FLICE-inhibitory Protein Expression during Macrophage Differentiation Confers Resistance to Fas-mediated Apoptosis

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Summary

Macrophages differentiated from circulating peripheral blood monocytes are essential for host immune responses and have been implicated in the pathogenesis of rheumatoid arthritis and atherosclerosis. In contrast to monocytes, macrophages are resistant to Fas-induced cell death by an unknown mechanism. FLICE (Fas-associated death domain–like interleukin 1β-converting enzyme)-inhibitory protein (Flip), a naturally occurring caspase-inhibitory protein that lacks the critical cysteine domain necessary for catalytic activity, is a negative regulator of Fas-induced apoptosis. Here, we show that monocyte differentiation into macrophages was associated with upregulation of Flip and a decrease in Fas-mediated apoptosis. Overexpression of Flip protected monocytes from Fas-mediated apoptosis, whereas acute Flip inhibition in macrophages induced apoptosis. Addition of an antagonistic Fas ligand antibody to Flip antisense–treated macrophages rescued cultures from apoptosis, demonstrating that endogenous Flip blocked Fas-induced cell death. Thus, the expression of Flip in macrophages conferred resistance to Fas-mediated apoptosis, which may contribute to the development of inflammatory disease.

Key words: monocytes • macrophages • apoptosis • Flip • Fas

Macrophages regulate host immune responses, contribute to acute and chronic inflammation, and protect against microbial infection and tumor formation (1). Before differentiation, peripheral blood monocytes are highly susceptible to Fas (CD95/Apo-1)–mediated apoptosis (2–5). In contrast, differentiated macrophages are resistant to numerous death stimuli, including death receptor ligation (5, 6), antineoplastic agents, and ionizing irradiation (7), suggesting upregulation of survival factor(s). The accumulation of activated macrophages in diseased tissues is involved in the pathogenesis of conditions such as rheumatoid arthritis (8) and atherosclerosis (9). The mechanism(s) responsible for macrophage resistance to apoptosis and persistence in disease states is unknown. Mice lacking a functional Fas-Fasl ligand (FasL) death receptor pathway displayed increased macrophage numbers and activity (10, 11), in addition to developing autoimmune disease and enhanced lymphocyte survival. Thus, regulation of the Fas-Fasl pathway may contribute to macrophage homeostasis.

Oligomerization of Fas induces the recruitment of Fas-associated protein with death domain (FADD [12, 13]), Fas-associated death domain–like IL-1β-converting enzyme (FLICE)–associated huge protein (FLASH [14]), and the cysteine protease caspase 8 (FLICE/MACH [15, 16]) to the death-inducing signaling complex (DISC). Aggregation of procaspase 8 results in auto- or trans-processing, which cleaves the inactive procaspase forming an active heterotetrameric caspase 8 (17–20). Once active, caspase 8 is released into the cytosol, inducing the proteolytic cascade of apoptosis (21). Direct inhibition of Fas-mediated apoptosis may oc-
cur through FLICE-inhibitory protein (Flip, also called CASPER, CLAR P, FLAM E-1, I-FLICE, CASH, or MR IT), a novel Fas pathway-inhibitory protein (22–28) which acts as a dominant negative caspase 8 (24, 29). Flip expression results in two gene products due to alternate splicing (24). The larger, Flip, possesses two death effector domains (DEDs) and a caspase-like domain in which tyrosine is substituted for the active cysteine residue necessary for enzymatic activity (24). The smaller protein, Flip, possesses two DEDs, but no caspase-like domain, similar to viral Flips (24). Thus, in cells resistant to Fas-induced apoptosis, Flip may confer protection from unwanted cell death.

The regulation of monocyte survival under serum-depleted conditions has been extensively investigated (2–5, 30–32). In vitro, the vast majority of monocytes cultured in the absence of serum undergo marked, spontaneous apoptosis, which was reduced by GM-CSF (5), IL-β, LPS, TNF-α (2, 5, 30, 31), or M-CSF (32). Inhibition of Fas or Fasl protected serum-deprived monocytes from apoptosis (3–5), indicating that monocytes may be deleted through the Fas-Fasl pathway. Nonetheless, even in the presence of serum, monocytes undergo spontaneous apoptosis (5) and are susceptible to Fas-induced cell death (4, 5). These data indicate that monocytes lack an apoptosis inhibitory factor of the death receptor pathway, which may be upregulated during monocyte to macrophage differentiation.

We examined the regulation of Fas-mediated apoptosis by Flip during monocyte differentiation into macrophages. Monocytes undergo spontaneous apoptosis in serum during days 1 and 2 after isolation, as indicated by terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) analysis and hypodiploid DNA content. Neutralization of Fasl or addition of the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (zVAD.fmk) rescued serum-treated monocytes from apoptosis (3–5), indicating that monocytes may be deleted through the Fas-Fasl pathway. Nonetheless, even in the presence of serum, monocytes undergo spontaneous apoptosis (5) and are susceptible to Fas-induced cell death (4, 5). These data indicate that monocytes lack an apoptosis inhibitory factor of the death receptor pathway, which may be upregulated during monocyte to macrophage differentiation.

Materials and Methods

Cell Isolation and Culture. Mononuclear cells were isolated by Histopaque (Sigma Chemical Co.) gradient centrifugation. Peripheral blood monocytes were then isolated from the mononuclear cells by either Percoll (Sigma Chemical Co.) gradient centrifugation (3, 7) or countercurrent centrifugal elutriation (Beckman-Coulter) (7, 33). All experiments were performed on monocytes that were isolated both ways, except where noted. There were no differences in the results due to the method of isolation. Monocyte purity was >90% as determined by morphology, CD14 staining, and nonspecific esterase staining. Monocytes were differentiated in RPMI containing 20% heat-inactivated fetal bovine serum (FBS) plus 1 μg/ml polymyxin B sulfate (Sigma Chemical Co.) (4, 5) in 24-well plates (Costar) except when noted.

Transient Transfection. For transient transfections, 3 × 10⁶ U937 cells were cultured in 100-mm plates, cotransfected for 4 h with 8, 6, or 4 μg of test plasmids and with 2 μg of CMV-e enhanced green fluorescent protein (EGFP) expression plasmid (Clontech), using the FuGENE™ procedure (1:5 ratio of DNA/FuGENE™; Roche Biochemicals). Empty vector was added to transfections to yield a total of 10 μg of DNA per transfection. After transfection, cultures were washed, incubated in 20% FBS/RPMI for 12 h, and treated with hamster anti-Fas antibody (500 ng/ml, clone CH11; MBL) for an additional 12 h. U937 cells were collected, and EGFP-expressing cells were quantified by flow cytometry. Nonspecifically excluded cells were excluded by propidium iodide (PI) incorporation.

TUNEL Labeling and Nuclear Condensation. Histopaque/Percoll-isolated monocytes were cultured on 60-mm plates containing glass coverslips treated with acetate acid ethanol (ETO H) (34, 35). At the indicated time points, cultures were fixed in 4% neutral buffered formalin for 5 min and subjected to two washes in PBS. Individual coverslips were treated with 0.5% NP-40 for 5 min, followed by two PBS washes. TdT enzyme and a cocktail containing dUTP conjugated to a fluorescein (FITC) were added to the coverslips according to the manufacturer’s specifications (In Situ Death Detection kit; Roche Biochemicals). Nuclei were counterstained with Hoechst 33258 (Sigma Chemical Co.), and mounted for examination using mounting media for fluorescence microscopy (Kirkgaard & Perry). Specimens were examined and photographed on a Zeiss microscope equipped with a phase-contrast and epifluorescence optics. Pictures were recorded on Zeiss software.

Flow Cytometric Analysis of Monocytes and Macrophages. At the indicated time points, cultures were harvested in 0.02% EDTA and fixed in 70% ETOH overnight (36–38). Cells were then stained with PI (Roche Biochemicals) as described previously (35). The subdiploid peak, immediately adjacent to the G0/G1 peak (2N), was determined by flow cytometry using an EpicSxL flow cytometer (Beckman-Coulter) and system 2 software (see Fig. 1 B). Objects with minimal light scatter were excluded since they may represent debris and would have inappropriately enhanced our estimate of the subdiploid population (38). For TUNEL analysis by flow cytometry, immediately isolated and 1-d macrophages were analyzed with the Apo-Direct™ apoptosis assay, according to the manufacturer’s specifications (PharMingen). In brief, cultures were fixed in 1% paraformaldehyde, permeabilized in 70% ETOH for 1 h, and incubated with FITC-dUTP, TdT, and reaction buffer for 1 h. Cells were then analyzed for FITC-dUTP incorporation by flow cytometry.

Fas expression was determined in immediately isolated monocytes and 7-d macrophages that were harvested in 0.02% EDTA, blocked in 50% human serum for 1 h, and then incubated with FITC-labeled anti-Fas antibody (clone UB2; Beckman-Coulter) or with FITC-labeled isotype control (Becton Dickinson). As additional negative controls, monocytes and macrophages were analyzed with FITC-conjugated anti-CD3 or anti-TCR-β/γ antibodies (Becton Dickinson) and the appropriate isotype control.
For surface FasL staining, monocytes or 7-d macrophages were blocked in 50% human serum for 1 h. Cells were then incubated in 33% human serum/33% goat serum with either rabbit anti-FasL (clone C-20; Santa Cruz Biotechnology), hamster anti-FasL (clone 4H9; Beckman-Coulter), or normal control IgG (Sigma Chemical Co.). Cells were then incubated with FITC-labeled goat anti-rabbit antibody (Kirkegaard & Perry) or rabbit anti-hamster antibody (Pel-Freez Biologicals). Mitocondrial permeability transition in macrophages after anti-Fas or TNF-α activation was analyzed by Rh123 (0.1 μM/ml; Molecular Probes). Rh123 was added to cultures for 30 min before analysis by flow cytometry, and live cells were determined by PI exclusion. Flow cytometry was conducted at the R obtet H. Lurie Comprehensive Cancer Center, Flow Cytometry Core Facility of the Northwestern University Medical School.

Modulation of M onocyte M acrophage A poptosis. M onocytes or macrophages were incubated with either anti-FasL antibody (clone C-20), anti-FasL antibody (clone 4H9), or anti-Fas antibody (clone CH11), control IgG (Dako), 2′,5′-DAdm (Enzyme System Products), or TNF-α (R & D Systems) for 24 h. Cultures were harvested in 0.02% EDTA and examined for apoptosis. To inhibit Flip expression, phosphorothioate oligodeoxynucleotides were created to include the Flip initiation codon (24; Flip antisense oligonucleotide 5′-GACTTCAAGCAGACATTAC-3′). Control nonsense phosphorothioate oligodeoxynucleotides have been described previously (39; 5′-TGATCGACATGTCAGA-3′). FITC-conjugated oligo nucleotides (10 or 20 μM) were added to monocytes or macrophages for 24 h. Cells were removed in 0.02% EDTA, fixed in 70% ETOH, stained with PI, and analyzed by flow cytometry. Parallel cultures were harvested for immunoblot analysis. Additionally, oligonucleotides in combination with either rabbit anti-FasL (clone C-20), hamster anti-FasL (clone 4H9), or control IgG (Dako) were also added to 7-d macrophages for 24 h. Cells were analyzed for DNA fragmentation by Cell Death ELISA (see below).

R everse T ranscriptase P CR A nalysis. Peripheral blood monocytes, isolated by Histopaque/Percoll gradient centrifugation, were differentiated in 20% FBS/RPMI/1 μg/ml polymyxin B and harvested for RNA preparation as described by Hockenbery et al. (40). 1 μg of total RNA was incubated in reaction buffer containing oligo(dT) primer, Moloney murine leukemia virus reverse transcriptase (RT), reaction buffer, and RNAse inhibitor for 1 h at 42°C according to the manufacturer's specifications (Clontech). The reaction was stopped at incubation at 94°C for 5 min. Primers specific for Flip were as follows: forward, 5′-GAT-GTCTGTCGATGGTATCAT-CACA-3′; reverse for Flip, 5′-CAC-TACCGCCACGCTTTTGG-3′, and reverse for Flip, 5′-AGT-AGAGGCGATTTGCA-CAT-3′. The reverse Flip primer is within the 3′ untranslated region of Flip, thus allowing for delineation between Flip and Flip. The PCR reaction was carried out with 5 U Taq polymerase (Perkin Elmer) in a total volume of 100 μl. Amplification was performed for 28 cycles (30 s denaturing at 94°C, 45 s annealing at 50°C, and 90 s extension at 72°C) in a thermal cycler. Control human β-actin primers were used under parallel conditions (Clontech). The 1,470-bp Flip, 620-bp Flip, and 838-bp β-actin amplified products were analyzed by 1.0% agarose gel electrophoresis and visualized under UV illumination after being stained with ethidium bromide.

W estern B lot A nal ysis. Whole-cell extracts were prepared from peripheral blood monocytes and differentiated in 20% FBS/RPMI/1 μg/ml polymyxin B. 25 or 50 μg of extract, as indicated, was analyzed by SDS-PAGE on 12.5% polyacrylamide gels and transferred to Immobilon P (Millipore) by semidry blotting. Filters were blocked for 1 h at room temperature in PBS/0.2% Tween 20/5% nonfat dry milk. The filters were then incubated with rabbit anti-Flip (41), which recognizes both Flip isoforms, with rabbit anti-caspase 8 antibody (Chemicon) or mouse anti-caspase 3 antibody (Transduction Laboratories) at 4°C in PBS/0.2% Tween 20/2% nonfat dry milk. Filters were washed in PBS/0.2% Tween 20/2% nonfat dry milk and incubated with either donkey anti-rabbit or anti–mouse secondary antibody (1:2,000 dilution) conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Visualization of the immunocomplex was performed using Enhanced Chemiluminescence Plus kit (Amersham Pharmacia Biotech).

R esults

S pontaneous A poptosis O ccurs in M onocytes, B ut M ono cytes Do Not Apoptose. Previous investigators demonstrated decreasing cell numbers over the first 3 d when monocytes were cultured in serum (32, 42–44). Here, we examined the occurrence of apoptosis during monocyte to macrophage differentiation in serum. TUNEL analysis, which measures DNA fragmentation, demonstrated peak apoptosis at 1 and 2 d after isolation, though few or no TUNEL-positive macrophages were observed at 0, 3, 7, and 14 d (Fig. 1A). Quantitative analyses of subdiploid DNA content in serum-treated monocytes (Fig. 1, B and C) revealed enhanced spontaneous apoptosis in the 0.5-, 1-, and 2-d cultures (12.1 ± 1.7, 26 ± 4.7, and 23 ± 9.0%, respectively). However, macrophages at 3, 7, and 14 d (Fig. 1, B and C) displayed minimal hypodiploid DNA content (<6%). To corroborate the quantification of apoptosis as determined by subdiploid DNA content, flow cytometry measuring FITC-dUTP incorporation (TUNEL) was performed. Since maximal apoptosis was exhibited in 1-d monocyte cultures, immediately isolated (day 0) and 1-d monocytes were examined. In the 1-d monocyte cultures, 25.8 ± 2% TUNEL-positive cells were detected (Fig. 1 D), similar to the analysis of subdiploid DNA (Fig. 1 B). Little or no TUNEL positivity (<1%) was observed in monocytes immediately after isolation. Furthermore, cell counting by trypan blue exclusion revealed a 50–60% reduction of monocyte cell numbers at 3 d after isolation (not shown), indicating that the apoptosis observed at 1 and 2 d resulted in the cumulative loss of cells. Consistent with previous reports (5, 30–32), >70% of serum-deprived monocytes underwent spontaneous apoptosis after 24 h (not shown). Collectively, these data demonstrate that in vitro–differentiated macrophages, unlike monocytes, undergo little or no spontaneous apoptosis.

I nhibition of Fas–FasL I nteraction R escues M onocytes from S pontaneous A poptosis. M onocytes cultured in serum undergo...
spontaneous apoptosis that may be mediated by the Fas-FasL pathway. Flow cytometric analyses of surface Fas and FasL revealed that Fas was present on essentially all the cells, although it was more strongly expressed on monocytes compared with macrophages (Fig. 2 A). FasL was also detected on the surface of both monocytes and macrophages, though more FasL was expressed on macrophages (Fig. 2 A).

To confirm that the detection of Fas and FasL was specific, flow cytometric analysis using FITC-conjugated anti-CD3 and anti-TCR-γ/δ antibodies demonstrated comparable staining to the isotype control in both monocytes and macrophages (Fig. 2 A). To define the mechanism(s) responsible for monocyte apoptosis, inhibitory FasL antibodies and the general caspase inhibitor zVAD.fmk were compared with TNF-α, which was previously shown to inhibit monocyte apoptosis (2, 5, 30, 31). Addition of either C-20 (82% inhibition) or 4H9 (41% inhibition, not shown) neutralizing anti-FasL antibodies to isolated monocytes in 20% serum significantly (P < 0.02) inhibited spontaneous apoptosis compared with IgG-treated or medium control–treated (mock) monocytes (Fig. 2 B). Similarly, TNF-α inhibited apoptosis by 62% (P < 0.02) while the general caspase inhibitor zVAD.fmk partially blocked spontaneous apoptosis (41%, P < 0.01) in 1-d (Fig. 2 B) and 2-d (not shown) monocyte cultures. The contribution of caspase activation to Fas-mediated apoptosis in monocytes was further examined by immunoblot analyses. Reduced detection of the pro-caspases 8 and 3 was observed in extracts from 0 and 1-d monocytes (Fig. 2 C) compared with 7-d macrophages. The intermediate active form of caspase 3 (p24[45]) was detected in extracts of monocytes on days 0 and 1, but not those from day 7 macrophages. As a control, Fas-agonistic antibody–treated Jurkat T cells also displayed the intermediate active caspase 3 (not shown). These data indicate that

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**Figure 1.** Peripheral blood monocytes, but not macrophages, undergo spontaneous apoptosis. (A) Representative photomicrographs of TUNEL–positive monocytes. Day 0 monocytes represent cells obtained within 2 h of isolation. Arrows identify cells that are positive for both Hoechst (top panels) and TUNEL (bottom panels) on days 1, 2, and 3. Original magnification of the digital photomicrographs: 800×. (B) Representative histogram of DNA content in monocytes and macrophages. Triplicate cultures were harvested at 0, 1, 2, 3, 7, and 14 d, fixed, and analyzed by flow cytometry after PI staining. Histograms were formed in the linear scale to identify subdiploid DNA (<2N), characteristic of apoptosis. (C) Monocytes exhibit hypodiploid DNA content. Triplicate cultures were harvested as described above. Values represent the mean ± SE of five independent determinations, which were compared for statistical significance by unpaired two-tailed Student’s t test. *P < 0.02 relative to day 0. (D) Representative histogram of FITC positivity in 0 and 1-d monocytes. Monocytes obtained immediately after isolation or 1 d later were fixed in 1% paraformaldehyde, permeabilized in 70% ETOH, and stained for TUNEL using FITC-labeled dUTP as described in Materials and Methods. TUNEL positivity was quantified by flow cytometry that measured FITC-labeled dUTP incorporation (x-axis) and cell number (y-axis).
the spontaneous monocyte apoptosis that occurs in serum was attributed to Fas–FasL interaction and caspase activation. Macrophage resistance to Fas-induced cell death is associated with Flip upregulation. The ability of agonistic Fas antibody and TNF-α to induce apoptosis in macrophages was examined. Macrophages were resistant to spontaneous and agonistic Fas antibody–induced apoptosis (Fig. 3 A) as indicated by normal DNA content and mitochondrial membrane integrity, suggesting that a potent inhibitor of the death receptor–mediated pathway, not present in monocytes, may be upregulated in macrophages. As a control, agonistic Fas antibody induced apoptosis in Jurkat T cells and U937 cells (not shown). In certain cell types, Flip overexpression has been shown to directly inhibit Fas–mediated apoptosis (29), indicating that Flip may function to promote survival during monocyte to macrophage differentiation. Previous investigations demonstrated that by day 3 in culture, monocytes begin to differentiate into macrophages based on morphology, cell surface markers including vitronectin (CD51 [32]), transferrin receptor (CD71; not shown), and cytolytic activity (46). Immunoblot analyses performed on isolated peripheral blood monocytes harvested at 0, 1, 2, 3, 7, and 14 d revealed that Flip upregulation was associated with macrophage differentiation and reduced apoptosis, beginning on day 3 (Fig. 3 B). Flip was detected at 7 and 14 d of macrophage differentiation, whereas Flip was highly expressed 3 d after isolation (Fig. 3 B). The ratio of Flip to Flip on days 3, 7, and 14 varied between individuals. Although low levels of Flip were detected before day 3 in some individuals, Flip upregulation at 3, 7, and 14 d was observed in every individual examined (n = 10). These data demonstrate that the decreased spontaneous apoptosis seen during and after macrophage differentiation is associated with Flip upregulation.

To determine if the Flip expression observed at 3 d is transcriptionally regulated, RT-PCR analyses performed with specific primers that differentiate between the two Flip isoforms revealed minimal Flip and Flip mRNA transcripts in immediately isolated monocytes (Day 0). However, Flip and Flip mRNA were upregulated on day 3 (Fig. 3 C). Equally expressed β-actin mRNA transcripts indicated that similar levels of mRNA were amplified in all samples. These data demonstrate that Flip is upregulated at the transcriptional level during monocyte differentiation into macrophages.
Flip overexpression rescues GFP-positive U937 cultures from Fas-mediated cell death. U937 cultures were transfected with 2 μg of the CMV-EGFP-expressing plasmid and either expression vectors 4, 6, or 8 μg of CMV-FlipL or CMV-FlipS using FuGENE™. Empty CMV vector was added to transfections to yield a total of 10 μg of DNA per transfection. After transfection, cultures were incubated in 20% FBS/RPMI for 12 h and treated with agonistic anti-Fas antibody (500 ng/ml) for an additional 12 h. U937 cells were collected, and EGFP-expressing cells were quantified by flow cytometry. Nontolive cells were excluded by PI incorporation. Before addition of Fas agonistic antibody, ∼6000 GFP-positive cells were observed. The data are representative of two independent experiments.

Figure 4. Flip overexpression rescues GFP-positive U937 cultures from Fas-mediated cell death. U937 cultures were transfected with 2 μg of the CMV-EGFP-expressing plasmid and either expression vectors 4, 6, or 8 μg of CMV-FlipL or CMV-FlipS using FuGENE™. Empty CMV vector was added to transfections to yield a total of 10 μg of DNA per transfection. After transfection, cultures were incubated in 20% FBS/RPMI for 12 h and treated with agonistic anti-Fas antibody (500 ng/ml) for an additional 12 h. U937 cells were collected, and EGFP-expressing cells were quantified by flow cytometry. Nontolive cells were excluded by PI incorporation. Before addition of Fas agonistic antibody, ∼6000 GFP-positive cells were observed. The data are representative of two independent experiments.
induced by Flip antisense treatment, as indicated by trypan blue exclusion (not shown) and by DNA fragmentation (Fig. 5 D). Addition of FasL antibodies or control IgG had no effect on the viability of control oligonucleotide–treated cultures (not shown). These data demonstrate that Flip contributes to macrophage survival and document for the first time the functional significance of endogenous Flip expression, which is necessary for survival during in vitro monocyte to macrophage differentiation.

Discussion

Isolated monocytes are highly sensitive to apoptosis induced by Fas–FasL ligation, whereas differentiated macrophages are resistant. Our data document that Fas–FasL interactions, which mediate caspase activation, contribute to the continued reduction of monocytes during the first 48–72 h. As early as 2 h after isolation (day 0), before the appearance of hypodiploid DNA or TUNEL positivity, caspases were already activated. This could be the result of activation of caspases, where the inactive procaspase has been cleaved to form the active protease (51). It is also possible that the increased procaspases detected in day 7 macrophages may be attributed to increased synthesis. While our data do not exclude this possibility, a previous study demonstrated caspase 8 activity in freshly isolated monocytes (52), suggesting that the reductions seen in our study (days 0 and 1) were at least in part due to caspase activation. We also observed the intermediate cleaved caspase 3 (p24) isoform on days 0 and 1, but not day 7, indicating that activation had occurred at the early time points. Protection of monocytes from apoptosis by the caspase inhibitor zVAD.fmk further supports the interpretation that caspases were activated immediately after isolation (<2 h; Fig. 2 B). The fact
that caspase activation appears to be initiated even during isolation may explain the lack of complete inhibition by neutralizing anti-Fasl or zVAD.fmk.

A decline in monocyte cell number by day 3 correlated with Flip expression. Both RT-PCR and immunoblot analyses demonstrated an upregulation in Flip expression by day 3. Although the ratios of the two isoforms, as determined by immunoblot analyses, varied between individuals at 3, 7, and 14 d, our data suggest that Flip expression was responsible for the protection against apoptosis observed on day 3 and thereafter. The incubation of macrophages with Flip antisense oligonucleotides reduced Flip expression by Western blot analyses and increased apoptosis as determined by hypodiploid DNA content. Furthermore, the concurrent reduction of procaspase 3 in the Flip antisense oligonucleotide–treated macrophages suggests that this process activated caspases. Additionally, the marked protection of U937 cells from Fas-mediated apoptosis by Flip, but not Bcl-2, supports the importance of Flip for macrophage survival. Recently, stably expressed Bcl-2 was shown to provide modest protection against Fas-mediated apoptosis in U937 cells (53), which was comparable to that observed in our study. Decreased numbers of GFP-positive U937 cells were not seen even when higher concentrations of the Flip expression plasmids were used. However, in other cell types, overexpression of Flip (22, 24, 25, 27; and data not shown) resulted in cell death, suggesting that the effects of Flip overexpression may be cell type specific. These data document an important role for Flip in the resistance of macrophages to Fas-Fasl-mediated cell death.

Surface Fas was expressed on essentially all day 7 macrophages, though less intensely compared with monocytes, suggesting that reduction in the amount of surface Fas on macrophages may have contributed to reduced apoptosis. However, in a recent investigation primary endothelial cells, which express low levels of Fas (54, 55) comparable to those observed on macrophages, were sensitized to Fas–FasL-induced apoptosis by oxidized lipids (55). Interestingly, the susceptibility of endothelial cells to Fas-mediated apoptosis induced by oxidized lipids correlated with Flip downregulation (41) even though the level of surface Fas remained unchanged. Fas–Fasl interactions also mediated apoptosis in macrophages after Flip inhibition, since neutralization of the Flip antisense oligonucleotides these data demonstrate that Flip, and not reduced surface Fas, was responsible for the absence of Fas-mediated macrophage apoptosis.

Activation of macrophages is an integral component of several host defense mechanisms, including activation of T cells, release of inflammatory cytokines, removal of virus-infected cells, and the antibody-dependent and -independent killing of tumorigenic cells (56). Although macrophages are resistant to Fas-mediated apoptosis, they may be rendered Fas sensitive. TNF-α or a combination of TNF-α plus INF-γ sensitized mouse peritoneal macrophages to Fas-mediated apoptosis in vitro. Additionally, Th1 CD4+ T cells induced apoptosis of antigen-pulsed, IFN-γ-treated peritoneal macrophages, which was mediated by Fas-Fasl interactions (57). Studies have yet to determine if decreased Flip expression contributed to macrophage apoptosis under these conditions. Fas on macrophages may not induce apoptosis of T cells for which they serve as the APCs, because unactivated T cells strongly express Flip (24, 58, 59). However, after stimulation, T cells downregulate Flip expression and undergo suicide, resulting in activation-induced cell death that is mediated by the Fas–Fasl pathway (24, 58-61). Thus, depending on the environmental milieu, T cell and macrophage sensitivity to Fas-mediated apoptosis appears to be regulated by Flip expression.

Circulating blood monocytes extravasate into tissues and differentiate into macrophages. Persistent expression of Fasl by adenoaviral delivery to endothelial cells prevented monocytes from emigrating into the inflamed tissue (54), suggesting that Fas is functional on monocyte surfaces in vivo. In addition, mice carrying functional mutations of Fas–Fasl displayed elevated macrophage cell numbers (11), indicating the importance of Fas–Fasl in regulating monocyte/macrophage homeostasis. Recently, we identified Flip expression in macrophages isolated from synovial fluid and in the synovial tissues (not shown) of patients with rheumatoid arthritis, demonstrating a potential significance for Flip expression in vivo. Additionally, animals with experimental arthritis also displayed increased numbers of synovial macrophages that were Flip positive (not shown). Thus, modulation of Flip expression may provide a novel therapeutic approach to diseases mediated by macrophages, such as rheumatoid arthritis or atherosclerosis.
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