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–Fas 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)[1]-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-

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H. Perlman and L.J. Pagliari contributed equally to this paper.

[1] *Abbreviations used in this paper:* EGFP, enhanced green fluorescent protein; ETOH, ethanol; FBS, fetal bovine serum; FLICE, Fas-associated death domain–like IL-1β–converting enzyme; Flip, FLICE-inhibitory protein; PI, propidium iodide; RT, reverse transcriptase; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT dUTP nick end labeling; zVAD.fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.

cur through FLICE-inhibitory protein (Flip, also called CASPER, CLARP, FLAME-1, I-FLICE, CASH, or MRIT), 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_L, 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_S, possesses two DEDs, but no caspase-like domain, similar to viral Flips (24). Thus, in cells refractory towards Fas-induced apoptosis, Flip may confer protection from unwarranted 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. Immunoblot analyses revealed undetectable Flip expression in monocytes, which was upregulated in macrophages. Additionally, Flip mRNA was present in macrophages, but not in monocytes, indicating that Flip was transcriptionally regulated. Procaspases 8 and 3 were reduced in monocytes compared with macrophages, suggesting that the procaspases were converted to the active state during monocyte apoptosis. Overexpression of Flip_L and Flip_S expression plasmids rescued U937 monocytic cells from Fas-mediated apoptosis. Acute inhibition of Flip mRNA by antisense oligonucleotides induced macrophage apoptosis, which was prevented by an antagonistic FasL antibody. These data indicate that Fas and FasL on neighboring macrophages interacted and that, under the conditions used, the apoptotic signal was blocked by Flip. Thus, during differentiation in vitro, Flip upregulation was responsible for inhibition of the Fas–FasL pathway, permitting macrophage survival.

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, 3×10^6 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–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. Nonviable 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 acetic acid/ethanol (ETOH) (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 (Kirkegaard & 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 monocytes 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-γ/δ 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). Mitochondrial permeability transition in macrophages after anti-Fas or TNF-α addition was analyzed by Rh123 (0.1 μg/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 Robert H. Lurie Comprehensive Cancer Center, Flow Cytometry Core Facility of the Northwestern University Medical School.

Modulation of Monocyte/Macrophage Apoptosis. Monocytes or macrophages were incubated with either anti-FasL antibody (clone C-20), anti-FasL antibody (clone 4H9), anti-Fas antibody (clone CH11), control IgG (Dako), zVAD.fmk (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′-GACTTCAGCAGACATCCTAC-3′). Control nonsense phosphorothioate oligodeoxynucleotides have been described previously (39; 5′-TGGATCCGACATGTCAGA-3′). FITC-conjugated oligonucleotides (10 or 20 μM) were added to 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).

Reverse Transcriptase PCR Analysis. 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 Chomczynski 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 by incubation at 94°C for 5 min. Primers specific for Flip were as follows: forward, 5′-GATGTCTGCTGAAGTCATCCATCA-3′; reverse for Flip_L, 5′-CACTACGCCCAGCCTTTTGG-3′, and reverse for Flip_S, 5′-AGTAGAGGCAGTTCCATG-3′. The reverse Flip_S primer is within the 3′ untranslated region of Flip_S, thus allowing for delineation between Flip_L and Flip_S. 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_L, 620-bp Flip_S, 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.

Western Blot Analysis. 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).

Cell Death ELISA. DNA fragmentation was detected using Cell Death ELISA Plus kit (Roche Biochemical) as recommended by the manufacturer. Mono- and oligonucleosomal DNA were detected in the cytoplasmic fraction of cell lysates. In brief, cell lysates were incubated in antihistone-coated microtiter plates. DNA attached to the bound histones was detected with peroxidase-conjugated anti-DNA antibody. After wash steps, substrate was added to the microtiter wells and color change was read at 405 nm by a microplate reader (Bio-Tek Instruments).

Results

Spontaneous Apoptosis Occurs in Monocytes, But Not Macrophages. 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. 1 A). 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.

Inhibition of Fas–FasL Interaction Rescues Monocytes from Spontaneous Apoptosis. Monocytes cultured in serum undergo

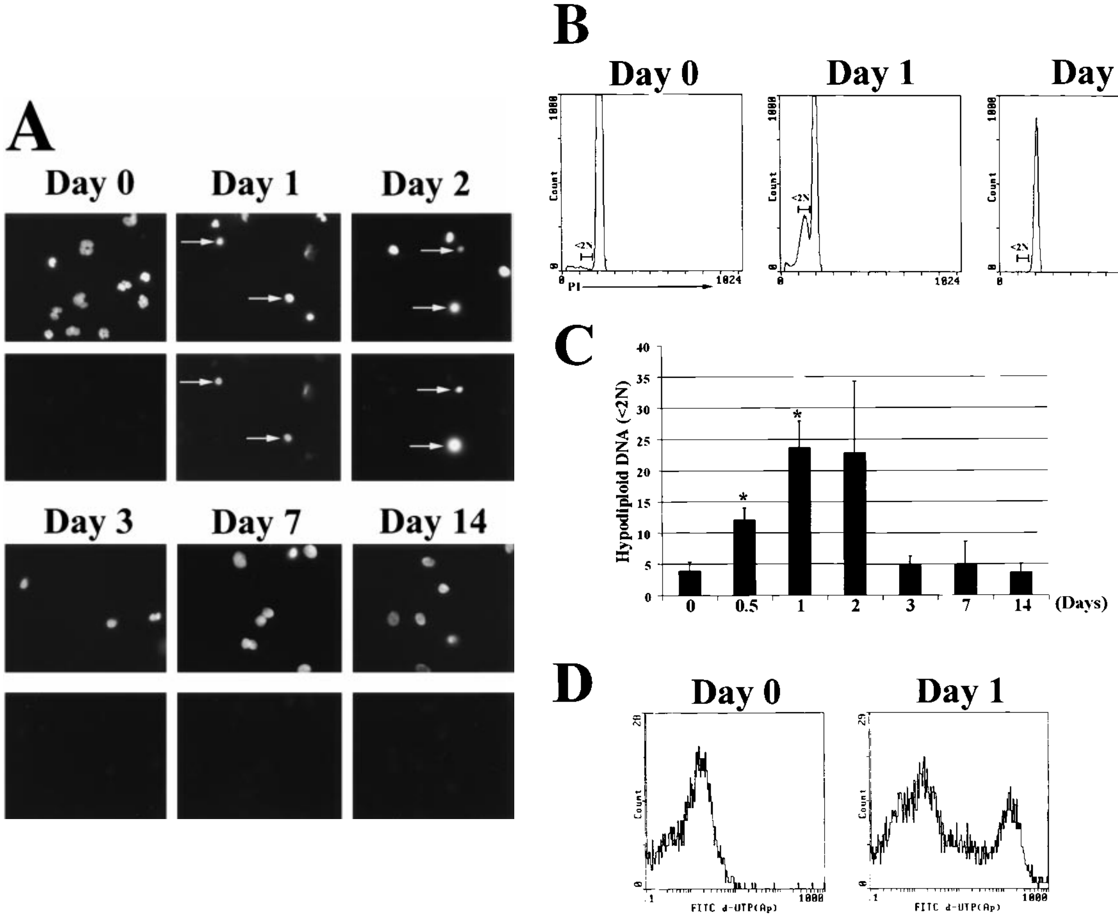

Figure 1. Peripheral blood monocytes, but not macrophages, undergo spontaneous apoptosis. (A) Representative photomicrographs of TUNEL-positive monocytes. Day 0 monocytes represent cells obtained within 2 h of isolation. Arrows identify cells that are positive for both Hoechst (top panels) and TUNEL (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).

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 procaspases 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

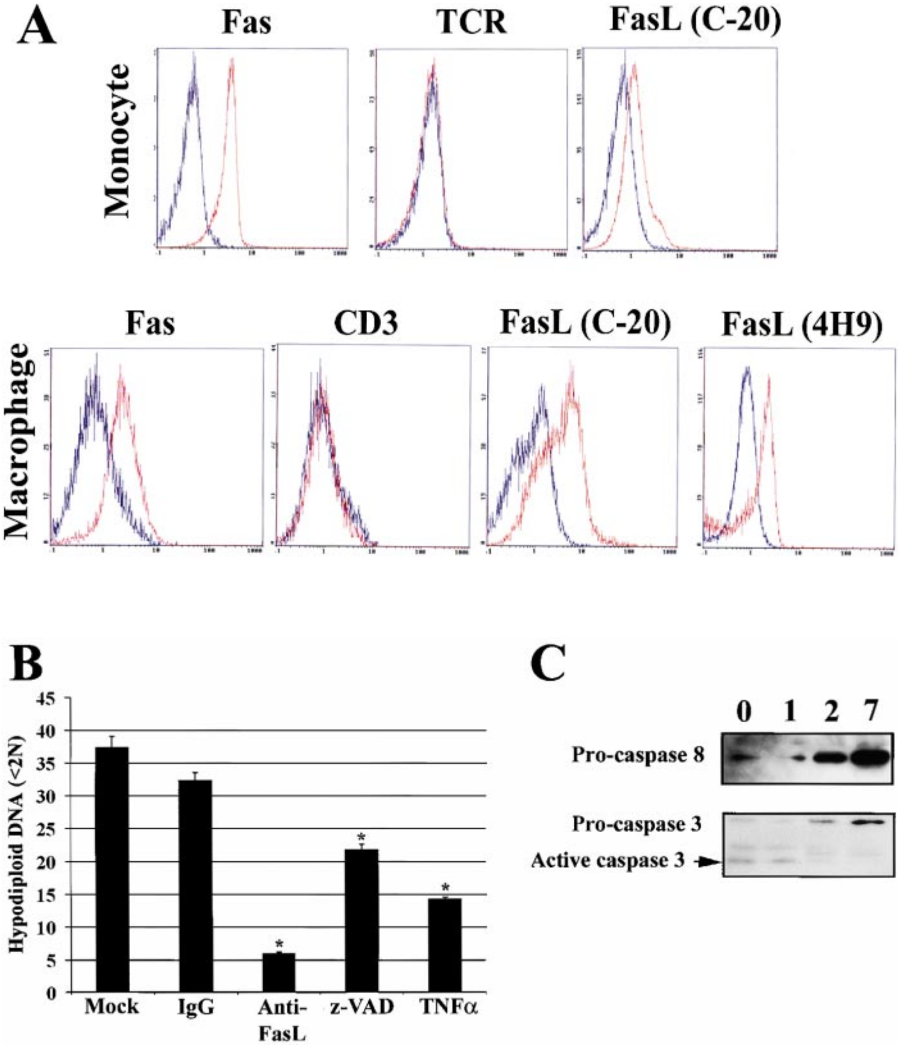

Figure 2. Spontaneous monocyte apoptosis is regulated by Fas–FasL. (A) Monocytes and macrophages express surface Fas and FasL. Cultures were blocked in human serum, stained with FITC-labeled anti-Fas, anti-CD3, anti–TCR-γ/δ, or isotype control. Additional cells were stained with anti-FasL (C-20), anti-FasL (4H9), or normal rabbit IgG, then with FITC-labeled goat anti–rabbit or rabbit anti–hamster antibody. All cultures were then analyzed by flow cytometry. Control Igs are in blue; the experimental antibodies (anti-Fas, anti-FasL, anti–TCR-γ/δ, or anti-CD3) are in red. (B) Neutralizing anti-FasL antibody protects monocytes cultured in serum from spontaneous apoptosis. Immediately after isolation, triplicate cultures of monocytes were treated with neutralizing anti-FasL (10 μg/ml), control IgG (10 μg/ml), zVAD.fmk (20 μM), TNF-α (10 ng/ml), or control medium (Mock) for 24 h. Cultures were fixed in 70% ETOH, incubated in PI (50 μg/ml), and analyzed by flow cytometry. Values represent the mean ± SE of three independent experiments. Anti-FasL and control IgG–treated cultures, and zVAD.fmk-, TNF-α–, and mock-treated cultures were compared for statistical significance by unpaired two-tailed Student's *t* test. $^*P < 0.0001$ for anti-FasL, $P < 0.01$ for zVAD.fmk, and $P < 0.02$ for TNF-α. (C) Whole cell extracts (25 μg) were prepared from peripheral blood monocytes and macrophages which had been cultured in 20% FBS/RPMI/1 μg/ml polymyxin B, and isolated at 0, 1, 2, and 7 d. Extracts were subjected to SDS-PAGE on 12.5% polyacrylamide gels. Gels were transferred to Immobilon *P* for immunoblot analysis with anti-caspases 8 and 3 antibodies. The anti-caspase 8 and 3 immunoblots are representative of three individuals.

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_L was detected at 7 and 14 d of macrophage differentiation, whereas Flip_S was highly expressed 3 d after isolation (Fig. 3 B). The ratio of Flip_L to Flip_S 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_S or Flip_L mRNA transcripts in immediately isolated monocytes (Day 0). However, Flip_S and Flip_L 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.

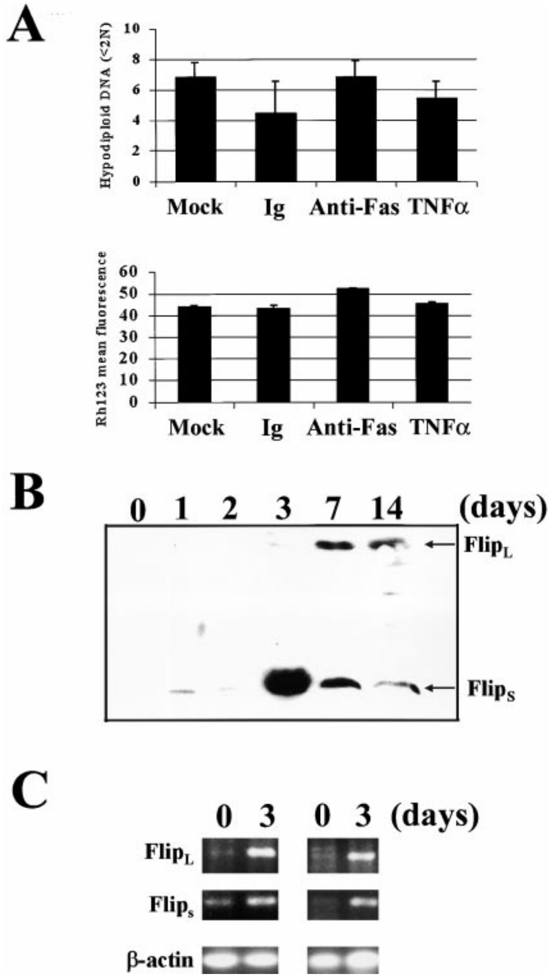

Figure 3. Macrophage resistance to Fas-mediated apoptosis is associated with Flip expression. (A) Macrophages are resistant to Fas-induced apoptosis. Triplicate cultures of macrophages differentiated for 7 d in 20% FBS/polymixin B were treated with activating anti-Fas mAb (250 ng/ml), control Ig, or TNF-α (10 ng/ml) for 1 d, fixed in 70% ETOH, and incubated in PI (50 μg/ml) for flow cytometry analysis to determine hypodiploid DNA content. Parallel cultures were incubated with Rh123 (0.1 μg/ml) for 30 min before analysis by flow cytometry. The results represent a mean of three experiments. (B) Flip is upregulated in macrophages. Whole cell extracts (25 μg) were prepared from peripheral blood monocytes and macrophages cultured in 20% FBS/RPMI/1 μg/ml polymyxin B and isolated at the indicated times. Extracts were subjected to SDS-PAGE on 12.5% polyacrylamide gels. Gels were transferred to Immobilon *P* for immunoblot analysis with anti-Flip. The anti-Flip immunoblot is a representative of 10 individuals. (C) Flip transcript levels are upregulated during monocyte to macrophage differentiation. RT-PCR analysis was performed on total RNA, and the 1,470-bp Flip_L, 620-bp Flip_S, and 838-bp β-actin amplified fragments were fractionated on 1.0% agarose gel and visualized by ethidium bromide staining.

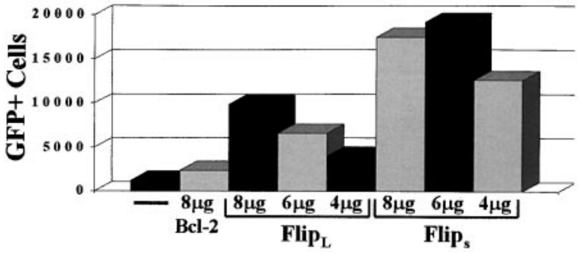

Figure 4. Flip overexpression rescues GFP-positive U937 cultures from Fas-mediated cell death. U937 cultures were transfected with 2 μg of the CMV-EGFP–expressing plasmid and either expression vectors 4, 6, or 8 μg of CMV-Flip_L or CMV-Flip_S using FuGENE™. Empty CMV vector was added to transfections 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. Nonviable cells were excluded by PI incorporation. Before addition of Fas agonistic antibody, ~6,000 GFP-positive cells were observed. The data are representative of two independent experiments.

Flip Overexpression Protects Monocytic Cells from Fas-mediated Apoptosis. Monocytes do not express Flip and are susceptible to Fas-mediated apoptosis, suggesting that forced expression of Flip may protect monocytes from apoptosis in response to Fas ligation. Since peripheral blood monocytes are difficult to transfect, the monocytic cell line U937, which expresses little or no Flip (not shown) (47) and undergoes apoptosis in response to agonistic Fas antibody (6), was used. An established cell death assay (48–50) was performed using expression plasmids encoding Flip_L, Flip_S (24), Bcl-2 (35), or empty vector transfected in combination with an EGFP expression plasmid. Flow cytometric analyses were used to determine changes in cell viability as indicated by the number of EGFP-positive cells. Equal levels of Flip_S and Flip_L expression were demonstrated by transient transfection of the expression plasmids in 293 cells (not shown). Expression vectors encoding Flip_L or Flip_S protected U937-transfected cells from Fas-mediated apoptosis in a dose-dependent manner (Fig. 4). In addition, Flip_S-transfected U937 cells displayed increased viability compared with Flip_L-transfected cells (15-fold vs. 8-fold). On the contrary, Bcl-2 provided little protection (twofold) compared with empty vector–transfected U937 cells (Fig. 4).

Acute Flip Inhibition Induces Macrophage Apoptosis. To determine if Flip is functionally significant for macrophage survival, FITC-conjugated antisense oligonucleotides were created complementary to a sequence that included the start site of the Flip open reading frame (24). Flow cytometry revealed similar uptake (Fig. 5 A) of the antisense and control nonsense oligonucleotides (39) in day 7 macrophages. Immunoblot analyses of oligonucleotide-treated macrophages demonstrated decreased expression of both Flip isoforms, using both 10 and 20 μM of the antisense oligonucleotides, compared with medium control (mock) or control oligonucleotide-treated macrophages (Fig. 5 B). Flow cytometric analyses revealed that 40 ± 3% of the antisense oligonucleotide–treated macrophages underwent apoptosis (Fig. 5 C) compared with 1 ± 0.1 and 3 ± 0.5% of mock- and control oligonucleotide–treated macrophages, respectively. Furthermore, reduction of the procaspase 3 (Fig. 5 B) was observed in the antisense oligonucleotide–treated macrophages compared with mock or control oligonucleotide–treated cells, supporting the role of caspase activation in macrophage cell death. Inhibition of Fas–FasL interactions by two different antagonistic FasL antibodies (40% with C-20 and 67% with 4H9) suppressed macrophage apoptosis

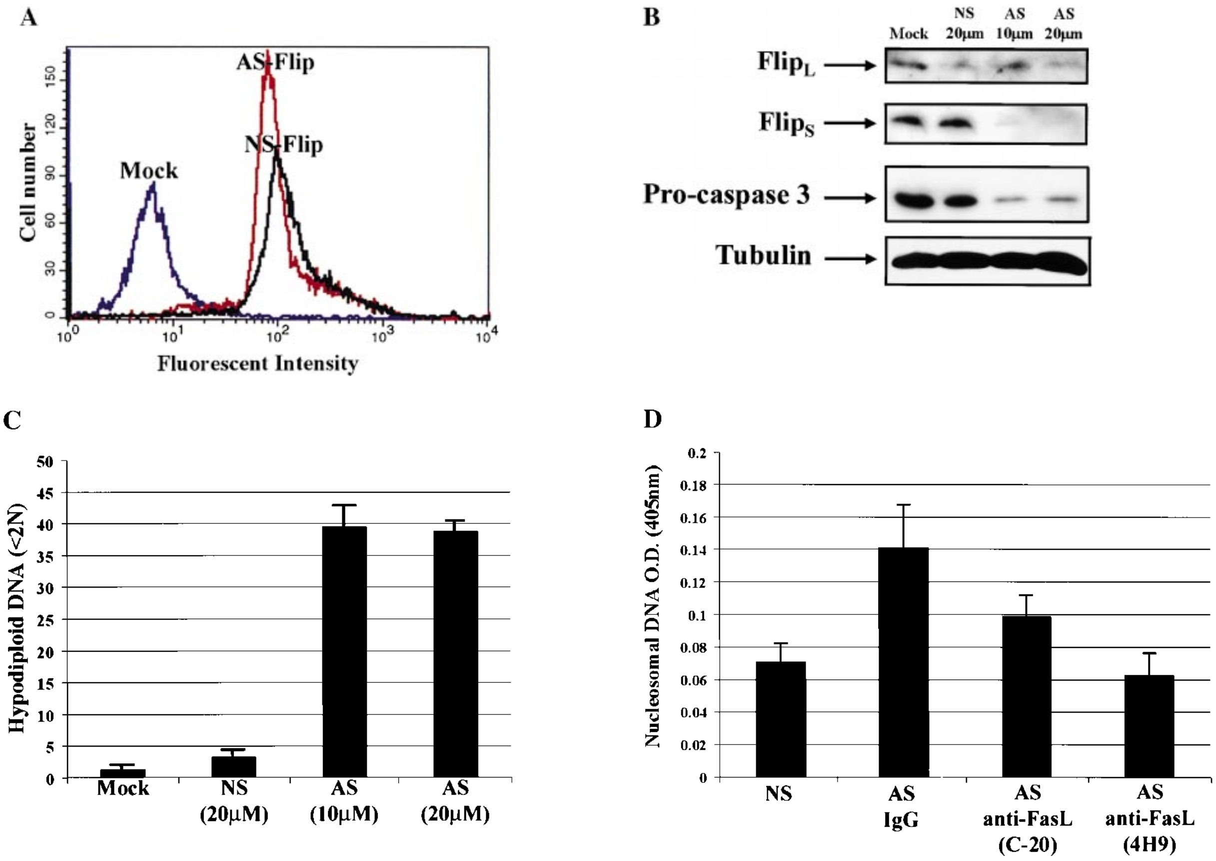

Figure 5. Acute inhibition of Flip in macrophages promotes apoptosis. (A) Flow cytometry demonstrates equal uptake of control nonsense (black, NS-Flip) and antisense (red, AS-Flip) oligonucleotides. Monocytes isolated by countercurrent centrifugal elutriation were differentiated in 20% FBS/RPMI/1 μg/ml polymyxin B for 7 d. FITC-conjugated oligonucleotides (10 and 20 μM) were added for 24 h. Cultures were then harvested, fixed in 70% ETOH, and analyzed by flow cytometry. (B) Flip antisense oligonucleotides reduced Flip expression associated with caspase 3 activation. Parallel cultures, as described above, treated with nonsense (NS) or antisense (AS) FITC-conjugated oligonucleotides were analyzed for Flip expression and caspase 3 expression. Whole cell extracts (50 μg) were subjected to SDS-PAGE on 12.5% polyacrylamide gels and transferred to Immobilon *P* for immunoblot analysis with anti-Flip, anti–caspase 8, and antitubulin antibodies. (C) Inhibition of Flip induces apoptosis in macrophages. Parallel cultures, as described above, were performed in triplicate and analyzed for hypodiploid DNA by flow cytometry. The results in this figure are representative of two experiments. Values represent the mean ± SE of triplicate determinations. (D) Neutralization of FasL blocks Flip antisense–induced macrophage cell death. 7 d–differentiated macrophages were incubated with anti-FasL (10 μg/ml, C-20 or 4H9) or control IgG for 1 h followed by the addition of the nonsense (NS) or antisense (AS) oligonucleotides (20 μM) for 24 h. DNA fragmentation was analyzed using the Cell Death ELISA Plus kit, which detects mono- and oligonucleosomal DNA fragments. Values represent the mean ± SE of triplicate determinations of a representative experiment.

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-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 FasL suppressed macrophage apoptosis in the presence 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. FasL 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 adenoviral 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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