Macrophages Require Constitutive NF-κB Activation To Maintain A1 Expression and Mitochondrial Homeostasis

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NF-κB is a critical mediator of macrophage inflammatory responses, but its role in regulating macrophage survival has yet to be elucidated. Here, we demonstrate that constitutive NF-κB activation is essential for macrophage survival. Blocking the constitutive activation of NF-κB with pyrrolidine dithiocarbamate or expression of IκBα induced apoptosis in macrophagelike RAW 264.7 cells and primary human macrophages. This apoptosis was independent of additional death-inducing stimuli, including Fas ligation. Suppression of NF-κB activation induced a time-dependent loss of mitochondrial transmembrane potential (ΔΨm) and DNA fragmentation. Examination of initiator caspases revealed the cleavage of caspase 9 but not caspase 8 or the effector caspase 3. Addition of a general caspase inhibitor, z-VAD.fmk, or a specific caspase 9 inhibitor reduced DNA fragmentation but had no effect on ΔΨm collapse, indicating this event was caspase independent. To determine the pathway leading to mitochondrial dysfunction, analysis of Bcl-2 family members established that only A1 mRNA levels were reduced prior to ΔΨm loss and that ectopic expression of A1 protected against cell death following inactivation of NF-κB. These data suggest that inhibition of NF-κB in macrophages initiates caspase 3-independent apoptosis through reduced A1 expression and mitochondrial dysfunction. Thus, constitutive NF-κB activation preserves macrophage viability by maintaining A1 expression and mitochondrial homeostasis.

The mechanism(s) by which the pleiotropic transcription factor nuclear factor kappa B (NF-κB) regulates cell survival remains unclear. Mice null homozygous for the p65 alleles or IkB kinase β are embryonic lethal due to extensive liver cell death (6, 40), demonstrating that NF-κB p65 or its activating kinase is essential for development. Embryonic macrophages and fibroblasts from p65 null mice are susceptible to tumor necrosis factor alpha (TNF-α)-induced apoptosis, which is rescued by overexpression of p65 but not p50 (5). Furthermore, inhibition of NF-κB by IκBα overexpression or by the chemical inhibitor pyrrolidine dithiocarbamate (PDTC) rendered many cell types normally resistant to the effects of TNF-α susceptible to TNF-α-induced apoptosis (21, 61, 62). In addition, suppression of NF-κB activation has been shown to enhance apoptosis following radiation or treatment with chemotherapeutic agents (59, 64, 66). Although many investigations have employed exogenous mediators to induce apoptosis following NF-κB inactivation, few have reported the occurrence of apoptosis in response to NF-κB inhibition in the absence of additional stimuli (16, 36, 38, 67).

Unlike monocytes, normal macrophages are long-lived cells resistant to many apoptotic stimuli, including Fas and TNF-α receptor ligation, ionizing radiation, and multiple antineoplastic or cytotoxic agents (32, 33, 49, 52). We recently demonstrated that expression of FLICE-inhibitory protein (Flip) protected differentiated macrophages from Fas-mediated apoptosis (52); however, the mechanisms responsible for macrophage survival have not been fully elucidated. In vitro, both monocytes and macrophages display constitutive activation of NF-κB p50 homodimers; however, p65-p50 heterodimers are present only in differentiated macrophages (13, 22). Hence, the constitutive presence of transcriptionally active p65-p50 heterodimers in macrophages may provide resistance to cell death.

Apoptosis may be initiated through two distinct mechanisms: (i) death receptor (DR) ligation (50, 63) or (ii) direct mitochondrial damage associated with a loss of mitochondrial transmembrane potential (ΔΨm), cytochrome c release, and activation of caspases 9 and 3 (34, 45, 70). DR signaling activates caspase 8 (7, 46), which can directly cleave caspase 3 and, in certain cell types, may also induce ΔΨm loss through activation of Bid (24). Other members of the Bcl-2 family, including the antiapoptotic proteins Bcl-2, Bcl-xL, and A1 and the proapoptotic proteins Bax and Bad, have also been implicated in regulating mitochondrial stability (23). Furthermore, both Bcl-xL and A1 may be regulated by NF-κB (11, 69), suggesting a role for NF-κB in regulating mitochondrial homeostasis.

In the present study, we have demonstrated that the constitutive activation of NF-κB is necessary for the survival of both the murine macrophagelike cell line RAW 264.7 and human monocyte-derived macrophages. Inhibiting NF-κB activation induced apoptosis associated with a loss of ΔΨm and caspase 9 activation. However, activation of caspase 8 was not observed and z-VAD.fmk or neutralizing anti-Fas ligand (FasL) antibody did not prevent ΔΨm collapse or cell death, indicating that apoptosis induced by NF-κB inhibition was not mediated by DR signaling. Moreover, a specific inhibitor of caspase 9 significantly reduced DNA fragmentation but not ΔΨm loss or cell death. These data suggest that while DNA fragmentation induced by NF-κB inhibition was caspase dependent, loss of ΔΨm and cell death were caspase independent. Furthermore, caspase 3 activation was not detected by either immunoblot analysis or cleavage of a DEVD substrate. Analysis of Bcl-2 family molecules revealed that A1 mRNA levels were reduced after 3 h of NF-κB inhibition and prior to ΔΨm loss. Additionally, ectopic expression of A1 provided protection from cell death induced by suppression of NF-κB. Our data demonstrate that blocking the constitutive activation of NF-κB in macro-
phages results in caspase 3-dependent apoptosis mediated by reduced A1 expression and the loss of ΔΨm.

MATERIALS AND METHODS

Materials. RTDC, trypan blue, L-tyrosine, and polyoxymethylene B sulfate were obtained from Sigma Chemical Co. (St. Louis, Mo.). RPMI, Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Opti-MEM, Lipofectamine, actinomycin D, propidium iodide, and streptomycin were obtained from Gibco (Gaithersburg, Md.). Propidium iodide (PI) was purchased from Roche Molecular Biochemicals (Mannheim, West Germany). DNA was digested with HindIII, HpaII, and SpeI and purified with QIAquick spin columns (Qiagen, Valencia, Calif.). Protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, N.J.) was used to purify recombinant fusion proteins. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) was purchased from Sigma Chemical Co. PI and Rh123 (3,3′-dihexyloxacarbocyanine iodide) were purchased from Molecular Probes (Eugene, Oreg.). Anti-FasL antibody (C-20) and rabbit immunoglobulin G (IgG) control were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.) and Jackson Laboratories (West Grove, Pa.), respectively.

Cell isolation and culture. Buffy coats (LeSueur, Glenview, Ill.) were obtained from healthy donors. Mononuclear cells, isolated by Histopaque (Sigma) gradient centrifugation, were separated by countercurrent centrifugal elutriation (JE-6B, Beckman Coulter, Palo Alto, Calif.) in the presence of 10 μg of polymyxin B sulfate/ml, as previously described (52). Isolated monocytes were washed twice with PBS and cultured in RPMI, 10% FBS, and 1% penicillin and streptomycin/ml (20% FBS-RPMI) (32). Seven-day differentiated macrophages that were treated as indicated. Extracts (25 or 30 μg, as noted) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) gels and transferred to Immobilon-P membranes (Millipore, Bedford, Mass.). Membranes were blocked for 1 h at room temperature in PBS–0.2% Tween 20%–5% nonfat dry milk (PBS-Tween milk). The membranes were then incubated overnight at 4°C in PBS-Tween-milk with various antibodies: mouse anti-caspase 8 (generous gift from Dr. C. Ron, Rockefeller University), rabbit anti-deltaFas (Clontech, Palo Alto, Calif.) and anti-FasL (Becton Dickinson, Franklin Lakes, N.J.) expression examined by flow cytometry, and the data are presented as the percentage of cell death (PI binding).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared, as previously described (10), from RAW 264.7 cells or primary macrophages incubated with control medium or medium containing 200 μg/ml of PI for 24 and 26 h. Macrophages infected with various MOI of either AdGFP or AdLacZ for 6 h were also analyzed. An oligonucleotide spanning the κB binding sites of human immunodeficiency virus LTR, previously shown to detect NF-κB binding, was obtained from Invitrogen and used for EMSA. The gel shift was blocked with unlabeled oligonucleotide or competed with 5 μg of a nuclear extract for 20 min at room temperature and electrophoresed on 5% to 6% polyacrylamide gels. The specificity of the signal for supershift assays, 1 to 2 μl of monospecific antibodies to p50 or p65 was added to the nuclear extract for 30 min before the addition of labeled oligonucleotide (10, 68). An unrelated antibody to c-Jun (Santa Cruz) was incubated with 5 μg of test plasmids (1:5 ratio of GFP to total test plasmids) previously described (12), and 1 μg of RNA was reverse transcribed with oligo(dt) primers according to the manufacturer’s specifications (Promega, Madison, Wis.). The PCR was performed with 2 μl of the cDNA samples (Roche Molecular Biochemicals) in a final volume of 50 μl. Amplification was carried out for 35 cycles (30 s of denaturing at 94°C, 45 s of annealing at 50°C, and 90 s of extension at 72°C) in a DNA thermal cycler. As a control, β-actin was also amplified under the same conditions. The A1, Bcl-xL, and Bcl-2 primers employed have been described previously (18, 26, 54). The amplified products were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) gels and transferred to Immobilon-P membranes. Autoradiography of the transferred membranes was carried out with an X-ray film and a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). The bands were visualized with UV light. The % band intensity was measured with NIH software (Image J) and expressed as a percentage of the control samples. Each experiment was repeated at least three times. The data were expressed as the mean ± standard deviation (SD). Statistical analysis were performed by Student’s paired t test. The significance level was set at P = 0.05.

RESULTS

Constitutive NF-κB activity in macrophages is inhibited by PDTC or expression of IκBα. To document the state of NF-κB activation in macrophages, EMSAs were performed on nuclear extracts from the macrophage-like cell line RAW 264.7 and primary human macrophages. Each cell type displayed a constitutive activation of NF-κB, which was diminished by treatment with PDTC (200 μM) (Fig. 1A). Supershift analyses, employing monospecific antibodies to p65 and p50 (data not shown), identified p65-p50 heterodimers and p50 homodimers. The ability of PDTC to inhibit NF-κB transcription was examined by transient transfection of murine macrophage-like RAW 264.7 cells with a luciferase reporter construct containing a promoter of three tandem κB sites (57). TFN-α-induced NF-κB tran-
A. **RAW 264.7**

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**FIG. 1**. Macrophages exhibit constitutive activation of NF-κB which is inhibited by PDTC and AdIκBα. (A) PDTC inhibits constitutive NF-κB activation in macrophages. RAW 264.7 cells and primary human macrophages differentiated for 7 days were treated with 200 μM PDTC for 6 and 24 h, as indicated. The cells were harvested, and nuclear extracts were prepared and analyzed by EMSA as described in Materials and Methods. Unlabeled oligonucleotide (Unlab. oligo) was added (+) to the indicated lanes. The locations of the NF-κB p65/p50 heterodimers and p50/p50 homodimers are designated by arrows. The results are representative of three experiments. (B) PDTC decreases TNF-α-induced NF-κB transcriptional activity as measured by luciferase expression. RAW 264.7 cells transiently transfected with a NF-κB promoter reporter (3X-WT-Luc) were cotransfected into RAW 264.7 cells with a plasmid expressing EGFP (pEFGFP). A 67% ± 10% decrease (P < 0.03) in the number of GFP+ cells was observed at 24 h following transient transfection with pIκBα compared to the control vector or one expressing wild-type NF-κB p65 (Fig. 2B). These data suggest that NF-κB activity is necessary for the survival of RAW 264.7 cells.

**B.**

![Graph showing promoter activity](image)

**C.**

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**FIG. 2**. Inhibition of NF-κB induces cell death of macrophage-like RAW 264.7 cells. (A) PDTC significantly decreases RAW 264.7 cell viability. Cultures were treated with control medium or 200 μM PDTC for 15 h and assessed for viability by trypan blue exclusion. The viability of control cells represents 100%, and by analysis of subdiploid DNA content (Fig. 3B). To determine the effect of specific inhibition of NF-κB, human macrophages were infected with AdIκBα, AdGFP, or a replication-defective adenovirus vector expressing β-galactosidase (Adβgal). Primary macrophages infected with AdIκBα for 72 h displayed a significant decrease in viability which was initially observed at an MOI of 20 and continued in a dose-dependent manner at MOI of 40, 100, and 200 (Fig. 3C).

NF-κB inhibition in primary macrophages induces apoptotic cell death. Since NF-κB inhibition may alter the viability of a proliferating cell line (i.e., RAW 264.7 cells) differently than that of noncycling primary cells, the effect of NF-κB suppression on terminally differentiated human macrophages was investigated. Similar to RAW 264.7 cells, PDTC-treated primary macrophages exhibited a significant increase in cell death, measured by PI incorporation, at 72 h compared to control cells (Fig. 3A). The loss of viability was due to apoptosis as determined by cell death enzyme-linked immunosorbent assay, which measures nucleosome-associated DNA fragments (data not shown), and by analysis of subdiploid DNA content (Fig. 3B).

Since NF-κB inhibition may alter the viability of proliferating cells differently than that of noncycling primary cells, the effect of NF-κB suppression on terminally differentiated human macrophages was investigated. Similar to RAW 264.7 cells, PDTC-treated primary macrophages exhibited a significant increase in cell death, measured by PI incorporation, at 72 h compared to control cells (Fig. 3A). The loss of viability was due to apoptosis as determined by cell death enzyme-linked immunosorbent assay, which measures nucleosome-associated DNA fragments (data not shown), and by analysis of subdiploid DNA content (Fig. 3B).
compared to Adβgal infection. The values are the means ± standard errors of triplicate cultures.

To determine if the collapse of ΔΨm in PDTC-treated macrophages was specifically due to NF-κB inhibition, primary macrophages were infected with AdIkBα and assessed for ΔΨm integrity. AdIkBα-infected macrophages displayed a time-dependent loss of ΔΨm (Rh123 decrease [Fig. 5A]) and subsequent increase in cell death (PI increase [Fig. 5A]) compared to Adβgal-infected cells. Rh123 retention was significantly reduced (P < 0.02) by 12 h in AdIkBα-infected cultures and continued to decrease over time (Fig. 5B). Parallel cultures revealed significant (P < 0.02) DNA fragmentation at 12 h post-AdIkBα infection compared to Adβgal-infected cells (Fig. 5C). Therefore, in contrast to PDTC-treated macrophages, infection of DNA fragmentation and loss of Rh123 retention were observed concurrently in AdIkBα-infected macrophages. The differences between the two methods of inhibiting NF-κB may be due to more effective inhibition of NF-κB by IkBα (Fig. 1A and C). In addition, the antioxidant effects of PDTC may have delayed DNA fragmentation by reducing reactive oxygen species (48), which have been shown to contribute to caspase-induced DNA degradation (29) and to macrophage apoptosis (2, 28). Other than the delay, all characteristics of cell death were comparable with either method of NF-κB inhibition.

To further confirm that inhibiting NF-κB activity in macrophages results in ΔΨm collapse and apoptosis, two additional methods were employed to suppress NF-κB activation. A proteasome inhibitor (MG132) or a peptide that blocks the NF-κB nuclear localization signal (SN50), both previously shown to inhibit constitutively active NF-κB in macrophages induced ΔΨm loss and apoptosis in a time-dependent manner (data not shown). Collectively, these data demonstrate that inhibiting constitutively active NF-κB in macrophages induced ΔΨm collapse and apoptosis.

**NF-κB inhibition induces caspase 9 activation and macrophage apoptosis independent of DR signaling.** Since macrophages express both Fas and FasL on their surfaces (52), the contribution of Fas signaling in NF-κB inactivation-induced apoptosis was investigated. Preincubation with neutralizing anti-FasL antibody did not protect primary macrophages from...
DNA fragmentation (data not shown) or ΔΨₘ collapse (Fig. 6A), suggesting that the mechanism did not involve Fas receptor signaling. Additionally, TNF-α, which can be produced by activated macrophages and has been shown to induce apoptosis in the presence of NF-κB inhibition (1, 5), was not detected in the culture supernatants of untreated or adenovirus-infected macrophages (data not shown).

To further characterize the mechanism of apoptosis induced by NF-κB inhibition, caspase activation was assessed. Seven-day macrophages were incubated with 200 μM PDTC and assayed for caspase 8 and caspase 9 activation at various times. Immunoblot analyses of PDTC-treated macrophages revealed that caspase 8 was not activated, as determined by the stable level of procaspase 8 (Fig. 6B) and a lack of cleaved active caspase 8 (data not shown), suggesting that DR signals had not been initiated. In contrast, active caspase 9 was identified at 12 h of PDTC treatment and was sustained through 72 h (Fig. 6B). Surprisingly, caspase 3 activation was not observed. Procaspase 3 levels were unaltered by the addition of 200 μM PDTC (Fig. 6C) or infection with AdIkBo (data not shown), and cleaved caspase 3 (not shown) was not detected by employing an antibody previously documented by us and others to recognize both the procaspase and cleaved forms of caspase 3 (30, 52). Furthermore, compared to control-treated cells, PDTC-treated primary macrophages did not exhibit increased caspase 3 activity at 24 (Fig. 6D) or 48 h (data not shown), as assessed by cleavage of fluorogenic DEVD-containing peptides. In contrast, treatment with the phosphatidylinositol 3-kinase inhibitor LY294002, previously shown to decrease macrophage viability (35), strongly induced caspase 3 activity (Fig. 6D). Despite a lack of caspase 3 activation, cleavage of cellular proteins indicative of apoptosis, such as PKCδ (Fig. 6E) and PARP (data not shown), was also observed. Collectively, these data indicate that inhibiting NF-κB resulted in caspase 9 activation and PKCδ cleavage, independent of DR signals or activation of caspase 8 or 3.

Caspase 9 inhibitors reduce PDTC-induced macrophage apoptosis. To determine if caspase activation is essential for cell death following NF-κB inactivation, RAW 264.7 cells were cultured with either a general caspase inhibitor (z-VAD.fmk) or a specific inhibitor of caspase 9 (z-LEHD.fmk). Treatment with 100 μg of the caspase 9 inhibitor or z-VAD.fmk/ml did not prevent PDTC-induced mitochondrial dysfunction (Fig. 7A), indicating that loss of ΔΨₘ occurred independently of caspase inhibition. These data further support the observation that DR signals and caspase 8 activation were not responsible for initiating PDTC-induced ΔΨₘ loss and apoptosis (Fig. 6A and B). In contrast, DNA fragmentation was significantly decreased by the addition of either the caspase 9 inhibitor (P < 0.004) or z-VAD.fmk (P < 0.02) (Fig. 7B). Furthermore, PDTC-treated RAW 264.7 cells displayed a loss of viability, which was not rescued by the addition of these caspase inhibitors (Fig. 7C). These data demonstrate that although the caspase 9 inhibitor and z-VAD.fmk provided protection from DNA fragmentation, they did not prevent ΔΨₘ collapse or cell death. Collectively, these data indicate that DNA fragmenta-
tion induced by NF-κB inhibition is caspase dependent while mitochondrial dysfunction and subsequent cell death are independent of caspase activation.

Expression of A1 protects macrophages from apoptosis induced by NF-κB inhibition. To elucidate the events leading to mitochondrial dysfunction in NF-κB-inactivated macrophages, the expression of Bcl-2 family members was assessed. Neither Bcl-2 nor Bcl-xL was decreased following the inhibition of NF-κB, as determined by RT-PCR analysis (Fig. 8A) or Western blotting (data not shown). In contrast, A1 mRNA was dramatically reduced in PDTC-treated (Fig. 8A) or AdIκBα-infected (data not shown) macrophages compared to control macrophages or neutrophils (51). RNase protection assays confirmed that A1 expression was diminished following NF-κB inhibition (data not shown). Furthermore, the expression of the proapoptotic proteins Bad and Bax was not increased in PDTC-treated macrophages (data not shown). These data suggest that a reduction in A1 expression may be responsible for inducing mitochondrial dysfunction following NF-κB inhibition.

If macrophage apoptosis induced by the inhibition of NF-κB is dependent on a decrease in A1, restoring A1 expression may be protective. An expression plasmid encoding A1 (pA1 [69]) was cotransfected with pEGFP into RAW 264.7 cells, which were then treated with PDTC for 24 h. Compared to those transfected with control vector, cells expressing A1 were significantly (P < 0.001) protected against PDTC-induced cell death (Fig. 8B). No difference was observed between the untreated control-transfected cells and PDTC-treated cells transfected with A1. These data indicate that A1 provided protection against cell death induced by NF-κB inhibition.

DISCUSSION

The constitutive activation of NF-κB is essential for macrophage viability. The requirement for NF-κB activation in the present study was not due to the fact that the primary human macrophages were terminally differentiated, since inhibition of constitutively activated NF-κB in proliferating macrophagelike RAW 264.7 cells also induced apoptosis. Previous investiga-
tions have focused on the effect of NF-κB inhibition in response to apoptotic stimuli, such as DR ligation, radiation, or chemotherapeutic compounds, in a variety of cell types, including macrophages (5, 59, 64, 66). In contrast, our data are novel in that NF-κB inhibition in the absence of additional apoptotic stimuli resulted in macrophage apoptosis, demonstrating that constitutive NF-κB activation is essential for macrophage survival.

The constitutive activation of NF-κB is not essential for the survival of all cell types. In contrast to macrophages, fibroblasts, endothelial cells, and epithelial cells did not undergo apoptosis following NF-κB inhibition by PDTC or IκBα (data not shown and references 31, 60, and 66). However, similar to macrophages, other cells of the immune system, including both B and T lymphocytes, exhibited constitutive NF-κB activation (36, 47) and underwent apoptosis following NF-κB inhibition (3, 36, 67), although the responsible mechanisms have not been well characterized.

Our data provide novel insights into the mechanism by which the constitutive activation of NF-κB protects macrophages from cell death. Here, we show that macrophage apoptosis induced by NF-κB inhibition was mediated by a loss of ΔΨm, activation of caspase 9, and cleavage of cellular proteins and DNA. DR ligation was not responsible for apoptosis, as caspase 8 was not activated and z-VAD.fmk did not protect against ΔΨm loss. Additionally, TNF-α was not detected in the culture supernatants (data not shown), and interruption of Fas-FasL interactions did not protect against macrophage apoptosis following NF-κB inhibition. This contrasts with previous studies, in which the expression of IκBα sensitized cells to ΔΨm collapse induced by TNF-α and mediated by caspase 8 activation (9, 65). Thus, our data document a direct role for constitutively activated NF-κB in maintaining macrophage viability by regulating mitochondrial homeostasis.

Unexpectedly, macrophage apoptosis induced by suppressing constitutively activated NF-κB occurred through a caspase
3-independent pathway. Although caspase 9 activation was documented following the initial loss of ΔΨm and the caspase 9 inhibitor effectively reduced DNA fragmentation, caspase 3 activation was not detected by either Western blot analysis or functional activity, regardless of the method employed to inhibit NF-κB. These are the first data to document a caspase 3-independent apoptotic pathway in primary macrophages. Similar to these results, a recent study demonstrated that inhibition of constitutive NF-κB activity in normal, human T lymphocytes resulted in apoptosis without activation of caspase

FIG. 7. Caspase inhibition reduces PDTC-induced DNA fragmentation but does not prevent ΔΨm collapse or cell death. RAW 264.7 cells were incubated, as indicated, with 100 μg of either a general caspase inhibitor (z-VAD.fmk) or a caspase 9 inhibitor (z-LEHD.fmk)/ml and 200 μM PDTC for 24 h. Analyses of mitochondrial dysfunction, DNA fragmentation, and cell viability were performed on each sample. (A) PDTC-induced loss of ΔΨm, determined by decreased Rh123 fluorescence, was not prevented by the presence of either caspase inhibitor. Cultures were incubated with Rh123 (0.4 μg/ml) for 30 min, harvested, and analyzed for Rh123 fluorescence by flow cytometry. Vehicle control cultures were designated 100% fluorescence. (B) Caspase inhibition reduces PDTC-induced DNA fragmentation. Following Rh123 analysis, cells were fixed in 70% ethanol and assessed for subdiploid (<2N) DNA content as described in Materials and Methods. *P < 0.02 compared to PDTC alone. (C) PDTC-induced cell death, as determined by PI (3 μg/ml) incorporation, was not prevented by caspase inhibition. All values represent the mean ± standard error of triplicate cultures. The results are representative of two independent experiments.

FIG. 8. A1 protects macrophages from apoptosis induced by NF-κB inhibition. (A) A1, but not Bcl-2 or Bcl-xL, mRNA levels were dramatically reduced following NF-κB inhibition. RT-PCR analysis was performed on 7-day macrophages treated with 200 μM PDTC for 0, 3, 6, or 12 h. Granulocyte RNA was employed as a positive control for A1 expression. As a control for quantification, β-actin was also amplified. (B) Transient expression of A1 prevented PDTC-induced RAW 264.7 cell death. The cells were cotransfected with pEGFP and either pA1 or control vector as described in Materials and Methods. Twenty-four hours after transfection, the indicated cultures were treated with 200 μM PDTC (+PDTC) for an additional 24 h. Viability was determined by the number of GFP+ cells. *P < 0.002 compared to control vector with PDTC. The data are presented as the mean ± standard error of triplicate cultures and are representative of three independent experiments.
3 (36). However, the mechanism responsible for apoptosis and the effect of caspase inhibitors on cell viability were not reported (36). It is possible that access to procaspase 3 was impeded (44) or an inhibitor of caspase 3 was present (14) under the conditions utilized, resulting in a lack of caspase 3 cleavage by activated caspase 9. Perhaps cleaved, proteolytically active PKCδ (20) or another caspase, such as caspase 7 (19), was responsible for DNA fragmentation following NF-κB suppression. The absence of caspase 3 activation by cleaved caspase 9 may be unique to NF-κB inhibition, because macrophage cell death induced by the phosphatidylinositol 3-kinase inhibitor LY294002 was associated with both mitochondrial dysfunction (data not shown) and caspase 3 activation (Fig. 6D). Likewise, we have observed caspase 3 activation in monocytic undergoing spontaneous apoptosis mediated by Fas-FasL interactions (52). Our data indicate that macrophage apoptosis induced by the inhibition of constitutive NF-κB activation was initiated by loss of ΔΨm and employed a caspase 3-independent pathway for DNA degradation.

The mechanism by which inhibition of NF-κB in macrophages initiates ΔΨm collapse was also examined. NF-κB inactivation resulted in the marked reduction of A1 expression prior to ΔΨm collapse, even though the expression of other Bcl-2 family members was unchanged (Fig. 8A). Previous investigations have documented induced A1 expression in macrophages following stimulation (51); however, our findings are novel, since they demonstrate an exquisite sensitivity of A1 regulation to constitutive NF-κB activation. Additionally, prior studies have reported that A1 may protect against apoptosis in the presence of NF-κB suppression (15, 37, 65, 69). In contrast to our data, however, apoptosis was observed only in response to exogenous death-inducing stimuli, such as TNF-α and anti-Fas antibody (15, 37, 65, 69). Supporting the importance of our observations, the ectopic expression of A1 protected against macrophage apoptosis induced by NF-κB inhibition. Additionally, A1 expression has been shown to contribute to myeloid differentiation (41), which may be due to protection against apoptosis, consistent with our observations in macrophages. In contrast to our results for A1, Bcl-2, another Bcl-2 family molecule regulated by NF-κB (11), was not reduced when constitutive NF-κB activation was blocked. Although the inhibitor of apoptosis proteins (IAPs) may also be regulated by NF-κB (65), IAPs arrest apoptosis by preventing the activation of caspases 9 and 3 (14) and therefore are unlikely to affect apoptosis initiated by a caspase-independent ΔΨm collapse (Fig. 7A). Our data provide important insight into the role of A1, suggesting that it may be an indispensable mitochondrial homeostatic molecule and mediator of the antiapoptotic function of constitutively activated NF-κB in macrophages.

Although the role of NF-κB in macrophage apoptosis has been previously investigated, the results differed from those presented here. Macrophages generated in vitro from hema-topoietic precursors of embryonic-lethal p65 knockout (p65−/−) mice were not reported to undergo spontaneous apoptosis but were sensitive to TNF-α-induced cell death (5). The precursors employed in this study were treated with macrophage growth factors that may have activated other transcription factors, including other NF-κB subunits. In hematopoietic lineages, c-Rel and p65 may serve redundant functions (25), suggesting that c-Rel activity may have compensated for the loss of p65. However, the ectopic expression of the IκBα employed in the present study, which avidly binds any NF-κB complex containing p65 or c-Rel (4), effectively inhibited all species of NF-κB detected (Fig. 1C). Another investigation utilizing a degradable form of IκBα did not observe macrophage apoptosis, even in the presence of TNF-α (17). Potential explanations for this discrepancy include the culturing of peripheral blood-derived monocytes in the presence of macrophage colony-stimulating factor, which may have induced other factors that protected against apoptosis (8), and the use of a degradable form of IκBα, which, when unbound, is unstable and rapidly degraded (39, 58). In contrast, our study employed monocytes differentiated in serum alone and a nondegradable, mutant IκBα that may have been more effective at preventing NF-κB activation. Our results were validated by utilizing four different methods to suppress NF-κB activation, all of which resulted in ΔΨm collapse and apoptosis.

In summary, we have shown that the constitutive activation of NF-κB is necessary for macrophage survival and that inhibition of NF-κB activity resulted in macrophage apoptosis initiated by a decrease in A1 expression and loss of ΔΨm, independent of DR ligation. The persistence of macrophages has been implicated in the pathogenesis of diseases such as rheumatoid arthritis and atherosclerosis. Consistent with our data, nuclear, activated NF-κB was identified in vivo in rheumatoid arthritis synovial tissue macrophages (27), indicating that activated NF-κB may prevent macrophage apoptosis. The data presented here provide important insights into the mechanism of macrophage survival and suggest a potential novel therapeutic approach through the inhibition of NF-κB or A1.

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