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Environmental Chemical-Induced Bone Marrow B Cell Apoptosis: Death Receptor-Independent Activation of a Caspase-3 to Caspase-8 Pathway

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Abbreviations: DISC: death inducing signaling complex · **DMBA:** 7,12-dimethylbenz[*a*]anthracene · **FADD:** fas-associated death domain · **FBS:** fetal bovine serum · **PAH:** polycyclic aromatic hydrocarbon

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Abstract

Programmed cell death is a critical process in B lymphocyte development. Premature apoptosis in developing B cells could affect the repertoire and number of mature B cells produced. Of particular concern is the ability of environmentally ubiquitous polycyclic aromatic hydrocarbons (PAH) to induce B cell apoptosis within the bone marrow microenvironment in a clonally non-specific way. Here, models of bone marrow B cell development were employed to assess the role of the "extrinsic" apoptosis pathway in PAH-induced apoptosis and to compare PAH-induced apoptosis with that induced during clonal deletion. As previously demonstrated with a non-transformed pro/pre-B cell line, primary pro-B cells cultured on bone marrow stromal cells underwent apoptosis following exposure to a prototypic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA). Apoptosis was preceded by cleavage of caspase-3 (4-6 h) and caspase-8 (6-8 h) and their respective substrates, α-fodrin and Bid. Inhibition of caspase-3 blocked caspase-8 activation and apoptosis. Furthermore, a pan-caspase inhibitor blocked apoptosis and activation of both caspases-3 and -8. Cells from mice defective in TNF-α, TNF-β, LT-β or TNFR1, TNFR2, Fas, or DR6 were as susceptible to apoptosis signaling as wildtype cells. These results suggest a complex death receptor-independent B cell apoptosis pathway in which caspase-8 is activated downstream of caspase-3.

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Introduction

Apoptosis is a critical event in the deletion of autoimmune B lymphocytes as they enter the periphery from the bone marrow (Defrance et al., 2002). Some of the signaling pathway leading to immature B cell death and clonal deletion has been mapped in model systems in which transformed cells (e.g. WEHI-231) (Andjelic and Liou, 1998; Doi et al., 1999; Ruiz-Vela et al., 1999; Wu et al., 1996a; Wu et al., 1998) or immature splenic B cells (Andjelic and Liou, 1998; Tian et al., 2001) were induced to undergo apoptosis following immunoglobulin cross-linking. In these systems, contributions of NF-κB and c-Myc down-regulation (Wu et al., 1996a; Wu et al., 1996b), p53, $p27^{Kip1}$ and $p21^{WAF1}$ up-regulation (Wu et al., 1998), mitochondrial activation (Doi et al., 1999) and protease (calpain, cathepsin and caspase) activation (Ruiz-Vela et al., 1999) have begun to be defined. Previously, our laboratory investigated whether B lymphocytes earlier in development are similarly susceptible to apoptosis (Mann et al., 1999; Mann et al., 2001; Ryu et al., 2003; Yamaguchi et al., 1997a). Since pro- and pre-B cells do not express surface Ig, prototypic polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene (B[*a*]P) or dimethylbenz[*a*]anthracene (DMBA) were used to induce apoptosis in these early B cells. Studies with PAH are particularly relevant since these ubiquitous environmental pollutants are profoundly immunosuppressive (Dean et al., 1986; Thurmond et al., 1987) and much of their immunotoxicity is directed toward B cells (Hardin et al., 1992; Page et al., 2003).

Using B cell/bone marrow stromal cell co-culture systems containing either primary pre-B cells in Whitlock/Witte cultures or a non-transformed, stromal cell-dependent primary $CD43^+$ pro/pre-B cell line (BU-11), it was shown that relatively low DMBA doses ($\geq$10 nM) rapidly induce pre- or pro/pre-B cell apoptosis (Mann et al., 1999; Mann et al., 2001; Ryu et al., 2003; Yamaguchi et al., 1997a). Like the clonal deletion pathway, down-regulation of NF-κB and c-

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Myc and upregulation of p53 contribute to PAH-induced pro/pre-B cell death (Mann et al., 2001; Ryu et al., 2003). However, unlike clonal deletion, upregulation of $p27^{kip1}$ and $p21^{waf1}$ plays no role in PAH-induced apoptosis (Ryu et al., 2003).

Caspase activation is a hallmark of apoptosis in many cell types including immature B lymphocytes undergoing clonal deletion (Ruiz-Vela et al., 1999). Caspases are grouped by phylogenetic analysis into three major classes, inflammatory (caspases-1, -4, -5, -11, -12), initiator (caspases-2, -8/10, and -9), and effector caspases (caspases-3, -6, -7). In many cases, caspase cascades can be assigned to one of two non-mutually exclusive pathways based on the initiator caspase activated and the contribution of death receptors in caspase activation (reviewed in (Nicholson, 1999)). The "extrinsic pathway" is frequently induced by ligation of TNFR family death receptors and requires early activation of caspase-8, the most proximal caspase in this pathway (Medema et al., 1997). Downstream targets of caspase-8 include pro-caspase-3 and Bid, the truncated form of which (tBid) translocates to and induces cytochrome c release from mitochondria (Gross et al., 1999). TNFR family members also may be activated independently of ligands resulting in caspase-8 activation (Aragane et al., 1998; Chen and Lai, 2001; Micheau et al., 1999).

The "intrinsic pathway" is thought to be induced by stress (e.g. cytotoxic agents, irradiation) rather than by specific extrinsic cytokines. This pathway involves caspase-8-independent mitochondrial membrane potential depolarization ($\Delta\Psi_m$) and/or permeabilization (reviewed in (Jiang and Wang, 2004)) and the formation of an "apoptosome," a death complex composed of cytochrome c, Apaf-1, and caspase-9. The apoptosome targets effector caspases-3 and -7. Importantly, caspase-6 may be activated by caspase-3 which in turn activates caspase-8

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in an apoptosis amplification loop (Belka et al., 2000; Cowling and Downward, 2002; Murphy et al., 2004; Slee et al., 1999; Wieder et al., 2001).

Given these models of caspase signaling, it was postulated that determination of a role for caspase-8 in apoptosis and the signal through which it may be activated (i.e. death receptors and/or caspase-3) would provide insight into whether and at what developmental stage developing B cells are mature enough to have functional “extrinsic” or “intrinsic” apoptosis pathways. Additionally, these studies could determine if the apoptotic pathway initiated during clonal deletion is activated inappropriately by environmental chemicals. Consequently, studies were designed to determine a putative role for TNFR family death receptors, caspase-8, and caspase-3 in PAH-induced bone marrow stromal cell-dependent B cell apoptosis, using a pro/pre-B cell line and primary pro-B cells.

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Materials and methods

Cell Culture

Stromal cell-dependent, $CD43^+$ (pro/pre-B) BU-11 cells expressing rearranged cytoplasmic Ig heavy chains (Mann et al., 1999; Yamaguchi et al., 1997a) were co-cultured on cloned BMS2 bone marrow-derived stromal cells (kindly provided by Dr. P. Kincade, Oklahoma Medical Research Foundation) in 50% RPMI and 50% DMEM (Mediatech, Washington, DC) containing 5% fetal bovine serum (FBS)(Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 0.01 mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 0.5 μg/ml of the anti-mycoplasma reagent plasmocin (Invivogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 incubator.

Primary bone marrow pro-B cell cultures were prepared from wildtype B6.129SF2/J and age-matched B6.129S6-Tnf^{tm1Gk1}/J (TNF-$\alpha^{-/-}$) or B6.129S-$Tnfrsf1a^{tm1Imx}$/$Tnfrsf1b^{tm1Imx}$/J ($TNFR1^{-/-}$/$TNFR2^{-/-}$) mice (Jackson Laboratories, Bar Harbor, ME), wildtype Balb/c and age-matched Balb/c-*lpr* mice (the generous gifts of Dr. A. Marshak-Rothstein, Boston University School of Medicine), B6.129-$DR6^{-/-}$ mice and their wildtype littermates (Schmidt et al., 2003), or C57BL/6 mice essentially as described (Tze et al., 2000). Bone marrow was flushed from the femurs of 4-6 week-old male mice. Red blood cells were lysed by incubation in 0.17 M NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA at 37ºC for 5 m. The remaining cells were cultured for 5-7 days in RPMI containing 10% FBS, penicillin/streptomycin (Mediatech), L-glutamine, 2-mercaptoethanol, and 16 ng/ml murine rIL-7 (RDI, Flanders, NJ). For isolation of stromal cells, murine rIL-7 was not included in the media. B cells were stained with FITC-conjugated B220-specific (Clone: RA3-6B2, Pharmingen, San Diego, CA) and PE-conjugated CD43-specific (Clone: S7, Pharmingen) antibodies or with FITC-conjugated rat IgG_{2a} and PE-conjugated rat IgG_{2a} (Clone: R35-95, Pharmingen) as controls, fixed in 1.5% paraformaldehyde, and analyzed

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on a Becton/Dickinson FACScan flow cytometer. At least 95% of the cells express CD43 and B220.

Experimental Treatment

BMS2 cells or primary bone marrow stromal cells were cultured for 24 h in 24-well plates or T75 flasks in DMEM containing 5% FBS to form a monolayer which was approximately 75% confluent. BU-11 cells or primary pro-B cells were added in RPMI containing 5% FBS and allowed to associate with the stromal cells for 24 h. Stromal cell monolayers or B cell/stromal cell co-cultures were treated in duplicate wells or flasks with vehicle (0.1% acetone) or DMBA (1 μM)(Sigma) for 2-24 h. DMSO (0.1%), the pan caspase inhibitor VAD-FMK, the caspase-3 inhibitor DEVD-FMK, or a control peptide FA-FMK (15-30 μM)(Calbiochem, San Diego, CA) was added to co-cultures 30 m prior to acetone (vehicle) or DMBA treatment.

Apoptosis Assays

BU-11 cells and primary pro-B cells were harvested and washed once with cold PBS containing 5% FBS and 0.01 M sodium azide (Sigma). For propidium iodide staining, cells were resuspended in 0.15 ml hypotonic buffer containing 50 μg/ml propidium iodide (Sigma), 0.1% sodium citrate and 0.1% Triton X-100 and analyzed by flow cytometry. Cells undergoing DNA fragmentation (i.e. apoptosis) were shown to have a lower propidium iodide fluorescence than those in the typical G_0/G_1 stages of cell cycle (Mann et al., 1999; Yamaguchi et al., 1997a). For Annexin V staining, cells were resuspended in 0.2 ml Annexin V binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$. 2.5 μl of Annexin V-PE (556422, Pharmingen) were added. Cells were incubated for 15 m in the dark at room temperature and analyzed by flow cytometry within an hour. Annexin V and propidium iodide staining yielded equivalent results. Data from duplicates were averaged and used as a single representation of the

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percentage of apoptotic cells for any given treatment. Experiments were performed with a minimum of three mice.

Immunoblotting

BU-11 cells or primary pro-B cells were harvested and washed once in cold PBS. Cells were resuspended in lysis buffer containing 50 mM Pipes/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, and Protease Inhibitor Cocktail for Mammalian Cells (1:200 dilution; Sigma) and incubated on ice for 15 m. The extracts were cleared by centrifugation at 14,000 rpm for 10 m at 4^{o}C. Supernatants were collected, aliquoted, and frozen at -80^{o}C until use. Protein concentrations were determined using the Bradford assay.

Total proteins (50-80 μg) were resolved on 6% (α-fodrin) or 15% gels, transferred to a 0.2 μm nitrocellulose membrane, and incubated with primary antibody. Primary antibodies included monoclonal mouse anti-α-fodrin (MAB1622, Chemicon International, Temecula, CA), polyclonal rat anti-Bid (MAB860, R&D Systems. Miineapolis, MN), polyclonal rabbit anti-cleaved caspase-3 (9661, Cell Signaling Technology, Beverly, MA), or polyclonal rat anti-caspase-8 (ALX-804-447, Axxora, San Diego, CA). Immunoreactive bands were detected using HRP-conjugated secondary antibodies (Biorad, Hercules, CA) followed by ECL. To control for equal protein loading, blots were stripped and re-probed with a β-actin-specific antibody (A5441, Sigma) or α-tubulin-specific antibody (CP06, EMD Biosciences, San Diego, CA) and analyzed as above.

Caspase Activity Assays

Bone marrow B cells were harvested and washed once in cold PBS. Cytosolic proteins were prepared according to the manufacturer's instructions (Apoalert, Clontech, Palo Alto, CA). Briefly, BU-11 cells were resuspended in 50 μl of chilled Cell Lysis Buffer, incubated on ice for

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10 m, and centrifuged at 14,000 rpm for 10 m at 4°C. Supernatants were collected and caspase activity determined immediately. Protein concentrations were determined using the Bradford assay. Cytosolic proteins (50 μg) were incubated with Reaction Buffer containing 10 mM DTT and chromophore p-nitroaniline-conjugated DEVD or IETD substrate (final concentration: 200 μM) at 37° C for 2 h. p-NA standard solution was diluted to a final concentration of 0–200 μM with Cell Lysis Buffer to generate a standard curve. The concentration (μM) of free p-nitroaniline released from caspase substrate was measured at 405 nm in a Bio-Tek Instruments microplate reader.

Analysis of TNFR ligand expression

For RNA analysis, stromal cells were trypsinized, washed once in complete medium and once in cold PBS. Immature dendritic cells were produced by culture of bone marrow cells with rGM-CSF and rIL-4 for 7 days. These cells were treated with LPS (1μg/ml) for 6 h as a positive control for *TNF-α*, *TNF-β*, and *LT-β* mRNA expression. Total RNA was isolated (RNAzol, TEL-TEST, Friendswood, TX), and 5 μg were reverse transcribed (Superscript First Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA). The cDNA was subjected to PCR amplification with *TNF-α*, *TNF*-β, *LT-β* (36 cycles) and *β-actin*-specific (26 cycles) primers. The primer sequences were as follows (Reddy et al., 2001):

TNF-α	sense	ATGAGCACAGAAAGCATGATCCGCGAC	(700bp)
	antisense	TCACAGAGCAATGACTCCAAAGTAGACCTG	
TNF-β	sense	CCCATGGCATCCTGAAAC	(485bp)
	antisense	GGAGGCCTGGAATCCAAT	
LT-β	sense	TCGGGTTGAGAAGATCATTGG	(640bp)
	antisense	GCTCGTGTACCATAACGACC	

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β-actin	sense	GTCGTCGACAACGGCTCCGGCATGTG	(256bp)
	antisense	CATTGTAGAAGGTGTGGTGCCAGATC	

For analysis of membrane-bound TNF-α, stromal cells were trypsinized for 5 m, washed once in complete medium and once in cold PBS. RAW 264.7 cells that were treated with LPS (1 μg/ml) for 4 h were included as a positive control. Cells were stained with anti-TNF-α-PE (Clone: MP6-XT22, Pharmingen) or PE-conjugated rat IgG_1 (Clone: R3-34, Pharmingen), fixed in 1.5% paraformaldehyde and analyzed by flow cytometry. For analysis of secreted TNF-α, cell-free supernatants were collected, and TNF-α production was determined by ELISA (Pharmingen).

Statistics

Statistical analyses were performed with Statview (SAS Institute, Cary, NC). At least three experiments were performed in each BU-11 cell protocol. Experiments with pro-B cells were performed with a minimum of three mice and cells from each mouse were maintained separately. Each treatment within an experiment using either BU-11 cells or primary pro-B cells was performed in duplicate wells and each well was assayed independently. Results from duplicate wells within each experiment were averaged prior to statistical analysis. Data from a minimum of three experiments were averaged and are presented as means ± standard errors (SE). The Student's T-Test and one-factor ANOVAs were used to analyze the data. For ANOVAs, the Dunnett's or Scheffe's multiple comparisons tests were used to determine significant differences.

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Results

DMBA rapidly induces apoptosis in primary pro-B lymphocytes

Previous studies demonstrated that a non-transformed pro/pre-B cell line (BU-11), or primary pre-B cells, co-cultured with bone marrow stromal cells, undergo apoptosis when the cultures are exposed to DMBA (Mann et al., 1999; Mann et al., 2001; Ryu et al., 2003; Yamaguchi et al., 1997a). To determine if earlier primary B cells, i.e. those at the pro-B cell stage, similarly express an intact apoptosis signaling pathway, bone marrow-derived $B220^{+}/CD43^{+}$ B cell populations were expanded in rIL-7. Culture of bone marrow cells with rIL-7 for at least 5 days resulted in a highly enriched pro-B cell population, >95% of which expressed B220 and CD43 (Fig. 1A). These B cells loosely adhered to and, in some cultures, grew under the stromal cell monolayer. Cultures of either primary pro-B or BU-11 cells on bone marrow stromal cell (BMS2) monolayers were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2-18 h. This dose of DMBA was chosen because it induces significant apoptosis that is completely AhR-dependent (Mann et al., 1999). Apoptosis was quantified by propidium iodide staining and flow cytometry.

Primary pro-B cells (Fig. 1B) and BU-11 cells (Fig. 1C) generally exhibited a relatively low level of background apoptosis (<5%). Treatment with DMBA for 12 h consistently induced apoptosis in a significant fraction of both bone marrow B cell types (Fig. 1, B and C). Time-course experiments indicated a trend towards increased apoptosis 6-8 h after DMBA treatment of BU-11 cultures that reached statistical significance 10 h after treatment (Fig. 1D). Similarly, significant apoptosis was induced in primary pro-B cells within 10 h of treatment (Vh: 4.4 ± 1.3%, DMBA: 16.5 ± 2.5%, $p<0.01$, Student's T test). These results demonstrate that bone marrow B cells become responsive to DMBA-dependent death signals at an early stage of

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development, i.e. at the pro-B cell stage. Furthermore, they support the use of primary pro-B cells from mice deficient in apoptosis signaling components to map out the PAH-induced apoptosis signaling pathway.

DMBA activates caspase-3 in developing B lymphocytes

Caspase-3 is considered to be the primary apoptosis executioner with the broadest substrate repertoire of the effector caspases (Slee et al., 2001). Among the substrates for caspase-3 are caspases-2 and -6, which may participate in an amplification loop leading to the activation of what is otherwise considered to be an initiator caspase, i.e. caspase-8 (Cowling and Downward, 2002; Slee et al., 1999). To determine the role of caspase-3 in DMBA-induced pro/pre-B cell apoptosis, BU-11/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2-18 h. B cells were analyzed for caspase-3 activation by immunblotting for cleaved caspase-3, by a colorimetric assay for cleavage of the caspase-3 peptide substrate DEVD, and by immunoblotting for endogenous cleaved α-fodrin, a specific caspase-3 substrate.

The appearance of the active 17 kDa caspase-3 fragment was detected in BU-11 cells 4-6 h after DMBA treatment (Fig. 2A). In the colorimetric assay, caspase-3-like activity increased 4-6 h after DMBA treatment and reached statistical significance 8 h after treatment (Fig. 2B). As expected from these results, endogenous cleavage of α-fodrin, a caspase-3 substrate, was observed after DMBA treatment (Fig. 2C, left panel). Similar data were obtained with primary pro-B cells (e.g. Fig. 2C, right panel). α-Fodrin cleavage was chosen as a marker for caspase-3 activity since its cleavage is mediated solely by caspase-3. While PARP cleavage occurs in both BU-11 cells and primary pro-B cells following DMBA treatment (data not shown), this cleavage may occur as a results of either caspase-3 or caspase-7 activation (Slee et al., 2001).

If caspase-3 activity plays a causal role in DMBA-induced B cell death, it would be

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predicted that a caspase-3 inhibitor, DEVD-FMK, would block apoptosis. To test this prediction, BU-11/BMS2 or primary pro-B/BMS2 cell co-cultures were treated with vehicle (0.1% DMSO) or DEVD-FMK (30 μM) for 30 m prior to treatment with acetone (0.1%) or DMBA (1 μM). BU-11 cells were harvested 24 h later and analyzed for apoptosis by flow cytometry.

DEVD-FMK reduced the level of DMBA-induced BU-11 cell death by 80% (Fig. 3A). Similarly, DEVD-FMK suppressed DMBA-induced apoptosis 67% in primary pro-B cells (Fig. 3B). Interestingly, DEVD-FMK also appeared to suppress the spontaneous apoptosis seen in the bone marrow B cell cultures (Fig. 3, A and B). FA-FMK, a peptide frequently used as a negative control but which can suppress cathepsin B and caspases-2 and -9 at higher doses (Lopez-Hernandez et al., 2003), had no effect on DMBA-induced apoptosis in BU-11 cells (data not shown). FA-FMK was significantly toxic to the primary pro-B cells and therefore could not be used as a control with these cells. These data are consistent with a role for caspase-3 in early bone marrow B cell apoptosis induced with DMBA.

Caspase-8 is activated during DMBA-induced apoptosis

Typically, the apoptotic process is activated by initiator caspases such as caspase-8. However, caspase-8 also can be activated by a caspase-3-dependent mechanism (Cowling and Downward, 2002; Slee et al., 1999). To determine if caspase-8 is involved in PAH-induced apoptosis in bone marrow B cells, BU-11/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 2-18 h. Caspase-8 activation in the B cells then was determined by immunoblotting for cleaved caspase-8, by a colorimetric assay for cleavage of the caspase-8 peptide substrate IETD, and by immunoblotting for truncated Bid, an endogenous caspase-8 substrate.

An increase in the formation of 40 kDa cleaved caspase-8 fragments was evident 6-8 h

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after DMBA treatment (Fig. 4A). In the colorimetric assay, an increase in caspase-8-like activity began 6-8 h after DMBA treatment, reached statistical significance after 10 h, and continued to increase through 20 h after treatment with DMBA (Fig. 4B). Furthermore, cleavage of Bid was evident in both BU-11 cells and primary pro-B cells 10 h after DMBA treatment (Fig. 4C). These data indicate that caspase-8 is activated after DMBA exposure.

Caspase-8 activation in bone marrow B cells is not mediated by TNF-α, TNF-β, LT-β, TNF receptors (TNFR1, TNFR2), Fas, or death receptor 6 (DR6).

The caspase-8-dependent "extrinsic" apoptosis pathway most commonly is activated by TNF family members through TNFR-like death receptors (Medema et al., 1997). BU-11 cells have a functional extrinsic apoptotic response as they undergo apoptosis when exposed to FasL (data not shown). To address the possible role of death receptors and their ligands in caspase-8 activation in specific and in DMBA-induced apoptosis in bone marrow B cells in general, the contributions of TNF-α, TNF-β, and lymphotoxin-β (LT-β) from stromal cells and of TNFR1, TNFR2, Fas, and DR6 on bone marrow B cells to PAH-induced apoptosis were investigated.

BMS2 cells were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 1-16 h, and steady-state levels of *TNF-α*, *TNF-β*, and *LT-β* mRNA were determined by RT-PCR. While significant levels of *TNF-α*, *TNF-β*, and *LT-β* mRNA were readily detected in LPS-activated primary murine dendritic cells, no signal was observed in vehicle or DMBA-treated BMS2 stromal cells (Fig. 5A). As would be expected from these results, TNF-α was detected on the surface of LPS-activated RAW 264.7 cells but not on BMS2 or primary bone marrow stromal cells (Fig. 5B). Similarly, DMBA did not induce TNF-α secretion, as measured by ELISA, in either BMS2 (Fig. 5C) or primary bone marrow stromal cells (data not shown). Furthermore, when BU-11 cells were co-cultured with primary bone marrow stromal cells isolated from

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wildtype or TNF-$\alpha^{-/-}$ mice and treated with DMBA (1 μM) for 24 h, there were no significant differences in the ability of primary bone marrow stromal cells from wildtype or TNF-$\alpha^{-/-}$ mice to contribute to BU-11 cell apoptosis (Fig. 5D). Finally, a potential TNFR ligand autocrine feedback loop described in other systems (Herr et al., 2000; Kasibhatla et al., 1998), appeared not to be involved in DMBA-induced primary pro-B cell apoptosis since pro-B cells from TNF-$\alpha^{-/-}$ mice were as sensitive to DMBA-dependent death signals as pro-B cells from wildtype controls (data not shown).

These results support the conclusion that these TNF family ligands do not play a role in DMBA-induced pro- or pro/pre-B cell death. However, caspase-8 activation also may occur in the absence of an exogenous death receptor ligand through FADD-dependent aggregation of TNFR family members followed by autocatalysis of caspase-8 (Aragane et al., 1998; Chen and Lai, 2001; Micheau et al., 1999). To determine the likelihood that such a mechanism contributes to apoptosis in the current system, primary pro-B cells from wildtype, TNFR1$^{-/-}$/TNFR2$^{-/-}$, or Balb/c-*lpr* mice were co-cultured with BMS2 stromal cells and treated with vehicle (0.1% acetone) or DMBA (1 μM) for 24 h. B cells were stained with propidium iodide, and apoptosis was quantified by flow cytometry.

DMBA induced a significant amount of apoptosis in age-matched wildtype B6.129SF2/J and TNFR1$^{-/-}$/R2$^{-/-}$ primary pro-B cells with no significant differences between the wildtype and TNFR$^{-/-}$/R2$^{-/-}$ primary pro-B cells (Fig. 6A). Similarly, DMBA induced significant levels of apoptosis in Balb/c wildtype and Balb/c-*lpr* primary pro-B cells with no significant differences observed between the wildtype and Balb/c-*lpr* primary pro-B cells (Fig. 6B).

Analysis of a potential role for DR6 was of particular interest since this recently described TNFR-like death receptor is expressed on resting, mature B cells (Sheikh and Fornace,

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2000) and since its genomic deletion results in increased mature B cell proliferation and reduced apoptosis (Schmidt et al., 2003). Furthermore, our preliminary studies indicated an up-regulation of DR6 on BU-11 cells following co-culture on BMS2 cells and treatment with DMBA (data not shown). To test a possible role for DR6 in PAH-induced apoptosis, primary pro-B cells from wildtype and DR6$^{-/-}$ littermates were co-cultured with BMS2 stromal cells, exposed to DMBA, and assayed for apoptosis as above.

While the percentage of pro-B cells undergoing apoptosis was somewhat lower in this series of experiments than was seen previously, a significant percentage of pro-B cells from both wildtype and DR6$^{-/-}$ littermates underwent apoptosis following exposure to DMBA (Fig. 6C). However, no differences were observed between the DMBA-treated wildtype and DR6$^{-/-}$ littermate groups. In addition to the fact that caspase-3 appears to be activated prior to caspase-8 (Figs. 2 and 4), results here are consistent with the hypothesis that DMBA-induced apoptosis and caspase-8 activation are not initiated by death signaling through TNFR1, TNFR2, Fas, or DR6.

Caspase-8 is not the initiator caspase in DMBA-induced pro/pre-B cell apoptosis

Caspase-8 also may be activated by other caspases, notably caspase-6 via caspase-3 (Belka et al., 2000; Cowling and Downward, 2002; Murphy et al., 2004; Slee et al., 1999; Wieder et al., 2001). Since death receptors did not appear to be involved in caspase-8 activation, the contribution of an alternative, caspase-3-dependent pathway was investigated. BU-11/BMS2 cell co-cultures were treated with vehicle (0.1% DMSO), FA-FMK (15 μM), as a putative negative control, VAD-FMK (15 μM), a pan-caspase inhibitor, or DEVD-FMK (15 μM), a caspase-3 inhibitor, 30 minutes prior to treatment with vehicle (0.1% acetone) or DMBA (1 μM). Limiting inhibitor doses (15 μM; eg. the lowest dose of DEVD-FMK that completely suppressed apoptosis at 10 hrs), which are significantly lower than those used in other publications (Andjelic

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and Liou, 1998; Doi et al., 1999), were used to maximize inhibitor specificity. BU-11 cells were harvested after a 10 h treatment with DMBA and analyzed for apoptosis by propidium iodide staining and flow cytometry, for caspase activation by immunblotting for cleaved caspase-3 and -8, and for caspase-8 activity by immunoblotting for truncated Bid.

A significant percentage of BU-11 cells underwent apoptosis following DMBA exposure at this early time point (Fig. 7A). Apoptosis was blocked by treatment with either VAD-FMK or DEVD-FMK but not with FA-FMK (Fig. 7A).

Formation of active caspase-3 fragments was reduced significantly in the presence of VAD-FMK (Fig. 7B) suggesting that an upstream caspase is required for caspase-3 activation. Since these peptide inhibitors block the activity and not the cleavage of caspases, DMBA-induced cleavage of caspase-3 was not expected to be and was not inhibited by the caspase-3 inhibitor DEVD-FMK (Fig. 7B). Unexpectedly, the "control" FA-FMK peptide slightly, though insignificantly, reduced caspase-3 formation without significantly reducing apoptosis (Fig. 7, A and B). This effect on caspase signaling may be due to its ability to suppress caspases-2 and -9 or cathepsin B (Lopez-Hernandez et al., 2003).

If caspase-8 cleavage is dependent on caspase-3 activity, then it would be predicted that inhibition of caspase-3 would decrease caspase-8 cleavage and activity. Indeed, 15 μM DEVD-FMK completely blocked cleavage of caspase-8 (Fig. 7C) and formation of truncated Bid (Fig. 8D). The pan-caspase inhibitor VAD-FMK also inhibited both caspase-8 and Bid cleavage, again supporting the hypothesis that upstream caspases appear to control both caspase-3 and caspase-8 activation. Consistent with its minimal inhibition of caspase-3 cleavage (Fig. 7B) and the hypothesis that caspase-3 lies upstream of caspase-8, FA-FMK slightly though insignificantly reduced cleavage of caspase-8 (Fig. 7C) and formation of truncated Bid (Fig. 7D). These results

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support the hypothesis that DMBA-induced caspase-8 activation occurs as part of an amplification loop rather than as an initiating event in the apoptotic process.

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Discussion

Studies performed with transformed and primary immature, sIg^+ B cells have begun mapping apoptosis pathways invoked during clonal deletion (Andjelic and Liou, 1998; Doi et al., 1999; Ruiz-Vela et al., 1999; Tian et al., 2001; Wu et al., 1996a; Wu et al., 1998). Our previous studies with non-transformed, bone marrow stromal cell-dependent, primary pre-B cells and pro/pre-B cell lines have shown that a similar but clearly distinct set of events leads to apoptosis at an earlier stage of B cell development when cultures are exposed to immunosuppressive environmental chemicals (Mann et al., 1999; Mann et al., 2001; Ryu et al., 2003; Yamaguchi et al., 1997a). The work presented herein was designed to extend these studies by analyzing the role of caspases in clonally non-restricted, PAH-induced apoptosis in bone marrow B cells. These studies contribute to our understanding of when the capacity to undergo apoptosis is acquired during B cell development and how environmental chemicals, represented by DMBA, inappropriately activate apoptotic pathways leading to immunosuppression (Dean et al., 1986; Thurmond et al., 1987).

To take advantage of mutant mouse strains defective in genes important to apoptosis, and to study earlier stages in B cell development, studies were extended to primary pro-B cells expanded from bone marrow. To model events taking place in the bone marrow microenvironment, these primary pro-B cells were maintained on bone marrow stromal cells during DMBA treatment. As with primary pre-B cells (Yamaguchi et al., 1997a) and the BU-11 pro/pre-B cell line (Mann et al., 1999), primary pro-B cells grown in rIL-7 in the absence of stromal cells were completely resistant to PAH-induced apoptosis (data not shown). This result is consistent with the hypothesis that stromal cells deliver a death signal to bone marrow B cells. However, the exact nature of this signal is unknown. Attempts to identify a soluble stromal cell

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"death factor" revealed what is likely to be DMBA metabolite-protein complexes in the supernatant of DMBA-treated stromal cells that can act at a distance but still require stromal cells to deliver a death signal to stromal cell-adherent B cells (Allan et al., 2003). This result, together with the inability of DMBA-treated stromal cells to kill B cells separated by permeable membranes (Yamaguchi et al., 1997b) suggests that either an as yet unidentified cytokine-like factor or a toxic DMBA metabolite is delivered through cell-cell contact to bone marrow B cells. While both of these possibilities are being considered, any putative death-inducing, membrane-bound cytokine is not likely to be among the TNF family members studied herein (see below).

In addition to the demonstration of stromal cell dependence, the validity of the primary pro-B cell system was supported further by similarities in the magnitude and kinetics of DMBA-induced apoptosis as compared with what had been observed in the BU-11 cell system. Similar caspase activation, shown by cleavage of endogenous caspase substrates and reduction in apoptosis by caspase inhibitors, also occurred in primary pro-B cells.

Caspases have been assigned to either to the "extrinsic" or "intrinsic" pathway. However, significant crossover can occur that leads to amplification of the apoptotic process. For example, caspase-8 can participate in either pathway. Caspase-8, activated by the "extrinsic" pathway through a death receptor, may directly activate caspase-3 or may activate Bid, leading to activation of the "intrinsic" mitochondrial pathway (Gross et al., 1999). Furthermore, once the "intrinsic" pathway has been activated, caspase-8 may be activated by a caspase-3-dependent mechanism through caspase-6 (Cowling and Downward, 2002; Murphy et al., 2004; Slee et al., 1999). The current studies, therefore, were centered on the possible activation of caspase-8 and the role of caspase-3 and/or death receptors in that activation.

Caspase-8 was activated within 6-8 h of DMBA exposure as assessed by: 1) appearance

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of a 40 kDa cleaved caspase-8 fragment, 2) cleavage of the caspase-8 peptide substrate IETD, and 3) cleavage of Bid, an endogenous caspase-8 substrate. Interestingly, activation of caspase-3, as assessed by similar criteria (i.e. appearance of the active caspase-3 fragment, cleavage of a peptide substrate, and endogenous α-fodrin cleavage), preceded that of caspase-8. These results suggest that caspase-8 activation occurs downstream of caspase-3 activation, presumably via the intrinsic pathway. Our observations are reminiscent of those obtained with a diverse group of toxicants including ionizing radiation (Belka et al., 2000), chemotherapeutic agents (Wieder et al., 2001), and celecoxib (Jendrossek et al., 2003), all of which induce apoptosis through a mitochondria-dependent process. As would be predicted if mitochondrial activation preceded caspase-8 activation, inhibition of caspase-3 with DEVD-FMK blocked caspase-8 activation and apoptosis. Inhibition of Bid cleavage with the caspase-3 inhibitor further suggests that caspase-8 may amplify a mitochondria-initiated "intrinsic" pathway through formation of tBid (Gross et al., 1999).

These results may be contrasted with those obtained with a transformed, stromal cell-independent pre-B cell line, 70Z3 (Page et al., 2002). In studies with these transformed pre-B cells, DMBA induced a minimal and transient level of caspase-8 activity that preceded the relatively late (approximately 15-20 h) induction of caspase-3 activity. Other characteristics, including a longer period of time until apoptosis is evident (e.g. 15-20 h), a smaller percentage of cells that undergo apoptosis (approximately 25% at 24 h), and the use of higher DMBA doses (e.g. 3 μM) to induce apoptosis suggest that the transformed cells are more resistant to apoptosis signals in general and that they may activate alternative pathways when exposed to PAH in specific.

Studies in several systems suggest how the "extrinsic" mitochondrial pathway activates

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caspase-8 through caspase-3. Most studies implicate caspase-6 as an intermediary. In cell-free studies, caspases-6 and -8 were activated following cytochrome c treatment (Slee et al., 1999). Expression of a catalytically inactive caspase-6 mutant prevented caspase-8 activation in COS-7 cells in response to serum starvation (Cowling and Downward, 2002). Caspase-8 activated by caspase-6 is catalytically competent, despite the lack of a dimerization stimulus (Murphy et al., 2004). Preliminary data obtained in the present system indicate that DMBA induces release of cytochrome c from mitochondria followed by caspase-6 activation and cleavage of its endogenous substrate lamin (data not shown). Studies are underway to determine if this putative caspase-6 activation is causally linked to caspase-8 activity.

The likely contribution of caspase-3 to caspase-8 activation in and of itself does not rule out a role for TNFR family members in DMBA-induced apoptosis. Indeed, *in vivo* studies with DMBA suggest a role for TNFRs in the elimination of at least some hematopoietic cell types in the bone marrow (Page et al., 2002). In the absence of specific analysis of the fate of bone marrow B cells in particular (Page et al., 2002), it is difficult to tell if TNFRs were in fact involved in DMBA-induced B cell death *in vivo*. However, several approaches described herein failed to implicate TNFR family members in early B cell apoptosis: 1) *TNF-α*, *TNF-β*, and *LT-β* mRNA were not detected by RT-PCR after DMBA treatment, 2) TNF-α, as assessed by ELISA or surface expression, was not induced in DMBA-treated primary bone marrow stromal cells or in BMS2 cells, 3) inhibitory TNF-Ig and Fas-Ig failed to block apoptosis (data not shown), 4) bone marrow stromal cells from TNF-$\alpha^{-/-}$ mice were as effective at inducing apoptosis as cells from wildtype mice, and 5) pro-B cells from TNFR1$^{-/-}$/TNFR2$^{-/-}$ double knock-out mice, Fas-defective Balb/c-*lpr* mice, or DR6$^{-/-}$ mice were as susceptible to DMBA-induced apoptosis as wildtype cells. Although these studies do not rule out the contribution of other, as yet

uncharacterized TNFR family members, they argue that at least the well-described death receptors do not contribute to DMBA-induced apoptosis under these conditions. The apparent disparity between the current studies and some *in vivo* studies (Page et al., 2002) could reflect the lack of information on the effects of DMBA treatment on B cell subsets *in vivo* or on a systemic stress response *in vivo* secondary to DMBA toxicity as measured relatively late (48 h) after DMBA exposure.

In summary, the studies presented herein strongly suggest that PAH-induced apoptosis is mediated primarily by activation of elements of the "intrinsic" pathway and not by death receptors. Similarly, the apoptotic pathway activated during B cell clonal deletion involves mitochondrial activation, followed by activation of caspases-3 and -9, PARP cleavage and DNA fragmentation (Doi et al., 1999; Ruiz-Vela et al., 1999). Caspase-8 activation following DMBA treatment appears to result from the activation of caspase-3, leading to the cleavage of Bid and the activation of a positive feedback loop. Since a pan caspase inhibitor blocked activation of both caspase-3 and caspase-8, it is postulated that an initiator caspase(s) upstream of caspase-3 is required for DMBA-induced bone marrow B cell apoptosis. Finally, these data and preliminary data indicating changes in cytochrome c release suggest that the apoptotic pathway induced by DMBA shares key elements with the mitochondria-dependent pathway activated during clonal deletion. Therefore, it appears as though immunosuppressive environmental chemicals activate some, but not all of the elements of the apoptosis pathway which signal clonal deletion.

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Figure Legends

Fig. 1. DMBA induces apoptosis in primary bone marrow pro-B cells and in a non-transformed pro/pre-B cell line (BU-11). (A) Bone marrow cells were cultured for 5 days with murine rIL-7 and then stained with FITC-conjugated rat anti-mouse B220 antibody and PE-conjugated rat anti-mouse CD43 antibody. Data are representative of 15 experiments. Co-cultures of BMS2 with primary pro-B cells (B) or BU-11 cells (C) were treated with vehicle (0.1% acetone) or DMBA (1 μM). B cells were harvested after 12 h, propidium iodide stained and analyzed for apoptosis by flow cytometry. (D) BU-11/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1 μM). BU-11 cells were harvested after 2-18 h and analyzed for apoptosis by propidium iodide staining. Data are presented as means ± SE from at least 4 experiments. *Statistically different from Vh ($p<0.05$, ANOVA, Dunnett's).

Fig. 2. Caspase-3 is activated in bone marrow B cells after DMBA treatment. BU-11/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1 μM), and BU-11 cells were harvested after 2-18 h. (A) Total proteins were extracted and analyzed for active caspase-3 fragments (arrow) and for β-actin by immunoblotting. Data are representative of 4 experiments. (B) Cytosolic proteins were extracted and caspase-3-like activity was measured using p-nitroaniline-conjugated DEVD substrate. Data are presented as the average fold increase in caspase-3-like activity relative to the activity in untreated cells ± SE from 3-4 experiments. The activity in untreated cells was 22.9 ± 2.8 μM p-nitroaniline. *Statistically different from Vh ($p<0.05$, ANOVA, Dunnett's). (C) Primary bone marrow pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were co-cultured with BMS2 cells, treated with vehicle (0.1% acetone) or DMBA (1

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μM), and were harvested after 10 h. Cytoplasmic proteins were extracted and analyzed for cleaved α-fodrin and for α-tubulin by immunoblotting. Data are representative of 3 experiments.

Fig. 3. DEVD-FMK blocks DMBA-induced apoptosis in BU-11 cells (A) and in primary pro-B cells (B). Primary bone marrow pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were co-cultured with BMS2 cells, pre-treated for 30 m with either vehicle (0.1% DMSO) or DEVD-FMK (30 μM) and treated with vehicle (0.1% acetone) or DMBA (1 μM). B cells were harvested after 24 h and analyzed for apoptosis by propidium iodide staining. Data are presented as the average percentage of cells undergoing apoptosis ± SE from 3-5 experiments. *Significantly different from cultures pretreated with DMSO and then with DMBA (Student's T test, $p<0.05$).

Fig. 4. Caspase-8 is activated in bone marrow B cells after DMBA treatment. BU-11/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1 μM) and BU-11 cells were harvested after 2-18 h. (A) Total proteins were extracted and analyzed for caspase-8 fragments and β-actin by immunoblotting. Data are representative of 4 experiments. (B) Cytosolic proteins were extracted and caspase-8-like activity was measured using p-nitroaniline-conjugated IETD substrate. Data are presented as the average fold increase in caspase-8-like activity relative to the activity in untreated cells ± SE from 4-6 experiments. The activity in untreated cells was 8.4 ± 1.3 μM p-nitroaniline. *Statistically different from Vh ($p<0.05$, ANOVA, Dunnett's). (C) Primary bone marrow pro-B cells were isolated from bone marrow from C57BL/6 mice by culturing with murine rIL-7 for 5 days. Primary pro-B cells or BU-11 cells were co-cultured with BMS2 cells, treated with vehicle (0.1% acetone) or DMBA (1 μM), and were harvested after 10

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h. Cytoplasmic proteins were extracted and analyzed for truncated Bid (tBid) and β-actin by immunoblotting. Data are representative of 3 experiments.

Fig. 5. TNF-α expression is not required for DMBA-induced bone marrow B cell apoptosis. Primary stromal cell cultures were prepared from mice by culturing cells expunged from bone marrow for 7 days. BMS2 cells or primary bone marrow stromal cells from C57BL/6 mice were treated with vehicle (0.1% acetone) or DMBA (1 μM) for 1-16 h. Stromal cells were harvested at the times indicated and analyzed for *TNF-α*, *TNF-β*, and *LT-β* mRNA by RT-PCR (A) or were harvested at 16 h to assay for membrane-bound TNF-α by flow cytometry (B). Primary mouse dendritic cells treated with LPS (1 μg/ml) for 6 h or RAW 264.7 cells treated with LPS for 4 h were included as positive controls. The data are representative of 3 experiments. (C) BU-11 cells were co-cultured with primary bone marrow stromal cells from either TNF-$\alpha^{-/-}$ or age-matched B6129F2/J mice and treated with vehicle (0.1% acetone) or DMBA (1 μM). BU-11 cells were harvested after 24 h and analyzed for apoptosis by Annexin V staining. The data are presented as the mean ± SE from 3-4 individual mice. **Significantly different from Vh-treated controls ($p<0.01$, ANOVA, Scheffe's). DMBA-induced apoptosis was not significantly different in BU-11 cells cultured with TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ primary stromal cells ($p>0.5$, ANOVA, Scheffe's).

Fig. 6. TNFR1/R2, Fas, and DR6 do not contribute to DMBA-induced B cell death. Primary bone marrow pro-B cells were isolated from bone marrow aspirate from TNFR1/R2$^{-/-}$ and wildtype, age-matched B6129F2/J wildtype mice, Balb/c-*lpr* and wild type, age matched Balb/c mice or from DR6$^{-/-}$ or wildtype littermates by culturing with murine rIL-7 for 5 days. Primary

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pro-B cell/BMS2 co-cultures were treated with vehicle (0.1% acetone) or DMBA (1μM) for 24 h and analyzed for apoptosis by Annexin V staining. The data are presented as mean ± SE from 4-10 individual mice. **Significantly different from Vh-treated controls ($p<0.01$, ANOVA, Scheffe's). DMBA-induced apoptosis was not significantly different in TNFR1/R2$^{+/+}$ as compared with TNFR1/R2$^{-/-}$ primary pro-B cells ($p>0.4$, ANOVA, Scheffe's), in Balb/c-*lpr* as compared with wildtype Balb/c primary pro-B cells ($p>0.9$, ANOVA, Scheffe's), or in wildtype littermate as compared with DR6$^{-/-}$ pro-B cells ($p>0.9$, ANOVA, Scheffe's).

Fig. 7. Inhibition of caspase-3 activation blocks caspase-8 cleavage. BU-11/BMS2 co-cultures were pre-treated for 30 m with vehicle (0.1% DMSO), or FA-FMK (15 μM, negative control peptide), VAD-FMK (15 μM), or DEVD-FMK (15 μM) prior to treatment with vehicle (0.1%, acetone) or DMBA (1 μM) for 10 h. An aliquot was analyzed for apoptosis by propidium iodide staining (A). Data are presented as the mean ± SE of 3-4 experiments. *Significantly different from Vh-treated controls ($p<0.05$, ANOVA, Dunnett's). Total proteins were extracted from an aliquot for analysis of caspase-3 cleavage (B), caspase-8 cleavage (C), or Bid cleavage (D), and β-actin by immunoblotting. Data are representative of 3-4 experiments.

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Figure 1

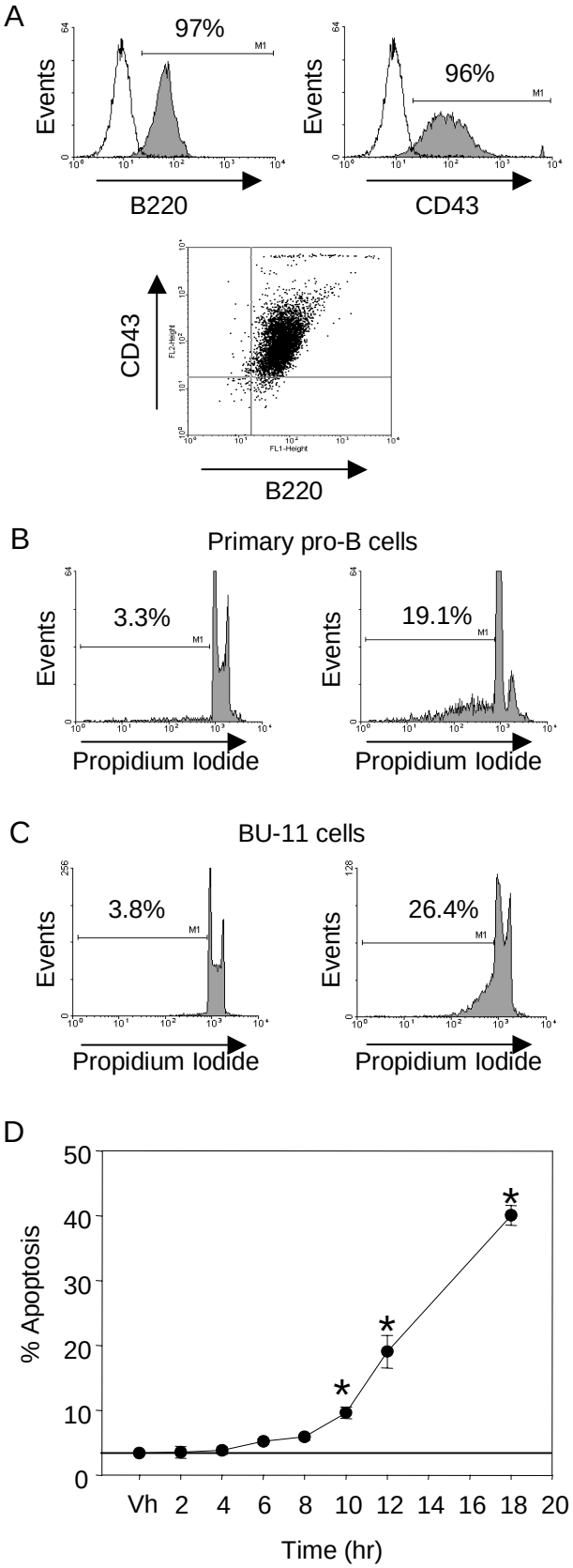

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Figure 2

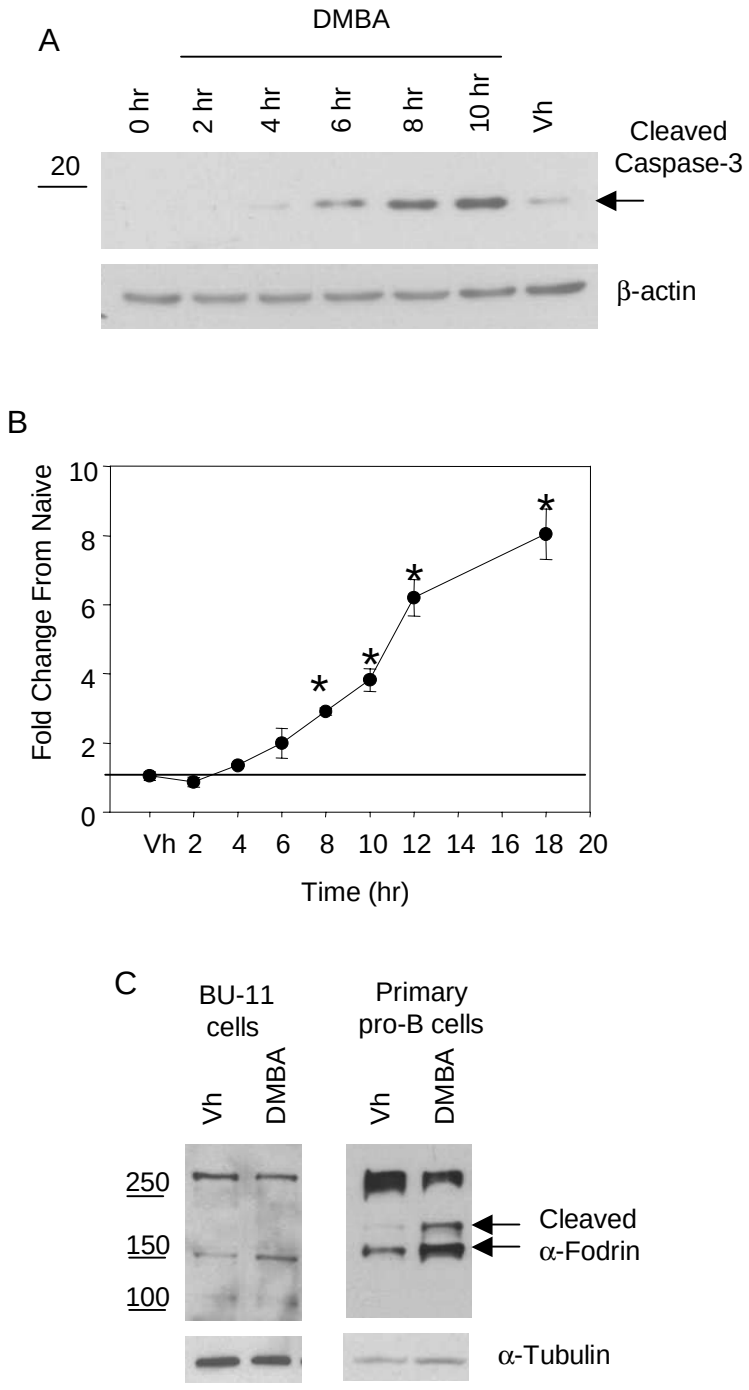

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Figure 3

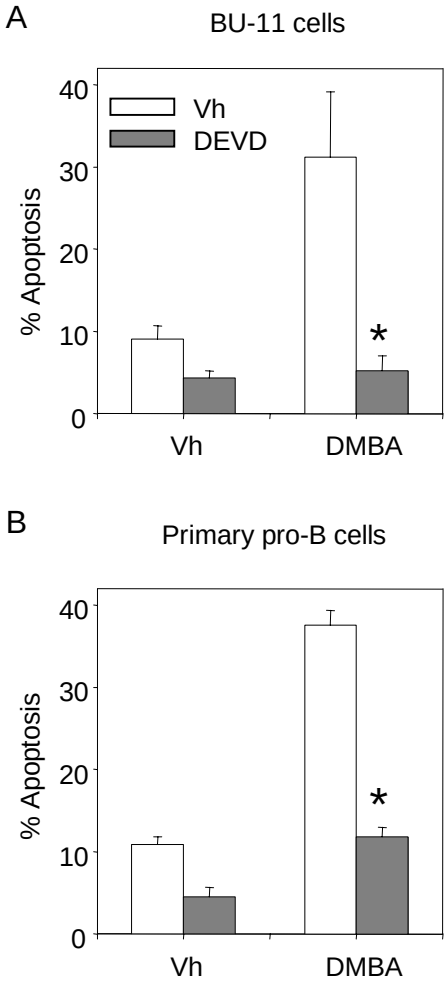

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Figure 4

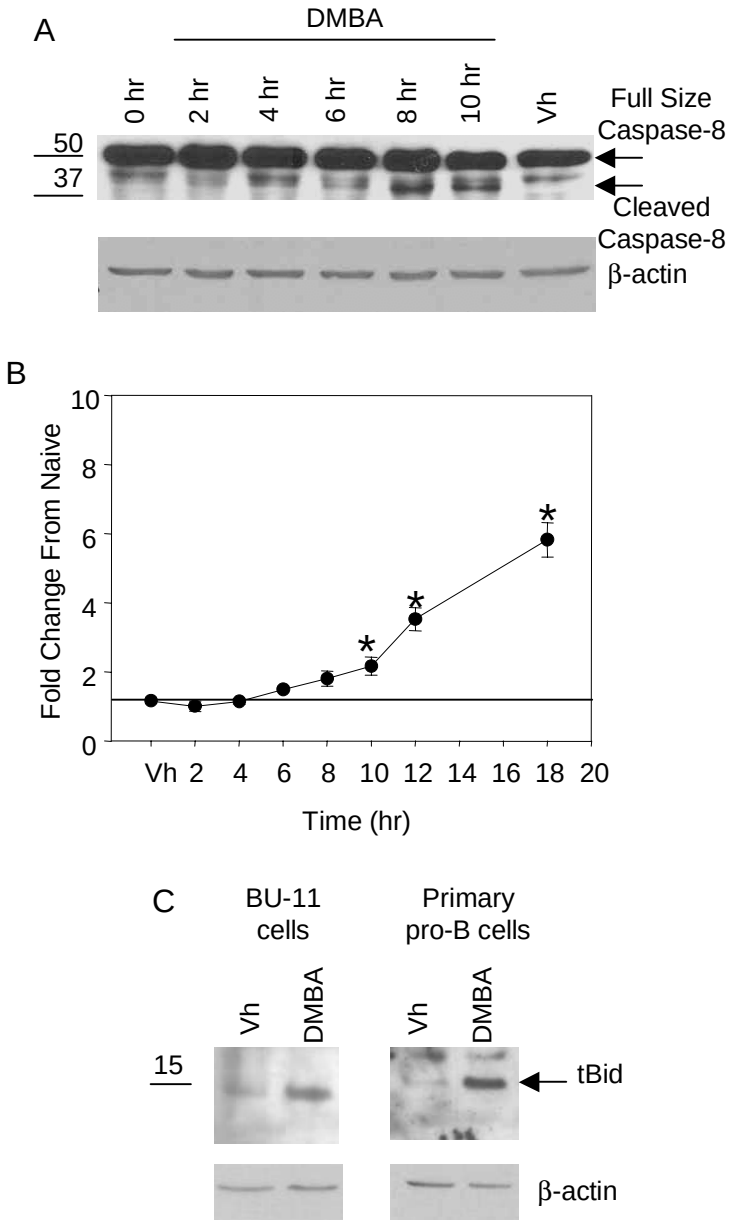

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Figure 5

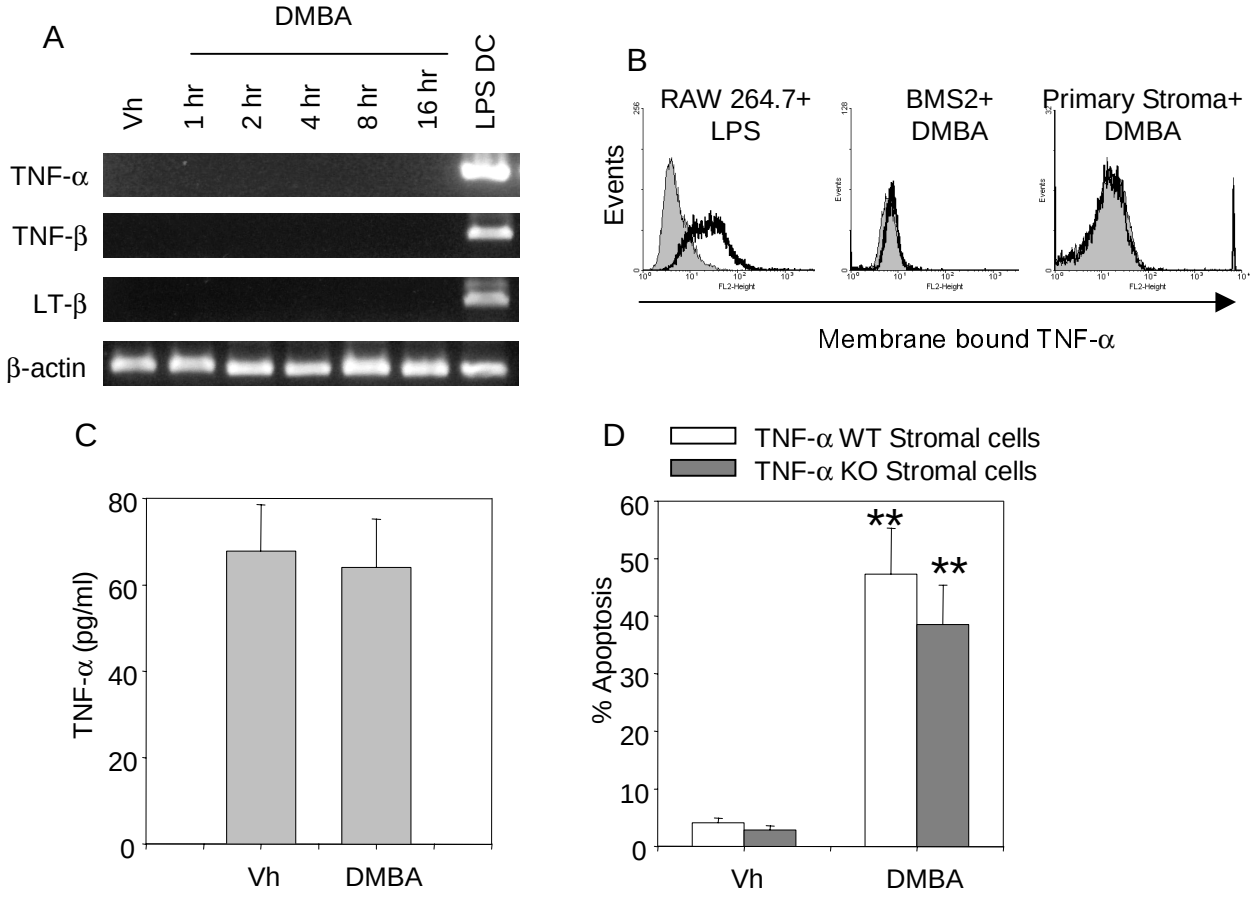

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Figure 6

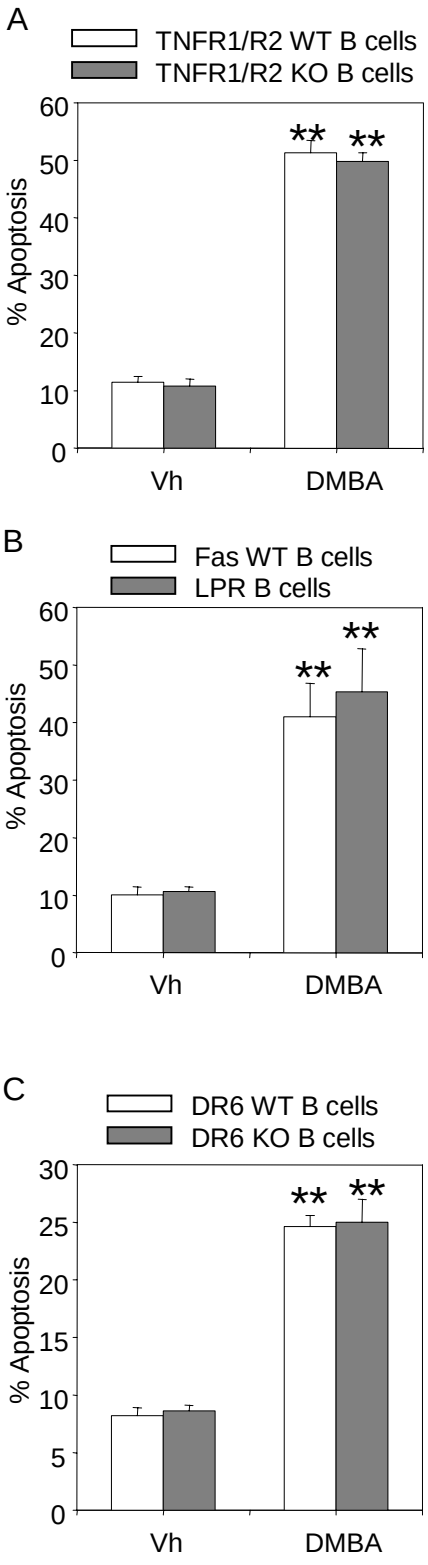

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Figure 7