

Lipopolysaccharides Indirectly Stimulate Apoptosis and Global Induction of Apoptotic Genes in Fibroblasts*

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Following Gram-negative bacterial infection there is a reduction in matrix-producing cells. The goal of the present study was to examine the apoptotic effects of lipopolysaccharide (LPS) on fibroblastic cells and to investigate the role that the host response plays in this reaction. This was accomplished *in vivo* by subcutaneous inoculation of LPS in wild type and TNFR1^{-/-}R2^{-/-} mice. The direct effects of LPS on fibroblast apoptosis was studied *in vitro* with normal diploid human fibroblasts. The results indicate that LPS *in vivo* induces apoptosis of fibroblasts. By RNA profiling we demonstrated that LPS stimulates global expression of apoptotic genes and down-regulates anti-apoptotic genes. Fluorometric studies demonstrated that LPS *in vivo* significantly increased caspase-8 and caspase-3 activity and by use of specific inhibitors, the activation of caspase-3 was shown to be initiated by caspase-8 with no contribution from caspase-9. *In vitro* studies demonstrated that LPS did not induce apoptosis of fibroblasts, whereas tumor necrosis factor (TNF) did. In addition, the pattern of apoptotic gene expression induced by TNF *in vitro* was nearly identical to that induced by LPS *in vivo*, as measured by RNase protection assay. Moreover, pre-treatment of cells with TNF greatly enhanced apoptosis induced by a second stimulation with TNF 24 h later, suggesting that the global induction of pro-apoptotic genes was functionally significant. Thus, LPS acts to modulate the expression of a large number of genes that favor apoptosis of fibroblastic cells that is dependent upon activation of caspase-8 and is largely mediated by TNF.

Tissue can be injured by a wide variety of conditions, including oxygen deprivation, physical trauma, chemical agents, and infectious microbes. Bacterial infection can cause cell and tissue damage through the production of toxins (1–3). Alternatively, damage may be induced indirectly through stimulation of an inflammatory response. Inflammatory cells are capable of damaging the host tissue by the release of lytic enzymes and reactive oxygen species (4–6).

An important source of inflammation from infection is LPS¹

that can be shed from the outer membrane of Gram-negative bacteria. Local reactions to LPS include vascular changes associated with recruitment of leukocytes and the subsequent release of proinflammatory mediators (7–9). It is believed that prolonged or excessive production of cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and IL-8 represents an important etiologic factor in inflammatory-based tissue injury (10–14).

A number of studies have investigated the impact of LPS and other bacterial products on apoptosis of white blood cells. *In vitro*, LPS has been shown to promote apoptosis in macrophages (15–17) but to inhibit apoptosis of neutrophils (18, 19). *In vivo* LPS has a pro-apoptotic effect on lymphocytes in Peyer's patches and thymocytes, whereas it has anti-apoptotic effects in peritoneal neutrophils (20–22).

Although apoptosis of white blood cells has been studied in considerable depth, the effect of LPS on the programmed cell death of other cells has not been as thoroughly studied. However, it has been reported that LPS directly induces apoptosis in hepatocytes, ventricular myocytes, and endothelial cells (23–28).

One of the crucial intracellular signaling events in apoptosis is the sequential activation of caspases, a family of cysteine proteases (29, 30). Caspases are produced as pro-enzymes and become activated by proteolytic cleavage at internal aspartate residues upon apoptotic stimulation. Two categories of caspases important for apoptosis have been recognized: the initiators and executioner caspases. The initiator caspases, such as caspase-8 and -9, are activated in the earlier phase of apoptosis; the executioner caspases, such as caspase-3, are activated by initiator caspases and are responsible for dismantling cells (31, 32). Caspase-3 appears to play a central role in LPS-mediated apoptosis (24, 33, 34). LPS also may modulate caspase-8 or caspase-9 activity (25, 35–38).

In contrast to the many *in vitro* studies showing that LPS induces apoptosis in macrophages, hepatocytes, or endothelial cells, there are no reports that we are aware of that investigated the direct effect of LPS on fibroblast apoptosis. To

caspase and RIP adaptor with death domain; *FADD*, Fas-associated protein with death domain; *FasL*, Fas ligand; *OX40L*, OX40 ligand; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TNFR1^{-/-}, TNF receptor 1-deficient mice; TNFR2, TNF receptor 2; TNFR2^{-/-}, TNF receptor 2-deficient mice; TNFR1^{-/-}R2^{-/-}, TNF receptor 1- and 2-deficient mice; *TRAF*, tumor necrosis factor receptor-associated factor; *TRADD*, TNFR1-associated death domain protein; *XIAP*, X-linked inhibitor of apoptosis; *WT*, wild type mice; *PBS*, phosphate-buffered saline; *TUNEL*, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; *AFC*, 7-amino-4-trifluoromethyl coumarin; *ELISA*, enzyme-linked immunosorbent assay; *TRAIL*, TNF-related apoptosis-inducing ligand; *IL-1*, interleukin 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *RPA*, RNase protection assay.

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¹ The abbreviations used are: LPS, lipopolysaccharide; *Apaf1*, apoptotic protease-activating factor 1; *Bcl-2*, B-cell lymphoma-2; *CD27L*, CD27 ligand; *CD30L*, CD30 ligand; *CD40L*, CD40 ligand; *CRADD*,

address this important topic, we undertook *in vitro* and *in vivo* studies. *In vivo* studies were carried out by subcutaneous injection of LPS into the connective tissue in the scalp. The results indicate that LPS *in vivo* induces apoptosis in fibroblasts. We demonstrated that LPS can stimulate a global expression of 37 apoptotic genes and down-regulation of several anti-apoptotic genes. LPS *in vivo* stimulated activation of caspase-3 through the action of caspase-8 but not caspase-9. Moreover, the apoptotic effect of LPS was largely mediated through TNFR1 signaling. Unlike reports for other cell types, LPS did not directly induce apoptosis of fibroblasts. However, TNF stimulated pro-apoptotic events in fibroblasts *in vitro* that were virtually identical to LPS-stimulated events *in vivo*.

EXPERIMENTAL PROCEDURES

Animals—There were two separate groups of experimental and matched wild type mice as follows: 1) TNFR1^{-/-}R2^{-/-} mice and matched wild type F2 C57BL/6 × 129 and 2) mice deficient in TNFR1 (TNFR1^{-/-}) or TNFR2 (TNFR2^{-/-}) and matched wild type C57BL/6J mice. Mice were purchased from Jackson laboratories (Bar Harbor, ME). All procedures involving mice were approved by the Boston University Medical Center Institutional Animal Care and Use Committee.

Injection of LPS—Purified *Escherichia coli* serotype 0111:B4 LPS was purchased from List Biologicals (Campbell, CA). Mice were anesthetized with injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) in sterile phosphate-buffered saline (PBS). LPS was inoculated adjacent to the periosteum at a point on the midline of the skull located between the ears. Injection at this anatomic site can be reproducibly achieved and leads to an inflammatory response in the loose connective tissue, which consists primarily of fibroblasts. For each data point, there were 6 mice ($n = 6$). We undertook preliminary experiments to identify a dose for LPS that gave a moderate number of apoptotic cells. Thus, 200 μ g of LPS or equal volume of vehicle alone, PBS (50 μ l) were injected. Mice were euthanized 6 and 24 h following injection. In addition to LPS, some animals were treated by intraperitoneal injection of caspase-8 or -9 inhibitor (1 mg/kg) 1 h before LPS injection and locally (25 μ g) at the time of LPS injection. The caspase-8 inhibitor, z-Ile-Glu(OMe)-Thr-Asp(OMe) fluoromethylketone and caspase-9 inhibitor, z-Leu-Glu(OMe)-His-Asp(OMe) fluoromethylketone were purchased from R&D Systems (Minneapolis, MN). Control mice received vehicle alone, sterile PBS containing 2% Me₂SO (Sigma-Aldrich, St. Louis, MO).

Preparation of Histologic Sections—Animals were euthanized by decapitation, and their heads were fixed for 72 h in cold 4% paraformaldehyde, decalcified by incubation of cold Immunocal (Decal Corp., Congers, NY) for ~12 days, with solution changed daily. Paraffin-embedded sagittal sections were prepared at a thickness of 5–6 μ m.

Immunohistochemistry and TUNEL Assay—Apoptotic cells were detected by an *in situ* TUNEL assay by means of a TACS 2 TdT-Blue Label kit purchased from Trevigen (Gaithersburg, MD), following the manufacturer's instructions. Apoptotic cells were detected by TACS Blue Label and counterstained with Red C. In some experiments apoptotic fibroblasts were identified by combining the TUNEL assay with immunohistochemistry. In these experiments, the TUNEL assay was performed as described above. Following TACS Blue Label, sections were incubated with polyclonal goat anti-vimentin (Cortex Biochem, San Leandro, CA). Primary antibody was localized by the avidin-biotin immunoperoxidase method using a kit from Vector Laboratories (Burlingame, CA). The signal was enhanced by tyramide signal amplification using a kit from PerkinElmer Life Sciences (Boston, MA).

Quantitative Histologic Analysis—The number of fibroblastic apoptotic cells was determined in the standard TUNEL assay by their characteristic microscopic appearance as we have previously described (39). The values were verified by double labeling. In the latter, cells counted were both vimentin-positive and TUNEL-positive. Counts and measurements were confirmed by re-analysis of all the specimens by one other independent examiner. The intra- and inter-examiner variation was less than 10%. Student's *t* test was used to determine significant differences between the experimental and control groups at the $p < 0.05$ level.

RNA Extraction—Following sacrifice at the indicated time points, the scalps of mice were immediately dissected from the calvaria and frozen in liquid nitrogen. Total RNA was extracted with TRIzol (Invitrogen, Rockville, MD) from pulverized frozen tissue, follow-

ing the manufacturer's instructions. The concentration and integrity of the extracted RNA were verified by denaturing agarose gel electrophoresis.

RNAse Protection Assay—³²P-Labeled riboprobes were incubated with 10 μ g of total RNA and then subjected to RNase digestion using a kit from BD Pharmingen (Franklin Lakes, NJ), following the manufacturer's instructions. Following electrophoresis on a 6% polyacrylamide gel, radiolabeled bands were visualized with a PhosphorImager (Amersham Biosciences). The optical density of the protected bands was measured with Image ProPlus Software (Media Cybernetics, Silver Spring, MD), which was then normalized by the value of GAPDH in the same lane. Three separate RNase protection assays were performed with similar results. Student's *t* test was used to determine significant differences between the experimental and control groups.

Microarray—The Mouse Apoptosis GEArray Q series kit, which includes 96 key genes involved in apoptosis, was purchased from SuperArray, Inc. (Bethesda, MD). Total RNA was used as the template for ³²P-labeled cDNA probe synthesis following the manufacturer's instructions. Following overnight hybridization with labeled probe, the GEArray membranes were exposed using a PhosphorImager. The resulting digital image was converted to raw data file using Scanalyze software (www.microarray.org/software.html). GEArray Analyzer software (SuperArray Inc.) was used for data analysis. Each experiment was performed twice with similar results, and the mean values of the two experiments are presented for each gene. The duplicate results for each gene was within 7% of the mean for that gene.

Caspases Activities—Caspases activities were assayed by a fluorometric kit purchased from R&D Systems. Briefly, following sacrifice at the indicated time points, murine scalps were immediately dissected from the calvaria and frozen in liquid nitrogen. Frozen tissues were pulverized, and lysates were prepared using cell lysis buffer provided by R&D Systems. Following centrifugation total protein was quantitated using a BCA Protein Assay kit (Pierce, Rockford, IL). Caspase-3 activity was detected by using the specific caspase-3 fluorogenic substrate, DEVD peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). Caspase-8 activity was detected by using the specific caspase-8 fluorogenic substrate, IETD-AFC. Caspase-9 activity was detected by using the specific caspase-9 fluorogenic substrate, LEHD-AFC. Measurements were made on a fluorescent microplate reader using filters for excitation (400 nm) and detection of emitted light (505 nm). In some assays, recombinant caspase-3 enzyme (R&D Systems Inc., Minneapolis, MN) was used as a positive control. Buffers without cell lysate and cell lysate without substrate were used as negative controls.

Cell Culture—Human adult dermal fibroblasts were purchased from Cambrex (Walkersville, MD). Cells were propagated and maintained in Dulbecco's modified Eagle's medium (Cambrex Co.) supplemented with 10% fetal bovine serum, Gentamicin (100 μ g/ml), and Amphotericin B (100 ng/ml) at 37 °C in a humidified atmosphere of 5% CO₂ in air. TNF- α was purchased from R&D Systems Inc. Experiments with TNF- α were performed in culture medium supplemented with 0.5% fetal bovine serum. Assays were performed when the cultures reached 75–85% confluent. Expression of apoptotic genes was measured as described above following extraction of total RNA with TRIzol. Apoptosis of fibroblasts was determined by measuring histone-associated DNA fragments (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. In some experiments cells were pre-treated with TNF- α to allow for induction of apoptotic genes and then stimulated a second time to induce apoptosis. In these studies, 20,000 fibroblasts/cm² were pre-treated with TNF- α (20 ng/ml) for 24 h. Control cells were plated identically but not pre-treated with TNF- α . Viable cells were then trypsinized and replated at 20,000 fibroblasts/cm² based upon trypan-blue dye exclusion counts (Sigma-Aldrich, St. Louis, MO). These cells were then incubated in culture medium supplemented with 0.5% serum or 0.5% serum plus TNF- α (10 ng/ml) for 24 h. Apoptosis was determined by ELISA and in corresponding wells, the cell number was assessed to normalize apoptosis measurements.

RESULTS

Based upon preliminary studies, it was determined that 200 μ g of LPS produced a moderate inflammatory response with apoptosis of fibroblastic cells. The induction of apoptosis following LPS inoculation was measured by the TUNEL assay. When vehicle alone was injected, few apoptotic cells with clearly denoted blue nuclei were detected. Apoptosis of fibroblasts was confirmed by cells that were double positive by the TUNEL assay and simultaneously for expression of the mesenchymal

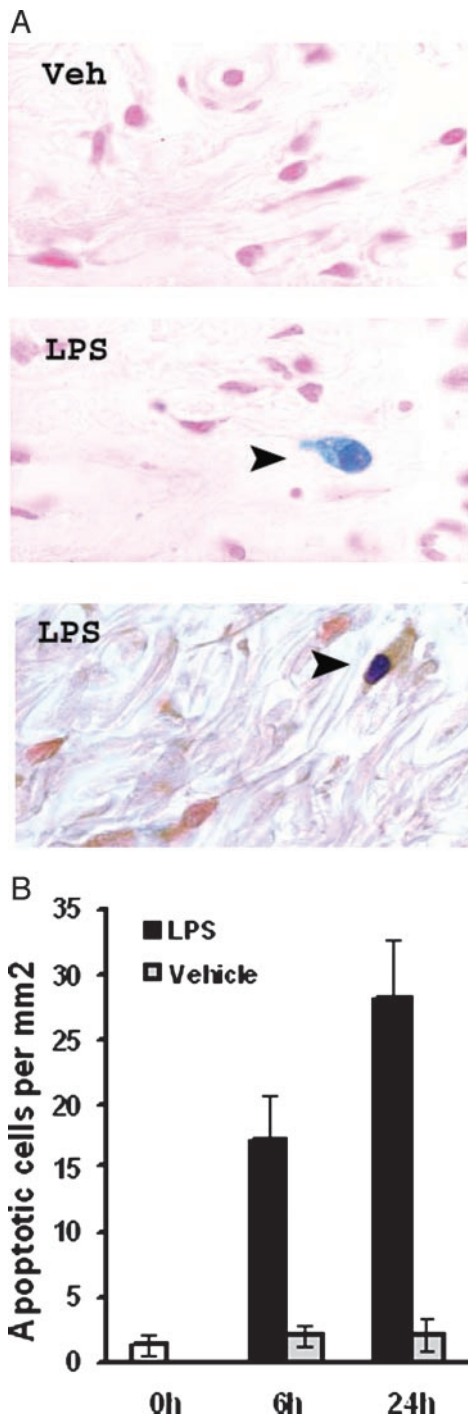


FIG. 1. Apoptotic effect of LPS. A, histologic sections of the site of LPS inoculation, the mouse scalp. Upper panel, TUNEL staining in wild type mice 24 h after inoculation with vehicle alone; middle panel, TUNEL staining of wild type mice 24 h after injection of LPS. Arrows point to apoptotic cells. Lower panel, apoptotic fibroblasts identified as double positive in the TUNEL assay and simultaneously for expression of vimentin. The arrow points to a cell that is positive for both. B, quantitative analysis of fibroblast apoptosis following LPS inoculation. The number of double-stained fibroblasts (both vimentin-positive and TUNEL-positive) was counted in wild type mice 0, 6, and 24 h after injection of LPS or vehicle alone. Each value represents the mean of six specimens \pm S.E.

cell marker, vimentin (Fig. 1A). Based on quantitative analysis of double-stained histologic sections LPS induced a statistically significant 8-fold level at 6 h and a 14-fold increase at 24 h in apoptotic fibroblastic cells compared with the vehicle alone injection ($p < 0.05$) (Fig. 1B).

TABLE I

Microarray analysis of apoptotic gene expression in wild type mice

Total RNA was isolated 24 h after injection of LPS or vehicle into the scalp of wild type mice. RNA was analyzed with a GEArray Q series kit. The relative expression of apoptotic genes was normalized to average signal of GAPDH and β -actin expression. Plasmid DNA (PUC18) was used as a negative control for background subtraction. Genes with more than 2-fold change in signal intensity were selected. The mean of duplicate results is shown.

Group	Gene	Relative density		
		24-h (PBS)	24-h (LPS)	
Ligands	<i>TNF</i>	0.0762123	0.2703511	
	<i>Trail</i>	0.0604328	0.2062432	
	<i>OX40L</i>	0.0274064	0.0642597	
	<i>CD40L</i>	0.0311190	0.0808486	
	<i>FasL</i>	0.0609219	0.2114744	
	<i>CD27L</i>	0.0345396	0.1123756	
	<i>CD30L</i>	0.0287830	0.0686745	
	<i>4-IBBL</i>	0.0358327	0.1009014	
	<i>Tnfsf12</i>	0.0339973	0.0922414	
	<i>LTα</i>	0.0639316	0.1638631	
Receptors	<i>TNFR2</i>	0.1653405	0.5946992	
	<i>TNFR1</i>	0.2137136	1.2135135	
	<i>CD27</i>	0.0692460	0.1973663	
	<i>CD40</i>	0.0318281	0.1073197	
	<i>Fas</i>	0.1191699	0.4239561	
	<i>OX40</i>	0.0226927	0.0542604	
	<i>Tnfrsf9</i>	0.0358327	0.1061401	
	Mitochondrial	<i>Bax</i>	0.0419835	0.1102827
		<i>Bak</i>	0.0390635	0.1253460
		<i>Bad</i>	0.0329001	0.1082736
<i>Bid</i>		0.0373662	0.1127503	
<i>Bcl-x</i>		0.4032156	0.1471590	
<i>Bcl-2</i>		0.3647554	0.1775225	
<i>Mcl-1</i>		0.5801648	0.2383997	
<i>Blk</i>		0.0354298	0.1079074	
Caspases	<i>Casp1</i>	0.0414642	0.1166716	
	<i>Casp2</i>	0.0786035	0.2840260	
	<i>Casp3</i>	0.0654875	0.2143876	
	<i>Casp6</i>	0.0585093	0.1922933	
	<i>Casp7</i>	0.0707060	0.2039648	
	<i>Casp8</i>	0.0946470	0.3136114	
	<i>Casp9</i>	0.0687037	0.1871880	
	<i>Casp11</i>	0.0348316	0.1061039	
	Adaptors	<i>TRAF2</i>	0.0923975	0.3753525
		<i>TRAF5</i>	0.0715403	0.2588839
		<i>TRAF6</i>	0.1375743	0.3732116
<i>Apaf-1</i>		0.0393785	0.1443492	
<i>FADD</i>		0.0710397	0.2591018	
Others	<i>CRADD</i>	0.1096642	0.3704377	
	<i>p53</i>	0.0692043	0.3525590	
	<i>XIAP</i>	0.0372583	0.0905102	
	<i>Bar-like</i>	0.0395036	0.1284657	
	<i>Hus1</i>	0.1014913	0.2507850	
	<i>Cide A</i>	0.0569183	0.1537364	

In Vivo Effect of LPS on Expression of Apoptotic Genes—To study pathways that might be modulated by LPS at the level of gene expression, microarrays were carried out (Table I). Gene expression was categorized into six groups based upon their function in apoptosis. LPS increased mRNA levels of apoptosis-inducing ligands 130% to 250% (*TNF*, *TRAIL*, *FasL*, *CD40L*, *CD30L*, *CD27L*, and *OX40L*). The expression of TNF receptors family increased 139% to 460% (*TNFR2*, *TNFR1*, *CD30*, *CD40*, *Fas*, and *OX40*). The expression of pro-apoptotic members of Bcl-2 family increased 160% to 230% (*Bax*, *Bak*, *Bad*, *Bid*, and *Blk*), whereas the expression of anti-apoptotic genes decreased 50–64% (*Bcl-x*, *Bcl-2*, and *Mcl-1*). The expression of initiator caspases (caspases 2, 8, and 9) and effector caspases (caspases 3, 6, and 7) increased 170% to 260%. The expression of death domain family of proteins such as FADD and CRADD and some of the TRAF family (*TRAF2* and *TRAF5*) increased 170% to 300%. LPS also increased by 270% the expression of *Apaf-1*, which participates in the cytochrome c-dependent activation of caspase-3, and the cell cycle regula-

TABLE II

List of apoptotic genes with less than 2-fold change in expression, 24 h after LPS injection in wild type mice

Total RNA was isolated 24 h after injection of LPS or vehicle into the scalp of wild type mice. RNA was analyzed with a GEArray Q series kit. The relative expression of apoptotic genes was normalized to average signal of GAPDH and β -actin expression. Plasmid DNA (PUC18) was used as a negative control for background subtraction. Fractions of gene families not induced by more than 100% are shown.

Family	Genes not induced by LPS	Fraction of gene family not induced by more than 100%
TNF ligand family	<i>Tnfsf11, Tnfsf14</i>	2/13
TNF receptor family	<i>Tnfrsf8, Tnfrsf10b, Tnfrsf11a, Tnfrsf11b, Tnfrsf12, April, Dr6</i>	7/15
Bcl-2 family	<i>Bcl2a1d, Bcl2110, Bcl212, Bokl, Bid3, Bim, Biklk, Bnip3</i>	8/16
Caspase family	<i>Casp12, Casp14</i>	2/10
IAP family	<i>Birc1a, Birc1b, Birc1d, Birc1e, Birc2, Birc3, Birc5, Birc6</i>	8/9
TRAF family	<i>Traf1, Traf3, Traf4, Traip, Tank</i>	5/8
CARD family	<i>Asc, Arc, BCL10, Nop30-like</i>	4/5
Death domain family	<i>Myd88, Ripk1, Dapk2, Daxx</i>	4/6
Death effector domain family	<i>Cash, Casp8ap2</i>	2/3
CIDE domain family	<i>Dffa, Dffb, Cideb</i>	3/4
p53 and ATM pathway	<i>Cdkn1a, Mdm2, Gadd45a, Atm, Rpa-like, chek 1, Rad53</i>	6/9

tor, *p53*, which is also pro-apoptotic, by 410%. Thus, LPS acted in a global way to enhance the expression of genes that promote apoptosis ranging from ligands to caspases or diminished those that inhibited apoptosis. Out of 96 genes examined that regulate apoptosis, the expression of 37 pro-apoptotic genes was increased by more than 100% following LPS inoculation. For each family of apoptotic genes there were some members whose expression were not modulated by more than 100% following LPS stimulation. These genes are summarized in Table II. Only anti-apoptotic genes of the Bcl-2 family exhibited decreased expression by 50%. The validity of the microarray results was confirmed by RNase protection assay as described below.

Direct Effect of LPS on Fibroblast Apoptosis in Vitro—To identify mechanisms through which LPS induced apoptosis, the direct effects of LPS were measured *in vitro*. Apoptosis of human adult primary fibroblasts was measured by the relative amount of histone-associated DNA fragments in the cytoplasm as determined by ELISA (Table III). Stimulation with LPS had no effect on fibroblast apoptosis. Because many of the genes identified in Table I are involved in TNF receptor signaling, the effect of TNF on fibroblast apoptosis was examined. TNF induced a dose-dependent increase in fibroblast apoptosis. At an optimal concentration, TNF (20 ng/ml) increased fibroblast apoptosis 350%.

Effect of LPS in Expression of Apoptotic Genes in *TNFR1^{-/-}R2^{-/-}* Mice—Given the capacity of TNF but not LPS to induce fibroblast apoptosis directly, the possibility that TNF-mediated LPS-induced fibroblast apoptosis *in vivo* was measured (Fig. 2). Quantitative analysis was utilized to measure the number of fibroblastic apoptotic cells in wild type and TNF receptor-ablated mice. LPS induced a statistically significant 5-fold increase at 6 h and a 7-fold increase at 24 h in apoptotic fibroblastic cells in wild type mice compared with TNF receptor-ablated mice ($p < 0.05$). Almost all of the LPS-induced apoptosis in fibroblasts can be accounted for by TNF. Similar results were obtained when TUNEL-positive fibroblasts were identified simultaneously by vimentin immunostaining.

To assess the functional role of TNF activity in the host response to LPS, the expression of apoptotic genes was studied by microarray in *TNFR1^{-/-}R2^{-/-}* compared with matched wild type mice (Fig. 3). Expression of the apoptotic mediators *TNF*, *TRAIL*, *FasL*, *OX40L*, *CD40L*, *CD27L*, and *CD30L* in wild type mice increased 130–250%, whereas in the TNF receptor-ablated mice increase in their expression was 29–98%. Expression of receptors *Fas*, *CD30*, *CD40*, and *OX40* in wild type mice increased 139–270%, whereas in TNF receptor-ablated mice this increase was 30–95%. Increase in expression of apoptotic members of Bcl-2 family in wild type mice was 160–230%,

TABLE III
Induction of fibroblast apoptosis by LPS or TNF

Human adult dermal fibroblasts were incubated for 24 h with increasing concentration of TNF (0–80 ng/ml) or LPS (0–1000 ng/ml). The extent of apoptosis was determined by ELISA. Each value represents the mean of five replicates \pm S.E.

LPS	Increased apoptosis after LPS stimulation	Increased apoptosis after TNF stimulation	TNF
ng/ml	%	%	ng/ml
0	0	0	0
0.1	7 \pm 5	264 \pm 10	10
1	3 \pm 2	350 \pm 10	20
10	5 \pm 2	325 \pm 30	40
100	5 \pm 1	345 \pm 20	60
1000	6 \pm 2	481 \pm 30	80

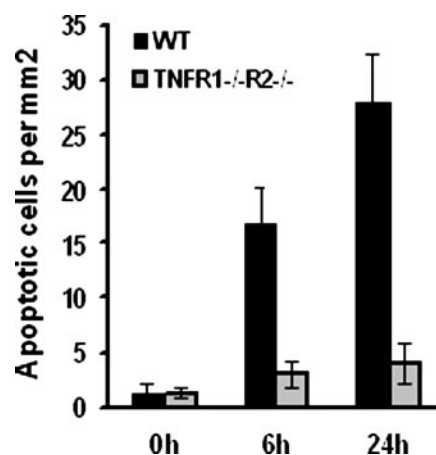


FIG. 2. Quantitative analysis of LPS-induced apoptosis of fibroblastic cells in *TNFR1^{-/-}R2^{-/-}* mice. The number of fibroblastic apoptotic cells was counted in matched wild type and *TNFR1^{-/-}R2^{-/-}* mice 0, 6, and 24 h after LPS. Each value represents the mean of six specimens \pm S.E.

whereas in *TNFR1^{-/-}R2^{-/-}* mice it was 22–55%. Decreased expression of anti-apoptotic members of bcl-2 family in wild type mice was 50–64%, whereas in *TNFR1^{-/-}R2^{-/-}* mice it was only 9–15%. Increase in expression of initiator caspases and executioner caspases in wild type mice was 170–260% in comparison to a 5–56% change *TNFR1^{-/-}R2^{-/-}*. The expression of adaptor proteins FADD and CRADD in wild type mice increased 245–270%, whereas in the *TNFR1^{-/-}R2^{-/-}* mice they increased 50–60%. LPS induced expression of TRAF family members *TRAF2*, *TRAF5*, *Apaf-1*, *p53*, and *XIAP* in wild type mice by 140–400%. In TNF receptor-ablated group induction was only 15–76%.

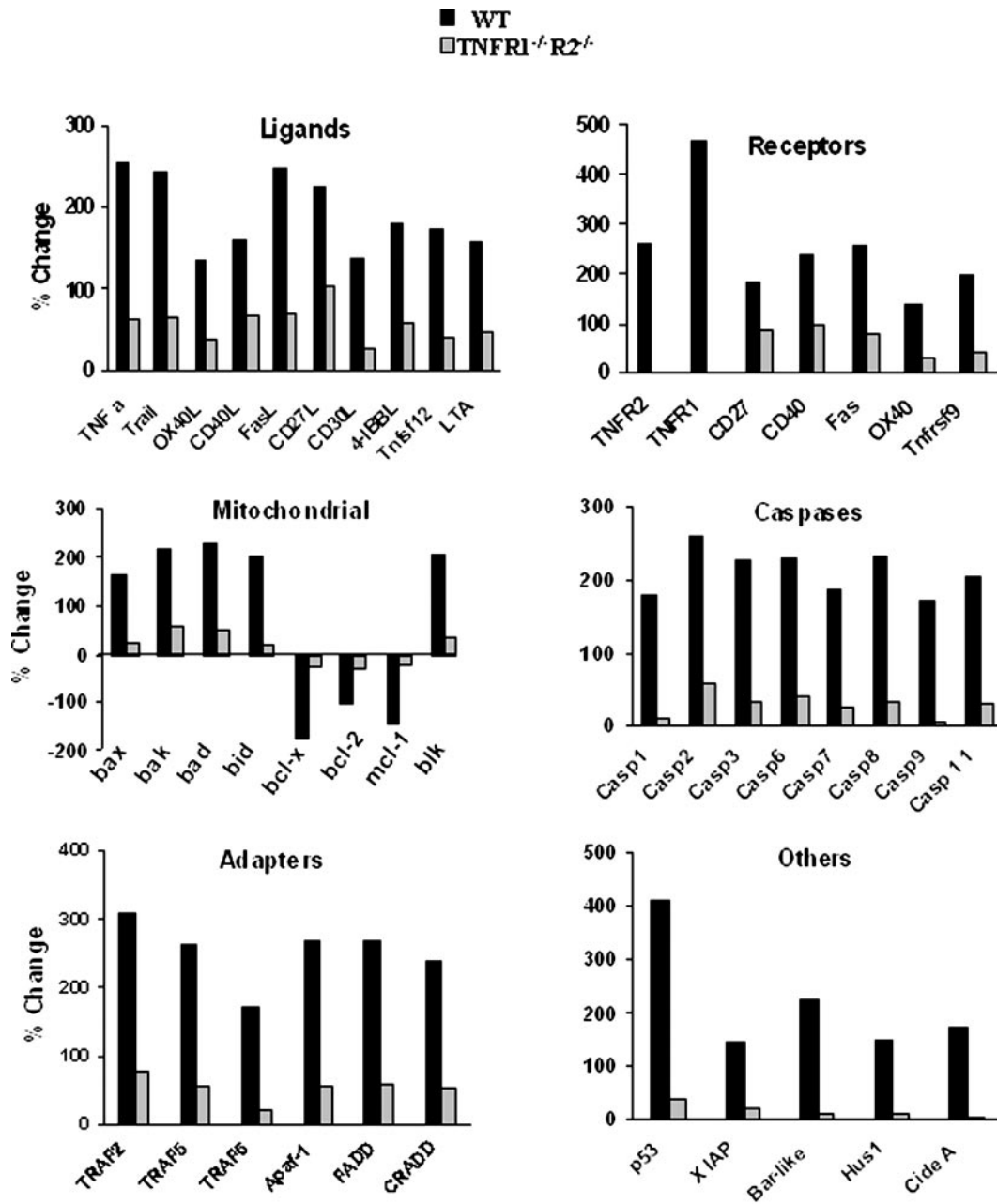


FIG. 3. Microarray analysis of apoptotic gene expression in wild type (WT) and TNFR1^{-/-}R2^{-/-} mice. Total RNA isolated 24 h after injection of LPS or vehicle alone as described in Fig. 1 was subjected to microarray analysis. The expression of apoptotic genes was normalized to the average signal of both GAPDH and β -actin expression. Plasmid DNA (PUC18) was used as a negative control for background subtraction. Genes with more than a 2-fold change in signal intensity are shown. Each value represents the mean of duplicate experiments.

To confirm data obtained with the microarray, RNase protection assays were carried out, and expression of selective apoptotic genes was compared at 24 h (Table IV). These assays demonstrated that in response to LPS, the expression of *TNF*, *FasL*, *TRAIL*, *Fas*, *caspase-2*, *caspase-3*, *caspase-6*, *caspase-7*, *caspase-8*, *TRADD*, *CRADD*, and *FADD* in wild type mice increased 159–274%. Moreover, the degree of up-regulation was very similar to that observed in microarrays (Table I). In TNF receptor-deficient mice this increase was far less ranging from only 20 to 86%. The difference between wild type mice and TNFR1^{-/-}R2^{-/-} mice for all genes was statistically significant ($p < 0.05$). The direct effect of TNF on expression of apoptotic genes in fibroblasts was assessed *in vitro* using the same set of riboprobes (Table IV). TNF increased the expression of *TNF*, *FasL*, *TRAIL*, *Fas*, *caspase-2*, *caspase-3*, *caspase-6*, *caspase-7*, *caspase-8*, *TRADD*, *CRADD*, and *FADD*

130–220%. It is striking that the same genes up-regulated by LPS *in vivo* were induced by TNF *in vitro* with the degree of stimulation being similar.

The Functional Significance of the Induction of Pro-apoptotic Genes—The functional significance of the induction of pro-apoptotic genes in fibroblasts was demonstrated by sensitizing fibroblasts prior to stimulation of apoptosis (Fig. 4). Pre-treatment of cells with TNF- α (20 ng/ml) for 24 h greatly increased the level of fibroblast apoptosis to a second incubation with TNF- α (10 ng/ml). This resulted in a 3.6-fold higher level of apoptosis compared with cells that received the second dose of TNF- α but not the first. Thus, stimulation with TNF- α twice showed considerably synergy when compared with the additive effect of the single incubations.

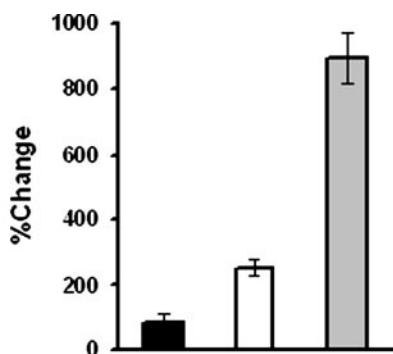
In Vivo Effects of LPS on Activation of Caspases-3, -8, and -9—To further investigate mechanisms of LPS-induced fibro-

TABLE IV

Apoptotic gene expression induced by LPS *in vivo* or TNF *in vitro*

Total RNA was extracted from tissue 24 h following inoculation of LPS (200 μ g) or vehicle alone or in fibroblast cell cultures, 24 h after TNF stimulation (20 ng/ml). Gene expression was measured by RPA, and the densitometric value of each band was normalized by the value for GAPDH in the same lane. The percent change shown for each experimental value is compared to vehicle alone. Each value represents the mean of 3 RPA \pm S.E.

Gene	LPS- <i>in vivo</i> , WT	LPS- <i>in vivo</i> , TNFR1 ^{-/-} R2 ^{-/-}	TNF- <i>in vitro</i>
		% Increase	
<i>Caspase-2</i>	274.0 \pm 11.9	68.9 \pm 8.3	198.9 \pm 9.1
<i>Caspase-3</i>	267.4 \pm 21.3	56.5 \pm 7.2	220.6 \pm 8.6
<i>Caspase-6</i>	191.9 \pm 17.1	24.8 \pm 5.4	141.5 \pm 18.2
<i>Caspase-7</i>	230.9 \pm 10.1	47.8 \pm 5.4	212.9 \pm 20.7
<i>Caspase-8</i>	255.2 \pm 22.2	20.2 \pm 9.1	180.1 \pm 18.7
<i>CRADD</i>	209.1 \pm 24.2	30.9 \pm 8.1	173.9 \pm 17.7
<i>FADD</i>	186.8 \pm 18.9	26.7 \pm 8.2	177.0 \pm 14.8
<i>Fas</i>	159.1 \pm 15.0	49.1 \pm 8.2	129.8 \pm 17.2
<i>FasL</i>	272.4 \pm 12.4	75.6 \pm 13.2	139.9 \pm 9.8
<i>TNF</i>	215.1 \pm 28.4	28.5 \pm 8.3	199.2 \pm 31.3
<i>TRADD</i>	232.0 \pm 15.4	31.3 \pm 6.3	151.4 \pm 16.3
<i>TRAIL</i>	274.7 \pm 24.5	86.4 \pm 14.5	200.5 \pm 21.7



TNF- α Pre-treatment	+	-	+
TNF- α Stimulation	-	+	+

FIG. 4. Pre-treatment with TNF- α causes a synergistic increase in apoptosis induced by a second stimulation with TNF- α . Cells were exposed to TNF- α (20 ng/ml) and then stimulated with a second dose of TNF- α (10 ng/ml) as described under "Experimental Procedures." For controls, one group of cells was stimulated with either the first or second dose of TNF- α but not both. The extent of apoptosis was determined by ELISA. Optical density was measured, and results were normalized based on cell number. The percent change values after TNF stimulation in compare with non-stimulated cells are displayed. Each value represents the mean of six replicates \pm S.E.

blast apoptosis, the activation of initiator and executioner caspases was measured following LPS injection *in vivo*. At 6 h LPS increased caspase-3 activity 450%, whereas it increased caspase-8 activity 580% but caspase-9 activity by only 90%. At 24 h a similar pattern was observed with LPS-stimulating caspase-3 activity by 730%, caspase-8 by 550%, and caspase-9 by 140% (Fig. 5A). To investigate the effect of caspase-8 or -9 on caspase-3 activity, mice were injected with LPS with or without specific inhibitors to caspase-8 or -9. Caspase-8 inhibitor was able to completely block caspase-3 activation, whereas the caspase-9 inhibitor had no effect (Fig. 5B).

DISCUSSION

It is well known that bacterial infection causes tissue destruction and is one of the frequently cited causes of tissue injury (40). Two separate processes are involved; one is the destruction of matrix and the other is the death of cells within the tissue. Fibroblasts are one of the principle cell types found

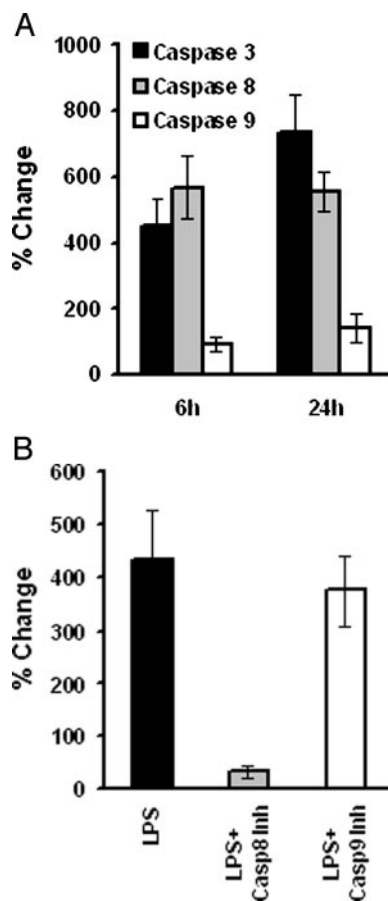


FIG. 5. Caspase activity in response to LPS injection. Caspase activity was measured using fluorometric assays in lysates from tissues obtained at different time points following inoculation of LPS or vehicle alone. A, caspase-3, -8, and -9 activities in wild type mice after LPS injection. B, caspase-3 activity in wild type mice after treatment with LPS, LPS, and caspase-8 inhibitor or LPS and caspase-9 inhibitor. The percent change in each experimental value is shown in comparison to vehicle alone. Each value represents the mean of six specimens \pm S.E.

in connective tissue and is responsible for the maintenance and repair of this tissue. To study how bacteria may affect fibroblasts, we injected LPS into the scalp and examined fibroblast apoptosis. Results with the TUNEL assay showed that LPS stimulated more than a 14-fold increase in fibroblast apoptosis, demonstrating that LPS has a potent effect on stimulating programmed cell death in this important connective tissue cell type *in vivo*.

To investigate mechanisms by which LPS could induce apoptosis we carried out *in vivo* and *in vitro* studies. The results indicate that LPS-stimulated fibroblast apoptosis is predominantly mediated through TNF receptor signaling, rather than through the direct effect of LPS. This is based on findings that *in vitro* LPS was not able to induce apoptosis, whereas *in vivo* studies demonstrated that LPS-induced fibroblast apoptosis was largely dependent on TNF, a cytokine with pro-apoptotic and anti-apoptotic effects (41–43). This may be significant, because it has been shown that TNF is elevated in chronic infection and delayed wound healing (44, 45).

In microarray assays, LPS *in vivo* stimulated global expression of 37 out of 71 tested pro-apoptotic genes and down-regulated 3 out of 25 anti-apoptotic genes. It is striking that in no instance was a pro-apoptotic gene substantially down-regulated or an anti-apoptotic gene up-regulated. Virtually identical results were obtained when the expression of 13 of these genes was re-examined by RPA, validating the results obtained by RNA profiling with microarrays. Increased ex-

pression of ligands such as *TNF*, *Fas*, *TRAIL*, and their related receptors was accompanied with enhanced gene expression of many adapter proteins that transfer signals from receptors to initiator caspases. This indicates that LPS is able to up-regulate expression of many genes that participate in the extrinsic (cytoplasmic) pathway of apoptosis. The intrinsic (mitochondrial) pathway was also modulated to enhance apoptosis as evidenced by increased expression of pro-apoptotic members of *bcl-2* family and down-regulation of anti-apoptotic members of this family. These results are the first that we are aware of that have examined the simultaneous regulation of members of several different gene families that participate in apoptosis following insult by bacteria or bacterial products.

Two approaches were taken to establish the mechanisms through which LPS induces fibroblast apoptosis *in vivo*. In the first, LPS was shown to induce apoptosis in wild type but not TNF receptor-ablated mice. In addition, the up-regulation of pro-apoptotic genes was greatly attenuated in TNFR1^{-/-}R2^{-/-} compared with wild type mice. Thus, TNF receptor signaling is an essential component of LPS-stimulated apoptosis. Consistent with this, LPS *in vitro* completely lacked the capacity to induce fibroblast apoptosis. This is in contrast to other cell types in which LPS is directly apoptotic (15, 23, 24, 26–28). In other studies this activity was further defined by demonstrating that LPS mediated apoptosis through TNFR1 rather than TNFR2 (data not shown). Because there are many cell types that are capable of producing TNF, it is possible that LPS-induced TNF acts in a paracrine manner to stimulate apoptosis. Thus, the target of LPS may be a cell type other than fibroblasts that induces fibroblast apoptosis through TNF.

In the second set of mechanistic studies, the specific caspase pathway of LPS-mediated apoptosis was examined *in vivo*. This was undertaken because microarray data indicated that LPS induced pro-apoptotic genes that participate in the cytoplasmic as well as the mitochondrial pathway. Furthermore, in other cells, LPS may modulate caspase-3 through either the cytoplasmic, caspase-8-dependent or mitochondrial, caspase-9-dependent pathways or both (25); (35–37). By using specific inhibitors we were able to show that activation of caspase-3 in response to LPS injection was depended on caspase-8 activation and completely independent of caspase-9. This would indicate that LPS-mediated apoptosis occurs in connective tissue via the cytoplasmic pathway without contribution from the mitochondrial pathway.

Injection of LPS induced a relatively high level of fibroblast apoptosis. Fibroblasts are responsible for synthesis and maintenance of the extracellular framework that provides structural support for various tissues and organs. The activity of fibroblasts is particularly important during growth and during healing following injury (46–49). It has been shown that during a chronic infection such as periodontal disease, there is a reduction in the number of fibroblasts (50–52). Fibroblast reduction also is reported following Gram-negative infection by *Helicobacter pylori* in human gastritis and gastroduodenal ulcers. This reduction is thought to contribute to ulcer formation (53). Thus, bacterial LPS may significantly contribute to tissue damage associated with infection by inducing TNF expression thereby stimulating expression of pro-apoptotic genes and increasing the probability that apoptosis occurs. That pre-treatment of fibroblasts *in vitro* with TNF- α caused a synergistic increase in apoptosis stimulated by a second exposure to TNF- α indicates that the global change in pro-apoptotic gene expression induced by the initial TNF- α stimulation is functionally significant. This may also explain a mechanism for the fre-

quently reported synergy that is observed between TNF and Fas ligand in inducing apoptosis (54, 55). TNF by global induction of pro-apoptotic genes and down-regulation of anti-apoptotic genes may enhance the sensitivity to Fas ligand at several steps in the Fas-mediated pathway. Similarly, LPS, by regulating the expression of multiple apoptotic gene families, can lower the threshold of surviving cells and sensitize them for future apoptotic signals.

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