Targeted Mutation of TNF Receptor I Rescues the RelA-Deficient Mouse and Reveals a Critical Role for NF-κB in Leukocyte Recruitment[1]

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NF-κB binding sites are present in the promoter regions of many acute phase and inflammatory response genes, suggesting that NF-κB plays an important role in the initiation of innate immune responses. However, targeted mutations of the various NF-κB family members have yet to identify members responsible for this critical role. RelA-deficient mice die on embryonic day 15 from TNF-α-induced liver degeneration. To investigate the importance of RelA in innate immunity, we genetically suppressed this embryonic lethality by breeding the RelA deficiency onto a TNFR type 1 (TNFR1)-deficient background. TNFR1/RelA-deficient mice were born healthy, but were susceptible to bacterial infections and bacteremia and died within a few weeks after birth. Hemopoiesis was intact in TNFR1/RelA-deficient newborns, but neutrophil emigration to alveoli during LPS-induced pneumonia was severely reduced relative to that in wild-type or TNFR1-deficient mice. In contrast, radiation chimeras reconstituted with RelA or TNFR1/RelA-deficient hemopoietic cells were healthy and demonstrated no defect in neutrophil emigration during LPS-induced pneumonia. Analysis of RNA harvested from the lungs of mice 4 h after LPS insufflation revealed that the induction of several genes important for neutrophil recruitment to the lung was significantly reduced in TNFR1/RelA-deficient mice relative to that in wild-type or TNFR1-deficient mice. These results suggest that TNFR1-independent activation of RelA is essential in cells of nonhemopoietic origin during the initiation of an innate immune response. *The Journal of Immunology,* **2001, 167: 1592–1600.**

The NF-κB/Rel family of transcription factors is believed to be an important regulator of innate immunity in species as diverse as insects and mammals (reviewed in Refs. 1–3). The mammalian transcription factors are homodimeric and heterodimeric complexes of five family members, p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB, and RelA (p65), that are held inactive in the cytoplasm by association with IκB inhibitory proteins. Activation of the cell with a wide variety of different stimuli leads to degradation of IκBα and nuclear translocation of NF-κB, resulting in the transcription of multiple target genes necessary for acute phase, inflammatory and immune responses (reviewed in Refs. 4–6). Mice deficient for one or more of these family members have been generated, revealing both redundant and nonredundant biological roles for the different proteins (7–17). These roles include promoting cell survival, regulating hemopoiesis, and controlling innate and adaptive immune responses (reviewed in Refs. 18 and 19).

The role of RelA in immune protection remains unclear because targeted mutation of RelA results in lethality on embryonic day 15 (E15)[5] from extensive liver degeneration (7). Studies of the RelA-deficient immune system using radiation chimeras generated with fetal liver-derived hemopoietic progenitors revealed that a RelA-deficient hemopoietic system develops normally, but that lymphocyte responses are impaired (9, 20). Nonetheless, these chimeras were relatively healthy, suggesting that RelA function is not essential in hemopoietic cells for innate immune protection. However, this does not obviate the potential importance of RelA in the response of nonhemopoietic tissue to infection.

Mouse embryonic fibroblast cells and 3T3 cells cultured from RelA-deficient animals undergo apoptosis when treated with TNF-α (21), as do Jurkat cells (22), human and mouse fibroblasts (22), and HT1080 cells (23) that express dominant-negative mutants of IκBα. These results revealed that TNF-α-induced activation of NF-κB, and specifically RelA, protects cells from the cytocidal effects of TNF-α in vitro and suggested that genetically abrogating the apoptotic TNF-α signal might suppress the extensive apoptosis in the RelA-deficient fetal liver and rescue the RelA-deficient mice. However, it was unclear which TNF receptor, TNFR1 (p55) or TNFR2 (p75), elicited the apoptotic response that must be counteracted by NF-κB activity for cells to survive. Both can mediate activation of NF-κB, and both have been implicated in programmed cell death.

The extensive apoptosis and subsequent liver degeneration observed in the RelA-deficient fetuses can be genetically suppressed

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[5] Abbreviations used in this paper: Ex, embryonic day x; TNFR1, TNF receptor 1; Px, postnatal day x; MIP2, macrophage inflammatory protein 2; mTNF-α, mouse TNF-α.

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by breeding the RelA deficiency into a TNF-α-deficient background (24), thereby illustrating that TNF-α is cytotoxic to cells in the absence of RelA in vivo as well as in vitro. Furthermore, it has been shown that the absence of TNFR1 suppresses RelA-like fetal liver apoptosis observed in mice deficient for IKK2 (25), one of the signaling molecules implicated in NF-κB activation. Finally, it has recently been reported that the absence of TNFR1 genetically suppresses the phenotype of the RelA-deficient mouse, indicating that TNFR1 is the mediator of this cytotoxic TNF-α signal (26), and that the resulting TNFR1/RelA-deficient mouse is susceptible to endogenous hepatic infection. We report these results as well, but also extend our observations to demonstrate that this susceptibility to infection is not liver specific, but, rather, is a more global phenomenon, revealing a critical role for the NF-κB family member RelA in the innate immune response.

Materials and Methods

Mice

TNFR1/RelA-deficient mice were established from TNFR1-deficient and RelA heterozygous mice (both 129/Sv $\times$ C57BL/6J) and subsequently maintained by intercrosses (27, 28). Mice were genotyped by PCR amplification of tail DNA. DNA was prepared by digesting samples 6 h to overnight at 55°C in 400 ml 67 mM Tris (pH 8.8), 16.6 mM ammonium sulfate, 6.5 mM $MgCl_2$, 0.5% Triton X-100, 1% 2-ME, and 0.2 mg/ml proteinase K, then heating at 95°C for 5 min. One microliter of the supernatant was used per 25 μl reaction. Amplification of the RelA locus was by PCR (1 min at 94°C and 2.5 min at 66°C for 30 cycles) using three primers, 5′-AAT CGG ATG TGA GAG GAC AGG-3′, 5′-CCT ATA GAG GAG CAG CGC GGG-3′, and 5′-AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG-3′, that recognized the intact *RelA* locus (oligonucleotides 1 and 2) and the targeted *RelA*-neo locus (oligonucleotides 2 and 3). Amplification of the *TNFR1* locus was achieved by PCR (1 min at 94°C, 0.5 min at 63°C, and 1.5 min at 72°C for 30 cycles) using three primers, 5′-TGT GAA AAG GGC ACC TTT ACG GC-3′, 5′-GGC TGC AGT CCA CGC ACT GG-3′, and 5′-ATT CGC CAA TGA CAA GAC GCT GG-3′, that recognized the intact *TNFR1* locus (oligonucleotides 1 and 2) and the neo-targeted *TNFR1* locus (oligonucleotides 2 and 3).

TUNEL assay

Wild-type, $RelA^{+/-}$, and $TNFR1^{-/-}RelA^{+/-}$ females were impregnated by matched males and sacrificed 15.5, 16.5, and 17.5 days postcoitum by cervical dislocation. Livers were harvested from fetuses and fixed in buffered formalin at 4°C for 2 h, embedded in paraffin, and sectioned (4–6 μm). Sections were deparaffinized and washed in water. They were then preincubated for 5 min in 10 mM Tris (pH 8.0)-20 mM EDTA, incubated for 10 min in 10 μg/ml proteinase K in 10 mM Tris (pH 8.0)-20 mM EDTA, and rinsed in water. Fragmented ends of DNA were labeled by incubating 1 h at 37°C in 1$\times$ TdT buffer with 15 U TdT (Life Technologies, Gaithersburg, MD) and 5 nM biotinylated dUTP (Roche, Indianapolis, IN). The reaction was stopped by washing twice in 2$\times$ SSC and rinsing in PBS. Sections were blocked for 1 h with 2% BSA in PBS, then rinsed again in PBS. Biotinylated dUTP was visualized using an alkaline phosphatase Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Tissue culture

Viability assays of fibroblastic and 3T3 cells were performed essentially as previously described (21). Briefly, 200,000 cells of each genotype were plated per well of a six-well dish in DMEM supplemented with 10% FBS. Twenty-four hours later the medium was replaced with DMEM-0.1% FBS with or without 10 ng/ml mouse TNF-α (mTNF-α; sp. act., 6.0×10^7 U/mg; Roche) and incubated 8 or 24 h. The cells were trypsinized and assayed for trypan blue exclusion. Percentage of viability was defined as the number of cells remaining per well following serum starvation in the presence of mTNF-α divided by the number of cells remaining per well following serum starvation in the absence of mTNF-α. Two cell lines were used per genotype, and each experiment was performed twice.

Histology

Moribund mice were sacrificed by CO_2 inhalation, and tissues were fixed by immersion in Bouin's solution (Sigma, St. Louis, MO) for 3 days. Tissues were embedded in paraffin, sectioned (4–6 μm thick), stained with H&E, and examined by light microscopy.

Flow cytometry

Spleen and thymus were harvested and prepared as single-cell suspensions by crushing between two slides and filtering through sterile mesh cell strainers (Applied Scientific, South San Francisco, CA). Bone marrow was harvested from the femur, passaged through a 26-gauge needle to make a single-cell suspension, and filtered through a cell strainer. Blood was collected from the severed necks of postnatal day (P) 3–5 pups and from the inferior vena cava in radiation chimeras. Bronchoalveolar lavage of radiation chimeras was performed as previously described (29). All samples were subjected to red cell lysis with ammonium chloride before staining.

For flow cytometric analysis, cells were first incubated with Fc-block (anti-CD32/CD16, FcγII/IIIR, 2.4G2; BD PharMingen, San Diego, CA) for 5 min. They were then incubated with combinations of the following primary and secondary Abs: anti-GR-1-biotin (Ly-6G; BD PharMingen), anti-Mac-1-FITC (M1/70; BD PharMingen), anti-TER-119-biotin (BD PharMingen), anti-Pan-NK-FITC (Dx5; BD PharMingen), anti-B220-biotin (RA3-6B; BD PharMingen), anti-IgM-FITC (R6-60.2; BD PharMingen), anti-CD4-PE (RM4-5; BD PharMingen), anti-CD8-FITC (Ly-2; Caltag, South San Francisco, CA), anti-F480-biotin (Caltag), anti-CD45.1-FITC (A20; BD PharMingen), anti-CD45.2-FITC (104; BD PharMingen), streptavidin-PE (BD PharMingen), and streptavidin Cy-Chrome (BD PharMingen; used with anti-F480-biotin only). Three mice of each genotype were analyzed; shown is a representative plot of each genotype.

Reconstitution of bone marrow with fetal liver cells

For radiation chimera experiments, $TNFR1^{+/-}RelA^{+/-}$ males and females were crossed to generate $TNFR^{+/+}RelA^{+/-}$ and $TNFR1^{-/-}RelA^{+/-}$ mice. Donor embryos were then generated by crossing $TNFR^{+/+}RelA^{+/-}$ males to females and $TNFR1^{-/-}RelA^{+/-}$ males to females. Fetal livers were harvested from day 14.5 embryos, and prepared and genotyped as previously described (12). Meanwhile, 6- to 8-wk-old C57BL/6 $CD45.1^+$ hosts were delivered two doses of irradiation (800 and 400 rad, separated by 3 h) using a ^{137}Cs source. Mice were anesthetized with avertin (2.5% solution of 2,2,2-tribromoethanol-tert amyl alcohol, 12 μl/g mouse) immediately after the second irradiation and transplanted with 1×10^6 liver cells from wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetuses in 200 μl medium by retro-orbital injection with a 26-gauge needle. The extent of reconstitution was analyzed by flow cytometry 4 wk after transplantation, and LPS-pneumonia experiments were performed 6 wk after transplantation.

Thioglycolate-induced peritonitis

Mice, aged 14–16 days, were anesthetized with an i.p. injection of avertin and retro-orbitally bled with Unopettes (BD Biosciences, Mountain View, CA). Blood cells were quantitated with a hemocytometer according to the manufacturer's instructions to determine total circulating leukocyte counts. Immediately after being bled, mice received an i.p. injection of sterile 2.98% thioglycolate broth (50 μl/g mouse; Sigma) to induce peritonitis. Mice were sacrificed with CO_2 6 or 48 h later, and the peritonea were washed with 3 ml ice-cold lavage solution (0.1% BSA-0.65 mM EDTA-heparin at 20 U/ml) delivered with a 26-gauge needle. After massaging for 30 s, 2 ml lavage solution was harvested from each peritoneum with an 18-gauge needle. Concentrations of leukocytes and cellular differentials were quantitated by hemocytometer counts and cytospin preparations, respectively.

LPS-induced pneumonia

Pneumonia was induced in mice, aged 3–5 days, by intranasal insufflation. Mice were anesthetized by inhalation of 2% halothane. Ten microliters of a solution of *Escherichia coli* LPS (2 mg/ml; Sigma L-2880) and colloidal carbon (5%) in PBS was placed into the nares and allowed to be inhaled. Six hours later, mice were sacrificed by an overdose of halothane. Lungs were removed, intratracheally instilled with 6% glutaraldehyde under 22 cm H_2O pressure, tied off, and submerged in 6% glutaraldehyde overnight. Fixed tissue was embedded in paraffin, sectioned (5–7 μm), and stained with H&E.

Pneumonia was induced in radiation chimeras by intratracheal instillation as previously described (30). Mice were anesthetized by i.m. injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg). The tracheas were surgically exposed, and 50 μl of a solution of *E. coli* LPS (2 mg/ml) and colloidal carbon (5%) was instilled intratracheally. Six hours later lungs were removed and prepared for analysis as described above.

Neutrophil emigration was quantitated by morphometry of histological sections essentially as previously described (30). Briefly, a multipurpose

test system containing 42 points was reflected onto the microscope field using a drawing tube. Fields of pneumonic peripheral lung were randomly selected for analysis. As LPS is only heterogeneously deposited into lungs following intranasal insufflation, sections that did not contain colloidal carbon-laden macrophages were not scored, and another region was randomly selected. The 42 points of the counting grid were classified as landing on 1) air space or tissue and 2) neutrophil or not a neutrophil. Ten fields (420 points) were assayed per lung. Statistical significance was determined by ANOVA.

Emigration was quantitated in at least six P3–5 mice per genotype, and in at least four radiation chimeras per donor genotype. P3–5 mice that were runted and lethargic were excluded from analysis, as were those with chronic organizing pneumonia, as these observations suggested preexisting illness. In addition, lungs of mice that had received a poor instillation of LPS, as defined by the macroscopic absence of colloidal carbon, were not analyzed.

RNA analysis

Mice, aged P3–5, were intranasally insufflated with *E. coli* LPS as described above. Four hours later pups were sacrificed by decapitation, and the lungs were excised and snap-frozen in liquid nitrogen. When all tissue samples had been dissected and frozen, samples were simultaneously thawed and homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) in 1 ml TRIzol reagent (Life Technologies). Total RNA was extracted and precipitated according to the manufacturer's instructions.

For Northern blot analysis, 20 μg RNA from five animals of each genotype was electrophoresed separately on a 1.2% agarose gel containing 0.38 M formaldehyde, and transferred overnight by standard capillary action to a Hybond N^+ membrane filter. ICAM-1 message was detected using a probe generated by RT-PCR that spanned from the third Ig repeat to the 3′ untranslated region. Superscript II reverse transcriptase (Life Technologies) and an oligo(dT) primer were used for first-strand synthesis of 10 μg total RNA from an LPS-treated wild-type lung sample. ICAM-1 cDNA was amplified using 1/25th of this reaction mix, Pfu polymerase (Stratagene, La Jolla, CA), and oligonucleotide primers (5′-GCG GAT CCG ATC TTC CAG CTA CCA TCC CAA AG-3′ and 5′-GCG AAT TCG TTC TGT GAC AGC CAG AGG AAG TG-3′). Probes for macrophage inflammatory protein 2 (MIP2) and KC were those used previously (31). The GAPDH transcript was detected with a probe to human cDNA (Clontech, Palo Alto, CA). All probes were randomly labeled to a sp. act. of at least 1×10^9 dpm/μg with a Prime-It RmT Random Primer Labeling Kit (Stratagene) and were cleaned over a Sephadex G-25 column (Roche). Prehybridization and hybridization were performed with Church buffer plus 10% dextran sulfate. Hybridization of all probes was conducted overnight at 65°C. After hybridization, blots of ICAM-1 and GAPDH were washed at 65°C with two 15-min low stringency washes in 2× SSC/1% SDS, followed by two 30-min high stringency washes in 0.1× SSC/1% SDS. Blots of MIP2 and KC received two low stringency washes and one high stringency wash. Blots were exposed to film as well as scanned on a phosphor imager for quantitation with ImageQuant software (BD Biosciences). Area was quantified by a line 11 pixels wide, after which background was subtracted. Three animals were quantitated per genotype.

Results

Inactivation of TNFR1 rescues RelA-deficient mice from embryonic lethality

RelA heterozygous mice (7) were bred with TNFR1 homozygous mutant mice (27), and the F_1 progeny subsequently bred to generate TNFR1/RelA homozygous mutant mice, which were born in the expected Mendelian ratios. Gross dissection and serial tissue sectioning and staining with H&E revealed the absence of lymph nodes and Peyer's patches and a disorganized splenic white pulp.[6] No other histological or morphological abnormalities were observed.

The viability of the TNFR1/RelA-deficient mice demonstrated that TNFR1 mediates the signals that induce embryonic lethality in the RelA-deficient mice. To determine whether and to what extent apoptosis in the fetal liver was suppressed, fetal livers from day 15–17 wild-type, RelA-deficient, or TNFR1/RelA-deficient embryos were harvested and analyzed by TUNEL and H&E staining. As previously reported (7), significant apoptosis was readily observed in the livers of most E15.5 RelA-deficient fetuses, and no viable E16 RelA fetuses could be recovered. In contrast, livers from wild-type and TNFR1/RelA-deficient E15.5, E16, and E17 fetuses displayed substantially less apoptosis (Fig. 1*A*). This suggests that TNFR1 mediates cytotoxicity of TNF-α in the RelA-deficient liver. To determine whether this were true in other cell types, we treated wild-type, RelA-deficient, TNFR1-deficient, and TNFR1/RelA-deficient mouse embryonic fibroblast and 3T3 lines with mTNF-α, which stimulates both TNFR1 and TNFR2, and

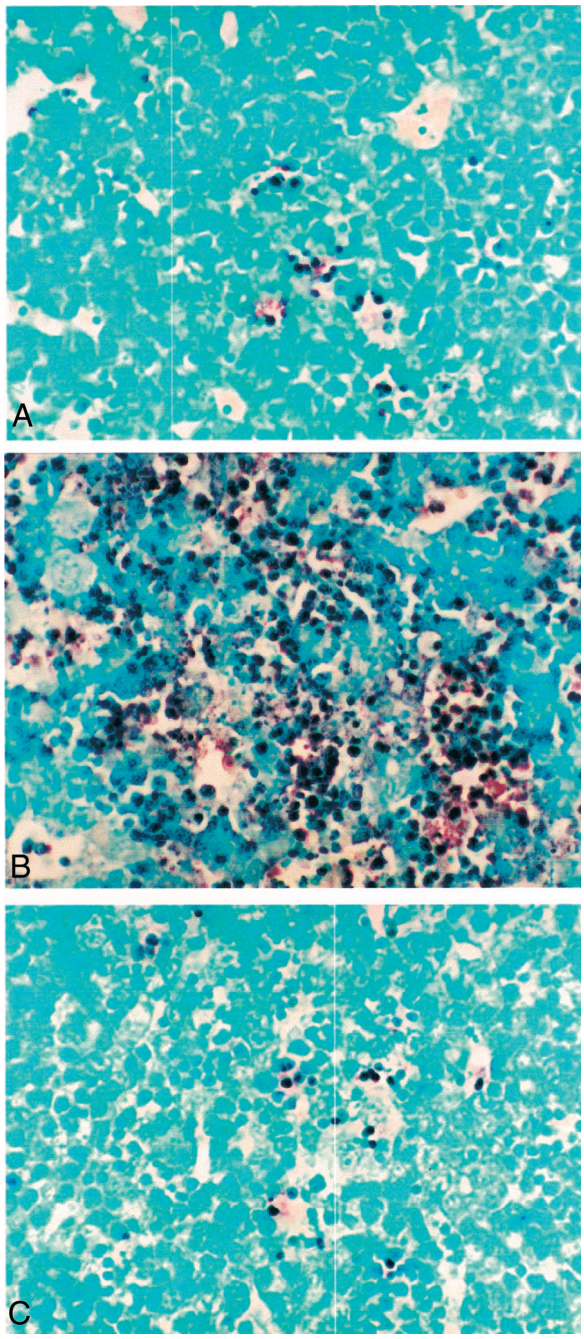

FIGURE 1. Apoptosis in wild-type, RelA-deficient, and TNFR1/RelA-deficient fetal livers. E15.5 fetal livers were harvested from viable RelA-deficient fetuses and compared with livers harvested from E15.5, E16, and E17 wild-type and TNFR1/RelA-deficient fetuses by TUNEL assay. *A*, Wild-type fetal liver on E16. *B*, RelA fetal liver on E15.5. *C*, TNFR1/RelA fetal liver on E16. Darkened nuclei are TUNEL-positive apoptotic cells. Sections are counterstained with methyl green.

[6] E. Alcamo, R. O. Hynes, and D. Baltimore. *Submitted for publication.*

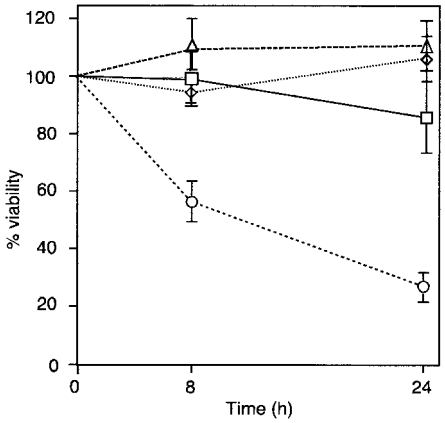

FIGURE 2. Sensitivity to TNF-α in wild-type, RelA-deficient, TNFR1-deficient, and TNFR1/RelA-deficient 3T3 cells. Cells were plated in DMEM-10% FBS at a high density and allowed to adhere for 24 h, after which the medium was changed to DMEM-0.1% FBS with or without 10 ng/ml mTNF-α. Viability was determined by trypan blue exclusion at the times indicated in the text. □, Wild-type cultures; ◇, TNFR1-deficient cultures; ○, RelA-deficient cultures; and △, TNFR1/RelA-deficient cultures.

assayed for viability by trypan blue exclusion. RelA-deficient cultures were significantly apoptotic 8 h following treatment with mTNF-α, but TNFR1/RelA-deficient mouse embryonic fibroblasts (data not shown) and 3T3s (Fig. 2) were as resistant to the cytotoxic potential of mTNF-α as wild-type or TNFR1-deficient cells.

Early mortality of TNFR1/RelA-deficient mice

Although TNFR1/RelA-deficient mice were born healthy, they began to appear runted, lethargic, and unkempt within a few days after birth, and 95% died by P20. Necropsy of moribund animals revealed the development of organized pneumonia (Fig. 3*A*) as well as bacterial aggregates associated with minimal inflammatory responses in the pharynx, s.c. fat, marrow, and gut (Fig. 3, *B* and *C*). Blood, lung, and liver tissue collected from five moribund mice was cultured for microorganisms; all preparations yielded *Pasteurella pneumotropica*.

To determine whether the susceptibility to bacterial infections was limited to a sensitivity to *P. pneumotropica*, the colony was treated with enrofloxacin, an antibiotic against Gram-positive bacteria, to clear the colony of *Pasteurella*, or was rederived into cleaner facilities, free of *Pasteurella, Pneumocystis*, and *Helicobacter pylori*. TNFR1/RelA-deficient mice still died very prematurely, although these measures delayed the onset of illness by 1 wk or more, and several mice survived >6 mo. Bacterial infections remained evident in moribund mice, although the affected tissues were predominantly the perivascular regions of the liver, kidney, and epicardium (Fig. 3*D*) instead of the lung. These lesions exhibited a paucity of neutrophils and an unusual accumulation of eosinophils. All five of the animals assayed from this cleaner facility were bacteremic as well, although blood microcultures yielded β-hemolytic streptococcus and *Staphylococcus aureus* instead of *Pasteurella*. Thus, the premature mortality of the TNFR1/RelA-deficient mice may be due to a susceptibility to infection by both common and uncommon mouse pathogens.

Hematopoiesis in TNFR1/RelA-deficient mice

The poor immune response to infection in TNFR1/RelA-deficient mice could be due to the aberrant production of leukocyte populations, as has been observed in PU.1-deficient (32) and C/EBPα-deficient mice (33). To determine whether hemopoiesis was intact in the absence of TNFR1 and RelA, the cellular compositions of primary and secondary hemopoietic tissues from P3 wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice were compared by flow cytometry (Fig. 4). The data indicate no substantial

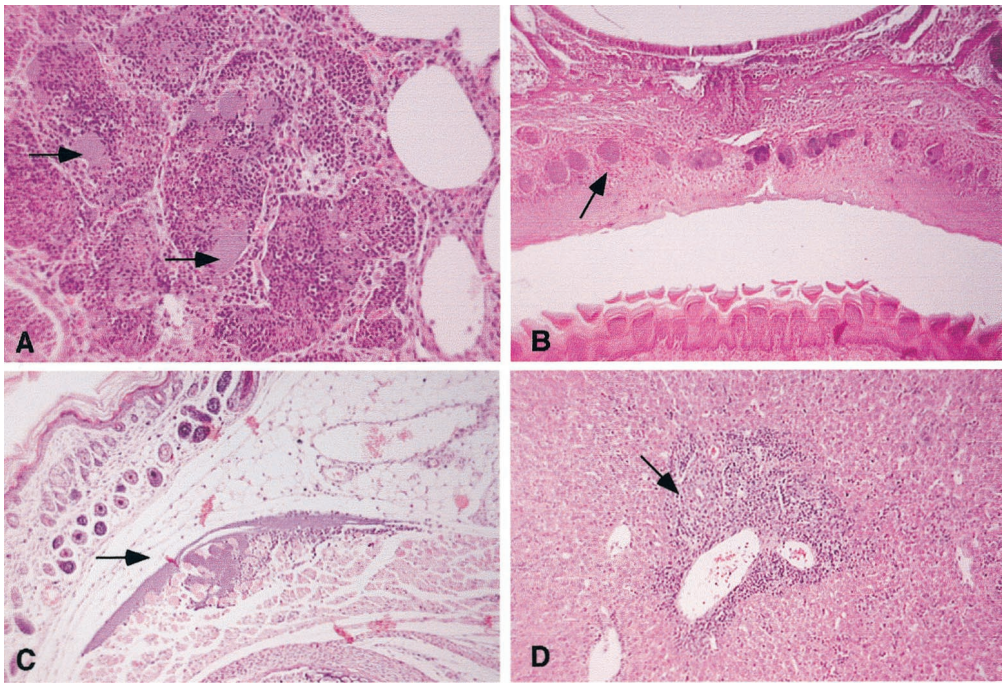

FIGURE 3. Histologic sections showing inflammatory foci in tissues from moribund TNFR1/RelA-deficient mice. Tissues were fixed with Bouin's fixative and stained by H&E. *A*, Organized pneumonia surrounding bacterial colonies (marked by arrows) in the lung; *B*, large bacterial colonies (arrows) in the pharynx; *C*, a large bacterial colony growing over the s.c. muscle of a *Pasteurella*-infected mouse. All mice were 8 days old. Note how minimal the inflammatory response is in the pharynx and muscle. *D*, Perivascular inflammation in the liver of a B-hemolytic streptococcus-infected mouse at 3.5 wk old. Arrows indicate bacterial infections. Original magnification, ×10.

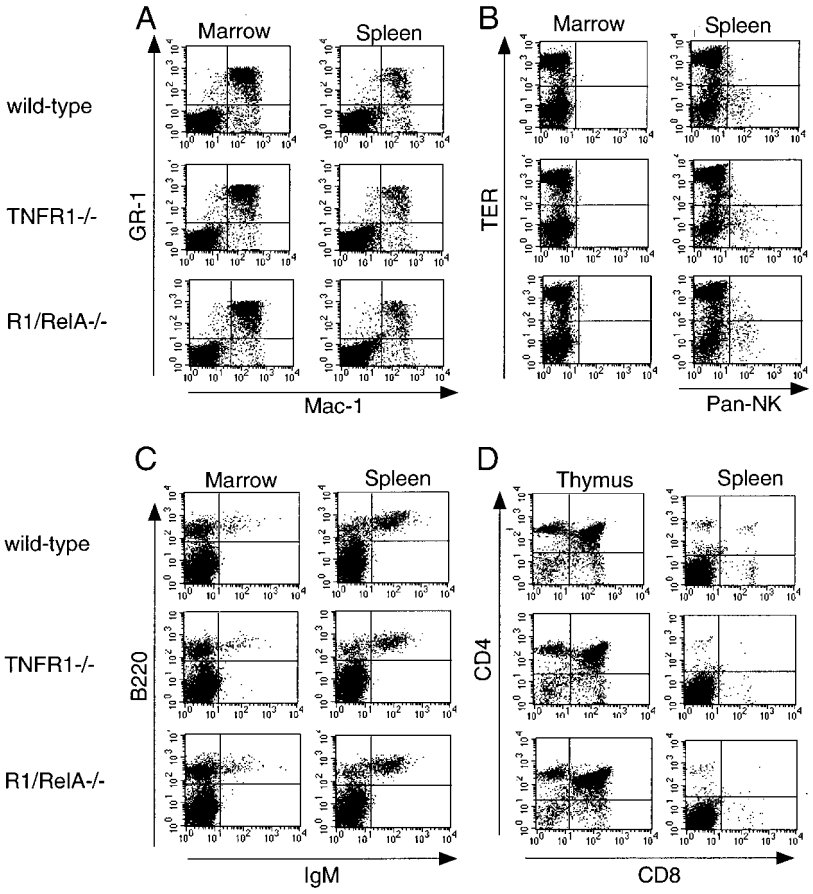

FIGURE 4. Hematopoietic cell populations in wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice at age P3. Flow cytometry was used to analyze the bone marrow, spleen, thymus, and peripheral blood (data not shown). *A*, Analysis of neutrophils (GR-1$^+$, Mac-1$^+$) and macrophages (GR-1$^-$, Mac-1$^+$). *B*, Analysis of erythrocytes (TER$^+$, Pan-NK$^-$) and NK cells (Ter$^-$, Pan-NK$^+$). *C*, Analysis of B-lineage cell (pro- and pre-B, B220$^+$, IgM$^-$; immature and mature, B220$^+$, IgM$^+$). *D*, Analysis of T-lineage cells (immature, CD4$^+$, CD8$^+$; mature, CD4$^+$, CD8$^-$ or CD4$^-$, CD8$^+$).

difference in the relative distribution of leukocyte populations in thymus, spleen, and peripheral blood in TNFR1/RelA-deficient mice. Total circulating leukocyte counts in peripheral blood were also comparable among newborns of the three genotypes (Table I), indicating no deficiency in generating appropriate numbers of circulating cells. However, leukocytosis was observed in the blood of TNFR1/RelA-deficient mice by P14 (Table I). In contrast, radiation chimeras generated with TNFR1/RelA-deficient hemopoietic progenitor cells did not have increased circulating leukocyte counts relative to those generated with wild-type or TNFR1-deficient progenitors (data not shown), suggesting that the leukocytosis in the TNFR1/RelA-deficient mice was not due to a cell intrinsic defect in hemopoietic cells.

Neutrophil emigration during thioglycolate-induced peritonitis

An attenuated inflammatory response to the spontaneous bacterial overgrowth observed in soft tissues could be due to a difficulty advancing leukocytes to sites of infection. To determine whether leukocyte recruitment was impaired in TNFR1/RelA-deficient mice, we measured neutrophil emigration into the peritoneal cavity of P15 mice following peritonitis induced by the sterile irritant thioglycolate. The number of leukocytes recovered by lavage before thioglycolate injection was elevated 2-fold in the TNFR1/RelA-deficient mice, although this was predominantly due to an increased number of eosinophils (data not shown). Substantial neutrophil accumulation in the peritoneal cavity of wild-type mice could be observed 6 h after thioglycolate injection; by 48 h these numbers had decreased to some extent (Fig. 5). In contrast, significantly fewer emigrated neutrophils were recovered from TNFR1-deficient mice at 6 h, and numbers comparable to wild-type numbers were recovered at 48 h (Fig. 5). These data demonstrated that TNFR1 is an important mediator of thioglycolate-induced neutrophil recruitment into the peritoneal cavity. TNFR1/RelA-deficient mice displayed a similar defect in neutrophil

Table I. *Total and differential leukocyte counts in wild-type, TNFR1-deficient, and TNFR1/RelA-deficient 2-wk-old pups*[a]

Genotype	Total Circulating Leukocytes	Neutrophils (%)	Eosinophils (%)	Mononuclear Cells (%)
Newborns				
Wild type	$3.1 \times 10^6 \pm 0.5 \times 10^6$	35.6 ± 8.0	1.7 ± 1.6	62.7 ± 7.4
TNFR1$^{-/-}$	$4.3 \times 10^6 \pm 0.9 \times 10^6$	32.2 ± 5.4	1.5 ± 1.1	66.3 ± 5.6
TNFR1$^{-/-}$RelA$^{-/-}$	$4.5 \times 10^6 \pm 1.5 \times 10^6$	32.4 ± 9.3	0.8 ± 0.9	66.8 ± 8.7
2-wk-old mice				
Wild type	$4.0 \times 10^6 \pm 1.3 \times 10^6$	13.2 ± 2.6	1.6 ± 0.9	85.2 ± 2.4
TNFR1$^{-/-}$	$4.0 \times 10^6 \pm 1.5 \times 10^6$	14.3 ± 5.6	1.3 ± 1.7	84.4 ± 5.9
TNFR1$^{-/-}$RelA$^{-/-}$	$25.9 \times 10^6 \pm 20.7 \times 10^6$	19.3 ± 11.5	2.5 ± 1.8	78.2 ± 12.0

[a] Blood was collected from wild-type, TNFR1-deficient, and TNFR1/RelA-deficient pups at P3 (collected from severed neck) or P14 (collected by eyebleed).

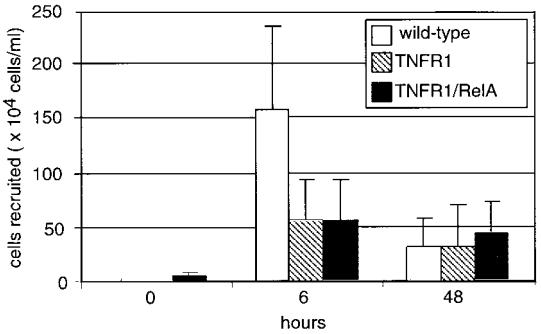

FIGURE 5. Neutrophil emigration during thioglycolate-induced peritonitis. Wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice, aged P14–16, were injected i.p. with thioglycolate. Neutrophil accumulation was quantitated 6 h later by lavage, followed by cytospin ($n = 20$).

emigration, suggesting that RelA activity independent of TNFR1 signaling is not important for neutrophil recruitment to the peritoneum.

Neutrophil emigration during LPS-induced pneumonia

To determine whether TNFR1-independent RelA activity was important for leukocyte recruitment to the lungs, we analyzed neutrophil emigration into pulmonary air spaces of P3–5 mice following delivery of *E. coli* LPS by intranasal insufflation. Before treatment, neutrophils were not observed in the uninflamed alveolar air spaces of the P3–5 TNFR1/RelA-deficient mice or wild-type controls (data not shown). Six hours after delivery, LPS-induced neutrophil emigration was observed in wild-type and TNFR1-deficient mice and did not significantly differ between these genotypes. In contrast, mice deficient for both TNFR1 and RelA showed significantly less neutrophil emigration compared with either wild-type or TNFR1-deficient mice (Fig. 6), suggesting that RelA activity independent of TNFR1 signaling is essential for initiating maximal neutrophil emigration in response to LPS in the pulmonary air spaces.

Neutrophil emigration during LPS-induced pneumonia in radiation chimeras

To determine whether TNFR1/RelA-deficient neutrophils were capable of being recruited into inflammatory foci, lethally irradiated C57BL/6 CD45.1$^+$ hosts were engrafted with wild-type or TNFR1-, RelA-, or TNFR1/RelA-deficient fetal liver hemopoietic progenitor cells, and the reconstituted mice were assayed for their ability to recruit neutrophils during LPS-induced pneumonia. Flow cytometry confirmed that virtually all peripheral blood neutrophils were donor derived (CD45.2$^+$; Fig. 7*A*). Six hours after delivery of LPS, neutrophil emigration was comparable in all radiation chimeras (Fig. 7*B*), suggesting that the emigration defect in the TNFR1/RelA-deficient mice is not a cell intrinsic defect of RelA-deficient neutrophils. Interestingly, the vast majority of alveolar macrophages, which are critical for inciting responses to bacteria in the lungs (34, 35), were donor derived as well (Fig. 7*A*). This suggests that RelA is not specifically required in alveolar macrophages for the induction of neutrophil emigration into the lungs in response to LPS.

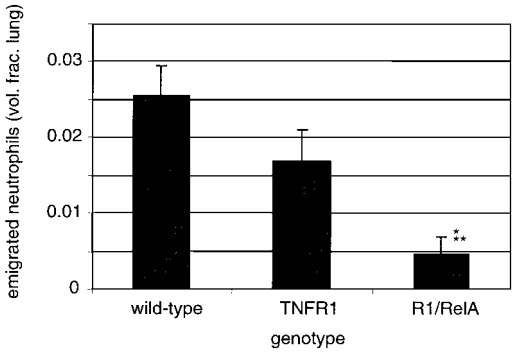

FIGURE 6. Neutrophil emigration during LPS-induced pneumonia. Wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice, aged P3–5, were intranasally insufflated with LPS. Neutrophil accumulation was quantitated in tissue 6 h later by morphometric analysis of histologic sections ($n = 6$). *, Statistically significant differences in response compared with wild-type and **, TNFR1-deficient tissues ($p < 0.05$).

Gene expression in TNFR1/RelA-deficient tissue during LPS-induced pneumonia

ICAM-1, KC, and MIP2 often regulate neutrophil recruitment to the lungs, particularly in response to LPS (36–38). To determine whether the transcriptional induction of these and other inflammatory genes by LPS was altered in the absence of RelA, RNA was collected from the lungs of wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice following insufflation of LPS and was analyzed by Northern blot. Four hours after LPS insufflation, levels of expression of ICAM-1, KC, and MIP2 were somewhat reduced in TNFR1-deficient mice relative to those in wild-type controls

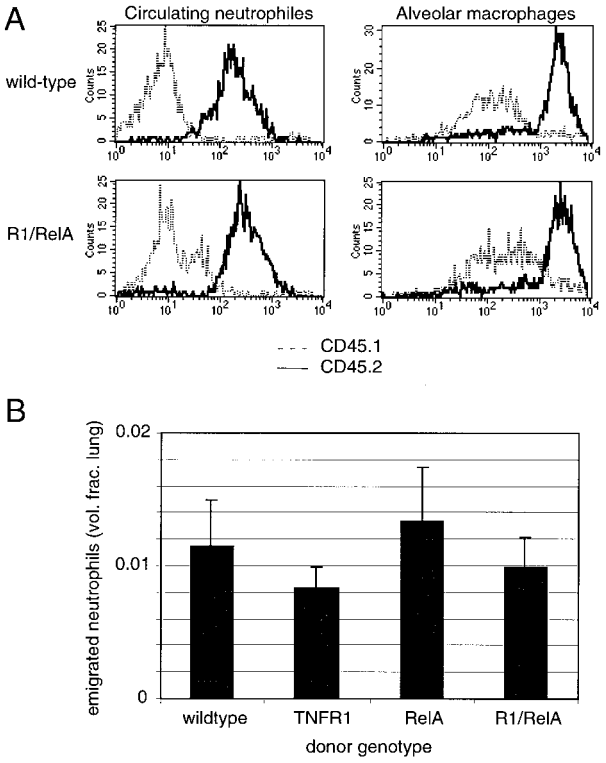

FIGURE 7. Neutrophil emigration in radiation chimeras during LPS-induced pneumonia. C57BL/6 CD45.1$^+$ mice were lethally irradiated and reconstituted with wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetal liver hemopoietic progenitor cells. *A*, Hemopoietic reconstitution. Origins of hemopoietic cells in peripheral blood and bronchoalveolar lavage of untreated radiation chimeras. Circulating neutrophils were defined as GR-1$^+$ cells in the blood, and alveolar microphages were identified as F4/80$^+$ cells in the BAL fluid. *B*, Neutrophil emigration. Mice were intratracheally instilled with LPS, and neutrophil accumulation was quantitated in tissue 6 h later by morphometric analysis of histologic sections ($n = 4$).

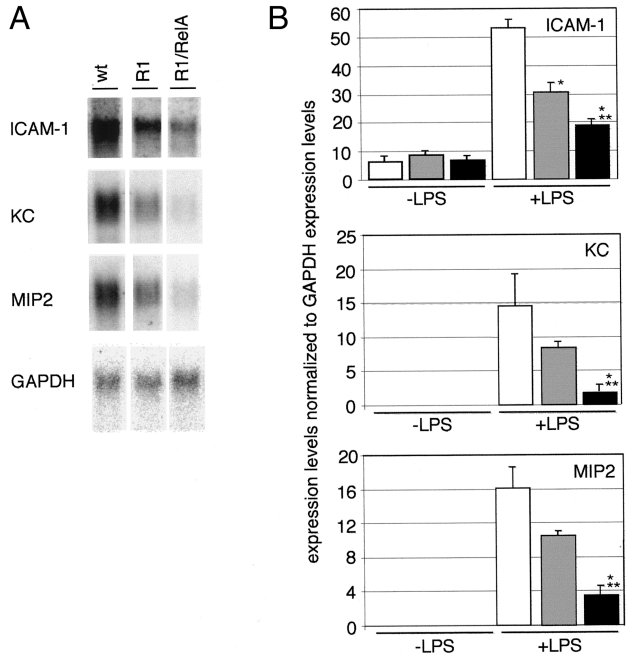

FIGURE 8. Gene expression in the lungs of wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice in response to LPS. Mice, aged P3–5, were intranasally insufflated with LPS. Four hours later mice were sacrificed, and RNA was prepared by Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction. *A*, Northern blot analysis. RNA (20 μg) was probed with ICAM-1, KC, MIP2, and GAPDH probes as discussed in *Materials and Methods*. *B*, Quantification of Northern blot analysis by ImageQuant ($n = 3$). $*$, Statistically significant differences in response compared with wild-type and $**$, TNFR1-deficient tissues ($p < 0.05$).

and were substantially reduced in TNFR1/RelA-deficient mice relative to those in wild-type TNFR1-deficient mice (Fig. 8). These data demonstrate that in lung stromal cells, representative genes for chemokine and adhesion proteins show reduced expression in the absence of RelA.

Discussion

We report here that RelA provides protection from TNFR1-mediated proapoptotic signaling in vivo. Viable TNFR1/RelA-deficient mice were protected from the hepatocyte apoptosis observed in RelA-deficient embryos. Furthermore, TNFR1/RelA-deficient fibroblasts and 3T3 cells were as resistant as wild-type cells to TNF-α-mediated apoptosis. Taken together, these results indicate that TNFR1 is the primary receptor mediating TNF-α cytotoxicity in RelA-deficient cells.

We also report here that the antibacterial host defenses of TNFR1/RelA-deficient mice were severely compromised. Unlike mice with targeted deficiency of the NF-κB family members RelB (8, 16) or p50/RelB (17) or the IκB family member IκBα (39), which develop chronic inflammation in the absence of bacterial infections, all moribund TNFR1/RelA-deficient mice presented with numerous inflammatory lesions and septicemia. The strains of infecting bacteria, the sites of infection, and the age of mortality were constant among animals raised in the same facility, but varied from facility to facility (data not shown). Therefore, progression of the infection was most likely directed by the type of bacteria to which the animal was first exposed, rather than by a susceptibility to bacterial infection in a specific organ or tissue. TNF-α/RelA-deficient mice exhibit a similar immune phenotype, although the onset of symptoms is delayed by several weeks (24). Given the variation in phenotype observed in our colony depending on the infecting pathogen, it is likely that the differences in phenotypes of the TNF-α/RelA-deficient and TNFR1/RelA-deficient mice are due to differences in pathogen exposure as well.

Although the development of secondary lymphoid organs was perturbed in TNFR1/RelA-deficient mice, such abnormalities should not compromise the innate immune response to the extent observed in these mice, suggesting that other aspects of host defense were also perturbed in the absence of TNFR1 and RelA. Large bacterial colonies devoid of leukocytes were present in various soft tissues despite normal or elevated circulating leukocyte counts, suggesting that leukocytes were unable to migrate to infected sites efficiently in the absence of TNFR1 and RelA. Therefore, we measured leukocyte recruitment in the TNFR1/RelA-deficient mice in two different tissues. Neutrophil emigration induced in the peritoneum by thioglycolate injection was significantly reduced by deficiency of TNFR1 alone, demonstrating that TNFR1 plays an important role in this inflammatory process. Thus, if RelA activity is important for neutrophil recruitment to the peritoneum, it is in response to TNFR1-mediated signaling. In contrast, inflammatory responses to LPS in the lungs were not reduced in the absence of TNFR1, allowing us to assess the importance of TNFR1-independent RelA activity in this inflammatory process. TNFR1/RelA-deficient newborns displayed a significant impairment in their ability to recruit neutrophils to the lung during a 6-h exposure to LPS relative to wild-type or TNFR1-deficient newborns, suggesting that early neutrophil emigration to the lung requires RelA activity that is independent of TNFR1 signaling.

To determine whether RelA-deficient neutrophils were capable of being recruited, neutrophil emigration was assayed in radiation chimeras. These experiments demonstrated that RelA is not intrinsically required in neutrophils or alveolar macrophages for neutrophil recruitment. Although a population of radiation-resistant host-derived T lymphocytes remained in the bronchoalveolar lavage fluid, which could have contributed to eliciting neutrophil emigration, these results more likely suggest that the production of recruitment mediators by parenchymal cells is sufficient for neutrophil recruitment and that the recruitment defect in TNFR1/RelA-deficient mice lies in this nonhemopoietic compartment. We attempted to confirm that the production of recruitment mediators by parenchymal cells was necessary by performing the reciprocal radiation chimera experiment, in which wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice were reconstituted with wild-type hemopoietic cells. However, although wild-type and TNFR1-deficient hosts were successfully reconstituted and appeared healthy 6 wk following transplant, transplanted TNFR1/RelA-deficient siblings did not survive the 2 wk necessary for reconstitution to be completed (E. Alcamo, unpublished observations), preventing further analysis of the importance of RelA in nonhemopoietic cells.

Previous studies have demonstrated the presence of TNFR1-independent pathways for recruitment in the lung (40–43). This study suggests that RelA is an important mediator of these pathways. In support of this, the induction of ICAM-1, MIP2, and KC, proteins that are required for LPS-induced neutrophil emigration into the lung, was reduced to a greater degree in the absence of RelA and TNFR1 than in the absence of TNFR1 alone. Thus, in addition to the TNFR1-dependent mechanisms for mounting an innate immune responses that may or may not require RelA, RelA is essential for regulating TNFR1-independent mechanisms of innate immunity that are indispensable for protection against pathogens.

Although leukocyte recruitment to the lungs 6 h following LPS insufflation is a RelA-dependent process, the spontaneous pneumonia that developed in TNFR1/RelA-deficient mice clearly demonstrates the presence of RelA-independent pathways mediating neutrophil emigration. It is possible that although the initiation of pulmonary inflammatory responses to LPS are RelA dependent, RelA-independent mechanisms for recruiting neutrophils develop over time, just as neutrophil emigration elicited by sterile irritants or bacteria in the peritoneal cavity progresses from being dependent on E-selectin, P-selectin, ICAM-1, and β_2 integrins at early time points to becoming independent of these same molecules after 24 h (44–46). Alternatively, it is possible that although LPS-induced inflammatory responses are RelA dependent, inflammatory responses induced by other organisms are not. For example, previous studies indicate that neutrophil emigration in the lung is CD18 dependent in response to LPS or *E. coli*, but CD18 independent in response to *Streptococcus pneumoniae* or *S. aureus* (reviewed in Ref. 47).

In conclusion, the work presented here demonstrates that TNFR1 is responsible for mediating TNF-α signals that in the absence of RelA are cytotoxic to fetal hepatocytes and result in embryonic lethality. Furthermore, the results demonstrate the importance of RelA in inducing an innate immune response. The innate host defense system is responsible for providing protection against the vast majority of infectious micro-organisms by recognizing and removing pathogens within hours of contact and activating the adaptive arm of the immune system should infection persist (reviewed in Refs. 48–50). As past studies have shown, deficiencies for hemopoietic cells of the myeloid lineage or molecules that are involved in mounting an innate immune response can be fatal (33, 51–54). The results presented here demonstrate that RelA plays a critical role in activating the transcription of molecules that are important for leukocyte emigration, an essential step in the development of an innate immune response.

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