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TNF Receptor-Deficient Mice Reveal Divergent Roles for p55 and p75 in Several Models of Inflammation

Jacques J. Peschon,^{1*} Dauphine S. Torrance,[†] Kim L. Stocking,^{*} Moira B. Glaccum,^{*} Carol Otten,[†] Cynthia R. Willis,^{*} Keith Charrier,^{*} Philip J. Morrissey,^{*} Carol B. Ware,^{2*} and Kendall M. Mohler[†]

The pleiotropic activities of the potent proinflammatory cytokine TNF are mediated by two structurally related, but functionally distinct, receptors, p55 and p75, that are coexpressed on most cell types. The majority of biologic responses classically attributed to TNF are mediated by p55. In contrast, p75 has been proposed to function as both a TNF antagonist by neutralizing TNF and as a TNF agonist by facilitating the interaction between TNF and p55 at the cell surface. We have examined the roles of p55 and p75 in mediating and modulating the activity of TNF *in vivo* by generating and examining mice genetically deficient in these receptors. Selective deficits in several host defense and inflammatory responses are observed in mice lacking p55 or both p55 and p75, but not in mice lacking p75. In these models, the activity of p55 is not impaired by the absence of p75, arguing against a physiologic role for p75 as an essential element of p55-mediated signaling. In contrast, exacerbated pulmonary inflammation and dramatically increased endotoxin induced serum TNF levels in mice lacking p75 suggest a dominant role for p75 in suppressing TNF-mediated inflammatory responses. In summary, these data help clarify the biologic roles of p55 and p75 in mediating and modulating the biologic activity of TNF and provide genetic evidence for an antagonistic role of p75 *in vivo*. *The Journal of Immunology*, 1998, 160: 943–952.

TNF is a potent proinflammatory cytokine released primarily from stimulated macrophages. TNF was originally identified and isolated based upon two unique activities: the ability to induce hemorrhagic necrosis of certain tumors and the ability to induce cachexia during states of chronic infection (1). TNF is now recognized as a key mediator of inflammatory responses. Many aspects of tissue damage following acute or chronic inflammatory reactions can be directly attributed to the concomitant induction of TNF biosynthesis and release, and provide the therapeutic rationale for developing TNF antagonists (2). TNF also plays a pivotal role in host defense mechanisms. A requirement for TNF in innate resistance to intracellular pathogens is well documented (3). Additionally, the existence of natural viruses encoding TNF antagonists within their genomes suggests that host defenses against specific viruses may in part be TNF dependent (4–6).

TNF belongs to a family of structurally related molecules including LT α ,³ and the ligands for CD27, CD30, CD40, OX40, 4-1BB, and Fas Ag (7, 8). When administered exogenously either *in vitro* or *in vivo*, LT α and TNF elicit similar biologic responses

and bind both known TNF receptors (TNFR) with comparable affinities and kinetics (9). However, the extent to which functional redundancy between endogenously produced TNF and LT α is physiologically relevant remains unclear. This issue is further complicated by the identification of a related, membrane-bound cytokine, LT β , that interacts with LT α (10). The predominantly expressed heteromeric complex of LT α and LT β ($\alpha_1\beta_2$) does not bind either of the known TNFR, but does bind to the LT β R gene product (11).

The biologic activities of TNF are mediated by two structurally related, but functionally distinct, receptors, p55 and p75, belonging to the TNFR gene family (6, 12–14). The two receptors are coexpressed on the surface of most cell types, and both are additionally proteolytically released as soluble molecules capable of binding TNF. A variety of approaches have been used to ascribe specific biologic functions to membrane-bound p55 and p75 receptors. Experiments using receptor-specific Abs (15, 16), receptor-specific ligands (17, 18), and mice genetically deficient in either p55 or p75 (19–21) indicate that p55 is the primary signaling receptor on most cell types through which the majority of inflammatory responses classically attributed to TNF occur. In contrast, TNF-induced thymocyte proliferation, TNF-mediated skin necrosis, and apoptosis of activated mature T lymphocytes are mediated by p75 (16, 22, 23). Two unique activities associated with membrane-bound p75 have recently been described. First, TNF associated with the cell surface is biologically active (24) and is superior to soluble TNF in triggering p75 (25). Additionally, surface-associated p75 is postulated to enhance p55-dependent responses by recruiting TNF to the cell surface and delivering the ligand to p55 in a ligand-passing model (26). However, it has recently been demonstrated that the enhancement of specific p55-dependent responses by p75 in fact does not involve ligand passing, but, rather, involves overlapping intracellular signaling events triggered by p55 and p75 (27).

A variety of inflammatory stimuli trigger the shedding of soluble p55 and p75 receptors from the cell surface into the circulation

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³ Abbreviations used in this paper: LT α , lymphotoxin α ; TNFR, tumor necrosis factor receptor; LT β , lymphotoxin β ; LT β R, lymphotoxin β receptor; PGK-neo, phosphoglycerol kinase-neomycin resistance gene; TK, thymidine kinase; ES cell, embryonic stem cell; BAL, bronchoalveolar lavage; D-gal, D-galactosamine; ICE, interleukin-1 β -converting enzyme.

through proteolytic processing (28, 29). However, the extent to which endogenously produced soluble TNFR modulate TNF bioactivity remains unclear. Soluble TNFR could ostensibly function either as TNF antagonists, capable of neutralizing TNF bioactivity, or as TNF agonists, by prolonging the circulating half-life of TNF or by facilitating the interaction of TNF with membrane-bound receptors. An antagonistic role for soluble TNFR is supported by the observation that TNF has antiviral activity, and that a pox virus-encoded soluble TNFR, structurally most similar to p75, is an essential virulence gene (30). Additionally, exogenously administered recombinant soluble p55 or p75 Ig fusion proteins are effective TNF antagonists in several models of inflammation known to involve TNF (30–34).

In this report we have taken a genetic approach toward characterizing the physiologic functions of p55 and p75 *in vivo* as either signaling receptors or molecules capable of modulating TNF activity. Mice lacking p55, p75, and both p55 and p75 were created by gene targeting and analyzed. The results argue against an obvious role for these receptors, and hence TNF, in normal mouse development and homeostasis. However, selective deficits in host defense and inflammatory responses are observed in mice lacking p55 or both p55 and p75. Responses mediated by p55 are not dependent upon p75, arguing against an obligatory role for p75 in facilitating the interaction between TNF and p55. In contrast, p75-deficient mice exhibit an exacerbated p55-dependent pulmonary inflammatory response, suggesting a dominant role for endogenous p75 in attenuating leukocyte accumulation within the lung. TNF and IL-1 have been postulated to be critical mediators of lethal endotoxemia. However, mice genetically unable to respond to both TNF and IL-1 remain fully sensitive to the lethal effects of endotoxin. These data help clarify the physiologic functions of and interactions between the p55 and p75 receptors in mediating and modulating the biologic activities of TNF and indicate that endotoxin-induced sepsis syndrome in the mouse is independent of both TNF and IL-1.

Materials and Methods

Targeting the p55 TNFR gene by homologous recombination

A genomic clone encoding murine p55 was isolated from a (CBA × C57BL/6)F₁ genomic library (Clontech, Palo Alto, CA) and mapped by a combination of PCR and restriction analyses. A targeting vector was constructed by replacing a 4-kb *SpeI* fragment encoding exons 2 to 5 (amino acids 30–184) with a PGK-neo cassette. A thymidine kinase cassette (MC-TK) was inserted into the 3' end of the vector. C57BL/6-derived ES cells (C. B. Ware, unpublished observations) were electroporated with the p55-targeting construct and selected as described previously (35). Approximately 1/20 G418 and ganciclovir-resistant clones carried a p55 allele disrupted by homologous recombination, as determined by PCR and genomic Southern blot analyses.

Targeting of the p75 TNFR gene by homologous recombination

A genomic clone encoding the ATG-containing exon of murine p75 was isolated from a 129 genomic library (Stratagene, La Jolla, CA) and mapped by PCR and restriction analyses. A targeting vector was constructed by replacing a 1-kb *KasI-BamHI* fragment encoding amino acids 3 to 26 with a PGK-neo cassette. A MC-TK cassette was inserted into the 5' end of the vector. 129-derived AB1 ES (36) cells were electroporated with the p75 targeting construct and selected as described above. Approximately 1/400 G418 and ganciclovir-resistant clones carried a p75 allele disrupted by homologous recombination as determined by PCR and genomic Southern blot analyses.

Generation of p55 and p75 TNFR-deficient mice

p55- and p75-targeted ES cell clones were injected into day 3.5 BALB/c and C57BL/6 blastocysts, respectively, and transferred to day 2.5 pseudopregnant Swiss-Webster recipients. Resulting male chimeras were bred to C57BL/6 females, and offspring were analyzed for germ-line transmission

of the mutant alleles by PCR and genomic Southern blot analyses. Mice heterozygous for either p55 or p75 mutations (p55^{+/-} and p75^{+/-}, respectively) were intercrossed to yield mice homozygous for the respective mutations (p55^{-/-} and p75^{-/-}). Mice heterozygous for both TNFR mutations (p55^{+/-}p75^{+/-}) were generated by crosses between p55^{+/-} and p75^{+/-} mice and subsequently intercrossed to derive p55 and p75 TNFR doubly deficient mice (p55^{-/-}p75^{-/-}). Genotyping was performed using a combination of PCR and genomic Southern analyses.

Mouse strains

The p55^{-/-} mice used in this study were C57BL/6 inbred. The p75^{-/-} and p55^{-/-}p75^{-/-} mice were maintained as random C57BL/6 × 129 hybrids, unless indicated otherwise. In some experiments, p75^{-/-} and p55^{-/-}p75^{-/-} mice on a C57BL/6 background (B6N5 p75^{-/-} and B6N4 p55^{-/-}p75^{-/-}, respectively) were generated by successive backcrossing to C57BL/6 for four (B6N4) or five (B6N5) generations and analyzed. C57BL/6 (Charles River, Wilmington, MA), 129/J, and (129/J × C57BL/6)F₁ (The Jackson Laboratory, Bar Harbor, ME) mice were used as controls as indicated in the text. Mice heterozygous for either TNFR mutation and the type 1 IL-1R mutation (p55^{+/-}IL-1R^{+/-}, p75^{+/-}IL-1R^{+/-}) were generated by crosses between, respectively, p55^{-/-} and p75^{-/-} mice and IL-1R^{-/-} mice (37) and subsequently intercrossed to derive p55^{-/-}IL-1R^{-/-} and p75^{-/-}IL-1R^{-/-} mice on random C57BL/6 × 129 hybrid backgrounds. Genotyping was performed using a combination of PCR and genomic Southern analyses. For bone marrow reconstitution experiments, 8-wk-old C57BL/6 females were lethally irradiated (950 rad) using a ¹³⁷Cs source (J. L. Shepard Co., Glendale, CA) at 89 rad/min and injected i.v. with 1 × 10⁶ bone marrow cells pooled from femurs of three C57BL/6 or three p55^{-/-} mice. Reconstituted mice were analyzed 8 wk following bone marrow transfer.

Binding analyses

Murine TNF- α (PeproTech, Rocky Hills, NJ) was biotinylated using biotin-X-NHS (Calbiochem, San Diego, CA) according to manufacturer's specifications. Approximately 2 × 10⁵ cells in a final volume of 25 μ l were incubated with biotinylated TNF and streptavidin-conjugated phycoerythrin (Becton Dickinson, San Jose, CA) and analyzed by flow cytometry (38). Thymocytes from adult mice were cultured in RPMI 1640 supplemented with 10% FCS, 0.5 mM β -ME, glutamine, and 10 μ g/ml Con A for 18 h before use in binding assays. Peritoneal exudate cells were lavaged from the peritoneal cavity in 10 ml of ice-cold RPMI 1640 supplemented with 10% FCS 3 days following i.p. injection of 1 ml of 5% thioglycolate broth (Sigma). Bronchoalveolar lavage (BAL) cells were prepared, as described below, 4 h after intranasal LPS administration.

Flow cytometric and histologic analyses of lymphoid organs

Thymic, splenic, and peripheral lymph node cellularities and subset distributions were determined by flow cytometric analyses using a variety of cell surface markers as described previously (39). Spleens were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Acute phase responses

Mice (8–12 wk of age) were injected i.p. with 2.4 μ g/g of body weight LPS (*Escherichia coli* strain 0127-B8, Difco, Detroit, MI). At the indicated times, mice were killed by cervical dislocation, and liver biopsies were collected. Total liver RNA was isolated and subjected to Northern blot analyses using PCR-generated cDNA probes encoding various acute phase reactants.

Listeria monocytogenes infection

Mice were lightly anesthetized with Metaflon (Pitman-Moore, Mundelein, IL), injected i.v. via the orbital plexus with live *Listeria monocytogenes* (40249, American Type Culture Collection, Rockville, MD) in PBS, and monitored twice daily for survival. Splenic bacterial loads were determined at various times postinfection by harvesting spleens from surviving animals, homogenizing in dH₂O/0.01% BSA, and plating out serial dilutions onto brain heart infusion agar plates. Colonies were enumerated after incubation for 24 h at 37°C.

Endotoxic shock

Susceptibility to LPS was assessed by injecting mice (8–16 wk of age) i.p. with 35 to 50 μ g/g of body weight LPS (*E. coli* strain 0127-B8, Difco) and monitoring twice daily for at least 10 days. Susceptibility to LPS in conjunction with D-galactosamine (D-gal) was assessed by i.v. coinjection into

the tail vein of 0.5 to 2.5 ng of LPS (*E. coli* strain 0127-B8, Difco)/g of body weight and 20 mg D-gal (Sigma) and monitoring twice daily for at least 5 days.

Intranasal instillation and BAL

Pulmonary inflammation was induced by intranasal administration of either LPS (*E. coli* strain 0127-B8, Difco) or *Micropolyspora faeni* (*M. faeni*, American Type Culture Collection 15347). LPS stock solutions were prepared at 1.8 mg/ml in saline. Lyophilized *M. faeni* Ag was prepared as described previously (40) from sonicated *M. faeni* cultures and reconstituted in saline at a concentration of 1.36 mg/ml. Endotoxin levels were determined to be 1.6 pg/mg *M. faeni* Ag using a *Limulus* amoebocyte lysate assay (Sigma). Age-matched animals were lightly anesthetized with Meta-fane before intranasal instillation of either LPS or *M. faeni* Ag stock solutions. Fifty-five microliters of LPS stock solution (100 μ g) was administered, and BAL was performed 22 h later. Fifty-five microliters of reconstituted *M. faeni* solution (75 μ g) was administered as described above daily for 3 days. BAL was performed either 2 or 24 h following the third instillation. BAL following either LPS or *M. faeni* instillations was performed by repeated lavage of airways with 1 ml of 0.6 μ M EDTA in PBS for a total of 5 ml. BAL cell counts were determined using a Coulter counter (HiLeah, FL). BAL compositions were determined on cytopspins stained using Diff-Quik (Baxter, McGaw Park, IL).

Cytokine determination

Levels of total TNF- α were determined by ELISA according to the manufacturer's specifications (Genzyme, Cambridge, MA). Levels of biologically active TNF- α were determined using the L929 cytotoxicity assay in conjunction with crystal violet (0.5% in 25% methanol) as described previously (33).

Results

Generation and gross characterization of TNFR-deficient mice

Mice lacking functional p55 and p75 TNFR genes were generated by gene targeting in ES cells using the constructs outlined in Figure 1, A and B. Southern blot analyses using diagnostic restriction digests in conjunction with probes lying outside the regions of homology contained within the targeting vectors confirm that the TNFR genes were disrupted by homologous recombination (Fig. 1, C and D). The p55 mutation was generated in a C57BL/6 ES cell. Germ-line transmission of the altered p55 allele from chimeras created with BALB/c blastocysts demonstrated that C57BL/6 ES cells were a practical alternative to the traditionally used, 129-derived ES cell lines. p55-deficient mice (p55^{-/-}) and p75-deficient mice (p75^{-/-}) were generated at the expected Mendelian frequencies from crosses between p55^{+/-} and p75^{+/-} parents, respectively (data not shown). p55 and p75 doubly deficient mice (p55^{-/-}p75^{-/-}) as well as p55^{-/-}IL-1R^{-/-} and p75^{-/-}IL-1R^{-/-} mice were generated at the expected frequencies from crosses of, respectively, p55^{+/-}-p75^{+/-}, p55^{+/-}IL-1R^{+/-}, and p75^{+/-}IL-1R^{+/-} parents (data not shown). None of the three TNFR-deficient or TNFR- and IL-1R-deficient strains displayed any gross phenotypic or reproductive anomalies.

Several binding analyses were performed to establish that the p55 and p75 mutations represented null mutations and to determine whether these receptors were the sole TNF binding molecules present on the cell types analyzed. As shown in Figure 2A, Con A-stimulated thymocytes from p55^{-/-}p75^{-/-} mice failed to bind murine TNF. It is also apparent that p75 was the predominant TNF binding moiety on these cells, as binding to p75^{-/-} cells was minimal, and binding to p55^{-/-} cells was comparable to that observed to wild-type cells. As observed with Con A-stimulated thymocytes, there was no detectable binding of murine TNF to thioglycolate-elicited peritoneal exudate cells from p55^{-/-}p75^{-/-} mice (Fig. 2B). The composition of cells from exudates of p55^{-/-}p75^{-/-} mice was indis-

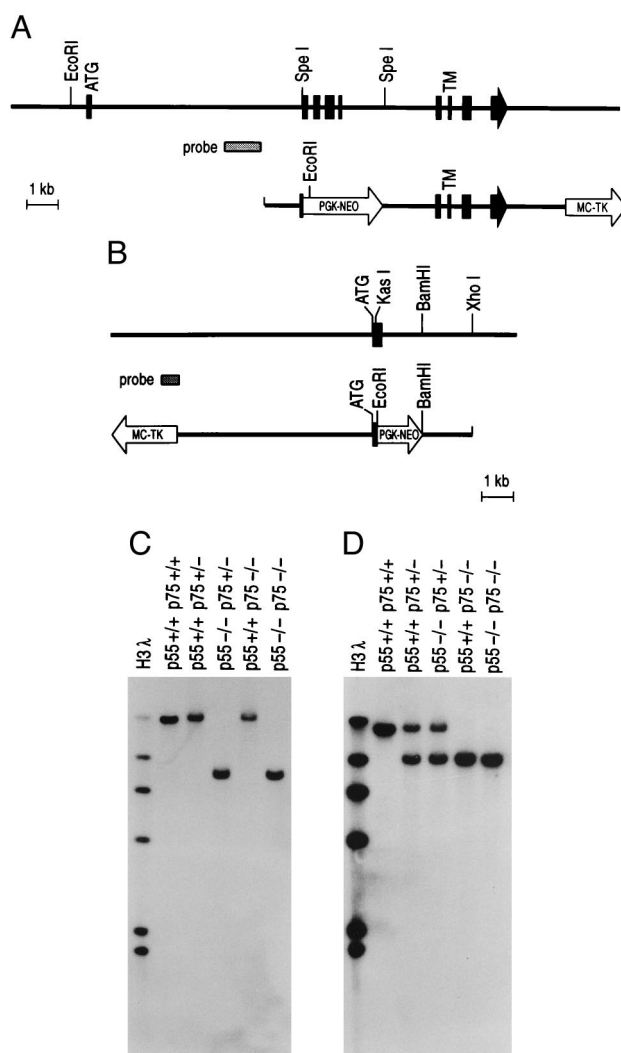


FIGURE 1. Generation and molecular characterization of mutant TNFR alleles. *A*, Schematic diagram of the wild-type p55 gene and p55-targeting vector. Exons are depicted in filled boxes. Relevant restriction sites as well as the probe used for genomic Southern analysis are shown. The positions of the initiator methionine (ATG) and the transmembrane domain (TM) are depicted. *B*, Schematic diagram of the 5' end of the p75 gene and p75-targeting vector. The initiator methionine (ATG)-containing exon is depicted as a filled box. Relevant restriction sites as well as the probe used for genomic Southern blot analysis are shown. Genomic DNAs from progeny of p55^{+/-}p75^{+/-} parents were digested with *EcoRI* and subjected to Southern blot analyses using p55-specific (*C*) or p75-specific (*D*) probes.

tinguishable from that of cells from exudates from control mice (data not shown). Additionally, exudate cells from p55 or p75 singly deficient mice displayed TNF binding profiles distinct from those of controls, indicating that both receptors were normally expressed on this population of cells and that TNF binding to either receptor was not strictly dependent upon the presence of the second receptor. Lastly, no binding of biotinylated murine TNF was observed to p55^{-/-}p75^{-/-} cells, predominantly neutrophils, lavaged from the lungs of mice 4 h after intranasal administration of LPS (data not shown). Collectively, these data indicated that p55 and p75 together comprised the sole TNF binding moieties on the surface of the cell types analyzed.

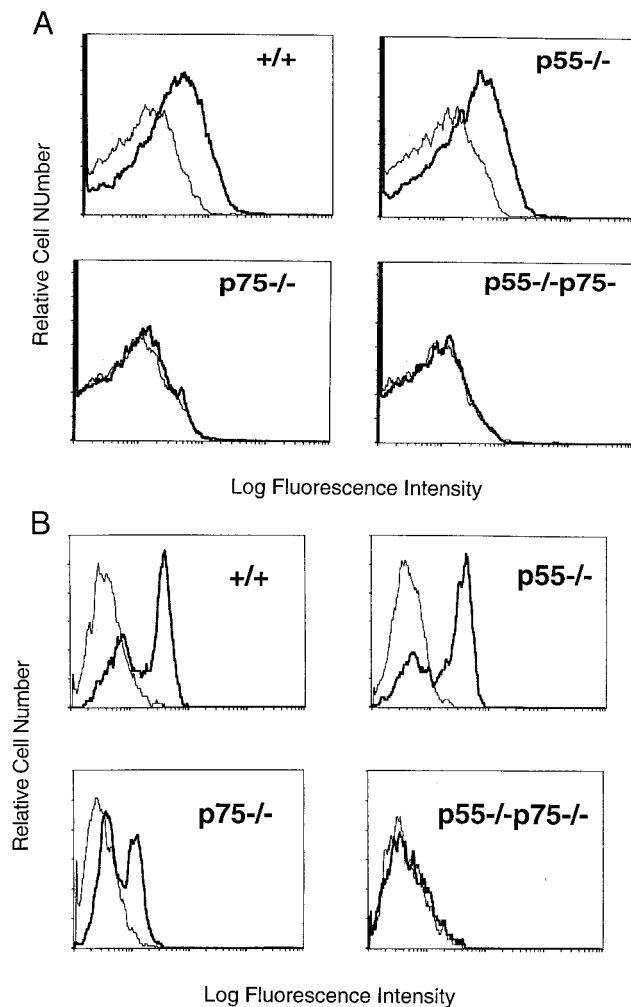


FIGURE 2. Analyses of TNF binding to wild-type and TNFR-deficient cells. The binding of biotinylated murine TNF to either Con A-stimulated thymocytes (A) or thioglycolate-elicited peritoneal exudate cells (B) isolated from wild-type (+/+) and TNFR-deficient mice was analyzed by flow cytometry using biotin TNF plus streptavidin-conjugated phycoerythrin (SA-PE; bold lines). Samples incubated with SA-PE alone (thin lines) are also shown.

Lymphoid development in TNFR-deficient mice

Analyses of thymic cellularity and subpopulations from young adult $p55^{-/-}$, $p75^{-/-}$, and $p55^{-/-}p75^{-/-}$ mice revealed that although TNF is constitutively expressed in the thymus and is capable of stimulating thymocyte proliferation, thymopoiesis was not critically dependent upon TNFR (Fig. 3A). Additionally, similar analyses of peripheral lymphoid organs of $p55^{-/-}p75^{-/-}$ mice using surface markers specific for B and T cell lineages indicated that lymphoid development was not dependent upon TNF (Fig. 3, B and C). Moreover, the total lymphoid cellularity of thymi, spleens, and mesenteric lymph nodes from $p55^{-/-}p75^{-/-}$ mice was comparable to that observed in control mice (data not shown). Lastly, gross histologic analyses of hematoxylin- and eosin-stained sections of spleens and mesenteric and inguinal lymph nodes derived from $p55^{-/-}$, $p75^{-/-}$, and $p55^{-/-}p75^{-/-}$ mice did not reveal any obvious anomalies (data not shown).

Resistance of TNFR-deficient mice to infection with *Listeria monocytogenes*

Injection of mice with the intracellular pathogen *L. monocytogenes* leads to an acute infection. Early resistance to infection requires activation of macrophage microbicidal activity and is critically dependent upon TNF and IFN- γ (3). Later developing sterilizing immunity is dependent upon functional T cells (41). As shown in Figure 4A and as previously reported (19, 20), mice genetically deficient in p55 succumbed to an otherwise sublethal infection of *L. monocytogenes*. In contrast, survival of $p75^{-/-}$ and B6N5 $p75^{-/-}$ mice following *L. monocytogenes* infection was comparable to that observed in controls (Fig. 4, A and B). Splenic bacterial loads in $p75^{-/-}$ mice 5 days postinfection were similar to those displayed by control mice, and by day 21, viable bacteria were no longer recovered from either $p75^{-/-}$ or control spleens (data not shown).

Serum TNF levels in TNFR-deficient mice

LPS is a potent inducer of TNF expression, and serum TNF levels rapidly rise following in vivo administration of LPS (1). The concentration of total serum TNF 2 h post-LPS challenge, as determined by ELISA, was elevated approximately 5-fold in mice lacking either p55 or p75 relative to that in wild-type mice and 15- to 20-fold in mice lacking both p55 and p75 (Fig. 5 and data not shown). Whereas the p55 and p75 mutations clearly influence the magnitude of the TNF response, the kinetics of TNF appearance and disappearance following LPS exposure are grossly unaffected in mice lacking either or both p55 or p75 (Fig. 5). The levels of biologically active TNF, as assessed by cytotoxic activity in the L929 bioassay, in wild-type mice and in mice lacking either or both p55 and p75 mirrored the levels determined by ELISA (data not shown).

Induction of the acute phase response in TNFR-deficient mice

LPS administration induces the synthesis of a series of acute phase reactants by the liver (42). This response has been shown to involve TNF, IL-1, and members of the IL-6 cytokine family. As shown in Figure 6, the magnitude and kinetics of induction of several acute phase reactants in the livers of $p55^{-/-}p75^{-/-}$ mice were comparable to those observed in controls. These data argue against an obligate participation of TNF in the induction of the hepatic acute phase response by LPS.

Lethal endotoxemia in TNFR-deficient mice

The lethal effects of LPS have been attributed to the massive induction of proinflammatory cytokine synthesis and release. TNF and IL-1 are thought to be critical mediators of endotoxin-induced septic shock. Mice treated with the hepatotoxin D-gal are rendered extremely sensitive to LPS (43, 44). As shown in Table I, $p55^{-/-}$ and $p55^{-/-}p75^{-/-}$ mice, but not $p75^{-/-}$ mice, were protected from a lethal dose of LPS (2.5 ng/g of body weight) plus D-gal. Although the mice were monitored for at least 5 days, all susceptible mice succumbed to LPS plus D-gal treatment within 2 days, whereas resistant mice displayed no obvious symptoms at any time following treatment. Similar results using independently derived p55- and p75-deficient mice have previously been reported (19–21). Lethality associated with LPS and D-gal is TNF dependent and requires p55. To examine the extent to which endogenous p75 modulates this response, control and TNFR-deficient mice were challenged with a sublethal dose of LPS (0.5 ng/g of body weight) plus D-gal. A slightly increased sensitivity to a sublethal dose of LPS plus D-gal was observed in p75-deficient mice (Table I).

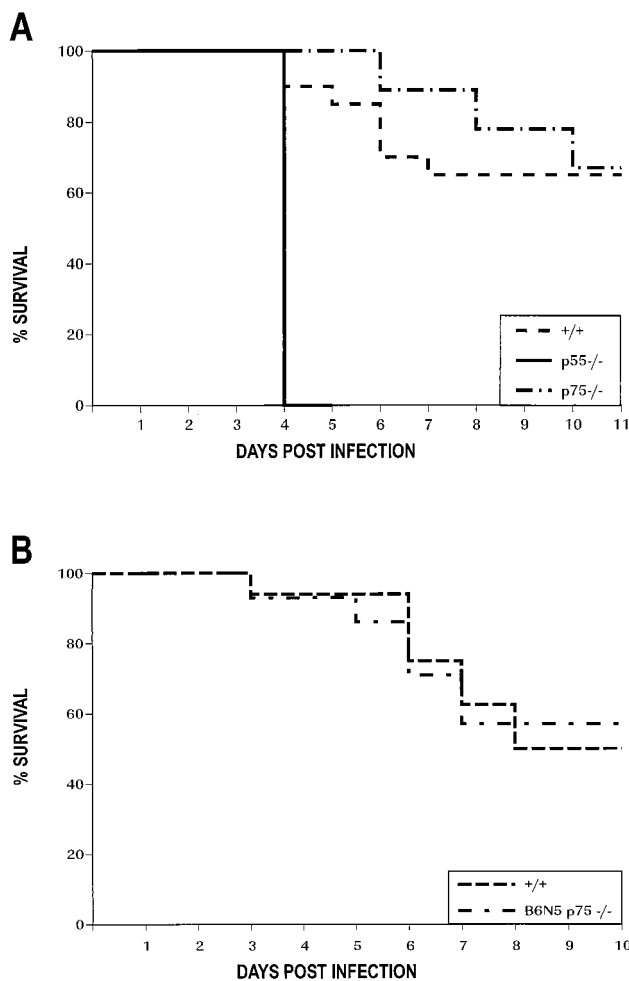
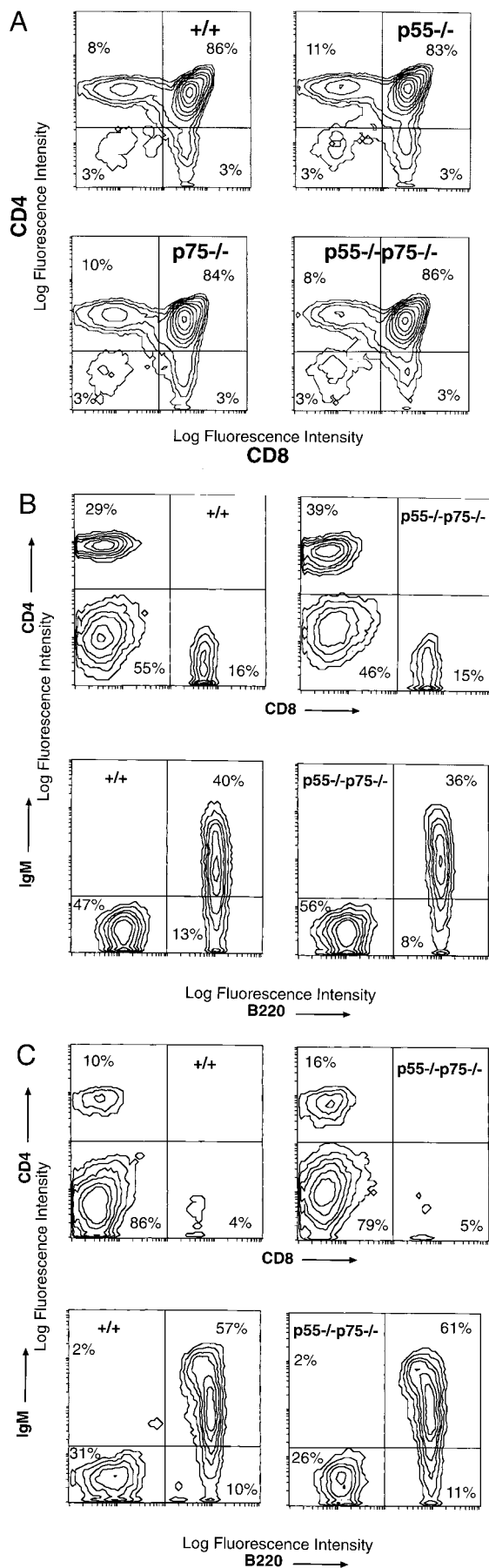


FIGURE 4. Susceptibility of TNFR-deficient mice to *L. monocytogenes* infection. *A*, Twelve- to 20-wk-old C57BL/6 and (C57BL/6 × 129)F₁ control (+/+; *n* = 20), p55^{-/-} (*n* = 9), and p75^{-/-} (*n* = 9) mice were infected i.v. with 1 × 10⁴ live CFU *L. monocytogenes* and monitored twice daily for deaths. *B*, Ten-week-old male C57BL/6 (+/+; *n* = 16) and B6N5 p75^{-/-} (*n* = 14) mice were infected i.v. with 1.5 × 10⁴ live CFU *L. monocytogenes* and monitored twice daily for deaths.

In the absence of D-gal, high levels of LPS are required to induce lethal endotoxemia in mice. p55^{-/-}, p75^{-/-}, and p55^{-/-}p75^{-/-} strains of mice remained susceptible to high and intermediate doses of LPS and succumbed to lethal endotoxemia with kinetics comparable to those observed in controls (Table II and data not shown). These data indicated that lethality in this model was not strictly dependent upon TNF. To investigate the contribution of IL-1 in the induction of lethal endotoxemia in mice lacking TNFR, mice lacking either p55 or p75 and the type 1 IL-1R were generated and analyzed. p55^{-/-}IL-1R^{-/-} and p75^{-/-}IL-1R^{-/-} mice remained susceptible to LPS and succumbed to lethal

FIGURE 3. Immunofluorescent profiles of lymphoid subpopulations defined by various cell surface markers within the thymus (*A*) of control (+/+), p55^{-/-}, p75^{-/-}, and p55^{-/-}p75^{-/-} mice and mesenteric lymph node (*B*) and spleen (*C*) of control (+/+) and p55^{-/-}p75^{-/-} mice. Experiments were performed on organs pooled from two or three age-matched mice. The percentages of the various subpopulations are indicated.

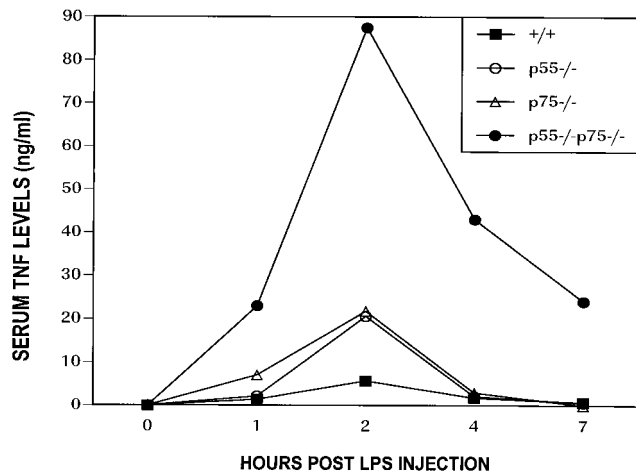


FIGURE 5. Serum TNF levels in LPS-challenged control and TNFR-deficient mice. Control (+/+), p55^{-/-}, p75^{-/-}, and p55^{-/-}p75^{-/-} mice (10- to 12-wk-old females; three or four mice per group) were challenged with 400 μ g of LPS i.v. Serum was collected at various times postchallenge and analyzed for TNF levels by ELISA. A representative experiment of two performed is shown.

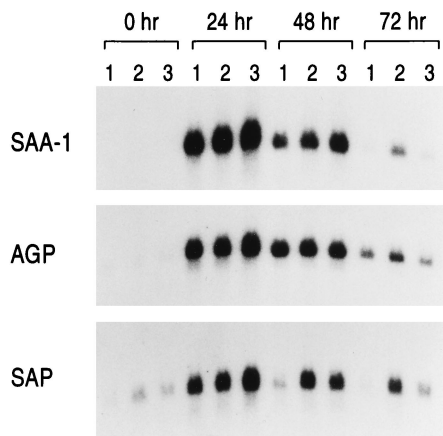


FIGURE 6. Acute phase responses in TNFR-deficient mice. C57BL/6 (lane 1), 129/J (lane 2), and p55^{-/-}p75^{-/-} (lane 3) mice were injected with 50 μ g of LPS i.p. Total RNA was prepared from livers collected at the indicated times postinjection. Ten micrograms of RNA was subjected to Northern blot analysis using cDNA probes for serum amyloid A (SAA-1), α_1 -acid glycoprotein (AGP), and serum amyloid P (SAP). Ethidium bromide staining of ribosomal RNA was used to establish that equivalent amounts of RNA were loaded (data not shown). A representative analysis from three independent experiments is depicted.

endotoxemia with kinetics comparable to those observed in controls (Fig. 7, A and B).

Pulmonary inflammation in TNFR-deficient mice

Leukocyte emigration into the lung is induced by systemic administration or direct inhalation of various inflammatory agents and is associated with pulmonary pathology. Intranasal instillation of heat-inactivated actinomycete *M. faeni* Ags into mice induces hypersensitivity pneumonitis. Disease induction is associated with elevated TNF production within the lung and can be prevented using neutralizing anti-TNF Abs (40, 45). The initial pulmonary influx of neutrophils in response to *M. faeni* was examined in p55- and p75-deficient mice. *M. faeni*-induced neutrophil accumulation within the lung was dramatically decreased in p55^{-/-} and

Table I. Susceptibility of p55 and p75 TNFR-deficient mice to septic shock induced by LPS and D-gal^a

Strain	Dose of LPS, deaths/total (% lethality)	
	2.5 ng/g	0.5 ng/g
C57BL/6	10/10 (100)	8/27 (30)
129/J	10/10 (100)	1/10 (10)
p55 ^{-/-}	0/10 (0)	ND
p75 ^{-/-}	10/11 (91)	5/14 (36)
B6N5p75 ^{-/-}	ND	8/14 (57)
p55 ^{-/-} p75 ^{-/-}	0/12 (0)	0/7 (0)

^a Mice were injected i.v. with 20 mg D-gal and either 0.5 ng or 2.5 ng LPS per g body weight and monitored twice daily for deaths.

Table II. Susceptibility of p55 and p75 TNFR-deficient mice to LPS induced septic shock^a

Strain	Dose of LPS, deaths/total (% lethality)	
	50 μ g/g	35 μ g/g
C57BL/6	25/30 (83)	14/20 (70)
(C57BL/6 \times 129)F ₁	17/18 (94)	9/10 (90)
p55 ^{-/-}	15/19 (79)	6/11 (55)
p75 ^{-/-}	19/26 (73)	7/10 (70)
B6N5p75 ^{-/-}	6/9 (67)	4/7 (57)
p55 ^{-/-} p75 ^{-/-}	9/14 (64)	6/10 (60)
B6N4p55 ^{-/-} p75 ^{-/-}	10/12 (83)	ND

^a Mice were injected i.p. with the indicated dose of LPS per g body weight and monitored twice daily for deaths. The data shown are a pool of three experiments for the 50- μ g/g dose and a pool of two experiments for the 35- μ g/g dose.

p55^{-/-}p75^{-/-} mice relative to that observed in control mice, whereas the levels of monocytes and lymphocytes within the lung were comparable in all strains (Fig. 8A). In contrast, an exacerbated neutrophil influx was observed in the lungs of p75^{-/-} mice, whereas the levels of monocytes and lymphocytes within the lung were not affected by the mutation.

Migration of neutrophils into the lung in response to *M. faeni* was clearly dependent upon p55. To determine whether *M. faeni*-induced neutrophil recruitment requires p55 expression by neutrophils or by cells within the lung microenvironment, lethally irradiated C57BL/6 mice were reconstituted with either C57BL/6^{+/+} or p55^{-/-} bone marrow and challenged with *M. faeni*. *M. faeni*-induced neutrophil accumulation was comparable in +/+ mice reconstituted with either +/+ or p55-deficient marrow (Fig. 8B). The genotypes of the emigrating cells were confirmed by genomic PCR analyses of BAL cells (Fig. 8C) and indicated that p55-deficient neutrophils could properly traffic into a wild-type lung.

To examine further the role of TNF in pulmonary inflammation, neutrophil emigration into the lungs of TNFR-deficient mice was examined following intranasal administration of LPS. In this model, neutrophil accumulation within the lung in mice lacking p55, p75, or both p55 and p75 was comparable to that observed in wild-type mice (data not shown).

Discussion

In this report we describe the generation and characterization of mice genetically deficient in p55 and p75 TNFR. We have used these mice to define the roles of p55 and p75 in mediating and modulating the effects of TNF during unperturbed and challenged conditions.

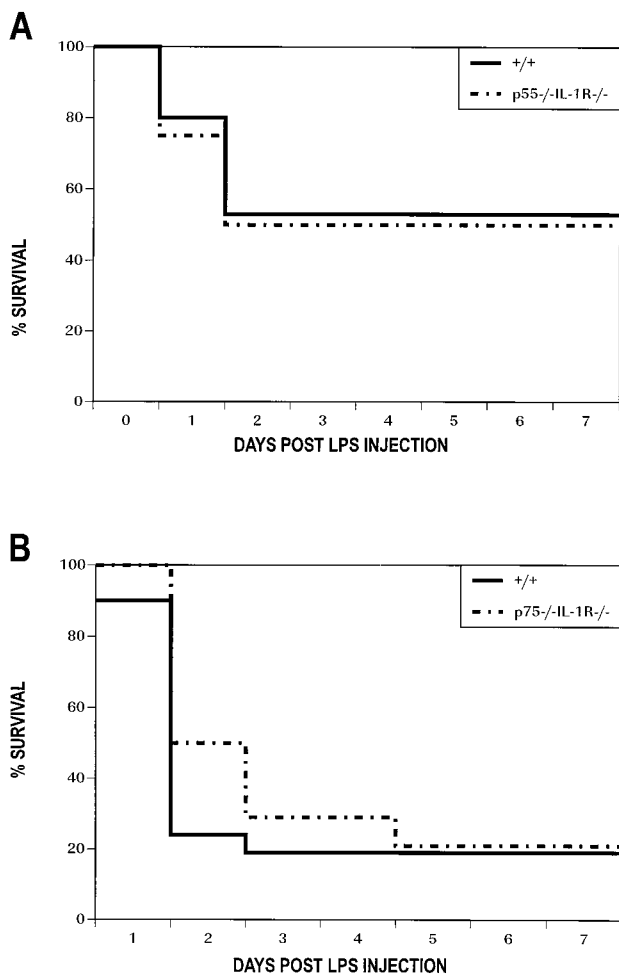


FIGURE 7. Susceptibility of TNFR and IL-1R-deficient mice to LPS. Eight- to 16-wk-old C57BL/6 (+/+; $n = 15$) and $p55^{-/-}$ IL-1R^{-/-} ($n = 12$) mice (A) or C57BL/6 (+/+; $n = 21$) and $p75^{-/-}$ IL-1R^{-/-} ($n = 14$) mice (B) were injected i.p. with 50 μ g of LPS/g of body weight and monitored at least twice daily for deaths.

$p55$ and $p75$ are expressed by most cell types. The observation that mice deficient in both $p55$ and $p75$ are overtly normal indicates that neither $p55$ nor $p75$, and hence TNF, are grossly required for normal mouse development and homeostasis under basal conditions. Recent analyses of mice specifically lacking TNF support these observations (46). These observations are in marked contrast to the phenotype observed in mice lacking $LT\alpha$, a cytokine that is structurally and functionally related to TNF. $LT\alpha$ -deficient mice lack lymph nodes (47). It is apparent from these studies as well as those previously described (19–21) that this phenotype is not shared by mice singly deficient in either $p55$ or $p75$. Moreover, we have extended these observations by demonstrating that mice lacking both $p55$ and $p75$ are also phenotypically distinct from $LT\alpha$ -deficient mice with regard to lymph node development. These observations clearly indicate that despite the functional redundancy between TNF and $LT\alpha$ defined *in vitro*, endogenous $LT\alpha$ can function independently of both $p55$ and $p75$ *in vivo*. $LT\alpha$ associates on the cell surface with $LT\beta$ (10). As the resulting heteromeric complex does not bind either $p55$ or $p75$, but, rather, interacts with the recently identified $LT\beta R$ (11), it is likely that defective lymph node development in $LT\alpha$ -deficient mice is due to an inability to trigger $LT\beta R$. These observations do not exclude a role for $p55$ or $p75$ in mediating other activities associated with $LT\alpha$ *in vivo*.

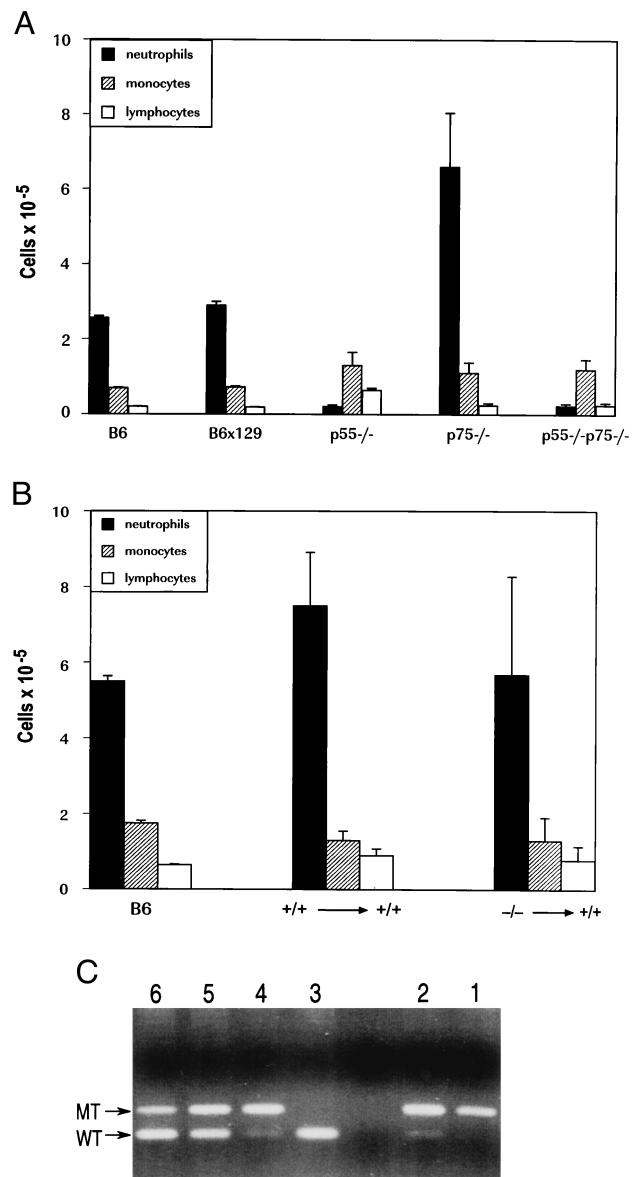


FIGURE 8. Pulmonary inflammatory responses in TNFR-deficient mice. A, BAL cellularity (mean \pm SD) in age-matched control C57BL/6 (B6) and (C57BL/6 \times 129)_{F1} hybrids (B6 \times 129), $p55^{-/-}$, $p75^{-/-}$, and $p55^{-/-}$ $p75^{-/-}$ mice (three mice per group) following intranasal administration of heat-killed *M. faeni* Ags is shown. BAL cellularity in mice receiving saline alone was comparable among all strains, ranging between 5.5 and 8 $\times 10^4$ cells, of which 97 to 98% were monocytes (data not shown). A representative experiment of several performed is shown. B, BAL cellularity (mean \pm SD) in age-matched unmanipulated C57BL/6 (B6) or in lethally irradiated C57BL/6 mice reconstituted with either +/+ (+/+ \rightarrow +/+) or $p55^{-/-}$ ($^{-/-}$ \rightarrow +/+) bone marrow (three mice per group) following intranasal administration of heat-killed *M. faeni* Ags is shown. C, Equivalent amounts of genomic DNA prepared from bone marrow (lane 1) and spleen (lane 2) of C57BL/6 mice reconstituted with $p55^{-/-}$ bone marrow as well as BAL cells lavaged following intranasal administration of *M. faeni* Ags to C57BL/6 mice reconstituted with either +/+ (lane 3) or $p55^{-/-}$ bone marrow (lane 4) were genotyped using a PCR assay that simultaneously detects both wild-type (WT) and mutant (MT) $p55$ alleles and were analyzed by gel electrophoresis. As quantitation controls, $p55^{-/-}$ and +/+ genomic DNA samples were mixed at ratios of 9:1 (lane 5) and 1:1 (lane 6) and analyzed.

Shared defects between $LT\alpha$ - and TNFR-deficient mice may define such activities. For example, we have recently demonstrated that germinal center formation is defective in mice lacking either

LT α or p55 (48). Additionally, it has recently been observed that the development of Peyer's patches is defective in p55-deficient mice (49) (J. J. Peschon, unpublished observation). The extent to which either of these shared defects reflects activities of LT α that require p55 remains to be established. Mice lacking p55 or TNF also display defects in primary B cell follicles and follicular dendritic cell networks, indicating yet additional roles for TNF and TNFR in lymphoid structure and function (46, 50). Future investigations using mice lacking TNF, lymphotoxin, and their receptors are directed at determining the extent to which signals transduced by TNFRs and LT β R might themselves be redundant. Phenotypic differences between these strains might in part represent exclusive or restricted profiles of expression of these molecules rather than functionally distinct signaling mechanisms.

The data presented in this report do not reveal a specific role for p75 in delivering a TNF-dependent signal in vivo. However, it is clear from a number of studies that p75 is a signaling receptor for TNF. For example, it has recently been demonstrated that activation-induced cell death of T cells is mediated by both p75 and Fas (23). Additionally, p75 is involved in the development of necrotizing skin lesions induced by s.c. administration of TNF (16, 21). The role of endogenous p75 in modulating the activity of TNF in vivo is less clear. p75 has been proposed to increase the sensitivity of cells to TNF through ligand passing to p55 (26), although an alternative explanation for the ability of p75 to enhance p55-mediated responses is that the two receptors can impinge upon overlapping or identical signal transduction pathways (27). Additionally, both soluble p55 and p75 can stabilize TNF and thus potentially function as TNF agonists (51). However, it is also apparent that soluble TNFRs can function as effective TNF antagonists. For example, administration of a soluble p75 Ig fusion protein is effective in reducing mortality in an animal model of endotoxemia (33) and decreases the incidence and the severity of disease in a model of collagen-induced arthritis (34). Additionally, pox virus genomes harbor open reading frames encoding soluble TNFRs structurally most similar to p75, which are essential for virulence (30). While inflammatory stimuli induce the proteolytic shedding of both p55 and p75, levels of soluble p75 greatly exceed those of soluble p55 and thus suggest a dominant role for endogenously produced soluble p75 in down-regulating TNF-driven responses (28, 29, 51). In this report we have used mice genetically deficient in either p55 or p75 to define, firstly, those responses that are dependent upon p55 and, secondly, the extent to which p75 can modulate these responses.

Mice lacking either p55 or p75 accumulate increased levels of TNF relative to controls following LPS administration. These observations indicate that both p55 and p75 play a role in regulating steady state TNF levels. It will be of interest to determine whether this regulation is dependent upon cell surface-associated or soluble TNFR. In this regard, it is of interest to note that the serum levels of LPS-induced soluble p55 TNFR are unaffected by the p75 mutation, and the serum levels of LPS-induced soluble p75 TNFR are unaffected by the p55 mutation (J. J. Peschon, unpublished observation). The elevated TNF levels in p55-deficient mice are predicted to be inconsequential with respect to most inflammatory responses involving TNF, as these appear to be predominantly mediated by p55 itself. However, the elevated levels of TNF observed in mice lacking p75 might lead to increased TNF sensitivity in responses that are mediated by p55. For example, we have demonstrated that neutrophil influx into the lung induced by intranasal administration of *M. faeni* Ags is TNF dependent and requires p55 expression within a radiation-resistant component of the lung microenvironment. Neutrophil influx in this model can be attenuated

by coadministration of a soluble p75 Ig fusion protein.⁴ In this model, mice lacking p75 display an exacerbated response, suggesting a role for endogenous p75 in down-modulating pulmonary inflammatory responses. The phenotype of p55^{-/-}p75^{-/-} mice resembles that observed in p55^{-/-} mice, indicating that the exacerbated response remains dependent upon a functional interaction between TNF and p55. In contrast, pulmonary neutrophil influx in response to LPS is normal in mice lacking either or both p55 and p75, suggesting that this response neither requires nor is modulated by TNF. The cytokines driving this latter response are not known, although molecules such as macrophage inflammatory protein-1 α are logical candidates, as macrophage inflammatory protein-1 α is expressed in the lung following inflammatory challenge and is a potent inducer of neutrophil infiltration into the lung (52, 53). Thus, there exist both TNF-dependent and TNF-independent pathways of leukocyte emigration into the lung. The clinical efficacy of TNF antagonists will certainly be influenced by which pathways are activated during disease states leading to pulmonary inflammation.

As described here and previously (19–21), lethal shock induced by LPS and D-gal coadministration requires p55 and not p75. Additionally, we demonstrate that p75-deficient mice remain susceptible to shock induced by a sublethal dose of LPS and D-gal, in which TNF is presumably limiting. This observation argues against agonistic or ligand-passing roles for p75 in this TNF-dependent response. In fact, the slightly elevated lethality observed in p75-deficient mice might, rather, reflect a role for p75 in attenuating the toxic effects of TNF in this model. In contrast to septic shock elicited specifically by D-gal and either endotoxin or enterotoxin treatment, in which lethality is clearly dependent upon TNF, it remains unclear which cytokines are critical for mediating the lethal effects of high doses of LPS alone. p75-deficient mice were reported to be less sensitive to the lethal effects of intermediate doses of LPS, and this reduced sensitivity was attributed to an enhancing or agonistic role of p75 in mediating the lethal effects of TNF through the p55 receptor (21). These data imply that TNF is involved in host sensitivity to LPS. However, as demonstrated here and reported previously (19, 20), mice lacking p55 remain sensitive to the lethal effects of intermediate or high dose LPS administration, indicating that lethality in this model is clearly p55 independent. Moreover, we find that p75-deficient mice are not less sensitive to high or intermediate doses of LPS. Although we cannot exclude the possibility that subtle variations in experimental regimens or genetic backgrounds contribute to this discrepancy, our observation that mice lacking both p55 and p75 remain as sensitive as controls to intermediate and high doses of LPS strongly indicates that lethality in this model is TNF independent. The cytokines mediating lethal sepsis in wild-type and TNFR-deficient mice remain undefined. We considered the involvement of IL-1, as IL-1 antagonists are protective in some animal models of endotoxemia and mice lacking the IL-1 β -converting enzyme (ICE), which fail to process IL-1 β and additionally show reduced TNF production, are resistant to high doses of LPS (54, 55). However, we have recently generated mice lacking the type 1 IL-1R (37) and used these to generate mice lacking both p55 and type 1 IL-1R and mice lacking both p75 and type 1 IL-1R. Surprisingly, these doubly deficient strains remain sensitive to the lethal effects of high doses of LPS. It has recently been reported that ICE is also required for the proteolytic processing of IFN- γ -inducing factor (56). In light of this observation and the data reported here, LPS

⁴ K. M. Mohler, D. Torrance, M. Glaccum, C. Otten, and R. A. Black. Neutrophil influx into the lungs of mice exposed to *Micropolyspora faeni* antigen is dependent upon TNF but not IL-1. Submitted for publication.

resistance in mice lacking ICE is probably independent of both IL-1 and TNF and instead is mediated by defects in the production of IFN- γ or other proinflammatory cytokines. These observations underscore the potential for cytokine redundancy in the inflammatory cascade leading to septic shock.

The role of TNF in innate resistance to bacterial infection is well established (57, 58), and as shown here and previously reported (19, 20), mice lacking p55 are rendered highly susceptible to infection by the intracellular pathogen *L. monocytogenes*. A percentage of mice deficient in p75, maintained on a mixed C57BL/6 \times 129 genetic background, were previously reported to have decreased resistance to *L. monocytogenes* infection due to a proposed requirement for p75 in facilitating an efficient interaction between TNF and p55 (21). However, 129 mice are naturally sensitive, and C57BL/6 mice are naturally resistant to *L. monocytogenes*, with resistance being genetically determined by the dominant Lsr-1^f allele (59). Thus, a fraction of random C57BL/6 \times 129 hybrid mice are expected to display increased sensitivity to *L. monocytogenes* independent of any targeted mutation. We have examined *L. monocytogenes* susceptibility of p75-deficient mice maintained on both C57BL/6 and hybrid C57BL/6 \times 129 genetic backgrounds and have found that p75-deficient mice do not display impaired resistance to *L. monocytogenes*. Moreover, administration of neutralizing Abs to either TNF or p55 renders mice extremely susceptible to *Listeria*, whereas administration of Abs against p75 does not impair *L. monocytogenes* resistance (16). Thus, the activity of TNF in innate resistance to *Listeria* requires p55 and is independent of p75.

In this report we have examined the physiologic function of p55 and p75 in vivo as either signaling receptors or as molecules capable of modulating TNF activity. We demonstrate that a p55-dependent response, namely neutrophil trafficking into the lung, is exacerbated in mice lacking p75. Our observation that the levels of TNF in the circulation of challenged p75-deficient mice are dramatically elevated relative to those observed in challenged wild-type mice provides a potential mechanism for this exacerbated response. The extent to which endogenous p75 regulates other p55-dependent responses in either acute or chronic inflammatory settings warrants further investigation.

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