

# A Critical Role of the p75 Tumor Necrosis Factor Receptor (p75TNF-R) in Organ Inflammation Independent of TNF, Lymphotoxin $\alpha$ , or the p55TNF-R

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## Summary

Despite overwhelming evidence that enhanced production of the p75 tumor necrosis factor receptor (p75TNF-R) accompanies development of specific human inflammatory pathologies such as multi-organ failure during sepsis, inflammatory liver disease, pancreatitis, respiratory distress syndrome, or AIDS, the function of this receptor remains poorly defined *in vivo*. We show here that at levels relevant to human disease, production of the human p75TNF-R in transgenic mice results in a severe inflammatory syndrome involving mainly the pancreas, liver, kidney, and lung, and characterized by constitutively increased NF- $\kappa$ B activity in the peripheral blood mononuclear cell compartment. This process is shown to evolve independently of the presence of TNF, lymphotoxin  $\alpha$ , or the p55TNF-R, although coexpression of a human TNF transgene accelerated pathology. These results establish an independent role for enhanced p75TNF-R production in the pathogenesis of inflammatory disease and implicate the direct involvement of this receptor in a wide range of human inflammatory pathologies.

Key words: transgenic model • sepsis • ligand-independent signaling • NF- $\kappa$ B activation

Tumor necrosis factor (TNF) is considered to be a potent proinflammatory molecule involved in the pathogenesis of chronic local or systemic inflammation *in vivo* (1). The effects of TNF are signaled via two cell surface receptors (TNF-R), designated p55 and p75TNF-R, which are capable of mediating, either in cooperation or independently, a wide spectrum of cellular responses ranging from direct cytotoxicity or apoptosis to cellular proliferation and differentiation (2, 3). Soluble forms of the two TNF-Rs (sTNF-R), which represent the extracellular portions of membrane-associated TNF-Rs and are shed from them by proteolytic partition, have been identified in serum and urine (4, 5). Using TNF or TNF-R knockout mice it has recently been demonstrated that the TNF/p55TNF-R pair is essential for many physiological processes such as lymphoid organ architecture (6, 7), immune cell activation and trafficking (8), and host defence against bacterial (6, 9, 10) or viral infections (11). Moreover, a dominant role of the p55TNF-R is also apparent in several TNF-mediated pathologies, including endotoxemic shock in the presence of TNF-sensitizing agents (9, 10), or in disease models where constitutively produced (12–16) or acutely administered levels of TNF are pathogenic (17). In contrast, using similar assay systems, there has been very little evidence for a specific role for the p75TNF-R in delivering TNF-dependent signals *in vivo* (2, 18). This may reflect either a well-documented preference of the p75TNF-R to signal upon bind-

ing to transmembrane (19) rather than soluble TNF (20), or an apparently essential requirement for this receptor to reach an induced density state in order to transmit an independent biological signal (21, 22). Indeed, a most important feature of the p75TNF-R, which distinguishes it from the p55TNF-R, has been its highly inducible production mainly on cells of hematopoietic origin (2, 23). Notably, chronic enhanced production of the soluble p75TNF-R demarcates many fatal human inflammatory and autoimmune conditions, including sepsis (24), chronic viral hepatic disease (25), acute respiratory distress syndrome (26), acute pancreatitis (27), lupus (28), rheumatoid arthritis (29), and AIDS (30). Perhaps most importantly, sustained production of the p75TNF-R during disease is rarely accompanied by chronically elevated levels of TNF indicating a regulatory and functional disengagement from TNF (31). However, an independent role for this receptor in the pathogenesis of inflammatory disease has never been suggested.

To assess the independent *in vivo* activities of the p75TNF-R we have generated and studied transgenic mice expressing constitutively enhanced, yet disease relevant levels of a wild-type human p75TNF-R. Our studies demonstrate that this receptor is capable of inducing a severe multi-organ inflammatory syndrome, affecting mainly the liver, pancreas, kidney, and lung. Similarly to the prolonged NF- $\kappa$ B activation observed in PBMC from human

septic patients and shown to cause pathology in models of endotoxemia (32), NF- $\kappa$ B binding activity is found constitutively increased in PBMC from hup75TNF-R transgenic mice suggesting an in vivo role for the p75TNF-R in triggering this pathogenic cascade. Interestingly, the severity of pathology developing in the human p75TNF-R transgenic mice was analogous to the levels of soluble p75TNF-R measured in the sera of these animals, simulating the quantitative correlation between levels of human soluble p75TNF-R production and severity of human disease (33). Remarkably, the pathogenic potential of this receptor is shown here to be exerted even in the absence of its known ligands, TNF or lymphotoxin  $\alpha$  (LT $\alpha$ ),<sup>1</sup> and independently of the presence of the p55TNF-R. These results establish an independent role for induced production of the p75TNF-R in inflammatory disease pathogenesis and suggest that antagonistic intervention with the functioning of this receptor may potentially be beneficial in a wide range of associated human pathologies.

## Materials and Methods

**Transgenic and Knockout Mice.** The hup75TNF-R gene was isolated from a human genomic P1-bacteriophage library by PCR screening (Genome Systems Inc., St. Louis, MO) using the primers 5'-CAT CCC TGG GAA TGC-3' and 5'-GAA GAG CGA AGT CGC-3' that amplify a 214-bp region of hup75TNF-R cDNA. A SalI-NotI fragment of  $\sim$ 70 kb containing both 5' and 3' sequences from the hup75TNF-R cDNA was prepared by centrifugation on a 5–25% (wt/vol) NaCl gradient and microinjected into CBA/C57BL/6J fertilized eggs, as described elsewhere (34). To identify transgenic founder mice, DNA was isolated from tail biopsies, digested with Sac I, and hybridized with a 640-bp XhoI-BglII fragment of hup75 cDNA. Transgenic progenies were identified by Southern and slot blot hybridization analysis. TNF (6), LT $\alpha$  (35; The Jackson Laboratory, Bar Harbor, ME), p55TNF-R (9; provided by Dr. Bluethmann, Hoffman-La Roche, Nutley, NJ), or p75TNF-R (18; provided by Dr. Moore, Genentech Inc., South San Francisco, CA) knockout mice were maintained on a mixed 129Sv  $\times$  C57Bl/6 genetic background in the animal facilities of the Hellenic Pasteur Institute.

**RNA Preparation and Analysis.** Total RNA was extracted from freshly dissected mouse tissues and S1 nuclease protection analysis was performed as described previously (36) by hybridizing 25  $\mu$ g of total RNA to a 3-kb 5'-<sup>32</sup>P-end-labeled BglII probe derived from the 5'-end of the hup75TNF-R cDNA plus vector sequences. Correct initiation of transcription from the hup75TNF-R gene produces a mRNA that protects 590 nt of the probe from S1 digestion. Endogenous mouse p75TNF-R expression was monitored by a 3.7 kb 5'-<sup>32</sup>P-end-labeled BglII probe derived from the 5'-end of mup75TNF-R cDNA plus vector sequences (protected fragment 137 bp). A 5'-end-labeled  $\beta$ -actin DNA probe (protected fragment 110 bp) was used to control for quantitative differences between RNA preparations.

**Thymocyte Proliferation Assay.** Freshly isolated murine thymocytes from 5-wk-old mice were cultured in 96-well flat-bottomed culture plates ( $6 \times 10^5/0.1$  ml; Costar Corp., Cambridge,

MA) in DMEM medium supplemented with 5% FCS (Globe-pharm Ltd, Esher, UK), L-glutamine, penicillin, streptomycin, nonessential amino acids (GIBCO BRL, Gaithersburg, MD) and 2-mercapto-ethanol (Sigma Chemical Co., La Verpilliere, France) in the presence of 1  $\mu$ g/ml Con A (Sigma Chemical Co.). Human rTNF (specific activity  $6 \times 10^7$  U/mg) was provided by the Genentech manufacturing group (Genentech Inc., South San Francisco, CA). Con A and human rTNF were added to a final volume of 0.2 ml. After 60 h at 37°C, cultures were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (25 Ci/mmol, 1 mCi = 37 MBq; Amersham Life Science Ltd, Little Chalfont, UK) for 18 h and harvested onto glass fiber filters (Skatron Instruments, Lier, Norway). [<sup>3</sup>H]thymidine incorporation (cpm) of triplicate cultures was determined using a liquid scintillation counter (LKB Wallac, Turku, Finland).

**TNF and LPS Administration.** Recombinant human TNF (Genentech Inc.) was administered intravenously at 60–150  $\mu$ g/mouse in 0.2 ml of PBS. Susceptibility to LPS was assessed by injecting mice (10–12 wk of age) intraperitoneally with 200–1,200  $\mu$ g/25 g of body weight with LPS (*Salmonella enteritis*; Sigma Chemical Co.) in 0.2 ml saline. Control and transgenic mice are littermates. Lethality was monitored for 5 d and indicated as lethality/total injected mice.

**Flow Cytometry.** Freshly isolated murine thymocytes were adjusted to  $2 \times 10^6$  cells/ml in DMEM (GIBCO BRL) supplemented as described above and activated with 1  $\mu$ g/ml Concanavalin A (Sigma Chemical Co.) for 24 h at 37°C. Whole blood was collected in heparinized tubes followed by erythrocyte depletion. To determine the expression of murine or human p75TNF-R on thymocytes or whole blood cells,  $10^6$  cells were stained with a specific anti-mup75TNF-R antibody (rabbit polyclonal biotin-conjugated 1:600, provided by Dr. Wim Buurman, University of Limburg, The Netherlands) or a specific anti-hup75TNF-R antibody (M80, rabbit polyclonal 1:500, provided by Dr. Matthias Grell, University of Stuttgart; reference 37) in 100  $\mu$ l PBA (0.1% BSA, 0.01% sodium azide in PBS) for 30 min at 4°C. After two washes with PBS, cells were incubated either by streptavidine-phycoerythrin (1:1,000; PharMingen, San Diego, CA) to detect mup75TNF-R or phycoerythrin-conjugated anti-rabbit IgG (1:100; Vector Laboratories, Inc., Burlingame, CA) to detect hup75TNF-R. Cells were analyzed on a Becton Dickinson Calibur flow cytometer, using the CellQuest software (Becton Dickinson & Co., Sparks, MD).

**ELISA for Murine and Human p75TNF-R.** Serum was collected 6 h after intraperitoneally injections of 100  $\mu$ g LPS (*Salmonella enteritis*; Sigma Chemical Co.). The ELISA assays for murine and human p75TNF-Rs were provided by Dr. Wim Buurman and performed as described earlier (38). In brief, 96-well Immuno-Maxisorp Plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with polyclonal antibodies specific for either receptor. Sera and standard titration samples were incubated for 2 h at room temperature. Subsequently, plates were incubated with biotin-labeled rabbit polyclonal anti-murine or anti-human p75TNF-R antibodies, followed by a final incubation with Horseradish Peroxidase Streptavidin (Vector Laboratories Inc.). ELISA was developed with 100  $\mu$ l of 0.5 mg/ml O-phenyldiamine dihydrochloride (Sigma Chemical Co.) containing 0.03% H<sub>2</sub>O<sub>2</sub>, and the reaction was terminated with 50  $\mu$ l of 2 N H<sub>2</sub>SO<sub>4</sub>. OD<sub>490</sub> was measured using a MRX microplate Reader (Dynatech, Chantilly, VA).

**Histopathology and Immunocytochemistry.** Tissues from freshly dissected mice were immersion-fixed overnight in neutral buffered formalin and embedded in paraplast (BDH Laboratory Supplies, Dorset, UK). Sections were cut and stained with hematoxy-

<sup>1</sup>Abbreviations used in this paper: DAB, diaminobenzidine; DTT, dithiothreitol; LT $\alpha$ , lymphotoxin  $\alpha$ .

lin and eosin according to standard procedures, dehydrated and mounted in DPX (BDH Laboratory Supplies).

Immunocytochemical analysis was performed on splenic cryostat sections. Immediately before use, sections were fixed for 10 min in acetone containing 0.03% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. For double immunostaining for IgM and CD3, sections were rehydrated in PBS and incubated with peroxidase-labeled goat anti-mouse IgM Ab (Sigma Chemical Co.) and rat anti-mouse CD3 mAb (clone KT [39] provided by Dr. S. Cobbold, Sir William Dunn School of Pathology, Oxford, UK) for 3 h at room temperature. Subsequently, sections were incubated with biotin-conjugated anti-rat IgG antibody (Southern Biotechnology Associates Inc., Birmingham, AL) followed by streptavidin-alkaline phosphatase (Vector Laboratories). Bound peroxidase activity was detected by staining with diaminobenzidine (DAB; Sigma Chemical Co.), and alkaline phosphatase activity was visualized with Fast Blue BB Base (Sigma Chemical Co.).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays.** Blood was collected by cardiac puncture in heparinized tubes and PBMC were isolated by centrifugation on Histopaque-1077 gradient (Sigma Chemical Co.) according to the manufacturer's instructions. The mononuclear band was aspirated, washed with PBS, and analyzed microscopically.

Nuclear proteins were harvested by the method of Dignam (40).  $2 \times 10^6$  PBMC were lysed in 1 vol of cold buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml type I-S soybean trypsin inhibitor), incubated for 15 min on ice and centrifuged in an Eppendorf microcentrifuge for 20 s at highest speed. The pellet was resuspended in 2/3 vol of cold buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml type I-S soybean trypsin inhibitor), incubated on ice for 30 min, and centrifuged for 5 min, 4°C, at highest speed. The supernatant was quick frozen at -80°C. Total protein concentration was determined according to the Bradford method (41).

Electrophoretic mobility shift assays were performed by incubating 10 µg of nuclear extract with 4 µg of poly (dI-dC; Sigma Chemical Co.) in a binding buffer (5 mM Hepes, pH 7.9, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.5 mM DTT, and 10% glycerol), at 20 µl final volume, for 20 min at RT. An end-labeled, double-stranded, NF-κB-specific oligonucleotide probe (MWG-Biotech, Ebersberg, Germany) containing the two tandemly arranged NF-κB binding sites of the human immunodeficiency virus long terminal repeat (5'-ATC AGG GAC TTT CCGCTG GGG ACT TTC CG-3') was used to assay for NF-κB binding activity (10), whereas an end-labeled double-stranded OCT1-specific oligonucleotide probe 5'-TGT CGA ATG CAA ATC ACT AGA A-3' (MWG-Biotech) was used as an internal quantitative control. Specificity of binding was ascertained by competition with a 150-fold molar excess of cold consensus NF-κB or OCT1 oligonucleotides. Protein-DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels.

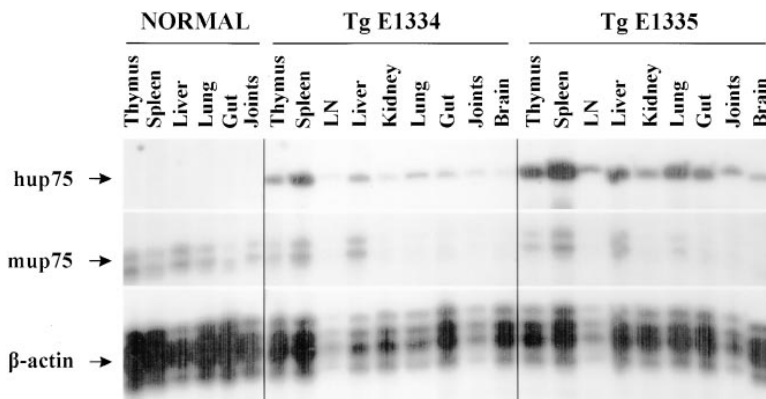
## Results

**Transgene Expression Patterns and Protein Production in Human p75TNF-R Transgenic Mice.** The hup75TNF-R gene was isolated from a human genomic P1-bacteriophage library by PCR screening (Genome Systems Inc., St. Louis, MO).

A large Sall-NotI insert of ~70 kb, was found to contain both 5' and 3' sequences from the hup75TNF-R cDNA and was used for transgenesis. Three transgenic lines were generated (TgE1322, TgE1334, and TgE1335) carrying various transgene copy numbers. The integrity of the inserted DNA was confirmed by Southern hybridization analysis (not shown). To assess whether regulation and tissue patterns of transgene expression were physiologically relevant, steady state hup75TNF-R mRNA levels were measured by S1-nuclease protection assays on total RNA from several transgenic tissues. Correctly initiated hup75TNF-R-specific transcripts could be detected in several tissues examined from transgenic mouse lines TgE1334 and TgE1335 (Fig. 1) or TgE1322 (not shown). Patterns of expression were comparable to those seen for the endogenous p75TNF-R mRNA with the highest levels seen in lymphoid tissues, liver, and lung (Fig. 1). Overall levels of transgene expression differed between lines and were dependent on transgene copy number. TgE1334 mice carrying low transgene copy numbers expressed lower levels of human p75TNF-R compared with the higher transgene copy number TgE1335 (Fig. 1) or TgE1322 mice (not shown). These results indicate that important *cis*-acting regulatory elements controlling the expression of the hup75TNF-R gene were included in the microinjected fragment and that correctly regulated patterns of transgene expression could be established in these transgenic mice.

Expression of cell surface p75TNF-Rs was assessed by flow cytometric analysis on ConA-activated transgenic thymocytes and on freshly isolated peripheral blood cells. Similar to the expression of murine p75TNF-R on normal activated thymocytes, induced expression of human p75TNF-R could be observed on the surface of ConA-activated transgenic thymocytes (Fig. 2 A), indicating correct regulation of the hup75TNF-R protein production. Moreover, transgenic PBMC (not shown) or total peripheral blood leukocytes were also found to express on their surface the human p75TNF-R protein (Fig. 3 B). Furthermore, using double immunostaining of liver sections with F4/80 and anti-p75TNF-R antibodies, the kupffer cell was identified as a source of both endogenous or transgenic p75TNF-Rs (not shown).

Serum levels of soluble human and murine p75TNF-Rs (sp75TNF-R) were measured in TgE1334 and TgE1335 transgenic mice before or after stimulation by LPS (38). Both transgenic lines were shown to produce in their serum human sp75TNF-R. TgE1334 mice produced the transgenic receptor at levels comparable to the endogenous sp75TNF-R in normal control mice ( $\sim 10 \pm 2.5$  ng/ml for either receptor, not shown), whereas TgE1335 mice expressed an overall three- to fourfold increased levels of transgenic sp75TNF-R ( $\sim 33 \pm 4.3$  ng/ml of transgenic versus  $10 \pm 2.5$  ng/ml of the endogenous in normal mice, Fig. 3 A). After challenge by LPS in vivo, human and murine sp75TNF-R levels were comparable in heterozygous TgE1334 (not shown) and TgE1335 mice versus normal control mice (Fig. 3 A), indicating correct regulation by LPS of the exogenous p75TNF-R protein production



**Figure 1.** S1 nuclease protection analysis of total RNA from tissues of normal, TgE1334 and TgE1335 mice shows correct patterns of expression for the transgenic p75TNF-R mRNA.  $\beta$ -actin acts as an internal control for sample loading.

and shedding. Interestingly, nonchallenged homozygous TgE1335 mice produce the transgenic hup75TNF-R protein at levels similar to those seen for endogenous p75TNF-R in LPS-treated normal control mice (Fig. 3 A).

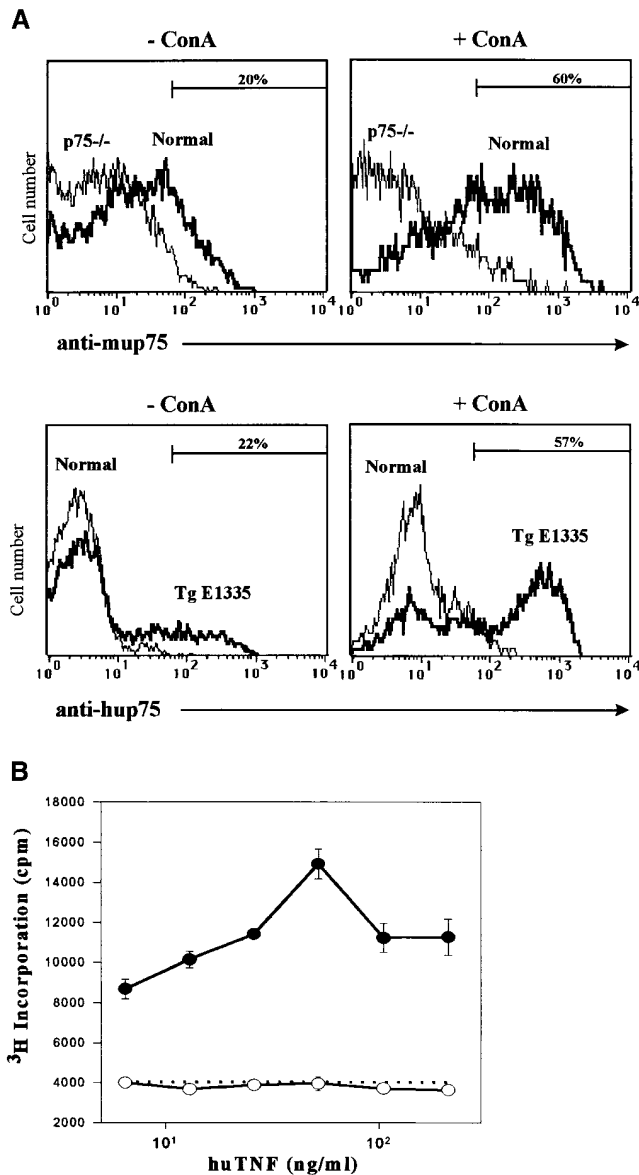
The functional integrity of the human p75TNF-R protein was assessed by measuring its activity in a thymocyte proliferation assay (42). Transgenic but not normal control thymocytes were induced to proliferate by exogenous recombinant human TNF demonstrating the presence of a functional human p75TNF-R (Fig. 2 B). Taken together, these results demonstrate that correctly regulated and physiologically relevant expression of a functional human p75TNF-R protein was established in these transgenic mice.

*Enhanced hup75TNF-R Expression Sensitizes Mice to the In Vivo Toxicity of rhuTNF and LPS.* Previous studies in p75TNF-R knockout mice have indicated an enhancing role for this receptor in the lethal toxicity of LPS or murine TNF (18). Additional studies however, have suggested a neutralizing potential of enhanced levels of soluble TNF-Rs in models of endotoxemia (38, 43). To assess whether expression of a human p75TNF-R protein would render mice more resistant or more susceptible to LPS or huTNF administration, and to determine the net in vivo effect of hup75TNF-R overexpression in endotoxemic mice, we measured lethality rates in TgE1335 and control animals challenged intravenously or intraperitoneally with different doses of recombinant huTNF or LPS respectively. Table 1 summarizes the results of these experiments. Human p75TNF-R expression is shown to potentially sensitize transgenic mice to the toxicity of an otherwise sublethal dose of either rhuTNF (90  $\mu$ g) or LPS (800  $\mu$ g), demonstrating that induced production of the hup75TNF-R protein contributes positively to the lethal outcome of endotoxemia.

*Sustained Overproduction of the p75TNF-R Triggers Multi-organ Inflammatory Pathology.* Mice heterozygous for the hup75TNF-R transgene from all three transgenic lines develop and grow normally and display no pathological changes with the exception of mice from the highest expressing line TgE1335 that develop a chronic but mild peri-vascular inflammatory pathology in liver, pancreas, and lung at 2-3 mo of age (Fig. 4). Notably, homozygous TgE1335 or TgE1322 mice develop a severe pathology

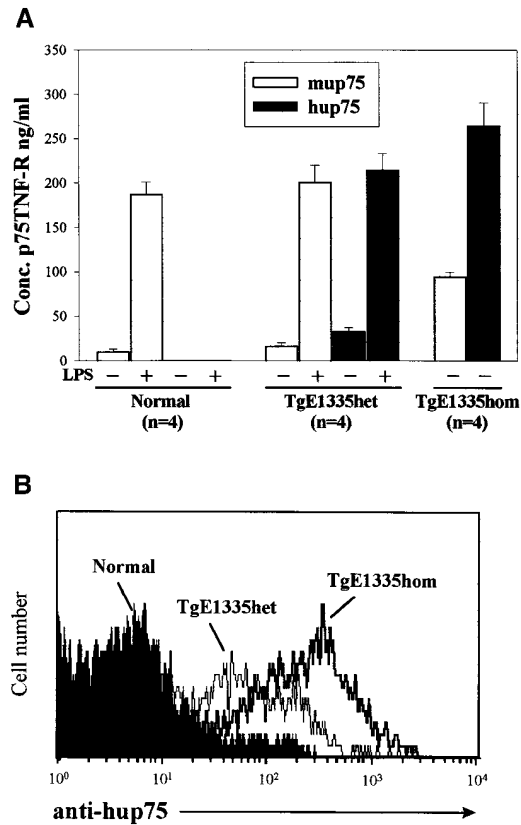
characterized by runting, lethargy, and abdominal distension and accompanied by a severely reduced weight gain (data not shown). The disease leads to the premature death of these animals between 2 and 4 wk of age. At necropsy of 20-d-old homozygous TgE1335 mice, thymic and pancreatic atrophy, splenomegaly, and extensive liver necrosis were observed. Histopathological analyses revealed heavy peri-vascular inflammatory lesions in several organs such as pancreas, liver, lung, and kidney. In addition to heavy inflammation, extensive ischemic tissue necrosis could be observed in the liver (Fig. 4). Other organs and sites such as muscle and brain meninges were also inflammatory in homozygous animals. Infiltrates in heterozygous TgE1335 animals consisted mainly of T and B cells, macrophages, and polymorphonuclear cells as assessed by immunocytochemical analysis using cell-specific markers (not shown). Interestingly, although a similar infiltrate was present in the heavily inflamed organs of 2-wk-old homozygous TgE1335 mice, a striking absence of B220-positive B cells was observed (not shown). IgM<sup>+</sup> B cells were also shown to be markedly decreased in sections of spleens from homozygous TgE1335 mice (Fig. 4). FACS<sup>®</sup>-analysis performed in peripheral blood, spleen, and bone marrow cells of homozygous TgE1335 mice confirmed the almost complete absence of B220<sup>+</sup>IgM<sup>-</sup> and B220<sup>+</sup>IgM<sup>+</sup> B cells, indicating impaired B cell development. In contrast, mature CD4<sup>+</sup> and CD8<sup>+</sup> single positive as well as Mac1<sup>+</sup>/Gr1<sup>+</sup> cells could readily be detected in both the inflammatory infiltrates and in spleen and blood of homoTgE1335 mice (not shown).

*p75TNF-R-induced Pathology Develops Even in the Absence of TNF, LT $\alpha$  or the p55TNF-R.* To examine the dependency of the observed p75TNF-R-mediated pathology on the presence of the TNF or LT $\alpha$  ligands, and to evaluate the contribution of a cooperation of the human p75TNF-R with the endogenous p75 or p55TNF-Rs, we have introduced the hup75TNF-R transgene, as a homozygous trait, into genetic backgrounds deficient in either TNF (6), LT $\alpha$  (35), the p75TNF-R (18), or the p55TNF-R (9). In all four deficient backgrounds, homozygous TgE1335 mice developed fully the lethal multi-organ pathology. Increased levels of the human p75TNF-R are therefore sufficient to induce disease even in the absence of the p55TNF-R (Fig. 5). Most importantly, the pathogenic potential of the hu-



**Figure 2.** Production of a functional hup75TNF-R protein on the surface of transgenic thymocytes. (A) Freshly isolated or ConA-stimulated thymocytes from normal, TgE1335, and mup75TNF-R knockout mice were analyzed by flow cytometry for the expression of murine and human p75TNF-R protein. Approximately 20 and 22% of freshly isolated normal and transgenic thymocytes were found to express endogenous or transgenic p75TNF-R, respectively. ConA stimulation resulted in the induction of both the murine p75TNF-R in normal mice (60% of cells positive) and the human p75TNF-R in transgenic mice (57% of cells positive). Thymocytes from normal or p75TNF-R-deficient mice are used as negative controls. (B) Proliferative response of ConA-treated TgE1335 (●) and normal (○) thymocytes to human rTNF reveals a functional human p75TNF-R protein. The amount of <sup>3</sup>H incorporation in either normal or transgenic thymocytes treated with ConA alone is indicated by a dashed line. Results are representative of three independent experiments.

man p75TNF-R could be exerted independent of the presence of TNF (Figs. 4 and 5) or LT $\alpha$  (not shown). Interestingly, however, although the p55TNF-R and LT $\alpha$  were dispensable for the development of pathology, a low but



**Figure 3.** Production of soluble and cell surface hup75TNF-R protein in transgenic sera and on peripheral blood leukocytes. (A) Serum levels of soluble mup75 and hup75TNF-Rs were measured by specific ELISAs, before or after LPS-administration in normal ( $n = 4$ ), and TgE1335 heterozygous ( $n = 4$ ) mice. Total (murine and human) sp75TNF-R production in heterozygous TgE1335 mice is increased approximately fivefold in comparison to the production of endogenous sp75TNF-R protein in normal mice ( $49 \pm 4.5$  ng/ml total p75TNF-R protein in transgenic mice versus  $10 \pm 2.5$  ng/ml of endogenous p75TNF-R protein in normal mice). Transgenic p75TNF-R production in LPS-treated TgE1335 mice is regulated similarly to normal LPS-treated mice. TgE1335 homozygous mice ( $n = 4$ ) spontaneously produce highly elevated levels of both soluble murine and human p75TNF-Rs. (B) Flow cytometric analysis of peripheral blood leukocytes from 3-wk-old TgE1335 heterozygous or homozygous mice shows the enhanced surface expression of hup75TNF-R on leukocytes taken from homozygous transgenic animals. Data are representative of three independent experiments.

measurable pathogenic contribution of the endogenous TNF could be observed, since in TNF-deficient backgrounds homozygous TgE1335 mice do succumb to disease but with a delay of a few weeks (Fig. 5). A measurable delay in disease progression was also evident in the absence of the endogenous p75TNF-R (not shown), especially at the histopathological level where homoTgE1335/p75TNF-R<sup>-/-</sup> mice displayed a generally milder phenotype (e.g., fewer inflammatory infiltrates in several organs and no evidence for necrosis in liver) in comparison to homoTgE1335 controls, confirming that development of pathology correlates with the level of p75TNF-Rs being produced. Consistent with the enhancing pathogenic involvement of endogenous TNF, diseased homozygous

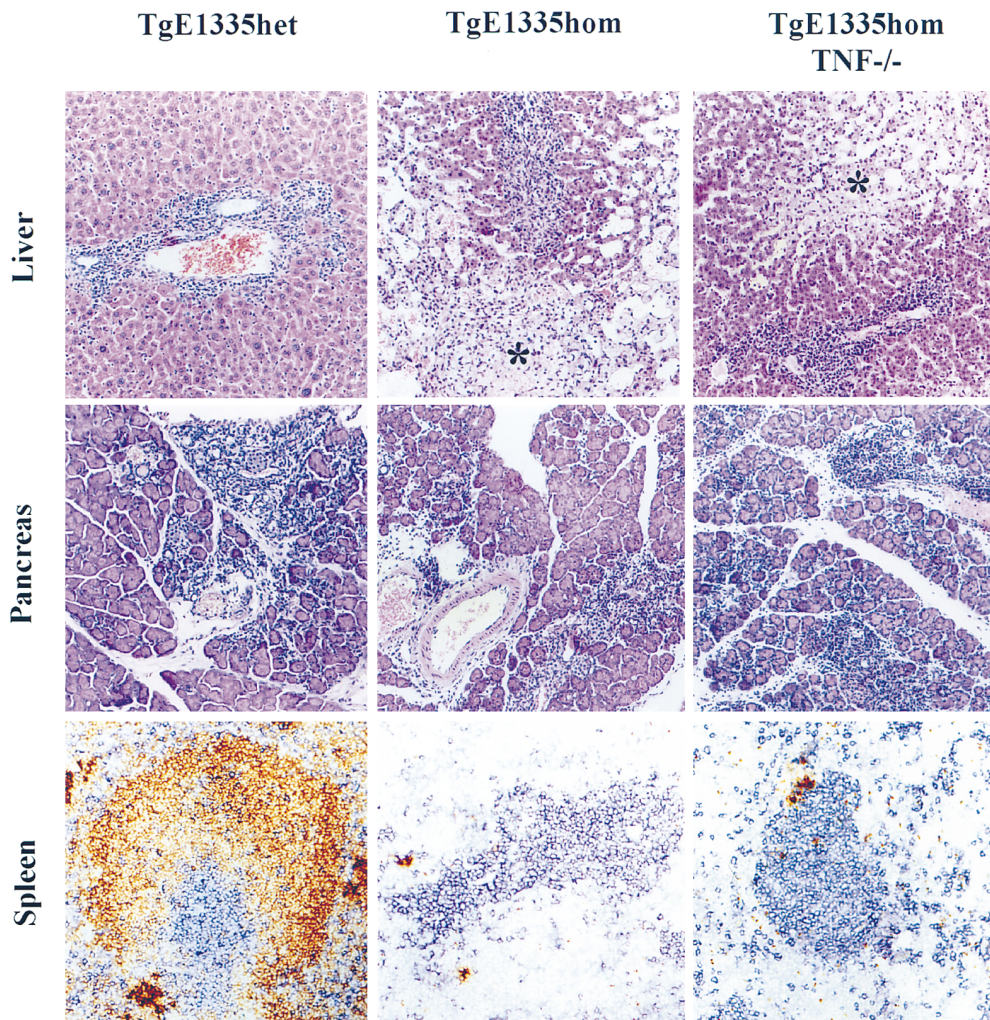
**Table 1.** Human p75TNF-R Transgenic Mice Are More Susceptible to Lethality After Administration of Human rTNF or LPS

| Dose                                 |                                   | Lethality |         |
|--------------------------------------|-----------------------------------|-----------|---------|
| huTNF ( $\mu\text{g}/\text{mouse}$ ) | LPS ( $\mu\text{g}/25\text{ g}$ ) | Control   | TgE1335 |
| 60                                   | -                                 | 0/3       | 1/3     |
| 90                                   | -                                 | 1/6       | 6/6     |
| 150                                  | -                                 | 3/3       | 3/3     |
| -                                    | 200                               | 0/7       | 1/7     |
| -                                    | 400                               | 0/7       | 2/7     |
| -                                    | 800                               | 2/7       | 7/7     |
| -                                    | 1200                              | 5/5       | 5/5     |

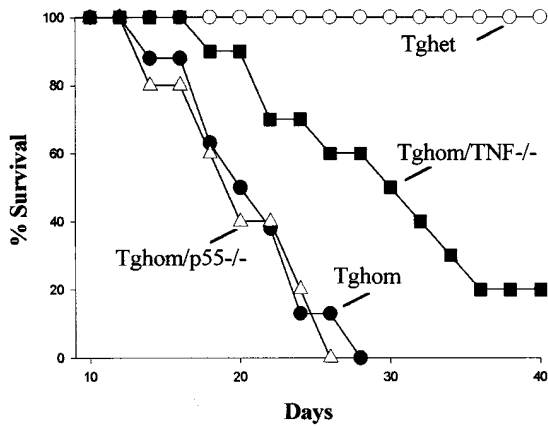
TgE1335 mice show dramatically increased levels of endogenous TNF in their sera ( $1.09\text{ ng/ml} \pm 0.15$ ,  $n = 4$ ). Notably, a similar lethal multi-organ inflammatory disease could be triggered at 4–8 wk of age, even in the absence of the p55TNF-R (i.e., in a p55TNF-R knockout back-

ground), in heterozygous TgE1335 mice bred with otherwise healthy transgenic mice overexpressing T cell targeted human wild-type (Tg7; reference 15), or transmembrane TNF (Tg5453; reference 16; not shown). This result shows that in this model, the pathogenic contribution of TNF results from direct interaction with the p75TNF-R and does not necessitate a functional p55TNF-R. The lethal phenotype of the Tg7/TgE1335 double heterozygotes, but not the baseline inflammatory complications of the heterozygous TgE1335 line could be completely neutralized by preventive treatment of mice with a specific anti-human TNF antibody (CB0006; Celltech Therapeutics Ltd, Slough, UK; not shown). These results demonstrate that, in vivo, the p75TNF-R mediates inflammatory signals independently of the presence or coactivation of the p55TNF-R. Moreover, although the presence of TNF could further enhance the spontaneous inflammatory activities of the p75TNF-R, its role in the activation of these deleterious functions was nonessential.

*Enhanced NF- $\kappa$ B Binding Activity in Nuclear Extracts of PBMC from hup75TNF-R Transgenic Mice.* NF- $\kappa$ B activation is believed to be important in the triggering of proinflammatory cytokine cascades (44) and it has recently been



**Figure 4.** p75TNF-R-triggered multi-organ inflammation and hematopoietic abnormalities develop even in the absence of TNF. Histopathological analysis (H/E) in liver and pancreas of 4-mo-old heterozygous TgE1335 mice (*TgE1335het*) and 3-wk-old TgE1335 heterozygous (*TgE1335het*) or homozygous TgE1335  $\times$  TNF $^{-/-}$  mice (*TgE1335hom/TNF $^{-/-}$* ). Lesions in heterozygous TgE1335 mice involve mainly the liver and pancreas where inflammatory infiltrates develop and persist chronically from 2–3 mo of age. In homozygous TgE1335 mice a more severe pathology develops, characterized by extensive periportal inflammation and tissue necrosis (asterisk) in the liver, and in a severely hypoplastic and inflamed pancreas. A similar histopathology evolves in homozygous TgE1335 transgenic mice bred into a null TNF background. Original magnification  $\times 108$ . Spleen sections from homozygous TgE1335 mice are characterized by markedly reduced numbers of IgM $^{+}$  B cells (brown) whereas CD3 $^{+}$  T cell localization (blue) seems unaffected. A similar phenotype occurs even in the absence of endogenous TNF. Original magnification  $\times 114$ .

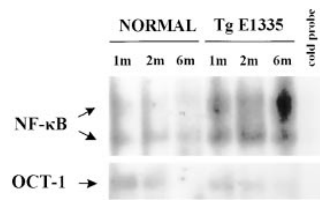


**Figure 5.** Survival of hup75TNF-R transgenic mice in the absence of TNF or the p55TNF-R. All TgE1335 heterozygous mice (*Tghet*,  $n = 10$ ,  $\circ$ ) survive usually past 10 mo of age whereas TgE1335 homozygous mice (*Tghom*,  $n = 10$ ,  $\bullet$ ) succumb to the disease during their first month of age even in the absence of the p55TNF-R (*Tghom/p55<sup>-/-</sup>*,  $n = 6$ ,  $\Delta$ ). The presence of TNF contributes positively but is not essential for the development of the lethal pathology (*Tghom/TNF<sup>-/-</sup>*,  $n = 10$ ,  $\blacksquare$ ). Survival is measured as a percentage of the initial number of animals in each genotype group.

shown that *in vivo* NF- $\kappa$ B activation in PBMC plays an important role in the lethality accompanying LPS-induced endotoxemia in mice (32). On the other hand, signaling through the p75TNF-R is known to involve NF- $\kappa$ B activating pathways (45). Therefore, it was important to assess the activation status of the NF- $\kappa$ B system in the p75TNF-R transgenic mice. NF- $\kappa$ B binding activity was determined by EMSA in nuclear extracts of PBMC from heterozygous TgE1335 mice at different developmental points. NF- $\kappa$ B binding activity was found consistently elevated in PBMC from transgenic versus control mice (Fig. 6), either before (1 mo of age), during (2 mo), or after (6 mo) development of pathology, as assessed by simultaneous histopathological analysis of all experimental mice (not shown). These results suggest that a possible target pathway of sustained p75TNF-R activation in PBMC is the NF- $\kappa$ B pathway and offer a mechanistic link to explain the observed *in vivo* inflammatory activities of this receptor.

## Discussion

Circulating levels of soluble TNF-Rs are constantly elevated in a variety of clinical conditions including malignant (46), infectious, and sub-acute or chronic inflammatory or autoimmune diseases (33, 47). In several of these conditions, measurement of sTNF-Rs, especially of the sp75TNF-R, correlates even better than classical disease-specific markers to the prognosis, symptoms, and clinical outcome of the disease (33). For example, sTNF-R levels have a strong and early prognostic value for disease progression in HIV-infected patients (30) and in several inflammatory diseases such as chronic hepatitis virus infections (25), acute pancreatitis (27), acute respiratory distress syndrome (26), SLE (28), and rheumatoid arthritis (29).



**Figure 6.** Enhanced NF- $\kappa$ B binding activity in PBMC from hup75 transgenic mice. EMSA of nuclear extracts from PBMC isolated from heterozygous TgE1335 mice or normal control littermates ( $n = 5$  per group) at 1, 2, and 6 mo of age. Specific NF- $\kappa$ B complexes are indicated by arrows, whereas OCT-1 probe acts as an internal control for sample loading. Cold NF- $\kappa$ B or OCT-1 probe was used to indicate specific NF- $\kappa$ B or OCT-1 binding, respectively.

The actual involvement of soluble TNF receptors in disease pathogenesis remains controversial and it has been suggested that they may act both as antagonists of TNF action by competing with its cell surface receptors, but also as agonists by protecting TNF from degradation and therefore stabilizing and enhancing its activity (47). Shedding of both TNF receptors occurs in both a constitutive and inducible manner, after appropriate stimulation by a plethora of immune activating signals, and in addition to providing the soluble receptors it is thought to serve in rendering cells temporarily unresponsive to TNF (47). However, a correlation of induced sTNF-R levels with enhanced expression of their cell surface precursors in specific cell types remains possible, especially in the case of the p75TNF-R, the expression of which is known to be highly inducible by the same signals that mediate its induced shedding (38, 47). Indeed, as shown clearly in this study, sustained upregulation of human p75TNF-R production in transgenic mice, leads to both an upregulated level of shed soluble receptors but also to a chronic accumulation of the receptor on the cell surface. Therefore, it is possible that increased levels of shed p75TNF-Rs, as seen in human diseases, reflect a similar upregulation of the cell surface form of the receptor, which may interfere with immune homeostasis and/or pathogenesis.

Notably, the severity of the inflammatory phenotypes developing in transgenic lines expressing hup75TNF-R transgenes correlates positively with the levels of soluble human p75TNF-R measured in diseased sera. For example, heterozygous TgE1334 mice constitutively expressing only double the physiological levels of p75TNF-Rs do not show any signs of pathology, whereas heterozygous TgE1335 mice producing four- to fivefold higher levels develop a chronic inflammatory disease. On the other hand, homozygous TgE1335 mice developing a most severe multi-organ inflammatory phenotype are found to constitutively produce twofold increased levels of total sp75TNF-Rs in comparison to the levels measured for endogenous p75TNF-Rs in sera from endotoxemic (LPS-treated) mice (Fig. 3 A). Interestingly, serum sp75TNF-R levels measured in several human inflammatory diseases, including AIDS, are usually two- to fourfold increased over normal individuals (25–30), whereas even higher levels have been recorded in septic shock patients (24). Taken together, our results show that at levels similar to those seen in human disease, chronic induced production of the p75TNF receptor *in vivo*, has detrimental effects to physi-

ological homeostasis and leads to a multi-organ inflammatory syndrome in mice. Furthermore, they demonstrate that the severity of the in vivo proinflammatory activities of the p75TNF-R correlate positively with its chronic level of production.

Although our current knowledge of the involvement of the TNF/p55TNF-R pair in disease pathogenesis is quite advanced, understanding of the in vivo function of the p75TNF-R remains vague. Recent studies in p75TNF-R knockout mice have failed to show a specific function for this receptor in physiology or in experimental models of TNF-mediated disease (18, 48) with the exception of its profound involvement in the cerebral complications of experimental malaria (49) or in the allergen-induced migration of Langerhans cells (50). Moreover, in view of the almost uniquely known in vitro activities of this receptor on thymocyte/T lymphocyte proliferation (42), or in the activation induced apoptosis of CD8<sup>+</sup> T cells (51), it was quite surprising that thymocytes and lymphocytes in p75TNF-R knockout mice were normal (18). The failure to demonstrate an in vivo independent activity of the p75TNF-R in the knockout system, together with ample evidence for a partial agonistic role of this receptor in TNF/p55TNF-R-mediated responses (14, 52, 53) has led to the hypothesis that the p75TNF-R serves an accessory role in enhancing p55TNF-R-mediated signaling. Interestingly, in all cases where p75TNF-R-specific signaling has been observed, a high density of this receptor on the cell surface was required, suggesting that inducibility of this receptor is a prerequisite for function (21, 22). Activation of receptors through induced aggregation is a widespread phenomenon in cytokine and growth factor signaling (54, 55). Ligand-induced clustering of receptors seems to be a primary control mechanism, in particular for constitutively produced receptors, such as the p55TNF-R. On the other hand, there is substantial evidence in vitro, that induced production of such members of the TNF-R family as the p75 (45, 56) or the p55TNF-R (57), Fas (57), CD40 (45, 58), or p75NGF-R (59) and the tyrosine kinase receptors for growth factors (60) may lead to spontaneous signaling even

in the absence of ligand. Our present data are in support of a physiologically significant role for ligand-independent signaling in vivo, especially for the p75TNF-R which is known to be highly induced in pathological conditions. However, it remains possible that yet unidentified ligands may contribute to its observed activation.

Our evidence that sustained p75TNF-R overproduction in mice may lead to inflammatory complications involving several vital tissues and organs, has important implications for inflammatory disease pathogenesis in humans. Interestingly, in a recent study addressing kinetics of production of soluble TNF-R after leakage of high doses of TNF in the circulation of patients undergoing isolated limb perfusion therapy (31), it has been observed that levels of soluble p75TNF-R remain high, long after TNF disappears from the circulation, suggesting a TNF-independent regulation of the production and perhaps function of this receptor. The surprising finding in this study, that the inflammatory activities of the p75TNF-R occur even in the absence of TNF, offers a novel mechanism for p75TNF-R functioning which may be of pivotal importance in many clinical conditions including sepsis. It is important to note that although the TNF/p55TNF-R system seems to operate only in an initial narrow window of time during clinical sepsis (61), sp75TNF-R levels are found constantly elevated, correlate positively with sepsis scores and show maximal values in patients that do not survive (24). After the disappointing neutral outcome of anti-TNF trials in sepsis, and taking into account the adverse effects of enhanced p75TNF-R production as presented in this study, it is tempting to speculate that specific antagonism of this receptor may be beneficial even at late phases of severe sepsis, but also during the course of many other human inflammatory pathologies where a positive correlation between soluble p75TNF-R and disease progression has been observed. The human p75TNF-R expressing transgenic lines presented in this study should offer a useful model system to develop and test the in vivo efficacy of such p75TNF-R antagonistic substances.

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