

## **Human Tumor Necrosis Factor Receptor (p55) and Interleukin 10 Gene Transfer in the Mouse Reduces Mortality to Lethal Endotoxemia and Also Attenuates Local Inflammatory Responses**

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### **Summary**

Anticytokine therapies have been promulgated in gram-negative sepsis as a means of preventing or neutralizing excessive production of proinflammatory cytokines. However, systemic administration of cytokine inhibitors is an inefficient means of targeting excessive production in individual tissue compartments. In the present study, human gene transfer was used to deliver to organs of the reticuloendothelial system antagonists that either inhibit tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) synthesis or block its interactions with cellular receptors. Mice were treated intraperitoneally with cationic liposomes containing 200  $\mu$ g of either a pCMV (cytomegalovirus)/p55 expression plasmid that contains the extracellular domain and transmembrane region of the human p55 TNF receptor, or a pcD-SR- $\alpha$ /hIL-10 expression plasmid containing the DNA for human interleukin 10. 48 h later, mice were challenged with lipopolysaccharide (LPS) and D-galactosamine. Pretreatment of mice with p55 or IL-10 cDNA-liposome complexes improved survival ( $p < 0.01$ ) to LPS-D-galactosamine. In additional studies, intratracheal administration of IL-10 DNA-liposome complexes 48 h before an intratracheal LPS challenge reduced pulmonary TNF- $\alpha$  levels by 62% and decreased neutrophil infiltration in the lung by 55% as measured by myeloperoxidase activity (both  $p < 0.05$ ). Gene transfer with cytokine inhibitors is a promising option for the treatment of both the systemic and local sequelae of septic shock.

It is now recognized that many of the pathologic consequences of septic shock result from the overproduction of proinflammatory cytokines (1). Two independent lines of evidence support the hypothesis that a single proinflammatory cytokine, TNF- $\alpha$ , contributes to many of the pathological changes that occur in sepsis. First, administration of TNF- $\alpha$  to laboratory animals and patients reproduces the clinical and pathological changes evident during overwhelming septic shock (2, 3). Second, neutralizing an exaggerated endogenous TNF response with soluble TNF receptor immunoadhesins or down-regulating TNF- $\alpha$  production with IL-10 pretreatment confers survival to otherwise lethal endotoxemia (4–6). Presently, clinical trials are under way with mAbs and immunoadhesins directed against TNF- $\alpha$  in patients with sepsis syndrome (7).

However, current therapeutic approaches are inherently inefficient. First, these natural antagonists or inhibitors of TNF and IL-1 have very short biological half-lives, ranging from minutes to hours (4, 8). In addition, TNF- $\alpha$  levels are often several times higher in the inflamed tissue compartment than in the plasma (9, 10), and inhibitors must therefore be given parenterally in large quantities to reach and saturate all tissue pools. Finally, exaggerated TNF- $\alpha$  production may contribute to the pathology in one body compartment, while, simultaneously, production in another compartment may actually have beneficial effects. Thus, systemic administration of cytokine inhibitors at levels sufficient to neutralize exaggerated TNF production in one tissue compartment may also block the presumably beneficial aspects of cytokine production in other tissue compartments.

To develop an alternative approach for anticytokine therapies, we investigated gene transfer of cytokine inhibitors as a novel drug delivery system. Although gene transfer and transfection studies are being actively pursued for patients

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with somatic gene disorders and for modulating the genetic basis of cancer, diabetes, and other chronic diseases, gene transfer as a therapeutic modality for sepsis and acute inflammation is not as actively pursued. However, the possibility of targeting specific organs to produce cytokine inhibitors or antagonists at local sites of inflammation makes transient gene transfer an attractive alternative to systemic administration.

## Materials and Methods

**Confirmation of Transgene Expression in Healthy Mice.** Healthy female C57BL/6j (15–17 g) mice were injected i.p. with 200  $\mu$ g of a pCMV expression plasmid that contains the open reading frame for the extracellular hinge regions of the human p55 DNA (11). The plasmid DNA (pCMV/p55) was mixed with 100 nmol of cationic liposomes containing equimolar ratios of dimethyldioctadecylammonium bromide (DDAB): dioleoylphosphatidylethanolamine (DOPE) (1 mM stock solution) in a total volume of 200  $\mu$ l (12, 13). This plasmid employs a human CMV immediate-early 1 promoter-enhancer element that has been previously shown to be an effective expression vector in a variety of murine tissues (14). Additional animals were injected with 200  $\mu$ g of pcD-SR- $\alpha$ /hIL-10 plasmid containing the open reading frame for the human IL-10 DNA (15) (obtained originally from DNAX, Inc., Palo Alto, CA) and 100 nmol of cationic liposomes. This plasmid uses an SV40 early promoter and the R-U5' sequence of the long terminal repeat of human T cell leukemia virus 1 as an enhancer element. The SV40-R-U5' promoter-enhancer sequence is shown to yield efficient expressions in a variety of cell lines (15). Our previous studies demonstrated that maximal expression of gene transfer by liposomes using similar expression vectors occurs after 48 h, and continued expression is observed in varying tissues for periods up to 14 d (12). As a control, mice were injected with 200  $\mu$ g of plasmid DNA (pCMV5) without the insert, and mixed with cationic liposomes (100 nmol) or administered liposomes alone.

After 48 h, the mice were killed by cervical dislocation, and the organs were rapidly removed and frozen immediately in liquid nitrogen. Total cellular RNA was isolated from frozen organs by homogenization in 10 wt/vol of a commercial acidic phenol, guanidinium isothiocyanate solution (RNAzol B<sup>®</sup>; Tel-Test, Inc., Friendswood, TX), and precipitation with isopropanol. Reverse transcription (RT)-PCR was performed on total cellular RNA according to the manufacturer's specifications (RNA-PCR; Perkin-Elmer Cetus Instruments, Norwalk, CT) with the following modifications: 1  $\mu$ g of total cellular RNA was reverse transcribed with 50 U Moloney murine leukemia virus reverse transcriptase for 10 min at room temperature and subsequently at 42°C for 30 min using oligo-d(T) as a template. The PCR was performed with 0.5 vol of the reverse transcriptase mixture, 1.25 U of Taq DNA polymerase, and 2.5  $\mu$ l of 20  $\mu$ M sense and antisense primers (sequences described below). The reaction was run at 30 cycles comprised of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. After completion of the 30 cycles, an additional primer extension period of 7 min at 72°C was performed. The DNA products were then run on a 1% agarose gel with 1  $\mu$ g/ml ethidium bromide.

The primer sequences used for IL-10 were 5'-CTG AAG ACT TTC TTT CAA ACA AAG-3' and 3'-CTG CTC CAC TGC CTT GCT CTT ATT-5', and were obtained from Stratagene, Inc. (La Jolla, CA). The region amplified corresponds to a 208-bp expected product size. Sense primer mismatch with murine IL-10 cDNA was 7 of 20 bp, and antisense primer mismatch was 4 of 20 bp.

The primer sequences used for p55 were 5'-CTG GTG CTC CTG GAG CTG TT-3' and 3'-TC TGT GAC GGA GTC GAC GAG-5'. The region amplified corresponds to a 271-bp expected product size.

Immune-reactive human p55 and IL-10 were determined by ELISA on cell-free fractions from homogenized organs. Very briefly, organs were homogenized in 5 wt/vol of PBS containing a protease inhibitor cocktail (16). After centrifugation at 19,000 g for 30 min at 4°C, the supernatant was assayed for human p55 and human IL-10 immunoactivity using ELISAs that have been previously shown not to cross-react with the murine p55 and IL-10, respectively.

**Endotoxemic Shock.** Three studies were performed. Healthy C57BL/6j mice were injected i.p. with 200  $\mu$ g of a pCMV/p55 or pcD-SR- $\alpha$ /hIL-10 plasmid mixed with 100 nmol of cationic liposomes containing equimolar ratios of DDAB:DOPE in a total volume of 200  $\mu$ l. As a control, mice were injected with 200  $\mu$ g of plasmid DNA (pCMV5) without an insert mixed with cationic liposomes (100 nmol) or administered liposomes alone.

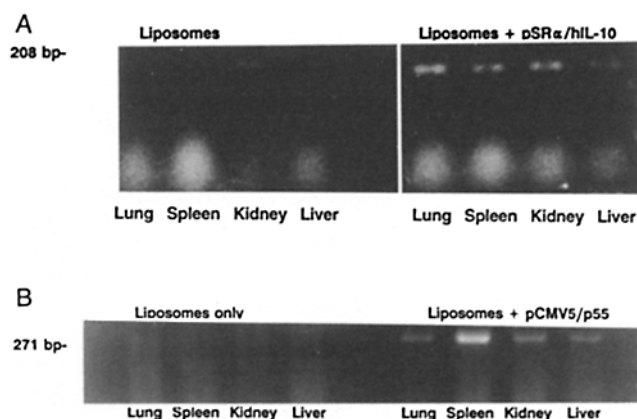
After 48 h, animals were challenged with 250 ng of *Escherichia coli* 0127:B8 LPS and 18 mg D-galactosamine (D-galN) administered i.p. At 90 min, mice were bled from the tail, and serum TNF bioactivity assayed using the WEHI 164 clone 13 cytotoxicity assay (4). Survival over the next 72 h was evaluated.

**Direct Lung Injury.** Under pentobarbital anesthesia (35 mg/kg body wt), a midline incision was made on the ventral aspect of the neck and the trachea identified after blunt dissection. The trachea was reflected forward by inserting a small plastic loop behind the trachea. Using a 30-gauge needle, 50  $\mu$ g of pCMV/p55 or pcD-SR- $\alpha$ /hIL-10 mixed with 25 nmol of DDAB:DOPE liposomes (in 25  $\mu$ l) was instilled directly into the trachea. The skin wound was closed with a surgical clip, and the animals were allowed to recover. 2 d later, the skin wound was reopened under general anesthesia, and 10  $\mu$ g of *E. coli* 0127:B8 LPS in 25  $\mu$ l of saline was reinstalled into the lung. 5 h later, the animals were killed and the lungs were removed and frozen in liquid nitrogen. TNF- $\alpha$  immunoactivity on the cell-free lung homogenates was determined by ELISA, and neutrophil infiltration was documented by myeloperoxidase (MPO) activity (17). The murine TNF- $\alpha$  ELISA incorporates an mAb (MP6-XT3; PharMingen, San Diego, CA) (1  $\mu$ g/ml) as the capture protein, a polyclonal rabbit antisera (A-0123; Cornell University Medical College, New York), as the primary antibody (1:100), and a commercially available horseradish peroxidase-conjugated, goat anti-rabbit antisera (Promega Corp., Madison, WI) (1:8,000) to visualize the protein. Recombinant murine TNF- $\alpha$  (Upstate Biotechnology, Inc., Lake Placid, NY) serves as the standard. Sensitivity of the assay is 34 pg/ml.

**Data Analysis.** Differences in survival between control and plasmid-infused animals were determined by Fisher's exact test. Differences in cytokine and MPO concentration were determined by ANOVA with Newman-Keuls post hoc multiple range test. Statistical significance was designated at the 95% confidence interval.

## Results and Discussion

48 h after plasmid/liposome injections, healthy C57BL/6j mice were killed, and human p55 or IL-10 mRNA expression in organs of the reticuloendothelial system was evaluated by RT-PCR using primers specific for the human proteins. As shown in Fig. 1, both human p55 and IL-10 mRNA expression was observed in spleen, liver, kidney, and lung of animals administered liposomes containing the appropriate



**Figure 1.** Confirmation of gene transfer by RT-PCR for human IL-10 and p55 mRNA. Mice received i.p. injections of 100  $\mu$ g DNA in 50 nmol DDAB:DOPE cationic liposomes, or cationic liposomes alone, as described in the manuscript. 48 h later, animals were killed and organs were removed. The presence of human IL-10 or p55 mRNA was confirmed by RT-PCR.

DNA. Immune-reactive IL-10 and p55 could also be detected in these organ homogenates. In the case of human p55, concentrations ranged from 0.4 to 7.1 ng/g of tissue, with highest levels in the spleen and lowest in the lung. For IL-10, concentrations ranged between 350 and 800 pg/g tissue. However, human IL-10 or p55 was not detected in the blood of these mice, thus confirming that gene transfer resulted only in the local tissue production of these mediators, and quantities produced were not sufficient to be detected in the systemic circulation.

The appearance of both human IL-10 and p55 mRNA in lungs from such animals was somewhat unexpected given the i.p. site of injection. However, transport of liposomes out of the peritoneal cavity via the lymphatics or the circulation could explain the gene transfer to the lungs.

To determine the effectiveness of the antagonists in inhibiting inflammatory responses, mice transfected with either human IL-10 or p55 were challenged 48 h later with 250 ng LPS and 18 mg D-galN. Lethality in this LPS-D-galN model is dependent on an exaggerated TNF- $\alpha$  response, since treatment of mice with TNF receptor immunoadhesins (18) or the use of TNF receptor (p55)-deficient mice (19) results in reduced mortality. Our results from three separate experiments demonstrated that gene transfer with IL-10 or p55 improves survival (Table 1). In addition, IL-10 gene transfer significantly reduced the plasma TNF- $\alpha$  response to LPS-D-galN. In the first experiment, mice were transfected with the pCMV5 expression plasmid without the p55 DNA insert, whereas, in the remaining two experiments, mice received only liposomes. In either case, survival was not affected by administration of either liposomes or liposomes and the expression plasmid alone. Thus, local delivery of these TNF- $\alpha$  inhibitors to organs of the reticuloendothelial system ameliorated the exaggerated TNF responses and improved survival.

In additional experiments, mice received the intratracheal instillation of liposomes/plasmids containing the DNA for IL-10 or p55. After 48 h, the trachea was reexposed, and

**Table 1.** Survival and Peak TNF- $\alpha$  Concentrations in LPS-D-galN Mice Pretreated with Liposomes Containing pCMV/p55 or pcD-SR- $\alpha$ /IL-10

Experiment number	pCMV/p55	pcD-SR- $\alpha$ /hIL-10	Irrelevant DNA/liposomes
	Survived/total	Survived/total	Survived/total
1	4/6	6/6	1/6
2	3/6	4/6	0/6
3	3/6	6/6	1/6
Totals	10/18 <sup>†</sup>	16/18 <sup>‡</sup>	2/18
TNF- $\alpha$ ,			
pg/ml	2,080 $\pm$ 810	190 $\pm$ 60 <sup>§</sup>	2,690 $\pm$ 660

Mice received the i.p. administration of 100  $\mu$ g of plasmid DNA containing either the pCMV/p55, pcD-SR- $\alpha$ /IL-10, or only the pCMV5 plasmid and/or 100 nmol of DDAB:DOPE liposomes. In all three experiments, survival was improved and serum TNF- $\alpha$  concentrations were significantly reduced by gene transfer with IL-10. p55 gene transfer also improved outcome but did not significantly affect serum TNF- $\alpha$  concentrations, as measured by WEHI bioassay.

\* In experiment number 1, control mice were pretreated with liposomes containing equivalent quantities of pCMV plasmid without the insert. In subsequent experiments, only liposomes were administered.

<sup>†</sup>  $p < 0.01$  versus control by Fisher's exact test.

<sup>§</sup>  $p < 0.05$  versus control by ANOVA and Newman-Keuls multiple range test.

10  $\mu$ g of *E. coli* 0127:B8 LPS in 25  $\mu$ l in normal saline was instilled. 5 h after the endotoxin challenge, the mice were killed, and lungs were removed for TNF- $\alpha$  content and neutrophil infiltration, as measured by myeloperoxidase levels (20). Gene transfer with IL-10, but not with p55, significantly reduced the TNF- $\alpha$  content of the lung homogenates (Table 2). Similarly, the degree of neutrophil infiltration was reduced by 55% in IL-10-treated animals but was not reduced in p55-treated animals.

In retrospect, it is not surprising that gene transfer with p55 was less effective than IL-10 after i.p. and intratracheal LPS challenge, given the magnitude of the TNF- $\alpha$  response. We have previously shown in primates that soluble p55 must be in a 100–500 molar excess to fully neutralize TNF- $\alpha$  (4). In the present studies, the quantities of soluble human p55 recovered from the lungs were only 1–10 times higher than the concentrations of TNF- $\alpha$ , suggesting that the quantities of p55 achieved were inadequate to completely inhibit TNF- $\alpha$ -mediated responses. In addition, the failure of p55 treatment to neutralize TNF- $\alpha$  activity in either study is not surprising, given the nature and stability of the p55-TNF- $\alpha$  complexes. For example, we showed that treatment of *E. coli* shock in baboons with recombinant p55 attenuated the inflammatory response but only delayed the appearance of plasma TNF- $\alpha$  bioactivity (4). Similarly, Mohler et al. observed increased quantities of TNF activity after an LPS challenge in mice administered recombinant p75 TNF receptor (21).

**Table 2.** Lung TNF and Myeloperoxidase Levels in Mice after Intratracheal LPS Administration and Gene Transfer

	pCMV/p55*	pcD-SR- $\alpha$ /hIL-10	Liposomes	Healthy, control
TNF- $\alpha$ , pg/g				
wet wt	500 $\pm$ 43	146 $\pm$ 58 <sup>†</sup>	382 $\pm$ 66	<100
MPO, U/g				
wet wt	53.2 $\pm$ 1.3	26.4 $\pm$ 5.0 <sup>§</sup>	58.7 $\pm$ 6.2	1.7 $\pm$ 1.7

Treatment of mice with pcD-SR- $\alpha$ /hIL-10, but not pCMV/p55 DNA, attenuates the TNF- $\alpha$  response and the neutrophil infiltration in the lung, as measured by myeloperoxidase activity.

\* The *n* for each group was 10 animals.

<sup>†</sup> *p* < 0.05 versus liposomes.

<sup>§</sup> *p* < 0.01 versus liposomes, by ANOVA and Newman-Keuls multiple range test.

In contrast to transfection with the modified p55, the increased effectiveness of IL-10 gene transfer highlights a therapeutic advantage associated with cytokines that directly inhibit proinflammatory cytokine production rather than competing for ligand binding. The concentrations of human IL-10 achieved in organs of the mice approximate the quantities required to down-regulate macrophage activation *in vitro* (22), whereas the levels of p55 are almost three logs lower than quantities presumed to neutralize fully the levels of TNF- $\alpha$  achieved (4).

Our results underscore several advantages for the use of gene transfer as a treatment option for septic shock or other acute inflammatory episodes. First, the specificity of targeting inhibitors to specific organs is greatly increased. Debs and colleagues have shown that aerosol delivery of plasmid DNA to rats results in transgene expression occurring only in the lung (23), an observation we observed with intratracheal instillation. Second, the gene transfer schema permits a continued expression of these inhibitors for several days, allowing for prolonged delivery of a short-lived antagonist. However, since the gene transfer is ultimately transient and the plasmid

DNA remains episomal, expression efficiencies decline after 48 h (12). Therefore, the risk of a stable transfection and incorporation of a gene for a potentially immunosuppressive agent under a constitutive promoter is remote. Finally, local cytokine inhibitors can be directed away from organs where the putative beneficial effects of proinflammatory cytokines are occurring.

It should be noted that, in the studies conducted here, the gene transfer was delivered 48 h before the inflammatory insult. The animals were exposed to increased levels of IL-10 and p55 for an extended period before the endotoxin challenge. It is unlikely that such therapeutic approaches would be as advantageous if they were given after the initial inflammatory insult, as is currently being proposed for clinical trials with TNF- $\alpha$  antibodies and immunoadhesins (7, 24). In fact, therapeutic efficacy with TNF- $\alpha$  inhibitors and IL-1 receptor antagonist in patients with sepsis syndrome have produced variable results when given several hours after the onset of symptomatology (7, 24). However, the current study confirms that gene transfer is a viable and potentially useful approach to targeting individual organs for anticytokine therapies.

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