# p53 is a suppressor of inflammatory response in mice

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## SPECIFIC AIMS

Chronic inflammation is known to promote cancer, suggesting that negative regulation of inflammation is likely to be tumor suppressive. p53 and NF-κB are two major players in regulation of tumor development and inflammation, respectively, and are competitors in trans-activation and apoptotic function. If suppression of trans-activation ability of NF-κB by p53 found in vitro would also occur in vivo, this might result in strong differences in the induction of inflammatory response between wild-type (WT) and p53-deficient mice. This work was devoted to testing this hypothesis.

## PRINCIPAL FINDINGS

### 1. p53 is an inhibitor of NF-κB-mediated trans-activation

We previously showed that suppression of p53 makes the human prostate carcinoma cell line LNCaP resistant to TNF, which in other systems is known to be associated with activation of NF-κB. This observation suggested that p53 could interfere with apoptosis-protecting function of NF-κB. Since both p53 and NF-κB exert their activities via regulation of transcription, we applied cDNA microarray-based gene expression profiling to estimate the effect of p53 status of cells on NF-κB-mediated transcription. Repression of p53 was achieved by transduction of LNCaP with a dominant negative mutant of p53, GSE56. NF-κB was induced by treating cells with TNF, and RNA was isolated 6 h later. Clusterization of genes according to their expression patterns revealed striking similarities between sets of genes that alter their expression in the presence of GSE56 and those induced by TNF (**Fig. 1*a***) and showed ~50% overlap between these subsets. A large proportion of TNF-responsive genes was stronger induced in GSE56-transduced rather than in control LNCaP after TNF treatment.

Thus, basal levels of p53 reduce the responsiveness of TNF-inducible genes to TNF, suggesting that p53 might be a general inhibitor of NF-κB-dependent transcription. To test this hypothesis, we compared the activity of three constructs, each containing different NF-κB binding sites placed upstream of minimal promoters, after transduction into the cells with or without the construct expressing WT p53 (Fig. 1*b*). Tumor-derived p53 mutant deficient in trans-activation ($p53^{175His}$) and insert-free vector served as controls. p53 reporter construct was used to monitor p53-mediated trans-activation. The results obtained demonstrate that WT, but not mutant p53, acts as an effective inhibitor of NF-κB-mediated trans-activation in all three different promoter constructs, indicating that p53 is a general suppressor of NF-κB-mediated transcription.

### 2. NF-κB activity is increased in tissues of p53 null mice

NF-κB regulates expression of proinflammatory cytokines and plays a key role in determining inflammatory response. If suppression of trans-activation ability of NF-κB by p53 found in vitro would occur in vivo, this might result in strong differences in the induction of inflammatory response between WT and p53-deficient mice. We tested this hypothesis by measuring the induction of mRNAs of proinflammatory cytokines in the thymuses of LPS-treated p53 null and p53 WT mice using inflammatory cytokine/receptor arrays including 94 genes. Higher levels of induction of many cytokines, chemokines, and chemokine receptors (including IL-1, IL-6, IL-12, TNF, CCR2, CCR5, Mig, and IP-10) were observed in p53 null mice (**Fig. 2*a***). The array hybridization results were confirmed using real-time RT PCR

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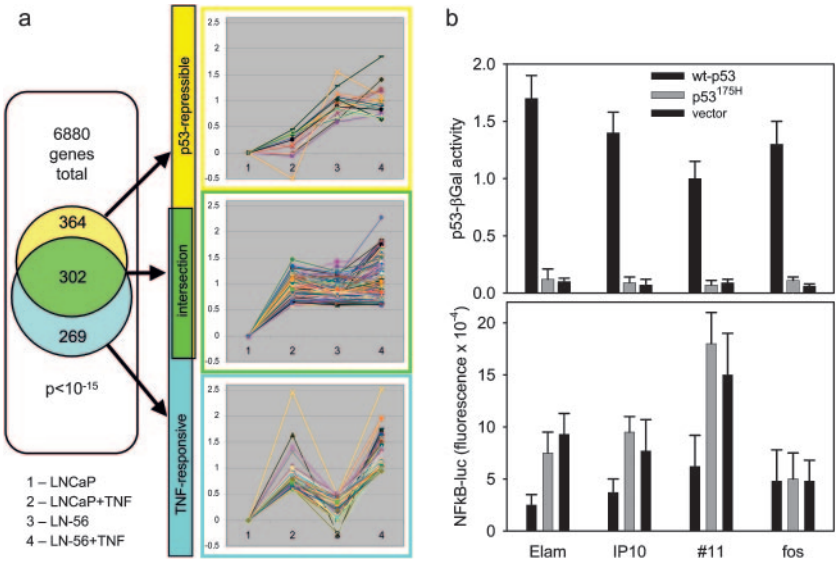
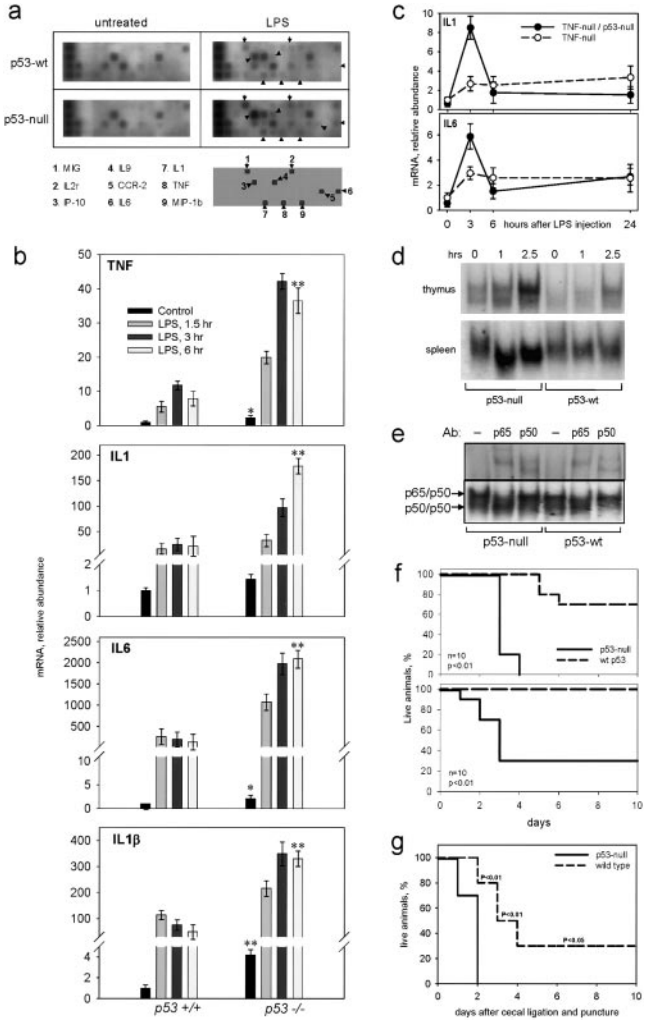



**Figure 1.** p53 is a general inhibitor of NF-κB-mediated transcription. *a*) Striking coincidence between changes in gene expression patterns caused by p53 inactivation and TNF treatment of LNCaP (LN) cells. cDNA microarray containing probes for 6880 annotated genes was used. Up- and down-regulated genes that significantly change in the same direction in both experiments are included. The 1.5-fold expression change was selected as a cutoff. Venn diagram illustrates the overlap between GSE56- and TNF-regulated genes. $P$ = probability of obtaining overlap of this size by random drawing (calculated as cumulative hypergeometric probability). Typical expression profiles are shown for each subset of genes. Y-axis reflects the log2 relative expressions (as compared with LNCaP baseline experiment). *b*) p53 represses NF-κB-mediated trans-activation. Lung carcinoma cell line H1299/CMV5 containing β-gal reporter under p53-responsive promoter (Con A) were transiently transfected with 3 different plasmids containing luciferase reporter under NF-κB dependent promoters from different genes (NF-κB binding sites from E-selectin: Elam, IP-10, or HIV LTR (#11), all cloned upstream of *fos* minimal promoter) in combination with plasmids expressing cDNAs for WT human p53, tumor-derived R175H p53 mutant ($p53^{175H}$), or empty vector. As a control NF-κB-independent reporter, we used a plasmid containing HIV LTR with a deleted NF-κB binding site. Luciferase and β-gal activity were measured in protein extracts 20 h after transfection.




(Fig. 2*b*), confirming that p53 deficiency results in an increase in responsiveness of NF-κB-inducible genes. These results correlated with stronger induction of

**Figure 2.** Increased NF-κB activity and sensitivity to septic shock in p53 null mice. *a*) LPS-induced (8 mg/kg, 3 h) expression of cytokines, chemokines, and chemokine receptors in the mouse thymuses was compared using inflammatory cytokine/receptor arrays (SuperArray, 94 genes). A fragment of arrays demonstrates the higher levels of induction of IL-1, IL-6, IL-9, IL-2r, TNF, CCR2, Mig, and IP-10 observed in p53 null compared with WT p53 mice. *b*) IL-1α, IL-1β, IL-6, and TNF mRNA expression in the thymuses of LPS-treated (8 mg/kg, 3 h) WT p53 and p53 null mice. Results of real-time RT PCR were expressed as relative units normalized to 18S RNA expression. Data represent means of 3 measurements ± SD. $*P < 0.05$, $**P < 0.01$ for p53 null vs. p53 WT mice by Student's $t$ test. *c*) Kinetics of IL-1 and IL-6 mRNA expression in TNF/p53 knockout mice in comparison with TNF knockout mice after treatment with LPS (8 mg/kg). *d*) NF-κB activation in the thymus and spleen of WT p53 and p53 knockout mice after LPS treatment. Results of gel shift assay are shown using 10 μg nuclear cellular extracts from mouse tissues either untreated or 1 and 2.5 h after LPS (8 mg/kg) treatment. *e*) Gel supershift analysis of molecular constituents of NF-κB activated by LPS in the thymus of WT p53 and p53 null mice. Antibodies against p65 or p50 were added to nuclear protein extracts (LPS, 2.5 h) normalized according to NF-κB DNA binding activity. Lower panel: area of the gel with the main NF-κB-specific bands. Upper panel: antibody-shifted bands. *f*) p53 null mice are hypersensitive to LPS-induced septic shock. Survival curves are shown for 2 doses of i.p.-injected LPS: 20 mg/kg (top) and 12 mg/kg (bottom). LPS was administered to 10 WT and 10 p53 null males (10–14 wk old, both on C57Bl/6 background). *g*) p53 null mice are more sensitive to cecal ligation and puncture (CLP) -induced peritonitis. 10 p53 null (on C57Bl/6 background) and 12 C57Bl/6 mice underwent CPL to induce sepsis.

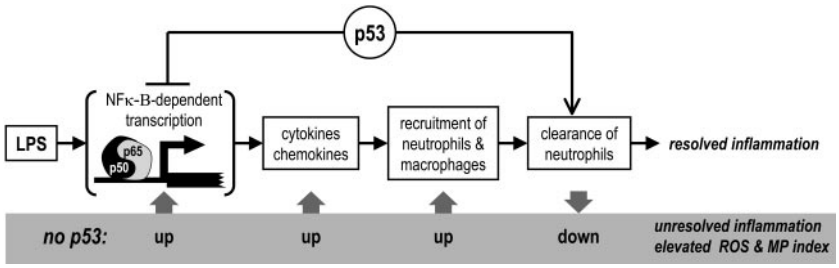



**Figure 3.** Scheme of p53-mediated regulation of inflammatory response. P53 acts in at least 2 stages of inflammation as a general inhibitor of NF-κB-dependent transcription and, through an unknown mechanism, as a positive regulator of neutrophil clearance by macrophages. Lack of these p53 functions results in overreaction to proinflammatory stimuli leading to hypersensitivity of p53 null mice.

DNA binding activity of NF-κB observed in the spleens and thymuses of p53 null animals (Fig. 2*d*). No changes in the composition of NF-κB DNA binding complexes (relative abundance of p65 and p50 isoforms) were found in p53 null vs. p53 WT mice (Fig. 2*e*).

Analysis of TNF knockout mice showed that lack of TNF did not change p53 dependence of LPS-induced expression of cytokines at least at early times after treatment (Fig. 2*c*). However, TNF might play the role of enhancer of cytokine induction, presumably by mediating a second wave of NF-κB activation.

### 3. p53-deficient mice are hypersensitive to septic shock

We suggested that abnormal inflammatory response found in p53 null mice might lead to accelerated tissue damage and increased sensitivity to LPS-induced septic shock. Increased lethality of p53 null mice was found after LPS treatment (Fig. 2*f*). Similar results were obtained in a more physiological model of peritonitis induced by surgical perforation of the cecum (Fig. 2*g*).

### 4. Increased macrophage recruitment and delayed neutrophil clearance in p53 null mice in response to inflammation-inducing agents

We measured another parameter of inflammatory response, the efflux of macrophages and granulocytes into the peritoneal cavity, of p53 WT and p53-deficient mice after intraperitoneal injections of thiyoglycolate (TG) or LPS. Higher numbers of macrophages were found in p53 null mice at all tested time points after injection of inflammation-inducing agents. Neutrophil clearance was delayed in p53 null animals compared with WT mice, which correlated with significantly reduced ability of peritoneal macrophages from p53 null mice to engulf apoptotic thymocytes. Binding of apoptotic thymocytes to p53-macrophages was also reduced, suggesting that the deficiency in phagocytosis may be partly attributable to a recognition defect of p53 null macrophages.

### 5. Biochemical markers of oxidation and myeloperoxidase index are increased in tissues and blood of p53 null mice

We tested the inflammatory status of tissues of p53 knockout mice by quantifying levels of myeloperoxidase (MPO), a major marker of inflammation, as well as multiple specific products formed by distinct oxidation pathways that participate in innate host defenses. MPO uses hydrogen peroxide and multiple organic and inorganic ions as cosubstrates to produce hypochlorous acid and other reactive oxidants, including those derived from oxidation of tyrosine and nitrite. In situ cytochemical staining of blood leukocytes for MPO was markedly enhanced in p53 knockout mice, as observed by the increased MPO index (a qualitative index of MPO content per leukocyte). Increased levels of ortho-tyrosine and nitro-tyrosine in the normal tissues of p53 knockout mice were noted, which was consistent with increased oxidative modification of proteins. We found that the levels of reactive oxygen species (ROS) (FACS analysis, DCF staining) were increased in the thymocytes of p53 null compared with WT p53 mice.

## CONCLUSIONS AND SIGNIFICANCE

We found that p53 is a general inhibitor of inflammation that acts as an antagonist of NF-κB. We first observed striking similarities in global gene expression profiles in human LNCaP prostate cancer cells transduced with p53 inhibitory genetic element or treated with TNF, suggesting that p53 inhibits transcription of TNF-inducible genes, which are largely regulated by NF-κB. Ectopically expressed p53 acts consistently as an inhibitor of transcription of NF-κB-dependent promoters. Suppression of inflammatory response by p53 was observed in vivo in mice by comparing WT and p53 null animals at molecular (inhibition of transcription of genes encoding cytokines and chemokines, reducing accumulation of reactive oxygen species and protein oxidation products), cellular (activation of macrophages and neutrophil clearance), and organismal (high levels of metabolic markers of inflammation in tissues of p53-deficient mice and their hypersensitivity to LPS) levels (**Fig. 3**). These observations indicate that p53, acting through suppression of NF-κB, plays the role of a general "buffer" of innate immune response in vivo that is well consistent with its tumor suppressor function and frequent constitutive activation of NF-κB in tumors. They also explain frequent death of p53 null mice from spontaneous unresolved inflammation. FJ