

Tumor Suppressor p53 Inhibits Autoimmune Inflammation and Macrophage Function

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The tumor suppressor p53 regulates apoptosis, cell cycle, and oncogenesis. To explore the roles of p53 in autoimmunity, we studied type 1 diabetes and innate immune responses using C57BL/6 mice deficient in p53. We found that p53-deficient mice were more susceptible to streptozotocin-induced diabetes than control mice, and they produced higher levels of interleukin-1, -6, and -12. The innate immune response of p53^{-/-} macrophages to lipopolysaccharides and γ -interferon was significantly enhanced compared with p53^{+/+} cells. p53^{-/-} macrophages produced more proinflammatory cytokines and higher levels of total and phosphorylated signal transducer and activator of transcription (STAT)-1. These results indicate that p53 inhibits autoimmune diabetes and innate immune responses through downregulating STAT-1 and proinflammatory cytokines. *Diabetes* 54: 1423–1428, 2005

p53 (transformation-related protein 53) is a member of the p53 transcription factor family, which also includes p63 and p73 (1–9). It is ubiquitously expressed at low levels in a variety of tissues and is significantly upregulated in tumors and inflamed or damaged tissues (1–7,10–13). Each p53 polypeptide contains a transactivation domain at its NH₂-terminus, a tetramerization domain at the COOH-terminus, and a DNA-binding domain at the center. The COOH-terminus also holds a regulatory domain that can negatively regulate the central DNA-binding domain. p53 has a short half-life and is normally present as a tetramer in association with the inhibitor protein MDM2 (mouse double minute 2). MDM2 binds to the NH₂-terminus of p53 and blocks its transcriptional activity. It also facilitates the export of p53 from the nucleus to cytoplasm, where it is degraded through the ubiquitin pathway (1–7). A wide variety of stimuli, including cytokines, growth factors, oncogenic stimuli,

hypoxia, and irradiation, can stabilize and activate p53. Although the exact mechanisms of p53 protein activation are not well characterized, phosphorylation and acetylation of p53 are believed to be important steps. Once activated, p53 can bind to specific DNA sequences located in the promoter regions of target genes. To date, a large number of p53 target genes have been identified in various cell types. These include genes that regulate 1) apoptosis (e.g., Fas, death receptor 5, Bax, PIG3, p85, PAG608, and IGF-Bp3) (14–20) and 2) cell cycle (e.g., p21, GADD45, B99, and 14–3–3 σ) (21–25). In addition to transactivating genes, p53 may also repress expression of certain genes, such as *c-fos*, *c-myc*, interleukin (IL)-4, and IL-6 (26,27). The mechanism of p53-mediated gene repression is not clear and may require the presence of the COOH-terminal domain. Additionally, it has been reported that p53 may be capable of inhibiting nuclear DNA replication by directly binding to DNA (1–7).

Despite intense investigations of p53 during the past few years, the physiological and pathological roles of p53 in vivo are still not well understood. Somatic mutations of p53 gene have been detected in many tumor cells and synovial cells of subjects with rheumatoid arthritis (1–7,28). Germline mutations of the p53 gene in humans and mice significantly increase the incidence of tumors of various cell lineages (2,3,29). These and other observations have led to the conclusion that p53 is responsible for preventing oncogenesis, presumably by inducing cell cycle arrest or apoptosis of proliferating cells. However, p53 gene expression is upregulated in a number of conditions not directly related to oncogenesis. These include inflammation, trauma, hypoxia, and infections (1–7,10–13). Additionally, p53 gene mutation has also been detected in synovial cells of subjects with rheumatoid arthritis (28), and p53 deficiency in mice accelerates—whereas p53 gene transfer ameliorates—autoimmune arthritis (10,30). Using microdissected rheumatoid arthritis synovial tissue sections, Firestein and colleagues (31) recently reported that p53 mutations were located mainly in the synovial intimal lining rather than the sublining. Regions with high rates of p53 mutations contained significantly greater amounts of IL-6 mRNA compared with the low-mutation samples, suggesting that p53 gene mutation may exacerbate arthritis by enhancing the production of inflammatory cytokines. Thus, in addition to oncogenesis, p53 may also play important roles in inflammatory disorders (13,32,33).

To directly test the roles of p53 in autoimmune diseases, we studied low-dose streptozotocin (STZ)-induced diabetes in p53-deficient C57BL/6 mice. We found that p53 plays crucial roles not only in the pathogenesis of autoimmune diabetes, but also in macrophage innate functions. Fur-

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DMEM, Dulbecco's modified Eagle's medium; IFN- γ , γ -interferon; IL, interleukin; STAT, signal transducer and activator of transcription; STZ, streptozotocin; TNF- α , tumor necrosis factor- α .

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thermore, we discovered that p53 may inhibit innate immunity through signal transducer and activator of transcription (STAT)-1, a transcription factor important for immunity and inflammation. Results reported here provide new insights into the roles of p53 in autoimmune inflammation, which may have important ramifications for the treatment or prevention of autoimmune diseases.

RESEARCH DESIGN AND METHODS

Breeding pairs of C57BL/6J (B6) p53^{-/-} mice (34) were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were used to generate p53^{+/+} and p53^{-/-} mice. The p53 gene mutation was tracked by PCR. All mice were housed in the University of Pennsylvania animal care facilities under pathogen-free conditions, and all procedures were approved by the animal care and use committee.

Reagents and cytokine assay. STZ was purchased from Sigma (St. Louis, MO). The following reagents were purchased from BD Pharmingen (San Diego, CA): purified rat anti-mouse IL-1 β , IL-6, IL-12, tumor necrosis factor- α (TNF- α), and γ -interferon (IFN- γ) monoclonal antibodies; recombinant mouse IL-1 β , IL-6, IL-12, TNF- α , and IFN- γ . Cytokines in the culture supernatant, serum, and organ extracts were measured by quantitative enzyme-linked immunosorbent assay per the manufacturer's recommendations.

Induction and evaluation of diabetes. Autoimmune diabetes was induced using multiple low doses of STZ as previously described (35). Briefly, 7- to 9-week-old male mice were injected intraperitoneally once a day for 5 consecutive days with 40 mg/kg body wt STZ dissolved in 25 mmol/l citrate buffer, pH 4.5. Mice were tested once every other day for urinary glucose levels using a Keto-Diastix kit (Bayer, Elkhart, IN). Mice were considered diabetic if the urinary glucose levels were ≥ 500 mg/dl on two consecutive tests. To determine the degree of insulinitis, pancreata were fixed in 10% formalin, sectioned, stained with hematoxylin/eosin, and examined by microscopy.

Macrophage cultures. To prepare macrophages from bone marrow, femurs and tibiae were surgically removed and the marrow flushed with Dulbecco's modified Eagle's medium (DMEM). After removing erythrocytes, bone marrow cells were cultured in 30% L-cell-conditioned medium at 10^6 /ml for 7–8 days. Adherent cells were collected and cultured in DMEM containing macrophage colony-stimulating factor (R&D Systems) for 24 h. In some experiments, macrophages were collected directly from peritoneum and cultured at 10^6 /ml, with or without 10 units/ml of IFN- γ and 200 μ g/ml of lipopolysaccharide, for different times.

Preparation of organ extracts. Spleen and pancreas were aseptically removed from the mice and homogenized in RPMI-1640 containing 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Calbiochem, La Jolla, CA) at a ratio of 1:10 (wt/vol) using a Dounce grinder. The homogenates were centrifuged at 2000g for 20 min, and the supernatant was collected and stored at -80°C before the cytokine assay.

Flow cytometry. Bone marrow-derived macrophages were cultured in DMEM with or without 10 ng/ml of recombinant mouse IFN- γ for 4 h followed by an overnight incubation with or without 1 μ g/ml of lipopolysaccharide in the presence of 2 μ mol/l of GolgiStop (BD Pharmingen). Cells were stained with anti-IL-12p40/p70- α (clone C15.6) and CD11b- α (clone M1/70.15; Caltag) per BD Pharmingen intracellular cytokine staining protocols and analyzed on a FACSCalibur (BD Biosciences) using the Cell-Quest program (BD Biosciences). Data were further processed using FlowJo software (Tree Star, San Carlos, CA).

Western blot. Cells were first incubated on ice for 20 min in a lysis buffer containing 0.05 mol/l Tris, pH 8.0, 0.15 mol/l NaCl, 0.01 mol/l EDTA, and 1% NP40 in the presence of a protease inhibitor. The cell lysate was centrifuged at 2300g for 10 min at 4°C , and the supernatant was fractionated by electrophoresis on a 12% PAGE and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was incubated with either rabbit anti-STAT-1 (Upstate Biotechnology, Lake Placid NY) or anti- β -actin and with horseradish peroxidase-labeled secondary antibodies (Amersham, NJ). Color was developed using enhanced chemiluminescence Western blotting detection reagents (Amersham/Pharmacia Biotech, Piscataway, NJ).

Statistical analysis. The significance of the differences in disease severity and immune parameters was determined by the Mann-Whitney test and ANOVA, respectively.

RESULTS

p53^{-/-} mice are more susceptible to STZ-induced diabetes. To determine whether p53 is required for the development of type 1 diabetes, wild-type control mice

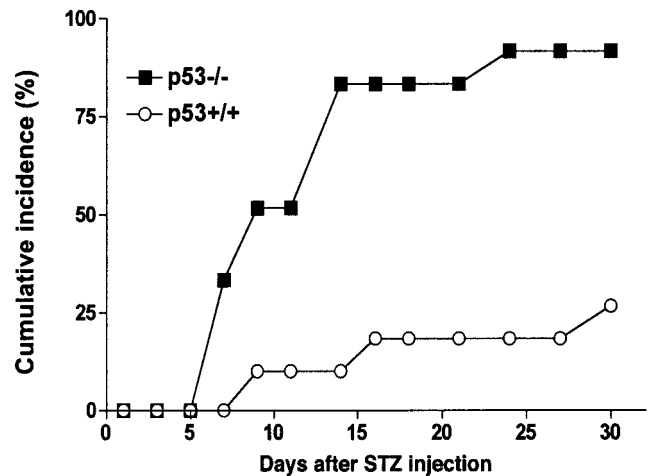


FIG. 1. p53^{-/-} mice are more susceptible to STZ-induced diabetes. p53^{+/+} (○, $n = 18$) and p53^{-/-} (■, $n = 18$) C57BL/6 male mice were injected with STZ to induce diabetes, as described in RESEARCH DESIGN AND METHODS. Data presented are accumulated diabetes incidences pooled from three independent experiments. The difference between the two groups is statistically significant ($P < 0.001$), as determined by the Mann-Whitney U test.

and mice homozygous for the p53 gene mutation were treated daily with a low dose of STZ (40 mg STZ/kg body wt) for 5 consecutive days (days 0–4) (36,37). Diabetes was monitored by both urine glucose levels and pancreatic islet histochemistry. As shown in Fig. 1, diabetes developed at a low frequency in control B6 mice ($\sim 25\%$), starting ~ 9 days after the first STZ injection. In contrast, the incidence of the disease was markedly increased in p53^{-/-} mice, reaching 95% by day 25 (Fig. 1). Consistent with these clinical findings, hematoxylin and eosin staining of pancreatic sections of control and p53^{-/-} mice revealed significant differences. Insulinitis, characterized by peri- and intraislet infiltration of inflammatory cells, was observed in most p53^{-/-} mice (Fig. 2). By contrast, the majority of pancreatic islets in control mice appeared normal, with no signs of inflammation. Therefore, p53 deficiency significantly increased the incidence of STZ-induced diabetes.

It has been reported that some of the p53^{-/-} mice develop tumors at ~ 15 weeks of age (29,34,38). To avoid this complication, we had planned our experiments in such a way that they could be completed before mice reached 13 weeks of age. Furthermore, we routinely performed mouse autopsy at the end of the experiments to search for signs of tumor growth in various organs. To date, we have detected no tumors in mice < 13 weeks of age, whereas tumors (principally lymphomas and osteosarcomas) were detected in some of the p53^{-/-} breeders that were > 17 weeks of age.

p53^{-/-} mice produce more proinflammatory cytokines. Low-dose STZ-induced diabetes is an autoimmune disease mediated by inflammatory cells and their cytokines. To determine whether p53 gene mutation alters cytokine production, we examined the levels of cytokines in the serum and spleen as well as the pancreas of p53^{+/+} and p53^{-/-} mice 30 days after STZ injection. We detected IL-1 and -6 in all of the samples and found that both were significantly increased in p53^{-/-} group compared with p53^{+/+} group (Fig. 3). Additionally, the concentration of

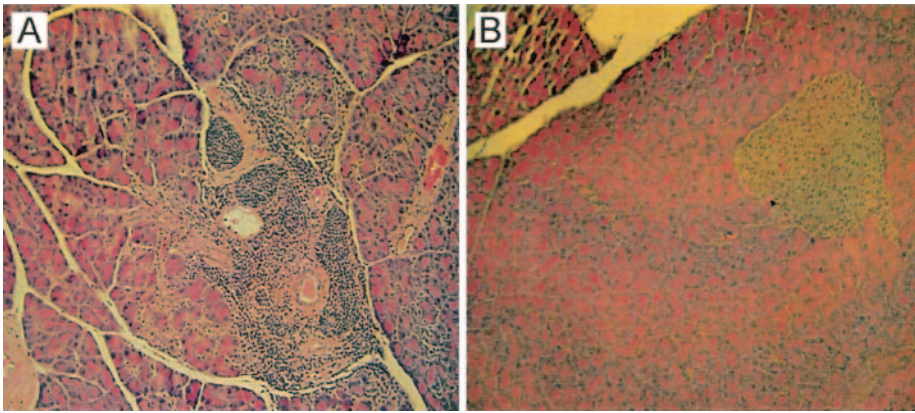


FIG. 2. Histological profiles of pancreas. Mice were treated as in Fig. 1 and killed 30 days after the first STZ injection. Pancreata were collected, fixed in 10% formalin, and embedded in paraffin. Paraffin sections 5 μm thick were stained with hematoxylin and eosin. **A:** Pancreas of a $p53^{-/-}$ mouse with severe inflammation of the islet. **B:** Pancreas of a $p53^{+/+}$ mouse with no infiltration.

IL-12 in the serum of $p53^{-/-}$ mice was also significantly higher than that of $p53^{+/+}$ mice (25 ± 1.5 vs. 7 ± 1.1 ng/ml, $P < 0.001$), although its concentrations in splenic and pancreatic extracts were essentially the same in the two groups (data not shown). By contrast, the levels of IFN- γ in all samples tested were not significantly different in the two groups (data not shown). These results indicate that $p53$ gene mutation may selectively affect the production of inflammatory cytokines.

$p53^{-/-}$ macrophages produce more proinflammatory cytokines. Because macrophages are major inflammatory cells that produce IL-1, -6, and -12, we next examined whether $p53$ gene mutation directly affected the functions of these cells. As shown in Fig. 4, after IFN- γ and lipopolysaccharide stimulation, $p53^{-/-}$ macrophages produced

significantly higher levels of IL-6 and IL-12p40 than control cells. By contrast, the production of TNF- α and nitric oxide was not significantly affected by $p53$ deficiency (data not shown).

To determine whether the increased IL-12 production resulted from an increase in the number of IL-12-producing cells, we performed flow cytometry analysis of macrophages. As shown in Fig. 5, the frequency of IL-12-producing cells was significantly increased in the $p53^{-/-}$ culture. Additionally, the relative IL-12 levels were also higher in $p53^{-/-}$ (mean fluorescence intensity = 214) than in $p53^{+/+}$ (mean fluorescence intensity = 163) cells. Taken together, these results indicate that $p53$ regulates proinflammatory cytokine production in macrophages.

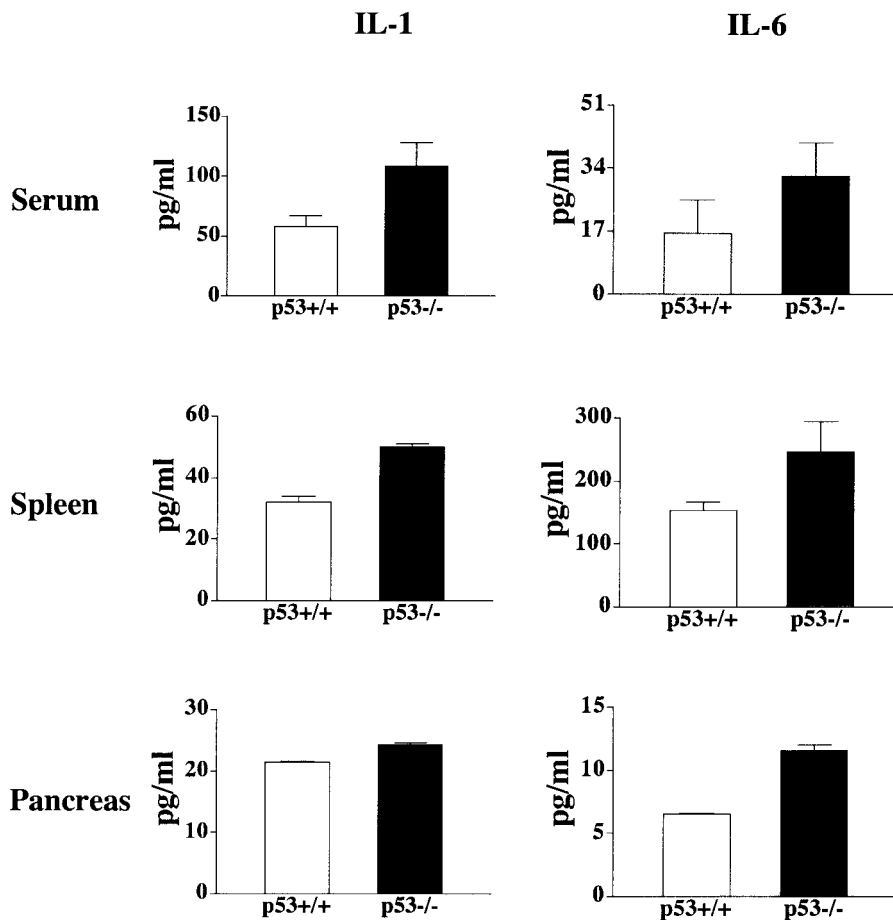


FIG. 3. Increased proinflammatory cytokines in $p53^{-/-}$ mice. Mice ($n = 6$) were treated and killed as in Fig. 2. Blood was collected at the time of death, and organ extracts were prepared as described in RESEARCH DESIGN AND METHODS. The levels of IL-1 β and -6 were determined by enzyme-linked immunosorbent assay as described in RESEARCH DESIGN AND METHODS. The differences between the two groups are statistically significant for all the panels ($P < 0.05$). The experiments were repeated three times with similar results.

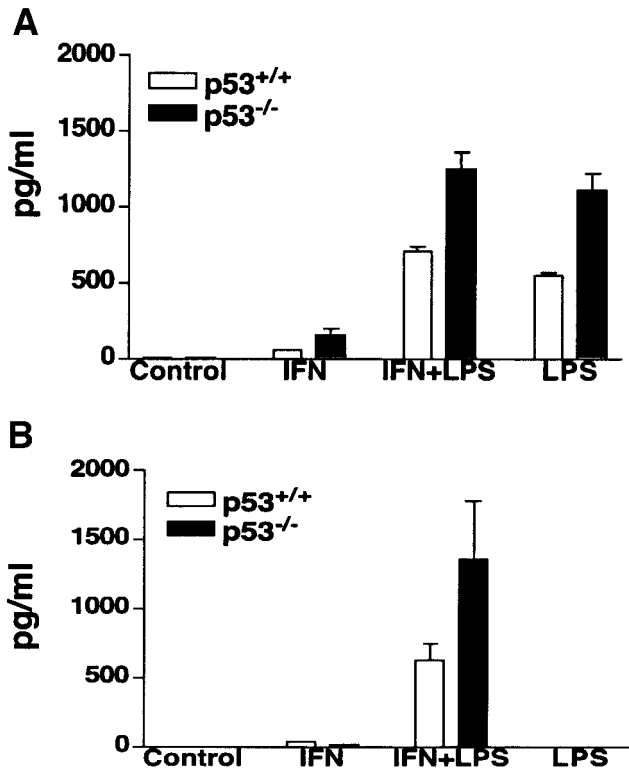


FIG. 4. p53^{-/-} macrophages produce increased levels of IL-6 (A) and IL-12 (B). Peritoneal macrophages from p53^{+/+} and p53^{-/-} mice ($n = 6$) were cultured at 10^6 /ml with or without 10 units/ml of IFN- γ and/or 200 pg/ml of lipopolysaccharide (LPS). Culture supernatants were collected 48 h later, and IL-6 and -12p40 concentrations determined by enzyme-linked immunosorbent assay. For IL-6, the differences between the two groups are statistically significant ($P < 0.01$) for cultures with lipopolysaccharide and/or IFN- γ (IFN). For IL-12, the differences between the two groups are statistically significant ($P < 0.01$) only for cultures with lipopolysaccharide and IFN- γ . Results are representative of two experiments.

p53 regulates STAT-1 expression in macrophages.

Both p53 and STAT-1 are latent transcription factors that can be activated by a variety of stimuli, including lipopolysaccharide and IFN- γ . To explore the potential mechanisms of p53 action in IL-6 and -12 cytokine expression after lipopolysaccharide/IFN- γ treatment (Figs. 4 and 5), we examined whether STAT-1 expression and phosphorylation were normal in p53^{-/-} macrophages. In p53^{+/+} macrophages, STAT-1 was expressed at high levels (Fig. 6) but was not phosphorylated without stimulation (Fig. 7, 0 min). Stimulation with lipopolysaccharide increased the basal levels of STAT-1 (Fig. 6) and induced its rapid phosphorylation (Fig. 7). Importantly, in p53^{-/-} macrophages, both total (Fig. 6, 0 min) and phosphorylated STAT-1 (Fig. 7, 20 min) levels were significantly increased. These results indicate that p53 may act upstream of STAT-1 in macrophages to modulate cytokine production after lipopolysaccharide stimulation.

DISCUSSION

STZ is a D-glucopyranose derivative of N-methyl-N-nitrosourea endowed with potent alkylation properties. In addition, it can also serve as a nitric oxide donor, promoting the production of reactive oxygen species. At high doses, STZ may directly destroy insulin-producing β -cells, leading to acute diabetes. At low doses (including the dose

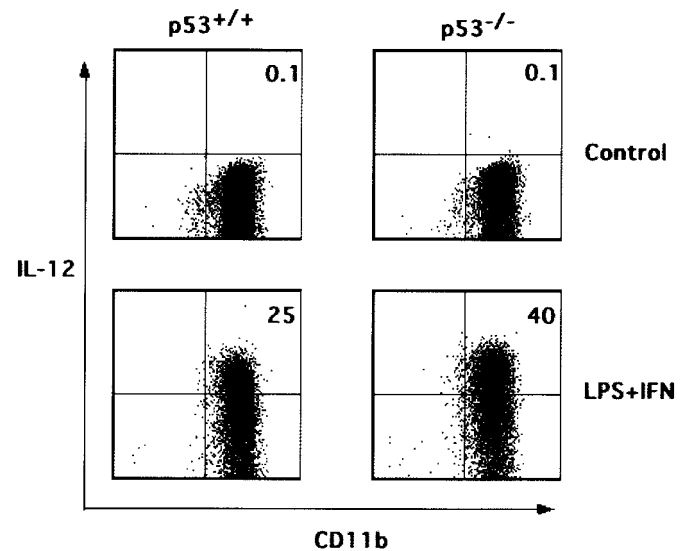


FIG. 5. Measuring IL-12-producing macrophages by flow cytometry. Bone marrow-derived macrophages from p53^{+/+} and p53^{-/-} mice ($n = 6$) were prepared and treated as described in RESEARCH DESIGN AND METHODS. Cells were stained with anti-IL-12p40 and anti-CD11b per BD PharMingen intracellular cytokine staining protocols and analyzed by flow cytometry. Data shown are representative of two experiments. IFN, IFN- γ ; LPS, lipopolysaccharide.

used in this study), however, STZ-induced β -cell damage may not be sufficient to cause diabetes, and participation of inflammatory cells is essential for the development of the disease (35,39,40). The mechanisms of inflammation in low-dose STZ-induced autoimmune diabetes are not clear. Results reported here clearly establish that the tumor suppressor p53 is crucial for this process. The importance of p53 in autoimmune inflammation can be generalized to other autoimmune disease models such as autoimmune encephalomyelitis in B6 mice (41) and collagen-induced arthritis in DBA/1 mice (10).

If p53 plays an inhibitory role in the development of autoimmune diseases, what are the mechanisms of its actions? Like many transcription factors, p53 is expressed by both the immune system and the nonimmune systems. On the one hand, p53 expressed by lymphocytes and cells of the innate immune system may activate or inactivate genes encoding apoptotic proteins, cell cycle inhibitors, and inflammatory cytokines. This would affect the functions of inflammatory cells. On the other hand, p53 expressed by the nonimmune systems, e.g., pancreatic islets, may also play important roles in the development of autoimmune diseases. Thus, the net effect of p53 in autoimmune diseases is likely determined by a balance between these two types of actions.

Results reported here support the view that p53-mediated gene repression in inflammatory cells plays a decisive role in the development of autoimmune diseases. Thus, it has been reported that p53 may directly repress the IL-4 and -6 promoter activities in certain cell lines (26,27). In the case of arthritis, it has been reported that IL-6 production in the arthritic synovium is upregulated when p53 gene mutation is present (10,31), although the cell type(s) producing IL-6 in this model is not clear. In this study, we discovered that p53 may repress not only IL-6 but also IL-1, and -12 expression in macrophages. Because these cytokines play crucial roles in the pathogenesis of autoimmune

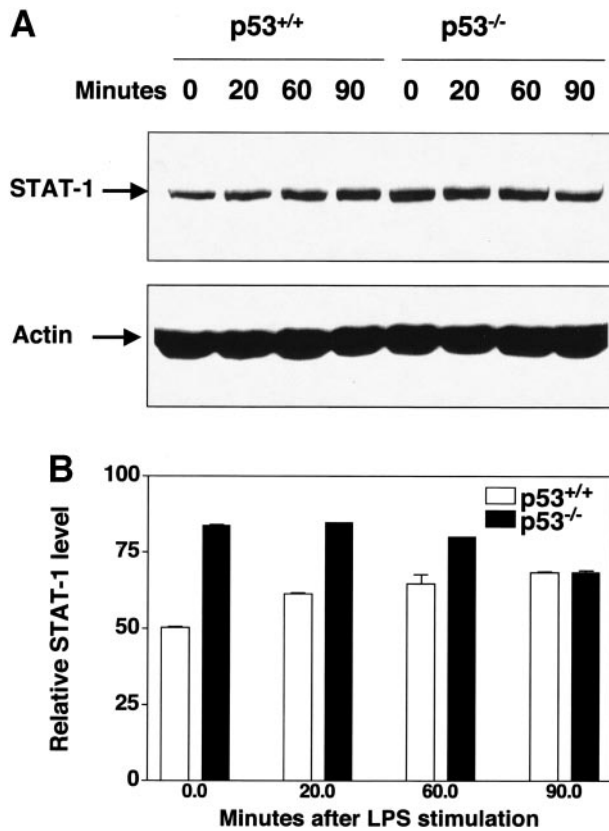


FIG. 6. Increased STAT-1 expression in p53^{-/-} macrophages. Bone marrow-derived macrophages from p53^{+/+} and p53^{-/-} mice ($n = 6$) were prepared as described in RESEARCH DESIGN AND METHODS and cultured in DMEM with or without 1 μ g/ml of lipopolysaccharide (LPS). Whole-cell extracts were prepared at indicated time points. **A:** STAT-1 and actin levels in the whole-cell extracts were determined by Western blot using anti-STAT-1 α and anti- β -actin, respectively. **B:** The relative level of STAT-1 expression, which is calculated as: (STAT-1 band density/actin band density of the same sample) \times 100.

diabetes (42,43), their upregulation in p53^{-/-} mice likely contributes to the exacerbated disease in these animals. Interestingly, IL-12 is upregulated in diabetes-prone NOD mice (44), and injection of recombinant IL-12 into NOD mice accelerated the disease (45). Conversely, blocking the functions of IL-12 using anti-IL-12 antibody or IL-12 antagonists significantly impeded the development of diabetes in NOD mice (43,46,47). In the current study, we found that IL-12 was increased in the serum and macrophages but not in the spleen and pancreas of p53^{-/-} mice. This indicates that the effect of p53 on IL-12 expression in vivo could be tissue- or cell-specific. Further studies are needed to address this issue. Nonetheless, results reported here support the theory that p53 inhibits autoimmune inflammation by inactivating genes encoding inflammatory cytokines. Additionally, our data also indicate that p53 may regulate type 1 diabetes through a separate mechanism involving STAT-1. STAT-1 is a crucial mediator of inflammatory cytokine signaling, and blocking STAT-1 activity using suppressors of cytokine signaling prevents type 1 diabetes (48). There are several potential mechanisms through which p53 may regulate the expression and activities of STAT-1. First, p53 may directly repress the promoter of STAT-1 gene, reducing its mRNA expression. Second, p53 may regulate the expres-

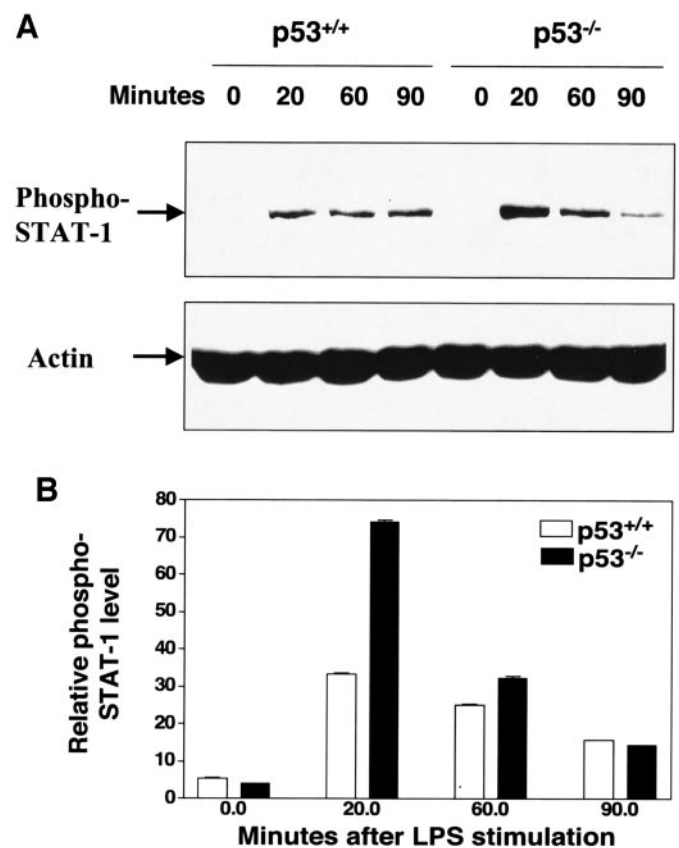


FIG. 7. Phosphorylation of STAT-1 is upregulated in p53^{-/-} macrophages. Macrophages were treated as described in Fig. 6. **A:** Phospho-STAT-1 and actin levels in whole-cell extracts were determined by Western blot using anti-phospho-STAT-1 α and anti- β -actin, respectively. **B:** The relative level of phospho-STAT-1 expression, which is calculated as follows: (phospho-STAT-1 band density/actin band density of the same sample) \times 100.

sion of genes important for the phosphorylation of STAT-1. Finally, it has been reported that p53 may directly interact with STAT-1 to enhance its proapoptotic activity (49). Regardless of the mechanisms, our observation that STAT-1 expression is enhanced in p53^{-/-} macrophages provides a novel link between p53 and STAT-1. Because macrophages play crucial roles in the development of innate immunity, results reported here also provide direct evidence that p53 may be involved in regulating innate immune responses.

As discussed above, development of low-dose STZ-induced diabetes requires the participation of both T-cells and macrophages (35,39,40). The roles of p53 in regulating T-cell functions have also been reported (50). Therefore, enhancement of diabetes in p53-deficient mice may be caused by the loss of p53 function not only in macrophages but also in T-cells. Further investigation is required to clarify this issue.

In summary, we have discovered that p53 deficiency significantly increased the incidence of autoimmune diabetes in mice and that this may be caused by the loss of p53-mediated inhibition of proinflammatory cytokines and STAT-1. These results provide new insights into the molecular mechanisms of autoimmune diabetes that may lead to the development of new strategies for the treatment of the disease.

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