P21 Regulates TGF-β_1–Induced Pulmonary Responses via a TNF-α–Signaling Pathway

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Transforming growth factor (TGF)-β_1 is an essential regulatory cytokine that has been implicated in the pathogenesis of diverse facets of the injury and repair responses in the lung. The types of responses that it elicits can be appreciated in studies from our laboratory that demonstrated that the transgenic (Tg) overexpression of TGF-β_1 in the murine lung causes epithelial apoptosis followed by fibrosis, inflammation, and parenchymal destruction. Because a cyclin-dependent kinase inhibitor, p21, is a key regulator of apoptosis, we hypothesized that p21 plays an important role in the pathogenesis of TGF-β_1–induced tissue responses. To test this hypothesis we evaluated the effect of TGF-β_1 on the expression of p21 in the murine lung. We also characterized the effects of transgenic TGF-β_1 in mice with wild-type and null mutant p21 loci. These studies demonstrate that TGF-β_1 is a potent stimulator of p21 expression in the epithelial cells and macrophages in the murine lung. They also demonstrate that TGF-β_1–induced lung inflammation, fibrosis, myofibroblast accumulation, and alveolar destruction are augmented in the absence of p21, and that these alterations are associated with exaggerated levels of apoptosis and caspase-3 activation. Finally, our studies further demonstrated that TGF-β_1 induces p21 via a TNF-α–signaling pathway and that p21 is a negative modulator of TGF-β_1–induced TNF-α expression. Collectively, our studies demonstrate that p21 regulates TGF-β_1–induced apoptosis, inflammation, fibrosis, and alveolar remodeling by interacting with TNF-α–signaling pathways.

Keywords: TGF-β; p21; apoptosis; fibrosis; emphysema

Transforming growth factor (TGF)-β family proteins are multifunctional cytokines that have been implicated in the pathogenesis of diverse biologic processes including cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, hematopoiesis, and tissue remodeling and repair (reviewed in Ref. 1). A number of studies demonstrate that, in the proper setting, TGF-β_1 is essential for wound healing, stimulates matrix molecule deposition and angiogenesis, and is an essential mediator of the pathologic scarring in fibrotic disorders (2–6). On the other hand, TGF-β_1 can also induce tissue injury and cellular apoptosis, decrease epithelialization, and inhibit wound healing (7–12). The "contradictory" and complex nature of these responses reflects an inadequate understanding of the mechanisms that TGF-β_1 uses to induce tissue responses. This is due, in part, to the lack of experimental

CLINICAL RELEVANCE

This is the first report directly demonstrating the *in vivo* role of p21 in fibrosis and alveolar destruction in association with TGF-β_1 expression. Intervention of p21 has therapeutic potential for the fibrotic and destructive lung disease as well.

systems in which the acute and chronic effects of TGF-β_1 can be characterized and their interrelationships can be investigated *in vivo*. We recently generated transgenic (Tg) mice in which bioactive TGF-β_1 is specifically overexpressed in the lung (13). These TGF-β_1 Tg mice manifest impressive bronchoalveolar and tissue inflammation, fibrosis, and pulmonary alveolar remodeling (13). Studies of these mice have also demonstrated that these TGF-β_1–induced tissue responses are dependent on an epithelial apoptotic response that precedes fibrosis and alveolar destruction. In spite of its importance, however, the molecular mechanisms and mediators that regulate this TGF-β_1–induced apoptosis response have not been adequately described.

p21$^{Cip1/WAF1/Sdi1}$ (p21), the first identified cyclin-dependent kinase (CDK) inhibitor, belongs to Cip/Kip family of CDK inhibitors and is an essential regulator of cell cycle progression, DNA repair, and apoptosis (14–17). In *in vitro* studies p21 has been shown to be induced by TGF-β_1 (18, 19) and to inhibit TGF-β_1–induced apoptosis (20). p21 has been shown to have protective effects in several animal models of pulmonary injury (21, 22), and it has also been shown that down-regulation of p21 expression caused enhanced Fas-mediated epithelial apoptosis by TGF-β_1 (23). However, the role(s) and the mechanism of p21 in *in vivo* TGF-β_1–induced tissue responses have not been adequately defined.

We hypothesized that p21 plays an important role in the pathogenesis of TGF-β_1–induced tissue alterations *in vivo*. To test this hypothesis, we characterized the expression of p21 in TGF-β_1 Tg mice and defined the effects of a null mutation of p21 on the phenotypes induced by transgenic TGF-β_1 in these animals. These studies demonstrated that TGF-β_1 is a potent stimulator of p21 expression in epithelial cells and macrophages in the lung. They also demonstrate that a deficiency of p21 significantly augments TGF-β_1–induced apoptotic tissue responses, inflammation, fibrosis, and alveolar destruction. Our studies further indicated that TGF-β_1 induces p21 *in vivo* via a TNF-α–signaling pathway and that p21 is a negative modulator of TGF-β_1–induced TNF-α expression. Collectively, our studies demonstrate that p21 significantly regulate TGF-β_1-induced tissue responses by interacting with TNF-α–signaling pathways.

MATERIALS AND METHODS

Overexpression Transgenic Mice

CC10-tTS-rtTA-TGF-β_1 Tg mice were generated in our laboratory, bred onto a C57BL/6 background, and used in these studies. These mice use the Clara cell 10-kD protein (CC10) promoter to specifically target TGF-β_1 to the lung. The methods that were used to generate and

characterize these mice were described previously (13). In this modeling system, TGF-β_1 caused macrophage-dominant bronchoalveolar lavage (BAL) and tissue inflammatory responses, subepithelial and parenchymal fibrosis, and alveolar destruction (13).

Breeding to p21 and TNF-α Receptor Null Mutant Mice

TGF-β_1 Tg mice were bred with mice with wild-type and null p21 or TNF-α receptor loci. p21(−/−), TNF-α Receptor I (TNFRI^{−/−}), and TNF-α Receptor II (TNFRII^{−/−}) null mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice with both TNF-α I and II receptors null mutant loci (TNFR^{−/−}) were generated by the mating the TNFRI(−/−) mice with TNFRII(−/−) mice. All the mice had been bred for over 10 generations onto a C57BL/6 genetic background. As a result of these crosses, TGF-β_1 Tg animals with (+/+) and (−/−) p21 or TNFR loci were obtained and used for analysis. Genotyping of TGF-β_1 Tg, p21(−/−), and TNFR(−/−) mice was accomplished according to the protocols established in our laboratory and provided by Jackson Laboratory, respectively (13). The phenotypes of these mice were compared as described below.

Doxycycline Water Administration

Six-week-old transgene (+) mice and transgene (−) littermate controls were randomized to normal water or water containing 0.5 mg/ml of doxycycline (dox) as described previously (13, 24). Phenotypic alterations were evaluated at intervals thereafter.

BAL and Lung Inflammation

Lung inflammation was assessed by BAL as described previously (13, 25). The BAL samples from each animal were pooled and centrifuged. The number and type of cells in the cell pellet were determined with light microscopy.

Quantification of Lung Collagen

Animals were anesthetized, a median sternotomy was performed, and right heart perfusion was accomplished with calcium- and magnesium-free PBS. The heart and lungs were then removed en bloc. The right lung was frozen in liquid nitrogen and stored at −80°C until used. Collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor) according to the manufacturer's instructions. The data are expressed as the collagen content of the entire right lung.

Histologic Analysis

The lungs were removed en bloc as described above, inflated at 25 cm pressure with PBS containing 0.5% low melting point agarose gel, fixd in Streck solution (Streck Laboratories, La Vista, NE), embedded in paraffin, sectioned, and stained. Hematoxylin and eosin, and Mallory's trichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at the Yale University School of Medicine. The paraffin-embedded sections were also used for immunohistochemistry and TdT-mediated dUTP nick-end labeling (TUNEL) evaluations as described below.

Morphometric Analysis

Alveolar remodeling was estimated from the mean cord length of the airspace. This measurement is similar to the mean linear intercept, a standard measure of air space size, but it has the advantage of being independent of alveolar septal thickness. These evaluations were performed as described previously by our laboratory (13, 26).

TUNEL Evaluations

End labeling of exposed 3′-OH ends of DNA fragments was undertaken with the TUNEL *in situ* cell death detection kit AP (Roche Diagnostics, Indianapolis, IN) as described by the manufacturer. After staining, 20 fields of alveoli were randomly chosen for examination. The labeled cells were expressed as a percentage of total nuclei.

Fluorescence-Activated Cell Sorter Analysis for Apoptotic Cells

Whole lung dispersed cells were prepared using the methods developed by Rice and coworkers (27). After anesthesia, the trachea was cannulated with 20-gauge tubing, the lungs were filled with 2 ml dispase (Roche

Diagnostics, Alameda, CA), followed by 0.5 ml of 1% low-melting-point agarose. The agarose was allowed to harden under crushed ice. The lungs were then placed in 2 ml dispase for 1 hour at room temperature and transferred to Dulbecco's modified Eagle's medium with 25 mM Hepes with 0.01% DNase I (Sigma-Aldrich, St. Louis, MO). After teasing apart the digested tissue, the resulting cell suspension was sequentially filtered through nylon mesh filters and collected after centrifugation for 8 minutes at $130 \times g$. In accord with the literature, the resulting cells were more than 97% viable as demonstrated by trypan blue dye exclusion (27). These cells were resuspended in $1\times$ binding buffer at 10^6 cells/ml for subsequent fluorescence-activated cell sorter analysis. Annexin V and propidium iodide staining were undertaken with the annexin V fluorescein isothiocyanate apoptosis detection kit (BD Biosciences, San Diego, CA). Analysis was undertaken by flow cytometry (Becton Dickinson, San Jose, CA).

mRNA Analysis

mRNA levels were assessed using conventional reverse transcription PCR assays as described by our laboratories (2, 13, 25). In these assays, total cellular RNA from lungs were obtained using trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The primer sequences used for amplification of p21 were used as previously described (28).

Immunoblot Analysis

Lung lysates were prepared and Western analysis was undertaken with antibodies that reacted selectively with p21, caspase-3, poly(ADP) ribose polymerase (PARP), Bcl-2, β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and ICAD (Chemicon International, Temecula, CA) as described previously (25).

Cytokine Measurements

The levels of TGF-β_1 in BAL fluids were evaluated by enzyme-linked immunosorbent assay using commercial assays (R&D Systems, Minneapolis, MN) as described by the manufacturer.

Immunohistochemistry

Immunohistochemistry (IHC) was undertaken to localize p21 (Santa Cruz Biotechnology), α-smooth muscle actin (DakoCytomation, Glostrup, Denmark), and α-smooth muscle myosin heavy chain (Biomedical Technology, Inc., Stoughton, MA). These assays were undertaken as described previously by our laboratories (13). We used lungs from p21(−/−) mice as a negative control for IHC, and no significant signals were detected in this setting.

Statistics

Normally distributed data are expressed as means ± SEM and assessed for significance by Student's t test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test.

RESULTS

TGF-β₁ Regulation of p21

To address the possibility that p21 contributes to TGF-β_1–induced responses in the murine lung, studies were first undertaken to determine if the expression of p21 was regulated by transgenic TGF-β_1. This was done by comparing the levels of p21 mRNA and protein in transgene (−) and transgene (+) mice at various times after transgene activation via the administration of dox. These studies demonstrate that TGF-β_1 is a potent stimulator of p21 mRNA and protein in lungs from dox-treated transgenic mice (Figure 1A). This induction was seen after as little as 1 day of dox administration and persisted throughout the 28-day study interval (Figure 1B). In these mice, immunohistochemistry demonstrated that the p21 was most prominently appreciated in airway epithelial cells, alveolar Type II cells, and macrophages (Figure 1C). Thus, TGF-β_1 is a potent stimulator of p21 in epithelial cells and macrophages in the murine lung.
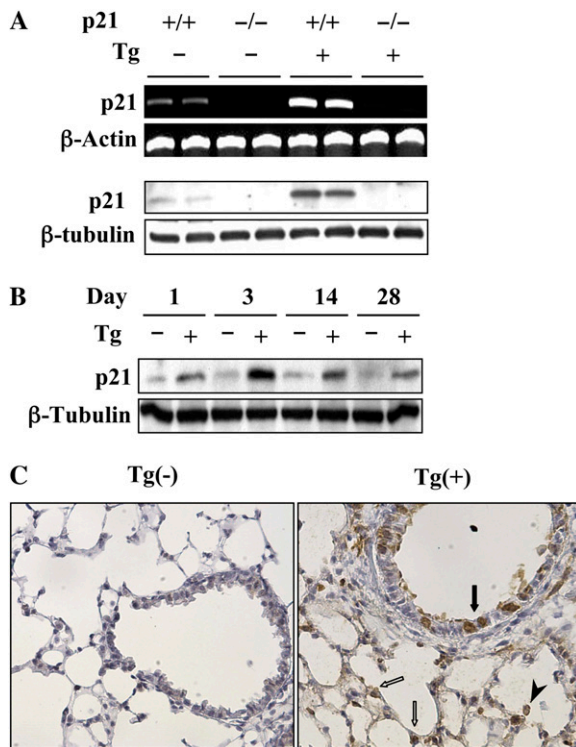
Figure 1. TGF-β_1 regulation of p21. TGF-β_1 Tg (+) and Tg (−) mice with (+/+) and without (−/−) p21 loci were given normal or doxycycline (dox) water for 1 to 28 days. RNA (*upper two lanes*) and protein (*lower two lanes*) expression levels at 2 weeks of dox incubation (*A*) and time kinetics of p21 protein expression (*B*) were detected by RT-PCR and Western blot analysis. The p21 expressions of the lung from mice given dox water for 3 days were localized using immuno-histochemistry (*C*) (*original magnification*: ×20). *Solid* and *open arrows* highlight epithelial and type II cells, respectively. *Arrowhead* denotes the alveolar macrophage. Each panel is illustrative of a minimum of three similar experiments.

p21 Regulation of TGF-β_1–Induced Inflammation

Studies were next undertaken to determine if p21 regulated TGF-β_1–induced tissue inflammation. This was done by comparing the inflammation in BAL and tissues from transgene (+) mice with wild-type and null p21 loci. These studies demonstrate that, in the absence of p21, the total cell recovery in BAL fluids was increased when compared with transgenic mice that produce p21 normally (Figure 2A). This effect was most prominent after 14 days of dox administration and could still be appreciated after 28 days of transgene activation (Figure 2A). At the 14-day time point, this effect was due to an increase in the recovery of macrophages, lymphocytes, and to a lesser degree, eosinophils and neutrophils in BAL fluids from p21 null animals (Figure 2B, and Figure E1 in the online supplement). Similar increases in inflammation were seen in tissue histologic evaluations (data not shown). Thus, these studies demonstrate that p21 inhibits TGF-β_1–induced inflammation in the murine lung. The expression level of transgene (human TGF-β_1) in the BALF of the Tg(+)/p21(−/−) was not significantly different from Tg(+)/p21(+/+) mice (Figure 2C).

Role of p21 in TGF-β_1–Induced Fibrosis

To determine if fibrotic effects of TGF-β_1 were altered in the absence of p21, we used biochemical (Sircol) and histologic approaches to quantitate the collagen in lungs from transgenic

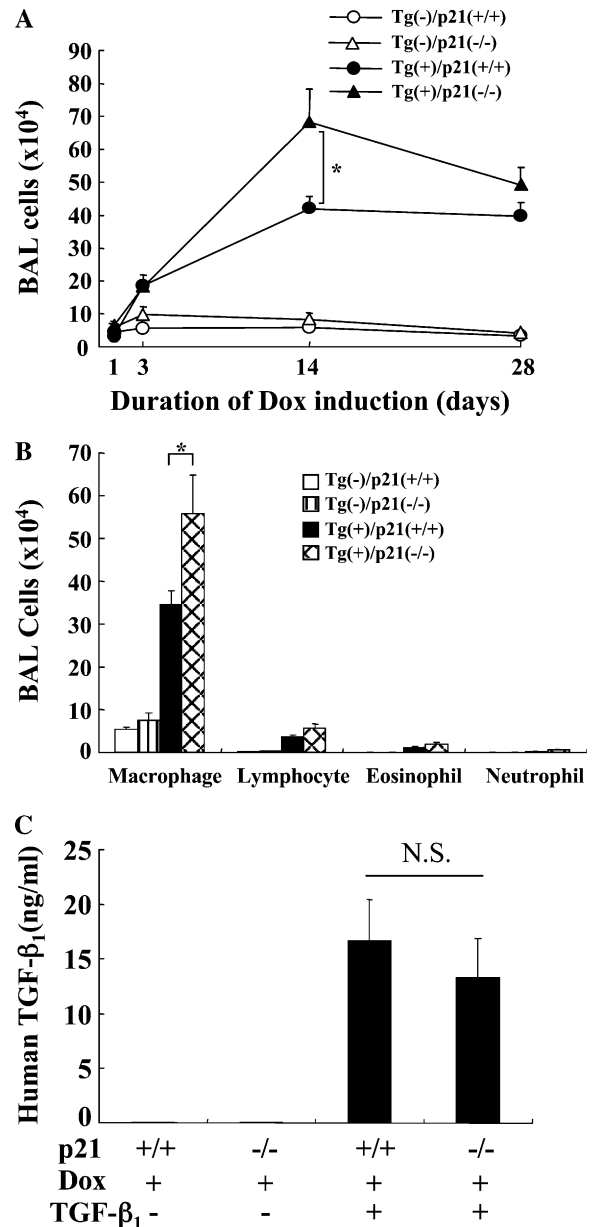**Figure 2.** p21 regulation of TGF-β_1–induced inflammation. TGF-β_1 Tg (+) and Tg (−) mice with (+/+) and without (−/−) p21 loci were given normal or dox water for 1 to 28 days. After dox incubation for the indicated period, time kinetic changes of bronchoalveolar lavage (BAL) cell recovery were evaluated (*A*) and a differential count on BAL cells after 2 weeks of dox induction was illustrated (*B*). The transgenic expression of TGF-β_1 in the BAL fluid was evaluated by enzyme-linked immunosorbent assay (*C*). The values represent the mean ± SEM of evaluations in a minimum of five animals (*$P < 0.05$; N.S., non-significant).

mice with wild-type and null p21 loci. In accord with previous studies from our laboratory (13), transgenic TGF-β_1 caused a significant increase in lung collagen content (Figures 3A and 3B). Interestingly, this fibrotic response was augmented in the absence of p21. This increase was most prominent after 2 to 4 weeks of dox administration. It was associated with a prominent increase in the accumulation of myofibroblasts based on an increase in α-smooth muscle actin (+) cells (Figure 3C). α-smooth muscle myosin containing myocytes were not simi-
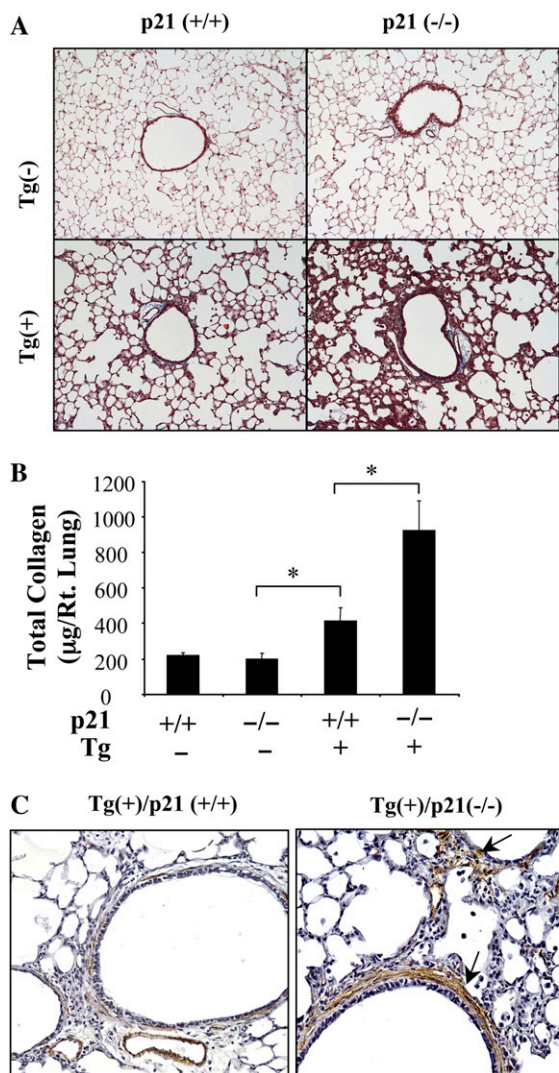
Figure 3. Role of p21 in the TGF-β₁–induced fibrosis. The collagen content of lungs from Tg (−) and Tg (+) mice with (+/+) and (−/−) p21 loci were compared using Mallory's trichrome (*A*; *original magnification*: ×10) and Sirchol collagen evaluations (*B*) after 2 weeks of dox induction. (*C*) Immunohistochemical localization of α-smooth muscle actin–positive cells (*arrows*) after 2 weeks of dox induction (*original magnification*: ×15). *A* and *C* are representative of a minimum of three similar evaluations. In *B*, each value represents the mean ± SEM of evaluations in a minimum of six mice (*P < 0.05).

larly altered (data not shown). These studies demonstrate that p21 is an important inhibitor of TGF-β₁–induced fibrosis and myofibroblast accumulation in the murine lung.

Role of p21 in Alveolar Remodeling

Previous studies from our laboratory demonstrated that TGF-β₁, in addition to inducing tissue fibrosis, also induced alveolar remodeling with septal destruction and an increase in alveolar chord length (13). To define the role of p21 in these responses, we compared the alveoli from transgenic mice with (+/+) and (−/−) p21 loci. In accord with our prior report (13), an increase in lung destruction was readily apparent in transgenic mice with normal p21 loci after 2 weeks of dox administration. At these time points, alveolar remodeling was heightened in the absence of p21. This is readily apparent in histologic evaluations (Figure 4A) and morphometric chord length evaluations (Figure 4B).
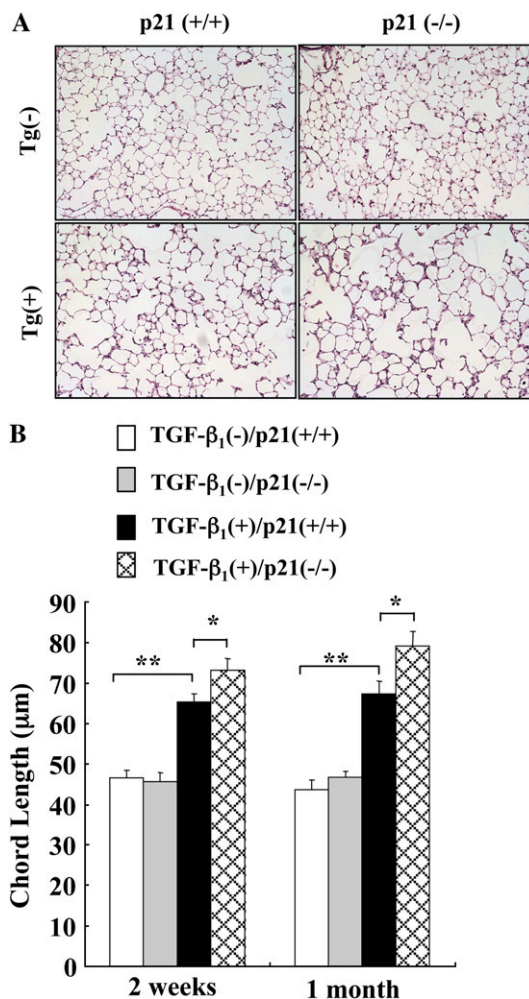

Figure 4. Role of p21 in TGF-β₁–induced alveolar remodeling. After 2 weeks of dox induction, lungs were obtained from Tg (−) and Tg (+) mice with (+/+) and (−/−) p21 loci, fixed to pressure, and hematoxylin and eosin histologic stains (*A*; *original magnification*: ×10) and chord length (*B*) assessments were undertaken. *A* is representative of a minimum of five similar experiments. In *B*, the values represent the mean ± SEM of evaluations in a minimum of five mice (*P < 0.05, **P < 0.01).

Thus, p21 is a potent inhibitor of TGF-β₁–induced alveolar rupture and remodeling in the murine lung.

p21 Regulation of TGF-β₁–Induced Apoptosis

To test the hypothesis that p21 regulates TGF-β₁–induced apoptosis, we compared the magnitude and kinetics of TGF-β₁–induced apoptosis in transgenic mice with wild-type and null p21 loci. In accord with our prior report (13), transgenic TGF-β₁ caused an impressive increase in TUNEL staining. These TUNEL (+) cells were largely epithelial cells, as evidenced by their histologic location and morphology (data not shown). This response was readily appreciated after 3 days of dox administration and decreased with longer periods of transgene activation (Figure 5A). PI/annexin V evaluations of total lung cells confirmed these findings and demonstrated that the majority of these cells were undergoing apoptosis and combined apoptosis and necrosis (Figure 5B). In all cases, p21 appeared to be an important regulator of this response, with the levels and chronicity of TUNEL staining and apoptosis being increased in transgene
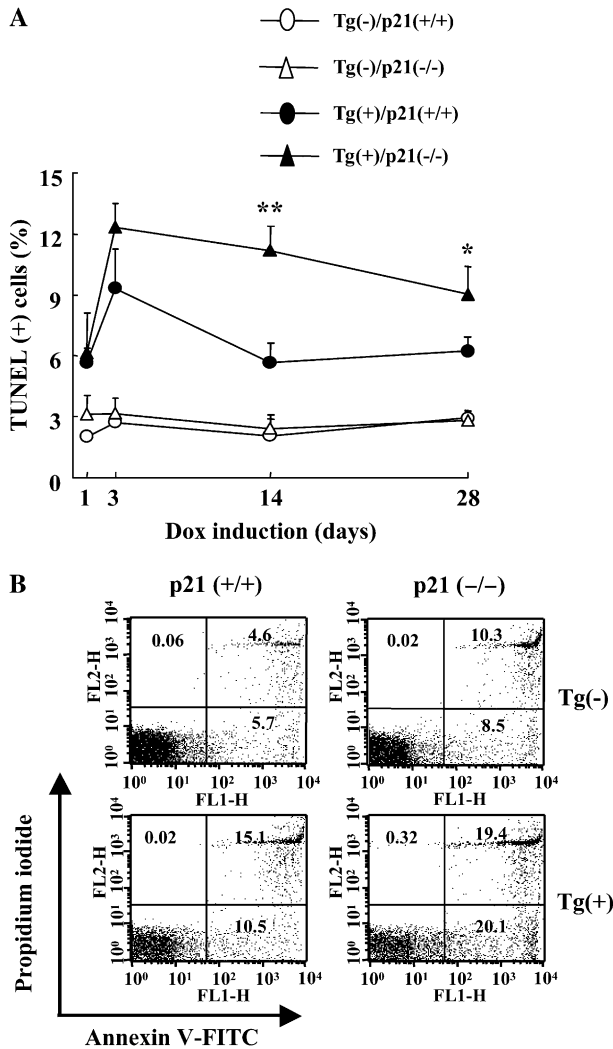
Figure 5. p21 regulation of TGF-β₁–induced apoptosis. After dox induction, lungs were obtained from Tg (−) and Tg (+) mice with (+/+) and (−/−) p21 loci. DNA injury and cell death were evaluated with TUNEL stains and time kinetic changes for the indicated dox induction period were illustrated (*A*). After 2 weeks of dox induction, cells were isolated from both lungs and fluorescence-activated cell sorter analysis was undertaken using annexin V and propidium iodide staining (*B*). In *B*, the values represent the mean ± SEM of evaluations in a minimum of six mice (*$P < 0.05$, **$P < 0.01$).

Figure 6. Regulation of TGF-β₁–induced TNF-α expression and signaling pathway of p21 induction. After 2 weeks of dox induction, TNF-α expression in the lung lysate (soluble TNF-α) obtained from Tg (−) and Tg (+) mice with (+/+) and (−/−) p21 loci was evaluated by Western analysis (*A*) and densitometric quantization (*B*). Expression of p21 and other apoptosis-related molecules in Tg (+) and Tg (−) mice were evaluated in the mice with wild-type and TNFR(−/−) null mutant loci. *A* and *C* are representative of a minimum of three similar experiments. In *B*, the values represent the mean ± SEM of evaluations in a minimum of six mice (**$P < 0.01$).

(+) mice with null p21 loci. Collectively, these studies demonstrate that p21 is an important inhibitor of TGF-β₁–induced epithelial apoptosis that contributes to the transient nature of this response in lungs from C57BL/6 TGF-β₁ transgenic mice. Western analyses demonstrated that TGF-β₁ caused a significant increase in caspase-3, -8, -9, and PARP cleavage (Figure E2). p21 appeared to be an important regulator of this response because Tg (+) mice with null p21 loci manifest a significant increase in caspase-3, -8, -9 activation and PARP cleavage compared with transgenic animals with wild-type p21 loci (Figure E2).

P21 Regulation of TGF-β₁–Induced TNF-α Expression and Signaling Pathway of p21 Induction

To understand the underlying mechanism of p21 in the TGF-β₁–induced tissue responses, first we have investigated the mRNA expression of selected chemokines, their receptors, and cytokines that possibly associated with BAL and tissue inflamma-
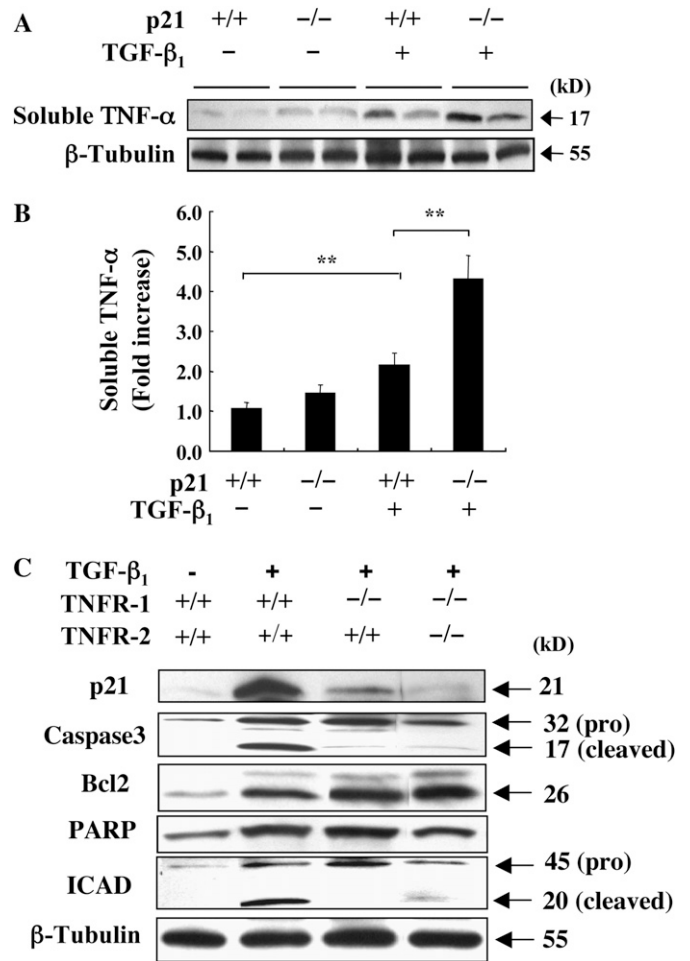
tion in the Tg (+) and Tg (−) mice with and without p21 null mutation. The mRNA expression of MCP-1, MCP-2, MIP-1α, CCR2, CCR5, and TARC were increased in the Tg(+)/p21 (+/+) mice, but significant changes were not detected between Tg(+)/p21(+/+) and Tg(+)/p21(−/−) mice (Figure E3). However, the expression of soluble TNF-α was significantly increased in the lung lysate of Tg(+)/p21(+/+) mice and further increased in the Tg(+)/p21(−/−) mice (Figures 6A and 6B). The significant role of TNF-α in the generation of pulmonary fibrosis and/or alveolar remodeling has been described in a number of papers (29–31). Thus, we investigated the interaction of TNF-α and p21 in our modeling system. Interestingly, as shown in Figure 6C, the increased expression of p21 in the Tg(+)/TNFR(+/+) mice was significantly reduced to the comparable level of the wild-type mice in Tg(+)/TNFR(−/−) mice. It is also interesting to note that expression of another anti-apoptotic molecule, Bcl-2, was not altered, while TGF-β₁–induced caspase 3 activation or ICAD cleavage were impressively decreased in the absence of TNF-α receptors. These studies demonstrated that reciprocal

interaction between TNF-α and p21: induction of p21 is largely regulated by TNF-α signaling and also p21 negatively regulate TNF-α expression in the lung of TGF-β_1 transgenic mice. These studies also demonstrate that TNF-α signaling mediate anti-apoptotic role of p21 in TGF-β_1–induced apoptosis and remodeling tissue responses.

DISCUSSION

To further understand the molecular events involved in TGF-β_1–induced phenotype generation, we used a transgenic system developed in our laboratory to characterize the role(s) of p21 in the pathogenesis of TGF-β_1–induced alterations in the lung. These studies demonstrate that TGF-β_1 is a potent stimulator of p21 expression. They also demonstrate that p21 plays a protective role in the pathogenesis of TGF-β_1–induced tissue responses because TGF-β_1–induced inflammation, fibrosis, myofibroblast accumulation, alveolar destruction, and cell death were markedly augmented by p21 ablation.

TGF-β_1 is a multifunctional cytokine that has the ability to regulate cell differentiation, survival, and death. In many cell types, TGF-β_1 inhibits cellular proliferation by causing growth arrest in the G1 phase of the cell cycle (reviewed in Ref. 32). This TGF-β_1–induced G1 cell cycle arrest has been attributed to the regulatory effects of TGF-β_1 on both the levels and activities of G1 cyclins and CDKs, which include p21, p27[kip1], p18, p16, and p15 (reviewed in Refs. 33 and 34). *In vitro*, TGF-β_1 stimulates p21 via a mechanism that is, at least in part, transcriptional and independent of p53 (19, 35). Consistent with these cell cycle and *in vitro* observations, our studies demonstrate that TGF-β_1 induces prominent increases in p21 mRNA and protein *in vivo*. Interestingly, in these studies p21 was most prominent in airway and alveolar epithelial cells and macrophages, the cells that have the highest levels of apoptosis in this modeling system. Our studies also demonstrate that p21 is a potent inhibitor of TGF-β_1–induced apoptosis with increased numbers of annexin V– and TUNEL-positive cells being seen in lungs from transgenic mice with null mutations of p21. This demonstration that p21 has *in vivo* cytoprotective effects is in accord with prior studies in other modeling systems. Specifically, O'Reilly and colleagues (36) demonstrated that bronchiolar and alveolar epithelial cells damaged by hyperoxia impressively express p21. They suggested that p21 protects the lung from oxidative stress by inhibiting DNA replication and thereby allowing additional time to repair damaged DNA (21). Similarly, epithelial p21 expression is also up-regulated during bleomycin-induced lung injury (28, 37, 38), adenoviral overexpression of p21 decreases bleomycin-induced pulmonary fibrosis (22), and p21 can directly regulate the activity of caspase-3 by formation of a pro–caspase 3–p21 complex *in vitro* (16, 17). In humans, up-regulation of p21 in fibrotic lung diseases has also been noted (38). Idiopathic pulmonary fibrosis is characterized by alternating zones of fibrosis, fibroplasia, and normal lung. The interface between these zones shows so-called fibroblastic foci in which epithelial cells have been shown to undergo apoptosis. These cells also show up-regulation of p21, while normal lung and lung away from these foci are negative (38). Nakashima and coworkers (39) showed that nonspecific interstitial pneumonia, considered a better prognosis variant of pulmonary fibrosis, as similar levels of p21 as IPF. However, not commented on in their text was that the figures illustrating the staining showed diffuse p21 staining rather than focal, consistent with the diffuse nature of the pathologic process in nonspecific interstitial pneumonia (NSIP). Diffuse alveolar damage, in which TGF-β has also been reported to play a role, also shows

diffuse high level staining with p21 (40). Good prognostic variants of chronic interstitial lung disease other than NSIP have not been reported. We can also detect increased and localized p21 expression in the fibrotic foci from patients with pulmonary fibrosis (Figure E4 and data not shown). Thus, the findings in humans are consistent with the idea that p21 expression reflects the degree and pattern of TGF-β expression. When viewed in combination, our studies suggest that p21 overexpression during tissue injury plays a protective role, at least in part, by regulating TGF-β_1–induced apoptosis.

Previous studies from our laboratory demonstrated that TGF-β_1 overexpression generates a macrophage-dominant inflammatory response in the lung (13). In the present studies, the absence of p21 significantly augmented this TGF-β_1–induced BAL and tissue inflammatory responses. p21 can exert its anti-inflammatory effects by modulating the expression of chemokines or chemokine receptors. However, we do not think this plays an important role in this modeling system because we did not detect significant alterations in the levels of mRNA encoding a number of TGF-β_1–induced chemokines and their receptors in comparisons of transgenic mice with (+/+) and null p21 loci. On the other hand, tissue and BAL expressions of a potent proinflammatory cytokine TNF-α were significantly increased in the absence of p21. Because the significant role of TNF-α in the pulmonary fibrosis and alveolar remodeling as well as in the inflammation has been described in a number of studies (29, 30), we sought the possible interactions between p21 and TNF-α in modulating TGF-β_1–induced tissue phenotypes. Interestingly, we have found that induction of p21 by TGF-β_1 is largely dependent on the TNF-α–signaling pathways. Also, our studies demonstrate that p21 negatively regulates the induction of TNF-α by TGF-β_1 *in vivo*, because absence of p21 augments TNF-α expression. This observation leads us to conclude that p21 is a negative regulator of inflammation and tissue remodeling responses, at least in part, by interacting with TNF-α signaling. Another interesting finding on the role of TNF-α in the TGF-β_1 Tg mice is that TGF-β_1–induced tissue apoptosis, fibrosis, and alveolar destructions were significantly reduced in the absence of TNF-α signaling (H.-R. Kang and C. G. Lee, unpublished data). Our studies suggest that there is an interacting feedback regulatory loop between TNF-α and p21 in the regulation of tissue phenotypes. However, the mechanisms of TNF-α regulation of p21 expression and their interaction with caspase 3 activation are largely unknown because in the absence of TNF-α signaling, both caspase 3 activation and p21 expression were greatly reduced (Figure 6). It is intriguing to speculate that up-regulation of another anti-apoptotic molecule like Bcl-2 can suppress the caspase 3 activation in the absence of p21 in this setting, but the exact mechanism(s) remains to be determined. When viewed in combination, these studies strongly suggest that regulatory effects of p21 in TGF-β_1–induced inflammatory and tissue responses are, at least in part, dependent, on TNF-α signaling. In the absence of p21, heightened expression of TNF-α increased the BAL and tissue inflammation and apoptosis of airway and alveolar epithelial cells. Increased alveolar type II cell apoptosis, as we can see in the Tg(+)/p21(−/−) mice, can further drive tissue inflammation (41). In addition, enhanced apoptotic responses of epithelial cells can be a primary cause of prominent pulmonary fibrosis and alveolar remodeling (13). As far as we know, this is the first report that demonstrate significant interactions between p21 and TNF-α in the *in vivo* regulation of TGF-β_1–induced tissue responses. However, the exact reciprocal regulatory mechanisms between p21 and TNF-α remain to be determined.

The essential role of TGF-β_1 in pulmonary fibrosis and remodeling has been relatively well characterized in a number

of studies. Idiopathic pulmonary fibrosis (IPF), a prototypic fibrotic disorder, is a fatal progressive lung disease characterized by epithelial damage, fibroproliferative matrix deposition, and parenchymal remodeling (42–44). For many years, it was believed that IPF was caused by chronic inflammation. Recent revisions, however, have highlighted the possibility that IPF is not an inflammatory disorder, but rather one characterized by an alteration in parenchymal homeostasis (44). TGF-β_1 is believed to play an important role in this dysregulation because it is expressed in an exaggerated fashion in IPF where, in contrast to controls, a sizable percentage of this cytokine is biologically active (45–47). In fact, the apposition of apoptosis, fibrosis, and the exaggerated expression of TGF-β_1 are well documented in IPF (48–51). TGF-β_1 is also a critical mediator of the pulmonary fibrosis that is seen after bleomycin administration, a commonly used animal model of IPF (5, 52). Recently, studies have demonstrated that Bid, a regulator of the mitochondrial cell death pathway, is required for bleomycin-induced pulmonary fibrosis (53). Our studies add to our knowledge in this area by demonstrating that p21 inhibits apoptosis, tissue fibrosis, and alveolar remodeling by interacting with TNF-α–signaling pathways in the TGF-β_1 transgenic model. It is tempting to speculate from these studies that the p21 in fibrotic foci represents a healing and/or protective response in these patients. It is also tempting to speculate that interventions that increase the expression of p21 can be used to control the destructive and fibrotic responses in the lungs from these patients.

A number of lines of evidence support the concept that TGF-β_1 plays an important role in alveolar remodeling in the lung. Many of these studies suggest that TGF-β_1 directly contributes to the genesis of emphysema. This includes studies that demonstrate that TGF-β_1 is expressed, in an exaggerated fashion, in lungs from smokers and patients with COPD (54, 55), studies that suggest that TGF-β_1 plays an important role in the emphysema that is seen in patients with Marfan syndrome (56), and studies that highlight the progressive increase in small airway wall thickness and fibrosis that are seen with COPD disease progression (57, 58). In contrast to these studies, there are studies that suggest that a deficiency of TGF-β_1 bioactivity contributes to emphysema generation (59). This includes studies that demonstrate that a null mutation of the β_6 integrin (which activates latent TGF-β) causes age-related emphysema via a matrix metalloproteinase-12–dependent mechanism (59) and studies that demonstrate that a null mutation of SMAD3 causes spontaneous emphysema (60). When viewed in combination, it is clear that TGF-β_1 is dysregulated in COPD, but its role in this disease remains controversial. The different outcomes that have been reported, however, may be due to the ability of TGF-β_1 to have simultaneous roles in injury and repair in the lung. In this regard, our studies demonstrate that TGF-β_1 simultaneously induces epithelial apoptosis while inducing the anti-apoptotic molecule p21 to control this response. It is intriguing to speculate that the balance between apoptosis and anti-apoptosis directs the fate of these cells and contributes to the balance of alveolar destruction and fibrosis that are induced in these animals.

In summary, using lung-specific overexpressing TGF-β_1 Tg mice and p21 null mice, we have demonstrated that p21 is an important negative regulator of TGF-β_1–induced inflammation, apoptosis, fibrosis, and alveolar destruction in the lung. Our studies demonstrate that p21 regulates TGF-β_1–induced tissue responses, at least in part, dependently via a TNF-α–signaling pathway. These studies suggest that interventions that enhance the expression of p21 can control tissue apoptosis and fibrosis and have therapeutic potential for the fibrotic and destructive lung diseases in which TGF-β_1 plays a major role. They also suggest that genetic polymorphisms, environmental exposures, or therapies that alter p21 expression can have major effects on the ability of an individual to tolerate and the natural history of TGF-β_1–mediated disorders.

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