The tumor suppressor p53 is a transcriptional master regulator that promotes DNA repair, cell cycle arrest, senescence, and apoptosis in response to challenges to genomic integrity, thereby guarding against oncogenesis (Junttila and Evan, 2009; Vousden and Prives, 2009). Maintained at low levels in the steady-state by proteasomal degradation, p53 is rapidly stabilized and activated in response to DNA damage and a variety of other metabolic stressors (Lavin and Gueven, 2006). In addition to classical genotoxic stress (e.g., γ-irradiation), p53 is also responsive to inflammatory stressors such as TNF (Donato and Perez, 1998) and reactive oxygen species (ROS; Lavin and Gueven, 2006; Voussen and Prives, 2009) and is up-regulated at sites of inflammation (Moon et al., 2000; Hofseth et al., 2003).

Recent studies have extended the purview of p53 as a suppressor gene by demonstrating that p53 also regulates inflammation. p53 suppresses NF-κB–dependent cytokine induction (Komarova et al., 2005; Liu et al., 2009; Ak and Levine, 2010). p53 also regulates cell migration through interactions with Rho GTPases (Sablina et al., 2003) and exerts complex effects on oxidant generation, promoting release of mitochondrial ROS during stress-induced apoptosis, but also buffering ROS under more physiological settings through induction of antioxidant genes and repression of inducible nitric oxide synthase (Polyak et al., 1997; Amb et al., 1998; Sablina et al., 2005). Cytokine induction, cell migration, and oxidant generation are not only...
hallmarks of inflammation, but also pivotal events in host defense. However, very few studies have investigated a possible role for p53 in infection. These investigations have been limited to viral inoculation (Takaoka et al., 2003; Turpin et al., 2005; Muñoz-Fontela et al., 2008), a setting in which cell-intrinsic hallmark functions of p53 established in the tumor suppressor literature (e.g., cell cycle arrest and apoptosis) had also already been established to play a central role in clearance of cells infected with oncogenic and nononcogenic viruses alike.

Host defense against extracellular as well as intracellular pathogens in complex organs such as the lung requires coordinated actions by multiple cell types. Alveolar macrophages, as sentinel cells, play a critical role through both cell-intrinsic antimicrobial functions (e.g., generation of oxidants) and production of cytokines that recruit microbicidal neutrophils (PMNs) into the infected airspace. Ultimately, death of macrophages and PMNs through apoptosis is coupled to their production of oxidants and is critical for successful bacterial clearance (Marriott et al., 2006). Given the importance of these various cellular functions to host defense, we speculated that the ubiquitously expressed tumor suppressor p53 might be positioned to regulate multiple critical checkpoints in the host defense response to extracellular pathogens in vivo.

Here, we show that mice with deletion or inhibition of p53 display enhanced clearance of both Gram-negative and -positive extracellular bacteria after intrapulmonary infection. p53-deficient mice have an expanded population of apoptosis-resistant, TLR-hyperresponsive alveolar macrophages, recruiting increased PMNs into the airspace, p53+/− PMNs, in turn, display coordinate enhancement of a suite of antimicrobial functions. Collectively, these findings suggest that p53 regulates host defense against extracellular pathogens through coordinate control of function and fate of phagocytes in the lung, thus positioning p53 as a fundamental link between defense of the organism and of the genome in the face of environmental insult.

RESULTS

p53 deletion and inhibition enhance clearance of extracellular bacteria during pneumonia

To determine whether p53 regulates antibacterial host defense in the lung, p53−/− and p53+/+ mice were inoculated intratracheally (i.t.) with the Gram-negative bacterium Klebsiella pneumoniae, and extrapulmonary bacterial dissemination was monitored by blood culture. As shown in Fig. 1A, p53−/− mice had significantly lower organism burden in blood than WT counterparts 24 and 48 h after lung infection. Similar findings were noted after lung infection with the Gram-positive bacterium Streptococcus pneumoniae (Fig. 1B). In parallel with the blood culture findings, p53−/− mice had a nearly 2-log reduction in splenic bacteria 48 h after i.t. infection with K. pneumoniae (Fig. 1C), consistent with reduced bacteremic seeding. WT mice pretreated with a single i.p. dose of the p53 inhibitor pifithrin-α (PFTα; Komarov et al., 1999) also had lower splenic K. pneumoniae CFUs than vehicle-treated counterparts, suggesting that pharmacologic antagonism of p53 is also effective at reducing pathogen burden in the setting of pneumonia. PFTα-treated p53−/− mice had higher splenic bacterial CFUs than vehicle-treated p53−/− mice, consistent with some off-target effects of the inhibitor; however, direct treatment of K. pneumoniae with PFTα in vitro revealed no effects on bacterial growth (not depicted). Confirming that bacterial clearance is indeed enhanced in the inoculated lung itself in the setting of p53 deficiency, both p53−/− mice and PFTα-treated p53−/− mice had significantly reduced organism burden in lung homogenates 24 h after i.t. K. pneumoniae (Fig. 1D). Collectively, these findings indicate that reduction of p53, either genetically or by inhibition, enhances pathogen clearance in the setting of bacterial pneumonia.

p53 deficiency enhances neutrophil recruitment to the infected lung

Recruitment of circulating PMNs to the infected lung is critical for successful clearance of bacteria. p53−/− mice recruited more leukocytes to the airspace than WT counterparts in the setting of infections with both K. pneumoniae and S. pneumoniae, reflecting increases in both PMNs and macrophages (Fig. 2, A and B). PFTα-treated WT mice similarly recruited...
increased PMNs to the airspace after *K. pneumoniae* infection (Fig. 2 C). In response to inhalation of LPS, the canonical ligand for TLR4 and the major immunostimulatory glycolipid of the Gram-negative bacterial cell wall, p53<sup>−/−</sup> mice also recruited increased PMNs to the airspace in a sustained manner over 48 h after exposure (Fig. 2 D).

Abundance of PMNs in the airway is regulated as a balance between PMN recruitment from the bloodstream on the one hand and PMN apoptosis and clearance on the other. As p53 is a proapoptotic factor and is expressed by PMNs (Hsieh et al., 1997), we queried whether neutrophilia in the infected p53<sup>−/−</sup> mice might in part stem from reduced PMN apoptosis. Intraalveolar PMNs, however, displayed equivalent rates of apoptosis in the p53<sup>+/+</sup> and p53<sup>−/−</sup> airway both in the naive state and 24 h after i.t. *K. pneumoniae* (Fig. 2 E), as indicated by flow cytometry of FLIVO, a poly-caspase reporter (Merrick et al., 2011). This finding suggests that the airway neutrophilia in infected p53<sup>−/−</sup> mice is not driven by reduced PMN apoptosis. Naive p53<sup>−/−</sup> mice also had normal numbers of circulating PMNs and other leukocyte subtypes (Table 1), thus ruling out steady-state peripheral neutrophilia, as might conceivably occur through an effect of p53 on cell cycle in the bone marrow (Leonova et al., 2010), as an explanation for increased numbers of PMNs recruited to the airspace of infected p53<sup>−/−</sup> mice.

**p53 deletion leads to genome-wide proinflammatory gene induction in the lung**

Given that p53 is a transcription factor with both activation and repression activity, we performed gene expression profiling on noninfected p53<sup>−/−</sup> and p53<sup>+/+</sup> mouse lung to screen for possible mechanisms underlying the enhanced inflammatory response in the p53-null lung. 258 genes were significantly (P < 0.01) up (n = 186) or down-regulated (n = 72) 2.0-fold in p53<sup>−/−</sup> as compared with p53<sup>+/+</sup> lung (Table S1).

Remarkably, gene ontology analysis (DAVID tool, http://david.abcc.ncifcrf.gov/tools.jsp) revealed striking enrichment of immune response genes (false discovery rate = 7.6 × 10<sup>−22</sup>; 152 regulated genes in immune gene ontology categories) in the naive p53<sup>−/−</sup> lung, including a wide variety of proinflammatory genes (cytokines, cytokine receptors, integrins, and pro-oxidant enzymes) that were up-regulated compared with WT lungs (Fig. 3 A). Given this, we analyzed the promoter regions of regulated genes to identify potential NF-κB–binding sites using the Genomatix MatInspector tool (Cartharius et al., 2005). 252 (97.7%) of the genes differentially expressed in the p53<sup>−/−</sup> lung were noted to possess high-confidence–predicted NF-κB–binding sites (5.0 ± 0.2 [mean ± SEM] sites per gene) in their promoters (Tables S2 and S3), suggesting that they may be NF-κB target genes. To more stringently test the in silico enrichment of NF-κB motifs in the promoter regions of genes up-regulated in the p53<sup>−/−</sup> lung, we performed a comprehensive, unbiased analysis. To do this, we first identified promoter sequences (1 kb) for the up-regulated genes in

### Table 1. Peripheral WBC count and differential in naive p53<sup>+/+</sup> and p53<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>p53&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>p53&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs</td>
<td>7.0 ± 2.34</td>
<td>7.1 ± 2.78</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.68 ± 0.26</td>
<td>1.03 ± 0.81</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.24 ± 0.11</td>
<td>0.22 ± 0.11</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.03 ± 2.41</td>
<td>5.72 ± 2.66</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.14 ± 0.11</td>
<td>0.18 ± 0.10</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Monocyte data were derived from n = 5/genotype; for all others, n = 10. All inter-genotype comparisons are P = NS.
Figure 3. Inflammatory priming of the p53-null lung and macrophages. (A) Proinflammatory genes up-regulated in naive p53−/− as compared with p53+/+ lung. Columns represent microarray data from separate mice. Mean fold change is shown at right and functional categories at left. Data are representative of one experiment involving n = 5 mice/genotype. (B) Alveolar macrophage (MΦ) number in naive p53+/+ and p53−/− mice (n = 14–15/genotype; representative of three independent experiments). (C) Number of F4/80+ macrophages in spleen of naive p53+/+ and p53−/− mice (n = 3/genotype; representative of two independent experiments). (D) Representative flow cytometry histograms of MHC class II, CD86, and IL-4Rα on alveolar macrophages.
were exposed to LPS and Pam3CSK4 (Pam) and conditioned media assayed by multiplex assay for the indicated cytokines (by ELISA for TNF release (n = 6/genotype for both assays; representative of two independent experiments). (F and G) Apoptosis rates of alveolar macrophages from naive p53+/+ and p53−/− mice. Shaded trace is isotype control, solid line is p53+/+, and dashed line is p53−/−.

16S rDNA analysis of aseptically harvested lung indicated no evidence for increased microbial colonization of naïve p53−/− lungs compared with p53+/+ lungs (not depicted). Moreover, bronchoalveolar lavage fluid (BALF) levels of a wide panel of cytokines and chemokines (MIP-2, IL-6, IL-17, keratinocyte-derived chemokine [KC], MCP-1, and TNF) were equivalent between naïve p53+/+ and p53−/− mice (not depicted), arguing against overt inflammation in naïve p53−/− lungs. Although the significance of the altered global gene expression in the naïve p53−/− lung was not fully clear; it was reminiscent of the transcriptional priming for secondary pro-inflammatory exposures that has been described with IFN-γ (El Chartouni and Rehli, 2010) and suggested to us the possibility that the p53−/− lung may be poised for a more robust immune response during infection. We thus more closely examined immune cell populations in the p53−/− lung.

**Expansion of TLR-hyperresponsive macrophages in the p53−/− lung**

Alveolar macrophages play a critical role in pulmonary host defense against *S. pneumoniae* and other extracellular pathogens (Dockrell et al., 2003). Naïve p53−/− mice had higher numbers of alveolar macrophages than p53+/+ mice (Fig. 3 B), whereas alveolar PMNs and lung parenchymal dendritic cells, CD4+ T cells, CD8+ T cells, and B cells were comparable in number with WT mice (not depicted), consistent with a macrophage-specific expansion of lung-resident leukocytes. In contrast, p53−/− mice had normal numbers of splenic F4/80+ macrophages (Fig. 3 C), ruling out a global expansion of tissue macrophages, and normal numbers of blood monocytes (Table 1), ruling out systemic monocytosis.

p53−/− alveolar macrophages appeared normal in terms of development and maturity, expressing WT levels of cell surface MHC class II, CD86, and IL-4Rα (Fig. 3 D) and also displaying normal morphology (not depicted) and phagocytic function (Fig. 3 E). However, p53−/− alveolar macrophages had a lower rate of constitutive in vivo apoptosis than p53+/+ counterparts, as indicated by both a poly-caspase activity reporter (Fig. 3 F) and a caspase-3/7—specific activity reporter (Fig. 3 G).

Notably, p53−/− alveolar macrophages produced higher levels of TNF than p53+/+ counterparts in response to LPS exposure (Fig. 3 H). p53−/− peritoneal elicited macrophages (PEMs) also produced higher levels of cytokines than WT controls in response to both LPS and Pam3CSK4, a synthetic TLR2 ligand used to model Gram-positive bacterial lipoproteins (Fig. 3 I). WT PEMs pretreated with PFTα also induced elevated TNF in response to LPS, whereas pretreatment with the p53 activator nutlin-3a diminished TNF induction (Fig. 3 J). p53−/− bone marrow–derived macrophages also induced higher IL-6 than WT counterparts in response to a yet wider panel of pathogen-associated molecular patterns, including stimuli for TLR2 (Pam3CSK4 and FSL1), TLR7 (imiquimod), TLR8 (siRNA40), and TLR9 (ODN; Fig. 3 K). Collectively, these findings suggest that p53 deletion coordinately enhances the number and global innate immune responsiveness of macrophages in the lung.

**Enhanced induction of NF-κB-dependent cytokines in the infected p53−/− lung**

Compared with WT counterparts, alveolar macrophages in p53−/− mice also had a reduced rate of programmed cell death, as well as higher cell surface display of the co-stimulatory protein CD86 24 h after i.t. inoculation with *K. pneumoniae* (Fig. 4, A and B), together suggesting that they may sustain higher viability and activation status during lung infection.

(CD11c autofluorescence) of naïve p53+/+ and p53−/− mice. Shaded trace is isotype control, solid line is p53+/+, and dashed line is p53−/−. Data are representative of two independent experiments. (E) Ex vivo phagocytosis of *E. coli* bioparticles by naïve p53+/+ and p53−/− alveolar macrophages (n = 5/genotype; representative of two independent experiments). (F and G) Apoptosis rates of alveolar macrophages from naïve p53+/+ and p53−/− mice, as quantified by flow cytometry of poly-caspase activation (FLUO; F) or caspase-3/7 activation (FLICA; G; n = 6/genotype for both assays; representative of two independent experiments). (H) Alveolar macrophages (>80%) were lavaged from p53+/+ and p53−/− mice 1 h after LPS inhalation and resuspended in culture. TNF production into media was evaluated 6 h later by ELISA (n = 8–10/genotype; representative of two independent experiments). (I) PEMs from p53+/+ and p53−/− mice were exposed to LPS and Pam3CSK4 (Pam) and conditioned media assayed by multiplex assay for the indicated cytokines (n = 4/genotype; data representative of two independent experiments). (J) p53−/− PEMs were pretreated with vehicle, 10 µM nutlin-3, or 40 µM PFTα, exposed to buffer or LPS, and then assayed by ELISA for TNF release (n = 2–3/condition; representative of two independent experiments). (K) Bone marrow–derived p53+/+ and p53−/− macrophages were either left unstimulated (NS) or exposed (24 h) to TLR ligands (1 µg/ml Pam3CSK4, 100 ng/ml FSL1, 10 µg/ml imiquimod, 1 µg/ml ssRNA40 [ssRNA41 is negative control for ssRNA40], and 5 µM ODN2395), after which normalized IL-6 mRNA expression was quantified by quantitative PCR. Data shown (log, scale) are representative of four independent experiments. Data are mean ± SEM. *, P ≤ 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4. Increased induction of NF-κB–dependent cytokines in the infected p53-null lung. (A) Programmed cell death (flow cytometric measurement of caspase activity [FLIVO]) of alveolar macrophages (MΦ) in p53+/+ and p53−/− mice 24 h after i.t. *K. pneumoniae* (*n* = 11/genotype; representative of three independent experiments). (B) CD86 surface display by flow cytometry on alveolar macrophages of unexposed (−*Kp*) and i.t. *K. pneumoniae*–exposed (+*Kp*) p53+/+ and p53−/− mice (representative of three experiments involving *n* = 9/genotype). (C and D) BALF cytokines 24 h after i.t. *K. pneumoniae* (C) or *S. pneumoniae* (D) in p53+/+ and p53−/− mice 48 h after i.t. *K. pneumoniae* (left; *n* = 5/genotype) and in conditioned media of p53−/− and p53−/− bone marrow–derived macrophages 24 h after treatment with 50 U/ml IFN-γ and 100 ng/ml LPS (right; *n* = 6/genotype). Data are representative of two to three independent experiments. (E) BALF total nitrite, an indicator of NO, in p53+/+ and p53−/− mice 48 h after i.t. *K. pneumoniae* (left; *n* = 5/genotype) and in conditioned media of p53−/− and p53−/− bone marrow–derived macrophages 24 h after treatment with 50 U/ml IFN-γ and 100 ng/ml LPS (right; *n* = 6/genotype). Data are representative of two to three independent experiments. (F) BALF cytokines in p53+/+ and p53−/− mice 2 h after LPS inhalation (*n* = 10/genotype; representative of two independent experiments). (G) p65 NF-κB activation in lung homogenates of p53+/+ and p53−/− mice 2 h after LPS inhalation (*n* = 6–10/genotype/time point; representative of two independent experiments). (H) NF-κB luciferase activity (RLU, relative light units) in buffer- or LPS-exposed CD14-MD2-TR4-HEK293 cells transfected with empty vector (EV) or p53 (representative of two independent experiments). (I) NF-κB luciferase activity in buffer- or LPS-exposed CD14-MD2-TR4-HEK293 cells transfected with p53 plus either negative control siRNA or p53 siRNA (representative of two independent experiments). Data are mean ± SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.
Given the enhanced ex vivo cytokine induction by p53−/− macrophages and increased number of viable and activated alveolar macrophages in the infected p53−/− lung, we examined BALF cytokine levels during infection. BALF of p53−/− mice infected with either K. pneumoniae or S. pneumoniae also contained elevated levels of a wide range of cytokines and chemokines of established importance to PMN recruitment (Fig. 4, C and D). In the case of K. pneumoniae, treatment of WT mice with PFTα produced a similar increase over vehicle treatment for all analytes other than IL-17 (Fig. 4 C). BALF of K. pneumoniae−infected p53−/− mice also contained elevated NO (Fig. 4 E), an oxidant integral to host defense against extracellular bacteria (Tsai et al., 1997). In support of p53−/− alveolar macrophages contributing to this finding, p53−/− bone marrow–derived macrophages displayed higher NO production after LPS/IFN-γ treatment than WT counterparts. To confirm the effect of p53 deletion on airspace cytokine induction in a more acute and simplified model, mice were exposed to inhaled LPS, p53−/− mice had higher BALF TNF, KC, and MIP-2 than WT counterparts 2 h after LPS inhalation, a time point which precedes significant influx of PMNs to the lung (not depicted), whereas IL-6 was equivalent to WT (Fig. 4 F).

NF-κB plays a critical role in induction of cytokines by lung-resident cells, PMN recruitment to the airspace, and bacterial clearance during pneumonia (Quinton et al., 2007) and is reportedly suppressed by p53 (Komarova et al., 2005; Ak and Levine, 2010). Thus, we next quantified NF-κB DNA binding in the nuclear fraction of lung parenchyma after LPS inhalation. LPS-induced p65 NF-κB DNA binding activity was higher in p53−/− than WT lung after LPS (Fig. 4 G). In further support of a suppressive effect of p53 on NF-κB transcriptional activity, overexpression of p53 attenuated both basal and LPS-induced NF-κB luciferase activity in TLR4-CD14-MD2-HEK293 cells, whereas p53 silencing enhanced basal and LPS-induced NF-κB luciferase (Fig. 4, H and I). Collectively, these findings suggest that the enhanced neutrophilia in the infected p53−/− airspace may stem from enhanced cytokine/chemokine induction in the airspace that in turn derives, at least in part, from enhanced NF-κB activation in lung-resident cells.

p53 regulates migration of PMNs to the lung in vivo
p53 is also expressed by both PMNs and the lung structural cells (i.e., epithelium and endothelium) that PMNs interact with during their complex integrated passage from blood to airspace. To test whether PMNs display a migratory advantage to the airspace in p53−/− mice, CXCL1/KC was instilled i.t. in p53+/+ and p53−/− mice, and PMN influx into the airspace was quantified. As shown in Fig. 5A, significantly more PMNs accumulated in the p53−/− airspace than in WT controls 4 h after KC. BALF KC was equivalent in p53+/+ and p53−/− mice at this time point, suggesting equal chemokine bioavailability, and BALF MIP-2 was also similar between genotypes, whereas G-CSF, TNF, and IL-6 were lower in the p53−/− airspace after i.t. KC (not depicted). Collectively, these findings indicate that (a) more PMNs enter p53−/− than WT airways even in the setting of an equal inoculum of chemokines, (b) enhanced PMN influx into the p53−/− airspace in response to KC is not explained by more robust secondary induction of chemokines, and (c) the effect of p53 on airspace cytokine induction is stimulus dependent.

p53-deficient PMNs display enhanced antimicrobial functions
Although enhanced airway neutrophilia and NO might suffice to explain the enhanced bacterial clearance in the p53−/− lung, we next sought to determine whether p53 deletion also alters PMN functions involved in phagocytic killing. As shown in Fig. 5 B, p53−/− peritoneal elicited PMNs were indeed observed to phagocytose bacterial bioparticles more effectively than WT counterparts. To model PMN phagocytic killing at inflammatory foci in vivo (Ledfond et al., 2007), K. pneumoniae was next injected i.p. into p53+/+ and p53−/− mice after thioglycollate eliciting PMNs to the peritoneum; peritoneal PMNs were then harvested 30 min after peritoneal infection and assayed for intracellular bacterial killing during a time course ex vivo. As shown in Fig. 5 C, p53−/− PMNs displayed enhanced killing of K. pneumoniae. Ex vivo treatment of the harvested peritoneal PMNs with BAY 11-7082, an NF-κB inhibitor, did not significantly alter intracellular killing by PMNs of either genotype (not depicted), arguing against an important role for PMN NF-κB in intracellular bacterial killing in this system.

Killing of bacteria in the PMN phagosome is thought to derive from NADPH oxidase 2 (Nox2)–derived superoxide (O2•−) as well as granule proteases such as elastase, although the major role of oxidants may be to license proteases by promoting their release from proteoglycan matrix (Reeves et al., 2002). p53−/− peritoneal elicited PMNs produced higher levels of O2•− than WT PMNs after stimulation with either the Nox2 activator PMA or the bacterial tripeptide formylated-Met-Leu-Phe (fMLF; Fig. 5 D). Consistent with this increased O2•− deriving from Nox2, O2•− induction by both stimuli was abolished in PMNs of both genotypes pretreated with the Nox2 inhibitor diphenyleneiodonium (Fig. 5 D), as well as in peritoneal PMNs harvested from gp91phox−/− (i.e., Nox2 deficient) mice (not depicted). p53−/− PMNs displayed increased expression of gp91phox and the cytosolic Nox2-activating protein p47phox (Fig. 5 E), consistent with up-regulation of the Nox2 complex. In contrast, p67phox was not differentially expressed.

Consistent with p40phox/Nfia, a positive regulator of Nox2, being a direct suppression target of p53, p53 chromatin immunoprecipitation (ChIP) in RAW 264.7 macrophages pulled down a sequence from the p40phox promoter in a manner that was enhanced by treatment with the p53 activator, nutlin-3 (Fig. 5 F). Indeed, in support of the possibility that p53 may directly regulate expression of multiple mouse Nox2 complex genes through transcriptional repression, promoter analysis revealed putative functional p53 response elements not only in p40phox/Nfia but also in gp91phox/Nox2, p47phox/Nox1, p22phox/Cyba, and Noxa1 (Table S4). In contrast, no
predicted functional p53 motif was found in the promoter of p67phox/Ncf2, consistent with our finding of unchanged expression of p67phox in p53−/− PMNs. Ncf1 (1.50-fold), Ncf4 (1.82-fold), and Noxa1 (2.0-fold) were also significantly upregulated in naive p53−/− as compared with p53+/+ lung by microarray analysis, suggesting that constitutive repression of Nox2 complex genes may also occur in the lung.

Increased O$_2^-$ production in response to both PMA and LPS/IMLF was also observed in PMNs isolated from bone marrow of naive p53−/− mice (Fig. 5, G and H). PFTα-treated primary human PMNs also produced higher levels of intracellular ROS than vehicle-treated controls after exposure to K. pneumoniae (Fig. 5 I). To test whether the enhanced intracellular killing of p53-deficient PMNs in fact derives from increased O$_2^-$, the K. pneumoniae killing assay was next performed in gp91phox+/+ and gp91 phox−/− mice pretreated systemically with a single dose of either vehicle or PFTα. Remarkably, as shown in Fig. 5 J (open and hatched open bars), PMNs harvested from gp91phox−/− mice that had been treated systemically with a single dose of PFTα displayed approximately
fournfold enhanced intracellular bacterial killing. Of note, however, PFTα treatment enhanced bacterial killing by gp91phox−/− PMNs comparably with its effect in gp91phox+/+ PMNs. This suggests that increased Nox2 function does not, at least by itself, underlie the enhanced bacterial killing phenotype of p53-deficient PMNs.

Pursuing the protease arm of PMN microbicidal function, we next tested the capacity of PMNs to release elastase by degranulation. Unlike the alternate PMN azurophilic granule serine protease cathepsin G, elastase has a proven role in PMN killing of *K. pneumoniae* (Belaaouaj et al., 1998). p53−/− PMNs indeed released significantly higher elastase activity than WT counterparts upon ex vivo treatment with the azurophilic granule–releasing dual stimulus, cytochalasin B/fMLF (Fig. 6 A). This suggested the possibility that the killing advantage of p53-deficient PMNs might stem from enhanced proteolytic activity: To test this hypothesis, we repeated the PMN intracellular killing assay in i.p. vehicle- and PFTα-treated WT mice, but this time incubated PMNs harvested from the peritonea of both treatment groups with either the cell-permeant general serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or vehicle (Standish and Weiser, 2009). AEBSF abolished the killing advantage of PMNs from PFTα-treated mice (Fig. 6 B), thereby suggesting that the enhanced killing phenotype of p53-deficient PMNs derives from enhanced serine protease activity.

Several cell types in the lung, in addition to recruited PMNs, are capable of producing NO. NO plays an important role in pulmonary host defense against several types of bacteria, including *K. pneumoniae* (Tsai et al., 1997). Given our finding of increased BALF NO in infected p53−/− mice, we thus questioned whether NO might also contribute to the enhanced clearance of *K. pneumoniae* from the p53−/− lung. To address this, p53+/+ and p53−/− mice were treated with either L-NAME (a NO synthase inhibitor) or vehicle and then infected with *K. pneumoniae* for 24 h. As is shown in Fig. 7, L-NAME had no significant effect on clearance of *K. pneumoniae* from p53+/+ lungs but dramatically impaired bacterial clearance from p53−/− lungs. This finding suggests that NO plays an important role in the enhanced antibacterial host defense of the p53−/− lung.

**p53−/− mice have reduced survival and aggravated lung injury during pneumonia**

PMN recruitment to the lung is required for successful host defense, but overexuberant inflammation can harm the host through bystander lung injury. We and others have reported various gene-deleted mouse strains that suffer increased mortality during bacterial pneumonia despite neutrophilia–enhanced pathogen clearance (Li et al., 2009; Draper et al., 2010). Notably, we found that p53−/− mice have lower survival than WT mice after i.t. *K. pneumoniae* (Fig. 8 A). In support of exacerbated lung injury contributing to this outcome, infected p53−/− mice had increased BALF protein (Fig. 8 B), a marker of pulmonary microvascular injury. Moreover, histopathologic analysis of *K. pneumoniae*-infected lungs confirmed significantly increased perivascular neutrophilic inflammation and edema in p53−/− mice 72 h after infection (Fig. 8, C and D).

**DISCUSSION**

p53 has been extensively studied as a tumor suppressor and more recently shown to be an inflammation suppressor. In the present study, we extend the domain of this master regulatory transcription factor to that of suppression of the host defense response, showing that p53-deficient mice have enhanced clearance of bacterial pneumonia associated with coordinate disinhibition of macrophage and PMN function. We propose that enhanced activation of NF-κB and induction of cytokines in the infected p53-null lung, originating at least in part from an expanded, TLR-hyperresponsive alveolar macrophage population, further augments recruitment of p53-null...
PMNs to the alveolus beyond a migratory advantage they already display in response to alveolar chemokines. p53−/− PMNs, in turn, display coordinate enhancement of a suite of antimicrobial functions, including phagocytosis, bacterial killing, oxidant generation, and granule release. That said, our data do not exclude possible important host defense roles for p53 in nonhematopoietic cells in the lung, such as alveolar epithelial cells. However, in the end, reminiscent of the case for some other gene deletions (e.g., Abg1 and Pten [Li et al., 2009; Draper et al., 2010]), enhanced pathogen clearance in the p53-null mouse is associated with worsened survival. p53’s integrated role may thus be to serve as a beneficial brake on the lung’s response to infection. We provide correlative data suggesting that p53−/− mice may suffer lower survival as the result of lung injury from an overexuberant immune response; however, it is very possible that p53 deletion may engender additional maladaptive responses that compromise survival.

We speculate that the p53-null lung is poised for a more robust host defense response at least in part through genome-wide effects of NF-κB dis inhibition. Our finding of an NF-κB response element–enriched transcriptional signature of inflammation in the naive p53-null lung is reminiscent of a recent study showing that p53 inhibition and TNF treatment elicit strikingly similar NF-κB target gene–enriched transcriptional profiles in LNCaP prostate cancer cells (Komarova et al., 2005). We are unaware, however, of any previous report that p53 deletion induces inflammatory gene programs in vivo in the steady-state. Of note, NF-κB can be activated by both ROS and DNA damage, and it has been reported that p53−/− mice have increased DNA damage in multiple tissues caused by increased ROS (Sabina et al., 2005). It is thus interesting to speculate that the genomic signature of the p53−/− lung in our study may in part stem from oxidative stress and that p53 may thus exert coordinate control over cancer and inflammation through regulation of ROS.

Alveolar macrophages were also increased in number in the naive p53−/− lung and displayed a reduced rate of constitutive apoptosis, consistent with a report that p53 promotes apoptosis of macrophages in other settings (Mercer et al., 2005). Given the equivalent expression (<1.5-fold difference) of monocyte/macrophage-attracting chemokines (CCL1, −2, −3, −4, −5, −7, −8, and −12) in naive p53−/− and p53+/- lungs (unpublished data) and the normal number of circulating monocytes in p53−/− mice, we speculate that the increase in alveolar macrophages stems, at least in part, from reduced local apoptosis, rather than increased trafficking to the lung. p53 has also been reported to suppress macrophage proliferation (Merched et al., 2003). Although we were unable to detect the proliferation marker PCNA in naive alveolar macrophages by either immunoblotting or flow cytometry, bone marrow–derived p53−/− macrophages expressed significantly increased PCNA compared with WT counterparts (unpublished data), suggesting that increased local proliferation may also possibly contribute to increased numbers of alveolar macrophages in the p53−/− lung. However, of interest, our finding of a normal number of splenic macrophages in p53−/− mice suggests that this regulatory effect of p53 on steady-state macrophage populations is tissue selective.

Macrophage apoptosis has been reported to be essential not only for resolution of inflammation but also for successful clearance of S. pneumoniae (Dockrell et al., 2003; Marriott et al., 2006). Although the reduced apoptosis of alveolar macrophages in the infected p53−/− lung was not associated with impaired microbial clearance, it is possible that sustained macrophage survival in the p53−/− lung nonetheless contributed to amplifying inflammation and organ injury. A prior report that p53−/− macrophages have defective efferocytosis (Komarova et al., 2005) may offer a unifying mechanism for the p53−/− pneumonia phenotype in our study, as efferocytosis impairs bacterial clearance (Medeiros et al., 2009) and represses inflammation (Huynh et al., 2002). Suggesting cell type specificity,
and consistent with a prior report that Bcl-2 family members play a dominant role in regulation of PMN apoptosis (Dzhagalov et al., 2007), PMN apoptosis was unaltered in vivo in infected p53−/− mice.

We report a critical role for the molecule NO in the enhanced bacterial clearance phenotype of the p53−/− lung. Treatment of p53−/− mice with the NO synthase inhibitor L-NAME markedly impaired clearance of *K. pneumoniae* from the lung, whereas no such effect was seen in p53+/+ mice. In addition to PMNs, several lung-resident cell types have the capacity to generate NO. In support of the possibility that alveolar macrophages may be the responsible cell type, we found that p53−/− bone marrow–derived macrophages produce elevated NO after in vitro stimulation. Thus, in addition to contributing to increased PMN influx during pneumonia via augmented cytokine induction, p53−/− alveolar macrophages may also contribute more directly to microbial killing through enhanced NO generation. Further studies are warranted to identify whether additional cell types in the p53−/− lung, including the alveolar epithelium, may also contribute to increased bacterial killing through augmented NO generation.

Our findings indicate a novel role for p53 as a master regulator of multiple hallmark host defense functions of the PMN. Remarkably, a single systemic injection of PFTα enhanced bacterial killing by PMNs and bacterial clearance in vivo, suggesting potential for p53 inhibitors as immunostimulatory adjuvants. Our use of bone marrow–purified PMNs suggests that at least some of these p53-regulated functions (e.g., O2− and elastase release) are, moreover, cell autonomous. Although others have demonstrated that p53 buffers ROS through induction of antioxidants (Sablina et al., 2005), we are unaware of prior reports that p53 regulates Nox-dependent generation of O2− in any cell type. p53 may modulate Nox function at least in part through directly repressing Nox2 complex genes. However, the finding that PFTα enhances PMN bacterial killing comparably in Nox2-sufficient and −deficient PMNs indicates that enhanced O2− does not, at least in isolation, explain the enhanced killing conferred by p53 deficiency. p53−/− PMNs also released increased elastase, a critical executor protease of bacterial killing (Belouatouj et al., 1998). We provide evidence that the killing advantage of p53-deficient PMNs derives from enhanced serine protease and, likely, elastase activity. As PMN elastase has been implicated in a wide array of lung disorders and shown to induce p53-dependent lung epithelial apoptosis (Suzuki et al., 2009), we speculate that p53 may represent an under-recognized central regulator of lung disease.

p53 in the lung is indeed broadly responsive to environmental stressors. Diesel exhaust, silica, and cigarette smoke all activate p53 in macrophages and/or other lung cell types (Wang et al., 2005; Yun et al., 2009; Damico et al., 2011), suggesting that these exposures may alter host defense functions at least in part through p53. p53 is also up-regulated in the alveolar epithelium in chronic obstructive lung disease (Siganaki et al., 2010). As small molecule p53 agonists are under development for human cancer therapy, future studies are urgently needed to define the effects of pharmacologic activation of p53 on the human innate immune response in vivo. Moreover, as chemotherapeutics already in wide clinical use as well as radiation therapy also activate p53, it is incumbent to determine whether these agents modify innate immunity and pneumonia risk in cancer patients via effects on p53. Finally, studies are warranted to determine whether genetic polymorphisms leading to hypofunction of the p53 pathway are associated with increased risk for lung injury and/or mortality during human pneumonia.

The recent recognition that bacteria and their products induce DNA damage and compromise DNA repair (Koturbash et al., 2002) has suggested that defense of the host and of the genome may be intrinsically interconnected to a degree not previously appreciated. We speculate that p53 may be centrally positioned to integrate these two fundamental responses to the environment and that this carries wide-ranging implications for human disease.

**MATERIALS AND METHODS**

**Reagents.** *Escherichia coli* 0111:B4 LPS, penicillin, streptomycin, and BAY 11–7082 were purchased from Sigma-Aldrich. *K. pneumoniae* 43816 (serotype 2), S. pneumoniae (serotype 3), DMEM, and FBS were purchased from the American Type Culture Collection. The Bio-Rad Laboratories protein assay was used. KC was purchased from R&D Systems. L-NAME was purchased from Cayman Chemical.

**Mice.** C57BL/6, B6.129S2-Trp53tm1T 66/J (backcrossed at least 7 times to C57BL/6), and B6.129S-Cybbtm1D in/J male mice (backcrossed >10 times to C57BL/6), 7–10-wk-old and weighing 18–22 g, were used and were obtained from the Jackson Laboratory. B6.129S-Cybbtm1D/J mice were provided by J.-S. Hong (National Institute of Environmental Health Sciences [NIEHS], Research Triangle Park, NC). All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the NIEHS.

**In vivo exposures.** Exposure to 300 μg/ml of aerosolized LPS (30 min) was performed as previously described (Draper et al., 2010). 0.5 μg/60 μl KC, 150–2,000 CFU/50 μl *K. pneumoniae*, and 2 × 10⁷ CFU/50 μl *S. pneumoniae* were delivered to lung by oropharyngeal aspiration during isoflurane anesthesia. In some experiments, 2.2 mg/kg PFTα or vehicle was injected i.p. 1 h preceding other exposures, as previously reported (Komarov et al., 1999).

**BALF collection and analysis.** BALF was collected immediately after sacrifice, and cells counts were performed as previously described (Draper et al., 2010). Total protein was quantified by the method of Bradford.

**PMN and macrophage harvests and culture.** Mature mouse bone marrow PMNs were isolated from mouse femurs and tibias by discontinuous Percoll gradient centrifugation as previously reported (Draper et al., 2010). Mouse bone marrow–derived macrophages were prepared and cultured in DMEM containing 10% (vol/vol) FBS and 10% (vol/vol) L cell-conditioned medium as a source of M-CSF for 5 d as previously described (Doan et al., 2004). Peritoneal exudate macrophages were harvested by peritoneal lavage 96 h after i.p. injection of 2 ml of 4% Brewer’s thioglycollate. Alveolar macrophages were harvested by airway lavage of naïve mice, followed by enrichment by adherence on plastic. Human PMNs were purified from peripheral blood of healthy human donors by discontinuous plasma-Percoll.
Bactericidal assays. Lung, spleen, and liver were excised after sacrifice and homogenized in PBS, and serial dilutions were plated on tryptic soy agar for bacterial quantification 16 h later, as previously described (Draper et al., 2010). Blood was collected from right ventricle and similarly plated after serial dilution. Intracellular killing capacity of PMNs against L. pneumophila was quantified as reported previously (Ledford et al., 2007). In brief, mice were infected with 1–2 mg/kg PFTα (i.p. or left untreated) received 2.5 ml of 4% thioglycollate i.p., followed by 4 h later by 10⁸ CFUs of L. pneumophila. PMNs were incubated (37°C for 60 min to 1:5,000 species-specific HRP-conjugated secondary antibody. Membranes were then washed in Tween Tris-buffered saline (TTBS) and exposed to ECL Western Blot Detection Reagents (GE Healthcare), followed by film exposure (GE Healthcare).

RNA isolation and quantitative PCR. Total RNA was isolated by the RNeasy kit (QiAGEN). cDNAs were generated from 1.5 μg of purified RNA using TaqMan reverse transcription reagents from Applied Biosystems. Real-time PCR was performed in triplicate with TaqMan PCR Mix (Applied Biosystems) in the 7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers were purchased from Applied Biosystems (information available upon request). Gene expression was normalized to 18S ribosomal RNA (18S rRNA) and expression levels in untreated control samples were set as one.

Microarray and bioinformatic analysis. Gene expression analysis was conducted using Agilent Whole Mouse Genome 4 × 44 multiplex format oligo arrays (014869; Agilent Technologies) according to the Agilent one-color microarray-based gene expression analysis protocol. Starting with 500 ng of total RNA, Cy3-labeled cRNA was produced according to the manufacturer's protocol. For each sample, 1.65 μg of Cy3-labeled cRNAs was fragmented and hybridized (17 h). Slides were washed and then scanned with an Agilent Technologies scanner. Data were obtained using the Agilent Technologies Feature Extraction software (version 9.5), using the one-color default parameters for all experiments. The Agilent Technologies Feature Extraction software performed error modeling, adjusting for additive and multiplicative noise. The resulting data were processed using the Rosetta Resolver system (version 7.2; Rosetta Biosoftware). ANOVA was performed followed by the Benjamini Hochberg false discovery rate multiple test correction. Next, a post-hoc analysis (Tukey-Kramer) was used to perform pairwise comparisons of the mean groups of the treatment groups to determine which specific pairs were statistically different. The microarray data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession no. GSE38420. Promoter regions (~3000 to 1000 from transcription start site) were evaluated for potential NF-κB-binding sites with the use of MatInspector Release professional software (Genomatix Software GmbH) using the MatInspector family matrix V$NFKB with 0.85 Optimized matrix threshold. Promoter regions were also analyzed for potential p53-binding sites by first identifying putative sites with the use of p35scan (Smeek et al., 2008) and then ranking these sites for predicted p53 responsiveness using established p53 response element functionality rules (Menendez et al., 2009).

Flow cytometry. Lungs and spleen were digested/disaggregated as previously reported (Draper et al., 2012). Anti–Gr-1 (FITC), -F4/80 (PE or APC), -CD11c (PE-Cy5), -CD8 (APC), -CD45R/B220 (APC), and -CD86 (APC) and isotype control antibodies were purchased from BioLegend. Anti–CD4 and -MHCII were purchased from eBioscience. Anti-CD3e (APC) and -CD124/IL-4Ra (PE) were purchased from BD. Cells were stained and fixed with 2% paraformaldehyde/PBS. Flow cytometry was performed using an LSR II (BD) and analyzed using FlowJo (Tree Star) and FCS Express software (De Novo Software).

Assessment of apoptosis. Macrophages and PMNs lavaged from the mouse airway were incubated (for 60 min at 37°C) ex vivo with 1 µl (1.5 DMSO) of either FLIVO, a cell-permeant fluorescent poly-caspase activity reporter, or FLICA, a caspase-3/7–specific activity reporter (ImmunoChemistry Technologies) and then washed twice (0.5% BSA in 1× PBS, pH 7.4). Fluorescence signal was quantified by flow cytometry.

Peripheral blood leukocyte typing and enumeration. The blood samples were analyzed using the HEMAVET 1700 hematology analyzer (Drew Scientific, Inc.). Manual white blood cell (WBC) differential counts were performed, and smear estimates were used to confirm values. Reticulocyte counts were performed using new methylene blue stain and the Miller Disc Method of determination.

Histopathologic analysis. Mice were inoculated i.t. with 2,000 CFUs of L. pneumophila and then sacrificed 3 d later. Lungs were fixed with 4% formaldehyde and embedded in paraffin, and H&E-stained sections were analyzed. Immunostaining was performed using a mouse monoclonal antibody against p53 (DO-1; Santa Cruz Biotechnology, Inc.) and an EnVision+ system (Dako, Carpinteria, CA) using the manufacturer's instructions. Immunohistochemical analysis was performed with a 1:50 dilution of primary antibody, and the slides were developed with diaminobenzidine. A DAKO autoclave system (DAKO, Carpinteria, CA) was used to perform antigen retrieval on formalin-fixed paraaffin-embedded tissues. Sections were then visualized with a Nikon L1 microscope. Image analysis was performed using Scion Image (NIH) software. Semi-quantitative analysis was performed using new methylene blue stain and the Miller Disc Method of determination.
paraformaldehyde using a body weight–based volume, embedded in paraffin, sectioned (5 μm), stained with hematoxylin and eosin (H&E), and then semi-quantitatively scored by a pathologist blinded to animal genotype. A scoring system which grades (scale 0–4) the degree of peri vascular edema and cellular infiltration (primarily PMNs in this study) in both peri vascular and alveolar regions was used, as previously reported (Titley et al., 2007). Slides were scanned using a ScanScope XT Scanner (Aperio Technologies, Inc.). Sections were viewed and images then captured at 10× using ImageScope software version 11.02.716 (Aperio Technologies, Inc.).

ChIP, RAW 264.7 cells were seeded in 150-mm tissue culture dishes and treated with either DMSO or 10 μM Nutilin-3 (for 16 h). ChIP assays were performed as described previously (Valouev et al., 2008). In brief, the cells were cross-linked and sonicated to yield DNA fragments 200–500 bp in size. Chromatin was immunoprecipitated with either a p53 (Santa Cruz Biotechnology, Inc.) or goat-IgG (Santa Cruz Biotechnology, Inc.) antibody  using Invitrogen Protein G Dynabeads. The precipitated DNA was purified with QIAquick PCR Purification (QIAGEN) kit and used as template DNA for SYBR Green PCR reactions (Applied Biosystems). The following primer sets were used: ncf-4 (FP 5′-CCCTGGCCCATTCCTCTCCTC-3′; and RP 5′-CCACACATCCTACACCGGTG-3′); msgn2 (FP 5′-GACGGGTGCGGAGAAGCCG-3′; and RP 5′-CCGGAGGCTAATGCCTGACA-3′); and gapdh (FP 5′-GTGGGGGTTGTGTTGTGTC-3′; and RP 5′-GATAGGGGCTACCTGTA-3′).

Statistical analysis: Analysis was performed using GraphPad Software Prism statistical software. Data are represented as mean ± SEM. Two-tailed Student’s t-test was applied for comparisons of two groups and ANOVA for comparisons of more than two groups. Survival was evaluated by log-rank test. For all tests, P<0.05 was considered significant.

Online supplemental material: Table S1, included as a separate Excel file, indicates genes differentially expressed in naive p53-null lung compared with naive WT lung. Table S2, included as a separate Excel file, shows predicted NF-κB-binding sites in genes differentially expressed in naive p53-null lung. Table S3, included as a separate Excel file, lists the number of predicted NF-κB-binding sites in genes differentially expressed in naive p53-null lung. Table S4, included as a separate Excel file, shows predicted transcription factor binding sites and promoter analysis based on transcription factor binding sites. Bioinformatics: 21:2933–2942. http://dx.doi.org/10.1093/bioinformatics/btq473


