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p53 Serves as a Host Antiviral Factor That Enhances Innate and Adaptive Immune Responses to Influenza A Virus

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Several direct target genes of the p53 tumor suppressor have been identified within pathways involved in viral sensing, cytokine production, and inflammation, suggesting a potential role of p53 in antiviral immunity. The increasing need to identify immune factors to devise host-targeted therapies against pandemic influenza A virus (IAV) led us to investigate the role of endogenous wild-type p53 on the immune response to IAV. We observed that the absence of p53 resulted in delayed cytokine and antiviral gene responses in lung and bone marrow, decreased dendritic cell activation, and reduced IAV-specific CD8+ T cell immunity. Consequently, p53−/− mice showed a more severe IAV-induced disease compared with their wild-type counterparts. These findings establish that p53 influences the antiviral response to IAV, affecting both innate and adaptive immunity. Thus, in addition to its established functions as a tumor suppressor gene, p53 serves as an IAV host antiviral factor that might be modulated to improve anti-IAV therapy and vaccines. The Journal of Immunology, 2011, 187: 6428–6436.

Influenza A virus (IAV) poses a global health and economic threat due to the emergence of IAV epidemics and pandemics at random intervals (1). The high rate of viral mutation (antigenic drift) and the putative emergence of reasortant strains (antigenic shift) have raised the need to find host-targeted therapeutic strategies to devise antiviral treatments (2, 3). Current efforts are focused on the identification of host factors with a role in the early inflammatory responses to IAV infection, including TLRs (4), C-type lectins (5), inflammasomes (6), and chemokine receptors (7). However, a possible limitation of such strategies is the fact that these protein families are mainly expressed in the hematopoietic compartment and not in the epithelial cells of the respiratory tract, which as primary targets of the virus play a key role in the induction of early cytokine and antiviral gene responses (8).

Recently, we identified a positive feedback loop involving p53-dependent enhancement of IFN signaling through transcriptional upregulation of IFN regulatory factor (IRF)9 (9). By doing so, p53 not only promotes the transactivation of IFN-stimulated genes (ISGs), but also enhances IFN production from virus-infected cells. Other reports indicate that in addition to IRF9, other genes involved in innate immunity are also p53 direct transcriptional targets, including pattern recognition receptors such as TLR3 (10), additional IRFs such as IRF5 (11, 12), antiviral genes such as ISG15 (13) and dsRNA-activated protein kinase R (14), and proinflammatory chemokines such as MCP-1 (15). These findings strongly suggest that p53, which is ubiquitously expressed both in the epithelium and the hematopoietic compartment, could play an important role in promoting host antiviral cytokine and antiviral responses to IAV. To test this hypothesis we evaluated the onset of immunity in response to IAV in wild-type (wt) and p53−/− mice by systematic analysis of the events that occur in the lungs, bone marrow, and draining mediastial lymph nodes (mLN) postinfection. Our findings indicate that p53 is a key regulator of antiviral immunity to IAV, influencing not only innate but adaptive immunity as well. Thus, p53 modulation could provide a novel strategy in efforts to enhance anti-IAV antiviral therapies and vaccines.

Materials and Methods

Mice infection and virus titration

The p53−/− mouse line B6.129S2-Tg5.5mTyr/J was purchased from The Jackson Laboratory and bred in the Mount Sinai School of Medicine Animal Facility, and this has been previously described (16). This line has been backcrossed with mice of pure C57BL/6 genetic background for >10 generations. Wild-type BALB/c and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice were also purchased from The Jackson Laboratory. Rag2+/−/− mice (B6.129S6-Rag2−/−mTim/Tim) were purchased from Taconic Farms. All of the experiments described were performed with males between 8 and 10 wk age. Animals were sacrificed according to guidelines of the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. Experimental infection with mouse-adapted influenza A/X-31 (H3N2) virus
was achieved by exposing the mice to aerosolized virus \(10^7\) virus particles/12 ml PBS for 30 min) in an infection chamber (Glass-Col model A4212) unless otherwise stated. For virus titration, the lungs were extracted, homogenized in PBS-gelatin (0.1%), and frozen in dry ice ethanol for preservation. Virus titers were determined by plaque assays in Madin–Darby canine kidney cells, as described elsewhere. To visualize plaques, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100. Cells were stained with rabbit anti-nuclear protein (NP) Abs and HRP-conjugated goat anti-rabbit IgG and then stained with TrueBlue peroxidase substrate (Kirkegaard & Perry Laboratories).

**Influenza A/Puerto Rico/8/1934 (PR8) (H1N1) and A/X-31 (H3N2)** were propagated in 10-d-old embryonated chicken eggs at 37°C. Recombinant influenza virus lacking the IFN-antagonist protein nonstructural protein 1 (ΔNS1) virus has been previously described (17) and was propagated in 8-d-old embryonated chicken eggs at 37°C. PR8-OT-I virus has been previously described (18) and was grown in 10-d-old embryonated chicken eggs at 37°C.

**Quantitative RT-PCR analysis**

For quantitative RT-PCR (qRT-PCR) analysis, RNA was isolated from lung tissue or bone marrow flushed from femur and tibiae by TRIZol (Invitrogen) following the manufacturer’s instructions. qRT-PCR was performed using 100 ng sample RNA and SYBR Green (Roche) in an Applied Biosystems Prism 7900HT instrument following the manufacturer’s instructions.

**OT-I T cell proliferation assay**

Mice were infected with PRS-OT-I virus as described above. Two hours postinfection mice were adoptively transferred with 2 \times 10^5 T cells isolated by negative selection from spleens of naive Rag2/OT-I mice using magnetic beads and specific Abs (Miltenyi Biotec). Before adoptive transfer, cells were labeled with 2 mM CFSE. Cell transfer was accomplished by i.v. injection of cells via retro-orbital sinus. Four days postinfection mice were sacrificed and OVA-specific T cell proliferation was analyzed by flow cytometry. The percentage of cells divided and the proliferation index were calculated using the FlowJo software proliferation platform (Tree Star).

**In vivo CTL assay**

Mice were infected with PRS-OT-I virus as described. Eight days postinfection, mice were adoptively transferred with splenocytes from congenic donor naive mice pulsed with OVA-specific H2-Kb–restricted SIINFEKL peptide or an irrelevant peptide. Target cells pulsed with SIINFEKL peptide were labeled with 0.25 mM CFSE. Sixteen hours after adoptive transfer mice were sacrificed and specific killing of target cells in spleen was analyzed by flow cytometry as previously described (19).

**Irradiation and bone marrow transplantation**

Eight-week-old recipient mice were lethally irradiated with 1100 rad delivered in two doses of 550 rad each, 4 h apart. Mice were irradiated i.v. via the retro-orbital sinus 2 h after the second irradiation with 4 \times 10^6 bone marrow cells obtained from wt and p53−/− congenic donor mice (1:1). After transplantation, mice were maintained on antibiotics for 2 wk. Hematopoietic engraftment was analyzed by measurement of blood chimerism 4 wk after transplantation by flow cytometry. Experiments were performed 6 wk after transplantation.

**Cell preparation and flow cytometry**

Single-cell suspensions were obtained from lungs cut into small fragments and digested for 45 min at 37°C with collagenase D (2 mg/ml) (Roche) in RPMI 1640. Single-cell suspensions were obtained from mLNs by mechanical disruption without enzymatic digestion. mLN tissue fragments and digested lungs were further disrupted by passage through a 70-μm nylon cell strainer (BD Biosciences). RBCs were lysed with BD Pharm Lyse buffer (BD Biosciences).

Fc receptors were blocked with CD16/CD32 Fc block Ab (BD Biosciences) followed by staining with fluorochrome-conjugated Abs. Fluorochrome-labeled anti-CD68 (GL1), anti-CD103 (biotin) (2E7), anti-CD8 (53-6.7), and PerCP-Cy5.5-conjugated streptavidin were from eBiosciences. Anti-CD11b (M1/70), anti-Ly6C (AL-21), and anti-CD11c (HL3) were from BD Biosciences. Anti-CD11b (FAB1124F), anti-B220 (FAB1217P), and anti-CD4 (FAB554F) were from R&D Systems. Anti-MHC class II and anti-Ly6C were from Miltenyi Biotec. Anti-MHC class II (M5/114.15.2), anti-CD11c (17A2), anti-F4/80 (BMS), and anti-CD45.1 (A20), and anti-CD45.2 (104) were from BioLegend. H-2Kb SIINFEKL pentamer was purchased from ProImmune. Labeling of IAV-specific dendritic cells (DCs) was accomplished by fixation and permeabilization of single-cell suspensions from mLNs using Cytotox/Cytoperm (BD Biosciences) followed by staining with FITC-conjugated anti-IAV NP Ab (Abcam). A FACScalibur or LSR II instrument (BD Biosciences) was used for flow cytometry. Analysis of data was performed with FlowJo software (Tree Star).

**Influenza virus-specific Ab ELISA**

Serum was collected from either naive mice or influenza virus–infected mice on day 9 postinfection and examined for influenza virus-specific Ab responses by ELISA. Briefly, 96-well plates were coated overnight at 4°C with 100 μl (10^5 PFU) of heat-inactivated (35°C) A/X-31 (H3N2) influenza virus. The plates were washed twice with PBS supplemented with 0.05% Tween 20 (PBST) and blocked with 100 μl 2% BSA in PBST for 1 h at room temperature. After washing the plates with PBST, 100 μl diluted serum was added to each well, in duplicate, and incubated for 2 h at room temperature. Bound Abs were detected by the incubation of HRP-conjugated anti-mouse IgG1 (1:1000; BD Pharmingen), IgG2a (R19-15) (1:1000; BD Biosciences), and IgG2b (LO-MG2b) (1:1000; Southern-Biotech) Abs at room temperature. After 1 h, the plates were washed with PBST, and 100 μl 3.3',5.5'-tetramethylbenzidine substrate solution (R&D Systems) was added into each well and incubated for 30 min. The enzyme reaction was stopped by adding 100 μl 2 N H2SO4 and OD values were determined at 450 nm using a plate reader (SpectraMax 384 Plus UV-VIS; MDS Analytical Technologies).

**Statistical analysis**

Statistical significance was evaluated using a two-tailed Student t test or Mann–Whitney nonparametric tests, as described for the individual experiments. A p value of <0.05 was considered statistically significant.

**Results**

**p53 absence causes delayed pulmonary antiviral gene expression after IAV infection**

To investigate the influence of the p53 transcriptional program on antiviral responses to IAV, we used qRT-PCR to study IFN-stimulated genes and cytokine profiles in the lungs of wt and p53−/− mice challenged with aerosolized IAV (A/X-31 [H3N2]). We observed a significantly higher expression of type I ISGs, including ISG54, OAS1, and IRF9, at early time points postinfection (day 2) in wt mice infected with IAV compared with p53−/− mice (Fig. 1A). These results suggested that p53 promotes the establishment of an IFN-dependent antiviral state in infected lungs. Consistent with this conclusion, wt mice also produced significantly higher amounts of pulmonary IFN-α in response to both A/X-31 (H3N2) and PR8 (H1N1) virus, as assessed by ELISA (data not shown). Moreover, we observed that p53−/− mice exhibited decreased expression of proinflammatory chemokines, including MCP-1, IFN-γ–induced protein of 10 kDa (IP-10), MIP-1α, and MIP-1β, at day 2 postinfection (Fig. 1B). This observation was confirmed at the protein level by analysis of chemokine expression in lungs of infected mice using multiplex ELISA (Supplemental Fig. 1). Whereas wt mice displayed significantly higher mRNA expression levels of proinflammatory chemokines and antiviral genes at day 2 postinfection, p53−/− mice exhibited a delayed but significant increase in mRNA transcripts at day 3 postinfection (Fig. 1C). To explore whether the known ability of p53 to inhibit viral replication (9, 20) contributed to the observed differences in chemokine kinetics, we evaluated the mRNA levels of IAV NP and nonstructural protein 1 (NS1) in the lungs of wt and p53−/− infected mice. Fig. 1C shows that whereas viral gene expression was similar in wt and p53−/− mice at day 2 postinfection, it was significantly higher in p53−/− mice at day 3 postinfection. Taken together, these results suggest that whereas the rapid innate immune response observed in wt mice was able to maintain low viral titers, in the absence of functional p53, viral titers peaked at day 3, provoking a later proinflammatory response (Fig. 1).
p53 enhances viral clearance and alleviates IAV-induced disease

To evaluate the biological consequences of p53 absence on IAV-induced disease, we analyzed viral titers in lung homogenates from wt and p53−/− IAV-infected mice by plaque assay. Consistent with our qRT-PCR data, viral titers were significantly higher in p53−/− compared with wt mice at day 3 postinfection (Fig. 2A). Moreover, whereas wt mice were able to clear virus from lungs by day 7 postinfection, there was still detectable virus in the lungs of p53−/− mice at this time point (Fig. 2A). These results suggested that the delayed expression of ISGs and chemokines in p53−/− mice impairs their ability to effectively clear virus from the lungs.

To determine the influence of p53 on IAV-associated pathogenesis, we infected wt and p53−/− mice with X-31 and evaluated virus-associated weight loss and pulmonary pathology. We observed that weight loss after X-31 infection was significantly greater in p53−/− mice, which lost 20% of their initial body weight (Fig. 2B). Survival of p53−/− mice was also significantly decreased compared with wt mice (Fig. 2C). These results suggested that the delayed expression of ISGs and chemokines in p53−/− mice impairs their ability to effectively clear virus from the lungs.
weight (Fig. 2B). These results indicated that the absence of p53 resulted in a more severe IAV-induced morbidity. To rule out viral strain-dependent effects and to test the effect of p53 absence in response to highly pathogenic IAV, we infected wt and p53\textsuperscript{−/−} mice with PR8 and evaluated survival in response to infection. As shown in Fig. 2C, p53 deficiency significantly increased mortality in response to PR8 challenge, with only 14.3% of p53\textsuperscript{−/−} mice surviving the infection compared with 42.8% of wt mice (Fig. 2C).

Moreover, in agreement with a delayed inflammatory response in the absence of p53, lung sections revealed a robust leukocyte infiltration in wt mice at day 3 postinfection, whereas p53\textsuperscript{−/−} mice did not show any signs of infiltration of immune cells at this time point (Supplemental Fig. 2). Conversely, p53\textsuperscript{−/−} mice exhibited a massive delayed leukocyte infiltration by day 6, as well as severe necrosis of the bronchial epithelium. These observations suggested that the absence of p53 delays the initial innate immune response to IAV, leading to increased IAV-induced disease.

**Defective immune response in p53\textsuperscript{−/−} mice impairs bone marrow antiviral instruction and pulmonary monocyte infiltration**

Previous studies have demonstrated that lung cytokines produced during IAV infection promote an antiviral state in the sterile, noninfected bone marrow and enhance migration of leukocytes to the infected lung (21). Thus, to evaluate whether p53 status also affected the establishment of an antiviral state in the bone marrow, we evaluated the expression of antiviral genes and proinflammatory chemokines in wt and p53\textsuperscript{−/−} mice with PR8 and evaluated survival in response to infection. As shown in Fig. 2C, p53 deficiency significantly increased mortality in response to PR8 challenge, with only 14.3% of p53\textsuperscript{−/−} mice surviving the infection compared with 42.8% of wt mice (Fig. 2C).

In the lungs, monocytes give rise to the two major populations of myeloid DCs, CD11c\textsuperscript{+} DCs and CD11b\textsuperscript{+} DCs (22, 23). To assess the influence of p53 on the immunoarchitecture of pulmonary DC subsets, we evaluated the kinetics of CD11c\textsuperscript{+} DCs and CD11b\textsuperscript{+} DCs in IAV-infected wt and p53\textsuperscript{−/−} mice. Because CD11c expression does not distinguish DCs and macrophages in the lungs (22), we gated DCs based on the expression of CD11c, MHC class II, and side scatter profiles, as previously described (24). We identified two main subsets of myeloid DCs, consisting of CD11c\textsuperscript{+} MHC class II\textsuperscript{+} CD11b\textsuperscript{+} CD103\textsuperscript{−} DCs (CD11b\textsuperscript{+} DCs) and CD11c\textsuperscript{+} MHC class II\textsuperscript{+} CD11b\textsuperscript{−} CD103\textsuperscript{−} CD200\textsuperscript{−} DCs (CD103\textsuperscript{+} DCs), which displayed similar steady-state frequencies in uninfected wt and p53\textsuperscript{−/−} mice (Fig. 4B).

When we compared the kinetics of the response of these DC subsets to IAV infection, we observed a very different dynamic profile for both DC populations. CD11b\textsuperscript{+} DCs accumulated in the lungs of both wt and p53\textsuperscript{−/−} infected mice, whereas CD103\textsuperscript{+} DC numbers decreased early in the course of infection (Fig. 4B). These differences in dynamics have been previously reported and are a consequence of greater numbers of CD103\textsuperscript{+} DCs migrating to the draining mLNs at early time points postinfection (25) as well as pulmonary infiltration of CD11b\textsuperscript{+} monocyte-derived DCs (moDCs) (25, 26). Of note, whereas pulmonary CD11b\textsuperscript{+} DCs accumulated to a greater extent in infected wt mice compared with p53\textsuperscript{−/−} mice, CD103\textsuperscript{+} DCs decreased to a greater extent in wt mice (Fig. 4B). These differences are consistent with our observations of reduced monocyte infiltration in p53\textsuperscript{−/−} mice as a result of a defective inflammatory response.

To evaluate whether the observed differences in DC kinetics influenced DC migration from lungs to mLNs, we analyzed the presence of migratory lung DCs in the mLNs in wt and p53\textsuperscript{−/−} mice following IAV challenge. Migratory DCs were identified in the mLNs as a population of CD11c\textsuperscript{med}MHC class II\textsuperscript{high} cells, as previously described (25). This cell population contained the two main subsets of lung DCs, CD11b\textsuperscript{+} and CD103\textsuperscript{+} DCs. In IAV-infected wt mice, the total accumulation of CD11c\textsuperscript{+} DCs in mLNs was significantly higher than in their p53\textsuperscript{−/−} counterparts (Fig. 4C). Further analysis revealed that CD103\textsuperscript{+} DCs arrived at
mLNs primarily at early time points postinfection (between days 3 and 5), whereas CD11b+ DCs accumulated in mLNs at later time points, in agreement with previous reports (25, 27). There was significantly less migration of CD103+ DCs in p53−/− mice infected with IAV for 6 d, which otherwise is prevented in the presence of this viral gene NS1, which in vitro prevents DC maturation (28). Supplemental Fig. 3 shows that after NS1 infection, wt BMDCs in the lungs, reduced DC migration to the mLNs, and impaired DC activation.

Effective DC migration to draining lymph nodes relies on their activation status, which in the context of viral infection depends to a great extent on type I IFN signaling (28, 29). To evaluate DC activation defects caused by the absence of p53, we quantified the expression of the activation marker CD86 in pulmonary DC subsets in mock-infected wt and p53−/− mice infected with IAV for 6 d. CD86 expression was significantly higher in CD11b+DCs in the presence of wt p53 (Supplemental Fig. 3). In wt and p53−/− mice infected with IAV, DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling. Taken together, our findings indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling (28). These results indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling. Taken together, our findings indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling (28). These results indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling. Taken together, our findings indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling.

p53−/− mice show defective antiviral T cell responses

DCs that capture Ags in the peripheral tissues migrate to the mLNs where they activate specific T cell immunity (30). To evaluate whether the differences in DC activation and migration observed in wt and p53−/− mice infected with IAV and p33−/− BMDCs with wt IAV, which prevented DC maturation in both wt and p53−/− BMDCs (Supplemental Fig. 3). These results indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling. Taken together, our findings indicated that virus-induced DC activation was impaired in the absence of p53 and that this effect depended at least to some extent on the ability of p53 to enhance type I IFN signaling (28). Supplemental Fig. 3 shows that after ∆NS1 infection, wt
BMDCs were more efficient inducers of T cell proliferation than were p53\(^{-/-}\) BMDCs, suggesting that the defective activation observed in p53\(^{-/-}\) BMDCs inhibited their ability to induce efficient T cell activation. To evaluate whether the differences in T cell activation observed in vitro influenced anti-IAV specific T cell immunity in vivo, we compared the cytolytic ability of IAV-specific CD8\(^{+}\) T cells from wt and p53\(^{-/-}\) mice. To do so, we infected mice with recombinant PR8 harboring MHC class I H-2K\(^{b}\)-restricted OVA-derived peptide (SIINFEKL), designated PR8-OT-I. At 8 d postinfection, mice were adoptively transferred with splenocytes isolated from naive wt donor mice that were pulsed with SIINFEKL peptide or an irrelevant peptide. Cells loaded with SIINFEKL or irrelevant peptide were labeled with different concentrations of the green dye CFSE to allow their identification by flow cytometry (see Materials and Methods). We observed that specific killing of cells expressing the SIINFEKL peptide was significantly higher in wt mice compared with p53\(^{-/-}\) mice, indicating that the absence of p53 function reduces the efficacy of anti-IAV-specific T cell immunity (Fig. 5A).

To evaluate the influence of p53 status on the capability of DCs to drive antiviral CD8\(^{+}\) T cell responses, we infected wt and p53\(^{-/-}\) mice with PR8-OT-I virus and evaluated OVA-specific CD8\(^{+}\) T cell proliferation. To do so, mice were adoptively transferred with CFSE-labeled T cells isolated from transgenic Rag2/OT-I mice (harboring OVA-specific CD8\(^{+}\) T cells) on the same day of PR8-OT-I challenge. Four days postinfection, mice were sacrificed and CFSE\(^{+}\) OT-I-specific CD8\(^{+}\) T cells were identified in the mLNs by flow cytometry. We observed that the absence of p53 significantly inhibited OVA-specific CD8\(^{+}\) T cell proliferation (Fig. 5B), suggesting that the defects in antiviral innate immunity associated with the absence of p53 ultimately reduced the effectiveness of adaptive T cell immunity. We observed no significant differences in IgG1, IgG2a, and IgG2b serum levels between wt and p53\(^{-/-}\) mice (Fig. 5C), indicating that p53-dependent enhancement of proinflammatory responses influences T cell immunity but not Ab responses to IAV.

**p53 deficiency in the nonhematopoietic compartment impairs the antiviral response**

To dissect the mechanisms by which p53 participates in the antiviral immune response in vivo, we next sought to determine the specific contribution of p53 to the antiviral response in the nonhematopoietic versus the hematopoietic compartment. To do so, we performed mixed bone marrow transplants (50% wt CD45.1\(^{+}\)/50% p53\(^{-/-}\) CD45.2\(^{+}\)) in lethally irradiated mice on a wt or p53\(^{-/-}\) background. Of note, analysis of chimerism 4 wk after transplant indicated that p53\(^{-/-}\) bone marrow engrafted significantly better than did wt bone marrow (Fig. 6A), presumably due to an enhanced resistance to apoptosis. This dominance of p53\(^{-/-}\) engraftment was even higher in p53\(^{-/-}\) recipients, in which p53\(^{-/-}\) hematopoietic cells accounted for up to 80% of blood leukocytes (Fig. 6A). To determine whether the observed defects in DC function associated with the absence of p53 resided in the nonhematopoietic or hematopoietic compartments, we next evaluated...
the migratory properties of IAV-specific DCs in mixed bone marrow chimeric mice following infection with X-31 virus. To do so, we infected mice with X-31 virus and evaluated the presence of IAV Ag-specific DCs in the mLNs between days 0 and 6 postinfection. We observed that the arrival of Ag-bearing DCs was significantly higher in wt compared with p53−/− recipients, regardless of their phenotype (CD45.1+ [wt] or CD45.2+ [p53−/−]) (Fig. 6B,6C). We also adoptively transferred chimeric mice with CFSE-labeled OT-I T cells after PR8-OT-I challenge. Four days postinfection, mice were sacrificed and mLNs extracted and prepared for flow cytometry. Left panel shows T cell proliferation as assessed by CFSE dilution. The red line shows the histogram profiles, and the blue lines show the peaks of proliferation detected using the FlowJo proliferation platform. The right panel indicates percentage of cells divided from the parent population of CD8+ T cells. *p < 0.05 as assessed by Student t test.

Discussion

Our present study demonstrates that in the innate immune response to pulmonary IAV infection, the absence of p53 severely impairs early expression of antiviral genes and proinflammatory chemokines, including p53-direct target genes such as IRF9 (9) and MCP-1 (15). These findings are consistent with a growing body of evidence indicating that the p53-dependent transcriptional program, in addition to its known influence on apoptosis and cell cycle arrest, enhances the expression of key regulators of innate immunity pathways (9, 10, 12, 14). Indeed, a recent study revealed that innate immune responses in the metazoan Caenorhabditis elegans is dependent on p53 function (31). All of these findings suggest that the highly conserved nature of p53 among eukaryotes may rely more on its role in host immunity than in its functions as a tumor suppressor gene.

Previous studies have indicated that p53-dependent apoptosis plays a role in inhibiting viral replication of several viruses in vitro, including IAV (20). However, the role of apoptosis in the pathogenesis of and the host response to IAV infection in vivo is not fully understood. Our study demonstrates that p53 promotes the expression of antiviral genes and proinflammatory chemokines in the bone marrow, which has been previously shown to contribute to the establishment of a systemic antiviral state (21). Replication of low pathogenic IAV in mice is restricted to the respiratory tract (21, 32), and thus bone marrow cells are not exposed to the virus. This evidence indicates that the observed p53-dependent effects
on antiviral gene induction in the bone marrow cannot be dependent on its ability to promote virus-induced apoptosis. Thus, our studies suggest that the role of p53 in the antiviral response to IAV in vivo is dependent on its ability to transcriptionally up-regulate pulmonary cytokine production rather than on its pro-apoptotic functions.

The defective cytokine responses in lungs and bone marrow observed in p53−/− mice were associated with reduced pulmonary monocyte infiltration in comparison with their wt counterparts. These findings are consistent with evidence that production of proinflammatory mediators is necessary for leukocyte infiltration at inflammation sites (8, 33, 34). The recent discovery that MCP-1, a known monocyte chemoattractant, is a bona fide p53 target gene (15) also raises the possibility that direct effects of p53 on MCP-1 expression may account to some extent for the differences observed in pulmonary monocyte infiltration. In fact, recent studies indicate that mice knockout for the MCP-1 receptor, CCR2, show defective monocyte migration to inflammation sites (Supplemental Fig. 4).

A slight decrease in splenic CD8+ T cells cellularity in bone marrow and spleens of 9-wk-old naive mice tirely normal immune system as assessed by analysis of leukocyte and 10 wk age in the C57BL/6 background, which showed an en- older than 15 wk (36). Of note, we used male mice between 8 and CBA/N aged mice, an effect that was particularly obvious in mice MCP-1 have been previously shown to regulate peripheral CD8+ T cell maintenance and bone marrow monocyte emigration (35, 37, 38). Further studies are required to evaluate the influence of aging on the ability of p53 to enhance host antiviral immunity.

Pulmonary IAV infection has been shown to promote infiltration of blood monocytes that differentiate into inflammatory CD11b+ monocytes both in lungs (23, 26) and mLNs (39), and these CD11b+ DCs have full potential to present microbial Ags and stimulate T cell responses (39, 40). Moreover, pulmonary accumulation of monocytes and CD11b+ DCs has been shown to be essential to ensure viral clearance postinfection with respiratory viruses by both T cell-dependent and independent mechanisms (21, 26). We observed that p53 absence significantly impaired monocyte accumulation in the lungs of infected mice, causing increased viral replication and delayed viral clearance. These findings strongly suggest that a rapid production of IFN-stimulated genes and cytokines during the early phase of IAV infection is needed for an effective control of viral replication, and that p53-dependent transcription is required for this effect.

p53 absence was associated with defective IAV-specific T cell immunity. These findings further suggest that defects associated with the innate antiviral response also ultimately affect the effectiveness of specific T cell antiviral immunity. A previous study by Grayson et al. (41) reported that p53 was not involved in antiviral T cell responses to lymphocytic choriomeningitis virus. However, note that IAV and lymphocytic choriomeningitis virus induce very different inflammatory responses. Whereas IAV induces inflammation through pathways influenced by p53 status such as RIG-I and TRLR3 (6, 42), lymphocytic choriomeningitis virus has been shown to primarily engage the TLR2-MyD88/MyD pathway (43). In any case, further studies will be required to evaluate the extent to which the impact of p53 on the immune response to IAV may be translatable to other viral infections.

Because p53 status affected CTL- but not IAV-specific Ab responses, it is conceivable that helper CD4+ T cell function is not affected by p53 status. This hypothesis is in agreement with recent reports indicating that innate immune responses to several viruses modulate CTL activity without the involvement of CD4+ T cells or cognate Ag stimulation (7, 37, 44, 45).

Our findings demonstrate that the antiviral effects exerted by p53 rely primarily on its functions in nonhematopoietic cells and are likely dependent on its ability to promote more rapid pro-inflammatory and antiviral gene expression. Of note, mixed bone marrow chimeric mice showed significantly higher engraftment of p53−/− hematopoietic cells compared with wt cells. Although further experiments will be needed to determine the basis for such differences, we hypothesize that apoptotic defects associated with p53 loss of function may provide an advantage in adaptation to the host environment (46, 47). Even though bone marrow-derived p53−/− cells outnumbered wt cells in both wt and p53−/− recipients, we still observed that the absence of p53 in nonhematopoietic cells inhibited migration of DCs carrying IAV Ags to the draining lymph nodes and prevented efficient T cell immunity. These findings strongly suggest that antiviral gene expression and cytokine production in nonhematopoietic cells infected with IAV play a major role in the recruitment of inflammatory cells, the activation of DC functions, and the onset of specific T cell immunity. This may help to explain the multifunctional role of cytokines such as type I IFNs and chemokines such as RANTES or MCP-1, which have been recently shown to fine-tune different aspects of the antiviral adaptive immune response, such as T cell cross-priming (38), peripheral CD8+ T cell maintenance (37), and Th2 polarization (48).

Our present studies strongly support the concept that enhancement of p53 functions as a host resistance factor against IAV infection may be used as a host-targeted therapeutic strategy to develop anti-IAV antiviral therapies and vaccine adjuvants. Indeed, recent studies indicate that the enforcement of innate immune responses through the use of TLR ligands serves to enhance IAV-specific Ab responses (4). These studies strongly suggest that modulation of innate immunity can increase the magnitude and persistence of adaptive immunity. A potential advantage of p53-based therapies in contrast to TLR-directed strategies is that p53 is expressed by both the epithelial cells primarily infected with IAV and the immune cell subsets involved in the antiviral response.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Cytokines from fresh lung homogenates of wt and p53-/- mice infected with X31 virus at the indicated time points assessed by multiplex ELISA. Whole lungs were ground in 1.8 ml 0.1% gelatin/PBS. Cytokine concentration in the lungs and serum was analyzed by 12 plex multiplex ELISA (Millipore). Mock (M)-infected animals were used as controls.
Supplementary Figure 2. Hematoxylin-Eosin staining of formalin-fixed lung tissue samples harvested from mice infected with IAV at the indicated time points. Lungs embedded in paraffin wax sections (5µM) were cut and stained with hematoxylin and then counterstained with eosin (H&E). The sections were analyzed for parameters indicative of pulmonary inflammation, the degree of interstitial leukocyte infiltration, and the presence of bronchial epithelial necrosis. Two investigators, who were blinded to the identity of each histological specimen, scored each section. Arrows indicate areas of leukocyte infiltration and asterisks indicate bronchial epithelial necrosis.
Supplementary Figure 3. Analysis of DC maturation in bone marrow-derived DCs (BMDCs). A, Flow cytometry analysis of CD86 expression in wt (black bars) and p53-/- (white bars) lung DC subsets in naïve mice (Mock) as well as mice infected with IAV for 6 days. Expression of CD86 is represented as Mean Fluorescence Intensity in the FL-1 channel. Asterisk denote statistical significance (p<0.05) as assessed by student’s T test. Results are represented as Mean ± SEM and are representative of three independent experiments. B, Flow cytometry analysis of MHC class II expression in bone marrow derived DCs (BMDCs) from wt (black bars) and p53-/- mice (white bars). Bone marrow hematopoietic progenitor cells were isolated as described in the Methods section and cultured with 40 ng of GM-CSF for 4 days. BMDCs were infected with PR8 or ΔNS1 virus at a multiplicity of infection (MOI) of 2 for 12 hours. BMDCs were defined as CD11c+ CD11b+ cells and MCH II expression was evaluated by Mean Fluorescence Intensity in the FL-2 channel. Results are represented as Mean ± SEM and are representative of three independent experiments. C, In vitro analysis of DC-induced T cell proliferation. BMDCs were obtained and infected with ΔNS1 virus as described. Isolation of naïve T cells from spleens of BALB/c mice was accomplished by negative selection of antigen presenting cells and B cells using specific antibodies. Infected BMDCs and T cells at the indicated ratios were co-cultured in 96-well plates for 48 h. T cell proliferation was assessed by incubation with Cell Proliferation Reagent WST-1 (Roche) and analysis of absorbance at 450 nm.
Supplementary Figure 4. Analysis of leukocyte cellularity in spleens and bone marrow of naïve wt and p53-/- mice. Single cell suspensions were obtained by mechanical disruption of tissues and use of a 70 mm cell strainer. Flow cytometry analysis was performed as described in the Materials and Methods section.