**Priority Report**

**Trp53 Inactivation in the Tumor Microenvironment Promotes Tumor Progression by Expanding the Immunosuppressive Lymphoid-like Stromal Network**

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**Abstract**

Inactivation of the tumor suppressor p53 through somatic mutations, observed in 50% of human cancers, is one of the leading causes of tumorigenesis. Clinical and experimental evidence also reveals that p53 mutations sometimes occur in tumor-associated fibroblasts, which correlate with an increased rate of metastases and poor prognosis, suggesting that p53 dysfunction in the tumor microenvironment (TME) favors tumor establishment and progression. To understand the impact of p53 inactivation in the TME in tumor progression, we compared the growth of subcutaneously inoculated B16F1 melanoma in p53null and wild-type (WT) mice. Interestingly, tumor growth in p53null mice was greatly accelerated, correlating with marked increases in CD11b⁺Gr-1⁺ myeloid-derived suppressor cells (MDSC), FoxP3⁺ regulatory T cells, and a loss of effector function, compared with those in WT mice. This augmented immunotolerant TME in p53null mice was associated with a marked expansion of a specialized stromal network in the tumor and spleen. These stromal cells expressed markers of fibroblastic reticular cells of lymphoid organs and were readily expanded in culture from p53null, but not WT, mice. They produced high levels of inflammatory cytokines/chemokines and immunosuppressive molecules, thereby enhancing MDSC differentiation. Furthermore, they significantly accelerated tumor progression in WT mice when co-injected with B16F1. Together, our results show that tumor-stroma interaction in hosts with dysfunctional p53 exacerbates immunosuppression by expanding the lymphoid-like stromal network that enhances MDSC differentiation and tumor progression. Cancer Res; 73(6); 1–8. ©2012 AACR.

**Introduction**

The tumor suppressor p53 (Trp53, p53) inhibits tumorigenesis by inducing apoptosis and senescence of aberrant cells (1–3). Trp53 somatic mutations, observed in about 50% of human cancers and some autoimmune pathologic tissues, are major causes of tumorigenesis (1–4). Besides p53 mutations, inactivation of the p53 pathway happens under various physiologic and pathologic conditions, including viral infection, oncogene activation, and aging (1–3). Furthermore, p53 inactivation also occurs in other components of the tumor microenvironment (TME), including cancer-associated fibroblasts (CAF), which is associated with an increased rate of metastases and poor prognosis (5, 6). The TME, a complex cellular and molecular network of immune cells, stromal cells, extracellular matrix, and cytokines/chemokines, is crucial in immunomodulation that impacts tumor development, progression, and metastases (7–9). Studies by our laboratory and others showed that innate and adaptive immunities in p53null hosts are skewed toward proinflammation (10, 11). However, it is largely unexplored whether p53 inactivation in the TME alters the immunologic milieu, which exacerbates tumor progression.

Here, we hypothesized that p53 inactivation in the TME favors tumor establishment and progression. By using B16F1 melanoma, which maintains functional p53, we showed that their growth in p53null mice upon subcutaneously (s.c.) inoculation was greatly accelerated, associated with an expansion of the lymphoid-like stromal network, compared with that in wild-type (WT) mice. The p53null stroma promoted tumor progression by expressing proinflammatory cytokines/chemokines, arginase I, and inducible nitric oxide synthase (iNOS) and by augmenting the accumulation of myeloid-derived suppressor cells (MDSC).

**Materials and Methods**

**Mice**

Trp53null (B6.129S2-Trp53tm1J) and C57BL/6 mice, purchased from the Jackson Laboratories, were maintained under specific pathogen-free conditions in the Louisiana State University Health Sciences Center (LSUHSC) animal care facility following approved LSUHSC-Institutional Animal Care and Use Committee protocols.

**Tumor inoculation**

B16F1, purchased from American Type Culture Collection, was maintained in Dulbecco’s Modified Eagle’s Medium...
(DMEM; Invitrogen) with 10% FBS and injected subcutaneously at $2 \times 10^5$ per mouse. Tumors were measured every other day and calculated as: volume = (length × width) × (length + width)/2.

**Histology and fluorescent immunohistochemistry**

Hematoxylin and eosin stain histology and immunohistochemistry (IHC) were conducted following standard protocols and viewed by a licensed pathologist. Antibody dilutions for IHC were: ER-TR7 (5 μg/mL), Lyve-1 (5 μg/mL), α-smooth muscle actin (1 μg/mL, Abcam), Gp38 (5 μg/mL), CD31 (5 μg/mL), CD11b (1 μg/mL, Biolegend), and Alexa Fluor-labeled secondary antibodies (2 μg/mL, Invitrogen). Vascular and stroma quantification was conducted using Slidebook software (Intelligent Imaging Innovations).

**Flow cytometry**

All antibodies were purchased from BD Biosciences unless otherwise specified. Tumors were cut into 1–2 mm$^3$ pieces and digested with liberase (0.5 wU/mL, Roche) and DNase I (100 μg/mL, Sigma) in DMEM at 37°C for 30 minutes. MDSCs were enriched using EasySep mouse CD11b positive selection kits (Stemcell Technologies). Regulatory T cells (Treg) were analyzed as CD4$^+$CD25$^{hi}$Foxp3$^+$ (ebioscience) cells. The effector cell function was determined via intracellular cytokine staining following standard protocols. Fluorescence-activated cell sorting acquisition was conducted using a FACSCalibur and analyzed using FlowJo (Tree Star Inc.).

**Splenic stromal cell isolation and expansion**

Spleens, cut and digested with liberase and DNase I at 37°C for 30 minutes, were subsequently cultured at $5 \times 10^5$/mL in DMEM with 10% FBS for 3–5 days. The CD45$^+$ population from adherent cells was enriched by depletion of CD11b$^+$-cells to >95% CD45$^+$ and was passaged twice a week. Their responses to B16F1-soluble factors were evaluated after a 6-hour exposure to B16F1-conditioned medium (50%). Their effects on tumor growth were evaluated by injection of $1 \times 10^5$ splenic stromal cell (SPSC) alone or mixed with $2 \times 10^5$ B16F1 s.c. to WT mice.

**Real-time reverse transcriptase-PCR**

Total RNA was harvested using Qiagen RNeasy Kits. Reverse transcription was conducted with 1 μg total RNA, followed by real-time PCR using PrimeTime qPCR primers (Integrated DNA Technologies) and a BioRad CFX96 (BioRad Life Science Research).

**Bone marrow-derived MDSCs**

Bone marrow-derived MDSCs (BM-MDSC) were cultured in DMEM containing 10% FBS, 100 ng/mL granulocyte colony-stimulating factor (G-CSF), and 10 ng/mL interleukin (IL)-6 (PeproTech) at $1 \times 10^6$/mL with or without SPSCs for 4 days.

**5-Bromodeoxyuridine labeling**

Tumor-bearing mice were injected intraperitoneally with 1 mg of bromodeoxyuridine (BrdUrd). BrdUrd$^+$ cells were analyzed via intracellular staining 24 hours postinjection (BD Biosciences). Cultured BM-MDSCs were labeled with 10 μmol/L BrdUrd for 2 hours.

**Multiplex cytokine assays**

Mouse serum was analyzed using the Millipore Mouse Cytokine/Chemokine-Premixed kit (MPXMCYTO70KPMX32) using Bio-Plex Manager (Bio-Rad Laboratories).

**Statistical analysis**

The differences between genotypes and/or treatments were analyzed via 2-tailed Student t tests using SigmaPlot (Systat Software Inc.). Statistical significance was set at $P < 0.05$.

**Results and Discussion**

*Tp53$^{null}$ hosts augment tolerogenic TME and promote tumor progression*

Previous studies suggest that p53 inactivation enhances inflammation (10–12). To investigate whether p53$^{null}$-mediated inflammation promotes tumor progression, we compared B16F1 progression in WT and p53$^{null}$ mice. As expected, B16F1 progressed more rapidly in p53$^{null}$ mice (Fig. 1A), associated with significant alterations in tumor-infiltrating leukocytes (CD4$^+$-TIL), a 50% reduction in TIL-CD8, and a reciprocal increase in CD11b$^+$ myeloid cells, compared with those in WT mice (Fig. 1B). These were in agreement with IHC results (Supplementary Fig. S1). Furthermore, IFN-γ- and IL-17A-producing effectors in p53$^{null}$-TILs were markedly suppressed despite a comparable percentage of TIL-CD4 and TNF-α producing cells between p53$^{null}$ and WT mice (Fig. 1B–D). It is noteworthy that the exacerbated immunotolerance in p53$^{null}$ mice was more profound in the TME, because the frequencies of effectors and Tregs in the spleen of WT and p53$^{null}$ mice were comparable (Supplementary Fig. S2). These results suggest that p53 inactivation in hosts TME favors tumor progression by augmenting immunotolerance.

**The accelerated tumor progression in p53$^{null}$ hosts is associated with an enhanced MDSC accumulation and expansion of lymphoid-like stromal network within the TME**

The CD11b$^+$ population usually comprises heterogeneous myeloid cells, including CD11b$^+$Gr-1$^+$ MDSCs, dendritic cells, granulocytes, and macrophages (13). Further analyses of B16F1-TILs CD11b$^+$ cells in WT and p53$^{null}$ mice showed that they were predominately MDSCs (TIL-MDSC) with comparable compositions of heterogeneous Ly6G$^+$Ly6C$^{mod/lo}$ granulocytic (G)-MDSCs and Ly6C$^+$Ly6G$^-$monocytic (M)-MDSCs (Fig. 2A and Supplementary Fig. S3A). Nevertheless, the absolute number of TIL-M-MDSCs and total TIL-MDSCs in p53$^{null}$ mice was significantly higher than that in WT mice (Fig. 2A). Functional analyses indicated that on a per cell basis, p53$^{null}$ TIL-MDSC showed a similar capacity in suppressing the proliferation of activated T cells to WT TIL-MDSC (Supplementary Fig. S3B), implying that the augmented immunosuppression in p53$^{null}$ mice mainly results from their increased MDSC number. *In vivo* BrdUrd labeling of proliferating cells showed that BrdUrd$^+$
TIL-MDSCs, but not TIL-CD11b^+ Gr-1^- cells, almost doubled in tumor-bearing p53null mice compared with those in WT counterparts (Fig. 2B), suggesting that p53 inactivation in the TME enhances the proliferation of MDSCs. Recent studies suggest that MDSCs in the TME augment angiogenesis. Pathologic examination of tumor specimens revealed that tumors growing in p53null, but not WT, mice appeared to be organized into isle-like structures with...
marked increases in vasculatures (Fig. 2C). Emerging evidence suggests that during inflammation and tumor progression, lymphatic vasculatures and fibroblasts form a network reminiscent of the specialized stroma of secondary lymphoid organs (14, 15). Analyses of B16F1 tumors showed marked increases in both blood endothelial cells (CD45^+CD31^+GP38^-Lyve-1^-) and lymphatic endothelial cells (LEC; CD45^-CD31^-GP38^-Lyve-1^-), associated with

Figure 2. The accelerated tumor progression in p53^null hosts is associated with an increase in MDSCs and expansion of lymphoid-like stromal network. A, TIL-MDSCs and subpopulations from WT and p53^null mice were analyzed (n = 5–10). B, proliferating TIL-MDSCs in tumor-bearing mice were analyzed as BrdUrd^+CD11b^+Gr-1^- cells 24 hours post-BrdUrd injection. C, representative tumor histology images from WT and p53^null mice. Scale bar, 100 mm. D, representative tumor IHC images from WT and p53^null mice revealing blood endothelial cells (LYVE-1^-Gp38^-CD31^-), LECs (LYVE-1^-Gp38^-CD31^-), FRCs (ER-TR7^-Gp38^-a-SMA^-), and CD11b^- cells. The relative area of CD31^- vasculatures and ER-TR7^-FRC-stroma was determined using a computer-assisted program. Data are mean ± SE (n = 3). *, significant difference (P < 0.05).
increased TIL-CD11b+ cells in p53null hosts (Fig. 2D). More strikingly, a fibroblast network, reminiscent of fibroblastic reticular cells (FRC) that are ER-TR7+α-SMA+ (α-smooth muscle actin)GP38+ (15), was expanded extensively in tumors from p53null mice compared with that from WT mice (Fig. 2D). These results suggest that p53 inactivation in the TME provides a permissive environment for angiogenesis and expansion of FRC-like network.

**B16F1 establishment in p53null hosts promotes systemic increases in proinflammatory cytokines, MDSCs, and stromal-network in the spleen**

To understand how p53null TME encourages MDSC proliferation/accumulation, we first examined host cytokine milieu as it provides essential signals for MDSC development (13). In WT tumor-bearing mice compared with nontumor-bearing mice, serum G-CSF and CXCL1 (keratinocyte chemoattractant) levels doubled and tripled, respectively (Fig. 3A). Likewise, serum G-CSF, CXCL1, and IL-6 in p53null tumor-bearing mice showed a 4- to 10-fold increases compared with nontumor-bearing mice (Fig. 3A). Further analysis of splenic MDSCs (SP-MDSC) in tumor-bearing WT and p53null mice showed that both M-MDSCs and G-MDSCs in the spleen of p53null mice were significantly higher because of their marked increases in proliferation, that is, BrdUrd+ SP-MDSCs (Fig. 3B and C). Further immunohistochemical analyses also confirmed a significant expansion of the FRC network in the spleen of tumor-bearing p53null mice compared with nontumor-bearing p53null mice and tumor-bearing WT mice (Fig. 3D). Thus, our results suggest that tumor-stroma interaction in the TME lacking functional p53 promotes inflammation, accumulation of MDSCs, and expansion of the FRC network.
Figure 4. *Trp53null* splenic fibroblastic stroma enhances MDSC differentiation and accelerates tumor progression in WT mice. A, a representative image (×400) and phenotypic analyses of CD45− SPSC from tumor-bearing *p53null* mice. Open lines indicate isotype controls. B, BM-MDSCs were differentiated in a 4-day culture with G-CSF and IL-6 with or without SPSCs. Their relative efficiency was normalized against that of WT BM-MDSCs without SPSCs, which was set as 100%. Proliferating (BrdUrd+) BM-MDSCs were examined on day 4 (*n* = 3). *, significant difference (*P* < 0.05) between groups with and without SPSCs; †, significant difference between WT and *p53null* BM-MDSCs. C, proinflammatory molecule expression in *p53null* SPSCs compared with those in MEF, as well as their alterations upon a 6-hour exposure to B16F1-conditioned medium, was determined via real-time RT-PCR (*n* = 3–5). D, the tumor size in WT mice that received 2 × 10⁵ B16F1 or 2 × 10⁵ B16F1 admixed with 1 × 10⁵ *p53null* SPSCs was compared (*n* = 5). *, significant difference (*P* < 0.05).
**Trp53**null splenic fibroblastic stroma enhances MDSC differentiation and markedly accelerates tumor progression in WT mice

Given the essential role of lymphoid FRC network in immune regulation (15) and the role of SP stroma in inducing regulatory dendritic cells (16), we postulated that **p53**null stroma augments MDSC development. Interestingly, splenic CD45- stromal cells, termed SPSCs, from tumor-bearing **p53**null, but not WT, mice were readily expanded in culture. Further analyses showed that SPSCs, differing from mouse embryonic fibroblasts (MEF; Supplementary Fig. S4), were CD106-CD54+GP388-Sca-1+broblastic lymphoid FRC-like cells (Fig. 4A). To assess the function of SPSCs in promoting MDSC differentiation under a cytokine milieu mimicking B16F1 tumor-bearing mice (Fig. 3A), we tested BM-MDSC differentiation in G-CSF + IL-6. Unlike G-CSF + GM-CSF (granulocyte macrophage-colony-stimulating factor; ref. 17), which predominantly induced M-MDSCs (Supplementary Fig. S5), G-CSF + IL-6 supported the differentiation of heterogeneous M-MDSCs and G-MDSCs, resembling the composition of TIL-MDSCs (Fig. 2A and Supplementary Fig. S3A). In the absence of SPSCs, comparable number of BM-MDSCs was derived from the bone marrow of WT and **p53**null cells (Fig. 4B). Strikingly, **p53**null SPSCs enhanced BM-MDSC differentiation of WT and **p53**null cells by 60% and 100%, respectively, mainly by enhancing their proliferation (Fig. 4B and Supplementary Fig. S6).

Further analyses of the cytokine/chemokine profile of **p53**null SPSCs via quantitative real-time RT-PCR showed that they expressed higher levels of proinflammatory cytokines/chemokines and immunosuppressive mediators, including IL-6, IL-10, CCL3, CCL21, Arg1, and iNOS, compared with **p53**null MEF (Fig. 4C). Moreover, exposure of SPSCs to B16F1 conditioned medium further enhanced the expression of CXCL1, CCL3, and CCL21 (Fig. 4D). To confirm that **p53**null stroma hastens tumor progression, we injected **p53**null SPSCs either alone or mixed with B16F1 to WT mice and showed that coinjection of SPSCs with B16F1 substantially accelerated tumor growth as early as day 8 postinjection (Fig. 4D). These results strongly suggest that the lymphoid-like stroma in **p53**null hosts is immunosuppressive and that tumor-stroma interaction exacerbates immunosuppression, augments MDSC development, and accelerates tumor progression.

Taken together, this study shows that **p53** inactivation in the TME promotes immunosuppression and tumor progression by expanding the lymphoid-like stromal network. The crucial role of the TME in tumor initiation and progression has been increasingly appreciated (7–9, 13). However, the immunologic consequence of **p53** inactivation in the TME involving tumor-stroma interaction has been unexplored. Early studies suggest that **p53** inactivation in CAFs enhances tumor progression via paracrine effects of CXCL12 (18). Our observed high-level CXCL12 expression in **p53**null SPSCs agrees with this notion. More importantly, our results further show that the **p53**null lymphoid-like stromal network skews the TME toward proinflammation via various inflammatory cytokines/chemokines and immunosuppressive molecules. Specifically, we postulate that the elevated G-CSF, IL-6, and IL-10 in circulation and/or the TME of **p53**null hosts provide crucial differentiation and survival signals for MDSCs and Tregs, respectively, while the increased expression of CXCL1, CCL3, and CCL21 plays a vital role in recruiting the immunosuppressive populations to the TME (9, 13, 19, 20). In addition, iNOS and Arg1 in the TME further augment the function of those immunosuppressors (9, 13). CXCL1, produced by melanomas or stroma, is a protumor chemokine either directly stimulates tumor via paracrine effect or indirectly via proinflammation-mediated recruitment of MDSCs (19, 20). Indeed, we detected a low level expression of CXCL1 in B16F1, which is further enhanced by tumor-stroma or tumor-immune cell interaction in the **p53**null TME. Therefore, the strong inflammation and resulted immunosuppressive effectors within the TME in **p53**null hosts are likely a result of concerted multidimensional interactions involving tumor, stroma, and immune cells.

The **p53**null mice used in this study best resemble those individuals with Li-Fraumeni syndrome (2). However, the immunologic mechanism revealed in this study by which **p53** inactivation/dysfunction in the TME enhances tumor progression should have broad clinical implications, because **p53** inactivation occurs frequently during physiologic and pathologic processes via various mechanisms, including the activation of oncogenes and viral proteins, in addition to somatic mutations (1–3, 5, 6).

In summary, this is the first study that elucidates the underlying mechanism by which **p53** inactivation immunomodulates the TME. These results underscore the importance and function of **p53** in maintaining proper immunologic microenvironment to suppress tumorigenesis and provide valuable information for novel immunotherapies by targeting **p53** activity in the stroma/TME.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Guo, L. Marrero, L.D. Valle, A.C. Ochoa, Y. Cui

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