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Lack of immunomodulatory Interleukin-27 enhances oncogenic properties of mutant p53 *in vivo*

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Abstract

Purpose—p53 is mutated in about 50% of human cancers, mostly through missense mutations. Expression of mutant p53 is associated with poor clinical outcomes or metastasis. Although mutant p53 is inherently instable, various stressors such as DNA damage or expression of the oncogenic Kras or c-myc affect the oncogenic properties of mutant p53. However, the effects of inflammation on mutant p53 are largely unknown. Interleukin-27 (IL-27) is an important immunomodulatory cytokine but its impact on mutant p53-driven tumorigenesis has not been reported.

Experimental Design—IL27RA$^{-/-}$ mice were bred with mutant p53 heterozygous (p53$^{R172H/+}$) mice to obtain IL27RA$^{-/-}$p53$^{H/+}$ and IL27RA$^{-/-}$p53$^{H/H}$ mice. Mouse survival and tumor spectra for the cohort were analyzed. Stability of p53 protein was analyzed via immunohistochemistry and western blot.

Results—this study unraveled that lack of IL-27 signaling significantly shortened the survival duration of mice with tumors expressing both copies of the mutant *p53* gene (Li-Fraumeni mouse

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model). Interestingly, in mice that were heterozygous for mutant *p53*, lack of IL-27 signaling not only significantly shortened survival time but also doubled the incidence of osteosarcomas. Furthermore, lack of IL-27 signaling is closely associated with increased mutant p53 stability *in vivo* from early age.

Conclusions—These results suggest that IL-27 signaling modulates the oncogenic properties of mutant p53 *in vivo*.

Keywords

mutant p53R172H; tumor incidence; survival; IL-27; IL27RA; osteosarcoma

INTRODUCTION

p53, which functions as a tumor suppressor, is mutated in about 50% of tumors, often through missense mutations occurring in the DNA-binding domain. Aside from affecting the transcriptional activity of p53, these missense mutations often are associated with an increase in the half-life of p53, causing mutant p53 to accumulate in the tumor cells and often acquire a gain-of-function phenotype. Although mutant p53 is inherently unstable, various stressors affect the oncogenic activation and properties of this protein. For example, DNA damage and oncogene activation such as RAS mutations, c-myc, or $p16^{INK4a}$ can alter the oncogenic properties of mutant p53 (1, 2).

Interleukin (IL)-27 is known to be an important pro-inflammatory or anti-inflammatory cytokine. IL-27 has been increasingly studied for its role in regulating the intensity and duration of T cell response. Briefly, IL-27 can antagonize IL-2 production (3) and GATA3 expression by limiting Th2 response (4), as well as decrease RoRγ expression by limiting Th17 (5) and increase IL-10 expression by inducing Th10 cells (6–8). IL-27 signaling through STAT1 and T-bet can also enhance Th1-type responses. Furthermore, IL-27 has been shown to have anti-tumor effects in subcutaneously injected neuroblastoma, colon carcinoma, lung carcinoma, and acute myeloid leukemia cells (9) (10, 11) (12) (13).. However, IL-27 can also increase PDL1, an important immune-suppressor checkpoint in both cancer and inflammation (14). Therefore, the biology of IL-27 is complex and context-dependent; IL-27 may either dampen or promote different types of inflammation or cancer.

Although inflammation plays a central role in all aspects of cancer, from tumor initiation to progression and metastasis, it remains unknown if or how inflammatory signals stabilize or promote oncogenic properties, such as tumor development and survival of oncogenes such as mutant *p53*. Recent mouse models have been developed to better dissect the role of mutant p53. Specifically, mice were bred with mutations at amino acid 172 of p53 (R172H, Li-Fraumeni mice), corresponding to the human p53 hot spot mutation at amino acid 175. However, how endogenous levels of IL-27 affect tumorigenesis or survival in these mice remains largely unknown. In the current study, we found that lack of IL-27 signaling significantly shortens survival duration in mice with both copies of the mutant *p53* gene (Li-Fraumeni mice, $p53^{H/H}$). Interestingly, in mice heterozygous for mutant p53 ($p53^{H/+}$), lack of IL-27 signaling not only significantly shortened survival but also doubled the incidence of osteosarcomas. This suggests that IL-27 signaling negatively modulates the oncogenic

properties of mutant p53 and lack of IL-27 signaling is closely associated with early mutant p53 stability *in vivo*.

MATERIALS AND METHODS

Transgenic Mice

We bred IL27RA$^{-/-}$ mice with p53$^{R172H/+}$ (generously donated by Dr. Lozano, University of Texas MD Anderson Cancer Center) mice to create IL27RA$^{-/-}$p53$^{R172H/H}$IL27RA$^{-/-}$p53$^{R172H/+}$ and IL27RA$^{-/-}$p53$^{+/+}$ mice, which were born at a Mendelian ratio. All mouse strains were maintained in C57Bl/6 cohorts. IL27RA$^{-/-}$ mice were previously donated by Dr. Frederic de Sauvage (Genentech). Mice were monitored over time for tumor development. Genotyping was performed as described below for both p53 and IL-27 receptor alpha (IL27RA). For the cohort studies, mice were euthanized when they met the criteria for euthanasia, mainly related to tumor burden or distress. For the time course study, mice from each genotype were euthanized at the indicated times. Tissue was fixed in neutralized paraformaldehyde prior to sectioning. All procedures involving animals were approved by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Hematoxylin-Eosin Staining and Pathologic Scoring

Sections of paraffin-embedded tissues were stained with hematoxylin and eosin. The pathologic readings for various abnormal growths were scored by a pathologist at MD Anderson. Images were captured using a Nikon Eclipse Ti microscope.

Micro-CT image Acquisition and Analysis

Computed tomography (CT) images were acquired as previously described (15). Briefly, images were acquired in 5 100-ms frames at 1° rotation increments with an X-ray tube potential of 80 kVp and a current of 450 μA using an RS-9 micro-CT scanner (GE Healthcare, London, Ontario, Canada). Two adjacent image volumes were acquired with an overlap of 40% of the field of view in the axial direction, and these images were stitched together after reconstruction. Images had a nominal isotropic resolution of 91 μm and required approximately 25 minutes of scan time. The bone was visualized using the GEHC Microview Software (GE Healthcare, London, ON, Canada). All image isosurfaces were visualized and analyzed for bone mineral density using the same image threshold value.

Immunohistochemistry Staining

Briefly, paraffin-embedded unstained slides from IL27RA$^{-/-}$ p53$^{R172H/+}$ or control (p53$^{R172H/+}$) mice were processed for heat-induced antigen retrieval in pH 6.0 citrate buffer for 40 minutes in the steamer. Slides were incubated with rabbit monoclonal anti-p53 (1:100, Novus Antibodies) or rabbit anti-p21 (1:50, Santa Cruz) antibodies diluted at 1:100 in goat serum/fish gel/BSA blocking buffer overnight at 4°C. The next day, tissue was washed, blocked for 15 minutes in blocking buffer, and incubated with anti-rabbit IgG-Biotin (diluted at 1:500 in blocking buffer) for 1 hour at room temperature, followed by 30 minutes of incubation in the ABC enhanced Vectastain Kit system (Vector Laboratories, Burlingame, CA). Similarly, other antibodies used for IHC staining included: Ki67 (1:800, Abcam); CD3

(1:100, Abcam), NCR1 (1:100, Abcam), B220 (1:50, BD Pharmingen), Foxp3 (1:100, Cell Signaling), Endoglin (1:100, Vector Laboratories).

Genotyping

DNA was extracted from ear tissue, and polymerase chain reaction (PCR) analysis for the *p53* gene was performed using the primer sequence described by Lang et al. (16). The presence of the *IL27RA* gene was confirmed using the forward primer sequence CAAGAAGAGGTCCCGTGCTG and the reverse primer sequence TTGAGCCCAGTCCACCACAT. PCR primers used to determine the absence of IL27RA were as follows: GCTTTCGTCTCCCGTGTGCT (forward) and TGAGCCCAGAAAGCGAAGGA (reverse).

Invasion and migration assays

The osteosarcoma H318 cell line derived from a $p53^{H/+}$ mouse and expresses functional levels of IL27RA was used for these studies (17). The invasion assay was performed using Transwell inserts (Greiner Bio-one) in 24-well plates. To perform invasion assays, inserts were pre-coated with 30 μl phenol red-free matrigel (BD Biosciences) for 1 hour at 37 C. Approximately, 10^5 cells in 100 μl heat-inactivated complete RPMI media (with or without rIL27) were plated on top of the matrigel-coated insert. was placed at each well of 24 well plate. Then the insert was placed on top of the 500 μl complete media containing 10% FBS in each well of 24 well plate overnight at 37C. 48hrs later, the media was removed from lower chamber and the insert was incubated with 500 μl media containing 8 μM Calcein-AM for 1 hour at 37 C. Then the insert was transferred to a new well containing 500 μl of pre-warmed 1 mM EDTA to detach the cells on the outer surface of the insert. 100 μl of cell suspension was transferred to each well (triplicate) of 96 well plate to measure fluorescence using spectramax. The migration assay was performed in a similar fashion with 10^4 cells in 100 μl (with or without rIL27) without coating the insert with matrigel. Cells inside the inserts were removed with cotton-tipped swabs on next day after overnight incubation.

Proliferation assay

H318 cell lines was serum starved overnight. Approximately 10,000 cells/well were treated with or without rIL27 (20ng/mL) containing heat inactivated serum media in quadruplicate for 24 or 48 hours. For 48 hrs treatment, fresh heat-inactivated media (with or without rIL27) was added to each well after 24hrs. To measure the viability of H318 cells after treating with or without rIL27, 100 μl of CyQUANT NF Cell Proliferation Assay reagent was added to each well after aspiration of medium. After incubation for 1 hour at 37°C, fluorescence was measured (excitation 485 nm, emission 538 nm) on a SpectraMax Geminin EM microplate reader (Molecular Devices) using Softmax Pro version 5.4.

Bone marrow isolation

Bone marrow cells from age-matched wildtype, $p53^{H/+}$ or $IL27RA^{-/-}p53^{H/+}$ mice were isolated as previously described (18) and seeded at $3x10^6$ in RPMI media in a 6 well plate. These cells were irradiated with 6Gy or left un-irradiated. These samples were harvested 4 and 24 hours post irradiation.

Flow cytometry analysis

Isolated bone marrow cells from 3 month old mice and were blocked for non-specific staining in 2% BSA and with anti-CD16 (5 μg/mL) for 20′ at 4°C. Afterwards, cells were stained in 2% BSA with anti-CD4-Violet421 (1:100, Pharminogen), anti-CD8-PE-Cy7 (1:100, Biolegend), anti-F4/80- Fitc (1:100, eBiosciences), anti-CD3-e450 (eBiosciences), anti-NK1.1- PE-Cy7 (eBiosciences), anti-CD19-Violet421 (1:100, Biolegend) and anti-B220-PerCP (1:100, Biolegend), anti-CD11c-Violet 421 (1:100, Biolegend), anti-MHCII-PerCP (1:100, Biolegend). At last, the cells were washed twice in 2% BSA and analyzed by flow cytometry using Attune (Invitrogen), and FlowJo.

Western Blot

Briefly, cells were lysed in lysis buffer, and supernatants were quantified for protein amount. Eighty microgram of cell lysate was loaded in 10% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibody overnight at 4° C (1000x dilution for anti-p53 (Novus), 1000x anti-actin.

Growth of primary tumor cells in NSG mice

Femur, tibia, and lumbar vertebras from mice were cut aseptically in 1.5 mm small pieces, rinsed in PBS, and placed in digestion media consisting 5 mg collagenase, 5 mg pronase, and 1 mg DNAse for 45 minutes at 37° C (19). Following digestion, single cell suspension was passed through a 70 μm strainers twice. And lastly, these cells were rinsed in PBS three times prior to intraosseous ($1*10^7$ in 15 μL PBS) or subcutaneous injections ($1*10^7$ in 30 μL PBS) as previously described (20).

Statistical Analysis

The Student *t* test was used to analyze differences between groups. $P < 0.05$ was considered statistically significant. GraphPad Prism for Windows was used to prepare and analyze the graphs (GraphPad Software, La Jolla, CA).

RESULTS AND DISCUSSION

The role of endogenous IL-27 during spontaneous tumorigenesis, and more specifically in a Li-Fraumeni mouse model, is not known. To understand the *in vivo* significance of IL-27 in regulating mutant p53-driven oncogenic properties, we crossed $IL27RA^{-/-}$ mice with heterozygous $p53^{H/+}$ mice, which allowed us to examine tumor incidence and survival between $IL27RA^{-/-}$ $p53^{H/H}$ mice and $p53^{H/H}$ mice. Interestingly, we found that mice lacking IL27RA signaling that carried both alleles of mutant p53 ($IL27RA^{-/-}$ $p53^{H/H}$ mice) had significantly shorter survival times than $p53^{H/H}$ mice (Figure 1A), suggesting that endogenous IL-27 signaling has antitumor properties. We observed a similar spectrum of tumor development between $IL27RA^{-/-}p53^{H/H}$ mice and $p53^{H/H}$ mice, including malignancies such as lymphoma and sarcoma (Figure 1B, C, D). However, the incidence of lymphomas in $IL27RA^{-/-}p53^{R172H/H}$ mice was 3-fold higher than in $p53^{R172H/H}$ mice.

Because $IL27RA^{-/-}p53^{R172H/H}$ mice have a short survival span, some important $IL27RA^{-/-}$-facilitated oncogenic interactions may be difficult to observe in these mice. Therefore, we

sought to investigate the role of IL-27 signaling in $p53^{R127H/+}$ heterozygote background. Therefore, we compared survival and tumor spectrum between $IL27RA^{-/-}p53^{H/+}$ and $p53^{H/+}$ mice ($IL27RA^{-/-}p53^{H/+}$ mice have a longer survival time than $p53^{H/H}$ mice because these mice carry only 1 mutant p53 allele). Similar to the $IL27RA^{-/-}$ $p53^{H/H}$ mice, $IL27RA^{-/-}p53^{H/+}$ mice had significantly shorter survival times than $p53^{H/+}$ mice (Figure 2A). Interestingly, the incidence of osteosarcomas in $IL27RA^{-/-}$ $p53^{R172H/+}$ mice (68%) was twice that in $p53^{R172H/+}$ mice (Figure 2B). More than 80% of these $IL27RA^{-/-}$ $p53^{H/+}$ osteosarcomas were osteblastic and 12% had fibroblastic and giant cell differentiation.

Lastly, 41 % of the $IL27RA^{-/-}p53^{H/+}$ mice that had osteosarcomas progressed to rear leg paresis (Supplementary Figure 1Figure 2B), while none of $p53^{H/+}$ mice in our cohort developed any of this phenotype.

Similar to human patients, micro-CT imaging showed that $IL27RA^{-/-}$ $p53^{H/+}$ mice developed tumors in various anatomical sites, including the spine, ribs, or tibia (Figure 2C). Since 41% of osteosarcoma-positive $IL27RA^{-/-}p53^{H/+}$ mice developed rear leg paresis, compressive myelopathy and Wallerian degeneration were observed histologically in these mice (Figure 2D).

Interestingly, osteosarcomas occurred in multiple locations simultaneously in the $IL27RA^{-/-}p53^{H/+}$ mice. Within the same mouse, we found multicentric osteosarcoma in the femur and throughout the spine, including the lumbar, thoracic, and cervical vertebrae (Figure 2D). These multicentric osteosarcomas were found in 41% of the $IL27RA^{-/-}p53^{H/+}$ mice. And lastly, these osteosarcomas also had the potential not only to invade into the spinal cord, spinal canal, or subjacent muscle, but also to form metastases to the lung (Figure 2D). However, the metastatic potential to the lung was not increased in $IL27RA^{-/-}p53^{H/+}$ mice when compared to $p53^{H/+}$. These observations suggest that lack of IL-27 signaling might generate a tumor-promoting environment.

To determine whether these osteosarcomas were aggressive, we transplanted the primary tumors into NSG mice. Briefly, we collected primary bone marrow from the femur or spine of mice that did not have visible tumor growth, minced the bone marrow, and placed it in enzymatic digestion media. Single cell suspensions were injected directly into NSG mice either subcutaneously or orthotopically via intra-osseous injections. Within 6–10 weeks, these mice developed visible tumors, which become aggressive osteosarcomas (Supplementary Figure 2A-C).

Mutant p53 protein stability is often associated with oncogenic functions of this protein. Because $IL27RA^{-/-}$ $p53^{H/+}$ mice have a higher incidence of osteosarcomas and shorter survival times than $p53^{H/+}$ mice, one possibility is that the lack of IL-27 signaling increases the number of cells with stable mutant p53 protein at a faster rate than under normal IL-27 signaling conditions. To examine the stability of mutant p53 over time, we stained for p53 healthy tumor-free bone marrow tissue derived from the spinal vertebrae of 8 month age-matched wild-type, $IL27RA^{-/-}p53^{H/+}$ and $IL27RA^{-/-}$ $p53^{H/+}$ mice (Figure 3A). Indeed, we found significant expression of p53 in $IL27RA^{-/-}$ $p53^{H/+}$ mice but not in controls. Stable p53 was detected as early as 2 months of age, but the levels of p21 were not detectable

(Figure 3B). These findings suggest that the absence of IL-27 signaling modulates the oncogenic properties of mutant p53 *in vivo* and is closely associated with early mutant p53 stability.

p53 is stabilized in response to stress signals, which could come from either irradiation, or from the stress of exposing primary cells in tissue culture growth and oxygen exposure. To test whether different stressor modulate p53 stability in absence of IL27, we exposed bone marrow cell from wildtype, $p53^{H/+}$ and $IL27RA^{-/-}p53^{H/+}$ age-matched mice to irradiation and growth in culture. We hypothesize that bone marrow cells derived from $IL27RA^{-/-}p53^{H/+}$ may have higher levels of p53 in response to in-vitro culture or due to irradiation. Indeed, bone marrow cells from $IL27RA^{-/-}p53^{H/+}$ had higher levels of p53 stability when grown in culture (compared to other genotypes at 24 hours) and when exposed to irradiation (See Figure 3C).

Additionally, the authors tested whether recombinant IL27 (rIL27) could reduce p53 levels in osteosarcomas cell lines derived from $p53^{H/+}$ mice (17). Indeed, short term treatment (30 minutes) with rIL27 has *modestly* reduced the p53 levels in these cells (Figure 3D).

Natividad and colleagues showed that absence of IL27 signaling enhanced tumor growth in a carcinogen-induced fibrosarcoma and oncogene-driven mammary carcinoma. Lack of IL27 signaling was associated with a decrease of interferon-γ-positive CD4 and CD8 T cells and absence of T regulatory cells (21). To understand which immune cell population may be involved in our model in absence of IL27 signaling, we performed an immune cell profiling among different mice at age 3 months in the bone marrow (pre-malignant niche). No significant changes were seen in immune cell subgroups amongst all genotypes, except the number of F4/80-positive cells were significantly increased in the bone marrow of $IL27RA^{-/-}p53^{H/+}$ mice when compared to the controls (Figure 4A).

Similarly, in end-stage osteosarcoma tumors derived from $p53^{H/+}$ vs. $IL27RA^{-/-}p53^{H/+}$ ones, we found no differences in immune cell infiltrates (CD3, Foxp3, NCR1, or B220-positive cells. We only found differences in the number of macrophages in these established osteosarcomas (Figure 4B). The immune cell infiltrates in these osteosarcomas are different from Natividad which saw a T cell dependent mechanism in carcinogen-induces fibrosarcomas. One possibility for this discrepancy may rely on the fact that carcinogen-induced tumors are more immunogenic than oncogene-induced spontaneously-developing tumors.

To better understand on a mechanistic basis the increased tumorigenicity and shorten survival that occurs in $p53^{H/+}$ mice lacking IL-27, we compared neo-angiogenesis markers and proliferation markers between $p53^{H/+}$ vs. $IL27RA^{-/-}p53^{H/+}$ osteosarcoma cells. We saw no differences in terms of proliferation, but increase in neo-angiogenesis marker endoglin was observed (Figure 4C, 4D). Endoglin has been suggested as an appropriate marker for tumor-related angiogenesis and neovascularization (22).

And lastly, to better understand the role of IL27 in osteosarcoma tumor cells, we determined the effects on proliferative capacity, migration and invasive properties on H318 osteosarcoma that is derived from $p53^{H/+}$ mice. H318 also contains IL27RA expression.

Interestingly, we found that *in vitro* rIL27 has a modest increase in osteosarcoma cell proliferation and invasion, but not migration (Supplementary Figure 3A-C). However, these effects are derived from cell lines in vitro while neglecting the immune response and in absence of the tumor microenvironment implying the importance of functionally understanding the biological significance of these cytokines in vivo.

Our study is one of the first to report that, in addition to other oncogenes such as Kras or c-myc, inflammatory signals such as IL-27 can negatively regulate the oncogenic properties of mutant p53.

The mouse studies reported here are in concordance with a study in human patients showing that IL27p28 levels were significantly lower in patients with osteosarcoma than in healthy controls (23). Furthermore, in that study, as the disease progressed, the levels of IL27p28 were further reduced. The antitumor properties of IL-27 in the Li-Fraumeni mouse model were also closely associated with mutant p53 stability in our study. One possible explanation is that inflammatory signals such as the absence of IL-27 signaling stabilize p53 *in vivo*. Another explanation could be that IL-27 promotes immunosurveillance to eradicate cells with excessive levels of stable p53.

In summary, endogenous levels of IL-27 affected the oncogenic properties of mutant p53 *in vivo* in a Li-Fraumeni mouse model. Lack of IL-27 signaling led to shortened survival times and increased incidence of osteosarcomas, suggesting that lack of IL-27 signaling was closely associated with early mutant p53 stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TRANSLATIONAL RELEVANCE

p53 is the most commonly mutated gene in cancer and the oncogenic properties of mutant p53 are highly dependent on protein stability. Understanding and targeting molecules that enhance mutant p53 stability and subsequently p53-driven oncogenesis is of utmost importance in cancer prevention and progression. Inflammatory signals play a crucial role in the initiation of tumorigenesis. It is not understood if and how inflammatory signals affect oncogenic properties of mutant p53. Here shows that immune-regulatory cytokine IL-27 modulates the oncogenic properties of mutant p53 (R172H, or short for H allele) *in vivo* and that lack of IL-27 signaling is closely associated with early mutant p53 stability *in vivo*. Of note, this study underlines the significance of IL27 signaling in osteosarcoma initiation by doubling the incidence of osteosarcoma in these $IL27RA^{-/-}p53^{H/+}$ mice, suggesting that IL27 could be a treatment modality to prevent or reduce osteosarcoma incidence.

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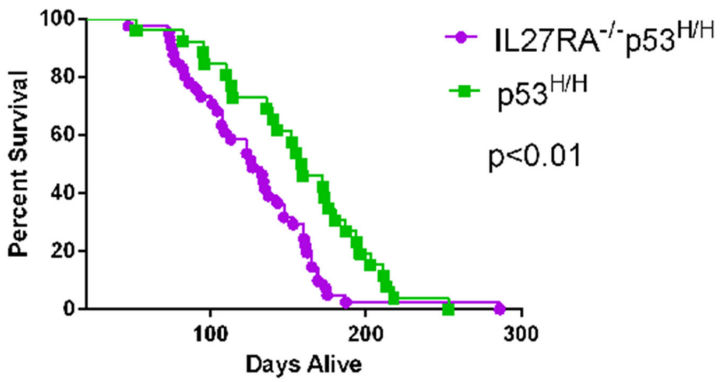

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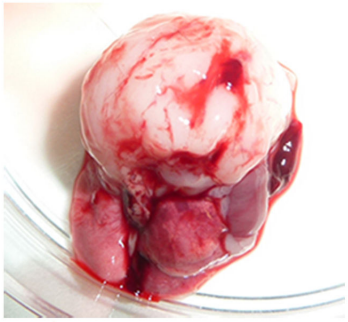

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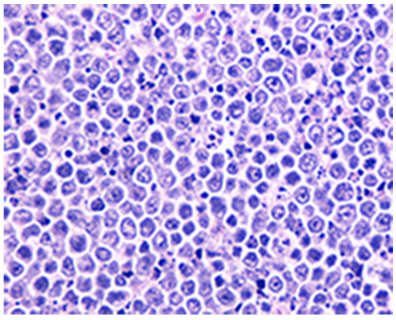

B

Tumor type	Mouse Strain (%) $p53^{H/H}$	$IL27RA^{-/-}$ $p53^{H/H}$
Lymphoma	17	52
Sarcoma	21	6
Hemangiosarcoma	17	12
Extramedullary hematopoeiesis	0	6
Hemartoma	4	0
Liver neoplasia	0	3
Lung hyperplasia	0	3

Figure 1. Increased tumorigenesis in homozygous $p53^{H/H}$ mice lacking IL27RA signaling

(A) Kaplan-Meier survival curve of $IL27RA^{-/-}$ $p53^{H/H}$ (N=41) compared with $p53^{H/H}$ mice (N=26). P< 0.05. (B) Tumor spectrum differences in tumor incidence between $IL27RA^{-/-}$ $p53^{R172H/H}$ compared with $p53^{R172H/H}$ mice. (C, D) Photomicrograph and hemotoxylin and eosin (HE) staining of thymus lymphoma tumor that occurs most frequently in $IL27RA^{-/-}$ $p53^{H/H}$.

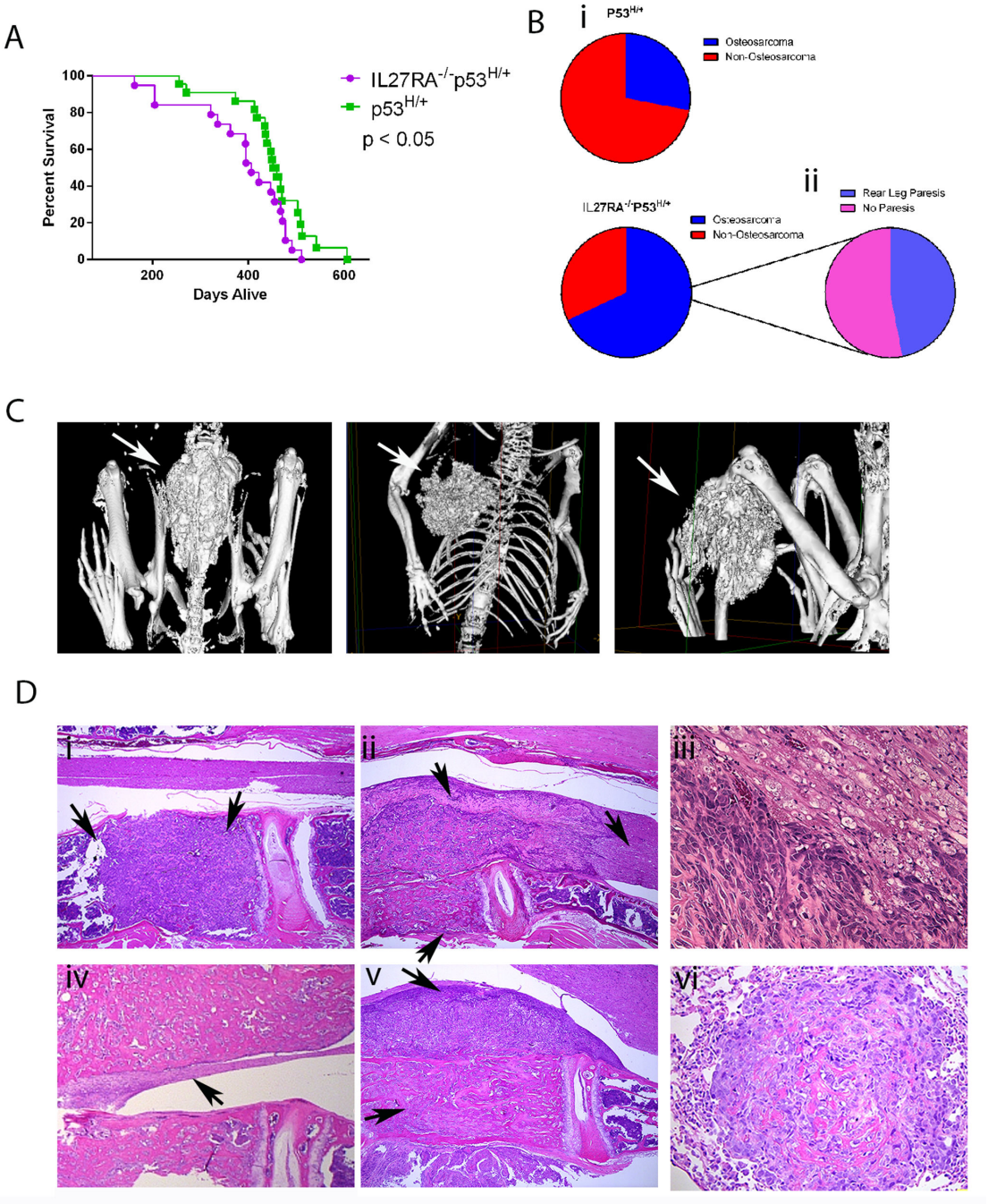

Figure 2. Increased tumorigenesis and doubling of osteosarcoma incidence in heterozygous $p53^{H/+}$ mice lacking IL27RA signaling when compared to $p53^{H/+}$ mice

(A) Kaplan-Meier survival curve of $IL27RA^{-/-}$ $p53^{H/+}$ (N=18) compared with $p53^{H/+}$ (N=22) mice. P< 0.05. (B) Difference in osteosarcoma incidence between $IL27RA^{-/-}$ $p53^{H/+}$ and $p53^{H/+}$ mice (i) and percentage of mice with osteosarcoma that develop rear leg paresis (ii). (C) micro-CT images portraying osteosarcomas in different anatomical positions. (D) Representative HE photomicrographs of multicentric osteosarcomas developing throughout different vertebral bodies through the spine (i) osteosarcoma from vertebral body infiltrated the spinal cord (ii), osteosarcoma causing Wallerian degeneration (iii), compressive

myelopathy (iv), osteosarcoma from vertebral body expended into spinal canal and infiltrated subjacent muscle (v), and HE photomicrographs from metastatic lesions of osteosarcomas in the lungs (vi).

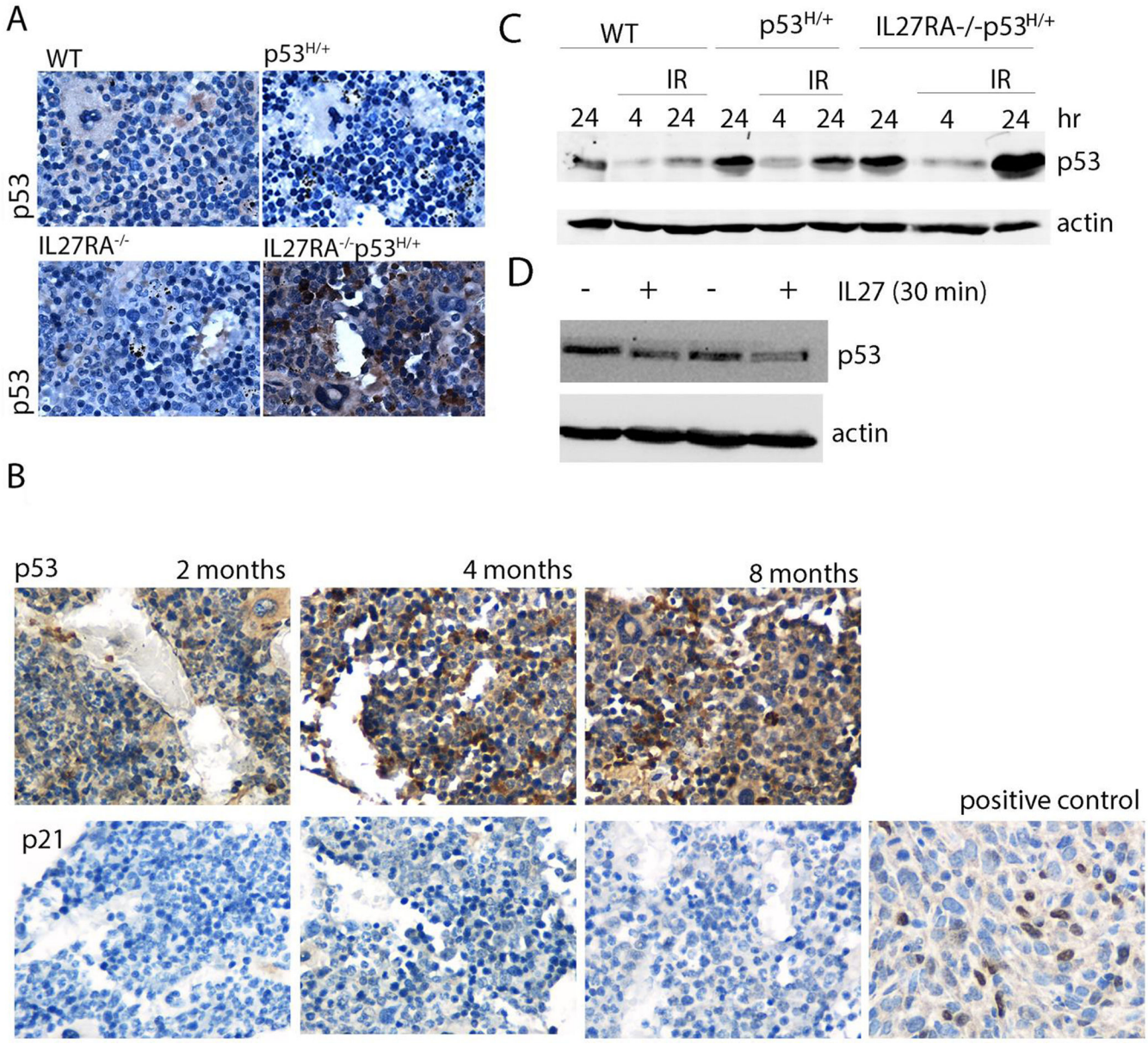

Figure 3. Lack of IL27 signaling stabilizes mutant p53 *in vivo and in vitro*

(A) p53 staining via immunohistochemistry of bone marrow vertebral bodies from 8 month old wildtype, IL27RA$^{-/-}$ p53$^{R172H/+}$, and IL27RA$^{-/-}$ p53$^{H/+}$ mice. (B) p53 and p21 staining via immunohistochemistry of bone marrow vertebral bodies from 2, 4, and 8 month old IL27RA$^{-/-}$ p53$^{H/+}$ mice including a positive control for p21. (C) Bone marrow cells derived from aged wildtype, p53$^{H/+}$ and IL27RA$^{-/-}$p53$^{H/+}$ mice were seeded in a 6-well plate and either irradiated (IR) or left un-irradiated. Cells were collected at the indicated time points and were probed for p53 and actin. (D) H318 and H76 osteosarcoma cells were treated with rIL27 for 30 mins at 20ng/ml and collected lysates were probed for p53 levels and actin,

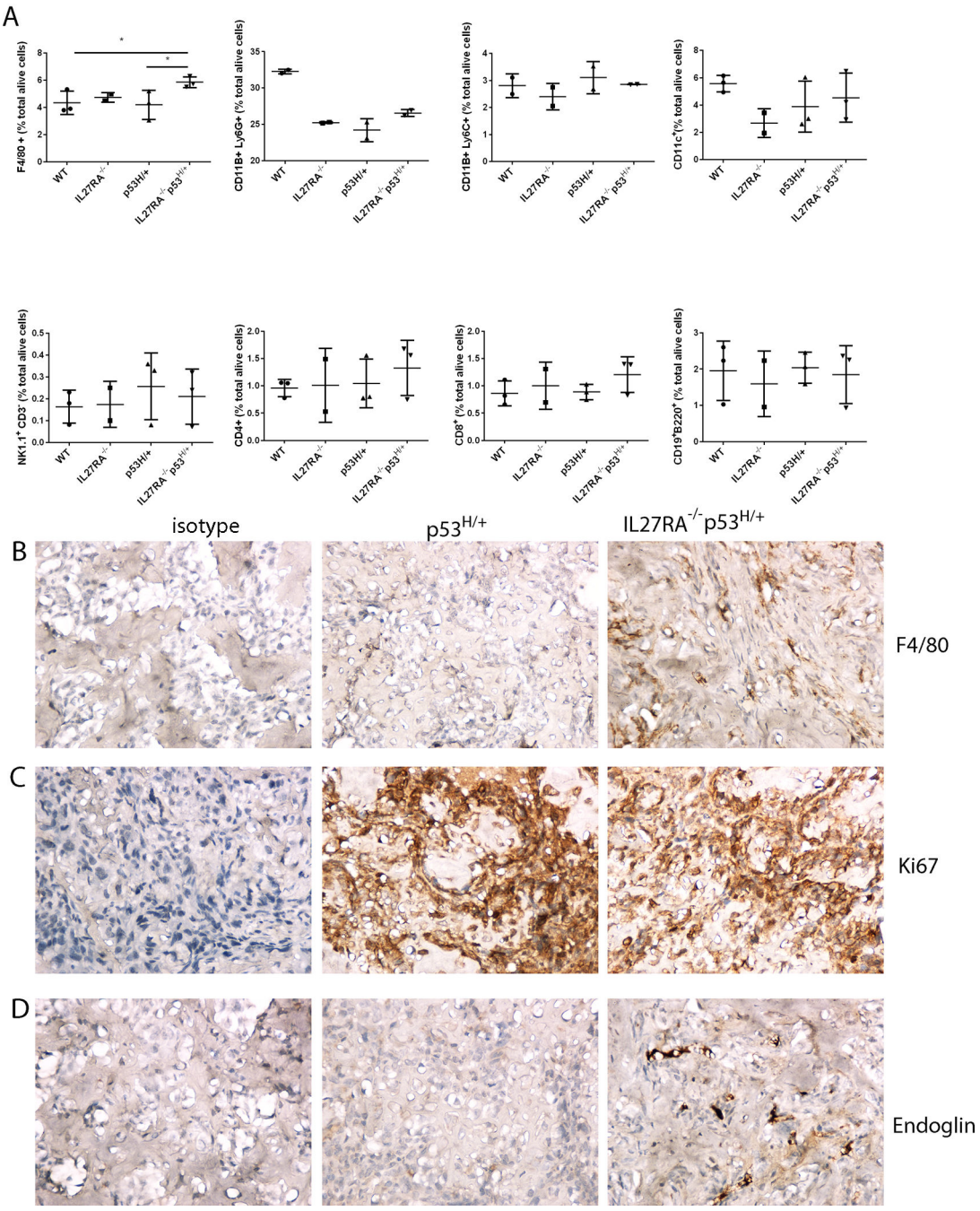

Figure 4. Absence of IL27 signaling increases macrophage and neo-angiogenesis marker Endoglin in mutant p53-driven osteosarcomas

(A) No significant changes were seen in mice of different genotypes in CD4, CD8, B, NK, myeloid, CD11c cells in the bone marrow of 3 month old mice bearing indicated genetic modifications as analyzed by flow cytometry. Significant changes were seen in the number of macrophages in the bone marrow (B) present in the aged IL27RA$^{-/-}$p53$^{H/+}$ mice when compared to age-matched control ones analyzed via immunohistochemistry. (C-D) No differences in the proliferation rate of these end-stage tumors were noted (C, Ki67 staining), but a higher number of endoglin-positive cells in osteosarcomas from IL27RA$^{-/-}$p53$^{H/+}$.

Representative migrographs from at least 3 independent mice. Dots indicate data from the bone marrow of individual mice. Pictures takes at 400X. *, $p<0.05$