Microenvironment and Immunology

Myeloid-Specific Expression of Ron Receptor Kinase Promotes Prostate Tumor Growth

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Abstract

Ron receptor kinase (MST1R) is important in promoting epithelial tumorigenesis, but the potential contributions of its specific expression in stromal cells have not been examined. Herein, we show that the Ron receptor is expressed in mouse and human stromal cells of the prostate tumor microenvironment. To test the significance of stromal Ron expression, prostate cancer cells were orthotopically implanted into the prostates of either wild-type or Ron tyrosine kinase deficient (TK⁻/⁻; Mst1r⁻/⁻) hosts. In TK⁻/⁻ hosts, prostate cancer cell growth was significantly reduced as compared with tumor growth in TK⁺/⁺ hosts. Prostate tumors in TK⁻/⁻ hosts exhibited an increase in tumor cell apoptosis, macrophage infiltration and altered cytokine expression. Reciprocal bone marrow transplantation studies and myeloid cell–specific ablation of Ron showed that loss of Ron in myeloid cells is sufficient to inhibit prostate cancer cell growth. Interestingly, depletion of CD8⁺ T cells, but not CD4⁺ T cells, was able to restore prostate tumor growth in hosts devoid of myeloid-specific Ron expression. These studies show a critical role for the Ron receptor in the tumor microenvironment, whereby Ron loss in tumor-associated macrophages inhibits prostate cancer cell growth, at least in part, by derepressing the activity of CD8⁺ T cells. Cancer Res; 73(6); 1752–63. ©2012 AACR.

Introduction

Coordinated signaling between different cell types of the stroma is required for the development and maintenance of an adult prostate secretory epithelium. The stroma also supports prostate tumor initiation and progression (1, 2). Inflammatory infiltrates are major cellular components of tumor stroma and include adaptive and innate immune cell types (3, 4). Tumor-associated immune cells support tumor growth by producing an immunosuppressive microenvironment capable of blocking productive antitumor immunity. Tumor-associated macrophages (TAMs) play pivotal roles in tumor progression and can promote or inhibit tumor growth depending on their activation state (5, 6).

The Ron receptor tyrosine kinase is expressed on several tissue-resident macrophage populations (7–11). Ron activation in peritoneal macrophages suppresses inflammation and promotes alternative macrophage activation (12, 13). Following lipopolysaccharide (LPS) stimulation in macrophages, Ron activation inhibits the expression of inducible nitric oxide synthase (iNOS) while promoting expression of arginase-1, an enzyme that competes with iNOS (14, 15). iNOS-mediated nitric oxide production is cytotoxic and tumor suppressive, whereas products of arginase-1 promote tissue repair and tumorigenesis (16).

Studies from our laboratory have shown Ron to be a negative regulator of inflammation in lung injury and bacterial peritonitis models (17, 18). Using mice with myeloid cell–specific Ron deletion, macrophages were identified as the major cell type regulating inflammatory responses in lung and liver injury models (19, 20). Ron signaling in peritoneal macrophages increases the phosphorylation of STAT3, suggesting an interaction between these two pathways (17, 21). Moreover, STAT3⁻/⁻ mice show similar phenotypic responses as that of Ron-deficient mice (22). Phosphorylation of STAT3 in TAMs is important for attenuating antitumor immunity by suppressing macrophage-mediated antigen presentation to cytotoxic CD8⁺ T lymphocytes (23, 24). Given that Ron regulates macrophage activation and inflammation, Ron signaling in TAMs may be important for maintenance of an immunosuppressive microenvironment.

Ron is overexpressed in more than 90% of human prostate cancers (25–27). Ron activation in tumor cells elicits a range of cellular responses including growth, differentiation, migration, and survival through various signaling pathways (27, 28). Gain- and loss-of-function studies in tumor epithelial cells, including human prostate cells, have shown that Ron expression is important for promoting tumor growth in vivo (29–32). Moreover, data from our laboratory have shown that mice containing a germline deletion of the Ron tyrosine kinase–signaling domain exhibit significant reduction in tumor mass when bred to mice predisposed to develop prostate cancer (32).
numerous studies have outlined the importance of epithelial-expressed Ron in supporting tumorigenesis, the importance of Ron expression in the tumor stroma has not been extensively investigated. Although a recently published study outlined the importance of Ron expression in the tumor microenvironment, the Ron-expressing stromal cell lineage that supported tumor growth was not identified (13).

This study identifies Ron expression in the stroma, particularly in TAMs, to be a critical factor that supports tumor growth through the regulation of apoptosis in tumor epithelial cells. In addition, we show that Ron expression in TAMs suppresses tumor immune surveillance through CD8+ T-cell regulation and that Ron-expressing TAMs have increased activation of STAT3. Our findings indicate that in addition to the well-established tumor-cell autonomous role, Ron plays a novel role in TAMs by promoting tumor cell survival through cytotoxic CD8+ T-cell regulation.

Materials and Methods

Mice

Wild-type (TK+/+), Ron tyrosine kinase–deficient mice (TK−/−), homozygous Ron-floxed mice (TK−/−), and LysMcrc (TK−/−LysMcrc−) mice were generated and maintained in a C57Bl/6 background as described (20, 33). All experiments used 8- to 12-week-old male mice. For bone marrow transplantation, donor bone marrow cells from TK−/− and TK−/− mice were injected into the tail vein of irradiated mice (Supplementary Methods). All mice were maintained under specific pathogen-free conditions and were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (Cincinnati, OH).

Cell lines and orthotopic injections

Murine TRAMP-C2Re3 cells were obtained from Dr. Zhongyu Dong (University of Cincinnati) and grown as previously described (34). The cells are of murine origin and were tested for growth in syngeneic mice; no further validation was conducted. Cells (2.5 × 10⁶) were injected into the ventrolateral prostates of mice and harvested after 30 days (26).

T-cell depletion

Mice were subcutaneously injected with 5 × 10⁵ TRAMP-C2Re3 cells. To deplete T-cell subsets, mice were injected on days 1, 7, 14, 21, and 24 with 1 mg/mL of rat monoclonal antibody that depleted specific lymphocyte subsets: mAb-GK1.5 (anti-CD4, IgG2b); mAb-2.43 (anti-CD8, IgG2b); and Rat-IgG2b isotype control antibody (Jc; Bio X Cell). Tumor volume was recorded biweekly and tumors were harvested on day 28.

Cell isolations

A single-cell suspension of normal or tumor-bearing prostate tissue was obtained by mechanical dissociation and enzymatic digestion. Enrichment for epithelial cells, fibroblasts, and immune cells was accomplished by differential centrifugation using standard procedures (Supplementary Methods). TAMs were enriched from the immune cell fraction by magnetic beads coated with mouse CD11b according to manufacturer’s instructions (Milteny Biotech). The isolated cells were more than 95% pure.

Flow analysis

Immune infiltrates were treated with Fc anti-CD16/CD32 antibody and stained with antibodies that detect macrophages (anti-mCD11b), granulocytes (anti-mGr-1), and CD8+ T cells (anti-mCD8a; eBiosciences). Epithelial cells were stained for Annexin V-propidium iodide (PI), as per manufacturer’s instructions (BD Biosciences). Cells were analyzed using the fluorescence-activated cell sorting (FACS) Aria and FACS Diva software (BD Biosciences).

Luminex array

Plasma was analyzed using the Milliplex Map Mouse Cytokine/Chemokine Panel with Luminex Map detection as per manufacturer’s instructions (Millipore, #MPXMCYTO-70).

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were stained for F4/80 (eBiosciences), CD-31 (Dako), and alpha smooth muscle actin (α-SMA; Sigma-Aldrich) using standard procedures.

Human tissue array and scoring

Immunohistochemistry for Ron was conducted on human prostate cancer tissue microarray specimens (IMH-303, ImageX; TMA1202-4, Chemicon/Millipore; 75–4063, Zyomed), using the Ron-α antibody (BD-Transduction Laboratories). The percentage of Ron-positive stromal cells was determined and staining intensity graded (0–3). The stromal staining index, a factor of the staining percentage and intensity, was obtained for each tissue section.

Quantitative real-time PCR and immunoblot analyses

Quantitative real-time PCR (qRT-PCR) was conducted using primer pairs listed in the Supplementary Methods as previously described (20). Expression levels were normalized to β-glucuronidase and relative gene expression reported. Western blot analyses used the following antibodies: Ron-C20 (Santa Cruz Biotechnologies) STAT3 and pSTAT3-Y705 (Cell Signaling) and arginase-1 (BD Biosciences).

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined by t tests for pairwise comparisons or ANOVA for comparison of multiple groups with Graph Pad Prism software. Significance was set at P < 0.05.

Results

Ron is expressed in stromal cells of normal and tumor-bearing prostates and is critical for promoting prostate tumor growth

Ron mRNA expression was detected in primary cells isolated from the prostates of TK−/− mice including resident prostate macrophages, epithelial cells, and fibroblasts (Fig. 1A). Ron expression in human stromal cells was examined by immunohistochemistry on a panel of human prostate tissues. As depicted in Fig. 1B, Ron was detected in the stromal...
cells of normal human prostates, benign prostate hyperplasia, and prostate adenocarcinoma samples at approximately similar levels. Interestingly, Ron expression in stromal cells did not parallel its expression in the prostate epithelium, which has been previously shown to increase with disease progression (26). While the levels of stromal Ron are not altered with prostate tumor progression, the functional role of this receptor may be regulated by the availability/processing of its ligand, hepatocyte growth factor-like protein, whose expression has been reported to increase with tumor progression (36–39).

The role of Ron expression in the tumor microenvironment was assessed after orthotopic transplantation of TRAMP-C2Re3 prostate cancer cells into the prostates of TK^+/+ and TK^-/- mice. TRAMP-C2Re3 cells were able to grow in TK^-/- animals; however, growth was markedly inhibited in the TK^-/- host microenvironment with an average prostate weight of 1.10 ± 0.20 g in TK^-/- mice as compared with 0.27 ± 0.05 g in TK^-/- mice (Fig. 1C). Histologic analysis of the TRAMP-C2Re3 cells from the prostates of TK^-/+ and TK^-/- hosts revealed similarities in tissue architecture (Fig. 1D). These findings show that Ron expression in the prostate tumor microenvironment is a critical mediator of prostate tumor growth.

**Comparison of the stromal compartment in TK^-/+ and TK^-/- prostates following TRAMP-C2Re3 cell implantation**

To characterize the host environment following transplantation of TRAMP-C2Re3 cells into the prostates of TK^-/+ and

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**Figure 1.** Ron is expressed in stromal cells of normal and tumor-bearing prostates and is important for promoting prostate tumor growth. A, qRT-PCR analysis of Ron mRNA expression in cells isolated from TK^-/+ mice. The expression of Ron in whole TK^-/+ and TK^-/- prostates is shown for comparison. Expression levels are representative of two independent isolations. B, Ron expression in human prostate tissue specimens. A representative human prostate adenocarcinoma section depicting positive Ron staining in both epithelial (E) and stromal (S) compartments. The horizontal line in the scatter plot represents the mean ± SE. C, TRAMP-C2Re3 cells were injected into the prostates of TK^-/+ and TK^-/- mice; tumor weights were determined 30 days postimplantation (TK^-/+; n = 36; TK^-/-; n = 33; *, P < 0.0001). Red line, average weight (0.18 ± 0.012 g) of normal prostates from TK^-/+ mice. D, hematoxylin and eosin–stained TK^-/+ and TK^-/- prostate tumor sections showing similar tissue architecture. Scale bar, 100 μm.

TK−/− mice, histologic sections were stained for cellular markers. F4/80 staining showed significantly more macrophage infiltration in tumors from TK−/− hosts compared with tumors from TK+/+ hosts (Fig. 2A). Interestingly, tumor macrophage distribution was distinct within each group, with macrophages observed around the tumor periphery in prostates from TK+/+ mice as opposed to diffuse intratumoral macrophages in tumors from TK−/− mice. Flow analyses confirmed increased macrophage infiltration in tumors from TK−/− hosts compared with controls (Supplementary Fig. S1A). To examine Ron-dependent macrophage migration in vitro, the murine macrophage cell line, MH-S cells, without (shNT) and with Ron knockdown (shRon) were used (11). Loss of Ron in these cells significantly increased their migration potential compared with the controls (Supplementary Fig. S1B).

**Figure 2.** Characterization of stromal cells in TRAMP-C2Re3 tumors from TK+/+ and TK−/− prostates. A, representative F4/80 staining of TRAMP-C2Re3 tumors from TK+/+ and TK−/− mice. Differences in the spatial distribution of macrophages are noted with red arrows depicting tumor peripheral infiltration, whereas green arrows depict intratumoral infiltration. Macrophages were quantitated, and the data represent the mean number of F4/80+ macrophages per 40× field (5 fields per tumor, TK+/+, n = 5; TK−/−, n = 5; *P < 0.05). Scale bar, 50 μm. B, representative images showing CD31 staining in microvessels and quantification of the microvessel density per tumor area (*P < 0.01). Scale bar, 100 μm. C, α-SMA-positive fibroblasts in the tumors and the average intensity of α-SMA expression per ×40 field (*, P < 0.01). Scale bar, 50 μm.
The impact of stromal Ron on tumor vascularization was determined by microvessel staining, which showed a significant increase in microvessel density in tumors from TK<sup>+/+</sup> hosts compared with tumors in TK<sup>−/−</sup> mice (Fig. 2B). Immunohistochemistry for tumor-associated myofibroblasts showed an increase in α-SMA staining in tumors from TK<sup>−/−</sup> hosts as compared with controls (Fig. 2C). Taken together, our data show that Ron signaling in the host regulates key events linked with tumor malignancy including angiogenesis, α-SMA expression, and macrophage infiltration.

**Lack of host Ron expression promotes prostate cancer cell apoptosis**

Given the significant difference in tumor size in TK<sup>+/+</sup> versus TK<sup>−/−</sup> host environments, we examined tumor cell proliferation and death. There was no difference in proliferation of the transplanted TRAMP-C2Re3 cells (data not shown). However, a 2-fold increase in necrosis was noted in histologic sections of tumors from TK<sup>−/−</sup> compared with TK<sup>+/+</sup> hosts (Fig. 3A). To evaluate differences in survival between groups, tumor cells isolated from TK<sup>−/−</sup> hosts were isolated and examined by Annexin V/PI staining. A significant increase in the number of apoptotic (Annexin V−PI<sup>+</sup>) and dead cells (Annexin V−PI<sup>−</sup> and Annexin V−PI<sup>−</sup>) was detected in TRAMP-C2Re3 tumor cells isolated from TK<sup>−/−</sup> hosts compared with controls (Fig. 3B). These data are consistent with immunohistochemistry for cleaved caspase-3, which showed an appreciable increase in the number of activated/cleaved caspase-3−positive tumor cells from TK<sup>−/−</sup> hosts compared with controls (data not shown). Furthermore, Annexin V/PI staining on the F4/80-positive macrophages showed a trend toward increased cell death in the TK<sup>−/−</sup> macrophages compared with TK<sup>+/+</sup>. However, this latter observation may be attributed to the increased necrotic environment observed in tumors from TK<sup>−/−</sup> hosts (Supplementary Figs. S2 and S3A).

**Ron regulation of the inflammatory tumor microenvironment is associated with STAT3 activation in TAMs**

We next examined the inflammatory milieu in tumor-bearing TK<sup>+/+</sup> and TK<sup>−/−</sup> hosts. A 3-fold increase in iNOS mRNA was observed in tumors isolated from TK<sup>−/−</sup> mice compared with controls (Fig. 4A). Increased iNOS protein from tumors in TK<sup>−/−</sup> hosts was confirmed by Western blot analysis (data not shown). Blood nitrite levels were also increased in the sera from tumor-bearing TK<sup>−/−</sup> animals (Fig. 4B). Expression of interleukin (IL)-9, monokine induced by gamma interferon (MIG/CXCL9), and IL-17 was significantly greater in tumor-bearing TK<sup>−/−</sup> sera, whereas macrophage colony-stimulating factor (M-CSF) was higher in TK<sup>+/+</sup> sera (Fig. 4C). Intratumoral gene expression examined by qRT-PCR showed significant increases in MIG and TNF-α expression in tumors from TK<sup>−/−</sup> hosts as

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**Figure 3.** Lack of Ron signaling in the host leads to increases in tumor necrosis and apoptosis. A, quantification of necrotic areas in TRAMP-C2Re3 tumor sections from TK<sup>+/+</sup> and TK<sup>−/−</sup> hosts. Representative tissue sections are shown. Scale bar, 1 cm. B, flow analysis for Annexin V/PI on TRAMP-C2Re3 tumor cells isolated from TK<sup>+/+</sup> and TK<sup>−/−</sup> hosts. A representative flow plot is shown with a histogram depicting data from all experiments (TK<sup>+/+</sup>, n = 4; TK<sup>−/−</sup>, n = 3). Data are the mean percentage of cells in early apoptosis (Annexin V−PI<sup>+</sup>), late apoptosis (Annexin V−PI<sup>−</sup>), dead cells (Annexin V−PI<sup>−</sup>), and live/viable cells (Annexin V−PI<sup>+</sup>) ± SE.
well as a significant increase in TGF-β in tumors from TK⁺/⁺ hosts (Supplementary Fig. S3A). Significant increases in the intratumoral expression of STAT1, CXCR3, IL-12b, and ICOS were observed in tumor cells from TK⁻/⁻ hosts compared with controls, whereas CD80 and IL-27 levels did not drift (Fig. 4D).

To examine signaling pathways in the TK⁺/⁺ versus TK⁻/⁻ microenvironment, TAMs were isolated from TK⁺/+ and TK⁻/⁻ tumors. Western blot analysis of TAMs from TK⁻/⁻ hosts tumors showed a significant reduction in phosphorylated (p)STAT3 compared with controls (Fig. 4E). TAMs were isolated by CD11b coated magnetic bead pulldown assays and pSTAT3-Y705 and total STAT3 in TAMs, whereas CD80 and IL-27 levels did not change (data not shown). qRT-PCR analysis of TAMs from TK⁻/⁻ hosts compared with controls, whereas CD80 and IL-27 levels did not change (Fig. 4E). TAMs were isolated by CD11b coated magnetic bead pulldown assays and cells from this isolation included both CD11b⁺Gr1⁻ (TAMs) and CD11b⁺Gr1⁺ [myeloid-derived suppressor cells (MDSC)] cells. We refer to the CD11b⁺ population as TAMs hereafter as flow cytometry analysis showed a 7.3-fold higher number of CD11b⁺Gr1⁻ compared with CD11b⁺Gr1⁺ cells (data not shown). qRT-PCR analysis of flow-sorted immune cells showed Ron mRNA expression in the various myeloid cell populations including CD11b⁺Gr1⁻ (TAMs), CD11b⁺Gr1⁺ (MDSCs) and to a lesser extent in CD11b⁺Gr1⁻ (granulocytes; Supplementary Fig. S3B). Ron protein expression in TAMs isolated from TRAMP-C2Re3 tumors from TK⁺/⁺ mice is also depicted by Western blot analysis (Supplementary Fig. S3B).

**TK⁻/⁻ bone marrow-derived cells inhibit the growth of TRAMP-C2Re3 cells in chimeric TK⁺/⁺ mice**

Given the role of Ron in regulating macrophage activation as well as the increase in proinflammatory cytokines observed in the TK⁻/⁻ microenvironment, we next determined whether the hematopoietic compartment of TK⁻/⁻ mice is sufficient to inhibit TRAMP-C2Re3 tumor growth in vivo. Using reciprocal bone marrow transplantation experiments, TK⁺/⁺ and TK⁻/⁻ mice were irradiated and reconstituted with either TK⁺/⁺ or TK⁻/⁻ donor bone marrow to generate the following groups TK⁺/⁺→TK⁻/⁻ (irradiated TK⁺/⁺ mice transplanted with TK⁻/⁻ donor marrow), TK⁻/⁻→TK⁻/⁻, TK⁺/⁺→TK⁻/⁻, and TK⁻/⁻→TK⁺/⁺. Following bone marrow reconstitution, the chimeras were intraprostatically injected with TRAMP-C2Re3 cells and tumor weight was measured at 30 days. TRAMP-C2Re3 cells injected into nonirradiated TK⁺/⁺ and TK⁻/⁻ mice served as controls. qRT-PCR analysis for Ron expression on whole bone marrow–derived cells and on CD11b⁺ TAMs isolated from tumors indicated efficient reconstitution (82%) of donor-derived bone marrow cells into recipient animals (Fig. 5A and B). At baseline, no appreciable differences in the number of myeloid precursor cells were noted between genotypes (Supplementary Fig. S4).

Irradiation had no effect on the growth of TRAMP-C2Re3 cells (Fig. 5C). However, tumor growth was significantly inhibited in TK⁻/⁻ donor mice (Fig. 5D). TAMs isolated from TK⁺/⁺ and TK⁻/⁻ mice showed a 7.3-fold higher number of CD11b⁺/Gr1⁻ cells (Fig. 5E). Interestingly, TAMs isolated from nonirradiated TK⁻/⁻ hosts showed a significant reduction in phosphorylated pSTAT3 compared with controls (Fig. 4E).

**Figure 4.** Loss of Ron leads to changes in the inflammatory tumor microenvironment and loss of STAT3 phosphorylation in TAMs. A, qRT-PCR analyses for iNOS mRNA in TRAMP-C2Re3 tumors from TK⁺/⁺ and TK⁻/⁻ hosts. Data represented as relative change compared with β-glucuronidase (TK⁺/⁺, n = 5; TK⁻/⁻, n = 5; *, P < 0.01). B, concentration of serum nitrite from TRAMP-C2Re3 tumor-bearing TK⁺/⁺ and TK⁻/⁻ mice (TK⁺/⁺, n = 5; TK⁻/⁻, n = 5; *, P < 0.05). C, levels of inflammatory cytokines observed in the serum of tumor-bearing mice (TK⁺/⁺, n = 5; TK⁻/⁻, n = 5; *, P < 0.05). D, gene expression for T-cell regulatory genes depicted as fold change in TK⁻/⁻ tumors over controls. E, Western blot analysis is for pSTAT3-Y705 and total STAT3 in TAMs isolated from orthotopic TRAMP-C2Re3 tumors from TK⁺/⁺ and TK⁻/⁻ mice and is representative of 4 independent isolations. TAMs, tumor-associated macrophages.
delayed in TK<sup>−/−</sup>%TK<sup>+/+</sup> and TK<sup>−/−</sup>%TK<sup>+/+</sup> chimeras relative to TK<sup>−/−</sup>%TK<sup>−/−</sup> mice. Interestingly, tumor growth in TK<sup>−/+</sup>%TK<sup>−/−</sup> and TK<sup>−/−</sup>%TK<sup>−/−</sup> mice was not significantly different from growth in TK<sup>−/+</sup>%TK<sup>−/−</sup> mice. These data indicate that tumor growth in Ron-deficient animals is due to contributions by hematopoietic and nonhematopoietic cells in the host microenvironment, as TK<sup>−/+</sup> bone marrow–derived cells were not sufficient to restore tumor growth in TK<sup>−/−</sup> animals. In contrast, TK<sup>−/−</sup> bone marrow–derived cells significantly reduced tumor growth in TK<sup>−/+</sup> animals. Analysis of tumor infiltrates from the chimeric mice showed a significant infiltration of TK<sup>−/−</sup> donor-derived macrophages compared with TK<sup>−/+</sup> donor macrophages (Fig. 5D) similar to that observed with TK<sup>−/−</sup> macrophages from tumors of TK<sup>−/−</sup> mice (Fig. 2A and Supplementary Fig. S1).

**Myeloid-specific Ron loss is sufficient to decrease prostate tumor cell growth**

To delineate the Ron-expressing hematopoietic cell type that influences tumor growth, we crossed LysMcre<sup>−/−</sup> mice with TK<sup>−/−</sup> to generate TK<sup>−/−</sup>LysMcre<sup>−/−</sup> mice. LysMcre<sup>−/−</sup> mice specifically express Cre recombinase in myeloid cells and efficiently delete Ron in this cell population (19, 20). To test the efficiency of Ron deletion in TAMs, TRAMP-C2Re3 cells were intraprostatically injected into TK<sup>−/−</sup> control and TK<sup>−/−</sup>LysMcre<sup>−/−</sup> mice and TAMs were isolated 30 days posttransplantation. A dramatic loss of Ron mRNA expression (>90%) was observed in TAMs isolated from TK<sup>−/−</sup>LysMcre<sup>−/−</sup> tumors compared with TAMs from TK<sup>−/−</sup> tumors (Fig. 6A). To test the functional importance of Ron-expressing myeloid cells, prostate tumor weight was compared across genotypes. Growth of TRAMP-C2Re3 cells was significantly reduced in TK<sup>−/−</sup>LysMcre<sup>−/−</sup> animals compared with controls (Fig. 6B). Flow analysis for Annexin V/PI on tumors from TK<sup>−/−</sup>LysMcre<sup>−/−</sup> mice showed a significant increase in total apoptotic and dead cells compared with controls, signifying that lack of Ron in myeloid cells is sufficient to inhibit tumor growth through a mechanism that induces tumor-cell apoptosis (Fig. 6C). Immunohistochemistry for macrophages on tumors from TK<sup>−/−</sup> and TK<sup>−/−</sup>LysMcre<sup>−/−</sup> mice indicated a significant increase in macrophage numbers in TK<sup>−/−</sup>LysMcre<sup>−/−</sup> tumors compared with controls, thus reafﬁrming studies, whereby Ron-deﬁcient macrophages localize to the tumors in greater numbers (Fig. 6D). Moreover, iNOS expression was increased in tumors from TK<sup>−/−</sup>LysMcre<sup>−/−</sup> mice.
arginase-1 expression than TK f/f TAMs (Fig. 6F and G).

Cells was important for reduced tumor burden in TK

were observed in microvessel density of tumors transplanted

Despite the similarities in tumor growth, no differences

arginase-1, and decreased iNOS.

signaling in macrophages/myeloid cells promotes tumor

Supplementary Fig. S5). Combined, these studies show that Ron

TRAMP-C2Re3 tumors from TKf/f and

mice 23 days post–tumor cell implantation (TKf/f, n = 4; TKf/fLysMcre −, n = 4; P < 0.01). Data represent two independent experiments. C, FACS analysis for Annexin V/PI on epithelial cells isolated from TKf/f and TKf/fLysMcre − mice. D, quantification for F4/80 on TRAMP-C2Re3 tumors sections from TKf/f and TKf/fLysMcre − mice. Data represented as mean F4/80− cells per 40× field (TKf/f, n = 5; TKf/fLysMcre −, n = 5; P > 0.05). E, qRT-PCR for INOS expression in TRAMP-C2Re3 tumors from TKf/f and TKf/fLysMcre − mice. F, Western blot analysis of pSTAT3-Y705 and total STAT3 in TAMs isolated from TRAMP-C2Re3 tumors from TKf/f and TKf/fLysMcre − mice. G, Western blot analysis of arginase-1 expression in TAMs isolated from TRAMP-C2Re3 tumors from TKf/f and TKf/fLysMcre − mice. F and G are representative of four independent TAM isolations. TAMs, tumor-associated macrophages.

Figure 6. Loss of Ron signaling in myeloid cells is sufficient to abrogate TRAMP-C2Re3 tumor growth. A, Ron mRNA in TAMs isolated from TRAMP-C2Re3 tumors from TKf/f and TKf/fLysMcre − mice. Data represented as relative change from two independent TAM isolations. B, TRAMP-C2Re3 cells were injected into the prostates of TKf/f and TKf/fLysMcre − mice and tumor weight determined after 30 days (TKf/f, n = 15; TKf/fLysMcre −, n = 13; *, P < 0.001). Data represent two independent experiments. C, FACS analysis for Annexin V/PI on epithelial cells isolated from TKf/f and TKf/fLysMcre − mice, suggesting that myeloid-specific Ron deletion does not replicate a complete Ron deficiency (Supplementary Fig. S6C). As shown in Fig. 7A, TRAMP-C2Re3 cells grew similarly in the TKf/f and TKf/f mice as compared with tumors from TKf/f mice, suggesting that myeloid-specific Ron deletion does not replicate a complete Ron deficiency (Supplementary Fig. S5). Combined, these studies show that Ron signaling in macrophages/myeloid cells promotes tumor growth and is associated with STAT3 activation, increased arginase-1, and decreased iNOS.

CD8⁺ T cells are essential for the antitumor immune response in TK−/− animals

Flow cytometry and qRT-PCR analyses of tumors for T cells showed an increase in CD8⁺ T cells in tumors from TK−/− hosts compared with the controls, although this number was not significant (Supplementary Fig. S6A and S6B). To determine if macrophage-mediated education of CD4⁺ and CD8⁺ T cells was important for reduced tumor burden in TK−/− mice, mice were depleted of CD4⁺, CD8⁺, or both T-cell subsets by injection of cytotoxic monoclonal antibodies. To allow for accurate measurement of tumor growth, TRAMP-C2Re3 cells were injected subcutaneously and tumor growth was assessed biweekly. The efficiency with which T-cell subsets were depleted was determined by flow analysis (>99%) for T-cell subset markers on splenocytes obtained at the time of sacrifice (Supplementary Fig. S6C). As shown in Fig. 7A, TRAMP-C2Re3 cells grew similarly in the TK−/− and TK⁺/− mice in the subcutaneous environment compared with the prostate microenvironment (Fig. 1C), wherein a 3-fold increase in tumor growth was observed in the TK−/− mice as compared with TK⁺/− mice; however, TRAMP-C2Re3 cells implanted orthotopically grew approximately 2-fold faster than cells implanted subcutaneously, despite the reduced inoculum cell numbers (40).

Depletion of CD4⁺ T cells had a limited effect on tumor growth in both genotypes, as tumor growth in TK−/− versus TK⁺/− mice was similar to that of isotype-treated controls (Fig. 7A and B). In contrast, depletion of CD8⁺ T cells restored tumor growth in TK−/− animals to that of controls. Depletion...
of CD4+ and CD8+ T-cell subsets resulted in similar growth rates in TK+/+ and TK− mice; however, a dramatic increase in tumor growth was observed in both genotypes.

Depletion of CD8+ T cells in TKfloLysMcre+ mice restores TRAMP-C2Re3 tumor growth

To determine if Ron loss in macrophages was sufficient to engage an antitumor immune response, CD8+ T-cell depletion studies were conducted in TK+/ and TKfloLysMcre- tumor-bearing mice. Depletion of CD8+ T cells in TKfloLysMcre mice abolished the antitumor immune response (Fig. 7C and D). Taken together, our results indicate that Ron expression in the tumor microenvironment, specifically in macrophages, aids in evasion of tumor immune surveillance by impacting CD8+ T-cell function.

Discussion

Tumors are regulated by complex signaling networks involving interactions between malignant epithelial cells and the adjacent stroma. While many publications have reported the importance of tumor-stromal interactions for supporting tumor growth, further investigations are required for the identification of the stromal cellular mediators and their associated signaling networks that promote tumorigenesis. In this report, we provide compelling evidence supporting the novel role for the Ron receptor in regulating tumor growth by virtue of its signaling in host bone marrow–derived cells, particularly myeloid cells, of the tumor microenvironment.

Myeloid cells and bone marrow–derived precursors are recruited to tumors and promote tumor growth, neovascularization, and aid in antitumor immune suppression (3, 41). Our
experiments indicate that Ron loss in macrophages increases their localization to tumors and is associated with decreased tumor growth. Interestingly, this influx of macrophages into tumors was only observed in the presence of TK−/− bone marrow–derived cells, even in the context of bone marrow transplantation experiments (Figs. 2A, 5D, and 6D). Furthermore, TK−/− mice were on par with wild-types as they exhibited similar numbers of bone marrow–derived cells and hematologic values (Supplemental Fig, S4 and ref. 33). These results suggest that Ron deficiency does not affect the number and development of the hematopoietic cell lineages, but instead improves localization of macrophages to tumors. Loss of Ron expression in the host environment also resulted in an increase in the expression of inflammatory cytokines including IL-9, MIG, IL-17, and TNF-α, which are known to promote macrophage infiltration and inhibit tumor growth through mechanisms that regulate phagocytic activity or support the activation of effector cells of the innate and adaptive immune responses. Alternatively, the increase in TK−/− TAMs in either host genotype may be due to the need to clear cellular debris. The underlying mechanism that leads to the increased localization of macrophages to tumors in the presence of TK−/− bone marrow–derived cells as well as the functional significances of increased macrophage numbers on tumor growth warrants further investigation.

In addition to the increased influx of Ron-deficient macrophages to tumors, our data show that a conditional loss of Ron in myeloid cells is sufficient to inhibit tumor growth in vivo. These studies are analogous to several previous studies, which showed decreased tumor growth in mice harboring myeloid-specific deletions of inhibitor of 1B kinase-β (IKK-β) (ref. 42), STAT3 (43, 44), hypoxia-inducible factor-1α (HIF-1α; ref. 45), and HIF-2α (46). A previous study on IKK-β has shown that deletion of this gene in either epithelial cells (enterocytes) or myeloid cells reduces colitis-associated cancer. In this study, enterocyte IKK-β expression promoted tumorigenesis by suppressing apoptosis, whereas myeloid expression enhanced tumor growth by controlling the production of inflammatory mediators, showing distinct roles for the protein in different cell types (42). The cell-type specific regulation of tumor growth by Ron is similar to that observed for IKK-β. Published studies have shown that loss of Ron in prostate cancer epithelial cells leads to a reduction in tumor growth by promoting epithelial cell apoptosis and limiting tumor vascularization (26, 32). In myeloid cells, Ron loss leads to increased tumor cell apoptosis associated with alterations in the inflammatory microenvironment and is independent of an effect on blood vessel formation. Of note, our data using bone marrow transplantation in chimeric mice suggested that in addition to hematopoietic cells, other nonhematopoietic Ron-expressing stromal cell types contribute to tumor progression. These data reinforce the notion of multiple cell-type specific roles for Ron in promoting tumor growth.

Previous studies from our laboratory in peritoneal macrophages have shown that Ron activation enhances macrophage STAT3 phosphorylation, whereas macrophages from TK−/− mice exhibit decreased STAT3 activation (17). Consistent with this data, our current study shows that Ron loss is associated with lower STAT3 activity in TAMs. STAT3 signaling in macrophages attenuates antitumor immunity in a variety of in vivo tumor models (23, 47). Similar to the studies in this report, disruption of STAT3 in myeloid cells through the use of LysMcre mice enhanced the ability of macrophages to stimulate CD8⁺ T-cell activity in response to tumor antigens (23). Ron signaling may also regulate T-cell activity through the induction of T-cell anergy by downregulating the expression of costimulatory molecules or regulating cytokine/chemokine production. A significant induction in costimulatory molecules CXCR3 and ICOS as well as an induction of IL-9, iNOS, and MIG were observed in tumors taken from TK−/− compared with wild-type hosts, suggesting multiple components downstream of Ron that may regulate T-cell activity. While further studies are needed to determine the mechanism(s) by which myeloid-specific Ron signaling regulates tumor growth, our experiments with depletion of CD8⁺ T cells in TK−/−LysMcre⁺ mice suggest that Ron loss in myeloid cells leads to enhanced tumor cell death through a mechanism that may depend upon STAT3 signaling and the activation of the immune response. Moreover, given the lack of current technologies to effectively target transcription factors such as STAT3, targeting Ron may serve as an important surrogate to activate the immune response for cancer therapy.

In addition to alterations in the immune response, Ron signaling has been shown to regulate key metabolites that support an immunosuppressive tumor microenvironment. Ron activation in macrophages results in the inhibition of iNOS and increased arginase-1 expression (15, 33). iNOS expression in tumor tissues has been shown to be either pro- or antitumorigenic, depending on the level of expression and the duration of NO (48). Consistent with published observations, Ron loss in the tumor microenvironment increased local expression of iNOS. This increase in iNOS expression may be partly attributed to the expression of Ron in myeloid cells, as tumors from TK−/−LysMcre⁺ mice also displayed increases in iNOS levels compared with controls. Taken together, our data suggest that Ron expression in myeloid cells is important for regulating the fine balance of iNOS expression, which may skew the microenvironment toward a tumor-promoting state. Conversely, expression of arginase-1 has been reported to protect tissue from damage. Consistently, our data show that Ron-deficient TAMs exhibited significant decreases in arginase-1 levels. The downregulation of arginase-1 in Ron-deficient TAMs may be significant, as arginase-1 and arginine have been shown to be important in regulating a variety of T-cell activities including proliferation, differentiation, and activation (49, 50).

In summary, our data suggest a new role for Ron in regulating tumor intrinsic and extrinsic circuits within the tumor microenvironment. In light of immune impairment in individuals with cancer, short-term blockade of Ron in a controlled manner, such as the infusion of Ron-inhibited macrophages, may reverse the immunosuppressive microenvironment and activate antitumor immune responses. Further research in dissecting bidirectional signaling and the context-dependent role of Ron in cancer may provide a novel therapeutic strategy to improve the efficacy of cancer therapy.
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No potential conflicts of interest were disclosed.

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Myeloid-Specific Expression of Ron Receptor Kinase Promotes Prostate Tumor Growth

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