TGF-β Signaling in Myeloid Cells Is Required for Tumor Metastasis

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ABSTRACT

TGF-β is overexpressed in advanced human cancers. It correlates with metastasis and poor prognosis. However, TGF-β functions as both a tumor suppressor and a tumor promoter. Here, we report for the first time that genetic deletion of Tgfbr2 specifically in myeloid cells (Tgfbr2^{Myleko}) significantly inhibited tumor metastasis. Reconstitution of tumor-bearing mice with Tgfbr2^{Myleko} bone marrow recapitulated the inhibited metastasis phenotype. This effect is mediated through decreased production of type II cytokines, TGF-β1, arginase 1, and inducible nitric oxide synthase, which promoted IFN-γ production and improved systemic immunity. Depletion of CD8 T cells diminished the metastasis defect in the Tgfbr2^{Myleko} mice. Consistent with animal studies, myeloid cells from patients with advanced-stage cancer showed increased TGF-β receptor II expression. Our studies show that myeloid-specific TGF-β signaling is an essential component of the metastasis-promoting puzzle of TGF-β. This is in contrast to the previously reported tumor-suppressing phenotypes in fibroblasts, epithelial cells, and T cells.

SIGNIFICANCE: Our study identifies myeloid-specific TGF-β signaling as a critical mediator in tumor metastasis, distinct from the tumor-suppressive effect of TGF-β signaling in epithelial cells, fibroblasts, and T cells. We further provide mechanistic insight into host antitumor immunity and suggest a cell type–specific cancer-targeting strategy. Cancer Discov; 3(8); 936–51. ©2013 AACR.


INTRODUCTION

TGF-β signaling plays an important role in tumor initiation and progression. Paradoxically, TGF-β can function as both a tumor suppressor and promoter (1, 2). The mechanisms underlying the dual role of TGF-β are very intricate and are poorly understood. In the past, most of the work dissecting the molecular mechanisms was focused on differential regulation of signaling pathways by tumor autonomous TGF-β signaling and cross-talk with other signaling networks (2–4). It was thought that changes in the signal intensity and connectivity of SMAD-dependent and -independent pathways, including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), as well as small GTPases, might explain the complex transition of TGF-β from a tumor suppressor to a tumor promoter (5).

Interestingly, disruption of TGF-β signaling in a number of epithelial cells results in a more malignant tumor phenotype in breast, intestinal, pancreatic, and colon cancers, and in head and neck squamous cell carcinoma (6). Deletion of Tgfbr2, the gene encoding TGF-beta receptor II (TβRII), in FSP1+ fibroblasts induces the development of invasive squamous cell carcinoma in the forestomach and intraepithelial neoplasia in the prostate (7, 8). In addition, deletion of SMAD4, an important downstream mediator of TGF-β signaling in T cells, induces development of gastrointestinal cancers (9). These studies suggest that TGF-β signaling in epithelial cells, fibroblasts, and T cells has a tumor-suppressive function. Recent work from our group and others showed that downregulation of TGF-β signaling, a frequent event observed in many tumor types, leads to increased CXCL1–CXCL5/CXCR2 and SDF-1/CXCR4 chemokine/chemokine receptor signaling, and subsequent recruitment of host-derived immature myeloid Gr-1+CD11b+ cells or myeloid-derived suppressor cells (MDSC) and macrophages (10, 11) into tumors. These infiltrating myeloid cells produce large quantities of TGF-β1 and matrix metalloproteinases (MMP) that suppress the host immune system and concurrently promote tumor invasion (10).

Myeloid cells play an important role in tumor progression. They suppress host immune surveillance (12–15) and influence the tumor microenvironment (10, 13, 14, 16). These cells are also present in the lungs before tumor cell arrival and contribute to premetastatic niche formation (17) and alteration of the local lung environment (18). These cells include tumor-associated macrophages (TAM; Mac-1+ or F4/80+ cells; ref. 14), Gr-1+CD11b+ MDSCs (12), and tumor-associated neutrophils (CD11b+Ly6G+; ref. 15). One of the most important properties of these cells is increased TGF-β production (10, 19). In fact, depletion of Gr-1+CD11b+ cells diminished the antitumor effect of TGF-β neutralization, suggesting that immature Gr-1+CD11b+ cells are responsible for the tumor-promoting effect of TGF-β in breast cancer progression (20). However, it is not known how TGF-β signaling in myeloid cells affects tumor phenotype. Delineation of TGF-β pathways in myeloid cells may unravel the paradoxical role of TGF-β in cancer.
report, we show that TGF-β signaling in myeloid cells of tumor-bearing hosts is fundamentally important for tumor metastasis. Genetic deletion of Tgfbr2 specifically in myeloid cells dramatically decreases tumor metastasis. Our data implicate myeloid TGF-β signaling as a potential novel therapeutic target.

**RESULTS**

**Increased Expression of TβRII in Myeloid Cells under Tumor Conditions, and LysM-Cre–Mediated Myeloid-Specific Tgfbr2 Deletion**

To assess the role of TGF-β signaling in tumor-associated myeloid cells, we used Gr-1+CD11b+ cells as samples for myeloid cells, as they constitute the majority of tumor-associated myeloid cells and produce high levels of TGF-β1. We used murine 4T1 mammary tumor and Lewis lung carcinoma (LLC) mouse models that are in Balb/c and C57Bl/6 backgrounds, respectively. For both models, we found that splenic Gr-1+CD11b+ cells from tumor-bearing mice express significantly higher levels of TβRII compared with their non-tumor-bearing counterparts (Fig. 1A and B; data not shown for LLC model). The impact of elevated TβRII expression is likely amplified, as the frequencies of these myeloid cells are also increased in the bone marrow, spleen, and peripheral blood of tumor-bearing mice (Supplementary Fig. S1A).

The overproduction of immature myeloid cells has also been reported in patients with a variety of cancers (16, 21), in which they are identified as CD33+, CD34+, or CD15+ cells (16, 22, 23). We used these markers to enrich the myeloid cells from the peripheral blood of 16 patients with metastatic non–small cell lung cancer. These myeloid cells, which include granulocytes, monocytes, and their precursors, accounted for approximately 82% of the total leukocytes, compared with 72% from healthy individuals (n = 11; P < 0.05; Supplementary Fig. S1B). Sorted myeloid cells from patients showed increased TβRII expression compared with healthy individuals (Fig. 1C). Further examination of human breast cancer and lung cancer datasets using GeneSpring GX 10.0 software showed a significant increase in TβRII and TGF-β1 mRNA in human peripheral blood mononuclear cells of patients with breast cancer compared with normal individuals (Fig. 1D; GSE27567; ref. 24). In lung cancer, TGF-β1 was increased at stages IIIA and IV compared with stages I and II, and correlated well with the modestly increased TβRII mRNA level (P > 0.05, but with a trend of increase) in the lung cancer patient cohort (GSE20189; ref. 25; Fig. 1D). Together, these data suggest that increased TβRII expression correlates with cancer progression in a clinical setting.

The overexpression of TβRII in both human and mouse myeloid cells from cancer hosts raised the possibility that TβRII signaling in myeloid cells affects tumor progression and metastasis. Although TGF-β3 has been recently implicated in tumor progression (26, 27), the TGF-β–induced and TGF-β3–mediated responses have not been shown to be independent of TβRII. On the other hand, overwhelming data from the field support a central role of TβRII in TGF-β signaling. Thus to test our hypothesis, mice with a targeted deletion of Tgfbr2 in myeloid cells (Tgfbr2<sup>myeKO</sup>) were generated through the cross-breeding of floxed Tgfbr2 (Tgfbr2<sup>lox/lox/­fox</sup>) mice with LysM-Cre transgenic mice. LysM-Cre transgenic mice have been well characterized and used in many studies to delete genes specifically in myeloid cells (28, 29). Indeed, sorted Gr-1+CD11b+ cells, but not B cells (B220+) or T cells (CD3+, more than 95% purity; Supplementary Fig. S1C) from Tgfbr2<sup>myeKO</sup> mice showed Tgfbr2 recombination (Fig. 1E), clearly decreased TβRII expression, and phosphorylation of SMAD2 (Fig. 1F and G). These data support that Tgfbr2 deletion was efficient and specific in myeloid cells.

**Deletion of Tgfbr2 in Myeloid Cells Significantly Inhibited Tumor Metastasis**

Tgfbr2<sup>myeKO</sup> mice appeared phenotypically normal with no alteration in the number or percentage of T (CD4+ and CD8+), B, natural killer (NK), or Gr-1+CD11b+ and F4/80+ cells in bone marrow, spleen, thymus, and lymph node (data not shown). However, Tgfbr2<sup>myeKO</sup> mice showed a decreased ability to develop tumor metastasis following injection of 4T1 mammary tumor cells into the #2 mammary fat pads (MFP, Fig. 2A), with a modest effect on 4T1 primary tumor growth (P > 0.05) and later stage of LLC growth (P < 0.05; Supplementary Fig. S2A and S2B). The 4T1 mammary tumor model shares many characteristics with human breast cancer, particularly its ability to spontaneously metastasize to the lungs. In an experimental metastasis design in which 2 × 10<sup>5</sup> 4T1 cells were injected into the tail vein, there was also a significant reduction in metastasis in Tgfbr2<sup>myeKO</sup> mice (Fig. 2B). This result was recapitulated in the B16 melanoma orthotopic and LLC experimental metastasis models in Tgfbr2<sup>myeKO</sup> mice in a C57Bl/6 background (Fig. 2C and D). Similar to the 4T1 tumor model, the primary tumor growth of B16 cells was not different between Tgfbr2<sup>myeKO</sup> and control mice (data not shown). Together, these data suggest that the major effect of myeloid Tgfbr2 deletion is on tumor metastasis. This was supported by two additional experimental metastasis tumor models, MC26 colon cancer and B16 melanoma, which showed a significantly decreased number of lung metastases in Tgfbr2<sup>myeKO</sup> mice compared with controls after tail vein injection of the tumor cells (Supplementary Fig. S2C and S2D).

To further confirm the inhibitory effect of myeloid-specific Tgfbr2 deletion on tumor metastasis, we transplanted bone marrow from Tgfbr2<sup>myeKO</sup> mice into wild-type mice bearing 4T1 tumors. To better model clinical metastatic disease, the primary tumor was surgically removed on day 15 and metastasis was allowed to continue until day 34, when the animals were irradiated and subjected to bone marrow transplantation (Fig. 2E). In these therapeutic experiments, 4T1 tumors were injected into the #4 MFP to allow surgical removal of tumors. We observed 100% survival of the mice that received bone marrow from Tgfbr2<sup>myeKO</sup> mice, whereas approximately 55% of the mice that received bone marrow from Tgfbr2<sup>lox/­fox</sup> control mice showed decreased survival (Fig. 2F). In addition, a significant reduction in the number of lung metastases was observed in mice that received Tgfbr2<sup>myeKO</sup> bone marrow relative to those that received control bone marrow (Fig. 2F). These data suggest that myeloid-specific TGF-β signaling constitutes an essential part of the metastasis-promoting role of TGF-β.
Myeloid-Specific TGF-β Signaling Promotes Metastasis

Figure 1. Increased expression of TβRII in myeloid cells under tumor conditions and mouse models for myeloid-specific deletion of Tgfbr2. A and B, Western blotting and immunofluorescence of TβRII. Sorted splenic Gr-1+CD11b+ cells were used for protein extraction or cytospin slides. Cells from mice bearing 4T1 tumors (Tu) were compared with nontumor-bearing normal mice (Nor). Scale bar, 20 μm. C, TβRII Western blotting of sorted human blood myeloid cells enriched using CD33+, CD34+, or CD15+ markers, from both normal individuals and patients with late-stage lung cancer. Right, magnetic-activated cell sorting of the myeloid cells, before (left) and after (right) sorting. D, heatmap (top) and dot plots (bottom) of Tgfb1 and Tgfbr2 mRNA in human peripheral blood mononuclear cells in cohorts of breast (GSE27567) and lung (GSE20189) cancers. The datasets were analyzed using GeneSpring GX 10.0 software. Red and green colors, increased and decreased expression, respectively. *, P < 0.05. E, Southern hybridization showing specific deletion of Tgfbr2 in sorted myeloid cells from 4T1 tumor-bearing Balb/c mice, but not in T or B cells. WT, wild-type. F, immunofluorescence of TβRII (red color) in splenic Gr-1+CD11b+ cells from Tgfbr2flox/flox and Tgfbr2flox/null mice bearing LLC (left) and 4T1 tumors (right). G, Western blotting of TβRII, p-SMAD2, and SMAD2 in sorted splenic Gr-1+CD11b+ cells from Tgfbr2flox/fl and Tgfbr2flox/null mice in Balb/c background bearing 4T1 tumors.
Figure 2. Deletion of Tgfbr2 in myeloid cells significantly inhibited tumor metastasis. **A, lung metastasis (mets) in Tgfbr2^{flox/flox} and Tgfbr2^{MyeKO} control mice 28 days after 4T1 injection (5 \times 10^4) in #2 MFP (n = 6 for each group). Shown is one of four experiments carried out. *, P < 0.05. **B, hematoxylin and eosin staining of representative butterfly sections of the lungs showing dramatic reduction of 4T1 lung metastasis in Tgfbr2^{MyeKO} mice (n = 12) compared with Tgfbr2^{flox/flox} mice (n = 5) receiving tail vein injection of 4T1 cells (2 \times 10^5) for 25 days. Quantitative data are on the right. ***, P < 0.01. **C, decreased metastasis in Tgfbr2^{MyeKO} mice following subcutaneous injection of B16 melanoma cells (1 \times 10^6; n = 7). Tumors were resected on day 16, lungs were harvested on day 37. *, P < 0.05. **D, a dramatic reduction of lung metastasis in Tgfbr2^{MyeKO} mice on a C57BL/6 background that received tail vein injection of LLC cells (2.5 \times 10^5; n = 5–6 mice). Quantitative data are on the right. One of two experiments is shown. ***, P < 0.01. **E, schematic experimental design for adoptive transfer of Tgfbr2^{MyeKO} bone marrow to wild-type 4T1 tumor-bearing mice. F, Kaplan-Meier survival curve (left) and metastasis counts (right) in mice that received bone marrow transplant from Tgfbr2^{MyeKO} and floxed control mice. Shown is one of two experiments carried out. *, P < 0.05. All data are represented as mean ± SEM.
Myeloid-Specific TGF-β Signaling Promotes Metastasis

Deletion of Tgfbr2 in Myeloid Cells Decreased Type II Cytokine Expression and Increased IFN-γ Production and Host Antitumor Immunity

TGF-β signaling is a critical mediator of immune cell polarization (19). It is not clear whether it has similar function in myeloid cells. We examined type I and II cytokine expression in sorted Gr-1+CD11b+ cells. Interestingly, the expression of type II cytokines, including interleukin (IL)-10 and IL-4, was reduced in myeloid cells with Tgfbr2 deletion compared with controls, with no difference in type I cytokine production (e.g., IL-12 and TNF-α; Fig. 3A). There was also reduced expression of arginase 1 and inducible nitric oxide synthase (iNOS; Fig. 3A), the critical mediators implicated in the immune suppression effects of Gr-1+CD11b+ cells. The decreased expression of type II cytokines was further confirmed in a cytokine protein array (Fig. 3B, top; with semiquantitative data of dot density at the bottom). Deletion of myeloid Tgfbr2 also decreased TGF-β1 production in sorted Gr-1+CD11b+ cells (Fig. 3C), suggesting an autocrine effect of TGF-β signaling. The decreased expression of iNOS and arginase 1 is consistent with decreased nitric oxide (NO) production and arginase 1 activity (Fig. 3D).

Tumor-associated myeloid cells inhibit multiple immune cell functions in tumor hosts (12). We therefore examined whether deletion of myeloid-specific Tgfbr2 resulted in improved immune function of CD4+, CD8+, B, NK, or macrophage cells. We observed an increased percentage of IFN-γ-positive (IFN-γ+) CD8+ T cells in the spleen of tumor-bearing Tgfbr2MyeKO mice compared with Tgfbr2flox/flox mice (Fig. 4A). No difference was found in other cytokines or cell types (data not shown). This was consistent with the increased number of IFN-γ-producing cells detected by enzyme-linked immunosorbent spot (ELISpot) in the spleens of Tgfbr2MyeKO mice (Supplementary Fig. S3A). To examine whether the enhanced T-cell immunity is antigen-specific, we cocultured Gr-1+ myeloid cells sorted from Tgfbr2MyeKO mice with T cells from OT1 transgenic mice, and observed increased IFN-γ production when we pulsed the T cells with a specific ovalbumin (OVA) peptide (Fig. 4B). Importantly, systemic neutralization of IFN-γ diminished the inhibitory effect of myeloid Tgfbr2 deletion on metastasis (Fig. 4C), with no significant effect on primary tumor size (Supplementary Fig. S3B).

We next overexpressed IL-4, IL-10, and TGF-β1, as well as arginase 1 and iNOS, in sorted myeloid cells with Tgfbr2 deletion (Supplementary Fig. S4A and S4B) to see whether this would result in a reduction of the IFN-γ-producing T cells. We found that overexpression of all IL-4, IL-10, and TGF-β1, or both arginase 1 and iNOS, but not either one alone in sorted Gr-1+CD11b+ cells with Tgfbr2 deletion decreased the number of IFN-γ+ T cells in the coculture by ELISpot assay (Fig. 5A and B), which was not the case in Gr-1+CD11b+ cells sorted from floxed control mice (data not shown). These data, together with the results showing that myeloid cells with Tgfbr2 deletion have decreased IL-4, IL-10, and TGF-β1, as well as arginase 1 and iNOS (Fig. 3A), suggest that IL-4+, IL-10+, TGF-β1-, arginase 1-, and iNOS-mediated mechanisms play a major role in enhancing IFN-γ-producing CD8+ T cells as a result of inhibition of TGF-β signaling in Gr-1+CD11b+ cells.

Local Innate Immunity in Premetastatic Lung Microenvironment of Tgfbr2MyeKO Mice

Tgfbr2MyeKO mice showed a decreased ability to develop tumor metastasis following tail vein injection of a number of tumor cell types, including 4T1 (Fig. 2B), LLC (Fig. 2D), MC26, and B16 (Supplementary Fig. S2C and S2D). We suspected there was an altered lung microenvironment in Tgfbr2MyeKO mice. When we examined the percentage and the number of B, CD4+ T, CD8+ T, and regulatory T cells (Treg), as well as different myeloid subsets including CD11b+Ly6G+ neutrophils, CD11b+Ly6C+ monocytes, CD11b+Ly6C–Ly6G+ macrophages, and CD11b+CD11c+ dendritic cells, no difference was found between Tgfbr2MyeKO and Tgfbr2flox/flox mice (Supplementary Fig. S5). This was true for both normal (Supplementary Fig. S5A) or tumor conditions (Supplementary Fig. S5B). In addition, the production of IFN-γ was similar in lung-residing CD8+ T cells in tumor-bearing Tgfbr2MyeKO and Tgfbr2flox/flox mice (Supplementary Fig. S5C). Interestingly, Tgfbr2 deletion in myeloid cells increased IFN-γ production in one subset of CD11b+Ly6C– cells (Fig. 5C, left and middle), but not other subsets (Supplementary Fig. S6A). These CD11b+Ly6C– cells express F4/80 and Ly6G and, thus, are likely macrophages and neutrophils (Fig. 5C, right). The production of the IFN-γ in these myeloid cells is likely mediated by IL-12 and IL-18, as neutralization of both IL-12 and IL-18 decreased IFN-γ levels in these myeloid cells stimulated with lipopolysaccharide (Supplementary Fig. S6B). This is consistent with previous reports (30, 31).

In addition, CD11b+CD11c+ cells in the lungs of Tgfbr2MyeKO mice showed increased expression of the costimulatory molecule CD86 (Supplementary Fig. S7), which was not observed in tumor tissues, spleens, or draining lymph nodes of tumor-bearing mice (Supplementary Fig. S7). This suggests an increased functional maturation of CD11b+CD11c+ myeloid cells in Tgfbr2MyeKO mice compared with control littermates. Therefore, our data suggest that improved innate immunity in the lung environment of Tgfbr2MyeKO mice may have a critical role in the decreased metastasis phenotype in these mice.

In contrast to metastasis, myeloid Tgfbr2 deletion had little effect on primary tumor growth. We carefully examined the number and percentage of infiltrating immune cells, including B, CD4+, and CD8+ T cells, Tregs, as well as different myeloid subsets including CD11b+Ly6G+ neutrophils, CD11b+Ly6C+ monocytes, CD11b+Ly6C–Ly6G+ macrophages, and CD11b+CD11c+ dendritic cells. Again, no difference was found between Tgfbr2MyeKO and Tgfbr2flox/flox mice (Supplementary Fig. S8A). In addition, there was no difference in IFN-γ production in CD8+ T cells (Supplementary Fig. S8B, left), or in myeloid cells (Supplementary Fig. S8B, right); the latter is different from our observations of the lung. Thus, despite a systemic increase in IFN-γ+CD8+ T cells and a local increase in IFN-γ+CD11b+Ly6G+ or IFN-γ+CD11b+Ly6C–Ly6G+ cells in the lung, the tumor microenvironment remained a sanctuary site to protect tumor cells from host antitumor immunity.
Figure 3. Myeloid-specific deletion of Tgfbr2 reduced the expression of type II cytokines, TGF-β1, arginase, and iNOS in myeloid cells. A, quantitative PCR of type I and II cytokines, arginase 1, and iNOS expression in Gr-1+ myeloid cells sorted from the spleens of Tgfbr2MyeKO and control mice bearing 4T1 tumors. Shown is the mean ± SEM of three samples per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, cytokine protein array analysis of sorted Gr-1+ myeloid cells (top), with semiquantitative data of dot density below. Shown is one of two experiments carried out. ***, P < 0.001. C, TGF-β1 ELISA of Gr-1+ myeloid cells sorted from the spleen of 4T1 tumor-bearing mice. Samples from three mice were collected and triplicates per sample were analyzed. D, decreased NO production and arginase 1 function. Samples were from 4T1 tumor-bearing Tgfbr2MyeKO and control mice. *, P < 0.05; **, P < 0.01.
Myeloid-Specific TGF-β Signaling Promotes Metastasis

Different myeloid cell subsets have been implicated in tumor progression. To investigate which subset of myeloid cells might be responsible for the decreased metastasis as a result of Tgfbr2 deletion, we sorted CD11b+Ly6G+, CD11b+Ly6C+, and CD11b+F4/80+ cells (Supplementary Fig. S9A and S9B). We first examined Tgifrii expression. We found that the CD11b+Ly6G+ subset expressed higher levels of Tgifrii compared with the CD11b+Ly6C+ and CD11b+F4/80+ subsets (Supplementary Fig. S9C). Consistent with this, CD11b+Ly6G+ cells with Tgfbr2 deletion also showed significantly decreased production of TGF-β1 and IL-10, as well as modestly decreased IL-4 (Fig. 6A), which is not observed in CD11b+Ly6C+ monocytes or CD11b+F4/80+ macrophages (Fig. 6A). The expression of iNOS and arginase I was decreased in both CD11b+Ly6G+ neutrophils and CD11b+Ly6C+ monocytes (Fig. 6B). In addition, both CD11b+Ly6G+ and CD11b+Ly6C+ myeloid cells showed inhibition of NO production when cocultured with T cells (Fig. 6C). These data suggest that TGF-β signaling affects the properties and function of both CD11b+Ly6G+ and CD11b+Ly6C+ myeloid subsets. However, it should be noted that the percentage of CD11b+Ly6G+ cells in Gr-1+ cells is significantly higher than the CD11b+Ly6C+ in the 4T1 tumor model (32). Therefore, the effect from the CD11b+Ly6G+ cells is likely major.

To further confirm the effect of these myeloid cell subsets in vivo, we injected sorted wild-type myeloid cell subsets into 4T1 tumor-bearing Tgfbr2−/− mice after injection with IFN-γ neutralizing antibody or immunoglobulin G (IgG) control. *, P < 0.05; ns, not significant; Ab, antibody.

Figure 4. Deletion of Tgfbr2 in myeloid cells elevated IFN-γ production in CD8+ T cells, and systemic IFN-γ neutralization diminished metastasis inhibition in Tgfbr2−/− mice. A, flow cytometry of IFN-γ intracellular staining of CD8+ T cells from the spleens of Tgfbr2−/− and Tgfbr2−/− mice bearing 4T1 tumors (left). Quantitative data are on the right. Shown is one of two experiments carried out. *, P < 0.05. B, IFN-γ ELISPOT of T cells from TGF-β−/− mice when cocultured with IFN-γ neutralizing antibody or IgG control. *, P < 0.01. C, lung metastasis (nps) of 4T1 tumor-bearing Tgfbr2−/− and Tgfbr2−/− mice after injection with IFN-γ neutralizing antibody or IgG control. *, P < 0.05; ns, not significant; Ab, antibody.
inhibiting metastasis in tumor models studied, such as MC26 colon cancer, LLC, constitute the majority of myeloid cells, including at least eight in inhibiting metastasis in Figure 5. Mechanisms of myeloid TGF-β-mediated immunosuppressive function. A and B, quantitative results of IFN-γ ELISPOT of T cells when cocultured with sorted Gr-1+ cells (from Tgfb2MyeKO mice) with overexpression of IL-4, IL-10, and TGF-β (A) or arginase 1 and iNOS (B). Gr-1+ cells were sorted from the spleens of 4T1 tumor-bearing mice in a Balb/c background. T cells were isolated from the spleens of CL4 transgenic mice. Shown is one of two experiments carried out. *, P < 0.05; **, P < 0.01; †††, P < 0.001. C, flow cytometry of IFN-γ intracellular staining of CD11b+Ly6C+ cells in the lungs of Tgfb2MyeKO mice, with gating on the left and quantitative data in the middle. They are mostly F4/80+ macrophages (65%) and some are Ly6G+ neutrophils (32%; right). Three mice each for Tgfb2MyeKO and Tgfb2MyeKO were examined. *, P < 0.05. All data are represented as mean ± SEM.

injection (Supplementary Fig. S1A). We found that the injection of CD11bLy6G+ cells reversed the metastasis defect in Tgfb2MyeKO mice (Fig. 6D). These data suggest that the CD11bLy6G+ myeloid subset is responsible for the inhibition of metastasis that results from the myeloid-specific deletion of Tgfb2. Our conclusion may more broadly apply to mouse models in which CD11bLy6G+ myeloid cells constitute the majority of myeloid cells, including at least eight tumor models studied, such as MC26 colon cancer, LLC, and B16 melanoma (32).

Cellular Mechanisms Underlying Improved Host Antitumor Immunity and Decreased Metastasis in Tgfb2MyeKO Mice

Our data clearly show an IFN-γ-dependent mechanism in inhibiting metastasis in Tgfb2MyeKO mice. We next asked the question, what cell type (CD8+ T or Ly6G+ neutrophils) is critical in this process? Tgfb2MyeKO mice were inoculated with 4T1 tumor cells in the MFP. The mice were also injected with CD8-neutralizing antibody or immunoglobulin G (IgG) control every 2 to 3 days to deplete CD8+ T cells (Fig. 7A). The depletion of CD8+ T cells in Tgfb2MyeKO mice diminished the metastasis inhibition compared to the IgG control-treated mice (Fig. 7B). In addition, to examine the direct contribution of myeloid-derived IFN-γ in the inhibition of the metastasis in the lung environment, we sorted IFN-γ-deficient or wild-type CD11bLy6G+ cells, and then coinjected them with tumor cells through the tail vein. No significant effect on tumor metastasis was found (data not shown). However, this observation may be limited by the small number of IFN-γ-producing myeloid cells within the Ly6G+ subset. Our data suggest that IFN-γ-producing CD8+ T cells are critical for the metastasis defect in Tgfb2MyeKO mice.

DISCUSSION

We report here for the first time that deletion of Tgfb2 specifically in myeloid cells significantly inhibited tumor metastasis, suggesting that myeloid-specific TGF-β signaling is an essential component of the metastasis-promoting puzzle of TGF-β. This is in contrast to the tumor-suppressing effect of TGF-β in epithelial cells, fibroblasts, and T cells (6, 7, 9, 33). Our study provides a new mechanistic
Figure 6. Reconstitution of the wild-type CD11b+Ly6G+ myeloid cell subset in Tgfbr2\textsuperscript{MyeKO} mice reversed the diminished metastasis phenotype. 

A. Decreased TGF-β1 (ELISA), IL-10, and IL-4 [quantitative PCR (qPCR)] were found in CD11b+Ly6G+ cells from Tgfbr2\textsuperscript{MyeKO} mice. B. qPCR showed decreased iNOS and arginase 1 in CD11b+Ly6G+ and CD11b+Ly6C myeloid cell subsets. For both A and B, myeloid subsets were sorted by fluorescence-activated cell sorting (FACS). RNA was extracted and subjected to qPCR analysis. Supernatant from cultured subsets was used for ELISA. Myeloid cells were isolated from the spleens of 4T1 tumor-bearing mice. C. NO production of myeloid cell subsets sorted from Tgfbr2\textsuperscript{MyeKO} mice (C57BL/6 background), when cocultured with OVA peptide-stimulated splenocytes from OT1 mice (C57BL/6 background). For A–C, three mice each for Tgfbr2\textsuperscript{fl ox/fl ox} and Tgfbr2\textsuperscript{MyeKO} were examined. D. Reconstitution of CD11b+Ly6G+ or Gr-1+CD11b+ cells, but not CD11b+Ly6C+ or CD11b+F4/80+ cells, diminished the decreased 4T1 tumor lung metastasis (mets) in Tgfbr2\textsuperscript{MyeKO} mice (n = 7–10). All data are represented as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 7. CD8+ depletion and IFN-γ-deficient myeloid cells and lung metastasis inhibition in Tgfbr2\textsuperscript{MYKO} mice. A, Flow cytometry shows significantly decreased CD8+ T cells in Tgfbr2\textsuperscript{MYKO} mice after receiving injection of CD8 neutralizing antibody. B, CD8 neutralizing antibody significantly decreased lung metastasis in Tgfbr2\textsuperscript{MYKO} mice. All data are represented as mean ± SEM. ***, P < 0.001. C, Schematic hypotheses for mechanisms underlying decreased metastasis in the Tgfbr2\textsuperscript{MYKO} mice. TGF-β1 production is enhanced in myeloid cells through autocrine (TGF-β1 produced in CD11b\textsuperscript{+}Ly6G\textsuperscript{+} cells) and/or paracrine mechanisms (TGF-β1 from tumor cells or other cells, such as CD4\textsuperscript{+}, Tregs, or stromal fibroblasts). Deletion of Tgfbr2 in myeloid cells decreased the production of type II cytokines, TGF-β1, Arginase 1, and iNOS, which in turn increased IFN-γ expression in CD8+ T cells. This likely improves systemic immune surveillance and results in decreased lung metastasis in the Tgfbr2\textsuperscript{MYKO} mice. In the metastatic lung, deletion of Tgfbr2 also enhances IFN-γ production in a subset of macrophages and neutrophils, and functional maturation of dendritic cells, which may improve local innate immunity. APC, antigen-presenting cells.
Myeloid-Specific TGF-β Signaling Promotes Metastasis

insight into TGF-β regulation of tumor metastases. Our data are reminiscent of those observed following blockade of TGF-β signaling in T cells using CD45bTGF-βRII mice, which confers resistance to an EL-4 lymphoma or a B16-F10 melanoma tumor challenge (34). However, those mice developed an autoimmune pathology that is not seen in our mouse model. The lack of pathology in our mouse model is likely due to the fact that myeloid cells are significantly expanded under tumor conditions (Supplementary Fig. S1A). Therefore, the specific deletion of myeloid Tgfbr2 produces a pronounced antitumor effect with very few adverse effects. A previous report showed CD8+ CTL-mediated suppression of melanoma and prostate metastasis upon the silencing of TGF-β signaling in total bone marrow cells by retroviral-mediated transfection of a dominant-negative TGF-βRII (35). Our findings indicate that the myeloid cell–associated TGF-β signaling may be the key player driving this effect.

Our studies show that TGF-β signaling is a critical mediator of the tumor-promoting effect of myeloid cells, supported by a recent report of the antitumor properties of myeloid Tgfbr2 deletion on primary tumor growth (36). Myeloid cells have been shown to promote tumor progression through modulation of host immune surveillance (12–15) and alteration of the tumor microenvironment (10, 13, 14, 16). Recent evidence also suggests that myeloid cells have a substantial impact on the premetastatic lung (17, 18, 37, 38). A number of studies have tried to identify the molecular mechanisms for these myeloid cells in tumor metastasis. One of the better-studied molecules involves the paracrine loop of colony-stimulating factor 1 (CSF-1) and EGF between tumor cells and TAMs (40, 41). Interestingly, in the lung, there was an increase in IFN-γ/CD11b+Ly6G+ or IFN-γ/CD11b+Ly6C+ cells, as well as CD11b+CD11c+CD86+ dendritic cells, which were not present in the tumor microenvironment. Apparently, deletion of Tgfbr2 decreased iNOS production in the splenic Gr-1+CD11b+ cells and improved systemic immunity, whereas deletion of Tgfbr2 also increased IFN-γ production in the lung-residing macrophages and neutrophils. This seems to be different from the observation that IFN-γ levels correlate with iNOS in macrophages (52). We want to point out that these two effects are in fact on very different populations of cells (myeloid immune suppressor cells versus lung-residing macrophages/neutrophils) and in two very different organ environments (spleen versus lung). A number of studies support the idea that regulation of iNOS in vivo may depend on the relative tempo with which the inflammatory and immune responses evolve. We believe this increased innate immunity in the lung may be important in the diminished tumor metastasis in Tgfbr2−/− mice. A recent study reported that type I IFN-α/β selectively within the innate immune compartment is essential for tumor-specific T-cell priming and tumor elimination (53). TAMs cross-talk with adaptive immune components (54). We found that the critical mediators in the improved IFN-γ-producing CD8+ T cells in the Tgfbr2−/− mice involve IL-4, IL-10, and TGF-β, as well as arginase 1 and iNOS. Overexpression of these factors in sorted Gr-1+CD11b+ cells with Tgfbr2 deletion decreased the number of IFN-γ+ T cells (Fig. 5A and B). Indeed, these type II cytokines as well as arginine 1 and iNOS are known for cancer-associated immune suppression by

lature and signaling to VEGFR2, and these changes act to retard, not promote, tumor progression (49). Our results provide insight into the molecular mechanisms for TGF-β regulation of myeloid cell tumor–promoting function (Fig. 7C). First, TGF-β is produced in high levels by tumor-associated myeloid cells including Gr-1+CD11b+ cells (10, 50, 51). In fact, deletion of Gr-1+CD11b+ cells diminished the antitumor effect of TGF-β neutralization (20). Deletion of Tgfbr2 significantly decreased TGF-β1 production, which is not the case for TGF-β2 or TGF-β3 (Supplementary Fig. S10B and S10C). In addition, the CD11b+Ly6G+ myeloid cell subset that we found plays a critical role in the decreased metastasis in Tgfbr2−/− mice showed significantly higher expression of TJR2 compared with CD11b+Ly6C+ and CD11b+F4/80+ cells (Supplementary Fig. S9C). Thus, our data suggest that there is likely an autocrine and/or paracrine loop that enhances TGF-β1 production and signaling in myeloid cells through the TJIR in the CD11b+Ly6G+ myeloid subset (Fig. 7C). Deletion or downregulation of TGF-β signaling in myeloid cells would disrupt TGF-β1 production. However, it is not clear whether TGF-β1 production directly converts the myeloid cells from a type I to type II phenotype or is the result of type II myeloid cell polarization. Second, myeloid TGF-β signaling is critical in tumor-associated immune suppression. The critical mediators downstream of TGF-β signaling include type II cytokines, arginase 1, and iNOS, which have a significant impact on both systemic adaptive immunity and innate immunity in the lung (Fig. 7C). It is very clear in our studies that TGF-β signaling is critical in shaping the type I/II phenotype of myeloid cells.

Interestingly, in the lung, there was an increase in IFN-γ/CD11b+Ly6G+ or IFN-γ/CD11b+F4/80+ cells, as well as CD11b+CD11c+CD86+ dendritic cells, which were not present in the tumor microenvironment. Apparently, deletion of Tgfbr2 decreased iNOS production in the splenic Gr-1+CD11b+ cells and improved systemic immunity, whereas deletion of Tgfbr2 also increased IFN-γ production in the lung-residing macrophages and neutrophils. This seems to be different from the observation that IFN-γ levels correlate with iNOS in macrophages (52). We want to point out that these two effects are in fact on very different populations of cells (myeloid immune suppressor cells versus lung-residing macrophages/neutrophils) and in two very different organ environments (spleen versus lung). A number of studies support the idea that regulation of iNOS in vivo may depend on the relative tempo with which the inflammatory and immune responses evolve. We believe this increased innate immunity in the lung may be important in the diminished tumor metastasis in Tgfbr2−/− mice. A recent study reported that type I IFN-α/β selectively within the innate immune compartment is essential for tumor-specific T-cell priming and tumor elimination (53). TAMs cross-talk with adaptive immune components (54). We found that the critical mediators in the improved IFN-γ-producing CD8+ T cells in the Tgfbr2−/− mice involve IL-4, IL-10, and TGF-β, as well as arginase 1 and iNOS. Overexpression of these factors in sorted Gr-1+CD11b+ cells with Tgfbr2 deletion decreased the number of IFN-γ+ T cells (Fig. 5A and B). Indeed, these type II cytokines as well as arginine 1 and iNOS are known for cancer-associated immune suppression by

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Gr-1+CD11b+ cells (12). Our data suggest that an improvement in both systemic adaptive immunity and local innate immunity in the lung is important for the reduced metastasis observed in Tgfbr2+myelko mice. However, our data support that CD8+ T cells play a prominent role in adaptive immunity that is critical in metastasis inhibition in Tgfbr2+myelko mice.

Consistent with the observations in tumor-bearing mice, myeloid cells from the peripheral blood of patients with advanced-stage lung cancer also showed increased TβRII expression, suggesting the clinical relevance of our studies. Importantly, the transplant of Tgfbr2+myelko bone marrow into wild-type mice bearing 4T1 tumors significantly increased survival and decreased lung metastasis. It is quite feasible with the current technology to manipulate TGF-β signaling in bone marrow–derived myeloid cells through viral vector–induced recombination and bone marrow transplantation. This novel approach, by targeting myeloid TGF-β signaling, may overcome some of the problems of systemic application of small-molecule inhibitors of TβRII kinase or neutralizing antibodies that often have adverse effects in normal healthy tissues. This is particularly exciting in two respects: (i) clinical inhibitors have not shown high rates of tumor response, and deletion of Tgfbr2 in bone marrow–derived cells may provide a novel option that has not been described before; (ii) the finding that myeloid-specific TGF-β signaling is a significant part of the tumor-promoting effects of TGF-β allows specific and effective targeting. This finding is particularly important because TGF-β functions as both a tumor suppressor and promoter. The reduced tumor metastasis that we have observed suggests that myeloid-specific Tgfbr2 deletion is likely to be effective in countering the tumor-promoting role of TGF-β. Taken together, our studies show that myeloid-specific TGF-β signaling is a significant part of the tumor-promoting effects of TGF-β. This may provide therapeutic opportunities for new approaches to cancer therapy.

**METHODS**

**Cell Lines and Generation of Tgfbr2+myelko Mice**

Murine 4T1, MC26, B16, and LLC cell lines were obtained from the American Type Culture Collection and kept in the liquid nitrogen when not in use. Cells were thawed, cultured, and passaged less than 6 months for experiments. These mouse cell lines have not been authenticated by the authors. The Tgfbr2flmex/fl mouse line was established as described previously (55, 56). LysM-Cre mice (C57BL/6 and 129 background) were obtained from The Jackson Laboratory. Tgfbr2flmex/fl mice were bred with LysM-Cre mice to generate the Tgfbr2 deletion in myeloid cells (Tgfbr2+myelko). Mice heterozygous for Tgfbr2 allele were further bred with wild-type Balb/c or C57BL/6 for 10 generations to generate a Balb/c or C57BL/6 background. The genotyping of Tgfbr2flmex/fl and Tgfbr2+myelko mice, as well as Southern blotting on sorted T and B lymphocytes and Gr-1+CD11b+ cells, were done as described previously (55). The OT1 and CL4 transgenic mice specific to OVA 257–264 peptide (SIINFEKL) and HA 518–526 peptide (YVYVTAASSL) were obtained from Taconic and The Jackson Laboratory, respectively. IFN-γ knockout mice in C57BL/6 background were provided by the Cancer Inflammation Program at the National Cancer Institute (NCI, Frederick, MD). All animal studies are approved by the NCI Animal Care and Use Committee.

**Flow Cytometry and Cell Sorting**

Single-cell suspensions were made from peripheral blood, lymph node, spleen, thymus, and bone marrow from normal and tumor-bearing mice (16), as well as tumor and lung tissues (57). Gr-1+CD11b+ cells, CD3+ T cells, B220+ B cells, CD11b+Ly6G+ cells, and CD11b+Ly6C+ cells, and CD11b+F4/80+ cells were sorted from splenocytes by the FACSaria flow cytometer (BD Biosciences) for various assays. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (1 μg/mL), and monensin (2 μm/L) for 4 hours, fixed, permeabilized using a BD Fixation/Permeabilization kit, and stained for cytokines. The cells were analyzed on a FACS caliber flow cytometer (BD Biosciences). For human immature myeloid cells, peripheral blood from patients with metastatic non–small cell lung cancer was collected at the NCI (Bethesda, MD), on an Institutional Review Board (IRB)–approved protocol. The cells were labeled with anti-CD33-PE, anti-CD15-PE, and anti-CD34-PE (BD Pharmingen) and sorted by magnetic-activated cell sorting (MACS) according to the manufacturer’s protocol (Miltenyi Biotec).

**Immunofluorescence Staining**

The sorted myeloid cells were centrifuged on cytoplasm slides, fixed with 4% paraformaldehyde, and incubated with a rat polyclonal anti-TβRII antibody (Santa Cruz Biotechnology) followed by Alexa Fluor 488 goat anti-rat or 594 goat anti-rabbit (Invitrogen) antibodies.

**TGF-β1 ELISA**

Conditioned media was collected from cultured Gr-1+CD11b+ cells, CD11b+Ly6G+ cells, CD11b+Ly6C+ cells, and CD11b+F4/80+ cells (2% FBS RPMI-1640, overnight). The samples were analyzed for TGF-β1 expression using an ELISA kit (R&D Systems).

**Cytokine Antibody Array**

Gr-1+CD11b+ cells were sorted from Tgfbr2+myelko and Tgfbr2+myelko mice bearing 4T1 tumors. The proteins were extracted and a cytokine antibody array was conducted as per manufacturer’s protocol (Raybiotech). The relative quantification was determined by dot density using ImageJ software.

**iNOS and Arginase 1 Functional Assays**

Fluorescence-activated cell sorting (FACS)–sorted myeloid cells were cocultured with splenocytes from OT1 mice with the stimulation of OVA peptide (Youn and colleagues [32]). Cell culture supernatant was collected and subjected to nitrite assay using the Nitric Oxide Quantitation Kit (Active Motif). For arginase 1 functional assay, sort myeloid cells (2% FBS RPMI-1640, overnight). The samples were analyzed for arginase activity with QuantiChrom Arginase Assay Kit (DARG-200).

**Electroporation**

MACS-sorted Gr-1+ myeloid cells (2 × 10⁹) from spleens of Tgfbr2flmex/fl and Tgfbr2+myelko mice were electroporated with overexpression plasmids of TGF-β1 (a gift from Dr. Lalage Wakefield, NCI, Bethesda, MD), IL-4 (pORF-Mll-4, InvivoGen), and IL-10 (pORF-Mll-10, InvivoGen) using an Amaxa mouse macrophage nucleofector kit according to the manufacturer’s instructions.

**IFN-γ ELISPOT**

Single-cell suspensions from the spleens of Tgfbr2flmex/fl and Tgfbr2+myelko mice bearing 4T1 tumors were prepared. Splenocytes (2 × 10⁹) were stimulated with CD3 (0.5 μg/mL, eBioscience) and cultured overnight as per manufacturer’s protocol (BD Biosciences). The
ELISPOT plate was scanned in ImmunoSpot (Cellular Technology Ltd.) and quantification was assessed using the CTL Scanning and CTL Counting 4.0. For antigen-specific T-cell response regulated by myeloid cells, Gr-1+ myeloid cells were sorted from Tgfb2fl/fl and Tgfb2+/- mice and cocultured with splenocytes (2 × 10^5) from OT1 or CL4 transgenic mice at 6:1 ratios (splenocytes:myeloid cells). Overexpression of arginase 1 (Orgione) and iNOS (a gift from Victor Laubach, University of Virginia, Charlottesville, VA) in myeloid cells isolated from Tgfb2fl/fl mice was done using a mouse macrophage nucleofector kit (Lonza). Irradiated splenocytes (2,000 rad, 5 × 10^5) were added as antigen-presenting cells. OVA257-264 peptide or HA 518-526 peptide (1 μg/mL) was added as a stimulator. After 24-hour culture, IFN-γ ELISPOT assays were conducted and spot numbers were counted as described earlier.

**Western Blotting**

Gr-1+CD11b+ cells sorted from the spleens or lung tissues of normal or 4T1 tumor-bearing mice were lysed and analyzed by Western blotting. Primary antibodies included TβRII, pSmad2, Smad2 (Cell Signaling Technology), and β-actin (Sigma).

**Quantitative Real-Time PCR**

Total RNA was extracted from sorted Gr-1+CD11b+ cells or subsets using an NucleoSpin Mini kit (Qagen), and cDNA was synthesized using the Invitrogen SuperScript First-Strand Synthesis System. Relative length was measured at 2- to 3-day intervals using calipers as: volume = length × width^2 × 0.5. The number of lung metastases was evaluated as described previously (58) or by hematoyxlin and eosin staining of lung sections when mice died or became moribund, or when the primary tumors reached a size of 2.0 cm in diameter.

**Spontaneous and Experimental Metastasis**

For orthotopic metastasis, mammary tumor 4T1 cells (5 × 10^5) were injected into the #2 MFP. The enumeration referred to: the neck to the groin, the neck (#1, left and right), the arm (#2, left and right), the thoracic (#3, left and right), and abdominal (#4 and #5, left and right). Mice were sacrificed 28 days later. For the B16 orthotopic model, 1 × 10^5 B16 cells were injected subcutaneously, tumors were removed at day 16, and mice were euthanized at day 37. For experimental metastasis, mice received a tail vein injection of 4T1 cells (2 × 10^5), LLC cells (2.5 × 10^5), MC26 cells (2 × 10^5), or B16 cells (2 × 10^5). Tumor size was measured at 2- to 3-day intervals using calipers as: volume = length × width^2 × 0.5. The number of lung metastases was evaluated as described previously (58) or by hematoyxlin and eosin staining of lung sections when mice died or became moribund, or when the primary tumors reached a size of 2.0 cm in diameter.

**IFN-γ Neutralization**

4T1 cells (5 × 10^5) were injected into #2 MFP of Tgfb2fl/fl and Tgfb2+/- mice. The mice were treated with IFN-γ-neutralizing antibody XMG-6 or IgG control by intraperitoneal injection. Doseage: 1 mg antibody or IgG per mouse on days 1, 3, and 6; and 0.5 mg on days 9, 12, 15, 18, 21, 24, and 27. Mice were sacrificed on day 28 after tumor injection. Lung metastases were evaluated as described earlier and tumors were weighed.

**CD8+ T-Cell Depletion**

For in vivo depletion of CD8+ T cells, CD8e-neutralizing antibody (2.43 clone) and IgG2b (100 μg/mouse) were intraperitoneally injected every 2 days starting from the day 0 of 4T1 injection until the mice were sacrificed and evaluated for lung metastasis.

**Myeloid Cell Reconstitution**

The Tgfb2fl/fl and Tgfb2+/- mice were injected with 2 × 10^5 4T1 cells through the tail vein (day 0). Tgfb2+/- mice were then injected with different myeloid subsets: CD11b+Ly6G+, CD11b+Ly6C+, CD11b+F4/80-, and Gr-1+CD11b+ cells on days -1, 1, 3, 6, 8, 10, 13, 15, and 17 via the tail vein. The mice received 3 × 10^6 myeloid cells for the first six injections, then 1 × 10^6 for CD11b+Ly6G+ and Gr-1+CD11b+ cells for the last three injections. This is based on an increase of those myeloid cells in the peripheral blood over the time after tumor injection. Mice were euthanized on day 20. Lung metastasis was evaluated.

**Ex vivo Pulmonary Metastasis Assay**

B16BL6-GFP cells (5 × 10^5) were co-injected with sorted myeloid cells (10%) through the tail vein. Mice were euthanized 5 minutes after injection, and the lungs were infused with agarose as described previously (59). Lung sections were sliced (1–2 mm thick) and placed on Gelofast (Pfizer-Pharmacia & Upjohn Co.) for culture. LEICA-DM IRB fluorescent inverted microscope (Leica) and R annonce-EXI Fast 1394 Mono Cooled CCD camera (Qimaging) were used to capture GFP-positive cells at ×10 magnification. The area was quantified using OpenLab software (Improvision; ref. 59). For the effect of IFN-γ-deficient myeloid cells on metastasis, the Ly6G cells were sorted from IFN-γ-knockout mice bearing B16 melanoma (C57BL/6 background). The cells were then injected with B16 cells through the tail vein. The lung section culture was conducted as described earlier. Metastasis was evaluated after 2 to 3 weeks.

**Bone Marrow Transplant**

4T1 cells (5 × 10^5) were injected into the #4 and #5 MFP of wild-type Balb/c mice (recipients). Fifteen days later, the primary tumors were surgically removed and weighed. The mice were left to recover until day 34 after tumor injection, which allowed them to develop invasive tumors and metastasis. On day 34, these mice were irradiated (900 cGy). Bone marrow cells (5 × 10^5) from Tgfb2fl/fl mice or Tgfb2+/- control mice (donor mice) were injected into the tail vein of recipient mice in 100 μL PBS. Acidified water (pH 1.3 to 2.0), autoclaved food, and autoclaved cages were used for the recipient mice for 2 weeks after irradiation. Lung metastases were evaluated starting on day 63.

**Statistical Analysis**

GraphPad Prism v5.04 was used for the graphs and for statistics. All data other than indicated were analyzed using the Student t test and were expressed as mean ± SE. Differences were considered statistically significant when the P value was less than 0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Acknowledgments**

The authors thank Drs. Glenn Merlino and Stuart Yuspa for their critical reading of the article, and Ana Chytil, Vanderbilt Cancer Center, Nashville, TN, for technical assistance to Hannah Yan on the Southern hybridization. The authors are grateful for technical assistance from FACS Core of the Center for Cancer Research, NCI.
Grant Support
This work was supported by NCI intramural funding (to L. Yang).

Received November 14, 2012; revised May 3, 2013; accepted May 6, 2013; published OnlineFirst May 9, 2013.

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TGF-β Signaling in Myeloid Cells Is Required for Tumor Metastasis

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Cancer Discovery 2013;3:936-951. Published OnlineFirst May 9, 2013.

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