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 Tgfr2 specifically in myeloid cells (Tgfr2\(^{myeKO}\)) significantly inhibited tumor metastasis. Reconstitution of tumor-bearing mice with Tgfr2\(^{myeKO}\) bone marrow recapitulated the inhibited metastasis phenotype. This effect is mediated through decreased production of type II cytokines, TGF-β1, arginine 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 Tgfr2\(^{myeKO}\) 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.

See related commentary by Souza-Fonseca-Guimaraes and Smyth, p. XX.

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 Tgfr2, 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. In this...
report, we show that TGF-β signaling in myeloid cells of tumor-bearing hosts is fundamentally important for tumor metastasis. Genetic deletion of Tgfr2 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 Tgfr2 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 nontumor-bearing counterparts (Fig. IA 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 breast tumors. We observed 100% survival of the mice that received bone marrow from MyeKO mice compared with controls after tail vein injection of the tumor cells (Supplementary Fig. S1C) from MyeKO+ (Fig. 2E). In these therapeutic experiments, 4T1 tumors were allowed to continue until day 34, when the animals were killed; tumor growth was measured to determine primary tumor size (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 × 10⁶ 4T1 cells were injected into the tail vein, there was also a significant reduction in metastasis in Tgfr2MyeKO 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 Tgfr2 deletion on tumor metastasis, we transplanted bone marrow from Tgfr2MyeKO 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 the primary tumor (Fig. 2F). This result was recapitulated in the B16 melanoma orthotopic and LLC experimental metastasis models in Tgfr2MyeKO 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 Tgfr2MyeKO and control mice (data not shown). Together, these data suggest that the major effect of myeloid Tgfr2 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 Tgfr2MyeKO 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 Tgfr2 deletion on tumor metastasis, we transplanted bone marrow from Tgfr2MyeKO 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 the primary tumor (Fig. 2F). These data suggest that myeloid-specific TGF-β signaling constitutes an essential part of the metastasis-promoting role of TGF-β.
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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 Tgfr2 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 Tgfr2^floxed/floxed and Tgfr2^MyeKO 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 Tgfr2^floxed/floxed and Tgfr2^MyeKO mice in Balb/c background bearing 4T1 tumors.
Deletion of Tgfbr2 in myeloid cells significantly inhibited tumor metastasis. A, lung metastasis (mets) in Tgfbr2<sup>MyeKO</sup> and Tgfbr2<sup>flox/flox</sup> control mice 28 days after 4T1 injection (5 × 10<sup>4</sup>) 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<sup>MyeKO</sup> mice (n = 12) compared with Tgfbr2<sup>flox/flox</sup> mice (n = 5) received tail vein injection of 4T1 cells (2 × 10<sup>5</sup>) for 25 days. Quantitative data are on the right. **, P < 0.01. C, decreased metastasis in Tgfbr2<sup>MyeKO</sup> mice following subcutaneous injection of B16 melanoma cells (1 × 10<sup>6</sup>; 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<sup>MyeKO</sup> mice on a C57BL/6 background that received tail vein injection of LLC cells (2.5 × 10<sup>5</sup>; n = 5–6 mice). Quantitative data are on the right. **, P < 0.01. E, schematic experimental design for adoptive transfer of Tgfbr2<sup>MyeKO</sup> 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<sup>MyeKO</sup> and floxed control mice. Shown is one of two experiments carried out. *, P < 0.05. All data are represented as mean ± SEM.
Deletion of Tgfrb2 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 Tgfrb2 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 we cocultured Gr-1\(^+\)cells sorted from flowex control mice (data not shown). This was 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 Tgfrb2 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 Tgfrb2\(^{Myko}\) mice compared with Tgfrb2\(^{Flx/Flx}\) 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 assay (ELISAPOT) in the spleens of Tgfrb2\(^{Myko}\) mice (Supplementary Fig. S3A). To examine whether the enhanced T-cell immunity is antigen-specific, we cocultured Gr-1\(^+\) myeloid cells sorted from Tgfrb2\(^{Myko}\) 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 Tgfrb2 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 Tgfrb2 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 Tgfrb2 deletion decreased the number of IFN-γ T cells in the coculture by ELISAPOT assay (Fig. 5A and B), which was not the case in Gr-1\(^+\)CD11b\(^+\) cells sorted from flowex control mice (data not shown). These data, together with the results showing that myeloid cells with Tgfrb2 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 Tgfrb2\(^{Myko}\) Mice

Tgfrb2\(^{Myko}\) 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 Tgfrb2\(^{Myko}\) 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\(^+\)F4/80\(^+\) macrophages, and CD11b\(^+\)CD11c\(^+\) dendritic cells, no difference was found between Tgfrb2\(^{Myko}\) and Tgfrb2\(^{Flx/Flx}\) 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 Tgfrb2\(^{Myko}\) and Tgfrb2\(^{Flx/Flx}\) mice (Supplementary Fig. S5C). Interestingly, Tgfrb2 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. S6). 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 Tgfrb2\(^{Myko}\) 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 Tgfrb2\(^{Myko}\) mice compared with control littermates. Therefore, our data suggest that improved innate immunity in the lung environment of Tgfrb2\(^{Myko}\) mice may have a critical role in the decreased metastasis phenotype in these mice.

In contrast to metastasis, myeloid Tgfrb2 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\(^+\)F4/80\(^+\) macrophages, and CD11b\(^+\)CD11c\(^+\) dendritic cells. Again, no difference was found between Tgfrb2\(^{Myko}\) and Tgfrb2\(^{Flx/Flx}\) 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 in CD8\(^+\) T cells and a local increase in IFN-γ in CD11b\(^+\)Ly6G\(^+\) or IFN-γ CD11b\(^+\)F4/80\(^+\) 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 Tgfr2 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 Tgfr2\textsuperscript{MyeKO} 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 Tgfr2\textsuperscript{MyeKO} and control mice. *, P < 0.05; **, P < 0.01.
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**Myeloid Cell Subsets Responsible for the Decreased Lung Metastasis in Tgfbr2\textsuperscript{MyeKO} Mice**

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 T\(\beta\)RII expression. We found that the CD11b\(^+\)Ly6G\(^+\) subset expressed higher levels of T\(\beta\)RII 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-\(\beta\)1 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 1 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-\(\beta\) 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\textsuperscript{MyeKO} mice through the tail vein. We first tested the homing efficiency of tail vein–injected cells using luciferase imaging. Approximately more than 65% to 75% of the luciferase-expressing TGF-\(\beta\)RII-expressing cells home to the lungs 24 hours after injection (Fig. 4A). In addition, we cocultured CD11b\(^+\)Ly6G\(^+\) and CD11b\(^+\)Ly6C\(^-\) myeloid cells with Gr-1\(^+\) cells, and the IFN-\(\gamma\)-neutralizing antibody diminished metastasis (Fig. 4B). These data suggest that TGF-\(\beta\)RII expression.
inhibiting metastasis in tumor models studied, such as MC26 colon cancer, LLC, and B16 melanoma (32). Our conclusion may more broadly apply to mouse models in which CD11b deletion of Tgfbr2 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 puzzle of TGF-β for the first time that deletion of Tgfbr2 in myeloid cells reversed the metastasis defect in Tgfbr2MyeKO mice (Fig. 6D). These data suggest that the CD11b+Ly6G+ myeloid subset is responsible for the inhibition of metastasis that results from the myeloid-specific deletion of Tgfbr2. Our conclusion may more broadly apply to mouse models in which CD11b+Ly6G+ 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 Tgfbr2MyeKO Mice**

Our data clearly show an IFN-γ-dependent mechanism in inhibiting metastasis in Tgfbr2MyeKO mice. We next asked the question, what cell type (CD8+ T or Ly6G+ neutrophils) is critical in this process? Tgfbr2MyeKO 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 Tgfbr2MyeKO 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 CD11b+Ly6G+ 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 Tgfbr2MyeKO mice.

**DISCUSSION**

We report here for the first time that deletion of Tgfbr2 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...
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Figure 6. Reconstitution of the wild-type CD11b+Ly6G+ myeloid cell subset in Tgfbr2^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^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^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^fl ox/fl ox^ and Tgfbr2^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^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-\(\gamma\)-deficient myeloid cells and lung metastasis inhibition in Tgfbr\(^{-}\)MyeKO mice. **A,** flow cytometry shows significantly decreased CD8\(^+\) T cells in Tgfbr\(^{-}\)MyeKO mice after receiving injection of CD8 neutralizing antibody. **B,** CD8 neutralizing antibody significantly decreased lung metastasis in Tgfbr\(^{-}\)MyeKO mice. All data are represented as mean ± SEM. **C**, schematic hypotheses for mechanisms underlying decreased metastasis in the Tgfbr\(^{-}\)MyeKO mice. TGF-\(\beta\)1 production is enhanced in myeloid cells through autocrine (TGF-\(\beta\)1 produced in CD11b\(^+\)Ly6G\(^+\) cells) and/or paracrine mechanisms (TGF-\(\beta\)1 from tumor cells or other cells, such as CD4\(^+\) Tregs, or stromal fibroblasts). Deletion of Tgfbr2 in myeloid cells decreased the production of type II cytokines, TGF-\(\beta\)1, as well as arginase 1 and iNOS, which in turn increased IFN-\(\gamma\) expression in CD8\(^+\) T cells. This likely improves systemic immune surveillance and results in decreased lung metastasis in the Tgfbr\(^{-}\)MyeKO mice. In the metastatic lung, deletion of Tgfbr2 also enhances IFN-\(\gamma\) production in a subset of macrophages and neutrophils, and functional maturation of dendritic cells, which may improve local innate immunity. APC, antigen-presenting cells.
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 CD4dnTGF-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 TgfbrII (35). Our findings indicate that the myeloid cell–associated TGF-β signaling may be the key driver 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 (13, 39). Apparently, CSF-1 is produced by tumor cells and acts on the CSF-1R on TAMs (40, 41). Interestingly, tumor cells also express EGF receptor that mediates the signaling of EGF produced by TAMs (39, 41–43). These two molecular pathways are critical for the effects of TAMs upon tumor metastasis (13, 39). Hypoxia-inducible factor 1α (HIF-1α) in myeloid cells was also shown to control the inflammatory response through the regulation of the metabolic switch to glycolysis (44), and contribution to T-cell suppression in the tumor microenvironment (45). Myeloid-specific HIF-2α promoted tumor cell proliferation and progression in murine hepatocellular and colitis-associated colon carcinoma models (46). In addition, the transcription factor Ets2 in myeloid cells is important in metastasis, as its deletion decreased the frequency and size of lung metastases in three different mouse models of breast cancer metastasis, which is mediated through inhibition of a gene program for angiogenesis (47). Recently, mice with myeloid-specific deletion of HuR, a RNA-binding protein that regulates mRNA transcription and translation, displayed enhanced sensitivity to endotoxemia, rapid progression of chemical-induced colitis, and increased severe susceptibility to colitis-associated cancer through increased inflammatory cytokines and enhanced chemokine (C-C motif) receptor 2–mediated macrophage chemotaxis (48). However, most of these reports did not focus on tumor metastasis. Of interest, the deletion of myeloid-specific VEGF-A resulted in accelerated tumor progression despite the fact that myeloid-derived VEGF-A is essential for the tumorigenic alteration of vascular 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 TLR2 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 TgfbrII 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+Ly6G2 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 1 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 arginase 1 and iNOS are known for cancer-associated immune suppression by
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<sup>2myKo</sup> mice. However, our data support that CD8+ T cells play a prominent role in adaptive immunity that is critical in metastasis inhibition in Tgfbr2<sup>2myKo</sup> 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<sup>2myKo</sup> bone marrow into wild-type mice bearing 4T1 tumors significantly increased survival and decreased lung metastasis. It is quite feasible that CD8+ T cells play a prominent role in adaptive immunity that is critical in metastasis inhibition in Tgfbr2<sup>2myKo</sup> mice.

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, CD8+ T cells, B220+ B cells, CD11b+Ly6G+ cells, and CD11b+Ly6C+ cells, and CD11b+F4/80<sup>-</sup> cells were sorted from spleenocytes 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 μmol/L) for 4 hours, fixed, permeabilized using a BD Fixation/Permeabilization kit, and stained for cytokines. The cells were analyzed on a FACSCalibur 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 Biotech).

Immunofluorescence Staining

The sorted myeloid cells were centrifuged on cytospin 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-β ELISA

Conditioned media was collected from cultured Gr-1+CD11b+ cells, CD11b+Ly6G+ cells, CD11b+Ly6C+ cells, and CD11b+F4/80<sup>-</sup> 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<sup>2myKo</sup> and Tgfbr2<sup>2fl ox/fl ox</sup> mice bearing 4T1 tumors. The proteins were extracted and a cytokine antibody array was conducted as per manufacturer’s protocol (RayBio). The relative quantification was determined by dot density using ImageJ software.

iNOS and Arginine 1 Functional Assays

Fluorescence-activated cell sorting (FACS)-sorted myeloid cells were cocultured with spleenocytes 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, myeloid cells (2 × 10<sup>6</sup>) from spleens of 4T1 tumor-bearing mice were cocultured with splenocytes from OT1 mice with the stimulation of OVA peptide (20 μg/mL), and monensin (2 μmol/L) for 4 hours, fixed, permeabilized using a BD Fixation/Permeabilization kit, and stained for cytokines. The cells were analyzed on a FACScan 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 Biotech).

Electroporation

MACS-sorted Gr-1+ myeloid cells (2 × 10<sup>6</sup>) from spleens of Tgfbr2<sup>2fl ox/fl ox</sup> and Tgfbr2<sup>2myKo</sup> mice were electroporated with overexpression plasmids of TGF-β1 (a gift from Dr. Lalage Wakefield, NCI, Bethesda, MD), IL-4 (pORF-mIL-4; InvivoGen), and IL-10 (pORF-mIL-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 Tgfbr2<sup>2myKo</sup> and Tgfbr2<sup>2fl ox/fl ox</sup> mice bearing 4T1 tumors were prepared. Splenocytes (2 × 10<sup>6</sup>) 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 × 10^6) from OT1 or CL4 transgenic mice at 6:1 ratios (splenocytes:myeloid cells). Overexpression of arginase 1 (Origene) and iNOS (a gift from Victor Laubach, University of Virginia, Charlottesville, VA) in myeloid cells isolated from Tgfb2−/− was done using a mouse macrophage nucleiectior kit (Lonza). Irradiated splenocytes (2,000 rad, 5 × 10^6) 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 lungs tissues of normal or 4T1 tumor-bearing mice were lysed and analyzed by Western blotting. Primary antibodies included TjR2, 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 RNeasy Mini kit (Qiagen), and cDNA was synthesized using the Invitrogen SuperScript First-Strand Synthesis System. Relative gene expression was determined using a Bio-Rad Cycler-iQ SYBR Green PCR kit. Primer sequences are available upon request.

Spontaneous and Experimental Metastasis

For orthotopic metastasis, mammary tumor 4T1 cells (5 × 10^5) were injected into the #2 MFP. The numeration 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 × 10^6 B16 cells were injected subcutaneously; tumors were removed at day 16, and mice were euthanized at day 37. For experimental metastasis, mice received 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 by 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 hematoxylin 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. Dosage: 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, 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 × 10^6) were coinjected 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 Gelfoam (Pfizer-Pharmacia & Upjohn Co.) for culture. LEICA-DM IRB fluorescent inverted microscope (Leica) and Ritteri-EXI Fast 1394 Mono Cooled CCD camera (Qlumaging) were used to capture GFP-positive cells at x10 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 (resipients). 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 × 10^6) from Tgfb2−/− mice or Tgfbr2−/− 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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