

# Abrogation of TGF $\beta$ Signaling in Mammary Carcinomas Recruits Gr-1+CD11b+ Myeloid Cells that Promote Metastasis

Li Yang,<sup>1,4,\*</sup> Jianhua Huang,<sup>3</sup> Xiubao Ren,<sup>5</sup> Agnieszka E. Gorska,<sup>1</sup> Anna Chytil,<sup>1</sup> Mary Aakre,<sup>1</sup> David P. Carbone,<sup>2,4</sup> Lynn M. Matrisian,<sup>1,4</sup> Ann Richmond,<sup>1,4</sup> P. Charles Lin,<sup>3,4</sup> and Harold L. Moses<sup>1,4,\*</sup>

<sup>1</sup>Department of Cancer Biology

<sup>2</sup>Department of Medicine

<sup>3</sup>Department of Radiation Oncology

<sup>4</sup>The Vanderbilt-Ingram Cancer Center

Vanderbilt University School of Medicine, Nashville, TN 37232, USA

<sup>5</sup>Department of Biotherapy, Tianjing Cancer Institute, Tianjing 300060, China

\*Correspondence: li.yang@vanderbilt.edu (L.Y.), hal.moses@vanderbilt.edu (H.L.M.)

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## SUMMARY

Aberrant TGF $\beta$  signaling is common in human cancers and contributes to tumor metastasis. Here, we demonstrate that Gr-1+CD11b+ myeloid cells are recruited into mammary carcinomas with type II TGF $\beta$  receptor gene (*Tgfr2*) deletion and directly promote tumor metastasis. Gr-1+CD11b+ cells infiltrate into the invasive front of tumor tissues and facilitate tumor cell invasion and metastasis through a process involving metalloproteinase activity. This infiltration of Gr-1+CD11b+ cells also results in increased abundance of TGF $\beta$ 1 in tumors with *Tgfr2* deletion. The recruitment of Gr-1+CD11b+ cells into tumors with *Tgfr2* deletion involves two chemokine receptor axes, the SDF-1/CXCR4 and CXCL5/CXCR2 axes. Together, these data indicate that Gr-1+CD11b+ cells contribute to TGF $\beta$ -mediated metastasis through enhancing tumor cell invasion and metastasis.

## INTRODUCTION

Mounting evidence indicates that alterations in TGF $\beta$  signaling have significant effects on tumor initiation and progression (Bierie and Moses, 2006). TGF $\beta$  signals through the type I and type II TGF $\beta$  receptors (T $\beta$ RI and T $\beta$ RII, respectively), resulting in phosphorylation of Smad2 and Smad3, which then combine with Smad4 to enter the nucleus to modulate transcription (Bierie and Moses, 2006). In addition, TGF $\beta$  binding to its receptors activates many noncanonical signaling pathways. TGF $\beta$  can function as a tumor suppressor or a tumor promoter depending on the context and stage of tumor progression. Mutations of the genes encoding T $\beta$ RI and T $\beta$ RII (*TGFBR1* and *TGFBR2*, respectively) have been reported in a number of human cancers

(Akhurst and Derynck, 2001). Mutations in *TGFBR2* are particularly frequent in tumors with microsatellite instability (Grady and Markowitz, 2002). Although mutations in TGF $\beta$  receptor genes are infrequent in human breast cancers, there is compelling evidence for impairment of TGF $\beta$  signaling in this disease (Bagadi et al., 2007; Chen et al., 1998, 2006; Seitz et al., 2003). Decreased expression of T $\beta$ RII occurs frequently both in early and advanced breast cancer (de Jong et al., 1998; Gobbi et al., 1999). Further, polymorphisms in *TGFBR1* that result in decreased TGF $\beta$  signaling are associated with an increased frequency and metastasis of breast cancer (Chen et al., 2006; Pasche et al., 2005). Transgenic animal studies indicate that a simple reduction of TGF $\beta$  signaling is sufficient to predispose to the development of mammary carcinomas (Tang et al., 1998).

## SIGNIFICANCE

TGF $\beta$  is a very important tumor suppressor. Inactivation of TGF $\beta$  signaling frequently occurs in human cancers and contributes to tumor metastasis. However, the contribution of host cells in this process is unclear. Here, we show that deletion of *Tgfr2* in mammary carcinoma cells results in increased chemokine signals that enhance Gr-1+CD11b+ myeloid cell infiltration into tumors, which leads to enhanced tumor invasion and metastasis. Gr-1+CD11b+ cell infiltration also results in increased TGF $\beta$  production in tumors with *Tgfr2* deletion. Thus, the tumor-suppressing role of TGF $\beta$  can be switched to tumor promoting through the recruitment of Gr-1+CD11b+ cells in the tumor microenvironment. Inhibition of Gr-1+CD11b+ cell production/recruitment could improve host immunosurveillance and inhibit tumor metastasis, having the effect of “killing two birds with one stone.”

Conditional knockout of *Tgfb2* in combination with expression of Kras<sup>G12D</sup> in pancreatic cancer (Ijichi et al., 2006) or with APC mutation in intestinal carcinomas (Munoz et al., 2006) resulted in the development of aggressive tumor phenotype. We have shown that conditional deletion of *Tgfb2* in mammary epithelial cells that also express the polyoma middle T antigen (PyVmT) under the MMTV promoter resulted in a shortened tumor latency and an increased metastases (Forrester et al., 2005; Ijichi et al., 2006; Munoz et al., 2006). However, others have shown that enhancement of TGF $\beta$  signaling by expression of a constitutively active TGF $\beta$ 1 (Muraoka et al., 2003; Muraoka-Cook et al., 2004) or T $\beta$ RI (Muraoka-Cook et al., 2004, 2006; Siegel et al., 2003) in mammary epithelial cells in conjunction with c-Neu of PyVmT expression increases pulmonary metastases creating a conundrum. It is unclear what mechanisms underlie these different observations.

Host-derived inflammatory cells infiltrate into tumor tissues and create an environment that favors tumor progression. They promote tumor angiogenesis and tumor progression by producing angiogenic factors and matrix-degrading enzymes (Balkwill and Coussens, 2004; Coussens and Werb, 2002). The recruitment of inflammatory cells to tumor sites is regulated by the interactions of chemokines and chemokine receptors. ENA-78/CXCL5, interacting with CXCR2, is responsible for the recruitment of polymorphonuclear neutrophils into inflamed lungs (Ahuja and Murphy, 1996; Bozic et al., 1996; Jeyaseelan et al., 2005; Zineh et al., 2006). In addition, stromal-derived factor 1 (SDF-1 or CXCL12) is considered one of the key regulators of hematopoietic stem and progenitor cell trafficking between the peripheral circulation and targeted tissues. SDF-1 mediates its effects on chemotaxis through its receptor, CXCR4, which is highly expressed on putative stem and progenitor cells (Balkwill and Coussens, 2004).

Gr-1+CD11b+ myeloid cells, also called myeloid immune suppressor cells (MISCs) or myeloid-derived suppressor cells (MDSCs), are significantly overproduced in the bone marrow and spleens of tumor-bearing mice (Melani et al., 2003; Serafini et al., 2006) as well as in peripheral blood of cancer patients (Almand et al., 2001; Young and Lathers, 1999). In mice they express CD11b, a marker for myeloid cells of the macrophage lineage, and a marker for granulocytes, Gr-1, thus they are also called Gr-1+CD11b+ cells. In human cancer patients, Gr-1+CD11b+ myeloid cells are identified as lineage (CD3, CD14, CD19, and CD57) and HLA-DR negative (Lin<sup>-</sup>HLA-DR<sup>-</sup>), or immature dendritic cells. Gr-1+CD11b+ myeloid cells have been demonstrated to be immune suppressive since the 1980s (Almand et al., 2001; Bronte et al., 2000; Gabilovich et al., 1996; Serafini et al., 2004, 2006; Young and Lathers, 1999). They represent one mechanism of tumor escape from immune system control and compromise the efficacy of cancer immunotherapy.

Recently, Gr-1+CD11b+ cells were found to infiltrate into tumors and promote tumor angiogenesis by producing high levels of MMP9 and by directly incorporating into tumor endothelium (Yang et al., 2004). Gr-1+CD11b+ cells have also been implicated in tumor refractoriness to anti-VEGF treatment (Shojaei et al., 2007). Despite the data defining the overproduction in tumor hosts and infiltration in tumor tissues, the molecular mechanisms for their recruitment and roles in tumor progression

remain to be investigated. Here we show that Gr-1+CD11b+ cells are specifically recruited into mammary carcinomas with conditional deletion of *Tgfb2* and 4T1 tumors through two distinct chemokine mechanisms, CXCL5/CXCR2 and SDF-1/CXCR4. These immature myeloid cells directly promote tumor invasion and metastasis through increased production and function of MMPs. In addition, Gr-1+CD11b+ cell infiltration also results in an increased TGF $\beta$  production in the microenvironment of tumors with *Tgfb2* deletion. These findings improve our understanding of tumor-host interaction. Therapeutic interventions on Gr-1+CD11b+ cells may not only enhance the host immune system but also inhibit tumor invasion and metastasis.

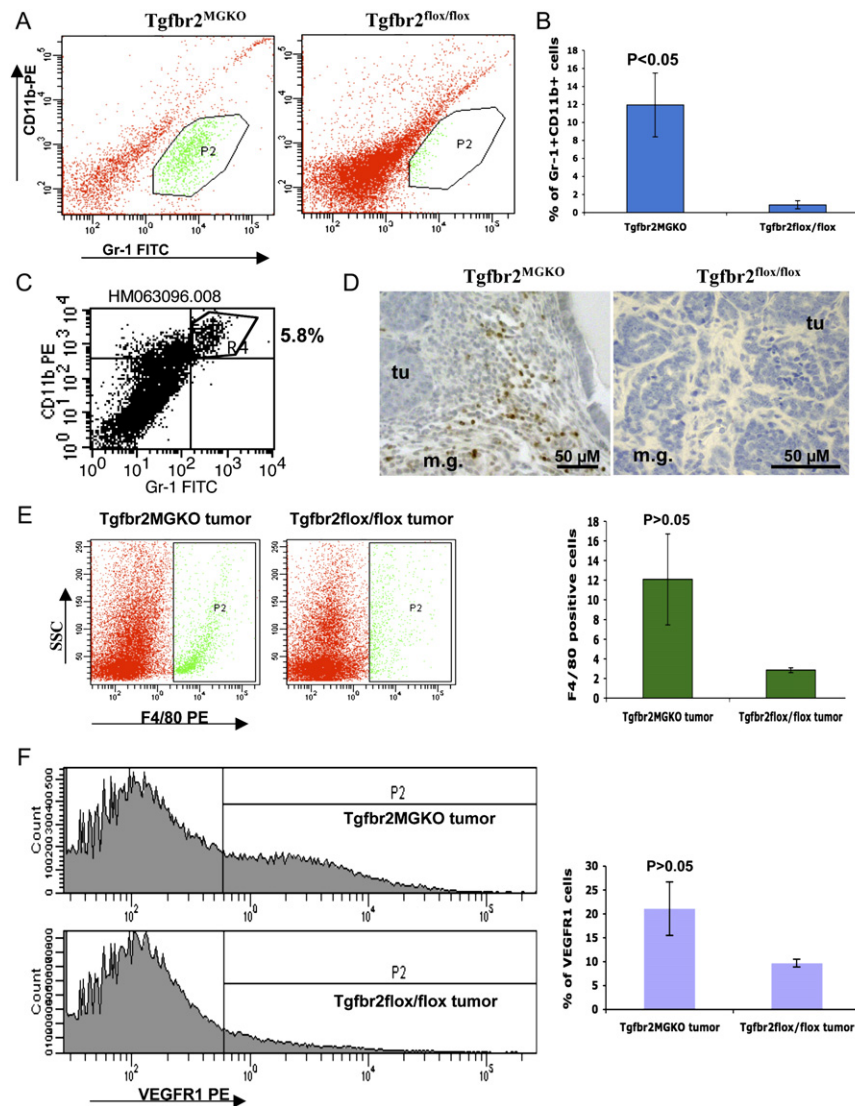
## RESULTS

### Gr-1+CD11b+ Cells Are Recruited to Mammary Carcinomas with Genetic Deletion of *Tgfb2*, Especially in the Invasive Front

We have previously reported that conditional deletion of *Tgfb2* in mammary epithelial cells also expressing PyVmT results in a shortened tumor latency and an increased pulmonary metastasis compared to PyVmT carcinomas with intact TGF $\beta$  signaling (Chytil et al., 2002; Forrester et al., 2005). We have also reported that Gr-1+CD11b+ myeloid cells infiltrate into tumor tissues and contribute to tumor angiogenesis (Yang et al., 2004). To examine the role of Gr-1+CD11b+ cells in TGF $\beta$ -mediated tumor progression, tumor tissues were collected 1 week after palpable tumors developed in a PyVmT genetic tumor model with *Tgfb2* knockout (*Tgfb2*<sup>MGKO</sup>). Single-cell suspension from tumor tissues of *Tgfb2*<sup>MGKO</sup> and floxed PyVmT control mice (*Tgfb2*<sup>flox/flox</sup>) were analyzed by flow cytometry. We observed a significant increase of Gr-1+CD11b+ cells in the PyVmT/*Tgfb2*<sup>MGKO</sup> compared with their *Tgfb2*<sup>flox/flox</sup> control tumors (Figures 1A and 1B). Gr-1+CD11b+ cells residing in tumor tissues express both Gr-1 and CD11b, and no distinct Gr-1 alone population was found (Figure 1C). This allowed us to examine the precise location of Gr-1+CD11b+ cells in tumor tissues with immunohistochemistry staining with anti-Gr-1 antibody. Very interestingly, Gr-1+CD11b+ cells were found mostly in the invasive front of PyVmT/*Tgfb2*<sup>MGKO</sup> mammary tumors (Figure 1D). In addition to Gr-1+CD11b+ cells, we also observed an increased infiltration of F4/80-positive and VEGFR1-positive cells in the PyVmT/*Tgfb2*<sup>MGKO</sup> tumors compared with their *Tgfb2*<sup>flox/flox</sup> control tumors (Figures 1E and 1F), although without statistical significance. We thus concentrated our studies on Gr-1+CD11b+ myeloid cells. The significant increase of Gr-1+CD11b+ cells is consistent with increased lung metastasis in those animals (Forrester et al., 2005), suggesting that these Gr-1+CD11b+ cells may play a role in breast tumor metastasis.

### Gr-1+CD11b+ Cells Promote Mammary Carcinoma Metastasis

To confirm our observation in PyVmT/*Tgfb2*<sup>MGKO</sup> tumors, we utilized a 4T1 orthotopic tumor model. This tumor model shares many characteristics with human breast cancer, particularly its ability to spontaneously metastasize to lungs. In addition, 4T1 tumors showed significantly decreased growth inhibition by TGF $\beta$  relative to normal mammary epithelial cells or PyVmT/*Tgfb2*<sup>flox/flox</sup> carcinoma cells (data not shown). Importantly,



**Figure 1. Gr-1+CD11b+ Myeloid Cells Are Recruited to Mammary Carcinomas with Genetic Deletion of *Tgfb2***

(A) Flow cytometry analysis of infiltrating Gr-1+CD11b+ cells in PyVmT/*Tgfb2*<sup>MGKO</sup> tumors and PyVmT/*Tgfb2*<sup>flox/flox</sup> control tumors. Shown are representative flow cytometry plots.

(B) Quantitative data for the presence of Gr-1+CD11b+ cells in tumors as shown in (A). Five- to seven-week-old mice were analyzed.

(C) Flow cytometry analysis of tumor-residing Gr-1+CD11b+ cells in PyVmT/*Tgfb2*<sup>MGKO</sup> tumors. (D) IHC of Gr-1+CD11b+ cells in the invasive front of PyVmT/*Tgfb2*<sup>MGKO</sup> mammary carcinomas compared with PyVmT/*Tgfb2*<sup>flox/flox</sup> control tumors. Scale bars are indicated in the panels.

(E and F) Flow cytometry analysis of infiltrating F4/80- and VEGFR1-positive cells in PyVmT/*Tgfb2*<sup>MGKO</sup> tumors and PyVmT/*Tgfb2*<sup>flox/flox</sup> control tumors. Representative flow cytometry plots are shown on the left and quantitative data on the right. m.g., adjacent mammary gland tissue; tu, tumor tissues. All quantitative data are presented as the mean ± standard error.

vested and the tumor nodules were counted after the mice showed evidence of distress. We observed a significant increase of tumor nodules in lungs from mice that received 4T1 coinjection with Gr-1+CD11b+ cells derived from tumor tissues or spleens of tumor-bearing animals compared with 4T1 alone or 4T1 with normal Gr-1+CD11b+ cells (Figure 2E). These data confirm that Gr-1+CD11b+ cells derived from tumor hosts enhance tumor metastasis.

In further characterization of Gr-1+CD11b+ cells, we found that, within the Gr-1+CD11b+ cell population, there were

Gr-1+CD11b+ cells were significantly overproduced in bone marrow and spleens of mice bearing large 4T1 tumors (Figure 2A). The number of Gr-1+CD11b+ cells increased with the growth of the tumor, with over 50% in the spleen, and over 80% in the bone marrow 28–35 days after tumor inoculation. This allows us to be able to sort large number of Gr-1+CD11b+ cells to investigate their roles in breast tumor metastasis using both in vivo and in vitro assays. In 4T1 breast tumors, Gr-1+CD11b+ cells comprised an average of 7.2% of total cells (Figure 2B). Gr-1+CD11b+ cells were mostly present in the invasive front (Figure 2C), which is similar to the MMTV PyVmT model.

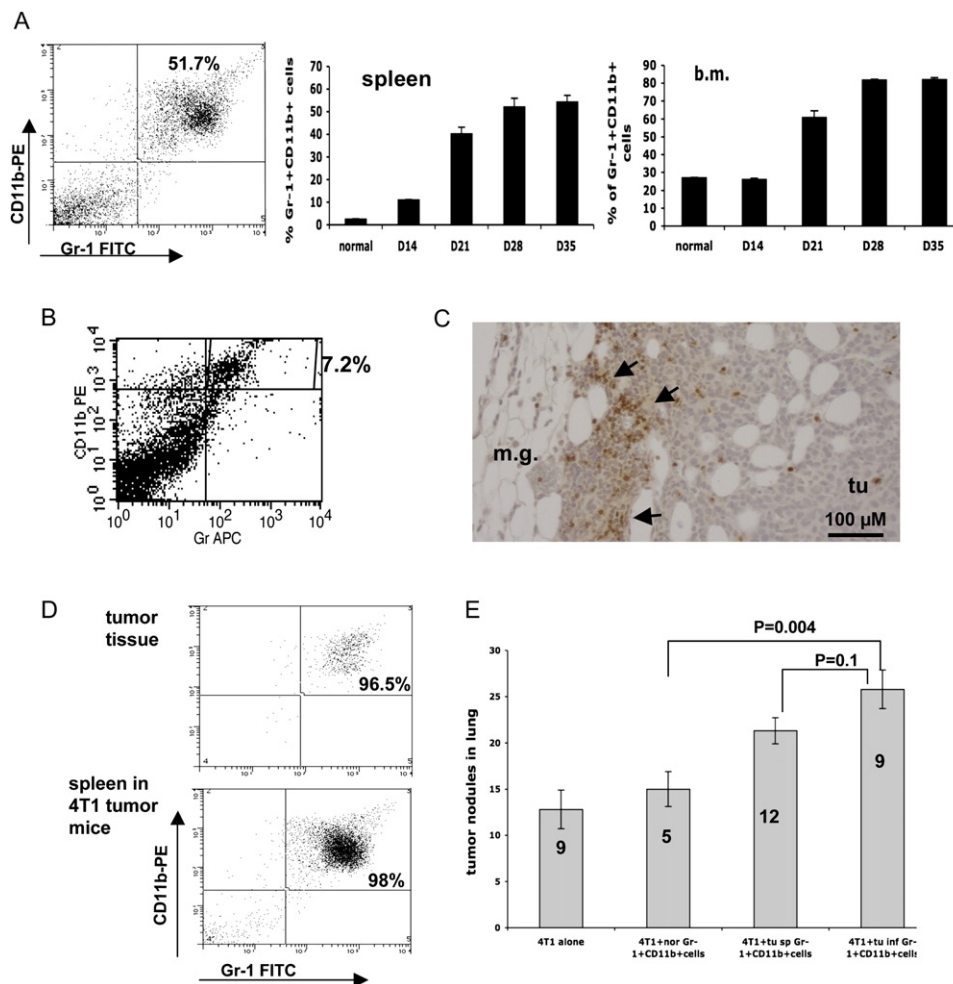
To examine the role of Gr-1+CD11b+ cells in tumor metastasis, 4T1 cells were injected into the mammary fat pad of female Balb/c mice, with or without Gr-1+CD11b+ cells sorted from 4T1 tumor tissues or spleens of mice bearing 4T1 tumors. Gr-1+CD11b+ cells from normal spleens were used as a control. We achieved a 95%–98% purity of sorted Gr-1+CD11b+ cells (Figure 2D). The primary tumors were removed after 12 to 14 days, which allows metastasis to develop in lungs avoiding morbidity or mortality caused by large primary tumors. Lungs were har-

vested and the tumor nodules were counted after the mice showed evidence of distress. We observed a significant increase of tumor nodules in lungs from mice that received 4T1 coinjection with Gr-1+CD11b+ cells derived from tumor tissues or spleens of tumor-bearing animals compared with 4T1 alone or 4T1 with normal Gr-1+CD11b+ cells (Figure 2E). These data confirm that Gr-1+CD11b+ cells derived from tumor hosts enhance tumor metastasis.

### Gr-1+CD11b+ Myeloid Cells Promote Mammary Carcinoma Cell Invasion In Vivo and In Vitro

Very interestingly, after surgical removal of primary tumors, at the primary injection site the recurrent tumors from 4T1 cells coinjected with tumor-derived Gr-1+CD11b+ cells were significantly larger than those from control groups (Figure 3A). In addition, we found no difference in primary tumor growth during the first 10–14 days after injection (data not shown). This indicates that Gr-1+CD11b+ cells derived from tumor tissues or tumor spleens increase tumor cell invasion to surrounding tissues, supporting our earlier observation that Gr-1+CD11b+ cells promote tumor metastasis, as invasion is the first step in the metastatic process.

To further investigate the effects of Gr-1+CD11b+ cells on tumor invasion, we performed in vitro invasion assays. 4T1 cells



**Figure 2. Gr-1+CD11b+ Cells Promote Tumor Metastasis**

(A) Increased Gr-1+CD11b+ cells in spleens and bone marrow of 4T1 tumor-bearing mice after tumor inoculation. (Left panel) Flow cytometry analysis of Gr-1+CD11b+ cells in the spleen of tumor-bearing mice 28 days following engraftment (four or more mice per time point). (Middle panel) Spleen. (Right panel) Bone marrow (b.m.).

(B) Gr-1+CD11b+ cells in 4T1 tumors with flow cytometry analysis.

(C) IHC showing Gr-1+CD11b+ cells in the invasive front of 4T1 tumors 15 days after tumor inoculation. Scale bar, 100  $\mu$ M.

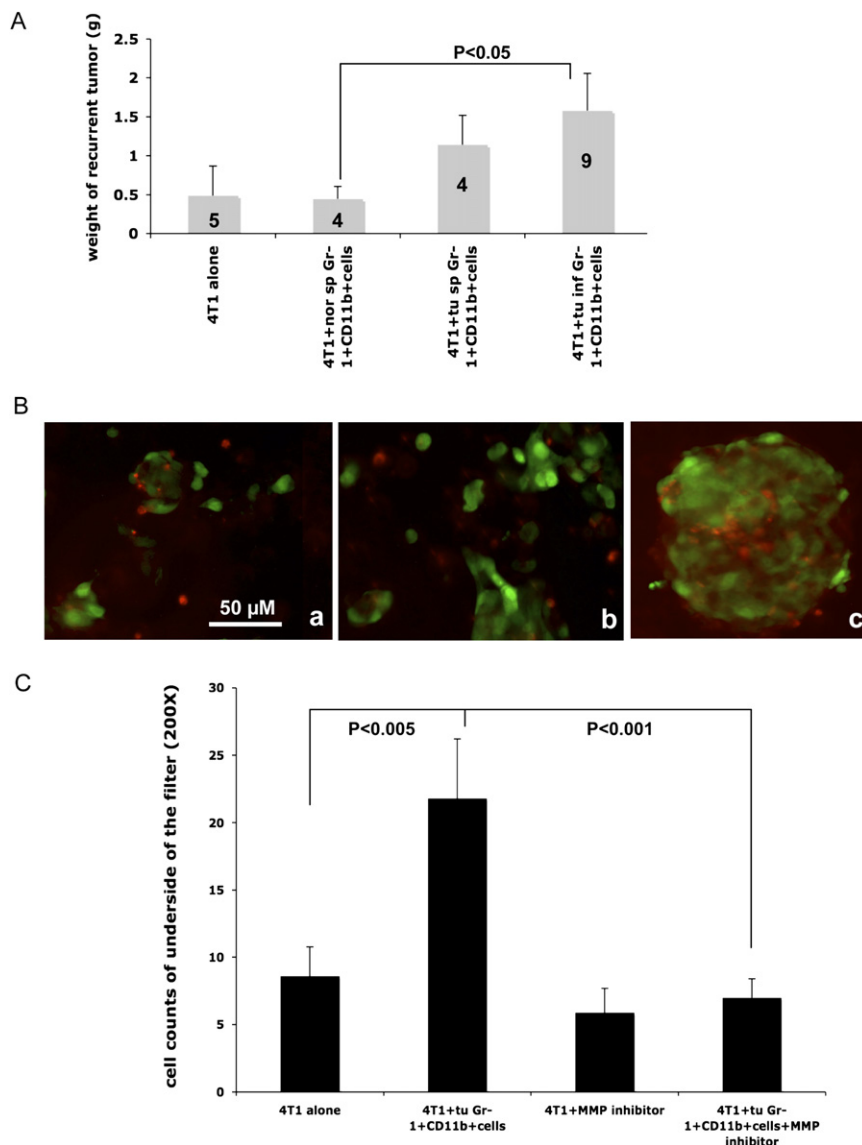
(D) Single-cell sorting of Gr-1+CD11b+ cells from tumors and spleens of 4T1 tumor-bearing mice 35 days after tumor inoculation. Flow cytometry analysis of Gr-1+CD11b+ cells after sorting is shown.

(E) Metastasis was significantly increased when 4T1 cells were coinjected with tumor-derived Gr-1+CD11b+ cells including Gr-1+CD11b+ cells derived from tumor tissues (tu inf Gr-1+CD11b+ cells) and spleens of tumor-bearing mice (tu sp Gr-1+CD11b+ cells). 4T1 cells alone and Gr-1+CD11b+ cells from normal mice (nor Gr-1+CD11b+ cells) were used as controls. Number of animals used is indicated in the bar. m.g., adjacent mammary gland tissue; tu, tumor tissues. Results are presented as the mean  $\pm$  SE.

were labeled with a green cell-tracking dye. Sorted Gr-1+CD11b+ cells were labeled with a red cell-tracking dye. 4T1 cells with or without Gr-1+CD11b+ cells were seeded into the top chamber of transwell filters coated with Matrigel. 4T1 cells that had invaded through the membrane were counted (eight to ten fields per filter) after overnight incubation. We found that 4T1 cells mixed with Gr-1+CD11b+ cells displayed a significant increase in tumor cell invasion (Figures 3B and 3C). Interestingly, GM6001 (1  $\mu$ M), a broad-spectrum MMP inhibitor, completely attenuated the observed invasion (Figure 3C). These results are in agreement with our in vivo findings that Gr-1+CD11b+ cells directly promote tumor cell invasion and metastasis.

Further, we observed significantly more invasive areas in the PyVmT/Tgfr2<sup>MGKO</sup> tumors than in the PyVmT/Tgfr2<sup>fllox.fllox</sup> tumors when the two tumor types were at the same stage and were similar in size (Figures S2A and S2C). The invasive areas in the PyVmT/Tgfr2<sup>MGKO</sup> tumors are often filled with a large number of Gr-1+CD11b+ cells (Figure S2B). Immunofluorescence staining of E-Cadherin did not show significant changes in E-Cadherin expression between the PyVmT/Tgfr2<sup>MGKO</sup> and control tumors, indicating that EMT is unlikely to be involved in PyVmT/Tgfr2<sup>MGKO</sup> tumor invasion (Figures S2D and S2E). These data suggest that PyVmT/Tgfr2<sup>MGKO</sup> tumor invasion may be aided by Gr-1+CD11b+ cells through a collective event between Gr-1+CD11b+ cells and tumor cells.





**Figure 3. Gr-1+CD11b+ Myeloid Cells Increased 4T1 Tumor Invasion In Vivo and In Vitro**

(A) Increased growth of recurrent tumors with 4T1 cells coinjected with Gr-1+CD11b+ cells derived from tumor tissues, or spleens of tumor-bearing mice. Number of animals is indicated in the bar. Results are presented as the mean  $\pm$  SE.

(B) Fluorescent microscopy of 4T1 cells that invaded through a Matrigel-coated transwell when cocultured with tumor-derived Gr-1+CD11b+ cells (Bb) compared to normal Gr-1+CD11b+ cells (Ba). 4T1 cells were labeled with a green tracking dye, and Gr-1+CD11b+ cells were labeled with a red tracking dye. (Bc) Close interaction between 4T1 cells and Gr-1+CD11b+ cells.

(C) An MMP inhibitor (GM6001, 1 mM) blocked Gr-1+CD11b+ cell-promoted 4T1 cell invasion in vitro. Results are from two experiments with triplicates for each group and are presented as the mean  $\pm$  SE. m.g., adjacent mammary gland tissue; tu, tumor tissues; tu inf Gr-1+CD11b+ cells, Gr-1+CD11b+ cells from tumor tissues; tu sp Gr-1+CD11b+ cells, Gr-1+CD11b+ cells from spleens of tumor-bearing mice; nor Gr-1+CD11b+ cells, Gr-1+CD11b+ cells from spleens of normal mice. Scale bar, 50  $\mu$ m for all panels.

demonstrating a requirement for metalloproteinase activity, we suggest that tumor-residing Gr-1+CD11b+ cells produce multiple MMPs that contribute to breast tumor cell invasion.

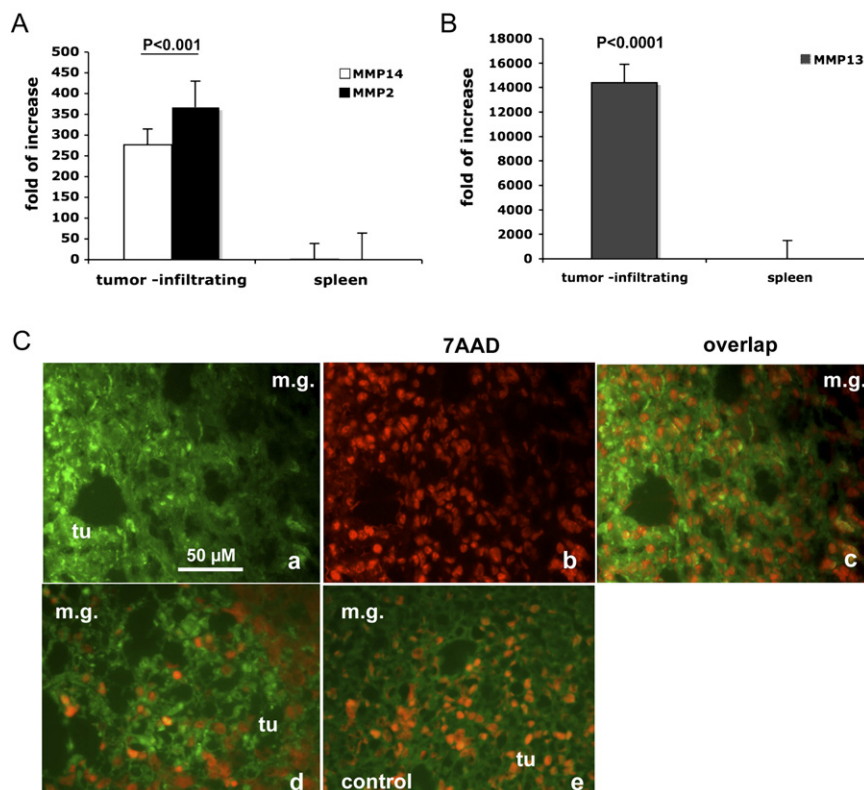
### Increased TGF $\beta$ Production in Mammary Carcinomas with Genetic Deletion of *Tgfb2*

In characterizing mammary carcinomas with *Tgfb2* deletion, we found that they contained significantly higher levels of TGF $\beta$ 1 than the control tumor without

### Tumor-Residing Gr-1+CD11b+ Cells Exhibit Elevated Production and Function of MMPs

MMPs contribute to tumor progression and metastasis (Lynch and Matrisian, 2002). Microarray analyses indicated that MMP14 (MT1-MMP), MMP13, and MMP2 are highly elevated in tumor-residing Gr-1+CD11b+ cells when compared with those from spleens (data not shown). We further confirmed the results with real-time RT-PCR (Figures 4A and 4B). Since inhibition of MMP activity abolishes Gr-1+CD11b+ cell-mediated tumor invasion in vitro (Figure 3C), we performed in situ zymography using a quenched fluorogenic substrate that releases fluorescent peptides upon cleavage by gelatinases. This assay allows us to examine the functionality of MMPs in Gr-1+CD11b+ cells directly in tumor tissues. As expected, very strong fluorescence intensity was localized in the tumor-mammary tissue interface where we had observed significant number of Gr-1+CD11b+ cells (Figures 4Ca–4Cc for 4T1 tumors and Figures 4Cd for MMTV-PyVmT/*Tgfb2*<sup>MGKO</sup> tumors). This was not observed in controls treated with EDTA (Figure 4Ce). Taken together with the in vitro data

*Tgfb2* deletion (Figure 5A). Because significantly more Gr-1+CD11b+ cells infiltrate into tumors with *Tgfb2* deletion, and our preliminary data from microarray analysis indicated that Gr-1+CD11b+ cells produce large amount of TGF $\beta$ 1, we reasoned that Gr-1+CD11b+ cells may contribute to increased TGF $\beta$ 1 production in these tumors. We measured TGF $\beta$ 1 in Gr-1+CD11b+ cells by ELISA. Gr-1+CD11b+ cells derived from tumor spleens produced significantly higher levels of TGF $\beta$ 1 than those from normal spleens (Figure 5B, left panel). No difference was found in TGF $\beta$ 1 production between tumor cell lines derived from mammary adenocarcinoma with or without *Tgfb2* deletion (Figure 5B, right panel). Immunohistochemistry of tumor sections further demonstrates that TGF $\beta$ 1-positive cells are mostly stromal cells (Figure 5C), especially in the invasive front where Gr-1+CD11b+ cells were mostly present (Figure 5C). These data support our observation that Gr-1+CD11b+ cells are likely one of the sources for increased TGF $\beta$ 1 production in mammary carcinomas with genetic deletion of *Tgfb2*.



**Figure 4. Increased Production and Function of MMPs in Tumor-Residing Gr-1+CD11b+ Cells**

(A and B) Real-time RT-PCR of MMP14 and MMP2 (A) as well as MMP13 (B) in Gr-1+CD11b+ cells derived from tumor tissues compared with those from spleens. Results are presented as the mean  $\pm$  SE.

(C) In situ zymography of MMPs in 4T1 mammary carcinomas and PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> carcinomas. Abundant green fluorescence (indicator of MMP activities) was observed in the invasive front of 4T1 tumors (Ca and Cc) and PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> carcinomas (Cd). Nuclei were labeled with 7AAD (red). Tumor sections treated with EDTA were used as negative controls (Ce). Scale bar, 50  $\mu$ m for all panels. m.g., adjacent mammary gland tissue; tu, tumor tissues.

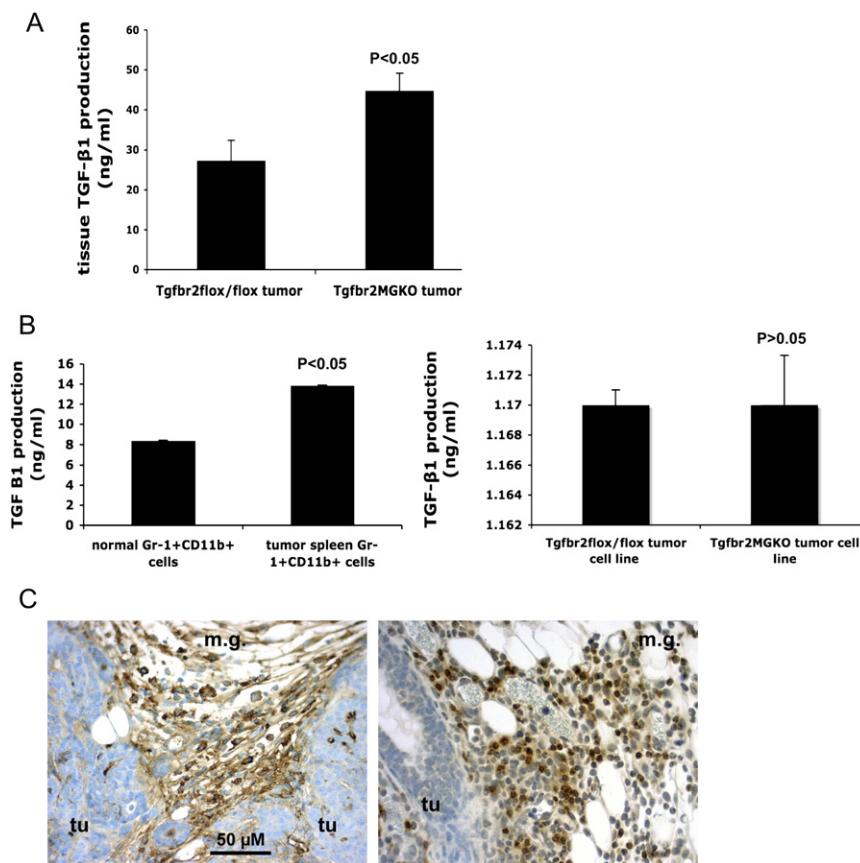
recruitment of Gr-1+CD11b+ cells to the mammary adenocarcinoma with *Tgfb $\beta$ 2* deletion but not the control tumors (Figure 6C), with no significant effect on tumor growth (data not shown). Gr-1+CD11b+ cells isolated from PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> tumor tissues express CXCR2, in levels that are modestly elevated compared to splenic Gr-1+CD11b+ cells (data not shown). These data indicate that elevated

CXCL5 production but not the corresponding CXCR2 receptor regulates the recruitment of Gr-1+CD11b+ cells in the PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> tumor microenvironment.

The SDF-1/CXCR4 chemokine axis mediates recruitment of several cell types, including hematopoietic progenitor cells (Burger and Kipps, 2006; Mohle et al., 1998; Peled et al., 1999; Urbich and Dimmeler, 2004). Thus, we examined CXCR4 expression in Gr-1+CD11b+ cells using flow cytometry analysis. Tumor-infiltrating Gr-1+CD11b+ cells express significantly increased CXCR4 when compared with those derived from spleens of tumor-bearing mice (Figure 6D). This includes Gr-1+CD11b+ cells from 4T1 and PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> tumors (Figure 6D), suggesting that upregulated CXCR4 expression is an intrinsic property of these cells regardless of differences in the tumor microenvironment to which they are recruited. Gr-1+CD11b+ cells isolated from tumor tissues migrated in response to recombinant SDF-1 in vitro (Figure 6E). Importantly, neutralizing CXCR4 using an antibody significantly blocked this process (Figure 6E). In addition to CXCR4, CXCR7 has been identified as another signaling receptor for SDF-1 (Balabanian et al., 2005; Burns et al., 2006). We found that approximately 19% of Gr-1+CD11b+ cells are CXCR7 positive (Figure S3B). Thus, Gr-1+CD11b+ cells could also be recruited through the SDF-1/CXCR7. Together, our data suggest that tumor-residing Gr-1+CD11b+ cells are recruited to tumor tissues through SDF-1/CXCR4 and potentially through CXCR7 interaction. In addition, PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> tumors have increased production of CXCL5 that is also responsible for the recruitment of Gr-1+CD11b+ cells to tumors with deleted or decreased TGF $\beta$  signaling.

#### Mechanisms of Gr-1+CD11b+ Cell Recruitment to Tumors

Our microarray analyses indicate that CXCL5 was significantly increased in PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> tumors compared with the PyVmT/Tgfb $\beta$ 2<sup>fllox/fllox</sup> tumors (data not shown). We further confirmed this observation with ELISA assays using cell culture supernatant from established cell lines (Figure 6A, left panel). Interestingly, we also observed elevated CXCL5 production in carcinoma cells with coexpression of a dominant-negative type II TGF $\beta$  receptor (DNIIR) with c-Neu under the MMTV promoter compared to c-Neu carcinoma cells (Figure S3A). In addition, pancreatic cancers with *Tgfb $\beta$ 2* deletion also showed increased CXCL5 production (H. Ijichi and H.L.M., unpublished data). These data indicate that CXCL5 may be a common mechanism in the tumor microenvironment with deleted or decreased TGF $\beta$  signaling. Further analysis confirmed that recombinant CXCL5 increased Gr-1+CD11b+ cell migration in vitro (Figure 6B), and neutralizing CXCL5 using a monoclonal antibody significantly blocked the migration of Gr-1+CD11b+ cells in response to PyVmT/Tgfb $\beta$ 2<sup>MGKO</sup> cell-conditioned medium (Figure 6B). To examine the role of the CXCL5/CXCR2 in Gr-1+CD11b+ cell recruitment to the tumor microenvironment in vivo, we transplanted breast adenocarcinoma with or without *Tgfb $\beta$ 2* deletion into the number four mammary gland in syngeneic FVB mice and allowed tumors to grow for 14 days. We then treated mice with equal tumor sizes with a specific CXCR2 antagonist, SB-265610, at 2 mg/kg/day for 2 weeks through i.p. injection. SB-265610 has been shown to have selective inhibition on neutrophil accumulation when used at 1–3 mg/kg/day (Auten et al., 2001). We found that the SB-265610 treatment significantly inhibited the



**Figure 5. Increased TGF $\beta$ 1 Production in Mammary Carcinomas with Genetic Deletion of *Tgfr2***

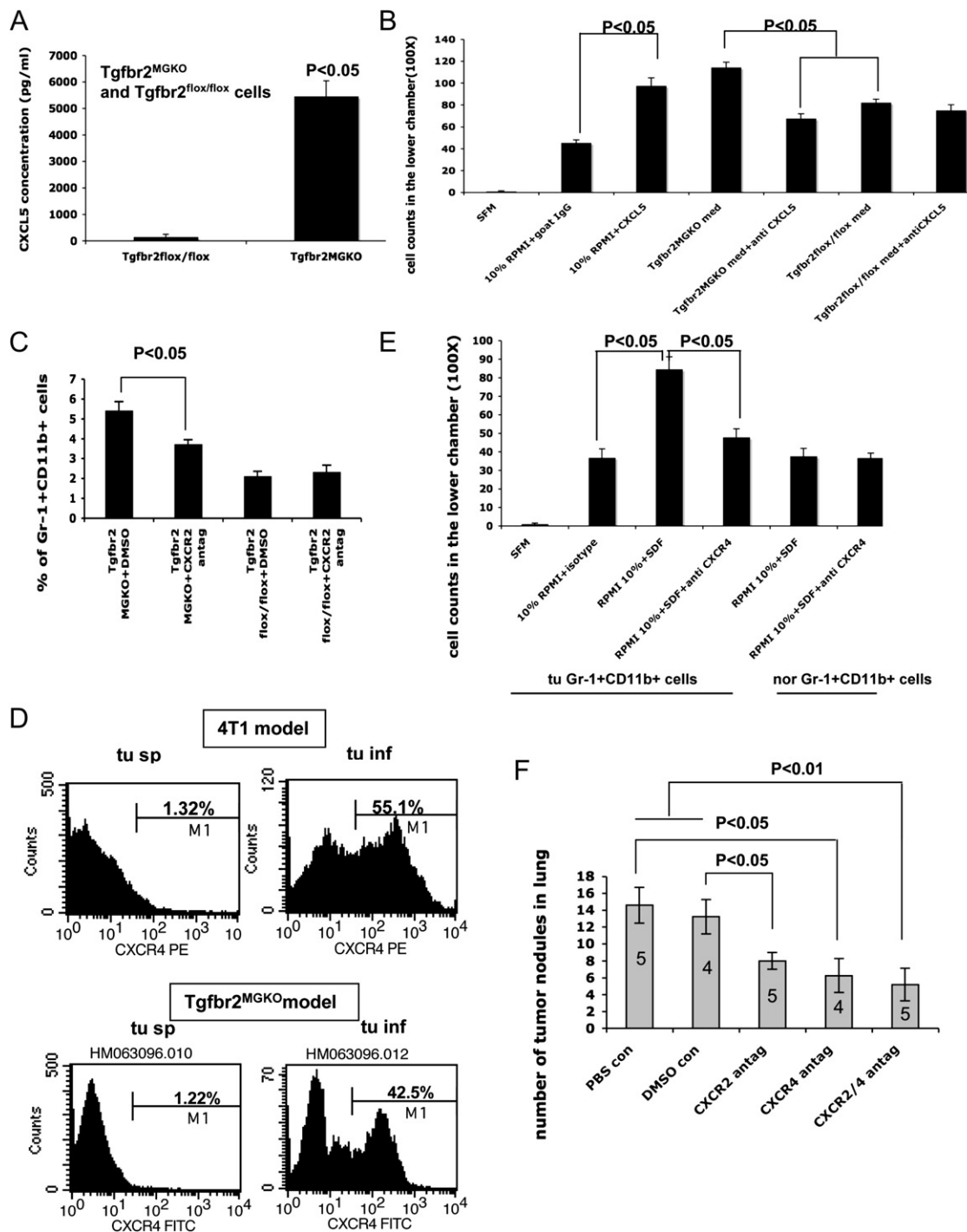
(A) Increased TGF $\beta$ 1 production in PyVmT/*Tgfr2*<sup>MGKO</sup> tumors compared with PyVmT/*Tgfr2*<sup>flox/flox</sup> tumors. *n* = 4 mice per group. (B) (Left panel) Gr-1+CD11b+ cells from spleens of mice bearing large 4T1 tumors exhibited significantly higher TGF $\beta$ 1 production when compared with Gr-1+CD11b+ cells from normal spleens. *n* = 2 mice per group. (Right panel) No difference of TGF $\beta$ 1 production was found between tumor cell lines derived from PyVmT/*Tgfr2*<sup>MGKO</sup> tumors versus PyVmT/*Tgfr2*<sup>flox/flox</sup> tumors. TGF $\beta$ 1 in conditioned media was measured by ELISA. Results are presented as the mean  $\pm$  SE. (C) Gr-1+CD11b+ cells are likely the resource for increased TGF $\beta$ 1 production in mammary carcinomas with genetic deletion of *Tgfr2*. IHC of TGF $\beta$ 1 (left panel) shows that TGF $\beta$ 1-positive cells are mostly in the invasive front where Gr-1+CD11b+ cells were mostly present (right panel with Gr-1 staining). Scale bar, 50  $\mu$ m for both panels. m.g., adjacent mammary gland tissue; tu, tumor tissues.

### Immature Myeloid Cells in Human Breast Tumors

Finally, we investigated human breast carcinomas for the presence of immature myeloid cells. We chose myeloperoxidase (MPO) as a marker for human immature myeloid cells for IHC. This is based on the following: (1) expression of MPO

To further examine the critical roles of Gr-1+CD11b+ cell recruitment in PyVmT/*Tgfr2*<sup>MGKO</sup> tumor invasion and metastases, we transplanted breast adenocarcinoma with *Tgfr2* deletion into the number four mammary gland in syngeneic FVB mice and allowed tumors to establish for 7 days. We then treated the mice with a CXCR2-specific antagonist, SB-265610, at 2 mg/kg/day, through i.p. injection or a CXCR4 antagonist, AMD3100, at 1.25 mg/kg/day twice daily through s.c. injection, or both for 10 days. The primary tumors were then surgically removed. The mice were allowed to live for another 3 weeks for tumor metastasis development in the lungs. The effects of the antagonist on tumor metastasis, tumor growth, and invasion were evaluated. We found that lung metastasis is significantly inhibited when mice are treated with the CXCR2 antagonist, the CXCR4 antagonist, or CXCR2 and CXCR4 antagonists when compared with vehicle controls (Figure 6F). No significant difference in primary tumor growth was found between the treatment and the controls (data not shown). H&E staining of the tumor tissues showed that the degree of tumor invasion in mice treated with CXCR2, CXCR4, or both was reduced when compared with the vehicle controls (Figures S2F–S2J). These data, together with data from in vitro coculture (Figures 3B and 3C) and the effect of in vivo coinjection of tumor cells with Gr-1+CD11b+ cells (Figures 3A and 2E) strongly suggest that Gr-1+CD11b+ cells are critical players in tumor cell invasion and metastasis formation.

is significantly elevated in Gr-1+CD11b+ immature myeloid cells from several mouse models by microarray analysis (data not shown); (2) MPO, a hemoprotein, is abundantly expressed in neutrophils. MPO possesses proinflammatory properties and participates in tumor initiation and progression (Knaapen et al., 2006). We examined eight cases of human breast carcinoma tissues. MPO-positive cells were clearly abundant in the invasive front of all samples examined (Figure 7A). These include the front in the collagen-rich area (Figures 7Aa–7Ac) and the front in normal mammary fat tissues (Figures 7Ad–7Af). MPO-positive cells were found in both situations (Figures 7Aa and 7Ad). H&E staining of these tumor tissues further supports our observations (Figures 7Ab and 7Ae). To further confirm the infiltrating myeloid cells in human breast tumor tissues, we utilized flow cytometry technology. We labeled single-cell suspensions from fresh human breast ductal adenocarcinomas (stages 2–3) with antibody cocktails. These include antibodies for lineage identification: CD3 for T cell; CD19 for B cell; CD56 for NK cell; CD40/CD86/HLA-DR for dendritic cells; and CD14 for monocytes; and antibodies for myeloid cell marker, including CD33, CD34, and CD15. The myeloid markers are typically used for examining the peripheral blood of patients with myelodysplasia. Tumor-infiltrating immature myeloid cells were identified as T, B, NK, monocyte, and dendritic cell lineage negative and positive for CD33, CD34, and CD15 myeloid markers. The percentage of immature myeloid cells was evaluated for each patient, with four patients total.



**Figure 6. Mechanisms of Recruitment of Gr-1+CD11b+ Cells to the Tumor Microenvironment**

(A) Elevated production of CXCL5 in cell culture supernatant of PyVmT/Tgfr2<sup>MGKO</sup> mammary carcinomas.

(B) In vitro migration of Gr-1+CD11b+ cells in response to CXCL5. Gr-1+CD11b+ cells that migrated after 6–8 hr incubation were counted and plotted. Shown is one of the representative experiments of three performed.

(C) Inhibition of Gr-1+CD11b+ cell recruitment to PyVmT/Tgfr2<sup>MGKO</sup> tumors with CXCR2 antagonism. Percentage of Gr-1+CD11b+ cells in all cells from PyVmT/Tgfr2<sup>MGKO</sup> and control tumors were plotted. Mice bearing PyVmT/Tgfr2<sup>MGKO</sup> and control tumors were treated with a CXCR2-specific antagonist, SB-265610, at 2 mg/kg/day for 2 weeks through i.p. injection.

(D) Flow cytometry analysis of CXCR4 expression in infiltrating Gr-1+CD11b+ cells from 4T1 tumors as well as PyVmT/Tgfr2<sup>MGKO</sup> tumors, compared with those from spleens of the same tumor-bearing mice (as labeled). Histogram of CXCR4 expression was gated on Gr-1+CD11b+ double-positive cells. Shown is one of the representative mice analyzed; n = 3–5 mice per group.

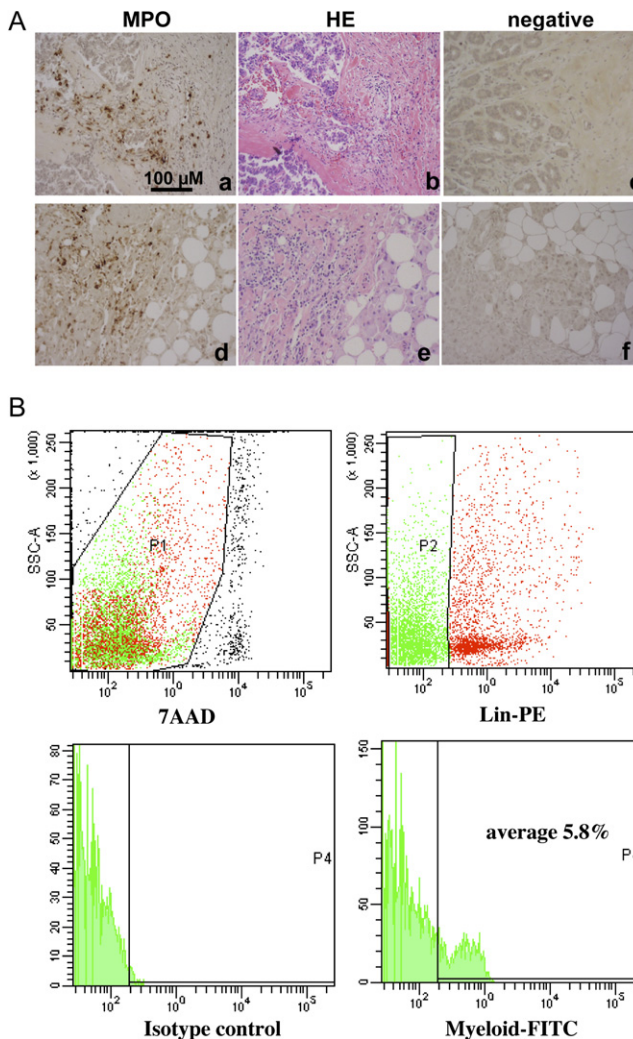


We found that an average of  $5.82\% \pm 2.3\%$  of total cells from tumor tissues are these myeloid cells (Figure 7B). Together, our data suggest that immature myeloid cells participate not only in tumor invasion in mouse models but also likely in human breast cancers. Collectively, our findings point out a strategy to target tumor metastasis by disrupting immature cell recruitment to tumors.

## DISCUSSION

Using the MMTV PyVmT mouse model we demonstrate that deletion of *Tgfb2* in mammary carcinomas specifically enhances recruitment of Gr-1<sup>+</sup>CD11b<sup>+</sup> myeloid cells to the invasive front. We further demonstrate that Gr-1<sup>+</sup>CD11b<sup>+</sup> cells promote tumor invasion and metastasis through increased MMP production. TGF $\beta$  functions as a tumor suppressor or a tumor promoter depending on the context and stage of tumor progression. We and others have shown that abrogation of TGF $\beta$  signaling with deletion of *Tgfb2* resulted in aggressive tumor progression in several mouse models (Biswas et al., 2004; Forrester et al., 2005; Ijichi et al., 2006; Munoz et al., 2006; Lu et al., 2006). However, other reports show that the enhancement of TGF $\beta$  signaling in mammary epithelial cells in conjunction with c-Neu or PyVmT increases pulmonary metastases (Muraoka et al., 2003; Muraoka-Cook et al., 2004, 2006). These seem to be contradicting observations, which now could be explained by the recruitment of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells into the tumor microenvironment. Our data suggest that autologous TGF $\beta$  signaling in mammary epithelial cells act as a tumor suppressor; when it is deleted or altered, it results in Gr-1<sup>+</sup>CD11b<sup>+</sup> cell recruitment. This leads to increased MMP and TGF $\beta$  production that enhances tumor invasion and metastasis. The switch of TGF $\beta$  signaling from tumor suppressor to tumor promoter thus involves an additional component, which is the recruitment of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in the tumor microenvironment. Indeed, inflammatory cells (CD45- and BM8-positive cells) have been observed in head-and-neck tumors lacking TGF $\beta$  signaling (Lu et al., 2006). In addition, inflammation causes precancerous lesions in mice with TGF $\beta$ 1 deficiency that can progress to colon cancer (Engle et al., 2002). Very recently, CCR1<sup>+</sup> myeloid cells (CD34<sup>+</sup>) have been shown to be recruited to colon cancers with deletion of Smad4 and to promote tumor invasion (Kitamura et al., 2007). These CCR1<sup>+</sup> cells seem to be different from Gr-1<sup>+</sup>CD11b<sup>+</sup> cells we described here, as they do not express high levels of Gr-1.

Several lines of evidence make the contribution of Gr-1<sup>+</sup>CD11b<sup>+</sup> to tumor metastasis particularly interesting. (1) These cells are overproduced in tumor hosts that include cancer patients with a variety of tumors. (2) Gr-1<sup>+</sup>CD11b<sup>+</sup> cells are composed of immature myeloid cells at the early stages of differentiation (Almand et al., 2001; Yang et al., 2004). They are different from terminally differentiated tumor-associated macrophages (TAM), identified as Mac-1 (CD11b) and F4/80<sup>+</sup>, which have been shown to promote tumor progression and metastasis through elevated CSF-1 production and enhanced EGF signaling



**Figure 7. Immature Myeloid Cells in Human Breast Tumor Tissues**

(A) IHC staining of myeloperoxidase to identify bone marrow-derived immature myeloid cells at the invasive front of human breast ductal adenocarcinomas (Aa and Ad). (Ab) is an H&E-stained section serial to (Aa); (Ae) is an H&E-stained section serial to (Ad). (Ac) and (Af) are negative controls. Scale bar, 100  $\mu$ M for all panels.

(B) Flow cytometry analysis of immature myeloid cells from single-cell suspension of human breast ductal adenocarcinomas (stages 2–3). The analyzed cells were gated as 7AAD negative (top left panel, P1); negative for lineage markers including CD3 for T cell, CD19 for B cell, CD56 for NK cell, CD40/CD86/HLA-DR for dendritic cells, and CD14 for monocytes; and CD33, CD34, and CD15 positive (top right panel, P2). A histogram for FITC-positive myeloid cells is shown in the lower right pane, with isotype control in the lower left panel.

in cancer cells (Condeelis and Pollard, 2006). Similarities between TAM and these immature myeloid cells were noticed from profiling work (Biswas et al., 2006); however, differences between the two populations were also evident. For example, myeloid suppressor cells produce high levels of TGF $\beta$ 1,

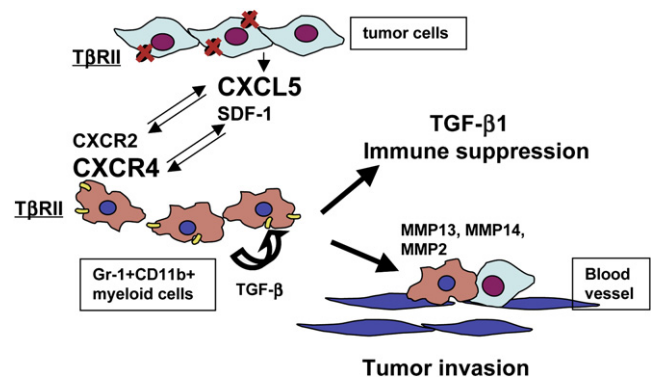
(E) In vitro migration of Gr-1<sup>+</sup>CD11b<sup>+</sup> cells in response to SDF-1. Gr-1<sup>+</sup>CD11b<sup>+</sup> cells that migrated after 6–8 hr incubation were counted and plotted. Shown is one representative experiment of three performed.

(F) PyVmT/*Tgfb2*<sup>MGKO</sup> tumor metastasis with blockade of CXCR2, CXCR4, or both. Tumor nodules in lung were counted after mice bearing 14 day tumors were treated with CXCR2 or CXCR4 antagonists or both for 3 weeks. All results are presented as the mean  $\pm$  SE.

whereas TGF- $\beta$ 1 expression in TAMs was restricted to unstimulated TAMs and was not further increased by M2-biasing cytokines (Biswas et al., 2006). Gr-1+CD11b+ cells are also different from two other cell types in the tumor microenvironment, neutrophils and mast cells, which express Gr-1 but not CD11b. (3) Gr-1+CD11b+ cells interact with other host immune cells including T, B, and NK cells. These immune cells may dictate the tumor microenvironment through a shift from inflammation/immune response to anti-inflammatory/immune-suppressive responses (Th1/Th2-like cytokine shift) and may be responsible in the metastatic liver milieu (Budhu et al., 2006). It is unclear whether systemic immune suppression and direct participation in tumor progression are two different properties or different manifestations of the same process.

Our data demonstrate that at least two distinct chemokine axes regulate the recruitment of Gr-1+CD11b+ cells in tumor tissues. One is SDF-1/CXCR4. Interestingly, tumor-residing Gr-1+CD11b+ cells, from both mammary tumors with *Tgfr2* deletion and 4T1 tumors, express high levels of CXCR4 when compared with controls (Figure 6D). This indicates that upregulation of CXCR4 in Gr-1+CD11b+ cells is an intrinsic property of Gr-1+CD11b+ cells regardless of the difference in the tumor microenvironments into which they are recruited. SDF-1/CXCR4 has been implicated in the recruitment of hematopoietic stem and progenitor cells (Balkwill and Coussens, 2004; Jin et al., 2006; Mohle et al., 1998; Peled et al., 1999; Urbich and Dimmeler, 2004), supporting our observation that Gr-1+CD11b+ cells are hematopoietic progenitors. In addition to the SDF-1/CXCR4 chemokine axis, mammary carcinomas with *Tgfr2* deletion showed an increased production of CXCL5 (Figure 6A). The role of CXCL5 in cancer-associated inflammation is indicated in the prostate (Hochreiter et al., 2000). CXCL5 also has profound angiogenic potential (Wente et al., 2006). Interestingly, elevated CXCL5 production was also observed in dominant-negative type II TGF $\beta$  receptor/MMTV-C-Neu cells (Figure S3), indicating CXCL5 may be a result of diminished TGF $\beta$  signaling in the tumor microenvironment. These data suggested two distinct mechanisms for Gr-1+CD11b+ cell recruitment: increased expression of CXCR4 in Gr-1+CD11b+ cells as an intrinsic property and elevated CXCL5 level in PyVmT/*Tgfr2*<sup>MGKO</sup> tumors as tumor microenvironment cues. When PyVmT/*Tgfr2*<sup>MGKO</sup> tumors were treated with a CXCR2-specific antagonist or a CXCR4 antagonist, there was a significantly decreased tumor metastasis, strongly suggesting a critical role of Gr-1+CD11b+ cells in PyVmT/*Tgfr2*<sup>MGKO</sup> tumor invasion and metastases.

Gr-1+CD11b+ cells promote tumor metastasis, likely through enhanced production and function of MMPs including MT1-MMP (MMP14), MMP13, and MMP2. Treatment with a pan-MMP inhibitor significantly attenuated tumor invasion in vitro. MT1-MMP plays a crucial role in degrading extracellular matrix and allows tumor cells to escape the matrix-enforced growth control effect (Hotary et al., 2003). MT1-MMP is known to be able to activate MMP2 and MMP13, both of which are associated with tumor invasion (d'Ortho et al., 1997; English et al., 2001; Leeman et al., 2002; Nelson et al., 2000). Results from our in vitro coculture (Figures 3B and 3C) and in vivo coinjection of tumor cells with Gr-1+CD11b+ cells (Figures 3A and 2E) strongly suggest a role for these cells in tumor invasion and metastasis. In addition, PyVmT/*Tgfr2*<sup>MGKO</sup> tumors, with a large



**Figure 8. Mechanisms for Enhanced Tumor Progression Resulting from Loss of TGF $\beta$  Signaling**

Deletion of the type II TGF $\beta$  receptor gene in mammary carcinomas results in elevated production of CXCL5, which interacts with CXCR2 on Gr-1+CD11b+ cells, thus recruiting the cells into the tumor microenvironment. Gr-1+CD11b+ cells, as hematopoietic cells, express high levels of CXCR4, which interacts with SDF-1 in the tumor microenvironment, and are also recruited. Gr-1+CD11b+ cells promote tumor invasion through high expression of MMP14, MMP13, and MMP2. In addition, Gr-1+CD11b+ cells produce high levels of TGF $\beta$ 1 that inhibit host immune surveillance.

number of Gr-1+CD11b+ cells, showed significantly more invasive area compared with PyVmT/*Tgfr2*<sup>flox.flox</sup> tumors. Further, tumors treated with CXCR2 or CXCR4 antagonists showed reduced invasiveness. These data suggest that the increased invasion of PyVmT/*Tgfr2*<sup>MGKO</sup> tumor may be aided by Gr-1+CD11b+ cells through a collective event between Gr-1+CD11b+ cells and tumor cells, with the myeloid cells providing MMPs. We cannot rule out the contribution of other factors produced by Gr-1+CD11b+ cells to tumor cell invasion, but our results strongly suggest that an induction of proteolytic activity is a major contributing factor. In addition, the observed increased tumor growth and metastasis-promoting effects of Gr-1+CD11b+ cells could also be due to their effect on angiogenesis, as we previously reported (Yang et al., 2004).

Tumor-infiltrating Gr-1+CD11b+ myeloid cells are likely one of the major sources for increased TGF $\beta$ 1 production observed in mammary carcinomas with genetic deletion of *Tgfr2* (Figure 5). This is demonstrated by the IHC of tumor sections showing that TGF $\beta$ 1-positive cells are mostly stromal cells (Figure 5C), especially in the invasive front where Gr-1+CD11b+ cells were most abundant. Our observation is supported by a recent publication showing that, in response to *Tgfr2* deletion, there was increased TGF $\beta$ 1 expression in the stroma (Lu et al., 2006). We have now shown that Gr-1+CD11b+ cells are components of these stromal cells. In addition, as Gr-1+CD11b+ cells produce high levels of TGF $\beta$ 1, they are likely to have a major effect on tumor evasion from host immune detection.

In summary, we have demonstrated that deletion of the type II TGF $\beta$  receptor gene in mammary carcinomas results in the recruitment of Gr-1+CD11b+ myeloid cells through two distinct mechanisms: SDF-1/CXCR4 as an intrinsic mechanism for Gr-1+CD11b+ cells and CXCL5/CXCR2. Gr-1+CD11b+ cells directly promote tumor metastasis through enhanced MMP and TGF $\beta$  production (Figure 8). Cellular and molecular targeting of Gr-1+CD11b+ cell production/recruitment should not only

improve host immunosurveillance but also inhibit tumor metastasis, having the effect of “killing two birds with one stone” and provide additional therapeutic options.

## EXPERIMENTAL PROCEDURES

### Cell Lines and Mice

The 4T1 breast cancer cell line was obtained from ATCC and maintained per standard cell culture techniques. MMTV-PyVmT/Tgfr2<sup>MGKO</sup> and floxed control cell lines were established as described (Forrester et al., 2005). Eight- to ten-week-old female Balb/c mice were purchased from Harlan Inc. (Indianapolis, IN). Tgfr2<sup>lox/flox</sup>, Tgfr2<sup>MGKO</sup>, MMTV-PyVmT/Tgfr2<sup>lox/flox</sup>, and MMTV-PyVmT/Tgfr2<sup>MGKO</sup> mice were established and maintained as described (Forrester et al., 2005). The studies were approved by IACUC at Vanderbilt University Medical Center.

### Flow Cytometry Analysis

Single-cell suspensions were made from spleens and BM of normal and tumor-bearing mice (Yang et al., 2004), and tumor tissues (Ljung et al., 1989). These cells were labeled with fluorescence-conjugated antibodies (BD PharMingen) and isotype-matched IgG controls. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

### Single-Cell Sorting

Splenocytes from normal or tumor-bearing mice were stained with fluorescence-labeled antibodies and sorted with a FACStarPlus flow cytometer (Becton Dickinson). Gr-1+CD11b+ cells were collected for tumor growth, invasion, and migration experiments.

### 4T1 Breast Tumor Model for Metastasis

4T1 ( $5 \times 10^5$  cells), without or with Gr-1+CD11b+ cells ( $0.5 \times 10^5$ ), was injected into the mammary fat pad of female Balb/c mice. Primary tumors were completely removed and weighed 12–14 days after tumor inoculation. The mice were sacrificed 4 weeks later, and the lung metastasis was examined using whole-lung mounting procedures. In addition, the weight of recurrent tumors at the primary injection sites was also recorded.

### Whole-Lung Mounting

Mice were sacrificed by anesthetic overdose. Lungs were processed as described (Jessen et al., 2004). The tumor nodules in lung were then counted.

### Immunohistochemistry and Immunofluorescence

Paraffin-embedded tumor sections were fixed in 4% paraformaldehyde and incubated with a rat polyclonal anti-Gr-1 antibody (BD PharMingen), rabbit polyclonal anti-myeloperoxidase (Abcam), or anti-TGF $\beta$ 1 (PharMingen). A biotinylated second antibody was applied, followed by incubation with streptavidin-conjugated HRP. Peroxidase activity was localized with diaminobenzidine (Vectastain ABC kit, Vector Laboratories). For immunofluorescence, anti-E-cadherin antibody (BD transduction laboratories, 1:500 dilution) was applied to paraffin-embedded tumor sections; Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, 10  $\mu$ g/ml) was used to detect E-cadherin expression.

### Immunoassay for Chemokines

Conditioned media and tissue lysates were analyzed for CXCL5 and TGF $\beta$ 1 expression by ELISA using commercial kits (R&D Systems).

### In Vitro Cell Migration and Invasion Assays

For cell migration, Gr-1+CD11b+ cells (50,000) were seeded onto the top chamber of transwell filters (8  $\mu$ m, VWR Scientific). The filters were placed in a 24-well plate that contains medium with recombinant mouse SDF-1 or isotype control at 100 ng/ml. To neutralize CXCR4, the CXCR4 monoclonal antibody at 10  $\mu$ g/ml was added to the top chamber of the transwell. Migrated Gr-1+CD11b+ cells were counted (five to seven fields per well, triplicate for each experimental groups) 6–8 hr after incubation. For in vitro invasion assays, 4T1 cells were labeled with green fluorescence dye CMFDA (Molecular Probes). Gr-1+CD11b+ cells were labeled with red fluorescence dye CMTMR.

4T1 cells (30,000) alone or with Gr-1+CD11b+ cells (30,000) were seeded into the top chamber of transwell filters coated with Matrigel (Becton Dickinson). The filters were placed in a 24-well plate that contains culture media of 10% FCS RPMI. Invaded 4T1 cells were counted (three fields per filter, triplicate for each experimental groups) 6–8 hr after incubation.

### In Vivo Inhibition of Gr-1+CD11b+ Cell Recruitment to Breast Adenocarcinomas

MMTV-PyVmT/Tgfr2<sup>MGKO</sup> and MMTV-PyVmT/Tgfr2<sup>lox/flox</sup> tumors from donor mice were cut into approximately 1 mm<sup>3</sup> and planted into the #4 mammary fat pad of syngeneic FVB recipient mice. Tumor size was determined 2 weeks later as described (Yang et al., 2004). Mice with similar tumor size were treated with the CXCR2-specific antagonist SB-265610 (GSK Pharmaceuticals) at 2 mg/kg/day through i.p. injection for 2 weeks (DMSO as vehicle). Tumor size was measured again at the end of the treatment. For the effect of CXCR2 and CXCR4 blockade on PyVmT/Tgfr2<sup>MGKO</sup> tumor metastasis, mice were implanted with tumors for 7 days and then were treated with SB-265610 as described above, or a CXCR4 antagonist (AMD3100, Sigma, at 1.25 mg/kg/day twice daily through s.c. injection, PBS as vehicle), or both for 10 days. The primary tumors were then surgically removed. The mice were allowed to live for another 3 weeks for lung metastasis development without further treatment. Metastasis nodules were counted. Primary tumors were removed, weighed, and processed for HE staining.

### In Situ Zymography of MMPs

Substrate (DQ-gelatin, Molecular Probes) was dissolved to a final concentration of 25  $\mu$ g/ml in a mixture of 2% gelatin and 2% sucrose in PBS + 0.02% sodium azide. One hundred microliters of this solution was then coated on a slide, set on ice for 10 min, then stored at room temperature until next day. Protease inhibitors including Aprotinin (2  $\mu$ g/ml), Leupeptin (100  $\mu$ M), and pepstatin were included in the substrate solution to exclude other protease activities in addition to MMPs. EDTA (25 mM), an inhibitor of metalloproteinases, was used in negative controls. Frozen tumor sections (5–10  $\mu$ m) were cut directly onto the coated slides, and the slides were incubated in dark at 30°C for 24–36 hr. The slides were then mounted using an aqueous fluorescence-saving mountant with 50 nM 7AAD, a nucleic acid stain (Molecular Probes). The slides were then examined under a fluorescence microscope.

### Quantitative RT-PCR

Total RNA was extracted from sorted Gr-1+CD11b+ cells as described using an RNeasy Mini Kit (QIAGEN Inc.). cDNA was synthesized using the Invitrogen SuperScript first-strand synthesis system for RT-PCR. Primers specific for MMP2, MMP13, and MT1-MMP were used, and relative gene expression was determined using BioRad iCycler-iQ SYBR Green PCR kit (BioRad Laboratories). The comparative threshold cycle method was used to calculate gene expression normalized to  $\beta$ -actin as a gene reference. Primers were designed using Beacon Designer 4 software and synthesized by Operon (a QIAGEN company). Primer sequences are available upon request.

### Human Breast Tumor Tissues

Human breast ductal adenocarcinomas (stages 2–3) were collected from patients under surgery in Tianjing Cancer Institute, China, with written consent. The samples were rinsed in cold PBS and put in RPMI supplemented with FCS. The samples were then processed to obtain single-cell suspension as described earlier. The cells were then labeled with fluorescence-conjugated antibodies (BD PharMingen) and isotype-matched IgG controls. The cells were analyzed on a FACSaria flow cytometer (Becton Dickinson, Mountain).

### Statistical Analysis

All data were analyzed by Student's *t* test and were expressed as means  $\pm$  SE, and differences were considered statistically significant when *p* < 0.05.

### Supplemental Data

The Supplemental Data include three supplemental figures and can be found with this article online at <http://www.cancer.org/cgi/content/full/13/1/23/DC1/>.



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