

# Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils

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**Expansion of myeloid cells associated with solid tumor development is a key contributor to neoplastic progression. Despite their clinical relevance, the mechanisms controlling myeloid cell production and activity in cancer remains poorly understood. Using a multistage mouse model of breast cancer, we show that production of atypical T cell-suppressive neutrophils occurs during early tumor progression, at the onset of malignant conversion, and that these cells preferentially accumulate in peripheral tissues but not in the primary tumor. Production of these cells results from activation of a myeloid differentiation program in bone marrow (BM) by a novel mechanism in which tumor-derived granulocyte-colony stimulating factor (G-CSF) directs expansion and differentiation of hematopoietic stem cells to skew hematopoiesis toward the myeloid lineage. Chronic skewing of myeloid production occurred in parallel to a decrease in erythropoiesis in BM in mice with progressive disease. Significantly, we reveal that prolonged G-CSF stimulation is both necessary and sufficient for the distinguishing characteristics of tumor-induced immunosuppressive neutrophils. These results demonstrate that prolonged G-CSF may be responsible for both the development and activity of immunosuppressive neutrophils in cancer.**

cancer | immunology | stem cell biology | hematopoiesis | myeloid-derived suppressor cells

Cancer is a systemic disease. Tumors secrete a variety of factors that not only regulate their local microenvironment, but also regulate peripheral tissues to promote metastasis (1–3). The tumor-promoting role of the immune system has long been recognized (4–7), and emerging evidence suggests that accumulation of circulating myeloid cells at peripheral sites promotes a permissive macro-environment for systemic tumor outgrowth.

In cancer patients with solid tumors, aberrant myeloid cells with T cell-suppressive activity are significantly increased in the circulation compared with healthy individuals and are highest in number in patients with extensive metastatic tumor burden (8). In murine transplantation models that mainly mimic the late stages of tumor development, T cell-suppressive myeloid cells expand in the circulation and also in the tumor, spleen, and premetastatic lungs (9). Of particular interest, these myeloid cells promote tumor progression by impairing adaptive immune responses (10, 11), modulating cytokine production by macrophages (12), and stimulating angiogenesis and remodeling of the extracellular matrix (13). In addition, expansion of these T cell-suppressive myeloid cells in premetastatic lung is associated with a local decrease in IFN $\gamma$  production, the major antitumor cytokine, and an increase in several protumor cytokines, along with vascular remodeling leading to abnormal leaky vessels (14, 15).

This population of myeloid cells that acquires the ability to suppress T-cell function in cancer is heterogeneous and includes monocytes, myeloid precursors, and neutrophils, leading to the collective term myeloid-derived suppressor cells (MDSCs) (16). The T cell-suppressive phenotype is mediated by increased ar-

ginase activity and the production of reactive oxygen species (ROS) (16). These cells share common myeloid surface markers with their naïve healthy counterparts (CD11b<sup>+</sup>Gr1<sup>+</sup> in mice or CD11b<sup>+</sup>CD33<sup>+</sup>HLADR<sup>-</sup> in humans), but differ in activity and gene expression. Although a link between poor prognosis in breast cancer and an increase in circulating myeloid cells in humans is well documented (17), how tumors regulate myeloid cell development remains largely unknown.

Hematopoietic cells are all produced from a rare population of self-renewing hematopoietic stem cells (HSCs), which reside in the bone marrow (BM) cavity (18). In mice, the lineage<sup>neg</sup> Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cell compartment contains the most immature hematopoietic stem and progenitor cells (HSPCs) and include HSCs (defined as LSK Flk2<sup>neg</sup>CD150<sup>+</sup>CD48<sup>neg</sup>) along with non-self-renewing multipotent progenitors (MPPs), which can be further divided into LSK Flk2<sup>+</sup> (MPP<sup>F+</sup>) and a less characterized LSK Flk2<sup>neg</sup>CD48<sup>+</sup> (MPP<sup>F-</sup>) subset (19). MPPs give rise to lineage-committed common lymphocyte progenitors (CLPs) and common myeloid progenitors (CMPs, defined as lineage<sup>neg</sup>Sca-1<sup>+</sup>c-Kit<sup>neg</sup>FcyR<sup>neg</sup>CD34<sup>+</sup>), which support the production of all circulating mature lymphoid and myeloid cells, respectively. CLPs give rise to distinct subsets of B cells, T cells, natural killer cells, and dendritic cells (DCs), whereas CMPs differentiate into granulocyte/macrophage progenitors (GMPs, defined as lineage<sup>neg</sup>Sca-1<sup>+</sup>c-Kit<sup>neg</sup>FcyR<sup>+</sup>CD34<sup>+</sup>) to support the produc-

## Significance

**We show that tumor reprogramming of hematopoiesis in bone marrow occurs at the onset of malignant conversion and results in systemic expansion of circulating activated neutrophils that preferentially accumulate in lungs. Our data are, to our knowledge, the first to show that activation and not inhibition of myeloid differentiation is responsible for expansion and activity of T cell-suppressive myeloid cells; a tumor-derived factor targets the immature hematopoietic compartment to drive myeloid expansion; granulocyte-colony stimulating factor (G-CSF) is the only hematopoietic growth factor to increase in serum during early tumor development; prolonged G-CSF induces production of Rb1<sup>low</sup> neutrophils and not short-term mobilization; and G-CSF acts in a cell intrinsic manner to expand multipotent progenitors to increase production of tumor-derived Ly6G<sup>+</sup> neutrophils.**

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tion of monocytic (macrophage) and granulocytic (neutrophils, eosinophils, and basophils) cells, as well as myeloid-derived DCs. CMPs also differentiate into megakaryocyte/erythrocyte progenitors (MEPs, defined as lineage<sup>neg</sup>Sca-1<sup>+</sup>c-Kit<sup>neg</sup>FcγR<sup>neg</sup>CD34<sup>neg</sup>) to give rise to red blood cells (RBCs) and megakaryocytes (platelets). Within this well-established hematopoietic tree, the origin of tumor-associated T cell-suppressive CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells is still a matter of debate.

The current model suggests that these cells arise from myeloid progenitors (MPs) that are past the GMP stage in a two-step process that includes a block in differentiation, resulting in an expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid precursors and an induction of T cell-suppressive activity (9). Tumor-derived myeloid differentiation cytokines including granulocyte (G)-colony stimulating factor (G-CSF), macrophage (M)-colony stimulating factor (CSF), and GM-CSF are thought to induce accumulation of immature myeloid progenitors, in combination with tumor-associated proinflammatory factors (e.g., IL-1β, IL-6, S100A8, S100A9). In addition, T cell-derived cytokines (IFNγ, IL-4, IL-10, and IL-13) likely commit T cell-suppressive activity to these cells (9). Epigenetic silencing of the gene that encodes the protein for Retinoblastoma-1 (Rb1) is also associated with the aberrant myeloid cell expansion that occurs in solid tumor cancers, and knockdown of Rb1 in the absence of tumor development is sufficient to induce the overproduction of similar myeloid cells, but without T cell-suppressive activity (20). Although these results provide a mechanism for the expansion of tumor-associated CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells, it remains unknown which tumor-secreted factors inhibit Rb1 expression, at which stage during myeloid commitment loss of Rb1 occurs, and how T cell-suppressive activity is acquired. Moreover, it remains unclear whether this effect is limited to MPs or also involves more immature HSPCs.

Our goal was to determine the driving factor(s) and identify the cell populations responsible for expansion of T cell-suppressive myeloid cells in cancer. We conducted a comprehensive *in vivo* evaluation of the HSPC and MP regulation in an autochthonous multistage mouse model of breast cancer. We identified a previously unidentified pathway of myeloid differentiation in cancer in which tumor-derived G-CSF directs myeloid differentiation at the primitive HSC to generate activated, Rb1<sup>low</sup>, T cell-suppressive neutrophils.

## Results

**A Coordinated Expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> Myeloid Cells in Peripheral Tissues Parallels Tumor Development.** In cancer patients, expansion of T cell-suppressive myeloid cells correlates with tumor stage (8). We used the PyMT transgenic mouse model (21), which expresses the polyomavirus middle T antigen driven by the mouse mammary tumor virus (MMTV) promoter, to map expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells during the multiple stages of breast cancer progression. PyMT mice develop a progressive disease that mimics human pathology (22), in which all mammary glands (MGs) display signs of breast cancer with an initial presentation of hyperplasia (6 wk), which then transitions to adenoma or mammary intraepithelial neoplasia (8 wk), early carcinoma (10 wk), and then late-stage carcinoma (11–15 wk) (Fig. S1 A and B). Similar to human disease, mammary tumor growth is associated with the dissemination of cancer cells in the periphery and notably the lungs. We detected disseminated tumor cells (DTCs) by real-time quantitative PCR (qPCR) in the lungs of three of five PyMT mice as early as 6 wk of age (Fig. S1C). At 14 wk, we detected metastases, and PyMT expression increased dramatically (Fig. S1D).

The expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> cells occurs within tumors, but also in blood, spleen, and premetastatic lung in murine transplantation models that mimic the late stages of tumor development (9). However, the kinetics of expansion and the lo-

cation of these myeloid cells during cancer initiation and the early stages of tumor progression remain unknown. To address this question, we analyzed the frequency and cell number of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in different tissues isolated from PyMT mice at the onset and during the course of disease (from 6 to 15 wk). To discriminate between circulating and tissue resident myeloid cells, we harvested tissues after vascular flushing. Interestingly, the proportion of CD11b<sup>+</sup>Gr1<sup>+</sup> cells within the developing primary tumors remained constant and below 0.4% of total cells for all tumor stages (Fig. 1A and Fig. S1C). In contrast, we detected a significant systemic increase in proportion and number of these cells in the lung, blood, and spleen at 10 wk, with a continued increase with age (Fig. 1A and Fig. S2A–C). Thus, we evaluated the proportion of CD11b<sup>+</sup>Gr1<sup>+</sup> cells within the CD45<sup>+</sup> immune compartment in the primary tumor and lung. CD11b<sup>+</sup>Gr1<sup>+</sup> cells represented 8% of CD45<sup>+</sup> cells in late-stage tumors, but they made up 39% in lungs (Fig. S2D). These data show a coordinated expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in peripheral tissues during early tumor development and provide a model system to decipher the mechanism for expansion of these cells in cancer.

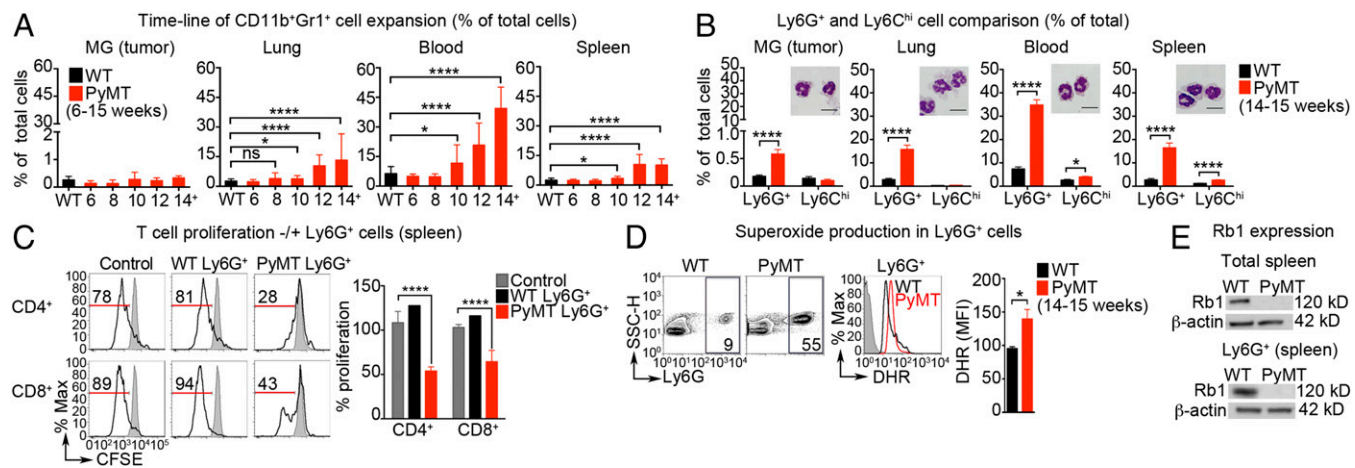
## Activated CD11b<sup>+</sup>Ly6G<sup>+</sup> Neutrophils Are the Predominant Myeloid Cell Type That Expand in Peripheral Tissues During Tumor Progression.

The Gr1 antibody recognizes two antigens: Ly6G, a specific marker for neutrophils (23), and Ly6C, which is expressed on myeloid and nonmyeloid cells (24). Two distinct subsets of Gr1<sup>+</sup> myeloid cells, polymorphonuclear (PMN) CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>int</sup> (referred to as Ly6G<sup>+</sup>) and monocytic (Mo) CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>hi</sup> (referred to as Ly6C<sup>hi</sup>) (also called PMN-MDSCs and Mo-MDSCs, respectively) (25) expand in cancer, both with T cell-suppressive activity. We found that Ly6G<sup>+</sup> cells were the predominant Gr1<sup>+</sup> cell subset that increased substantially in all tissues from PyMT mice (Fig. 1B and Fig. S3A and B). Ly6G<sup>+</sup> cells from PyMT mice showed typical neutrophil morphology (Fig. 1B).

A distinguishing characteristic of Ly6G<sup>+</sup> cells in tumor-bearing mice is their ability to suppress T-cell function (26). To determine whether Ly6G<sup>+</sup> cells acquired this ability in these mice, we isolated them by flow cytometry from spleens and cocultured them with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes, in the presence of anti-CD3 and anti-CD28 activating antibodies to stimulate T-cell proliferation. Ly6G<sup>+</sup> cells from PyMT mice reduced both CD4 and CD8 T-cell proliferation by ~50%, whereas Ly6G<sup>+</sup> cells from WT mice did not (Fig. 1C and Fig. S3C). Because T-cell suppression by Ly6G<sup>+</sup> cells is attributed to enhanced production of ROS (27), we next used a flow cytometry-based assay to assess superoxide production. In the absence of exogenous stimulation, Ly6G<sup>+</sup> cells in blood and lung of tumor-bearing mice showed increased dihydrorhodamine (DHR) fluorescence, a probe that detects intracellular superoxide, indicating that neutrophils in tumor-bearing mice actively produce more ROS compared with WT mice (Fig. 1D and Fig. S3D). We also observed that total splenocytes and FACS-sorted Ly6G<sup>+</sup> cells from PyMT mice had lost Rb1 expression, confirming that the loss of Rb1 is a distinct phenotype of Ly6G<sup>+</sup> cells from tumor-bearing mice (20) (Fig. 1E). Taken together, these data demonstrate that breast cancer development in PyMT mice leads to the generation of T cell-suppressive, activated Ly6G<sup>+</sup> neutrophils in peripheral tissues.

## Breast Cancer Development Results in Profound Remodeling of BM Hematopoiesis.

To determine the origin of Ly6G<sup>+</sup> cells associated with tumor development, we assessed proliferation of Ly6G<sup>+</sup> cells by BrdU incorporation (Fig. 2, A–C and Fig. S4A–C). We did not detect any BrdU<sup>+</sup> Ly6G<sup>+</sup> cells in blood, lung, or MG (tumor) from WT or PyMT mice (14–15 wk) after a 2-h BrdU chase, suggesting that these organs do not significantly contribute to their production (Fig. S4A and B). In contrast, ~5% of Ly6G<sup>+</sup> cells in BM were BrdU<sup>+</sup> in both WT and tumor-bearing



**Fig. 1.** Activated, T cell-suppressive  $Ly6G^+$  neutrophils are the predominant myeloid cells to expand in peripheral tissues during early tumor progression. (A) Quantification of the frequency (% of total) of  $CD11b^+Gr1^+$  cells in WT and PyMT mice (6–15 wk).  $P > 0.05$  was not significant (ns). (B) Quantification of the frequency (% of total) of  $Ly6G^+$  or  $Ly6C^{hi}$  cells. Wright-Giemsa (WG) staining of FACS-sorted  $Ly6G^+$  cells from PyMT mice (*Insets*). (Scale bar, 10  $\mu m$ .) (C) Representative histograms show CFSE fluorescence in unstimulated (gray-shaded) and  $CD3/CD28$ -stimulated (black line)  $CD4^+$  and  $CD8^+$  T cells. Values shown are the % of  $CD4^+$  or  $CD8^+$  T cells that proliferated (red bar) in the absence (served as the control) or presence of  $Ly6G^+$  cells from WT or PyMT spleen. Quantification of  $CD4^+$  and  $CD8^+$  T-cell proliferation in the absence (control) or presence of WT or PyMT  $Ly6G^+$  cells from the spleen. Values shown are the frequency of T cells that proliferated normalized to the control. (D) Superoxide production in  $Ly6G^+$  cells in blood was assessed using DHR and flow cytometry. FACS plots illustrate the frequency (% of total) of  $Ly6G^+$  cells and the histogram shows DHR fluorescence in  $Ly6G^+$  cells. Bar graphs summarize the median fluorescent intensity (MFI) of DHR in  $Ly6G^+$  cells. (E) Rb1 protein expression in total splenocytes (*Left*) and FACS-sorted  $Ly6G^+$  cells (*Right*) was assessed by Western blot. Data are representative of (A and B) six experiments at each time point (mean  $\pm$  SEM,  $n = 8$ –15) or two experiments (WG staining,  $n = 2$ ), (C) two experiments (mean  $\pm$  SEM of four samples), (D) three experiments (mean  $\pm$  SEM,  $n = 5$ –6), and (E) four experiments (blots for total splenocytes;  $n = 4$ –6 biological samples) and two experiments (blots for FACS-sorted  $Ly6G^+$  cells;  $n = 2$  biological samples). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ .

mice after a 2-h chase (Fig. 2B), suggesting that  $Ly6G^+$  cells are generated in BM. After a 24-h chase,  $40 \pm 5\%$   $Ly6G^+$  cells were  $BrdU^+$  in BM of PyMT mice, which was significantly more than in BM of WT mice in which  $25 \pm 2\%$  were  $BrdU^+$  (Fig. 2C). Of note, only  $Ly6G^{low}$  cells incorporated  $BrdU$  after a 2-h  $BrdU$  chase, whereas  $Ly6G^{med/hi}$  cells showed  $BrdU$  incorporation after a 24-h chase. Thus, these data indicate that  $Ly6G^{low}$  cells are immature granulocytes that give rise to mature  $Ly6G^{hi}$  neutrophils with little proliferation potential (28). Consistent with this idea, we found that  $CD11b^+Gr1^+$  cells dramatically expand in BM at 10 wk due to a predominant increase in  $Ly6G^+$  cells (Fig. 2D and Fig. S4D and E). We also detected  $BrdU^+ Ly6G^+$  cells in spleen following a 2-h chase (Fig. 2B), but the frequency was approximately one third in spleen of PyMT mice compared with WT after a 24-h chase (Fig. 2, B and C), and the cell number was less than 10% compared with BM (Fig. S4C). These data demonstrate that the BM is the main production site of  $Ly6G^+$  cells during breast tumor development.

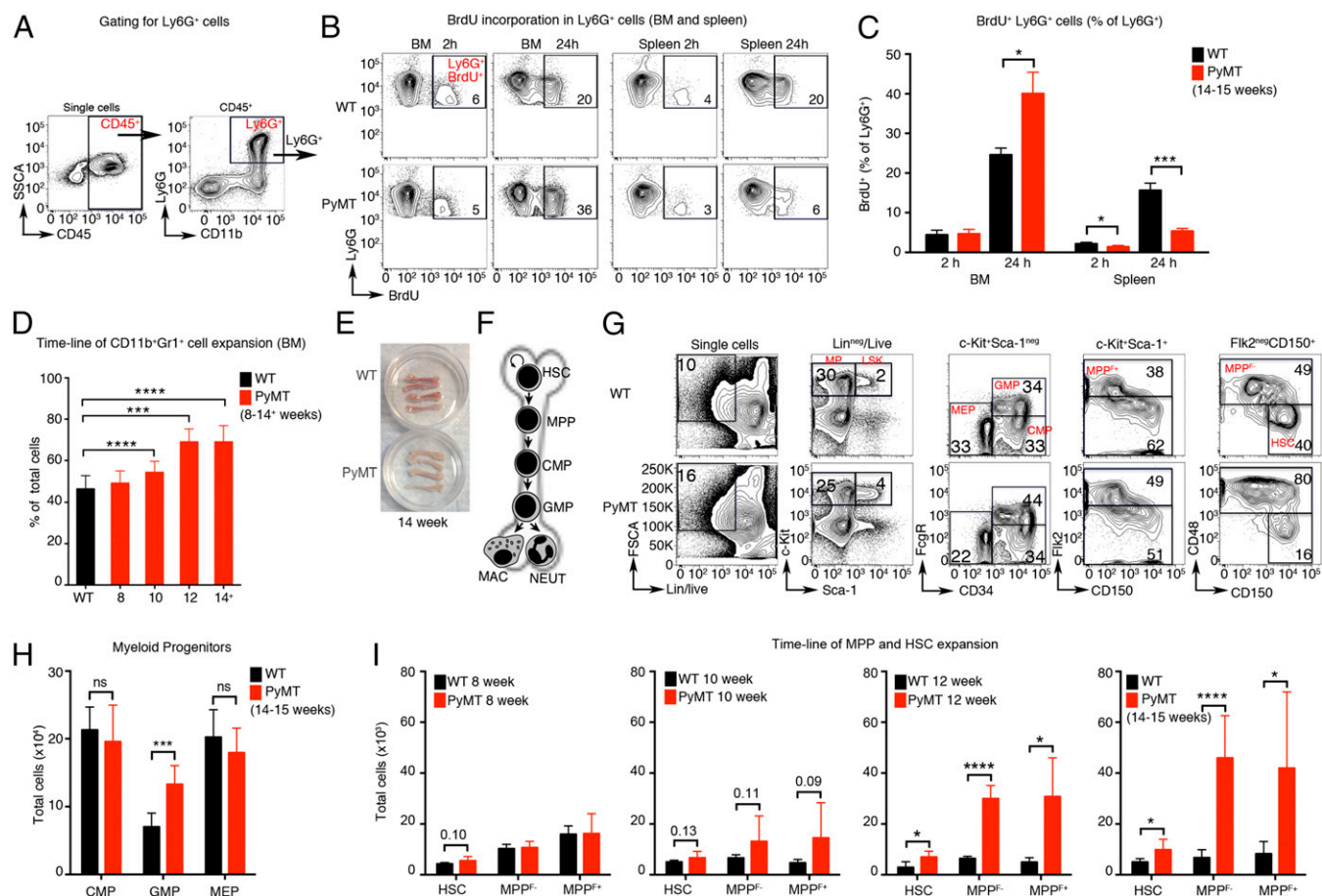
The pale appearance of the BM from late-stage, tumor-bearing PyMT mice (Fig. 2E and Fig. S4F) and their severe anemia (Fig. S4G) suggested that tumor growth in the breast induces a profound alteration of BM hematopoiesis. Therefore, we examined changes in MPs and more immature HSPCs in BM of late-stage PyMT mice (Fig. 2F). We detected higher frequencies of GMPs within the MP population associated with an overall increase of their absolute numbers in PyMT mice compared with WT controls (Fig. 2, G and H). Although there was no significant change in CMPs, the LSK population expanded approximately twofold, with a significant increase in cell number of HSCs,  $MPP^{F-}$ , and  $MPP^{F+}$  populations (Fig. 2, G and I). We next performed longitudinal studies to determine the kinetics of HSPC expansion in early tumor-bearing PyMT mice. Strikingly, we found that HSCs expanded as early as 8 wk, whereas both  $MPP^{F+}$  and  $MPP^{F-}$  expanded at 10 wk in PyMT mice (Fig. 2I and Fig. S4H), concomitant with the increased  $CD11b^+Gr1^+$  cells in peripheral tissues (Figs. 2D and 1). Thus, expansion of HSCs occurred during early tumor development, followed by expansion of MPPs

and  $CD11b^+Gr1^+$  myeloid cells as early as 10 wk, suggesting activation of HSCs leads to increased production of MPPs, which then gives rise to an expanded myeloid compartment.

Expansion of T cell-suppressive myeloid cells in cancer is associated with enlargement of the spleen (29), which may act as a reservoir for extramedullary hematopoiesis (30). We also observed an enlarged spleen in late-stage PyMT mice (Fig. S5A); thus, we also assessed MP and HSPC populations in the spleen. Unlike BM, the majority (80–90%) of spleen-residing MPs were MEPs (Fig. S5B), which increased in numbers approximately sixfold in PyMT mice (Fig. S5C and D). No difference in the frequencies of  $MPP^{F+}$ ,  $MPP^{F-}$ , or HSCs was observed (Fig. S5B), although the total number of these cells was higher than in WT mice (Fig. S5E) due to the increased size of the spleen in late-stage PyMT mice (Fig. S5A and C). Increased production of  $Ly6G^+$  cells combined with an increase in the frequency of HSCs, MPPs, and GMPs in BM, but not spleen, suggests activation of a specific myeloid differentiation pathway in BM of tumor-bearing mice.

#### G-CSF Is Necessary and Sufficient for the Activated and $Rb1^{low}$ Phenotype of Immunosuppressive Neutrophils.

We next hypothesized that the factor(s) driving expansion of  $Ly6G^+$  cells would be present in the serum of PyMT mice. When we assessed the serum concentration of several cytokines and chemokines, only a few factors were increased systemically during early carcinoma formation (10 wk), whereas multiple factors were deregulated during late-stage tumor progression (>12 wk) (Fig. 3A and Table S1). Notably, G-CSF and the neutrophil-attracting chemokine CXCL1 (KC), and to a lesser extent CCL2 (MCP-1), increased early during disease development (Fig. 3A). In contrast, the related myeloid growth factors GM-CSF and M-CSF and chemokines CCL3 and CCL4 did not increase during the early phase of tumor progression (Fig. 3A). We also detected abundant secretion of G-CSF and CXCL1, but not CCL2, in the supernatants of cultured primary PyMT tumor cells (Fig. 3B) and the VO-PyMT



**Fig. 2.** Breast cancer development results in profound remodeling of BM hematopoiesis. (A) Gating strategy to evaluate BrdU incorporation in Ly6G<sup>+</sup> cells by flow cytometry. (B) Representative FACS plots of BrdU incorporation (2 and 24 h after i.p. injection) in Ly6G<sup>+</sup> cells in BM and spleen of WT and PyMT FVB/n mice (14–15 wk). (C) Summary of the % of Ly6G<sup>+</sup> cells that are BrdU<sup>+</sup> in BM and spleen at 2 and 24 h. (D) Frequency (% of total) of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in BM of WT and PyMT FVB/n mice during tumor progression (8–15 wk). (E) Representative image of femurs and tibias from WT and PyMT FVB/n mice. (F) Schematic of myeloid differentiation. (G–I) The frequency and total cell numbers of MPs and HSPCs were evaluated in BM of WT and PyMT FVB/n mice (14–15 wk) by flow cytometry. (G) FACS plots illustrate the gating for MP, MPP, and HSC populations. Values shown are the frequency of cells within each parent gate. (H) Total cell numbers of MP subpopulations (CMP, GMP, and MEP). (I) Total cell numbers of HSCs and MPPs (MPP<sup>-</sup> and MPP<sup>+</sup>) in BM of WT and PyMT FVB/n mice (8–12 wk). Data are representative of (A–C) two experiments (mean  $\pm$  SD,  $n = 3$ –4), (D) four experiments at each time point (mean  $\pm$  SD,  $n = 10$ –12), (E) six experiments ( $n = 8$ –15), (H) three experiments (mean  $\pm$  SD,  $n = 5$ –7), (I) three experiments (8 and 10 wk; mean  $\pm$  SD,  $n = 5$ ), two experiments (12 wk; mean  $\pm$  SD,  $n = 6$ –7), and five experiments (14–15 weeks; mean  $\pm$  SD,  $n = 5$ –9). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ .

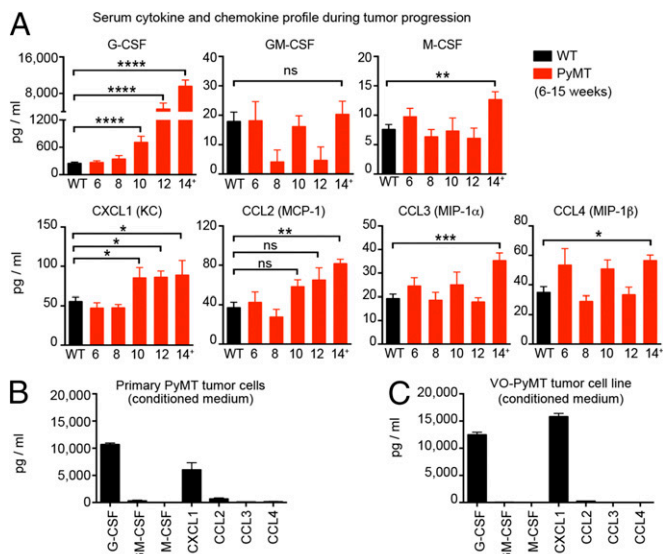
cell line (Fig. 3C), pointing to the tumor cells as the source of these systemic factors.

Given that G-CSF was recently shown to promote tumor progression by contributing to the T cell-suppressive activity of neutrophils (31), we asked whether G-CSF contributed to the activated phenotype of Ly6G<sup>+</sup> cells in PyMT mice. Although Ly6G<sup>+</sup> cells from control antibody-treated PyMT mice had increased ROS production compared with WT mice, Ly6G<sup>+</sup> cells from PyMT mice treated with a neutralizing antibody to G-CSF did not (Fig. 4A). Anti-G-CSF treatment also reduced the frequency of circulating Ly6G<sup>+</sup> cells (Fig. 4A) and the concentration of G-CSF in the serum of PyMT mice (Fig. 4B). Next, we assessed whether G-CSF was sufficient to induce ROS production. We stimulated WT mice for 5 consecutive days with G-CSF (2  $\mu$ g/mouse), a dose that recapitulates the persistent high levels detected in late-stage PyMT mice (Fig. 4C). Indeed, G-CSF stimulation alone increased ROS production in Ly6G<sup>+</sup> cells (Fig. 4D). Thus, tumor-derived G-CSF was both necessary and sufficient for enhanced ROS production in Ly6G<sup>+</sup> cells in PyMT mice.

We then investigated whether tumor-derived G-CSF also regulated the loss of Rb1 in activated Ly6G<sup>+</sup> cells. Anti-G-CSF

treatment of late-stage PyMT mice restored Rb1 protein expression in splenocytes (Fig. 4E). In addition, Rb1 expression decreased in total splenocytes and isolated Ly6G<sup>+</sup> cells from WT mice stimulated with G-CSF for 3 d, but not 1 d, and decreased in a time-dependent manner thereafter to levels similar to late-stage PyMT mice (Fig. 4F). To assess the kinetics in the loss of Rb1 expression in Ly6G<sup>+</sup> cells from multiple tissues, we used a more sensitive intracellular flow cytometry assay (Fig. 4G). Rb1 expression was strongly expressed in circulating Ly6G<sup>+</sup> cells in spleen and blood of WT mice. Surprisingly, Ly6G<sup>+</sup> cells from BM of WT and PyMT mice did not express Rb1. These results suggest that Rb1 expression is normally acquired in circulating Ly6G<sup>+</sup> cells after they leave the BM in WT mice, and its acquisition is inhibited by tumor-derived G-CSF. Thus, tumor-derived G-CSF is the key factor responsible for the two distinguishing characteristics of tumor-derived immunosuppressive neutrophils: enhanced ROS production and loss of Rb1 expression.

**Tumor-Derived G-CSF Targets Hematopoietic Stem and Early Progenitors to Drive Myeloid Cell Production in BM.** To test the involvement of G-CSF in activating myeloid cell production in tumor-bearing mice, we treated WT and PyMT mice with the



**Fig. 3.** Tumor-derived G-CSF, not GM-CSF or M-CSF, increases systemically during early tumor progression. Concentrations of G-CSF, GM-CSF, M-CSF, CXCL1, CCL2, CCL3, and CCL4 in (A) serum from WT and PyMT FVB/n mice (6–15 wk) and conditioned medium collected from (B) primary PyMT C57BL/6 tumor cells and (C) FVB/n VO-PyMT cell line were determined using multiplex laser bead technology (Eve Technologies). Data are representative of (A) two or three experiments (mean  $\pm$  SEM,  $n = 4$ –7) and (B and C) two experiments, each sample in duplicate (mean  $\pm$  SD of four biological samples). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ .

anti-G-CSF antibody. As expected, blocking G-CSF reduced systemic levels of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in all tissues in WT and PyMT mice (Fig. S6A–C). Importantly, blocking G-CSF not only reduced Ly6G<sup>+</sup> neutrophils, but also returned Ly6C<sup>hi</sup>, MPP<sup>F+</sup>, MPP<sup>F-</sup>, and HSC numbers to WT levels in BM of tumor-bearing mice while having no significant effect on MPP and HSC numbers in WT mice (Fig. 5A and Fig. S6D). In addition, the altered phenotypes of the BM and spleen that are characteristic of myeloid dysregulation were restored to WT conditions after anti-G-CSF treatment (Fig. 5B).

Next, we assessed whether G-CSF stimulation alone was sufficient to expand mature myeloid cells, as well as early progenitors in WT mice. After 1 d of G-CSF stimulation, Gr1<sup>+</sup> cells and specifically Ly6G<sup>+</sup> cells increased in blood, but not in BM or spleen (Fig. 5, C and D and Fig. S7A), supporting mobilization of Ly6G<sup>+</sup> cells from BM by G-CSF. Of note, an increase in Ly6G<sup>low</sup> cells was observed in BM after 1 d and was more evident after 3 d of G-CSF stimulation (Fig. 5D), consistent with production of immature neutrophils (32, 33). We also observed a similar increase in Ly6G<sup>low</sup> cells in blood and spleen, but not until 3 d of G-CSF stimulation (Fig. 5D), which suggests that this increase was due to increased production of Ly6G<sup>+</sup> cells from the BM. In addition, an increase in GMPs and the loss of red pigment in the BM was also observed after 3 d of G-CSF stimulation (Fig. 5, E and F and Fig. S7B). Importantly, 12 h and 1 d of G-CSF stimulation was sufficient to increase MPPs, which continued to increase after 3 and 5 d of stimulation (Fig. 5G and Fig. S7B). During most of the treatment, the number of HSCs did not significantly change despite a minor and transient increase after 12 h of G-CSF stimulation (Fig. 5G). In contrast to BM, 12-h G-CSF stimulation did not increase HSCs or MPPs in the spleen (Fig. 5H). The kinetics of expansion of these BM populations suggests a rapid activation of a myeloid differentiation program in the early hematopoietic compartment by prolonged G-CSF stimulation (>1 d). These data show that G-CSF is necessary and

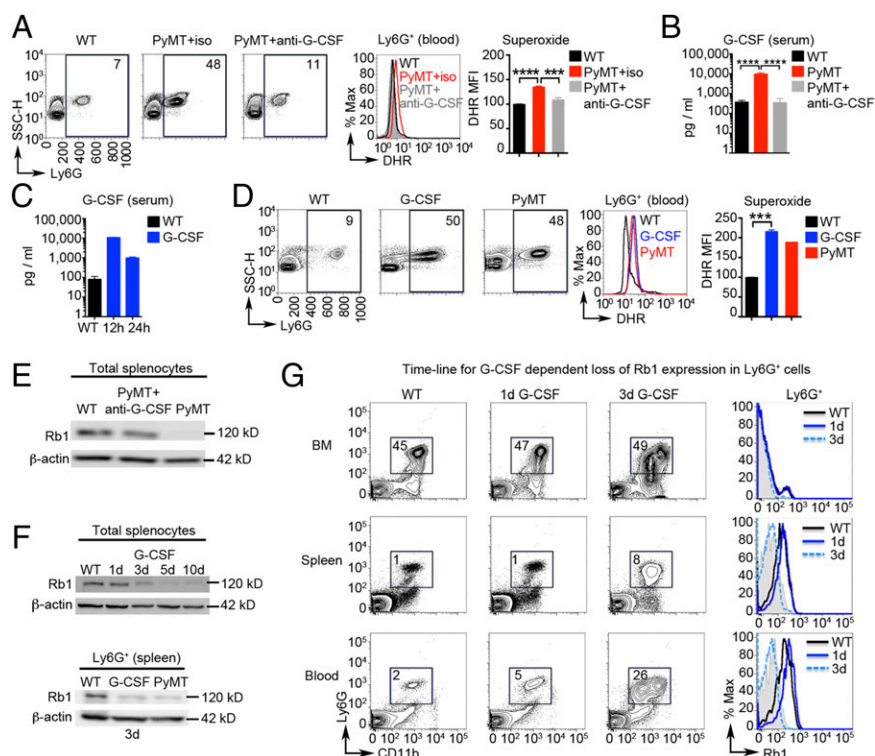
sufficient for tumor reprogramming of hematopoietic differentiation in the BM in cancer.

**G-CSF Acts in a Cell Intrinsic Manner to Drive Altered Myeloid Differentiation in Cancer.** To demonstrate the role of G-CSF in activating myeloid differentiation in the early hematopoietic compartments in tumor-bearing mice directly, we developed an orthotopic transplantation model (Fig. 6A). First we established mixed BM chimeras by transplanting lethally irradiated WT (CD45.1) mice with a 1:1 ratio of WT (CD45.1) and G-CSF-R<sup>-/-</sup> (CD45.2) BM cells. Then, following BM reconstitution, we orthotopically transplanted primary C57BL/6 late-stage PyMT tumor cells or vehicle (sham-treated) into chimeric mice. Similar to PyMT transgenic mice, we found expansion of Ly6G<sup>+</sup> cells in lung, blood, spleen, and BM (Fig. 6B), consistent with elevated G-CSF production by primary C57BL/6 late-stage PyMT tumor cells (Fig. 3B). We observed a highly significant expansion of GMPs, MPPs, and HSCs, but no expansion of CMPs in orthotopically transplanted mice (Fig. 6C), similar to changes in FVB/n PyMT transgenic mice (Fig. 2, H and I). Chimerism analyses showed a defect in the ability of G-CSF-R<sup>-/-</sup> cells to contribute to the expanded GMP compartment in tumor-bearing mice (Fig. 6D), which was expected because G-CSF is known to regulate GMPs directly (34). However, G-CSF-R<sup>-/-</sup> cells did not contribute to the expanded MPP compartment, and there was an overrepresentation of these cells in the expanded HSC compartment in tumor-bearing mice (Fig. 6E). These results confirm the engraftment of G-CSF-R<sup>-/-</sup> HSCs and demonstrate their inability to contribute to the activated myeloid differentiation in the BM of tumor-bearing mice. The specific expansion of G-CSF-R<sup>-/-</sup> HSCs may result from their inability to differentiate into MPPs or their inadequate mobilization compared with WT HSCs in the G-CSF-rich milieu of tumor-bearing mice, although we cannot rule out that other tumor-derived factors may increase HSC numbers in these animals. Taken together, these data directly demonstrate that G-CSF produced by tumor cells activates a myeloid differentiation program in the early hematopoietic compartment, before the more committed CMPs, to skew hematopoiesis toward the myeloid lineage in the BM, resulting in increased production of a specific neutrophil subset, which on circulation in the periphery, acquires an activated, T cell-suppressive, and Rb1<sup>low</sup> phenotype (Fig. 6F).

## Discussion

In our study, we show that tumor-induced T cell-suppressive Ly6G<sup>+</sup> myeloid cells are generated from an expanded stem and early progenitor compartment, which includes HSCs, MPPs<sup>F+</sup>, and MPPs<sup>F-</sup>, along with GMPs in BM of tumor-bearing mice. Using longitudinal studies in a multistage transgenic mouse model, we documented an activated myeloid differentiation pathway in which HSCs and MPPs expand in parallel with Ly6G<sup>+</sup> and Ly6C<sup>hi</sup> cells at the onset of malignant conversion (8–10 wk) and continue to expand during tumor development. We confirmed activation of a similar myeloid differentiation pathway in an orthotopic transplant model of breast cancer. Although expansion of tumor-induced T cell-suppressive Ly6C<sup>hi</sup> and Ly6G<sup>+</sup> myeloid cells has been hypothesized to result from expansion of monocyte and granulocyte precursors due to a block in myeloid differentiation downstream of CMPs (27), our data show that expansion of T cell-suppressive neutrophils in cancer is not the result of a significant block in differentiation but rather targeted reprogramming of myeloid differentiation from an early hematopoietic compartment.

By defining the time-dependent expansion of T cell-suppressive myeloid cells that occurred during tumor development in PyMT mice, we showed that the myeloid differentiation factor G-CSF, and not M-CSF or GM-CSF, increases in the serum during early tumor development. Using a loss-of-function ap-



**Fig. 4.** G-CSF is sufficient for the enhanced ROS activity and Rb1<sup>low</sup> phenotype of tumor-induced Ly6G<sup>+</sup> neutrophils. (A) Superoxide production was assessed by DHR fluorescence in Ly6G<sup>+</sup> cells in blood from WT and isotype or anti-G-CSF-treated PyMT FVB/n mice (14–15 wk). Representative FACS plots illustrate the frequency (% of total) of Ly6G<sup>+</sup> cells (Left), histogram illustrates DHR fluorescence in Ly6G<sup>+</sup> cells (Center), and bar graphs summarize the MFI of DHR in Ly6G<sup>+</sup> cells (Right). Multiplex laser capture technology (Eve Technologies) was used to measure the concentration of G-CSF in the serum from (B) WT, PyMT, and anti-G-CSF-treated PyMT FVB/n mice (14–15 wk) and (C) PBS control treated or G-CSF-stimulated (12 or 24 h) WT mice. (D) Superoxide production was assessed by DHR fluorescence in Ly6G<sup>+</sup> cells in blood from WT, G-CSF-stimulated, and PyMT FVB/n mice (14–15 wk). Representative FACS plots illustrate the frequency (% of total) of Ly6G<sup>+</sup> cells (Left), histogram illustrates DHR fluorescence in Ly6G<sup>+</sup> cells (Center), and bar graphs summarize the MFI of DHR in Ly6G<sup>+</sup> cells (Right). (E and F) Rb1 expression was assessed by Western blot in (E) total splenocytes and (F) total splenocytes (Upper) or FACS-sorted Ly6G<sup>+</sup> cells from the spleen (Lower). WT or PyMT FVB/n mice (14–15 wk). (G) FACS plots illustrate the frequency (% of total) of Ly6G<sup>+</sup> cells and histograms illustrate intracellular Rb1 expression in Ly6G<sup>+</sup> cells, assessed by flow cytometry. Data are representative of (A) two experiments (mean ± SEM, *n* = 3–4), (B) two experiments (mean ± SEM, *n* = 6), (C) one experiment (*n* = 2–4), (D) two experiments (mean ± SEM, *n* = 3–4), (E and F) two experiments (*n* = 2–3 biological samples), and (G) two experiments (*n* = 4). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005, \*\*\*\**P* < 0.001.

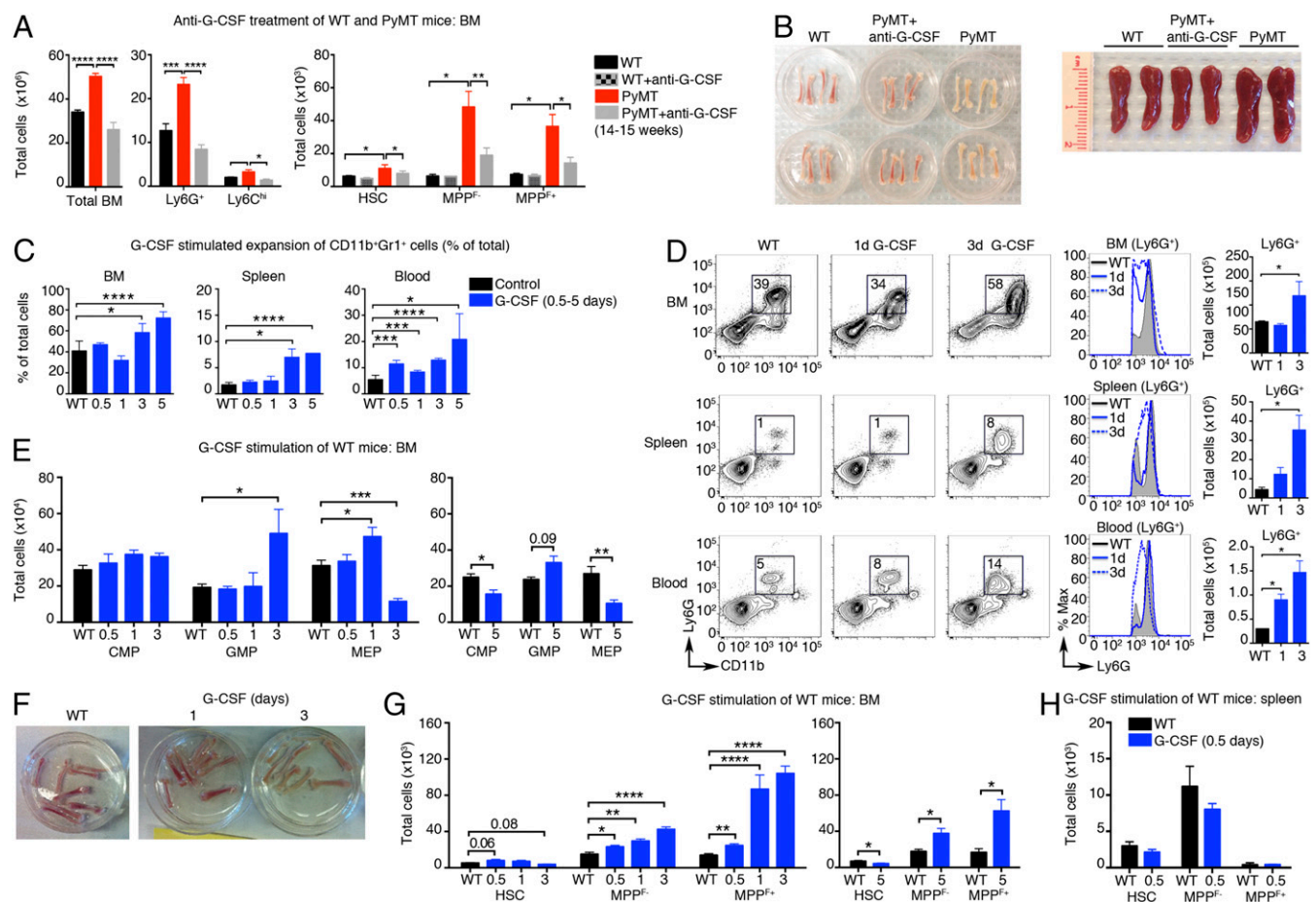
proach with a blocking antibody to G-CSF, we demonstrated that tumor-derived G-CSF is necessary for the expansion of HSC, MPP<sup>F+</sup>, MPP<sup>F-</sup>, GMP, and more mature Ly6G<sup>+</sup> and Ly6C<sup>hi</sup> cells in the BM of tumor-bearing mice. Furthermore, G-CSF is sufficient to rapidly expand these populations. A thorough analysis of the kinetics in myeloid expansion following G-CSF stimulation showed HSCs, along with MPP<sup>F+</sup> and MPP<sup>F-</sup> populations increased as early as 12 h, whereas GMPs did not increase until 3 d after stimulation, revealing that the early hematopoietic compartment is the primary target of tumor-derived G-CSF and a novel mechanism by which tumors expand Ly6G<sup>+</sup> and Ly6C<sup>hi</sup> myeloid cells in cancer.

G-CSF is a complex pleiotropic cytokine that regulates neutrophil production and function, along with HSC mobilization and proliferation, although less is known about the latter (35, 36). G-CSF is believed primarily to regulate the more committed CMP and GMP populations to increase neutrophil production (35), but we demonstrated that G-CSF expands the less committed HSC and MPP populations. We found G-CSF to act in a cell intrinsic manner to expand MPPs and GMPs, but HSC expansion appeared to occur indirectly in tumor-bearing mice. Although prolonged G-CSF stimulation alone may induce quiescence and inhibit HSC function (36), we found increasing levels of G-CSF induced a linear expansion of MPP, GMP, and mature neutrophil populations while maintaining an increase in HSC numbers over the course of tumor development (10–15

wk). Thus, it is likely that the tumor microenvironment provides an additional factor(s) to maintain HSC numbers.

We showed that G-CSF drives expansion and differentiation of the early hematopoietic compartment to generate Rb1<sup>low</sup>, Ly6G<sup>low</sup> neutrophils in PyMT mice. Importantly, the loss of Rb1 expression was not observed after mobilization of mature neutrophils following short-term G-CSF stimulation, but was associated with new production of Ly6G<sup>low</sup> immature neutrophils. Because G-CSF is known to shorten the passage time of neutrophils in the BM (37), the Rb1<sup>low</sup> phenotype may reflect new production of less mature Ly6G<sup>+</sup> neutrophils by G-CSF. In two different mouse models of breast cancer (4T1 and PyMT), Kowanetz et al. also showed tumor-derived G-CSF can induce systemic accumulation of Ly6G<sup>+</sup> cells (15); however, the mechanism by which G-CSF induced expansion was not investigated and presumed to result from mobilization of mature neutrophils. Our data provide a previously unidentified mechanism of how tumor-derived G-CSF induces a systemic expansion of neutrophils that are phenotypically different from mature neutrophils and the development of Rb1<sup>low</sup> neutrophils in cancer. In addition, we showed that a single tumor-derived factor regulates both the development and activity of T cell-suppressive Ly6G<sup>+</sup> neutrophils in cancer.

Although we showed that G-CSF is the driving factor inducing the production and systemic expansion of T cell-suppressive neutrophils in the PyMT mouse model of cancer, other tumor-derived hematopoietic cytokines such as GM-CSF and IL-6 can



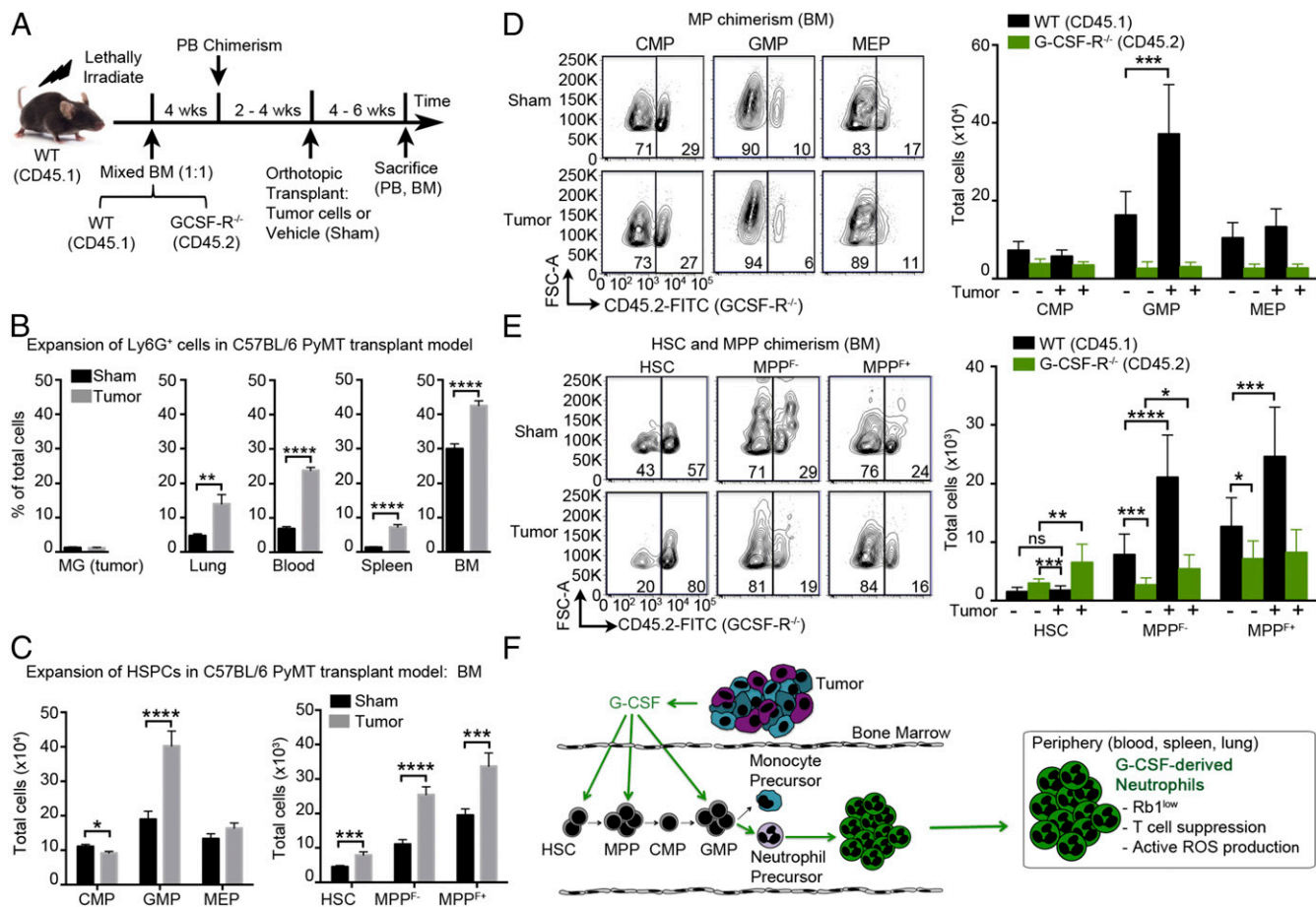
**Fig. 5.** Kinetics of G-CSF stimulation reveal G-CSF targets expansion of HSC and MPP populations in the BM. (A and B) BM, blood, spleen, lung, and MG (tumor) were harvested from WT FVB/n and PyMT mice (14–15 wk) or anti-G-CSF-treated mice. (A) Quantification of total BM cells (Left), cell numbers of Ly6G<sup>+</sup> and Ly6C<sup>hi</sup> cells (Center), and cell numbers of HSC, MPP<sup>5+</sup>, and MPP<sup>5-</sup> populations (Right) in BM. (B) Representative images of femurs and tibias (Left) and spleens (Right). (C–H) BM, spleen, and blood were harvested from WT and G-CSF-stimulated (0.5–5 consecutive days) FVB/n mice. (C) Summary of the frequency (% total) of CD11b<sup>+</sup>Gr1<sup>+</sup> cells assessed by flow cytometry. (D) FACS plots illustrate the frequency (% of total) of Ly6G<sup>+</sup> cells in BM, spleen, and blood (Left), histograms of Ly6G expression in Ly6G<sup>+</sup> cells (Center), and total cell numbers of Ly6G<sup>+</sup> cells (Right). (E) Cell numbers of CMPs, GMPs, and MEPs in BM. (F) Representative images of femurs and tibias. Cell numbers of (G) HSCs, MPP<sup>5+</sup>, and MPP<sup>5-</sup> in BM and (H) HSCs, MPP<sup>5+</sup>, and MPP<sup>5-</sup> in spleen. Data are representative of (A and B) three experiments (mean ± SEM, *n* = 3–7) and (C–H) two experiments (mean ± SEM, *n* = 3–10). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005, \*\*\*\**P* < 0.001.

generate T cell-suppressive CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells (or MDSCs) (27). In addition, other myeloid populations, such as CSF-1-dependent tumor-associated macrophages (38, 39), can impair T-cell function and contribute to immune suppression. In an eloquent study by Elpek et al. (40), two different types of tumors with distinct myeloid cell compositions were implanted into the same mouse, and yet the immune compositions were unaffected by the presence of the other tumor type. This study shows that the tumor type dictates the immune composition of myeloid cells within the tumor and suggests different tumor types may elicit different cytokines.

In human cancer, multiple reports are now revealing that the T cell-suppressive myeloid cells that copurify in the mononuclear fraction, but are negative for macrophage and dendritic mature myeloid markers, are in fact activated, ROS-producing CD15<sup>+</sup>CD66b<sup>+</sup> neutrophils (41, 42). Of note, T-cell proliferation is highly suppressed by a subset of CD15<sup>+</sup>/CD16<sup>low</sup> neutrophils that expand in the blood of terminal cancer patients (43). Similar to Ly6G in mice, CD16 expression increases on human neutrophils with maturation (44) and is decreased on newly produced, but not mobilized, mature neutrophils in healthy volunteers after G-CSF stimulation (45). It is intriguing to speculate that G-CSF could be driving the

production of CD16<sup>low</sup> immunosuppressive neutrophils in cancer patients and that targeting G-CSF in these patients in combination with an immunotherapy that targets T-cell activation may increase antitumor efficacy and possibly even NK function (46). However, neutrophils in healthy individuals, with low levels of G-CSF, do not inhibit T-cell function and are essential for wound repair and protection from infection. Thus, future studies are warranted to assess the relationship between G-CSF concentration, neutrophil-mediated T-cell inhibition, and the protumor role of G-CSF.

Interestingly, ROS-producing neutrophils from tumor-bearing mice and human cancer patients can kill tumor cells directly *ex vivo* and when adoptively transferred into mice can inhibit lung metastasis (47). However, endogenous neutrophils in tumor-bearing mice do not appear to exhibit this antitumor phenotype and, in fact, exhibit a protumor phenotype (15). One possible explanation for these somewhat contradictory phenotypes is that the first study evaluated the potential of neutrophils to kill tumor cells when removed from the tumor microenvironment, whereas the latter study evaluated their ability. ROS production by neutrophils can be inhibited in hypoxic environments (48), and thus, it is possible that the hypoxic tumor microenvironment prevents neutrophils from killing tumor cells. Of note, we found late-stage tumor-bearing



**Fig. 6.** Intrinsic G-CSF signaling is required in MPPs for altered myeloid differentiation in breast cancer. Mixed BM chimera (WT:G-CSF-R<sup>-/-</sup> at 1:1) C57BL/6 mice were transplanted with C57BL/6 PyMT primary tumor cells to evaluate chimerism. (A) Schematic illustrating experimental approach. (B) Summary of the frequency (% of total) of Ly6G<sup>+</sup> cells in lung, blood, spleen, and BM. (C) Cell numbers of CMP, GMP, MEP, HSC, MPP<sup>F-</sup>, and MPP<sup>F+</sup> populations in BM of tumor transplanted or sham-treated mixed BM chimera mice. (D and E) Representative FACS plots illustrate chimerism (Left) and quantification of cell numbers (Right) of CMP, GMP, MEP, HSC, MPP<sup>F-</sup>, and MPP<sup>F+</sup> populations in BM of tumor transplanted or sham-treated mixed BM chimera mice. (F) Model for the generation of activated, T cell-suppressive neutrophils in breast cancer. Tumor-derived G-CSF induces expansion of immature hematopoietic compartments, before the level of CMPs, resulting in reprogramming of the BM and expansion of activated, Rb1<sup>low</sup> and T cell-suppressive neutrophils in peripheral tissues. Data are representative of (A–E) four experiments (mean ± SEM, *n* = 8). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005, \*\*\*\**P* < 0.001.

mice to succumb to anemia due to a decrease in RBC production in BM, which paralleled the increasing myeloid production. Anemia could further contribute to a hypoxic environment and exacerbate the protumor role of neutrophils in cancer.

Skewing of HSPC differentiation toward the myeloid lineage has been reported in mouse models (30, 49) and in human patients (50) with different types of solid tumors, but until now the mechanism responsible was not known. Our data demonstrate a previously unidentified mechanism for the production of T cell-suppressive neutrophils in which early compartments of the hematopoietic hierarchy in BM are regulated by tumor-derived G-CSF. Expansion of these early BM compartments by G-CSF could provide a constant supply of lineage-committed progenitors during tumor-induced production of Ly6G<sup>+</sup> neutrophils. This would also explain the increased number of granulocyte-macrophage colony-forming units observed in BM of HER2 transgenic BALB/c tumor-bearing mice (49), and the increased numbers of circulating HSPCs observed in the blood of human patients with different types of carcinomas that also show elevated G-CSF levels (50). The pleiotropic mechanisms by which G-CSF regulates myeloid progenitor differentiation and proliferation (51), apoptosis of mature neutrophils (52), and priming of neutrophil activation (53), together with our previously un-

identified observations of expansion of HSCs and MPPs, support the hypothesis that pathways involved in immune activation are also linked to early hematopoiesis to fulfill the demand of the immune system (35).

The role of neutrophils in human cancer has just begun to emerge, but a strong relationship between an increase in circulating neutrophils, referred to as a high neutrophil-to-lymphocyte ratio (NLR), and poor prognosis has been reported for decades; however, only recently were these reports compiled in a meta-analysis and shown to be an independent prognostic factor for poor survival in solid tumor cancers in more than 100 studies and 40,000 patients (54). In mice, tumor-associated neutrophils (TANs) and immunosuppressive neutrophils (PMN-MDSCs) promote tumor progression and metastasis by T cell-dependent and independent mechanisms, including promoting angiogenesis and remodeling of the extracellular matrix (55). Of interest, the tumor-promoting activity of TANs can be reversed to an anti-tumor role with anti-TGFβ blockade (56). Although these two populations of neutrophils are reported as distinct populations, it is becoming increasingly clear that T cell-suppressive neutrophils are closely related to TANs (55). Thus, future studies are warranted to evaluate the effect of anti-TGFβ blockade on the T cell-suppressive role of neutrophils in cancer.



The essential role of BM-derived precursors of myeloid origin in promoting early tumor progression was first shown by Lyden et al. (57), who revealed that tumor-secreted VEGF recruits endothelial precursors that are essential for tumor angiogenesis. Little is known about the long-range systemic effects of tumor-derived factors on distant microenvironments (58), although there is evidence that tumor-derived factors can activate BM cells to provide a supportive systemic environment to promote growth of distant tumors (59–62). We demonstrated that tumor production of G-CSF induces chronic activation of myeloid differentiation in the BM, which ultimately results in inefficient erythropoiesis, anemia, and enlarged spleens to meet the demands of neutrophil and RBC cell production during tumor progression. Our data significantly increase our understanding of how tumors regulate the BM to generate T cell-suppressive neutrophils in cancer.

## Materials and Methods

**Mice.** All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco, in accordance with the guidelines of the National Institutes of Health. Transgenic PyMT (MMTV-PyMT<sup>634M/J</sup>) mice (21, 22) were maintained on FVB/n and C57BL/6 backgrounds; littermate controls were used for all experiments. WT FVB/n female mice were purchased from Charles River and WT C57BL/6 female (CD45.1 or CD45.2) mice were purchased from the Jackson (JAX) Laboratories for cross-breeding, in vivo G-CSF stimulation, or BM transplants. Homozygous C57BL/6 mice lacking expression of the receptor for G-CSF (G-CSF-R<sup>-/-</sup>) (63) were provided by Daniel Link, Washington University, St. Louis, and then later acquired from JAX [B6.129 × 1(Cg)-Csf3<sup>tm1Link/J</sup>], with age-matched WT C57BL/6 (JAX) mice used as controls.

**Flow Cytometry.** All antibodies are listed in Table S2. Cells were blocked with rat IgG (10 µg/mL; Sigma) for at least 20 min on ice, washed with staining media [2% (vol/vol) HI, FBS in HBSS (BSS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>, denoted SM], and then stained with fluorescently conjugated antibodies in SM for 30 min on ice, unless stated otherwise. For evaluation of MPs and HSPCs in BM and spleen, cells were stained as previously described (64). In brief, a six-step staining procedure was used that included the following combinations of antibodies: (i) mixture of unconjugated rat antibodies to lineage specific markers (B220, CD3, CD4, CD5, CD8, CD11b, Ter119, Gr1); (ii) fluorescently-tagged (PE-Cy5 or PE-Cy5.5) anti-rat secondary antibody; (iii) rat IgG (as described above) to block nonspecific binding; (iv) mixture of biotin- or fluorophore-conjugated antibodies to c-Kit, Sca-1, FcγR, and CD34 (for staining MPs) or c-Kit, Sca-1, Flk2, CD48, and CD150 (for staining HSPCs); (v) streptavidin conjugated to a fluorophore; and (vi) propidium iodide to identify live and dead cells. In some experiments evaluating mature myeloid cells (i.e., Gr1<sup>+</sup>, Ly6G<sup>+</sup>, or Ly6C<sup>hi</sup>), stained cells were fixed with 2% (vol/vol) PFA at 4 °C overnight and analyzed 24–48 h later. For evaluation of intracellular Rb1 protein expression using flow cytometry, cell were stained for extracellular cell surface markers, fixed in 4% (vol/vol) PFA, permeabilized briefly (5 min) in 0.1% Triton X-100, blocked for 20 min at 4 °C with the 2.4G2 Fc-blocking antibody, and then stained with an anti-Rb1 antibody for 30 min on ice. The data were acquired with the FACSCalibur, LSRII, or FACSAria instruments (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Inc.).

**Isolation of Epithelial-Enriched Cells from Tumors.** Tumors were digested in collagenase as described above, with several modifications. Tumors were digested for 30 min in collagenase media, centrifuged, DNase I treated, and then resuspended in DMEM complete. Single cells (which consisted primarily of stromal cells) were then removed from the cell suspension by a series of brief centrifugations (500 × g for 1 s, four times), which resulted in epithelial-enriched organoids (65). Organoids were then further digested in DMEM complete with trypsin (0.05% in EDTA) and DNase I (20 U/mL) for 20 min at

37 °C. Digestion was stopped by the addition of 10% (vol/vol) heat inactivated (HI) FBS in DMEM complete. Resulting epithelial-enriched cells were washed, filtered (as described above) and then cultured for in vitro assays or frozen in DMEM/F12 with 30% (vol/vol) HI FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (P/S), and 10% (vol/vol) DMSO for in vivo transplant experiments.

**Cell Lines.** An MMTV-PyMT (FVB/n) cell line (66), which we refer to as VO-PyMT, was stably transduced with GFP and luciferase, as previously described (67).

**Conditioned Media from Tumor Cells.** Primary epithelial-enriched tumor cells (C57BL/6 PyMT) were cultured in 24-well plates at 4 × 10<sup>5</sup> cells/mL for 48 h in DMEM/F-12 medium with 1% FBS and P/S. VO-PyMT cells were cultured for 48 h in DMEM/F-12 medium and P/S (no FBS) with a final concentration of ~1 × 10<sup>6</sup> cells/mL. Tumor cell-conditioned media were centrifuged at 800 × g for 5 min, and then supernatant was collected and stored at –80 °C.

**In Vivo Studies and Reagents.** Murine G-CSF (PeproTech) and a blocking antibody to murine G-CSF (R&D Diagnostics), along with the isotype control antibody, were prepared per manufacturer's instructions. For in vivo stimulation, 2 µg (in 200 µL) of G-CSF were injected s.c. daily, unless indicated otherwise. Fifty micrograms (in 100 µL) anti-G-CSF or isotype control antibody was injected i.p. every 48 h (total of four injections) for ~1 wk, unless stated otherwise.

**In Vivo BrdU Proliferation.** Mice were injected i.p. with 100 µL (10 mg/mL) BrdU (BD Pharmingen) 2 or 24 h before death. Cells, isolated from multiple tissues, were first stained for extracellular surface markers, fixed, and permeabilized using the BD Cytotfix/Cytoperm buffer (15 min at room temperature), washed, and then stored at 4 °C overnight. The next day, intracellular BrdU was stained according to the FITC BrdU Flow Kit Staining Protocol (BD Pharmingen). Following BrdU staining, cells were stored at 4 °C overnight, and data were then acquired on a BD LSR II flow cytometer and analyzed using FlowJo software.

**BM Transplantation.** Total BM cells from donor mice (CD45.1 WT and CD45.2 G-CSF-R<sup>-/-</sup> C57BL/6) (63) were collected from femurs by flushing. Cells were passed through a 70-µm filter, RBCs were lysed, and then cells were resuspended at 1:1 with a total of 3 × 10<sup>6</sup> cells/100 µL in SM. Congenic recipient mice (CD45.1 WT C57BL/6) were irradiated using a cesium source irradiator with a lethal dose (9 Gy) delivered in two doses (4.5 Gy) 3 h apart and were given antibiotic-containing water (1.1 g/L neomycin sulfate and 10 U/L of polymyxin B sulfate) for 4 wk after irradiation as described previously (64).

**Statistics.** All of the data are expressed as mean ± SD or SEM as indicated. GraphPad Prism6 was used for all statistical analysis. *P* values were generated using the unpaired Student *t* test and considered significant when <0.05. In analysis of some experiments, normalization was performed by dividing individual raw data values (such as median fluorescent intensity values acquired by flow cytometry) by the mean of WT samples in that experiment.

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