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Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: "N1" versus "N2" TAN

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SUMMARY

TGF-β blockade significantly slows tumor growth through many mechanisms, including activation of $CD8^+$ T cells and macrophages. Here, we show that TGF-β blockade also increases neutrophil-attracting chemokines, resulting in an influx of $CD11b^+/Ly6G^+$ tumor-associated neutrophils (TANs) that are hypersegmented, more cytotoxic to tumor cells, and express higher levels of proinflammatory cytokines. Accordingly, following TGF-β blockade, depletion of these neutrophils significantly blunts antitumor effects of treatment and reduces $CD8^+$ T cell activation. In contrast, in control tumors, neutrophil depletion decreases tumor growth and results in more activated $CD8^+$ T cells intratumorally. Together, these data suggest that TGF-β within the tumor microenvironment induces a population of TAN with a protumor phenotype. TGF-β blockade results in the recruitment and activation of TANs with an antitumor phenotype.

INTRODUCTION

Mounting evidence suggests that the immunosuppressive cytokine TGF-β is overexpressed by tumors and plays a significant role in blocking immune responses and affecting tumor progression. The pivotal role of TGF-β in suppressing antitumor immune responses has made it a logical target for the development of antagonists (Bierie and Moses, 2006). TGF−β blockers (soluble receptors/antibodies) and TGF−β receptor inhibitors have antitumor effects that, in several models, are due primarily to $CD8^+$ T cell-dependent immunologic mechanisms (Ge et al., 2006; Nam et al., 2008; Suzuki et al., 2007).

In addition to suppressing T cell functions, it has been shown that TGF-β also has an impact on myeloid cell functions. The tumor microenvironment polarizes TAMs toward a protumor (M2) versus an antitumor (M1) phenotype (Allavena et al., 2008). Since TGF-β can alter macrophage cell function and phenotype in vitro (Lee et al., 2007; Tsunawaki et al., 1988), it may play an important role in regulating macrophage phenotype in vivo as well. Although it is less well studied, TGF-β has also been noted to inhibit neutrophil activity (i.e., degranulation) (Shen et al., 2007). Early studies suggested that TGF-β had chemoattractant activity for neutrophils at very low concentrations (Reibman et al., 1991), and more recent studies have suggested that blocking the TGF-β pathway increases the recruitment of neutrophils in some types of chronic disease states (Allen et al., 2008).

In recently published studies, we used a small, orally available type I TGF−β receptor (Alk-5/Alk-4) kinase inhibitor (SM16) and showed that TGF-β receptor blockade increased the percentage and activation of intratumoral $CD8^+$ T cells and was able to augment immunotherapy (Kim et al., 2008; Suzuki et al., 2007). In addition, blockade of TGF-β function led to an influx of myeloid cells (marked by CD11b positivity on FACS) into tumors. The goals of this study were to evaluate the effect of SM16 on the myeloid cell phenotype of tumors and to explore how these changes might affect $CD8^+$ T cell function.

SIGNIFICANCE

The role of tumor-associated neutrophils (TANs) in tumor biology is unclear. It is well established that the tumor microenvironment polarizes tumor-associated macrophages (TAMs) toward a protumor (M2) versus an antitumor (M1) phenotype. Our data support a paradigm in which resident TANs acquire a protumor phenotype (similar to M2), largely driven by TGF-β to become "N2" neutrophils. After TGF-β blockade, neutrophils acquire an antitumor phenotype to become "N1" TANs (similar to M1). This paradigm suggests that TANs are a double-edged sword, capable of being pro- or antitumorigenic, depending on the tumor microenvironment. Our study also shows another mechanism by which TGF-β can enhance tumor growth and supports the potential utility of TGF-β blockade to inhibit tumor growth.

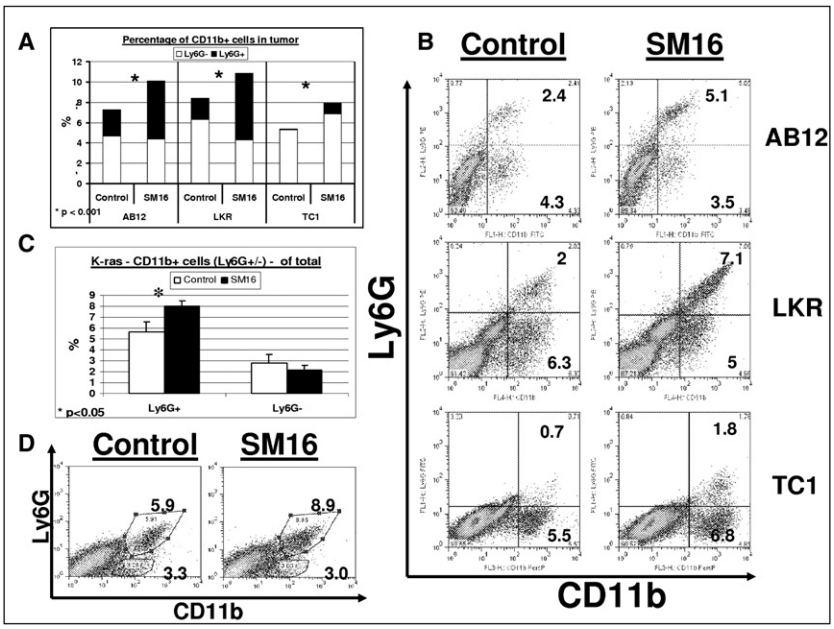

Figure 1. SM16 Causes an Influx of CD11b$^+$ Ly6G$^+$ Granulocytic Cells into Tumors

(A and B) Flow cytometry was performed on digested tumors from animals treated for 1 week with control chow (left columns) or SM16 chow (right columns) in each of the three flank tumors: AB12 (n = 26), LKR (n = 5) and TC-1 (n = 9–10). (A) summarizes the percentage of CD11b$^+$ cells out of all tumor cells in the three cell lines, in both groups (total). This is divided to Ly6G$^-$ cells (bottom section of each bar, white), and Ly6G$^+$ (granulocytic) cells (top section of each bar, black). *p < 0.001. (B) shows representative FACS tracings of CD11b versus Ly6G expression in each of the lines. The number in each quadrant is the percentage of the total tumor cells.

(C and D) Flow cytometry was performed on digested lungs with orthotopic tumors from mice with the conditionally expressed, K-*ras*G12D allele, treated for 1 week with control (white) or SM16 (black) chow (n = 5 per group). (C) summarizes the percentage of the different CD11b$^+$ cells out of all lung cells ± SEM—Ly6G$^+$ (granulocytic) cells (left) and Ly6G$^-$ cells (right). *p < 0.05. (D) shows representative FACS tracings of CD11b versus Ly6G expression in the two groups. The numbers shown are the percentage of total lung cells.

RESULTS

Inhibition of TGF-β Signaling Increases Intratumoral CD11b$^+$ Cells that Express Neutrophil, Ly6G$^+$, Rather than Macrophage, Ly6G$^-$, Markers

To evaluate the role of myeloid (CD11b$^+$) cells, mice bearing established flank tumors from three syngeneic models were fed with chow containing SM16 or control chow. Tumors were harvested and subjected to FACS to detect CD11b$^+$ cells and different myeloid cell markers.

As shown in Figures 1A and 1B, administration of SM16 increased the percentage of CD11b$^+$ cells in the tumors by 30%–45% ($p < 0.02$). To differentiate macrophages from neutrophils, we used the 1A8 anti-Ly6G antibody, which is found only on neutrophils (Daley et al., 2008). SM16 treatment led to significant increases in the percentage of intratumoral Ly6G$^+$ cells and only minor changes in the Ly6G$^-$ cells (mostly macrophages). As seen in Figure 1B, virtually all the Ly6G$^+$ cells were also CD11b$^+$.

To determine whether neutrophils travel to areas of tumor necrosis, we performed immunohistochemistry of tumors using the Ly6G antibody. We found an increased number of Ly6G$^+$ cells in tumors from SM16-treated mice and found that the cells were primarily in the non-necrotic areas of the tumors (see Figure S1 available with this article online). We also blocked TGF-β activity using a neutralizing anti-TGF-β monoclonal antibody (1D11) in the AB12 cell line and confirmed significantly increased levels of intratumoral neutrophils (CD11b$^+$/ Ly6G$^+$) (data not shown).

Evaluation of myeloid cell populations in the spleens of mice treated with SM16 versus control showed no significant changes in the percentage of CD11b$^+$ cells (12.1 ± 4.7 in control-treated versus 13 ± 0.7 in SM16-treated mice), CD11b$^+$/GR1$^+$ myeloid-derived suppressor cells (10.7 ± 4.3 versus 11.7 ± 0.7), or CD11b$^+$/Ly6G$^+$ cells (9.2 ± 3.8 versus 9.6 ± 0.6). There was no change in the percentage of CD11b$^+$/Ly6G$^+$ neutrophils in the blood in control tumor-bearing mice (41.3% of leukocytes) versus SM16-treated mice (38.3% of leukocytes). The percentage of CD11b$^+$/Ly6G$^-$ in the blood was negligible in both groups of mice. These data suggest that the changes in TAN were not systemic, but rather due to a change in recruitment and/or persistence within the tumors.

To evaluate the morphology of the TANs, intratumoral CD11b$^+$/Ly6G$^+$ cells were isolated. As seen in Figure 2, the Ly6G$^+$ cells isolated from flank tumors from both control untreated mice and SM16-treated mice had a clear neutrophil-like morphology. Interestingly, however, most of the neutrophils in the SM16-treated tumors were more lobulated and hypersegmented (bottom panel), losing some of the characteristic circular nuclei appearance typical of blood or bone marrow murine neutrophils (top panel), that was relatively maintained in control TAN (middle panel).

We further evaluated the pulmonary influx of CD11b$^+$ cells in the orthotopic transgenic activated K-ras model of bronchogenic adenocarcinoma of the lung. Eight to nine weeks after activation of the K-ras mutation, we treated the mice with SM16 or control chow followed by flow cytometry of the whole lung. As seen in Figures 1C and 1D, we found a 43% increase in the percentage of neutrophils in the lungs of the SM16 mice (8 ± 0.5) compared with the control mice (5.6 ± 0.9) ($p = 0.03$). Similar to the results in the flank models, the percentage of CD11b$^+$/Ly6G$^-$ cells in control (2.8 ± 0.7) versus SM16-treated (2.1 ± 0.5) mice did not increase.

TGF-β-Blockade Increases the mRNA for Neutrophil Chemoattractants

We next used real-time RT-PCR to measure the level of cytokines, chemokines, and cell adhesion molecules in flank tumors derived from AB12, LKR, and TC1 cells. As we have shown previously (Kim et al., 2008; Suzuki et al., 2007), SM16 treatment resulted in changes in the tumor microenvironment manifested