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CD11b+Ly6G+ cells inhibit tumor growth by suppressing IL-17 production at early stages of tumorigenesis

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Neutrophils are important innate immune cells involved in microbial clearance at the sites of infection. However, their role in cancer development is unclear. We hypothesized that neutrophils mediate antitumor effects in early tumorigenesis. To test this, we first studied the cytotoxic effects of neutrophils *in vitro*. Neutrophils were cytotoxic against tumor cells, with neutrophils isolated from tumor-bearing mice trending to have increased cytotoxic activities. We then injected an ELR+ CXC chemokine-producing tumor cell line into C57BL/6 and *Cxcr2*−/− mice, the latter lacking the receptors for neutrophil chemokines. We observed increased tumor growth in *Cxcr2*−/− mice. As expected, tumors from *Cxcr2*−/− mice contained fewer neutrophils. Surprisingly, these tumors also contained fewer $CD8^+$ T cells, but more IL-17-producing cells. Replenishment of functional neutrophils was correlated with decreased IL-17-producing cells, increased $CD8^+$ T cells, and decreased tumor size in *Cxcr2*−/− mice, while depletion of neutrophils in C57BL/6 mice showed the opposite effects. Results from a non-ELR+ CXC chemokine producing tumor further supported that functional neutrophils indirectly mediate tumor control by suppressing IL-17A production. We further studied the correlation of IL-17A and $CD8^+$ T cells *in vitro*. IL-17A suppressed proliferation and IFNγ production of $CD8^+$ T cells, while CD11b+Ly6G+ neutrophils did not suppress $CD8^+$ T cell function. Taken together, these data demonstrate that, while neutrophils could control tumor growth by direct cytotoxic effects, the primary mechanism by which neutrophils exert antitumor effects is to regulate IL-17 production, through which they indirectly promote $CD8^+$ T cell responses.

Introduction

The tumor environment is composed of tumor cells, stromal cells and tumor-infiltrating immune cells of both innate and adaptive lineages. These diverse cells communicate with each other by means of direct contact and/or indirect signaling that can be mediated by cytokine and chemokine secretion. This interplay may functionally alter and polarize immune cells in a way which either favors tumorigenesis or limits tumor growth.[1,2]

There is an emerging interest in the role of neutrophils in cancer. Recent evidence suggests that neutrophils can adopt either protumor or antitumor activity.[2-9] Protumor neutrophils are thought to be functionally related to the recently described myeloid-derived suppressor cells (MDSCs).[4,10-12] In mice, MDSCs are characterized by the co-expression of the myeloid lineage differentiation antigens Gr-1 and CD11b (Ly6C/G and α M-integrin, respectively).[13,14] However, neutrophils, whether suppressive or not, express the same markers. While some properties of antitumor neutrophils have been described,[7,8] the mechanisms by which neutrophils exert these effects have not yet been defined. As normal or tumor infiltrating neutrophils are indistinguishable by cell surface markers from g-MDSC, mechanistic

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studies on antitumor neutrophil function have been difficult. Thus, the effect of neutrophils on early tumor development remains obscure.

A considerable body of work suggests that neutrophil trafficking in the circulation is controlled, at least in part, by chemokines. Based on their structural properties, neutrophil-attracting chemokines are categorized in the ELR+ CXC chemokine family. In mice, there are five members, including CXCL1/KC, CXCL2/MIP-2 and CXCL5/LIX, that interact with a single receptor, CXCR2.[15] These chemokines are critically involved not just in migration to inflammatory sites but in homeostatic regulation of neutrophil development and release from marrow. Despite considerable interest in these signals, no consensus has been reached as to their effect on tumor growth.

One important cytokine in inducing chemokine releases is IL-17. IL-17 plays a pivotal role in protecting the host against certain infectious microorganism through neutrophil recruitment, by inducing the production of granulocyte colony-stimulating factor (GCSF) and increasing the expression of CXC chemokines.[16-21] Although IL-17 is detected in cancer patients and tumor-bearing mice,[22-25] as with neutrophils the precise role of IL-17 during tumor development remains unclear. IL-17 has been shown to exert pro-tumor properties, as local blockade of IL-17A in a model of lung cancer enhanced antitumor immunity, characterized by increased IFNγ, diminished T-regulatory cell number and reduced tumor growth.[26,27] We have previously demonstrated an intimate connection between neutrophil homeostasis and IL-17-producing cells.[17] Not only does IL-17 induce granulopoiesis through G-CSF, but IL-17 responds rapidly to a perceived deficiency of neutrophils as one critical component of a feedback loop. Since neutrophils and IL-17-producing cells have been both shown in the tumor microenvironment, we sought to examine the neutrophil/IL-17 relationship within the context of early control of tumor growth.

In this paper, we demonstrate that deficiency of neutrophils, either by depletion or presence of chemotaxis-defective neutrophils, enhances early tumor growth. Furthermore, under such circumstances the tumor environment is altered, such that the tumor contains fewer $CD8^+$ T cells and more IL-17-producing cells. Additionally, the major IL-17-producing cell population is altered from αβ T cells to γδ T cells. Adoptive transfer of active neutrophils inhibits IL-17 production, and increases $CD8^+$ T cell number, with an associated decrease in tumor growth. *In vitro* T cell culture experiments indicate that IL-17A alone is sufficient to limit $CD8^+$ T cell functionality, and that CD11b+Ly6G+ neutrophils, isolated from bone marrow of naive or tumor-bearing mice, do not show suppressive effects on $CD8^+$ T cell function. While neutrophils may control tumor growth through direct cytotoxic effects, as other groups[28] and our own *in vitro* experiments have shown, we believe that this is unlikely to be the primary mechanism by which neutrophils promote antitumor immunity. Rather we suggest that the primary role of neutrophils in promoting antitumor immunity is to control IL-17 secretion, and thus indirectly suppress tumor growth by promoting $CD8^+$ T cell function. Since many cancer therapies may involve depletion of neutrophils, these findings provide new insights that can be employed for the development of novel cancer immunotherapies.

Materials and Methods

Animals

All mice were kept in SPF conditions in the animal facility of the Children's Hospital of Philadelphia. *Cxcr2*−/− and WT control mice were on a C57BL/6J background. Sex- and age-matched 6- to 10-week-old mice were used for experiments. Mouse experiments were conducted under oversight of the Institutional Appropriate Animal Care and Use Committee.

Cell line

The murine Lewis lung carcinoma (LLC) cell line (American Type Culture Collection) was propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mmol/L L-glutamine and 10 ug/mL penicillin/streptomycin. Mouse TC-1 lung cancer cells,[29] were maintained in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum (Georgia Biotechnology), 2 mmol/L L-glutamine and 10 ug/mL penicillin/streptomycin. AE-17 cells were also maintained in RPMI 1640 medium (with 2 mmol/L L-glutamine, 10 ug/mL penicillin/streptomycin, 25mM HEPES and 5% FCS).

Animal flank tumor models

Mice were injected on the right flank with 2×10^6 LLC cells or 1.2×10^6 TC-1 cells in C57B6 and *Cxcr2*−/− mice. 2×10^6 AE-17 cells were injected in BALB/c mice.

ELISA assay

Cytokines and chemokines were quantified by ELISA using kits specific for CXCL1/KC, CXCL2/MIP-2, CXCL5/LIX, G-CSF, and IL-17A according to the manufacturer's specifications (R&D Systems or eBioscience).

In vitro killing assay

In vitro killing assay was performed following the protocol of Reise and colleagues.[30] Briefly, luciferase labeled cells (5000/well) were plated on a 96-well in MEM 0.5% FBS. Four hours later, purified neutrophils (100,000/well or 200,000/well) were added to the plated tumor cells and cocultured overnight. Following overnight incubation, luciferase activity was measured using the Clarity (Bio-Tek) microplate luminescence reader. *In vitro* killing experiments were repeated at least three times.

In vivo depletion of Ly-6G+ neutrophils

Neutrophil depletion was achieved using daily intraperitoneal injections of 250 ug 1A8 monoclonal Ab (anti-Ly-6G; BioXcell) starting one day before LLC flank injection. Control mice were injected with 250 ug 2A3 isotype control Ab (Rat IgG2a; BioXcell). Systemic neutrophil depletion was evaluated periodically with CBC count and manual blood differentials. Tumor and splenic neutrophil depletion was confirmed at the end of each experiment using flow cytometry.

Adoptive transfer of neutrophils

Mice were injected on the right flank with 2×10^6 LLC in *Cxcr2*$^{-/-}$ mice. 4 h later, mice were intravenously injected with 1×10^7 isolated BM neutrophils from C57BL/6 mice or with vehicle control (PBS). I.V. injections were repeated on day 2, 8 and 11.

Flow cytometry

To examine cytokine production, single cell suspensions of tumor, lung, bone marrow, and spleen were cultured at 37°C in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin and 1 ug/mL BFA (Sigma-Aldrich) for 4 h with 30 ng/mL PMA (Sigma-Aldrich) and 1 ug/mL ionomycin (Sigma-Aldrich). Unstimulated single cell suspensions were stained directly *ex vivo*. Cells were washed in PBS and stained with live/dead blue viability dye (Invitrogen) prior to surface staining. Fixation and intracellular staining were done using the FoxP3 fix/perm buffer kit (Ebioscience). The following antibodies were purchased from BioLegend: CD11b (M1/70), Ly6G (1A8), CD45.2 (104), TCRβ (H57–597), TCRγδ (GL3), IFNγ (XMG1.2), CD8α (53–6.7), CD4 (GK1.5). Antibody against IL-17A (eBio17B7) was purchased from Ebioscience. Flow cytometry was performed on an LSR Fortessa Flow Cytometer (BD Biosciences). Data were analyzed using Flowjo (Treestar).

T-cell proliferation and intracellular IFNγ production

For proliferation assay, purified splenic CD8$^+$ T cells from C57BL/6 mice were labeled with 2.5 uM CellTrace ef670 (eBioscience) in PBS for 6 min at 37°C. The labeling reaction was quenched by addition of cold DMEM medium with 10% FCS. Sorted CD11b+Ly6G+ cells from spleens of LLC tumor-bearing mice or isolated neutrophils from bone marrows of naive C57BL/6 mice were cocultured with polyclonal-stimulated (5 ug/mL plate-bound anti-CD3ε (2C11) and 5 ug/mL anti-CD28 (37.51)) and ef670-labeled splenic CD8$^+$ T cells at a series of ratios (4:1, 1:1, 1:4) in the presence or absence of 10ng/mL of rmIL-17A (Peprotech). The proliferation and IFNγ production of CD8$^+$ T cells was evaluated 3 d later by flow cytometry as described above.

H&E and immunohistochemical staining

Portions of tumor were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H&E) for light microscopy. Additional tissue sections for immunohistochemistry were stained with primary antibodies for myeloperoxidase (MPO) (Dako A0398) with DAB (DAKO Cytomation) for antigen detection. Only cells with 2+ and 3+ intensity of staining were selected for analysis.

Statistical analyses

We performed all statistical analyses with the GraphPad Prism software (version 4). Data are presented as means ± SEM, and a p value less than 0.05 was considered significant. The number of mice used in each experiment is noted in the figure legends. We used a 1-way ANOVA, 2-way ANOVA or 2-tailed Student's t-test to compare data sets, as appropriate.

Results

LLC cells induce neutrophil chemotaxis by secreting ELR+CXC chemokine and neutrophils had a cytotoxic effect on these cells *in vitro*

A number of lung cancers have been described to produce neutrophil chemoattractants.[31,32] In order to examine the role of neutrophils in early tumorigenesis, we first compared a variety of cell lines for the production of ELR+ CXC neutrophil chemokines. As seen in **Fig. 1A**, not all mesothelioma and NSCLC cell lines tested produced detectable amounts of both CXCL1 and CXCL5, suggesting that production of ELR+ CXC chemokines is highly cell line dependent. Of these cell lines, only one expressed both CXCL1 and CXCL5 and is syngeneic to our C57BL/6 recipient mice: the lung cancer cell line, LLC.[33]

To test the chemotactic function of chemokines secreted by LLC cells, we utilized a transwell assay. Neutrophils were purified from the bone marrow of C57BL/6 or *Cxcr2*−/− mice, which lack the primary neutrophil receptor for ELR+ CXC chemokines, and tested for their ability to migrate toward tumor cells (**Fig. 1B, C**). After 16 h of incubation, neutrophils were collected from both top and bottom chambers and quantified. As seen in **Fig. 1C**, neutrophils isolated from C57BL/6 mice showed enhanced migration toward LLC cells, while neutrophils isolated from *Cxcr2*−/− mice were unresponsive. These data indicate that chemokine expression by LLC tumor cells induces CXCR2-dependent neutrophil chemotaxis.

To test whether neutrophils have a direct cytotoxic effect on LLC cells, we performed *in vitro* cell killing assays as previously described.[30] Neutrophils isolated from bone marrow exerted cytotoxic effects and there was a trend for neutrophils isolated from LLC tumor-bearing mice to induce more cell killing than those from naive mice (**Fig. 1D**). The cytotoxic effect was cell number dependent (**Fig. 1D**). A similar killing pattern was observed with LLC cells cocultured with neutrophils from AE17-tumor bearing mice (**Fig. 1E**). We further tested whether inflammatory cytokines, specifically TNF-α and IL-1β, affected neutrophil cytotoxicity. Both TNF-α and IL-1β enhanced neutrophil-mediated cancer cell killing in our coculture model (**Fig. 1F**).

These results establish use of the LLC tumor cell line as an appropriate model for investigating the role of tumor infiltrating neutrophils. Further, they indicate that neutrophils may exert a direct cytotoxic effect on cancer cells, and this effect may be enhanced by cytokines such as TNF-α and IL-1β.

Heterotopic LLC tumors fail to recruit neutrophils and show enhanced tumorigenicity in *Cxcr2*−/− mice at early stages of tumorigenesis

Since LLC cells express neutrophil chemokines and attract neutrophils *in vitro* in a CXCR2 dependent manner, we

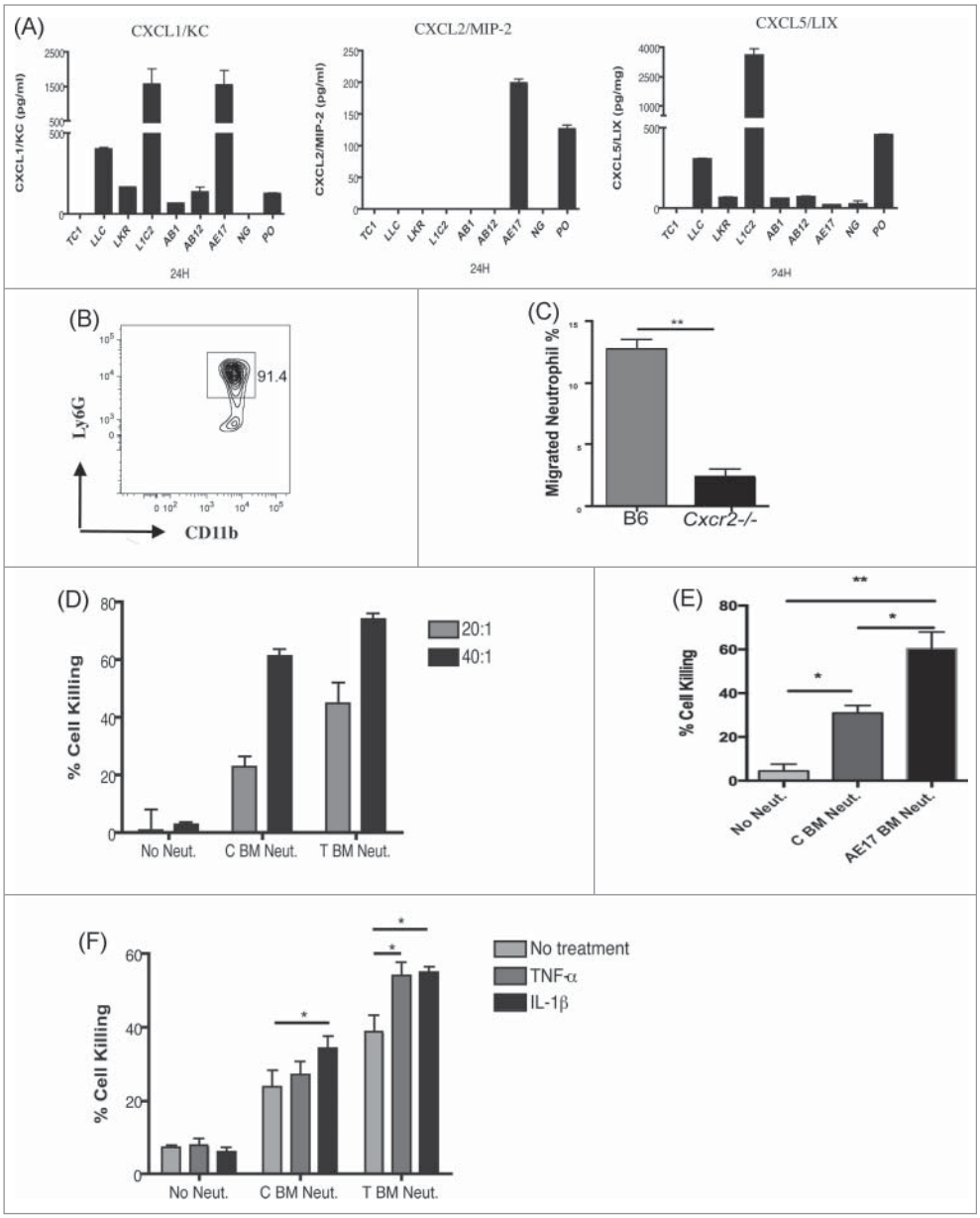


Figure 1. LLC cells induce neutrophil chemotaxis via secreting ELR+CXC chemokine and neutrophils had a cytotoxic effect on these cells *in vitro*. (**A**) The production of CXCL1, CXCL2 and CXCL5 by NSCLC and mesothelioma lines was measured by ELISA. (**B**) Neutrophils were purified from bone marrow of C57BL/6 mice and were analyzed by flow cytometry for purity by CD11b+Ly6G+ staining. (**C**) Purified neutrophils from C57BL/6 or *Cxcr2*−/− mice were assayed by transwell migration for chemotaxis toward LLC cells. Negative control was neutrophil migration toward a non-neutrophil secretion lung cancer cell line. Cells were counted from both the top and bottom of transwell plate after 16 h. (**D**) Neutrophils isolated from bone marrow of naive (C BM Neut) or tumor-bearing (T BM Neut) mice were cocultured with luciferase-labeled LLC cells at 20:1 and 40:1 neutrophil to tumor cell ratio. Following overnight incubation, luciferase activity was measured using the Clarity (Bio-Tek) microplate luminescence reader as a measure of cytotoxicity. (**E**) As above neutrophils isolated from bone marrow of naive (C BM Neut) or AE17 tumor bearing (AE17 BM Neut) mice were assayed for cytotoxicity against LLC cells, at a 20:1 neutrophil:tumor cell ratio. (**F**) Addition of 10ng/mL recombinant TNF-α or IL-1β induced enhanced neutrophil cytotoxic effect, measured as in D. **p* <0.05 ***p* <0.01; ****p* <0.001. Values are mean ± SEM, n = 3/experiment, representative of three experiments).

hypothesized that neutrophils might directly affect tumor growth in mice injected with LLC cells. To test the role of neutrophils in controlling LLC tumors, we injected 2×10^6 mouse LLC cells into the flanks of C57BL/6 and *Cxcr2*−/− mice. We observed increased growth of tumors in *Cxcr2*−/− recipients as compared to C57BL/6 controls within 2 weeks of injection (**Fig. 2A**).

Recently, it has become clear that many cells labeled with Gr-1, often used as a neutrophil marker, actually represent heterogeneous cell populations. The percent of CD11b+Gr-1+ cells within tumors from LLC-injected C57BL/6 and *Cxcr2*−/− mice was not significantly different (29.8 ± 2.7% and 30.8 ± 2.1% CD11b + Gr-1+ cells in C57BL/6 and *Cxcr2*−/− tumors respectively, **Fig. 2B, C**), indicating that the accumulation of the large Gr-1+ population is not dependent on CXCR2. However, the population of bona fide neutrophils, which are CD11b + Ly6G+ and can also be identified by high expression of Gr-1, was significantly different between LLC-injected C57BL/6 and *Cxcr2*−/− mice (3.5 ± 0.3% and 0.2 ± 0.0% respectively, **Fig. 2B, C**).

In order to determine where neutrophils were localized within the LLC tumors, we used immunohistochemical staining of MPO to identify neutrophil activity within the tumor 14 d after injection. As seen in **Fig. 2D**, neutrophils were found largely within necrotic areas of the tumor tissue, and the percentages of MPO+ cells in the tumor was consistent with the percent of CD11b + Ly6G+ cells in the tumors as identified by flow cytometry (**Fig. 2E**). These results indicate that only the infiltration of CD11b + Ly6G+ neutrophils is CXCR2-dependent, and influx of other Gr-1+ cells is intact in CXCR2 deficient mice. We also observed that, consistent with decreased MPO+ cells in the tumor and increased tumor volume, the percent of tumor necrosis in LLC injected *Cxcr2*−/− mice was decreased relative to controls (**Fig. S1A** However, when tumors

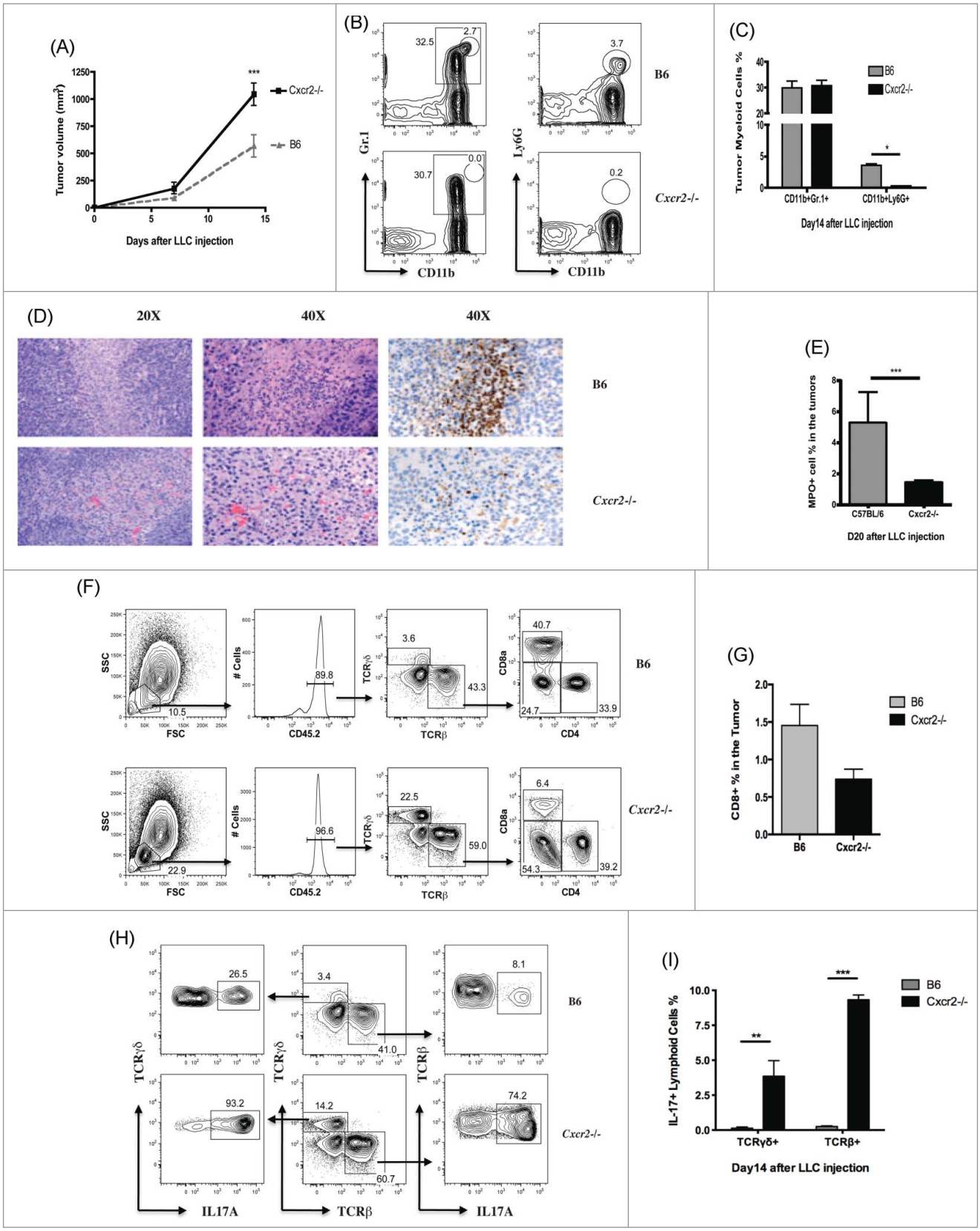


Figure 2. For figure legend, see page 6.

were examined 21 d after inoculation, we found that necrosis was increased in *Cxcr2*−/− mice (**Fig. S1B**). This phenomenon was previously observed by Kean and colleagues, and is likely related to loss of pro-angiogenic effects of CXCR2.[15,34,35]

In addition to increased tumor size, tumors isolated from *Cxcr2*−/− mice showed alterations in lymphoid populations other than neutrophils (**Fig. 2F**). The total $CD8^+$ T cell percentage in the tumors of C57BL/6 mice was two-fold more than that of *Cxcr2*−/− mice (1.45 ± 0.28% and 0.74 ± 0.13% respectively), while the population of TCRγδ+ T cells was expanded in tumors from *Cxcr2*−/− mice (**Fig. 2F, G**). Strikingly, most of the lymphoid cells isolated from tumors of *Cxcr2*−/− mice (70%) were IL-17A positive; only about 3% of the same population was IL-17A positive in C57BL/6 mice (**Fig. S1C**). These IL-17 producers were enriched for TCRβ+ $CD4^+$ cells (**Fig. S1C**). Of TCRγδ+ T cells found in the CD45.2+ lymphoid population of tumors from C57BL/6 (3% total lymphoid cells) and *Cxcr2*−/− (15% total lymphoid cells) mice, almost all of these TCRγδ+ T cells in *Cxcr2*−/− mice (90%), and many in C57BL/6 mice (25%), were IL-17-producing cells. There was a significant increase in both TCRβ and TCRγδ IL-17 producing cells between C57BL/6 and *Cxcr2*−/− mice (**Fig. 2H, I**).

Taken together, these data indicate that dysfunction of bona fide neutrophils (CD11b+ Ly6G+ cells), and no other CD11b+Gr-1+ cell populations, is associated with decreased $CD8^+$T cell numbers, increased IL-17 production from several cell lineages, and enhanced tumor growth. However, from these data it is unclear whether the effect of neutrophil loss on tumor $CD8^+$ T cells and IL-17-producing lymphoid cells is exerted within the tumor microenvironment, in the systemic compartment, or in other tissue sites.

Neutrophil depletion augments tumor growth rate, increases percentages of IL-17-producing cells, and reduces percentages of $CD8^+$ T cells in the tumors

Since recruitment of CD11b + Ly6G+ cells is CXCR2-dependent, and these cells may affect tumor growth by altering the balance of tumor infiltrating IL-17-producing cells and $CD8^+$T cells, we next tested directly the effects of CD11b + Ly6G+ cell depletion on tumor growth in C57BL/6 mice. Based on published[7] and our own unpublished data, intermittent depletion of neutrophils by anti-Ly6G antibody post-tumor engraftment does not decrease, and may actually increase, neutrophil numbers in the circulation. On the other hand, daily neutrophil depletion appears highly effective until day 14 post-tumor engraftment.[7] Based on these findings, we developed a new approach for neutrophil depletion. Neutrophil depletion was started the day before LLC flank injection via intraperitoneal injection of 250 ug anti-Ly6G antibody and depletion was performed daily until mice were sacrificed on day 12. Control mice were injected with 250 ug isotype control antibody.

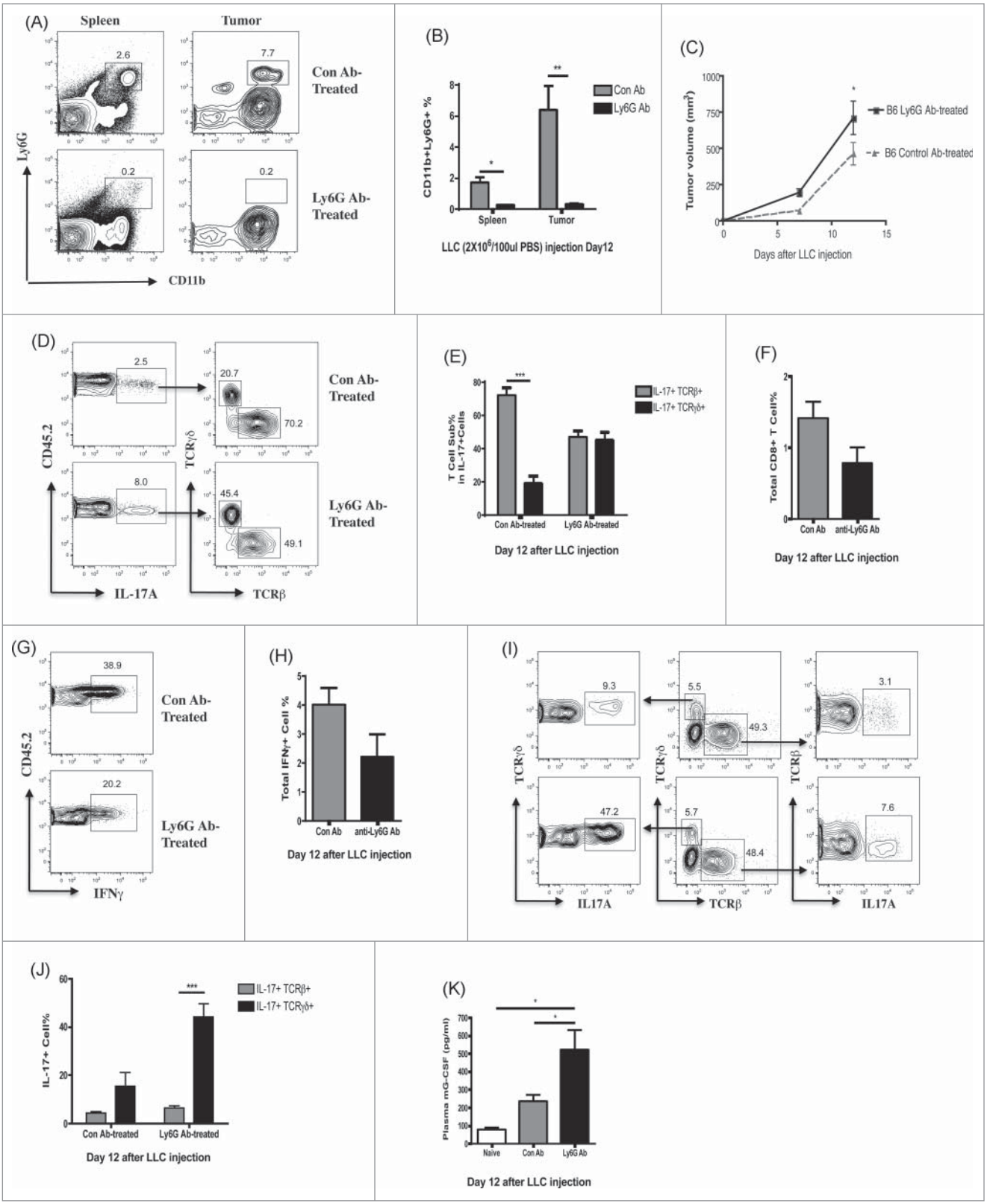
Analysis of spleens and tumors showed that CD11b + Ly6G+ cells were dramatically reduced after antibody, but not isotype control, treatment (**Fig. 3A, B**). The CD11b + $Gr\text{-}1^{high}$ population was also specifically diminished after anti-Ly6G antibody treatment, again indicating that the $Gr\text{-}1^{high}$ cells represent the CD11b + Ly6G+ cell population (data not shown). Tumor progression and lymphocyte infiltration in neutrophil depleted tumor-bearing C57BL/6 mice phenocopied our observations of tumor-bearing *Cxcr2*−/− mice. Neutrophil depletion significantly augmented tumor growth (**Fig. 3C**) and increased the percentages of CD45.2 + IL-17-producing cells (from 3.11 ± 0.55% to 7.26 ± 1.66%) within the tumor (**Fig. 3D**). IL-17-producing cells were predominantly TCRβ+ T cells in isotype control treated mice, but TCRγδ+ and TCRβ+ T cells contributed equally to the IL-17 production in neutrophil-depleted mice (**Fig. 3D, E**). Neutrophil depletion also decreased the total percentages of tumor infiltrating $CD8^+$ T cells (from 1.41 ± 0.23 to 0.78 ± 0.22) (**Fig 3F**). Consistent with a loss of $CD8^+$ infiltrating T cells, neutrophil depletion led to decreased total IFNγ producing cells in the tumors (**Fig. 3G, H**). Although neutrophil depleted mice did not show skewing toward TCRγδ+ T cells within the tumor T cell population, TCRγδ+ T cells were much more likely to be IL-17-producing cells, similar to findings in tumor-bearing *Cxcr2*−/− mice (**Fig. 3I, J**). These higher levels of IL-17 in the tumors corresponded with increased plasma levels of G-CSF in the neutrophil-depleted group (**Fig. 3K**).

These results are consistent with the concept that the absence of tissue-infiltrating neutrophils, whether through neutrophil deficiency or dysfunction, modifies the tumor microenvironment to increase IL-17 production and decrease $CD8^+$ T cell number, leading to failure to control early tumorigenesis. This still leaves open the question, however, of whether this effect is exerted locally in the tumor, or at other sites.

Replenishment of functional neutrophils inhibits IL-17A production and reduces tumor growth

Our results from *Cxcr2*−/− mice and neutrophil-depleted WT mice suggested that neutrophils can condition the tumor

Figure 2 (See previous page). Heterotopic LLC tumors fail to recruit neutrophils and show enhanced tumorigenicity in *Cxcr2*−/− mice at early stages of tumorigenesis. (**A**) 2×10^6 mouse LLC cells were injected into flanks of C57BL/6 and *Cxcr2*−/− mice. Tumor growth was monitored, data show the mean tumor volume ± SD, (B) Representative flow plots showing CD11b+Gr-1+ or CD11b+Ly6G+ cells in the tumors of C57BL/6 mice and *Cxcr2*−/− mice. Previously gated on live singlets. (**C**) Quantification of B. D, Histological (Left & Middle panels) and immunohistochemical (Right panels) analysis of tumor-infiltrating MPO+ cells from isolated solid tumors. (**E**) Quantification of MPO+ cell percentages in the tumors of C57BL/6 and *Cxcr2*−/− mice. (**F**) Representative flow cytometry plots showing CD45.2+ TCRβ/TCRγδ lymphocytes, and TCRβ+ $CD8^+$/$CD4^+$ T cells in the tumors of C57BL/6 mice and *Cxcr2*−/− mice. Previously gated on live singlets. (**G**) Quantification of %$CD8^+$ T cells (CD45.2+, TCRβ+) relative to total tumor cells, in *Cxcr2*−/− and control mice. (**H**) Representative flow cytometry plots of TCRγδ+ or TCRβ+ IL-17A producing lymphocytes in *ex vivo* stimulated tumors from *Cxcr2*−/− mice and C57BL/6 mice. Previous gates: live, singlet, lymphocyte, CD45.2+. I, Quantification of %IL-17A+ cells gated as in H. *$p < 0.05$ **$p < 0.01$; ***$p < 0.001$, values shown as mean +/− SEM unless otherwise noted. 5mice/group, representative of five experiments.



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microenvironment to limit IL-17A production and promote $CD8^+$ T cell function. Therefore, we tested whether CXCR2 sufficient neutrophils could rescue the loss of tumor control observed in tumor-bearing *Cxcr2*−/− mice. We first compared the roles of normal neutrophils from C57BL/6 mice and chemotaxic-defective neutrophils from *Cxcr2*−/− mice by adoptively transferring 1×10^7 purified bone marrow neutrophils, either from WT or *Cxcr2*−/− mice, into *Cxcr2*−/− recipients. 24 h later, the *Cxcr2*−/− mice injected with WT neutrophils showed significantly reduced plasma levels of IL-17A and G-CSF compared with those injected with *Cxcr2*−/− neutrophils (**Fig. 4A, B**). These data indicate that only functional neutrophils can control the IL-17A/G-CSF axis, and that this is a systemic effect.

To test whether functional neutrophils can limit tumor growth *in vivo*, we intravenously injected 1×10^7 bone marrow neutrophils isolated from naive C57BL/6 mice on day 1, 2, 8 and 11 after LLC tumor engraftment in *Cxcr2*−/− mice. Control tumor-bearing *Cxcr2*−/− mice were injected with PBS. As seen in **Fig.4C**, adoptive transfer of neutrophils limited tumor growth (**Fig. 4C**), led to decreased percentages of IL-17-producing cells (**Fig. 4D, E**) and increased $CD8^+$ T cells in the tumors (**Fig. 4F, G**). Similar changes in cell populations were also found in the spleens (**Fig. 4H–J**). Surprisingly, neutrophil percentages were not significantly different in the tumors of control and neutrophil-replenished *Cxcr2*−/− mice (data not shown), suggesting that infused neutrophils did not contribute significantly to the tumor-associated neutrophils (TAN) population. Hence, the effects of these infused neutrophils on tumor control are likely indirect, due to the ability of functional neutrophils to limit IL-17A production systemically.

This suggests that increased IL-17A due to dysfunction or depletion of neutrophils will lead to loss of early tumor control independent of neutrophils directly infiltrating tumor. To test this, we injected *Cxcr2*−/− and C57BL/6 mice with a cell line that produces very little ELR+CXC neutrophil chemokine, the TC-1 cell line (**Fig. 1A**). As this tumor should not robustly recruit neutrophils via CXCL1/5, direct effects of neutrophils on limiting tumor growth should be minimal, allowing observation of indirect effects. We hypothesized that, similar to our LLC tumor observations, *Cxcr2*−/− mice would fail to control TC-1 tumors. Consistent with this, we observed that TC-1 injected *Cxcr2*−/− mice had increased tumor burden (**Fig. S2A**). TC-1 injected C57BL/6 mice did show some neutrophil infiltration into the tumor that was CXCR2-dependent, as this small population was further decreased in *Cxcr2*−/− mice (**Fig. S2B, C**). However, the neutrophil recruitment to TC-1 tumors was approximately five-fold lower than the recruitment observed in LLC tumors, suggesting a minimal role for direct cytotoxicity as an effective mechanism of neutrophil-mediated tumor control. Similar to our observations in the LLC tumor model, in TC-1 tumors from *Cxcr2*−/− mice we observed increased IL-17-producing cells, decreased $CD8^+$ cells, and decreased IFNγ+ CD8 cells (**Fig. S2D–F**). Thus, in a model in which direct effects of neutrophils within the tumor are limited, dysregulation of IL-17 by defective neutrophils in *Cxcr2*−/− mice indirectly leads to loss of tumor control.

IL-17A suppresses proliferation and IFNγ production of $CD8^+$ T cells *in vitro*

Our previous results indicate that neutrophils promote antitumor immunity by limiting IL-17A production systemically. However, it is unclear what role IL-17A plays in promoting tumor growth. To study mechanisms by which IL-17A affects tumor development, we first investigated whether IL-17A could directly promote tumor growth *in vitro*. IL-17A had no direct effect on LLC cell viability or growth (data not shown) after 24 h of stimulation. This indicates that IL-17A is unlikely to directly promote LLC tumor growth, but that IL-17 could lead to a protumor microenvironment by affecting other cell types.

As our data indicated a correlation between IL-17A producing cells and $CD8^+$ T cells, we focused on whether IL-17A might affect $CD8^+$ T cells. We cocultured neutrophils from bone marrow of naive mice with anti-CD3/CD28 stimulated $CD8^+$ T cells in the presence or absence of exogenous IL-17A with or without CD11b+Ly6G+ neutrophils. CD11b+Ly6G+ cells isolated from the bone marrow of naive C57BL/6 mice did not affect proliferation and IFNγ production of $CD8^+$ T cells, consistent with CD11b+Ly6G+ neutrophils being functionally distinct from related MDSCs (**Fig. 5A**). Surprisingly, IL-17 alone decreased proliferation and IFNγ production of $CD8^+$ T cells, in the presence or absence of neutrophils (**Fig. 5B**). To further

Figure 3 (See previous page). Neutrophil depletion augments tumor growth rate, increases percentages of IL-17-producing cells, and reduces percentages of $CD8^+$ T cells in the tumors. (**A**) Representative flow cytometry plots of CD11b+Ly6G+ cells in the spleens and tumors in Ly6G antibody or isotype control treated C57BL/6 mice 12 d after injection with LLC tumor cells. Previously gated on live singlets. (**B**) Quantification of CD11b+Ly6G+ cell percentages in the spleens and tumors of control and anti-Ly6G antibody-treated LLC tumor-bearing C57BL/6 mice. (**C**) Tumor measurement of LLC-injected C57BL/6 mice treated with anti-Ly6G antibody or isotype control antibody. Data show the mean tumor volume ± SD. (**D**) Representative flow cytometry plots of CD45.2+IL-17+ cells, and TCRβ/TCRγδ staining on IL-17+ positive cells in *ex vivo* stimulated tumors from anti-Ly6G treated tumor-bearing C57BL/6 mice compared to control mice. Previous gates: live, singlet, lymphocyte, CD45.2+. (**E**) Quantification of of CD45.2+ IL-17+ cells stained from TCRγδ and TCRβ+ from tumors of neutrophil-depleted mice and controls. (**F**) Quantification of %$CD8^+$ T cells (CD45.2+, TCRβ+) relative to total tumor cells of control mice and anti-Ly6G treated tumor-bearing mice. (**G**) Representative flow cytometry plots of CD45.2+IFNγ+ cells in the *ex vivo* stimulated tumors of control mice and anti-Ly6G treated tumor-bearing mice. Previous gates: live, singlet, lymphocyte, CD45.2+. (**H**) Quantification of percentages of IFNγ+ cells in the *ex vivo* stimulated tumors of control and anti-Ly6G treated tumor-bearing mice, relative to total tumor cells analyzed. (**I**) Representative flow cytometry plots of TCRγδ+ or TCRβ+ IL-17-producing cells from *ex vivo* stimulated tumors of anti-Ly6G treated or untreated tumor-bearing mice. Previous gates: live, singlet, lymphocyte, CD45.2+. (**J**) Quantification of CD45.2+IL-17-producing cell percentages in different T cell subpopulations, gated as in I. (**K**) Quantification of plasma levels of G-CSF from serum of treated and untreated tumor bearing mice relative to naive controls, as measured by ELISA. *p <0.05 **p <0.01; ***p <0.001, values shown as mean +/− SEM unless otherwise noted. 5 mice/group.

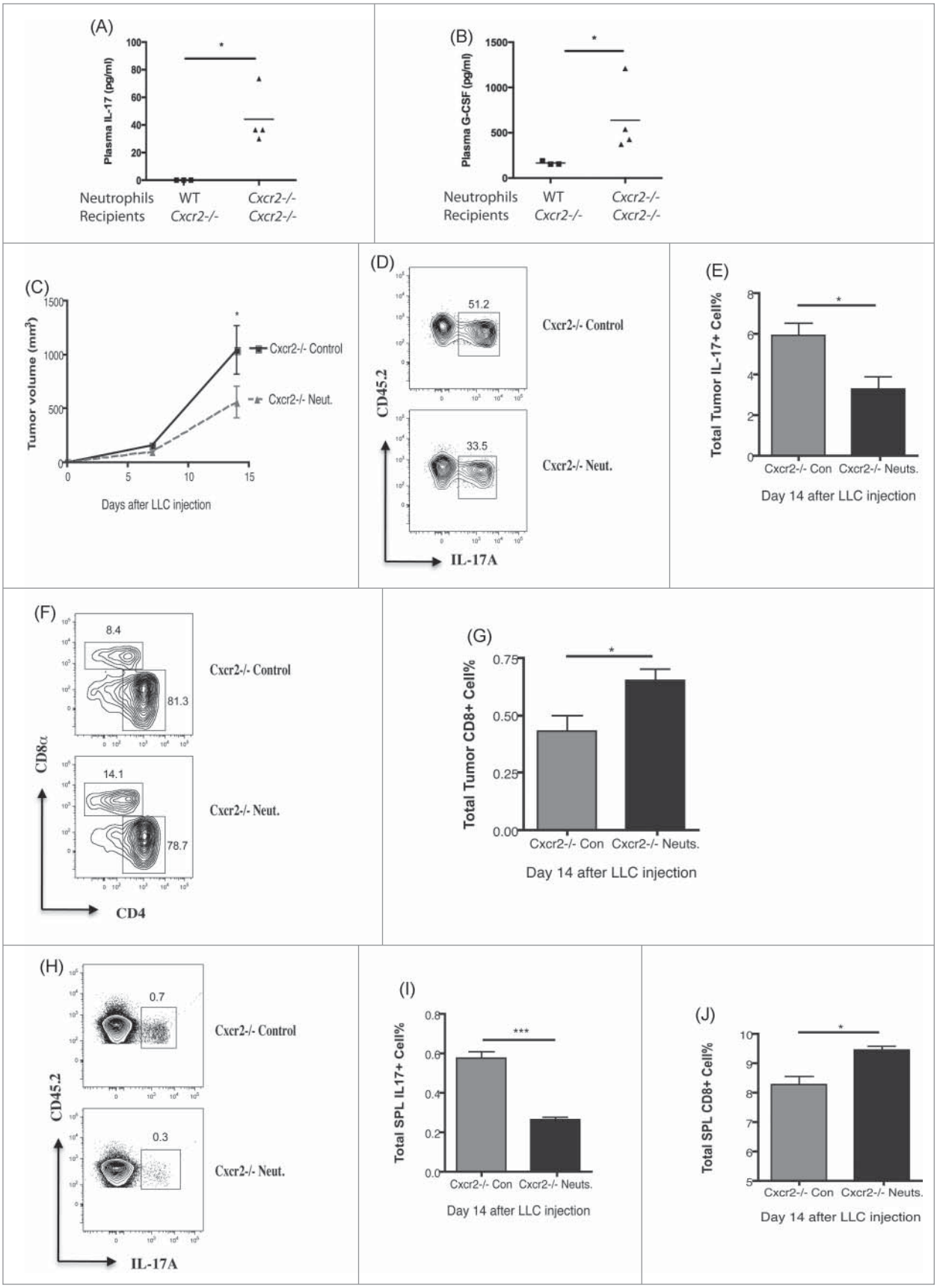

Figure 4. For figure legend, see page 10.

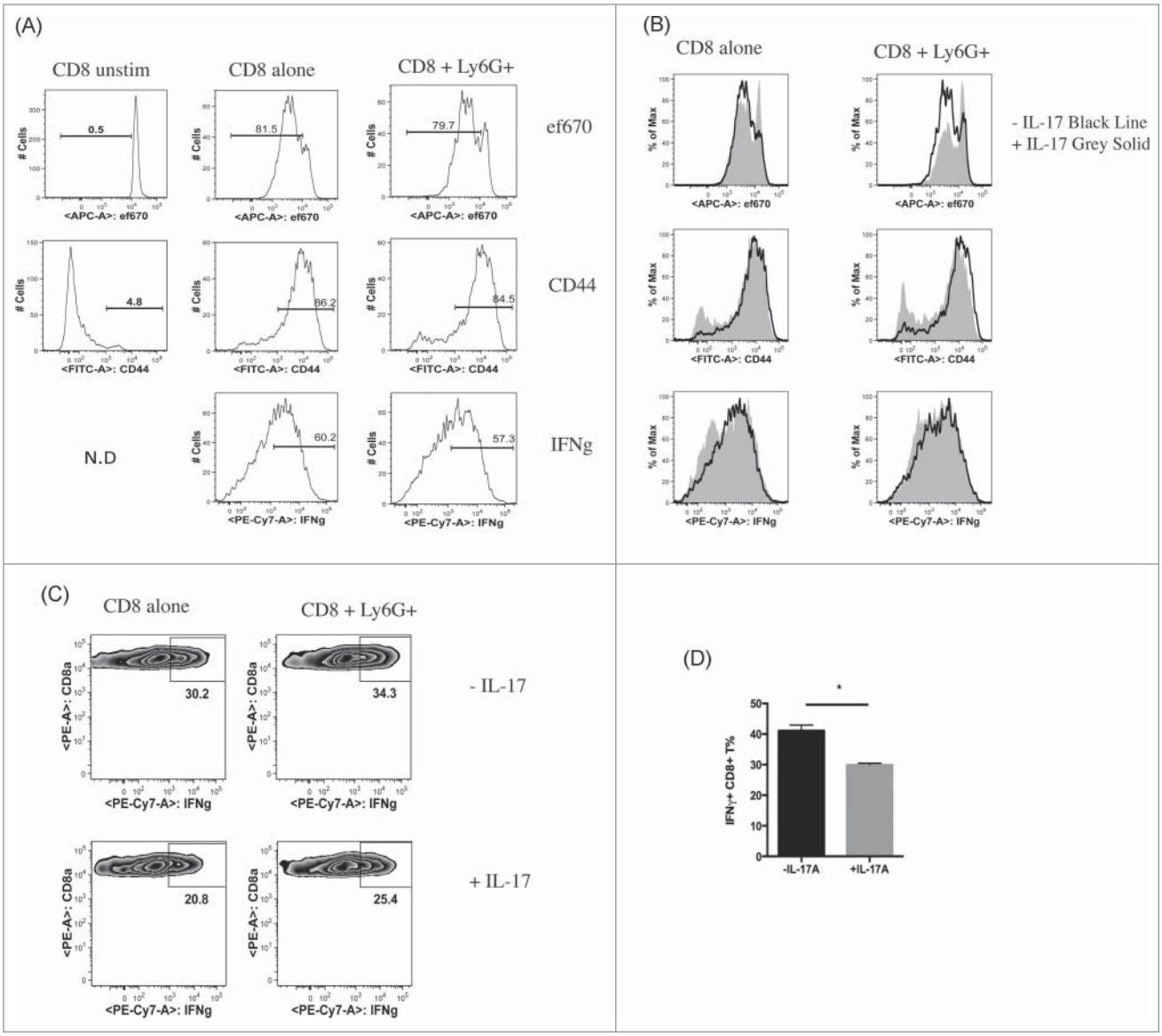

Figure 5. IL-17A suppresses proliferation and IFNγ production of $CD8^+$ T cells *in vitro*. (**A**) Representative flow cytometry histograms of $CD8^+$ T cells from naive C57BL/6 mice cultured with or without CD11b+Ly6G+ cells isolated from the bone marrow of naive C57BL/6 mice. Proliferation, CD44 expression, IFNγ production of $CD8^+$ T cells was measured by flow cytometry after anti-CD3/CD28 stimulation. (**B**) Representative flow cytometry histograms of $CD8^+$ T cells from naive C57BL/6 mice cultured with or without CD11b+Ly6G+ cells, isolated from the bone marrow of naive C57BL/6 mice, in the presence or absence of IL-17A, and assayed by flow cytometry as in A. (**C**) Representative flow cytometry histograms of IFNγ production from $CD8^+$ T cells cultured in the presence or absence of CD11b+Ly6G+ cells isolated from the spleens of LLC tumor-bearing C57BL/6 mice, with or without IL-17A. (**D**) Quantification of IFNγ production of $CD8^+$ T cells cultured in the presence or absence of IL-17A. Values are mean ± SEM (n = 3).

Figure 4 (See previous page). Replenishment of functional neutrophils inhibits IL-17A production and reduces tumor growth. (A–B) 24 h post neutrophil i.v. injection, serum was isolated from control or *Cxcr2*−/− mice that received CXCR2 deficient or sufficient neutrophils. IL-17A/G-CSF were measured by ELISA. (**C**) 1×10^7 bone marrow neutrophils isolated from naive C57BL6 mice were i.v injected into LLC tumor-bearing *Cxcr2*−/− mice on day 1, 2, 8 and 11. Tumor size was measured, data shown mean tumor volume +/-SD. (**D**) Representative flow cytometry plots of CD45.2+IL-17-producing cells from *ex vivo* stimulated tumors of neutrophil injected or uninjected *Cxcr2*−/− mice. Previous gates: live, singlet, lymphocyte, CD45.2+. (**E**) Quantification of total percentages of IL-17-producing cells, relative to the all tumor cell populations, in the tumors of PBS and neutrophil-injected *Cxcr2*−/− mice. (**F**) Representative flow cytometry plots of $CD8^+$/$CD4^+$ T cells in the lymphocyte population from tumors of neutrophil injected and uninjected *Cxcr2*−/− mice. Previous gates: live, singlet, lymphocyte, CD45.2+, TCRβ+. (**G**) Quantification of percentages of $CD8^+$ T cells in the tumors of PBS and neutrophil-injected Cxcr2−/− mice, relative to the total tumor. (**H**) Representative flow cytometry plots of CD45.2+IL-17-producing cells in *ex vivo* stimulated spleens of neutrophil-injected Cxcr2−/− mice, gated as in D. (**I**) Quantification of percentages of IL-17-producing cells in the spleens of PBS and neutrophil-injected Cxcr2−/− mice, among all splenocytes. (**J**) Quantification of percentages of $CD8^+$ T cells in the spleens of PBS and neutrophil-injected Cxcr2−/− mice, among all splenocytes. *p <0.05 **p <0.01; ***p <0.001, values shown as mean +/− SEM unless otherwise noted. 5 mice/group.

examine the effect of IL-17A in the context of cells derived from tumor-bearing mice, we sorted CD11b+Ly6G+ cells from the spleens of 14 day LLC tumor-bearing mice, and cocultured them with $CD8^+$ T cells from naive mice in the presence or absence of IL-17A. CD11b+Ly6G+ cells isolated from tumor-bearing mice did not show strong suppressive effects on T cell proliferation or IFNγ production (**Fig. 5C** and data not shown). We again observed that IL-17A suppressed IFNγ production of $CD8^+$ T cells; this effect was not altered by coculture with CD11b+Ly6G+ cells from tumor bearing mice (**Fig. 5C, D**).

Together with our neutrophil depletion and repletion experiments, in which IL-17A was correlated with tumor control, these results demonstrate that IL-17A could impair the antitumor immune response through suppressing proliferation and IFNγ production of tumor-infiltrating $CD8^+$ T cells.

Discussion

A considerable body of evidence supports the importance of inflammatory cells in the control or progression of different types of cancer.[36-39] Neutrophils are primary inflammatory cells and essential to protect the host during the early phases of microbial infection. However, the role of neutrophils in tumor development remains controversial.[3,4,34,40-45] Here, we suggest that early control of the growth of syngeneic heterotopic tumors in mice is facilitated by neutrophils via an indirect mechanism that, to the best of our knowledge, has not heretofore been suggested.

Neutrophils are found within many human and murine tumors, and research thus far on the role of neutrophils in cancer has focused largely on these tumor associated neutrophils (TAN).[3,6,46-50] The apparent function of these neutrophils may vary depending on the tumor context. Typically, the microenvironment surrounding a solid tumor possesses many of the characteristics of chronic inflammation, a condition considered favorable for tumor growth and spread. However, inducing a shift from this chronic inflammatory state toward an acute inflammatory response may convert neutrophils into anticancer effector cells.[46] To date, a clear consensus on the relationship of neutrophil pro/antitumor function with the tumor microenvironment eludes the field.

The infiltration and effects of neutrophils within a tumor may be related to intrinsic properties of both the tumor and the host. In this regard, considerable interest has focused on the vascular supply to the tumor. CXCR2 on endothelial cells functions as an angiogenic receptor.[15,35] Keane and coworkers reported that heterotopic LLC tumor growth was attenuated in *Cxcr2*−/− mice over a longer time course than that used in these studies, in which angiogenesis may indeed play a role.[34] In our model, we observed less necrosis in tumors from day 14 LLC-injected *Cxcr2*−/− mice, but on day 21 somewhat more necrosis were found in tumors from *Cxcr2*−/− mice (**Fig. S1A, B**), suggesting that the angiogenic effects of CXCR2 are more important at later stages of tumor control. At both timepoints, regions of necrosis were associated with neutrophil infiltration in tumors of B6 mice, but not in *Cxcr2*−/− (**Fig. 2D**), further suggesting that direct cytotoxicity of neutrophils did not fully account for restricting tumor growth in CXCR2 sufficient mice.

To date, a systemic antitumor role for neutrophils has not been described. Rather, many groups have suggested that systemic MDSCs, including granulocytic MDSCS, function as a major protumor cellular compartment.[4,10,51] Here, we show that functional CD11b+Ly6G+ cells with tissue-infiltrating potential, regardless of presence or absence in the tumor, possess not protumor, but antitumor ability at the early phases of tumorigenesis.

Direct cytotoxicity of neutrophils for cancer cells has been shown in a number of settings,[2,52] and we have recapitulated these findings. We show here that bone marrow-derived neutrophils from naive C57BL/6 mice are capable of direct cytotoxicity, that neutrophils from tumor-bearing C57BL/6 mice are more directly toxic on cancer cells *in vitro*, and neutrophils from tumor-bearing mice are more readily stimulated by IL-1β/TNFα to enhance killing. These data are more consistent with a primed or partially activated neutrophil than with one that is suppressive. While these data suggest neutrophils can be cytotoxic against tumor cells, the artificial nature of these *in vitro* killing assays makes it questionable whether this is an important antitumor mechanism *in vivo*. However, for neutrophils, this may complement more robust indirect mechanisms by which they may promote an antitumor microenvironment.

It has been shown that IL-17A and F are capable of inducing neutrophil generation via induction of G-CSF and upregulating neutrophil chemokines.[16-21] We have recently shown that IL-17 is part of a feedback control system to regulate neutrophil availability in tissues.[17] Our data here show defective neutrophil trafficking induced more IL-17-producing cells in the tumors of *Cxcr2*−/− mice. Furthermore, TCRγδ+ T cells appear to become major IL-17-producing cells. Depletion of neutrophils, as we show here, acutely induces IL-17-producing cells (**Fig. 3D**). Thus, in both neutrophil dysfunction (*Cxcr2*−/− mice) and deficiency (neutrophil-depleted C57BL/6 mice), there is evidence of a compensatory increase in IL-17-producing cells, which can be detected in the tumor itself. Adoptive transfer of neutrophils induces a reciprocal change by decreasing numbers of IL-17-producing cells. By using two tumor cell lines, one which induces neutrophil chemotaxis into the tumor itself and one that does not, we have found that the conditioning effect of neutrophils on IL-17 production, systemically and in the tumor, is a primary driving force on early tumor control.

Availability of neutrophils directly affected local and systemic IL-17 production, however IL-17 had no direct effect on cancer cell viability. Since effector $CD8^+$ T cells have been recognized as a main player in antitumor immune responses, we further examined the relationship of IL-17A and $CD8^+$ T cells *in vitro*. IL-17 alone suppressed proliferation and IFNγ production of $CD8^+$ T cells in the presence and absence of neutrophils. Neither CD11b+Ly6G+ cells isolated from the bone marrow of naive C57BL/6 mice, nor sorted CD11b+Ly6G+ cells from the spleens of 14 day LLC tumor-bearing mice affected $CD8^+$ T cell function. These data indicate that the CD11b+Ly6G+ population, whether naive or primed, at early stages of tumorigenesis is

functionally distinct from g-MDSCs, and that they play an antitumor role by regulating IL-17 production. In the absence of neutrophil-mediated control of IL-17 production, IL-17 exerts direct, negative effects on $CD8^+$ T cell function.

This work raises several questions for further research. While focusing on early tumor growth allowed us to concentrate on the effects of neutrophils on tumor control, as opposed to angiogenesis, this required a larger inoculum which is a limitation of this study design. Specifically, in control mice this high inoculum may have supported a more pro-inflammatory, increased antitumor immune response than might be observed otherwise. Further, the suppressive effect of IL-17A on $CD8^+$ T cells, which our *in vitro* work identified, could be occurring in peripheral lymphoid organs or in the tumor. Further work will be needed to clarify the role of IL-17A in $CD8^+$ T cell dysfunction. Similarly, inferences of this study for cancer in humans is complex. However, the implication that chemotherapy-induced neutropenia might increase IL-17-producing cells in humans should be investigated as a confounding variable in the development of cancer immunotherapies.

In summary, neutrophil depletion and dysfunction, via deletion of CXCR2, are associated with enhanced tumor growth, increased IL-17-producing cells, and decreased $CD8^+$T cells. Replenishment of *Cxcr2*−/− mice with functional neutrophils reverses these phenomenon. Our data suggest that availability of functional neutrophils orchestrates a complex immune response by regulating IL-17 production to modify tumor growth. Specifically, we determined that IL-17A suppresses proliferation and IFNγ production of $CD8^+$ T cells *in vitro*, suggesting that neutrophils limit early tumor growth by indirectly promoting antitumor $CD8^+$ T cell responses. Although MDSCs have been well recognized as playing a protumor role, our data indicate that CD11b+ Ly6G+ neutrophils are not suppressive on $CD8^+$ T cell response. Moreover, they do not have to be found within the tumor to exert profound antitumor effects. This indirect effect of neutrophils on tumor control via limiting IL-17-producing cells fits well with data from several groups suggesting that IL-17-producing cells promote tumor growth.[22,25, 53] Taken together, these data demonstrated that, while neutrophils could control tumor growth by direct cytotoxic effects, the primary mechanism by which neutrophils exert antitumor effects is to regulate IL-17 production, through which they indirectly promote $CD8^+$ T cell responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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