Tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) can control cancer growth and exist in almost all solid neoplasms. The cells are known to descend from immature monocytic and granulocytic cells, respectively, which are produced in the bone marrow. However, the spleen is also a recently identified reservoir of monocyes, which can play a significant role in the inflammatory response that follows acute injury. Here, we evaluated the role of the spleen reservoir in a genetic mouse model of lung adenocarcinoma driven by activation of oncogenic Kras and inactivation of p53. We found that high numbers of TAM and TAN precursors physically relocated from the spleen to the tumor stroma, and that recruitment of tumor-promoting spleen-derived TAMs required signaling of the chemokine receptor CCR2. Also, removal of the spleen, either before or after tumor initiation, reduced TAM and TAN responses significantly and delayed tumor growth. The mechanism by which the spleen was able to maintain its reservoir capacity throughout tumor progression involved, in part, local accumulation in the splenic red pulp of typically rare extramedullary hematopoietic stem and progenitor cells, notably granulocyte and macrophage progenitors, which produced CD11b+ Ly-6C^hi monocytes and CD11b+ Ly-6G^hi granulocytic cells locally. Splenic granulocyte and macrophage progenitors and their descendants were identified in clinical specimens. The present study sheds light on the origins of TAMs and TANs, and positions the spleen as an important extramedullary site, which can continuously supply growing tumors with these cells.
new CD11b+ Ly-6C^hi monocytic and CD11b+ Ly-6G^hi granulocytic cells locally, and participate in the continuous replenishment of the myeloid cell reservoir. The identification of these processes should contribute to our understanding of myeloid responses in cancer and provide new vantage points for therapy.

Results

Spleen Contributes TAMs and TANs. The lung contains different populations of macrophages, which can express CD11b at variable levels (20) and include CD11b+ CD11c+ F4/80+ alveolar macrophages. To test whether the spleen participates in macrophage responses during tumor progression, we splenectomized KP mice at week 0 (at tumor initiation) (Fig. 1A) via a procedure that preserves bone marrow and blood cell pools (17). The procedure reduced both CD11b+ and CD11b− lung macrophages (TAMs) by week 11 in a large fraction of animals (Fig. 1B and Fig. S1). Splenectomy also significantly reduced the numbers of CD11b− Gr1+ Ly-6G+ lung tissue neutrophils (TANs) (Fig. 1B).

The experiment was repeated with mice that were splenectomized at week 8 instead of week 0 (Fig. L4) (i.e., mice with preestablished cancer). These mice likewise showed impaired TAM and TAN responses by week 11 (Fig. 1B). This finding supports the previous notion that TAMs and TANs are continuously replaced during tumor progression (14, 15) and suggests that the spleen acts to contribute these cells at least after week 8. The spleen was also found to be required for full accumulation of TAMs and TANs in two grafted tumor models (Fig. S2).

TAMs produce proteases that degrade ECM proteins and enhance tumor cell invasion and intravasation (21). Ex vivo flow cytometry analysis of KP mice that received a cysteine Cathepsin B-targeted optical sensor revealed high cathepsin activity in TAMs, as expected, but also in splenic monocytes (Fig. S3). We next compared proteolytic activity in vivo by combining the optical sensor with fluorescent-mediated tomography/computed tomography (CT) fusion imaging (2). The lungs of spleen-deficient animals showed significantly reduced proteolytic activity (Fig. 1C), in line with the above findings that splenectomy reduced the TAM and TAN responses. Other tumor-promoting functions have been identified for TAMs (5) and may also be relevant in KP mice.

Spleen Contributes to Tumor Growth. Given that removal of the spleen prevented full-fledged TAM accumulation and TAM-associated tumor-promoting activity, we investigated whether it also affected tumor progression. To do this, spleen-sufficient and spleen-deficient animals were monitored by CT at weeks 7, 9, and 11 after tumor initiation. The majority of KP mice from which the spleen was removed showed decreased numbers of detectable tumor nodules and reduced total tumor volumes over time (Fig. 1D and E). Histopathological analysis at week 11 (Fig. S4) confirmed reduced tumor progression in the majority of spleen-deficient mice. This same phenotype was observed regardless of whether mice were splenectomized at week 0 or at week 8. In both cases, splenectomized mice often presented with fewer and smaller tumor nodules, which were either grade 1 adenomatous hyperplasias or grade 2 adenomas compared with grade 2 or grade 3 adenocarcinomas in age-matched control animals.

Splenectomy decreased the TAM response before changes in tumor progression could occur (Fig. S5A), which is in accordance with a causal association between TAMs and tumor progression. Also, in support of the notion that the spleen contributed cells to tumors directly, the number of blood CD11b+ Ly-6C^hi mononcytic cells decreased shortly after splenectomy in tumor-bearing KP animals (Fig. S5B and C). Spleenectomy likely did not affect bone marrow hematopoiesis because bone marrow leukocyte counts remain normal in steady state (17) and in the presence of growing tumors (Fig. SSD).

Histopathological analysis indicated that the tumor burden at week 11 in spleen-deficient KP mice was similar (i.e., not lower) to that at week 8 in spleen-sufficient mice (Fig. S4); thus, splenectomy delayed tumor progression but did not induce tumor rejection. Antitumor cytotoxic T lymphocytes (CTLs) seemed uninvolved because they remained rare in the tumor stroma of KP mice even in spleen-deficient animals (Fig. S5E). Likewise, the CTLs that did accumulate in tumors did not exhibit enhanced effector functions (Fig. S5F). Spleenectomy also did not affect the number of tumor-infiltrating FoxP3+ regulatory T cells (Fig. S5G). In contrast, impaired tumor progression in spleen-deficient KP mice was associated with decreased numbers of TAMs and TANs (Fig. S5H and I).

Inflammatory Ly-6C^hi monocytes express the chemokine receptor CCR2, which is involved in the amplification of TAM

Fig. 1. Spleen removal impairs TAM and TAN responses and tumor growth. (A) Experimental procedures. (B) Total CD11b+ and CD11b− macrophage and neutrophil counts in lungs of mice 11 wk after tumor initiation [+adeno virus expressing Cre recombinase (AdCre), n = 17] and in mice from which the spleen was removed either immediately before tumor initiation [+AdCre SP (wk 0), n = 19] or after tumors became apparent [+AdCre SP (wk 8), n = 7]. Control mice (−AdCre, n = 13) were age-matched. Histograms show median values, and dots represent single mice. (C) In vivo fluorescent-mediated tomography/CT fusion images and quantification of Cathepsin B activity at week 11 after AdCre infection in KP mice with [+AdCre +SP] or without [+AdCre −SP (wk 0)] or [−AdCre −SP (wk 0)] or without [+AdCre −SP (wk 0)] a spleen (n = 3). Data are presented as the mean ± SEM. (D and E) High-resolution CT analysis of tumor progression at weeks 7, 9, and 11 in mice with [+AdCre, n = 7] or without [+AdCre −SP (wk 0), n = 12] a spleen. (D) Number and volume of tumor nodules in each group. Histograms show median values, and dots represent single mice. (E) Representative 3D volume renderings of tumors for each group. Tumors are shown in red, and lungs are shown in green. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
responses (15, 22). To test whether CCR2 controlled spleen-derived TAM accumulation, we used a lipid nanoparticle that contains short interfering (si) CCR2 RNA sequences and that selectively silences CCR2 expression in splenic Ly-6C<sup>hi</sup> (CCR2<sup>+</sup>) monocytes (23). KP mice received injections of CCR2-silencing siRNA (siCCR2) or control-silencing (siCON) nanoparticles starting on week 12 after tumor initiation and over 12 d (Fig. 2A). The siCCR2-treated mice showed an approximately twofold reduction of TAMs (Fig. 2B). Spleenic granulocytic cells do not express CCR2 and, accordingly, siCCR2 treatment did not alter TAN responses (Fig. 2B). Further evaluation of lung tissue showed reduced tumor progression in mice that received siCCR2 nanoparticles (Fig. 2C and Fig. S6). These data indicate that spleen-derived TAMs were recruited via CCR2 and were causally associated with tumor progression. The apparently unaltered splenic-derived TAMs were recruited via CCR2 and were causally associated with tumor progression. The apparently unaltered splenic-derived TAMs were recruited via CCR2 and were causally associated with tumor progression. The apparently unaltered splenic-derived TAMs were recruited via CCR2 and were causally associated with tumor progression. The apparently unaltered splenic-derived TAMs were recruited via CCR2 and were causally associated with tumor progression.

Splenocytic Monocytic and Granulocytic Cells Relocate to Cancer Sites. Based on these results, we attempted to define whether reservoir splenocytes physically relocate to tumor sites. We used a method involving transplantation of an anastomosed spleen and tracking of the cells originating from the transplanted spleen (17) (Fig. 3A). Perfusion of the transplant (Fig. 3B) allowed donor splenocytes to enter the recipient’s bloodstream directly, and thus to relocate elsewhere. Spleens transplanted into tumor-free animals did not trigger a measurable cell release (17) (Fig. 3C). However, spleens transplanted into tumor-bearing KP mice (11 wk after tumor initiation) triggered relocation of numerous cells to tumors. Namely, 1.9 ± 1.2 × 10<sup>5</sup> TANs and 5.1 ± 3.2 × 10<sup>5</sup> TAMs redistributed within 24 h from the donor spleen to the recipient’s tumor stroma. A total of 66 ± 4% and 61 ± 17% of spleen-derived TAMs down-regulated Ly-6C<sup>hi</sup> and up-regulated F4/80, respectively, in line with the initiation of a differentiation program of these cells on recruitment to tissue. This approach did not permit us to assess the relative contribution of spleen-vs. bone marrow-derived cells; however, it showed that within 24 h, the spleen replaced as many as 4% and 8%-10% of the preexisting endogenous TAM and TAN repertoires, respectively (Fig. 3D). Because these repertoires increased, on average, only 2% daily, it is likely that a small fraction of older cells either died locally or exited the tumor stroma, in part, counteracting the contribution of newly arrived splenic cells. Future studies will have to determine whether TAMs and TANs are short-lived or exit the tumor stroma. Either way, these experiments positioned the spleen as a significant contributor to TAM and TAN precursors.

Spleen Produces CD11b<sup>+</sup> Ly-6C<sup>hi</sup> Monocytic and CD11b<sup>+</sup> Ly-6C<sup>lo</sup> Granulocytic Cells Locally During Cancer Progression. The spleen may enhance TAM and TAN responses by controlling the quantity and/or quality of immature myeloid precursor/progenitor cells released to tumors. For example, splenic monocytic and granulocytic cells may acquire unique functions locally by receiving microenvironmental instruction signals, which differ from those in bone marrow. Although this mechanism cannot be formally excluded, a comparative analysis of (CD11b<sup>+</sup> Ly-6C<sup>hi</sup>) monocytic and (CD11b<sup>+</sup> Ly-6C<sup>lo</sup>) granulocytic cells in bone marrow vs. spleen of tumor-bearing hosts (Fig. S7A) and of splenic monocytic and granulocytic cells in tumor-bearing vs. control mice (Fig. S7 B and C) did not reveal notable differences. Phenotypic changes were detected mostly only on cell recruitment to the tumor stroma (Fig. S7C and Table S1).

The spleen may also control TAM and TAN responses quantitatively by supplying new monocytic and granulocytic cells continuously during tumor progression. Continuous support of the spleen, however, requires its constant replenishment with new reservoir cells to compensate for the loss of those that are mobilized. Interestingly, the spleen of tumor-bearing mice contained a significantly larger number of cells in the S/G2 phase of the cell cycle, which indicated active cell proliferation within the organ. The proliferating splenocytes exhibited a CD11b<sup>+</sup> Lin<sup>−</sup> phenotype (Fig. 4A). Thus, cancer induced the accumulation of proliferating splenocytes that were neither monocytic nor granulocytic but could include their lineage progenitors. Accordingly, in vitro cultures of splenocytes from tumor-bearing mice produced more granulocyte/macrophage colonies than from controls (Fig. 4B).

The established paradigm states the following paths of lineage differentiation for monocytes and neutrophils: HSC→CMP (common myeloid progenitor)→GMP→MDP (macrophage/dendritic cell progenitor)→monocyte (24) and HSC→CMP→GMP→neutrophil (10). The presence of these populations was tested by flow cytometry with a combination of markers used for cell phenotyping in bone marrow (10–12). HSCs, CMPs, GMPs, and MDPs increased largely in the spleen (Fig. 4 C and D and Fig. S8A) in comparison to bone marrow (Fig. S8 B and C) in KP mice at 11 wk after tumor initiation. Similar splenic responses were detected in two other graft tumor models (Fig. S8D).

Subsequent experiments were restricted to the analysis of HSCs and GMPs. These studies indicated that their expansion in the spleen of tumor-bearing mice was likely the consequence of both their continuous recruitment and their local proliferation. To analyze recruitment, parabiosis was established between two tumor-bearing mice for 16 d until HSC and progenitor cell equilibrium. The spleens contained a significant fraction of HSCs (28.5 ± 2%) and GMPs (24 ± 4%) that originated from the other parabiont; thus, circulating HSCs and progenitor cells
remained relatively low within the bone marrow as well as the splenic reservoir in tumor-bearing mice. The ability of splenic HSCs and GMPs to produce myeloid progeny was highest when these cells were in the S/G2 phase of the cell cycle, similar to their bone marrow counterparts (Fig. S8). Splenic HSCs and GMPs were functionally active in vitro, because they formed colonies as efficiently as their bone marrow counterparts.

We performed additional experiments to evaluate the fate of GMPs in vivo. Intravital microscopy 5 d after injection of fluorescently tagged GMPs indicated that the cells gave rise to large numbers of colonies in the splenic red pulp parenchyma next to, but outside, collecting venous sinuses (Fig. 5A). Characterization of the cells by flow cytometry identified that GMP descendants were virtually all monocytic or granulocytic (Fig. 5B). The ability of transferred GMPs to produce myeloid progeny was highest within the spleen of KP animals with cancer. The activity remained relatively low within the bone marrow as well as the liver (another extramedullary site that can exhibit myelopoietic activity) even in splenectomized mice (Fig. 5B and Fig. S8F). We made similar observations on adoptive transfer of HSCs instead of GMPs, although, as expected, the appearance of HSC-derived monocytic and granulocytic cells was delayed by several days. Finally, GMPs had a similar fate in a grafted mouse tumor model (Fig. S8G).

Spleenic GMPs Contribute to the TAM and TAN Responses. Tracking of the GMP progeny in the lungs of KP mice also identified that these cells produced tissue macrophages (TAMs) and neutrophils (TANs) (Fig. 5C). The capacity of the transferred GMPs to generate these cells markedly increased in mice with cancer (11 wk after tumor initiation). To test whether the GMP-derived TAMs and TANs required the spleen, we repeated the GMP adoptive experiments (11 wk after tumor initiation); however, we used spleen-sufficient or spleen-deficient mice as recipients this time. Five days after transfer, GMPs failed to produce full TAM and TAN responses in the spleen-deficient mice (Fig. 5C). This result supports the role of splenic GMPs as TAM and TAN progenitors and positions the spleen as an important site for amplification of these cells.

Human Patients with Cancer Expand Splenic Myeloid Progenitor Cells. Mouse and human spleens are physiologically very similar (30), and the spleen of patients with chronic inflammatory disorders is capable of producing leukocytes (31-33). However, whether the extramedullary myeloid response seen in animal models also occurs in human patients has not been addressed. Fresh spleen tissue was collected from seven control patients and seven patients with pathological evidence of invasive cancer. Splenic GMP-like cells were sorted based on the expression of cell surface markers used previously to identify human bone marrow GMPs (11) (i.e., CD117+ CD34+ CD38+ IL-3Ra+) and seven patients with pathological evidence of invasive cancer. Mouse and human spleens are physiologically very similar (30), and the spleen of patients with chronic inflammatory disorders is capable of producing leukocytes (31-33). However, whether the extramedullary myeloid response seen in animal models also occurs in human patients has not been addressed. Fresh spleen tissue was collected from seven control patients and seven patients with pathological evidence of invasive cancer. Splenic GMP-like cells were sorted based on the expression of cell surface markers used previously to identify human bone marrow GMPs (11) (i.e., CD117+ CD34+ CD38+ IL-3Ra+) and seven patients with pathological evidence of invasive cancer. Mouse and human spleens are physiologically very similar (30), and the spleen of patients with chronic inflammatory disorders is capable of producing leukocytes (31-33). However, whether the extramedullary myeloid response seen in animal models also occurs in human patients has not been addressed. Fresh spleen tissue was collected from seven control patients and seven patients with pathological evidence of invasive cancer. Splenic GMP-like cells were sorted based on the expression of cell surface markers used previously to identify human bone marrow GMPs (11) (i.e., CD117+ CD34+ CD38+ IL-3Ra+) and seven patients with pathological evidence of invasive cancer.
GATA binding protein 3, von Willebrand factor, or IL-7 receptor α (IL-7Ra), all of which are involved in developmental lineages disparate from neutrophils and monocytes (Fig. 6B). In vitro, purified splenic GMP-like cells produced colonies and generated cells that were mostly CD14+ CD115+ CD68+ CD11b+/−/ (Fig. S9A). Because we could not detect granulocytes, it is possible that the culture conditions skewed maturation toward the mononuclear phagocyte lineage or that the splenic population was already committed to this lineage (and thus may also qualify as human MDPs).

Patients with invasive cancer had significantly higher numbers of splenic GMPs ex vivo compared with controls (P < 0.05; Fig. 6C), and in vitro cultures of splenocytes of patients with cancer (n = 2) produced higher numbers of granulocyte/macrophage colonies than splenocytes of a control patient (Fig. S9B). Also, a flow cytometry protocol established to detect endogenous human splenic monocytes and neutrophils ex vivo (Fig. S9C) showed increased numbers of these cells in patients with cancer (Fig. 6D). These findings suggest that patients with cancer can mount active GMP responses and produce monocyctic and granulocytic cells locally.

To directly address whether human splenic GMPs produce monocyctic and granulocytic cells in vivo, we purified human splenic progenitor cells obtained ex vivo from a patient with invasive cancer and injected the cells into a nonobese diabetic SCID mouse bearing a lung carcinoma xenograft. Lin, lineage. (F) Table showing spleen weight and total numbers of splenic GMPs and monocyte- and neutrophil-like cells in tumor-bearing mice and in patients with cancer. The fold difference between species is also shown. Data are presented as the mean ± SEM. *P < 0.05.

**Discussion**

Here, we show that the spleen significantly amplifies tumor-promoting TAM responses in KP mice and can do so over time during cancer progression. Our findings in favor of this notion are as follows: (i) the impaired TAM response and tumor growth in spleenectomized animals, (ii) the impaired TAM response and tumor growth on CCR2 silencing in splenic monocyctic cells, (iii) the redistribution of splenic TAM precursors to tumors in the spleen transplant experiments, (iv) the expansion of splenic myeloid progenitors in tumor-bearing mice, and (v) the reduced capacity of these progenitors to produce TAM when transferred into spleen-deficient mice. Future studies should address whether manipulation of the splenic reservoir not only suppresses primary tumor growth but also delays metastasis and increases survival. The functions of splenic-derived TANs also remain to be determined.

The constant “demand” for TAMs is met, at least in part, by active and chronic proliferation of splenic HSCs and progenitor cells, which continuously replenish the reservoir with new monocyctic cells. The latter cells, produced within the spleen, do not necessarily become local resident cells but are capable of relocating to tumor sites. These findings shed light on the mechanisms of immune cell production and relocation in cancer. They also prompt a number of questions, such as the following: (i) "Why does the bone marrow ‘farm out’ the production of TAM (and TAN) precursors?" (ii) "How do cancers induce the extramedullary response?" (iii) "Does a splenectomy in cancer help or hurt?" (iv) "How does chemotherapy affect splenic myeloid cell production?" and (v) "How can the HSC→TAM/TAN axis be modulated to create more efficient anticancer therapies?"

The data presented here agree with the established notion that the bone marrow in the steady state autonomously replaces and maintains the pools of relatively short-lived monocytes and neutrophils (13, 17, 24, 34). However, during chronic inflammation, our findings suggest that the spleen can also continuously contribute Ly-6Chigh monocyctic and Ly-6Chigh granulocytic cells. Others have shown the liver as a site for extramedullary myelopoiesis in cancer (35) and other inflammatory processes (29). It is likely that the outsourcing process occurs when demand for macrophages/neutrophils is high. It may also be that medullary and extramedullary environments differ significantly from one another that they generate cells with divergent functions. Currently, our phenotyping studies suggest that bone marrow- and spleen-derived monocyctic cells are similar. However, it is worth noting that CD11b+ Gr-1+ MDSCs, which often accumulate within the spleen of tumor-bearing animals, exhibit a distinct immunosuppressive phenotype (36). GMP-derived splenic monocyctic and granulocytic cells are also CD11b+ Gr-1+; thus, at least a fraction of bona fide MDSCs likely derive from splenic (CD11b+) GMPs. Future work should identify the splenic signals that instruct HSCs and progenitor cells and their lineage descendants and also whether these signals differ from those in the bone marrow.

The extramedullary hematopoietic response triggered in cancer could be host-controlled and/or tumor-controlled: The loss of splenic cells on mobilization to tumors may activate host-controlled feedback homeostatic mechanisms to replenish the reservoir, although tumors may also actively secrete long-range signals that trigger/amplify the response. Growth factors, such as GM-CSF (37) and the glycoprotein osteopontin (38), have already been implicated as long-range signals controlling hematopoietic cells in the bone marrow. GM-CSF and other factors control the mobilization of bone marrow HSCs to the periphery and should next be investigated for their roles in extramedullary hematopoietic responses. Interestingly, adenocarcinomas driven by oncogenic Kras only, as opposed to KP cancers, fail to evoke a
splenic monocyte/granulocytic response through all stages of tumor progression. Thus, a comparative analysis of Krasfl/fl;G12D+ and KP mice may be useful to identify remote cross-talk instigators.

Although this study has indicated that splenectomy can delay tumor growth in KP mice, the consequences of such a procedure in humans remain unclear. Retrospective population-based studies (39–41) have shown that patients whose spleens were removed because of traumatic rupture do not show a reduced risk for developing cancer. However, the spleen is a multicellular organ, capable of accommodating many major functions for the host, including immune protection against pathogens as well as orchestration of healing responses (17). Thus, although removal of the entire spleen may carry positive effects in some circumstances, it would be preferable to identify specific tumor-promoting activity in the organ, against which therapeutic approaches could be designed.

Many chemotherapeutic drugs not only target tumor cells but eliminate proliferating HSCs in the bone marrow. Because TAMs and TANs derive from HSCs, one could imagine that the drugs act to suppress the tumor-promoting immune response. However, chemotherapeutic drugs can also mobilize bone marrow HSCs to the periphery, resulting in an increase in the circulating HSC repertoire (42). It would therefore be useful to determine whether chemotherapies suppress or enhance tumor-promoting extramedullary hematopoietic responses. With accumulating evidence indicating the immune system as critical for tumorigenesis, an emerging concept for fighting established cancer involves identifying drug combinations that not only kill cancer cells but influence the immune system in such a way as to be favorable to the host (43). In the present study, in view of their tumor-promoting effects, we highlight extramedullary stem and progenitor cells as relevant targets for drug therapy.

Methods

Detailed methods are available in SI Methods. KP mice in a 129 background were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). To induce lung adenocarcinoma, KP mice were injected with an adenovirus expressing Cre recombinase by intranasal instillation (18). Spleen transplants were performed using KP donor and recipient mice 11 wk after AdCre infection.

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Supporting Information

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SI Methods

Animals. Kras<sup>LSL-G12D/+</sup>;<p53<sup>−/−</sup> (referred to as KP) and Kras<sup>LSL-G12D/+</sup> (referred to as K) mice in a 129 background were used for conditional mouse models of non-small cell lung cancer and were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). (ACTB-eYFP)<sup>7ACSNavy</sup> (referred to as EYFP<sup>+</sup>) mice in a 129 background were a gift from Andras Nagy (Mount Sinai Hospital, Toronto, ON, Canada). Agr1a<sup>−/−</sup> B6.129P2-Agrimat1Unc/J (referred to as AT-1) mice (NanoZoomer 2.0RS; Hamamatsu). For histopathological analysis, lung tissue was harvested and tissue sections were treated for 5 min in 1% SDS (Boston Bio-Products) and incubated in RPMI 1640 (GIBCO; Invitrogen) containing 10% FBS (vol/vol) for 4 h at 37 °C. Digested lungs were filtered, and single-cell suspensions were washed and resuspended in PBS with 0.5% BSA. The same procedure was adapted for recovery of cells from stroma of tumor cell lines that were grafted by s.c. injection.

Histology. For histopathological analysis, lung tissue was harvested, fixed in 10% (vol/vol) formalin solution, and embedded in paraffin. Serial paraffin-embedded sections were prepared and then stained with H&E. For immunohistochemistry, all sections used were incubated in 1% hydrogen peroxide solution to block endogenous peroxidase activity before antibody staining. To detect macrophages, tissue sections were deparaffinized and rehydrated for heat-induced epitope retrieval using a retrieval solution (pH 6.0; BD Biosciences) and then incubated with a rat anti-mouse Mac-3 mAb (BD Biosciences). To detect neutrophils, tissue sections were treated for 5 min in 1% SDS (Boston Bio-Products) in PBS and then stained with the rat anti-mouse NIMP-R14 mAb (Santa Cruz Biotechnology). A biotinylated anti-rat IgG antibody (Vector Laboratories) was used to detect primary antibodies, and the Vectastain ABC kit (Vector Laboratories) was used to detect the secondary antibody. Finally, color development was performed with a 3-amin-9-ethylcarbazole substrate (Dako Cytomation) to reveal the staining, and all sections were counterstained with Harris Hematoxylin solution (Sigma-Aldrich). Images of slides were captured and digitized automatically at a magnification of 40× (NanoZoomer 2.0RS; Hamamatsu).

mAbs and Flow Cytometry. The following antibodies were used: phycoerythrin (PE)-conjugated anti-CD90 (clone 53-2.1), PE-conjugated anti-B220 (clone RA3-6B2), PE-conjugated anti-CD49b (clone DX5), PE-conjugated anti-NK1.1 (clone PK136), PE-conjugated anti-Ly-6G (clone 1A8), PE-conjugated anti-CD11c (clone N418), Allophycocyanin (APC)-conjugated anti-CD11b (clone M1/70), APC-Cy7–conjugated anti-CD11b (clone M1/70), PE-Cy7–conjugated anti-F4/80 (clone BM8), Alexa Fluor 700-conjugated anti-CD11c (clone HL3), APC-conjugated anti-CD117 (clone 2B8), APC-Cy7–conjugated anti-CD16/32 (clone 2.4G2), PE-Cy7–conjugated anti-Sc1 (clone D7), Alexa Fluor 700-conjugated anti-CD34 (clone RAM34), FITC-conjugated anti-CD34 (clone RAM34), PE-conjugated anti-CD127 [IL-7 receptor α (IL-7Ra); clone A7R34], FITC-conjugated anti-BrdU, APC-conjugated anti-BrdU, APC-conjugated anti-CD45.1 (clone A20), peridinin chlorophyll protein (PerCP)-Cy5.5–conjugated anti-CD45.2 (clone 104), biotin-conjugated anti-CD115 (clone AFS98), and PerCP-conjugated streptavidin (all from BD Biosciences); PE-conjugated anti-CD19 (clone 1D3), PE-conjugated anti-CD11b (clone M1/70), APC-conjugated anti-mouse TNF-α, and FITC-conjugated anti-mouse CD62L (clone MEL-14) (all from BD Pharmingen); FITC-conjugated anti-mouse Granzyme B (clone 16G6; eBioscience); and APC-conjugated anti-mouse IFN-γ (R&D Systems). For progenitor staining, the lineage (Lin<sup>−</sup>) antibody mix included the following PE-conjugated antibodies: anti-CD90, anti-B220, anti-CD19, anti-CD49b, anti-NK1.1, anti-Ly-6G, anti-CD11b, anti-CD11c, and anti-CD127. For cell labeling, single-cell suspensions were labeled for 30 min at 4 °C with appropriate antibodies in PBS supplemented with 1% FBS. For cell cycle analysis, FxCycle violet stain (Invitrogen) was used. The number of monocytes/macrophages, dendritic cells, neutrophils, progenitors, and other cells was defined as the total number of cells per organ multiplied by the percentage of each cell type identified by flow cytometry (LSRII; BD Biosciences). Data were analyzed with FlowJo v.8.8.7 (Tree Star, Inc.). For adoptive transfer experiments, cell suspensions obtained from spleen or bone marrow were stained with appropriate antibodies; cells of interest were sorted (FACS Aria; BD Biosciences) and injected i.v. without delay. Typically, 6.5 × 10<sup>6</sup> GMPs or HSCs were injected. For cytokine detection, cells were stimulated in RPMI + 10% FBS (vol/vol) for 4 h at 37 °C with 0.05 μM or 0.1 μM phorbol 12-myristate 13-acetone (BD Biosciences). Golgi-plug (1 μL/mL; BD Biosciences) was added after 1 h into the reaction.

Surgical Removal of the Spleen. Skin covering the spleen was shaved and cleansed with 70% ethyl alcohol (vol/vol). A splenectomy was performed under isoflurane anesthesia by making an incision in the abdominal left hypochondrium. The spleen was gently prodded away from the connective tissue, while cauterizing associated vessels. The incision was thereafter sutured.

Spleen Transplantation. Spleen transplants were performed using KP donor and recipient mice 11 wk after AdCre infection. The surgical procedure of spleen transplantation and the enumeration of cells deployed from the donor spleen to distant tissue were performed as previously described (2). We did not have syngeneic KP mice expressing different allelic markers, which could be used for tracking donor cells in the recipient. Instead, donor splenocytes were “tagged” by administering BrdU into the donor mice before transplantation (daily injections of 1 mg of BrdU in the peritoneal cavity for 3 d). BrdU was incorporated into the DNA during proliferation, and control experiments using flow cytometry indicated that 60% of splenic monocytes and neutrophils became BrdU<sup>hi</sup>. To define the number of cells that migrated from the spleen to the lung, the number of BrdU<sup>hi</sup> TAMs and TANs (these cells originated from the donor spleen) were counted, and this number was multiplied by 1.67 to correct for the fact that not all splenic monocytes and neutrophils were...
To assess the

For parabiosis experiments, weight-matched WT and + %GFP CD45.2 + %GFP cells and C57BL/6 mice or GMP cells were adoptively trans-

quanti

vein catheter to increase vascular contrast. Tumor size was expressed as a nanomolar measure. For CT imaging, Isovue-370 (Bracco Diagnostics) was infused i.v. at 55 L/min through a tail

modal imaging cartridge containing fiducial markers detectable by FMT and CT (4, 5). For FMT imaging, mice received 2 nmol of ProSense-680 as described above. Mice were shaved before imaging to reduce the attenuation effects of fur on the optical signal and anesthetized with iso- fluorane during imaging. A 3D FMT dataset was reconstructed, where fluorescence per voxel was expressed as a nanomolar measure. For CT imaging, Isovue-370 (Bracco Diagnostics) was infused i.v. at 55 μL/min through a tail vein catheter to increase vascular contrast. Tumor size was quantified using OsiriX imaging software (OsiriX Foundation).

Noninvasive Evaluation of Tumor Burden by High-Resolution CT. Mice infected with AdCre, and splenectomized or not, were anonym-

ized and subjected to high-resolution CT at different time points. For imaging, mice were anesthetized with isoflurane gas inhalation and had a respiratory pillow placed underneath the abdomen to provide gating of X-ray exposures on expiration of the respiratory cycle (to prevent motion artifacts). The CT data were acquired on an Inveon PET-CT system (50-μm isotropic spatial resolution; Siemens) and analyzed blindly by a radiologist. Multiplanar and 3D reconstructions were obtained using OsiriX and AMIRA (Visage Imaging, Inc.).

Intravitral Microscopy. EYFP+ GMP cells were adoptively transferred and visualized in the spleen 5 d later using an Olympus FV1000 confocal laser scanning system on an upright BX61-W1 microscope (Olympus) running Fluoview 1000 version 2.1 software (Olympus). For imaging, the mice were placed onto a temperature-regulated heating plate (Olympus Corporation) set to 37 °C. The spleen was exposed surgically and kept moist during imaging as previously described (2), and it was imaged with an Olympus objective (0.5x N.A.) with a magnification of 20x. EYFP+ cells and blood vessels (labeled with AngioSense-680; VisEn Medical–PerkinElmer) were visualized through excitation at 488 nm and 635 nm, respectively, also as previously described (2).

Parabiosis. For parabiosis experiments, weight-matched WT and GFP C57BL/6 mice (or CD45.1 and CD45.2 C57BL/6 mice or WT 129 and EYFP+ mice) were used. Mice were anesthetized with isoflurane and joined by a technique adapted from Bunster et al. (6). Mice were then surgically separated by a reversal of the procedure. The percentage of chimerism for each studied cell population was calculated as %GFP/(%GFP+ %GFP) in C57BL/6 mice and as %GFP/(%GFP+ %GFP) in GFP mice.

Detection of Protease Activity by Flow Cytometry. To assess the proteolytic activity of monocytes and neutrophils in the spleen and lung, mice were injected i.v. with 2 nmol of ProSense-680 (VisEn Medical–PerkinElmer) as a cathepsin activatable probe (3). Mice were killed 24 h after administration of the agent and analyzed by flow cytometry as described above.

Fluorescent Mediated Tomography/CT Fusion Imaging. Fluorescent-mediated tomography (FMT)/CT fusion imaging was done on an FMT-2500 instrument (VisEn Medical–PerkinElmer) as described previously (3). Mice were placed on a highly purified reduced-manganese diet (Harlan) to reduce autofluorescence caused by normal mouse chow diet when imaging. For efficient image fusion and reconstruction, mice were placed in a multimodal imaging cartridge containing fiducial markers detectable by FMT and CT (4, 5). For FMT imaging, mice received 2 nmol of ProSense-680 as described above. Mice were shaved before imaging to reduce the attenuation effects of fur on the optical signal and anesthetized with isoflurane during imaging. A 3D FMT dataset was reconstructed, where fluorescence per voxel was expressed as a nanomolar measure. For CT imaging, Isovue-370 (Bracco Diagnostics) was infused i.v. at 55 μL/min through a tail vein catheter to increase vascular contrast. Tumor size was quantified using OsiriX imaging software (OsiriX Foundation).

Human Spleen Processing and Flow Cytometry. De-identified human spleen samples were obtained from the Massachusetts General Hospital Department of Pathology following an approved institutional review board protocol. Specimens were processed under Biosafety Level 2 conditions in compliance with the Harvard Medical School Committee on Microbiological Safety. All samples were processed identically, and patients were subsequently divided into two groups based on pathological and/or clinical findings. Group 1 included samples from patients with no evidence of malignant cancer (e.g., biopsied patients with benign pathological findings, patients who required a splenectomy following a physical trauma), and group 2 included patients with invasive cancer (e.g., pancreatic ductal adenocarcinoma, colon carcinoma). Spleen sections weighing 1–3 g were cut into small pieces and incubated in RPMI 1640 containing 20 units/mL hyalurondiase (Sigma–Aldrich), 41.6 units/mL collagenase type XI (Sigma–Aldrich), 150 units/mL collagenase type I (Sigma–Aldrich), DNase I (Sigma–Aldrich), 6.67 μL/mL of Heps Buffer (Mediatech), and 305.43 μL/mL of PBS without calcium or magnesium (Lonza) for 1 h at 37 °C. Digested spleens were filtered, and cells were washed in RPMI and resuspended in red blood cell lysis buffer for 2 min. For flow cytometry analysis, cell suspensions were washed and fixed/permeabilized following the BD Cytofix/Cytoperp (BD Biosciences) protocol for intracel-

ular antibody labeling. The following mAbs were used: FITC-conjugated antibodies specific for lineage markers anti-CD3e (clone ucht1) and NKP46 (clone 195314) (both from R&D Systems); anti-CD15 (clone h98), anti-CD19 (clone hib9), and anti-CD56 (clone mem188) (all from BD Biosciences); PE-conjugated anti-CD11c (clone b-ly6); anti-CD68 (clone y1/82a); PerCP-conjugated anti-CD14 (clone mpf9); APC-conjugated anti-CCR2 (clone 48607); anti-GLD1 (clone 1243); PE-Cy7-conjugated anti-CD16 (clone 5c8); APC-conjugated anti-CD11b (clone icrf44); biotinylated anti-CD120a (clone mbtnfr1-b1); and anti-CD115 (clone 12-3a3-b10). For analysis and sorting of myeloid progenitors, cells were stained with PE-C5–conjugated antibodies specific for the following lineage markers: CD2 (clone RPA-2.10), CD11b (clone ICRF-44), CD20 (clone 2H7), and CD36 (clone B159) (all from BD Pharmingen); CD3 (clone S4), CD4 (clone S3.5), CD8 (clone 3B5), and CD19 (clone SJ25-C1) (all from Invitrogen); CD7 (clone CD7-687) and CD10 (clone 5-1B4) (both from Biolegend); and CD14 (clone TU.K.4) (from eBioscience). The cells were also stained with FITC-conjugated anti-CD45RA (clone MEM56; Abcam), PE-conjugated anti-IL-3RA (clone 9F5; Biolegend), biotinylated anti-CD38 (clone HIT2; Biolegend), PE–Cy–conjugated anti-CD117 (clone A3C6E2; Biolegend), and APC-conjugated anti-CD34 (clone HPCA-2; Biolegend). Alexa Fluor 700 conjugated to streptavidin (Invitrogen) was used for visualization of biotinylated antibodies.

Gene Expression Profile Signature of Human Progenitor Cells. RT-PCR analysis on spleen-derived human progenitors of a represen-

PCR analysis on spleen-derived human progenitors of a representative set of genes expressed in progenitor cells was performed as described by Manz et al. (7). Briefly, human progenitor popu-

ations were isolated by FACs from cell suspensions of human spleens. Approximately 1,000 cells were directly sorted into 350 μL of RNeasy Lysis Buffer (RLT) (Qiagen) supplemented with 1% 2-mercaptoethanol. RNA was extracted using the RNAeasy micro Kit (Qiagen). The entire sample was used for cDNA generation using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed with prede-

signed and validated primer sets (Applied Biosystems) (Table S1).
siCCR2 or control (siCON; siRNA targeting luciferase as a control) per injection (8). Animals were killed 1 day after the last injection.

**Statistics.** Results were expressed as the median or mean ± SEM. Statistical tests included an unpaired two-tailed Student t test and one-way ANOVA, followed by Tukey’s multiple comparison test (for more than 2 groups). Otherwise, for data not normally distributed, we applied the nonparametric Kruskal–Wallis test, followed by Dunn’s multiple comparison test. P values of 0.05 or less were considered to denote significance.


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**Fig. S1.** Gating scheme for identification of CD11b+ and CD11b− macrophages and of neutrophils in mouse lungs by flow cytometry. (Insets) Gated neutrophils are Ly-6G+, whereas CD11b+ macrophages are F4/80+. FSC, Forward Scatter; SSC, Side Scatter.

**Fig. S2.** Effect of splenectomy on the TAM and TAN responses in grafted EL-4 and CT26 tumors. EL-4 T-cell lymphoma (1 × 10^6 cells) and CT26 colon carcinoma (1 × 10^6 cells) cell lines were injected s.c. into C57BL/6 mice and BALB/c mice, respectively, from which the spleen was either kept (+SP) or removed (−SP). The data show total counts of TAMs and TANs in tumors on day 14 after implantation of EL-4 tumors (n = 4 for each condition) and on day 7 after implantation of CT26 tumors (n = 7 for each condition). Data shown in the graph are representative of two independent experiments. Data are presented as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

**Fig. S3.** Quantification of Cathepsin B activity in diverse cell populations obtained ex vivo from tumor-bearing KP mice. Single-cell suspensions were either obtained from the lung (Upper) or spleen (Lower). Monocytes were defined as CD11b+ Lin− Ly-6G− (F4/80/CD11c/I-Ab)Lo Ly-6C+. Neutrophils were defined as CD11b+ Gr-1+Ly-6G+. Lineage (Lin) refers to the following combination: B220/DX5/NK1.1/CD90.2/Ly6G. Experiments used KP mice at week 11 after tumor initiation (n = 4). Data shown in the graph are representative of two independent experiments. Data are presented as the mean ± SEM. MFI, mean fluorescent intensity.

* Cortez-Retamozo et al.  www.pnas.org/cgi/content/short/1113744109
Fig. S4. Histological identification of tumor burden in the lungs of KP mice. The images show H&E staining of lungs obtained from multiple animals, which belong to five different cohorts. The five cohorts are as follows: (i) +AdCre: positive controls, KP animals infected with AdCre at week 0 and analyzed at week 8 (n = 4); (ii) −AdCre: negative controls, KP animals not infected with AdCre (n = 3); (iii) +AdCre: positive controls, KP animals infected with AdCre at week 0 and analyzed at week 11 (n = 8); (iv) +AdCre−SP (wk 0): KP animals splenectomized and infected with AdCre at week 0 and analyzed at week 11 (n = 8); and (v) +AdCre−SP (wk 8): KP animals infected with AdCre at week 0, splenectomized at week 8, and analyzed at week 11 (n = 4). Scale bar: 5 mm.
Splenectomy in KP mice has an impact on TAM and TAN responses but does not improve cytotoxic CD8 T-cell responses or suppress regulatory CD4 T-cell responses. (A) Accumulation of TAMs and TANs in mice shortly after splenectomy. KP mice were infected with AdCre to induce lung tumors; 11 wk later, they were either splenectomized (−SP, n = 4) or sham operated (+SP, n = 4). All animals received BrdU i.p. and were killed 24 h later for analysis by flow cytometry. Histograms show the total number of BrdU+ TAMs and BrdU+ TANs in the lungs. (B and C) Number of blood monocytes in KP mice shortly after splenectomy. (B) Number of blood monocytes in control mice (ø, n = 4), KP mice 11 wk after tumor initiation (KP +SP, n = 4), and KP mice 11 wk after tumor initiation and shortly after splenectomy (KP −SP, n = 4). (C) Number of BrdU+ blood monocytes in KP mice 11 wk after tumor initiation; on week 11, the KP mice were either sham operated (KP +SP, n = 4) or splenectomized (KP −SP, n = 4). Animals received BrdU i.p. and were killed 24 h later for analysis by flow cytometry (same as for A). (D) Number of bone marrow monocytes and neutrophils in KP mice with or without a spleen during tumor development. Monocytes (Left) and neutrophils (Right) are shown in the bone marrow of KP animals that were splenectomized (−SP, n = 19) or not splenectomized (+SP, n = 17) at the time of tumor initiation (week 0). Mice were killed on week 11. (E) Total number of CD8+ T cells retrieved in the lungs of control mice (−AdCre, n = 8), mice infected with AdCre (+AdCre, n = 8), and mice infected and splenectomized at week 0 (+AdCre −SP wk 0, n = 8). (F) Determination of the production of IFN-γ (IFN-g) and Granzyme b (GZMB) by flow cytometry on CD8 T cells in the animal cohorts described in E. The graphs show the mean fluorescent intensity (MFI) as a measure of cytokine production. (G) Total number of FoxP3+ CD4+ regulatory T cells retrieved in the lungs of control mice (−AdCre, n = 8), mice infected with AdCre (+AdCre, n = 8), and mice infected and splenectomized at week 0 (+AdCre −SP wk 0, n = 8). (H) Lung weight as a measure of tumor burden. Lung weight correlates positively with the number of tumors detected by high-resolution CT in animals analyzed at week 11 after AdCre infection. Data shown are representative of two independent experiments. Data are presented as the mean ± SEM. (I) Relationship between tumor burden (here, based on lung weight; Fig. S6) and number of TAMs and TANs on week 11. Mice with a spleen (+AdCre +SP, brown diamonds, n = 17), mice splenectomized on week 0 (+AdCre −SP, blue diamonds, n = 19), and mice splenectomized on week 8 (+AdCre −SP, green diamonds, n = 3) are shown. Dotted lines represent maximal lung weight and macrophage/neutrophil numbers observed in age-matched animals without cancer (n = 12).

CCR2-dependent tumor growth. KP mice were infected with AdCre to induce lung tumors; 12 wk later, they received nanoparticles containing either siCCR2 (CCR2-silencing siRNA) or siCON (control nanoparticles), as described by Leuschner et al. (8). KP mice received nanoparticle injections every 4 d for 12 d. (A) Weight of lungs from tumor-free mice (Control) or tumor-bearing mice treated with siCCR2 (n = 4) or siCON (n = 4). (B) Representative pictures of lungs from mice treated with siCON or siCCR2. Scale bar: 5 mm.
Fig. S7. Phenotypic characterization of splenic HSCs, GMPs, monocytes, and neutrophils. (A) Comparison of splenic and bone marrow cells isolated from KP mice (+AdCre, week 11, n = 3). The markers characterize different progenitor populations (CD117, Sca-1, CD16/32, CD34, and CD115) or monocytes and neutrophils (CD11b, Ly-6C, F4/80, CD11c, and CD62L). Lineage-positive cells (shown in gray) were investigated in parallel as internal controls. The populations analyzed did not show notable phenotypic differences. (B) Comparison of splenic cells (monocytes and neutrophils) in tumor-free (−AdCre, n = 3) and tumor-bearing (+AdCre, week 11, n = 3) mice. The markers were chosen to provide information on enzymatic activity, phagocytic capacity, cytokine production, and activation state. For the determination of enzymatic activity, a pan-cathepsin activatable probe (Prosense-680) was injected 24 h before flow cytometry analysis. In a similar fashion, phagocytosis was determined by injection of a fluorescently labeled nanoparticle (CLIO-VT680; CLIO nanoparticles are synthesized in house and are not commercially available, the particles are conjugated to the dye VT 680 purchased from Visen Medical/Perkin Elmer) 24 h before analysis. For intracellular detection of TNF-α production, splenic cell suspensions were restimulated for 4 h with phorbol 12-myristate 13-acetone. Lineage-positive cells (shown in gray) were investigated in parallel as internal controls. We did not observe notable differences between the two cohorts of mice. (C) Further comparison of splenic monocytes in tumor-free (group 1) and tumor-bearing (group 2) mice and of TAMs (group 3) (n = 4 for each group). The nine genes were chosen based on their reported role in monocyte/macrophage function and/or differentiation. Relative gene expression was measured by Taqman real-time PCR and normalized to 18s rRNA. Analysis of the gene expression profile showed that the presence of tumors did not significantly alter the phenotype of monocytes in the spleen (except for IFN-γ (IFN-g)) but that splenic monocytes differ from TAMs. Data shown in the graph are representative of two independent experiments. Data are presented as the mean ± SEM. ***P < 0.001.
Fig. S8. Quantification of HSC and progenitor responses. (A) Quantification of CMPs and MDPs in the spleen of tumor-bearing (+AdCre) KP animals (n = 17) in comparison to age-matched tumor-free mice (−AdCre, n = 12) on week 11. (B) Quantification of HSCs, CMPs, GMPs, and MDPs in the bone marrow of tumor-bearing (+AdCre) KP animals (n = 17) in comparison to age-matched tumor-free mice (−AdCre, n = 12) on week 11. (C) Fold increase in the total number of hematopoietic progenitor cells (HSCs, CMPs, GMPs, and MDPs) in the bone marrow and spleen of KP mice infected with AdCre (KP +AdCre) at week 11. The data are compared with those in syngeneic tumor-free control mice. (D) Fold increase accumulation of GMPs in bone marrow and spleen in three tumor mouse models. (Left to Right) KP mice infected with AdCre (KP +AdCre, n = 12) analyzed at week 11, C57BL/6 mice grafted with EL-4 tumors (B6 +EL-4, n = 10) analyzed at day 14, and BALB/c mice grafted with CT26 tumors (BALB/c +CT26, n = 4) analyzed at day 8. The data are compared with those on syngeneic tumor-free mice. (E) Percentage of HSCs and GMPs in the S/G2 phase in the bone marrow and spleen of tumor-bearing KP mice at week 11 (n = 4). (F) Total numbers of HSCs (Left), GMPs (Center), and the progeny of adoptively transferred GMPs (Right) in the liver of tumor-free control mice and mice infected with AdCre and from which the spleen was either retained (+AdCre +Spleen, wk 0) or not retained (-AdCre +Spleen, wk 0). Analyses were performed 11 wk after AdCre infection. (G) Fate mapping of GMPs transferred into C57BL/6 mice grafted with EL-4 tumors (+EL-4, n = 10) and tumor-free mice (−EL-4, n = 6). (Left) Experimental procedure. (Right) Quantification of GMP-derived monocytes and neutrophils in the bone marrow (BM) and spleen (SP). Data shown in the graph are representative of four independent experiments (A–D) or two independent experiments (E–G). Data are presented as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

Fig. S9. Spleen of human patients with invasive cancer accumulates myeloid progenitor cells and lineage descendants. (A) (Upper) Photograph of a colony derived from human GMP-like cells. Colonies were harvested, and the cells were analyzed by H&E staining on cytospins (Lower Left) and by flow cytometry (Lower Right) (n = 3). Scale bars: Top, 50 μm; Bottom, 10 μm. Data are presented as the mean ± SD. (B) Total number of GM-CFU colonies estimated to derive from the spleen of a control patient and of two patients with invasive cancer. (C) (Upper) Identification by flow cytometry of monocytes (red) and neutrophils (green) in human spleen samples. (Lower) Histograms show the expression of eight cell surface markers for the same cells. FSC, Forward Scatter; SSC, Side Scatter.
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