Cancer progression is associated with alterations of intra- and extramedullary hematopoiesis to support a systemic tumor-promoting myeloid response. However, the functional specialty, mechanism, and clinical relevance of extramedullary hematopoiesis (EMH) remain unclear. Here we showed that the heightened splenic myelopoiesis in tumor-bearing hosts was not only characterized by the accumulation of myeloid precursors, but also associated with profound functional alterations of splenic early hematopoietic stem/progenitor cells (HSPCs). With the distinct capability to produce and respond to granulocyte-macrophage colony-stimulating factor (GM-CSF), these splenic HSPCs were “primed” and committed to generating immunosuppressive myeloid cells. Mechanistically, the CCL2-CCR2 axis-dependent recruitment and the subsequent local education by the splenic stroma were critical for eliciting this splenic HSPC response. Selective abrogation of this splenic EMH was sufficient to synergistically enhance the therapeutic efficacy of immune checkpoint blockade. Clinically, patients with different types of solid tumors exhibited increased splenic HSPC levels associated with poor survival. These findings reveal a unique and important role of splenic hematopoiesis in the tumor-associated myelopoiesis.
Spleen Mediates A Distinct Hematopoietic Progenitor Response
Supporting Tumor-Promoting Myelopoiesis

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ABSTRACT

Cancer progression is associated with alterations of intra- and extramedullary hematopoiesis to support a systemic tumor-promoting myeloid response. However, the functional specialty, mechanism, and clinical relevance of extramedullary hematopoiesis (EMH) remain unclear. Here we showed that the heightened splenic myelopoiesis in tumor-bearing hosts was not only characterized by the accumulation of myeloid precursors, but also associated with profound functional alterations of splenic early hematopoietic stem/progenitor cells (HSPCs). With the distinct capability to produce and respond to granulocyte-macrophage colony-stimulating factor (GM-CSF), these splenic HSPCs were “primed” and committed to generating immunosuppressive myeloid cells. Mechanistically, the CCL2-CCR2 axis-dependent recruitment and the subsequent local education by the splenic stroma were critical for eliciting this splenic HSPC response. Selective abrogation of this splenic EMH was sufficient to synergistically enhance the therapeutic efficacy of immune checkpoint blockade. Clinically, patients with different types of solid tumors exhibited increased splenic HSPC levels associated with poor survival. These findings reveal a unique and important role of splenic hematopoiesis in the tumor-associated myelopoiesis.
INTRODUCTION

Cancer is associated with a profound myeloid response resulting in the expansion of tumor-associated myeloid cells, including myeloid-derived suppressor cells (MDSCs), neutrophils, and macrophages, to promote disease progression (1–3). These myeloid cells modulate adaptive immune responses against tumors (3–5), enhance cancer cell stemness (6, 7), and create conditions that support angiogenesis, invasion, and metastasis (2, 3, 8–10). However, these cells are generally short-lived (11) and must be continuously replenished throughout the progression of cancer. Therefore, to guarantee a sufficient pool of myeloid cells, cancer interferes with hematopoietic stem and progenitor cell (HSC and HPC, or HSPC combined) activity and reroutes host’s hematopoiesis to generate cells of myeloid lineages with profound tumor-promoting functions (12, 13). These findings consist with clinical observations that the progression of different types of solid tumors is associated with an increased peripheral neutrophil-to-lymphocyte ratio (14, 15) and an elevated level of circulating granulocyte-macrophage progenitors (GMPs) (16). Thus, defining the nature and characteristics of the altered hematopoiesis in cancer is crucial for understanding the systemic tumor-promoting myeloid response.

In the steady state, the postnatal development of blood cell lineages primarily occurs in bone marrow (BM). However, recent studies have revealed that tumor-derived factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (17–19), granulocyte colony-stimulating factor (G-CSF) (12), and peptide hormone Angiotensin II (20), mediate systemic deviation of hematopoiesis not only in the BM but also in extramedullary tissues such as the spleen (12, 20–22). Yet, it is unclear whether different sites of hematopoiesis are established for distinct purposes. In particular, little is known about the functional differences between intra- and extramedullary HSPCs, and between the myeloid cells derived from the BM and spleen of tumor-bearing hosts (22). Also, it remains to be addressed whether and how the
spleen could selectively accumulate HSPC subsets in a population- and organ-specific manner, and what roles the local environmental factors play in regulating the functional properties of splenic HSPCs. Identifying the specific mechanisms regulating the tumor-induced splenic extramedullary hematopoiesis (EMH) might provide a novel strategy for anticancer therapy.

In the present study, we investigated the function, mechanism, and clinical relevance of cancer-induced splenic EMH. We found that splenic EMH is not a mere quantitative supplement to BM hematopoiesis in tumor-bearing hosts; in contrast, it exerts unique and significant function by selectively amplifying a specific HSPC response committed to generating immunosuppressive myeloid cells.
RESULTS

Myeloid-biased splenic early HSPCs in tumor-bearing mice

Hepatocellular carcinoma (HCC) represents a type of cancers that are associated with negligible/low levels of systemic myeloid differentiation cytokines but evident extramedullary HSPC activities (16). To investigate the cancer-associated EMH in such tumor settings, we used an orthotopic Hepa1-6 hepatoma mouse model (hereafter referred to as Hepa mice) which reproduces many features of human HCC (23, 24). In this model, the spleen was the major site of EMH (Supplemental Figure 1, A and B). With slight splenomegaly, the spleens of Hepa mice accumulated both lineage\textsuperscript{lin}\textsuperscript{low/−}Sca-1\textsuperscript{−}c-Kit\textsuperscript{high} (LSK) and line\textsuperscript{lin}\textsuperscript{low/−}Sca-1\textsuperscript{−}c-Kit\textsuperscript{high} (LK) HSPCs in a progression-dependent manner (Supplemental Figure 1, C–E) and at levels comparable to other transplanted or genetic mouse tumor models (Supplemental Figure 1, F and G).

To describe and compare the cancer-associated hematopoietic alterations in BM and spleen, we initially performed competitive reconstitution experiments. BM from Hepa mice reconstituted myeloid cells and lymphocytes at levels similar to its control counterpart (Figure 1A, left). In contrast, the transfer of splenocytes from Hepa mice led to a rapid and more robust reconstitution compared to control spleen, with a myelopoiesis preference (Figure 1A, right). The fold changes of myeloid reconstitution peaked at two weeks after transplantation showing six-fold greater chimerisms compared to control spleen, and gradually decreased afterwards. In accordance with the boost of myeloid cell reconstitution, colony-forming units (CFUs) assays exhibited a fifteen-fold increase in CFU-granulocyte/macrophage (CFU-GM) in the spleens of Hepa mice (Figure 1B), whereas the enrichment of multi-lineage CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) was insignificant. These findings suggested that the spleens of Hepa mice accommodated a myeloid-biased hematopoiesis facilitating rapid myeloid immune cell generation.
Heightened splenic myelopoiesis in cancer has been linked to the accumulation of multiple HSPC populations (13, 20, 21), but the functional preference of early HSPCs, the LSK cells, in the spleen of tumor-bearing host remains unclear. These LSK cells are highly heterogeneous, containing various HSC and HPC subpopulations with different lineage potential (25–27). Although BM and splenic LSK cells from Hepa mice differentiated into FcγR\textsubscript{low}CD34\textsuperscript{+} common myeloid progenitors (CMPs) and FcγR\textsubscript{high}CD34\textsuperscript{+} GMPs at similar kinetics in vitro (Supplemental Figure 1, H and I), LSK cells from the spleen produced markedly fewer trilineage spleen colony-forming units in vivo (CFU-S\textsubscript{12}; Figure 1C), suggesting a reduced proportion of multipotent HSCs (28). To corroborate the differentiation potential of splenic LSK cells at the single-cell level, we adopted a two-step colony-forming assay (29). The daughter cells from >85% single BM LSK cells simultaneously formed both GM-type and MegE-type colonies, indicating that most BM LSK cells were multipotent (Figure 1D). In stark contrast, the daughter cells from >70% single splenic LSK cells formed GM-type colonies only, suggesting that the majority of splenic LSK cells were myeloid immune cell-restricted. These findings indicate that the heightened splenic myelopoiesis in tumor-bearing mice is not only characterized by the accumulation of HSPCs, but also associated with a significant myeloid skew within the LSK population.

**Accumulation of GM-CSF-expressing LSK cells in the spleens of tumor-bearing mice**

Emerging evidence has suggested that the biased lineage potential of LSK cells may be associated with their altered cytokine production capacity (30). Along this line, we found that a significantly higher proportion of splenic LSK cells from Hepa mice expressed GM-CSF, an important myeloid differentiation cytokine (Figure 2A). The enhanced GM-CSF expression was associated with up-regulated NF-κB activation and down-regulation of p38 MAPK (Figure 2B). These GM-CSF-expressing HSPCs were commonly present in the spleens of tumor-bearing mice, including in another hepatoma model induced by N-nitrosodiethylamine (DEN)
and CCl₄, a transplantable melanoma model, and an intestinal neoplasia model caused by the Apc gene mutation (Supplemental Figure 2A). However, these cells were rarely detected in the BM, in the control spleen (Figure 2A), or in an EMH model induced by repeated bleeding (Supplemental Figure 2A). Moreover, the level of GM-CSF was not increased in the serum or in the splenic microenvironment of Hepa mice (Supplemental Figure 2, B–E) and another important cytokine G-CSF was also not increased in this setting (Supplemental Figure 2F). These findings suggested that the endogenous GM-CSF signal might be functionally significant for splenic LSK HSPC differentiation.

To confirm the function of the endogenous GM-CSF, we found that neutralizing antibodies (Abs) against GM-CSF impaired the CFU-GM activity (with SCF, IL-3, IL-6, and erythropoietin in the methylcellulose-based culture) of splenic, but not BM, LSK cells (Figure 2C). In addition, neutralizing the endogenous GM-CSF in an essential HSPC culture condition (serum-free medium supplemented with SCF only) attenuated splenic LSK cells’ proliferation and differentiation into myeloid cells in a dose-dependent manner (Figure 2D). These data suggested an important role of this endogenous signal in mediating LSK cell myelopoiesis in both simple and complex cytokine environments. Moreover, the presence of anti–GM-CSF Abs in the culture also attenuated the intracellular GM-CSF expression of these splenic LSK cells, suggesting a positive feedback of this endogenous GM-CSF signal (Figure 2E). Together, these findings indicate that a significant fraction of splenic LSK cells upregulate the GM-CSF expression potentiating their myeloid differentiation, which may represent a distinct stress-induced HSPC response in tumor-bearing mice.

**Endogenous GM-CSF drives splenic HSPCs to generate myeloid suppressors**

We next investigated whether and how this splenic HSPC response contributed to the immunosuppressive function of tumor-associated myeloid cells. To evaluate the overall effect of the splenic EMH, we first applied splenectomy. Splenectomy did not change the frequencies
or the distribution of tumor myeloid cells (Supplemental Figure 3, A and B). However, it did abolish the suppressive activity of tumor CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sub>low</sub> polymorphonuclear (PMN)-like cells (or tumor PMN-MDSCs; Figure 3, A and B), which was the major suppressive MDSC subset in Hepa tumors (Supplemental Figure 3, C and D). The abrogation of the splenic EMH by splenectomy impaired the suppressive function of tumor-infiltrating PMN-like cells on the proliferation and the antigen-induced cytotoxic activity of T cells (Figure 3, A and B). We observed that splenectomy suppressed the expression of arginase 1 (Arg1) in tumor-infiltrating PMN-like cells (Supplemental Figure 3, E–G), which was associated with the inhibition of STAT3 activation (Supplemental Figure 3G). In addition, splenectomy increased the frequencies of tumor-infiltrating IFNγ<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) by two-fold (Figure 3C). These findings suggested an important role of the splenic EMH in mediating the suppressive myeloid response.

GM-CSF is a critical regulator of MDSC generation and activation (8, 19). However, it has been established that de novo induction of a naïve progenitor/precursor to generate fully functional MDSCs requires at least two signals (31, 32). GM-CSF alone failed to induce BM progenitors from Hepa mice to produce functional CD11b<sup>+</sup>Gr-1<sup>+</sup> MDSCs in vitro (Supplemental Figure 3, H and I), consistent with a previous report (17). In contrast, GM-CSF effectively induced splenic LSK and GMP cells to generate myeloid cells with potent immunosuppressive activity both in serum-free and complete media (Supplemental Figure 3, H and I). These data suggested that the splenic HSPCs from Hepa mice, but not their BM phenotypic counterparts, included “primed” progenitors of myeloid suppressor cells. Based on this finding, we tested whether the endogenous GM-CSF produced by LSK cells was sufficient for the generation of MDSCs. As expected, splenic LSK cells from Hepa mice generated highly suppressive myeloid descendants without exogenous supplementation with GM-CSF, which could be completely prevented by the inclusion of anti–GM-CSF Abs in the culture (Figure 3,
D and E). Consistently, similar results were obtained using splenic LSK cells isolated from mice with DEN and CCl₄–induced hepatoma and Apc\(^{\text{Min/+}}\) mice (Supplemental Figure 3, J and K).

Given the distinct capability of splenic HSPCs to produce and respond to GM-CSF, we speculated that these “primed” progenitors could readily generate myeloid suppressors in vivo, even independent of the presence of tumors. To test this hypothesis, we transferred CD45.1\(^-\) splenic or BM LSK cells from Hepa mice into the spleens of naïve CD45.1\(^+\) recipients. Five days after the adoptive transfer, donor cells were isolated and tested for their immunosuppressive activity (Figure 3F). Consistent with above in vitro findings, only the CD45.1\(^-\)CD11b\(^+\)Gr-1\(^+\) myeloid descendant cells of splenic LSK cells, but not those BM-derived, could effectively inhibit anti-CD3/28 stimulated proliferation and antigen-induced cytotoxic activity of T cells (Figure 3, G–I). These ex vivo data, together with the findings of the endogenous GM-CSF expression and effects of neutralizing Ab, indicate that the splenic HSPCs in Hepa mice include “primed” progenitors of potent myeloid suppressors, which can readily respond to the endogenous GM-CSF signal and support the suppressive myeloid response.

**Spleens of tumor-bearing mice recruit BM-derived circulating HSPCs**

To investigate the underlying mechanism regulating the distinct properties of splenic LSK cells in Hepa mice, we first determined the cellular origin of these splenic HSPCs. The splenic HSPC population could be originated from the BM and/or the clonal expansion of local residents. To find out the original source(s) of these accumulated HSPCs, we generated tumor-bearing parabiotic pairs by joining wild-type B6 and EGFP\(^+\) mice (Supplemental Figure 4A). After four weeks of parabiosis, the tumor dramatically induced partner-derived (EGFP\(^+\)) HSPCs accumulation in the spleens of B6 mice (Supplemental Figure 4, B and C), suggesting circulating HSPCs as an important income. Importantly, removal of the spleen of the EGFP\(^+\)
partner barely altered the number, composition, and CFU-C activity of the partner-derived splenic HSPCs, excluding that re-circulating splenic resident population was the major source. In addition, the spleens of tumor-bearing mice showed two-fold stronger capacity for recruiting circulating BM-derived CFU-GM progenitors (Supplemental Figure 4, D and E). Thus, these results suggested that the majority of splenic HSPCs in Hepa mice were derived from the circulating population that originated from BM.

**Splenic niche modulates LSK HSPC functional properties**

Since the majority of splenic HSPCs are BM-derived, we sought to investigate the mechanism(s) accounting for their distinct functions from their counterparts in BM. We reasoned that this difference could possibly result from the local “education” by the cancer-conditioned splenic niche, and/or the selective recruitment of circulating HSPCs.

We first tested the “local education” hypothesis by co-culturing naïve BM LSK HSPCs with BM or splenic stromal cells isolated from control or Hepa mice (Figure 4A). The results showed that only the splenic stromal cells from Hepa mice could induce an increase in the CFU-GM activity (Figure 4B) and the endogenous GM-CSF expression (Figure 4C) of BM-derived naïve LSK cells. To determine whether splenic stromal cells could alter the capability of these naïve early HSPCs in vivo, we adoptively transferred naïve BM LSK cells into the spleens of control or Hepa mice (Figure 4D). Splenic stroma of Hepa mice, but not that of control mice, induced these early progenitors to produce highly suppressive myeloid descendants in vivo (Figure 4E). These data indicate that cancer-conditioned splenic niche is capable of “educating” naïve LSK cells to generate myeloid immune suppressors.

This “education” effect was retained in the transwell culture system (Figure 4, F–H), suggesting that this effect was mediated by soluble factor(s). We found that IL-6 was up-regulated in the splenic stromal cells of Hepa mice (Supplemental Figure 4, F and G). The inclusion of anti–IL-6 neutralizing Abs in the transwell assays attenuated the CFU-GM activity
(Figure 4F) and the GM-CSF expression (Figure 4G) of LSK cells cultured with Hepa splenic stromal cells, and impaired the suppressive function of their myeloid descendants (Figure 4H). In support, recombinant mouse IL-6 induced the GM-CSF expression in LSK cells (Supplemental Figure 4H). Together, these data demonstrate that splenic stromal cells of tumor-bearing mice can induce LSK cell functional alteration via soluble factors such as IL-6.

**Upregulation of CCL2-CCR2 signaling in the spleen**

Having revealing the role of the “local education”, we next investigated whether a selective recruitment mechanism might also contribute to this splenic HSPC response. Interestingly, we observed higher chimerisms in both splenic LSK and LK cells in Hepa tumor-bearing parabiotic pairs (Supplemental Figure 5A), suggesting a faster turnover of splenic HSPCs which contradicted with their accumulation. To further define the kinetics of HSPC turnover in the spleen, we surgically separated pairs of mice after four weeks of parabiosis. After separation, splenic HSPC chimerisms in tumor-free pairs were unchanged during the 96-hr observation, whereas the chimerisms in tumor-bearing mice significantly decreased (Supplemental Figure 5B). These results indicate a cancer-driven increase in splenic HSPC turnover, possibly due to their faster differentiation since the apoptotic rates of splenic HSPCs were similar between control and Hepa mice (Supplemental Figure 5, C–E). Thus, we reasoned that the accumulation of splenic HSPCs in Hepa mice might rely on the cancer-endowed stronger attraction for BM-derived circulating progenitor cells (Supplemental Figure 4E), which outstrips the accelerated HSPC consumption.

To probe the mechanism involved in the recruitment of circulating HSPCs into spleen, we examined the expression profiles of cytokines and chemokines (Supplemental Figure 6A). CXCR4 ligand CXCL12 (also known as stromal cell-derived factor 1) is not only a critical mediator for HSPC recruitment and retention in BM, but also mediates splenic EMH induced by myeloablation, blood loss, or pregnancy (33). We were surprised to find that CXCL12 was
down-regulated in the spleens of tumor-bearing mice (Figure 5A and Supplemental Figure 6B). In contrast, CCR2 ligand CCL2, along with CXCR2 ligands CXCL2 and CXCL5, was among the most up-regulated chemokines in the spleens of Hepa mice (Figure 5B and Supplemental Figure 6C). Since CXCR2 was not expressed on BM or splenic HSPCs (Supplemental Figure 6D), we focused on the CCL2-CCR2 axis in our study. To determine the potential source(s) of splenic CCL2, we analyzed its production in different splenic cell populations. Splenic stromal cells, especially VE-cadherin+ endothelial cells, markedly increased CCL2 production in Hepa mice (Figure 5C and Supplemental Figure 6E). We then analyzed the expression of the corresponding chemokine receptor CCR2 on HSPCs. A remarkably larger proportion of splenic LSK HSPCs from Hepa mice expressed CCR2, compared to those from BM or control spleen (Figure 5D).

**Role of CCL2-CCR2 axis in splenic recruitment of myeloid HSPCs**

To investigate the role of CCL2-CCR2 axis in mediating splenic recruitment of circulating myeloid progenitors, we measured CFU-GM homing. RS 504393, a highly selective CCR2 antagonist, effectively reduced the splenic recruitment of circulating CFU-GM progenitors in tumor-bearing mice to the basal level (Figure 6A). To corroborate the direct action of CCL2 on HSPCs, we transferred CCR2-deficient (EGFP-) and CCR2-competent (EGFP+) BM cells simultaneously into control (Group #1 and #3) or tumor-bearing (Group #2 and #4) recipients (Figure 6B). Irrespective of the CCR2 expression status in recipient mice, the ratios of CCR2-/EGFP- versus CCR2+/EGFP+ CFU-GM progenitors homing to the spleens of tumor-bearing hosts were at least three-fold lower, compared to the ratios of input and that in control spleens (Figure 6C).

Next, we tracked CCR2-deficient and CCR2-competent HSPCs in parabiotic pairs with or without Hepa tumors (Figure 6D). The proportion of CCR2-competent partner-derived (CD45.2+) splenic HSPCs was significantly increased in the tumor-bearing pairs. As expected,
this substantial elevation in the partner-derived HSPC proportion was abrogated if partner-derived circulating progenitor cells lacked CCR2 expression (Figure 6E). Similarly, the tumor-elicited splenic accumulation of LSK and GMP cells was attenuated in CCR2<sup>−/−</sup> Hepa mice, while little change was observed in the BM (Figure 6F). These results indicate that CCL2-CCR2 interaction directly mediates the splenic recruitment of circulating HSPCs with myeloid potential in tumor-bearing hosts. More importantly, the remaining splenic LSK cells in CCR2-deficient Hepa mice exhibited markedly decreased levels of endogenous GM-CSF expression (Figure 6G), suggesting that the “education” by the spleen required the peripheral CCR2<sup>+</sup> LSK subset (34).

Accordingly, lack of CCR2 expression on HSPCs recapitulated the effect of splenectomy on the tumor-promoting myeloid response. The absence of the endogenous GM-CSF-driven splenic EMH impaired the suppressive activity of tumor PMN-like cells in CCR2-deficient Hepa mice (Figure 6H and Supplemental Figure 6F), which allowed an increase in the number of tumor-infiltrating IFNγ<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> CTLs (Figure 6I). Although knock-out of CCR2 also reduced the tumor-infiltrating CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>high</sup> monocytic cells (Supplemental Figure 6G), this was supposed to be a minor effect compared to the functional alteration of PMN-like cells, given the relatively smaller number and less suppressive ability of these cells in Hepa model (Supplemental Figure 3, A–D). Thus, these findings indicate that the recruitment of HSPCs via the CCL2-CCR2 axis, followed by the splenic “education”, is an integral and essential process for inducing the distinct splenic EMH promoting the systemic myeloid response.

**Abrogation of splenic EMH synergistically enhances anti–PD-L1 therapy**

Based on the above findings that the abrogation of splenic EMH enhanced antitumor immunity (Figure 3), we expected that it might potentially promote immunotherapy. Hepa mice were treated with splenectomy or sham-surgery, and sacrificed when the tumor reached to a diameter
of approximately 1.5 cm. In the general setting of Hepa mice, the impact of splenectomy on mouse survival was mild but statistically significant (Figure 7A). The effect of splenectomy was more robust when the initiating tumor cells were reduced (Supplemental Figure 7A). This agrees with previous reports suggesting that the impact of splenectomy on cancer progression was dependent on the tumor settings in animal models and is conditional in patients (21, 35-37). We noticed that splenectomy did not decrease the expression of PD-L1 on tumor PMN-like cells (Supplemental Figure 3F), which might suggest an opportunity of combined therapy with anti–PD-L1 (38, 39). Monotherapy of anti–PD-L1 treatment did not affect the number or the phenotype of splenic HSPCs (Supplemental Figure 7, B-D), and only elicited marginal therapeutic effects in Hepa mice (Figure 7A) and in another hepatoma mouse model based on a BALB/c background (Supplemental Figure 7, E and F). However, splenectomy synergistically enhanced the therapeutic efficacy of anti–PD-L1 treatment (Figure 7A and Supplemental Figure 7F). Complete regression of established cancer was achieved in over 50% of Hepa mice. These results suggest a potential role of targeting splenic EMH in enhancing immunotherapy.

In order to preserve the normal physiological functions of the spleen while inhibiting splenic EMH, we tested whether selectively targeting splenic HSPCs could also synergize with immunotherapies. Low-dose sorafenib, a c-Kit inhibitor (40), induced splenic HSPC apoptosis (Supplemental Figure 7G), inhibited their proliferation (Supplemental Figure 7H), and reduced the numbers of HSPCs in the spleens but had much less effect on those in the BM (Figure 7B and Supplemental Figure 7I). Moreover, low-dose sorafenib treatment attenuated the endogenous GM-CSF expression in the splenic LSK cells (Figure 7C). Consistent with splenectomy, low-dose sorafenib inhibited the suppressive functions of tumor PMN-MDSCs on T cell proliferation and cytotoxic activity (Figure 7, D and E), increased the frequencies of infiltrating IFNγ+CD3+CD8+ CTLs (Figure 7F), and recapitulated the synergistic effect when
combined with PD-L1 blockade (Figure 7G). Importantly, as shown in Figure 7G, this synergistic effect was abrogated by restoring the splenic lin<sup>low</sup>c-Kit<sup>high</sup> population including LSK and downstream myeloid progenitor cells which are capable of generating myeloid suppressor cells (Supplemental Figure 3, H and I), but not by transferring splenic MDSCs possibly due to their short life-span (11). These data demonstrate the important role of splenic EMH in impeding anti-tumor immunity and immunotherapy. Similarly, anti–PD-L1 treatment elicited profound therapeutic responses in CCR2-deficient Hepa mice (Figure 7H). Together, these findings demonstrate that targeted therapies against splenic EMH can effectively synergize with immune checkpoint blockade, and thus, may represent a novel strategy to enhance present immunotherapy.

**Splenic EMH in patients with different types of solid tumors**

Having established the function, mechanism, and significance of tumor-induced splenic EMH in mouse models, we sought to assess the clinical relevance of splenic EMH. CD133 is a marker for human HSPCs (41). We confirmed that splenic CD133<sup>+</sup> cells had a linCD34<sup>high</sup> phenotype (Supplemental Figure 8A), and were highly enriched for CFU-GM activity (Supplemental Figure 8B). We examined the frequencies of splenic HSPCs in situ by staining for CD133 in formalin-fixed, paraffin-embedded spleen tissues from 135 patients with different types of solid tumors and 34 non-cancer patients (Supplemental Table 1). The frequencies of splenic CD133<sup>+</sup> cells in cancer patients were significantly higher than those in non-cancer patients (Figure 8A and 8B; see also Supplemental Figure 8C). The densities of CD11b<sup>+</sup> myeloid cells were also markedly increased in the spleens of cancer patients (Figure 8A and 8C), which significantly correlated with the splenic CD133<sup>+</sup> HSPC levels (Figure 8D).

Finally, we evaluated the clinical significance of cancer-induced splenic accumulation of HSPCs. Kaplan-Meier analyses revealed that the high frequency of CD133<sup>+</sup> progenitors in the spleen negatively impacted the overall survival of patients with gastric cancer (\(P = 0.0145, HR\))
Patients with lower splenic CD133+ cell frequencies had a significantly longer survival (median: 29 months) than patients with higher splenic CD133+ cell frequencies (median: 18 months). This analysis on patients with other types of tumor was not applicable yet, either due to an insufficient sample size or because the majority of the patients were recently diagnosed at early stage (Supplemental Table 1). Overall, in line with our findings in mouse models, the clinical data presented so far suggest the functional and clinical importance of splenic EMH in cancer patients.
DISCUSSION

Cancer is associated with deviations of host hematopoiesis in both BM and extramedullary niches (20, 21, 42). The present study indicates that the spleen, as a major site of cancer-induced EMH, actively recruits HSPCs via the CCL2-CCR2 signaling pathway, and supports their endogenous GM-CSF-driven differentiation into potent myeloid suppressor cells. These findings demonstrate that the spleen could serve as a distinct niche for “priming” myeloid HSPCs to fuel the tumor-promoting myeloid response.

Extramedullary niches of hematopoiesis can be established to exert critical influence to alleviate (43, 44) or deteriorate (34, 45–48) pathological conditions. However, our knowledge about the nature and properties of the extramedullary HSPCs is very limited, and the necessity and advantages to outsource hematopoiesis, especially under chronic pathological circumstances, remain unclear. In cancer, the splenic EMH is generally perceived as a supplementary mechanism serving to fulfill the increased demand of myeloid cells (22). Thus far, the relative contribution of splenic EMH, compared to BM myelopoiesis, to the systemic tumor-associated myeloid response seems dependent on model set-ups. This EMH has been attributed for the generation of the majority of tumor-associated macrophages in a genetic mouse model of lung adenocarcinoma (20, 21, 49), while represents a quantitatively minor source of tumor-infiltrating myeloid cells in some other models (42, 50) including our Hepa mice. Apart from the comparison on production capacity, we herein provide evidence that cancer-induced splenic myelopoiesis functionally and mechanistically differs from its BM counterpart and plays a significant role in the tumor-promoting myeloid response. In tumor-bearing mice, splenic LSK HSPCs, selectively recruited via the CCL2-CCR2 axis, are “educated” by the splenic niche to express higher levels of GM-CSF, acquire heightened proliferative activity, and become committed to generating potent immunosuppressive myeloid cells. Therapeutically, targeted abrogation of splenic EMH alone (without affecting the BM) is
sufficient to synergistically enhance the therapeutic efficacy of immune checkpoint blockade, suggesting an important role for this EMH in tipping the balance of tumor immune responses. Taken together, our findings provide novel insights into the functional specialty of splenic hematopoiesis in cancer.

HSPCs are not only able to sense stress signals, but are also capable of converting environmental cues into versatile cytokine signals to regulate hematopoiesis in an autocrine/paracrine manner (30). The present study reveals a positive feedback mechanism of endogenous GM-CSF in splenic LSK HSPCs, which primes their myeloid differentiation and fully exploits their profound potential for generating myeloid suppressors. The regulations of HSPC activity and MDSC generation have long been attributed to systemic signals derived from tumors (12, 18–20, 31, 32). Complementary to these findings, the present study suggests an alternative mode of regulation, in which one crucial endogenous signal from “primed” progenitor cells could be required and sufficient. However, this “priming” process is tightly regulated, as recruiting circulating HSPCs via the CCL2-CCR2 axis and niche signals provided by the splenic stromal cells are both essential preconditions for eliciting such a HSPC response. These findings may help to elucidate the mechanisms regulating divergent HSPC activities in different sites of hematopoiesis.

An effective and durable anti-tumor immune response requires an intact “cancer-immunity cycle”, which allows a series of immune events to initiate, proceed and expand iteratively (51). In some patients, monotherapy approaches are sufficient to restore and re-initiate such a self-sustaining cycle of cancer immunity, whereas others may require combined therapies (38, 39, 52–54). Although the impact of splenectomy on cancer progression has been shown to be generally limited in the clinic (35, 37), the beneficial effect is more robust in a subset of patients with higher levels of myelopoiesis (36). In combination with immunotherapies such as adoptive transfer of tumor antigen-specific CTLs (42) or immune checkpoint blockade (the present
study), abrogation of splenic myelopoiesis exhibits promising effect in enhancing the therapeutic anti-tumor immune responses. Moreover, by ways of targeted inhibitor or blockade of CCL2-CCR2 signaling, intervening splenic EMH while preserving other physiological functions of the spleen can be equally effective and may be more appropriate for clinical practice. These findings indicate the important role of splenic EMH in tumor immunity, suggesting that selective abrogation of this process may create conditions potentiating anticancer immunotherapy.

Although splenic EMH in cancer patients has been suggested to be analogous to that in tumor-bearing mice (21), its clinical relevance remains unclear (22, 35, 37), largely due to sample restriction and technical limitation. The present study demonstrates that CD133 could serve as a useful marker for splenic HSPCs in humans. Using this marker, our findings unveil the general existence and clinical relevance of cancer-associated splenic EMH in patients. Importantly, higher splenic CD133+ cell frequencies are associated with poorer patient prognosis at least in gastric cancer. Still, further investigation into the relationship between splenic EMH and the immunosuppressive systemic (macro-) and tumor (micro-) environment is warranted. In addition, clinical data from larger patient cohorts and more types of cancers are required to further address the exact extent of involvement of splenic EMH in tumor immunity, which will be an area of significant interest for future studies.

In summary, our work describes previously unrecognized features of cancer-induced splenic EMH, and suggests its unique and important role in both mouse models and cancer patients. Our findings propose a two-step model for the distinct cancer-induced splenic HSPC response: 1) the CCR2-dependent recruitment of HSPCs; followed by 2) the local induction of endogenous GM-CSF signaling, which drives HSPC myeloid commitment and differentiation into potent immunosuppressive myeloid cells. Identifying these unique signals and mechanisms
that instruct splenic EMH may provide a novel strategy for cancer therapy, by targeting the
tumor-promoting myeloid response at its source.
METHODS

Animals. All mice were maintained under specific pathogen-free conditions in the animal facilities of Sun Yat-sen University Cancer Center (Guangzhou, China). Details of mice are provided in Supplemental Methods.

Tumor models and treatments. Orthotopic hepatic tumor was established by a subcapsular intrahepatic injection of $7.5 \times 10^5$ Hepa1-6 tumor cells (unless indicated otherwise) suspended in 25 μl of 50% Basement Membrane Extract (Trevigen) into the left lobe of livers of anesthetized B6 mice (6–8 weeks of age). Mice were sacrificed when the tumor reached to a diameter of approximately 1.5 cm or show any sign of agony. Details of other mouse models used in this study are provided in Supplemental Methods. For therapeutic anti–PD-L1 treatment, 200 μg anti–PD-L1 (clone 10F.9G2; Bio X Cell) or corresponding IgG2b isotype control (Bio X Cell) in 100 μl PBS was administered intraperitoneally to mice since 2 weeks after tumor cell transplantation, for a total of four times with intervals of three days. For sorafenib treatment, the mice received two doses of 20 mg/kg sorafenib (Nexavar, Bayer) or vehicle (100 μl sterile water) by oral gavage with an interval of three days. For CCR2 antagonist treatment, mice received 2 mg/kg RS 504393 (Sigma-Aldrich) or vehicle (1% DMSO in PBS) by oral gavage 3 hr before the adoptive cell transfer.

Patients. Spleen samples were obtained from patients who underwent a splenectomy at the Sun Yat-sen University Cancer Center or The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). None of the patients had received anticancer therapy prior to the sampling, and no splenic metastasis was observed. Individuals with concurrent autoimmune disease, HIV, or syphilis were excluded. Clinical stages were classified according to the guidelines of the
International Union against Cancer. Details of patient information are provided in Supplemental Table 1 and Supplemental Methods.

**Flow cytometry.** Flow cytometry was performed as previously described (55, 56). Details of the antibodies used for flow cytometry are provided in the Supplemental Table 2. The procedures of staining are described in Supplemental Methods. Data were acquired on a Gallios or a Cytoflex S flow cytometer (Beckman Coulter), and analyzed with Kaluza Analysis (Beckman Coulter) and FlowJo Software (Tree Star).

**Colony-forming unit assays.** CFU-S\textsubscript{12} assays were performed as previously described (57, 58). Briefly, isolated LSK cells from the BM or spleen were intravenously transferred into lethally irradiated recipient mice. After 12 days, the spleens of the recipients were fixed in Bouin’s solution (Sigma-Aldrich), and the colonies were counted. CFU-C assays were carried out using complete methylcellulose-based medium (MethoCult GF M3434 or H4034, Stem Cell Technologies), following the manufacturer’s instructions. In some experiments, anti–mouse GM-CSF (3 μg/ml; eBioscience) or corresponding isotype control Ab was added to the medium. Colony numbers and morphology were assessed after 12–14 days of culture. For quantification, BM CFU activity was estimated by calculating the colonies in the BM of one femur and one tibia per mouse. A two-step culture assay was also performed to examine the multilineage differentiation capability of single HSPCs. Single LSKs or GMPs were deposited into wells of 96-well plates containing 100 μl of Serum-Free Expansion Medium (StemSpan SFEM, Stem Cell Technologies) supplemented with SCF (50 ng/ml) and TPO (10 ng/ml). After 4.5 days of culture, single cell–derived daughter cells were transferred into methylcellulose-based medium (MethoCult GF M3434; containing SCF, EPO, IL-3, and IL-6) supplemented with TPO (10 ng/ml).
ng/ml). Secondary colonies were counted after 10 days. All cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere.

**Immunohistochemistry.** Paraffin-embedded samples were cut into 3 to 4-μm sections, which were processed for immunohistochemistry as previously described (55, 56) with minor modifications. The detailed procedures are provided in Supplemental Methods. Slides were imaged using Vectra Intelligent Analysis System (PerkinElmer). Quantification of positive signals and evaluation of spleen red and white pulp areas were performed with InForm 2.1 (PerkinElmer). For human CD133⁺ cell quantification, evaluation of CD133⁺ cells was performed by two independent observers who were blinded to the diagnosis and clinical outcome.

**HSPC culture.** HSPCs were isolated by FACS and cultured in Serum-Free Expansion Medium (StemSpan SFEM, Stem Cell Technologies) supplemented with indicated recombinant cytokines and/or antibodies, unless indicated otherwise. Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. Details of cell culture are provided in Supplemental Methods.

**T cell proliferation assay.** Naïve splenocytes were labeled with 2 μm CFSE (Molecular Probes) and cultured in RPMI 1640 supplemented with 10% FBS, 20 U/ml recombinant IL-2 (eBioscience), 1 μg/ml anti-CD3 (eBioscience) and 5 μg/ml anti-CD28 (eBioscience). Freshly isolated myeloid cells or Gr-1⁺ cells derived from cultured HSPCs were incubated with the splenocytes at the indicated ratios. After 72 hr of co-culture, CFSE dilution was assessed and splenocyte proliferation was analyzed. Within each experiment, all test groups were internally controlled and compared with the “splenocyte alone” control group (shown as shaded in histograms). Division index is the average number of cell divisions that a cell in the original
population has undergone, which was calculated using FlowJo and normalized to “splenocyte alone” group in each experiment.

**T cell cytotoxicity assay.** The CTL assay we performed was based on a mixed-leukocyte peptide culture as previously described (17, 59). The culture was set up with $6 \times 10^5$ irradiated B6 splenocytes pulsed with 1 μg/ml OVA257-264 peptide as feeder cells and incubated together with $1.2 \times 10^4$ OVA-specific CD8$^+$ T effectors isolated from the spleens of OT-I mice. $2.5 \times 10^4$ freshly isolated myeloid cells or Gr-1$^+$ cells derived from cultured HSPCs were added as a third component in the culture, except for the effector alone (Eff alone) control group. After five days, cultures were tested for the ability to lyse the OVA257-264 peptide-pulsed Hepa 1-6 cells in a non-radioactive cytotoxicity assay measuring lactate dehydrogenase release (Promega). The percentage of specific lysis was calculated from triplicate samples as follows: 

\[
\frac{\text{experimental OD}_{490} - \text{spontaneous OD}_{490}}{\text{maximal OD}_{490} - \text{spontaneous OD}_{490}} \times 100\%.
\]

**Homing assays.** BM cells from donor mice were intravenously transferred into lethally irradiated recipient mice. After 16 hr, the splenocytes of recipients were harvested. Homing efficiency was evaluated by calculating the percentage of injected CFU-GM that was retrieved in the recipients’ spleens.

**Statistics.** All statistical tests were performed as two-sided. For data normally distributed, we applied the Student’s $t$ test; and the nonparametric exact Wilcoxon signed-rank test was used to compare data not normally distributed. For multiple comparisons (including multiple two-group comparisons shown in the same panel), a one-way or two-way ANOVA (for parametric data) followed by the Bonferroni’s correction (only two groups were compared), the Dunnett's test
(all groups were compared to one control group), or the Tukey’s multiple comparison test (all groups were compared to each other); or a Kruskal-Wallis test (for nonparametric data) followed by the Dunn’s multiple comparison test was applied. Cumulative survival time was estimated by the Kaplan-Meier method, and the log-rank test was applied to compare the groups. \( P \) values less than 0.05 were considered statistically significant.

**Study approval.** All animal experiments were performed according to state guidelines and approved by the IACUC of Sun Yat-sen University Cancer Center (Guangzhou, China). For experiments using human samples, all samples were anonymously coded in accordance with the local ethical guidelines (as stipulated by the Declaration of Helsinki). Written informed consents were obtained from the patients, and the protocol was approved by the IRB of Sun Yat-sen University Cancer Center (Guangzhou, China).
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AUTHOR CONTRIBUTIONS

CW, HN, ML, and LZ designed experiments. CW, HN, ML, JLin, SL, WZ, and JR performed experiments, analyzed and interpreted the data. JX, WCW, JLiang, CKS, BW, JC, and MSC provided mice, clinical resources, and technical support. LZ supported and supervised the research. CW and LZ wrote the manuscript, and all authors contributed to manuscript editing.
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Figure 1. The myeloid-biased differentiation of splenic early HSPCs from Hepa mice. (A) Analysis of peripheral blood myeloid cell (CD11b+Gr-1+) and lymphocyte (CD3+ T and B220+ B cells) reconstitution chimerisms in recipients. (BM, N = 7 mice per group; spleen, N = 9 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001 (two-way ANOVA, corrected by Bonferroni’s method). (B) Quantification of CFU-C activities in the BM and spleens from control (N = 7) or Hepa (N = 8) mice. ***P < 0.001 (Student’s t-test). BFU-E: burst-forming unit-erythroid; CFU-GM: colony-forming unit-granulocyte/macrophage; CFU-GEMM: colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte. (C) CFU-S_{12} activity of 500 BM-(recipient: N = 15) or spleen-derived (recipient: N = 21) LSK cells from Hepa mice. ***P < 0.001 (Student’s t-test). (D) The scheme of the two-step single-cell colony-forming assay, representative colonies, and the percentage of different types of lineage read-outs of the secondary CFU-C. Scale bar, 500 μm. The numbers above columns represent the sample size of initiating single cells in each group. MegE: megakaryocyte/erythrocyte; GM: granulocyte/macrophage. Result are shown as mean and s.e.m. of mice in each group (A–C). Data are from two experiments (A,B), or from three experiments with cells pooled from 6–10 mice (C,D).
Figure 2. Accumulation of GM-CSF-expressing LSK cells in the spleens of tumor-bearing mice. (A) The endogenous GM-CSF expression in freshly isolated LSK and GMP (FcyRI/CD34+ LK) cells (N = 6 per group). ***(P < 0.001 (two-way ANOVA followed by Dunnett's test). (B) Immunoblot for NF-kappab p65 and MAPK p38 activation in LSK cells isolated from BM and spleens of Hepa mice. p: phosphorylated; t: total. (C) The clonogenic ability of 500 BM or splenic LSK cells isolated from Hepa mice (N = 6 per group) in the methylcellulose-based assay. aGM-CSF: 3μg/ml. ***(P < 0.001 (two-way ANOVA followed by Dunnett's test). BFU-E: burst-forming unit-erythroid; CFU-GM: colony-forming unit-granulocyte/macrophage; CFU-GEMM: colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte. (D) LSK cells were CFSE-stained, and cultured in serum-free medium for five days, supplemented with SCF and indicated concentration of anti–GM-CSF Abs in the cultures. The proliferation and differentiation HSPCs into myeloid cells was shown by CFSE dilution. (E) The GM-CSF expression in LSK cells was examined after 24 hr of cultures described in (D). Numbers in flow cytometric plots indicate the proportions of gated cells (A,D,E). Result are shown as mean and s.e.m. of mice in each group (A,C). Data are from two experiments (A), from three experiments with cells pooled from 6–10 mice (C), or representative of at least three experiments (B,D,E).
Figure 3. Endogenous GM-CSF drives splenic HSPCs to produce myeloid suppressor cells. (A and B) The immunosuppressive activity of tumor-infiltrating CD11b⁺Ly6G⁺Ly6C⁻ (PMN-like) myeloid cells on anti-CD3/28 stimulated T cell proliferation (A) and on the cytotoxic activity of CD8⁺ T cells elicited by antigen-specific stimulation in vitro (B). Hepa mice were subjected to a sham surgery (Sham) or splenectomy (SPx). **P < 0.01; ***P < 0.001 (two-way ANOVA, corrected by Bonferroni’s method). (C) Frequencies of tumor-infiltrating IFNγ⁺CD3⁺CD8⁺ CTLs in Hepa mice subjected to a sham surgery or splenectomy. ***P < 0.001 (Student’s t-test). (D and E) Splenic LSK cells were cultured in serum-free medium with SCF for four days. The Gr-1⁺ myeloid descendants were isolated and tested for their suppressive function on anti-CD3/28 stimulated T cell proliferation at the ratio of 1:4 (D), and on the antigen-induced cytotoxic activity of CD8⁺ T cells (E). ***P < 0.001 (two-way ANOVA, corrected by Bonferroni’s method). (F) Cartoon depicting the adoptive transfer of CD45.1⁻ BM or splenic LSK cells into the spleens of CD45.1⁺ tumor-free recipients. (G–I) Representative phenotype (G) and the suppressive activity (H and I) of donor-derived CD11b⁺Gr-1⁺ myeloid cells retrieved from the spleens of recipient mice (N = 3 per group) as described in (F). **P < 0.01, ***P < 0.001 (two-way ANOVA corrected by Bonferroni’s method). Result are shown as mean and s.e.m. (A–C,E,H,I). CFSE⁺ splenocyte proliferation when co-cultured with myeloid descendant cells at indicated ratios (solid lines) or cultured alone (shaded) is shown (A,D,H). Numbers in flow cytometric plots indicate the proportions of gated cells (A,D,G,H). Data are representative of two experiments (A–C, N = 3 per group in each experiment), or from three experiments with cells pooled from 6–10 mice (D–I).
Figure 4. Splenic stroma of tumor-bearing host supports LSK cells to produce MDSCs. (A) Cartoon depicting the scheme of the co-culture experiments. (B and C) The CFU-C activity (B) and the endogenous GM-CSF expression (C) of BM naïve LSK cells after co-cultured with CD45- stromal cells from indicated tissues. ***P < 0.001 (two-way ANOVA followed by Dunnett's test). (D) Cartoon depicting the adoptive transfer of CD45.1 naïve BM LSK cells into the spleens of CD45.1 tumor-free or tumor-bearing recipients. (E) The suppressive activity of donor-derived CD11b+Gr-1+ myeloid cells retrieved from the spleens of recipient mice as described in (D). ***P < 0.001 (two-way ANOVA corrected by Bonferroni's method). (F and G) The CFU-C activity (F) and the endogenous GM-CSF expression (G) of BM naïve LSK cells after co-cultured with splenic stromal cells from Hepa mice in transwells, with indicated Abs (1 μg/ml) in the cultures. ***P < 0.001 (two-way ANOVA corrected by Bonferroni's method). (H) After four days of co-culture in transwells as described in (F and G), lin- HSPCs were isolated and transferred into serum-free medium supplemented with SCF only. After another 3 days of culture, the Gr-1+ myeloid descendants were isolated and tested for their suppressive activity. CFSE+ splenocytes proliferation when co-cultured with myeloid descendant cells at indicated ratios (solid lines) or cultured alone (shaded) is shown (E, H). Numbers in flow cytometric plots indicate the proportions of gated cells (C, E, G, H). Data are shown as mean and s.e.m. of three experiments with cells pooled from 6–8 mice (B, E, F), or representative of three experiments with cells pooled from 6–8 mice (C, G, H).
Figure 5. The enhanced CCL2-CCR2 signaling in the spleens of Hepa mice. (A and B) Splenic Cxcl12 (A) and Ccl2 (B) mRNA expression levels. Values are relative to the Actb mRNA expression ($N = 6$ per group). **$P < 0.01$; ***$P < 0.001$ (one-way ANOVA followed by Dunnett's test). (C) CCL2 release was measured in the supernatants of 48-hr cultures of indicated cell populations isolated from the spleens of control or Hepa mice ($N = 3$ per group; evaluated in triplicates), by ELISA. ***$P < 0.001$ (two-way ANOVA followed by Bonferroni's test). (D) Flow cytometric analysis of the surface CCR2 expression on BM and splenic LSK and GMP cells from control ($N = 7$) and Hepa mice ($N = 6$). Numbers in cytometric plots indicate the proportions of gated cells. ***$P < 0.001$ (two-way ANOVA followed by Tukey's test). Data are representative of at least two experiments and presented as mean and s.e.m. of mice in each group (A–D).
Figure 6. CCL2-CCR2 axis mediates the enhanced splenic recruitment of circulating HSPCs in Hepa mice. (A) Percentages of transferred BM-derived CFU-GM progenitors homing to the spleens of control or Hepa mice. **P < 0.01; ***P < 0.001; ns, not significant (one-way ANOVA followed by Tukey's test). (B) Scheme of competitive homing assays. (C) Ratios of EGFP-CCR2−/− vs. EGFP+CCR2+/+ BM-derived CFU-GM progenitors homing to the spleens of control or Hepa mice (N = 8 for CCR2+/+ recipient groups; N = 4 for CCR2−/− recipient groups) as described in (B). ***P < 0.001; ns, not significant (one-way ANOVA followed by Tukey's test). (D) Cartoon depicting the generation of control or Hepa-bearing parabiotic pairs. i.h., intrahepatic. (E) Quantification of CD45.2+ partner-derived splenic LSK and GMP cells from parabiotic mice pairs described in (D), by flow cytometry. ***P < 0.001 (two-way ANOVA followed by Tukey's test). (F) Quantification of BM (left) and splenic (right) LSK and GMP cells in CCR2+/+ or CCR2−/− Hepa mice. ***P < 0.001 (Student’s t-test). (G) The GM-CSF expression in splenic LSK cells from CCR2+/+ or CCR2−/− Hepa mice was measured by flow cytometry. *P < 0.05 (Student’s t-test). (H) The immunosuppressive activity of tumor-infiltrating CD11b+Ly6G+Ly6C− PMN-like cells. **P < 0.01; ***P < 0.001 (two-way ANOVA, corrected by Bonferroni’s method). (I) Frequencies of tumor-infiltrating IFNγ+CD3+CD8+ CTLs in CCR2+/+ and CCR2−/− Hepa mice. **P < 0.01 (Student’s t-test). Data are representative of two experiments and presented as mean and s.e.m. (A–E: N = 3 per group in each experiment; F: N = 4 for the CCR2+/+ group, N = 5 for the CCR2−/− group in each experiment; G–I: N = 3 per group in each experiment).
Figure 7. Abrogation of splenic EMH synergistically enhances anti–PD-L1 efficacy. (A) Survival of Hepa mice subjected to a sham surgery (Sham) or splenectomy (SPx) with or without anti–PD-L1 (αPD-L1) treatment. *P < 0.001, ***P < 0.001 compared to “Sham” group (log-rank test); ###P < 0.001 compared to “SPx” group (log-rank test); &&&P < 0.001 compared to “Sham + αPD-L1” group (log-rank test). (B) Fold changes in the numbers of splenic LSK and GMP cells in Hepa mice. ***P < 0.001 (two-way ANOVA followed by Dunnett’s test). (C) Proportions of GM-CSF+ splenic LSK cells in Hepa mice. ***P < 0.001 (two-way ANOVA followed by Dunnett’s test). (D–F) The immunosuppressive activity of tumor-infiltrating CD11b+Ly6G+Ly6Cdim (PMN-like) cells (D and E) and the frequencies of tumor-infiltrating IFNγ+CD3+CD8+ CTLs (F). Hepa mice were treated with low-dose sorafenib (Sora) 7 days prior to examination or left untreated as controls. ***P < 0.001 (two-way ANOVA, corrected by Bonferroni’s method). (G) Survival of Hepa mice receiving none, either, or both of the low-dose sorafenib (Sora) and anti-PD-L1 treatments. For some groups, mice received adoptive transfers of 1 × 10^6 splenic HSPCs or 5 × 10^6 MDSCs three and ten days after the latter dose of sorafenib. ***P < 0.001 compared to “Isotype + Sora” group (log-rank test); ###P < 0.001 compared to “αPD-L1” group (log-rank test); &&&P < 0.001 compared to “αPD-L1 + Sora” group (log-rank test). (H) Survival of CCR2+/+ or CCR2-/- Hepa mice treated with anti–PD-L1 or isotype antibodies. ***P < 0.001 compared to “CCR2-/- + isotype” group (log-rank test); #P < 0.05 compared to “CCR2+/+ + αPD-L1” group (log-rank test). Data are representative of two experiments (B–F; N = 3 per group in each experiment; mean and s.e.m.), or pooled from two experiments (N = 7–11 per group as indicated; A,G,H).
Figure 8. Splenic EMH in patients with different types of solid tumors. (A) Representative immunohistochemical staining of CD133+ (upper) and CD11b+ (lower) cells in spleen specimens from a total of 135 patients with hepatocellular carcinoma (HCC; N = 22), gastric cancer (GC; N = 86), kidney cancer (KC; N = 16), or pancreatic cancer (PC; N = 11), and 34 non-cancer patients with splenic hemangioma (N = 6) or cirrhosis (N = 28). Red arrows point to the CD133+ cells in the fields. Scale bar, 50 μm. (B and C) Quantification of CD133+ HSPCs (B) and CD11b+ myeloid cells (C) in spleen specimens described in (A). ***P < 0.001; ns, not significant (Kruskal-Wallis test). (D) Correlation between the splenic CD133+ HSPC and splenic CD11b+ myeloid cell densities. Result of a Spearman’s rank correlation analysis is indicated. (E) Patients with gastric cancer were divided into two groups according to the median value of the splenic CD133+ cell frequencies. The time of overall survival was estimated using the Kaplan-Meier method and compared by the log-rank test (P = 0.0145).