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Osteoblasts remotely supply lung tumors with cancer-promoting Siglec^F^{high} neutrophils

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Bone marrow-derived myeloid cells can accumulate in high numbers within solid tumors and mediate functions that foster cancer outgrowth. Immune-neoplastic interactions in the local tumor microenvironment have been intensively investigated, but the contribution of the host systemic environment to tumor growth remains poorly understood. Here, we show in mice and in cancer patients (n= 70) that lung adenocarcinomas increase bone stromal activity even in the absence of local metastasis. Animal studies further reveal that the cancer-induced bone phenotype involves bone-resident osteocalcin-expressing (Ocn⁺) osteoblastic cells. These cells promote cancer by remotely supplying a distinct subset of tumor-infiltrating Siglec^F^{high} neutrophils, which exhibit discrete cancer-promoting properties. Experimentally-induced reduction in the number of Ocn⁺ cells suppresses both the neutrophil response and lung tumor outgrowth. These observations uncover a role for osteoblasts as remote regulators of lung cancer and identify Siglec^F^{high} neutrophils as myeloid cell effectors of the osteoblast-driven pro-tumoral response.

Myeloid cells have emerged as key regulators of cancer growth due to their abundance in the tumor stroma in a broad range of cancers, association with patient disease outcome and ability to modulate tumor progression {Coffelt et al., 2016, Nat Rev Cancer, 16, 431-46; Hanahan and Coussens, 2012, Cancer Cell, 21, 309-22; Gabrilovich et al., 2012, Nat Rev Immunol, 12, 253-68; Engblom et al., 2016, Nat Rev Cancer, 16, 447-62}.

Most tumor-infiltrating myeloid cells are continuously replenished by circulating precursors, which are produced in distant tissues {Engblom et al., 2016, Nat Rev Cancer, 16, 447-62; McAllister and Weinberg, 2014, Nat Cell Biol, 16, 717-27} and some tumors amplify myeloid cell activity by skewing hematopoiesis toward the myeloid lineage or increasing myeloid cell populations in the periphery {Casbon et al., 2015, Proc Natl Acad Sci U S A, 112, E566-75; Bayne et al., 2012, Cancer Cell, 21, 822-35; Cortez-Retamozo et al., 2012, Proc Natl Acad Sci U S A, 109, 2491-6}. For example, patients across cancer types present with elevated levels of hematopoietic myeloid progenitor cells in peripheral blood {Wu et al., 2014, Proc Natl Acad Sci U S A, 111, 4221-6}. Additionally, increased numbers of circulating myeloid cells, such as neutrophils, often correlate with poorer clinical outcome {Templeton et al., 2014, J Natl Cancer Inst, 106, dju124; Jiang et al., 2015, Oncotarget, 6, 9542-50; Huang et al., 2015, Cancer, 121, 545-55}. It is therefore important to consider host changes that occur away from the tumor stroma to more fully understand the biological processes underlying tumor growth.

The bone marrow is a tissue of particular interest as it is the main [site of](#) hematopoietic cell production [site](#) for all circulating blood lineages in the adult {Hoggatt et al., 2016, Annu Rev Pathol, 11, 555-81}. The marrow contains resident cell components that not only participate in bone maintenance but also regulate hematopoiesis and immune cell fate, at least at steady-state {Morrison and Scadden, 2014, Nature, 505, 327-34; Mendelson and Frenette, 2014, Nat Med, 20, 833-46; Reagan and Rosen, 2016, Nat Rev Rheumatol, 12, 154-68}. For example, osteoblasts, which are bone-forming cells, were the first bone-resident cells identified to regulate hematopoiesis {Hoggatt et al., 2016, Annu Rev Pathol, 11, 555-81; Morrison and Scadden, 2014, Nature, 505, 327-34; Calvi et al., 2003, Nature, 425, 841-6}. However, our understanding of bone dynamics in the context of cancer ([that grows at sites distant to from](#) the local bone microenvironment) and related immune responses remains limited. To address this knowledge gap, we explored whether a common solid cancer—lung adenocarcinoma—affects bone tissue and how this might shape tumor-associated hematopoietic responses and distant tumor growth.

Results

Lung tumors modulate bones in mice and patients

To test whether lung tumors disrupt bone homeostatic activity, we initially used a fluorescent bisphosphonate derivative (OsteoSense-750EX) {Zaheer et al., 2001, Nat Biotechnol, 19, 1148-54} that binds hydroxyapatite minerals in areas of active bone formation and is detectable *in vivo* by fluorescence-mediated tomography (FMT) {Weissleder and Pittet, 2008, Nature, 452, 580-9}. We considered a mouse model of lung adenocarcinoma in which tumors are induced by intratracheal delivery of Adenovirus-Cre, which activates oncogenic *Kras* and deletes the tumor suppressor *Trp53* (hereafter referred to as [KP](#); fig. S1A-C), and whose growth recapitulates key aspects of the human disease {DuPage et al., 2009, Nat Protoc, 4, 1064-72}. We also used the KP1.9 tumor cell line, which derives from KP lung tumors and behaves similarly to its autochthonous counterpart {Pfirschke et al., 2016, Immunity, 44, 343-54}, and the Lewis Lung Carcinoma (LLC) cell line, a commonly used murine lung tumor model. *In vivo* FMT analysis of the femoral-tibial joint (fig. S2A) showed significantly elevated OsteoSense activity in both KP (Fig. 1A and B) and LLC (Fig. 1C and fig. S2B) [lung tumor-bearing](#) mice [bearing lung tumors](#), when compared to tumor-free controls.

Commented [ROH1]: Usually called KPC – for the Cre

Ex vivo analysis of explanted bones from KP tumor-bearing mice further revealed that this activity extended across all compartments analyzed, including the elbow joint, sternum, ribs, vertebrae and pelvic bone (fig. S2C-E). Bone metastases have not been reported for mice bearing KP tumors {DuPage et al., 2009, Nat

Protoc, 4, 1064-72}, which we confirmed by histology and PCR-based methods(fig. S3A-E). These studies indicate that lung tumors can disrupt bone stromal activity in absence of local metastasis.

By using confocal microscopy, we also found increased OsteoSense signal in the sternum (Fig. 1D and fig. S4A) and distal femur (fig. S4B) of KP1.9 tumor-bearing mice. The signal's location was separate from vasculature and enriched in areas of active bone remodeling, including the edges of sternebrae, which are associated with areas of increased bone in the sternum (Fig. 1D and fig. S4A). OsteoSense signal was also found in the metaphysis of the femur, which is an area of active trabecular bone formation adjacent to the growth plate (fig. S4B) as well as the epiphysis and diaphysis of the femur (fig. S4B).

These data suggested that KP tumors might influence bone microarchitecture. Accordingly, high-resolution μ CT showed increased trabecular bone volume (Fig. 1E and fig. S5A-C) and higher mineral density of distal femoral metaphysis (fig. S5D) in mice bearing KP tumors. The same mice also showed more (fig. S5E) and thicker (fig. S5F) trabeculae and decreased space between those trabeculae (fig. S5G). Cortical bone morphology in the femoral mid-diaphysis showed similar tumor-induced phenotypes (fig. S5H). Fig. S5I includes a complete tabulation of the μ CT results.

To investigate the relevance of our findings to human disease, we examined 35 *KRAS*⁺ non-small cell lung cancer (NSCLC) patients who had undergone non-contrast chest CT prior to cancer therapy and did not have osseous metastases. These patients were matched by age, sex, body mass index, and smoking status to 35 control individuals who did not have active malignancy or chronic illness and who did not use medications known to affect bone metabolism (table S1). This analysis revealed significantly higher trabecular bone density in the thoracic vertebrae of *KRAS*⁺ NSCLC patients compared to controls (Fig. 1F and table S1). Similarly, equivalent analysis of 35 *KRAS* non-mutant NSCLC patients revealed increased bone density compared to their ~~uniquely~~-matched controls (Fig. 1G). Thus, ~~lung-lung~~-tumor-induced changes in bone occur in both mice and humans.

~~Lung Lung~~-tumor-induced bone alterations involve osteoblasts

The bone phenotypes may be explained by altered osteoblast and/or osteoclast activity {Karsenty et al., 2009, *Annu Rev Cell Dev Biol*, 25, 629-48}. Histological analysis of these cell lineages in the distal femur identified more osteoblasts in KP tumor-bearing mice than in tumor-free controls (Fig. 2A and B). Osteoblasts in tumor-bearing mice also exhibited features of increased activity, including cuboidal shape and association with newly formed osteoid (Fig. 2A). Accordingly, the osteoid surface, characterizing newly formed bone, expanded in tumor-bearing mice (fig. S6A). By contrast, osteoclast numbers (fig. S6B) and eroded bone surfaces (fig. S6C) were not significantly different in tumor-bearing mice, although there was a trend for lower osteoclast-related indices compared to tumor-free controls.

To trace osteoblastic lineage cells by genetic means, we generated mice that expressed Cre-driven yellow fluorescence protein (YFP) under the control of the osteoblastic cell reporter osteocalcin (*Ocn*). *Ocn* is mainly expressed by mature osteoblasts and constitutes the major non-collagenous protein in the bone {Wu et al., 2009, *J Bone Miner Res*, 24, 759-64; Weinreb et al., 1990, *J Bone Miner Res*, 5, 831-42}. We found that *Ocn*-YFP⁺ cells expanded in KP tumor-bearing mice when compared to tumor-free controls (Fig. 2C). Femurs of tumor-bearing mice showed increased mineralized bone and bone formation, as assessed by von Kossa staining (Fig. 2D) and dynamic histomorphometry (Fig. 2E; fig. S7).

We next compared the transcriptome of Ocn^+ cells from tumor-free and lung cancer-bearing mice to ask whether Ocn^+ cells in lung cancer-bearing mice acquire unique phenotypes. Specifically, we generated triple transgenic KP- Ocn -GFP reporter mice (i.e., genetically engineered KP mice in which GFP expression is driven by the Ocn promoter) {DuPage et al., 2009, Nat Protoc, 4, 1064-72; Bilic-Curcic et al., 2005, Genesis, 43, 87-98}, sorted $Lin^- CD45^- Ter119^- CD31^- GFP^+$ (Ocn^+) cells from mice with or without tumors, and subjected these cells to RNAseq analysis (fig. S8A). Control experiments showed that the GFP^+ cells expressed 1000-fold higher levels of *Ocn*, *Osteopontin*, *Runx2* and other osteoblast-associated genes, when compared to $CD45^- Ter119^- CD31^- GFP^-$ cells from the same mice, and thus were highly enriched for Ocn -expressing osteoblasts. RNAseq analysis identified distinct changes in Ocn^+ cells from tumor-bearing mice (101 and 207 genes were significantly upregulated and downregulated, respectively. Some of these genes were associated with bone phenotypes (fig. S8B and C)). For example, Ocn^+ cells in tumor-bearing mice upregulated *Fosl2*, whose overexpression leads to increased trabecular bone mass {Bozec et al., 2010, J Cell Biol, 190, 1093-106; Bozec et al., 2013, J Cell Sci, 126, 5432-40}. Decreased expression of genes, such as *Dlk1* {Abdallah et al., 2011, J Bone Miner Res, 26, 1457-71} and *Ndrp1* {Watari et al., 2016, Sci Rep, 6, 19470}, with ascribed bone inhibitory functions, could also be relevant for the tumor-induced bone activity. Based on our combined data, we conclude that KP tumors increase osteoblast numbers, bone formation, and bone mineralization *in vivo* and induce discrete changes in Ocn^+ cell gene expression of genes related to bone phenotypes.

Commented [ROH2]: This paragraph now much better than before

Ocn^+ osteoblasts foster lung tumor growth

To investigate whether bone marrow osteoblastic cells remotely regulate lung cancer growth, we examined tumor progression in $Ocn^{Cre;Dtr}$ mice, a model in which Ocn^+ cells can be reduced by diphtheria toxin (DT) injection. We also used $Ocn^{Cre;Dtr/Yfp}$ mice to track Ocn^+ cells based on YFP expression. DT treatment did not affect body weight (fig. S9A and B) but significantly reduced Ocn^+ cell numbers, as detected by flow cytometry, immunohistochemistry, *in situ* microscopy and bone histomorphometry (fig. S9C-F). These analyses confirmed that DT treatment of $Ocn^{Cre;Dtr}$ mice resulted in efficient reduction of osteoblastic lineage cells. Importantly, DT treatment in $Ocn^{Cre;Dtr}$ mice was sufficient to interrupt the progression of established KP lung tumors (Fig. 3A and B). Control experiments further indicated that tumor reduction required Ocn^+ cell targeting because DT treatment did not suppress KP tumor progression in mice lacking the Ocn^{Cre} or DT receptor (DTR) transgenes (Fig. 3A). Suppression of tumor growth was not due to nonspecific DT-induced cell death in the bone marrow because DT targeting of $CD169^+$ bone marrow cells did not suppress KP lung tumor progression in $Cd169^{Dtr}$ mice (fig. S10). These findings indicate that Ocn^+ cells affect lung tumor progression.

Ocn^+ osteoblasts supply tumor-infiltrating neutrophils

We hypothesized that Ocn^+ cells may affect lung cancer growth by supplying specific hematopoietic cells to the tumor microenvironment. We thus compared KP lung tumor immune infiltrates in mice with either unmanipulated or reduced Ocn^+ cell numbers. We found similar pools of monocytes, lung macrophages, B cells and T cells in both cohorts; however, mice with fewer Ocn^+ cells showed a ~2-3-fold reduction in $CD11b^+ Ly-6G^+$ neutrophils (Fig. 3C and fig. S11). These mice also had more $CD49b^+ NK1.1^+$ NK cells (fig. S11), which were likely not required for KP tumor control because NK cell depletion did not restore cancer growth in these mice (fig. S12A-D). Importantly, DT did not target neutrophils directly because wild-type mice treated with DT maintained their neutrophil counts (fig. S13A and B). Also, $CD11b^+$ myeloid cells from $Ocn^{Cre;Dtr}$ mice were not killed by DT *in vitro*, confirming no functionally relevant DT receptor expression by these cells (fig. S13C and D), whereas positive control experiments showed DT's ability to kill DTR^+ cells *in vitro* (fig. S13E and F).

We next considered whether controlled KP tumor progression in Ocn⁺ cell-reduced mice involves the altered neutrophil response. In this scenario, removing neutrophils should delay growth of the primary tumor even in presence of Ocn⁺ cells. Accordingly, targeting neutrophils with depleting antibodies (Fig. 3D and fig. S14A and B) significantly suppressed KP lung tumor progression in Ocn⁺ cell-sufficient mice, as defined by longitudinal and noninvasive μ CT monitoring of lung tumor nodules (Fig. 3D).

To assess Ocn⁺ cells' impact on the tumor-induced systemic neutrophil response, we compared the number of circulating neutrophils in presence or absence of KP lung tumors when Ocn⁺ cells were depleted or not. We found that the presence of KP tumors was associated with a significant increase in the number of (CD11b⁺Ly6G⁺) neutrophils in the blood (Fig. 3E). This response required Ocn⁺ cells because the number of circulating neutrophils did not increase when Ocn⁺ cells were depleted. These data indicate that Ocn⁺ cells are required for the amplification of tumor-associated circulating neutrophils.

To further test this idea, we assessed tumor microenvironments when the circulatory systems of mice were joined by parabiosis. We found that joining osteoblast-reduced mice to osteoblast-sufficient mice increased tumor-infiltrating CD11b⁺Ly-6G⁺ neutrophil numbers in the former by 2.6 ± 0.3 fold (mean \pm SEM) (Fig. 3F). These neutrophil numbers were comparable to those seen in control (osteoblast-sufficient, nonparabiosed) mice (2.2 ± 0.4 fold; $p = n.s.$). Importantly, tumors in osteoblast-reduced mice grew faster when joined to osteoblast-sufficient parabionts (Fig. 3E) and similarly to tumors in control (osteoblast-sufficient, nonparabiosed) mice (lung weights: 557.1 ± 68.34 mg and 551.4 ± 30.24 , respectively, $p = n.s.$). Thus, both tumor neutrophil counts and tumor progression were restored in osteoblast-reduced mice when parabiosed to Ocn⁺ cell sufficient mice. Combined, these data not only show that Ocn⁺ cells ~~contribute~~ recruit tumor-infiltrating neutrophils, but also suggest that these cells display tumor-promoting functions.

Ocn⁺ cell-driven neutrophils promote cancer growth

Neutrophils are heterogenous (Coffelt et al., 2016, Nat Rev Cancer, 16, 431-46) and we therefore wondered whether those supplied by Ocn⁺ cells have distinct attributes that can accelerate tumor progression. To address this question, we delved deeper into lung neutrophil phenotypes and found that CD11b⁺ Ly-6G⁺ cells can be divided into two subsets according to expression levels of the lectin SiglecF (Fig. 4A). The SiglecF^{low} subset appeared ~~at~~ in high numbers in healthy lungs and expanded only slightly in lungs from tumor-bearing mice; by contrast, the SiglecF^{high} subset was rare in the healthy tissue but expanded ~70-fold in tumor-bearing lungs (Fig. 4A and B). The SiglecF^{high}/SiglecF^{low} cell subset ratio positively correlated with KP lung tumor burden (fig. S15A and B), further indicating that the SiglecF^{high} subset continued to accumulate in growing tumors.

Both the cell surface phenotype and forward/side scatter profiles of the SiglecF^{high} cells closely resembled those of neutrophils and were distinct from those of other myeloid cell types including SiglecF⁺ eosinophils and SiglecF⁺ alveolar macrophages (fig. S16). Immunohistochemical SiglecF and Ly-6G staining further revealed the presence of Ly-6G⁺ and SiglecF^{high} neutrophil-like cells within tumor nodules (Fig. 4C and fig. S17A and B), suggesting that the SiglecF^{high} neutrophils localize proximal to tumor cells. SiglecF⁺ cells outside the tumor stroma instead mainly resembled alveolar macrophages based on their morphology and Ly-6G⁻ phenotype (fig. S17C-F).

Commented [ROH3]: Do you want to mention the CXCL14 down-regulation – maybe not unless you have some data that the recruited SiglecF+ cells have CXCR4

To study whether osteoblasts preferentially ~~contribute-recruit~~ SiglecF^{high} lung neutrophils, we quantified both SiglecF^{high} and SiglecF^{low} subsets in tumor-bearing mice with reduced or unchanged Ocn⁺ cell numbers. We found that Ocn⁺ cell deficiency significantly reduced the percentage of SiglecF^{high}, but not SiglecF^{low}, neutrophils (Fig. 4D). These data suggest that Ocn⁺ cells promote SiglecF^{high} neutrophil accumulation in tumors. To further investigate whether SiglecF^{high} neutrophil accumulation in tumors requires Ocn⁺ cells, we mapped the fate of wild-type donor CD45.1⁺ c-Kit⁺ hematopoietic cells upon adoptive transfer into CD45.2⁺ tumor-bearing recipient mice that had either reduced or unchanged Ocn⁺ cell numbers. We found that the c-Kit⁺ donor cells' ability to produce SiglecF^{high} lung neutrophils was impaired in Ocn⁺ cell-reduced mice (Fig. 4E). These findings indicate that SiglecF^{high} neutrophil accumulation in tumors depends on Ocn⁺ osteoblastic cells. By contrast, the c-Kit⁺ donor cells were equally able to produce tumor-infiltrating SiglecF^{low} neutrophils (Fig. 4E), as well as macrophages (fig. S18A) and B cells (albeit at frequencies >25-times lower than myeloid cells; fig. S18B), in Ocn⁺ cell-reduced and sufficient mice. Donor-derived T cells were very rare or undetectable in the tumor stroma. These findings indicate that KP tumor accumulation of SiglecF^{high} neutrophils, in contrast to other immune cells, depends on Ocn⁺ cells.

SiglecF^{high} neutrophil profiling reveals cancer-promoting phenotypes

We next asked whether SiglecF^{high} neutrophils in mice have cancer-promoting properties. To this end, we interrogated single-cell transcriptomic data of neutrophils from healthy lungs or KP tumors. By defining a single-cell SiglecF expression score (table S2; detailed in the Methods section) we confirmed that neutrophils in healthy lungs were SiglecF^{low}, whereas tumor tissue contained both SiglecF^{low} and SiglecF^{high} subsets (fig. S19A). We thus compared gene expression of three neutrophil populations: SiglecF^{high} cells in tumor-bearing lung (T-SiglecF^{high}; n = 1,502 cells), SiglecF^{low} cells in tumor-bearing lung (T-SiglecF^{low}; n = 273), and SiglecF^{low} cells in healthy lung (H-SiglecF^{low}; n = 4,245). Differential gene expression analysis revealed that T-SiglecF^{high} cells substantially diverged ~~from both between~~ T-SiglecF^{low} and H-SiglecF^{low} cells (1,769 and 1,798 differentially expressed genes, respectively; Fig. 5A, table S3 and fig. S19B). T-SiglecF^{low} and H-SiglecF^{low} cells were more similar (123 differentially expressed genes; fig. S19C).

T-SiglecF^{high} cells ~~uniquely~~ upregulated the expression of genes associated with tumor-promoting processes (Fig. 5B, fig. S19D and table S4), including angiogenesis (*Vegfa*, *Hif1a*, *Sema4d*), myeloid cell differentiation and recruitment (*Csf1*, *Ccl3*, *Mif*), extracellular matrix remodeling (*Adamdec1*, *Adam17*, various cathepsins), suppression of T cell responses (*Cd274/PDL1*, *Fcgr2b*, *Havcr2*) and tumor cell proliferation and growth (*Tnf*, *Tgfb1*, *Il1a*). T-SiglecF^{high} cells also showed decreased expression of genes involved in cytotoxicity (*Cd244*, *Itgal*, *Fas*) (Fig. 5B). Other genes overexpressed in T-SiglecF^{high} cells included the ~~ER-ER-stress-stress-~~response gene and transcription factor *Xbp1* and the short fatty acid receptor *Ffar2* (Fig. 5A); *Xbp1* impairs myeloid antitumor functions (Cubillos-Ruiz et al., 2015, Cell, 161, 1527-38) and positively regulates *Ffar2* expression (Ang et al., 2015, Sci Rep, 5, 8134). Gene set enrichment analysis indicated upregulation of genes involved in oxidative phosphorylation, fatty acid metabolism and glycolysis, suggesting that T-SiglecF^{high} cells undergo a metabolic switch (fig. S20A). Genes involved in Myc signaling and E2F gene targets were also overexpressed, suggesting that T-SiglecF^{high} cells are more proliferative and resistant to apoptosis (fig. S20A). Taken together, these findings suggest that SiglecF^{high} neutrophils undergo metabolic changes in the tumor microenvironment and are poised to support tumor-promoting functions, including tumor angiogenesis, tumor cell proliferation, extracellular matrix remodeling and immunosuppressive myeloid cell recruitment.

Commented [ROH4]: Not a good word to use – how about selectively?

Tumor-infiltrating neutrophils are replenished by circulating cells. We thus explored whether differentiated Siglec^F^{high} neutrophils already exist in the blood of tumor-bearing mice. Specifically, we sorted blood-circulating CD45⁺ CD11b⁺ Ly6G⁺ neutrophils from either tumor-free or lung tumor-bearing mice and assessed the expression of several genes that we identified to be selectively upregulated by tumor-infiltrating Siglec^F^{high} neutrophils. This analysis revealed increased expression of transcripts corresponding to *SiglecF*, *Xbp1* and *Clec4n* (and, to a lesser extent, to *Ltc4s*) in circulating neutrophils from tumor-bearing mice (fig. S21A). In contrast, the expression of *Vegfa* and *Clec5a* was unchanged and flow cytometry analysis showed comparable expression of Siglec^F^{high}-associated cell surface proteins (fig.S21B). These findings suggest that at least some circulating neutrophils acquire molecular features of Siglec^F^{high} neutrophils prior to arrival at the tumor site. However, the acquisition of full-fledged Siglec^F^{high} neutrophil phenotypes occurs only after the cells have reached their destination tissue (which likely limits execution of the cells' effector functions to that site).

Siglec^F^{high} neutrophils exhibit cancer-promoting functions

Next, we evaluated the functions of Siglec^F^{high} compared to Siglec^F^{low} neutrophils. First, we tested the capacity of the different neutrophil populations (T-Siglec^F^{high}, T-Siglec^F^{low} and H-Siglec^F^{low} cells) to produce reactive oxygen species (ROS), which drive diverse pro-tumorigenic inflammatory responses {Casbon et al., 2015, Proc Natl Acad Sci U S A, 112, E566-75; Schmielau and Finn, 2001, Cancer Res, 61, 4756-60; Kusmartsev et al., 2004, J Immunol, 172, 989-99}. To this end, we measured intracellular ROS using an imaging probe that becomes fluorescent upon activation by ROS {Casbon et al., 2015, Proc Natl Acad Sci U S A, 112, E566-75}. Siglec^F^{high} neutrophils showed increased ROS activity compared to Siglec^F^{low} neutrophils in tumor or tumor-free tissue (Fig. 5C), indicating that ROS activity provided by neutrophils is contributed mainly by the Siglec^F^{high} subset.

Second, we assessed the ability of Siglec^F^{high} neutrophils to support other tumor-promoting myeloid cells. The neutrophil RNAseq analysis (Fig. 5A) revealed that T-Siglec^F^{high} neutrophils expressed high levels of the mRNA encoding colony-stimulating factor 1 (CSF-1), which is critical for the differentiation of macrophages from monocytes. In addition, monocyte-derived tumor-associated macrophages drive KP tumor growth {Cortez-Retamozo et al., 2012, Proc Natl Acad Sci U S A, 109, 2491-6}. Thus, we hypothesized that Siglec^F^{high} neutrophils support cancer progression by promoting the differentiation of tumor-associated macrophages. To test whether T-Siglec^F^{high} neutrophils favor macrophage differentiation, we isolated monocytes and myeloid precursors from the spleens of tumor-bearing mice and cultured these cells together with either T-Siglec^F^{high}, T-Siglec^F^{low}, or H-Siglec^F^{low} neutrophils. Splenic cells cultured with exogenous CSF-1 (instead of neutrophils) served as a positive control. We found that the presence of T-Siglec^F^{high} cells, compared to T-Siglec^F^{low} and H-Siglec^F^{low} neutrophils increased the proportion and number (Fig. 5D and E) of F4/80-expressing cells. These findings indicate that Siglec^F^{high} neutrophils promote monocyte differentiation into F4/80⁺ macrophages.

Third, we asked whether Siglec^F^{high} neutrophils promote cancer growth in vivo. To this end, we isolated tumor-associated Siglec^F^{high} neutrophils from tumor-bearing mice, and, as controls, Siglec^F^{low} neutrophils from either tumor-bearing mice or healthy tissue. Each neutrophil population was mixed with KP tumor cells and the mixture injected intradermally into mice. We then assessed the relative abilities of these various neutrophil populations to promote KP tumor progression. Importantly, we found that T-Siglec^F^{high} cells accelerated tumor growth compared to either T-Siglec^F^{low} or H-Siglec^F^{low} cells (Fig. 5F). These data suggest that the KP tumor-promoting effects provided by neutrophils are contributed largely by T-Siglec^F^{high} cells. Thus, Siglec^F^{high} neutrophils exhibit a tumor-promoting transcriptional profile, have increased ROS production, promote macrophage differentiation

and boost tumor progression *in vivo*. Overall our findings indicate that Siglec^F^{high} neutrophils have **unique** tumor-promoting functions compared to their Siglec^F^{low} counterparts.

Commented [ROH5]: Again, I am not sure "unique" is the appropriate word – you could just delete it.

We next determined whether the mouse Siglec^F^{high} neutrophil signature (detailed in Methods, table S5) might have clinical value. To do this, we **harnessed** patient tumor transcriptome and survival data (table S6) {Director's et al., 2008, Nat Med, 14, 822-7; Nguyen et al., 2009, Cell, 138, 51-62} and asked whether the expression of a Siglec^F^{high} neutrophil gene signature was associated with disease outcome in patients with lung adenocarcinoma. A Cox proportional hazards model, controlling for confounding variables, revealed a statistically significant ($p = 0.0017$) association of the Siglec^F^{high} neutrophil signature with worse patient survival (Fig. 5G, fig. S22 and table S6). In contrast, the Siglec^F^{low} neutrophil signature did not associate with disease outcome in lung cancer patients. The survival of the top 25% versus bottom 25% of Siglec^F^{high} and Siglec^F^{low} neutrophil signature expressers is shown in Kaplan-Meier plots (Fig. 5G and table S7). If these results are confirmed in **future studies of independent cohorts of lung adenocarcinoma patients**, the T-Siglec^F^{high} gene signature may prove to be a valuable biomarker of poorer prognosis.

Commented [ROH6]: A little unusual use of this verb – what about "analyzed"?

Commented [ROH7]: Could delete if you need to shorten

sRAGE contributes to the osteoblast-induced neutrophil response

The activation of bone marrow resident cells by distant lung tumors likely involves tumor-induced signals that act over extended distances and stimulate Ocn⁺ osteoblasts. To begin to address the underlying molecular and cellular mechanisms, we first set up *in vitro* experiments to define whether the blood of lung-tumor bearing mice contains factors that increase osteoblastic lineage cell activity. We collected serum from either ~~lung-lung-~~ tumor-bearing or tumor-free mice and added the serum to bone marrow cells cultured under osteogenic conditions. We then quantified the number of osteoblastic colonies measured by alkaline phosphatase enzymatic staining, as a proxy ~~ef for~~ osteoblastic activity. As shown in Fig 6A, serum from tumor-bearing animals significantly increased the number of osteoblastic colonies compared to serum from tumor-free mice. Thus, blood components from tumor-bearing mice are sufficient to promote osteoblastic lineage cells.

To identify specific serum factors that contribute to increased osteoblast activity, we used a protein array to quantify 111 cytokines and growth factors in the blood collected from tumor-bearing or tumor-free mice. We found that the concentration of the majority of factors tested was similar in tumor-bearing and tumor-free mice (including the myeloid growth factors G-CSF, GM-CSF, and M-CSF) whereas some factors (e.g. C1qR1, CCL21, Complement factor D) were slightly enriched in tumor-bearing mice. Notably, the receptor for advanced glycation endproducts (RAGE) was upregulated ~two-fold in the blood of tumor-bearing mice when compared to tumor-free mice (Fig. 6B and fig. S23A and B). We confirmed this finding using an independent ELISA assay (fig. S23C).

Various ligands can activate membrane-bound RAGE to trigger pro-inflammatory cascades, a process which has been implicated in several diseases, including diabetes, Alzheimer's and cancer. The circulating form of RAGE, referred to as soluble RAGE (sRAGE), can prevent the binding of ligands, including advanced glycation end products, to the RAGE receptor (Schmidt, 2015, Vascul Pharmacol, 72, 1-8). Interestingly, previous studies have connected sRAGE and RAGE ligands to bone regulation (Zhou and Xiong, 2011, Front Biosci (Schol Ed), 3, 768-76; Lalla et al., 2000, J Clin Invest, 105, 1117-24; Mercer et al., 2007, Mol Cell Biochem, 306, 87-94). To test the possibility that sRAGE contributes at least in part to increasing osteoblastic activity, we repeated the *in vitro* osteoblast culture experiment described above, but this time we specifically asked whether supplementing serum from tumor-free mice with sRAGE was sufficient to stimulate osteoblast

activation. Indeed, this experimental condition significantly increased osteoblastic colony forming units when compared to control conditions (Fig. 6C).

Finally, we investigated whether sRAGE could stimulate bone marrow neutrophil maturation and whether this process involved stromal osteoblastic cells. Because developing bone marrow neutrophils require upregulation of CXCR2 expression for release into blood {Coffelt et al., 2016, Nat Rev Cancer, 16, 431-46}, we tested neutrophil expression of this chemokine receptor. To this end, we cultured Lin⁻ cKit⁺ bone marrow hematopoietic cells with or without the ST2 pre-osteoblastic cell line and in the presence of increasing doses of exogenous sRAGE (Fig. 6D). This experiment indicated that the presence of sRAGE increases CXCR2 expression on developing neutrophils. Furthermore, we found that CXCR2 expression increased only in the presence of bone marrow stromal cells. These preliminary data support the notion that tumor-associated factors can stimulate osteoblastic cells, which in turn regulate immune responses, and suggest sRAGE as an interesting candidate for further investigation.

Commented [ROH8]: Might be another place to mention CXCR4 and CXCL14 if you have any data to implicate that arm as another means of recruitment?

Discussion

This study identifies systemic crosstalk between lung tumors and bones: lung adenocarcinomas can remotely activate Ocn⁺ osteoblasts in bones even in the absence of local metastasis. In turn, these osteoblasts supply tumors with SiglecF^{high} neutrophils, which foster cancer progression. The tumor-promoting functions of SiglecF^{high} neutrophils align with previous experimental data showing that neutrophils can promote cancer in various animal models {Casbon et al., 2015, Proc Natl Acad Sci U S A, 112, E566-75; Nozawa et al., 2006, Proc Natl Acad Sci U S A, 103, 12493-8; Shojaei et al., 2007, Nature, 450, 825-31; Kowanetz et al., 2010, Proc Natl Acad Sci U S A, 107, 21248-55; Fridlender et al., 2009, Cancer Cell, 16, 183-94; Wculek and Malanchi, 2015, Nature, 528, 413-7; Coffelt et al., 2015, Nature, 522, 345-8}. The findings also align with human studies, which indicate that a high neutrophil-to-lymphocyte ratio in blood is associated with adverse overall survival in many solid cancers, including that of the lung {Templeton et al., 2014, J Natl Cancer Inst, 106, dju124}, and that lung adenocarcinoma infiltration by neutrophils is strongly linked to poorer clinical outcome {Gentles et al., 2015, Nat Med, 21, 938-45}. Interestingly, both ~~single-single~~-cell transcriptomics and functional studies suggest that SiglecF^{high} neutrophils, but not their SiglecF^{low} counterparts, promote primary tumor growth. These data support the idea that tumor-infiltrating neutrophil populations encompass cell subsets with heterogeneous functions {Coffelt et al., 2016, Nat Rev Cancer, 16, 431-46} and suggest new ways to interrogate the neutrophil response to cancer. Interestingly, the phenotype exhibited by SiglecF^{high} neutrophils resembles at least in part that of granulocytic myeloid-derived suppressor cells (MDSCs) {Gabrilovich, 2017, Cancer Immunol Res, 5, 3-8}. For example, both granulocytic MDSCs and SiglecF^{high} neutrophils express the CD11b and Ly-6G surface markers, upregulate pro-angiogenic factors (e.g. *Vegf*) and produce high levels of ROS {Gabrilovich, 2017, Cancer Immunol Res, 5, 3-8}.

Osteoblastic-lineage cells are mostly known for their role in the control of bone formation {Karsenty et al., 2009, Annu Rev Cell Dev Biol, 25, 629-48; Visnjic et al., 2001, J Bone Miner Res, 16, 2222-31; Zhang et al., 2002, J Biol Chem, 277, 44005-12}, but increasing evidence indicates that these cells can also regulate hematopoiesis {Calvi et al., 2003, Nature, 425, 841-6; Visnjic et al., 2004, Blood, 103, 3258-64} with reported impacts on both B cell {Greenbaum et al., 2013, Nature, 495, 227-30; Ding and Morrison, 2013, Nature, 495, 231-5; Wu et al., 2008, Proc Natl Acad Sci U S A, 105, 16976-81; Zhu et al., 2007, Blood, 109, 3706-12} and T cell {Ding and Morrison, 2013, Nature, 495, 231-5; Yu et al., 2015, J Exp Med, 212, 759-74} production at

steady-state. Also, genetic perturbations of osteoblast-lineage cells deregulate myelopoiesis and can instigate myeloid hematopoietic malignancies {Fulzele et al., 2013, Blood, 121, 930-9; Raaijmakers et al., 2010, Nature, 464, 852-7; Kode et al., 2014, Nature, 506, 240-4}. Here we further report that osteoblastic cells can control tumor-infiltrating Siglec^F^{high} neutrophils, i.e. a discrete immune cell subset of the tumor microenvironment. We did not find evidence that osteoblastic cells control tumor-infiltrating B or T cells, although it is possible that osteoblast-mediated regulation of lymphocytes or other immune cells occurs in other cancer types. Furthermore, the bone marrow is composed of many different resident cell populations including adipocytes, endothelial cells, hematopoietic cells and nerves, which together with osteoblasts form a complex network that is critical to the production, maturation and egress of hematopoietic populations {Hoggatt et al., 2016, Annu Rev Pathol, 11, 555-81}. It is conceivable that some cancers affect multiple ~~bone-bone~~-resident cell populations, which in turn regulate distinct tumor-associated immune events. The study of additional bone marrow resident cells, for example with Cre-based models {Hoggatt et al., 2016, Annu Rev Pathol, 11, 555-81}, may help to ~~capture~~ more fully ~~capture~~ the complexities of systemic tumor-associated host responses. Additionally, fully understanding how lung tumors activate osteoblasts will require further study. Beside the effects of sRAGE identified in this study, it is possible that tumor-bone interactions involve additional components, which remain to be identified.

In accordance with amplified osteoblastic activity in tumor-bearing mice, we observed increased bone density in lung adenocarcinoma patients. This contrasts with the decrease in bone density that is known to occur in patients with other cancer types [for example, those with Parathyroid hormone-related protein (PTHrP) secreting tumors {Broadus et al., 1988, N Engl J Med, 319, 556-63}] or more broadly in cancer patients following certain anticancer therapies {Coleman et al., 2014, Ann Oncol, 25 Suppl 3, iii124-37; Rizzoli et al., 2013, Osteoporos Int, 24, 2929-53}. In this study we focused on patients prior to cancer therapy and we excluded individuals with chronic conditions (e.g. rheumatoid arthritis), medication use (e.g. glucocorticoids, bisphosphonates and prior cancer treatment), paraneoplastic syndromes, and osseous metastases, as these variables can all influence bone density. Because cancer cachexia or smoking can also lead to bone loss, we carefully matched our lung cancer patients to control individuals with similar body mass index, age and smoking history. Furthermore, because most lung cancer patients undergo chest CT scans with the administration of intravenous contrast, which can artificially increase bone density measurements {Pompe et al., 2015, Eur Radiol, 25, 283-9}, we limited our analysis to non-contrast CT scans. Our findings, based on the analysis of 140 individuals, indicate that in patients with non-small cell lung cancer, the primary tumor alters bone metabolism, resulting in increased bone density. It will be important to investigate bone parameters in more patients and in various clinical conditions, since the systemic manifestation of cancer is complex and may vary depending on disease stage, tumor type and the tumor's secretory profile.

This study underscores the importance of studying cancer as a systemic disease. Interrogating tumor-associated host responses through this lens should be important to fully address fundamental mechanisms of tumor immunity and effects of cancer therapies. Specifically, considering immune cells as critical therapeutic targets, it will be relevant to broadly investigate hematopoietic organs distant ~~to-from~~ the primary tumor to uncover ways in which cellular and molecular components at those sites control hematopoietic cell production, maturation and activation in cancer, and how these parameters can be manipulated. Given their involvement in shaping tumor progression, our study posits ~~bone-bone-marrow-marrow~~-resident Ocn⁺ cells and Siglec^F^{high} neutrophils as relevant clinical biomarkers and candidate vantage points for anticancer therapy.

Materials and Methods

Mice

Kras^{LSL-G12D/WT}; *p53*^{Flox/Flox} (referred to as KP) mice were used as a conditional mouse model of non-small cell lung cancer (NSCLC) {DuPage et al., 2009, Nat Protoc, 4, 1064-72} and bred in our laboratory in the C57BL/6 background or in the laboratory of Dr. Meylan. To track and deplete osteoblastic lineage cells by genetic means, we generated mice that expressed Cre-driven yellow ~~fluorescence-fluorescent~~ protein (YFP) under the control of the osteoblastic cell reporter osteocalcin (Ocn) {Zhang et al., 2002, J Biol Chem, 277, 44005-12; Srinivas et al., 2001, BMC Dev Biol, 1, 4; Buch et al., 2005, Nat Methods, 2, 419-26}. In detail, *Ocn*^{Cre} (B6N.FVB-Tg(BGLAP-cre)1Clem/J) transgenic mice were bred to *Rosa*^{Dtr} (C57BL/6-Gt(ROSA)26Sor^{tm1(HBEGF)Awai/J}) and *Rosa*^{Yfp} mice (B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP)Cos/J}) (Jackson Laboratory) to generate *Ocn*^{Cre;Dtr}, *Ocn*^{Cre;Dtr/Yfp} or *Ocn*^{Cre;Yfp} mice, respectively. Moreover, we generated *KPOcn*^{Gfp} mice by breeding KP mice with *Ocn*^{Gfp-topaz} (C57BL6/Tg(BGLAP-Topaz)1Rowe/J) mice {Bilic-Curcic et al., 2005, Genesis, 43, 87-98}. *Cd11c*^{Dtr} mice (B6.FVB-Tg(ltgax-DTR/EGFP)57Lan/J) were obtained from Jackson Laboratory. *Cd169*^{Dtr} transgenic mice (*Siglec1*^{tm1(HBEGF)Mka}) were kindly provided by the Riken Institute (Japan). Wild type and CD45.1 C57BL/6 mice were purchased from Jackson Laboratory. All animal experiments were performed according to approved IACUC guidelines, except experiments in KP mice for anti-Gr-1 antibody (Ab) depletion that were approved by the Veterinary Authority of the Canton de Vaud, Switzerland (license number VD2391) and the Réseau des animaleries lémaniques (RESAL) competent ethic committee.

Commented [ROH9]: Did you actually use these mice to deplete Siglec+ cells and, if so, wouldn't they deplete other cell types – Siglec1 is not the same as SiglecF? Or was this just used as a control?

Following primers were used for genotyping *Ocn*^{Cre;Dtr/Yfp} and *Ocn*^{Gfp} mice:

iDTR = WSS-F: 5'-GGCTACTGCTGACTCTCAACATT-3'; DTR-R: TCATGGTGGCGAATTCGAT
Cre = *Ocn*Cre-F: CAA ATA GCC CTG GCA GAT TC; *Ocn*Cre-R: TGA TAC AAG GGA CAT CTT CC
GFP (Jackson Laboratory) = oIMR0872: TTC ATC TGC ACC ACC G; oIMR1416: TTG AAG AAG ATG GTG CG

Tumor models

Adenovirus-Cre (AdCre) was delivered intratracheally (i.t.) to KP mice as previously described {DuPage et al., 2009, Nat Protoc, 4, 1064-72; Pfirschke et al., 2016, Immunity, 44, 343-54}. Mice were analyzed for bone or tumor phenotypes 12-14 weeks post-tumor initiation. Tumor burden was scored by measuring post-mortem lung weight and by histological analyses of lung tissue using hematoxylin and eosin (H&E) stainings. For some experiments micro-computed tomography (μ CT) was used to monitor tumor burden in the lung. The lung adenocarcinoma cell line KP1.9 was used to induce lung tumors in male wild-type C57BL/6, *Ocn*^{Cre;Dtr} or *Ocn*^{Cre;Yfp} mice via intravenous (i.v.) ~~tail-tail~~-vein injection (0.25×10^6 cells in 100 μ l PBS). Male mice with KP1.9 tumors were typically euthanized between 28-41 days ~~post-after~~ tumor cell injection. Cells of the ~~lewis-Lewis~~ lung cancer line (LLC, 1.5×10^6 cells in 150 μ l PBS) were injected i.v. into wild-type C57BL/6 mice and the mice were euthanized 32 days post-tumor cell injection. Diphtheria toxin (DT) was used to deplete *Ocn*⁺ cells in *Ocn*^{Cre;Dtr} and *Ocn*^{Cre;Dtr/Yfp} mice; for the detailed depletion protocol see section: *In vivo* osteoblast depletion.

Cell lines

The KP1.9 cell line, derived from ~~lung-lung~~-tumor nodules of a C57BL/6 KP mouse, was kindly provided by Dr. Zippelius (University Hospital Basel, Switzerland). ~~GFP-GFP~~-positive KP1.9 cells (KP-GFP cell line) were established in our laboratory. The LLC cell line was obtained from ATCC and ST2 cells were kindly provided by Marc Wein (MGH). All cell lines were maintained in Iscove's DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Patient bone density measurements

The study was conducted using IRB approval (2016P000394/MGH) and complied with HIPAA guidelines with exemption status for individual informed consent. A retrospective search was performed to identify patients with non-small cell lung cancer (NSCLC) who were KRAS positive and KRAS negative, and who had undergone non-contrast chest CT prior to therapy at MGH between 2011 and 2017. Patients with osseous metastases, paraneoplastic syndrome or therapy prior to or at time of chest CT were excluded. Control subjects (referred to as control patients) who had undergone non-contrast chest CT using the same imaging protocols as the patient group were identified and 1:1 matched for sex, age within ± 2 years, BMI within ± 2 kg/m², and smoking (pack-years). Potential controls with active malignancy, significant chronic illness or medication use known to affect bone metabolism were excluded. Trabecular bone density was determined from non-contrast chest CT (16- or 64-MDCT scanner Biograph 16 or 64, Siemens Healthcare; or Discovery CT750HD, GE Healthcare) using an axial slice thickness of 2.5 or 5 mm, 120 kVp and 11-40 mAs. Scans were then reviewed offline on an IMPAX workstation (AGFA Diagnostic Software, version 4, Afga). Circular regions of interest (exemplarily shown in Figure 2.1F) within trabecular bone of the T6, T8, T10 and T12 vertebral bodies were placed manually, avoiding cortical bone and posterior veins. The mean trabecular bone density of each vertebral body in Hounsfield Units (HU) was determined and an average thoracic trabecular bone density of the four vertebral bodies was calculated.

Fluorescence molecular tomography (FMT)

OsteoSense-750EX {Zaheer et al., 2001, Nat Biotechnol, 19, 1148-54} was injected retro-orbitally (4nmol/100 μ l, Perkin Elmer) according to manufacturer's instructions. The mice (for *in vivo* study) or cleaned bones (for *ex vivo* investigation) were imaged with FMT no earlier than 4 h and no later than 24 h post OsteoSense injection using an FMT imaging system (VisEn Medical). For *in vivo* imaging, hair from hind legs and lower abdomen were removed by shaving and chemical depilation. Mice were anaesthetized using isoflurane during the entire scanning procedure. The positioning of the mice relative to the detector were kept consistent throughout the experiments and groups. Detected OsteoSense signal in the femoral-tibial joint (region of interest, ROI) was analyzed using TrueQuant software and normalized against age and sex-matched control values.

Micro-computed tomography (μ CT) for lung tumor measurements

Lung tumor volumes were received through repeated μ CT measurements and pre- versus post-treatment measurements calculated. Mice were anaesthetized using isoflurane during the entire scanning procedure. Lungs were imaged with a CT (Quantum FX, PerkinElmer) at a 50- μ m voxel size, with retrospective respiratory gating. Individual tumor volumes were measured and calculated using the Analyze software (PerkinElmer).

Micro-computed tomography (μ CT) for bone microarchitecture

Femurs from KP1.9 tumor-bearing versus age- and sex-matched tumor-free controls were dissected out, cleaned, fixed in 10% formalin for 24 h, washed in PBS and transferred to 70% ethanol (EtOH) prior to μ CT analysis. Trabecular bone microarchitecture and cortical bone morphology in the distal femoral metaphysis and mid-diaphysis, respectively, were quantified using a high-resolution desktop micro-tomographic imaging system (μ CT40, Scanco Medical AG). The scans were performed using the following settings: 10 μ m³ isotropic voxel size, 70 kVp peak x-ray tube intensity, 114 mA x-ray tube current, 200 ms integration time, and were subjected to Gaussian filtration and segmentation. Image

acquisition and analysis protocols were performed according to μ CT guidelines for the assessment of bone microstructure in rodents {Bouxsein et al., 2010, J Bone Miner Res, 25, 1468-86}. Trabecular bone was analyzed in a region (1500 μ m; 150 transverse slices) extending proximally from 200 μ m above the peak of the distal growth plate. A threshold of 339 mgHA/cm³ was used to segment trabecular bone from soft-tissue and then, trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm⁻¹), trabecular separation (Tb.Sp, mm), and trabecular bone mineral density (Tb.BMD, mgHA/cm³) were measured using the Scanco Evaluation program trabecular morphology script. Cortical bone was evaluated in a 500 μ m long (50 transverse slices) region at the femoral mid-diaphysis and was segmented using a threshold of 700 mgHA/cm³ and then analyzed using the Scanco mid-shaft evaluation script to measure total cross-sectional area (Tt.Ar, mm²), cortical bone area (Ct.Ar, mm²), medullary area (Ma.Ar, mm²), bone area fraction (Ct.Ar/Tt.Ar, %), cortical tissue mineral density (Ct.TMD, mgHA/cm³), cortical thickness (Ct.Th, mm), cortical porosity (%), as well as the maximum, minimum and polar moments of inertia (I_{max} , I_{min} , and J , mm⁴).

Histology and immunohistochemistry (IHC)

For histological analysis of tumor burden in mice, lung tissues and femurs were harvested, formaldehyde-fixed and paraffin-embedded following standard procedures and consecutive sections were prepared. Lung tissue sections were stained with H&E to define tumor tissue areas in the lung as described earlier {Pfirschke et al., 2016, Immunity, 44, 343-54}.

IHC on mouse tissue sections was performed as previously described {Pfirschke et al., 2016, Immunity, 44, 343-54}. Briefly, mouse lung and bone sections were prepared using a Leica RM2255 rotary microtome (Leica Biosystems), dried at 60°C for 1 h, dewaxed and rehydrated before heat-induced epitope-retrieval (HIER) prior to immunostaining. ~~Therefore, the~~ The sections were incubated in 10mM Tris (pH9.0) or 10mM sodium-citrate (pH6.0) buffered solution containing 0.05% Tween and, depending on the Ab used, if needed heated at 120°C for 2 min using a pressure cooker. To obtain consistent and reliable staining the LabVision Autostainer 360 (Thermo Fisher Scientific) was used. The sections were pretreated using BLOXALL endogenous enzyme blocking solution (Vector Laboratories) for 10 min to destroy all endogenous peroxidase activity. After blocking with normal goat serum, the sections were incubated with rat anti-mouse Ly-6G (clone 1A8, Biolegend) or anti-mouse osteocalcin (clone M-15, Santa Cruz) monoclonal Abs (mAbs) for 1 h followed by several washes and secondary ImmPRESS polymer detection system (Vector Laboratories) according to the manufacturer's protocol. DAB Quanto (Thermo Fisher Scientific) was applied as substrate and hematoxylin used as counterstain.

Prior to preparation of bone tissue sections, femurs were harvested and cleaned, fixed for 24 h in 10% formalin, washed in PBS and transferred to 70% EtOH. The samples were then decalcified in 14% EDTA for up to two weeks and stored in 70% EtOH until paraffin embedding.

For anti-SiglecF stainings (rat anti-mouse Siglec-F mAb, clone E50-2440, BD Pharmingen), IHC on murine lung tissue of KP tumor-bearing or tumor-free mice was performed on frozen tissue sections. Spleen tissue sections were prepared for reference ~~positive-positive~~ control stainings. Frozen tissue sections were generated as described before {Pfirschke et al., 2016, Immunity, 44, 343-54}, air-dried and fixed in acetone (-20°C) for 10 min. The sections were rehydrated and treated as described above, but without HIER.

For histological evaluation of tumor metastases (femur), 10 regions of interest (n = 8) on ~~paraffin-paraffin-~~ embedded decalcified H&E-stained femur sections were defined at 20x and blindly scored for the presence or absence of tumor cell clusters. Positive control evaluations were done on histological sections from KP tumor-bearing lungs.

For cytopspins, SiglecF^{high} neutrophils (CD45⁺CD11b⁺Ly-6G⁺SiglecF⁺), SiglecF^{low} (CD45⁺CD11b⁺Ly-6G⁺SiglecF⁻) neutrophils and lung alveolar macrophages (CD45⁺CD11b⁺F4/80⁺SiglecF⁺CD11c⁺) were FACS sorted from lung tissue of KP tumor-bearing or tumor-free mice based on marker expression using the following anti-mouse mAbs: CD45 (clone 30-F11, Biolegend), Ly-6G (clone 1A8, Biolegend), CD11b (clone M1/70, BD), SiglecF (clone E50-2440, BD), Ly-6C (clone HK1.4, Biolegend), CD11c (clone N418, eBioscience). Cytopspins were performed using a Shandon Cytospin 4 centrifuge (Thermo Fisher Scientific). In detail, 5x10⁴ cells were centrifuged (500 rpm, 3 min) onto Tissue Path Superfrost Plus Gold microscope slides (Thermo Fisher Scientific) and dried overnight at RT. Cytopspins were then fixed in 4% formaldehyde-buffered solution and H&E stained following standard procedures.

For all histological sections, image documentation was performed using the NanoZoomer 2.0-RS slide scanner system (Hamamatsu).

Bone histomorphometry

Bone histomorphometric analysis was performed on femurs from KP1.9 tumor-bearing or age and sex-matched tumor-free controls as previously described {Wein et al., 2016, Nat Commun, 7, 13176}. In brief, calcein (20mg/kg; Sigma) and demeclocycline (50mg/kg, Sigma) were injected at 9 days and 2 days prior to animal euthanasia, respectively. Dissected, cleaned, formalin-fixed (10%, 24 h) femurs were washed in PBS and transferred to 70% EtOH. Fixed non-decalcified femurs were dehydrated (graded ethanol) and subsequently infiltrated and embedded in methylmethacrylate. Longitudinal sections (5µM) were cut using a microtome (RM2255, Leica) and stained with Goldner Trichrome for measurements of cellular parameters and by the method of von Kossa {Liu et al., 2016, J Bone Miner Res, 31, 929-39} to evaluate bone mineralization. Dynamic bone parameters were evaluated on unstained sections by measuring the extent and the distance between double labels using the Osteomeasure analyzing system (Osteometrics Inc.). Measurements were made in the area 200µm below the growth plate. Quantification of bone parameters was done in a blinded manner. The structural, dynamic and cellular parameters were evaluated using standardized guidelines {Dempster et al., 2013, J Bone Miner Res, 28, 2-17}.

Confocal microscopy

Confocal microscopy was performed based on a previously published protocol {Courties et al., 2015, Circ Res, 116, 407-17}. The mice were injected with OsteoSense retro-orbitally to label bone (< 24 h before mice were sacrificed) and with fluorescently conjugated mAbs (anti-CD31 (clone MEC13.3, Biolegend), anti-CD144 (clone BV13, Biolegend), anti-Sca1 (clone D7, eBioscience)) 30 min prior to euthanasia via cardiac perfusion with PBS and subsequent 4% methanol-free paraformaldehyde (Alfa Aesar). After fixation, the femurs or sternums were quickly dissected out, cleaned of tissue and cut for imaging. In brief, sternum marrow was exposed by cutting longitudinally along the bone and subsequently scanned at 10x (3-4 partially overlapping field of views). Femurs were OCT embedded, frozen at -80°C (>1 h), and marrow tissue was exposed using a cryostat. Z-stack images from femur and sternum were immediately acquired at 2-5µM steps (Olympus IV100 confocal

microscope) and analyzed in FIJI (ImageJ). Non-injected controls or non-fluorescent mice were used as staining controls.

***In vivo* Gr-1⁺ cell depletion**

Twelve-to-fourteen ~~weeks-week~~-old KP mice were infected i.t. with 1500 Cre-active lentiviral units using a protocol described earlier {DuPage et al., 2009, Nat Protoc, 4, 1064-72}. KP mice bearing well-established tumors (identified by μ CT) were treated 20 weeks post tumor initiation with anti-Gr-1 mAb (10 mg/kg, clone RB6-8C5, BioXcell) intraperitoneally (i.p.) three times per week for 2 weeks. Neutrophil depletion was validated by tail-vein blood sampling at day 7 followed by flow cytometry analyses of SSC^{hi} Ly-6G⁺ circulating cells. Control mice were injected with IgG control mAb (10 mg/kg, clone 2A3, Jackson Immunoresearch). At the end of the experiment tumor-bearing lungs were collected and ~~single-single~~-cell suspensions were obtained using the GentleMACS tissue octo dissociator (Miltenyi) and an enzymatic digestion mix composed of DMEM, 0.02 mg/ml DNase I (Sigma) and 1 mg/ml collagenase (Sigma) applied for 35 min at 37°C. Cells were washed with medium then resuspended in PBS supplemented by 2% FBS and 0.5mM EDTA. To obtain ~~single-single~~-cell suspensions, cells were passed through 70 μ m cell strainer. Cell number was determined and 1×10^7 cells were used for flow cytometry staining. Cells were first stained with live and dead blue dye (Life Technologies) in PBS containing Fc-Block reagent (Miltenyi) for 20 min at 4°C. After washing, mAb staining (anti-Ly-6G-FITC, clone RB6-8C5; anti-CD11b-BV711, clone M1/70; anti-Ly-6C-AlexaFluor700, clone HK1.4; anti-CD11c-BV450, clone N418 and anti-CD45-PerCP, clone 30-F11; all from Biolegend) was performed on ice or a 4°C in PBS supplemented with 2% FBS and 0.5mM EDTA for 15 min. All acquisitions were performed using the LSRII SORP (BD), a 5-laser and 18-detector analyzer at the EPFL Flow Cytometry Core Facility. Data analyses were performed using FlowJo X (FlowJo LLC).

***In vivo* osteoblast depletion**

Ocn^{Cre;Dtr}, Ocn^{Cre;Dtr/Yfp} and control mice lacking either transgene were treated i.p. with DT (100 μ l; 20 μ g/kg, Sigma-Aldrich) every other day for 9 days with a total of five injections per mouse. In some experiments, osteoblast depletion was performed for 3 consecutive days using DT. For both DT treatment protocols, osteoblast depletion was verified. Body weight was monitored to control for DT-induced toxicity. Osteoblast depletion was verified using histological evaluation of femurs, IHC for osteocalcin and *ex vivo* whole mount immunofluorescence of Ocn^{Cre;Dtr/Yfp} mice. Performing *in vitro* DT titration studies and *in vivo* cellular stainings using flow cytometry, we ensured that the used DT concentration did not effect the viability of hematopoietic cells in this murine model.

***In vivo* NK cell depletion**

NK cells were depleted in tumor-bearing Ocn^{Cre;Dtr} or control mice performing i.p. injections of anti-NK1.1 Ab (clone PK136, BioXcell, 200 μ g/mouse, i.p.) every fourth day. The detailed treatment schema is outlined in Fig. S11A. NK cell mAb depletion in ~~osteoblast-osteoblast~~-reduced tumor-bearing mice was evaluated using flow cytometry and was efficient in substantially decreasing lung NK cells (detected by CD49b and NKp46 double staining since the NK1.1 epitope may be masked by the depleting mAb).

***In vitro* assay to test potential DT-mediated direct effects on hematopoietic cells**

Splenocytes from WT or Ocn^{Cre;Dtr} mice were harvested by gently meshing a spleen through a 40 μ m filter. The cells were washed, plated in medium (RPMI, 10% FBS, 1% P/S) and treated with 0, 1, 10, 100, 1000 ng/ml of

DT. Cells were harvested after 20 h of incubation at 37°C and stained with mAbs for flow cytometry, see section on flow cytometry for staining procedure.

Parabiosis

In some experiments, parabiosis was used to study the contribution of circulating cells to osteoblast-controlled tumor-infiltrating immune cells. The experimental procedure was performed as previously described {Pucci et al., 2016, Science, 352, 242-6}. In brief, one week post-tumor injection, lung tumor-bearing $Ocn^{Cre;Dtr}$ mice were parabiosed to $Ocn^{Cre;Dtr}$ or control mice (lacking either transgene). Both types of parabionts were treated with DT following the procedure described in section: *In vivo* osteoblast depletion.

Neutrophil single cell RNAseq

~~Single-Single~~-cell RNA sequencing (scRNA-Seq) data were obtained from CD45⁺ cells collected from either tumor-free or KP tumor-bearing lungs from two independent experimental replicates using droplet microfluidic barcoding technology (inDrops) as previously described {Klein et al., 2015, Cell, 161, 1187-201; Zilionis et al., 2017, Nat Protoc, 12, 44-73}. WHERE WILL RAW DATA BE. Due to the limited sensitivity of scRNA-Seq at the ~~single-single~~-cell level, which leads to gene 'drop-out' events, SiglecF expression alone could not be reliably used to distinguish SiglecF^{high} and SiglecF^{low} cells. Therefore, we defined a SiglecF expression score, among granulocytes (n = 6,020 cells), for each single cell k as $S_k = (X_k - Y_k)$, where $X_k = \sum_{i=1}^{50} r_{k,i}$ and $r_{k,i}$ is the percentile gene expression (dense ranking) of cell k for gene i , for the 50 most correlated genes to SiglecF

(Spearman correlation), and $Y_k = \sum_{i=N-49}^N r_{k,i}$ is the corresponding sum of percentiles of the 50 most anticorrelated genes to SiglecF (table S2). As anticipated from FACS data, the distribution of granulocytes by SiglecF expression score was bimodal in tumor, with SiglecF^{low} cells overlapping with healthy granulocytes. By visual inspection of fig. S18A, we set a threshold of -7 to separate between SiglecF^{high} and SiglecF^{low} granulocytes in tumor tissue.

For differential gene expression (DGE) analysis of healthy, tumor SiglecF^{low}, and tumor SiglecF^{high} granulocyte populations, we used a parameter-free permutation-based test to calculate p-values, with the difference in means as the test statistic. We accounted for multiple hypothesis testing with a false discovery rate of 5% using the Benjamini-Hochberg procedure {Benjamini and Hochberg, 1995, Journal of the Royal Statistical Society, 57, 289-300}. To be considered for differential gene expression analysis, genes had to be expressed at least by 5% of cells in at least one of the two groups of cells compared. Significantly differentially expressed genes with an absolute fold-change of 2 were selected for further analysis. Genes judged as significant by the permutation test but with a p-value less than the specified accuracy of the permutation test were assigned an approximate p value using a t-test assuming unequal variances for representation on volcano plots.

For gene set enrichment analysis {Subramanian et al., 2005, Proc Natl Acad Sci U S A, 102, 15545-50; Mootha et al., 2003, Nat Genet, 34, 267-73}, we performed the same pre-filtering as for DGE analysis: only genes expressed by at least 5% of cells in at least on the two groups in a comparison were considered. Then we used the GSEA PreRanked tool {Subramanian et al., 2005, Proc Natl Acad Sci U S A, 102, 15545-50; Mootha et al., 2003, Nat Genet, 34, 267-73} on genes ranked by log₂ (fold-change) and considered gene sets that were enriched based on an FDR of 25%.

Osteoblast low-input bulk RNAseq

KP-Ocn^{Gfp} mice were infected with Ad-Cre i.t. and euthanized when high tumor burden was detectable (at 14 weeks post AdCre). All bones were harvested, cleaned and pooled from each single mouse. The bones were crushed gently and the released cells were collected (fraction 1). Red blood cells were lysed using ACK buffer (Lonza) and cells were depleted of mature cells using the lin-depletion kit (Stem cell technologies). In parallel, the bone fragments (fraction 2) were cut finely with scissors, filtered through a 70µm cell strainer (BD), digested for 1h at 37°C (0.25% collagenase type I (Worthington Biochemical Corporation) in FBS), washed and pooled with fraction 1. Ocn-expressing cells were FACS sorted (FACSAria) based on the following parameters: Lin⁻CD45⁻CD31⁻Ter119⁻GFP⁺. Approximately 2000 cells were sorted per mouse into Trizol and frozen at -80°C. RNA was isolated using a Trizol extraction protocol according to the Immgen standard <https://www.immgen.org/Protocols/Total%20RNA%20Extraction%20with%20Trizol.pdf>

Libraries were made following the protocol by Meredith et al. {Meredith et al., 2015, Nat Immunol, 16, 942-9}. RNA was reverse transcribed using ArrayScript (Ambion) using a specific primer containing T7 promoter, the 5' TruSeq Illumina adapter, a 8-positions with random nucleotide assignment as a unique molecular identifier (UMI), and a oligo-dT sequence. Second-strand synthesis was performed using the mRNA Second strand synthesis module (NEBNext #E6111L). After cDNA size selection using AMPure XP beads (0.8x and 1x, BeckmanCoulter- A63987), the product was amplified via in vitro transcription (MEGAscript, Invitrogen) for 12 hours and then fragmented (Magnesium RNA Fragmentation Module, New England Biolabs). 3' indexing adaptor was ligated (truncated T4 RNA ligase 2 -Enzymatics), reverse-transcribed (Superscript II, Invitrogen), and amplified by PCR for 18 cycles (HiFi hotstart PCR kit, Kapa). cDNA cleanup and size selection were performed on AMPure XP beads. Libraries were quantitated by BioAnalyzer using the Agilent High Sensitivity DNA Kit (Agilent 5067-4626) and qPCR using Kapa library quantification kits, and sequenced on a MiSeq (nano kit) and HiSeq 2500 (rapid mode).

Raw sequencing reads were processed using custom scripts. Read 1 contains the transcript sequence, Read 2 the UMIs. Raw reads were first trimmed using the FASTX-Tollkit v0.0.13 (fastx_trimmer -Q 33) {Gordon and Hannon, 2010, Fastx-toolkit. Computer program distributed by the author, website <http://hannonlab...>}. Read 2 was trimmed in order to extract the UMI (5-12bp), and Read 1 was trimmed to 30bp eliminate a potential oligo-dT sequence. Reads were filtered for quality (more than 80% of the sequence having a Sanger Phred+33 quality score > 33) using fastq_quality_filter -v -Q 33 -q 20 -p 80. Mapping was performed with Tophat2 to the mm10 mouse transcriptome {Kim et al., 2013, Genome Biol, 14, R36} keeping the strand information with the following options: tophat -p 2 - -library-type fr-firststrand --read- mismatches 5 --read-gap-length 5 --read-edit-dist 5 --nocoverage-search --segment-length 15 --transcriptome-index. Reads mapping at multiple positions were discarded using samtools flag 256 {Li et al., 2009, Bioinformatics, 25, 2078-9}. Duplicated mapping reads were filtered out using the UMIs with custom R scripts as follows. Reads were first assigned to genes. For each gene, only reads with distinct UMIs were kept. To take into account mutations in UMIs, distinct UMIs but with a Hamming distance of 1 were also collapsed to 1 read. Samples were normalized with DESeq using the estimateSizeFactors function {Love et al., 2014, Genome Biol, 15, 550}. Multiplot studio was used to define differentially expressed genes in Ocn⁺ cells between tumor-bearing and tumor free mice ($p < 0.05$). Osteoblast RNAseq data has been deposited under accession number [ADD]...

Survival analysis of lung adenocarcinoma patients

Analyses were performed using tumor microarray data and survival outcome in lung adenocarcinoma patients. Raw microarray CEL files along with patient annotations were obtained from two sources:

- 1) GSE68465 {Director's et al., 2008, Nat Med, 14, 822-7}
- 2) http://cbio.mskcc.org/public/lung_array_data/ {Nguyen et al., 2009, Cell, 138, 51-62}

CEL files from individual patients were converted into a single expression matrix using ExpressionFileCreator (v12.3, method=MAS5), followed by quantile normalization using the array NCI_U133A_61L as a reference as described before {Director's et al., 2008, Nat Med, 14, 822-7}. Probes were collapsed to gene symbols by selecting the probe with maximum mean expression after excluding probes mapping to multiple gene symbols {Miller et al., 2011, BMC Bioinformatics, 12, 322}. From the list of differentially expressed genes (table S3) between T-SiglecF^{high} and T-SiglecF^{low} neutrophils, we selected genes with a minimum expression of 50 transcripts per million and > 5 times higher expression in T-SiglecF^{high} neutrophils. The resulting 305 mouse genes were mapped to human orthologs using the HCOP tool (<http://www.genenames.org/cgi-bin/hcop>), including orthology predictions from Ensembl, NCBI, HGNC, Panther, HomoloGene, OrthoDB, OrthoMCL, OMA, PhylomeDB, TreeFam, Inparanoid, EggNOG. All orthologs were included for mouse genes mapping to multiple human genes. The conversion yielded 302 human orthologs (table S4). Using human patient microarray data, each patient was attributed a 'T-SiglecF^{high} neutrophil signature' value, defined as the sum rank transformed expression of the 302 human orthologs of genes enriched in T-SiglecF^{high} neutrophils in mouse. Here, rank transformation refers to the process by which the expression of gene *i* in patient *j* in the microarray data is replaced with the rank for patient *j* among other patients based on the expression of *i* (dense ranking). The signature was rescaled to have values from 0 to 1. Cox regression analysis was performed using the T-SiglecF^{high} neutrophil signature, sex, age, T stage, and N stage as predictor variables. Other sample characteristics, which were not documented for a fraction of patients, were used as strata; these included M stage, source of data, histological grade, smoking history, treatment with adjuvant chemotherapy and radiotherapy, tumor relapse, and positive surgical margin (table S5). All predictor variables satisfied the proportional hazards assumption as validated by a Schoenfeld residual tests (cox.zph function in R). To further validate the statistically significant p-value returned by the Cox Hazard test (p value = 0.0017), we randomly sampled 302 genes present in the microarray data, and recorded the number of times a Cox p-value smaller than the one observed was obtained. If the Cox Hazard model is accurate, we would expect approximately 0.17% of random trials to give the observed p-value or less. 7 out 1000 random samplings (0.7%) yielded a Cox *p*-value <0.0017, indicating a slight underestimate of the p-value by the Cox Hazard model, but nonetheless allowing to reject the null hypothesis of the gene selection being random with *p*<0.01. The T-SiglecF^{low} gene signature was defined in an analogous way, using the same number (n=302) of human orthologs of genes most enriched in T-SiglecF^{low} neutrophils. T-SiglecF^{low} neutrophil gene signature showed no significant association with survival. For Kaplan-Meier plots, survival data of top 25% and bottom 25% SiglecF^{high} signature expressers was used. Survival analysis was performed using the "survival" package in R {Therneau, 2015, version 2.38, A Package for Survival Analysis in S} and "Lifelines" package in Python {Davidson-Pilon, 2016, Lifelines. Github respository}.

***In vivo* cell fate mapping**

To track the progeny of hematopoietic precursors in tumor-bearing control or Ocn depleted mice, we performed cell fate mapping experiments. We used bead enrichment (Miltenyi) followed by FACS-based sorting of live lineage negative congenic CD45.1 cKIT⁺ (CD117) cells (here lineage = B220, CD19, Ter119, CD11c, CD11b, NK1.1, CD49b, CD127, Ly-6G, CD90.2). The purity of the sorted CD45.1⁺ cKIT⁺ cells was above 95%. 2.5 x 10⁵ cells were injected i.v. into tumor-bearing control or Ocn depleted mice (both CD45.2 genotypes) at 29 days post tumor-injection. 7 days post-cKit⁺ cell transfer, lung tumor tissue was harvested and CD45.1⁺

immune cell infiltrates were quantified using flow cytometry. Non-injected biological controls, Fluorescence Minus One (FMO)-staining controls and unstained cells were used to analyze the CD45.1⁺ cell progeny in the tissue.

***In vivo* tumor cell and neutrophil co-injection experiment**

To investigate whether SiglecF^{high} neutrophils are able to support the growth of tumor cells *in vivo*, we co-injected KP-GFP tumor cells with different neutrophil subpopulations (T-SiglecF^{high}, T-SiglecF^{low} or H-SiglecF^{low}) intradermally (i.d.) to the flank of C57BL/6 mice. Neutrophils were FACS sorted based on cell surface marker expression (CD45⁺CD11b⁺Ly-6G⁺SiglecF⁺ or SiglecF⁻) from lungs of KP1.9 tumor-bearing or tumor-free mice as detailed in the Flow cytometry methods section. Tumor cells (2x10⁵) and the respective neutrophil population (2x10⁵) were mixed in 50µl 1xPBS before i.d. injection (1:1 ratio). Tumor growth was recorded over time with a digital caliper and tumor volumes defined as $\pi/6 \times \text{length} \times \text{width}^2$.

***Ex vivo* ROS activity assay**

Neutrophils were analyzed *ex vivo* for their reactive oxygen species (ROS) content. Single cell suspensions were generated from KP tumor-bearing lungs or lungs of tumor-free mice as described in the Flow cytometry methods section. Cells were resuspended in HBSS containing 0.1% BSA followed by FACS antibody surface marker staining for 30min on ice as detailed below. Then cells were washed and resuspended in PBS-EGG buffer (1mM EDTA, 0.05% gelatin, 0.09% glucose) and 0.5µM DHR123 probe (Thermo Fisher Scientific) was added for 30min at 37°C. The reaction was stopped by moving the tubes to ice and washing the cells with PBS-EGG buffer. Cells were resuspended in PBS containing 0.1% BSA and activated rhodamine 123 signal (activated DHR 123) was analyzed in the FITC channel on a LSRII flow cytometer (BD) within 30min. Hydrogen peroxide added to cells served as a positive control.

***In vitro* macrophage differentiation experiment**

Monocytes and neutrophil were co-cultured to investigate if neutrophils can help to mature macrophages from their monocytic precursors. Neutrophils were FACS sorted based on cell surface marker expression (CD45⁺CD11b⁺Ly-6G⁺SiglecF⁺ or SiglecF⁻) from lungs of KP1.9 tumor-bearing (T-SiglecF^{low} or T-SiglecF^{high}) or tumor-free mice (H-SiglecF^{low}) as detailed in the Flow cytometry methods section. Murine spleens were harvested from tumor-bearing mice and were used to enrich for monocytes. In detail, spleens were harvested, meshed through a 40µm cell strainer and ACK lysed to remove erythrocytes. Splenic monocytes were enriched through a MACS based negative isolation protocol by incubating single cells with PE conjugated Abs specific for CD90.2, CD3, B220, CD19 and Ly-6G followed by anti-PE MACS beads. Both incubation steps were performed for 20min on ice. The negative isolation resulted in a 20-fold enrichment of monocytes based on flow cytometry measurements. This population likely also include myeloid precursors since these accumulate in spleens of tumor-bearing mice {Cortez-Retamozo et al., 2012, Proc Natl Acad Sci U S A, 109, 2491-6}. For the co-culture, 4x10⁴ monocytes and 8x10⁴ neutrophils were incubated in Iscove's DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin in 96-well cell culture plates for 6 days. Monocytes only and monocytes together with CSF-1 (1µl/1ml) were used as controls. Cells were removed from the plate and investigated by flow cytometry for F4/80 and CD11b expression in order to assess myeloid cell differentiation.

***In vitro* alkaline phosphatase assay for osteoblastic colony formation**

To study whether tumor derived circulating factors can affect the osteogenic potential, osteoblastic colony formation was investigated after addition of serum pooled from individual mice that were either tumor-bearing or tumor-free. In some experiments tumor-free serum +/- sRAGE was added. Long bones (femur and tibia) and vertebrae of tumor-free C57BL/6 mice were harvested and flushed. Single cell suspensions were generated using 70µm cell strainer and red blood cells removed in a ACK lysis step. Cells were counted and resuspended (4×10^7 /ml) in osteogenic medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, 10mM B-glycerophosphate and 50µg/ml ascorbic acid). 4×10^6 cells were transferred to 6 well cell culture plates and 200µl serum of tumor-bearing or tumor-free mice added. Medium was refreshed every second day and non-adherent cells were removed. Cells were fixed after 9-10 days by adding 4% PFA for 8min followed by 2 washing steps with H₂O and the alkaline phosphate substrate reaction (B5655, Sigma) according to manufactures procedure. As counter stain, Nucleofast Red (Polysciences, Inc) was used. The number of ALP positive colonies was evaluated by 2-3 independent persons in a blinded manner.

***In vitro* co-culture of hematopoietic precursors and bone marrow stromal cells +/- sRAGE**

To test whether sRAGE altered neutrophil maturation from hematopoietic precursors via stromal cells, we performed co-culture experiments. In detail, 1×10^3 ST2 cells per well were cultured in 96 well plates for two days. Then, bone marrow was harvested from tumor-free mice and depleted of differentiated cells using negative MACS bead separation (Abs specific for B220, CD19, Ter119, CD11c, CD11b, NK1.1, Dx5, CD127, Ly-6G and CD90.2 were utilized). The flowthrough was collected. The following conditions were tested: ST2 cells +/- 1×10^4 lineage negative bone marrow cells +/-sRAGE (0.01 µg/ml, 0.1 µg/ml, 1µg/ml). Neutrophil maturation was evaluated after three days by staining for CD11b, Ly-6G and CXCR2 surface expression using flow cytometry {Coffelt et al., 2016, Nat Rev Cancer, 16, 431-46}.

p53 recombined PCR for tumor cell detection

Detection of p53 recombined locus (only present in KP tumor cells after exposure to Cre recombinase) was used to survey bone and marrow tissues for KP tumor cell metastases. In brief, DNA was extracted from bone marrow or calvarial bone (after digestion) using DNeasy blood and tissue kit (Qiagen) according to manufacturer's instructions. KP1.9 tumor cells were used as positive control. Different DNA concentrations from KP1.9 tumor cells were used to determine PCR detection limit to <10 cells (with the estimate of ~6pg DNA/cell). DNA was isolated from Gel PCR products from a primary PCR run. A second PCR amplification run on these DNA samples was performed to detect low levels of DNA in the isolated tissues. The following primers were used: A: 5' CAC AAA AAC AGG TTA AAC CCA G 3'; B: 5' AGC ACA TAG GAG GCA GAG AC 3'; C: 5' GAA GAC AGA AAA GGG GAG GG 3'. Following bands were amplified: p53 recombined 1lox: 612bp, WT band: 288bp and a background band: 400bp.

Real-time PCR for analysis of blood samples

Neutrophils from the blood of KP lung tumor-bearing or tumor-free mice were investigated in order to define whether these cells exhibited transcriptional characteristics of tumor-infiltrating SiglecF^{high} cells outside the tumor microenvironment. Neutrophils were FACS sorted based on surface marker expression (CD45⁺ CD11b⁺ Ly-6G⁺) and RNA was isolated from the sorted cells using the RNeasy Micro Kit (Qiagen) according to manufactures procedures. Afterwards, cDNA was generated utilizing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Real time PCR assays performed using the TaqMan Fast Advanced MasterMix together with TaqMan probes at the 7500 Fast Real-Time PCR System (Applied Biosystems). β2-Microglobulin was used as a housekeeping gene.

Protein array

To investigate soluble factors in serum or plasma of KP and KP1.9 lung tumor-bearing or tumor-free control mice, a membrane-based sandwich immunoassay with 111 different cytokine and chemokine antibodies was performed using the Proteome Profiler Mouse XL Cytokine Array Kit (R&D Systems) according to manufactures procedures. The captured soluble factors were visualized in duplicate using a chemiluminescent detection readout with an exposure time of 3 min. The signal intensity from each protein spot on the array was quantified using the Microarray_Profile plugin in FIJI (http://www.optinav.info/MicroArray_Profile.htm). The intensity was normalized against six reference spots on each array. From two independent experiments, fold change of tumor versus tumor-free soluble factors was calculated and the resulting values presented in a heat map. Factors below detection level and that failed to alter reproducibly were excluded from further analysis.

sRAGE ELISA

Mouse sRAGE levels in the serum of KP1.9 tumor-bearing or tumor-free mice were quantified using ELISA according to manufacturer's instructions (MRG00, R&D systems). OD values were measured at 450 and 570nm (reference value) using a Tecan microplate reader. Murine blood was harvested and transferred to BD Microtainer tubes, incubated at room temperature for 30min, spun at 1000g for 15min and the serum was stored at -80°C until ELISA analysis. The concentration of sRAGE in serum samples, investigated in duplicate, was calculated by extrapolating values of a standard curve following manufactures guidelines.

Flow cytometry

Single cell suspensions were obtained from lung tumors, bone marrow, spleen and bone tissue. The respective tissues and isolated single cell fractions were kept on ice for all steps if not stated otherwise. Tumor tissue was received by dissecting out tumor-bearing lungs. Small tissue pieces were generated using scissors and digested (RPMI containing 0.2 mg/ml collagenase type I, Worthington Biochemical Corporation) for 1 h at 37°C while shaking. Femurs and for some experiments tibias were harvested, cleaned and the bone marrow flushed out using cold staining buffer (PBS containing 0.5% BSA and 2mM EDTA). Digested lung tissue and harvested bone marrow were gently meshed through 40µM cell strainers using a plunger. Spleens were harvested and also meshed through 40µM cell strainer as described before.

Red blood cells were removed using 1 ml ACK lysis buffer (Lonza) per cell pellet for 1 min (for lung cells) or 2 min (for spleen cells) and the reaction was stopped with RPMI media. In some experiments blood was collected from the cheek or if mice were euthanized via cardiac puncture and directly treated with 5 ml ACK lysis buffer for 5 min to remove red blood cells. The resulting single-cell suspensions were washed and resuspended in staining buffer. In order to investigate bone cells by flow cytometry, in general, long bones were harvested, cleaned and crushed gently and the released cells were collected (fraction 1) and lysed with ACK lysis buffer. In parallel, the bone fragments (fraction 2) were cut into small pieces with scissors, filtered through 70µM cell strainer, digested (PBS containing 20% FBS and 0.25% collagenase type I) for 1 h at 37°C, washed and finally pooled with the cells derived from fraction 1.

Single cell suspensions were incubated with FcBlock (clone 93, Biolegend) for 15 min at 4°C, followed by staining with fluorescent conjugated Abs for 45 min at 4°C. The cells were washed with staining buffer and analyzed on a LSRII flow cytometer (BD). 7-aminoactinomycin (7AAD, Sigma) positivity was used to exclude

dead cells. Flow Cytometry Absolute Count Standard (Bangs Laboratories) were used to quantify circulating neutrophils.

Following cell populations were identified based on cell marker expression: Ocn⁺ cells (Lin⁻CD45⁻CD31⁻Ter119⁻YFP⁺), neutrophils (CD45⁺CD11b⁺Ly-6G⁺), SiglecF^{high} neutrophils (CD45⁺CD11b⁺Ly-6G⁺SiglecF^{high}), SiglecF^{low} neutrophils (CD45⁺CD11b⁺Ly-6G⁺SiglecF^{low}), monocytes (CD45⁺CD11b⁺Ly-6G⁻Ly-6C^{high}), CD11b⁻ alveolar macrophages (CD45⁺CD11b⁻F4/80⁺SiglecF⁺CD11c⁺), CD11b⁺ macrophage-like cells (CD45⁺CD11b⁺Ly6G⁻Ly6C⁻), T cells (CD45⁺CD3⁺CD4⁺ or CD8⁺), B cells (CD45⁺B220⁺CD19⁺), NK cells (CD45⁺CD49b⁺NK1.1⁺ or CD45⁺CD49b⁺Nkp46⁺).

The lineage (Lin) Ab mix contained the following Abs unless otherwise noted: B220, CD19, Ter119, CD11c, CD11b, NK1.1, CD49b, CD127, Ly-6G, CD90.2.

Following Abs were purchased from BD if not mentioned differently: B220 (553089, clone RA3-6B2); CD19 (553786, clone 1D3); Ter119 (553673, clone TER-119); CD11c (12-0114-83, clone N418, eBioscience); CD11b (557397, clone M1/70); NK1.1 (553165, 550627, clone PK136); CD49b (553858, clone DX5); CD127 (12-1271-82, clone A7R34, eBioscience); Ly-6G (551461, 560599, clone 1A8); CD90.2 (553006, clone 53-2.1); SiglecF (564514, clone E50-2440); CD4 (557956, clone RM4-5), Biolegend: CD117 (105812, clone 2B8); F4/80 (123115, clone BM8); CD45.1 (110738, clone A20); CD45.2 (109831, clone 104); CD3e (100306, clone 145-2C11); CD8 (100725, clone 53-6.7); CD19 (115530, clone 6D5); CD11c (117333, clone N418); Nkp46 (137619, clone 29A1.4); CD45 (103126, clone 30-F11); CXCR2/CD182 (149303, clone SA044G4) or R&D Systems: CLEC5a/MDL-1 (FAB1639P, clone 226402).

Statistical methods

Unpaired t-test was used to compare two groups. Multiple t-test was performed to compare several cell populations between two groups and false discovery rate was accounted for using the Benjamini–Hochberg–Yekutieli procedure with Q=1%. One-way or Two-way ANOVA with subsequent post-hoc analysis was done to compare three or more groups. GraphPad Prism was used to test for statistical significance except for when noted. Matlab and Python were used for scRNAseq analysis, corresponding statistical testing is described above in section 'Neutrophil single cell RNAseq'. Python was used for patient survival analysis as detailed in section 'Bioinformatical analyses of lung adenocarcinoma patients'. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s. not significant.

REFERENCES AND NOTES

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Fig. 1. Lung tumors increase bone density in mouse models and in cancer patients. (A) Fluorescence molecular tomography-based detection of OsteoSense signal (marking areas of active bone formation) in the femoral-tibial joint of KP lung tumor-bearing mice compared to their respective age- and sex-matched littermate tumor-free controls. (B) Quantification of (A) (n = 10-12 femoral-tibial joints per group). (C) Detection of OsteoSense signal as in (A) but in LLC lung tumor-bearing mice and their tumor-free controls (n = 4 femoral-tibial joints per group). (D) *Ex vivo* confocal microscopy of representative OsteoSense signal (white) and vasculature signal (red; labeled with anti-Sca-1, anti-CD31 and anti-CD144 mAbs) in the sternum of tumor-free mice (top) and KP lung tumor-bearing mice (bottom). (E) 3D reconstruction of micro-computed tomography (CT) scans (left) and quantification of trabecular bone volume fraction (BV/TV) (right) in the distal femoral metaphysis of KP1.9 lung tumor-bearing and control mice (n = 4 mice per group). (F) CT-based trabecular bone density in patients with NSCLC and in control individuals. Left: representative axial non-contrast CT image of the 10th thoracic vertebra (T10) in a 53-year-old healthy woman who underwent non-contrast chest CT for cough and was found to have no abnormalities (control patient). Middle: a 53-year-old woman with *KRAS*⁺ NSCLC. Images are presented using the same window and level. The mean trabecular bone density of the region of interest depicted by a black oval was calculated in Hounsfield Units (HU) for all investigated individuals. Right: quantitative data from control (n = 35) and *KRAS*⁺ NSCLC (n = 35) patients. (G) As in (F), but showing mean trabecular bone density of *KRAS*⁻ (negative) NSCLC patients (n = 35) and matched controls (n = 35). All figures show mean ± SEM. Statistical significance was calculated using an unpaired t-test. **p*<0.05, ***p*<0.01, ****p*<0.001. Abbreviations: AdCre: adenovirus-Cre; KP: Kras and p53 mutant lung tumors; LLC: Lewis Lung Carcinoma; NSCLC: non-small cell lung cancer.

Fig. 2. Lung tumors increase osteoblast activity in mice. (A) Representative Goldner's Trichrome staining of distal femur sections from a tumor-free mouse (top) and a KP lung tumor-bearing mouse (bottom) (n = 4 mice per group). Osteoblasts are indicated with white arrowheads. See fig. S6A-D. (B) Number of osteoblasts per bone surface in distal femur trabecular bone from the same mice as in (A) (n = 4 mice per group). (C) Flow cytometry-based quantification of the percentage of bone marrow Ocn-YFP⁺ cells isolated from tumor-free mice and KP lung tumor-bearing *Ocn^{Cre:Yfp}* mice (n = 6 mice per group). Ocn-YFP⁺ cells were defined as 7AAD⁻ Lin⁻ CD45⁻ CD31⁻ Ter119⁻ YFP⁺. (D) Representative von Kossa staining (left) and quantification of mineralized bone (% von Kossa area, right) in femurs from the same mice as in (A) (n = 4 mice per group). (E) Left: representative images of bone formation in trabecular bone of femurs from tumor-free mice and KP lung tumor-bearing mice. Double arrows depict distance between sequential injections of calcein (green) and demeclocycline (red). # denotes trabecular bone. Right: quantification of mineral apposition rate (n = 3-4 mice per group). See fig. S7 for additional measurements. All figures show mean ± SEM. Statistical significance was calculated using an unpaired t-test. **p*<0.05, ***p*<0.01. Abbreviations: KP: Kras and p53 mutant lung tumors; Ocn: osteocalcin; YFP: yellow fluorescent protein.

Fig. 3. Ocn⁺ cells foster a tumor-promoting neutrophil response in mice. (A) Comparison of lung weight (proxy of tumor burden) in KP1.9 tumor-bearing mice with reduced numbers of Ocn⁺ cells (green: *Ocn^{Cre;Dtr}* mice treated with DT) or in tumor-bearing control mice (pink: mice lacking Cre or Dtr and treated with DT). DT was administered three weeks after tumor injection, i.e. when tumors were established. *Ocn^{Cre;Dtr}* mice that did not receive DT were used as additional controls (grey). Data show delta lung weights (pre/post DT treatment) and are pooled from four separate experiments (n = 8-29 mice per group). Statistical significance was calculated using one-way ANOVA and Tukey's multiple comparisons test. (B) Tumor burden in control mice or in mice with reduced numbers of Ocn⁺ cells. Mice are defined as in (A). Left: representative H&E-stained lung tissue sections; right: quantification of percent change in tumor area following DT treatment. Statistical significance was calculated using an unpaired t-test. Data are pooled from three independent experiments (n = 13 mice per group). (C) *Ex vivo* flow cytometry-based evaluation of neutrophils, monocytes and macrophages in lungs of tumor-bearing control mice or in mice with reduced numbers of Ocn⁺ cells, as defined in (A). Data were normalized to control (Ocn-sufficient) tumor-bearing mice and pooled from three independent experiments (n = 14-29 mice per group). Statistical significance was calculated using multiple t-tests. (D) Fold change in volume of KP lung tumor nodules pre and post anti-Gr-1 or isotype mAb treatment. Tumors were detected noninvasively by micro-computed tomography (n = 2-3 tumor nodules per mouse, 4-5 mice per group). Statistical significance was calculated using an unpaired t-test. (E) Number of CD11b⁺ Ly6G⁺ neutrophils per ml blood in KP1.9 tumor-bearing control mice (pink) or in mice with reduced numbers of Ocn⁺ cells (green). Mice are defined as in (A). Mice were analyzed three days after DT treatment (n = 4-5 mice per group) and cells were quantified by flow cytometry. Tumor-free *Ocn^{Cre;Dtr}* mice were used as additional controls (grey). Statistical significance was calculated using one-way ANOVA and Tukey's multiple comparisons test. (F) Tumor-bearing mice with reduced numbers of Ocn⁺ cells (mice depicted in black) were parabiosed with mice that had either normal numbers of Ocn⁺ cells (mouse in pink, control parabiont) or reduced numbers of Ocn⁺ cells (mouse in green, *Ocn^{Cre;Dtr}* parabiont). Left: outline of the parabiosis experiments. Middle: quantification by flow cytometry of lung tumor-infiltrating granulocytes in tumor-bearing *Ocn^{Cre;Dtr}* mice (depicted in black). Right: lung weight of the same mice (n = 4-6 mice per group). Statistical significance was calculated using an unpaired t-test. All figures show mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, n.s. not significant. Abbreviations: DT: diphtheria toxin; KP: Kras and p53 mutant lung tumors; Ocn: osteocalcin.

Fig. 4. Ocn⁺ cell-driven neutrophils show discrete tumor-promoting phenotypes and functions in mice.

(A) Flow cytometry-based detection (left) of Ly-6G⁺ SiglecF^{high or low} neutrophils from healthy lung tissue (top) and KP1.9 lung tumors (bottom). Plots are shown for gated live CD45⁺ CD11b⁺ cells. Representative cyospin images (right) are from FACS-sorted populations further stained with H&E. (B) Fold change Ly-6G⁺ SiglecF^{high} and Ly-6G⁺ SiglecF^{low} cell number in lungs from tumor bearing-mice when compared to tumor-free mice. Cells were assessed by flow cytometry (n = 6 mice per group). (C) Representative SiglecF mAb staining on cryo-preserved KP ~~lung-lung~~-tumor tissue. Tumor areas are highlighted by dotted purple lines. (D) Flow cytometry-based quantification of Ly-6G⁺ SiglecF^{high} and Ly-6G⁺ SiglecF^{low} cells in tumor-bearing lungs of mice with either preserved Ocn⁺ cells (pink: control mice treated with DT) or reduced numbers of these cells (green: *Ocn^{Cre;Dtr}* mice treated with DT) (n = 7-9 mice per group). (E) Ability of CD45.1⁺ Lin⁻ cKit⁺ hematopoietic precursors to produce tumor-infiltrating SiglecF^{high} and SiglecF^{low} neutrophils upon transfer into KP tumor-bearing CD45.2⁺ recipient control mice (pink) or mice with reduced numbers of Ocn⁺ cells (green). Mice were treated as in (D). Results are shown as fold change relative to control mice. All figures show mean ± SEM and significance values were calculated using multiple t-tests. **p*<0.05, ***p*<0.01, *****p*<0.0001, n.s. not significant. Abbreviations: KP: Kras and p53 mutant lung tumors; Lin.: Lineage; Ocn: osteocalcin.

Fig. 5. SiglecF^{high} neutrophils show tumor-promoting phenotypes and functions in mice. (A) Volcano plot showing differential gene expression between T-SiglecF^{high} and T-SiglecF^{low} cells. Genes with false discovery rate (FDR) <5% and an absolute fold change (FC) >2 are highlighted in blue and red, denoting down- and up-regulated genes, respectively, in T-SiglecF^{high} cells versus T-SiglecF^{low} cells. Statistical analysis is outlined in materials and methods. (B) Average expression levels of genes involved in angiogenesis, myeloid cell recruitment, tumor proliferation, cytotoxicity, extracellular matrix remodeling and immunosuppression in T-SiglecF^{high}, T-SiglecF^{low} and H-SiglecF^{low} cells. (C) Representative histogram (left) and quantification of gMFI (right) of ROS activity, measured by rhodamine 123 fluorescence (oxidized Dihydroamine 123) using flow cytometry, in T-SiglecF^{high}, T-SiglecF^{low} and H-SiglecF^{low} cells (n = 4-5 mice per group). (D) Representative flow cytometric dot plots showing CD11b⁺F4/80⁺ macrophages derived from splenic monocytes and cultured with T-SiglecF^{high}, T-SiglecF^{low} or H-SiglecF^{low} cells (all gated on live CD45⁺ cells). Cultures in medium alone or with CSF-1 were used as negative and positive controls, respectively. Mean macrophage frequency ± SEM are shown in parentheses. (E) Quantification of macrophage numbers as in (D) with 4-5 replicates per condition. (F) KP1.9 tumor growth in mice following tumor cell co-injection with either T-SiglecF^{high}, T-SiglecF^{low} or H-SiglecF^{low} cells (n = 4-5 mice per group). (G) Survival (Kaplan-Meier) plots of lung adenocarcinoma patients. Patients were stratified based on high (SiglecF^{high}, top 25%) versus low (SiglecF^{low}, bottom 25%) expression of the humanized SiglecF neutrophil gene signature. *p* valued calculated using Cox regression method. See Methods for details. Panels (C-F) show mean ± SEM. ***p*<0.01, *****p*<0.0001, n.s. not significant. Statistical values were calculated using one-way ANOVA (C and E) or two-way ANOVA (F). Abbreviations: CSF-1: colony-stimulating factor-1; gMFI: geometric mean fluorescence intensity; H: Healthy; KP: Kras and p53 mutant lung tumors; ROS: reactive oxygen species; T: Tumor.

Fig. 6. sRAGE contributes to the osteoblast-induced neutrophil response. (A) Bone marrow cells were cultured in osteogenic medium with serum from either tumor-free or lung tumor-bearing mice. Osteoblastic colonies were detected by alkaline phosphatase (ALP) staining. Graph shows the change in ALP⁺ (osteoblastic) colonies upon culture with serum from tumor-bearing mice compared to serum from tumor-free mice (n = 4 replicates per condition). (B) Protein content was investigated in the blood of lung tumor-bearing (TB) and tumor-free (TF) mice using protein arrays. Heat-map shows relative protein content that was detectable above background levels and reproducibly altered between two individual protein arrays. Heat map shows pooled results from the two arrays and are normalized to blood from tumor-free mice. Scale: 0.5- to 2.0-fold change. (C) Osteoblastic colony formation measured as in (A) but using bone marrow cells exposed or not to sRAGE. Graph shows the change in ALP⁺ (osteoblastic) colonies upon exposure to sRAGE compared to serum alone (n = 6 replicates per condition). (D) Flow cytometric evaluation of CXCR2 expression on developing neutrophils derived from bone marrow HSPCs of tumor-free mice. The cells were cultured without (left) or with (right) ST2 stromal cells, and with increased amounts of sRAGE (n = 3 replicates per condition). Abbreviations: CXCR2: C-X-C chemokine receptor 2; HSPCs: hematopoietic stem and progenitor cells; sRAGE: soluble receptor for advanced glycation endproducts.