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Abstract

Cancer patients with high serum squamous cell carcinoma antigen (SCCA1/SERPINB3) are commonly associated with treatment resistance and poor prognosis. Despite being a clinical biomarker, the modulation of SERPINB3 in tumor immunity is poorly understood. We found positive correlations of SERPINB3 with CXCL1/8, S100A8/A9 and myeloid cell infiltration through RNAseq analysis of human primary cervix tumors. Induction of SERPINB3 resulted in increased CXCL1/8 and S100A8/A9, which promoted monocyte and MDSC migration in vitro. In mouse models, Serpinb3a-tumors showed increased MDSC and TAM infiltration contributing to T cell inhibition and this was further augmented upon radiation. Intratumoral knockdown of Serpinb3a demonstrated tumor growth inhibition and reduced CXCL1, S100A8/A9, MDSC, and M2 macrophage infiltration. These changes led to enhanced cytotoxic T cell function and sensitized tumors to radiotherapy. We further revealed SERPINB3 promoted STAT-dependent suppressive chemokine expression, whereby inhibiting STAT activation by ruxolitinib or siRNA abrogated CXCL1/8 and S100A8/A9 in SERPINB3 cells. Patients with elevated pre-treatment SCCA and high pSTAT3 had increased intratumoral CD11b+ myeloid cell compared to patients with low SCCA and pSTAT3 cohort that had overall improved survival after radiotherapy. These findings provide a preclinical rationale for targeting SERPINB3 in tumors to counteract the immunosuppression and improve response to radiation.
Introduction

Radiotherapy (RT) is commonly used in the treatment of patients with squamous cell carcinomas, including the head and neck, esophageal, lung, and cervical cancer (1). RT can have both immunostimulatory and immunosuppressive effects, which in part determines the prognosis of cancer (1). The activation and infiltration of cytotoxic T cells post-radiation is critical to the curative activity of RT. However, tumors with an immunosuppressive tumor microenvironment (TME), dominated by myeloid cells, such as M2 macrophage polarization, myeloid-derived suppressor cell (MDSC), tend to diminish T cell activity and may be more susceptible to the suppressive immune response induced by RT (2, 3). Chemokines are a subclass of cytokines with chemotactic properties that control the migration of cells and influence the composition of tumor immune microenvironment (4). Some chemokines promote an immunostimulatory environment, such as CXCL9, CXCL10, CXCL11, CXCL16, which improve dendritic cell activation and T cell trafficking to tumors (4, 5). Conversely, CCL2, CCL5, CXCL1, CXCL8, and CXCL12 can be induced by RT and have the opposite effect to recruit suppressive immune cells, inhibit effector T cells, and often correlate with poor treatment outcome (6-8).

Squamous cell carcinoma antigen 1 (SCCA), encoded by the SERPINB3 gene locus and now known as SERPINB3, is a highly conserved cysteine proteinase inhibitor that interacts with lysosomal proteases upon lysosomal leakage and prevents cell death (9). We have recently demonstrated that SERPINB3 also protected cervix tumor cells against RT-induced cell death by preventing lysoptosis (10). In many cancers, SERPINB3/SCCA (The ELISA-based clinical assay used to measure circulating SERPINB3 is still referred to as “SCCA”) was highly expressed in tumors or in the circulation of cancer patients, including cervical, head and neck, lung, breast, and esophageal cancers, often associated with poor prognosis, treatment outcomes and recurrence (11-15). In addition, elevated SERPINB3 expression was also found in autoimmune disorders and implicated
in the induction of inflammatory cytokines (16). However, in both tumors and autoimmune
diseases the mechanistic link between SERPINB3 and immune regulation remains poorly
understood. Considering the increasing number of studies reporting the association of SERPINB3
with tumorigenesis (17), metastasis (18), prognosis and recurrence, additional roles of SERPINB3,
independent of proteinase-inhibitory activity, in tumor progression and resistance to therapy are
likely.
We have previously demonstrated that patients with persistently high levels of SCCA before
treatment and throughout the course of definitive RT had increased risk of recurrence and death
(14). Prospective cohort studies also showed the prognostic value of SCCA for monitoring the
response to RT and clinical outcome post-RT in cervical cancer patients (19). Given the
unfavorable outcomes of patients with high SERPINB3 expression, we hypothesized that
SERPINB3 promotes immune evasion by modulating suppressive immune responses that alter
tumor microenvironment and impede RT-induced anti-tumor immunity. Our data showed that
SERPINB3 tumors secreted high levels of chemokines that attract myeloid cells. These myeloid cell
populations in SERPINB3 tumors possessed potent immunosuppressive activity and inhibited T
cell activation, leading to a resistant environment to RT. Targeting CD11b+ myeloid cells or
SERPINB3 both reduced tumor growth, however, the latter in combination with RT demonstrated
more sustained inhibition of tumor growth and remodeling of infiltrating myeloid cells. We further
discovered that STAT signaling plays an essential role in inducing suppressive chemokine
expression in SERPINB3-expressing cells. Cervical cancer patients with high SERPINB3/SCCA were
associated with increased pSTAT3 and CD11b expression. Here, we present a regulatory function
of SERPINB3 in establishing a pro-tumor microenvironment and the clinical importance of
targeting SERPINB3 to improve RT-induced antitumor immunity.
Results

SERPINB3 tumors are marked by myeloid cell-rich and suppressive immune profile.

RNAseq was performed on 66 cervical tumor biopsies collected prior to (chemo)-RT. Patient and tumor characteristics of this cohort have been previously described, and are summarized in Supplemental Table 1. Patients were divided into three groups based on the distribution of SERPINB3 transcript levels; SERPINB3-low (B3/L, n=22), SERPINB3-intermediate (B3/Int, n=22), and SERPINB3-high (B3/H, n=22) groups (Figure 1A). To investigate the distinct immune signature associated with SERPINB3 expression in tumors, we focused our analysis on B3/L versus B3/H patient groups. The Immune Score (IS) was determined by xCell (20), via gene signature-based single-sample gene set enrichment analysis with the overall score representing a ranking of tumors in the dataset by lowest (IS of 0) to highest immune infiltrate. B3/H tumors showed overall higher immune scores than B3/L tumors, and this was true for patients whose cancer recurred (R) or did not recur (NR) indicating enrichment of infiltrating immune cells in the tumor microenvironment of B3/H tumors (Figure 1B and Supplemental Figure 1A). Immune cell content showed that B3/H tumors were characterized by increased myeloid cell subsets, including macrophages, monocytes, plasmacytoid dendritic cells and a small subset of CD8 T lymphocytes. In contrast, T-helper type 1 (Th1), Th2 and natural killer T cells were lower in B3/H compared to B3/L tumors (Figure 1C and Supplemental Figure 1B).

We then investigated differential expression of two major human chemokine subfamilies, CC and CXC chemokines, in the three groups (Supplemental Figure 1C). Two chemokines associated with recruitment of myeloid cells - CXCL1 and CXCL8, correlated with SERPINB3 expression (Figure 1, D and E). In contrast, expression of T- and NK-cell recruiting chemokines CXCL9, CXCL10 and CXCL16, were not associated with SERPINB3 expression (Supplemental Figure 1C). Further analysis of chemokines that attract myeloid cells demonstrated a positive correlation between SERPINB3 and
S100A8/S100A9 expression (Figure 1, F and G). These correlations were validated in the TCGA-CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma, n=306) dataset (Supplemental Figure 1D). Notably, analysis of the TCGA PanCancer Atlas showed a consistent positive correlation between SERPINB3 and CXCL1, CXCL8, S100A8, and S100A9 across multiple tumor types including bladder, breast, head and neck, lung, prostate and uterine cancers (Figure 1H). These same tumor types have high levels of SERPINB3 expression (Supplemental Figure 1E).

Of note, HPV subtype was varied in the 66 patient RNAseq cohort (Supplemental Table 1), and there was no obvious correlation between HPV positive/negative or HPV subtype (Supplemental Figure 1F). This is consistent with our previous finding that both HPV positive and HPV negative tumors and tumor cell lines express SERPINB3 (14).

SERPINB3 results in upregulation of CXCL1/8 and S100A8/A9 chemoattractants, promoting myeloid cell migration from patient-derived peripheral blood.

To study the mechanistic link between SERPINB3 and chemokine expression, we genetically altered SERPINB3 levels in human cervical cancer cells, Caski and SW756 cells (Supplemental Figure 2A), and examined the effect on chemokine production. Caski and SW756 with stable expression of SERPINB3 (Caski/B3, SW756/B3) showed increased CXCL1/8 and S100A8/A9 gene expression (Figure 2A) while downregulating SERPINB3 using shRNA (Caski/shB3) or CRISPR-Cas9-mediated deletion (SW756/CRISPR-B3KO) significantly reduced CXCL1/8 and S100A8/A9 expression (Figure 2B), when compared to their control counterparts. In addition to gene expression, significantly higher CXCL1/8 and S100A8/A9 protein expression and secretion was detected in Caski/B3 vs. Caski/Ctrl as well as SW756/B3 vs. SW756/Ctrl (Figure 2, C and D). Because Caski and SW756 are positive for HPV16 and HPV18 respectively, whether SERPINB3-induced chemokine expression is associated with HPV infection was examined using HPV-negative
cervical cancer cells, C33A. Similar to the observation in HPV-positive cells, C33A with SERPINB3 upregulation (C33A/B3) showed increased CXCL1/8 and S100A8/A9 expression (Supplemental Figure 2B). We next examined the chemotactic response of human peripheral blood mononuclear cells (PBMCs), obtained from seven patients with biopsy-proven cervical cancer prior to delivery of any treatment, to the chemokines secreted by tumor cells with high SERPINB3 expression. Supernatant collected from Caski/B3 and SW756/B3 promoted the migration of CD11b+ myeloid cells, with an average of 1.9-fold increase in Caski/B3 vs. Caski/Ctrl and 2.1-fold increase in SW756/B3 vs. SW756/Ctrl, whereas the migration of CD4+ and CD8+ T cells showed no statistical difference (Figure 2, E and F). Further analysis of migrated CD11b+ cells showed that populations migrating in response to both Caski/B3 and SW756/B3 supernatant were enriched in monocytes, and monocytic and polymorphonuclear myeloid-derived suppressor cells (Mo-/PMN-MDSCs) with an approximately 1.5-2 fold increase compared to Ctrl supernatant (Figure 2G and Supplemental Figure 3, B and C).

**SERPINB3 tumors show accumulated myeloid cells and increased tumor growth.**

Since SERPINB3 upregulated the expression of myeloid chemoattractant in vitro, we hypothesized that tumor expressing SERPINB3 attract myeloid cell infiltration and mediate in vivo TME. Human Caski/Ctrl or Caski/B3 cells were injected subcutaneously on the flank of athymic nude mice and tumor-infiltrating immune cells were analyzed (Supplemental Figure 4A). Tumor growth showed no difference between Caski/Ctrl and Caski/B3 over the course of the experiment (Supplemental Figure 4B); however, Caski/B3 tumors had a significant increase in infiltrating CD11b+ myeloid cells compared to Caski/Ctrl tumors at days 22 and 40 post-injection (Supplemental Figure 4C). M-MDSCs, TAMs, and M2 macrophages were significantly increased in Caski/B3 at both days 22 and 40, while no difference was seen in DCs, PMN-MDSCs and B cells (Supplemental Figure 4D).
Given that lymphocyte-mediated immune activity plays a role in tumor response to radiotherapy and that RT is known to reshape the TME, SERPINB3-mediated TME and its response to radiation was characterized in an immunocompetent murine model. However, there are no murine cervical tumor lines, and the commonly used alternative, TC1 cells with HPV E6/E7 gene expression, were derived from normal lung epithelial cells with relatively low chemokine expression (Supplemental Figure 5A). Therefore, constructs driving murine Serpinb3a, homologous to human SERPINB3 (21), were expressed in LL2 murine lung carcinoma cells (LL2/B3a) and an empty vector was used as a control (LL2/Ctrl) (Supplemental Figure 5B). Of note, SERPINB3 is also expressed in lung cancer (supplemental Figure 1C) and negatively associated with prognosis, providing credence to this model (11). The expression murine CXCL1/3, functionally corresponding to human CXCL1/8, and murine S100A8/9, homologous to human S100A8/9, were induced by Serpinb3a, whereas chemokines associated with T cell migration, Cxcl9 and Cxcl10 were not affected by Serpinb3a expression (Supplemental Figure 5C).

LL2/Ctrl and LL2/B3a cells were injected subcutaneously into C57/BL6 mice which were then randomized to receive a single dose of 10 Gy or sham RT (14 days post-injection). Tumor growth curves showed that RT-LL2/Ctrl had the smallest tumor volumes and RT-LL2/B3a tumor growth curve overlapped with sham-LL2/Ctrl tumors, while sham-LL2/B3a showed the fastest tumor growth (Figure 3A). This is consistent with our prior study showing that human cervical tumor cell lines expressing SERPINB3 are more radioresistant than control tumors in an athymic nude murine model (10). Tumor weights showed no statistical differences in all groups at 2-day post-RT while a more substantial increase in sham- and RT- LL2/B3a tumor growth was corresponded to increased tumor weight at 7-day post-RT compared to LL2/Ctrl counterpart (Supplemental Figure 6A). The visualization of t-distributed stochastic neighbor embedding (viSNE) plots show the unsupervised clustering of CD45+ immune cell subsets based on pre-defined markers in LL2/Ctrl
and LL2/B3a tumors (Supplemental Figure 6B). The viSNE analysis revealed that LL2/B3a tumors had an overall higher M-/PMN- MDSCs than LL2/Ctrl tumors at both pre-RT and post-RT time points. Both irradiated LL2/Ctrl and LL2/B3a had increased numbers of total CD11b+ myeloid cells, but different subsets were represented (Figure 3B). Thus, we sought to examine further the dynamic change of immune cell subsets in LL2/Ctrl and LL2/B3a tumors at different time points.

**SERPINB3 tumors are enriched for suppressive myeloid cells, further augmented by radiation.**

Similar to in vitro findings, LL2/B3a tumors had higher levels of intra-tumoral CXCL1 and S100A8/A9 expression over time, compared to sham-LL2/Ctrl (Figure 3, C and D). Radiation promoted further CXCL1 production in RT-LL2/B3a but not in RT-LL2/Ctrl (Figure 3C). Although radiation induced S100A8/A9 in both RT-LL2/Ctrl and RT-LL2/B3a tumors at 2 days post-RT, the magnitude of chemokine induction was greater in RT-LL2/B3a than RT-LL2/Ctrl, with an average of 2.3-fold and 1.8-fold increase, respectively (Figure 4D). Higher and more persistent expression of immunosuppressive chemokines in the tumor milieu of irradiated LL2/B3a tumors led us to hypothesize that the increased myeloid compartment summarized by viSNE plots differed specifically in immunosuppressive myeloid cell subtypes. Indeed, sham-LL2/B3a tumors showed consistently higher infiltration of M-MDSCs and PMN-MDSCs compared to LL2/Ctrl while radiation induced an early increase of infiltrating M-/PMN-MDSCs at 2-day post-RT in both groups; however, MDSCs in irradiated tumors remained elevated compared to sham-treated tumors at 7-days post-RT only in RT-LL2/B3a tumors (Figure 3, E and F). The number of infiltrating TAMs and M2 macrophages was higher in sham LL2/B3a versus LL2/Ctrl, with a gradual increase in both groups as the tumors grew, but no statistical change with irradiation in either genetic background (Figure 3, G and H).
To assess immunosuppressive activity of myeloid cells from LL2/Ctrl and LL2/B3a tumors, we isolated intratumoral CD11b+ myeloid cells, Ly6C+ M-MDSCs, Ly6G+ PMN-MDSCs, F4/80+ TAMs and cocultured with splenic T cells derived from non-tumor bearing mice. Intratumoral Ly6C+ M-MDSCs from both LL2/Ctrl and LL2/B3a tumors demonstrated strong inhibition towards T cell proliferation. Notably, Ly6G+ PMN-MDSCs and F4/80+ TAMs derived from LL2/B3a tumors had more significant inhibitory effects than those from LL2/Ctrl tumors (Figure 3, I and J).

Cytotoxic T cells from SERPINB3 tumors display impaired proliferation and exhausted phenotypes. With evidence of immunosuppressive TME, T cell recruitment and function is likely to be compromised in LL2/B3a tumors. CD8+ TILs showed significantly lower in sham-/RT- LL2/B3a vs LL2/Ctrl tumors at 7-day post-RT. In RT-LL2/Ctrl tumors, CD8+ TILs doubled compared to sham-treated tumors, and while statistically increased, the magnitude of increase was less in RT-LL2/B3a (Figure 4A). No difference was seen in CD4+ TILs between LL2/Ctrl and LL2/B3a tumors and a significant decrease at 2-day post-RT in both groups was associated with radiation effect (Figure 4B), consistent with radiosensitivity of in-field lymphocytes (22). The ratio of CD8+ T to Treg (CD4+CD25+FoxP3+) was significantly decreased in RT-LL2/B3a compared to sham-LL2/B3a, indicating an increase of Treg cells in LL2/B3a tumors shortly after radiation. In contrast, increased CD8+ TILs in RT-LL2/Ctrl at 7-day post-RT correlated with higher CD8+ T/Treg ratio compared to sham-LL2/Ctrl tumors (Figure 4C). Moreover, the proliferation marker Ki-67 showed lower expression in CD8+ TILs from LL2/B3a compared to LL2/Ctrl tumors, suggesting that despite of increased infiltration of CD8+ TILs, tumor-directed radiation did not promote proliferation of CD8+ T cells (Figure 4D).

Cytotoxic CD8+ T cells were further evaluated by the production of interferon gamma (IFNy) and tumor necrosis factor alpha (TNFα) following ex vivo stimulation with PMA/ionomycin. An average
of 20% of sham-treated CD8+ TILs from LL2/Ctrl and 15% from LL2/B3a tumors showed IFNγ production while the frequencies of IFNγ-producing CD8+ TILs were reduced with tumor growth in both groups (Figure 4E). CD8+ TILs taken from RT-LL2/Ctrl tumors at 2-day post-RT showed significant enhancement of both IFNγ and TNFα production following stimulation, whereas radiation-boosted IFNγ and TNFα production was not observed in CD8+ TILs from RT-LL2/B3a tumors (Figure 4, E and F). T cell receptor (TCR)-mediated activation was examined by the proliferation of CellTrace-labeled CD8+ TILs stimulated with anti-CD3/28 antibody. CD8+ TILs from sham- and RT- LL2/Ctrl tumors demonstrated stronger proliferative capacity then those derived from LL2/B3a tumors. Radiation did not have significant effects on TCR-mediated proliferation of CD8+ TILs from LL2/Ctrl while decreased proliferation was observed in CD8+ TILs from LL2/B3a tumors (Figure 4G). The impaired proliferation and decreased IFNγ and TNFα production might suggest an exhausted phenotype. Indeed, increased expression of PD-1 and CTLA-4 was observed in LL2/B3a-derived CD8+ TILs compared with LL2/Ctrl-derived CD8+ TILs. Radiation further promoted CTLA-4 expression in LL2/B3a-derived CD8+ TILs, indicating increased T cell exhaustion in LL2/B3a tumors (Figure 4H).

**Depleting CD11b+ myeloid cells in SERPINB3 tumors improves T cell activity.**

To determine whether impaired T cell activity in LL2/B3a tumors was associated with high infiltration of immunosuppressive myeloid cells, we treated LL2/B3a-tumor bearing mice with CD11b neutralizing antibody to deplete myeloid cells or IgG2b isotype control starting on day 9 post-tumor inoculation. Splenic and intratumoral depletion of CD11b+ cells was examined on day 15 and 21, where efficient depletion was observed in spleen on both days but slightly recovered in tumors on day 21 (Figure 5A). The growth of LL2/B3a tumors significantly reduced by anti-CD11b antibody treatment compared to LL2/B3a treated with IgG2b control or LL2/Ctrl tumors
A decreased total number of CD8$^+$ T cells in LL2/B3a compared to LL2/Ctrl tumors was reversed by the depletion of CD11b$^+$ cells (Figure 5C). This also relieved the suppression of CD8$^+$ T cells to enhance their activity in LL2/B3a tumors, where less responsive CD8$^+$ T cells to CD3/28-induced activation was increased in anti-CD11b-treated LL2/B3a tumors (Figure 5D). The expression of cytotoxic granules, perforin and granzyme B were significantly increased in CD8$^+$ T cells from anti-CD11b-treated LL2/B3a tumors compared with IgG2b-treated LL2/B3a and LL2/Ctrl tumors (Figure 5E). The improved T cell activity in anti-CD11b-treated LL2/B3a tumors was accompanied by reduced PD-1 and CTLA-4 expression, which were highly expressed in IgG2b-treated LL2/B3a tumors (Figure 5F).

High numbers of myeloid cells in LL2/B3a tumors can be a therapeutic target to restore T cell anti-tumor response; however, clinical trials targeting myeloid cell integrins such as CD11b/CD18 have failed to yield therapeutic benefits, due to the limitation in tolerable doses in human (23, 24). We also found that even though tumor size were smaller by anti-CD11b antibody treatment, tumor doubling time remained the same from day 14-21, suggesting that once tumors were established, the growth of tumor cells were not inhibited by CD11b$^+$ cell depletion (Supplemental Figure 7). This may be in part due to a multifaceted role of CXCL1 and S100A8/A9 secreted by LL2/B3a tumors in promoting tumor cell proliferation and survival (25). Therefore, targeting SERPINB3 may be an alternative approach to reduce tumor growth and provide therapeutic potential.

 Targeting SERPINB3 sensitizes tumors to radiation therapy and enhances T cell response.

We sought to understand the potential of targeting SERPINB3 in tumor growth inhibition and whether combination with radiation therapy could enhance antitumor immunity. To this end, we treated LL2/B3a tumors with Serpinb3a siRNA (siB3) or negative control siRNA (siNC) on day 9 post-tumor inoculation with repeated injection every 2-3 days and a single dose of 10 Gy or sham
RT was given on day 14. Knockdown of *Serpinc3a* showed an average of 65% decrease in siB3-treated tumors (Supplemental Figure 8A). Reduced tumor growth was observed in sham siB3-treated compared to sham siNC-treated tumors, and the combination of *Serpinc3a* knockdown with RT (RT/siB3) resulted in more significant tumor growth inhibition (Figure 6A and Supplemental Figure 8B). The high expression of CXCL1, and S100A8/A9 in LL2/B3a tumors showed significant decrease by *Serpinc3a* knockdown (sham/siNC vs. sham/siB3). RT induced S100A8/A9 in both RT/siNC and RT/siB3 tumors but not CXCL1, which showed induction only in RT/siNC tumors, compared to their sham counterparts (Figure 6B). Reduced suppressive chemokine secretions in siB3 tumors also led to decreased myeloid cell infiltration, where significant reduction in M-MDSCs, PMN-MDSCs, and M2 macrophages in sham/siB3 tumors was observed. Increased PMN-MDSCs after radiation was seen in both RT/siNC and RT/siB3 tumors, whereas increased M-MDSCs by RT was only observed in RT/siNC but not RT/siB3 tumors (Figure 6C). In addition, radiation-induced antitumor immunity relies on cytotoxic T cell infiltration. We observed an increase in CD8⁺ T cells and CD8⁺T/Treg ratio in sham/siB3 tumors vs. sham/siNC as well as higher ratio of CD8⁺T/Treg in RT/siB3 vs sham/siB3, suggesting increased CD8⁺ T and reduced Treg infiltration by *Serpinc3a* knockdown, and that RT-induced CD8⁺ T cell infiltration was not accompanied by significant Treg expansion in *Serpinc3a*-knockdown tumors (Figure 6D). CD8⁺ T cells in siB3 tumors also demonstrated improved cytotoxic potential with increased expression of granzyme B and perforin, which were further enhanced by radiation treatment (Figure 6E). Similarly, improved T cell activity upon anti-CD3/28 stimulation was observed in siB3 tumor-derived CD8⁺ T cells that shown markedly higher proliferative capacity than those derived from siNC tumors (Figure 6F). This correlated with lower PD-1 in RT/siB3 vs RT/siNC and CTLA-4 in sham/RT- siB3 vs siNC tumors, indicating less exhausted CD8⁺ T cells when silencing *Serpinc3a* in tumors (Figure 6G). Collectively, targeting *Serpinc3a* resulted in remolding
infiltrating myeloid cells and reduction of immunosuppressive chemokines, together with enhanced T cell function, and when combining Serpinb3a knockdown and radiation therapy, it achieved more significant inhibition in tumor progression and improved radiation-induced antitumor immunity.

**SERPINB3 mediates suppressive chemokine production through promoting STAT activation.**

Although SERPINB3 has been implicated in pro-inflammatory signaling in pancreatic cancer and Kras mutant tumors (26), the underlying molecular mechanism is unknown. To provide further insight of SERPINB3-mediated suppressive immune response, we employed a human phosphorylation pathway profiling array that contained five cancer-associated pathways - MAPK, AKT, JAK/STAT, NF-κB and TGF-β, and identified 14 proteins with upregulated phosphorylation (fold change ≥ 2) and 4 proteins with downregulation (fold change ≤ 0.5) in Caski/B3 compared to Caski/Ctrl cells (Supplemental Figure 9). Among those with increased phosphorylation, signal transducer and activator of transcription (STATs) proteins, including STAT1/2/3/5, showed the highest magnitude of change in phosphorylation (Figure 7A). Phosphorylation of STAT1 and STAT3 in response to SERPINB3 expression was examined in Caski and SW756 cells, where the induction of SERPINB3 resulted in increased pSTAT1 and pSTAT3 (untreated, Figure 7B and Supplemental Figure 10A). In contrast, knockdown of SERPINB3 led to reduced pSTAT1 and pSTAT3 expression (Supplemental Figure 10B). Thus, we hypothesized that SERPINB3 mediates suppressive chemokine production through promoting STAT signaling activation. FDA-approved small molecule inhibitor, ruxolitinib, inhibiting the phosphorylation of STAT1 and STAT3 was confirmed by immunoblotting (Figure 7B and Supplemental Figure 10A). The initially high secretion of CXCL1/8 and S100A8/A9 in Caski/B3 and SW756/B3 cells was significantly suppressed by ruxolitinib, suggesting an essential role of STAT activation in chemokine production in SERPINB3
cells (Figure 7C and Supplemental Figure 10C). To further understand whether STAT signaling directly regulates chemokine expression in SERPINB3 cells, we used siRNA to individually silence STAT1 or STAT3 (Figure 7D and Supplemental Figure 10D). The expression of CXCL1/8 and S100A8/A9 was decreased in SERPINB3 cells by silencing either STAT1 or STAT3, and the simultaneous knockdown of both STAT1 and STAT3 did not lead to more significant suppression in CXCL1 and CXCL8. However, knockdown of both STAT1 and STAT3 achieved more effective inhibition of S100A8 and S100A9 in SERPINB3 cells (Figure 7E and Supplemental Figure 10E).

Moreover, both STAT1 and STAT3 proteins showed increased phosphorylation and nuclear translocation in SERPINB3 cells, indicating upregulated transcriptional activity in promoting downstream gene expression (Figure 7F). Notably, an increase in phosphorylated STAT1/3 was not only observed in nucleus but also cytoplasm, which suggests SERPINB3 may be involved in mediating upstream cytoplasmic kinase of the signaling cascade to promote STAT activation. Thus, we performed co-immunoprecipitation of JAK1 and found increased interaction of JAK1 with STAT1 and STAT3 in Caski/B3 and SW756/B3 compared to Caski/Ctrl and SW756/Ctrl cells (Figure 7G). These data show that SERPINB3 mediated STAT activation through promoting JAK/STAT interaction, leading to increased STAT transcriptional activity and chemokine production.

Elevated serum SCCA and high tumor pSTAT3 is associated with CD11b expression and poor cancer-specific survival after CRT.

In corresponding to in vitro findings, mouse LL2/B3a tumors with initially high pSTAT3 expression was significantly reduced by the intratumoral knockdown of Serpinb3a (LL2/B3a+siB3), evident by the immunostaining of pSTAT3(Tyr705) (Figure 8A and Supplemental Figure 11A). The reduction of pSTAT3 in Serpinb3a-knockdown tumors further correlated with reduced CD11b⁺ myeloid cell expression (Figure 8B and Supplemental Figure 11A).
To evaluate the clinical implication in our findings, tissue microarray containing pre-treatment cervix tumor biopsy specimens obtained from patients with biopsy-proven invasive cervical carcinoma were immunostained for pSTAT3(Tyr705) and myeloid cell marker CD11b. Pretreatment serum SCCA value from 72 cancer patients with an average of 9.16 ng/ml presented a significant cutoff point for cancer specific survival in our patient population. Patients with elevated pretreatment SCCA (≥9.16 ng/ml) had worse survival than those with low SCCA at the time of diagnosis (Figure 8C and Supplemental Figure 11B), in agreement with our previous study reporting SCCA as a clinical biomarker. The histoscore of pSTAT3 evaluated by immunohistochemistry was determined through the combined factors of the intensity and percentage of stained cells within the tumor proportion of TMA cores using an attribute cutoff of 100 for high or low pSTAT3 expression (Figure 8D). In high pretreatment SCCA patient cohort (≥9.16 ng/ml), 71% of the population showed high pSTAT3 histoscore as opposed to 41% of high pSTAT3 in low pretreatment SCCA patients (<9.16 ng/ml) (Figure 8E). Although pSTAT3 was not an independent prognostic factor for survival in our cohort, the patients with elevated serum SCCA, along with high pSTAT3 were associated with increased CD11b expression (Figure 8F) and poor cancer-specific survival on both Univariate and Multivariate analysis, along with FIGO Stage (Figure 8C, Supplemental Table 2). In contrast, the majority of patients with pretreatment SCCA <9.16 ng/ml had low pSTAT3 histoscore and was correlated with low CD11b expression. This cohort had the highest cancer-specific survival (Figure 8C). Overall, SCCA is a strong clinical biomarker and when combining with pSTAT3 expression, it may indicate an unfavorable tumor microenvironment and provide an opportunity for selection of patients for anti-STAT and/or anti-SERPINB3 directed therapies.
Discussion

In this study, we revealed that SERPINB3 modulated the TME towards an immunosuppressive phenotype by upregulating CXCL1/8 and S100A8/A9 production, to facilitate tumor growth and impede the success of RT. These chemokines were increased in the tumors of both human SERPINB3-expressing Caski xenograft and murine Serpinb3a-expressing LL2 syngeneic mouse models, resulting in increased infiltrating M-MDSCs, PMN-MDSCs, and M2 macrophages. Radiation-induced T cell responses were compromised by the suppressive microenvironment in SERPINB3 tumors. In contrast, targeting SERPINB3 in tumors reversed these effects on the TME, which demonstrated reduced immunosuppressive chemokine production and myeloid cell infiltration, leading to enhanced T cell activity. More importantly, targeting SERPINB3 in combination with RT showed significant tumor growth inhibition and improved RT-induce T cell immunity. It is worth noting that the correlation between SERPINB3 and CXCL1/8, S100A8/A9 was conserved across several cancers known to have high SERPINB3 expression and often associated with poor treatment outcomes, suggesting wide application of our study to a variety of tumors with SERPINB3 expression.

The association between SERPINB3 and chemokines has been reported in atopic dermatitis and psoriasis, whereby downregulation of SERPINB3 in keratinocytes was associated with reduced expression of CXCL1, 5, 8 (27), and S100A8 (16). Catanzaro and colleagues showed that SERPINB3 was a downstream mediator of mutant Ras-induced tumorigenesis and knockdown of SERPINB3 led to decreased IL-6, CXCL1, CXCL8 production suppressing tumorigenesis (26). In patients with cervical cancer and esophageal squamous cell carcinoma, high expression of SERPINB3 was associated with lymph node metastasis (28-30); however, the underlying causes are unknown. Given the crosstalk between chemokines and immune cells, we revealed that the secretion of
CXCL1/8 and S100A8/A9 by SERPINB3-expressing tumors resulted in increased immunosuppressive myeloid cell infiltration and these populations have been shown to promote tumor progression by disrupting T cell activation signaling (31), facilitating tumor angiogenesis (32), neoplastic cell invasion and forming a pre-metastatic niche (33). The increased immunosuppressive myeloid cell populations provided a possible mechanism for high metastatic tendency of SERPINB3 tumors and represent a potential target for tumor control.

Strategies of targeting myeloid cells to improve T cell-mediated immunity or alter myeloid cell polarization and infiltration have been studied extensively in the preclinical space. For instance, CXCR1/2 and CCR2 inhibitors interrupted CXCL8/CXCR1-2 and CCL2/CCR2 axis, blocking the recruitment of TAMs and MDSCs (34). Colony-stimulating factor 1 receptor (CSF-1) inhibitor repolarized TAMs from M2-like to M1-like phenotype and depleted TAMs to reduce suppressive immune responses but studies also found increased PMN-MDSC infiltration through a CXCR2-dependent manner (35-37). Other compensatory actions, such as expansion of monocytes and macrophages when targeting granulocytes, and compensatory upregulation of PD-L1 and CTLA-4 by untargeted myeloid cells have also been reported (38, 39). This limits the therapeutic efficacy of myeloid cell-targeting strategies. Similarly, when we depleted CD11b+ myeloid cells in SERPINB3 tumors, initial tumor growth inhibition and improved T cell activities were observed. However, after initial tumor growth delay, we noticed no difference in tumor doubling time between CD11b-depleted and non-depleted tumors and an immune cell population with intermediate levels of CD11b expression appeared in CD11b-depleted tumors. This suggests a combined therapy or additional strategies to overcome compensatory mechanisms triggered by myeloid cell-targeting therapies might be required.
Hyperactivated STAT3 signaling has been shown to mediate immunosuppression through tumor cell intrinsic and extrinsic mechanisms, and associated with poor clinical prognosis in many cancers, including cervical, lung and head and neck cancer (40-42), where elevated SERPINB3 expression is also frequently observed. Increased STAT3 activity was found to inhibit the production of immunogenic cytokines/chemokines, induce the expression of PD-1/PD-L1, and regulate the suppressive immune activities in Tregs and MDSCs (43-45). Therefore, the potential of inhibiting STAT activity to improve therapeutic responses have been explored in many preclinical studies. Ruxolitinib, the first FDA-approved JAK/STAT pathway inhibitor, successfully triggered tumor regression in preclinical mouse models; however, clinical trials of pancreatic adenocarcinoma (46), breast cancer (47), colorectal cancer (48), and lung cancer (49) showed lack of efficacy and very limited or no overall survival benefit. Among few other ongoing trials, a phase I study in glioblastoma (NCT03514069) showed a promising preliminary result from combing ruxolitinib with radiation and temozolomide (50). The consideration of using STAT inhibitor in combination with radiation was also found in a recently completed trial (NCT01904123) using STAT3 inhibitor - WP1066, for patients with recurrent malignant glioma. Their preclinical study showed that STAT3 inhibitor and irradiation reprogrammed the immunosuppressive glioma TME by improving dendritic cell maturation and interactions with T cells (51). Of note, HPV-related cancers including cervical and head and neck, often showed hyperactivated STAT3 due to virus-associated inflammatory responses (52). Ruxolitinib demonstrated in-vitro effects on facilitating Cisplatin-induced cell death in HPV-positive cervical cancer cells (53); however, in vivo efficacy has not been investigated and whether this success can transition into clinical trials remain unclear.
In addition, knowing that STAT transcriptional activity is also involved in T-cell function (54), and other facets of the immune response, direct inhibition of this pathway may unintentionally tip the immune axis back in favor of the tumor. Here, we provided the rationale for targeting an upstream, tumor-specific signal - SERPINB3, to be a more effective approach in the clinical setting. Silencing SERPINB3 leads to a reduction in infiltrating immunosuppressive myeloid cells and in return, enhances T cell responses. The changes in TME further render radiation treatment effective in previously radioresistant SERPINB3 tumors. In addition to impacting myeloid-derived cell recruitment, SERPINB3 silencing also abrogated CTLA4 expression. The potential connection between SERPINB3 and immune checkpoints has also been reported in HPV-negative head and neck squamous cell carcinoma, where patients with high SERPINB3 expression corresponded to increased PD-L1 and PD-L2 (55). Similarly, genome-level and IHC showed upregulated PD-L1 in SERPINB3-high ovarian and esophageal tumors (56). Although SERPINB3 knockdown improved cytotoxic T cell function, an increase in PMN-MDSCs and slightly higher PD-1 expression were observed following RT. Therefore, a combined therapy of targeting SERPINB3 and immune checkpoint inhibitors may simultaneously reduce immunosuppressive chemokine-associated induction of myeloid cells and prevent T cell exclusion and dysfunction, leading to maximal RT-induced antitumor immunity. HPV subtype or positivity did not appear to be associated with SERPINB3 expression in our cohort, thus this may be a viable approach for patients with both types of cervical cancer.

Our findings that SERPINB3 modulates the crosstalk between immune and cancer cells via secretion of CXCL1/8 and S100A8/A9 implicates this protease inhibitor member of the SERPIN superfamily in a key tumor strategy to evade the anti-tumor immune responses and resist therapies such as radiation. This study implicates SERPINB3 in promoting interaction with and
activation of STATs by upstream kinases, and the direct molecular mechanism is major focus of
current study. Targeting SERPINB3 reprograms immunosuppressive environment and sensitizes
tumor to radiation therapy. This also presents several potential therapeutic combinations, such
as STAT inhibitors or immune checkpoint blockade, to further improve treatment responses for
cancers with elevated SERPINB3 expression.
Methods

Cell lines and plasmids

Caski, SW756, C33a were purchased from the ATCC and Lewis lung carcinoma (LL2/LLC) were gifted by Dr. Dinesh Thotala (Washington University School of Medicine, St Louis). Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) or Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin-streptomycin. Generation of SERPINB3 CRISPR-Cas9 knockout cells was described previously (10). SERPINB3 stable expression cells were generated using pULTRA lentiviral vector (Addgene #24129) containing human SERPINB3 or pLV-C-GFPSpark vector (Sino Biological LVCV-35) containing mouse Serpinb3a GFP-tagged fusion proteins. SERPINB3 knockdown cells were transduced with scramble shRNA (Addgene #1684) or SERPINB3 shRNA (Sigma Mission shRNA, TRCN0000052400). Genetically modified cells were generated through a lentivirus system by transfection of human 293T packaging cells. All cell lines were grown in monolayer at 37°C with 5% CO2 and periodically tested for Mycoplasma contamination.

RNA sequencing and TCGA data analysis

RNA sequencing (RNAseq) was performed on pre-treatment tumor biopsies obtained from patients enrolled on a prospective tumor banking study with written informed consent (Washington University IRB No. 201105374). Tumor samples with extracted RNA exceeding thresholds for quality and quantity as defined by The Cancer Genome Atlas were submitted for whole transcriptome sequencing (n=66). PolyA selection was performed before multiplexed sequencing (Illumina HiSeq 3000, 1 × 50 nt, approximately 40 million reads per sample). Sequenced reads were aligned to human reference genome GRCh38 (r90) using STAR v2.7.0f and aligned reads were sorted and indexed using sambamba v0.6.9. Gene expression was quantified
using featureCounts v1.6.4 to obtain read counts and cufflinks v2.2.1 to obtain normalized FPKM. In downstream analyses, genes with consistently low expression (i.e., <1 FPKM or <200 reads) in at least 95% of samples were excluded, as reported previously (57). Raw sequencing reads and expression data are available on GeneExpression Omnibus (GEO): accession number: GSE151666. The Cancer Genome Atlas (TCGA) RNAseq data were obtained through cBioPortal (https://www.cbioportal.org/). Correlation between SERPINB3 expression and that of other genes was evaluated by Spearman’s correlation coefficient. P-values were adjusted for multiple testing by the method of Benjamini & Hochberg, with adjusted p-value < 0.05 considered to be significant. Immune cell population and enrichment score were analyzed using xCell analysis, a gene signature-based method to estimate cell composition in bulk transcriptomic data (20). For correlation analysis and enriched immune cell gene signature, p-value were corrected with the false discovery rate < 0.05. Heatmaps were generated using GraphPad Prism base on the average scores for each immune cell subtypes in our predefined patient groups.

PBMC isolation and transwell assay

Fresh primary PBMCs were obtained from patients planned to undergo radiation therapy with brachytherapy for cervical cancer and had enrolled on a prospective biospecimen banking protocol (Institutional Review Board Protocol #201105374). Fresh blood was collected in EDTA separator tubes and peripheral blood mononuclear cells (PBMC) were immediately isolated using Lymphoprep and SepMate-50 (Stemcell Technologies) centrifugation tubes, according to the manufacture’s instruction. Transwell assay was performed using 8-µm Transwells (Falcon). Supernatants were collected from cells cultured in complete growth media for 48 h and loaded in the lower chamber of the transwell. PBMCs were loaded to the upper transwell for a 4 h migration period. Migrated cells were phenotyped by flow cytometry.
Flow cytometry and data analysis

Single cell suspensions were blocked with either Human TruStain FcX Solution (422301, Biolegend) or mouse TruStain FcX PLUS (anti-mouse CD16/32) Antibody (S17011E, Biolegend) to avoid nonspecific Fc receptor binding and stained with LIVE/DEAD Fixable Dead Cell Stain Kit (MACS) to exclude dead cells. For surface staining, cells were incubated with the appropriate antibodies for 30 min at 4 °C. Intracellular cytokine and nuclear staining was performed after surface staining using Cyto-Fast Fix/Perm Buffer Set and True-Nuclear Transcription Factor Buffer Set, respectively (BioLegend). Stained cells were analyzed using MACSQuant Analyzer 10 Flow Cytometer. Antibody information is shown in Supplemental Table 3. Data analysis including viSNE and FlowJo plugin FlowSOM were performed on FlowJo v.10 (TreeStar). A range of 20,000 to 60,000 live cells was acquired and individual flow cytometry data from each group were combined into a single data file for generating viSNE. Color-coded subpopulations were gated by pre-defined markers for each immune cell types and overlaid to the viSNE plots for total CD45+ cells from tumors. All flow cytometry gating plots, histograms, and statistics were generated on FlowJo.

Mouse tumors with anti-CD11b antibody, siRNA and/or radiation treatment

For xenograft models, female athymic nude mice aged 6-7 weeks old (Charles River) were injected subcutaneously with 5×10⁶ Caski/Ctrl or Caski/B3 cells suspended in serum-free IMDM and 50% Matrigel Basement Membrane Matrix (Corning) to a final volume of 100μl on their flank. For immunocompetent models, female C57/BL6 mice aged 7-8 weeks (Charles River) were injected subcutaneously with 5×10⁵ LL2/Ctrl or LL2/B3a cells suspended in 100μl of PBS into the right flank. To deplete myeloid cells, mice were treated with anti-CD11b antibody (Ultra-LEAF purified anti-mouse CD11b, Biolegend) or isotype IgG2b as control (Ultra-LEAF purified rat IgG2b, Biolegend).
Antibodies were administered through intraperitoneal injection at the initial dose of 300µg in 150µl PBS on day 10 post-tumor inoculation, and a subsequent dose of 150µg in 100µl PBS every three days. To knock down Serpinb3a, mice received either mSerpinb3a siRNA (5’-ACAUCGAAUUAAACUUCAUt-3’; 5’-AUGAAGUUAAAUUCGAUGUtt-3’; ID:s73336, Thermo) or control siRNA (Ambion in vivo negative control #1, Thermo) complexed with Invivofectamine 3 (Thermo Fisher) as per manufacturer’s protocol. Mice received three intratumoral injections of 10µg siRNA on day 9, 11, 13 post-tumor inoculation before radiation treatment performed on day 14. On day 16 and 19, mice received 20µg siRNA via intraperitoneal injection. For radiation treatment, mice were randomized to receive sham or 10Gy RT using the Xstrahl Small Animal Radiation Research Platform (SARRP) 200 (Xstrahl Life Sciences). Tumor volume was measured twice weekly and calculated by \( \text{length} \times \text{width}^2 / 2 \). For tissue dissociation, tumors were manually dissected and digested with 1 mg/ml Collagenase, 0.5 mg/ml hyaluronidase, and 10 mg/mL DNase I type IV (Sigma), and transferred to a tissue disaggregator Medicon (Becton Dickinson) using CTSV Medimachine II (Becton Dickinson). Animal work was approved by the Washington University Institute Institutional Animal Care and Use Committee (Protocol #20-0470).

**T cell suppression assay**

Intratumoral myeloid cells were isolated from dissociated tumors using MojoSort mouse CD11b selection kit, biotin anti-mouse Ly6C, biotin anti-mouse Ly6G, biotin anti-mouse F4/80 antibodies, and streptavidin nanobeads (Biolegend) through magnetic purification. Splenic T cells were isolated from non-tumor bearing mice and labeled with CellTrace Violet (Thermo Fisher) to evaluate proliferation. Purified myeloid cells were co-cultured with anti-CD3/CD28-activated T cells at a ratio of 1:1 for 4 days. The suppression was determined by CellTrace dilution using FACS comparing to the proliferation of anti-CD3/CD28-activated T cells without myeloid cell co-cultured.
Ex vivo T cell stimulation

T cells isolated using MojoSort mouse CD3 T cell isolation kit (Biolegend) were labeled with CellTrace violet and activated with CD3/CD28 Dynabeads (Thermo Fisher) for 4 days to evaluate proliferation. To examine TNF and IFNγ, T cells were stimulated with 500x Cell Activation Cocktail containing 40.5 µM phorbol-12-myristate 13-acetate (PMA) and 669.3 µM ionomycin (Biolegend) in the presence of 5 µg/mL BFA (Biolegend) for 5 h and stained with surface/intracellular markers for FACS analysis.

Serum SCCA and tissue microarray immunohistochemistry

Pretreatment serum SCCA was evaluated by ARUP National Reference Laboratory (Salt Lake City, UT, USA) using ELISA and tissue microarray was generated from untreated human tumor specimens, as described previously (14). TMA sections were sent to HistoWiz Inc for IHC staining for CD11b (1:100, Abcam ab224800) and IHC for pSTAT3 (1:200, Sigma SAB4300033) was performed by Washington University AMP Core Labs. Mouse tumor sections were stained with pSTAT3 (1:150, Invitrogen PA5-121259) and CD11b (1:500, Invitrogen PA5-79532), using Pierce peroxidase IHC detection kit (Thermo Fisher). QuPath V0.3.2 software was used for automated analysis using surface and cytoplasmic staining to determine percent cells positive for CD11b. The staining scores for pSTAT3 were evaluated by the pathologist and calculated by (% of positive stained tumor cells x staining intensity ranged from 0-3). Values from at least two cores from each sample were considered valid and an average score was taken.

siRNA knockdown, RNA extraction and qPCR
Lipofectamine RNAiMAX (Thermo Fisher) was used for STAT1 siRNA (ID: SASI_Hs02_00343387, SASI_Hs01_00098937, Sigma), STAT3 siRNA (ID: SASI_Hs01_00121206, SASI_Hs01_00061860, Sigma), and negative control siRNA (SIC001, Sigma) transfection, according to the manufacturer's instructions. RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma) and reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative polymerase chain reaction (qPCR) was performed using PowerUp SYBR green PCR Master Mix (Applied Biosystems) and the Applied Biosystems 7900 Fast real-time PCR system and software. Each sample was performed in triplicate, gene expression levels were normalized to GAPDH and fold changes were calculated using the \( \Delta\Delta C_t \) method. Sequences of primers are detailed in Supplemental Table 4.

**Enzyme-linked immunosorbent assay (ELISA)**

Cell culture supernatant was collected at 48 h after fresh media was added to the adherent cells in monolayer. Quantification of human/mouse chemokines in tissue culture supernatants and tissue homogenates was performed using a commercially available human CXCL1/GRO alpha, human IL-8/CXCL8, human S100A8/S100A9 Heterodimer, mouse CXCL1/KC, and mouse S100A8/S100A9 Heterodimer DuoSet ELISA kit from R&D Systems. Mouse GRO gamma ELISA Kit was obtained from Abcam. Chemokine concentration in samples was determined by interpolation from a standard curve.

**Phosphorylation protein array**

Human Phosphorylation Pathway Profiling Array C55 consisted of the detection of 55 phosphorylated proteins (RayBiotech). Same amount of protein from each sample was used for screening and assays were performed according to the manufacturer's instruction. Array blots
were scanned with Bio-Rad ChemiDoc MP imaging system and images were processed using Protein Array Analyzer plug-in (http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.html) of the ImageJ program.

Co-immunoprecipitation and immunoblotting

Immunoprecipitation of Jak1 was performed using Pierce co-immunoprecipitation kit (Thermo Fisher). Cell fractionation was carried out using NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Fisher). The purity of non-nuclear and nuclear fractions was determined using GAPDH and lamin A/C, respectively. For immunoblotting, cells were lysed with RIPA buffer (Cell signaling) supplemented with proteinase/phosphatase inhibitors (Thermo Fisher). Proteins concentration was determined using BCA (Thermo Fisher) and proteins were electrophoresed on 4–20% gradient gels (Bio-Rad), transferred to PVDF blot using the Trans-Blot TurboTransfer system (Bio-Rad), and incubated with antibodies shown in Supplemental Table 3. Chemiluminescence was detected by using ECL reagent (Cytiva) and visualized using the Bio-Rad ChemiDoc MP imaging system and Image Lab software (Bio-Rad).

Statistics

Statistical analyses was performed using GraphPad Prism version 8 and all values are reported as mean ± SEM. Two-tailed unpaired t test or Mann-Whitney U test was used for two groups comparisons. One-way or two-way ANOVA was used for multiple comparisons, followed by post hoc analysis. Statistics used are specified in the figure legends. P values of less than 0.05 were considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Study approval
All experiments were performed in accordance with relevant guidelines and regulations and approved by the Washington University Institutional Biological & Chemical Safety Committee under protocol #12737 (Ver.2.1). All mouse experiments were approved by the Washington University Institute Institutional Animal Care and Use Committee (Protocol #20-0470). Human subjects research was approved with informed consent by the Washington University Institutional Review Board (No. 201105374).

Data Availability
The data generated in this study are available within the article and its supplementary data files. RNA sequencing data are available on GeneExpression Omnibus (GEO): accession number: GSE151666. RNA sequencing from The Cancer Genome Atlas (TCGA) consortium was obtained through cBioPortal (http://www.cbioportal.org/). This study did not generate new analytic code.

Author contributions
LC and SM conceptualized the study. LC designed and conducted experiments, acquired data, performed formal analysis, and wrote the manuscript. VS, SW, RF and JY conducted experiments and acquired data. MI, FR, KJ, JZ, and PC contributed to RANseq data curation and analysis. LS interpreted and analyzed histology data. YH and JL assisted with statistical analysis. SG, CJL, CS, PWG, and JKS provided resources. SM supervised the study, acquired data, performed formal analysis, acquired funding, and wrote the manuscript. All authors reviewed and edited the manuscript.

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References


Figure 1

Figure 1. SERPINB3 tumors are marked by myeloid cell-rich and suppressive immune profile. (A) Normalized SERPINB3 transcript in cervical tumor biopsies from RNAseq was distributed by reads per kilobase of transcript per million mapped reads (RPKM). (B) Boxplots along with individual data points show xCell immune scores in recurrent (R)/non-recurrent (NR) SERPINB3-low (B3/L) and SERPINB3-high (B3/H) tumors. *P < 0.05, one-way ANOVA test. (C) Heatmap of enriched immune cell subpopulation was generated through xCell immune infiltrate prediction. Color intensity is proportional to average xCell score for each population across samples. (D-G) Spearman’s correlation of SERPINB3 with the expression of (D) CXCL1, (E) CXCL8, (F) S100A8, (G)
S100A9 was performed using RNAseq from 66 cervical tumor biopsies collected prior to (chemo)-RT. (H) SERPINB3 expression correlated with CXCL1, CXCL8, S100A8, S100A9 expression in multiple cancer types. Analysis was performed using TCGA PanCancer Atlas and numeric values indicate Spearman’s correlation coefficient. BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; HNSC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; UCEC, uterine corpus endometrial carcinoma.
Figure 2. SERPINB3 results in upregulation of CXCL1/8 and S100A8/A9 chemoattractants, promoting myeloid cell migration from patient-derived peripheral blood. (A) Cells were transduced with pUltra vector (Caski/Ctrl, SW756/Ctrl) or pUltra-SERPINB3 (Caski/B3, SW756/B3) and CXCL1/8 and S100A8/A9 expression was examined by qPCR. (B) Caski cells were transfected with scrambled negative control shRNA (Caski/shCtrl) or shRNAs specific SERPINB3 (Caski/shB3); SW756 cells were transduced with CRISPR control vector (SW756/CRISPR-Ctrl) or CRISPR-Cas9 for SERPINB3 knockdown (SW756/CRISPR-B3KO). The expression of CXCL1/8 and S100A8/A9 was examined by qPCR. Gene expression were normalized to GAPDH and fold changes were calculated by comparing to the expression levels in parental cells (Caski WT or SW756 WT). (C) Intracellular chemokine protein was measured by ELISA and the expression levels were normalized to total...
protein concentration. (D) Supernatant was collected from adherent cells in monolayer and chemokine secretion was measured by ELISA. Data are presented as mean ± SEM of n = 3 independent experiments, *P < 0.05, **P < 0.01, ***P < 0.001 using (A-B) Mann-Whitney test (C-D) one-way ANOVA with Tukey’s post hoc test. (E-G) PBMC migration towards supernatant collected from cancer cells was examined by Transwell assays and the migrated PMBC populations were analyzed by flow cytometry (Supplemental Figure 3A). Fold changes were calculated as the percentage of migrated (E) T and myeloid cells, (F) T cell subsets and (G) myeloid cell subsets in Caski/B3 or SW756/B3 relative to Caski/Ctrl or SW756/Ctrl supernatant. Data are shown as mean ± SEM, ns, no significance; *P < 0.05, **P < 0.01, ***P < 0.001 using two-tailed one sample T test against 1. Each dot represents the mean of duplicate values for a single donor sample (n=7).
Figure 3. SERPINB3 tumors are enriched for suppressive myeloid cells, further augmented by radiation. (A) Tumor growth of C57/BL6 mice with LL2/Ctrl tumors (blue lines) and LL2/B3a tumors (red lines) randomized to receive sham-treated (solid lines) or 10Gy RT at day 14 (dotted lines). Significance was determined by two-way ANOVA, *P < 0.05; ***P < 0.001. (B) viSNE plots show flow cytometry analysis of total viable CD45+ immune cells from tumors with separate clustering by pre-defined cell surface markers, including M-MDSCs (CD11b+Ly6G-Ly6Chigh), PMN-MDSCs (CD11b+Ly6G+), TAM (CD11b+Ly6G-F4/80+), M2 macrophages (CD11b+Ly6G-F4/80+CD163+) and lymphocytes (CD45+CD11b-). (C-D) Chemokines, CXCL1 and S100A8/A9 in
tumor homogenates were examined by ELISA. Data was normalized to the protein concentration for each tumor homogenate. (E-H) Cumulative data from FACS analysis show alteration of immune cell infiltration by SERPINB3 expression and radiation in LL2 tumors. The graphs represent the frequencies of (E) CD11b+Ly6G-Ly6Chigh M-MDSCs, (F) CD11b+Ly6G+ PMN-MDSCs, (G) CD11b+Ly6G-F4/80+ TAMs, (H) CD11b+Ly6G-F4/80+CD163+ M2 macrophages in total tumor infiltrating leukocytes (TILs). Data in C-H are shown as mean ± SEM and each dot represents a biologically independent animal; * indicates comparisons between LL2/Ctrl and LL2/B3a; † indicates comparisons between sham-treated and RT; *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA with Tukey’s post hoc test. (I-J) Myeloid cell subtypes were isolated from tumors and cocultured with CellTrace-labeled splenic T cells at a ratio of 1:1 for 4 days. Anti-CD3/28 antibodies were added to stimulate T cell proliferation. Histograms show the percentage of divided cells. Percentages of suppression were calculated by comparing to the dilution of CellTrace in splenic T cell without myeloid cell co-culture. Data in I-J are shown as mean ± SEM; *P < 0.05 using Mann-Whitney test.
Figure 4. Cytotoxic T cells from SERPINB3 tumors display impaired proliferation and exhausted phenotypes. Cumulative data from FACS analysis of (A) CD3+CD8+ T cells and (B) CD3+CD4+ T cells in tumors. (C) The ratio of CD8/Treg represented the infiltrating percentage of CD8+ T cells relative to CD4+CD25+FoxP3+ regulatory T (Treg) cells. (D) Frequencies of Ki-67+ CD8+ T cells in total infiltrating CD8+ T cell population were analyzed by flow cytometry. (E-F) Intratumoral T cells were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 5 h and the expression of IFN-γ and TNF was examined by intracellular staining using flow cytometry. Protein transport inhibitor, brefeldin A was used to block the protein transport processes and cytokine release. Positive expression was normalized to cells without PMA/ionomycin stimulation (basal
levels). Box plot whiskers span minimum and maximum; lines represent median. (G) CellTrace-labeled intratumoral T cells were stimulated with anti-CD3/28 antibody for 4 days and cell proliferation was determined by the dilution of CellTrace. (H) The expression of PD-1 and CTLA-4 was examined by flow cytometry, shown in mean fluorescence intensity (MFI). Data are shown as mean ± SEM and each dot represents a biologically independent sample; * indicates comparisons between LL2/Ctrl and LL2/m; † indicates comparisons between sham-treated and RT; *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA with Tukey’s post hoc test.
Figure 5. Depleting CD11b+ myeloid cells in SERPINB3 tumors improves T cell activity. (A) Representative plots show the depletion of CD11b+ cells in tumors and spleens on day 15 and day 21 post-tumor inoculation, gated on CD45+CD11b+ cells. (B) Tumor growth of LL2/ Ctrl tumors (blue line), and LL2/B3a tumors treated with CD11b antibody (red dotted line) or IgG2b antibody (red solid line). Significance was determined by two-way ANOVA, ***P < 0.001. (C) The numbers of infiltrating CD8+ T cells in 5x10^5 total tumor cells were determined by flow cytometry. (D) CellTrace-labeled intratumoral T cells were stimulated with anti-CD3/28 antibody for 4 days and cell proliferation was determined by the dilution of CellTrace. (E) Representative histograms of
intracellular cytokine staining of granzyme B and perforin in CD8+ T cells. (F) The expression of PD-1 and CTLA-4 was examined by flow cytometry, shown in mean fluorescence intensity (MFI). Data in C-F are shown as mean ± SEM and each dot represents a biologically independent sample; *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA with Tukey’s post hoc test.
Figure 6. Targeting SERPINB3 sensitizes tumors to radiation therapy and enhances T cell response. (A) Tumor growth curves of LL2/B3 treated with negative control siRNA (siNC, red lines) and Serpinb3a siRNA (siB3, green lines) with or without radiation treatment (sham-solid lines; RT-
dotted lines). Significance was determined by two-way ANOVA, ***P < 0.001. (B) Chemokines, CXCL1 and S100A8/A9 in tumor homogenates were examined by ELISA. Data was normalized to the protein concentration for each tumor homogenate. (C-D) Cumulative data from FACS analysis show the frequencies of immune cell populations including CD11b+Ly6G-Ly6Chigh M-MDSCs, CD11b+Ly6G+ PMN-MDSCs, CD11b+Ly6G-F4/80+ TAMs, CD11b+Ly6G-F4/80+CD163+ M2 macrophages, CD3+CD8+ T cells in total tumor infiltrating leukocytes (TILs) and the ratio of CD3+CD8+ T cells to CD4+CD25+Foxp3+ Treg cells. (E) Intracellular cytokine staining of granzyme B and perforin in CD8+ T cells was analyzed by flow cytometry. (F) CellTrace-labeled intratumoral T cells were stimulated with anti-CD3/28 antibody for 4 days and cell proliferation was determined by the dilution of CellTrace. (G) The expression of PD-1 and CTLA-4 was examined by flow cytometry, shown in mean fluorescence intensity (MFI). Data in B-G are shown as mean ± SEM and each dot represents a biologically independent sample; *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA with Tukey’s post hoc test.
Figure 7. **SERPINB3 mediates suppressive chemokine production through promoting STAT activation.**

(A) Activation of JAK/STAT pathway-associated proteins was evaluated using phosphorylation antibody array. Fold changes in phosphorylation were calculated by normalizing the intensity to the expression levels in Caski parental cells and comparing the phosphorylation intensity in Caski/B3 to the levels in Caski/Ctrl cells. Red line indicates fold change ≥ 2 and blue line indicates fold change ≤ 0.5. (B) Immunoblotting (left) and quantification (right) show the inhibition of STAT1/3 phosphorylation after treating Caski parental cells (WT), Caski/Ctrl (C), and Caski/B3 (B3#1, B3#2) with 1uM Ruxolitinib for 48 h. (C) Caski/WT, Caski/Ctrl, and Caski/B3 were
treated with 1uM Ruxolitinib and the secretion CXCL1, CXCL8 and S100A8/A9 was examined by ELISA. (D) Immunoblotting shows the knockdown of STAT1/3 by siRNA in Caski cells (E) The expression CXCL1/8 and S100A8/A9 mRNA was examined by qPCR. Gene expression were normalized to GAPDH. Fold changes and significance were calculated by comparing to the expression levels in Caski/Ctrl transfected with negative control siRNA. (F) The expression of pSTAT1/3 in nucleus (Nuc.), cytoplasm (Cyt.), and total cell lysates (input, Inp.) was examined by immunoblotting. (G) Immunoprecipitation using anti-JAK1 antibody shows increased interaction with STAT1 and STAT3 in Caski/B3 and SW756/B3 compared with Caski/Ctrl and SW756/Ctrl cells, respectively. Data are shown as mean ± SEM of n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA with Tukey’s post hoc test.
Figure 8

A. pSTAT3

B. CD11b

C. Disease Specific Survival

D. SCCA < 9.16
   pSTAT3 < 100 (L)
   pSTAT3 ≥ 100 (H)

E. pSTAT3

F. CD11b

P-values and comparisons are provided for each panel.
Figure 8. Elevated serum SCCA and high tumor pSTAT3 is associated with CD11b expression and poor cancer-specific survival after CRT. (A-B) Quantification of immunostaining for pSTAT3 and CD11b expression in mouse tumors treated with negative control (siNC) or Serpinb3a siRNA (siB3). Box plots show pSTAT3 staining score and percentage of CD11b positive staining from 8-12 representative fields each for n = 6 or 7 mice per group. Box plot whiskers span minimum and maximum; lines represent median. (C) Kaplan-Meier plot show overall survival in patients with serum SCCA <9.16 ng/ml with either pSTAT3 histoscore <100 (n=30) or ≥100 (n=21), compared to patients with serum SCCA ≥9.16 ng/ml with either pSTAT3 histoscore <100 (n=6) or ≥100 (n=15). The average pretreatment serum SCCA value of 9.16 ng/ml from 72 cancer patients was used as a cutoff. (D) Representative images of pSTAT3 and CD11b staining from patients with SCCA < or ≥ 9.16 ng/ml (E) Phosphorylated STAT3 staining score (histoscore) in patients with serum SCCA <9.16 ng/ml vs. SCCA ≥9.16 ng/ml. (F) Percentage of myeloid cell marker, CD11b staining in patients with serum SCCA < or ≥9.16 ng/ml and pSTAT3 histoscore <100 (low) or ≥100 (high). Each dot represents an individual patient. Data are shown as mean ± SEM, using Mann-Whitney test.