IL6/STAT3 Signaling Orchestrates Premetastatic Niche Formation and Immunosuppressive Traits in Lung

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ABSTRACT

Cancer cells that succeed in forming metastasis need to be reprogrammed to evade immune surveillance and survive in a new microenvironment. This is facilitated by metastatic niches that are either postformed through reciprocal signaling between tumor cells and local stromal cells or preformed as premetastatic niches before tumor cell arrival. IL6/STAT3 signaling is aberrantly activated in lung tumorigenesis and metastasis, however, the roles and mechanisms of action of IL6 remain controversial. Here, we showed that blockade of intrinsic STAT3 signaling in lung tumor cells suppressed lung metastasis in immune-competent syngeneic mice, but not in immune-deficient nude mice. Consistently, repression of STAT3 signaling in tumor cells made them susceptible to T-cell–mediated cytotoxicity. Thus, STAT3-mediated immunosuppression is crucial for metastasis. Notably, lung metastasis was greatly increased in Gprc5a-knockout (ko; 5a−/−) mice compared with wild-type mice, which correlated with upregulated IL6 in the tumor microenvironment. Depletion of IL6 via combined deletion of Il6 and Gprc5a genes almost completely eliminated lung metastasis in Gprc5a-ko/Il6-ko (5a−/−;Il6−/−) mice. Mechanistically, dysregulated IL6 reprogrammed the STAT3 pathway in metastatic tumor cells, and induced recruitment of myeloid-derived suppressor cells and polarized macrophages to evade host immunity. Consistently, IHC staining showed that activated STAT3 correlated with repressed infiltration of CD8+ T cells in non–small cell lung cancer. Therefore, IL6/STAT3 signaling is crucial for orchestrating premetastatic niche formation and immunosuppression in lung.

Significance: IL6 plays important roles not only in cell autonomous propensity for metastasis, but also in establishing the metastatic niche.

Introduction

Tumor metastasis is a complex process consisting of multiple steps, involving dissemination of cancer cells to local and distant organ sites, and their adaptation to new environments (1, 2). The processes of metastasis are driven by the interplay between cancer cells and the metastasis-supportive microenvironment (3). On one hand, proinflammatory cytokines in microenvironment can induce cancer cells to program epithelial-to-mesenchymal transition (EMT), a key step of the metastatic process (4, 5); on other hand, cancer cells can release factors or cytokines to recruit immunosuppressive lymphocytes, which facilitate the metastatic niche formation (6). Cancer cells may thus be reprogrammed to become invasive and immunosuppressive during the process. Recently, cancer cells undergoing EMT process were found to acquire immunosuppressive functions during metastasis (7, 8). These observations provide a functional link between metastatic potential and immunosuppressive traits. Because most of studies on EMT and metastasis are based on the analyses in vitro and/or in immune-deficient nude mice, the interplay between EMT-like traits of tumor cells and host immunity in metastasis remains poorly characterized.

The metastasis-supportive microenvironment, or metastatic niches, can be either postformed by reciprocal signaling between the metastasized tumor and stromal cells or preformed as premetastatic niches in tissue microenvironment before tumor cells arrived. Emerging evidences suggest that inflammatory stimuli provoke premetastatic niche formation. For example, inflammatory response induced by endotoxin via Toll-like receptor 4 (TLR4) increases vascular permeability and leukocyte mobilization to the lungs (9). Tumor exosomal RNAs promote lung premetastatic niche formation by activating alveolar epithelial TLR3 via recruitment of neutrophils (10). These studies suggest that the proinflammatory stimuli, either via TLR4 or TLR3, facilitate lung premetastatic niche formation. Alternatively, the oncogenic program in the metastasized cells can be activated by the signaling network in target tissue microenvironment.

IL6, a cytokine of the chemokine family, is widely expressed in a variety of immune cells and malignant tumors including lung cancer (11–13). IL6 activates JAK/STAT3 signaling by binding its receptor, and subsequently dimerizes with its co-receptor gp130, followed by recruitment of cellular signaling proteins, including JAKs...
and STAT3 (14–16). Elevated levels of IL6 or activated STAT3 have been observed in chronic inflammatory tissues and many solid tumors (17). Importantly, elevated levels of systemic and pulmonary IL6 are associated with poor survival of patients with non–small cell lung cancer (NSCLC; refs. 18, 19). In parallel, activated STAT3 can also promote IL6 gene expression, resulting in a feed-forward autocrine feedback loop (20). Intrinsically, IL6 can act directly on tumor cells to induce the expression of STAT3 target genes, to drive proliferation (such as via cyclin D1; ref. 21), and survival (such as via BCL2-like protein 1 (BCL-xl; ref. 22)). Extrinsically, IL6/STAT3 signaling has a profound effect on tumor-infiltrating immune cells in the microenvironment. STAT3 negatively regulates neutrophils, natural killer (NK) cells, effector T cells, and dendritic cells (DC); STAT3 positively regulates regulatory T (Treg) cells and myeloid-derived suppressor cell (MDSC) populations (23). Because metastasis is an outcome of tumor cell survival in a new tissue microenvironment under selection pressure, we reasoned that, the major oncogenic program of metastatic tumor cells should be compatible to the signaling network in the target tissue microenvironment. Consequently, the signaling network in the tissue microenvironment should have a great impact on the outcome of metastatic potential and immunosuppressive traits via reprogramming. Therefore, targeting the key player of the signaling network may greatly facilitate the inhibition of metastasis.

GPRC5A (G-protein–coupled receptor, family C, member 5A), also known as RAIG1 or RAI3, is a retinoic acid–inducible gene and is predominately expressed in lung tissue (24). Gprc5a–knockout (ko) mice are prone to develop spontaneous and carcinogen-induced lung cancer, indicating that Gprc5a is a lung tumor suppressor gene (24–26). Importantly, lung tumor development in Gprc5a–ko mice is associated with chronic inflammation (25, 27). Of note, GPRC5A is repressed in tissues of most human NSCLC and all chronic obstructive pulmonary disease (COPD; refs. 25, 27, 28), supporting that GPRC5A repression contributes to lung cancer development. Thus, Gprc5a–ko mice provide a unique immune-competent mouse model with chronic inflammation in lung.

In this study, we investigated the roles and mechanisms of IL6/STAT3 signaling in lung metastasis in Gprc5a–ko mouse model. Our study shows that immunosuppression induced by IL6/STAT3 signaling is critical for lung metastasis. We found that IL6/STAT3 signaling facilitates lung metastasis by orchestrating the premetastatic niche formation and immunosuppressive traits.

Materials and Methods

Cell lines and cell culture

Primary mouse lung cancer cells (SJ1601) were obtained from Gprc5a–ko mouse lung tumor at the age of 14 months, and SJ1601-luc was obtained from stable transfection of luciferase in SJ1601 cells, Lewis lung carcinoma (LLC) cell line was obtained from Zhejiang University (Hangzhou, China), and Calu-1 cells were obtained from ATCC. All the cells were cultured with DMEM (Hyclone) supplemented with 10% FBS (Gibco).

Reagents and antibodies

Detailed information is provided in the Supplementary Experimental Procedures.

qRT-PCR

tRNA was extracted from lung tissue using RNA Extract Kit (TIANGEN). Then, cDNA was obtained by Fast Quant Kit (TIANGEN). The qRT-PCR were performed using SuperReal Premix Plus SYBR Green Kit (TIANGEN). All experiments were performed according to the manufacturer’s instructions. Primers are listed in Supporting Information, Table S1.

Migration and wound healing assay

Detailed information is provided in the Supplementary Experimental Procedures.

Sphere culture assay

A total of 5,000 cells were added to each well of a 96-well low-adherence culture plate and suspended in culture medium to simulate a three-dimensional culture environment. The medium was changed every 3 days. Spheroid size was monitored to avoid necrosis in the center of the spheres. Images of the spheroids were captured by Nikon camera.

Soft agar colony formation assay

Detailed information is provided in the Supplementary Experimental Procedures.

Western blot analysis

Cells were lysed with RIPA lysis buffer (29). Experiments were performed as described previously (30). Briefly, whole cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were probed with indicated antibodies and protein expression was detected by chemiluminescence (GE Healthcare).

RNA interference and transfection

Pairs of complementary oligonucleotides against Gp130 or non-specific (NS) control shRNA were synthesized by Sangon Biotech, annealed, and ligated to the PGIPZ Lentiviral Vector (Clontech Laboratories, Inc.). The shRNA-carrying retroviruses produced in 293T cells were used to infect cells.

Flow cytometry analysis

Mice were sacrificed, and the lungs lavaged three times with ice-cold PBS. Then lung tissues were scissored and digested with 150 μl/mL Collagenase IV (Sigma-Aldrich) and 0.001% DNase1 (Sigma-Aldrich) in PBS for 45 minutes at 37°C; single-cell suspensions were prepared by passing the cell digest through 70 μm and then 40 μm nylon mesh. Cells were incubated with Red Blood Cell Lysate (Sigma-Aldrich) for 5 minutes at room temperature and washed twice with PBS containing 10% FBS. Cells were stained with indicated fluorescently labeled antibodies on ice for 30 minutes. Then cells were analyzed by FACS (BD Biosciences), and the data were further analyzed by Flowjo7.6.1 software. Antibodies information is listed in Supporting Information, Table S2.

Mouse models

Gprc5a–ko mice were generated in a mixed background of 129sv C57BL/6 as described previously (29) and were bred with Gprc5a–ko/IL6–ko mice were generated by cross-breeding Gprc5a–ko mice with IL6–ko mice. Mice were maintained according to a protocol approved by Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee [experimental animal use permission no.: SYXK (Shanghai) 2008-0050] in the specific pathogen-free animal facility in the university.

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Tumorigenicity and migration experiment in xenograft
For migration study, SJT-1601/SJT-1601-Luc and Lewis cells were injected into the tail vein (5 × 10^6/0.2 mL cells in PBS) of designated mice and were sacrificed after 3 weeks. In a separate study, SJT-1601-NS, SJT-1601-Gp130 were injected into the tail vein (5 × 10^6/0.2 mL cells in PBS) of SJ7BL/6 (WT), Gprc5a-ko mice; and (1 × 10^6/0.2 mL cells in PBS) were injected into nude mice. These animals were sacrificed after 3 weeks. For tumorigenicity study, SJT-1601-NS, SJT-1601-Gp130, SJT-1601-dnStat3 were injected subcutaneously (2 × 10^6/0.2 mL cells in PBS) in the left and right hind leg of mice in C57BL/6 and nude mice respectively. After the tumor was palpable (approximately 100 mm^3), the tumor volume was measured twice a week. Tumor volume (cm^3) was calculated by the formula: (a × b^2)/2, where “a” is the long diameter and “b” is the short diameter (mm). For in vivo IL6 blockade assay, SJT-1601 cells were injected into the tail vein (5 × 10^6/0.2 mL cells in PBS) of Gprc5a-ko mice, 1 week later, 20 mg/kg dose of Tocilizumab (A2012, Selleck) or control IgG1 (Sigma-Aldrich) were intraperitoneally injected twice a week for 2 weeks; these animals were sacrificed and lung metastasis were assessed after 3 weeks.

Ex vivo experiment
Mouse lung tumor cells, SJT-1601-GFP cells (1.5 × 10^7/0.2 mL cells in PBS) were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Ile-ko mice. Ten days later, single-cell suspensions from mouse lung tissues were obtained and then GFP^+ cells were harvested via FACS-sorting. For immunofluorescence experiments, the procedure was done as described previously (25).

IHC
A tissue microarray composed of tumor and adjacent normal tissue from NSCLC samples were obtained from Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). The tissue microarray and fixed mouse lung tissue samples were processed for IHC; the detailed protocol and score method were performed as described previously (25). Shanghai Chest Hospital (Shanghai, China) approved the use of the NSCLC samples in this study.

T-cell–mediated tumor cell–killing assay (cytotoxic T-cell lymphocyte)
Detailed information is provided in the Supplementary Experimental Procedures.

Statistical analysis
Comparisons among groups were performed by the Student t test or Tukey–Kramer comparison test followed by analysis with GraphPad Prism Software (GraphPad Software). A P < 0.05 was considered significant (* P < 0.05; ** P < 0.01; *** P < 0.001; ****, P < 0.0001).

Results
Aberrantly activated IL6/STAT3 signaling is highly correlated with tumorigenesis and metastasis in Gprc5a-ko mouse lungs
Tobacco carcinogen 4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung tumorigenesis in Gprc5a-ko (5a^-/-) mice (100%), but not in wild-type (WT) mice (0%; Fig. 1A and B; ref. 25). IHC staining showed that active STAT3 (p-STAT3) was strongly positive in lung tumors of Gprc5a-ko mice, but not in WT lungs (Fig. 1B). This finding suggests that STAT3 signaling is involved in lung tumorigenesis in Gprc5a-ko mice. Of note, p-STAT3 was also positive in adjacent normal lung tissues of Gprc5a-ko-NNK-12m mice with lung tumors, especially in the small/terminal bronchioles region (S7B; Fig. 1C). This observation implies a paracrine effect by cytokines from tumor microenvironment (TME). Further analysis via qRT–PCR and ELISA showed that the IL6 level was indeed significantly upregulated in the tumors from Gprc5a-ko mouse lungs compared with adjacent normal lungs and WT lungs (Fig. 1D and E). These data are consistent with the previous observation, in which Gprc5a-ko mouse lungs are sensitive to proinflammatory stimuli (27, 31). Because IL6/STAT3 signaling is involved in a variety of solid cancers, we questioned, whether the IL6-enriched inflammatory tissue microenvironment of Gprc5a-ko mouse lung facilitates metastasis.

To determine the impact of the tissue microenvironment on metastasis, we performed an experimental metastasis analysis. The mouse lung tumor cell line SJT-1601 (1601), derived from Gprc5a-ko mouse lung tumor, was intravenously injected into syngeneic C57BL/6 (WT) and Gprc5a-ko (5a^-/-) mice (Fig. 1F). Lung metastatic nodules were assessed 3 weeks later. Hematoxylin and eosin (H&E) staining analysis showed that SJT-1601 cells formed significantly more metastatic nodules in Gprc5a-ko mouse lungs than in WT mouse lungs (Fig. 1G–I), suggesting that premetastatic niches were increased in Gprc5a-ko mouse lungs compared with those in WT ones. In addition, IHC staining showed that p-STAT3 staining was more intense in the metastatic tumor tissues of Gprc5a-ko mouse lungs than in WT ones (Fig. 1G and J), and ELISA analysis showed that the IL6 level was significantly higher in Gprc5a-ko mouse lungs than that in WT ones (Fig. 1K). Taken together, these results suggest that upregulated IL6/STAT3 signaling is associated with metastasis in Gprc5a-ko mouse lungs.

IL6/Gp130/STAT3 signaling is essential for the prometastatic traits of lung cancer cells
To determine the role of IL6/STAT3 signaling in the metastatic features of lung cancer cells, we knocked down Gp130 in SJT-1601, a mouse lung cancer cell line derived from Gprc5a-ko mouse lung adenocarcinoma and human lung cancer cell line Calu-1, and established the stable transfectants, 1601-shGp130 (Fig. 2A) and Calu-1-shGp130 (Fig. 2B). Immunoblot analysis showed that the levels of mesenchymal markers, including N-cadherin and Twist, were reduced in 1601-shGp130 and Calu-1-shGp130 cells, and the level of epithelial marker E-cadherin was increased in 1601-shGp130, although there was no significant change in vimentin level (Fig. 2A and B). These findings suggest that IL6/Gp130 signaling is essential for the induction of the EMT-like features in these lung cancer cells. Next, we examined the effect of IL6 on cell migration and invasion activities in vitro using wound healing and transwell assays. Addition of exogenous IL6 significantly enhanced the migration (Fig. 2C and D) and invasion (Fig. 2E and F) of 1601-NS and Calu-1-NS cells, but not 1601-shGp130 and Calu-1-shGp130 cells (Fig. 2C–F), which indicates that IL6 signaling is essential for migration and invasion. Furthermore, we examined the role of the IL6/Gp130 pathway on lung metastasis in vivo. The results showed that metastasis of 1601-NS cells was significantly increased in Gprc5a-ko mice compared with WT mice (Fig. 2G–I). And, the number of metastatic nodules of 1601-shGp130 cells was significantly reduced compared with those of 1601-NS cells in both WT and Gprc5a-ko mouse lungs (Fig. 2G–I), suggesting that the intrinsic IL6/Gp130/STAT3 signaling pathway of lung tumor cells is essential for the metastatic potential. Interestingly, the numbers of metastatic nodules of 1601-NS and 1601-shGp130 in immune-
deficient nude mice were similar (Fig. 2J–L), suggesting that immune-evasion is a major mechanism of IL6/Gp130/STAT3-mediated metastatic potential. Thus, although the IL6/Gp130/STAT3 pathway alters many features of lung tumor cells in vitro, Gp130/STAT3-mediated immunosuppression is critical for lung metastasis in the immune-competent host in vivo.

Gp130/STAT3 signaling is intrinsically linked to the stem-like and immunosuppressive traits of lung cancer cells

EMT-like features are functionally linked to stemness, metastatic potential, and immunosuppressive features of cancer cells (6). To determine the roles of IL6/Gp130 signaling in regulation of these features in lung tumor cells, we examined the stem-like markers in the...
Figure 2. IL6/Gp130/STAT3 signaling is essential for induction of the EMT-like features of NSCLC cells. A and B, Western blot analysis of E-cadherin, N-cadherin, vimentin, Twist, and Gp130 protein levels in 1601-NS and 1601-shGp130 cells (A), and Calu-1-NS and Calu-1-shGp130 cells (B). C and D, Migration analysis of 1601-NS and 1601-shGp130 (C), and Calu-1-NS and Calu-1-shGp130 cells (D) with or without IL6 by wound healing. E, Transwell migration analysis of 1601-NS and 1601-shGp130 cells, and Calu-1-NS and Calu-1-shGp130 cells with or without IL6. F, Statistical analysis of E, G and H. 1601-NS or 1601-shGp130 cells were intravenously injected into WT and Gprc5a-ko mice; representative images of lung tissues with H&E (n = 5). I, Number of metastatic nodules in G and H. J and K, 1601-NS or 1601-shGp130 cells were intravenously injected into nude mice; representative images of lung tissues (J) and tissues stained with H&E (K; n = 5). L, Number of metastatic nodules in nude mice. *, P < 0.05; **, P < 0.01.
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NS and shGp130 stable transfectants of 1601 and Calu-1 cells. Immunoblot showed that p-STAT3, the stem-like markers (Abcg2, CD44, Sox2), and immune checkpoint marker PD-L1 were significantly reduced in shGp130 transfectants compared with the NS control (Fig. 3A and B), whereas p-ERK and p-AKT of these cells remained at similar levels. This suggests that Gp130 knockdown mainly inhibits STAT3 signaling and stemness in these lung cancer cells. Consistently, three-dimensional sphere formation (Fig. 3C and D), colony formation in soft agarose (Fig. 3E and F), and clonogenic assay (Supplementary Fig. S1A and S1B) were also greatly suppressed in 1601-shGp130 and Calu-1-shGp130 cells compared with their NS transfectants. Thus, Gp130/STAT3 signaling is intrinsically essential for the stem-like features of lung cancer cells in vitro. Next, we examined the tumorigenicity of these cells in vivo. Interestingly, 1601-NS and 1601-shGp130 cells generated tumors with similar efficiency in immune-deficient nude mice (Fig. 3G–I). However, the tumorigenicity of 1601-shGp130 cells was greatly suppressed compared with 1601-NS in immune-competent syngeneic WT/C57 mice (Fig. 3J–L). In addition, we examined the biological features of 1601 cells expressing dominant negative STAT3 transfectants (1601-dnStat3; Supplementary Fig. S1C). The tumorigenicity of 1601-dnStat3 was similar, as compared with 1601 cells, in immune-deficient nude mice (Supplementary Fig S1D–S1F), but significantly suppressed in WT/C57 mice (Supplementary Fig S1G–S1I). Taken together, these results suggest that the immunity of immune-competent C57 mice is responsible for suppression of the tumorigenicity of SJT-1601-shGp130 cells, which is similar to lung metastasis in these mice (Fig. 2G–I). Thus, the major impact of STAT3 signaling in tumorigenicity and lung metastasis is mainly through immunosuppression.

PD-L1 overexpression was reportedly associated with poor prognosis (32, 33). Immunoblot showed that PD-L1 levels in 1601-shGp130, Calu-1-shGp130, and 1601-dnStat3 cells were significantly reduced compared with NS cells (Fig. 3A and B; Supplementary Fig. S1C). Importantly, 1601-shGp130 and 1601-dnStat3 cells were much more sensitive to T-cell–mediated cytotoxicity than 1601-NS cells in cytotoxic T-cell lymphocyte (CTL) assay (Fig. 3M). These results suggest that intrinsic STAT3 signaling, possibly through overexpression of immunosuppressive molecules like PD-L1, in tumor cells endows the resistance to cytotoxic T (Tc) cells. Thus, intrinsic IL6/Gp130/STAT3 signaling is critical for the immunosuppressive features of the lung cancer cells.

IL6 signaling is extrinsically crucial for premetastatic niche formation in Gprc5a-kO mouse lungs

We reasoned that STAT3 signaling in metastatic tumor cells was activated by IL6 in microenvironment. To determine the role of IL6 signaling in lung metastasis, we generated Gprc5a-kO/Ile6-kO mice by cross-breeding Gprc5a-kO mice with Ile6-kO mice (Fig. 4A). Next, we performed lung metastasis in these mice by intravenous injection of 1601-luc (luciferase) cells followed by in vivo imaging. Lung metastasis was dramatically increased in Gprc5a-kO mice compared with WT mice. Strikingly, lung metastases in Gprc5a-kO/Ile6-kO mice were almost completely eliminated (Fig. 4B–D). IL6 appears to be the major cytokine responsible for STAT3 activation because the expression of other IL6 family cytokines remains unchanged, and upregulated IL6 was correlated with increased metastatic potential (Fig. 4E and F). These observations suggest that although tumor cells or “seeds” are the same, tissue microenvironment or “soil” has great impact on their metastatic potential. To determine the effect of tissue microenvironment on reprogramming the oncogenic signaling of the metastatic tumor cells in vivo, we examined the gene expression in the STAT3 and EMT pathways in lung tissues. Immunoblot showed that IL6, p-STAT3, N-cadherin, vimentin, PD-L1, and Twist were significantly upregulated, whereas E-cadherin expression was reduced in lung tissues from Gprc5a-kO mice injected with 1601 tumor cells compared with those from WT and Gprc5a-kO/Ile6-kO mice (Fig. 4G, right). The difference of the gene expression in normal control lungs is negligible (Fig. 4G, left). Collectively, these results suggest that (i) IL6/STAT3 signaling in tumor-containing lung tissues is activated via the reciprocal interaction between the metastatic tumor cells and stromal cells in tissue microenvironment; and (ii) dysregulated IL6 is essential for the effects. To exclude the noise of stromal cells in background in detection and to specifically define the status of STAT3 signaling in the metastatic tumor cells from different tissue microenvironment, we performed an ex vivo experiment. Mouse lung tumor cells, 1601-GFP, were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Ile6-kO mice. Ten days later, GFP+ cells were harvested from mouse lungs via FACS-sorting (Supplementary Fig. S2A and S2B). Immunofluorescent analysis showed that PD-L1–staining intensity in 1601-GFP cells isolated from Gprc5a-ko mouse lung was significantly enhanced (red in Fig. 4H) as compared with those isolated from WT and Gprc5a-ko/Ile6-kO mouse lungs (Fig. 4H and 4I). These results suggest that the metastatic tumor cells are reprogrammed to exhibit the active STAT3 pathway by tissue microenvironment of Gprc5a-ko mouse lungs, whereas IL6 is essential for the effect.

Consistently, IHC staining showed that Ki-67, p-STAT3, and PD-L1 were all increased while CD8 was decreased in the lung tumors from Gprc5a-ko mice compared with lung tissues from WT and Gprc5a-ko/Ile6-kO mice (Fig. 4J; Supplementary Fig. S2C). These results suggest that activated STAT3 is associated with increased proliferation and PD-L1 upregulation. Metastasis is known to associate with angiogenesis and cell proliferation (34). qRT-PCR showed that the angiogenesis biomarkers, BV8 and ANGPT, but not VEGF, were significantly upregulated in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/Ile6-kO mice (Fig. 4K). These findings further support the role of IL6 in premetastatic niche formation.

To extend this observation, we repeated the metastasis experiments using LLC cells in these mice and observed similar results. Biologically, lung metastasis via LLC cells was greatly increased in Gprc5a-ko mice compared with WT mice and Gprc5a-ko/Ile6-kO mice (Supplementary Figs. S3A–S3C). Biochemically, IL6, p-STAT3, PD-L1, N-cadherin, vimentin, and Twist were all increased while E-cadherin was decreased in Gprc5a-ko mice compared with WT mice and Gprc5a-ko/Ile6-kO mice (Supplementary Fig. S3D). Taken together, lung metastasis, via either SJT-1601 cells or LLC cells, is greatly enhanced in Gprc5a-ko mouse lungs in which IL6/STAT3 signaling plays a critical role.

IL6 signaling mediates the inhibition of innate and adaptive immunity in Gprc5a-ko mouse lungs

MDSCs and tumor-associated macrophages (TAM) are important negative regulators of host innate immunity in tumor microenvironment. Uregulation of these cells is associated with metastasis (35, 36). MDSCs can be subclassified into granulocyte-like myeloid derived suppressor cells (G-MDSC) and monocytic myeloid derived suppressor cells (M-MDSC). To determine the extrinsic roles of IL6 on innate immunity, we assessed MDSC levels in mouse lung tissues from lung metastasis experiments via FACS analysis. G-MDSCs were significantly increased while M-MDSCs were significantly reduced in Gprc5a-ko mouse lungs compared with WT mouse lungs (Fig. 5A and B). Of note, Il6 gene knockout in Gprc5a-ko/Ile6-kO mice completely reversed the G-MDSC upregulation and M-MDSC downregulation.
in Gprc5a-knockout (Gprc5a-ko) mice (Fig. 5B). Consistently, G-MDSC–related markers, S100a8 and S100a9, were significantly upregulated in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/Ile6-ko mouse lungs (Fig. 5C). These data suggest that IL6 is essential for upregulated G-MDSCs in Gprc5a-ko mouse lungs. Next, we examined via qRT-PCR the expression of a panel of soluble factors that are potentially involved in MDSCs recruitment. IL6, G-CSF, and prostaglandin E synthase (Ptges) were highly upregulated (red), whereas IL10 was significantly downregulated (blue) in Gprc5a-ko mouse lungs compared with the WT, and all of these alterations were reversed in Gprc5a-ko/Ile6-ko mouse lungs (Fig. 5D). These results suggest that IL6 is essential for recruitment of MDSCs in Gprc5a-ko mouse lungs.
IL6 signaling is essential for lung metastasis in Gprc5a-ko mice lung tissues. SJT-1601-luc cells were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Il6-ko mice. A, Model diagram of Gprc5a-ko/Il6-ko mice. B, Fluorescence images of excised lung tissues from mice. C, The quantitative value of fluorescence intensity of B. D, Lung weight of mice. E, The mRNA level of IL6 family cytokines in mice lung tissues. F, ELISA analysis of IL6 level in mice lung tissues. G, Western blot analysis of mouse lung tissues. H, Immunofluorescence staining for PD-L1 in 1601-GFP cells cultured in vitro and 1601-GFP cells sorted from indicated mouse lung tissues with tumors. I, Quantification of relative fluorescence intensity (PD-L1/DAPI) from H. J, IHC scores from lung metastatic nodules of Ki-67, p-STAT3, PD-L1, and CD8. K, The mRNA level of Bv8, Angpt2, and Vegf in lung tissues. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
IL6 signaling mediates the inhibition on innate and adaptive immunity in Gprc5a-ko mouse lungs. SJT-1601-luc cells were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Il6-ko mice. A, Gating strategy for G-MDSCs and M-MDSCs staining in tumor lung tissues and representative FACS images. B, Quantitative analysis of G-MDSCs and M-MDSCs by FACS analysis. C, The mRNA level of S100a8 and S100a9 in tumor lung tissues. D, The mRNA level of inflammatory genes responsible for MDSCs recruitment in tumor lung tissues. E, Quantitative analysis of TAM by FACS analysis. F, The mRNA level of type I macrophage biomarkers: Ccl2, Nos2, and Cxcl10; and type II macrophage biomarkers: Arg, Fizzl, and Mrc in tumor lung tissues. G, Quantitative analysis of T cells by FACS analysis. H, The mRNA level of TNFα, Ccl5, Ifng, Gzmb, and Tbx21, which are associated with CD8+ T-cell cytotoxic activation in tumor lung tissues. I, T-cell-mediated cytotoxicity for 1601-shGp130 and 1601-dnStat3 cells with or without MDSCs. *P < 0.05; **P < 0.01; ***P < 0.001.
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M-MDSCs recruited to tumor tissues will rapidly differentiate to TAMs (37). Consistent with this pattern, we found that the infiltrated macrophages in tumors were significantly increased in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko ones (Fig. 5E, Supplementary Fig. 5A). This suggests that IL6 is responsible for increased macrophages in Gprc5a-ko mouse lungs. Further analysis by qRT-PCR showed that the markers of polarized type II macrophages, Arg, Fizzl, and Mrc, were greatly increased, while the markers of type I macrophages, Ccl2, Nos2, and Cxcl10, were greatly suppressed in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko mouse lungs (Fig. 5F). Thus, Gprc5a-ko mouse lungs are enriched with TAMs that exhibit significant polarization of type II macrophages.

G-MDSC has the potential to inhibit CD8+ T, DC, and NK cells (38, 39). To determine the effects of IL6 on adaptive immunity, we examined the T-cell composition and related gene expression. FACS analysis showed that both CD4+ and CD8+ T cells were significantly reduced in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko mouse lungs (Fig. 5G; Supplementary Fig. 5B).

Consistently, qRT-PCR analysis showed that T-cell activation gene products, such as TNFα, chemokine ligand 15 (Ccl5), IFNγ (Ifng), Granzyme B (GzmB), and T-box transcription factor 21 (Tbx21), were all significantly suppressed in Gprc5a-ko mouse lungs compared with levels in WT and Gprc5a-ko/I6-ko lungs (Fig. 5H).

To evaluate the biological impact of dysregulated MDSCs on T-cell immunity, we performed CTI analysis. While 1601-shGp130 and 1601-dnStat3 cells were sensitive to T cells (T), addition of exogenous MDSCs (T + MDSCs) greatly suppressed T-cell-mediated cytotoxicity (Fig. 5I). This observation indicates that MDSCs are the major component of immunosuppression to T-cell-mediated cytotoxicity. In addition, NK cells were reduced in Gprc5a-ko mice (Supplementary Fig. 5C and 5D), whereas DC cells were similar among these groups (Supplementary Fig. 5E and 5F). Consistent with the status of the immune cells, the immune organs including spleen and thymus exhibited obvious atrophy in Gprc5a-ko mice compared with WT tissues. However, these alterations were reversed in Gprc5a-ko/I6-ko mice (Supplementary Fig. 5G and 5H).

IL6-mediated regulation of innate and adaptive immunity was also observed in lung metastasis induced by LLC. LLC-induced metastasis was correlated with increased G-MDSC (Supplementary Fig. 5A and 5B) and TAM (Supplementary Fig. 5C and 5D), and suppressed CD8+ T cells (Supplementary Fig. 5E and 5F) and NK cells (Supplementary Fig. 5G and 5H). Collectively, these findings suggest that IL6 is crucial in inducing enhancement of MDSC and TAM, and suppression of T cells and NK cells in Gprc5a-ko mouse lung. Thus, IL6 is a promising target for restoration of host immunity.

Targeting IL6 suppresses MDSCs, restores T-cell cytotoxicity, and inhibits lung metastasis in Gprc5a-ko mice

Because IL6 plays a central role in the suppression of host immunity and enhancement of lung metastasis, we investigated the therapeutic effects of IL6 targeting on lung metastasis. SJT-1601 cells were intravenously injected into Gprc5a-ko mice. One week later, anti-IL6 antibody was intraperitoneally injected twice a week for 2 weeks. On day 21, mice were sacrificed for analysis of lung metastasis (Fig. 6A). We found that targeting IL6 significantly inhibited lung metastasis compared with control mouse lungs (Fig. 6B and C). Functionally, G-MDSCs were also significantly reduced following anti-IL6 treatment, although M-MDSCs remained at a similar level, as analyzed by FACS (Fig. 6D and E). TAMs were also significantly reduced following anti-IL6 treatment (Fig. 6F and G). In contrast, CD8+ T cells were significantly increased following anti-IL6 treatment, although CD4+ T cells remained at a similar level (Fig. 6H and I). Furthermore, NK cells were also upregulated significantly following anti-IL6 treatment, although NK-T cells remained unchanged (Fig. 6J and K). Taken together, these observations demonstrate that targeting IL6 restores the innate and adaptive immunity in Gprc5a-ko mouse lungs and inhibits lung metastasis. Thus, IL6 signaling in lung is crucial for premetastatic niche formation and immunosuppressive traits.

Dysregulated IL6/STAT3 is correlated with reduced CD8+ T-lymphocyte infiltration in NSCLCs

Next, we asked if the gene products of an active IL6/STAT3 axis can serve as biomarkers for the immunosuppressive features in human NSCLC. By IHC staining analysis, we examined the levels of IL6, p-STAT3, PD-L1, and CD8 in 140 NSCLC samples. The expression levels of IL6, p-STAT3, and PD-L1 were significantly higher in tumors than in adjacent normal tissues, whereas CD8+ expression was higher in adjacent normal tissues than tumor tissues (Fig. 7A and B). Of note, areas of tumor tissues that were low in IL6/p-STAT3 were also low in PD-L1, but high in CD8+, whereas areas that were high in IL6/p-STAT3 were also high in PD-L1, but low in CD8 (Fig. 7A). These observations support the notion that activated IL6/STAT3 induces PD-L1 expression and other immunosuppressive features that lead to the suppression of CD8+ T-cell infiltration. Statistical analysis showed that IL6 was highly correlated with p-STAT3 in NSCLC as expected (Fig. 7C). Similarly, IL6 and p-STAT3 were significantly correlated with PD-L1 (Fig. 7C). Importantly, p-STAT3 was inversely correlated with CD8 in expression (Fig. 7C), which suggests that the activated IL6/STAT3 axis induces T-cell infiltration of CD8+ T cells in NSCLC.

Finally, by The Cancer Genome Atlas analysis, we found that increased IL6 and increased PD-L1 predict poor overall survival and disease-free survival rates (Fig. 7D and E). These observations further support the model that activated IL6/STAT3 contributes to immunosuppression, including upregulation of PD-L1, and reduced infiltration of CD8+ T cells in NSCLCs.

Discussion

In this study, we showed first that STAT3-mediated immunosuppressive trait in tumor cells is mainly responsible for lung metastasis in vivo. Second, dysregulated IL6 reprogrammed the oncogenic signaling in the metastatic tumor cells for increased metastasis in Gprc5a-ko mouse lungs. Third, IL6 induces recruitment of MDSC and macrophage polarization, which inhibits host immunity. And fourth, the active IL6/STAT3 axis can be used as a biomarker for suppressed CD8 T-lymphocyte infiltration in NSCLC samples. Thus, IL6 plays a key role in orchestrating premetastatic niche formation and immunosuppression in lung.

In this study, we showed that although 1601-shGp130 cells exhibited suppressed EMT-like features, still they formed metastasis and tumors in immune-deficient mice, but did not do so in immune-competent mice. This suggests that STAT3-induced immunosuppression is functionally critical for the metastatic potential in immune-competent mice. Indeed, 1601-shGp130 or -dnStat3 cells are susceptible to T-cell–mediated cytotoxicity. Previously, Snail-induced EMT and immunosuppression were functionally linked in melanoma metastasis model (40). Consistently, a strong correlation between EMT and expression of immunosuppressive genes was revealed by an integrated, global analysis of genomic and proteomic profiles (8). Thus, the EMT-like features are functionally linked to the immunosuppressive traits in cancer cells.
GPRC5A repression is prevalent in NSCLCs. On one hand, lungs from Gprc5a-ko mice are prone to a variety of inflammatory stimuli as well as to spontaneous and carcinogen-induced lung tumorigenesis (27, 31). On the other hand, inflammation via NF-κB signaling inhibits GPRC5A expression. Thus, repressed GPRC5A is an indication of the inflammatory status in lung tissue such as in COPD (41). We showed that lung metastasis via the same tumor cells was greatly increased in Gprc5a-ko mouse lungs compared with lungs from WT mice. This finding suggests that although the tumor cells or "seeds" are the same, different tissue microenvironments or "soils" would have a great impact on lung metastasis. Thus, upregulated IL6 in Gprc5a-ko mouse lungs is essential for premetastatic niche formation as compared with those in WT and Gprc5a-ko/Il6-ko mice.

An inflammatory microenvironment is implicated as a contributory factor to tumor development and metastasis in many cases. For example, the incidence of lung cancer in patients with COPD is 6-fold higher than that in control group (42). Obesity, a chronic, low-grade inflammation was found to enhance breast cancer metastases to lung by altering lung myeloid cells (43). Smoking, an inflammatory stimulus, increases the incidence of pulmonary metastasis in colorectal cancer (44). And fatty liver facilitates liver metastases in patients with NSCLC (45). Although the normal lung...
serves as a defensive barrier against foreign pathogens and particulates, the microenvironment of lung tissue can be reprogrammed, such as by chronic inflammation or smoking, to a status that is protumorigenic or prometastatic. In fact, lung is an organ in which metastases are frequently formed from a variety of malignancies. In this study, inflammatory microenvironment of Gprc5a-knockout mouse lungs was shown to enhance lung metastasis, supporting the notion.

Figure 7. Dysregulated IL6/p-STAT3 is correlated with suppressed CD8+ T lymphocytes in NSCLC. A, Representative images from a microarray of human lung tissue that includes tumor and adjacent normal tissues (n = 140) stained by IHC for IL6, p-STAT3, PD-L1, and CD8. B, IHC scores from human lung tissues chip of A. C, Correlation analysis for the expression of IL6, p-STAT3, PD-L1, and CD8 in the microarray of human lung tissue. D, IL6 and PD-L1 expression were negatively correlated with patient overall survival rate. E, IL6 and PD-L1 expression were negatively correlated with patient disease-free survival rate. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Upregulated IL6 is linked to increased metastasis in Gprc5a-ko mouse lungs, whereas IL6 gene deletion in Gprc5a-ko/IIf6-ko mice almost completely eliminated lung metastases. This observation strongly supports the impact of the extrinsic roles of IL6 in lung metastasis. Persistent STAT3 activation was previously shown to promote chronic inflammation (17). However, most of studies were focused on the intrinsic features of cancer cells such as EMT-like features or biological characterization in immune-deficient mice in vivo. In this study, we hypothesize that the major oncogenic program in metastatic tumor cells should be compatible with the signaling network in tumor microenvironment. In support, the metastatic tumor cells are reprogrammed to an activated STAT3 status by tissue microenvironment in Gprc5a-ko mouse lungs, whereas depletion of IL6 in Gprc5a-ko/IIf6-ko mice abrogated the effect. Thus, the compatibility between the oncogenic pathway in metastatic tumor cells and the extrinsic signaling in tissue microenvironment dictates the efficiency of metastasis. Similar in concept, a recent study showed that the intrinsic programs of cancer cells are dictated by tissue microenvironment (46). Thus, cancer cells that succeed in forming metastasis should be reprogrammed to evade immune surveillance, which is compatible to the target tissue microenvironment. Here, IL6 is shown to play a critical role in lung metastasis. In this study, IL6 targeting therapy significantly inhibited lung metastasis in Gprc5a-ko mice. This suggests that the metastatic tumor cells are addicted to IL6 signaling, whereas neutralization of IL6 by antibody therapy disrupts the oncogenic program that is required for metastatic process. The IL6 targeting strategy was also effective in the treatment of a mouse model with IL6-expressing LLC cells, which caused cachexia (47). In the clinic, serum IL6 levels were reportedly higher in patients with cachexia than in those without. And a high IL6 serum level strongly correlated with short survival in patients with chemotherapy-resistant lung cancer (47). However, anti-IL6 mono-therapy in clinical trials appears to be tolerated and shows no clinical activity in several solid tumors, including colorectal, ovarian, and pancreatic cancers. It is possible that the early stages of tumor formation may be IL6-dependent, whereas late-stage disease may be not. Practically, human solid tumors are more complex than mouse model and multiple clones of cancer cells could be another factor for tolerance. Thus, IL6 inhibition alone may not be sufficient in treating advanced solid tumors (48). Interestingly, a recent study showed that treatment of ovarian cancer cells with neutralizing IL6 antibodies resulted in upregulated EGFR, whereas combination of neutralizing IL6 antibodies and the EGFR inhibitor gefitinib exhibited enhanced anticancer activity (49). Thus, it is expected that combination of IL6/STAT3-targeted therapy with other therapy, such as chemo- or immunotherapy, will be more efficient than monotherapy.

Generally, tumors can be subclassified into “hot” and “cold,” which reflects the status of T-lymphocyte infiltration and the ability to respond to immunotherapy. In this study, we showed that an active IL6/STAT3 signaling system is inversely correlated with CD8+ T-lymphocyte infiltration in NSCLC samples. This finding suggests that the IL6/STAT3 axis provides the candidate markers for “hot” and “cold” tumors, which is potentially used for therapeutic application. Taken together, we found that IL6/STAT3 signaling promotes lung metastasis by orchestrating premetastatic niche formation and immunosuppressive traits (Supplementary Fig. S6).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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