Semaphorin4D Inhibition Improves Response to Immune-Checkpoint Blockade via Attenuation of MDSC Recruitment and Function

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Abstract

Tumor infiltration by immunosuppressive myeloid cells, such as myeloid-derived suppressor cells (MDSCs), causes resistance to immunotherapy. Semaphorin4D, originally characterized for its axonal guidance properties, also contributes to endothelial cell migration and survival and modulates global immune cytokine profiles and myeloid cell polarization within the tumor microenvironment. Here, we show how a therapeutic murine Sema4D mAb improves responses to immune-checkpoint blockade (ICB) in two murine carcinoma models. Treatment of tumor-bearing mice with Sema4D mAb abrogated Ly6Ghi PMN-MDSC infiltration by tumor cells in Murine oral cancer-1 (MOC1) tumors. PMN-MDSC suppressive capacity was reduced through inhibition of Sema4D-driven arginase expression. These changes led to enhanced tumor infiltration by CD8+ TIL and activation of tumor-draining lymph node T lymphocytes in response to tumor antigen. Sema4D mAb in combination with either CTLA-4 or PD-1 blockade enhanced rejection of tumors or tumor growth delay, resulting in prolonged survival with either treatment. This function of Sema4D mAb provides a rationale for its evaluation in combination with ICB to treat tumors with immunosuppressive myeloid infiltration.

Introduction

Despite advances in surgical techniques and standard anticancer therapeutics, outcomes for advanced head and neck carcinomas remain poor [1]. A subset of patients with recurrent disease after standard treatment demonstrate durable antitumor responses to immune-checkpoint blockade (ICB) immunotherapy, though the majority of patients with recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) do not respond to ICB [2, 3]. Both extrinsic and intrinsic resistance to ICB immunotherapy occurs [4]. One resistance mechanism appears to be due to immunosuppressive myeloid cells within the tumor microenvironment (TME; refs. 5–7). Many preclinical approaches to alter myeloid cell–driven immunosuppression within the TME, which may enhance effector immune cell activity and responses to ICB, have been investigated [5, 6, 8, 9].

Semaphorins are a family of transmembrane and soluble proteins that guide axonal sprouting [10, 11]. Signaling through its cognate receptor Plexin B, Semaphorin4D (Sema4D, CD100), a class-4 semaphorin, regulates endothelial cell migration and survival and tumor vascularization [12, 13]. A therapeutic murine Sema4D mAb shifts immunity toward a type I antitumor response with enhanced infiltration of CD11c+ myeloid cells and CD8+ tumor-infiltrating lymphocytes (TILs) into syngeneic murine Colon26 tumors [14]. Tumor cell–derived Sema4D induces formation of myeloid-derived suppressor cells (MDSCs), a type of immunosuppressive myeloid cell, from PBMCs in an in vitro setting [15]. Sema4D expression may drive carcinoma cell peripheral invasion [16], and Sema4D expression correlates with poor outcome in multiple carcinoma types [17].

Given these data, we hypothesized that blockade of Sema4D with a therapeutic mAb could alter MDSCs within the TME and sensitize tumors to ICB. Murine oral cancer-1 (MOC1) is a carcinogen-induced, HPV-negative model of oral cavity carcinoma [18]. The MOC1 TME is infiltrated with immunosuppressive Ly6G+ granulocytic MDSC (PMN-MDSC) but not FoxP3+ CD4+ regulatory T lymphocytes (Treg; refs. 5, 8). Herein, we describe effects of a murine Sema4D mAb on PMN-MDSC. Treatment with Sema4D mAb sensitized MOC1 tumors to rejection following CTLA-4 blockade and growth delay following PD-1 blockade. Enhanced sensitivity to ICB following Sema4D mAb treatment was separately validated in Lewis lung carcinomas (LLC). The ICB sensitization was not due to significant changes in tumor vascularity or direct inhibition of tumor cell proliferation or survival. Following Sema4D mAb treatment, TME infiltration of PMN-MDSCs was significantly reduced, due in part to reduced expression of the myeloid chemokines CXCL1, 2, and 5, but not due to inhibited PMN-MDSC expansion within the TME. The ability of PMN-MDSC to suppress T-cell proliferation and tumor antigen–specific killing was reduced due to blockade of Sema4D-induced PMN-MDSC arginase expression. These effects resulted in enhanced infiltration of CD8+ TIL in the MOC1 TME and...
enhanced tumor antigen–specific responses in CD8+ T lymphocytes from mice treated with Sema4D mAb. A phase I trial of a human Sema4D mAb (vX15) demonstrated a good safety profile in patients with advanced solid cancers (19). Thus, a treatment that combines Sema4D mAb with immune-checkpoint blockade may be useful against tumors infiltrated with MDSCs such as HN5SCC.

Materials and Methods

Cell culture

MOC1 cells from the same stock that has been genomically characterized (20) were obtained from Ravindra Uppaluri (Washington University, St. Louis, Missouri) in 2014 and cultured as described (21). LLC cells were obtained from James Hodge (National Cancer Institute) in 2016 and cultured as described (22). Both cell lines were used for experiments within 20 passages and serially tested to ensure negativity for murine pathogens and mycoplasma. Cell lines were not reauthenticated within the past year. Recombinant Sema4D (rSema4D), Sema4D mAb, and isotype mAb were obtained from Vaccinex under a Cooperative Research and Drug Agreement. U0126 was purchased from Selleckchem.

Animal studies

All in vivo treatments were approved by the NIH animal care and use committee. MOC1 or LLC tumors were established via subcutaneous injection of MOC1 (5 × 10^6) or LLC (1.5 × 10^6) cells suspended in 30% matrigel (Corning) in the right flank of wild-type C57BL/6 (B6) mice (Tacoxn). Sema4D (clone 67-2, 200 μg/injection once every 7 days for 4 total doses, starting at day 4) or isotype control mAb treatments were performed via intraperitoneal injection (i.p.) and started on day 4. PD-1 (clone RMP1-14, 200 μg/injection once every 4 days for 4 total doses, starting at day 10) or CTLA-4 (clone 9H10, 100 μg/injection once every 5 days for total doses, starting at day 10) mAb (Bio X Cell) injections were performed via i.p. and started on day 10. For tumor growth experiments, tumor measurements and weights were obtained 2 to 3 times per week, and volume was calculated as (length^2 × width^2)/2. Tumors, draining lymph nodes, and spleens were harvested and assayed as described. Digestion of tumors into single-cell suspensions was performed using a murine tumor dissociation kit and a gentleMACS dissociator (Miltenyi) per the manufacturer’s recommendations. When performed, leukocyte separation from whole tumor digestes was achieved using a standard 80%/40% Percoll (Sigma-Aldrich) gradient. The >95% purity of CD45.2+ cells after gradient was verified by flow cytometry. For some experiments, primary T lymphocytes were isolated and negative selection of CD45.2+ T-lymphocytes (pan T-cell isolation kit II) was performed on an autoMACS (Miltenyi).

Flow cytometry

Only fresh cultured cells or tissues prepared into single-cell suspensions were analyzed. Non-specific staining was minimized by staining with CD16/32 (FcR) blocking antibodies. Primary antibodies were applied for 30 minutes at concentrations titrated for each antibody. PlexinB1 antibody was purchased from Proteintech, phospho-AKT antibody was purchased from Cell Signaling Technology, phospho-ERK and phospho-STAT3 antibodies were from Thermo Fisher, and all other antibodies were from BioLegend or eBioscience. Dead cells were excluded via 7AAD uptake or a fixable viability dye and a “fluorescence-minus-one” technique was used to validate specific staining of all antibody combinations. Fixation and permeation for Ki67 and transcription factor staining was achieved with the transcription factor staining buffer set (eBioscience) per the manufacturer’s recommendations. Apoptosis was assessed using the annexin V apoptosis detection kit (eBioscience) per the manufacturer’s protocol. All analyses were performed on a BD FACSCanto analyzer running FACSDiva software and interpreted using FlowJo (vX10.0.7r2).

Tumor-draining lymph node T-lymphocyte responses

From tumor-draining lymph nodes, T lymphocytes were isolated by negative magnetic selection on an autoMACS (Miltenyi) and combined with MOC1 tumor cells that had been irradiated (50 Gy was required to reverse tumor cell–mediated suppression
of T-lymphocyte activation) and treated with IFN (20 ng/mL IFNγ × 24 hours to increase MHC class I expression). The cells were combined at a ratio of 3:1 T lymphocyte:MOC1. Supernatants were collected at 24 hours and analyzed for IFNγ concentration by ELISA. Other lymph node T lymphocytes were stimulated with CD3/28 dynabeads (Thermo Fisher) at a 1:1 T lymphocyte bead ratio. Supernatant IFNγ concentration was measured by ELISA.

Immunofluorescence

Tumor sections (5 μm) were fixed for 7 minutes in ice-cold methanol at −20°C. Following several washes, samples were blocked with RTU normal goat serum (2.5%; Vector Laboratories) and Renaissance Antibody diluent (Biocare Medical). Anti-CD31 conjugated to APC (clone 390; eBioscience) was diluted 1:50 in Renaissance antibody diluent, and sections were stained for 1 hour on an orbital shaker (80 rpm). Slides were washed, mounted with DAPI VECTASHIELD mounting media, cover-slipped, and sealed. Images were obtained on a Zeiss LSM780 confocal microscope.

qRT-PCR

Cells were lysed or whole tumor lysates were generated using the Tissue Lyser II. RNA was purified using the RNaseasy Mini Kit (Qiagen) per the manufacturer’s protocol. cDNA was synthesized utilizing a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems). A TaqMan Universal PCR master mix was used to assess the relative expression of target genes compared with GAPDH on a Viia7 qPCR analyzer (Applied Biosystems. master mix was used to assess the relative expression of target genes compared with GAPDH on a Viia7 qPCR analyzer (Applied Biosystems). All primers were commercially available and purchased from Thermo Scientific.

Western analysis

Whole-cell lysates were obtained using NP40 lysis buffer, mixed with NuPAGE LDS sample buffer and reducing agent (Life Technologies), heated at 95°C for 5 minutes, and subjected to electrophoresis using Bis-Tris precast gels (Life Technologies) at 150 V for 100 minutes. The Invitrogen iBlot Dry Blotting System was used to transfer proteins onto a PVDF membrane. Primary antibodies were diluted in 5% BSA prepared from Tween 20-TBS (dilutions: pErk1/2 1:1,000; pAKT 1:1,000; pSTAT3 1:2,000; ERK1/2 1:1,000; Actin 1:5,000). Secondary antibodies were diluted in 5% BSA prepared from Tween 20-TBS (dilutions: pErk1/2 1:1,000; pAKT 1:1,000; pSTAT3 1:2,000; ERK1/2 1:1,000; Actin 1:5,000). Primary antibodies were purchased from Cell Signaling Technologies. Blots were incubated with Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific Inc.) and imaged using Image Studio software (LI-COR Biosciences).

ELISA

CXCL1 (R&D) and IFNγ (eBioscience) ELISAs were used per the manufacturer’s recommendations.

Statistical analysis

Tests of significance between pairs of data are reported as P values, derived using a Student t test with a two-tailed distribution and calculated at 95% confidence. Comparison of multiple sets of data was achieved with analysis of variance with Tukey multiple comparisons. Survival analysis was determined by log-rank (Mantel–Cox) analysis. All error bars indicate standard deviation. Statistical significance was set to P < 0.05. All analysis was performed using GraphPad Prism v7.

Results

Sema4D mAb sensitized carcinomas to CTLA-4 or PD-1 ICB

Sema4D mAb can polarize the TME toward antitumor activity (14) and carcinomas have an immunosuppressive microenvironment (5, 8). We therefore hypothesized that treatment of mice bearing MOC1 tumors with Sema4D mAb would sensitize them to growth control with CTLA-4 mAb. Although treatment of MOC1 tumor-bearing mice with Sema4D mAb alone had little effect, Sema4D mAb treatment enhanced the rate of tumor rejection after CTLA-4 mAb treatment from 50% to 90% (Fig. 1A), resulting in enhanced survival (Fig. 1B). Mice that rejected MOC1
tumors after combination treatment were rechallenged with MOC1 cells in the opposite flank. Mice that previously rejected MOC1 did not engraft tumors (Fig. 1C), suggesting the presence of immunologic memory.

We hypothesized that Sema4D mAb treatment could sensitize mice bearing MOC1 tumors to growth control with PD-1 mAb. Although neither treatment alone altered tumor growth, the combination of Sema4D mAb and PD-1 delayed primary tumor growth (Fig. 1D) and significantly prolonged survival of mice (Fig. 1E). Together, these data suggested that Sema4D mAb enhanced the rate of rejection of MOC1 tumors following CTLA-4 blockade, and sensitized resistant MOC1 tumors to growth delay following PD-1 blockade.

To validate these findings in a separate carcinoma model, these experiments were repeated in mice bearing LLC tumors. Although treatment with Sema4D or CTLA-4 mAb alone induced no tumor growth delay (Fig. 2A), combination treatment delayed tumor growth in 5 of 10 (50%) LLC tumors, leading to significantly prolonged survival (Fig. 2B). Treatment with PD-1 mAb alone initially delayed growth in some tumors, but these quickly rebounded. However, the combination of Sema4D and PD-1 mAb induced rejection of 2 of 10 (20%) established LLC tumors and slowed growth in the remaining tumors (Fig. 2C), leading to significantly prolonged survival (Fig. 2D). Thus, treatment with Sema4D mAb enhanced tumor control or tumor rejection following treatment with CTLA-4 or PD-1 ICB in two murine carcinoma models.

Sema4D mAb did not affect tumor cell growth or immunogenicity or tumor vascularity

To understand the mechanisms of enhanced responses to ICB following Sema4D mAb treatment, we explored the expression of Sema4D and its high-affinity receptor PlexinB1 in normal oral mucosa from B6 mice and MOC1 carcinomas from B6 mice (Fig. 3A). Compared with epithelial cells from the oral mucosa, MOC1 tumor cells expressed more cell-surface Sema4D and PlexinB1. Consistent with prior reports (12, 13), CD31 + endothelial cells expressed high levels of PlexinB1, and this expression was greater on tumor than oral mucosa endothelial cells. Although both CD11b + myeloid cells and CD3 + T lymphocytes highly expressed Sema4D, Ly6GLy6CintF4/80 + myeloid cells expressed more PlexinB1 than other leukocytes.

We investigated how Sema4D mAb treatment altered MOC1 tumor cell growth or viability by assessing the effect of Sema4D mAb on the viability of MOC1 cells in vitro. Although MOC1 cells express both Sema4D and PlexinB1 (Fig. 3B), real-time impedance analysis suggested that neither recombinant Sema4D (rSema) nor Sema4D mAb significantly altered MOC1 tumor cell growth or proliferation (Fig. 3C). Similarly, treatment with Sema4D mAb did not induce MOC1 cell necrosis or apoptosis (Fig. 3D). To rule out the possibility that MOC1 tumor cells were made more susceptible to T-lymphocyte killing by Sema4D mAb, we assayed for expression of components of immunogenicity (Fig. 3E). Expression of MOC1 cell MHC class I, PD-L1, or related costimulatory factors or death receptors, such as ICAM, CD80, Fas, or TNFR, was not altered after Sema4D mAb treatment. Thus, despite expression of Sema4D and PlexinB1 on many cell types within MOC1 tumors, enhanced responses to ICB were not due to direct inhibition of proliferation or survival of MOC1 tumor cells or direct alteration of tumor cell immunogenicity that sensitizes cells to immune elimination.

Given expression of both Sema4D and PlexinB1 on endothelial cells, we next investigated alterations in CD31 + tumor endothelial accumulation within MOC1 tumors following Sema4D mAb treatment (Fig. 3F) and in CD31 + vessel density (Fig. 3G). Neither was significantly altered, suggesting that reduction in tumor...
same mice were digested into single-cell suspensions, and cellular subsets were analyzed for Sema4D and PlexinB1 expression by representative dot plot of a digested MOC1 tumor is shown. Expression levels on CD45.2 and peripheral myeloid cells with Ki67 to assess proliferation.

Sema4D mAb reduced myeloid cell infiltration by reducing chemokine production.

MOC1 tumors recruit Ly6GhiLy6Cint myeloid cells into the TME between days 10 and 20 of tumor progression (5). Evaluation of MOC1 tumors after Sema4D mAb treatment revealed a significant reduction in tumor infiltration of Ly6GhiLy6Cint myeloid cells (Fig. 4A). Accumulation of either myeloid cell type in the periphery of MOC1 tumor–bearing mice was not significantly altered after Sema4D mAb treatment (Supplementary Fig. S1). Given reports that tumor cell–derived Sema4D can induce expansion of myeloid cell populations, we stained both tumor-infiltrating and peripheral myeloid cells with Ki67 to assess proliferation (Fig. 4B). Reduction in Ki67 positivity was not statistically significant following Sema4D treatment, suggesting that the reduced Ly6GhiLy6Cint myeloid cell accumulation in MOC1 tumors was not due to reduced expansion within the TME. We and others have reported the constitutive expression of the chemokine receptor CXCR2 on Ly6GhiLy6Cint myeloid cells (5). We next assessed expression of the ligands for CXCR2, myeloid chemokines CXCL1, 2, and 5, within the MOC1 TME (Fig. 4C). Given evidence that Ly6GhiLy6Cint myeloid cells were also reduced within the TME following Sema4D mAb treatment, we also assessed changes in expression of the CCR2 ligand and myeloid chemokine CCL2. PCR analysis of whole tumor digests demonstrated reductions in all three CXCR2 ligands but not in CCL2 following Sema4D mAb treatment. Analysis of the immune compartment alone demonstrated a reduction in CXCL1 but not in other myeloid chemokines, suggesting that reduced chemokine expression was primarily due to Sema4D mAb-induced alterations in the tumor and/or stromal cell compartments. Treatment of MOC1 cells in vitro with Sema4D mAb significantly reduced expression of CXCR2 ligands.
PMN-MDSCs. This led us to the hypothesis that Sema4D mAb not only reduced \( \text{Ly6G}^+\text{Ly6C}^+ \) myeloid cell recruitment into MOC1 tumors, but also reversed their immune-suppressive capacity. Evaluation of the ability of \( \text{Ly6G}^+\text{Ly6C}^+ \) myeloid cells to suppress T-lymphocyte proliferation in an \textit{ex vivo} T-lymphocyte proliferation assay demonstrated that these cells are immunosuppressive, and that both sorted peripheral and tumor-infiltrating \( \text{Ly6G}^+\text{Ly6C}^+ \) myeloid cells were less immunosuppressive following Sema4D mAb treatment (Fig. 5A). To be identified as PMN-MDSC, distinct from tumor-associated neutrophils, \( \text{Ly6C}^+ \) myeloid cells must suppress T-cell antigen-specific function (28, 29). We assessed the ability of \( \text{Ly6G}^+\text{Ly6C}^+ \) myeloid cells sorted from MOC1 tumors to be able to suppress MOC1 tumor cell killing by TIL cultured from MOC1 tumors (Fig. 5B). \( \text{Ly6C}^+\text{Ly6C}^+ \) myeloid cells at a 3:1 ratio to TIL (there are about three times as many \( \text{Ly6C}^+\text{Ly6C}^+ \) myeloid cells in MOC1 tumors as there are CD8\(^+\) T cells) suppressed killing of MOC1 cells, validating them as PMN-MDSC. PMN-MDSCs sorted from MOC1 tumors following Sema4D mAb treatment were less suppressive compared with control. Accordingly, PCR analysis of whole tumor digests revealed less arginase expression following Sema4D mAb treatment (Fig. 5C). To explore signaling alterations within PMN-MDSCs following Sema4D mAb treatment, whole leukocytes were sorted from tumors and analyzed for phosphorylation of AKT, ERK, and STAT3 by flow cytometry (Fig. 5D). Although phosphorylation of these signaling...
proteins was unaltered in CD3⁺ lymphocytes, phosphorylation of ERK and STAT3 was reduced in PMN-MDSCs following Sema4D mAb treatment. To validate these findings, we treated PMN-MDSCs sorted from treatment-naïve MOC1 tumors ex vivo with rSema4D or Sema4D mAb and assessed their suppressive capacity (Fig. 5E). Exposure of PMN-MDSCs to rSema4D enhanced suppressive capacity. Addition of Sema4D mAb reduced baseline and reversed rSema4D-induced suppressive capacity. Expression of arginase and phosphorylation of ERK and STAT3 followed a similar pattern (Fig. 5F and G), suggesting that arginase expression within PMN-MDSCs was dependent at least in part on ERK and STAT3 signaling downstream of PlexinB1. Cumulatively, these data suggest that in addition to reduced PMN-MDSC recruitment, Sema4D mAb treatment suppressed ERK- and STAT3-dependent arginase production downstream of PlexinB1 and PMN-MDSC suppressive capacity.

Sema4D mAb enhanced tumor antigen–specific T-lymphocyte responses

To characterize the effect of reduced PMN-MDSC infiltration and suppressive capacity on effector immune cells, we assessed changes in infiltration of T lymphocytes and natural killer (NK) cells after Sema4D mAb treatment (Fig. 6A). Tumors were infiltrated with more CD8⁺ TILs after Sema4D mAb. Infiltrating CD8⁺ TIL did not display significant differences in PD-1 or CTLA-4 expression. Tumor infiltration of CD4⁺ TIL was also enhanced to a degree, but there was no change in infiltration of NK cells after Sema4D treatment (Supplementary Fig. S2). Ki67 expression was greater after Sema4D mAb treatment (Fig. 6B), indicating enhanced expansion of CD8⁺ TIL. Direct exposure of Sema4D mAb to T lymphocytes ex vivo did not alter proliferation (Supplementary Fig. S3), suggesting that the enhanced proliferation of T lymphocytes in vivo was not due to the antibody. To assess T-lymphocyte tumor antigen–specific responses, we combined...
T lymphocytes sorted from tumor-draining lymph nodes with irradiated MOC1 cells as an antigen source. T lymphocytes from mice treated with Sema4D mAb demonstrated greater IFNγ production upon exposure to MOC1 antigen (Fig. 6C). As an additional verification of enhanced T-lymphocyte responses, we stimulated sorted T lymphocytes nonspecifically with CD3/28 mAbs, and found similar increased responses in T lymphocytes from mice treated with Sema4D mAb. Lastly, we assessed MOC1 tumor cells for expression of known IFNγ-responsive proteins H-2Kb/Db and PD-L1 (Fig. 6D). Expression of both was elevated in mice treated with Sema4D mAb, indicating increased IFNγ production in the microenvironment of these tumors.

Discussion

In this study, we describe mechanisms of action of a therapeutic Sema4D mAb in murine models of carcinoma. Blockade of Sema4D appeared to both reduce PMN-MDSC accumulation in MOC1 carcinomas through reduced myeloid chemokine expression from MOC1 tumor cells and decrease PMN-MDSC suppressive capacity through inhibition of PMN-MDSC arginase expression. These changes in PMN-MDSC accumulation and function resulted in enhanced T-lymphocyte tumor antigen–specific responses that enhanced responses to either CTLA-4 or PD-1 ICB. We assessed the performance of a Sema4D mAb in the MOC1 model given previous data that targeting Sema4D altered the myeloid immune compartment (14, 30, 31). Our previous work demonstrated the role of MDSCs in local immunosuppression within MOC1 tumors (5, 8). To ensure that these findings were not model specific, sensitization to ICB following Sema4D blockade was validated in mice bearing LLC tumors. Similar effects of Sema4D blockade have been demonstrated in models of colon and breast carcinoma (14).

This work enhances our understanding of how Sema4D blockade sensitizes carcinomas to ICB. Work by Evans and colleagues demonstrated enhanced tumor penetration of CD11c+ antigen-presenting myeloid cells, cytokine polarization to a type I anti-tumor profile, and enhanced infiltration of IFN-producing antigen-specific CD8+ TILs after Sema4D mAb treatment of Colon26 tumors (14). Our work suggests that, in addition to global alterations in immune profiles, disruption of Sema4D and PlexinB1 signaling within the TME affects tumor cells and PMN-MDSC.

Our finding that Sema4D mAb alters signaling through ERK in tumor cells validates the presence of MAPK pathway signaling downstream of PlexinB1 (25, 27). Although MAPK driven expression of CXCL1 in MOC1 cells was known, here we demonstrate regulation of a more diverse myeloid chemokine expression profile with interruption of signaling downstream of PlexinB1. PlexinB1 forms a signaling complex with the receptor tyrosine kinase "Semaphorin4D Blockade Inhibits MDSC Recruitment and Function"
kinases c-Met or ErbB-2 (32, 33). Intracellular signaling networks downstream of c-Met regulate CXCL1 expression in murine cells (34), and work by Van Waes and colleagues in human HNSCC cells demonstrates the role of c-Met in the regulation of expression of IL8, the human homologue of murine CXCL1 (35, 36). Whether Sema4D mAb disrupts CXCL1 expression downstream of PlexinB1 or alters signaling downstream of c-Met or ErbB-2 due to disrupted receptor tyrosine kinase:plexinB1 interaction remains to be determined. Sema4D blockade and the resulting decreased chemokine expression seemed to slow the accumulation of PMN-MDSCs to a greater degree compared with Ly6C<sup>hi</sup> myeloid cells. This can be explained by more reduction in CXCR2 ligands CXCL1, 2, and 5 within the TME following Sema4D blockade, suggesting Sema4D-independent expression of CCL2 by multiple cell types. PMN-MDSCs are more susceptible to depletion given the high baseline infiltration present in MOC1 tumors. Among tumor-infiltrating myeloid cells, PlexinB1 expression was greater on PMN-MDSCs compared with Ly6C<sup>low</sup>Ly6<sup>chi</sup> cells or macrophages. This may explain why Sema4D blockade appeared to have the greatest impact on PMN-MDSC function. Given the expression of PlexinB1 on PMN-MDSCs, we cannot rule out that Sema4D mAb blocked recruitment of PMN-MDSCs into MOC1 tumors independent of reduced myeloid chemokine expression. CXCL1 expression drives recruitment of CXCR2<sup>+</sup> PMN-MDSCs in multiple tumor models (9, 37, 38), but control of migration of myeloid cells by semaphorin family members has also been demonstrated in other models (30, 31).

Sema4D-driven expression of arginase in PMN-MDSCs was first demonstrated by Younis and colleagues in vitro (15). Our work validates this finding in vivo. Reduced STAT3 phosphorylation and arginase expression in MOC1 tumor-infiltrating PMN-MDSCs after Sema4D treatment supports the work of Vasquez-Dunddel and colleagues demonstrating STAT3-dependent arginase expression in human MDSCs (6). Reduced ERK phosphorylation in MOC1 tumor-infiltrating PMN-MDSCs observed after Sema4D mAb treatment suggests that MAPK signaling may regulate expression of arginase as well. In addition to promoting chemotaxis, signaling through CXCR2 after CXCL1 ligation directly regulates arginase expression in MDSCs (39), offering the possibility that reduced PMN-MDSC suppressive capacity and arginase expression after Sema4D mAb treatment in vivo was due to reduced CXCL1 expression. This is unlikely, however, given that ex vivo rSema4D enhanced the suppressive capacity of sorted PMN-MDSCs, and that this suppressive capacity was abrogated in the presence of Sema4D mAb.

Previous work showed that tumor cell-derived or macrophage-derived Sema4D participates in tumor vascularization by recruiting and promoting the survival of PlexinB1<sup>+</sup> endothelial cells (12, 13, 40). Induction of Sema4D expression by hypoxia-inducible factor-1 is likely required (41). However, signaling through PlexinB1 could regulate independent processes in both myeloid and endothelial cells despite their common embryologic origin (42). Here, we demonstrate that accumulation of CD31<sup>+</sup> endothelial cells and CD31<sup>+</sup> vessels in MOC1 tumors was not altered by Sema4D mAb treatment.

In conclusion, we found reduced PMN-MDSC accumulation and suppressive capacity within the TME of carcinogen-induced, HPV-negative MOC1 oral cavity carcinomas. This adds mechanistic insight into how Sema4D alters the TME to enhance responses to ICB. With low overall response rates to ICB alone in patients with recurrent or metastatic HNSCC, adjuvant therapies are needed to improve outcomes in this patient cohort. These and previously described effects on tumor vasculature and myeloid cells within the TME provide a rationale for the combination of Sema4D mAb blockade and ICB in a clinical trial setting.

Disclosure of Potential Conflicts of Interest

E.S. Smith is Chief Scientific Officer at and has ownership interest in Vaccinex, Inc. M. Zauderer reports receiving commercial research funding from and has ownership interest in Vaccinex, Inc. No potential conflicts of interest were disclosed by the other authors.

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