Human Head and Neck Squamous Cell Carcinoma–Associated Semaphorin 4D Induces Expansion of Myeloid-Derived Suppressor Cells

Rania H. Younis,*,†,[1] Kyu Lee Han,*,[1] and Tonya J. Webb†,‡

One of the mechanisms by which malignancies can induce immune suppression is through the production of cytokines that affect the maturation and differentiation of inflammatory cells in the tumor microenvironment. Semaphorin 4D (Sema4D) is a proangiogenic cytokine produced by several malignancies, which has been described in the regulation of the immune system. In the present study, we examined the role of human head and neck squamous cell carcinoma (HNSCC)–secreted Sema4D on myeloid cell differentiation. $CD33^+$ cells cultured in HNSCC cell line–derived conditioned medium differentiated into myeloid derived suppressor cells (MDSC) ($CD33^+CD11b^+HLA\text{-}DR^{-/low}$). The addition of anti-Sema4D Ab to HNSCC conditioned medium significantly reduced the expansion of the MDSC population. Similarly, knockdown of Sema4D in an HNSCC cell line resulted in a loss of MDSC function as shown by a decrease in the production of the immune-suppressive cytokines arginase-1, TGF-β, and IL-10 by MDSC, concomitant with recovery of T cell proliferation and IFN-γ production following stimulation of CD3/CD28. Importantly, $CD33^+$ myeloid and T cells cultured in conditioned medium of HNSCC cells in which Sema4D was knocked down promoted antitumor inflammatory profile, through recovery of the effector T cells ($CD4^+$T-bet^+ and $CD8^+$T-bet^+), as well as a decrease in regulatory T cells ($CD4^+CD25^+FOXP3^+$). We also showed that Sema4D was comparable to GM-CSF in its induction of MDSC. Collectively, this study describes a novel immunosuppressive role for Sema4D in HNSCC through induction of MDSC, and it highlights Sema4D as a therapeutic target for future studies to enhance the antitumorigenic inflammatory response in HNSCC and other epithelial malignancies. ***The Journal of Immunology*****, 2016, 196: 1419–1429.**

Head and neck squamous cell carcinoma (HNSCC) is a malignancy of high morbidity and mortality, with 45,780 new cases and 8,650 estimated deaths of oral and pharyngeal cancer estimated to occur in the United States in the year 2015 (1). There is accumulating evidence indicating the immunomodulatory effects of HNSCC by which it can escape and/or suppress the immune system (2–6).

Myeloid-derived suppressor cells (MDSC) have been described in peripheral blood, draining lymphoid tissue, and tumor tissue of several malignancies (5, 7–10). Circulating MDSC correlated with advanced stages of HNSCC (stages III and IV) as well as other carcinomas (8, 10, 11). MDSC represent a key player in immune regulation in the tumor microenvironment. It is generally agreed that they comprise a heterogeneous population of myeloid progenitor cells and immature myeloid cells that have a suppressive function on T cells (12, 13). MDSC described in human malignancies have the phenotype of $CD33^+$, $CD11b^+$, and non–lineage determined with poor Ag presentation abilities ($HLA\text{-}DR^{-/low}$). They can have a progranulocytic phenotype expressing CD66b or CD15 (polymorphonuclear leukocyte–MDSC) or monocytic features expressing CD14 (10, 14, 15). MDSC induce their immune-suppressive effect mainly through production of arginase-1 and inducible NO synthase, which consume extracellular arginine and accordingly suppress T cell activation in an Ag-nonspecific manner in the tumor microenvironment. However, they mediate Ag-specific suppression by NADPH oxidase production of reactive oxygen and nitrogen species, particularly in peripheral lymphoid tissue, as well as by other mechanisms (12, 15–17). In addition to direct T cell suppression, recent evidence suggests a role for MDSC in the expansion of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) in the tumor microenvironment through both TGF-β–dependent and independent pathways (11, 18). Although several mechanisms have been described by which tumor cells induce MDSC, the specific pathways by which HNSCC recruit, expand, and activate MDSC remain to be investigated (15, 19, 20).

Tumor cells overexpress several cytokines to manipulate their own microenvironment, among which are multiple semaphorins, which have the potential to act on different stromal cells (18). Semaphorin 4D (Sema4D; CD100) is a transmembrane glycoprotein belonging to the fourth group of the semaphorin family that can also be found in a soluble form following proteolytic cleavage. It was initially identified as an evolutionarily conserved chemorepellent protein that regulates axonal guidance in the developing nervous system (21). Later on, its interactions in other systems were emphasized, including the cardiovascular system and immune system. In the immune system, Sema4D is described

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Abbreviations used in this article: Ctl, control; HNSCC, head and neck squamous cell carcinoma; MDSC, myeloid-derived suppressor cell; NM, normal medium; NOK, normal oral keratinocyte; Sema4D, semaphorin 4D; shRNA, short hairpin RNA; Treg, regulatory T cell.

as being expressed abundantly on resting T cells and weakly on resting B cells and APCs (22–26). Two opposing roles of Sema4D have been described in the immune system. One role is a proinflammatory response where, for example, in the humoral and cell-mediated immune system, Sema4D acts on B cells and dendritic cells, respectively, promoting proinflammatory cytokines (25–27). Sema4D expressed by T cells and NK cells has also been implicated in their activation through a Sema4D-associated tyrosine kinase (28), and it has been shown to play a role in T cell priming and accordingly in the pathogenesis of autoimmune diseases (29). Alternatively, an anti-inflammatory role of Sema4D in the immune system has also been described. On monocytes and immature dendritic cells, Sema4D can act on plexin C1 and plexin B1, respectively, inhibiting their migration, but not that of mature dendritic cells, which can provide more interaction between immature myeloid cells and T cells (30, 31). Furthermore, in vitro studies have shown that Sema4D can modulate cytokine production by monocytes and dendritic cells, and it can induce a significant increase in the anti-inflammatory cytokine IL-10 and a decrease in the proinflammatory cytokines IL-6, IL-8, and TNF-α (26, 30, 31).

The role of Sema4D produced by tumor cells in inducing tumor angiogenesis, invasiveness, and progression has been demonstrated in several malignancies, both in human and animal models, and correlates with poor prognosis (32–38). Sema4D overexpression has been described in a large number of human HNSCC tumor tissue samples and in a panel of primary and metastatic cell lines (32). In the tumor microenvironment, tumor-associated macrophages were reported to be the main producers of Sema4D in a mouse breast cancer model (39). Taken together, the hypothesis tested in the present study was that the net inflammatory profile orchestrated by Sema4D in the tumor microenvironment is protumorigenic through induction of immune-suppressive cells.

In this study, we investigated whether HNSCC-derived Sema4D plays a role in inducing MDSC and examined its effect on T cell phenotype and function. We found that Sema4D produced by the HNSCC HN6 and HN13 cell lines polarized myeloid cells into an MDSC phenotype, which corresponded with a reduction in T cell proliferation and IFN-γ production. Furthermore, short hairpin RNA (shRNA) inhibition of Sema4D in HN6 resulted in a decrease in the production of the immunosuppressive factors arginase-1, TGF-β, and IL-10 by myeloid cells and the recovery of autologous T cell proliferation, as well as IFN-γ production, specifically leading to an increase in the effector T cell population and decrease in Tregs. Our findings describe HNSCC-associated Sema4D production as one of the mechanisms by which the tumors induce MDSC, which subsequently mediate their immunosuppression effects on T cells.

Materials and Methods

Cell lines and tissue culture

Human normal oral keratinocytes (NOK) and human-derived HNSCC cell lines WSU-HN6 and WSU-HN13 underwent DNA authentication (Johns Hopkins Genetic Resources Core Facility, Baltimore, MD) to ensure consistency in cell identity in comparison with their source (40). SCC-9 was obtained from American Type Culture Collection (Manassas, VA). The WSU-HN4 cell line was a gift from Dr. Silvio Gutkind (National Institute of Dental and Craniofacial Research, Bethesda, MD) (40). All HNSCC cell lines used were derived from primary carcinomas of the tongue. All cell lines were grown and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma-Aldrich, St. Louis, MO) at 37°C in humidified air with 5% CO_2. For treatment with conditioned medium, culture medium was collected from confluent HNSCC cells grown in 5 ml DMEM for 24 h. For myeloid and T cell coculture experiments, conditioned medium supplemented with 5% FBS was used.

Separation of myeloid and T cells from human peripheral blood

Mononuclear cell suspension was prepared from peripheral blood of healthy donors obtained from commercial vendors, that is, Biological Specialty (Colmar, PA) or New York Blood Center (New York, NY), using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Life Sciences, Pittsburgh, PA). $CD33^+$ myeloid cells were separated from the PBMC using MACS CD33 microbeads (catalog no.130-045-501), and $HLA\text{-}DR^+$ cells were separated using anti-HLA-DR microbeads (catalog no.130-046-100; Miltenyi Biotec, Auburn, CA). Autologous T cells were enriched by negative selection with the human pan T cell isolation kit (catalog no. 19051; Stemcell Technologies, Vancouver, BC, Canada).

Flow cytometry characterization of MDSC and T cells

MDSC were characterized using multicolor staining fluorochrome-labeled anti-human CD33-PerCP/Cy5.5 (catalog no. 303414), HLA-DR-allophycocyanin (catalog no. 307610), CD11b-PE (catalog no.301306), and CD14-FITC (catalog no. 325604) (BioLegend, San Diego, CA). For T cell analysis, CD3-allophycocyanin/Cy7 (catalog no. 300317) (BioLegend), CD8a-PE-Cy7 (catalog no. 25-0088-42), CD25-allophycocyanin (catalog no. 17-0259-42), FOXP3-PE (catalog no. 12-4776-42), and T-bet–PerCP-Cy5.5 (catalog no. 45-5825-82) (eBioscience, San Diego, CA) were used. For anti–FOXP3-PE and anti–T-bet-PerCP/Cy5.5, fixation and permeabilization buffer (catalog no. 00-8333) eBioscience was used. For T cell proliferation, T cells were stained with CFSE (catalog no. C34554) (Life Technologies, Grand Island, NY), prior to plating. Dynabeads human T-activator CD3/CD28 (Life Technologies) were used at a 1:3 (T cell/bead) ratio after coculture with myeloid cells for at least 4 h. Data were acquired using a BD LSR II flow cytometer, FlowJo software (Tree Star, Ashland, OR), and analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA).

Immunoblot, Abs, and reagents

Cells were harvested in cell lysis buffer (20mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) (catalog no. 9803) (Cell Signaling Technology, Danvers, MA) with the addition of 1× protease inhibitor cOmplete, Mini, EDTA-free (catalog no. 11836170001) (Roche Diagnostics, Indianapolis, IN). Whole-cell lysate was separated using SDS-PAGE. The primary Abs Sema4D (30/CD100, catalog no. 610670) (BD Biosciences/Pharmingen, San Diego, CA), arginase-1 (A-2; sc-365547), GAPDH (8C2; sc-81545), and β-actin (catalog no. sc-2301) (Santa Cruz Biotechnology, Dallas, TX) were used. The secondary Abs used were anti-rabbit IgG (catalog no. sc-2301) and anti-mouse IgG (catalog no. sc-2302) (Santa Cruz Biotechnology). For arginase-1 detection, 15 μg protein was loaded, whereas for Sema4D detection, 70–100 μg was loaded. For detection of Sema4D in the tumor conditioned medium, equal aliquots of the medium or of a concentrate of the medium using Millipore Amicon Ultra centrifugal filter units were loaded after adding the sample buffer. Cell viability was measured using the cell proliferation reagent WST-1 (Roche Diagnostics). The plate was read at 450 nm on a BioTek Epoch microplate spectrophotometer (BioTek, Winooski, VT). The recombinant human Sema4D (sCD100) (catalog no. 310-29) was obtained from (PeproTech, Rocky Hill, NJ). Recombinant human GM-CSF (catalog no. 215-GM-010) (R&D Systems Minneapolis, MN) and IL-6 recombinant human (catalog no. I1395) from (Sigma-Aldrich) were also used. Griess reagent (catalog no. G2930) (Promega, Madison, WI) was used to measure the nitrite concentration as a stable nonvolatile byproduct of NO in the media, read at absorbance of 520 nm using a BioTek Epoch microplate spectrophotometer.

Anti-Sema4D treatment and immune depletion

For target protein inhibition in the conditioned medium, anti-Sema4D (catalog no.610670) (BD Biosciences) was used at a concentration of 10 μg/ml. For immune depletion, immunoprecipitation of Sema4D or GM-CSF was carried out by overnight incubation of the conditioned media with Sema4D (catalog no. ab39710) (Abcam, Cambridge, MA) or GM-CSF (clone BVD2-21C11; catalog no.502301) (BioLegend; 10 μg/ml) mAbs using protein A beads, followed by precipitation.

Sema4D shRNA and lentivirus infections

The lentivirus and shRNA system were a gift of Dr. John R. Basile (University of Maryland, Baltimore) (32). In brief, the shRNA sequences for human Sema4D were obtained from Cold Spring Harbor Laboratory's

RNAi library (RNAi Codex; http:katahdin.cshl.org:9331_homepage_portal_scripts_main2.pl). The oligonucleotides (Invitrogen, Grand Island, NY) used to knockdown Sema4D protein levels were 5′-GGCCTGAGGACCTTG-CAGAAGA-3′. The Sema4D shRNA oligonucleotides were cloned into lentiviral expression vector pWPI GW as previously described (32). pWPI (empty) vector was used as negative control. Infections were performed using FuGENE HD transfection reagent (catalog no. E2311; Promega).

ELISA

Human ELISA MAX IFN-γ (catalog no. 430103), TGF-β1 total (catalog no. 436707), IL-10 (catalog no. 430601), and IL-4 (catalog no. 430301) were obtained from BioLegend. Samples were run in triplicates and read using a BioTek Epoch microplate spectrophotometer at 450 nm wavelength.

Statistical analyis

Student paired *t* tests were performed as appropriate. All data analysis was presented with custom SD. The *p* values are $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Results

Sema4D expressed by HNSCC cell lines induces CD33$^+$, CD11b$^+$, HLA-DR$^{-/low}$ cells

To examine the effects of HNSCC-derived condition medium on myeloid differentiation, total human PBMC or total myeloid cells ($CD33^+$) isolated from PBMC were plated in conditioned medium from HNSCC cell lines HN4, HN6, HN13, and SCC-9. Total PBMC or separated myeloid cells cultured in HNSCC conditioned medium displayed markers characteristic of MDSC, namely $CD33^+$ $CD11b^+HLA\text{-}DR^{-/low}$ compared with cells cultured in normal medium (NM) or NOK conditioned medium. The percentage of MDSC developed from total PBMC was ∼20% (Supplemental Fig. 1A), whereas that generated from $CD33^+$ myeloid cells averaged 30% depending on the HNSCC cell line and density used (Fig. 1A, Supplemental Fig. 1B). Moreover, titration of HN6- and HN13-derived conditioned medium with NM corresponded with a dose-dependent decrease in the MDSC population (Fig. 1B, 1C).

Several groups have recently described an immunomodulatory role for tumor-produced proangiogenic factors (41). Given the immune modulatory role previously described for Sema4D in the immune system (27, 30), we sought to investigate whether the proangiogenic factor Sema4D, secreted by HNSCC (32), plays a role in the induction of $CD33^+CD11b^+HLA\text{-}DR^{-/low}$ myeloid cells. Immunoblotting was performed on conditioned medium from HN6 and HN13 HNSCC cell lines, and their cellular lysates served as controls. We detected the cleaved form of Sema4D in the medium at ∼120 kDa (Fig. 2A) as previously reported (42). We then neutralized Sema4D in the HN4-, HN6-, HN13-, and SCC-9 conditioned medium using anti-Sema4D Ab (clone 30/CD100) and then used the medium to culture myeloid cells. Blocking Sema4D in HNSCC conditioned medium resulted in an ∼50% reduction in the MDSC population compared with cells growing in HNSCC conditioned medium pretreated with the isotype control mAb (Fig. 2B, 2C, Supplemental Fig. 1B).

Sema4D inhibition rescues MDSC-induced T cell suppression

We next sought to ascertain whether HNSCC-derived Sema4D mediated its suppressive effects when myeloid cells were cultured with primary T cells. The induction of $HLA\text{-}DR^{-/low}$ cells by HN6 conditioned media was still observed upon coculture of the $CD33^+$

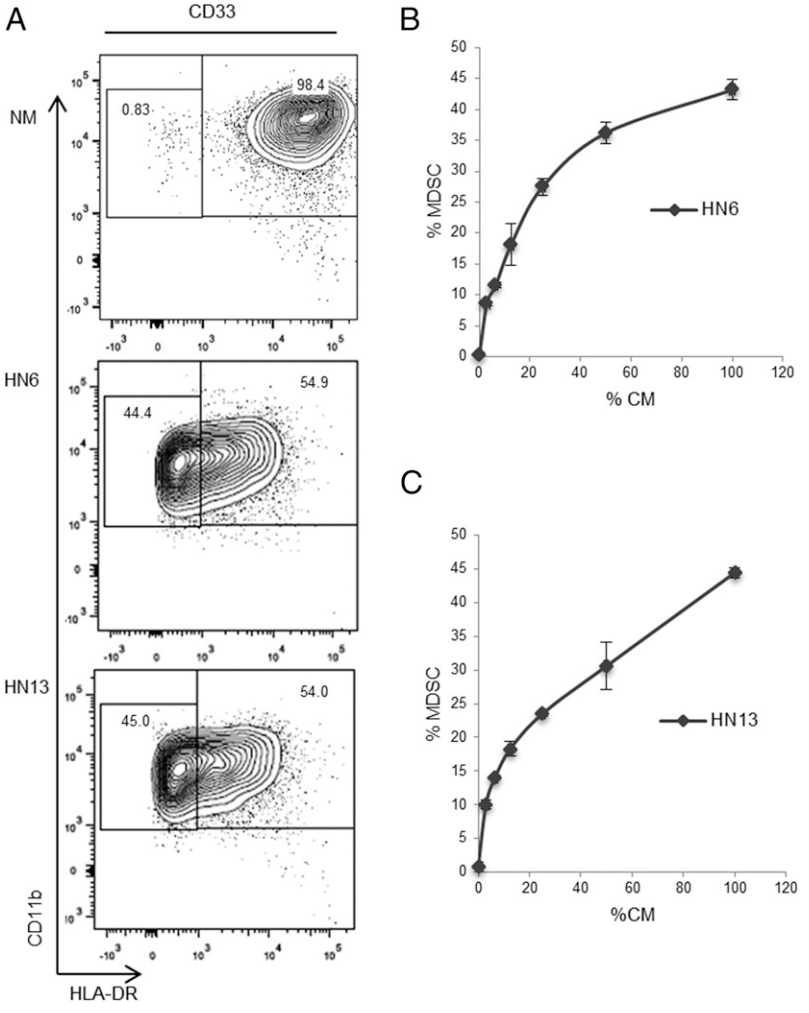

FIGURE 1. HNSCC conditioned media polarizes myeloid cells toward an MDSC phenotype. (**A**) $CD33^+$ cells were cultured in NM or HNSCC conditioned media (HN6, HN13) for 72 h and then analyzed by flow cytometry. (**B**) Dose-dependent induction of MDSC following culture in HNSCC conditioned media. $CD33^+$ cells were cultured in serial dilutions of HN6 conditioned medium (CM) and (**C**) HN13 CM. Cells were first gated for $CD33^+$ then for $CD11b^+$, followed by a third gate for $CD11b^+HLA\text{-}DR^{-/low}$. CD33 cells were isolated using MACS from PBMC separated by centrifugation gradient of peripheral blood. All samples were run in duplicates. CM, conditioned medium.

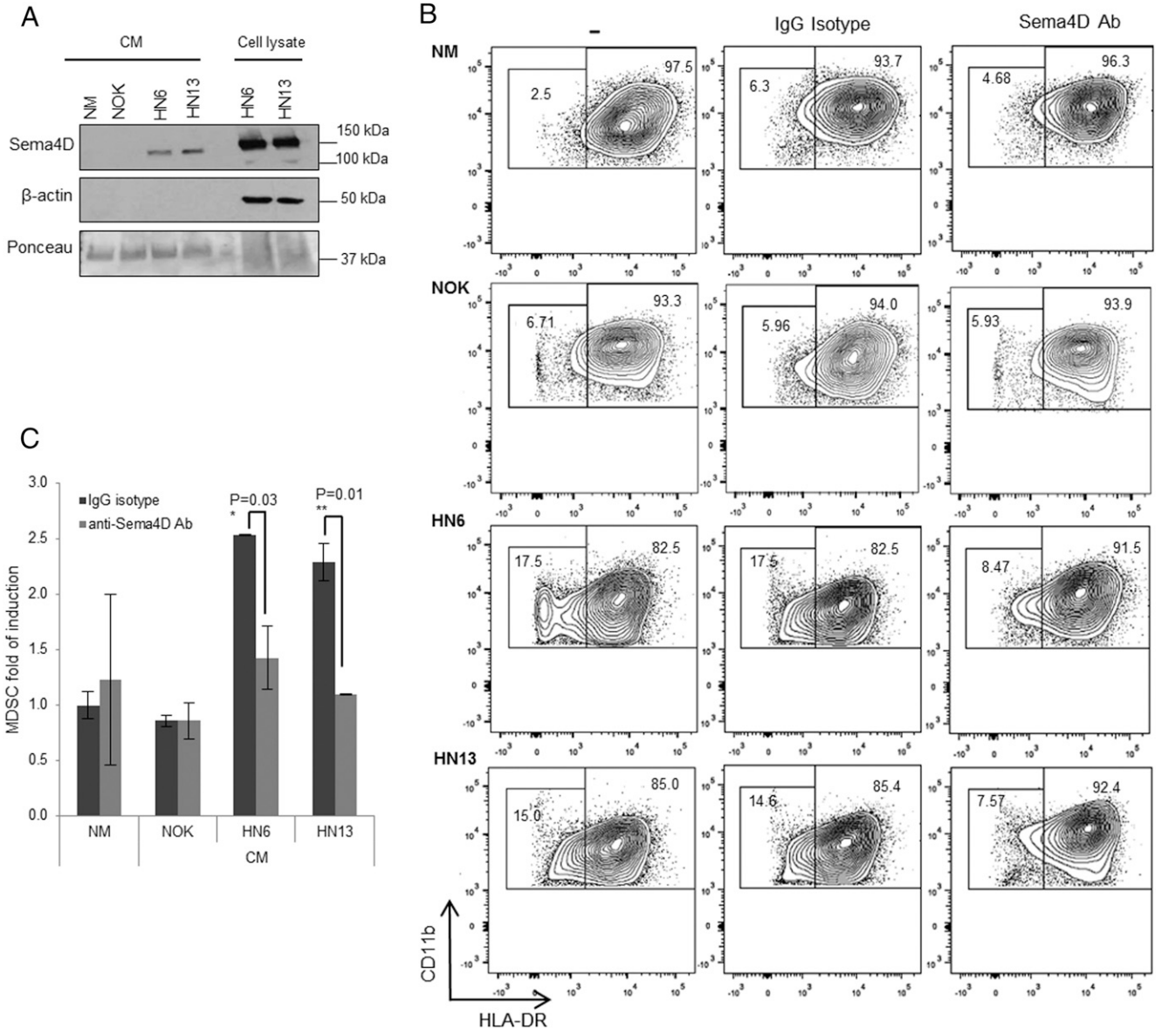

FIGURE 2. Sema4D produced by HNSCC plays a role in MDSC induction. (**A**) Sema4D is secreted by HN6 and HN13 cell lines. Conditioned medium (CM) and total cell lysates from HN6 and HN13 HNSCC cell lines were analyzed by Western blot. Ponceau staining was used as a control for protein loading prior to immunoblotting. β-Actin served as control for cell lysates. (**B**) Anti-Sema4D Ab treatment downregulates MDSC induction by HNSCC cells. $CD33^+$ cells were cultured in NM, NOK, HN6, and HN13 CM in the presence or absence of anti-Sema4D mAb (10 μg/ml) or isotype control mAb for 72 h. Cells were analyzed by flow cytometry by gating on $CD33^+CD11b^+$, then analyzed for $CD11b^+HLA\text{-}DR^{-/low}$. The experiment was independently repeated twice. (**C**) Graphical presentation of two combined experiments showing MDSC reduction ($CD33^+CD11b^+HLA\text{-}DR^{-/low}$) upon anti-Sema4D Ab treatment of HN6 and HN13 CM. Data were normalized to MDSC in NM treated with IgG isotype. CM, conditioned medium.

cells with autologous T cells (Supplemental Fig. 2A). To determine whether Sema4D induction of MDSC induces T cell suppression, we constructed Sema4D-shRNA to knock down Sema4D in HN6 cells and accordingly in the conditioned medium (Supplemental Fig. 2B, 2C). Significant recovery of T cell proliferation was observed upon coculture of myeloid and T cells in the HN6 Sema4D-shRNA compared with the control (Ctl)-shRNA medium (Fig. 3A, 3B). T cells grown in HN6 Ctl-shRNA medium were suppressed upon coculture with the myeloid cells, but not when cultured in NM, indicating that the suppression is mediated by the induction of MDSC (Fig. 3B). Notably, the knockdown of Sema4D produced by HN6 resulted in significant recovery of the T cells at different myeloid/T cell ratios (Fig. 3A, 3B). This was paralleled with recovery of IFN-γ production (Fig. 3C) as well as inhibition of IL-4 in the supernatant (Fig. 3D).

HN6-secreted Sema4D induces an immunosuppressive T cell phenotype

To investigate which T cell subset is rescued following blockade of Sema4D, a phenotypic analysis of the T cells was performed. We found that inhibition of Sema4D restored effector Th1 cells ($CD4^+$ $T\text{-}bet^+$) and cytotoxic T cells ($CD8^+T\text{-}bet^+$) and significantly decreased Tregs ($CD4^+CD25^+FOXP3^+$) at different myeloid/T cell ratios (Fig. 4, Supplemental Fig. 2D–F). These findings indicate that inhibition of Sema4D can rescue effector T cells while suppressing the Treg population in the presence of myeloid cells.

Sema4D enhances GM-CSF plus IL-6–mediated induction of MDSC

To better assess the role of HNSCC-derived Sema4D on myeloid differentiation, we compared Sema4D with another well-described

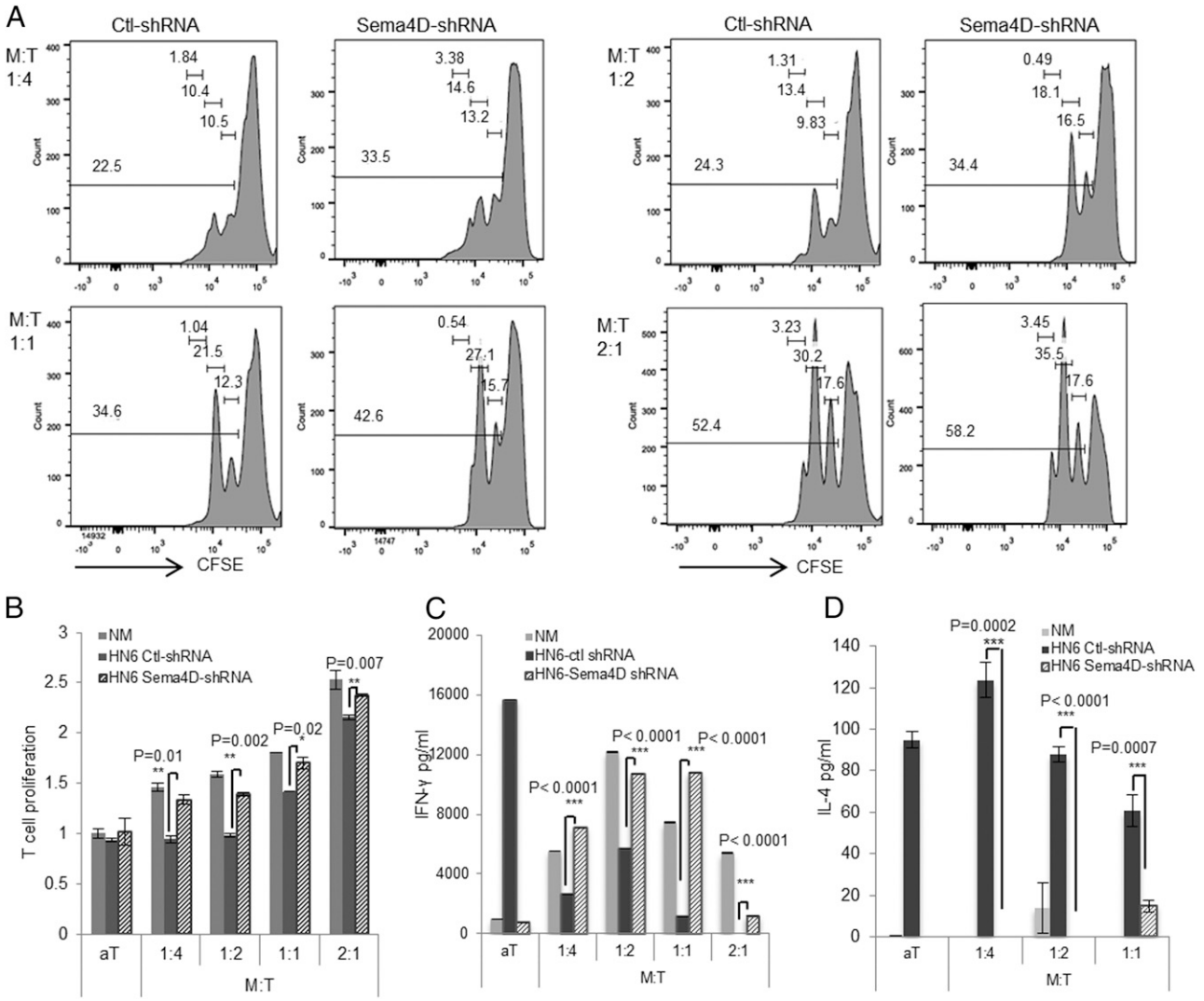

FIGURE 3. Inhibition of Sema4D produced by HN6 rescues MDSC-mediated T cell suppression. (**A**) Recovery of T cell proliferation following inhibition of Sema4D in HN6 cell line using lentivirus shRNA. T cells stained with CFSE and myeloid cells were added at the indicated ratios (myeloid/T cell [M:T]). Cells were cocultured in conditioned medium (CM) from HN6 Sema4D-shRNA or HN6 Ctl-shRNA and activated by anti-CD3/CD28 microbeads for 72 h. CFSE dilution in T cells was analyzed by FACS. (**B**) Graphical representation of flow cytometry data shown in (A). Data were normalized to activated T cells cultured alone in NM. The supernatants were collected to assess IFN-γ (**C**) and IL-4 (**D**) production using ELISA. Error bars indicate SD of triplicate assays.

MDSC inducer, GM-CSF (14). GM-CSF has been shown to be produced by several MDSC-inducing human solid tumors, including HNSCC, and to best induce human $CD33^+$ suppressive cells upon combination with IL-6 (14, 16, 19, 20). In fact, we tested the HN6 HNSCC cell line using ELISA and it showed production of both GM-CSF and IL-6 (data not shown). To compare the effect of tumor-secreted Sema4D on the induction of MDSC to GM-CSF–mediated induction, $CD33^+$ cells were cocultured with T cells in HN6 medium with immune depletion of Sema4D versus immune depletion of GM-CSF. Immune depletion of Sema4D resulted in a significant reduction in the MDSC population that was comparable to that observed upon depletion of GM-CSF in HN6 conditioned medium (Fig. 5A, 5B). The reduction in MDSC corresponded with a significant increase in T cell proliferation (Fig. 5C, 5D) and IFN-γ production (Fig. 5E). These data strongly support a role for Sema4D produced by HN6 cells in the induction of MDSC and its subsequent T cell suppression. To further study the role of Sema4D in conjunction with other tumor-produced cytokines in MDSC induction, we grew $CD33^+$ cells in NM to which the three recombinant proteins (Sema4D, IL-6, and GM-CSF) were added. The three-cytokine induction mixture of Sema4D plus GM-CSF plus IL-6 resulted in a 30% increase in MDSC induction compared with the GM-CSF plus IL-6 mixture. This indicated that Sema4D acts synergistically with GM-CSF plus IL-6 to increase the repertoire of the MDSC in the HNSCC tumor microenvironment (Fig. 5F, 5G).

Sema4D promotes the production of immunosuppressive mediators by MDSC

To investigate whether HNSCC-associated Sema4D has an effect on MDSC viability, a WST cell proliferation assay and a CFSE proliferation tracking experiment were performed using $CD33^+$ cells grown in conditioned medium from the HN6 cells in which Sema4D has been knocked down using shRNA. The loss of Sema4D resulted in a significant decrease in the proliferation of total $CD33^+$ cells, as well as that of the $CD33^+$ HLA-$DR^{-/low}$ population compared with conditioned medium from controls (Fig. 6A, 6B). Interestingly, reconstitution of the Sema4D-shRNA HN6 conditioned media with human recombinant Sema4D protein recovered the $CD33^+$HLA-$DR^{-/low}$ proliferation as shown by

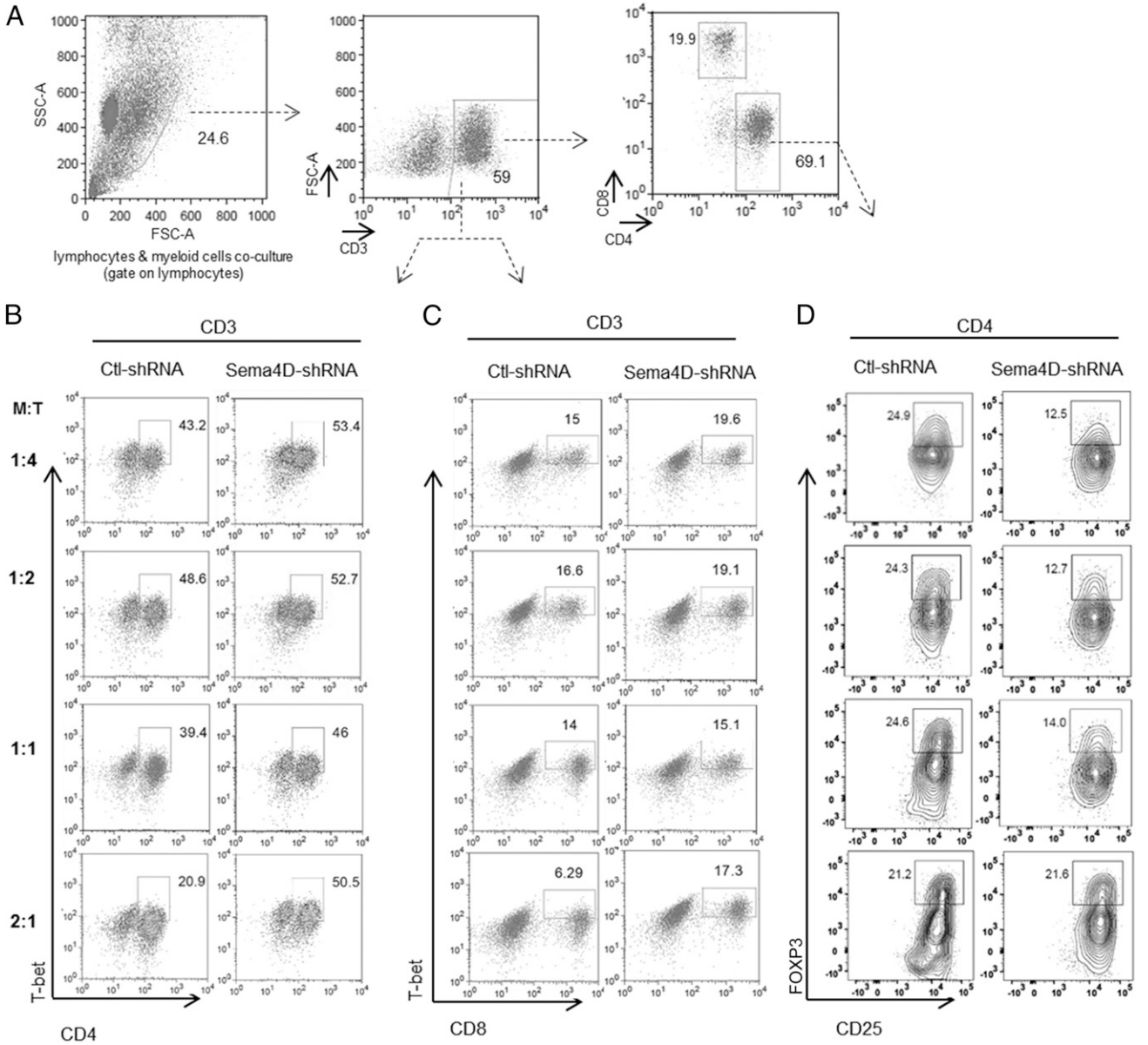

FIGURE 4. Sema4D produced by HN6 induces an immune-suppressive T cell phenotype. (**A**) T cells were cocultured with myeloid cells at the indicated ratios, followed by CD3/CD28 activation for 72 h. Then cells were analyzed by flow cytometry. (A) The cells were gated on the T cell subpopulation, followed by a second gate on $CD3^+$ cells. $CD3^+$ cells were then gated on (**B**) $CD4^+$T-bet^+ and (**C**) $CD8^+$T-bet^+. The $CD4^+$ population was gated on $CD25^+$ $FOXP3^+$. Representative flow cytometry plots displaying percentage of recovery of (B) $CD3^+CD4^+$T-bet^+ effector cells, (C) $CD3^+CD8^+$T-bet^+ effector cells, and (**D**) decrease in $CD4^+CD25^+FOXP3^+$ Tregs in HN6 conditioned medium (CM) of Sema4D-shRNA versus Ctl-shRNA.

CFSE analysis (Fig. 6C). Taken together, these findings showed that Sema4D produced by HNSCC plays a significant role in the proliferation and viability of MDSC.

The myeloid cells mediate their suppressive effects by various mechanisms. Among these, arginase-1 and NO play a major role in the MDSC suppressive effect. To investigate whether Sema4D produced by HNSCC-HN6 can affect arginase-1 production, $CD33^+$-enriched myeloid cells were grown in conditioned media from Sema4D-shRNA HN6 and then checked for arginase-1 expression. A significant reduction in arginase-1 production by the $CD33^+$ cells was observed when grown in Sema4D-shRNA HN6 conditioned media (Fig. 6D). To check for the effect of Sema4D knockdown on NO production by myeloid cells, $CD33^+$ cells were grown in HN6 Sema4D-shRNA– versus Ctl-shRNA conditioned media, and then the media were collected after 72 h to check for nitrite concentration. Interestingly, there was a significant reduction in nitrite concentration produced by the myeloid cells in the media upon Sema4D knockdown in HN6 (Fig. 6E).

MDSC can also indirectly induce a suppressive effect through production of other immune-suppressive cytokines such as IL-10 and expansion of Tregs in a TGF-β–dependent pathway (11, 18). These cytokines were assessed by ELISA, specifically to measure IL-10 and TGF-β produced by MDSC (43). Interestingly, there was a significant reduction in both IL-10 and TGF-β production by $CD33^+$ cells upon inhibition of Sema4D in HN6 (Fig. 6F, 6G).

Discussion

Understanding the specific mechanisms underlying tumor-mediated immunosuppression is important for the development of effective antitumor immunotherapy (44). Myeloid progenitor cells have been shown to mediate T cell suppression in patients

with HNSCC (8, 45, 46), and even inducing differentiation of these immature myeloid cells can indirectly activate tumor-infiltrating T cells (47). The link between angiogenesis and immune suppression has been emphasized recently to play a crucial role in tumor development, progression, and metastases. In this study, we investigated Sema4D, a cytokine expressed by several epithelial malignancies and known to induce tumor angiogenesis, for its role in induction of MDSC, in the tumor microenvironment of HNSCC (24, 32, 33). We show Sema4D produced by HNSCC-induced MDSC and its immune-suppressive cytokines, with subsequent suppression of T cell proliferation and IFN-γ production. These findings are supported by others, who reported that Sema4D acts on immature myeloid cells and dendritic cells by inhibiting their migration and inducing significant increases in the immune-suppressive profile (26, 30, 31). They are also in good agreement with a recent report showing that Sema4D modulates the inflammatory cytokine milieu in a murine colon and mammary carcinoma model (48).

Several clinical trials showed that advanced cancers can be controlled by immunotherapy. However, these therapies are often compromised by the immune-suppressive tumor microenvironment (49, 50). Interestingly, the composition of T cells reported in

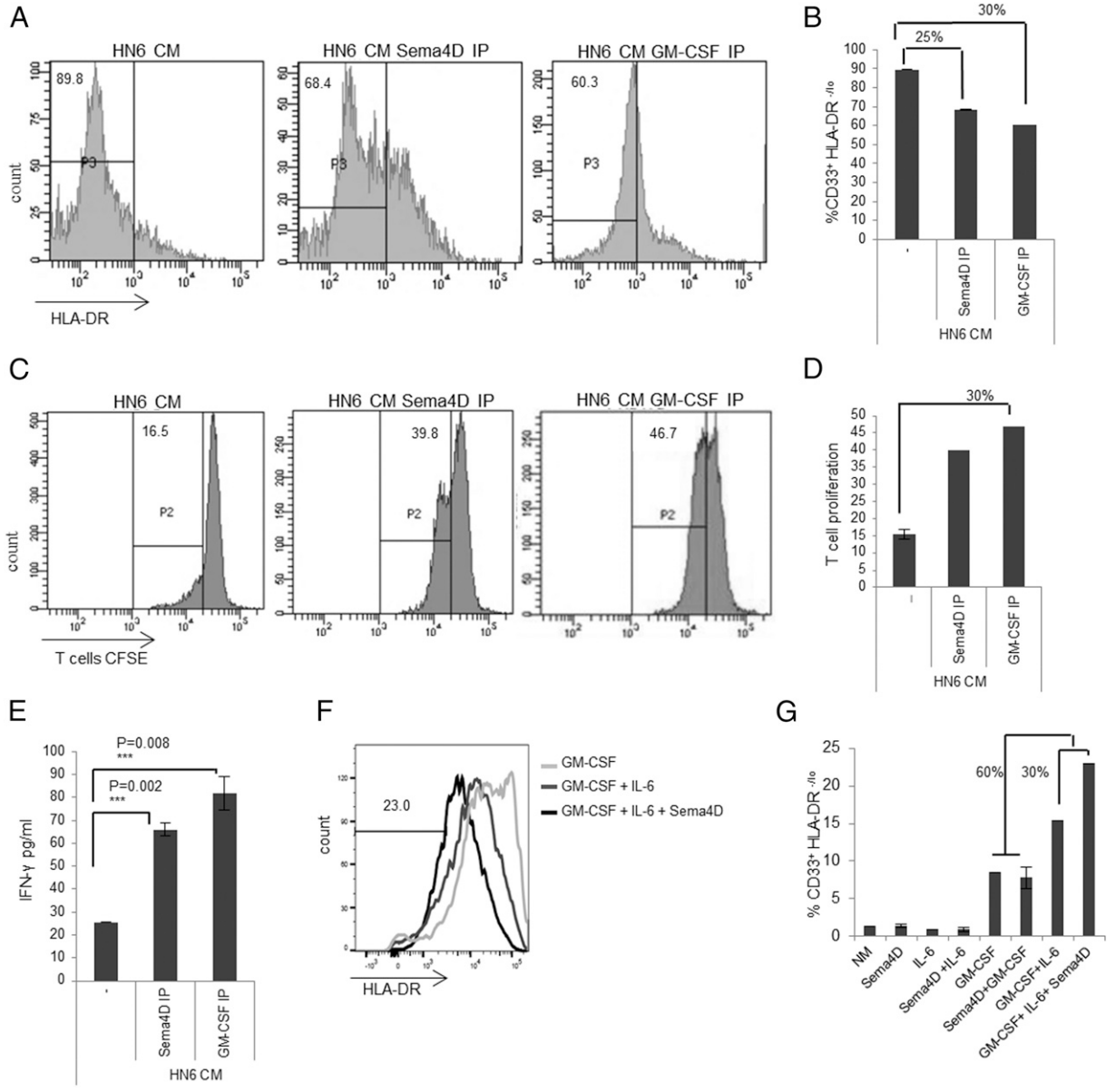

FIGURE 5. Sema4D enhances GM-CSF plus IL-6–mediated induction of MDSC. (**A**) The percentage of $CD33^{+}HLA\text{-}DR^{-/low}$ MDSC induced by culture in HN6 conditioned medium (CM) is reduced following Sema4D or GM-CSF immunoprecipitation (IP) HN6 CM. (**B**) Graph shows comparable decrease in the $CD33^{+}HLA\text{-}DR^{-/low}$ population following immune depletion of Sema4D or GM-CSF, compared with the control HN6 CM. (**C**) HN6 CM was depleted of Sema4D or GM-CSF by IP and then used to culture myeloid cells and T cells, followed by anti-CD3/CD28 stimulation. After 72 h, the cells were analyzed by flow cytometry analysis, and the supernatants were collected for ELISA. CFSE dilution in T cells was analyzed by flow cytometry. (**D**) Graph shows comparable recovery of T cell proliferation upon immune depletion of Sema4D or GM-CSF in HN6 CM. (**E**) IFN-γ production following immune depletion of Sema4D is comparable to GM-CSF–depleted CM. (**F**) Sema4D is synergistic to GM-CSF plus IL-6 in MDSC induction. $CD33^{+}$ cells separated from PBMC of normal donors were cultured in NM with the recombinant protein GM-CSF alone, GM-CSF plus IL-6, or the cytokine induction mixture of GM-CSF plus IL-6 plus Sema4D. (**G**) $CD33^{+}HLA\text{-}DR^{-/low}$ induction in response to treatment with Sema4D, IL-6, and GM-CSF alone or in combination. For (F) and (G), each of the recombinant proteins was used at a concentration of 10 ng/ml. CM, conditioned medium; IP, immunoprecipitation.

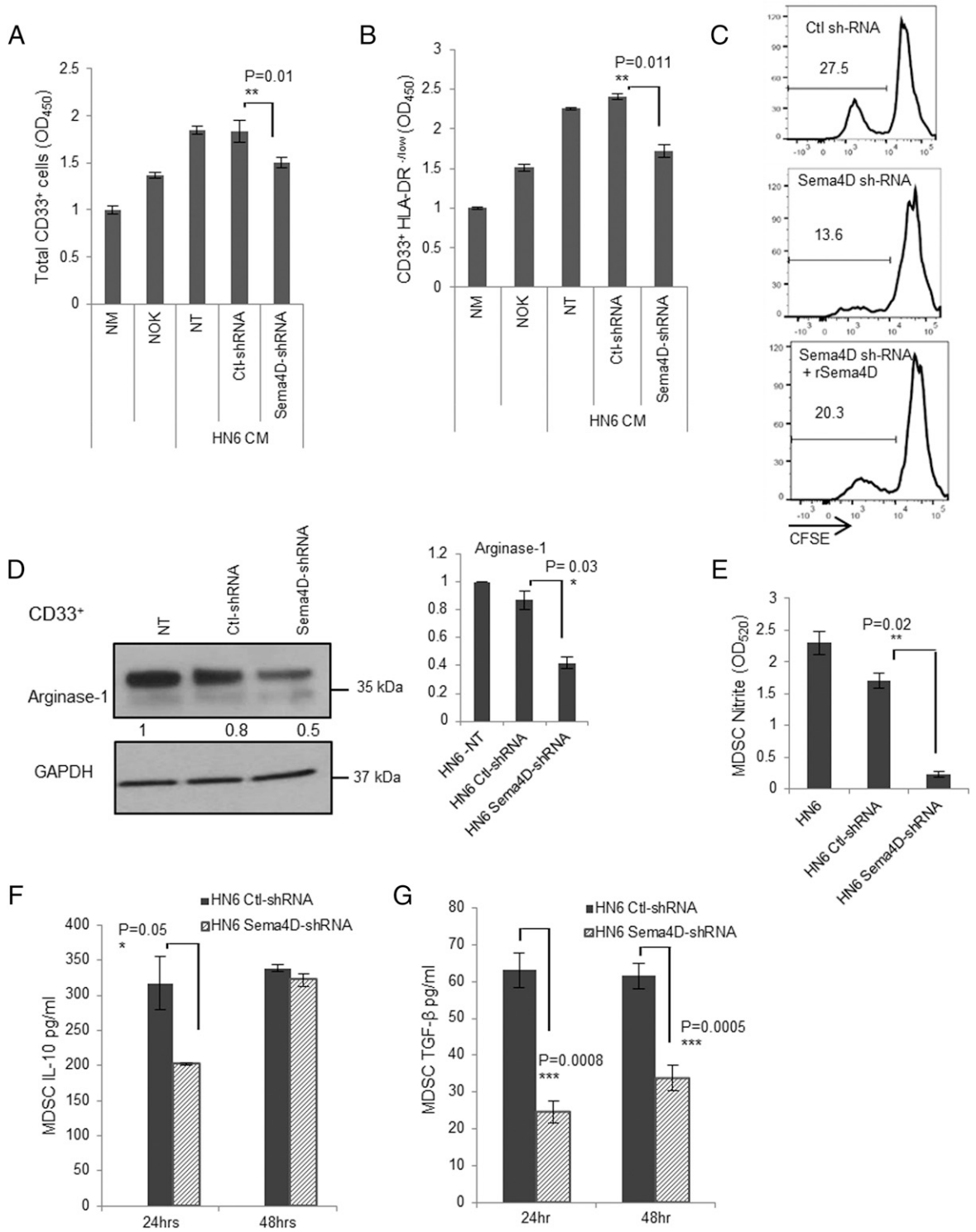

FIGURE 6. HN6-secreted Sema4D induces production of immune-suppressive mediators by MDSC. (**A**) Sema4D promotes proliferation of total myeloid cells. Bulk $CD33^+$ cells cultured in HN6 control or Sema4D-shRNA conditioned medium (CM) for 24 h were analyzed using the WST-1 proliferation assay. (**B**) Sema4D promotes proliferation of $CD33^+HLA\text{-}DR^{-/low}$ cells. $CD33^+HLA\text{-}DR^{-/low}$ cells were sorted using magnetic beads and analyzed by WST-1 proliferation assay. All readings were normalized to cells growing in NM. (**C**) Reconstitution of HN6 Sema4D-shRNA CM with human recombinant Sema4D rescues $CD33^+HLA\text{-}DR^{-/low}$ MDSC proliferation. $CD33^+HLA\text{-}DR^{-/low}$ cells labeled with CFSE were analyzed by FACS. (**D**) Sema4D inhibition in HN6 decreases arginase-1 expression in myeloid cells. $CD33^+$ cells were cultured in nontransfected (NT), Ctl-shRNA, or Sema4D-shRNA HN6 CM for 24 h and then analyzed by Western blot for arginase-1 expression. GAPDH served as a loading control. Densitometric analyses included for Western blot data were determined using ImageJ. (**E**) Sema4D inhibition in HN6 decreases NO production by myeloid cells. HN6 Sema4D-shRNA CM inhibits nitrite production by myeloid cells. Total $CD33^+$ cells were cultured in CM from HN6 Ctl-shRNA or HN6 Sema4D-shRNA for 72 h, and then the media were collected to detect the nitrite concentration using Griess reagent. (**F**) $CD33^+$ cells were cultured in HN6 Sema4D-shRNA or Ctl-shRNA CM, and cytokine production was assessed by ELISA. Knockdown of Sema4D results in a decrease in IL-10 production by $CD33^+$ cells. (**G**) A reduction in Sema4D results in a concomitant decrease in TGF-β production by $CD33^+$ cells. Data are shown with background levels of cytokine subtracted to demonstrate myeloid cell–specific cytokine production. CM, conditioned medium; NT, nontransfected.

human tumor-infiltrating lymphocytes in a cohort of colorectal cancer liver metastases and ovarian cancer indicated that whereas the $CD8^+$ T cells showed signs of proliferation and activation, still there was no tumor rejection (50). This was attributed to several factors, including, but not limited to, presence of MDSC and Tregs. In this context, our data show promising results, in which inhibition of Sema4D in HN6 cells cocultured at several ratios of myeloid/T cells resulted in enhanced IFN-γ production (Fig. 3C) and effector CD4 and CD8 T cells (Fig. 4B, 4C), concomitant with a significant decrease in both Tregs (Fig. 4D) and MDSC (Fig. 5A).

Our data indicate that inhibition of Sema4D produced by the tumor cells may recover T cell activation to Ag-nonspecific stimuli and avoid T cell anergy. This would be of importance in adoptive immunotherapy in which the transferred activated T cells are Ag specific, yet the presence of MDSC in the tumor microenvironment might induce anergy (51). This is further supported by others showing that although Sema4D knockout mice display severe impairments in the Ag priming of T cells, interestingly, Sema4D-deficient T cells still retained the ability to respond normally to either mitogens or anti-CD3 stimulation, and, more importantly, the development of dendritic cells is not defective in cells lacking Sema4D (25, 52).

Intriguingly, the opposing cellular responses played by Sema4D in the immune system (53), either as an anti-inflammatory or a proinflammatory cytokine, seem to be dependent on several factors, including, but not restricted to, its concentration, the specific cell type present in the microenvironment, and its stage of maturation upon which Sema4D acts (27, 30, 37, 54). Also, note that in the studies showing the immunosuppressive effect and inhibition of myeloid cell migration, Sema4D recombinant protein was effective at <10 ng/ml and ranged up to 80 ng/ml (30, 31), and the concentrations of Sema4D used to show its proangiogenic effect in tumor microenvironment were in the range of 400 ng/ml (55). In contrast, concentrations used to show increased activation of the immune system, induction of proinflammatory cytokines, and maturation and activation of dendritic cells were in the micromolar range of soluble Sema4D (4–20 μg/ml), suggesting that the Sema4D mechanism of action postulated in other pathological conditions, such as autoimmune-mediated diseases (52, 56), may differ from those in the tumor microenvironment, depending on its concentration and presence of tumor-educated cells in the microenvironment.

There is evidence for the involvement of multiple pathways by which malignancies induce MDSC: several cytokines, including IL-6, cyclooxygenase 2–generated PGE_2, GM-CSF, vascular endothelial growth factor, IL-10, matrix metalloproteinases, and others (7, 12, 57–59), acting through several cellular receptors on myeloid cells and stimulating transcription factors that involve STAT3, STAT6, STAT1, NF-kβ, HIF1-α, C/EBPβ have been described. Our data do provide evidence that Sema4D acts synergistically with GM-CSF and IL-6 (Fig. 5F, 5G) to increase the repertoire of MDSC in the tumor microenvironment and accordingly the downstream effectors arginase-1, NO, TGF-β, and IL-10. We also did detect Sema4D receptors CD72 and plexin-B1 on myeloid cells treated with recombinant Sema4D, as well as plexin-C1, a Sema7A receptor (unpublished observations); however, we are currently investigating the exact receptor and underlying pathway by which Sema4D may activate downstream transcription factors and induce the effector cytokines in MDSC (18, 60–62).

MDSC have proangiogenic activities that can mediate refractoriness to antiangiogenic therapies (49, 63, 64). Our data suggest an important role for Sema4D in the continuous positive feedback mechanism between MDSC induction and angiogenesis that is to an extent comparable to GM-CSF and reminiscent of the angiogenic role ascribed to vascular endothelial growth factor in the tumor microenvironment (41). Of particular note is the ability of MDSC to differentiate further into the immune-suppressive M2 macrophage, which has also been reported to produce Sema4D (39). This has significant implications for tumor resistance to antiangiogenic and immune therapy (41, 63–66) and indicates that Sema4D inhibition might be required to disrupt this positive feedback loop between angiogenesis and immune suppression.

MDSC represent a cell population that contributes to tumor immune suppression. It responds to a variety of proinflammatory and anti-inflammatory stimuli, which drive their recruitment and activation. Understanding how the inflammatory milieu favors immune escape through the accumulation of MDSC could be very useful to improve the efficacy of cancer immunotherapy (67). In addition to the well-described proangiogenic role of Sema4D in the tumor microenvironment, in the present study we describe, to our knowledge for the first time, its immunosuppressive effect through the induction of MDSC. This novel role described for Sema4D may open new avenues for understanding and reversing tumor-associated immunosuppressive mechanisms, with the ultimate goal of increasing T cell activation and enhancing antitumorigenic immune responses in HNSCC, either as a component of immune-based therapy or as a complement to conventional treatment regimens.

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Disclosures

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