Myeloid-Derived Suppressor Cells Are a Major Source of Wnt5A in the Melanoma Microenvironment and Depend on Wnt5A for Full Suppressive Activity

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ABSTRACT

Metastatic dissemination remains a significant barrier to successful therapy for melanoma. Wnt5A is a potent driver of invasion in melanoma and is believed to be secreted from the tumor microenvironment (TME). Our data suggest that myeloid-derived suppressor cells (MDSC) in the TME are a major source of Wnt5A and are reliant upon Wnt5A for multiple actions. Knockdown of Wnt5A specifically in the myeloid cells demonstrated a clear decrease in Wnt5A expression within the TME in vivo as well as a decrease in intratumoral MDSC and regulatory T cell (Treg). Wnt5A knockdown also decreased the immunosuppressive nature of MDSC and decreased expression of TGFβ1 and arginase 1. In the presence of Wnt5A-depleted MDSC, tumor-infiltrating lymphocytes expressed decreased PD-1 and LAG3, suggesting a more exhausted phenotype. Myeloid-specific Wnt5A knockdown also led to decreased lung metastasis. Tumor-infiltrating MDSC from control animals showed a strong positive correlation with Treg, which was completely ablated in animals with Wnt5A-negative MDSC. Overall, our data suggest that while MDSC contribute to an immunosuppressive and less immunogenic environment, they exhibit an additional function as the major source of Wnt5A in the TME.

Significance: These findings demonstrate that myeloid cells provide a major source of Wnt5A to facilitate metastatic potential in melanoma cells and rely on Wnt5A for their immunosuppressive function.

Introduction

Over the last decade, mortality rates in patients with melanoma have increased by approximately 50% predominantly due to metastatic spread of the primary tumor. Despite breakthroughs in targeted therapies and immunotherapies, enhancing the immune microenvironment within a tumor remains a major hurdle to overcome to achieve therapeutic success. Recent work from our group and others has demonstrated that noncanonical Wnt ligands such as Wnt5A drive the metastatic spread of melanoma, and interact with canonical Wnt upon microenvironmental cues to drive phenotype switching (1). Phenotype switching occurs when the canonical and noncanonical Wnt pathways play antagonistic roles, where canonical signaling drives proliferation of melanoma cells, while Wnt5A, contributes to a slower growth rate in melanoma cells, increasing their invasive and migratory nature (2, 3). In addition, Wnt signaling plays critical roles in the immune microenvironment (4). We have previously shown that melanoma cells with high Wnt5A have lowered expression of antigens such as MART1 and DCT (5), and that Wnt5A is highly expressed at sites of chronic inflammation and has roles in immune regulation and cytokine production from leukocytes (4, 6). The canonical Wnt pathway also plays multiple roles in T-cell development and has also been shown to antagonize the differentiation of myeloid-derived suppressor cells (MDSC), and inhibition of β-catenin promotes the activity and expansion of MDSCs (7).

MDSCs are cells that develop as a result of inflammatory mediators activating immature myeloid cells. These cells are frequently reported in numerous pathologies, especially in cancer. In mice, MDSCs can be subdivided on the basis of their myeloid lineage, resulting in the generation of monocyctic-MDSCs (M-MDSC), which are Ly6Chigh and Ly6Cneg; and polymorphonuclear-MDSCs (PMN-MDSC), which are Ly6CINT and Ly6Gpos (8). Once activated, these cells develop a suppressive capacity and are therefore associated with a worse prognosis in patients with cancer as they increase with disease stage and negatively correlate with the success of immunotherapy treatment in melanoma (9, 10). As potent immunosuppressive cells, MDSCs decrease immunogenicity mainly through inhibiting T-cell activation (11–13), induction of regulatory T cells (Treg; ref. 14) and secretion of anti-inflammatory cytokines such as TGFβ1 (15, 16) and arginase 1 (17).

Recent studies have demonstrated that Wnt5A is expressed within myeloid lineages (18). Several proinflammatory cytokines such as TNFα, IL6, and IL1β can increase Wnt5A expression in macrophages (19). A hypothesis based on the fact that certain myeloid cells express the Wnt5A receptor Fzd5 (20, 21), is that Wnt5A signals in an autocrine manner and is a proinflammatory factor enhancing the expression of IL1β, IL6, and IL10 in these cells (19).

Given the role of Wnt5A in the inhibition of the canonical Wnt pathway, and the observation that Wnt5A can have effects on myeloid cells, we queried whether Wnt5A could play a role in the myeloid...
compartment of the melanoma microenvironment. Using novel transgenic animal models with myeloid-specific knockdown of Wnt5A, we examined the contribution of Wnt5A from MDSCs in promoting melanoma metastasis, its influence on the immune dynamics of the TME, and the effects of Wnt5A on the suppressive machinery of MDSCs.

Materials and Methods

The Cancer Genome Atlas
RNA sequencing data were downloaded from The Cancer Genome Atlas (TCGA) database using cBioPortal (http://www.cbioportal.org). Individual gene expression values for genes of interest were retrieved as normalized RNA-seq by expectation maximization (RSEM) read counts processed through TCGA cBioportal. Heatmap data were then transformed such that Wnt5A gene expression was separated into two groups based on the top 25 percentile and bottom 25 percentile of gene expression within the melanoma samples from TCGA dataset (N = 120). A Students t test (unpaired) was then performed to individually analyze the difference in the mean expression of MDSC and immune-related markers in these low versus high percentile melanoma samples for our specific gene of interest. The data are presented as a heatmap of the mean Z-score.

Cell lines
YUMM1.7 cells were obtained from Marcus Bosenberg, Yale University (New Haven, CT), and cultured in DMEM/F12 media containing 10% FCS, 1% Pen/Strep, and 1% L-glutamine. BSC9AJ2 cells were obtained from Richard Marais, CRUK Manchester (Alderley Park, United Kingdom), and cultured in RPMI media containing 10% FCS, 1% Pen/Strep, and 1% L-glutamine. Cell lines were MAP tested before use in animals. All the cell lines were cultured at 37°C in 5% CO₂. Short tandem repeat profiling was done for melanoma cell line authentication and compared against our internal control of over 200 melanoma cell lines as well as control lines such as HeLa and 293T and the results are available upon request. Mycoplasma testing was carried out using a Lonza MycoAlert assay at the University of Pennsylvania Cell Center Services (Philadelphia, PA).

Animals
All animal experiments were performed at the Wistar Institute (Philadelphia, PA; Association for Assessment and Accreditation of Laboratory Animal Care International accredited) and approved by the Institutional Animal Care and Use Committee. C57Bl/6 mice were purchased from the Charles River NCI facility. C57Bl/6 Mice with Wnt5A knockdown in myeloid cells were generated by breeding Wnt5A<sup>Smo<sup>fl/fl</sup></sup> (JAX stock: #026626; ref. 22) with LyzM<sup>−/−</sup> (JAX stock: #004781; ref. 23) purchased from the Charles River NCI facility. C57Bl/6 Mice with LyzM<sup>−/−</sup> were incubated for 45 minutes at 37°C in the precoated ELISA plate wells. The plate was washed five times followed by incubating for 30 minutes at 37°C with an HRP-conjugated detection antibody. The plate was washed a further five times before finally developing with chromogen. After 15 minutes at 37°C, the reaction was stopped, and the plate was read using a microtiter plate reader at 450 nm.

IHC
Sections were deparaffinized using xylene followed by rehydration through series of alcohol washes and finally PBS. Heat-mediated antigen retrieval was performed using citrate-based retrieval buffer (Vector Labs, H-3300). Samples were blocked in peroxide blocking buffer (Thermo Fisher Scientific), followed by protein block (Thermo Fisher Scientific) and incubated in appropriate antibody (Ki67; Novus,

Western blot analysis
Total protein lysate was quantified using BCA assay (Pierce) and 50 µg was loaded and run on precast 4%–12% NuPAGE Bis Tris gels (Invitrogen). Proteins were then transferred onto polyvinylidene difluoride membrane using the iBlot system (Invitrogen), and blocked in 5% milk/Tris-buffered saline with TWEEN 20 (TBST) for 1 hour. Primary antibodies were diluted in 5% milk/TBST and incubated at 4°C overnight. The membranes were washed in TBST and probed with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at 0.2 µg/mL. Proteins were visualized using ECL prime (Amersham). Primary antibodies used were Wnt5A (1:2,000, biotin labeled, BAf645 R&D Systems), HSP90 (1:10,000, #4874, Cell Signaling Technology), and β-actin (1:10,000, #4970, Cell Signaling Technology).

Isolation of mouse cells and flow cytometry
Flow cytometry was performed on the LSRII, 18 color flow cytometer by BD Biosciences at the Wistar Institute Flow Facility. Tumors were harvested when volumes were no larger than 1,500 mm³ by isolating tumors from the stromal capsule and skin, chopping into small fragments, and incubating at 37°C for 1 hour in the Miltenyi Tumor Dissociation Kit, mouse (#130-096-730). Spleens, lungs, and dissociated tumors were mashed through 70 µm cell strainers directly into MACS buffer (PBS containing 0.5% FCS and 2.5 mmol/L EDTA) and incubated with ammonium-chloride-potassium (ACK) lysis buffer for 1 minute on ice. All single-cell suspensions were washed and blocked using TrueStain FCX (BioLegend, #101320) for 15 minutes at room temperature. Cells were washed and stained with the appropriate concentrations of antibodies in MACS buffer for 1 hour at 37°C. Intracellular staining was performed following cell membrane staining as described in ref. 24. Intracellular cytokines were stained after cells were incubated for 5 hours with BD GolgiBlock (#554724). Intracellular staining then consisted of cells being fixed and permeabilized using the True-Nuclear Transcription Factor Buffer Set from BioLegend (#424401) and stained with the appropriate antibody for 1 hour at 37°C. Cells were rinsed and resuspended in PBS prior to FACS analysis. Antibodies used are listed in Supplementary Table SI. Immunophenotyping analysis was focused on changes based on leukocyte infiltration and to demonstrate changes in these populations were proportionate, equal numbers of CD45<sup>+</sup> cells (for lymphocytes) and CD11b<sup>+</sup> (for myelocytes) were collected during FACS.

ELISA
Wnt5A quantification was determined using a mouse Wnt5A ELISA Kit (Abkine, KTE70006). Single-cell suspensions were isolated from tumors as described previously. ELISA was carried out according to the manufacturer’s protocol. Briefly, equal concentrations of sample were incubated for 45 minutes at 37°C in the precoated ELISA plate wells. The plate was washed five times followed by incubating for 30 minutes at 37°C with an HRP-conjugated detection antibody. The plate was washed a further five times before finally developing with chromogen. After 15 minutes at 37°C, the reaction was stopped, and the plate was read using a microtiter plate reader at 450 nm.
NB600-1252, Wnt5A; R&D Systems, mab645) at 4°C overnight in a humidified chamber. Following day, samples were washed and incubated with a biotinylated secondary antibody (Thermo Fisher Scientific) followed by streptavidin-HRP incubation. Samples were then washed in PBS and incubated in 3-amino-9-ethyl-1-caroazoole chromogen and counterstained with Mayer’s hematoxylin (Millipore Sigma), rinsed with water and mounted in aquamount.

**TUNEL stain**

Slides were stained using the TUNEL Assay Kit–HRP–DAB Kit (Abcam, ab206386) according to the manufacturer’s protocol. Briefly, sections were deparaffinized and rehydrated as described above. Samples were permeabilized using proteinase K (1:100) followed by endogenous peroxidase quenching and equilibration using a TdT buffer. The reaction was labeled using a TdT enzyme and incubated for 1.5 hours at room temperature. The reaction was terminated and slides were washed before blocking and developing using 3,3’-diaminobenzidine solution and finally counterstained using Methyl Green, rinsed and mounted in aquamount.

**Quantification and statistical analysis**

All data points reflect biological replicates. Error bars are representative of SEM. P values were determined using nonparametric Mann–Whitney tests for comparisons between two groups. Significance of tumor growth was determined using a linear mixed effects model. Spearman rank-order correlation was used to analyze correlation of two variables. All statistical tests were performed with Graph Pad Prism V8 software (GraphPad Software, Inc.) and R software. (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, and N.S., not significant).

**Results**

**Wnt5A expression is associated with MDSC infiltration**

The presence of Wnt5A and MDSCs within the tumor microenvironment (TME) has been independently associated with an aggressive phenotype in various cancers (2, 9). We analyzed TCGA database to examine changes in MDSC markers in patients with Wnt5A-low versus Wnt5A-high melanoma across all samples (Fig. 1A). These data were also investigated in primary and regional subcutaneous metastasis (Supplementary Fig. S1A) as well as distant and lymph node metastasis (Supplementary Fig. S1B). Because of the heterogeneity of MDSCs, we used a panel of markers closely associated with MDSC expression and function including arginase 1, nitric oxide synthase, TGFβ1, and CD11b, and found that many of the selected genes were significantly increased in Wnt5A-high tumors (Fig. 1A). These data suggest a link between the infiltration of MDSCs and Wnt5A expression in melanoma.

Building on this finding, we subdermally injected C57Bl/6 mice with YUMM1.7 melanoma cells and isolated MDSCs from the bone marrow, spleens, and tumors. Results suggested that MDSCs extracted from tumors, but not other sites, expressed very high levels of Wnt5A even in comparison with the Wnt5A-high cell lines FS4 and 1205Lu (Fig. 1B; ref. 25). In addition, we performed an ELISA to demonstrate MDSC expression of Wnt5A was approximately eight times higher in MDSCs derived from tumors than those derived from spleens (Fig. 1C). To analyze the impact of Wnt5A on MDSCs in the tumor, we implanted YUMM1.7 cells into mice and treated with either 200 ng/mL recombinant Wnt5A (rWnt5A) or PBS every 3–4 days, as we have previously reported that this dose is effective in eliciting in vivo effects of Wnt5A (26). As reported by several groups including our own (25, 27), the administration of rWnt5A slowed tumor growth compared with PBS control mice, decreasing tumor cell proliferation (Fig. 1D; Supplementary Fig. S1C). Despite this decrease in tumor volume, we found that animals treated with rWnt5A had significantly increased levels of tumor MDSCs (Fig. 1E; Supplementary Fig. S1D). Interestingly, no significant changes were witnessed in CD8+ T-cell or Treg infiltration (Supplementary Fig. S1E). Changes observed were tumor specific as no change was evident in the levels of MDSCs within spleens of animals treated with rWnt5A (Fig. 1F). We also performed IHC on tumors to confirm increased levels of Wnt5A in the treatment group (Supplementary Fig. S1F). Our lab and others have previously shown that Wnt5A inhibits melanoma tumor growth by promoting a slow-cycling phenotype, albeit driving metastasis and therapy resistance through phenotype switching (26, 28). We evaluated the correlation between the infiltration of tumor-derived MDSCs and tumor volume from control and rWnt5A-treated animals. Control animals showed a trend but no significant relationship between MDSCs and tumor volume (Fig. 1F). However, when animals were treated with rWnt5A, there was a significant negative correlation between tumor growth and MDSC infiltration (Fig. 1F). Similar analysis on the ratios of MDSCs in the spleen showed no correlation to tumor volume in either PBS or rWnt5A groups (Supplementary Fig. S1G). We and other reports demonstrate that rWnt5A can act to decrease cancer cell proliferation, while increasing Wnt5A-expressing MDSC infiltration into the tumor, augmenting a slower tumor growth rate (Fig. 1D; ref. 25). These data suggest that while Wnt5A can increase MDSC infiltration into the tumor, the impact of Wnt5A on tumor cell proliferation overrides the impact of an increased immunosuppressive microenvironment due to the MDSC infiltration.

**Myeloid-specific knockdown of Wnt5A decreases lung metastasis in vivo**

In addition to Wnt5A being able to enhance MDSC infiltration into the tumor, our data show that MDSCs themselves express large amounts of Wnt5A within the TME. Therefore, to better understand the effects of Wnt5A specifically from MDSCs in melanoma tumors, we generated novel transgenic animal models utilizing cre/lox recombination. Animals generated with LoxP sites inserted flanking exon 2 in the Wnt5A gene (Wnt5A<sup><sup>−/−<sub>cre<sub>Lox<sub>P</sub></sub></sup></sup>) (22) were crossed with animals that have a nuclear-localized Cre recombinase inserted into the myeloid-specific Ly6c<sup>+</sup> promoter gene (Ly6c<sup><sup>−/−<sub>cre<sub>Lox<sub>P</sub></sub></sup></sup>) (Supplementary Fig. S2A and S2B). Wnt5A<sup><sup>−/−<sub>cre<sub>Lox<sub>P</sub></sub></sup></sup> animals are from herein referred to as control, whereas Wnt5AA<sup>−/−<sub>cre<sub>Lox<sub>P</sub></sub></sup></sup> animals crossed with Ly6c<sup><sup>−/−<sub>cre<sub>Lox<sub>P</sub></sub></sup></sup> were positive for both the Wnt5A-mutant allele and the LysM-Cre–mutant allele are referred to as Wnt5A<sup><sup>−/−<sub>LysM-Cre<sub></sub></sub></sup></sup>. This model was validated by isolating CD11b<sup>+</sup> GR1<sup>+</sup> cells from bone marrow of offspring that were positive for the Wnt5A-mutant allele
in the control Wnt5A<sup>+/+/+</sup> mice, and either positive or negative for the LysM-Cre<sup>−/−</sup> mutant by performing RT-PCR for Wnt5A exon 2 (Supplementary Fig. S2C) as well as ELISA (Fig. 2E and F). Control mice and animals negative for the LysM-Cre<sup>−/−</sup> mutant allele had detectable Wnt5A levels, whereas the levels were noticeably diminished in the offspring containing both the Wnt5A- and LysM-Cre<sup>−/−</sup> mutant allele (Supplementary Fig. S2C).

As described previously, melanoma cell lines exhibit different levels of Wnt5A expression. We found that BSC9AJ2 cells have lower levels of endogenous Wnt5A than YUMM1.7. Using this cell line with our myeloid Wnt5A knockout model allowed us to gain a better idea on the role of Wnt5A secreted from tumor-derived myelocytes did indeed decrease proliferation (Fig. 2C and D). Moreover, no
Figure 2.
Effects of myeloid-specific Wnt5A knockdown on melanoma phenotype switching in vivo. A, Tumor growth rate of Wnt5A−/− and control animals implanted with BSC9AJ2 melanoma cells (n = 8). B, Representative IHC for Wnt5A on tumors from Wnt5A−/− and control animals at ×40 magnification and no primary antibody control images (NPC). C, Representative IHC for Ki67 on tumors from Wnt5A−/− and control animals at ×10 and ×20 magnification and NPC. D, Quantitation of Ki67+ cells in mouse tumors. E, ELISA for intracellular Wnt5A on MDSCs isolated from tumors and spleens from Wnt5A−/− and control animals implanted with BSC9AJ2 cells (n = 3). F, ELISA for secreted levels of Wnt5A from MDSCs populations isolated from tumors. MDSCs were isolated and cultured for 48 hours and Wnt5A within culture media was measured (n = 3). G, M-MDSCs present in the lungs of Wnt5A−/− and control animals implanted with BSC9AJ2 cells (n = 8). H, Wnt5A−/− and control animals were implanted with YUMM1.7-GFP cells. Tumors and lungs were harvested after 4 weeks postinjection. GFP+ cells were analyzed by FACS. Representative plots for GFP+ cells. I, Quantification of FACS analysis from GFP+ cells within the lungs of Wnt5A−/− and control animals (n = 13). **, P < 0.01; ****, P < 0.0001.
changes were witnessed in samples when stained for TUNEL suggesting changes in tumor growth were not due to increased apoptosis (Supplementary Fig. S3B). These data suggest that MDSCs are the major source of TME-derived Wnt5A, affecting melanoma tumor growth in vivo. When investigating the levels of Wnt5A in YUMM1.7 cells grown in two-dimensional culture and lysates of YUMM1.7 tumors, the expression of Wnt5A in the tumor lysate was considerably higher, further suggesting that the increased expression of Wnt5A within the TME came from sources beyond the tumor cells (Supplementary Fig. S3C). Moreover, when MDSCs were isolated from the spleens and tumors of control and Wnt5A−/− animals, Wnt5A was considerably decreased in the MDSCs of tumors in Wnt5A−/− compared with control animals, but levels were barely detectable in either control or Wnt5A−/− animal spleens as shown by ELISA (Fig. 2E). Knockdown of Wnt5A in these cells also diminished the secretion of this protein from both M- and PMN-MDSCs (Fig. 2F).

As previously mentioned, Wnt5A is an important driver of a phenotype switching in melanoma, promoting a slow growing but highly metastatic phenotype. We found that M-MDSCs were decreased in the lungs of animals following Wnt5A knockdown (Fig. 2G), suggesting a role for Wnt5A in the migration of MDSCs to the premetastatic niche. PMN-MDSCs did not show a statistically significant difference (Supplementary Fig. S4A). To investigate the role that myeloid-derived Wnt5A has in contributing to metastatic spread, we subdermally injected YUMM1.7-GFP cells into Wnt5A−/− and control animals. After reaching tumor burden, we quantified the number of GFP+ melanoma cells that had metastasized by measuring positive cells in the lung by flow cytometry. Wnt5A−/− animals had decreased YUMM1.7-GFP+ cells within the lung compared with the control (Fig. 2H and I). These data show that MDSC-derived Wnt5A contributes to metastatic spread and decreases melanoma proliferation in vivo thus supporting the phenotype switching phenomenon.

**Myeloid-specific knockdown of Wnt5A decreases accumulation of tumor M-MDSCs and Tregs**

To determine whether Wnt5A affected tumor infiltration of leukocytes, we assessed the levels of T cells, MDSCs, and Tregs from control and Wnt5A−/− animals. Despite no changes in PMN-MDSCs, we found approximately a 50% decrease in the number of M-MDSCs in Wnt5A−/− mice compared with control animals (Fig. 3A). Interestingly, when we looked at correlations from the Wnt5A-negative MDSCs with tumor volume, the strong negative correlation associated with tumor size previously described was abrogated (Supplementary Fig. S4B). This suggests that Wnt5A specifically from MDSCs may be an important regulator of melanoma growth rate.

In addition to MDSCs, Tregs play important roles in increasing immunosuppression by inhibiting the activity of T cells. Moreover, one of the key mechanisms involved with MDSC-mediated suppression is via the recruitment of Tregs. We investigated whether decreases in tumor-derived MDSCs in Wnt5A−/− animals had an impact on infiltration of lymphocytes including CD4+ , CD8+ T cells, and Tregs. In keeping with MDSC biology, there was a significant decrease in tumor Tregs in the Wnt5A−/− animals (Fig. 3B). This finding was also validated through TCGA data, which suggested that Wnt5A-low melanoma tumors have decreased expression of the predominant Treg marker, FOXP3, whereas, Wnt5A-high tumors have increased expression of this gene (shown in Fig. 1A). Despite no changes in CD4+ or CD8+ T cells (Fig. 3C), the decrease in M-MDSC and Tregs suggests that myeloid-derived Wnt5A knockdown can increase the immunogenicity of tumors. These data suggest a more favorable antitumor environment due to decreased infiltration of immunosuppressive cell populations. We looked at the correlation between M-MDSCs and Tregs in tumors of animals from control and Wnt5A−/− animals. In keeping with MDSC biology, control animals had a strong positive correlation between intratumor M-MDSCs and Tregs; however, this correlation was completely lost in the Wnt5A−/− animals (Fig. 3D).

In keeping with the notion that Wnt5A can influence immunologic tolerance and promote metastasis, animals treated with rWnt5A had decreased levels of T cells and increased Tregs in the lungs, which correspond with significantly decreased ratios of CD4+ and CD8+ T cells to Tregs in rWnt5A-treated animals (Supplementary Fig. S4C). Inversely, we found that there was a significant increase in the CD8+ T cells in peripheral blood of Wnt5A−/− animals, which resulted in an increased CD8:M-MDSC ratio (Supplementary Fig. S4D).

**Myeloid-specific knockdown of Wnt5A decreases T-cell exhaustion markers PD-1 and LAG3 on cytotoxic T cells in primary tumors**

In addition to low infiltration of effector T-cell levels, inhibited T-cell activation is a major factor in determining response to immunotherapy. High-level expression of immune checkpoint molecules including PD-1 on T cells are associated with inhibited T-cell activation through interaction of PD-L1. High-level expression of PD-1 and LAG3 on T cells is a key marker for exhausted T cells, which affect tumor progression and therapy success. Despite observing no changes in the levels of CD4+ or CD8+ T cells between the groups, a significant decrease in PD-1 (Fig. 4A) and LAG3 (Fig. 4B) expression on CD8+ T cells was observed in Wnt5A−/− animals; however, only PD-1 expression decreased on CD4+ T cells, suggesting this is predominantly a change in the cytotoxic T-cell subset. We analyzed the expression of PD-1 and LAG3 on T cells within the spleens of tumor-bearing mice and found considerably lower levels of these markers in the spleens, with a modest increase in PD-1 on CD8+ T cells within Wnt5A−/− animals (Fig. 4C and D), suggesting that Wnt5A knockdown decreases T-cell exhaustion markers in a tumor-specific manner.

**Wnt5A contributes to suppressive mechanisms of MDSCs**

As described previously, the expression of Wnt5A in MDSCs was predominantly expressed within the TME. The suppressive capacity of MDSCs is also well known to be localized to the TME compared with other sites such as the spleen and the bone marrow (29, 30). On the basis of these findings, we wanted to see whether the site-specific expression of Wnt5A in MDSCs was associated with the suppressive mechanisms of these cells.

The secretion of TGFβ and arginase 1 are established methods of MDSC-mediated suppression that are well described in the literature. When we harvested the tumors and spleens of mice, we found a significant decrease in the levels of TGFβ1 within both MDSC subtypes in tumors (Fig. 5A) but no changes within the spleen (Fig. 5B). Similar results were observed for arginase 1 as both M- and PMN-MDSCs had significantly reduced arginase 1 expression only in tumor-derived MDSCs of the knockdown animals (Fig. 5C and D).

It is reported that MDSCs express high levels of CXCR2 (31). Data within the literature suggest that MDSCs may utilize the CXCR2-CXCL1 axis as a method of site-specific chemokine-mediated migration of MDSCs to metastatic sites such rich in CXCL1 (32, 33). However, when we examined CXCR2 expression on MDSCs, we found no changes in the expression of this chemokine receptor, suggesting altered MDSC infiltration was not driven through a CXCR2 mechanism (Supplementary Fig. S5).
To determine whether the suppressive capacity of MDSCs is affected by Wnt5A knockdown, we isolated M-MDSC and PMN-MDSCs from tumors of control and Wnt5A−/− mice and measured the proliferation of antigen-specific splenocytes from transgenic mice in culture. Isolated M-MDSC and PMN-MDSCs were independently cocultured with activated T cells stimulated with an

Figure 3.
Immunosuppressive changes in tumors following Wnt5A knockdown in myeloid cells. Wnt5A−/− and control animals were intradermally implanted with BSC9AJ2 cells. A–C, Changes in MDSCs (A), Tregs (B), and T cells (C) were assessed using FACS. Representative FACS plots included for each immune subset (n = 8). D, Correlation of Tregs and M-MDSCs infiltrating tumors. Tregs and M-MDSCs were normalized before significance was determined using Spearman rank correlation. **, P < 0.01.
antigen-specific peptide for 48 hours. We found that the ability of both PMN-MDSCs and M-MDSCs to suppress stimulated T cells was decreased by approximately 50% when Wnt5A was knocked down in these cells (Fig. 5E).

These data suggest that tumors of Wnt5A−/− animals not only have decreased numbers of MDSCs, but that the function of MDSCs may also be abrogated making them less responsive within tumors. Taken together, these data suggest that Wnt5A may influence, at the least, suppressive mechanisms such as arginase 1 and TGFβ1 in MDSCs, which could indirectly affect MDSC suppression via this cytokine predominantly within the tumors, as well as directly decreasing T-cell function and proliferation.

**Discussion**

In these studies, we investigated the role of MDSC-derived Wnt5A on melanoma proliferation and metastasis via noncanonical Wnt signaling in melanoma and the role it plays in the immune component of the TME. Both MDSCs and Wnt5A have independently been linked to aggressive forms of metastatic melanoma promoting therapy resistance. Using novel transgenic animal models, we made the surprising finding that a major source of Wnt5A in the TME comes from suppressive myelocytes. This increase in Wnt5A from exogenous sources results in increased expression of Wnt5A within the tumor cell through a positive feedback signaling mechanism (described by O’Connell and colleagues; ref. 34). This can largely be attributed to the fact that Wnt5A signals via Fzd5 and ROR2 to activate protein kinase C and calcium signaling. Activation of protein kinase C results in the ultimate stabilization of Wnt5A mRNA, resulting in increased tumor cell–specific Wnt5A (3). While control animals retain expression of Wnt5A in melanoma cells, which slows their growth rate, increases their metastatic capacity, and decreases the immunogenicity of these tumors (summarized as a schematic in Fig. 6A), depleting Wnt5A in the MDSCs inhibits Treg infiltration and inhibits metastasis (Fig. 6B).
Figure 5.
Effects of Wnt5A on MDSC suppressive mechanisms. A–D, FACS analysis of MDSCs from mice implanted with BSC9AJ2 cells were assessed using FACS for changes in TGFβ1 expression in tumors (A) and spleen (n = 5; B) and arginase 1 in tumors (C) and spleen (n = 8; D). E, MDSCs from tumors of animals implanted with BSC9AJ2 cells were isolated and cocultured with antigen-stimulated T cells from PMEL animals. Results were plotted as a percentage of T-cell suppression based on positive controls consisting of stimulated T cells only (n = 6). *, P < 0.05; **, P < 0.01.
These findings are important in several contexts. First, they expand the role of MDSCs from being uniquely suppressive myeloid cells providing tumor immunosuppression, to directly affecting the growth rate and invasive capacity of cancer cells in vivo through the secretion of Wnt5A. Second, we show here that Wnt5A is important in directing the recruitment of MDSCs into tumors. Recent data have suggested that cytokines such as IL6 are important in both the generation and function of MDSCs (35). Analysis of TCGA data demonstrated that there was a significant association between Wnt5A and IL6 in melanoma patient samples (Fig. 1A). In addition to this, both CXCR2, which is a potent chemokine receptor expressed on MDSCs and involved in site-specific migration, and its main ligand CXCL1 were significantly associated with Wnt5A expression in melanoma tumors (Fig. 1A). Third, we found myeloid-Wnt5A was also an important factor in regulating the immune axis within the TME. Not only does Wnt5A secretion from myeloid cells act directly on melanoma cells, we found that Wnt5A is important in MDSC-mediated suppression of T cells. When Wnt5A was knocked down in myeloid cells, there was a decrease in the number of the MDSC subsets and Tregs, thus, decreasing immunosuppression within the TME. Significant changes were not observed in the spleen, suggesting the primary effect of Wnt5A on MDSCs is to affect their recruitment to the microenvironment of the tumor.

MDSCs have several mechanisms of immunosuppression, including the recruitment of Tregs. Interestingly, we found that in control animals the levels of M-MDSCs positively correlated with the number of Tregs in tumors; however, when Wnt5A was knocked down the correlation was ablated. We asked whether Wnt5A was required for Treg recruitment by MDSCs. However, we found that rWnt5A did not cause a change in Treg migration in vitro (Supplementary Fig. S6), suggesting that instead, Wnt5A has indirect effects on Treg migration. Interestingly, TGFβ1 expression was significantly decreased within both MDSC subsets following myeloid Wnt5A knockout. TGFβ1 has been demonstrated to induce an adaptive Treg phenotype in naïve CD4+ T cells (36, 37). Therefore, these data could imply that TGFβ1 release from MDSCs could promote the development of adaptive Tregs, whereas decreased TGFβ1 from Wnt5A−/− animals potentially decreases Treg induction from naïve CD4+ T cells. MDSC-mediated TME migration may be related to Wnt5A via changes in TGFβ1 production. We also found that arginase 1 was decreased in MDSCs following Wnt5A knockdown. Both arginase 1 and TGFβ1 have been reported to have strong interplay with Wnt5A (5, 38).

The decrease in TGFβ1 and arginase 1 in Wnt5A-depleted MDSCs also suggest that the suppressive function could be diminished. This notion was confirmed using an antigen-specific T-cell proliferation assay. When M-MDSCs and PMN-MDSCs were cocultured with antigen-stimulated T cells, both Wnt5A-depleted MDSC subsets had significantly decreased ability to suppress T-cell proliferation in vitro. These data suggest Wnt5A has a dual effect on MDSC-mediated TME suppression, first, by inhibiting the number of tumor-infiltrating suppressive cells, and second, by decreasing the suppressive capacity of MDSCs. It should be noted that MDSCs have a wide variety of reported suppressive mechanisms including the secretion of nitric oxide synthase, reactive oxygen species,
depletion of l-arginine and cystine, and various cell–cell contact mechanisms (39). Therefore, the decreased suppression may be attributed to other mechanisms.

We found that myeloid-derived Wnt5A has a dual effect on tumor-infiltrating lymphocytes decreasing tumor immunosuppression. First, due to a decrease in the number of immunosuppressive MDSCs and Treg cells infiltrating the tumor, the TME may go from a less-favorable cold tumor characterized by high levels of Tregs, MDSCs, and Wnt5A, to a hot tumor with significantly decreased immunosuppression. These data have key translational significance as depletion of MDSCs (31) and Tregs (40, 41) improves responses to immunotherapy. Second, following MDSC-Wnt5A knockdown levels of PD-1 and LAG3 were both significantly decreased on cytotoxic T cells. This decreases the ability of T cells to utilize inhibitory immune checkpoints via PD-L1 + cells such as tumor cells, increasing the potential of these effector T cells to elicit an antitumor response. High-level expression of both PD-1 and LAG3 are strongly associated with T cell exhaustion, therefore a decrease in these markers on cytotoxic T cells could suggest a modest reversal in T-cell fatigue.

The secretion of cytokines including IFNγ is associated with antigen-stimulated T cells (42). Within a complex TME, IFNγ is known to have potent antitumor effects particularly within the immune system where it can activate antitumor myeloid cells including macrophages and neutrophils, as well as upregulating MHC class II. We found that patients with melanoma with high expression of IFNγ had increased survival, whereas survival decreased when the levels of IFNγ declined (Supplementary Fig. S7A). As T cells become more exhausted, their levels of PD-1 and LAG3 increase, whereas the production of proinflammatory cytokines including IFNγ diminishes (43). In addition to decreases in PD-1 and LAG3, we found Wnt5A−/− MDSCs had a modest increase in IFNγ expression in CD4+ and CD8+ T cells (Supplementary Fig. S7B). As well as having potent antitumor effects, the role of IFNγ can be a double-edged-sword. Melanoma cells can utilize this cytokine as a mechanism to evade the immune system by upregulating PD-L1. We treated BSC9A2J melanoma cells with 50 ng/mL of IFNγ for 16 hours. Untreated melanoma cells had moderately high levels of PD-L1; however, treatment with IFNγ greatly increased the levels of PD-L1 (Supplementary Fig. S7C, left). IFNγ treatment demonstrated no proapoptotic effects on these cells in vitro as there was no change in annexin V staining (Supplementary Fig. S7C, right). These data suggest that the regulation of IFNγ within a TME may require a fine balance of control, which was achieved within our model, between the activation of innate and adaptive immunity, and cancer cell immune evasion. The potential reversal of T-cell exhaustion is not believed to be dictated directly by the effects of Wnt5A on T cells, but due instead to the decrease in the TME of immunosuppressive cells such as MDSCs, which are known to promote T-cell exhaustion (44, 45).

Poorly immunogenic tumors are well known to be negatively associated with immunotherapy success. These data could positively contribute to the potential success of therapies designed to inhibit MDSCs. MDSCs are an extremely heterogeneous population of cells that cannot be identified with one or two markers, but are generally classified by their ability to suppress immune reactions and express immunosuppressive cytokines. Given the importance of MDSCs in many cancers, innovative methods to target these cells have emerged in numerous clinical trials. Studies show that the exhaustion and functional impairment of T cells in the TME is a defining feature in the prognosis of many cancers. Infiltrating antitumor CD8+ T cells coexist with a wide variety of other factors including cancer cells, immunosuppressive leukocytes, and a combination of proinflammatory and antiflammatory cytokines. Immunosuppressive leukocytes such as MDSCs are key contributors to antiflammatory TME cytokines including arginase 1, IL10, and TGFβ augmenting CD8+ T-cell exhaustion while decreasing IL2 and IFNγ secretion from these cells. Targeting antiflammatory cytokines secreted from tolerogenic leukocytes have been used in numerous therapies to help reduce T-cell exhaustion. The use of IL10 inhibitors have been shown to increase the efficiency of PD-1 blockade in patients with advanced melanoma enhancing the activity of antigen-specific CD8+ T cells (46). STAT3 is a transcription factor responsible for most immunosuppressive factors in myeloid cells (47). STAT3 inhibitor, AZD9150, was used in combination with checkpoint blockade in patients with hepatocellular carcinoma (NCT01839604), pancreatic, non–small cell lung, and colorectal (NCT02983578) and squamous cell carcinoma of head and neck (NCT02499328). Another study targeted TLR7 as a method of decreasing immunosuppressive functions of MDSCs in CT26 models (48). As exhausted T cells lose their secretion and responsiveness to T-cell expansion factors such as IL2, trials have also investigated using high doses of IL2 as an adjuvant in PD-1 immunotherapy to overcome anti-PD1-resistant metastatic melanoma and renal cell carcinoma (NCT03991130). A strong objective of this trial was to correlate MDSC and T-cell subsets in the peripheral blood during therapy. Other methods targeting MDSCs prevent their differentiation through targeting the inflammatory cytokines frequently found to cause their activation and their migration to distal sites by blocking chemokine receptors implicated in site-specific chemokine-mediated migration. These trials are described in more detail by Fleming and colleagues (49). Targeting Wnt5A might provide one more mechanism by which to disrupt the immunosuppressive effects of MDSCs and increase the efficacy of immunotherapy.

Authors’ Disclosures
D.J. Gabrilovich reports funding from AstraZeneca outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

Acknowledgments
We thank the outstanding Core Facilities of the Wistar Institute, supported by P30CA110515 and of the Johns Hopkins Kimball Cancer Center, P30CA069756. A.T. Weeraratna, S.M. Douglass, M.E. Fane, and G.M. Alicea are supported by R01CA174746 and R01CA207935. Q. Liu and A.T. Weeraratna are also supported by P01 CA114046. M. Webster is supported by R01 CA280812. R. Kuruvilla is supported by R01 NS107342 and R01 NS114478. A.T. Weeraratna is also supported by R01CA232256, a Bloomberg Distinguished Professorship, and the EV McCollum Endowed Chair.

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Received April 14, 2020; revised November 20, 2020; accepted November 24, 2020, published first December 1, 2020.
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References


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