Guided migration analyses at the single-clone level uncover cellular targets of interest in tumor-associated myeloid-derived suppressor cell populations

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Myeloid-derived suppressor cells (MDSCs) are immune cells that exert immunosuppression within the tumor, protecting cancer cells from the host's immune system and/or exogenous immunotherapies. While current research has been mostly focused in countering MDSC-driven immunosuppression, little is known about the mechanisms by which MDSCs disseminate/infiltrate cancerous tissue. This study looks into the use of microtextured surfaces, coupled with in vitro and in vivo cellular and molecular analysis tools, to videoscopically evaluate the dissemination patterns of MDSCs under structurally guided migration, at the single-cell level. MDSCs exhibited topographically driven migration, showing significant intra- and inter-population differences in motility, with velocities reaching ~40 μm h⁻¹. Downstream analyses coupled with single-cell migration uncovered the presence of specific MDSC subpopulations with different degrees of tumor-infiltrating and anti-inflammatory capabilities. Granulocytic MDSCs showed a ~≥3-fold increase in maximum dissemination velocities and traveled distances, and a ~10-fold difference in the expression of pro- and anti-inflammatory markers. Prolonged culture also revealed that purified subpopulations of MDSCs exhibit remarkable plasticity, with homogeneous/sorted subpopulations giving rise to heterogenous cultures that represented the entire hierarchy of MDSC phenotypes within 7 days. These studies point towards the granulocytic subtype as a potential cellular target of interest given their superior dissemination ability and enhanced anti-inflammatory activity.

The tumor microenvironment is highly heterogeneous in nature, with cancerous cells co-habiting with both stromal and immune cells. Such complex cellular interplay plays a central role in modulating tumor progression. Myeloid-derived suppressor cells (MDSCs), in particular, have been known to exert immunosuppressive activity in the tumor niche, which protects cancerous cells from the host immune system and/or different therapeutic modalities. While a lot of research has been devoted to developing advanced drugs and drug delivery systems to target cancerous cells and/or blocking MDSC-driven immunosuppression within the tumor niche, less is known about the motility mechanisms by which MDSCs disseminate and colonize the tumor in the first place.

MDSCs are innate immune cells that are highly expanded in cancer patients. These cells tend to infiltrate tumors and lymphoid tissues, and their levels correlate with increased tumor burden and limited survival in a variety of malignancies. MDSCs specifically contribute to the loss of immune effector cell function and reduce the efficacy of immunotherapies. As such, MDSCs have emerged as an attractive therapeutic target in

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cancer. Drugs that inhibit MDSC effector functions or proliferation within the tumor could potentially lead to an enhanced host anti-tumor immune response and clearance of the cancer burden. However, efforts to effectively target MDSCs within the tumor niche have been hampered by a lack of robust “druggable” targets at the cellular and/or molecular level. While targeting the dissemination-based mechanisms by which MDSCs infiltrate the tumor niche could be a viable alternative strategy against MDSC-driven immunosuppression at the tumor site, our understanding of such mechanisms for MDSCs is limited compared to what we know about the dissemination modalities of cancerous tumor cells. Structurally guided migration has been known to play a key role in the escape of cancerous cells from the primary tumor, as well as in dissemination and metastasis. Nevertheless, to the best of our knowledge, no study has probed MDSC motility under structurally guided dissemination conditions. Here we used microscale engineering tools, coupled with cellular and molecular biology analysis tools, to probe the dissemination capabilities of MDSCs at the single-clone level under guided migration conditions, and to identify MDSC subpopulations of interest based on their disseminative and suppressive capabilities.

Results and Discussion

MDSCs respond to topographical cues and exhibit structurally guided dissemination patterns. Structurally guided cell dissemination has been known to play a role in the escape of cancerous cells from the primary tumor and the establishment of metastatic outgrowths in peripheral organs and tissues. Highly aggressive cancer cells tend to exhibit distinct spreading patterns, disseminating preferentially along pre-aligned anatomical microstructures within the tissues, including radially oriented fibrils from the extracellular matrix (ECM), white matter tracts, the basal lamina of blood vessels, and the subpial/subperitoneal spaces, among others. While topographical or cell confinement cues have been used to mimic rapid and highly directional motility in a wide variety of cancerous cells, no studies have probed MDSC motility under structurally guided dissemination conditions. Here we used microscale engineering tools, coupled with cellular and molecular biology analysis tools, to probe the dissemination capabilities of MDSCs at the single-clone level under guided migration conditions, and to identify MDSC subpopulations of interest based on their disseminative and suppressive capabilities.

Figure 1. MDSCs are responsive to aligned structural cues and exhibit guided dissemination patterns. (A) Schematic diagram of the tumor microenvironment showing invasive cancer cells and infiltrative MDSCs using pre-aligned structural cues (e.g., remodeled ECM, blood vessel walls) to escape and invade the tumor stroma, respectively. (B) SEM micrograph (with superimposed MDSC mock-ups) of a PDMS-based biomimetic textured surface used to evaluate structurally guided MDSC migration at the single-clone level. (C) Actin (green) – Nuclei (blue) staining of MDSCs cultured on textured vs. control/TCP surfaces. MDSCs assume an aligned/more migratory morphology on the textured surfaces compared to TCP. (D) Single-clone dissemination tracks and (E) quantification of MDSCs on textured vs. control/TCP surfaces confirming enhanced dissemination capabilities (i.e., average single-clone velocity and net track distance) for MDSCs when exposed to pre-aligned structural cues. The net track distance is a reflection of the geometrical distance traveled by a cell during the tracking period. *p < 0.01 and ‡p < 0.02 (t-test, n = 4).
MDSC subpopulations exhibit different dissemination capabilities. Based on the clear inter-clonal variability in motility, we proceeded to further stratify and probe the MDSC population via flow cytometry-based sorting into granulocytic (CD11b+Ly6C+Ly6G−) and monocytic (CD11b+Ly6C−Ly6G+) subpopulations (Fig. 2A–C) based on standard MDSC nomenclature. A subpopulation of CD11b+Ly6C+Ly6G− cells was also identified from the flow cytometry data and included in our analyses. Flow-sorted subpopulations were then subjected to structurally guided motility studies on textured surfaces, as described above, in addition to qRT-PCR analyses of pro- and anti-inflammatory markers. Single-clone dissemination studies indicate that when probed in isolation, granulocytic MDSCs have superior dissemination capabilities compared to monocytic MDSCs and the CD11b+Ly6C−Ly6G+ subpopulation (Fig. 2D) (Videos S3–5), with single clones reaching in some cases average velocities and net displacements of ~400 μm·h⁻¹ and ~20 mm over a period of 16 hours. However, we also found that certain populations of patient-derived circulating MDSCs exhibited limited overall motility, which could potentially be a direct reflection of the underlying malignancy (e.g., type, stage, mutations) and/or concurrent treatment modalities (Tables S1–S3).

MDSC subpopulations show phenotypic plasticity that drives populational homeostasis under prolonged culture conditions. Following flow-based purification of the MSC-2 cells into distinct subpopulations of granulocytic and monocytic MDSCs, as well as CD11b+Ly6C+Ly6G− cells, the cells were maintained in culture for 1–7 days. Phenotypic plasticity was evaluated via flow cytometry at days 1 and 7. Single-clone motility assays and gene expression analyses were run at day 7 (Fig. 3A). Surprisingly, and in contrast to what we found immediately after flow-based sorting, no significant differences were detected in the dissemination characteristics across all three populations by day 7 (Fig. 3B). Average single-clone velocities stayed within ~50 μm·h⁻¹ for all populations, while the overall net track distance stayed below ~200 μm. Flow cytometry analyses indicated that 1 day post-sorting the purified populations still comprised the majority (~80%) of the culture, however, by day 7 the whole hierarchy of populations had been reestablished (Fig. 3C–E), possibly suggesting a role for cellular plasticity in the maintenance of populational homeostasis/heterogeneity in MDSC populations. Following flow-based purification of the MSC-2 cells into distinct subpopulations, as well as CD11b+Ly6C+Ly6G− cells, the cells were maintained in culture for 1–7 days. Phenotypic plasticity was evaluated via flow cytometry at days 1 and 7. Single-clone motility assays and gene expression analyses were run at day 7 (Fig. 3A). Surprisingly, and in contrast to what we found immediately after flow-based sorting: no significant differences were detected in the dissemination characteristics across all three populations by day 7 (Fig. 3B). Average single-clone velocities stayed within ~50 μm·h⁻¹ for all populations, while the overall net track distance stayed below ~200 μm. Flow cytometry analyses indicated that 1 day post-sorting the purified populations still comprised the majority (~80%) of the culture, however, by day 7 the whole hierarchy of populations had been reestablished (Fig. 3C–E), possibly suggesting a role for cellular plasticity in the maintenance of populational homeostasis/heterogeneity in MDSC populations. Cell cultures derived from the purified granulocytic subpopulation (Fig. 3C), for example, gave rise to monocytic MDSCs and CD11b+Ly6C+Ly6G− cells, with the monocytic subpopulation showing the sharpest increase from day 1 to 7 (~7-fold change), and the CD11b+Ly6C−Ly6G+ subpopulation showing a ~3-fold increase by day 7. Cultures derived from purified monocytic MDSCs, on the other hand, were more prone to giving rise to the CD11b+Ly6C+Ly6G+ population by day 7 (~2.5-fold increase) compared to the granulocytic population. Finally, cultures derived from the purified CD11b+Ly6C+Ly6G− population were more prone to giving rise to granulocytic MDSCs by day 7 (~3-fold increase) compared to the monocytic MDSCs, which did not show a significant increase between days 1 and 7. Gene expression profiles of pro-(Fig. 3F) and anti-inflammatory (Fig. 3G) markers at day 7 showed more subtle differences across populations, with decreased and increased iNOS and IL-6 expression, respectively, in the “fresh” MDSC population relative to the sorted/purified subpopulations. However, when comparing the expression profiles between day 0 (i.e., day of sorting/purification) and day 7, a more pronounced difference was noted, with an overall increase in the expression of pro-inflammatory iNOS for all three populations, and a significant decrease in arginase 1 and IL-10 for the granulocytic subpopulation only (Fig. S3).
Micro- and nanoscale technologies have been used extensively to probe and/or modulate various aspects of cell biology for medical applications, especially in cancer therapy and diagnostics. Here we used microscale engineering tools to demonstrate that tumor-associated MDSCs exhibit structurally guided migration patterns, similar to invasive cancerous cells. Single-clone motility analyses unmasked clear heterogeneities within and across patient-derived MDSC populations, confirming the presence of clonal subsets with enhanced dissemination capabilities in both murine and patient-derived MDSCs.

**Conclusions**

Micro- and nanoscale technologies have been used extensively to probe and/or modulate various aspects of cell biology for medical applications, especially in cancer therapy and diagnostics. Here we used microscale engineering tools to demonstrate that tumor-associated MDSCs exhibit structurally guided migration patterns, similar to invasive cancerous cells. Single-clone motility analyses unmasked clear heterogeneities within and across patient-derived MDSCs, confirming the presence of clonal subsets with enhanced dissemination capabilities in both murine and patient-derived MDSCs. Follow-up motility analyses showed distinct dissemination and gene expression patterns among different MDSC subpopulations. Further studies are needed to fully understand the role of MDSCs in tumor progression and potential therapeutic targets.
Figure 3. Single MDSC subpopulations appear to show phenotypic plasticity that can drive the replenishment of the entire phenotypic spectrum. (A) Schematic diagram of the experimental design. (B) Single-clone dissemination (i.e., average velocities and net track distances) studies did not show significant differences between all three populations by day 7. (C–E) Flow cytometry analyses indicate that while by day 1 post-sorting all subpopulations remained relatively pure, by day 7 the entire spectrum of phenotypes had been replenished regardless of the phenotype of the starting cell population. *p < 0.0001, †p = 0.01, ‡p = 0.03, §p = 0.0001 (2-way ANOVA/Tukey’s multiple comparisons, n = 3–4). qRT-PCR analyses of (F) pro-inflammatory and (G) anti-inflammatory genes at day 7 post-sorting. *p = 0.006, **p = 0.01 (2-way ANOVA/Tukey’s multiple comparisons, n = 3–6).
studies coupled with flow cytometry-based sorting, gene expression analyses, and orthotopic tumor xenograft experiments in nude mice, suggest that the granulocytic subpopulation is more prone to exhibiting increased dissemination and tumor-infiltrative ability, as well as enhanced anti-inflammatory activity, which could make this population an attractive cellular target in cancer research and therapeutic development. Subsequent studies, however, highlight the remarkably dynamic and plastic nature of such clonal subsets, with purified MDSC subpopulations quickly reaching populational homeostasis by giving rise to the full spectrum of MDSC phenotypes. While there have been conflicting reports regarding the dominant phenotype of tumor-resident MDSCs (i.e., granulocytic vs. monocytic)41–47, our single-clone dissemination and phenotypic plasticity results point towards a potential mechanism by which granulocytic MDSCs are presumably better equipped to infiltrate the tumor niche, where they could then remain as granulocytic and/or give rise to monocytic MDSCs depending on multiple factors, including the tumor type. Interestingly, single-clone dissemination studies with circulating MDSCs derived from cancer patients suggest that MDSC motility could potentially be impacted by the patient’s background (e.g., type/stage of cancer, treatment modalities, etc.), and as such, additional studies are needed to determine whether the dissemination patterns of circulating MDSCs, ex vivo, could be used to monitor disease and/or treatment progression.

**Materials and Methods**

**Textured PDMS surfaces.** Microtextured PDMS surfaces were fabricated from photolithographically patterned silicon masters via a replica molding process. A parallel array of ridges and grooves (2 μm wide, 2 μm tall, spaced by 2 μm) was first patterned on a silicon master via standard UV photolithography using S1813 photoresist. A 10:1 mixture of PDMS with curing agent was then cast on the master and allowed de-gas and cure for several hours. The PDMS was then demolded from the master, sterilized and placed on multi-well plates for single-cell migration experiments. Scanning electron microscopy (SEM) was used to characterize the surface morphology.

**MDSC cultures.** The mouse MDSC cell line (MSC-2) was a kind donation from Gregoire Mignot. MSC-2 cells were cultured in RPMI 1640 media supplemented with 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic, and 1 mM sodium pyruvate. Patient-derived MDSCs were enriched from peripheral blood using the RosetteSep HLA-myeloid cell enrichment kit (Stemcell Technologies) followed by Ficoll-Paque centrifugation (GE healthcare). MDSC were isolated by subsequent negative selection of HLA-DRneg cells using anti-HLA-DR MicroBeads (Miltenyi Biotec) for 15 minutes at 4 °C and isolated using a MS-MACS column. Patient-derived MDSCs were acquired with informed consent under institutional review board (IRB)-approved protocols for human subject research at The Ohio State University, in accordance with the Declaration of Helsinki.

**Single-cell migration assays.** ~1.5 × 10⁵ MSC-2 cells were seeded and allowed to adhere on the textured PDMS surfaces or TCP controls in regular culture media for several hours. Cells were imaged via time-lapse microscopy every 10 minutes for over 16 h using a cell culture chamber (Okolab) mounted on an inverted microscope. Images were analyzed using the manual tracker plugin in Fiji. Single-cell displacement data were then analyzed via MATLAB to determine velocities and net track traveled distances.

**Flow cytometry-based analysis and sorting.** The following antibodies were used for the MSC-2 cells: anti-CD11b-FITC, anti-Ly6-C-APC and anti-Ly6-G-PE, all purchased from Beckman Coulter. For patient-derived MDSCs, we used anti-CD33-APC, anti-CD11b-AP, and anti-HLA-DR-PECy7, purchased from Beckman Coulter. Data were acquired using an LSRII flow cytometer (BD Biosciences). All colors were evaluated against their respective isotype controls and samples with no staining.

**Gene expression analyses.** Total RNA was extracted using the Trizol reagent (ThermoFisher). Reverse transcription reactions were performed using 500–1000 ng RNA in a 20 μl reaction with the superscript VILO cDNA synthesis kit (ThermoFisher). cDNA was used as a template to measure the expression levels of pro- and anti-inflammatory genes by quantitative real-time PCR using predesigned primers. Real-time PCR reactions were performed using the QuantiStudio 3 Real-Time PCR System with TaqMan fast advance chemistry (Thermo Scientific) with the following conditions: 95 °C 10 min, 40 cycles of 95 °C 1 min, 60 °C 1 min, and 72 °C 1 min. Gene expression was normalized against the house keeping genes GAPDH and ATP-6.

**Orthotopic tumor xenografts.** Immunodeficient nude mice (Jackson Laboratory), 6–8-week-old, were first injected with 1 million human breast cancer cells (MDA-MB-231, ATCC) in the mammary fat pad to generate tumors. After 4 weeks of tumor development, sorted MDSC subpopulations were stained using PKH67 green fluorescent cell linker kit for general cell membrane labeling (Millipore Sigma) following the instructions suggested by the manufacturer. Tumor-bearing mice were then injected with ~2.5 × 10⁵ MDSCs via the tail vein. The mice were then collected 1-day post-injection, and the tumors, lungs and spleens were characterized with an IVIS Imaging System (Xenogen Imaging Technologies). All animal studies were performed in accordance with protocols approved by the Laboratory Animal Care and Use Committee of The Ohio State University.

**Statistical analysis.** All statistical analyses were run in Sigma Plot 12 or GraphPad Prism 7. We used n = 3–6 replicates per experiment. Specific information on the number replicates, statistical tests, and levels of significance can be found in the figure legends.
Data availability

The data generated through this study are available from the corresponding author upon reasonable request.

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Author contributions

S.D.-S., W.E.C. and D.G.-P. conceived the idea. S.D.-S., V.S., B.B., J.M., G.G.-V., N.H.-C., N.I. and S.G. were involved in microtexture fabrication, characterization, cell migration experiments and analyses. S.D.-S., L.L., A.P. and S.W., contributed with qRT-PCR characterization. S.D.-S., S.G. and W.L. were in charge of *in vivo* experiments. D.G.-P. prepared the manuscript with input from S.D.-S., W.E.C., B.B. and S.G.

Competing interests

The authors declare no competing interests.

Additional information

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