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Characterization of GM-CSF and G-CSF expressing cell subtypes in the tumor microenvironment using the Paletrra™ multiplex immunofluorescence assay integrated with RNAscope™

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are cytokines known to stimulate differentiation and activation of granulocytes, monocytes, macrophages, and dendritic cells (DCs) as well as non-immune cells including endothelial, epithelial and fibroblasts. GM-CSF and G-CSF can be produced by many different cell types, including immune cells, fibroblasts, endothelial cells and have also been shown to be upregulated in multiple human cancers. Studies have further demonstrated the immune-modulatory effects of GM-CSF/G-CSF can promote both anti-tumorigenic or pro-tumorigenic activity through regulation of neutrophils/M1 macrophages/T-effectors or myeloid-derived suppressor cells (MDSCs)/M2 macrophages/Tregs, respectively. Therefore, modulators to promote or reduce the activity of GM-CSF/G-CSF have been explored for tumor therapeutic intervention, often in combination with other therapies such as chemotherapy. However, to further dissect and target anti or pro-tumor GM-CSF/G-CSF modulation requires a better understanding of the distribution and identity of GM-CSF/G-CSF secreting cells within the tumor micro-environment (TME).

To characterize GM-CSF/G-CSF expression in the TME, we use the Integrated Paletrra™-RNAscope™ workflow. Paletrra (NeoGenomics Laboratories, Inc) is a proprietary multiplex immunofluorescence (mIF) service for the visualization and characterization of up to 60 protein biomarkers in a single formalin-fixed paraffin-embedded (FFPE) section and offers high-resolution spatial and quantitative analysis of protein expression in tissue samples. RNAscope (Bio-Techne) Multiplex is a highly sensitive fluorescent in-situ hybridization (ISH) assay that can detect up to 3 RNA markers in a single FFPE section. The Integrated Paletrra-RNAscope assay allows for simultaneous detection of both protein and RNA markers in a single sample. Herein we report the design and use of a novel panel of commercially-available RNAscope ISH probes and IF antibodies broad enough to characterize GM-CSF and G-CSF subpopulations in the TME of a variety of tumor indications including lung, prostate, melanoma, and colon cancer. Using this panel in combination with proprietary deep-learning based image analysis, GM-CSF and G-CSF expressing cells can be characterized into different immune and TME subtypes. Understanding of the variety and phenotype of GM-CSF and G-CSF expressing cells in the TME will help improve the understanding and development of targeted therapies for cancer treatment.

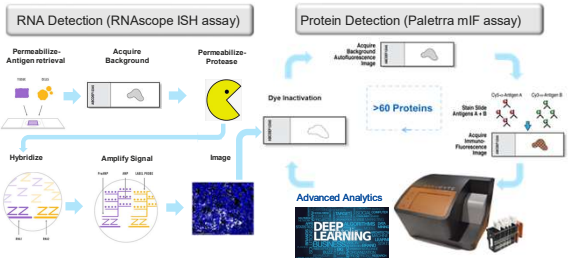

Figure 1: Integrated Paletrra-RNAscope overview and workflow. Untreated colon adenocarcinoma (64 cases), lung adenocarcinoma (64 cases), malignant melanoma (73 cases), and prostate adenocarcinoma (52 cases) TMA samples were used in this study. All TMA slides were prepared and stained using the Integrated Paletrra-RNAscope workflow, which combines RNAscope ISH and Paletrra mIF staining protocols. Slides were first cleared and then undergo pretreatment, which includes both heat activated epitope retrieval and protease steps. Then RNAscope ISH probe hybridization, amplification, and imaging was performed before proceeding to IF multiplexing. For each round of IF staining, conjugated fluorescent antibodies were applied to the slide, followed by imaging acquisition of stained slides. The dye was erased, enabling additional rounds of staining with another pair of fluorescent antibodies. For this study, IF biomarkers were included to characterize multiple immune (CD3, CD68, CD20, Nkp46, CD11c), stromal (CD31, FAP), and tumor (PanCK, SOX10) populations.

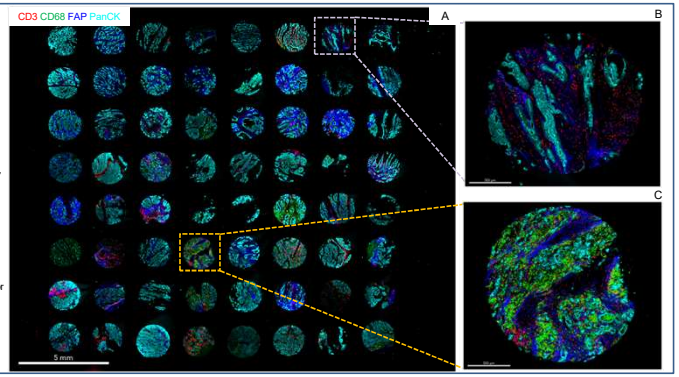

Figure 2: Whole slide TMA imaging allows for visualization and characterization of TME heterogeneity. (A) Whole slide representative color overlay from colon adenocarcinoma TMA of CD3 (red), CD68 (green), FAP (blue), and PanCK (cyan). Magnified views corresponding to dotted green (B) and yellow C) box reveals detailed resolution of immune and tumor expression within the TME.

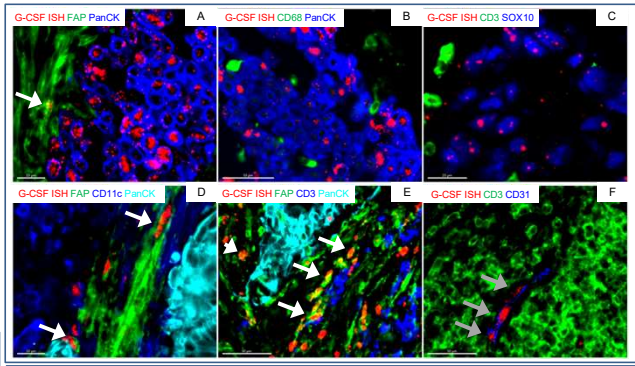

Figure 4: Characterization of G-CSF ISH in different cell subtypes. Representative color overlay images showing expression of G-CSF ISH (red) in different cell populations. (A-C) G-CSF+ tumor cells are seen as red cytoplasmic dots within blue PanCK membrane in colon (A) and lung (B) TMA samples or blue nuclear SOX10 in melanoma (C) TMA. (A, D, E) White arrows point to examples of G-CSF (red) positive FAP+ fibroblast cells (green) in colon (A, D) or prostate (E) TMAs. (F) Gray arrows show examples of G-CSF ISH (red) overlay with CD31 (blue) in melanoma TMA.

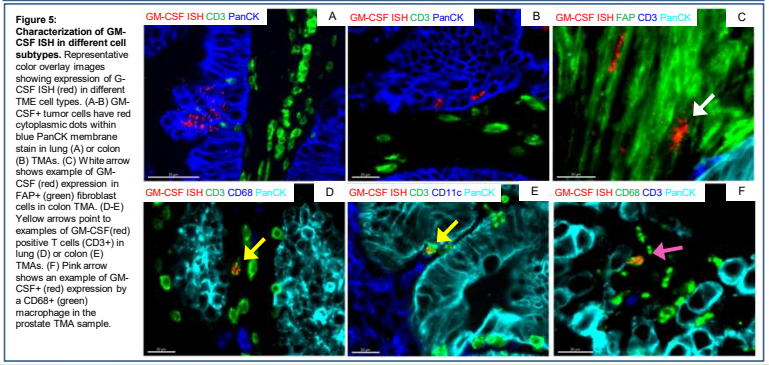

Figure 5: Characterization of GM-CSF ISH in different cell subtypes. Representative color overlay images showing expression of G-CSF ISH (red) in different TME cell types. (A-B) GM-CSF+ tumor cells have red cytoplasmic dots within blue PanCK membrane stain in lung (A) or colon (B) TMAs. (C) White arrow shows example of GM-CSF (red) expression in FAP+ (green) fibroblast cells in colon TMA. (D-E) Yellow arrows point to examples of GM-CSF(red) positive T cells (CD3+) in lung (D) or colon (E) TMAs. (F) Pink arrow shows an example of GM-CSF+ (red) expression by a CD68+ (green) macrophage in the prostate TMA sample.

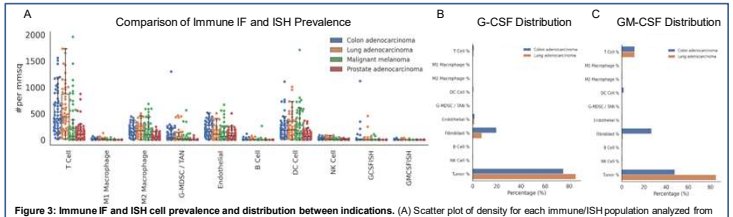

Figure 3: Immune IF and ISH cell prevalence and distribution between indications. (A) Scatter plot of density for each immune/ISH population analyzed from all 4 TMA samples. Each dot represents results from a single core from colon (blue), lung (orange), melanoma (green), or prostate (red) TMA. Overall prevalence of GM-CSF ISH and G-CSF ISH was fairly low in all four indications evaluated. In general, G-CSF prevalence was higher than GM-CSF. Colon adenocarcinoma and lung adenocarcinoma TMAs also showed highest prevalence of both G-CSF/GM-CSF. (B-C) Barplots showing overall distribution of immune, stromal, or tumor populations positive for G-CSF (B) or GM-CSF (C) in colon (blue) or lung (orange) adenocarcinoma. G-CSF is mostly expressed by tumor or fibroblasts while GM-CSF is expressed by T cells, fibroblasts, and tumor.

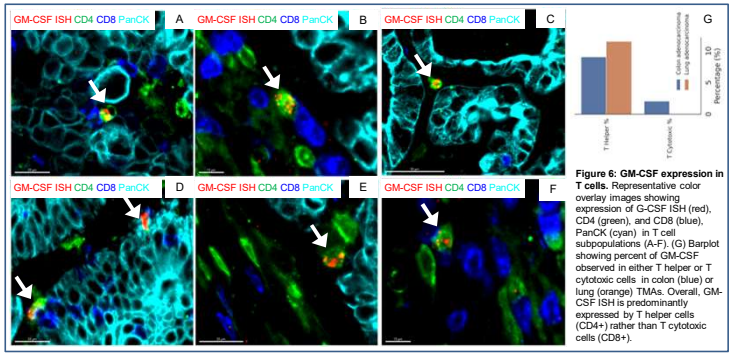

Figure 6: GM-CSF expression in T cells. Representative color overlay images showing expression of G-CSF ISH (red), CD4 (green), and CD8 (blue), PanCK (cyan) in T cell subpopulations (A-F). (G) Barplot showing percent of GM-CSF observed in either T helper or T cytotoxic cells in colon (blue) or lung (orange) TMAs. Overall, GM-CSF ISH is predominantly expressed by T helper cells (CD4+) rather than T cytotoxic cells (CD8+).

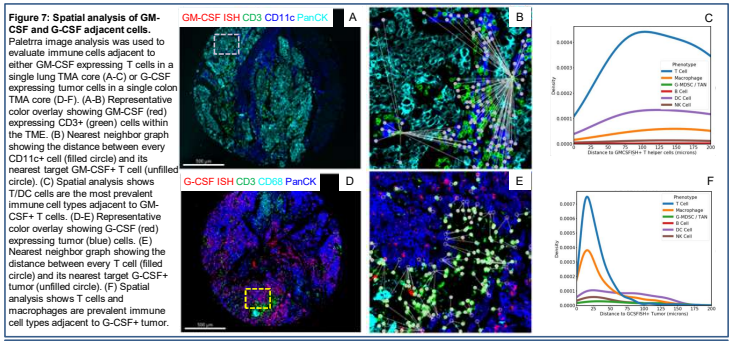

Figure 7: Spatial analysis of GM-CSF and G-CSF adjacent cells. Paletrra image analysis was used to evaluate immune cells adjacent to either GM-CSF expressing T cells in a single lung TMA core (A-C) or G-CSF expressing tumor cells in a single colon TMA core (D-F). (A-B) Representative color overlay showing GM-CSF (red) expressing CD3+ (green) cells within the TME. (B) Nearest neighbor graph showing the distance between every CD11c+ cell (filled circle) and its nearest target GM-CSF+ T cell (unfilled circle). (C) Spatial analysis shows T/DC cells are the most prevalent immune cell types adjacent to GM-CSF+ T cells. (D-E) Representative color overlay showing G-CSF (red) expressing tumor (blue) cells. (E) Nearest neighbor graph showing the distance between every T cell (filled circle) and its nearest target G-CSF+ tumor (unfilled circle). (F) Spatial analysis shows T cells and macrophages are prevalent immune cell types adjacent to G-CSF+ tumor.

Summary

- GM-CSF and G-CSF expressing cells were characterized in the TME of colon, lung, prostate, and melanoma TMAs using the Integrated Paletrra-RNAscope assay.
- Both GM-CSF and G-CSF showed overall low prevalence in all TMA indications evaluated, however lung and colon TMAs showed higher prevalence than melanoma and prostate.
- GM-CSF signal was mostly observed in T cells, tumor, and fibroblasts while G-CSF was mostly observed in tumor and fibroblasts.
- The majority of GM-CSF T cell expression was observed specifically in CD4+ T helper cells.
- Paletrra spatial analysis quantified proximity of target immune populations to GM-CSF/G-CSF expressing cells.

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