The complexity of myeloid-derived suppressor cells in non-small cell lung cancer: A combinatorial multiplex IHC and flow cytometry approach

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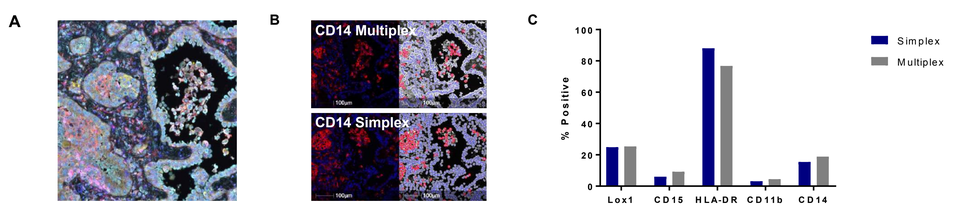
Background: Lung cancer is the leading cause of cancer deaths worldwide. Non-small cell lung cancer represents the majority of lung cancer cases and can be divided into further pathological subtypes: large cell carcinoma, adenocarcinoma, and squamous cell carcinoma. The architectural and genetic profiles of these subtypes varies as well as their immune cell composition and function. Myeloid derived suppressor cells are immature myeloid cells that are pro-tumor through their suppression of innate and adaptive immunity. Increased MDSC have been reported in the circulation of NSCLC patients and correlated with poor response to treatment and disease progression. MDSC are heterogenous and the phenotyping is complex. Until now, MDSC have been analyzed primarily by flow cytometry or with limited markers in immunohistochemistry (IHC). Multiplex IHC offers a possibility of visualizing the MDSC within the tumor environment with the high level of phenotyping required to identify the different subpopulations.

Objective: Compare MDSC populations in NSCLC subtypes with Histalim's HISTOPROFILE® -MDSC multiplex immunofluorescence panel on FFPE tissue resections and in paired peripheral blood samples by flow cytometry.

Methods:

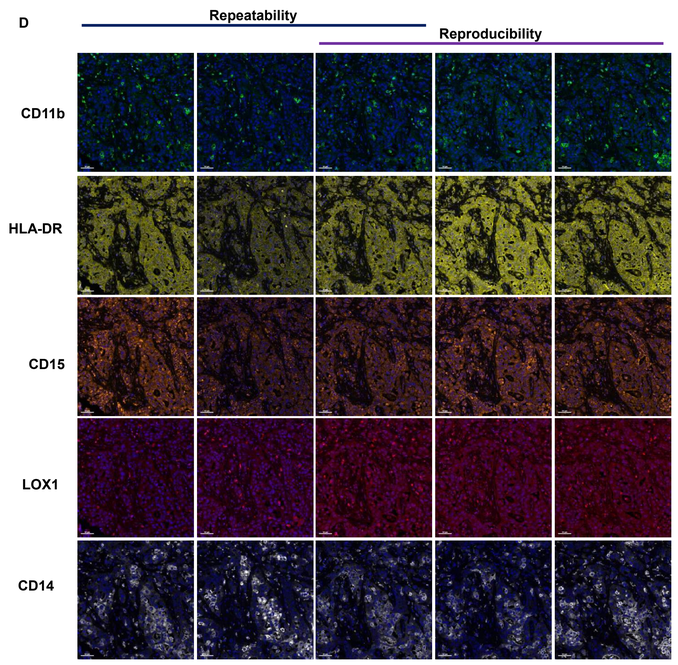
- Panel Design and Validation
  - Targets – CD11b/CD14/CD15/LOX1/HLA-DR
    - Polymorphonuclear MDSC – $CD11b^{+}/CD15^{+}/LOX1^{+}$
    - Monocytic MDSC – $CD11b^{+}/CD14^{+}/HLA\text{-}Dr^{lo/-}$
- Paired tissue resections and peripheral blood samples were collected from five NSCLC patients
- A pathologist determined the NSCLC subtype on hematoxylin & eosin stained slides
- Peripheral blood samples were stained with an eleven color flow cytometry panel and analyzed on a BD FACS Canto® (BD Biosciences)
  - Compensation setting were determined with BD Comp Beads and 100,000 leukocyte events were acquired
  - Leukocytes, neutrophils, monocytes, and M-MDSC were analyzed with BD FACSDiva software
- Sequential multiplex protocol with Opal® (Akoya Biosciences) fluorophores on the BOND RX® (Leica) slide stainer
  - Multispectral images acquired with the VECTRA® Polaris™ (Akoya Biosciences) slide scanner.
  - Images were spectrally deconvoluted with INFORM® (Akoya Biosciences) software.
  - Image Analysis with the HALO® (Indica Labs) Highplex module on NSCLC
    - Total myeloid cells, monocytes, neutrophils, PMN-MDSC, and M-MDSC were quantified

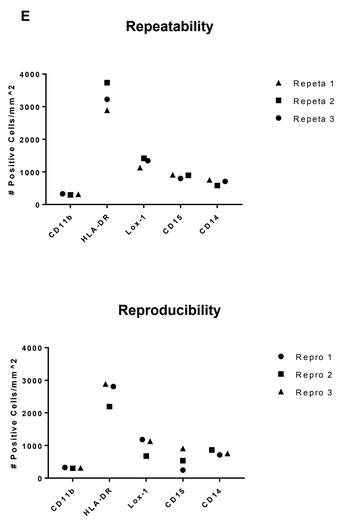
HISTOPROFILE® -MDSC Panel Validation

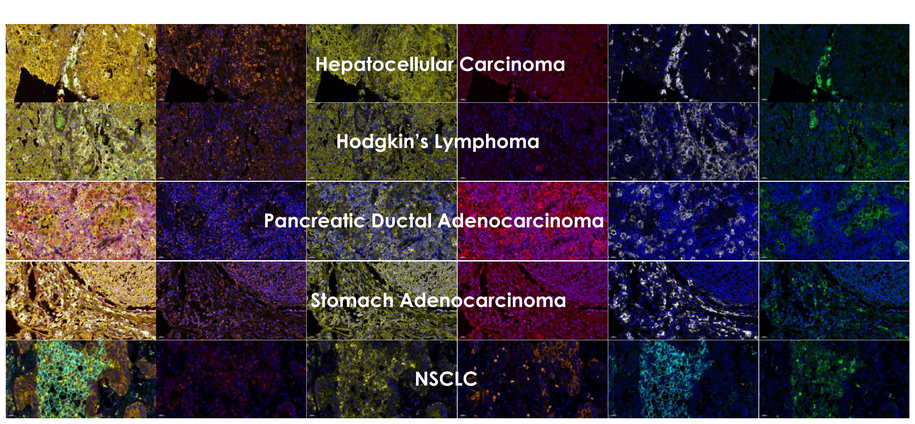


Simplex slides were stained for each individual biomarker in a simplex protocol and compared to a serial section stained with the multiplex HISTOPROFILE® -MDSC IHC panel. Staining concordance between the multiplex slide and simplex slides was determined by analysis with HALO® after multispectral deconvolution. A) Example image of a HISTOPROFILE® -MDSC multiplex stained slide (left). B) Example images corresponding to the multiplex slide for CD14 (top) and the simplex slide (bottom) and the corresponding HALO® masks identifying positive cells. C) Results from the simplex vs multiplex panel analysis. The percentage of positive cells from the simplex slide (blue bars) is compared to the multiplex slide (grey bars). Satisfactory results were obtained for the HISTOPROFILE® -MDSC panel.

The precision of the protocol was determined by inter-run repeatability analysis and intra-run reproducibility analysis. D) Example images from the repeatability and reproducibility slides. E) Analysis with HALO® demonstrated satisfactory results on the panel's performance. F) The robustness of the panel was demonstrated on a variety of tissues including hepatocellular carcinoma, Hodgkin's lymphoma, pancreatic ductal adenocarcinoma, stomach adenocarcinoma, and NSCLC.

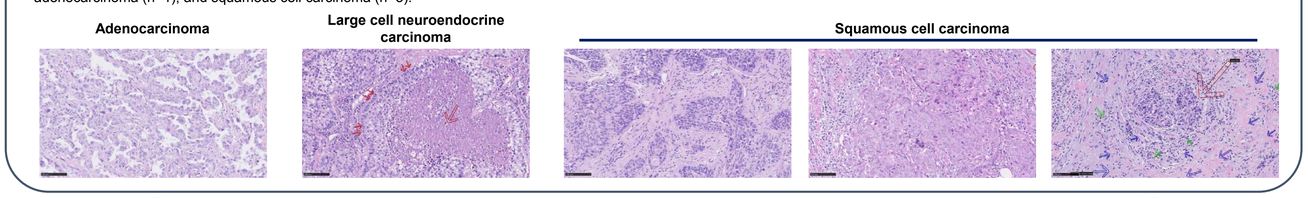






Pathologist Examination

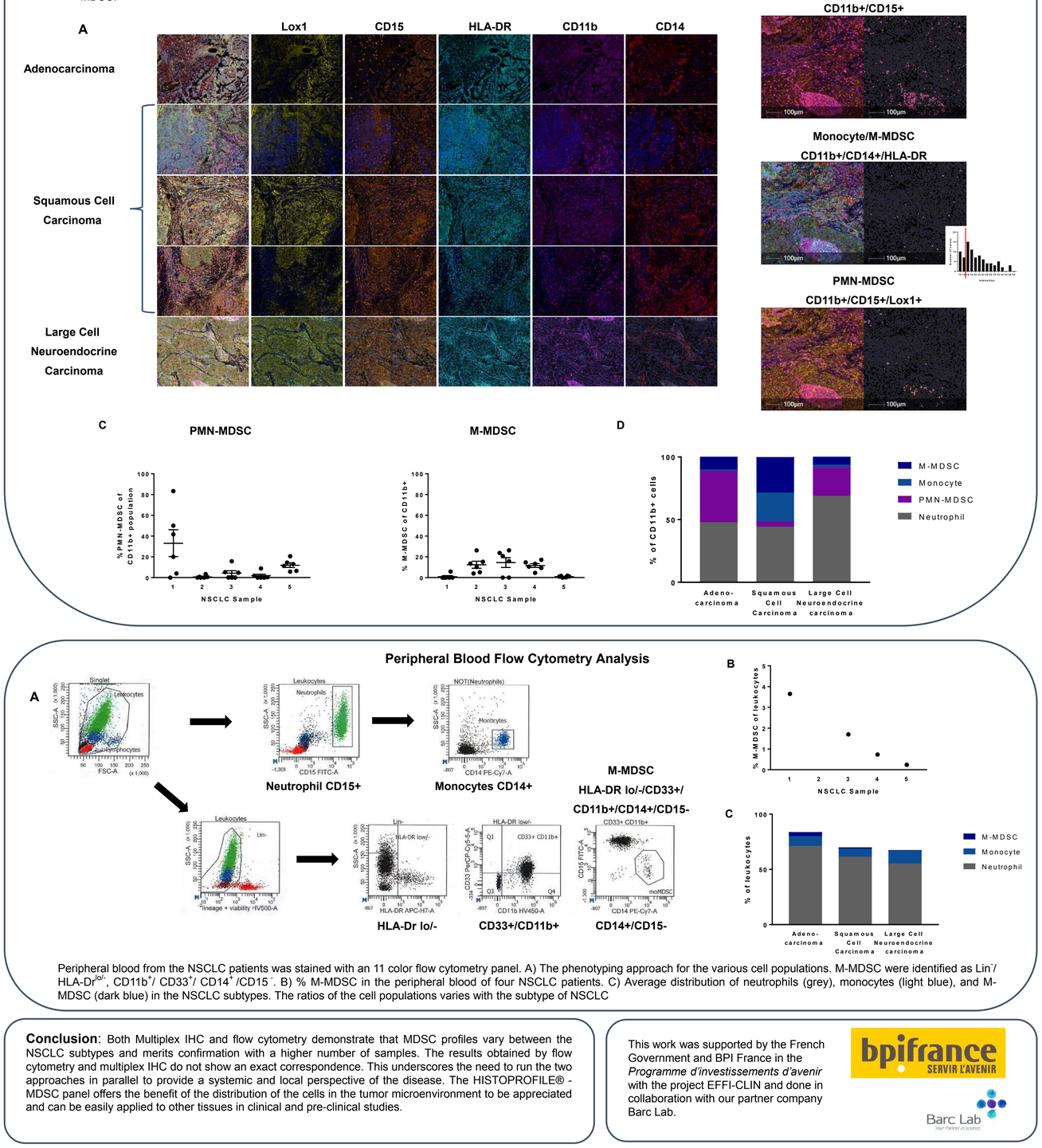
Hematoxylin and eosin slides were examined by a pathologist to determine the NSCLC subtype. The five samples were distributed between the three subgroups: large cell carcinoma (n=1), adenocarcinoma (n=1), and squamous cell carcinoma (n=3).



HISTOPROFILE® -MDSC NSCLC Analysis

The five NSCLC samples were stained with the HISTOPROFILE® -MDSC panel and imaged with th VECTRA® Polaris™. Six multispectral images from each sample were post-processed with INFORM® and analyzed with HALO® A) Example deconvoluted multispectral images of the five NSCLC samples grouped by pathology.

The frequency of myeloid cells ($CD11b^{+}$), neutrophils ($CD11b^{+}/CD15^{+}$), monocytes ($CD11b^{+}/CD14^{+}/HLA\text{-}DR^{+}$), M-MDSC ($CD11b^{+}/CD14^{+}/HLA\text{-}Dr^{lo/-}$), and PMN-MDSC ($CD11b^{+}/CD15^{+}/LOX1^{+}$) was analyzed. B) Example images of the individual populations with the fluorescent image (left) and the corresponding cell masks (right). The threshold for $HLA\text{-}Dr^{lo/-}$ was placed after the peak of HLA-DR fluorescence above background as seen by the red line on the histogram. C) The distribution of PMN-MDSC and M-MDSC in the five NSCLC samples. There is a clear heterogeneity of the cells within the tissues. D) Average distribution of neutrophils (grey), monocytes (light blue), M-MDSC (dark blue), and PMN-MDSC (purple) in the NSCLC subtypes. The ratios of the cell populations varies with the subtype of NSCLC. Squamous cell carcinoma has a larger presence *in situ* of M-MDSC.



Peripheral Blood Flow Cytometry Analysis

Peripheral blood from the NSCLC patients was stained with an 11 color flow cytometry panel. A) The phenotyping approach for the various cell populations. M-MDSC were identified as $Lin^{-}/HLA\text{-}Dr^{lo/-}$, $CD11b^{+}/CD33^{+}/CD14^{+}/CD15^{-}$. B) % M-MDSC in the peripheral blood of four NSCLC patients. C) Average distribution of neutrophils (grey), monocytes (light blue), and M-MDSC (dark blue) in the NSCLC subtypes. The ratios of the cell populations varies with the subtype of NSCLC

Conclusion: Both Multiplex IHC and flow cytometry demonstrate that MDSC profiles vary between the NSCLC subtypes and merits confirmation with a higher number of samples. The results obtained by flow cytometry and multiplex IHC do not show an exact correspondence. This underscores the need to run the two approaches in parallel to provide a systemic and local perspective of the disease. The HISTOPROFILE® -MDSC panel offers the benefit of the distribution of the cells in the tumor microenvironment to be appreciated and can be easily applied to other tissues in clinical and pre-clinical studies.

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