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# BLEACH&STAIN 15 marker multiplex fluorescence immunohistochemistry revealed six major PD-L1 immune phenotypes with distinct spatial orchestration.

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## Introduction and Objectives

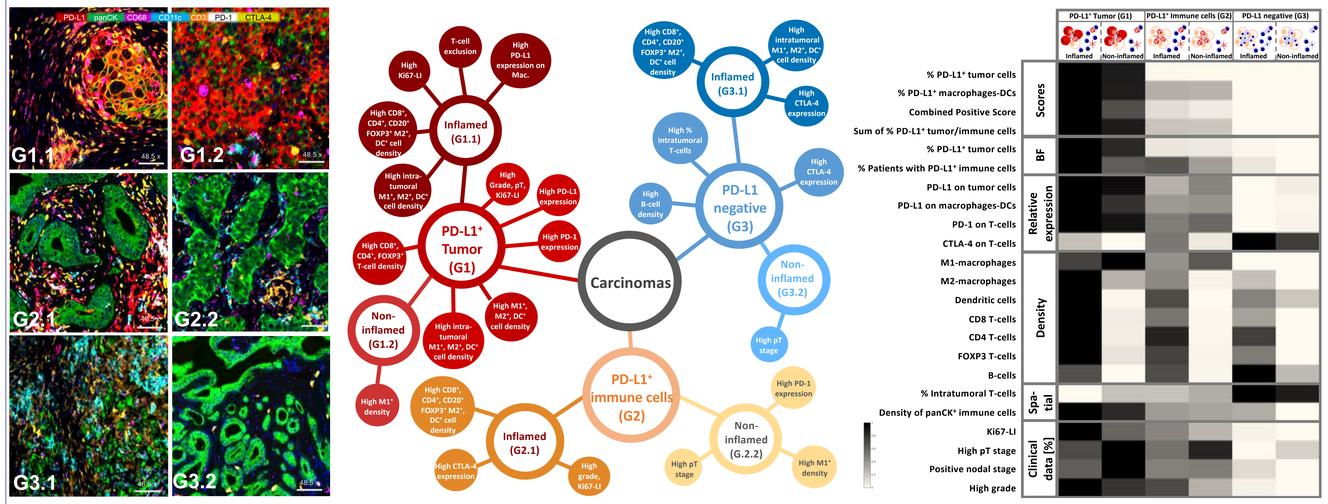
Highly multiplexed fluorescence immunohistochemistry (IHC) enables quantification of immune checkpoints such as PD-L1 (programmed cell death ligand 1), PD-1 (programmed cell death protein 1) or CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) in the tumor microenvironment (TME). However, a framework to assess the spatial orchestration of these markers and immune cells has yet to be established.

## Materials & Methods

To study the impact of PD-L1, PD-1 and CTLA-4 expression on the TME and patient's outcome, a framework for automated immune checkpoint quantification on tumor and immune cells was established and validated. Automated immune checkpoint quantification was facilitated by incorporating three different deep learning steps for the analysis of 44 different human carcinomas from 3098 tumor specimens using a bleach & stain 15-marker multiplex fluorescence IHC panel was used for this study (i.e., PD-L1, PD-1, CTLA-4, panCK, CD68, CD163, CD11c, iNOS, CD3, CD8, CD4, FOXP3, CD20, Ki67, CD31).

## RESULTS

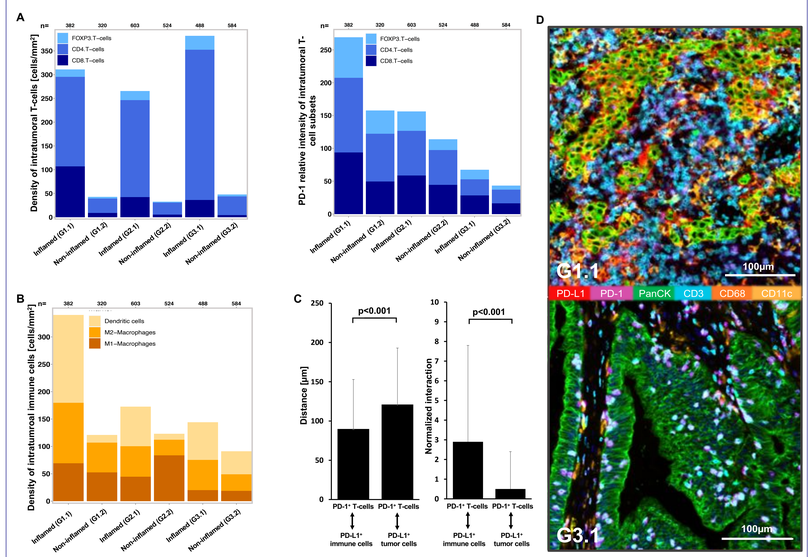
### Immune landscape of 3098 human carcinoma samples




Unsupervised clustering showed that a major proportion of the three PD-L1 phenotypes (i.e., PD-L1$^+$ tumor and immune cells [G1], PD-L1$^+$ immune cells [G2], PD-L1 negative [G3]) were either inflamed (G1.1, G2.1, G3.1) or non-inflamed (G1.2, G2.2, G3.2).

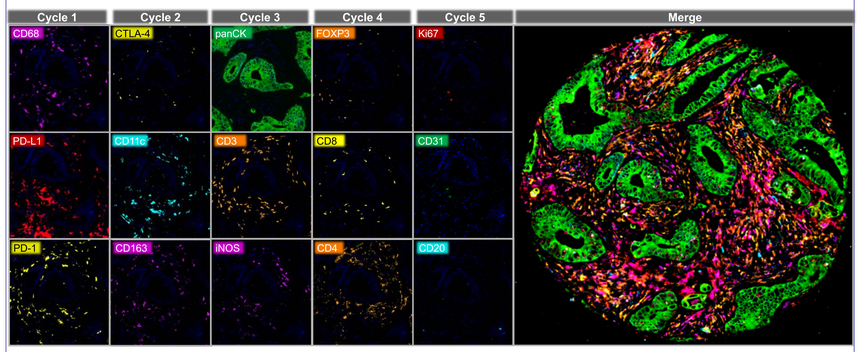
A high intratumoral macrophage density, a high degree of T-cell exclusion, a high PD-1 expression on T-cells, and a low CTLA-4 expression level on T-cells was significantly associated with the inflamed PD-L1 immune phenotype (p<0.001 each). A high M1 macrophage (CD68$^+$iNOS$^+$CD163$^-$) density was linked to the non-inflamed PD-L1$^+$ macrophage phenotype (p=0.048). All other macrophage and DC subsets (identified by CD68, CD163, CD11c, iNOS) as well as T-cell subsets (identified by CD3, CD8, CD4, FOXP3) were linked to the inflamed PD-L1 immune phenotypes (p<0.001 each).

### Spatial orchestration of immune cells across the PD-L1 immune phenotypes



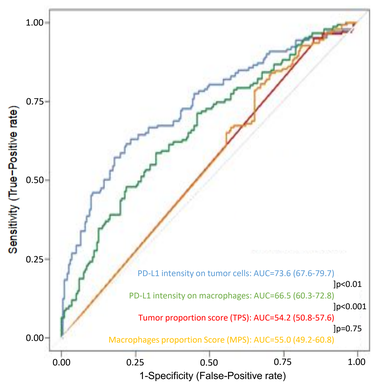

In the inflamed PD-L1$^+$ patients (G1.1), spatial analysis revealed that an elevated intratumoral CD68$^+$CD163$^+$ M2 macrophages as well as CD11c$^+$ dendritic cell infiltration (p<0.001 each) was associated with a high (CD3$^+$CD4$^\pm$CD8$^\pm$FOXP3$^\pm$) T-cell exclusion and a high PD-1 expression on T-cells (p<0.001 each).

### 15+1 BLEACH&STAIN




We developed and validated a novel BLEACH&STAIN multiplex fluorescence immunohistochemistry approach that enables high throughput 15 marker staining. Bleeding, crosstalk, or leftover staining was not detected across the 5 staining cycles.

### Higher overall survival rate for PD-L1 relative expression (intensity) on tumor cells




In breast cancer, the PD-L1 intensity on tumor cells showed a higher predictive performance for overall survival with an area under receiver operating curves (AUC) of 0.72 (p<0.0001) than the percentage of PD-L1$^+$ tumor cells (AUC: 0.54)

## Conclusions

- The highly multiplexed fluorescence IHC **BLEACH&STAIN** framework for automated PD-L1, PD-1 and CTLA-4 assessment revealed that **PD-L1 positive tumor samples** can be also associated with a **non-inflamed TME and high spatial T-cell exclusion**.

- 6 major PD-L1 phenotypes were identified ranging from:
  - an inflamed PD-L1$^+$ tumor cell phenotype with a **spatial T-cell exclusion** (G1.1)
  - to a non-inflamed PD-L1$^+$ immune cell phenotype showing a **particular poor prognosis** (G2.2)
  - to a non-inflamed PD-L1$^-$ negative phenotype (G3.2).

CD68 PD-L1 PD-1 CTLA-4 CD11c CD163 panCK CD3 iNOS FOXP3 CD8 CD4 Ki67 CD31 CD20

Conflicts of interest: The PD-L1, CTLA-4, panCK, CD4, Ki67, CD31, and CD20 antibody clones were provided by MS Validated Antibodies GmbH (owned by a family member of GS)

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