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In situ detection of various activated immune checkpoints via next-generation proximity ligation assays in tumor tissue

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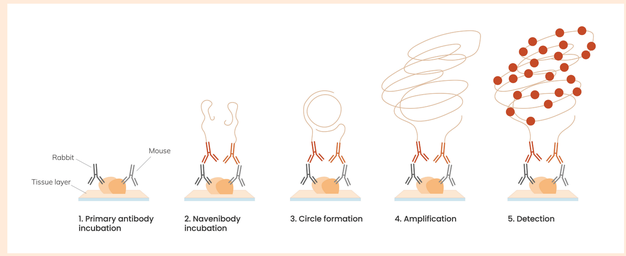
Introduction

Immune checkpoints (ICs), such as the PD1/PD-L1 axis, are inhibitory signaling pathways that down-regulate the immune responses of T cells. They play a crucial role in maintaining immune self-tolerance in peripheral tissues. In addition to antigen-presenting and other immune cells, many tumors express PD-L1, which allows them to evade the immune system. Thus, IC inhibition has revolutionized the field of cancer therapy. However, its effectiveness has been limited to a poorly defined subset of patients. High levels of PD1/PD-L1 interaction and other hallmarks of pathway activation, such as PD1 phosphorylation and recruitment of SHP-2, may be better predictors of patient outcomes than the overexpression of one IC protein alone. Therefore, a better understanding of the complex interactions between IC proteins and the immune system is needed to improve cancer therapy outcomes.

Technology and methods

Optimized *in situ* proximity ligation methods were developed for the NaveniBright™ kit (brightfield readout) and the NaveniFlex™ Tissue kit (fluorescent readout). To visualize the target interactions, FFPE tissues (Biomax) were incubated with pairs of monoclonal antibodies specific to the target of interest, followed by incubation with Navenibodies (affinity reagents conjugated to proprietary oligo arms). Strong and distinct signal is generated, provided that the Navenibodies are in close enough proximity to generate a rolling circle amplification reaction. This signal can be detected using chromogens or fluorescence and assessed by bright-field or fluorescence microscopy, respectively. For co-staining with additional markers in brightfield, an HRP-labeled antibody targeting cytokeratin was combined with Naveni™ PD1/PD-L1 AP. For fluorescent co-staining, different fluorescently labeled antibodies (such as pan-cytokeratin, CD8a, CD20, and CD3d) were added during the detection step, and the signal was assessed by epifluorescence microscopy.



Results

For a T cell to become activated, its T cell receptor (TCR) needs to be introduced to an antigen. However, priming leads not only to immune response induction, but also to the initiation of an inhibitory program facilitated by the binding of PD1 to its ligand PD-L1. The phosphorylation of the PD1 cytoplasmic tail is necessary for its inhibitory function, leading to the recruitment of the phosphatase SHP-2. We created an immuno-profiling line of ultrasensitive assays based on proximity ligation to improve the predictive value of tissue staining and visualize the tumor microenvironment:

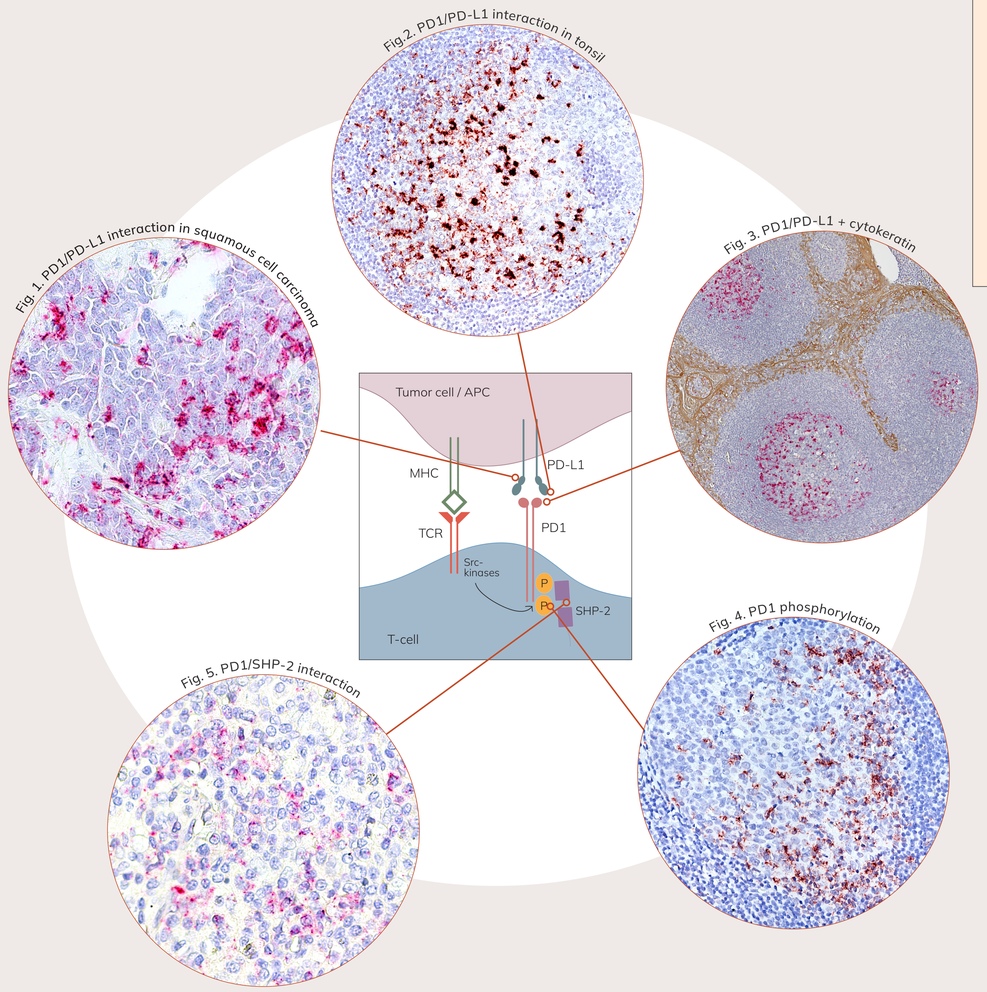
Fig. 1-2. PD1/PD-L1 interaction using Naveni™ PD1/PD-L1. Directly visualizes the interaction between PD1 and PD-L1 in squamous cell carcinoma (lung, AP readout, Fig. 1) or tonsil (HRP readout, Fig. 2).

Fig. 3. PD1/PD-L1 interaction + cytokeratin using Naveni™ PD1/PD-L1 AP and a cytokeratin-HRP co-stain in tonsil.

Fig. 4. PD1 phosphorylation using Naveni™ pY PD1 HRP in tonsil. Detects the first step in the PD1/PD-L1 inhibitory pathway activation.

Fig. 5. PD1/SHP-2 interaction, using the NaveniBright™ AP kit with primary antibodies against PD1 and SHP-2 in a lymph node (diffuse T cell lymphoma of right neck).

Fig. 6-7. PD1/PD-L1 immune profiling: we developed multiplex assays based on the NaveniFlex™ Tissue kit for detection of PD1/PD-L1 interactions, and co-stains of relevant biomarkers with fluorescent readout.



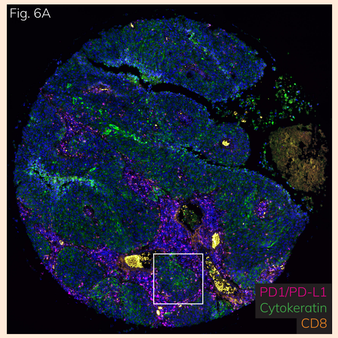


Fig. 6. PD1/PD-L1 interaction and co-staining against cytokeratin and CD8 in non-small-cell lung cancer TMA. Tumor cells are visualized by cytokeratin co-staining (green), along with abundant PD1/PD-L1 interactions (magenta), and infiltrating CD8 T cells (rusty red). (A) Tissue overview. White square depicts zoomed-in area. (B-D) Zoom-in showing different combinations of markers for easier visualization. Arrows indicate PD1/PD-L1 interaction between cytokeratin-positive and CD8-positive cells, suggesting that tumor cells are inducing T cell inhibition.

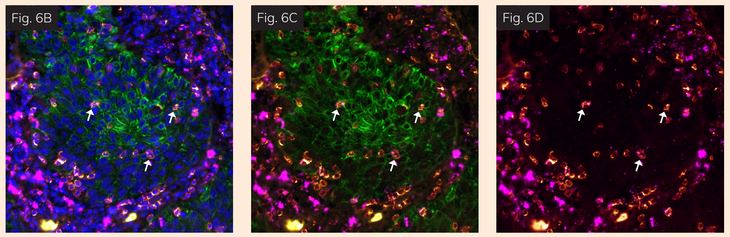
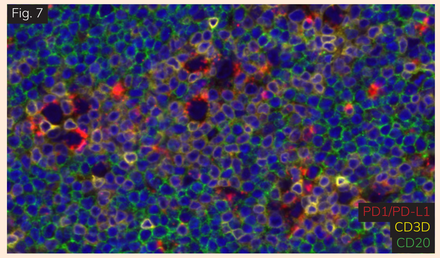


Fig. 7. PD1/PD-L1 interaction and co-staining against CD3 delta and CD20 in Hodgkin's lymphoma (mixed cellularity, harvest site lymph node, 10% cancer). Large multinucleated cells interact via PD1/PD-L1 (red) with T cells (CD3 delta, yellow). B cells positive for CD20 in green.



Conclusion

It is crucial to determine responders to IC inhibition prior to treatment, but current diagnostic methods, such as PD-L1 IHC, have limited accuracy in identifying patients who would benefit. To this end, we developed multiple kits and assays for immune profiling that allow the detection of key interactions in the PD1/PD-L1 immune checkpoint axis, along with various relevant biomarkers that visualize the tumor microenvironment. Visualizing activated ICs and tumor-infiltrating lymphocytes or tumor markers can help push the frontiers of cancer research forward and opens possibilities for deeper understanding of the mechanisms of IC inhibition therapy.

Have more questions?
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