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WHITE PAPER

Identifying PD-1/PD-L1 interactions

Implications for clinical research and ICI patient stratification

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Background

Immune checkpoints, which are inhibitory signaling pathways that can down-modulate the immune responses of T cells, are pivotal in peripheral tissues and for maintaining immune self-tolerance. The involvement of T cells as tools in cancer treatment has emerged as an attractive therapy option, as T cell specificity, memory, and adaptability to tumor heterogeneity makes the treatment broadly applicable to different types of cancer (1). Among the many molecularly defined checkpoint proteins (2,3), two of the most studied targets are programmed cell death 1 (PD-1) receptor, also known as CD279 (cluster of differentiation 279), and its ligand PD-L1, also known as B7-H1 or CD274 (4–7), which are the main focus of this paper.

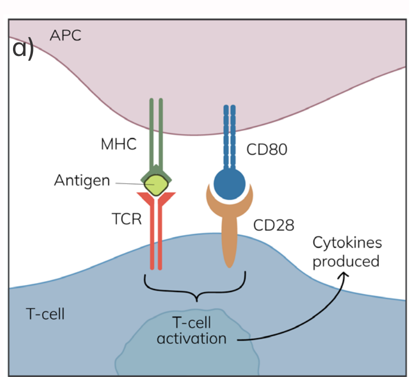
For a T cell to become activated, its T cell receptor (TCR) needs to be introduced to an antigen. Yet, priming is not complete until the CD28 co-receptor on the T cell binds to CD80 or CD86 on the antigen-presenting cell (APC) (8) (Fig. 1a). However, priming of T cells leads not only to immune response induction, but also to the initiation of an inhibitory program that, in time, can deter that same response to prevent autoimmunity. Inhibition starts with the upregulation of CTLA-4 on the surface of the T cell. CTLA-4 can outcompete the TCR co-activator CD28, generating a negative regulatory effect (9). A subsequent inhibitory signal is then initiated by the *trans*-binding of PD-1 on the T cell to its ligand PD-L1. The inhibitory function of PD-1 depends on the phosphorylation of its cytoplasmic tail, leading to the recruitment of the phosphatase SHP-2 (Fig. 1c). SHP-2 dephosphorylates its downstream targets and downregulates other signaling pathways (10,11), eventually promoting anergy, exhaustion or apoptosis in antigen-specific T cells (4,12).

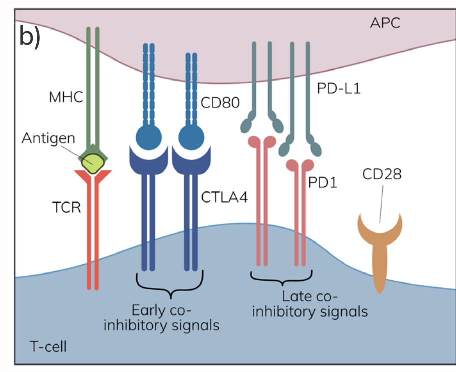
Immune response can be further fine-tuned by the recently described crosstalk between the CTLA-4/CD80 and PD-1/PD-L1 inhibitory pathways through an interaction between PD-L1 and CD80 in *cis* (13,14). As a result, both the CTLA-4 and the PD-1 axes are inhibited, while CD28 co-stimulation remains active (15).

Notably, in addition to APCs and other immune cells, many tumors also express PD-L1 on their surface, which facilitates tumor evasion from the immune system (16,17). Therefore, to promote sustained T cell response and pro-inflammatory cytokine production, and to keep tumors “visible” to the immune system, immune checkpoint proteins, including PD-1 and PD-L1, are considered suitable targets for blocking with monoclonal antibodies (Table 1).

Factors affecting immune checkpoint inhibition and clinical outcomes: progress and challenges

To date, immune checkpoint inhibitors (ICIs) have significantly increased the survival rates for patients with cancers such as metastatic melanoma (19,20), non-small-cell lung cancer (NSCLC) (21,22), and renal cell carcinoma (23).





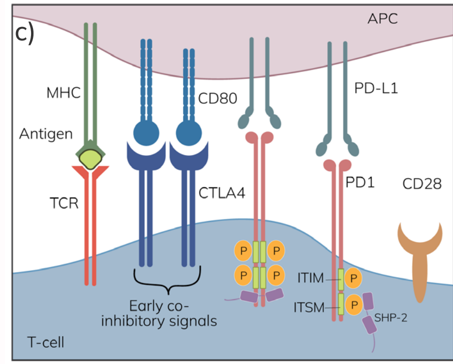


Fig. 1. T cell activation.

a) An APC presents a peptide to the T-cell via its MHC glycoprotein, and the T cell recognizes it with the help of the TCR. Co-receptor CD28 aids priming by interacting with CD80. In time, this will promote the production of cytokines by cytotoxic T cells.

b) Early inhibition begins by CTLA-4 outcompeting CD28 for the binding of CD80. PD-1 is subsequently expressed and binds to its ligand PD-L1, starting the late co-inhibitory program.

c) The PD-1/PD-L1 axis is activated when the cytoplasmic tail of PD-1 becomes phosphorylated, which recruits SHP-2 and results in downstream inhibition. its MHC glycoprotein, and the T cell recognizes it with the help of the TCR. Co-receptor CD28 aids priming by interacting with CD80. In time, this will promote the production of cytokines by cytotoxic T cells.

Table 1. Current FDA-approved immune checkpoint inhibitors (ICIs). Modified from (18).

Agent (US brand name)	Target IC	Approved conditions
Ipilimumab (Yervoy)	CTLA-4	Melanoma, MSI-H/dMMR colorectal cancer, renal cell carcinoma (in combination with nivolumab)
Nivolumab (Opdivo)	PD-1	MSI-H or dMMR colorectal cancer, head and neck squamous cell carcinomas, hepatocellular carcinoma, melanoma, Classic Hodgkin lymphoma, non-small-cell lung carcinoma, renal cell carcinoma, urothelial cancer, small-cell lung carcinoma
Pembrolizumab (Keytruda)	PD-1	Cervical cancer, gastric cancer, head and neck squamous cell carcinomas, hepatocellular carcinoma, Classic Hodgkin lymphoma, melanoma, Merkel cell carcinoma, MSI-H or dMMR colorectal cancer, non-small-cell lung carcinoma, diffuse large B-cell lymphoma, urothelial cancer
Cemiplimab (Libtayo)	PD-1	Cutaneous squamous cell carcinoma
Atezolizumab (Tecentriq)	PD-L1	Non-small-cell lung carcinoma, urothelial cancer
Avelumab (Bavencio)	PD-L1	Merkel cell carcinoma, urothelial cancer
Durvalumab (Imfinzi)	PD-L1	Non-small-cell lung carcinoma, urothelial cancer

Although a subset of patients receiving PD-1/PD-L1 inhibitors has an impressive response to the treatment, for many, the outcomes are less positive, potentially including significant adverse effects (24). In fact, less than half of those treated with ICIs benefit from disease stability or improvement (25).

Ultimately, the reason immunotherapy does not succeed is the failure to prime CD4 and CD8 T-cell responses against the tumor (26). This can be caused various factors including lack of tumor-specific antigens (particularly in tumors with a low tumor mutational burden (TMB)), insufficient antigen presentation, suppressive signals coming from the tumor microenvironment (TME), CD8 T-cell exhaustion, and insufficient colocalization of T cells and APCs (26–28). In addition, it has recently been shown that a subset of cancer cells can express both PD-1 and PD-L1, with similar pathway activation in the tumor as observed in immune cells. Thus, treatment of such patients with ICIs would produce paradoxical effects (29). Approaches to circumvent or overcome these obstacles include the use of bispecific antibodies (bsAbs) to aid immunotherapy, and the discovery and usage of relevant biomarkers to help define patients that will respond to treatment.

Bispecific antibodies give new hopes for treatment, emphasizing the importance of interactions

BsAbs are artificially designed antibody-based molecules that have two target-binding sites, each recognizing a different epitope or antigen (30). They are categorized as cell-bridging bsAbs, which connect immune and malignant cells, and antigen-crosslinking bsAbs that are similar to monoclonal antibody ICIs but bind two epitopes instead of one. The antigens to be crosslinked can be single targets such as HER2/HER2 or two different proteins e.g., PD-1/PD-L1, PD-1/CTLA-4, PD-L1/CTLA-4 (31). Multiple bsAbs are currently in trial (albeit almost all are still in phase I or II), in the hope that they will outperform monoclonal antibodies that have shown poor efficiency in cold tumors and have elicited resistance after treatment (31). Currently, two bsAbs, KN046 (against PD-L1/CTLA-4) and tebotelimab (against PD-1/LAG-3), are in phase III trials for solid tumor treatment (32,33).

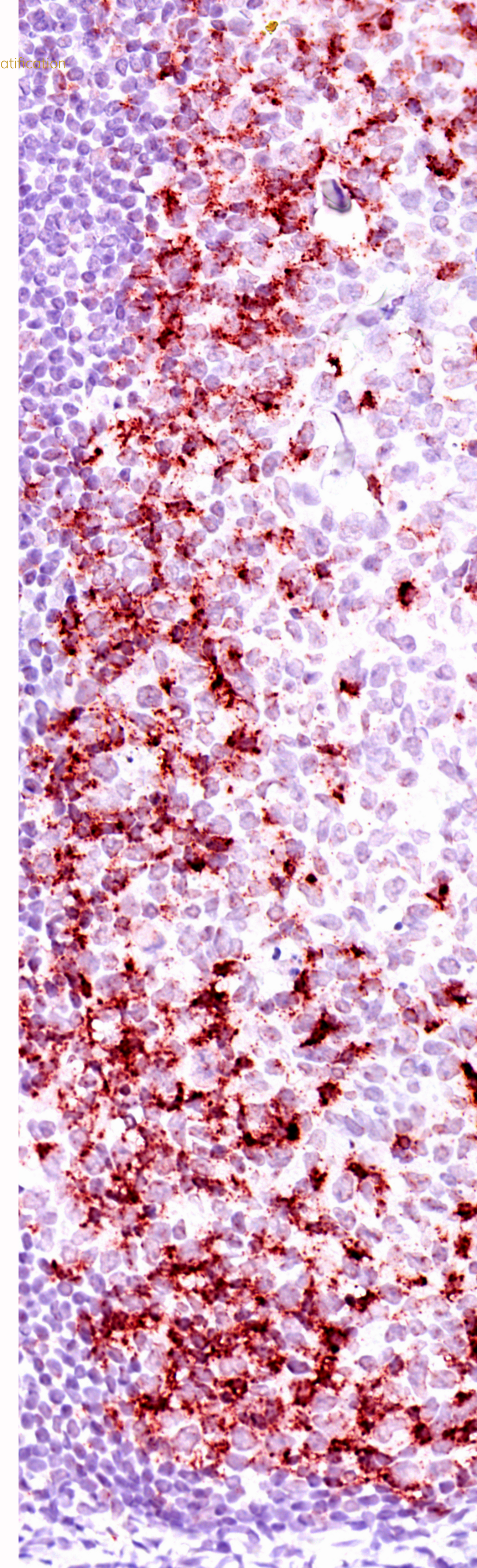
Understanding the complex effects of the TME

With respect to biomarkers, the TME plays a pivotal role in how effective immune checkpoint therapy will be. In responders, tumors present an abundance of neoantigens, have a high load of tumor-infiltrating lymphocytes (TILs), and increased cytokine secretion, among others. In contrast, in non-responders the levels of immunosuppressive cells are high, and the levels of NK cells and activated lymphocytes are very low (34). In addition, determining TMB along with PD-L1 expression may have predictive value for identifying responders. According to the CheckMate 227 trial, a TMB of ≥10 mutations per megabase combined with PD-L1 expression appear to indicate longer progression-free survival in some tumors, such as NSCLC, in response to treatment with ipilimumab and nivolumab (35). Notably, in contrast to these results, a meta-analysis of ten studies with ICIs used for patients with advanced urothelial carcinoma indicated that PD-L1 expression was associated with objective response rates but not overall survival, suggesting that further investigation is needed to identify patients that will benefit from ICI therapy (36).

Current approaches to assessing patient responses to ICI therapy

Despite indications that the expression levels of a single checkpoint are insufficiently informative, and that PD-L1 abundance alone is a poor guideline of responsiveness, the leading diagnostic tool currently used to identify patients eligible for PD-1/PD-LI inhibition is still PD-L1 immunostaining (34). When combined with an estimation of TILs, it allows patient tissues to be classified into four groups: type I (PD-L1-positive with TILs indicative of adaptive immune resistance), type II (PD-L1-negative with no TILs, indicating immune ignorance), type III (PD-L1-positive with no TILs, indicating intrinsic induction), and type IV (PD-L1-negative with TILs, indicating the role of other suppressor(s) in promoting immune tolerance) (37,38). There are several different staining options available, with varying predictive value due to the fact that PD-L1 evaluation by immunohistochemistry (IHC) is not sufficiently standardized. On the one hand, different criteria, as well as a variety of antibodies and tumor types are being used, and on the other, there is an intrinsic heterogeneity in the expression of PD-L1 across different tumors (39). The commercially available antibodies produce staining that varies considerably in intensity and patterns between different products (40).

Further variability in results can also be introduced by



the lack of standardization concerning how samples are handled and processed. The KEYNOTE 010 and other trials confirmed that archival tissue (as opposed to fresh) can be used for PD-L1 staining. Yet, the lack of unification between platforms may be an additional reason PD-L1 positivity has had such widely varying clinical significance (40–42).

The Blueprint PD-L1 IHC Assay Comparison Project, an industrial-academic collaboration, aimed to compare the analytical and clinical performance of four primary PD-L1 companion or complementary assays used in clinical trials, with the goal of establishing a cross-platform standard for PD-L1 positivity (43). Three expert pathologists reviewed 39 NSCLC formalin-fixed paraffin-embedded (FFPE) tissue samples using the FDA and EMA-approved assays outlined in Table 2. The analysis of tumor cell staining demonstrated that Ventana's SP263 and the two Dako assays produced comparable results, whereas SP142 tended to stain fewer cells. Concerning the immune cell stain, there was higher interobserver variability across all assays (40,43). Notably, 37% of the evaluated samples showed discordance between clinical levels of PD-L1 when the assay-specific cut-off scores were applied, indicating that the classification of these patients as PD-L1-positive would depend on the assay used (43). These observations emphasize the fact that the choice of an assay and, to some degree, the user's individual interpretation can lead to bias towards false positives or false negatives.

The importance of interaction detection

Clinical studies report conflicting data about the association between high PD-L1 expression in tumor tissue, be it on the surface of immune or cancer cells, and clinical outcomes. PD-1 expression might be better at predicting overall survival, but high PD-L1 positivity alone is not a clear indicator of whether PD-1 will also be highly expressed or not, and PD-1/PD-L1 pathway activation in the absence of immune cells speaks for a negative prognosis (29,46–48). To a degree, the inconsistencies in the observed role of PD-L1 could be explained by the fact that PD-L1 positivity in patient samples is not directly correlated to either interaction with PD-1, or pathway activation (i.e., phosphorylation of PD-1 and effector proteins). For example, one study reported that multiple patients who tested negative for PD-L1 expression with an FDA-approved IHC assay scored high for PD-1/PD-L1 interaction. Conversely, one patient sample with a positive PD-L1 status showed a minimal number of interactions (49). PD-L1-negative tumors can still respond to ICI, as PD-L1 expression is inducible upon activation of the interferon pathway. At the same time, regardless of PD-L1 status, tumors with low infiltration of TILs are unlikely to

Table 2. US Food and Drug Administration (FDA) and European Medical Agency (EMA) approved PD-L1 assays and example cutoffs relevant for non-small-cell lung cancer (modified from (43–45)).
TC = Tumor cell score, TPS = tumor proportion score, IC = immune cell score, 1L = first line, 2L = second line, NSCLC = non-small-cell lung cancer.

Therapeutic agent (Developer)	Antibody clone used in companion/complementary assay	Interpretative scoring	IHC assay positivity (cut-off for NSCLC)	Instrument and detection system required
Nivolumab ± Ipilimumab (Bristol-Myers Squibb)	28-8 (Dako)	Tumor cell membrane	1L TC≥1%	EnVision Flex on AutostainerLink 48
Pembrolizumab (Merck)	22C3 (Dako)	Tumor cell membrane	1L TPS≥1%	EnVision Flex on AutostainerLink 48
Atezolizumab (Roche or Genentech)	SP142 (Ventana)	Tumor cell membrane Infiltrating immune cells	1L TC≥50% or IC≥10%	OptiView detection and amplification on Benchmark ULTRA
Durvalumab (AstraZeneca)	SP263 (Ventana)	Tumor cell membrane	2L TPS≥1%	OptiView detection on Benchmark ULTRA

respond to treatment (50). In line with this, when Sánchez-Magraner et al. then assessed patients with known disease outcomes, they did not observe a correlation between PD-L1 status and prognosis. Yet, they found that a low number of PD-1/PD-L1 interactions was indicative of worse overall and progression-free survival, making interaction a suitable criterion for treatment with ICI (49), and possibly with bsAbs.

In light of this, it would be useful to not only use a combination of biomarkers for predicting response and prognosis, but also to evaluate the activation status of the PD-1/PD-L1 axis (as well as other immune checkpoint pathways). A further benefit would be the addition of a stain for either immune, or tumor cells (or both), which would elucidate whether the detected interaction occurs between two immune cells, between an immune and a tumor cell, or between tumor cells. An assay that quantifies PD-1/PD-L1 interaction, for example, could therefore be of great interest to basic research, but also to the clinic for patient stratification and the approval of new companion diagnostic assays, as well as for personalized medicine, development of bsAbs and to decrease treatment-associated adverse effects. However, studies based on PD-1/PD-L1 interaction and pathway activation are almost entirely lacking. The unavailability of straightforward, sensitive and robust methods that detect interactions without disrupting tissue morphology is the most likely culprit for this.

Navinci Diagnostics' kits detect protein interactions and post-translational modifications of key players in ICI inhibition

Considering the indicated value of detecting PD-1/PD-L1 axis activation for potential patient selection and/or treatment, Navinci Diagnostics now provides a comprehensive package of products that could become an alternative to the FDA and EMA-approved PD-L1 IHC assays.

Naveni PD1/PD-L1 is a tool for direct visualization of the interaction between PD-1 and PD-L1 that has been verified in various FFPE tumor tissue sections. The proprietary Naveni technology relies on the use of a carefully selected set of Navenibodies with optimized concentrations and performance (Fig. 2). Dual target recognition is advantageous over traditional IHC, as it reduces the effects of antibody cross-reactivity and in this way improves selectivity. Proximity ligation is facilitated solely when target proteins are positioned close to one another, ensuring sensitive detection exclusively of interacting proteins. Furthermore, the signal is augmented via rolling circle amplification (RCA), which results in strong staining even when targets are of low abundance and interactions are few. This makes the assay highly sensitive. Visualization is conducted on a regular brightfield microscope, and no additional instruments are needed.

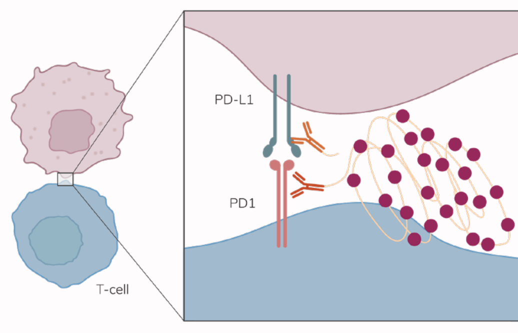


Fig. 2. The Naveni PD1/PD-L1 assay detects the interaction between two cells expressing the receptor and its ligand.

The Navenibodies bind to their target proteins. If they are in close proximity, this allows the formation of a DNA circle amplifiable by RCA. Individual proteins are thus not able to produce signal. Detection is chromogenic and could either be HRP or AP-based, which allows flexibility in case co-staining is used. The result is visualized with standard brightfield microscopy.

Table 3 shows FFPE tissue samples from different patients with a type of NSCLC evaluated by a pathologist for the expression of relevant biomarkers. All tissues were stained with two of the approved IHC assays (SP142 and SP263, Ventana) and given a respective TC grade. Consecutive sections from the same samples were then stained with the Naveni PD1/PD-L1 kit. Notably, samples No. 101-102 show high expression of PD-L1 (based on both TC scores) but are CD8-negative, suggesting the absence of cytotoxic T cells from the tumor. Nonetheless, the high expression of CD3 is evidence for the presence of other types of lymphocytes that may express PD-1. This could explain the strong and ubiquitous interaction staining observed throughout the tissue with the Naveni kit and may be an indication that these patients would be responders to ICI therapy.

Table 3. FFPE tissue sections from 8 NSCLC patient samples were evaluated by a pathologist for various biomarkers and stained either with the standard IHC protocols for PD-L1, or with the Naveni PD1/PD-L1 kit.

No	Anatomic Site/ Pathology diagnosis	Grade Stage Type	PD-L1 (SP142)	PD-L1 (SP263)	CD70	HLA1	MPO	CD3	CD8	Naveni PD1/PD-L1 AP stain
102	Lung/Squamous cell lung carcinoma	3 IIB Malignant	TC≥ 50%	TC≥25%	+++	+++	-	+++	-	
101	Lung/Squamous cell lung carcinoma	3 IIB Malignant	TC≥ 50%	TC≥25%	+++	+++	-	+++	-	
100	Lung/Squamous cell lung carcinoma	3 IIA Malignant	TC≥25%	-	+++	-	++	-	-	
5	Lung/Squamous cell lung carcinoma	1 IB Malignant	-	-	++	+++	-	++	-	
43	Lung/Squamous cell lung carcinoma	2 IB Malignant	TC < 50% and IC< 10%	TC≥25%	+++	+++	-	+++	+	
64	Lung/Squamous cell lung carcinoma	2 IIA Malignant	TC < 50% and IC< 10%	-	+++	+++	-	+++	-	
109	Lung/Squamous cell lung carcinoma	3 IB Malignant	TC<50% and IC< 10%	TC≥25%	+++	+++	-	+++	++	
110	Lung/Squamous cell lung carcinoma	3 IB Malignant	TC<50% and IC< 10%	TC≥25%	+++	+++	-	+++	++	

(acquired from US Biomax, https://www.biomax.us/tissue-arrays/Lung/LC1461)
LC1461_lung_EH33-SP142_PD3-2-36

Even more interesting is sample No. 100, which is negative for lymphocyte markers (CD3-, CD8-) and antigen presentation (HLA1-) and has a moderate TC score with Ventana SP142 staining indicating some – but not high – PD-L1 expression. While sparse, the sensitive Naveni PD1/PD-L1 assay detects interactions in the tumor, suggesting the presence of both PD-1 and PD-L1 in the TME, but not on the surface of T cells. At the same time, the tissue appears to be infiltrated by neutrophils as evidenced by the relatively high expression of MPO. While neutrophils in the TME can be conditionally associated with either a favorable or an unfavorable phenotype, PD-L1+ neutrophils are known to have tumor-promoting properties as they can suppress cytotoxic T cells (51). Considering this patient's high-grade tumor and the absence of cytotoxic cells, it is possible that the infiltrating neutrophils are expressing PD-L1. Alternatively, both PD-L1 and PD-1 may be expressed on the tumor cells (29). The detection of PD-1/PD-L1 interactions and putative pathway activation in cancer cells might indicate a patient who would not benefit from ICI treatment (29), but further studies are necessary in order to make a confident claim.

Furthermore, patient No. 109 is PD-L1-positive and presents with high immune cell infiltration, but also high expression of CD70, which is an unfavorable marker associated with T cell exhaustion (52). There are virtually no PD-1/PD-L1 interactions detected in the tumor. In contrast, patient No. 110 presents with identical biomarker expression, but a higher activation in the PD-1/PD-L1 pathway as detected by the Naveni technology. These results suggest that a high expression of PD-L1 as indicated by IHC could be but is not necessarily evidence for the activation of the inhibitory pathway. In addition, these observations may indicate different therapeutic potential for ICI treatment in these two patients. Clinical studies that include detection of PD-1/PD-L1 interactions along with standard biomarker and IHC stains, as well as follow-up on patient survival and prognosis would be extremely valuable and have the potential to uncover more precise approaches for patient stratification.

As two alternatives, or to complement the Naveni PD1/PD-L1 kit, Naveni pY PD1 and Naveni PD1/SHP-2 sensitively detect different steps in the PD-1/PD-L1 inhibitory pathway activation in FFPE tissues. Like all Naveni-assays, Naveni pY PD1 uses dual target recognition to identify tyrosine phosphorylation of PD-1, which is the first step of PD-1-mediated inhibition (Fig. 3a and c). In comparison to traditional IHC (Fig. 3b), the assay produces a much clearer signal over background. To study the subsequent recruitment of SHP-2 to the activated PD-1 receptor,

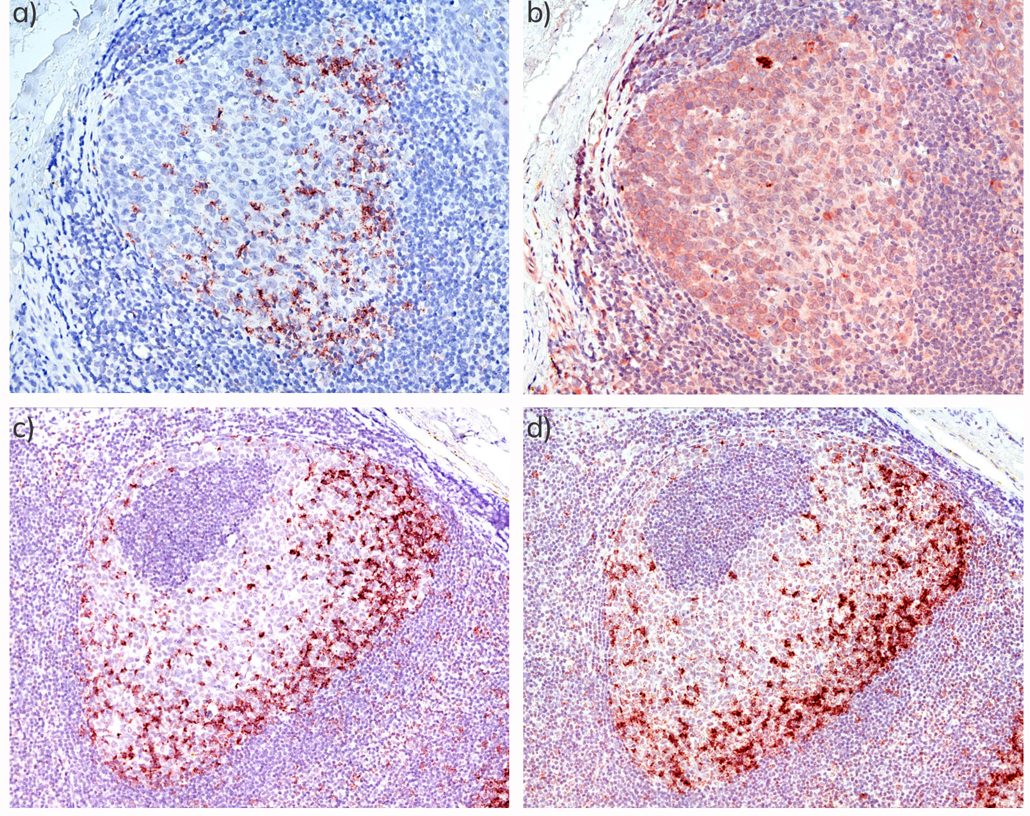


Fig. 3. Naveni pY PD1 HRP and Naveni PD1/SHP-2 HRP performance.

Naveni pY PD1 (a) sensitively and specifically detects phosphorylated Tyr residues on PD1 in the germinal center of a human tonsil. In comparison, pY PD1 IHC staining (b) gives considerably higher background.

Consecutive FFPE sections stained with Naveni pY PD1 (c) and PD1/SHP-2 (d) consistently demonstrate the activation of the PD1/PD-L1 inhibitory pathway in the germinal center.

which is crucial in immune cells but dispensable when the pathway is activated between two tumor cells (29), one can use the upcoming Naveni PD1/SHP-2 kit (Fig. 3d). The three kits can be used on consecutive tissue sections to obtain a comprehensive picture of the pathway activation (Fig. 3c and d), which may not always correspond to PD-1 or PD-L1 expression and localization.

Conclusions

PD-1/PD-L1 immune checkpoint blockade has revolutionized the field of cancer therapy, but its efficiency has been limited to a poorly defined subset of patients. This has led to the approval of ICI companion assays which rely on IHC detection of PD-L1 in tumor tissue with the hope of identifying responders to ICI therapy. However, these assays are not standardized, fail to consider important aspects of the complex TME, and have shown an unreliable correlation to patient response and survival. To improve the predictive value of PD-L1, it is likely that different or additional biomarkers should also be evaluated(53), creating standardized biomarker panels. Furthermore, patient outcomes have a stronger correlation to high levels of PD-1/PD-L1 interaction and other hallmarks of pathway activation such as PD-1 phosphorylation and recruitment of SHP-2, than to the expression of either PD-1 or PD-L1. Navinci Diagnostics has established three assays based on proximity ligation technology that allow the reliable and sensitive detection of these three biomarkers in tumor tissue. The assays can be applied in basic research to elucidate the interplay of immune checkpoint axes and downstream molecules, in pre-clinical and clinical research to compare stainings with the existing IHC assays and evaluate the potential prognostic value of interaction detection, and in pharma, aiding the development of new drugs or bsAbs.

Available Products:

- Naveni PD1/PD-L1
- Naveni pY PD1

Coming soon:

- Naveni PD1/SHP-2

In development:

- Naveni CTLA-4/CD80
- Naveni CTLA-4/CD86
- Naveni CD28/CD80
- Naveni CD28/CD86

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