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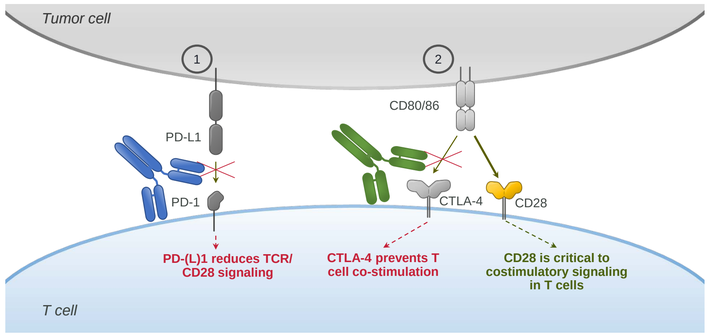
Development of Novel Monoclonal Antibodies for the Robust Detection of CD28, CD80, and CD86 by Immunohistochemistry in Human Tumors

Gary D. Means[1], Russell J. Sanderson[1], Brian Johnson[2], Megan Larmore[2], Mark Maurer[1], Stanford L. Peng[1]
[1]Alpine Immune Sciences, Seattle, WA; [2] University of Washington Histology and Imaging Core, Seattle, WA.

Abstract

- **Background**: The CD28-CD80/CD86 costimulatory pathway plays a critical role in T cell activation and is implicated in the prognosis of cancers and in their response to immunotherapies. Expression of CD28, CD80, and/or CD86 may therefore serve as clinically significant prognostic biomarkers. However, no validated reagents are currently available for the assessment of these targets by immunohistochemistry (IHC). The present effort was undertaken to identify and to develop robust IHC reagents for these targets, in part to enable a diagnostic assay for our novel immuno-oncology drug candidate, ALPN-202, a conditional CD28 co-stimulator and dual PD-L1/CTLA-4 checkpoint inhibitor currently in a phase 1 clinical trial.
- **Design**: Commercially available monoclonal antibodies (mAb) reported to detect CD28, CD80 or CD86 by IHC were evaluated in FFPE tonsil, thymus and cerebellum using multiple antigen retrieval conditions. Proprietary mouse and rabbit mAbs directed against CD28, CD80 and CD86- identified during hybridoma campaigns and selected via ELISA, western blot, and flow cytometry- were similarly assessed for suitability in IHC. Target reactive mAbs were further evaluated using a range of tumor sections and tumor microarrays (TMA)(628 individual samples). IHC results were compared to mRNA expression as assessed by PCR on duplicate slides.
- **Results**: Eight mAbs (two commercial, six proprietary) demonstrated IHC staining in thymus and tonsil with sufficient sensitivity and precision to advance to evaluation in tumor sections and microarrays. At least one proprietary mAb specific for each target, respectively, was identified and demonstrated robust staining across multiple tissues with excellent inter-reader precision.

Figure 1: Current checkpoint inhibitors work by augmenting T cell costimulation or TCR signaling, essentially disinhibiting PD-1/CTLA-4 functions.



1 PD-L1 engages PD-1 which suppresses T cells by antagonizing CD28 signaling, the most important of the T cell costimulatory molecules[1]

PD-L1 expression is an important biomarker positively associated with clinical response.[1]

1. Krummel & Allison, *J Exp Med* 1995;182:459-65.

2 CTLA-4 reduces T cell signaling by preventing CD80 / CD86 costimulation of CD28.

CPIs that block these two pathways reverse these inhibitions, but in the absence of sufficient CD28 ligands and/or CD28 receptor expression, costimulation may be insufficient to promote anti-tumor immunity.[2]

2. Kamphorst, A.O., et al. *Science* 2017;355:1423-1427.

Combining checkpoint inhibition of both PD-1 and CTLA-4 with costimulation of CD28 may provide improved anti-tumor immunity. Expression of CD80, CD86 and CD28 may be important to identification of responders to this strategy.

Table 1: Hybridoma campaigns in mouse and rabbit identified antibodies with specificities for CD28, CD86, CD80. Antibodies were required to be positive by ELISA in order to be advanced for additional testing. Antibodies that stained tonsil and were negative for staining in cerebellum were evaluated on normal and tumor tissue (data not shown) as well as TMAs. Each antibody was scored for appropriate membrane staining and were further characterized to identify clones with infrequent non-specific staining.

Hybridoma campaign	ELISA-positive antibodies advanced for IHC testing			Tonsilar structure positive; Cerebellum negative			Specific staining in TMAs		
Target	CD28	CD86	CD80	CD28	CD86	CD80	CD28	CD86	CD80
Mouse	20	24	16	3	1	1	1	0	1
Rabbit	nd	8	8	1	1	1	0	1	1

Figure 2: Anti-CD28, anti-CD86 and anti-CD80 IHC candidates were evaluated in cerebellum, thymus and tonsil. In tonsils, CD28 stained in the T cell rich regions surrounding germinal centers, while CD86 and CD80 stained predominantly in the germinal centers. Staining was observed for CD28 in thymic medulla. Punctate staining consistent with antigen presenting cells was observed for CD80 and CD86 in thymus.

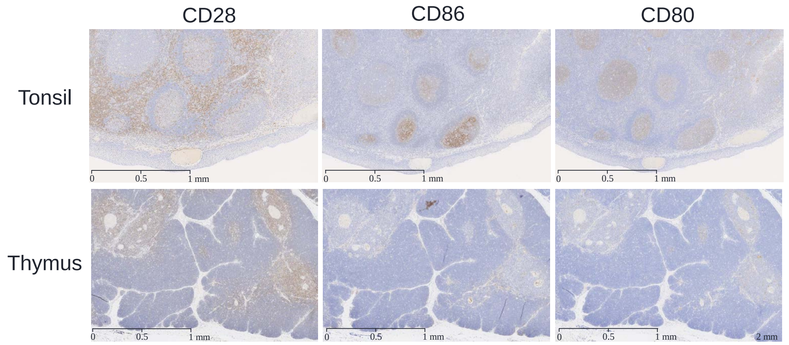
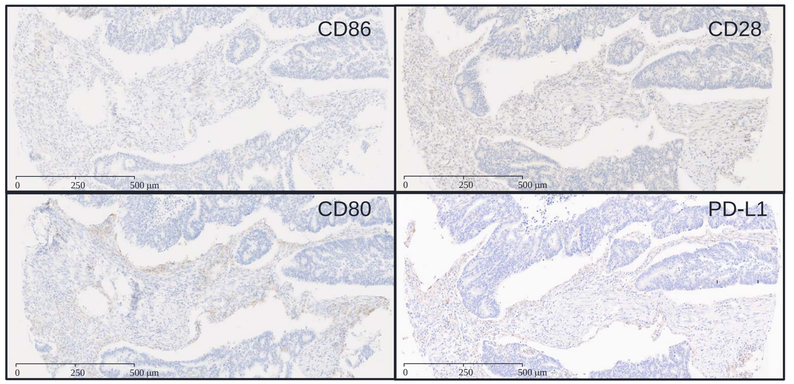


Figure 3. CD28, CD86, CD80 and PD-L1 staining of a representative colorectal carcinoma. CD28, CD86 and CD80 IHC was performed on a Leica Bond III using antigen retrieval conditions unique to each target. PD-L1 was assessed on a Dako LINK 48 platform using PharmDx 22C3.



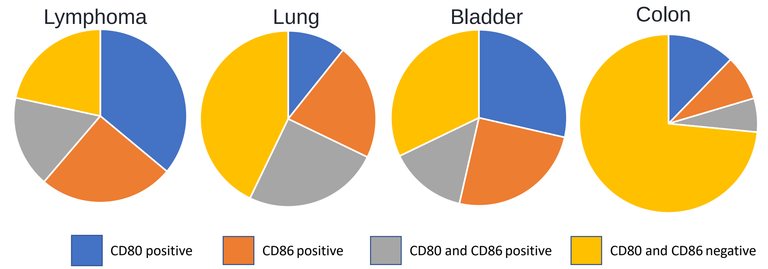
In solid tumors, CD28 expression was most often within tumor adjacent microenvironment (TME) or in tertiary lymphoid structures. While CD80 and CD86 were often found the TME, expression on immune cells such as macrophages within tumors was also observed. We observed rare membranous staining with CD80 and CD86 on tumor cells except for Lymphomas (where staining was common).

Table 2: Expression of PD-L1, CD28, CD80, and CD86 were evaluated on TMAs (US Biomax). Representative data from lung (HLugC120PT01) and lymphoma (LY2087a) are summarized below.

Marker	Lung (N= 59) % positive	Lymphoma (N= 204) % positive	Scoring Criteria
PD-L1	47%	59%	*TPS ≥ 1%
CD28	39%	31%	Positive cores were defined as those with a membrane staining of any intensity in ≥ 1% of tumor or immune cells
CD80 only	10%	27%	
CD86 only	20%	28%	
CD80 and CD86	27%	7%	

* TPS, Tumor Proportion Score, defined as the percentage of viable tumor cells showing partial or complete membrane staining at any intensity (PD-L1 IHC 22C3 pharmDx Interpretation Manual).

Figure 4. Pie charts summarizing the detection of CD80 and CD86 (or their co-expression) in TMAs derived from lymphoma, lung, bladder and colon in PD-L1 positive tumors



Conclusions:

- Successful development of proprietary monoclonal antibodies against CD28, CD80, and CD86 has been accomplished with promising performance characteristics for IHC and potentially companion diagnostic applications.
- Significant proportions of some tumors appear to exhibit reduced or no CD28, CD80 and/or CD86 staining, which may account for a lack of responsiveness to CPI-only therapies. Novel therapies providing costimulation of CD28 on tumor-resident and/or recruited T cells may be more effective than CPI alone.
- These reagents will likely be particularly useful in future studies to assess the prognostic relevance of these biomarkers in cancer and other diseases; and also may be incorporated in companion diagnostic strategies for patient stratification in trials of drugs involving checkpoint inhibition and/or T cell co-stimulation

Sponsor: Alpine Immune Sciences, Inc. @AlpineImmuneSci