# Immune Cell Expression of LAG3 and TIM3 In The Tumor Micro Environment Of PD-L1 Positive/Negative Bladder Cancer, Melanoma, And Lung Cancer.

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## Abstract

PD-L1/PD1 immune therapy has been very successful for some patients. Treatment is usually based on PD-L1 tumor expression levels in tumors; however, some patients with positive tumors have shown no response to therapy. Recent clinical trials have shown that treating patients with more than one immune therapy target results in better outcome suggesting the importance of understanding PD-L1 tumor micro environment. In this study, we look at a number of immune cell markers with PD-L1 expression to see if patterns of the immune cells infiltrating the tumor micro environment vary. PD-L1 expression in the tumors was assessed by multiple PD-L1 antibodies since no single PD-L1 antibody has been FDA approved for all tumor types. Immuno-histo chemistry (IHC) screen used 2 recombinant rabbit monoclonal antibodies (clone OR-5E3 and OR-5H8), one mouse anti PD-L1 clone UMAB229, and the FDA approved antibodies clones (SP142 and 28-8). Immune cell markers used in the IHC screen were CD3, CD8A, CD20, CD68, FOXP3, LAG3 and TIM3. The screen was done on sequential sections of bladder, melanoma, and lung tumors. Variation existed between five PD-L1 antibodies in their sensitivity and specificity to detect PD-L1 in the different tumor types; however, the differences were usually associated with the ability to detect low expressing tumors. Immune cell markers CD3, CD8A, CD20, CD68, FOXP3, LAG3, and TIM3 cell markers all produced strong staining if positive cells were present; however, they vary in number and distribution pattern throughout the three types of tumor. For example, when lung or bladder tumors presented a strong PD-L1 staining at the edge of the tumor they often had a number of CD3E or CD8A immune positive cells present, this was not the case for CD20 which were foci clusters or scattered through the tumors. FOXP3 and CD68 staining were scarce event in all three tumor types. Although this study was limited to small sample size, a 10 tumors each it did show differences in the amount and distribution of immune cells in the 3 different types of tumor positive for PD-L1 expression.

## Introduction

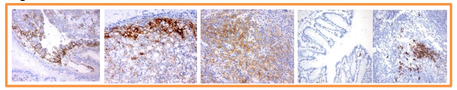
Targeted PD-L1 and PD-1 therapy successes in preventing the progression of melanoma has expanded to include treatment of non-small-cell lung cancer, bladder cancer, head and neck cancer, renal cell cancer, with clinical trials of other solid tumor on going as seen in Figure1. Clinical studies have shown that positive PD-L1 protein expression in these tumors are associated with higher response rates from targeted PD-L1 /PD-1 immunotherapy. However, tumor responses are not mediated by the antibody per se, but by tumor PD-L1 antigen interaction with specific T cells that had been previously blocked by the PD-1–PD-L1 interaction (1). Here we looked at the expression profile of immune cell markers CD3, CD8A, CD20, CD68, FOXP3, LAG3 and TIM3 with OriGene rabbit mono PD-L1 antibodies clone OR-5E3 and OR-5H8. Both PD-L1 antibody clone OR-5E3 and OR-5H8 have previously been shown to have similar staining as the FDA approved rabbit mono clonal anti-PDL1 clone SP142 and clone 28-8 for targeted immunotherapy drugs atezolizumab and OPDIVO® (nivolumab) respectively as shown in figure 2. NSCLC, Bladder Cancer, and Melanoma immune cells, as indicated by the CD3, CD8A, CD20, CD68 and FOXP3 staining, generated different distribution patterns in the three tumor types. The five PD-L1 antibodies showed variation in detection of both immune and tumor cells.

## Design & Methods

### Rabbit PD-L1 Monoclonal Antibody Development

Rabbit recombinant monoclonal antibody platform was developed using B cells from peripheral blood. Briefly, B cells were isolated from the whole blood of rabbits immunized with PD-L1 peptides. Immune response positive cells were selected after they were cultured for 7-10 days. Rabbit IgG variable light and heavy chain were PCR amplified and cloned into vectors. Positive clones were sequenced. Both light and heavy chain were co- transfected into 293 cells for antibody expression. More than 10 positive PD-L1 clones, were first screened by immunocytochemistry and then immunohistochemistry. Two clones (OR-5E3 and OR-5H3) for IHC were shown to work on both human and mouse tissues Figure 1.

**Figure 1 Positive PD-L1 OR-5H8 On Human & Mouse Tissues**



BCell Lymphoma HER2+ Breast Cancer Stomach Cancer / Ms Colon Ms Spleen

Fig. 1 Shows rabbit monoclonal PD-L1 clone OR-5H8 staining on both human and mouse tissues. The antibody can pick up strong and weak staining easily with in the same tissue.

**Table 1 Antibody Information and Dilution**

| Target | Clone | OriGene Cat # | Dilution |
|---|---|---|---|
| CD3e | UMAB54 | UM500048 | 1:200dil |
| CD8A | UMAB241 | UM800133 | 1:200dil |
| CD20 | UMAB37 | UM800001 | 1:200dil |
| CD68 | UMAB150 | UM800047 | 1:200dil |
| FOXP3 | UMAB248 | UM800140 | 1:200dil |
| LAG3 | OTI10E7 | TA807146 | 1:250dil |
| TIM3 | OTI5C8 | TA812325 | 1:250dil |
| PD-L1 | UMAB229 | UM800121 | 1:200dil |
| PD-L1 | OR-5H8 | TA591003 | 1:100dil |
| PD-L1 | OR-5E3 | TA591004 | 1:100dil |

Manual IHC staining of paraffin-embedded human and mouse tissues using anti PD-L1 rabbit mono antibodies clone SP142 [Spring Biosciences -Pleasanton, CA] , clone 28-8 [Abcam - Cambridge, MA], clone OR-5E3 and OR-5H8, [C/N TA591003 & TA591004] OriGene Technologies-Rockville MD]. The immune marker antibodies from OriGene of CD3e, CD8A, CD20, CD68, FOXP3, LAG3, and TIM3 are listed on the Table1 .

### Immunohistochemistry:

All antibodies required heat induced epitope retrieval HIER using OriGene-ACCEL Tris-EDTA buffer pH8.7 for clone OR-5H8 or OR-5E3; OriGene TEE pH9.0 for clone SP142; BioCare DIVA DeCloaker for clone 28-8 at 120C for 3 minutes in BioCare Decloaker chamber. PD-L1 clones SP142 and 28-8 were diluted 1:50; PD-L1 clones OR-5H8 & OR-5E3 were diluted 1:100 and incubated for 1hr at room temperature. OriGene Polink-1 a one step anti- rabbit polymer HRP detection (Cat# D13-100) was used except for clone 28-8 which used 2 step Rb Polymer Polink2Plus (D39) and DAB chromogen according to manufacture's protocol. The seven immune marker antibodies also required antigen retrieval.

### Immunohistochemistry Scoring:

For this study PD-L1 intensity of stain was not incorporated into the overall score 20% 1+ and a 20% 3+ was considered 20% positive tumor. Tissues were score under two assessments. First pass they were given an overall score of positive tumor cell and positive immune cells. Intensity of the stain was not evaluated. Second the same cases were compared to each other to see if the scores were correct and adjusted against each clone for that tissue. This was to insure that similar staining patterns received a similar score.

## Results

**Figure 2: PD-L1 on NSCLC, Bladder Cancer & Melanoma**

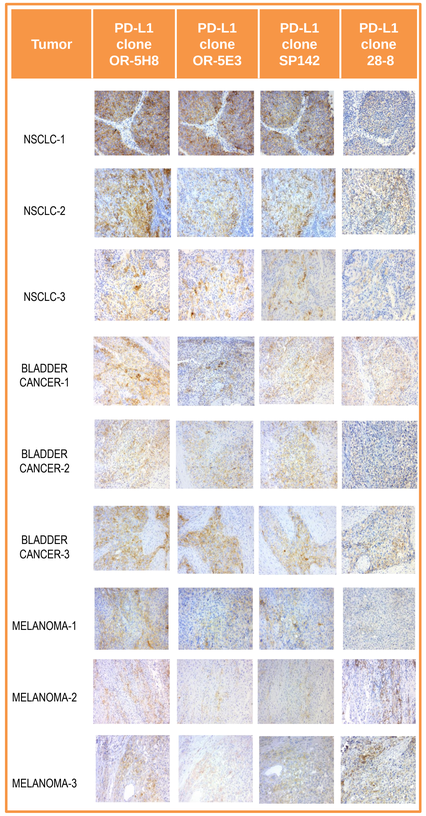



**Table 2: PD-L1 Antibodies IHC Results**

| PD-L1 Clone Results | TC > 1% | TC 1 > 50% | TC >50% |
|---|---|---|---|
| OR-5H8 NSCLC | 4/13 | 4/13 | 5/13 |
| OR-5E3 NSCLC | 3/13 | 6/13 | 3/13 |
| UMAB229 NSCLC | 5/13 | 4/13 | 4/13 |
| SP142 NSCLC | 4/13 | 8/13 | 3/13 |
| 28-8 NSCLC | 5/13 | 5/13 | 3/13 |
| OR-5H8 Bladder Cancer | 8/13 | 3/13 | 2/13 |
| OR-5E3 Bladder Cancer | 6/14 | 3/14 | 2/14 |
| UMAB229 Bladder Cancer | 6/14 | 5/14 | 3/14 |
| SP142 Bladder Cancer | 7/14 | 3/14 | 1/14 |
| 28-8 Bladder Cancer | 7/14 | 3/14 | 1/14 |
| OR-5H8 Melanoma | 0/10 | 9/10 | 1/10 |
| OR-5E3 Melanoma | 3/9 | 5/9 | 1/9 |
| UMAB229 Melanoma | 3/10 | 6/10 | 1/10 |
| SP142 Melanoma | 2/10 | 8/10 | 0/10 |
| 28-8 Melanoma | 4/10 | 6/10 | 0/10 |

**Fig-2** Show examples of tumors stained with new and FDA approved rabbit PD-L1 clones on NSCLC (lung), bladder cancer, and melanoma. Results show new rabbit clones stain stronger. Comparison was done using manual staining. PD-L1 clone 28-8 required 2-step polymer amplification one step resulted in weak or no staining.

**Fig-3** Shows various staining pattern of immune cell markers with PD-L1 expression in NSCLC (lung), bladder cancer, and melanoma.

**Figure 3: PD-L1, CD3E, CD8A, CD20, CD68, FOXP3, LAG3 and TIM3 IHC Stain on NSCLC, Bladder Cancer, & Melanoma**

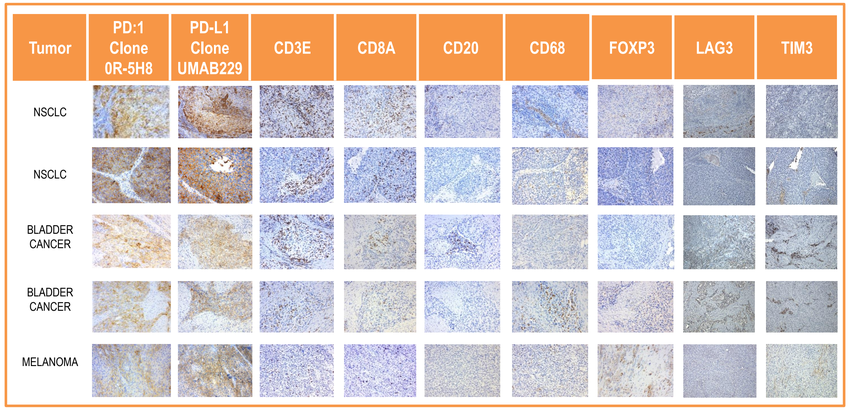
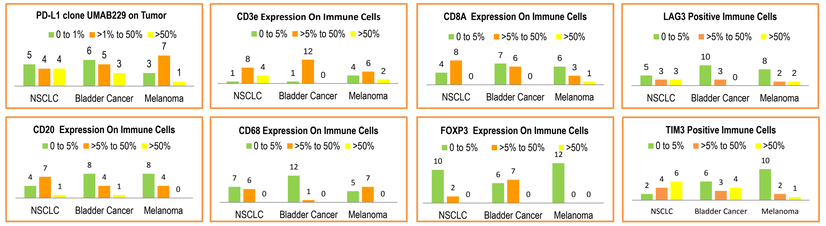



**Figure 4: PD-L1, CD3E, CD8A, CD20, CD68, FOXP3, LAG3, TIM3 IHC Summary on NSCLC, Bladder Cancer, & Melanoma**

(Score is based on immune cells present not stain on total tissue.)




## Conclusion

The rabbit monoclonal PD-L1 clones OR-5H8 and OR-5E3 antibodies stained human and mouse tissue and can be used for screening PD-L1 positive tumor and immune cells when compared to the FDA approved clones. For example the bladder cancer case 3, PD-L1 clone OR-5H8 stains 90% of the tumor but both the FDA approved antibodies show less positive PDL1 expression in this tumor. We observed the rabbit antibody clones OR-5H8 and OR-5E3 were easier to see low expression of PD-L1 positive tumor cells in all three cancer types used in this study.

PDL1 positive tumors the immune CD3, CD8A, CD20, CD68, FOXP3, LAG3 and TIM3 cell markers presented different expression levels and distribution pattern throughout the tumor. The overall expression pattern of these markers is complicated. CD3 and CD8 can be seen scattered throughout thePDL1 positive lung tumors however CD8 positive cells were mostly observed at the edge of bladder tumor but not scattered in the bladder tumor. CD20 rare to see in the PD-L1 tumor but rather foci with of several hundred positive cells would be seen in the some of the tumor sections. CD68 and FOXP3 were rare and seen scattered though out the tumor. In this sample we could not see enough events to conclude their role. LAG3 and TIM3 show strong staining on lung macrophages and weak signal on the tumor cells; the other immune markers evaluated did not show expression by the tumor cells. In bladder cancer we rarely observed TIM3 expression in the tumor (3of14) but LAG3 we observed 10 of 14 tumors to have at least low levels of expression. In melanoma we observed only 4 cases with immune positive LAG3 and/ or TIM3 cells and these same cases show weak positive staining in the tumor cells.