Flagship BIOSCIENCES

IMAGE ANALYSIS-BASED IDENTIFICATION AND QUANTIFICATION OF MACROPHAGES IN PD-L1 (SP263) STAINED NSCLC TISSUE SECTIONS WITHOUT ADDITIONAL MACROPHAGE-SPECIFIC STAINING

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3879

ABSTRACT

PD-L1 positivity in tumor-associated macrophages has been related to a favorable response to anti-PD-1 and PD-L1 targeted therapies. This concept has driven interest in specifically identifying macrophages in PD-L1 stained tissues, but pathologists often have difficulty in performing this task reliably. Thus, there is a clear need for tools capable of detecting and classifying tumor-associated macrophages in the context of a standardized PDL1 assay. One such method relies on the utilization of image analysis (IA) which measures hundreds of defining cellular features. This is necessary because macrophages identification based on few features would be difficult due to their varying morphologies and similarity with tumor cells. In order to isolate macrophages using multiple aspects of their cellular characteristics, AI and machine learning algorithms were leveraged to provide robust models for their identification. In this study we compare two artificial intelligence (AI) assisted macrophage classification methods for their accuracy in specifically identifying CD68 positive macrophages. Both approaches are based on interrogating a cohort of 23 non-small cell lung cancer (NSCLC) sections with two tumor-associated macrophage recognition algorithms, based on a decision tree (DT) model. These classifiers were developed by training a learning model on the morphometric, spatial, and chromogenic PD-L1 staining features measured on detected cells, using their fluorescent CD68 expression profile as an indicator of their macrophage lineage. These classification algorithms were trained on images developed using procedures wherein tissue sections were stained by immunofluorescence with antibody to the macrophage associated CD68 marker and subsequently scanned to obtain a digital image. After removal of the coverslip and stripping of the CD68 antibodies, the sections were then stained with the anti-PDL1 SP263 antibody and rescanned to obtain a second digital image. Co-registration of the two digital images allowed for the identification of CD68 positive cells in the context of only the SP263 PDL1 assay. The accuracy of each classification algorithm was determined by comparing the positivity for macrophage lineage among algorithm predicted macrophage to CD68 positivity. For the decision tree classifier, we demonstrated a > 90% accuracy classification of macrophage identification. Application of the SP263 model to NSCLC tissues stained with the 22C3 PD-L1 assay demonstrates the need for assay-specific detection models. The decision tree method achieved highly accurate predictions. These data indicate that sophisticated assay and data science methods allow for independent identification of macrophages using only a SP263 immunohistochemical PD-L1 assay, making it possible to isolate the contribution of PD-L1 macrophage positivity to successful checkpoint therapy response.

MATERIALS & METHODS

23 NSCLC tissue sections containing >12,000,000 quantifiable cells were first stained for CD68 by immunofluorescence and imaged. Once fluorescent imaging was complete, the coverslip was removed, the CD68 antibody was stripped, and the tissues were stained for PD-L1 using the SP263 in vitro diagnostic kit (Roche). PD-L1 staining in these samples was compared to prior PD-L1 stains in the same sections to confirm there was no loss of sensitivity due to the CD68 staining. Additional studies (not shown) confirmed no cross-reactivity between the CD68 and PD-L1 stains. After PD-L1 staining was complete, the slides were imaged on brightfield whole-slide scanners. The two tissue images generated (CD68 FL, PD-L1 BF) were overlain in Flagship Image Analysis software in order to co-localize staining between FL and brightfield images. Image analysis using Flagship's proprietary software generated per-cell data sets for each tissue, measuring hundreds of morphometric, staining, and spatial features on each cell. Cell measures were generated only on the brightfield PD-L1 image, while the CD68 status was retained for model training. An iterated random forest classifier was trained on blocks of total cell data (>12,000,000 cells) at intervals of 500,000 observations, separated into a training set (475,000 cells) and a test set (25,000 cells). The trained model was implemented in tissues outside of the training set. Test tissues were additionally separated in to tumor and stroma compartments to localize macrophage quantifications in to each tissue area.

RESULTS & CONCLUSIONS

- An average 96.83% accurate model for detecting and quantifying macrophages (CD68-positive cells) in NSCLC tissues stained for PD-L1 (SP263) was achieved, improving on a top manual accuracy of 25%.
- Per-cell data profiles achieved via proprietary image analysis can be combined with thoughtful biological approaches and assays to remove manual bias for training cell detection models.
- Models built using SP263 staining are non-transferrable to 22C3-stained tissues, meaning models must consider assay and indication when used in practice.
- Application of the macrophage detection model in PD-L1 stained tissues allows investigators to interrogate PD-L1 status of macrophages without the need for additional stains. Pathologist review of markup accuracy is challenging based on difficulty of manual evaluations
- Combinatorial assay design and data science approaches to image analysis modeling will be expanded to multiple assays and tissues in future studies

ESTABLISHING A CELL-BASED MORPHOLOGY AND STAINING FEATURE PROFILE TO PREDICT CD68 STATUS

While manual pathology scoring of tissue images is often referred to as the gold standard when it come to scoring tissue-based assays, recognition of challenging cellular presentation, such as macrophages in PD-L1 stained tissues, is very challenging to do reliably by eye alone. Figure 1 shows the challenge associated with recognizing CD68-positive cells in PD-L1 stained images by eye alone, with manual accuracy ranging anywhere from 1 to 25% accurate. The use of sophisticated image analysis, which measures hundreds of cellular features not discernable by eye, can be paired with cell-type specific staining in order to establish a reliable ground truth for model training (Fig 2).

MANUAL PATHOLOGY STRUGGLES TO IDENTIFY MACROPHAGES IN PD-L1 STAINED NSCLC SECTIONS

Sample ID	Macrophages		Non-macrophages		Total Cell #	Accuracy
	#	%	#	%		
NSCLC 20	29282	4.2%	661820	95.8%	691102	25
NSCLC 58	6498	1.0%	632469	99.0%	638967	1
NSCLC 76	7378	5.8%	120348	94.2%	127726	5
NSCLC 95	112668	9.1%	1121843	90.9%	1234511	1
NSCLC 101	37312	10.2%	330086	89.8%	367398	1
NSCLC 125	162191	14.8%	933704	85.2%	1095895	20

Fig. 1 | Pathologist Manual Identification of Macrophages is an Unreliable Ground Truth. In NSCLC tissues co-stained for CD68 (fluorescence) and PD-L1 (brightfield), pathologists were asked to identify CD68 positive cells from PD-L1 brightfield images alone. Manual accuracy measured in 5 samples shows accuracy ranging from 1-25%, with a bias towards selection of alveolar-type macrophages.

ESTABLISHING DEFINITIVE GROUND TRUTH TO REMOVE MANUAL INACCURACY

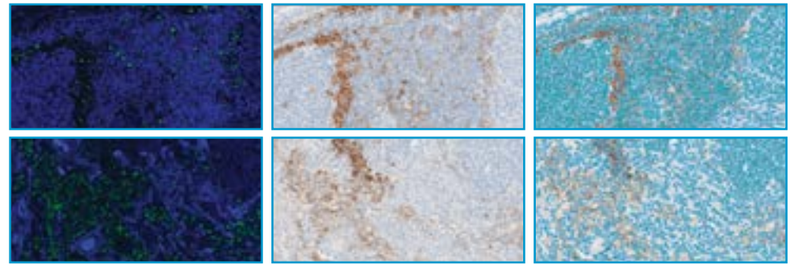

Fig. 2 | Image Analysis of PD-L1 Stained Tissues Co-Stained for CD68. NSCLC tissues co-stained for CD68 (green, fluorescence) and PD-L1 (brown, brightfield) were analyzed via cell-based image analysis. Per-cell data profiles of morphology, staining, and spatial characteristics were generated using the PD-L1-stained images (teal, digital cell recognition and measurement). Cellular data profiles incorporated CD68 status in order to build predictive models using the PD-L1 brightfield cell features only.

TESTING MACROPHAGE DETECTION MODEL IN SP263 AND 22C3 STAINED NSCLC SECTIONS

Cellular feature data describing the morphometric, staining, and contextual characteristics of detected cells from 23 NSCLC tissue blocks were generated. CD68 immunofluorescent staining was used to label cells as either Macrophage or Non-Macrophage classes in the complete dataset with a total model accuracy of 96.83% (Table 1). Three of the top five most important features within this model (comprising ~20% of the total feature importance) were related to cytoplasmic and nuclear PDL1 staining. This observation corroborates general pathology descriptions of macrophages, suggesting that SP263 PDL1 expression in macrophages appears to mostly localize in the nuclear and cytoplasmic compartments. Cell predicted to be macrophages in tissues outside of the training set were marked up, quantified, and scored with other cell populations (Figure 4). Images of NSCLC sections stained with PD-L1 22C3 assay were tested with this model but gave a null result (not shown), indicating discrepancies between assays in macrophage staining.

MODEL IS BUILT IN TRAINING SAMPLE ITERATIONS

Train Model to Identify CD68 Cells

Cell ID	Feature 1	Feature 2	Feature 3	Feature n	CD68
001	1.2	0.55	32	200	0
002	1.1	0.18	22	225	0
003	2.3	0.002	11	142	1
004	1.6	0.22	49	267	0
005	2.8	0.01	64	133	1

Replace and Repeat

Test Model in Outside Datatset Against Ground Truth

Cell ID	Feature 1	Feature 2	Feature 3	Feature n	CD68	Model Predicted CD68
006	1.2	0.55	29	200	0	0
007	1.1	0.18	34	225	0	1
008	2.3	0.002	22	142	1	1

Fig 3 | Schematic Workflow for Model Training and Testing. Data set was continually split in to training and test sets of cellular data. Once trained and tested, data was randomly re-split, trained, and tested

MODEL ACCURACY IS ASSESSED IN TEST SAMPLE ITERATIONS

Model Parameter	Value
Total Model Accuracy, CD68 Prediction	**96.83%**
95% CI Per Iteration, CD68 Prediction	91.09-94.42%
Total Data Set, Number of Cells	12,672,146
Iteration Size, Number of Cells	500,000
Estimators Generated Per Run	500

Table 1 | Macrophage Prediction Model Results. Classification accuracy on test sets against CD68 labeling were recorded per interval (Mean accuracy: 96.83 %, CI: 91.09% – 94.42%). When tested against the complete data set, model accuracy was shown to be 96.83%.

PREDICTED MACROPHAGES ARE MARKED UP IN TISSUES EXCLUDED FROM MODELING DATA

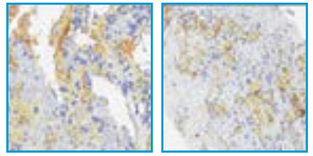

Fig 4 | Applying Model in New Tissues. The macrophage prediction model can be applied to other NSCLC tissues stained with SP263 to assess the macrophage population and quantify PD-L1 in these cell types (blue = negative, yellow = 1+, orange = 2+, red = 3+). Application to 22C3-stained tissues g (not shown) gave a null result and is indicative of the differential staining properties of each assay.

PD-L1 STAINING CHARACTERISTICS OF MODEL-DETERMINED MACROPHAGES

BINNED PD-L1 INTENSITY OF MODEL-DETERMINED MACROPHAGES IN SP263-STAINED NSCLC SECTIONS

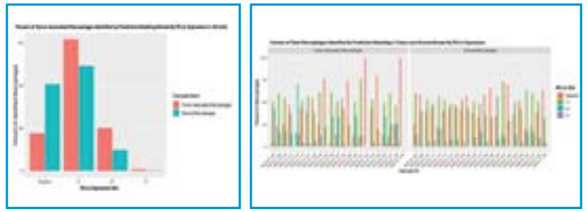

Figure 5 | PD-L1 Intensity Profiles of Model-determined Macrophages. Once the macrophage identification model was run on additional NSCLC tissues stained with SP263, this specific population was scored for PD-L1 expression. Shown here is the overall average expression of PD-L1 in macrophages across the entirety of the tissue cohort. Stromal macrophages tended to skew toward being either negative or 1+ expression for PD-L1, while tumor-associated macrophages (TAMs) had a higher overall percentage of positivity. PD-L1 expression in TAMs tended to show 1+ or 2+ bin intensity, with a small population showing 3+ intensity. Expression across the entire range of tissues is also shown, demonstrating a range of macrophage staining characteristics throughout the cohort.

LOCALIZATION OF PD-L1 EXPRESSION IN NSCLC TISSUES EXHIBITING A RANGE OF MACROPHAGE CONTENT

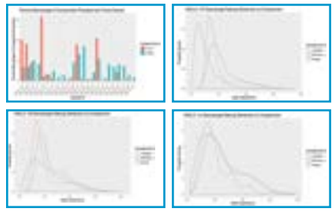

Figure 6 | Total Macrophage Content and Localizing PD-L1 Expression in Cellular Compartments. Here the total macrophage population in the tumor and stroma of each tissue is quantified and represented (top left). Based on expression patterns, we show the differential characteristics of PD-L1 expression in macrophages. NSCLC 137, for example, shows almost exclusive stromal macrophage presence, whereas NSCLC 135 is more balanced, and NSCLC 114 shows very high tumor expression of macrophages (~15%). Looking at PD-L1 localization in the cellular profiles of each tissue, we can see that in the mostly stromal macrophage sample, NSCLC 137, the expression of PD-L1 is mostly contained in the membrane and cytoplasm, with little overlap on the nucleus. NSCLC 114, which is mostly tumor-associated macrophages, reveals a different staining profile, with PD-L1 expression overlapping into the nucleus at the low end of staining, and demonstrating higher mean absorbances in the membrane and cytoplasm.