Visualize Immune Infiltrate & Checkpoint Expression in Murine Syngeneic Models

Advantages of CST Rabbit Monoclonal Antibodies for IHC

Minimize Background	Avoid the pitfalls of mouse-on-mouse background.
Disease Relevance	Targets relevant for preclinical cancer research in mouse.
Validation	Comprehensive data package and IHC protocol testing to ensure antibody specificity and performance.
Versatility	Work in FFPE tissue samples, automated IHC platforms and both chromogenic and multiplexed fluorescent IHC protocols.
Value	Get more insights from precious tissue samples, including co-localization.

Introduction

Advances in immuno-oncology have successfully led to novel cancer therapeutics with favorable patient responses that are more durable than conventional cytotoxic chemotherapy (1). To enable the development of immunotherapeutic strategies and personalized medicines, investigators are trying to understand drug mechanisms of action and patient responses using clinically relevant biomarkers. Murine syngeneic tumor models are increasingly utilized for preclinical immuno-oncology studies and to characterize immunologic features of the tumor microenvironment (TME) (2).

Spatial localization of multiple biomarkers is critical when cataloging subsets of immune infiltrate and cancer cells and their interactions in the TME. Multiplexed assays are required for investigations of multiple therapeutic targets and predictive biomarkers in limited and valuable samples. Mouse-on-mouse background staining, which often obscures interpretation of staining results, may be avoided by using rabbit monoclonal antibodies in serial labeling strategies. For these reasons, tyramide-based fluorescent multiplex immunohistochemistry (mIHC), which enables detection of 6 or more proteins/biomarkers in formalin-fixed, paraffin-embedded (FFPE) tissue samples, is a valuable tool for immuno-oncology.

In mIHC, as well as in single/dual staining chromogenic IHC approaches, using application-validated antibodies against relevant targets is crucial in order to obtain reliable results. Antibodies validated for IHC from Cell Signaling Technology (CST) enable investigations of biomarker expression, localization, interaction, and disease context.

This application note explores considerations for selecting and using antibodies in mIHC to characterize immune infiltrate in murine syngeneic tumor models.

Background and Results

In the TME, evolving cancer cells interact with a range of immune cells, which may include subsets of T cells and macrophages in various stages of activation. The number and types of immune cells present in the TME may vary among different cancers, stages of tumor progression, host tissue and between primary and metastatic sites. Molecular hallmarks of tumor immunogenicity and immunosuppression, including checkpoint proteins such as PD-1 and PD-L1, are sources of additional diversity within and among tumors. Visualization of the underlying differences in the immunologic landscapes of tumors is needed to develop tailored immunotherapeutic approaches and combination strategies. Unlike xenograft models, which rely on immunocompromised hosts, syngeneic mouse models have intact immune functions, making them advantageous for preclinical investigations of tumor-immune interactions.

In this study, we applied a 7-color (mIHC) panel (**Table 1**) to visualize and quantify the immune infiltrate within (FFPE) tissue sections from LL/2, Renca, and CT26.WT tumors and from 4T1 primary and metastatic tumors. The multiplex panel included antibodies detecting CD3 and CD8 as T cell markers, F4/80 as a myeloid cell marker, the immunosuppressive receptor PD-1 and its ligand PD-L1, pan-keratin (cytokeratin, CK) as a tumor mask, and DAPI as a nuclear counterstain.

Table 1 – Immune Infiltrate/Checkpoint Protein Antibody Selections

Product #	Antibody	Host	Dilution	Fluorophore	Order
#99940	**CD3ε (D4V8L)**	Rabbit	1:300	Cy™5	1st
#84651	**PD-1 (D7D5W) XP®**	Rabbit	1:100	FITC	2nd
#98941	**CD8α (D4W2Z) XP®**	Rabbit	1:300	Alexa Fluor® 594	3rd
#64988	**PD-L1 (D5V3B)**	Rabbit	1:50	Alexa Fluor® 555	4th
#70076	**F4/80 (D2S9R) XP®**	Rabbit	1:500	Cy™5.5	5th
#4279	**Pan-Keratin (C11)**	Mouse	1:50	Alexa Fluor® 350	6th

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Figure 1

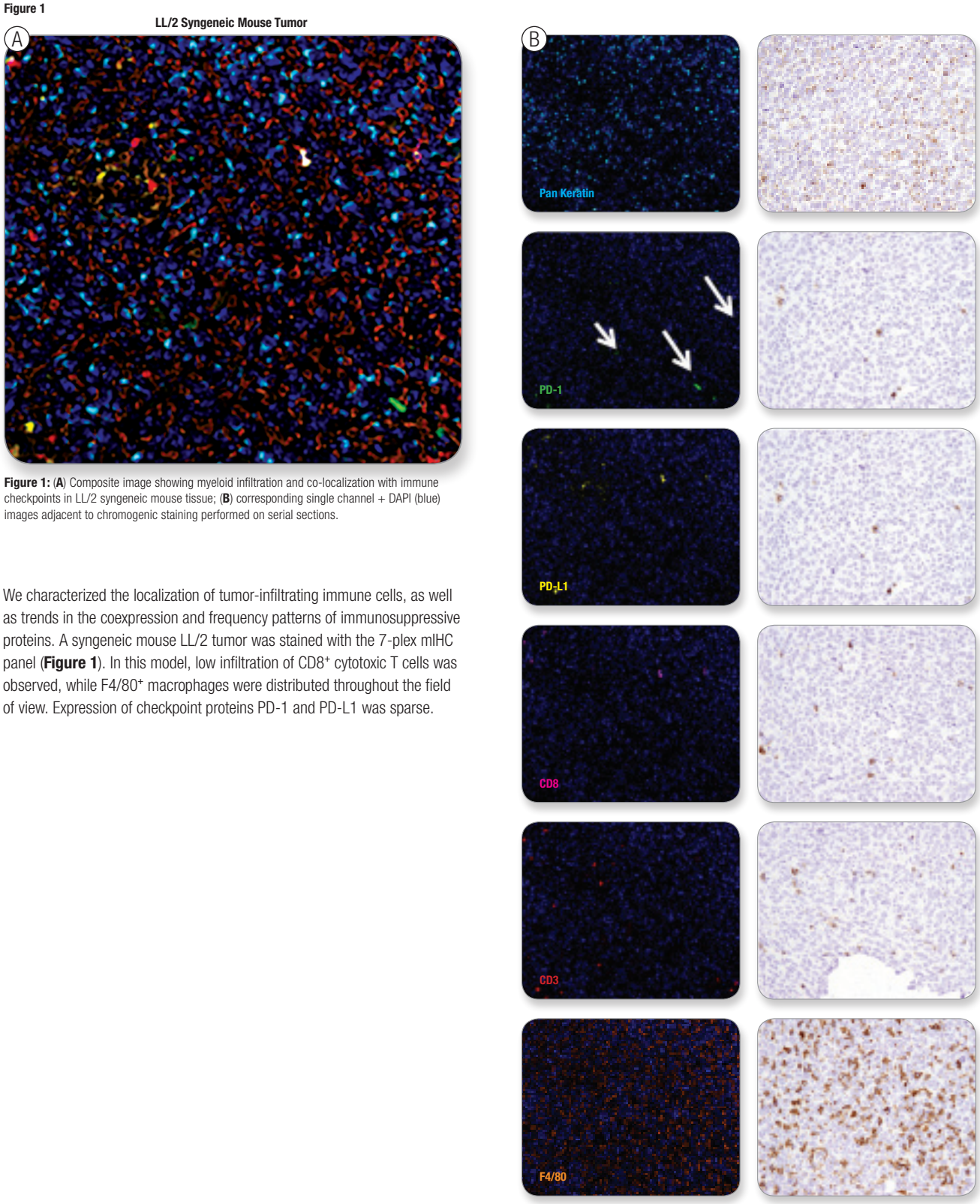

Figure 1: (**A**) Composite image showing myeloid infiltration and co-localization with immune checkpoints in LL/2 syngeneic mouse tissue; (**B**) corresponding single channel + DAPI (blue) images adjacent to chromogenic staining performed on serial sections.

We characterized the localization of tumor-infiltrating immune cells, as well as trends in the coexpression and frequency patterns of immunosuppressive proteins. A syngeneic mouse LL/2 tumor was stained with the 7-plex mIHC panel (**Figure 1**). In this model, low infiltration of $CD8^+$ cytotoxic T cells was observed, while $F4/80^+$ macrophages were distributed throughout the field of view. Expression of checkpoint proteins PD-1 and PD-L1 was sparse.

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Figure 2

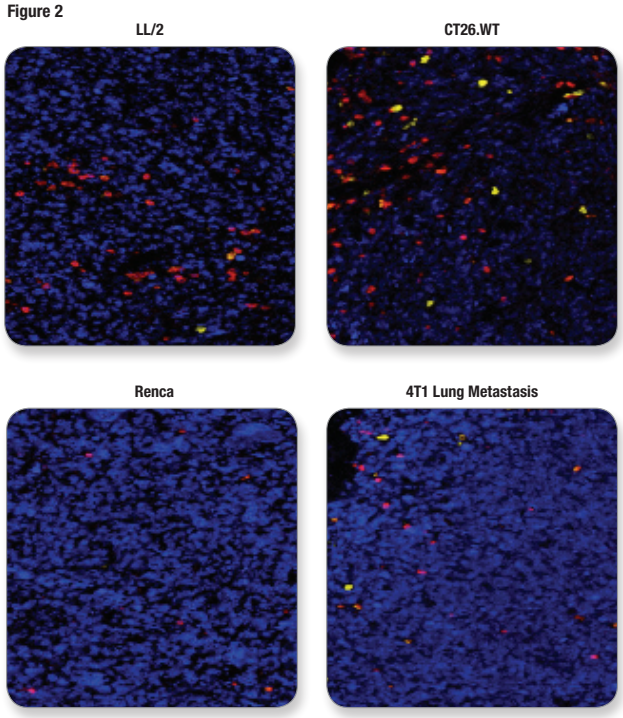

Figure 2: Representative images of $CD3^+$ (red) and $CD8^+$ (yellow) cells present in LL/2, CT26.WT, Renca, 4T1 lung metastasis, and 4T1 mammary tumor. Blue pseudocolor = DAPI (fluorescent DNA dye).

Figure 3

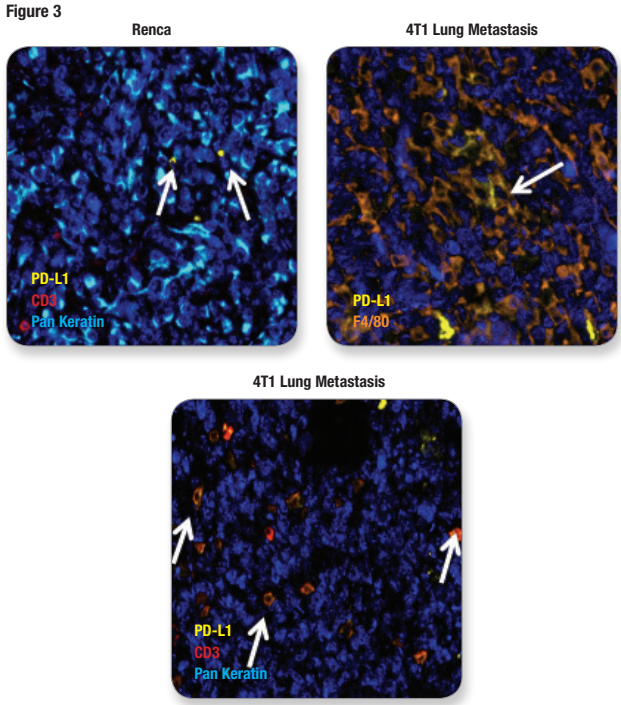

Figure 3: Representative images of the various patterns of PD-L1 expression. From top to bottom: PD-L1 (yellow) CD3-/CK- cells in the Renca tumor, PD-L1 (yellow) on $F4/80^+$ cells in the 4T1 lung metastasis, and PD-L1 (yellow) on $CD3^+$ (red) cells in the 4T1 lung metastasis. Blue pseudocolor = DAPI (fluorescent DNA dye).

Tumor infiltration in various syngeneic models was assessed using the CD3 and CD8 antibodies. LL/2, CT26.WT, Renca, and 4T1 primary (mammary) and metastatic (lung) tumors exhibited varying degrees of T cell invasion (**Figure 2**).

Combinatorial evaluation of protein expression can be performed with mIHC. **Figure 3** denotes observations of $PD\text{-}L1^+/CD3$- cells in Renca tumor, $PD\text{-}L1^+/F4/80^+$ macrophages in 4T1 lung metastasis, and $PD\text{-}L1/CD3^+$ cells in 4T1 lung metastasis.

mIHC analysis of a 4T1 orthotopic mammary tumor and 4T1 lung metastasis is shown in **Figure 4A** (next page). Myeloid infiltration as measured by the number of $F4/80^+$ cells was greater in the 4T1 lung metastasis than in the primary tumor (**Figure 4B**). $PD\text{-}L1^+$ cell counts were higher in the primary mammary tumor. In addition, higher coregistration of F4/80 and PD-L1 was observed in the lung metastasis.

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Figure 4

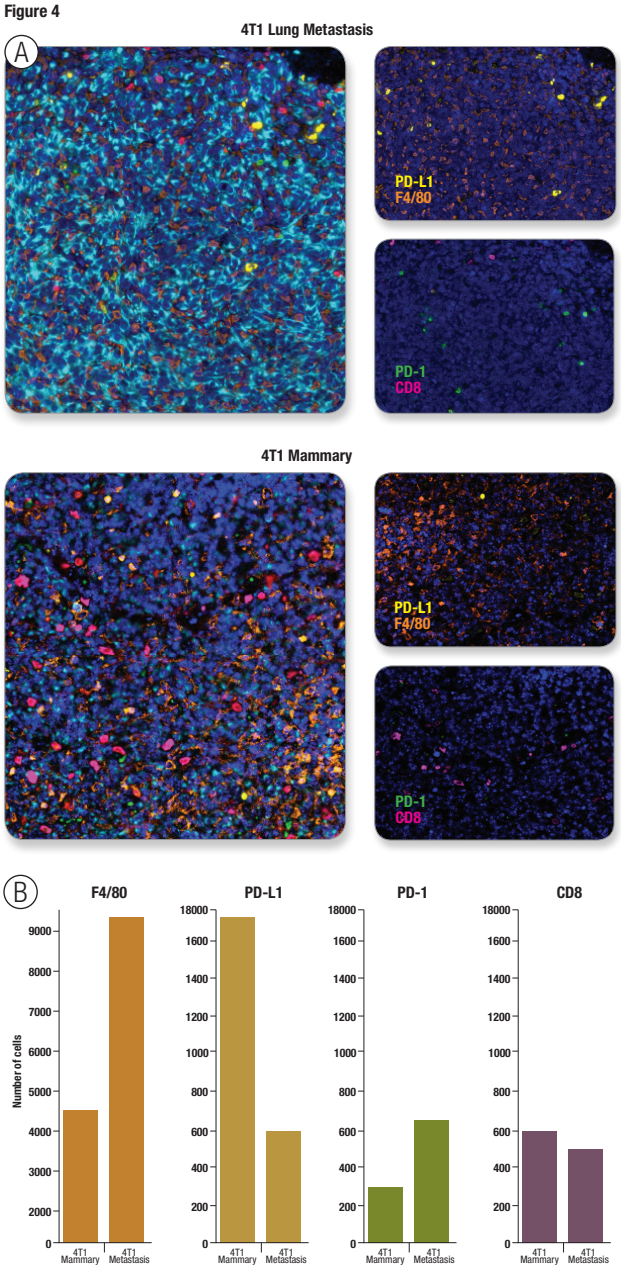
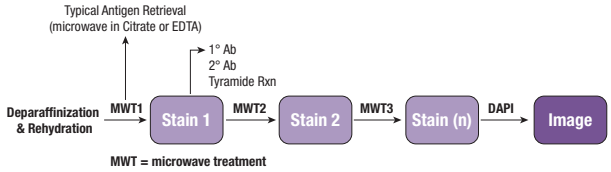


Figure 4: A. Composite mIHC images of 4T1 lung metastasis (top) and primary mammary tumor (bottom), with PD-L1/F4/80 and PD-1/CD8 signals isolated (right). **B.** Total cell counts positive for F4/80 (orange), PD-L1 (yellow), PD-1 (green), and CD-8 (purple) stained across 3 fields imaged of 4T1 mammary (primary) tumors and 4T1 lung (metastatic) tumors.

Methods

For questions about how to customize your protocol using our full catalog of nearly 1000 antibodies approved for IHC, please contact technical support: www.cellsignal.com/support.



Tyramide-based fluorescent mIHC employs a serial labeling strategy. Following incubation of tissue with primary antibody, HRP-conjugated secondary antibodies catalyze deposition of tyramide-fluorophore complexes, which subsequently remain covalently bound to the tissue while the antibodies are removed via via microwave-mediated heating between labels. Note that the removal of primary antibodies after each labeling round allows for the use of multiple antibodies from the same host species. All antibodies used in this panel were raised in rabbit, with the exception of Pan-Keratin, which was a biotinylated mouse antibody that was detected via HRP-streptavidin followed by an Alexa Fluor® 350 tyramide conjugate. This approach effectively avoids mouse-on-mouse background staining that can complicate the interpretation of results.

A mIHC panel was optimized and applied to 5 FFPE murine syngeneic tumor sections as well as to 1 normal murine lung section. Images were acquired and spectrally unmixed with a Mantra™ Quantitative Pathology Workstation (PerkinElmer). Three fields per tissue were imaged and used for quantification. This quantification included analysis of parameters such as total cell counts positive for each phenotypic marker and colocalization or coregistration of signal.

Conclusions

This study highlights the diverse immune landscape among murine syngeneic tumor models with respect to unique cell subsets and PD-1/PD-L1 expression patterns. A set of robustly validated mouse-reactive IHC antibodies, along with in-depth optimization of the multiplex panel, is required for the accurate identification and analysis of these subsets. Mouse-on-mouse background is avoided through mIHC panel design incorporating rabbit monoclonal antibodies.

References

1. Sharma, P. and Allison, J. P. (2015) Cell 161, 205–14
2. Mosely, S. I. S. et al. (2016) Cancer Immunol Res 5, 1–13

For more on pre-clinical IHC tools for mouse models, go to:
www.cellsignal.com/IHCmousemodels

Technical Support

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

For questions about how to customize your protocol using our full catalog of nearly 1000 antibodies approved for IHC, please contact technical support:
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