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OMIP-031: Immunologic Checkpoint Expression on Murine Effector and Memory T-Cell Subsets

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Purpose and Appropriate Sample Types

This panel was designed to assess the expression levels of cell surface inhibitory receptors known as "immune checkpoints" within the context of multiple naïve, activated, memory, and effector phenotypes among T-cells for subsequent adoptive transfer using the CD45.1/CD45.2 congenic system in C57BL/6 mice. It can be easily adapted to other congenic systems, or may be used without any congenic marker. While many panels have been published that analyze T-cell activation, memory phenotypes, or effector differentiation states, few, if any, are comprehensive enough to assess these compartments simultaneously while measuring inhibitory immune checkpoint receptor expression. The ability to do so within a congenic system creates a powerful tool for investigating the evolution of T-cell based immune responses in a broad range of contexts. Here, the panel is used to analyze the T-cell compartment in normal spleen, or T-cells infiltrating subcutaneous murine colon adenocarcinoma, MC38. However, any murine source of T-cells would serve as an appropriate sample source for this panel.

Background

Upon activation, T-cells undergo a high rate of proliferation, and alter the expression of numerous genes associated with effector response, or with supportive function. This is followed by a contraction period in which the majority of activated T-cells die off and a remaining minority of antigen-specific T-cells transition into one of multiple longer-lived memory T-cell phenotypes capable of future reactivation in the event of antigen re-encounter. Concomitant with T-cell activation and subsequent memory formation are alterations in cell surface protein expression that can be used to classify T-cell subsets and identify their activation or memory state. This panel is built upon a basic framework that allows for a comprehensive overview of these subpopulations (Table 1). It includes CD69, which is a traditional marker for T-cell activation that is one of the earliest inducible proteins on the cell surface following activation of both helper T-cells (T_H), and cytotoxic T-cells (T_C), which can be identified using CD3 and CD4, or CD3 and CD8, respectively. Following activation, T-cells down-regulate CD45RA and up-regulate other higher molecular weight CD45 isoforms (1). While several cases of

Table 1. Summary table for application of OMIP-031

Purpose	Checkpoint expression on subsets of memory T-cells
Species	Mouse
Cell types	Any source containing murine T-cells
Cross-references	No similar OMIPs

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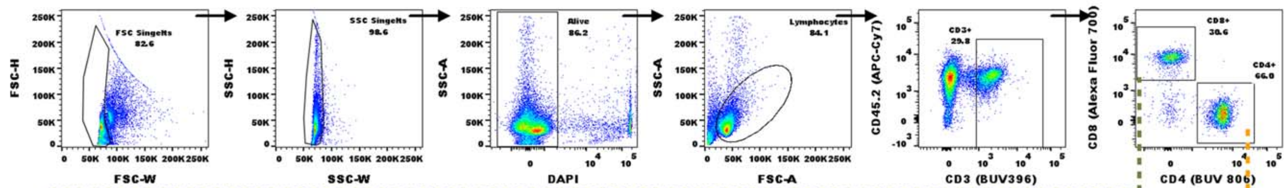

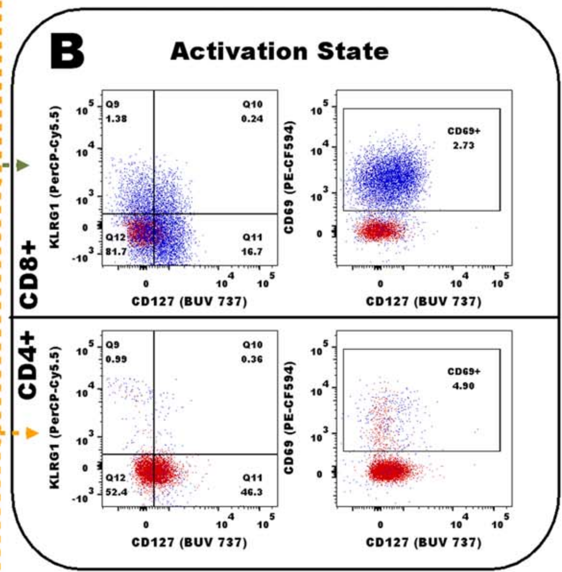

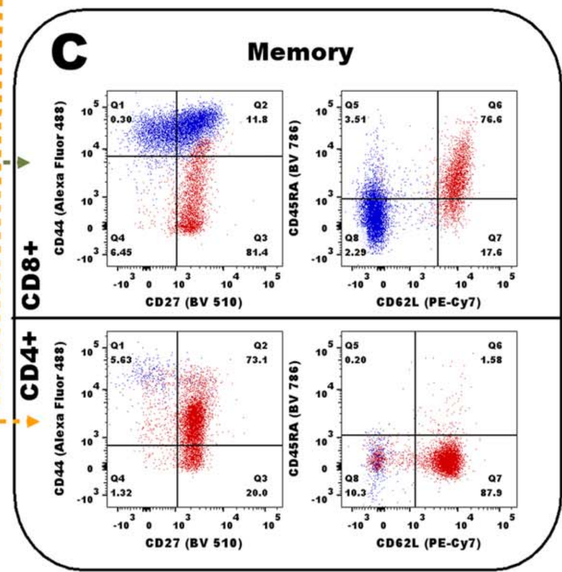

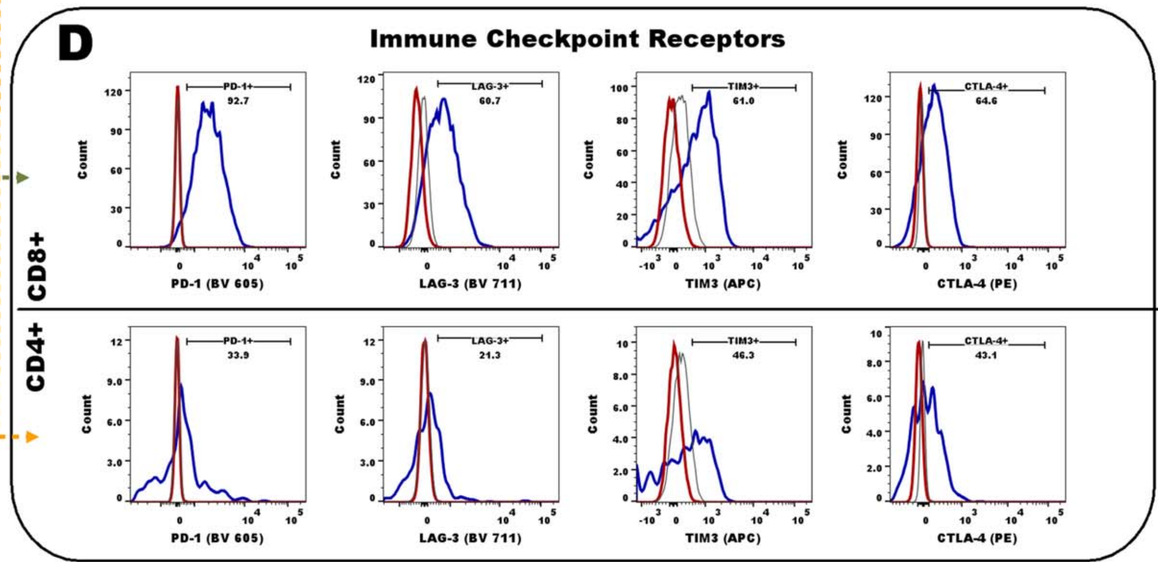

Figure 1. Example gating schema. **A**: Single cells were gated using forward scatter height versus width parameters followed by side scatter height versus width parameters. Lymphocytes were then gated using forward scatter area versus side scatter area, followed by viability gating using DAPI. T_H and T_C cells were then selected by gating on CD45.2$^+$ CD3$^+$ cells followed by gating on CD4$^+$ or CD8$^+$ cells. Black arrows = gated cells. **B**: Early activation and differentiation states can be analyzed using KLRG1 versus CD127 to define SLEC and MPEC populations and CD69 can be used to demark currently activated T-cells. T-cells from healthy C57BL/6 spleen are shown in red, and MC-38 tumor-infiltrating T-cells are overlaid in blue to demonstrate changes in phenotype and differentiation state that may be observed in this panel. Green arrows = gated CD8$^+$ T-cells, orange arrows = gated CD4$^+$ cells. **C**: Conventional memory phenotypes including naïve (N), central memory (CM), effector memory (EM), and effector T-cells can be analyzed using a combination of CD44 or CD45RA versus CD27 or CD62L staining. **D**: Four immune checkpoint markers may also be analyzed using this panel: PD-1, LAG-3, TIM3, and CTLA-4. Gate percentages in overlaid plots represent analysis from MC-38 tumor-infiltrating T-cell samples. Gates were placed according to fluorescence-minus-one (FMO) controls.

Table 2. Reagents for OMIP-031

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	145-2C11	BUV 395	T-cell
CD4	GK1.5	BUV 805	T_H
CD8	53-6.7	Alexa Fluor 700	T_C
CD69	H1.2F3	PE-CF594	Activation
CD44	IM7	Alexa Fluor 488	Memory
CD45RA	14.8	BV 786	Memory
CD27	LG.3A10	BV 510	Memory
CD62L	MEL-14	PE-Cy7	Memory
KLRG1	2F1/KLRG1	PerCP-Cy5.5	Differentiation
CD127	SB/199	BUV 737	Differentiation
PD-1	J43	BV 605	Checkpoint
CTLA4	UC10-4B9	PE	Checkpoint
TIM-3	B8.2C12	APC	Checkpoint
LAG-3	C9B7W	BV 711	Checkpoint
CD45.2	104	APC-Cy7	Congenic
DAPI	-	DAPI	Viability

BUV, Brilliant Ultra Violet™; PE, R-phycoerythrin; BV, Brilliant Violet™; Cy, cyanine; PerCP-Cy5.5, Peridinin-chlorophyll Cy-5.5; APC, allophycocyanin; DAPI 4′,6-diamidino-2-phenylindole; T_H, T helper cell; T_C, T cytotoxic cell.

CD45RA re-expression have been reported on chronically stimulated T-cells (2,3), CD45RA is generally considered a naïve T-cell marker. As T-cells transition into memory phenotypes, the expression of CD44 increases from low to high. Thus, naïve and memory T helper (T_H) and T cytotoxic (T_C) cells can be identified by discriminating $CD44^{low}$ and $CD45^{high}$ populations and by $CD45RA^+$ and $CD45RA^-$ populations.

The two major subtypes of memory T-cells, central memory (CM), and effector memory (EM), have been conventionally defined by differential expression of CD62L and CD27. While a number of cell surface molecules have been reported to discriminate these memory subpopulations, it is also appreciated that a degree of plasticity exists between CM and EM T-cells resulting in incomplete overlap among CM/EM markers. This is particularly true among T_H cells (4). Therefore, the use of multiple EM/CM markers increases the power to effectively resolve these populations or identify transitional phenotypes. In this panel, CM can be identified as $CD62L^+$ and/or $CD27^+$ cells, while EM can be identified as $CD62L^-$ and/or $CD27^-$.

How it is determined which activated effector T-cells die off during contraction, and which go on to persist as memory T-cells has been an outstanding question in immunology. Recent work has identified two subsets of effector T-cells that can be identified by CD127 and KLRG1 that are associated heightened cytotoxic function and subsequent die off or pre-destination for memory formation. Activated T-cells undergo a transient down-regulation of both KLRG1 and CD127. This brief state is followed by up-regulation of either CD127 or KLRG1. Short-lived effector cells (SLECs) express lower levels of CD127 and heightened levels of KLRG1 while memory precursor cells (MPEC) express higher levels of CD127 and lower levels of KLRG1 (5,6).

Finally, this panel also measures the levels of inhibitory receptors recently defined as immunological "checkpoint," due to the transient nature of their expression following activation, and their potent inhibitory potential upon interaction with cognate ligands (7–12). Included in this panel are the inhibitory checkpoint receptors TIM3, LAG-3, PD-1, and CTLA-4 (Table 2). To demonstrate how overall naïve, activated, memory, and effector phenotypes can change, labeled infiltrating T-cells from a single cell suspension of resected orthotropic MC38 tumor grown in C57BL/6 mice are overlaid on labeled T-cells from normal murine C57BL/6 spleen (Fig. 1). Please refer to the online Supporting Information for details regarding panel development.

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