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# OMIP-036: Co-inhibitory Receptor (Immune Checkpoint) Expression Analysis in Human T Cell Subsets

**Zachary R. Healy,* David M. Murdoch**

Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Duke University Hospital, Durham, North Carolina

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Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Z. R. Healy, DUMC Box 103000, Duke University Medical Center, Durham, NC 27710, USA. E-mail: zachary.healy@duke.edu

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• **Key terms**
T cell exhaustion; immune checkpoint; co-inhibitory receptor; flow cytometry; chronic antigenic exposure

## Purpose and Appropriate Sample Types

**This** panel was optimized to quantify inhibitory receptor expression on CD4 and CD8 T cells from differentiation and activation subsets. Six inhibitory (i.e., immune checkpoint) receptors (PD-1, TIM-3, LAG-3, CD160, BTLA, CTLA-4) were chosen based upon previously published observations suggesting their role in modulating CD4 and CD8 T cell activation in response to persistent antigen exposure (1–3). Furthermore, given the important observations that inhibitory receptor expression varies by differentiation and prior antigen experience, markers of T cell differentiation status and prior antigen experience (CCR7, CD45RA, CD28, CD127, KLRG1) were also included (4–6). This panel was developed and optimized for use in cryopreserved human peripheral blood mononuclear cells (PBMCs), although it has also been applied in fresh PBMCs as well as other bodily fluids (e.g., malignant ascites) (Table 1).

## Introduction

Following initial antigen exposure, the generation of highly functional antigen-specific T cells is dependent on the ability of the immune system to clear antigen in a timely manner. In situations where antigen cannot be effectively cleared (e.g., malignancy, HIV, HCV), chronic antigenic stimulation leads to T cell exhaustion, a dysfunctional state marked by poor effector function, cytokine production, proliferative ability, and the sustained expression of inhibitory co-receptors (e.g., PD-1, CTLA-4) (1,3,7). This state of T cell exhaustion appears to have a molecular signature distinct from T cell anergy and senescence, representing a potentially reversible process regulated by the balance between co-stimulatory and co-inhibitory receptor activation (2,8). The expression of these inhibitory receptors has become a major focus in cancer immunotherapy, with several novel chemotherapeutics introduced in the last several years (9). However, work from several laboratories has demonstrated that inhibitory receptor expression does not necessarily identify an exhausted phenotype, with expression dependent on differentiation, activation, prior antigen experience, and the molecular microenvironment (5,10).

## Background

Adequate T cell activation requires engagement of both the T cell receptor (TCR) as well as co-stimulatory receptors (e.g., CD28) during antigen presentation;

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**Table 1.** Summary table for application of OMIP-036

| | |
|---|---|
| Purpose | Co-inhibitory Receptor Expression on CD4+ and CD8+ T cell maturation subsets |
| Species | Human |
| Cell Types | PBMC |
| Cross References | None |

additionally, T cell activation may be attenuated through the binding and activation of co-inhibitory receptors, providing an important feedback control over the degree of stimulation in response to antigen (8,11). The majority of T cell co-inhibitory receptors are members of the immunoglobulin (Ig) superfamily, which when engaged by the corresponding ligand expressed on antigen-presenting cells or tumors cells, attenuates cell activation via protein tyrosine phosphatases

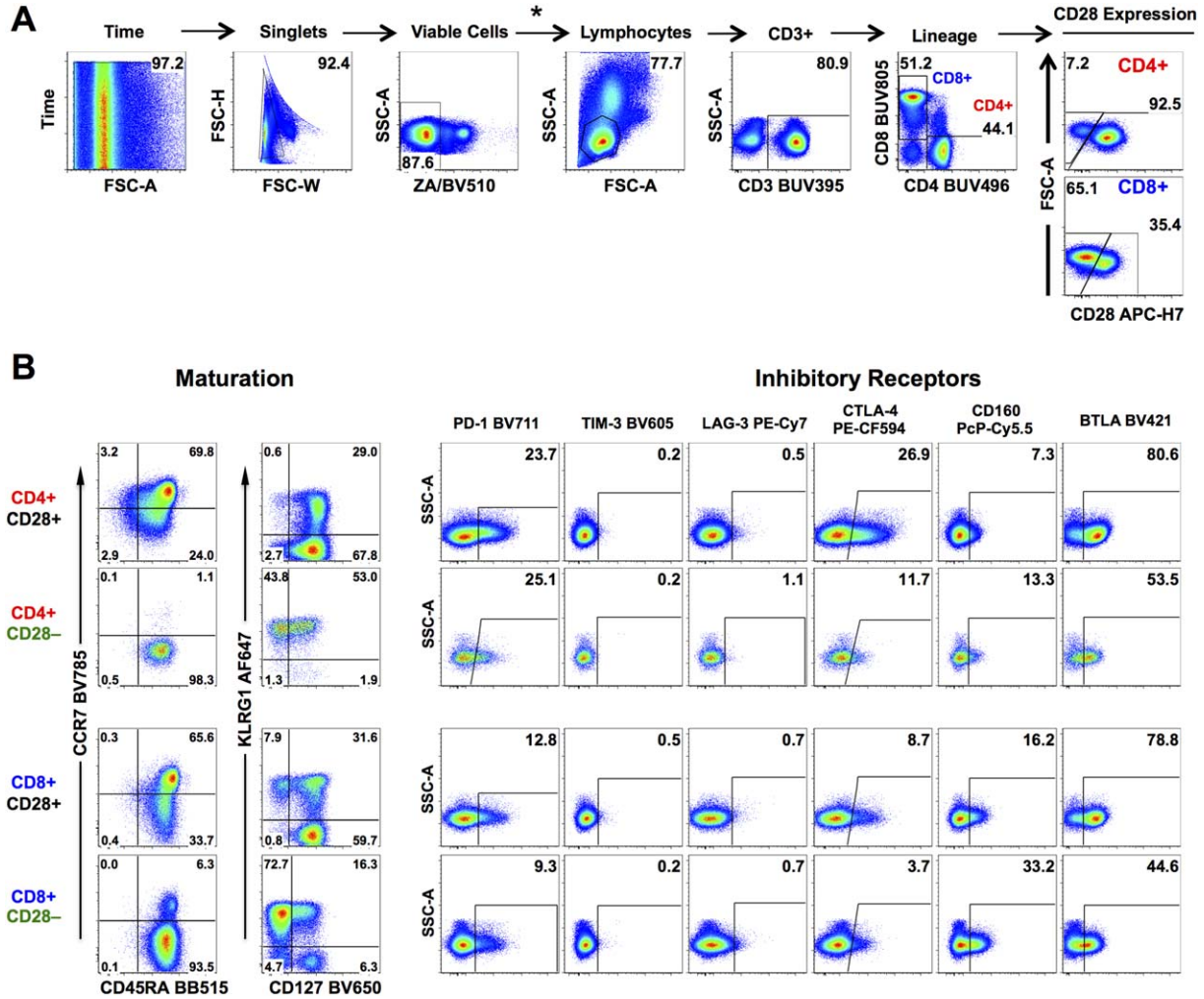


**Figure 1.** Proposed gating strategy of adult human PBMC in the quiescent state. (**A**) Identification of CD4+ and CD8+ T cell subsets. Following identification of singlets, viable cells are differentiated from nonviable cells (Zombie Aqua) as well as monocytes (CD14+ BV510) and B-cells (CD19+ BV510). Aggregate gates (*) are used to exclude cell and/or dye aggregates prior to identification of lymphocytes (please refer to Supplemental Figure 11, available online). Afterward, CD3+ followed by CD4+ and CD8+ T cell subsets are identified and further differentiated by CD28 expression, as the loss of CD28 expression has been associated with chronic viral persistence in HIV and CMV. (**B**) Determination of T cell differentiation and co-inhibitory receptor expression within CD4+ and CD8+ subsets. For previously identified T cell subset (CD4+/CD28+, CD4+/CD28-, CD8+/CD28+, CD8+/CD28-), differentiation and activation status (CCR7 vs CD45RA, KLRG1 vs CD127) as well as co-inhibitory receptor expression may then be quantified. Overall, there is minimal expression of TIM-3 and LAG-3 in the resting state. Alternatively, the basal expressions of CD160, CTLA-4, PD-1, and BTLA vary considerably between subsets and donors. Additionally, co-inhibitory receptor expression may also be analyzed within multiple subsets including naïve (CCR7+/CD45RA+), central memory (CCR7+/CD45RA-), effector memory (CCR7-/CD45RA-), EMRA (CCR7-/CD45RA+) as well as in the short-lived effector cell (SLECs, KLRG1+/CD127-) and memory precursor effector cell (MPEC, KLRG1-/CD127+) populations during acute infections. An antigen specific tetramer may also be used on the PE channel for added analysis. Boolean gating may be implemented to determine co-expression of differentiation markers and co-inhibitory receptors for further characterization. For an example of panel performance in an activated state, please refer to Supplemental Figure 1 (available online). **Note**: the donor displayed in this figure was selected for their CD160 expression, and this donor has an abnormal CD4+ memory subset distribution with only naïve and EMRA cells present. Please note that this is a physiologic finding and not an effect of inappropriate CCR7-CD45RA staining. Please see Supplemental Figure 4 (available online) for a representation of CCR7- CD45RA staining across several donors. The donor represented in Figure 1 and Supplemental Figure 1 is shown as donor 1 in Supplemental Figure 4.

**Table 2.** Reagents used for OMIP-036

| SPECIFICITY | CLONE | FLUOROCHROME | PURPOSE |
|---|---|---|---|
| Viability Dye | – | Zombie Aqua | Dump |
| CD14 | M5E2 | BV510 | |
| CD19 | HIB19 | BV510 | |
| CD3 | SK7 | BUV395 | Phenotype |
| CD4 | SK3 | BUV496 | |
| CD8 | SK1 | BUV805 | |
| CCR7 | G043H7 | BV785 | Differentiation |
| CD45RA | HI100 | BB515 | |
| CD28 | CD28.2 | APC-H7 | |
| CD127 | A019D5 | BV650 | |
| KLRG1 | SA231A2 | AF647 | |
| PD-1 | EH12.2H7 | BV711 | Co-inhibitory Receptors |
| TIM-3 | F38-2E2 | BV605 | |
| LAG-3 | 3DS223H | PE-Cy7 | |
| CD160 | BY55 | PerCP-Cy5.5 | |
| BTLA | MIH26 | BV421 | |
| CTLA-4 | BNI3 | PE-CF594 | |

(e.g., SHIP-1, SHP-2), ultimately leading to reduced effector function and the expression of inhibitory genes (1,12). Additionally, co-inhibitory receptors may also interfere with co-stimulatory receptor function by competing for ligands as well as by disrupting the formation of lipid rafts and microclusters necessary for appropriate antigen response (8,11).

T cell co-inhibitory receptors, as well as their corresponding ligands, may be expressed on a number of other cell types, most notably natural killer (NK) cells, B cells, antigen presenting cells (APCs), as well as on certain somatic tumors (e.g., melanoma; non-small cell lung cancer, NSCLC; renal cell carcinoma, RCC) (11). Most recently, the introduction of immunotherapy aimed at the reversal of T cell exhaustion in the setting of cancer has generated significant interest in this field (9). The process of antigen-specific T cell exhaustion is thought to involve dysfunctional effector cell contraction and memory differentiation in the face of persistent antigenic exposure, ultimately leading to accumulated expression of several co-inhibitory receptors coinciding with reductions in effector function and proliferative ability (3,9,12–15). Importantly, the expression of a co-inhibitory receptor in isolation does not adequately define an exhausted state, as many factors may influence co-inhibitory receptor expression without leading to reductions in functionality and proliferative ability. For example, ex vivo studies have demonstrated that T cell differentiation status dictates basal co-inhibitory receptor expression levels (1,3,12,16); studies in healthy human subjects have demonstrated significant variations in basal co-inhibitory receptor expression between naïve, memory, and effector subsets, particularly with respect to PD-1 and CTLA-4. Furthermore, studies have also shown co-inhibitory receptor expression is also transiently modified during T cell activation. Several ex-vivo studies have shown that prolonged stimulation (αCD3/αCD28, 16–72 h) of PBMCs from healthy donors leads to the transient expression of several co-inhibitory receptors, which may represent an important feedback loop for control over inflammation. Therefore, inclusion of markers for differentiation (CCR7, CD45RA) and antigen experience (CD28, CD127, KLRG1) provides critical information when interpreting changes in co-inhibitory receptor expression (Figure 1).

To this end, we developed a comprehensive polychromatic flow cytometry panel for the characterization and quantification of inhibitory receptor expression (PD-1, TIM-3, LAG-3, CTLA-4, BTLA, CD160) on CD4 and CD8 T cells, including markers for prior antigen exposure (CD127, CD28, KLRG1) and differentiation status (CCR7, CD45RA) to allow for additional subset analysis with the potential to improve our molecular understanding of this important immune mechanism (Table 2). The channel for PE remains open for use of an antigen-specific marker or other high priority markers of interest. Please refer to supplemental material available online for technical information and additional figures.

## Human Subjects Research

Human PBMCs were obtained from healthy donors using protocols approved by the Duke institutional review board (IRB).

## Similarity to Published OMIPs

This panel is related to that recently published by Nemoto et al. (OMIP-031), with the important exception that this panel has been developed for use in human PBMCs rather than in murine cells (17). Prior panels have also examined T cell memory subsets in human cells as well, although none have examined co-inhibitory receptor expression in these subsets.

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