Evaluating the expression patterns of multiple inhibitory receptors associated with T-cell exhaustion using multicolor flow cytometry

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Introduction

T-cell exhaustion is a dysfunctional state caused by persistent antigen stimulation and defined by loss of antigen-specific $CD8^+$ T-cell effector function.[1] Exhausted T cells were originally identified in the context of chronic murine lymphocytic choriomeningitis virus *(LCMV)* infection.[2] Exhausted T cells have also been observed in humans affected with chronic viral infections, such as human immunodeficiency virus *(HIV)*, hepatitis B virus *(HBV)* and hepatitis C virus *(HCV)*, as well as cancer.[3-8]

During an acute infection, antigen-specific $CD8^+$ T cells are activated through T-cell receptor *(TCR)* stimulation and signaling mediated by co-stimulatory molecules and inflammatory cytokines. Activated $CD8^+$ T cells proliferate, produce cytokines and cytotoxic molecules that kill the infected cells. Upon infection clearance, most effector $CD8^+$ T cells undergo programmed cell death, whereas a small subset of cells differentiates into long-lived, self-renewing memory T cells, which are able to rapidly mount robust recall responses upon re-infection.

During chronic infection, or in the tumor microenvironment, $CD8^+$ T cells are persistently stimulated, leading to T-cell exhaustion characterized by the progressive and hierarchical loss of proliferative capacity, secretion of cytokines and cytotoxic molecules and ability to differentiate into long-lived memory T cells, ultimately culminating in clonal deletion.

Increased expression of inhibitory receptors is another hallmark of T-cell exhaustion.[9] Inhibitory receptors serve as immune checkpoints transiently expressed by activated $CD8^+$ T cells that play a key role in maintaining immune homeostasis and preventing autoimmunity. Upon binding to their cognate ligands, inhibitory receptors attenuate $CD8^+$ T cell activation via different mechanisms, including competition for co-stimulatory molecules, inhibition of proliferation, cytokine secretion and metabolic pathways. Persistent stimulation induces a sustained upregulation of inhibitory receptors that is exploited, for example, by tumor cells. Inhibitory ligands expressed by tumor

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cells bind to the inhibitory receptors highly expressed on exhausted CD8+ T cells, thus progressively inhibiting T-cell function and resulting in immune surveillance escape.

Cytotoxic T-lymphocyte-associated protein 4 *(CTLA-4, CD152)* and programmed cell death 1 *(PD-1, CD279)* are two well-characterized inhibitory receptors. Both are associated with T-cell exhaustion, although they exert their inhibitory function through different mechanisms.[10] Pre-clinical studies showing that inhibition of CTLA-4 and/or PD-1 signaling reverses T-cell exhaustion, limits tumor growth and improves survival have paved the way for the development of immunotherapy strategies based on the blockade of these immune checkpoint markers in a growing number of solid and hematological tumors.[11] Despite successful outcomes in different cancers and chronic viral infections, the blockade of CTLA-4 or PD-1 still remains ineffective for a significant number of patients and for certain tissue-specific tumors.

This observation suggests that the immune system has developed multiple, non-redundant inhibitory mechanisms to ensure self-tolerance. Other inhibitory receptors have been linked to T-cell exhaustion, as shown in **Table 1**.[10] Deciphering the mechanism*(s)* of action and synergy among these inhibitory receptors resulting in tumor growth and expansion has become an intensive area of research in immuno-oncology.

Studies in both mice and humans have shown complex patterns of inhibitory receptors' co-expression dependent on T-cell function and differentiation, viral infection severity and tumor aggressiveness.[12-15] Cumulative expression of up to five inhibitory receptors *(PD-1, BTLA, TIM-3, LAG-3 and CTLA-4)* was specifically associated with increased T-cell loss of function and tumor progression in non-small cell lung cancer.[16] These studies overall indicate that exhausted T cells are highly heterogeneous and a combinatorial analysis of multiple inhibitory receptors would enable a more precise assessment of T-cell dysfunction.

Inhibitory receptor	Cognate ligands
CD152 *(CTLA-4)*	CD80/CD86
CD279 *(PD-1)*	CD274/CD273 *(PD-L1/PD-L2)*
CD223 *(LAG-3)*	MHC class II
CD366 *(TIM-3)*	Galectin 9
TIGIT	CD112/CD155
CD244 *(2B4)*	CD48
CD160	CD270 *(HVEM)*
CD272 *(BTLA)*	CD270 *(HVEM)*
VISTA	VSIG-3
CD96	CD155

Table 1. List of inhibitory receptors upregulated on exhausted T cells, and cognate ligands expressed on antigen-presenting cells or tumor cells.

T-cell dysfunction is also responsible for poor efficacy of cellular immunotherapies relying, for example, on the use of chimeric antigen receptor T *(CAR T)* cells. Despite the success shown by clinical trials using CD19 CAR T cells for the treatment of B-cell malignancies, limited and inconsistent anti-tumor efficiency has been observed. This has been primarily attributed to the lack of in vivo persistency due to T-cell terminal differentiation and exhaustion associated with the composition and state of the starting cell population, activation protocols and CAR engineering and design.[17] A comprehensive immunophenotypic analysis of T-cell differentiation and expression of inhibitory receptors would therefore enable a deeper characterization of CAR T cells.

12-color flow cytometry panel design for the analysis of multiple T-cell inhibitory receptors in fresh and in vitro stimulated T cells.

Multicolor flow cytometry is exceptionally well-suited for a comprehensive immunophenotypic analysis of exhausted T cells because of its unique ability to simultaneously analyze the expression of multiple parameters at the single-cell level and delineate complex expression patterns in a single-tube assay. In order to demonstrate the validity and utility of a flow-cytometry-based assay, we have developed a 12-color panel for the simultaneous analysis of 6 inhibitory receptors associated with T-cell exhaustion within distinct T-cell subsets. In this study, we analyzed fresh, unstimulated peripheral blood mononuclear cells *(PBMCs)* as well as magnetically enriched T cells stimulated in vitro for 9 days with Dynabeads® Human Activator CD3/CD28 and human recombinant IL-2. The multicolor panel was designed following panel-design principles that take into account antigen co-expression and relative expression, fluorochrome brightness and spillover. High-performing antibodies and fluorochromes, in combination with the highly sensitive flow cytometer BD FACSCelesta™, ensured optimal resolution and quantitation of all the populations of interest. The multicolor panel included antibodies recognizing the inhibitory receptors CD279 *(PD-1)*, CD152 *(CTLA-4)*, CD223 *(LAG-3)*, CD366 *(TIM-3)*, CD272 *(BTLA)* and TIGIT ***(Table 2)***. The inclusion of CD4, CD8, CD45RA, CD197 and CD95 in the panel allowed for the assessment of the expression of each inhibitory receptor within different CD4 and CD8 T-cell subsets at different stages of differentiation. Either CD3 or the viability dye 7-AAD were included in the panel for the analysis of fresh PBMCs or cultured T cells, respectively.

Marker	Fluorochrome or dye	Clone	Cat. No.
CD3 or Live/Dead	BD Pharmingen™ PerCP-Cy™ 5.5 BD Pharmingen 7-AAD	UCHT1 N/A	560835 559925
CD4	BD Horizon™ APC-R700	RPA-T4	564975
CD8	BD Pharmingen APC-H7	SK1	560179
CD45RA	BD Horizon BV650	HI100	563963
CD197 *(CCR7)*	BD Pharmingen FITC	150503	561271
CD95	BD OptiBuild™ BV480	DX2	746675
TIGIT	BD Optibuild BV421	741182	747844
CD152 *(CTLA-4)*	BD Pharmingen PE	BNI3	555853
CD223 *(LAG-3)*	BD Pharmingen Alexa Fluor® 647	T47-530	565716
CD272 *(BTLA)*	BD OptiBuild BV605	J168-540	743986
CD279 *(PD-1)*	BD Pharmingen PE-Cy™ 7	EH12.1	561272
CD366 *(TIM-3)*	BD OptiBuild BV786	7D3	742857

Table 2. A 12-color flow cytometry panel was developed for the analysis of inhibitory receptors within fresh or cultured T-cell subsets on a BD FACSCelesta *(BVR configuration).*

The panel was designed following best panel-design principles. For the analysis of fresh, unstimulated PBMCs, CD3 was included in the panel to exclude contaminants such as monocytes and NK cells. CD4 and CD8 were used to broadly identify helper and cytotoxic T cells, respectively. CD45RA, CD197 and CD95 enabled the identification of naïve, stem cell memory, central memory, effector memory and effector memory RA T-cell subsets. CTLA-4, LAG-3, BTLA, PD-1, TIGIT and TIM-3 were included to determine the expression patterns of these inhibitory receptors within different T-cell subsets as well as their combinatorial expression. No viability dye was included as fresh, unstimulated PBMCs are mostly viable. For the analysis of magnetically-enriched and in vitro stimulated T cells, CD3 was substituted with the viability dye 7-AAD to exclude dead cells resulting from stimulation and in vitro culture. The magnetic pre-enrichment of T cells, together with the nonpermissive culture conditions, eliminated potential contaminants, hence the omission of CD3 from this panel.

Results

We first delineated the expression patterns of the six inhibitory receptors in fresh, unstimulated PBMCs from a healthy donor. **Figure 1A** shows a representative gating strategy for the identification of CD8⁺ naïve *(T_N)*, stem cell memory *(T_{SCM})*, central memory *(T_{CM})*, effector memory *(T_{EM})* and effector memory RA *(T_{EMRA})* cells based on the differential expression of CD45RA, CD197 and CD95, as previously described.[18] **Figure 1B** shows a representative analysis of the inhibitory receptor TIGIT within the CD8⁺ T-cell subsets. TIGIT is expressed by a discrete subset of total CD8⁺ T cells. Expression analysis within different subpopulations further revealed that TIGIT is not expressed on naïve cells, whereas its expression was observed in a subset of T_{SCM} cells and the percentage of TIGIT⁺ cells remained constant throughout differentiation. **Figure 1C** summarizes the individual expression analysis of each of the six tested inhibitory receptors within CD8⁺ T-cell subsets and shows unique distribution patterns. For example, BTLA is the only inhibitory receptor expressed by nearly all CD8⁺ cells in the different stages of differentiation *(from naïve to T_{EMRA} cells)*. Similar to TIGIT, CD8⁺ T cells expressed PD-1 at the T_{SCM} cell stage. The percentage of PD-1⁺ cells gradually increased as cells differentiated into T_{CM} and T_{EM} cells and eventually decreased in T_{EMRA} cells. Low percentage of TIM-3⁺ cells was only observed within CD8⁺ T_N and T_{SCM} cells, whereas no expression of LAG-3 and CTLA-4 was observed at any differentiation stage. A similar analysis was performed on cells stimulated in vitro for 9 days. Persistent stimulation resulted in an accumulation of cells phenotypically resembling T_{CM} cells, concomitant with a significant reduction in T_{SCM} and T_{EMRA} cells and complete depletion of naïve cells *(**Figure 1D**)*. T-cell subsets were defined using CD45RA, CCR7 and CD95 markers, and using the same gating strategy as for fresh, unstimulated PBMCs. Upon persistent stimulation, TIGIT was downregulated in total CD8⁺ T cells and subsets thereof, as compared to fresh PBMCs *(**Figure 1E**)*. **Figure 1F** shows the changes in the individual distribution of the inhibitory receptors tested within the different CD8⁺ T-cell subsets, as compared with fresh PBMCs. Notably, CTLA-4, LAG-3 and TIM-3 were upregulated upon persistent stimulation, although with different levels of expression in different CD8⁺ T-cell subsets.

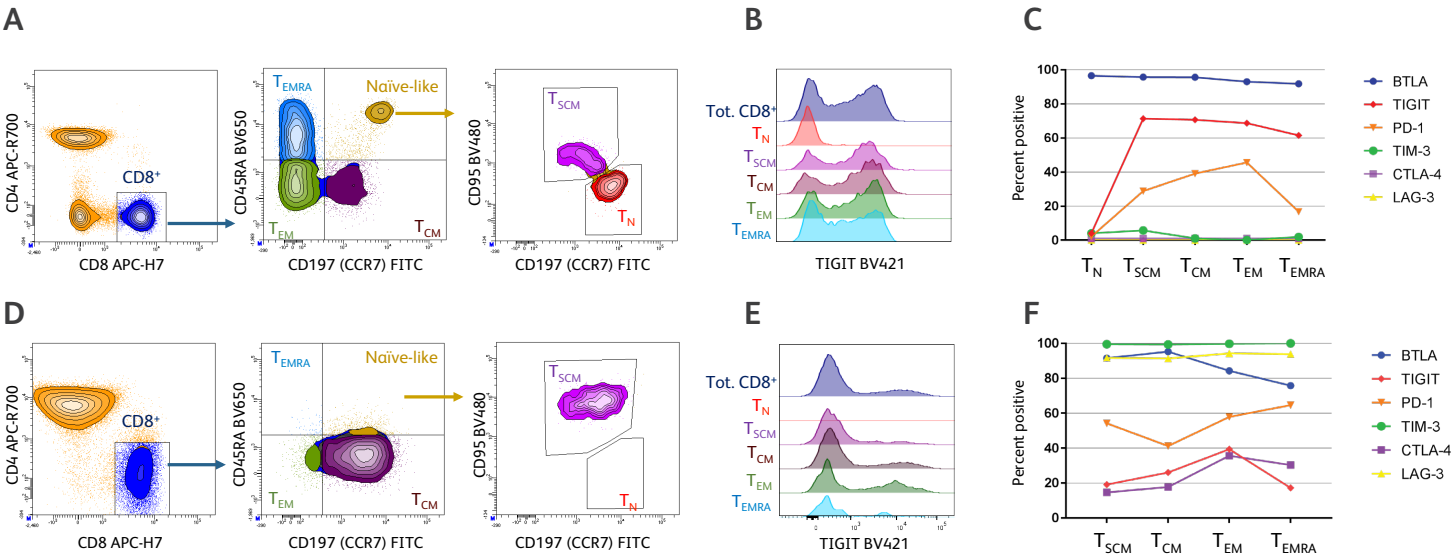

Figure 1. Analysis of the expression of multiple inhibitory receptors within distinct subsets of CD8⁺ T cells

Fresh PBMCs were isolated from a healthy donor and stained with the 12-color panel described in Table 1, including CD3 in place of 7-AAD. Not all the reagents were used at the recommended concentration as optimal assay- and sample-specific stain conditions were determined. Cells were not stimulated prior to stain and analysis. Cells were acquired on a BD FACSCelesta flow cytometer *(BVR configuration)*. Lymphocytes were first gated based on light-scatter properties and CD3 expression *(not shown)*. *(A)* Upon gating on CD8⁺ CD4⁻ cells, T_{CM}, T_{EM} and T_{EMRA} cells were identified based on the differential expression of CD45RA and CD197 *(CCR7)*. From the CD45RA⁺CD197⁺ naïve-like population, CD95⁺ T_{SCM} and CD95⁻ T_N cells were further detected. *(B)* Representative analysis of TIGIT expression within total CD8⁺ T cells and subsets thereof. *(C)* Representation of the distribution of cells expressing each inhibitory receptor, showing unique expression patterns throughout CD8⁺ T-cell differentiation. *(D-F)* T cells were magnetically enriched from the same PBMC preparation using the BD IMag™ Human T Lymphocyte Enrichment Set-DM and cultured for 9 days in the presence of Dynabeads® Human Activator CD3/CD28 *(1:1 ratio; Invitrogen)* and human recombinant IL-2 *(25 U/ml; Roche)*. At the end of the culture, cells were stained with the panel described in Table 1, including 7-AAD in place of CD3 for exclusion of dead cells *(not shown)*. T-cell subsets were defined based on phenotypic resemblance to fresh PBMC subsets. Persistent stimulation resulted in loss of naïve cells and significant decrease in T_{SCM} and T_{EMRA} subsets. Unique patterns of expression were observed for each inhibitory receptor, with overall upregulation induced by activation, as compared to fresh PBMCs, with the only exception for BTLA and TIGIT. This data shows the utility of a multicolor flow cytometry assay for the assessment of changes in T-cell differentiation, as well as expression and distribution of multiple inhibitory receptors within distinct T-cell subsets.

Figure 2 shows the analysis of the same data set using bivariate plots in order to assess the co-expression and combinatorial patterns of the tested inhibitory receptors in total CD8⁺ and CD4⁺ cells from unstimulated PBMCs and persistently stimulated T cells. Consistent with the data shown in **Figure 1C**, clear expression of TIGIT, PD-1, BTLA, but not TIM-3, LAG-3 and CTLA-4 was observed in total unstimulated CD8⁺ T cells (***Figure 2A***). The use of bivariate plot analysis further enabled the identification of distinct subpopulations expressing different combinations of inhibitory receptors. For example, within the total CD8⁺ T cells, distinct subsets of TIGIT⁺PD-1⁻ TIGIT⁺PD-1⁺ and TIGIT⁻PD-1⁺ were detected. Interestingly, persistent stimulation induced a reduction in the overall percentage of CD8⁺ TIGIT⁺ cells and a concomitant increase in the specific subset of CD8⁺ TIGIT⁺PD-1⁺ cells (***Figure 2B***). As expected, persistent stimulation resulted in increased percentage of CD8⁺ T cells expressing PD-1, LAG-3, TIM-3 and CTLA-4, whereas no change in the frequency of BTLA⁺ T cells was observed. Despite an overall increase in the percentage of CD8⁺ T cells expressing PD-1, TIM-3, LAG-3 and CTLA-4, we observed different patterns of expression for the different inhibitory receptors. While LAG-3 and TIM-3 were upregulated in nearly all CD8⁺ T cells, only a small subset of cells upregulated CTLA-4. Similar observations were made for CD4⁺ cells from unstimulated PBMCs and persistently stimulated T cells (***Figures 2C and D***). This data shows that different inhibitory receptors are differentially regulated and co-expressed upon in vitro persistent stimulation.

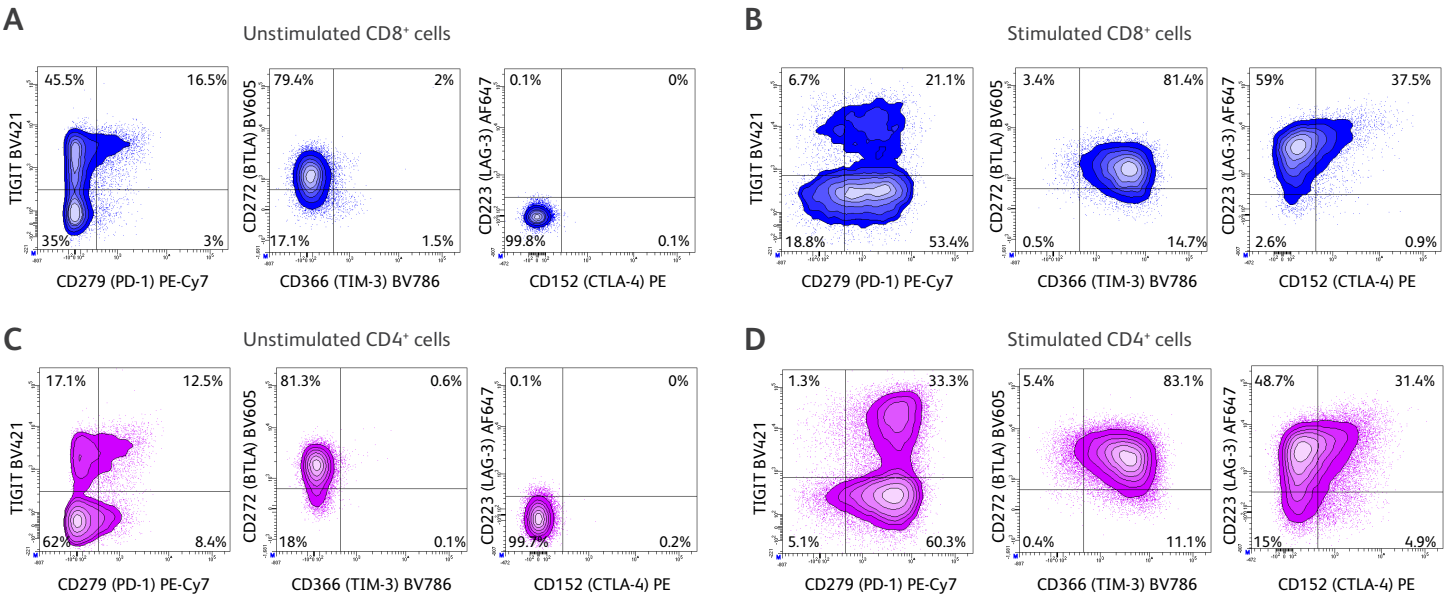

Figure 2. Co-expression patterns of inhibitory receptors in unstimulated and in vitro stimulated CD8⁺ and CD4⁺ T cells

Bivariate plot analysis was performed to identify subsets of total CD8⁺ T and CD4⁺ cells co-expressing inhibitory receptors within fresh, unstimulated PBMCs and T cells persistently stimulated in vitro with Dynabeads Human Activator CD3/CD28 and human recombinant IL-2 for 9 days. *(A)* Bivariate plot analysis provided information on the heterogeneity of CD8⁺ T cells co-expressing inhibitory receptors. For example, distinct subsets of cells expressing only TIGIT, only PD-1 or co-expressing both inhibitory receptors were detected. As shown in Figure 1C, nearly all CD8⁺ T cells expressed BTLA, whereas very little to no expression was observed for TIM-3, LAG-3 and CTLA-4. *(B)* More complex patterns of expression were observed upon persistent stimulation in vitro that resulted in differential regulation of the inhibitory receptors tested. For example, while the overall percentage of CD8⁺TIGIT⁺ cells decreased, an increase in cells co-expressing PD-1 and TIGIT was observed. Interestingly, only a small, discrete subset of CD8⁺ cells upregulated CTLA-4 expression, thus confirming the heterogeneity of cells persistently stimulated. *(C-D)* Similar observations were made for CD4⁺ T-cell subsets. Fresh and stimulated cells showed difference in autofluorescence, gates were determined for each sample using unstained and fluorescence minus one controls. The use of bivariate plots enables the identification of complex co-expression patterns of inhibitory receptors and highlights the heterogeneous phenotype of in vitro persistently stimulated T cells.

Conclusions

We have developed a 12-color flow cytometry panel that enables a comprehensive assessment of the expression of multiple inhibitory receptors associated with T-cell exhaustion within distinct T-cell subsets. Analysis of fresh, unstimulated PBMCs was performed to demonstrate the ability of the 12-color panel to identify the different T-cell differentiation subsets expressing different inhibitory receptors. Furthermore, an artificial in vitro system was utilized to mimic a chronic, persistent stimulation and induce upregulation of inhibitory receptors. While more comprehensive functional and genomic analyses would be required to confirm whether the in vitro persistent stimulation recapitulates a bona fide exhausted phenotype, this approach allowed us to demonstrate the utility of multicolor flow cytometry and the validity of our optimized panel for the delineation of complex, heterogeneous expression patterns and the quantification of cells cumulatively expressing multiple inhibitory receptors. The ability to simultaneously analyze six inhibitory receptors within five differentiation subsets of both CD4⁺ and CD8⁺ cells within a single tube assay, while retaining optimal resolution of all the populations of interest, provides a powerful tool for the efficient determination of the differentiation and exhaustion state of chronically stimulated T cells.

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