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Use of novel fluorescent dyes enables in depth analysis of checkpoint inhibitors CTLA-4 (CD152) and PD-1 (CD279) through spectral flow cytometry

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ABSTRACT

PD-1 (CD279) and CTLA-4 (CD152) are prominent checkpoint inhibitors in T cell responses and their inhibition has emerged as a successful route to boost anti-tumor immune responses in cancer patients. Clinical trials involving CAR-T cell therapy with anti-CTLA-4 and PD-1 antibodies seem to be improving the tumor directed T cell response.

Regulation of surface vs cytoplasmic CTLA-4 pools is crucial for balancing stimulatory and inhibitory signals in T-cell responses. Surface CTLA-4 contributes to tumor escape by inhibiting T cell responses, and upregulation of CTLA-4 expression from the cytoplasm to the surface is highly regulated. Therefore, it might be of interest to monitor these CTLA-4 pools as this might indicate the efficacy of CAR-T cell therapeutic approaches. Here we show how novel fluorophores enable the composition of a spectral flow cytometry panel to interrogate the presence of surface versus cytoplasmic CTLA-4 pools and the dynamics of PD-1 expression in a single panel. Combining these parameters into a single panel allows for less workflow steps and permit conservative sample use.

INTRODUCTION

T cell checkpoint inhibitors are an important target of immunotherapeutic approaches. CTLA-4 is one of the first checkpoint inhibitors that was targeted to treat cancer. In steady state it is stored intracellularly in T cells but upregulated to the surface of T cells upon stimulation. While binding of CD28 to costimulatory molecules CD80/86 mediates T cell activation, surface-bound CTLA-4 (sfCTLA-4) provides an inhibitory signal and essentially shuts down T cell responses. Consequently, sfCTLA-4 favors tumor escape by inhibiting anti-tumor T cell responses whereas treatment of T cell driven autoimmune responses calls for increased CTLA-4 signaling. Hence, monitoring the dynamics of sfCTLA-4 and intracellular CTLA-4 (iCTLA-4) pools in different scenarios could be of interest, e.g., to observe the efficacy of drugs aiming to modulate CTLA-4 surface expression or to interrogate the functionality of inhibitory CAR-T cells.

We composed a flow cytometry panel that enables differentiation between sfCTLA-4 and iCTLA-4 using our newly released Brilliant Ultra Violet and NovaFluor conjugated antibodies. As the optimum concentration of CTLA-4 BUV805 for staining sfCTLA-4 might not be sufficient to saturate all sfCTLA-4 molecules this might lead to a false CTLA-4 BUV737 signal from the remaining unbound sfCTLA-4 during the intracellular staining (Figure 1). To this aim, we developed a three-step protocol in which stimulated and unstimulated cells were stained for CTLA-4 using the same clone conjugated to two different fluorophores (Figure 2).

Our new products are geared towards more flexibility in panel design by reserving space against targets of interest that are conjugated to more traditional fluorophores, such as PE, Alexa Fluor 647, or APC-eFluor 780.

MATERIALS AND METHODS

Antibodies and buffers: All antibodies used are products of Thermo Fisher Scientific (see: Table 1) and were used at optimum concentration (5µL), unless stated otherwise. eBioscience™ Flow Cytometry Staining Buffer (part number: 00-4222-26), IC Fixation Buffer (part number: 00-8222-49) and Permeabilization Buffer (part number: 00-8333-56) were used for surface or intracellular staining, respectively.
Cell culture: Normal human PBMCs were isolated from whole blood and either stimulated for three days with PHA-L (part number: 00-4977-93) in complete RPMI or left untreated.
Flow Cytometry: Cells were incubated on ice with viability followed by surface staining with fluorophore-conjugated antibodies to CTLA-4, CD3, CD4, and PD-1. Cells were washed and then stained with purified CTLA-4. Lastly, cells were fixed and permeabilized before intracellular staining with CTLA-4 (Figure 3).

RESULTS

Figure 1. Reliable differentiation between surface and intracellular CTLA-4 pools requires saturation of sfCTLA-4

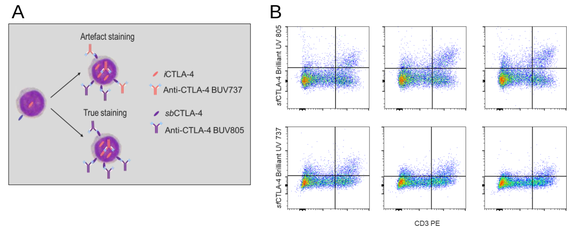

Figure 1. **A**. Unbound epitope after surface staining may cause false positive intracellular staining. **B**. Antibody titration is used to determine application dependent optimal concentrations of anti-human CTLA-4 (clone 14D3). Cells were surface stained with 1x, 2x, or 4x of the optimal concentration of CTLA-4 BUV805, followed by a second staining step with CTLA-4 BUV737 at the optimal concentration only. Higher concentrations of CTLA-4 BUV805 reduce CTLA-4 BUV737 signal (bottom) but also result in increased background.

Figure 2. Purified CTLA-4 antibody sufficiently blocks free sfCTLA-4 and minimizes non-specific signal

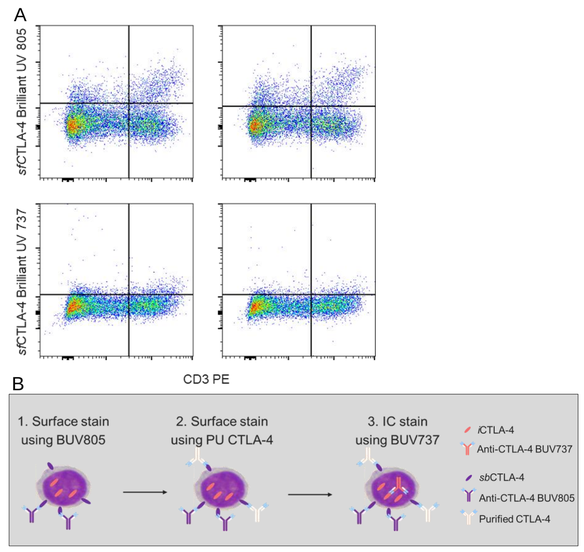

Figure 2. A. Antibody titration is used to determine the required concentration of purified CTLA-4 antibody to block unbound sfCTLA-4 before intracellular CTLA-4 staining. Cells were sequentially surface stained with CTLA-4 BUV805, followed by purified CTLA-4 at either 1.0 ug/mL or 2.0 ug/mL, and finally with CTLA-4 BUV737. **B.** Blocking with purified CTLA-4 after surface staining provides saturation of unbound sfCTLA-4 and prevents CTLA-4 BUV737 from binding to sfCTLA-4.

Figure 3. T cell stimulation increases PD-1 expression and both surface and intracellular pools of CTLA-4 in CD3⁺CD4⁺ subpopulation

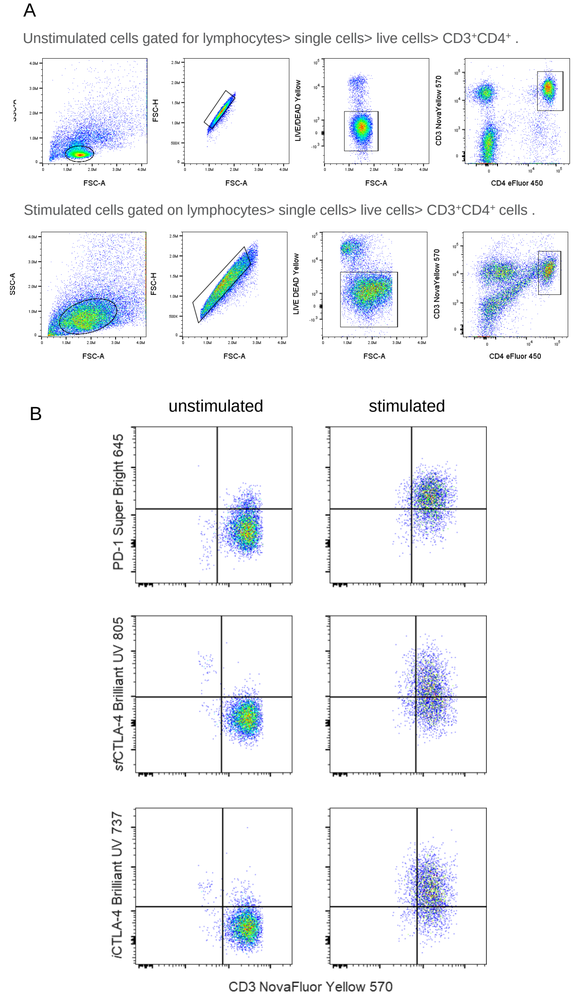

Figure 3. Cells were stained as described in the materials and methods. **A.** Gating strategy unstimulated and stimulated PBMC. **B.** Expression of PD-1 (top), surface CTLA-4 (middle), and iCTLA-4 (bottom) on CD3⁺CD4⁺ cells in unstimulated (left) or PHA-L-stimulated (right) PBMC.

Table 1. Antibody panel

Specificity	Fluorochrome	Catalog Number
CTLA-4	BUV 805	368-1529-42
CTLA-4	BUV 737	367-1529-42
CTLA-4	Purified	14-1529-82
PD-1	Super Bright 645	64-2799-42
CD3	NovaFluor Yellow 570	H002T03Y01
CD4	eFluor 450	48-0049-42
LIVE/DEAD Yellow	N/A	L34959

Table 1. Summary of antibodies and viability used for CTLA-4 panel

CONCLUSIONS

- Stimulation of PBMC with PHA-L increases both surface and intracellular pools of CTLA-4
- Optimal resolution of surface staining for CTLA-4 by flow cytometry is not saturating
- Differentiation of surface and intracellular pools of CTLA-4 is enabled by blocking residual, unlabeled surface CTLA-4 with purified antibody
- Differentiating between sfCTLA-4 and iCTLA-4 pools allows a more targeted approached to understanding drug efficacy for autoimmune disorders or CAR-T cell functionality in oncology research
- New fluorophores offered by Thermo Fisher Scientific, including Brilliant Ultra Violet 737, Brilliant Ultra Violet 805, and NovaFluor Yellow 570, add flexibility and expand the options for building multi-color panels

REFERENCES

Jago CB, Yates J, Câmara NO, Lechler RI, Lombardi G. Differential expression of CTLA-4 among T cell subsets. Clin Exp Immunol. 2004 Jun;136(3):463-71

ACKNOWLEDGEMENTS

Castle Funatake, Natalie Ruiz, Kanako Lewis for scientific feedback.

TRADEMARKS/LICENSING

SNAP ME FOR MORE INFORMATION

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