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

Nathalie Brambila, Jessica Ellsworth, Kendall DeFelippi, Jazmine Stenger-Smith, and Sonja Zahner, Thermo Fisher Scientific, 5781 Van Allen Way, Carlsbad, CA, USA, 92008

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 antitumor 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 responses. Regulation of surface vs cytoplastic 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 they might indicate the efficacy of CAR-T cell therapeutic approaches. Here we show how novel fluorophores enable the simultaneous visualization of surface and intracellular pools of the same epitope by comparing various cytoplastic CTLA-4 pools and the dynamics of PD-1 expression in a single panel. Combining these parameters into a single panel allows for a workflow step 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 is upregulated on the surface of T cells upon stimulation. While binding of CD80 to costimulatory molecules CD86 molecules T cell activation, surface-bound CTLA-4 (sCTLA-4) provides an inhibitory signal and essentially shuts down T cell responses. Consequently, sCTLA-4 favors tumor escape by inhibiting anti-tumor T cell responses whereas treatment of T cells with an antibody against sCTLA-4 results in increased CTLA-4 signaling. Hence, monitoring the dynamics of sCTLA-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 investigate the functionality of inhibitory CAR-T cells.

We composed a flow cytometry panel that enables differentiation between sCTLA-4 and CTLA-4 using our newly released Brilliant Ultra Violet and NovaFluor conjugated antibodies. As the optimum concentration of CTLA-4 (BUV737) for staining iCTLA-4 might not be sufficient to saturate all CTLA-4 molecules this might lead to a false positive iCTLA-4 signal from the remaining unbound sCTLA-4 during the intracellular staining (Figure 1). To this end, 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 targeted of interest that are conjugated to more traditional fluorophores, such as PE, Alexa Fluor 647, or APC-eFluor 750.

MATERIALS AND METHODS

Antibodies and buffers: All antibodies are products of Thermo Fisher Scientific (see Table 1) and were used at optimum concentration (μL), unless stated otherwise. eBioscience® Flow Cytometry Staining Buffer (part number: 00-4071-80) was 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: 10-4077-80) at 2.5 μM or untreated for 24 hours at 37°C.

Flow Cytometry: Cells were incubated on ice with viability followed by surface staining with fluorescein-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 4).

RESULTS

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

Figure 2. Purified CTLA-4 antibody sufficiently blocks free sCTLA-4 and maintains non-specific signal

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

CONCLUSIONS

• Simulation 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 unbound, unsurface BUV737 with purified antibody

• Differentiating between iCTLA-4 and sCTLA-4 pools allows a more targeted approach to understanding drug efficacy for autoimmune disorders or CAR-T cell functionality in oncology research

REFERENCES

ACKNOWLEDGEMENTS
Casie Furnalke, Natalie Ruiz, Karan Lewis for scientific feedback.

TRADEMARKS/LICENSING
For Research Use Only. Not intended for use in diagnostic procedures. © 2022 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.

SNAP ME FOR MORE INFORMATION

Table 1. Antibody panel

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog Number</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>BUV 93</td>
<td>386-1529-42</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>BUV 737</td>
<td>386-1530-42</td>
</tr>
<tr>
<td>PD-1</td>
<td>Purified</td>
<td>14-1529-82</td>
</tr>
<tr>
<td>Fluor-5</td>
<td>Super Bright</td>
<td>84-2799-42</td>
</tr>
<tr>
<td>CD4</td>
<td>NovaFluor 570</td>
<td>497-1529-11</td>
</tr>
<tr>
<td>CD4</td>
<td>eFluor 450</td>
<td>48-1299-42</td>
</tr>
</tbody>
</table>

Table 1. Summary of antibodies and usability critical panel

Figure 1. Unstimulated after surface staining may cause false positive intracellular staining A. Antigenic function is used to determine application-dependent epitope concentrations of anti-human CTLA-4 (clone 14G3). Cells were surface stained with 1x, 2x, or 4x of the optimal concentration of CTLA-4 (BVU93). Figure 2. Unstimulated and with optimal BUV737 signal (bottom) but also result in increased BUV737 signal. Figure 3. Unstimulated and with optimal BUV737 signal (bottom) but also result in increased BUV737 signal. Figure 4. Unstimulated only. Higher BUV737 signal (bottom) but also result in increased BUV737 signal.