Simultaneous Measurement of Cell Surface Markers with T-bet and Stat5 (pY694) in IL-2–Stimulated Human Whole Blood using BD Phosflow™ Lyse/Fix Buffer and BD Phosflow™ Perm Buffer III

Reagents Required

Reagent Full Name	Short Name	Catalog Number
Mouse Anti-Stat5 (pY694) Alexa Fluor® 647, Clone 47	Stat5 (pY694)	612599
Mouse Anti–T-bet PE, Clone O4-46	T-bet	561268
Mouse Anti-Human CD3 Alexa Fluor® 488, Clone UCHT1	CD3	567694
Mouse Anti-Human CD4 PE-Cy™7, Clone SK3	CD4	560909
Mouse Anti-Human CD45RO PerCP-Cy™5.5, Clone UCHL1	CD45RO	560607
Mouse Anti-Human CD45RA BD Horizon™ V450, Clone HI100	CD45RA	560363
BD Phosflow™ Lyse/Fix Buffer, 5X	Lyse/Fix Buffer	558049
BD Phosflow™ Perm Buffer III	Perm Buffer III	558050
BD Pharmingen™ Stain Buffer (FBS)	Stain Buffer	554656
Recombinant Human IL-2	IL-2	554603

Procedural Notes

Detailed procedures for reagent preparation, stimulation, fixation, permeabilization, and staining are described here: BD Phosflow™ Protocols for Human Whole Blood and Mouse Primary Cells.

Cells

Human whole blood was collected in the presence of EDTA or heparin.

Stimulation

Cells were either untreated or stimulated with various concentrations of recombinant human IL-2 (0.05, 0.1, 0.2, 0.5, 1, 5, 10, 50, or 100 ng/mL) for 15 minutes in a 37°C water bath.

Fixation and Permeabilization

Cells were fixed using Lyse/Fix Buffer and permeabilized using Perm Buffer III as described here:
BD Phosflow™ Protocols for Human Whole Blood and Mouse Primary Cells.

Staining with Antibodies for Intracellular and Cell Surface Markers

Cells were stained for 60 minutes at room temperature with the recommended or determined optimal concentration of each fluorochrome-conjugated antibody.

Antibody titration is critical for successful post-permeabilization staining of CD markers and other cell surface antigens. For more information, see the BD FACSelect™ Buffer Compatibility Resource (http://cytobank.org/facselect/) and the Tested Surface Markers chart (http://www.bdbiosciences.com/documents/antibodies_human_cellsurface_marker.pdf).

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The optimal concentration of each antibody used in this protocol is listed below. The 1X concentration represents cells stained with the recommended test size for live cell stains (see the product Technical Data Sheets for test size information). The 1/2X or 1/4X concentrations represent cells stained with 1/2 or 1/4 the antibody recommended for live cell stains.

Stat5 (pY694)	1X
T-bet	1X
CD3	1X
CD4	1X
CD45RO	1/4X
CD45RA	1X

Flow Cytometric Analysis of Stained Cell Samples

Flow cytometric analysis of the samples and controls was performed on a BD™ LSR II flow cytometer equipped with three lasers: a 488-nm blue laser, a 633-nm red laser, and a 405-nm violet laser. For each cell sample, ~100,000 events were collected in the lymphocyte gate. Prior to sample collection, fluorescence compensation settings were established using single-color BD™ CompBead control samples and the BD FACSDiva™ software compensation procedure.

Figure 1 shows one representative example of T-bet and Stat5 (pY694) signal analysis in stimulated human whole blood cells analyzed using this protocol.

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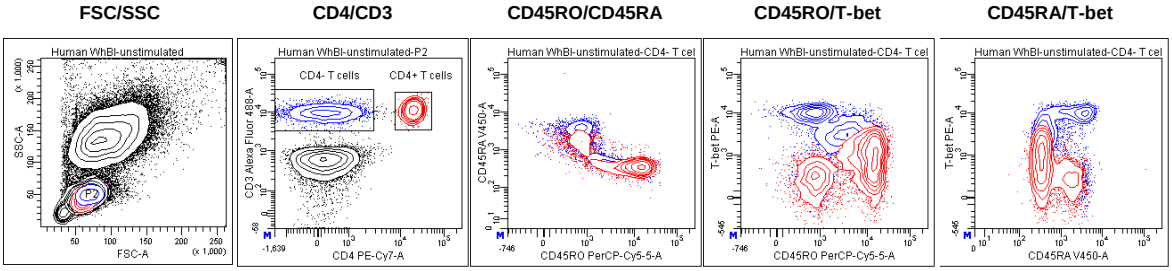
Figure 1

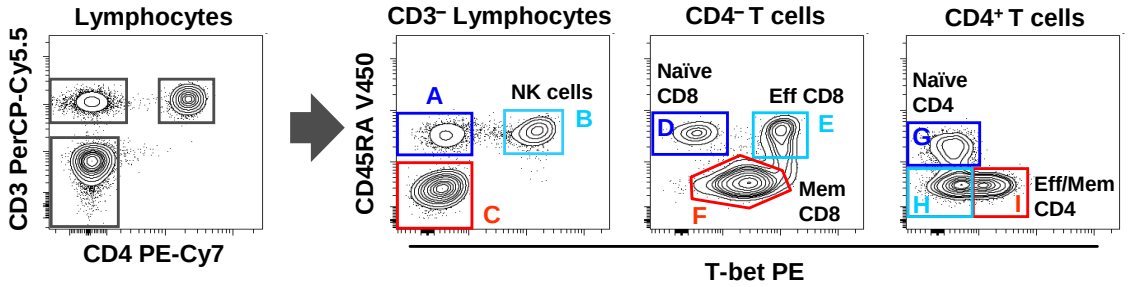
Human whole blood was either not treated or stimulated with various concentrations of IL-2 (0.05, 0.1, 0.2, 0.5, 1, 5, 10, 50, or 100 ng/mL) for 15 minutes at 37°C. Following stimulation, cells were fixed with Lyse/Fix Buffer, permeabilized with Perm Buffer III, and stained with optimal concentrations of antibodies against CD3, CD4, CD45RA, CD45RO, T-bet, and Stat5 (pY694). Approximately 100,000 events were collected in the lymphocyte gate for each sample.

A. CD45RA, CD45RO, and T-bet expression in $CD4^+$ T cells and $CD4^-$ T cells



A. Lymphocytes were identified based on scatter characteristics, and $CD4^+$ T cells and $CD4^-$ T cells were identified as $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ lymphocytes, respectively. Contour plots showing CD45RA, CD45RO, and T-bet staining patterns were generated for $CD4^+$ T cells (red) and $CD4^-$ T cells (blue) using BD FACSDiva analysis software.

B. Identification of lymphocyte subsets based on intracellular and surface protein expression



B. Lymphocytes were identified based on scatter characteristics, and $CD3^-$ lymphocytes, $CD4^+$ T cells, and $CD4^-$ T cells were identified based on expression of CD3 and CD4. Multiple lymphocyte subpopulations were identified based on expression of CD45RA and T-bet, including cells expressing the T-bet transcription factor at high levels (NK cells, population B; effector CD8 T cells, population E), cells expressing intermediate levels of T-bet (memory CD8 T cells, population F; Th1-like CD4 T cells, population I), and T-bet^- cells ($CD3^-$ lymphocyte populations A and B; naïve CD8 and CD4 T cells, populations D and G, respectively; non–Th1-like effector/memory CD4 T cells, population H). Subset identification was confirmed using additional surface markers, including CD45RO (data not shown). Contour plots were generated using Cytobank analysis software.

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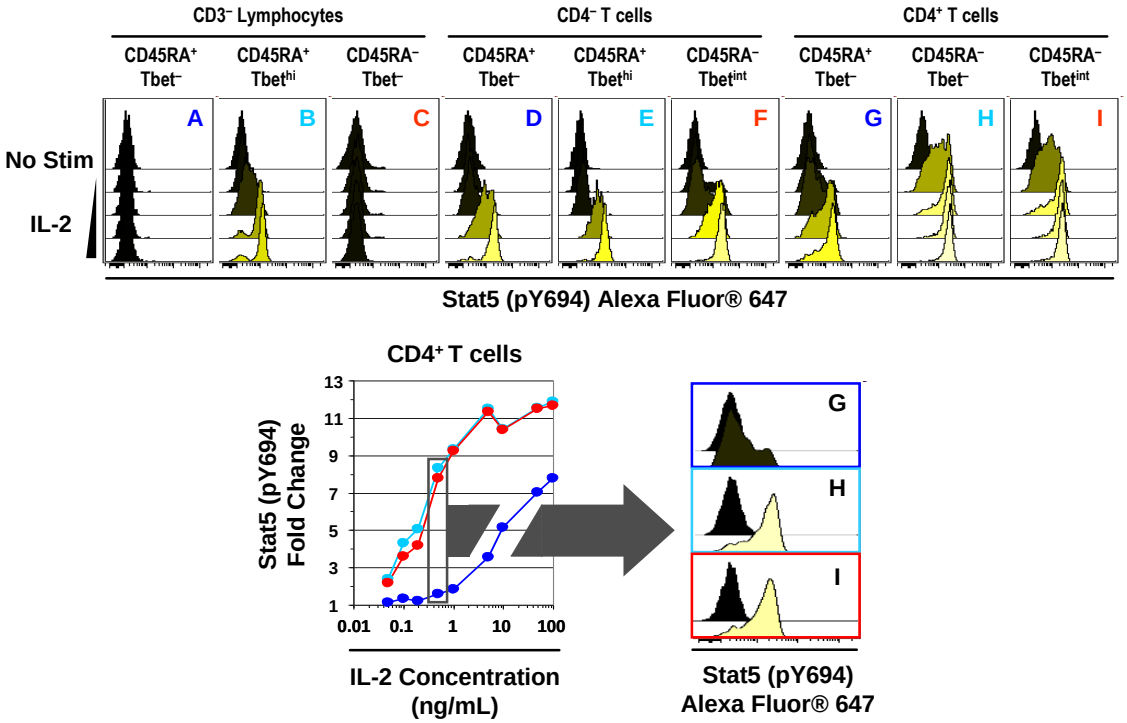
C. Dose-response analyses reveal differences in IL-2–induced Stat5 (pY694) phosphorylation among lymphocyte subsets



C. Stat5 (pY694) phosphorylation responses to IL-2 stimulation were assessed in each lymphocyte subpopulation. Dose-response analyses revealed differences in sensitivity to IL-2 stimulation. For instance, effector/memory CD4 T-cell subsets (populations H and I) responded to stimulation with very low concentrations of IL-2, whereas naïve CD4 T cells required higher IL-2 concentrations to induce Stat5 phosphorylation. Histogram overlays were generated using Cytobank analysis software.