NovoCyte® Flow Cytometer

Application Note No. 13

App Note No. 2

Determination of the Inhibitory Activity of Ibrutinib on Cytokine Secretion by Primary Monocytes Using a Bead-based Multiplex Assay

Flow cytometry is an established method used in bead-based multiplexing assays allowing for the simultaneous measurement of multiple targets in a single sample. Quantitative measurements of secreted and intracellular proteins, including cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins have broad applications in both basic research and clinical diagnostics. In this Application Note, we describe how to utilize the NovoCyte Flow Cytometer with a commercially available bead-based multiplexing assay designed to measure multiple human pro-inflammatory cytokines.

The Becton-Dickinson CBA Human Inflammatory Cytokines Kit (catalog no. 551811) provides a method for measuring multiple soluble analytes in a single sample using a mixture of bead populations of varying fluorescence intensities. Each bead population in the kit has been conjugated with a capture antibody specific for a single human cytokine. Cytokine-specific phycoerythrin (PE)–conjugated antibodies serve as detection reagents generating fluorescent signals proportional to the amount of bound analyte. After the capture beads and detection antibodies are incubated with samples containing soluble cytokines, the complexes are then measured using flow cytometry to resolve particles with fluorescence characteristics of both, the bead and the detection antibody.

ACEA NovoCyte Flow Cytometers combine outstanding detection sensitivity and resolution with multiple fluorescence detection channels, thus making quantification of soluble cytokines using a bead-based multiplex assay a straight forward approach. Using the BD CBA kit, we determined the cytokine secretion by primary human monocytes and the inhibitory activity of Ibrutinib on cytokine production. Ibrutinib is a highly selective Brutons tyrosine kinase (BTK) inhibitor, primarily used for the treatment of a rare and aggressive leukemia, mantle cell lymphoma. Ibrutinib irreversibly binds BTK in a variety of immune cells thereby inhibiting cytokine secretion. The CBA kit used in this study provides detection antibodies specific for the pro-inflammatory cytokines IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70.

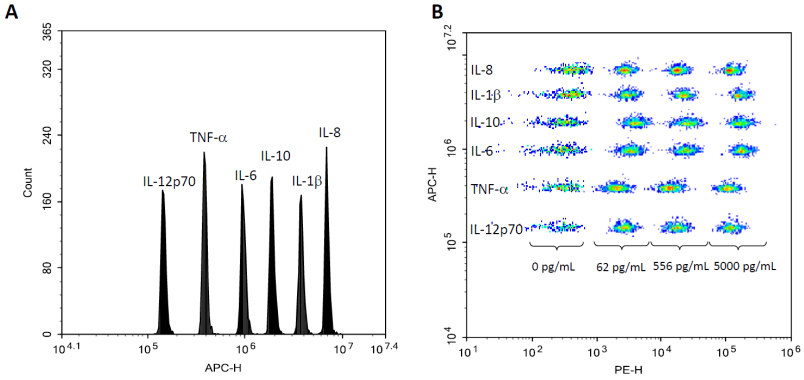

Figure 1. Resolution of bead populations on ACEA NovoCyte. A. Histogram plot of antibody-coated capture beads using the APC detection channel (Ex 640nm/Em 675nm). From left to right (lowest to highest APC MFI), beads coated with antibodies against IL-12p70, TNF-a, IL-6, IL-10, IL-1β, and IL-8, respectively. B. Two parameter plot of the indicated cytokines (APC channel) vs. increasing concentrations of the corresponding standard cytokines (PE channel, Ex 488 nm/Em 572 nm). Here, 4 different standard concentrations are overlayered where the PE MFI corresponding to different concentrations of diluted standards vs. APC fluorescence of the different bead populations is shown for a few standard concentrations. The fluorescence ranges of capture beads and analytes were well separated using the NovoCyte flow cytometer.

Cytokine Standard Curves

Representative plots identifying the cytokine specific beads based on APC fluorescence are shown in **Figure 1**. Detection reagents with PE fluorescence are used to detect cytokine abundance. To determine cytokine concentrations in unknown samples, a standard curve for each cytokine was generated using 1:3 serially diluted standards covering a concentration range of 20 to 5,000 pg/mL. and differing concentrations of analyte bound based on PE fluorescence are shown in As shown in **Figure 2**, a five-parameter curve fit was applied and the fitted curve was then used for extrapolating the concentration of each analyte in the sample. PE fluorescence intensity was determined to be proportional to the concentration of the cytokine standard and the R^2 value of each standard curve was above 0.999.

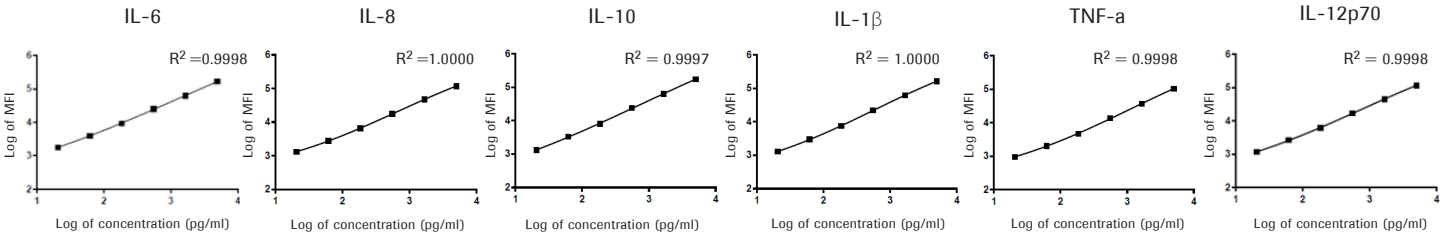

Figure 2. Cytokine standard curves generated using the NovoCyte. Initial data analysis was performed using ACEA's NovoExpress acquisition and analysis software. A standard curve for each cytokine was obtained by plotting the concentration of cytokine standard versus the MFI (mean fluorescence intensity) obtained with the NovoCyte. A curve was fitted to the data points and R^2 values are reported on each plot.

6779 Mesa Ridge Road Ste 100 San Diego, CA 92121 | 866.308.2232 | www.aceabio.com

NovoCyte® Flow Cytometer

Application Note No. 13

App Note No. 2

Effect of Ibrutinib on Cytokine Secretion

Monocytes were obtained from primary PBMCs using magnetic bead sorting (EasySep Human CD14 Positive Selection Kit, StemCell Technologies). Purified monocytes were then seeded in IgG coated plates to stimulate pro-inflammatory cytokine secretion and treated with different concentrations of the BTK inhibitor Ibrutinib. After 18 hours, supernatants were processed following the CBA protocol. Briefly, supernatants were mixed with the pool of antibody coated beads, washed, then added to the antibody-PE cytokine detection reagent, washed again, and analyzed on a NovoCyte flow cytometer to quantify secreted cytokines under different treatment conditions. As shown in **Figure 3**, Ibrutinib inhibited the secretion of IL-8, IL-1b, IL-6 and TNF-α in primary monocytes in a dose-dependent manner. The IC50 values for IL-8, IL-1β, IL-6 and TNF-α were at 2.5 uμ, 0.65 nM 0.28 nM and 0.69 nM, respectively (**Figure 3A**). IL-10 and IL-12p70 were not induced upon IgG stimulation. Raw data plots for the different treatment conditions are shown in **Figure 3B**. Interestingly, IL-8 production was induced by DMSO treatment alone and even more so with DMSO and IgG stimulation. Even though it is difficult to visualize using the log scale and high IL-8 background levels with DMSO alone, there was a dose dependent affect with increasing Ibrutinib concentrations **(Figure 3A&B)**.

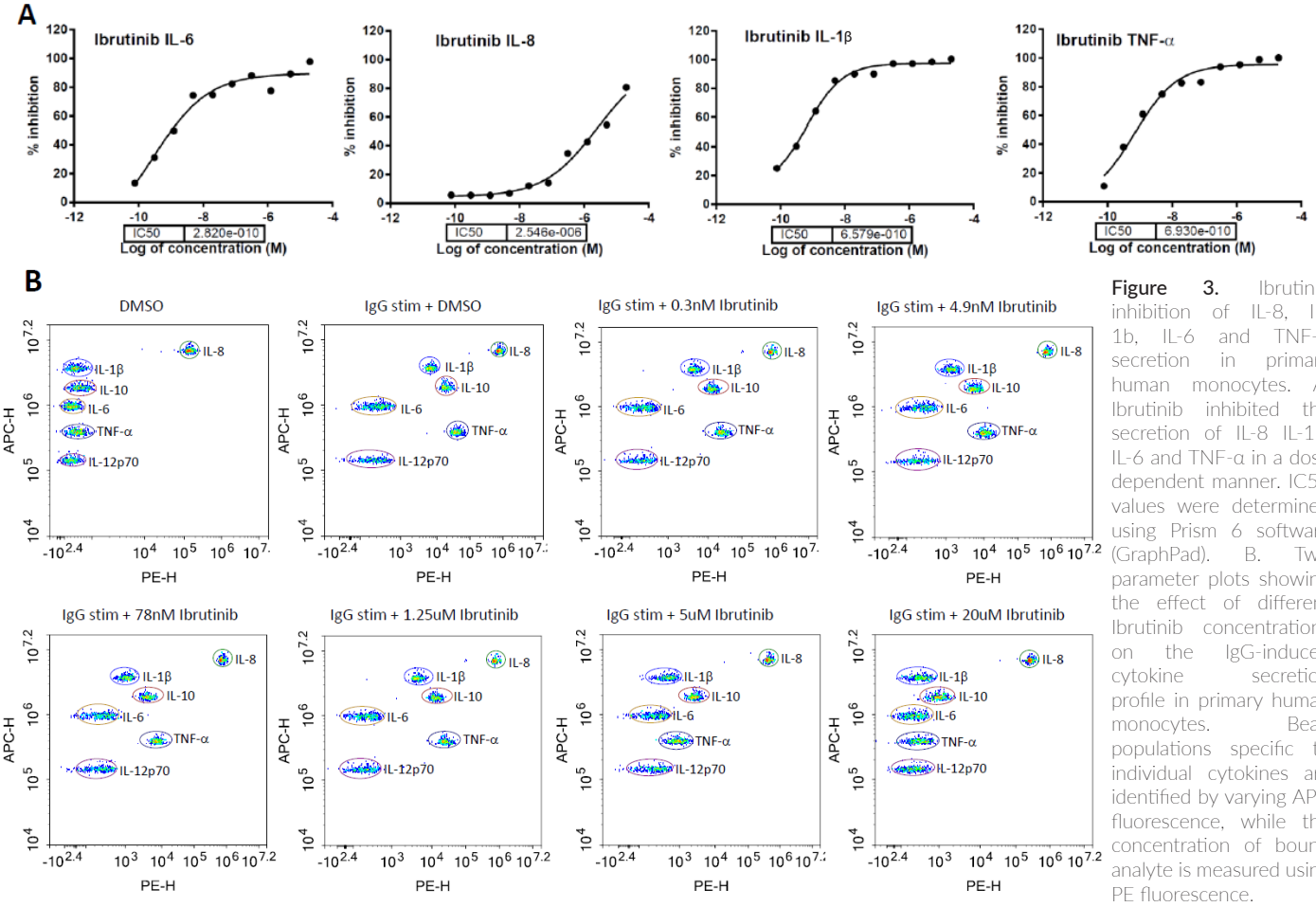

Figure 3. Ibrutinib inhibition of IL-8, IL-1b, IL-6 and TNF-α secretion in primary human monocytes. A. Ibrutinib inhibited the secretion of IL-8 IL-1β, IL-6 and TNF-α in a dose dependent manner. IC50 values were determined using Prism 6 software (GraphPad). B. Two parameter plots showing the effect of different Ibrutinib concentrations on the IgG-induced cytokine secretion profile in primary human monocytes. Bead populations specific to individual cytokines are identified by varying APC fluorescence, while the concentration of bound analyte is measured using PE fluorescence.

Effect of Ibrutinib on Cytokine Secretion

Flow cytometry is a more sensitive, powerful, and quantitative tool for measuring analyte concentrations than other currently available assays. In this study, we demonstrated the simultaneous analysis of six cytokines from cell culture supernatants and detected changes in concentration caused by the addition of a small molecule inhibitor. Using this method of analyte detection, research and diagnostic laboratories alike can increase their multiplexing capabilities and thereby decrease required sample volumes. Current commercially available kits for this type of assay can include up to 30 different analyte measurments simultaneously. Combining multiplexed analyte detection with an easy to use flow cytometer like the NovoCyte can provide faster and more accurate results.

For life science research only.
Not for use in diagnostic procedures.

For more information, visit www.aceabio.com or email info@aceabio.com