**Immunostaining**

Primary antibodies: murine anti-ABCD (IgG1κ; clone N45-961; BD Biosciences 535251), rat anti-EFGH (IgG1κ, Clone J58-61; BD Biosciences 616263).

Isotype controls: Murine and rat IgG1κ (BD Biosciences 595857 and 6060670 respectively).

Secondary antibodies: APC-conjugated goat anti-murine IgG (BD Biosciences B56749875); PE-conjugated rabbit anti-rat IgG (BD Biosciences B573672).

All antibodies were previously titrated using an 8-point, 2-fold serial dilution series. The starting dilution for each series was 1/50 (2.4 µL of the antibody in 120 µL of staining buffer). Subsequent dilutions were prepared by transferring 60 µL from the preceding dilution into 60 µL of fresh staining buffer. Each tube received 60 µL of cell suspension at a concentration of 1 x 10^6 cells/mL in staining buffer. The tubes were incubated on ice in the dark for 30 minutes. Subsequently, cells were washed by adding 3 mL of staining buffer to each tube, followed by centrifugation at 1500 rpm for 5 minutes at 4°C. The supernatant was carefully aspirated without disturbing the cell pellet. Cell pellets were resuspended in 100 µL of staining buffer. A minimum of 10,000 events were collected for each sample. The Mean Fluorescence Intensity (MFI) for each dilution was recorded. Data were plotted against the corresponding antibody concentrations to determine the optimal dilution, defined as the lowest concentration that achieved signal saturation

Peripheral Blood Mononuclear Cells (PBMCs) were fixed in a 4% paraformaldehyde solution prepared in phosphate-buffered saline (PBS) for 10 minutes at 4°C to preserve cellular morphology and maintain the protein epitopes. The cells were then washed twice with staining buffer: 1X PBS (Sigma-Aldrich D8537) containing 2% Fetal Bovine Serum (FBS; Gibco 10082-147), to remove excess fixative and to minimize nonspecific antibody binding. Subsequently, the cells were permeabilized for 30 minutes on ice using:

*OR BD™ Phosflow Perm Buffer III (BD Biosciences 567493-60)*

*OR 0.1% Triton X-100 in PBS*

*OR 0.1% saponin in PBS*

OR other,

allowing for the intracellular staining of proteins.

Following permeabilization, cells underwent Fc receptor blocking to prevent nonspecific binding of immunoglobulins to Fc receptors. This was achieved by incubating the cells with Human Fc Block (BD Biosciences 564220) for 15 minutes at room temperature.

After blocking, the cells were stained with primary antibodies or corresponding isotype controls to assess nonspecific binding for 1 hour on ice, then washed, and secondary antibodies were applied for 30 minutes on ice in the dark. Cells were then washed three times with PBS containing 2% FBS to remove unbound antibodies

 …. and resuspended in 100 μL of staining buffer.

*If you use live, non-fixed, non-permiabilized cells :*

…. and resuspended in 100 μL of staining buffer containing 10 μg/mL of cell non-permeable propidium iodide (or 2 um/ ml of DAPI ; or DRAQ7 etc) to allow exclusion of dead cells.

Prior to analysis, each sample was filtered through a 40 um nylon mesh ( U-CMN-40) using homemade filters.

**Fluorescence-Activated Cell Scanning (FACS; Flow cytometry)**

Flow cytometric analysis was performed using a an automatic 96-well-plate loader equipped CytoFLEX flow cytometer (Beckman Coulter Life Sciences) *Violet-Blue-Red (V-B-R) Series* equipped with 405 nm, 488 nm, and 638 nm lasers

*OR b) Blue-Red (V-B-R) Series* equipped with 488 nm, and 638 nm lasers

*OR c) CytoFLEX S Flow Cytometer (N-V-B-R) Series* equipped with 375 nm, 405 nm, 488 nm, and 638 nm lasers

Data acquisition was set to collect a minimum of 10,000 events per sample in the gate of single cells. As sheath fluid deionized water was used and the sample flow rate was 30 μL/min Forward scatter area (FSC-A); side scattered area (SSC-A) and SSC-width signals were used to establish the live gates to exclude debris, and cell clumps. Compensation controls were prepared using single-stained beads (CompBeads, Beckman Coulter Life Sciences A12345), single-stained samples, and fluorescence minus one (FMO) controls to correct for spectral overlap.

Data analysis was conducted using CytExpert software (Beckman Coulter), where gating strategies were applied to identify populations of interest based on forward and side scatter properties, as well as specific fluorescence intensities. The mean fuorescence intensity recorded for a particularly labeled blood cell was subtracted by the respective auto- fluorescence observed in the control.

*OR a)*Results are expressed as relative change in signal intensity compared to unstimulated control cells unless stated otherwise

*OR b)*Results are expressed as the percentage of positive cells.

*OR c)* The percentages of cells in different phases of cell cycle were calculated by ModFit software (Verity Software House, Topsham, ME).

*OR other*

The results were statistically analyzed using GraphPad Prism (GraphPad Software, Inc.) to determine the significance of the observed differences between experimental groups, employing appropriate statistical tests such as ANOVA or t-tests, as dictated by the data distribution and experimental design.