# Effects of Resting Cryopreserved PBMCs on Viability, Subset Frequency, Chemokine Receptor Expression, and Cytokine Secretion

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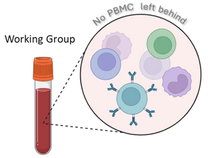


## BACKGROUND

Monitoring cellular immune responses during drug development and clinical trials is critical for assessing the safety and efficacy of therapeutic interventions. Flow cytometry–based intracellular cytokine staining (ICS) and ELISpot assays are widely used for this purpose, each with distinct advantages and limitations. Both platforms generally use cryopreserved peripheral blood mononuclear cells (PBMCs) as starting material, followed by stimulation with peptide pools for a defined period, and subsequent detection of cytokine secretion or production using either fluorescence-conjugated antibodies or enzyme-linked antibody–mediated colorimetric detection. An important topic of discussion concerns whether cryopreserved PBMCs should undergo a resting period following thawing prior to downstream functional assays.

Herein, PBMC resting periods of 0, 2, 6, 18, and 22 hours were investigated. At each time point, cell viability and recovery, early apoptotic and late apoptotic/dead cells, frequencies of major immune cell subsets (B cells, NK cells, NKT-like cells, total T cells, $CD4^+$ T cells, and $CD8^+$ T cells), chemokine receptor expression (CCR4, CCR6, CCR7, CXCR3, and CXCR5), and IFN-γ secretion were evaluated.

## METHODS

Cryopreserved PBMC samples were thawed in a 37 °C water bath, washed twice with RPMI 1640 complete medium, and resuspended in an appropriate volume of medium. Cell counts and viability were determined using an imaging-based cell counter (Cellaca MX) with AO/PI staining. Cell concentrations were adjusted to 1 × $10^7$ live cells/mL. Each sample was allocated to different assays, including flow cytometry immunophenotyping, ELISpot assay, and/or PBMC resting. Cell counts were performed after each resting period, followed by sample allocation for flow cytometry and cell stimulation for ELISpot analysis. For the ELISpot assay, each sample was plated at 2 × $10^5$ cells per well in triplicate and stimulated with mock control, CEF peptide pool, or anti-CD3 for 22 - 24 hours. ELISpot staining was performed using the Mabtech IFN-γ (HRP) ELISpot Plus kit, and spots were analyzed using the Mabtech IRIS 2 system to determine spot-forming units (SFU). In summary, at each resting time point, including no-resting samples, PBMCs were assessed for cell viability and recovery by cell counts, early and late apoptotic/dead cells, major immune cell subset frequencies, chemokine receptor expression by flow cytometry, and IFN-γ secretion by ELISpot assay.

## SAMPLES

Cryopreserved healthy donor PBMCs were sourced from BioIVT and performed in accordance with the human resources ethics committee.

## PANEL INFORMATION

**Table 1: Immunophenotyping Panel with Apoptosis Detection**

| Description | Vendor | Cat # | Clone | Purpose |
|---|---|---|---|---|
| LD Zombie NIR | BioLegend | 423105 | NA | Exclusion marker |
| Annexin V FITC | ThermoFisher | 88-8005-74 | NA | Apoptosis marker |
| CD45 Spark UV387 | BioLegend | 304085 | HI30 | Leukocyte marker |
| CD14 AF647 | BioLegend | 301818 | M5E2 | Monocyte marker |
| CD19 RB545 | BD Biosciences | 569195 | SJ25C1 | B cell marker |
| CD3 SB436 | Thermofisher | 62-0036-42 | SK7 | Pan T cell marker |
| CD8 BUV805 | BD Biosciences | 612890 | SK1 | CD8+ T cell marker |
| CD4 Spark Plus UV395 | BioLegend | 344627 | SK3 | CD4+ T cell marker |
| CD56 BUV563 | BD Biosciences | 612929 | NCAM16.2 | NK cells marker |
| CD16 Pacific Blue | BioLegend | 302024 | 3G8 | NK/monocyte subset marker |
| CD183 (CXCR3) PE Cy7 | BioLegend | 353720 | G025H7 | Chemokine receptor |
| CD185 (CXCR5) BV605 | BD OptiBuild | 569174 | RF8B2 | Chemokine receptor |
| CD194 (CCR4) BV785 | BioLegend | 359447 | L291H4 | Chemokine receptor |
| CD196 (CCR6) PE | BioLegend | 353409 | G034E3 | Chemokine receptor |
| CD197 (CCR7) BV750 | BioLegend | 353254 | G043H7 | Chemokine receptor |

## EXPERIMENTAL WORKFLOW

**Figure 1: An Overview of the Experimental Workflow**

| | Tue | | | Wed | | | | | | Thu |
|---|---|---|---|---|---|---|---|---|---|---|
| | Assays | | Procedure | Assays | | Procedure | | Assays | | Assays |
| | Cell thawing | Cell counting & Viability | Resting | Cell thawing | Cell counting & Viability | Resting | Stimulation for ELISpot | Cell counting & Viability | Immunophenotyping | ELISpot |
| 18hr Resting | 16:00 | | 17:00 | | | | 11:00 | 11:00 | 11:00 | 08:00 |
| 22hr Resting | | | 17:00 | | | | 15:00 | 15:00 | 15:00 | 14:00 |
| 2hr Resting | | | | 08:00 | | 09:00 | 11:00 | 11:00 | 11:00 | 08:00 |
| 6hr Resting | | | | | | 09:00 | 15:00 | 15:00 | 15:00 | 14:00 |
| 0hr Resting | | | | 10:00 | | 11:00 | 11:00 | 11:00 | 11:00 | 08:00 |

**Figure 1.** The experimental workflow was divided into two parts: one for assay preparation and the other for conducting the assays. The experimental design aimed to minimize the number of assays performed across all resting time points.

## RESULTS

**Figure 2: Cell Count, Viability, and Apoptosis at Different Resting Time**

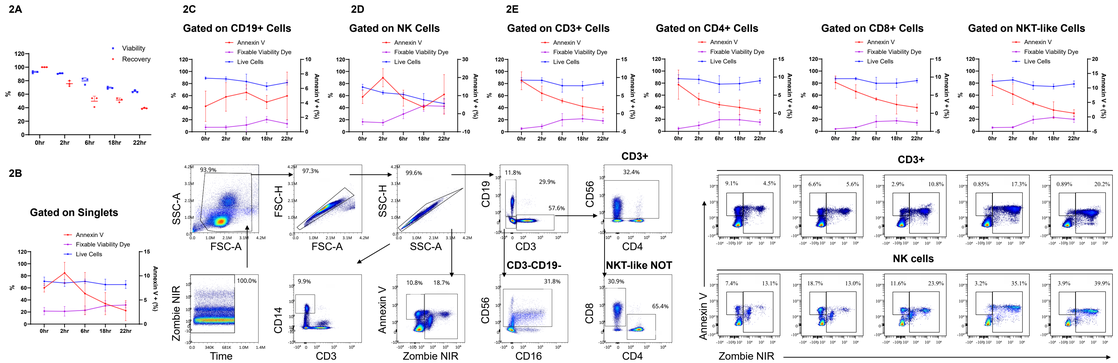


**Figure 2. (A)** Cell viability and recovery after different resting periods were assessed using an imaging-based cell counter. **(B - E)** Early apoptosis, late apoptosis/dead cells, and live cells were investigated on different subsets using a flow cytometry-based assay. The gating strategy for different subsets is shown in the middle of the figure. Representative 2D plots of showing Annexin V versus Fixable Viability dye in CD3+ cells and NK cells are shown in the lower right corner.

**Figure 3: Chemokine Receptor Expression at Different Resting Time**

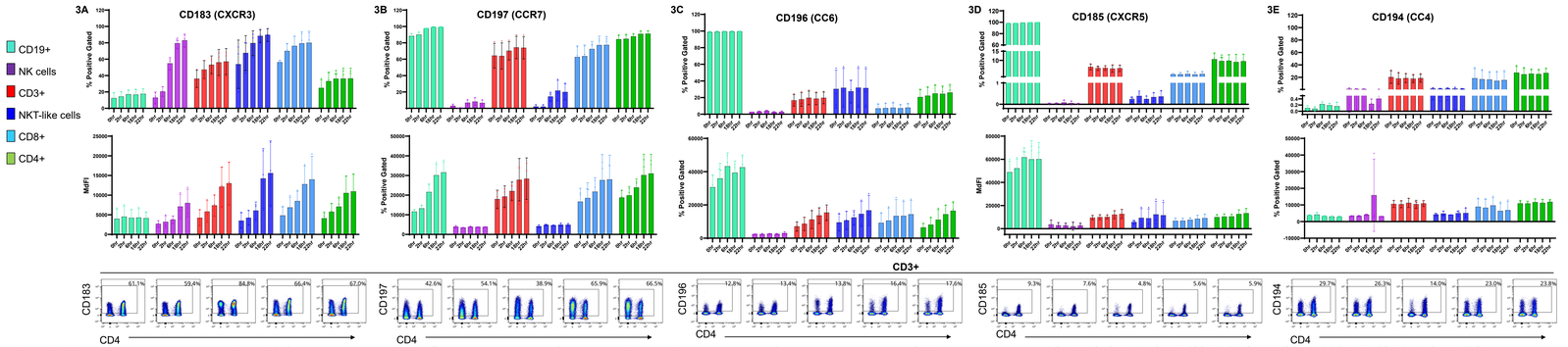


**Figure 3.** Both the percentage and signal intensity (MdFI) of each chemokine receptor on different subsets are shown after various resting times. Representative plots of each chemokine receptor on $CD3^+$ cells are also shown. (**A**) CD183 (CXCR3). (**B**) CD197 (CCR7). (**C**) CD196 (CCR6). (**D**) CD185 (CCR5). (**E**) CD194 (CCR4).

**Figure 4: IFNγ ELISpot Assay**

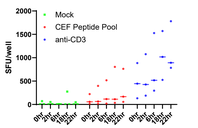


**Figure 4.** Spot-forming units (SFUs) are shown after 22–24 hours of stimulation, including mock control, CEF peptide pool, and anti-CD3, following different resting times.

**Figure 5: Correlations between Chemokine Receptor and IFNγ Secretion**

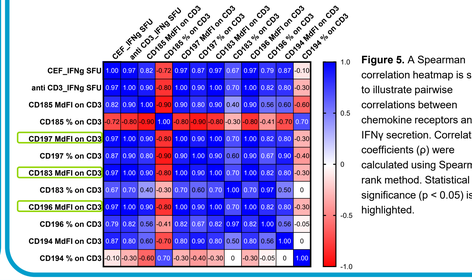


**Figure 5.** A Spearman correlation heatmap is shown to illustrate pairwise correlations between chemokine receptors and IFNγ secretion. Correlation coefficients (ρ) were calculated using Spearman's rank method. Statistical significance ($p < 0.05$) is highlighted.

## CONCLUSION

- Overnight resting of cryopreserved PBMCs improves functional assay quality, particularly for the IFN-γ ELISpot assay. During resting, early apoptotic cells were reduced, while live cell proportions remained stable across most subsets, although NK cells showed some decline after 18 hours. In addition, expression of certain chemokine receptors (CD183, CD197) increased after resting, which may partly explain the enhanced magnitude of responses after stimulation. Further investigation with more samples and varied stimulation conditions is warranted to strengthen these findings. Overall, these results support using an 18 - 22 hour resting period to optimize PBMC viability and functional responses.