

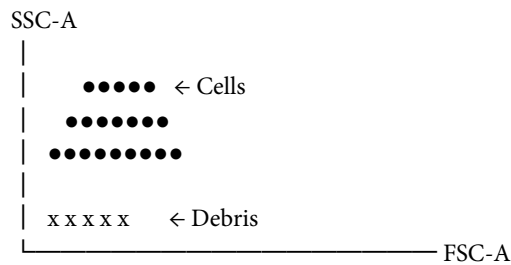
Supplementary Document:

Gating Strategy Used for Flow Cytometry Analysis

To ensure reproducibility and transparent interpretation, flow cytometry data were analyzed using a standardized, sequential gating strategy. All analyses were performed on compensated data, using fluorescence-minus-one (FMO) controls where applicable.

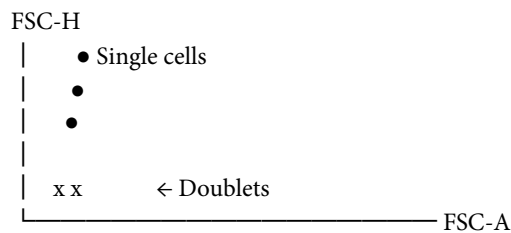
Step 1. Exclusion of debris

Initial gating was performed on **Forward Scatter (FSC-A) versus Side Scatter (SSC-A)** to exclude debris and non-cellular events, and the main cell population was selected based on size and granularity.



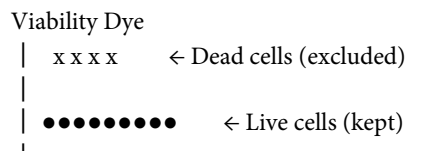
Step 2. Doublet discrimination

To ensure analysis of single cells only, **doublets were excluded** using FSC-A versus FSC-H (or FSC-W).



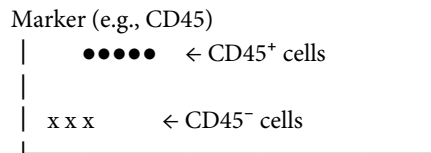
Step 3. Live/dead cell discrimination

Dead cells were excluded using a **viability dye** (Live/Dead Fixable dyes). Only **viability dye-negative cells** were included.



Step 4. Lineage or pan-population identification

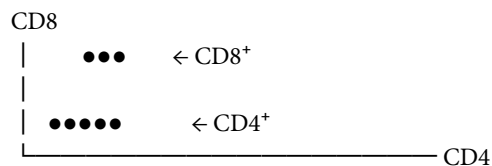
Live singlets were gated on a **lineage or pan-cell marker**, depending on the experiment (e.g., CD45⁺ leukocytes).



Step 5. Subpopulation identification

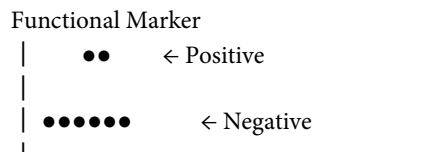
Within the parent population, sequential gates were applied to define specific subsets using combinations of markers. Example for immune profiling:

- CD3⁺ T cells
- CD4⁺ and CD8⁺ T cells
- Further functional or activation markers (e.g., PD-1)



Step 6. Functional or phenotypic analysis

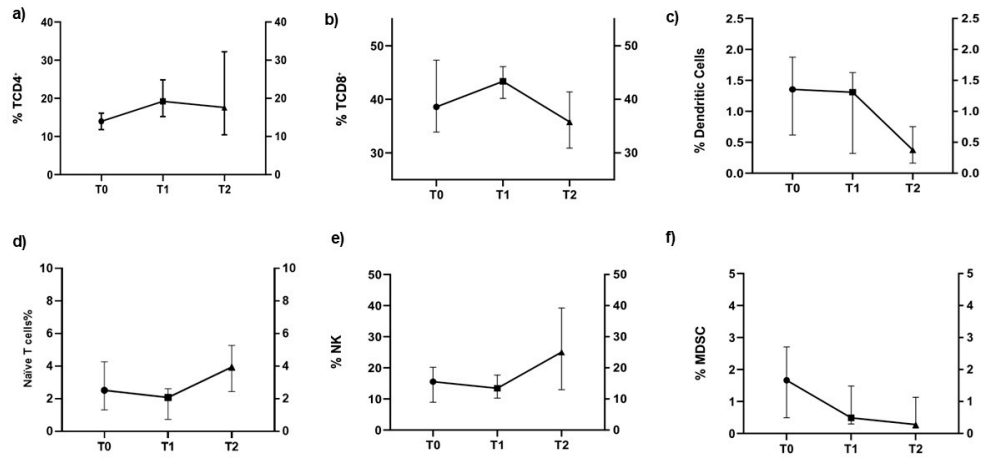
Functional markers (e.g., checkpoint molecules - PD-1) were quantified within the final gated populations. Gates were defined using **FMO controls** to minimize subjective thresholding.



Quality Control and Reproducibility Measures

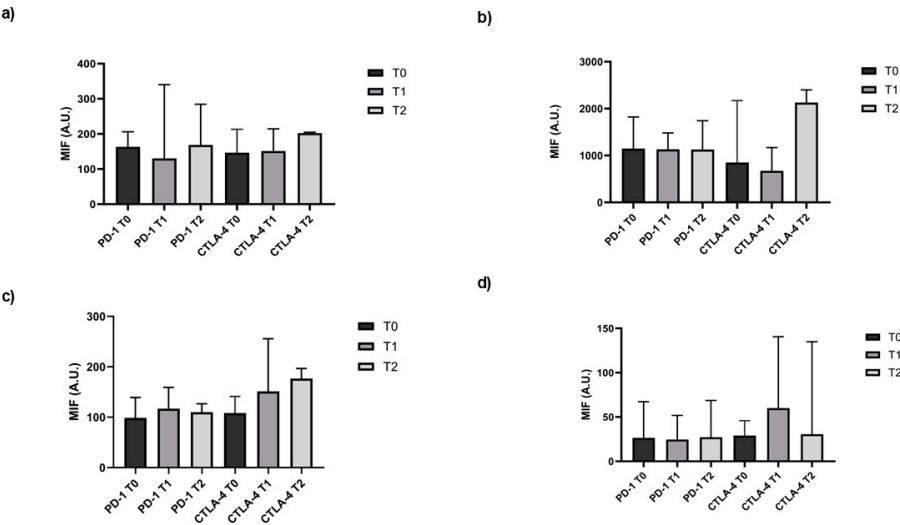
- Instrument performance was monitored using standardized calibration beads.
- Compensation matrices were generated using single-stained controls.
- FMO controls were used for all multicolor panels to define positive populations.
- Gating templates were applied uniformly across all samples.
- Analysis was performed using FlowJo, v11.0.2

S1. Changes in the frequency of circulating immune cell populations during intravesical BCG immunotherapy.



Panels A–F show the distribution of CD4⁺ T helper cells (A), CD8⁺ cytotoxic T cells (B), dendritic cells (C), naïve T cells (D), natural killer (NK) cells (E), and myeloid-derived suppressor cells (MDSCs) (F) in peripheral blood at three treatment timepoints: T0 (unexposed), T1 (partially exposed), and T2 (fully exposed) of BCG induction course. Data are presented as medians with corresponding interquartile ranges (25th to 75th percentiles).

S2. PD-1 and CTLA-4 Expression in CD8⁺ T Cells During BCG Therapy



Mean fluorescence intensity (MFI) of PD-1 and CTLA-4 on dendritic cells (A), naïve T cells (B), natural killer (NK) cells (C), and myeloid-derived suppressor cells (MDSCs) D) at baseline (T0), mid-treatment (T1), and after completion of BCG therapy (T2). Data are shown as median frequencies and interquartile ranges across treatment timepoints.