Detection of CD4-/CD8- Double Negative T-cells by Routine 6-color Flow Cytometry in Paediatric Patients: Technical Aspects

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Introduction. Double negative cells (DNC) are a small population of T-cells that express neither CD4, nor CD8. DNC are heterogeneous: they express TCRαβ, TCRγδ or are TCR-negative, may exhibit helper (particularly in AIDS) and regulatory and suppressive properties. Increased TCRαβ+ DNC are the marker of autoimmune lymphoproliferative syndrome in children. Little is known about the significance of DNC population in general paediatric practice.

Flow cytometry with at least 4 fluorochromes is necessary for DNC detection. BD Multitest 6-Color TBNK kit defines main T-cell subpopulations (CD3+/CD4+ and CD3+/CD8+) and double-positive CD3+/CD4+/CD8+ cells, and calculates an additional parameter – "T-sum" (TS) as the difference between the sum of the 3 mentioned populations and the total number of CD3+ T-cells. By definition, this parameter should be equal to DNC count. The software flags abnormal finding if TS is above 10%.

Aim. The aim of the study is to retrospectively analyze the TS parameter in paediatric lymphocyte subpopulations tests, to prove its relation to DNC and to check the pre-programmed critical value of 10%.

Material and Methods. 2563 consecutive paediatric samples (age 0–17) tested in 2012–2014 at the Children's Clinical University Hospital were assessed. The test was performed by BD FACSCanto II flow cytometer (Becton-Dickinson) with 6-Color TBNK reagent kit and BD FACSCanto Software. Files of tests with TS > 10% were evaluated by manual software INFINICYT v.1.5 (Cytognos) that had been designed for multicolor flow cytometry experiments. A sub cohort of 981 patients with normal blood counts and lymphocyte subpopulations was selected to determine reference values for DNC relative and absolute counts. IBM SPSS v.21 software was used for non-parametric analysis (Spearman for correlations, Wilcoxon for differences).

Results. TS was above 10% in 131 sample (5.0%). The files' analysis by INFINICYT revealed a DNC population in 129 cases (the automatic software failed in 2 cases of T-/B-/NK+ SCID, with high DNC counts due to false CD3 positivity). Discrepancy between automatic TS and manually defined DNC count was minimal, median difference 0.03×10^9/L (1.1% lymphocytes). Still, in 122 cases (94%) manual analysis returned higher counts ($p = 6 \times 10^{-16}$). In all cases, DNC formed a well-defined cluster on CD4/CD8 and CD19/CD16+CD56 plots, clearly separated from CD4+, CD8+ and B-cells and uniformly CD16+CD56-negative. DNC differed from the rest of T cells by higher FS (in 99%) and stronger CD3 expression (92%). Median FS difference was 5.5% ($p = 8 \times 10^{-14}$), median difference in CD3 fluorescence was 48.5% ($p = 1 \times 10^{-15}$).

Median DNC content in normal samples was 3.4%, 95% percentile range 0.8–11.2%; median absolute count 0.12×10^9/L, 95% percentile range $0.03–0.35 \times 10^9$/L. From entire cohort, 88 samples (3.4%) were outside the newly defined margin of 11.2%, and 122 samples (4.7%) had DNC above 0.35×10^9/L.

Conclusions. The study demonstrated that routine 6-TBNK test is suitable for detecting and counting DNC, with very few exceptions. Manual analysis proved the high precision of automatic DNC count as well as suggested that DNC by nature are rather a distinct subpopulation of T-cells than a maturation stage. The study results suggest that the pre-programmed critical limit of 10% may be too narrow for paediatric population, as well as established the previously unknown critical absolute level for DNC absolute count at 0.35×10^9/L.