Monitoring Human T Cell Activation in the Context of Immunotherapeutic Approaches
Determination of cell growth and proliferation using CASY

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Introduction

Immunotherapeutic approaches, such as the application of checkpoint inhibitors or adoptive T cell transfer are emerging strategies for cancer therapy (*1, 2, 3*). The success of these immunotherapies relies on optimal activation and expansion of T cells *in vitro* and *in vivo*.

The application of checkpoint inhibitors should overcome T cell anergy driven by the expression of inhibitory surface molecules on tumor cells (PD-L1) or T cells (PD-1, CTLA4) (*2*). However, it became more and more evident, that several tumoral factors, e.g. the metabolic tumor microenvironment, blunt the anti-tumor immune response of T cells (*4, 5, 6, 7, 8, 9*).

For the transfer of genetically engineered T cells into patients (adoptive T cell transfer), high numbers of viable and functioning T cells are required, expanded *in vitro* under optimal conditions (*10*).

When human T cells get activated they go through an initial growth phase - the so called "*on-blast*" formation, characterized by high cytokine production, followed by the clonal expansion phase. Measuring T cell growth and proliferation is a feasible method to monitor T cell stimulation when expanding T cells for adoptive T cell transfer. Moreover, those parameters also allow a first insight whether nutrient conditions found in the tumor affect T cell stimulation, thereby limiting the efficacy of checkpoint inhibitors.

Methods

Human T cells were isolated from peripheral blood mononuclear cells (PBMC) from healthy donors by leukapheresis, followed by density gradient centrifugation over Ficoll/Hypaque. Different cell populations of PBMCs (Figure 1, green line) were further separated by elutriation based on their cell size. Cell number and cell size were monitored in each elutriation fraction (Figure 1) to determine the fractions containing B cells (yellow line, mean diameter 7 µm), lymphocytes (red line, 8 µm) or monocytes (blue line, 10 µm). 10 µl of each fraction was diluted in 10 ml CASYton (dilution factor 1001) and measured. Left normalization cursor was set to 3.8 µm, right normalization cursor to 20 µm, left evaluation cursor to 6.0 µm and right evaluation cursor to 18 µm.

CD4 and CD8 T cells were isolated from the respective fraction (IIa) by magnetic bead separation (Miltenyi Biotec GmbH), achieving more than 98% purity.

T cells were activated by stimulation as described in (*11*). The impact of glucose deprivation was analyzed by T cell stimulation in glucose free RPMI 1640 (Sigma-Aldrich)

supplemented with all additives listed above including 10% AB serum. Mitochondrial ATP production was inhibited by applying the complex V inhibitor oligomycin (Sigma-Aldrich, final concentration 5 µM). T cells were activated in the presence or absence of 15 mM lactic acid (Sigma-Aldrich).

T cell growth and proliferation were monitored with the CASY TT (OLS) in a time dependent manner. After removing cells from the magnetic Dynabeads, 50 µl of cell suspension was diluted in 10 ml CASYton (dilution factor 201) and measured. Left normalization cursor was set to 4 µm, right normalization cursor to 20 µm, left evaluation cursor to 6.0 µm and right evaluation cursor to 20 µm.

Graphs were created using CASYworX 1.2 software (OLS).

Cluster formation was evaluated and documented by transmission light microscopy using the EVOS Xl (Thermo Fisher Scientific).
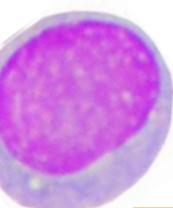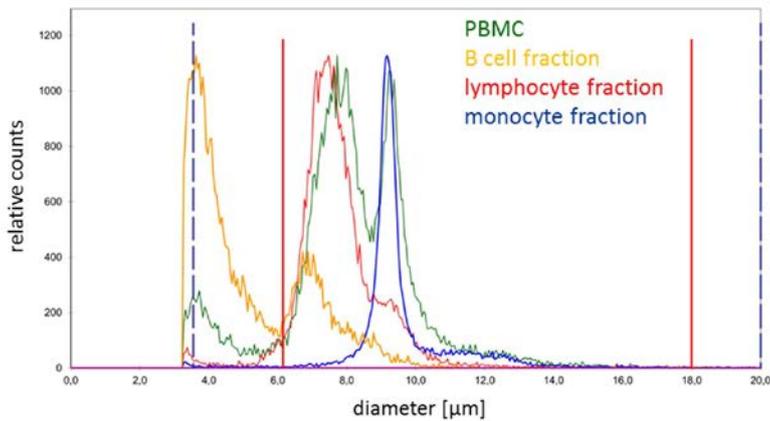
Fig. 1: **Determination of fraction containing T cells by monitoring cell size distribution with the CASY system.** Cell number and cell size distribution in single elutriation fractions. Cell size distribution of PBMC (green line), fraction I containing mainly B-cells (yellow line), fraction IIa containing lymphocytes (red line) and fraction III containing monocytes (blue line).
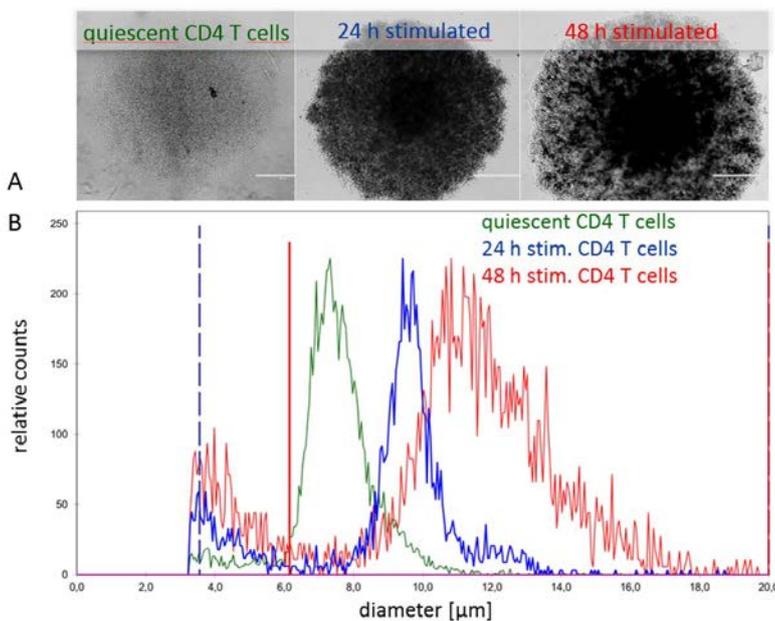

Fig. 2: **Cluster formation and cell size distribution during CD4 T cell stimulation.** CD4 T cell populations were stimulated with anti-CD3/CD28 Dynabeads. (A) Cluster formation in the initial phase of activation of human CD4 T cells. (B) CD4 T cells increased their cell size from 8 µm in freshly isolated quiescent T cells (green line) to 10 µm after 24 h (blue line) finally to 12 µm after 48 h of stimulation (red line). Shown are CD4 T cells of one representative donor.

- **Results**

Proliferation and Cluster Formation

When human T cells were activated they formed clusters and started to proliferate beyond 24 h (Fig. 2A). Cell size was increased from a mean diameter of 8 µm to 12 µm during the first 48 h. Fig. 2B shows cell size distribution of CD4 T cells of one representative donor over time.

After 48 h, T cells reached their final cell size and proliferation started. Figure 3 summarizes the data for CD4 and CD8 T cells of more than 10 different human donors, taken from (*11*) with kind permission. There was no difference between CD4 and CD8 T cells detectable.
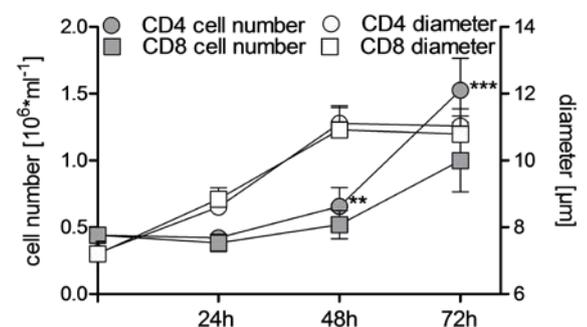

Fig. 3: **Cell growth and proliferation during the early phase of CD4 and CD8 T cell activation.** Cell size [µm] and cell number were analyzed with the CASY system in a time dependent manner. Data are shown as mean + SEM of n ≥ 10 different donors; * show significant differences between control and treatment. **$p<0.01$, ***$p<0.001$ (ANOVA and post hoc by Bonferroni's Multiple Comparison Test). Taken from Renner et al., Eur J Immunol. 45(9):2504-16 Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

T Cell Stimulation is Nutrient Dependent

Nutrient restriction, as lack of oxygen or low glucose levels, are frequently observed in tumors and might limit T cell activation. Moreover, high amounts of lactic acid (≥ 15 mM) are detected in different tumors due to their high glycolytic activity. We analyzed cluster formation, T cell growth and proliferation under conditions of glucose restriction, the accumulation of lactic acid and mitochondrial inhibition to mimic the situation in the tumor microenvironment. In CD4 T cells glucose restriction reduced "on-blast" formation only slightly (Figure 4A), however cell growth was almost blocked in the presence of 15 mM lactic acid, the glycolytic end product (Figure 4B). Inhibition of mitochondrial ATP production with oligomycin (oligo) resulted also in reduced CD4 T cell growth (Figure 4C). Cluster formation was reduced by lactic acid and by oligomycin treatment (Figure 4) when compared to 48 h control cultures (Figure 2A).

All conditions diminished proliferation after 72 h (starting concentration 0.4×10^6/ml) from 1.279×10^6/ml in control T cells to 0.48×10^6/ml under glucose deprivation, to 0.42×10^6/ml in the presence of 15 mM lactic acid and to 0.5076×10^6/ml by oligomycin treatment.

Fig. 4: **Cluster formation and T cell growth monitored under different metabolic conditions.** CD4 T cell populations were stimulated with anti-CD3/CD28 Dynabeads either (A) in the absence of glucose (w/o glucose, yellow line), (B) in the presence of 15 mM lactic acid (LA, yellow line) or (C) in the presence of oligomycin (oligo, yellow line). T cells stimulated under standard culture conditions for 24 h are displayed in blue and for 48 h in red.
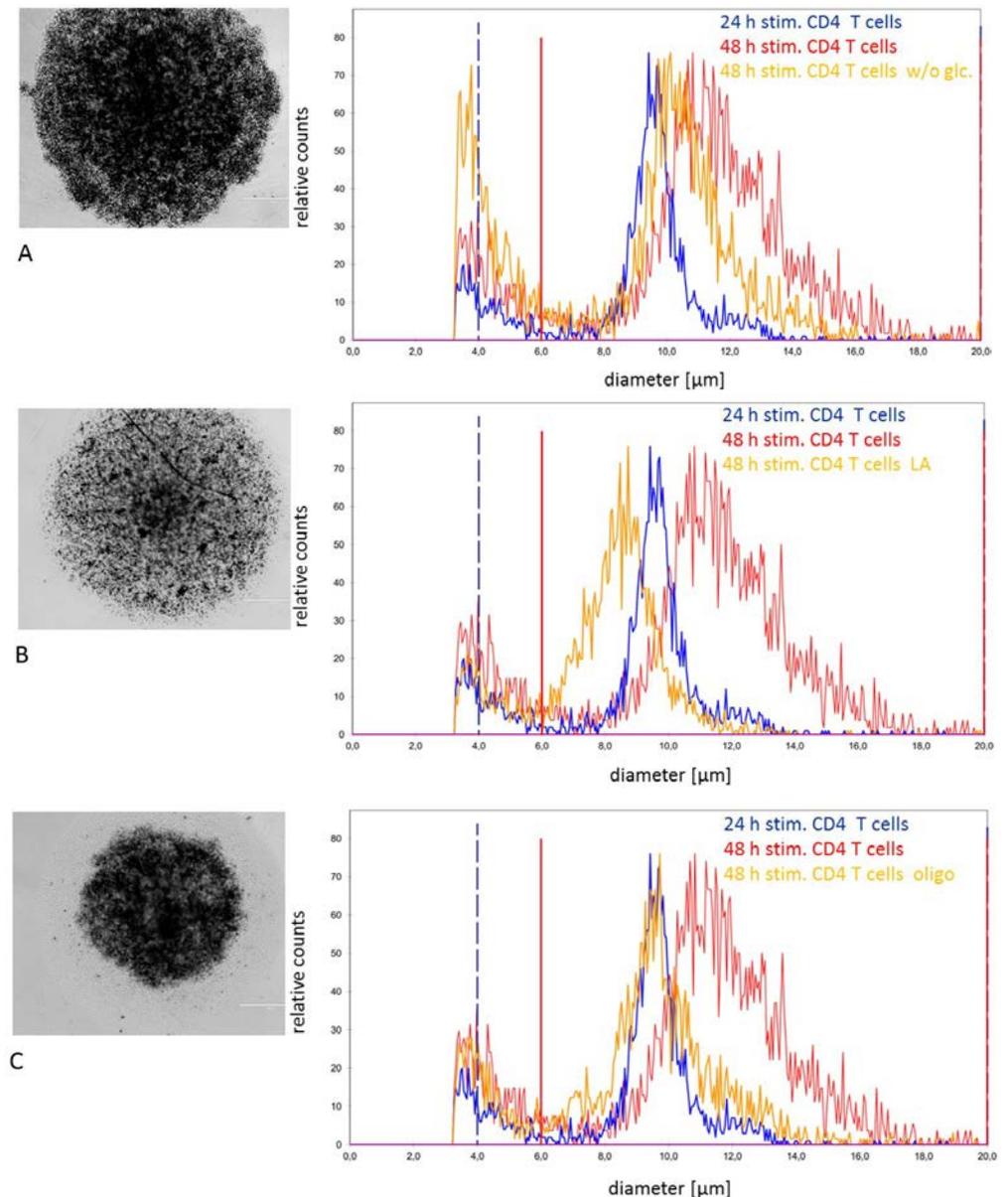
Summary

Monitoring Cell Size and Proliferation using CASY

T cell growth and proliferation are two important indicators for T cell function. The CASY system allows a reliable, reproducible and label free determination of both parameters providing first insights whether T cell function is preserved under pathophysiologic conditions.

Moreover, determination of cell size and proliferation are reliable indicators to assess the quality of T cell cultures during expansion for adoptive T cell transfer.

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