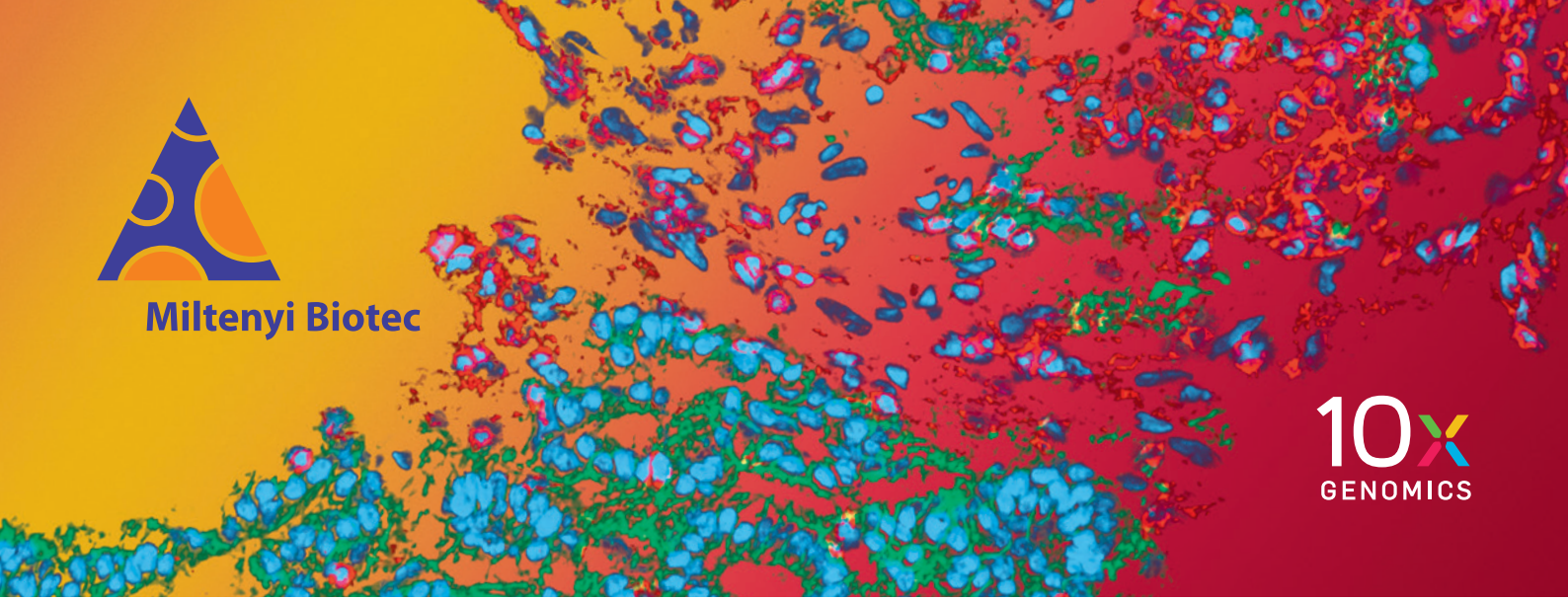

Isolation of TILs after tumor dissociation significantly improves the sensitivity of single-cell immune profiling

Anne Richter, Ramona Siemer, Elvira Criado-Moronati, Anna Baranska, Philipp Gert, David Agorku, Olaf Hardt, Daniela Vorholt, Aparajita Singh, Ruth Kläver, Christian Dose, Bianca Heemskerk, and César Evaristo
Miltenyi Biotec, Bergisch Gladbach, Germany

Background

Immunotherapy has proven clinical efficacy and has tremendous potential, but actual clinical benefit is experienced by only a small subset of patients. There is a growing necessity of additional research to improve beneficial clinical outcomes. It is particularly important to analyze steady-state anti-tumor immunity and monitor the effects of therapy on the tumor microenvironment, including tumor-infiltrating leukocytes (TILs). However, TIL numbers can be very low and small subpopulations might escape analysis when they are lost in background noise. This can be especially challenging when performing single-cell analysis. Moreover, a prerequisite for single-cell analysis is a debris free single-cell suspension with a viability above 80%.[1] Here we demonstrate, that our workflow solution, which includes tissue storage, tumor dissociation, dead cell removal, and cell isolation, yields enriched viable T and B cell populations that significantly increase single-cell immune profiling results.[2] An overview of the workflow is shown in figure 1.

Materials and methods

Tumor storage and dissociation materials

- MACS® Tissue Storage Solution
- gentleMACS™ Octo Dissociator with Heaters
- gentleMACS C Tubes
- Tumor Dissociation Kit, human

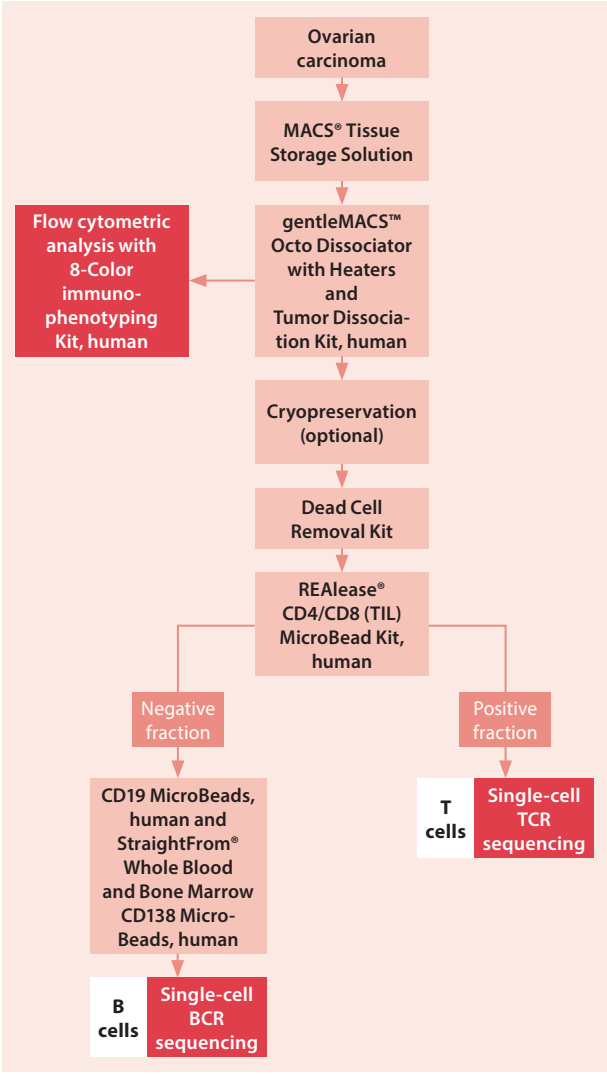

Figure 1: Overview of workflow for the preparation of immune cells for single-cell immune porfiling.

Sample cleaning materials
- MACS® SmartStrainers (70 µm)
- Dead Cell Removal Kit

Cell isolation
- REAlease® CD4/CD8 (TIL) MicroBead Kit, human
- CD19 MicroBeads, human
- StraightFrom® Whole Blood and Bone Marrow CD138 MicroBeads, human

Flow cytometry
- MACSQuant® Analyzer 16
- 8-Color Immunophenotyping Kit, human
- 7-AAD Staining Solution
- CD3-Viogreen, human (REA613) antibody

Single-cell receptor sequencing (10x Genomics)
- Chromium™ Controller instrument
- Chromium Single Cell 5' Library & Gel Bead Kit
- Chromium Single Cell 5' Library Construction Kit
- Chromium Single Cell V(D)J Enrichment Kit, Human T Cell
- Chromium Single Cell V(D)J Enrichment Kit, Human B Cell
- Chromium Single Cell A Chip Kit
- Chromium i7 Multiplex Kit

Tumor storage and dissociation
Human tumor tissue was stored in MACS Tissue Storage Solution and maintained at 4 °C overnight until further processing. Tumors were dissociated using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, human, as per manufacturer's instructions.

TILs isolation
One aliquot of the dissociated tumor sample was used for the analysis of immune cells with the 8-Color-Immunophenotyping Kit. The remaining cell suspension was cryopreserved in vials containing 1×10^7 cells to assess the impact of freezing on the workflow. The freezing protocol followed has been optimized for the preservation of immune cells. Later, 1×10^7 cells were thawed, dead cells were removed using the Dead Cell Removal Kit, and T cells were isolated using the REAlease CD4/CD8 TIL MicroBead Kit. The T cell depleted fraction was used for sequential B cell isolation using CD19 MicroBeads, human and StraightFrom Whole Blood and Bone Marrow CD138 MicroBeads, human.

Flow cytometry
One aliquot of the dissociated tumor sample was stained with the 8-Color Immunophenotyping Kit, human to determine the immune cell composition. Aliquots of thawed cells prior to and after dead cell removal and isolation of CD4/CD8 positive cells were stained for flow cytometric analysis with CD3-Viogreen antibody and 7-AAD according to the datasheets.

Single-cell receptor sequencing
Both isolated T and B cells, as well as unseparated bulk samples were subjected to single-cell TCR- or BCR-sequencing using the 10x Genomics Platform and the Chromium Single Cell V(D)J Reagent Kits according to the manufacturer's instruction. The target cell number for sequencing was 10,000 cells per sample. Sequencing was performed on the MiSeq™-System (Illumina).

Results

Immunophenotyping of TILs by flow cytometry reveals low T and B cell frequencies
In the dissociated bulk tumor sample only 14% of the cells were leukocytes. Using the appropriate gating strategy, leukocytes were further subclassified as T cells, B cells, neutrophils, eosinophils, and NK cells.

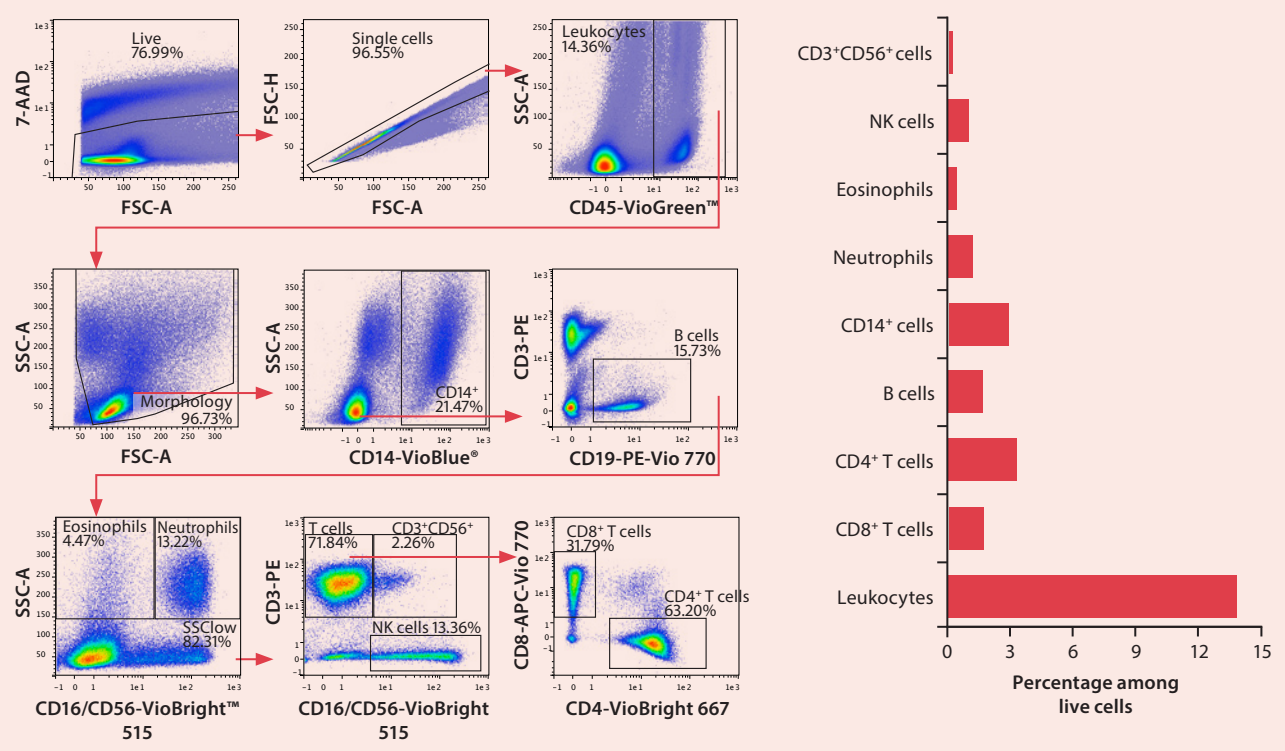

Figure 2: Flow analysis of immune cell composition in a dissociated tumor sample using the 8-Color Immunophenotyping Kit.

Magnetic isolation of TILs increases the sensitivity of single-cell receptor sequencing and immunoprofiling

For the single-cell immune profiling assay, the frozen tumor cell suspension was thawed and dead cells were removed. The percentage of viable cells in the sample was increased from only 26% prior to dead cell removal to over 80%. After dead cell removal, T cells were enriched to over 80% using REAlease® CD4/CD8 (TIL) MicroBeads (fig. 3). B cells were subsequently isolated from the T cell depleted fraction by labeling the cells with CD138 and CD19 MicroBeads (data not shown).

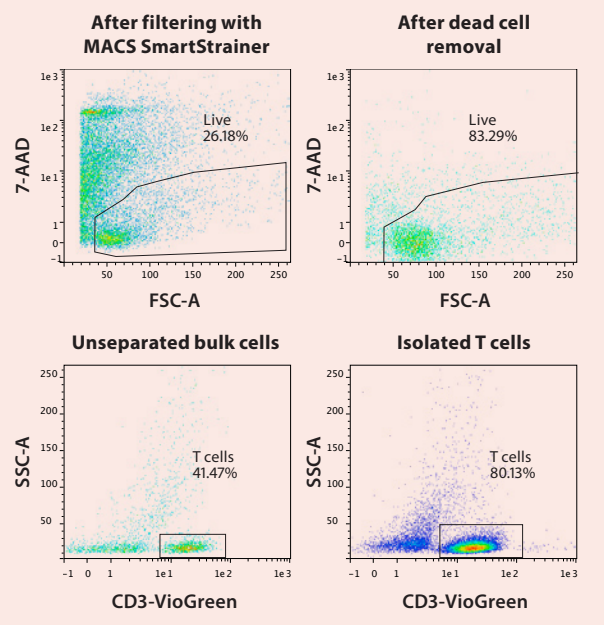

Figure 3: Thawed cells from a dissociated tumor sample were applied to dead cell removal using the Dead Cell Removal Kit (upper panel). Subsequently, CD4/CD8 positive cells were isolated using the REAlease CD4/CD8 (TIL) Microbead Kit, human (lower panel). B cell were isolated from the T-cell depleted fraction, using CD19 MicroBeads, human and StraightFrom Whole Blood and Bone Marrow CD138 MicroBeads, human (data not shown).

The clonality of TILs was assessed via single-cell receptor sequencing, either performed with unseparated bulk cells or with isolated TILs. The comparison proved that the magnetic isolation of TILs improved the resolution of the RNA sequencing, highlighting clonotypes otherwise poorly or altogether unrepresented. In figure 4, the top 50 TCRβ (A) and BCR (B) CDR3 clonotypes identified via RNA sequencing in the isolated TIL population (purple) are represented together with the corresponding number of cells. The red bars show the number of cells that showed the same TCRβ (A) and BCR (B) CDR3 clonotypes in the bulk sample.

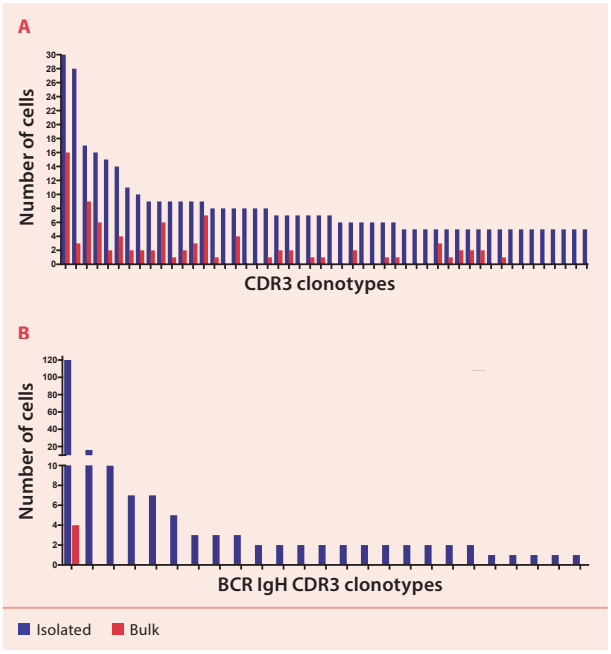

Figure 4: Single-cell TCRs (A) and BCRs (B) were sequenced prior to and after the isolation of T or B cells from a tumor sample. The graphs show the top 50 TCR (A) and top 25 BCR (B) CD3 clonotypes ranked by their abundancy in the isolated T or B cell fraction (blue). The red bars show the number of cells containing the same CDR3 clonotype of the respective receptor in the bulk sample.

Conclusions

- T cell isolation significantly reduces the time required for flow cytometric analysis and flow sorting of rare cell populations.
- We developed an innovative workflow to improve single-cell sequencing of TILs, which can significantly increase the quality of the data obtained in immuno-oncology and immunotherapy research.
- Single-cell analysis requires a cell viability above 80%. For samples with low viability, this can be easily achieved with the Dead Cell Removal Kit.

References

1. Application Note by Miltenyi Biotec in collaboration with 10x Genomics: Dissociation of tumor tissue samples for single-cell genomics.
2. Richter, A. *et al.* "Complete workflows allow comprehensive tumor microenvironment analysis and culture of cell subsets of limited tumor patient samples." In: Proceedings of the American Association for Cancer Research Annual Meeting, Atlanta, Mar 29–Apr 3, 2019, 79 (13 Suppl): Abstract nr LB-138. Philadelphia, Cancer Res, 2019.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macs@miltenyibiotec.de | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

V.01