Complete workflows allow comprehensive tumor microenvironment analysis of cell subsets from limited tumor patient samples

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 GmbH, Bergisch Gladbach, Germany

Introduction

Immunotherapy has proven clinical efficacy and tremendous potential, but clinical benefit is experienced by only a subset of patients, such that additional research is necessary to improve 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. Flow cytometry–based phenotyping of different cell populations requires dividing limited tumor material into multiple samples, thus reducing the number of cells available for analysis. Therefore, innovative tools and workflows are needed to maximize the quality of data obtained from limited tumor patient samples.

Materials and methods

1 Isolation and characterization of TIL subsets from limited tumor samples

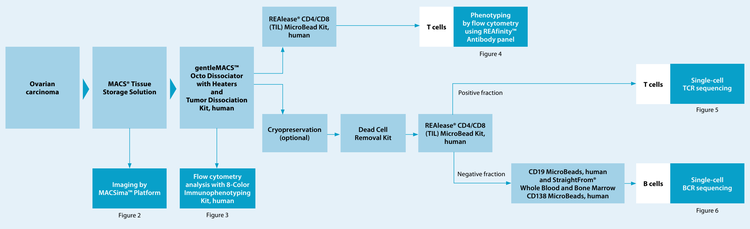

Figure 1

Results

1 Characterization of tumor microenvironment by immunohistochemistry, using the MACSima™ Imaging Platform

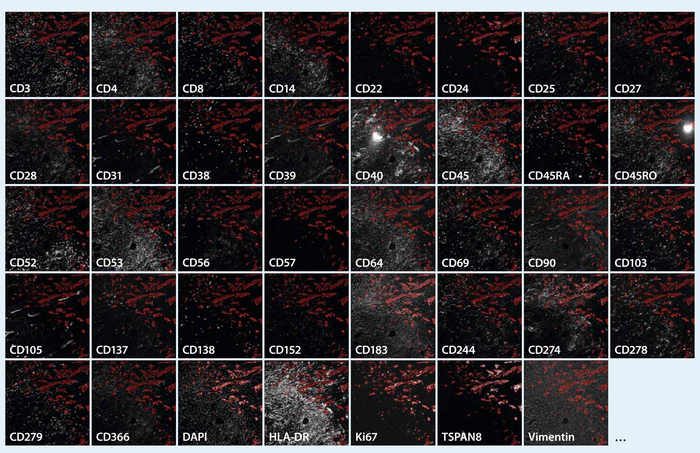

Figure 2

One tissue section was snap-frozen, acetone-fixed, and analyzed by immunohistochemistry using the MACSima™ Imaging Platform. The underlying MICS (multiparameter imaging cell screen) technology enables staining of hundreds of markers on a single sample. It uses the principle of an iterative staining process comprising three main steps, i) fluorescent staining, ii) image acquisition, and iii) erasure of the fluorescence signal, all of which are conducted by the MACSima Imaging Platform in a completely automated manner.

Figure 2 shows the expression of CD326 (EpCAM, in red) overlaid with the indicated marker (in white) within the same tumor section.

2 TIL immunophenotyping by flow cytometry reveals low frequency of T cells within the tumor microenvironment

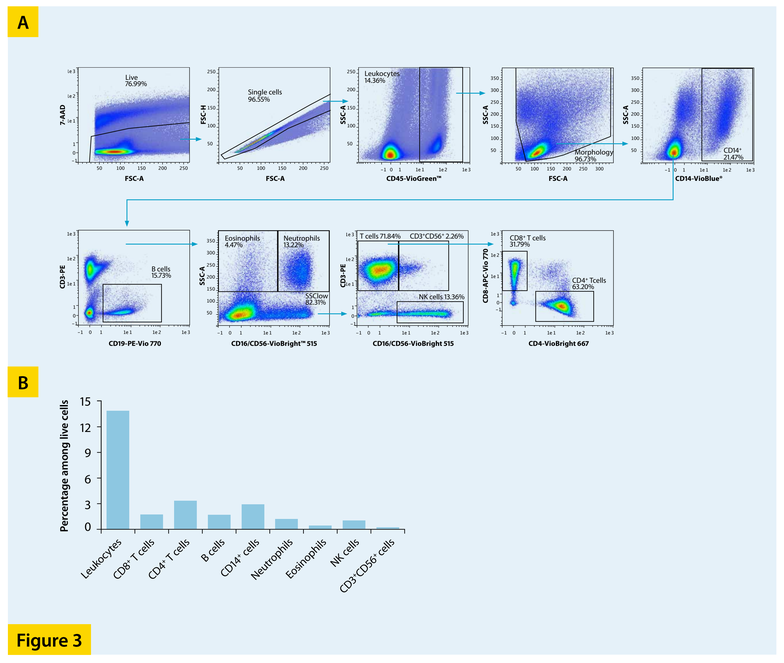

Figure 3

Dissociated tumor was stained with the 8-Color Immunophenotyping Kit, human and analyzed by flow cytometry using the MACSQuant® Analyzer 16 to determine the immune cell composition. The gating strategy used to identify the indicated cell types (fig. 3A) and their frequency among total living cells (fig. 3B) are displayed. Cells were pre-gated for live, single, CD45+ cells, and FSC/SSC.

3 Effective isolation of tumor-infiltrating T cells using the REAlease® CD4/CD8 (TIL) MicroBead Kit, human enables improved analysis

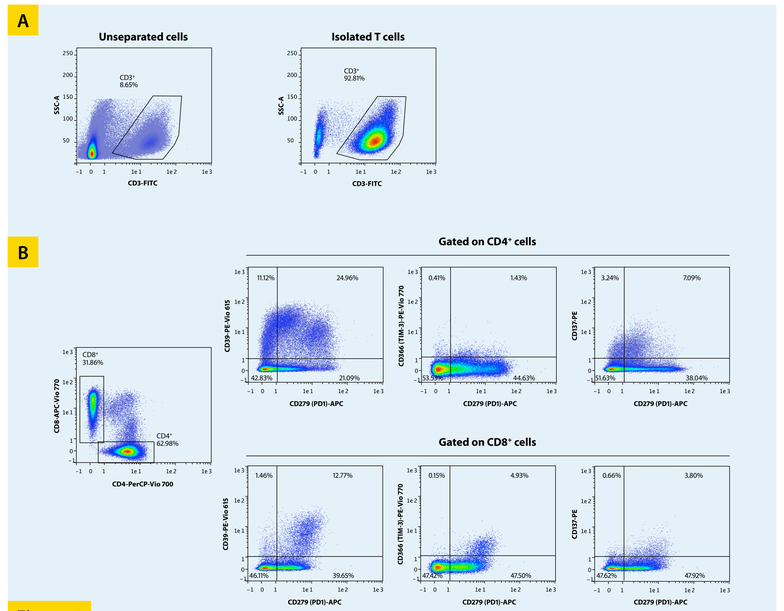

Figure 4

T cells were isolated from the dissociated tumor sample, using the REAlease® CD4/CD8 (TIL) MicroBead Kit, human. Frequencies of T cells after gating on i) live cells, ii) FSC/SSC, iii) single cells was increased by more than 10-fold in the isolated T cell fraction compared to the unseparated cells (fig. 4A). Within the isolated population, critical tumor-specific subpopulations such as CD279(PD1)^{hi}CD366(TIM-3)$^{+}$CD39^{+}CD8^{+} T cells and CD279(PD1)^{hi}CD137(4-1BB)$^{+}$ CD39^{+}CD4^{+} T cells could be identified easily (fig. 4B). Using enriched target cells, the number of events that needed to be acquired for proper flow cytometry analysis was 10-fold lower than with unseparated bulk tumor cells. This greatly reduced the time required for analysis.

4 T and B cell isolation increases sensitivity of single-cell receptor sequencing

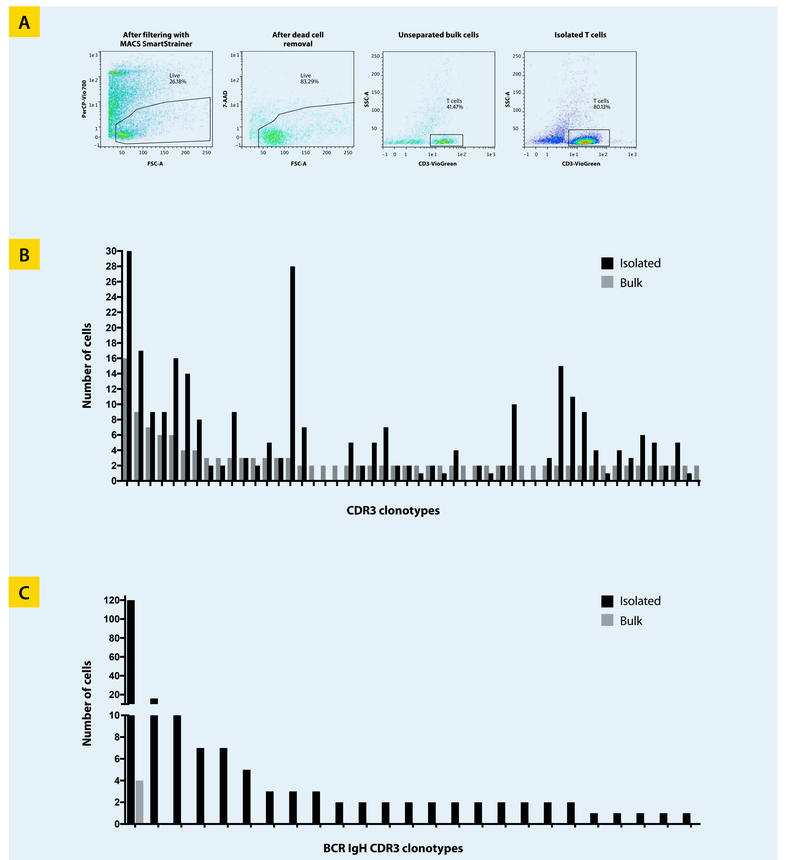

Figure 5

Cryopreserved dissociated tumor sample was thawed and filtered using a MACS SmartStrainer (70 µm). Cell viability in the thawed sample was below 30%. By eliminating dead cells using the Dead Cell Removal Kit, the viability was increased to 83%. T cells were magnetically isolated using the REAlease CD4/CD8 (TIL) MicroBead Kit, human to a purity of 80% (fig. 5A). To maximize the number of cells available for analysis, we used the T cell–depleted fraction for sequential B cell isolation. B cells were isolated using CD19 MicroBeads, human and StraightFrom Whole Blood and Bone Marrow CD138 MicroBeads, human, to obtain all possible B cells and plasma cells. Single-cell TCR (fig. 5B) and BCR (fig. 5C) sequencing was then performed to assess clonality of tumor-infiltrating T and B cells, either using unseparated bulk cells or isolated cells. For single-cell sequencing on the 10x Genomics Platform, 17,000 cells per sample were loaded in order to obtain 10,000 cells within droplets. Only T and B cells were sequenced, and so the number of analyzed cells differed greatly between the unseparated bulk cell samples and the samples of isolated cells (table 1). (B) The top 50 TCR CDR3 clonotypes identified in the bulk sample were ranked by order of abundancy from left to right on the x-axis (gray bars), and the corresponding number of cells was plotted on the y-axis. The number of cells with the same TCR CDR3 clonotype in the isolated T cell population is displayed in black bars. (C) The top 25 BCR clonotypes identified in the isolated B cell population were ranked by order of abundancy (black bars). The number of cells with the same BCR in the bulk sample is displayed in gray bars.

Sample	Number of analyzed cells
Bulk (T cells)	616
Isolated T cells	2,049
Bulk (B cells)	268
Isolated B cells	1,588

Table 1

Conclusion and outlook

- We developed novel tools that allow the comprehensive analysis of the tumor microenvironement.
- The MACSima Imaging Platform allows identification and phenotyping of multiple cell types while maintaining their spatial distribution in the tumor sample.
- T cell isolation improved phenotypic analysis by flow cytometry and, due to REAlease Technology, can be used to isolate further subpopulations of interest.
- Sequential isolation of the different cell types maximized the number of cells of each lineage available for analysis.
- T and B cell isolation greatly increased the sensitivity of single-cell immunoprofiling.
- These workflows greatly reduce experimental time and allow the performance of more complex experimental setups. We believe the use of these innovative tools and workflows can significantly increase the quality of the data obtained in immuno-oncology and immunotherapy research.