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

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Introduction

Immunotherapy approaches that engage T cells to attack tumors have proven clinical efficacy and tremendous potential in multiple cancers. However, there are several mechanisms of resistance to current therapeutic strategies. Therefore, clinical benefit is experienced by only a subset of patients, and often only partial responses are achieved. Thus, additional research is necessary to improve immunotherapeutic strategies. In particular, it is important to analyze steady-state anti-tumor immunity and monitor the effects of therapeutic interventions on tumor-infiltrating leukocytes (TILs) and other cell populations within the tumor microenvironment.

However, the phenotypic and functional analysis of TILs is technically challenging and labor intensive. The number of TILs can be very low and small subpopulations might escape analysis as they are lost in the background noise. Importantly, flow cytometry–based phenotyping of different cell populations requires dividing the limited tumor material into multiple samples for analysis using population-specific antibody panels, which dramatically reduces the number of cells available for analysis. Therefore, it is fundamental to use tools and workflows that can maximize the amount and quality of the data obtained from limited tumor patient samples.

Materials and methods

1 Isolation and characterization of TIL subsets from limited tumor samples

Resected ovarian carcinoma was stored and shipped overnight in MACS® Tissue Storage Solution that was shown to maintain cell viability and phenotype up to 48 h after collection. One small tissue section was analyzed by immunohistochemistry, using the MACSima™ Imaging Platform. Dissociation of the remaining tumor tissue was automated and optimized for epitope preservation using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, human. One aliquot of the dissociated tumor sample was stained with the 8-Color Immunophenotyping Kit, human to determine the immune cell composition. T cells were magnetically isolated from 1×10^7 total dissociated cells using the REAlease® CD4/CD8 (TIL) MicroBead Kit, human. Isolated T cells were phenotyped by flow cytometry to assess expression of activation and exhaustion markers. The remaining cell suspension was cryopreserved in vials containing 1×10^7 cells.

At a later time point, 1×10^7 cells were thawed, and dead cells were removed using the Dead Cell Removal Kit. T cells were isolated using the REAlease CD4/CD8 TIL MicroBead Kit and used for single-cell TCR sequencing using the 10x Genomics Platform. To maximize the number of cells available for analysis, we used the T cell–depleted fraction for sequential B cell isolation using CD19 MicroBeads, human and StraightFrom Whole Blood and Bone Marrow CD138 MicroBeads, human. Isolated B cells were subjected to single-cell BCR sequencing using the 10x Genomics Platform. For comparison, single-cell TCR and BCR sequencing was also performed on unseparated bulk cell samples.

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. An example is shown in table 1.

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

An overview of the workflow is shown in figure 1.

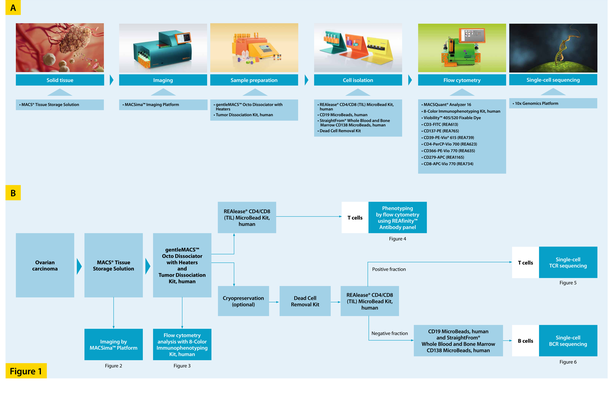

Figure 1

Results

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

One tissue section (approx. 1 cm wide) was snap-frozen and later fixed with acetone 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.

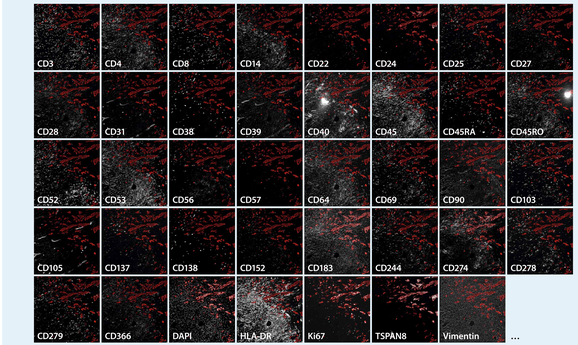

Figure 2

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

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.

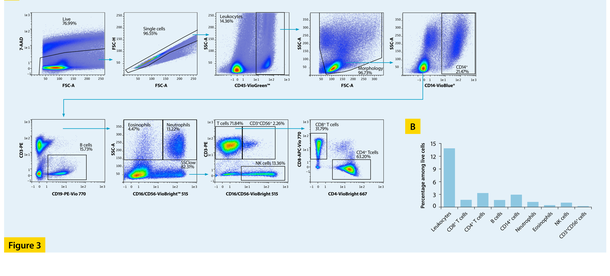

Figure 3

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

REAlease® Technology relies on recombinantly engineered antibody fragments instead of antibodies to label specific cell surface markers. The antibody fragments are engineered to have a low affinity for markers when present as monomers. However, when the fragments are multimerized as a complex they bind markers with high avidity. REAlease Technology can control the multimer/monomer state of the fragments and thus allows for a controlled release, where monomerized antibody fragments dissociate from the cell surface. Therefore, the technology enables users to obtain cells that are free from antibody fragments and magnetic label (fig. 4A).

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. 4B). (C) 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. 4C). 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.

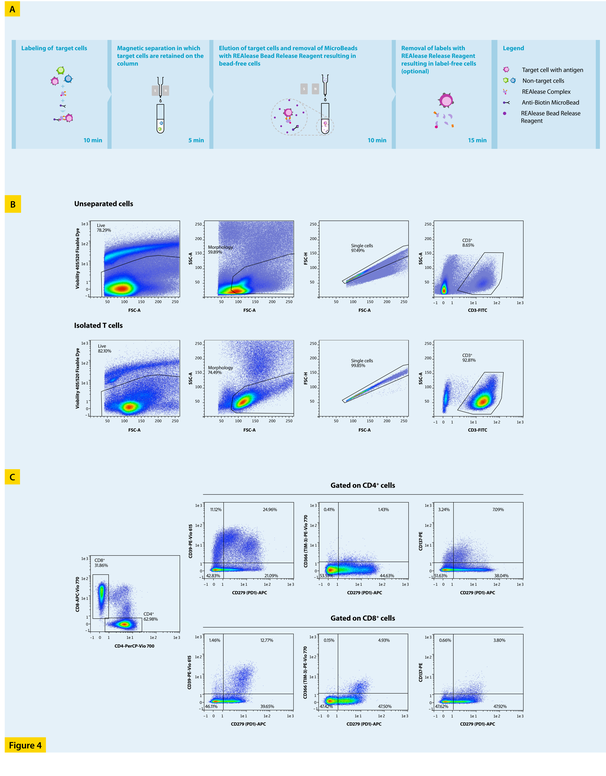

Figure 4

4 T cell isolation increases sensitivity of single-cell TCR sequencing

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 rate was increased to 83% (fig. 5A). T cells were magnetically isolated using the REAlease CD4/CD8 (TIL) MicroBead Kit, human to a purity of 80%. Single-cell TCR sequencing was then performed to assess clonality of tumor-infiltrating T cells, either using unseparated bulk cells or isolated T cells. Target cell number for sequencing was 10,000/sample.

The top 50 TCRβ 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 TCRβ CDR3 clonotype in the isolated T cell population is displayed in black bars (fig. 5B). Conversely, the top 50 TCRβ CDR3 clonotypes identified in the isolated T cell population were ranked by order of abundancy (black bars). The number of cells with the same clonotype in the bulk sample is displayed in gray bars (fig. 5C).

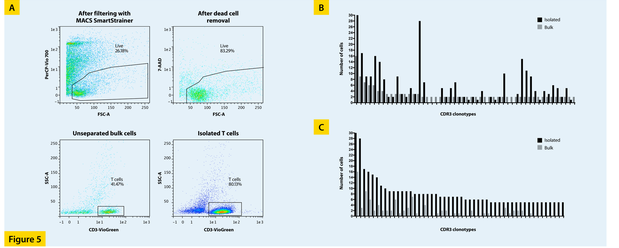

Figure 5

5 B cell isolation increases sensitivity of single-cell BCR sequencing

To maximize the number of cells available for analysis, we used the T cell–depleted fraction (obtained during T cell isolation, fig. 5A) 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 BCR sequencing was then performed to assess clonality of tumor-infiltrating B cells, either using unseparated bulk cells or isolated B cells. Target cell number for sequencing was 10,000/sample. The top 25 heavy-chain BCR 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 BCR identified in the isolated B cell population is displayed in black bars (fig. 6A). Conversely, 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 (fig. 6B).

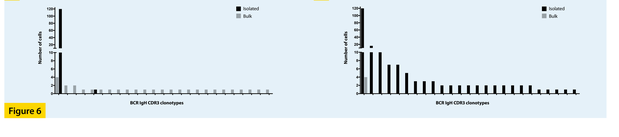

Figure 6

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.