Optimized workflow improves the characterization of tumor-infiltrating T cells

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Introduction

Immunotherapy has proven clinical efficacy and tremendous potential in multiple cancers. Syngeneic mouse tumor models represent the gold standard to analyze effects of immunotherapy, as they possess a fully competent immune repertoire. However, the amount and composition of tumor-infiltrating leukocytes (TILs) is highly variable, complicating the analysis of individual subpopulations. In particular, small subpopulations might escape analysis as they could get lost in the background noise. When working with large cohort sizes, even immunophenotyping of TILs by flow cytometry is time consuming and data processing highly work intensive. Therefore, pre-enrichment of TILs is highly desirable to increase the sensitivity of analysis and save time and effort during flow cytometry.

Results

1 Reliable and fast isolation of T cells from syngeneic mouse tumors

We have developed new CD4+, CD8+, and pan T cell specific enrichment reagents for magnetic cell isolation (MACS® Technology) directly from dissociated tumor tissue (fig. 1A). Tumors derived from B16-F10 (left and center columns) or B16-OVA (right column) melanoma cells were inoculated subcutaneously into the right flank of C57BL/6 mice. After 11–15 days, tumors were collected and dissociated using the gentleMACS™ Octo Dissociator and the mouse Tumor Dissociation Kit (TDK) optimized for epitope preservation. CD4+, CD8+, and pan T cells were isolated using the new mouse CD4 (TIL) MicroBeads, CD8 (TIL) MicroBeads, and CD4/CD8 (TIL) MicroBeads, respectively (fig. 1B). Frequencies of tumor-infiltrating T cells among living cells before or after isolation are shown for different tumor models, as assessed by flow cytometry. Magnetic cell isolation resulted in purities above 80%, which represents an up to 500-fold enrichment of the target cell population (fig. 1C), dramatically decreasing time of analysis (table 1).

Cell type	Cells to analyze	Events to collect	Flow cytometry time/ sample*	Total flow cytometry time**
CD4+ T cells				
Bulk	5,000	7.96×10^6	66.3 min	>10 h
Isolated***	5,000	5.41×10^4	0.5 min	~11 min
CD8+ T cells				
Bulk	5,000	2.80×10^6	23.3 min	>3.5 h
Isolated***	5,000	4.37×10^4	0.4 min	~10 min
T cells				
Bulk	10,000	8.13×10^5	6.8 min	>1 h
Isolated***	10,000	3.24×10^4	0.3 min	<10 min

* Flow rate: 2,000 events/s
** Considering 9 samples (3 experimental groups × 3 replicas/group). Includes 45 s automated mixing and rinsing between samples on the MACSQuant Instrument
*** Isolation using CD8 (TIL), CD4 (TIL), or CD4/CD8 (TIL) MicroBeads, respectively

Table 1

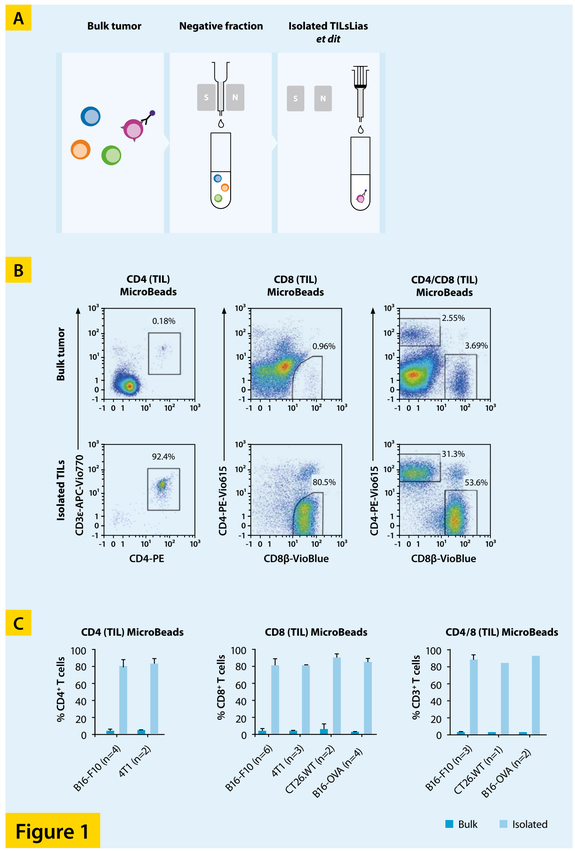


Figure 1

2 Using recombinantly engineered REAfinity™ Antibodies decreases background in flow cytometry analysis of tumor-derived samples

REAfinity™ Recombinant Antibodies have specifically mutated human IgG1 Fc regions, which virtually eliminate unspecific binding to Fcγ receptors. Splenocytes and tumor cells were isolated from BALB/c mice harboring H8N8 breast cancer tumors. Respective cells were subsequently labeled with Viobility™ 405/520 Fixable Dye, REA clones CD45-FITC, and CD3-APC, and either REA clone CD8α-PE or hybridoma clone CD8α-PE. Staining was performed in either presence or absence of FcR blocking reagent and viable $CD45^+CD3^-$ cells are shown.

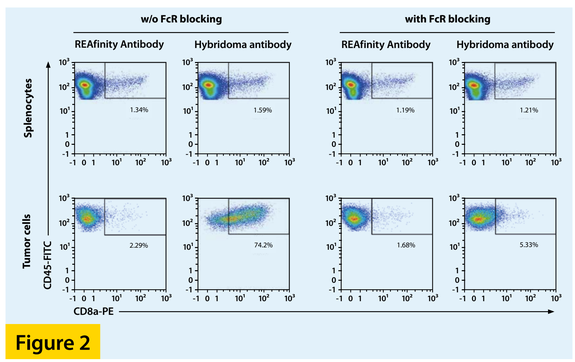


Figure 2

3 Magnetic isolation improves the quality of flow cytometry analysis of tumor-infiltrating CD8+ T cells

We used optimized panels of fluorochrome-conjugated REAfinity Antibodies to phenotypically characterize tumor-infiltrating CD8+ T cells (table 2). Total (fig. 3A) or magnetically isolated CD8+ T cells (fig. 3B) from B16-OVA tumors were labeled with the reagents described in table 2 and analyzed by flow cytometry using the MACSQuant® Analyzer 10. Gated populations show viable cells.

Panel 1	Clone	Fluorochrome	Panel 2	Clone	Fluorochrome
CD8β	REA793	VioBlue	CD8β	REA793	VioBlue
Viobility		405/520	Viobility		405/520
Lag-3	REA776	VioBright™ 515	CD103	REA789	VioBright™ 515
TIM-3	REA602	PE	CXCR3	REA724	PE
CD4	REA604	PE-Vio615	CD4	REA604	PE-Vio615
PD-1	REA802	APC	CD39	REA870	APC
CD44	REA664	APC-Vio770	CD44	REA664	APC-Vio770

Table 2

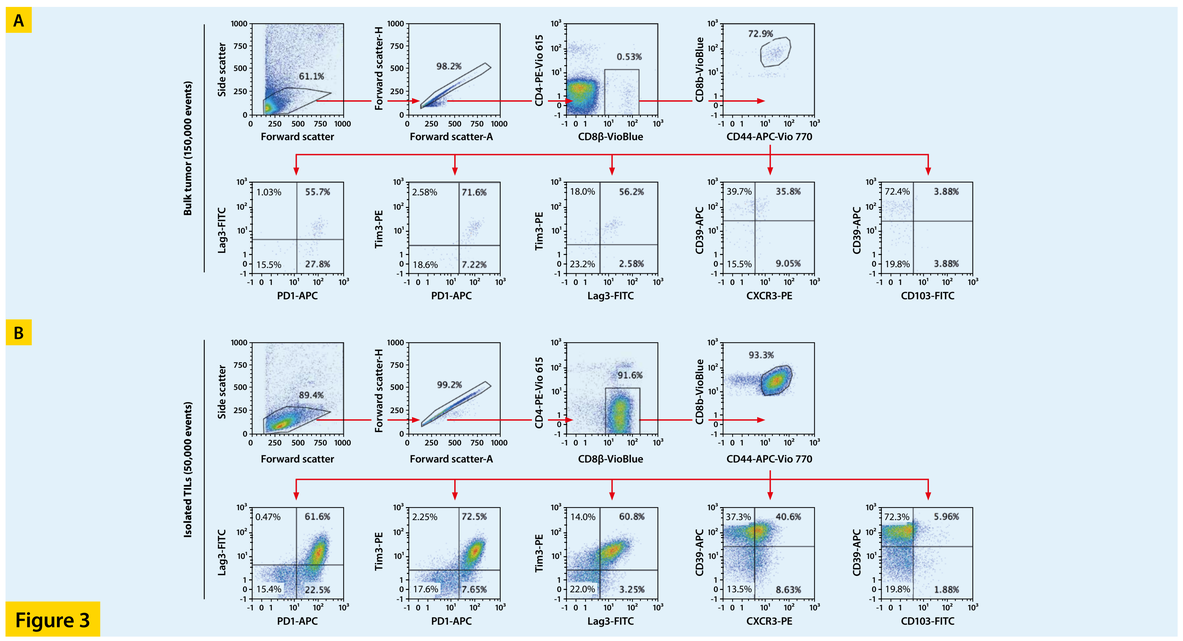


Figure 3

4 Enzymatic tumor dissociation is essential for efficient recovery of key populations of tumor-infiltrating CD8+ T cells

B16-F10 tumors were collected and dissociated using the gentleMACS™ Octo Dissociator in the presence (fig. 4A) or absence (fig. 4B) of Tumor Dissociation Kit enzymes. Cells were subsequently labeled with REAfinity™ Antibodies (table 1). Gating of cell populations was performed as previously mentioned (fig. 3).

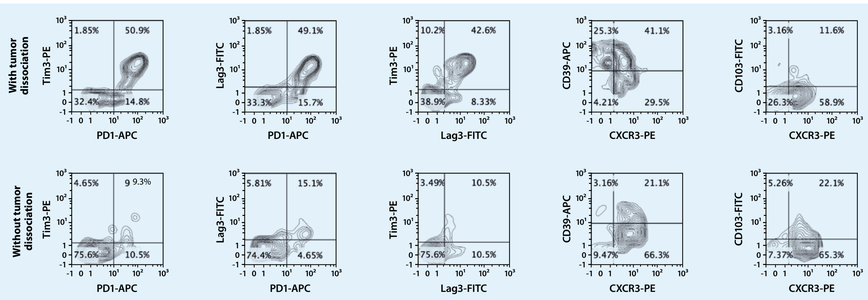


Figure 4

Conclusion

- Standardized processing of tumor samples using TDK enhanced reproducibility and was essential for recovery of key populations of TILs.
- Enrichment of tumor-infiltrating T cells by magnetic cell separation greatly reduced time and costs of downstream analysis while the quality of data obtained from TIL analysis was significantly increased.
- In comparison to hybridoma-derived antibodies, recombinantly engineered REAfinity Antibodies virtually eliminated unspecific binding to Fcγ receptors leading to improved quality of flow cytometry analysis.
- Complete workflows for TIL analysis, validated in different mouse tumor models, significantly reduced the processing and analysis time while data quality was greatly improved.

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