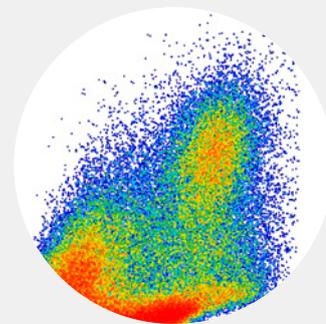# Flow cytometric analysis of tumor infiltrating lymphocytes (TILs) in human cancer tissue

Introduction

Flow cytometry is one of the most successful single cell analytical tools used to characterize immune cell phenotypes. Multiparameter flow cytometry makes detailed analysis of cell subsets possible for all types of immuno-oncology studies.

The examples described here apply Indivumed's procedure and ability to analyze tumor infiltrating lymphocytes (TILs) via flow cytometry in our high-quality human cancer tissue samples.

Material and Methods

Collection of human tumor tissue and adjacent normal tissue from cancer patients: Human tumor and adjacent normal tissue from non-small cell lung cancer (NSCLC) or colorectal cancer (CRC) patients were collected immediately after resection by Indivumed and processed to single cell suspensions for either further enrichment of TILs or direct flow cytometric analysis.

Preparation of single cell suspensions: Single cell suspensions were prepared using the Miltenyi tumor dissociation kit and the gentleMACS™ Dissociator under standardized procedures for highly reproducible results. The viability of the cells is routinely assessed by trypan blue staining and usually varies between 70–95%.

Enrichment of TILs by either EpCAM depletion or CD45 selection: For the enrichment of TILs prior to flow cytometric analysis different techniques were established. By either EpCAM positive depletion of epithelial cells or CD45 positive cell selection of immune cells, TILs were enriched with a magnetic-based, column-free method from Stemcell Technologies. The selected cells (positive fraction) were separated through the use of antibodies specific for EpCAM or CD45, bound to dextran-coated magnetic particles. The negative fraction contained all cells negative for the selected target i.e. EpCAM or CD45. The purity of the cell fractions was controlled using flow cytometry.

Flow Cytometry: Flow cytometric analysis was performed with the CyFlow® Space from Sysmex. This cytometer is equipped with four lasers (violet laser: 405 nm, blue laser: 488 nm, red laser: 640 nm, UV laser) as well as 12 filters to detect a wide spectrum of different emitted wavelengths. Multiplex staining for flow cytometric analysis was carried out according to Indivumed's standard procedures. Doublets and dead cells were excluded from analysis and gates for low expressed markers like CD137 and OX40 were set on FMO (Fluorescence Minus One) controls.

Results

Preparation and viability of single cell suspensions from human cancer tissue: After preparation of single cell suspension from a human NSCLC patient tissue sample, cell viability was determined via pre-gating of Hoechst positive events followed by Propidium Iodide (PI) staining of cells **(Figure 1)**.

As human tissue samples are complex samples that need to be broken down to single cells prior to analysis via flow cytometry, single cell suspensions of tissue samples are composed of different cell types as well as debris resulting in a very heterogeneous suspension (Figure 1a). After exclusion of doublets (Figure 1b), pre-gating of Hoechst positive events is useful to exclude cell debris to receive suitable results for cell viability

(Figure 1c). About 57% of all measured events depicted cells while 43% made up debris. Detected negativity for PI, the cell viability was assessed to 86% for case DP008.6307 (Figure 1d). The viability of individual cases differs and usually varies between 70 and 95%.

Enrichment of TILs from single cell suspensions through selection of CD45 positive cells: After preparation of single cell suspension from a human CRC patient tissue sample, CD45 positive cells were selected via the magnetic-based, column-free method by Stemcell Technologies. The purity of the CD45 positive and negative fraction was analyzed via flow cytometry **(Figure 2)**.
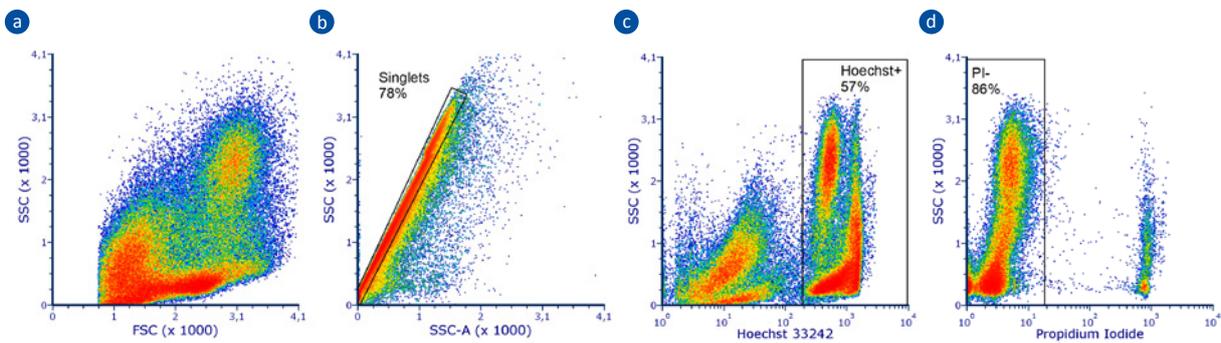

Figure 1: Flow cytometric analysis of single cell suspensions from human NSCLC patient tissue sample in respect to viability (Case DP008.6307). Shown is the scatter plot (a), exclusion of doublets (b), pre-gating of Hoechst positive events (c) followed by analysis of viability via detection of PI negative cells (d).
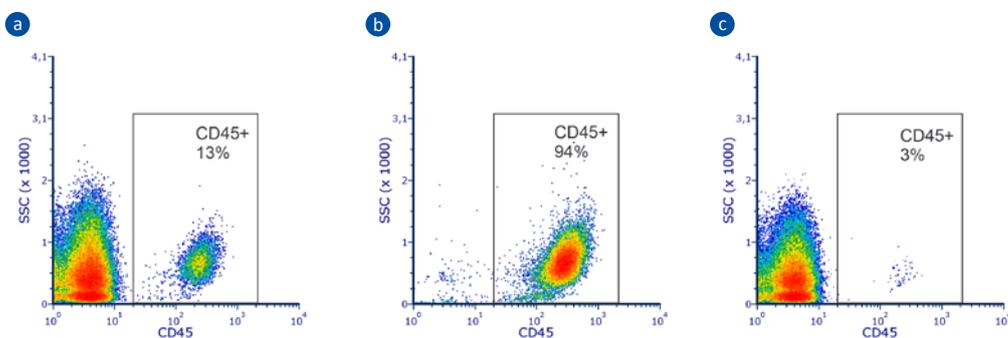

Figure 2: Staining of CD45 positive cells in single cell suspension of CRC case INT0916.912 (a). Staining of CD45 positive cells after selection of TILs in the CD45 enriched fraction (b) and the depleted fraction (c).

After exclusion of doublets and dead cells (data not shown), CD45 positive cells were analyzed within the single cell suspension **(Figure 2a)**. CD45 positive cells accounted for 13% of the total suspension; this fraction also included the TILs. After selection of CD45 positive cells via the magnetic-based, column-free method, the purity of the positive fraction was 94% **(Figure 2b)** while the recovery within the negative fraction was 3% **(Figure 2c)**. Taken together these results demonstrate that the enrichment of CD45 positive cells was highly efficient and allows even rare cell populations within TILs in human tumor tissue samples to be further analyzed.

Analysis of the activation markers CD137 and OX40 on TILs from tumor and adjacent normal tissue of NSCLC patients: After preparation of single cell suspensions from human NSCLC tumor and adjacent normal tissue samples, EpCAM positive cells were depleted via the magnetic-based, column-free method by Stemcell Technologies for enrichment of TILs. Samples were analyzed with a 7-plex flow cytometry panel comprising of a live/dead marker, CD45, CD3, CD4, CD8, CD137, and OX40. Distribution of CD4 and CD8 positive T-cells was compared between adjacent normal and the corresponding tumor tissue **(Figure 3a)**.
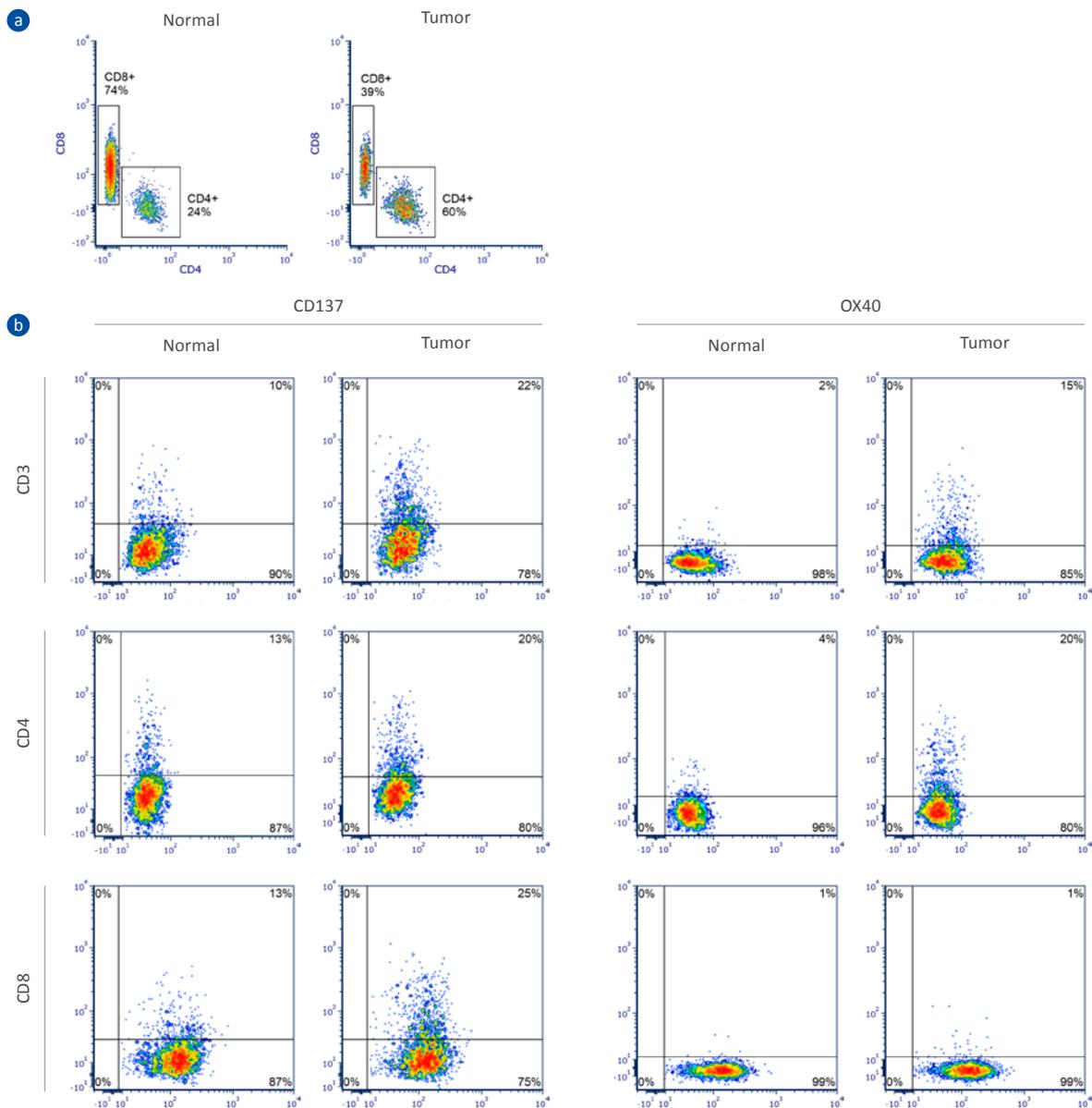

Figure 3: Multiplex panel normal vs. tumor of one representative case FC020.6344. Shown is the expression of CD4 and CD8 on CD3 positive cells (a) and the activation marker CD137 as well as OX40 on CD3, CD4, and CD8 positive cells (b).

In three out of four cases CD4 positive T-cells were increased in tumor tissue samples compared to the corresponding adjacent normal tissue samples while CD8 positive T-cells were decreased **(Table 1).**

Furthermore, the expression of T-cell activation markers CD137 and OX40 was detected on T-cells and compared between adjacent normal and the corresponding tumor tissue samples (Figure 3b). CD137 and OX40 were upregulated in tumor tissue

samples on T-cells in all cases analyzed except one case (FC020.2751) in which OX40 was unchanged **(Table 2).**

In accordance with the literature the expression and upregulation of OX40 were observed on CD4 positive T-cells only (data not shown). These results indicate higher expression levels of the T-cell activation markers CD137 and OX40 in TILs compared to infiltrating immune cells in adjacent normal tissue of NSCLC patients.

Table 1: CD4 and CD8 positive cells [% of viable CD3]

	CD4 [% of viable CD3]		CD8 [% of viable CD3]	
Case	**normal**	**tumor**	**normal**	**tumor**
FC020.6416	27	54	72	44
FC020.2750	24	33	73	63
FC020.6344	24	60	74	39
FC020.2751	65	34	34	65

Table 2: CD137 and OX40 positive cells [% of viable CD3]

	CD137 [% of viable CD3]		OX40 [% of viable CD3]	
Case	**normal**	**tumor**	**normal**	**tumor**
FC020.6416	8	16	2	11
FC020.2750	6	23	5	16
FC020.6344	10	22	2	15
FC020.2751	8	23	3	3

Conclusions

Here, we exemplify Indivumed's ability to analyze TILs in complex human tissue samples using multiplex flow cytometry analysis. Through efficient enrichment of TILs via different selection strategies even rare cell populations can be analyzed indicating active, infiltrating immune cells within the tumor. Indivumed offers multiplex flow cytometry analysis of established as well as customized developed panels of high-quality tissue samples

from different tumor entities. Furthermore, detailed analysis of PBMCs or even matched PBMCs is possible at Indivumed via prospective, customized sample collection by the Indivumed clinical network.

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