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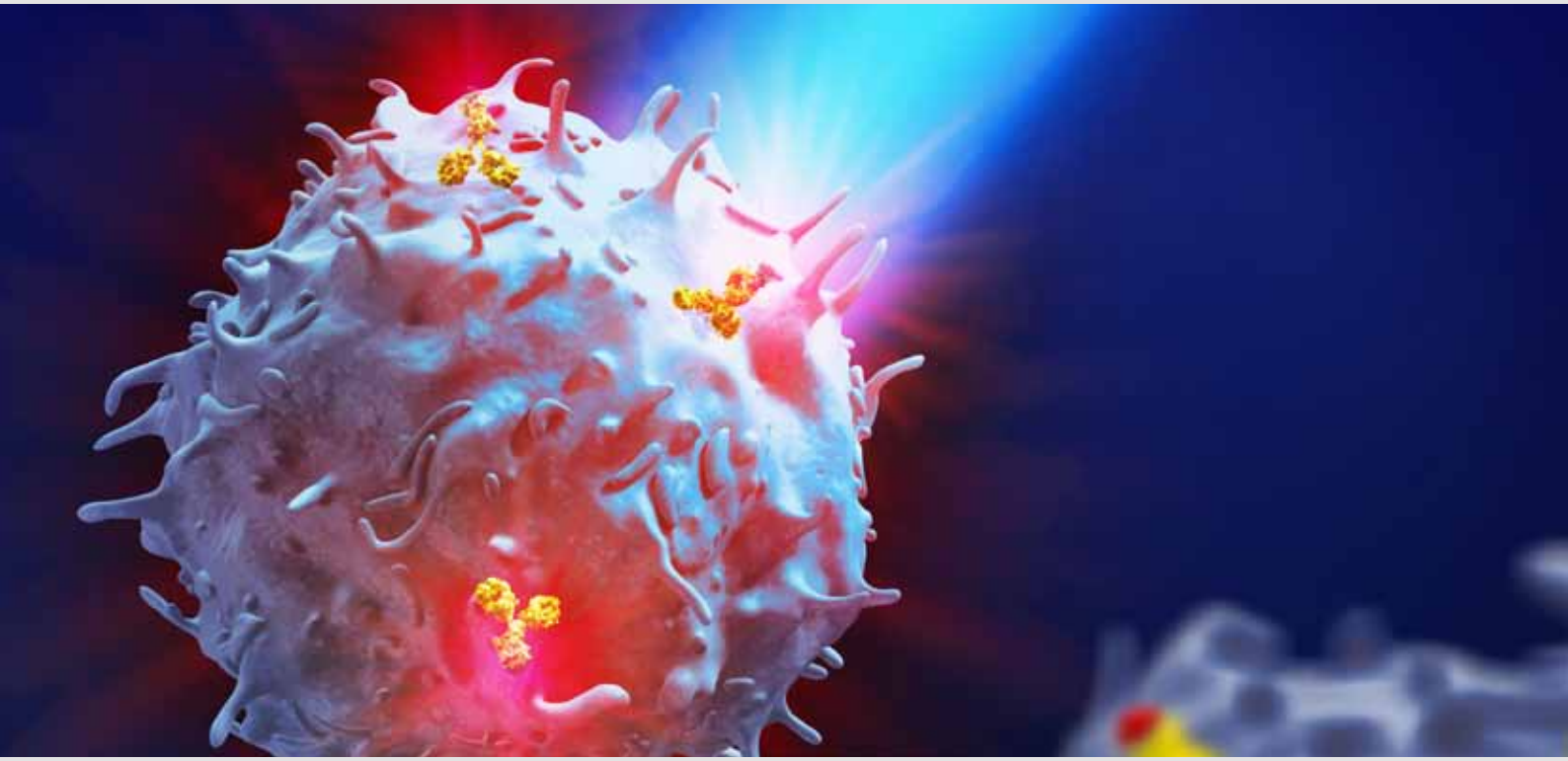

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Identification and enumeration of melanoma-specific tumor-infiltrating lymphocytes and cancer stem cells

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Introduction

Many of the different cell types found in melanomas are of paramount interest for research into tumor biology and ultimately the development of potential treatment options. Adoptive cell therapy with tumor-infiltrating lymphocytes (TIL) has shown promising results in various trials[1]. Cancer stem cells (CSCs) are critically involved in cancer development and metastasis. They seem to be resistant to various cancer therapies and this might explain why complete eradication of the disease is mostly impossible[2].

The unambiguous identification and enumeration of different melanoma cell types by flow cytometry requires i) a reliable method for tissue dissociation, ii) specific antibody labeling, and iii) dependable gating strategies. Recently, it was shown that inconsistency among results with melanoma CSCs were at least partially due to the use of aggressive proteases during dissociation, such as trypsin, which cleaves off several CSC markers, e.g., CD44 and ABCB5[3]. We used the Tumor Dissociation Kit (Miltenyi Biotec) for the preparation of single-cell suspensions from primary tumor tissue preserving epitopes and leading to high viability rates. We also developed a gating strategy for the enumeration of TIL and CSCs using MACS® Antibodies and the MACSQuant® Analyzer.

Materials and methods

Melanoma dissociation

Primary human melanomas were resected and the surrounding healthy tissue was removed. Single-cell suspensions were prepared by using the Tumor Dissociation Kit, human and the gentleMACS™ Dissociator from Miltenyi Biotec according to the accompanying standard protocol.

Flow cytometric analysis

Single-cell suspensions derived from melanoma were labeled with the following antibodies: CD45-FITC, Anti-MSCA-1-PE, CD271 (LNGFR)-APC, and a PE-conjugated CD235a (Glycophorin A) antibody. Flow cytometry was performed on the MACSQuant Analyzer by using standard settings with <2000 events/s.

Results and discussion

Determination of absolute cell counts and viability in melanoma-derived cell suspensions

Single-cell suspensions from melanoma were prepared in a semi-automated procedure by using the Tumor Dissociation Kit and the gentleMACS Dissociator. The resulting suspension contained significant amounts of red blood cells (RBC) in most of the cases (16% in this example, fig. 1A), hampering the accurate determination of cell count for TILs, CSCs, and other melanoma tissue cells. There are two common methods of eliminating RBC from cell suspensions: RBC lysis and density gradient centrifugation. However, both approaches are very time-consuming and lead to a significant cell loss. We therefore labeled

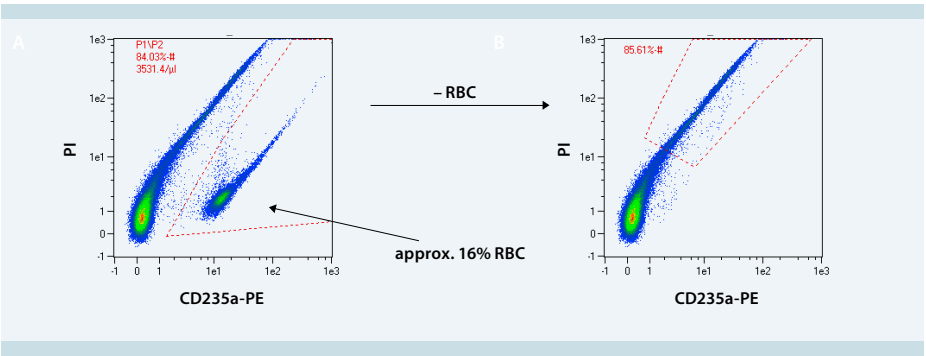

Figure 1 Determination of viability and absolute cell count in a melanoma-derived single-cell suspension. RBC and dead cells were excluded from the analysis by gating off CD235a⁺ and PI⁺ cells, respectively.

the suspension with a CD235a (glycophorin A) antibody and propidium iodide (PI), which allowed us to exclude RBC and dead cells from the analysis. Exclusion of RBC by SSC/FSC gating is not feasible after most tissue dissociation procedures due to the broad variability of cell size and granularity within the heterogeneous cell population. Without exclusion, RBC would appear in the PI-negative fraction and the overall viability as well as the cell count would be overestimated. After excluding RBC by gating off CD235a$^+$ cells, the remaining fraction of viable cells amounted to 86% with a yield of 5.8×10^7 cells per gram of tissue (fig. 1B).

Quantification of TIL

In the melanoma cell suspension shown in figure 2A, RBC amounted to 25%, tumor cells to 70%, and TIL to 4.5%. RBC affect the accurate determination of the TIL fraction within the total tumor cell population as they increase the number of CD45$^-$ cells. Eliminating RBC as well as dead cells from flow cytometric analysis led to fractions of 6% TIL and 94% tumor cells (fig. 2B).

Simultaneous quantification of CSCs and TIL

CSCs in melanoma express CD271 (LNGFR).[4] Staining with CD271 (LNGFR)-APC and CD45-FITC therefore allowed us to distinguish between CD271 (LNGFR)$^+$ CD45$^-$ CSCs and CD45$^+$ TIL (fig. 3A). By staining the cells with CD271 (LNGFR) and Anti-MSCA-1 antibodies (fig. 3B), we were able to distinguish between CD271 (LNGFR)$^+$MSCA-1$^-$ CSCs and CD271 (LNGFR)$^+$MSCA-1$^+$ tumor-infiltrating MSCs for an accurate quantification of true CSCs. RBC and dead cells were excluded from the analysis.

Conclusion

The Tumor Dissociation Kit, human in combination with the gentleMACS Dissociator allows the dissociation of melanoma tissue into single-cell suspensions with high viability rates, high yields, and preserved cell surface epitopes. By using a variety of MACS Antibodies in combination with the MACSQuant Analyzer, we were able to develop an adapted gating strategy for the accurate enumeration of TIL and CSCs in melanoma.

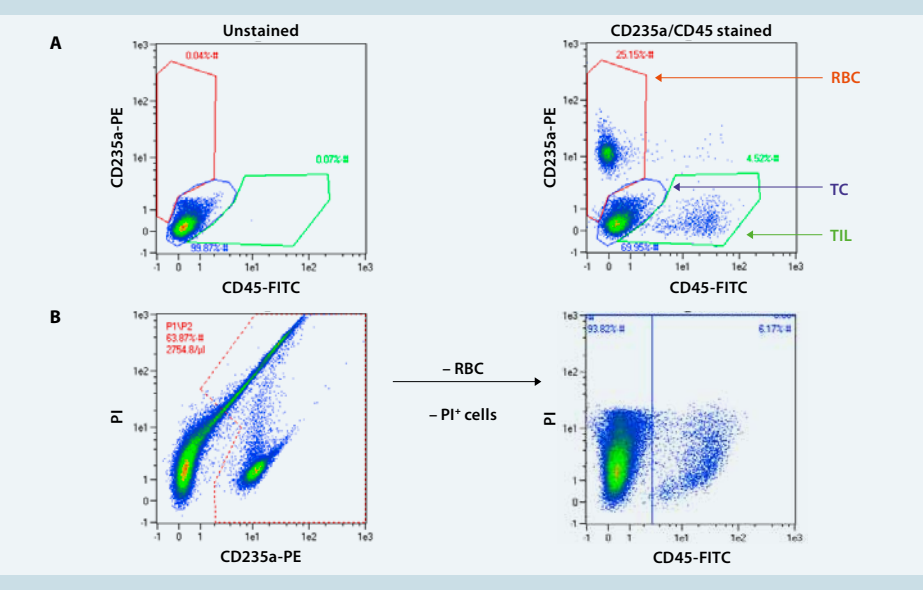

Figure 2 Quantification of TIL. Cells were stained with CD235a-PE, CD45-FITC, and PI, except for the upper left dot plot, where unstained cells were analyzed. RBC and dead cells were excluded from the analysis by gating off CD235a$^+$ and PI$^+$ cells, respectively. TC: tumor cells.

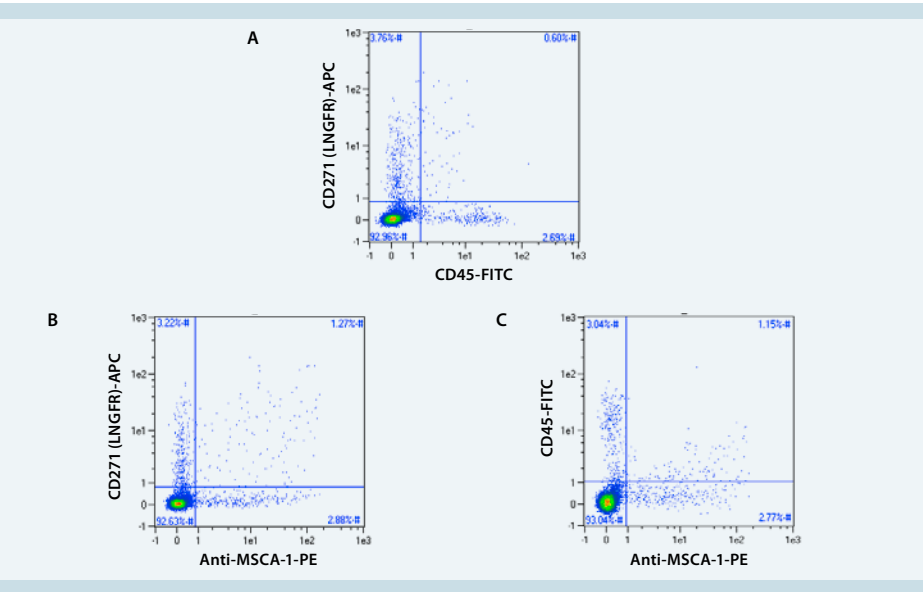

Figure 3 Quantification of TIL and CSCs. Cells were labeled with CD271 (LNGFR)-APC, Anti-MSCA-1-PE, and CD45-FITC. RBC and dead cells were excluded from the analysis by gating off CD235a$^+$ and PI$^+$ cells, respectively.

References

1. Rosenberg, S.A. *et al.* (2008) Nat. Rev. Cancer 8: 299–308.
2. Trumpp, A. and Wiestler, O.D. (2008) Nat. Clin. Pract. Oncol. 5: 337–347.
3. Civenni, G. *et al.* (2011) Cancer Res. 71: 3098–3109.
4. Boiko, A. *et al.* (2010) Nature 466: 133–137.

MACS Product	Order no.
MACSQuant Analyzer	130-092-197
gentleMACS Starting Kit	130-093-235
Tumor Dissociation Kit, human	130-095-929
Anti-MSCA (W8B2)-1-PE, human	130-093-587
CD45-FITC, human	130-080-202
CD271 (LNGFR)-APC, human	130-091-884