Miltenyi Biotec

REAfinity™ Recombinant Antibodies

Background-free analysis of mouse TILs

Background

Syngeneic mouse tumor models represent the gold standard to analyze effects of immunotherapy, as they possess a fully competent immune repertoire. Such analyses routinely rely on the characterization of tumor-infiltrating leukocytes (TILs) and their phenotype. However, the amount and composition of TILs is highly variable and often very low, complicating the flow cytometric analysis of individual subpopulations.

To make things more difficult, traditional, hybridoma-derived antibody clones used for flow cytometric evaluation of TILs show a tendency of unspecific binding to immune cells, such as myeloid cells, via naturally expressing Fcγ receptors (FcγR) or induced expression of FcγRs on T cells in the context of the tumor microenvironment[1,2,3,4]. This can lead to an inaccurate and biased estimation of TIL populations. To avoid FcγR-mediated background binding, blocking reagents, including commercially available immunoglobulins are used. This adds additional complexity to the analysis as there is no defined consensus on the best titer and type of blocking reagent to be used for complex tissues like tumors. In addition, blocking reagents increase staining time and hinder scale up or automation of multi-sample analyses.

Here, we describe the benefits of using REAfinity™ Recombinant Antibody clones for the analysis of different TIL populations. REAfinity Antibodies provide several benefits (fig. 1) over hybridoma-derived antibody clones, for example a specifically mutated human IgG1 Fc region that abolishes their binding to FcγRs and therefore enables more reliable and reproducible flow cytometry analyses of immune cells, as described in this application note.

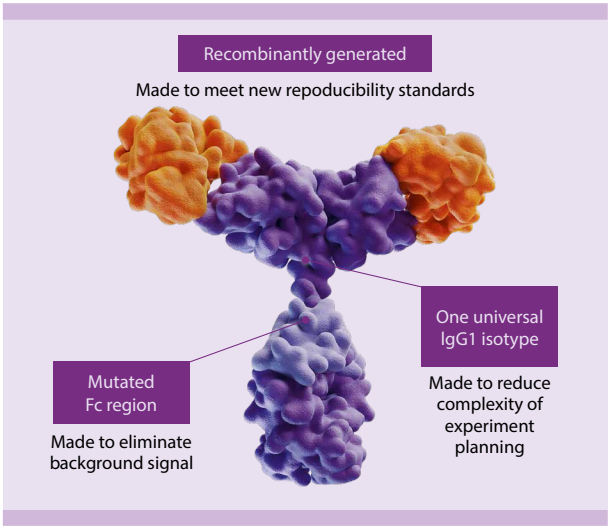

Figure 1: REAfinity Recombinant Antibody model

Materials and methods

Tumor dissociation

WAP-T mice were orthotopically transplanted with 1×10^6 H8N8 mammary carcinoma cells. Balb/c mice were subcutaneously transplanted with 5×10^5 CT26.WT colon carcinoma cells. Upon reaching a tumor volume of 0.5 cm^3, mice were sacrificed, tumors were resected, and stored in Tissue Storage Solution. Tumors were dissociated using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, mouse (both Miltenyi Biotec).

Cell staining and flow cytometry

All cells were stained with Viobility 405/520 Fixable Dye to assess cell viability. Furthermore, cells were stained with CD16/CD32-APC, mouse (clone REA377) and CD64-PE-Vio 770, mouse (clone REA286) to evaluate expression of FcγRs (fig. 2), or as indicated with CD3-APC, mouse (clone REA641), CD45-FITC, mouse (clone REA737), and/or CD8b-VioBlue, mouse (clone REA793) (fig. 4 and 5). To compare antibody types side by side, cells were stained with either PE-conjugated REAfinity Antibody clones or PE-conjugated hybridoma-derived clones that detect identical cellular markers (table 1, fig. 3 and 5). Staining was performed in the absence or presence of FcR blocking reagent. All antibodies

were from Miltenyi Biotec and cells were stained according to recommended protocols from respective datasheets. Cells were analyzed by flow cytometry on a MACSQuant® Analyzer 10 using the MACSQuantify™ Software (both from Miltenyi Biotec).

Cellular target	REAfinity Antibody Clone	Hybridoma clone
CD4	REA604	GK1.5
CD8a	REA601	53-6.7
CD8b	REA793	H35-17.2
CD11c	REA754	N418
CD19	REA749	6D5
CD27	REA499	LG.3A10
CD134 (OX40)	REA625	OX-86
TIM-3 (CD366)	REA602	RMT3-23

Table 1: Antibody clones used for cell staining.

Results

Substantial FcγRI expression in syngeneic tumor tissue

Our results indicate that a majority of viable, single cells in the investigated tumor tissue express FcγRI, also known as CD64 (fig. 2A), and FcγRII / FcγRIII, also known as CD16/CD32 (fig. 2B). Therefore, background binding of antibodies to FcγRs is a considerable concern that has to be evaluated when analyzing TILs by flow cytometry.

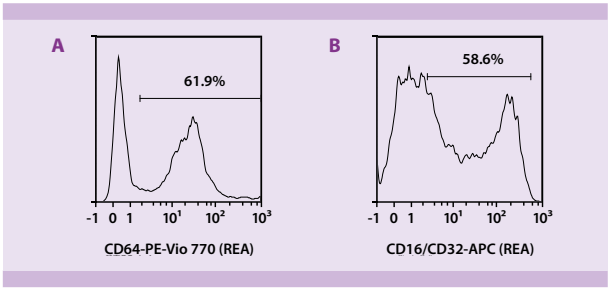

Figure 2: Expression of FcγRs in xenografted tumor tissue. Cells were labeled with Viobility 405/520 Fixable Dye to assess viability, and CD16/CD32-APC, mouse (clone REA377) and CD64-PE-Vio 770, mouse (clone REA286) to evaluate expression of FcγRs. Viable single cells were gated for analysis on the MACSQuant Analyzer 10.

REAfinity™ Recombinant Antibodies enable an accurate analysis of TIL populations

Using hybridoma-derived antibodies to identify TILs lead to a gross overestimation of the frequency of immune cell subpopulations present in the tumor (fig. 3). In particular, it resulted in false identification of CD8a⁺ cells within the CD3⁻ cell population (fig. 4). This experimental artifact was most likely caused by unspecific binding of hybridoma-derived antibodies to FcγRs on immune cells, as it was significantly reduced when using an FcR blocking reagent. In contrast, assessment of TIL frequency using REAfinity™ Antibodies was unchanged in the presence or absence of FcR blocking; therefore, providing a more exact analysis of respective cell populations, even in the absence of FcR blocking.

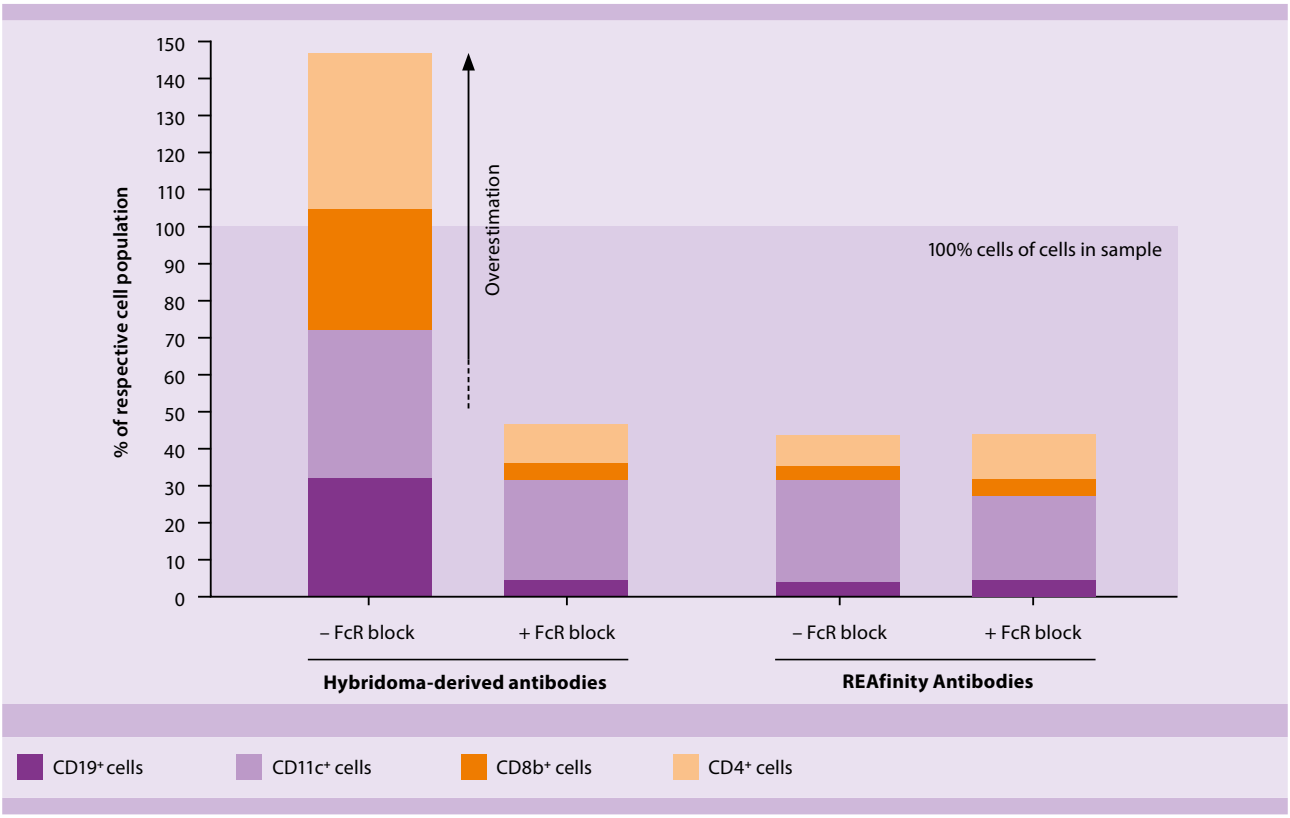

Figure 3: Background staining leads to gross overestimation of different immune cell subsets in the tumor microenvironment when using hybridoma-derived antibodies. H8N8 tumor single-cell suspensions were labeled with Viobility 405/520 Fixable Dye and the indicated (table 1) PE-labeled hybridoma-derived antibodies or REAfinity Recombinant Antibodies in the presence or absence of FcR blocking. Staining with each PE-labeled antibody was done individually. Viable single cells were gated for the analysis on the MACSQuant® Analyzer 10.

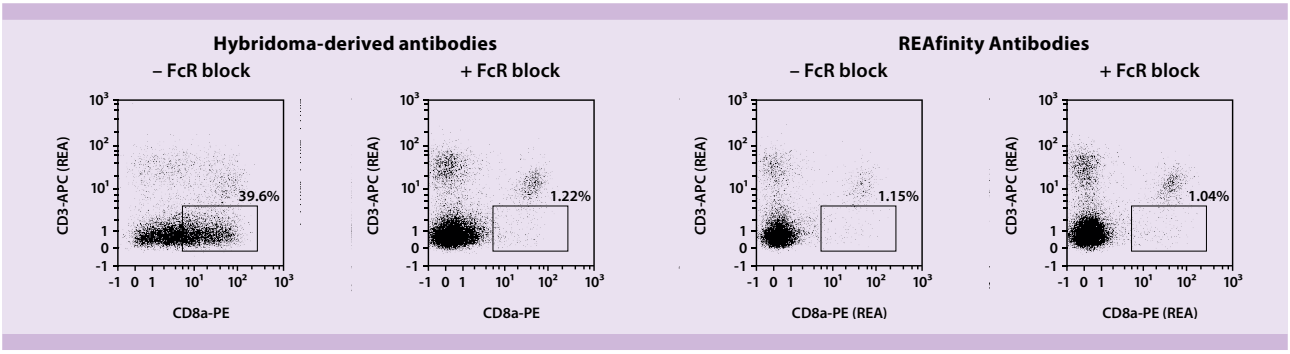

Figure 4: Background staining using hybridoma-derived antibodies leads to identification of artifact populations.
H8N8 single-cell suspensions were labeled with Viobility 405/520 Fixable Dye, CD3-APC (clone REA641) and the indicated PE-labeled hybridoma-derived antibody or REAfinity Antibody detecting CD8a. Staining was performed in the presence and absence of FcR blocking. Viable single cells were gated for the analysis on the MACSQuant® Analyzer 10.

Precise analysis of T cell populations without the need for FcR blocking

It has been shown that chronically-activated T cells, such as tumor-reactive T cells, can express FcγRs[4], which is relevant for T cell function within the tumor microenvironment[1]. Our results show that FcγR expression on T cells lead to unspecific binding of hybridoma-derived antibodies, resulting in mischaracterization of T cell subtypes (fig. 5). Addition of a FcR blocking reagent reduced the amount of background binding; however, FcγR blocking is often suboptimal, as it has been shown to affect T cell function *in vivo*[1]. This issue can be resolved by using REAfinity™ Antibodies, which enable an accurate analysis of T cell subtypes without the need for FcR blocking.

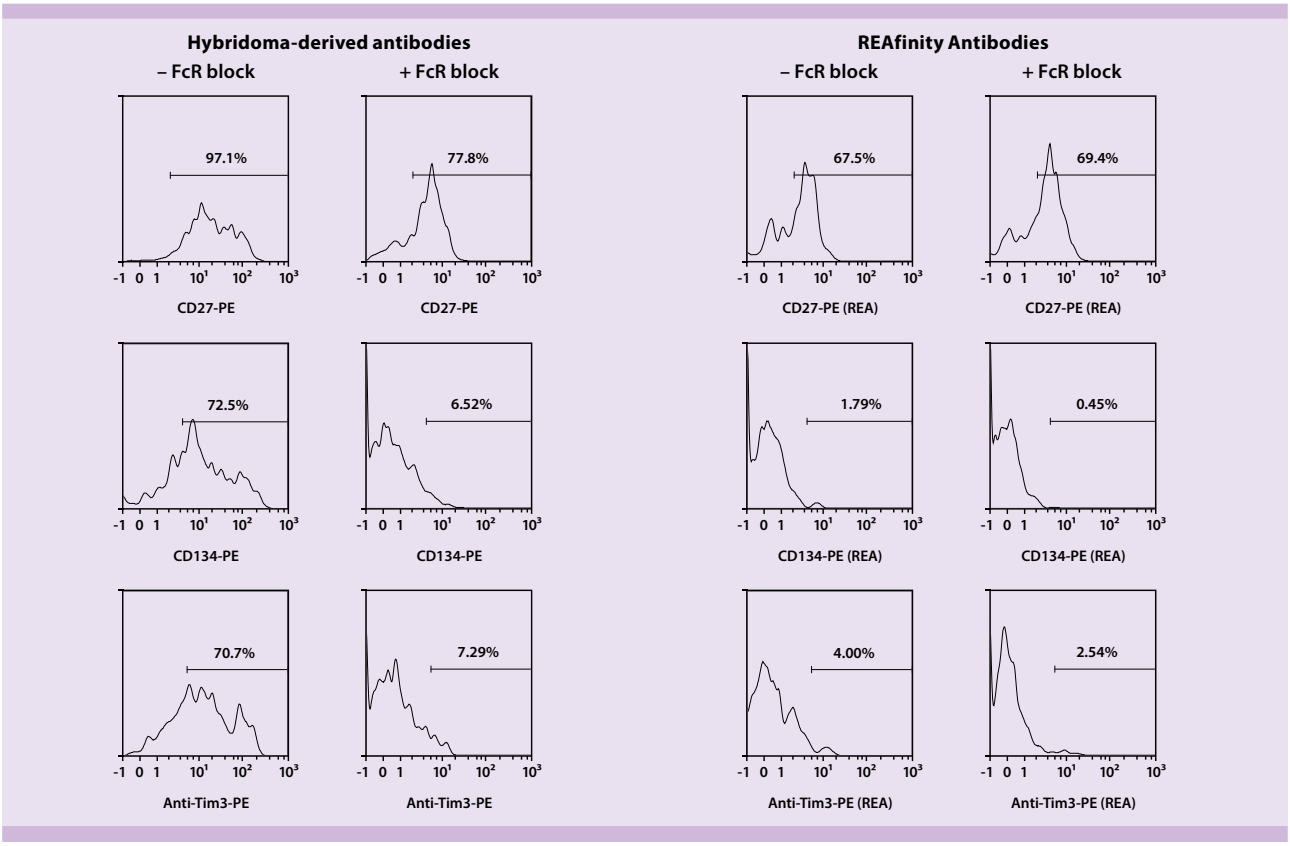

Figure 5: Background staining with hybridoma-derived antibodies leads to biased phenotypical characterization of critical tumor-infiltrating T cell subpopulations. Cells were labeled with CD45-FITC (clone REA737), CD3-APC (clone REA641), CD8b-VioBlue (clone REA793), and the indicated PE-labeled hybridoma-derived antibodies or REAfinity Recombinant Antibodies in the presence and absence of FcR blocking. Analyzed cell populations were gated on viable, CD45⁺, CD3⁺, CD8b⁺ single cells for analysis on the MACSQuant® Analyzer 10.

Conclusions

- REAfinity™ Recombinant Antibodies enable background-free, accurate flow analyses even in the absence of FcR blocking.
- Flow cytometry experiments without the need for FcR blocking allow for analysis of FcγRs on immune cells within the tumor microenvironment.
- In contrast to traditional hybridoma-derived antibodies, REAfinity Antibodies can be used for analysis across different tumor types without investing time and resources in establishing the right blocking reagent and condition.
- Use of REAfinity Antibodies allows unbiased analysis of leukocyte populations, a pre-requisite for consistent analysis over time.

References

1. Starbeck-Miller, G. R. *et al.* (2012) Cutting edge: Expression of Fc RIIB tempers memory CD8 T cell function *in vivo*. J. Immunol. 192: 35–39.
2. Hudrisier, D. *et al.* (2009) Ligand binding but undetected functional response of FcR after their capture by T cells via trogocytosis. J. Immunol. 183(10): 6102–13.
3. Lee, S. T. & Paraskevas, F. (1978) Macrophage – T cell interactions. I. The uptake by T cells of Fc receptors released from macrophages. Cell. Immunol. 40(1): 141–53.
4. Wirth, T. C. *et al.* (2010) Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8⁺ T cell differentiation. Immunity 33, 128–40.

Acknowledgments

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Product	Order no.
REAfinity Antibodies	
CD16/CD32-APC, mouse	130-102-390
CD64-PE-Vio 770, mouse	130-103-810
CD3-APC, mouse	130-109-838
CD45-FITC, mouse	130-110-658
CD8b-VioBlue, mouse	130-111-638
CD4-PE, mouse	130-116-539
CD8a-PE, mouse	130-109-247
CD8b-PE, mouse	130-111-633
CD11c-PE, mouse	130-110-701
CD19-PE, mouse	130-111-883
CD27-PE, human and mouse	130-113-640
CD134 (OX40)-PE, mouse	130-109-741
Anti-TIM-3-PE, mouse	130-118-563
Hybridoma antibodies	
CD4-PE, mouse	130-102-619
CD8a-PE, mouse	130-102-595
CD8b-PE, mouse	130-106-314
CD11c-PE, mouse	130-102-545
CD19-PE, mouse	130-102-598
CD27-PE, human and mouse	130-097-223
CD134 (OX40)-PE, mouse	130-102-572
Anti-TIM-3-PE, mouse	130-102-415
Other	
gentleMACS™ Octo Dissociator with Heaters	130-096-427
MACSQuant Analyzer 10	130-096-343
Tumor Dissociation Kit, mouse	130-096-730
Viobility 405/520 Fixable Dye	130-109-814

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