

Characterization of antigen-specific naive and memory T cell subsets

Detection, enrichment, and direct phenotypic and functional characterization of antigen-specific naive and memory T cell subsets

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Introduction

Infection-related mortality is a considerable clinical challenge in immunocompromised individuals, e.g., after hematopoietic stem cell transplantation or chemotherapy. Pathogen-specific T cells are crucial mediators of immune protection as shown for example by adoptive transfer of antigen-specific T cells. However, for both predicting or diagnosing infectious complications as well as for the development of effective therapies it is crucial to have reliable methods to phenotypically and functionally characterize the antigen-specific T cells. In general, multicolor flow cytometry is a robust technique to enumerate and characterize cells according to a multitude of parameters simultaneously. However, antigen-specific T cells are very rare in the naive compartment of peripheral blood (0.2–60 cells/10⁶ naive T cells; ref. 1 and references therein), and even in the memory compartment their proportion is well below 1%.¹ Therefore, the number of naive antigen-specific T cells, for example, is too low for the direct *ex vivo* characterization by conventional flow cytometry. To overcome these limitations, we developed a straightforward method for the fast and specific antigen-reactive T cell enrichment (ARTE) based on MACS® Technology. This technique greatly increases the sensitivity of detection in flow cytometry, and enables the comprehensive analysis of extremely rare T cell subsets.² The method is based on the immunomagnetic enrichment of activated CD154⁺ or CD137⁺ cells. For the detailed flow cytometric analysis of the enriched antigen-specific T cells, we designed comprehensive panels of fluorochrome-conjugated antibodies and gating strategies. CD154 (CD40L) is expressed specifically on all antigen-activated conventional CD4⁺ T cells upon TCR stimulation.³

We detected CD154⁺ cells after stimulation of PBMCs from healthy individuals with antigens from *Aspergillus fumigatus*, *Candida albicans*, cytomegalovirus (CMV), and adenovirus (AdV) and with tetanus toxoid. For cell enrichment and detailed characterization, we focused on *A. fumigatus*-specific T cells as an example.

Regulatory T (Treg) cells contribute to maintaining the balance between pro- and anti-inflammatory immune responses. We showed recently that *A. fumigatus* causes a robust Treg cell response *in vivo*,⁴ counteracting inappropriately strong immune responses in healthy individuals. Six hours after stimulation with antigen, Treg cells express CD137.⁵ Using the ARTE technique to enrich CD137⁺ and CD154⁺ cells from PBMCs stimulated with *A. fumigatus* lysate *in vitro*, we were able to simultaneously identify CD137⁺CD154⁺ conventional (Tcon) T cells and CD137⁺CD154⁻ Treg cells.

ARTE in combination with multicolor flow cytometry will be a valuable tool for sensitive monitoring of antigen-specific T cells in various important immune-mediated diseases, such as autoimmunity, inflammatory bowel disease, allergy, and tumor immunology as well as for the development of specific immunotherapies.

Materials and methods

Stimulation of antigen-specific T cells

PBMCs were prepared by density gradient centrifugation from blood obtained from healthy donors. All donors gave their informed consent. PBMCs (1–2×10⁷ cells) from healthy volunteers were stimulated for 7 h in RPMI 1640, supplemented with 5% AB serum, with the following antigens: *A. fumigatus* lysate (40 µg/mL, Miltenyi Biotec), *C. albicans* lysate (20 µg/mL, Greer Laboratories), PepTivator® CMV pp65, PepTivator AdV5 Hexon (0.6 nmol/mL; both from Miltenyi Biotec), or tetanus toxoid (10 µg/mL; Statens Serum Institute) in the presence of CD40 pure – functional grade and CD28 pure – functional grade antibodies (1 µg/mL each; both from Miltenyi Biotec). For some experiments, cells were stained intracellularly with anti-cytokine antibodies. In this case, 1 µg/mL of brefeldin A was added to the cells for the last two hours of stimulation.²

Enrichment of CD154⁺ and CD137⁺ antigen-specific T cells

After stimulation, the antigen-specific cells were isolated using the CD154 MicroBead Kit alone or in combination with the CD137 MicroBead Kit (both from Miltenyi Biotec) according to the manufacturer's instructions. Briefly, cells were magnetically labeled with CD154-Biotin/Anti-Biotin MicroBeads or CD137-PE/Anti-PE MicroBeads and isolated using two sequential MS Columns.²

Cell staining and flow cytometry

Cells were stained for multiparametric flow cytometry with different panels of fluorochrome-conjugated antibodies (table 1), depending on the subset to be analyzed. All antibodies were from Miltenyi Biotec except CD25-Brilliant Violet™ 421 (BioLegend®).

For flow cytometry analysis of cytokine-secreting antigen-specific T cells, the stimulated cells were labeled fluorescently during the enrichment procedure: Cells labeled with MACS MicroBeads were applied to the first MS Column and subsequently stained on the column with fluorochrome-conjugated antibodies. Cells were eluted for fixation (Inside Stain Kit, Miltenyi Biotec) and subsequently applied to the second MS Column, where they were permeabilized (Inside Stain Kit, Miltenyi Biotec). Intracellular cytokines were stained while the cells were still on the column. The transcription factors FoxP3 and Helios were stained using the respective antibodies in combination with the FoxP3 Staining Buffer Set (Miltenyi Biotec). After elution from the second column, the cells were analyzed by flow cytometry on a MACSQuant® Analyzer 10 with the MACSQuantify™ Software (both from Miltenyi Biotec).²

Naive T cell subsets	Memory T cell subsets	Cytokine-producing T cells (panel 1)	Cytokine-producing T cells (panel 2)	CD154 ⁺ Tcon and CD137 ⁺ Treg cells
CD8/14/20-VioGreen™	CD8/14/20-VioGreen	CD8/14/20-VioGreen	CD8/14/20-VioGreen	CD8/14/20-VioGreen
CD4-APC-Vio® 770	CD4-APC-Vio 770	CD4-APC-Vio 770	CD4-APC-Vio 770	CD4-APC-Vio 770
CD154-VioBlue®	CD154-VioBlue	CD154-VioBlue	CD154-VioBlue	CD154-PE-Vio 770
CD45RO-PE-Vio 770	CD45RO-PE-Vio 770	CD45RO-PE-Vio 770	CD45RO-PE-Vio 770	CD25-Brilliant Violet 421
CD197 (CCR7)-FITC	CD197 (CCR7)-FITC	Anti-TNF-α-FITC	Anti-IL-17-FITC	CD137-PE
CD45RA-APC	CD27-APC	Anti-IL-10-APC	Anti-IL-5-APC	Anti-FoxP3-APC
CD31-PE	CD95-PE	Anti-IFN-γ-PE	Anti-IL-4-PE	Anti-Helios-FITC

Table 1: Antibody panels for the analysis of naive/memory T cell subsets, cytokine-producing T cells, as well as CD154⁺ Tcon and CD137⁺ Treg cells. Tandem Signal Enhancer was added to all panels.

Results

Detection of antigen-specific CD4⁺CD154⁺ T cells

The CD154 antigen is a reliable marker for the detection of activated antigen-specific T cells.³ We first determined the proportion of CD154⁺ T cells in PBMCs from healthy donors, upon stimulation with antigens from *A. fumigatus*, *C. albicans*, CMV, and AdV and with tetanus toxoid for 7 h.

The percentage of the entire population of activated CD154⁺ cells among CD4⁺ cells could be determined reliably (range: 0.03%–0.64%; fig. 1). However, the total number of CD154⁺ cells in the samples was too low to characterize smaller subpopulations, such as naive and memory T cells, by flow cytometry.

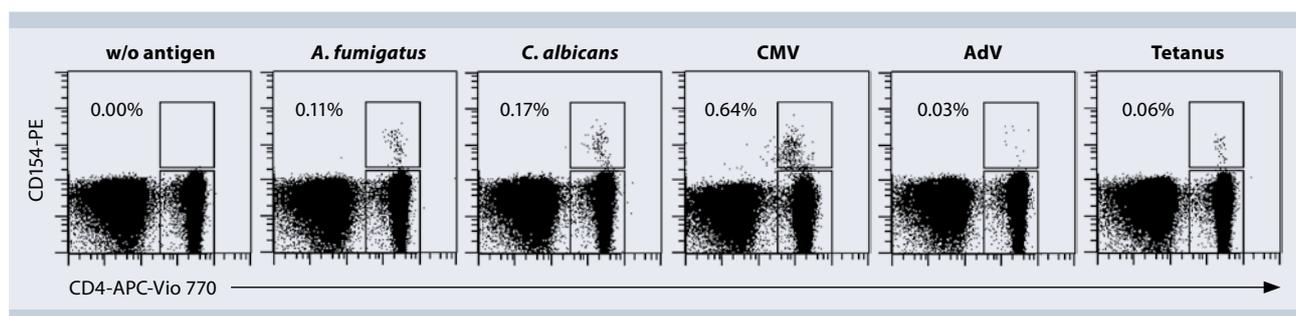


Figure 1: CD154 is a reliable marker for the detection of antigen-specific CD4⁺ T cells. PBMCs from healthy donors were stimulated with *A. fumigatus* or *C. albicans* lysates, CMV or AdV peptide pools, or tetanus toxoid for 7 h. CD154⁺ expression was analyzed by flow cytometry. Numbers indicate the frequencies of antigen-reactive CD154⁺ cells among CD4⁺ T cells. Data originally published in: Bacher *et al.* (2013) *J. Immunol.* 190: 3967–3976. Copyright © 2013 The American Association of Immunologists, Inc.

Highly sensitive enumeration and characterization of rare antigen-specific CD4⁺ T cell subsets

To enable the sensitive analysis of rare antigen-specific T cell subsets by flow cytometry, we magnetically enriched the activated CD154⁺ cells after stimulation with *A. fumigatus* lysate. In the example shown in figure 2, only about 120 CD154⁺ cells were detected after acquiring 4×10⁵ PBMCs. In contrast, after enrichment of CD154⁺ cells from 1.5×10⁷ PBMCs and acquisition of the entire positive fraction, more than 3,600 CD154⁺ cells were detected among only approx. 5×10⁴ total events, whereas background levels in the non-stimulated sample remained low (<100 cells). These results indicate that enrichment of CD154⁺ T cells prior to flow cytometry greatly increases the signal-to-noise ratio for a sensitive analysis of antigen-specific T cell subsets.

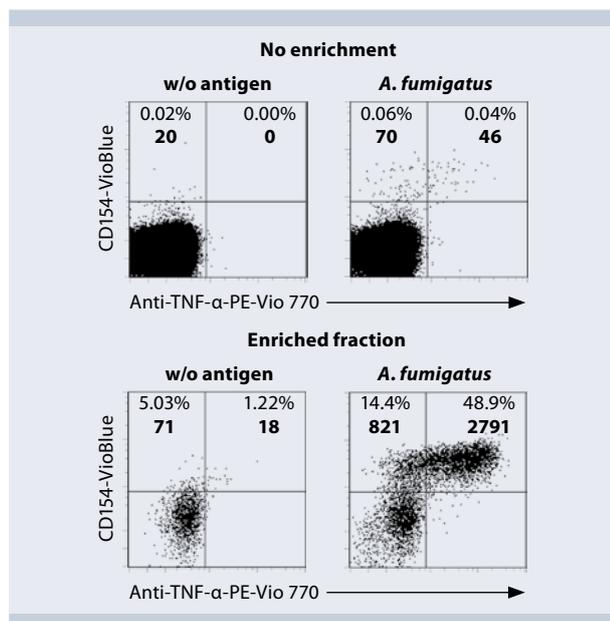


Figure 2: Enrichment of CD154⁺ cells allows for highly sensitive enumeration and characterization of rare antigen-specific CD4⁺ T cells. PBMCs from a healthy donor were stimulated with *A. fumigatus* lysate or left unstimulated. CD154 expression was assessed among CD4⁺ T cells without prior enrichment (upper plots) and after CD154⁺ cell enrichment (lower plots). Bold numbers indicate the total count of CD154⁺ cells after acquiring 4×10⁵ PBMCs (upper plots) or the enriched fraction obtained from 1.5×10⁷ PBMCs (lower plots).

Phenotypic characterization of antigen-specific naive and memory subsets

To further dissect the enriched CD154⁺ *A. fumigatus*-specific T cell population by flow cytometry, we designed two antibody panels (see materials and methods section). The gating strategy illustrated in figure 3 allowed us to easily distinguish naive from memory T cells. Moreover, we were able to determine the proportions of recent thymic emigrants (RTE, 22.7% of CD154⁺ cells), peripheral circulating

naive T cells (31.2%), stem cell memory T cells (T_{scm}; 0.2%), central memory T cells (T_{cm}; 15.2%), and effector memory T cells (T_{em}; 4.7%). These data show that the possibility of measuring a large number of rare target cells pre-enriched from a large blood sample greatly improves the significance of the multiparameter approach, permitting identification of small cell subsets at high resolution. Small subsets, such as T_{scm}, would not be detectable without prior enrichment of the antigen-specific T cells.

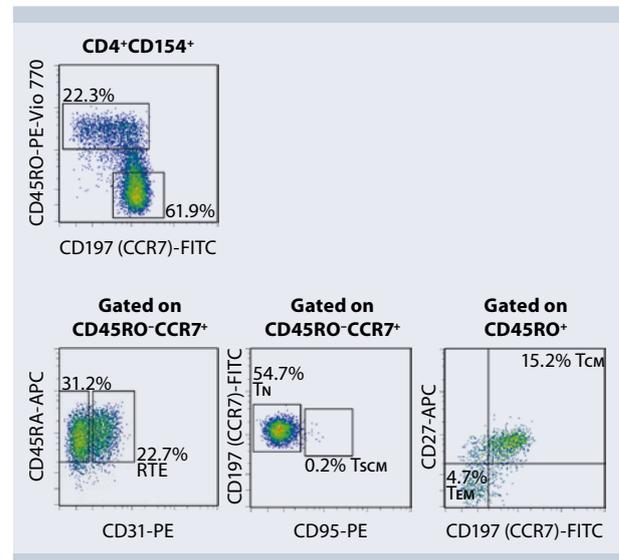


Figure 3: Characterization of naive and memory T cell subsets. *A. fumigatus*-specific CD154⁺ T cells were enriched as described and counterstained for phenotypic markers to discriminate between naive and memory T cell subsets. Percentages of the respective cell subsets among all reactive CD154⁺ T cells are shown. RTE: recent thymic emigrants; CD31⁻ naive cells: peripheral circulating naive T cells; T_n: naive T cells; T_{scm}: stem cell memory T cells; T_{cm}: central memory T cells; T_{em}: effector memory T cells

Characterization of cytokine production in antigen-specific naive and memory T cells

The high resolution is also important for the analysis of cytokine-producing subsets. We compared the cytokine production capacity of the naive and memory T cell subsets by flow cytometry, based on two antibody panels (see materials and methods section). These panels enabled us to determine the percentages of cell subsets producing TNF-α, IFN-γ, IL-10, IL-17, IL-4, or IL-5. In the example shown in figure 4, the majority (70.4%) of naive CD4⁺CD154⁺ T cells produced TNF-α, whereas effector cytokines were almost absent (<1%). Likewise, the majority (63.6%) of memory CD4⁺CD154⁺ T cells produced TNF-α. More than 10% of the memory subset produced IFN-γ, indicating the presence of Th1 cells. However, only small but still significant proportions of memory but not naive T cells produced IL-10, IL-17, or IL-4.

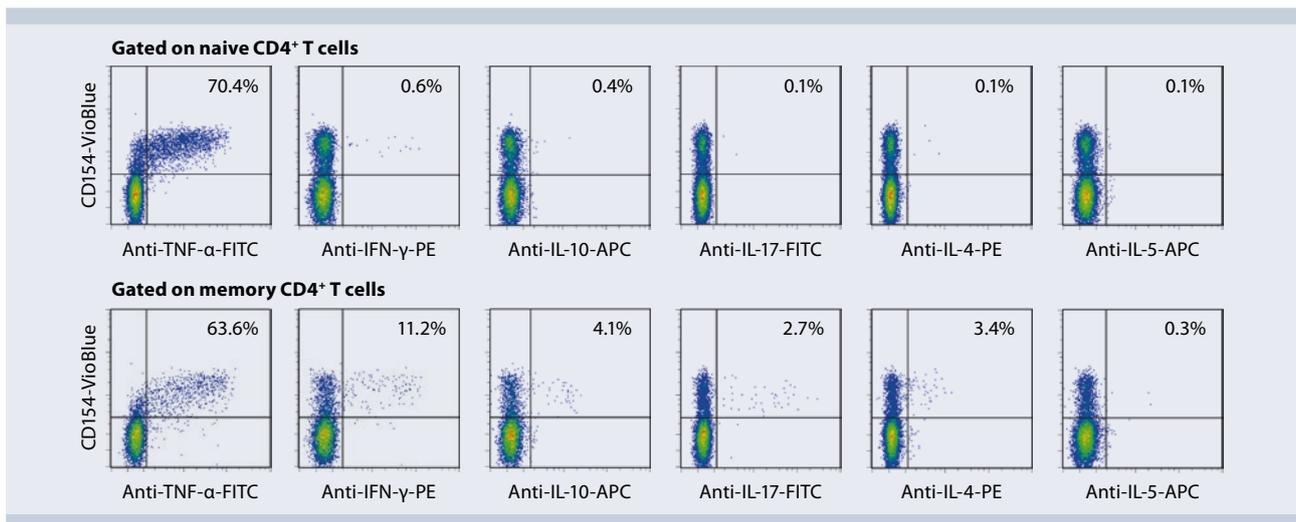


Figure 4: Characterization of cytokine production in antigen-specific naive and memory T cells. *A. fumigatus*-specific CD154⁺ T cells were enriched by ARTE and analyzed for cytokine expression within the antigen-specific naive and memory compartments. Cells were gated on CD4⁺ lymphocytes. Percentages of cytokine-expressing cells among CD154⁺ T cells are shown.

Enrichment of CD154⁺ and CD137⁺ cells enables identification of antigen-specific Tcon and Treg cells in parallel

Using the ARTE technique based on the expression of CD154 and CD137^{4,5}, we were able to differentiate between CD137⁺CD154⁺ Tcon and CD137⁺CD154⁻ Treg cells⁶. Almost all CD137⁺ cells co-expressed FoxP3, whereas FoxP3 expression was absent in CD154⁺ cells (fig. 5, upper row). Around 90%

of the CD137⁺ cells were positive for FoxP3 and CD25, and the majority of FoxP3⁺ cells co-expressed the transcription factor Helios. In contrast, CD154⁺ T cells had a conventional T cell phenotype (CD25⁻FoxP3⁻Helios⁻). These data are in line with our findings that *A. fumigatus* induces a significant Treg response *in vivo*⁴, which can control excessive immune responses, such as allergies.

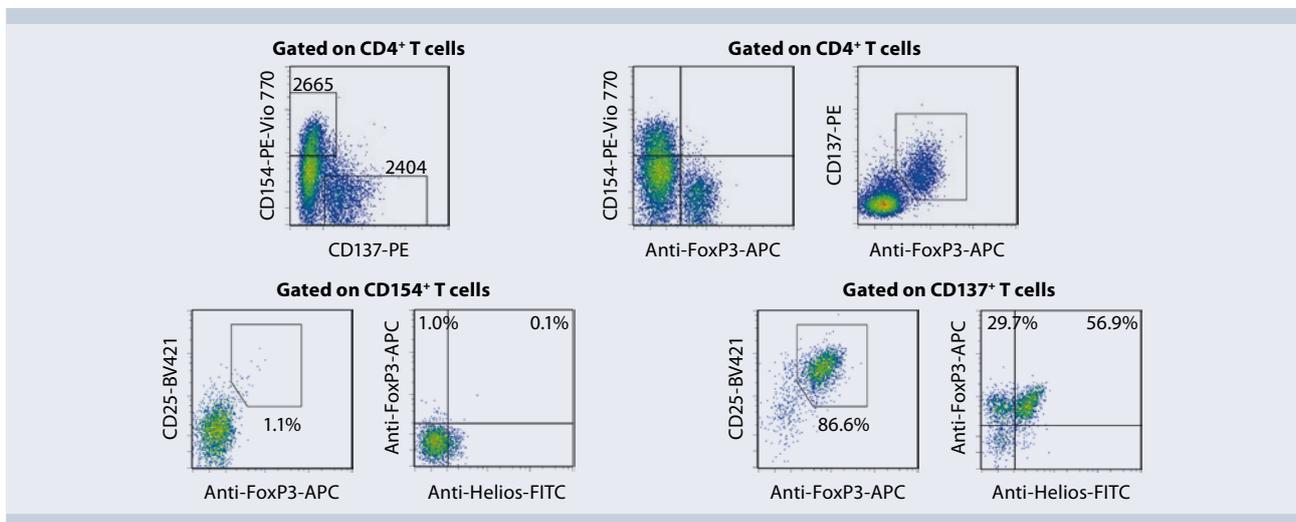


Figure 5: Combined enrichment of CD154⁺ and CD137⁺ cells enables parallel identification of antigen-specific Tcon and Treg cells. PBMCs were stimulated with *A. fumigatus* lysate and reactive CD154⁺ and CD137⁺ T cells were enriched by ARTE. Enriched CD154⁺ and CD137⁺ were counterstained for typical Treg cell markers CD25, FoxP3, and Helios.

Conclusion

- Enrichment of antigen-reactive T cells, based on MACS Technology, enhances the signal-to-noise ratio for sensitive multicolor flow cytometry.
- The collection of large numbers of rare target cells following magnetic pre-enrichment greatly improves the resolution of downstream multiparameter flow cytometric analyses.
- Comprehensive panels of MACS Antibodies enable the detailed phenotypic characterization of the enriched antigen-specific CD154⁺ T cells for the distinction between naive and memory T cell subsets as well as the analysis of cytokine production in naive and memory T cells.
- The parallel enrichment of CD137⁺ and CD154⁺ cells and a specific antibody panel allow for the characterization of CD137⁺CD154⁺ Tcon cells and CD137⁺CD154⁻ Treg cells.

References

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4. Bacher, P. *et al.* (2014) *Mucosal Immunol.* 7: 916–928.
5. Schoenbrunn, A. *et al.* (2012) *J. Immunol.* 189: 5985–5994.
6. Bacher, P. *et al.* (2014) *J. Immunol.* 193: 3332–3343.

MACS Product	Order no.
Cell isolation	
CD154 MicroBead Kit, human	130-092-658
CD137 MicroBead Kit, human	130-093-476
Cell culture and stimulation	
<i>A. fumigatus</i> Lysate	130-098-170
PepTivator CMV pp65, human	130-093-435
PepTivator Adv5 Hexon	130-093-496
CD28 pure – functional grade, human	130-093-375
CD40 pure – functional grade, human	130-094-133
Flow cytometry	
MACSQuant Analyzer 10	130-096-343
MACSQuantify Software	130-094-556
Anti-FoxP3-APC, human and mouse (clone: 3G3)	130-093-013
Anti-Helios-FITC, human and mouse (clone: 22F6)	130-104-000
Anti-IFN- γ -PE (clone: 45-15)	130-091-653
Anti-IL-4-PE (clone: 7A3-3)	130-091-647
Anti-IL-5-APC (clone: JES1-39D10)	130-091-834
Anti-IL-10-APC (clone: JES3-9D7)	130-096-042
Anti-IL-17-FITC (clone: CZ8-23G1)	130-094-520
Anti-TNF- α -FITC (clone: cA2)	130-091-650
CD4-APC-Vio 770 (clone: VIT4)	130-096-652
CD8-VioGreen (clone: BW135/80)	130-096-902
CD14-VioGreen (clone: TÜK4)	130-096-875
CD20-VioGreen (clone: LT20)	130-096-904
CD27-APC (clone: LG.3A10)	130-097-218
CD31-PE (clone: AC128)	130-092-653
CD45RA-APC (clone: T6D11)	130-092-249
CD45RO-PE-Vio 770 (clone: UCHL1)	130-096-739
CD95-PE (clone: DX2)	130-092-416
CD137-PE (clone: 4B4-1)	130-093-475
CD154-VioBlue (clone: 5C8)	130-096-217
CD154-PE-Vio 770 (clone: 5C8)	130-096-793
CD197 (CCR7)-FITC, human (clone: REA108)	130-099-172
FoxP3 Staining Buffer Set	130-093-142
Inside Stain Kit	130-090-477
Tandem Signal Enhancer, human	130-099-888



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