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Magnetic enrichment of antigen-specific CD4+ T cells using the MACSQuant® Analyzer 10

Magnetic enrichment of antigen-specific CD4+ T cells enables the in-depth characterization of vaccine-induced circulating follicular T helper cells

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Introduction

Importance of circulating follicular T helper cells (cTFH)

Follicular T helper cells (TFH) play a crucial role in supporting germinal center induction and responses by providing B cell help needed for affinity maturation and generation of B cell memory. Circulating (cTFH) have moved to the center stage of immunological research since it becomes increasingly apparent that these cells are providing insights into the immune responses occurring in the germinal centers of lymphoid organs. Typically, these lymphatic tissues are not accessible when studying human immune responses, thus making cTFH an attractive target for surrogate markers of immunity. The main identifying marker is CXCR5 which is a chemokine receptor required for lymphocytes to enter the B cell compartments of lymphoid tissues, while other markers such as CXCR3 and CCR6 have been used to characterize cTFH subsets[1,2]. Here, we report on the feasibility of an in-depth characterization of cTFH subsets in humans immunized once with a promising Ebola (rVSV-ZEBOV) vaccine[3]. The described method allowed us to demonstrate successfully that the frequency of cTFH and in particular, cTFH17, was associated with ZEBOV-specific antibody titers[4]. Moreover, applying this analysis panel may reveal similar associations and therefore, advance our understanding of immune mechanisms associated with protective immunity.

Results

Enrichment of antigen-specific cTFH cells

The main hurdle for the characterization of antigen-specific cells, and in particular, subsets within these cells, is the low frequency of cells which requires very large numbers of cells to be acquired for flow cytometric analysis[5]. To overcome this limitation, we adapted the enrichment of CD4+CD154+ antigen-specific T cells to a panel that identifies subsets of cTFH cells. Multiple antigen-specific T cell enrichments were performed via the use of the integrated pre-enrichment column of the MACSQuant® Analyzer 10. This workflow allowed us to stain, enrich, and analyze multiple samples in an automated fashion increasing the through-put and reproducibility of the experimental set-up.

The objective was to determine whether this T cell subset serves as immune correlates or surrogate marker of protection for vaccines that supposedly mediate protection through antibodies. We tested the analysis strategy first on samples from a recent Ebola vaccine, where study subjects received a single immunization with the vaccine[6]. The results of the study attest to the high sensitivity of this experimental approach.

Cryopreserved peripheral blood mononuclear cells (PBMCs) were cultured with a ZEBOV-GP peptide pool at 1.0 µg/mL or medium alone (control stimulation). Cells were cultured for 16 hours (37 °C, 5% CO_2) in RPMI-1640 containing 10% human serum at a concentration of 5×10^6 cells/mL. CD196 (CCR6)-APC, human and CD40 pure – functional grade, human were added to the culture at a 1:10 and 1:100 dilution, respectively. Following stimulation, cells were washed and stained with CD154-Biotin for 15 minutes at 4 °C in solution (0.5% human serum and 0.1% sodium azide in phosphate-buffered saline (PBS)). Cells were further incubated with Anti-Biotin MicroBeads UltraPure for 15 minutes at 4 °C. After washing, a pre-titrated and optimized antibody cocktail with fluorochrome-conjugated antibodies against CD3-VioBlue®, CD4-PerCP-Vio® 700, CD185 (CXCR5)-PE-Vio770, CD183 (CXCR3)-VioBright™ FITC, CD154-PE, and Zombie Aqua™ Fixable dye was added and incubated for 45 minutes at 4 °C.

Cells were enriched (program Enrich-S) and acquired on a MACSQuant® Analyzer 10. Refer to figure 1 for the gating strategy. Data analysis was performed using Flowlogic™ flow cytometry analysis software.

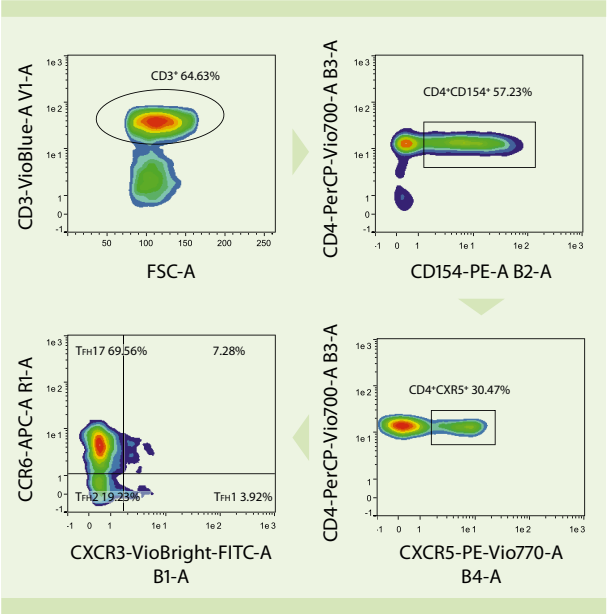

Figure 1: Gating strategy for flow cytometric analysis. After antigen stimulation, cells were gated based on viability (not shown here) and expression of CD3. This population was then further gated based on the expression of the activation marker CD154 (cells are considered "antigen-specific") and lineage marker CD4. Antigen-specific CXCR5⁺ cells were then analyzed for the concomitant expression of CXCR3 (TFH1) and CCR6 (TFH17).

Enrichment increased the signal of antigen-specific cells more than thirty fold (representative enrichment results depicted in figure 2).

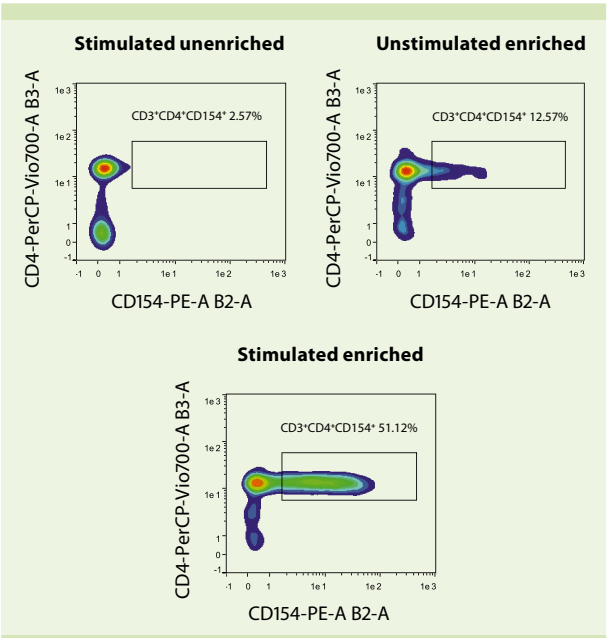

Figure 2: Frequency of CD154⁺ CD3⁺CD4⁺ T cells after control stimulation (left panel) or after antigen stimulation prior (middle panel) and after enrichment (right panel). Both, manual and automated enrichment (MACSQuant Analyzer 10 integrated separation column) resulted in similar yields of CD154⁺ cells.

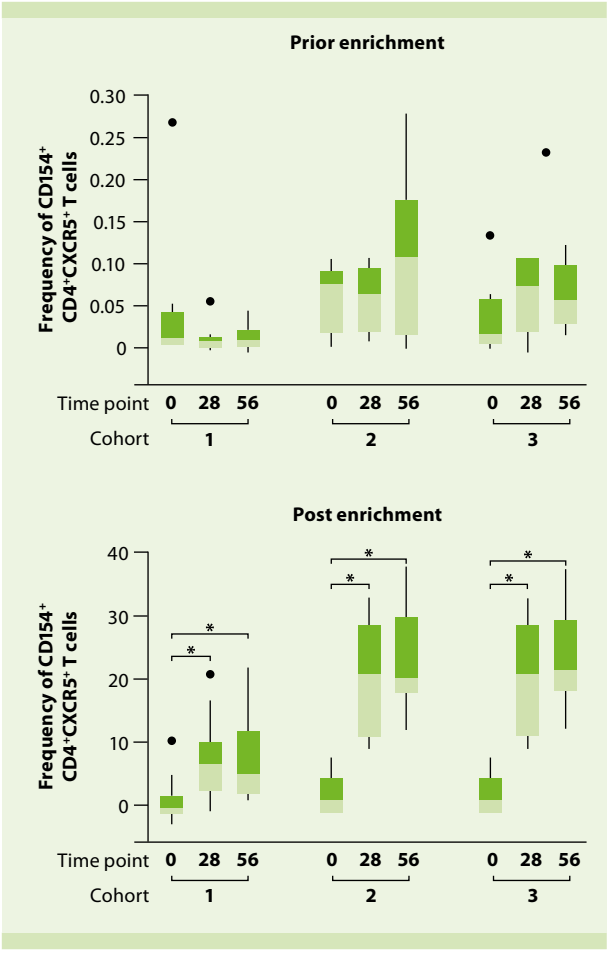

Figure 3: Enrichment of antigen-specific cTFH reveals significant changes induced by vaccination. Samples from anti-ZEBOV vaccinated subjects were acquired without (left column) or with enrichment (right column) of CD154⁺ cells. The gating strategy shown in figure 2 was applied. Box plots represent n=10 subjects/dose group (three dose groups). Cohorts 1, 2, and 3 represent low, medium, and high dose vaccine groups, respectively.

Identification of antigen-specific cTFH subsets

The enrichment of CD154⁺ cells enabled the subsequent in-depth characterization of the cTFH subsets as originally described[1] and was modified to enable the analysis using a MACSQuant Analyzer 10: CD3⁺CD4⁺CD154⁺CXCR5⁺CXCR3⁻ CCR6⁻ cells are considered to be cTFH2, CD3⁺CD4⁺CD154⁺CXCR5⁺CXCR3⁺CCR6⁻cells are cTFH1, and CD3⁺CD4⁺CD154⁺CXCR5⁺CXCR3-CCR6⁺cells are cTFH17.

We applied this experimental strategy to the evaluation of cTFH induction via a viral vaccine (figure 3). To determine the impact of enrichment on the ability to detect significant changes after vaccination, we acquired and analyzed aliquots of the samples prior and after enrichment.

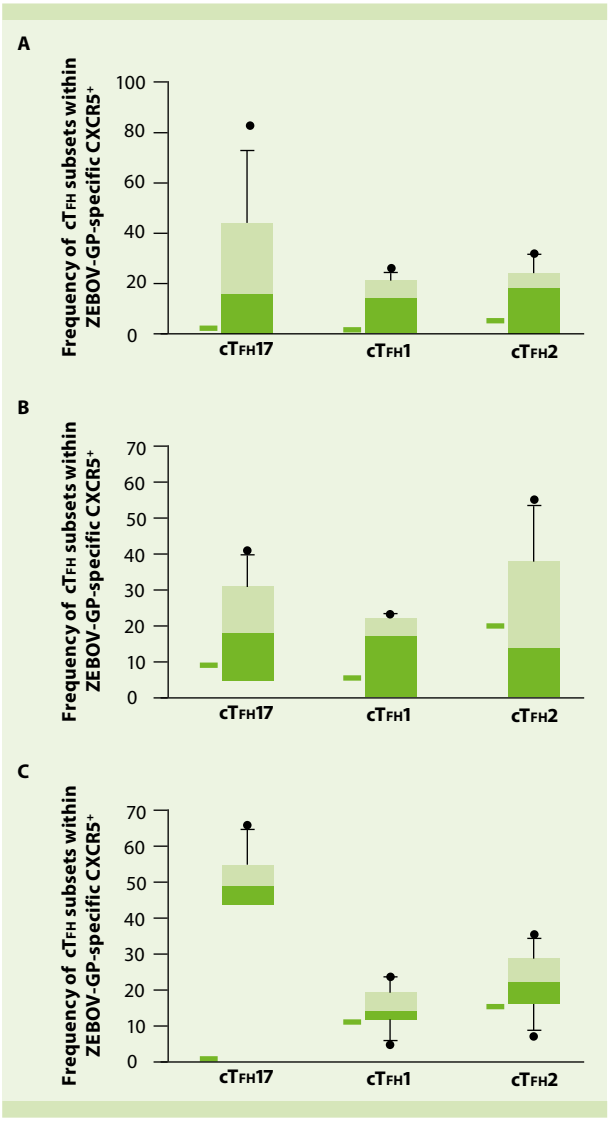

Figure 4: Differences in the composition of cTFH as a function of vaccine dose. 28 days post vaccination, PBMCs enriched based on the expression of the activation marker CD154 were subsequently analyzed by flow cytometry for the expression of CD3, CD4, CXCR5, and the subset-specific markers CCR6 and CXCR3. Responses in the dose escalation cohorts are shown in box plots (4A: low dose; 4B: medium dose; 4C: high dose). The bold line next to each box plot represents the median frequency of each cTFH population at day 0. Data expressed as absolute numbers of $CD3^+CD4^+CXCR5^+CCR6^-CXCR3^-$ (TFH2), $CD3^+CD4^+CXCR5^+CCR6^-CXCR3^+$ (TFH1), and $CD3^+CD4^+CXCR5^+CCR6^+CXCR3^-$ (TFH17) within $CD4^+CD154^+$ T cells. The original data were published in Farooq *et al.* (2016), Scientific Reports 6, 27944.

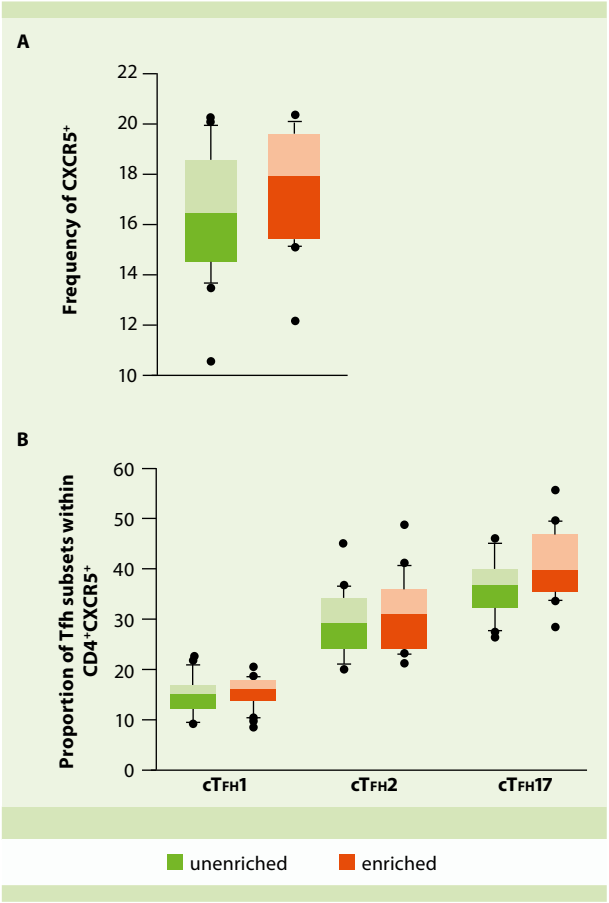

Figure 5: Enrichment of $CD154^+$ $CD4^+$ T cells does not skew the composition of the cTFH subsets. PBMCs stimulated with antigen and then either acquired ("unenriched") or enriched based on CD154 ("enriched") were analyzed for (A) the frequency of $CXCR5^+$ T cells and (B) the composition of the cTFH populations based on the expression of chemokine markers (see figure 1 for gating strategy). Boxplots represent data from 18 different samples obtained from subjects immunized with a viral vaccine.

Conclusion

(1) The enrichment of antigen-specific CXCR5$^+$ T cells based on CD154 via the integrated pre-enrichment column on the MACSQuant® Analyzer 10 enables for a detailed characterization of cTFH subsets (using MACS® Antibodies) and results in statistically significant frequencies compared to pre-immune time point for the rVSV-ZEBOV vaccine.
(2) The enrichment did not skew the composition of the cTFH subsets thus allowing an unbiased analysis with a much higher resolution.

References

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Product	Clone	Order number
CD3-VioBlue, human	BW264/56	130-094-363
CD4-PerCP-Vio700, human	M-T466	130-103-793
CD40 pure – functional grade, human	HB14	HB14
CD154-Biotin, human	5C8	130-092-690
CD154-PE, human	5C8	130-092-289
CD185 (CXCR5)-PE-Vio770, human	REA103	130-105-459
CD183 (CXCR3) -VioBright FITC, human	REA232	130-106-009
CD196 (CCR6)-APC, human	REA190	130-100-373
Anti-Biotin MicroBeads UltraPure	–	130-105-637
Viobility™ 405/520 Fixable Dye*	–	130-109-814
MACSQuant Column	–	130-094-458
MACSQuant Analyzer 10	–	130-096-343

* equivalent to Zombie Aqua™ Fixable dye

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