Microchip-based fluorescence-activated cell sorting of antigen-specific CD137^-CD8^+ T cells in a disposable and closed cartridge system using the MACSQuant® Tyto™ Sorter

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Introduction
Adoptive T cell therapy has been shown to be a promising new treatment strategy for a variety of malignancies. Exploitation of this potent therapeutic approach increases the need for easy and effective isolation of antigen-specific T cells in a clinical setting. Recent technological advances like the MACSQuant® Tyto™, a microfluidic flow sorter that is fully closed, sterile, and easy to use, enable purification of target cells in compliance with GMP, cell manufacturing requirements. The heart of the Tyto System is the MACSQuant TytoCard, a single-use cartridge carrying a sorting microchip, which allows for completely aseptic sorting conditions without cross-contamination between samples. Here we demonstrate the capacity of the MACSQuant Tyto to sort CD8^-HBB^- antigen-specific cytotoxic T cells. CD137 is a member of the TNFR family and functions as a marker for activated T cells by promoting T cell proliferation and survival. CD137 might be a promising selection marker for identifying and validating T cell responses to unknown antigens or epitopes since the method is not restricted to the knowledge of the immunogenic peptide or HLA alleles, which is necessary in the widely used peptide/MHC (pMHC) multimer or dye-based isolation of antigen-specific T cells. As a model system, we used fresh peripheral blood mononuclear cells (PBMCs) and stimulated them overnight with different peptides to induce a CD137 expression. CD137-FITC^- cytotoxic T cells were sorted in high purity and showed good viability as well as activation marker expression profiles in subsequent cell culture. These results demonstrate the applicability of the MACSQuant Tyto system for future medical scale isolation of antigen-specific T cells.

Methods
1. Workflow overview
An overview of the work-flow is shown in figure 1. Whole blood or buffy coat was obtained from healthy blood donors, and PBMCs were isolated by density gradient centrifugation. Cells were suspended in RPMI 1640 medium supplemented with 5% human AB serum at 1-10 x 10^6/mL in 24-well plates either with 1 µg/mL SEB or 1 µg/mL PepTivator® EBV Consensus and stimulated overnight. The Tyto System is the MACSQuant TytoCard, a single-use cartridge carrying a sorting microchip, which allows for completely aseptic sorting conditions without cross-contamination between samples. Here we demonstrate the capacity of the MACSQuant Tyto to sort CD8^-HBB^- antigen-specific cytotoxic T cells. CD137 is a member of the TNFR family and functions as a marker for activated T cells by promoting T cell proliferation and survival. CD137 might be a promising selection marker for identifying and validating T cell responses to unknown antigens or epitopes since the method is not restricted to the knowledge of the immunogenic peptide or HLA alleles, which is necessary in the widely used peptide/MHC (pMHC) multimer or dye-based isolation of antigen-specific T cells. As a model system, we used fresh peripheral blood mononuclear cells (PBMCs) and stimulated them overnight with different peptides to induce a CD137 expression. CD137-FITC^- cytotoxic T cells were sorted in high purity and showed good viability as well as activation marker expression profiles in subsequent cell culture. These results demonstrate the applicability of the MACSQuant Tyto system for future medical scale isolation of antigen-specific T cells.

2. Sorting of antigen-specific CD137^-CD8^+ T cells
Isolation of antigen-specific T cells was performed entirely on the microfluidic flow sorter MACSQuant Tyto using a single-use cartridge. In total, 2.5 x 10^7 cells with 0.4-3.5% target cells were used and CD137^-CD8^+ CTLs were isolated with (fig. 2A) or without (fig. 2B) exclusion of CD94^+, CD20^+, and CD56^+ cells. The sample was sorted at 4 mL/hour and a pressure of approximately 130 mbar. Depending on the sorted volume, the sort took 1 to 2.5 h to complete.

3. Cultivation of enriched antigen-specific T cells and activation marker expression
CD137^+CD8^+ T cells from PBMCs (woven donor) with stimulation frequencies ranging from 1.8% to 12.1% were sorted on the MACSQuant Tyto. The average purity of enriched cells amounted to 96% (among viable leukocytes) and 91% (among viable lymphocytes) as shown in figure 4. The different symbols represent samples from individual donors. Samples were used for one or more experiments. The average yield of target cells in the enriched fraction and depletion of target cells in the non-target cell fraction was around 60%. For some samples however yields of up to 80% could be achieved. Consequently, sorting of 2.1 x 10^7 total cells containing 6% CD137^-CD8^+ T cells resulted in the recovery of about 1.1 x 10^6 target cells from a complete 10-ml sort. The addition of a dump channel for exclusion of CD19^+, CD4^+ and CD56^+ cells as part of the sorting strategy, led to an increased purity of more than 98% (94% viability). The viability of sorted cells was greater than 98% in all sorts, which demonstrates the gentleness of the sorting process.

Results
1. Flow cytometry analysis of cell sorting
Flow cytometry analysis of the sorted fractions was performed on the MACSQuant Analyzer 10 using the MACSQuantify™ Software. Data was gated on viable single cells (fig. A) and then plotted to show CD137-FITC^- vs. CD8-VioBlue (fig. B). Cell viability was assessed by staining with propidium iodide (PI) prior to the measurement. For the analysis of isolated cells obtained by the depletion strategy, CD94^+, CD20^+, and CD56^+ cells were evaluated separately during flow cytometry analysis (fig. 1D).

2. Cell sorting performance
CD137^+CD8^+ T cells from PBMCs (woven donor) with stimulation frequencies ranging from 1.8% to 12.1% were sorted on the MACSQuant Tyto. The average purity of enriched cells amounted to 96% (among viable leukocytes) and 91% (among viable lymphocytes) as shown in figure 4. The different symbols represent samples from individual donors. Samples were used for one or more experiments. The average yield of target cells in the enriched fraction and depletion of target cells in the non-target cell fraction was around 60%. For some samples however yields of up to 80% could be achieved. Consequently, sorting of 2.1 x 10^7 total cells containing 6% CD137^-CD8^+ T cells resulted in the recovery of about 1.1 x 10^6 target cells from a complete 10-ml sort. The addition of a dump channel for exclusion of CD19^+, CD4^+ and CD56^+ cells as part of the sorting strategy, led to an increased purity of more than 98% (94% viability). The viability of sorted cells was greater than 98% in all sorts, which demonstrates the gentleness of the sorting process.

Conclusion
• The MACSQuant Tyto System enables isolation of antigen-specific (cytotoxic) T cells based on the expression of the activation marker CD137.
• Sorting strategies with additional depletion of CD94^- monocytes, CD20^- B cells, and CD56^- NK cells achieved purities of around 95%.

• Sorting of 2-5 x 10^7 cells containing 6-12% CD137^-CD8^+ T cells results in a target cell recovery of up to 80%.
• Upon cultivation SEB-stimulated sorted cells showed high viability rates and an activation marker expression profile similar to unsorted cells.