The MHC MACSimer technology combines flexibility, quality and releasability for reliable detection of antigen-specific T cells

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Introduction

Antigen (Ag)-specific T cells play an essential role in the immune responses towards cancer, infectious diseases, and autoimmunity. Furthermore, the precise analysis, isolation and characterization of rare Ag-specific T cells is crucial for the development of cellular therapies, cancer- or virus-specific vaccinations, and patient monitoring. Given the individual component of Ag-specific T cell responses ("MHC/HLA" restriction), researchers need innovative and flexible tools providing them with high-quality results to enable effortless integration of Ag-specific T cell responses into translational workflows.

We have therefore designed new reagents, the MHC MACSimer FleX Kits and peptide-loaded MACSimers. The novel construct design of the MHC multimer product is overcoming major challenges of Ag-specific T cell analytics. MHC MACSimers feature superior specificity and fluorescence brightness through an optimal balance of MHC molecules and fluorophores, enabling the detection of even extremely rare T cell subsets with highest reliability.

To enable the integration into translational workflows our MHC MACSimer reagents feature

a) A flexible peptide-loading technology in the case of our *MHC MACSimer FleX Kits*, allowing for extensive T cell epitope screening.

b) The REAlease® technology for our MHC MACSimer FleX Kits and *peptide-loaded MACSimers* to obtain label-free T cells after enrichment, e.g., for multiple sequential sorting steps or functional downstream assays.

c) A comprehensive collection of *MACSpep Single Peptides* covering a broad variety of T cell epitopes related to cancer, infection, and autoimmunity, delivering the ideal accessory to our MHC MACSimer FleX Kits.

Overview of the MHC MACSimer and MACSpep portfolio

MHC MACSimer FleX Kits	MACSpep Single Peptides	Peptide-loaded MACSimers
"Load-it-yourself" MACSimers	MHC class I and class II binding peptides	Pre-loaded "off-the-shelf" MACSimers
Flexible TCR and epitope screening	For MACSimer FleX Kit loading	For highly standardized T cell analytics
Two colors for flow cytometry	Expansion of Ag-specific T cells	Two colors for flow cytometry
(PE/Vio® 667, more will follow)		(PE/ Vio® 667, more will follow)

Results

1 Performance of MHC MACSimers

Figure 1

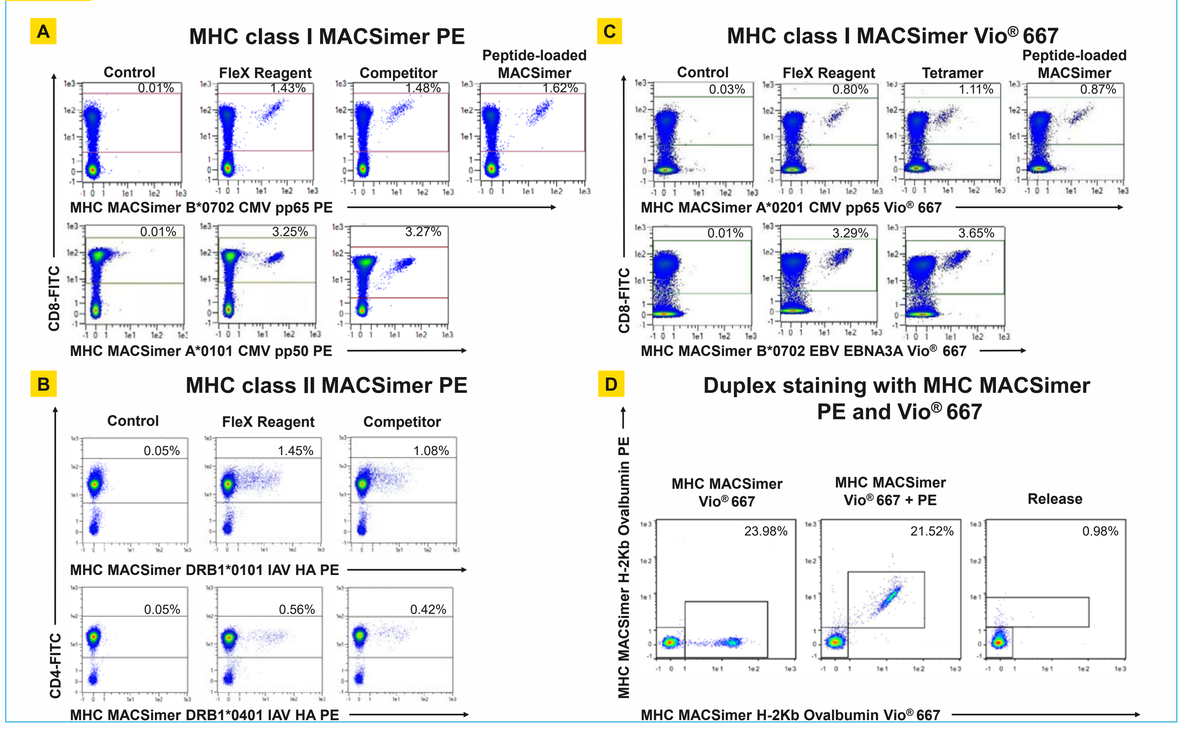


Figure 1: Detection of human and murine Ag -specific CD4+ and CD8+ T cells with MHC MACSimer FleX Reagents and peptide-loaded MACSimers in comparison to a competitor product. Frequencies are given for MHC class I MACSimer-stained cells under CD8+ T cells and MHC class II MACSimer-stained cells under CD4+ T cells. As control, allele-matched MHC MACSimer control reagents were used. CD8-FITC staining was performed with CD8 antibody, anti-human, REAfinity™ or CD8a antibody, anti-mouse. CD4 co-staining was performed with CD4 antibody, anti-human, REAfinity™. Flow cytometry analyses were performed using the MACSQuant® Analyzer. *A) MHC class I MACSimer PE*: Staining with MHC MACSimer B*0702 FleX Reagent, human, PE loaded with MACSpep CMV pp65 (TPRVTGGGAM) HLA-B*07; a competitor product; and peptide-loaded MHC MACSimer B*0702 CMV pp65 (TPRVTGGGAM), human, PE. The stainings were performed on HLA-B*0702+ CMV pp65-reactive human PBMC (upper row). Staining with MHC MACSimers A*0101 FleX Reagent, human, PE loaded with MACSpep CMV pp50 (VTEHDTLLY) HLA-A*01; and a competitor product. The stainings were performed on HLA-A*0101+ CMV pp50-reactive human PBMC (lower row). *B) MHC class II MACSimer PE*: Staining with MHC MACSimer DRB1*0101 FleX Reagent, human, PE and MHC MACSimers DRB1*0401 FleX Reagent, human, PE loaded with MACSpep IAV HA (PKYVKQNTLKLAT) HLA-DR; and a competitor product. The stainings with MHC MACSimers DRB1*0101 were performed on expanded HLA-DRB1*0101+ IAV HA-reactive CD4+ T cells, spiked into PBMC (upper row). The stainings with MHC MACSimers DRB1*0401 were performed on expanded HLA-DRB1*0401+ IAV HA-reactive CD4+ T cells, which were sorted for positive cells with the MACSQuant® Tyto® Cell Sorter and spiked into human PBMC (lower row). *C) MHC class I MACSimer Vio® 667*: Staining with MHC MACSimer A*0201 FleX Reagent, human, *Vio® 667* loaded with MACSpep CMV pp65 (NLVPMVATV) HLA-A*02; with an in-house reagent A*0201 CMV pp65 Tetramer Vio® 667; and peptide-loaded MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, Vio® 667. The stainings were performed on HLA-A*0201+ CMV pp65-reactive human PBMC (upper row). Staining with MHC MACSimer B*0702 FleX Reagent, human, Vio® 667 loaded with MACSpep EBV EBNA3A (RPPIFIRRL) HLA-B*07; and with an in-house B*0702 EBV EBNA43A Tetramer Vio® 667. The stainings were performed on HLA-B*0702+ EBV EBNA3A-reactive human PBMC (lower row). *D) Duplex staining with MHC MACSimer PE and Vio® 667*: Detection of murine Ovalbumin-reactive CD8+ T cells with MHC MACSimer H-2Kb FleX Reagent, muouse, PE and Vio® 667 loaded with MACSpep Ova (SIINFEKL) H-2Kb. Single stained MHC MACSimer Vio® 667 (left), double-stained MHC MACSimer Vio® 667 and PE (middle), and release of the double-stained MHC MACSimer Vio® 667 and PE (right). The stainings were performed on H-2Kb+ OVA-reactive OT1 mouse splenocytes which were spiked into C57BL/6J wildtype mouse splenocytes.

2 Releasability of MHC MACSimers by the REAlease® technology

Figure 2

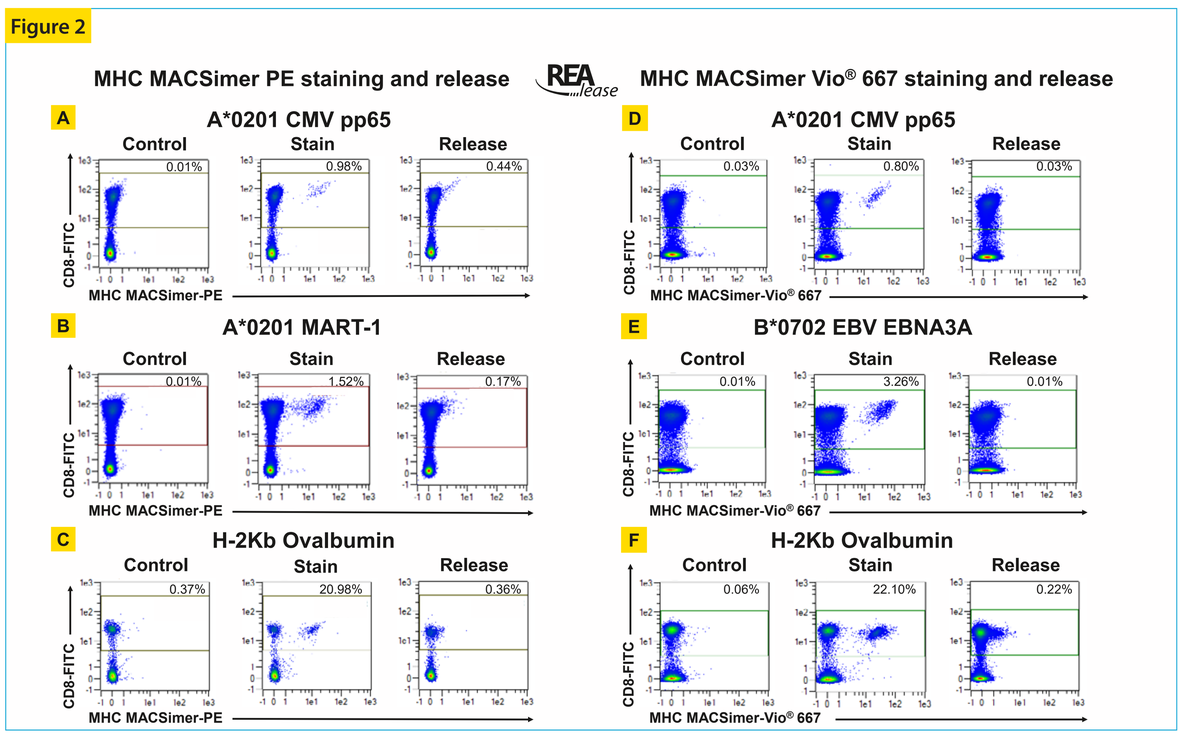


Figure 2: Staining and release of human as well as murine Ag-specific CD8+ T cells using MHC MACSimer FleX Reagents. Frequencies are given for MHC MACSimer-stained cells under CD8+ T cells. As control, allele-matched MHC MACSimer control reagents were used. CD8-FITC staining was performed with CD8 antibody, anti-human, REAfinity™ or CD8a antibody, anti-mouse. MHC MACSimers were released by the REAlease® Technology. Flow cytometry analyses were performed using the MACSQuant® Analyzer. *A)* Staining of expanded human HLA-A*0201+ CMV pp65-reactive T cells spiked into PBMC with MHC MACSimers A*0201 FleX Reagent, human, PE loaded with MACSpep CMV pp65 (NLVPMVATV) HLA-A*02. *B)* Staining of expanded human HLA-A*0201+ MART-1-reactive T cells spiked into PBMC with MHC MACSimers A*0201 FleX Reagent, human, PE loaded with MACSpep MART-1 (ELAGIGILTV) HLA-A*02. *C)* Staining of murine H-2Kb+ Ovalbumin (Ova)-reactive OT1 splenocytes spiked into C57BL/6J splenocytes with MHC MACSimers H-2Kb FleX Reagent, mouse, PE loaded with MACSpep Ova (SIINFEKL) H-2Kb. *D)* Staining of expanded human HLA-A*0201+ CMV pp65-reactive T cells spiked into PBMC with MHC MACSimers A*0201 FleX Reagent, human, Vio® 667 loaded with MACSpep CMV pp65 (NLVPMVATV) HLA-A*02. *E)* Staining of expanded HLA-B*0702+ EBV EBNA3A-reactive T cells spiked into PBMC with MHC MACSimers B*0702 FleX Reagent, human, Vio® 667 loaded with MACSpep EBV EBNA3A (RPPIFIRRL) HLA-B*07. *F)* Staining of murine H-2Kb+ Ovalbumin (Ova)-reactive OT1 splenocytes spiked into C57BL/6J splenocytes with MHC MACSimers H-2Kb FleX Reagent, mouse, Vio® 667 loaded with MACSpep Ova (SIINFEKL) H-2Kb.

3 MHC MACSimers stain Ag-specific T cells reliably on different cell materials

Figure 3

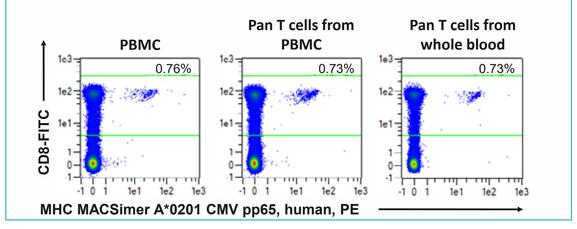


Figure 3: MHC MACSimer staining on different cell materials. Frequencies are given for MHC MACSimer-stained cells under CD8+ T cells. CD8-FITC staining was performed with CD8 antibody, anti-human, REAfinity™. Flow cytometry analyses were performed using the MACSQuant® Analyzer. The staining of Ag-specific T cells with MHC MACSimers is reliable and of equivalent precision on different cell materials. Shown are MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE stainings on three different cell materials from an HLA-A*0201+ CMV pp65-reactive donor. Staining of PBMC isolated from buffy coat by density gradient centrifugation (left), of Pan T cells isolated with the Pan T cell Isolation Kit (#130-096-535) from PBMC (middle), and of Pan T cells isolated with the MACSxpress® Whole Blood Pan T Cell Isolation Kit, human (#130-098-193) from whole blood (right).

4 Increased Ag-specific T cell frequencies by MACSpep stimulation, MACSQuant Tyto sort or magnetic enrichment

Figure 4

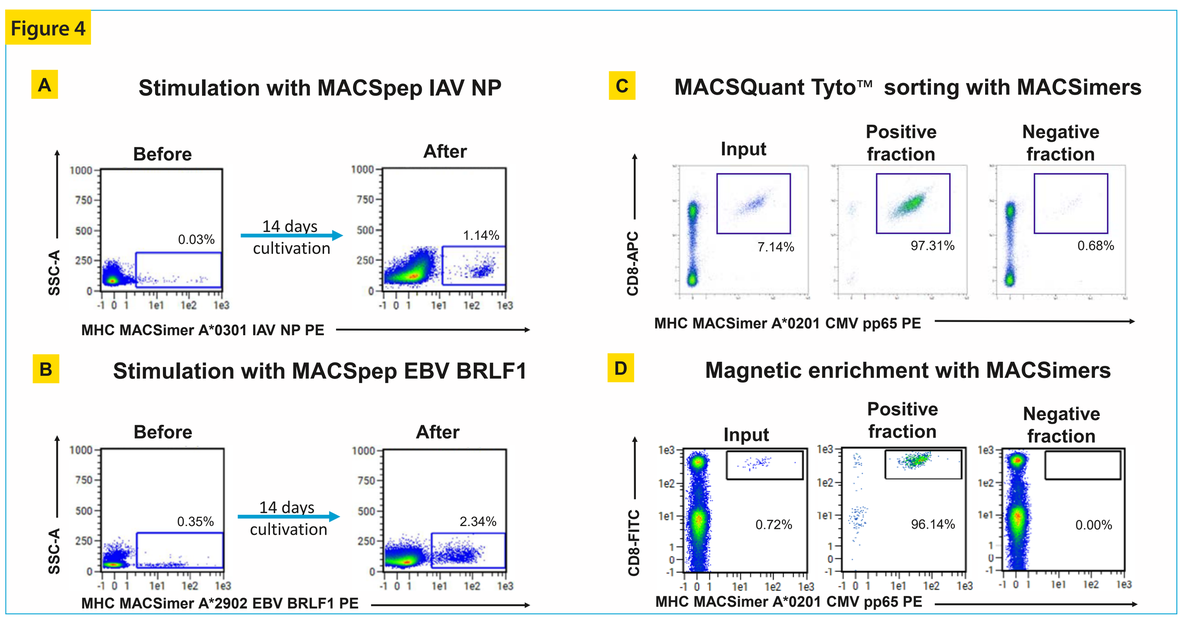


Figure 4: Increased Ag-specific T cell frequency by MACSpep stimulation, MACSQuant Tyto sort, or magnetic enrichment. Frequencies are given for MHC MACSimer-stained cells under CD8+ T cells. CD8-FITC staining was performed with CD8 antibody, anti-human, REAfinity™. Flow cytometry analyses were performed using the MACSQuant® Analyzer. Prior the MACSpep Single Peptide stimulation, PBMC from HLA-allele+ and peptide-reactive donors were isolated from whole blood via density gradient centrifugation. *A)* HLA-A*0301+ IAV NP-reactive human PBMC were stained with MHC MACSimer A*0301 FleX Reagent, human, PE loaded with MACSpep IAV NP (ILRGSVAHK) HLA-A*03. A starting frequency of 0.03% was measured. The isolated PBMC were stimulated with the MACSpep single peptide and expanded with supplemented medium. After cultivation for 14 days, the frequency of IAV NP-reactive cells under CD8+ T cells was increased to 1.14%. *B)* HLA-A*2902+ EBV BRLF1-reactive human PBMC were stained with MHC MACSimer A*2902 Flex Reagent, human, PE loaded with MACSpep EBV BRLF1 (IACPIVMRY) HLA-A*29. A starting frequency of 0.35% was measured. The isolated PBMC were stimulated with the MACSpep single peptide and expanded with supplemented medium. After cultivation for 14 days, the frequency of EBV BRLF1-reactive cells under CD8+ T cells was increased to 2.34%. *C)* MACSQuant® Tyto® sort of A*0201+ CMV pp65-reactive T cells stained with MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE. HLA-A*0201+ CMV pp65-reactive human PBMC were stained with MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE and applied to the MACSQuant Tyto™ Cartridge. Flow cytometric analysis demonstrates that the input fraction contained a frequency of 7,14% CMV pp65-reactive cells under CD8+ T cells (left). After flow sorting, the frequency of CMV pp65-reactive cells under CD8+ T cells was enriched to 97.31% in the positive fraction (middle) with correlation to very low unspecific staining in the negative fraction with 0.68% (right). *D)* Magnetic enrichment of A*0201+ CMV pp65-reactive T cells stained with MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE. HLA-A*0201+ CMV pp65-reactive human PBMC were enriched by primary staining with MHC MACSimer A*0201 CMV pp65, human, PE combined with secondary staining of anti-PE MicroBeads UltraPure (#130-105-639) and subsequent separation on the QuadroMACS Separator™ with LS columns. The input sample contained 0.72% CMV pp65-reactive cells under CD8+ T cell (left). After magnetic enrichment, the frequency of CMV pp65-reactive cells under CD8+ T cells was increased to 96.14% in the positive fraction (middle) with correlation to very low unspecific staining in the negative fraction with 0.00% (right).

5 MHC MACSimers show high lot-to-lot consistency

Figure 5

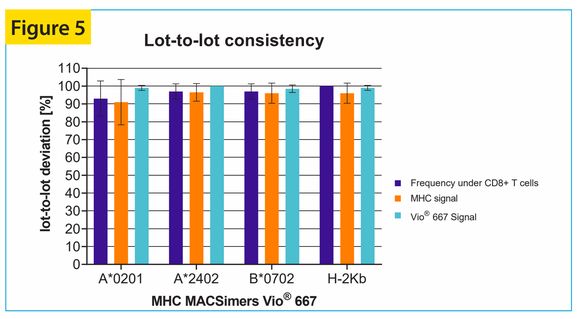


*Figure 5: Allele-specific lot-to-lot comparison of MHC MACSimer A*0201, A*2402, B*0702 and H-2Kb Vio® 667 shown for 3 parameters:*
(I) frequency of detected Ag-specific T cells (cell staining analysis)
(II) verification of MHC amount and integrity on MACSimer constructs (quality control assay)
(III) validation of Mean Fluorescence Intensity (MFI) of the Vio® 667 for MHC MACSimer lots (quality control assay).
Values were normalized to one lot (first lot) and set in relation. Plotted are the mean of the relative values for each parameter and the error bars indicate the parameter-specific standard deviation (SD). The comparison of allele-specific lots demonstrates a high consistency and precision regarding the staining performance. This makes MHC MACSimer staining reagents highly suitable for reliable IPC and QC monitoring.

6 Characterisation of Ag-specific T cell phenotypes in combination with MHC MACSimer duplex staining

Figure 6

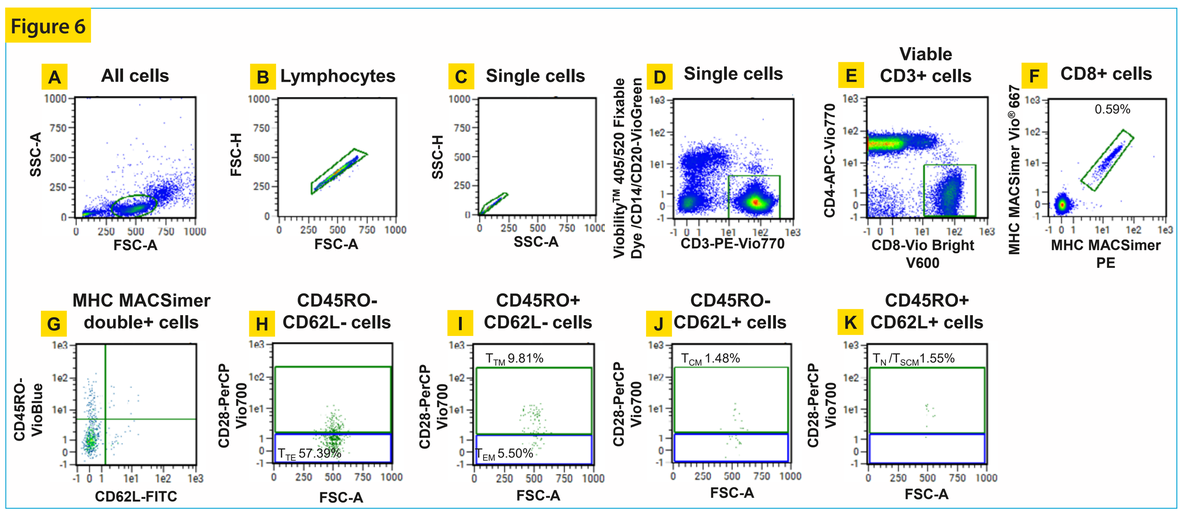


Figure 6: Characterization of T cell maturation phenotypes of Ag-specific T cells. HLA-A*0201+ CMV pp65-reactive T cells within PBMC were stained with MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE and further characterized with a counter staining. Flow cytometry analysis was performed using the MACSQuant® Analyzer. Within PBMC, lymphocytes were selected by an SSC low gate (A). Lymphocytes were plotted in FSC-H against FSC-A (B) and SSC-A against SSC-H (C) for the exclusion of doublets. Next, dead cells, monocytes, B cells, and dendritic cells were excluded by gating on CD3 PE-Vio770+ and Viability™ 405/520 Fixable Dye-, CD14 VioGreen-, and CD20 VioGreen- cells (D). Within the CD3+ T cells, CD8+ cells were identified by expression of CD8 VioBright V600 and exclusion of CD4 APC-Vio770 (E). Within the CD8+ T cells, Ag-specific T cells were identified by double staining with MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE and Vio® 667 with a frequency of 0.59% (F). MHC MACSimer double+ cells were further analysed with CD45RO-VioBlue against CD62L-FITC and divided by a quadrant gate (G). Every quarter of the quadrant gate was plotted against CD28 PerCP-Vio700. The following T cell maturation phenotypes could be identified via CD28+ and CD28- gated cells: CD62L-, CD45RO-, CD28- expressing terminal effector T cells (T_{TE}) with a frequency of 57.39% under MHC MACSimer double+ cells (H); CD62L-, CD45RO+ CD28- expressing effector memory T cells (T_{EM}) with a frequency of 5.50% under MHC MACSimer double+ cells; CD62L-, CD45RO+, CD28+ expressing terminal memory T cells (T_{TM}) with a frequency of 9.81% under MHC MACSimer double + cells (I); CD62L+, CD45RO-, CD28+ expressing central memory T cell (T_{CM}) with a frequency of 1.48% under MHC MACSimer double+ cells (J); CD62L+, CD45RO+, CD28+ expressing naïve T cells (T_N) and/or stem cell-like memory T cells (T_{SCM}) with a frequency of 1.55% under MHC MACSimer double + cells (K).

Conclusion

We show that both, our *MHC MACSimer FleX Kits* as well as our *peptide-loaded MHC MACSimers* feature superior performance when staining and analyzing shared cancer antigens, or virus-specific T cells for both, CD4+ and CD8+ T cells of different origins (PBMC, whole blood samples, or dissociated organs) and comparing them to other state-of-the-art MHC multimer reagents. Furthermore, we demonstrate that MHC MACSimer reagents can be perfectly combined with antibody panels allowing for analysis of e.g., T cell exhaustion or differentiation to get the most information out of the rarest T cells.

Combining them with fluorochrome-specific magnetic beads using MACS technology leads to extremely pure (>90%) and high-yield-enriched T cell populations, displaying the intended T cell phenotype. *MACSpep Single Peptides* come with a tailored MHC loading protocol and alternatively can be used for epitope-specific activation and expansion of T cells *ex vivo*. In conclusion, our three reagent classes enable Ag-specific T cell analysis, enumeration, and isolation and cover the need for flexibility, stability, and standardization.

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