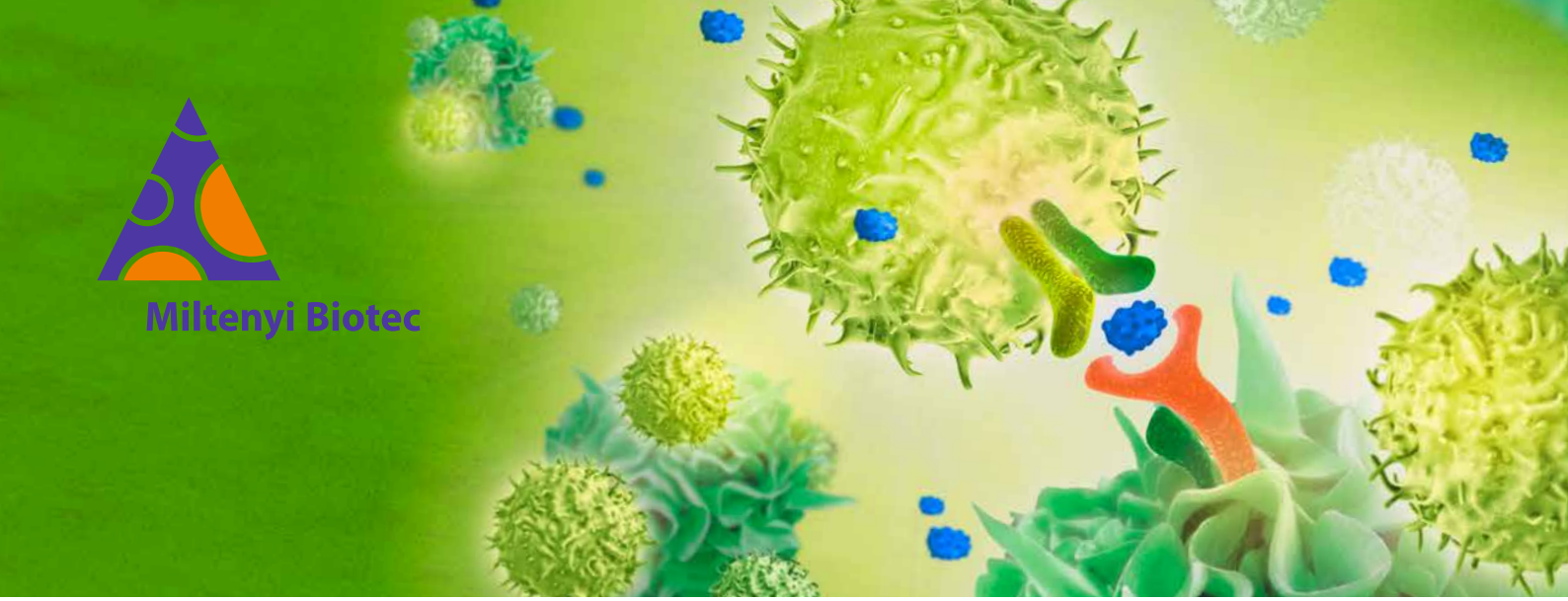


Sensitive detection and isolation of HCMV-specific T cells in the context of stem cell transplantation using MHC multimers

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Background

Human cytomegalovirus (HCMV) reactivation remains the leading cause of morbidity following allogeneic hematopoietic stem cell transplantation (alloSCT), posing serious risks to patient recovery and survival. Understanding the T cell–mediated immune responses to HCMV is therefore essential for advancing post-transplant care. Yet, isolating and analyzing HCMV-specific T cells has long been a technical and clinical bottleneck, due to their rarity, patient-to-patient variability, and fragility to experimental conditions. In this study, we break through those barriers by leveraging MHC MACSimers, cutting-edge tools that enable ultra-sensitive detection and gentle isolation of rare, virus-specific T cells from HLA-typed alloSCT patients. Remarkably, MHC MACSimers enable simultaneous detection of CD4$^+$ and CD8$^+$ antigen-specific T cells from a single patient sample, maximizing data yield and unveiling deeper, unprecedented insights from every drop.

Methods

Epitope selection and staining

To identify immunodominant HCMV epitopes, HLA-matched peptides were selected based on immune epitope databases and HLA affinity prediction tools. Candidate peptides matching the patient's HLA allotypes were loaded onto MHC MACSimer FleX Kits (class I and II) by following the MHC MACSimer loading protocol.

Peripheral blood mononuclear cells (PBMCs) from alloSCT patients were stained with peptide-loaded MHC MACSimers according to Miltenyi Biotec's MHC multimer staining protocol.

Simultaneous CD8$^+$ and CD4$^+$ T cell detection

For simultaneous detection of CD8$^+$ and CD4$^+$ HCMV–pp65-specific T cells, double staining of PBMCs with MHC class I and II MACSimers was performed (table 1).

PE-conjugated MHC MACSimer FleX Kit	MHC class	Epitope
HLA-A*0201	I	NLVPMVATV
HLA-DRB1*0401	II	AQTFAATH-NPWASQA

Table 1: MHC MACSimers used for the simultaneous detection of CD8$^+$ and CD4$^+$ responses in PBMCs from alloSCT patients.

Samples were co-stained with anti-CD3, anti-CD4, anti-CD8, and anti-CD14/CD20 antibodies for flow cytometry analysis.

T cell isolation

Two enrichment approaches were tested for the isolation of antigen-specific T cells:

- Magnetic separation: positive selection of CMV-reactive CD4$^+$ T cells was performed using MACS® Technology, by combining PE-conjugated MHC multimers with anti-PE MicroBeads, following the protocol for isolating CMV-specific antigen-reactive T cells.
- Flow sorting: the MACSQuant® Tyto® Cell Sorter was used for high-purity and gentle sorting based on PE fluorescence, following the protocol for sorting CMV-reactive antigen-specific T cells with PE-conjugated MHC MACSimers.

For both methods, frequencies of antigen-specific T cells before and after sorting were assessed by flow cytometry.

TCR internalization

To assess the impact of peptide stimulation on MHC MACSimer staining, T cells were stimulated *in vitro* with peptide antigens. TCR expression was monitored over time to assess surface reexpression after stimulation.

Gating strategy
Using live/dead staining and forward/side scatter gating, living single lymphocytes were selected first. Monocytes and B cells were excluded using anti-CD14/CD20 antibodies. T cells were selected using anti-CD3 antibody. To discriminate T cell subsets, anti-CD8 and anti-CD4 antibodies were used. MACSimer-positive cells were identified by PE fluorescence (table 2).

Antibody	Clone	Gating	Fluorochrome
Anti-CD14	REA599	Macrophages and monocytes	PerCP-Vio® 700
Anti-CD20	LT20	B cells and dendritic cells	PerCP-Vio 700
Anti-CD3	REA613	T cells	VioGreen™
Anti-CD4	REA623	Helper T cells	Vio Bright V600
Anti-CD8	REA734	Cytotoxic T cells	Vio Bright R720
MHC MACSimer	–	TCR staining	PE

Table 2: Antibody panel and gating description used for antigen-specific T cell analysis in combination with MHC MACSimers.

Results

MHC MACSimers enable sensitive detection of CD8⁺ and CD4⁺ T cell responses in alloSCT patient samples
To identify immunodominant HCMV epitopes, defined as the viral peptide sequences most strongly recognized by a patient's T cells, HLA-matched candidate epitopes were first selected using immune epitope databases and HLA affinity prediction tools. These online resources are valuable for narrowing down potential epitopes based on known immunogenicity and predicted MHC binding affinity.

MHC MACSimer staining was then used to validate these predictions. When loaded with the preselected candidate epitopes, MHC MACSimers enable direct confirmation of whether a patient's T cells express functional TCRs that specifically recognize and bind the corresponding peptide-MHC complex. This approach not only determines which predicted epitopes are truly immunodominant in each patient, but also allows highly sensitive detection of even rare virus-specific CD4⁺ and CD8⁺ T cell responses.

To detect HCMV-specific CD4⁺ and CD8⁺ T cell responses in alloSCT patients, PBMCs were stained with allotype-matched MHC class I and class II MACSimers loaded with the different preselected epitope candidates (table 3).

PE-conjugated MHC MACSimer FleX Kit	MHC class	Candidate epitopes
HLA-B*0702	I	1) RPHERNGFTVL 2) RPHERNGFTV
HLA-DRB1*0401	II	1) AQTFAATHNPWASQA 2) AGILARNLVPMVATV

Table 3: Epitope screening to detect HCMV-specific CD8⁺ and CD4⁺ T cell responses using MHC class I and class II MACSimers.

MHC MACSimer staining enabled robust detection of CD8⁺ and CD4⁺ T cell populations in alloSCT patient samples (fig. 1), demonstrating sensitivity and specificity in identifying immunodominant TCRs within polyclonal populations.

In the MHC class I panel (B*0702) (fig. 1A), MHC MACSimer staining with a dominant epitope (epitope 1) enabled the detection of HCMV-specific CD8⁺ T cells (4.37%), while there was no TCR staining detected against epitope 2.
Similarly, in the MHC class II panel (DRB1*0401) (fig. 1B), a strong HCMV-specific CD4⁺ T cell response (5.79%) was identified in an alloSCT patient samples against epitope 1, while epitope 2 was non-reactive.

These results demonstrate that MHC MACSimers can be seamlessly integrated into flow cytometry panels, providing a sensitive and reliable method for rapid screening of candidate epitopes and validation of individual TCR specificities in patient samples.

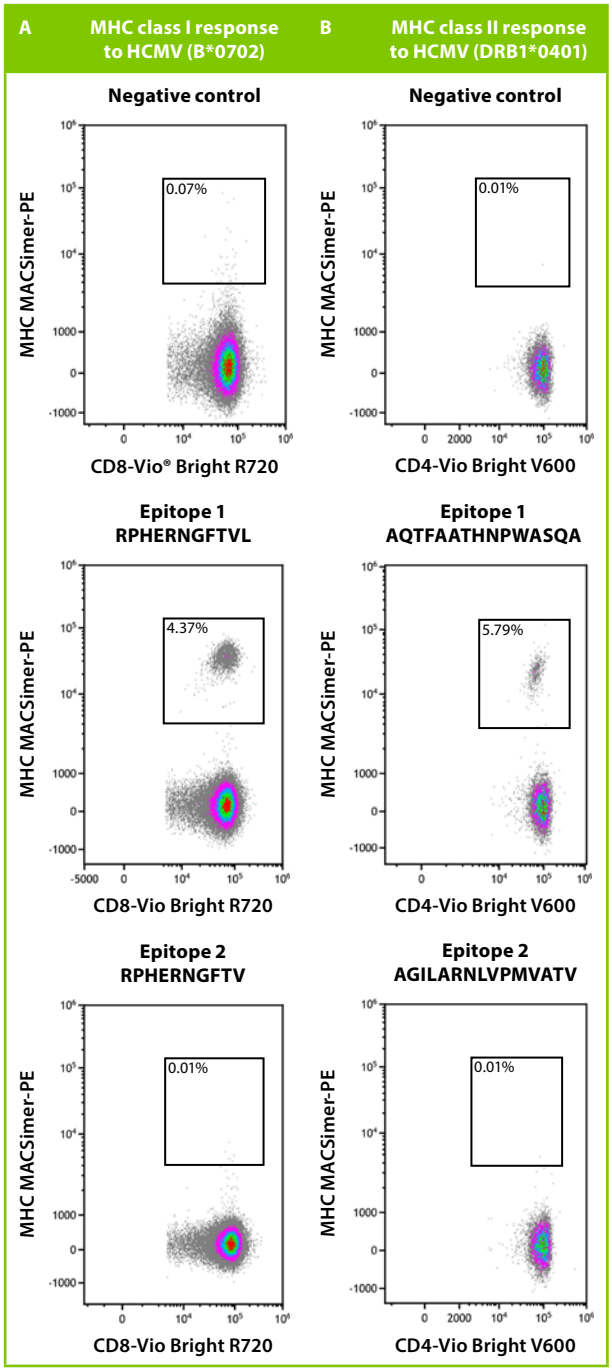


Figure 1: HCMV-specific CD8 and CD4 T cell responses in patient samples. PE-conjugated MHC MACSimer FleX Kit (A) B*0702 and (B) DRB1*0401 were loaded with different epitope candidates of interest and used to stain allotype-matched PBMCs. Antigen-specific T cell frequencies were assessed via flow cytometry.

Given the limited availability of patient material, obtaining sufficient cells to perform both MHC class I and class II stainings can be challenging. To overcome this, a double-staining approach was applied, simultaneously using MHC MACSimer A*0201 (NLVPMVATV) and DRB1*0401 (AQTFAATHNPWASQA) on a single sample.

Both class I and class II MHC multimer single staining effectively detected HCMV-specific CD8⁺ T cells (fig. 2A, 5.87%) and CD4⁺ T cells (fig. 2B, 6.85%), respectively.

Importantly, the dual staining yielded comparable frequencies of antigen-specific T cells (fig. 2C), identifying 6.27% of CD8⁺ T cells and 7.28% of CD4⁺ T cells as HCMV-specific.

This combined staining strategy enables the simultaneous detection and discrimination of CD8⁺ and CD4⁺ T cell responses within a single sample, thereby maximizing data acquisition from limited patient material without compromising sensitivity.

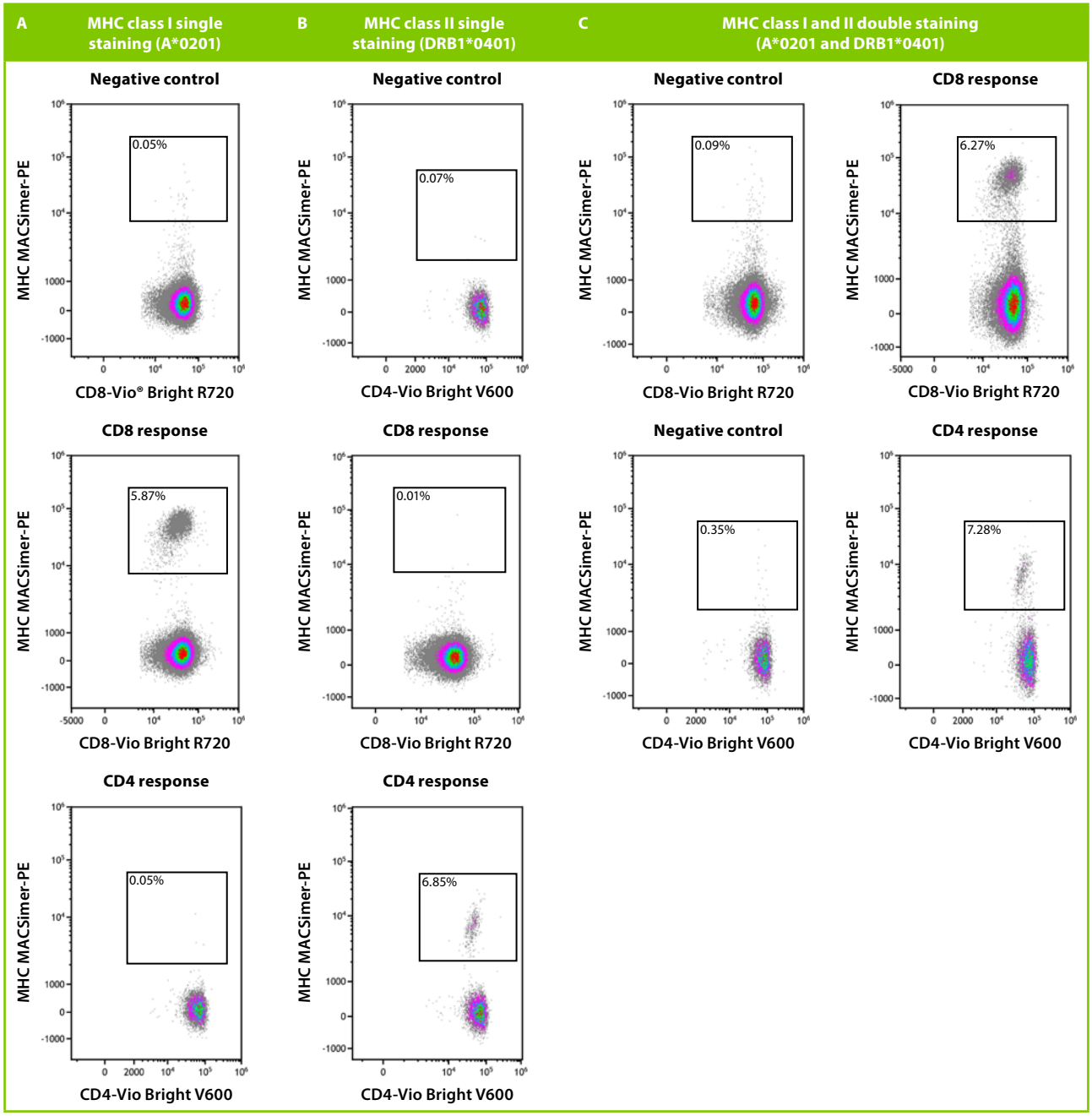


Figure 2: Simultaneous detection of antigen-specific CD8 and CD4 T cell responses in one single sample. MHC class I (HLA-A*0201 loaded with NLVPMVATV) and class II (HLA-DRB1*0401 loaded with AQTFAATHNPWASQA) MACSimers were used to stain PBMCs from allotype-matched patients. As controls, the CD8 and CD4 responses were individually analyzed by single stainings using MHC class I (A) and class II (B) MACSimers, respectively. A double-staining approach enables simultaneous detection of CD8 and CD4 responses from a single sample, without compromising precision (C). Antigen-specific responses were assessed via flow cytometry.

In summary, MHC MACSimers enable sensitive and reliable detection of both CD8⁺ and CD4⁺ T cell responses in alloSCT patient samples. Their flexibility and compatibility with double-staining strategies make them an indispensable tool for obtaining comprehensive, high-value immune insights from limited patient material.

MHC MACSimers enable sensitive and gentle isolation of antigen-specific T cells for downstream assays

HCMV-specific T cells are rare, patient-specific, and highly sensitive, making their isolation and analysis technically demanding, especially when preparing samples for sensitive downstream assays like single-cell RNA sequencing (scRNA-seq), TCR sequencing, or functional profiling. These applications require high-quality input material, in which cells must not only be enriched but also remain viable, transcriptionally intact, and functionally competent.

To proceed with enrichment of HCMV-specific T cells from HLA-typed alloSCT patient samples, two approaches were employed: magnetic separation using MACS Technology and flow sorting with the MACSQuant Tyto Cell Sorter. MACS Technology offers a fast and gentle method for positive selection of antigen-specific T cells using PE-conjugated MACSimers and anti-PE MicroBeads, ideal for preserving cell viability in high-throughput workflows. Alternatively, the MACSQuant Tyto Cell Sorter enables high-purity microfluidic sorting in a closed cartridge system. Its gentle microchip technology minimizes cellular stress and preserves cell viability and functionality, making it particularly well-suited for sensitive molecular assays.

Both methods are suitable for isolating antigen-specific T cell populations stained with MHC MACSimers, with representative results showing that magnetic separation and flow sorting yielded highly enriched populations of HCMV-specific CD4⁺ and CD8⁺ T cells, respectively (fig. 3). CD4⁺ T cells were enriched from a frequency of 20.80% in the input fraction to 95.3% purity following magnetic separation (fig. 3A). Using the MACSQuant Tyto Cell Sorter, CD8⁺ T cells were enriched from 2.88% in the input fraction to 87.73% purity in the positive fraction (fig. 3B).

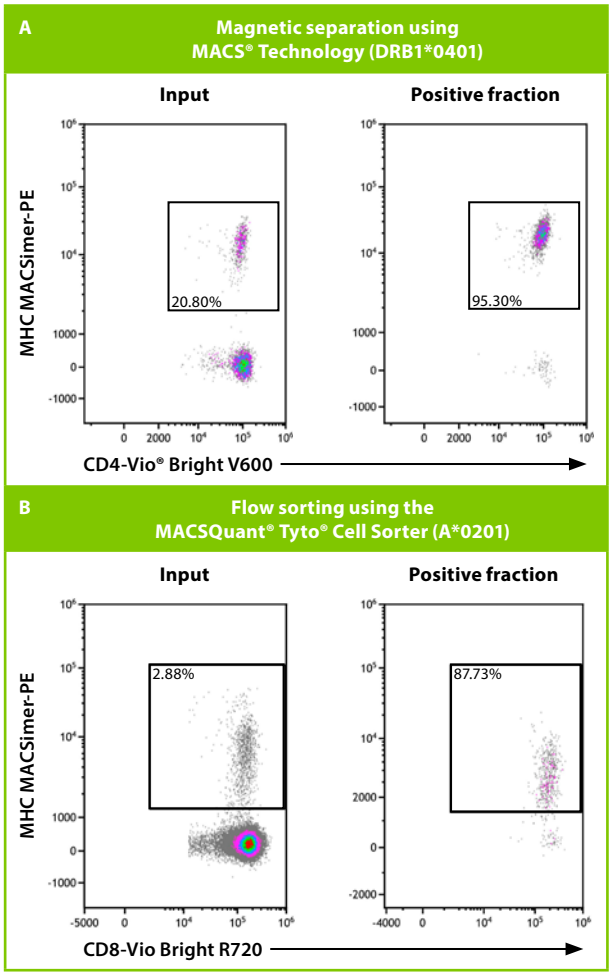


Figure 3: Sorting of HCMV-specific T cells from patient samples using MHC MACSimers. PE-conjugated MHC class I (A*0201 loaded with NLVPMVATV) and MHC class II (DRB1*0401 loaded with AQTFAATHNPWASQA) MACSimers were used to stain PBMCs from allotype-matched patients. Subsequently, MACSimer-positive cells were sorted. Representative results are shown for magnetic separation of CD4⁺ T cells using anti-PE MACS MicroBeads (A) and for flow sorting of CD8⁺ T cells using the MACSQuant Tyto Cell Sorter (B). Antigen-specific T cell frequencies before (input) and after (positive fraction) sorting were determined by flow cytometry.

These results show that MHC MACSimer staining, combined with gentle sorting technologies, offers a robust and flexible workflow for isolating even the rarest antigen-specific T cells with confidence and precision, yielding high-quality samples ready for advanced immune and molecular analyses.

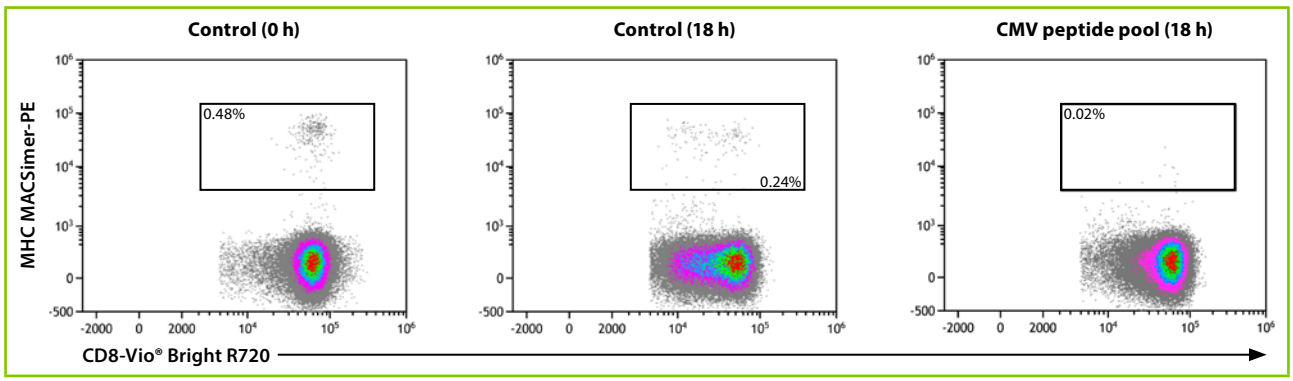


Figure 4: Peptide-based stimulation of HCMV-reactive T cells. Frequencies of HCMV-specific T cells at 0 hours of incubation (original cell fraction unstimulated, A), after 18 hours of incubation without peptide (B), and after 18 hours of incubation in the presence of CMV peptide pool (C) are shown.

Peptide-based stimulation of HCMV-specific T cells leads to TCR internalization

The relative frequency of TCR-expressing cells in a sample, as well as the expression levels of surface TCRs, are decisive factors for the technical success of MHC MACSimer staining. Since MACSimer reagents directly bind to the TCR, reduced surface availability of TCRs can significantly compromise the sensitivity and reliability of antigen-specific T cell detection.

One factor known to impact TCR surface expression is peptide-based stimulation of T cells, used to activate and expand antigen-specific T cells when working with low-frequency populations. TCR engagement during stimulation initiates TCR internalization as part of the canonical activation response, making T cells temporarily undetectable by peptide-MHC multimers. To assess the extent of this effect, we followed the MHC MACSimer staining after 18 hours of stimulation using CMV peptide pools. In comparison with the unstimulated controls (0 and 18 hours), antigen-specific T cells cannot be detected directly after peptide-based stimulation (fig. 4).

This experiment provides a critical technical insight: performing MHC MACSimer staining immediately after peptide stimulation will lead to false-negative results, even when antigen-specific T cells are present. For accurate multimer-based identification of antigen-specific T cells following peptide stimulation, we recommend including a resting phase of 5–7 days in cell culture. This allows sufficient time for reexpression of surface TCRs and robust detection with MHC MACSimer reagents (not shown).

Conclusion

MHC multimers offer a sensitive and reliable platform for detecting and isolating HCMV-specific CD4$^+$ and CD8$^+$ T cells in alloSCT patient samples, enabling researchers to extract critical data from limited patient material with confidence and precision:

Native TCR detection

MHC MACSimers move beyond conventional peptide-based assays by identifying antigen-specific T cells through their native TCRs, providing high sensitivity and epitope-level specificity.

Highly sensitive detection, analysis, and isolation

Engineered for optimal TCR avidity and stability, MHC MACSimers deliver exceptional brightness and superior staining performance.

Deeper insights from limited samples

Double-staining strategies allow simultaneous detection of CD4$^+$ and CD8$^+$ T cells from a single sample, making every precious cell count.

Seamless integration into T cell analysis workflows

With optimized protocols and a versatile, application-ready portfolio, MHC multimers fit effortlessly into existing T cell workflows.

By combining precision, scalability, and flexibility, MHC MACSimers provide an application-ready platform for advancing immune monitoring research, epitope discovery, and TCR-based immunotherapy development, making them a powerful tool for immunological studies in alloSCT and beyond.

Product	Order no.
MHC MACSimer B*0702 FleX Kit, human, PE	130-134-686
MHC MACSimer B*0702 Control, human, PE	130-136-346
MHC MACSimer A*0201 FleX Kit, human, PE	130-133-926
MHC MACSimer A*0201 Control, human, PE	130-134-668
MHC MACSimer DRB1*0401 FleX Kit, human, PE	N.A.*
CD14 Antibody, anti-human, PerCP-Vio 700, REAfinity®	130-110-523
CD20 Antibody, anti-human, PerCP-Vio 700, REAfinity	130-111-342
CD3 Antibody, anti-human, VioGreen, REAfinity	130-113-142
CD8 Antibody, anti-human, Vio Bright R720, REAfinity	130-127-379
CD4 Antibody, anti-human, Vio Bright V600, REAfinity	130-129-579
Anti-PE MicroBeads UltraPure	130-105-639
MACSQuant Tyto Cell Sorter	130-103-931
PepTivator® CMV pp65	130-093-435
MACSpep CMV pp65 (RPHERNGFTV)	130-134-299
MACSpep CMV pp65 (RPHERNGFTVL)	130-134-298

*This product is not yet commercially available and was provided to the authors as part of a beta-test agreement.

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Santamorena, M. M. *et al.* (2024) HLA. 103(6):e15541.

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