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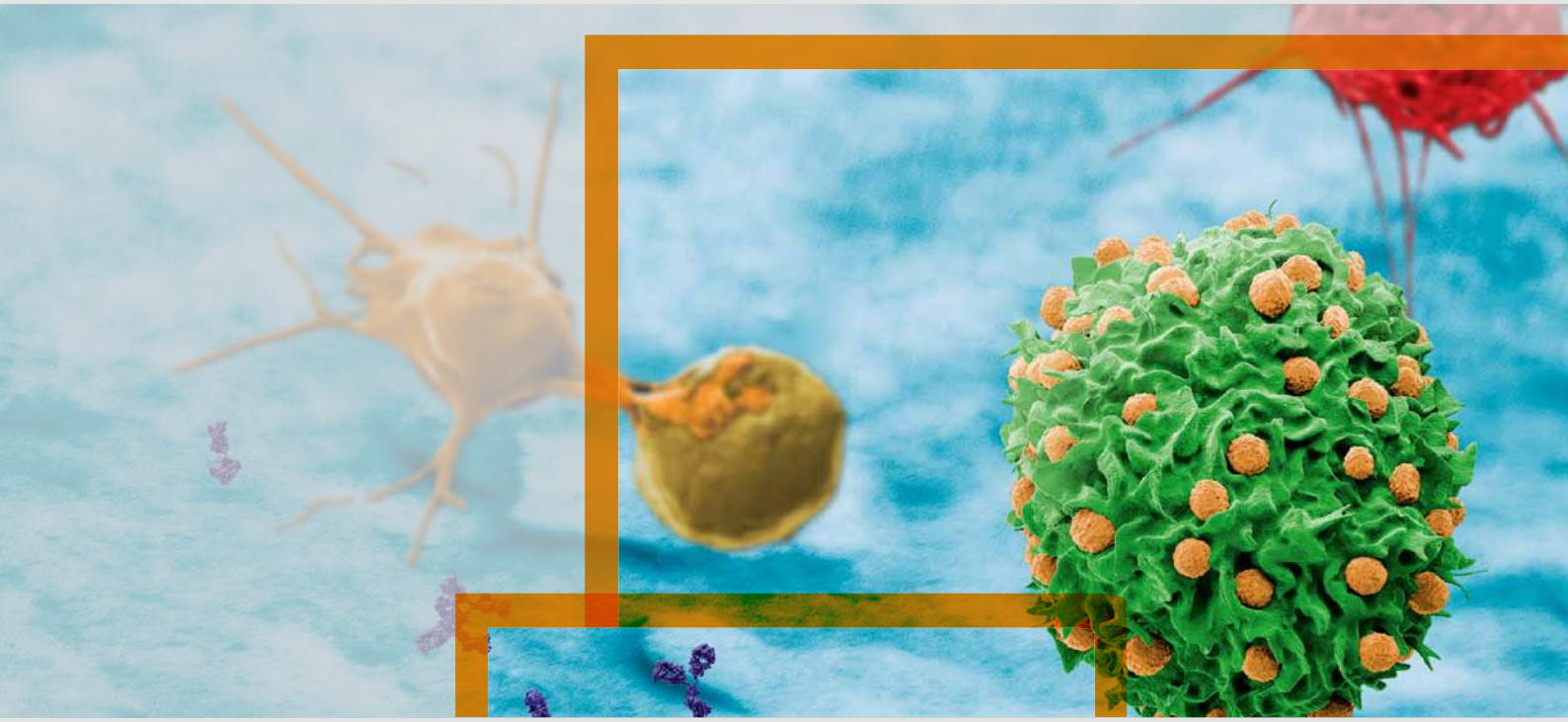

Efficient and rapid *in vitro* generation of fully functional multi-virus-specific CD4+ and CD8+ T cells

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Introduction

Antigen-specific T cells play a critical role in the regulation of immune responses and the elimination of virus-infected or malignant cells in the human body. T cells are essential, e.g., for the control of the outgrowth of Epstein-Barr virus (EBV)-infected B cells. CD4+ and CD8+ T cells specific for the EBV antigens BZLF1[1-4] and EBNA-1[5-11] have been found in EBV-infected individuals. Virus-specific T cells also hold great potential for clinical use. The adoptive transfer of clinical-grade CD4+ and CD8+ T cells specific for adenovirus (AdV) hexon, cytomegalovirus (CMV) pp65, and EBV antigens is a valuable tool for the treatment of AdV[12], CMV[13-16], and EBV[17,18] infections after hematopoietic stem cell transplantation. To further enhance research on virus-specific T cells, we have established a protocol for the efficient and rapid generation of EBV-, AdV-, and CMV-specific (tri-virus-specific) T cells. The virus-specific CD4+ and CD8+ T cells were stimulated *in vitro* using peptide pools, and subsequently magnetically enriched according to their IFN-γ secretion. The tri-virus-specific T cell population could be easily expanded without major alterations in the proportions of the respective specificities.

Materials and methods

Generation of multi-virus-specific T cells

For the generation of multi-virus-specific T cells we stimulated 10^9 PBMCs from leukapheresis products of several healthy donors with a combination of four PepTivator® Peptide Pools (Miltenyi Biotec) covering CMV pp65 or IE-1, AdV hexon, or EBV EBNA-1 or BZLF-1 for four hours. The simultaneous addition of four peptide pools to a single

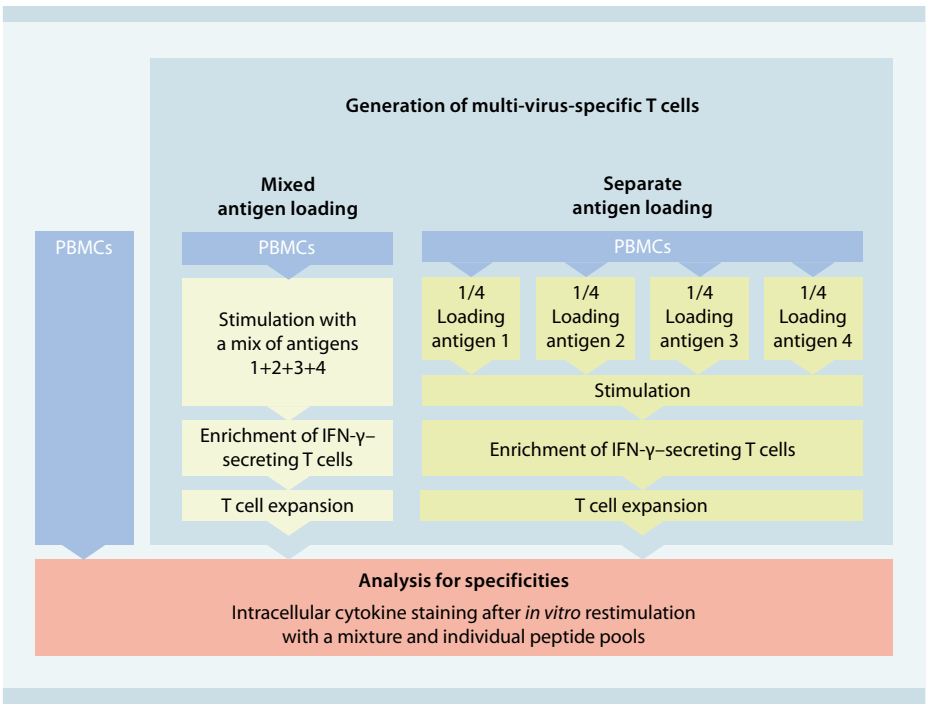

Figure 1 **Experimental procedure for the generation of multi-virus-specific T cells.** For details see the materials and methods section.

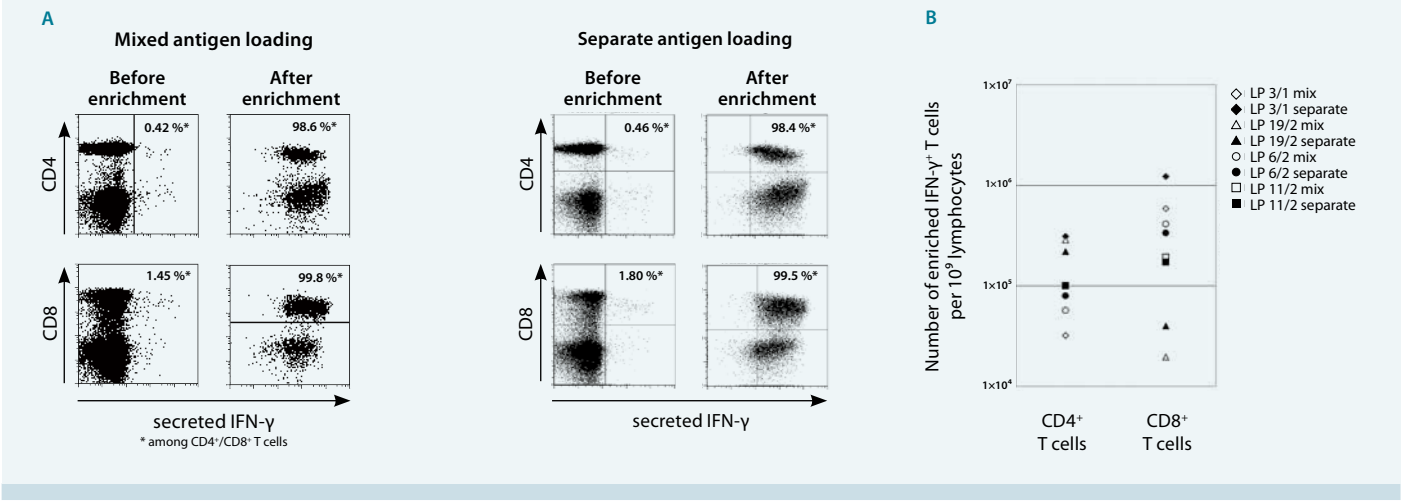

Figure 2 **Enrichment of multi-virus-specific T cells after stimulation with individual peptide pools or a combination thereof.** PBMCs from four different donors were stimulated in two different ways as described in the materials and methods section: i) Cells were stimulated with a combination of pp65, IE-1, hexon, and BZLF1 peptide pools in a single batch (mixed antigen loading), or ii) cells were loaded with the individual peptide pools in four aliquots, and aliquots were subsequently combined for further stimulation (separate antigen loading). Subsequently, virus-specific T cells were enriched according to IFN-γ secretion. Before and after enrichment IFN-γ–secreting CD4+ and CD8+ T cells were analyzed by flow cytometry. (A) Results from one representative donor are shown. Numbers indicate frequencies among CD4+ or CD8+ cells. (B) Numbers of IFN-γ–positive CD4+ and CD8+ T cells enriched from PBMCs of four different donors.

sample might decrease the activation efficacy for each peptide pool due to competition of peptides for MHC binding. As a control, we divided the PBMC samples into four aliquots, incubated each aliquot with a single peptide pool for two hours, and recombined the four aliquots for T cell stimulation for another four hours. Afterwards, we used the Large Scale IFN-γ Secretion Assay – Enrichment Kit, human, from Miltenyi Biotec to magnetically select antigen-activated IFN-γ–secreting CD4+ and CD8+ T cells. Purities of enriched cells were determined by flow cytometry using the MACSQuant® Analyzer (Miltenyi Biotec). Enriched multi-virus-specific CD4+ and CD8+ T cells were expanded in the presence of IL-2 and feeder cells for 9–13 days. A flowchart illustrating the experimental procedure is shown in figure 1.

Analysis of PBMCs and multi-virus-specific T cells for antigen specificity

For the analysis of antigen specificity and functionality of the enriched, expanded multi-virus-specific T cells, we examined the IFN-γ response after short-term *in vitro* restimulation with individual peptide pools or a mixture thereof. For comparison, we also restimulated PBMCs. The IFN-γ response was determined by intracellular cytokine staining using an

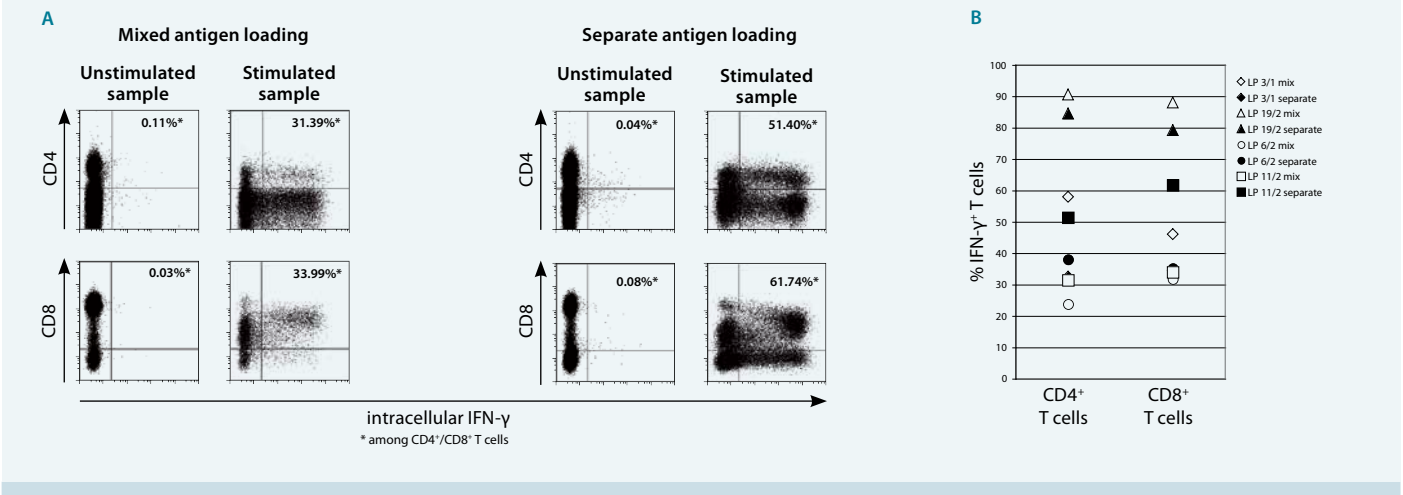

Figure 3 **Restimulation of enriched and expanded multi-virus-specific T cells.** Enriched IFN-γ–secreting cells were expanded for 9 to 13 days. Subsequently, cells were restimulated with a mixture of peptide pools or were left unstimulated. Cells were analyzed for IFN-γ production by intracellular staining and flow cytometry. (A) Results from one representative donor are shown. Numbers indicate frequencies among CD4+ and CD8+ T cells, respectively. (B) Frequencies of IFN-γ–positive CD4+ and CD8+ T cells. Results from four different donors are shown.

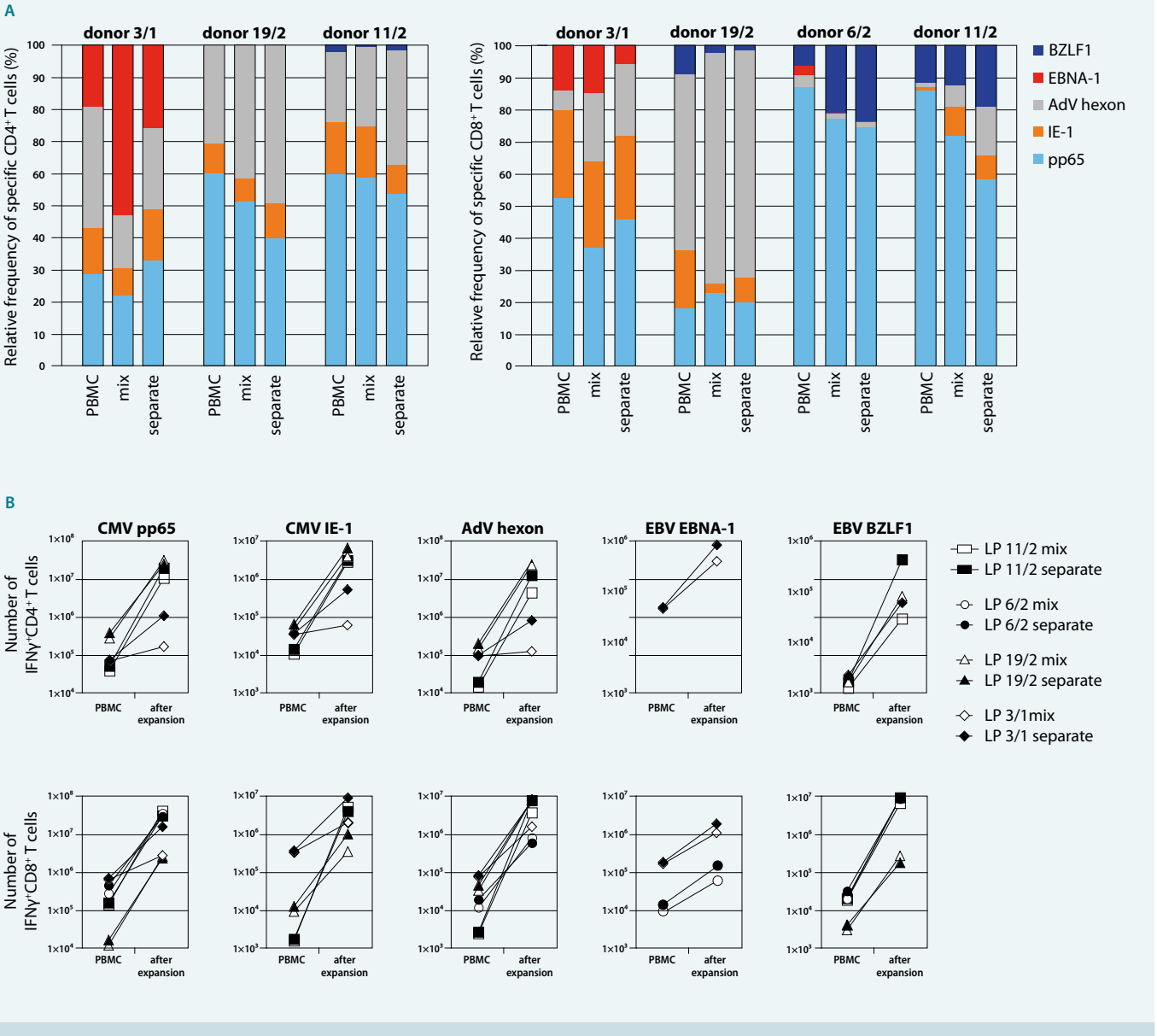

Figure 4 **Analysis of individual virus-specific T cell populations.** Multi-virus-specific T cells were enriched as indicated in figure 2 and expanded. PBMCs and multi-virus-specific T cell lines were restimulated with individual peptide pools or a mixture thereof. The relative frequencies and absolute cell numbers of T cells with a single antigen specificity were calculated based on total cell numbers and the frequencies of IFN-γ–positive T cells among PBMCs and multi-virus-specific T cells upon restimulation with individual peptide pools. Data from different donors are shown as indicated.

Anti-IFN-γ-PE antibody (Miltenyi Biotec). $CD4^+$ and $CD8^+$ cells were then analyzed by flow cytometry using the MACSQuant Analyzer. Based on total cell numbers and the frequencies of IFN-γ–positive T cells among PBMCs and multi-virus-specific T cells upon restimulation with individual peptide pools, we calculated the relative frequencies and absolute cell numbers of T cells with a single antigen specificity.

Results and discussion

Magnetic enrichment of multi-virus-specific $CD4^+$ and $CD8^+$ T cells

Using the Large Scale IFN-γ Secretion Assay – Enrichment Kit, we were able to consistently enrich virus-specific $CD4^+$ and $CD8^+$ T cells to purities higher than 90%. Flow cytometric analyses of IFN-γ–secreting $CD4^+$ and $CD8^+$ T cells in PBMCs before and after magnetic enrichment are shown in figure 2A for one representative donor. Prior to enrichment, cells were stimulated in two different ways: Cells were either stimulated with a combination of pp65, IE-1, hexon, and BZLF1 peptide pools in a single batch, or cells were loaded with the individual peptide pools in four aliquots, and aliquots were subsequently combined for further stimulation. Both stimulation procedures resulted in comparable frequencies of IFN-γ–secreting T cells before enrichment

(fig. 2A). Likewise, purities of enriched cells (fig. 2A and data not shown) and numbers of enriched cells (fig. 2B) were similar in the respective samples from all tested donors, regardless of whether the samples underwent mixed or separate antigen loading.

Specificity and functionality of the enriched and expanded multi-virus-specific T cells

Within 9 to 13 days cell populations that were generated by either mixed or separate antigen loading expanded between 25- and 496-fold (data not shown). Both cell lines were restimulated with a mixture of peptide pools and analyzed for intracellular IFN-γ production. The percentage of IFN-γ–re-expressing $CD4^+$ T cells derived from mixed antigen loading and separate antigen loading amounted to 23.8–90.5% and 32.5–84.8%, respectively. Similarly, the percentage of $CD8^+$ T cells amounted to 31.8–88.0% and 34.0–79.2%, respectively (fig. 3A,B). These results confirm the high specificity and functionality of the T cell lines. T cell expansion rates and frequencies of T cells re-expressing IFN-γ were similar, regardless of whether the cell lines were originally generated by mixed or separate antigen loading (fig. 3B).

Individual virus-specific T cell populations show similar enrichment and expansion rates

The strategy for the enrichment of virus-specific T cells from PBMCs is based on IFN-γ expression induced by stimulation with the peptide pools. Therefore, differences between stimulation efficacies of individual peptide pools would result in differences in IFN-γ responses of the respective virus-specific T cells, and ultimately in a biased proportion of specificities within the enriched multi-virus-specific T cell population. To ensure that the proportions of the different specificities are similar before and after enrichment, we compared the relative frequencies (fig. 4A) and absolute numbers (fig. 4B) of T cells specific for each single antigen in PBMCs and in the T cell lines. The relative frequencies of T cells specific for the individual CMV, EBV, and AdV antigens were about the same in PBMCs and in both T cell lines. This demonstrates that all individual virus-specific T cell populations within PBMCs were effectively stimulated and enriched. Moreover, regardless of whether the original PBMCs were loaded with a mixture of peptide pools or separately with single peptide pools, the obtained T cell lines were comparable with respect to the expansion rate of the individual specificities.

Conclusion

- A combination of several PepTivator Peptide Pools enables the simultaneous, effective activation of CMV-, EBV-, and AdV-specific (tri-virus-specific) $CD4^+$ and $CD8^+$ T cells.
- Activated tri-virus-specific T cells can be co-enriched using the Large Scale IFN-γ Secretion Assay – Enrichment Kit.
- Enriched tri-virus-specific T cells can be expanded without significantly altering the proportion of the individual specificities.
- Enriched tri-virus-specific T cells are fully functional and re-express IFN-γ upon restimulation.

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