RESEARCH ARTICLE

Temperature-based MHC class I multimer peptide exchange for human HLA-A, -B, and -C

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eLife Assessment

This is a very helpful study that presents an easy-to-use and reliable alternative to conventional and UV-based tetramers for the identification of antigen-specific CD8 T cells. The authors demonstrate that tetramers for a number of HLA alleles can be subjected to specific temperatures that facilitate peptide exchange while maintaining structural integrity, thereby generating detection reagents. They provide **convincing** evidence for this method, and the approach is likely to provide a **useful** alternative to generating reagents for examining T cell specificities.

Abstract T cell recognition of specific antigens presented by major histocompatibility complex class I (MHC-I) can play an important role during immune responses against pathogens and cancer cells. Detection of T cell immunity is based on assessing the presence of antigen-specific cytotoxic CD8+ T cells using MHC-I multimer technology. Previously, we have designed conditional peptides for HLA-A*02:01, H-2K^{b}, and HLA-E that form stable peptide-MHC-I-complexes at low temperatures and dissociate when exposed to a defined elevated temperature. The resulting conditional MHC-I complex can easily and without additional handling be exchanged with a peptide of interest, allowing to exchange peptides in a ready-to-use multimer and a high-throughput manner. Here, we present data that this peptide-exchange technology is a generally applicable, ready-to-use, and fast approach to load many different peptides in MHC-I multimers for alleles of the HLA-A, HLA-B, and HLA-C loci. We describe the development of conditional peptides for HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02 that only form stable peptide-MHC-I complexes at low temperatures, allowing peptide exchange at higher defined temperatures. We document the ease and flexibility of this technology by monitoring CD8+T cell responses to virus-specific peptide-MHC complexes in patients.

Introduction

An essential component of cellular immunity is mediated through the T cell receptor (TCR) of CD8+ T cell that can bind major histocompatibility complex class I (MHC-I) complexes. The MHC-I complex consists of three components: a polymorphic heavy chain, the β2-microglobulin (β2M) protein, and an 8–10-amino-acid peptide ligand (***Bjorkman et al., 1987***). Peptide selection occurs through docking of the peptide C-terminus in MHC-I and occupation of the pockets in MHC-I followed by a conformational change that preferably traps the peptides with the highest MHC-binding affinity. As a result, peptides with low MHC-binding affinity are replaced by peptides with a high MHC-binding affinity, a phenomenon used in this work (***Garstka et al., 2015***). The peptide ligand is an essential part of the MHC-I complex; without it, the complex cannot remain stable and dissociate (***Ljunggren et al., 1990***). Pathogens, such as viruses, can infect human cells, and a primary infection often leads to disease and immunity. Recurrent infections typically do not cause severe disease and are cleared, in part, by T cells recognizing viral peptides presented by MHC-I, as recently demonstrated for SARS-CoV-2 (***Roukens et al., 2022***; ***Nesamari et al., 2024***). The emergence of viral variants can potentially give rise to a partial change in immunogenic viral peptides, thereby affecting disease progression. Moreover, immune-compromised patients who lack proper B cell immunity are fully dependent on T cell-mediated immunity. Therefore, monitoring antigen-specific T cells is crucial for tracking the impact of recurrent infections and determining whether therapeutic intervention is required.

The detection and visualization of antigen-specific T cells were first achieved by using peptide-loaded MHC-I multimers with a fluorochrome (***Altman et al., 1996***). To generate such labeled multimers, soluble MHC-I monomers complexed with a peptide are multimerized through biotinylating and fluorochrome-conjugated streptavidin. This process is time-consuming and involves multiple steps for each individual peptide-MHC-I complex. Several technologies have since been developed to allow the parallel production of multiple MHC-I multimers, facilitating high-throughput analysis. For instance, dipeptides have been used as catalysts for peptide exchange (***Saini et al., 2015***; ***Saini et al., 2019***; ***Sun et al., 2023***; ***Anjanappa et al., 2020***), UV-sensitive conditional MHC ligands have been a widely adopted approach (***Rodenko et al., 2006***; ***Toebes et al., 2006***), in which a photocleavable peptide is cleaved upon UV exposure. Additional peptide-exchange technologies include chaperone-mediated peptide exchange (***Overall et al., 2020***; ***Ilca et al., 2018***), enzymatic cleavage of low-affinity peptides that are covalently linked (***Jurewicz et al., 2019***), and small alcohol peptide exchange (***Hadeler et al., 2022***). This results in an unloaded MHC-I monomer, which can subsequently be loaded with a high-affinity peptide and multimerized (***Rodenko et al., 2006***; ***Toebes et al., 2006***). However, UV exposure can cause photodamage to the peptide MHC-I complex, which leads to sample evaporation and is incompatible with fluorescently labeled multimers, requiring multimerization to be performed after the peptide exchange (***Pattison et al., 2012***). Additionally, more recent methods involve empty MHC-I stabilized by the introduction of a disulfide bond, which can be loaded with the desired peptide of choice. It is currently unknown how these mutations and structural alterations affect the peptide binding repertoire of these disulfide-stabilized MHC-I molecules (***Saini et al., 2019***; ***Sun et al., 2023***).

One major limitation of current MHC-I multimer-based technologies is the complex, multistep protocols to obtain ready-to-use peptide MHC-I complexes, without inducing mutations in the HLA class I heavy chain or β2M. This limits the broad clinical usage of MHC-I multimer technology. To address this, we previously developed a temperature-based peptide-exchangeable MHC-I multimer technology for human HLA-A*02:01, HLA-E, and murine H2Kb, which simplifies and accelerates peptide exchange on MHC-I multimers (***Luimstra et al., 2018***; ***Ruibal et al., 2023***; ***Ruibal et al., 2022***). This technology allows the generation of multimers within hours with ease, simply by incubating selected peptides at a defined temperature with a single standardized batch of fluorescently labeled MHC-I multimers (***Luimstra et al., 2019***). Depending on the platform, peptide exchange may be performed either before or after multimerization and labeling. In this study, we aimed to determine whether this technology could be developed into a robust and broadly applicable system for analyzing CD8+ T cell immunity. To this end, we developed temperature-based peptide-exchange MHC-I multimers for human MHC gene product HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02, representing all major MHC-I gene families.

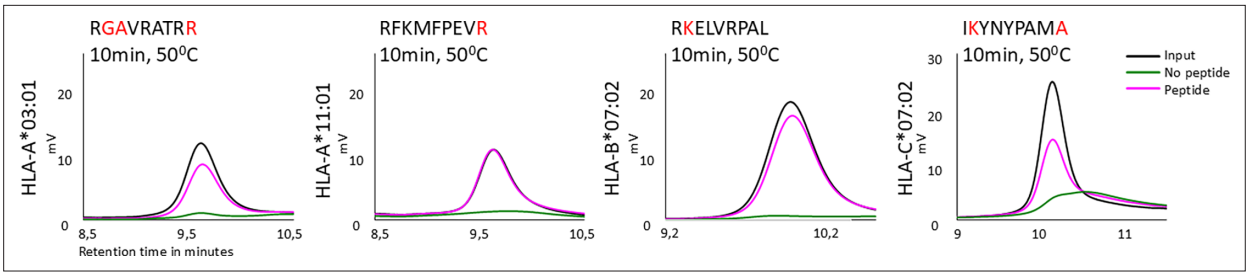

Figure 1. Thermal stability of MHC-I monomers with template peptide. Thermal stability of HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, HLA-C*07:01, and HLA-C*07:02 complexed with their template peptides was assessed at 50°C using analytical size-exclusion HPLC. Peptide-MHC-I monomers were incubated with indicated peptides at 50°C for 10 minutes. Peptides used are listed in ***Table 1***. One of at least three representative experiments is shown. The black line represents primary data without thermal exchange, the green line shows thermal exchange without peptide (resulting in complex instability), and the pink line represents thermal exchange in the presence of peptide (yielding a stable complex).

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Thermal stability of the template peptide-MHC-I complex at 50°C assessed by analytical size-exclusion HPLC.

Results

Design of temperature-exchangeable template peptide

We set out to extend our toolbox of HLA alleles that allow temperature-based template peptide exchange using multimerized, ready-to-use MHC-I complexes. Our goal was to design peptides that form stable complexes with MHC-I at low temperature and are unstable at higher temperatures, allowing replacement of the template peptide by a desired high-affinity peptide. The peptide off-rate is the primary determinant for the MHC-I stability (***De Silva et al., 1999***). For HLA-A*03:01, we designed peptides based on a hepatitis C virus epitope (HCV43-51; RLGVRATRK) (dissociation constant [Kd] 11.7 nM) (***Reynisson et al., 2020***) and modified the residues to decrease the dissociation constant (***Scognamiglio et al., 1999***). HLA-A*03:01 complexes were produced with wild-type or modified peptide, and thermal stability was assessed at 50°C using analytical size-exclusion-high-performance liquid chromatography (HPLC). With the modified peptide, RGAVRATRR (Kd 7098.44 nM), the complex was unstable at 50°C, resulting in denaturation, as shown by the absence of a MHC-I peak in a size-exclusion HPLC (***Figure 1***, ***Figure 1—figure supplement 1***). In contrast, the presence of the high-affinity epitope (RLGVRATRK, 11.7 nM) produced a distinct peak, demonstrating that the HLA-A*03:01 complex could be rescued from unfolding. A similar approach was applied to HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02. For each allele, we designed peptides for each respective allele and modified the residues to decrease the affinity (***Table 1***; ***Reynisson et al., 2020***; ***Kubo et al., 1994***; ***Axelsson-Robertson et al., 2013***). HLA complexes with the modified peptides were produced, and their thermal stability was evaluated at 50°C using analytical size-exclusion HPLC. For each allele, we identified a thermal template peptide that was unstable at 50°C, which could be rescued by the addition of a high-affinity peptide (***Figure 1***, ***Figure 1—figure supplement 1***). We proceeded with peptides that exhibited thermo-instability at 50°C that showed a rescued HLA complex in the presence of the high-affinity peptide. The peptides that formed stable complexes at 4°C but unstable complexes at 20–32 °C and 50 °C displayed affinities ranging from 4 to 11 µM. Such low affinities are uncommon for endogenous peptides and are unlikely to have physiological relevance, given that the complexes are highly unstable at 37°C.

Next, we optimized the temperature and time for peptide exchange for each allele. For HLA-A*03:01, we tested three template peptides that were unstable at 50°C and could be rescued in the presence of a high-affinity peptide. Thermal stability was assessed by incubating the HLA-A*03:01 complex with a high-affinity peptide (RGAVRATRR) at various temperatures for 1 hour (***Figure 2***, ***Figure 2—figure supplement 1***). We selected the template peptide RGAVRATKR, which had the lowest optimal exchange temperature (23°C) for 1 hour. This condition resulted in a nearly complete unfolding of the complex in the absence of a high-affinity peptide and full rescue of the HLA-A*03:01 complex in the presence of a high-affinity peptide (***Figure 2***). Similar results were obtained when the complexes were incubated at 30°C. A similar approach was used for HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02, where the optimal peptide-exchange conditions entailed a 1-hour incubation at 30°C

Table 1. Peptides used for template development.

MHC-I allele	Source	Epitope	Sequence	K_d (nM)
A*03:01	Hepatitis C virus	$HCV_{43–51}$	RLGVRATRK	11.7 (***Scognamiglio et al., 1999***)
			RGAVRATRR	7098.44 (***Reynisson et al., 2020***)
			RAGVRATKR	8206.05 (***Reynisson et al., 2020***)
			RSGVRATRH	8293.07 (***Reynisson et al., 2020***)
			RGAVRATKR	8654.53 (***Reynisson et al., 2020***)
			RAGVRATRH	13381.76 (***Reynisson et al., 2020***)
A*11:01	*Homo sapiens*	PLA-$X_{26–34}$	KVVNPLFEK	8.1 (***Kubo et al., 1994***)
	Homo sapiens	GAD-$65_{255–263}$	RFKMFPEVK	3898.11 (***Reynisson et al., 2020***)
			RRKMFPEVK	21628.45 (***Reynisson et al., 2020***)
			RFKMFPEVR	11988.30 (***Reynisson et al., 2020***)
	Dengue virus, genome polyprotein	$_{2007–2015}$	KTFVELMRR	30.08 (***Reynisson et al., 2020***)
			KAFVDLMRH	3871.92 (***Reynisson et al., 2020***)
			KTFVDLGRH	3277.65 (***Reynisson et al., 2020***)
			KTFVDLARH	1659.70 (***Reynisson et al., 2020***)
			KTFVDLMRH	414.23 (***Reynisson et al., 2020***)
B*07:02	*Homo sapiens*	AKR1C3$_{91–99}$	RPELVRPAL	4.38 (***Reynisson et al., 2020***)
			LAELVRPAL	732.89 (***Reynisson et al., 2020***)
			VAELVRPAL	826.71 (***Reynisson et al., 2020***)
			RKELVRPAL	4118.37 (***Reynisson et al., 2020***)
C*07:02	*M. tuberculosis*	$EsxH_{4–12}$	IMYNYPAML	67 (***Axelsson-Robertson et al., 2013***)
			IAYNYPAMA	5158.68 (***Reynisson et al., 2020***)
			IKYNYPAMA	9139.38 (***Reynisson et al., 2020***)

(***Figure 2***, ***Figure 2—figure supplement 1***). The exchange efficiency was then examined in detail for all four of the alleles included in this study. We found that replacing the template peptide with the high-affinity peptide and incubating for approximately 1–1.5 hours at the specified temperature resulted in nearly complete peptide exchange, as shown in ***Figure 2B***. This pattern was consistent across the different HLA alleles tested and aligns with the high-exchange efficiencies previously documented for HLA-A*02:01 and H2Kb (***Luimstra et al., 2018***). For three alleles, the measured exchange efficiency exceeded 100%. This effect arises from the intrinsic instability of the input complex at temperatures above 4°C, which partially dissociates during the short preparation and measurement steps. In the condition when a high-affinity peptide is present, the peptide stabilizes the complex. Thus, the efficiency of thermal exchange may be underestimated due to technical issues. Collectively, these findings establish robust conditions for efficient thermal peptide exchange across multiple HLA class I alleles and expand the applicability of thermal peptide exchange for multimer production. To verify the quality of the thermally exchanged peptide-MHC (pMHC) complexes, we incorporated an additional control based on LILRB1 binding. Stability of each pMHC complex was assessed using K562 cells retrovirally transduced to overexpress LILRB1 (***Figure 2C***). Because LILRB1 recognizes structural elements of β2-microglobulin (β2M) and the α3 domain of the MHC heavy chain, it selectively interacts with properly assembled and stable pMHC complexes. We have previously demonstrated this for both thermally exchanged HLA-E tetramers and UV-mediated peptide-exchange reagents (***Fuchs et al., 2024***; ***Ruibal et al., 2023***). Consistent with this, four conventional PE-conjugated tetramers stained only K562-LILRB1^{OE} cells (***Figure 2D***). We then applied the thermal peptide-exchange tetramers,

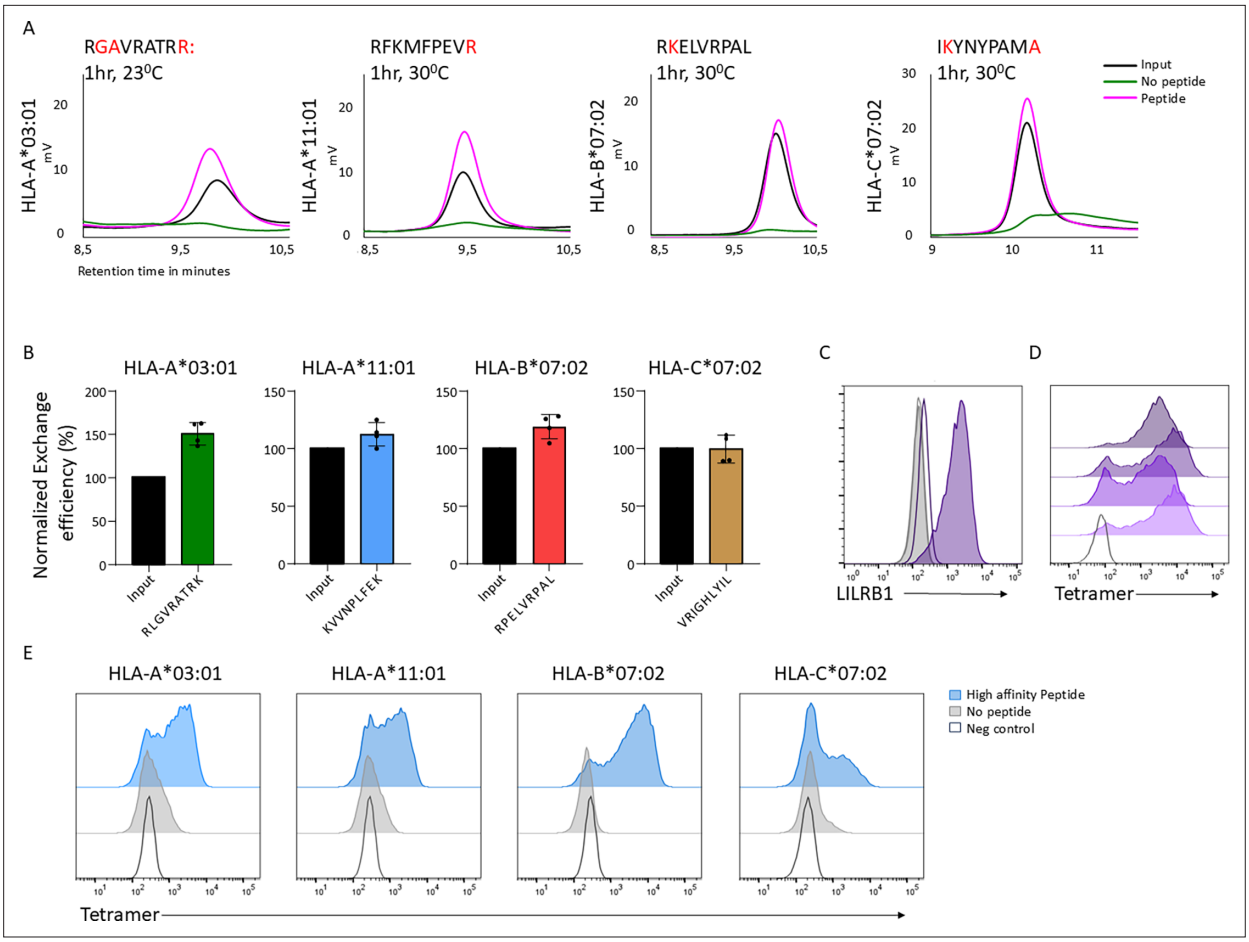

Figure 2. Optimal exchange conditions for template-peptide MHC-I monomers. (**A**) Thermal stability of the template peptide-MHC-I complex to determine optimal exchange temperature with one high-affinity peptide for all alleles tested. Primary data of temperature-based peptide exchange analyzed by gel filtration chromatography at indicated temperatures. The black line represents primary data without thermal exchange, the green line shows thermal exchange without peptide (resulting in complex instability), and the pink line represents thermal exchange in the presence of peptide (yielding a stable complex). The thermal exchange temperatures corresponding to each histogram are indicated above the panels and are 23, 30, 30, and 30°C, respectively. Peptides used are depicted in ***Table 1***. One of at least three representative experiments is shown. (**B**) The exchange efficiency was calculated from the area under the curve measured by HPLC and normalized to binding of the optimal peptide and timing as indicated in each histogram. Mean values ± SD from three independent experiments are shown. (**C**) LILRB1 over-expression (OE) in K562. Gray: negative control; purple line: K562-WT plus anti-LILRB1-Fitc; purple filled: K562-LILRB1^{OE} plus anti-LILRB1-Fitc (N=3). (**D**) Four different conventional tetramer staining on K562-LILRB1^{OE}. Open black line: negative control (N=6). (**E**) Thermal exchange using a high-affinity peptide or no peptide, followed by staining of the K562-LILRB1^{OE} and flow cytometric analysis (N=4).

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Thermal stability of the template peptide-MHC-I complex to determine the optimal exchange temperature using a high-affinity peptide for each allele tested.

representing four HLA alleles. When a high-affinity peptide formed a stable complex with the PE-conjugated tetramer, robust staining of K562-LILRB1^{OE} cells was observed (***Figure 2E***). In the absence of peptide, unstable association with the MHC heavy chain led to β2M dissociation from the fluorescent tetramer, and no LILRB1-dependent staining was observed. Collectively, these optimizations and validation steps confirm that thermal peptide exchange is a robust and versatile method for generating stable pMHC complexes, enabling reliable downstream detection of antigen-specific T cells.

Temperature-exchanged MHC-I multimers for detection of antigen-specific CD8+ T cells

Previously, we demonstrated that HLA-A*02:01 MHC-I multimers generated via temperature-based peptide exchange performed comparably to those produced using conventional methods (***Luimstra***

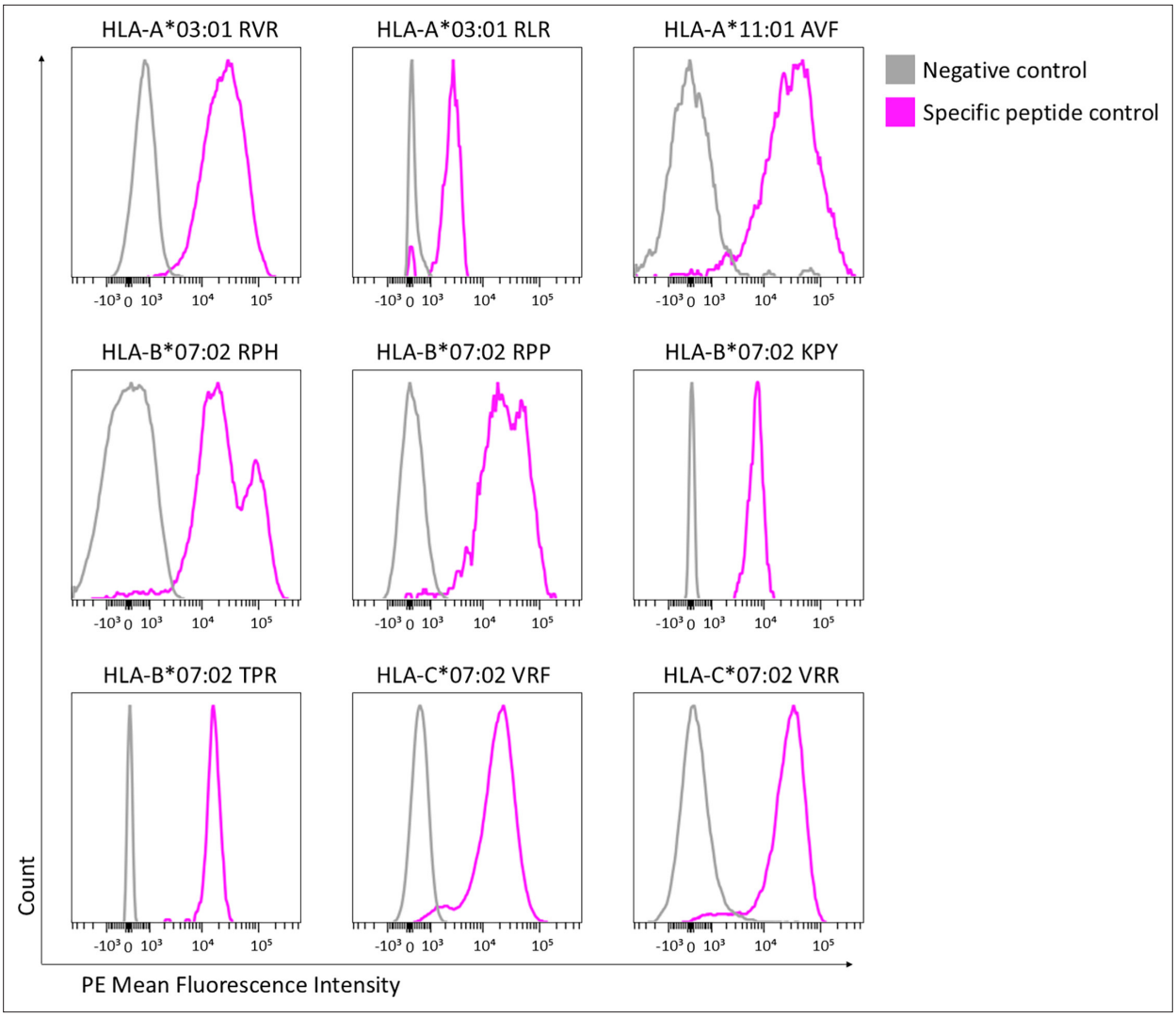

Figure 3. Temperature exchange tetramer binding to clonal T cell lines. Clonal CD8+ T cell lines stained with PE-conjugated temperature exchange multimers that either contained a relevant (pink) or irrelevant peptide (gray). The specificities of the T cell lines are depicted above each figure, as well as HLA-type and first three amino acids of the peptide. y-axis shows the individually scaled counts and x-axis shows the mean fluorescent intensity of PE.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Dilution of temperature exchange multimers on T cell lines.

et al., 2018; ***Luimstra et al., 2019***). We also established that peptide exchange can be directly applied to multimers themselves, resulting in a more user-friendly protocol with reduced handling time (***Luimstra et al., 2018***; ***Luimstra et al., 2019***). To evaluate the effectiveness of the newly developed temperature-exchanged MHC-I multimers for detection of antigen-specific T cell responses, we performed temperature exchange reactions using a panel of high-affinity peptides specific for HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02. These temperature-exchanged MHC-I multimers efficiently labeled clonal T cell lines (***Figure 3***), in a concentration-dependent manner (***Figure 3—figure supplement 1***). This evaluation included two HLA-A*03:01-restricted T cell lines, one HLA-A*11:01-restricted T cell line, two HLA-B*07:02-restricted T cell lines, and one HLA-C*07:02-restricted T cell line. A complete list of peptides used for temperature-based peptide exchange is provided in ***Table 1***. We then compared the staining of T cells by temperature-exchanged MHC-I multimers to those stained with classical folded MHC-I multimers. In the latter case, each MHC-I multimer has to be produced individually, while temperature exchange allows for a batch that can be taken from the freezer and warmed up in the presence of intended peptides before flow analyses. This procedure takes only 1 hour. For this comparison, we generated temperature-exchanged MHC-I

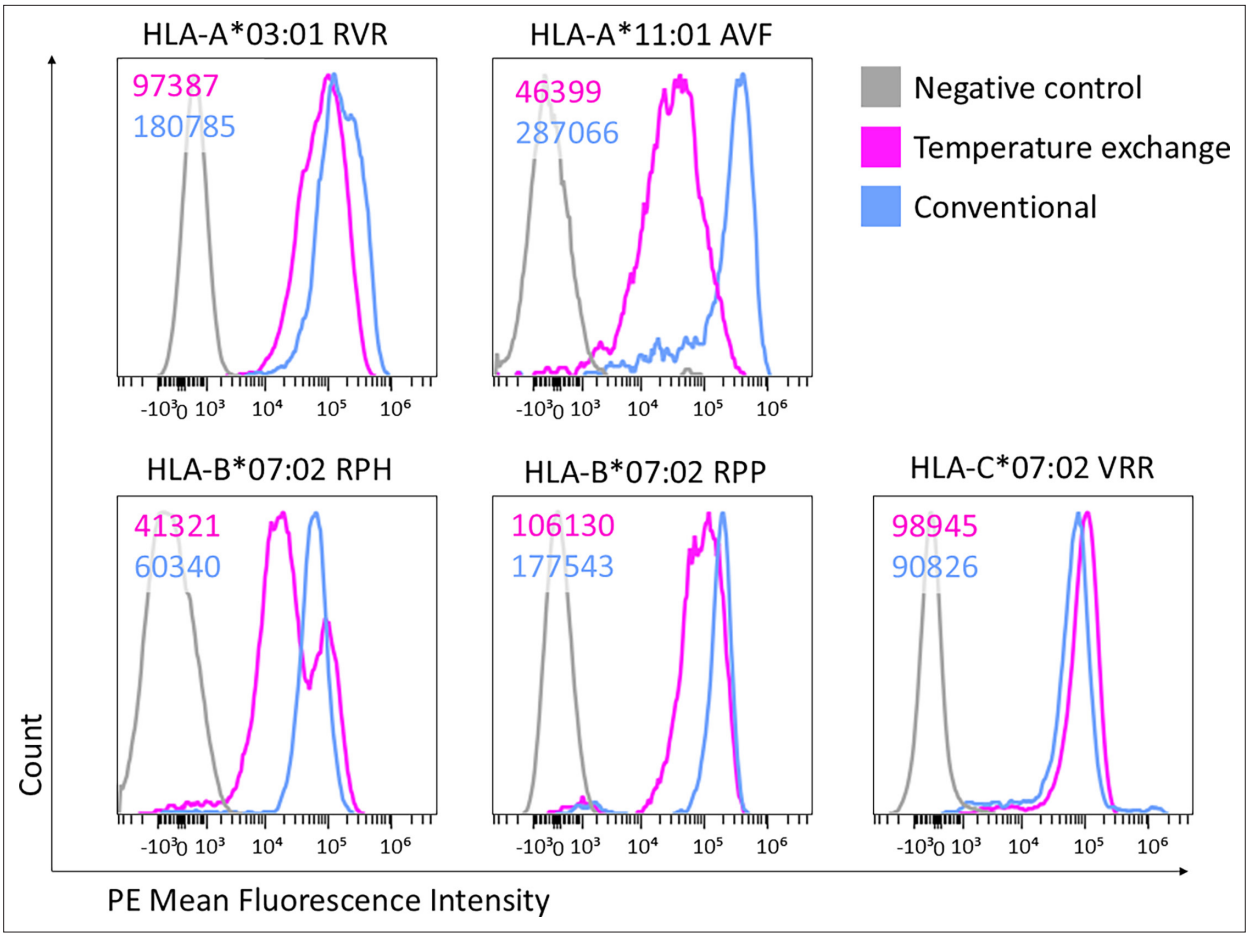

Figure 4. Comparison of temperature exchange multimers to conventional multimers on clonal T cell lines. Clonal CD8+ T cell lines stained with 13.3 ng/µL of PE-conjugated temperature exchange tetramers (pink) or with conventional tetramers (blue). The specificities of the T cell lines are depicted with the HLA-type and first three amino acids of the peptide for each line. y-axis shows the individually scaled counts and x-axis shows mean fluorescent intensity of PE.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Comparison of temperature exchange (TE) multimers to conventional multimers at different concentrations.

multimers for each of the four newly developed alleles and compared their staining performance to that of the classical folded MHC-I multimers at isomass for the same peptide (***Figure 4***). Additionally, staining intensity was evaluated across multiple dilutions using isomass MHC-I multimer concentrations (***Figure 4—figure supplement 1***). Overall, the temperature-exchanged MHC-I multimers showed distinct binding to clonal T cells in an antigen-specific manner and exhibited only a minor decrease in performance compared to the classical MHC-I multimers, particularly for the HLA-A11:01 and HLA-B07:02 complexes. Temperature-exchanged multimers for HLA-A*03:01 and HLA-C*07:02 showed similar or slightly lower staining intensities compared to their classical counterparts. Higher concentrations of both multimer types can be used to further enhance staining intensity without any technical complications.

Detection of virus-specific CD8+ T cells in peripheral blood

To evaluate the practical utility of our additional HLA alleles for temperature-based peptide-exchange MHC-I multimers using human peripheral blood samples, we compared the performance of temperature-based peptide-exchanged multimers to that of classical multimers within an immune monitoring framework. This is particularly relevant in the context of monitoring virus-specific T cells in immunocompromised patients, such as those undergoing T cell-depleted allogeneic stem cell transplantation and adoptive cell therapy. Herpesviruses, including human cytomegalovirus (HCMV) and

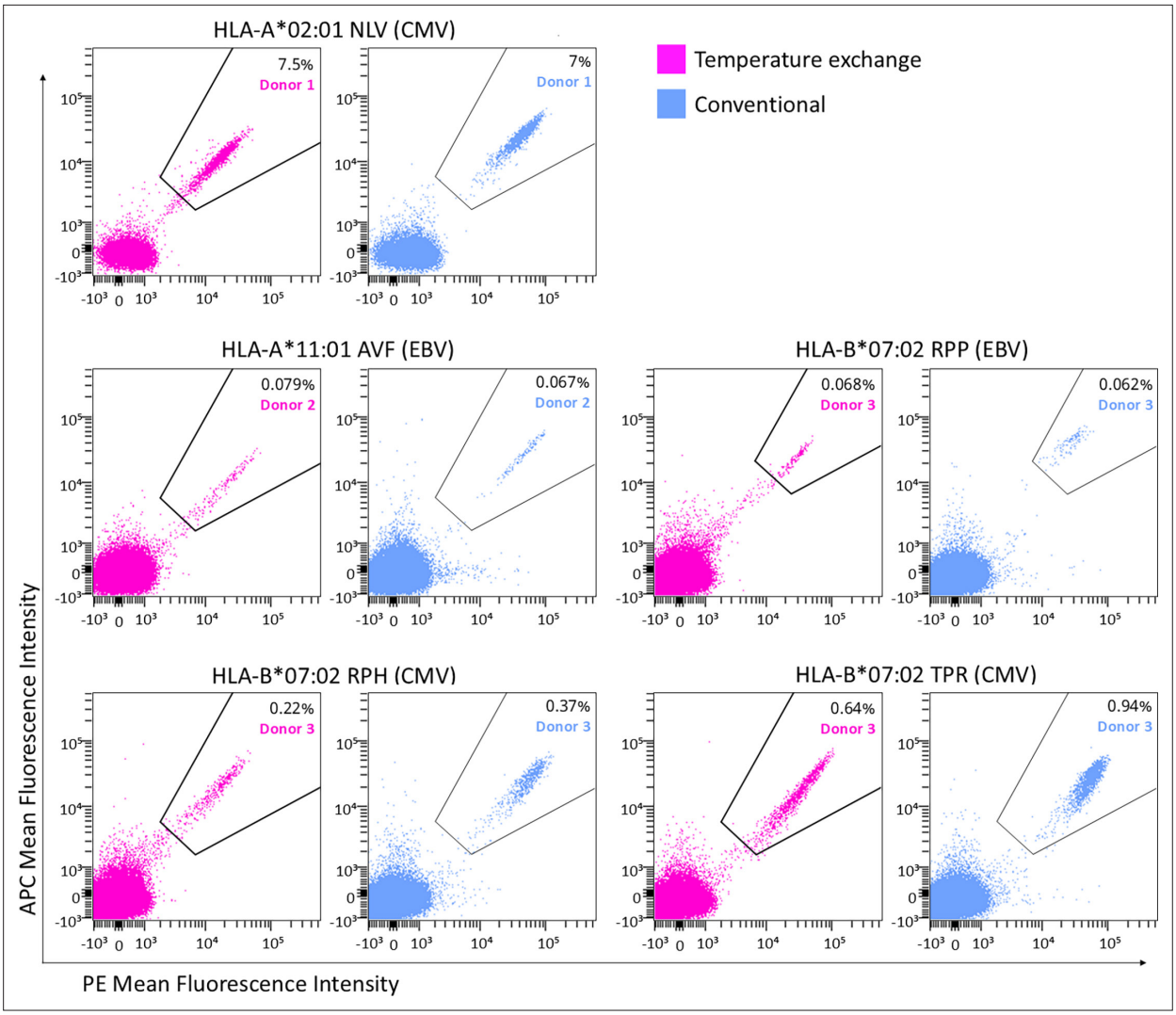

Figure 5. Comparison of temperature exchange tetramers to conventional tetramers on PBMCs. PBMCs of healthy individuals were incubated with peptide exchange (pink) or conventional (blue) tetramers. Donor PBMCs were CMV- or EBV-positive based on serology and were incubated with the relevant tetramer based on donor HLA-type. Tetramers were conjugated to APC (y-axis) as well as PE (x-axis). Gate shows the tetramer-binding CD8+ T cells and the percentage of total CD8+ T cells is depicted in the top-right corner.

The online version of this article includes the following figure supplement(s) for figure 5:

Figure supplement 1. Comparison of temperature exchange multimers to conventional multimers on irrelevant PBMCs.

Figure supplement 2. Flow cytometry gating strategy example.

Epstein–Barr virus (EBV), can cause severe infections in these patients. Restoring T cells is essential to prevent these infections and to minimize complications and mortality. We analyzed peptides derived from HCMV and EBV for the HLA types HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02 in peripheral blood samples from healthy donors. Additionally, we included the previously developed temperature-based peptide-exchange multimers for HLA-A*02:01 in this analysis (***Luimstra et al., 2018***). We exchanged PE- and APC-labeled multimers for a selection of these peptides to measure T cell frequencies in peripheral blood mononuclear cells (PBMCs) and compared them to traditional folded multimers. The antigen-specific T cells identified with temperature-exchanged multimers stained a distinct cell population (***Figure 5***). Importantly, we did not observe any MHC-I multimer-positive T cells in two irrelevant donors (***Figure 5—figure supplement 1***). The percentages observed are comparable to those observed with conventional multimers (***Figure 5***). The peptides are listed in ***Supplementary file 1a***, and the gating strategy of the immune monitoring framework is shown in ***Figure 5—figure supplement 2***. These results illustrate the efficiency and versatility of our technology

in rapidly generating diverse MHC-I multimers from a ready-to-use stock, enabling the detection of antigen-specific T cells, even at the low frequencies commonly observed in primary immune monitoring samples.

Discussion

The use of multimeric versions of peptide MHC-I complexes has become an important technology to detect and quantify antigen-specific CD8+ T cells (***Tippalagama et al., 2023***; ***Rapoport et al., 2015***; ***Tran et al., 2016***; ***Moravec et al., 2025***). To identify antigen-specific T cells using flow cytometry, fluorescently labeled MHC multimers are widely employed. There is an increasing interest in the detection of CD8+ T cells immune responses since they are important for the protection against pathogens or cancer and can be used to define antigens for vaccination purposes. Two challenges in advancing this field are the need for high-throughput approaches and the lack of ready-to-use reagents. Classical peptide-MHC-I complexes are each produced in a separate production run, required for each unique peptide-HLA complex. The process is elaborate, time consuming, and demands highly skilled people. This restricts the generation of a larger library of peptide-HLA combinations and thereby results in incomplete detection of the full CD8+ T cell response against an antigen. Previously, it has been shown that peptide-exchange technologies, such as UV-based peptide exchange, are able to upscale towards higher throughput approaches. While the stability of UV-exchanged pMHC complexes can be assessed using methods such as thermal shift assays and size-exclusion chromatography, the efficiency of peptide exchange is more difficult to quantify directly. Temperature-based peptide exchange is controlled, simple, fast, and quantitative if the correct conditions are identified, as for the HLA alleles in this article. Moreover, the broader availability and simplicity of equipment needed for thermal exchange compared to UV sources enables wider adoption of this approach. The time to prepare the materials for flow analyses is around 1 hour and consists of taking the exchange MHC-I multimer from the fridge and warming it up in the presence of peptides of interest. This can be immediately followed by staining the $CD8^+$ T cells for flow cytometric analysis.

Additionally, MHC-I molecules can interact with other surface proteins in both a peptide-dependent and peptide-independent manner. For example, they bind to the inhibitory receptor Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1), independently of the sequence of the presented peptide, while interactions with receptors such as Killer Immunoglobulin-like Receptors and CD55 are peptide-dependent (***Sim et al., 2023***; ***Cosman et al., 1997***; ***Jones et al., 2011***; ***Schoufour et al., 2024***). Consequently, a positive flow cytometry signal does not necessarily indicate interaction with the TCR of CD8+ T cells. It is therefore essential to exercise caution when interpreting MHC-I multimer results to ensure accurate conclusions. These diverse MHC-I-based interactions may provide further utility for studying various immune cell subsets beyond T cells, including natural killer (NK) cells and other innate immune cells from a peptide MHC-I specific perspective.

We have set out to determine whether temperature-based peptide-exchange technology can be developed into a broadly applicable platform for the screening of human CD8+ T cells. For this purpose, we designed conditional peptide ligands for four different human MHC-I alleles across the three human HLA loci: HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, and HLA-C*07:02. Refolding reactions with the temperature-based peptide are efficient and result in stable peptide MHC-I complexes at 4°C, which are unstable with increased temperature. The presence of a high-affinity peptide during temperature-based peptide exchange ensures the replacement of the temperature conditional ligand in the MHC-I peptide-binding groove. This leads to the generation of the peptide-MHC-I complex with a desired antigen specificity. Importantly, we show that the temperature-based peptide exchange can be performed using the multimerized and fluorescently labeled MHC-I complexes. This allows the development of ready-to-use MHC-I multimers, with high-throughput abilities demonstrating the ease of use of temperature-based peptide-exchange technology.

The temperature-based peptide-exchange method for generating MHC-I multimers has several limitations. First, effective peptide exchange necessitates the design of HLA-specific ligands, adding complexity compared to approaches that utilize empty-loadable MHC-I multimers. This reliance on specific ligands could also affect the specificity and functionality of T cell responses, particularly when low-affinity peptides are involved. Additionally, although the method allows for rapid ready-to-use multimer generation, the inherent stability of some MHC-I complexes could limit the types of peptides that can be successfully exchanged. Furthermore, while this system is designed to be accessible to

a wide range of laboratories, the initial need for appropriate peptide selection and optimization might still pose a challenge for some users. This technology is most likely not optimal for low-affinity peptides, as the template peptide itself must also have low affinity. Lastly, despite the benefits of temperature-based peptide-exchange technology, there is still a risk that some combinations may not provide the desired specificity and functionality. This highlights the need for ongoing refinement and validation of the method across different MHC-I alleles. Additionally, using complementary peptide-exchange methods may enhance overall effectiveness as has been done for UV-exchange multimers and empty disulfide-stabilized multimers (***Hansen et al., 2024***).

Current peptide-exchange technologies predominantly target HLA alleles common in individuals of Western European descent. However, we have started expanding this work to include HLA-A*11:01, which is most prevalent in East and Southeast Asian populations (***de Miranda and Scheeren, 2024***; ***Slieker et al., 2024***). Currently, there are temperature-based peptide-exchange multimers for six HLA alleles, namely HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, HLA-B*07:02, HLA-C*07:02, and HLA-E (***Ruibal et al., 2023***; ***Ruibal et al., 2022***). With this set of MHC-I reagents, a deep analysis could be possible for people with a European genetic background.

A variety of MHC-I reagents are currently available for use in CD8+ T cell diagnostics, including classical folded MHC-I multimers, empty stabilized multimers (***Sun et al., 2023***), and UV-exchange multimers. These MHC-I reagents are being used to determine virus-specific T cell diagnostics, personalized neoantigen-specific T cell diagnostics, and more. We propose that the ready-to-use temperature-based peptide-exchange MHC-I technology could further advance the field, as these reagents simplify the process by eliminating additional steps typically required for MHC-I multimers production.

Materials and methods

PBMC isolation

PBMCs were isolated from healthy donors and obtained via Sanquin or biobanked by the Department of Hematology (LUMC) after informed consent, in accordance with the Declaration of Helsinki. PBMCs were isolated from buffy coats or whole blood using Ficoll-Isopaque and used directly or stored using cryo-preservation. Biobanked PBMCs were thawed in Iscove Modified Dulbecco Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, Saint Louis, MO, USA), 2.7 mM L-glutamine (Lonza), 100 U/mL penicillin (Lonza), and 100 µg/mL streptomycin (Lonza; 1% p/s), and subsequently treated with 1.33 mg/mL DNAse to minimize cell clumping. PBMCs were either used for isolation of clonal T cell lines or for flow cytometry analysis.

Clonal T cell lines and cell lines

Clonal T cell lines were isolated and expanded as previously described (***Huisman et al., 2021***; ***de Rooij et al., 2023***). In brief, PBMCs were incubated with peptide-HLA multimers and antibodies targeting CD14, CD8, and CD4 to isolate CD14-CD8+CD4- multimer-binding cells using fluorescence-activated cell sorting (FACS). In some cases, cells were pre-selected prior to FACS using magnetic-activated cell sorting (MACS) based on peptide-HLA multimer-binding. Obtained cells were further expanded either as bulk (***Huisman et al., 2021***) or single cell per well (***de Rooij et al., 2023***), in the presence of irradiated feeders with or without irradiated Epstein–Barr virus lymphoblastoid cell lines (EBV-LCLs), phytohemagglutinin (PHA), or specific peptides. See ***Supplementary file 1b*** for T cell line details. HEK293T cells (ATCC, cat# CRL-3216; RRID:CVCL_0063) were used for retroviral production. K562 cells (RRID:CVCL_0004) were obtained from Dr. B. Pang and used for generation of the LILRB1-expressing cell line. Cell cultures were routinely tested for mycoplasma contamination and were negative. Cell line identity was based on acquisition from the indicated sources.

Flow cytometry

1–2 × 10^6 PBMCs or 0.05–0.1 × 10^6 T cells were seeded in a round bottom 96-well plate. Cells were stained with the viability dye Zombie-Red (BioLegend, San Diego, CA, USA) for 25 minutes at room temperature after which the cells were washed in PBS containing 0.8 mg/mL albumin (FACS buffer). Cells were stained with 20 µL multimer staining mix, incubated for 30 minutes at 4°C. Conventional multimers were generated in-house, as previously described (***Huisman et al., 2021***). After washing,

cells were stained with 20 µL of an antibody cocktail containing: αCD8-pacific blue (cat#558207, BD Biosciences), αCD4-BV510 (cat#562970, BD Biosciences), αCD14-FITC (cat#555397, BD Biosciences), αCD19-FITC (cat#555412, BD Biosciences), and αCD16-FITC (cat#335035, BD Biosciences). After incubation for 20 minutes at 4°C, cells were washed and measured on a 3-laser Aurora (Cytek Biosciences) or LSR II (BD Biosciences) flow cytometer. Flow cytometry data analysis was performed using OMIQ.ai. See ***Supplementary file 1b*** for patient details regarding CMV- and EBV status and HLA typing.

K562 LILRB1^{OE} cells were harvested, washed, and 100K cells were stained in 50 uL facs buffer (1% FCS in PBS) supplemented with multimers (1:80 dilution for temp exchange or 200 ng conventional tetramer) or anti-LILRB1 FITC (1:40, clone REA998, Miltenyi Biotec) for 30 minutes on ice shielded from light. After two rounds of washing, cells were measured in 100 uL facs buffer on a BD FACS LSR II 4L Full (BD Biosciences, San Jose, CA, USA) flow cytometer, and data was analyzed using FlowJo FlowJo (V10.8.1).

Peptide synthesis and purification

Peptides were synthesized at the LUMC Peptide Facility by standard solid-phase peptide synthesis using Syro I and Syro II synthesizers. Peptides were purified using reversed-phase HPLC (Waters) using a mobile phase of water/acetonitrile gradient containing 0,1% TFA on a C18 column (X-bridge, Waters). Purity of the peptides was confirmed using LC-MS (Micromass LCT Premier; Waters).

Generation of temperature-sensitive monomers and multimerization

Temperature-based sensitive monomers were produced as previously described (***Luimstra et al., 2019***). Briefly, recombinant HLA-A/B/C heavy chain and β2M light chain were produced as inclusion bodies in bacterial strain BL21 (DE3). These purified inclusion bodies containing 2.5 mg of heavy chain were solubilized in 8 M urea and folded for 5 days at 10°C with 1.2 mg prefolded β2M and 3 mg temperature-sensitive peptide (A3: RGAVRATKR; A11: RFKMFPEVR; B07: RKELVRPAL; C07: IKYNYPAMA) in 50 mL folding buffer (400 mM L-arginine, 0,5 mM oxidized glutathione, 5 mM reduced glutathione, 2 mM EDTA, 100 mM Tris-HCl pH 8, glycerol 5%, and half a tablet of protease inhibitor cocktail [complete, Roche]). Temperature-based sensitive monomers were concentrated on a 30 kDa filter (Amicon Ultra-15) and biotinylated with BirA ligase overnight at 4°C. Biotinylated temperature-based sensitive monomers were purified with size-exclusion chromatography (Superdex 75 10/300 GL) on a NGC system (Bio-Rad). Properly folded complexes were concentrated on a 30 kDa filter (Amicon Ultra-15), and the concentration was measured on a NanoDrop (Thermo Fisher Scientific) and stored in 300 mM NaCl and 20 mM Tris-HCl, pH 8.0 with 15% glycerol at –80°C. Next, the monomers were multimerized with PE-labeled streptavidin and/or APC-labeled streptavidin (SA-PE, SA-APC; Thermo Fisher Scientific, Invitrogen) to a final concentration of 0,625 µM. For peptide exchange, temperature-based monomers were incubated at a final concentration of 0.5 µM and 50 µM of selected peptide. This was diluted 1:20 with PBS to be added to CD8+T cells.

HPLC analysis of temperature-mediated peptide exchange

To initiate temperature-based peptide exchange, a 0.5 µM peptide-MHC-I complex was mixed with 50 µM exchange peptide in 110 µL PBS and incubated under defined conditions. After centrifugation at 14,000 × *g* for 1 minute, the supernatant was analyzed using size-exclusion chromatography on a Shimadzu Prominence HPLC system with a 300×7.8 mm BioSep SEC–s3000 column and PBS as mobile phase. Data were analyzed using Shimadzu LabSolutions software.

LILRB1 overexpressing K562 cell line

The ECD of LILRB1 was fused to the platelet-derived growth factor receptor transmembrane spanning domain and cloned into pCDH (IRES Puro) vector (deposited at Addgene). Virus production, HEK 293T cells were transfected with packaging plasmids pMDLg/pRRE (Addgene), pRSV-Rev (Addgene), pCMV-VSV-G (Addgene) together with the d_LILRB1 plasmid using polyethyleneimine (Polyscience Inc). Virus was harvested, filtered, and K562 cells were transduced with LILRB1 in the presence of 8 µg/mL polybrene (Millipore). Transduced cells were selected using puromycin (2 µg/mL, Thermo Fisher) and sorted twice based on high LILRB1 antibody staining (clone REA998, Miltenyi Biotec, FITC) on a CytoFLEX SRT Benchtop cell sorter (Beckman Coulter, Brea, CA, USA). K562 cells transduced with

LILRB1 were cultured in IMDM supplemented with 10% FBS (Bodinco), 1.5% glutamine (200 mM; Lonza), and 1% penicillin/streptomycin (200 mM; Lonza). The stability of PE fluorescent tetramers was indicated by PE-positive K562 cells (***Fuchs et al., 2024***; ***Schoufour et al., 2024***).

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Author contributions

Cilia R Pothast, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing – original draft; Ian Derksen, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration; Anneloes van der Plas-van Duijn, Conceptualization, Data curation, Formal analysis, Supervision, Validation, Investigation, Methodology, Writing – original draft, Project administration; Angela el Hebieshy, Conceptualization, Supervision, Investigation; Wesley Huisman, Formal analysis; Kees LMC Franken, Resources; Jacques Neefjes, Conceptualization, Supervision; Jolien J Luimstra, Conceptualization, Methodology; Marieke Griffioen, Supervision; Michel GD Kester, Data curation, Formal analysis, Investigation, Methodology; Maarten H Vermeer, Supervision, Funding acquisition, Methodology, Writing – original draft; Marcus Ostholm, Resources, Validation, Investigation; Sine Reker Hadrup, Resources, Methodology; Mirjam HM Heemskerk, Supervision, Writing – original draft; Ferenc A Scheeren, Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Investigation, Visualization, Methodology, Writing – original draft, Project administration, Writing – review and editing

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Additional files

Supplementary files

Supplementary file 1. Supplementary tables. (a) contains clonal CD8+ T cell line and exchange peptide details. (b) contains donors used for detection of virus-specific CD8+ T cells using temperature-based sensitive multimers.

MDAR checklist

Source data 1. Numerical source data underlying the quantitative analyses presented in the figures and figure supplements.

Data availability

All data generated or analyzed during this study are included in the manuscript, supplementary information files, and Source Data files. Raw flow cytometry data (.fcs files) underlying the study have been deposited in Zenodo and are publicly available at https://doi.org/10.5281/zenodo.24293358. Raw HPLC data, including original Shimadzu LabSolutions (.lcd) files and corresponding exported text files (.txt), have been deposited in Zenodo and are publicly available at https://doi.org/10.5281/zenodo.24364453. Source data underlying all figures are provided with the article. No custom code was used to analyse the data.
The following datasets were generated: ***Scheeren, 2026a***, ***Scheeren, 2026b***

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