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Thermal-exchange HLA-E multimers reveal specificity in HLA-E and NKG2A/CD94 complex interactions

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Abstract

There is growing interest in HLA-E-restricted T-cell responses as a possible novel, highly conserved, vaccination targets in the context of infectious and malignant diseases. The developing field of HLA multimers for the detection and study of peptide-specific T cells has allowed the in-depth study of TCR repertoires and molecular requirements for efficient antigen presentation and T-cell activation. In this study, we developed a method for efficient peptide thermal exchange on HLA-E monomers and multimers allowing the high-throughput production of HLA-E multimers. We optimized the thermal-mediated peptide exchange, and flow cytometry staining conditions for the detection of TCR and NKG2A/CD94 receptors, showing that this novel approach can be used for high-throughput identification and analysis of HLA-E-binding peptides which could be involved in T-cell and NK cell-mediated immune responses. Importantly, our analysis of NKG2A/CD94 interaction in the presence of modified peptides led to new molecular insights governing the interaction of HLA-E with this receptor. In particular, our results reveal that interactions of HLA-E with NKG2A/CD94 and the TCR involve different residues. Altogether, we present a novel HLA-E multimer technology based on thermal-mediated peptide exchange allowing us to investigate the molecular requirements for HLA-E/peptide interaction with its receptors.

KEYWORDS
antigen presentation/processing, antigens/peptides/epitopes, MHC/HLA, NK cell, T-cell

INTRODUCTION

Antigen presentation via the highly polymorphic HLA system in humans is a key process bridging innate and adaptive immune responses against pathogens and

Ferenc A. Scheeren and Simone A. Joosten contributed equally to this study.

malignancies [1]. Classical HLA class I and HLA class II molecules are involved in pathways leading to the activation of $CD8^+$ and $CD4^+$ T cells, respectively. One unique element of the HLA system, the nonclassical and monomorphic HLA-E, with only two functional variants expressed in humans, HLA-E*01:01 and HLA-E*01:03, has recently been highlighted as a potential target for preventive and therapeutic treatments in the context of cancer and infectious diseases [2]. HLA-E was initially thought to bind only a limited number of peptides, namely HLA class Ia-derived leader peptides [3,4]. These leader peptides are presented by HLA-E for NKG2A/CD94 recognition on NK cells, serving as an indicator of cell health and a regulator of NK cell function as part of the self/missing-self surveillance by NK cells [5]. However, more recent studies have shown that a wider repertoire of self- and pathogen-derived peptides can also be presented by HLA-E for T-cell recognition via TCR, suggesting an important role for HLA-E-restricted T cells in the adaptive response [2]. This dual role of HLA-E in regulating NK cell function and mediating T-cell activation has raised interest in this molecule as a target for vaccines and therapeutics aimed against infectious and malignant diseases [2]. The study of peptide-specific HLA-restricted T-cell responses hinges on the availability of high-quality fluorescently labelled HLA multimers [6]. Indeed, HLA-E tetramers have already been instrumental in identifying pathogen-specific HLA-E-restricted T cells in the context of *Mycobacterium tuberculosis* (Mtb) [7], human cytomegalovirus (HCMV) [8], and simian immunodeficiency virus [9], amongst others. In addition, such tools can be equally valuable to understand the interaction of HLA-E/peptide complexes with NKG2A/CD94 and help identify optimal peptides to be included in HLA-E vaccines [2].

The growing interest in the high-throughput analysis of diverse, polyclonal TCR repertoires has resulted in the recent development of novel technologies to facilitate the production of multimer libraries. Recent advances in the development of multimers include the increase in valency for the detection of T cells with lower affinity [10–12] and the establishment of alternative multimer loading systems to allow the detection of a greater repertoire of peptide-specific T cells [13,14]. Contrary to conventional tetramers, which need a purification step after refolding in the presence of each peptide [15], peptide exchange allows the generation of a library of tetramers loaded with different peptides in a shorter amount of time, as one single refolding and purification step is needed in the presence of the template peptide. Peptide exchange for the loading of alternative peptides on murine H2 and human HLA molecules has been demonstrated by cleavage of UV-labile peptide [16,17], thermal-sensitive peptide [13,14] or through dipeptides or chemical catalyzers [18–20]. In addition, disulphide-stabilized multimers which are peptide-receptive have also been recently described [21]. Recent studies have demonstrated that peptide exchange on HLA-E is possible through UV-mediated cleavage of a photosensitive peptide leading to applications such as peptide/HLA-E binding assays and the production of HLA-E tetramers [15,22–24]. However, UV exposure to cleave the photosensitive peptide can potentially induce alterations in the proteins and damage the fluorescent labels on tetramers, motivating the search for alternative peptide exchange methods [13,25]. Developing the thermal exchange of peptides for other MHC molecules requires the identification of suitable template peptides which bind MHC at low temperatures and are able to dissociate at higher temperatures [13]. To facilitate the study of HLA-E-restricted T-cell repertoires we developed thermal-mediated peptide exchange for HLA-E multimers, to enable the screening of a large variety of peptides in the context of T-cell recognition. Here we describe the identification of a template peptide suitable for thermal-mediated peptide exchange on HLA-E monomers and multimers, as well as the optimization for thermal exchange. The establishment of this tool allows the detection of peptide-specific HLA-E-restricted T cells and NK cells through the staining of both TCR and NKG2A/CD94, which could be of value for the identification of peptides involved in HLA-E-restricted immune responses in the context of both infectious and malignant diseases. Finally, we exploited this novel thermal-exchange HLA-E multimer technology to interrogate the molecular requirements for NKG2A/CD94-mediated peptide recognition. We could confirm the relevance of arginine in peptide position 5 and 6 for NKG2A/CD94 recognition of HLA-E-binding peptides, and identified modifications in peptide positions 7 and 9 which led to reduced peptide recognition, while substitution of peptide position 8 had no effect in binding.

MATERIALS AND METHODS

Peptide synthesis and purification

The peptides were synthesized at Leiden University Medical Center Peptide Facility by standard solid-phase peptide synthesis using Syro I and Syro II synthesizers. Peptides were purified using reversed-phase high-performance liquid chromatography (HPLC) (Waters) using a mobile phase of water/acetonitrile gradient containing 0.1% TFA on a C18 column (X-bridge, Waters). The purity of the peptides was confirmed on LC–MS using a mass spectrometer (micromass LCT Premier; Waters).

Generation of thermal-sensitive monomers and multimerization

Thermal-sensitive monomers were produced as previously described [14]. Briefly, recombinant HLA-E*01:03/01:01 heavy chain and β_2M light chain were produced as inclusion bodies in bacterial strain BL21 (DE3). These purified inclusion bodies containing 2.5 mg of the heavy chain were solubilized in 8 M urea and folded for 5 days at 10°C with 1.2 mg prefolded β_2M and 3 mg temperature-sensitive peptide (VLRPGGHFAA) 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]). Thermal-sensitive monomers were concentrated on a 30 kDa filter (Amicon Ultra-15) and biotinylated with BirA ligase overnight at 4°C. Biotinylated thermal-sensitive monomers were purified with size exclusion chromatography (Superdex 75 10/300 GL) on an NGC system (Bio-Rad). Properly folded complexes were concentrated on a 30 kDa filter (Amicon Ultra-15) and stored in 300 mM NaCl and 20 mM Tris–HCl, pH 8.0 with 15% glycerol at −80°C. For peptide exchange, thermal-sensitive monomers were diluted 1:20 with phosphate-buffered saline (PBS) containing a 100-fold concentration of selected peptide. The exchange reaction was incubated in a thermoblock or incubator for 1 h at 30°C. The concentration was measured on a NanoDrop (Thermo Fisher Scientific). Next, the monomers were tetramerized with PE-labelled streptavidin (SA-PE; Thermo Fisher Scientific, Invitrogen) to a final concentration of 0.5 μM.

Detection of peptide exchange by sandwich ELISA-binding assay

A binding assay was performed as previously described [22,23]. Briefly, 96-well half-area ELISA microplates (Greiner Bio-One) were coated with 10 mg/ml of purified anti-human HLA-E antibody (clone 3D12; BioLegend). After the thermal exchange, the resulting exchanged monomers were added to plates previously blocked with 2% IgG-free BSA in PBS and washed with 0.05% Tween 20 in PBS. Detection of β_2M-associated complexes was performed using 2 mg/ml horseradish peroxidase (HRP)-conjugated anti-β_2M antibody (Thermo Fisher Scientific) in blocking buffer, and the signal was amplified with a 1:15 dilution of HRP-coupled goat anti-rabbit IgG (Dako) before developing with tetramethylbenzidine substrate (Invitrogen) and terminating the reaction with H_2SO_4 stop solution. Absorbance readings were obtained at 450 nm using a SpectraMax i3x Reader. Sample-to-positive (s/p) ratio was calculated by normalizing values to positive control (pCMV) after subtraction of the background obtained in the absence of test peptide (no rescue): s/p ratio = (value − no rescue)/(pCMV − no rescue).

Transfections and transductions

For the generation of TCR-expressing cells, human 293-based Phoenix GALV packaging cells were transfected using Fugene HD transfection reagent (Promega) in serum-free Optimem I medium (Invitrogen) with 2.6 μg MP71flex retroviral vector containing published HLA-E restricted T-cell receptor α- and β-chain and CDR3αβ sequences derived from the T-cell clone KK50.4 recognizing the HCMV UL40-derived epitope sequence VMAPRTLIL [26]. Virus supernatant was harvested 48 h posttransfection and used to transduce $CD8^+$ human Jurkat E6.1 derived subline (J76, lacking expression of endogenous TCR) using 30 μg/ml RetroNectin (TaKaRa) coated plates for at least 24 h. Transduced Jurkat cells were subsequently cultured in IMDM (Gibco) supplemented with 10% heat-inactivated FCS and 100 μg/ml streptomycin. TCR expression was monitored 3 days posttransduction using hamster anti-mouse TCRβ chain (APC-Cy7) and mouse anti-human CD3 (Amcyan) mAbs on a flow cytometer.

For the generation of LILRB1-expressing cells, HEK 293 T cells were transfected using polyethyleneimine (Polyscience Inc.) with packaging plasmids pMDLg/pRRE (Addgene #12251), pRSV-Rev (Addgene #12253), pCMV-VSV-G (Addgene #8454) combined with the lentiviral construct. The virus was harvested and used to transduce target cells using 10 μg/ml polybrene (Millipore). To generate LILRB1 overexpressing cells, K562 cells were first stably transduced with the lenti dCas-VP64 vector (Addgene plasmid # 61425) and selected using blasticidin. These cells were consecutively transduced with the pXPR_502 vector (Addgene plasmid # 96923) containing a gRNA sequence targeting LILRB1, and selected using puromycin. The guide sequence used for LILRB1 in the pXPR_502 vector was: 5′-AAGACTCAGAGATTTGTTCC-3′.

Tetramer staining and flow cytometry analysis

Jurkat cells expressing the KK50.4 TCR were stained at 2×10^5 cells per sample with 50 μl of a 1:50 dilution of the conventional tetramer or 40 μl of different dilutions of the thermal-exchange tetramer (both containing either the VMAPRTLIL peptide or the VMAPRTLLL peptide as control and conjugated to PE) at 37°C for 30 min in the

dark. Tetramers were diluted in FACS buffer (PBS with 0.1% BSA). After washing with FACS buffer, cells were fixated with 1% paraformaldehyde for 10 min at room temperature and then washed again. The cells were then further stained with anti-CD3-BV510 (clone UCHT1; BioLegend) and anti-CD8a-FITC (clone HIT8a; BioLegend) in 100 µl FACS buffer for 15 min at 4°C in the dark. After another wash with FACS buffer, the cells were fixated with 1% paraformaldehyde and the cells were acquired using an LSR Fortessa (BD Biosciences). NKG2A/CD94- and LILRB1-expressing K562 cells, as well as control K562 cells, were stained with 50 µl of a 1:50 dilution of the conventional tetramer or 25 µl of thermal-exchange tetramer for 30 min at 37°C. After washing, cells were acquired on a FACSLyric (BD Biosciences). All data were analysed using FlowJo software v10.7.2.

RESULTS

Design of temperature-sensitive peptide for exchange on HLA-E

For HLA-E*01:03 we selected four peptides that had a low dissociation constant, namely, GLDSRAYRL, VMAPJTVL, QLAPGLQLII and the Mtb68 epitope VLRPGGHFL [27] (dissociation constant [Kd] 4435.48 M) which we modified at position 9 and extended to further decrease the dissociation constant (VLRPGGHFAA, Kd 35995.11 M) (Figure S1). HLA-E*01:03 monomer complexes with these peptides were produced and thermal stability was assessed at 50°C using analytical size exclusion HPLC, in the presence or absence of the high-affinity peptide pCMV-LLL (VLAPRTLLL, Kd 211.08 M). Using the peptides GLDSRAYRL (Kd 23801.87 M) and VMAPJTVL (Kd 10514.47 M) we found that these complexes were too stable at 50°C, as illustrated by an MHC-I peak in absence of peptide when analysed by size exclusion HPLC (Figure S1). The QLAPGLQLII peptide complex was unstable at 50°C, however, the quality of the input sample, which contains thawed monomer complex at 4°C, resulted in a low output peak due to aggregation (Figure S1). On the other hand, the VLRPGGHFAA HLA-E*01:03 complex was unstable at 50°C and the input sample showed optimal output signal. Importantly in the presence of the high-affinity peptide pCMV-LLL, a clear peak can be observed, showing that the HLA-E*01:03 complex could be rescued from unfolding (Figures 1 and S1). Next, we optimized the temperature and timing of the peptide exchange for HLA-E*01:03 in a complex with VLRPGGHFAA. The thermal stability of the complex was determined by incubation with the HLA-E*01:03 high-affinity peptide pCMV-LLL at different temperatures for 1 h, as indicated (Figure 1A). We chose the lowest optimal exchange temperature (30°C) for 1 h as this resulted in almost complete unfolding in the absence of peptide while the superior rescue of the HLA-E*01:03 complex was observed in the presence of pCMV-LLL (Figure 1a). Next, we set out to determine the exchange efficiencies for a number of HLA-E*01:03 binding peptides. To test this, we exchanged HLA-E*01:03–VLRPGGHFAA for either Mtb44 (RLPAKAPLL, Kd 52.1 nM), pCMV-LLL, Mtb34 (VMTTVLATL, Kd 9203.29 nM), Mtb62 (RMPPLGHEL, Kd 622.64 nM), Mtb68 (VLRPGGHFL, Kd 4435.48 nM) and Mtb55 (VMATRRNVL, Kd 6430.40 nM) for 1 h at 30°C. The exchange efficiency reached 90%–100% of the level observed for Mtb44 for high HLA-E-binding peptides such as pCMV-LLL and 40%–60% for moderate and lower HLA-E-binding peptides such as Mtb34 and Mtb68 (Figure 1b).

Despite the highly monomorphic characteristic of HLA-E, previous studies have shown that differences exist in peptide binding repertoires to HLA-E*01:01 and HLA-E*01:03 [28,29], and some dissimilarities have been observed in peptide binding motifs to each molecule [23]. Therefore, to confirm that the thermal-exchange works similarly in both HLA-E*01:01 and HLA-E*01:03, we next optimized the temperature and timing of the peptide exchange for HLA-E*01:01. The VLRPGGHFAA HLA–E*01:01 complex gave similar results as the VLRPGGHFAA HLA-E*01:03: at 30°C the complex was unstable in absence of peptide and showed optimal input signal. Importantly, in the presence of the high-affinity peptide Mtb44, the HLA-E*01:01 could be rescued similarly to the HLA-E*01:03 complex (Figure 1c). Together this illustrates the efficiency and flexibility of our peptide exchange technology to rapidly produce many different HLA-E monomers and multimers.

Given the observed differences in exchange efficiency based on the peptide used, we set out to develop a fast and easy quality control for the peptide exchange of the HLA-E tetramers using flow cytometric analysis. LILRB1 binds the conserved β_2M and α3 domains of HLA-I molecules regardless of the peptide bound [30,31]. Therefore, we reasoned that HLA-E tetramer binding to LILRB1 only occurred when peptide exchange was successful and the HLA-E heavy chain is in complex with β_2M. To test this, we overexpressed LILRB1 on K562 cells by using a dCas9 expression system (Figure S2A). After validating LILRB1 staining with conventional HLA-E tetramers, we observed strong staining of LILRB1-expressing K562 cells with thermal-exchange HLA-E TMs as determined by flow cytometry while dCas9-K562 cells did not show staining (Figure 1d). Indeed, the staining of LILRB1-expressing cells proved an efficient method to validate the complex formation of our thermal exchange

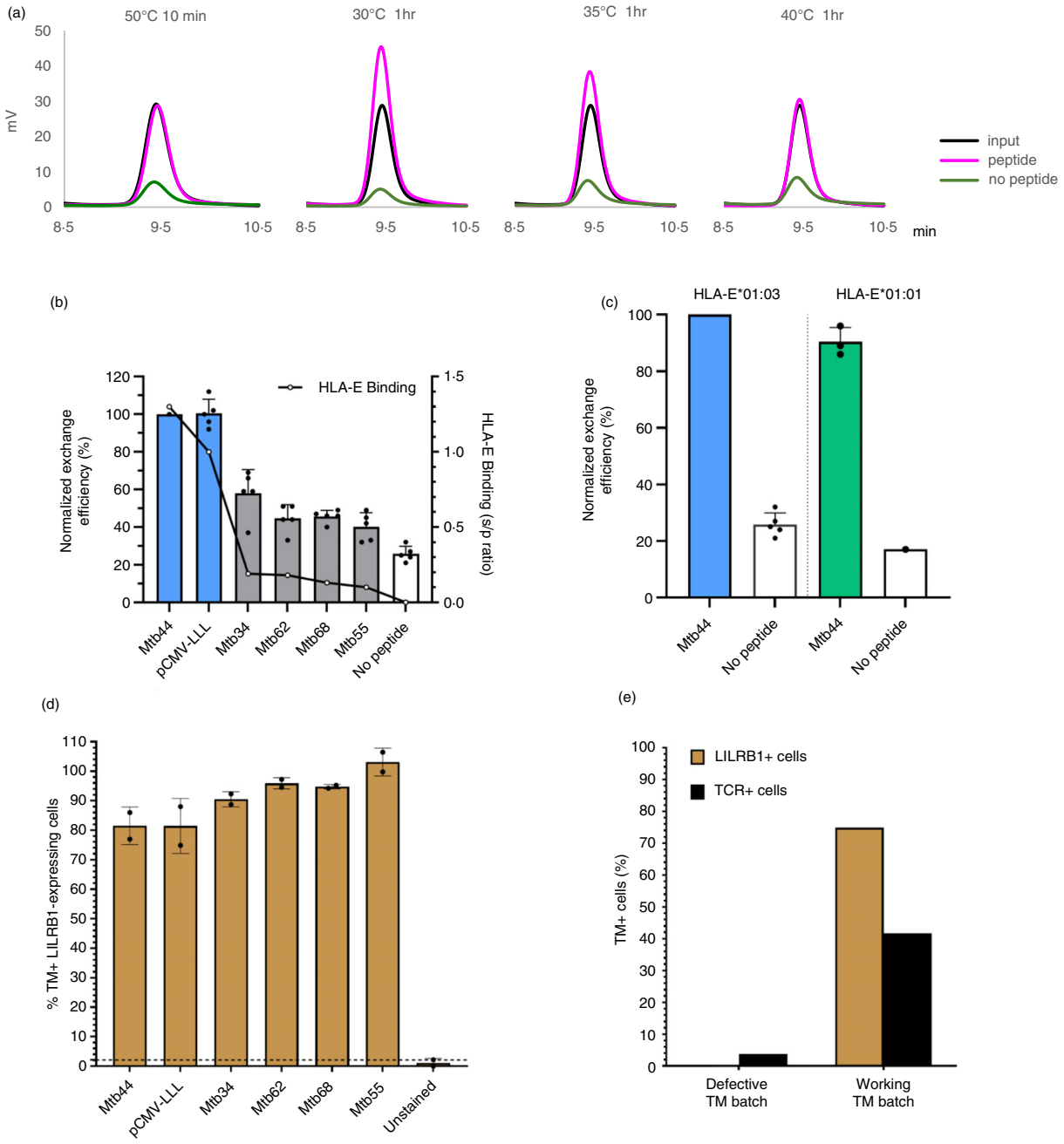

FIGURE 1 Optimization and exchange efficiency of HLA-E*01:03 and HLA-E*01:01. (a) HLA-E*01:03-VLRPGGHFAA monomers were exchanged for high-affinity peptide VLAPRTLLL for optimization. Chromatograms of monomers analysed by gel filtration chromatography at indicated temperatures and time. (b) Exchange efficiency of peptides with high (blue) and low (grey) HLA-E*01:03-binding affinity calculated from the area under the curve from HPLC chromatograms and normalized to monomers folded with Mtb44/RLPAKAPLL. Monomers were also exchanged for pCMV/VLAPRTLLL, Mtb34/VMTTVLATL, Mtb62/RMPPLGHEL, Mtb68/VLRPGGHFL and Mtb55/VMATRRNVL for 1 h at 30°C. high HLA-E*01:03-binding peptides are shown in blue and moderate to low HLA-E*01:03-binding peptides are shown in grey. Peptide binding was also determined in the UV exchange based binding assay and results expressed as the sample to positive (s/p ratio), based on sample as well plate positive and negative controls (black line, right *y*-axis). (c) Exchange and dissociation comparison between HLA-E*01:01 and HLA-E*01:03, both folded with template peptide VLRPGGHFAA. Monomers were exchanged with peptide Mtb44/RLPAKAPLL for 1 h at 30°C for recovery and in absence of any peptide for dissociation. Efficiency and dissociation were calculated from the area under the curve from HPLC chromatograms normalized to Mtb44/RLPAKAPLL. Mean values ± SD from minimal three independent experiments are shown. (d) Bar plots represent the frequency of LILRB1-expressing K562 cells stained with thermal-exchange HLA-E tetramers. dcas9 cells were used as negative staining control with an average staining indicated by the dotted line. (e) Bar plots represent the frequency of LILRB1-expressing K562 cells (brown) and TCR-expressing cells (black) stained with two different conventional HLA-E tetramer batches.

as well as our conventional tetramers. A conventional TM batch that failed to stain a TCR transduced cell line, which worked with previous batches, illustrated that lack of TCR and LILRB1 staining can be an indicator of poor complex formation (Figure 1e). Therefore, peptides in complex with an HLA-E TM can bind LILRB1 and this is a good internal quality control method for peptide exchange.

Optimization of HLA-E thermal exchange and detection of TCR-expressing cells with fresh and freeze-stored thermal-exchange HLA-E tetramers

To confirm and optimize the TCR staining capacity of thermal-exchange multimer we used a KK50.4 TCR-expressing Jurkat cell line with known restriction and specificity for HLA-E-presented pCMV-LIL (VMAPRTLIL) peptide [26] and we compared the staining of these cells with thermal-exchange HLA-E tetramer to that of conventional HLA-E tetramer (cTM). In addition, multimerization first thermal-exchange tetramers allow a fast and easy production of tetramers loaded with different peptides, as staining can be performed directly after peptide exchange on the multimers. Therefore, we compared the exchange first (Figure 2a) and multimerization first (Figure 2b) methods for the production of thermal-exchange TMs in terms of TCR staining. Our results indicate equal staining intensity and specificity when using tetramers built by thermal exchange before multimerization compared to cTM, in both total lymphocyte and specific TCR-expressing cell populations (Figure 2c). However, our results showed that the peptide thermal exchange after multimerization resulted in lower staining intensity (Figures 2c and S2C) and frequency (Figure 2f) of positive cells compared to before multimerization and cTM. Therefore, thermal-exchange HLA-E tetramers are optimally produced by peptide exchange first and multimerization second.

For an optimized peptide thermal exchange and staining, we tested multiple conditions. First, we tested different peptide thermal-exchange incubation times (1 vs. 3 h) and temperatures (30°C vs. 40°C) and observed that the optimal staining was obtained with thermal-exchange HLA-E tetramers after 1 h peptide exchange at 30°C (Figure 2d). All subsequent peptide exchanges were performed under these conditions. Next, we tested different thermal-exchange HLA-E tetramer dilutions to find the staining conditions for optimal signal-to-noise ratio and selected 1:4 as the optimal dilution as it more closely resembled the staining with cTM (Figure 2e).

Conventional monomers and tetramers are usually stored at −80°C for several months without any detriment to their staining capacity. We, therefore, were interested in confirming whether the same storage conditions could be applied to thermal-exchange HLA-E tetramers. We tested the staining of the TCR-expressing Jurkat cell line after storage of thermal-exchange HLA-E tetramers at 4°C, −20°C, and −80°C. Our results showed that similar to conventional tetramers, thermal-exchange tetramers could still properly stain TCR-expressing cells after up to 6 months of storage in these conditions, with optimal staining being maintained after storage at −20°C and −80°C (Figure S3).

Screening of HLA-E-binding peptides for recognition by NKG2A/CD94 with thermal-exchange HLA-E tetramer staining

The interaction of HLA-E-presented peptides with NKG2A/CD94 can lead to important functional consequences on both T cells and NK cells expressing this heterodimer receptor, which is known to be involved in regulating cell activation through the transmission of inhibitory signals [32]. Therefore, we applied our thermal-exchange tetramers for high-throughput screening of NKG2A/CD94 recognition of HLA-E-binding peptides. In an attempt to reduce the tetramer manipulation steps after thermal exchange and to explore a faster pipeline for the production of thermal-exchange HLA-E tetramers for large screening of NKG2A/CD94-binding, we investigated whether, differently to our results above on staining of TCR, staining of NKG2A/CD94-expressing cells could be performed equally well with thermal-exchange tetramers when peptide thermal exchange was performed before or after multimerization. For this, we stained NKG2A/CD94-expressing K562 cells with both types of tetramers, as well as conventional tetramers (Figure S4).

Our results on the staining of LILRB1-expressing cells indicate that the exchange first approach leads to more optimal production of thermal-exchange tetramers compared to the multimerization first approach. This could either be due to a more efficient peptide exchange in monomers compared to multimers or due to the selection of non-aggregated, properly folded monomers for the tetramerization. (Figure 3a,b). Importantly, thermal-exchange tetramers obtained by exchange first performed equally well as cTMs (Figure 3a,c), supporting the notion that thermal-exchange tetramers are an ideal platform to generate large libraries of tetramers to explore HLA-E/peptide interactions with NKG2A/CD94. The similar binding of tetramers created with all three methods to NKG2A/CD94, with only pCMV-LLL (VMAPRTLLL) and pCMV-LIL (VMAPRTLIL) loaded tetramers being

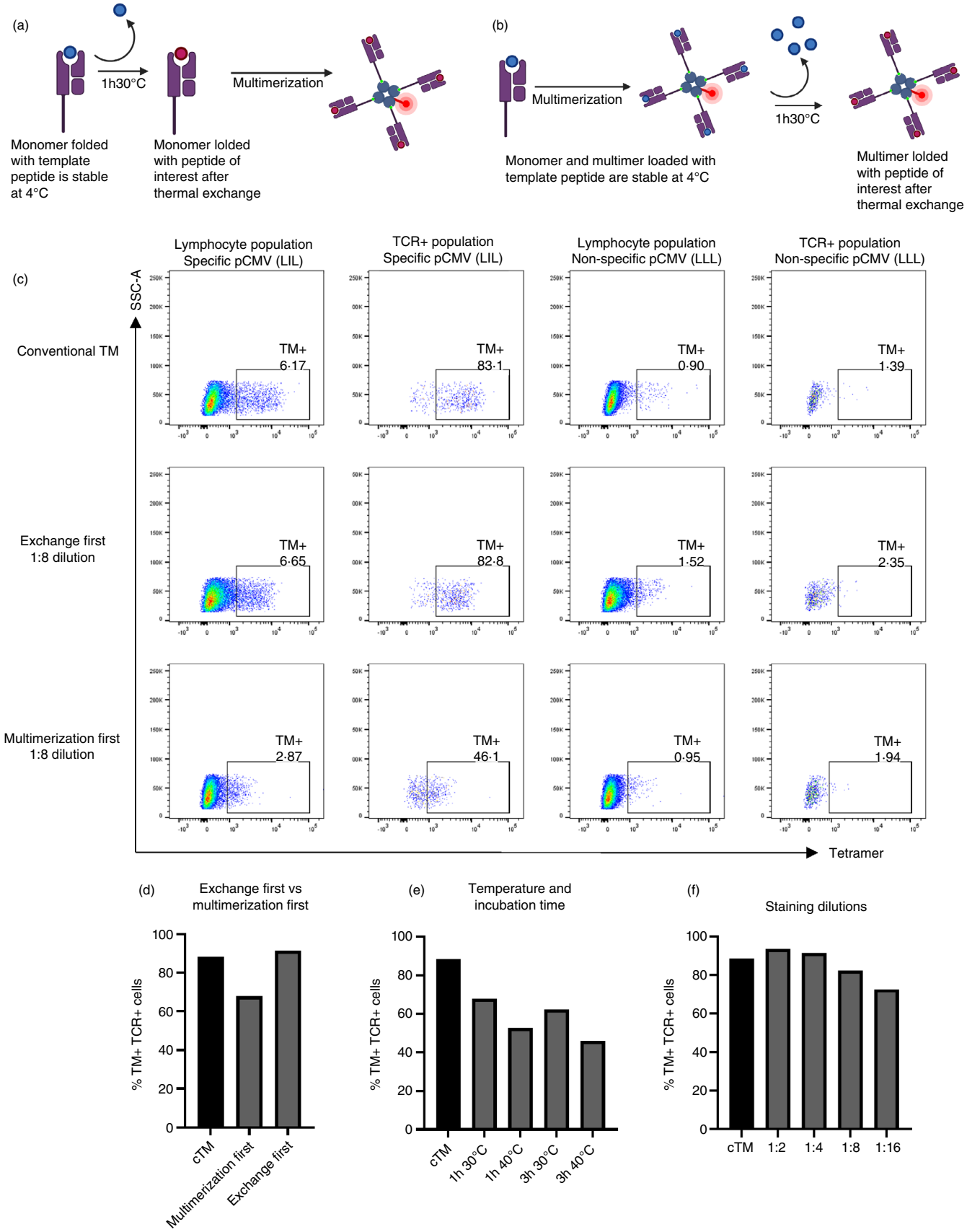

FIGURE 2 Legend on next page.

recognized by this receptor, suggest that all three approaches could be suitable for the screening of HLA-E/peptide-NKG2A/CD94 interactions. However, the exchange first approach is optimal and the reduced tetramer complex formation in the multimerization first approach would only allow to evaluate the interaction of tetramers loaded with high HLA-E-binding peptides, such as Mtb44, pCMV-LLL and pCMV-LIL, as indicated by ELISA-binding assay (Figure 3d) and LILRB1 staining. When using the multimerization first approach, to further optimize the use of thermal-exchange tetramers for a peptide screening purpose, we tested whether thermal peptide exchange could also be performed on previously diluted HLA-E multimers. In this way, the same amount of HLA-E multimers could be used to produce a larger set of peptide-loaded tetramers, allowing the evaluation of more peptides simultaneously. We tested three different dilutions (1:25, 1:50 and 1:75) and saw that, in all dilutions, pCMV-LLL and pCMV-LIL loaded tetramers could similarly stain NKG2A/CD94- and LILRB1-expressing cells, with Mtb44-loaded tetramers additionally staining LILRB1-expressing cells, in line with our results above (data not shown).

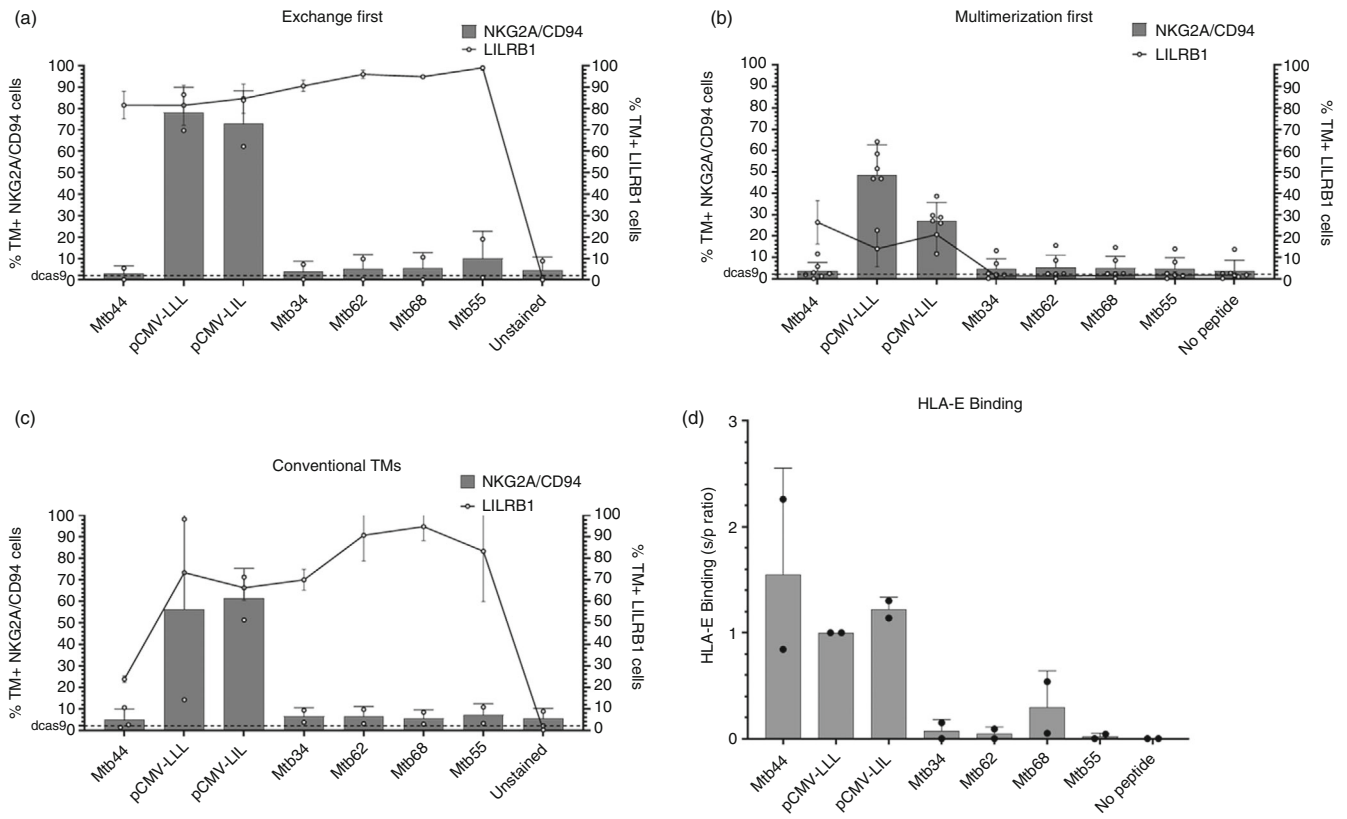

FIGURE 3 NKG2A/CD94 staining with thermal exchanged HLA-E tetramers. (a–c) Bar plots represent the frequency of NKG2A/CD94-expressing K562 cells (left *y*-axis) and symbols and lines represent the frequency of LILRB1-expressing K562 cells (right *y*-axis) stained with thermal-exchange HLA-E tetramers obtained by exchange first (a) or multimerization first (b), and with conventional HLA-E tetramers (c). In all figures dcas9 cells were used as negative staining control with an average staining indicated by the dotted line. (d) s/p ratio represent the binding of the indicated peptides to HLA-E*01:03 as measured by ELISA-binding assay.

FIGURE 2 Thermal-exchange HLA-E tetramers can stain TCR-expressing cells. (a and b) Schematic representations of the thermal peptide exchange before (a) or after (b) multimerization. (c) Representative staining of TCR-expressing Jurkat cells with conventional, exchange first and multimerization first thermal-exchange HLA-E tetramers are shown for the specific (pCMV-LIL) and an unspecific (pCMV-LLL) peptide, in both total lymphocyte and TCR-expressing populations as indicated. (d–f) Bar plots represent the frequency of TCR-expressing cells which were stained with thermal-exchange HLA-E tetramers obtained through multimerization vs. exchange first approaches (f), different peptide exchange temperatures and incubation times (d), and FACS staining dilutions (e), compared to conventional tetramers (cTM).

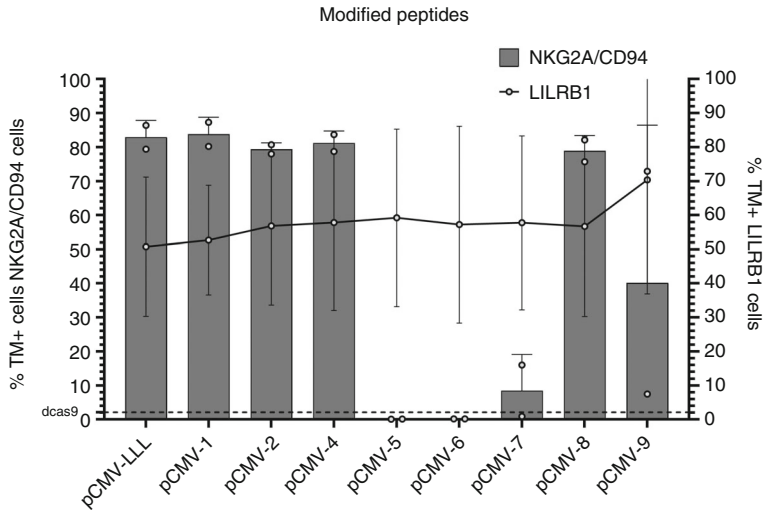

FIGURE 4 Alanine substitutions in pCMV-LLL. Bar plots represent the frequency of NKG2A/CD94-expressing K562 cells (left *y*-axis) and circles and lines represent the frequency of LILRB1-expressing K562 cells (right *y*-axis) stained with thermal-exchange HLA-E tetramers loaded with alanine substitutions of pCMV-LLL peptide as indicated on the *x*-axis. dcas9 cells were used as negative staining control with an average staining indicated by the dotted line.

Thermal-exchange HLA-E tetramers can be used to explore molecular requirements for the NKG2A/CD94 recognition of HLA-E-binding peptides

Previous studies have shown that NKG2A/CD94 recognition of HLA-E-binding peptides is governed by primary contacts with an arginine residue in position 5, as well as a hydrophobic residue in position 8 [33,34]. Moreover, residues present in positions 6 and 7 have been shown to have a measurable effect on NKG2A/CD94 recognition of HLA-E-binding peptides [35]. To further explore the molecular requirement for NKG2A/CD94 recognition of HLA-E-binding peptides we produced thermal-exchange HLA-E tetramers loaded with pCMV-LLL-derived peptides having alanine substitutions. This replacement is expected to reduce recognition by the receptor when substituting relevant residues for this interaction with the small and chemically inert alanine residue. In line with previous findings, our results indicate that NKG2A/CD94 recognition of HLA-E-binding peptides is lost when the arginine in position 5 or the threonine at position 6 is removed (Figure 4). In addition, NKG2A/CD94 interaction with HLA-E-binding peptides is influenced by the peptide residues in positions 7 and 9, as shown by the loss of multimer binding, while substitution of residue in position 8 for an alanine had no effect, suggesting higher flexibility for the residues present at this position (Figure 4). Altogether these results demonstrate the value of the thermal-exchange tetramers as a rapid and straightforward tool to investigate the recognition of HLA-E-binding peptides and suggest new insights into the molecular requirements for NKG2A/CD94 receptor binding.

DISCUSSION

The increasing knowledge of HLA-E-binding peptides and their role in the activation [7,8,27,36] and modulation [32,37] of immune responses against infections and tumours calls for the development of multimer technologies based on peptide exchange. Recently, several studies have described a novel approach to produce peptide-loaded HLA-E monomers through UV exchange which could be used to measure peptide HLA-E interactions through ELISA-binding assay or to stain specific HLA-E-restricted T cells through flow cytometry [15,22–24]. Alternatively, the use of temperature to induce peptide exchange has the advantage of avoiding possible damage to the proteins and fluorescent labels which could be induced by exposure to UV light or chemicals [13,25]. In this study, we demonstrate an alternative approach using thermal exchange for the generation of functional HLA-E tetramers which we have shown can be used to interrogate HLA-E/peptide interactions with receptors such as TCR and NKG2A/CD94. For the generation of thermal-exchange tetramers, we refolded HLA-E*01:03 and HLA-E*01:01 monomers at low temperature in the presence of binding template peptide VLRPGGHFAA which loses binding to HLA-E once the temperature is increased, allowing for alternative peptides to occupy the available peptide binding groove. In addition, we demonstrated that thermal-exchange HLA-E tetramers can be stored at −20°C or −80°C for at least 3 months without detriment to their staining capacity. On the other hand, the disadvantage of the thermal-exchange HLA-E monomer is the relatively low yield of refolded monomers which could be obtained in the initial refolding step in the presence of a thermal-sensitive peptide, due to its relatively low HLA-E

binding affinity. Nevertheless, the quantity of produced monomer was sufficient to perform multiple thermal-mediated peptide exchanges with each batch. Our efforts to further optimize the use of thermal-sensitive HLA-E multimers instead of monomers resulted in an adequate peptide exchange only when the alternative peptide is a high HLA-E binder. Therefore, we suggest to perform the thermal peptide exchange on HLA-E monomers before multimerization when the goal is to obtain tetramers for screening the recognition of HLA-E-binding peptides and, if the peptides of interest obtained from this screen are good HLA-E binders, the thermal peptide exchange on HLA-E multimers could be envisaged to produce a larger batch of tetramers. Nevertheless, proper peptide exchange on HLA-E multimers should always be confirmed by analysing the conformational integrity of the HLA-E/peptide complex by staining of LILRB1-expressing cells, by ELISA-binding assay or through biochemical assays such as peptide elution in combination with mass spectrometry, especially in the case of peptides with known low binding affinity to HLA-E [15,38].

Tetramer libraries have already been used in chaperone-mediated peptide exchange [39] or in vitro transcription and translation [40] and would be a beneficial improvement for HLA-E tetramers as well. Indeed, recent advances in recombinant 68-1 RhCMV-vectored vaccines have shown the induction of broad and diverse MHC-E-restricted T-cell responses despite the restricted polymorphism of MHC-E presenting molecules, encouraging the study of MHC-E-restricted T-cell repertoires in more detail [9]. More recently, the in vitro expansion of HLA-E-restricted RL9HIV-specific $CD8^+$ T cells failed to identify a dominant TCR sequence further supporting the need to perform more extensive HLA-E-restricted T-cell repertoire profiling with advanced tetramer technologies [41]. In addition to its relevance for infectious diseases, the study of peptides presented by HLA-E, an antigen-presenting molecule whose expression, contrary to classical HLA molecules, has been shown to be maintained in the context of certain malignancies [32], could benefit from such a tool for the identification of neoepitopes and other relevant peptides to target protective immune responses against cancerous cells or tissues. The thermal-exchange HLA-E multimers we describe here can be of value to interrogate in greater detail HLA-E-restricted TCR repertoires and phenotypes in several diseases and to further understand the relevance of this cell population in immune protection.

In addition to TCR, NKG2A/CD94 is a major receptor involved in the recognition of HLA-E-presented self-peptides, an interaction which leads to the inhibition of NK cell-mediated lysis [42]. NKG2A/CD94 recognition of HLA-E-presented peptides has been shown to be selective and dependent on primary contacts with arginine in peptide position 5 and hydrophobic amino acids in position 8 [33,34]. Our results of NKG2A/CD94 staining with thermal-exchange HLA-E tetramers loaded with an alanine substitution at position 5 and 6 of pCMV-LLL peptide confirms that these are relevant positions for NKG2A/CD94 recognition of HLA-E-binding peptides. However, substitution at position 8 led to no changes in NKG2A/CD94 binding, suggesting that this position is more flexible than previously thought. Surface plasmon resonance measurements additionally suggested that peptide positions 6 and 7 have an effect on NKG2A/CD94 recognition of HLA-E-binding peptides [35]. In line with this, we observed that substitutions at positions 6 and 7 led to the loss of NKG2A/CD94 recognition. Additionally, with the use of our thermal-exchange HLA-E tetramers, we identified position 9 as an additional important player for NKG2A/CD94 recognition of HLA-E-binding peptides, as its substitution led to reduced tetramer staining. Using a similar strategy, we are currently investigating the molecular requirements for TCR recognition of HLA-E-binding peptides. Importantly, this novel information will be of great value for the identification of novel HLA-E epitopes which could be considered for vaccine development efforts [43].

We have developed and optimized a new technology for the production of HLA-E tetramers through thermal-mediated peptide exchange. We have shown that this novel technology can be used to interrogate the sequence-specific requirements driving the interactions with relevant receptors such as TCR and NKG2A/CD94, which could help identify HLA-E-binding peptides based on this. Finally, we highlight new insights on the peptide sequence requirement for interaction with NKG2A/CD94. Altogether, this novel tool and these results will be of relevance in the future study of HLA-E-restricted T-cell responses and the identification of novel pathogen- or self-derived peptides in the field of vaccine design [2].

AUTHOR CONTRIBUTIONS

Paula Ruibal, Ian Derksen, Marjolein van Wolfswinkel, Linda Voogd, Tom A. W. Schoufour and Ruud H. Wijdeven performed the experiments and analysed the data. Kees L. M. C. Franken and Angela F. El Hebieshy provided technical support. Paula Ruibal wrote the original manuscript, Thorbald van Hall, Tom H. M. Ottenhoff, Ferenc A. Scheeren and Simone A. Joosten edited and commented on the manuscript. All authors have read and approved the manuscript. Ferenc A. Scheeren and Simone A. Joosten contributed equally to this work.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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SUPPORTING INFORMATION

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