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Immune responses to citrullinated and homocitrullinated peptides in healthy donors are not restricted to the HLA SE shared allele and can be selected into the memory pool

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Abstract

Citrullination and homocitrullination are stress induced post-translational modifications (siPTMs) which can be recognized by T cells. Peripheral blood mononuclear cells isolated from healthy donors and rheumatoid arthritis (RA) patients were stimulated with nine siPTM-peptides. CD45RA/CD45RO depletion was employed to determine if peptide-specific responses are naïve or memory. Human leucocyte antigen (HLA)-DP4 and HLA-DR4 transgenic mice were immunized with siPTM-peptides and immune responses were determined with ex vivo ELISpot assays. The majority (24 out of 25) of healthy donors showed CD4 T cell-specific proliferation to at least 1 siPTM-peptide, 19 to 2 siPTM-peptides, 14 to 3 siPTM-peptides, 9 to 4 siPTM-peptides, 6 to 5 siPTM-peptides and 4 to 6 siPTM-peptides. More donors responded to Vim28-49cit (68%) and Bip189-208cit (75%) compared with Vim415-433cit (33%). In RA patients, the presentation of citrullinated epitopes is associated with HLA-SE alleles; however, we witnessed responses in healthy donors who did not express the SE allele. The majority of responding T cells were effector memory cells with a Th1/cytotoxic phenotype. Responses to Vim28-49cit and Eno241-260cit originated in the memory pool, while the response to Vim415-433cit was naïve. In the HLA-DP4 and HLA-DR4 transgenic models, Vim28cit generated a memory response. Peptide-specific T cells were capable of Epstein–Barr virus transformed lymphoblastoid cell line recognition suggesting a link with stress due to infection. These results suggest

Abbreviations: ACPA, anti-citrullinated protein antibody; APCs, antigen presenting cells; AS, acid stripped; ATCC, American Type Culture Collection; CFSE, carboxyfluorescein succinimidyl ester; CM, central memory; EM, effector memory; FCS, fetal calf serum; LCL, lymphoblastoid cell line; LPS, lipopolysaccharide; MPO, myeloperoxidase; PADs, peptidylarginine deiminases; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; S/S, serum starved; siPTMs, stress induced post-translational modifications; TEMRA, terminally differentiated population of effector memory cells.

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siPTM-peptides are presented under conditions of cellular stress and inflammation and drive cytotoxic CD4 T cell responses that aid in the removal of stressed cells. The presentation of such siPTM-peptides is not restricted to HLA-SE in both humans and animal models.

KEYWORDS
citrullination, HLA SE allele, homocitrullination, memory response, stress

INTRODUCTION

T cells undergo thymic education to distinguish between self and non-self in order to mount an immune response to foreign antigens without harming the host. Tolerance to self-antigens is achieved by two mechanisms: central tolerance, through deletion of autoreactive lymphocytes during maturation in the central lymphoid organs and, peripheral tolerance, by functional suppression of autoreactive lymphocytes that have escaped elimination, in the periphery [1]. T cells developing in the thymus are subjected to 'positive' selection, in which cells binding to major histocompatibility complex (MHC) molecules with moderate affinity survive, and 'negative' selection, where high avidity T cells to self-antigens undergo apoptosis [1]. As a result, some low-affinity self-reactive T cells escape clonal deletion, as evidenced by the presence of self-specific T lymphocytes in the periphery [2].

Citrullination involves the conversion of the positively charged aldimine group of arginine to the neutrally charged ketone group of citrulline. Citrullination is mediated by peptidylarginine deiminases (PADs), a family of calcium-dependent enzymes found in a variety of tissues. This modification is thought to occur during times of cellular stress and/or apoptosis leading to the precipitation of proteins and stimulation of both CD4 and antibody responses that are the hallmarks of autoimmune diseases such as rheumatoid arthritis (RA). Citrullination can occur as a result of protein degradation and recycling during autophagy, a process that is induced in stressed cells and during inflammation which also induces MHC-II expression [3]. Ireland and Unanue demonstrated that citrullinated peptide epitopes can be presented on MHC-II via an autophagy and PAD dependent mechanism in both antigen-presenting cells (APCs) and epithelial cells [3]. Autophagy is constitutive in APCs but minimal in other cells, where it is only induced by stress such as hypoxia and nutrient starvation [4].

Another post-translational modification also shown to be detected during autoimmune diseases is homocitrullination, modification involving the addition of isocyanic acid to free functional protein groups resulting in the conversion of lysine to homocitrulline [5]. Isocyanic acid can originate from the spontaneous decomposition of urea or from thiocyanate catalysed by myeloperoxidase [5], a component of neutrophil extracellular traps that are evident during inflammation [6]. Furthermore, increased protein homocitrullination is associated with oxidative stress and inflammation in ageing individuals [7]. Similar to citrulline, autoantibodies to homocitrulline have been detected in RA patients suggesting a potential link between these modified peptides and the pathogenesis of RA [8].

CD4 T cells specific for PTM proteins play a major role in the pathogenesis of several autoimmune diseases, including RA [9–11]. CD4 T cells specific for citrullinated peptides in RA patients escape tolerance and, in combination with anti-citrullinated protein antibody (ACPA) drive the pathogenesis of the disease [12]. In RA, the presentation of citrullinated epitopes is largely associated with SE alleles such as human leucocyte antigen (HLA)-DR*0401 and HLA-DR*0101 [13–15]. However, if citrullination is a normal stress response, it cannot be restricted to only HLA-DR4^{+ve} and HLA-DR1^{+ve} individuals. We, and others, have previously demonstrated that citrullinated peptides can bind to both HLA-DP4 [16] and HLA-DQ alleles [17] implying a wider HLA presentation profile for citrullinated and homocitrullinated peptides [18, 19].

In mouse models, vaccination in combination with Toll-Like receptor (TLR) ligands induces potent cytotoxic CD4 T cells [16, 20]. Here we show similar responses are evident in healthy human donors. CD4 responses to citrullinated and homocitrullinated peptides can be detected in healthy donors which are not restricted to the HLA-SE shared allele and can be found in the memory pool.

METHODS

Peptides

Enolase aa241-260wt (VIGMDVAASEFFRSGKYDLD), enolase aa241-260cit (VIGMDVAASEFFcitSGKYDLD), vimentin aa28-49cit (cit-SYVTTST-cit-TYSLGSAL-cit-PSTS), vimentin aa415-433cit (LPNFSSLNLcitETNLDSLPL), binding immunoglobulin protein aa189-208wt (TIAGLNVMRIINEPTAAAIA), binding immunoglobulin protein aa189-208cit (TIAGLNVM-cit-IINEPTAAAIA), nucleophosmin aa266-285cit (AKFINYVKNCFCitMTDQEAIQ), cytokeratin

8 aa101-120cit (KFASFIDKV-cit-FLEQQNKMLE), aldolase aa74-93wt (IGGVILFHETLYQKADDGRP), aldolase aa74-93hcit (IGGVILFHETLYQhcitADDGRP), aldolase aa140-157hcit (hcitDGADFAhcitWRCVLhcitIGEH), vimentin aa116-135wt (NYIDKVRFLEQQNKILLAEL), vimentin aa116-135hcit (NYIDhcitVRFLEQQNhcitILLAEL) and Hepatitis B aa181-193 (GFFLLTRILTIPQ) were synthesized by Genscript at >95% purity and stored in lyophilized form at −80°C. Peptides were dissolved in *N*,*N*-dimethylformamide (227056, Sigma) and made into a 10% solution with dH_2O (10977, Invitrogen) at a final concentration of 1 mg/mL.

PBMC isolation, CD25 depletion, CD45RA$^+$ and CD45RO$^+$ depletion

Sixty millilitre of blood was collected from 25 healthy donors at Nottingham City Hospital. All donors provided a written and informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated and depleted of CD25 T cells as previously described [21]. Naive T cells (CD45RA^{+ve}) or memory T cells (CD45RO^{+ve}) were depleted with 90%–99% purity using Miltenyi MicroBeads (Miltenyi, 130-046-901 and 130-045-901) in accordance with the manufacturer's instructions. CD14 cells were positively isolated (Miltenyi, 130-050-201) from PBMCs and then reintroduced to the PBMCs following CD45RO depletion as these cells express CD45RO.

About 40–60 mL of blood was also obtained from 18 RA patients from different sites. Written and informed consent was obtained from all patients, ethical approval was obtained from the South West – Cornwall & Plymouth Research Ethics Committee (IRAS 194833). PBMCs were isolated and depleted of various T cell sub-sets as described above.

CFSE proliferation assay and restimulation assay

Following depletion of target cells, PBMCs were carboxyfluorescein succinimidyl ester (CFSE) loaded and stimulated with antigen as previously described [16, 20]. Briefly, CD25-depleted PBMCs were loaded with CellTrace, CFSE cell proliferation kit (C34554, ThermoFisher Scientific). CFSE was dissolved in 18 µL of dimethyl sulphoxide then diluted with phosphate-buffered saline (PBS) 1:100. For CFSE loading, 100 µL of CFSE solution was added to 1–5 × 10^7 PBMCs resuspended in 1 mL loading buffer (PBS containing 5% fetal calf serum [FCS]). PBMCs were plated out at 2 × 10^6 per well in 2 mL RPMI (supplemented with 10% autologous serum) in a 24-well plate and stimulated with peptides at 10 µg/mL. Donor cells were challenged with as many individual peptides as the final PBMCs yield allowed [21]. Phytohaemagglutinin and vehicle were used as positive and negative controls, respectively. Data are presented as proliferation ratio, where percentage of proliferating cells to peptides was divided by the percentage of proliferating cells to unstimulated control. A donor's response to a peptide was considered significant if proliferation was twice that seen in vehicle and greater than 1%.

PBMCs from healthy donors were also stimulated with 10 µg/mL peptides without CFSE loading. On Days 9–11, the cells were restimulated with citrullinated peptides or wild type peptides with/without anti-pan HLA class I, anti-DR, anti-DP and anti-DQ antibodies (see Table S1). The responses were measured using an ELISpot assay with human IFNγ capture and detection reagents according to manufacturer's instructions (3420-2A, MABTECH).

Flow cytometry staining

CD25, CD14, CD45RA and CD45RO depletion was confirmed by staining approximately 1 × 10^5 cells with anti-CD4 eFluo 450, anti-CD25-PE, anti-CD14-VioGreen, anti-CD45RA-VioGreen and anti-CD45RO-VioGreen antibodies (Table S1). Immunostaining for activation and cytotoxic markers was carried out using anti-CD134-PEcy7, anti-IFNy-APCe780 and anti-GranB-PE antibodies (Table S1). For phenotyping proliferating and non-proliferating PBMCs, CD45RA-VioGreen and CCR7-PE-vio-770 were used (Table S1). PBMCs were stained as described previously [22]. Samples were assayed on a MACSQuant 10 flow cytometer equipped with MACSQuant software version 2.8.168.16380. Unstained samples were used for gating strategies.

Sorting and TCR analysis

To analyse the specificity of CD4 T cell expansion in response to siPTM peptides, bulk sorted proliferating (CFSE low) and non-proliferating (CFSE high) CD4 cells were sent to iRepertoire Inc. (Huntsville, AL, USA) for Next generation sequencing of the T-cell receptor (TCR; α and β chains) as described previously [16]. Briefly, RNA was isolated and reverse transcribed from both groups of sorted cells. Amplicon rescue multiplex polymerase chain reaction was carried out with cDNA using human TCRα and β 250 PER primers patent (nos. 7,999,092 and 9,012,148B2; iRepertoire Inc.). The PCR products were sequenced using Illumina MiSeq platform (Illumina, San Diego, CA, USA). IRweb software (iRepertoire) was used to analyse raw data table. CDR3 of α and β chains was sequenced, analysed and plotted in a diversity graph showing the total and unique numbers of TCRs. IRweb tools

also produced a diversity index score (D50); a measure of diversity of the TCR of the CD4 population. The lower the D50 score, the more clonal a population is, a score of 50 is highly diverse.

Cell lines and culture

B16F1 cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI medium 1640 (GIBCO/BRL) supplemented with 10% FCS, L-glutamine (2 mM) and sodium bicarbonate buffered. B16F1 cell lines were engineered and cultured as described previously [23]. PER 255 EBV transformed lymphoblastoid cell line (LCL; HLA type: HLA-A: 2,24, HLA-B: 27,35, HLA-DR: 4,53 and HLA-DQ: 8) was a kind gift from Prof. Alan Rickinson & Dr Graham Taylor, University of Birmingham. PER 255 LCL was cultured in RPMI medium 1640 (ThermoFisher Scientific) supplemented with 10% FCS.

Immunization protocol

HLA-DR4 mice (Model #4149, Taconic) and HHDII/HLA-DP4 mice (EM:02221, European Mouse Mutant Archive) aged between 8 and 12 weeks old were used. All work was carried out under a Home Office approved project licence. For all the studies, mice were randomized into different groups and not blinded to the investigators. Peptides were dissolved in PBS and then mixed with adjuvant and delivered at a 25 μg dose subcutaneously at the base of the tail, unless stated otherwise. CpG ODN 1826 (TLR9; InvivoGen) and MPLA (TLR4; InvivoGen) at 5 μg were used as the adjuvant. Mice were immunized with Eno241cit, Vim28cit or Vim415cit three times on Days 1, 8 and 15, and spleens removed for analysis on Day 21. Groups of $n = 3$ selected through statistical power calculations were used on independent occasions.

Groups of mice were also immunized on Days 0, 7 and 14, the spleens were removed and splenocytes were harvested for analysis at Day 20 unless stated otherwise. For memory response analysis, mice were immunized with a single immunization on Day 0 or Day 11 and spleens were removed for analysis on Day 13.

Ex vivo ELISpot assay

ELISpot assays were carried out using murine IFNγ capture and detection reagents (MABTECH, #3321-2A) according to the manufacturer's instructions. Briefly, IFNγ specific capture antibodies at 10 μg/mL were coated onto wells of 96-well Immobilin-P plate and quadruplicate wells were seeded with 5×10^5 splenocytes. Synthetic peptides at final concentration of 10 μg/mL were added to appropriate wells. For serum starvation experiments, B16F1 DR4 and T2 DR4 cells were acid stripped (AS) (treated with 500 μL cold citric acid pH 2) followed by incubation at 4°C for 2 min, washed and then resuspended in RPMI (without serum) and cultured overnight at 37°C, 5% CO_2. B16F1, B16F1 acid stripped and serum starved (AS and S/S), B16F1 DR4, B16F1 DR4 AS and S/S, T2 DR4, T2 DR4 AS and S/S and PER 255 LCL were added at 5×10^5 with splenocytes in appropriate wells. Lipopolysaccharide at 5 μg/mL was used as a positive control. Following incubation for 40 h at 37°C, 5% CO_2 captured antibody was detected by a biotinylated anti-IFNγ antibody at 1 μg/mL and development with a streptavidin alkaline phosphatase (1/1000) and chromogenic substrate. Spots were analysed and counted using an automated plate reader (CTL Europe GmbH, Aalen, Germany).

Western blot

B16F1, T2 DR4 and PER 255 LCL cell lines were lysed with RIPA buffer supplemented with 1% of protease and phosphatase inhibitors cocktail (Thermo Scientific™, 78440). Twenty microgram reduced protein samples were loaded on a precast polyacrylamide gel (Invitrogen, NW04125BOX) and run at 130 V for 50 min followed by a transfer to nitrocellulose membranes at 30 V for 90 min. After blocking with 5% non-fat milk the membrane was incubated with relevant antibodies (Table S1) overnight at 4°C on a roller. Following 3× wash the membrane was incubated with secondary antibodies (Table S1) for 60 min in the dark at room temperature. The membranes were then washed and analysed using LI-COR ODYSSEY scanner.

Statistics

Statistical analyses were carried out using GraphPad Prism software version 8. The Wilcoxon test was used for the comparison of peptide responses between CD25 depleted and non-depleted PBMCs and for assessing the difference in cytokine expression between proliferating and non-proliferating cells. The Mann–Whitney test was used to assess the frequency of responses to the peptides between RA patients and healthy donors. The Kruskal–Wallis test was used to assess ratio of granzyme B to IFNγ responses between peptides. Diversity index of TCRα and TCRβ chain between CFSE high and CFSE low groups were assessed using the Wilcoxon test. Ex vivo IFNy responses between control and various groups were compared by the Wilcoxon test.

RESULTS

CD4 repertoire to citrullinated peptides exists in healthy donors that is subject to T cell regulatory control

Citrullination is a common modification known to be prevalent in autoimmune disease and cancer. We have previously identified and screened peptides from cellular proteins that are known to be citrullinated or homocitrullinated for CD4 T cell responses by vaccination in combination with TLR ligands in mouse models [16, 18, 20–23]. Peptides that induced potent CD4 T cells responses were further investigated in this paper. An earlier study also showed that depletion of CD25 T cells can enhance antigen specific T cell responses [24]. To determine if responses to citrullinated peptides could be detected in healthy donors, we assessed the CD4 T cell proliferative response with or without CD25 T cell depletion. There was a significant ($p = 0.0313$) increase in response to the citrullinated peptides following CD25 depletion (Figure 1a). Initially, the donors BD76 and BD08 did not show responses to Eno241cit (ratio of vehicle to peptide stimulated of 1) and Vim28cit (ratio of 0.8) peptides, however, upon CD25 depletion CD4 proliferative responses (ratio of 25.92 and 17.9) were observed (Figure 1b). Similarly, BD16 and BD26 only showed responses to Vim415cit following CD25 depletion (ratio of 0–91.7 and 1.1–17.9; Figure 1b). Response to Eno241cit peptide was also observed in non-CD25 depleted PBMCs from BD16 (ratio of 3.2; Figure 1b) but after depletion this was enhanced (ratio of 3.9). A similar trend was also observed in response to stimulation with a peptide from Hepatitis B in a Hep B vaccinated donor BD16 (2.1–2.8) [25]. An example of flow cytometric analysis of response with and without CD25 deletion is shown in Figure 1c. Together these data suggest that CD25 T cells can influence the in vitro assessment of CD4 T cell repertoire to citrullinated peptides, which is consistent with a previous report showing depletion of CD25 Treg enhances detection of antigen specific immune responses [24].

Citrullinated and homocitrullinated peptides induce CD4 responses in healthy donors and RA patients

As regulatory T cells can influence the detection of responses to self-antigens [24], we next examined the responses to citrullinated and homocitrullinated peptides following depletion of CD25 T cells to better assess the available T cell repertoire without the influence of Tregs. For this, 25 healthy donors and 18 RA patients were screened for reactivity to 6 citrullinated and 3 homocitrullinated peptides. Of the 25 healthy individuals tested, 24 donors responded to at least one peptide, 19 to 2, 14 to 3, 9 to 4, 6 to 5 and 4 to 6 peptides (Table 1, Figure 2). More donors responded to Vim28-49cit (68%), Bip189-208cit (75%) and Vim116-135hcit (67%) than to Vim415-433cit (33%). Eno241-260cit, NPM266-285cit and Aldo140-157hcit all stimulated a greater than 50% response rate. In RA patients, the presentation of citrullinated epitopes and ACPA is associated with HLA-SE, commonly among subtypes of DRB1*04 and DRB1*01 allele [14, 15]. In this study, 7 of the 25 healthy donors were either DRB1*04 or DRB1*01, and hence the responses to siPTMs in these individuals were also compared against the full cohort. Healthy donors responded to significantly more peptides ($p < 0.0001$) compared with those with the DRB1*04 or DRB1*01 allele (Table S2). There were multiple T cell responses to citrullinated peptides in donors who lacked an HLA-SE allele, demonstrating that other HLA types can present siPTM peptides. Screening RA patients with the same peptides showed that only 8 out of 18 responded to at least 1 peptide, 5 to 3 peptides and 2 to 7 peptides. The frequency of responses to siPTM peptides between healthy donors and RA patients was compared, the response to Vim415cit was similar in the two groups 33% versus 28%, but there was a significantly lower frequency of responses in RA patients to Eno241cit (52% vs. 11%, $p = 0.0088$), Vim28cit (64% vs. 22%, $p = 0.0124$) and all peptides (54.5% vs. 20.83%, $p < 0.0001$; Figure 1b) which may be related to their biologic treatment (Table S3) [9].

Restimulation responses to citrullinated peptides in healthy donors is citrulline specific and is not restricted to HLA-SE alleles

Our previous work demonstrated healthy donors' responses to citrullinated and homocitrullinated peptides but not to their respective wild type versions [18, 20–22]. We next investigated if the expanded CD4 T cells were specific only to the modification. Following an initial culture of PBMCs isolated from BD07 in Eno241-260cit PBMCs were restimulated with either the same peptide or its wild type equivalent. In an ELISpot assay IFNγ release was only observed in response to re-stimulation with the Eno241-260cit peptide and not to the wild type equivalent indicating that these T cells were citrulline-specific (Figure 3a). The assay was also repeated for Eno241-260cit and Bip189-208cit in donor BD20 and similar results were observed. Since BD07 and BD20 lack the

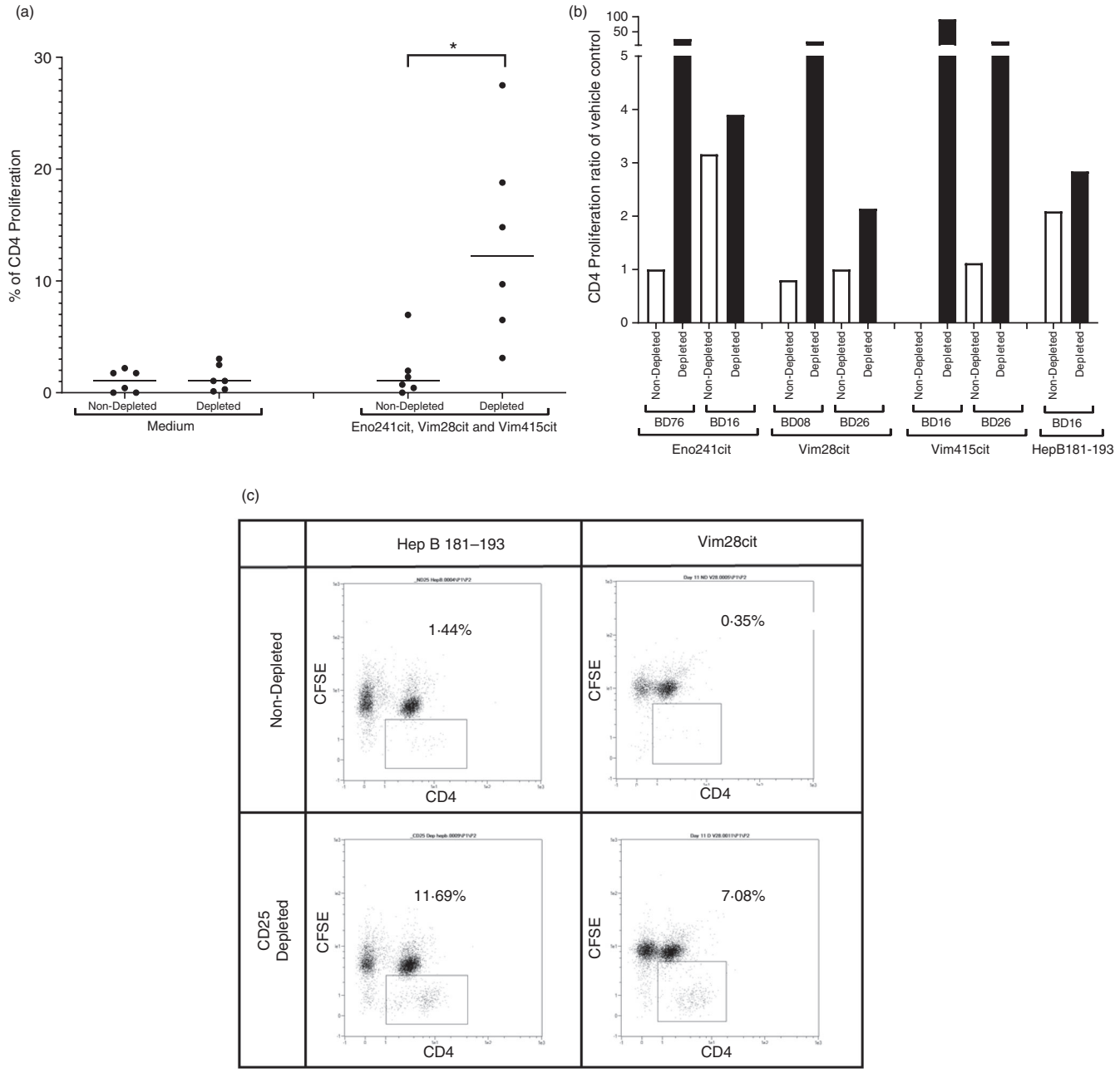

FIGURE 1 CD4 responses to citrullinated peptides in healthy donors are subject T regulatory control. Responses were measured between Days 7 and 10. (a) Proliferation to vehicle control and to citrullinated peptides with/without CD25 depletion. $n = 6$. (b) Bar chart shows proliferation ratio with control. Eno241cit, Vim28cit and Vim415cit were tested in two donors each, Hep B 181–193 peptide was tested in one donor. (c) Example staining of CD4 response characterization to Hep B 181–193 and Vim28cit with and without CD25 depletion in healthy donor BD0016.

HLA-SE alleles (Table S2) the suggestion is that Eno241-260cit is presented via HLA alleles that are not associated with the specific HLA-SE alleles. BD07 is DRB1*13:02 which is known to contain the protective sequence DERAA that allows binding and presentation of the arginine and citrulline-containing epitopes [14].

Donor BD16 showed responses to modified peptides Bip189-208cit and Vim116-135hcit while BD02 showed responses to Aldo74-93hcit, indicating expansion of citrulline and homocitrulline-specific CD4 T cells (Figure 3a).

In blocking experiments, IFNγ secretion from the Eno241-260cit specific response was blocked with an anti-DR antibody but not with an isotype control, anti-pan class I, anti-DQ or anti-DP antibody, indicating the peptide is presented by DR (Figure 3b). Since the donor

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TABLE 1 Responses to all peptides in (a) healthy donors and (b) RA patients.

(a) Healthy donors

BD	Eno241cit	Vim28cit	Vim415cit	Bip189cit	Npm266cit	Cyk8 (101)cit	A74hcit	A140hcit	Vim116hcit
1	x	√	x	x	√	x	x	x	x
2	√	√	x	√	√	x			
3	x	x	x	√	x	x			
7	√	√	x						
8	x	x	√						
11	x	√	√	x		√			
13	√	√	√	√					
14	√	√	x	√	x	√	x	√	√
15	x	x	x	x		x	√	x	x
16	√	√	√	√	√	√		x	√
17	x	x	x	√			x	√	√
18	√	√	x	x		√	x	x	x
19	x	x	x	√	x	x	√	x	x
20	√	√	x						
22	x	x	√	√	√	x	x	√	
24	√	√			√	x			
25	x	x	x						
26	x	√	√						
31	x	x	x	√	x				
38	√	√	√		√	√	√	√	√
41	√	√	x			x	x	x	√
44	x	√	x	√		√	√	√	√
51	√	√	x	√	√	√	√	√	√
76	√	√	√						
95	√	√	x	x	x		√	√	√
Total	13/25 52%)	17/25 (68%)	8/24 (33%)	12/16 (75%)	7/12 (58%)	7/15 (47%)	6/12 (50%)	7/13 (54%)	8/12 (67%)

(b) RA patients

BD	Eno241cit	Vim28cit	Vim415cit	Bip189cit	Npm266cit	Cyk8(101)cit	A74hcit	A140hcit	Vim116hcit
SA1	x	√	√	√	√	√	x	√	√
SA2	√	√	√	√	√	√	√	x	√
SA3	x	x	x	x	x	x	x	x	x
SA4	x	x	x	x	x	x	x	x	x
B2.1	x	x	x	x	x	x	√	√	√
B.2.2	√	x	√	√	x	x	x	x	x
B2.3	x	x	√						
B2.4	x	√	x					x	
B2.5	x	x	x	x	x	x	x	x	x
F1	x	√	x			x			
F2	x	x	x		x	x			

(Continues)

TABLE 1 (Continued)

(b) RA patients									
BD	**Eno241cit**	**Vim28cit**	**Vim415cit**	**Bip189cit**	**Npm266cit**	**Cyk8(101)cit**	**A74hcit**	**A140hcit**	**Vim116hcit**
F3	x	x	x		x	x	x	x	x
F4	x	x	x		x				
F5	x	x	x						
B1	x	x	x	x	x	x	x	x	x
B2	x	x	√	√	x	x	x	√	x
B3	x	x	x				x	x	
B4	x	x	x						
Total	2/18 (11%)	4/18 (22%)	5/18 (28%)	4/9 (44%)	2/12 (16%)	2/12 (16%)	2/11 (18%)	3/12 (25%)	3/10 (30%)

Note: √ denotes response and x denotes no response.

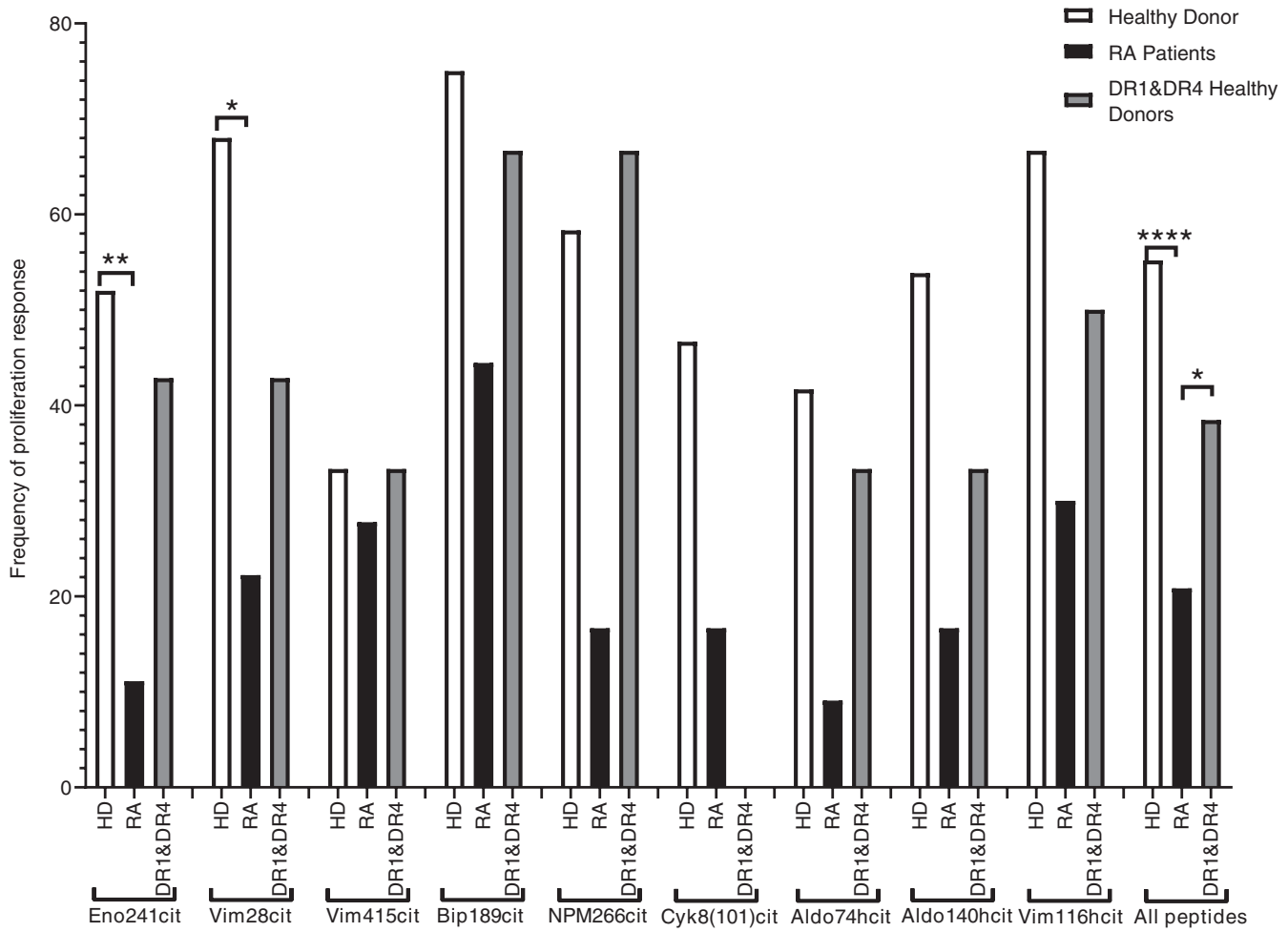
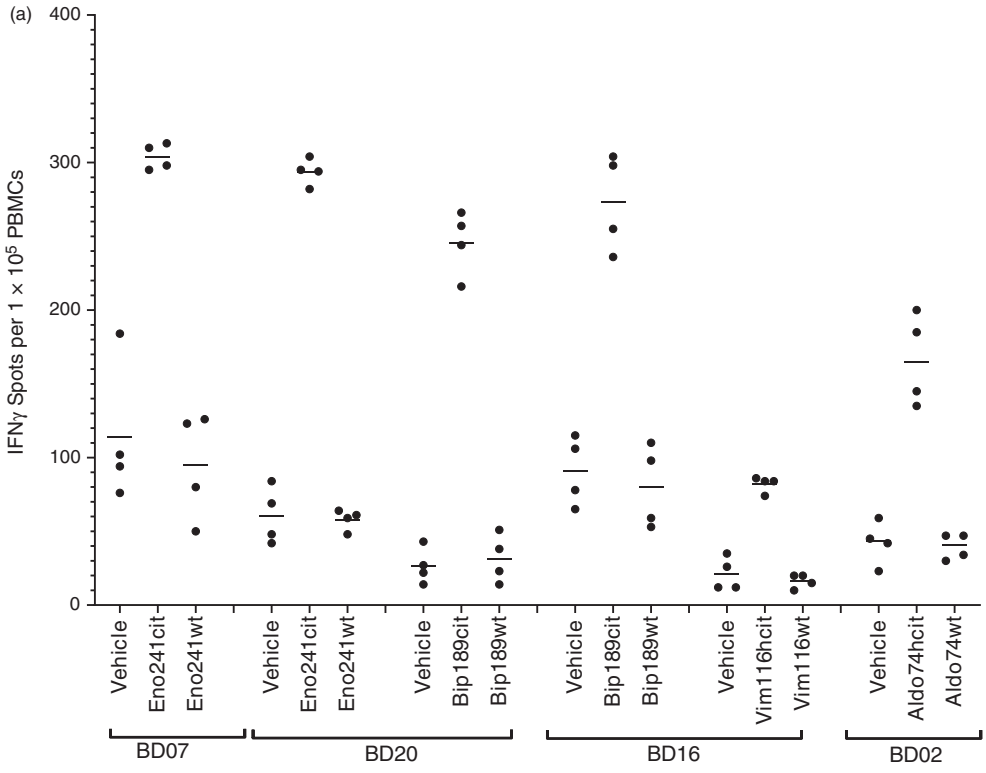


FIGURE 2 Characterization of CD4 responses to citrullinated and homocitrullinated peptides in healthy donors (HD) and rheumatoid arthritis (RA) patients. Responses were measured between Days 7 and 10. Bar chart shows frequency of responses to citrullinated and homocitrullinated peptides among all HD, RA patients and DR1 and DR4 positive HD. HD $n = 25$, RA patients $n = 18$ and, DR1 and DR4 HD $n = 7$.

was vaccinated against Hep B, as an additional control, PBMCs were also cultured with a DP4-restricted Hep B peptide (Hep B 181-193) [20] and re-stimulated with the same peptide. In this instance, IFNγ release was blocked only by the anti-DP antibody (Figure 3b) supporting the validity of this assay.



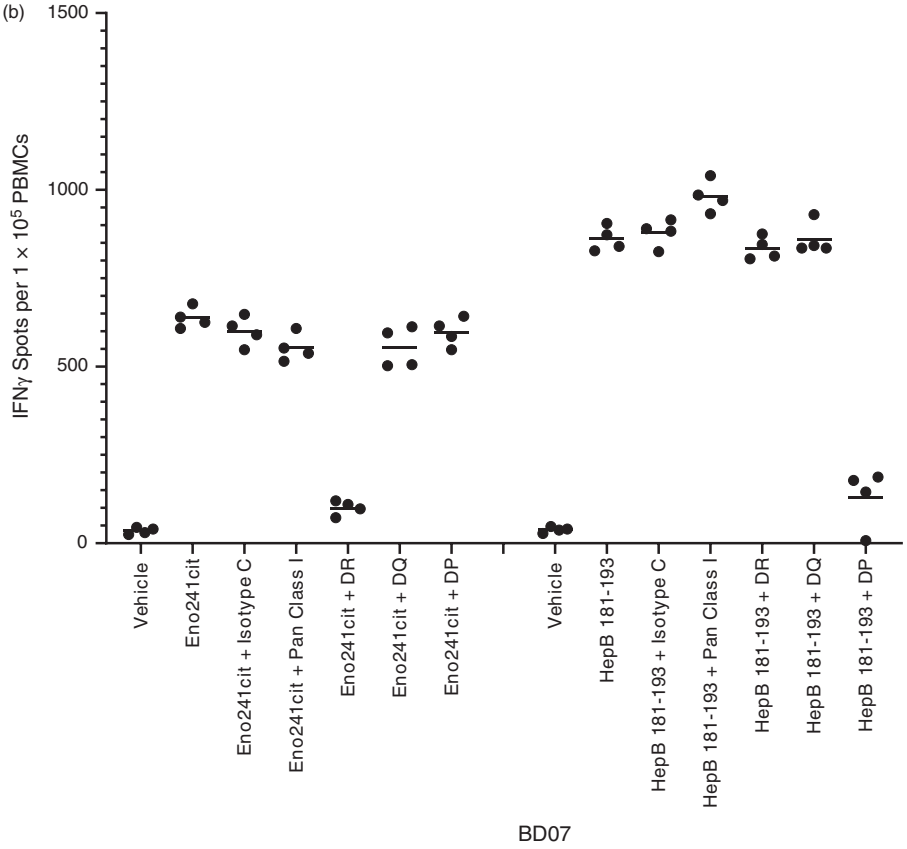


FIGURE 3 Restimulation responses to citrullinated and homocitrullinated peptides in healthy donors are citrulline/homocitrulline specific and are not restricted to HLA-SE alleles. (a) Peripheral blood mononuclear cells (PBMCs) from BD07, BD20, BD16 and BD02 were restimulated with citrullinated, homocitrullinated or wild type peptides following initial stimulation with the respective modified peptides. Immune responses were assessed to Eno241cit and Eno241wt in BD007 and BD20, to Bip189cit and Bip189wt in BD20 and BD16, to Aldo74hcit and Aldo74wt in BD02 and, to Vim116hcit and Vim116wt in BD16. (b) Immune responses to Eno241cit and Eno241wt peptides were assessed with/without human leucocyte antigen blocking antibodies by IFNγ ELISpot at Days 10–12. (B) Hep B 181–193 was used as positive control. Quadruplicate wells were seeded, $n = 1$, mean.

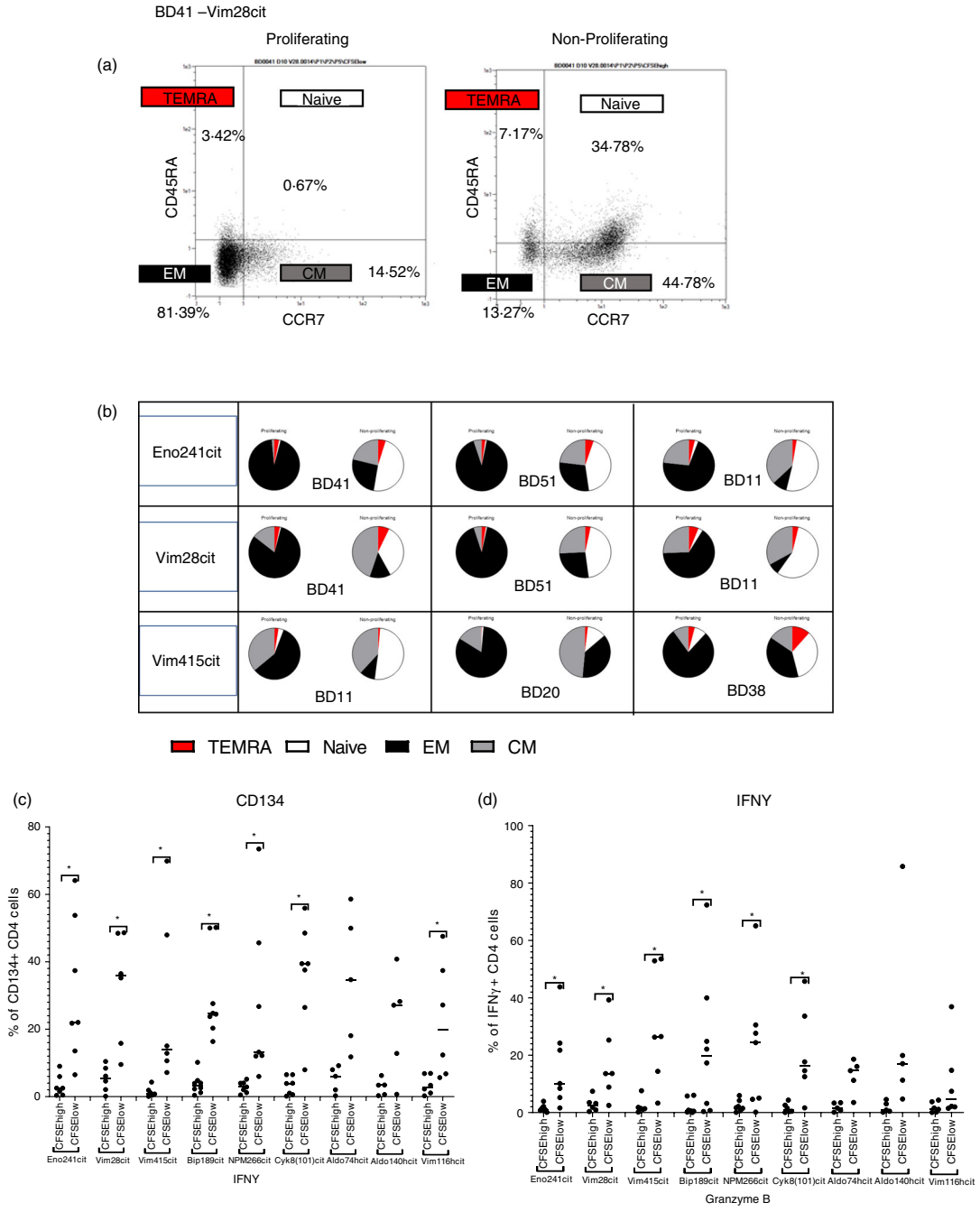


FIGURE 4 Citrulline specific CD4 responses in healthy donors display an effector memory/TEMRA Th1 phenotype. Proliferating and non-proliferating CD4 cells were phenotyped using CCR7 and CD45RA on Days 9 and 10. (a) Example of staining showing phenotype of the proliferating and non-proliferating CD4 T cells in BD41 upon Vim28cit stimulation. (b) Pie charts showing proportion of naïve, central memory, effector memory and TEMRA population in proliferating and non-proliferating CD4 T cells in response to Eno241cit, Vim28cit and Vim415cit stimulation in donors BD0041, BD0011, BD0051, BD0020 and BD0038. Expression of cytokines and cytotoxic markers was assessed on Days 7–10. Comparison of (c) CD134 (d) IFNγ and (e) granzyme B expression in proliferating and non-proliferating cells in response Eno241cit, Vim28cit, Vim415cit, Bip189cit, Npm266cit, Cyk8(101)cit, Aldo74hcit, Aldo140hcit and Vim116hcit peptide stimulation. $n = 5–7$ *$p < 0.05$, median. (F) Example of staining plot showing CD134, IFNγ and granzyme B expression in proliferating and non-proliferating cells. (G) Ratio of granzyme B to IFNγ in response to citrullinated and homocitrullinated peptides. $n = 6–7$. CM, central memory; EM, effector memory; TEMRA, terminally differentiated population of effector memory cells.

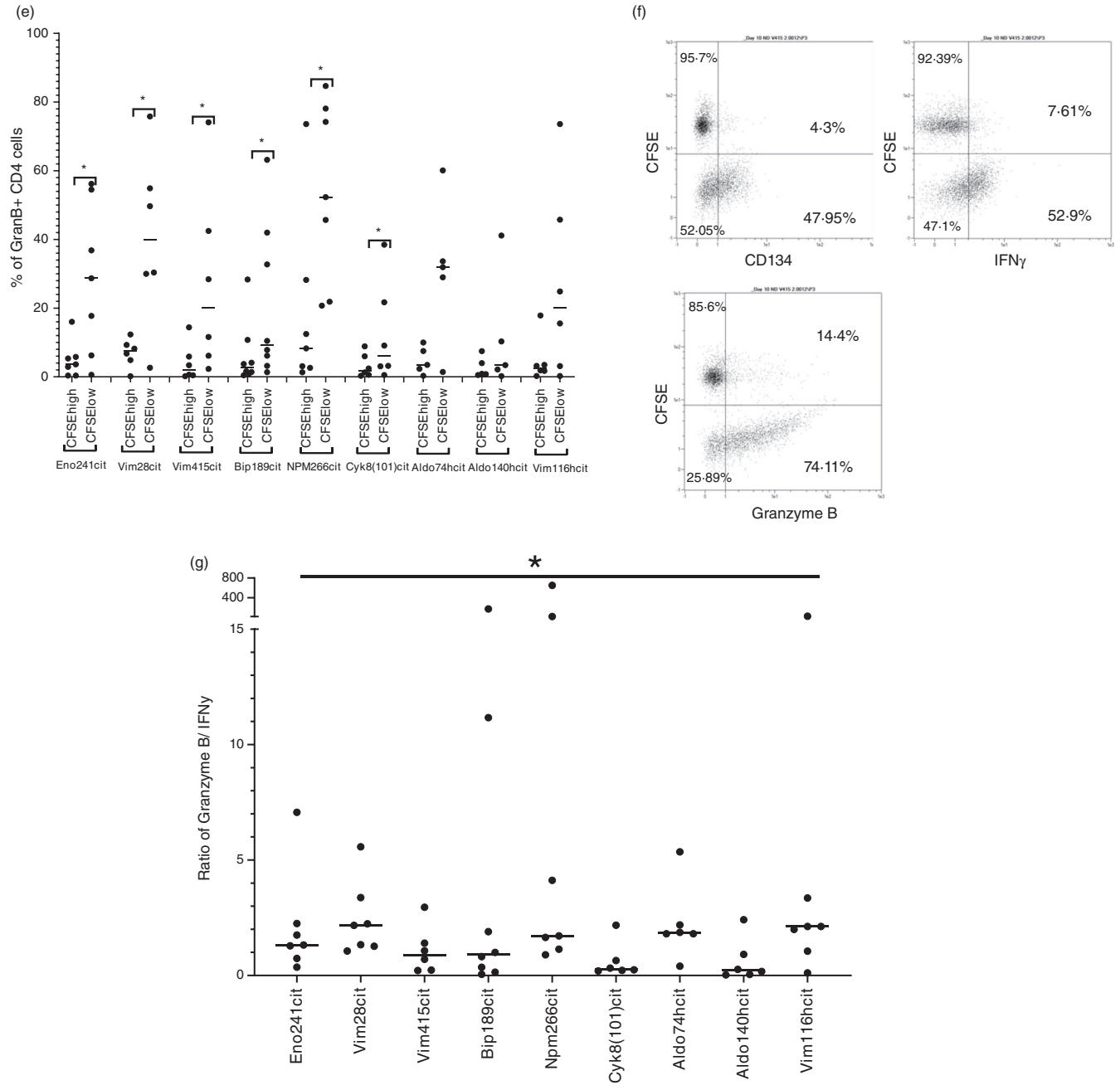


FIGURE 4 (Continued)

Citrulline specific CD4 responses in healthy donors display an effector memory Th1 phenotype

We next sought to phenotype T cells from healthy donors that proliferate in response to different citrullinated peptides. The phenotype of the proliferating and non-proliferating CD4 cells was characterized using CD45RA and CCR7 as markers to distinguish effector memory (EM), central memory (CM), effector memory expressing CD45RA (TEMRA), and naive populations (Figure 4a) [26]. The majority (58.2%–94.2%) of CD4 T cells proliferating in response to Eno241-260cit, Vim28-49cit and Vim415-433cit stimulation were of an EM phenotype ($CD45RA^{-}CCR7^{-}$) whereas 11.9%–51.22% and 15.51%–48.48% of non-proliferating CD4 cells showed a naïve ($CD45RA^{+}CCR7^{+}$) or central memory ($CD45RA^{-}CCR7^{+}$) phenotype, respectively. The terminally differentiated population of TEMRA cells ($CD45RA^{-}CCR7^{+}$) were of similar frequency in proliferating and non-proliferating populations (Figure 4b).

To determine the activation state and cytokine secretion of the CD4 T cells proliferating in response to citrullinated peptides in healthy donors we measured

the expression of CD134, IFNγ and granzyme B. The expression of these markers was analysed in T cells isolated from 5 to 7 donors that showed significant proliferation for each siPTM-peptide investigated. Although variable expression of these markers was observed across donors, a significantly higher proportion of proliferating cells expressed CD134 ($p < 0.05$), IFNγ ($p < 0.05$) and granzyme B ($p < 0.05$) compared with the non-proliferating population in response to Eno241-260cit, Vim28-49cit, Vim415-433cit, Bip189-208cit, NPM266-285cit and Cyk8101-120cit (Figure 4c–e). For T cells responding to homocitrulline peptides, there was also higher expression of IFNγ and granzyme B in proliferating cells compared with non-proliferating cells in response to Aldo74-93hcit, Aldo140-157hcit and Vim116-135hcit (Figure 4de). Generally, higher CD134 expression was observed in proliferating cells compared with non-proliferating cells in response to homocitrulline peptides, although the difference was only significant for Vim116-135hcit (Figure 4c, $p = 0.0313$). Example staining for these markers in donor BD011 is shown in Figure 4f. Variations in the ratio of granzyme B to IFNγ in proliferating CD4 T cells were also observed between different peptides (Figure 4g, $p = 0.0458$). Higher granzyme B:IFNγ ratio for Vim28-49cit, NPM266-285cit, Aldo74-93hcit and Vim116-135hcit suggested that these peptides were inducing the T cells to differentiate to cytotoxic CD4 T cells whereas the ratio was less than 0.5 for Cyk8101-120cit and Aldo140-157hcit suggesting these were more Th1 responses (Figure 4g).

Citrulline specific CD4 responses can be stimulated from both the naïve and memory T cell pools and show an oligoclonal TCR expansion

To investigate whether CD4 responses to siPTM-peptides were naïve or reflective of a possible repertoire of memory cells that had been previously activated in vivo, responses were screened following depletion of different T cell subsets. PBMCs from known responders were stimulated with their respective peptide following depletion of naïve (CD45RA) cells (Figure S1B). The proliferative response to Eno241-260cit in T cells from BD13 and BD38 persisted following CD45RA naïve cell depletion indicating the response originated in the circulating memory pool (Figure 5a). Similarly, CD4 proliferation in response to Vim28-49cit persisted in T cells isolated from BD16, BD51 and BD01 after CD45RA depletion suggesting the presence of a memory response. However, responses to Vim415-433cit in all three donors (BD11, BD22, BD38) were lost following naïve cell depletion indicating these responses originate from a naïve population. These observations were confirmed with CD45RO memory cell depletion (Figure S1C). Since monocytes can potentially act as APCs and express CD45RO they were isolated prior to CD45RO depletion and reintroduced back into the culture system. Under these conditions, the response to Vim415-433cit persisted in T cells isolated from BD22 and BD11, suggesting it was naïve in nature (Figure 5b). To confirm that our experimental approach to this question was valid we repeated our assays using the Hep B 181–193 peptide. As expected, responses in BD01 and BD38 (who were previously immunized for Hepatitis B) persisted after CD45RA depletion indicating a memory response (Figure 5a). An example of staining plots showing the presence of a response to Eno241-260cit in BD38, despite CD45RA depletion and loss of response to Vim415-433cit upon CD45RA depletion, is depicted in Figure 5c,d.

Next, we analysed the TCR repertoire of CD4 T cells that proliferated in response to Eno241-260cit, Vim28-49cit and Vim415-433cit. The TCR repertoire of the siPTM peptide specific proliferating CD4 cells was highly clonal with respect to the non-proliferating population with sequencing showing a much higher diversity in the α and β chains of the non-proliferating cells with respect to the proliferating populations (Figure 5e). Oligoclonal response to Hepatitis B 181–193 peptide and polyclonal response to anti-CD3 antibody stimulation were used as controls as previously demonstrated (data not shown [18]). Overall TRα and TRβ diversity scores were significantly lower in the proliferating CD4 cells compared with non-proliferating CD4 cells in six donors stimulated with citrullinated peptides ($p = 0.0078$; Figure 5f). Of note, in vitro culture of T cells may skew the phenotype and TCR analysis to an extent however due to low frequency citrulline specific T cells and in the absence of citrullinated peptide specific tetramer reagents, the cells were profiled from the whole PBMC population based on proliferation as a marker of response 7–11 days post stimulation. Taken together these data suggest that in healthy donors citrullinated peptides can stimulate pre-existing CD4 populations of cells to proliferate and induce oligoclonal TCR expansion.

HLA transgenic mice show rapidly detectable responses to citrullinated peptide vaccination

We have previously shown that citrullinated peptides stimulate responses in HLA-DR4 and HLA-DP4 transgenic mice [16, 20, 22]. Here HLA-DP4 and HLA-DR4

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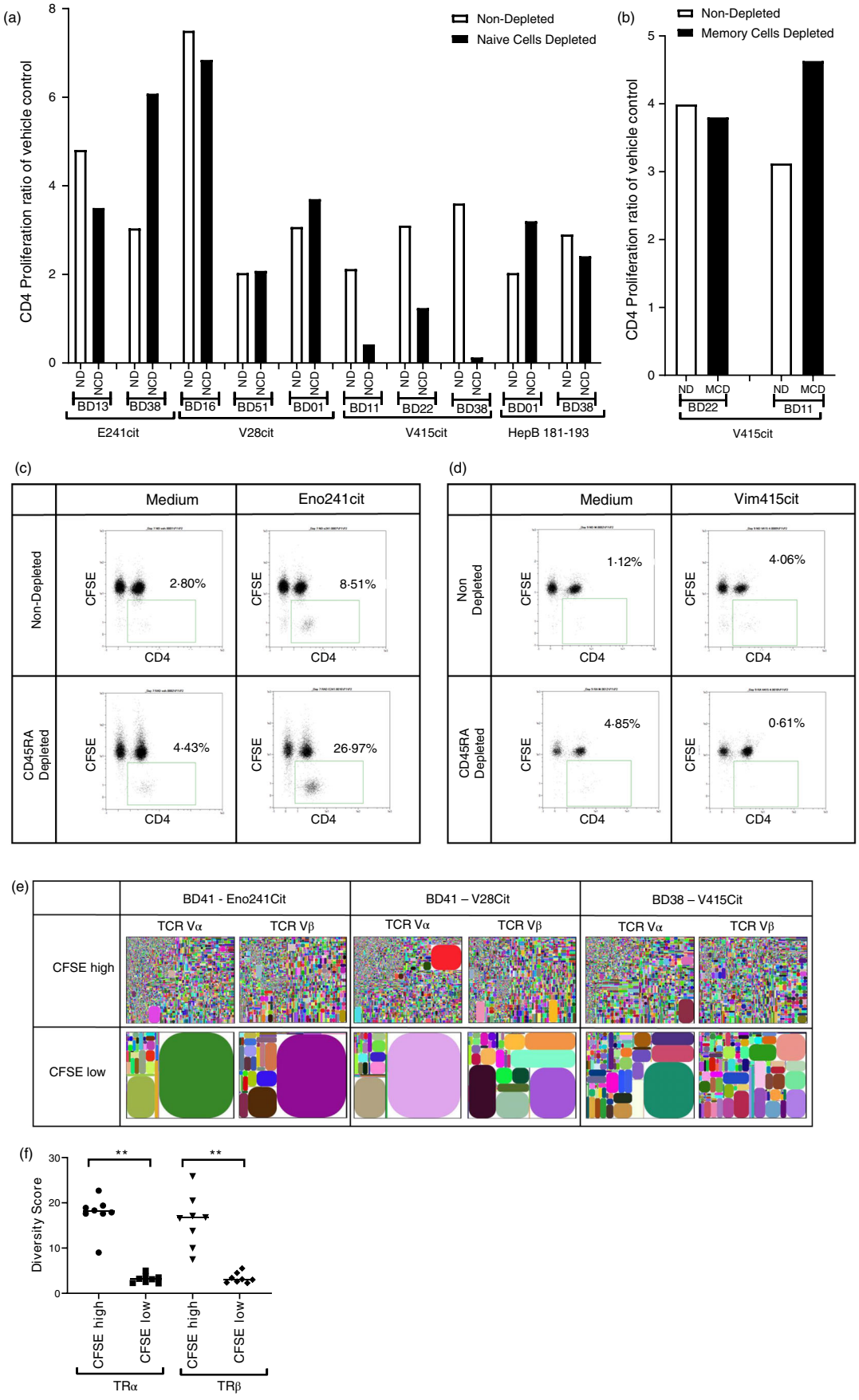

FIGURE 5 Legend on next page.

mice immunized with Eno241cit, Vim28it and Vim415cit peptides were assessed to see if IFNγ release was specific to the citrullinated peptide. Strong IFNγ responses were detected for all three citrullinated peptides that were significant ($p < 0.0313$) when compared with their respective unmodified wild type versions (Figure 6a,b). We also investigated if the responses to citrullinated peptides observed in these animal models, like the human donors, were memory responses. HLA-DP4 or HLA-DR4 mice were immunized with a single vaccination of citrullinated peptide and immune responses were assessed 2 days and 12 days later. Immune responses to Vim28-49cit and Vim415-433cit were observed in HLA-DP4 transgenic mice 2 days post vaccination suggesting a rapid reactivation of a memory population (Figure 6c). However, only Vim28-49cit stimulated a similar rapid response in HLA-DR4. Responses to Eno241-260cit and Vim415-433cit were only observed 12 days after immunization (Figure 6D). As a comparison, Hep B 181–193 was used as a foreign antigen control in HLA-DP4 mice. The responses to this peptide were only observed 12 days post immunization suggesting that stimulation of a naïve CD4 T cell population requires between 2 and 12 days to establish (Figure 6c). The responses to citrullinated peptides in HLA-DP4 and HLA-DR4 mice demonstrate different kinetics, we therefore hypothesized that different HLA alleles present citrullinated peptides under different physiological conditions and perhaps stress and inflammation drive variation in HLA restricted memory CD4 responses.

Citrullinated peptide specific responses show recognition of EBV transformed B cells

We hypothesized that cellular stress or infection can lead to the presentation of citrullinated epitopes and subsequent stimulation of CD4 T cells. To further investigate this possibility, splenocytes from HLA-DR4 mice immunized with Vim28-49cit or Vim415-433cit were assessed for IFNγ release following culture with a HLA DP4+ve EBV transformed cell line, namely PER 255 LCL, HLA matched B16F1 DR4 tumour line and T2 DR4 lymphoblasts or the original immunising peptide. IFNγ release was observed to both citrullinated peptides and to PER 255 LCL but not to the B16F1 DR4 tumour cells and T2 DR4 lymphoblasts unless they were first stressed by acid stripping of surface MHC and serum starvation (A/S SS; Figure 7a). The strong IFNγ responses observed to PER 255 LCL suggest that this cell line was naturally presenting at least one of the citrullinated peptides used for immunization (Figure 7a). No responses to A/S SS B16F1 cell line indicated the relevance of DR4 in the presentation of the peptide. Western blot also confirmed the presence of vimentin and PAD2, the enzyme responsible for citrullination of vimentin, in all cell lines (Figure 7b) [27, 28]. Although vimentin bands were observed at the expected molecular weight (∼50 kDa), PAD2 bands were ∼5 kDa lower than expected, possibly due to post-translational modification resulting in differential mobilities on the gel. Together these observations support our previous work demonstrating that tumour cells present citrullinated peptides under conditions of cellular stress and, in addition, that cellular stress induced by EBV infection can cause citrullination of vimentin and the presentation of citrullinated vimentin epitopes.

DISCUSSION

Here we present evidence to show that there is a repertoire of CD4 T cells in healthy donors that can recognize Eno241-260cit, Vim28-49cit and Vim415-433cit epitopes [11–14]. This is consistent with our animal studies demonstrating that we can induce responses to modified peptides in both HLA-DR4 and HLA-DP4 transgenic mice through vaccination. In addition, we show that there is a CD4 T cell repertoire specific to three new citrullinated and three homocitrullinated epitopes present in healthy donors, namely Bip189-208cit, NPM266-285cit, Cyk8 (101-120)cit, Aldo74-93hcit, Aldo140-157hcit and Vim116-135hcit. All T cell responses were predominantly Th1 with

FIGURE 5 Healthy donors showed naïve and memory responses to citrullinated peptides. (a) CD4 responses to citrullinated peptides or Hep B 181–193 peptide in healthy donors were assessed with or without CD45RA depletion to investigate of the responses were naïve or memory. Responses were measured between Days 7 and 11. Bar chart shows CD4 proliferation ratio to control. (b) CD45RO depletion and Vim415cit peptide stimulation with BD22 and BD11 peripheral blood mononuclear cells. Example staining plot from BD38 of CD4 proliferation response to (c) Eno241cit and (d) Vim415cit with and without CD45RA depletion. Tree plots for sorted $CD4^+$ carboxyfluorescein succinimidyl ester (CFSE) high and CFSE low TRα and TRβ chain in response to (e) Eno241cit and Vim28cit in BD41 and Vim415cit in BD38. Each rectangle represents a CDR3 peptide sequence and the size of the shape relates to the relative frequency of the peptide sequence. (f) Lower diversity scores in TRα and TRβ chains in CFSE low compared to CFSE high populations. $n = 8$. MCD, memory cells depleted; ND, non-depleted; NCD, naïve cells depleted.

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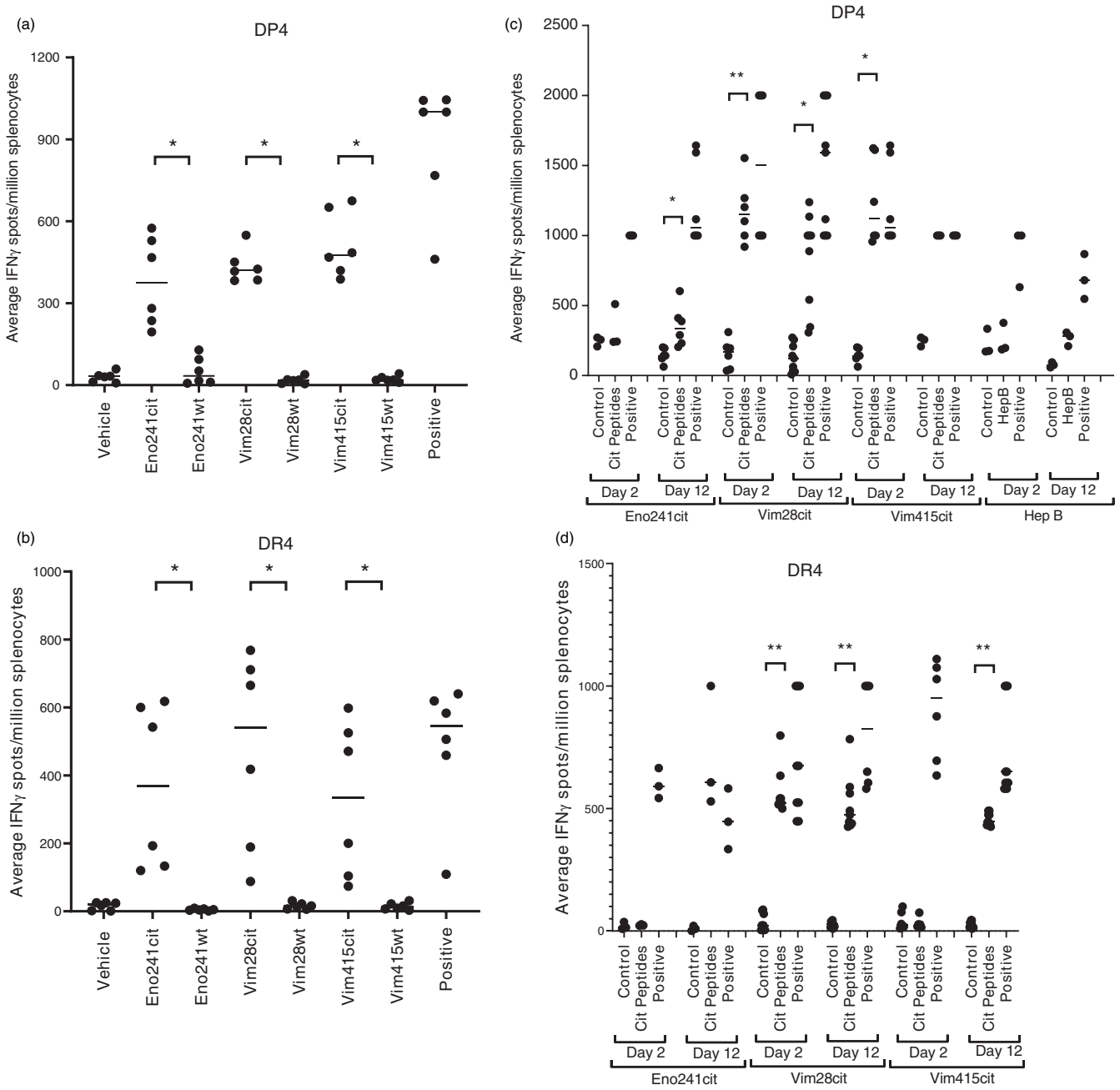
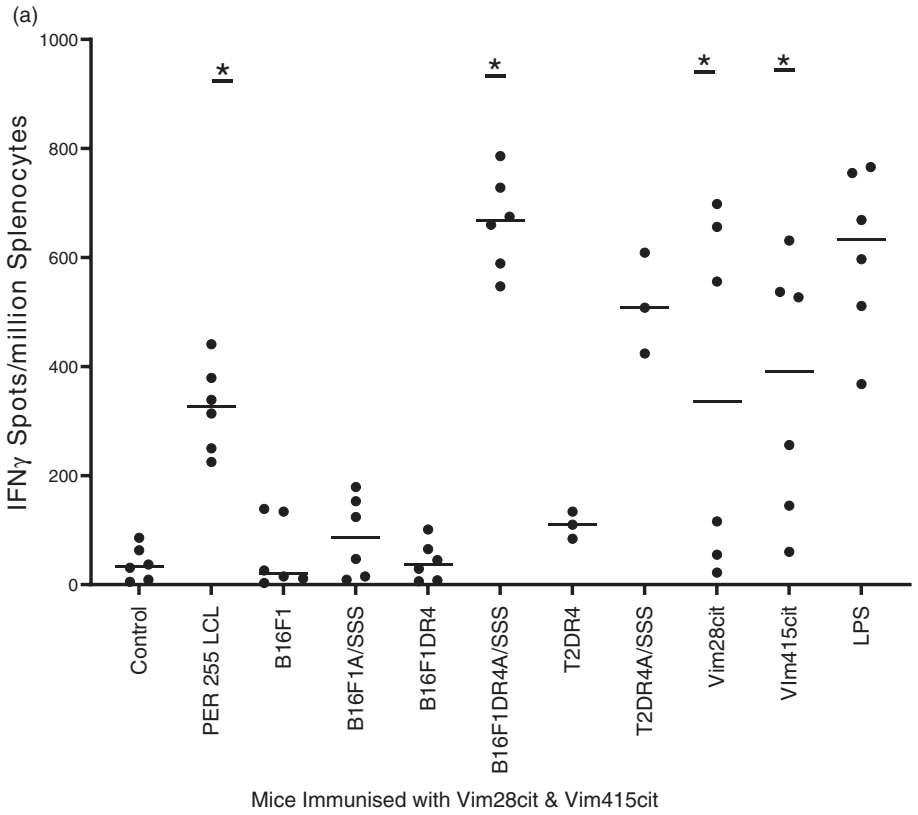


FIGURE 6 Human leucocyte antigen (HLA) transgenic mice show rapid generation of responses to citrullinated peptide vaccination. (a) HLA-DP4 and (b) HLA-DR4 mice were immunized with Eno241cit, Vim28cit and Vim415cit three times on Days 1, 8 and 15 and immune responses were assessed by IFNγ ELISpot on Day 21. $n = 6$. (c) HLA-DP4 and (d) HLA-DR4 mice were immunized with Eno241cit, Vim28cit and Vim415cit once on Day 0 or Day 11. Immune responses to specific citrullinated peptides were assessed at 2 or 12 days post vaccination. DP4 restricted Hepatitis B 181–193 peptide was used as a positive control. $n = 3$–6.

those specific to Vim28-49cit, NPM266-285cit, Aldo74-93hcit and Vim116-135hcit further differentiating to produce higher levels of granzyme B than IFNγ suggesting that they were cytotoxic T cells. This is in agreement with recent evidence indicating the direct cytotoxic function of CD4 T cells against viral infection [29–31] and tumours [32–34] in addition to their traditional helper role. The cytotoxicity of CD4 cells is restricted by expression of MHC-II on the target cells. While cells constitutively express MHC-II upon infection by some viruses such as EBV, the majority of other viruses do not follow the same mechanism [35]. However, upregulation of MHC-II expression under inflammation or in the presence of IFNγ has been reported in several studies [36–38]. Hence, CD4 cytotoxicity is not only restricted to cells with constitutive MHC-II expression. Furthermore, single cell transcriptome



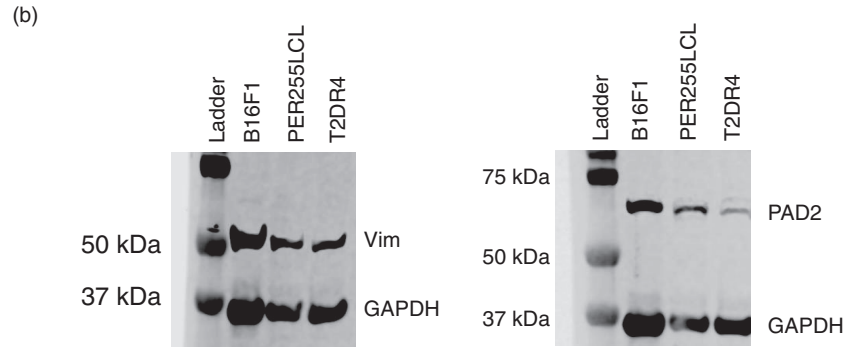


FIGURE 7 (a) Human leucocyte antigen (HLA) transgenic mice immunized with citrullinated vimentin peptides show response to lymphoblastoid cell line. HLA-DR4 transgenic mice were immunized with Vim28cit and Vim415cit peptides and splenocytes tested in IFNγ ELISpot assay for responses to citrullinated peptides, PER 255 LCL, B16F1 DR4 and T2 DR4 cell targets. $n = 3$–6. A/S SS, acid stripped serum starved. (b) Western blot of lysate from B16F1, PER 255 LCL and T2 DR4. The bands for vimentin (53 kDa), PAD2 (~70 kDa) and GAPDH (36 kDa) is shown.

analysis by Patil et al. showed CD45RA$^+$ effector memory CD4 T cell subsets in human peripheral blood express several CD8 cytotoxic and NK cell linked transcripts, including granzyme B, perforin, KLRG1 and NKG7 indicating cytotoxicity of CD4 T cells can be similar to CD8 [26].

Here we demonstrate that many healthy individuals have a repertoire of T cells that will recognize several siPTMs, for example, nine of the donors responded to at least four modified peptides, including one homocitrullinated peptide. We also show that CD25 cell depletion uncovers or enhances the T cell response to citrullinated peptides. As well T regs, the depleted population also include recently activated T cells [39], but this is likely to have minimal effects on citrulline-specific response in healthy donors. Our data provide more evidence to support the theory that autoreactive T cells can escape thymic negative selection and are likely supressed in the periphery to avoid tissue damage [40]. Dysregulation of Tregs is observed in autoimmune conditions and dormant pathogenic CD4 clones with TCRs specific to autoantigens are constrained by T regs in the periphery [41]. More than 30% of all CD4 T cells are controlled by peripheral tolerance [41] suggesting the repertoire to many self-antigens may not be deleted as previously thought. This is consistent with a study by James et al. demonstrating citrulline specific T cells in the peripheral blood of healthy donors and RA patients [9].

Interestingly, the T cell responses to Eno241-260cit and Vim28-49cit peptides in normal donors reside in the memory pool suggesting that they had previously been activated in vivo. In contrast, the T cell response to Vim415-433cit peptide was naïve. This suggests that even when vimentin is citrullinated certain epitopes are preferentially expressed and stimulate autoreactive T cells.

These results further suggest that citrullination could be a common occurrence in healthy donors, possibly as a result of cellular stress. Indeed, internal stress such as nutrient deprivation, hypoxia, DNA damage, reactive oxygen species and protein aggregation induce autophagy which is known to play a role in citrullination [42]. Furthermore, environmental stress such as smoking [13], diesel exhaust particles [43], cadmium [44], nickel [45] and coal exposure [46] are all associated with citrullination and homocitrullination of proteins [47]. In this scenario, if this is accompanied by inflammation, and in particular secretion of IFNγ which activates the pIV promoter of CIITA to induce expression of MHC-II on most other cells including epithelia, endothelia, fibroblasts and astrocytes [48], then citrullinated epitopes can be presented and stressed cells removed by cytotoxic CD4 T cells. Viral infection also induces ER stress and inflammation and can result in autophagy and citrullination [49, 50]. Anti-cyclic citrullinated antibodies were found in tuberculosis [51] and hepatitis [50] patients, suggesting a role of infection in citrullination. EBV infection has been linked to citrullination of myelin oligodendrocyte glycoprotein which can contribute to multiple sclerosis disease progression [52]. Furthermore, antibodies to citrullinated EBV peptides were identified in early RA patients [53]. In this study, we show that EBV immortalization of B cells can result in expression of citrullinated vimentin which then becomes a target for citrullinated vimentin specific T cells. This may be a more generic mechanism for removal of any virally infected cell which uses vimentin as its major cytoskeletal protein. The limitation of the study was the use of only class II matched (DR4) transgenic mice to assess citrulline specific responses against EBV cell lines as generating a complete HLA matched transgenic mice is challenging. However, citrullinated peptides primed splenocytes also showed response to B16F1 DR4 and T2 DR4 cells when stressed indicating stress induced citrullination of vimentin in these cells. Citrullination and presentation of cytokeratin epitopes may play a similar role in epithelial cells. These results support the hypothesis that stress, inflammation and/or infection can induce citrullination and homocitrullination of proteins which are presented to CD4 cells.

Contrary to the narrative that the HLA SE allele facilitates citrullinated antigen presentation, recent evidence demonstrates the shared epitope is a ligand for calreticulin and their interaction causes activation of intracellular PAD via an influx of calcium and enhanced citrullination [54]. Therefore, individuals with the SE may have a greatly enhanced propensity to citrullination rather than simply the right HLA allele for presentation. This study further confirms that the presentation of citrullinated and homocitrullinated epitopes is not restricted to a particular HLA allele, consistent with this being a normal stress response. We observed citrulline and homocitrulline specific responses in several donors without any wild type cross-reactivity, indicating citrulline is involved for TCR recognition. Although, this was assessed on only four donors including one 'RA protected' donor, the results were consistent with our previous reports [16, 21, 22]. Similarly, responses in HLA-DP4 and DR4 transgenic mice were citrulline specific further suggesting that citrulline is part of the core sequence of the antigen. It has also been suggested that the conversion of arginine to citrulline enhances the binding of some peptides to HLA-DR and DQ alleles [9, 17]. In contrast, the conversion of arginine to citrulline does not always lead to enhanced peptide MHC-II binding affinity [55]. Increasing evidence is emerging that HLA-DP molecules can present peptides in the context of infectious disease, allergy and cancer [56–59]. HLA-DP alleles appear more conserved than DR or DQ alleles, with five alleles frequently expressed in the worldwide population that cover approximately 90% of individuals [60]. Therefore, the reduced polymorphism among HLA-DP alleles suggests these as prime candidates for the presentation of peptides in this universal process and may point to a role of HLA-DP alleles in the clearance of stressed cells. Indeed, van Lith et al. have shown that HLA-DP does not require invariant chain or HLA-DM to form stable dimers making it more accessible to peptides produced during autophagy [61, 62]. Other studies have shown that HLA-DP does not bind CLIP fragments [63], and known HLA-DP peptide-binding motifs differ from those of ER-loaded MHC molecules, so HLA-DP is not likely to compete for classical class II-binding peptides [64]. The reported lower expression of HLA-DP molecules [65, 66] and Treg regulation most likely play a role to avoid autoimmunity and promote self-tolerance in the absence of stress or inflammation. In contrast, co-expression on HLA-DP4 and HLA-DR4 may push T cells over the threshold and result in autoimmune disease. Interestingly, in this study the responses in healthy donors were more frequent than in RA patients. Although, due to inaccessibility of the patients HLA data, we were unable to perform an appropriate matched analysis against healthy donors. The lower frequency responses in RA patients could be as a result of patient's biological therapies which are known to reduce citrulline specific T cells [9]. Besides, patient population were considerably older than healthy donors, which further could impact the response rate.

In summary, we showed that normal healthy donors have a repertoire of memory CD4 T cells that recognize and respond to citrullinated peptides. We hypothesize that stress and/or inflammation may drive presentation of citrullinated peptides and induce cytotoxic T cells which can remove stressed cells.

AUTHOR CONTRIBUTIONS

Ruhul H. Choudhury: Methodology, investigation, writing–original draft, review and editing. **Ian Daniels**: Methodology, investigation. **Poonam Vaghela**: Methodology, investigation. **Suha Atabani**: Methodology, investigation. **Thomas Kirk**: Methodology, investigation. **Peter Symonds**: Methodology, investigation. **Katherine W. Cook**: Methodology, investigation. **Abdullah Al-Omari**: Investigation and data collection. **Daisy Weston**: Investigation and data collection. **Sabaria Shah**: Investigation and data collection. **David Hutchinson**: writing–review editing. **Samantha J. Paston**: writing–review editing. **Rachael L. Metheringham**: Conceptualization, methodology, resources. **Victoria A. Brentville**: Conceptualization, visualisation, supervision, writing–review editing. **Lindy G. Durrant**: Conceptualization, visualization, supervision, writing–review editing.

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CONFLICT OF INTEREST STATEMENT

Victoria A. Brentville, Rachael L. Metheringham and Lindy G. Durrant have ownership interest in patent WO2017013425 A1. Lindy G. Durrant is a director, shareholder and CSO of Scancell Ltd. All authors except David Hutchinson are employees of Scancell Ltd.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

ETHICS STATEMENT

All donors provided written and informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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