SHORT COMMUNICATION

Circulating gluten-specific, but not CMV-specific, CD39+ regulatory T cells have an oligoclonal TCR repertoire

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Abstract

Objectives. Understanding the T cell receptor (TCR) repertoire of regulatory CD4+ T-cell (Treg) populations is important for strategies aiming to re-establish tolerance in autoimmune diseases. We studied circulating deamidated gluten-epitope-specific CD39+ Tregs in patients with coeliac disease following an oral gluten challenge, and we used cytomegalovirus (CMV)-specific CD39+ Tregs from healthy controls as a comparator population. **Methods.** We used the OX40 assay to isolate antigen-specific Tregs by induced surface co-expression of CD25, OX40 and CD39. RACE PCR amplification and Sanger sequencing of the TCR β chain were used to analyse repertoire diversity. **Results.** We found that, following oral gluten challenge, circulating gluten-specific CD39+ Tregs had an oligoclonal TCR repertoire that contained public clonotypes. Conversely, the TCR repertoire of CMV-epitope-specific CD39+ Tregs from healthy controls was polyclonal. **Discussion.** These data indicate that a biased TCR repertoire is not inherent to CD39+ Tregs, and, in this case, is apparently driven by the HLA-DQ2.5-restricted deamidated gluten peptide in coeliac disease patients. **Conclusion.** This is the first assessment of the TCR repertoire within circulating human Tregs specific for foreign antigen. These data enhance our understanding of antigen-specific CD4+ responses in the settings of chronic inflammation and infection and may help guide immunomonitoring strategies for CD4+ T cell-based therapies, particularly for coeliac disease.

Keywords: CD4+ T cells, CMV, coeliac disease, gluten, regulatory T cells, TCR repertoire

INTRODUCTION

The T cell receptor (TCR) repertoire diversity of memory T-cell populations is important for determining clearance of pathogens, is useful as a biomarker for monitoring immunotherapies in cancer and autoimmunity, for classifying various inflammatory diseases, and is an important factor in vaccine design.[1] Although antigen-specific CD8^{+} T cells have been well studied,[2,3] the TCR repertoire of antigen-specific CD4^{+} T cells, particularly regulatory T cells (Tregs), remains underexplored. Similarly, very few human studies have directly compared antigen-specific TCR repertoires of effector and regulatory memory CD4^{+} T cells, with evidence for both distinct repertoires[4] and clonotype sharing.[5]

We chose to investigate the TCR repertoire of antigen-specific Tregs in the context of coeliac disease, a gluten-dependent enteropathy associated with an acquired memory CD4^{+} T-cell response against deamidated gluten peptides.[6] The immunodominant hierarchy of wheat gliadin T-cell epitopes in HLA-DQ2.5 (DQA1*05:DQB1*02)[+] coeliac disease patients has been well characterised,[7] and gluten-responsive effector CD4^{+} T cells can be detected in the peripheral blood of patients with coeliac disease on a gluten-free diet, following oral gluten challenge.[8] We have previously shown that the majority (> 80%) of circulating gluten-specific CD4^{+} T cells in patients with coeliac disease are CD39^{+} Tregs and that the TCR repertoire of these cells may have biases, as we identified a public TCR clonotype (TRBV7-2; CASSLRYTDTQYF) expressed by a DQ2.5-glia-α1/α2-specific clone.[9] Previous TCR repertoire studies of gluten-peptide-specific effector CD4^{+} T cells have demonstrated biased usage of TRBV7-2 within DQ2.5-glia-α2-specific responses, with several public clonotypes also detected.[10–12]

To confirm whether our findings in the context of coeliac disease could be generally applied to foreign antigen-specific CD39^{+} Tregs, we analysed cytomegalovirus (CMV)-specific CD39^{+} Tregs as a comparator population, utilising reponses to 15mer pp65 epitopes with known restriction elements.[13,14] These cells were obtained from healthy individuals as there was insufficient blood volume available from patients with coeliac disease. Previous studies of the TCR repertoire of CMV-specific CD4^{+} T cells in humans are limited and have largely relied on *ex vivo* expansion of these cells, which introduces bias into the repertoire.[5,15] Our methodology to obtain these cells (the OX40 assay) uses 44-h antigen-stimulated upregulation of the activation markers CD25 and OX40 (CD134), so cells are isolated prior to any proliferation occurring.[16] To our knowledge, this is the first description of the TCR repertoire within human circulating regulatory T cells specific for foreign antigens.

RESULTS AND DISCUSSION

The TCR repertoire of circulating gluten-specific CD39^{+} Tregs is oligoclonal

To characterise the TCR repertoire of gluten-specific CD4^{+} Tregs and Tconv cells, we utilised our previously described OX40 assay, which detects antigen-specific cells by induced co-expression of CD25 and OX40 following 44-h antigen stimulation *in vitro*[16] (Figure 1a). In this assay, we have validated that CD39 co-expression identifies a sub-population of antigen-specific cells (CD25^{+}OX40^{+}CD39^{+}) highly enriched (> 80%) for CD25^{high}CD127^{low}FOXP3^{+} Tregs.[17] In patients with coeliac disease, we confirmed that > 85% of the DQ2.5-glia-α1/α2-specific CD25^{+}OX40^{+}CD39^{+} cells originate from peripheral CD25^{high}CD127^{low}FOXP3^{+} Tregs.[9] Notably, CD39^{−} Tconv cells comprised < 10% of DQ2.5-glia-α1/α2-specific responses in the patients with coeliac disease, providing insufficient cell numbers for analysis in this study. Additionally, as previously described,[9] gluten-specific OX40 assay responses could only be detected in post-gluten challenge, and not pre-challenge, blood, restricting our analysis of gluten-specific cells to CD39^{+} Tregs isolated post-gluten challenge.

We isolated DQ2.5-glia-α1/α2-specific CD25^{+}OX40^{+}CD39^{+} Tregs from three patients with coeliac disease (Supplementary table 1) 6 days after oral gluten challenge as previously described[9] and performed TCR clonotype analysis (Figure 1b). In this study of TCR repertoire diversity, only the TCR β chain was sequenced and analysed. TRBV and TRBJ segment usage, along with the translated CDR3 region for all clonotypes, is listed in Table 1, and there were no silent mutations observed. The TCR repertoire of DQ2.5-glia-α1/α2-specific CD39^{+} T cells from each individual was oligoclonal, that is containing few clones and dominated by a few expanded clonotypes. For individuals #0174 and #0251,
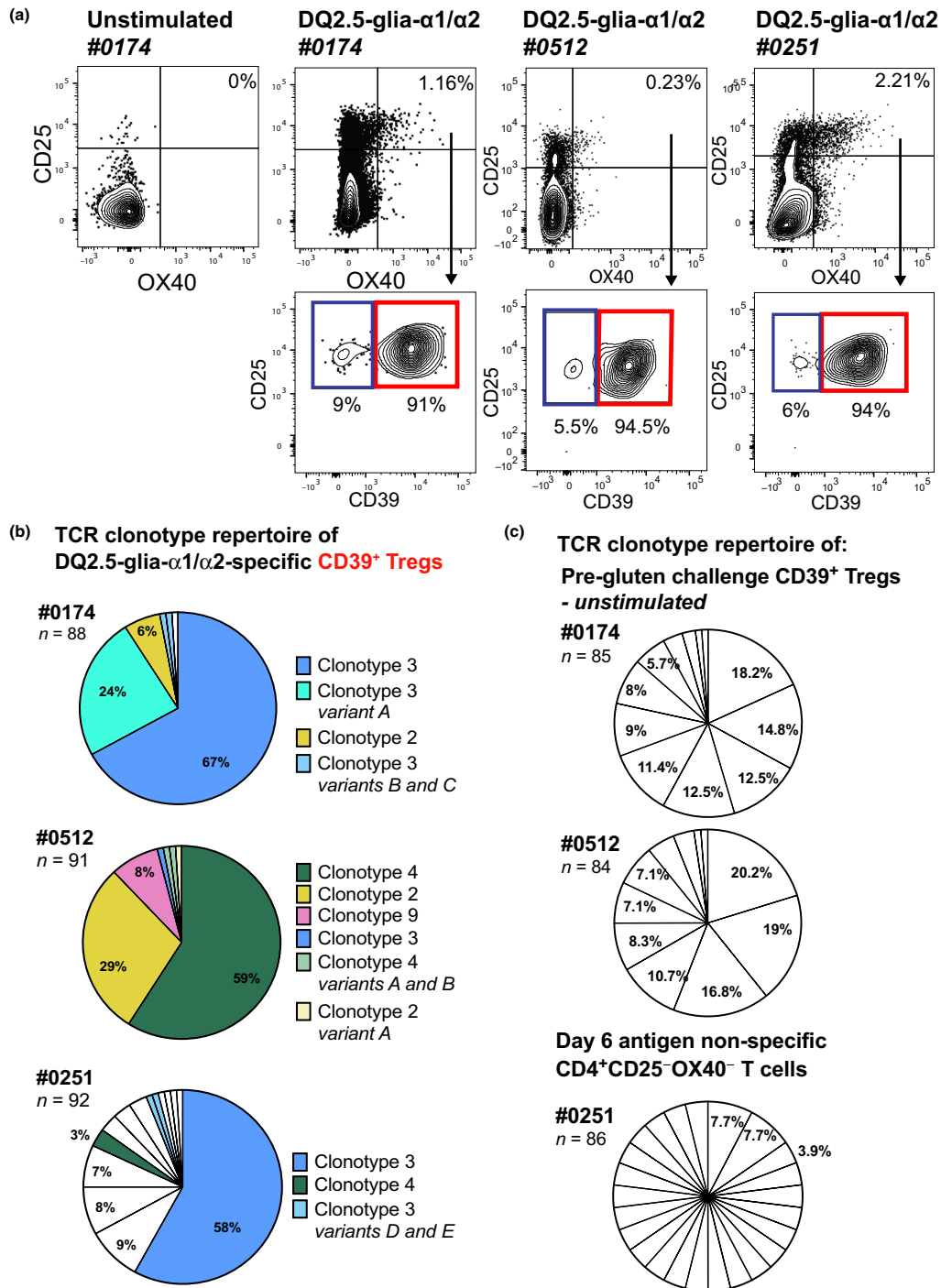
Figure 1. TCR repertoire of gluten-peptide-specific CD39^{+} T cells. **(a)** PBMCs were isolated from $n = 3$ coeliac disease patients (#0174, #0512 and #0251) 6 days following oral gluten challenge, stimulated with gluten peptide DQ2.5-glia-α1/α2 for 44-h and the antigen-specific CD4^{+}CD25^{+}OX40^{+}CD39^{+} Tregs sorted. **(b)** The TCR clonotype repertoires of the CD4^{+}CD25^{+}OX40^{+}CD39^{+} Treg populations are shown along with the number of clonotypes analysed for each patient. Coloured segments indicate clonotypes (including variants of these clonotypes that differed by a single residue in the CDR3 region) detected in more than one individual (this study and Qiao *et al.*[10]), and clonotype sequences are in Table 1. **(c)** TCR clonotype repertoires of unstimulated total CD39^{+} Tregs sorted from coeliac disease patients #0174 and #0512 prior to gluten challenge, and CD4^{+} cells not specific for gluten peptides from coeliac patient #0251 that were sorted from an OX40 assay as CD25^{−}OX40^{−} cells post-gluten challenge (n = number of clonotypes analysed for each patient). Clonotype sequences are in Supplementary tables 2–4; data are from a single experiment for each patient.

Table 1. TCR clonotypes expressed by DQ2.5-glia-α1/α2-specific CD39^{+} T cells isolated from coeliac disease patients post-gluten challenge

Clonotype ID	Expression	TRBV	TRBJ	CDR3
Coeliac disease patient #0174				
Clonotype 3	67%	TRBV4-1	TRBJ2-5	CASSQVTLPTETQYF
Clonotype 3A	24%	TRBV4-1	TRBJ2-5	CASGQVTLPTETQYF
Clonotype 2	6%	TRBV3-1	TRBJ2-3	CASSSLNTQYF
Clonotype 3B	1%	TRBV4-1	TRBJ2-5	CTSSQVTLPTETQYF
Clonotype 3C	1%	TRBV4-1	TRBJ2-5	CASSQVTLPTGTQYF
Clonotype 8	1%	TRBV4-1	TRBJ2-5	CASGQVILPTETQYF
Coeliac disease patient #0512				
Clonotype 4	59%	TRBV7-2	TRBJ2-7	CASSFGVEDEQYF
Clonotype 2	29%	TRBV3-1	TRBJ2-3	CASSSLNTQYF
Clonotype 9	8%	TRBV7-3	TRBJ2-3	CASSIRSTDTQYF
Clonotype 3	1%	TRBV4-1	TRBJ2-5	CASSQVTLPTETQYF
Clonotype 4A	1%	TRBV7-2	TRBJ2-7	CASNFGVEDEQYF
Clonotype 4B	1%	TRBV7-2	TRBJ2-7	CASSLGVEDEQYF
Clonotype 2A	1%	TRBV3-1	TRBJ2-3	CTSSSLNTQYF
Coeliac disease patient #0251				
Clonotype 3	58%	TRBV4-1	TRBJ2-5	CASSQVTLPTETQYF
Clonotype 10	9%	TRBV7-3	TRBJ2-3	CASSLGGSTDTQYF
Clonotype 11	8%	TRBV20-1	TRBJ2-1	CSASSSGGASYNEQFF
Clonotype 12	7%	TRBV4-2	TRBJ2-7	CASSRTGQGGETQYF
Clonotype 4	3%	TRBV7-2	TRBJ2-7	CASSFGVEDEQYF
Clonotype 13	3%	TRBV7-3	TRBJ1-6	CASSLSFDSPLHF
Clonotype 14	3%	TRBV6-2	TRBJ1-2	CASSRSYGYTF
Clonotype 15	3%	TRBV5-1	TRBJ1-6	CASSLSGADNSPLHF
Clonotype 16	1%	TRBV12-4	TRBJ1-4	CASSVQGITNEKLFF
Clonotype 3D	1%	TRBV4-1	TRBJ2-5	CASSQVTLPTETRYF
Clonotype 3E	1%	TRBV4-1	TRBJ2-5	CASSQVTLPTETQHF
Clonotype 17	1%	TRBV6-2	TRBV1-5	CASSWGQGYQPQHF
Clonotype 18	1%	TRBV28	TRBJ2-4	CPSFRGDIQYF
Clonotype 19	1%	TRBV2	TRBJ2-5	CASSPLSFGGGQETQYF

Coloured rows match Figure 1b and indicate TCR clonotypes (including variants that differed by a single residue (underlined) in the CDR3 region) detected in more than one individual in this study and Qiao *et al.* [10]

clonotype 3 comprised 67% and 58%, respectively, of all clonotypes analysed. For individual #0512, 59% of the sampled TCR repertoire was composed of one clonotype (clonotype 4). For each individual, only 6–14 unique clonotypes were detected within all clonotypes analysed (range: 88–92 clonotypes). No conserved CDR3 motifs or residues were detected.

Similar to previous studies,[10,11] we observed public clonotypes within DQ2.5-glia-α1/α2-specific CD4^{+} T cells. One TCR clonotype (clonotype 9; TRBV7-3; CASSIRSTDTQYF; individual #0512) was detected that has been previously described.[10] We also identified three novel public clonotypes (clonotypes 2, 3 and 4) that may be unique to the DQ2.5-glia-α1/α2-specific CD39^{+} Treg population. Clonotype 2 (TRBV3-1; CASSSLNTQYF), clonotype 3 (TRBV4-1; CASSQVTLPTETQYF) and clonotype 4 (TRBV7-2; CASSFGVEDEQYF), each identified in at least two individuals, were also previously found

expressed by *in vitro* expanded DQ2.5-glia-α1/α2-specific CD39^{+} T-cell lines and clones derived from individual #0174.[9]

To ensure the repertoire skewing observed within DQ2.5-glia-α1/α2-specific T cells was not due to any inherent TCR repertoire bias within these individuals, TCR analysis was also performed on control populations. For individuals #0174 and #0512, total memory CD39^{+} Tregs (CD45RO^{+}CD25^{high}CD127^{low}CD39^{+}) were sorted prior to gluten challenge. Additionally, for individual #0251 the DQ2.5-glia-α1/α2-non-specific CD4^{+}CD25^{−}OX40^{−} T cells were sorted from an OX40 assay. Importantly, none of the TCR clonotypes identified in either our previously described *in vitro* expanded DQ2.5-glia-α1/α2-specific T-cell lines and clones,[9] or the *ex vivo* DQ2.5-glia-α1/α2-specific CD25^{+}OX40^{+}CD39^{+} T cells reported here, were detectable in these control populations (Figure 1c and Supplementary tables

2–4). Therefore, the T-cell receptor and public clonotype bias observed within DQ2.5-glia-α1/α2-specific CD39$^+$ Treg populations post-gluten challenge are apparently driven by the gluten antigen, as clonotypes detected in this population do not occur at high frequencies within circulating memory CD39$^+$ Tregs prior to gluten challenge. Additionally, there was no degeneracy in the public clonotype sequences, suggesting that convergent recombination processes were not the key mechanism generating these clonotypes.[18] It is more likely the nature of the deamidated gliadin epitope, and its restricted presentation by HLA-DQ2 molecules is the key determinant of the biased TCR repertoire in coeliac disease.[2]

These data extend our previous findings that gluten-specific, but not polyclonal, CD39$^+$ Tregs in coeliac disease have impaired suppressive function,[9] by demonstrating these cells also have an oligoclonal TCR repertoire that contains public clonotypes. It will be important for future studies to assess whether the circulating gluten-specific CD39$^-$ effector T cells have any clonotype sharing with CD39$^+$ Tregs, potentially by utilising single-cell sequencing technologies.

CMV-specific CD39$^+$ Tregs exhibit a type 1 TCR bias and have a polyclonal repertoire

Importantly, as we are describing a novel population using the OX40 assay, it was important to ascertain whether an oligoclonal TCR repertoire was a feature of circulating CD39$^+$ Tregs with specificity for foreign antigen. To do this, we utilised healthy individuals ($n = 3$) with known responses to either of two 15mer peptides derived from CMV pp65: CMV-P1 (LLQTGIHVRVSQPSL), reported to be restricted by HLA-DRB1*15,[13] and CMV-P4 (EHPTFTSQYRIQGKL), reported to be restricted to HLA-DRB1*11:01.[14] We chose CMV-peptide-specific CD39$^+$ Tregs as our comparator population as previous work had indicated that chronic infection with CMV can result in a biased TCR repertoire in CD8$^+$ and CD4$^+$ T cells.[19–21] Additionally, from our previous work we had identified healthy donors with robust CD39$^+$ Treg and CD39$^-$ Tconv cell responses to these CMV epitopes.[17] Donors 1 and 2 had responses to CMV-P1, and donor 3 had a response to CMV-P4 (Figure 2 and Supplementary table 1). OX40 assays (44-h antigen stimulation, 10 μg mL^{-1} of antigen) were performed, and CMV-peptide-specific CD4$^+$CD25$^+$OX40$^+$CD39$^+$ Tregs (and

CD4$^+$CD25$^+$OX40$^+$CD39$^-$ Tconv cells) were sorted and TCR repertoire analysed.

We observed that CMV-peptide-specific CD39$^+$ Tregs from all three donors had a polyclonal TCR repertoire, being a repertoire containing many clones and without dominant clonal expansions, although there was some bias towards usage of particular TRBV segments (Figure 2 and Supplementary tables 5–9). For donors 1 and 2, we also sorted CD39$^-$ Tconv cells and observed that, although the pattern of TRBV segment usage was similar, there were no shared clonotypes with CD39$^+$ Tregs, consistent with these being distinct populations, as previously described.[4] Although no clonotype sharing was observed between regulatory and effector populations, the individual frequencies of CMV-specific clonotypes were too small to enable statistical analysis of similarity. We used flow cytometry to confirm a normal distribution of TCR Vβ family usage within *ex vivo* unstimulated CD4$^+$ T cells for donors 1 and 2 using TCR Vβ mAbs (IOTest® Beta Mark Kit, Beckman Coulter, Brea, CA, USA; Supplementary figure 1). In all donors, regulatory and effector CD4$^+$ T-cell responses to CMV peptides exhibited a type 1 TCR bias, as indicated by preferred TRBV segment usage,[2] with a polyclonal clonotype repertoire with high diversity mostly composed of low-frequency clonotypes.

CD39$^+$ Treg populations with higher avidity for CMV peptides also exhibit a polyclonal repertoire

The CD4$^+$ T-cell response to deamidated gluten peptide DQ2.5-glia-α1/α2 in patients with coeliac disease is highly specific and has likely developed over repeated antigen exposure; therefore, we asked whether a population of CMV-peptide-specific CD39$^+$ Tregs selected for higher avidity might exhibit a biased TCR repertoire, particularly as this has been described for CMV-specific CD8$^+$ T-cell responses.[20] We confirmed using a dose titration that CMV-peptide-specific CD39$^+$ Tregs had higher avidity for antigen than CD39$^-$ Tconv cells (Figure 3a).

CMV-P1-specific T regulatory and conventional cell populations containing higher avidity clonotypes were isolated from donor 1 following an OX40 assay stimulated with 2.5 μg mL^{-1} of CMV peptides, being the dose where the maximal OX40 assay response was reduced by 75% (Figure 3a). Analysis of TCR sequences from cell populations
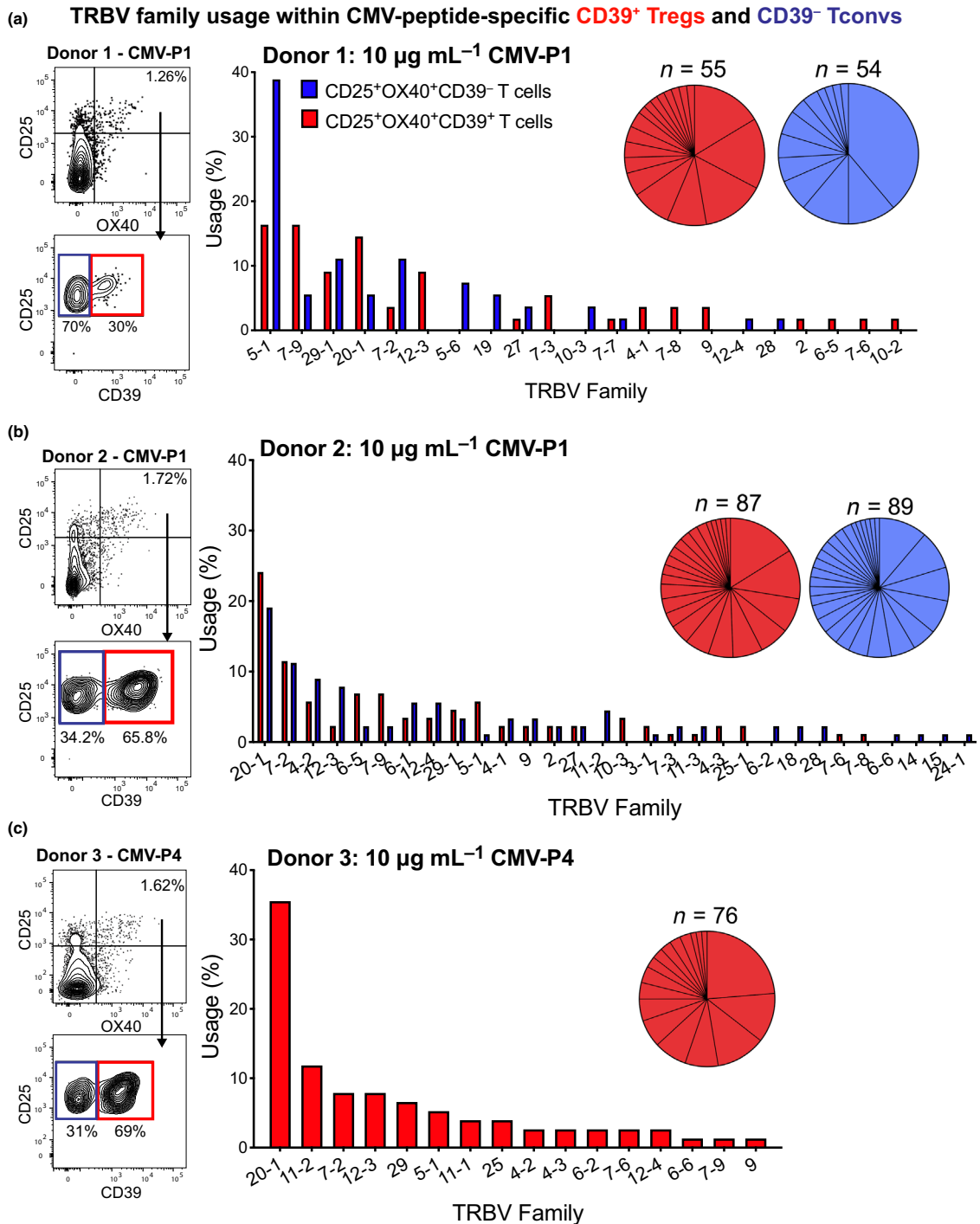
Figure 2. TCR repertoire of CMV-peptide-specific CD39$^+$ and CD39$^-$ CD4$^+$ T cells. For n = 3 healthy donors, PBMCs were stimulated with 10 µg mL^{-1} antigen for 44 h and the antigen-specific CD4$^+$CD25$^+$OX40$^+$ cells sorted into CD39$^-$ cells and CD39$^+$ Tregs, as shown in the FACS plots. TCR repertoire was assessed by 5'RACE and Sanger sequencing, and the percentage usage of each TCR Vβ family is shown for **(a)** donor 1 CMV-P1-specific cells, **(b)** donor 2 CMV-P1-specific cells and **(c)** donor 3 CMV-P4-specific cells. The number of TCR clonotype sequences analysed is represented by n for each subset. There was insufficient CMV-P4-specific CD39$^-$ cells sorted from donor 3 to enable analysis. Clonotype sequences are in Supplementary tables 5–9; data are from a single experiment for each donor.

(a) Dose-dependent CD39⁺ Treg and CD39^{−} Tconv proportions within CMV-peptide responses
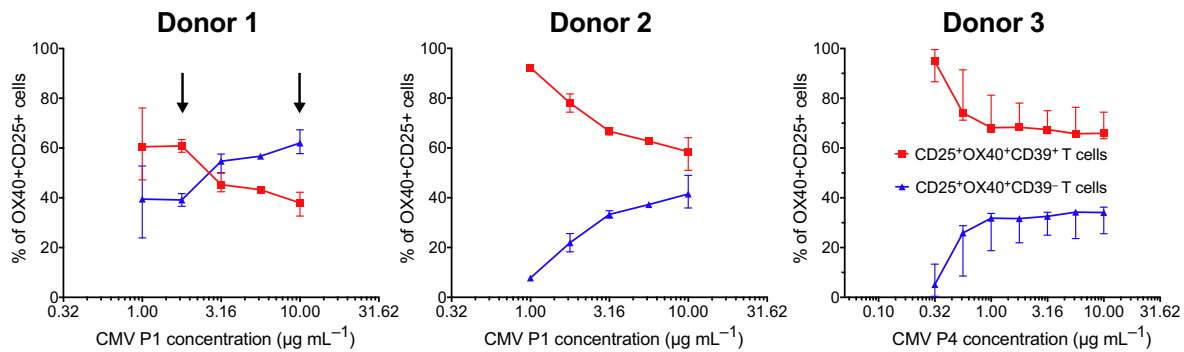
(b) TRBV family usage within CMV-P1-specific CD39⁺ Tregs
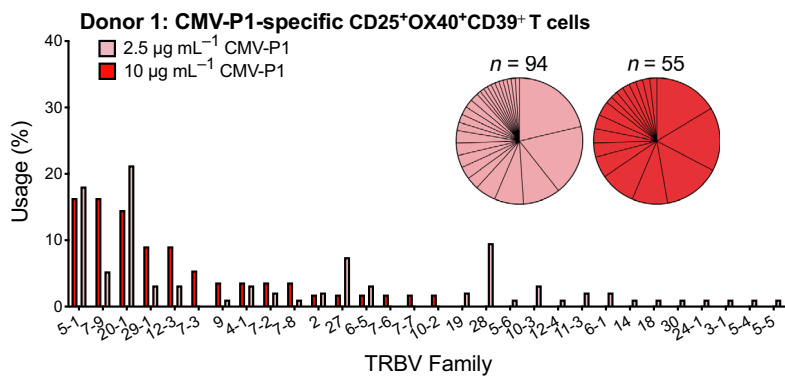
TRBV family usage within CMV-P1-specific CD39^{−} Tconvs
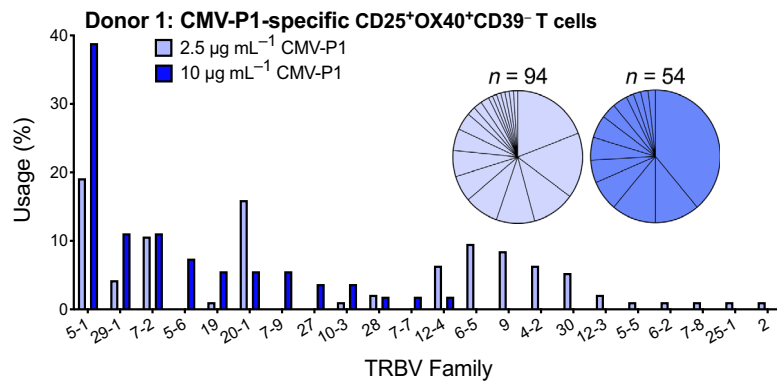

Figure 3. Effect of antigen concentration on proportions of CD39$^+$ Tregs in recall responses. **(a)** For CMV-P1 and CMV-P4 responses, the proportion of CD39$^+$ Tregs and CD39$^−$ Tconv cells are shown for a log dilution of antigen concentrations from 10 to 1 µg mL^{-1}. Data represent 3–4 independent experiments for each donor, median ± interquartile range. Black arrows indicate the CMV-P1 concentrations used to sort higher and lower avidity responses for donor 1. **(b)** Percentage usage of TCR Vβ families is shown for CD39$^+$ cells and CD39$^−$ cells responding to 2.5 and 10 µg mL^{-1} of CMV-P1 from donor 1. The number of TCR clonotype sequences analysed is shown (Supplementary tables 5, 6, 10 and 11); data are from a single experiment.

containing higher avidity clonotypes revealed that, for donor 1, the TCR repertoire was equally diverse as seen in populations inclusive of lower avidity responses (10 µg mL^{-1}), with no overlap in TCR clonotypes observed (Supplementary tables 10 and 11). Within CD39$^+$ Treg and CD39$^−$ Tconv cells responding to either high- or low-dose antigen, the TRBV usage hierarchy did not alter

substantially, indicating that the higher avidity TCRs utilise TRBV segments that are also dominant after stimulation with high-dose antigen (Figure 3b). These preliminary data indicate that CMV-P1-specific CD39^{+} Treg populations containing both high- and low-avidity clonotypes had a polyclonal TCR repertoire that was distinct from CD39^{−} Tconv cells. Of note, previous studies of CMV pp65- and glycoprotein B- epitope-specific CD4^{+} T cells, that defined these cells by IFNγ production or cytotoxicity, observed a more biased TCR repertoire containing clonal expansions.[19,21] In contrast, we show that using the OX40 assay to sample a more functionally diverse epitope-specific CD4^{+} T-cell population reveals a highly diverse, polyclonal TCR repertoire. The TCR repertoire of CD4^{+} T cells specific for CMV peptides P1 and P4 has not been previously assessed, and it is known that differences in peptide–MHC complexes can drive either a polyclonal or an oligoclonal TCR repertoire.[22] Our data support previous reports of polyclonal CD4^{+} T-cell TCR repertoires in populations specific for other viral antigens from Epstein–Barr virus and influenza A.[23,24] However, our data require confirmation in a larger cohort to provide a more accurate description of the TCR repertoire diversity of CMV-pp65-epitope-specific CD4^{+} T-cell populations.

Conclusion

In conclusion, our data indicate the TCR repertoire of gluten-specific CD39^{+} Tregs in patients with coeliac disease is oligoclonal and may contain unique public clonotypes, whilst CMV-peptide-specific CD39^{+} Tregs contain a polyclonal TCR clonotype profile. These data suggest that the oligoclonal repertoire of gluten-specific CD39^{+} Tregs is not inherent to CD39^{+} Tregs with specificity for foreign antigen, but is instead driven by the antigen and its precise HLA restriction. This exploratory study extends upon our previous work identifying that CD39^{+} Tregs dominate recall responses to gluten and have impaired suppressive function.[9] Further work should validate the presence of gluten-specific public clonotypes unique to Treg cell populations in a larger coeliac disease cohort. These data, detailing the restricted TCR repertoire of gluten-specific Tregs, may be useful in monitoring the effectiveness of novel immunotherapies aiming to re-establish tolerance in patients with coeliac disease.

METHODS

Subjects and samples

Patients with coeliac disease were recruited after provision of informed consent (Human Research Ethics Committees: Royal Melbourne Hospital ID: 2003.009; The Walter and Eliza Hall Institute of Medical Research ID: 03/04). Enrolment criteria were as follows: biopsy-proven disease conforming to ESPGHAN guidelines, HLA-DQ2.5[+] and compliant on gluten-free diet for ≥ 6 months (Supplementary table 1). All patients with coeliac disease undertook a 3-day gluten challenge by consuming four slices of commercial white bread daily (approximately 10 g/day of wheat gluten).[8] Healthy donor blood was collected from volunteers (St Vincent's Hospital Human Research Ethics Committee ID: HREC/13/SVH/145). Peripheral blood was collected into lithium heparin vacutainers (Becton Dickinson (BD), Franklin Lakes, NJ, USA), transported at ambient temperature and processed within 8 h of collection. Mononuclear cells were obtained by centrifugation over Ficoll-Paque (GE Healthcare, Chicago, IL, USA).

HLA typing

For coeliac disease patients #0174 and #0251, HLA-DQB1 and HLA-DQA1 alleles were determined using PCR–sequence-specific oligonucleotide hybridisation (Victorian Transplantation and Immunogenetics Service, Melbourne, Australia). For coeliac patient #0512, the presence of alleles encoding HLA-DQ2.5, DQ2.2 and DQ8 was determined by detecting five single-nucleotide polymorphisms (rs2187668, rs2395182, rs4713586, rs7454108 and rs7775228) as per our previous study.[9] HLA genotyping of healthy individuals was performed by the Institute for Immunology & Infectious Diseases using Illumina next-generation sequencing (Murdoch University, Perth, Australia).

Antigens

A HLA-DQ2.5-restricted 15mer (5′ – LQPFPQPELPYPQPQ – 3′) that encompasses two overlapping immunodominant deamidated wheat gliadin T-cell epitopes, DQ2.5-glia-α1a (PFPQPELPY) and DQ2.5-glia-α2 (PQPELPYPQ),[7] synthesised to ≥ 95% purity (Pepscan, Lelystad, The Netherlands) was used at 50 μg mL^{−1}, as previously optimised.[9] Two previously described[13,14] 15-mer CMV-peptides, CMV-P1 (5′ – LLQTGIHV RVSQPSL – 3′) and CMV-P4 (5′ – EHPTFTSQYRIQGKL – 3′), synthesised to > 95% purity (Mimotopes, Mulgrave, Australia), were used at 10 μg mL^{−1} (unless specified otherwise).

OX40 assay

The OX40 assay was performed as previously described[16,17] using PBMCs at 2×10^6 cells mL^{−1} in RPMI supplemented with 5% human serum, 1% L-glutamine and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were either left unstimulated or incubated with antigen for 44 h at 37°C (5% CO_2).

Flow cytometry

Staining was performed as previously described[9] using: from BD anti-CD3-PerCP-Cy5.5 (SK7), CD4-AF700 (RPA-T4), CD25-APC (2A3) and OX40 (CD134)-PE (L106); CD45RO-ECD (UCHL1; Beckman Coulter) and from eBioscience, San Diego, CA, USA, CD127-eFluor450 (eBioRDR5) and CD39-PECy7 (A1). The IOTest® Beta Mark Kit (Beckman Coulter) was used according to manufacturer's instructions, in combination with anti-CD3 and anti-CD4 mAbs. A four-laser LSRII flow cytometer (BD) was used and analysis performed using FlowJo software (v8.8.7 Treestar Inc, Ashland, OR, USA).

Cell sorting

Cell sorting was performed using a three-laser FACS Aria II cell sorter (BD) to > 95% purity. For analysis of TCR diversity, 10 000 cells were sorted from each population.

Analysis of TCR diversity by 5′RACE PCR

Total RNA was extracted from sorted cell populations utilising TRIzol reagent (Invitrogen) as per the manufacturers' protocol. T-cell receptor clonotypes were analysed using 5′ Rapid Amplification of cDNA Ends (RACE) PCR (Clontech), as previously described.[25] Briefly, RNA was reverse transcribed using the SMARTer™ cDNA RACE Kit (Clontech Laboratories, Inc, Mountain View, CA, USA) and then purified using the Wizard® SV gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to manufacturers' instructions. The TRBV region was amplified using the Advantage® 2 PCR enzyme system and the SMARTer™ cDNA RACE Kit (Clontech Laboratories, Inc) and the MBC2 reverse primer (5′-TGCTTCTGATGGCTCAAACAC AGCGACCT-3′; Sigma-Aldrich, St Louis, MO, USA). Gel-purified TRBV DNA was ligated by TA cloning into the pCR®4-TOPO®vector, using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) and transformed into OneShot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen). Transformed cells were grown on LB agar plates containing 100 μg mL^{-1} ampicillin (selective for transformed cells containing a ligated vector). Colonies were transferred to each well in a 96-well PCR plate and vector inserts amplified using Platinum® Taq DNA Polymerase High Fidelity PCR (Invitrogen) with M13 forward (5′-TTTTCCCAGTCACGAC-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers (Sigma-Aldrich). Sanger sequencing of purified TRBV DNA was performed at the Australian Genome Research Facility (Sydney, Australia). Sequences were analysed by using the ImmunoGenetics (IMGT) V-quest database and are reported using IMGT nomenclature.[26] TRBV sequences were only included in analysis if they were functional rearranged coding sequences.

CONFLICT OF INTEREST

RPA and JT-D are co-inventors of patents pertaining to the use of gluten peptides in therapeutics, diagnostics and non-toxic gluten; both are shareholders of Nexpep Pty Ltd and RPA also of ImmusanT, Inc. (USA). RPA is Chief Scientific Officer, and JT-D is a consultant to ImmusanT, Inc. NS, JZ and ADK are named inventors on a patent for the use of CD39 and the OX40 assay to identify antigen-specific Tregs, held by St Vincent's Hospital, Sydney, Australia. Full disclosure was provided to all study participants.

AUTHOR CONTRIBUTIONS

LC performed experiments, acquired, analysed and interpreted data and wrote the manuscript; CMLM, NS, MYH and JZ contributed to experiment design and optimisation and critically reviewed the manuscript; RPA and JT-D recruited coeliac disease patients, provided reagents, contributed to experiment design and critically reviewed the manuscript; and ADK and DVB contributed to study concept, design, supervision and critical revision of manuscript.

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Supporting Information

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