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C-terminal citrullinated peptide alters antigen-specific APC:T cell interactions leading to breach of immune tolerance

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A B S T R A C T

In rheumatoid arthritis, the emergence of anti-citrullinated autoimmunity is associated with HLA-antigen-T cell receptor complexes. The precise mechanisms underpinning this breach of tolerance are not well understood. *Porphyromonas gingivalis* expresses an enzyme capable of non-endogenous C-terminal citrullination with potential to generate citrullinated autoantigens. Here we document how C-terminal citrullination of ovalbumin peptide$^{323-339}$ alters the interaction between antigen-presenting cells and OTII T cells to induce functional changes in responding T cells. These data reveal that C-terminal citrullination is sufficient to breach T cell peripheral tolerance *in vivo* and reveal the potential of C-terminal citrullination to lower the threshold for T cell activation. Finally, we demonstrate a role for the IL-2/STAT5/CD25 signalling axis in breach of tolerance. Together, our data identify a tractable mechanism and targetable pathways underpinning breach of tolerance in rheumatoid arthritis and provide new conceptual insight into the origins of anti-citrullinated autoimmunity.

1. Introduction

Citrullination is a post-translational modification catalysed by peptidylarginine deiminases (PAD), which convert the native amino acid arginine to non-encoded citrulline, and in doing so alter the structure, function, and potential immunogenicity of modified proteins [1]. For reasons not fully understood, two-thirds of patients with rheumatoid arthritis (RA) develop autoimmunity to citrullinated proteins [2]. This autoimmunity is evidenced by the emergence of autoantibodies, known as anti-citrullinated protein antibodies (ACPA) [3], which recognise host proteins containing citrulline residues [4]. Over time, this breach of tolerance culminates in dysfunctional immune signalling and the emergence of altered immune cell phenotypes capable of infiltrating joints. Once in the joint, these cells perpetuate altered stromal cell activation, leading to synovitis and later chronic destructive arthritis [2,5]. For many patients, disease progression can be controlled through immunotherapies targeted toward the dysfunctional immune signalling associated with later stages of the disease [6]. However, the precise mechanisms underpinning breach of immune tolerance and the emergence of anti-citrullinated autoimmunity are not well understood. Elucidation of the very early events initiating breach of tolerance to citrullination are crucial to develop novel therapies capable of preventing breach of tolerance or re-instating self-tolerance.

Genetic risk factors for RA implicate human leukocyte antigen (HLA) and effector CD4 T cells in disease pathogenesis [7]. The strongest genetic risk for ACPA positive RA is conferred through HLA class II alleles, specifically HLA-DRB1 alleles containing an amino acid sequence known as the shared epitope (SE) [8]. These HLA-DRB1-SE alleles are capable of presenting citrullinated peptides to autoreactive T cells more efficiently than native peptides [9,10]. Meanwhile, non-HLA risk alleles are

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preferentially expressed in CD4 T cells [11], and both experimental and clinical research indicate a role for autoreactive CD4 T cells in the pathogenesis of RA [12,13]. As such, presentation of citrullinated antigens to T cells in the periphery is implicated in disease pathogenesis.

Genetic risk factors alone are insufficient to account for the prevalence of anti-citrullinated autoimmunity in RA [14]. The interaction between genetic risk factors with environmental exposures, such as smoking or microbial dysbiosis, substantially increase the risk of anti-citrullinated autoimmunity [15,16]. This strong dependence on environmental context, together with the observation that anti-citrullinated protein antibodies (ACPA) can appear in patient serum up to 10 years prior to the onset of clinical symptoms [17], have led to the hypothesis that breach of tolerance originates outside the joint at mucosal surfaces; specifically in the lung, gut and/or periodontal tissues [18]. Following the recent observation that smoking and HLA-SE risk alleles operate at different stages of RA development [19], it has been postulated that the emergence of anti-citrullinated autoimmunity is not necessarily restricted to HLA-SE-alleles. Rather, HLA-SE alleles are predicted to operate after the initial breach of tolerance by driving the maturation of anti-citrullinated autoimmunity via epitope spreading and fuelling the progression towards inflammatory arthritis [19,20]. Therefore, understanding how environmental risk factors drive citrullination at mucosal surfaces and how this alters immune recognition are crucial to understand the mechanisms underpinning breach of immune tolerance.

Porphyromonas gingivalis is a bacterial species associated with microbial dysbiosis and chronic inflammation of the periodontium, leading to periodontitis. Periodontitis shares pathogenic features and is epidemiologically associated with RA [21]. Mechanistically, infection with *P. gingivalis* is hypothesised as a potential trigger for anti-citrullinated immunity [22]. *P. gingivalis* is the only bacterial species known to express its own PAD enzyme, referred to as PPAD [23]. Unlike human PADs, PPAD preferentially citrullinates C-terminal arginine residues, giving rise to non-endogenous citrullinated peptides. Conveniently, *P. gingivalis* also expresses an arginine gingipain capable of cleaving host proteins at arginine peptide bonds [24]. Incubation of candidate autoantigens with *P. gingivalis* results in the generation of host-peptides with C-terminal citrullination [25], demonstrating the potential of PPAD to generate citrullinated neoepitopes. However, it remains unclear whether C-terminal citrullination is sufficient to breach T cell peripheral tolerance.

Here we used T cell receptor (TCR) transgenic OTII mice, whose CD4 T cells express a TCR specific for ovalbumin peptide$^{323\text{-}339}$ (pOVA) to demonstrate the capacity for C-terminal citrullinated pOVA (pOVA:cit) to alter APC:T cell interactions and breach antigen-induced T cell peripheral tolerance *in vivo*. We propose that pOVA:cit lowers the threshold for T cell activation and show that this is dependent upon the IL-2-STAT5-CD25 signalling axis. Together our data reveal C-terminal citrullination alters peptide recognition with marked functional changes in responding T cells.

2. Materials and methods

2.1. Animals

Female C57bL/6 mice, aged 6 weeks, were purchased from Envigo, UK. CD45.1^{+} OTII mice were produced in house (Central Research facility, University of Glasgow, UK). Animals were maintained on a 12-h light/dark cycle, with food and water provided *ad libitum*. All procedures were performed in accordance with a UK Home Office Licence and with the Animals Scientific Procedures Act (ASPA) 1986.

2.2. Peptides

The peptides used in this study (pOVA, amino acid sequence ISQAVHAAHAEINEAGR, and pOVA:Cit, amino acid sequence ISQAVHAAHAEINEAG[cit]) were synthesised by ProImmune Limited (Oxford, UK). Peptides were aliquoted and stored at −80 °C and were thawed the day of the assay and diluted to the required concentration. They were never frozen or thawed more than once. The peptides were quantified before use by absorbance at 205 nM using nanodrop.

2.3. In vitro T cell proliferation assay

Spleens were isolated from OTII mice. Single cell suspensions were treated with red cell lysis buffer (eBioscience). Splenocytes were resuspended at 2.5 × 10^{6} cells/ml in complete RPMI (Gibco) containing 2 mM L-glutamine (Invitrogen, UK), penicillin (100 μg/ml), streptomycin μg/ml and 10% foetal calf serum (all Invitrogen, UK). To round bottom 96-well plates (Corning, UK), 100 μl splenocytes was added to each well. Peptides were added to cells to give final concentration of 0.01, 0.1, 1 and 10 μg/ml in a final volume of 300 μl. In each independent assay, each peptide, at each concentration was assessed in duplicate. The proliferative response and activation of OTII T cells were immediately evaluated by flow cytometry at 72- and 120-h post-stimulation. Cell culture supernatants were collected at stored at −20 °C until required for cytokine quantification by enzyme-linked immunosorbent assay (ELISA).

2.4. Optical trapping system

The system, optimisation and validation of the optical trapping system used here has previously been described [26]. Briefly, a triple-spot optical trap is used to bring a T-cell into contact with a denditic cell, setting the time and location of the interaction. Once the cells are in contact the optical trap is turned off and re-instated 120 s later 5 μm from the T cell. The laser beam power is slowly increased until the T cell separates from the dendritic cell. The relative interaction force is determined from the laser power at which the T cell returned to the optical trap.

Dendritic cells were generated from bone marrow of C57BL/6 mice by culture in cRPMI and 10% of culture supernatant from X63 myeloma cells transfected with mouse GM-CSF, as previously described [27]. Mature dendritic cells were plated at a final concentration of 5 × 10^{6}/ml and activated with 1 μg/ml LPS (Sigma-Aldrich) and/or antigen pulsed with 1 μg/ml pOVA or pOVA:cit for 6 h at 37 °C in 5% CO_2. Following stimulation or antigen pulsing, DCs were washed in cRPMI medium to remove soluble antigen and re-plated onto microscope chamber slides for optical trapping experiments or tissue culture plates for 72 h for measurement of IFNγ from cell culture supernatants by ELISA.

2.5. Isolation and purification of OTII T cells

Single cell suspensions were prepared from the lymph nodes (excluding mesenterics) and red blood cell lysed spleens of OTII mice. Pooled single cell suspensions were subject to immunomagnetic purification using the CD4^{+} T cell negative isolation kit for mice (Miltenyi Biotech, UK). The purity of CD3^{+}CD4^{+} T cells was confirmed by expression of Vα2 and Vβ5 by flow cytometry.

2.6. Adoptive transfer of transgenic CD45.1 OTII T cells

In some experiments purified OTII T cells were stained with Invitrogen™ CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry, according to the manufacturer's instructions. For transfer into female C57BL/6 syngeneic recipients, CFSE labelled or unlabelled purified OTII T cells were resuspended in sterile phosphate buffered saline (PBS) at a concentration of 1.5 × 10^{7}/ml, and 200 μl cell suspension was transferred to individual mice via injection into the lateral tail vein.

2.7. In vivo T cell proliferation

Following transfer of purified transgenic OTII T cells labelled with CFSE on day 0, 24-h later, each mouse was challenged intravenously with either 10 μg pOVA or pOVA:cit +50 ng LPS (Sigma-Aldrich, UK) in 200 μl PBS. Transferred cells were left to expand *in vivo* for 3 further days, or to expand and contract *in vivo* for 10 days. On post-transfer days 4 and 10, mice were euthanised by CO_2 asphyxiation and major lymph nodes harvested to prepare single cell suspensions for immediate analysis by flow cytometry or *ex vivo* restimulation assay.

2.8. Ex vivo restimulation to pOVA

At the end of certain *in vivo* experiments, single lymph node cell suspensions were assessed for recall responses to pOVA. Lymphocyte cell suspensions were added at a concentration of 5×10^5 cells/well in 96-well round bottom plates. pOVA was added at a concentration of 1 μg/ml and assays were assessed after 96-h culture. At the end of the assay, cells were analysed by flow cytometry and cell culture supernatants were collected and stored at −20 °C until required for cytokine quantification by ELISA.

2.9. Induction of oral tolerance to ovalbumin

Peripheral tolerance to ovalbumin was induced via oral administration of ovalbumin in drinking water as previously described [28,29]. Ovalbumin (Sigma-Aldrich, UK) was dissolved in sterile water at 50 mg/ml, sterile filtered through a 0.22 μM membrane and added to sterile water bottles. Mice in treatment groups were offered ovalbumin in the drinking water, *ad libitum*, for 10 days, refreshed daily. Mice in control groups received sterile distilled water.

2.10. Induction of delayed-type hypersensitivity reaction

DTH was induced in accordance with previous studies [30,31]. On day 16, post transfer of transgenic $CD45^+$ OTII T cells, ovalbumin-fed and control mice were immunised in the scruff with 40 μg pOVA or pOVA:cit emulsified in Complete Freund's adjuvant (MD Bioproducts, UK). On day 35, mice were challenged in the right footpad with 50 μl PBS containing 100 μg heat-aggregated OVA (HAO). Mice were weighed and monitored daily for signs of footpad swelling. Contralateral control and challenged footpads were measured using callipurs. The degree of footpad swelling for each mouse was calculated by subtracting the measurement of control footpads from challenged footpads. At the end of the experiment, mice were sacrificed by CO_2 asphyxiation. Axillary and brachial lymph nodes were collected for immediate analysis by flow cytometry or *ex vivo* restimulation to pOVA.

2.11. Generation of mouse bone marrow-derived DCs and co-culture with $CD4^+$ T cells

Femurs and tibiae of C57BL/6 mice were removed and purified from the surrounding muscle tissue; thereafter, intact bones were left in 70% ethanol for 2–5 min for disinfection and washed with cRPMI. The bone marrow was flushed with RPMI with 100 units/ml penicillin and 100 μg/ml streptomycin using a syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting and the cells were washed twice in RPMI with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were filtered with a 70 μm filter before cell counting and plating.

The cells were plated at 1.5×10^6 cells/ml per well of a 6-well plate. Cells were cultured at 37 °C with 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Sigma-Aldrich) and 20 ng/ml GM-CSF or 20 ng/ml GM-CSF + 20 ng/ml IL-10 (PeproTech). Cells were incubated for 6 days; cultures were refreshed on days 3 and 6 with a complete RPMI medium supplemented with GM-CSF or GM-CSF + IL-10. On day 6, the DCs with GM-CSF only were treated with 0.1 μg/ml LPS (Sigma-Aldrich) and incubated for 16 h; these were matured BMDCs (mat-DC). Tolerogenic BMDCs (tol-DC) are BMDCs that were generated using GM-CSF + IL-10 during maturation. The confirmation of DC phenotype was confirmed using FACS (Supplementary Fig. 2).

On day 7, Mat-DCs and Tol-DCs were harvested, washed, counted, and then treated with 0.1 μg/ml pOVA and pOVA:cit. The cells were incubated for 2 h and then co-cultured with CFSE-labelled $CD4^+$ T cells from OTII mice at a 1:10 (DC/T cell) ratio. The cells were incubated for 72- and 120-h and then harvested for flow cytometry analysis. The supernatants were collected at the end of each incubation time point to quantify secreted cytokines using Luminex.

In some experiments, anti-mouse CD25 antibody (PC61.5) and IgG1 isotype control (eBRG1) were added to co-cultures tol-DC + pOVA:cit co-cultures at 2 μg/ml (both eBioscience). Mouse recombinant IL-2 (RP-8605 - Thermo-fisher scientific) was mixed 1:10 with IL-2 Monoclonal Antibody (S4B6 -eBioscience) prior to adding to tol-DC + pOVA co-cultures at a final concentration of 1.5 μg IL-2: 15 μg IL-2 mAB.

2.12. Flow cytometry

Single cell suspensions of lymphocytes or splenocytes were washed in PBS with anti-CD16/32 (eBioscience, UK) for 15 min at 4 °C before incubation was antibody cocktails in FACs buffer (PBS + 2 mM EDTA) for 20 min. Dead cells were excluded by use of a Live/Dead Fixable viability dye (Molecular Probes, UK). Lymphocytes were stained with combinations of the following antibodies to detect expression of cell surface markers. Antibodies were obtained from eBioscience, Biolegend and BD PharMingen: CD3ε (17A2), CD4 (GK1.5), CD45.1 (A20), CD71 (R17217), CD25 (PC61), CD62L (DREG-56), CXCR5 (L138D7), CD44 (IM7). For intracellular staining, cells were fixed and permeabilised in BD Permwash and stained with combinations of c-myc (D84C12 – New England Biolabs LTD), IRF4 (3E4) and/or FoxP3 (MF-14). Samples were acquired on a BD LSRFortessa (BD Biosciences) or Macsquant (Miltenyi) and analysed with FlowJo software (TreeStar).

2.13. Phosflow

Following 24 h of BMDC:OTII T cell co-culture, cells were harvested and fixed immediately by addition of 20 vol warmed Phosflow Lyse/Fix buffer (BD Biosciences, UK) and incubation at 37 °C for 10 min. Cells were permeabilised using Perm Buffer III (BD Biosciences, UK) for 30 min on ice. Cells were washed in staining buffer (BD Biosciences, UK) and incubated with anti-CD16/32 on ice. Antibodies compatible with phosflow reagents were obtained from BD Biosciences UK: CD3 (17A2), CD4 (RM4-5), CD25 (7D4), Monoclonal (47/Stat5(pY694). Samples were acquired on a BD LSRFortessa (BD Biosciences).

2.14. Cytokine analysis

Cell culture supernatants were collected from co-cultures at times indicated for each experiment and assessed for concentration of cytokines using Mouse IL-2 Duo set ELISA and Mouse IFNγ Duo set ELISA (both R&D Systems, UK). In some experiments, cytokines in cell culture supernatants were quantified using Luminex (Biotechne, UK).

2.15. Statistical analysis

All graphs and statistical analyses were produced using GraphPad Prism 9 (GraphPad Software Inc, San Diego, CA, USA. All data are presented as mean and standard deviation, unless otherwise indicated. Statistical testing between 2 independent groups was performed using Students *t*-test. Statistical testing between a single-independent variable and more than 2 groups was performed using analysis of variance (ANOVA) with Tukey multiple comparison. Statistical testing between

two-independent variables and 2 or more groups was performed using Two-Way ANOVA with Holm-Sidak multiple comparison.

3. Results

3.1. C-terminal citrullinated peptide is recognised by and differentially activates OTII T cells

The proliferative response of $CD3^+CD4^+$ T cells from OTII mice to titrated doses of native pOVA or pOVA:cit was compared over time. OTII

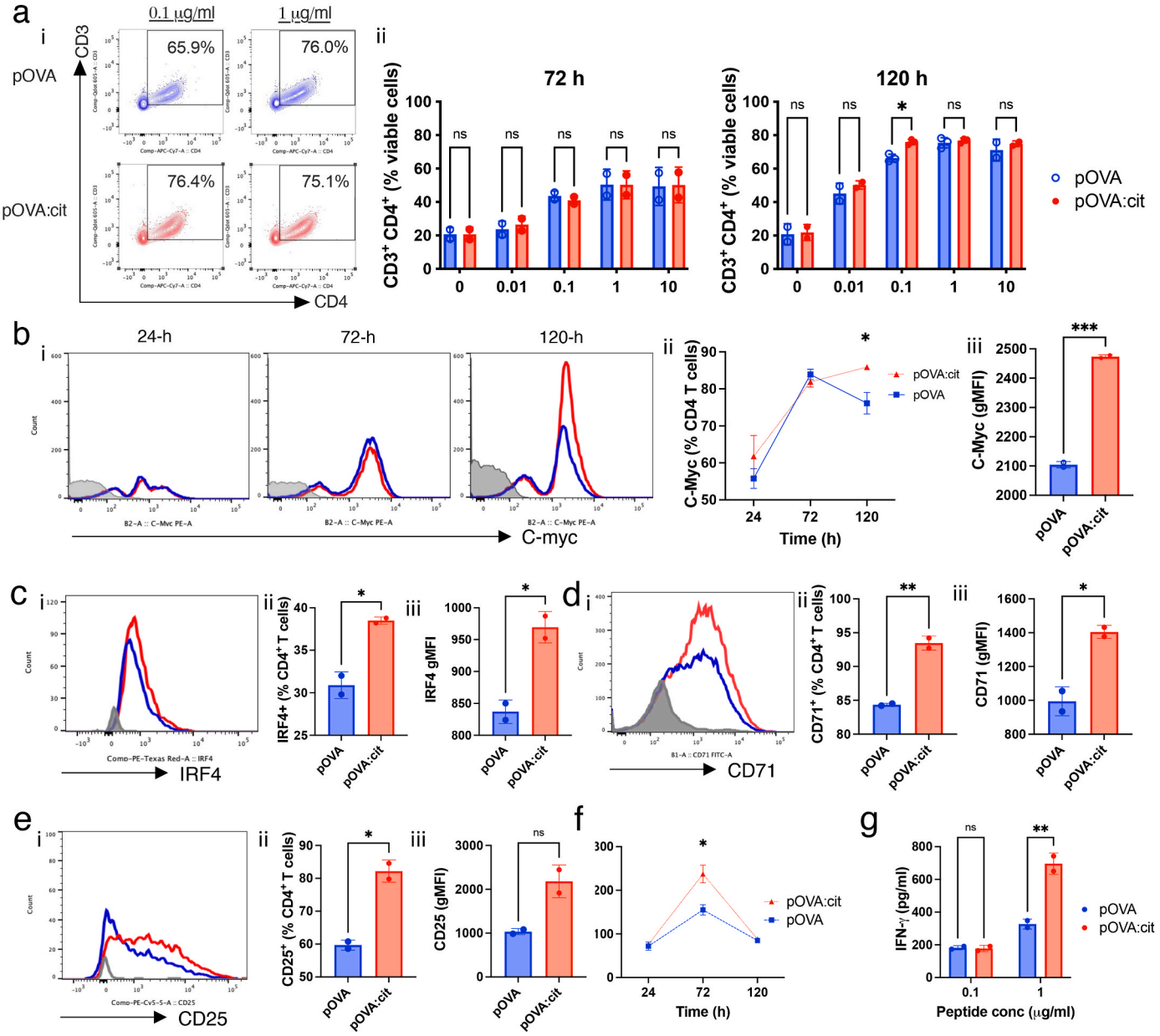

Fig. 1. C-terminal citrullinated peptide is recognised by and differentially activates OTII T cells.
a) Representative flow cytometry plots, with proportions inset, of $CD3^+CD4^+$ T cells at 120-h post-stimulation with pOVA or pOVA:cit at indicated concentrations (gated on viable cells)(i) average proportion of $CD3^+CD4^+$ T cells by peptide dose and time (ii). b) Representative histograms of c-myc staining on $CD3^+CD4^+$ T cells at indicated times following stimulation with 0.1 µg/ml pOVA or pOVA:cit (grey: media control, blue: pOVA, red: pOVA:cit) (i), line graph showing average proportion of c-myc^+ CD4 T cells over time (ii), average gMFI of c-myc^+ CD4 T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit (iii). **c)** Representative histograms of IRF4 staining on $CD3^+CD4^+$ T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit (grey: media control, blue:pOVA, red:pOVA:cit) (i), average proportion (ii) and gMFI (iii) of $IRF4^+$ CD4 T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit. d) Representative histograms of CD71 staining on $CD3^+CD4^+$ T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit (grey: media control, blue:pOVA, red:pOVA:cit) (i), average proportion (ii) and gMFI (iii) of $CD71^+$ CD4 T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit. **e)** Representative histograms of CD25 staining on $CD3^+CD4^+$ T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit (grey: media control, blue:pOVA, red:pOVA:cit) (i), average proportion(ii) and gMFI (iii) of $CD25^+$ CD4 T cells at 120-h post-stimulation with 0.1 µg/ml pOVA or pOVA:cit. **f)** The concentration of IL-2 (pg/ml) in cell culture supernatants over time. **g)** The concentration of IFN- γ (pg/ml) in cell culture supernatants at 120-h stimulation by peptide dose. All data are mean and SD, n = 2/group, representative of 2–4 independent experiments.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by Two-way Anova with Sidak's multiple comparison, or Students t-test, ns = not significant.

T cells recognised and proliferated in response to both pOVA and pOVA:cit (Fig. 1a). At each peptide dose examined, the proliferative response to pOVA and pOVA:cit initially proceeded at the same rate, with equivalent proportions of T cells up to the 72-h timepoint (Fig. 1a). Stimulation with 0.1 μg/ml pOVA:cit resulted in an increased proportion of T cells compared with pOVA at the 120-h timepoint (Fig. 1a). There was no indication of a differential T cell response to pOVA:cit at peptide concentrations above or below 0.1 μg/ml. The 1 μg/ml peptide dose appeared to represent the maximal response, with no measurable increase in the proportion of T cells at higher dose of either peptide, while the 0.01 μg/ml peptide failed to induce a substantial increase in the proportion of T cells for either peptide.

The extent of signalling through the T cell receptor (TCR) is determined by the quantity and quality of peptide:MHC interactions with the TCR [32]. Subsequently, the extent of TCR signalling influences downstream signalling pathways to determine the potency of T cell activation and proliferation [32]. To investigate whether the increased proliferative capacity of OTII T cells stimulated with 0.1 μg/ml pOVA:cit was associated with altered peptide:MHC interaction compared with pOVA, we examined the expression of a range of transcription factors and activation markers known to be associated with quantitative and/or qualitative changes in TCR signalling strength.

The transcription factor myelocytomatosis oncogene (c-myc) is critical for controlling the metabolic reprogramming required to sustain T cell effector expansion [33,34], with the proportion of CD4 T cells expressing c-myc directly proportional to the strength of the stimulus through the TCR [35]. Following stimulation with 0.1 μg/ml pOVA or pOVA:cit, the proportion of T cells expressing c-myc increased concurrently from 24- to 72-h (Fig. 1b). By 120-h post stimulation there was a divergence in the proportion of T cells expressing c-myc, with a significantly higher proportion in cultures stimulated with pOVA:cit compared with pOVA (Fig. 1b). While TCR signalling strength determines the frequency of cells expressing c-myc, at later time points co-stimulatory signals sustain and determine the quantity of c-myc expression per cell [35]. The geometric mean fluorescence intensity (gMFI) of c-myc$^+$ OTII T cells at 120-h was significantly higher per cell in pOVA:cit stimulated T cells compared with pOVA stimulated (Fig. 1b).

The transcription factor IRF4 co-ordinates the metabolic changes required to sustain effector functions, and the quantity of IRF4 expression is directly proportional to TCR signalling strength [36,37]. The proportion and per cell expression of IRF4 was significantly higher in pOVA:cit stimulated OTII T cells compared with pOVA stimulated T cells (Fig. 1c).

We next investigated the expression of cell surface activation markers CD71 and CD25, whose expression is upregulated upon, and correlated with, the strength of TCR signalling [35,38]. The proportion and per cell expression of T cells expressing the transferrin receptor CD71 at 120-h post-stimulation with pOVA:cit was significantly higher compared with pOVA stimulated cells (Fig. 1d). Similarly, the proportion of T cells expressing CD25 was significantly higher following stimulation with pOVA:cit compared with pOVA; however, the elevated per cell expression of CD25 on pOVA:cit stimulated cells did not reach statistical significance (Fig. 1e).

Autocrine IL-2 co-stimulation is crucial for maintaining proliferation of T cells in response to antigen [39]. The concentration of IL-2 in cell culture supernatants from both pOVA and pOVA:cit stimulated cultures increased from 24- to 72-h and then decreased by 120-h, presumably due to consumption by T cells during the proliferative response (Fig. 1e). At 72-h the concentration of IL-2 was significantly higher in cell culture supernatants from pOVA:cit stimulated cultures compared with pOVA stimulated (Fig. 1f).

Activation of OTII T cells with pOVA, is associated with differentiation to Th1 and release of IFNγ. Stimulation with either peptide at 0.1 μg/ml resulted in a similar concentration of IFNγ in the cell culture supernatants (Fig. 1g). However, at 1 μg/ml peptide we observed a significantly higher secretion of IFNγ into the cell culture supernatants of pOVA:cit stimulated compared with pOVA stimulated (Fig. 1a). These data suggest that where the proliferative response was maximal, pOVA:cit altered OTII T cell recognition leading to enhanced effector function compared with OTIIs stimulated with pOVA. Together, these data demonstrate that pOVA:cit is recognised by and differentially activates OTII T cells and further indicate that pOVA:cit is associated with quantitative and/or qualitative changes in the interaction with the OTII TCR compared with pOVA.

3.2. C-terminal citrullinated peptide enhances the interaction between dendritic cells and OTII T cells and is associated with enhanced effector function

Quantitative and qualitative changes in peptide:MHC interactions with the TCR are associated with altered physical interactions between antigen-presenting cells (APCs) and T cells [32,40]. Optical trapping is a non-invasive approach to measure the interaction force between single cell pairs and can provide a relative measure of the strength of physical interaction between APCs and T cells [26]. Within the detectable range of the assay, OTII T cells incubated with bone marrow-derived dendritic cells (BMDCs) presenting pOVA, required greater force to separate the T cell:BMDC pairs compared with OTII T cells incubated with (BMDCs) in the absence of antigen (Fig. 2a). OTII T cells incubated with BMDCs presenting pOVA:cit required significantly greater force to separate the T cell:BMDC pairs compared with BMDCs presenting pOVA. Commensurate with initial observations in whole spleen cell cultures, purified OTII T cells cultured with BMDC presenting pOVA:cit released significantly greater levels of IFNγ compared with OTII T cells responding to BMDCs with pOVA (Fig. 2b).

3.3. C-terminal citrullinated peptide enhances proliferation of OTII T cells in vivo

OTII T cells differentially respond to pOVA:cit *in vitro*, and this differential response was associated with enhanced interaction between OTII T cells and APCs presenting pOVA:cit. We next investigated whether pOVA:cit differentially activates OTII T cells *in vivo*. Following adoptive transfer of CFSE labelled OTII CD45.1$^+$ T cells into syngeneic hosts, mice were immunised with either pOVA, pOVA:cit or PBS control. At 3 days post-immunisation, OTII T cells were harvested from major lymph nodes and examined for CFSE divisions (Fig. 3a).

The average proportion of CD45.1$^+$ OTII T cells was significantly higher in pOVA:cit immunised mice compared with pOVA immunised mice (Fig. 3b). Visual inspection of representative CFSE divisions within these populations revealed a striking difference in the division pattern between the two peptides. At 3 days post-immunisation, both peptides had induced at least 7 rounds of cell division as indicated by the number of CFSE peaks (Fig. 3c). CFSE histograms from mice immunised with pOVA:cit were substantially skewed to the left, relative to mice immunised with pOVA. Quantification of the average proportion of CD45.1$^+$ OTII T cells at each cell division revealed that pOVA:cit immunisation resulted in fewer CD45.1$^+$ cells having divided only 2 or 3 times, while a higher proportion had undergone 6 or more rounds of cell division compared with pOVA immunised mice (Fig. 3c). The proliferation index revealed that on average, across 3 independent experiments, CD45.1$^+$ T cells recovered from mice immunised with pOVA had expanded 4.86-fold relative to naïve transfer control (NTC) mice. In comparison, expansion of CD45.1$^+$ T cells recovered from mice immunised with pOVA:cit was significantly higher at 8.54-fold relative to NTC mice (Fig. 3c, proliferation index). Despite these substantial differences in proliferative capacity, we found no difference in the proportion or per cell expression of CD25, CD71, IRF4 or Ki67 at 3 days post-immunisation (data not shown).

In separate, replicate experiments, CD45.1$^+$ T cells were evaluated 10 days after immunisation (Fig. 3d). The proportion of CD45.1$^+$ T cells

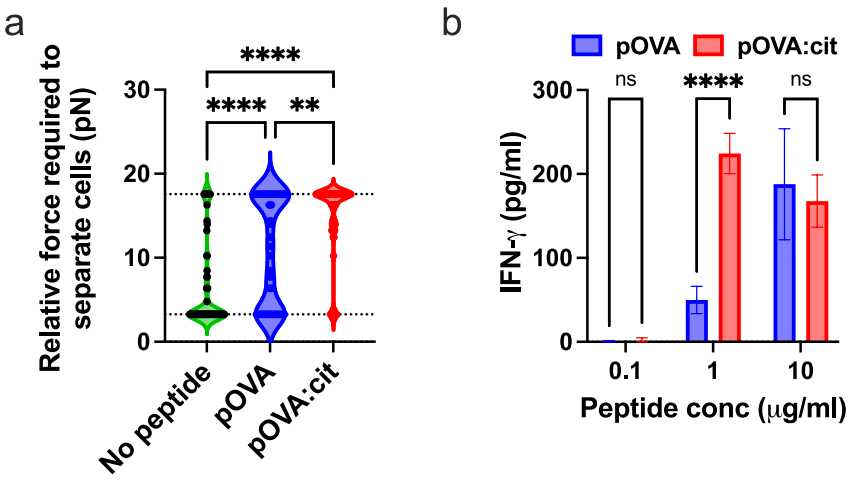

Fig. 2. C-terminal citrullinated peptide enhances the interaction between BMDCs and OTII T cells and is associated with enhanced effector function **a)** Violin plot depicting the relative force (picoNewtons) required to separate the interaction between OTII T cells and BMDCs in the absence of peptide, or presence of 1 μg/ml pOVA or pOVA:cit. Each data point represents the force required to separate an individual cell pair, n = 80/group. Dotted black lines represent the upper and lower limits of forces detectable by the optical trapping system. ****p < 0.001, p < 0.01 by Kruskal-Wallis with Dunn's multiple comparisons. **b)** Concentration of IFN- γ (pg/ml) in cell culture supernatants assessed by ELISA after 72-h co-culture of OTIIs with bone-marrow-derived dendritic cells (BMDCs) with varying concentrations of pOVA or pOVA:cit. Data shown are mean and SD, n = 3/group. ****p < 0.001 by two-way ANOVA with Holm-Sidak comparison.

in the pooled lymph nodes of pOVA:cit-immunised mice was significantly higher compared with pOVA-immunised mice (Fig. 3e). Thus, the increased proliferative response following immunisation with pOVA:cit gave rise to a larger sustained population of transgenic cells *in vivo*. We next investigated the recall response of these cells to pOVA. Following 96 h *ex vivo* re-stimulation with pOVA, the proportion of pOVA:cit-primed cells increased to a significantly greater extent compared with pOVA primed cells (Fig. 2F). Thus, OTII T cells stimulated with pOVA:cit retained the ability to respond with enhanced proliferation to pOVA. Furthermore, we recorded significantly higher concentrations of IFNγ and IL-2 in the cell culture supernatants from pOVA:cit-primed CD45.1$^+$ cells recalled to pOVA compared with pOVA-primed CD45.1$^+$ cells (Fig. 2F).

3.4. Administration of C-terminal citrullinated peptide breaches antigen-induced peripheral T cell tolerance in vivo

We next examined whether OTII T cells exposed to tolerogenic stimuli *in vivo* subsequently elicit a differential response to pOVA:cit compared with pOVA. To establish a functional output to measure T cell tolerance, we employed ovalbumin-induced oral tolerance [28,29], combined with a delayed-type hypersensitivity (DTH) model [30,31]. Following transfer of OTII T cells, tolerance was induced by addition of ovalbumin to drinking water for 10 days prior to systemic immunisation with either pOVA or pOVA:cit. Later, the DTH response was assessed by measuring the extent of footpad swelling following footpad administration of heat-aggregated ovalbumin (HAO) (Fig. 4a). In mice that did not consume ovalbumin in the drinking water, systemic immunisation with pOVA, followed by HAO in the footpad (pOVA-primed) caused substantial paw swelling relative to contralateral control paws. In comparison, mice who consumed ovalbumin and were subsequently immunised systemically with pOVA, followed by HAO in the footpad (tolerised-pOVA) had significantly less paw swelling (Fig. 4b). These data demonstrate that feeding ovalbumin induced functional tolerance.

Mice who consumed ovalbumin and were subsequently immunised systemically with pOVA:cit, followed by HAO in the footpad (tolerised-pOVA:cit) presented with significantly greater paw swelling compared with tolerised-pOVA mice at 24-h post HAO (Fig. 4b). Indeed, there was no discernible difference in the extent of paw swelling between tolerised-pOVA:cit mice compared with pOVA:cit-primed mice. These data suggest that the interaction between OTII T cells and pOVA:cit was sufficient to breach antigen-induced peripheral T cell tolerance in this model.

Lymph nodes draining the immunisation site were harvested and immunophenotyped by flow cytometry. There was no statistical difference in the proportion of endogenous CD4$^+$ T cells or transferred CD45.1$^+$ T cells (Supplementary figure 2). We next performed *ex vivo* restimulation to pOVA on cell suspensions isolated from draining lymph nodes. There was no statistically significant difference in the proportion of transgenic CD45.1$^+$ T cells or endogenous CD4$^+$ T cells following *ex vivo* restimulation (Fig. 4c), or in the proportion of CD45.1$^+$ T cells expressing CD25. However, the quantity of CD25 expressed per cell (gMFI) was significantly higher in tolerised-pOVA:cit compared with all other groups (Fig. 4d). A higher proportion of cells expressed CD71 in tolerised-pOVA compared with pOVA-primed. However, the per cell expression of CD71 was significantly higher in tolerised-pOVA:cit compared with pOVA:cit-primed (Fig. 4e). In cell culture supernatants, the concentration of IFNγ was significantly higher from tolerised-pOVA:cit cultures restimulated to pOVA compared with pOVA-primed cultures (Fig. 4f). There was no difference in the concentration of IL-2 between any of the groups.

3.5. C-terminal citrullinated peptide induces proliferation and activation of OTII T cells cultured with tolerogenic BMDCs

To further dissect the phenotype of tolerised OTII T cells responding to pOVA:cit we used a tolerogenic DC model. BMDCs were cultured under conditions to induce mature (mat-DC) or tolerogenic DCs (tol-DC). Phenotypic analysis revealed that mat-DC expressed high levels of MHCII, CD80, CD86, and IL-12, meanwhile tol-DC expressed low levels of these markers and high levels of IL-10 (Supplementary figure 1). Either mat-DC or tol-DC were co-cultured with CFSE-labelled OTII T cells in the presence of either pOVA or pOVA:cit. The phenotype of the DCs remained unchanged irrespective of peptide used in the culture (not shown). Inspection of CFSE histograms at 72- and 120-h post-stimulation revealed that OTII T cells cultured with tol-DCs + pOVA failed to divide to the same degree as OTII T cells cultured with mat-DCs + pOVA (Fig. 5a). In contrast, OTII T cells cultured with tol-DCs in the presence of pOVA:cit underwent several rounds of cell division.

The proliferative response by OTII T cells cultured with tol-DCs + pOVA:cit was accompanied by robust T cell activation compared with tol-DCs + pOVA. At both 72- and 120-h post-stimulation, the quantity of CD71 and CD25 expression on OTII T cells cultured with tol-DCs + pOVA:cit was equivalent to the level expressed by OTII T cells cultured with mat-DCs + pOVA, and was significantly higher compared with OTII T cells cultured with tol-DCs + pOVA (Fig. 5b and c). These data indicate that OTII T cells responding to pOVA:cit are less dependent on, and can potentially bypass co-stimulatory signals normally required to stabilize signalling through the OTII TCR to drive activation and proliferation.

The concentration of IL-2 was significantly lower in cell culture supernatants from tol-DC co-cultures compared with mat-DCs co-cultures for both peptides and there was no difference in the concentration of IL-2

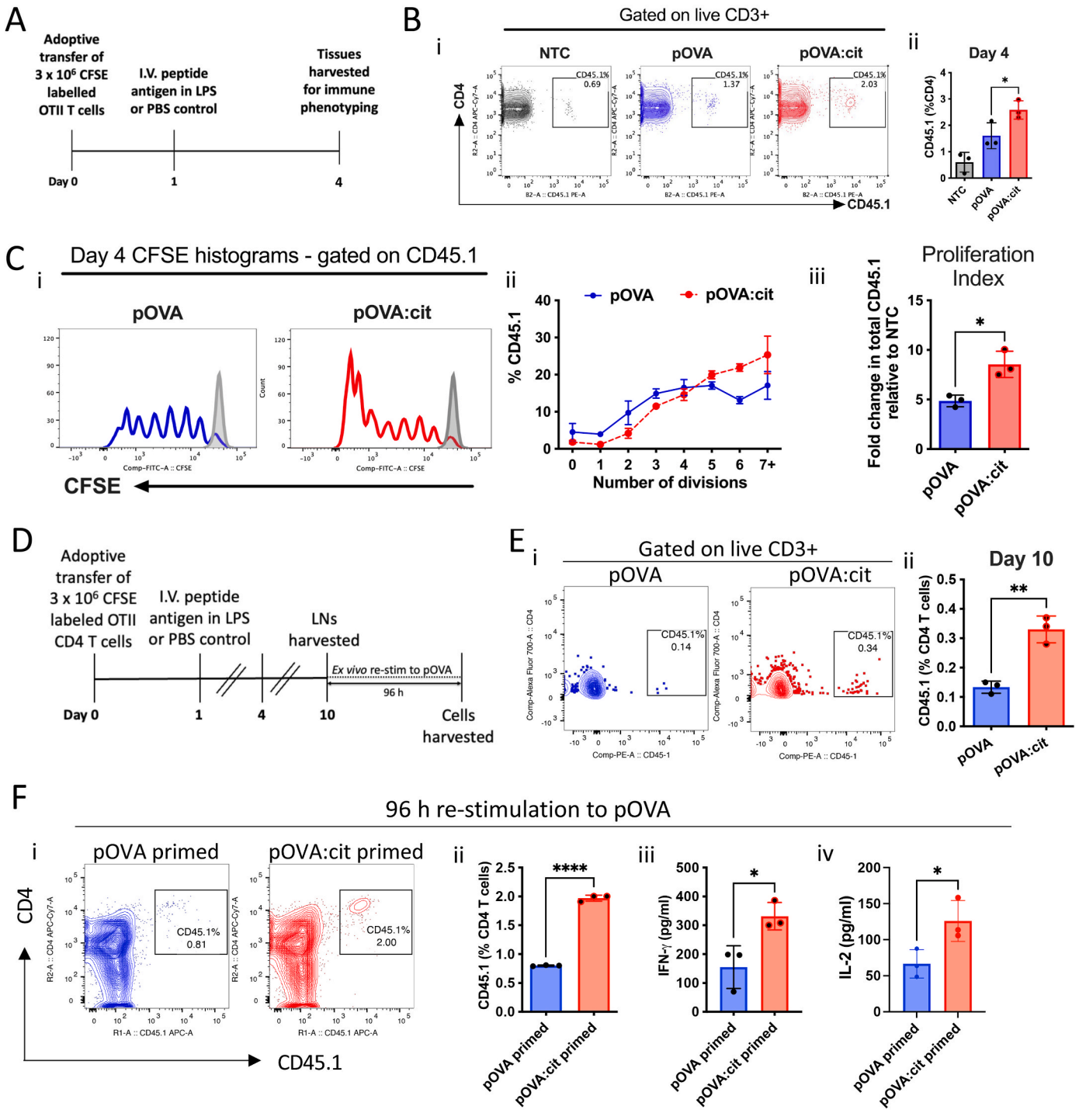

Fig. 3. C-terminal citrullinated peptide enhances proliferation of OTII T cells *in vivo*.
a) Female C57bl/6J recipient mice received 3×10^6 CFSE labelled CD45.1$^+$ OTII T cells on day 0. Transfer recipient mice received 10 μg/ml pOVA or pOVA:cit, each with 50 ng LPS, or PBS control 24 h later by IV administration into the lateral tail vein. Lymph nodes were harvested 3 days post-immunisation (PI) and CD45.1$^+$ cells were examined by flow cytometry for CFSE divisions and expression of activation markers. **b)** Representative flow cytometry plots (with proportions inset (i)), average proportion of total CD45.1$^+$ T cells in each group (ii). Data are n = 3 mice/group and are representative 3 independent experiments, *p > 0.05 by Students t-test. **c)** Representative histograms showing CFSE divisions of CD45.1$^+$ lymphocytes in mice challenged with pOVA or pOVA:cit. Grey histograms represent CFSE divisions in naïve transfer recipient control mice (NTC) (i). Line graph showing the proportion of CD45.1$^+$ cells at each CFSE division, n = 3 mice/group and representative of 3 independent experiments (ii). Proliferation index of fold change in the number of CD45.1$^+$ cells at 3-day PI, data are average of 3 independent experiments, relative to the number of CD45.1+ T cells in NTC mice, *p > 0.05 by Students t-test (iii). d) In separate, replicate experiments mice were left for 10-days PI, lymph nodes were harvested and analysed immediately by flow cytometry and cell suspensions restimulated to pOVA (1 μg/ml) for 96 h to investigate *ex vivo* recall response. e) The proportion of CD45.1$^+$ T cells at 10 day PI from pOVA and pOVA:cit immunised mice. Representative flow cytometry plots of CD45.1 proportion of CD4 T cells with proportions inset (i). Average CD45.1 proportion, data are mean and SD, n = 3 mice/group, **p > 0.005 by Students t-test (ii). f) Ex vivo restimulation with pOVA. Representative flow cytometry plots showing proportion of CD45.1$^+$ T cells 96-h post recall to pOVA (i), the average proportion of CD45.1$^+$ T cells in pOVA and pOVA:cit primed mice recalled to pOVA, data are mean and SD (3 mice/group), ****p < 0.0001 by Students t-test (ii). The concentration of IFN-γ (iii) and IL-2 (iv) in cell culture supernatants at 72-h. Data are mean and SD, n = 3/group, *p < 0.05 by Students t-test.

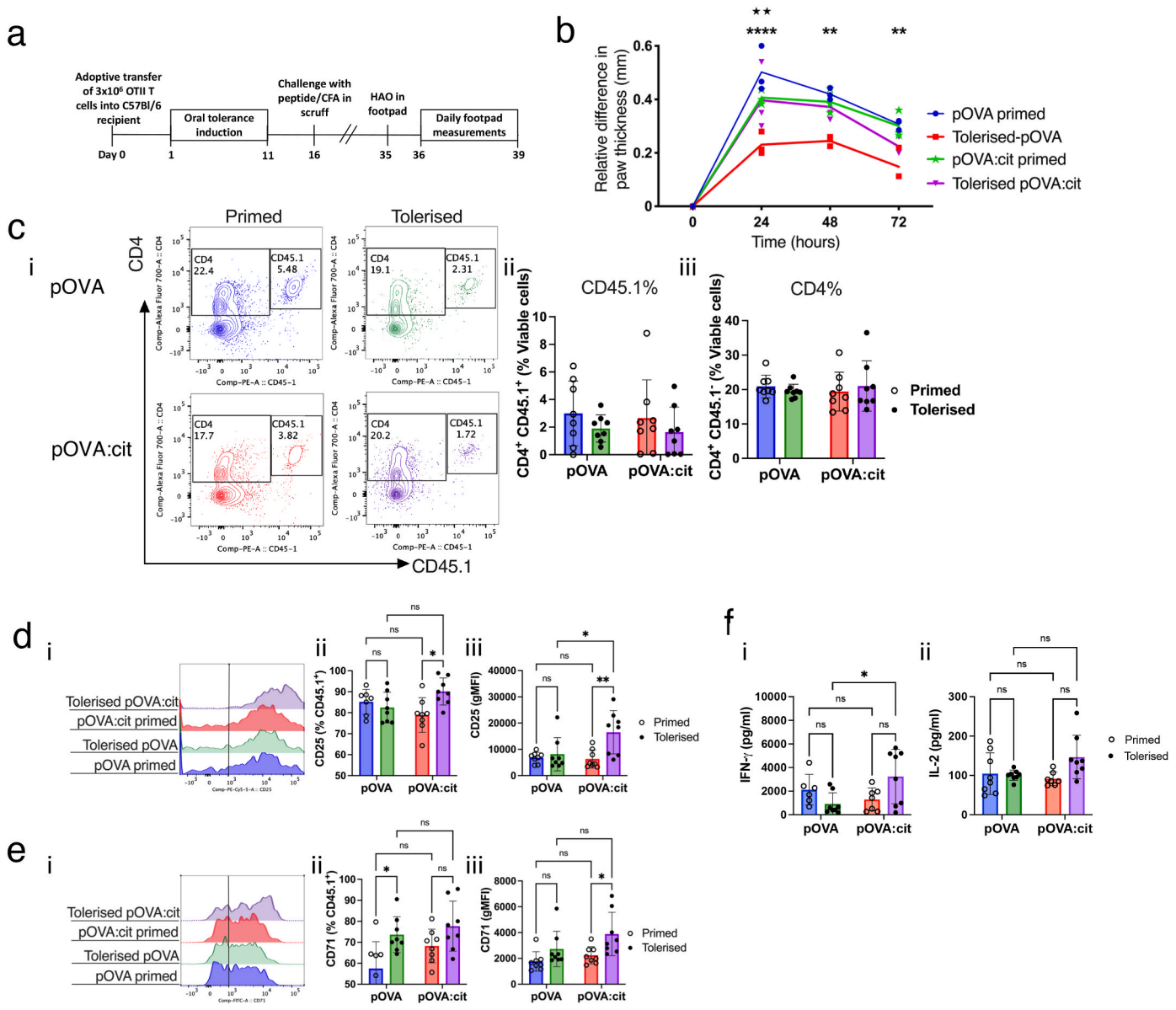

Fig. 4. Administration of C-terminal citrullinated peptide breaches antigen-induced peripheral tolerance *in vivo*.
a) Female C57bl/6J recipient mice received 3×10^6 CD45.1$^+$ OTII T cells on day 0. Oral tolerance was induced by adding 50 mg/ml ovalbumin in drinking water for 10 days. Control groups did not receive ovalbumin in drinking water. On day 16, mice were challenged with pOVA or pOVA:cit (40 μg/ml) emulsified in CFA in the scruff. On day 35, mice were challenged with heat-aggregated OVA (HAO) in the footpad. Daily footpad measurements were performed for contralateral control and test paws for each mouse prior to HAO challenge on day 35 and daily thereafter from 36 to 39 using calipurs. b) Relative difference in paw thickness (mm). Each data point represents the average of a single independent experiment, each with n = 8 mice/group, with a total of 3 independent experiments. Lines represent the average group mean over time. ★★$p < 0.01$: tolerised pOVA vs tolerised pOVA:cit, ****$p < 0.001$, **$p < 0.01$: pOVA primed vs tolerised pOVA by Two-way ANOVA with Holm-Sidak comparison. c) The proportion of endogenous CD4$^+$CD45.1$^-$ and transferred CD4$^+$CD45.1$^+$ cells following 96-h *ex vivo* restimulation to pOVA. Representative flow cytometry plots (with proportions inset) (i), the average proportion of total CD4$^+$CD45.1$^-$ (ii) and CD4$^+$CD45.1$^+$ cells in each group (iii). Data are n = 8/group, with no significant differences by Two-Way ANOVA. d) Representative histograms of CD25 staining (i), average proportion of CD25$^+$ (% CD45.1$^+$) (ii), average CD25 gMFI (iii). Data are n = 8/group, *$p < 0.05$, **$p < 0.01$ by Two way-ANOVA with Holm-Sidak multiple-comparison. e) Representative histograms of CD71 staining (i), average proportion of CD71$^+$ (% CD45.1+) (ii), average CD71 gMFI (iii). Data are n = 8/group, *$p < 0.05$, by Two way-ANOVA with Holm-Sidak multiple-comparison. f) The concentration (pg/ml) of IFN-γ (i) and IL-2 (ii) in cell culture supernatants at 96-h. Data are mean and SD, n = 8/group, *$p > 0.05$ Two way-ANOVA with Holm-Sidak multiple-comparison. ns = not significant.

between tol-DC co-cultures for either peptide at 72-h post-stimulation (Fig. 5d). There was no difference in the secretion of IFNγ into cell culture supernatants in the presence of either peptide (Fig. 5d).

Despite low levels of IL-2 from tol-DC + pOVA:cit co-cultures, the high level of CD25 expression on OTII T cells – and robust proliferative response - strongly indicate that TCR ligation and IL-2/CD25 signalling have likely occurred.

3.6. Proliferation and activation of OTII T cells cultured with tolerogenic BMDCs is associated with IL-2 signalling via CD25

We next examined the IL-2/CD25 signalling capacity of OTII T cells cultured with tol-DCs. Expression of CD25 is regulated at the transcriptional level by TCR signal strength and later via IL-2 in a positive feedback loop directly regulated by STAT5 [41,42]. We hypothesised that altered TCR ligation by pOVA:cit is a sufficient stabilising signalling

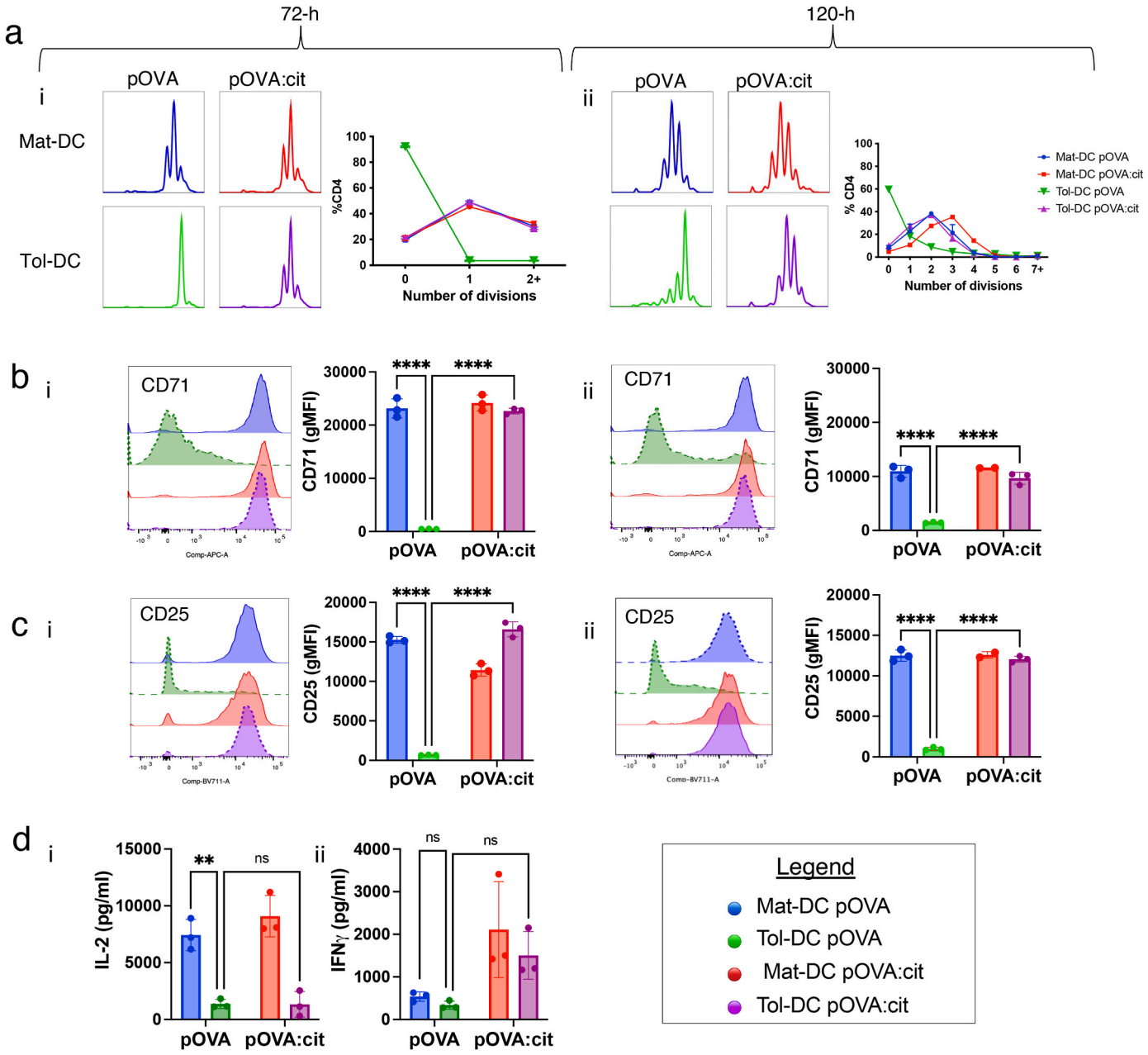

Fig. 5. C-terminal citrullinated peptide induces proliferation and activation of OTIIs cultured with tolerogenic BMDCs a) Representative histograms of CFSE divisions of OTII T cells following 72-h (i) and 120-h (iii) co-culture with mat-DC or tol-DC in the presence of either pOVA or pOVA:cit at 0.1 μg/ml, with line graphs showing the average proportion of $CD4^+$ OTII T cells per CFSE division peak. b) Representative histograms of CD71 staining, average CD71 gMFI at 72-h (i) and 120-h (ii) post-stimulation with peptide. c) Representative histograms of CD25 staining, average CD25 gMFI at 72-h (i) and 120-h (ii) post-stimulation with peptide. **d**) The concentration (pg/ml) of IFN-γ (i) and IL-2 (ii) in cell culture supernatants at 72-h post-stimulation with 0.1 μg/ml peptide, as measured by Luminex. All data are n = 3/group. Day 3 data are representative of 2 independent experiments. $^{**}p < 0.05$, $^{****}p < 0.001$ by Two-way ANOVA with Holm-Sidak comparison.

through the TCR, even in the absence of robust co-stimulation, to support early IL-2/CD25 signalling, which in turn, supports the ability of OTII T cells to proliferate under tolerising conditions. We examined changes in the quantity of IL-2 and CD25 expression at an early timepoint, and quantified STAT5 phosphorylation as a read out of IL-2/CD25 signalling. OTII T cells were co-cultured with tol-DCs + pOVA:cit and a CD25 blocking antibody or isotype control antibody [35]. Meanwhile, we used an IL-2/CD25 antibody complex [43] to attempt to replicate IL-2/CD25 signalling in OTIIs co-cultured with tol-DCs + pOVA.

We did not observe any difference in the concentration of IL-2 from the cell culture supernatants of tol-DC co-cultures with either pOVA or pOVA:cit at 24-h post-stimulation (Fig. 6a). However, in tol-DC + pOVA:cit co-cultures containing the CD25 blocking antibody - but not isotype control antibody - the concentration of IL-2 was significantly higher in the cell culture supernatants compared with tol-DC + pOVA:cit co-cultures (Fig. 6a). To determine whether the low levels of IL-2 in cell culture supernatants from tol-DC co-cultures with either pOVA or pOVA:cit might be associated with consumption due to IL-2R signalling, we quantified the cell surface expression of CD25. The proportion of OTII T cells expressing CD25 was significantly higher following 24 h co-culture with tol-DCs + pOVA:cit + isotype control compared with pOVA (Fig. 6b). There was no difference in the proportion of OTII T cells positive for CD25 in tol-DCs + pOVA:cit with and without isotype control (not shown). Compared with the isotype control, addition of the CD25 blocking antibody to OTII:Tol-DC + pOVA:cit co-cultures significantly reduced the proportion of cells positive for CD25. In contrast,

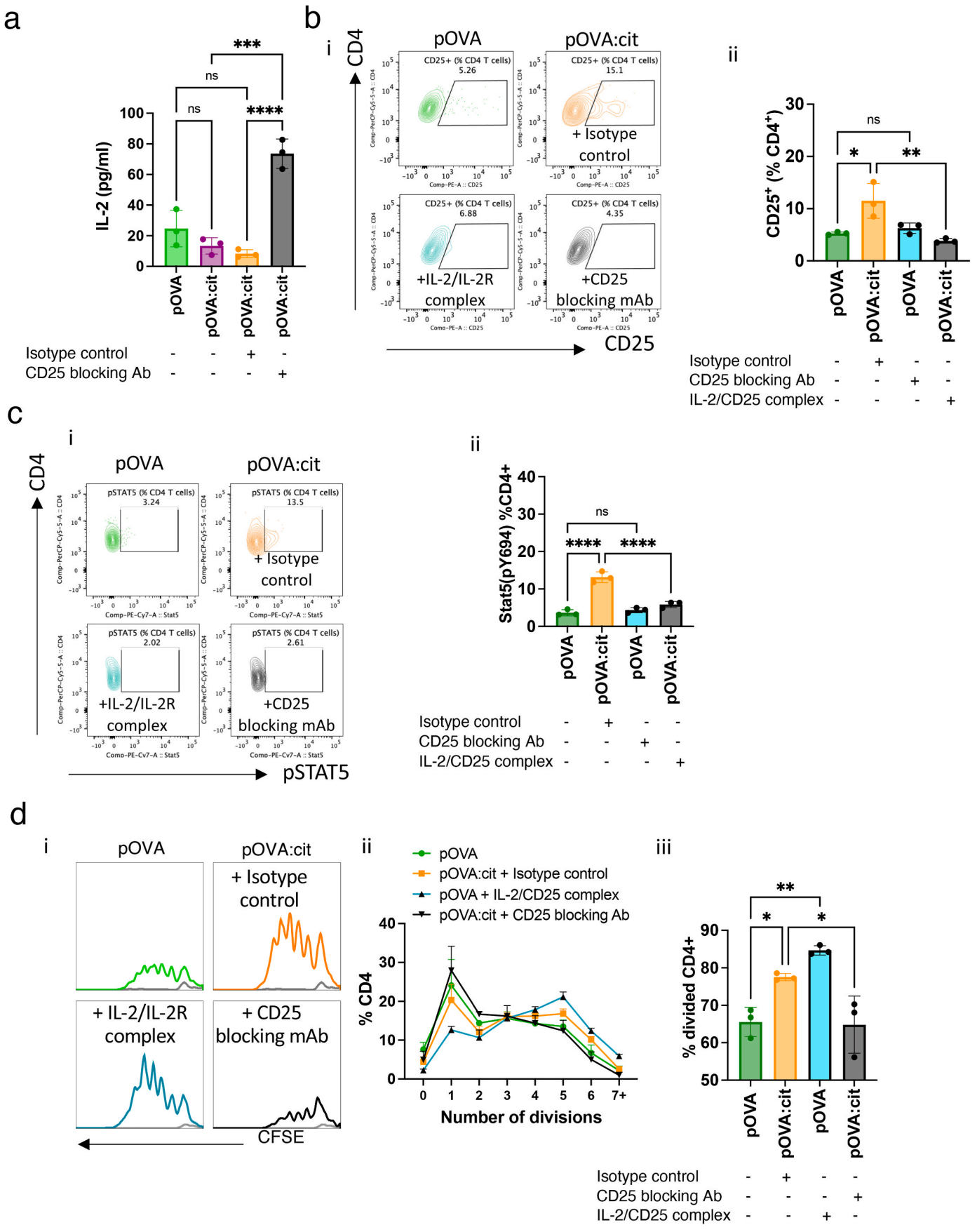

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Fig. 6. C-terminal citrullinated peptide induced proliferation and activation of OTIIs cultured with tolerogenic BMDCs is blocked by inhibition of signalling via CD25 a) Concentration of IL-2(pg/ml) in cell culture supernatants from OTII:tol-DC co-cultures after 24-h stimulation with 0.1 μg/ml pOVA, pOVA:cit, pOVA:cit + isotype control antibody (eBRG1) or pOVA:cit + CD25 blocking antibody (PC61.5). b) Representative flow cytometry plots (i), and the average proportion (ii) of OTIIs expressing CD25 at 24-h post-stimulation with 0.1 μg/ml pOVA, pOVA:cit + isotype control antibody (eBRG1), pOVA + IL-2/CD25 (S4B6) or pOVA:cit + CD25 blocking antibody (PC61.5). c) Representative flow cytometry plots (i), and the average proportion (ii) of OTIIs positive for Stat5(pY694) at 24-h post-stimulation with 0.1 μg/ml pOVA, pOVA:cit + isotype control antibody (eBRG1), pOVA + IL-2/CD25 (S4B6 or pOVA:cit + CD25 blocking antibody (PC61.5). d) Representative histograms of CFSE divisions (i), average proportion of $CD4^+$ T cells per CFSE division (ii), and total divided OTII T cells (iii) at 72-h following stimulation with 0.1 μg/ml pOVA, pOVA:cit + isotype control antibody (eBRG1), pOVA + IL-2/CD25 (S4B6 or pOVA:cit + CD25 blocking antibody (PC61.5). Data are mean and SD, n = 3/group. *p < 0.05, **p > 0.01, ***p < 0.005, ****p < 0.001 by Two-way ANOVA with Holm-Sidak comparison. ns = not significant.

addition of IL-2/CD25 antibody complex had no impact on CD25 expression at this time (Fig. 6b). These data indicate that the low levels of IL-2 in cell culture supernatants from tol-DC + pOVA:cit - but not tol-DC + pOVA – are likely due to signalling via IL-2R. This conclusion was supported by the observation that the proportion of OTIIs positive for STAT5 (pY694) following 24 h co-culture with tol-DCs + pOVA:cit + isotype was significantly higher compared with OTIIs cultured with tol-DCs + pOVA (Fig. 6c). Moreover, the proportion of cells positive for STAT5 phosphorylation was significantly reduced following administration of CD25 blocking antibody to OTII:Tol-DC + pOVA:cit co-cultures. Addition of the IL-2/CD25 antibody complex had no impact on the proportion of OTIIs positive for pSTAT5 at this timepoint (Fig. 6a).

To determine the impact of manipulating IL-2/CD25/pSTAT5 signalling on proliferation, we investigated the proliferative response of OTII T cells at 72-h post-stimulation. Inspection of CFSE division plots revealed that OTIIs cultured with Tol-DCs + pOVA:cit + isotype divided to a greater extent compared with OTIIs cultured with Tol-DCs + pOVA (Fig. 6d). This proliferative response was significantly reduced, but not ablated in the presence of the CD25 blocking antibody. Meanwhile, addition of IL-2/CD25 complex enhanced the proliferative response of OTIIs co-cultured with Tol-DCs + pOVA, leading to a significantly greater proportion of total divided cells compared with tol-DCs + pOVA without the IL-2/CD25 complex (Fig. 6d).

4. Discussion

Here we document, hitherto unknown capacity of C-terminal citrullinated peptide to alter the outcome of APC:T cell interactions, leading to breach of peripheral T cell tolerance. OTII T cells recognise and respond to C-terminal citrullinated pOVA with enhanced proliferative capacity, activation status and effector function compared with the response to native peptide. This was associated with stronger physical interactions between APCs and OTII T cells in the presence of pOVA:cit compared with pOVA. We further document that OTII T cells primed with pOVA:cit retained the capacity to respond with enhanced proliferative capacity and effector function to subsequent challenge with pOVA. Together these data demonstrate that pOVA:cit is associated with quantitative and qualitative changes in the interaction between APCs and OTII T cells, and we demonstrate that the outcome of this interaction was sufficient to breach functional T cell tolerance. We propose that the threshold for T cell activation is, in effect lowered by presentation of pOVA:cit, since OTII T cells cultured with tolerogenic DCs expressing low levels of co-stimulatory signals was sufficient to prevent T cell activation and proliferation in response to pOVA but not pOVA:cit. Finally, our data implicate the IL-2/STAT5/CD25 signalling axis in breach of tolerance and suggest that this pathway may be amenable for therapeutic manipulation.

Citrullination (and other PTMs relevant to RA autoimmunity) are not unique to RA, they occur naturally *in vivo*, are essential for normal physiological functioning, and in health are not overtly immunogenic [44]. Yet, recognition of citrullinated epitopes is arguably the earliest initiating event mediating breach of self-tolerance in RA. There are several mechanisms through which citrullination is proposed to modify the immunogenicity of proteins. In this regard, the bulk of evidence exists in relation to presentation of citrullinated peptides by HLA-DRB1-SE alleles, where peptides with citrulline residues are accommodated in SE binding pockets with higher affinity than peptides with native arginine residues [9,10,45,46]. The premise of this autoreactivity is that citrullination - in the gums of patients with periodontitis or the lungs of smokers - generates neo-epitopes, which in the context of HLA-DRB1-SE are recognised by autoreactive T cells that may have escaped thymic deletion [22]. A role for citrulline specific autoreactive T cells has been demonstrated in patients with established RA [45], patients at risk of RA and with early untreated RA [47].

To investigate functional breach of tolerance, we employed an OVA-induced DTH model combined with ovalbumin-induced oral tolerance. This combined model produces a transient monoarticular arthritis that enables interrogation of the role of antigen specificity in breach of tolerance [30,31]. A recent study using the same combined model corroborated our data by demonstrating that ovalbumin feeding prior to antigenic challenge successfully induced T cell tolerance [48]. We show that the OTII TCR can recognise and respond to both native and citrullinated versions of antigen, and that in the context of both naivety and peripheral tolerance, the different versions of the peptide induce functional changes in responding T cells. Thus, our data support the hypothesis that T cells that escape thymic deletion to enter the periphery, but which remain unresponsive to self-antigens due to weak affinity and/or active regulation, may be capable of recognising and responding to citrullinated self-antigens. An alternative hypothesis, which our data do not address but also do not exclude, is the potential for non-autoreactive T cells to provide help to cross-reactive B cells, which could be a driver for ACPA generation. Future studies could use transgenic TCR models to evaluate whether B cell activation and antibody responses differ following T cell exposure to citrullinated self antigen or microbial antigens.

Notably, our combined model was not restricted to HLA-SE alleles, as both the transgenic and syngeneic mice expressed the I-Ab haplotype. However, the ability to present citrullinated peptides with enhanced binding affinity is neither a prerequisite of, nor fully restricted to, HLA-SE alleles [49]. This is supported by the observation that around 20% of seropositive RA patients do not express HLA-SE alleles [8]. Therefore, there are likely to be additional alternative pathogenic events initiating anti-citrullinated autoimmunity, at least in a subset of RA patients. Indeed, several studies have documented that citrullination within the 9-aa MHC binding 'core' only infrequently impacts HLA-SE binding capacity and that citrullinated residues are often found in non-anchor positions, including the C-terminus positions P10 and P11 [41,46]. Furthermore, enhanced MHCII binding does not reliably predict enhanced T cell responses [46]. Recently, it has been suggested that HLA-SE independent T cell responses are involved in the initial breach of tolerance to citrullination; while the observation that HLA-SE risk alleles associate more strongly with the transition to pre-clinical symptomatic RA suggests a role for HLA-SE-restricted T cell responses specifically in the maturation of the anti-citrulline immune response [19,20]. Our data highlight the potential for non-endogenous C-terminal citrullination to precipitate breach of immune tolerance, which may be unrestricted to HLA-SE.

Many CD4 T cells are critically dependent on residues that lie out with the core 9-aa epitope. These critical but non-anchoring peptide-flanking regions are often enriched in the C-terminus at positions P10 and P11 [50,51] and are proposed to help stabilize peptide MHC:TCR

complexes, for example by forming hairpin turns [52–54]. The OTII TCR recognises a 9-aa core epitope (329–337) of pOVA [55]. Thus, the citrullinated residue (339) on pOVA:cit is predicted to be in the peptide-flanking region, and available to interact with the OTII TCR. Truncation of pOVA at the C-terminal appeared to have no effect on OTII T cell function [55]; while citrullination of the C-terminal of a haemaglutinin peptide from influenza A resulted in modified CD4 T cell response [56]. Our data demonstrate that C-terminal citrullination altered T cell recognition, and intriguingly illustrate the potential for C-terminal citrullination to bypass co-stimulatory immune checkpoints. Thus, the capacity of *P. gingivalis* to degrade proteins and C-terminal citrullinate host- or microbial-derived peptides in periodontal tissues, represent processes with potential to precipitate breach of immune tolerance.

There is evidence that breach of tolerance to citrullination can occur within periodontal tissues. Host PADs and PPAD, together with both host and bacterial citrullinated proteins are detected at higher levels in the periodontal tissues of patients with periodontitis, and/or RA, compared with healthy controls [57–59]. Antibodies to citrullinated human α-enolase, an immunodominant autoantigen in RA, were shown to cross-react with antibodies to citrullinated *P. gingivalis* enolase [60]. Additionally, gingival reactive B cells displaying cross-reactivity with *P. gingivalis* and citrullinated host peptides have been identified [61]. These studies indicate that immunity to citrullination may result from molecular mimicry, whereby an immune response directed towards bacterial citrullinated epitopes cross-react with human citrullinated epitopes. While these studies focussed on antibody and B cell cross-reactivity, the identification of class-switched IgG antibodies cross-reactive with bacterial and host citrullinated epitopes implicates T cell help with the emergence of cross-reactive autoantibodies. Currently, the precise autoantigens responsible for breach of tolerance remain obscure - and identification and isolation of autoreactive T cells in the gingiva remains a significant challenge. However, our model system demonstrates the potential for an antigen-specific T cell to cross-react with both the native and C-terminal citrullinated epitope. We suggest our data support the hypothesis that C-terminal citrullination represents an alternative pathogenic mechanism with potential to initiate anti-citrullinated autoimmunity and are congruent with the mucosal origin, neo-epitope, and molecular mimicry hypotheses.

Our primary aim was to investigate the impact of non-endogenous C-terminal citrullination on the interaction between APC and CD4 T cells in the context of immunity and tolerance. We selected the pOVA:OTII model antigen system, as pOVA contains a native C-terminal arginine available for citrullination. We did not investigate the impact of citrullination at internal sites of the pOVA peptide, (which has no internal arginine residues) nor did we investigate the impact of C-terminal citrullination in other model antigen systems, and these could merit further investigation. Peptide modifications have the potential to enhance or diminish the T cell:APC interaction [62]. In our model system, the C-terminal citrullination of pOVA appeared to reduce the need for co-stimulation for T cell activation and proliferation. The nuances of this phenomenon *in vivo* and in patients require detailed exploration. Our data provide evidence that C-terminal modification (not directly impacting peptide binding within the MHC Class II binding groove [55]) may influence the interaction between the TCR with MHC. The *in vivo* situation is likely to be impacted by the dose, persistence, and context of antigen presentation, which are likely important factors in overcoming peripheral T cell tolerance mechanisms [63].

Both *in vitro* and *in vivo*, breach of tolerance to pOVA:cit was associated with high levels of CD25 expression on responding T cells. We propose that pOVA:cit enhances signalling strength through the TCR, even in the absence of robust co-stimulation, leading to IL-2 signalling via CD25. Thus, this signalling axis is implicated in breach of tolerance. Further investigation of this pathway is required to fully elucidate its contribution to breach of tolerance *in vivo*. Notably, activation of STAT5 is mediated via activation of Janus Kinase 3 (JAK3) in response to IL-2 signalling [64], and this pathway is essential for IFNγ production by CD4 T cells [65]. Recently, JAK inhibitors have demonstrated efficacy in the treatment of RA patients [66]. In particular, the JAK3 inhibitor tofacitinib has been shown to reduce proliferation and IFNγ production by CD4 T cells [67] and reduce the T cell stimulatory capacity of DCs [68]. Thus, tofacitinib directly targets the pathways and cellular mechanisms we have identified in breach of tolerance to C-terminal citrullinated antigen. It will be of interest to elucidate whether tofacitinib has the potential to prevent autoimmunity or re-instate immune tolerance in periodontal tissues and whether this halts the subsequent dysfunctional immune signalling which eventually culminates in joint infiltration and destructive arthritis. Inhibition of citrullination has been explored through use of PAD inhibitors, developed to inhibit human PAD and thus reduce citrullination of internal arginine residues. This approach has shown efficacy in animal models and is associated with modulation of the T cell response [69]. Studies exploring inhibition of C-terminal citrullination by *P. gingivalis* PAD (PPAD) show that the periodontitis associated exacerbation of arthritis can be ameliorated by targeting PPAD [70] Thus, the pathways identified in these studies suggest there is merit in further exploring targeting of both T cell activation and citrullination as therapeutic avenues.

Author contributions

JM, MHN, JLB, AAP, LC and KP performed laboratory work. JPB and DG performed, collected and analysed the optical trapping data with support and supervision from AJW and ORM. JM had overall responsibility for data analysis with support and guidance from SC. SC, ORM and IBM conceived, and supervised the study. JM and SC wrote the manuscript. All authors have read, critically reviewed, and approved the manuscript.

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Declaration of competing interest

IBM has received grants and honoraria from BMS, Pfizer, Lilly, Abbvie, Gilead and Galapagos.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2023.102994.

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