On the connection between HLA and rheumatoid arthritis

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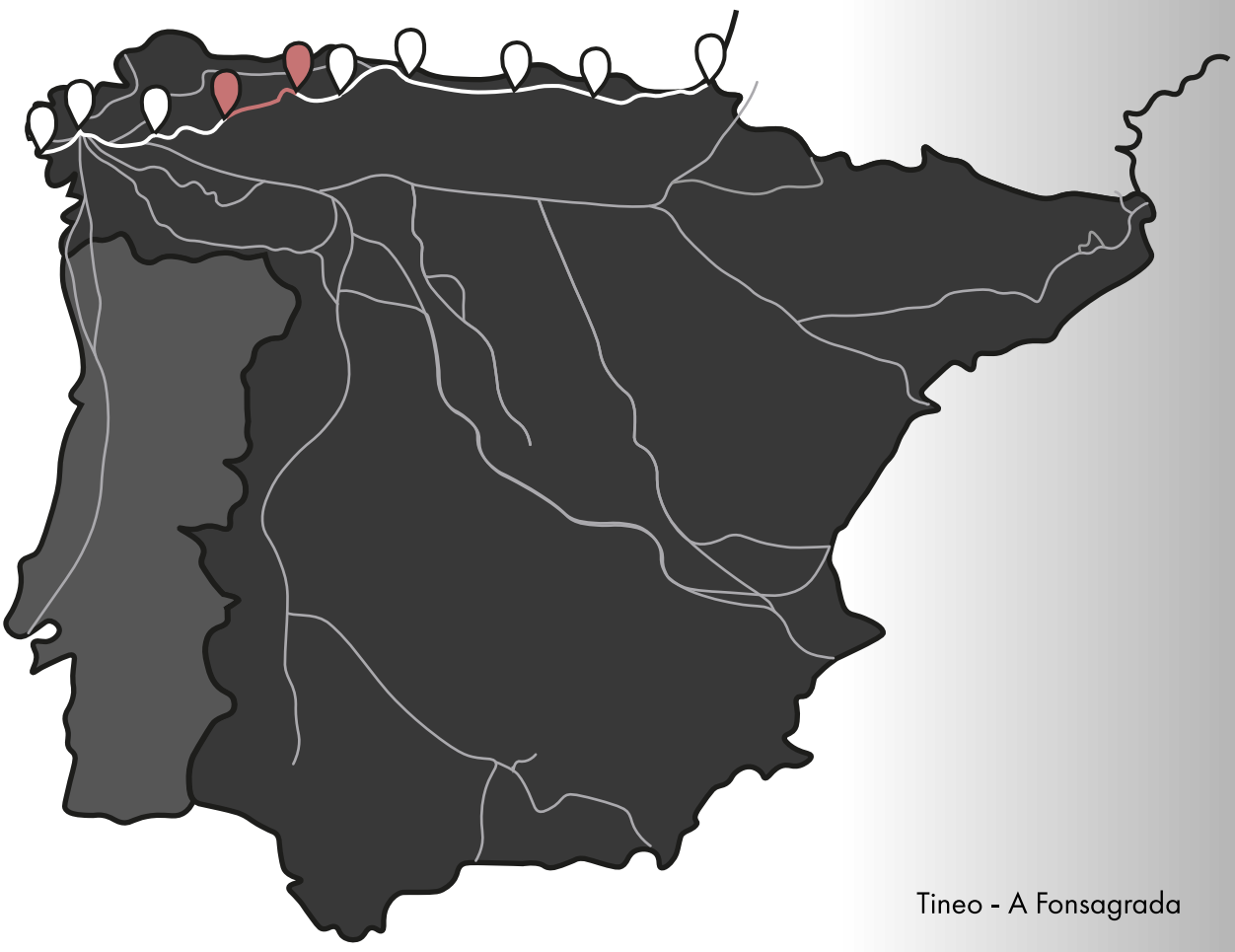

Tineo - A Fonsagrada

Chapter 6

Vinculin is an autoantigen that is processed and presented by human antigen-presenting cells

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Manuscript

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterised by autoantibodies targeting self-proteins. For B cells to receive proper T-cell help, they are required to take up and process antigens for presentation to T cells. Antigen-specific T cells are usually detected by the utilisation of synthetic peptides. Although detection of T cells by peptide-based approaches is important, they do not show whether the peptide can also be processed from the entire protein and presented by antigen-presenting cells (APCs). Vinculin has been implicated as an autoantigen in RA and an vinculin-derived epitope has been shown to be recognised by T cells. Here, we analysed whether a T-cell epitope from vinculin, "VCL-DERAA", can be naturally processed and presented by professional APC to T cells. To this end, a VCL-DERAA-directed T-cell receptor was introduced into a reporter T-cell line and cocultured with antigen-presenting cells loaded with the vinculin protein. We show that the reporter T-cell line expressing the VCL-DERAA T-cell receptor is activated after stimulation with the vinculin-loaded APCs. Thus, these data illustrate the VCL-DERAA epitope can be processed from the protein for presentation to T cells, furthering our understanding of the mechanism behind the involvement of T cells in the development of RA.

Introduction

Rheumatoid Arthritis (RA) is an autoinflammatory disease targeting the synovial joints. The disease can result in the destruction of the joints if not treated properly. One of the hallmarks of RA is the presence of autoantibodies, for example Rheumatoid Factor (RF) or Anti-Citrullinated Protein Antibodies (ACPA)(1, 2). Based on antibody status, RA patients can be subdivided into two groups, seropositive patients and seronegative patients(1, 3). These two patient groups differ from each other in disease severity as well as risk factors.(4) The risk factors, both environmental and genetic, are less defined for seronegative patients as compared to seropositive patients. For seropositive patients, studies have shown that among others, smoking (environmental), gender and the HLA locus (genetic) have an influence on the risk of developing seropositive RA disease(5). The HLA locus consists of many different genes involved in antigen-presentation and immune regulation. The most prominent genes shown to be involved in the increased risk of developing RA disease are the HLA class II genes(6, 7). More specifically, certain HLA-DRB1 alleles have been associated with increased risk for specifically seropositive RA disease development(8). Interestingly, a few other HLA-DRB1 alleles, mainly HLA-DRB1*13:01 and *13:02, have been shown to decrease risk of developing disease(8, 9). The protection associated with these alleles show dominance over the alleles that associate with increased risk. The mechanisms behind protection are not yet understood, though several hypotheses have been described (reviewed in (10)). The commonality between the protective HLA-DRB1 alleles is the DERAA-sequence located at position 70-74 of the HLA-beta chain. The DERAA-sequence has been found in a few other human proteins of which vinculin has received the most attention(11). Vinculin is a cytoskeletal protein which is present in all cells. This protein has been found to be citrullinated in synovial joints of RA patients, and can be recognised when citrullinated by monoclonal ACPA as well as ACPA derived from patient sera(11, 12). Therefore, citrullinated vinculin has been proposed as candidate autoantigen in RA that is recognised by both autoreactive T and B cells. Recently, a T-cell clone recognising an epitope containing the vinculin-derived DERAA (VCL-DERAA) epitope was isolated from a healthy individual(11). This T-cell clone was able to support ACPA production from B cells when the B cells were pulsed with the VCL-DERAA peptide(11). Interestingly, these T cells could also recognise three DERAA-containing epitopes derived from microbes(11). It is however not known whether the (citrullinated) vinculin is also endogenously processed and presented to T cells as the studies described above used exogenously added peptides in the assays presented.

An essential element for a proper T-cell response is not only the ability of a T cell to recognise a given epitope *in vitro*, but also that this epitope is naturally processed and presented by cells. This is especially relevant in case predicted T-cell epitopes are being analysed. Often, as is the case in the design of tumour vaccines, HLA-binding and T cell-activation studies are performed to validate a particular predicted (tumour) T-cell epitope(13-16). However, these studies do not show the capacity of peptide-reactive T cells to recognise naturally processed antigen presented by cells of the body,

such as professional Antigen-Presenting Cells (APCs). Similarly, also in the case of autoimmunity, T-cell epitopes are often identified on their ability to bind to HLA molecules and recognition by T cells, but experimental evidence showing that these epitopes are actually processed and presented endogenously by cells is often lacking(17-19). Nonetheless, peptide-reactive T cells are not always able to recognise antigen-expressing cells as recognition of "natural" antigens also depends on e.g. correct processing and presentation of the epitope in the HLA class II pathway as well as on the avidity of the peptide epitope to its HLA:T-cell receptor complex. Indeed, several studies have shown that higher T-cell precursor frequency and robust T-cell responses to a synthetic peptide do not always correlate with the ability of T cells to recognise the naturally processed antigens(20, 21). These studies emphasise that it is crucial to analyse whether T cells reactive towards self-peptides *in vitro* can also recognise endogenously presented antigens as selection of peptides based on HLA binding affinity and robustness of T-cell responses to synthetic peptides only may be insufficient and even misleading in identifying "bonafide" T-cell antigens.

Previously, it was shown that a T-cell clone against a DERAA-containing epitope derived from the protective HLA-DRB1 molecules could recognise not only the HLA-DERAA-peptide, but also cells expressing both HLA-DR13 and HLA-DQ8, the HLA-DQ molecule linked to HLA-DRB1*04(22). These data indicate that this particular DERAA-peptide is endogenously processed and presented by the HLA-expressing APC. These data are important as they indicate that in individuals expressing both the predisposing and protective HLA molecules, DERAA-reactive T cells are possibly tolerised within the thymus by the DERAA-derived epitope from HLA-DRB1*13(22). Such a tolerising phenomenon could potentially underlie the protective effects against RA development that are associated with HLA-DRB1*13(11, 22).

Indeed, Van Heemst *et al* have shown that individuals carrying HLA-DRB1*13 display a reduced T-cell response to either VCL-DERAA and microbial-DERAA, indicating that the presence of HLA-DRB1*13 prohibited the generation of a productive DERAA-directed T-cell response(11). In the same publication, it was also indicated that especially VCL-DERAA-reactive T cells could underlie the long known association between the HLA system and RA(11). However, in case VCL-DERAA-directed T cells are involved in the T-cell response underlying ACPA-positive disease, they should be able to recognise endogenously processed antigen as well. As these responses were analysed with peptides containing a "DERAA-epitope", it is unknown whether the VCL-DERAA peptide can be presented upon endogenous vinculin-protein processing. Likewise, it is not known whether post-translational modifications influence the magnitude of processing or presentation of proteins like vinculin as it is conceivable that the introduction of citrulline or homocitrulline modifies the overall protein-cleavage pattern of proteases present in endo-lysosomes. Nonetheless, these aspects are important to comprehend whether (citrullinated/carbamylated) vinculin within the joints could

represent an autoantigen involved in RA pathogenesis by steering T-cell help to ACPA-expressing B cells.

To determine whether vinculin could represent a "bona fide" autoantigen that can be recognised by T cells, we sequenced the T-cell receptor (TCR) of a vinculin-peptide-directed T-cell clone and transduced it into a T-cell reporter line(23). Using this reporter-cell line, we studied the recognition of DERAA-peptide as well as protein by the TCR-transduced cells. We show here that the TCR can recognise both DERAA-peptides derived from microbes and vinculin, as well as that vinculin protein-pulsed dendritic cells were recognised by the TCR-transduced reporter cells. Our study shows that vinculin can be processed and presented by professional APCs for recognition by autoreactive T cells potentially involved in provision of help to ACPA-expressing B cells.

Materials and methods

JP-T57 TCR-vector design To acquire the sequence of the VCL-DERAA-specific TCR, a vinculin-directed T-cell clone (JP-T57) was expanded and subsequently lysed with TriZol lysis reagent. mRNA was isolated and converted into cDNA. With a TCR-specific primer, the TCR genes were specifically amplified for sequencing. The variable part of the sequenced TCR genes were combined with a murine constant part and inserted in an MP71-Flex vector. The MP71-Flex vector was then used to transduce the TCR-negative, $CD4^+$ Jurkat T-cell line.

Jurkat triple-parameter reporter (TPR)-cell line A Jurkat76-cell line, positive for CD4, NFAT-eGFP, NFkB-CFP and AP1-mCherry was kindly provided by Dr. Mirjam Heemskerk (Dept. Haematology, LUMC)(23). The cell line was transduced with VCL-DERAA-directed TCR as previously described(23). The transduced Jurkat cells were selected on TCR expression and sorted in single cells. The Jurkat cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 1% glutamax, 1% Pencilin/streptomycin and 8% Fetal Calf Serum.

Vinculin expression The plasmid (pET3d, AmpR, Novagen) encoding Vinculin435-742 (hereafter referred to as VCL) was transformed into the methionine auxotroph *E. coli* B834(DE3) (Novagen, cat# 69041). The protein was expressed from the overnight culture of a single colony. 100mL of this overnight culture (o.n. 50µg/mL with Ampicillin) was used for the inoculation per 1L LB medium (Ampicillin 50µg/mL). The cells were grown to an OD600 of 0.6-1.0 prior to the addition of 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 18hrs. The cells were harvested by centrifugation (4000x*g*, 20min, 4°C) and washed once with Tris-buffered saline (TBS; 50mM Tris-HCl, 300mM NaCl, pH 8.0)

Cell pellets were weighed, resuspended in TBS to 1-4g/mL, prior to lysis by French Press

(1.5-kbar, Stansted, Pressure Cell Homogeniser). Lysed cells were centrifuged (15.000x*g*, 1hr, 4°C). The soluble fraction was then loaded onto a Ni-NTA affinity resin (Thermo Fisher Scientific, cat# 88221) pre-equilibrated with TBS. After incubation for 1hr under gentle rotation, the beads were first washed with 2 column volumes (CV) of washing buffer 1 (TBS, 10mM Imidazole, pH 8.0) and subsequently with 3 CV washing buffer 2 (TBS with 50mM Imidazole). The resin was then treated with elution buffer (TBS, 250mM Imidazole, pH 8.0, 2mL) to obtain the VCL in ~10mg/mL, which was then exchanged into TBS using a Sephadex G25 resin (PD-10 column; GE Healthcare).

Vinculin modification Recombinant vinculin was used for two different post-translational modifications. Citrullination of the protein was performed as has been described before(24). Summarised, the protein was incubated with 10mM $CaCl_2$, 100mM Tris-HCL (pH 7.6), and 2U/mg rabbit skeletal muscle-derived PeptidylArginine Deiminase (PAD)4 enzyme (Sigma Aldrich, cat# P1584) o/n for at least 18hrs at 53°C. The carbamylation protocol utilised has been described before(25). In short, the VCL protein was diluted to 2mg/mL and incubated with 0.5M potassium cyanate (Sigma Aldrich, cat# 215074) for 12hrs at 37°C. After incubation, the potassium cyanate was removed by gel-filtration columns (Thermo Scientific, cat# 89890) according to manufacturer's protocol and protein was kept in PBS.

Dendritic cell generation PBMCs were isolated by Ficoll-paque gradient from healthy HLA-DQ8-positive donors, acquired from the Sanquin bloodbank. $CD14^+$ monocytes were subsequently isolated using CD14-based positive selection using MACS beads (Miltenyi Biotec, cat# 130-050-201) according to the manufacturer's protocol (with minor alterations). In short, PBMCs were incubated with 10uL/10^7 cells $CD14^+$ microbeads and incubated for 15' at 4°C. Coated PBMCs were run over an LS column twice to purify the $CD14^+$ fraction. To differentiate the $CD14^+$ cells to dendritic cells, the $CD14^+$ cells were counted and seeded 0.5x10^6 cells/well in a 24-wells plate, in medium (Iscove's Modified Dulbecco's Medium, Gibco, cat#:12440-053) supplemented with 1% Glutamax, 1% Pencilin/streptomycin, 8% Fetal Calf Serum (FCS, Bodinco), 800U/mL GM-CSF (Peprotech, cat#:300-03) and 500U/mL IL-4 (Peprotech, cat#:200-04) and cultured for 6 days. After differentiation of the $CD14^+$ cells to immature dendritic cells, the cells were fed with 200µg VCL per well and were allowed to take it up for ~18hrs. Subsequently, the dendritic cells were matured with a maturation cocktail: 100ng/mL GM-CSF (Peprotech, cat# 300-03), 15ng/mL TNF-α (R&D, cat# 210-TA-005), 10ng/mL IL-1β (R&D,cat# 201-LB-005), 10ng/mL IL-6 (Peprotech, cat# 200-06), 1µg/mL PGE2 (Cayman Chemical, cat# 14010), 500U/mL IFN-γ (Peprotech, cat# 300-02). Dendritic cells were incubated for ~30hrs with the maturation cocktail before they were harvested and cocultured with Jurkat T cells.

Jurkat-cell stimulation An EBV-transformed B-cell line (L-BCL) or mature DCs were used for peptide-presentation to TCR-transduced Jurkat cells. These APCs were pulsed with 10µM peptide for 1 to

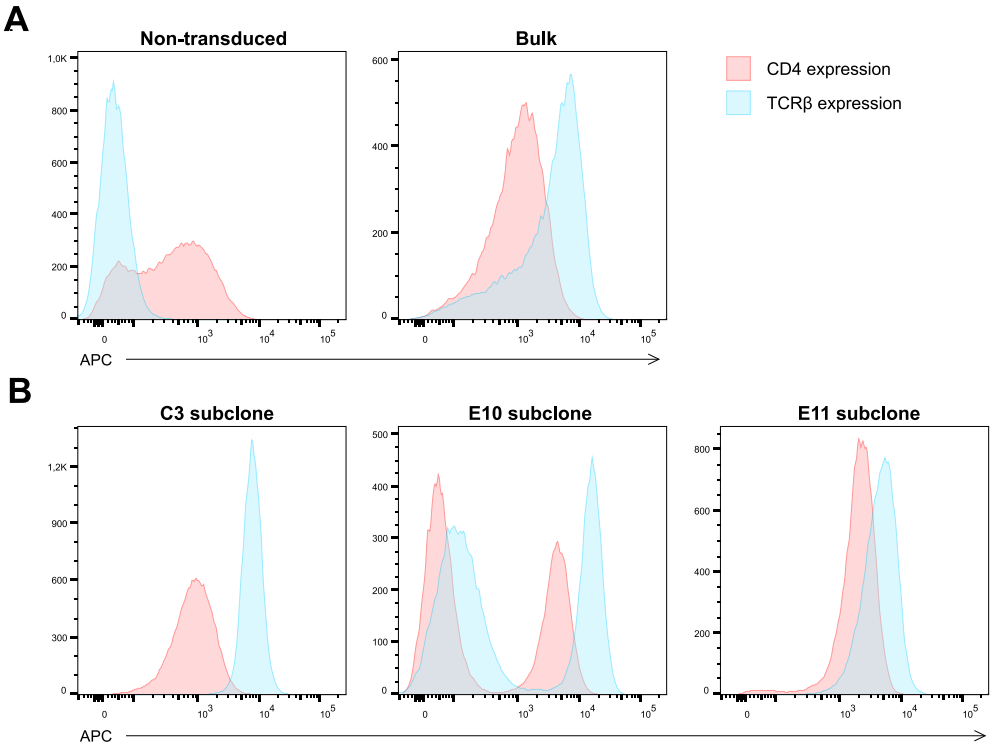

Figure 1: FACS analysis of CD4 and murine TCR-beta chain expression on Jurkat reporter cells after transduction

The Jurkat reporter-cell line expresses only CD4 when no TCR is transduced (A, non-transduced cells) whereas expression of the murine TCR-beta chain is induced after transduction (A, Bulk cells). From the bulk cells, single-cell cultures were established through sorting the cells based on TCR expression. These subclones were analysed on their CD4 and murine TCR beta-chain expression (B). CD4 expression is depicted in red, whereas the murine TCR beta-chain expression is depicted in blue. TCR, T-cell receptor; CD4, cluster of differentiation 4; APC, allophycocyanin

3 hours prior to coculture with the T cells in a 1:1 ratio. For the protein-presentation experiments, dendritic cells were harvested and transferred to a 96-wells plate, 3 wells per condition, except for the "Jurkat cell only condition". Jurkat cells were added to the wells in a 1:1 or a 1:2 ratio, keeping the number of Jurkat cells equal. For both peptide and protein stimulations, DC and Jurkat cells were cultured for ~16-18hrs before the T cells were harvested, stained with Zombie NIR Fixable viability dye (BioLegend, Cat#: 423105) (and CD19-APC-Cy7 (BD Biosciences, Cat#: 557791) in the B cell conditions) according to manufacturer's protocol and fixed with 3% paraformaldehyde. Cells were measured using the BD LSRFortessa. Results were analysed using FlowJo Version 10.4.2.

Results

Establishing Jurkat cells expressing a DERAA-directed TCR

Jurkat cells provide a versatile reporter-cell line to study the presentation of antigens after expression of a TCR of interest. To obtain a reporter-cell line expressing a "DERAA"-directed TCR, Jurkat reporter

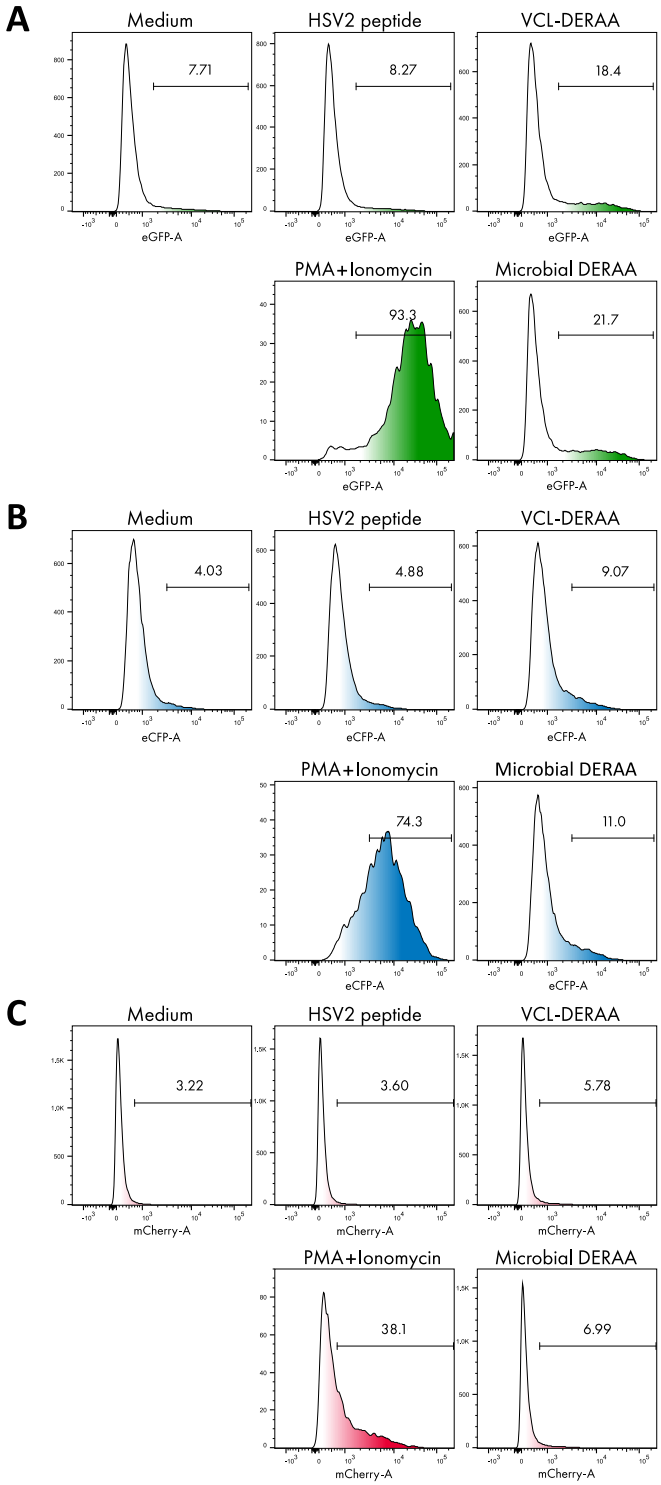

Figure 2: Activation of transcription factors after stimulation of TCR-transduced Jurkat cells

Analysis of eGFP (A), eCFP (B) and mCherry (C) expression after stimulation of transduced bulk Jurkat-T cells with different peptides presented by an HLA-DQ8-positive EBV-transformed B-cell line. eGFP expression is associated with NFAT activation, eCFP expression is associated with NFκB activation and mCherry is associated with AP1 activation. HSV2 served as negative control, PMA + ionomycin as positive control. HSV2, herpes simplex virus 2; VCL, vinculin; PMA, phorbol myristate acetate; eGFP, enhanced green fluorescent protein; eCFP, enhanced cyan fluorescent protein; mCherry, monomeric cherry; TCR, T-cell receptor; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa B; AP1, activator protein 1.

T-cell line J76 was transduced to express the VCL-DERAA specific TCR by lentiviral-gene transfer. The cells obtained after transduction were sorted based on CD4 and TCR-beta chain expression (figure 1a). From these TCR-positive cells, three subclones were established (figure 1b; subclone C3, E10 and E11). Before cell-stimulation assays, the cells were analysed by FACS to validate TCR expression on the cell surface. All cell lines expressed a TCR and hence were successfully transduced (data not shown). As depicted in fig 1B, the T cells exhibit a high degree of TCR expression, as well as CD4. The E10 subclone, however, showed decreased TCR expression, despite sorting the TCR-positive cells on several occasions. Therefore, this subclone was excluded for the remainder of the study. The clone and subclones E11 and C3 were used for subsequent stimulation assays.

Stimulation of DERAA-TCR-bearing Jurkat cells with peptide-pulsed B cells

To verify whether the transduced TCR recognised the peptide-antigen, the DERAA-TCR-expressing Jurkat cells (bulk) were stimulated with peptide-pulsed B-LCLs. In comparison with the negative controls (medium or Herpes Simplex Virus 2 peptide), the VCL-DERAA peptide induced the expression of eGFP and eCFP sufficiently (figure 2a and b). As eGFP was under control of the NFAT promoter and eCFP of the NFκB promoter, these results indicate that the TCR-transduced cells recognised the peptide. In contrast, the expression of mCherry, which is under the control of the AP1 promoter, only showed a minor increase after stimulation (figure 2c), indicating that a read-out measuring mCherry expression was suboptimal.

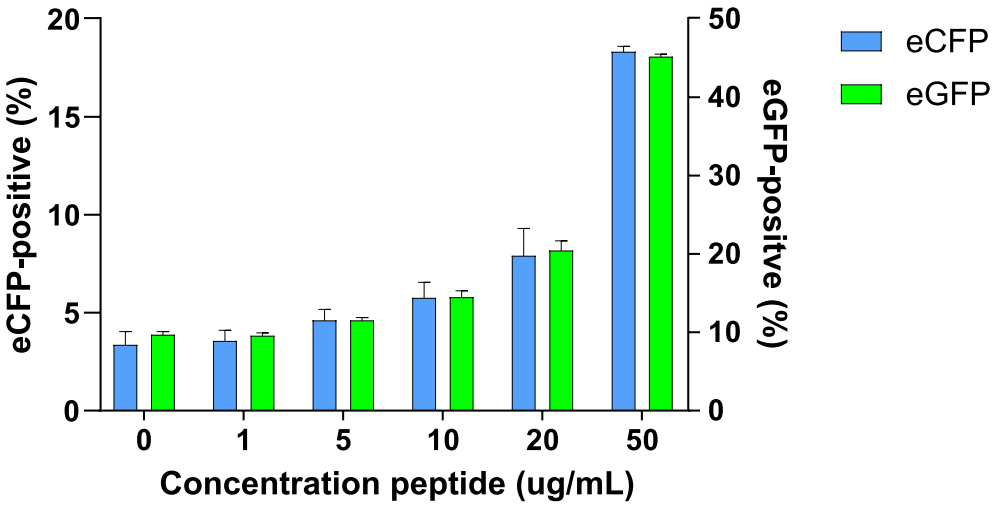

Figure 3: Dose-dependent induction of transcription factor activation

The C3 Jurkat subclone was stimulated with different concentrations of VCL-DERAA peptide to analyse the induction of NFAT and NFκB. The expression of eCFP and eGFP was analysed and depicted in blue and green respectively. VCL, vinculin; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein; eGFP, enhanced green fluorescent protein.

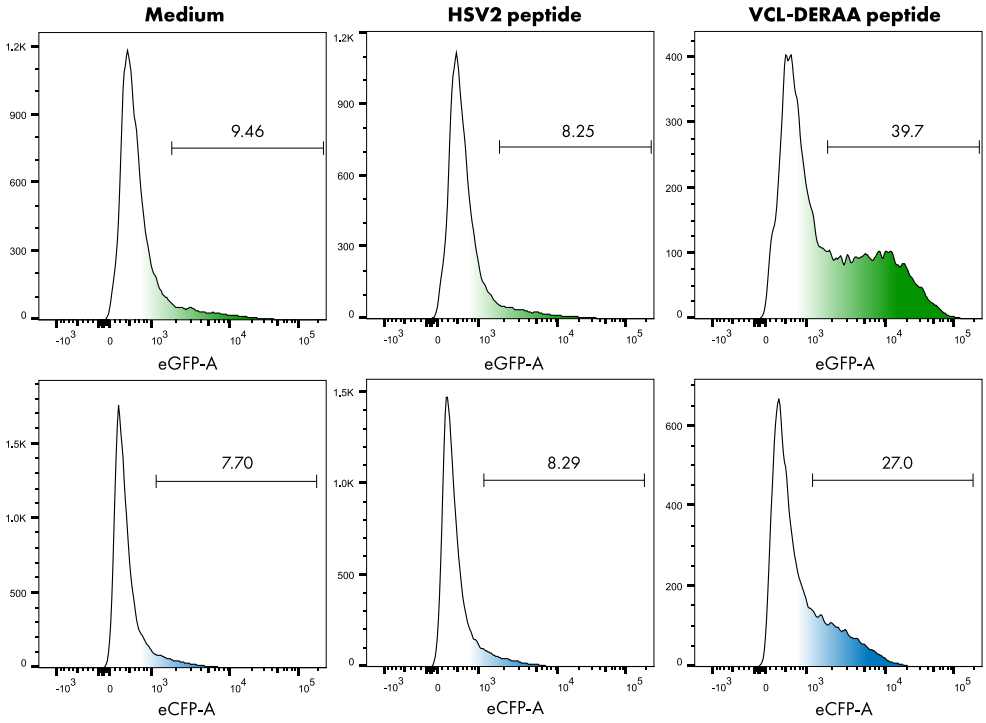

Figure 4: Activation of transduced Jurkat cells by pulsed dendritic cells
The C3 Jurkat subclone was stimulated by human monocyte-derived dendritic cells pulsed with peptides. The expression of eGFP (top) and eCFP (bottom) were both analysed by flow cytometry as depiction of NFAT and NFκB activation respectively. HSV2, herpes simplex virus 2; VCL, vinculin; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein; eGFP, enhanced green fluorescent protein.

Next, we stimulated the Jurkat cells with microbial-derived DERAA-peptides (crossDERAA) to verify whether also these peptides are recognised by the transduced Jurkat cells. These crossDERAA peptides were able to induce a stronger activation of the transcription factors as compared to VCL-DERAA (figure 2). Additionally, the subclones C3 and E11 were tested using the VCL-DERAA and crossDERAA peptides, showing more pronounced activation of the TCR (supplementary figure 1A and 1B respectively).

To validate the sensitivity of the read out system, we performed a titration series on the C3 subclone with the VCL-DERAA peptide, as this population demonstrated the most abundant activation after stimulation. As shown in figure 3, increasing concentrations of VCL-DERAA peptide induces increases the expression of eGFP and eCFP, showing the specificity and sensitivity of stimulation-associated transcription factor activation. Additional controls were performed with Jurkat cells with no TCR expression (non-transduced Jurkat-reporter cells) and with CMV-peptide-specific $CD8^+$ Jurkat cells (supplementary figure 2). Stimulation with medium or HSV2 peptide did not induce expression of the fluorescent proteins. Likewise, the VCL-DERAA peptide or the crossDERAA-peptide mixture

were unable to induce the activation of the transcription factors NFAT nor NFκB, showing that the peptides do not stimulate Jurkat cells in a non-specific manner. Altogether, these data indicate that the DERAA-TCR transduced Jurkat cells can be utilised to analyse VCL-DERAA presentation. More importantly, they also show that the TCR obtained from JP-T57 clone is directed against VCL-DERAA and crossreactive towards other DERAA-peptides.

Presentation of VCL-DERAA after protein processing by DCs

Besides B cells, dendritic cells (DCs) constitute an important population of antigen-presenting cells. They are efficient in processing and presentation of antigens. To establish whether the DERAA T-cell epitope can be processed and presented by human APC, we next determined the ability of DCs to endogenously process and present the DERAA-epitope from vinculin. Immature DCs were primed with vinculin or as control protein, tetanus toxoid, and matured with cytokines. Peptide-pulsed DCs with either the control peptide, VCL-DERAA peptide or crossDERAA peptides were used as controls. Similar to the peptide-pulsed B cells, peptide-pulsed DCs were able to induce NFAT and NFκB activation after stimulation with the crossDERAA and VCL-DERAA peptides, but not towards the HLA-DQ8-binding HSV2-control peptide. As depicted in figure 4, VCL-DERAA-pulsed DCs readily stimulated TCR-expressing Jurkat cells. More importantly, tetanus toxoid-primed DCs were unable to activate the Jurkat-T cells, as represented by a limited T-cell activation comparable to the medium control (figure 5a). In contrast, VCL-pulsed DCs were able to activate the DERAA-TCR-expressing T cells, primarily through NFκB induction as was determined by eCFP expression (figure 5b).

Intriguingly, NFAT was barely activated upon stimulation with processed vinculin, whereas the transcription factor was readily activated when the DCs were pulsed with peptide (supplementary figure 3).

Since we did not observe convincing activation of eGFP, we wished to perform additional controls to rule out e.g. non-specific activation of the transduced Jurkat cells with e.g. pyogenes that could potentially be present in the vinculin protein. However, no activation was observed of control Jurkat cells cultured in the presence of VCL-pulsed DCs (data not shown). Previously, we have shown that the JP-T57 clone does not recognise the "DERAA"-epitope when citrullinated. Therefore, it is predicted that citrullination, but not carbamylation, of the vinculin protein will abolish activation of TCR-transduced Jurkat cells as the VCL-derived DERAA-epitope is prone to be citrullinated and not carbamylated, due to the presence of arginines and absence of lysines. To further confirm the data obtained with JP-T57, we first determined whether citrullination of the DERAA-epitope would abrogate T-cell recognition of the DERAA-epitope. As is shown in supplementary figure 4, the citrullinated VCL-DERAA epitope abrogates TCR recognition of the epitope, showing the impact of citrullination on TCR triggering. Importantly, a similar finding was made when DCs were primed with citrullinated VCL

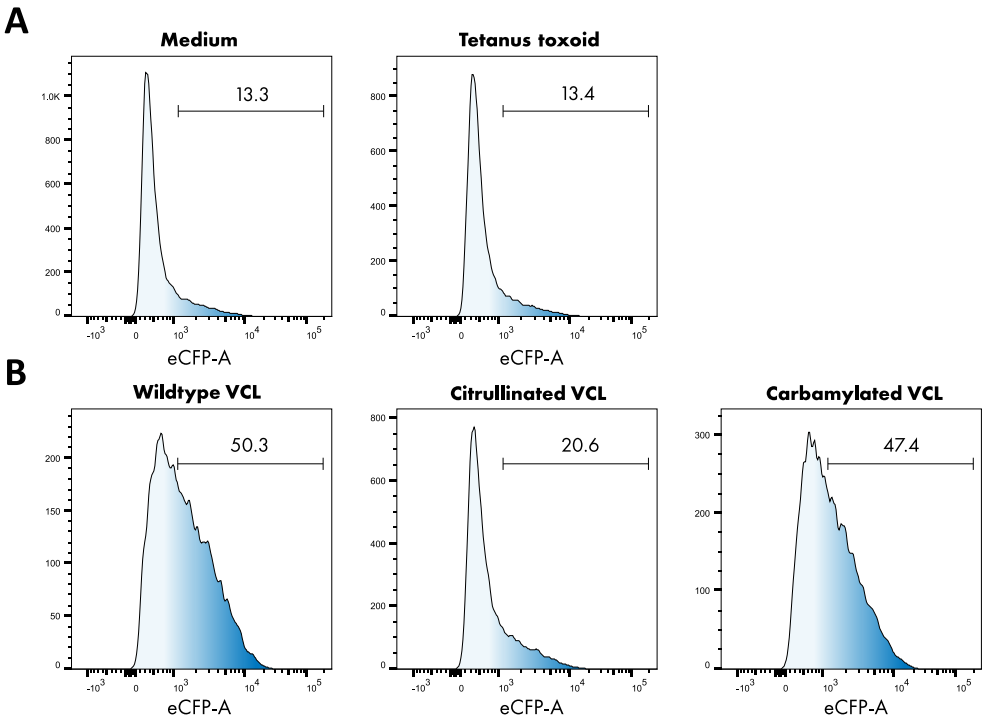

Figure 5: Presentation of vinculin-derived peptides by dendritic cells to Jurkat-T cells
Dendritic cells were pulsed with proteins before maturation with cytokines and lipids. After culturing C3 Jurkat-T cells with the pulsed and mature DCs, NFκB activation was analysed based on eCFP expression. Medium and tetanus toxoid protein served as negative control (A), whereas vinculin and its post-translational modified counterparts (B) were the test conditions. VCL, vinculin; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein.

as activation of the T cells was strongly affected by citrullinated of vinculin (figure 5c). In contrast, carbamylation did not affect the recognition of the VCL-DERAA epitope as no lysine is present in the epitope (figure 5c). Together, these results show that post-translational modifications can obscure T-cell reactivity. More importantly, these results confirm that the DERAA-epitope is efficiently processed for presentation to T cells by professional APCs.

Discussion

Rheumatoid arthritis is a multifactorial disease with a clear contribution of the HLA system as both predisposing and protective HLA alleles have been described(26). The protective alleles have an amino acid sequence in common, DERAA, which can be presented by other HLA molecules when simultaneously present(22). It has been described that the presence of DERAA-bearing HLA molecules is associated with diminished IFN-γ responses of T cells towards other DERAA-peptides (self or foreign) (11). These observations were hypothesised to be explained by the notion that the presentation of the HLA-DERAA peptide from the protective HLA molecules (mainly HLA-DR13) lead to the deletion of DERAA-directed T cells in the thymus, including T cells crossreactive to microbial antigens expressing

the DERAA-sequence(11, 22). As a consequence, B cells reactive towards citrullinated self proteins containing DERAA, such as vinculin, cannot receive help from "DERAA-reactive" T cells, explaining the protective effects associated with HLA-DR13 in RA.

Like all T cells, also T cells recognising DERAA-epitopes will only be activated *in vivo* when the epitope recognised is processed and presented from the protein. For VCL, as is the case for many putative autoantigens, this has not been shown. To determine whether the VCL-DERAA epitope can become processed and presented, we established a Jurkat T-cell line bearing the VCL-DERAA-specific TCR, derived from a primary T-cell clone, as read-out. By isolating and cloning a defined TCR into Jurkat, it can be excluded that activation of the T-cell clone from which the TCR is isolated, is a result of other non-TCR-related triggers provided by the APC. The Jurkat T cells used for the analyses carry a triple parameter, facilitating easy activation measurements, as the activation of three different transcription factors leads to the expression of fluorescent proteins(23). By generating a TCR-transduced Jurkat cell, the opportunity was created to determine whether vinculin is processed and presented using a well-defined and controlled setting in which the transduced TCR is primarily responsible for cell activation.

The primary T-cell clone reacted, in a crossreactive manner, towards both VCL-DERAA and microbial-DERAA. The isolation and sequencing of the TCR has further validated the presence of a single TCR harbouring both reactivities. Using peptide-pulsed APCs, we were able to show that both VCL-DERAA and crossDERAA peptides induced the expression of the transcription factor-controlled fluorescent proteins, while they remained inactive when stimulated with negative control peptides. Intriguingly, we demonstrated that the transduced Jurkat cells, like the T-cell clone, did not respond to the HLA-DERAA peptide. This observation was unexpected as it was predicted that the recognition of DERAA-containing epitopes derived from protective HLA class II molecules, VCL-DERAA-specific T cells would be depleted from the immune system. However, if the TCR does not crossreact between the HLA-DERAA and the VCL-DERAA peptide, it is conceivable that VCL-DERAA-directed T cells will not (completely) be depleted by recognition of the HLA-DERAA peptide in the thymus. Therefore, additional studies are necessary to investigate how the presence of protective HLA class II molecules could exert their protection. For instance, the expression of DERAA-bearing HLA class II molecules in the thymus can induce the differentiation of HLA-DERAA specific T cells into regulatory T cells (Tregs)(27). These Tregs could, potentially, modulate inflammatory responses due to the presentation of HLA-DERAA by APCs in those areas, as the majority of presented peptides are derived from endogenous (including membrane-based) proteins(28, 29). Tregs display a varying array of immunomodulatory capacities, including the restriction of IL-2 production at the inflammatory site, decreasing the availability of IL-2 for effector T cells(30, 31). Moreover, they have been shown to inhibit immune cells by cytolysis through the expression of granzymes and perforins(32, 33). It is however still unclear how cell-cell contact is facilitated. Additionally, Tregs produce a wide variety of anti-inflammatory cytokines, including IL-10,

IL-35 and TGF-β, which in turn transform the inflammatory milieu to an anti-inflammatory milieu(34). Considering this proposed mechanism of protection, the absence of VCL-DERAA or microbial-DERAA-specific IFN-γ response by T cells shown in a recent study can be explained, as HLA-DERAA-specific Tregs able to inhibit effector T-cell functions *ex vivo*, would only be found in HLA-DR13 (or "HLA-DERAA")-positive subjects(11). Nonetheless, a different hypothesis involving crossreactivity between different protein-derived DERAA-peptides can also be postulated. Recently, it has been shown that two different diabetes-related regulatory T cells recognise a self antigen in an unconventional pHLA complex conformation(35). Normally, the alpha chain of the TCR lines up with the beta chain of the HLA class II molecule. However, for these two TCRs, the TCR recognised the pHLA complex in a 180° reversed fashion, bringing the alpha chain of the TCR in line with the HLA-alpha chain(35). The mode of TCR docking on its cognate antigen will definitely have impact on the TCR-signalling strength and, accordingly, T-cell phenotype (regulatory or effector). Therefore, it would be interesting to study the docking mechanism of a DERAA-specific TCR onto HLA-DERAA in comparison to VCL-DERAA in HLA-DR13 positive and negative individuals to reveal whether the mode of docking has an influence on the function of DERAA-directed T cells.

As indicated above, it is important that putative autoantigens are not only recognised when presented as a peptide to T cells, but also that the epitope is processed from the self protein. Several mechanisms might prevent such processing and presentation such as the cleavage of the peptide-epitope by intracellular proteases, or the inability of proteins to reach the HLA class II-processing pathway/loading compartments(36-39). In case peptides derived from autoantigens, although recognised by T cells when provided synthetically *in vitro*, are not endogenously processed and presented by HLA class II-positive cells from proteins, they are likely not involved in the regulation of the autoimmune response present in patients. Our data demonstrate that human DCs are able take up the vinculin protein and process it for presentation of the VCL-DERAA epitope to human T cells. These data are relevant as they indicate that VCL could represent a self protein involved in the autoimmune response in RA patients.

Our data also indicate that post-translational modification of vinculin does not affect the processing of the DERAA-epitope for presentation in the context of HLA class II. Although we could not analyse the effect of citrullination on antigen-processing because the arginine present in DERAA is crucial for recognition of the epitope by the TCR used in these studies, carbamylation of vinculin did not lead to an apparent increase or loss in antigen recognition. Nonetheless, recently it was demonstrated, by the analyses of the effects of post-translational modifications on proteolysis by protease mixtures and *in cellulo*, that carbamylated proteins are more resistant to proteolysis (manuscript submitted). This resistance is likely due to a tightened protein structure due to the carbamylated lysines, compared to the native protein. To the contrary, citrullination did not influence proteolysis significantly, suggesting

that the protein was subject to processing for peptide presentation. Similar to carbamylated vinculin, the lack of effect of carbamylation on antigen processing and presentation was also observed when the response of OTII cells was analysed in mice challenged with unmodified or carbamylated Ovalbumin (OVA). Carbamylation of the protein did not alter T-cell proliferation significantly, as there are no lysines within the OTII epitope (data unpublished). Together, these results suggest that depending on protein context and epitope, carbamylation might affect antigen presentation and thereby (autoreactive) T-cell responses. In this context, unpublished data from our group indicate that carbamylation of mouse albumin can lead to the induction of an albumin-specific immune response, possibly by the formation of neo-epitopes to which tolerance has not been established.

The studies described in this manuscript have been performed in a Jurkat triple-parameter reporter-cell line(23). This cell line originates from a tumour T-cell line and as such, has the tendency to alter the genetic information rapidly. This was, for example, evident for the subclone E10, which, after culture, started to lose TCR expression gradually as well as CD4 expression. In addition, it was shown that DCs after processing of VCL, were unable to induce eGFP expression in the Jurkat-T cells expressing the VCL-DERAA-specific TCR, even though eCFP expression was readily accomplished. We can only speculate about the cause of this difference in read-out after stimulation, which could be of physiological relevance or due to an "artefact". Future experiments to study the DERAA-specific TCR include the utilisation of TCR-transduced donor-derived $CD4^+$ T cells and analyse other read-outs (expression of activation markers or cytokine production)(40, 41).

In summary, we have shown that the VCL-DERAA T-cell clone expresses a single TCR crossreactive towards multiple DERAA-containing epitopes, derived from foreign and self origin. In addition, we have shown that processing of the VCL protein releases the VCL-DERAA peptide for presentation to T cells. These findings are important for furthering the understanding of the mechanism behind T-cell involvement in RA. Nevertheless, our knowledge regarding relevant T-cell epitopes in RA and their role in the pathogenesis displays large gaps that need to be filled in future studies.

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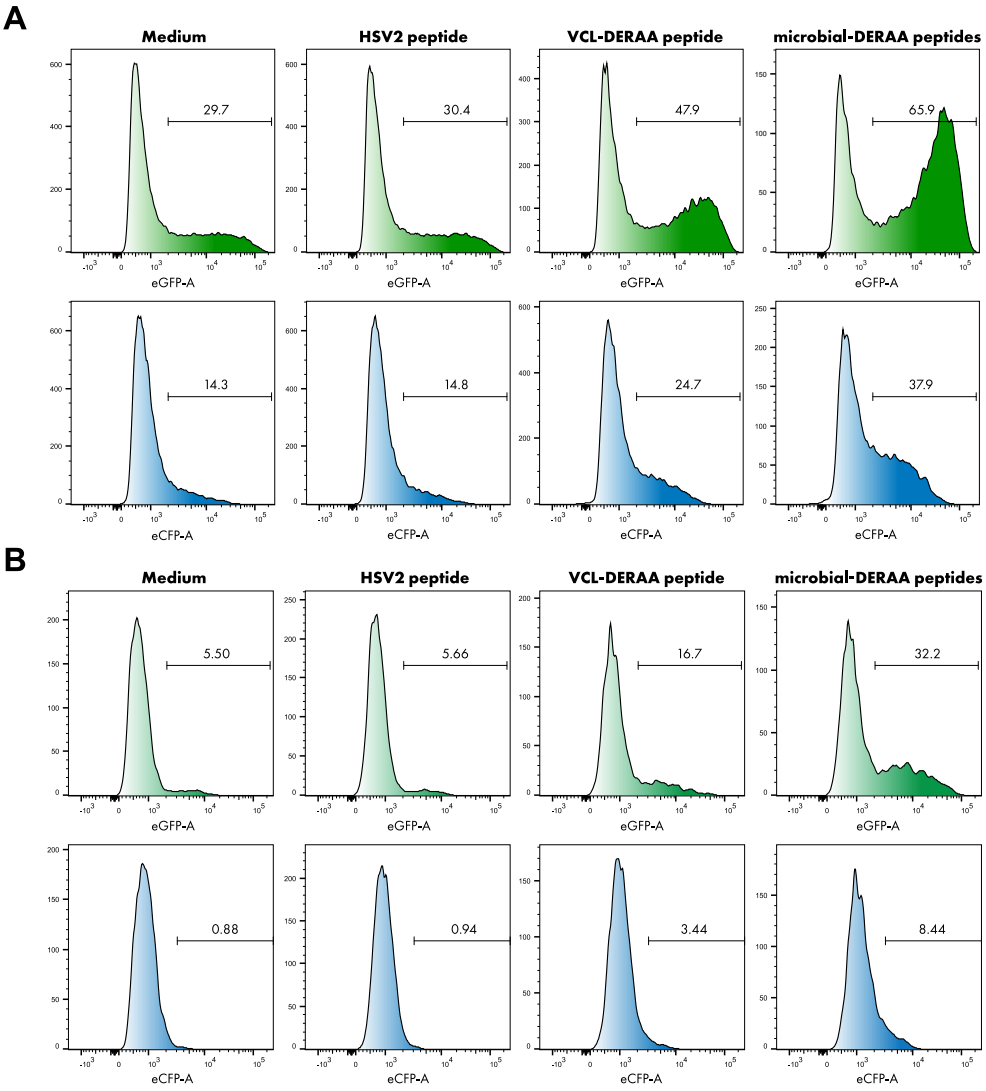
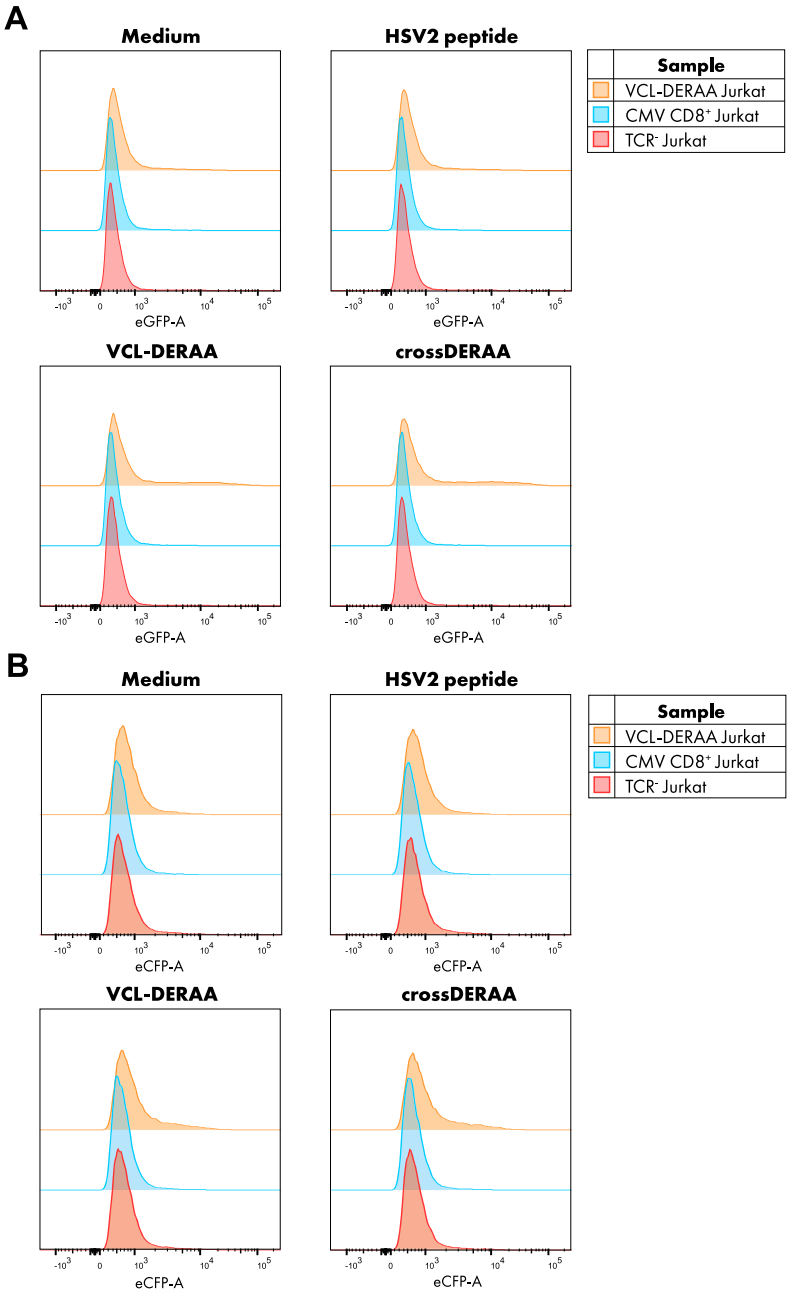


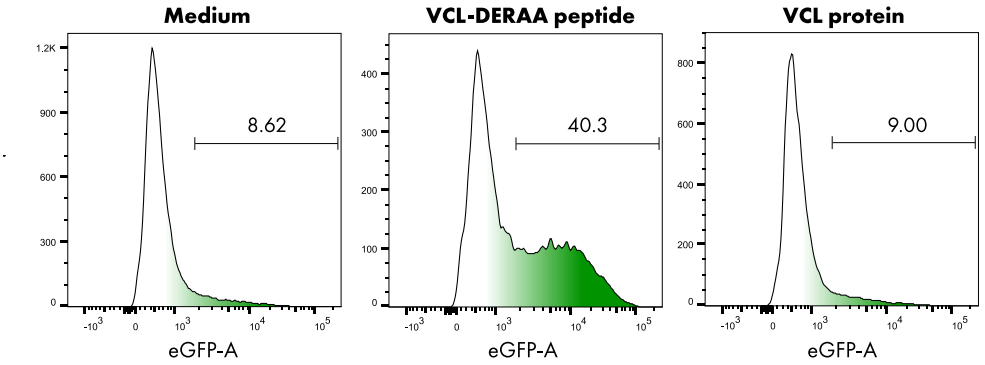
Supplementary figure 1: Activation of Jurkat subclones C3 and E11 by peptides

Both C3 (A) and E11 (B) subclones were stimulated with EBV-transformed B-cell lines pulsed with control peptide, VCL--DERAA or microbial-DERAA peptides. Activation of NFAT (eGFP, upper panels) and NFκB (eCFP, bottom panels) was analysed with flow cytometry. HSV2, herpes simplex virus 2; VCL, vinculin; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein; eGFP, enhanced green fluorescent protein



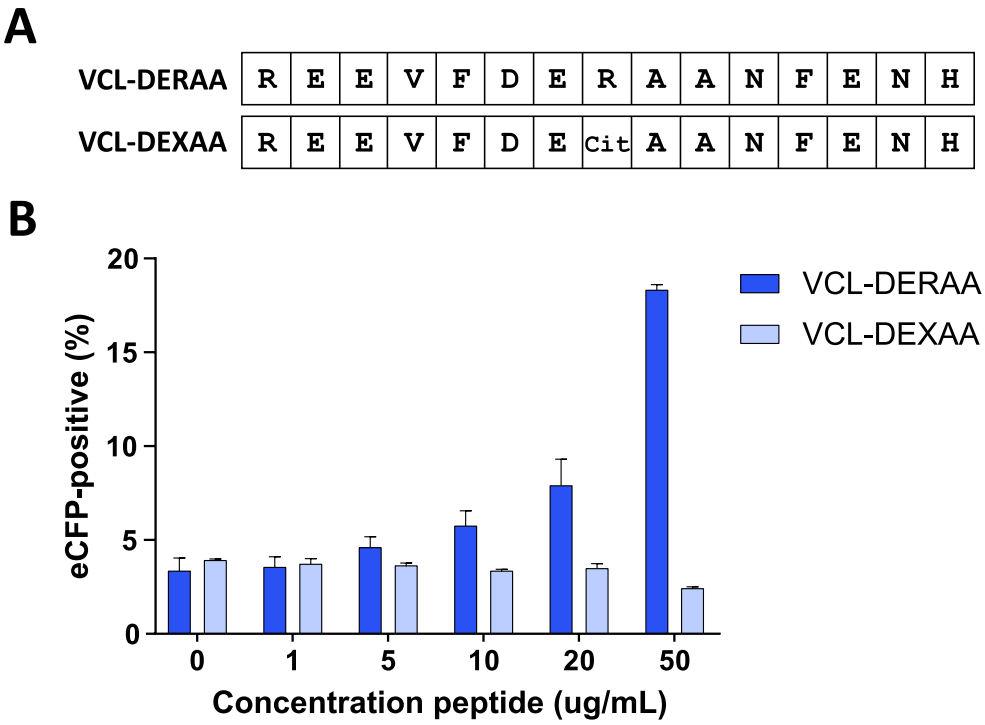
Supplementary figure 2: Activation of non-DERAA-directed TCR transduced Jurkat T cells

TCR- CD4+ Jurkat T cells (red) and CMV-directed CD8+ Jurkat-T cells (blue) were stimulated with B-LCLs pulsed with HSV2, VCL-DERAA or microbial-DERAA peptides. The NFAT (A) and NFκB (B) activation was compared with stimulation-induced activation in VCL-DERAA TCR-transduced C3 Jurkat-T cells (yellow), analysed based on eGFP and eCFP expression respectively. HSV2, herpes simplex virus 2; VCL, vinculin; TCR, T-cell receptor; CMV, cytomegalovirus; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein; eGFP, enhanced green fluorescent protein



Supplementary figure 3: NFAT activation after stimulation with VCL-pulsed DCs

C3 Jurkat subclone was cultured with mature DCs pulsed with VCL-DERAA peptide or primed with VCL protein. The eGFP expression was determined with flow cytometry to analyse NFAT activation after stimulation. VCL, vinculin; DC, dendritic cell; NFAT, nuclear factor of activated T cells; eGFP, enhanced green fluorescent protein.



Supplementary figure 4: Effect of citrullination of the DERAA-epitope on DERAA-directed TCR activation

The DERAA-epitope contains an arginine within the peptide core (A). This arginine can be converted to a citrulline during citrullination (A). The effect of citrullinating the DERAA-epitope was analysed by measuring eCFP expression upon stimulation of the C3 Jurkat subclone with increasing concentrations of the unmodified VCL-DERAA peptide (dark blue) in contrast to the citrullinated peptide (light blue). VCL, vinculin; TCR, T-cell receptor; NFκB, nuclear factor kappa B; eCFP, enhanced cyan fluorescent protein.