Memory T cells specific to citrullinated α-enolase are enriched in the rheumatic joint

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ABSTRACT

ACPA-positive rheumatoid arthritis (RA) is associated with distinct HLA-DR alleles and immune responses to many citrullinated self-antigens. Herein we investigated the T cell epitope confined within α-enolase$_{326-340}$ in the context of HLA-DRB1*04:01 and assessed the corresponding CD4$^+$ T cells in both the circulation and in the rheumatic joint. Comparative crystallographic analyses were performed for the native and citrullinated α-enolase$_{326-340}$ peptides in complex with HLA-DRB1*04:01. HLA-tetramers assembled with either the native or citrullinated peptide were used for *ex vivo* and *in vitro* assessment of α–enolase-specific T cells in peripheral blood, synovial fluid and synovial tissue by flow cytometry. The native and modified peptides take a completely conserved structural conformation within the peptide-binding cleft of HLA-DRB1*04:01. The citrulline residue-327 was located N-terminally, protruding towards TCRs. The frequencies of T cells recognizing native eno$_{326-340}$ were similar in synovial fluid and peripheral blood, while in contrast, the frequency of T cells recognizing cit-eno$_{326-340}$ was significantly elevated in synovial fluid compared to peripheral blood (3.6-fold, p=0.0150). Additionally, citrulline-specific T cells with a memory phenotype were also significantly increased (1.6-fold, p=0.0052) in synovial fluid compared to peripheral blood. The native T cell epitope confined within α-enolase$_{326-340}$ does not appear to lead to complete negative selection of cognate CD4$^+$ T cells. In RA patient samples, only T cells recognizing the citrullinated version of α-enolase$_{326-340}$ were found at elevated frequencies implicating that neo-antigen formation is critical for breach of tolerance.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that can be divided in at least two subsets based on the presence of antibodies to citrullinated proteins (ACPAs) [1]. Importantly, such ACPA target proteins [2-5] also represent candidate autoantigens for $CD4^+$ T cell responses, as further substantiated by the strong genetic association between ACPA-positive RA and the so called shared epitope HLA-DR alleles [6-8]. Indeed, previous studies of peripheral blood from ACPA-positive RA patients have demonstrated autoreactive $CD4^+$ T cells against several citrullinated self-proteins [9-13].

Alpha-enolase represents an interesting autoantigen in RA as the presence of autoantibodies against citrullinated α-enolase is preferentially linked to HLA-DRB1*04 [14], which is the most studied HLA class II allele in the context of RA. Although α-enolase is considered a ubiquitous protein, it is citrullinated and overexpressed in the inflamed synovium [4], and antibodies to the immunodominant citrullinated B cell epitope CEP-1 are present in approximately half of all ACPA-positive patients [14, 15]. These citrullinated α-enolase-specific autoantibodies are highly specific for RA [4, 14] and significantly enriched in synovial fluid [16].

$CD4^+$ T cells play an important role in RA by secreting pro-inflammatory cytokines and activating B cells, which contribute to the inflammatory perpetuation. A major technical hurdle, regarding the characterization of T cells that specifically recognize potential RA-specific autoantigens, is related to the fact that autoantigen-specific T cells are generally present in very low frequencies [17-21]. Through recent improvements in the field of HLA class

II/peptide tetramers, based on bead enrichment, it is now possible to assess rare antigen-specific $CD4^+$ T cells directly *ex vivo* without prior *in vitro* expansion [13, 22-24].

Furthermore, several T cell epitopes have been identified based on the notion that the P4 pocket of RA-associated HLA-DR alleles cannot accommodate a positively charged arginine side chain but can fit a neutral citrulline [25]. However, a growing list of citrulline-specific T cell epitopes have demonstrated that the citrulline can also be located in positions contributing to TCR contact [13, 26, 27]. We have recently scanned the entire α-enolase protein for T cell epitopes and demonstrated the existence of several peptides for which the citrulline modification is not confined to the classical P4 position in the HLA binding cleft [26]. Since this alternative repertoire of T cell epitopes has been less studied, we have here focused on the α-enolase peptide KRIAKAVNEKSCNCL, spanning residue 326 to 340 which comprises an arginine residue at the N-terminus. We have previously demonstrated the suitability of this epitope for HLA-tetramer studies aiming at characterization of specific autoreactive T cell populations [13]. Moreover, we have demonstrated in peptide competition assays that both peptide versions, native $eno_{326-340}$ and modified cit-$eno_{326-340}$ have similar binding affinities to HLA-DRB1*04:01 [26]. This provided us with a unique opportunity to assess the contribution of the non-conventionally located arginine/citrulline residue of the peptide to T cell receptor (TCR) recognition. To achieve this, we determined the crystal structures of the HLA-DRB1*04:01/$eno_{326-340}$ and HLA-DRB1*04:01/cit-$eno_{326-340}$ complexes at 1.33Å and 1.35Å resolution, respectively, and demonstrated the existence of both specific as well as cross-reactive T cells. Strikingly, only

cit-α-eno$_{326-340}$ specific T cells were found to be enriched in the RA joint implicating their involvement in the HLA-restricted immune response driving joint inflammation.

2. Material and methods

2.1 Patients and healthy control subjects

RA patients (n=38) were recruited under the auspices of the Karolinska University Hospital Rheumatology clinic, the BRI rheumatic disease registry and the BRI immune-mediated disease registry and had an age range of 22-78 with 84% being CCP-positive. Control subjects (n=15) were recruited from the BRI IMD registry and the Uppsala Bioresource and had an age range of 27-82. Informed consent was obtained from all patients and control subjects based on local ethical permits. All patients were diagnosed with RA by a rheumatologist in accordance with the 1987 American College of Rheumatology criteria [28]. All subjects had at least one copy of the HLA-DRB1*04:01 allele. Peripheral blood mononuclear cells (PBMC), obtained from heparinized blood and synovial fluid mononuclear cells (SFMC), were prepared by centrifugation over Ficoll-Hypaque gradients. Frozen samples were cryopreserved in liquid nitrogen in 10% DMSO and 90% heat-inactivated FBS. Frozen samples were utilized for studies carried out at KI and fresh samples were used for all analyses carried out at BRI. Synovial biopsies were obtained through ultrasound-guided arthroscopy from two HLA-DRB1*04:01 RA patients. The synovial tissues were digested via incubation with Collagenase A and DNAse I (Roche).

*2.2 Production of HLA-DRB1*04:01/peptide tetramers*

Recombinant HLA-DRB1*04:01/peptide complexes were produced as previously described [29]. All peptides were purchased at purity higher than

95% from GenScript, Inc. Biotinylated HLA-DRB1*04:01 monomers were loaded with either the influenza-hemagglutinin $HA_{306-318}$, the native or the citrullinated versions of the α-enolase peptide $eno_{326-340}$ by incubation in the presence of n-octyl-β-D-glucopyranoside and Pefabloc SC (Sigma-Aldrich). Peptide-loaded monomers were subsequently conjugated to tetramers using either R-PE streptavidin (Invitrogen) or APC streptavidin (BD).

2.3 In vitro detection of native and citrullinated α-enolase-specific T cells using HLA class II tetramers

For tetramer assays PBMCs from healthy controls or synovial biopsies (n=2) from DRB1*04:01 RA subjects were cultured in RPMI-1640 + 10% pooled human serum with 10μg/ml of $HA_{306-318}$, native or citrullinated $eno_{326-340}$ peptides. Interleukin-2 (IL-2) (Proleukin, Novartis) was added on day 6. After 14 days, cells were stained according to table S1. Data was analyzed using FlowJo software (Treestar).

2.4 Ex vivo detection of native and citrullinated α-enolase-specific T cells using HLA class II tetramers

PBMC and SFMC samples from RA patients were labeled according to table S1 as previously described [29]. Samples were run on a BD-LSRII flow cytometer or a Beckman Coulter Gallios, and data was analyzed using FlowJo software. The frequency of antigen-specific cells was calculated as the total number of tetramer-positive cells in the bound fraction divided by the total number of $CD4^+$ T cells. A cut-off of $1/10^6$ $CD4^+$ T cells was applied.

2.5 Murine Assays

HLA-DRB1*04:01-IE transgenic mice on a class II deficient C57Bl/6 (I-Abo/o) background were obtained from Taconic Biosciences (Hudson, NY, USA) and housed under specific pathogen-free conditions. Mice were immunized with 100μg of peptide, and splenocytes, harvested at day 14, were used for studying recall responses as previously reported [13]. All animal work was approved by the BRI Animal Care and Use Committee (ACUC). Animals were housed in the BRI AAALAC-accredited animal facility.

*2.6 Production and isolation of HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes*

The extracellular domains of the HLA-DRB1*04:01 α- and β-chains with an acidic and basic leucine zipper, respectively, as well as C-terminal hexa-histidine tags were cloned into the pET28a vector and expressed separately in *E. coli* BL21 (DE3) STAR cells (Novagen). Inclusion bodies were dissolved in 8M urea, 50mM Tris-HCl (pH8) and purified on a HiTrapQ-HP anion exchange column (GE Healthcare). The purified α- and β-chains were diluted to a final concentration of 2mg/ml each in a refolding solution containing 50mM Tris-Citrate buffer pH7.5, 25%(w/v) glycerol, 0.01% Pluronic F-68 and 5μM corresponding peptide. The mixture was incubated for 72h at RT and centrifuged 20min at 40,000g. The supernatant was concentrated on a 10kDa cut-off Vivaspin Turbo 15 (Sartorius) and run through size-exclusion chromatography on a Superdex200-10/300 GL column (GE Healthcare). The monomeric HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes were concentrated to 1mg/ml in 25mM Tris-HCl (pH8) and

subjected to thrombin cleavage in order to remove the leucine zippers. The HLA/peptide complexes were thereafter separated from leucine zippers using a HiTrap Chelating HP column (GE Healthcare). The flow-through from IMAC was pooled, concentrated using a Vivaspin (Sartorius) to 2-3mg/ml and further purified on a Superdex200-10/300 GL column. Fractions containing monomeric HLA/peptide complexes were concentrated to a final concentration of 6-8mg/ml and used in crystallization trials.

2.7 Crystallization and structure determination

Crystals of the HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes were grown using the hanging-drop vapor diffusion method at 20°C. Protein solution and a mother liquor of 10% (vol/vol) MPD, 15% (vol/vol) PEG3350 and 100mM MES buffer pH6.5 were mixed at a 1:1 ratio. Rod-like crystals typically grew within five to fifteen days. Crystals were flash frozen in liquid nitrogen without additional cryoprotection. X-ray diffraction data from a single crystal for each data set were collected at the automatic ID30A-1 (MASSIF-1) beam line [30] at the European Synchrotron Research Facility and processed using the programs XDS [31] and XDSAPP [32]. The crystals diffract up to 1.33Å and 1.35Å for HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$, respectively. The crystal structures were determined by molecular replacement method using the program Phaser [33] with the three-dimensional structure of HLA-DRB1*04:01 (PDB ID:4MCY) [34] without peptide as a search model. The models were subsequently refined using Refmac5 and iterations of manual rebuilding using COOT [35] (Table 1). The structures were validated using MOLPROBITY [36]. The atomic coordinates

and structure factors for the crystal structures of the HLA-DRB1*04:01 in complex with eno$_{326-340}$ and cit-eno$_{326-340}$ have been deposited to the Protein Data Bank under accession codes 5NI9 and 5NIG, respectively.

2.8 Statistics

Statistical tests used for this paper include Wilcoxon matched pairs ranked test and Mann-Whitney tests. Analyses were performed using Prism software (version 5.0 or higher). Values <0.05 were considered as significant and marked with an asterisk. Two asterisks denote values <0.01.

3. Results

*3.1 The crystal structures of HLA-DRB1*04:01 in complex with eno$_{326-340}$ and cit-eno$_{326-340}$ reveal conserved peptide binding registry and conformation*

The crystal structures of HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes were refined to 1.33Å and 1.35Å resolution, respectively (figure 1 and suppl table 2). The electron-density maps characteristic for such high-resolution structures allow for unambiguous modeling of the peptides in the HLA binding cleft. In both structures, the HLA-DRB1*04:01 binding groove is fully occupied by the peptides in canonical orientations with residues p328I, p331A, p333N and p336S anchoring in pockets P1, P4, P6 and P9, respectively (figure 1B-C). The arginine/citrulline-327 occupies position P-1 in the HLA groove preceding the main anchor residue p328I buried in pocket P1. The side chains of the arginine/citrulline-327 are solvent-exposed, extending towards the TCRs (figure 1A). In addition to p327R/Cit, four other residues p330K, p332V, p335K and p338N all extend towards the solvent ready for interactions with TCRs (suppl figure 1).

Modification of the arginine residue p327R to citrulline does not alter the binding register nor the conformation of the cit-eno$_{326-340}$ peptide (figure 1A). However, the electrostatic surface potentials of the HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes are significantly different (figure 1C) due to the removal of the positive charge upon modification of arginine to citrulline. Such a change in the charge distribution on the interacting surface of a peptide-HLA complex is likely to affect TCR binding to this complex. Thus, citrullination

of peptide eno$_{326-340}$ creates a potential neo-antigen that could interact with an entirely different (autoreactive) T cell repertoire that focuses on the N-terminal section of the cit-eno$_{326-340}$ peptide. Conversely, nearly identical conformations of the entire peptide section stretching from p328I to p338N in both eno$_{326-340}$ and cit-eno$_{326-340}$ structures (figure 1A) enable for cross-reactivity of TCRs that focus on the central sections of HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$.

The crystal structures of HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ complexes revealed two interesting additional features. First, residue p334E forms two interactions with “shared epitope” residues in HLA-DRB1*04:01, including a salt bridge with K71B and a water-mediated hydrogen bond with Q70B (suppl figure 2). These interactions possibly compensate for the relatively weak binding of the non-optimal peptide anchor residues p331A and p336S to pockets P4 and P9, respectively. Secondly, the two crystal structures are to our knowledge the first to describe the presence of a disulfide bridge between peptide cysteine residues. It is not clear if the disulfide bridge between p337C and p339C has any functional consequence although a mutated version of the eno$_{326-340}$ peptide, with both cysteine residues replaced to serine, displays one order of magnitude lower affinity towards HLA-DRB1*04:01 compared to native eno$_{326-340}$ (supplementary figure 3).

*3.2 Use of peptide-HLA-DRB1*04:01 tetramers for visualization of antigen-specific CD4$^+$ T cells in vitro and ex vivo*

Before utilizing peptide-HLA tetramers for *ex vivo* assessment of autoreactive T cells in RA patient samples, we first validated our reagents by use of peripheral blood from healthy blood donors carrying the same HLA-DR allele. As shown in figure 2A, both influenza-HA- and α-eno$_{326\text{-}340}$-specific T cells can be readily visualized following 2 weeks *in vitro* propagation with the corresponding peptide. The HLA tetramers were also successfully used for direct *ex vivo* staining, *i.e.* without any prior *in vitro* cell manipulation. As exemplified in figure 2B, influenza-HA specific T cells could, as expected, be detected in RA synovial fluid. In this particular example, the antigen-specific cells could be enumerated to 20 per million CD4$^+$ T cells, and based on the phenotypic markers, the proportion of tetramer-positive cells being of memory CD45RO$^+$ phenotype could be determined (here 85%), as well as additional phenotypic markers (0% CD25+ and 100% CD28+).

*3.3 CD4$^+$ T cells recognizing the native α-enolase$_{326\text{-}340}$ peptide in complex with HLA-DRB1*04:01 are part of the normal T cell repertoire but mostly display a naïve phenotype*

We next continued with *ex vivo* staining using HLA tetramers assembled with either the positive control influenza-HA peptide or with the native arginine-containing eno$_{326\text{-}340}$ peptide. Both T cell specificities were found to be part of the peripheral T cell repertoire of both healthy donors and RA patients carrying the HLA-DRB1*04:01 allele (figure 2C, D). For HA, all healthy controls and all RA subjects displayed influenza-specific T cells with an average of 26 per million CD4$^+$ cells in each group. In contrast, α-eno$_{326\text{-}340}$-specific T cells were

more rare and only detected in around 66% of RA patients with an average of 4 per million $CD4^+$, and 2 per million in healthy individuals. When it comes to the phenotype of this autoantigen-specific T cell population, it was distinctly different from HA-specific T cells which in both healthy subjects and RA patients were primarily (80%) $CD45RO^+$ memory. The T cells recognizing the native version of $eno_{326-340}$ were mostly naïve with only 20% memory in peripheral blood of both healthy donors and RA patients (figure 2E).

*3.4 $CD4^+$ T cells recognizing the citrullinated α-enolase$_{326-340}$ peptide in complex with HLA-DRB1*04:01 are rare in healthy subjects, but enriched in the RA joint*

We next turned our attention to T cells specific for the modified peptide $eno_{326-340}$ carrying a citrulline in aa-position 327. Such T cells were only present in peripheral blood of three out of the eleven studied healthy controls (27%), implicating that this population is not commonly part of the normal T cell repertoire (figure 3A) and when found, T cells were at frequencies of 3-4 per million $CD4^+$ (figure 3B). A similar frequency was found also in peripheral blood of RA patients, but now the autoreactive T cells were detected in 62% (13/21) of the RA subjects. Even more strikingly, such cit-$eno_{326-340}$-specific T cells were significantly elevated in synovial fluid with an average of 15 per million and were found in 50% (9/18) of the RA patients. Moreover, these cells were consistently of a memory T cell phenotype (average 95%), implying previous cognate interaction with the antigen (figure 3C).

As synovial fluids and synovial tissues are two discrete compartments of the rheumatic joint, we investigated whether α-enolase-specific T cells could be found also in the tissue. Due to the size limitation of the biopsy material, we *in vitro* propagated single cell suspensions with the peptide mix and IL-2 prior to staining. Hereby, T cells specific for HLA-DRB1*04:01/eno$_{326-340}$ and HLA-DRB1*04:01/cit-eno$_{326-340}$ could be found in one out of two tested biopsy samples, implicating that autoreactive T cells are not restricted to synovial fluids but are also present in synovial tissues (figure 3D). N.B. as the tissue cells required cell culture expansion, we could not calculate the original frequencies nor analyse the unmanipulated phenotypes.

Lastly, we had the possibility to assess Tmr+ T cells in paired samples of peripheral blood and synovial fluid taken from the same patients. Here we found that, in four out of seven paired samples, the numbers of native eno$_{326-340}$ specific T cells are increased in SF compared to PB when gating at the CD45RO+ population. For cit-eno$_{326-340}$ specific T cells, an increase was only seen in two out of six sample pairs, but when present, the frequency was much higher than those for the native peptide (figure 3E).

3.5 Presence of both specific and cross-reactive T cells recognizing native and citrullinated eno$_{326-340}$

Our crystal structures of HLA-DRB1*04:01 in complex with eno$_{326-340}$ or cit-eno$_{326-340}$ indicated that dependent on where the TCR docks, T cells could either be specific for the respective peptides (by binding to the N-terminal part of the peptide-HLA complex) or cross-react (when interacting with the central

parts of the peptide-HLA complexes). To visualize this, we overlayed our crystal structure of HLA-DRB1*04:01/eno$_{326-340}$ with three ternary complexes with known three-dimensional structures: HLA-DRB1*04:01 with a TCR specific for a viral peptide [37], a tumor-specific TCR [38] and a TCR specific for the multiple sclerosis-associated autoantigen myelin basic protein [39] (figure 4). The superposition clearly demonstrates that peptide residue p327R could interact with the α-chain of a TCR. Since this residue protrudes away from the HLA molecule, its conformation can change after TCR binding resulting in interaction either with CDR3α or with CDR1α. Modeling of the interaction surface area found in three crystal structures of ternary complexes DRB1*0401/TCR [37] clearly demonstrates that in all cases TCR come close to the amino acid preceding the first anchor residue (Figure 4a and b).

To further assess this from a functional point of view, we assembled HLA-DRB1*04:01 tetramers using two different fluorophores. Dual-color tetramer staining was performed on peripheral blood T cells from several HLA-DRB1*04:01-positive RA patients, following *in vitro* stimulation with either the native eno$_{326-340}$ or the cit-eno$_{326-340}$ peptide. Here, we could detect two types of patterns: samples with double-positive, *i.e.* cross-reactive, T cells (figure 5A), and samples without double positives, *i.e.* selective T cells recognizing either the native or the citrullinated peptide (figure 5B). As a positive control, *in vitro*-expanded cells stimulated with HA$_{306-318}$ were stained simultaneously with two differently labeled HLA-DRB1*04:01/HA$_{306-318}$-tetramers and a clear double stained population was observed as expected (figures 5A and B, far right panels). These results implicate that the α-enolase-specific T cells we detect

with tetramers represent a diverse repertoire with some being dependent on interactions with the N-terminal arginine/citrulline residues, and others not.

As a next step, we immunized HLA-DRB1*04:01-IE transgenic mice with the native $eno_{326-340}$ peptide and examined recall responses to both the native and citrullinated versions of the α-enolase peptide. Mice immunized with $eno_{326-340}$ displayed similar proliferation responses irrespective if they were re-stimulated with $eno_{326-340}$ or cit-$eno_{326-340}$ (figure 5C), suggesting high degree of T cell cross-reactivity between the two peptides in a setting of active immunization. As we previously reported, the opposite observation is also true, *i.e.* mice immunized with cit-$eno_{326-340}$ displayed recall responses to both versions of the peptide [13].

Finally, dual color tetramers were also used for *ex vivo* staining of synovial fluid T cells obtained from three HLA-DRB1*04:01-positive RA patients. Interestingly, one of these samples contained T cells cross-reacting between HLA-DRB1*04:01/$eno_{326-340}$ and HLA-DRB1*04:01/cit-$eno_{326-340}$ (figure 5D), demonstrating that this was not only an *in vitro* phenomenon and that this cross-reactivity occurs in active RA as well.

4. Discussion

HLA-DRB1*04:01 is the most studied RA-associated HLA class II molecule. In the present study we have determined the crystal structures of HLA-DRB1*04:01 in complex with a 15-mer peptide derived from an RA candidate autoantigen, *i.e.* peptide 326-340 of α-enolase, in both its native and citrullinated forms. Whereas the native peptide is an abundantly available self-peptide, the citrullinated version represents a neo-antigen not necessarily present during thymic T cell selection. This is an important distinction since the posttranslational citrulline modification did not alter peptide binding to HLA, but instead would bias TCR binding based on contacts made with either the positively charged side chain of the arginine or the neutrally charged citrulline residue.

Upon assembly of HLA tetramers loaded with either of the two peptide versions, we could query the T cell repertoires of RA subjects and healthy individuals for the presence of cognate T cells recognizing the respective peptide-HLA complexes. We found no significant difference in frequencies of T cells recognizing the native $eno_{326-340}$ between peripheral blood of RA patients and healthy controls, implicating that such cells are usually not fully eliminated during negative selection, but rather part of the normal repertoire. T cells recognizing the native peptide further lacked a memory phenotype and signs of expansion, thus implying that they are naïve (*i.e.* have not encountered their cognate antigen(s)). In contrast, elevated frequencies of T cells specific for the citrullinated version of the α-enolase peptide in complex with HLA-DRB1*04:01 were observed in RA synovial fluid. These were

primarily of memory phenotype indicating that they have been previously activated and expanded.

Rheumatoid arthritis manifests in the joints both as synovitis, *i.e.* the growth of the synovial lining layer, and as joint swelling with exudates in the synovial joint space. We retrieved most of the data on synovial fluid, the edema of inflamed joints, but also had the possibility to assess inflamed synovial tissue. Although direct tissue staining is still not available for HLA class II tetramers, digesting synovial tissues and assaying single cell suspensions makes it still possible to interrogate the RA joint. As a proof of principle, we could demonstrate the presence of auto-reactive $CD4^+$ T cells in synovial tissue.

Two findings in this study are of particular interest. First, T cells reactive to HLA-DRB1*04:01 in complex with either the native α-enolase-derived $eno_{326-340}$ or the post translational modified version $cit\text{-}eno_{326-340}$ are part of the normal circulating T cell repertoire. Secondly, citrulline-reactive memory T cells were not only found elevated in synovial fluid but also in peripheral blood, which is compatible with the notion that the primary activation of auto-reactive T cells may take place outside the joints, for example in the gums [40, 41] or in the lungs [8, 42, 43]. These memory T cells may subsequently migrate to the joints in response to some 'local insult' [44], where they may be reactivated since extracellular citrullination is abundant during any kind of inflammation. These effector memory T cells may then contribute to the maintenance of an inflammatory milieu.

Moreover, our structural data allow the dissection of cross-reactivity and the possibility to predict TCR interactions. The vast majority of crystal structures of ternary TCR-peptide-HLA class II complexes indicate that the α-chain of

the TCR is landing in the vicinity of the first anchor residue [37-39]. The residue preceding the first anchor position is always protruding towards the TCR and during classical TCR-landing is typically located between the CDR3α and CDR1α loops. Our crystal structures demonstrate that the native and citrullinated versions of the peptide adopt nearly identical conformations throughout the entire length of the binding cleft of HLA-DRB1*04:01. This explains the similar binding affinities of the two peptide versions to HLA-DRB1*04:01 and hence that the arginine/citrulline residue serves as a TCR contact; it is therefore not surprising that citrullination may expand a new fraction of the T cell repertoire. Furthermore, the partial cross-reactivity, which we also functionally detected, could be taken to suggest the existence of three different types of TCRs: One type of TCR recognizes both peptides, *i.e.* without making direct contact with the citrulline or arginine, while the other two TCR types interacting directly to either the arginine or citrulline, hence exhibiting a charge preference only for the positive arginine or the neutral citrulline. It will be of interest to investigate in further detail to which extent cross-reactive and single-reactive T cells give different functional responses (*e.g.* cytokine secretion or proliferation) in response to the native or citrullinated peptide [45-47] and to what extent single-reactive T cells exhibit different TCR usage.

Obviously, there are some limitations in our study; due to sample constraints, we had to focus our efforts on one specific T cell epitope from one candidate autoantigen. Optimally, we could have studied several different peptides in parallel. Additionally, all RA patients included in the study have a chronic disease, and all of them are immunosuppressed, which could affect the

observed frequency and functionality of their T cells [13]. However, our cohort is representative for real life scenario patients. In fact, our data on peripheral blood are in good agreement with other studies using both *in vitro* and *ex vivo* approaches [13, 48]. Our results using synovial fluid cells, furthermore, corroborate the findings from the borrelia-induced Lyme arthritis, showing higher frequency of antigen-(borrelia)-reactive T cells in synovial fluid compared to peripheral blood [49, 50].

5. Conclusions

We identified and characterized autoreactive $CD4^+$ T cells specific for HLA-DRB1*04:01 in complex with the RA autoantigen α-enolase-derived native $eno_{326\text{-}340}$ and citrullinated cit-$eno_{326\text{-}340}$ peptides in the periphery, synovial fluids and synovial tissues of RA patients. Importantly, citrulline-α-enolase-specific T cells were more often of a memory phenotype in the circulation and were enriched in the synovial fluid compared to those recognizing the native variant of the peptide. This study highlights the added value of interrogating the inflamed joint and not only peripheral blood when studying autoimmunity.

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Suppl Table 1. **Flow cytometry panel used for *in vitro* and *ex vivo* peptide-HLA-DR tetramer phenotyping. (BL=Biolegend, eBio=eBioscience)**

Panel	Machine	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	eFluor650 NC	Alexa Fluor 700	APC-Cy7/H7	BV421/ Pacific Blue	V500
in vitro BRI	BD FACSCalibur	CD25 (BD)	Tmr	CD4 (BL)		Annexin V, CD14, CD19 (BD)					
in vitro KI	Beckman Coulter Gallios or BD Influx	Annexin V, CD14, CD19 (BD)	Tmr	CD25 (BL)							CD4 (BD)
ex vivo BRI	BD LSR II	Annexin V (BD) CD14, CD19 (BL)	Tmr	CCR6 (BD)	CD28 (Biol)		CD45RO (eBio)	CCR7 (BD)		CXCR3 (BL)	CD4 (BD)
ex vivo KI	Beckman Coulter Gallios	Annexin V, CD14, CD19 (BD)	Tmr	CD25 (BL)	CD62L (BD)			CD28 (BL)	CD45RO (BL)		CD4 (BD)

Suppl Table 2. **Data collection and refinement statistics of the crystal structures of HLA-DRB1*04:01 in complex with cit-eno$_{326-340}$ or eno$_{326-340}$**

	cit-eno$_{326-340}$	eno$_{326-340}$
PDB code	5NIG	5NI9
Data collection:		
Beamline	ID30A-1/MASSIF-1, ESRF	ID30A-1/MASSIF-1, ESRF
Wavelength (Å)	0.966	0.966
Space group	$P2_12_12$	$P2_12_12$
a (Å) b (Å) c (Å)	68.6 128.3 53.5	68.5 128.1 53.6
Resolution (Å)	46.86 – 1.35 (1.43 – 1.35)	46.77 – 1.33 (1.4 – 1.33)
No. of observed reflections	455890	451740
No. of unique reflections	103630	108263
Multiplicity	4.4	4.2
Completeness (%)	98.9 (97.6)	99.3 (98.9)
[1]R_{meas} (%)	8.3 (70.2)	5.0 (70.1)
I/σ(I)	9.9 (1.9)	13.7 (1.8)
CC(1/2) (%)	99.6(78.0)	99.9 (80.5)
Wilson B-value (Å^2)	23.5	26.0
Refinement statistics:		
Resolution of Data (Å)	46.86 – 1.35	49.43 – 1.33
[2]R_{cryst} (%)	14.0	14.7
[3]R_{free} (%)	17.8	17.5
Number of protein atoms	3183	3141
Number of peptide atoms	121	121
Water molecules	414	309
Rmsd from ideal geometry		
Bond length (º)	0.011	0.011
Bond angles (deg.)	1.56	1.48
Ramachandran Plot (%):		
Residues in preferred regions	98.2	98.3
Residues in allowed regions	1.8	1.7
Outliers	0	0
Average B- value (Å2)	23.9	25.2
Protein	21.8	23.8
Peptide	26.8	27.0
Water	38.4	36.5

Values in parentheses are for the highest resolution shell

[1] $R_{meas} = \frac{\sum_{hkl} \sqrt{\frac{n}{n-1}} \sum_{j=1}^{n} |I_{hkl,j} - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_{j} I_{hkl,j}}$ redundancy independent R-factor (intensities) [36]

[2]R_{cryst} = Σ||F_o| - |F_c||/Σ|F_o|, where |F_o| and |F_c| are the observed and calculated structure factor amplitudes of a particular reflection and the summation is over 95% of the reflections in the specified resolution range. The remaining ~5% of the reflections were randomly selected (test set) before the structure refinement and not included in the structure refinement.

[3]R_{free} was calculated over these reflections using the same equation as for R_{cryst}.

FIGURE LEGENDS

Fig. 1. Crystal structures of HLA-DRB1*04:01 in complex with eno$_{326-340}$ or with cit-eno$_{326-340}$ show conventional peptide binding with p328I as anchor residue in pocket P1.

(**A**) Superposition (overlay) of eno$_{326-340}$ (orange) and cit-eno$_{326-340}$ (light blue) in complex with HLA-DRB1*04:01 demonstrates nearly identical conformation of both peptides in the peptide binding cleft. The HLA-DRB1*04:01 is shown as ribbon diagram. The peptides are shown in sticks representation.

(**B**) Electron-density maps calculated for eno$_{326-340}$ (left) and cit-eno$_{326-340}$ (right) allow unambiguous modelling of peptides in the binding cleft.

(**C**). Comparison of the electrostatically colored surface of HLA-DRB1*04:01 in complex with the native eno$_{326-340}$ (left) and modified cit-eno$_{326-340}$ (right) illustrates the difference in property of the TCR landing surface. Red – negatively charged and blue – positively charged residues. Positions of p327Arg/Cit are labeled.

Fig. 2. peptide-HLA tetramers for *in vitro* and *ex vivo* analyses.

(**A**) PBMC from HLA-DRB1*04:01 healthy subjects were stimulated with peptides, cultured for 14 days and stained with indicated tetramers. Left plot: Negative control; cells were stimulated with eno$_{326-340}$ and stained with HA$_{306-318}$-loaded tetramer**.** Middle and right plot: Cells were stimulated with HA$_{306-318}$ (middle) or eno$_{326-340}$ and cit-eno$_{326-340}$ peptides (right) and stained with corresponding tetramers.

(**B**) Representative example of *ex vivo* analysis of influenza-(HA)-specific T cells from synovial fluid of a DRB1*04:01 positive RA subject after staining with $HA_{306-318}$ tetramer along with CD4, CD45RO, CD25 and CD28 antibodies.
(**C**) Proportion (%) of positive tested patients for $HA_{306-318}$ or $eno_{326-340}$ in peripheral blood of control subjects (white bars) and RA patients (grey bars). The numbers of positive tested individuals/totally tested individuals is depicted on top of the bars.
(**D**) The frequency of antigen-specific $CD4^+$ T cells in blood from healthy controls (unfilled symbols) and RA-patients (grey symbols). The y-axis depicts tetramer-positive T cells per 1 Million $CD4^+$ T cells. Cut-off for positivity is 1 per 1 x 10^6.
(**E**) Phenotypic comparison of tetramer positive cells from blood and synovial fluid. Each symbol depicts the percentage of tetramer-positive cells that are positive for the memory marker CD45RO.

Fig. 3. Frequency and characterization of T cells specific for the citrullinated neo-antigen cit-$eno_{326-340}$.
(**A**) Proportion (%) of positive tested patients for cit-$eno_{326-340}$ in peripheral blood of control subjects (white bars), peripheral blood of RA patients (grey bars) and synovial fluid of RA patients (black bars). The numbers of positive tested individuals/totally tested individuals is depicted on top of the bars.
(**B**) The frequency of antigen-specific $CD4^+$ T cells in blood from healthy controls (unfilled symbols) and RA-patients (grey symbols) and synovial fluid from RA-patients (black symbols). The y-axis depicts tetramer-positive T cells

per 1 Million $CD4^+$ T cells. Cut-off for positivity is 1 per 1 x 10^6. p values <0.05 are considered significant and marked with an asterisk.

(**C**) Phenotypic comparison of tetramer positive cells from blood and synovial fluid. Each symbol depicts the percentage of tetramer-positive cells that are positive for the memory marker CD45RO. p values<0.05 are considered significant and marked with an asterisk. Two asterisks depicts p values <0.01.

(**D**) Cells from a synovial biopsy obtained from an HLA-DRB1*04:01 RA patient and enzymatically digested were stimulated with $eno_{326-340}$ and cit-$eno_{326-340}$, cultured for 14 days and stained with a cocktail of the two tetramers.

(**E**) The frequency of antigen-specific $CD4^+$ T cells in peripheral blood (PB) and synovial fluid (SF) in paired samples from RA patients (n=7 for $eno_{326-340}$ and n=6 for cit-$eno_{326-340}$) is shown. Plotted are Tmr+ memory cells per 1 Million $CD4^+$ T cells. Left: cells specific for $eno_{326-340}$, right: cit- $eno_{326-340}$ specific cells.

Fig. 4. Peptide residue p327 can participate in TCR binding.

Arginine/citruline-327 precedes the first peptide anchor residue and is available to contact the CDR1α and/or CDR3α loops.

(**A**). $Eno_{326-340}$ is shown as sticks and two possible conformations of p327R are depicted to demonstrate different potential binding scenarios. CDR1α and CDR3α loops from crystal structures of three ternary complexes of HLA-DRB1*04:01 in complex with TCR HA1.7, specific for influenza hemagglutinin HA1 peptide (magenta), TCR MS2-3C8, specific for myelin basic protein (pink) and tumor-specific TCR G4 (green) are shown.

(**B**). Arginine/citrulline-327 is located in the vicinity of the HLA-DRB1*04:01 surface area (colored in blue) interacting with HLA-DRB1*04:01-specific TCRs mentioned in figure 4A.

Fig. 5. Dual-tetramer staining with PE and APC-Tmr in peripheral blood and synovial fluid.

(**A, B**) PBMC were expanded for 14 days with either $HA_{306-318}$, $eno_{326-340}$ or cit-$eno_{326-340}$ and stained with corresponding tetramers. Examples from two representative HLA-DRB1*04:01 RA patients are shown.

(**C**) HLA-DRB1*04:01-IE transgenic mice were immunized with $eno_{326-340}$ peptide and their proliferation responses measured after re-stimulation with either $eno_{326-340}$ or cit-$eno_{326-340}$ peptide.

(**D**) *Ex vivo* staining of synovial fluid from an HLA-DRB1*04:01 RA patient. $Eno_{326-340}$-Tmr was conjugated to APC and cit-$eno_{326-340}$-Tmr was conjugated to PE.

Supplementary Figure 1.

Binding of $eno_{326-340}$ peptide in the HLA-DRB1*04:01 peptide binding cleft. The HLA-DR is shown as a surface colored with electrostatic potential (red – negatively charged and blue – positively charged residues). The peptide is shown in sticks representation. Peptide residues and binding pockets are labeled.

Supplementary Figure 2.

Interactions of the two eno$_{326-340}$ peptides with shared epitope residues Q70B and K71B of HLA-DRB1*04:01. The HLA-DR is shown as a ribbon diagram. The peptides eno$_{326-340}$ (figure A, orange) and cit-eno$_{326-340}$ (figure B, light blue) are shown in sticks representation. Residues p334E and K71B were modelled in two alternative conformations. Both conformations allow for salt bridge formation between side chains of residues p334E and K71B.

Supplementary Figure 3.

Competition binding assay demonstrates the decreased capacity of mutated α-enolase peptide KRIAKAVNEKS**S**N**S**L (two cystein residues replaced by serine) to bind HLA-DRB1*04:01 compared to the native eno$_{326-340}$ peptide KRIAKAVNEKS**C**N**C**L. The data for the reference influenza hemagglutinin HA$_{306-318}$ (circles, solid gray line), native eno$_{326-340}$ (triangles, dotted black line) and mutated eno$_{326-340}$ (squares, dashed black line) peptides are shown. The lines represent fitting of experimental data to the one site competition model using SigmaPlot software v.13 (Systat software). The calculated IC$_{50}$ values were 0.047±0.006μM for HA$_{306-318}$, 0.073±0.013μM for native eno$_{326-340}$ and 0.67±0.1μM for double cysteine-to-serine mutant of eno$_{326-340}$ peptide. Increasing concentrations of the peptides were incubated in 384-well polypropylene plate in the presence of 30nM HLA-DRB1*04:01 and 5nM biotin-labeled HA$_{306-318}$ peptide overnight at 37°C in a humidified incubator. Reaction mixture was transferred to a polystyrene plate coated with anti-HLA-DR mAb L243 and incubated overnight at +4°C. Bound peptide-HLA complexes were developed in DELFIA® time-resolved fluorescence assay using europium-labeled streptavidin (PerkinElmer).