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HLA-DRB1*04:01 restricted T cells in blood and synovial fluid of RA patients

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Abstract

Objective—ACPA-positive RA is associated with distinct HLA-DR alleles and with antibodies targeting citrullinated α-enolase. We screened α-enolase for tentative T cell epitopes and focused the present study on aa326–340. Both the frequency and quality of T cell responses were studied in blood and synovial fluid of RA patients.

Methods—Binding of native and citrullinated α-eno$_{326–340}$ HLA-DRB1*04:01 was studied by in vitro competition assays and by DSC, and T cell responses by in vitro culture. HLA-DRB1*04:01 tetramers were used for assessing ex vivo frequency and phenotype of α-enolase-specific T cells. Cross-reactivity, i.e. whether the same T cells could recognize both peptides, was addressed utilizing RA patient samples and HLA-DRB1*04:01-transgenic mice.

Results—Frequencies of T cells recognizing native eno$_{326–340}$ were similar in blood and synovial fluid, whereas the frequency of cit-eno$_{326–340}$ T cells was elevated in synovial fluid. The cit-eno-specific, but not native-eno-specific T cells in blood displayed a memory CD45RO phenotype indicating previous exposure to citrullinated enolase. In vitro assays revealed functional responses to both peptides in patient samples and immunized HLA-DRB1*04:01-IE-transgenic mice. Cross-reactivity to the two peptides was observed but was not universal.

Conclusions—HLA-DRB1*04:01-restricted CD4 T cells recognizing native and cit-eno$_{326–340}$ peptides are part of the normal circulating T cell repertoire. We observed more T cells specific for the citrullinated peptide with a memory phenotype in the circulation, which supports the concept that such T cells might be activated outside the joints and subsequently recruited and possibly re-activated in inflamed joints.

Keywords

Rheumatoid arthritis; CD4+ T cells; MHC class II tetramer; ACPA; alpha-enolase

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that can be divided into subsets based on the presence of antibodies to citrullinated proteins (ACPAs) [1]. Antibody targets represent candidate autoantigens for T cell responses [2–5]. This is further substantiated by the observation that ACPA-positive RA is strongly associated with HLA-DR-shared epitope alleles [6–8]. Previous studies of peripheral blood from ACPA-positive RA patients have demonstrated auto-reactive T cells against several citrullinated self-proteins [9–13]. In this context α-enolase represents an interesting autoantigen as the presence of autoantibodies against citrullinated α-enolase is preferentially linked to HLA-DRB1*04 [14].

α-enolase is an ubiquitous protein but is also abundantly expressed in the inflamed synovium [4] and antibodies to the immunodominant B cell epitope of citrullinated α-enolase (CEP-1) are present in approximately 40% of ACPA-positive patients [14, 15]. These autoantibodies are highly specific for RA [4, 14] and enriched in synovial fluid [16].

CD4+ T cells play an important role in RA by providing B cell help and secreting proinflammatory cytokines, which may contribute to the perpetuation of the inflammation in RA. A major hurdle in research aimed at characterizing T cells that specifically recognize potential RA-specific autoantigens is the fact that autoantigen-specific T cells, as shown in different disease settings, are generally present in very low frequencies [17–21]. Through recent improvements in the field of MHC class II tetramers it is now possible to look specifically for rare antigen-specific CD4+ T cells directly ex vivo without the need of in vitro expansion [13, 22–24], utilizing a bead enrichment method. Herein we have utilized this technology to study a-enolase specific T cells in blood and synovial fluid of RA patients.

MATERIAL AND METHODS

Patients and healthy control subjects

RA patients were recruited under the auspices of the Karolinska University Hospital Rheumatology clinic, the Benaroya Research Institute (BRI) rheumatic disease registry, and the BRI immune-mediated disease registry. Control subjects were recruited from the BRI IMD registry or the Uppsala blood bank (table S1). Informed consent was obtained from everyone based on local ethical permits. All patients were diagnosed as having RA by a rheumatologist in accordance with the 1987 American College of Rheumatology criteria [25]. All subjects recruited in this study 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 biopsy was obtained through ultrasound guided arthroscopy from one RA patient. Synovial tissue was digested via incubation with Collagenase A (Roche) and DNAse (Roche). The tissue was then sieved through a 100μm filter and washed before staining.

Peptides and selection

Peptides used for intracellular cytokine staining were synthesized by ProImmune. Peptides used for all other purposes were synthesized by Genscript. For peptide binding assays increasing concentrations of each non-biotinylated test peptide were incubated in competition with 0.01μM biotinylated influenza-$HA_{306–318}$ peptide in wells coated with HLA-DRB1*04:01 protein as previously described [26]. Peptide binding curves were fitted by non-linear regression with a sigmoidal dose response curve model using Prism software (version 5.0, GraphPad Software Inc.).

Tetramers

Recombinant DRB1*04:01 protein was produced as previously described [27]. The biotinylated monomer was loaded with either influenza-$HA_{306–318}$ or the α-enolase peptide of interest by incubating 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).

In vitro detection of influenza-hemagglutinin (HA) and α-enolase-specific T cells by HLA class II tetramers

For tetramer assays PBMCs from healthy controls or a synovial biopsy from a DRB1*04:01 RA subject were cultured in RPMI-1640 + 10% pooled human serum with 10μg/ml of either $HA_{306–318}$ or the α-enolase peptides. Interleukin-2 (IL-2) (Proleukin, Novartis) was added on day 6. After 14 days cells were stained according to table S2. The data was analyzed by FlowJo software version 9.3.3. or higher (Treestar Inc.).

Ex vivo detection of influenza-hemagglutin and α-enolase-specific T cells by HLA class II tetramers

PBMC and SFMC samples from RA patients were labeled according to table S2 as previously described [27]. Samples were run on a BD LSRII flow cytometer or Beckman Coulter Gallios, and data was analyzed using FlowJo software version 9.3.3. or higher. The frequency of antigen-specific cells was calculated as the total number of Tmr+ cells in the bound fraction divided by the total number of CD4+ T cells. A cut-off of 1 per $1x10^6$ CD4+ T cells was applied.

Intracellular cytokine staining

Functional T cell assays were performed as previously described [11]. Cells were treated with LIVE/DEAD Fixable Green Dead Cell Stain (Invitrogen) and stained according to table S2. Intracellular cytokine staining (ICS) was performed using Cytofix/Cytoperm Kit (BD) according to the manufacturers instructions. Cells were run on a Beckman Coulter CyAn. Analyses were performed with FlowJo software, version 7.5.1 (Treestar Inc.).

Murine Assays

HLA-DR04:01-IE transgenic mice on a class II deficient C57Bl/6 (I-Abo/o) background were obtained from Taconic and were housed under specific pathogen-free conditions. Mice were immunized with 100μg of peptide and spleenocytes harvested 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) and animals were housed in the BRI AAALAC-accredited animal facility.

Statistics

Statistical tests used for this paper include Fischer exact test, Wilcoxon matched pairs ranked test and Mann-Whitney tests. Spearman's rank correlation coefficient was used to analyze correlations. Analyses were performed using prism software (version 5.0). Values <0.05 were considered significant.

RESULTS

Alpha-enolase$_{326–340}$ can be presented by HLA-DRB1*04:01 in both its native and citrullinated form

Overlapping peptides covering the entire length of α-enolase were screened for HLA-DRB1*04:01 binding. Among the peptides identified to contain binding epitopes for HLA-DRB1*04:01, three were successfully used to generate HLA-DRB1*04:01 tetramers. The sequences for these are shown in figure 1A and supplementary figure S1A-B. We subsequently performed in vitro culture experiments with the candidate peptides to validate whether T cells recognizing the different peptide-MHC complexes were indeed present in HLA-matched patient samples. We focused our study on the peptide pair eno$_{326–340}$ and cit-eno$_{326–340}$, because these specificities were reproducibly seen in the pilot experiments. As shown in figure 1B both peptides bound with equal avidity to the HLA-DRB1*04:01 molecule.

In vitro detection of antigen-specific T cells by tetramer

To substantiate that antigen-specific T cells can be found in HLA-DRB1*04:01-positive samples and to validate the autoantigen-loaded tetramers, PBMCs from healthy subjects were stimulated with either influenza-HA or α-enolase peptides. After 14 days of culture, during which both naïve and memory T cells specific for the respective peptides can expand, we stained with tetramers for the presence of antigen-specific T cells. Hereby we could demonstrate the presence CD4+ T cells specific for both HA (figure 1D) and the two α-enolase peptides (figure 1E).

Frequencies of autoantigen-specific T cells

Studying expanded cell populations involves in vitro culture steps, which can alter phenotypes and does not allow for the enumeration of the frequency of cells at the time of sampling. To avoid these complications we continued to study antigen-specific cells directly ex vivo in the peripheral blood and synovial fluid of our study subjects.

HA$_{306–318}$ was used as a control tetramer (figure 2A). HA-specific T cells were found in similar numbers and frequency in the peripheral blood of healthy controls, RA peripheral blood, and in RA synovial fluid (figure 2B, supplementary figure S2).

Native α-enolase specific CD4+ T cells were found in the blood of all healthy controls (10/10), in the blood of 12/18 investigated patients and in 13/18 of the investigated synovial fluid samples (supplementary figure S2). There was no significant difference in the frequencies of T cells recognizing native $eno_{326–340}$ in RA blood as compared with synovial fluid, nor between peripheral blood of RA patients and healthy controls.

T cells specific for the citrullinated version of $eno_{326–340}$ were present in the blood of 3/11 controls and in 13/21 RA patients and in 9/18 synovial fluids from RA joint effusions (figure S2). In contrast to the case for native enolase reactive T cells, there was an increased frequency of T cells reactive to the citrullinated enolase peptide in the synovial fluid samples as compared with peripheral blood, p<x (figure 2B). The same tendency was seen also by direct comparison in paired samples from synovial fluid and peripheral blood (figure 2C).

Phenotype of autoantigen-specific T cells

We next investigated the phenotypes of the tetramer-positive T cells. Peripheral blood T cells specific for cit-$eno_{326–340}$ from RA patients displayed a higher proportion of CD45RO-positive (memory-type) T cells as compared to the native $eno_{326–340}$-specific T cells, p<x (figure 2D). The expression of CD25, a cell surface marker that can either depict recently activated T cells or those of regulatory function, was generally low on the tetramer+ cells, although variable, with no bias toward a $CD25^{high}$ phenotype (figure 2E). We also investigated whether the tetramer+ T cells belonged to the proinflammatory $CD4+CD28^{null}$ phenotype, which can be enriched in the blood of some RA patients [28], We saw no differences, with most tetramer-positive T cells expressing CD28 both in peripheral blood and synovial fluid (data not shown).

Both native and cit-$eno_{326–340}$ elicit functional T cell responses

The presence of memory T cells specific for citrullinated-α-enolase in RA patients suggests they have seen their cognate antigen in vivo. To investigate any functional differences of α-enolase recognition we stimulated PBMC from RA patients with the two α-enolase peptides. After 5 days we measured surface expression of CD40L, as a marker for antigen-recognition, as well as intracellular levels of IFN-γ, IL-17A and TNF. We detected immune responses to both peptides, with some variation in the degree of CD40L upregulation and cytokine pattern (figure 3A). This response was HLA-DR restricted as it could be blocked by antibodies against HLA-DR, but not by antibodies against HLA-DQ or HLA-DP (data not shown). As seen in figure 3A, some patients had T cells favoring a cit-eno response, others preferentially responded to the native peptide, and some patients responded similarly to both peptides.

Our data suggests that the T cells from some of our patients fail to distinguish the two versions of the α-enolase peptide. To dissect this further, we immunized HLA-DRB1*04:01-IE transgenic mice with the native $eno_{326–340}$ peptide and examined their recall response to both enolase peptides by proliferation assays. Mice immunized with $eno_{326–340}$ showed a similar proliferation response irrespective if they were restimulated with $eno_{326–340}$ or cit-$eno_{326–340}$ (figure 3B), suggesting cross-recognition between the peptides in a setting of active immunization. As we have reported previously, also the opposite is true, i.e. mice

immunized with the citrullinated peptide display recall responses to both versions of the peptide (13).

T cells can cross-recognize native and cit-eno$_{326–340}$

To further elucidate if an individual T cells could recognize both forms of the α-enolase peptide we manufactured our α-enolase tetramers in two different flourophores to allow co-staining of samples and used these tetramers for ex vivo stainings of T cells obtained from three synovial fluids of DRB1*04:01 positive RA patients. One of these samples contained T cells reactive to enolase and in this case, all stained cells were positive for both tetramers (figure 4).

We continued to investigate this cross-recognition by in vitro assays, where more Tmr-positive events can be expected. We performed in vitro restimulation experiments with T cells from peripheral blood of several DRB1*04:01 positive RA patients using both the native and citrullinated enolase peptides as well as the control HA peptide. Dual-color Tmr stainings were then performed on these in vitro expanded cells. As a positive control, in vitro expanded cells stimulated with HA$_{306–318}$ were stained simultaneously with the two different labeled HA-tetramers. As depicted in figure 5, two types of reactivity patterns were found. Some patients had many double-positive T cells (figure 5A), while other patients had mainly T cells that specifically recognized only one of the peptides without cross-recognition (figure 5B).

Alpha-enolase-specific T cells in synovial tissue

As synovial fluid and synovial tissue are two discrete compartments of the rheumatic joint, we wanted to investigate whether autoantigen-specific T cells were also present in the tissue. To this end, we digested a synovial biopsy specimen from an HLA-DRB1*04:01 RA patient, and performed in vitro tetramer staining following in vitro stimulation with both peptides. Hereby, T cells specific for eno$_{326–340}$ and cit-eno$_{326–340}$ could be visualized (figure 6).

DISCUSSION

Direct investigation of human antigen-specific CD4+ T cells has become feasible in recent years due to improvements in tetramer technology, allowing for enumeration of frequencies and ex vivo phenotyping of even rare T cells e.g. in the setting of autoimmune diseases [29]. Tissue staining is still not available for HLA class II tetramers, but the RA joint can be interrogated via cell suspensions by sampling synovial fluid and digesting synovial tissue.

In the present study we have focused on parallel studies of CD4+ T cells from the blood and inflamed joints of RA patients to assess whether auto-reactive T cells are enriched and their phenotypes change at the site of inflammation. Our primary material was synovial fluid, where α-enolase reactive T cells were readily found, but as a proof of principle, we could also demonstrate auto-reactive CD4+ T cells in synovial tissue. We chose α-enolase as our model autoantigen in this study because it represents one of the major autoantigens recognized by autoantibodies in RA [4, 14, 30], and because the response to citrullinated α-enolase is strongly associated with a specific HLA-DR allotype DRB1*04:01 [14]. We

further focused our study on $eno_{326–340}$, a peptide that binds to HLA-DRB1*04:01 in both its native as well as its citrullinated form.

Two findings in this study are of particular interest. First, we showed a skewing towards a memory phenotype in circulating T cells that recognized the citrullinated α-enolase peptide compared to those that recognized the native version. Secondly, even though both α-enolase reactive T cell subsets in synovial fluid were of the memory phenotype, we found a higher frequency of citrullinated α-enolase T cells from inflamed RA joints.

Our findings indicate that T cells reactive to both the native and citrullinated α-enolase peptides are part of the normal circulating T cell repertoire. The observation that RA patients have more circulating memory T cells to cit-α-enolase 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 [31, 32] or in the lungs [8, 33, 34]. These memory T cells may subsequently migrate to joints in response to some ‘local insult’, where they may be reactivated since extracellular citrullination is abundant during any kind of inflammation. These effector T cells may then contribute to the maintenance of an inflammatory milieu.

Our study also addresses the question of whether TCRs recognizing these α-enolase peptides are cross-reactive or specific for either the native or citrullinated form. In this context, it is essential to note that the post-translationally modified arginine is in a position that is just outside the binding grove of the HLA-DRB1*04:01 molecule (P-1), which explains why binding to this HLA-DR molecule is similar for the two peptides. Provided the position of the citrullinated residue, it’s not surprising that some cross-reactivity is seen for some T cell receptors. The partial cross-reactivity we detected could be explained by the existence of three different types of TCRs. One type of TCR recognizes both peptides, irrespective of the charge difference between the positive arginine and neutral citrulline, and two other TCR types only recognize either the arginine or the citrulline at P-1. It will be of interest to investigate in further detail to which extent cross-reactive and single-reactive T cells give different responses (e.g. cytokine secretion or proliferation) in response to the native or citrullinated peptide [35–37].

Obviously, there are also some limitations in our study; we only investigated one autoantigen. Additionally, all RA patients studied have chronic disease, and all of them are immunosuppressed, which could affect the frequency and functionality of the T cells [13]. However, our cohort is thus representative of real life scenario (typical) patients. In fact, our data on peripheral blood are in good agreement with other studies using both in vitro and ex vivo approaches [38] [13]. Concerning data from synovial fluid cells, there are two previous reports that have utilized tetramer-technology to enumerate antigen-reactive T cells, both in the setting of Lyme arthritis. Similar to our results, both of these reports found a higher frequency of antigen-(borrelia)-reactive T cells in synovial fluid as compared to peripheral blood [39, 40].

CONCLUSIONS

We identified and characterized auto-reactive CD4+ T cells specific for the RA candidate autoantigen α-enolase from the periphery, synovial fluid, and synovial tissue 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 need to look at native and citrullinated -α-enolase T cell epitopes in future studies dissecting RA initiation and propagation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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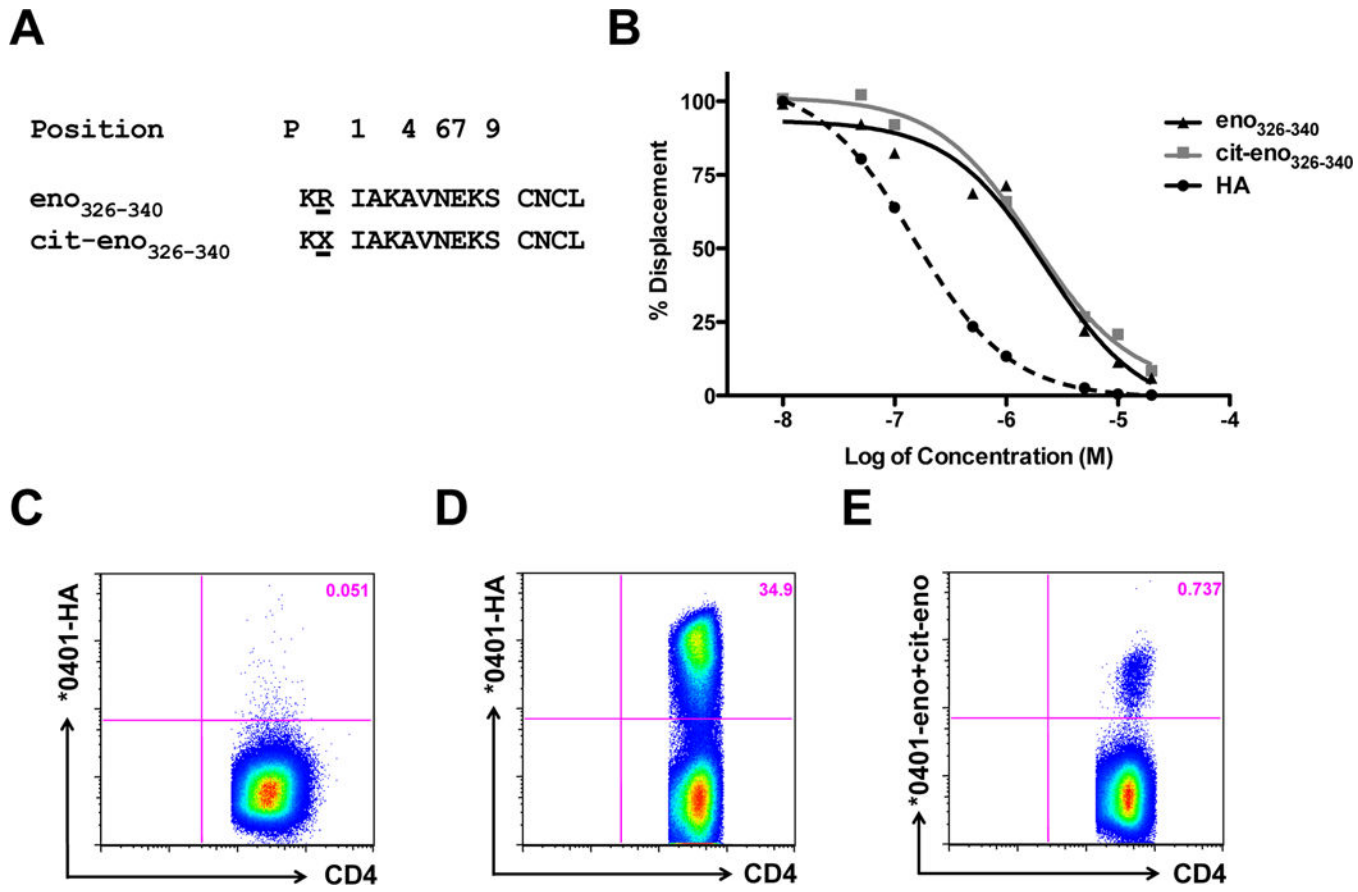

Figure 1. Peptide sequence, binding to HLA-DRB1*04:01 and tetramer staining.

(A) Amino acid sequence from α-enolase aa326–340 in the native ($eno_{326–340}$) and citrullinated form ($cit\text{-}eno_{326–340}$). Numbers indicate the tentative binding position for P1, P4, P6, P7 and P9. **(B)** Binding affinity to HLA-DRB1*04:01, as determined by europium-based peptide competition assay, was done for the native arginine and the citrullinated version of α-enolase. HA: $hemagglutinin_{306–318}$ antigen as a positive control. **(C-E)** PBMC from DRB1*04:01 healthy subjects were stimulated with peptides, cultured for 14 days and stained with tetramers. **(C)** Negative control, cells were stimulated with $eno_{326–340}$ and stained with $HA_{306–318}$ tetramer. **(D+E)** Cells were stimulated with $HA_{306–318}$ **(D)** or $eno_{326–340}$ and $cit\text{-}eno_{326–340}$ peptides **(E)** and stained with corresponding tetramers.

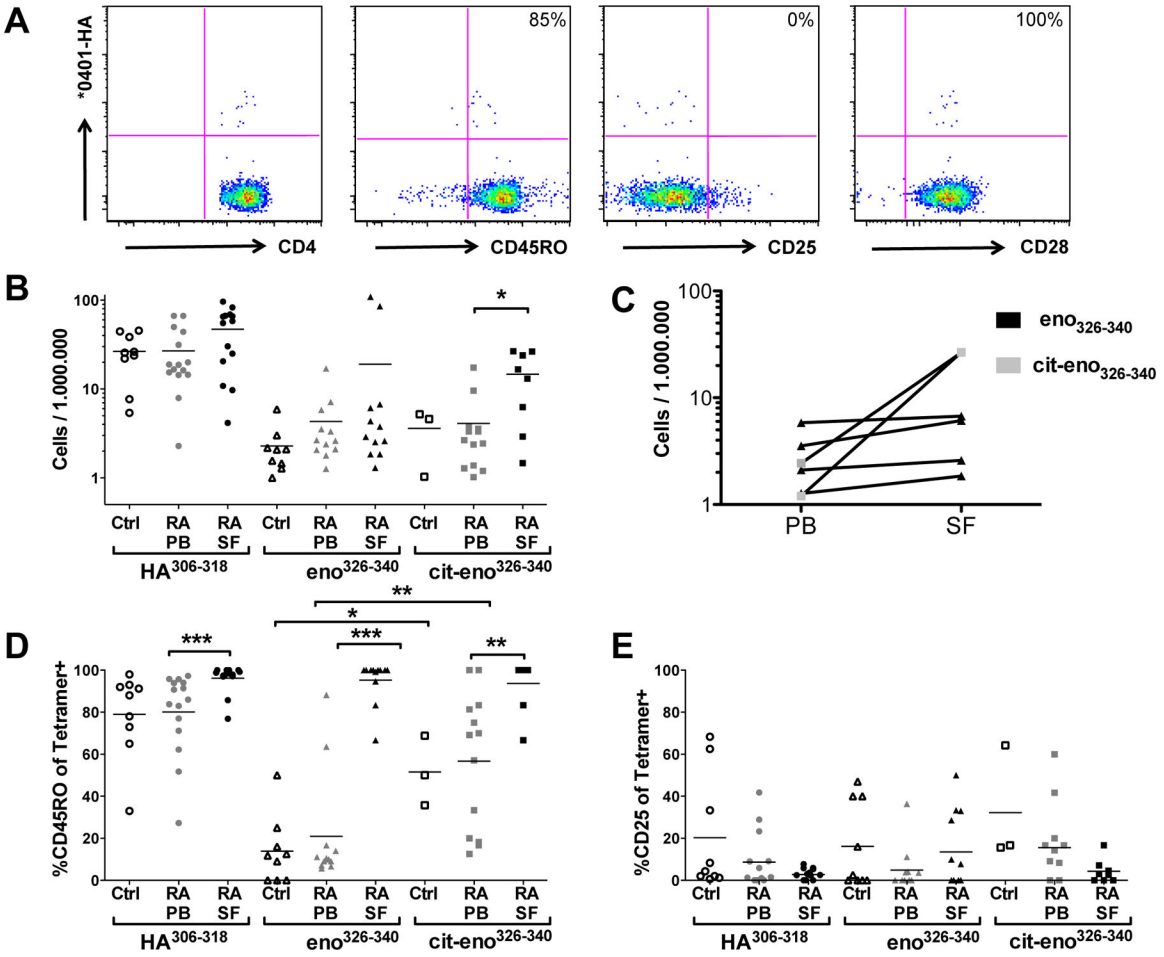

Figure 2. Frequency and characterization of tetramer-positive cells.

(A) Representative example of ex vivo analysis for flu specific T cells from SFMC of a DRB1*04:01 RA subject after staining with $HA_{306–318}$ tetramer along with CD4, CD45RO, CD25 and CD28 antibodies. **(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). Plotted are positive cells per 1 x 10^6 CD4+ T cells. Cut-off for positivity is 1/1 x 10^6. **(C)** The frequency of antigen-specific CD4+ T cells in peripheral blood (PB) and synovial fluid (SF) in paired samples from RA patients is shown. Plotted are positive cells per 1 x 10^6 CD4+ T cells. Black: cells specific for $eno_{326–340}$, grey: cit- $eno_{326–340}$ specific cells. **(D-E)** Further characterization of tetramer positive cells from blood and synovial fluid. Depicted is the percentage of Tetramer-positive cells that are positive for CD45RO **(D)** or CD25 **(E).**

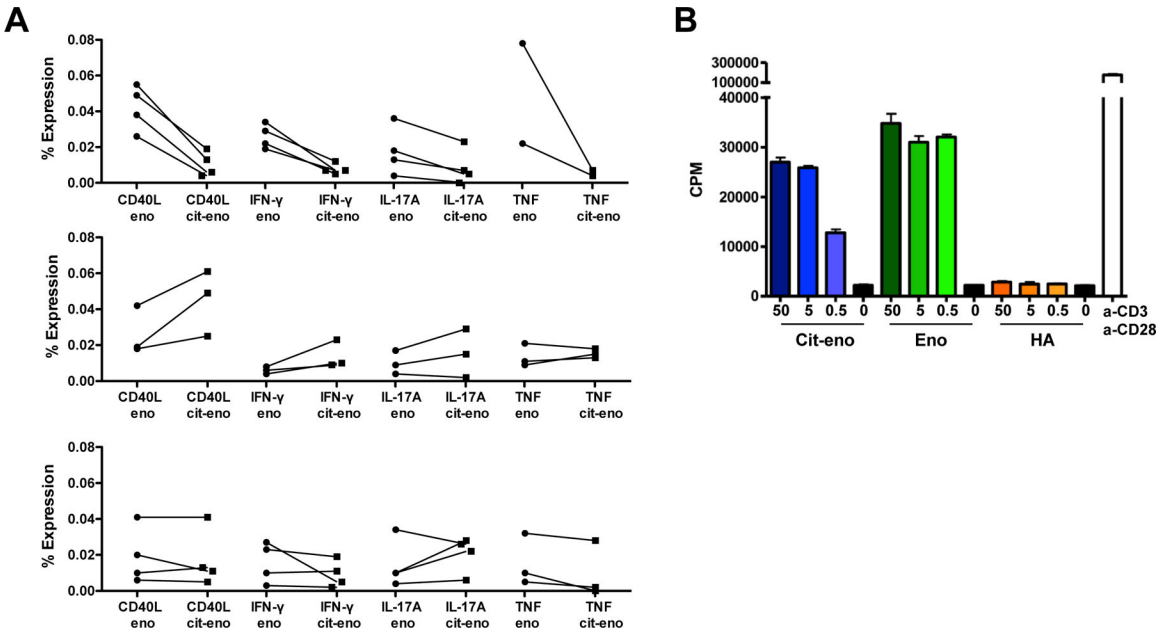

Figure 3. Functional T cell assay and murine studies.

(A) PBMC were stimulated with eno$_{326–340}$ or cit-eno$_{326–340}$ peptides for 5 days. -17A and TNF. We detected immunePatients were then divided into 3 groups (tertiles) based on response pattern: generally more stimulation with eno$_{326–340}$peptide (upper panel), generally more stimulation with cit-eno$_{326–340}$ peptide (centered panel) and patients with same or mixed stimulation (lower panel). Visualized is the % of expression on CD4+ T cells. **(B)** HLA-DRB1*04:01-IE transgenic mice were immunized with eno$_{326–340}$ peptide, restimulated with either eno$_{326–340}$ or cit-eno$_{326–340}$ peptide and proliferation was measured.

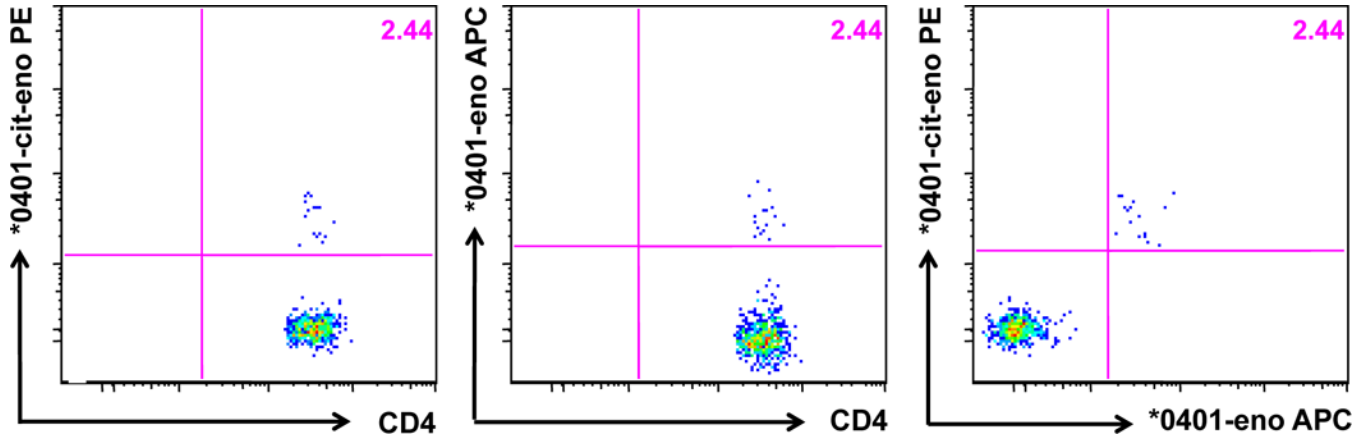

Figure 4. Tetramer stainings in synovial fluid.

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

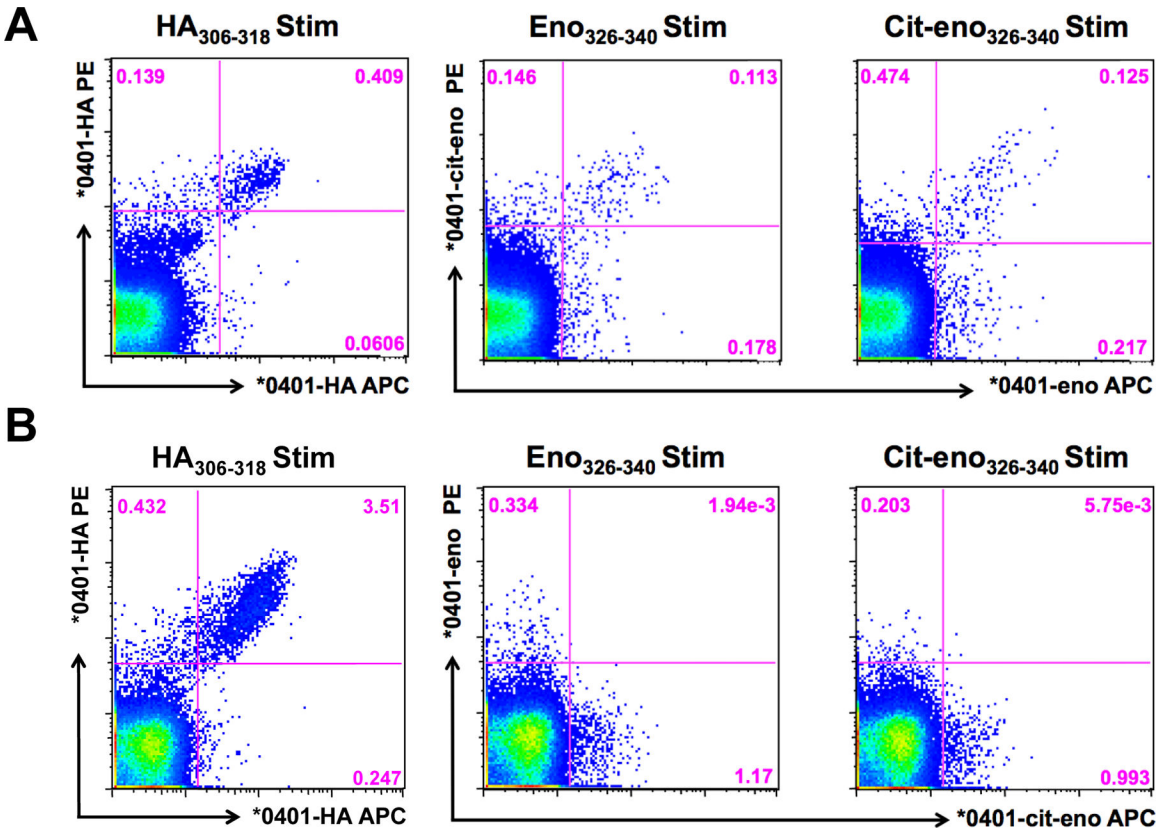

Figure 5. Dual-tetramer staining with PE and APC-Tmr in peripheral blood.

PBMC were expanded for 14 days with either $HA_{306–318}$, $eno_{326–340}$ or cit-$eno_{326–340}$ and stained with corresponding tetramers. Two examples from different HLA-DRB1*04:01 RA patients.

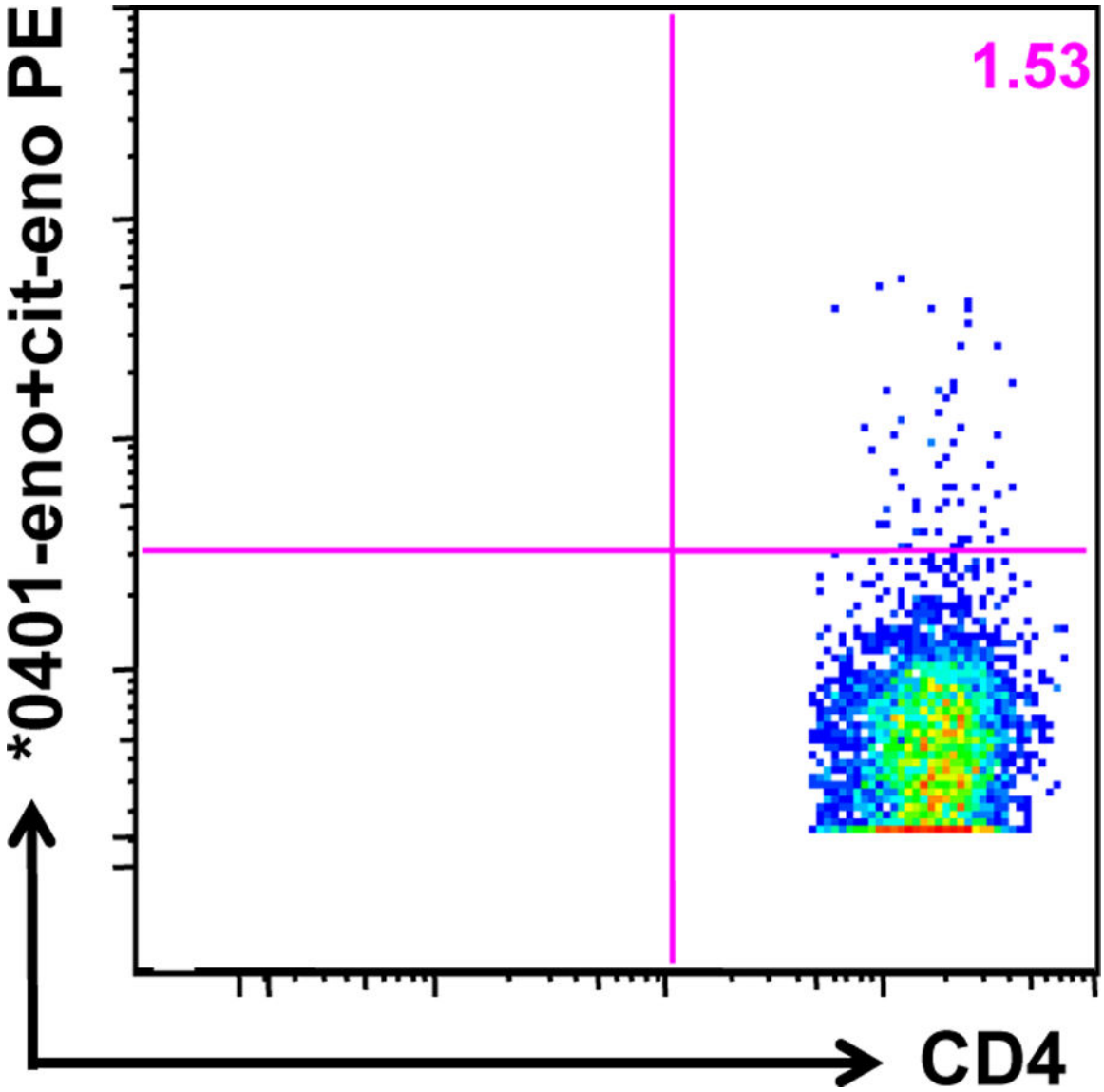

Figure 6. Enolase-specific T cells in synovial tissue.

A synovial biopsy was obtained from a DRB1*04:01 RA patient. The tissue was enzymatically digested and the cells were stimulated with $eno_{326–340}$ and cit-$eno_{326–340}$, cultured for 14 days and stained with the tetramers.