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Multifunctional T Cell Reactivity With Native and Glycosylated Type II Collagen in Rheumatoid Arthritis

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Objective. **Type II collagen (CII) is a cartilage-specific protein to which a loss of immune tolerance may trigger autoimmune reactions and cause arthritis. The major T cell epitope on CII, amino acids 259–273, can be presented by several HLA–DRB1*04 alleles in its native or posttranslational glycosylated form. The present study was undertaken to functionally explore and compare CII-autoreactive T cells from blood and synovial fluid of patients with rheumatoid arthritis (RA).**

Methods. **Peripheral blood was obtained from HLA–DRB1*04–positive RA patients (n = 10) and control subjects (n = 10) and stimulated in vitro with several variants of the $CII_{259–273}$ epitope, i.e., unmodified, glycosylated on Lys-264, glycosylated on Lys-270, or glycosylated on both Lys-264 and Lys-270. Upregulation of CD154 was used to identify responding T cells. These cells were further characterized by intracellular staining for interleukin-17 (IL-17), interferon-γ (IFNγ), and IL-2 by flow cytometry. Synovial T cells from RA patients were investigated in parallel.**

Results. **Multifunctional T cell responses toward all examined variants of the $CII_{259–273}$ peptide could be detected in RA patients and, to a lesser extent, also in healthy HLA-matched controls ($P < 0.001$). In RA patients, a comparison between blood- and joint-derived T cell function revealed a significant increase in levels of the proinflammatory cytokine IFNγ in synovial T cells ($P = 0.027$). Studies of longitudinally obtained samples showed that T cell responses were sustained over the course of disease, and even included epitope spreading.**

Conclusion. **The identification of inflammatory T cell responses to both glycosylated and nonglycosylated variants of the major CII epitope in RA patients suggests that CII autoreactivity in RA may be more common than previously recognized.**

CD4+ T cells are believed to have a central role in the autoimmune pathogenic mechanisms that occur in rheumatoid arthritis (RA). This notion is supported by findings of high numbers of activated CD4+ T cells in the inflamed joint and a strong genetic association with several HLA–DRB1 alleles (1). Type II collagen (CII), the main constituent of articular cartilage, is a possible autoantigen in RA. Indeed, an RA-like disease, collagen-induced arthritis (CIA), can be induced in rodents and monkeys following immunization with CII in adjuvant (2), and in humans with RA, autoantibodies toward both native and citrullinated CII are present in the serum and synovial fluid (SF) (3,4).

Susceptibility to CIA is associated with the murine class II major histocompatibility complex (MHC) molecule A^q (5), and also with transgenic expression of the human RA-associated class II MHC molecule HLA–DRB1*0401 (DR4) (5,6). Importantly, these class II

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MHC molecules (from mice and humans) select and present amino acids 259–273 from CII as the immunodominant T cell epitope in a similar manner. Likewise, epitopes recognized by CII-specific antibodies are shared between mice affected by CIA and patients with RA (4). Hence, MHC presentation as well as T and B cell recognition of CII show striking similarities in mice and humans.

Interestingly, $CII_{259–273}$ represents a T cell epitope with several variants. It contains 2 lysines, at positions 264 and 270, that can be hydroxylated and further glycosylated with mono- or disaccharides. These modifications are recognized by T cells and play an important role in the development of CIA, as well as influencing T cell tolerance to self CII in both A^q- and DR4-expressing mice (7–9).

Only a few published studies have addressed T cell responses to glycosylated and nonglycosylated CII in humans (7,10). In the present study, we investigated the frequencies and characteristics of T cell responses to different variants of the dominant HLA–DR*04–restricted T cell epitope on CII in RA patients. Our approach consisted of 1) parallel studies of several variants of the epitope, 2) detection of multiple effector functions, i.e., interleukin-2 (IL-2), IL-17, and interferon-γ (IFNγ) production, 3) comparison of these in T cells purified from paired blood and SF samples, and 4) longitudinal studies to assess changes occurring during the course of disease.

MATERIALS AND METHODS

Patients. Nine HLA–DRB1*0401–positive and 1 HLA–DRB1*0404–positive RA patients who were seropositive for anti-CII antibodies and had established disease according to the 1987 American College of Rheumatology criteria (11) and 10 HLA–DRB1*0401–positive healthy controls were recruited for this study. RA disease activity according to the 28-joint Disease Activity Score (DAS28) (12) was determined within 2 weeks of sampling when possible. Mononuclear cells were obtained from heparinized peripheral blood (PB) and SF collected from RA patients at 1 time point (n = 5) or up to 4 time points (n = 5) and passed over Ficoll-Hypaque gradients. Control PB mononuclear cells (PBMCs) were obtained at 1 time point. Isolated PBMCs and SFMCs were cryopreserved in liquid nitrogen in 10% DMSO and 90% heat-inactivated fetal bovine serum and thawed before use. Informed consent was obtained from all subjects under protocols approved by the Karolinska University Hospital Ethical Review Board or the Benaroya Research Institute Institutional Review Board.

Peptides. The following native and galactosylated variants of $CII_{259–273}$were synthesized as previously described (13): 1) CII-K, an unmodified $CII_{259–273}$ with lysine residues at positions 264 and 270, 2) CII-Gal264, galactosylated with a β-D-galactopyranose residue on l-hydroxylysine at position 264, 3) CII-Gal270, galactosylated exclusively at position 270, and 4) CII-Gal264/270, galactosylated at positions 264 and 270.

Functional cellular assay. PBMCs and SFMCs were thawed and rested overnight in RPMI 1640 supplemented with 10% pooled human AB serum (1.5×10^6 cell/well). Cells were stimulated for 7 hours with the respective peptide variants at a concentration of 50 μg/ml together with anti-CD28 (BioLegend) at 1 μg/ml. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 5 hours. As a positive control and for determination of CD154 assay sensitivity, staphylococcal enterotoxin B (SEB; Sigma-Aldrich) was added to a separate culture at 1 μg/ml. For the sensitivity assay, SEB-stimulated cells were diluted in 13 consecutive 2-fold dilutions, down to 1:8,192. Following stimulation, cells were treated with Live/Dead Fixable Green Dead Cell Stain (Invitrogen) and then stained for surface expression of CD3 and CD4 for positive gating, and for CD19 and CD14 for exclusion of monocytes and B cells. Cells were then fixed, permeabilized (BD Biosciences), and stained for IL-17, IFNγ, IL-2 (all from BioLegend), and CD154 (BD Biosciences). Background levels were determined using unstimulated cells (treated with anti-CD28 and brefeldin A), and the values were subtracted from those obtained in CII-stimulated cultures. Samples were run on a CyAn ADP Analyzer (Beckman Coulter), and the data were analyzed using FlowJo software, version 8.6.36 (Tree Star).

Statistical analysis. One-way analysis of variance (by Kruskal-Wallis test) followed by Dunn's multiple comparison test was used to analyze the significance of differences between groups, and paired *t*-test (Wilcoxon signed rank test) was used for comparing levels of cytokine production by T cells from SF and PB. Spearman's rank correlation was used for correlation analysis between disease activity (DAS28) and T cell responses. *P* values less than 0.05 were considered significant.

RESULTS

RA T cell reactivity with both native and glycosylated $CII_{259–273}$. It was recently demonstrated that activated antigen-specific CD4+ T cells could be detected based on up-regulation of CD154 (14). In this study we used CD154 up-regulation to study autoreactive CD4+ T cell responses to CII in RA. We first defined the lowest level of detection of CD154 up-regulation (i.e., the sensitivity of the assay). To this aim, cells were stimulated with the superantigen SEB and further diluted in 2-fold serial dilutions, demonstrating a linear reduction and a clear positive CD154 signal from CD4+ T cells at frequencies of <0.01% (Figure 1A). We then stimulated PBMCs from 10 RA patients and 10 healthy controls carrying HLA–DRB1*04 alleles with the different variants of the $CII_{259–273}$ epitope and investigated for positive responses. As depicted in Figures 1B and C, T cells from both RA patients and healthy controls responded to CII, with CII-Gal264 evoking the strongest response. Data on disease activity

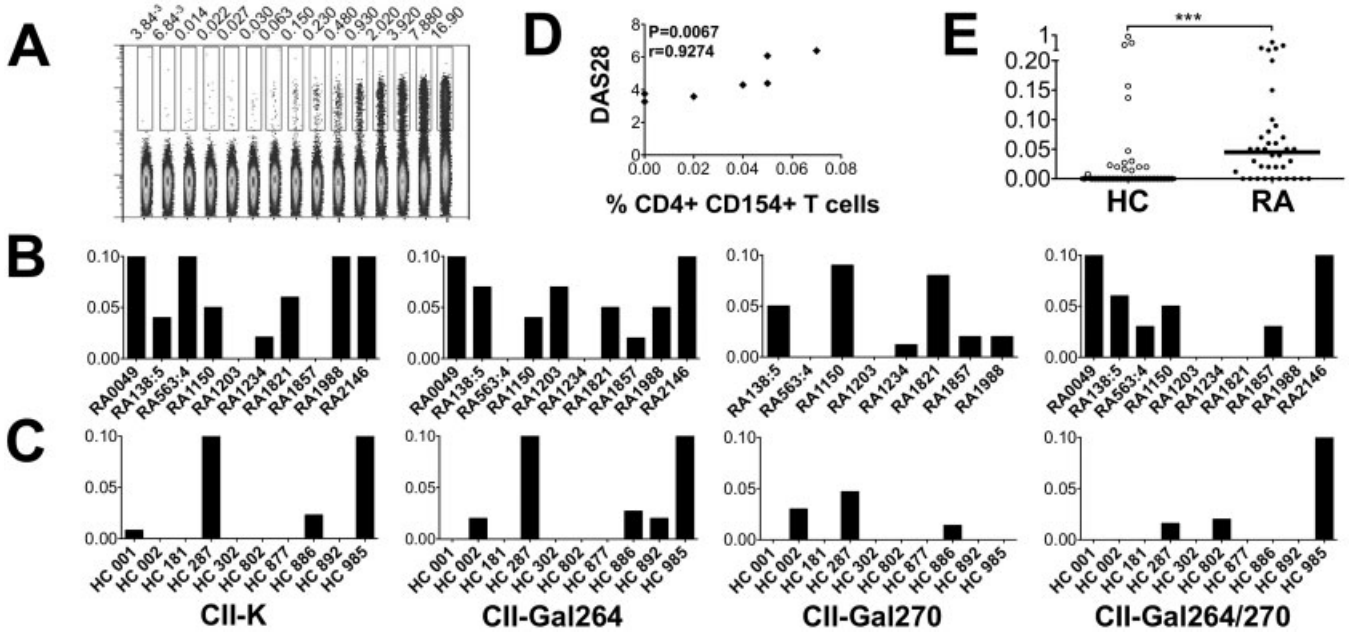

Figure 1. CD154 up-regulation in response to the different variants of the 259–273 epitope on type II collagen ($CII_{259–273}$). **A,** Expression of CD154 in CD4+ T cells stimulated with the superantigen staphylococcal enterotoxin B and further diluted in 2-fold serial dilutions from 1:1 down to 1:8,192. The threshold of detection was set at <0.01%. **B** and **C,** CD154 responses of T cells from 10 rheumatoid arthritis (RA) patients (**B**) and 10 HLA–DR*0401–matched healthy controls (HC) (**C**) to the 4 variants of $CII_{259–273}$ (CII-K, an unmodified $CII_{259–273}$ with lysine residues at positions 264 and 270, CII-Gal264, galactosylated with a β-D-galactopyranose residue on l-hydroxylysine at position 264, CII-Gal270, galactosylated exclusively at position 270, and CII-Gal264/270, galactosylated at positions 264 and 270). Response was monitored by multicolor flow cytometry after short-term in vitro stimulation of peripheral blood mononuclear cells. Values are the expression levels (percent) of CD154 after subtraction of background levels. Bars have been cut off at 0.1% to emphasize the lower responses. **D,** Association between RA disease activity as determined by the 28-joint Disease Activity Score (DAS28) and the magnitude of CD154 up-regulation following stimulation with CII-Gal264. **E,** Comparison of CD154 up-regulation in healthy controls versus RA patients following stimulation with all 4 $CII_{259–273}$ variants. Each circle represents a sample from an individual subject stimulated with one of the $CII_{259–273}$ variants. Horizontal bars show the median. *** = $P < 0.001$.

were available for 7 of the 10 RA patients, and revealed a significant correlation (r = 0.93, $P < 0.007$) between the DAS28 and the degree of CD154 up-regulation following stimulation with the CII-Gal264 peptide (Figure 1D). The responses against all 4 $CII_{259–273}$ variants taken together were stronger in the patient samples than in the samples from healthy controls ($P < 0.001$) (Figure 1E).

Diverse cytokine response of RA T cells to CII. Having established the presence of CII-reactive T cells in patients with RA, we next wished to determine the effector functions of these T cells. Using a multiparameter flow cytometry approach, we examined cytokine production together with CD154 up-regulation. The combination of the two readouts reduces background and was postulated to increase sensitivity. Again, we first determined the detection levels for the assay, i.e., for the cytokines IL-17, IFNγ, and IL-2. Following SEB stimulation we concluded that the detection limit of this assay was also <0.01% (Figure 2A).

We then investigated the production of IL-17, IFNγ, and IL-2 in response to the different variants of the $CII_{259–273}$ epitope. As can be seen in Figures 2B and C, CD4+ T cells from both patients and healthy donors secreted cytokines in response to all variants of $CII_{259–273}$. Again, CII-K and CII-Gal264 induced stronger responses in comparison with the 2 remaining $CII_{259–273}$ variants. With regard to the magnitude of production of individual cytokines, there was a trend toward higher production of IFNγ and IL-2 in the patient samples than in samples from controls, although the difference did not reach statistical significance. However, when the results for IL-17, IFNγ and IL-2 were plotted together, the overall production of cytokines was found to be significantly higher in samples from RA patients compared with healthy controls ($P < 0.05$) (Figure 2D).

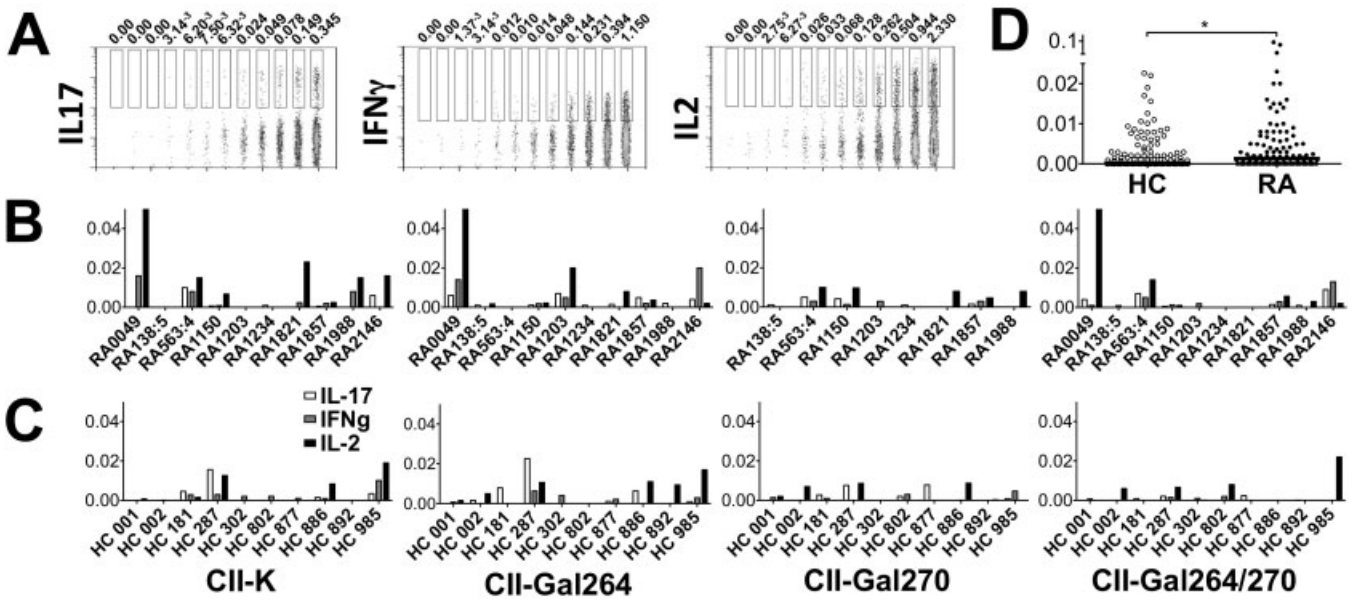

Figure 2. Cytokine production in response to $CII_{259–273}$ variants. **A,** Intracellular expression of interleukin-17 (IL-17), interferon-γ (IFNγ), and IL-2 in CD4+ CD154+ T cells stimulated with the superantigen staphylococcal enterotoxin B and further diluted 10 times in 2-fold serial dilutions. The threshold of detection was set at <0.01%. **B** and **C,** Cytokine responses of T cells from 10 RA patients (**B**) and 10 HLA–DR*0401–matched healthy controls (**C**) to the 4 variants of $CII_{259–273}$. Response was monitored by multicolor flow cytometry after short-term in vitro stimulation of peripheral blood mononuclear cells. Values are the expression levels (percent) of IL-17, IFNγ, and IL-2 after subtraction of background levels. **D,** Comparison of cytokine production in healthy controls versus RA patients following stimulation with all 4 $CII_{259–273}$ variants. Each circle represents a sample from an individual subject stimulated with one of the $CII_{259–273}$ variants. Horizontal bars show the mean. $* = P < 0.05$. See Figure 1 for explanations and other definitions.

Prominent production of IFNγ by RA synovial T cells. We next examined T cell reactivity with the $CII_{259–273}$ variants at the site of inflammation and compared responses in the circulation with those in affected joints. T cell responses to the 4 variants of $CII_{259–273}$ were examined in 7 paired sets of PB and SF samples from 5 different patients. The level of CD154 upregulation varied between patients (Figure 3A), but a robust proportion of the CD154+ T cells produced cytokines in response to peptide stimulation. Production of IFNγ was significantly higher in SF than in PB ($P = 0.027$) (Figure 3B).

Persistence of CII-reactive T cells over time. Finally, we investigated changes in T cell reactivity toward CII during the course of RA. In longitudinally obtained samples from 3 HLA–DRB1*0401–positive RA patients, we examined T cell responses in relation to disease activity. From patient 1 we had access to 4 samples obtained over a 5-year period; from patients 2 and 3 we had 3 samples each, obtained over 4 years and 8 years, respectively. As shown in Figure 4, the T cell responses varied in both magnitude and fine specificity. Patient 1 exhibited multireactivity throughout the study period, while patient 2 had a dramatic increase in Gal264/270 reactivity that coincided with high disease activity. Patient 3 developed new subspecificities over the study period even though this patient already had disease of long duration (40 years) at the start of the study.

DISCUSSION

Understanding of the role of T cells in RA has increased in recent years, due to both improved methods for detecting rare antigen-specific T cells (14) and the emergence of new candidate autoantigens dependent on posttranslationally modified epitopes (15,16). Hence, detailed T cell studies using well-characterized patient samples have good potential to enhance our current knowledge about autoimmune responses in different subsets of RA patients, and their contribution to disease development and/or chronicity. Here we studied T cell responses to native and glycosylated variants of the immunodominant T cell epitope from type II collagen, $CII_{259–273}$, in HLA-DRB1*0401–positive and HLA-DRB1*0404–positive RA patients. We showed that CII-

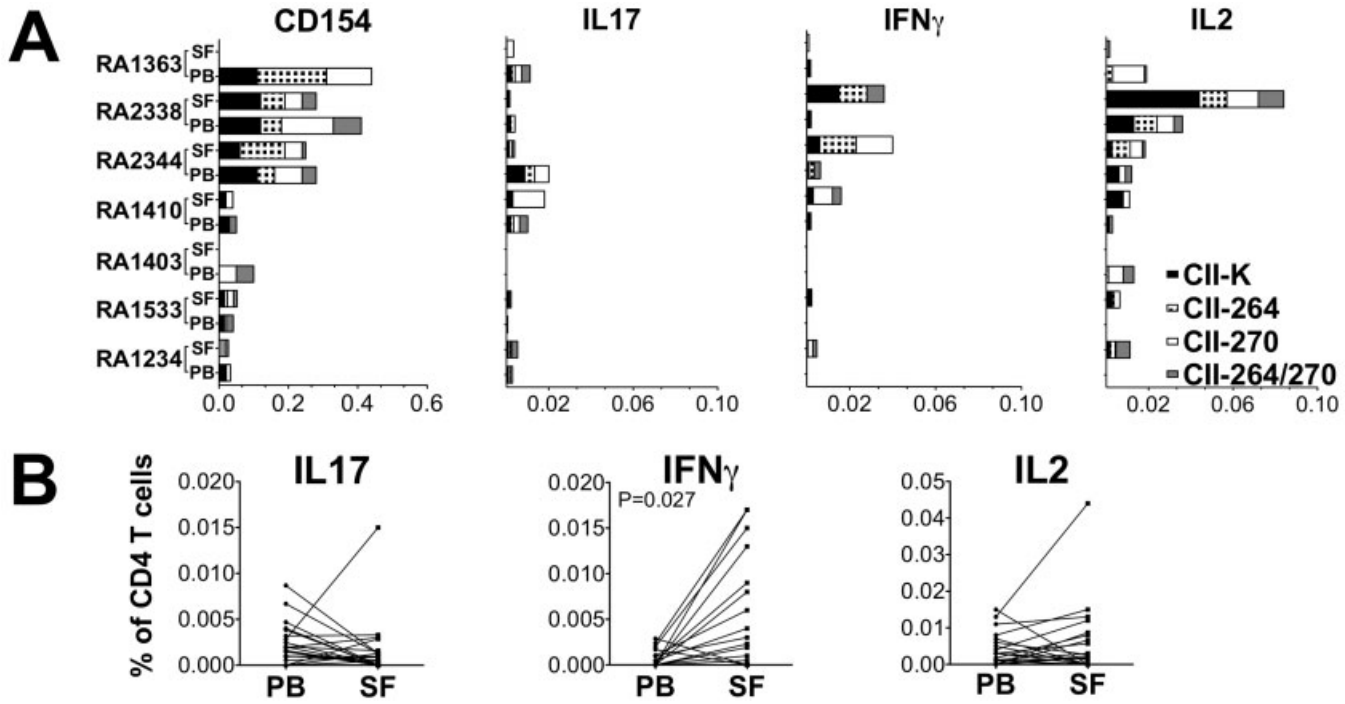

Figure 3. Comparative analysis of T cell reactivity with CII in peripheral blood (PB) and synovial fluid (SF). **A,** Paired PB- and SF-derived mononuclear cells from 5 different RA patients were stimulated with CII-K, CII-Gal264, CII-270, and CII-Gal264/270, and the frequency of T cell response to these peptides in terms of CD154, interleukin-17 (IL-17), interferon-γ (IFNγ), and IL-2 expression was measured by flow cytometry. To ensure correct detection of T cell reactivity, patients with low CII responses were reanalyzed; thus, RA1403 and RA1410 are consecutive cell samples from one RA patient, and RA1234 and RA1533 from another. **B,** The frequency of positive response in terms of IL-17, IFNγ, and IL-2 expression in PB versus SF was compared. Expression of IFNγ was significantly higher in SF versus PB. See Figure 1 for explanations and other definitions.

specific T cells recognizing different variants of $CII_{259–273}$ are frequent in HLA-DRB*04–positive patients with RA and are also found in HLA-matched control subjects. Importantly, the magnitude of the

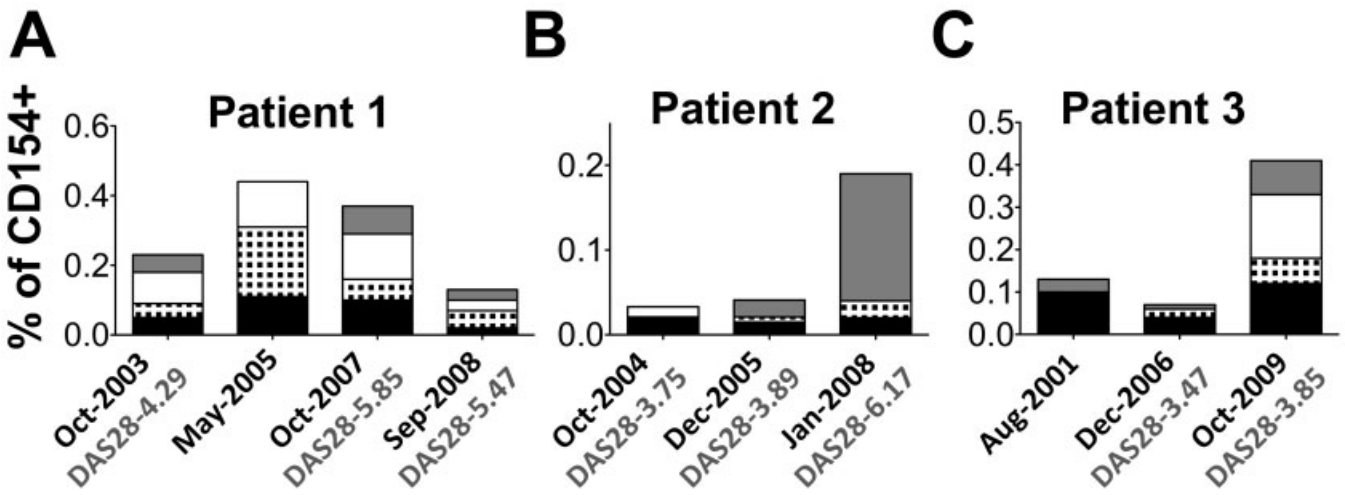

Figure 4. Longitudinal assessment of T cell responses to CII. T cell responses to each of the 4 variants of $CII_{259–273}$, i.e., CII-K (black), CII-Gal264 (checkered), CII-270 (white), and CII-Gal264/270 (gray), were monitored by measuring up-regulation of CD154 on CD4+ T cells in 3 of the 5 RA patients with cell samples obtained at multiple time points. DAS28 data are shown where available. See Figure 1 for explanations and definitions.

response was significantly higher in samples derived from RA patients. In fact, most samples displayed CII reactivity, and for one of the peptides (CII-264) we were able to correlate the observed response with disease activity levels (DAS28) in this small study population.

When we performed similar studies at the site of inflammation, comparing the magnitude of response between peripheral blood and synovial fluid from patients with RA, we found that IFNγ production in response to CII stimulation was significantly higher in SF as compared to PB in our patient samples. In these experiments we also attempted to correlate T cell effector function with disease activity. Disease activity measurements were available for 4 of the 7 patient samples, but the magnitude of response could not be correlated with DAS28 values in this small cohort.

Our data show that many RA patients display T cell responses to both native and glycosylated CII, with few patients responding exclusively to either native or glycosylated CII. This type of response pattern mirrors previously reported observations in A^q mice (8), while DR4-transgenic mice display a biased T cell response against the nonglycosylated self CII peptide (9). Whether T cell responses to the different CII epitopes contribute to early RA (i.e., disease development) or whether they are a consequence of joint destruction is still unknown. Notably, we demonstrated in the present study that DR4-restricted CII recognition toward all variants of the CII epitope occurred in the human setting (thus differing from the findings in the DR4-transgenic mouse model) and that the functional outcome of CII recognition was cytokine production (most prominently IFNγ, but also IL-17 and IL-2).

Our study was not powered to assess in detail the influence of disease activity, but notably, the sample with the highest T cell responses was obtained at a time of high disease activity (DAS28 6.63), suggesting that the level of anti-CII reactivity may directly influence, or be indirectly influenced by, disease activity in RA. Hence, T cells residing at the site of inflammation might have higher pathogenic capacity than those in the periphery. We also were not able to address the effect of treatment in the present study. All of our study patients had chronic RA, and the majority were receiving methotrexate in combination with anti–tumor necrosis factor therapy. The observed differences between the RA patients and the healthy controls could potentially have been even greater had the patients not been immunocompromised by treatment.

Our results in samples obtained longitudinally from the same patients at different times in the disease course also demonstrate that CII reactivity may vary over time, but clearly the responses were persistent and even included epitope spreading late in disease. Patient 3 exhibited T cell autoreactivity to new versions of the CII epitope after a disease duration of 40 years.

RA is a heterogeneous disorder, with different antigens, genes, and environmental factors likely involved in various subsets of the disease. In this work we focused on the cartilage-restricted candidate autoantigen type II collagen, and studied the function of PB- and SF-derived T cells in HLA–DRB1*04–positive RA patients. Our study demonstrates the feasibility of renewed investigation of T cell reactivities with candidate autoantigens in RA using recently available refined methods. In addition to glycosylation, citrullination is a critical posttranslational modification in RA, and future T cell studies obviously should be focused also on citrullinated antigens. Studies of T and B cell reactivities with different potential arthritogenic autoantigens, including those that have undergone various posttranslational modifications, in different subsets of RA and in different genetic contexts may bring about better understanding of which T and B cell reactivities may contribute to disease development and/or chronicity in different subsets of RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Malmström had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Snir, Bäcklund, Boström, Andersson, Klareskog, Holmdahl.

Analysis and interpretation of data. Snir, Bäcklund, Kihlberg, Buckner, Klareskog, Holmdahl, Malmström.

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