Title: Shared recognition of citrullinated tenascin-C peptides by T and B cells in rheumatoid arthritis

Authors: Jing Song¹, Anja Schwenzer², Alicia Wong²*, Sara Turcinov³*, Cliff Rims¹, Lorena Rodriguez Martinez², David Arribas-Layton¹, Christina Gerstner³, Virginia S. Muir⁴, Kim S Midwood¹†, Vivianne Malmström³†, Eddie A. James¹ and Jane H. Buckner¹

Affiliations:
¹Center for Translational Immunology, Benaroya Research Institute at Virginia Mason, Seattle, WA, USA
²Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, UK
³Division of Rheumatology, Department of Medicine, Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm, Sweden
⁴Center for Systems Immunology, Benaroya Research Institute at Virginia Mason, Seattle, WA, USA

*, These authors contributed equally: Alicia Wong and Sara Turcinov

†, These authors contributed equally: Kim S. Midwood and Vivianne Malmström

Corresponding Author: Jane H. Buckner, Benaroya Research Institute at Virginia Mason, 1201 Ninth Avenue, Seattle, WA 98101. Telephone: 206.287.1033. Fax: 206.342.6581. Email:
jbuckner@benaroyaresearch.org
Conflict of interest statement

V.S.M has left the Benaroya Research Institute since completing the study and now works at Janssen. V.M. has had research projects sponsored by Pfizer. E.A.J has two joint patents on Factor VIII epitopes with BloodWorks Northwest, and has past and current research projects sponsored by Pfizer, Janssen, Sanofi and Novartis. J.H.B is President of the Benaroya Research Institute at Virginia Mason, a Scientific Co-Founder and Scientific Advisory Board member of GentiBio, a consultant for Bristol-Myers Squibb and has past and current research projects sponsored by Amgen, Bristol-Myers Squib, Janssen, Novo Nordisk, and Pfizer. J.H.B also has a patent on methods of generating antigen-specific CD4+CD25+ regulatory T cells. All other authors have declared no conflict of interest exists.
Abstract

Tenascin-C, an extracellular matrix protein that has proinflammatory properties, is a recently described antibody target in rheumatoid arthritis. In this study, we utilized a systematic discovery process and identified five novel citrullinated tenascin-C (cit-TNC) T cell epitopes. CD4+ T cells specific for these epitopes were elevated in the peripheral blood of subjects with rheumatoid arthritis and showed signs of activation. Cit-TNC-specific T cells were also present among synovial fluid T cells and secreted interferon-γ. Two of these cit-TNC T cell epitopes were also recognized by antibodies within the serum and synovial fluid of individuals with RA. Detectable serum levels of cit-TNC reactive antibodies were prevalent among subjects with RA and positively associated with cyclic citrullinated peptide (CCP) reactivity and the HLA shared epitope. Furthermore, cit-TNC reactive antibodies were correlated with rheumatoid factor and elevated in subjects with a history of smoking. Taken together this work confirms cit-TNC as an autoantigen that is targeted by autoreactive CD4+ T cells and autoantibodies in patients with RA. Furthermore, our findings raise the possibility that coinciding epitopes recognized by both CD4+ T cells and B cells have the potential to amplify autoimmunity and promote the development and progression of rheumatoid arthritis.
Introduction

Rheumatoid arthritis (RA) is a chronic debilitating disease with a prevalence between 0.3 and 1% (1). It is a systemic, inflammatory autoimmune disease, with joints as the primary site of inflammation, and its progression leads to significant morbidity and increased mortality in affected individuals. Anti-citrullinated protein antibodies (ACPA) are a hallmark of RA and are predictive of developing disease, thereby defining an at-risk population (2-4). RA has a strong genetic association with the shared epitope (SE), a group of HLA-DRB1 alleles with a common motif in residues 70-74 of their peptide binding groove (5). The high level of somatic mutation in ACPA suggests an important role for ongoing T cell help in the disease (5-7). Indeed, a variety of CD4+ T cell specificities have been implicated in disease pathogenesis, including multiple citrullinated peptides from self-proteins that have been shown to be recognized not only by peripheral blood T cells but also by T cells from synovial fluid (8-11). In spite of this knowledge, the immunologic factors that initiate and perpetuate RA are not completely understood. Perhaps due to discovery bias, there is broad overlap between antigens that are known ACPA targets and known sources of T cell epitopes. However, this overlap, coupled with the observation of compartmentalized T and B cell structures could indicate that interactions between antigen specific T cells and B cells play an important role in the etiology of RA (12).

Among the diverse array of citrullinated antigens that have been reported, published work implicates extracellular matrix proteins (e.g. fibrinogen, cartilage intermediate layer protein, fibronectin, type II collagen, and aggregan) as an important class of citrullinated antigens, implying an important role in disease etiology (9, 10, 13-15). There is growing evidence that the extracellular matrix protein, tenascin-C (TNC) is an important citrullinated antigen in RA. TNC protein is largely absent in healthy tissues, but its expression is upregulated in response to inflammation in
multiple autoimmune disease settings (16-19). In patients with RA, TNC protein levels are elevated in serum as well as in the synovium of knee joints, correlating with disease activity (17). In addition to its known effects as a TLR4 agonist (20), the C-terminal fibrinogen-like domain of TNC has been shown to be citrullinated within synovial fluid (21), and immunodominant peptides within this domain are recognized by serum ACPA from subjects with RA (22). Notably, the presence of cit-TNC autoantibodies in an early synovitis cohort was associated with development of RA (23). These studies establish TNC as a relevant autoantigen in RA and highlight the potential application of measuring TNC specific immunity as a biomarker of disease activity and a means of understanding the loss of tolerance. However, it remains unclear which domains of this complex protein are immunogenic and whether antibody and T cell responses are most prevalent in a particular subset of subjects with RA.

Therefore, in this study we sought to examine CD4$^+$ T cell responses to citrullinated TNC (cit-TNC) by screening the entire length of the TNC protein to identify novel T cell epitopes recognized in the context of the prevalent RA susceptible HLA-DRB1*04:01 allele. We identified five immunogenic cit-TNC peptides that elicited T cell responses in the peripheral blood and synovial fluid of RA subjects. Additionally, two of these immunogenic cit-TNC peptides were also antibody targets that were detectable within peripheral blood and synovial fluid samples from individuals with RA. Taken together, this work demonstrates the importance of cit-TNC as an autoantigen in RA and establishes a link between epitopes that drive CD4$^+$ T cell responses restricted by a high risk shared epitope allele (HLA-DRB1*04:01) and antibody epitopes that are recognized in ACPA positive RA.
Results

Citrullinated-tenascin C peptides bind HLA-DRB1*04:01 and are immunogenic

TNC is a large protein (2,110 amino acids in length) comprised of an N-terminal assembly domain, 14.5 epidermal growth-factor-like repeats (EGF-L), 8 constant fibronectin type III-like repeats (FNIII) plus an additional 9 alternatively spliced FNIII elements, and a C-terminal fibrinogen-like globe (FBG) (16). Using a previously described algorithm (9, 24), we scanned the entire length of the TNC monomer and identified 64 arginine-containing peptides with motifs that would be predicted to permit binding to HLA-DRB1*04:01 in either their non-citrullinated native form or citrullinated form or both. Based on these predictions, we synthesized the citrullinated versions of these peptides and tested their ability to bind HLA-DRB1*04:01 and to elicit CD4+ T cell expansion in vitro. Of the peptides synthesized, 8 cit-TNC peptides bound to HLA-DRB1*04:01 with moderate to high affinity (Table 1). Among these, cit-TNC17, cit-TNC22, cit-TNC45, cit-TNC50 and cit-TNC56 were determined to be the most immunogenic, based on their ability to drive epitope-specific T cell expansion, as measured by HLA Class II tetramer staining of peripheral blood mononuclear cells (PBMC) from HLA-DRB1*04:01 positive subjects following two weeks of in vitro expansion (Table 1 and Supplemental Figure 1).

To assess whether citrullination is required for the HLA binding and immunogenicity of these peptides, the corresponding unmodified arginine-containing peptides (arg-TNC) were synthesized. Among the arg-TNC peptides synthesized, arg-TNC22, arg-TNC45 and arg-TNC50 bound to HLA-DRB1*04:01, whereas arg-TNC17 and arg-TNC56 did not (Figure 1A, Supplemental Table 1). This suggests that cit-TNC17 and cit-TNC56 are citrullinated at sites that are critical for binding. Indeed, the cit-TNC17 and cit-TNC56 peptides each contain a citrulline residue that is predicted to bind in pocket 4 of HLA-DRB1*04:01. To determine whether any of
the citrullinated sites are critical for TCR recognition, we assessed the immunogenicity of the arg-TNC22, arg-TNC45 and arg-TNC50, observing that each of these non-citrullinated versions had the ability to elicit T cell responses in vitro (Supplemental Table 1). We also investigated partially citrullinated versions of cit-TNC17 and cit-TNC56, showing that each contains a predicted TCR contact that is associated with increased immunogenicity (Figure 1B, Table 2). These findings indicate that TNC has the ability to promote T cell responses in both its citrullinated and non-citrullinated forms but that some epitopes are uniquely immunogenic when citrullinated.

The frequency of cit-TNC-specific CD4+ memory T cells is increased in RA subjects

We next asked whether cit-TNC-specific T cells are present in individuals with RA (n=9) and healthy control (HC) subjects (n=7) (Supplemental Table 2 T Cell Cohort). We directly assessed the frequency and surface phenotype of the cit-TNC-specific T cells using a multiplex HLA class II tetramer staining approach that allows ex vivo enrichment and detection of multiple tetramer specificities in a single peripheral blood sample and co-staining with cell surface marker antibodies (10). The flow cytometry panel included the five immunogenic cit-TNC specificities: cit-TNC17, cit-TNC22, cit-TNC45, cit-TNC50, and cit-TNC56, plus an influenza peptide (MP 97-116) as a positive control. Combining all cit-TNC specificities, there was a significant increase in the frequency of memory cit-TNC-specific T cells in patients with RA compared to HC subjects (Figure 2A). In contrast, the frequency of memory influenza-specific T cells did not differ between RA and HC subjects. Notably, cit-TNC45, cit-TNC50 and cit-TNC56, contributed the most to the increased frequency of cit-TNC-specific T cells, with a trend toward higher cit-TNC17-specific T cells that did not reach statistical significance (Figures 2B-C, Supplemental Table 3). In contrast, the frequency of memory influenza-specific T cells did not differ between RA and HC subjects.
Notably, the frequencies of cit-TNC45, cit-TNC50 and cit-TNC56 specific T cells were all significantly increased, while the other specificities were only detected in a few of the RA patient samples (Figures 2B-C, Supplemental Table 3). As can be seen in Figure 2C, each patient sample contained several cit-TNC T cell specificities, but with variable frequencies.

Cit-TNC-specific T cells are recently activated and have predominantly Th2 and Th17 phenotypes

We utilized the cell surface phenotype of cit-TNC-specific T cells in peripheral blood to draw inferences about T cell lineage, observing key differences between RA subjects and HC subjects. The cell surface markers included CD45RA as a marker of naïve T cells, CD38 as a marker of recent activation, and CXCR3, CCR4, CXCR5 and CCR6 to define Th1, Th2, Tfh, and Th17 T helper subsets, respectively (25-27). Most notably, the frequency and percentage of CD38+ cit-TNC-tetramer (Tmr)⁺ CD4 memory T cells were increased in RA compared to HC subjects (Figures 2D-E). On memory cells, this marker has been shown to indicate in vivo activation due to antigen exposure (28, 29). Therefore, the presence of CD38 suggests that a substantial proportion of cit-TNC specific T cells in RA subjects were recently activated. In addition, there was a significant increase in the frequency of both CCR4+ and CCR6+ cit-TNC-specific T cells in RA subjects compared to HC subjects (Figure 2D). Further gating of these Tmr⁺ cells indicated that this represented a significant increase in the Th2 (CCR4⁺CCR6⁻CXCR3⁻) and Th17 (CCR4⁺CCR6⁺CXCR3⁻) population within the RA cohort (Figure 2F). Therefore, our results suggest that cit-TNC-specific T cells are more frequent in subjects with RA than in controls and exhibit an activated Th2/Th17 phenotype in peripheral blood.

Synovial fluid mononuclear cells secrete cytokines in response to cit-TNC peptides
Having established that cit-TNC specific T cells are present in peripheral blood, we next sought to evaluate their presence at disease relevant sites of inflammation. Synovial fluid mononuclear cells (SFMC) from HLA-DRB1*04:01 RA patients (n=7) were stimulated with the five immunogenic cit-TNC peptides or their corresponding arg-TNC peptides, and cytokine secretion (IFN-γ/IL-17/IL-10) was then assessed using a FluoroSpot assay. Citrullinated peptides derived from α-enolase, fibrinogen, vimentin and CILP were included as reference epitopes, since these proteins were previously reported as RA autoantigens (9, 11, 30-32) (Supplemental Table 4). Cit-TNC17, cit-TNC22, cit-TNC45 and cit-TNC56 (but not cit-TNC50) elicited IFN-γ secretion by SFMC (Figure 3A). Even though arg-TNC peptides could promote peripheral blood T cell expansion, we rarely observed cytokine responses to the unmodified peptides in the synovial fluid samples, while the cit-TNC peptides displayed a robust IFN-γ output (Figure 3A). Strikingly, the observed responses toward cit-TNC was almost an order of magnitude greater than that seen for cit-enolase, cit-fibrinogen, cit-vimentin and cit-CILP (Figure 3B) – in some cases approaching the level of response seen for the influenza peptide. As expected, the observed SFMC response to cit-TNC peptides was HLA-DR restricted, as HLA-DR blocking, but not HLA-DQ blocking, completely abrogated the induction of IFN-γ secretion (Figure 3C). The cit-TNC peptides also elicited modest IL-10 and IL-17 secretion by SFMC (including some IFN-γ and IL-10 double positives), although this appeared to be epitope and subject specific (Supplemental Figure 2).

*Cit-TNC17 and cit-TNC56 are recognized by antibodies in RA subjects*

Given the importance of anti-citrullinated protein antibodies (ACPA) as markers for the diagnosis of RA and prior observations that a citrullinated peptide from the C-terminal FBG domain of TNC is recognized by serum ACPA (18), we were curious whether the peptides found to promote the T
cell response to TNC were also antibody epitopes. To assess this, antibody responses to cit-TNC and arg-TNC peptides (plus the previously published cit-TNC5 peptide as a positive control) were measured in serum samples from HLA-DRB1*04:01 and CCP positive RA (n=17) and HLA-DRB1*04:01 matched HC (n=24) subjects (Supplemental Table 2 Autoantibody Cohort 1, Supplemental Table 5). By necessity, this autoantibody cohort had only partial overlap with the T cell cohort. Antibodies to the previously reported cit-TNC5 were present in 47% of the RA sera, consistent with the levels previously seen in two independent cohorts (22). In contrast, antibodies to cit-TNC17 were present in 100% of the RA sera while antibodies to cit-TNC56, cit-22 and cit-TNC45 were present in 53%, 35%, and 29% of the RA sera, respectively. Antibody titers were highest for TNC17, TNC5 and TNC56 respectively, while more modest reactivity was observed for TNC22 and TNC45 (Figure 4A). Notably, there was little or no antibody reactivity directed against the corresponding arg-TNC peptides (Figure 4A). Furthermore, antibody reactivity was virtually absent among the HC sera. Together, these observations suggest that TNC is selectively recognized as a citrullinated antigen by antibodies present in the sera from RA patients that are essentially absent from HLA matched controls.

To further probe their potential significance, we correlated observed levels of cit-TNC reactive antibodies with anti-CCP2 antibody levels (Figure 4B), observing a positive correlation in this cohort between anti-CCP2 and antibody levels for both cit-TNC17 (r=0.6478, p=0.006) and cit-TNC5 (r=0.55, p=0.0223) but that trend did not reach significance for cit-TNC56 (r=0.4539, p=0.0685). Since ACPA are thought to be directly involved in joint pathology, we next tested polyclonal anti-CCP-pools purified from both plasma and synovial fluid samples for reactivity to an array of TNC peptides covering every arginine/citrrulline residue within the protein (33). Among the peptides tested, we could verify reactivity to cit-TNC17, cit-TNC56 and cit-TNC5 in plasma
and observed a strong positive signal in synovial fluid derived anti-CCP pools (Figure 4C, Supplemental Table 6). The elevated levels of TNC reactivity in synovial fluid versus plasma was further replicated in a set of individually paired serum and synovial fluid samples taken 10 years apart, suggesting that cit-TNC antibodies persist over time (Figure 4D, Supplemental Table 6).

Since it has been recently shown that ACPA can cross-react against multiple peptides (especially those that contain a common citrulline-glycine motif (34)), we examined the specificity and potential cross-reactivity between cit-TNC17 and cit-TNC56 reactive antibodies by performing inhibition experiments using sera from subjects that were double-reactive for cit-TNC17 and cit-TNC56 antibodies. Absorption by the homologous peptide was efficient for both cit-TNC17 and cit-TNC56, (Figure 5, left hand panels), whilst cross-reactivity was limited; only partial inhibition was observed in serum 2 (maximum inhibition 24.8%: 56 vs 17) and serum 6 (maximum inhibition 22%: 17 vs 56 and 43% for 56 vs 17). (Figure 5, middle panels). There was also no cross-reactivity between cit-TNC17 and cit-TNC5, and only limited cross-reactivity between cit-TNC56 and cit-TNC5 (maximum inhibition of serum 2:38.7%) (Figure 5, right hand panels). Cumulatively, this minimal cross-reactivity suggests that distinct antibodies recognize these different cit-TNC peptides.

Cit-TNC17 and cit-TNC56 antibodies are associated with clinical features of RA

Given the prevalence of antibody reactivity toward cit-TNC17 and cit-TNC56 in subjects with RA, we investigated whether levels of these antibodies correlate with specific clinical features. To facilitate this analysis, we examined cit-TNC seropositivity in an independent cohort of RA subjects. This cohort included CCP⁺ RA (n=55) and CCP⁻ RA (n=43) subjects and who were either
positive or negative for the HLA DRB1*04:01 haplotype (Supplemental Table 2 Autoantibody Cohort 2). In this cohort, cit-TNC17 antibodies were present in 64% of CCP+ subjects but only 2.3% of CCP- subjects, and cit-TNC56 antibodies were present in 46% of the CCP+ cohort and 12% of the CCP- subjects (Figure 6A). The antibody reactivity for arg-TNC17 and arg-TNC56 peptides was below the threshold for positivity in almost all subjects (Figure 6A). Therefore, data from this patient cohort supports preferred antibody reactivity toward cit-TNC and an association with CCP positivity.

We next combined the clinical and serologic data from Autoantibody Cohorts 1 and 2 to maximize our ability to assess relationships between cit-TNC17 and cit-TNC56 reactive antibodies with specific features of RA. In the combined cohort 79% of the CCP+ subjects exhibited positive reactivity toward at least one of the cit-TNC peptide. Of these seropositive subjects, 28% were single positive for anti-cit-TNC17 antibodies, 11% were single positive for anti-cit-TNC56 antibodies, 40% were dual positive for both antibodies (Figure 6B). Furthermore, there was a positive association between cit-TNC seropositivity and the HLA shared epitope (SE) (Figure 6B). This was further confirmed using a logistic regression model in which age and sex were used as co-variates to calculate odds ratios (OR) (Table 3). In addition, cit-TNC17 seropositivity was strongly associated with both rheumatoid factor (OR = 14.2, FDR = 0.00002) and CCP seropositivity (OR = 92.98, FDR = 0.00009) (Table 3). To a lesser extent, cit-TNC56 seropositivity was also associated with rheumatoid factor (OR = 3.49, FDR = 0.04345) and CCP seropositivity (OR = 7.92, FDR = 0.0012) (Table 3). In addition, cit-TNC17 seropositivity was associated with smoking (current smoking OR = 6.5, FDR 0.001) (Table 3), but not associated with disease activity and disease duration. Applying the model exclusively to shared epitope positive RA subjects, the associations between cit-TNC17 seropositivity and rheumatoid factor,
CCP seropositivity and smoking remained strong and significant (Supplemental Table 7). The association of antibodies against cit-TNC17 and TNC56 with the shared epitope, smoking and rheumatoid factor mirrors the characteristics described in ACPA+ subjects who go on to develop RA (3, 15, 35-41). Therefore, our results suggest that cit-TNC responsiveness is most pronounced in subjects with high risk HLA genotypes and who have a history of smoking.
Discussion

There is ample evidence to suggest that failure of both B cell and T cell tolerance contributes to the pathogenesis of seropositive RA. Indeed, in light of the observation of intimate interactions between T cells and B cells in the synovium (12) and the fact that antigen specific B cells can be uniquely potent antigen presenting cells, these are likely to be mutually reinforcing phenomena. ACPA and rheumatoid factor are diagnostic in RA, can precede disease by up to 10 years, and are thought to participate in joint pathology (2-4). ACPA have been shown to target multiple citrullinated antigens, with some exhibiting narrow or single antigen specificity and others demonstrating substantial cross-reactivity toward shared consensus motifs (34). Notably, the number of ACPA specificities expands as individuals progress from pre-RA to develop inflammatory arthritis (4). CD4+ T cells are implicated through the strong genetic association of RA with HLA Class II SE alleles, by their presence in the RA joint, and due to the affinity maturation and somatic mutation seen in RA autoantibodies indicating T-B cell collaboration (5-9, 11). This apparent collaboration, and observations that ACPA target multiple citrullinated antigens, have prompted the investigation of several citrullinated proteins as T cell targets in RA, including vimentin, α-enolase, cartilage intermediate-layer protein (CILP), fibrinogen, and aggrecan (42-47). The epitopes identified through these studies were typically recognized in the context of SE alleles and in many cases, their immunogenicity depended on citrullination at key HLA-DR binding residues (in particular binding pocket 4). Cumulatively, these studies have shown that T cells specific to citrullinated peptides are present in RA patients and exhibit an expanded memory phenotype.

Likewise, in this study, we explored the T cell response to TNC, an extracellular matrix protein with unique features that have been implicated in the development of RA, in the context
of the RA risk allele HLA DRB1*04:01. Building on prior work that established cit-TNC5 as an important antibody epitope, we searched the entire TNC protein sequence to define immunogenic epitopes, identifying five novel immunogenic T cell epitopes, two of which required citrulline modification for HLA binding and T cell recognition. We assessed the frequency of cit-TNC specific T cells in peripheral blood through direct staining with HLA class II tetramers, observing increased frequencies in RA subjects as compared to HC. Notably, a significant proportion of cit-TNC reactive T cells in subjects with RA expressed CD38 (an activation marker on memory CD4 T cells) suggesting expansion and recent activation. Furthermore, although some caution is advisable when comparing results obtained from different cohorts of patients, the observed frequency of TNC reactive T cells was substantially higher than those recently reported for other citrullinated antigens (e.g. memory frequencies of 2-6 cells per million for cit-aggrecan (10)). T cells that recognized cit-TNC were also present among synovial fluid derived T cells, exhibiting cytokine responses that were substantially more robust than established citrullinated epitopes. These findings suggest that cit-TNC is an important T cell target in RA.

Among the T cell epitopes identified in our study, two (TNC17 and TNC56) were also found to be antibody epitopes that were commonly recognized by serum antibodies from subjects with RA. These two epitopes (cit-TNC17 and cit-TNC56) were unique in that citrullination was required for peptide binding and immunogenicity. The relevance of these peptides as antibody targets was first demonstrated in a cohort of 17 HLA-DRB1*04:01 positive and CCP positive RA patients. In those subjects, cit-TNC17 reactive antibodies were universally present, whereas cit-TNC56 reactive antibodies were present in just over half of the subjects (essentially the same proportion as the observed antibody reactivity against the previously published cit-TNC5 epitope). However, cit-TNC17 and cit-TNC56 reactive antibodies remained prevalent (64% and 43%,
respectively) among CCP+ subjects in a larger cohort of 55 RA patients with more diverse HLA genotypes. Antibody recognition of these peptides was rare in CCP negative subjects, essentially absent in controls, and required citrullination. This requirement for citrullination of these peptides for both T cell and antibody recognition, along with the relatively high T cell frequencies observed, opens up the possibility of linked T cell and antibody responses that synergize in the setting of inflammation and tissue injury (known to promote protein citrullination by peptidyl arginine deiminase enzymes) to elicit uniquely robust responses. Naturally, these observations obtained through the use of citrullinated peptides should be interpreted with caution, given that relatively short peptides are not typically the natural targets of antibodies in vivo. However, although it could be argued that experiments using citrullinated wild type recombinant TNC and recombinant TNC with mutations at key arginine residues are necessary to rigorously prove that these citrullinated sites are specifically targeted by antibodies in RA, our current observations are suggestive and informative.

One key insight which can be drawn from our study are the similarities and differences observed between peripheral blood and synovial samples. With the exception of cit-TNC50, each of the citrullinated TNC epitopes defined in peripheral blood drove strong cytokine responses in synovial fluid. However, although arg-TNC22, arg-TNC45, and arg-TNC50 specific T cells could be detected in peripheral blood, synovial T cell reactivity was exclusively observed in response to citrullinated TNC peptides. Further study would be needed to draw firm conclusions, but given that some studies suggest an enriched T cell repertoire in synovial fluid (48, 49), citrulline reactive TCRs may be selectively retained within inflamed joints. Notably, although cit-TNC specific T cells exhibited a memory phenotype in peripheral blood and synovial fluid, T cells with different phenotypes may home to different sites or be critical at different stages of disease. Specifically, in
peripheral blood cit-TNC-specific memory CD4+ T cells in RA subjects predominantly exhibited Th2 (CCR4+CCR6-CXCR3-) and Th17 (CCR4+CCR6+CXCR3-) associated surface phenotypes, but very low proportions of Th1 (CXCR3+CXCR4-CCR6-) cells. These two phenotypes could be interpreted to have opposing effects, given that Type 2 responses have been associated with remission (50), whereas IL-17 is thought to promote disease (51). Regrettably, our fluorospot assays were not designed to detect Th2 cytokines with the exception of IL-10 which was only seen in a limited number of samples, whereas cit-TNC peptides did elicit robust IFN-γ responses from synovial fluid mononuclear cells. Furthermore, prior studies have reported that the vast majority of T cells in synovial fluid are CXCR3+ (52). Therefore, it could be argued that cit-TNC specific CXCR3+ T cells are enriched within inflamed joints and selectively reduced in the periphery. It is notable that antibody reactivity to both cit-TNC17 and cit-TNC56 was observed in RA serum and in polyclonal anti-CCP-pools purified from both plasma and synovial fluid samples (including matched samples). Based on the consistently higher levels of reactivity seen for synovial fluid samples, it could be asserted that some B cells present within synovial infiltrates (53) produce cit-TNC reactive antibodies.

In addition to documenting cit-TNC reactivity in general, our study sought to draw conclusions about the clinical significance of such responses. The observed associations between cit-TNC reactivity and CCP, RF, the shared epitope, and smoking history are notable. In some sense, the positive relationship between cit-TNC reactive antibodies and anti-CCP positivity is expected, given that polyclonal anti-CCP-pools reacted to cit-TNC17, cit-TNC56 and cit-TNC5. Indeed, one confounding factor for cit-TNC17 in particular is the presence of a highly conserved citrulline-glycine “shared motif” within this peptide sequence that has been shown be recognized by highly multi-reactive ACPA (7, 34), such that these antibodies cannot be strictly defined as
being cit-TNC specific. Therefore, it could be argued that an unknown proportion of cit-TNC17 reactive antibodies are multireactive ACPA with no discernable “primary specificity”. However, the cit-TNC56 peptide lacks the citrulline-glycine motif and is less widely recognized, but still exhibits parallel positive relationships with CCP, RF, and the shared epitope. This increases our confidence in the idea that cit-TNC responsiveness is most pronounced in subjects with high-risk HLA genotypes and who are RF and CCP positive – factors which have already been associated with erosive disease (2, 40, 54).

In summary, we have utilized complementary T cell and antibody measures to investigate cit-TNC as a relevant self-antigen in RA. We identified five immunogenic cit-TNC peptides that elicited T cell responses in the peripheral blood and synovial fluid of RA subjects, two of which were also recognized by ACPA from serum and synovial fluid. Cit-TNC responses were positively correlated with RF and CCP, and the shared epitope, thereby linking these responses with a subgroup of patients known to have higher risk of severe joint destruction. It is clear that for multiple citrullinated RA autoantigens, CD4+ T cell responses and corresponding ACPA responses to the same antigen have been documented. However, for cit-TNC in particular we observe that the exact same cit-TNC peptides are recognized by both CD4+ T cells and B cells. We conclude that such shared recognition, coupled with the capacity for enhanced presentation by B cells where the BCR is specific for a peptide that then reinforces antigen-specific CD4+ T cell help to that same peptide, creates a unique scenario in which T-B cell cooperation in joints can amplify autoimmunity and promote the development and progression of RA.
Methods

Human subjects

All RA subjects met the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 Rheumatoid Arthritis Classification Criteria and had at least one HLA-DRB1*04:01 allele, and ACPA positivity was determined based on clinical testing for CCP. All HC subjects had no history of autoimmune disease themselves or among their first-degree relatives, had at least one HLA-DRB1*04:01 allele, and were ACPA negative. The characteristics of each of the RA and HC cohorts are summarized in Supplemental Table 2. PBMC and serum samples from both RA and HC subjects were from the Benaroya Research Institute Immune-Mediated Disease Registry and Repository. Synovial fluid samples were obtained from patients with RA undergoing arthrocentesis as part of clinical treatment at the Karolinska University Hospital. For the study testing polyclonal anti-CCP-pools purified from both plasma/serum and synovial fluid samples for reactivity to an array of TNC peptides, the plasma and serum samples were obtained from RA patients attending the Karolinska University Hospital as previously described (7, 33).

Isolation and cryopreservation of PBMC and SFMC

PBMC and SFMC were isolated from heparinized blood and synovial fluid respectively by centrifugation over Ficoll-Hypaque gradients, and frozen in liquid nitrogen in 10% DMSO and 90% heat-inactivated FBS. Cryopreserved PBMC were thawed in a 37°C water bath and prepared in drip-wise addition of RPMI-1640 media supplemented with 10% FBS and 0.001% benzonase nuclease (Sigma-Aldrich), with a final suspension in complete medium (RPMI-1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 10mM
HEPES) supplemented with 10% commercial human pooled serum (HPS). SFMC were thawed in a 37°C water bath and rapidly resuspended in complete medium with ≥5 U/ml benzonase nuclease (Sigma-Aldrich) and 10% FBS. Before in vitro stimulation, the cells were resuspended in complete medium with 10% HPS (Sigma-Aldrich).

**Epitope prediction and peptide synthesis**

Putative cit-TNC epitopes were predicted using our scanning algorithm as previously described (9, 24). Briefly, motif scores were calculated by multiplying coefficients corresponding to each anchor residue for all possible core 9-mers within the protein that included an internal or flanking arginine or citrulline residue. A total of 64 peptides with motif scores of 0.1 or higher were synthesized and purified by the manufacturer (Sigma-Aldrich). All peptides were dissolved in DMSO to a stock concentration of 20mg/ml. For tetramer production and further studies, selected citrullinated peptides and their corresponding native peptides were resynthesized by Sigma-Aldrich, GenScript or Pepceuticals.

**Peptide binding to HLA-DRB1*04:01**

To assess peptide binding to HLA-DRB1*04:01, increasing concentrations of each non-biotinylated test peptide were incubated in competition with 0.01µM biotinylated influenza HA306–318 (PKYVKQNTLKLAT) in wells coated with anti-HLA-DR antibody (Clone L243, supplied by the BRI Tetramer Core) as previously described (55). Europium-conjugated streptavidin (PerkinElmer) was used to label residual biotinylated peptide bound to the HLA-DR protein and was quantified using a Victor2 multi-label time resolved-fluorometer (PerkinElmer). Binding curves were fitted by non-linear regression with a sigmoidal dose response curve model.
using GraphPad Prism 7.0 and EC50 values were calculated as the peptide concentration needed to displace 50% of the reference peptide. Peptides selected for further study based on positive binding results were re-synthesized at a higher purity by another manufacturer (GenScript or Pepceuticals).

**HLA Class-II tetramer production**

Recombinant HLA-DRB1*04:01 protein was produced by the BRI Tetramer Core as previously described (56). Soluble HLA-DRB1*04:01 monomer was purified from insect cell culture supernatants and biotinylated at a sequence-specific site using biotin ligase (Avidity) prior to dialysis into phosphate storage buffer. The biotinylated monomer was loaded with 0.2mg/ml of peptide by incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl-β-D-glucopyranoside and 1mM Pefabloc SC (Sigma-Aldrich). Peptide loaded monomers were conjugated into tetramers using fluorescently labeled streptavidin (Invitrogen) for 6-18 hours at room temperature at a molar ratio of 8:1.

**In vitro expansion cit-TNC–specific T cells and HLA class-II tetramer staining**

Cryopreserved PBMCs from RA and healthy subjects were cultured at 5 × 10^6 cells/well in a 48 well plate in RPMI-1640 + 10% HPS with 10μg/ml of peptide. IL-2 (Novartis) was added at 325 IU/ml on day 6. On day 14, cells were stained for expression of tetramer-PE, CD25 APC (BD) and CD4 FITC (BioLegend) (Supplemental Table 8), and then run on a FACSCanto. The data was analyzed by FlowJo software version 10.

**SFMC secretion of cytokines in response to cit-TNC peptides**
Cryopreserved SFMC from seven patients were stimulated with cit-TNC17, cit-TNC 22, cit-TNC 45, cit-TNC 50, cit-TNC56 peptides and their arginine counterparts. Peptide stimulation with citrullinated α-enolase, vimentin, CILP and fibrinogen peptides was also made as a comparison for four of these seven patients (n=2 at the same time-point, n=2 at a different time-point and sample), and additionally three patients. Non-TNC peptides used in the assay were synthesized by GenScript and are described in Supplemental Table 4. One additional patient was only included in a TNC HLA-blocking experiment. A three color fluorospot assay was performed using the Human IFN-γ/IL-10/IL-17A FluoroSpotPLUS kit from Mabtech (Catalog # FSP-010703) for all tenascin-C experiments and the IFNγ/IL-22/IL-17 kit for the additional experiments with other citrullinated peptides. Briefly, SFMC were thawed and 250 – 850 thousand cells were plated per well, in complete medium with 10 % human serum (Sigma-Aldrich), in a pre-coated fluorospot plate and stimulated with 20μg/ml of the separate peptides for 48 hours at 37°C, 5% CO₂. 20μg/ml influenza peptide (MP97-116) and anti-CD3 were used as positive control. 10μg/ml HLA-DR (BioLegend) and HLA-DQ (Beckman Coulter) blocking antibodies were used in addition to the cit-TNC peptides for three patients. After incubation, the plate was washed in PBS and stained with antibodies according to the manufactures protocol. The plates were read using an ELISpot/Fluorospot reader (iSpot Spectrum, AID, Strassberg, Germany), software version 7.0 (build 151117) with automated spot count. Number of spots were normalized to spots per million cells and spots seen in the unstimulated wells were subtracted from spot count in the stimulated wells prior to further analyses. The Wilcoxon matched pairs signed rank test was used for the fluorospot assays and p < 0.05 was considered significant.

*Ex vivo detection of flu and cit-TNC- reactive T cells by HLA class-II tetramers*
Ex vivo tetramer staining and enrichment was accomplished using previously published protocols (10, 57). A total of 40-60 million PBMCs were thawed and rested overnight in a tissue culture incubator at 37°C, 5% CO₂. PBMCs were equally divided into 2 FACS tubes for MP97-116 and citrulline TNC Tetramer tests in 200μl of T cell culture medium following Dasatinib treatment for 10 minutes at 37°C. PBMCs were then stained with 6μl of each TNC-PE, TNC-PE-CY5, TNC-PE-CF or MP97-116-BV421-labeled tetramers at room temperature for 90 minutes. Cells were washed and incubated with 40μl anti-PE and 10μl anti-Myc magnetic beads (Miltenyi Biotec) at 4°C for 20 minutes, washed again, and a 1/100th fraction was saved for antibody staining (“Pre”). The other fraction was passed through a MS magnetic column (Miltenyi Biotec). Bound, PE, PE-CY5, PE-CF or BV421-labeled cells were flushed and collected. Both enriched (“Post”) and non-enriched (“Pre”) fractions were labeled with Sytox, CD4 V500, CD45RA AF700, CD38 BUV395 (BD Bioscience) CXCR3 AF647, CCR4 BV605, CXCR5 PE-Cy7, CCR6 BV650, CCR7 APC-Cy7, CD14 FITC and CD19 FITC (BioLegend) (Supplemental Table 8). Samples were run on a BD LSRII flow cytometer, and data was analyzed using FlowJo software version 10.

The frequency (F) of epitope-specific T cells per million memory CD4+ T cells was calculated as follows: F=(1,000,000 × tetramer-positive events from enriched tube)/(100 × number of memory CD4+ T cells from the “Pre” fraction). Single T cells were gated CD4+/CD14−CD19−sytox− and a triple-negative population was gated for each of the three possible tetramer fluorophores. These served as the parent populations from which one of the four tetramer positive T-cell populations were then gated on and analyzed for surface receptor expression. T-cell lineage was assigned on CD45RA− memory cells as follows: Th1 (CXCR3+, CCR4− and CCR6−); Th2 (CXCR3−, CCR4+ and CCR6−); Th17 (CXCR3−, CCR4+ and CCR6+); Th1* (CXCR3+, CCR4− and CCR6+) (25, 26).
**ELISAs for detection of antibodies**

ELISAs were used to detect antibodies against citrullinated peptides in sera from patients in Autoantibody Cohorts 1 and 2 (Supplemental Table 2). Peptides used in the assay were synthesized by either GenScript or Pepceutical and are described in Supplemental Table 5. The ELISAs were conducted as previously described (18, 26). In brief, 96-well Nunc Maxisorp plates were coated with 10μg/ml peptide in PBS, blocked with 2% BSA and incubated with sera diluted 1:100. Bound antibodies were detected with an HRP-conjugated anti-human IgG Fc monoclonal antibody (6043HRP, Stratech). A standard curve of positive sera was used to calculate relative antibody titers in arbitrary units (AU) for each sample. The CCP2 ELISA for Autoantibody Cohort 1 was performed as by the manufacturer’s instruction (FCCP600, Axis-Shield). Cross-reactivity was analyzed in human sera that were reactive to both cit-TNC56 or cit-TNC17 and cit-TNC5. Sera were diluted 1:100, incubated with 1, 10 and 100μg/ml of peptides for 2 hours, centrifuged at 10,000 g for 10 min and the supernatant added to peptide-coated plates for analysis by ELISA as described above. Positivity was defined by the cut-off of the 98% percentile of healthy control samples from Autoantibody Cohort 1.

**Extracellular matrix peptide microarray.**

The custom-made microarray was performed as previously described (7). In brief, 16-aa long peptides that covered all arginine residues of 1,610 extracellular matrix proteins and RA related proteins were synthesized in situ (Roche NimbleGen). For tenascin C, there were a total of 217 peptides including both native and citrullinated variants of each peptide synthesized. Samples tested for reactivity against TNC derived peptides included synovial and plasma ACPA pools containing CCP-reactive antibodies from 26 and 38 RA patients, respectively (33, 34) Paired
synovial fluid and serum samples from one RA patient taken at two time points 10 years apart were also screened. ACPA pools were run at a concentration of 15μg/ml, whereas synovial fluid and plasma or serum samples were diluted 1/100 and tested for citrulline reactivity using a NimbleGen MS200 Scanner (Roche NimbleGen). A peptide signal intensity variation (spot size) of 25 pixels was used as a basis for calculating the median fluorescence intensities. The cutoff for positive signals was defined as 5 times the fluorescence intensity of the 98th percentile of values for a set of monoclonal antibodies without citrulline reactivity. These were included in the same experiment and were tested at the same time.

Statistics

All statistical tests were performed using GraphPad Prism version 7.02. Tests that were used (as appropriate) included paired two-tailed Student t tests, Wilcoxon signed ranked test, Spearman correlations, Mann-Whitney tests, and Kruskal Wallis test with Dunn’s multiple comparison test. P values < 0.05 were considered significant. To determine the association of Tenascin-C seropositivity with clinical variables – including smoking, HLA-DRB1 SE, Rapid3 disease activity scores, disease duration, and PTPN22 C1858T SNP, odds ratios (OR) with 95% Wald confidence intervals (95% CI) were calculated using logistic regression models including age and sex as covariates. p-values were corrected for multiple testing with the Benjamini & Hochberg method.

Study approval

The study was approved by Benaroya Research Institute’s Institutional Review Board (Protocol # IRB07109) and the Karolinska University Hospital’s Institutional Review Board (Protocol # dnr 03-138). All samples were obtained under approved research protocols with informed consent.
Author Contributions

JS, KSM, VM, EAJ and JHB conceptualized and designed the study. JHB and JS was responsible for subject selection and clinical data collection. JS performed the T cell studies. CR and DAL performed peptide binding assay. ST selected samples for and performed the synovial fluid studies. CG performed the analyses of the peptide array data. AS, AW and LRM performed the autoantibody studies. VSM performed the statistical modeling. JS, EAJ and JHB wrote the manuscript with assistance from all co-authors. JHB obtained funding and was responsible for the entire project.
Acknowledgements

This work was supported by NIH U01 AI101990 and the Office of the Assistant Secretary of Defense for Health Affairs through the Peer Reviewed Medical Research Program (PRMRP) Investigator-Initiated Research Award under Award No. W81XWH-15-1-0003 to Dr. Buckner. All opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense. ST was supported by the Stockholm County Council (combined residency and PhD training program) while the experimental costs in Stockholm were supported by the Swedish Research Council, the Swedish Rheumatism Association and the King Gustaf V’s 80-year foundation. LRM was supported by Xunta de Galicia (Spain) through a Gain pre-doctoral contract. This funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

We thank the patients and doctors at the Virginia Mason Medical Center, Seattle, USA and at the Karolinska University Hospital, Solna, Sweden. We also thank the staff in the rheumatology research laboratory for sample processing. We are also grateful for Anne Hocking for editing the manuscript. The graphic abstract was created with Biorender.com.
References


42. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, et al. Citrullinated proteins have increased immunogenicity and arthritogenicity and their


eosinophils induce the resolution of experimental arthritis and appear in remission state of

51. Gaffen SL. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. *Current

52. Patel DD, Zachariah JP, and Whichard LP. CXCR3 and CCR5 ligands in rheumatoid


Autoantibodies can be prognostic markers of an erosive disease in early rheumatoid

55. Ettinger RA, Papadopoulos GK, Moustakas AK, Nepom GT, and Kwok WW. Allelic
variation in key peptide-binding pockets discriminates between closely related diabetes-

56. Novak EJ, Liu AW, Nepom GT, and Kwok WW. MHC class II tetramers identify peptide-
specific human CD4(+) T cells proliferating in response to influenza A antigen. *The

vivo analysis of CD4+ T-cell responses using combinatorial HLA class II tetramer staining.
Figure legends

Figure 1. Citrulline residues are required in cit-TNC17 and cit-TNC56 peptides for HLA-DRB1*0401 binding and immunogenicity. (A) The binding of cit-TNC and their counterpart arginine peptides (arg-TNC) to DRB1*04:01 was tested using a peptide competition assay; each peptide was tested in triplicate. Europium-conjugated streptavidin was used to label residual biotinylated peptide bound to the HLA-DR protein. Binding curves were fitted by non-linear regression with a sigmoidal dose response curve model and EC50 values were calculated as the peptide concentration needed to displace 50% of the reference peptide. The HA306-318 peptide was used as a positive control. The EC50 cutoff for measurable binding was 50µM. Results indicated that citrullination at one specific residue was required for cit-TNC17 and cit-TNC56 binding. X stands for one citrullinated amino acid in cit-TNC, R stands for one arginine amino acid in arg-TNC. (B) Citrullination of a distinct T cell contact residue was associated with cit-TNC17 and cit-TNC56 immunogenicity. Representative plots showing representative CD4+ T cell response to TNC peptide citrullinated at either both residues (TNC17XX and TNC56XX) or only one residue (TNC17RX and TNC56XR); n=14 RA subjects tested for all peptides.

Figure 2. T cells that recognize tenasin-C epitopes are more frequent and have a distinct phenotype in patients with RA. The frequency and phenotype of tetramer+ cit-TNC-specific T cells was determined ex vivo using a multiplex HLA class II tetramer approach including cell surface marker antibodies to define the phenotype. Frequencies are expressed as the number of antigen-specific cells per million memory CD4+ T cells. Each symbol represents an individual subject (n=7 for HC, n=9 for RA), and the horizontal bar shows the median. (A) The frequency of the combined cit-TNC-specific CD4 memory T cells were elevated in patients with RA compared
to HC subjects, but the frequency of influenza-specific memory CD4+ T cells were similar between RA and HC subjects. (B) The increased frequency of cit-TNC-specific CD4 memory T cells appeared to be mainly due to cit-TNC45, cit-TNC50 and cit-TNC56. (C) The heatmap shows the cit-TNC T cell frequency for each epitope in each subject. Each row stands for a single epitope and each column stands for one RA subject; intensity based on the number of cit-TNC T cells per million memory CD4 T cells. (D) The frequency of CCR4+, CCR6+ and CD38+ cit-TNC-specific CD4 memory T cells were elevated in patients with RA compared to HC subjects. (E) The percent of CD38+ cells among total cit-TNC-specific CD4 memory T is increased in patients with RA compared to HC subjects. (F) The lineage of cit-TNC-specific memory CD4+ T cells in RA subjects was predominantly Th2 (CCR4+CCR6-CXCR3-) and Th17 (CCR4+CCR6+CXCR3-), but not Th1 (CXCR3+CCR4-CCR6-). P-values were calculated using an unpaired non-parametric Mann-Whitney test.

**Figure 3. Synovial fluid mononuclear cells from patients with RA secrete IFN-γ in response to cit-TNC peptides.** Synovial fluid mononuclear cells (SFMC) from patients with RA (n=7) were stimulated with cit-TNC peptides or their arginine (arg) counterparts, or other known cit-epitopes: cit-enolase (eno), cit-fibrinogen (Fib), cit-vimentin (vim), cit-cartilage intermediate layer protein (CILP) for 48 hours. Influenza peptide (MP97-116) and anti-CD3 were used as positive controls. IFN-γ was measured by a three-color fluorospot. Number of spots were normalized to spots per million cells and spots seen in the unstimulated wells were subtracted from count in the stimulated wells prior to further analyses. Each symbol represents an individual subject, and the horizontal line shows the median (A) SFMC from patients with RA produced IFN-γ in response to cit-TNC17, cit-TNC22, cit-TNC45 and cit-TNC56 peptides (B) The level of IFN-γ produced was an order of

37
magnitude greater than that produced in SFMC in response to cit-eno, cit-vim and cit-CILP. Note this experiment was done side by side with the experiment in (A) but due to limited sample volumes only 4 of the 7 subjects in (A) were included here. Furthermore, apart from cit-eno26 and cit-eno326, the peptides between samples varied. (C) HLA-DR blocking antibodies (DR), but not HLA-DQ blocking antibodies (DQ), completely abrogated cit-TNC induction of IFN-γ secretion by SFMC from RA patients (n=3). P-values were calculated using a Wilcoxon matched pairs signed ranked test.

**Figure 4. cit-TNC17 and cit-TNC56 are citrullinated epitopes that are recognized by ACPA in patients with RA.** Antibody responses to cit-TNC peptides or their arginine counterparts in serum from patients with RA and HC subjects were measured by ELISA. Numbers below x-axis indicate percentage of each cohort that were seropositive. Each symbol represents an individual subject, and the horizontal line shows the median. (A) Antibodies to cit-TNC17, cit-TNC56 and cit-TNC5 were detected in 100%, 53% and 47% of CCP positive RA subjects (n=17), respectively, but not in HC subjects (n=24), with little or no antibody response against the corresponding arg-TNC peptides. Each peptide was run on one plate and each peptide pair (arg/cit) was run at the same day. P-values were calculated using a Mann-Whitney test. (**p<0.01, ***p<0.001 and ****p<0.0001). (B) CCP2 antibody levels correlated significantly with the antibody levels for both cit-TNC17 and cit-TNC5 but not cit-TNC56. (Spearman r test *p < 0.05). (C) Antibody reactivity to cit-TNC peptides was stronger in polyclonal anti-CCP pools from synovial fluid than plasma. Cutoff for positivity is 1,000, and P-values were calculated using a paired two tailed Student t test. (D) cit-TNC antibody reactivity over time in paired serum and synovial fluid samples from a single
RA subject. T = time point. The different colors in Figures 4C and 4D indicate the specific peptides tested in the microarray see Supplemental Table 6.

**Figure 5. Anti-cit-TNC antibodies do not cross-react with each other.** Sera which were double-reactive with peptides cit-TNC17 or cit-TNC56, cit-TNC17 or cit-TNC5, respectively, were diluted 1:100, incubated with 1, 10 and 100μg/ml of peptides for 2 hours, centrifuged at 10,000g for 10 minutes and the supernatant then added to peptide-coated plates for analysis by ELISA. (A) No cross-reactivity between anti-cit-TNC17 and both anti-cit-TNC56 and anti-cit-TNC5. (B) Limited cross-reactivity between anti-cit-TNC56 and both anti-cit-TNC17 and anti-cit-TNC5. Each panel was run on a separate plate.

**Figure 6. cit-TNC antibodies are associated with CCP seropositivity and the shared epitope.** Antibody responses to cit-TNC peptides or their arginine counterparts in serum from patients with RA and HC subjects were measured by ELISA. (A) cit-TNC17 and cit-TNC56 seropositivity was more prevalent in CCP⁺ RA subjects (n=55) than CCP⁻ RA subjects (n=43). Each symbol represents an individual subject, and the horizontal line shows the median. Dotted lines indicate cut-off for positivity. Numbers below x-axis indicate percentage of each cohort that were seropositive. Left panel, cit-TNC17, cut off AU=29.51; Right panel, cit-TNC56, cut off AU=91.98. P-values were calculated using a Kruskal Wallis test with Dunn’s multiple comparison test; (*p<0.05 and ****p<0.001). (B) Heatmaps showing cit-TNC antibody seropositivity and its association with the HLA shared epitope (SE) in CCP⁺ RA subjects (n=72; Autoantibody Cohorts 1 and 2 combined) and CCP⁻ RA patients (n=43).
Table 1. cit-TNC peptides that bind HLA-DRB1*04:01 and are immunogenic *in vitro*.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Cit Location/Peptide Length</th>
<th>Protein Location&lt;sup&gt;B&lt;/sup&gt;</th>
<th>EC50&lt;sup&gt;C&lt;/sup&gt; (μM)</th>
<th>Responders (% in RA)&lt;sup&gt;D&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC56</td>
<td>[H]QGQ&lt;sub&gt;YEL[Cit]VDL[Cit]DHGE&lt;/sub&gt;[OH]</td>
<td>TNC-2073-2077-cit(2067-2081)</td>
<td>FBG</td>
<td>9</td>
<td>79%</td>
</tr>
<tr>
<td>TNC17</td>
<td>[H]VSL&lt;sub&gt;IS[Cit][Cit]GDMSSNPA&lt;/sub&gt;[OH]</td>
<td>TNC-876-877-cit(871-885)</td>
<td>3rd to 4th Fn3</td>
<td>3.6</td>
<td>64%</td>
</tr>
<tr>
<td>TNC45</td>
<td>[H]PDGF&lt;sub&gt;[Cit]LSWTADEGV&lt;/sub&gt;[OH]</td>
<td>TNC-1637-cit(1633-1647)</td>
<td>12th Fn3</td>
<td>17.1</td>
<td>50%</td>
</tr>
<tr>
<td>TNC50</td>
<td>[H]VES&lt;sub&gt;[Cit]ITYVPI&lt;/sub&gt;GTG[OH]</td>
<td>TNC-1741-cit(1737-1751)</td>
<td>13th Fn3</td>
<td>1</td>
<td>50%</td>
</tr>
<tr>
<td>TNC22</td>
<td>[H]FD&lt;sub&gt;[Cit]Y[Cit]LNYSLPTGQW&lt;/sub&gt;[OH]</td>
<td>TNC-1014-1016-cit(1012-1026)</td>
<td>5th Fn3</td>
<td>0.7</td>
<td>46%</td>
</tr>
<tr>
<td>TNC52</td>
<td>[H]LA&lt;sub&gt;[Cit]WOPAIATVCS&lt;/sub&gt;YV[OH]</td>
<td>TNC-1818-cit(1816-1830)</td>
<td>14th Fn3</td>
<td>28.8</td>
<td>33%</td>
</tr>
<tr>
<td>TNC44</td>
<td>[H]TKP&lt;sub&gt;[Cit]AEIVTEA&lt;/sub&gt;EPE[OH]</td>
<td>TNC-1795-cit(1791-1805)</td>
<td>13th Fn3</td>
<td>33.3</td>
<td>33%</td>
</tr>
<tr>
<td>TNC59</td>
<td>[H]SMA&lt;sub&gt;YHNGC[Cit]ISFST&lt;/sub&gt;FD[OH]</td>
<td>TNC-2303-cit(2296-2310)</td>
<td>FBG</td>
<td>25</td>
<td>33%</td>
</tr>
<tr>
<td>Influenza HA306-318</td>
<td>PKYVKQNTLKLAT</td>
<td></td>
<td></td>
<td>0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

A. Predicted binding register underlined with anchor residues in red

B. FBG: Fibrinogen domain; Fn3: Fibronectin type III domain

C. Cutoff in the peptide binding assay is 50μM

D. Percentages were calculated based on the number of individuals with a positive *in vitro* response out of the total number of individuals tested; n=14 RA subjects tested for all peptides except TNC44 and TNC59 where n=6. The cutoff for a positive in vitro response is more than 0.1% of CD4+ T cells stained with tetramer.
Table 2. Chi-squared test showing that citrullination of a distinct T cell contact residue was associated with cit-TNC17 and cit-TNC56 immunogenicity.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>&lt;0.1%(^B)</th>
<th>&gt;0.1%(^B)</th>
<th>Total</th>
<th>(\chi^2) p-value(^B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC17RX</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>TNC17XX</td>
<td>15</td>
<td>1</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>9</td>
<td>32</td>
<td>0.0059</td>
</tr>
<tr>
<td>TNC56XR</td>
<td>2</td>
<td>15</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>TNC56XX</td>
<td>13</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>18</td>
<td>33</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

A. X stands for one citrullinated amino acid in cit-TNC, R stands for one arginine amino acid in arg-TNC.

B. The cutoff for a positive in vitro response is more than 0.1% of CD4+ T cells stained with tetramer; n=14 RA subjects tested for all peptides.
Table 3. cit-TNC antibodies are associated with clinical measures and smoking in rheumatoid arthritis.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>CIT-TNC specificity</th>
<th>Comparison</th>
<th>Odds Ratio</th>
<th>95% Confidence Intervals</th>
<th>P-Value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Rheumatoid Factor (+/-)</td>
<td>14.2</td>
<td>5.14 - 47.49</td>
<td>2.00E-06</td>
<td>2.00E-05</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Anti-CCP (+/-)</td>
<td>92.98</td>
<td>18.12 - 1712.79</td>
<td>2.00E-05</td>
<td>9.00E-05</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Disease Duration (years)</td>
<td>0.94</td>
<td>0.90 - 0.99</td>
<td>2.20E-02</td>
<td>4.83E-02</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Smoking Currently</td>
<td>6.5</td>
<td>2.47 - 18.91</td>
<td>3.00E-04</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Smoke Ever</td>
<td>2.67</td>
<td>1.17 - 6.23</td>
<td>2.07E-02</td>
<td>4.83E-02</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC17</td>
<td>Shared Epitope</td>
<td>2.29</td>
<td>0.99-5.58</td>
<td>5.80E-02</td>
<td>1.07E-01</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Shared Epitope</td>
<td>3.41</td>
<td>1.37 - 9.45</td>
<td>1.19E-02</td>
<td>4.35E-02</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Rheumatoid Factor (+/-)</td>
<td>3.49</td>
<td>1.43 - 9.32</td>
<td>8.20E-03</td>
<td>4.35E-02</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Anti-CCP (+/-)</td>
<td>7.92</td>
<td>2.99 - 25.21</td>
<td>1.00E-04</td>
<td>1.20E-03</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Disease Duration (years)</td>
<td>1</td>
<td>0.96-1.05</td>
<td>8.31E-01</td>
<td>9.99E-01</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Smoking Currently</td>
<td>1.35</td>
<td>0.53-3.38</td>
<td>5.21E-01</td>
<td>8.19E-01</td>
</tr>
<tr>
<td>Combined RA</td>
<td>cit-TNC56</td>
<td>Smoke Ever</td>
<td>0.99</td>
<td>0.42-2.29</td>
<td>9.78E-01</td>
<td>9.99E-01</td>
</tr>
</tbody>
</table>
Figure 1. Citrulline residues are required in cit-TNC17 and cit-TNC56 peptides for HLA-DRB1*0401 binding and immunogenicity. (A) The binding of cit-TNC and their counterpart arginine peptides (arg-TNC) to DRB1*04:01 was tested using a peptide competition assay. Europium-conjugated streptavidin was used to label residual biotinylated peptide bound to the HLA-DR protein. Binding curves were fitted by non-linear regression with a sigmoidal dose response curve model and EC50 values were calculated as the peptide concentration needed to displace 50% of the reference peptide. The HA306-318 peptide was used as a positive control. The EC50 cutoff for measurable binding was 50µM. Results indicated that citrullination at one specific residue was required for cit-TNC17 and cit-TNC56 binding. X stands for one citrullinated amino acid in cit-TNC, R stands for one arginine amino acid in arg-TNC. (B) Citrullination of a distinct T cell contact residue was associated with cit-TNC17 and cit-TNC56 immunogenicity. Representative plots showing representative CD4+ T cell response to TNC peptide citrullinated at either both residues (TNC17XX and TNC56XX) or only one residue (TNC17RX and TNC56XR).
Figure 2. T cells that recognize tenascin-C epitopes are more frequent and have a distinct phenotype in patients with RA. The frequency and phenotype of tetramer+ cit-TNC-specific T cells was determined ex vivo using a multiplex HLA class II tetramer approach including cell surface marker antibodies to define the phenotype. Frequencies are expressed as the number of antigen-specific cells per million memory CD4+ T cells. Each symbol represents an individual subject (n=7 for HC, n=9 for RA), and the horizontal bar shows the median. (A) The frequency of the combined cit-TNC-specific CD4 memory T cells were elevated in patients with RA compared to HC subjects, but the frequency of influenza-specific memory CD4+ T cells were similar between RA and HC subjects. (B) The increased frequency of cit-TNC-specific CD4 memory T cells appeared to be mainly due to cit-TNC45, cit-TNC50 and cit-TNC56. (C) The heatmap shows the cit-TNC T cell frequency for each epitope in each subject. Each row stands for a single epitope and each column stands for one RA subject; intensity based on the number of cit-TNC T cells per million memory CD4 T cells. (D) The frequency of CCR4+, CCR6+ and CD38+ cit-TNC-specific CD4 memory T cells were elevated in patients with RA compared to HC subjects. (E) The percent of CD38+ cells among total cit-TNC-specific CD4 memory T is increased in patients with RA compared to HC subjects. (F) The lineage of cit-TNC-specific memory CD4+ T cells in RA subjects was predominantly Th2 (CCR4+CCR6-CXCR3-) and Th17 (CCR4+CCR6+CXCR3-), but not Th1 (CXCR3+CCR4-CCR6-). P-values were calculated using an unpaired non-parametric Mann-Whitney test.
Figure 3. Synovial fluid mononuclear cells from patients with RA secrete IFN-γ in response to cit-TNC peptides. Synovial fluid mononuclear cells (SFMC) from patients with RA (n=7) were stimulated with cit-TNC peptides or their arginine (arg) counterparts, or other known cit-epitopes: cit-enolase (eno), cit-fibrinogen (Fib), cit-vimentin (vim), cit-cartilage intermediate layer protein (CILP) for 48 hours. Influenza peptide (MP97-116) and anti-CD3 were used as positive controls. IFN-γ was measured by a three-color fluorospot. Number of spots were normalized to spots per million cells and spots seen in the unstimulated wells were subtracted from count in the stimulated wells prior to further analyses. Each symbol represents an individual subject, and the horizontal line shows the median (A) SFMC from patients with RA produced IFN-γ in response to cit-TNC17, cit-TNC22, cit-TNC45 and cit-TNC56 peptides (B) The level of IFN-γ produced was an order of magnitude greater than that produced in SFMC in response to cit-eno, cit-vim and cit-CILP. Note this experiment was done side by side with the experiment in (A) but due to limited sample volumes only 4 of the 7 subjects in (A) were included here. Furthermore, apart from cit-eno26 and cit-eno326, the peptides between samples varied. (C) HLA-DR blocking antibodies (DR), but not HLA-DQ blocking antibodies (DQ), completely abrogated cit-TNC induction of IFN-γ secretion by SFMC from RA patients (n=3). P-values were calculated using a Wilcoxon matched pairs signed ranked test.
Figure 4. cit-TNC17 and cit-TNC56 are citrullinated epitopes that are recognized by ACPA in patients with RA. Antibody responses to cit-TNC peptides or their arginine counterparts in serum from patients with RA and HC subjects were measured by ELISA. Numbers below x-axis indicate percentage of each cohort that were seropositive. Each symbol represents an individual subject, and the horizontal line shows the median. (A) Antibodies to cit-TNC17, cit-TNC56 and cit-TNC5 were detected in 100%, 53% and 47% of CCP positive RA subjects (n=17), respectively, but not in HC subjects (n=24), with little or no antibody response against the corresponding arg-TNC peptides. Each peptide was run on one plate and each peptide pair (arg/cit) was run at the same day. $P$-values were calculated using a Mann-Whitney test. ($**p<0.01$, ***$p<0.001$ and ****$p<0.001$). (B) CCP2 antibody levels correlated significantly with the antibody levels for both cit-TNC17 and cit-TNC5 but not cit-TNC56. (Spearman r test *$p < 0.05$). (C) Antibody reactivity to cit-TNC peptides was stronger in polyclonal anti-CCP pools from synovial fluid than plasma. Cutoff for positivity is 1,000, and $P$-values were calculated using a paired two tailed Student t test. (D) cit-TNC antibody reactivity over time in paired serum and synovial fluid samples from a single RA subject. T = time point. The different colors in Figures 4C and 4D indicate the specific peptides tested in the microarray see Supplemental Table 7.
Figure 5. Anti-cit-TNC antibodies do not cross-react with each other. Sera which were double-reactive with peptides cit-TNC17 or cit-TNC56, cit-TNC17 or cit-TNC5, respectively, were diluted 1:100, incubated with 1, 10 and 100μg/ml of peptides for 2 hours, centrifuged at 10,000g for 10 minutes and the supernatant then added to peptide-coated plates for analysis by ELISA. (A) No cross-reactivity between anti-cit-TNC17 and both anti-cit-TNC56 and anti-cit-TNC5. (B) Limited cross-reactivity between anti-cit-TNC56 and both anti-cit-TNC17 and anti-cit-TNC5. Each panel was run on a separate plate.
Figure 6. cit-TNC antibodies are associated with CCP seropositivity and the shared epitope. Antibody responses to cit-TNC peptides or their arginine counterparts in serum from patients with RA and HC subjects were measured by ELISA. (A) cit-TNC17 and cit-TNC56 seropositivity was more prevalent in CCP+ RA subjects (n=55) than CCP- RA subjects (n=43). Each symbol represents an individual subject, and the horizontal line shows the median. Dotted lines indicate cut-off for positivity. Numbers below x-axis indicate percentage of each cohort that were seropositive. Left panel, cit-TNC17, cut off AU=29.51; Right panel, cit-TNC56, cut off AU=91.98. P-values were calculated using a Kruskal Wallis test with Dunn’s multiple comparison test; (*p<0.05 and ****p<0.001). (B) Heatmaps showing cit-TNC antibody seropositivity and its association with the HLA shared epitope (SE) in CCP+ RA subjects (n=72; Autoantibody Cohorts 1 and 2 combined) and CCP- RA patients (n=43).