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Citrullinated Aggrecan Epitopes as Targets of Auto-reactive CD4+ T cells in Patients with Rheumatoid Arthritis

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Abstract

Objective.—Recognition of citrullinated antigens such as vimentin, fibrinogen, and alpha-enolase is associated with rheumatoid arthritis (RA). Emerging data suggests that the matrix protein aggrecan is also recognized as a citrullinated antigen. The goal of this study was to directly visualize cit-aggrecan specific T cells and characterize them in subjects with RA.

Methods.—Citrullinated aggrecan peptides with likely DRB1*04:01 binding motifs were predicted using a previously published scanning algorithm. Peptides with detectable binding were assessed for immunogenicity by HLA tetramer staining, followed by single cell cloning. Selectivity for citrullinated peptide was assessed through tetramer staining and proliferation assays. *Ex vivo* tetramer staining was then employed to assess frequencies of aggrecan specific T cells in peripheral blood. Finally, disease association was assessed by comparing T cell frequencies in RA patients and controls and correlating aggrecan specific T cells with levels of aggrecan specific antibodies.

Results.—We identified six immunogenic peptides, two of which were the predominant T cell targets in peripheral blood. These two epitopes were citrullinated at HLA binding residues and

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shared homologous sequences. RA patients had significantly higher frequencies of cit-aggrecan-specific T cells than healthy subjects. Furthermore, T cell frequencies were significantly correlated with antibodies against citrullinated aggrecan.

Conclusion.—Our findings indicate that T cells that recognize citrullinated aggrecan are present in subjects with RA and correlate with antibodies that target this same antigen. Consequently, aggrecan-specific T cells and antibodies are potentially relevant markers that could be utilized to monitor patients with RA or at risk subjects.

Rheumatoid arthritis (RA) is a chronic disease in which joints are destroyed through inflammatory processes (1). Serological markers, including rheumatoid factor and anti-citrullinated protein antibodies (ACPA) and strong association with high risk alleles such as *HLA-DRB1*04:01* implicate autoreactive CD4+ T cells as an important facet of disease etiology (2, 3). Detailed studies of ACPA specificity establish that vimentin, fibrinogen, and α -enolase are recognized as citrullinated antigens (4–6). These proteins are also recognized by autoreactive CD4+ T cells, supporting the notion that T cells provide help for antibody responses in RA (7). Furthermore, T cell frequency has been shown to be influenced by disease duration and therapy, suggesting that changes in T cell number and function are reflective of changes in the overall disease process (8).

Emerging data suggests that the matrix protein proteoglycan aggrecan is recognized as a citrullinated antigen (7, 9). Aggrecan is an abundant component of extracellular matrix within the joints (along with proteins such as tenascin-C and type II collagen) and has been conclusively shown to be citrullinated in human articular cartilage (10). The presence of aggrecan fragments has been documented within synovial fluid and is reported to increase with age (11, 12). Furthermore, immunization with aggrecan was shown to induce arthritis in murine models (13). Autoantibodies to citrullinated epitopes within the G1 domain are elevated in RA but not in Osteoarthritis, indicating that such antibodies are specifically associated with autoimmunity rather than merely accompanying joint damage (10). Citrullinated CD4+ T cell epitopes from aggrecan have been described and elevated responses to some were shown to be associated with RA (9, 14, 15). However, a systematic HLA-specific characterization of aggrecan derived T cell epitopes has yet to be performed and tools to directly visualize aggrecan specific T cells have yet to be developed. The goals of our study were to define citrullinated aggrecan epitopes in the context of DRB1*04:01, to visualize and characterize cit-aggrecan-specific T cells, and to investigate their relevance in subjects with RA.

MATERIALS AND METHODS

Epitope prediction and peptide synthesis.

A previously described prediction method was utilized to identify citrullinated aggrecan peptides with motifs likely bind to HLA-DRB1*04:01 (henceforth DR0401) (8, 16). 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 (R) or citrulline (Cit) residue. A total of 28 peptides with motif scores of 0.1 or higher were synthesized by Mimotopes. For tetramer production and further studies,

selected citrullinated peptides and their corresponding native peptides were re-synthesized by Sigma. All peptides were dissolved in DMSO to a stock concentration of 20 mg/ml.

Peptide binding to DR0401.

The binding capacity of citrullinated aggrecan peptides to DR0401 was assessed using a previously described assay (17). Briefly, candidate peptides were plated at increasing concentrations against a fixed concentration of a biotinylated reference peptide, influenza HA₃₀₆₋₃₁₈ (PKYVKQNTLKLAT) in wells coated with anti-HLA-DR antibody (Clone L243, supplied by the Benaroya Research Institute (BRI) Tetramer Core). Europium conjugated streptavidin was used to label residual HLA-bound biotinylated peptide (PerkinElmer) and quantified using a Wallac Victor 2 Multi-label Counter. Binding curves were fitted by a sigmoidal regression model using Graphpad Prism 7.0 and EC50 values were calculated as the peptide concentration needed to displace 50% of the reference peptide.

Subject recruitment.

Control and RA subjects were recruited with informed consent through the BRI healthy control and rheumatic disease registries. Sample use was approved by the BRI Institutional Review Board. All subjects had *HLA-DRB1*04:01* haplotypes. Control subjects had no autoimmune disease, no first degree relatives with RA, and ranged in age from 23 to 66 years (mean $\pm$ SD; 50.2 $\pm$ 10.9 years). All RA patients were ACPA positive, ranged in age from 25 to 71 years (mean $\pm$ SD; 52.5 $\pm$ 11.3 years), and met 1987 American College of Rheumatology criteria (1).

PBMC isolation and preparation.

PBMC were separated from whole blood over Ficoll-Hypaque gradient, cryopreserved in heat inactivated fetal bovine serum supplemented with 10% DMSO, and stored in liquid nitrogen. PBMC were subsequently thawed at 37°C, washed with RPMI media supplemented with 10% FBS and 0.001% DNase/Benzonase (Sigma Aldrich), and re-suspended in RPMI media supplemented with 10% human serum (Gemini Bio-Products).

Tetramer production.

Class-II tetramers were generated by the BRI Tetramer Core as previously described (18). Briefly, DR0401 monomer was purified from insect cell cultures and biotinylated at a sequence-specific site. Biotinylated monomer was loaded with 0.2 mg/ml of peptide, incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl- β -D-glucopyranoside and 1 mM Pefabloc SC (Sigma-Aldrich). Loaded monomers were conjugated into tetramers with fluorescently labeled streptavidin for 6-18 hours at room temperature at a molar ratio of 8:1.

In vitro PBMC expansion, HLA class-II tetramer staining and T cell clone isolation.

PBMC were cultured at 5 $\times$ 10⁶ cells/well in 48-well plates in 1 ml RPMI supplemented with 10% human serum and 10 μ g/mL of peptide. On day 6, cells were transferred to fresh wells and 10 U/ml of rIL-2 (Roche) was added. After 14 days, cultures were screened by staining a 50 μ l aliquot with 0.5 μ l of tetramer (final concentration 5 ng/ml) at 37°C for 1 hour, co-

stained with anti-CD4 APC (BD Biosciences) for 30 minutes at 4°C in the dark, washed, and analyzed by flow cytometry. To isolate T cell clones, staining was repeated and tetramer-labeled cells were single cell sorted using a BD FACS Aria II into 96-well round bottom plates containing 150 µl of human T cell media and expanded by adding 10⁵ irradiated feeders, 10 U/ml IL-2 and 2 µg/ml phytohaemagglutinin (PHA, Thermo). After 10-14 days, expanded cells were transferred to 96-well flat bottom plates and split as needed for an additional 14 days. The resulting clones were re-screened by tetramer staining. Positive clones were further expanded by additional rounds of PHA stimulation and then cryopreserved.

Proliferation assays.

To assess proliferation in response to citrullinated, partially citrullinated, or unmodified peptide, clones or cells were plated at 2.5×10⁴ cells/well in 96-well round bottom plates in human T cell media with 10⁵ irradiated antigen presenting cells from a *HLA-DRB1*04:01* positive donor plus peptide and incubated at 37°C. After 72 hours, 1 µCi of [³H]-thymidine was added and incubated for 24 hours. Cells were then washed, water lysed, and DNA was collected onto glass fiber filter membranes (PerkinElmer) using a plate harvester. Each filter mat was immersed in Betaplate Scint (PerkinElmer) and counts collected on a Wallac 1450 LSC and Luminescence Counter (PerkinElmer). Stimulation index was determined by calculating the ratio of counts of peptide-stimulated cells to counts of non-stimulated cells.

Intracellular cytokine staining of T cell clones.

To assess their functional profiles, T cell clones were activated with 50 ng/mL phorbol myristic acid (PMA) and 1 µg/mL ionomycin for 30 min, treated with brefeldin A (eBioscience) and incubated for 3 h at 37°C. Cells were fixed using fixation/permeabilization buffer (eBioscience), washed in permeabilization buffer (eBioscience), stained using antibodies to GM-CSF (PerCP-Cy5.5), granzyme B (FITC), IFNγ (AF700), IL-4 (AF647), IL-21 (PE) (all from Biolegend), collected on an LSR II flow cytometer (BD Biosciences), and analyzed using FlowJo (TreeStar).

Activation of T cell clones by fibroblast-like synoviocytes (FLS).

Human FLS were HLA typed and isolated as previously described (19), were co-cultured with T cell clones by plating 1 × 10⁵ FLS (Passage 10 0401, passage 12 non-0401) with 2 × 10⁵ T cells per well, and incubated in the presence or absence of anti-DR antibody (L243, BRI Tetramer Core, 20 µg/ml) or corresponding peptide as a positive control for 5 days. Clones and FLS were also cultured individually to assess background. On day 4, 1 µCi of [³H]-thymidine was added to each well. Cells were harvested after 24 hours and counts collected on day 5 as described above.

Gene expression of aggrecan by fibroblast-like synoviocytes (FLS)—Human FLS were plated on a single 100mm tissue culture dish (BD). Once 80% confluent, cells were treated with 1000 Units/ml interferon gamma (RnD #285-IF) for 72 hours upon which cells were treated with trypsin and RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen #74134) following manufacturer's protocol. RNA was quantified using a Nanodrop, cDNA was synthesized using ThermoFisher SuperScript IV reverse transcriptase kit

(Thermo #18091050) as per manufacturer instructions in a PTC-225 Peltier thermal cycler. cDNA was probed using TaqMan Fast Advanced Master Mix (Applied Biosystems #4444557) in an Applied Biosystems 7500 Fast Real-Time PCR System cycler with the following Applied Biosystems Taqman primers: VIC-MGB GAPDH Hs02786624_g1, FAM-MGB PDPN Hs00366766_m1, FAM-MGB CD3E Hs01062241_m1, FAM-MGB ACAN Hs00153936_m1, FAM-MGB VCAN Hs00171642_m1. Gene expression were assessed by comparing mean Ct values to mean Ct of GAPDH. Ct values defined as “undetermined” by 7500 software v2.3 assigned a default value of “40” or above 35 were considered “undetectable”.

Ex vivo detection of cit-Aggrecan-specific T cells.

For *ex vivo* detection of antigen-specific T cells, 3.5×10^7 PBMCs were thawed and rested for 2 hours at 37°C, re-suspended in 200 µl of T cell media and treated with Dasatanib for 10 minutes at 37°C to prevent internalization of T cell receptors. Cells were stained by adding 4.5 µl of each tetramer (final concentration 11 ng/ml) at room temperature for 90 minutes, with gentle manual shaking every 15 minutes. Cells were then labeled with anti-PE and anti-Myc magnetic beads (Miltenyi) for 20 minutes at 4°C, enriched on a magnetic column according to manufacturer’s protocols (Miltenyi), reserving a 1% cell fraction before enrichment to estimate the total number of CD4+ T cells in the sample. Cells were surface-stained for 30 minutes at 4°C with CD14/CD19/Annexin V-FITC (all from Biolegend), CD4-V500 (BD), CD45RA-AF700 (BD), and CCR7-APC/Cy7 (Biolegend). Samples were collected to completion on a BD FACS Canto II. Flow cytometry data was analyzed using FlowJo v10 and Graphpad Prism 7.0. The frequency (F) of antigen specific T cells was calculated as: $F = (1,000,000 \times \text{tetramer positive events from enriched sample}) / (100 \times \text{number of CD4+ cells from the non-enriched fraction})$. Supplemental Figure 1 depicts the gating strategy.

IgM Depletion and Serum Antibody Detection.

IgM from serum samples of RA patients and healthy control subjects (HC) were depleted via immuno-absorption. Briefly, diluted sera were incubated with goat anti-human IgM (µ chain-specific) antibody conjugated to agarose beads (Millipore Sigma) for 90 minutes at 4°C on a rotary wheel. IgM depletion was verified by anti-IgM Western blot. For ELISA, wells of 96-well ELISA plates (Nunc) were coated overnight with recombinant human (rh)-aggrecan G1 or citrullinated rh-aggrecan G1 domain (0.2 µg/well each) in 100 µl/well of 0.15 M sodium carbonate buffer (pH 9.6) at room temperature (10). Unbound antigen was removed by washing with HRP wash buffer (Inova Diagnostics). Wells were blocked with heat-inactivated normal goat serum (R & D Systems) diluted to 1:10 in HRP sample diluent (Inova Diagnostics) at for 2 hours at room temperature. IgM-depleted serum samples were diluted 1:100 in sample diluent and incubated with the antigen-coated wells (100 µl/well, duplicate wells) for 2 hours at room temperature. Bound IgG was detected by incubation with 100 µl/well of HRP-conjugated polyclonal goat anti-human IgG (Abcam) at 1:3,000 dilution for 1 hour at room temperature. Unbound material was removed with HRP wash buffer between each of these steps. The color reaction was developed by incubation with 100 µl/well 3,3',5,5'-tetramethylbenzidine (BD OptEIA TMB substrate set) for 10 minutes in the dark at room temperature and stopped with 25 µl/well stop solution (4N HCl). Absorbance at

450 nm was read in a Synergy 2 ELISA reader (BioTek Instruments). Net optical density (OD) values were calculated by subtracting the OD of wells not containing samples (but coated with rhG1 or CitrhG1 and reacted with HRP secondary antibody) from the OD values of serum samples.

Anti-IgM Western Blot—Paired non-depleted and IgM-depleted serum samples (60 µg protein each) were run in a 7.5% SDS-PAGE gel under reducing conditions. The proteins were transferred to a PVDF membrane, probed with HRP-conjugated goat anti-human IgM µ chain-specific antibody (Abcam) and visualized by enhanced chemiluminescence (ECL, Amersham)

Statistical Methods.

All statistical tests were performed using PRISM version 7 software (GraphPad). Tests that were used (as appropriate) included unpaired t tests, unpaired t tests with Welch's correction, Mann-Whitney tests, or ANOVA with Sidak's multiple comparisons test, and Spearman correlation. P values < 0.05 were considered significant. Data are reported as the mean ± standard error of the mean.

RESULTS

Aggrecan peptides bind to DR0401 and are immunogenic.

Following our previously described approach for identifying epitopes from joint-associated antigens (8, 16), we synthesized 28 citrullinated peptides corresponding to aggrecan sequences that contain DR0401 binding motifs (Supplemental Table 1). Detectable *in vitro* binding to recombinant DR0401 protein was confirmed for thirteen of these peptides; among these, three were preferentially bound in their citrullinated form (Table 1). To assess the immunogenicity of these arginine-containing (arg-Agg) or citrullinated aggrecan (cit-Agg) peptides, PBMC from DR0401 positive RA patients and healthy controls were stimulated *in vitro*. For cit-Agg or arg-Agg peptides with detectable HLA binding, expansion of epitope specific T cells was evaluated through HLA class II tetramer staining. Possible responses to arg-agg peptides that corresponded to cit-Agg peptides but lacked detectable binding were evaluated through proliferation assays, but no responses above background proliferation were observed (Supplemental Figure 2A). Tetramer staining indicated that aggrecan peptides display varying degrees of immunogenicity (Figure 1A). Two arg-Agg peptides occasionally elicited responses (Figure 1B), but all median responses were well below our established cutoff and consequently were not advanced for further study. In total, seven cit-Agg elicited median responses in subjects with RA that were above a previously established cutoff (Figure 1C). Among these, six were verified through isolation T cell clones (Supplemental Figure 2B) and were advanced for further study. Examining *in vitro* responses to cit-Agg peptides, responses were also detectable in HLA matched controls (Figure 1D) but were more frequently seen in subjects with RA (Figure 1E).

In toto, our approach identified six citrullinated aggrecan peptides that bound to DR0401 and elicited *in-vitro* T cell responses. Two of these epitopes exhibited preferential binding to DR0401. For the remaining peptides, the citrulline residues within the predicted binding

register are positioned as T cell contacts (Table 2). Some of these immunogenic cit-Agg peptides overlapped with previously described epitopes. In particular, our cit-Agg₂₀₀, cit-Agg₂₉₈ and cit-Agg₆₂₁ peptides correspond to epitopes described by Boots et al. (20). The cit-Agg₂₉₈ peptide also overlaps with the p49 aggrecan sequence reported by Markovics et al. (MDMCSAGWLAD(Cit)SVR) (9). Surprisingly, the cit-Agg₈₄ peptide (VLLVATEG(Cit)VRVNSAYQDK) described by Law et al bound with very low affinity. (21) (Not shown).

Aggrecan specific CD4+ T cells are citrulline selective and respond to FLS-derived antigens.

To assess the selectivity of aggrecan specific T cells, clones corresponding to each specificity were tested by proliferation assay for their responsiveness to cit-Agg or the corresponding arg-Agg peptide. Each clone preferentially responded to citrullinated peptide (Figure 2A). To assess their function, we characterized the cytokine profiles of aggrecan-specific T cell clones by intracellular staining. Following activation with PMA/ionomycin, the clones predominantly secreted IFN γ and GM-CSF with lesser amounts of granzyme B and IL-4 (Supplemental Figure 2C). It is possible that a less robust antigen specific activation of the clones would have elicited a more focused cytokine response. To assess whether the cit-Agg epitopes that we identified are naturally processed and presented, we utilized co-culture experiments with aggrecan-specific clones with human FLS cells. FLS lines isolated from DR0401+ and DR0401- donors had detectable levels of aggrecan mRNA (albeit lower than versican), expressed podoplanin, and (as expected) lacked CD3e expression (Figure 2B). Each clone showed increased proliferation when co-cultured with DR0401+ FLS cells alone or pulsed with peptide or with cognate peptide presented by irradiated DR0401+ feeder cells and this proliferation was blocked by an anti-HLA-DR antibody (Figure 2C and 2D). Clones with all six specificities (cit-Agg₁₆₁, cit-Agg₂₀₀, cit-Agg₂₂₅, cit-Agg₅₂₀, cit-Agg₅₅₃, and cit-Agg₂₀₀) had significantly higher levels of proliferation in response to DR0401+ FLS cells as compared with DR0401- FLS cells (Figure 2D). However, a negative control T cell clone that recognized an influenza epitope did not proliferate in response to FLS cells. These observations demonstrate that aggrecan is produced by FLS cells and that epitopes corresponding to cit-Agg peptides can be processed and presented by FLS cells.

T cells that recognize the dominant cit-aggrecan epitopes are more frequent in the peripheral blood of RA patients.

To investigate the frequency of CD4+ T cells specific for citrullinated aggrecan epitopes in the peripheral blood of RA patients, *ex vivo* tetramer staining was performed. As an initial screen, we performed a grouped analysis, examining the combined T cell frequencies for groups of tetramers: cit-Agg₂₂₅ and cit-Agg₅₅₃ (both of which preferentially bound in their citrullinated form); cit-Agg₁₆₁, cit-Agg₂₀₀, cit-Agg₅₂₀ and cit-Agg₆₂₁ (which bound in both their citrullinated and unmodified forms), and cit-Agg₈₄ (which bound with very low affinity to DR0401). Previously published work (22) and our preliminary assays (Supplemental Figure 3) demonstrate that this tetramer staining approach is reliable. The observed frequencies demonstrated a hierarchy in which the cit-Agg₂₂₅ and cit-Agg₅₅₃ epitopes stood out as the dominant specificities. T cells specific for the other aggrecan epitopes were

present, but at lower frequencies that in some cases was near the threshold of detection (Figure 3A). Additional *ex vivo* analysis of 12 *HLA-DRB1*04:01* positive RA subjects showed that cit-Agg₅₅₃-specific T cells were significantly higher in frequency than cit-Agg₂₂₅-specific cells suggesting that the cit-Agg₅₅₃ epitope is the most prominent specificity (Figure 3B).

Previous studies have reported immunogenic aggrecan peptides and demonstrated responses to citrullinated aggrecan in samples from patients with RA (9, 10, 23). To corroborate the relevance of citrullinated aggrecan epitope-specific T cells, we compared the frequency of T cells specific for cit-Agg₂₂₅ and cit-Agg₅₅₃ in RA patients and healthy controls with *DRB1*04:01* haplotypes. We observed that patients had significantly higher T cell frequencies than controls (Figure 3C). These differences did not merely reflect global differences between patients and controls, as no difference was observed for T cells specific for the immunodominant influenza epitope.

The dominant citrullinated aggrecan epitopes have similar sequences but are recognized by distinct T cell clones.

The cit-Agg₂₂₅ and cit-Agg₅₅₃ peptides are derived from the G1 and G2 domains respectively, but have homologous sequences, raising the possibility that these peptides could be cross-recognized by overlapping sets of T cells. Based on binding assays and proliferation assays with variants of these two sequences, we identified truncated peptides that met minimum requirements for binding to DR0401 and recognition by cit-Agg₂₂₅ or cit-Agg₅₅₃ specific T cell clones. These results (Supplemental Table 2) were consistent with the predicted minimal motifs: YGI(Cit)DTNET for cit-Agg₂₂₅ and YGV(Cit)PSTET for cit-Agg₅₅₃. Based on these results, both peptides bind to DR0401 in similar registers. These registers predict that a citrulline residue would be positioned within binding pocket 4. This supposition is further supported by the fact that citrullination of the corresponding arginine residue is required for binding. Despite sharing 10 of 14 residues within their minimal peptides (Supplemental Figure 4A), the cit-Agg₂₂₅ and cit-Agg₅₅₃ peptides were not cross-recognized by T cell clones (Supplemental Figure 4B). Furthermore, *ex vivo* tetramer staining showed no significant signs of cross-reactivity (Supplemental Figure 4C). Therefore, these sequences appear to represent distinct epitopes, in spite of their considerable homology.

Citrullinated aggrecan-specific T cell frequency correlates with aggrecan-specific antibodies.

A recent study demonstrated that, although ACPA+ RA patients had increased levels of antibodies against a citrullinated aggrecan epitope, there was no clear correlation between antibodies and T cell reactivity with the same epitope in these patients (9). Having identified two dominant cit-Agg T cell epitopes, we were curious to explore correlations between T cell and antibody responses against aggrecan. Therefore, we measured citrullinated aggrecan antibody levels in the serum and the frequency of T cells that recognize cit-Agg₂₂₅ and cit-Agg₅₅₃ in matched samples. IgM-depleted serum samples (Supplemental Figure 5A) from 18 healthy control and 21 ACPA+ RA subjects were screened for the presence of IgG antibodies against native and citrullinated G1 domain of aggrecan (an assay for G2 domain

antibodies has yet to be developed). Based on either background adjusted OD (Figure 4A) or raw OD (Supplemental Figure 5B) RA patients had significantly higher levels of antibodies against both the native and citrullinated G1 domain of aggrecan than HLA matched controls. Levels of antibody recognition of citrullinated and native aggrecan were not different. Notably, we observed a positive correlation between the frequency of aggrecan-specific memory T cells and antibody levels in subjects with RA (Figure 4B). This correlation was absent in controls, due at least in part to their decreased levels of antibody and lower T cell frequencies.

DISCUSSION

Previous studies have indicated that T cell and antibody responses against citrullinated aggrecan contribute to the loss of peripheral tolerance in patients with RA. Of note, Boots et al. assessed the HLA binding of native (non-citrullinated) aggrecan peptides to DR0401 (and also DRB1*04:04 and DRB1*01:01) and identified multiple aggrecan peptides as being immunogenic in the context of DR0401 (20). That study, which focused on unmodified (rather than citrullinated) peptides, observed proliferative responses that were lower in RA patients than in controls. Buzas et al. identified the Agg₈₄₋₁₀₃ peptide as an immunodominant epitope recognized by BALB/C mice immunized with human aggrecan, and observed that some aggrecan peptides were “conditionally immunogenic” in that responses were only elicited in arthritic animals (15). Subsequently, Law et al. examined responses to citrullinated collagen₁₂₃₇₋₁₂₄₉, vimentin₆₆₋₇₈, aggrecan₈₄₋₁₀₃ and fibrinogen₇₉₋₉₁ in RA patients and in a limited number of controls, observing that the citrullinated aggrecan peptide was the most immunogenic, eliciting IL-6 and TNF- α secretion that was not observed in response to the corresponding unmodified peptide (21). This result appears to imply that functional responses to aggrecan are citrulline selective. However, the T cell responses observed in the study by Law et al. were not exclusively restricted to DR0401; 10 of the 21 RA subjects sampled were not DR0401 positive and among those 4 were DR4 negative.

Our present study provides new evidence that CD4⁺ T cells selectively recognize citrullinated aggrecan epitopes in the context of the high risk *DRB1*04:01* haplotype. We identified six aggrecan peptides that are recognized by T cell clones from subjects with RA when crucial arginine residues are converted to citrulline. Citrullinated residues could increase recognition by enhancing HLA binding or alter peptide recognition by modulating TCR interactions (depending on the positioning of the residue within its HLA binding motif). Indeed, two of the aggrecan epitopes identified through our study (cit-Agg₂₂₅ and cit-Agg₅₅₃) bound to DR0401 and elicited T cell responses only in their citrullinated form. The remaining aggrecan epitopes (cit-Agg₁₆₁, cit-Agg₂₀₀, cit-Agg₅₂₀, and cit-Agg₆₂₁) bound comparably in their citrullinated and unmodified forms and were citrullinated at predicted TCR contact residues. Among these Agg₂₀₀ and Agg₆₂₁ were able to elicit T cell responses in a limited number of subjects. Since cit-Agg₂₂₅ and cit-Agg₅₅₃ specific T cells were present at significantly higher frequencies in RA patients than other cit-Agg specificities, it could be suggested that the poor HLA binding of the unmodified Agg₂₂₅ and Agg₅₅₃ peptides may lead to impaired T cell tolerance to the corresponding cit-epitopes. In spite of sharing considerable sequence homology, the cit-Agg₂₂₅ and cit-Agg₅₅₃ peptides originate

from different domains of aggrecan and T cells specific for these peptides showed no evidence of cross-reactivity.

Cit-Agg-specific T cells were present at elevated frequencies in RA patients in comparison with HLA-DR matched healthy controls. The mere presence of CD4+ T cells that recognize citrullinated aggrecan does not guarantee that these cells play a role in disease. However, T cell clones that recognize citrullinated aggrecan epitopes exhibited a Th1-like phenotype that is consistent with the functional phenotype that we previously observed for more established citrullinated antigens such as vimentin, fibrinogen, and enolase (8). In addition, T cell clones specific for all six of the citrullinated aggrecan peptides proliferated in response to DR0401+ FLS cells, suggesting that these epitopes can be naturally processed and presented to T cells by synovial cells. Interestingly, these same clones failed to proliferate in response to the corresponding arg-Agg peptides (Figure 2A), suggesting that aggrecan is citrullinated by FLS.

A recent study documented the presence of citrullinated aggrecan in human cartilage extracts and established an ELISA assay to detect unmodified or citrullinated aggrecan G1 domain-specific serum autoantibodies (10). Applying this methodology to analyze serum autoantibodies in patients and controls from our cohort, aggrecan G1 domain specific antibodies were present at significantly higher levels in RA patients than in HLA matched controls, but cit-Agg- and arg-Agg were recognized at similar levels, suggesting either that G1 domain specific antibodies cross-recognize native and citrullinated aggrecan or that there are roughly equivalent levels of antibodies that uniquely recognize native and citrullinated aggrecan respectively. We observed a significant positive correlation between cit-Agg-specific memory T cell frequency and antibody levels in RA patients (but not controls). This correlation raises the possibility that antigen specific T cell help may contribute to antibody responses. Cumulatively, our results demonstrate that T cell responses to cit-Agg are associated with RA. Such responses are of interest because aggrecan is an extracellular matrix proteoglycan that interacts with cells and extracellular molecules through its globular domains. Given that aggrecan fragments are present within synovial fluid, this protein is apparently subject to fragmentation and release in inflamed joints, where it can be presented to T cells as a citrullinated antigen by either FLS or professional APCs.

Our study does have limitations. Although we were able to demonstrate differences in the frequency of cit-Agg specific CD4+ T cells in RA patients and HLA matched controls, the characteristics of the RA patients selected for our study did not allow us to effectively examine variations with respect to disease activity or severity. In general, our patients had well controlled disease, which may explain in part the overlap in aggrecan-specific T cell frequencies observed between controls and some patients. In addition, although it would be of considerable interest to document the presence of cit-Agg specific T cells within inflamed joints, synovial fluid and tissue samples from HLA typed subjects were not available for our study. However our *ex vivo* tetramer staining approach should be applicable to address these questions in future studies that utilize such samples.

Although previous studies have documented T cell responses to citrullinated aggrecan, our study represents the first HLA-controlled study to define disease relevant citrullinated

aggrecan epitopes and to characterize T cells that recognize these peptides in subjects with established RA. To our knowledge, this is also the first study to demonstrate a direct correlation between T cell frequency and antibody responses against the same joint-associated antigen. Together, these results suggest that cit-Agg specific CD4⁺ T cells could play a role in the etiology of RA and that future studies of the frequency, phenotype, and role of cit-Agg-specific T cells in various stages of disease are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Assessing the immunogenicity of aggrecan peptides.

Peptides with positive binding to DR0401 were evaluated for immunogenicity by tetramer staining after 14 days of peptide stimulation. As demonstrated by the representative FACS plots, peptides elicited diverse levels of T cell expansion (A) that ranged from negligible expansion (upper left), modest expansion (upper middle), moderate expansion (upper right and lower right), to robust expansion (lower left); expansion in response to an immunodominant influenza peptide was used as reference (lower middle). (B) The candidate arg-agg epitopes exhibited relative low immunogenicity, with median responses that were less than 0.05% of total CD4+ T cells. (C) Several candidate cit-agg epitopes were immunogenic, exhibiting median responses that were above 0.05% of total CD4+ T cells. (D) The six most promising cit-agg epitopes elicited *in vitro* responses in some HLA matched controls. (E) The percentage of subjects with responses above the 0.05% cutoff was always higher for RA patients. Differences between RA patients and controls were significant for cit-161 and cit-520 (p-values 0.028 and 0.021 respectively) and approached significance for cit-553 and cit-621 (p-values 0.08 and 0.01 respectively, Fisher Exact test). Dotted lines in panels B-D indicate a threshold value of 0.05% used to define positive responses.

Figure 2. AggreCAN-specific T-cell clones respond when co-cultured with fibroblast-like-synoviocytes (FLS).

(A) As a specificity control, each clone was stimulated with cit-agg or arg-agg peptide in the presence of HLA-DR0401+ APCs. (B) AggreCAN expression by DR0401 and Non-DR4 FLS lines was assessed by qPCR with GAPDH (reference), podoplanin (PDPN; positive control), CD3e (negative control), and versican (related proteoglycan). Ct is inversely correlated with mRNA levels and values over 35 (dotted line) were considered undetectable. (C) Representative raw proliferation results for an aggreCAN specific T cell clone co-cultured with an FLS line from a DR0401+ RA subject, a DR0401- subject, and each of these cell types individually, or the same T cell clone co-cultured with irradiated DR0401+ PBMC. Antigen presentation via HLA-DR0401 was required to elicit proliferation above background. (D) Stimulation indices for T cell clones co-cultured with DR0401- FLS (white bars), DR0401+ FLS (shaded bars), or DR0401+ FLS plus peptide (solid gray bars). Clones specific for cit-Agg 161, 200, 225, 520, 553, and 621 showed higher expansion when co-cultured with DR0401+ FLS as compared to DR0401- FLS (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$). In contrast, the influenza (MP54) specific clone could not be activated by DR0401+ FLS cells in the absence of peptide.

Figure 3. T cells that recognize dominant aggrecan epitopes are more prevalent in RA patients than in healthy subjects.

(A) The ex-vivo frequencies of T cells specific for cit-Agg₂₂₅ + cit-Agg₅₅₃ were significantly higher in RA patients (n=14 DRB1*04:01 subjects) than cit-Agg₁₆₁ + cit-Agg₂₀₀ + cit-Agg₅₂₀ + cit-Agg₆₂₀ or cit-Agg₈₄ (P=0.0002; two tailed Mann Whitney test). An influenza tetramer (MP54) was used as a positive control. (B) In a separate comparison, the frequencies of T cells specific for cit-Agg₅₅₃ were significantly higher in RA patients (n=12 DRB1*04:01 subjects) than cit-Agg₂₂₅ (p=0.0037; two tailed Mann Whitney test). (C) Ex-vivo frequencies of cit-Agg₂₂₅ and cit-Agg₅₅₃ specific T cells were significantly higher in RA patients (open circles, n=16 DRB1*04:01 subjects) than in healthy subjects (filled circles, n=15 DRB1*04:01 subjects) (p=0.0395; two-tailed Mann-Whitney test).

Figure 4. Aggrecan-specific antibodies are elevated in patients and positively correlate with T cell frequency.

(A) Based on the background adjusted delta OD, the levels of antibodies against the native or citrullinated aggrecan-G1 domain in IgM-depleted serum were significantly higher in RA patients (open circles, n=21 subjects) than in healthy subjects (filled circles, n=18 subjects) (* indicates $p < 0.05$; two tailed Mann-Whitney test). (B) Serum levels of aggrecan-G1 domain antibodies and combined frequencies of memory positive cit-Agg 225 + cit-Agg553 specific T cells showed a significant correlation ($r^2 = 0.7982$, $p = 0.0128$) in RA subjects (N=10) when compared to healthy subjects ($r^2 = 0.01008$, $p = 0.5598$, N=8). Correlation by two-tailed Pearson r, memory cells defined as CD45RA negative.

Table 1.

Binding characteristics of aggrecan peptides chosen for further study

Peptide [*]	Sequence	Cit (μM)	Arg (μM)
agg 3-20	TLLWVFVTL(Cit)VITAAVTV	35.30	36.06
agg 153-168	IVFHY(Cit)AIST(Cit)YTLDF	0.98	50.96
agg 161-178	ST(Cit)YTLDFD(Cit)AQ(Cit)ACLQ	0.99	1.02
agg 200-215	DAGWLADQTV(Cit)YPIHT	2.95	2.53
agg 225-244	DEFPGV(Cit)TYGI(Cit)DTNETYDV	2.18	>100
agg 298-313	SAGWLAD(Cit)SV(Cit)YPIISK	1.90	2.94
agg 477	GVVFHY(Cit)PGPT(Cit)YSLTF	14.69	>100
agg 520	GYEQCDAGWL(Cit)DQTV(Cit)YPIV	12.62	15.61
agg 553	PGV(Cit)TYGV(Cit)PSTETYDVY	7.96	>100
agg 568	DVYCFVD(Cit)LEGEVFFA	23.42	3.06
agg 579	EVFFAT(Cit)LEQFTFQE	78.43	15.33
agg 621	KCYAGWLADGSL(Cit)YPIV	6.90	4.66
agg 684	NSPFCLE(Cit)TPLGSPDPA	0.12	0.13

* Sequences that bind better as citrullinated peptides are shown in boldface

Table 2.

Predicted motifs for immunogenic aggrecan peptides

Peptide	Sequence*
agg 161-178	ST(CIT) <u>Y</u> TLDFD(Cit) <u>AQ</u> (Cit)ACLQ
agg 200-215	DAG <u>WL</u> ADQTV(Cit)YPIHT
agg 225-244	DEFPGV(Cit) <u>TYGI</u> (Cit) <u>DTNET</u> YDV
agg 520	G <u>YEQCDAGWL</u> (Cit)DQTV(Cit)YPIV
agg 553	PGV(CIT) <u>TYGV</u> (Cit) <u>PSTET</u> YDVY
agg 621	KCYAG <u>WLADGSL</u> (Cit)YPIV

* Predicted DR0401 binding motifs are underlined with the first anchor shown in boldface