Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice

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Rheumatoid arthritis (RA) is a chronic autoimmune disease that afflicts the synovium of diarthrodial joints. The pathogenic mechanisms inciting this disease are not fully characterized, but may involve the loss of tolerance to posttranslationally modified (citrullinated) antigens. We have demonstrated that this modification leads to a selective increase in antigenic peptide affinity for major histocompatibility complex (MHC) class II molecules that carry the RA-associated shared epitope, such as HLA-DRB1*0401 (DR4). We describe the induction of arthritis in DR4-IE transgenic (tg) mice with citrullinated fibrinogen, a protein commonly found in inflamed synovial tissue and a frequent target of autoantibodies in RA patients. The disease induced in these mice was characterized by synovial hyperplasia followed by ankylosis, but lacked a conspicuous polymorphonuclear cell infiltrate. Immunological analysis of these mice through T cell epitope scanning and antibody microarray analysis identified a unique profile of citrulline-specific reactivity that was not found in DR4-IE tg mice immunized with unmodified fibrinogen or in wild-type C57BL/6 mice immunized with citrullinated fibrinogen, two conditions where arthritis was not observed. These observations directly implicate citrullinated fibrinogen as arthritogenic in the context of RA-associated MHC class II molecules.

Rheumatoid arthritis (RA) is a chronic disease affecting the peripheral joints in which abnormalities in the synovium precipitate a destructive process that often leads to cartilage and bone erosion. The autoimmune nature of this disease has been defined, in part, through the presence of IgG autoantibodies such as rheumatoid factor and a tight genetic association with MHC class II molecules that contain a motif known as the shared epitope (1, 2). This SE forms one of the major MHC class II anchoring pockets (known as P4) and imparts the ability to preferentially interact with certain amino acid side chains from antigenic peptides for subsequent presentation to CD4 T cells (3). Because of these properties, the adaptive arm of the immune system has been implicated in driving disease pathogenesis through autoantigen recognition.

Although many candidate autoantigens have been investigated in RA, a frequent target of the immune response found predominantly in this patient population has been lacking until recently. The discovery of serum IgG autoantibodies from RA patients that bind posttranslationally modified arginine (citrulline) within the context of certain proteins/peptides has provided an excellent diagnostic tool due in large part to their disease specificity (4–7). The propensity to develop anti-citrulline antibodies is also associated with the expression of the SE, suggesting that an MHC class II–restricted
mechanism may initiate this immune response (8–10). We have shown that the conversion of arginine to citrulline at the peptide side chain position that interacts with the P4 pocket formed by the SE leads to a profound increase in MHC–peptide affinity and to the subsequent activation of CD4 T cells (11). This phenomenon is caused by the different charge interactions made between the MHC class II P4 pocket (positively charged because of arginine or lysine at position 71 of the β chain) and either peptide-bound arginine (positively charged because of the terminal amino group) or citrulline (polar and uncharged because of the terminal carbonyl group), where the latter interaction is preferred. These observations suggest that MHC class II–restricted CD4 T cells may propagate the autoimmune response to citrullinated self-antigens found in RA patients.

Although the substrate of anti-citrulline antibodies was initially identified as citrullinated filaggrin (a protein that is found in the cornified layer of the skin, but not the joint), further investigation determined that citrullinated fibrinogen is a synovial–derived target (12). Because the expression of peptidylarginine deiminase, the enzyme responsible for converting protein–bound arginine to citrulline, has been found to colocalize with fibrin deposits and other intracellular citrullinated proteins (possibly vimentin) within RA synovial tissue (13–15), it is likely that these autoantigens can be generated in the rheumatoid lesion. This, in addition to fact that autoantibodies that bind citrullinated fibrinogen are frequently and specifically found in RA patients, implicate this autoantigen in disease etiology (16–18).

We provide evidence that citrullinated fibrinogen is arthritogenic in mice made tg for the RA-associated MHC class II molecule DRB1*0401 (DR4–IE tg mice). Immunization of DR4–IE tg mice with citrullinated, but not unmodified, human fibrinogen (hFib) induced a progressive arthritic condition characterized by synovial fibroblast–like cell hyperplasia and the transient appearance of citrullinated proteins in the joints, but lacked significant inflammatory cell infiltration. Notably, wild-type C57BL/6 (B6) mice expressing murine H–2b were not susceptible to this disease, potentially owing to the fact that distinct differences in the immune response were found to be mediated by the HLA transgene. Although these results implicate citrullinated fibrinogen as an arthritogenic antigen in the context of the RA-associated MHC class II molecule DRB1*0401, they also suggest that this HLA–restricted immune response may provoke arthritis in the absence of a robust and persistent polymorphonuclear cell infiltrate.

RESULTS

Induction of arthritis in DR4–IE tg mice

To determine the MHC class II–restricted arthritogenicity of citrullinated antigens, we chose to explore the immune response to citrullinated fibrinogen, a protein that is found in the diseased synovium of RA patients and is a target of autoantibodies in this disease (12, 14–18). DR4–IE tg mice were immunized with either citrullinated hFib (CithFib) or unmodified hFib and assessed for clinical signs of arthritis. Although DR4–IE tg mice immunized with hFib did not develop arthritis during an extended observation period (200 d), immunization with CithFib induced arthritis in ~35% of tg mice. Joint swelling typically ensued 10 wk after primary immunization (Fig. 1 and Fig. 2 A) and was almost completely restricted to the ankles (one arthritic mouse also developed dactylitis in one forepaw). The disease had a progressive and persistent course, beginning with mild swelling followed by severe erythema, eventually leading to ankylosis. Clinically evident swelling lasted for up to 6 wk, and restricted plantarflexion persisted in arthritic mice that were observed for an extended period (200 d).

To determine whether this arthritis was restricted by expression of the human DR4 transgene, wild-type C57BL/6 (B6) mice were also immunized with these antigens and observed for arthritis. Clinical signs of disease did not develop in B6 mice after immunization with these proteins. Arthritis did not develop in either strain after immunization with

![Figure 1. Clinical and histological evaluation of arthritis induced by CithFib in DR4–IE tg mice.](image)
citrullinated KLH, citrullinated mFib (CitmFib), or unmodified mFib (Table I). The development of arthritis was therefore dependent on the posttranslational modification (citrullination) of hFib and the expression of the RA-associated DRB1*0401 MHC class II molecule.

Histological and immunohistochemical assessment of joint pathology

Histological analysis of tissues from arthritic mice revealed prominent synovial hyperplasia, with some pannus formation at bone and cartilage interfaces (Fig. 1 and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1). Surprisingly, although synovial sublining inflammatory cells were evident in arthritic DR4-IE tg mice, their presence was less conspicuous compared with other disease models, such as streptococcal cell wall–induced arthritis (unpublished data) (19) or the arthritis seen in K/BxN tg mice (20). To accurately quantitate the degree and type of infiltrate in the synovial tissue, we compared arthritic DR4-IE tg mice with the well-characterized K/BxN model. Contrasting cellular content by high-power view quantification revealed a stark difference in the degree of polymorphonuclear cell infiltration that dominated the infiltrate in K/BxN mice, whereas arthritic DR4-IE tg mice showed a small but significant increase in lymphocytes (Fig. 2 B).

Histological abnormalities persisted in the ankle joints of arthritic DR4-IE tg mice assessed at later stages of disease (>150 d after primary immunization; Fig. 1 F) and showed fibrotic synovial tissue in joint spaces, which is consistent with the clinical appearance of ankylosis. Other histological abnormalities were rarely seen outside of the ankle joint of arthritic mice, as the spine and major organs appeared normal (unpublished data).

We next addressed whether citrullinated protein was evident in the joints of arthritic mice by immunohistochemistry using an anti-modified citrulline (AMC) antibody (Fig. 3, A–C). The most intense staining was found intracellularly in synovial fibroblast-like cells from hyperplastic tissue of mice with arthritis of recent onset. Some chondrocytes in the superficial zone of articular surfaces were also found to stain with AMC; however, no citrullinated protein was identified at sites of cartilage or bone erosion or in ankylosed joints (unpublished data). Detection of fibrin in serial sections (Fig. 3, D–F) showed mainly perivascular deposition in synovial tissues, but minimal colocalization with citrullinated protein, suggesting that other intracellular citrullinated proteins (possibly citrullinated vimentin) could be present in the joints of these arthritic mice.

Table I. Summary of immunizations and incidence of arthritis in DR4-IE tg and B6 mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Immunizing antigen</th>
<th>Mice immunized</th>
<th>Arthritic mice %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR4-IE tg</td>
<td>CithFib</td>
<td>135</td>
<td>48 (35.5)</td>
</tr>
<tr>
<td>DR4-IE tg</td>
<td>hFib</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>DR4-IE tg</td>
<td>CitmFib</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>DR4-IE tg</td>
<td>mFib</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>DR4-IE tg</td>
<td>CitKLH</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>B6</td>
<td>CithFib</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>B6</td>
<td>hFib</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>B6</td>
<td>CitmFib</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>B6</td>
<td>mFib</td>
<td>8</td>
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Immunizations were conducted as outlined in Materials and methods. All mice were observed for at least 70 d after primary immunization, with the maximum observation period for some groups being 200 d.
to murine H-2b, the HLA-DRB1 transgene can generate a strong citrulline-specific T cell response characterized by the production of high levels of IFN-γ and IL-10 and mediates cross-reactivity to CitmFib.

To understand the pathogenic potential of this citrulline-specific T cell response in DR4-IE tg mice, we established a cell transfer system. Here, we immunized and boosted DR4-IE tg mice with CithFib, harvested splenocytes at day 31, and activated them in vitro with CithFib for an additional 4 d. At the end of this culture, viable cells were isolated (of which >85% were CD4+ by FACS) and transferred IP to DR4-IE tg recipients that received an intraarticular injection of CithFib, hFib, CitmFib, mFib, or BSA (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1).

In this transfer system, only recipients that received an intraarticular injection of CithFib developed arthritis, suggesting that CithFib-specific T cells can drive disease, but only when the cognate antigen is found within the joint.

Identification of DR4-restricted T cell epitopes

Using a predictive model for identifying DR4-binding epitopes (23), we selected all peptides within the α, β, and γ chain of hFib that would contain the required P1 anchor (aliphatic or aromatic amino acid), an arginine or citrulline at the P4 SE position, and a noninhibitory amino acid at the P6 anchor. This resulted in the identification of 10 peptides, 7 originating from the α chain, 2 from the β chain, and 1 from the γ chain of fibrinogen. To monitor the immune response to heterologous regions of hFib, we also synthesized a DR4-binding peptide (Fib371-383) that is found exclusively in the α chain of hFib and lacks arginine (Fig. 5 C). These peptides were used to monitor T cell recall responses in the two strains of mice at day 70 after immunization with CithFib or hFib.

Figure 3. Immunohistochemical localization of citrullinated proteins and fibrinogen deposition in arthritic DR4-IE tg mice. Citrullinated protein was identified in arthritic mice 70 d after immunization with CithFib by staining with AMC antibodies in areas of synovial hyperplasia (A), with the most intense staining identifying intracellular proteins in synovial fibroblast cells (B). (C) Nonarthritic mice immunized with hFib did not stain positive for AMC in these regions. Although fibrin deposition was evident in perivascular regions of synovial tissue in arthritic mice (D), colocalization with citrullinated protein was minimal and virtually absent in areas of intense AMC staining (E). (F) No fibrin deposition was detected in joints of nonarthritic mice immunized with hFib. Control staining without the primary antibody for each section is shown in the insets. Bar, 100 μm.

Assessment of T cell responses

Splenic T cell responses to various forms of fibrinogen were assessed by proliferation and cytokine production in both DR4-IE tg and B6 mice 70 d after primary immunization. Antigen-specific proliferation in response to CithFib in DR4-IE tg mice immunized with this antigen was prominent and consistently higher than the response to hFib in individual mice (Fig. 4 A). B6 mice also showed citrulline-specific reactivity in this context; however, proliferation was approximately twofold lower than that seen in DR4-IE tg mice. Recall proliferative responses were detected in both strains after immunization with hFib, but in this circumstance, augmented reactivity to CithFib was not detected.

Clear differences in cytokine production were also evident, and parallelled responses identified by proliferation. After immunization with CithFib, DR4-IE tg mice produced high levels of IFN-γ after in vitro challenge with CithFib; again, much of this response appeared to be citrulline specific (Fig. 4 B). These levels of IFN-γ production were also increased ~10-fold compared with B6 mice immunized with the same antigen or with either strain after immunization with hFib (Fig. 4 C). A similar trend in cytokine production was also observed for IL-10 (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1), suggesting that a highly polarized Th1 response occurred (21, 22). IL-4 production was not detectable in these cultures (unpublished data).

We also tested for T cell reactivity to the endogenous antigens CitmFib and mFib and found that only DR4-IE tg mice that were immunized with CithFib produced an immune response to CitmFib (Fig. 4, A, D, and E). Of particular note, although T cell reactivity to unmodified hFib was frequently detected in DR4-IE tg mice, no responses to unmodified mFib occurred. These results show that in contrast to murine H-2b, the HLA-DRB1 transgene can generate a strong citrulline-specific T cell response characterized by the production of high levels of IFN-γ and IL-10 and mediates cross-reactivity to CitmFib.
Splenocytes were cultured in the presence of 50 μg/ml of CithFib or hFib (day 70). Induction in DR4-IE tg and B6 mice immunized with CithFib or hFib (day 70). * indicates statistically significant difference in cytokine production between DR4-IE tg and B6 mice. P < 0.05, paired Student’s t test.

Assessment of antibody responses
To determine if anti-citrulline antibodies were produced in the two strains of mice, we tested for serum IgG reactivity to citrullinated and unmodified forms of mFib by ELISA. These antigens were chosen because initial studies using hFib immunized mice with each peptide and characterizing the recall proliferative response to these antigens. Both Fibα371-383 and FibαR84Cit induced T cell proliferation after in vitro challenge with the cognate peptide, whereas Fibα79-91 did not (Fig. 5 B). Although T cell reactivity to Fibα371-383 and FibαR84Cit was evident in DR4-IE tg mice after peptide immunization, neither peptide induced arthritis (observed until day 70). No response was seen in B6 mice. These data clearly confirm the DR4-restricted T cell response to heterologous regions of hFib and identify a citrulline-specific epitope restricted by this human MHC class II molecule.

IgG profiling using synovial proteome microarray
To gain further insight into IgG specificity, we assessed serum reactivity to hundreds of unique protein and peptide antigens implicated in RA pathogenesis using antibody microarray. These arrays contained overlapping peptides spanning the full length of the α and β chains of fibrinogen in both citrullinated

As expected, HLA-DRB1*0401–restricted T cell reactivity to Fibα371-383 (measured by proliferation and IFN-γ production) was evident in DR4-IE tg mice after immunization with either CithFib or hFib, whereas no recall response to this peptide was seen in B6 mice (Fig. 5 A). Of the 10 fibrinogen peptides containing citrulline at the P4 position, only one peptide from the α chain (FibαR84Cit) consistently induced strong IFN-γ production in DR4-IE tg mice immunized with CithFib, whereas no response was seen against the corresponding arginine-containing peptide Fibα79-91 (Fig. 5 A and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1). Notably, this particular sequence is identical in both the human and mFib α chain (Fig. 5 C). T cell reactivity to FibαR84Cit was absent in DR4-IE tg mice immunized with hFib and in B6 mice immunized with either antigen. The HLA-restricted response to the α chain peptide in CithFib immunized DR4-IE tg mice was further confirmed by incubating splenocytes in the presence of anti-DR antibody and FibαR84Cit, which completely inhibited proliferation (Fig. S4).

The immunogenicity of the peptides Fibα371-383, FibαR84Cit, and Fibα79-91 was also assessed by immunizing DR4-IE tg mice with each peptide and characterizing the recall proliferative response to these antigens. Both Fibα371-383 and FibαR84Cit induced T cell proliferation after in vitro challenge with the cognate peptide, whereas Fibα79-91 did not (Fig. 5 B). Although T cell reactivity to Fibα371-383 and FibαR84Cit was evident in DR4-IE tg mice after peptide immunization, neither peptide induced arthritis (observed until day 70). No response was seen in B6 mice. These data clearly confirm the DR4-restricted T cell response to heterologous regions of hFib and identify a citrulline-specific epitope restricted by this human MHC class II molecule.
and unmodified forms, in addition to peptides from other candidate citrullinated autoantigens, notably vimentin. Serum samples from DR4-IE tg and B6 mice immunized with CithFib or hFib (day 70) were used to identify patterns of epitope recognition within and between strains.

We initially looked for differential reactivity to fibrinogen-derived antigens contained on the array and found that the HLA transgene, indeed, skewed the antibody response to multiple epitopes (Fig. 6 C and Table S1, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1). This is highlighted by the fact that both strains responded to unique clusters of peptides after immunization with either Fib/H9251 371-383 (left), Fib/H9251 R84Cit (right), or Fib/H9251 79-91 (closed circle; Fib 79-91, open circle; Fib 371-383, inverted closed triangle). Data represent the mean response ± the SEM of six mice per immunizing antigen. Amino acids differing between mouse Fib 371–383 are indicated in red. P4 amino acid from Fib/H9251 R84Cit and Fib/H9251 79–81 predicted to be positioned at the SE are indicated in green and red, respectively, with the corresponding sequence from the α chain of mFib.

We next addressed IgG reactivity to vimentin in these sera because autoantibodies to this citrullinated antigen (also known as the Sa antigen) are a specific marker of disease in RA patients (8, 15, 24). Differential responses were noted for multiple citrullinated vimentin peptides, with increased antibody reactivity clearly biased in favor of DR4-IE tg mice after immunization with CithFib (Fig. 5 D and Table S3, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1). Vimentin-derived targets that were significantly different between DR4-IE tg and B6 mice immunized with CithFib were vim286–305cit and vim256–275cit. Additional analysis through peptide-based ELISA assay with vim256–275cit confirmed the specific HLA-restricted antibody reactivity seen by microarray (unpublished data).

Finally, pairwise comparison between DR4-IE tg and B6 mice immunized with CithFib was conducted to identify all significant differences in antibody reactivity. Several epitopes outside of vimentin and fibrinogen were highlighted (Fig. 6 E). Of the epitopes specifically targeted in DR4-IE tg mice, >50% were citrullinated, whereas the two targets of antibodies in B6 mice were derived from unmodified fibrinogen.

These results provide evidence that the HLA transgene mediates a distinct skewing in anti-citrulline antibody reactivity, and suggests that immunization with CithFib can perpetuate a wide range of IgG target recognition, which is indicative of broad cross-reactivity or epitope spreading.

Figure 5. T cell responses to fibrinogen derived peptides in DR4-IE tg and B6 mice. (A) Splenocytes from DR4-IE tg and B6 mice immunized with CithFib or hFib (day 70) were cultured in the presence of 50 μg/ml of either Fib 371–383 (left) or Fib R84Cit (right). Supernatants from these cultures were removed at 72 h and tested for the production of IFN-γ by ELISA. Individual (filled circles) and mean (open boxes) responses ± the SD are shown (n = 6 mice/strain/immunizing antigen). Labels above boxes indicate the antigen used for in vitro challenge, and labels within boxes indicate the immunizing antigen. * indicates significant difference in cytokine production between DR4-IE tg immunized with CithFib and hFib (P < 0.05, paired Student’s t test). (B) Confirmation of peptide immunogenicity by antigen-specific T cell proliferation. DR4-IE tg mice were immunized with either Fib 371–383 (left), Fib R84Cit (middle), or Fib 79–91 (right); 10 d later, draining lymph node cells were cultured with various concentrations of peptide (Fib R84Cit, closed circle; Fib 79–91, open circle; Fib 371–383, inverted closed triangle). Data represent the mean response ± the SEM of six mice per immunizing antigen.

(C) Peptides corresponding to human α chain sequences used in A and B for determining T cell recall responses. Amino acids differing between mouse Fib 371–383 are indicated in red. P4 amino acid from Fib R84Cit and Fib 79–81 predicted to be positioned at the SE are indicated in green and red, respectively, with the corresponding sequence from the α chain of mFib.
In total, there were 16 arginine residues from the α chain and 4 from the β chain of hFib that were not present in mouse. Many of these arginines were shown to be citrullinated in vitro by PAD2 or 4 (Table S4, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1, and unpublished data) (25). We next searched for citrulline-specific antibody reactivity to these regions (found in CithFib– but not hFib–immunized DR4-IE tg mice), and then stratified for antibody reactivity that was higher in DR4-IE tg versus B6 mice immunized with hFib. Because arthritis was found after immunization with citrullinated human, but not mouse, fibrinogen in DR4-IE tg mice, we further characterized aspects of the xenoreactive immune response that may be important in this process by using antibody microarray data in combination with mass spectrometry sequencing of citrullinated fibrinogen that was previously described (25). We first aligned the human and mFib sequences to identify heterologous regions where arginine substitutions occur.

Xenoreactive antibody responses are evident to CithFib

Because arthritis was found after immunization with citrullinated human, but not mouse, fibrinogen in DR4-IE tg mice, we further characterized aspects of the xenoreactive immune response that may be important in this process by using antibody microarray data in combination with mass spectrometry sequencing of citrullinated fibrinogen that was previously described (25). We first aligned the human and mFib sequences to identify heterologous regions where arginine substitutions occur.

In total, there were 16 arginine residues from the α chain and 4 from the β chain of hFib that were not present in mouse. Many of these arginines were shown to be citrullinated in vitro by PAD2 or 4 (Table S4, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1, and unpublished data) (25). We next searched for citrulline-specific antibody reactivity to these regions (found in CithFib– but not hFib–immunized DR4-IE tg mice), and then stratified for antibody reactivity that was higher in DR4-IE tg versus B6 mice immunized
with CithFib. From the antibody microarray data, and using the preceding selection criteria, we identified the α chain sequence 121-140Cit (Fig. 7 A) as a candidate target for a xenoreactive antibody response in DR4-IE tg mice (Fig. 7, B and C, and Table S4). To confirm the species specificity in regard to the immunizing antigen, we synthesized a region of this peptide to test serum from DR4-IE tg mice immunized with CithFib or mFib by ELISA. As predicted from the sequencing data, antibody reactivity to the Fibα123Cit peptide was only found in DR4-IE tg mice immunized with CithFib, but not with CitmFib (Fig. 7 D). These results identify a xenospecific citrullinated B cell epitope that is targeted by the antibody response in HLA-DR4-IE tg mice immunized with CithFib.

DISCUSSION
In this study, we show that citrullinated fibrinogen, an antigen that is frequently targeted by autoantibodies in RA patients, can induce arthritis in mice that carry the RA-associated MHC class II SE allele DRB1*0401. This disease was restricted by both the posttranslational modification of the antigen and the HLA transgene, as arthritis was not seen in mice administered unmodified fibrinogen or in wild-type (B6) mice administered either form of fibrinogen. Citrulline-specific T and B cell responses were prominent in DR4-IE tg mice, but they were not absolutely restricted by the SE, as antibody reactivity to some citrullinated antigens could be detected by ELISA and antibody microarray profiling in B6 mice. Further, although the arthritis in DR4-IE tg mice led to joint damage, and like RA, was accompanied by synovial cell hyperplasia, we did not find a robust and persistent polymorphonuclear cell infiltrate within joint tissues, suggesting that in this model, HLA-restricted responses to citrullinated fibrinogen can recapitulate some, but not all, aspects of the rheumatoid pathology.

Evidence that, in large part, the SE regulates the production of anti-citrulline antibodies in RA patients implies a causal relationship with disease pathogenesis mediated through CD4 T helper cell activation (26, 27). We have shown previously that the increased affinity of peptide-bound citrulline versus arginine for RA-associated MHC class II molecules correlates with CD4 T cell activation, but whether these peptides can be processed from citrullinated fibrinogen was not known. In this study, we addressed this question by assessing T cell recall responses in DR4-IE tg and B6 mice after immunization with CithFib. The presence of the HLA SE transgene afforded citrulline-specific T cell proliferation accompanied by IFN-γ production after in vitro challenge with CithFib and CitmFib. We also assessed peptide-specific recall responses to predicted DR4-binding epitopes and confirmed that a strong HLA SE-restricted T cell response was

with CithFib (n = 9), hFib (n = 5), CitmFib (n = 4), and mFib (n = 5) detected by ELISA. Mean OD of duplicates for individual serum are shown.
generated against both heterologous regions of hFib, and to
a region of the α chain that was identical between species.
The later peptide (Fibα R84Cit) contained citrulline at the
critical P4 SE position and T cell responses in DR4-IE tg
mice were restricted by this posttranslational modification.
Notably, this was the only peptide within fibrinogen that we
identified with this stimulatory property after conversion
from arginine to citrulline at P4. The significance of T cell
reactivity to this epitope is not known, but sequencing data
from in vitro citrullination shows that modification of argi-
nine at position 84 of the α chain is variable for hFib, whereas
the same amino acid is consistently converted to citrulline
after in vitro modification of mFib (unpublished data) (25).
Site-specific citrullination within antigens highlights another
level of variability that could impact the development of au-
toimmune responses. Moreover, whereas epitope specificity
of the PAD enzymes have been assessed in vitro, naturally
occurring citrullination sites within fibrinogen (or other anti-
gens) have not been determined in vivo, or in other disease
conditions besides RA.

In regard to HLA-restricted antibody responses, we found
that characteristics of RA serum reactivity toward citrul-
nated antigens were paralleled in these mice. First, as in
human patients (28), anti-citrulline antibodies were not
completely restricted by the MHC class II SE, but instead
levels were significantly higher in DR-4-IE tg mice. This was
evident by ELISA and antibody microarray profiling when
assessing reactivity to CitnFib. Second, antibody reactivity
to citrullinated antigens was broad, targeting sequences not
only within fibrinogen, but also antigens such as vimentin
(the target of anti-Sa antibodies in RA patients), fibromodu-
lin, cartilage oligomeric protein (COMP), vitronectin, bigly-
can, and clusterin. Some of these proteins remain to be
confirmed as targets of PAD in vivo, but all have been impli-
cated either in RA pathogenesis or synovial/cartilage ho-
meostasis. An unresolved issue with respect to anti-citrulline
antibody specificity and evolution in RA patients is whether
this broad reactivity is the result of epitope spreading or merely
cross-reactivity, which is a question that is not directly ad-
dressed in this study, but one that is currently being pursued.
If epitope spreading is the cause, it would suggest that many
of the proteins targeted by anti-citrulline antibodies are func-
tionally and/or physically associated. This may be true for
some of the citrullinated aforementioned antigens, as fibrino-
gen, vimentin, and vitronectin have been shown to associate
during platelet activation, whereas fibromodulin, COMP,
and biglycan are integral components of cartilage (29–31).
The polyreactive nature of this antibody response also con-
found the interpretation of the potential arthritogenicity of
autoantibodies solely directed against citrullinated fibrinogen
in this model and in RA.

It was interesting that several DR4-IE tg mice immu-
nized with CitFib responded uniquely to citrullinated pep-
tides from vimentin. The reactivity to Cit vimentin peptides
derived from the C-terminal region of vimentin downstream
of a caspase 6/8 cleavage site of the protein (e.g., vim 256–275cit)
was noteworthy. Autoantibodies to citrullinated vimentin or
Sa are strongly associated with disease severity and SE car-
riage in RA patients (8), and a role in disease pathogenesis has
been speculated for years. Vimentin’s role as an intracellular
intermediate filament protein has been extensively studied;
however, it is now evident that this protein is also present
extracellularly, and could therefore interact directly with au-
toantibodies. Extracellular vimentin has been detected on
platelets, macrophages, neutrophils, and T cells (30, 32–35).
It is not known if extracellular vimentin is citrullinated, but
vimentin is seen in situations of cellular activation and apop-
tosis, two conditions where intracellular calcium fluxes oc-
cur, a process necessary to activate peptidylarginine deiminase.

A distinct feature of the arthritis seen in DR4-IE tg mice
was pronounced synoviocyte dysregulation, but a relative
paucity of polymorphonuclear cells within the joints com-
pared with other mouse models of arthritis. The reason for
this is not known, but it is possible that a transient and self-
limiting inflammatory infiltrate (not captured at the time
points of our histological examination) could have provided
a stimulus for synovial hyperplasia. A discordant relationship
between anti-citrulline immune responses and synovial in-
flammatory cell infiltration has, however, been reported in
RA patients. Baeten et al. have shown that anti-citrulline an-
tibody titers do not correlate with histological parameters
of synovial inflammation, nor does local inflammation correlate
with SE carriage (14). Although not formally addressed in
the current study, autoantibodies in this model could perpetuate
disease by directly altering synovial fibroblast or chondrocyte
cellular homeostasis, as has been shown previously with IgG
(36, 37). Alternatively, a persistent and robust inflammatory
cell infiltrate in the joint could be facilitated by additional ge-
netic insults (possibly in PTPN22 or FcγRIII) that are inde-
pendent of the SE (38–41).

This is the first description of citrulline-dependent arthri-
tis in mice. Previous studies in mice have shown that the state
of citrullination can influence disease severity in collagen-
induced arthritis and that citrullination can break tolerance,
leading to the development of citrulline-specific antibodies
(42–44). Recent work has also shown that murine mono-
clonal antibodies specific for citrullinated fibrinogen can aug-
ment arthritis through passive transfer with anti-collagen II
antibodies (45). Our studies show that the influence of the
HLA transgene in citrulline-specific immune responses influ-
ences the magnitude and diversity of reactivity rather than
the mere presence or absence of a response. These factors
likely influence disease expression, as thresholds for T cell re-
activity and antibody titers, as well as IgG epitope specificity,
are limiting factors in other murine models of arthritis (46–49).
Threshold effects may also be relevant to human disease, as
prospective analysis of anti–cyclic citrullinated peptide anti-
odies in healthy individuals who later go on to develop RA
show a marked increase in titer before disease onset (50, 51).

There are still unresolved issues pertaining to the model
of arthritis presented in this study that require further explo-
ration. For instance, it is not known why <40% of the mice

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develop disease, and we have not identified any 1 parameter of the immune response that significantly differentiates arthritic and nonarthritic DR4-IE tg mice immunized with CithFib (although arthritic mice did show a trend to higher cytokine responses and antibody production to certain antigens). In preliminary studies, we have induced a transient arthritis in DR4-IE tg mice after the transfer of citrulline-specific CD4+ T cells or anti-citrulline antibodies, directly implicating these mediators in the disease process (Fig. S3 and unpublished data) (52).

We have also found that CitmFib, an antigen that can evoke a citrulline-specific immune response in DR4-IE tg mice, is not arthritogenic. Our examination of heterologous regions of fibrinogen that have been found to be citrullinated by mass spectrometry (25), coupled with IgG antibody microarray and ELISA data, does suggest that reactivity to Fbx(121–140)Cit may be important to this species-specific response. It is possible that reactivity to this sequence provides a platform for cross-reactivity to other proteins (such as vimentin), but a role in pathogenesis requires further examination.

In conclusion, these results indicate that in genetically susceptible DR4-IE tg mice, citrullinated fibrinogen can drive an autoimmune response that is associated with the development of arthritis. Further exploration of this model will help elucidate the role and contribution of SE-restricted T and B cell responses to citrullinated antigens in the pathogenesis of RA.

MATERIALS AND METHODS

Mice. DR4-IE tg murine MHC class II-deficient mice (53) were bred at the John P. Robarts Barrier Facility and C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Both strains were housed in the Animal Care and Veterinary Services Barrier Facility at the University of Western Ontario under specific pathogen-free conditions. The work with these mice was performed according to the guidelines established by the Canadian Council on Animal Care. 12–14-wk-old male mice were used in these experiments, as initial studies showed the incidence of arthritis to be higher than in females. 8-wk-old K/BxN mice used for histological analysis were maintained as previously described, and all procedures were approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

Immunizations. Mice were immunized subcutaneously on the inner thigh/flank with 100 μg protein or peptide antigen in CFA (1:1 volume of antigen and IFA supplemented with 4 mg/ml Mycobacterium tuberculosis HA37) in a total volume of 100 μl. Boosting immunizations with protein antigens were conducted with the same concentration and volume of antigen in IFA 21 d later.

Protein antigens. Purified human (Calbiochem) and mFib (Innovative Research, Inc.) were used in both citrullinated and unmodified form, whereas KLH (Sigma-Aldrich) was used in the citrullinated form. Citrullination of fibrinogen was performed as previously described (18) and confirmed by mobility shift in SDS-PAGE (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20072051/DC1), Western blotting with human RA sera, and antigen-specific ELISA.

Peptide antigens. Peptides used in these studies were synthesized and purified by the manufacturer (Genemed Synthesis). Peptides were selected based on their predicted affinity for DRB1*0401 according to the method of Hammer et al. (23). Because citrulline is not accounted for in the predictive algorithm of Hammer et al., the value of glutamine was substituted for arginine when identifying a candidate T cell epitope (glutamine has the same terminal side chain group as citrulline). Fibrinogen-derived peptides spanning the sequences Fbα 79–91, Fbα 135–150, Fbα 173–185, Fbα 507–519, Fbα 673–685, Fbα 715–727, Fb α 738–750, Fbβ 69–81, Fbβ 280–292, and Fbbγ 118–130 were synthesized in both arginine- and citrulline-containing forms, except for Fbα 371–383, which does not contain arginine.

Assessment of arthritis. Mice were monitored twice weekly for clinical signs of arthritis, and caliper measurements were taken once weekly. Three clinically apparent stages of disease were apparent, beginning with mild joint swelling, followed by severe erythema, and ending with reduced swelling accompanied by plantarflexion restriction upon physical examination. These clinical observations closely followed caliper measurements. Some arthritic mice killed at day 70 that showed mild clinical swelling were further confirmed to have synovial abnormalities by histology.

Histological and immunohistological evaluation. Mice were killed at the indicated time points, and the hind paws were dissected and fixed in 10% buffered formalin solution (WVR). Fixed tissues were decalcified for 5–7 d in 14% EDTA, followed by dehydration and paraffin embedding. Sagittal sections (8 μm) were stained with hematoxylin and eosin (HE) or safranin O, or further processed for immunohistochemical staining. HE-stained sections were blindly assessed for joint abnormalities in DR-4-IE tg mice immunized with CithFib or hFib at day 10, 31, and 70 after immunization (4 mice/immunizing antigen/time point). Additional arthritic mice were also assessed at day 70, 150, and 200. Sections from B6 mice immunized with CithFib or hFib (day 70) and DR4-IE tg mice immunized with CitmFib or mFib (day 100) were blindly assessed (4 mice/immunizing antigen), but did not show abnormal pathology. Joints including knee, shoulder, elbow, wrist, and spine were assessed in representative arthritic and control mice by histology, but did not show abnormal pathology. Citrullinated proteins were detected as previously described (54) using an AMC staining kit (Millipore) in combination with Vectastain ABC reagent (Vector Laboratories). Fibrinogen was detected using an anti-fibrinogen antibody (DAKO) according to the manufacturer’s instructions, in combination with Vectastain ABC reagent.

T cell cultures. Cell suspensions were prepared from the spleen or draining lymph nodes of mice killed at the indicated time points and cultured in 96-well plates at a concentration of 4 × 10^5 cells/well in the presence or absence of protein or peptide antigen for 4 d. 18 h before culture termination, 0.5 μCi of [3H]thymidine (ICN Biomedicals) was added to each well to assess T cell proliferation. Proliferation experiments were conducted in triplicate, and results are presented as the mean stimulation index (cpm of experimental sample/cpm of control sample) ± SD. Supernatants were removed from cultures after 72 h for measuring cytokine production by ELISA (BD Biosciences), as previously described (55). Cytokine production was measured in duplicate and represents the mean antigen-specific cytokine production (cytokine production in control samples [no antigen in vitro] + 2 SD were subtracted from the protein- or peptide-specific cytokine production) ± SD.

T cell transfers. DR4-IE tg mice were immunized and boosted, as described in Immunizations. Splenocytes were harvested at day 31, and processed for in vitro stimulation with CithFib, as described in T cell cultures. d after in vitro stimulation, viable cells were harvested by Ficoll gradient centrifugation and assessed for CD4 content by FACS (>85% positive). Cells then were transferred i.p. (10 × 10^6/mouse) and recipients received an intraperitoneal injection of 5 μg of the indicated antigen in a total volume of 5 μl. Caliper measurements were taken on the ankles every day for 2 wk, and data are presented as mean ± SD ankle width (mm) ± SEM for the indicated number of mice.

ELISA detection of antigen-specific serum IgG. Serum was collected from blood obtained by heart puncture from mice killed at the indicated time points. Antigen-specific ELISA was performed as previously described (18).


In brief, MaxiSorp (Nunc) plates were coated with citrullinated and unmodified forms of fibrinogen, or the Fibs123Ct peptide (ESANNCaDN-TNYNR), at a concentration of 10 μg/ml (100 μl/well) in carbonate coating buffer overnight at 4°C. After washing with PBS and 0.05% Tween (PBST) and blocking with PBS 0.1% BSA, serum samples were diluted 1:100 in PBST 0.1%BSA and were incubated in duplicate for 2 h at room temperature. After washing again with PBST, biotin-conjugated anti-mouse IgG secondary antibodies (Sigma-Aldrich) were diluted 1:2,000 in PBST 0.1% BSA containing streptavidin/horseradish peroxidase diluted 1:2,500 (Sigma-Aldrich) and incubated in the wells for 1 h at room temperature. After further washing, wells were developed with tetramethyl benzidine substrate (Sigma-Aldrich) for 10 min, after which the reaction was stopped with 2 M H2SO4 and absorbance was read at OD 450 nm. All samples were tested in parallel to standardize results and presented as the mean OD for each sample tested in duplicate.

Synovial proteome microarray analysis of sera. Arrays were generated and probed with mouse sera as previously described (56, 57). Significance analysis of microarrays was used to identify patterns of antigen reactivity, followed by relationship arrangement with Cluster and Tree View software (58, 59). Direct Student’s t test calculations were used to identify significantly different antibody reactivity between DR4-IE tg and B6 mice immunized with CithFib and stratified based on showing greater than fourfold increased reactivity compared with immunization with hFib. Raw data and peptide sequences can be found in Table S1–S3.

Online supplemental material. Fig. S1 shows histological evaluation of arthritis in DR4-IE tg mice highlighting proteoglycan changes by safranin O staining. Fig. S2 shows that splenocytes from DR4-IE tg mice immunized with CithFib (day 70) produce IL-10 after in vitro challenge with CithFib or hFib. Fig. S3 shows that T cells from CithFib-immunized DR4-IE tg mice can induce arthritis after transfer to naive DR4-IE tg hosts that received intraarticular injection of CithFib. Fig. S4 shows the HLA-DR-restricted immune response to Fibs R84Ct in DR4-IE tg mice immunized with CithFib. Fig. S5 shows the citrulline-specific antibody reactivity determined by inhibition. Fig. S6 shows the typical mobility shift of CithFib seen by SDS-PAGE. Table S1–S3 show the raw data and peptide sequences for the synovial proteome microarray analysis presented in Fig. 5 (C–E), respectively. Table S4 shows the location of arginine substitutions between human and mFib sequences, in addition to citrullination sites (25) and antibody reactivity by synovial microarray analysis. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072051/DC1.

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