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Tracking of Proinflammatory Collagen-Specific T Cells in Early and Late Collagen-Induced Arthritis in Humanized Mice

Pia Svendsen,* Claus B. Andersen,† Nick Willcox,‡ Anthony J. Coyle,§ Rikard Holmdahl,¶ Thomas Kamradt,¶ and Lars Fugger2*‡

Rheumatoid arthritis is a chronic inflammatory disease associated with certain HLA-DR4 subtypes. The target autoantigen(s) is unknown, but type II collagen (CII) is a candidate, with a single immunodominant DR4-restricted 261–273 T cell epitope (CII(261–273)). In the present study, we have prepared HLA-DR4:CII(261–273) tetramers and analyzed peripheral blood, lymph node, and synovial fluid cells from DR4-transgenic mice with early and late collagen-induced arthritis to draw a fuller picture of the role of CII-reactive T cells in disease development. Their frequencies increased ~20-fold in blood 1–2 wk postimmunization, and even more in acutely arthritic joints. Our data strongly suggest that CII-specific Th cells are necessary, but not sufficient for collagen-induced arthritis. The CII-specific Th cells displayed an activated proinflammatory Th1 phenotype, and their expansion correlated with onset and severity of arthritis and also with anti-CII Ab levels. Surprisingly, shortly after the first clinical signs of arthritis, activated HLA-DR4:CII tetramer+ cells became undetectable in the synovial fluid and rare in the blood, but persisted in lymph nodes. Consequently, future human studies should focus on patients with early arthritis, and on their synovial cells, to re-evaluate the occurrence and pathogenic importance of CII-specific or other Th cells in rheumatoid arthritis. The Journal of Immunology, 2004, 173: 7037–7045.

The pathogenesis of RA is not well understood, but the HLA-DR association strongly suggests that it is Th cell dependent, and that the associated DR molecules present autoantigenic peptides to autoreactive Th cells, which initiate the inflammatory process that ultimately leads to RA. This assumption is supported by the presence of T cells in the RA synovial compartment (5, 6); more recently also by the clinical benefits of treating RA patients with a fusion protein, CTLA4–IgG1, that binds to CD80 and CD86 on APC, and thereby blocks Th cell costimulation, so selectively inhibiting their activation (7). However, the specificity of the pathogenic Th cells in RA is largely unknown. Collagen type II (CII) is one candidate because it is the major protein in articular cartilage and because it is arthritogenic in animals. Anti-CII autoantibodies are important players in this collagen-induced arthritis (CIA). They are also found in the blood and joints in RA (8, 9), whereas CII-specific T cells have been difficult to demonstrate in either compartment (10, 11). However, reflecting this search mainly in patients with long durations of both RA and immunosuppressive drug treatments probably biases against detection of the T cells that initiate the disease process, which may well be rare and/or short-lived.

To investigate the role of RA-associated DR molecules in autoimmune T cell responses in a more controlled biological setting, we have generated transgenic mice expressing DR4 (DRA1*0101/ DRB1*0401) and human CD4 molecules (12). Analysis of their responses against bovine CII showed that DR4-restricted T cells almost exclusively recognize a single 261–273 epitope in CII (CII(261–273)) (13). The corresponding human CII sequence is identical and is the major DR4-binding CII epitope; in the mouse, it differs only by a conservative 266E→D substitution buried deep in pocket 4 of DR4 (14). Moreover, by defining MHC and TCR contacts in this CII(261–273) peptide, and by modeling its structure in the DRB1*0401 molecule, we clearly showed that it conforms to established requirements for presentation by DR4 (15). When DR4-transgenic mice are immunized with native CII, ~75%
of them develop an inflammatory arthritis with similarities to RA (15). This humanized mouse model makes it possible to track DR4-restricted CII(261–273)-reactive T cells at any time point of disease without the complicating influence of immunosuppressive therapy. Using DR4-CII(261–273) tetramers and analyzing blood, lymph nodes, and synovial fluid from DR4-transgenic mice, we show that activated specific Th cells are much more evident in the early stages, and are greatly enriched in newly arthritic joints; however, their frequencies decline soon afterward, but remain above background for months in both blood and lymph nodes.

Materials and Methods

Construction of baculovirus vectors for production of soluble HLA-DR4 molecules with covalently bound peptides

Soluble HLA-DR4:CII(261–273) and HLA-DR4:CII(261–273) tetramers were produced using a baculovirus expression system. The bicistronic pAcAB3 transfer vector (BD Pharmingen, San Diego, CA), using two AcNPV p10 promoters, was chosen to allow for simultaneous expression of recombinant DRα and DRβ genes. The hydrophobic transmembrane regions of the DRα and DRβ chains were replaced by leucine zipper dimerization domains from the transcription factors Fos and Jun to promote DR αβ assembly (16). Cytoplasmic cDNA sequences of DRA1*0101 and DRB1*0401 were ligated to fos- and jun-encoding sequences in the TA vector pCR2.1, and the resulting plasmids were used as templates for further modifications. A birA site (GLNDIFEAQKIEWH) was added to the 3′ end of the DRA1*0101-fos template, and a 5′ end BglII restriction site, as well as a 3′ end Cell restriction site, was introduced by PCR for cloning of the complete DRA1*0101-fos-birA insert between the BglII and Cell restriction sites of the baculovirus expression vector pAcAB3. Covalently bound peptides HA307–319 (PKYVKQNTLKLAT) and CII261–273 (AGFKGEQGPKGEP) were genetically attached by a flexible linker-DRB1*0401 leader-peptide-linker and a 3′ fragment ( linker-DRB1*0401 cytoplasmic-jun), produced as previously described (18). The resulting DRB1*0401 leader-peptide-linker-cytoplasmic-jun PCR product was amplified by PCR using primers introducing a 5′ SmaI site and 3′ BamHI site for cloning between the SmaI and BamHI sites of the baculovirus vector. Finally, the modified DRα and DRβ inserts were cloned into the BglII/Cell and SmaI/BamHI sites under control of the two p10 promoters of the pAcAB3 vector, and correct insertions were confirmed by restriction digestions as well as sequencing of the final baculovirus constructs.

Baculovirus expression and purification of HLA-DR4-peptide complexes

The pAcAB3-DRB-DRα plasmids were cotransfected with linearized baculovirus DNA (BD Pharmingen; BaculoGold kit) into Sf9 insect cells, according to the manufacturer’s instructions. Following two rounds of plaque purification, clonal virus isolates were further amplified three times before preparation of high-titer virus (10⁷–10⁸/ml). These stocks were used to infect High Five or serum-free S21 insect cells (Invitrogen Life Technologies, Carlsbad, CA) for protein production. Spinner cultures (2–20 ml) were incubated with 25 μg of BirA at room temperature for 18–20 h in 10 mM Tris (pH 8) and biotin-containing buffer provided by the manufacturer. Unbound biotin was then removed by gel filtration using a Superdex 200 column (Amersham Biosciences, Hillerød, Denmark). Biotinylated HLA-DR4:peptide complexes were concentrated to 0.7–1 ml using Centricron-10 concentrators (Millipore), and the biotinylation efficiency was estimated by an ELISA. Fluorescent DR4:peptide tetramer complexes were assembled by the addition of ultra-avidin-PE (Leinco Technologies, St. Louis, MO) at a final molar ratio of HLA-DR4 peptide biotin to avidin-PE of 6:1. The resulting DR4:peptide multimer complexes were subjected to size exclusion on a Superdex-200 column to separate the tetramer complexes from protein aggregates as well as lower m.w. complexes and excess free HLA-DR4:peptide. The tetramer complexes were concentrated using Centricon-30 concentrators (Millipore) and stored at 0.1–0.3 mg/ml in a mixture of protease inhibitors.

T cell hybridomas and T cell assays

The CII(261–273)- and HA(309–317)-specific T cell hybridomas have been described previously (15, 19). We assayed their direct recognition of immobilized tetramer complexes by measuring their IL-2 responses in an IL-2-specific sandwich ELISA. Serial dilutions of tetramer complexes (5–0.05 μg/well) were coated overnight at 4°C before addition of the hybridoma cells (5 × 10⁴/well) and incubation for 24 h at 37°C. Subsequently, 100 μl of the supernatant was transferred to an anti-IL-2 Ab-coated microtiterplate, and the IL-2 produced was determined using an Eu³⁺-labeled streptavidin detection system, as previously described (15). A time-resolved fluorometer (PerkinElmer Wallac, Gaithersburg, MD) was used to measure the resulting fluorescence.

Flow cytometry

Hybridoma T cells (2 × 10⁵) were incubated with HLA-DR4:peptide tetramers (10–20 μg/ml) in a balanced salt solution (2% FCS, 0.1% NaN₃) for 1 h at room temperature. Anti-CD3 (clone 145-2C11) or anti-TCR (H5-579) (BD Pharmingen) (5–7 μg/ml) was added to the incubation for the last 20 min. To exclude CD8⁺ T cells, monocytes, and B lymphocytes from further analysis, mouse PBMC, lymph node cell suspensions (0.5–2 × 10⁶ cells), and synovial fluid cells (1–2 × 10⁵ cells) were incubated with biotinylated anti-CD8, anti-CD14, and anti-CD19 (BD Pharmingen) for 30 min at room temperature, washed, and subsequently incubated with CyChrome-conjugated streptavidin (BD Pharmingen) for 20 min. CD3⁵⁷ / CD8⁺/CD14⁺/CD19⁻ T cells were shown to be CD4⁺ T cells in a separate analysis and subsequently designated CD4⁺ T cells. Following extensive washing of the cell pellet, the PE-conjugated HLA-DR4:peptide tetramers were added at 1–2 μg/10⁵ cells in 100–200 μl of RPMI 1640 plus 2% FCS for 1 h, and finally allophycocyanin anti-CD3 and sometimes FITC anti-CD44 (BD Pharmingen) were added for a further 30 min at room temperature. RBCs were lysed, and stained cells were washed twice to three times in balanced salt solution before analysis on a FACSCalibur (BD Biosciences, San Jose, CA).

Transgenic mice and induction of arthritis

HLA-DR4- and human CD4-DRA4-transgenic mice on a mouse MHC class II-deficient DBA1/J background (15) were selected for this study by flow cytometric analysis of PBMC stained with mAbs to HLA-DR, human CD4, I-A<sup>+</sup>, and I-A<sub>old</sub> (BD Biosciences and BD Pharmingen). For induction of arthritis, 8- to 12-wk-old mice were immunized intradermally at the base of the tail with 100–150 μg of bovine CII (Chondrex; MD Biosciences, Zurich, Switzerland) emulsified in CFA, and boosted with 50–100 μg of CII in IFA at day 40. From day 20, the mice were scored three times per week for clinical signs of arthritis. Peptides were injected at the base of tail as well as in both rear paws using 100 and 50 μg of peptide in CFA, respectively.

Scoring system

The mice were scored by examining forepaws and hindpaws three times per week from day 20. Clinical severity of CIA was determined by the swelling of individual joints and the number of affected joints in the front and rear paws. Each paw was scored from 1 to 3, so the maximum score, including all four paws, was 12. Histopathologic arthritis activity/severity was scored according to pannus formation and cartilage and/or bone destruction (score from 1 to 3).

Histopathology

Mouse legs were fixed in 10% Formalin and then decalcified with EDTA at 56°C. Tissue samples were paraffin embedded, sectioned, and stained with H&E.

Serum analyses

The level of anti-CII Abs was detected by ELISA. The 96-well flat-bottom plates were coated with 100 μl of mouse CII (5 μg/ml in PBS, pH 7.4, at
4°C overnight). After blocking with 1% skimmed milk/0.05% Tween 20
PBS at 20°C for 1 h, the plates were washed, and 100 μl of diluted serum
(10⁻³, 10⁻⁴, and 10⁻⁵) was added to duplicate wells for 1 h at 20°C. After
repeated washing, 100 μl of biotinylated Abs to IgG1, IgG2a, IgG2b, or
IgG3 (each at 10⁻³; BD Pharmingen) was added, and the plates were
further incubated for 45 min. After repeated washing and incubation with
Eu³⁺-streptavidin (10⁻⁰⁷) for 1 h, the fluorescence of bound IgG was mea-
sured using a time-resolved fluorometer (PerkinElmer Wallac). The exper-
iments were performed in duplicates. Amounts of anti-CII Abs
(calibrated using known amounts of purified mouse IgG isotype stan-
dards (BD Pharmingen) in each assay.

Statistics
All datasets were analyzed for normal distribution in Q-Q plots using SPSS
software (Chicago, IL). Normally distributed data are presented as means
and others as medians. Statistical differences in the frequencies of tet-
ramer + CD4 + T cells, disease onset and severity, and anti-CII Ab levels
were determined by Mann-Whitney U or Student’s t test using SPSS soft-
ware, and all correlations with the Spearman rank order test. Values of p <
0.05 were considered statistically significant.

Results
Characterization of HLA-DR4:CII(261–273) and HLA-DR4:
HA(307–319) tetramers
HLA-DR4 (DRA1*0101/DRB1*0401) molecules loaded with co-
valently bound CII(261–273) or HA(307–319) peptides were ex-
bressed from a baculovirus vector, affinity purified, biotinylated,
and tetramerized with ultra-avidin-R-PE.

These tetramers clearly interacted directly with specific Th cells
in the absence of APC. When immobilized on microtiter plates, the
homologous, but not the heterologous, DR4 tetramers all stimu-
lated strong dose-dependent responses by the five different hybrid-
omas specific for HA(307–319) and the 15 for CII(261–273) (Fig.
1, a–c). They also proved to bind very specifically to the DR4-
restricted T cell hybridomas in flow cytometry assays. The
DR4:HA tetramer bound strongly to the five HA(307–319)-spe-
cific T cell hybridomas, but not to any of the CII-specific T cell
hybridomas, as illustrated in Fig. 1, d–f. With both tetramers, spe-
cificity was greatest at 20 μg/ml, whereas backgrounds were higher
at 50 μg/ml.

Notably, 9 of the 15 CII-specific hybridomas recognize the un-
modified CII(261–273) peptide (19), whereas 6 greatly prefer a
modified peptide glycosylated at positions 264K(Gal²⁶⁴) or
270K(Gal²⁷⁰). Maximal responses of the former to stimulation with
the DR4:CII tetramers differed by up to 100-fold (data not shown),
but those of the glycospecific hybridomas were consistently 3- to
10-fold lower (Fig. 1, b and c). Similarly, the DR4:CII tetramer
bound strongly to all 9 CII(261–273)-specific T cell hybridomas
and more weakly, but significantly to all of the 6 glycospecific-
CII(259–273) clones (Fig. 1, e vs f), but not to any of the HA(307–
319)-specific T cell hybridomas (e.g., Fig. 1, d–f).

The binding intensity varied between T cell hybridomas, but
correlated broadly with their TCR expression levels and respon-
siveness to stimulation with tetramer (data not shown). Impor-
tantly, tetramer staining was still detectable with all six glycospe-
cific clones, despite very low TCR expression in three of them
(Fig. 1f).

Because we expected Ag-specific Th cells to be extremely rare
in vivo, we next determined the sensitivity of DR4-peptide tet-
ramer staining in mixtures of CII(261–273)- and HA(307–319)-
specific T cells. The threshold proved to be as low as 0.01–0.05% (Fig.
2). Thus, the DR4-peptide tetramers bind specifically and
with high sensitivity to DR4-restricted T cell hybridomas, even
when TCR levels are low.

![FIGURE 1.](http://www.jimmunol.org/)

**a–c,** DR4 tetramers directly stimulate T cell hybridomas specifically. Plate-bound DR4:HA(307–319) (circles) and DR4:CII(261–273)
(triangles) tetramers were assayed for their ability to stimulate HA(307–319)-specific HA3.3 (a), CII(261–273)-specific CII3838 (b), and CII (259–273)
glycopeptide-specific CII hDR4.5 (c) hybridomas in the absence of APC. Supernatants from cells stimulated overnight were analyzed for IL-2
production using an IL-2-specific sandwich ELISA. IL-2 production is given in Eu³⁺/H11001
units. **d–f,** DR4 tetramer-specific staining of T cell hybridomas.
HA3.3 (d), CII3838 (e), and CII hDR4.5 (f) hybridoma cells (2 × 10⁵) were incubated with 2 μg of PE-conjugated DR4:HA(307–319) tetramer (gray line)
and DR4:CII(261–273) tetramer (black stippled line) for 1 h at room temperature and analyzed for bound PE fluorescence by flow cytometry. Separate
aliquots of the cells were also labeled with the anti-mouse Ab H57–597 (solid thick line) to measure total surface αβTCR expression. Cells incubated
without Ab are shown as thin solid lines.
In other words, 37 of the 61 (60.6%) tetramer-positive mice responded to CII in the blood before, during, and after the development of CIA. This peak was significantly higher (median 0.46%) in the PBMC (Fig. 3, a) compared to those that never developed CIA (median 0.00–0.02%) (median 0.01%) (Fig. 3, b–d). Notably, cross-binding of either heterologous tetramer was minimal, ranging from 0.0 to 0.02% (median 0.01%) in PBMC (Fig. 3, e–g) to 0.02 to 0.08% (median 0.04%) in draining lymph nodes (Fig. 3, h–j), i.e., not significantly higher than in mice injected with PBS or CFA alone (0.00–0.02%) (median 0.01%) (Fig. 3, e–h, and data not shown).

Serial tetramer analysis in PBMC predicts development and severity of CIA

Having thus established that the tetramers were able to detect rare cells in heterogeneous populations with very high specificity and sensitivity, we next focused on CIA. We immunized 74 DR4-transgenic mice twice (days 0 and 40) with native CII, and then enumerated and characterized DR4:CII tetramer-positive CD4+ T cells in the blood before, during, and after the development of CIA.

Arthritis was seen in 50% of the 74 immunized mice, as expected (15). A response by CII tetramer-positive CD4+ T cells was evidently necessary, but not sufficient for its development: specific Th cell frequencies declined gradually, but remained above background in the blood even at day 150. The arthritis ranged from mild swelling of one paw to complete destruction of the joint and bone, and arthritis was seen in 50% of the 74 immunized mice, as expected (15).

Frequency and severity of CIA

Next, we examined the ability of our tetramers to detect rare specific cells in mixed populations ex vivo. Mice transgenic for HLA-DR4 and human CD4 were immunized with CII(261–273), or HA(307–319) with CFA or with PBS alone. Thirteen days later, we determined the frequency of tetramer-positive cells in peripheral blood and draining lymph nodes. Frequencies of DR4:CII tetramer-positive CD4+ T cells ranged from 0.2 to 0.6% (median 0.35%) in PBMC (Fig. 3a) and 0.5 to 1.5% (median 0.9%) in draining lymph nodes (Fig. 3i). They were similar for HA(307–319)-responding T cells (Fig. 3, d and f). Notably, cross-binding of either heterologous tetramer was minimal, ranging from 0.0 to 0.02% (median 0.01%) in PBMC (Fig. 3, c and b) to 0.02 to 0.08% (median 0.04%) in draining lymph nodes (Fig. 3, j and k), i.e., not significantly higher than in mice injected with PBS or CFA alone (0.00–0.02%) (median 0.01%) (Fig. 3, e–h, and data not shown).

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The frequency of tetramer CII(261–273)-specific T cells by flow cytometry before and after immunization with mice (74) were analyzed for the presence of CII(261–273) and HA(307–319)-specific T cells. Fresh blood samples from HLA-DR4-transgenic mice (74) were analyzed for the presence of CII(261–273) tetramer frequencies are indicated (74). The 75% percentiles are shown in bars, but only for days 13, 49, 56, (74); the 25 and 75 percentiles are shown in parentheses. The immunized mice that developed detectable DR4:CII(261–273) tetramer staining was detected during the course of the experiments. The immunized mice that developed detectable DR4:CII(261–273) tetramer T cells were compared according to development of CIA (no CIA/CIA). We show the median frequencies of DR4:CII(261–273) tetramer T cells (a) as well as the median percentage of these cells that were also CD44+ (b). Significant differences between data points are indicated (*). The 75% percentiles are shown in bars, but only for days 13, 49, 56, and 76 (for simplicity). c, Severity of CIA. Each paw of each immunized mouse was scored (0–3) for clinical signs of inflammation three times per week, resulting in a clinical score for each mouse (0–12). Mean scores (± SD) of arthritis are shown on indicated days for comparison with the severities on those days (p = 0.009 and 0.007). Histopathologic changes also correlated significantly with tetramer + T cell frequencies (Table II). These were graded according to pannus formation and cartilage and/or bone destruction (one point for each; mean score = 2). Notably, in mice with a score of 3, frequencies of tetramer + T cells were significantly higher on both days 13 and 56 than in those with a score of 1 or 2 (Table II). Taken together, these data show that frequencies of CIA(261–273) tetramer + T cells are significantly higher in mice destined to develop arthritis, and also predict its clinical and histopathologic severity, and that the majority of tetramer + T cells were activated (CD44+) before and at disease onset.

Mice with tetramer + T cells have persistently high serum anti-CII IgG levels

Serum was analyzed for autoantibodies in the different IgG subclasses against autologous mouse CII before (day 20), during (day 60), and after (day 120) disease development (Table I). Levels were high in all subclasses, except IgG3 at all three time points. At day 60, they were consistently higher in the mice that developed arthritis (p < 0.02 vs nonarthritic mice). Likewise, among those with no arthritis, they were higher in the tetramer responders than the nonresponders (p < 0.04). Moreover, levels of IgG1, IgG2a, and IgG2b autoantibodies correlated significantly with tetramer + T cell frequencies at the time when arthritis was peaking (−days 60/56; p < 0.03).

Progression of CIA and frequencies of peripheral and synovial CIA(261–273)-reactive T cells

Histopathologically, the acute phase (the first 2 wk after onset) was characterized by different degrees of acute and chronic arthritis.

or several toes (score 1) to severe swelling of both front and rear paws (score 11). The mean maximum severity was 5, and the first mice reached this score by day 59; in all of them, the CIA was at its worst by day 76 (Fig. 4c), whereafter we saw no new cases of arthritis.

Strikingly, on both days 13 and 56, tetramer + T cell frequencies correlated significantly with the maximum disease severity in individual mice (p = 0.015 and 0.004, respectively). In addition, the frequencies on days 49 and 56 also correlated with the severities on those days (p = 0.009 and 0.007).

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### Table 1. Development of DR4-CII tetramer-specific T cells and mouse anticollagen levels in collagen-immunized mice

<table>
<thead>
<tr>
<th>Anti-Mouse CII Ab (μg/ml)</th>
<th>Day 20</th>
<th>Day 60</th>
<th>Day 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>17 (9–50) 133 (56–175) 16 (4–120)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2a</td>
<td>55 (12–98) 1195 (261–2094) 73 (24–425)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2b</td>
<td>97 (31–247) 382 (61–588) 81 (17–293)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>0.5 (0.1–2) 0.1 (0.05–0.3) 0.15 (0.1–0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−CIA/+4-mer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>10 (4–30) 32 (9–46) 8 (0.7–113)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2a</td>
<td>17 (6–129) 285 (58–966) 62 (19–307)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2b</td>
<td>38 (5–160) 169 (87–241) 73 (13–300)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>0.3 (0.2–0.7) 0.2 (0.1–0.4) 0.25 (0.2–0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−CIA−4-mer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>5 (4.5–6) 6 (2–13) 0.9 (0.6–3.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2a</td>
<td>14 (4–64) 166 (123–179) 26 (12–37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2b</td>
<td>22 (17–28) 17 (6–29) 9 (3–200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>0.07 (0.5–0.1) 0.01 (0.0–0.01) 0.05 (0.01–0.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Serum samples were analyzed in a sandwich ELISA for the presence of mouse anti-CII Abs 20, 60, and 120 days post-CII immunization. Anti-CII IgG levels (μg/ml) are presented as median values from arthritic mice (+CIA/+4-mer, n = 47) as well as nonarthritic mice that developed tetramer-positive T cells (+CIA/+4-mer, n = 29) and nonarthritic mice that never developed tetramer-positive T cells (+CIA−4-mer, n = 21). The 25 and 75 percentiles are shown in parentheses.
with or without cartilage and bone destruction. During the next 2–4 wk, the picture changed to one of chronic inflammation with severe cartilage and bone destruction as well as remodeling. Furthermore, synovitis and pannus formation were now more pronounced. End-stage disease (2–3 mo after onset) was characterized by pannus growth, bone and cartilage remodeling, and deformity.

In a new cohort of 41 mice, clinical arthritis was further characterized according to disease progression; it was acute in 16 (1–14 days after onset), progressive in 14 mice (2–4 wk), and at the end stage in 11 mice (2–3 mo). Representative FACS data are shown in Fig. 5; frequencies of tetramer+ cells were highest in the acute phase, and declined slowly thereafter. Moreover, 100% of the CD4+ tetramer+ cells also expressed CD44 in the acute phase, but only 10% by the end stage. These data demonstrate a significant decrease in both total and activated CII(261–273)-specific T cells in the circulation just a few weeks after arthritis onset ($p < 0.001$).

In lymph nodes, frequencies of CII(261–273)-specific Th cells were much more stable, with a median frequency of 0.25% in the acute phase (e.g., Fig. 5c), 0.4% at 2–4 wk, and 0.36% thereafter, i.e., persistently 10–20× background (data not shown). Notably, however, 76% of these tetramer+ cells were CD44+ in the acute phase, 25% 2–4 wk later, and only 14% by the end stage (Fig. 5c, and data not shown).

We were able to obtain small numbers of synovial fluid cells from the ankle joints at the peak of arthritis (e.g., Fig. 5e). The frequencies of CII(261–273)-specific T cells were consistently higher there than in blood from the same mice. Their median was 4.2% (range 1.7–17% of all CD4+ T cells), and 83% of them were CD44+. Strikingly, however, we were no longer able to detect any CD4+ T cells in the synovial fluids after 2 wk, but only B cells, macrophages, and very few CD8+ T cells.

Thus, by the end stage (2–3 mo), CII(261–273)-specific T cells were rare or undetectable in the circulation, and especially in synovial fluid from damaged joints, but persisted in lymph nodes. Significantly, in all three populations, >75% of them were CD44+ effector cells at onset, strongly implicating them in pathogenesis.

**Th phenotype of CII(261–273)-specific T cells in the synovial fluid**

We next analyzed Th1/Th2 markers on days 1–7 after onset, when synovial T cell numbers were still adequate. We made use of well-established surrogate markers, which are stably expressed on the surface of Th1 (20) or Th2 cells (21, 22), i.e., the Th2-specific IL-1R family member T1/ST2 and the Th1-associated molecule TIM-3. Almost all (79–95%) of the synovial CII(261–273)-specific T cells proved to be TIM-3+ and T1/ST2−, clearly indicating a proinflammatory potential (Fig. 6).

**Discussion**

In this study, we show that activated, proinflammatory, CII-specific Th cells can be detected and tracked in a humanized model of inflammatory arthritis by using DR4:CII(261–273) tetramers. These cells are detectable in the blood and joints of HLA-DR4-transgenic mice at the early stage of clinically overt arthritis. Interestingly, shortly afterward, they become undetectable in the synovial fluid and rare in the blood, but merely decline in lymph nodes, even though the arthritis persists. These findings have important novel implications both for the pathogenesis of CIA and for approaches for studying this process in human RA.

**The role of Th cells in the pathogenesis of CIA**

CIA is the most intensively studied murine model for human inflammatory arthritis (23). The pathogenic importance of anti-CII

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**Table II. The frequency of CII(261–273)-specific T cells and its correlation with histopathologic severity**

<table>
<thead>
<tr>
<th>Score</th>
<th>Frequency Day 13</th>
<th>Frequency Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.25</td>
<td>0.54</td>
</tr>
<tr>
<td>II</td>
<td>0.24</td>
<td>0.55</td>
</tr>
<tr>
<td>III</td>
<td>0.53</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*p Values for the different comparisons were determined by Mann-Whitney test.

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**FIGURE 6.** Th1 phenotype of synovial DR4:CII tetramer+ T cells. Tetramer+ T cells in synovial fluids pooled from arthritic mice 1–7 days after onset of arthritis were labeled with mAbs against the Th1-specific TIM-3 and the Th2-specific T1/ST2 molecules. Numbers are the percentage of CD3+/CD4+/tetramer+ cells in the indicated quadrants.

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**FIGURE 5.** Acute arthritis in transgenic mice; examples of CII-specific T cells in PBMC (a and b), lymph nodes (c and d), and synovial fluids (e and f) from a representative mouse 1 wk after CIA onset. They were labeled as for Fig. 4b, and gated as described in Fig. 3. Numbers are the percentage of CD3+/CD4+ cells in the indicated quadrants.
Abs is clearly established in this model. The disease can be induced by passive transfer of either polyclonal IgG Abs purified from the sera of arthritic mice (24–26) or combinations of mAbs against CII (27), even in mouse strains that are not susceptible to actively induced CIA (24). In contrast, the role of Th cells is less clear. Activated T cells appear in the earliest detectable arthritic lesions before any joint swelling (28). Whereas attempts to induce CIA in mice by T cell transfer have largely been unsuccessful (29), arthritis was induced in naive DBA/1 mice after the inoculation of CII-reactive CD4+ T cell lines (30), although no CII-specific pathogenic T cell clone(s) has been isolated (31, 32). One study showed that early and repeated injections of anti-CII Abs prevented CIA (33), whereas others reported unaltered CIA incidence and severity in CD4-deficient mice, while CD8-deficient mice showed a decreased incidence, but unaltered severity (34). In one study, even rag-deficient DBA/1 mice developed arthritis, albeit less severe than in wild-type littermates, on injection of CIA (35).

The DR4-restricted responses to human/bovine CII(261–273) in the humanized mice seem almost certain to cross-react with mouse CII, which differs only by a conservative 266E→D substitution at position p4; it lies deep in the peptide binding site and points into its pocket 4, which can expand to accommodate side chains of different sizes (14). Moreover, because this region is also immunodominant in CIA in DBA/1 (H2d) mice, it must be processed naturally from murine CII (36). Because immunization with CII evokes arthritis as well as Th and Ab responses, human-mouse cross-reactivity seems almost certain for both Th and B cells. Indeed, similar cross-reactions have been strongly incriminated in the autoimmune-mediated arthritis in monkeys induced by avian, human, and primate CII (37).

Interestingly, glycosylation of CII(261–273) seems to be important for recognition by some T cells, and for at least some of the arthritis, in both I-A\(^d\) DBA/1 (38) mice and HLA-DR4-transgenic mice (19). Indeed, recent data suggest that both glycosylated and nonglycosylated CII epitopes are important, and that CII glycosylation also varies during the disease course (our unpublished results). We demonstrate in this study that the DR4:CII tetramers are capable of detecting T cells specific for the glycosylated as well as the unmodified CII peptide, even when TCR expression is low, suggesting cross-reactivity of the DR4:CII tetramer. Also, the enrichment/activation of tetramer+ Th cells in diseased synovial fluid strongly suggests that they can recognize autoprotective native glycosylated collagen, and are, in fact, responding to it. Indeed, preliminary analysis of our HLA-DR4+CII monomer showed glycosylation of the linked CII peptide (data not shown). Thus, it is quite possible that the DR4:CII(261–273) tetramers represent a mixture of glycosylated and unmodified CII peptide; to ensure adequate concentrations of any minority CII peptide glycoform, we always used saturating concentrations of tetramers.

Our data strongly suggest that CII-specific Th cells are necessary, but not sufficient for the development of CIA. HLA-DR4:CII tetramer staining demonstrated their expansion in HLA-DR4-transgenic mouse PBMC after both primary and secondary immunizations with CII. These expansions preceded the onset of CIA, which was never seen in their absence. Moreover, the size of the primary T cell expansion and the T cell activation predicted the development of CIA. Even higher (activated) Th cell frequencies were seen during the second expansion; its magnitude again correlated with disease onset and severity, and with anti-CII Ab levels, while its rapid subsequent decline in the blood correlated with disease progression and persistence of anti-CII Abs. These latter are further evidence of responses to autologous native Ag.

Apparently, therefore, the total number of circulating activated CII-specific CD4\(^+\) T cells is an important determinant of development of CIA, a certain threshold frequency being required for initiation of disease. Immediately after onset, the numbers of HLA-DR4:CII tetramer+ Th cells began to drop in the peripheral blood. At that time, an even higher percentage (up to 17%) of the Th cells isolated from the synovial fluid was HLA-DR4:CII tetramer+. Moreover, these cells were T1/ST2-/TIM-3+, indicating a proinflammatory Th1 phenotype (20–22, 39). Interestingly, in preliminary experiments, lymph node and synovial fluid DR4:CII tetramer+ cells expressed TNF-α more consistently than IFN-γ, another parallel with human RA (2). Shortly after the acute inflammation, activated HLA-DR4:CII tetramer+ cells became rare in the synovial fluid and the peripheral blood of the arthritic mice. Currently, it is not clear whether that is due to their activation-induced cell death, TCR down-regulation, dilution, or homing into the pannus/synovial matrix and/or lymph nodes. Although we cannot exclude spreading to other epitopes, there is no evidence for its involvement in CIA in mice.

Whatever the explanation, our findings agree well with the conclusions from other groups that specific Th cells are especially important during the initiation of autoimmune diseases. By contrast, during the maintenance phase, long-lived autonomous plasma cells may also make major contributions in many autoantibody-mediated disorders (40).

Our data must have implications for researchers trying to detect pathogenic Th cells in RA patients with established disease (for example, see Refs. 11 and 41).

Are CII-specific T cells relevant to RA pathogenesis?

The key autoantigen(s) that is recognized in chronic inflammatory arthritides such as RA is unknown (42). T cells and Abs against a variety of self Ags, including CII, occur in patients with RA (8, 9, 19, 43). Some of these Abs, such as the rheumatoid factors that recognize IgG, have diagnostic significance. Nevertheless, there is little solid evidence yet for collagen as a pathogenetically relevant autoantigen, a diagnostic marker, or a therapeutic target in RA (43–46).

In addition, there has been controversy for several decades about the major pathogenic cell type(s); many candidates have been implicated (47–49). After an initial focus on B cells and Abs (47), Th cells became prime suspects largely because of the HLA-DR associations in RA (1). Together, the difficulty in detecting cellular immune responses against autoantigens in RA patients (41), the failure of some treatment strategies directed against T cells, and the impressive successes of therapeutic TNF-α blockade all seemed to implicate macrophages as the major effector cells in the clinically overt stages of RA (50). However, most recently, two different lines of evidence reassert the importance of T cells. First, a large clinical trial showed clear clinical benefits from treating active RA by blocking T cell co-stimulation and activation (8). Second, a spontaneous point mutation of the gene encoding an Src homology 2 domain of ZAP-70, a key signal transduction molecule in T cells, causes chronic autoimmune arthritis in mice that resembles human RA in many aspects (51).

Our data in the CIA model demonstrate that CII-specific T cells are readily detectable in the acute phase, especially in affected joints, and persist in lymph nodes in the chronic stage. These results may have important implications for RA in humans. For example, they might predict that CII-specific Th cells will be hard to detect in the blood in patients with ongoing RA, as others have indeed found (11, 41). Instead they argue that it would be more informative to focus on patients with early rather than established RA, and before treatment, and on synovial T cells in newly affected joints. One great advantage of MHC:epitope tetramers is...
that specific Th cells can be detected independently of their activation status. This may be an important point even in patients with very early RA, because T cells from RA patients show diminished proliferative capacity (52), which is probably due to TCR-signaling defects (53, 54). RA is a heterogeneous syndrome, and that is reflected in the variety of experimental animal models using different target Ags and demonstrating different pathogenic pathways (reviewed in Refs. 23 and 36). Indeed, none of the existing models explains the complexity of RA, although each contributes valuably toward our understanding of the underlying cellular and molecular responses/mechanisms.

Thus, our findings in a humanized mouse CIA model are consistent with the proposed initiating role for specific Th cells in inflammatory arthritides. Indeed, they strongly suggest that CIA-specific Th cells and autoantibodies are each necessary, but not sufficient for arthritis induction in CIA. These CIA-specific Th cells have proinflammatory capacities and are readily detectable in the blood only very early in the disease course. Consequently, future studies should focus on patients with early arthritis and/or newly affected joints to re-evaluate the presence and pathogenicity of CIA-specific or other Th cells in RA or other chronic inflammatory arthritides.

Acknowledgment

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References