Ex Vivo Characterization of the Autoimmune T Cell Response in the HLA-DR1 Mouse Model of Collagen-Induced Arthritis Reveals Long-Term Activation of Type II Collagen-Specific Cells and Their Presence in Arthritic Joints

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Susceptibility to autoimmunity diseases such as rheumatoid arthritis (RA), type I diabetes, multiple sclerosis, and celiac disease is associated with the expression of particular HLA class II alleles (1–4). Implicit in this association is the involvement of CD4+ T cells, presumably through interaction with autoantigenic peptides presented by these particular HLA alleles, although other class II-mediated mechanisms may also play a role in disease susceptibility (5). Based on these associations with class II expression, T cells have become a focal point of study for defining pathogenic mechanisms of autoimmunity and for the development of novel therapeutic approaches to the treatment of autoimmune diseases. However, much remains unknown regarding the functional roles these pathogenic T cells play, the peptide ligands for which they are specific, the stages in disease pathogenesis to which they contribute, and in many cases whether these autoimmune T cells specifically migrate to sites of inflammation and directly participate in the pathogenesis.

To answer some of these questions, a number of HLA transgenic (Tg) mouse models have been developed with pathogenic characteristics that closely resemble human autoimmune diseases (6–9). The advantage of these models is that the functional role of the HLA molecule associated with the human disease can be studied in the context of a defined autoantigen. Despite these advantages, identifying the autoantigen-specific T cells and studying their physical and functional characteristics throughout the development of the autoimmune response and its associated pathology have remained difficult. One approach to identifying populations of Ag-specific T cells that has shown a great deal of promise is the use of tetrameric MHC molecules (10–12). These MHC tetramers (or multimers) consist of four biotinylated MHC molecules that are encoded at the DNA level (13) or loaded into physical and functional characteristics throughout the development of the autoimmune disease models (16–18).

Using a similar approach, we have developed an HLA-DRB1*0101 class II tetramer containing a covalently linked type II collagen (CII) peptide that binds specifically to DR1-restricted, CII-specific T cells ex vivo. In combination with HLA-DR1 Tg mice that are susceptible to collagen-induced autoimmune arthritis (CIA) (7, 19), we have used this tetramer-based approach to study the development of the CII-specific autoimmune T cell response...
that mediates the development of arthritis, focusing on ex vivo analyses of activation marker expression, TCR use, and cytokine production. Our data indicated that only a small population of CII-specific T cells is required to mediate the development of arthritis, and that the numbers of these cells in the periphery wane before the appearance of arthritic limbs, although a highly enriched population of tetramer-positive cells could be detected in the arthritic joints of these mice. In addition, these CII-specific T cells express high levels of mRNA for a number of Th1 and inflammatory cytokines and maintain their expression of activation markers for at least 130 days. In all, these data indicate that CII-specific T cells maintain an activated state throughout and well after the development of arthritis and that they may have a direct role in the pathogenesis of CIA by either selective migration or retention in the inflamed synovium of arthritic joints.

Materials and Methods

Generation of Tg mice expressing DR

Mice expressing the chimeric (human/mouse) DRB1*0101 construct were either raised in our onsite pathogen-free facility or purchased from Taconic Farms. The chimeric DRB1*0101 construct has been previously described, as has the production of Tg mice expressing these constructs (7, 19, 20). In brief, both the α- and β-chains of the DR1 construct contain murine I-E leader sequences, followed by DRα and DRβ first domains and murine I-Eα and I-Eβ second, transmembrane, and cytoplasmic domains.

Production of soluble HLA-DR protein

Soluble DR1 was purified from culture supernatants of S2 Drosophila cells transfected with DRB1*0101 and DRA1*0101. Both the α- and β-chains of the DR1 constructs contain murine I-E leader sequences, followed by DRα or DRβ first domains and murine I-Eα and I-Eβ second domains. The immunodominant collagen peptide, residues 259–273, was inserted after the third residue of the β-chain, and a flexible (Gly)4-Ser3 linker was added that allows the peptide to fold into the binding groove of the DR molecule (Fig. 1) (13). The sequences encoding the cytoplasmic and transmembrane portions of these molecules were deleted from the cDNA using PCR, and for the α-chain, a new stop codon was inserted at the 3′ end of the second domain. The β-chains were altered by adding a linker and a biotinylation site 3′ to the β-chain second domain, as described by Kappler et al. (11), to allow for site-specific addition of biotin and tetramerization using streptavidin. The resulting cDNA was cloned into the Drosophila expression vector pRmHA-3 (gift from Dr. D. Zaller, Merck, Rahway, NJ). S2 cells were transfected with a 10:10:1 ratio of chimeric DRB1 and DRA1 to pUCsNeo using calcium phosphate precipitation. Soluble DR1 production was induced by 1 mM CuSO4, and 5 days later, the culture supernatant was collected and adjusted to 0.05% o-cetyl glucoside (OcG). The soluble DR was purified by passage of the culture supernatant over an affinity column coupled with the anti-DR Ab LB3.1. The column was washed with 0.05% OcG and 0.15 M NaCl in phosphate buffer, pH 7.5, followed by 0.05% OcG and 0.5 M NaCl in phosphate buffer, pH 7.5, and a final wash with 10 mM Tris in 0.5 M NaCl, pH 7.5. The DR was eluted with 100 mM Tris and 0.5 M NaCl, pH 11.2, and the fractions were immediately neutralized with acetic acid. The DR recovered was concentrated using an Amicon Stirred Cell, quantitated by OD280 absorption and ELISA, and was evaluated by SDS-PAGE for purity before use.

T cell hybridoma stimulation assay

Recombinant DR1-CII protein, diluted to 400 μg/ml in PBS, was added at 40 μg/well to the first well of the top row of a 96-well plate (Costar 3595) and was serially diluted across the plate. After an overnight incubation at 4°C, the plate was washed three times with 200 μl of PBS/well, and 10 T hybridoma cells in 200 μl of DMEM complete (DMEM supplemented with 10% FBS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine) were added to each well. After a 24-h incubation, 80 μl of supernatant was removed from each well, and 2-fold serial dilutions were made down the rows of the plate. IL-2-dependent HT-2 cells (6 × 104) were then added to each well of the 96-well plate, and after an 18-h incubation, HT-2 cell viability was assessed by cleavage of MTT and quantitation of OD at 690 nm, with background absorption at 560 nm subtracted (21, 22). IL-2 titers were quantified by the reciprocal of the highest 2-fold serial dilution maintaining HT-2 cell viability >2-fold over that in control cultures. Results are presented as units of IL-2 per milliliter of undiluted supernatant, as originally described by Kappler et al. (23).

Tetramer production

Tetramers were formed using biotinylated DR1 protein and PE-conjugated streptavidin as described by Crawford et al. (11). Before biotinylation, the protein buffer was exchanged with 0.01 M Tris, pH 8, and the protein solutions were diluted to 2.5 mg/ml in Tris, pH 8. The protein was then biotinylated overnight using the BirA enzyme at a ratio of 15 μg of BirA to 38 nmol of protein as instructed by the manufacturer (Avidity). After biotinylation, the protein buffer was exchanged with PBS supplemented with 0.1% Na3, and the protein was tetramerized with PE-conjugated streptavidin (PE-SA; Rockland Laboratories) at a final ratio of 66 μg of DR protein to 10 μg of PE-SA. To favor the formation of tetrameric molecules, PE-SA was added in two separate steps, 12 h apart.

Tetramer binding

For tetramer staining of T cells, lymph node cells were diluted to a concentration of 2.5 × 107/ml in HL-1 medium (BioWhittaker) supplemented with 50 U/ml penicillin G sodium, 50 μg/ml streptomycin sulfate, 0.05 mM 2-ME, 2 mM L-glutamine, and 0.1% BSA (complete HL-1), and T hybridoma cells were diluted to a concentration of 2.5 × 107/ml in DMEM complete. One million lymph node cells or 107 T hybridoma cells were then aliquoted into a 96-well plate and were incubated with 1 μg of tetramer in 10 μl of complete medium supplemented with 5% Na3. Cells were incubated at 37°C for 2.5 h, at which time Abs to cell surface markers were added, and cells were incubated for an additional 30 min at 4°C. In some experiments two different Abs conjugated to the same fluorochrome were used for purposes of inclusion or exclusion of specific cell populations for data analysis. For example, Abs to BV8 and BV14 were frequently used simultaneously in the fluorescence 1 (FL1) channel to select all cells expressing either TCR for data analysis. Similarly, exclusion of CD8+ T cells and CD19+ B cells was accomplished by using anti-CD8- allophycocyanin and anti-CD19-allophycocyanin simultaneously in the FL4 channel and subsequently gating on the CD8+ and CD19+ population of cells. Populations that were positive for either BV8 or BV14 and were negative for both CD8 and CD19 are referred to in the text as BV8+/ BV14- /CD8+/ CD19- /CD8 /CD19 populations. After incubation with Abs to cell surface markers, samples were washed three times with 200 μl of PBS supplemented with 0.1% Na3, and 2% FBS, resuspended in 200 μl of PBS supplemented with 0.1% Na3, and 2% FBS, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). For sorting of cells and subsequent RNA analysis, cells from the draining lymph nodes of DR1 mice were harvested 10 days postimmunization with CII and were incubated with anti-CD8 and anti-CD19 magnetic beads (Miltenyi Biotec) and subjected to magnetic sorting to remove CD8+ T cells and CD19+ B cells, thereby enriching for CD4+ T cells. Cells were then washed and stained with tetramer and cell surface markers as described above, except for the use of only 0.5 μg of tetramer/106 cells. The cells were then separated into tetramer-negative and tetramer-positive populations using a MoFlo FACS (DakoCytomation).

For recovery and staining of mononuclear cells infiltrating arthritic joints, arthritic hind limbs were amputated about the ankle joint, and the

![FIGURE 1](image-url)
skin around the arthritic joint was removed. Care was taken to avoid contamination of the cell preparation with bone marrow and peripheral blood. The inflamed edematous tissue surrounding and infiltrating the arthritic joint was carefully removed using a scalpel blade (no. 10) and forceps and placed into culture medium, and the tissue was dissociated with forceps. Single cell populations were separated from large tissue masses by 1×g sedimentation, and the cells were washed in culture medium. For tetramer staining and immunofluorescence, the same procedures were used as described above for cells recovered from lymph nodes.

**RT-PCR**

RNA was isolated from cells using the RNAqueous-4PCR kit according to the manufacturer’s protocol (Ambion). RT was conducted at 25°C for 10 min, 37°C for 1 h, and 95°C for 5 min using MultiScribe Reverse Transcriptase (Applied Biosystems). RT-PCR was performed using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Amplification was conducted for 40 cycles using Taq Gold Enzyme (Applied Biosystems) and AmpErase (Applied Biosystems) according to the following protocol: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of the following two steps: 95°C for 15 s and 60°C for 1 min. The optimized ABI probe/primer pairs (Applied Biosystems) used were IL-2 (no. 4329585T), IL-4 (no. 4329590T), IL-6 (no. 4329592T), IL-10 (no. 4329593T), IFN-γ (no. 4339850T), TNF-α (no. 4329596T), IL-17 (no. Mm00439619_ml), CCR3 (no. 4329569T), and CCR5 (no. 4329571T). Probes were labeled with a 6FAM reporter dye and a minor groove binding/nonfluorescent quencher.

**Immunization and primary cell culture**

Mice were immunized with 100 µl of an emulsion consisting of equal parts bovine CII at 4 mg/ml and CFA (Difco). Ten days later, draining lymph nodes were removed, and cells were cultured in 96-well plates at 5×10⁵ cells/well in 300 µl of complete HL-1 and 100 µg/well bovine CII 257-274. On day 4 of culture, a sample of cells was collected, washed, and resuspended for tetramer staining, and the remaining cells were layered over a Lympholyte-M (Cedarlane Laboratories) gradient to remove dead cells. On day 4 of culture, a sample of cells was collected, washed, and resuspended for tetramer staining, and the remaining cells were layered over a Lympholyte-M (Cedarlane Laboratories) gradient to remove dead cells. Live cells collected from the interface were resuspended at a concentration of 5×10⁶/ml in complete HL-1 supplemented with 20 U of IL-2/ml (PeproTech). Cells were cultured in a 24-well plate at 10⁶ cells/well. This process was repeated for analysis on days 7 and 10. In some experiments lymph node cells were labeled with CFSE (Molecular Probes) before stimulation in culture. For CFSE labeling, cells were resuspended at 1×10⁶/ml in prewarmed (37°C) sterile PBS with 0.8 µM CFSE, incubated for 30 min at 37°C, washed again, and used in the stimulation assay described above.

**Results**

**Functional analysis of recombinant soluble DR molecules**

To mediate the elicitation of CIA in DR1 Tg mice, we developed a chimeric DR1 (DRB1*0101) recombinant molecule (Fig. 1) to function as an Ag-specific TCR ligand. When the ability of this recombinant DR1 molecule to stimulate DR1-restricted, CII-specific T cells was tested by allowing it to adhere to the wells of a 96-well plate, the immobilized recombinant DR protein was found to be very efficient at stimulating IL-2 production by the Ag and DR restriction. For example, T cell hybridoma lines DR1-16 and DR1-22.7 bound tetrameric DR1-CII, whereas the DR4-restricted T hybridoma, DR4-61.7, did not (Fig. 2B). This binding was Ag specific, because the tetrameric DR1-CII failed to bind to HA-specific hybridomas that are restricted to DR1 (HA33.6-6; Fig. 2). The CII-specific T cell response of DR1 mice was initially characterized by analysis of T cells ex vivo and after short periods of stimulation in culture. DR1 mice were immunized with CII, and 10 days later T cells from draining lymph nodes were stained with tetramer and anti-CD4 either immediately or after several days of restimulation in culture with the immunodominant CII peptide. As shown in Fig. 3, a clear increase in the percentage and number of CD4⁺ T cells that bound the DR1-CII tetramer could be observed both ex vivo and after stimulation in culture compared with controls. Although the ex vivo analyses of DR1-restricted, CII-specific T cells revealed only a small population of tetramer⁺/CD4⁺ T cells compared with controls, by day 4 of stimulation in culture a clearly discernable population, representing an ~10-fold expansion of cells, was visible. Additional evidence of the specificity of the tetrameric DR1 molecule for CII-specific cells was provided by CFSE staining of cultured cells, which showed that 90% of the tetramer-positive, DR1-specific T cells present on day 4 of culture...
were actively dividing (Fig. 3). By day 7 of culture, the tetramer-positive, DR1 CII-specific T cells reached >30% of the total cells. Again, the specificity of the tetramer staining was quite clear, because nonspecific binding by cells derived from DR1 mice immunized with CII, but stained with a DR1-HA peptide tetramer (Fig. 3, lower panels), was extremely low.

After identification of CII-specific cells with tetramer, BV usage of these cells was analyzed. PCR analysis indicated that these T cells express the same TCR BV gene segments that are preferentially used by the CII259–273-specific, DR1-restricted T cell hybridomas (24). As shown in Fig. 4, virtually all the tetramer+ T cells that responded to CII stimulation in vitro expressed either the BV14 or BV8 (BV8.1, BV8.2, or BV8.3) gene segment. The ratio of tetramer+ T cells expressing BV14 to those expressing BV8 (~2:1) was very similar to that observed using panels of T cell hybridomas derived from DR1 Tg mice immunized with CII (24). Collectively, these two TCR chains accounted for >90% of the tetramer+ T cells, thus identifying them as the major T cell population responding to the CII259–273 determinant presented by DR1.

Ex vivo analysis of the developing autoimmune T cell response

Based on the data reported above indicating that CII-specific T cells could be detected ex vivo (Fig. 3) and that they expressed a limited TCR BV repertoire (Fig. 4), these approaches were combined with the use of the DR1-CII tetramer to study ex vivo the development and characteristics of the autoimmune T cell response during elicitation of CIA. DR1 mice were immunized with CII, and the numbers of CD4+/BV8+/BV14+/CII259–273-specific T cells harvested from draining lymph nodes and their expression of several CD markers were measured ex vivo at several time points after immunization. As depicted in Fig. 5, the development of the CII259–273-specific T cell autoimmune response can be clearly visualized by this approach. In comparison with control DR1 mice immunized with OVA, a significant number of T cells specific for the CII peptide could be detected as early as 5 days after immunization. The peak expansion of CII-specific T cells was observed on day 10, reaching ~5-fold higher numbers than on day 5. Thereafter, the numbers of tetramer+ T cells steadily, but slowly, declined (Figs. 5 and 6A), although a very small tetramer+ T cell population was still discernable on day 130 postimmunization.

In addition to quantitating the CII-specific T cell response over time, we used this approach to measure the expression of several CD markers associated with activated T cells (Fig. 6B). CD62L, an activation marker involved in the trafficking of naive T cells to lymph nodes early in the immune response, was expressed by 45% of the CII-specific T cells on day 5, but expression decreased markedly between days 5 and 10 and remained low at the rest of the time points studied. In contrast, CD44, an activation marker that is expressed at low levels in naive T cells and at much higher levels in activated and memory T cells, was significantly up-regulated over time. Less than 50% of the CII-specific T cells expressed CD44high on day 5, and this number increased to nearly

FIGURE 3. Flow cytometric analysis of CII-specific T cells from DR1 mice immunized with CII. Draining lymph nodes from DR1 mice were removed 10 days after immunization with CII, and cells were either stained ex vivo with DR1-CII tetramer (top panel) or a DR1-HA tetramer control (bottom panel) or were restimulated in culture with CII257–274 or HA peptide. After day 4 of culture, T cells were expanded with IL-2. Restimulated T cells were collected on days 4 and 7 of culture and stained with DR1-CII tetramer, followed by anti-CD4, anti-CD8, and anti-B220. Data shown are gated on CD8+/CD4−/CD4+ cells. For the CFSE experiment (top right panel), lymph node cells were recovered from DR1 mice immunized with CII, labeled with CFSE, and then restimulated with 100 μg/well CII or cultured without stimulation (○) for 4 days, then analyzed by flow cytometry. More than 90% of the tetramer+/CD4+ T cells had undergone cellular division.

FIGURE 4. DR1-CII tetramer-positive cells preferentially use TCR BV8 and BV14. Lymph node cells from DR1 Tg mice immunized with CII were cultured with CII peptide for 4 days, then stained with DR1-CII tetramer, followed by anti-CD4, anti-BV8, and anti-BV14 Abs. Data shown are BV14+ cells (A) and BV8+ cells (B) and are gated on CD4+/tetramer+ T cells.
CD8-derived from these tetramer-positive and -negative populations of at least five mice per time point and are based on a study as described for Fig. 5. The data in the top panel of FIGURE 6. Development of the CII-specific T cell response in vivo. To analyze cytokine expression by tetramer+ cells, lymph node cells from mice immunized with CII 10 days earlier were stained with DR1-CII tetramer and several Abs ex vivo and sorted into CD4+/CD19−/CD8−/BV8+/BV14+/tetramer+ and CD4+/CD19+ /CD8+/BV8+/BV14+/tetramer− populations using FACS. mRNA derived from these tetramer-positive and -negative populations was then subjected to real-time PCR analyses. As demonstrated in Fig. 7, T cells that bound the DR1-CII tetramer expressed a 5- to 10-fold higher level of inflammatory and Th1 cytokine transcripts, including IL-2, IFN-γ, IL-6, TNF-α, and IL-17. Somewhat surprisingly, levels of CCR5, a chemokine receptor associated with Th1 responses, were only marginally elevated in tetramer-positive cells, although the expression of this chemokine may be associated only with acquisition of a memory phenotype (25). Levels of the Th2 cytokine IL-10 were slightly higher in the tetramer-positive population, although transcripts of IL-4 and Th2-associated chemokine receptor CCR3 were barely detectable in either population (data not shown). The cytokines expressed in tetramer+ cells in combination with the CD markers expressed on tetramer− cells over time indicate that these T cells probably play an active inflammatory role in disease pathogenesis and appear to be resistant to homeostatic down-regulation of their activation state.

**Analysis of T cells in arthritic joints**

Although it is known that CII-specific T cells play an important role in promoting the activation of B cells to produce Ab to CII, it has been difficult to determine whether CD4+ CII-specific T cells...
migrate to arthritic joints and participate directly in the pathogenesis of CIA. To determine whether CII-specific T cells can be found in the arthritic joints of these mice, synovial fluids and tissue were recovered from arthritic limbs within 3 days of onset of disease. Tissue was carefully excised to avoid contamination by bone marrow or peripheral blood, dissociated into single cells, and stained with DR1-CII tetramer, followed by Abs to CD19, CD8, BV8, BV14, and CD4. A. Percentage of T cells expressing either BV8 or BV14 (70%) after gating on CD4+/CD19+/CD8- cells. B and C. Percentage of BV8+/BV14+ or BV8- /BV14- cells that bind the DR1-CII tetramer. D and E. Tetramer binding was negligible in the CD19- and CD8 populations, respectively.

Discussion

Using a highly specific HLA-DR1-CII tetramer to identify CII-specific T cells, we have advanced our understanding of the role and function of these T cells in a DR1 Tg mouse model of arthritis. Ex vivo studies of these cells in real time revealed the expansion and function of CII-specific T cells in the lymph nodes of these mice and demonstrated for the first time the presence of CII-specific, CD4+ T cells in the arthritic joints of these animals. Through these studies, we have demonstrated that the CII-specific T cell response that mediates the development of autoimmune arthritis in DR1 Tg mice constitutes only 1% or less of the CD4+ T cell population (1% or less), this still represents at least a 1000-fold expansion of these cells given an estimated Ag-specific T cell precursor frequency of $10^{-5}$–$10^{-6}$. The development of the autoimmune T cell response was also evident through phenotypic changes in CII-specific T cells. These T cells quickly gained expression of the activation marker CD69 and the memory marker CD44B+H6, and the majority of the DR1-CII tetramer+ T cells maintained expression of these markers for at least 130 days. Similarly, activation of these T cells was also evident by the sharp drop in CD62L expression, a ligand that regulates T cell migration and is downregulated by T cell activation (26). We also examined the expression of CD25, but very few of the tetramer+ CII-specific T cells expressed CD25 at early time points, and no significant changes in this expression were observed over time. In all, this approach demonstrates that a detailed ex vivo image of the developing autoimmune T cell response can be achieved using class II tetramers, and our data suggest that autoimmune T cells mediating CIA remain activated long after their initial B cell help function has been conducted. Alternatively, these data may indicate that new CII-specific T cells are being generated, perhaps in response to joint destruction and the subsequent availability of murine collagen to the immune system, although the advent of arthritis does not appear to result in a re-expansion of CII-specific T cells in the periphery.

The T cells identified by the DR1-CII tetramer expressed high levels of cytokines that are associated with autoimmune pathogenesis. The cytokines expressed by the DR1-CII tetramer+ BV8+/BV14+ T cell population, compared with BV8-/BV14+ tetramer- cells, were overwhelmingly skewed toward the Th1 type. Levels of IFN-γ and IL-2 mRNA were 10-fold higher in the tetramer+ population compared with the tetramer- cells. Somewhat surprisingly, expression levels of TNF-α were increased only 5-fold in tetramer+ cells, whereas mRNA levels of IL-17 were increased >15-fold. TNF-α is a key inflammatory mediator in the pathogenesis of both CIA and RA, as has been shown by the lack of susceptibility to CIA in TNF-α knockouts and the success of anti-TNF-α and soluble TNF-α receptor therapies in both CIA and RA (27–30). Similarly, IL-17 has been shown to be an important pathogenic cytokine in the CIA model, because mice genetically
deficient for IL-17 are protected from CIA (31), whereas the administration of IL-17 to mice causes an increase in CIA disease severity (32). Additionally, increased levels of IL-17 have been found in the inflamed synovium of RA patients (33). These elevated levels of Th1 and inflammatory cytokine mRNA in the tetramer+ T cells support their identification as CII-specific T cells that are probably playing a significant role in promoting the development of disease in this arthritis model.

The primary mechanism of immunopathogenesis in the CIA model is the production of CII-specific Ab. Passive transfer of sera or a mixture of mAb to CII induces severe arthritis within days (34, 35). Although a direct pathogenic role of T cells has been suggested for CIA, passive transfer of arthritis with CII-primed T cells has been largely unsuccessful (36). Our analysis of T cells in the arthritic joints of these mice, however, suggests that these cells are specifically recruited or retained in the inflamed synovium. Although PMNs appear to be the predominant cell type recovered from these joints, the majority of the lymphocyte population were CD4+ T cells and B cells. Seventy percent of the CD4+ T cells in these joints expressed the TCR-BV8 or BV14 chain, and the vast majority of these BV8+ or BV14+ cells bound the DR1-CII tetramer. The frequency of BV8- and BV14-expressing T cells in the synovium was 2-fold higher than that found in peripheral blood (37), and CDR3 analysis of these T cells indicates they are of limited clonality (Z. Qian, K. Latham, and E. Rosloniec, manuscript in preparation). It is not clear whether these cells are preferentially retained in the inflamed synovium because of their specificity for CII or if once there they are activated and proliferate in response to the murine CII peptides generated by the inflammatory response. The peptide representing the immunodominant determinant in bovine and murine CII differs by only one amino acid, Glu and Asp, respectively, at position 266, and cross-reactivity between these peptides has been frequently observed for CII-specific T cells (38). This raises the possibility that the T cells found in the joint are either supporting the local stimulation of B cells and the production of CII-specific Ab or are participating directly in the pathogenesis of the disease, perhaps through their production of cytokines that alter the function of the synovial cells.

This tetramer-based approach to the analysis of an autoimmune T cell response offers several advantages over conventional approaches. First, cells are studied ex vivo, thus avoiding any tissue preparation. It is not clear whether these cells are preferentially retained in the inflamed synovium because of their specificity for CII or if once there they are activated and proliferate in response to the murine CII peptides generated by the inflammatory response. The peptide representing the immunodominant determinant in bovine and murine CII differs by only one amino acid, Glu and Asp, respectively, at position 266, and cross-reactivity between these peptides has been frequently observed for CII-specific T cells (38). This raises the possibility that the T cells found in the joint are either supporting the local stimulation of B cells and the production of CII-specific Ab or are participating directly in the pathogenesis of the disease, perhaps through their production of cytokines that alter the function of the synovial cells.

This tetramer-based approach to the analysis of an autoimmune T cell response offers several advantages over conventional approaches. First, cells are studied ex vivo, thus avoiding any tissue culture effects on gene expression and T cell function. Stable tetramer binding to T cells can be achieved within two hours and the assay is performed at physiological temperatures. Second, all measurements of phenotype and function are focused on T cells of interest instead of an averaged analysis of the total T population derived from the lymphoid tissue. Although the frequency of the Ag-specific CD4+ T cells of interest is low both in our analyses and others (12, 14, 17, 39), this problem can be overcome by analysis of large numbers of cells and by focusing the analysis on specific subpopulations. In our analysis, identification of CII-specific T cells was possible relating only CD4+ T cells and tetramer binding, but the sensitivity of detection was vastly improved by the use of Abs specific for the TCR BV chains preferentially used by CII-specific T cells. In terms of data acquisition and analysis, we found that care must be taken in quantitating these low frequency events, because background or nonspecific fluorescence can significantly compete with a 1% or less positive population. The use of both inclusion (CD4+/BV8+ or BV14+) and exclusion (CD8+/CD19-) gates for data analysis proved to be critical to ensure the high degree of sensitivity and reproducibility in identifying these low percentage events.

Although the CII-specific T cell response could be clearly visualized by our DR-CII tetramers, some questions remain as to whether these tetramers detect all the DR1-restricted CD4+ T cells specific for the CII260-273 antigenic peptide. We have seen no evidence of epitope spreading in this model, and the T cell response is strongly focused on this one dominant determinant, indicating that we are probably measuring the vast majority of the CII-specific T cell response (7, 19). In addition, the frequency of CII-specific T cells we observed is similar to that reported in other MHC class II tetramer-based studies of Ag-specific T cells (12, 14, 17, 39). Nevertheless, it is still possible that we are failing to identify some small population of CII-specific T cells that either have low affinity for DR1-CII or for some other unknown reason fail to bind the tetramer. In our analysis of tetramer binding by T cell hybridomas we found that 10% of these cells do not stably bind the DR1-CII tetramer despite their ability to recognize the same CII peptide presented by APC. Whether this is an artifact of T cell hybridomas or represents actual events that occur in the ex vivo analysis of T cells with tetramers is unclear and will probably require the establishment of cloned T cell lines to determine. In all, this tetramer-based approach has significantly broadened our understanding of the role and function of autoimmune T cells during the development of CIA. The discovery of both long term, CII-specific T cell activation and the presence of CII-specific T cells in the joints of arthritic animals indicates that these cells may play a more direct role in disease pathogenesis beyond their originally described role in promoting an anti-CII B cell response.

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