Nucleotide Sequencing of Psoriatic Arthritis Tissue before and during Methotrexate Administration Reveals a Complex Inflammatory T Cell Infiltrate with Very Few Clones Exhibiting Features That Suggest They Drive the Inflammatory Process by Recognizing Autoantigens

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Nucleotide Sequencing of Psoriatic Arthritis Tissue before and during Methotrexate Administration Reveals a Complex Inflammatory T Cell Infiltrate with Very Few Clones Exhibiting Features That Suggest They Drive the Inflammatory Process by Recognizing Autoantigens

Shane A. Curran,*† Oliver M. FitzGerald,† Patrick J. Costello,*† Jeanette M. Selby,* David J. Kane,† Barry Bresnihan,† and Robert Winchester2*†

Psoriatic arthritis is an interesting MHC class I allele associated autoimmune disease where injury is likely mediated exclusively by T cells. We used TCR β-chain nucleotide sequencing to gain insight into the adaptive immune events responsible for this injury and determine whether the numerous oligoclonal expansions of this disease represent extreme determinant spreading among driving clones that recognize autoantigen or were non-Ag-driven, inflammation-related expansions. Because methotrexate suppresses but does not eliminate this inflammation, we hypothesized that clones persisting during methotrexate treatment would likely drive the inflammation. Seventy-six percent of the T cell clones in active tissue were polyclonal and unexpanded, accounting for 31% of transcripts. They were decreased greatly by methotrexate. Strikingly, most expanded clones in the inflamed joint did not persist during methotrexate treatment, were found only in inflammatory sites, exhibited no structural homology to one another, and were either CD4 or CD8 in lineage, suggesting they were non-autoantigen-driven, inflammation-related expansions. Only 12% of the expanded clones could be grouped into clonal sets distinguished by structurally homologous CDR3 β-chain amino acid motifs suggesting Ag drive. These were exclusively CD8 in lineage, persisted during methotrexate administration, and were present in both joint fluid and blood implying they were candidate driver clones that recognized an autoantigen. However, a major set of putative driver clones exhibited a previously described EBV-specific β-chain motif, emphasizing that the dominant feature of the disease was activation of multiple clones apparently lacking specificity for an inciting autoantigen.


1 Abbreviation used in this paper: CDR3, complementarity determining region 3.
receptors differing in nucleotide sequence (9–15). Studies in a number of autoimmune diseases reveal that another distinguishing feature of Ag-specific T cell clones driving inflammation is that they often have highly expanded clonal blood precursor pools (16–18). Accordingly, one would hypothesize that the clones driving the inflammation in psoriatic arthritis would be CD8 in lineage, be present in each site of inflammation, have an expanded presence in blood, and exhibit evidence of Ag-driven determinant selection. Indeed, identification of CD8 T cell clones with these properties should facilitate the search for the identity of the elusive inciting peptide(s) (5).

However, there is increasing evidence that some T cells in an inflammatory infiltrate are unrelated to the inciting peptide. These include both clonally unexpanded polyclonal T cells (19, 20) and, importantly, a category of inflammation-related expanded T cell clones identified in the inflammatory site that do not demonstrably recognize the inciting peptides designated “bystander clones” (21). Some of these expanded bystander clones reflect the attraction, activation, and proliferation of memory/effector T cells induced primarily by immunoreactants found in the inflammatory milieu (21). Moreover at a structural level, these clones cannot be placed in clonal sets with homologous TCR structures that would reflect the signature of Ag drive suggesting that their presence in the inflammatory site is not a consequence of clonal selection by TCR engagement (21). In a study of psoriatic arthritis, the presence of these cognitively irrelevant clones would complicate the search for the inciting peptide that drives the inflammatory response because the peptides that they recognize are apparently unrelated to those driving the inflammation.

Earlier studies in psoriatic arthritis (22) showed that joint fluid in an inflamed joint contained a large number of oligoclonal expansions of either CD4 or CD8 T cell lineage demonstrated by CDR3 β-chain length analysis. One CD8 oligoclonal expansion present in both blood and joint fluid was sequenced and shown to be composed of a set of several clones predominantly exhibiting the CDR3 β-chain amino acid motif SQSPGGTQY, implying the operation of extensive Ag drive and determinant spread (22). Each of these clones was distinguished from the other by multiple differences at the nucleotide sequence. In another study in psoriatic arthritis, Tassiuas et al. (23) showed oligoclonal expansions in the tissue, some of which were shared with the skin. Nucleotide sequencing showed instances of homologous amino acid CDR3 motifs of T cells in both inflammatory skin and joint tissue in individual patients suggesting Ag drive, although clonal lineage was not determined. Thus, a major question is how to relate the large number of oligoclonal expansions found in either CD4 or CD8 T cell lineages (22) to the imputed recognition of a self-peptide by CD8 T cells. Were the many oligoclonal expansions due to extensive determinant spread typical of clones driven by an Ag (8)? If so, further study would identify multiple examples of CD8 T cell clones that could best be explained by autoantigen drive in each of the oligoclonal expansions along with the presence of the same clone in a highly expanded precursor pool in blood, as has been emphasized by Tian et al. (17). Or conversely, in view of the finding that both psoriatic and rheumatoid arthritis joints contain expanded clones specific for various viral peptides not directly implicated in the pathogenesis of the disease (24–26), would many of the identified clonal expansions prove to have the features of non-Ag-driven bystander inflammation-related clones?

The analysis of the TCR repertoire in a site of inflammation has been facilitated by the relative ease of CDR3 β-chain length analysis, a technique sometimes termed spectratyping or immunoscope, used extensively to identify oligoclonal expansions (27, 28). However, determining the clonotypic TCR β-chain nucleotide sequence has three advantages over CDR3 length analysis. It provides a more exact enumeration of clonal composition in a repertoire and the degree of expansion of each constituent clone. It permits determining whether an oligoclonal expansion is composed of a single homogeneous clone or a set of multiple clones from the same TRBV family, with the same CDR3 length, that upon translation of the nucleotide sequence reveals a homologous CDR3 motif suggesting they reflect clones driven by the same peptide Ag. Lastly, by unambiguously identifying a particular clone it allows tracking the presence of the clone in multiple sites and its persistence at different time points.

Because the synovial tissue and not the joint fluid is the principal site of the inflammation and joint destruction in psoriatic arthritis (1, 2), we sought to use newer high throughput nucleotide sequencing techniques as the primary analytical tool to take advantage of a clinical trial in which synovial tissue biopsies were obtained during acute arthritis and during extensive treatment with the disease-modifying drug methotrexate. This provided an opportunity to gain insight into the components of an inflammatory site before and during the effect of a disease-modifying drug that greatly diminishes, but does not totally abolish, inflammation (1) and to follow the fate of specific clones during this intervention. Moreover, because psoriatic arthritis often redevelops after methotrexate withdrawal (1), we reasoned that those clones present in acute inflammation and that persisted in the joint during treatment might be of particular interest as candidates to be driver clones responsible for disease chronicity and its recrudescence after discontinuing methotrexate.

**Materials and Methods**

**Study subjects**

Synovial tissue biopsies were obtained with informed consent at the time of active disease from seven male and two female patients undergoing arthroscopy. Repeat biopsies were obtained from five of these individuals 6–18 mo later when inflammation was reduced during methotrexate treatment as seen by a significant improvement in the Ritchie articular index, EULAR swollen joint count, and disease activity score (29). Paired blood and joint fluid were also obtained at the time of tissue biopsy from five patients during active disease and the purification of CD4 and CD8 T cell subsets by positive selection using CD4 and CD8 magnetic beads was performed as described (22). Determination of CD4 or CD8 T cell lineage from the synovial tissue biopsy was accomplished by identifying the presence or absence of the same clone with identical β-chain sequence in the separated CD4 or CD8 T cell subsets of blood or joint fluid, because efficient elution of lymphocytes from the small tissue biopsy samples was not feasible.

**CDR3 β-chain length spectratype analysis**

RNA was extracted and a cDNA repertoire representation was prepared from whole synovial tissue biopsies and separated CD4/CD8 subpopulations of blood and joint fluid as described (22). β-chain length spectratype analysis was conducted by PCR amplification using 23 different TRBV family primers on all samples (30). For representations of TRBV family repertoires for analysis by sequence, selected TRBV region families were cloned as described (30). High throughput selection and amplification of clones from each TRBV-specific TCR colony library was performed. Bacterial colonies were picked using toothpicks and the toothpick placed in 25 μl of water in a 96-well PCR plate. They were shaken for 3 min at 250 rpm. The toothpicks were removed and 1 μl was added to a 20 μl PCR mix containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.02 mM TRBV and TRBC primer, 0.02 mM dNTP, and 0.6 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Initial incubation at 95°C for 10 s was followed by 30 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 60 s, with a final extension of 72°C for 600 s. One microliter of this product was then used as template in a dye terminator sequencing reaction (30). The 3100 sequences obtained in this analysis were analyzed as described previously (22) with the exception that the earlier nomenclature of BV families proposed by Arden et al. (31) has been supplanted by both the new nomenclature using the TRBV families proposed by Arden et al. (31).
For length spectratype analyses across all TRBV families, the degree of clonality was calculated by defining the number and size of oligoclonal expansions exactly as described (22). Briefly, an oligoclonal expansion in a TRBV family was considered to be present at a particular CDR3 length when the area of a given CDR3 length was 5% greater than the 95th percentile of the area of that CDR3 length determined by averaging 140 normal distribution profiles for that particular TRBV family. Because the absolute number of T cells in blood, joint fluid and tissue, the quantity of total TCR message, and the relative abundance of each TRBV family could not be reliably measured, each site studied during active arthritis was treated as if it were the same size in terms of total number of transcripts with equal proportions of each TRBV family. To create the summary of repertoire composition from the sequence data, the number of sequences and clonal size were normalized as if 150 sequences per TRBV family had been obtained at each site, although the actual number of sequences sometimes varied from this number for technical reasons. The nucleotide sequences were then ordered by TRBV family, CDR3 length and the CDR3 sequence motif with identical sequences grouped together. The number of identical sequences was used to determine the relative clonal size in sequences per clone. For simplicity, a clone was considered expanded if two or more instances of identical TCR β-chain sequences were found during sequence analysis. It should be pointed out that this is not strictly accurate because two identical TCR β-chain sequences might not necessarily be derived from the same clone. For example, two T cells may share the same TCR β-chain but have different α-chains and be clonally distinct. Also, TCR α-chains but not β-chains may undergo secondary rearrangements in the periphery (33) and furthermore occasional dual TCR-bearing T cells are found (34). These sequence results were plotted as histograms, with the total area of the histogram equal to the total number of clones identified in the particular site for each TRBV family.

To obtain an average estimate across all TRBV families in all tissue samples for the number of clones, their size and the percentage of the repertoire they occupy, and the ratio of expanded to unexpanded clones, we first established that in 15 TRBV families in 7 active tissue samples the number of clones identified by nucleotide sequencing was 9.0 times greater than the number of clones that were identified by spectratyping. To describe the number of expanded clones of defined lineage and distribution found in active tissue, two samples that also had pooled joint fluid and blood as well as tissue obtained during methotrexate treatment were used and the actual number of expanded clones found by spectratyping across all TRBV families was averaged and multiplied by the adjustment factor of 9.0. This projected number of expanded clones that would be found across all TRBV families in a representative sample is multiplied by the proportion of expanded to unexpanded clones observed in the seven active tissue samples to give the projected number of nonexpanded single sequence clones.

To relate the repertoire in tissue obtained during methotrexate treatment to that obtained during active arthritis, we took advantage of the observation that certain clones persisted in both sites and used this number to adjust the size of the repertoire in tissue obtained during methotrexate treatment. The number of transcripts in the methotrexate sample was calculated with the assumption that the clonal size was equal to that in the active tissue. In tissue obtained during methotrexate treatment, the ratio of expanded to unexpanded clones from 12 TRBV families in 5 samples found by sequencing was 3.5 times that observed by spectratyping. The total number of clones observed by spectratyping in the two samples that also had blood, joint fluid, and active arthritis tissue was multiplied by 3.5 to give the total projected number of expanded clones in the tissue during methotrexate treatment.

To examine the question of the possibility of clonal selection by the same presented peptide Ag reflected by identification of clonal sets, the nucleotide sequence of each clone was translated using the ImMunoGeNeTics Web site (http://imgt.cines.fr/). This provided the germline TRBV, TRBD, TRBJ, and TRBC elements, the CDR3 length, and the somatically encoded N or P regions. For each CDR3 length and TRBV family, the amino acid sequence motifs of the CDR3 regions of each clone were compared with those of all other clones within each CDR3 length and TRBV family for homologous amino acids or identical amino acids encoded by codons made up from different nucleotides. The extent of homology was assigned according to the Dayhoff matrix (35). Different clones with identical or highly homologous amino acid motifs that differed at the nucleotide level were grouped into clonal sets, suggesting that they could have been selected by the same peptide Ag (9–15).

**Description of TCR repertoire clonality and clonal sets**

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Statistics

The Mann-Whitney U Wilcoxon rank sum test was used to compare the difference between TRBV families that were selected for sequencing and the remaining TRBV families analyzed by spectratyping. The χ² test was used to compare the proportion of unexpanded to expanded clones in the tissue in active disease and during methotrexate treatment. Both tests were done using SPSS 11 (SPSS, Chicago, IL). A binomial probability was used to test the chance of not obtaining a CD4 T cell sequence that persisted in the tissue during methotrexate treatment.

**Results**

Clonally unexpanded polyclonal CD4 T cells are the major component of the inflammatory infiltrate in the tissue during active arthritis

To obtain an overall description of the total αβ TCR repertoire in synovial tissue biopsies obtained during active psoriatic arthritis, we first used the intermediate resolution technique of β-chain CDR3 length spectratype analysis, illustrated in Fig. 1A. This revealed a predominance of quasi-Gaussian repertoire β-chain lengths in most TRBV families from nine samples indicating polyclonal T cells constituted a large proportion of the infiltrating T cells. Fig. 2A shows that the β-chain length spectratype of the inflammatory infiltrate in tissue most resembled that of the polyclonal CD4 T cell repertoire of paired blood or joint fluid. In addition to the polyclonal component, the infiltrating T cell repertoire had an average of 20 (range 11–29) oligoclonal expansions

![FIGURE 1. Clonally unexpanded polyclonal T cells are the major component of the inflammatory infiltrate in the tissue during active arthritis](http://www.jimmunol.org/)

![Tissue during active arthritis](http://www.jimmunol.org/)

![Tissue during methotrexate treatment](http://www.jimmunol.org/)

A Tissue during active arthritis

B Tissue during methotrexate treatment

Pl1 BV27

Pl1 BV12

Pl1 BV14

CDR3 Length (AA)

9 10 11 12 13 14 15 16

9 10 11 12 13 14 15 16

9 10 11 12 13 14 15 16 17 18 19

9 10 11 12 13 14 15 16 17 18 19

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across the total repertoire in all studied samples with Figs. 1A and 2A illustrating representative examples.

Fig. 2B illustrates β-chain nucleotide sequences from the same TRBV family and sample grouped together according to CDR3 length revealing active tissue was dominated by single sequence polyclonal T cells (gray rectangles in Fig. 2B). The pattern in Fig. 2B resembled the CDR3 length analysis (Fig. 2A). Expanded clones are shown by rectangles proportional in size to the number
of identical sequences forming the clone. The number of expansions found by spectratype analysis of the TRBV families that were sequenced did not differ from the number of expansions found in TRBV families studied only by spectratyping (p = NS). Some expanded clones found by sequencing were not evident by spectratype analysis, e.g., in the top panels in Fig. 2, A and B, two expanded clonal groups at CDR3 length 11 in active tissue were only identified by sequencing.

Grouping the 879 sequences obtained from 15 TRBV families in 7 active tissue samples into clones showed that 76% of clones were unexpanded single sequence clones with the balance of the repertoire consisting of expanded clones. Six of these TRBV families in two samples were also sequenced in blood, joint fluid, and tissue during methotrexate treatment. The results from the active tissue in these latter samples were averaged and extrapolated to account for all 23 TRBV families to give a total of 2077 T cell transcripts (Fig. 2D) present in the active tissue for the purpose of analysis. These transcripts were grouped into 845 T cell clones (Fig. 2C). Fig. 2C and Table I using the proportion of unexpanded to expanded clones observed in the 15 active tissue TRBV families shows that 643 of 845 (76%) of all clones in active tissue were unexpanded single sequence clones, accounting for 31% (643 of 2077) of all transcripts. Fig. 2C and Table I show an inferred 202 expanded clones in active tissue, comprising 1434 β-chain sequences (Fig. 2D); average clonal size 7.1 sequences per clone (Table I).

**Methotrexate treatment markedly diminished the proportion of the infiltrate composed of polyclonal T cells**

Spectratype analysis revealed that synovial tissue biopsies obtained after 6–18 mo of methotrexate treatment as seen in Figs. 1B and 2A had a substantial decrease in the polyclonal component of the synovial T cell infiltrate compared with the repertoire in the inflamed tissue. This decrease in the polyclonal component revealed oligoclonal expansions as the predominant feature (Fig. 1B), with an average of 33 (24–42) oligoclonal expansions identified in each tissue biopsy across all TRBV families.

Nucleotide sequencing of selected TRBV families revealed that (Fig. 2C, Table I), the number of single sequence clones in the tissue during methotrexate treatment decreased markedly from 643 to 43, while expanded clones decreased only from 202 to 53. The proportion of single sequence to expanded clones decreased from 3:1 in the active tissue to ~1:1 in the tissue during methotrexate treatment (p < 0.001). Fig. 2, A and B, shows that the sequence and spectratype profiles of tissue obtained during methotrexate treatment most resembled the highly oligoclonal CD8 T cell repertoires of paired joint fluid obtained during active arthritis.

The majority of expanded clones in active arthritis tissue were found exclusively in active tissue and were not present in any other site

The 202 expanded clones calculated to be present in the active tissue were placed in four major groups according to their distribution (Fig. 2E), based on whether the transcripts were also found in blood or joint fluid, their lineage and whether they persisted in the tissue during methotrexate treatment. Fig. 2E shows that 91 of the 202 expanded clones were only found in tissue and were not identified in blood or joint fluid during active disease. Eighty-six of these 91 clones (colored green in Fig. 2, B and E), found exclusively in the inflamed tissue, did not persist during methotrexate treatment and had a clonal size of 6.1 sequences per clone, Table II. Only 5 of these 91 expanded clones persisted in tissue obtained during methotrexate treatment with an average clonal size of 3.3 sequences per clone (Fig. 2E and Table II).

**Some expanded CD8 clones identified in the active tissue persisted during methotrexate treatment and were also present in both blood and joint fluid**

Because clonal lineage was determined by the presence of a clone from the tissue in the separated blood or joint fluid, or both, we were able to determine lineage in the remaining inferred 111 of 202 expanded clones found in active arthritis tissue. Fig. 2E and Table II showed that 22 of the subset of 111 expanded clones persisted during methotrexate treatment and were exclusively composed of transcripts identified in CD8 lineage T cells. Another 44 clones present in blood or joint fluid or both were also identified as CD8 but these did not persist in the tissue during methotrexate treatment. Interestingly, no sequences from clones identified as CD4 lineage were found to persist in the tissue during methotrexate treatment. Because this lineage ascertainment was based on an actual sample of 59 β-chain sequences that were all identified as originating from clones shown to be of CD8 lineage, the chance of not obtaining a CD4 sequence among a sample of 59 sequences in the tissue during methotrexate treatment was p = 8.15e−4.

Fig. 2E showed that 18 (colored blue) of the 22 persisting CD8 lineage clones were of particular interest because they were expanded in both blood and joint fluid taken during active disease. They exhibited the greatest clonal expansion, 9.2 sequences per clone, although representing only 2% (18 of 845) of the total clonal repertoire in active tissue. Moreover, these 18 clones were highly expanded in blood (20.4 sequences per clone). Fig. 2E shows two minor components of the inflammatory repertoire that were identified in the blood or in the joint fluid (two clones each) that persisted during methotrexate treatment but which were not further studied.

**Table I. Comparison of clonal composition of tissue during active arthritis and methotrexate treatment**

<table>
<thead>
<tr>
<th>Disease State</th>
<th>Unexpanded single sequence clones</th>
<th>Expanded clones*</th>
<th>No. of Clones</th>
<th>Size</th>
<th>No. of Clones</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active arthritis</td>
<td>643</td>
<td>1.0</td>
<td>202</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate treated</td>
<td>43</td>
<td>1.0</td>
<td>53</td>
<td>26.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expanded clones contain two or more identical β-chain sequences.

**Table II. Expanded clones in the inflammatory infiltrate during active arthritis exhibit different patterns of distribution**

<table>
<thead>
<tr>
<th>Persistence of Tissue Clones during Methotrexate Treatment</th>
<th>Exclusively found in active arthritis tissue</th>
<th>Found in tissue and also present in joint fluid or blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Clones</td>
<td>Size</td>
<td>No. of Clones</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Do not persist</td>
<td>86</td>
<td>6.1</td>
</tr>
<tr>
<td>Persist</td>
<td>5</td>
<td>3.3</td>
</tr>
</tbody>
</table>
Table III. Homologous CDR3 structure suggesting Ag drive identified among sets of expanded CD8 clones distributed across blood, joint fluid, and tissue during active psoriatic arthritis and methotrexate treatment.

<table>
<thead>
<tr>
<th>Name</th>
<th>Pt</th>
<th>BV</th>
<th>CDR3 Length</th>
<th>CDR3 Region Sequence</th>
<th>BJ</th>
<th>Act Tis</th>
<th>Mtx Tis</th>
<th>PB CD8</th>
<th>JF CD8</th>
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</thead>
<tbody>
<tr>
<td>CS1a</td>
<td>1</td>
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<td>11</td>
<td>GCC AGC AGC Cag tcc cCA GGC ggt ACG CAG TAT</td>
<td>BJ2-3</td>
<td>5</td>
<td>24</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>CS1b</td>
<td></td>
<td>BV14</td>
<td>11</td>
<td>GCC AGC AGC CAA tcc ccc GGG GGG AGC CAG TAT</td>
<td>BJ2-3</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
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<td></td>
<td>BV14</td>
<td>11</td>
<td>GCC AGC AGC Cag agc cCA GGC ggt ACG CAG TAT</td>
<td>BJ2-3</td>
<td>1</td>
<td>0</td>
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<td>7</td>
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<tr>
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<td></td>
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<td>11</td>
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<td>BJ2-5</td>
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<td>10</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
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<td>BV14</td>
<td>11</td>
<td>GCC AGC AGC Cag tcc cca Gac ggt ACG CAG TAT</td>
<td>BJ2-3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
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<tr>
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<td>BV14</td>
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<td>GCC AGC AGC Cag tca acG GGG GGG ACC CAG TAC</td>
<td>BJ2-5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td></td>
<td>BV14</td>
<td>19</td>
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<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>CS1h</td>
<td></td>
<td>BV14</td>
<td>19</td>
<td>GCC AGC AGC Cac ctc cca ggg GGG GGG ctc ggc ggG CAG TAT</td>
<td>BJ2-1</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>CS1i</td>
<td></td>
<td>BV14</td>
<td>19</td>
<td>GCC AGC AGC CAA tcc ccc ggg GGG ACA GGG tcc cgc aCG ACC GGG GAG CTG TGG</td>
<td>BJ2-2</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
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<tr>
<td>CS2a</td>
<td>1</td>
<td>BV4</td>
<td>14</td>
<td>GCC AGC AGC CAA Gct ggg GGG GGG tct ggg ggg CAG TAT</td>
<td>BJ2-3</td>
<td>21</td>
<td>30</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>CS2b</td>
<td></td>
<td>BV4</td>
<td>14</td>
<td>GCC AGC AGC CAA Cac ctc cca ggg GGG GGG ctc ggc ggg GGG ACC CAG TAC</td>
<td>BJ2-5</td>
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<td>0</td>
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<td>1</td>
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<tr>
<td>CS2c</td>
<td></td>
<td>BV4</td>
<td>14</td>
<td>GCC AGC AGC CAA Gct ccc ggg GGG GGG cca AAT GAG CAG TAC</td>
<td>BJ2-1</td>
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<td>CS3a</td>
<td>2</td>
<td>BV18</td>
<td>11</td>
<td>GCC AGC TCA Cct ATG GGG GGG GGG tcc ggg ggg CAG TAT</td>
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<td>BV18</td>
<td>11</td>
<td>GCC AGC TCA Cct ccc ggg ggg ggg gcc cca ACC GAG CAG TAC</td>
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<tr>
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<td>BV28</td>
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<td>BJ2-1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>21</td>
</tr>
</tbody>
</table>

*The nongermline encoded N region is shown in lower case while the germline D region is in upper case and underlined. The flanking V and J regions are shown in upper case. Degeneratively encoded amino acids and those differing among clones in an oligoclonal expansion are highlighted in bold. Correspondence between the IMGT and Arden TCR nomenclatures: TRBV14/16; TRBV4–2 = BV7S1; TRBV18 = BV18; TRBV28 = BV3; TRBV4–1 = BV7S1. CS: clonal set; C: clone; Pt: patient sample; BV: β-chain variable gene element; BJ: β-chain joining gene element; Act Tis: tissue during active arthritis; Mtx Tis: tissue during methotrexate treatment; PB: blood; JF: joint fluid.
Table IV. Differences in lineage, distribution, and clonal size of expanded T cell clones found in the tissue and also in blood or joint fluid that persisted during methotrexate treatment compared to clones that did not persist

<table>
<thead>
<tr>
<th>CD8 Lineage Active Arthritis Tissue Clones</th>
<th>CD4 Lineage Active Arthritis Tissue Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persisted</td>
<td>Did not persist</td>
</tr>
<tr>
<td>JF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PB&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Size&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>No. of clones&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> JF: joint fluid; PB: blood; clonal size: average number of sequences per clone.

<sup>b</sup> Average number of clones determined by nucleotide sequencing adjusted to include all BV families.

<sup>c</sup> Includes clones structurally homologous to persistent CD8 clones depicted in Table III.

*Only this subset of persistently expanded CD8 T cell clones or clonal sets present in the tissue both during active arthritis and methotrexate treatment exhibited evidence of Ag drive*

Table III shows examples of these calculated 18 persisting clones that were sequenced. These could be grouped together into four principal clonal sets (CS1 through CS4) based on their sequence structure homology, indicating the possibility that all members of each set had been selected by the same peptide Ag (9–15). Each clonal set was composed of one or more large clones and several smaller clones.

A total of six homologous clones made up the CS1 set that formed a dominant oligoclonal expansion, TRBV14 CDR3 length 11 on spectratyping analysis, (Fig. 1, A and B). This was previously identified in joint fluid and blood (22). Highlighted in bold are the amino acids, in single letter code, that are encoded differently at the nucleotide level or that have an amino acid substitution between constituent clones. CS1 contains three clones (CS1a, b, c) that encode the SQSPGGTQY motif previously identified in joint fluid and blood (22). The remaining three clones were highly homologous and differed from this motif by one amino acid. Clones CS1g, h, and i, although differing in CDR3 length, similarly shared homologues to this CDR3 motif.

CS2 formed another oligoclonal expansion, TRBV4 CDR3 length 14, in spectratyping analysis, that was similarly composed of a set of structurally homologous clones (illustrated in Fig. 2, A and B). CS3 and CS4 (Table III) were also oligoclonal expansions composed of structurally homologous clones. Similar clonal sets with Ag-driven features were identified in the tissue both during active arthritis and methotrexate treatment in each of two other subjects (data not shown), although no blood or joint fluid was available with which to determine lineage. The remaining two clones at the bottom of the table numbered C5 and C6 illustrate a possible public Ag-driven homology at the amino acid level shared between CDR3 regions of single CD8 T cell clones differing in nucleotide sequence, obtained from two HLA-B unmatched cases.

*Subeditorial Note: Reference Cited, Fig. 2B*

A subset of expanded CD4 or CD8 lineage clones was found only in sites of inflammation

We finally sought to characterize the 89 expanded clones in the active tissue whose lineage could be determined by the presence of an identical T cell clone in separated blood or joint fluid but that disappeared during methotrexate treatment (Fig. 2E and Table II). Fig. 2E and Table IV show that this expanded subset contained nearly equal numbers of CD4 (45) and CD8 (44) lineage clones with the majority, 64% (57 of 89), found only in the inflammatory sites of active tissue and joint fluid. These 57 clones were not detectable in the noninflammatory blood or tissue obtained during methotrexate treatment, emphasizing the inflammation-related properties of these clones. The lineage and distribution of these inflammation-related clones colored red (CD4) and orange (CD8) is shown in Fig. 2, B and E. For example, Fig. 2B shows a TRBV4 CD4 clone at CDR3 length 15 that was found shared between the active tissue and the joint fluid, where it was greatly expanded, a result not obvious by spectratype analysis. It was not identified in the blood nor did it persist during methotrexate treatment. CD8 T cell clones with the same distribution pattern were identified as shown by the clone at CDR3 length 13 that was shared between joint fluid and active arthritis tissue but not found in blood or the tissue during methotrexate treatment. Table IV shows that inflammation-related CD4 and CD8 clones in comparison to the clonal size of 9.2 of the persistent CD8 T cell clones found in blood and joint fluid had only an average clonal size of 6.8 and 4.6 sequences per clone, respectively. Combining these 57 clones with the 86 expanded clones found exclusively in the inflamed tissue accounts for 71% (143 of 202) of all active arthritis tissue-expanded clones, suggesting that the majority of expanded clones in inflamed tissue are inflammation related. Table IV also shows two minor components of the inflammatory repertoire that were identified in the blood (9 CD8 clones) or in both blood and joint fluid (10 CD8 and 13 CD4 clones) that were not further studied. Importantly, with the exception of CS1c, CS1h, and CS3b (Table III) that were included in Table IV for calculation purposes in the groups denoted with the footnote “C”, no other sequences found only in active arthritis tissue but not persisting during methotrexate treatment could be grouped into putatively Ag-driven clonal sets. Furthermore, CS1c, CS1h, and CS3b each belonged to clonal sets with other members identical or highly homologous in CDR3 motif that persisted during methotrexate treatment. No CD4 T cell sequences identified in any site, despite several having blood precursor pools, could be grouped into putatively Ag-driven clonal sets.

Discussion

In this study, the use of a simplified method of higher throughput nucleotide sequencing permitted following the distribution and fate of clones and allowed the identification of Ag drive in T cells infiltrating the inflamed joint tissue. Three contrasting populations of T cells were identified, of which one major population consisted of nonclonally expanded polyclonal CD4 T cells that did not persist in the tissue during methotrexate treatment. The most surprising finding was the size of the second major population of moderately expanded inflammation-related clones identified only in active arthritis tissue and/or active joint fluid that were either of CD4 or CD8 lineage. These clones were only present as single clonal expansions and could not be grouped together by structural homology among their CDR3 regions to form clonal sets that indicated Ag drive and potential determinant spread. The third and smallest population consisted of highly expanded clones that were exclusively CD8 in lineage, distinguished by marked expansion in
both blood and joint fluid and persisted during methotrexate treatment to become conspicuous features in the tissue. Providing further evidence for the importance of CD8 T cells in psoriatic arthritis, these persisting CD8 clones could be grouped into clonal sets characterized by a group of clones that shared the same TRBV family and CDR3 length and had completely identical or highly homologous inferred CDR3 amino acid sequences (Table III). This suggested that the clonal set was driven by the same antigenic peptide and that clones with these features would be the best candidates to be potential driver clones. However, they were curiously few in number, accounting for only 2% of all clones in the tissue and, although highly expanded, made up only 12% of the transcripts among all expanded clones. Other functional interpretations for this third population can be envisioned, but each accounts for fewer disease features and leaves unexplained the identity of clones driving the inflammation. Regulatory T cell clones remain an interesting possibility. Although the majority of regulatory T cells are CD4 in lineage and polyclonal (36), CD8 regulatory T cells also operate to fine-tune the autoreactive repertoire in experimental murine autoimmunity (37).

In grouping these clonal sets, several issues were raised. First, while each set had at least one dominant member present in blood, joint fluid, and tissue during active disease and persisting in methotrexate treatment, there were several minor members of each clonal set that had identical or highly homologous CDR3 motifs with differing distributions (Table III), likely accounted for by sampling small numbers. Two CD8 clones from patient samples 1 and 2, numbered C5 and C6 in Table III, were found in blood, joint fluid, and tissue during active disease and methotrexate treatment, but lacked homologous sets of clones within the individual to suggest they were Ag driven. However, their close amino acid homology to one another implied they could be examples of driver clones with public specificities (8). An interesting subset of clonal set CS1, CS1g, h and i, (Table III) is made up of three clones of the unusual CDR3 length of 19, however, CS1g shared the identical amino acid CDR3 motif and the same TRBV and TRBJ element suggesting that despite the different CDR3 length all the clones in this set likely recognized the same peptide.

The major polyclonal population of single sequence αβ T cell clones accounted for 76% of all clones and 31% of all T cell transcripts in the inflammatory tissue. These expanded T cell clones were most likely recruited by inflammatory chemokines released by other clonally expanded T cell populations. We inferred that most of this polyclonal T cell population was CD4 in lineage and derived from blood because the β-chain CDR3 length polyclonal spectratype resembled those of the CD4, but not those of the more oligoclonal CD8 T cell blood subsets. Their inferred CD4 lineage explains much of the perplexing predominance of CD4 T cells found in the tissue infiltrate by immunostaining (6, 7). The lack of clonal expansion in this population suggests it does not play a role in driving the inflammation and presumably traffics through the joint. An analogous population of polyclonal T cells is present in multiple sclerosis plaques (19, 20). Methotrexate administration profoundly decreased this component of the inflammatory infiltrate 15-fold (Fig. 2C).

The intriguing second major population designated as inflammation-related clones outnumbered the persisting Ag-driven CD8 population of clones identified in active arthritis tissue by a ratio of nearly 8:1. The numerous clones comprising this population were distinguished from the putatively Ag-driven population by several features. In terms of distribution, they were mainly found in the actively inflamed sites of joint fluid and/or tissue, and nearly all were not detectably expanded in blood. They were either CD4 or CD8 in lineage, depicted by the colors red and orange, respectively, in Fig. 2, and were less expanded (Table IV). Importantly, none of these clones could be grouped into sets that would have provided evidence to suggest they were Ag driven. Moreover, because none of these clones persisted during methotrexate treatment they are not likely implicated in the chronicity of the arthritis nor in its recrudescence after cessation of methotrexate treatment. Accordingly, the large majority appeared inflammation related and likely entered and proliferated in the inflammatory site by a mechanism other than TCR-mediated selection. The features of these clones resemble those of bystander clones (21). This bystander-type population is likely a secondary consequence of the inflammation induced by other proliferating clones (21, 38). This population presumably includes clones specific for viral Ags previously reported in psoriatic and rheumatoid arthritis that were postulated to not be centrally involved in the disease (24–26, 39). Of course, the absence of structural evidence of homology among any of these clones does not exclude that some are driven by an Ag in the joint, but emphasizes that the diversity of many hundreds of different stimulating peptides would have to be very large and not easily reconciled with the notion of driving clones responding to a few autoantigenic peptides expressed in sites of inflammation.

These bystander type clones account for most of the puzzling high number of oligoclonal expansions seen in this disease (22). However, because they appear to have preferentially expanded in the tissue we presume they contribute importantly to intensification of the inflammation and likely play a major role in disease pathogenesis. Although their antigenic specificity remains unknown, their large number raises the question of whether one feature in the pathogenesis of psoriatic arthritis/psoriasis is an over-generation of this type of inflammatory clone in response to minimal inflammatory stimulation. We speculate that their large number in this disease suggests the threshold of clones to be triggered by engagement of receptors other than their TCR might be reduced in psoriatic arthritis/psoriasis, perhaps also accounting for the Koebner phenomenon of mechanically induced inflammation (40). The presence of these many clones apparently directed to unrelated Ags could explain difficulties by other investigators in using clones from inflammatory sites in efforts to recognize the inciting psoriatic arthritis autoantigen (5).

The analysis of the T cell repertoire during methotrexate treatment provided considerable insight into how this disease-modifying drug modulates immunologic mechanisms and lymphocyte homeostasis in psoriatic arthritis. It also provided an objective measurement of the effect of the drug action on immune events in target tissues, as illustrated in Fig. 2, C and D. Methotrexate relatively decreased the proportion of inflammation-related expanded CD4 or CD8 T cell bystander clones and strikingly decreased the polyclonal expanded T cell infiltrate of the tissue. This effect on repertoire composition is highly consistent with the cytokine blockade mechanism of action proposed for methotrexate by Chan and Cronstein (41) as opposed to the classic mode of action of the drug as a DNA antimetabolite. We presume the cytokine blockade down-regulated the proinflammatory mediators of joint injury responsible for the accumulation and expansion of T cells. Because joint inflammation is significantly decreased but not abolished during methotrexate treatment this partial efficacy likely accounts for the reduced but still continued presence of bystander and the few unexpanded polyclonal T cells. We speculate the persistence of these Ag-driven clones is primarily due to engagement of their TCR by inciting peptides and a correspondingly decreased dependency on cytokines. It was also of interest to observe the continued recruitment of a new group of expanded clones in the tissue during methotrexate treatment that accounted for 30% of the tissue repertoire. Because these new clones had no identified antecedent
clone during the acute arthritis, either in blood, joint fluid, or tissue, and could not be grouped into clonal sets that indicated Ag drive, this suggested they were also inflammation-related, bystander type clones newly recruited into the joint by the low level of sustained inflammation, and further emphasized the predominance of this type of clone in psoriatic arthritis.

This study was motivated by the search for clones with the molecular signature of autoantigen drive to ultimately use them in identifying the driving peptides. Although it was not designed to ascertain functional data on these clones, to our surprise a literature search of the sequences revealed the \( \beta \)-chain CDR3 amino acid motif SQSPGQTQY of three of the clones that comprised the clonal set CS1 (Table III) was identical to the \( \beta \)-chain of an EBV-specific T cell clone, despite being encoded by different nucleotides (15, 39, 42). The EBV-specific clone used the same TRBV and TRBJ gene elements as CS1 (BV16 is now redesignated TRBV14; Ref. 32). This \( \beta \)-chain identity strongly suggests that CS1 is an EBV clone. \( \beta \)-chain homologies similar to this have been used to predict the recognition properties of clones (43). The EBV-specific clone recognizes a peptide from the early lytic protein BMLF1 in the context of HLA-A2 (15, 39, 42), an allele also reported increased in individuals with psoriasis (44). Because B cells, likely attracted by inflammatory chemokines, are abundant in the psoriatic arthritis joint tissue (6) presumably the EBV T cell clones respond to these sites of local EBV replication. Perhaps methotrexate diminishes the efficiency of T cell suppression through its blockade effect, thus enhancing EBV replication and permitting a change from a latent form of infection to a lytic cycle including expression of immediately early genes such as BMLF1 (45). This presumably accounts for the higher proportion of EBV-specific T cell clones found in the tissue during methotrexate treatment. The expression of these lytic phase proteins during therapy with methotrexate could set the stage for a strong and broad intrasynovial EBV T cell response once immunosuppression therapy was discontinued. The T cell response to newly expressed EBV proteins might account for some of the recrudescence of arthritis after therapy with the drug is stopped. However, the unexpected finding that the best candidates to be driving clones capable of recognizing a putative autoantigen appear to be specific for an EBV peptide categorizes this subset of clones as bystander clones but only in the limited sense that they are not driven by a putative peptide autoantigen, not in the sense that they exhibit the distribution of other inflammation-related clones. The distribution of these putatively EBV-specific clones contrast sharply with that of the inflammation-related clones. These putatively EBV-specific clones have the distribution and characteristic features of driver clones (8), including, CD8 lineage, evidence of Ag drive and persistence during methotrexate administration. This emphasizes these clones likely have a more fundamental role in driving inflammation that is different from the typical population of bystander type clones that more likely appear to respond to inflammation. Emphasizing the potential complexity of stimulating peptides in autoimmunity, the T cell response to EBV could play a more central role in the development of psoriatic arthritis by driving the inflammatory process through recognition of a nonself EBV peptide. Indeed, EBV has also been postulated to be implicated in the pathogenesis of a variety of other autoimmune diseases including rheumatoid arthritis and lupus (46, 47). However, clones with structural features suggesting they recognize EBV peptides were found only in this individual, implying this potential mechanism could be particular to this individual and raising the possibility that immune responses to different exogenous Ags may have the features of a driving response in other samples. These results strongly suggest the importance of making an extensive search using Ag-specific methodologies to confirm the presence of EBV reactivity in driving clones in this individual and to ascertain whether EBV is common to all driver clones in other cases of psoriatic arthritis. Clones specific for EBV or other viral peptides may also help to initiate or intensify the psoriatic autoimmune response either through a molecular mimicry mechanism (48, 49) or perhaps more likely by activating elements of the innate immune system that regulate clonal tolerance of self-peptides (50). This latter mechanism resembles that postulated to occur in type I diabetes induced by Coxsackie virus infection (51) and herpetic stromal keratitis resulting from HSV infection (52) where autoreactive clones are activated by the antiviral response. Were this found to be the case, it would blur the seemingly precise theoretic distinction between driver and bystander clones and suggest the importance of inappropriately activated innate immune mechanisms in inducing expansion of numerous T cell clones of highly diverse Ag specificities in the pathogenesis of psoriatic arthritis.

Intriguingly, because the peptide recognized by this EBV-specific clone is presented by HLA-A2 alleles, this further suggests the CS1 clones are likely also restricted on HLA-A2. Because the individual in whom this clone was identified is HLA-B*5701 and HLA-B*27052, two major susceptibility alleles in psoriatic arthritis, extremely few putatively Ag-driven clones among those identified in this study remain that could be restricted on these latter HLA molecules and recognize putative inciting autoantigenic peptides. These findings suggest that if MHC molecules encoded by susceptibility alleles present self peptides to clones in psoriatic arthritis that become driving clones capable of mediating the disease, these clones must be exceedingly infrequent (<0.1%) and have not been convincingly identified in this sequencing study that included 3100 TCR \( \beta \)-chain sequences. Alternatively, it appears more likely that the involvement of these class I susceptibility alleles in the disease may occur either through features of MHC molecules unrelated to peptide binding that result in CD8 T cell clonal activation, such as via killer Ig-related (KIR) receptor engagement or possibly through the effect of linked genes.

### Acknowledgments

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