How is autoimmunity against citrullinated proteins regulated?
Cantaert, T.

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CHAPTER 9

T cell involvement in the ACPA response

anti-citrullinated protein antibodies define a subset of rheumatoid arthritis with preferential T lymphocyte involvement.

pathogenic subset of distinct antibodies

Submitted for publication
Abstract

Objectives

Genetic and epidemiologic evidence suggests that rheumatoid arthritis with and without anti-citrullinated protein antibodies (ACPA) are distinct pathogenic entities. The association of HLA-DRB1 alleles with ACPA+ patients points towards involvement of T lymphocytes in the pathophysiology of this subtype. We assessed at the molecular levels if ACPA+ and ACPA- RA are distinct disease entities and if T cells are preferentially involved in the pathophysiology of the former subset.

Patients and Methods

Synovial biopsies were obtained from actively inflamed knee joints of 54 RA patients. Inflammation was assessed by standard synovial histology. RNA was isolated from synovial tissue biopsies of 11 ACPA+, 7 ACPA-, and 10 SpA patients with similar levels of synovial B and T cell inflammation. T cell receptor clonality was assessed by the TcLandscape technology. Alterations of the normal TCR length distribution were analyzed for all Vβ families, after correction for the number of specific transcripts and using Cβ as internal control.

Results

Qualitative CDR3-LD alterations without marked quantitative expansions of the clonal T cell size were specifically increased in ACPA+ synovitis, although clinical disease features and synovial histopathology were similar in both subsets. Both the overall skewing of the Vβ family usage and the qualitative CDR3-LD alterations, which were confirmed by Jβ analysis, were specific for the synovial compartment, indicating selective recruitment and/or local expansion of T cells. These clonal T cell alterations did not appear to result directly from specific T-B cell interactions in the synovial membrane as they did not parallel B cell clonal expansions and were inversely associated with synovial lymphoid neogenesis.

Conclusions

These data support at the molecular level the novel concept that ACPA+ and ACPA- RA are distinct pathogenic entities with preferential involvement of T cells in ACPA+ disease.
Introduction

Rheumatoid arthritis (RA) is a clinically heterogeneous disease. The American College of Rheumatology criteria are used to characterize patients in clinical trials and for reference in daily practice but do not define one homogeneous patient population (1,2). Also at the immunological level the disease is heterogeneous as approximately two thirds of the RA patients show signs of B cell autoimmunity with the presence of the rheumatoid factor and/or the highly RA-specific anti-citrullinated protein antibodies (ACPA) (3;4). These autoantibodies are directed against protein epitopes modified by citrullination, a posttranslational modification which can be triggered by inflammation but also occurs in physiological conditions in healthy individuals (5-8). Increasing evidence defines the ACPA+ RA patients as a distinct subtype of RA. Firstly, ACPA can be detected years before the first clinical symptoms of RA and only rarely appear or disappear after disease onset (9-11). Secondly, the presence of ACPA is associated with more severe joint destruction (3;11). Thirdly, ACPA+ RA appear to respond better to B cell depletion by rituximab than ACPA-RA (12). Fourthly, the long established genetic association between RA and a subset of HLA-DRB1 alleles (the so-called shared epitope) can be refined to an association with ACPA+ but not ACPA- RA (13). Furthermore, other RA predisposing genes such as PTPN22 or IRF-5 are also confined to ACPA+ and ACPA- RA, respectively (14;15). Finally, a gene-environment interaction between smoking and the shared epitope plays a role in the development of ACPA+ but not ACPA- arthritis (16).

The association of specific HLA-DRB1 alleles (HLA-DRB1*0401, *0101, *0404) with RA supports the concept that specific antigenic epitopes are presented to T cells in a HLA-restricted manner (17-19). The refinement of this genetic association with ACPA+ but not ACPA- RA and the fact that ACPA are classed-switched antibodies directed against a peptide antigen suggests that T cells are specifically involved in the pathogenesis of ACPA+ RA and, eventually, contribute to the maturation of this humoral autoimmune response. Therefore, the hypothesis was proposed that T lymphocytes in ACPA+ RA may specifically recognize citrullinated peptides presented in the context of the shared epitope. Indeed, in comparison with its native counterpart, a citrullinated peptide of vimentin binds with higher affinity to the HLA-DRB1*0401, *0404 and *0101 but not to HLA-DRB1 alleles not encoding the shared epitope (20). This concept was recently supported by preferential T cell reactivity to citrullinated epitopes in HLA-DR4 transgenic mice immunized with citrullinated human fibrinogen, a model in which one third of the animals developed mild arthritis of the ankle joints 10 weeks after immunization (21). In contrast, extensive studies with peptides derived from fibrinogen could not confirm that citrullination influences the HLA-DR binding or the T cell reactivity in human RA (22). Alternatively, T cells
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may recognize a non-citrullinated epitope of a larger protein complex in which a distinct citrullinated part is recognized by the B cell receptor, thereby allowing adequate antigen-specific T-B cell collaboration (23). Formal demonstration of such mechanism is complicated by the fact that the driving autoantigenic epitopes have not been fully identified in human RA (23). As a consequence, direct evidence for specific T cell reactivity in ACPA+ RA has not been yet provided.

Peptides presented in the context of MHC are recognised by the T cell receptor (TCR). To be able to recognise a diverse repertoire of peptides, the TCR is generated by a complex process of gene segment rearrangements in which nucleotides can be removed or added randomly at the junctions between the segments, generating as much as 2.5*10^7 to 1*10^8 different TCRs in one single individual (24;25). Most variability can be found in the complementarity determining region 3 (CDR3), which is the major recognition site for the peptide-MHC complex. After antigenic stimulation, a given T cell will expand and form a clonally identical population, each cell bearing the same TCR. These selections and expansions lead to modifications of the presumed Gaussian-like CDR3 length distribution (CDR3-LD) encountered in physiological conditions (26-28). Therefore, studying the CDR3-LD can provide direct evidence for the involvement of T cells in specific diseases or disease subsets (29-31). As synovial tissue is the major target organ of RA and since ACPA are produced locally in the inflamed synovium (32-34), we used here a novel technique for qualitative and quantitative TCR analysis to study clonal alterations at the primary site of inflammation in ACPA+ RA versus ACPA- RA using spondyloarthritis (SpA) as arthritic control.

Patients and Methods

Patients

Synovial tissue biopsies were obtained by needle arthroscopy from a clinically swollen knee, ankle or wrist joint (35;36) in 158 RA patients fulfilling the American College of Rheumatology criteria for RA (37). As a control population for the TCR analysis, synovial tissue biopsies were also obtained from 10 spondyloarthritis (SpA) patients fulfilling the European Spondyloarthropathy Study Group criteria for SpA (38). Six to eight biopsies per patient were snap frozen in liquid nitrogen for immunohistochemistry and 8 biopsies were stored in Trizol (Invitrogen, Carlsbad, CA) at -80°C for RNA extraction. Additionally, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient (Sigma-Aldrich, St. Louis, MO) from 12 healthy individuals, 13 RA, and 8 SpA patients. Patient characteristics are
Clinical and demographic description of the patient cohorts. Values are depicted as median (interquartile range) or as the number of patients (%) or joints. Patient’s assessment of global disease activity was scored on a 100 mm Visual Analog Scale (VAS disease activity). ST: synovial tissue, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: disease activity score, DMARD: Disease-modifying antirheumatic drugs, NSAID: non-steroidal anti-inflammatory drugs, cortico: corticosteroids. N/A: not applicable.

### Clinical analysis

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>ACPA -</th>
<th>ACPA +</th>
<th>RA - PBMC</th>
<th>SpA - PBMC</th>
<th>RA - ST</th>
<th>SpA -ST</th>
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<tbody>
<tr>
<td></td>
<td>46</td>
<td>112</td>
<td>9</td>
<td>4</td>
<td>8</td>
<td>11</td>
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<tr>
<td>Number of patients</td>
<td>46</td>
<td>112</td>
<td>9</td>
<td>4</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 (42 – 62)</td>
<td>56 (48 – 65)</td>
<td>57 (51 – 65)</td>
<td>44 (35 – 62)</td>
<td>50 (42-58)</td>
<td>57 (50 – 75)</td>
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<tr>
<td>Gender (m/f)</td>
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<td>36/76</td>
<td>1/9</td>
<td>2/4</td>
<td>2/6</td>
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<tr>
<td>Rheumatoid factor (% positive)</td>
<td>10 (22%)</td>
<td>95 (85%)</td>
<td>8 (89%)</td>
<td>2 (50%)</td>
<td>0</td>
<td>8 (72%)</td>
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<td>Disease Duration</td>
<td>5 (1 – 14)</td>
<td>8 (3 – 16)</td>
<td>13 (2 – 16)</td>
<td>10 (5 – 15)</td>
<td>12 (5 – 19)</td>
<td>13 (2 – 18)</td>
</tr>
<tr>
<td>DAS28</td>
<td>6.0 (5.0 – 6.9)</td>
<td>5.6 (4.9 – 6.6)</td>
<td>5.1 (4.9 – 6.4)</td>
<td>6.1 (3.8 – 6.8)</td>
<td>N/A</td>
<td>5.0 (4.8 – 6.9)</td>
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<td>VAS disease activity</td>
<td>65 (48 – 77)</td>
<td>59 (34 – 75)</td>
<td>47 (18 – 68)</td>
<td>67 (40 – 88)</td>
<td>55 (50 – 70)</td>
<td>78 (47 – 90)</td>
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<td>CRP (mg/L)</td>
<td>8.0 (3.0 – 32.5)</td>
<td>16.5 (3.5 – 12.0)</td>
<td>11.0 (24.0 – 100.0)</td>
<td>30.5 (9.8-88.3)</td>
<td>17.5 (11.1 – 85.0)</td>
<td>13.0 (7.0 – 98.0)</td>
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<td>ESR (mm/h)</td>
<td>270 (12.0 – 45.0)</td>
<td>35.5 (22.3 – 49.8)</td>
<td>38.0 (20.1 – 48.5)</td>
<td>50.0 (41.1 – 71.0)</td>
<td>20.5 (8.5 – 26.0)</td>
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<td>Tender joint count</td>
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<td>7 (3 – 13)</td>
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<td>12 (7 – 13)</td>
<td>0 (0-3)</td>
<td>6 (1 – 9)</td>
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<td>Therapy DMARD</td>
<td>40 (87%)</td>
<td>98 (88%)</td>
<td>9 (100%)</td>
<td>4 (100%)</td>
<td>4 (50%)</td>
<td>10 (90%)</td>
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<tr>
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<td>39 (85%)</td>
<td>65 (58%)</td>
<td>8 (89%)</td>
<td>3 (75%)</td>
<td>8 (100%)</td>
<td>3 (27%)</td>
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<tr>
<td>Therapy Cortico</td>
<td>16 (35%)</td>
<td>35 (31%)</td>
<td>1 (11%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (18%)</td>
</tr>
</tbody>
</table>

Table 1: Clinical and demographic description of the patient cohorts. Values are depicted as median (interquartile range) or as the number of patients (%) or joints. Patient’s assessment of global disease activity was scored on a 100 mm Visual Analog Scale (VAS disease activity). ST: synovial tissue, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: disease activity score, DMARD: Disease-modifying antirheumatic drugs, NSAID: non-steroidal anti-inflammatory drugs, cortico: corticosteroids. N/A: not applicable.
summarized in Table 1. The healthy individuals were age-matched with the patients, with a median age of 46 years (42-69 years). The presence of ACPA was determined with the anti-CCP2 kit, using the diagnostic cut-off of 25 U/ml to define positivity. All patients and healthy controls gave written informed consent before inclusion as approved by the local Medical Ethics Committee.

**Synovial histology and immunohistochemistry**

Synovial tissue biopsies were processed as described previously (35;36;39). In a first cohort of 54 RA patients as well as 10 control SpA patients, synovial tissue was evaluated for histologic parameters such as lining layer thickness, vascularity, and global inflammatory infiltration. Lymphoid aggregates were evaluated as described previously (40-42). Furthermore, infiltrating lymphocytic cells were phenotyped by immunohistochemistry with antibodies directed towards CD3 (clone UCHT1, DakoCytomation, Glostrup, Denmark) for T lymphocytes, CD20 (clone L26, DakoCytomation) for B lymphocytes, and CD138 (clone CBL455, Chemicon, CA) for plasma cells. Sections were blocked with 10% swine serum for 30 min, and incubated with primary antibody for 30 min. After blocking endogenous peroxidase with 1% H2O2, stainings were developed with the LSAB+ kit and AEC substrate (DakoCytomation). Concentration- and isotype-matched control antibodies were included as negative control and tonsil sections were included as positive control. Histology and immunostainings were scored on a semi-quantitative four-point scale by two independent observers blinded for diagnosis and clinical data (39).

In a second independent cohort of RA patients (n = 104), the lymphocytic infiltrate was characterized by immunohistochemical analysis using the following monoclonal antibodies: anti-CD3 (clone SK7, Beckton Dickinson and Company, NJ) for T cells, anti-CD22 (clone CLB-B-ly/1,6B11, Sanquin, the Netherlands) for B cells, and anti-CD38 (clone HB-7; Beckton Dickinson and Company) for plasma cells. Staining was performed using a 3-step immunoperoxidase method as described previously (43). All coded sections were randomly analyzed by computer-assisted image analysis and the total number of positive cells per square millimeter (counts/mm²) was determined. The images of the high-power fields were analyzed using the Qwin analysis system (Leica, Cambridge, UK), as described previously in detail (43).
TCR repertoire analysis

The TCR repertoire was analyzed by TcLandscape technology as extensively described and validated previously (30;44-49). For the synovial T cell analysis, patient samples were selected on the basis of comparable synovial histology and with a high semi-quantitative score of infiltrating T cells, to exclude biases related to composition of the synovial tissue. RNA was extracted from PBMC and homogenized synovial tissue using Trizol reagents (Invitrogen) according to the manufacturer’s instructions. The concentration and quality of RNA for each sample was checked using nanoRNA Chips (Agilent®, UK). Two μg of RNA was reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100 μl. cDNA was subsequently amplified by PCR using a Cβ primer and one of the 26 Vβ specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA). Each amplification product was used for an elongation reaction using a dye-labeled Cβ primer, then heat denatured, loaded onto a 6% acrylamide–8 M urea gel, and electrophoresed for 9 h using an Applied Biosystems 373A DNA Sequencer (Perkin-Elmer). Each specific Vβ family transcript amount was divided by the number of hydroxyphosphoribosyltransferase (HPRT) transcripts to correct for variability in the initial concentration and quality of total RNA and in the conversion efficiency of the reverse transcription reaction. To confirm clonal alterations revealed by spectratyping with one of the 26 Vβ specific primers, we reanalyzed these amplified samples with specific clonal alterations using Jβ specific primers.

TcLandscape data analysis

Analysis of CDR3-LD was performed using Immunoscope® software (30;44-48). A drawback of classical spectratyping is that a low transcript number for a given Vβ family increases the chances of false positive deviation from the Gaussian distribution. Using the quantitative RT-PCR data (Vβ/HPRT transcript ratio), previous dilution experiments on peripheral blood T cells allowed to define for each Vβ family a minimal number of transcripts needed for reliable assessment of the CDR3-LD profile (Brouard et al, unpublished observations). Those Vβ families not reaching this quantitative threshold were excluded from the CDR3-LD analysis in order to avoid false positive high CDR3-LD alteration. The percentage of CDR3-LD alteration for each Vβ family and the global percentage of CDR3-LD alteration for each individual or each group was calculated as described (29;50). Furthermore, we analyzed the CDR3-LD dichotomously, using 25% alteration as cut-off (30). Combined CDR3-LD alterations for each Vβ family and the quantitative Vβ/HPRT
data was represented in integrated landscapes, referred to as TcLandscape. This gives a direct assessment of the contribution and alteration of each Vβ family: the x-axis displays the 26 Vβ families analyzed, the y-axis the CDR3 lengths, and the z-axis the Vβ/HPRT transcript ratio. Colors represent the percentage of alterations from deep blue (≤−30%) to dark red (≥+30%) compared to healthy controls.

**Immunglobulin variable heavy chain gene sequencing**

For direct comparisons of synovial T cell and B cell clonality, we performed immunoglobulin variable heavy chain (Ig VH) gene analysis of 8 RA synovial tissue samples as described previously (41;51). In brief, total RNA was extracted from RA synovial biopsies (6 biopsies per patient) using an RNAeasy kit (QIAGEN Inc Chatsworth, CA). Ig VH and VDJ genes were amplified by PCR and subsequently ligated into the pGEM T vector (Promega, La Jolla, CA) and transfected into Escherichia coli DH5a. For each sample, 15 white colonies were picked at random and grown overnight in LB (Luria-Bertani) medium. The double-stranded DNA template from the colonies containing VH gene inserts was confirmed by Eco RI digestion and then sequenced using a high efficiency DNA sequencer (MCLab Inc,CA). A mucosal B cell lymphoma and peripheral blood cells from a healthy individual were used as positive and negative control, respectively, for the B cell clonality analysis.

**Statistical analysis**

Since clinical and histologic data were not normally distributed, the results were expressed as median and interquartile range and analyzed with the Mann-Whitney U test. The CDR3-LD and Vβ/Cβ ratio’s passed normality tests and are represented as mean ± standard deviation and analyzed with unpaired or paired 2-sided Student t-tests, as appropriate. All data points fell between mean ± 2 x standard deviation and were included in analysis (no outliers). Dichotomous data were analysed using a Fisher’s exact test. P values < 0.05 were considered statistically significant. When sample size < 5 non-parametric testing was used for analysis.
CHAPTER 9

Table 2: Comparison between ACPA positive and ACPA negative patients for synovial histology. The presence of ACPA was determined with the anti-CCP2 kit, using the diagnostic cut-off of 25 U/ml to define positivity. P values were obtained by Mann-Whitney U test. Synovial histology was scored semi-quantitatively on a four-point scale by 2 independent observers in cohort 1 and was assessed quantitatively by digital image analysis for cohort 2. NA: not available, NS: not significant.

Results

Similar systemic disease activity in ACPA+ and ACPA- RA

As the aim of the study was to detect qualitative differences in T cell involvement in ACPA+ versus ACPA- RA, we first investigated if both patient groups were similar in terms of disease activity. Therefore, we studied a cross-sectional cohort of 158 patients with biological naïve, active RA of which 46 were ACPA negative and 112 were ACPA positive. As shown in Table 1, age and gender distribution were similar in both groups but the disease duration was shorter in the ACPA- group compared to the ACPA+ group (P = 0.027). Disease activity as measured by the DAS28 was similar in both subgroups, with a score of 6.0 (5.0 – 6.9) in ACPA+ RA and 5.6 (4.9 – 6.6) in ACPA- RA. Accordingly, there were no significant differences for physician’s assessment of global disease activity (visual analog scale), erythrocyte sedimentation rate, C-reactive protein serum levels, and swollen joint count. The tender joint count was lower in the ACPA+ patients (P = 0.04). There was no difference in treatment regimen between both groups (Table 1). Taken together these data confirm that the clinical disease activity is similar in ACPA+ and ACPA- RA, even if the former subset has a worse prognosis in terms of structural damage (52).
Similar inflammation and T cell infiltration in ACPA+ and ACPA- synovitis

In order to ascertain that not only systemic activity but also local disease activity in the joint of interest was similar in both RA subsets before proceeding with the synovial TCR analysis, we performed a detailed histologic analysis of the synovial tissue biopsies. Assessment of 54 RA synovial tissue samples, consisting of 37 ACPA+ and 17 ACPA- samples, revealed no differences in intimal lining layer hyperplasia, degree of vascularisation, and global cellular infiltration (Table 2). Furthermore, there was no difference in the number of infiltrating CD3+ T cells, CD20+ B cells, and CD138+ plasma cells (Table 2). As these data are discrepant with a recent report demonstrating increased T cell infiltration in ACPA+ versus ACPA- synovitis (53), we analyzed the lymphocytic cell infiltrate in an additional set of 104 RA synovial tissue samples using different antibodies for immunostaining in order to exclude technical biases and using digital image analysis for scoring in order to be more sensitive in detecting small differences (43). Also in this independent set, there was no difference in the presence of CD3+ T cells, CD22+ B cells, and CD38+ plasma cells (Table 2). This extensive study of two large, independent sets of RA synovial tissue indicates that, with exception of synovial lymphoid neogenesis, the global synovial tissue architecture as well as the infiltration with T and B lymphocytes is comparable in ACPA+ and ACPA- RA.

Marked CDR3-LD alterations in ACPA+ synovitis

As histologic enumeration of T cells in synovium does not reflect functional involvement, we next analyzed synovial tissue biopsies obtained from an actively inflamed knee joint with the combined quantitative and qualitative TcLandscape technology to compare synovial T cell CDR3-LD in ACPA+ RA versus ACPA- RA, using SpA as arthritic control (Figure 1A-E). To avoid false positive clonal alterations that can be observed with classical spectratyping, CDR3-LD alterations were excluded if quantitative analysis showed that the cDNA copy number was below the threshold for CDR3-LD analysis (see materials and methods section for details). Thus, although all Vβ families were clearly expressed by peripheral blood T cells, the cDNA copy numbers for Vβ 13.5, Vβ 16, Vβ 23 and Vβ 24 were below the threshold for CDR3-LD analysis in all synovial tissue specimens. Other Vβ families with low transcript numbers were variable between individual patients (Figure 1E, white squares). When analyzing qualitatively those families with sufficient transcript numbers, thereby avoiding false positive clonal alterations that can be observed with classical spectratyping, the CDR3-LD alterations were significantly higher in the ACPA+ versus the ACPA- patients (29.1 ± 8.6% versus 19.5 ± 4.6%, P = 0.011)
Figure 1 (left): A-C: TcLandscape representation of the integrated analysis of qualitative and quantitative alterations in the TCR repertoire. The X axis represent individual Vβ families, the Y axis shows the CDR3 length, and the Z axis shows the Vβ/HPRT ratio. A: representative TcLandscape of the synovial T cells of an ACPA A+ RA samples. B: representative TcLandscape of the synovial T cells of an ACPA A- RA sample. C: representative TcLandscape of the synovial T cells of a SpA sample as arthritic control. D: Comparison of global CDR3-LD alteration between ACPA A+, ACPA A– and SpA synovial T cells. Each dot represents the mean global CDR3-LD alteration of 1 patient. Data passed normality tests, therefore P values were calculated using an unpaired T-test. E: Significant differences between ACPA A+ and ACPA A– TCR length distribution alteration was observed in several Vβ families and was different in each individual sample. Each row represents one patient; each column represents one Vβ family. Red squares represent alteration of more than 25%, white squares represents non available data due to low amount of cDNA of that Vβ family. Bright yellow squares show significant differences between ACPA+ and ACPA- patients analyzed for each Vβ family separately. P values were obtained with Fisher’s exact test. ACPA: anti-citrullinated protein antibody, RA: rheumatoid arthritis, SpA: spondyloarthritis, CDR3: complementarity determining region, TCR: T cell receptor.

TCR CDR3-LD alterations in ACPA+ RA are specific for the inflamed synovium.

RA is a systemic disease with the synovial tissue as primary target. Previous studies in organ-specific immune-mediated inflammatory diseases such as multiple sclerosis and solid organ transplantation (30;46) have demonstrated that specific alterations of the CDR3-LD can be observed in the peripheral blood compartment. However, we could not detect such alterations in RA peripheral blood T cells. Firstly, the degree of CDR3-LD alteration was significantly lower in peripheral blood T cells than in synovial tissue in both RA (16.0 ± 3.7 versus 25.4 ± 8.3, P < 0.001) and SpA.
Figure 2: A: Comparison of global CDR3-LD alteration between HC PBMC, RA PBMC and synovial tissue, and SpA PBMC and synovial tissue. Each dot represents the mean global CDR3-LD alteration of 1 patient. P values were calculated using an unpaired T-test, as in only a subgroup of the patients we obtained paired samples. B: Comparison of global CDR3-LD alteration between peripheral T cells of ACPA+ and ACPA− RA patients. C-D: TcLandscape representation of integrated analysis of qualitative and quantitative alterations in the TCR repertoire. The X axis represents individual Vβ families, the Y axis shows the CDR3 length, and the Z axis shows the Vβ/HPRT ratio. C: ACPA+ peripheral T cells. D: ACPA- peripheral T cells. E-F: direct comparison of CDR3-LD alteration between synovial and peripheral T cells. Each dot represents CDR3-LD alteration of one Vβ family. E: ACPA+ RA patient. F: ACPA- RA patient. ACPA: anti-citrullinated protein antibody, RA: rheumatoid arthritis, SpA: spondyloarthritis, CDR3: complementarity determining region, TCR: T cell receptor, HC: healthy control, ST: synovial tissue, PBMC: peripheral blood mononuclear cells.
as control chronic arthritic disease (13.9 ± 2.3 versus 22.6 ± 4.2, P < 0.001) (Figure 2A). Furthermore, RA peripheral blood T cells showed a similar pattern of CDR3-LD as peripheral blood T cells of SpA and age-matched healthy controls (Figure 2A), indicating that in arthritic diseases in general and in RA in particular T cell clonal alterations are more pronounced in the primary target organ of the disease than in the peripheral compartment. Secondly, there was no difference for the global alteration of the CDR3-LD of peripheral blood T cells in ACPA+ versus ACPA− RA (16.6 ± 3.5% versus 14.6 ± 4.1%) (Figure 2B-D). Accordingly, there was no difference in the number of Vβ families with a CDR3-LD deviation of more than 25% between ACPA+ and ACPA− RA patients (10.3 % versus 9.5 %). Finally, direct comparison of the CDR3-LD alteration of paired peripheral blood and synovial tissue T cells in 5 RA patients (3 ACPA+ and 2 ACPA−) indicated that, whereas most Vβ families displayed a much higher CDR3-LD alteration in the synovium than in the blood, some specific Vβ families have a lower degree of CDR3-LD alteration in the inflamed joint as illustrated in Figure 2E and F. These data demonstrate that the T cell populations found in the inflamed synovium are not merely a reflection of bystander infiltration of peripheral blood T cells, but that there is a selective recruitment and/or local expansion of specific T cell clones in ACPA+ RA.

Characterization of the oligoclonal T cell population in ACPA+ synovitis

As the observed difference in CDR3-LD in ACPA+ versus ACPA− synovial T cells argues for a distinct role of synovial T cells in ACPA+ RA pathogenesis, we further characterized these synovial T cells in terms of clonality and clonal size. To confirm that the TCR CDR3-LD alterations found in specific Vβ families resulted from genuine oligoclonal alterations, we first performed an additional spectratyping analysis of the Jβ gene segments of 9 strongly altered synovial Vβ families (3 from ACPA+ RA, 4 from ACPA− RA, and 2 from SpA). All Vβ families depicting a high CDR3-LD alteration showed also a discrete usage of Jβ segments, as illustrated for Vβ17 of an ACPA+ RA patient synovial tissue sample in Figure 3A. In order to assess the preferential alteration of specific Vβ families and their clonal size, we calculated the number of transcripts for each Vβ family in function of the total number of Cβ transcripts for each sample. As illustrated in Figure 1E, there was no restriction of pronounced clonal alterations to a particular family, neither in one single sample nor across the different patients. Analysis of the Vβ/Cβ ratio for each Vβ family showed that there is a quantitatively similar Vβ usage in ACPA+ and ACPA− RA synovitis as well as in SpA synovitis (Figure 3B). Moreover, comparison for each separate Vβ family of the Vβ/Cβ ratio between ACPA+ RA synovial samples with and without a high degree of CDR3-LD alteration did not show preferential quantitative expansion
Figure 3: A: Oligoclonality observed in synovial tissue T cells was confirmed by Jβ analysis. Spectratyping of one representative Vβ family (Vβ17) of 1 ACPA+ RA patient is shown; B: Relative usage of different Vβ families by synovial T cells of ACPA+ versus ACPA- RA patients versus arthritic controls (SpA). The relative usage was calculated by dividing the amount of Vβ transcripts for each family by the total number of Cβ transcripts for each patient separately. ACPA: anti-citrullinated protein antibody, RA: rheumatoid arthritis, SpA: spondyloarthritis
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in samples with qualitative alterations, with exception of the Vβ6.5 family (mean Vβ/Cβ ratio of 0.049 versus 0.028 in samples with, respectively, more and less than 25% CDR3-LD alteration; P = 0.019). Taken together, these data confirm the qualitative clonal alterations in ACPA+ synovitis but suggest that they are not paralleled by marked quantitative expansions of the clonal T cell size.

TCR CDR3-LD alterations in ACPA+ RA are not driven by ectopic lymphoid neogenesis

As our data point towards distinct T cell clonal alterations in ACPA+ compared to ACPA- RA, we next aimed to explore a direct relationship with B cell autoimmunity in the inflamed synovium. Although difficult to assess in human translational studies, we approached this question by extensive sequencing of Ig VH genes in 8 RA synovial tissue samples, of which 4 were ACPA+ and 4 were ACPA-. Although single and dual expansions of B cell clones were observed in 4 and 3 samples, respectively, there was no correlation with TCR CDR3-LD alterations (data not shown). As direct interaction between antigen-specific T and B lymphocytes and resulting clonal expansions may be facilitated by a dedicated microarchitecture in the inflamed tissue, we also explored the relationship between T cell clonal alterations and synovial lymphoid neogenesis. Whereas the degree of TCR CDR3-LD alterations tended to be similar in samples with versus without synovial lymphoid neogenesis in SpA (P = 0.117) (Fig 4A), this relationship was inversed in RA: the degree of TCR CDR3-LD alterations was significantly higher in RA synovium without ectopic lymphoid neogenesis (31.0
± 9.3%) than in samples with ectopic lymphoid neogenesis (19.6 ± 2.4%) (P = 0.005) (Fig 4B). Accordingly, in our combined larger RA cohort used for histologic analysis, synovial lymphoid neogenesis was less frequently observed in ACPA+ (25/113, 22%) than in ACPA- (21/44, 48%, P =0.031) RA.

Discussion

Based on the strong association with HLA-DRB1 alleles, T cells have been proposed to play a pivotal role in the pathogenesis of RA and have been the subject of extensive molecular analyses in the nineties. Although recent advances in therapies targeting macrophage-derived cytokines and depleting B cells have emphasized the complexity of the disease process, these T cell studies have provided a number of crucial insights (55). Firstly, both the Vβ usage and the T cell clonality appeared to be skewed in RA (56-59). Secondly, this skewing is a primary event rather than merely a consequence of inflammation as unaffected siblings of RA patients also display expanded T cell clonotypes (60;61). Thirdly, although alterations have been reported in the peripheral compartment (62), they are much more pronounced in the joint, are similar between different joints of the same patient, and are stable over time (63-68) Finally, the transfer of disease by synovial T lymphocytes to severe combined immunodeficient mice suggests a pathogenic role for these cells (69). Unfortunately, further translation of these features into mechanisms of disease has been hampered by the large heterogeneity between patients, the potential biases related to the origin of the samples (IL-2 derived cell lines, synovial fluid rather than synovial tissue T cells, synovial tissue from end-stage, destructive disease), and technical constraints such as false positive spectratyping results due to low transcript numbers.

Here, we combined state-of-the-art synovial biopsy sampling and integrated quantitative and qualitative CDR3-LD analysis to revisit the role of T cells in RA synovitis in the context of the growing evidence that ACPA+ and ACPA- RA are distinct pathogenic entities. This approach allowed the confirmation of a number of essential findings, such as the skewing of the Vβ usage and the preferential clonal alteration in the synovial versus peripheral compartment, but also revealed new features. The use of SpA synovial biopsies as an appropriate inflammatory control indeed demonstrated that synovial TCR alterations are not specific for RA but are also found in other chronic inflammatory joint diseases. The major novel finding of the present study, however, is that T cell clonal alterations present in the inflamed RA synovium are significantly increased in ACPA+ versus ACPA- RA despite similar clinical and histologic characteristics. This increase was specific for ACPA+ RA as it was confirmed by comparison with SpA synovitis. In line with previous data (66),
T cell involvement in the ACPA response

This marked CDR3-LD alteration is not accompanied by an increase in clonal size, although this should be confirmed by more sensitive techniques. More importantly, the CDR3-LD alteration is specific for synovial T cells as we did not detect elevated CDR3-LD in peripheral T cells of ACPA+ RA patients. These data confirm at the molecular level the novel concept that ACPA+ and ACPA- RA are distinct pathogenic entities and suggest a preferential involvement of T cells in the former subset.

A first important question raised by this observation is which T cell subset harbors these clonal alterations? In this study, we chose to analyze the unselected synovial T cell RNA as isolation of T cells from synovial tissue by either IL-2 driven expansion or extraction with enzymatic agents induces severe biases in the T cell pool or phenotypic markers, respectively (70). Unfortunately, double immunostaining on paired synovial sections of phenotypic markers and specific Vβ families with high CDR3-LD alterations did not allow us to draw firm conclusions with regard to naïve versus memory and CD4 versus CD8 subsets (data not shown). However, previous studies have demonstrated that naïve T cells are undetectable in synovial tissue and that most synovial T cells are antigen experienced, memory T cells expressing CD45RO (67;71) suggesting that the clonal alterations are present in this population. On the other hand, the data on CD4 versus CD8 clonal alterations in RA are conflicting: the most prominent clonal expansions are often found in the CD8+ populations in RA (72;73) as well in other pathologies (30;44) whereas other studies demonstrated clear clonal alterations in CD4+ populations (58;61;66). This issue is under further investigation in the context of the MHC class II association in ACPA+ RA versus the MHC class I association in SpA.

A related question is the origin of these clonal alterations. The most widely accepted concept is that the presence of clonal T cell expansions in the synovial tissue reflects activation and/or proliferation in response to a local antigen (58;65;74;75). This would be in line with our finding that the profiles in synovium are not merely a reflection of peripheral blood alterations as well as with our previous demonstrations of HLA-DRB-restricted presentation of autoantigens in RA synovitis (17-19). This hypothesis, however, raises a number of additional issues. Firstly, the exact nature of the antigens driving this process in RA is unknown. More specifically, it is not yet clear if the specific presence of these TCR alterations in ACPA+ synovitis implies that the antigen is citrullinated (20-23). Secondly, the strong association between ACPA+ RA and HLA-DRB1 alleles does not allow to determine to what extent the restriction of the TCR repertoire is shaped by the HLA-DRB-restricted antigen or by the class II molecule itself (65). HLA-DR4+ healthy controls did not differ from HLA-DR4- healthy controls with respect to size of clonal expansions or expanded families (61) but unaffected siblings of RA patients display clonal CD4+ T cell specificities (61), suggesting that the TCR repertoire may be intrinsically shaped by the broader genetic background (55;60). Thirdly, we previously showed low numbers of Ki67 positive
lymphocytes in synovial tissue, thereby arguing against strong local proliferation (41). Alternatively, the T cells could have proliferated in the secondary lymphoid organs and migrated to the inflamed synovium where they specifically accumulate. In this context, a most intriguing finding is that not only antigen-driven proliferation but also homeostatic proliferation leads to a marked contraction of the T cell repertoire in RA (76;77). The development of a novel microarray-based methodology to monitor the TCR repertoire at the single-clone resolution now enables us to test this hypothesis formally (78). Although the scenario of homeostatic contraction is not mutually exclusive with antigen-driven or genetically controlled TCR alterations, it implies that not only the presence but also the absence of specific TCR reactivities may be relevant to the disease process and thus that the mere presence of clonal TCR alterations is not per se an argument in favor of T cell directed therapies such as CTLA-4 blockade.

Finally, our observation raises the question whether T cells are directly involved in the ACPA response. As indicated before, the hypothesis that T cells recognizing citrullinated antigens provide help to autoreactive, ACPA-specific B cells needs further confirmation (20-22). Alternatively, the T cells could react to a non-citrullinated epitope included in the protein complex recognized by ACPA+ B cells (23). Whereas better experimental models are required to address this issue formally, our translational approach did not allow us to detect a clear relationship between TCR CDR3-LD alterations and clonal B cell expansions in rheumatoid synovium. Of interest, however, our data plea against the concept that ectopic lymphoid neogenesis contributes to this process by facilitating T-B cell interactions as, in contrast to SpA, the degree of TCR alteration was lower in RA samples with than without clear lymphoid aggregate formation. This observation fits with our recent reports that synovial ectopic lymphoid neogenesis does not determine humoral autoimmunity in RA (41;42) and even argues that this specific microarchitecture is associated with a better preservation of the T cell diversity in the inflamed synovium.

In conclusion, our study indicates selective T cell alterations in ACPA+ versus ACPA-RA synovitis despite similar clinical and histologic features. This strongly supports the concept that both subsets are distinct disease entities and illustrates how state-of-the-art molecular techniques can contribute to pathophysiological rather than phenotypical disease characterization and classification.
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