Clinical studies and tissue analyses in the earliest phases of rheumatoid arthritis: In search of the transition from being at risk to having clinically apparent disease

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INFLAMED TARGET TISSUE PROVIDES A SPECIFIC NICHE FOR HIGHLY EXPANDED T-CELL CLONES IN EARLY HUMAN AUTOIMMUNE DISEASE

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ABSTRACT

Objective

To quantitatively profile the T-cell repertoire in multiple joints and peripheral blood of patients with recent onset (early) or established rheumatoid arthritis (RA) using a novel next-generation sequencing protocol to identify potential autoreactive clones.

Methods

Synovium of patients with recent onset (early) RA (<6 months) (n=6) or established RA (>18 months) (n=6) was screened for T-cell clones by sequencing >10,000 T-cell receptors (TCR) per sample. T-cells from paired blood samples were analyzed for comparison. From 2 patients synovial T-cells were obtained from multiple inflamed joints. The degree of expansion of each individual clone was based on its unique CDR3 sequence frequency within a sample. Clones with a frequency of >0.5% were considered as highly expanded clones (HECs).

Results

In early RA synovium, the T-cell repertoire was dominated by 35 HECs (median; range 2-70) accounting for 56% of the TCRs sequenced. The clonal dominance in the synovium was patient-specific and significantly greater than in established RA (median of 11 HECs (range 5-24) in established RA synovium accounting for 9.8% of T-cells, $p<0.01$). Thirty-four percent (range (28-40%)) of the most expanded T-cell clones were shared between different joints in the same patients, compared to only 4% (range 0-8%) between synovium and blood ($p=0.01$).

Conclusions

In RA, a systemic autoimmune disease, the inflamed synovium forms a niche for specific expanded T-cell clones, especially in early disease. This suggests that – at least in RA – autoreactive T-cells should be addressed specifically in the inflammatory tissue – preferably in the early phase of the disease.

Autoreactive T-cells are likely to play an important role in many autoimmune diseases but the identification and quantification of these cells in autoimmune tissue inflammation has proven difficult in humans[1-3]. Identifying these T-cells is important to understand their role in autoimmune inflammation, to identify auto-antigens, and might help to develop strategies to selectively induce tolerance against auto-antigens.

A strong rationale for the involvement of autoreactive T-cells comes from genome wide association studies in autoimmune disease such as rheumatoid arthritis (RA), autoimmune diabetes (T1D), systemic lupus erythematosus (SLE) and multiple sclerosis (MS). These studies all confirmed that the most influential genetic predispositions to these diseases are located in the HLA-locus[4-7]. Furthermore, a pivotal role for autoreactive T-cells has been demonstrated in many models of autoimmune diseases such as collagen induced arthritis, non-obese diabetic mice and experimental auto-immune encephalitis[8-10]. In several human auto-immune diseases autoreactive T-cell clones have been observed in peripheral blood (T1D, MS)[11, 12]. However, only few studies have demonstrated the presence of autoreactive T-cells in the primary target organ or in its surrounding lymph nodes[13]. Therefore, the relationship between autoreactive clones in blood and the inflamed tissue is poorly understood.

Identification of autoreactive T-cell clones in human tissue is hampered by several factors. Most target tissues in autoimmune diseases are difficult to obtain, especially in the early phase of inflammation. Secondly, in most autoimmune diseases the autoantigens are either unknown (e.g. RA, SLE) or consist of candidate antigens (T1D, MS). In this setting many studies focus on identification of clonally expanded T-cells, since this approach does not require knowledge of putative autoantigens. Classically this approach uses a broad screening method (e.g. spectratyping) to find major repertoire-aberrations followed by cloning and sequencing of TCRs within the aberrations to identify expanded clones. Unfortunately, this approach has a keyhole perspective that has important drawbacks. First, it does not give a full repertoire perspective. Secondly, it cannot quantitatively compare the clonal aberrations found across the repertoire. Third, it is prone to either miss clonal aberrations in the first screening step or suffer from false positive aberrations due to low input[1, 14, 15].

To overcome these limitations we and others recently developed protocols based on next-generation sequencing (NGS) that provide quantitative data on the degree of expansion of individual clones within the complete TCR-repertoire[15-18]. Thus it not only identifies expanded clones, but also obtains detailed information on the impact of these clones on the repertoire as a whole. Furthermore, these protocols for the first time allow quantitative comparisons of clonality between different samples.

Here, we utilize this approach to investigate the TCR-repertoire in early autoimmune inflammatory tissue. We selected RA as a model immune-mediated inflammatory disease as it allows for sampling of the primary target tissue, the synovium. Previous studies showed clonal expansions in RA synovitis leading to a ‘skewed’ repertoire compared to peripheral blood[14, 19-24]. Whereas these studies indicate the presence of expanded synovial T-cell clones, their frequency and relation to the total repertoire in synovium and blood are unknown. Using NGS to visualize the TCR-repertoire, we addressed this challenge by

assessing: (1) Which part of the repertoire is taken up by expanded clones? (2) Does the repertoire change over the course of disease? (3) How does the repertoire in synovium compare to the repertoire in peripheral blood, (4) Does the repertoire overlap between different sites of inflamed tissue? And finally (5) Can we find clones that are shared between different patients?

MATERIALS AND METHODS

Patients

We included 6 patients with a disease duration of <6 months (median 3.5 months; IQR=2.5-4.3 months) who were naïve to treatment with disease-modifying antirheumatic drugs (DMARDs) and 6 RA patients with a disease duration of >1.5 years (median=166 months; IQR=96-274 months) using methotrexate (MTX). The first group is denoted as 'early RA' patients while the latter is denoted as 'established RA' patients. All patients fulfilled the classification criteria for RA, were autoantibody positive (anti-cyclic citrullinated peptide test >25 kAU/L and/or IgM-rheumatoid factor >12.5 kU/L), had active RA (disease activity score evaluated in 28 joints (DAS28) >3.2) and were naïve for treatment with biologicals (Table S1 & S2 for characteristics and MHC-typing)[25]. One additional patient was included for synovial fluid analysis. This patient, (pt13), had established active disease despite treatment with rituximab (last infusion was 9 months before sampling). The study was performed according to the Declaration of Helsinki and approved by the local medical ethics committee. All patients gave written informed consent.

Synovial biopsy, peripheral blood and synovial fluid sampling

At inclusion a peripheral blood sample was obtained and an arthroscopic synovial biopsy was performed from a clinically inflamed joint as described previously[26]. From 3 early RA patients who had persistent arthritis after inclusion despite MTX treatment (7.5-25 mg/wk), additional synovial biopsies were obtained from the same joint after 6 months, when these joints were still inflamed. From one of the patients with established RA synovial biopsies were obtained from both inflamed knee joints within a 10-day period, to study overlap of clones. From pt13 synovial fluid was obtained by arthrocentesis from an inflamed elbow and knee at the same time.

Linear amplification and next-generation sequencing

The linear amplification and next-generation sequencing protocol has been described extensively before[15, 27]. Details are described in the supplementary methods.

Statistics

Values are either expressed as mean or median depending on criteria for (non-) normal distribution. Differences between groups were analyzed using the Student's t-test or Mann-Whitney U tests if appropriate. P-values of <0.05 (two-sided test) were considered statistically significant. Graphpad Prism Software was used to perform the analyses (GraphPad Software, version 5.1, 2007, La Jolla, CA).

RESULTS

First we profiled the TCR repertoire in synovium of 6 patients with early RA (<6 months) who were naive to DMARDs or biologicals. Per patient >10,000 TCR sequences were obtained. We detected multiple clearly expanded clones at the full-repertoire level in all synovium samples in a background of hundreds of less expanded clones (Fig. 1A). A frequency distribution showed a right skewed distribution in which the majority of the clones (84% of clones (mean; SEM 5,9%)) was of low frequency (<0.1% of total TCRs analyzed) (Fig. 1B). Above this value the distribution decreases quickly towards the X-axis, which it approaches at a clonal frequency of 0.4-0.5%. Therefore, we here defined clones with a frequency of >0.5% of the analyzed TCRs to be highly expanded clones (HECs). Only 8.4% (mean; SEM 4.0%) of the clones was expanded beyond this value. In absolute numbers this corresponded to a median of 35 clones (range 2-70).

Subsequently, we determined the contribution of HECs to the total T-cell repertoire. We took a step-wise approach in which we included an ever increasing number of clones starting with the most expanded one, working our way down through the 250 most expanded clones (Fig 1C). Strikingly, we found that the majority of the repertoire was taken up by a small number of HECs. Together, the 6 most expanded clones accounted for 27% of the T-cell sequences present (median; SD 5.2%), while the 35 most expanded clones (median number of HECs) constituted 56% of the T-cell sequences (median; SD 10.4%).

To investigate whether these findings were specific for the synovium, we performed the same analyses on peripheral blood in 3 of the same patients (Fig. 2 and extensive description of data in the supplementary results). In blood only 0.08% of the clones had a frequency of >0.5%. The impact of the most expanded clones was significantly less compared to synovium. Collectively, these findings show that although hundreds of clones were observed, the T-cell repertoire in the inflamed synovium in early RA is dominated by a few HECs only.

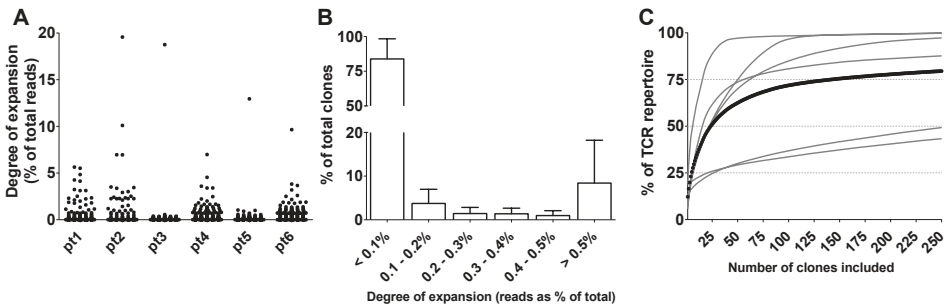

Figure 1. Degree of expansion and impact of T-cell clones in synovium of patients with early rheumatoid arthritis. (A) Scatterplot showing all clones recovered from the synovial samples (6 patients), in which each dot represents one clone. The size of the clones is depicted as percentage of the total T-cell receptors (TCR). (B) Frequency distribution of synovial clones showing a right-skewed distribution. Mean and SD are shown. (C) Cumulative size of most expanded clones (mean (black dots) and individual measurements (grey lines) are shown) from 6 patients. The x-axis depicts the number of clones included (always starting from the most expanded clones). The y-axis shows the percentage of TCR sequences that are attributable to these clones.

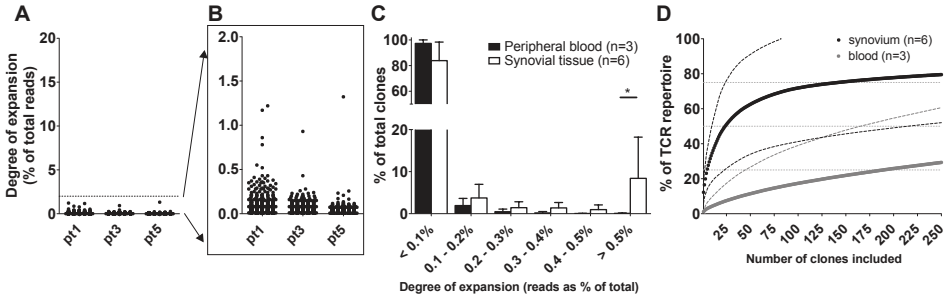

Figure 2. Degree of expansion and impact of T-cell clones in blood in patients with early rheumatoid arthritis, compared with synovium. (A) Scatterplot showing all clones recovered from peripheral blood in early rheumatoid arthritis (RA). Each dot represents one clone. The degree of expansion of the clones is depicted as % of the total T-cell receptors (TCR). (B) Enlargement of 0-2% showing that clones are present, but less expanded. (C) Frequency distribution of T-cell clones. Blood (black bars) is compared with synovium (white bars). Mean and SD are shown. *p<0.05 (Mann-Whitney test). (D) Cumulative size of most expanded clones of peripheral blood (black) and synovium (grey) (mean and 95% CI (dashed lines) are shown). The x-axis depicts the number of clones included (always starting from the most expanded clones). The y-axis depicts the percentage of TCR sequences that are covered by the included clones. The impact of the expanded clones is significantly higher in the synovium compared with the peripheral blood for any number of clones up to 125 clones (Student's t-test).

The available literature is undecided about the question whether prolonged inflammation influences clonality[22, 28, 29]. We therefore analyzed the T-cell repertoire in synovium of 6 patients with 'established' RA (disease duration >1.5 years) who also had active disease (mean DAS 7.3, SD 0.8). In these patients we did observe clonal expansions, but the degree of expansion was less compared to that in early RA patients (Fig. 3A&B). Illustrative was the finding that the median frequency of the most expanded clone was lower compared to synovium of early RA patients (2.2% (SD 0.5) and 12.2% (SD 2.4) respectively (p<0.001)) (Fig. 3C). To exclude the possibility that this difference was due to use of methotrexate (MTX) in the established RA patients, we analyzed additional synovial biopsies from 3 of the early RA patients after 6 months of (ineffective) MTX treatment. These additional samples all showed expanded clones with frequencies of 6-27% and were comparable to the samples described in figure 1 (Fig. S1).

The overall frequency distribution of clones in established RA synovium did not differ significantly from that in early RA, although there was a trend towards a lower number of HECs (mean 0.8% (SD 0.7%) in established RA versus 8.4% (SD 9.7%) in early RA; p=0.11) (Fig. 3D). In established RA this corresponded to a mean of 11 HECs.

Finally, we determined the impact of the expanded clones on the total repertoire (Fig. 3E). The cumulative size of the 11 HECs corresponded to a mean percentage of the TCR-repertoire of 9.8%, which was significantly less compared to the 35 HECs in early RA (56%; p<0.01). When we included the 35 most expanded clones for comparison we could only explain 18.4% of the TCRs, which is significantly less compared to early RA (p<0.001). These data show that there are still HECs present during established RA, but the degree of expansion of these HECS is significantly less than in early RA.

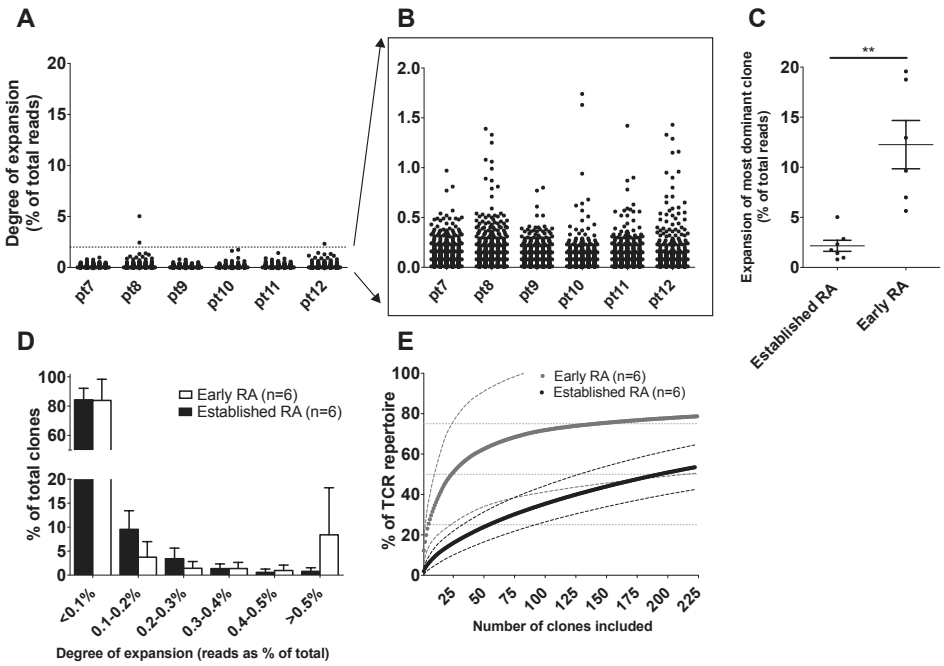

Figure 3. Degree of expansion and impact of T-cell clones in established rheumatoid arthritis compared with early rheumatoid arthritis. (A) Scatterplot showing all clones recovered from synovial tissue in established rheumatoid arthritis (RA). Each dot represents one clone. The size of the clones is depicted as percentage of the total T-cell receptors (TCR). (B) Enlargement of clonal size 0-2% showing that clones are present, but that they are less expanded. (C) Comparison of the degree of expansion of the most expanded clones in each sample shows that clones in early RA are significantly more expanded (median shown, Mann-Whitney test, ** $p<0.01$). (D) Frequency distribution of T-cell clones. Early RA (white bars) is compared with established RA (black bars). Mean and SD are shown. (E) Cumulative size of most expanded clones (mean and 95% CI (dashed lines) are shown). The x-axis depicts the number of clones included (always starting from the most expanded clones). The y-axis shows the percentage of TCR sequences that are covered by the included clones. The impact of the expanded clones is significantly higher in early compared with established RA for any number of clones up to 73 clones (Student's t-test).

Next we investigated whether the HECs overlap between multiple joints of the same patient and between joints and blood. To study overlap between joints we analyzed (rare) synovial samples that were obtained from multiple joints at the same time. From one patient synovial tissue was obtained from both inflamed knees within a 10-day interval. From the second patient synovial fluid T-cells were obtained from an inflamed elbow and knee joint at the same time. To be able to compare synovium, synovial fluid and blood we arbitrarily chose to study the 25 most expanded clones in each sample. When comparing synovium we observed that 15 (60%) of the top 25 clones from the left knee could also be recovered in the right knee joint (Fig 4A). Vice versa 9 of the 25 (36%) selected clones from the right knee could be recovered in the left knee. In both knees 7 (28%) of the 25 most expanded clones overlapped with the 25 most expanded clones from the other joint. In

synovial fluid we observed similar findings; all 25 selected clones (100%) from the elbow could be recovered in the knee and vice versa (Fig 4B). In both joints 10 of the 25 most expanded clones (40%) were among the 25 most expanded clones from the other joint.

To compare synovium with blood we studied paired synovium and peripheral blood from 3 of the early RA patients and 4 of the established RA patients (Fig 4C&D). In this comparison we found that only 4% (median value; range 0-8%) of the 25 most expanded synovium clones could be detected among the 25 most expanded clones in blood, which is significantly less compared to the overlap between joints 34% (range 28-40%; p=0.01) (Fig 4E). These findings illustrate that there is considerable overlap of highly expanded T-cell clones between different joints, but not between joints and peripheral blood.

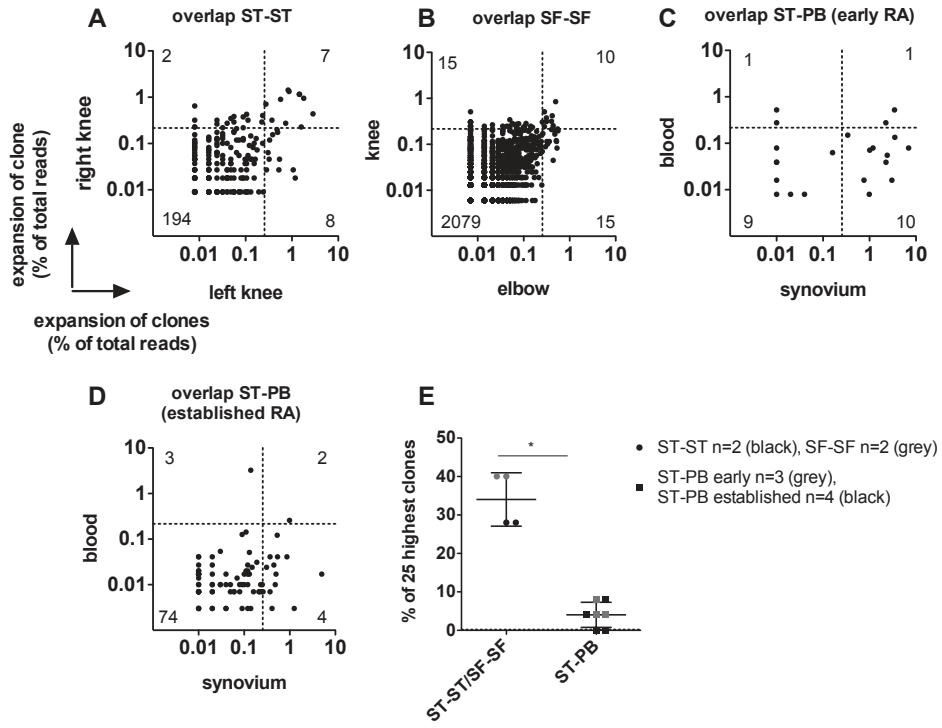

Figure 4. Overlap of T-cell clones between different joints and between synoval tissue and blood. (A) Overlapping clones in 2 synovital tissue (ST) samples taken within 10 days from right and left knee respectively in a patient with rheumatoid arthritis (RA). Each dot represents 1 clone. The frequency (percentege of repertoire) in each knee is depicted on both axes. The lines delineate the frequency of the 25 highest expanded clones; clones depicted above or to the right of these lines represent the clones that are among the 25 highly expanded clones in each knee. Clones in the upper right quadrant represent clones that are found among the 25 highest clones in both knees. (B) Overlapping clones in synovial fluid (SF) taken at the same time from an elbow and knee in a patient with established RA. (C) Representative graph of overlapping clones between peripheral blood (PB) and ST in an early RA patient (n=3 analyses) (D) Representative graph of overlapping clones between PB and ST in an established RA patient (n=4 analysed) (E) Percentage of the 25 most expanded clones that is found in the upper right quadrant of each comparison (ST-ST, SF-SF, ST-PB) (*p<0.05).

Finally, we addressed the question whether the HECs identified in the synovium showed similarities in their TCR-sequence between different patients. Such similarities have been suggested by previous studies although findings are highly contradictory[30]. To this end we compared the TCR sequences of all the synovial HECs detected in this study. We found no clones that were shared by different patients (all 275 CDR3 sequences are shown in Table S3). Moreover, we found great variation in the use of the (V)ariable and the (J)oining genes within individual patients and between different patients (Fig. 5). No preferential use of certain VJ combinations could be observed. Therefore, on the sequence and peptide level these findings do not provide evidence of strong similarities of HECs between different patients despite the presence of at least one DRB1 04-allele in at least 9 of 12 patients.

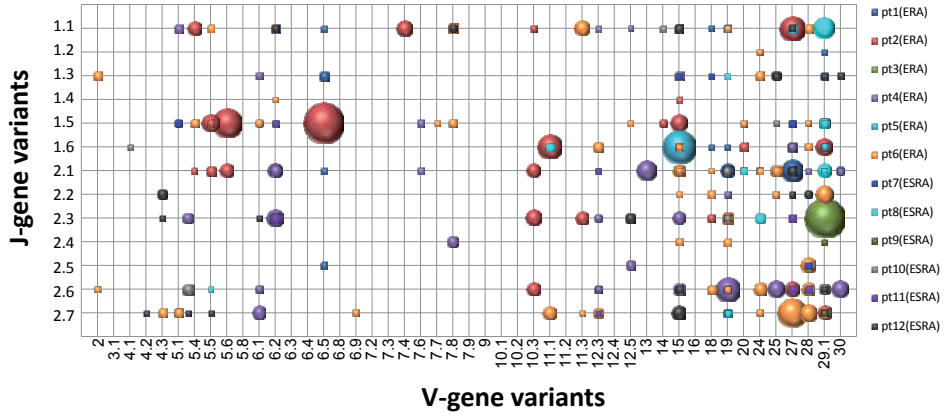

Figure 5. Structural similarity in T-cell receptors of highly expanded clones derived from different patients. All 275 recovered highly expanded clones from all 12 patients with early RA (ERA) or established RA (ESRA) are shown. A balloon represents each HEC. The x-axis shows the V(ariable)-gene segment used by the clones, the y-axis the J(oining)-gene segment. The balloon is proportional to the degree of expansion of the clone.

DISCUSSION

We described the findings of a quantitative and sensitive full-repertoire analysis of inflamed tissue in the context of an auto-immune disease (RA). Our observations suggest that the inflamed synovium in multiple joints forms a specific inflammatory niche for expanded T-cell clones especially in early stages of RA.

Previous studies showed that expanded T-cell clones could be found in the synovium[14, 19-24]. However, both the extent of clonal expansion and the degree of repertoire skewing varied greatly between the studies[30]. Overlap of clones between different joints was reported but the exact relation between clones in different joints and peripheral blood could not be addressed[21, 29]. Our data show clear oligoclonal expansions in the synovium, with great overlap between different joints. In contrast the TCR repertoire in peripheral blood is polyclonal and there is hardly any overlap between synovium and blood. In fact,

many of the HECs from the synovium could not be recovered from the peripheral blood suggesting that – if they are present in the blood – their frequency is lower than 0.01% (lower detection limit). This observation is in accordance with previous studies in T1D reporting an extremely low frequency of pancreas-specific T-cells in the blood (<0.001%) and enrichment for autoreactive T-cells in the (T1D) pancreas compared to the peripheral blood[3, 31]. These findings imply that analysis of clonality in blood samples might not be informative on the T-cell repertoire in the inflamed synovium. The observed selective oligoclonality in synovium implies that there is highly effective retention and/or proliferation of certain T-cell clones in the synovial niche. The notion that this process is not random is supported by the observed overlap of expanded clones between different inflamed joints. It is tempting to speculate that this retention is caused by the presence of specific (auto)antigens in the synovium.

A striking observation is the difference in the degree of expansion in synovium when comparing early and established RA. Previous literature on such temporal differences in synovial T-cell clonality is conflicting[29]. Here, looking at the full repertoire using the quantitative NGS approach we observed clear and significant differences. A possible explanation for this temporal difference might be 'epitope spreading', in which chronic inflammation leads to neo-epitopes that trigger new autoreactive clones. This phenomenon has been demonstrated in models of autoimmune disease, including models of RA and T1D[32]. In accordance with this model, Monach *et al.* recently showed that in synovium of established RA patients many different autoantigens are being recognized by multiple immunoglobulins[33]. If epitope spreading would be the leading process behind the differences observed in our study, the HECs in early RA would lose their dominance during chronic inflammation as more clones are being triggered by neo-epitopes. If epitope spreading does take place it is a strong rationale for very early intervention in RA to prevent the disease from spreading. Another theoretical explanation could be that the differences are caused by a change in the presence of T-cell subsets that are known for their vigorous expansions (e.g. CD8, CD28null, or CD57+)[15, 23, 34]. However, in well-controlled studies such differences between early and longstanding disease have never been detected for phenotypic markers, cytokines or other markers[35-37]. Future studies might combine immunophenotyping with repertoire sequencing to further characterize the HECs. Finally, although we did not observe reduced clonality after 6 months of MTX-treatment, the influence of medication cannot be fully ruled out at this point. These effects, and the effects of disease activity itself (both systemically and locally) should be explored in detail in additional (longitudinal) studies.

To estimate the degree of expansion of clones we chose to measure TCR mRNA over TCR DNA as an mRNA-based analysis uses 13 times less primer combinations and does not require a multiplex PCR amplification. Measuring mRNA as read-out assumes that TCR mRNA levels are equal in different T cells. Although this assumption has been used for many years, it has not been studied extensively. In-vitro studies showed that TCR mRNA levels and TCR surface expression are equal for quiescent CD4+ and CD8+ T cells (including memory cells). Effector T cells are thought to have at most two time higher levels of TCR surface expression than quiescent cells, with related mRNA levels[38, 39]. In our study this means that the estimates of clonal expansions 'as percentage of all TCRs present' are

likely to be accurate, but estimates on clonal expansions in terms of number of cells might be slightly inflated up to a factor of 2.

The search for autoreactive T-cell clones in autoimmune disease, especially in RA, has proven more difficult than in models of autoimmune disease. The lack of knowledge on both the autoantigens and the autoreactive T-cells forms a dilemma. For instance, it has been shown that clones against latent viruses such as Epstein Barr virus also reside in inflamed synovium[40-41]. Therefore, to prove that expanded T-cells are truly autoreactive one needs auto-antigens, but to find these antigens one needs the autoreactive T-cells. Therefore, the advent of new approaches as described in this study may help to identify potential autoreactive clones that subsequently need to be coupled to autoantigens. Our results clearly show that such 'coupling' studies (e.g. using stimulation approaches) need to take into consideration the fact that there are hundreds of small clones in the synovium. These clones might represent 'bystander' clones as they do not overlap between different joints. Therefore, the most sensible approach may be to separate the HECs from the small clones before characterizing these clones and interrogating their specificity.

The inflamed tissue niche therefore seems an attractive location to identify and further characterize potential autoreactive clones especially in early RA. This in turn will help to develop novel therapies targeting the underlying autoimmune processes in a more selective and effective way.

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SUPPLEMENTARY METHODS

Linear amplification protocol:

Preparation: Synovial biopsies were snap-frozen in liquid nitrogen and cryo-preserved until use. RNA was isolated from synovial tissue samples using a polytron tissue homogenizer (Kinematica AG, Littau-Lucerne, Switserland) in the presence of STAT60 RNA reagent (Tel-test Inc, Friendswood, TX) according to the manufacturer's protocol. After isolation RNA was purified using the RNeasy Mini System (Clean-up-protocol (#74106, Qiagen, Venlo, the Netherlands). Blood samples consisted either of heparinized blood (established RA-patients) or blood that was collected directly in PAXgene lysis buffer for RNA-isolation (#762165, Preanalytix, Hombrechtikon, Switzerland), and stored at -80°C according to the manufacturer's instructions (early RA patients and 1 established RA patient). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using standard density gradient centrifugation techniques by use of Lymphoprep (Nycomed Pharma, Oslo, Norway) and cryo-preserved until used. RNA was isolated from blood samples using the RNeasy Mini System. RNA quality was checked using the Bioanalyzer 2100 system (Agilent) and quantified using the Qubit1.0-platform (#Q32857, Invitrogen Life Technologies, Breda, the Netherlands).

Linear amplification: Samples were processed as described previously[1, 2]. cDNA was synthesized with Superscript RT-III and oligo-dT primers according to the manufacturer's protocol (#18080-051, Invitrogen). Linear amplification (LA) of the complete TCR repertoire was performed as described earlier[1]. In the first step of LA cDNA was amplified using a modified version of the V-beta primerset described by van Dongen *et al*.[3]. All V-primers contain the primerB sequence needed for sequencing according to the 454-titanium protocol for Amplicon sequencing (version 2010) (Roche Diagnostics, Mannheim, Germany (G)). Here we use the HUGO-nomenclature according to reference[4]. In the first step of LA the cDNA was amplified in the presence of 5 pmol of each of the 23 V-primers, 1x buffer B (Solis BioDyne, Tartu, Estonia), 1mM $MgCl_2$, 0.1 mM dNTPs and 3U of Hotfire (Solis BioDyne) in a volume of 20 uL using a T-Professional thermocycler (Biometra, Goettingen, Germany) (96°C (900s), 40× (96°C (30s), 60°C (60s), 72°C (60s)), 72°C (600s)). Amplified products were purified using AMPure XP SPRI-beads (#A63881, Agencourt-Bioscience, Beverly, MA) in a template:bead ratio of 0.9. After LA a generic PCR was performed to prepare the samples for sequencing. In this second reaction primerB was used as generic forward primer and a generic primer specific for the TCRβ-Constant gene segment (5'-CTCAAACACAGCGACCTC-3') was used as reverse primer. The reverse primer contains a multiplex identifier and primerA as described in the amplicon-sequencing manual (Roche). The PCR was performed with 25% of the purified LA-product in the presence of 10 pmol of each of the primers, 1x buffer B, 1mM $MgCl_2$, 0.1 mM dNTPs and 3U of Hotfire in a volume of 40 uL using a T-Professional thermocycler (96 ◦C (900s), 35× (96°C (30s), 60°C (60s), 72°C (60s)), 72°C (600s)). After amplification, samples were again purified using the AMPure beads and quantified using fluorospectometry (Quant-iT dsDNA HS Assay Kit (#Q32851, Invitrogen). Samples were prepared for sequencing according to

the manufacturer's protocol for Amplicon Sequencing. NGS was performer on a Roche Sequencer FLX using the Titanium platform. For each sample > 60,000 (bead-bound) TCR sequences were analyzed.

Bioinformatics and data-analysis: The bioinformatics pipeline used to obtain the TCR-sequence was described previously in detail and contains 4 modules: (1) MID-sorting, (2) Identification of gene segments, (3) CDR3 detection, and (4) Removal of artifacts[1]. To prevent bias that might be caused by differences in the number of T-cells within the synovial biopsies, we analyzed the background frequency of non-expanded clones in all of the samples (Fig. S4). In none of the samples the mean clonal frequency of non-expanded clones differed > 2 times SD of the grand mean.

SUPPLEMENTARY RESULTS

Extensive description of comparison of synovial tissue repertoire to repertoire in peripheral blood

To investigate whether the make-up of the repertoire described in Fig. 1 is specific for the synovium we performed the same analyses in peripheral blood. We used paired peripheral blood samples from the same patients (n=3) taken at the time of the synovial biopsy. The peripheral blood did not show the same degree of clonality as the synovium (Fig. 2A & B). The most dominant clone observed had a size of 1.2% of the TCRs analyzed. The frequency distribution of clones with a given clonal size in peripheral blood and synovium showed that the distribution of small clones was similar in both compartments, while the TCR repertoire in the synovium contained significantly more expanded clones than the peripheral blood (mean 0.97% (synovium) of clones vs 0.04% (blood) in the histogram range 0.4% - 0.5% ($p < 0.05$) and 8.4% (synovium) vs 0.08% (blood) in the histogram range > 0.5% ($p < 0.05$)) (Fig 2C). In another approach, we analyzed the impact of the most expanded clones on the repertoire (Fig 2D). This again showed that most expanded clones in the peripheral blood had a significantly smaller impact on the T-cell repertoire when compared to synovium. This difference was most pronounced for the most expanded clones and remained statistically significant up to the 125 most expanded clones. The 35 most expanded clones (mean number of HECs in synovium) constituted only 9.46% (median; SD 4.4%) of the T-cells present in the peripheral blood, compared to 56% in the synovium ($p < 0.05$). These findings show that the difference in T-cell repertoire in early RA between peripheral blood and synovium is mostly confined to the expanded clones, which are hardly present in the blood.

SUPPLEMENTARY FIGURES

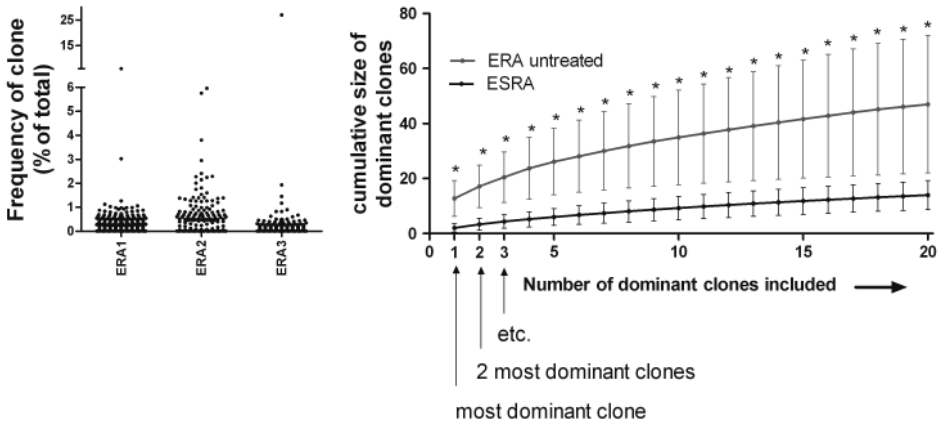

Figure S1. Comparison of T-cell clones in synovial tissue of early RA patients (ERA) after MTX treatment with T-cell clones of established RA patients. Left: A scatterplot shows that the size of the most expanded clones is comparable with that of early RA patients without MTX. Right: Cumulative size of most expanded clones (mean and 95% CI are shown). The x-axis depicts the number of clones included (always starting from the most expanded clones). The y-axis shows the percentage of T-cell receptor sequences that are covered by these clones. The impact of the expanded clones is significantly higher in early compared with established RA for any number of clones up to 125 clones.

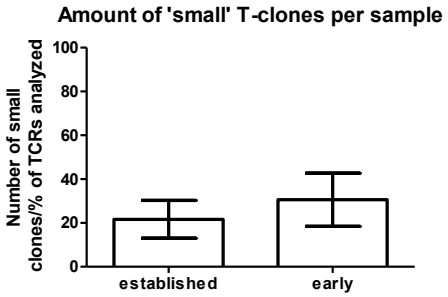

Figure S2. Comparison of amount of 'small' clones in the synovial tissue. To exclude that the difference in clonality found between early and established RA patients was based on a difference in T-cell infiltration we performed the following analysis: we removed all the highly expanded clones (clonal size >0.5% of the repertoire) (HECs) from the datasets as they can make up large parts of the cells analysed (example: HECs can take up 40% of all TCR sequences). In the remaining datasets we determined the amount of clones that were recovered (example 1200 clones). We then divided the number of recovered clones by the percentage of data that was left after removal of the HECs (example: 1200/60% = 20 clones/% of data). This way the background-frequency of (small) clones can be determined. If there are less T-cells in the biopsy than there were sequences analysed, then the number of 'small' clones/% of data will decrease. As can be seen in the figure this number is equal for both patients groups. Samples in which the mean clonal frequency of non-expanded clones differed >2 times standard deviation (SD) from the grand mean (of all samples analyzed in this study) were excluded.

SUPPLEMENTARY TABLES

Table S1. Patient characteristics

	Early RA	Established RA
Number of patients	6	6
Female, no. (%)	5 (83)	4 (67)
DAS28, mean (SD)	5.3 (1.3)	7.3 (0.8)
ACPA positive, no. (%)	5 (83)	6 (100)
IgM-RF positive, no. (%)	5 (83)	6 (100)
Disease duration, months (IQR)	3.5 (2.5-4.3)	166 (96-274)

DAS28 = Disease activity score 28-joint assessment, SD = standard deviation, ACPA = anti-citrullinated protein antibodies, IgM-RF = IgM Rheumatoid factor. The cohorts were well matched. The DAS28 score was slightly lower in the ERA cohort, but this did not reach significance. The disease duration was significantly different as expected ($p<0.005$, Mann-Whitney test).

Table S2. HLA-typing

Pt	group	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1*
1	Early RA	02/11	27/51	02/03	04/16	03/05
2	Early RA	30/68	35/44	04/05	04/04	03/03
3	Early RA	34/34	13/15	03/04	04/15	04/06
4	Early RA	02/32	15/55	03/03	04/04	03/03
5	Early RA	02/68	44/51	05/15	04/11	03/03
6	Early RA	02/11	07/07	07/07	04/15	03/06
7	Established RA	02/02	35/35	03/03	-	03/03
8	Established RA	01/26	56/57	01/07	04/04	03/03
9	Established RA	11/74	46/49	01/07	04/12	04/05
10	Established RA	02/03	07/55	07/03	04/04	03/03
11	Established RA	-	-	-	-	-
12	Established RA	-	-	-	-	-

Two-digit HLA typing was performed Sanquin (Amsterdam, the Netherlands) using sequence specific primers (PCR-SSP). In pt 7 DRB1 could not be characterized. From patient 11 and 12 no DNA was available for analysis. Strikingly, 9/10 patients carried at least one DRB1 4-allele.

Table S3. T-cell receptors of highly expanded clones in synovium of 12 patients

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
1	27	2,1	CASKKEQGSYNEQFF	5,56%
1	19	2,1	CASSITSGDYNEQFF	2,53%
1	27	1,2	CASSTGQGRDYGYTF	2,10%
1	27	2,7	CASSLRTTPTYEQYF	1,97%
1	19	2,1	CASRGLAGEGEQFF	1,47%
1	6,5	2,5	CASSYRSWETQYF	1,41%
1	27	2,1	CASRVYLGGNEQFF	1,41%
1	27	1,2	CASSLYRTDYGYTF	1,25%
1	29,1	2,5	CSVVHSAPAQETQYF	1,01%
1	27	2,1	CASSSAGEYNEQFF	1,00%
1	30	2,1	CAWSVSSYNEQFF	0,96%
1	19	1,1	CASSTTTGRTEAFF	0,96%
1	29,1	2,3	CSPTGQSTDTQYF	0,92%
1	27	2,2	CASSPRQGATGELFF	0,92%
1	6,5	1,3	CASSSGRGNTIYF	0,90%
1	27	2,7	CASSLSLTRTGTYEQYF	0,90%
1	12,3	1,2	CASSGLDGYTF	0,89%
1	19	1,5	CASSISVSQPQHF	0,89%
1	19	2,1	CASSLTSGSYNEQFF	0,87%
1	24,1	2,1	CATSDDTSGRHNEQFF	0,84%
1	29,1	2,3	CSVETGGTDTQYF	0,80%
1	6,2	2,1	CASSYSTLYEQFF	0,80%
1	29,1	2,3	CSVRAGGGDTQYF	0,71%
1	6,5	2,7	CASSYSIRGHEQYF	0,68%
1	6,5	2,1	CASSYSIVPQGANVFF	0,67%
1	19	2,7	CASRQTSGSFHEQYF	0,63%
1	18	2,2	CASSPPTNTGELFF	0,63%
1	15	2,7	CATSRGDLANSYEQYF	0,62%
1	6,2	2,1	CAIGEGTEAFF	0,61%
1	19	2,1	CASGLTSGSYNEQFF	0,60%
1	19	2,2	CASSMGLAGRGGELFF	0,57%
1	29,1	2,7	CSVEVGSNEQYF	0,57%
1	29,1	2,7	CSVEVKPYEQYF	0,55%
1	29,1	2,6	CSVEDPGSGANVLTF	0,55%
1	18	2,7	CASSPPARSVSEQYF	0,54%
1	27	1,5	CASSLFGQGVYQPQHF	0,53%
2	6,5	2,3	CASTSGRGADTQYF	19,57%
2	5,6	2,3	CASSLKGLDTQYF	10,10%
2	11,1	2,2	CASSLSSWESNTGELFF	6,96%
2	27	2,7	CASSLSPRVHEQYF	6,95%
2	5,5	2,3	CASSLASGEDTQYF	3,51%
2	29,1	2,2	CSVPRTSGSTGELFF	3,45%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
2	15	2,3	CATSLAVGAADTQYF	3,37%
2	10,3	1,5	CATSTGDSNQPQHF	3,02%
2	7,4	2,7	CASSLSTLDDRYEQYF	2,92%
2	27	1,2	CASSRSWQNYGYTF	2,52%
2	10,3	2,1	CATQGPQIGGNEQFF	2,41%
2	12,3	2,7	CASSLTSGYEQYF	2,35%
2	12,3	1,5	CASSQTLQVQPQHF	2,28%
2	5,6	2,1	CASSFITSEDNEQFF	2,26%
2	29,1	1,1	CSAPPGQGNTEAFF	2,26%
2	5,4	2,7	CASSYPGLGHEQYF	2,26%
2	10,3	1,2	CATNNGQGAESYTF	2,25%
2	6,2	2,1	CASSYSDRGLSSYNEQFF	2,11%
2	6,6	2,3	CASSHQGKTDTQYF	1,54%
2	5,5	2,1	CASGRGNEQFF	1,42%
2	20,1	2,2	CSARASLMGNTGELFF	1,19%
2	18	1,5	CASSPPGSGNQPQHF	1,05%
2	29,1	2,2	CSATPSTGELFF	1,01%
2	14	2,3	CASKGTSGYTDTQYF	0,99%
2	12,3	1,1	CASSFQGVGTEAFF	0,91%
2	5,5	2,3	CASSQQVSLSQTQYF	0,79%
2	6,2	2,1	CASSYAGQGGYNEQFF	0,76%
2	10,3	2,7	CATKQERAGEYEQYF	0,70%
2	15	2,4	CATSRDGAISGRLYF	0,67%
2	19	2,7	CASTRTSGDSYEQYF	0,64%
2	5,4	2,1	CASSSRGERGEQFF	0,60%
2	6,2	2,1	CASSHLTGSQGQFF	0,58%
3	29,1	1,5	CSVDDGDSNQPQHF	18,76%
3	19	1,5	CSVDDGDSNQPQHF	0,56%
4	19	1,2	CASSPGSYGYTF	6,99%
4	13	2,1	CASREGFGNWANEQFF	4,54%
4	6,2	1,5	CASSYRTGRAQHF	3,59%
4	11,2	1,2	CATSTGGHGYTF	3,41%
4	5,6	1,2	CASSPGTGAYGYTF	3,40%
4	6,2	2,1	CASMGGRNEQFF	2,47%
4	6,1	1,1	CASSYQDREAFF	2,23%
4	15	1,5	CATSRVGGHQPQHF	2,01%
4	6,5	1,2	CASSYGTGVYGYTF	1,80%
4	6,6	1,5	CASSGGWGDQPQHF	1,67%
4	7,8	1,4	CASSPTATDALKKLFF	1,65%
4	6,2	2,1	CASSYWGGGNEQFF	1,65%
4	29,1	2,3	CSVGQVLITDTQYF	1,56%
4	7,8	2,7	CASSPRQGPSYEQYF	1,53%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
4	27	2,2	CATGGHGRAGELFF	1,45%
4	6,2	1,5	CASSSTGRNQPQHF	1,39%
4	12,5	1,3	CASGLILPRTKLHNTIYF	1,37%
4	29,1	1,2	CSVEGSAGPNYGYTF	1,27%
4	29,1	2,7	CSVNLADSYEQYF	1,26%
4	6,2	2,7	CAGSSASLYEQYF	1,22%
4	6,5	2,5	CASSYSTGRSWETQYF	1,21%
4	6,5	1,1	QP	1,20%
4	5,1	2,7	CASSTRDSGYEQYF	1,14%
4	19	1,5	CASRGANQPQHF	1,02%
4	6,1	1,2	CASSELRTGNYGYTF	1,01%
4	19	2,1	CASSANPRTGGEQFF	1,00%
4	27	2,2	CASSLQWTGGKAGELFF	0,99%
4	12,3	1,5	CASRPDRGGNQPQHF	0,96%
4	29,1	1,6	CSVSLSSYNSPLHF	0,94%
4	19	1,2	CASSRDRGNYGYTF	0,94%
4	7,6	2,1	CASSLQAGQPNEQFF	0,86%
4	7,6	2,3	CASSLAEGQGFRNTQYF	0,79%
4	6,2	2,7	CASSYSMWTTYEQYF	0,78%
4	6,1	2,5	CASMAGRGAGPPETQYF	0,77%
4	6,2	2,3	CASSYRQGTDTQYF	0,74%
4	6,5	2,1	CASSYLGQYNEQFF	0,73%
4	19	1,6	CASSIRPRINSPLHF	0,72%
4	12,3	1,2	CASSSGQRFNYGYTF	0,71%
4	5,1	2,3	CASSPNTDTQYF	0,70%
4	6,5	2,2	CASSYLGGSGELFF	0,70%
4	27	2,7	CASSPGTGFYEQYF	0,69%
4	12,3	2,7	CASSFLTGQGREQYF	0,66%
4	27	2,3	CASSPYQGGTDTQYF	0,60%
4	11,2	2,1	CASSLNRGNNEQFF	0,58%
4	10,3	2,1	CAISPMATANEQFF	0,58%
4	12,3	1,1	CASSSRQETQYF	0,53%
4	12,3	2,1	CASSRQAGAYNEQFF	0,52%
4	19	2,1	CATSHRGGAVEQFF	0,52%
4	12,5	2,7	CASSLGFSYEQYF	0,50%
5	6,5	2,2	CASSYSAGNTGELFF	12,95%
5	19	1,1	CASLPGTGHTEAFF	1,04%
5	6,5	2,7	CASSSQREFYEQYF	1,00%
5	19	2,7	CASSTRASTYEQYF	0,91%
5	29,1	2,2	CSGRNTGELFF	0,70%
5	18	1,2	CASSPLKGGVEGYTF	0,57%
5	27	2,1	CASSLLAGRSDEQFF	0,57%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
5	19	1,1	CASSPRTERGDTEAFF	0,55%
5	19	2,7	CANGDSLSYEQYF	0,52%
6	27	1,1	CASSLGREGGMNTEAFF	11,09%
6	29,1	1,6	CSVTTGQGNSPLHF	4,39%
6	10,3	1,1	CAISESRGQGANTEAFF	4,21%
6	5,7	2,7	CASSTRPSGGQYF	3,67%
6	10,3	1,3	CAISEWDIHSSGNTIYF	2,99%
6	11,3	2,7	CASSLDGPGVYEQYF	2,88%
6	11,1	1,1	CASSLAEYTEAFF	2,49%
6	24,1	1,2	CATRDSHGYTF	2,17%
6	6,5	2,1	CASSTTSFAYNEQFF	2,15%
6	10,3	1,2	CAISQQQGDFGYTF	2,15%
6	5,6	2,1	CASSFKGGPVSYNEQFF	2,08%
6	19	1,5	CASSIDFHSNQPQHF	2,02%
6	7,8	2,7	CASSPSQGPSYEQYF	1,77%
6	12,3	2,2	CASSWGGDTGELFF	1,58%
6	6,5	2,1	CASRTGQRNEQFF	1,57%
6	19	1,5	CASSIMADFNQPQHF	1,56%
6	12,3	1,1	CASSLRENTEAFF	1,54%
6	5,1	1,1	CASSRDAGLNTEAFF	1,41%
6	29,1	2,7	CSVAGVVSREQYF	1,39%
6	4,3	1,1	CASSQADRARSEAFF	1,36%
6	10,3	2,7	CAISESNRGYEQYF	1,33%
6	2	2,5	CASSGNRGRETQYF	1,31%
6	7,2	1,2	CASSLAGSHYGYTF	1,30%
6	6,5	2,7	CASGREQYF	1,30%
6	24,1	2,5	CATSDLYAGRETQYF	1,26%
6	7,8	2,3	CASSLNRGGTDTQYF	1,25%
6	5,1	1,1	CASSLVGAREAFF	1,25%
6	5,4	2,3	CASSLEVAGGTQYF	1,24%
6	27	2,7	CASSLSTGPNEQYF	1,18%
6	6,6	1,5	CASSRDRHNQPQHF	1,15%
6	6,2	2,7	CASSRDRHNQPQHF	1,15%
6	6,1	2,3	CASSEYVDTQYF	1,07%
6	19	1,4	CASRRGTNRVLSNEKLFF	1,07%
6	5,7	2,7	CASSTRPSGGQYF	1,06%
6	19	2,1	CASSIRSSNEQFF	1,05%
6	5,7	2,1	CASSLSGGMMSYNEQFF	1,04%
6	15	2,2	CATSRDNLRGTTGELFF	1,02%
6	29,1	1,1	CSVEERMNTEAFF	1,01%
6	7,2	1,6	CASSALTLELYNSPLHF	0,99%
6	6,5	1,4	CASSYGGRTEKLFF	0,97%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
6	15	2,7	CATSRTGSSYEQYF	0,94%
6	10,3	2,2	CAIGKGQAINTGELFF	0,93%
6	6,9	1,1	CASSYYGRDTEAFF	0,87%
6	7,9	1,1	CASSPGTGGWLNTEAFF	0,87%
6	19	1,5	CASSITRGVNQPQHF	0,84%
6	20,1	2,1	CSARENDRPGRDEQFF	0,84%
6	5,6	1,6	CASSMRMLHSPLHF	0,83%
6	27	2,3	CASSFRDRESYTQYF	0,83%
6	6,5	2,2	CASSYPAWGGYTGELFF	0,83%
6	19	2,7	CASSPETGIIYEQYF	0,82%
6	29,1	1,1	CSVGSANTEAFF	0,82%
6	20,1	2,3	CSAIPISTDTQYF	0,82%
6	7,9	2,6	CASSFPGRRVSGANVLTF	0,80%
6	19	1,5	CASSIRTGASNQPQHF	0,80%
6	10,3	2,3	CAIRDRGDTQYF	0,80%
6	2	1,2	CASSARDRGNGYTF	0,79%
6	24,1	2,1	CATSESGTKDNEQFF	0,76%
6	6,5	1,1	CASSYSPGRMNTEAFF	0,75%
6	11,1	2,2	CASSLARNTGELFF	0,73%
6	19	1,2	CASSIDRGTVYGYTF	0,69%
6	2	1,2	CASSDFNVVANYGYTF	0,66%
6	12,5	2,3	CASGPDRGRRDTQYF	0,65%
6	6,9	1,6	CARGGRGDHSPLHF	0,65%
6	7,2	2,1	CASSPPGLGGAYNEQFF	0,62%
6	6,2	2,4	CASIDRGRANIQYF	0,62%
6	19	1,1	CASSIRSNTEAFF	0,61%
6	7,7	2,3	CASSLTQRVRADTQYF	0,61%
6	15	2,1	CATARTRNEQFF	0,60%
6	7,3	1,1	CASSLKVRDEAFF	0,60%
6	6,6	2,2	CASSYLGQGNTGELFF	0,60%
7	5,1	2,3	CASSLFGATDTQYF	0,97%
7	10,3	1,3	CAISGPGNTIYF	0,81%
7	18	2,5	CASSPDRFGTQYF	0,57%
7	6,5	2,5	CASRRGWAQYF	0,54%
7	6,6	2,7	CASSTLSGRGGNEQFF	0,52%
8	29,1	2,7	CSVERIGNNEQYF	5,03%
8	29,1	2,1	CSVVQALYNEQFF	2,44%
8	29,1	2,3	CSVGGGVPDTQYF	1,39%
8	24,1	1,5	CATSPTSSNQPQHF	1,33%
8	5,7	2,2	CASSSGGSNTGELFF	1,25%
8	27	2,7	CASSLYPLGLRSYEQYF	1,08%
8	11,1	2,2	CASRWTSGGNTGELFF	1,06%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
8	19	2,1	CASSINGGYNEQFF	0,99%
8	6,5	1,1	CASSNNQEGAEAFF	0,89%
8	21,1	2,7	CASSKARGLYEQYF	0,87%
8	6,5	1,1	CASSYHDTRTEAFF	0,81%
8	20,1	2,1	CSARDDRGPEQFF	0,79%
8	29,1	2,3	CSSEYTDTQYF	0,71%
8	19	2,5	CASSPGQGGETQYF	0,59%
8	27	2,7	CASSLGTYHEQYF	0,54%
8	29,1	2,1	CSSRVSYNEQFF	0,54%
8	6,5	1,2	CASRHSRTAGYGYTF	0,53%
8	29,1	1,2	CSVLSTENYGYTF	0,52%
8	27	1,2	CASRGAGTGHLLNYGYTF	0,52%
8	19	1,1	CATDAGTTEAFF	0,52%
8	5,5	1,2	CASNRESAYTF	0,51%
8	5,7	2,1	CASSLLAGAYNEQFF	0,51%
8	27	2,7	CASSLTTASYEQYF	0,50%
8	29,1	2,1	CSVVDSHSYNEQFF	0,50%
9	29,1	1,1	CSVEDPGSNTEAFF	0,80%
9	6,5	1,2	CAPTPEAYGYTF	0,77%
9	29,1	1,4	CSVGTGGTNEKLFF	0,61%
9	27	2,1	CASSFRTSGGVGEQFF	0,52%
9	29,1	1,1	CSVIGDWNTEAFF	0,52%
10	6,6	1,2	CASSRQFNYGYTF	1,74%
10	29,1	1,2	CSATMDRDYGYTF	1,63%
10	19	2,1	CASSIDYISYNEQFF	0,94%
10	14	2,7	CASSPTGTSTHEQYF	0,68%
10	4,1	2,2	CASGLAGNTGELFF	0,64%
10	27	1,5	CASSSQYSNQPQHF	0,62%
10	19	1,5	CASGTGDSNQPQHF	0,57%
10	5,6	2,3	CASSLGLAIDTQYF	0,51%
11	10,3	1,2	CAISVQGASYTF	0,89%
11	27	1,2	CASSPGTGFGGYTF	0,86%
11	27	1,5	CASRPTGTGEGQPQHF	0,78%
11	12,3	1,1	CASRYIGVTTEAFF	0,63%
11	6,5	1,1	CASRRTGMNTEAFF	0,58%
11	6,2	2,3	CASSYSQGWDTQYF	0,57%
11	6,6	2,7	SYSGQVSYEQYF	0,56%
11	6,5	1,2	CASSYIGSNYGYTF	0,56%
11	12,3	2,1	CASRLDYNNEQFF	0,55%
11	6,5	2,7	CASSCTQGSPSYEQYF	0,52%
11	6,6	2,5	CASSSTGGGETQYF	0,52%
12	6,5	1,1	CASLPGTGTEAFF	2,32%

Table S3. continued

Patient	V-gene	J-gene	CDR3 sequence (Amino Acids)	Frequency in synovium
12	12,5	1,5	CASGLDTESNQPQHF	1,43%
12	4,3	1,6	CASSQDQGRGSPLHF	1,33%
12	5,6	2,5	CASSLASFGQETQYF	1,29%
12	15	2,7	CATSRDPEGGYEQYF	1,16%
12	27	2,1	CASSLLSGFNEQFF	1,15%
12	10,3	1,6	CASGQAYEQYF	0,96%
12	7,8	2,7	CASSLGQAYEQYF	0,95%
12	15	1,2	CATSRDQEGIYGYTF	0,90%
12	30	2,5	CAWTGREETQYF	0,80%
12	6,2	2,7	CASRILAGGYEQYF	0,71%
12	27	1,6	CASSPAPGGSPLHF	0,71%
12	29,1	1,2	CSVEGWTLYGYTF	0,65%
12	4,2	1,1	CASSHDRDATEAFF	0,65%
12	29,1	2,5	CSVLGQAGETQYF	0,60%
12	5,6	2,5	CASSLARALLYQETQYF	0,59%
12	5,5	1,1	CASSGQTNTEAFF	0,57%
12	27	2,7	TGNDYEQYF	0,55%
12	4,3	1,5	CASSQVWTEANQPQHF	0,54%
12	29,1	1,2	CSVEGRAGGSNYGYTF	0,54%
12	6,1	1,5	CASSGTGHNQPQHF	0,53%
12	6,6	1,1	CASSPGTEARTEAFF	0,52%

Frequency of highly expanded clones (HECs)>0.5% of total reads of a sample. Shown: V(ariable) gene segment, J(oining) gene segment, CDR3 and frequency of clone in synovium (percentage of all T-cell receptor sequences in sample). The CDR3-amino acid sequence was determined using V-quest (Brochet, X. et al., Nucl. Acids Res. 36, W503-508 (2008).

SUPPLEMENTARY REFERENCES

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