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

de Hair, M.J.H.

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THE CELLULAR COMPOSITION OF LYMPH NODES IN THE EARLIEST PHASE OF INFLAMMATORY ARTHRITIS

L.G.M van Baarsen¹,²*, M.J.H. de Hair¹*, T.H. Ramwadhoebé¹,², I.J.A.J. Zijlstra³, M. Maas³, D.M. Gerlag¹, P.P. Tak¹

¹Division of Clinical Immunology and Rheumatology, ²Department of Experimental Immunology, ³Department of Radiology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands, *Both authors contributed equally

Submitted for publication
ABSTRACT

Objectives
Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease of unknown etiology. Recent work has shown that systemic autoimmunity precedes synovial inflammation and animal models have suggested that changes in the lymph nodes may precede those in the synovial tissue. Therefore, we investigated the cellular composition of the lymph node in the earliest phases of inflammatory arthritis.

Methods
Thirteen individuals positive for IgM rheumatoid factor and/or anti-citrullinated protein antibodies without arthritis were included. Additionally, we studied 15 early arthritis patients (arthritis duration ≤6 months, naïve for disease-modifying antirheumatic drugs), and 8 healthy controls. All subjects underwent ultrasound-guided inguinal lymph node biopsy. Different T- and B-lymphocyte subsets were analysed by multi-color flow cytometry.

Results
There was an increase in activated CD8+CD69+ T cells and CD19+ B cells in early arthritis patients compared to healthy controls. We also observed a trend towards increased CD19+ B cells in autoantibody-positive individuals without arthritis compared to healthy controls.

Conclusions
This exploratory study suggests that there is increased immune cell activation within lymph nodes of early arthritis patients as well as in autoantibody-positive individuals at risk of developing RA. This method provides a unique tool to investigate immunological changes in the lymph node compartment in the earliest phases of inflammatory arthritis.
Rheumatoid arthritis (RA) is a prototypic inflammatory autoimmune disease with a poorly understood etiopathogenesis. Given the destructive nature of the disease, early diagnosis and start of treatment is highly important\(^1\)-\(^3\). Several studies have shown that elevated acute phase proteins, chemokines, cytokines and RA-specific autoantibodies (rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)) can be detected in peripheral blood years before the onset of arthritis\(^4\)-\(^9\). In prospective cohort studies, these autoantibody-positive individuals can be defined as having systemic autoimmunity associated with RA and being at risk of developing RA\(^10\). A recent study showed that the cellular composition of the primary target of RA, the synovium, is comparable to that of healthy controls during this phase\(^11\). Thus, systemic autoimmunity appears to precede the development of synovial inflammation. Since the RA-specific autoantibodies can be present for years without disease symptoms and without increased synovial cellularity, factors outside the synovial compartment should be responsible for the initial changes leading to RA.

As a general principle, the recruitment of activated immune cells to the site of inflammation is initiated after informing a nearby lymph node of a danger signal. Thus, the immune reaction in lymph nodes generally precedes the influx of effector cells into the target tissue. Indeed, animal models have shown that the onset of arthritis is preceded by phenotypic changes in the cellular compartment of draining lymph nodes, indicating a primary role for lymph nodes in the initiation of arthritis\(^12\)-\(^14\). However, very little is known about the initial events that occur in lymph nodes before disease onset in patients with arthritis. In the current study we investigated the cellular composition of lymph node biopsies obtained from autoantibody-positive individuals at risk of developing RA and compared the results with those observed in early arthritis patients and healthy controls.

**METHODS**

**Study subjects and lymph node biopsy sampling**

Individuals with elevated IgM-RF and/or ACPA levels without arthritis were included in the study. These individuals have systemic autoimmunity associated with RA and are at risk of developing RA (phase c, ref.\(^10\)). In addition early arthritis patients (arthritis duration ≤6 months; disease-modifying antirheumatic drug naive) and healthy controls without any joint complaints and without RA-specific antibodies were included. Ultrasound-guided inguinal lymph node biopsies were obtained by a radiologist using a 16G core needle as previously described\(^15\) and immediately processed for flow cytometry analysis. The study was approved by the local ethical committee and all study subjects gave written informed consent.

**Flow cytometry analysis**

Lymph node biopsy samples were put through a 70 μm cell strainer (BD Falcon) to obtain a single cell suspension. Subsequently, cells were washed with PBS containing 0.01% NaN\(_3\) and 0.5% BSA. Cells were stained for 30 minutes at 4°C and protected from light using the following directly labelled antibodies: CD3 FITC (Sanquin, Amsterdam, the Netherlands), CD45 V500, CD69 PerCP, CD27 PerCP-Cy5.5, IgD FITC (BD Biosciences, Breda, Netherlands),
CD19 eFluor 450, CD4 Pe-Cy7, CD45RO PE, CD45RA eFluor 450 and CD8 APC eFluor 780 (eBioscience). After incubation cells were washed and measured on a FACS CANTO II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc. Ashland, OR).

Statistics
Not normally distributed data were presented as medians (IQR), and differences between study groups were analyzed using Kruskal-Wallis with post-Dunn’s multiple comparison tests or Mann-Whitney U-test where appropriate. Categorical data were presented as numbers (percentages), and differences between groups were analyzed using Chi-square test. GraphPad Prism Software (version 5, GraphPad Software, Inc. La Jolla, CA) was used for statistical analysis.

RESULTS
Lymph node biopsies were obtained from 13 IgM-RF and/or ACPA positive individuals without arthritis (referred to as ‘at risk’ individuals). None of these individuals has developed arthritis after a follow-up time of median (IQR) 12 (9-14) months. For comparison, lymph node biopsies were obtained from 15 early arthritis patients (8 RA according to the 2010 ACR/EULAR criteria for RA, 1 psoriatic arthritis, 6 unclassified arthritis; median (IQR) arthritis duration 1 (0-2) months) and 8 autoantibody-negative healthy controls). Table 1 shows the demographic data of the study participants.

<table>
<thead>
<tr>
<th>Table 1. Demographic data of the study participants</th>
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<tbody>
<tr>
<td>Healthy controls N= 8</td>
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<tr>
<td>Sex, female (%)</td>
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<tr>
<td>Age (years) (median (IQR))</td>
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<tr>
<td>IgM-RF positive (n (%))</td>
</tr>
<tr>
<td>IgM-RF level * (kU/L) (median (IQR))</td>
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<tr>
<td>ACPA positive (n (%))</td>
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<tr>
<td>ACPA level * (kAU/L) (median (IQR))</td>
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<tr>
<td>IgM-RF and ACPA both pos (n (%))</td>
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<tr>
<td>ESR (mm/hr) (median (IQR))</td>
</tr>
<tr>
<td>CRP (mg/L) (median (IQR))</td>
</tr>
<tr>
<td>68 TJC (n) (median (IQR))</td>
</tr>
<tr>
<td>66 SJC (n) (median (IQR))</td>
</tr>
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</table>

IgM-RF= IgM rheumatoid factor; ACPA= anti-citrullinated protein antibodies; *: levels only if positive status; ESR= erythrocyte sedimentation rate; ** level missing from 1 individual; CRP= C-reactive protein; 68 TJC= tender joint count of 68 joints; 66 SJC= swollen joint count of 66 joints. Categorical variables: n (%) and Chi-square test. Continuous variables (data not normally distributed): median (IQR) and Kruskal Wallis or Mann Whitney U test where appropriate. * Levels only in positive patients.
To explore the cellular composition of the lymph node compartment, freshly collected lymph node specimens were directly analyzed by multi-color flow cytometry for the presence of specific T and B lymphocytes including their activation or differentiation state. The frequencies of CD4+ and CD8+ T cells were within the expected range (~80% CD4+ and ~20% CD8+ within CD3+ T cells) and no differences were observed between at risk individuals, early arthritis patients and healthy controls (Figure 1AB). Subsequently, the percentage of activated T cells was determined by analyzing co-expression of CD69 (Figure 1CD). Interestingly, the percentage of activated CD8+ T cells was different between the three study groups. The frequency of activated CD4+CD69+ T cells (C) was highly variable between subjects, but significantly more activated CD8+CD69+ T cells (D) were observed in lymph node samples from early arthritis patients compared to healthy controls.

**Figure 1.** T-cell frequencies in lymph node biopsies. Fresh lymph node biopsies were immediately processed for flow cytometry analysis using T-cell surface markers including CD3, CD4, CD8, CD45 and CD69. For inter-individual comparisons frequencies were determined as percentage of CD45+ cells. The frequencies of CD4+ (A) and CD8+ (B) T cells were as expected and not different between the three study groups. The frequency of activated CD4+CD69+ T cells (C) was highly variable between subjects, but significantly more activated CD8+CD69+ T cells (D) were observed in lymph node samples from early arthritis patients compared to healthy controls.
study groups (p=0.017), and specifically increased in early arthritis patients compared to healthy controls (median (IQR) 32.2 (23.7-42.8) vs. 22.2 (15.8-26.6), p<0.05)). The increase in percentage of activated T cells was not dependent of the presence of arthritis in the lower limb on the ipsilateral side of the biopsied lymph node (data not shown).

Next, we analyzed the percentage of CD19+ B cells which showed a trend towards a difference between the three study groups (p=0.066) and, using post-Dunn’s multiple comparison test, observed that it was significantly higher in early arthritis patients compared to healthy controls (median (IQR) 37.7 (21.9-48.3) vs. 20.1 (13.0-28.3), p<0.05) (Figure 2A). We also observed a trend towards increased CD19+ B cells in at risk individuals compared to healthy controls (median (IQR) 29.6 (19.9-39.7) vs. 20.1 (13.0-28.3), p>0.05). The increased number of B cells was independent of the levels of ACPA or RF (Figure 2B). In the group of early arthritis patients no differences were observed between the unclassified arthritis and RA patients and the increased number of B cells was independent of the presence of arthritis in the lower limb on the ipsilateral side of the biopsied lymph node (data not shown). The percentage of different subsets of B cells, naïve, memory switched and memory non-switched (based on IgD and CD27), was comparable between the different study groups (data not shown).

Figure 2. B-cell frequencies in lymph node biopsies. Fresh lymph node biopsies were immediately processed for flow cytometry analysis using B-cell surface markers including CD45 and CD19. For inter-individual comparisons frequencies were determined as percentage of CD45+ cells. The frequency of CD19+ B cells was significantly increased in patients with arthritis compared to healthy controls (A). The number of CD19+ B cells was not dependent of anti-citrullinated protein antibody (ACPA) status (B).

**DISCUSSION**

This explorative study was undertaken to explore for the first time the cellular composition of lymph nodes in at risk individuals having systemic autoimmunity associated with RA and early arthritis patients compared to healthy controls. First, the results indicate that flow cytometry analysis of lymph node biopsies is a feasible method for studying the
cellular composition and activation of lymph node tissue in the earliest phases of arthritis. Second, we observed more CD19+ B cells and activated CD8+ T cells in early arthritis patients compared to healthy controls. Third, there was a trend towards an increase in CD19+ B cells in at risk individuals compared to healthy controls.

During an immune response the egress of T lymphocytes from lymph nodes is shut down transiently by downregulation of S1P1 and upregulation of CD69 leading to lymphocyte retention, maturation and proliferation. The results of this explorative study suggest increased activation of T cells, as shown by co-expression of CD69, within lymph nodes of arthritis patients during the earliest phase of disease. Of interest, the percentage of activated CD8+ T cells is increased. These results are in line with animal models of arthritis where a skewed CD4/CD8 ratio is observed in regional lymph nodes before the onset of arthritis. These results support the notion that T cells are intimately involved in the initiation of seropositive RA. Future research should focus on the identification of T-cell subset(s) and antigen-specificity associated with development of RA.

Of interest, an increased percentage of CD19+ B cells was observed in lymph nodes of early arthritis patients and autoantibody positive subjects at risk of developing RA. These results of the lymph node analyses differ from those in a previous study on peripheral blood, in which gene expression profiling of at risk individuals revealed a low B-cell signature especially in those individuals who developed arthritis after follow up. It is tempting to speculate that the B cells are retained in the lymph nodes, to ensure maturation and differentiation during the immune response, which would be in line with findings in animal models of arthritis.

An obvious limitation of the current study is the small sample size and the short follow up period of the ‘at risk’ individuals. Of note, it has been challenging to obtain lymph node biopsy samples from these patients and controls. We now have all the tools available to first identify those individuals at risk of developing arthritis and to prospectively analyse the immune cells in lymph node tissues. This will create a framework for studying the molecular events taking place in lymph nodes before the onset of arthritis that can be potentially related to the processes involved in the pathogenic changes in synovial tissues.

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REFERENCE LIST


