Lymphoid neogenesis in juvenile idiopathic arthritis correlates with ANA positivity and plasma cells infiltration

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Objective. The aim of the study was to evaluate the pattern of the lymphoid organization in the synovial tissue of patients affected with juvenile idiopathic arthritis (JIA).

Methods. A total of 40 JIA patients who underwent synovectomy or synovial biopsies were enrolled. The mean age at surgery was 15.1 yrs (range 6–30 yrs) and the mean disease duration was 6.7 yrs (range 3 months to 22.2 yrs). Tissue specimens were grouped according to the following criteria: (i) diffuse perivascular infiltrate without lymphoid organization, (ii) T cell–B cell aggregates with or without germinal centre reaction.

Results. Synovial tissues from 12 JIA patients did not show any sign of lymphoid organization, whereas 28 patients displayed a variable number of T–B cell aggregates. Typical features consistent with a germinal centre reaction were present in two JIA patients only. Lymphoid organization in JIA patients did not correlate with the duration and severity of the disease or with the degree of synovial inflammation, but did positively correlate with the presence of anti-nuclear antibodies. Moreover, a diffuse lymphocyte infiltration was significantly related to the presence of an acute phase of inflammation and the presence of lymphoid aggregates correlated with the degree of plasma cells infiltration.

Conclusions. Lymphoid neogenesis in JIA represents a phase in the immunopathological process that characterize the development of inflammatory synovitis. It is not related to disease activity or severity, but appears to be more frequent in patients with circulating anti-nuclear antibodies.

KEY WORDS: Synovial tissue, Lymphocyte infiltration, B cells.

Introduction

Rheumatoid synovitis is characterized by a massive infiltration of inflammatory cells that may organize into complex lymphoid microstructures that share many features with secondary lymphoid tissue, including the formation of T cell–B cell follicles with germinal centre (GC) reaction [1].

Lymphoid organization in the inflamed synovium is regulated by a number of soluble and tissue factors, including pro-inflammatory cytokines (such as lymphotoxin-α and -β) and homeostatic chemokines (CCL21/CCL19 and CXCL13) that have been shown to be up-regulated in the inflamed synovium. The combined expression of these factors appears to influence the pattern of the lymphoid organization in patients affected with chronic arthritides [2–5].

Three different pattern of lymphoid organization can be found in adult patients with rheumatoid arthritis (RA): (i) T–B cell aggregates with GC reaction, (ii) T–B cell aggregates without GC reaction and (iii) diffuse infiltration of T and B cells in the absence of lymphoid organization [2]. The possible clinical relevance of the presence of different patterns among RA patients is still a matter of debate and relatively few data are available on this particular issue.

According to some authors, each individual RA patient displays a stable pattern of lymphoid organization [2, 6] and a more aggressive disease course has been postulated for those patients showing more organized lymphoid structures [7, 8].

The alternative hypothesis is that the aforementioned diversity could merely reflect different developmental stages of the inflammatory process. In fact, a diffuse pattern of cell infiltration could simply be the precursor of more organized lymphoid structures [9]. In this line, it has also been proposed that synovial cell infiltration could not be considered as a stable, but as a dynamic process [5]. Notably, adult RA patients with a longstanding disease display an higher presence of lymphoid aggregates with respect to those with early RA [10].

JIA is an heterogeneous condition gathering together all forms of chronic arthritis of unknown origin with onset before 16 yrs of age [11]. It is currently divided into different subtypes according to symptoms at onset [11]. So far, no information is available on the pattern of lymphoid neogenesis in the synovial tissue of the different JIA subtypes. Moreover, the variability in the severity of the disease course among the various JIA subtypes represents an interesting opportunity to test the hypothesis of a possible influence of the pattern of lymphoid organization on the disease outcome [6, 7].

Thus, the aim of the present study was to analyse whether different patterns of lymphoid organization found in JIA patients could be related to the disease subtype, the clinical course of the
disease and/or to the histological features at the moment of the tissue sampling.

Patients and methods

Patient population

A total of 40 JIA patients (27 female, 13 male) underwent synovectomy or synovial biopsies from 1998 to 2005 at ‘G. Gaslini’ or ‘G. Pini’ Institutes.

The median age at surgery was 14 yrs (range 6–30 yrs) and the median disease duration was 7.3 yrs (range 3 months to 22.2yrs). According to the ILAR criteria [11], 21 patients had a persistent oligoarticular form, five had an extended oligoarticular form, seven had polyarticular RF+ form, one had polyarticular RF− form, three had systemic form, two had an enthesis-related arthritis and one had psoriatic arthritis. At the moment of surgery, clinical (disease duration, number of active joints, number of joints with limited range of motion, Steinbrocker Class) and laboratory parameters (erythrocyte sedimentation rate, ESR; antinuclear antibodies, ANA; rheumatoid factor, RF), as well as treatment, were recorded. Tissue samples were taken after patients and/or parents’ permission according to the informed consent approved by the Ethical Committee of the ‘G. Gaslini’ Institute.

Twenty-two patients (21 with an oligoarticular form and one with psoriatic arthritis) displayed a benign persistent oligoarticular form, whereas 18 patients displayed a more aggressive polyarticular course. Eight patients displayed a severe functional impairment (Steinbrocker class 3 or 4), whereas the majority of the patients were able to exert their normal daily activities with any or minor limitations.

Twenty-four patients were positive for ANA. One patient with a polyarticular onset was positive for RF. ANA were detected by immunofluorescence on HEP-2 cells. ANA positivity was defined by the finding of high titre ANA (>1:160) in at least two occasions, three months apart in the first 6 months of the disease and confirmed to be persistent during follow-up.

Tissue collection and analysis

Synovial tissue for the study was derived from arthroscopic biopsy (20 patients) or synovectomy (20 patients). Most of the patients (14) who underwent synovectomy displayed an oligoarticular form. The diameter of a single biopsy ranged from 0.2 to 0.5 cm. In order to avoid variability due to the heterogeneity of synovial tissue, at least 6 different biopsies were taken from a single joint [12, 13]. Larger samples (mean maximum diameter 1.2–1.5 cm) were obtained from synovectomy and considered as representative. Synovial tissues were taken from knees (34 patients), wrists (three patients), ankles (two patients) and hip (one patient).

All specimens were fixed in 10% formalin for no longer than 4 h and subsequently embedded in paraffin. Immunohistochemical staining was performed in serial sections by a three-step indirect immunoperoxidase technique, using 3,3'-diaminobenzidine as chromogen substrate. Primary monoclonal or polyclonal antibodies included anti-CD3 (polyclonal, Dako, Denmark) for T-cells, anti-CD20 (clone L26, Dako) for B-cells, anti-CD138 (clone M115, Dako) for plasma cells, anti-CD21 (clone 1F8, Dako) for follicular dendritic cells.

After independent evaluation of two observers (A.G. and M.G.), tissue specimens were grouped according to the following criteria: (i) diffuse perivascular infiltrate without lymphoid organization (Fig. 1A–C), (ii) T–B cell aggregates without lymphoid organization (Fig. 1D–F) or with (Fig. 1G–I) GC reaction (presence of a network of CD21+ follicular dendritic cells and/or presence of clusters of centrocytes and centroblasts with tingible body macrophages) [2, 14]. In the presence of at least one T–B cell aggregate the tissue analysed was automatically classified as T–B.

Tissues were semi-quantitatively scored by two independent observers (A.G. and M.G.) according to degree of infiltration by lymphocytes (CD3+ cells) and plasma cells (CD138+ cells). A score of 1 represented minimal infiltration, while a score of 3 represented infiltration by numerous inflammatory cells (Fig. 1J–L) [15]. Each tissue was assigned a score for hyperplasia of the synovial lining layer (0: 1–2, 1: 3–4, 2: 5–6, 3: >7 cell layers) [16].

Moreover, an histological characterization of each sample was performed by an expert pathologist (C.G.) into two broad categories: (i) tissues with histological features consistent with an acute/exudative stage of inflammation (fresh deposits of fibrin with or without neutrophils infiltration) (Fig. 1M) and (ii) tissues in a chronic/quiescent stage (non-fibrin deposit, fibrosed stroma, plus low-grade lining layer hyperlasia) (Fig. 1N) (adapted from [9]).

Statistical analysis

Comparisons were carried out using Mann–Whitney U-test for continuous variables and chi-square test for categorical variables.

Results

Pattern of lymphocyte infiltration in JIA patients and correlation with clinical parameters

Synovial tissues from 12 patients were characterized by a diffuse infiltration of CD3+ T-lymphocytes with scattered CD20+ B-cells (Fig. 1A–C), without any sign of lymphoid organization (group A) (Table 1).

Conversely, synovial tissue form 28 patients (group B) displayed a variable number of T–B cell aggregates (Fig. 1D–F). A GC reaction, namely presence of a network of CD21+ follicular dendritic cells (Fig. 1G–I), was present in two JIA patients only (Fig. 1). Notably, in 10 out of 28 patients, few (three or less) T–B aggregates were interposed to a diffuse lymphocyte infiltration (Fig. 1J–L), whereas other patients displayed an higher number of T–B cell aggregates that markedly characterized the whole area of analysed tissue. In some patients, different patterns of lymphocyte infiltration were observed in single biopsies coming from the same joint (data not shown), as already reported by other authors [5].

No difference was observed in terms of pattern of lymphocyte infiltration according to the type of procedure performed (biopsy vs synovectomy). In fact, variable degree of lymphoid aggregation was observed in 15 synovial tissues coming from synovectomy and in 13 from biopsy.

In Table 2, the main clinical characteristics of JIA patients belonging to groups A and B are reported.

Most patients displayed a very long disease course (mean disease duration 6.7yrs, range 0.3–22.2yrs), which in 15 patients was longer than 10yrs. In seven patients, a synovial biopsy was performed for diagnostic purposes during the first year of the disease. Statistical analysis did not show any difference in disease duration between the two groups of patients (Table 2). Notably, among the JIA patients who underwent synovial biopsies during the first year of the disease, four presented T–B aggregates, whereas three displayed a diffuse infiltrate.

Similarly, no substantial differences were observed between groups A and B in the distribution of the various JIA onset subtypes (Table 2).

Conversely, the prevalence of ANA positivity, a feature that is common in patients with oligoarticular onset JIA and suggested to define an homogeneous subset among JIA subtypes [17, 18], was significantly higher in group B (71.4%) than in group A (33.3%, chi-square test P = 0.03) (Table 2).
One of the aims of the present study was to evaluate whether the pattern of lymphoid organization could be related to the degree of disease activity and disease severity at the moment of surgery.

No differences were found between groups A and B in terms of ESR and number of active joints at the time of surgery (Table 2). Moreover, the pattern of lymphoid organization was not related to disease severity, as expressed by the number of joints with limited range of motion and Steinbrocker functional class. In this respect, among eight patients with severe limitation in their daily life, five presented lymphoid aggregates, whereas three had a diffuse infiltration (Table 2).

The two patients in whom a GC reaction was found (nos. 31 and 34, Table 1) presented different forms of JIA. Patient no. 31 presented a mild persistent ANA⁺ oligoarticular form with frequent relapses of synovitis at the left knee. Conversely, patient no. 34 had a systemic onset JIA with aggressive and destructive polyarticular course. Both patients had a very long disease course (7 and 18 yrs, respectively).

Finally, the pattern of lymphoid organization in synovial tissue did not appear to be related to the type of treatment. When patients were subdivided according to the presence or absence in their personal history of treatment with disease-modifying anti-rheumatic drugs (DMARDs) and oral steroids, no difference was found between the two subgroups (Table 2).

Correlation between the pattern of lymphoid organization and concomitant histological features at the moment of the study

In the majority of the patients (31 out of 40) synovial tissues presented the histological characteristics of an active/exudative phase of inflammation, whereas a chronic/quiescent stage was observed in nine patients only. Interestingly, while the nine tissue presenting a quiescent stage state presented a variable degree of lymphoid organization, all the 12 tissues with a diffuse lymphocyte infiltration displayed histological features consistent with an acute/exudative synovitis (Table 1 and Fig. 2A) \( (P = 0.037, \text{Fisher’s exact test}) \).
Conversely, a correlation was found between the presence of a lymphoid organization and the degree of plasma cell infiltration, with a significantly higher plasma cell infiltration in group B in comparison with group A (chi-square test $P = 0.003$, Fig. 2D).

Similarly, synovial tissues of ANA positive patients displayed the same higher degree of plasma cells infiltration with respect to ANA negative patients ($P = 0.001$).

Thus, the presence of a lymphoid organization is not influenced by the degree of synovial infiltration of inflammatory cells, but seems to be more related to the phase of the inflammatory process taking place in the synovial tissue. Moreover, the presence of lymphoid neogenesis, as well as ANA positivity, is clearly correlated to the degree of plasma cells infiltration.

### Discussion

In the present study, 70% of the synovial tissues obtained from JIA patients was characterized by the presence of a distinct lymphoid organization, a percentage higher with respect to previous studies performed in adult RA in which only 40–44% of patients displayed a T–B cell aggregates either in presence or in absence of a GC reaction [2, 7].

However, in a recent study conducted on a smaller group of RA patients, 17 out of 20 samples (85%) displayed lymphoid structures with different degree of organization [5]. It is therefore also possible that the higher number of lymphoid aggregates found in this latter and in the present study could be related to the large area of tissue that we had the possibility to analyse in order to assure the highest possible representativeness of synovial samples taken during arthroscopic procedures [12, 13].

The histological changes seen in rheumatoid synovial membrane are the results of continuous exacerbations and remissions.

### Table 2. Clinical features of JIA patients presenting a diffuse lymphocyte infiltration (group A) or T–B cell aggregates (group B)

<table>
<thead>
<tr>
<th></th>
<th>Group A ($n = 12$)</th>
<th>Group B ($n = 28$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease duration (yrs)</td>
<td>8.6 (0.3–22.2)</td>
<td>7.8 (0.3–20.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Age at onset (yrs)</td>
<td>7.2 (1.4–16)</td>
<td>4.4 (1.6–15.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Form</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligoarticular persistent</td>
<td>5/12</td>
<td>16/28</td>
<td></td>
</tr>
<tr>
<td>Oligoarticular extended</td>
<td>2/12</td>
<td>3/28</td>
<td></td>
</tr>
<tr>
<td>Polyarticular RF$^+$</td>
<td>2/12</td>
<td>5/28</td>
<td>NS</td>
</tr>
<tr>
<td>Polyarticular RF$^-$</td>
<td>1/12</td>
<td>0/28</td>
<td></td>
</tr>
<tr>
<td>Systemic</td>
<td>0/12</td>
<td>3/28</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2/12</td>
<td>1/28</td>
<td></td>
</tr>
<tr>
<td>ANA positive (%)</td>
<td>4/12 (33.3)</td>
<td>20/28 (71.4)</td>
<td>0.03</td>
</tr>
<tr>
<td>ESR (mm/1st h) median; range</td>
<td>13.5 (5–48)</td>
<td>20 (6–87)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of active joints</td>
<td>2 (1–33)</td>
<td>2 (1–28)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of joint with LRM</td>
<td>2 (0–20)</td>
<td>1 (0–35)</td>
<td>NS</td>
</tr>
<tr>
<td>Steinbrotker Class</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4/12</td>
<td>15/28</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>3/12</td>
<td>6/28</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2/12</td>
<td>3/28</td>
<td>NS</td>
</tr>
<tr>
<td>IV</td>
<td>1/12</td>
<td>2/28</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>2/12</td>
<td>2/28</td>
<td></td>
</tr>
<tr>
<td>Treatment with DMARDs (%)</td>
<td>7/12 (58.3)</td>
<td>16/28 (57.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-TNF + methotrexate</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Ciclosporin A</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Salazopyrin</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Treatment with oral steroids</td>
<td>2/12 (16.6)</td>
<td>4/28 (14.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Others; enthesitis–arthritis syndrome or psoriatic arthritis; RF, Rheumatoid factor; ANA, anti-nuclear antibodies; ESR, erythrocyte sedimentation rate; LRM, limited range of motion; DMARDs, disease-modifying anti-rheumatic drugs; TNF, tumour necrosis factor. $P$-values were calculated with Mann–Whitney U-test and chi-square test. NS, not significant.
that characterize the disease course, ranging from an acute and exudative stage to a chronic and quiescent phase [9].

It is still debated whether the presence of a lymphoid organization in synovial tissue might be related to an individual pattern of response [2, 6] or, alternatively, if it might simply represent a step in a dynamic process [5, 19]. According to this second hypothesis, it is possible that the study of synovial tissue for each single patient simply represents a picture of the ongoing inflammatory process in that particular moment.

For these reasons, together with the characterization of the pattern of lymphoid organization, we also analysed the concomitant histological features at the moment of tissue sampling. The presence of a lymphoid organization was not related to the degree of tissue inflammation in terms of lymphocyte infiltration and lining layer hyperplasia. However, a diffuse lymphocytic infiltration without any sign of aggregation was only seen in samples presenting an acute and exudative stage of inflammation, whereas all tissues presenting a chronic and quiescent stage displayed a variable degree of lymphoid organization. These results may support the hypothesis that lymphoid neogenesis can be considered as a step in the dynamic immunopathological process that characterize the development of inflammatory synovitis [5, 9].

The lack of any correlation between the pattern of lymphoid organization in synovial tissue and clinical and laboratory parameters of disease activity or severity observed in the present study do not support its possible prognostic value at least in JIA.

Interestingly, in the present study, the presence of a lymphoid organization was associated with the capacity to constantly produce antinuclear antibodies.

It has been suggested that the presence of ANA positivity could be able to identify among JIA subsets an homogeneous subgroup of patients that is characterized by asymmetric arthritis, early disease onset, high risk to develop anterior uveitis and association with DRB1*0801 [16, 18]. Thus, the higher prevalence of patients showing lymphoid aggregates found in the present study can also be related to the presence of a large subgroup of ANA positive patients in JIA. It is also possible that the development of a lymphoid organization in synovial tissue could be related to the individual capacity to produce auto-antibodies [2].

Accordingly, it is interesting to note that presence of a lymphoid organization in synovial tissue of our JIA patients was strongly related to the concomitant degree of plasma cells infiltration. This finding is in line with a previous study in adult RA showing a close relationship between the width of T-cell infiltration and the degree of B-cells and plasma cells infiltration [19]. The existence of a positive correlation between ANA positivity and synovial tissue plasma cells infiltration, may also suggest the possibility of a local production of autoantibodies at the site of tissue inflammation in JIA patients [2].

In conclusion, our study suggest that lymphoid organization in synovial tissue in JIA patients represents a particular phase of tissue inflammation which is not related to disease activity or severity, but appears to be more common in patients characterized by the presence of circulating ANA.

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The authors have declared no conflicts of interest.

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