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Anatomic Localization of Immature and Mature Dendritic Cells in an Ectopic Lymphoid Organ: Correlation with Selective Chemokine Expression in Rheumatoid Synovium

Guillaume Page,* Serge Lebecque, † and Pierre Miossec1∗

It remains to be clarified whether dendritic cells (DC) reach the rheumatoid arthritis (RA) synovium, considered an ectopic lymphoid organ, as mature cells or undergo local maturation. We characterized by immunohistochemistry the DC subsets and used tonsils as a control. Immature and mature DC were defined by CD1a and DC-lyso-some-associated membrane protein/CD83 expression, respectively. Immature DC were mainly detected in the lining layer in RA synovium. Mature DC were exclusively detected in the lymphocytic infiltrates. The DC-lyso-some-associated membrane protein/CD1a ratio was 1.1 in RA synovium and 5.3 in tonsils, suggesting the relative accumulation of immature DC in RA synovium. We then focused on the expression of CCL20/CCR6 and CCL19/CCR7, CCL21/CCR7 chemokine/receptor complex, which control immature and mature DC migration respectively. A close association was observed between CCL20-producing cells and CD1a+ cells, suggesting the contribution of CCL20 to CCR6+ cell homing. Conversely, CCL21 and CCL19 expression was only detected in perivascular infiltrates. The association among CCL19/21-producing cells, CCR7 expression, and mature DC accumulation is in line with the roles of these chemokines in mature CCR7+ DC homing to lymphocytic infiltrates. The role of DC in disease initiation and perpetuation makes chemokines involved in DC migration a potential therapeutic target. The Journal of Immunology, 2002, 168: 5333–5341.

Dendritic cells (DC)2 are the most potent APCs and play a central role in the processing and presentation of Ags to T cells during the immune response (1). After Ag exposure, immature DC migrate to draining lymph nodes where they differentiate into mature DC to present Ags to T cells (1).

DC are found in the rheumatoid arthritis (RA) synovium and are likely to play an important role in its pathogenesis. This conclusion is supported by the histological observations demonstrating MHC class II+ APC clustered with T cells around blood vessels, sometimes in germinatal center (GC) structures, and the association of HLA-DR alleles with disease severity (2–6). However, the function of DC according to phenotype and subset in RA synovium remains to be clarified, in particular regarding their mode of migration and accumulation (7–10). Recently, differentiated DC expressing nuclear RelB have been detected in RA synovium, predominantly located in perivascular mononuclear cell aggregates (11).

RA synovium has several, but not all, of the characteristics of lymphoid organs with regard to cellular composition and organization (12), but it is unknown whether DC reach the synovium as mature cells or undergo local maturation. In particular, an accumulation of immature DC could be secondary to inflammation. Such studies are difficult to perform because of the low frequency of DC in such tissue. In addition, synovium dissociation with enzymes may affect phenotypes and functions with a difficult recovery of such rare cells. Accordingly, we have selected immunohistochemical techniques to investigate the different DC subsets found in RA synovium. We used several newly defined markers of the immature and mature DC subsets. CD1a, first described as a marker of Langerhans cells (LC) in skin epithelium, was later described as a marker of immature DC (13). Conversely, DC-lyso-some-associated membrane protein (LAMP) and CD83 have been associated with more mature DC subsets. In vitro studies of DC differentiation have shown that CD83 expression appears before that of DC-LAMP, suggesting that CD83+ DC are less mature than DC-LAMP+ DC (14, 15). We selected tonsils, an active lymphoid organ, as a control. We analyzed DC frequency and localization with the aim of studying the respective immature/mature DC compartmentalization pattern in RA synovium. Such compartmentalization has been observed in breast carcinoma tissue where immature DC were detected within the tumor and mature DC in peritumoral areas (16).

To study the mode of migration of DC, we also looked at the expression of several chemokines and their associated receptors. Macrophage-inflammatory protein-3α, a CC chemokine, recently renamed CCL20 (17), and its receptor CCR6 (18) are critical for the recruitment of immature DC (19). CCL20 plays a major role in epithelial colonization by LC in response to inflammation (20). Macrophage-inflammatory protein-3β/CCL19 and 6-chemokine/CCL21 contribute through their associated receptor CCR7 to the accumulation of Ag-loaded mature DC in T cell-rich areas of lymphoid organs (19). These chemokines attract in vitro-derived DC following CCR7 induction by CD40 ligand, TNF-α, or LPS stimulation (19, 21). In situ detection of CCL19 showed its expression within the T cell areas of secondary lymphoid organs (22), with CCL21 having a wider distribution.

In this work we report the coexistence of both immature and mature DC subsets in RA synovium. The close association between immature CD1a+ DC and CCL20-producing cells suggests

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2 Abbreviations used in this paper: DC, dendritic cell; AEC, 3-amino 9-ethylcarbazole; GC, germinal center; LAMP, lysosome-associated membrane protein; LC, Langerhans cell; RA, rheumatoid arthritis.
the contribution of CCL20 to the homing of immature CCR6$^+$ cells into RA synovium. Conversely, the colocalization of CCL21/ CCL19, CCR7, and mature DC in perivascular infiltrates suggests a role for these chemokines in the homing of mature CCR7$^+$ DC in lymphocytic infiltrates.

Materials and Methods

**Collection of samples**

Synovial samples were obtained from 12 patients with RA, defined according to the revised criteria of the American College of Rheumatology (formerly the American Rheumatism Association) (23). To assess inflammatory synovium, samples were obtained from patients undergoing knee or wrist synovectomy, and not at a late stage, such as for joint replacement. Tonsils were obtained from children undergoing tonsillectomy.

The study was first initiated with frozen sections. However, immunostaining using frozen sections, despite good Ag preservation, was not sufficient to clearly define the morphology of DC and to allow their quantification. Accordingly, an effort was made to develop immunostaining in paraffin-embedded sections, which allows a better preservation of histological patterns. Samples were fixed in 4% phosphate-buffered paraformaldehyde and then embedded in paraffin. Four-micrometer sections were cut and mounted on glass slides (adhesive slides, SuperFrost; CML, Nemours, France). To detect Ag expression in paraffin-embedded sections, Ag retrieval procedures were performed, including incubation in either citrate buffer (10 mM, pH 6) or EDTA buffer (1 mM, pH 8), followed by microwave oven incubation (three times for 3 min each time).

Following staining with hematoxylin, samples were classified according to the following criteria (24): diffuse infiltration of T cells and B cells and absence of lymphoid organization (n = 4), T cell-B cell aggregates without GC (n = 5), and presence of GC (n = 3).

Detection of DC subsets

For single staining, an immunoperoxidase technique using 3-amino 9-ethylcarbazole (AEC; red color; DAKO, Glostrup, Denmark) or 3,3′-diaminobenzidine tetrahydrochloride (DAKO) as chromogen was performed. Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were incubated with several mouse mAbs recognizing the following molecules (see also Table I): CD1a (IgG2b; BD Biosciences, Dardilly, France), CD11c (IgG2b; Immunotech, San Jose, CA), Langerin (IgG1; Schering-Plough, Dardilly, France), CD40 (IgG1; Schering-Plough), HLA-DR (IgG2b; BioSource, Camarillo, CA), CD83 (IgG2a; Immunootech, Marseilles, France), and DC-LAMP (IgG1; Immunotech). (Refs. 13–15 and 25–28, see Table I for details). After overnight incubation at 4°C and washing, the sections were incubated with biotinylated anti-goat IgG and streptavidin-peroxidase. CCR6 and CCR7 were detected using anti-CCR6 (R&D Systems, Abingdon, U.K.) and anti-CCR7 (BD Biosciences) mouse mAbs.

For detection of chemokines, goat polyclonal Abs to CCL19, CCL20, or CCL21 (R&D Systems, Abingdon, U.K.) were used, followed by biotinylated rabbit anti-goat IgG (DAKO), and was developed using streptavidin-peroxidase. Peroxidase was developed by AEC. Mouse mAb to CD1a (IgG2b) was followed by rat anti-mouse IgG2a/2b (BD Biosciences) and mouse alkaline phosphatase anti-alkaline phosphatase (DAKO). Alkaline phosphatase was revealed using Fast Blue as chromogen (blue color; Vector Laboratories, Burlingame, CA). In negative control sections, one of the two primary Abs was omitted.

Detection of chemokines and their associated receptors

For detection of chemokines, goat polyclonal Abs to CCL19, CCL20, or CCL21 (R&D Systems, Abingdon, U.K.) were used, followed by biotinylated rabbit anti-goat IgG (DAKO), and was developed using streptavidin-peroxidase. Peroxidase was developed by AEC. Mouse mAb to CD1a (IgG2b) was followed by rat anti-mouse IgG2a/2b (BD Biosciences) and mouse alkaline phosphatase anti-alkaline phosphatase (DAKO). Alkaline phosphatase was revealed using Fast Blue as chromogen (blue color; Vector Laboratories).

Quantification of positive cells

Because of the high heterogeneity of RA synovium and the low frequency of DC subsets, quantification was performed with a method first used for the estimation of new blood vessel formation in breast tumor (29). This method allows quantification of cells in hot spots, defined in this study as synovium areas containing the highest density of positive cells. Accordingly, two hot spots per rheumatoid synovium section were selected. In each spot positive cells were counted in 10 consecutive high-power fields (∼500). One field corresponded to 0.3 mm$^2$, and thus one spot to 3 mm$^2$. The number of positive cells per two hot spots was averaged, and results are expressed as the number of positive cells per square millimeter.

**Statistical analysis**

Results were expressed as the mean ± SD. Levels of marker expression were compared using the nonparametric Mann-Whitney U test between RA synovium and tonsils and between subsets of RA synovium.

**Results**

Expression of HLA-DR, CD11c, and CD40 in RA synovium

We investigated DC subsets in sections obtained from 12 RA patients and used tonsils, an active secondary lymphoid organ as a control. We used several mAbs against specific and nonspecific DC markers (Table I). HLA-DR, CD11c, and CD40, known to be expressed by DC, were detected in all RA synovium samples. HLA-DR, CD11c, and CD40 were highly expressed in every sample (44 ± 11, 32 ± 11, and 35 ± 12 positive cells/mm$^2$, using the hot spot analysis, respectively; Table II). Similar expression was observed in tonsils (not significantly different vs RA synovium). These markers were detected in many cell types other than DC, such as T cells, B cells, and synoviocytes, and localized in the lining and sublining layers as well as in infiltrates.

Immature DC are present within RA synovium

We then focused on more DC-specific markers. Immature DC were defined by CD1a expression. Immature CD1a$^+$ DC were

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**Table I. Markers and Abs used for DC subset detection**

<table>
<thead>
<tr>
<th>Markers</th>
<th>Expression by DC</th>
<th>Abs (origin/isotype)</th>
<th>Catalog References</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>+ + + (13)</td>
<td>Mouse/IgG2b</td>
<td>347430</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11c</td>
<td>+ + + (25)</td>
<td>Mouse/IgG2b</td>
<td>550004</td>
<td>CamFolio</td>
</tr>
<tr>
<td>CD40</td>
<td>+ + + (26)</td>
<td>Mouse/IgG1</td>
<td>2069</td>
<td>Schering-Plough</td>
</tr>
<tr>
<td>CD83</td>
<td>+ + + (14)</td>
<td>Mouse/IgG2a</td>
<td>AHU0182</td>
<td>Immunootech</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>+ + + (27)</td>
<td>Mouse/IgG2b</td>
<td>IM3448</td>
<td>BioSource</td>
</tr>
<tr>
<td>DC-LAMP</td>
<td>+ + + (15)</td>
<td>Mouse/IgG1</td>
<td>IM3448</td>
<td>Immunootech</td>
</tr>
</tbody>
</table>

* Corresponding references are listed in parentheses.

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Results were expressed as the mean ± SD. Levels of marker expression were compared using the nonparametric Mann-Whitney U test between RA synovium and tonsils and between subsets of RA synovium.

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detected in 10 of 12 RA synovium samples. The number of CD1a<sup>+</sup> cells per square millimeter in RA synovium ranged from 1 to 26 cells (mean ± SD, 7 ± 7; Table II). In tonsils, that number was ~2-fold higher (15 ± 2 positive cells/mm<sup>2</sup>), but without reaching significance (not significantly different vs RA synovium).

The LC subset, also expressing CD1a marker in skin epithelium (13), has been characterized by the specific expression of langerin/DCGM4. This represents a marker of epithelial tissue. As expected, no LC were found in RA synovium. Conversely, LC in tonsils (14 ± 2 positive cells/mm<sup>2</sup>; p < 0.001 vs RA synovium) were exclusively present in the epithelium. Such finding excludes the migration of epithelial-derived DC to the synovium.

In RA synovium immature CD1a<sup>+</sup> DC were preferentially localized in the lining (inset of Fig. 1A) or sublining layer as well as at the periphery of perivascular infiltrates (Fig. 1A). CD1a<sup>+</sup> cells were detected in the lining layer of all 10 CD1a<sup>+</sup> samples, whereas immature DC in perivascular infiltrates were present in only 4 of 12 samples (patients 4, 8, 10, and 12). In tonsils, CD1a<sup>+</sup> DC were detected in the T cell zone and the epithelium (Fig. 1B).

Most of the immature CD1a<sup>+</sup> DC showed a different dendritic morphology compared with the stellate shape of LC in skin epithelium, with large veils expanding in every direction from the cell body with long (10 μm) and thin processes. In RA synovium, immature CD1a<sup>+</sup> DC had only a few long processes (Fig. 1A).

Mature DC are preferentially localized in the perivascular or lymphocytic infiltrates

Mature DC were analyzed for the expression of CD83 and DC-LAMP. In vitro studies of DC differentiation have shown that CD83 expression appears before that of DC-LAMP, suggesting that CD83<sup>+</sup> DC are less mature than DC-LAMP<sup>+</sup> DC (15). CD83<sup>+</sup> cells were detected in 9 of 12 samples, and DC-LAMP<sup>+</sup> cells were found in 11 of 12 samples. The mean numbers of CD83<sup>+</sup> cells and DC-LAMP<sup>+</sup> cells per square millimeter in RA synovium were 5 ± 6 and 8 ± 5, respectively (Table II). In tonsils the expression of CD83 and DC-LAMP was 7-fold higher (35 ± 7 positive cells/mm<sup>2</sup>; p < 0.001 vs RA synovium) and 10-fold higher (79 ± 3 positive cells/mm<sup>2</sup>; p < 0.001 vs RA synovium) compared with RA synovium, and the DC-LAMP/CD83 ratio was 79/35 = 2.3, indicating a relative accumulation of the most mature subset. In RA synovium, that ratio was 8/5 = 1.6 (not significant vs tonsils).

Mature CD83<sup>+</sup> cells were mainly detected at the periphery of the perivascular or lymphocytic infiltrates (Fig. 1C). In contrast, DC-LAMP<sup>+</sup> DC were not observed at the periphery, but inside the lymphocytic infiltrates (Fig. 1E), the site of interactions between mature DC and lymphocytes. In tonsils, DC-LAMP<sup>+</sup> DC were exclusively detected in the T cell zone (Fig. 1F), whereas CD83<sup>+</sup> cells were found in both T cell and GC zones (Fig. 1D).

In synovium, most of the mature DC also had a dendritic-like morphology (inset of Fig. 1, C and E). Some of the DC-LAMP<sup>+</sup> or CD83<sup>+</sup> cells had a less common aspect, with a plasmacytoid-like appearance and a remote nucleus in a large cytoplasm (Fig. 1E, inset).

To further clarify the dichotomy between immature and mature DC subsets in RA synovium, double staining using anti-CD1a and anti-CD83 Abs was performed. The lack of coexpression of CD-LAMP and CD1a confirms the presence of independent immature and mature DC subsets (Fig. 1, G and H). The DC-LAMP/CD1a ratio was 8/7 = 1.1 in RA synovium vs 79/15 = 5.3 in tonsils (p < 0.001 vs RA synovium), suggesting the relative accumulation of immature DC in RA synovium.

CCL20 is expressed in RA synovium

To study the mode of migration of DC in response to chemokines, we first analyzed the expression pattern of CCL20 and its receptor CCR6, which is known to control the migration of immature DC. In RA synovium CCL20 was mainly expressed in the lining layer (Fig. 2A), but also in the perivascular infiltrates (Fig. 2C). CCL20 expression showed a large degree of heterogeneity between the different RA synovium samples. In 4 of 12 samples no CCL20 expression was detected. In the eight CCL20<sup>+</sup> RA synovium, the densities of CCL20<sup>+</sup> cells per square millimeter in the lining layer and/or perivascular infiltrates were 9 ± 9 and 9 ± 11, respectively, with a large degree of heterogeneity (Table III). In samples 11 and 12, CCL20 was only expressed in the lining layer. In tonsils, CCL20-producing cells were more common (38 ± 5 positive cells/mm<sup>2</sup>; p < 0.001 vs RA synovium) and were exclusively detected in the epithelial crypts (Fig. 2E). In RA synovium CCL20-producing cells detected in the lining layer extend the demonstration of secretion of CCL20 by synoviocytes (30). In tonsils CCL20-producing cells in epithelial crypts were identified as epithelial cells characterized by their elongated shape.

### Table II. Quantification of DC subsets in RA synovium and tonsils<sup>a</sup>

<table>
<thead>
<tr>
<th>RA Patients (histological classification)</th>
<th>Immature DC</th>
<th>Mature DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>Langerin</td>
<td>CD83</td>
</tr>
<tr>
<td>1 (lymphoid follicles)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2 (lymphoid follicles)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3 (diffuse)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>4 (GC)</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>5 (lymphoid follicles)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6 (diffuse)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7 (diffuse)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>8 (lymphoid follicles)</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>9 (diffuse)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 (GC)</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>11 (lymphoid follicles)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>12 (GC)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>All (mean ± SD)</td>
<td>7 ± 7*</td>
<td>0**</td>
</tr>
<tr>
<td>Control (tonsils)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 ± 2</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as mean of DC marker-positive cells per square millimeter based on the hot spot analysis (see Materials and Methods for details). RA synovium vs tonsils: *, NS; ***, p < 0.001.

<sup>b</sup> n = 3. Shown are the means ± SD.
CCR6, the receptor for CCL20, is highly expressed in RA synovium

CCR6 expression was detected in all RA synovium samples. CCR6 was highly expressed (39 ± 20 positive cells/mm²) in lymphocytic infiltrates and at the periphery of blood vessels (Fig. 2D). In some cases CCR6 was also observed in the lining layer where CCL20 was highly expressed (Fig. 2B). In tonsils, CCR6 was highly detected (66 ± 20 positive cells/mm²; not significant vs RA synovium) in the T cell zone, and accumulation of CCR6⁺ cells was directly adjacent to CCL20-expressing epithelial cells (Fig. 2F). In both RA synovium and tonsils, CCR6-expressing cells had various morphotypes, including cells with a dendritic morphology.

Close association between CCL20-producing cells and immature CD1α⁺ DC

As observed in Table II, the lack of CCL20 expression was associated with the absence (samples 3 and 9) or low number (patients 6 and 7) of CD1α⁺ cells. This suggested a link between the accumulation of immature CD1α⁺ DC and the expression of CCL20 in RA synovium. This observation was extended by double staining
with anti-CCL20 and anti-CD1a Abs. In both RA synovium and tonsils we observed a close association between immature CD1a+ cells in the lining layer and the perivascular infiltrates in RA synovium and tonsils. Immunostaining with anti-CCL20 (A and C) and anti-CCR6 (B and D) Abs of serial paraffin-embedded RA synovium sections shows the association between CCL20-producing cells and CCR6+ cells in the lining layer, as well as in the perivascular infiltrates. In tonsils CCL20 expression is found only in epithelial crypts (E), and that of its associated receptor, CCR6, is found in the T cell zone adjacent to the CCL20-producing cells (F). Double staining with anti-CCL20 (red) and anti-CD1a (blue) Abs shows the association of CCL20-producing cells and immature CD1a+ cells in the lining layer (G) and in perivascular infiltrates (H). Such an association is also observed in tonsils where immature CD1a+ cells are adjacent to CCL20-expressing epithelial cells (I). Magnification for all: ×400.

CCL19 and CCL21 are mainly localized in perivascular infiltrates

We then looked at CCL19 and CCL21 expression, the CCR7 ligands known to control the migration of mature DC. In RA synovium the frequencies of CCL21+ and CCL19+ cells were 13 ± 14 and 7 ± 10/mm², respectively (Table III). Staining of CCL21+ cells was more intense than that of CCL19+ cells. As observed for
CCL20, the expression of CCL21 and CCL19 revealed a large degree of heterogeneity between samples. No expression of CCL21 and CCL19 was detected in samples characterized by a diffuse infiltrate without organization of the lymphocytic aggregates (patients 3, 6, 7, and 9). All samples with such features (patients 1, 2, 5, 8, and 11) showed the expression of CCL21, whereas CCL19 was only detected in two of those samples (patients 1 and 2). Both chemokines were detected in samples showing lymphocytic infiltrates with GC (patients 4, 10, and 12). More finely lymphocytic infiltrates (patients 1 and 2). Both chemokines were detected in samples showing lymphocytic infiltrates with GC (patients 4, 10, and 12). In RA synovium CCL21 and CCL19 were mainly localized in perivascular or lymphocytic infiltrates. Immature DC are highly efficient APC and were localized in lymphocytic infiltrates of the synovium, where they can interact with T and B cells. Such interaction could occur at various stages of the RA process.

**Discussion**

We demonstrate in this work the presence in RA synovium of independent immature and mature DC subsets using the CD1a- and DC-LAMP/CD83-specific markers, respectively. We focused on these three markers as reflecting the different steps of in vitro DC differentiation, with CD1a and DC-LAMP linked to early and late stages of differentiation, respectively, CD83 expression appearing before that of DC-LAMP.

Compared with tonsils, accumulation of DC in RA synovium was lower, with a relative accumulation of immature DC. In tonsils, the mature/immature ratios (DC-LAMP/CD1a) were 5.3 and 1.1 in RA synovium. Such a difference may lead to functional changes between a lymphoid organ in a normal position (in this study, the tonsils) or an ectopic position (in this study, the synovium). Immature DC are highly efficient in Ag uptake. In the context of RA, further clarification is difficult because the causative Ag remains unknown. Immature DC could take up Ag directly in synovium, as suggested by the presence of a long process from CD1a+ cells, but immature DC could have already loaded Ag before their entry into the synovium. Conversely, mature DC are highly efficient APC and were localized in lymphocytic infiltrates of the synovium, where they can interact with T and B cells. Such interaction could occur at various stages of the RA process.

Immature CD1a+ DC were localized in the lining or sublining layers, as well as in the perivascular infiltrates. Mature DC were mainly detected in perivascular infiltrates. In RA synovium, detection of both immature and mature DC subsets in perivascular infiltrates argues for the lack of compartmentalization, such as is observed for breast carcinoma tissue, where immature DC were found within the tumor and mature DC in peritumoral areas. However, in RA synovium different localization patterns were observed for DC-LAMP+ and CD83+ DC in these infiltrates. CD83+ cells, considered less mature than DC-LAMP+ cells, were exclusively detected at the periphery, whereas the fully mature DC-LAMP+ cells were also observed inside the lymphocytic infiltrates. Differential localization of CD83+ and DC-LAMP+ cells could reflect the effect of maturation on the response of DC to the chemokines that control their homing into infiltrates.

Such differences in the mature/immature ratio suggest changes in the chemokine contribution. Indeed, chemokines are nonspecific soluble factors that control the migration of DC and many other cells into the synovium. We analyzed the expression of CCL20,
the ligand of CCR6, and CCL19 and CCL21, the ligands of CCR7. These chemokines have been classified as chemoattractant for in vitro-derived immature and mature DC, respectively (19, 32). It was critical to demonstrate whether such findings are in line with the in vivo situation. The close association between CCL20-producing cells and CD1a<sup>+</sup> immature DC observed in this study was indeed an in situ confirmation of these previous results obtained in vitro. CCL20 was highly expressed in the lining layer, extending our recent results where in vitro incubation of synoviocytes with IL-1, IL-17, and TNF-α was associated with an up-regulation of CCL20 production (30). Synoviocytes are predominant in the lining layer and represent the leading CCL20-producing cells, similar to keratinocytes in psoriasis skin and epithelial cells in tonsil crypts, which are the sites of CCL20 production (19, 20, 33). The expression of CCR6 near the lining layer suggests that it represents one site of entry of immature CCR6<sup>+</sup> DC into the RA synovium. Indeed, such DC were observed inside the lining layer. However, high endothelial venules should also be considered a site of

FIGURE 3. CCL19 and CCL21 are detected in perivascular infltrates and vascular endothelium in RA synovium and tonsils. CCR7, their associated receptor, is expressed in the lymphocytic infltrates. Immunostaining of paraffin-embedded sections was performed with anti-CCL21 (A and C, RA synovium; G, tonsils) and anti-CCL19 (B and D, RA synovium; H, tonsils) Abs. CCL21<sup>+</sup> (A) and CCL19<sup>+</sup> cells (B) are found in the perivascular infltrates, with a stronger expression for CCL21. Some vascular endothelial cells in RA synovium are also CCL21<sup>+</sup> (C) or CCL19<sup>+</sup> (D), again with a higher intensity for CCL21. In tonsils CCL21 (G) is highly expressed only in the T cell zone, where CCL19-producing cells are less common (H). Staining using anti-CCR7 Ab (brown) shows CCR7<sup>+</sup> cells in the lymphocytic infltrates in RA synovium (E). Some CCR7<sup>+</sup> cells have a dendritic morphology (inset of E). In tonsils, most CCR7<sup>+</sup> cells are detected in the GC and some in the T cell zone (F), with a dendritic morphology (inset of F). Immunostaining of serial RA synovium sections with anti-CCR7 (I), anti-DC-LAMP (J), and anti-CCL21 (K) Abs shows that CCL21, a chemoattractant for mature DC, and its associated receptor, CCR7, colocalize in the lymphocytic infltrates with mature DC. Magnification: A–C, inset of E and F, and I–K ×400; G and H, ×100; E and F, ×250; D, ×600.
immature DC entry, as shown by the high expression of CCR6 and the close association between CCL20-producing cells and CD1a+ cells in the perivascular infiltrates.

Compared with tonsils, where mature DC represent the major DC subset, the RA synovium was characterized by the relative accumulation of immature DC. This suggests a relative defect in DC maturation in RA synovium. When considering the various cytokines used for in vitro generation of hemopoietic CD34+–derived DC (GM-CSF and TNF-α) and monocyte-derived DC (GM-CSF and IL-4) (34, 35), the lack of IL-4 in RA synovium contrasts with the high levels of GM-CSF and TNF-α (36). The defect in DC differentiation compared with tonsils could be linked to the lack of IL-4 leading to defective Ag processing. In addition, T cell-derived cytokines are characterized by high IL-17 and low IL-4 production, resulting in a proinflammatory cytokine pattern (37). IL-10, another anti-inflammatory cytokine known to inhibit the capacity of synovial macrophages to function as APC (38), had no immunosuppressive effect on rheumatoid synovial fluid DC compared with peripheral blood DC (39). These results indicate the contribution of synovium cytokine microenvironment that results in complex changes in DC recruitment and function.

The potent local defect of maturation of mature DC in RA synovium led to evaluation of the expression of CCR7 and its ligands, CCL19 and CCL21, known chemoattractants for in vitro-derived mature DC. The detection of both chemokines in vascular endothelium and perivascular infiltrates suggests that migration of mature CCR7+ DC occurs from blood to RA synovium through vascular endothelium. The expression of CCL19 and CCL21 in RA samples with GC and their absence in samples with diffuse infiltrates suggest that these chemokines are implicated in GC formation and migration of mature DC in these lymphocytic aggregates. A similar conclusion was reached with the B cell-attracting chemokine-1 (40). However, stronger expression of CCL21 compared with CCL19 in RA synovium may indicate a greater contribution of CCL21 to the migration of mature DC. In addition, CCL19 expression (5 of 12 samples) was less common than that of CCL20 production (9 of 12). When considering the quantification of their associated receptors, the CCR6/CCR7 ratio was 39/10 = 3.9 in RA synovium, suggesting a higher contribution of CCL20/CCR6 to that of CCR7 and its ligands, CCL19 and CCL21, in the homing of DC. Such differences could result in a reduced migration of mature DC from blood. The combined results observed here are depicted in Fig. 4, which represents a model of DC subsets and migration patterns in RA synovium.

Cheomokines acting as chemoattractants of DC are key factors in stimulating immunity against tumor Ags. Adenovirus-mediated gene transfer of CCL20 to murine tumors induced the local accumulation of DC, leading to tumor-specific cellular immunity and suppression of tumor (41). Vaccination of mice with the CCL19-transduced C3L5 resulted in the rejection of CCL19-transduced tumor, showing an antitumor activity for CCL19 (42). The role of DC in disease initiation and perpetuation makes DC subsets a potential therapeutic target in RA. This also suggests the control of chemokines involved in DC migration. The link between CCL20 production and inflammation makes this chemokine a potential therapeutic target.

FIGURE 4. A model of DC subsets and migration patterns in RA synovium. In response to local inflammation and the production of proinflammatory cytokines (1) following an unknown event, immature DC are attracted to the synovium in response to the local production of CCL20 in the perivascular infiltrates and the lining layer (2). CCR6+ immature DC migrate from blood through vascular endothelium and possibly also from synovial fluid through the lining layer. It is also possible that CCR6+ cells migrate to synovial fluid toward CCL20 expressed within the lining layer (3). The presence of mature DC in synovium results from the combined effects of cell interactions and cytokine microenvironment (4). A defect in production of differentiation factors, such as IL-4, may favor a relative accumulation of immature DC. However, the detection of CCL19 and CCL21 in perivascular infiltrates and vascular endothelium argues for a direct migration of CCR7+ mature DC from blood into the RA synovium, where they can interact with lymphocytes, leading to potent local lymphocyte activation.