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*J Immunol* 2004; 173:2815-2824; doi: 10.4049/jimmunol.173.4.2815

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Characterization and Recruitment of Plasmacytoid Dendritic Cells in Synovial Fluid and Tissue of Patients with Chronic Inflammatory Arthritis

Roberto Lande,* Elena Giacomini,* Barbara Serafini,† Barbara Rosicarelli,† Gian Domenico Sebastiani,† Giovanni Minisola,§ Umberto Tarantino,§ Valeria Riccieri,¶ Guido Valesini,¶ and Eliana M. Coccia²*

Dendritic cells (DCs) are thought to play a key role in driving the immunopathogenic response underlying chronic inflammatory arthritis. In this study, we have examined the presence and phenotype of plasmacytoid DCs (pDCs) in the synovial fluids (SF) of patients with rheumatoid arthritis (RA), psoriatic arthritis (PA), and osteoarthritis (OA) and determined the chemotactic properties of SF from these patients toward pDCs. Flow cytometry analysis showed that the percentage of pDCs, identified as a population of Lin⁻CD123⁺ cells, is 4- to 5-fold higher in RA SF and PA SF than in OA SF. The morphological and immunophenotypic characterization of pDCs isolated from PA and RA SF indicates that they are in an immature state, most likely due to inhibitory factors present in RA SF, but are still able to undergo maturation when exposed ex vivo to viral agent or unmethylated DNA. CD123⁺ and BDCA2⁺ pDCs were detected by immunohistochemistry in RA synovial tissue in which expression of the IFN-α-inducible protein MxA was also found, suggesting production of type I IFN by maturing pDCs. We also show that CXCR3 and CXCR4 are expressed by both blood-derived pDCs and pDCs isolated from RA and PA SF and that CXCL-10, CXCL-11, and CXCL-12 present in RA and PA SF stimulate chemotaxis of blood-derived pDCs. Altogether, these findings suggest that chemokine-driven recruitment of pDCs from the blood to the inflamed synovium could be important in the regulation of the immune response in chronic inflammatory arthritis. The Journal of Immunology, 2004, 173: 2815–2824.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial joint inflammation and infiltration of activated CD4⁺ T cells, NK, granulocytes, macrophages, and dendritic cells (DCs) in the synovial fluids (SF) and synovial tissues (ST). DCs are the most potent APCs and are thought to regulate processing and presentation of arthritogenic Ags to autoreactive T cells, leading to their sustained activation in rheumatoid arthritis (RA) (1). Previous immunohistochemical studies have shown that immature myeloid DCs accumulate in both RA SF and ST, whereas mature DCs have been detected predominantly in perivascular location and ectopic lymphoid-like structures within RA ST (1–5). Little is known on the presence and role of DCs in the etiopathogenesis of psoriatic arthritis (PA), a chronic inflammatory disease characterized by inflammatory cell infiltrates and angiogenesis in the connective tissue of the skin and joints (6, 7).

Among DC subsets, plasmacytoid DCs (pDCs) constitute a rare population of immature DCs, which lack myeloid markers, show a plasmacytoid morphology, and differentiate in vitro into mature DC2 following stimulation with CD40L, viruses, and bacterial DNA (8, 9). The maturation process into DC2 involves increased cell surface expression of MHC and costimulatory molecules as well as production of elevated amounts of type I IFN. Recent studies performed in inflammatory neurological and skin diseases have provided evidence that pDCs accumulate in the target tissues, suggesting that pDCs might be involved in the establishment and/or maintenance of chronic inflammatory processes (10, 11). In particular, pDCs accumulating in cutaneous systemic lupus erythematosus (SLE) lesions are thought to play a key role in the pathogenesis of this autoimmune disease in which disease progression is associated with high systemic levels of IFN-α (12–14).

To get more insights into the role of pDCs in the pathogenesis of chronic inflammatory arthritis, we have analyzed the number, frequency, and immunophenotype of pDCs in the SF of patients affected by RA and PA and performed immunohistochemical stainings to identify pDCs and to detect the expression of the IFN-α-inducible protein MxA in RA ST. As control, SF and ST samples from patients with osteoarthritis (OA), a degenerative joint disease with no immunopathological alterations, were analyzed. We found that increased numbers of pDCs expressing an immature phenotype are present in RA and PA SF as compared with OA SF and that numerous pDCs accumulate in the ST of RA but not OA patients. The marked MxA expression detected in RA ST is indicative of local production of type I IFN, suggesting that acquisition of a mature phenotype by pDCs might be induced by appropriate maturative stimuli in the inflamed synovium. We then

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Received for publication October 23, 2003. Accepted for publication June 15, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants (1AC/F4, 2A/IF, and 2132/RI) from the Istituto Superiore di Sanità (to E.M.C.).

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; DC, dendritic cell; FSC, forward scatter; Lin, lineage-associated markers; MC, mononuclear cell; OA, osteoarthritis; PA, psoriatic arthritis; PB, peripheral blood; pDC, plasmacytoid DC; SSC, side scatter; SF, synovial fluid; SLE, systemic lupus erythematosus; ST, synovial tissue; MFI, mean fluorescence intensity.

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investigated the mechanisms driving migration of pDCs to the inflamed joints. We find that the levels of CXCL-10, CXCL-11, and CXCL-12 are higher in RA and PA SF than in OA SF. Moreover, we show that CXCL-10, CXCL-11, and CXCL-12 contained in RA and PA SF promote the in vitro migration of peripheral blood (PB)-derived pDCs, indicating a role for these chemokines in the homing of immature pDCs into the inflamed synovium.

Materials and Methods

Patients and specimens

We studied a total of 45 SF samples obtained during therapeutic arthrocentesis from the knee joint of 19 patients with RA, 11 patients with PA, and 15 patients with OA. The first SF samples used for immunohistochemical studies were obtained from two patients with RA and from two patients with OA. A tonsil from a child undergoing tonsillectomy was used as positive control for the immunostaining of pDCs.

The diagnoses of RA and OA were made according to the criteria of the American College of Rheumatology (15, 16). PA was diagnosed according to the criteria proposed by Moll and Wright (17). SF and blood samples, taken at the same time, were collected in plastic heparinized tubes and immediately processed. Selected demographic and laboratory data of the patients are presented in Table I. The majority of patients with RA and PA were being treated with nonsteroidal anti-inflammatory drugs. Some were treated with low-dose glucocorticoids and a disease-modifying antirheumatic drug (sulfasalazine, hydroxychloroquine, methotrexate). None of the patients were being treated with nonsteroidal anti-inflammatory drugs.

Isolation and stimulation of pDCs

SF were enzymatically processed by a three-step method to reduce the viscosity: 1) hyaluronidase type IV-S (15 μg/ml; Sigma-Aldrich, St. Louis, MO) for 15 min at 37°C; 2) collagenase type IV-S (250 ng/ml; Sigma-Aldrich) for 15 min at 37°C; and 3) hyaluronidase as in step 1. After each step, cells were centrifuged at 300 × g for 10 min. Resuspended cells were isolated by density gradient centrifugation using Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada) followed, occasionally, by lysis of erythrocytes in isotonic ammonium chloride buffer (Sigma-Aldrich). No patient had undergone intra-articular injection of corticosteroids within the 3 mo of study entry. Informed consent was obtained from all subjects who entered the study.

Table I. Selected demographic and laboratory data

<table>
<thead>
<tr>
<th>RA</th>
<th>PA</th>
<th>OA</th>
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<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Male/Female</td>
<td>2/17</td>
<td>8/3</td>
</tr>
<tr>
<td>Age, mean (range) years</td>
<td>52.4 (25–75)</td>
<td>42.9 (24–72)</td>
</tr>
<tr>
<td>Disease duration, mean (range) mo</td>
<td>74.3 (6–240)</td>
<td>85.3 (6–288)</td>
</tr>
<tr>
<td>SF leukocyte counts × 10³, mean (range)/μl</td>
<td>9.2 (1–20)</td>
<td>8.3 (1.8–24)</td>
</tr>
<tr>
<td>Serum rheumatoid factor positive</td>
<td>0</td>
<td>0</td>
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</table>

Morphological and FACS analysis

SF pDCs (1 × 10⁴) were cytospin centrifuged onto microscope slides, stained with May-Grünewald-Giemsa, and then examined by light microscopy. The following mAbs, conjugated with FITC, PE, or Cy5, were used for flow cytometry analyses. mAbs for CD3, CD4, CD14, CD16, CD19, CD20, CD56, CD80, CD83, CD86, CD45RA, CD54, CD58, CD38, CD45L, HLA-DR, and CXCR3, as well as IgG1, IgG2a, and IgG2b control isotypes were purchased from BD Pharmingen (San Diego, CA) or R&D Systems (IL-3Ra) and anti-BDCA2 mAbs were obtained from Miltenyi Biotec. Anti-CXCR4 mAb was purchased from R&D Systems. Cells were washed once in PBS containing 2% FCS and then incubated with purified mAbs at 4°C for 45 min. The cells were then washed and fixed with 1% paraformaldehyde. The cells (5 × 10⁴) were acquired on a BD FACSCalibur. Data were analyzed using CellQuest software (BD Biosciences, Mountain View, CA). Where indicated, the physical parameters of pDCs, forward scatter (FSC) signals and side scatter (SSC) signals were also examined.

Immunohistochemistry

All tissues were embedded in Tissue-Tek OCT compound, snap frozen in dry ice-chilled isopentane, and stored at −80°C until use. Ten-μm-thick serial sections were cut with a cryostat, mounted on SuperFrost Plus slides, dried overnight at room temperature, and fixed in acetone for 10 min at 4°C. After blocking endogenous peroxidase with 0.1% H₂O₂ in PBS for 20 min, sections were incubated with 10% normal rabbit serum for 1 h and then overnight at 4°C with anti-BDCA2 mAb (Miltenyi Biotec) and anti-CD123 (IL-3Ra) mAb (BD Biosciences) diluted 1/15 and 1/100 in PBS, respectively, or anti-CD3 mAb (Immunotech, Marseille, France). For MxA immunostaining, sections were incubated for 1 h at room temperature with anti-MxA mAb (clone M143, courtesy of Dr. O. Haller, Abteilung Virologie, Institut fur Medizinische Mikrobiologie und Hygiene, Universitat Freiburg, Freiburg, Germany) diluted 1/400 in PBS containing 2% BSA (18). The binding of biotinylated secondary Ab (rabbit anti-mouse IgG from The Jackson Laboratory, Bar Harbor, ME) was visualized with the avidin-biotin-HRP complex technique (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (Sigma-Aldrich) as substrate. Sections were counterstained with hematoxylin and viewed with a Zeiss Axiophot phase microscope (Zeiss, Oberkochen, Germany). Negative controls included the use of IgG isotype controls and the omission of the primary Ab.

ELISA

Supernatants from control and CpG- or flu-treated pDC cultures were harvested and stored at −80°C. ELISA for IFN-α was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). ELISA kits for CXCL-10, CXCL-11, and CXCL-12 were obtained from R&D Systems. Supernatants from 6 to 10 separate experiments were analyzed. The ELISA were conducted according to the manufacturer’s instructions. The minimum detectable dose ranged from 0.4 to 4 pg/ml for CXCL-10, from 4 to 40 pg/ml for CXCL-11, from 1 to 47 pg/ml for CXCL-12, and from 10 to 500 pg/ml for IFN-α.

ELISPOT

The ELISPOT test, used to enumerate cells secreting IFN-α, was based on the method described by Versteegen et al. (19) with some modifications. Briefly, flat-bottom microtiter plates (96 wells, Nunc-ImmunoPlate Maxi-sorp; Nunc, Roskilde, Denmark) were coated with 0.1 ml/well mouse anti-human IFN-α mAbs (10 μg/ml in carbonate-bicarbonate buffer (pH 9.7); PBL Biomedical Laboratories) and incubated overnight at 4°C. Plates were washed with PBS-0.05% Tween 20 and then blocked with 3% gelatin (both from Sigma-Aldrich) in PBS. After exhaustive washings, the cells were added to the wells and incubated for the indicated times at 37°C in a 5% CO₂ incubator in the presence or absence of flu virus or CpG. After removal of the cells, the wells were incubated for 90 min at 37°C with an optimal dilution of rabbit anti-human IFN-α (PBL Biomedical Laboratories) and then washed and incubated for 90 min at 37°C with an optimal dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich). After extensive washing, 0.1 ml of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate (1 mg/ml) in 1 M 2-amino-2-methyl-1-propanol buffer (Sigma-Aldrich) was added. After 1 h incubation at room temperature, the plates were rinsed with water and allowed to dry.

National Public Health Institute, Helsinki, Finland). AAC-30 was added twice at 0 and 4 h to compensate for degradation by DNase activity in the medium. To evaluate the presence of inhibitory factors in RA SF, a 1/5 dilution of the pooled SF from four RA patients (at a final concentration of 20%) was added along with flu virus to pDCs from PB.
The blue spots were counted under a stereomicroscope with ×40 magnification.

**Chemotaxis assay**

pDC chemotaxis assays were performed using a 24-well Transwell chamber with a 5-μm pore size (Corning, Cambridge, MA). pDCs isolated from PB were resuspended at a concentration of 1 × 10^6/ml in RPMI 1640 containing 0.5% BSA. SF from four RA and four OA patients were pooled separately. A 1/2 dilution of the pooled SF (final volume, 600 μl) was added to the bottom chamber of the Transwell, while 1 × 10^6 cells were added to the top chamber (input cells). Where indicated, CXCL-12 (100 ng/ml; PeproTech) was added to the SF or the cells were preincubated for 30 min with the CXCR4 antagonist AMD 3100 (25 μg/ml; Sigma-Aldrich). As control chemotactic stimuli, CXCL-11 and CXCL-12 (100 ng/ml; PeproTech) were added to the bottom chamber either alone or in combination. All SF and chemokine dilutions were performed in RPMI 1640 containing 0.5% BSA. The plates were then incubated for 3 h at 37°C. Migrated cells were counted by microscopy and flow cytometry. All experimental points were performed in duplicate.

**Statistical analysis**

Data are expressed as means ± SE. Statistical significance of differences was determined via the nonparametric Wilcoxon test (p < 0.05 was considered to be significant).

**Results**

**Detection of pDCs in SF of patients with RA and PA**

Chronic inflammatory arthritis is associated with the migration of a large number of leukocytes in the synovium. Despite recent data indicating that myeloid DCs accumulate in the SF and ST of RA patients (1–5), nothing is known on the presence of pDCs in the diseased tissue. To evaluate the presence of pDCs in the SF obtained from RA and PA patients, a phenotypical analysis was performed based on the expression of CD4, CD123, CD45RA, and HLA-DR, and the lack of CD11c and hemopoietic lineage markers (CD3 for T cells, CD14 for monocytes, CD16 and CD56 for NK cells, and CD19 and CD20 for B cells). As control, SF from patients with OA, a degenerative joint disease with no immunopathological involvement, was examined. Cells from RA, PA, and OA SF were stained with a mixture of FITC-labeled mAbs specific for lineage-associated markers (Lin) and with PE-labeled anti-CD123 mAb. Using flow cytometry, a small homogeneous cell population was identified in RA SF, which exhibited a size similar to that of activated lymphocytes. These cells did not express Lin markers and were highly positive for CD123 (Fig. 1A). To exclude contamination by small size granulocytes, like basophils, which share with pDCs the expression of high levels of CD123, we performed a three-color staining with markers that are not expressed on granulocytes, such as CD45RA and HLA-DR. PE-labeled anti-CD123, FITC-labeled anti-CD45RA, and Cy5-labeled anti-HLA-DR mAbs unequivocally identified the pDC population (gate R1 in Fig. 1C; gate R2 in Fig. 1D). By performing double stainings with anti-CD123 and Lin mAbs, pDCs were also identified in the SF of patients with PA and OA (Fig. 1, E and G). Furthermore, gating Lin−CD123++ cells (gate R) showed that the physical parameters of pDCs from SF correspond to those of pDCs from PB of a healthy donor (FSC and SSC in Fig. 1, B, F, H, and L).

We then evaluated the percentage of pDCs, identified as a Lin−CD123++ population, in the SF from RA, PA, and OA patients (Fig. 2). Interestingly, we noticed that the percentage of pDCs was significantly higher in RA (0.8 ± 0.12% of total mononuclear cells (MC), n = 19) and PA SF (0.98 ± 0.22% of MC, n = 10) than in OA SF (0.19 ± 0.06% of MC, n = 15). The frequencies of Lin−CD123++ cells in SF from PA and RA patients were also significantly higher compared with the percentage of pDCs in PB of healthy controls (0.3 ± 0.07%, n = 10) and RA patients (0.2 ± 0.06%, n = 7).
pDCs from PB of healthy donors with cell-free RA or PA SF failed to inhibit the expression of CD62L (data not shown), suggesting that the down-regulation of this molecule is not due to factors present in RA and PA SF but may occur upon interaction of pDCs with inflamed endothelial cells during their migration from PB into the synovial compartment.

**Induction of pDC maturation by flu virus infection or unmethylated DNA (CpG)**

To analyze whether the absence of a mature phenotype was due to an intrinsic defect of SF pDCs or to the lack of appropriate stimuli in the RA or PA SF, we stimulated SF pDCs with conventional maturative stimuli, such as flu virus and unmethylated DNA (9). Fresh, highly purified pDCs from RA SF, stimulated for 36 h with CpG oligodeoxynucleotide AAC-30 or for 18 h with flu virus, acquired a mature phenotype as demonstrated by a marked increase of HLA-DR, CD86, CD83, and CD40 expression (Fig. 4A). The acquisition of a mature phenotype was also confirmed by morphologic analysis performed with May-Grünwald-Giemsa staining (Fig. 4B). Freshly sorted cells displayed the typical lymphoplasmacytoid morphology and appeared as medium-sized round cells with an oval or kidney-shaped nucleus, unruffled or scarcely ruffled plasma membrane, and no dendritic processes. After the stimulation with CpG, pDCs acquired an irregular shape and a larger size and developed dendritic processes, all of these features being characteristic of mature pDCs. IFN-α production was also analyzed by measuring cytokine accumulation in the culture supernatants by ELISA (Fig. 5A) or by counting the number of IFN-α-producing cells with ELISPOT (Fig. 5B). Both assays showed that pDCs were able to produce large amounts of type I IFN after stimulation with CpG or infection with flu virus. Conversely, no production of IL-12 was observed in the supernatants of mature pDC cultures (data not shown). Because IFN-αβ has been reported to induce chemokine gene expression (21), we also analyzed CXCL-10 and CXCL-11 production in flu-infected and CpG-stimulated pDCs. Fig. 5, C and D) shows that both chemokines were significantly induced in response to such stimuli.

To investigate whether inhibitory factors affecting the maturation of pDCs are present in RA SF, we stimulated pDCs from PB with flu infection or with RA SF. Conversely, no production of IL-12 was observed in the supernatants of mature pDC cultures (data not shown). Because IFN-αβ has been reported to induce chemokine gene expression (21), we also analyzed CXCL-10 and CXCL-11 production in flu-infected and CpG-stimulated pDCs. Fig. 5, C and D) shows that both chemokines were significantly induced in response to such stimuli.

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used as positive control tissue. The spatial distribution and morphological appearance of BDCA2⁺ cells in the tonsil tissue closely paralleled that of CD123⁺ cells, with immunopositive cells essentially localized in the interfollicular T cell zones (Fig. 7, A and B). Stainings performed on serial sections revealed that CD123⁺ cells were more numerous than BDCA2⁺ cells, which is in line with the finding that anti-BDCA2 mAb is more selective for immature pDCs than anti-CD123 mAb (11, 22). Both CD123⁺ and BDCA2⁺ cells appeared as medium-sized cells with a round cell body and cytoplasmic ramifications (Fig. 7, insets in A and B). We did not observe any staining on vascular endothelia. The histological evaluation of the two RA surgical samples, performed using the conventional H&E staining, showed scarce or moderate inflammatory infiltrates and a varying degree of fibrosis extending from the synovial membrane to the deepest layers of the tissue (data not shown). These histological patterns are compatible with tissue damage associated with the chronic phases of the disease. Numerous CD123⁺ and fewer BDCA2⁺ cells were found in RA ST beneath the surface of the synovium (Fig. 7, D and F), in association with T cell infiltrates (Fig. 7, E, G, and H) as well as scattered in fibrotic areas (data not shown). CD123⁺ but not BDCA2⁺ cells were significantly more numerous in the vicinity of immune cell infiltrates than in the fibrotic areas of the ST (data not shown). Both CD123⁺ and BDCA2⁺ cells showed a dendritic morphology with long cytoplasmic processes which closely resembles that observed in tonsil tissue (Fig. 7; compare D–G with the insets in A and B). In agreement with the finding that surface expression of BDCA2 on pDCs is markedly reduced after maturation (23), the observation that BDCA2⁺ are less numerous than CD123⁺ cells suggests that most pDCs found in RA ST might display a mature phenotype. To confirm this hypothesis, we immunostained both RA and OA ST for MxA, an IFN-α-inducible protein frequently used as a surrogate marker for local IFN-α production (24). Control staining performed on tonsils showed that MxA reactivity was localized on cells in interfollicular T cell areas (Fig. 7C) with a distribution which closely parallels that of CD123⁺ and BDCA2⁺ pDCs (Fig. 7, A and B). In RA ST, but not in OA ST, numerous MxA-expressing cells were detected both beneath the synovial surface and within the T cell infiltrates (Fig. 7, I and L). Expression of MxA was found in cells showing an irregular and ramified shape similar to that of CD123⁺-positive cells observed in RA ST (arrows) and in additional cells with a round cell body, suggesting that in inflamed RA synovium pDCs may produce type I IFN. In agreement with the flow cytometry data, neither BDCA2⁺ nor CD123⁺ cells were detected in OA ST (Fig. 7, J and K).
Chemokine levels in SF of RA, PA, and OA patients

Recent studies have shown that pDCs are able to migrate in response to a combination of inflammatory (CXCL-10, CXCL-11) and constitutive (CXCL-12) chemokines (25, 26). To study the mechanisms underlying pDC recruitment to the inflamed synovium, the SF of RA, PA, and OA patients were then analyzed for the presence of the above-mentioned chemokines. Using ELISA, we detected considerable amounts of CXCL-10, CXCL-11, and CXCL-12 in SF from RA patients (Fig. 8). Except for CXCL-11, similar amounts of these chemokines were detected in SF of PA patients. Conversely, only CXCL-12 was detectable in OA SF, whereas CXCL-10 and CXCL-11 were present in very low amounts.

Recruitment of PB-derived pDCs by chemokines present in RA SF

We next investigated whether CXCL-10, CXCL-11, and CXCL-12 present in the SF of RA and PA patients (Fig. 8) could be involved in the recruitment of pDCs from PB into the inflamed synovium. Flow cytometry analysis showed that human blood pDCs express CXCR3 and CXCR4 (Fig. 9A), as previously described (27). Migration assays with human blood pDCs were performed using SF from RA and OA patients (Fig. 9B). A 2-fold increase in the percentage of pDCs migrated in the presence of RA SF compared with OA SF was observed (p = 0.02). Similar results were obtained when SF from PA patients were used (data not shown). The modest but significant migration of pDCs in response to CXCL-10, CXCL-11, and CXCL-12 present in RA SF was potentiated by the addition of CXCL-12 (100 ng/ml) and was blocked by pretreatment of pDCs with a CXCR4 antagonist (AMD 3100, 25 μg/ml), indicating that pDCs require CXCL-12 to respond to CXCR3 ligands (Fig. 9B). In line with this, exposure of pDCs to recombinant CXCL-11 and CXCL-12 elicited no significant and only little pDC migration, respectively, while the combination of the two chemokines induced a massive migration of pDCs (Fig. 9B), as recently described (25–28).

Discussion

This study provides the first evidence for the recruitment of pDCs in the SF and ST of patients with chronic inflammatory arthritis. We found that the percentage of pDCs is significantly higher in RA and PA SF compared with OA SF and to RA PB, indicating a preferential accumulation of pDCs in the inflamed joints. By immunohistochemical analysis, BDCA2 and CD123+ pDCs were also detected in ST from long-standing RA patients, but not in OA synovium. The presence of pDCs in RA synovium and their persistence during chronic disease phases suggest that these cells might be important in sustaining immunopathological alterations underlying chronic inflammatory arthritis. These findings are in line with previous observations on the presence of pDCs in target tissues in inflammatory skin and neurological diseases (10, 11, 13), SLE cutaneous lesions (12, 14, 29), and nasal mucosa of allergic individuals (30, 31).

The immunocytochemical analysis of the phenotype of pDCs isolated from RA and PA SF indicates that they are similar to immature pDCs present in PB, as they fail to express costimulatory molecules, like CD40, CD83, and CD86, and to produce type I IFN. However, the absence of a mature phenotype does not appear to be due to an intrinsic functional defect of pDCs in RA or PA SF but to the presence of inhibitory factors in such fluids. In fact, in the presence of RA SF, ex vivo treatment of PB-derived pDCs with a conventional maturative stimulus, such as flu virus (9), reduced the expression of the above molecules as well as the production of type I IFN. These results are consistent with the lack of type I IFN in SF from RA and PA patients. We have also shown that, in contrast to circulating pDCs, RA- and PA SF-derived pDCs do not express CD62L. This finding suggests that pDCs accumulating in the inflamed synovia have extravasated through the inflamed endothelia and, possibly, high endothelial venules that are present in ectopic lymphoid organs in the ST of a proportion of RA patients (32, 33). Adhesion and rolling of lymphocytes and pDCs on HEV is mediated by CD62L (20, 34), which is known to be cleaved after interaction with endothelial cells (20, 35).

Because only mature pDCs are able to induce the activation of memory CD4+ and CD8+ T cells (8, 36–38) and to promote the differentiation of B cells into Ab-producing plasma cells (39), the above results would suggest that pDCs accumulating in RA and PA SF might fail to promote pathogenic immune responses due to the presence of inhibitory factors as well as to the lack of appropriate maturation stimuli. However, it cannot be excluded that pDCs undergo maturation after entering the inflamed ST, as recently shown for myeloid DCs (1, 3). This possibility is indirectly supported by the finding that in the inflamed RA ST pDCs expressing BDCA2, a marker that is down-regulated upon pDC maturation, are substantially less numerous than cells expressing CD123, which is uniformly expressed by immature and mature pDCs (23). It is conceivable that in the inflamed ST the final maturation of pDCs might be induced by the combination of different
stimuli, such as a cytokine-rich microenvironment (1, 40–42), interaction with CD40L-expressing T lymphocytes (43), and/or binding of FcyRII expressed on pDCs by immune complexes containing autoantibodies. The latter mechanism has been recently proposed as a major pathogenic event in RA and SLE (44, 45). Although we have observed that cells with dendritic morphology expressing the DC maturation marker CD83 are present in RA ST (B.S. and E.M.C., unpublished data), technical limitations in performing double-immunohistochemical stainings of frozen tissue sections with anti-BDCA2 and anti-CD83 mAbs did not allow us to evaluate directly the maturation state of pDCs. However, the conspicuous number of MxA-positive cells detected by immunohistochemistry in inflamed RA ST well correlates with the distribution of pDC in RA ST, indicating that pDCs may produce significant amounts of type I IFN which in turn stimulate, in a paracrine and autocrine fashion, the expression of IFN-induced genes.

Persistent production of type I IFN by mature pDCs could have pathogenic relevance in the inflammatory process that characterizes RA and PA, as shown in SLE (14, 46). Type I

FIGURE 7. Immunohistochemical detection of pDCs in RA synovium. Control immunostainings of serial sections of human tonsils with anti-CD123 (A), anti-BDCA2 (B), and anti-MxA (C) mAbs. CD123⁺ and BDCA2⁺ pDCs with dendritic morphology (insets) and MxA-expressing cells are located in the interfollicular T cell areas of the tonsil (F, follicle). Immunostainings of a representative specimen from long-standing RA synovium with anti-CD123 (D and E), anti-BDCA2 (F and G), anti-CD3 (H), and anti-MxA (I) mAbs. Numerous CD123⁺ and rare BDCA2⁺ cells, both displaying cytoplasmic ramifications (arrows), were found in the RA ST beneath the synovial membrane (D and F) and in close association with CD3⁺ T cell infiltrates (E and G). MxA-expressing cells show a similar distribution within RA ST (I) and an analogous dendritic morphology (arrows). ST from OA were negative for CD123 (J), BDCA2 (K), and MxA (L) immunostainings. All sections were counterstained with hematoxylin. E, G, H and I, Asterisks identify the same T cell infiltrate in serial sections. I, A field slightly rotated with respect to the same field shown in H. Original magnifications: ×500 (A, B, J, K, and L); ×1000 (C–I and insets).
IFNs, which are potent antiviral agents, have been shown to promote immune responses by regulating the proliferation, differentiation, and activation of different leukocyte populations, including DCs, B cells, NK cells, CD4⁺, and memory CD8⁺ T lymphocytes (47-51). The expression of type I IFN, previously demonstrated in joint tissue of RA patients, may promote the survival and accumulation of T lymphocytes in the synovial compartment by inhibiting the apoptotic process (49, 52). Moreover, type I IFN produced by pDCs appears to be directly implicated in plasma cell differentiation and Ab production (39).

In this study, we also show that pDCs isolated from RA and PA SF express CXCR3 and CXCR4, the receptors for CXCL-10 and CXCL-11, and for CXCL-12, respectively, and that the levels of these chemokines are significantly increased in RA and PA SF, but not in OA SF. The latter finding confirms previous studies showing that CXCL-10, CXCL-11, and CXCL-12 are expressed in RA ST and SF (53-56). Interestingly, the expression of CXCL-10 and CXCL-11 is induced by type I IFN, suggesting a possible role for pDC-derived type I IFN in facilitating leukocyte recruitment to the inflamed joint (21, 57, 58). When the functional relevance of CXCL-10, CXCL-11, and CXCL-12 contained in RA and PA SF was evaluated, we found that RA SF displayed a significant chemotactic activity toward PB-derived pDCs expressing CXCR3 and CXCR4 receptors. Conversely, OA SF, which contains CXCL-12 but very low levels of CXCL-10 and CXCL-11, only slightly induced pDC migration. Notably, treatment of pDCs with AMD 3100, a specific inhibitor of CXCR4 (59), prevented the migration of pDCs toward RA SF by blocking specifically the activity of CXCL12 and, in turn, that of CXCL-10 and CXCL-11. Our results are in line with recent observations in an experimental model of collagen-induced arthritis where the administration of AMD 3100 strongly reduced the infiltration of CXCR4⁺Mac-1⁺ synovial cells, including immature and mature monocyte/macrophage and neutrophils, into the inflamed joints (60). Our results extend the anti-inflammatory effects of AMD 3100 to pDCs and confirm that CXCL-12 is required for the response of pDCs to CXCR3 ligands, as previously shown by Krug et al. (25) and Penna et al. (27). The sustained production of CXCL-10 and CXCL-11 in the RA and PA SF could also orchestrate the migration of other CXCR3-expressing leukocytes, such as T lymphocytes and NK cells, in the inflamed tissue (55, 61-63).

In conclusion, the present study provides new insights into the presence and mechanisms of recruitment of pDCs in the synovium of RA and PA patients. Although the accumulation of pDCs has been reported in various chronic inflammatory diseases, their exact role in regulating T and B cell responses mediating immunopathological alterations in target tissues remains to be defined. It can be envisaged that a better understanding of these events could lead to the development of new therapeutic strategies aimed at blocking the infiltration and functions of pDCs in the inflamed joints.
Acknowledgments

We thank M. Eloranta, F. Barone, C. Gagliardi, and F. Aloi for helpful discussions and critical reading. The technical assistance of T. Grassi is acknowledged. We are grateful to E. Morassi for preparing drawings and P. Monteolene for editorial assistance.

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