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Dendritic Cells (DCs) in Rheumatoid Arthritis (RA): Progenitor Cells and Soluble Factors Contained in RA Synovial Fluid Yield a Subset of Myeloid DCs That Preferentially Activate Th1 Inflammatory-Type Responses

Frances Santiago-Schwarz,* Prachi Anand,† Sean Liu,* and Steven E. Carsons*

There is evidence that mature dendritic cells (DCs) present in the rheumatoid arthritis (RA) joint mediate immunopathology in RA. In this study, we indicate that early myeloid progenitors for DCs and DC growth factors existing in RA synovial fluid (SF) are also likely participants in the RA disease process. A fraction of cells lacking markers associated with mature DCs or DC precursors and enriched in CD34<sup>negative</sup> myeloid progenitors was isolated from RA SF. These cells proliferated extensively when cultured in vitro with cytokines that promote the growth of myeloid DCs (GM-CSF/TNF/SCF/stem cell factor/IL-4) and, to a lesser degree, when cultured with monocyte/granulocyte-restricted growth factors (M-CSF/GM-CSF). Mature DCs derived from RA SF progenitors with CD14-DC cytokines known to be prevalent in the inflamed RA joint (GM-CSF/TNF/stem cell factor/IL-13) were potent stimulators of allogeneic T cells and inflammatory-type Th1 responses and included CD14-DC subtypes. Cell-free RA SF facilitated DC maturation from myeloid progenitors, providing direct evidence that the inflamed RA joint environment instructs DC growth. Enhanced development of CD14-derived DCs was correlated with the presence of soluble TNFR (p55), raising the possibility that soluble TNFR also regulate CD14-derived DC growth in vivo. SF from patients with osteoarthritis contained neither myeloid DC progenitors nor DC growth factors. The existence of DC progenitors and myeloid DC growth factors in RA SF supports the concept that RA SF may be a reservoir for joint-associated DCs and reveals a compelling mechanism for the amplification and perpetuation of DC-driven responses in the RA joint, including inflammatory-type Th1 responses. The Journal of Immunology, 2001, 167: 1758–1768.

Dendritic cells (DCs)<sup>3</sup> are Ag handling cells that regulate a wide spectrum of immune responses (1–5). Emerging concepts indicate that DCs are not a single cell type, but a system of cells that arise from both the myeloid and lymphoid hematopoietic lineages. Various DC subtypes are thought to differ in their capacity to either stimulate or inhibit the immune response (1–7). For instance, in humans, fully matured myeloid (CD14-derived) DCs may preferentially activate inflammatory-type Th1 responses (IFN-γ, IL-1, TNF) over Th2 (IL-4) responses, whereas lymphoid DCs may facilitate the activation of Th2 responses and promote negative selection in the thymus (4, 8–10). Importantly, DC functions are also related to stages of maturity. Fully matured DCs are equipped to deliver antigenic signals to T cells in a MHC-restricted manner, but do not efficiently handle Ag. Conversely, immature DCs present Ag poorly, but avidly internalize and process Ag (2, 4). Recent evidence also indicates that immature DCs may directly activate regulatory T cells, which suppress the development of effector T cells (2, 11, 12). Arguably, DC function is not fixed, but is highly influenced by extracellular signals. While immature DCs and DC precursors/progenitors may be highly susceptible to external instruction, mature DCs appear to be more resistant (9).

Within the myeloid DC lineage, two distinct DC pathways have been demonstrated (5–7). One pathway yields the CD14-derived (monocyte-derived) DC subtype. Intermediate developmental stages of this pathway include: myeloid dendritic progenitor cells exhibiting monocyte-, granulocyte-, and DC-differentiating potential, CD14<sub>dim</sub> precursors with either DC- or monocyte-differentiating potential, and CD14<sub>dim</sub>CD1a<sup>+</sup> precursors that are DC committed and that develop from CD14<sub>dim</sub> precursors (5–7, 13). The second myeloid DC pathway produces the CD14-independent DC subtype (Langerhans cell) and includes CD14<sub>negative</sub>CD1a<sup>+</sup> precursors (6, 7, 13). TNF/GM-CSF +/- TGFβ appear to be primary growth factors for the CD14-independent pathway (14, 15), while the CD14-derived DC pathway develops with GM-CSF + IL-4/IL-13 (16–18). A third DC subtype, the lymphoid-related DC, may stem from common NK-T cell precursors under the influence of IL-3/CD40 ligand, but not GM-CSF (1, 2, 4, 19–21).

DCs are thought to play an important role in driving immunopathogenic responses that lead to the establishment of chronic proliferative synovitis and joint destruction in rheumatoid arthritis (RA) (22–31). Recent studies concerning DC activity in RA have revealed that different stages of DCs and distinct DC subtypes may...
be represented in various synovial microenvironments (30). In inflamed RA synovial tissue, most APCs are fully differentiated DCs expressing high levels of class I and II MHC and T cell costimulatory molecules (30, 31). Because these DCs are clustered around activated T cells, it has been proposed that they are directly involved in the generation of destructive autoimmune responses. Unlike RA synovial tissue, RA synovial fluid (SF) is enriched in DCs expressing a less mature phenotype, similar to that exhibited by CD14<sup>dim</sup> DC precursors (30). When isolated and cultured in vitro with DC growth factors (GM-CSF/IL-4), these SF precursors do not proliferate, but mature into DCs expressing high levels of MHC and T cell costimulatory molecules. It has been speculated that immature DCs present in RA SF DCs are a source for DCs involved in the DC-lymphocyte interactions of synovial tissue and that suppressive factors such as TGFβ modulate DC maturity/function, while DCs are contained in the SF space (29, 30).

Despite recent data indicating that myeloid DCs may be overrepresented in RA, little is known about the mechanisms promoting differentiation along specific DC pathways within distinct joint microenvironments or the association of specific DC subtypes with lymphocyte abnormalities, including abnormal Th1 responses. Moreover, even though mature DCs are likely major contributors to inflammatory responses in the RA joint, the potential contribution of early self-renewing or proliferating DC progenitors to joint pathology has not been described. Conceivably, the existence of progenitors with proliferative and DC-differentiating potential in the joint, along with factors that mediate DC growth and function, would greatly amplify local DC-driven events.

Materials and Methods

Subjects

Twenty-four patients with RA diagnosed according to the 1987 revised criteria of the American College of Rheumatology were studied. All patients were receiving treatment at the time of sample collection. Most (>80%) of the patients were receiving disease-modifying agents such as gold, penicillamine, and methotrexate. A few were being treated with steroids and nonsteroidal antiinflammatory agents. None were receiving TNF antagonist therapy. Eight patients were male; mean age was 67 (± 42–85). SF and peripheral blood (PB) samples were obtained as part of routine clinical care. Cell-free SF and serum were cleared of any precipitate by centrifugation (× 15 g for 15 min at 4°C). Six patients with osteoarthritis (OA) and ten normals (six females) were also included. The study was conducted according to Winthrop University Hospital institutional guidelines.

Enrichment and culture of precursors/progenitors

Because early self-renewing CD34<sup>+</sup> progenitors for specific DC lineages are still not completely well characterized, we studied cell fractions known to be lacking in mature DCs, but enriched in DC precursors/progenitors (32). The strategy we used limited manipulation of the cells, which also diminished the problem of DC maturation initiated by excessive handling (33). Due to strong effects of endotoxins on myeloid cell/DC development and function, all phases of cell handling and culture were performed under low endotoxin conditions. SF or PB were collected into sterile heparinized containers, diluted in RPMI media, and layered on density gradients (lymphoprep) in a Teflon vials at 37°C in a 5% CO2 humidified incubator. Cultures were supplemented with various combinations of cytokines, including cytokines known to sustain myeloid/DC development and to be prevalent in the RA joint (GM-CSF/TNFα/stem cell factor (SCF)/IL-13) (34–37). Although conflicting reports exist related to IL-13 levels in RA (35, 38), we have confirmed by ELISA (Immunotech/Coultier, Hialeah, FL) that IL-13 is abundant in RA SF and serum, compared with NHS, OA SF, and serum. After dose analysis, human rSCF (Genzyme, Boston, MA) was utilized at 50 ng/ml, human rTNF-α (Knoll Pharmaceuticals, Whippany, NJ) at 500 U/ml, human rGM-CSF (Genzyme) at 100 U/ml, human rIL-1 (Genzyme) at 10 ng/ml, human rM-CSF (Genzyme) at 50 ng/ml, and human rIL-4 (Genzyme) at 200 U/ml. To assess nonspecific effects of T/NK cell activation, NWX1 cells were also cultured in NHS/RPMI media containing 300 U/ml human rIL-2 (Hoffman-LaRoche, Nutley, NJ).

Enrichment of neonatal cord blood progenitor cells

Cord blood was collected from healthy full-term infants into sterile heparinized containers during repeat caesarean sections, according to institutional guidelines. MNCs were prepared by density centrifugation on Lymphoprep gradients and placed on NW columns for the isolation of nonadherent cells (32). Further separation of CD34<sup>+</sup> progenitor used positive immunoselection using immunomagnetic beads (Dynabeads; Dynal, Great Neck, NY), as previously detailed (32, 39). Myeloid DC hemopoiesis (both the CD14-derived and CD14-independent pathways) from these progenitors was instituted by adding GM-CSF (100 U/ml)/TNF (500 U/ml)/ stem cell factor (SCF)/IL-4 (final) and human rIL-13 (Peprotech, Rocky Hill, NJ) at 10 ng/ml, human rIL-1 (Genzyme) at 50 ng/ml, and human rGM-CSF (Genzyme) at 50 ng/ml, and human rIL-4 (Genzyme) at 200 U/ml. To assess nonspecific effects of T/NK cell activation, NWX1 cells were also cultured in NHS/RPMI media containing 300 U/ml human rIL-2 (Hoffman-LaRoche, Nutley, NJ).

Myelodendritic progenitor cells

Myelodendritic cells, representing intermediate (CD34<sup>−</sup>CD3<sup>+</sup>CD123<sup>+</sup>DR<sup>−</sup>CD11<sup>+</sup>CD14<sup>+</sup>) progenitors of the CD14-derived DC pathway in the cord blood model (13), were used to test the differentiating effects of RA SF in serum-free and cytokine-free RPMI media. These cells differentiate into either monocytes, granulocytes, or DCs when cultured with M-CSF, G-CSF, or CD14-DC growth factors (GM-CSF/IL-4 or GM-CSF/IL-13 ± SCF, ± TNF), respectively, and were obtained by treating GTS-instituted cord blood cultures on day 3 with 15 μg/ml rabbit polyclonal anti-TNF Ab (Genzyme), as previously described (13). After anti-TNF Ab treatment, cultures were supplemented with fresh NHS/RPMI media without exogenous cytokines on a weekly basis so as to maintain myelodendritic cells in a progenitor state (13). After 10–21 days, myelodendritic cells were removed from culture, centrifuged, and adjusted to 0.4 × 10<sup>5</sup> cells/ml in fresh serum-free RPMI 1640 medium containing 2 mM l-glutamine, 10 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. Myeloid DC cytokines, 10% NHS, 10% RA SF, 10% RA serum, or 10% OA SF were included as indicated.

Proliferation

Proliferation was measured by the uptake of [1<sup>4</sup>H]thymidine and by manual hemacytometer-assisted cell counts (Improved Neubauer). For thymidine uptake, 0.5 μCi [1<sup>4</sup>H]thymidine (sp. act., 25 Ci/mmol; Amersham, Arlington, IL) was added to 100-μl aliquots taken from Teflon cultures and placed into 96-well microtiter plates. After 5 h, cells were harvested using an automated sample harvester and counted in a liquid scintillation counter. Results are expressed as the mean of triplicate samples; the SE was <25% in all experiments.

Immunofluorescence (IF) analysis

Abs to DR, CD13, CD33, and CD34 were obtained from Becton Dickinson in all experiments. Immunofluorescence microscopy analysis was performed in indirect assays, reactivity was detected with either mouse or PE-linked anti-mouse Ig and contained IgG1, IgA, and IgM. Boehringer Mannheim, Indianapolis, IN; Cappel, West Chester, PA; Jackson ImmunoResearch Laboratories, West Grove, PA). Matched nonimmune mouse or rabbit Ig were used as negative controls (Becton Dickinson; Coulter). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) calibrated with Calbrite beads (Becton Dickinson for FITC and PE. The distribution of debris, dead cells, and any contaminating RBCs was assessed on the basis of forward (FSC) and right angle (side) scatter (SSC) before proceeding with the analysis. A total of 5,000–10,000 events was examined using a 488-nm wavelength excitation. Acquired events were
analyzed using CellQuest Software (Becton Dickinson). Results are expressed as percentage of positive cells after subtracting negative control values.

**Mixed leukocyte reaction (MLR)**

Stimulator cells were removed from Teflon cultures, centrifuged in RPMI, adjusted to equal concentrations in 5% NHS/RPMI, and irradiated (60Co source, 2000 rad total). Varying numbers of stimulator cells were added to 96-well microtiter plates containing 5 × 10^4 responder cells/well (NW-enriched T cells obtained from normal PB). Proliferation was measured on days 6–7 by adding 0.5 μCi [3H]thymidine to each well and harvesting the cells, as described above. Control cultures containing irradiated stimulator or responder cells alone yielded <200 cpm.

**T cell activation and measurement of Th1/Th2 responses**

Putative DCs were irradiated as above, combined with allogeneic T cells (RA NW nonadherent cells) at various DC:T cell ratios in 5% NHS/RPMI, and incubated for 6 days in a humidified 5% CO2 incubator (10, 40). Cells were then incubated with anti-CD3 PerCP (Becton Dickinson), permeabilized (FACS-lyzing and permeabilizing solutions; Becton Dickinson), and stained with FITC anti-IFN-γ (Th1-restricted) and PE anti-IL-4 (Th2-restricted) Abs (Becton Dickinson). Isotype control samples received nonimmune mouse IgG1 labeled with PE and FITC instead of anti-cytokine Abs. After staining, cells were fixed in 10% buffered Formalin in PBS and stored in the dark until analyzed. Approximately 15–30,000 cells were collected on the FACScan, as indicated above. Calibrite beads (Becton Dickinson) were used to calibrate the FACSScan for FITC, PE, and PerCP. Region analysis was set according to CD3 reactivity; positive values were determined according to isotype controls.

**TNFR ELISA**

Soluble (s) TNFR I (p55) content was measured using a commercial sandwich ELISA with a sensitivity of 1 pg/ml (Genzyme; Predicta, ELISA human TNFR1). Experiments were performed exactly as recommended. In some instances, repeat determinations were performed with diluted samples. Results were detected using a microplate ELISA reader (Titerek) at 450 nm and are expressed as pg/ml sTNFR.

**Blocking studies**

Neutralization of sTNFR activity in RA SF was performed with TNF (500 U/ml) and mAb anti-human TNFR (p55) (2 μg/ml; Genzyme). Before being added to progenitor cells, RA SF was incubated for 45 min at room temperature with TNF, anti-sTNFR Ab, or control Abs nonimmune mouse IgG1.

**Statistics**

Student's t test or the Mann-Whitney rank sum test was used to analyze data using Sigma Stat software (Jandel Scientific, San Rafael, CA).

**Results**

**Distribution of various stages of myeloid DCs in subsets of RA SF and PB**

A higher proportion of RA SF MNCs than RA PB MNCs expressed CD14^hi^, DR, CD86, and CD33 (Table I, all regions), confirming that relatively mature DCs are increased in RA SF vs RA PB (30, 31). Two subregimes of RA SF and RA PB MNCs, one exhibiting high SSC, the other exhibiting high FSC, were identified (Fig. 1A). The high SSC area was comprised mostly of CD33^CD13^ cells, some DR^CD86^ cells, and few CD14^CD1a^ cells (Table I). The lack of monocyte and DC markers in the high SSC area, together with the known high SSC profile of PMN cells, indicated that the majority of the high SSC area cells were CD33^CD13^ PMN cells. Wright stain analysis confirmed the presence (~20%) of PMN cells in the RA SF MNC subset. While most of cells in the high FSC subregime were also CD33^CD13^, a much larger proportion (>60%) were DCs, as indicated by the expression of CD14 (dim), DR, and CD86.

Cell passage of RA SF MNCs over NW resulted in the enrichment of myeloid precursors/progenitors (Table I) and in the removal of mature cells, including PMN cells and DCs, residing in the high SSC area (Fig. 1A, right plots). The NW nonadherent SF FSC area (Table I) was particularly enriched in a myelodendritic cell-like progenitor compared with the SF SSC MNC area, as revealed by increases in CD113 (3-fold, p = 0.01), decreases in CD14 and CD86 (p < 0.01), and DR, CD13, and CD33 reactivity (13, 41). Neither the RA SF MNCs nor the RA SF nonadherent cells expressed CD34, which is consistent with the lack of CD34 on hemopoietic cells in the RA joint (30). As previously shown by us (26), higher levels of CD33^CD13^ myeloid progenitors were detected in the RA PB nonadherent cells vs normal PB nonadherent cells (data not shown). Thus, a very immature (CD34^+^) myeloid progenitor is present in RA PB, but not RA SF.

As reported (32), passage of normal PB MNCs over NW produced mostly T and NK cells and decreases in CD14-, DR-, CD86-, and CD33-positive cells (p < 0.025, n = 3, data not shown). The high SSC region (containing CD33^CD13^ PMN

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**Table I. The distribution of myeloid/DC-associated Ags on freshly prepared RA SF and RA PB cell subsets**

<table>
<thead>
<tr>
<th>Cell Subset</th>
<th>Region Analyzed</th>
<th>CD14</th>
<th>CD1a</th>
<th>DR</th>
<th>CD86</th>
<th>CD33</th>
<th>CD13</th>
<th>CD115</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA SF MNC</td>
<td>All</td>
<td>23 ± 7</td>
<td></td>
<td>5 ± 0.5</td>
<td>49 ± 5.5</td>
<td>48 ± 19</td>
<td>51 ± 8</td>
<td>52 ± 15</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>↑ SSC</td>
<td>6 ± 4</td>
<td></td>
<td>1 ± 0.6</td>
<td>22 ± 7</td>
<td>16 ± 3.5</td>
<td>77 ± 12</td>
<td>88 ± 11</td>
<td>1.4 ± 1</td>
</tr>
<tr>
<td></td>
<td>↓ FSC</td>
<td>67 ± 11</td>
<td></td>
<td>8 ± 2</td>
<td>92 ± 2</td>
<td>87 ± 2</td>
<td>86 ± 7</td>
<td>82 ± 11</td>
<td>3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>↓ SSC</td>
<td>5 ± 3</td>
<td></td>
<td>4 ± 3</td>
<td>59 ± 11</td>
<td>25 ± 9</td>
<td>10 ± 5</td>
<td>8 ± 7.6</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>RA PB MNC</td>
<td>All</td>
<td>13 ± 3</td>
<td></td>
<td>3 ± 1</td>
<td>20 ± 1</td>
<td>16 ± 2</td>
<td>28 ± 7</td>
<td>27 ± 14</td>
<td>3.5 ± 3</td>
</tr>
<tr>
<td></td>
<td>↑ SSC</td>
<td>9 ± 2</td>
<td></td>
<td>3 ± 1</td>
<td>5 ± 1.5</td>
<td>5 ± 2.5</td>
<td>81 ± 10</td>
<td>78 ± 18</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>↓ FSC</td>
<td>66 ± 8</td>
<td></td>
<td>5 ± 2</td>
<td>73 ± 11</td>
<td>62 ± 12</td>
<td>86 ± 5</td>
<td>80 ± 8.6</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>↓ SSC</td>
<td>2 ± 1.6</td>
<td></td>
<td>0.6 ± 0.4</td>
<td>11 ± 1</td>
<td>9 ± 2</td>
<td>7.5 ± 7</td>
<td>8 ± 7.5</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>RA SF NWX1</td>
<td>All</td>
<td>3 ± 3</td>
<td></td>
<td>4 ± 1</td>
<td>41 ± 6</td>
<td>7.5 ± 23</td>
<td>6 ± 1</td>
<td>4 ± 1</td>
<td>10 ± 5</td>
</tr>
<tr>
<td></td>
<td>↑ FSC</td>
<td>3 ± 3</td>
<td></td>
<td>3 ± 2</td>
<td>67 ± 8</td>
<td>31 ± 8</td>
<td>39 ± 7</td>
<td>33 ± 15</td>
<td>15 ± 2</td>
</tr>
<tr>
<td></td>
<td>↓ FSC</td>
<td>0.04 ± 0.2</td>
<td></td>
<td>0.7 ± 0.6</td>
<td>41 ± 2</td>
<td>1.6 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 1.4</td>
<td>7.3 ± 12</td>
</tr>
<tr>
<td>RA PB NWX1</td>
<td>All (↓ FSC)</td>
<td>0.02 ± 0.02</td>
<td></td>
<td>0.5 ± 1.6</td>
<td>3.5 ± 2</td>
<td>2.6 ± 1</td>
<td>2 ± 0.4</td>
<td>3 ± 1</td>
<td>CD14^-^CD1a^-^DR^-^CD86^-^CD33^-^CD13^-^CD115^-^-</td>
</tr>
<tr>
<td></td>
<td>↓ SSC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* The cell subsets analyzed were: MNC prepared from RA SF (RA SF MNC) and RA PB (RA PB MNC) and progenitor cell-enriched, NW nonadherent, NW nonadherent cell fractions prepared from RA SF MNC (RA SF NWX1) and RA PB MNC (RA PB NWX1). Cell regions were designated according to the light scatter patterns depicted in Fig. 1. All, All events (excluding debris) were analyzed. ↑ SSC, High SSC. ↑ FSC, high FSC. +, >50% of the cells were positive; +, 6–49% of the cells were positive; +, 1–5% of the cells were positive, neg, <1% of the cells were positive. Values represent the mean percentage ± SEM of three or more separate determinations for each marker. The ↑ SSC area was lacking in the RA SF and RA PB NWX1 subsets and the ↓ FSC area was lacking in the RA PB NWX1 subset.

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cells) present in RA SF and RA PB MNCs was not present in normal PB MNCs (data not shown). Unlike RA SF nonadherent cells, normal PB nonadherent cells were not enriched in myeloid-dendritic progenitor cells (<3% were DR, CD13, CD33, or CD115 positive).

Fig. 1B demonstrates that the APC potential of RA SF and PB cell fractions was hierarchical, correlating with DC content. The strongest MLR-stimulatory capacity was displayed by SF MNCs containing the highest levels of DCs. Compared with RA SF MNCs, the MLR generated by SF nonadherent progenitors was weak (p = 0.04), confirming that a large proportion of mature DCs had been removed from the MNCs. Both RA PB MNCs and RA PB nonadherent cells exhibited poor MLR-stimulatory potential. Nonetheless, unfractionated PB MNCs exhibited higher stimulatory capacity than nonadherent (NWX1) cells (Fig. 1B), further demonstrating the efficiency of the separation strategy in removing mature APCs. As expected from the low mature APC content in the samples (<10% and <1% DR”CD86” cells in MNC and non-adherent cells, respectively), freshly prepared normal PB MNC cells produced weak MLRs (data not shown).

**Lineage-specific growth of SF progenitors in response to cytokines**

We evaluated the growth response of RA SF cell subsets to various combinations of hemopoietically active myeloid cytokines. In support of the existence of myeloid progenitor cells in RA SF, non-adherent cells treated with myeloid DC cytokines produced marked proliferation. On day 7 (Fig. 2A), the level of proliferation was higher in the nonadherent progenitor cell-enriched fractions (NWX1) of RA SF than in total MNC fractions with either GTS or GTS/IL-4 treatment (GTS MNC vs NWX1 = 1303 ± 621 vs 2980 ± 2278 cpm, respectively, and GTS/IL-4 MNC vs NWX1 = 3007 ± 1724 vs 4942 ± 2807 cpm, respectively, n = 4–7). Within the progenitor cell group, six of seven of the samples displayed higher levels of proliferation with GTS/IL-4 vs GTS (n = 7, p = 0.023), suggesting a preferential growth response to IL-4. In the remaining sample, no change in growth occurred between treatment groups until later time points. Temporal analysis of cell growth (Fig. 2B) demonstrated that proliferation persisted on days 14 and 18 in GTS/IL-4 cultures, but not in GTS cultures (p = 0.04 for GTS vs GTS/IL-4 on day 18). Hemacytometer-assisted cell counts of progenitor cell cultures revealed that the total number of cells was higher in the GTS/IL-4 cultures than in the GTS cultures, in further support of sustained growth with GTS/IL-4 (data not shown). Because the growth of particular DC progenitors/precursors (CD14-derived DCs) may be TNF independent at certain developmental phases (13), cultures were also established in the absence of TNF. Progenitor cells cultured with GM-CSF/SCF/IL-4 displayed increased proliferation vs GTS (p = 0.04, n = 3), indicating that a growth response may be achieved independently of exogenous TNF (data not shown).

These results indicate that myeloid progenitors with persistent proliferative capacity exist in RA SF. Because GTS/IL-4 is a cytokine combination that yields CD14-derived DCs, the sustained growth response to GTS/IL-4 provides specific evidence that early progenitors for the CD14-derived DC pathway are contained in RA SF. IL-13, which shares many biological properties with IL-4 and which may substitute for IL-4 as a CD14-derived CD14 growth factor (16–18), also promoted proliferation of RA SF progenitors when combined with GTS (data not shown). Treatment of RA SF progenitors with non-DC-restricted myeloid cytokines that yield...
monocytes and monocytes/neutrophils (GM-CSF/M-CSF and GM-CSF/SCF, respectively) (39) produced levels of proliferation that were not increased over GTS (1428 ± 388 cpm for GM-CSF/M-CSF, and 1621 ± 935 cpm for GM-CSF/SCF, n = 3–4, p > 0.05).

An equivalent progenitor cell subset could not be demonstrated in OA SF. As reported (42), we noted that fewer MNCs were present in OA SF than in RA SF. Most of the OA SF MNCs were adherent macrophages, and few nonadherent cells (lymphocytes and precursor/progenitor cells) were present (data not shown). Because of the lack of nonadherent cells in OA SF, cultures could not be established from OA samples for comparison with RA SF.

**Phenotypic and functional features of cultured SF progenitor cells**

On day 0, nonadherent RA SF cells contained few CD14dim cells (3% ± 1), CD1a+ cells (4% ± 1) (Table 1), and CD14+CD1a+ cells (1% ± 0.5), which are transient intermediates of the CD14-derived DC pathway developing from CD14dim cells. Thus, fresh RA SF nonadherent cells were not enriched in late CD14-derived DC precursors. After 8–12 days in IL-4, the proportion of total CD1a+ cells increased (12% ± 3), and transient CD14+CD1a+ cells were noted in some cultures (Fig. 3A, middle plot). Without the addition of CD14-derived DC-restricted cytokines (GTS alone), fewer (6% ± 1) CD1a+ cells were observed (Fig. 3A, right plot). Fig. 3B depicts typical DC clusters developing from RA SF progenitors cultured in myeloid DC cytokines (GTS ± IL-4 or IL-13).

The development of myeloid DCs from progenitors present in RA SF was also demonstrated by increases in the number of cells falling in the light scatter areas corresponding to DCs (Fig. 3C). Treatment of progenitors with GTS/IL-13 (or GTS/IL-4) resulted in the expansion of myeloid DCs residing in the high FSC area (Fig. 3C, right plot). These cultures contained more (>2×, p < 0.02) cells falling in the high FSC DC area than freshly isolated RA SF nonadherent cells (Fig. 3C, middle plot), denoting the extensive potential of the RA SF progenitors to yield DC progeny.

We assessed the MLR- and Th1/Th2-stimulatory capacity of mature DCs developing from the RA SF progenitor cells (Fig. 4). Culture with GTS +/− IL-4 (Fig. 4A) produced progeny with potent T cell-stimulatory capacity in the MLR. In 67% of paired comparisons (six of nine), the T cell response was higher (~1.3-fold, p = 0.04) when cells were cultured with GTS/IL-4 vs GTS.

In the remaining 33% of the samples, no difference in the MLR occurred between treatment groups. IL-13, which is much more abundant than IL-4 in the RA joint (35), produced results similar to IL-4 when combined with GTS (75% of the samples exhibited increased MLRs vs GTS, Fig. 4B). Culture of RA SF progenitors with yet other myeloid cytokines prevalent in the RA joint (IL-1) also produced progeny with MLR-stimulatory potential (Fig. 4C) (GM-CSF/SCF/IL-1 vs GTS, p > 0.05).

The RA SF progenitor cell subsets cultured in NHS alone did not exhibit growth and failed to stimulate T cell responses (data not shown). This indicates that exogenous factors were required for APC development. IL-2, an irrelevant cytokine with no direct effects on myeloid progenitor maturation, produced strong proliferation, but yielded cells devoid of MLR-stimulatory potential (Fig. 4A). Because IL-2 would further activate and expand contaminating T cells present in the progenitor cell-enriched fractions, the lack of stimulatory capacity of the IL-2-treated cultures argues against RA SF T cells acting as APCs in our system.

In Fig. 4D, we compared the ability of fresh and cultured SF progenitor cells to elicit Th1/Th2/Th0 responses during the stimulation of naive T cells in the allogeneic MLR. Fresh RA SF progenitor cells (Fig. 4, middle plot) produced only weak Th1 (IFN-γ), Th2 (IL-4), and Th0 (IFN-γ/IL-4) responses. This was in contrast to fresh RA SF MNCs (Fig. 4, left plot), which preferentially produced strong Th1-type responses (NWX1 vs MNC, p = 0.004). Growth of progenitors with GTS/IL-13 (Fig. 4, right plot) yielded increases in the number of Th1-type cells over day 0 (p = 0.04) and a similar Th1 profile as that promoted by RA SF MNCs on day 0 (Fig. 4D). In all samples studied (n = 5), GTS/IL-13-treated cells lacked Th2-activating potential. Cells cultured in GTS and GTS/IL-4 also produced strong Th1 responses (data not shown). Thus, DC progenitors present in RA SF mature, after in vitro culture with DC cytokines prevalent in the inflamed RA joint (GTS/IL-13), into potent APCs that preferentially activate inflammatory-type Th1 responses over Th2 and Th0 responses.

**RA SF skews the development of CD34+ progenitors toward the CD14-derived DC pathway**

In the remaining sections, we describe the impact of the RA microenvironment on the in vitro growth of DC progenitors. GTS-driven cord blood CD34+ progenitors were utilized as a model for...
DC growth. DC hemopoiesis was instituted from these progenitors with GTS and 10% NHS (39), or with GTS and either 10% RA SF, 10% RA serum, or 10% OA SF. A subgroup of the RA SF samples tested (five of eight) produced higher levels of proliferation around day 7, compared with NHS ($p = 0.013$, Fig. 5A). With three of the eight RA SF samples, proliferation was not increased over NHS ($p > 0.05$). With RA serum, trends toward increased proliferation were noted, but differences were not significant.

DCs obtained from cultures containing GTS/RA SF and GTS/RA serum, but not GTS/OA SF, were more effective than DCs obtained from cultures containing NHS in stimulating an MLR (Fig. 5B, $p = 0.04$). Increases in DR$^+$CD86$^+$ cells occurred with four of five RA SF and five of six RA serum samples tested vs NHS (Fig. 6, $p < 0.05$). With RA serum, trends toward increased proliferation were noted, but differences were not significant.

The capacity of RA SF to promote the CD14-derived DC pathway is linked to increases in sTNFR.

The expansion of the CD14-derived DC pathway promoted by RA SF was analogous to that previously described with an anti-TNF Ab (13). Naturally occurring TNF antagonists are presumed to down-regulate normal TNF-mediated responses. In chronic inflammation, however, it has been suggested that increases in TNF antagonists such as sTNFRs contribute to the disregulation of inflammatory responses via an unknown mechanism (43). We associated high levels of sTNFRs (p55) with the ability of RA SF to enhance the development of the CD14-derived DC pathway from cord blood progenitors. An ELISA (Fig. 7) showed that p55 sTNFRs were significantly ($p = 0.0027$) increased in the RA SF samples ($n = 10$) reported in this work compared with NHS samples ($n = 5$), which is consistent with previous reports (44, 45). Pretreatment of RA SF with either Abs to sTNFRs (p55) or excess TNF ligand inhibited the development of CD14-derived DCs (Fig. 7). Thus, while untreated RA SF increased the number of proliferation was initially lower on day 3 with RA SF and SE vs NHS, subsequent increases in proliferation occurred. B, DCs obtained with RA SF or SE displayed potent MLR-stimulatory capacity. C, Compared with RA SF, the addition of OA SF to GTS-driven cord blood progenitor cultures yielded progeny with markedly decreased MLR-stimulatory capacity. A representative comparison is shown. A, $n = 6–8$; B, $n = 7–8$; C, $n = 3$, for each growth condition.
intermediates of the CD14 pathway, this ability was lost when the binding capacity of sTNFRs in RA SF was blocked. Pretreatment with isotypic Ab controls did not produce these effects, further supporting that sTNFRs were specifically neutralizing TNF bioactivity.

RA SF alone promotes the maturation of intermediate progenitors committed to the CD14 DC pathway

In the experiments depicted in Figs. 5–7, RA SF augmented the development of the CD14-derived DC pathway in cultures that were initially instituted from CD34<sup>+</sup> progenitors with DC cytokines (GTS). Thus, SF appears to enhance the effects of the DC cytokines used. Hemopoiesis from CD34<sup>+</sup> progenitors did not proceed with RA SF alone (except in one instance), suggesting the absence of essential differentiation factors, decreased distribution of preferred target cells, and/or the presence of inhibitory factors. We tested these possibilities by studying the differentiating effects of RA SF alone (i.e., no exogenous cytokines) on intermediate (CD34<sup>negative</sup>) myelodendritic progenitor cells of the CD14 DC pathway (13). Phase microscopy revealed that myelodendritic cells placed in RA SF/RPMI media developed typical branched and veiled DC morphology (Fig. 8B). In contrast, cells placed in NHS/RPMI media (Fig. 8A) remained mostly undifferentiated (as expected) (13), and cells placed in OA SF/RPMI yielded scant progeny (Fig. 8C). Of the few viable cells that had developed in OA SF, most resembled monocytes/macrophages and a minority (<1%) were DCs (Fig. 8C). Dual label IF analysis showed that myelo-
dendritic cells placed in RA SF alone contained an increased number of CD14<sup>+</sup>CD1a<sup>+</sup> intermediates and DR<sup>+</sup>CD86<sup>+</sup> cells, compared with cultures containing NHS alone (<i>p</i> < 0.05, Fig. 9A), which is consistent with the development of CD14-derived DCs (6, 7, 13). DCs generated from myelodendritic cells in the presence of RA SF or RA serum alone stimulated potent MLRs (Fig. 9B, <i>p</i> <

![FIGURE 6.](image)

**FIGURE 6.** The addition of RA SF and serum (SE) to GTS-driven cord blood cultures skewed DC development toward the CD14-derived pathway, as revealed by increases in CD14<sup>+</sup>CD1a<sup>+</sup> cells (day 10). Increases in DR<sup>+</sup>CD86<sup>+</sup> DCs were also noted. A representative comparison for each marker pair is shown (n = 5–11 for each growth condition). Quadrant markers were set according to isotype controls.

![FIGURE 7.](image)

**FIGURE 7.** The ability of RA SF to provoke the growth of CD14-derived DCs is associated with high levels of soluble TNFRs. An ELISA (left panel) revealed that sTNFRs (p55) were significantly increased in RA SF samples (n = 10) vs NHS samples (n = 5). In support for the involvement of sTNFRs, pretreatment of RA SF with TNF or Abs to sTNFRs (p55) inhibited the development of CD14-derived DCs from cord blood progenitors, as revealed by reduced expression of CD14<sup>+</sup>CD1a<sup>+</sup> (n = 2). Pretreatment with isotypic Ab controls did not produce these effects.

![FIGURE 8.](image)

**FIGURE 8.** Phase microscopy revealed that myelodendritic progenitors placed in RPMI media with NHS alone (A) remained mostly undifferentiated after 5 days, whereas those cells placed in RPMI with RA SF alone (B) developed typical branched veiled DC morphology. OA SF alone (C) produced few progeny, which mostly resembled large macrophages. Myelodendritic progenitors represent CD34<sup>negative</sup> intermediates of the CD14 pathway that display the capacity to differentiate into monocytes, granulocytes, or CD14-derived DCs, and were expanded originally from cord blood CD34<sup>+</sup> cultures treated with GTS. Original magnification, ×40.
0.02 vs NHS), whereas cells obtained from myelodendritic cell cultures maintained in OA SF alone produced weak MLRs. Thus, the RA microenvironment is capable of sustaining the in vitro growth of early myeloid progenitor cells committed to DC development.

**Discussion**

In this study, we demonstrate that RA SF contains specific subsets of myeloid progenitor cells, which in the correct microenvironment can differentiate into mature DCs. We also report the presence of growth and differentiation factors in RA SF that support the proliferation and maturation of such progenitors. The existence of myeloid progenitors/precursors with extensive proliferating potential in the inflamed RA joint, although assumed (26, 46, 47), had not been conclusively demonstrated.

We used a comparative strategy using light scatter subregion analysis coupled with cell surface IF analysis to assess the distribution of DCs and DC progenitors/precursors in RA SF cell subsets (Fig. 1A, Table I). A high FSC region in RA SF MNCs contained a large proportion of CD33<sup>+</sup>CD13<sup>+</sup>DR<sup>+</sup>CD86<sup>+</sup>CD14<sup>+</sup>CD1a<sup>+</sup> cells, which is consistent with a myeloid DC (immature and mature) phenotype (30, 41). Passage of SF MNCs over NW produced a nonadherent cell subset that lacked mature myeloid DCs (DR<sup>−</sup>CD86<sup>−</sup> cells) and myeloid DC precursors, and that was enriched in myeloid progenitors (CD13<sup>−</sup>CD33<sup>−</sup>DR<sup>−</sup>CD86<sup>−</sup>CD11<sup>−</sup>CD14<sup>−</sup>/CD1a<sup>−</sup> cells).

The degree of MLR activity generated by fresh RA SF and PB cell subsets correlated with mature DC content (Fig. 1, Table I). RA SF MNCs contained higher levels of mature DCs than RA PB MNCs and were potent stimulators of an MLR, which is in agreement with prior studies (30, 31). In addition to stimulating a potent MLR, RA SF MNCs stimulated Th1-inflammatory responses over Th2/Th0 responses (Fig. 4D). In contrast, RA PB MNCs were weak stimulators of either Th1/Th2 or Th0 responses (data not shown). The polarization toward Th1 responses induced by freshly isolated RA SF DCs, but not by RA PB cells, provides new evidence that DCs are instructed within the joint to acquire functions associated with the selective activation of inflammatory T cells.

The fresh RA SF progenitor cells displayed poor MLR- and Th1/Th2-stimulatory potential. A proliferative response, potent MLR- and Th1-stimulatory capacity, and large increases in the number of cells residing in the high FSC DC areas were produced only after in vitro culture of these cells with myeloid DC growth factors (Figs. 2–4). The ability of RA SF progenitor cell-derived DCs obtained with DC growth factors to selectively promote Th1-type responses resembled that of mature DCs present in fresh RA SF MNCs (Fig. 4D). Because inflammatory-type Th1 responses have been associated with immunopathology in RA (48–50), it is conceivable that mature myeloid DCs arising from RA SF progenitors in situ would activate pathogenic Th1 responses (Fig. 10). Besides driving Th1-type responses, myeloid DCs might mediate other immune responses within the RA joint, such as Ig (rheumatoid factor) synthesis by B cells and the production of IFN-γ by CD8<sup>+</sup> T cells (6, 7, 51).

Our data substantiate that diverse developmental stages of myeloid DCs are represented in RA SF. These include early (CD34<sup>−</sup>CD33<sup>−</sup>CD13<sup>−</sup>DR<sup>−</sup>CD11<sup>−</sup>/−) myeloid progenitors with proliferative capacity and DC-differentiating potential, intermediate (CD33<sup>−</sup>DR<sup>−</sup>CD14<sup>dim</sup>/CD1a<sup>+</sup>/−) precursors committed to mono/DC development but lacking proliferative capacity, and late stage (CD14<sup>−</sup>CD1a<sup>−</sup>/− DR<sup>high</sup>CD86<sup>high</sup>) DCs exhibiting potent MLR- and Th1-stimulatory capacity. The emergence of CD14<sup>+</sup>CD1a<sup>+</sup> cells restricted to the CD14-derived DC pathway from RA SF progenitors after culture with CD14-derived DC growth factors (IL-4 or IL-13) indicates the existence of progenitors for CD14-derived DCs in RA SF. The low numbers of CD14<sup>dim</sup> cells present initially, the sustained proliferation exhibited by the cultures in the presence of IL-4/IL-13, and the transient nature of CD14<sup>+</sup>CD1a<sup>+</sup> cells in culture (6, 7, 13) imply that new CD14<sup>+</sup>CD1a<sup>+</sup> cells developed from CD33<sup>−</sup>CD13<sup>−</sup>DR<sup>−</sup>CD14<sup>−</sup>/CD1a<sup>−</sup>/CD11<sup>−</sup> progenitors contained in RA SF.

A reservoir of myeloid DC progenitors in RA SF could provide a pool of mature DCs exhibiting T cell-activating potential, and could also be a plentiful source of immature DCs in the joint. In the context of the RA-inflammatory environment, the ability of immature DCs to acquire Ag from autologous apoptotic and/or necrotic cells for subsequent cross-priming of T cells might be favored (Fig. 10) (52–54). Such events have been linked to the autoimmune process in lupus erythematosus and autoimmune diabetes (52–54) and could perpetuate autoimmune responses, even in the absence of the original instigating Ag. In theory, the process of acquiring Ags from apoptotic/necrotic cells in the RA SF space (55) by immature DCs would be facilitated by CD36, α, β<sub>1</sub>, and α, β<sub>2</sub> (53, 56), and would prompt maturation. Further maturation would be favored by an excess of myeloid DC growth factors and...
IL-1 found in RA SF (35, 36, 56). Since immature DCs are incapable of Ag presentation and might directly activate regulatory T cells that suppress self-reactivity (11, 12), we propose that the intraarticular regulation of DC maturation is a critical control point in the RA disease process.

RA SF facilitated the maturation of DCs from myeloid progenitors, providing direct evidence that the inflamed RA environment instructs DC differentiation (Figs. 5–9). While the addition of RA SF to GTS-driven CD34+ progenitors enhanced the effects of the cytokines used (Figs. 5–7), RA SF alone (in the absence of exogenous growth factors) did not generally induce DC growth from CD34+ progenitors. In contrast, RA SF directly promoted the differentiation of myeloid DCs from CD34−CD14+CD33+ progenitors (intermediate myelodendritic cells, Figs. 8–9), indicating that the factors present in SF were targeting specific progenitors/precursors that differentiate immediately upon entry into the inflamed joint (Fig. 10). Because GM-CSF, TNF, IL-13, and SCF are abundant in the RA joint (34–37), they most likely facilitate the local growth of myeloid DCs, especially CD14-derived DCs. IL-4, which has been consistently shown to be present at low levels in the RA joint (36), is unlikely to be a major participant in this process. Mature DCs derived from progenitor cells in vitro with myeloid DC growth factors and IFN-γ exhibit a greater capacity to activate IFN-γ-producing T cells (Th1-type cells) than cells cultured with myeloid DC growth factors alone (57). Thus, IFN-γ-existing in RA SF (58, 59) may also skew DC development and function toward a Th1 response. Other factors known to promote CD14-DC growth and that are prevalent in RA SF, such as hyaluronan and fibronectin (1, 2, 60, 61), may contribute to the formation of a Th1 response as well.

The role of TNF and TNF antagonists in the regulation of myeloid DC hemopoiesis is complex and varies with stages of DC development (5). While TNF is required to ensure DC hemopoiesis from CD34+ progenitors, the addition of polyclonal anti-TNF Ab to GTS-treated progenitor cells after 3 days of culture produces amplification of the CD14-derived pathway (13). In the present study, we noted that the effects of RA serum/SF on the myeloid DC pathway were analogous to those achieved with polyclonal anti-TNF Ab. That is, RA serum/SF altered DC developmental responses to yield increases in CD14+CD1a+ intermediates and mature CD14-derived DCs with strong MLR potential. The capacity of RA SF to induce the development of CD14-derived DCs correlated with high levels of sTNFRs (p55) (Fig. 7), which share with anti-TNF Abs the ability to bind TNF and inhibit TNF activity. Thus, within the RA joint, it is possible that sTNFRs (p55) also regulate the growth of CD14-derived DCs. This suggests that...
TNF blockade, including therapeutic TNF inhibition, may have important suppressive and stimulatory effects on DC activity in RA.

Notwithstanding the positive effects of the RA extracellular environment on DC development, factors present in RA SF such as TGFβ may negatively regulate DC-mediated functions, both at the level of immature DCs (late DC precursors) and during DC-T cell interactions (29, 30). We noted that the substitution of TGFβ for IL-13 or IL-4 (GM-CSF/SF/TGFβ) in RA SF progenitor cell cultures appeared to interfere with DC maturation and yielded cells with a diminished potential to induce Th1 responses (~40% decreases vs IL-13 and IL-4; data not shown). Because TGFβ is the preferred growth factor for CD14-independent DCs (Langerhans cell type), we cannot discount the possibility that the reduced Th1 response was related to a lack of TGFβ-responsive progenitor/precuror cells.

The inability to obtain nonadherent progenitor cells from OA SF is consistent with differences in the distribution of MNC types in OA SF vs RA SF (42). Reduced levels of sTNFRs (p55) and proinflammatory cytokines (TNF, IL-1, IL-13) in OA SF vs RA SF (44, 45) further imply that OA SF lacks factors that would facilitate the maturation of myeloid (CD14+) DCs. Our findings do not exclude the possibility that other DC subtypes with distinct effector functions are amplified in RA.

In summary, we provide new insight into how selection of specific DC progenitor cells by the RA synovial microenvironment may play a central role in the RA disease process (Fig. 10). The final outcome of DC-driven events in the various joint microenvironments may ultimately be determined by the proportion of factors promoting and inhibiting the maturation and function of particular members of the DC lineage system, and by the nature of factors regulating the trafficking of DCs into the synovial space. By understanding the precise mechanisms regulating these events, it may ultimately be possible to provide some basis for the heterogeneity of the immune response in RA and to devise highly directed therapeutic strategies aimed at either blocking and/or skewing the differentiation and/or functions of particular DC subtypes.

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