Minimal Recruitment and Activation of Dendritic Cells within Renal Cell Carcinoma

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ABSTRACT

Dendritic cells (DCs) are predicted to participate in natural tumor immunity by migrating into tumors, where they acquire antigen, undergo activation, and migrate to lymph nodes to initiate a T-lymphocyte response against tumor-associated antigens. The presence of DCs using defined lineage markers and their function in human tumors has not been assessed previously. The monoclonal antibodies against CMRF-44 and CD83, which are differentiation/activation antigens on DCs, were used in immunohistological and flow cytometry studies to analyze the DC subtypes infiltrating 14 cases of human renal cell carcinoma (RCC). The functional immunocompetence of the DCs isolated from RCC was assessed by testing their ability to stimulate an allogeneic mixed leukocyte reaction. The majority of leukocytes present within the RCC were macrophages (62% ± 14.7) or T lymphocytes (19% ± 9.5), with CD45+ HLA-DR+ lineage-negative putative DCs accounting for less than 10% of the leukocytes present. Of these, a monoclonal antibody against CMRF-44 or CD83+ DC phenotype. Activated CMRF-44+ and CD83+ DCs were more evident outside the tumor in association with T-lymphocyte clusters. The number of CMRF-44+ DCs correlated closely with the number of S-100-positive DCs. Isolation of DCs from eight RCCs was achieved, and flow cytometry studies confirmed the small proportion of activated CMRF-44+ DCs. The CMRF-44+ DCs stimulated an allogeneic mixed leukocyte reaction, but the CMRF-44- DCs (normal tissue DC precursors and other cells) failed to do so. These results suggest that RCCs recruit few DCs into the tumor substance, and the tumor environment fails to initiate the expected protective activation of DCs. These two mechanisms, amongst others, may contribute to tumor escape from immunosurveillance. In vitro loading of DCs with tumor-associated antigens may be a useful therapeutic maneuver.

INTRODUCTION

DCs are specialist antigen-presenting cells that are found in the interstitium of tissues and within epithelial surfaces (1, 2). If DCs are to play a part in natural tumor immunity, DCs would be expected to migrate into tumors, derive antigen, undergo activation, and migrate to central lymphoid tissue to initiate any T-lymphocyte response against TAAs. The failure of DCs to migrate from the blood into malignant tissue or to be activated and migrate out into draining lymph nodes represents a potential tumor escape mechanism. Little is known about the migration of DCs into human tumors, their state of activation and their interactions, if any, with responding T lymphocytes. The immunohistological studies performed to date using S-100 staining have shown that an increased number of DCs located within tumors correlate with a better prognosis in colorectal adenocarcinoma (3); adenocarcinoma of the lung (4, 5); gastric (6), esophageal (7), and nasopharyngeal (8) carcinomas; and papillary carcinoma of the thyroid (9). These studies have not been correlated with more detailed immunohistological analyses using mAbs. Furthermore, the widespread interest in using blood DCs as “nature’s adjuvant” for tumor immunotherapy (10) makes an understanding of how DCs function within tumors vital for evolving strategies to use DCs in effective tumor vaccines.

RCC is the most common malignancy of the kidney and accounts for approximately 2–3% of all adult neoplasms (11). The observation that large numbers of lymphocytes are often found in RCC has encouraged investigators to develop immuno-therapeutic treatments but with limited success (12, 13). The presence or absence of DCs in RCC is unknown, despite the fact that interstitial DCs were first defined in normal kidney some time ago (14, 15). A recent study (16) described infiltration of S-100-positive cells into RCC but not into benign adenomas. Difficulties with the specificity of S-100 expression and the solubility of S-100 has limited its use for characterizing DCs further.

DCs have been identified in blood (17) and normal tissues (14, 15) by demonstrating their high density expression of MHC class II antigens and their lack of other mature hematopoietic lineage markers (lin−) using immunocytological techniques. The recent availability of the relatively more DC-specific, mAbs

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3 The abbreviations used are: DC, dendritic cell; TAA, tumor-associated antigen; mAb, monoclonal antibody; RCC, renal cell carcinoma; NK, natural killer; HLA, human leukocyte antigen; FACS, fluorescence-activated cell sorter; MLR, mixed leukocyte reaction.
CMRF-44 (18) and HB15a (19), combined with double-labeling techniques, allows more accurate identification of DCs, as well as providing information on their state of differentiation/activation. The CMRF-44 antibody recognizes an early activation antigen, which is expressed on blood DCs, only after a short period of culture (18, 20). HB15a detects the CD83 antigen, a member of the immunoglobulin superfamily with an as yet unknown function (21), which is expressed by activated DCs and monococyte-derived DCs generated in culture with cytokines (Mo-DC; Ref. 22).

In this study on DCs in RCC, we identified the CD45^+/HLA-DR^-/lin^ DC population in RCC and adjacent normal kidney, and used the CMRF-44 and HB15a mAbs to define the activated DC subsets. Expression of the CD1a antigen (23), a marker of the Langerhans cell subset of DCs as well as the up-regulation of the critical costimulator molecules, CD80 and CD86 (24), were also assessed. These results and the functional data on the DCs isolated from RCC suggest that deficient DC activation and migration may contribute to RCC escape from immune surveillance.

MATERIALS AND METHODS

Tissue Samples. Fresh tumor tissue was collected, following informed consent, from 14 radical nephrectomy specimens, all removed for RCC. All patients were well at the time of surgery with no evidence of metastases, and only one case (2) had invasion of the resection margin of Gerota's fascia. There were nine clear cell tumors, three papillary tumors, and two granular tumors. After initial assessment by a histopathologist, samples were processed for routine histopathology. Samples for immunohistochemistry were mounted in OCT compound (Tissue Tek), snap-frozen in liquid nitrogen, and stored at -80°C until required for sectioning. Additional fresh tumor tissue was processed immediately when available for further studies on the isolated cell populations. For S-100 staining, tissues obtained from the same specimens were embedded in paraffin.

mAbs. The anti-CD3 (OKT3, IgG2a), anti-CD19 (FMC63, IgG2a), and anti-HLA-DR (L243, IgG2a) antibodies were purchased from Becton Dickinson (Australia). Phycoerythrin-conjugated CD14, CD19, and anti-HLA-DR (L243, IgG2a) antibodies were purchased from Becton Dickinson (Australia), and the anti-CD80 (IgG1) mAb was purchased from Prof. I. F. C. McKenzie (Melbourne, Australia), and the Na1/34 mAb (CD1a, IgG2a) was purchased from Becton Dickinson (Australia).

Immunoperoxidase (Single-Label) Staining. Cryostat-cut sections (~20°C; 4–6 μm) were placed on gelatin-coated slides and air-dried overnight, prior to fixing for 10 min in cold acetone (4°C). Sections were preblocked with 10% human AB serum for 30 min, followed by the application of the primary mAb for 30 min at room temperature. Sections were then washed three times with PBS and then incubated with peroxidase-conjugated goat anti-mouse IgG (Dako) for an additional 30 min. After washing twice in PBS and once in Tris/HCl buffer (pH 7.6), the sections were developed with 0.1% 3,3′-diaminobenzidine for 3–10 min before counterstaining with hematoxylin.

S-100 staining was performed on sections cut from formalin-fixed, paraffin-embedded tissues. These were dewaxed and rehydrated before staining as for fresh tissue, except that rabbit anti-S-100 and peroxidase-conjugated goat anti-rabbit IgG (Bio-source International, CA) were used.

Immunoperoxidase/Alkaline Phosphatase Staining (Double-Label) Staining. Sections were treated as for single staining up to the first primary mAb incubation. Following this, sections were incubated with biotinylated goat anti-mouse IgG (Dako) diluted 1:200 for 30 min, washed three times in PBS, and then incubated for 30 min in Extravidin® alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) at 1:200 dilution. After washing two times in PBS and once in Tris/HCl buffer (pH 7.6), the sections were developed with Fast Blue (Sigma) for 5–10 min. Sections were then washed with PBS before a 30-min incubation in 10% mouse serum. Incubation with the second mAb was carried out as for single immunoperoxidase staining, including developing the color reaction with 3,3′-diaminobenzidine.

Immunohistological Assessment. T lymphocytes (CD3), B lymphocytes (CD19), NK cells (CD16), and monocyte/macrophages (CD14 and CD11b) were identified and counted as cells when identifiable nuclei were present. Potential DCs were identified as CD45^-, CD1a^-, lin^- (CD3, CD19, CD16, CD14, and CD11b^) using double-labeling and further confirmed to be HLA-DR positive. CMRF-44^ DC and CD1a^ LC were identified by double-labeling with CD14 to exclude macrophages, which may also express these antigens. CMRF-44^- and CD83^- DCs were considered on the basis of data published previously (18, 19) to be partially activated.

Sections were examined at ×400 for cell types present in large numbers and at ×100 for cell types present in low numbers through an eyepiece graticule, giving a field of view of 0.044 and 0.772 mm², respectively. All counts were converted to number per mm² for comparison. Positively stained cells were counted in 5–10 random, nonoverlapping fields of tumor tissue, excluding areas containing dense stroma or large lymphoid aggregates. Cell numbers are expressed as a percentage of the total leukocyte count (mean ± SD), as determined by CD45 staining. Separate counts were made within the area of lymphoid aggregates when present.

Isolation of Tumor Containing Leukocyte Populations. Tissues were minced into small pieces in sterile 10% FCS/RPMI media (RPMI 1640 supplemented with 100 units/ml penicillin, 1 mm glutamine, and 100 units/ml streptomycin) containing collagenase (0.5 mg/ml) and DNase (1.0 μg/ml) and incubated at 37°C for 1–2 h with gentle agitation. The resulting cell suspension was washed in PBS twice before filtering through a
Cell counts are expressed as the number of cells per mm² and are also expressed as a percentage of total CD45 leukocytes (boldface). S-100 counts were not converted to percentages, because these were obtained from different samples of the same tumor. Counts were averaged over 5 fields for CD45 and CD14 and 10 fields for CMRF-44 and CD83. Individual T-lymphocyte and NK cell counts have been omitted for clarity (they represented 8–40% of leukocytes; see Fig. 3A).

<table>
<thead>
<tr>
<th>Histology(*)</th>
<th>CC</th>
<th>CC</th>
<th>CC</th>
<th>P</th>
<th>CC</th>
<th>CC</th>
<th>CC</th>
<th>CC</th>
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<th>P</th>
<th>CC</th>
<th>P</th>
<th>G</th>
<th>G</th>
<th>Mean ± SD</th>
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<tr>
<td>Stage (TNM)(**)</td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>T4</td>
<td>T5</td>
<td>T6</td>
<td>T7</td>
<td>T8</td>
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<td>T16</td>
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<tr>
<td>Total lin(a)</td>
<td>23</td>
<td>21</td>
<td>20</td>
<td>16</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>17</td>
<td>8</td>
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<td>16</td>
<td>7</td>
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<tr>
<td>Total leukocytes</td>
<td>531</td>
<td>603</td>
<td>349</td>
<td>413</td>
<td>363</td>
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<td>619</td>
<td>513</td>
<td>925</td>
<td>308</td>
<td>354</td>
<td>581 ± 239</td>
<td></td>
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\*CC, clear cell; P, papillary clear cell; G, granular clear cell.
\(**)Cystic variety of clear cell.
\(a\)TNM, Tumor-Node-Metastasis classification.
\(b\)NA, not available.

Flow Cytometry Analysis and Sorting. Mononuclear cells were labeled with primary mAb for 30 min at 4°C. Following one wash in PBS, labeled cells were detected by incubation with FITC-conjugated sheep anti-mouse IgG (Silenus Laboratories, Australia) for 30 min at 4°C. Following blocking for 10 min in 10% mouse serum, cells were double-labeled with phycoerythrin-conjugated CD14 mAb. Appropriate gating and flow cytometry analysis were performed using Lysis software on a FACS (FACS Vantage; Becton Dickinson). Contaminating flow cytometry analysis were performed using Lysis software.

RESULTS

Immunohistological Analysis of the Leukocytes Infiltrating RCC

Identification of Leukocyte Types within Cryostat Sections. A summary of the results of the immunostaining of the 14 tumors analyzed are shown in Tables 1 and 2 and Figs. 1 and 2. The majority of the leukocytes infiltrating the RCCs were either CD14\(^+\) macrophages or CD3\(^+\) T lymphocytes, accounting for 62% ± 14.7 and 19% ± 9.5, respectively, of the leukocytes present. NK cells (CD16\(^+\) lymphoid cells) were present in small numbers (11% ± 8.1), whereas B lymphocytes were virtually never detected. Total CD45\(^-\)/lin\(^-\) cells, which include the DCs, comprised 13.1% ± 4.1 of the total leukocyte population in RCCs. These numbers were not significantly different from the proportion of CD45\(^-\)/lin\(^-\) leukocytes seen in normal kidney (12.7% ± 3.0). Morphologically, at least 25% of these were large cells with dendritic processes, suggesting a degree of activation (Fig. 1A). When only these cells were counted, the potential DCs were found to comprise 3.4% ± 0.9 in RCCs and 4.4% ± 2.1 in normal kidney. Double-labeling to identify lin\(^-\) and HLA-DR\(^+\) cells, although difficult to analyze accurately due to HLA-DR expression on endothelium and the tumor cells, gave cell counts similar to the CD45\(^-\)/lin\(^-\) counts, with a minimum estimate of 50% of these cells having a CD45\(^-\)/HLA-DR\(^-/\)lin\(^-\) phenotype. CD1a\(^-\)/CD14\(^+\) LC ac-

70 μm nylon cell strainer (Falcon; Becton Dickinson, Australia) to remove undigested material. This single-cell suspension was layered over a Ficoll-Hypaque density gradient (density, 1.077; Pharmacia, Uppsala, Sweden) and centrifuged at 400 × g for 20 min to obtain mononuclear cells. This step excluded granulocytes but retained some tumor cells (identified as CD45\(^-\) cells). Cell viability was always >80%, as confirmed by trypan blue exclusion.

Flow Cytometry Analysis and Sorting. Mononuclear cells were labeled with primary mAb for 30 min at 4°C. Following one wash in PBS, labeled cells were detected by incubation with FITC-conjugated sheep anti-mouse IgG (Silenus Laboratories, Australia) for 30 min at 4°C. Following blocking for 10 min in 10% mouse serum, cells were double-labeled with phycoerythrin-conjugated CD14 mAb. Appropriate gating and flow cytometry analysis were performed using Lysis software on a FACS (FACS Vantage; Becton Dickinson). Contaminating CMRF-44\(^+\)-activated B lymphocytes were not considered, because B lymphocytes were not present in the mononuclear cell populations. For analysis of DC phenotype, mononuclear cells were labeled with a mix of lineage markers (CD3\(^+\), CD11b, CD14, CD16, and CD19), and the lin\(^-\) population was sorted and then reanalyzed for the expression of DC activation antigens.

For functional studies, the mononuclear cell suspension was sorted after labeling, as described in the results. Single labeling was used for three experiments. In an additional five double-labeling studies, the four different populations consisting of CMRF-44\(^+\)/CD14\(^+\) (activated DCs), CMRF-44\(^+\)/CD14\(^+\) (activated monocytes), CMRF-44\(^-\)/CD14\(^+\) (resting monocytes), and CMRF-44\(^-\)/CD14\(^+\) (other cells, including resting DCs) were isolated.

MLR. Tumor cell suspension and the sorted leukocyte populations were cocultured with 1 × 10\(^5\) allogeneic normal peripheral blood T lymphocytes. Cultures were maintained at 37°C and 5% CO\(_2\) in 96-microwell, round-bottomed plates for 6 days. Wells were pulsed with \([\text{H}]\)thymidine (5 Ci/mmol; Amersham) in the last 16–18 h of culture. \([\text{H}]\)Thymidine incorporation was measured on a liquid scintillation analyzer to give a direct readout of T-lymphocyte proliferation. Responses were reported as the mean value ± SE of triplicate wells.
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Table 2  DC numbers in T-lymphocyte aggregates at tumor margins

<table>
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<th>Case number</th>
<th>DC phenotype</th>
<th>2</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>14</th>
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<tr>
<td>CMRF-44+ CD14-</td>
<td>136</td>
<td>556</td>
<td>447</td>
<td>415</td>
<td>513</td>
<td>386</td>
<td>284</td>
<td>358</td>
<td>449</td>
<td></td>
</tr>
<tr>
<td>CD83+ CD14-</td>
<td>113</td>
<td>426</td>
<td>295</td>
<td>243</td>
<td>333</td>
<td>147</td>
<td>159</td>
<td>352</td>
<td>277</td>
<td></td>
</tr>
<tr>
<td>S-100</td>
<td>NA*</td>
<td>NA</td>
<td>NA</td>
<td>95</td>
<td>177</td>
<td>150</td>
<td>132</td>
<td>95</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

* NA, not available.

The results for the S-100 antigen staining in paraffin sections cannot be compared directly with the frozen sections because the former are necessarily cut from a slightly different area of the tumor. Nonetheless, staining for S-100 antigen identified only small numbers of positive cells in 9 of 10 cases (Fig. 1D; Table 1). They were present in greater numbers where T lymphocytes had accumulated. In over one-half the cases, weak S-100 staining of tumor cells was noted.

Identification of S-100 Cells in Paraffin Sections. The results for the S-100 antigen staining in paraffin sections cannot be compared directly with the frozen sections because the former are necessarily cut from a slightly different area of the tumor. Nonetheless, staining for S-100 antigen identified only small numbers of positive cells in 9 of 10 cases (Fig. 1D; Table 1). They were present in greater numbers where T lymphocytes had accumulated. In over one-half the cases, weak S-100 staining of tumor cells was noted.

Identification of Activated DCs. CD14- leukocytes, which expressed the CMRF-44 antigen, i.e., activated DCs, accounted for only 0.15% ± 0.13 of the total leukocytes infiltrating the tumors. The CD83 antigen, which is induced after the CMRF-44 antigen (20) and may undergo some subsequent down-regulation (26), was expressed by a similar number (0.23% ± 0.16) of tumor leukocytes (Fig. 1C). These data suggested that only a small proportion (5-10%) of the lin- / HLA-DR+ putative DCs identified within the RCCs are activated.

The number of activated DCs in the adjacent normal kidney was much less, with only 0.04% ± 0.04 CMRF-44+/CD14- cells and 0.04% ± 0.04 CD83+ cells present. No specific localization of DCs was found within the tumors with respect to necrotic or inflammatory areas.

Staining for the p55 antigen (25) was also performed, but accurate assessment was difficult because of tumor cell staining in close to one-half of the cases. In those cases that could be assessed, the number of p55-positive cells was similar to the number of CMRF-44- and CD83-staining cells.

The expression of the CD80 and CD86 costimulator molecules was examined. No CD80+ cells were detected in either normal or RCC tissue. Small numbers of CD86+ cells were present in many of the cases, but when double staining with CD14 was performed to exclude CD86+ macrophages, only one case was found to have CD86+/CD14- cells present.

Although the number of activated DCs within the RCCs was limited, CMRF-44+ or CD83+ DCs were more evident just outside the tumor in the pseudocapsule. Here, relatively large numbers of activated DCs were seen in the T-lymphocyte clusters that develop. In nine tumors, in which sufficiently well-defined T-cell aggregates had formed, it was possible to count the number of DCs in the clusters (Fig. 1, E and F; Table 2). Perhaps consistent with the earlier induction of the CMRF-44 antigen on blood DCs in vitro (20), more CMRF-44+/CD14- cells than CD83+/CD14- cells were present in the lymphoid areas.

FACS Analysis of Leukocytes in Single-Cell Tumor Suspensions

Flow cytometric analyses on the mononuclear cell suspensions isolated from digested tumor tissue were performed on eight tumors (Table 3). Side scatter and forward scatter were used to define typical "lymphoid" and "monocytic-macrophage" electronic gates. The lymphoid gate contained >95% CD45+ leukocytes and consisted predominantly of CD3- cells. The monocytic-macrophage gate, containing >85% CD45+ leukocytes, was analyzed more extensively to identify potential DC populations. CD14+ cells accounted for over 50% of the monocytic-macrophage gate. A variable number of these macrophages expressed the CMRF-44 antigen and accounted for the bulk of the CMRF-44+ cells within the tumors (Fig. 3A). Activated CMRF-44+/CD14- DC comprised only 1.5% ± 1.02 of the total macrophage-gated cell population. The contaminating CD45- tumor cells did not express the CMRF-44 or CD14 antigens. The remaining population of CD14-/CMRF-44- cells was presumed to contain unactivated DCs, contaminating tumor cells, and occasional CD14- lymphoid cells. Overall, all leukocyte populations occurred in similar proportions to that observed in direct tissue staining.

To facilitate a more direct comparison of the flow cytometry data with the immunohistological results, lin- cells were sorted, and the CD45+ cells were analyzed in five cases (Fig. 3B). These represented 8.1% ± 2.4 of the total leukocytes (lymphoid and monocyte-macrophage gates). Approximately one-half these cells were HLA-DR-, i.e., approaching the proportion of total DCs identified by tissue staining. The CMRF-44 antigen was expressed on only a subpopulation of lin- cells (15.6% ± 9.0), i.e., ~1% of total leukocytes, a figure that relates closely to the low percentage of CMRF-44+ DCs estimated by direct staining. The CD83 antigen was not detected by this method, possibly because of its sensitivity to proteases.

Functional Studies

To examine the functional capacity of these tumor leukocyte populations as antigen-presenting cells, various cell populations were tested as stimulators in an allogeneic MLR. Limited cells were available in each case; therefore, only certain sorted...
Fig. 1 Immunohistological analysis identifies a CD45+/HLA-DR+/lin− population of putative DCs, a subset of which express the CMRF-44 CD83 differentiation/activation antigens within RCC. A, a putative DC (arrow) stained for CD45 (golden brown) but not CD3, CD11b, CD14, CD16, or CD19 (lin−, blue black) compared with the CD14-positive macrophage (arrowhead, ×400). B, single CMRF-44+/CD14− DC (arrow) surrounded by many CD14+ macrophages (blue, ×400). C, CD83+ DCs were seen among RCC cells (single-label immunoperoxidase). D, S-100+ DC (white arrow) in RCC (single-label immunoperoxidase). Note weak S-100+ tumor cells. DCs are found in lymphoid aggregates at the margins of RCCs. E, CMRF-44+/CD14− DC (yellow) and CD14+ macrophages (blue) are identified (no counterstain). F, CD83+ DCs are seen among the lymphocytes (counterstained) in a lymphoid aggregate.

populations could be tested. The first two analyses established that the RCC leukocyte population was allostimulatory (data not shown) and that the lin− population contained the allostimulatory cells (data not shown). An additional case was analyzed after sorting the tumor mononuclear cell suspension into CMRF-44+ and CMRF-44− populations. The CMRF-44+ population was clearly the most effective at stimulating the allo-MLR (Fig. 4A). Five additional cases were sorted using double-labeling to identify the CMRF-44+/CD14− DCs. The CMRF-44+/CD14− DCs were found to be at least three to five times more potent stimulators of the allogeneic T-lymphocyte response than the CD14+ macrophages (Fig. 4B). The CMRF-44− cells, i.e., the population that included the presumed resting or unactivated tumor-associated DCs (and some contaminating tumor cells), failed to stimulate T lymphocytes in all five cases tested. Blood DCs, sorted from two additional patients, were found to fully stimulate T lymphocytes in an allogeneic MLR (Fig. 4D).
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DISCUSSION

These studies suggest that there is minimal recruitment and activation of DCs within RCCs and that the CMRF-44− DCs present may not function normally. To date, no marker for resting human DCs, or less differentiated members of the DC lineage, has been discovered; therefore, unactivated putative DCs were defined as CD45+ cells lacking mature lineage-specific markers and the activated DC subset defined by CMRF-44 and CD83 staining. This is the first study to use these techniques to describe the DC response in human cancer. Relatively few putative DCs compared with macrophages were present in the tumors, and most of these were present in a resting state with a normal tissue DC phenotype, i.e., CMRF-44+/CD83−. Only a minority of the DCs showed signs of activation, phenotyped as CMRF-44+/CD83+. Conversely, the majority of DCs located in the T-cell aggregates within the normal tissue surrounding the tumor had an activated CMRF-44+/CD83+ phenotype consistent with that documented for interdigitating DCs in normal lymph nodes (18, 19).

Tumors are known to produce a modified inflammatory response, and it was expected that the majority of leukocytes present would be either macrophages or T lymphocytes. We established that 13.1% ± 4.1 of the leukocytes present did not express the lineage markers of macrophages, T lymphocytes, B lymphocytes, or NK cells and have a phenotype consistent with DCs. Admittedly, not all of these lin− cells will be DCs, particularly the proportion with lymphoid features, and these may represent cells similar to the lin− lymphoid cells noted in blood DC preparations (20). Nonetheless, the CD45+/HLA-DR+ lin− phenotype of the majority of these cells and the fact that ~25% of them had a dendritic morphology raise the probability that a significant proportion are DCs. When morphology was taken into consideration and only large 45+/lin− cells were counted, these cells were found to constitute 3.4% ± 0.9 of total leukocyte numbers. The number of these lin− cells in RCCs, when compared with numbers within normal kidney, showed no significant difference.

The number of activated DCs present in RCCs was readily assessed using the CMRF-44 and HB15a antibodies. These mAbs detect activation/differentiation antigens induced on blood DCs and found on DCs in the T-lymphoid areas of lymph nodes (18, 19). Activated CMRF-44+ or CD83+ DCs were present in comparatively small numbers in RCCs, representing 0.13 and 0.23% of tumor leukocytes, respectively. The numbers using either antibody are very similar, showing a strong correlation reminiscent of the dual staining CMRF-44+/CD83+ blood DC population (20). These activated DC counts represent 5–10% of the total (lin−) population of DCs. We speculate that these DCs are likely to have been activated either by inflammatory stimuli from the tumor or as a result of an interaction with T lymphocytes. The proportion of activated DCs, although small, was several times larger than that seen within the normal kidney. No expression of CD80 on DCs was detected, and with the exception of one case, no CD86+/14 cells could be identified.

Similar numbers of CD1a+ cells were identified in the tumors. Whereas p55 staining was difficult to assess, it also appeared to identify similar numbers of activated DCs. The number of CMRF-44+ and CD83+ DCs identified in frozen sections correlated with the number of S-100 staining DCs in paraffin sections. It has been unclear at what stage S-100 is expressed in normal DC differentiation/activation, but this first attempt at a comparison suggests that this may be a relatively late event. Another study, which used the S-100 antibody to define DCs within RCCs, demonstrated three or less S-100-positive cells per ×100 field in 6 of 10 tumors (16). We found this to be the case in 8 of 10 tumors. It is noteworthy that the numbers of S-100+ cells are also considerably fewer in RCCs than in tumors of epithelial organs (3, 5–9).

The ability to isolate viable leukocytes from RCCs was of major benefit to the study. Using flow cytometry, it was possible to confirm our immunohistology findings by demonstrating a significant lin−/HLA-DR+/CD45+ population of cells with the scatter characteristics of DCs. The sensitivity of flow cytometry
Table 3  FACS analysis of tumor mononuclear cell suspension (myeloid gate) \(^a\)

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<tr>
<th>DC phenotype</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>3.3</td>
<td>5.3</td>
<td>41.0</td>
<td>24.0 ± 14.4</td>
</tr>
<tr>
<td>CMRF-44(^+)/CD14(^+) macrophages</td>
<td>65.1</td>
<td>42.7</td>
<td>24.4</td>
<td>21.5</td>
<td>39.8</td>
<td>9.8</td>
<td>42.6</td>
<td>41.0</td>
<td>35.9 ± 16.9</td>
</tr>
<tr>
<td>CMRF-44(^+)/CD14(^-) cells(^b)</td>
<td>15.9</td>
<td>30.3</td>
<td>42.7</td>
<td>52.6</td>
<td>14.7</td>
<td>78.9</td>
<td>46.4</td>
<td>17.0</td>
<td>37.3 ± 22.4</td>
</tr>
</tbody>
</table>

\(^a\) Results are expressed as percentages of each cell type in the myeloid gate. The myeloid gate included between 5 and 30% of total cells, and when analyzed, >85% of CD45\(^+\) cells. The nonleukocytes fall in the CMRF-44\(^+\) CD14\(^-\) population.

\(^b\) Heterogeneous population including DCs (~50% HLA-DR\(^+\) cells).

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Fig. 3  FACS analysis of tumor leukocyte suspension. In A, the analysis was performed on the gated mononuclear population. Within the mononuclear gate, the majority of the cells are CD14\(^+\) macrophages. Only 1.59% of this sample are CD14\(^+\)/CMRF-44\(^+\) DC (one of eight representative analyses; see Table 3). B, analysis of sorted lin\(^-\) cells sorted from the mononuclear gate. In this sample, lin\(^-\) cells account for 7.06%. The majority are CD45\(^+\) leukocytes, with close to 50% of these being HLA-DR\(^+\), probable DCs. Only a small proportion (15%) of lin\(^-\) cells are activated DCs expressing CMRF-44. Solid line, the test mAb; broken line, the control mAb labeling. Parentheses, mean channel fluorescence.

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excludes the possibility that the lin\(^-\) cells included low density CD14\(^+\) or CD3\(^+\) cells, which were not detected by immunohistological techniques. Flow cytometry, likewise, confirmed that CMRF-44\(^+\)-activated DCs comprise a very small portion of the tumor leukocyte infiltrate. We also compared the stimulatory abilities of DCs and macrophages, albeit with difficulty, because only limited numbers of cells were obtained from finite tissue samples.

It is of some significance that the CMRF-44\(^+\) DCs obtained from RCCs were relatively poor stimulators in our experiments, although an additional effect from contaminating tumor cells in the MLR cannot be readily excluded. Interstitial DCs isolated from mouse and rat require a period of \textit{in vitro} culture to induce allostimulatory (costimulatory) activity (27). However, in humans, functional allostimulatory activity in the DC lineage arises early with the CD34\(^+\) precursor (28) and blood DCs (including the CMRF-44\(^+\), freshly isolated DCs; Ref. 29) stimulate a strong MLR. Isolated human Langerhans cells also act as potent allostimulatory cells (30). Thus, the failure of RCC-associated interstitial CMRF-44\(^+\) DCs to stimulate an allogeneic MLR is remarkable. Indeed, these results have parallels with the situation in rheumatoid arthritis, where it appears that despite an inflammatory environment, inhibitory factors prevent full DC activation and expression of a costimulator phenotype (31). Any effect on the DCs within RCCs appears to be localized to the tumor because fresh blood DCs from RCC patients were able to stimulate T lymphocytes maximally. Both interleukin 10 (32) and transforming growth factor \(\beta\) (33) have negative effects on DCs, and both have been shown to be produced by RCCs (34).

The CD14\(^+\)/CMRF-44\(^+\) DCs were considerably more potent stimulators of allogeneic T-lymphocyte proliferation than were isolated CD14\(^+\) tumor macrophages, and this suggests that at least some DCs avoid active tumor suppression of their function. A predominantly activated CMRF-44\(^+\) and CD83\(^+\) DC population was noted in the presence of T-lymphoid aggregates. This population may represent the DCs induced to migrate out of the tumor. We speculate that the interaction between these CMRF-44\(^+\) DCs and the corresponding T lymphocytes may involve recognition of TAA. It is possible that these aggregates represent a peripheral version of DC-T-lymphocyte interactions normally seen in the lymph node. Equally, these T lymphocytes (specific or nonspecific) may represent an efferent response reacting to the DCs that have not yet reached regional lymph nodes. Whether the relatively few CMRF-44\(^+\) DC or T lymphocytes noted in these clusters are capable of trafficking...
Fig. 4 Analysis of tumor-infiltrating DC allostimulatory potential. Individual tumor cell mononuclear cell suspensions were prepared, and the electronically gated lymphoid and monocyte-macrophage cell populations were sorted after labeling. A, CMRF-44+ cells but not CMRF-44− cells are allostimulatory (one experiment). B, CMRF-44−CD14+ DCs are potent allostimulatory cells. CMRF-44−CD14+ cells stimulated minimally in this experiment only. CMRF-44− cells were not allostimulatory (one of two experiments). Results are presented as [3H]thymidine uptake (mean cpm) using 2 × 10^5 responders and the absolute stimulator cell numbers as indicated. C, CMRF-44+ tumor DCs stimulate as potently as blood DCs, whereas CMRF-44− tumor DCs stimulate poorly. In D, fresh blood DCs from two patients with stage T tumors were able to stimulate T lymphocytes maximally (as determined by phorbol myristate acetate and ionomycin stimulation). A–D, bars, SE.

normally is another important question. It has to be conceded that little is known about the reaction of DCs in pathological circumstances and to what extent these results reflect normal or abnormal DC responses to tissue damage.

It seems that RCCs do not recruit DCs into the tumor above the numbers seen in normal tissue: (a) these first clinical data reinforce a similar concept proposed as a result of rat studies in vivo (27), and recent experiments demonstrate that a tumor cell line secreting vascular-endothelial growth factor suppressed in vitro DC production (35); (b) our functional studies on the CMRF-44+ tumor DC population also raise the possibility that there is a component of the tumor environment that suppresses their normal costimulatory function. This is supported by the finding of the lack of CD80 or CD86 up-regulation; and (c) the paucity of CMRF-44+ DCs in RCCs also prompts us to speculate that RCCs lack the inflammatory/noxious signals, such as lipopolysaccharide, tumor necrosis factor-α, or chemokines, required to initiate significant DC activation/migration (36). If DC function is compromised at these three levels, tumor escape from immunosurveillance is likely to result.

These data establish that the new reagents CMRF-44 and CD83 can be exploited to study DCs in tumor biology, and extended studies on breast cancer are now in progress. Furthermore, it encourages the concept that in vitro loading of autologous DCs with TAA and subsequent vaccination of patients with these DCs may reinforce one phase of the immune response to RCCs sufficiently to initiate a therapeutic immune response.

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