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All-trans Retinoic Acid Enhances Murine Dendritic Cell Migration to Draining Lymph Nodes via the Balance of Matrix Metalloproteinases and Their Inhibitors

Stephanie Darmanin,* Jian Chen,2 Songji Zhao,† Hongyan Cui,§ Reza Shirkoohi,‡ Naoki Kubo,¶ Yuji Kuge,|| Nagarai Tamaki,‡ Koji Nakagawa,§ Jun-ichi Hamada,* Tetsuya Moriuchi,* and Masanobu Kobayashi3§#

Cancers escape immune surveillance through the manipulation of the host’s immune system. Sequestration of dendritic cells (DCs) within tumor tissues and the subsequent inhibition of their migration is one of the several mechanisms by which tumors induce immunosuppression. In view of recent findings depicting the improvement of tumor immune responses in cancer patients following all-trans retinoic acid (ATRA) treatment, we sought to identify the effects of ATRA on DC mobility in the context of tumor immunotherapy. Our results demonstrate that ATRA, added to differentiating murine bone marrow progenitor cells, enhances the invasive capacity of the resulting DCs. Immature DCs injected intratumorally in mice show increased accumulation in draining lymph nodes, but not in nondraining lymph nodes and spleens, when differentiated in the presence of ATRA. The in vitro migration of mature DCs through the basement membrane matrix toward the lymphoid chemokines CCL19 and CCL21 is enhanced in these cells, albeit not in the presence of a matrix metalloproteinase (MMP) inhibitor. An increase in MMP production with a simultaneous decrease in the production of their inhibitors (tissue inhibitors of matrix metalloproteinase or TIMPs) is provoked by ATRA. This affects the MMP/TIMP balance in DCs, in particular that of MMP-9 and TIMP-1, favoring protease activity and thus allowing for enhanced DC mobilization. In conclusion, this study demonstrates that ATRA is capable of improving DC trafficking in a tumor milieu and, in view of the encouraging results obtained in the clinic, further supports the notion that ATRA might be a valuable chemical adjuvant to current immunotherapeutic strategies for cancer. The Journal of Immunology, 2007, 179: 4616–4625.

Cancers exert systemic impact on immune cell function through various mechanisms (1, 2). Among these, altered dendritic cell (DC)4 differentiation, maturation, and function are of utmost importance because DCs are the most potent APCs of the immune system. They act as sentinels, initiating and controlling adaptive immune responses, and in view of their potent effects on primary T cell differentiation and activation (3, 4) they are of central relevance to antitumor immune responses and vaccine development.

Localizaton in tumor tissues and migration to lymphoid organs are essential steps in DC immunobiology that are linked to their differentiation and T cell stimulatory function (2). However, within the tumor microenvironment several factors contribute to the sequestration of DCs within tumor tissues and the subsequent inhibition of their migration. It has been reported that melanoma cell lines can effectively chemotract DCs, modulate their phenotype, and eventually damage DC mobility. Melanoma-conditioned DCs exhibited an increased adhesion capacity to melanoma cell lines in vitro and did not migrate in response to lymphoid chemokines (5). Furthermore, in vitro-generated DCs failed to home into draining lymph nodes (DLNs) following intratumoral injection in patients with metastatic melanomas (6). One potential explanation for this might be the production of IL-8, which is shown to be produced by several kinds of solid tumors, including hepatocellular carcinoma as well as colorectal and pancreatic cancers, and contributes to the retention of DCs inside malignant lesions and the impairment of DC migration toward CCR7 ligands (7). In our laboratories we found that hypoxia, which is a characteristic of solid tumors, inhibits monocyte-derived DC migration through the suppression of matrix metalloproteinase (MMP) production (8), and recently it was reported that melanoma-derived gangliosides can also impair DC migratory function through the down-regulation of CCR7 expression (9). Neuroblastoma has been shown to hinder chemokine-mediated DC migration as well, probably by interfering with CCR7-CCL19 intracellular signal transduction pathways (10). In view of these findings, it seems that the development of a strategy to enhance DC mobilization from tumor tissues, particularly when using cancer vaccines, is highly desirable.

All-trans retinoic acid (ATRA) is a vitamin A derivative used in the therapy of acute promyelocytic leukemia (APL) that significantly improves the outcome of the disease by differentiating

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4 Abbreviations used in this paper: DC, dendritic cell; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; DLN, draining lymph node; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT, membrane type; TIMP, tissue inhibitor of matrix metalloproteinase.

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leukemic promyelocytes into mature cells. Organ infiltration by leukemic cells, however, produces a life-threatening therapeutic complication known as retinoic acid syndrome that affects many patients with APL (11–13). ATRA-induced APL cell extravasation was found to be due to the increased adhesion and motility of these cells as compared with their undifferentiated counterparts (14–16). Because enhanced motility is desirable in DCs located within the tumor microenvironment, we hypothesized that ATRA might also have this effect on DCs, these cells being also of myeloid lineage. It has been recently established that the accumulation of immature myeloid suppressor cells in patients with solid tumors is a limiting factor in cancer immunotherapy (17, 18). ATRA was successfully exploited in this scenario for its ability to promote the differentiation of myeloid progenitors in vivo, both in animal studies and in a clinical trial (19, 20), suggesting that ATRA could be a promising tool in the endeavor to improve immune responses to tumors.

In the present study we investigate the effect of ATRA, added to differentiating bone marrow progenitor cell cultures, on the resulting DC migratory properties. Immature DCs, injected intratumorally in mice, show increased accumulation in DLNs when differentiated in the presence of ATRA, and the in vitro migration of mature DCs through the basement membrane matrix toward lymphoid chemokines is enhanced in these cells. We found that this happens due to an increase in MMP production with a simultaneous decrease in the production of tissue inhibitors of metalloproteinases (TIMPs), allowing for enhanced DC mobilization. In consideration of the encouraging results obtained in cancer patients, our findings further warrant the use of ATRA as a chemical adjuvant in cancer immunotherapy.

Materials and Methods

Mice and tumor cells

C57BL/6 female mice (6–8 wk old) were purchased from Sankyo Laboratory Service and kept in specific pathogen-free conditions within the animal housing facility of the Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan. Studies were performed in accordance with local ethical guidelines.

B16-BL6 melanoma cells (21, 22) were maintained in DMEM (Nissui Pharmaceutical) supplemented with 10% heat-inactivated FBS (Japan Bio-Serum), penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively) (Invitrogen Life Technologies), 2 mM l-glutamine (MP Biomedicals), 200 μM sodium pyruvate, and 10 mM sodium bicarbonate (both from Wako Pure Chemical Industries). The cells were grown in a humidified 5% CO2/air incubator at 37°C.

ATRA

ATRA was obtained from Sigma-Aldrich, dissolved in DMSO, flushed with argon, and stored in light-proof containers at −20°C. When ATRA was used during experiments it was done in subdued light.

Bone marrow-derived DC cultures

Bone marrow-derived DCs were cultured in complete medium consisting of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% charcoal-treated FCS (HyClone), 50 μM 2-ME (Wako Pure Chemical Industries), penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively), 2 mM l-glutamine, and 2 g/L sodium bicarbonate. Bone marrow progenitor cells were obtained from bone marrow according to previously described methods (23, 24). Briefly, femurs were removed from the mice and the bone marrows were flushed with complete medium containing 50 U/ml heparin using a 21-gauge needle. Bone marrow cells were depleted of RBC, re-suspended in complete medium, and cultured in bacteriological grade dishes at a density of 2 × 10^6 cells in 10 ml of complete medium containing 20 ng/ml GM-CSF (Chemicon) and 10 ng/ml IL-4 (Peprotech) in a humidified 5% CO2/air incubator at 37°C. Medium, including cytokines, was added on day 3 of culture, and half the medium, including cytokines, was replaced on day 6 of culture. Where appropriate, ATRA (1 μM) was added to the cultures on days 3 and 6 together with the medium and cytokines. Cells were harvested on day 8 and used as immature DCs, or LPS (1 μg/ml; Sigma-Aldrich) was added and cells were harvested on day 9 and used as mature DCs.

In vitro extracellular matrix (ECM) (Matrigel) invasion assay

ECM (Matrigel) invasion assays for mature DCs were performed using Transwell cell culture chambers (pore diameter, 5-μm pore size; Costar). The filters were coated with 40 μl of Matrigel (BD Biosciences) (diluted 1/3 (v/v) in cold serum-free medium) and incubated at 37°C for 60 min to allow for gelling of the matrix according to the manufacturer’s instructions. Recombinant murine CCL19/MIP-3β and CCL21/6Ckine (Exodus-2/SLC) (both from R&D Systems) were diluted in complete medium at a concentration of 100 ng/ml and placed in the lower chamber of the Transwell in a volume of 600 μl. Mature DCs (1 × 10^6) were suspended in 100 μl of complete medium and placed in the upper chamber of the Transwell. To assess random migration, in some experiments chemokines were placed in both the upper and lower chambers of the Transwell. Also, to block MMP activity, some previously selected fields from each well were used with the broad-spectrum MMP inhibitor GM6001 (V-1(2R)-(2-hydroxymethyl-docosanoyl)methyl)-4-methylpentanoyl-l-tryptophan methylamide) at 100 μM or its inactive control peptide, N-t-butoxycarbonyl-l-leucyl-l-tryptophan methylamide (both from Calbiochem) for 30 min at 37°C and then added, together with the GM6001 or inactive control peptide, to the upper chamber of the Transwell. Standard migration assays through filters not covered with Matrigel were run in parallel to the experiments described above. Complete medium alone was used as a control for all of these experiments. After 6 to 8 h the migrated cells were collected from the lower chamber of the Transwell, cytopsinn onto glass slides by means of a cyto centrifuge (Cytospin II; Shandon), fixed with methanol for 1 min, and then stained with Giemsa solution (5% Giemsa in phosphate buffer (pH 7.4)) for 10 min. Slides were rinsed in tap water and viewed under a light microscope (Nikon ECLIPSE 80i) using low-power (×100) magnification. Cells were counted in three randomly selected fields from each slide. Each test condition had three replicates per experiment and thus a total of nine fields were used to calculate the average number of cells that invaded through the Matrigel. Results are presented as a relative ratio in arbitrary units (DCs migrating in the presence of ATRA/DCs migrating in control medium) compared with the number of cells migrating in control medium. Data were analyzed using the Student’s t test.

In vivo migration experiments

Immature DCs were removed from culture, washed twice in FBS-free culture medium, and checked for cell viability by trypan blue dye exclusion. Cells (0.7–1 × 10^6) were labeled with radioactive indium, 111In-oxine (Nihon Medi-Physics), at 100 μCi for 20 min at room temperature or with radioactive chromium, 51Cr (Amersham Biosciences), at 150 μCi for 1 h at 37°C, mixed occasionally by hand, and washed three times in PBS. More than 90% of the cells were viable following radioactive labeling in all of our experiments as determined by trypan blue dye exclusion. Radioactively labeled DCs or the radiolabeled compound only (for control experiments), were injected into B16-BL6 s.c. melanomas (100–150 mm^3) located in the right flanks of C57BL/6 female mice (8-wk old). The tumor cells had been injected 1 wk earlier and allowed to form melanomas in the mice. Each test group was composed of at least five mice. At this time, to determine the spontaneous release of the radioisotopes from the DCs, 111In-labeled and 51Cr-labeled DC samples (same as injected samples) were kept in slightly opened 15-ml centrifuge tubes in 5 ml of complete medium without cytokines in a humidified 5% CO2/air incubator at 37°C. After 24 h the mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and scintigraphic images of the 111In groups were recorded with a GCA-602A gamma camera (Analytic Imaging Systems), equipped with a parallel hole, medium-energy collimator. Images were acquired for 20 min and were digitally stored in a 512 × 512 matrix. After 48 h the mice were sacrificed and the radioactivity counts in DLNs, non-DLNs, and spleens were measured by means of an automatic well-type gamma counter (1480 WIZARD; Wallac). To correct for radioactive decay, radioactivity emissions from test injection standards, which had been stored for 48 h at 4°C, were measured simultaneously. This was taken to be the value of radioactivity associated with the initially injected DCs. Results were calculated by expressing the radioactive emission from lymph nodes and spleens as a percentage of the radioactive emission from the DC injection standards. Also, after 24 and 48 h the cells from the spontaneous release assay were spun down, the supernatant was discarded, and the cell pellet was resuspended in fresh medium. The emitted radioactivity was measured by means of the gamma counter and the cell-associated radioactivity was expressed as a percentage of the DC injection standards.
represents the absence of chemokines or Matrigel.

Table I. Primer sequences for murine mRNA quantification by real-time PCR

<table>
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<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Primer Sequences (5′ to 3′)†</th>
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</thead>
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<td>β-Actin</td>
<td>X03672</td>
<td>AGGAAATCTGTCGGATGATACAT (F)</td>
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<tr>
<td></td>
<td></td>
<td>GAACTGCCTGTTGGCAATAG (R)</td>
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<td>MMP-9</td>
<td>NM_013599</td>
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<td>TCACCCCTAAGTGAGGAGACATG (R)</td>
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<tr>
<td>MMP-14</td>
<td>NM_008608</td>
<td>ACCCCCTAAGTGAGGAGACATG (F)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTACACCCGAGGAGACATG (R)</td>
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<tr>
<td>MMP-2</td>
<td>NM_008610</td>
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<td>AAGACCGGTTTTAATTGGGAC (R)</td>
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<td>TIMP-1</td>
<td>NM_011593</td>
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<td>GAAGGACCCACGACTGCTGACA (R)</td>
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</table>

† F, Forward primer; R, reverse primer.

Immunofluorescence analysis for CCR7 surface expression

Immunofluorescence experiments were performed by incubating cells (1 × 10^6) in staining buffer (100 µl) containing PBS supplemented with 5% FBS and 0.1% sodium azide (Wako Pure Chemical Industries) for 30 min at 37°C with appropriate concentrations of PE-conjugated mouse anti-CCR7 (eBioscience) according to the manufacturer’s instructions. A PE-conjugated, isotype-matched Ab was purchased from the respective company and used for control staining throughout these experiments. After staining, cells were washed three times in staining buffer, resuspended in 500 µl of staining buffer, and analyzed by flow cytometry. Flow cytometric analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences).

Real-time PCR

Total RNA was isolated from 5 × 10^6 DCs using the RNeasy Mini kit (Qiagen). cDNA was obtained from RNA (10 µg) by means of reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. Primers for the quantitative detection of target mRNAs were designed by means of Primer Express computer software (Applied Biosystems) (Table I). Real-time PCR was then performed by means of a Platinum SYBR Green kit (Invitrogen Life Technologies) on an ABI Prism 7900HT machine. After an initial incubation step at 95°C for 15 min, 40 PCR cycles were performed. The cycling conditions consisted of a denaturation step at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Relative gene expressions of the genes of interest were expressed as a ratio of the expression level of the gene of interest to that of β-actin. The mRNA expression levels of β-actin showed no significant changes between the DCs cultured in the absence of ATRA and those cultured in its presence.

Gelatin zymography

Enzymatic activity in cell culture supernatants was analyzed by electrophoresis in polyacrylamide gels containing SDS and gelatin as described previously (25), with some modifications. Cells were harvested by gentle pipetting, washed in serum-free medium, and incubated at a density of 5 × 10^6 cells per plate in bacteriological grade dishes in 10 ml serum-free medium. After 24 h the cells were spun down and the culture supernatants were collected, centrifuged at 15,000 rpm for 15 min, and stored at −80°C. The supernatants (2 ml) were concentrated using centrifugal filter devices for <30 kDa (Centricron; Millipore), resuspended in equal amounts of sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, and 0.05% bromophenol blue (pH 6.8)) and applied to 10% (w/v) polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich) followed by electrophoresis. The gels were renatured in 2.5% (v/v) Triton X-100 in buffer (50 mM Tris-HCl and 0.05% sodium azide) for 3 h at room temperature and then incubated overnight at 37°C in developing buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl_2, and 0.05% sodium azide). Staining was done with Coomassie brilliant blue (Bio-Rad Laboratories) at 0.1% (w/v) in a 10% acetic acid and 10% isopropanol solution, and destaining was done in the same solution without the stain. Destaining was done until the bands with diminished staining appeared on a uniformly stained background. Gels were then photographed by means of a digital camera (Nikon Coolpix S1; Nikon). Zones of proteolytic activity are evident as clear bands against a dark blue background (Coomassie blue staining). The molecular mass (kDa) of the gelatinases was estimated against markers of known molecular mass (Sigma-Aldrich).

FIGURE 1. In vitro migration of mature DCs toward lymphoid chemokines. The migration of mature DCs generated in the absence or presence of ATRA toward lymphoid chemokines was assessed by means of Transwell chamber migration assays in the presence or absence of Matrigel. CCL21/6Ckine (100 ng/ml) or CCL-19/MIP-3β (100 ng/ml) in complete medium were placed in the lower chamber (A) or in both chambers to assess random migration and invasion (B), whereas DCs (1 × 10^6) were placed in the upper chamber. After 6–8 h of incubation cells were recovered from the lower chamber, cytoplasm onto glass slides, stained with Giemsa stain, and counted using a light microscope under low power (×100). Triplicates were used for each experiment and three fields were chosen randomly from each slide. Values are expressed as a relative ratio in arbitrary units for the mean ± SEM for nine fields. Representative results for at least three experiments are shown. The asterisk (*) represents statistically significant differences (p ≤ 0.05); values are determined by comparing DCs generated in the presence of ATRA vs control DCs. The positive sign (+) represents presence while the negative sign (−) represents the absence of chemokines or Matrigel.
Statistical analyses

Two-tailed, unpaired Student’s *t* tests were used to compare differences in the properties of DCs cultured in the presence or absence of ATRA. A two-tailed Mann-Whitney *U* test was used to compare the in vivo migration of the two groups. Using either test, *p* < 0.05 was considered significant and is represented by an asterisk over the error bars in the figures.

Results

**ATRA enhances DC migration both in vitro and in vivo**

Tumor-induced defects in the host immune system are currently believed to be critical in hindering tumor-specific immune responses, thereby limiting the benefits of cancer immunotherapy (26). A recent study by Mirza et al. (20) reports the successful use of ATRA in reducing the numbers of immature myeloid suppressor cells in cancer patients, the accumulation of such cells being a major tumor escape mechanism, as well as improving DC function. Because the mobilization of DCs from tumor tissues and their homing to DLNs is essential for the elicitation of an appropriate immune response, we hypothesized that ATRA might also be affecting the migratory behavior of the DCs in these patients apart from their differentiation, thus contributing to the improved DC functions observed in this study. To investigate this possibility experimentally, Transwell invasion assays were used to mimic the passage of DCs that have taken up Ag within tumor tissues through the basement membrane matrix on their way to lymphoid organs. Mature DCs generated in the presence of ATRA showed markedly increased migration through Matrigel toward the lymphoid chemokines CCL19/MIP-3β and CCL21/6Ckine as compared with their untreated counterparts (Fig. 1A). This happens only along a concentration gradient, however, because this increase was not observed when the chemokines were present in both the upper and lower chambers of the Transwell (Fig. 1B), indicating that this enhanced migration is not random but occurs only toward chemoattractant signals. Furthermore, when these experiments were performed in the absence of Matrigel there were no significant differences between the migration patterns of DCs generated in the
presence of ATRA and those generated in its absence, which demonstrates that the increase in migration is, in fact, due to the enhanced capacity of these cells to traverse ECM (Fig. 1).

These results were further confirmed in vivo by using a B16-BL6 melanoma tumor model. Melanoma cells were injected s.c. in C57Bl/6 mice and allowed to grow for 1 wk until the tumor was palpable. \(^{111}\)In-labeled or \(^{51}\)Cr-labeled immature DCs were then injected intratumorally and their accumulation in DLNs, non-DLNs, and spleens was analyzed after 48 h by means of a gamma counter. Migration of DCs to DLNs in both the \(^{111}\)In (Fig. 2A) and \(^{51}\)Cr groups (Fig. 2C), but not to non-DLNs or spleens (Fig. 2D), was significantly enhanced for DCs differentiated in the presence of ATRA when compared with the control DCs. Enhanced migration to DLNs could also be easily detected by means of scintographic images obtained from mice in the \(^{111}\)In group (Fig. 2B). In vitro spontaneous release assays were conducted concurrently to make sure that the increase in radioactivity observed in the organs of mice injected with ATRA-treated DCs was not a result of an increased spontaneous release of the radioisotope by these cells and its subsequent accumulation in the DLNs. Results for both of the radioisotopes used in these experiments show that the spontaneous release is similar for both DCs generated in the presence of ATRA as well as the control DCs (Fig. 2, E and F).

Previous studies have shown that \(~0.1\%\) of DCs administered intratumorally can migrate to the DLNs within 24 h (27, 28). Although we obtained similar results for the control DC groups, the percentage of injected DCs that reached the DLNs in the ATRA-treated group averaged \(~0.5\%\) (Fig. 2, A–C), representing a significant increase. Importantly, the effective plasma concentrations of ATRA in the patients who participated in the study of Mirza et al. (20) were in the same range as the concentration we used throughout our experiments, namely 1 mM (300 ng/ml). The dose levels used have been identified to be generally safe and well-tolerated by patients, thus supporting the clinical relevance of the results obtained in our experiments. Furthermore, in our experiments the mRNA expression of CCR7 by real-time PCR to check whether the mechanism by which the DCs generated in the presence of ATRA showed increased migratory features was, in fact, through the up-regulation of CCR7. CCR7 mRNA expression in mature ATRA-treated DCs was not significantly enhanced (Fig. 3A). We then analyzed the DC surface expression of CCR7 by flow cytometry and found that it was not significantly affected in any of our experiments (Fig. 3B), thus confirming that the migration of ATRA-treated DCs to DLNs is not enhanced due to CCR7 up-regulation and, therefore, is not due to an increased chemotactic response to lymphoid chemokines.

**FIGURE 3.** CCR7 mRNA and surface expression in mature DCs. A, mRNA expression of CCR7 in mature DCs generated in the absence or presence of ATRA was determined by real-time PCR. CCR7 mRNA expression levels were normalized to that of \(\beta\)-actin. Representative values for at least three independent experiments are expressed as the mean \(\pm\) SD for three replicates. The asterisk (*) represents statistically significant differences (\(p \leq 0.05\)); values are determined by comparing DCs generated in the presence of ATRA vs control DCs. B, Surface expression of CCR7 on mature DCs generated in the absence (thick line) or presence (thin line) of ATRA was determined by flow cytometry. Representative histograms for three separate experiments are shown.

**CCR7 expression is not responsible for the increase in DC migration induced by ATRA**

During DC maturation the expression of the chemokine receptor CCR7 is up-regulated, guiding the DCs into DLNs in response to the chemotactic signals from its ligands CCL19/MIP-3\(\beta\), produced by stromal cells and mature DCs in the lymph node, and CCL21/6Ckine; produced by afferent lymphatic cells (29). Lack of CCR7 expression by DCs leads to failure in migrating to the DLNs, whereas increased CCR7 expression is correlated with an increased migration to the lymph nodes (30). We therefore examined the mRNA expression of CCR7 by real-time PCR to check whether the mechanism by which the DCs generated in the presence of ATRA showed increased migratory features was, in fact, through the up-regulation of CCR7. CCR7 mRNA expression in mature ATRA-treated DCs was not significantly enhanced (Fig. 3A). We then analyzed the DC surface expression of CCR7 by flow cytometry and found that it was not significantly affected in any of our experiments (Fig. 3B), thus confirming that the migration of ATRA-treated DCs to DLNs is not enhanced due to CCR7 up-regulation and, therefore, is not due to an increased chemotactic response to lymphoid chemokines.

**ATRA increases MMP mRNA expression and secretion while simultaneously decreasing TIMP mRNA expression in DCs**

MMPs are a family of structurally related, zinc-containing enzymes that have been identified as key players in the degradation and remodeling of all components of the ECM. The members of the MMP family have been further subdivided into groups according to their substrate specificity and cellular location and include the collagenases, gelatinases, stromelysins, and membrane-type MMPs, among others (31). Notably, the gelatinases, MMP-2 and MMP-9 cleave type IV collagen, which constitutes a backbone for the attachment of other basement membrane components. MMP-9 in particular has been implicated in the migration of DCs through...
the ECM, and it has been shown that changes in the balance between MMPs and TIMPs can modulate DC migratory capacity (32–34). TIMPs are endogenous inhibitors of MMPs and can inhibit most MMPs without major selectivity between them (with the exception of TIMP-1, which is a very poor inhibitor of membrane type (MT)1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MMP-19). Importantly, it is widely recognized that the balance between MMPs and TIMPs is critical for the eventual ECM remodeling within tissues (35); hence, its significance in DC migration.

In our experiments both CCR7 mRNA levels (Fig. 3A) and CCR7 surface expression (Fig. 3B) were not significantly altered in ATRA-differentiated DCs when compared with the control DCs. Moreover, when we checked DC adhesion to Matrigel by means of in vitro adhesion assays, it was very similar in both control as well as ATRA-differentiated DCs (data not shown). Therefore, to

**FIGURE 4.** MMP mRNA expression and secretion increases in mature DCs generated in the presence of ATRA. A, C, and D, mRNA expression of MMP-9 (A), MMP-2 (C), and MMP-14 (D) in mature DCs generated in the absence or presence of ATRA was determined by real-time PCR. The expression levels of the genes of interest were normalized to that of β-actin. Values are expressed as the mean ± SD for three replicates. Representative results for at least three separate experiments are shown. The asterisk (+) represents statistically significant differences (p ≤ 0.05); values are determined by comparing DCs generated in the presence of ATRA vs control DCs. B, Secreted MMP-9 and MMP-2 proteins in conditioned medium of mature DCs generated in the absence (−) or presence (+) of ATRA was determined by gelatin zymography. Representative results for at least three separate experiments are shown.

**FIGURE 5.** Enhanced in vitro migration of mature DCs generated in the presence of ATRA is dependent on MMPs. Migration of mature DCs generated in the absence or presence of ATRA toward lymphoid chemokines was assessed by means of Transwell chamber migration assays in the presence or absence of the broad-spectrum MMP inhibitor GM6001 or its inactive control peptide. CCL21/6Ckine (100 ng/ml) or CCL-19/MIP-3β (100 ng/ml) in complete medium was placed in the lower chamber, whereas DCs (1 × 10^6) were placed in the upper chamber. Assays were conducted in the presence (A) or absence (B) of Matrigel. After 6–8 h of incubation, cells were recovered from the lower chamber, cytopun onto glass slides, stained with Giemsa stain, and counted using a light microscope under low power (×100). Triplicates were used for each experiment and three fields were chosen randomly from each slide. Values are expressed as a relative ratio in arbitrary units for the mean ± SEM for nine fields. Representative results for at least three experiments are shown. The asterisk (+) represents statistically significant differences (p ≤ 0.05); values are determined by comparing DCs generated in the presence of ATRA vs control DCs. The positive sign (+) represents the presence whereas the negative sign (−) represents the absence of chemokines, GM6001, or its inactive control peptide.
were normalized to that of TIMP-3 (C). TIMP-2 and TIMP-3 mRNA expressions were also decreased in ATRA-treated cells than in control cells throughout our study (Fig. 6). The secretion of MMP-9 by the ATRA-treated cells was also enhanced in all of our experiments compared with the control cells as determined by gelatin zymography (Fig. 4B). MMP-2 mRNA expression (Fig. 4C) and secretion (Fig. 4B), however, was either not affected or showed a very slight increase in DCs generated in the presence of ATRA compared with the control cells. MMP-14 mRNA expression, in contrast, was significantly increased (Fig. 4D). Apart from promoting cell invasion and motility through pericellular ECM degradation, MMP-14 or MT1-MMP is essential for the activation of pro-MMP-2 on the cell surface and the consequent generation of the fully active enzyme (36). Moreover, it has been recently reported that MMP-14 is required for the migration of both monocytes and dendritic cells (37, 38), thereby highlighting the importance of this result within this context.

The role of MMPs in the ATRA-mediated increase in DC migration obtained in our experiments was further confirmed by Transwell invasion assays conducted in the presence of a broad spectrum MMP inhibitor, GM6001. Although an inactive control peptide allowed for an enhanced migration of DCs generated in the presence of ATRA when compared with the untreated cells, the trend of the increase being similar to that obtained in the original assays that had no control peptide (Fig. 1A), GM6001 completely inhibited this enhanced migration (Fig. 5A). Moreover, when the same assays were conducted in parallel in the absence of Matrigel (Fig. 5B), no significant difference in migration could be observed between DCs generated in the presence of ATRA and their untreated counterparts. Hence, these results ascertain that the increase in DC migration brought about by ATRA is dependent on MMPs. Also, when DCs were cocultured with B16-BL6 melanoma cells for up to 48 h the expression of MMPs was not diminished, whereas that of TIMPs was not enhanced in ATRA-treated DCs when compared with control DCs as revealed by real-time PCR, which is particularly relevant to the in vivo situation (data not shown).

Interestingly, the mRNA levels of TIMP-1, the natural selective inhibitor of MMP-9, were consistently at least three times lower in ATRA-treated cells than in control cells throughout our study (Fig. 6A). TIMP-2 and TIMP-3 mRNA expressions were also decreased in the DCs differentiated in the presence of ATRA as compared with their control counterparts (Fig. 6, B and C). This reduction in TIMP mRNA levels, together with the increase in MMP mRNA expression and production obtained in our experiments, confirm that ATRA, when added to differentiating bone marrow progenitor cultures, regulates the balance between proteases and their inhibitors in the resulting DCs and, in so doing, enhances their traffic through the ECM and subsequent accumulation within the DLNs.

Discussion

MMPs have been shown to be essential for DC movement through basement membranes and the ECM (33, 34), and several studies have described the importance of MMP-9 in this process both in immature as well as in mature DCs (39–41). In fact, it has also been reported that MMP-9-deficient DCs have impaired migration through tracheal epithelial junctions (42), further substantiating its involvement in this process. The balance in the expression of MMPs and TIMPs has been reported to define the net migratory capacity of DCs and, consequently, their imbalance may result in disease (32, 34). Our results confirm that regulation of the MMP/TIMP balance in DCs, particularly MMP-9 and TIMP-1, leads to an increase in directional migration to DLNs following intratumoral administration. This knowledge, therefore, can be potentially exploited in a cancer scenario, where it has been shown that DC mobility is impaired due to several elements present within the tumor microenvironment.

There are, however, certain limitations for augmenting MMP levels within tumor tissues, because these enzymes are also associated with many types and stages of cancer; they are thought to be essential for basement membrane penetration during cancer cell invasion and thus play an important role in metastatic disease. Moreover, the functions of MMPs within the tumor mass are not restricted solely to ECM degradation, which in turn leads to invasion and metastasis, but also include the regulation of cancer cell growth, differentiation, and apoptosis as well as tumor angiogenesis and immune surveillance (43). The involvement of MMPs in tumor progression, therefore, starts at the early stages of tumor growth such as angiogenesis, whereas their role in metastasis occurs in the later stages of cancer development. In view of the above, we hypothesized that if MMPs are secreted within the tumor microenvironment by, say, immune cells such as in our case, these might aid the tumor in its growth and invasion. The B16-BL6 melanoma is a B16 variant selected for increased invasive capacity, specifically pulmonary metastases (21, 22). To check whether
the enhanced MMP production by the DCs obtained in our experiments was contributing to tumor cell metastases, we injected B16-BL6 melanoma cells subcutaneously in C57BL/6 mice, allowed the tumor to reach a palpable size, and injected DCs differentiated in the absence or presence of ATRA or PBS alone intratumorally. After ~2 wk following DC or PBS injection the tumor size was smaller for the mice injected with DCs than for the mice injected with PBS alone, although there was no reduction in tumor size for the mice injected with the ATRA-treated DCs when compared with the control DCs (data not shown). Interestingly, there was no significant difference in the number of pulmonary metastases between the ATRA group and the control group; rather, there was a tendency for fewer metastases in the ATRA group (data not shown). This proves that the increased production of MMPs by DCs is not helping the process of metastasis but is most probably hindering it through a boost in T cell response as a result of enhanced DC accumulation in the DLNs. Furthermore, leukocyte MMPs have been defined as potentially difficult molecules to safely target in an anticancer therapy approach using inhibitors of MMPs because they sometimes have roles in preventing tumor progression depending on the stage of the tumor, such as with MMP-9, and at other times their inhibition might actually promote metastasis, as is the case with MMP-14 (44), thus confirming that the enhanced MMP production brought about by ATRA might safely be taken advantage of in DC immunotherapies.

Cancer immunotherapy is a field of intense research aiming to induce active immunity with either therapeutic or adjuvant intent. The identification and molecular characterization of tumor-associated Ags has resulted in the development of tumor-specific immunotherapy. Also, the characterization of biological adjuvants to further improve vaccine immunogenicity as well as the investigation of multimodal therapies in which vaccines are being combined with other oncological treatments such as radiation and chemotherapy are current areas of study that aspire to generate cancer vaccines with enhanced clinical efficacy (45, 46). DCs, being the most potent APCs of the immune system, represent a promising tool in therapeutic vaccination against cancer. Clinical trials have indicated that DC vaccines are feasible and safe with minimal side effects and effective in some patients, particularly if the DCs have been appropriately matured and activated (47, 48). Hence, numerous immunization strategies involving DCs are in various stages of investigation. Immunotherapeutic vaccines have been prepared by loading tumor Ags in DCs via tumor cell lysates, lysate fractions, or apoptotic cell bodies from tumor cells (49), by fusing allogeneic DCs with tumor cells (50), and by the transfer of RNAs (51) as well as genomic DNA transfection (52) from tumor cells into DCs. The administration of genetically modified DCs to express CD40L (53) and a drug-inducible reengineered CD40 receptor (54), as well as cytokine genes such as IL-2, IL-12, and IL-18, has also been conducted (55, 56). Despite the success obtained in animal studies and to a lesser extent in clinical trials by using the above-mentioned methods, manipulation of the migration of ex vivo generated DCs from the site of injection to the lymph nodes remains a major challenge in this field. Because murine studies show that DC migration correlates with T cell proliferation (30), it is not wrong to assume that T cell distribution and tumor control might be linked to injection route-dependent localization of DCs in lymphoid organs. In fact, the use of multiple routes of vaccination in cancer patients, such as intradermal and i.v. as well as intranodal administration, has been recommended to induce a truly systemic immune response able to eradicate tumor foci at different locations in the body. Additionally, as a result of the difficulties associated with ex vivo generated DC vaccines, the notion of in situ targeting of DCs and thus use of the efficient migratory properties of DCs in vivo is gaining popularity (57). Of course, proper DC maturation and activation is mandatory to induce immunity in such a case, because the absence of inflammation will induce Ag tolerance (58). Among the attempts to target DCs in situ and at the same time provide appropriate maturation and activation stimuli, the use of a synthetic CpG oligonucleotide, which is known to trigger TLR 9, to immunize melanoma patients with a Melan-A peptide in a clinical trial yielded particularly successful results (59). Thanks to such work, it is now established that there is great promise in the use of chemical adjuvants, both in combination with cancer vaccines as well as to elicit antitumor immune responses in general (60).

As a naturally occurring isomer of retinoic acid, ATRA is a well-known factor capable of inducing the differentiation of APL cells and was thus successfully used in the treatment of APL patients (13). At the molecular level, ATRA activates nuclear receptors belonging to the family of steroid/thyroid-retinoid-activated transcriptional regulators. Two classes of retinoid receptors have been identified, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each family consisting of three isoforms, α, β, and γ, which are encoded by separate genes. These receptors activate the transduction of target genes via complex genetic and epigenetic mechanisms in response to the retinoid signal (61). In this study, we show that ATRA enhances MMP mRNA expression while decreasing that of TIMPs; this suggests that both MMP and TIMP genes might possibly be retinoid target genes in DCs, although the effects of ATRA on MMP and TIMP regulation have also been observed in other cell types. ATRA treatment resulted in a reduction of MMP-9 plasma levels in patients with emphysema, and alveolar macrophages from these patients had a similar reduction in MMP-9 activity with a significant increase in TIMP-1 levels following exposure to ATRA in vitro (62). In contrast, ATRA induced NF-κB activation in human neuroblastoma cells that, in turn, induced MMP-9 expression and increased the basement membrane invasive capacity of these cells (63). This effect, however, is not observed in all types of cancer cells. In two studies using human breast cancer cells, for instance, ATRA treatment resulted in decreased invasive properties either through the up-regulation of TIMP-1 and consequently the impairment of MMP-9 gelatinolytic activities (64) or through the direct down-regulation of MMP-9 secretion (65), whereas the effect of ATRA on MMP-9 mRNA expression and secretion in two human myeloid leukemia cell lines was very similar to that obtained in our results (66). It thus seems to be clear that modulation of the protease/antiprotease balance by ATRA may have an impact on disease pathogenesis and that, moreover, this characteristic of ATRA can be exploited in DCs, thus making ATRA an attractive candidate for use as a chemical adjuvant within the cancer scenario.

In this study we sought to identify the effects of ATRA on DC mobility in the context of tumor immunotherapy. Our results demonstrate that ATRA, added to differentiating murine bone marrow progenitor cells, enhances the invasive capacity of the resulting DCs through the ECM and, as a result, their in vivo migration to DLNs following intratumoral injection is significantly increased. We show that this happens through modulation of the expression of both MMP and TIMP, in particular, that of MMP-9 and TIMP-1, which affects the MMP/TIMP balance in the DCs, favoring protease activity. Considering the implications of DC migration in a tumor milieu, ATRA might conceivably be a valuable addition both to cancer vaccines as well as to cancer immunotherapy overall. In conclusion, our findings, together with the encouraging results obtained in the clinic, support the rationale for integrating ATRA into the standard therapeutic arsenal used in cancer patient care.
Disclosures

The authors have no financial conflict of interest.

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