

All-trans-Retinoic Acid Improves Differentiation of Myeloid Cells and Immune Response in Cancer Patients

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Abstract

Abnormal dendritic cell differentiation and accumulation of immature myeloid suppressor cells (ImC) is one of the major mechanisms of tumor escape. We tested the possibility of pharmacologic regulation of myeloid cell differentiation using all-trans-retinoic acid (ATRA). Eighteen patients with metastatic renal cell carcinoma were treated with ATRA followed by s.c. interleukin 2 (IL-2). Eight healthy individuals comprised a control group. As expected, the cancer patients had substantially elevated levels of ImC. We observed that ATRA dramatically reduced the number of ImC. This effect was observed only in patients with high plasma concentration of ATRA (>150 ng/mL), but not in patients with lower ATRA concentrations (<135 ng/mL). Effects of ATRA on the proportions of different dendritic cell populations were minor. However, ATRA significantly improved myeloid/lymphoid dendritic cell ratio and the ability of patients' mononuclear cells to stimulate allogeneic T cells. This effect was associated with significant improvement of tetanus-toxoid-specific T-cell response. During the IL-2 treatment, the ATRA effect was completely eliminated. To assess the role of IL-2, specimens from 15 patients with metastatic renal cell carcinoma who had been treated with i.v. IL-2 alone were analyzed. In this group also, IL-2 significantly reduced the number and function of dendritic cells as well as T-cell function. These data indicate that ATRA at effective concentrations eliminated ImC, improved myeloid/lymphoid dendritic cell ratio, dendritic cell function, and antigen-specific T-cell response. ATRA treatment did not result in significant toxicity and it could be tested in therapeutic combination with cancer vaccines. (Cancer Res 2006; 66(18): 9299-307)

Introduction

Tumor-induced defects in the host immune system play a critical role in impeding tumor-specific immune responses and limiting the effect of cancer immunotherapy (1). Abnormal differentiation of myeloid cells is one of the major mechanisms of these defects. It manifests in decreased presence of mature, functionally competent dendritic cells, accumulation of immature dendritic cell, and accumulation of immature myeloid-suppressive cells (ImC; ref. 2). In mice, transplantable tumor (3–5) or spontaneous development of tumors in transgenic mice with tissue-restricted expression of oncogenes (6) resulted in marked

systemic expansion of these cells. The ImC may contribute to the failure of immune therapy in patients with advanced cancer and in tumor-bearing mice.

Recent data from a number of groups have shown that ImC accumulated in tumor-bearing hosts play an important role in tumor nonresponsiveness by suppressing antigen-specific T-cell responses (2, 6–8). Earlier experiments from H. Schreiber's group (9) and from V. Bronte and N. Restifo (4) showed that depletion of murine Gr-1⁺ cells significantly improved CD8⁺ T-cell immune response and allowed for eradication of tumor. More recent work of J. Berzofsky's group showed that depleting Gr-1⁺ myeloid cells *in vivo* prevented tumor recurrence (10). ImC suppress immune response via number of different mechanisms, including loss or significant decrease of the expression of the T-cell receptor ξ chain (CD3 ξ), which is the principal part of the T-cell receptor complex (11); inhibition of CD3/CD28-induced T-cell activation/proliferation by production of reactive nitrogen and oxygen intermediates (12); inhibition of IFN- γ production by CD8⁺ T cells in response to the specific peptide presented by MHC class I molecules (5); prevention of the development of CTLs *in vitro* (13, 14); and induction of antigen-specific CD8⁺ T-cell tolerance *in vivo* (15). When cultured *in vitro* in the presence of appropriate growth factors, ImC could be differentiated into dendritic cell or macrophages (16–18). However, their differentiation is blocked when cells are transferred into tumor-bearing mice (17), indicating that tumor-derived factors play critical role in preventing differentiation of these cells. The role of these factors was further supported by the fact that surgical resection of the murine tumor decreased the number of ImC (19).

In cancer patients, ImC are defined as cells that express the common myeloid marker CD33 but lack expression of markers of mature myeloid and lymphoid cells and of the MHC class II molecule HLA-DR (7). An accumulation of phenotypic ImC was associated with the decreased number of dendritic cells in the peripheral blood of patients with head and neck, lung, or breast cancer (20). In functional testing, ImC isolated from peripheral blood of HLA-A2–positive cancer patients inhibited production of IFN- γ by CD8⁺ T cells restimulated with specific peptide-pulsed dendritic cells (7). Cells with similar phenotype were shown to suppress T-cell function in patients with advanced cancer patients (21). Thus, accumulation of ImC could be one of the mechanisms by which a growing tumor may induce an antigen-specific CD8⁺ T-cell unresponsiveness. It seems logical that elimination of those immune-suppressive cells may help to enhance the immune antitumor mechanisms in patients.

A promising, clinically relevant approach to reduce the proportion of ImC in tumor-bearing hosts may be use of agents that promote the differentiation of myeloid progenitors. Retinoic acids, ligands of the retinoic acid receptors [RAR; retinoid X receptor (RXR)], are among the compounds that may stimulate

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differentiation of myeloid progenitors into myeloid dendritic cells (5, 22). Mice with vitamin A deficiency (23) and mice treated with a pan-RAR antagonist (24) show accumulation of CD11b⁺Gr-1⁺ myeloid cells similar to the ImC that accumulate in cancer patients. Physiologic concentrations of *all-trans* retinoic acid (ATRA) induced *in vitro* differentiation of ImC from tumor-bearing mice into CD11c⁺MHC class II⁺ myeloid dendritic cell (5). *In vivo*, parenteral or oral administration of ATRA significantly reduced the presence of ImC in two different tested tumor models (25), independent of a direct antitumor effect of ATRA or decreased production of growth factors by tumor cells. Experiments with adoptive transfer showed that ATRA differentiated ImC *in vivo* into mature dendritic cell, macrophages, and granulocytes. The decreased presence of ImC in tumor-bearing mice noticeably improved CD4- and CD8-mediated tumor-specific immune responses. The combination of ATRA with two different types of cancer vaccines, in two different tumor models, significantly prolonged the antitumor effect of the treatment (25). These data suggest that elimination of ImC with ATRA could be a promising approach to improvement of immune response in cancer.

However, all these studies were done *in vitro* or *in vivo* in mice. The critical questions are if and how to use ATRA therapeutically to eliminate ImC in cancer patients. For the first time, in this study, we evaluate the effect of ATRA treatment on phenotype and function of dendritic cells, the presence of ImC, and antigen-specific immune response in metastatic, clear cell type, and kidney cancer patients.

Materials and Methods

Patient selection and treatment. Competent adults (>18) with metastatic kidney cancer were eligible to participate in this study, which was approved by the University of South Florida Institutional Review Board. All patients in the ATRA-treated group had histologically confirmed renal carcinoma with "clear cell component," identifiable metastatic disease, Eastern Cooperative Oncology Group performance status ≤ 2 , adequate renal and liver function, and no signs of anemia and leukopenia. Primary tumor was removed via nephrectomy or partial nephrectomy at least 90 days before start of treatment, other tumor debulking surgery at least 30 days before start, radiation therapy completed at least 7 days before start, and no required steroid treatment. Main exclusion criteria were the presence of not-resected primary tumor; brain metastasis with <90 days of stability after the end of active treatment; ≥ 3 days of steroids at greater than physiologic replacement dose within the last 90 days; HIV; pregnancy (ATRA is a known teratogen); seizure medication that was contraindicated with ATRA; or other medical contraindication such as nonhealing wound, peripheral vascular disease, or congestive heart failure worse than New York Heart Association class I, or myocardial infarction or hypertension within 1 year.

ATRA (Vesanoid) was obtained from commercial supply and provided to the subjects. A range of ATRA dose based on the experience in treatment of promyelocytic leukemia patients was selected as follows: 50, 100, and 150 mg/m²/d. The ATRA treatment dose was divided in three portions using 10 mg Vesanoid capsules, with instructions to take three a day, with at least 6 hours between each dose; missed doses were not made up for 21 doses. One week after the finish of ATRA, therapy with interleukin-2 (IL-2) started. The IL-2 (Proleukin) treatment included 30 planned doses, given as five doses per week with 2-day breaks (weekends), over 6 weeks. The first five doses were 250,000 IU/kg, and 125,000 IU/kg for the next 25 doses (26). Observed toxicity was graded using CTCAE3 criteria and tabulated. Management of toxicity was based on dose omission (ATRA, IL-2) or dose reduction. Following the completion of the IL-2, there was a 2- to 3-week break, with clinical and radiologic evaluation of the treatment response. Eight healthy donors comprised a control group.

Fifteen subjects received i.v., high-dose IL-2 for therapy of kidney cancer or melanoma among patients on a separate trial testing a novel schedule using courses of five doses of 600,000 units/kg/dose i.v. IL-2 at 8-hour intervals, repeated on 4 consecutive weeks (clinical results to be reported separately). The specimens collected at baseline and at the start of the 3rd week (after planned 10 doses of 600,000 units/kg IL-2) comprised the specimens used for an IL-2-only treatment group. These are at a 14-day interval, for the purpose of assessing IL-2 effect, to the day 14 and day 28 specimens from the ATRA-treated subjects.

Collection of blood samples and ATRA plasma level testing. For evaluation of immunologic variables, blood was collected before start of ATRA treatment, after the end of ATRA treatment (7 days after start), 1 week after the end of ATRA treatment before start of IL-2 therapy (14 days after start), and then every week for 4 weeks. Mononuclear cells (MNC) isolated at each of these time points were cryopreserved and stored for future analysis.

Specimens for ATRA pharmacokinetics were collected after the 1st (day 1), 10th (day 4), and 21st (day 7) doses, at time points "baseline," 1, 2, 3, and 4 hours after ATRA administration. These time points were selected based on previously published pharmacokinetics of ATRA (27, 28). The measurement of ATRA level in plasma was done at the Clinical Pharmacology Laboratory at the H. Lee Moffitt Cancer Center & Research Institute. Heparinized plasma samples that had been stored frozen were thawed and processed as described (29, 30). ATRA used to prepare solutions for standards was obtained from Sigma-Aldrich (St. Louis, MO). The internal standard chosen was retinol acetate (Sigma-Aldrich). The chromatography was done on an Agilent 1100 high-performance liquid chromatography system using a Zorbax 5 μ m RX-C₁₈ column (Agilent Technologies, Wilmington, DE) with a mobile phase consisting of a gradient of acetic acid (0.1%, v/v)/methanol/methyl *tert*-butyl ether (20:65.5:14.5, v/v/v) that increased in organic content to 10:74:16 (v/v/v) over 5 minutes, then held constant for 6 minutes, and returned to original conditions in 2 additional minutes. The run time was 16.5 minutes at a flow rate of 0.8 mL/min. The compounds were detected at a wavelength of 354 nm, with a linear range from 10 to 500 ng/mL. Quality control samples were checked at 30, 80, and 250 ng/mL and the limit of quantitation for the assay was 10 ng/mL. Calibration curves were calculated by linear regression with 1/y weighting to determine the slopes, intercepts, and correlation coefficients. Unknowns were plotted against the regression analysis of each assay to determine the concentration.

Evaluation of cell phenotype. All samples from one patient were analyzed simultaneously. Peripheral blood MNCs were thawed, cultured overnight in complete culture medium (RPMI 1640 and 10% FCS), and then used for the analysis. Cells were labeled with the antibodies for 40 minutes on ice and analyzed the same day by flow cytometry. The cocktail of lineage (Lin)-specific antibodies included phycoerythrin-conjugated antibodies against CD3, CD19, CD56, and CD14 (all from BD PharMingen, Franklin Lakes, NJ). In addition, we used PerCP-conjugated anti-HLA-DR antibody; antigen-presenting cell-conjugated anti-CD33 and CD11c antibodies; and FITC-conjugated anti-CD86, CD83, and CD40 antibodies (all from BD PharMingen). FITC-conjugated antibodies against CCR7 and CD123 were obtained from R&D Systems (Minneapolis, MN) and Miltenyi (Auburn, CA) respectively. The phenotype of the cells was evaluated by multicolor flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). One hundred thousand cells were collected from each variable to obtain reliable data. The following subsets of cells were evaluated: (a) immature myeloid cells: Lin⁻HLA⁻DR⁻CD33⁺; (b) dendritic cells: Lin⁻HLA⁺DR⁺; (c) myeloid dendritic cells: Lin⁻HLA⁺DR⁺CD11c⁺CD123⁻; (d) plasmacytoid dendritic cells: Lin⁻HLA⁺DR⁺CD11c⁻CD123⁺; (e) mature dendritic cells: Lin⁺HLA⁺DR⁺ and either CD86⁺, CD40⁺, CD83⁺, or CCR7⁺; (f) regulatory T cells: the presence of T regulatory cells was determined by staining the cells with antigen-presenting cell-conjugated anti-CD4, FITC conjugated anti-CD25 (both of which were from BD PharMingen), and phycoerythrin-conjugated anti-GITR (R&D Systems, Minneapolis, MN) antibodies.

Allogeneic mixed leukocyte reaction. T cells were purified from a leukocyte-enriched buffy coat from healthy donors using T-cell enrichment column (R&D Systems) according to the instructions from the

manufacturer. MNCs obtained from patients were first irradiated at 25 Gy and then incubated with donor T cells in triplicates in round-bottomed, 96-well plates at T cells/MNC ratios 1:1, 1:2, 1:4, and 1:8. On day 4, ³[H]thymidine was added (1 μCi/well) and the cells were harvested 18 hours later. Thymidine uptake was measured using a liquid scintillation counter (Packard Instrument, Meriden, CT). T cells in culture medium alone were used as a background control.

Evaluation of T-cell function. MNCs were cultured in triplicates in round-bottomed, 96-well plates (10⁵ MNC/well) for 4 days with either 0.1 μg/mL tetanus-toxoid (List Biological Labs, Campbell, CA) or 5 μg/mL phytohemagglutinin (PHA; Sigma). These concentrations of tetanus-toxoid and PHA were selected after initial testing. [³H]thymidine (1 μCi/well) was added 18 hours before cell harvest. Thymidine uptake was measured using a liquid scintillation counter (Packard Instrument). MNC in culture medium without any antigen were used as background controls. To measure T-cell response to stimulation with immobilized anti-CD3 antibody, round-bottomed, 96-wells plates were coated overnight at 4°C with 1 μg/mL anti-CD3 antibody (BD PharMingen) diluted in 1× Dulbecco's PBS. Excess of unbound anti-CD3 antibody was washed off with Dulbecco's PBS. Then, 1 × 10⁵ MNC in 100 μL were added in triplicates and incubated for 4 days. T-cell proliferation was measured as described above.

Cytokine bead array assay. Irradiated patient MNCs were cultured with donors' T cells at 1:1 ratio for 48 hours. The supernatants were collected and stored at -80°C, until the analysis. The profile of cytokines secreted by T cells after stimulation with patient's MNC was evaluated using the BD Cytometric Bead Array kit (BD Biosciences). Each sample tube was analyzed on the BD FACSCalibur flow cytometer using the BD FACSComp Software.

Statistical analysis. Patients were assigned to one of three ATRA dose levels, at a 1:1:1 ratio, using a randomly permuted list assignments, with the assignment generally being made on the initial day of treatment. The present report describes the experience in treatment of all 18 subjects; the original protocol allowed treatment of up to 36 subjects. The purpose of the randomization was to provide an unbiased assignment, not to compare the arms. Data were analyzed using two-sided Wilcoxon-Mann-Whitney test or Student's paired *t* test.

Results

Clinical results and ATRA pharmacokinetics. Between September 2004 and November 2005, 18 patients with metastatic kidney cancer were treated with ATRA followed by course of IL-2. There were 11 men and 7 women, with an age range 45 to 79 years (median 66 years), with performance status range of 0 to 1. Disease

features included metastatic spread to lungs (16), liver (7), bone (8), brain (1), lymph nodes (7), and other (9). Among blood test identified to be prognostic, the hemoglobin range was 9.2 to 16.0, median (13.0); the serum calcium 8.4 to 10.0 (median 9.0); and lactate dehydrogenase 279 to 978 (median 430).

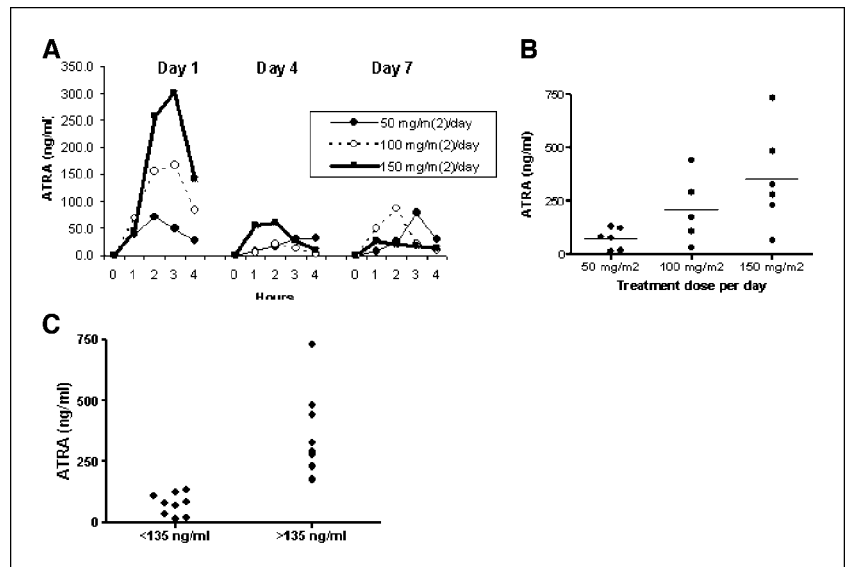
Among the side effects observed during the ATRA portion of the treatment were dry skin, myalgia, and headache, all mild to moderate grades; these typically remitted within a day or two of end of ATRA administration. Per-patient observed best clinical responses, using Response Evaluation Criteria in Solid Tumors, were evaluated after 11 to 12 weeks of treatment. Among 18 treated subjects, there were 1 complete response (lung, adrenal, and lymph nodes) ongoing at 9+ months, no partial responses, 11 stable diseases, and 3 progression. Three patients could not tolerate IL-2 treatment and it was discontinued before finishing planned treatment. Two of these three patients had progressive disease and one had stable disease.

Consistent with previous reports (31, 32), the peak of the plasma ATRA concentration was observed 2 to 3 hours after oral administration. Importantly, when assayed on day 4 or 7 of ATRA treatment, a smaller peak was observed (Fig. 1A) in the same time frame. ATRA concentration in patients' plasma varied depending on dose of the drug (Fig. 1B). However, based on the day 1 peak ATRA level, the subjects were easily separated into two groups. Nine patients had peak of ATRA concentration <135 ng/mL and eight patients had ATRA concentration >150 ng/mL (one patient specimen not collected; Fig. 1C). These two groups were composed of patients who received different doses of ATRA (Fig. 1B and C). Subsequent analysis suggests a critical role of plasma ATRA concentration in the biological effects of the drug. In further discussion, we refer to the patients with lower ATRA concentrations (<135 ng/mL) as LC patients and patients with higher ATRA concentrations (>150 ng/mL) as HC patients.

Effect of ATRA on immature myeloid cells and dendritic cells in cancer patients. Blood samples were collected before start of ATRA treatment (day 0), at the end of the treatment (day 7), before start of IL-2 therapy (day 14), and then weekly during IL-2 therapy (days 21, 28, and 35).

First, we evaluated the effect of ATRA on the number of WBC. Treatment with ATRA did not affect the total number of WBC,

Figure 1. ATRA pharmacokinetics. Patients with renal cell carcinoma were treated with indicated doses of ATRA and the level of the compounds was measured in plasma as described in Materials and Methods. Six patients had received 50 mg/m²/d dose, six patients 100 mg/m²/d, and six patients 150 mg/m²/d. ATRA concentrations were measured in 17 of 18 treated patients. *A*, plasma ATRA concentrations at different time points after administration of the single dose of the drug. Measurements were done on days 1, 4, and 7 during the course of the treatment. *B*, peak level of ATRA after first dose. Individual results and mean values in the groups receiving different doses. *C*, peaks of ATRA concentrations in all patients.



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Table 1. Effect of ATRA and IL-2 treatment on the number of WBC

Variables	Day 0 (before treatment)	Day 7 (end of ATRA treatment)	Day 14 (before start of IL-2)	Day 28 (2 wk of IL-2)	Day 42 (4 wk of IL-2)
WBC ($\times 10^6$ /mL)	6.22 \pm 1.55	5.41 \pm 1.53 (<i>P</i> = 0.07)	6.71 \pm 1.92 (<i>P</i> = 0.31)	15.75 \pm 6.32 (<i>P</i> = 0.01)	10.01 \pm 3.07 (<i>P</i> = 0.07)
Neutrophils ($\times 10^6$ /mL)	3.99 \pm 1.18	3.66 \pm 1.49 (<i>P</i> = 0.14)	4.50 \pm 1.60 (<i>P</i> = 0.18)	5.51 \pm 2.64 (<i>P</i> = 0.02)	3.06 \pm 1.47 (<i>P</i> = 0.26)
Lymphocytes ($\times 10^6$ /mL)	1.62 \pm 0.52	1.47 \pm 0.29 (<i>P</i> = 0.15)	1.53 \pm 0.68 (<i>P</i> = 0.85)	4.40 \pm 1.73 (<i>P</i> = 0.005)	3.50 \pm 1.40 (<i>P</i> = 0.001)
Monocytes ($\times 10^6$ /mL)	0.58 \pm 0.19	0.40 \pm 0.15 (<i>P</i> = 0.04)	0.53 \pm 0.17 (<i>P</i> = 0.23)	0.66 \pm 0.52 (<i>P</i> = 0.73)	0.57 \pm 0.22 (<i>P</i> = 0.71)

NOTE: Eighteen patients were evaluated. Mean \pm SD deviation are shown. *P* values were calculated using two-sided Student's *t* test. Each time point was compared with pretreatment level (day 0).

neutrophils, or lymphocytes but induced slight decrease in the number of monocytes, which was restored to pretreatment level 1 week after finish of the treatment (Table 1). IL-2 treatment resulted in significant increased in total number of WBC, neutrophils lymphocytes, but not monocytes (Table 1).

ImCs were defined as lineage-negative, HLA-DR-negative cells that express myeloid marker CD33. Patients with metastatic renal cell carcinoma had significant increase in this cell population (Fig. 2A). After 7 days of ATRA treatment, their proportion was significantly reduced and remained at that level 7 days later. IL-2 treatment eliminated this effect and increased ImC to the pretreatment level (Fig. 2A). The effect of ATRA on ImC was dependent on achieved ATRA concentration. Patients with LC and HC had similar background levels of ImC population (Fig. 2B). Only slight decrease in ImC was observed in LC patients, whereas proportion of ImC in HC patients was reduced dramatically and reached the control level. Subsequent IL-2 treatment increased the proportion of ImC in both groups of patients (Fig. 2B).

The presence of Lin⁻HLA-DR⁺ dendritic cells was significantly reduced in the cancer patients. In contrast to the effect on ImC,

ATRA did not change the proportion of dendritic cells (Fig. 2C and D). There was a trend for an increased proportion of dendritic cells but it did not reach statistical significance. IL-2 treatment significantly reduced the proportion of these cells (Fig. 2C and D). As a result of the changes in the proportion of ImC and dendritic cells, the cancer patients had significantly reduced dendritic cell/ImC ratio from 27.3 to 16.3 (*P* < 0.05). ATRA restored it to the control level (25.4 on day 7 and 29.6 on day 14, *P* > 0.1). Subsequent treatment of patients with IL-2 had just opposite effect by significantly reducing this ratio (17.8 on day 21, 10.1 on day 28, and 13.0 on day 35, *P* < 0.05).

We evaluated the presence of two major subtypes of dendritic cells: myeloid dendritic cells and plasmacytoid dendritic cells. The proportion of mature dendritic cells was evaluated within the population Lin⁻HLA-DR⁺ cells based on the expression of four markers typically associated with the phenotype of mature cells: CD86, CD40, CD83, and CCR7. The proportion of myeloid dendritic cells in renal cell carcinoma patients was significantly reduced (Fig. 3A), whereas the proportion of plasmacytoid dendritic cells remained at the control level (Fig. 3B). This resulted in significant

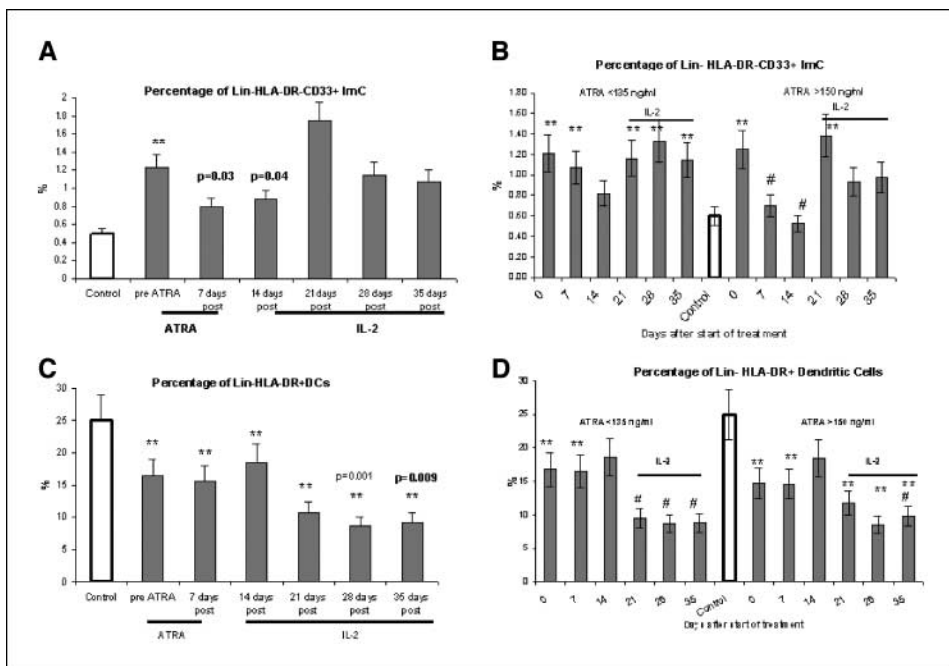
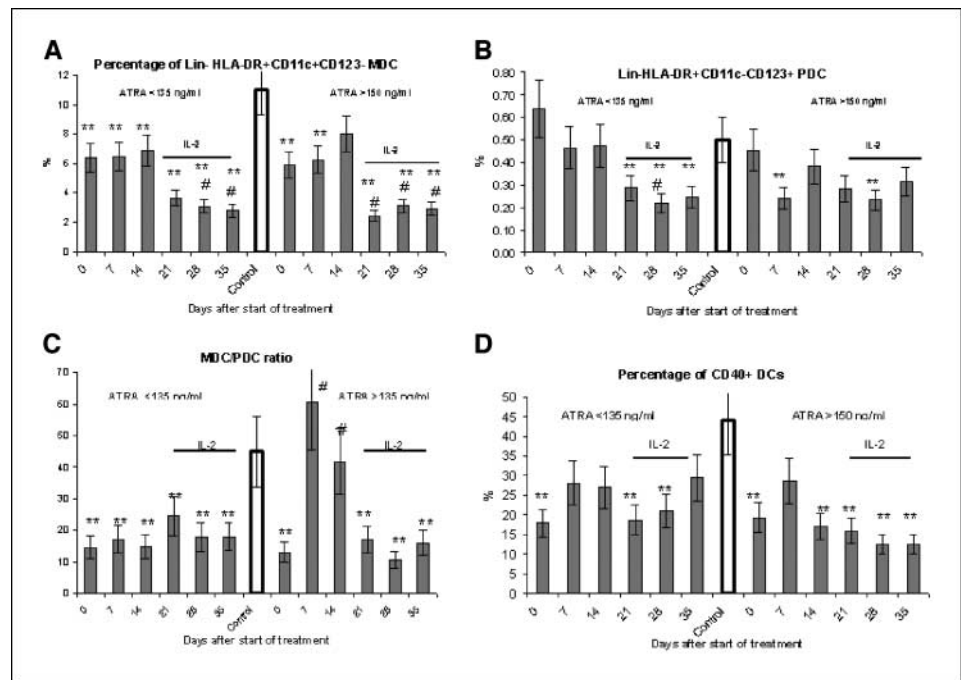


Figure 2. Effect of ATRA treatment on the proportion of ImC and dendritic cells. MNCs from cancer patients and healthy donors were stored in liquid nitrogen. All samples from the same patient or donor were thawed simultaneously and cells were incubated in complete culture medium overnight at 37°C and 5% CO₂. After that time, cells were labeled with antibodies and analyzed by multicolor flow cytometry as described in Materials and Methods. A and C, proportion of ImC and dendritic cells in all 18 patients treated with ATRA. Control, data from eight healthy volunteers and donors. Columns, mean; bars, SE. **, *P* < 0.05, statistically significant differences from control group. Actual *P* values are shown in groups that statistically different (*P* < 0.05) from pretreatment level. B and D, proportion of ImC and dendritic cells in patients with different ATRA concentration in plasma. Nine patients had lower ATRA concentrations (<135 ng/mL) and eight patients had higher ATRA concentrations (>150 ng/mL). **, *P* < 0.05, statistically significant differences from control group. #, *P* < 0.05, statistically significant difference from pretreatment level.

Figure 3. Effect of ATRA treatment on the proportion of dendritic cell populations. A to D, results in patients with different levels of ATRA in plasma. All abbreviations are as in Fig. 2B and D. MDC, myeloid dendritic cells; PDC, plasmacytoid dendritic cells.



decrease in myeloid dendritic cell/plasmacytoid dendritic cell ratio (Fig. 3C). No changes in the levels of these dendritic cell populations were observed in patients with ATRA LC (Fig. 3A-C). Patients with ATRA HC showed slight increase in the proportion of myeloid dendritic cells and decrease in the proportion of plasmacytoid dendritic cells. Although these changes were not statistically significant, they resulted in a substantial improvement of myeloid dendritic cell/plasmacytoid dendritic cell ratio (Fig. 3A-C). Subsequent treatment with IL-2 equally reduced the proportion of both these populations and therefore did not change the proportion of myeloid dendritic cell/plasmacytoid dendritic cell (Fig. 3A-C). The proportion of mature dendritic cells was significantly reduced in patients with renal cell carcinoma. Treatment with ATRA resulted in slight increase in their presence. However, it did not reach statistical significance (Fig. 3D and data not shown).

ATRA effect on dendritic cell function and immune response in cancer patients. Next, we evaluated the function of dendritic cells in allogeneic MLR, a hallmark of dendritic cell activity. MNC obtained from cancer patients had significantly reduced ability to stimulate control allogeneic T cells (Fig. 4A). ATRA substantially improved MNC-induced stimulation of allogeneic T cells. This effect was observed only in patients with HC but not in those with LC. IL-2 therapy reversed this effect (Fig. 4A). We studied the type of cytokines produced by T cells after stimulation with patients' MNC in allogeneic MLR. MNC from patients treated with ATRA stimulated T-cell production of IFN- γ and IL-2 and decreased the production of Th2-type cytokine (IL-5). Another Th2-type cytokine IL-10 was below the level of detection. These changes resulted in increase of Th1/Th2-type cytokines ratios from 4.6 before ATRA treatment to 51.2, 7 days after ATRA treatment ($P < 0.05$) and 69.3 after 14 days of ATRA treatment ($P < 0.05$).

To evaluate antigen-specific T-cell response in cancer patients, MNCs were stimulated with tetanus-toxoid. Consistent with previous observations (reviewed in ref. 1), tetanus-toxoid-specific

response was significantly reduced in cancer patients (Fig. 4B). In LC group, no effect of ATRA on tetanus-toxoid-specific T-cell proliferation was found. In HC group, ATRA significantly increased this response, which was restored to the control level (Fig. 4B). In contrast, anti-CD3 antibody or PHA-induced T-cell responses were not affected in these patients, and treatment with ATRA did not result in significant changes in T-cell proliferation (Fig. 4C and D).

ATRA treatment did not affect the proportion of CD4⁺CD25⁺GITR⁺ regulatory T cells (data not shown). IL-2 therapy initially increased that proportion albeit the change was not statistically significant (from 0.89 ± 1.09 before start of the treatment to 4.70 ± 7.42 on day 21; $P = 0.06$). It decreased slightly later during the course of IL-2 treatment (2.88 ± 4.24 on day 28 and 1.87 ± 1.72 on day 35, $P > 0.06$ in both cases).

The effect of IL-2 therapy alone on ImC, dendritic cells, and T-cell responses. The data presented above showed that effects of ATRA on ImC, dendritic cell subsets, and function were eliminated after start of IL-2 therapy. It was not clear whether those changes were indeed the effect of IL-2 therapy or the result of a time-dependent attenuation of the ATRA effect. The original design of the trial did not allow for long observation period between ATRA and IL-2 treatment. Therefore, to evaluate potential role of IL-2 on immunologic variables, we tested samples of blood obtained from patients enrolled in separate clinical trial.

Fifteen patients with metastatic cancer were treated as described in Materials and Methods. They have received several cycles of IL-2 therapy alone. Blood was collected before treatment and after the first 3-week cycle. Treatment with IL-2 alone produced significant decrease in the proportion of dendritic cells and myeloid dendritic cells but not plasmacytoid dendritic cells (Fig. 5). Slight increase in the proportion of ImC was observed. As a result, dendritic cell/ImC and myeloid dendritic cell/plasmacytoid dendritic cell ratios were reduced significantly (Fig. 5). IL-2 therapy also significantly decreased the ability of MNC to stimulate allogeneic T cells. The response of T cells to stimulation with

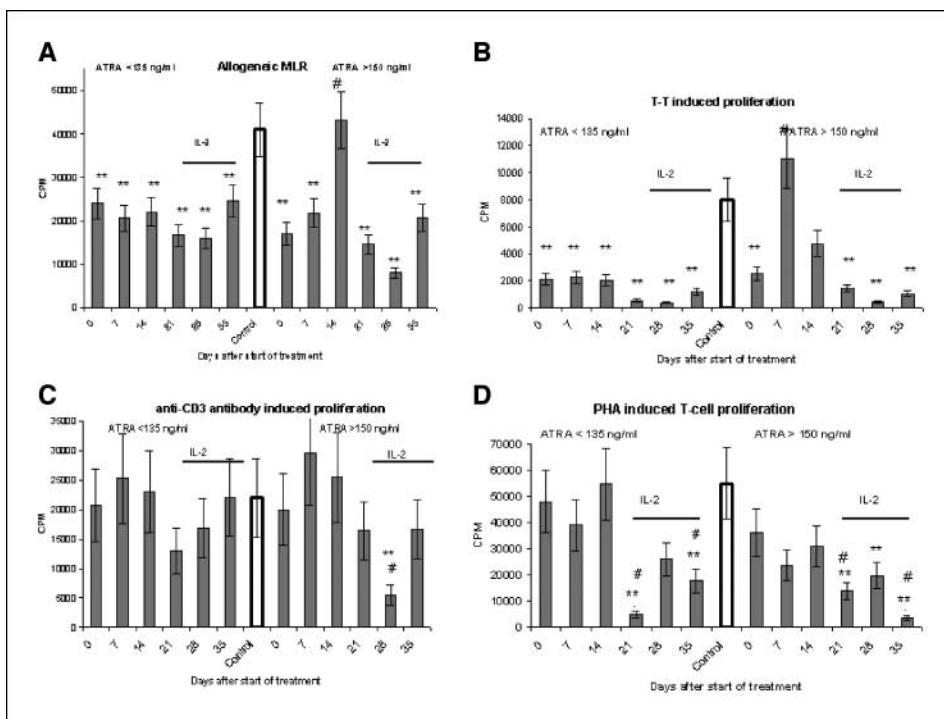


Figure 4. Effect of ATRA treatment on dendritic cell function and antigen-specific immune response. A, MNC isolated from patients and donors were irradiated and cultured for 5 days in triplicates in U-bottomed, 96-well plates with 10^5 allogeneic T cells at different ratios. [3 H]thymidine (1 μ Ci) was added to each well 18 hours before cell harvesting. Thymidine uptake was measured in liquid scintillation counter as described in Materials and Methods. The background values of T-cell proliferation were subtracted. Only one (1:1) ratio is shown. **, $P < 0.05$, statistically significant differences from control group. #, $P < 0.05$, statistically significant difference from pretreatment level. B to D, MNC (2×10^5 /well) were incubated in triplicates with 0.1 μ g/mL tetanus-toxoid (B), immobilized anti-CD3 antibody (C), or 5 μ g/mL PHA (D) for 4 days. [3 H]thymidine (1 μ Ci) was added to each well 18 hours before cell harvesting. The background values (MNC incubated with medium alone) were subtracted. All abbreviations are as in Fig. 2.

tetanus-toxoid or anti-CD3 antibody was reduced. However, those differences did not reach statistical significance (Fig. 5). In addition, IL-2 significantly increased the proportion of regulatory T cells.

Discussion

It is well established that tumor growth is associated with decreased presence of dendritic cells and accumulation of ImC. Studies from many groups have identified immunosuppressive ImC as one of the major factors contributing to tumor evasion of T-cell-mediated rejection (2, 33, 34). Elimination of these cells could improve antitumor immune responses and enhance the effect of the vaccines. Previous studies have shown that ATRA could differentiate mouse ImC *in vitro* (5) and *in vivo* (25) and patients' ImC *in vitro* (7, 35). A naturally occurring isomer of retinoic acid, ATRA is a molecule capable of induction of differentiation of the human leukemia cell line HL-60, freshly isolated acute promyelocytic leukemia cells and of affecting the growth of hematopoietic progenitors in acute myelogenous leukemia (36–38). It is successfully used in differentiation induction therapy in patients with acute promyelocytic leukemia (39, 40). At the molecular level, ATRA activates nuclear receptors, which belong to the family of steroid/thyroid/retinoid-activated transcriptional regulators. Two classes of retinoid receptors—RAR α , RAR β , RAR γ and RXR α , RXR β , RXR γ —activate transduction of target genes via complex genetic and epigenetic mechanisms (41). In therapeutic concentrations, ATRA does not directly inhibit the growth of most solid tumors. This could be partly explained by decreased expression of the receptors (42). In this study, we addressed, for the first time, the question whether ATRA can affect ImC and dendritic cells in cancer patients. This question is critical not only for understanding the biology of ATRA effects on human myeloid cells but also for the potential use of this treatment in association with cancer vaccines. The trial was done

in patients with metastatic clear cell kidney cancer. The ATRA treatment was followed by an IL-2 schedule for which there is extensive experience.

Although generally well tolerated, ATRA has pharmacokinetics that are complicated by heterogeneity of absorption and clearance between individuals, and the capacity to induce its own metabolism with chronic dosing. Therefore, it was important to establish an optimal dose schedule of the drug. We used a 3-fold range of dose levels, encompassing doses previously identified to be generally well tolerated and safe as well as having a biological effect (induced maturation and apoptosis of APL cells of promyelocytic leukemia; refs. 31, 43). As anticipated, ATRA pharmacokinetics exhibited wide interpatient variability. Median peak levels declined over the 7-day ATRA treatment period, consistent with previous observations that continuous administration of ATRA resulted in decreased plasma level of this drug (31). Analysis of ATRA levels in patients' blood revealed the existence of two groups with relatively low and high peak ATRA concentrations. It is important that low concentration group was composed of all six patients that received lowest dose of ATRA (50 $\text{mg}/\text{m}^2/\text{d}$) but also two patients who received the 100 $\text{mg}/\text{m}^2/\text{d}$ dose and one patient with 150 $\text{mg}/\text{m}^2/\text{d}$ dose. To evaluate the relationship between ATRA concentration and its effect on ImC and dendritic cells, we compared ATRA HC and LC patients. This plasma level-based analysis is more logical than the dose-assigned one, because of the heterogeneity issue. Each dose group was relatively small (only six patients) and some patients in the groups with higher dose had low ATRA concentration in plasma. The mechanism of this heterogeneity is not entirely clear. ATRA does not require a specific transport mechanism; therefore, general conditions that influence absorption of the drug during oral administration like quantity and quality of recent food intake, the condition of gastrointestinal tract, etc., may affect the absorption and serum concentration of ATRA. The heterogeneity observed within dose

cohorts seems comparable with that seen in previous studies. It is likely that in a larger cohort, these differences will be less prominent and patients can be separated based only on dose administered. Our data indicated that ATRA concentration below 135 ng/mL did not significantly affect either phenotype or function of dendritic cells and ImC, whereas higher concentration (>150 ng/mL) showed a significant effect. This points to the specific nature of the observed effects of ATRA and suggests that ATRA dose of 150 mg/m²/d could be the best for achieving a biological effect. At the highest dose level, ATRA concentrations in plasma on days 4 and 7 were similar to the ineffective level observed on day 1 of the lower ATRA dose. This provides a basis to conjecture that a shorter duration at the higher dose level could be sufficient to achieve optimal effect.

ATRA treatment did not change the number of WBC. Only slight decrease in monocyte count was found immediately after finish of the treatment. It returned to pretreatment level 7 days later. To reduce interexperimental variations, MNC collected at different time points from the same patient were thawed and analyzed at the same time. Although no additional cell purification was done, MNCs were cultured overnight before the analysis. This was important for two reasons. First, the phenotype and especially the function and of the cells analyzed immediately after the thawing can be significantly affected. Second, normal peripheral blood contains small proportion of immature myeloid cells and immature dendritic cells that quickly differentiate during overnight culture. This probably reflects the process that is taking place in tissues. We wanted to distinguish those cells from abnormal ImC accumulated in cancer patients. Overnight incubation would help to achieve this

goal. As previously observed in other advanced cancer patients (7, 20), subjects with metastatic kidney cancer have almost 3-fold excess of ImC. ATRA significantly reduced that proportion. This was evident when samples from all patients on trial were analyzed together. However, the differences became stronger when we compared patients with low and high ATRA plasma concentration. Slight decrease in ImC was found in former group, whereas the latter one showed dramatic reduction of the proportion of these cells, restoring them to the control level. Thus, these data confirmed previous *in vitro* observations (7, 35). ATRA had little effect on the proportion of total dendritic cells. The ratio between myeloid and lymphoid dendritic cells may play an important role in tumor-associated immune defects in light of observations that myeloid dendritic cells primarily responsible for induction of immune response and plasmacytoid dendritic cells under certain circumstances can have immunosuppressive features (2, 44–46). In a number of studies, preferential loss of myeloid dendritic cells but not plasmacytoid dendritic cells in cancer patients was reported (20, 47, 48). A similar effect was observed in the patients with metastatic renal cell carcinoma. The proportion of myeloid dendritic cells was significantly reduced, whereas plasmacytoid dendritic cells were not affected. This resulted in dramatic drop in myeloid dendritic cell/plasmacytoid dendritic cell ratio. At “effective” concentrations, ATRA slightly increased the proportion of myeloid dendritic cells and decreased the proportion of plasmacytoid dendritic cells that restored myeloid dendritic cell/plasmacytoid dendritic cell ratio to the control level. Our previous study *in vitro* has shown that ImC isolated from cancer patients suppressed allogeneic MLR and antigen-specific T-cell

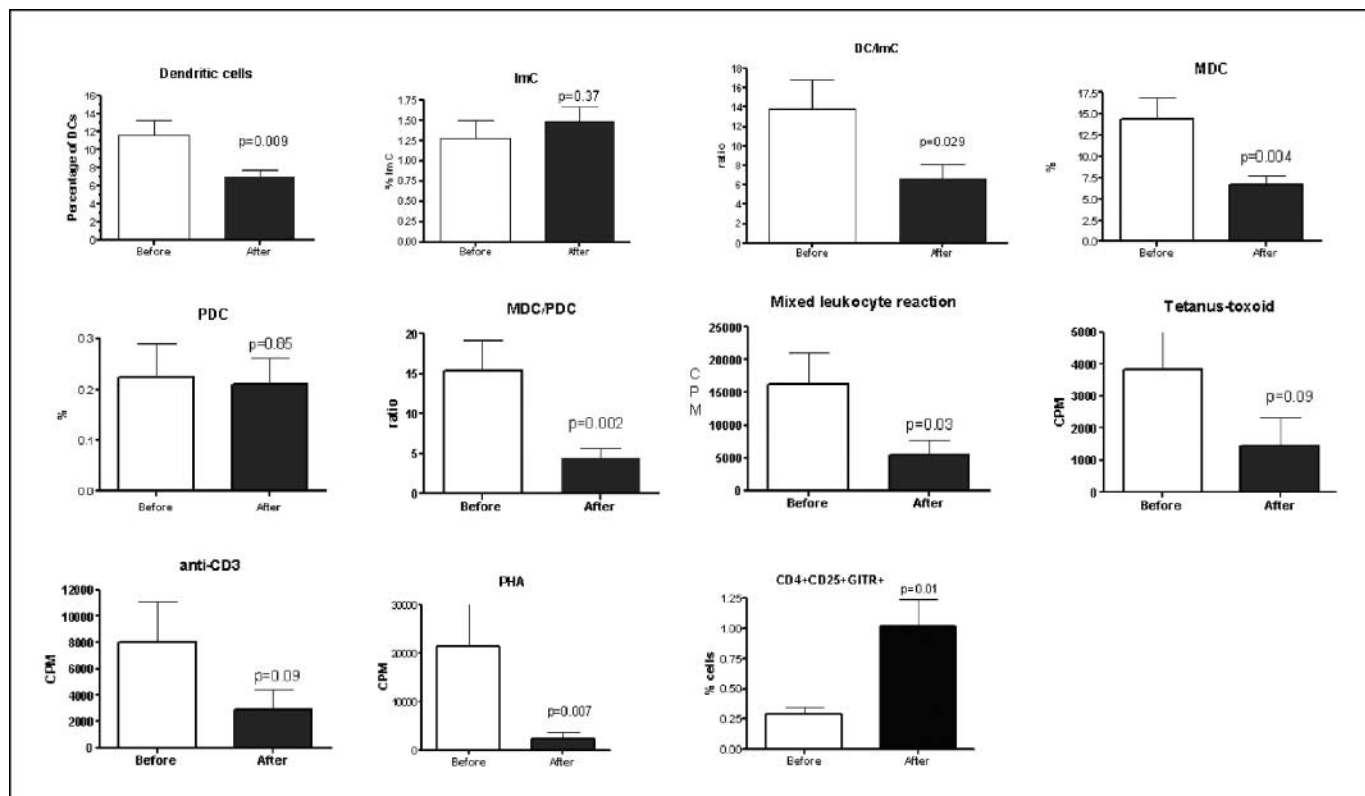


Figure 5. Effect of IL-2 on ImC and dendritic cells. Fifteen patients with renal cell carcinoma were treated with IL-2 alone. Blood samples were collected before the start of the treatment and after finish of one 4-week cycle of the therapy. Immunologic variables were evaluated as described above. Actual P values in two-sided paired Wilcoxon-Mann-Whitney tests are shown.

responses (7). Elimination of ImC and increase in myeloid dendritic cell/plasmacytoid dendritic cell ratio after ATRA treatment apparently was responsible for the significant improvement of allogeneic MLR and antigen-specific T-cell response (tetanus-toxoid), which depends on adequate function of antigen-presenting cells. ATRA did not affect T-cell response to immobilized CD3 antibody and PHA-induced T-cell proliferation that requires little or no antigen-presenting cell participation. Consistent with preferential effect on myeloid rather than T-cell compartment, ATRA did not affect regulatory T cells.

All patients on this trial were treated with s.c. IL-2 therapy following ATRA. Therefore, the duration of ATRA effect could not be clearly established in this study. IL-2 therapy substantially increased the total number of lymphocytes. This effect was reported in previous clinical trials and was attributed to accumulation of natural killer (NK) and lymphokine-activated killer (LAK) cells (reviewed in ref. 49). Seven days after start of IL-2 therapy, the proportion of ImC was significantly increased. It was especially dramatic when taking into account the significant increase in the total number of MNCs. Apparently, in addition to stimulation of lymphocyte production, IL-2 also stimulates myeloid lineage that resulted in increased production of immature cells and decreased proportion of dendritic cells. Increased number of neutrophils observed in these patients was consistent with this fact. Importantly, IL-2 also decreased antigen- and mitogen-driven T-cell proliferation. Although the precise mechanism of this effect is not clear, it could be attributed to a significant increase in the proportion of regulatory T cells observed in these patients. Investigation of the effect of IL-2 was not a goal of this study. However, it is consistent with a hypothesis that limited clinical success of IL-2 therapy could be attributable to the accumulation of immunosuppressive ImC, which are able to suppress the very same antigen-specific immune response IL-2 therapy is trying to enhance. It has been previously reported that some patients showed profound activation of NK and LAK without any evidence of tumor regression (50).

Observed IL-2 effects raised important question, whether elimination of the positive effect of ATRA on ImC and dendritic cells was due to IL-2 effect or due to the short duration of ATRA effect. Precise analysis would require new trial in a different group of patients where the effect of ATRA could be monitored for a longer period of time. However, in a framework on this study, we could partially address this question by evaluating the effect of IL-2 in patients who did not receive ATRA treatment. Our data showed that IL-2 alone exerted exactly the same effect as in combination with ATRA. This strongly suggests that abrogation of ATRA effect was probably due to the effect of IL-2. Our study suggests that for the purpose of improvement of host immune responses and to provide the support for cancer vaccines ATRA probably should not be combined with IL-2. However, IL-2 therapy can have a major therapeutic effect in a subset of patients with renal cell cancer. It is possible that accumulation of ImS and Treg in patients treated with IL-2 could limit the clinical efficacy of this therapy. If this is the case, then ATRA could be considered as potentially useful addition to IL-2. Specifically designed clinical trials are needed to address this question.

Thus, at an effective dose, ATRA treatment substantially reduced the presence of ImC and improved myeloid/lymphoid dendritic cell ratio. This resulted in improved dendritic cell function and antigen-specific immune response. This indicates that impaired dendritic cell phenotype and immune function in patients with advanced cancer can be effectively modulated pharmacologically and provides strong rationale for combining ATRA treatment with different cancer vaccines.

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