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All-*trans*-Retinoic Acid Eliminates Immature Myeloid Cells from Tumor-bearing Mice and Improves the Effect of Vaccination

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ABSTRACT

Tumor-induced immunosuppression is one of the crucial mechanisms of tumor evasion of immune surveillance. It contributes greatly to the failure of cancer vaccines. Immature myeloid cells (ImCs) play an important role in tumor-induced immunosuppression. These cells accumulate in large numbers in tumor-bearing hosts and directly inhibit T-cell functions via various mechanisms. In this study, we tried to eliminate ImCs in an attempt to improve antitumor response. *In vivo* administration of all-*trans*-retinoic acid (ATRA) dramatically reduced the presence of ImCs in all tested tumor models. This effect was not because of a direct antitumor effect of ATRA or decreased production of growth factors by tumor cells. Experiments with adoptive transfer demonstrated that ATRA differentiated ImC *in vivo* into mature dendritic cells, macrophages, and granulocytes. Decreased presence of ImC in tumor-bearing mice noticeably improved CD4- and CD8-mediated tumor-specific immune response. Combination of ATRA with two different types of cancer vaccines in two different tumor models significantly prolonged the antitumor effect of the treatment. These data suggest that elimination of ImC with ATRA may open an opportunity to improve the effect of cancer vaccines.

INTRODUCTION

Tumor-induced immunosuppression is one of the critical mechanisms of tumor escape. It is probably one of the major factors responsible for the failure of cancer vaccines, especially in patients with advanced disease. Recent studies have identified ImCs[3] as one of the important elements in tumor-associated immunosuppression (1–8). ImCs accumulate in all tested animal tumor models and in patients with all tested types of cancer. In mice, these ImCs have the phenotype of Gr-1$^+$CD11b$^+$ cells and inhibit antigen-specific and nonspecific T-cell functions via several different mechanisms (8–11). Thus, ImCs may be responsible for the inability of T cells from tumor-bearing hosts to effectively recognize and eliminate tumor cells in an antigen-specific manner. Increased presence of ImCs could be one of the reasons for vaccine failure in patients with advanced stage cancer because these cells can inhibit the very same antigen-specific immune response that a cancer vaccine is trying to generate. The increased presence of ImCs capable of inhibiting T-cell responses could also be a major factor contributing to the development of tumor-induced T-cell tolerance previously described in tumor-bearing hosts (12, 13). All these data suggest that elimination of ImCs may significantly improve antitumor immune response and enhance the effect of cancer vaccines. One of the approaches to achieve this goal could be the differentiation of ImCs. In our previous studies, we have demonstrated that several combinations of growth factors that promote differentiation of DCs, macrophages, and granulocytes provide a rather minor effect on the population of ImCs (3, 11). As a naturally occurring isomer of retinoic acid, ATRA is a well-known factor capable of induction of differentiation of human leukemia cell line HL-60 and freshly isolated acute promyelocytic leukemia cells (14, 15). It has been successfully used in differentiation induction therapy in patients with acute promyelocytic leukemia (16, 17). ATRA may also affect growth of normal hematopoietic progenitors and blast progenitors in acute myelogeneous leukemia (18–20). In our *in vitro* experiments, ATRA dramatically reduced the presence of ImCs in humans and mice (3, 11). These data suggested that ATRA could be an effective differentiation agent for ImCs. In this study, we have tested this hypothesis in several experimental tumor models. We asked whether ATRA is capable of reducing the presence of ImCs in tumor-bearing mice and whether such reduction has any effect on the development of tumor-associated immune tolerance and response to vaccination.

MATERIALS AND METHODS

Mice. Female C57BL/6 and BALB/c mice 6–8 weeks old were obtained from the National Cancer Institute (Frederick, MD). B6.SJL-PtrcaPep3b/BoyJ mice (CD45.1$^+$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TCR-transgenic mice expressing an α/β TCR specific for MHC class II-restricted SFERFEIFPKE peptide, derived from influenza HA, were originally obtained from Harold von Boehmer (Basel Institute for Immunology, Basel, Switzerland) and then were crossed to a BALB/c background for $>$10 generations.

Reagents. Pellets with ATRA and placebo were obtained from Innovative Research of America (Sarasota, FL). Control MHC class I (K^d)-restricted (IYSTVASSL) and MHC class II (I-A^d)-restricted (SFERFEIFPKE) HA-derived peptides, MHC class I-restricted mutant p53-derived peptide (KYICNSSCM), HPV-16-derived E7 peptide (H-2K^d-restricted, aa 49–57, RAHYNIVTF), as well as OVA control peptide (H-2K^b-restricted, aa 257–264, SIINFEKL) were purchased from SynPep Corporation (Dublin, CA). A recombinant vaccinia virus-encoding HA from the 1934 PR8 strain of influenza was a gift from Frank Guarneri (Johns Hopkins Institute, Baltimore, MD). A recombinant adenovirus encoding full open-reading frame of wild-type p53 gene was described elsewhere (21). The mAbs used for flow cytometry were CD45.2 (clone 104), Gr-1 (clone RB6–8C5), CD11b (clone M1/70), CD11c (clone HL3), I-A^b (clone AF6-120.1), CD86 (clone GL1) from BD PharMingen; CD4 (clone GK1.5) and anticlonotypic TCR (clone 6.5) are from Caltag (Burlingame, CA) and F4/80 from Serotec, Inc. (Raleigh, NC).

Tumor Models. DA3, 7,12-dimethylbenz(*a*)anthracene-induced mammalian adenocarcinoma, was obtained from Dr. Diana Lopez (University of Miami, Miami, FL). DA3-HA tumor was generated by transfection of DA3 tumor cells with pIHA, which encodes the full open-reading frame of HA from the influenza virus A/PR/8/34. C3 fibrosarcoma was made by transformation of B6 mouse embryonic cells with HPV-16-induced (22) and kindly provided by Dr. Martin Kast (Loyola University of Chicago, Maywood, IL). MHC class I- restricted HPV-16-derived peptide RAHYNIVTF expressed by this tumor was shown to elicit potent antitumor immune response (23). MethA cells were obtained from Dr. Lloyd J. Old. MethA tumor is methylcholantrene-induced sarcoma developed in BALB/c mice and passaged as an ascitic tumor. It is a

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[3] The abbreviations used are: ImC, immature myeloid cell; DC, dendritic cell; TCR, T-cell receptor; HA, hemagglutinin; ATRA, all-*trans*-retinoic acid; HPV, human papillomavirus; mAb, monoclonal antibody; CFA, complete Freund adjuvant; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ad-p53, adenovirus containing wild-type p53 gene; RAR, retinoic acid receptor; RXR, retinoid X receptor.

relatively immunogenic tumor that carries a mutant endogenous p53 gene. The cell cultures were maintained at 37°C in a 5% CO_2-humidified atmosphere. DA3-HA and C3 cell lines were maintained in a DMEM culture medium supplemented with OPI medium supplement (for DA3-HA), 10% FCS, 100 units/mg penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 2 mM glutamine (all from Life Technologies, Inc., Grand Island, NY). MethA cells were passaged *in vivo* as an ascitic tumor in BALB/c mice. To initiate the experiments, tumor cells in 0.1 ml of PBS were injected s.c. into the shaved right flank of each mouse. To establish tumor, mice were injected with 10^6 DA3-HA cells, 5×10^5 C3 cells, or 10^5 MethA cells.

ATRA Pellets Implantation and Treatment Protocols. ATRA pellets (21-day release, 5 mg) or placebo pellets were implanted s.c. by trocar injection at the side contralateral to the tumor (into left flank). For *in vivo* assessment of ATRA effect on tumor-antigen specific $CD4^+$ T-cell-mediated immune response, we used DA3-HA tumor model. ATRA (or placebo) pellets were implanted into BALB/c mice on day 4 after tumor inoculation. Seven days later, 2.5×10^6 double-positive CD4 and clonotypic TCR lymphocytes from HA-TCR-transgenic mice were injected i.v. Four days later (day 15 after tumor implantation), mice were *in vivo* primed by s.c. inoculation with 1×10^7 pfu of recombinant vaccinia encoding HA in 0.1 ml of PBS. Mice were sacrificed 1 week later, and antigen-specific response was evaluated.

For evaluation of ATRA effect on $CD8^+$ T-cell tumor antigen-specific response we used a C3 tumor model. On day 5 after tumor cell inoculation, C57Bl/6 mice were immunized with s.c. injection of 50 μg of C3 peptide (RAHYNIVTF) emulsified in CFA. ATRA (or placebo) pellets were implanted 7 days later, and the same day mice were given second immunization with peptide. On day 17 after tumor inoculation, mice were immunized with C3 peptide the third time, and 10 days later, ice was sacrificed.

To study antitumor effect of ATRA in combination with immunotherapy, we used the MethA tumor model. For immunization, DCs were generated from bone marrow of BALB/c mice and infected with Ad-p53 as described below. DCs were injected s.c. into mice (4×10^5 cells/mouse) on days 5, 10, and 15 after tumor cell inoculation. ATRA pellet (or placebo pellet) was implanted on day 7 after tumor cell injection.

Cell Isolation. Mice were sacrificed by cervical dislocation, and their spleens, lymph nodes, tibias, and femurs were harvested under sterile conditions. Spleen cell suspensions were prepared, and red cells were depleted by incubation with ACK buffer. Cells were washed and resuspended in complete culture medium. Bone marrow cells were obtained by flushing the contents of the mouse femora and tibia with cold PBS. T lymphocytes were purified from spleens of naïve TCR-HA-transgenic mice using T-cell enrichment columns (MTCC-525; R&D Systems, Minneapolis, MN). $Gr\text{-}1^+$ cells were isolated from spleens of C3 tumor-bearing mice using magnetic beads. Briefly, spleen cell suspension (10^7 splenocytes/ml) was incubated for 15 min at 4°C with biotinylated anti-Gr-1 mAbs (clone RB6-8C5; BD PharMingen). Cells were washed to remove unbound antibodies and then incubated with streptavidin microbeads for 15 min at 4°C. Positive $Gr\text{-}1^+$ cell population was isolated on a MS+ MACS column according to the manufacturer's instructions (Miltenyi Biotec GmbH, Auburn, CA). Purity of Gr-1+ cell population was evaluated by flow cytometry and exceeded 90%.

Generation of Bone Marrow-derived DC and Infection with p53-Adenovirus. Generation of DCs and their infection with Ad-p53 was performed as described before (21). Briefly, DCs were generated from murine bone marrow using RPMI 1640 supplemented with 10% FCS, 20 ng/ml murine recombinant GM-CSF, and 10 ng/ml IL-4 (both from RDI, Flanders, NJ). The cultures were maintained at 37°C in 5% CO_2-humidified atmosphere in 24-well plates. On day 3 of culture, floating cells were gently removed, and fresh medium with cytokines was replaced. On day 5, cells were collected, and DCs were enriched by centrifugation over a 13.5% metrizamide gradient (Accurate Chemicals, Westbury, NY). DCs were washed in serum-free medium and infected with Ad-p53 (20,000 viral particles/cell) for 2 h in 0.5 ml of serum-free medium supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 in 24-well plates followed by culturing in complete culture medium with the cytokines for an additional 24 h.

ELISPOT Assay. ELISPOT assay was performed as described earlier (11). Briefly, MultiScreen-HA plates (Millipore Corporation, Berford, MA) were precoated with anti-IFN-γ mAbs (cloneR4-A2; BD PharMingen, San Diego, CA) by overnight incubation in PBS at 4°C. Splenocytes from immunized mice were plated in wells (2×10^5 cells/well) in complete media and cultured for 24 h at 37°C in the presence of the specific (RAHYNIVTF) or control (SIINFEKL) peptide (10 μg/ml). Wells were gently rinsed with PBS containing 0.1% Tween 20 and after adding of biotinylated anti-IFN-γ mAbs (BD PharMingen), plates were incubated overnight at 4°C. Results were visualized using avidine-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Sigma, St. Louis, MO). The number of spots in each well was scored by in blind fashion by two investigators and then recalculated per 10^6 cells.

ELISA. ELISA was performed using antibodies and protocol developed by BD PharMingen. The sensitivity of the assay was 6 pg/ml.

Flow Cytometry. Cells (10^6 in PBS containing 0.1% FCS) were incubated for 30 min with optimal concentration of antibodies on ice and then washed twice with cold PBS. Fluorescence-activated cell sorting data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Fluorescence-activated cell sorting data were analyzed using CellQuest software (BD Biosciences).

RNase Protection Assay. RNA was extracted from C3 and DA3-HA tumor cells using Trizol reagent (Life Technologies, Inc.). Twelve μg of each sample RNA was dried and resuspended in hybridization buffer. RNase protection assay was performed using BD PharMingen RiboQuant kit according to manufacturer's protocol. Briefly, $\alpha[^{32}P]$UTP-labeled multitemplate probe was synthesized in an *in vitro* transcription reaction using a template set that contained DNA templates for murine IL-3, IL-6, IL-10, IL-11, GM-CSF, M-CSF, G-CSF, VEGF, as well as L32 and GAPDH. The probe (2.8×10^5 cpm) was added to each sample. After hybridization, samples were treated with RNase, then hybridized probes were extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. Samples were subjected to electrophoreses on 5% denaturing polyacrylamide gel. Gel was dried and exposed to phosphor screen. Quantitation was performed using ImageQuaNT software (Molecular Dynamics, Inc., Sunnyvale, CA). Each protected fragment was normalized against two housekeeping genes L32 and GAPDH.

Statistical Analysis. Statistical analysis was performed using JMP statistical software (SAS Institute, Cary, NC).

RESULTS

ATRA has a very short half-life. Therefore, we used pellets (Innovative Research of America, Sarasota, FL), which were implanted s.c. to maintain a constant concentration of ATRA in circulation. These pellets lasted for 21 days. After preliminary experiments, we selected pellets with 5 mg of ATRA. This concentration was not toxic and provided a level of ATRA < 0.1 μM (level of sensitivity of the detection, data not shown). In control, mice were implanted with pellets containing vehicle alone (placebo). Several tumor models were used in this study. DA3-HA is a 7,12-dimethylbenz(*a*)anthracene-induced mammalian adenocarcinoma transfected with the HA molecule of the influenza virus A/PR/8/34 (see "Materials and Methods"). C3 fibrosarcoma was made by HPV-16-induced transformation of C57BL/6 mouse embryonic cells (22).

Treatment of Mice with ATRA Reduces the Number of ImCs. In agreement with observations made by a number of research groups, growth of C3 fibrosarcoma or DA3-HA mammary adenocarcinoma was associated with substantial accumulation of $Gr\text{-}1^+/CD11b^+$ ImC in spleens (Fig. 1*A*). Three weeks after tumor cell injection, almost one-third of all splenocytes had the phenotype of ImCs. Absolute number of ImCs increased >15-fold (data not shown). Treatment with ATRA did not affect the total number of splenocytes in tumor-bearing mice, but it significantly decreased the proportion of ImCs. In C3 tumor-bearing mice treated with ATRA, the percentage of ImC was only $10.9 \pm 2.4\%$, whereas ImCs comprised $27.2 \pm 7.8\%$ of the total splenocytes in mice treated with placebo ($P < 0.01$). In DA3-HA tumor-bearing mice, treatment with ATRA decreased the proportion of ImC from 34.1 ± 3.4 to $18.5 \pm 3.9\%$ ($P < 0.05$). In control tumor-free mice, $Gr\text{-}1^+CD11b^+$ ImCs represented >30% of bone marrow cells and <1.5% of lymph node cells (Fig. 1, *B* and *C*). In C3 tumor-bearing, mice the presence of ImCs increased 2-fold in both

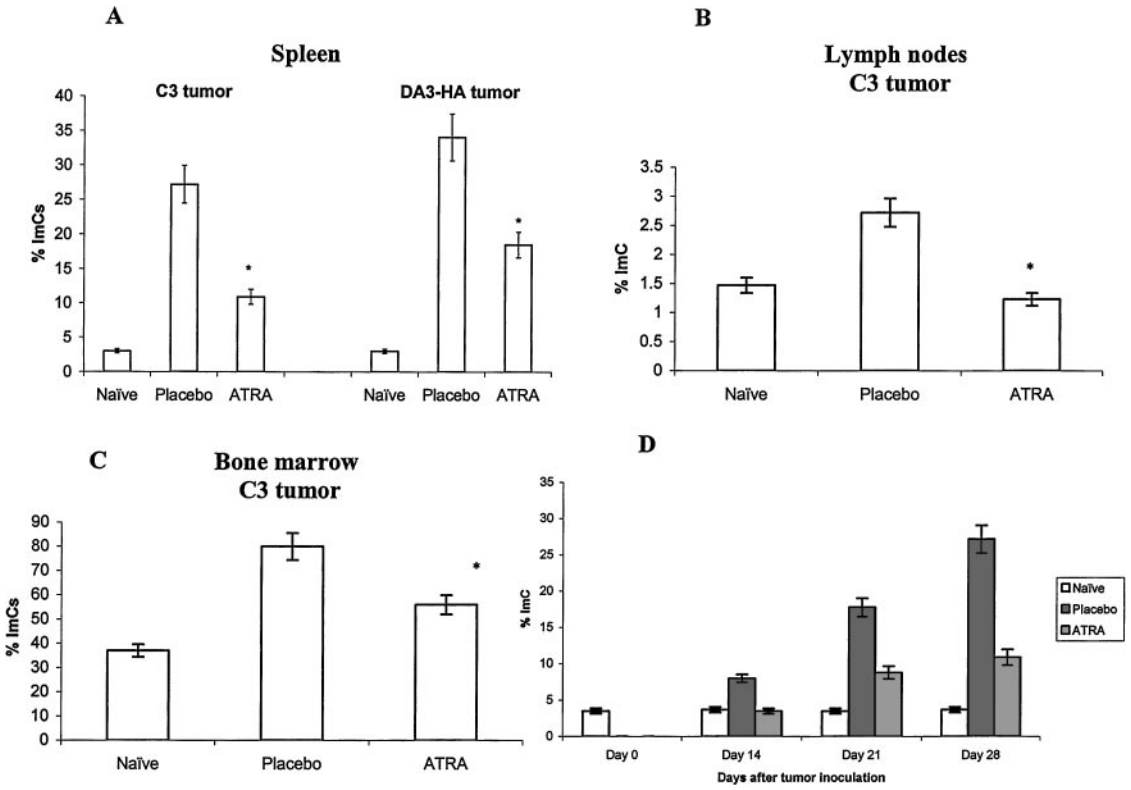

Fig. 1. ATRA decreases presence of ImC in tumor-bearing mice. C3 or DA3-HA tumors were established in C57BL/6 and BALB/c mice, respectively. When tumors reached 4–5 mm in diameter, ATRA or placebo pellets were implanted s.c. ATRA pellets contained 5 mg of the compound. Mice were sacrificed 3 weeks later, and the proportion of Gr-1$^+$CD11b$^+$ ImC was evaluated in spleens (*A*), lymph nodes (*B*), and bone marrow (*C*) using anti-Gr-1 and anti-CD11b mAbs and flow cytometry. Naïve, placebo untreated tumor-free mice. Each group included four mice. Average ± SE is shown. ∗, statistically significant differences between tumor-bearing mice treated with placebo and ATRA ($P < 0.05$). *D*, C3 tumor-bearing mice were treated with placebo or ATRA pellets starting from day 7 after tumor inoculation. Mice were sacrificed 1, 2, and 3 weeks after start of the treatment (14, 21, and 28 days after tumor inoculation, respectively). Splenocytes were labeled with anti-Gr-1-APC and anti-CD11b-PE antibodies, and the proportion of Gr-1$^+$CD11b$^+$ImCs was evaluated. Three mice/group were analyzed. Average ± SE is shown. ∗, statistically significant differences between tumor-bearing mice treated with placebo and ATRA ($P < 0.05$).

bone marrow and lymph nodes. Treatment of C3 tumor-bearing mice with ATRA significantly reduced the population of ImCs in both lymph nodes and bone marrow (Fig. 1, *B* and *C*). We investigated the time course of ATRA effect on the population of ImCs. Treatment of C3 tumor-bearing mice with ATRA or placebo was started 7 days after tumor inoculation. Mice were sacrificed 7, 14, and 21 days after the start of the treatment, and the proportion of Gr-1$^+$CD11b$^+$ ImCs was evaluated. The presence of ImCs in C3 tumor-bearing mice was significantly increased as early as 2 weeks after tumor inoculation (Fig. 1*D*). ATRA significantly reduced the presence of ImCs at all tested time points (Fig. 1*D*).

Direct Effect of ATRA on Tumor Cells Is not Responsible for the Decrease in ImC Production. Our data described above indicate that treatment of tumor-bearing mice with ATRA significantly reduced the presence of ImCs in different organs. We asked whether ATRA at the selected dose directly affected tumor cells. Concentrations as high as 2 μM ATRA did not affect growth of these two experimental tumors *in vitro* as measured by the MTT test (Fig. 2*A*). Tumor growth *in vivo* was also unchanged (Fig. 2*B*). This is consistent with previously reported observations that in therapeutic concentrations, ATRA does not directly inhibit growth of solid tumors (24). Thus, changes in tumor volume were not responsible for the effect of ATRA on ImCs.

It is known that different tumors produce growth factors and cytokines (GM-CSF, M-CSF, G-CSF, and VEGF) able to affect myelopoiesis. It is possible that ATRA could decrease the production of one or several of these factors and, thus, affect the generation of ImCs. We investigated the effect of ATRA on tumor cell production of the various growth factors able to affect myelopoiesis. C3 or DA3-HA tumor cells were cultured for 48 h in the presence of different concentrations of ATRA. RNA was isolated and expression of the genes for IL-3, IL-6, IL-10, IL-11, GM-CSF, M-CSF, G-CSF, and VEGF was analyzed using multitemplate set in RNase protection assay. Both tumor cell lines had undetectable levels of IL-3, IL-6, and IL-10. In C3 tumor cells, ATRA did not affect the expression of any of the genes. In DA3-HA, ATRA increased the expression of *G-CSF* in a dose-dependent manner (Fig. 2*C*).

ATRA Induces Differentiation of ImCs. Next, we evaluated the effect of ATRA on ImC differentiation *in vivo* in adoptive transfer experiments. Gr-1$^+$ cells were isolated from spleens of C3 tumor-bearing C57BL/6 (CD45.2$^+$) mice using a magnetic bead separation technique. The purity of Gr-1$^+$CD11b$^+$ ImCs was >90% (data not shown). ImCs (3×10^6) were transferred i.v. into B6.SJL-PtrcaPep3b/BoyJ congenic (CD45.1$^+$) mice. Pellets containing ATRA or vehicle alone (placebo) were implanted into recipient mice 1 week before the transfer of ImCs. The proportion of donors' CD45.2$^+$ cells in the total population of splenocytes, and the presence of immature and mature myeloid cells within the population of CD45.2$^+$ cells was evaluated 3 and 5 days after the transfer using multicolor flow cytometry. Three days after adoptive transfer into mice treated with placebo donors' CD45.2$^+$ cells represented 1.1 ± 0.3% of nucleated cells in recipients' spleens. Five days after the transfer, their presence slightly reduced to 0.8 ± 0.3% ($P > 0.1$). On day 3 and 5 after the transfer into ATRA-treated mice, the proportion of the donors' CD45.2$^+$ cells in the spleens was similar to that in placebo treated mice (1.2 ± 0.3 and 0.9 ± 0.3%, respectively). After transfer into control mice, a significant proportion of donors' cells (25%) retained the phenotype of

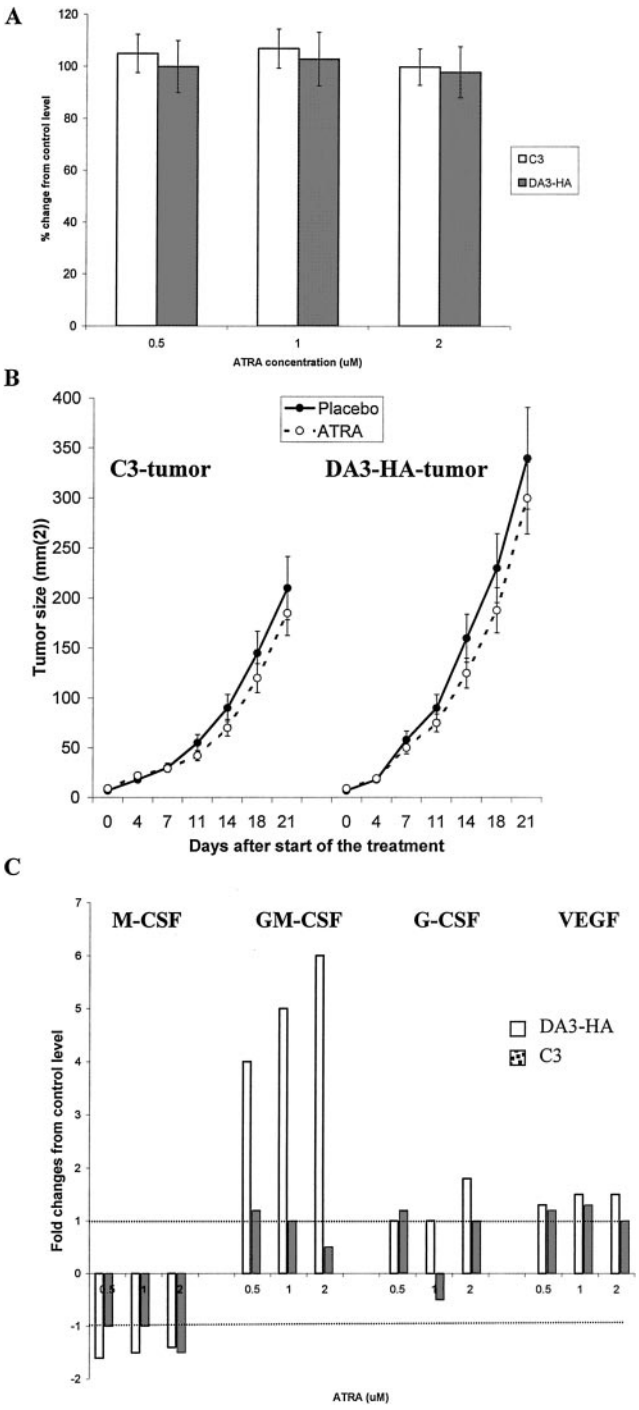

Fig. 2. ATRA does not directly affect tumor cells. *A*, a total of 1×10^4 C3 or DA3-HA tumor cells was cultured in the presence of different concentrations of ATRA for 96 h in triplicates in 96-well plates. The growth of tumor cells was evaluated in triplicates in a standard 4-h MTT test. MTT level in cells treated with vehicle alone was used as 100%. *B*, C3 or DA3-HA tumor-bearing mice were treated with ATRA or placebo started from days 10 to 12 after tumor inoculation. Each group included six mice. *C*, C3 and DA3-HA tumor cells were cultured for 48 h in the presence of different concentrations of ATRA or vehicle alone. RNA was extracted, and RNase protection assay was performed with multitemplate set as described in "Materials and Methods." The relative level of expression of each gene was normalized to the level of house keeping L32 and GAPDH genes. Changes in gene expression from the levels observed in tumor cells treated with vehicle alone are presented. Two experiments with the same results were performed.

ImCs ($Gr\text{-}1^+CD11b^+$; Fig. 3). The rest of the cells consisted of $CD11c^+IA^{b+}$ DCs, $F4/80^+$ macrophages, and a small proportion of $Gr\text{-}1^+CD11b^-$ granulocytes (Fig. 3). In the presence of ATRA, the proportion of ImCs decreased rapidly (16.3% on day 3, $P < 0.05$), and by day 5, it reached the level observed in naïve tumor-free mice (3.3%). The proportions of DCs, macrophages, and granulocytes among donors' $CD45.2^+$ cells were significantly higher in ATRA-treated mice than in mice treated with placebo (Fig. 3).

These data indicate that ATRA may induce differentiation of ImCs. To verify these findings, we evaluated the presence of DC, macrophages, and granulocytes in spleens and lymph nodes of C3 tumor-bearing mice treated with placebo or ATRA. Mice were sacrificed 7 and 14 days after start of the treatment (17 and 24 days after tumor inoculation). The number of DCs in spleens of tumor-bearing mice was significantly decreased at the first time point tested (17 days after tumor inoculation). It was especially evident in the population of $CD11c^+CD86^+$ or $IA^{b+}CD86^+$ mature DCs (Fig. 4*A*). No changes in the populations of $CD11c^+B220^+Gr\text{-}1^+$ plasmocytoid DCs or $F4/80^+CD11b^-$ mature macrophages were found. This was associated with significant increase in the number of granulocytes (Fig. 4*A*). Seven days of ATRA treatment resulted in a dramatic increase in the number of DCs. It was restored to the control level (Fig. 4*A*). Because ATRA did not significantly affect the total number of splenocytes (data not shown), all changes in the absolute numbers of DCs were caused by an increased proportion of these cells. ATRA did not change the number of mature macrophages or granulocytes in spleens (Fig. 4*A*). The same effect of ATRA was seen in spleens isolated after 14 days of treatment (24 days after tumor inoculation; data not shown). Lymph nodes isolated from C3 tumor-bearing mice 17 days after tumor inoculation did not show significant changes in the populations of myeloid cells (data not shown). This is consistent with previously reported data (25) and reflects the fact that DC turnover in spleens is significantly higher than in lymph nodes (26, 27). However, by day 24 after tumor inoculation, a decrease in the population of DCs became evident in lymph nodes as well (Fig. 4*B*). Treatment of mice with ATRA significantly increased the number of DCs and granulocytes (Fig. 4*B*). Thus, it appears that ATRA induces differentiation of ImCs into mature cells, preferentially DCs.

ATRA Effect on Tumor-induced $CD4^+$ T-Cell Tolerance. Next, we asked whether elimination of ImCs might improve the immune response in cancer. To address this question we used the previously described model of tumor-induced T-cell tolerance. In this model, tumors expressing HA antigens induce antigen-specific tolerance of $CD4^+$ T cells (12, 13). ATRA or placebo pellets were implanted into control (tumor-free) or DA3-HA tumor-bearing mice. Seven days later, transgenic T cells with TCR specific for one HA-derived epitope were adoptively transferred into all mice. Four days after T-cell transfer, mice were immunized with a recombinant vaccinia virus-encoding influenza virus HA and evaluated 7 days later. Antigen-specific response in spleens and lymph nodes was determined by measurement of the proportion of clonotypic TCR-positive $CD4^+$ cells and by cytokine production after *in vitro* restimulation with specific MHC class II-restricted HA-peptide.

As expected, immunization of control mice resulted in significant expansion of the clonotype-positive $CD4^+$ T cells (Fig. 5*A*). These T cells produced large amounts of IL-2 and IFN-γ in response to restimulation with HA-specific peptide (Fig. 5, *B* and *C*). There was no expansion of antigen-specific T lymphocytes obtained from tumor-bearing mice treated with placebo pellets. These cells did not respond to stimulation with specific peptide (Fig. 5, *A* and *B*). These data are consistent with previously reported characteristics of this model of tumor-induced immune tolerance. Treatment of tumor-bearing mice with ATRA resulted in significant expansion of antigen-specific $CD4^+$ T cells. Vaccination with vaccinia-HA increased the proportion of these cells in spleen even further (Fig. 5*A*). Production of IL-2 in

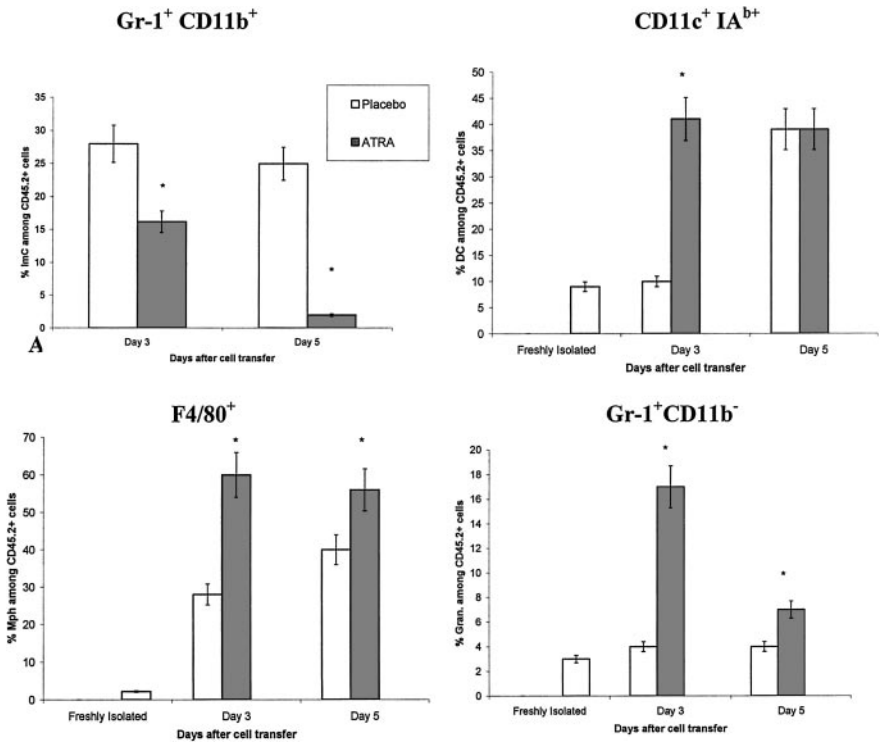

Fig. 3. ATRA induces differentiation of ImC in adoptive transfer model. Gr-1$^+$ was isolated from C3 tumor-bearing C57BL6/mice (CD45.2) using magnetic bead separation technique. These cells (3×10^6) were injected i.v. into congenic (CD45.1) mice. ATRA or placebo pellets were implanted into congenic mice 1 week prior adoptive transfer. Mice were sacrificed 3 and 5 days after the transfer, and CD45.2$^+$ donors' cells were evaluated using multicolor flow cytometry. The proportions of Gr-1$^+$CD11b$^+$ ImCs, CD11c$^+$IA^{b+} DCs, F4/80$^+$ macrophages, and Gr-1$^+$CD11b$^-$ granulocytes within the population of CD45.2$^+$ cells were analyzed. Each group included three mice. Average ± SD is shown. *, statistically significant differences between the groups ($P < 0.05$).

response to specific peptide was restored to the control level both in spleen and in lymph nodes of these mice. (Fig. 5*B*). IFN-γ production was increased at a lesser extent and did not reach control level (Fig. 5*C*). These results indicate that ATRA administration can substantially reduce tumor-induced CD4$^+$ T-cell tolerance.

ATRA Treatment Increases CD8-mediated T-Cell Response in Tumor-bearing Mice and Improves the Effect of Cancer Vaccine. Next, we asked whether ATRA treatment may affect CD8-mediated T-cell response and the success of cancer vaccines. C3 tumors were established s.c. in C57BL/6 mice. Treatment was initiated when

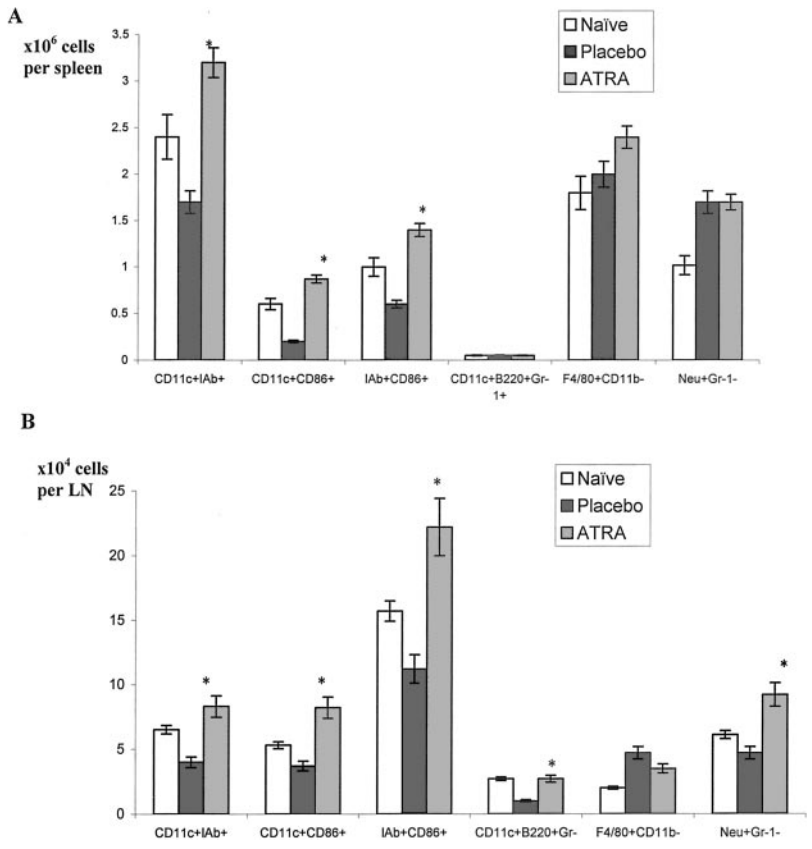

Fig. 4. ATRA effect on myeloid cell differentiation in tumor-bearing mice. C3 tumor-bearing C57BL/6 mice were treated with placebo or ATRA pellet. Different populations of cells were analyzed using flow cytometry and combination of antibodies against indicated cell surface markers in spleens (*A*) 7 days after the start of the treatment (17 days after tumor inoculation) and in lymph nodes (*B*) 14 days after the start of the treatment (24 days since tumor inoculation). Absolute number of cells are presented. Average ± SE is shown. *, statistically significant differences between tumor-bearing mice treated with placebo and ATRA ($P < 0.05$).

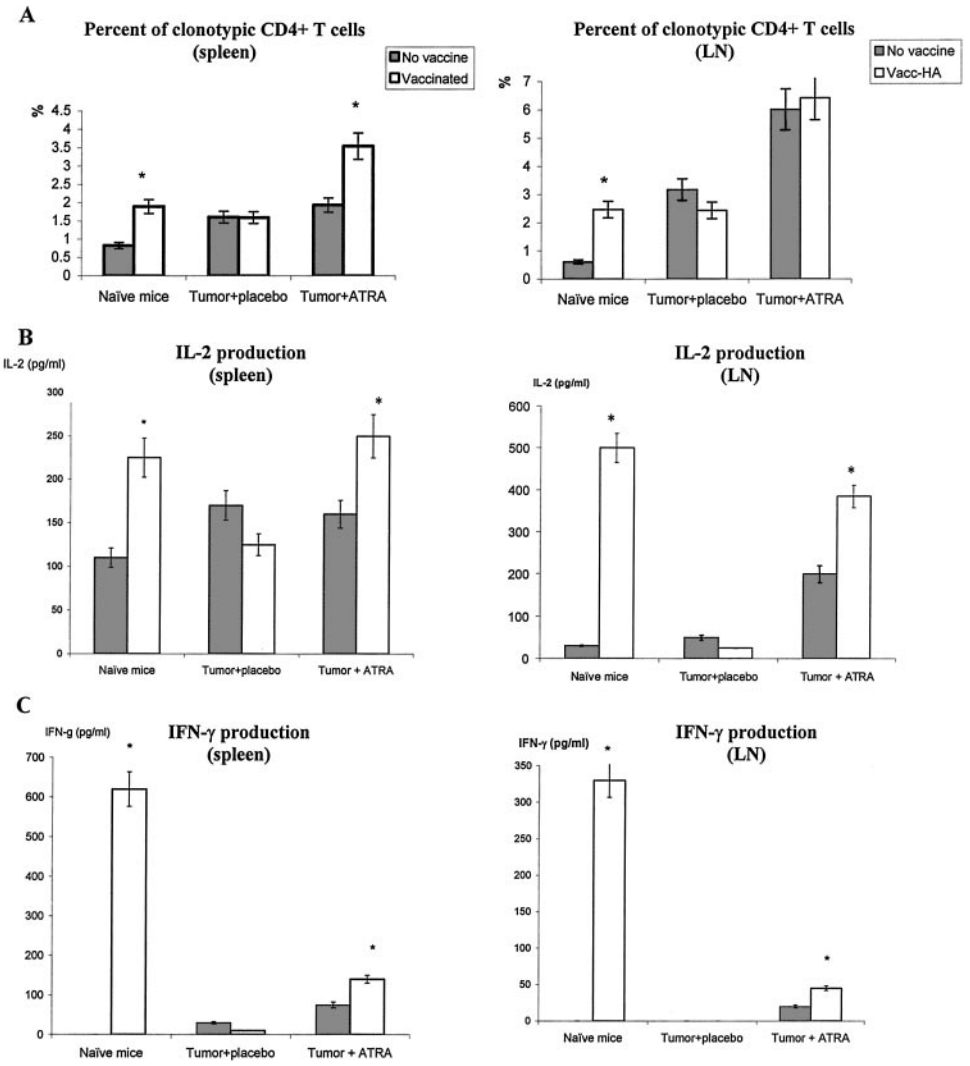

Fig. 5. ATRA effect on tumor-induced T-cell tolerance. ATRA or placebo pellets were implanted into naïve (tumor-free) and DA3-HA tumor-bearing mice. Seven days later, 2.5×10^6 transgenic T cells with TCR specific for one HA-derived epitope were adoptively transferred into all mice. Four days later, mice were immunized with a recombinant vaccinia virus-encoding influenza virus HA (Vacc-HA). Mice were evaluated 7 days later. Each group included 4 mice. *A*, proportion of clonotypic TCR^+ cells within the population of $CD4^+$ lymphocytes in spleens and lymph nodes. Less than 0.1% of clonotypic TCR^+ cells were detected among $CD4^+$ cells in BALB/c mice without adoptive transfer of the lymphocytes. *B*, IL-2 production by T cells from spleens and lymph nodes in response to the specific peptide. T cells isolated from treated mice were cultured at concentration of 5×10^4/well in 96-well plates with 10^5/well irradiated splenocytes from control BALB/c mice in the presence of 12.5 μg/ml of the HA-specific or control peptides (OVA-derived). Supernatants were collected after 48 h, and IL-2 was measured in ELISA as described in "Materials and Methods." *, statistically significant differences between the vaccinated and nonvaccinated mice ($P < 0.05$). IL-2 level in T cells cultured with control peptide was below 50 pg/ml. *C*, IFN-γ production by T cells from spleens and lymph nodes in response to the stimulation with the specific peptide. IFN-γ was measured in 48-h supernatants from T cells as described above using ELISA assay. No IFN-γ production was detected when T cells were cultured with control OVA-derived peptide. *, statistically significant differences between the vaccinated and nonvaccinated mice ($P < 0.05$).

tumors reached 4–5 mm in diameter (day 5 after tumor implantation). Mice were immunized three times (on day 5, 12, and 17) with C3 tumor-specific peptide (RAHYNIVTF) in CFA as described in "Materials and Methods." This peptide contains H-2D^b-restricted epitope of the HPV-16 E7 protein expressed by C3 tumor and has been shown to elicit potent protective antitumor immune response (23). ovalbumine-derived peptide was used as control. ATRA or control (placebo) pellets were implanted s.c. on day 12. Each group included 7 mice. Treatment with specific peptide had a limited effect on growth of this poorly immunogeneic tumor (Fig. 6*A*). This was consistent with many previous observations that immunotherapy of established tumors is often ineffective. In contrast, in the presence of ATRA, the decrease in tumor growth was much more sustained. By day 35, there was more than a 3-fold difference in tumor size between the group of mice treated with combination of ATRA and immunization and all other groups ($P < 0.01$). In a separate set of experiments, we evaluated the immune response to vaccination. On day 27, mice were sacrificed, and the presence of antigen-specific CTL precursors was evaluated by IFN-γ production in ELISPOT assay. T cells derived from spleens of placebo-treated, placebo-treated and immunized with peptide, or ATRA-treated mice did not respond to *in vitro* restimulation with the specific peptide (Fig. 6*B*). In contrast, a significant number of antigen-specific IFN-γ-producing T cells was found in the spleens of ATRA-treated tumor-bearing mice that had been immunized with C3-derived peptide (Fig. 6*B*).

To confirm the positive effect of ATRA on cancer immunotherapy, we used a different experimental model, MethA sarcoma. MethA is an immunogenic 3-methylcholantrene-induced sarcoma, which carries a carcinogen-induced mutant endogenous p53 gene. Our previous study has shown that treatment of tumor-bearing mice with DCs transduced with wild-type p53 gene significantly delayed tumor growth (21). However, tumor growth resumed 1 week after the finish of the treatment. We hypothesized that if ATRA effectively eliminates immunosuppressive ImCs, this should lead to an enhanced antitumor effect of immunization. MethA sarcoma was established in BALB/c mice. Our preliminary data demonstrated that ATRA reduced the presence of ImC in this model > 3-fold (data not shown). Treatment was started on day 5 when tumor reached 3–5 mm in diameter. For immunization, we used DCs transduced with Ad-p53. In control, DCs were infected with adenovirus containing empty vector (Ad-c). Pellets with ATRA or vehicle (placebo) were implanted on day 6. Four groups of mice were treated (eight mice/group). The first control group included mice treated with Ad-c DCs and placebo; the second group was comprised of mice treated with Ad-c DCs and ATRA; the third group was mice immunized with Ad-p53 DCs; the fourth group was mice treated with Ad-p53 DCs and ATRA. Mice were immunized on days 5, 10, and 15 after tumor inoculation. Immunization with Ad-p53 DCs significantly slowed the tumor growth. One week after the last immunization, the tumor size in this group

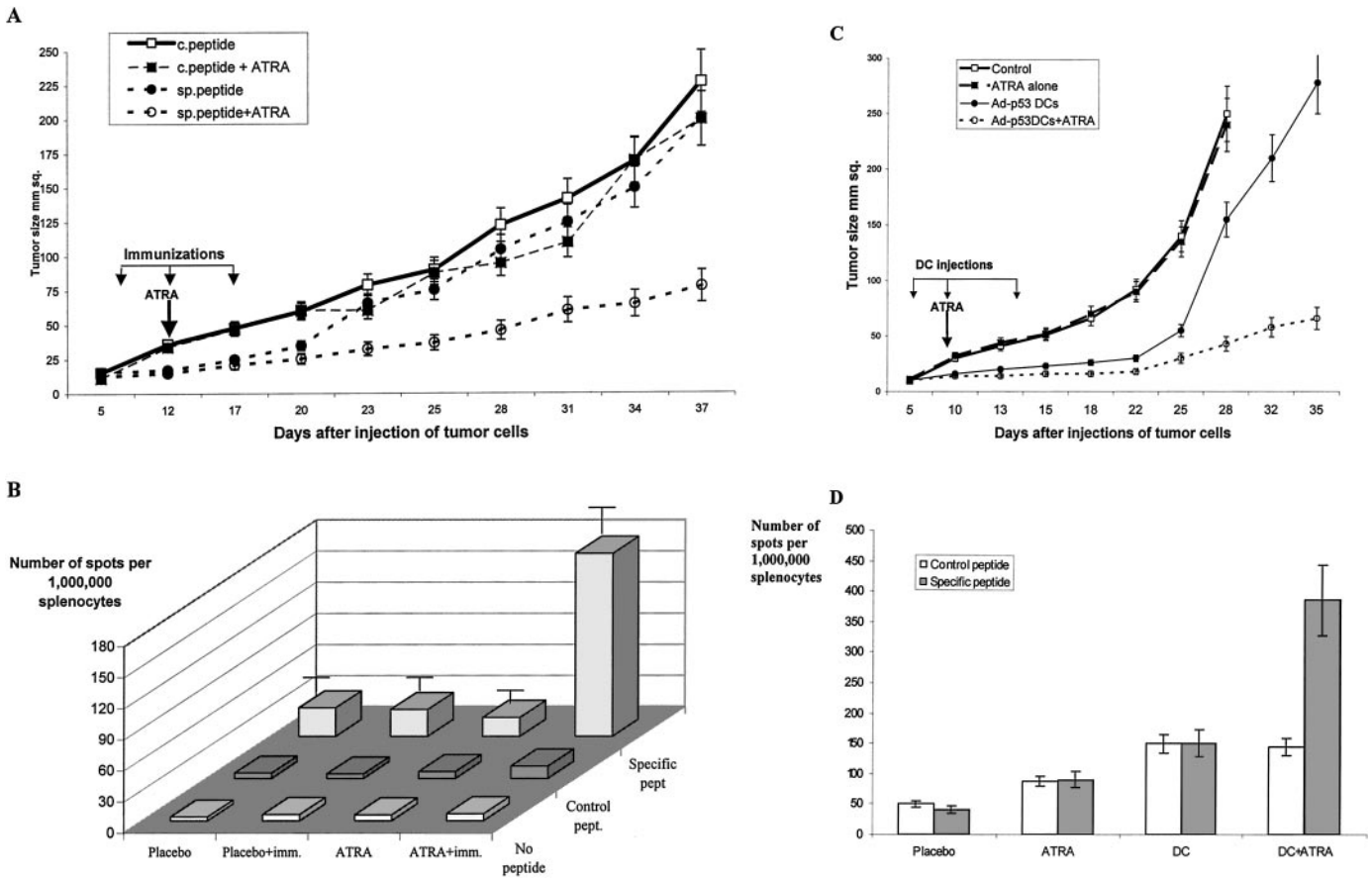

Fig. 6. ATRA and antitumor effect of vaccination. *A*, C3 tumor-bearing mice were treated with three rounds of immunization with control or specific peptides in CFA as indicated in combination with ATRA or placebo pellets. ATRA pellets lasted for 21 days. Each group included seven mice. Differences in tumor size between the group of mice treated with ATRA in combination with the specific peptide and the other three groups became statistically significant on day 23. *B*, tumor antigen-specific immune response in treated mice. C3 tumor-bearing mice were treated with three rounds of immunization in combination with ATRA or placebo treatment as described in Fig. 6*A*. On day 27 (10 days after the last immunization), mice were sacrificed, and the response of T cells to the specific C3 or control OVA-derived peptides was evaluated in ELISPOT assay as described in "Materials and Methods." Both peptides matched $H2K^b$. Each group included three mice. Each experiment was performed in triplicates. Average ± SD from all experiments are shown. *C*, MethA sarcoma-bearing BALB/c mice were treated as indicated with ATRA or placebo and three rounds of immunization with 4×10^5 Ad-c or Ad-p53 DCs. Each group included eight mice. Differences in tumor size between mice treated with Ad-c DCs (control) and mice treated with Ad-p53 DCs became statistically significant on day 15. Differences between the group of mice treated with Ad-p53 DCs and mice treated with Ad-p53 DCs in combination with ATRA became statistically significant on day 28. *D*, tumor antigen-specific immune response in treated mice. MethA sarcoma-bearing mice were treated as described in Fig. 6*C*. Mice were sacrificed on day 28, and the response of T cells to MethA sarcoma-specific (KYICNSSCM) or control HA-derived (IYSTVASSL) peptides was evaluated in ELISPOT assay as described in "Materials and Methods." Both peptides matched $H2K^d$. Each group included three mice. Each experiment was performed in triplicates. Average ± SD from all experiments are shown.

was three times smaller than in mice from the control group and from the group treated with ATRA and Ad-c DCs ($P < 0.01$). However, consistent with the results of many previous experiments, tumor growth quickly resumed, and within a week, tumor size became comparable with that in the other two groups. In contrast, tumor growth in mice treated with Ad-p53 DCs and ATRA was significantly delayed. Two weeks after the last immunization, the tumor size in this group of mice was ~5-fold smaller than in mice from any other group ($P < 0.001$; Fig. 6*C*). In a separate group of experiments, we evaluated the immune response to vaccination. Four weeks after tumor inoculation, mice were sacrificed, and the presence of antigen-specific CTL precursors was evaluated by IFN-γ production in ELISPOT assay. As specific peptide, we used $H2K^d$-restricted mutant p53-derived peptide (KYICNSSCM). This peptide is specific for MethA sarcoma. Previous study demonstrated that treatment of MethA sarcoma with DCs in combination with γ-irradiation induced potent immune response against this particular epitope, probably via mechanism of epitope spreading (28). In control, $H2K^d$-restricted HA-derived peptide (IYSTVASSL) was used. Only T cells derived from spleens of immunized mice treated with ATRA demonstrated significant level of antigen-specific immune response 4 weeks after tumor inoculation (Fig. 6*D*).

DISCUSSION

This study demonstrates that ATRA dramatically reduces the presence of ImCs in tumor-bearing mice and improves immune response to vaccination. ATRA is a well-known compound capable of inducing the differentiation of freshly isolated acute promyelocytic leukemia cells and normal hematopoietic progenitors, as well as blast progenitors in acute myelogeneous leukemia (14, 15, 18–20). Our previous study *in vitro* showed that ATRA is able to differentiate a significant proportion of human ImC into DCs (3). Treatment of mouse ImCs with ATRA *in vitro* resulted in induction of myeloid cell differentiation. However, in contrast to human ImCs, most of mouse ImCs differentiated into macrophages (11). 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 have been described, the RAR receptors (RARα, RARβ, RARγ) and the RXRs (RXRα, RXRβ, RXRγ). Each receptor subtype is encoded by a separate gene (29). In most cases, an efficient

transduction of the ATRA signal requires heterodimerization between RAR and RXR. Our previous experiments showed that ImCs expressed both RAR and RXR types of ATRA receptors (V. Gorelov, unpublished observation).

In this study, we first investigated whether *in vitro* observations can be confirmed *in vivo*. We selected pellets containing 5 mg of ATRA because this dose did not result in toxicity but still provided a clear effect on ImC. This dose resulted in <0.1 μM serum concentration of ATRA. In all three tested tumor models, ATRA administration substantially reduced the proportion and absolute number of ImCs in spleens. There could be several possible explanations of this fact: (*a*) ATRA has an antitumor effect in our experimental tumor models, and decreased tumor mass produces less factors able to stimulate ImC production; (*b*) ATRA does not affect tumor growth but inhibits production by tumors of ImC growth factors; (*c*) ATRA induces differentiation of ImCs; and (*d*) ATRA induces apoptosis of ImCs.

ATRA at concentration as high as 2 μM did not affect the growth of selected tumor cells *in vitro*. It was possible that ATRA could affect tumor growth not directly but via endothelial cells, which could interfere with tumor vascular supply. To test this possibility, we evaluated tumor growth *in vivo* in mice treated with 5 mg of ATRA pellets. No differences from the control group were found in any of the used animal tumor models. It has been reported that at higher concentrations, ATRA was able to suppress growth of some solid tumors. However, clinical trials performed in the past several years yielded a very limited effect. This could be partly explained by decreased expression of the specific receptors (24). Hyperproduction of myeloid cell growth factors such as M-CSF, G-CSF, GM-CSF, or factors able to affect myelopoiesis such as VEGF by tumor cells could be responsible for an accumulation of ImCs in cancer. Therefore, we asked whether ATRA effects on ImCs in tumor-bearing mice could be explained by the decreased production of some of these growth factors. In our experiments, ATRA did not inhibit expression of any of the growth factors. Moreover, it increased expression of G-CSF in DA3-HA tumor cells. These findings were consistent with previous observations that ATRA might increase the production of some myeloid cell growth factors by bone marrow stromal cells (30) and in acute promyelocytic leukemia (31). However, the fact that ATRA did not suppress the expression of some myeloid growth factors and VEGF, by itself, was not sufficient to rule out this mechanism because there are number of other factors that potentially could be involved in that process. Therefore, we directly evaluated ATRA effect on differentiation of ImCs *in vivo* using adoptive transfer of ImCs into congenic mice. Our experiments demonstrated that ATRA induces rapid differentiation of ImCs into mature DCs, macrophages, and granulocytes. Five days after the transfer, the presence of donors' ImCs in ATRA-treated mice was reduced to the normal level, whereas in placebo-treated mice, one-fourth of the donors' cells still expressed the phenotype of ImCs. This was consistent with our previous observations *in vitro* (11) and the fact that ATRA treatment did not affect the total number of splenocytes *in vitro* or *in vivo*. These data also argue against the possibility that ATRA-induced apoptosis of ImCs may be responsible for the elimination of these cells from spleens. Tumor-bearing mice had a reduced number of DCs in spleens and lymph nodes. This was in agreement with previously reported observations (25, 32). ATRA treatment did not significantly affect the total number of splenocytes or lymph node cells but increased the number of DCs in these mice. Because this increase was associated with a decreased presence of ImCs in these mice, the results of these experiments demonstrated that ATRA might reduce presence of ImCs by differentiating them into mature myeloid cells.

Next, we asked whether this effect had any consequences for antitumor immune response. Freshly isolated ImCs expressed MHC class I but not MHC class II molecules and suppressed only CD8-mediated but not CD4-mediated responses (11). However, within several days after adoptive transfer into control or tumor-bearing mice, these cells up-regulated MHC class II expression (33). In addition, after several days in culture, ImCs differentiate into immunosuppressive macrophages that produce high levels of NO and suppress both $CD4^+$ and $CD8^+$ T cells (8, 34). Therefore, we suggested that elimination of ImCs *in vivo* might improve not only CD8-mediated but also CD4-mediated immune responses. To test this hypothesis, two experimental tumor models were used. HA-expressing tumor abrogated the response of antigen-specific $CD4^+$T cells to vaccination. It manifested in lack of expansion of antigen-specific $CD4^+$T cells and diminished production of IL-2 and IFN-γ in response to the stimulation with MHC class II-matched specific antigen. This phenomenon has been previously described in this type of experimental system (12, 35). ATRA treatment increased the proportion of antigen-specific T cells and IL-2 production to the control level. IFN-γ production by T cells was significantly increased. However, it did not reach the control level. A similar effect was observed in MHC class I-restricted T-cell response to vaccination. Tumor-bearing mice immunized with MHC class I-bound specific peptide failed to maintain antigen-specific response 10 days after the last immunization, whereas T cells from immunized mice treated with ATRA showed significant response. These data indicate that ATRA considerably improves antitumor immune response.

In two experimental systems, using two different methods of immunization, ATRA dramatically enhanced the antitumor immune effect of vaccination. It appears that this treatment increases not the strength of the responses because it did not increase the number of mice rejecting tumors but the longevity of the responses. One of the well-described problems of immunotherapy of established tumors is the limited duration of its effect. Tumor growth resumes soon after the finish of the therapy. That has seriously weakened the overall efficacy of the treatment. This is associated with inhibition of tumor-specific immune response. In a previous study, we directly confirmed that by implanting tumor to mice previously immunized with irrelevant antigen, preexisted strong CTL response diminished within 10 days after tumor implantation (36). In tumor models, used in this study, tumor growth resumed in about a week after the last immunization. However, in both models, in mice treated with ATRA, tumor growth remained suppressed a long time after that. It is consistent with the mechanism of ATRA effects on ImC. Decreased presence of ImC in tumor-bearing mice reduces their immunosuppressive effect on T cells, which allow CTLs persist longer and keep tumor growth under control. It appears that the treatment with ATRA alone will not be sufficient to provide sustained long-lasting effect of immunization. Repeated vaccination will be required to maintain clonal outgrowth of antigen-specific T cells. However, ATRA may be a critical factor supporting the persistence of these CTLs and, thus, dramatically improving the effect of vaccination.

In summary, here we demonstrated that decreased presence of ImCs might dramatically improve antitumor immune response and enhance the effect of vaccination. ATRA provides such an effect and may be a valuable addition to different cancer vaccines.

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