

## Reversal of Myeloid Cell – Mediated Immunosuppression in Patients with Metastatic Renal Cell Carcinoma

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**Abstract Purpose:** Tumor-induced immunosuppression remains a significant obstacle that limits the efficacy of biological therapy for renal cell carcinoma. Here we evaluate the role of CD33 myeloid-derived suppressor cells (MDSC) in the regulation of T-cell responses in renal cell carcinoma patients. We also examine effect of *all-trans*-retinoic acid (ATRA) on MDSC-mediated immune suppression.

**Experimental Design:** CD33-positive myeloid cells were isolated from the peripheral blood of renal cell carcinoma patients with magnetic beads and tested *in vitro* for their ability to inhibit T-cell responses. T-cell function was evaluated using ELISPOT and CTL assays.

**Results:** MDSC isolated from renal cell carcinoma patients, but not from healthy donors, were capable of suppressing antigen-specific T-cell responses *in vitro* through the secretion of reactive oxygen species and nitric oxide upon interaction with CTL. MDSC-mediated immune suppression and IFN- $\gamma$  down-regulation was reversible *in vitro* by exposing cells to the reactive oxygen species inhibitors. Moreover, ATRA was capable of abrogating MDSC-mediated immunosuppression and improving T-cell function by direct differentiation into antigen-presenting cell precursors.

**Conclusions:** These results may have significant implications regarding the future design of active immunotherapy protocols that may include differentiation agents as part of a multimodal approach to renal cell carcinoma immunotherapy.

The incidence of renal cell carcinoma is increasing, with ~35,000 new cases detected annually in the United States. Like melanoma, renal cell carcinoma has shown to respond to immunotherapeutic intervention, but tumors are capable of evading immune recognition, thus limiting the efficacy of biological therapy (1–3). Previous studies have shown that secretion of tumor-mediated factors such as vascular endothelial growth factor, transforming growth factor- $\beta$ , granulocyte macrophage colony-stimulating factor (GM-CSF), and prostaglandin E2 results in altered hematopoiesis and accumulation of myeloid-derived suppressor cells (MDSC) in the tumor-bearing host (4–11). MDSC have shown to interfere with the differentiation, function, and survival of antigen-

presenting cells (APC) and effector T cells by using enzymes involved in arginine metabolism, namely inducible nitric oxide synthase, which generates nitric oxide (NO), and arginase I, which acts by L-arginine depletion (12–14). Moreover, MDSC produce elevated levels of reactive oxygen species (15, 16) and trigger T-cell tolerance through T-cell receptor-dependent or independent mechanisms (17–19). Although several studies have analyzed the phenotype and function of MDSC in murine systems, much less is known regarding the relevance and immunosuppressive action of MDSC in cancer patients.

In this study, we found that the frequencies of CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC isolated from the peripheral blood of patients with metastatic renal cell carcinoma are significantly elevated when compared with CD33<sup>+</sup>HLA-DR<sup>-</sup> cells from healthy donors. MDSC isolated from the peripheral blood of renal cell carcinoma patients, but not from healthy donors, were capable of suppressing antigen-specific T-cell responses *in vitro* through the secretion of reactive oxygen species and NO upon interaction with CTL. MDSC-mediated immune suppression and INF- $\gamma$  down-regulation was reversible by adding the reactive oxygen species inhibitors catalase and superoxide dismutase to MDSC. Moreover, *all-trans* retinoic acid (ATRA) was capable of abrogating MDSC-mediated immunosuppression and improving T-cell function by direct differentiation into APC precursors. These results may have significant implications regarding the future design of active immunotherapy protocols that may include differentiation agents as part of a multimodal approach to the immunotherapy of renal cell carcinoma patients.

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## Translational Relevance

Many studies have shown that the growth of cancers, including renal cell carcinoma, is often associated with a decline in immune function, and therapeutic vaccines alone are often ineffective in overcoming tumor-mediated immune suppression. Previous studies have highlighted the role of myeloid-derived suppressor cells (MDSC) in cancer-associated immune nonresponsiveness in patients with metastatic renal cell carcinoma. Accumulation of these cells in tumor hosts is promoted by tumor-derived factors, and this tumor-driven expansion of MDSC contributes to tumor escape from the immune system. In this study we analyzed the mechanisms by which MDSC inhibit T-cell responses and show a reversal of this effect by ATRA treatment. Our results suggest that ATRA treatment could help in the depletion of these immunosuppressive cells; however, more studies are needed to find the most optimal individual approach to improve the effect of cancer vaccination.

## Materials and Methods

**Biological samples.** Peripheral blood was collected from patients with diagnosis of a metastatic renal cell carcinoma stages III to IV (pT<sub>4</sub>N<sub>x</sub>M<sub>x</sub>) and healthy donors through the Department of Urology at the University of Florida, Gainesville, Florida and the Department of Surgery at Duke University Medical Center, Durham, NC. All specimens were obtained after informed consent and approval by the institutional review boards. Excluded from the study were patients with central nervous system metastases, a history of autoimmune disease, serious intercurrent chronic or acute illnesses, concurrent second malignancy other than nonmelanoma skin cancer, or controlled superficial bladder cancer. Also excluded were patients on immunosuppressive agents. The ages of donors and patients were matched. Peripheral blood mononuclear cells (PBMC) were separated by Histopaque gradient centrifugation.

**Reagents and antibodies.** The MART-1-specific CTL clone was originally obtained from S.A. Rosenberg (National Cancer Institute) and kindly provided by S.K. Pruitt (Department of Surgery, Duke University). MART-1<sub>26-35</sub> native peptide (EAAGIGILTV) and MART-1<sub>26-35</sub> A27L peptide analogue (ELAGIGILTV) were synthesized by New England Peptides, Inc. GM-CSF was obtained from Immunex. Dichlorodihydrofluorescein diacetate (DCFDA) and 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM) were purchased from Molecular Probes. Superoxide dismutase, catalase, and uric acid were purchased from Calbiochem. Tetanus toxoid was purchased from List Biological Labs. ATRA (Tretinoin) was obtained from Roche Pharmaceuticals. <sup>51</sup>Cr and [<sup>3</sup>H]thymidine were from Amersham-Buchler. The following fluorochrome-conjugated antibodies were used for flow cytometry: HLA-DR, HLA-ABC, CD1a, CD3, CD10, CD11c, CD13, CD14, CD15, CD16b, CD18, CD19, CD31, CD33, CD40, CD56, CD86, and CD115 (all from BD Pharmingen or Caltag Laboratories).

**Isolation of CD33<sup>+</sup>HLA-DR<sup>+</sup> cells.** CD33<sup>+</sup>HLA-DR<sup>+</sup> cells were isolated from the peripheral blood of renal cell carcinoma patients using MACS microbeads and columns (Miltenyi Biotec). Briefly, freshly isolated PBMC were resuspended in cold MACS buffer and incubated with HLA-DR microbeads (Miltenyi Biotec) for 15 min on ice. Then cells were washed with cold MACS buffer to remove unbound beads and subsequently subjected to depletion of HLA-DR<sup>+</sup> cells on a MACS column according to the manufacturer's instructions (Miltenyi Biotec).

The negative cell fraction was collected, washed, and then incubated with CD33 microbeads. MACS column was used for positive selection of CD33<sup>+</sup>HLA-DR<sup>+</sup> cells. The purity of the CD33<sup>+</sup> cell population was evaluated by flow cytometry and exceeded 90%.

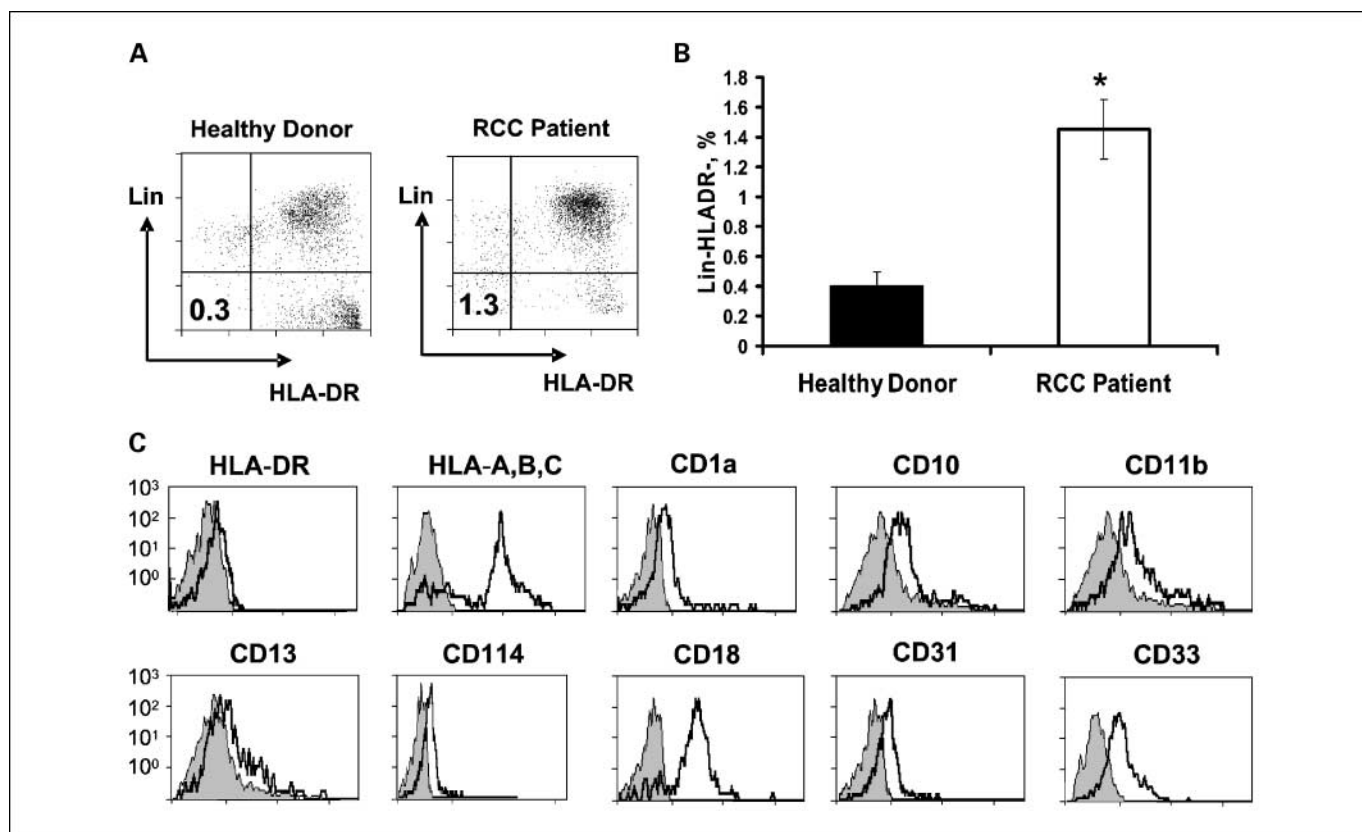
**Flow cytometry.** A total of  $1 \times 10^6$  cells were suspended in PBS buffer and incubated for 20 min at 4°C with the antibody and then washed twice with cold PBS. Fluorochrome-conjugated antibodies as well as isotype control antibodies were used for cell staining. Nonspecific staining was prevented by blocking Fc receptors. To block Fc receptors, cells were incubated for 5 min at 4°C with anti-CD16/CD32 monoclonal antibodies. Fluorescence-activated cell sorting data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and were analyzed using CellQuest software (BD Biosciences). Results were expressed as the percentage of positive cells and mean fluorescence intensity.

**ELISPOT assay.** To test whether CD33<sup>+</sup>HLA-DR<sup>+</sup> myeloid cells are capable of inhibiting a CD8 T-cell response, we used ELISPOT assay (20, 21). Briefly, freshly isolated myeloid cell subsets from a HLA-A0201-positive cancer patient or from a healthy donor were pulsed with MART-1 peptide and incubated with MART-1-specific CTL clone at a ratio of 1:1. A cell mixture of CD33<sup>+</sup>HLA-DR<sup>+</sup> cells, CTLs, and T2 cells (loaded with peptide) was added to 96-well plates for ELISPOT (Millipore), which were precoated with 2 µg/mL IFN-γ capture antibody. Plates were incubated for 20 h at 37°C, and biotinylated IFN-γ detection antibody was added to each well. Cells were then incubated for an additional 2 h at room temperature, then with streptavidin-alkaline phosphatase (1 µg/mL; Sigma-Aldrich); plates were developed with substrate (Kirkegaard & Perry Laboratories). After washing, spots were counted using an automated ELISPOT counter.

**CTL assay.** For CTL assay, HLA-A201-restricted MART-1-specific CTLs (MART-1 CTL clone) were cultured for 5 h with <sup>51</sup>Cr-labeled, MART-1 peptide-loaded T2 target cells at a 1:1 ratio. Similar numbers of CD33<sup>+</sup>HLA-DR<sup>+</sup> (pulsed with peptide) cells from HLA-A201-positive renal cell carcinoma patients, or control CD33<sup>+</sup>HLA-DR<sup>+</sup> (pulsed with peptide) from healthy control donors, were then added to wells containing effector/target cells. As controls, peptide-loaded T2 cells (Cold Target) were added to individual cultures. Target cells were labeled with 200 µCi of NaCrO<sub>4</sub> (Amersham-Buchler) in 0.5 mL of complete medium for 1 h. They were washed three times with complete medium and added at a concentration of  $5 \times 10^3$  cells per well in round-bottom microplates. Effector cells were added at various E:T-cell ratios in a final volume of 200 µL per well. The plates were incubated for 4 h at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Maximum chromium release was ensured by the addition of 10% Triton-X, and spontaneous release was assessed by adding complete medium (RPMI 1640 plus 10% FCS) to the target cells. The culture supernatant was harvested semiautomatically with a Scatron Titertek System (Scatron) and counted in a γ counter (Beckman). The percentage of specific lysis was calculated as [(experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm)] × 100. All determinations were made in triplicate.

**Production of reactive oxygen species and NO.** Oxidation-sensitive dye dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) and NO-specific fluorogenic probe DAF-FM were used for the measurement of reactive oxygen species and NO production, respectively, by CD33<sup>+</sup> myeloid cells. Freshly isolated CD33<sup>+</sup>HLA-DR<sup>+</sup> cells from an HLA-A0201-positive renal cell carcinoma patients (or healthy donors) were pulsed with MART-1 peptide, loaded with H<sub>2</sub>DCFDA (5 µmol/L) or DAF-FM (5 µmol/L), and then incubated with a MART-1 specific CTL clone for 1 h (at a cell ratio of 1:1). After washing with cold PBS, CD3-negative cells were gated and analyzed by flow cytometry.

**Reverse transcription-PCR.** Total RNA was isolated using TRIZOL reagent (Invitrogen). Reverse transcription was done at 25°C for 10 min, 42°C for 2 h, and 72°C for 5 min from 100 ng of total RNA using Superscript II reverse transcriptase (Invitrogen) and random primers (3 µg/µL; Invitrogen).



**Fig. 1.** Characterization of Lin<sup>-</sup>HLA-DR<sup>-</sup> myeloid cell population in the peripheral blood of renal cell carcinoma patients. *A*, MDSC were isolated from the PBMC of healthy donors and renal cell carcinoma patients (one representative pair of donor-patient from 9 are shown) by selecting lineage negative (CD3, CD14, CD19, CD56) and HLA-DR<sup>-</sup> negative cells as described previously (22). *B*, proportion of Lin<sup>-</sup>HLA-DR<sup>-</sup> cells in the peripheral blood of renal cell carcinoma patients is significantly increased. Freshly isolated PBMC from renal cell carcinoma patients and healthy donors were stained with CD3, CD14, CD19, CD56, and HLA-DR, and then analyzed by flow cytometry for presence of Lin<sup>-</sup>HLA-DR<sup>-</sup> cell population. *C*, Lin<sup>-</sup>HLA-DR<sup>-</sup> MDSC populations were characterized by flow cytometry. White histograms, cell surface marker expression; gray histograms, isotype controls.

**Statistical analysis.** The statistical significance between values was determined by Student's *t* test. All data were expressed as the mean SD. *P* values  $\geq 0.05$  were considered not significant. Flow cytometry data shown are representative of at least two separate determinations.

## Results

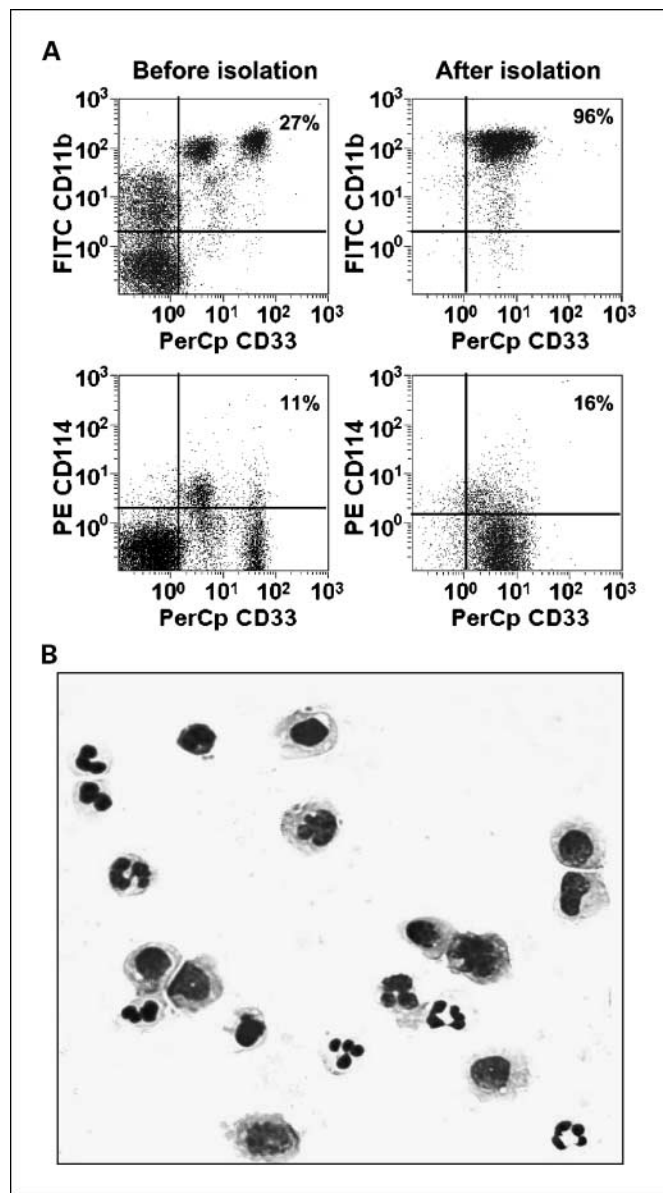
**Isolation of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid cell populations.** For initial phenotypic analysis of MDSC in the peripheral blood of renal cell carcinoma patients, we adapted techniques described previously (22). We isolated PBMC from renal cell carcinoma patients and control healthy donors and then analyzed for presence of lineage-negative/HLA-DR<sup>-</sup> negative cell populations (Lin<sup>-</sup>/DR<sup>-</sup>). As shown in Fig. 1A and B, the number of Lin<sup>-</sup>HLA-DR<sup>-</sup> cells was significantly increased in cancer patients. Subsequently, Lin<sup>-</sup>/DR<sup>-</sup> cells were analyzed for cell surface expression of CD1a, CD10, CD11b, CD13, CD114, CD18, CD31, CD33, HLA ABC, and HLA-DR. As shown in Fig. 1C, Lin<sup>-</sup>/DR<sup>-</sup> cells exhibited high expression levels of HLA class I, CD18, and CD33, and intermediate cell surface expression for CD1a, CD10, CD13, CD31, and CD11b, consistent with the MDSC phenotype previously described (22).

Because most of Lin<sup>-</sup>HLA-DR<sup>-</sup> MDSC expressed CD33, we developed an isolation method of MDSC peripheral blood of

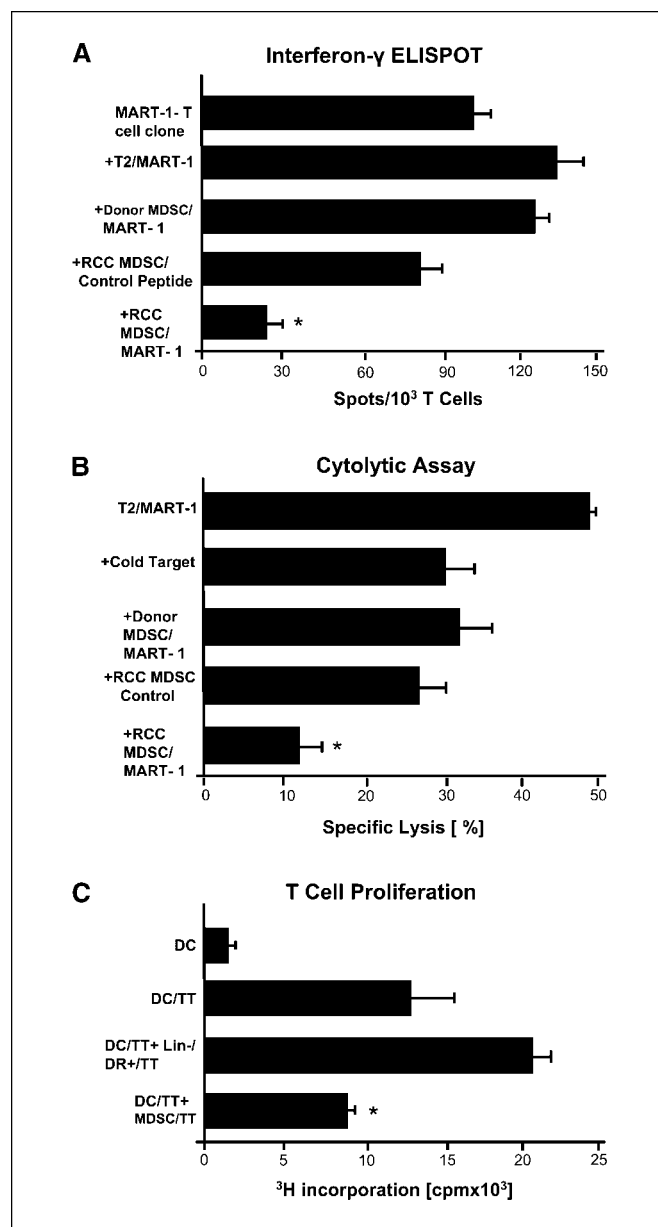
cancer patients using CD33 as positive selection marker. PBMC from renal cell carcinoma patients and healthy donors were first depleted of HLA-DR<sup>+</sup> positive cells, and then subsequent positive selection of CD33<sup>+</sup> cell population resulted in depletion exhibiting high expression of HLA class I in both donor and renal cell carcinoma samples. Practically all isolated CD33<sup>+</sup> MDSC also coexpressed CD11b (Fig. 2A, top right), and only a minor portion of these cells (16%) expressed granulocytic marker CD114 (Fig. 2A, bottom right). CD115, the receptor for CSF-1 (macrophage colony stimulating factor), was only expressed in renal cell carcinoma-derived CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC (data not shown). Cytologic analysis of CD33<sup>+</sup> cells revealed the heterogeneous cell morphology consisting of two major cell types, including mononuclear "monocytic" cells with one large nucleus and polymorphonuclear "granulocytic" cells with segmented nuclei (Fig. 2B). It should be noted that the proportion of monocytic and granulocytic cell types among CD33<sup>+</sup> MDSC has varied from patient to patient. In summary, we show that the myeloid marker CD33 can be used for isolating and enumerating MDSC from the PBMC of renal cell carcinoma patients and that CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC are significantly elevated in cancer patients but not healthy volunteers.

**Functional analysis of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid cells.** We next evaluated the functional capacity of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid

cells to suppress antigen-specific T-cell responses using INF- $\gamma$  ELISPOT analysis. CD33<sup>+</sup> MDSC from a HLA-A0201-positive cancer patient or from a healthy donor were pulsed with MART-1 peptide and incubated with a MART-1 – specific CTL clone at a ratio of 1:1. As shown in Fig. 3A, the addition of renal cell carcinoma patient – derived, but not donor-derived CD33<sup>+</sup>HLA-DR<sup>+</sup> cells to the ELISPOT reaction significantly suppressed the numbers of INF- $\gamma$  secreting effector T cells. In separate experiments, we tested whether CD33<sup>+</sup>HLA-DR<sup>+</sup> cells are capable of suppressing CTL activity and T-cell proliferation. As shown in Fig. 3B, CD33<sup>+</sup>HLA-DR<sup>+</sup> cells from renal cell carcinoma patients significantly inhibited lysis of T2 tumor

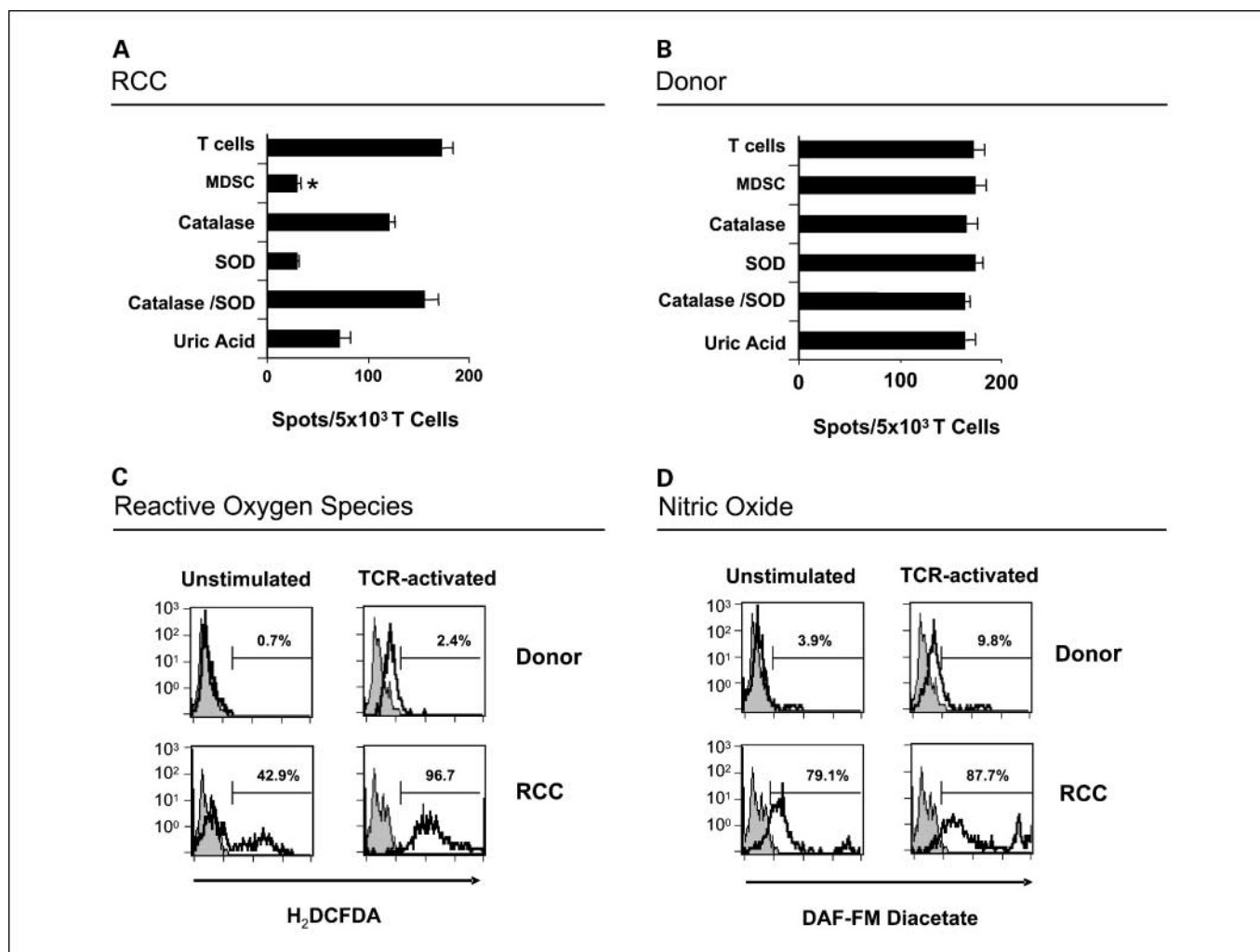


**Fig. 2.** Isolation of CD33<sup>+</sup>HLA-DR<sup>+</sup> MDSC populations. *A*, PBMC obtained from a healthy volunteer and a renal cell carcinoma patient were labeled with anti-HLA-DR magnetic beads and subjected to magnetic separation. HLA-DR – negative cells were labeled with anti-CD33 magnetic beads and CD33<sup>+</sup> cells were isolated by positive selection. *A*, whole PBMC and purified CD33<sup>+</sup> cells from a renal cell carcinoma patient were stained with anti-CD33 and anti-CD11b or anti-CD114 antibody and then analyzed for cell surface marker expression by fluorescence-activated cell sorting. *B*, cytologic analysis of CD33<sup>+</sup>HLA-DR<sup>+</sup> cells. Cytopsin with CD33-positive cells were prepared and stained with H&E.



**Fig. 3.** Renal cell carcinoma – derived CD33<sup>+</sup>HLA-DR<sup>+</sup> cells inhibit T-cell responses. *A*, ELISPOT assay. MDSC isolated from a HLA-A0201-positive healthy volunteer (*Donor*) or a renal cell carcinoma patient (*RCC*) were pulsed with MART-1 peptide and incubated with a MART-1 – specific CTL clone at a ratio of 1:1 for 20 h. Assay was developed as described in Materials and Methods. After washing, spots were counted using an automated ELISPOT counter. *B*, CTL assays were done in the presence of MDSC isolated from a renal cell carcinoma patient as described in the legend to Fig. 2B. We show that MDSC isolated from a renal cell carcinoma patient (*RCC MDSC*), but not from a healthy donor (donor CD33<sup>+</sup> cells) inhibited cytolytic activity of MART-1 – specific CTL. Data were obtained from triplicates of single experiment representative of two. *C*, effect of renal cell carcinoma-derived MDSC on tetanus toxoid-induced T-cell proliferation. Dendritic cells derived from a HLA-A0201-positive healthy volunteer were pulsed with tetanus toxoid (0.1 mg/mL for 1 h in 37<sup>o</sup>C), washed and then cultured with autologous T cells (1:5) in round-bottomed 96-well plates for 4 d in the presence or absence of renal cell carcinoma – derived MDSC. [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) was added 18 h before cell harvest. Thymidine uptake was measured using a liquid scintilla.

target cells mediated by MART-1 – specific CTLs. Additionally, renal cell carcinoma – derived myeloid cells efficiently inhibited tetanus toxoid – induced proliferation of T cells from healthy donors (Fig. 3C).



**Fig. 4.** Involvement of reactive oxygen species and NO in mechanisms of immune suppression mediated by  $CD33^+HLA-DR^+$  cells. To evaluate the role of reactive oxygen species in MDSC-mediated immunosuppression, catalase and/or superoxide dismutase (SOD) or uric acid were added to IFN- $\gamma$  ELISPOT assays in the presence of MDSC isolated from a renal cell carcinoma donor (A) and a healthy volunteer (B). MDSC isolated from an HLA-A0201-positive healthy volunteer (Donor) or a renal cell carcinoma patient (RCC) were pulsed with MART-1 peptide and incubated with a MART-1-specific CTL clone at a ratio of 1:1 for 20 h. Assay was developed as described in Material and Methods. After washing, spots were counted using an automated ELISPOT counter. Antigen-specific production of reactive oxygen species and NO was measured by flow cytometry. MDSC were incubated with MART-1 specific clone, and after 1 h,  $5 \mu\text{mol/L}$  of  $H_2DCFDA$  (C) or  $5 \mu\text{mol/L}$  DAF-FM diacetate (D) were added for 20 min. After washing, CD3-negative cells were analyzed by fluorescence-activated cell sorting (MDSC incubated with MART-1-specific CTL in the absence of fluorogenic probes are shown as gray histograms). Results of representative experiment out of two are shown.

It has been previously reported that production of IFN- $\gamma$  by T cells in response to antigenic stimuli is regulated by reactive oxygen species that are produced by MDSC derived from cancer patients (15) or tumor-bearing mice (23). To test whether reactive oxygen species are involved in inhibition of T-cell responses by  $CD33^+HLA-DR^+$  myeloid cells, we used specific inhibitors of reactive oxygen species production (Fig. 4A). The addition of catalase (500 units/mL) significantly reduced MDSC-mediated T-cell suppression, suggesting that  $H_2O_2$  contributes to MDSC-mediated T-cell inhibitory function. Superoxide dismutase, which converts superoxide anion into hydrogen peroxide, alone did not significantly reverse myeloid cell-mediated immune suppression; however, the combination of catalase and superoxide dismutase reversed immune suppression in a synergistic fashion.

Because the production of reactive oxygen species was responsible for T-cell inhibition, we further sought to answer

in a separate experiment whether direct interaction between  $CD33^+HLA-DR^+$  myeloid suppressor cells (loaded with antigen) and antigen-specific T cells could promote a burst of reactive oxygen species production. Therefore, we incubated MART-1 peptide pulsed  $CD33^+$  myeloid cells with a MART-1 peptide specific CTL clone and subsequently stained these cells with the fluorogenic reactive oxygen species-sensitive probe  $H_2DCFDA$ . As shown in Fig. 4C, MDSC isolated from a renal cell carcinoma patient constitutively produced reactive oxygen species, and its production was further enhanced after coculture with CTL. In contrast, only low levels of reactive oxygen species production could be detected in a similar control cell population ( $CD33^+HLA-DR^+$  cells isolated from a healthy donor) after coculture with MART-1-specific CTL that only insignificantly increased after antigen-specific stimulation. Thus, consistent with the experiments shown in Fig. 4A and B, renal cell carcinoma patient-derived  $CD33^+$  cells

constitutively produced reactive oxygen species and its levels were greatly enhanced after coculture with T cells.

Because NO has also been shown to be involved in mechanisms of MDSC-mediated immune suppression (8, 24–26), we measured NO production by CD33<sup>+</sup>HLA-DR<sup>-</sup> cells using the same experimental conditions described above. For detection of NO produced by myeloid cells, the fluorogenic diacetate DAF-FM was used. DAF-FM diacetate is a cell permeable molecule that forms a fluorescent product benzotriazole after reaction with endogenous NO (27). We found that CD33<sup>+</sup>HLA-DR<sup>-</sup> cells derived from cancer patients but not from healthy donors constitutively produce substantial levels of NO (Fig. 4D). Moreover, NO production can be further enhanced by MDSC upon interaction with CTLs in an antigen-specific manner. In summary, we show here that reactive oxygen species and NO are major factors contributing to T-cell suppression mediated by CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid suppressor cells. In renal cell carcinoma patients, both reactive oxygen species and NO production increased significantly after antigen-specific T-cell interaction, whereas in healthy volunteer-derived, no significant production of reactive oxygen species or NO could be observed.

**In vitro and in vivo effect of ATRA on immune suppression mediated by CD33<sup>+</sup>HLA-DR<sup>-</sup> cells.** ATRA is a naturally occurring metabolite of vitamin A. It is capable of inducing the differentiation of the leukemic cell line HL6020 (28) and is clinically used for induction therapy of patients with acute promyelocytic leukemia. It has also previously been shown that ATRA can promote differentiation of human immature myeloid cells into monocyte/macrophages and dendritic cells (22, 29). Murine studies suggested that ATRA can significantly decrease numbers of MDSC in the tumor-bearing host by promoting its differentiation into a mature CD11c<sup>+</sup> DC, thereby improving the efficacy of the cancer vaccination (30). Therefore, we investigated whether ATRA could promote *in vitro* differentiation of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid suppressor cells derived from cancer patients and reduce or revert its inhibitory effects on T cells.

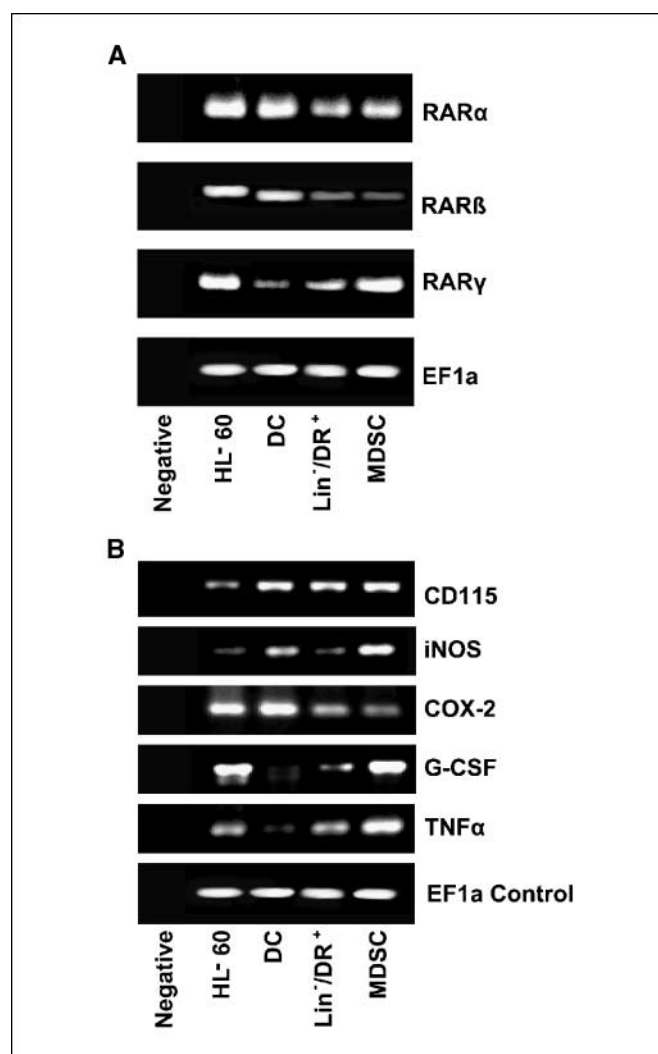
Because ATRA binds to specific receptors, we first measured expression of retinoic acid receptor (RAR)- $\alpha$ , RAR- $\beta$ , and RAR- $\gamma$  in MDSC derived from renal cell carcinoma patients. As shown in Fig. 5A, the renal cell carcinoma-derived MDSC population has increased expression of RAR- $\gamma$ . We next asked whether ATRA can reduce the T-cell inhibition mediated by CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid suppressor cells. CTL assay was done in the presence of MDSC isolated from a renal cell carcinoma patient as described in Materials and Methods. As shown in Fig. 6A, MART-1 peptide-loaded CD33<sup>+</sup>HLA-DR<sup>-</sup> cells, isolated from a renal cell carcinoma patient, significantly inhibited lysis by MART-1-specific CTL, whereas the same cell population from healthy donor-derived exhibited only a modest T-cell suppressive function. Exposure of MDSC to ATRA (1  $\mu$ mol/L) was capable of abrogating the immunosuppressive effect of CD33<sup>+</sup>HLA-DR<sup>-</sup> cells. Thus, CD33<sup>+</sup> MDSC significantly inhibited antigen-specific CTL responses *in vitro*, but their immunosuppressive action could be reversed by presence in culture of ATRA.

To evaluate the effect of ATRA on *in vitro* differentiation of MDSC, PBMC from a renal cell carcinoma patient were subjected to HLA-DR-negative depletion, followed by positive selection for CD33 marker. After 4 days of culture in GM-CSF-

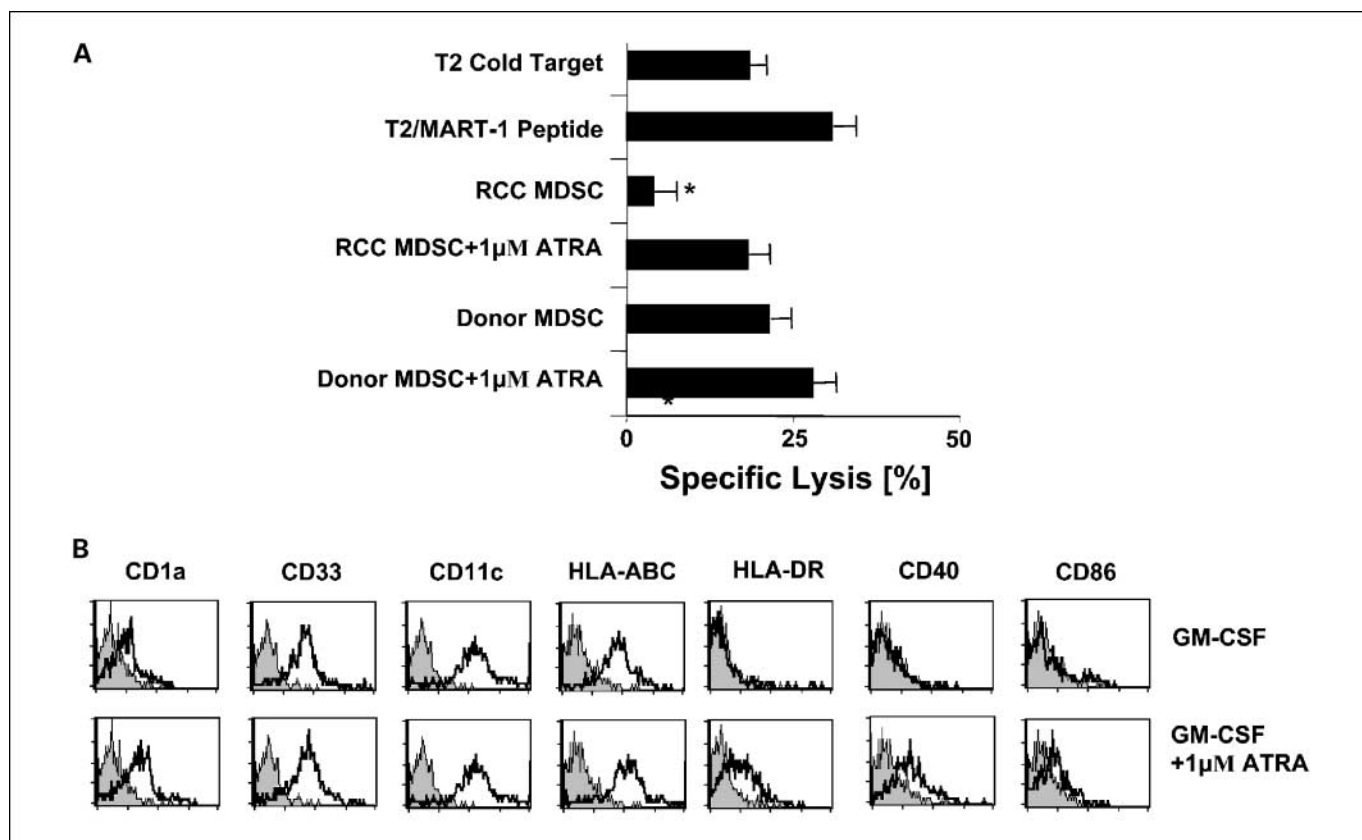
containing media supplemented with or without 1  $\mu$ mol/L ATRA, the phenotype of isolated cells was analyzed by fluorescence-activated cell sorting. Figure 6B shows that presence of ATRA in culture could significantly improve GM-CSF-induced differentiation of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid cells as evidenced by enhanced acquisition of the cell surface markers CD1a, HLA-DR, and CD40. Taken together, we have shown that *in vitro* culture of CD33<sup>+</sup>HLA-DR<sup>-</sup> myeloid suppressor cells with ATRA abrogates its immune suppressive effect on T-cell function through the promotion of APC differentiation and up-regulating of MHC class II and costimulatory molecules.

## Discussion

Many studies have shown that the growth of cancers, including renal cell carcinoma, is often associated with a



**Fig. 5.** Reverse transcription-PCR analysis of renal cell carcinoma-derived MDSC. Total RNA from dendritic cells (DC), HL-60 cell line, MDSC, and lineage-negative/DR<sup>+</sup> renal cell carcinoma-derived cells (Lin<sup>-</sup>/DR<sup>+</sup>) were isolated using Trizol reagent (Invitrogen). Reverse transcription was done at 25°C for 10 min, 42°C for 2 h, and 72°C for 5 min from 100 ng of total RNA using Superscript II reverse transcriptase (Invitrogen) and random primers (3  $\mu$ g/ $\mu$ L; Invitrogen). *A*, expression of receptors for ATRA; *B*, expression of MDSC-related genes.



**Fig. 6.** MDSC-mediated T-cell inhibition *in vitro* can be reversed by ATRA. **A**, CTL assays were done in the presence of MDSC isolated from a renal cell carcinoma patient as described in the legend to Fig. 2B. We show that MDSC isolated from a renal cell carcinoma patient (*RCC MDSC*), but not from a healthy donor (*Donor MDSC*), inhibited cytolytic activity of MART-1-specific CTL. Exposure of MDSC to ATRA (1  $\mu$ mol/L) was capable of abrogating the immunosuppressive function of CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC (*RCC MDSC+ATRA*; *Donor MDSC+ATRA*). Data (specific lysis percent  $\pm$  SD) are derived from triplicate wells of single experiment representative of three. **B**, ATRA enhances *in vitro* differentiation of CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC into APC. PBMC from a renal cell carcinoma patient were subjected to HLA-DR<sup>+</sup> cell depletion, followed by positive selection for CD33. After 4 d of culture in GM-CSF-containing medium supplemented without (**B**, top) or with 1  $\mu$ mol/L ATRA (**B**, bottom), the phenotype of isolated cells was analyzed by fluorescence-activated cell sorting.

decline in immune function, and therapeutic vaccines alone are often ineffective in overcoming tumor-mediated immune suppression. Also, most vaccination strategies lack sufficient activity to counteract antigen-specific immune tolerance and to generate a potent and durable T-cell response in cancer patients (3, 31, 32). Previous studies have highlighted the role of MDSC in cancer-associated immune nonresponsiveness (reviewed in ref. 33). Accumulation of these cells in tumor hosts is promoted by tumor-derived factors, and this tumor-driven expansion of MDSC contributes to tumor escape from the immune system. Recently Mirza et al. showed that ATRA treatment of patients with renal cell carcinoma reduced the number of MDSC and improved T-cell proliferation after antigen stimulation (29). In this study we analyzed the mechanisms by which MDSC inhibit T-cell responses and show a reversal of this effect by ATRA treatment.

We show that the proportion of Lin<sup>-</sup>HLA-DR<sup>-</sup> myeloid cells is considerably elevated in the peripheral blood of renal cell carcinoma patients, when compared with healthy volunteers. These cells display characteristics of immature myeloid cells consistent with the MDSC phenotype previously described by Almand and coworkers, and are capable

of inhibiting T cells (29). Importantly, these cells also express the myeloid cell marker CD33. We show that the MDSC population from renal cell carcinoma patients could be enriched through the isolation of CD33<sup>+</sup>HLA-DR<sup>-</sup> cells. Consistent with the data shown in Figs. 1 and 2, CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC represent a homogeneous cell population that is significantly elevated in renal cell carcinoma patients when compared with healthy volunteers. Purified CD33<sup>+</sup> myeloid cells from renal cell carcinoma patients are characterized by considerable T-cell suppressive activity *in vitro*. Thus, CD33<sup>+</sup>HLA-DR<sup>-</sup> cells derived from renal cell carcinoma patients inhibit phytohemagglutinin-induced T-cell proliferation in a dose-dependent manner. To provide evidence that CD33<sup>+</sup>HLA-DR<sup>-</sup>MDSC are also capable of inhibiting antigen-specific CD8<sup>+</sup> T-cell responses, we tested their immunosuppressive activity in CTL and INF- $\gamma$  ELISPOT assay using isolated and peptide-loaded CD33<sup>+</sup> cells from a HLA-A2<sup>+</sup> renal cell carcinoma patient. MART-1 peptide-loaded MDSC significantly inhibited CTL-mediated lysis (Fig. 6) or INF- $\gamma$  secretion (Fig. 4) in an antigen-specific fashion whereas the control peptide-loaded MDSC, MART-1 peptide-loaded healthy donor MDSC, and unloaded T2 cells exhibited no or only modest inhibitory activity. These

results support the notion that freshly derived CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC show significant T-cell inhibitory potential.

It has been previously established that MDSC derived from cancer patients as well as from tumor-bearing mice produce high levels of reactive oxygen species (15). Moreover, through the production of reactive oxygen species the tumor-derived myeloid cells can regulate T-cell responses. To evaluate the contribution of reactive oxygen species in MDSC-mediated mechanisms of T-cell inhibition in renal cell carcinoma patients, purified CD33<sup>+</sup> cells were pulsed with MART-1 peptide, loaded with DCFDA, and then cultured with MART-1-specific CTLs. Obtained results show that (a) CD33<sup>+</sup> myeloid cells derived from renal cell carcinoma patients constitutively produced reactive oxygen species, whereas no significant production was observed in their cell counterparts isolated from healthy donors; (b) production of reactive oxygen species by CD33<sup>+</sup> cells was increased upon antigen-specific contact of myeloid cells with T cells; and (c) inhibition of IFN- $\gamma$  production by T cells by CD33<sup>+</sup>HLA-DR<sup>-</sup> cells could be abrogated by specific reactive oxygen species scavengers such as catalase. Another characteristic of CD33<sup>+</sup>HLA-DR<sup>-</sup> MDSC is the production of NO. Again, these myeloid cells derived from renal cell carcinoma patients but not from healthy donors constitutively produced NO. The amount of NO production was substantially increased by CD33-positive cells after contact with T cells.

Interestingly, all the characteristics indicated above of CD33<sup>+</sup>HLA-DR<sup>-</sup> suppressive cells (such as the ability to inhibit T-cell proliferation and CTL activity, and increased production of reactive oxygen species and nitric oxide upon contact with activated T cells) are similar to the MDSC population (Gr-1<sup>+</sup>CD11b<sup>+</sup> cells) whose expansion is described in various murine tumor models (8, 34). Moreover, similar to MDSC derived from animal tumor hosts, CD33<sup>+</sup> MDSC are precursors of APC because they are capable of differentiating *in vitro* into mature APC after culture in the presence of GM-CSF (16, 34). Taken together, these results support the hypothesis that cancers in humans and animals may use the same or similar mechanisms to evade the immune system. The accumulation of immune suppressive cells in cancer patients favors conditions that allow tumors to escape immune recognition and promote progressive growth of malignant cells.

Among the strategies that can overcome MDSC-mediated immune suppression is the use of differentiation agents, such as ATRA. ATRA promotes *in vitro* differentiation of MDSC into

mature APC (22, 29). Studies in mice suggest that systemic treatment with ATRA is capable of reducing the frequencies of MDSC, thereby abrogating their inhibitory activity *in vivo* (30). ATRA-mediated reduction of MDSC improved both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses and significantly enhanced the efficacy of the cancer vaccination. These findings form a rationale to augment a vaccine-induced T-cell response with pretreatment of ATRA that can lead to the reduction or elimination of MDSC in cancer patients. In the present study we tested the effect of ATRA on *in vitro* differentiation of CD33<sup>+</sup> myeloid cells derived from renal cell carcinoma patients as well on their immune suppressive potential. We show that the addition of ATRA enhances GM-CSF-stimulated differentiation that results in the acquisition of HLA-DR and costimulatory molecules (CD40). Furthermore, the presence of ATRA abrogated the immune suppressive effect of CD33<sup>+</sup> cells on T-cell response presumably by promoting myeloid cell differentiation. In separate experiments we measured expression of nuclear RAR by immune suppressive cells by reverse transcription-PCR. These cells express high levels of nuclear RAR- $\alpha$  and nuclear RAR- $\gamma$ , but nuclear RAR- $\beta$  expression was almost negligible.

Cumulatively, we have identified that CD33<sup>+</sup>HLA-DR<sup>-</sup> cells in renal cell carcinoma patients represent a MDSC population. Additionally we have shown that reactive oxygen species and NO are major mechanisms by which MDSC inhibit a T-cell response. MDSC may greatly contribute to immune evasion thus promoting tumor growth. These immunosuppressive cells represent a significant obstacle for cancer immune therapy because they may inhibit the vaccine-induced T-cell response. Therefore, there is considerable interest in developing strategies that allow targeting and eliminating MDSC in immunotherapy protocols. Our results suggest that ATRA treatment could help in the depletion of these suppressor cells; however, more studies are needed to find the most optimal individual approach to improve the effect of cancer vaccination.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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