

Mechanism of All-*Trans* Retinoic Acid Effect on Tumor-Associated Myeloid-Derived Suppressor Cells

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Abstract

Myeloid-derived suppressor cells (MDSC) play an important role in tumor escape by suppressing T-cell responses. MDSC represent a group of cells of myeloid lineage at different stages of differentiation. Increased arginase activity and production of reactive oxygen species (ROS) are among the main functional characteristics of these cells. Recent studies have shown that all-*trans* retinoic acid (ATRA) had a potent activity in eliminating MDSC in cancer patients and in tumor-bearing mice. ATRA differentiates these cells into mature myeloid cells. However, the mechanism of this effect is unclear. Here, we have shown that ATRA dramatically and specifically up-regulated gene expression and protein level of glutathione synthase (GSS) in MDSC. This resulted in accumulation of glutathione (GSH) in these cells, observed in both mice and cancer patients. Blockade of GSH synthesis cancelled the effect of ATRA on MDSC. Accumulation of GSH in these cells using *N*-acetyl-L-cysteine mimicked the effect of ATRA on MDSC differentiation. Analysis of potential mechanisms of ATRA effect on GSS revealed that ATRA regulates its expression not by directly binding to the promoter but primarily via activation of extracellular signal-regulated kinase 1/2. Thus, ATRA induced differentiation of MDSC primarily via neutralization of high ROS production in these cells. This novel mechanism involves specific up-regulation of GSS and accumulation of GSH and could be used in developing and monitoring therapeutic application of ATRA. [Cancer Res 2007;67(22):11021–8]

Introduction

Myeloid-derived suppressor cells (MDSC) are produced in large numbers in patients with many different types of cancer and in practically all tested mouse tumor models. These cells play an important role in mechanisms of tumor escape by suppressing T-cell responses (1–3). MDSC belong to myeloid lineage and represent a group of relatively immature cells at different stages of differentiation. In mice they have a phenotype of Gr-1⁺CD11b⁺ cells, and in humans the MDSC are identifiable as lineage (CD3, CD14, CD19, CD56)–negative, HLA-DR–negative, and CD33–positive or CD33⁺CD14[–]CD11b⁺ cells (4). Increased arginase activity and production of reactive oxygen species (ROS) are among the

main functional characteristics of these cells. ROS were shown to be not only directly involved in MDSC-mediated T-cell suppression but also as one of the major factors contributing to the inability of MDSC to differentiate into mature myeloid cells (5, 6). Because of the important role of MDSC in tumor-associated immune suppression, intensive studies in recent years are focused on the identification of the therapeutic means to eliminate these cells. It has been shown that all-*trans* retinoic acid (ATRA) had a potent activity in differentiation of MDSC *in vitro* (7, 8). Treatment of cancer patients and tumor-bearing mice with ATRA resulted in substantial decrease of these cells and improvement of immune responses (9, 10). In mice, treatment with ATRA significantly enhanced the effect of cancer vaccines (9). ATRA has also been shown to improve differentiation of dendritic cells in cancer patients (11). ATRA and granulocyte macrophage colony-stimulating factor (GM-CSF) could drive the differentiation of monocytes into dendritic cell–like cells. These cells exhibited dendritic cell morphology, secreted interleukin (IL)-12p70, and could induce a proliferative response in naïve CD4⁺ T cells (12).

Thus, ATRA could be a valuable addition to cancer immunotherapy, which prompted efforts in testing this compound in ongoing clinical trials. However, a critical question that remained unresolved is the mechanism of the ATRA effect on MDSC. It is known that ATRA, a member of the retinoid family of molecules structurally related to vitamin A, exerts profound effects on control of cell proliferation, induction of differentiation, and apoptosis in normal cells and various cancer cells (13). The biological effects of retinoids are modulated through families of nuclear receptors, retinoic acid receptors (RAR), and retinoid X receptors (RXR), which work as RXR/RAR heterodimers. These heterodimers have two distinct functions: first, they modulate the frequency of transcription initiation of target genes after binding to retinoic acid response elements (RARE) in their promoters; second, they affect the efficiency of other signaling pathways by mechanisms that remain elusive (13). RAREs have been identified in the promoters of a large number of retinoid target genes implicated in a wide variety of functions (14). Acute promyelocytic leukemia is the most well-defined target of ATRA activity. Acute promyelocytic leukemia is associated with chromosomal translocation involving chromosome 15 (*PML* gene) and chromosome 17 (*RAR α* gene). The consequence of this translocation is the expression of PML-RAR α fusion protein, which leads to an arrested differentiation of the myeloid cell development. Treatment of acute promyelocytic leukemia with ATRA results in displacement of the chimeric PML-RAR α protein. Restored PML and RAR α overcome the differentiation block and commit acute promyelocytic leukemia cells to differentiation. The effect of ATRA on nontransformed myeloid cells is much less clear. It seems that it is highly dependent on cell type and specific conditions.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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In this study, we described novel molecular mechanism of ATRA effect on MDSC via up-regulation of GSH synthesis and down-regulation of increased ROS level in these cells.

Materials and Methods

Mice, cell culture, and reagents. Female BALB/c and C57BL/6 mice, ages 6 to 8 weeks, were obtained from the National Cancer Institute or Harlan. All mice were handled in accordance with the Guidelines for Animal Experiments requirements. CT-26 colon carcinoma was established in BALB/c mice and MC38 and EL-4 tumors were established in C57BL/6 mice by s.c. inoculation of 5×10^5 tumor cells. To generate tumor cell conditioned medium (TCCM), cells were kept for 48 h in medium with reduced (3%) fetal bovine serum (FBS) concentration. RPMI 1640, DMEM, FBS, and antibiotics were obtained from Invitrogen; recombinant murine IL-4 and human GM-CSF were from RDI; and murine GM-CSF was from Biosource (Invitrogen). DL-Buthionine-(S,R)-sulfoximine (BSO), *N*-acetyl-L-cysteine (NAC), and ATRA were purchased from Sigma. BSO was dissolved in PBS, NAC in serum-free RPMI, and ATRA was diluted in ethanol. Antibodies against phosphorylated and total signal transducers and activators of transcription 3 (STAT3), Akt, c-jun NH₂-terminal kinase (JNK), and phosphorylated extracellular signal-regulated kinase (ERK) were obtained from Cell Signaling Technologies; against total ERK from BD Transduction Laboratories (BD Biosciences, San Jose, CA); and against nuclear factor E2-related factor 2 (Nrf-2) and GSS from Santa Cruz Biotechnology (Santa Cruz, CA). The mitogen-activated protein kinase (MAPK) kinase (MEK)-1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and the JNK inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone (SP 600125) were obtained from Calbiochem (EMD Chemicals San Diego, CA).

Preparation of cells. Dendritic cells were generated from bone marrow, as described earlier (15), followed by isolation of CD11c-positive dendritic cells using magnetic beads separation technique (Miltenyi Biotec). Purity of CD11c-positive population was >95% as determined by flow cytometry. MDSC were isolated from spleens of tumor-bearing mice using anti-Gr-1 antibody as described earlier (15, 16). The purity of the Gr-1⁺CD11b⁺ MDSC population was evaluated by flow cytometry and exceeded 90%.

Peripheral blood was obtained from three patients with renal carcinoma who had consented to donate it in accordance with University of South Florida Institutional Review Board guidelines, consistent with the Declaration of Helsinki. The mononuclear cells were isolated by Ficoll gradient centrifugation. Cells were resuspended in RPMI 1640 supplemented with 25 mmol/L HEPES, 10% FBS, and 10 ng/mL human GM-CSF; plated at concentration 1.2×10^6 /mL in 24-well plate; and treated with 1.5 μmol/L ATRA or vehicle control for 48 h. After that time, cells were collected and surface staining followed by intracellular GSH staining was done.

Measurement of intracellular GSH and ROS. GSH level was determined using Colorimetric Glutathione Detection Kit (BioVision) according to the manufacturer's protocol. Absorbance was read at 405 nm using a microplate reader. GSH level was expressed as nanograms per 10^6 cells.

Flow cytometric measurement of GSH was done using monochlorobimane (Molecular Probes, Invitrogen Corp.) as described previously (17, 18). Cells were analyzed using LSRII (BD). Monochlorobimane was excited at 405 nm (violet laser) and emission was detected at 515 to 520 nm. ROS production by MDSC was measured as previously described (5) using the oxidation-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes, Invitrogen).

Western blotting. Cells were collected, washed with ice-cold PBS, and lysed in 30 mmol/L HEPES (pH 7.5), 10 mmol/L NaCl, 5 mmol/L MgCl₂, 25 mmol/L NaF, 1 mmol/L EGTA, 1% Triton X-100, 10% glycerol, and protease and phosphatase inhibitor cocktails (Sigma). To detect Nrf-2 protein expression, cells were collected, washed with ice-cold PBS, followed by isolation of cytoplasmic and nuclear fractions using CelLytic NUCLEAR Extraction Kit (Sigma). Western blotting was done as described earlier (15).

Electromobility shift assay. MDSC isolated from spleens of CT-26 tumor-bearing mice were plated with 10 ng/mL GM-CSF and CT-26 TCCM and treated with 1.5 μmol/L ATRA or vehicle control (ethanol) for 0.5, 1, 1.5, 3, and 24 h. Cells were then collected, washed with ice-cold PBS, and resuspended in 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.3% NP40, and protease and phosphatase inhibitors. Cells were incubated on ice for 5 min and then centrifuged for 5 min at 4°C. The supernatant representing cytoplasmic fraction was removed and the pellet was resuspended in 20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 25% glycerol, and protease and phosphatase inhibitors; incubated on ice for 10 min; mixed briefly on a Vortex; and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant representing nuclear fraction was collected and electromobility shift assay was done using 5 μg of nuclear extract as described previously (19). Samples were resolved in 6% polyacrylamide gel.

Quantitative real-time PCR. MDSC isolated from spleens of naïve or tumor-bearing mice were cultured overnight in the presence of GM-CSF and TCCM with or without ATRA. After that time, cells were collected and RNA was extracted using Trizol reagent (Invitrogen). In some experiments, RNA was extracted from MDSC immediately after their isolation. The cDNA was synthesized and real-time PCR was done with 2.0-μL cDNA template using TaqMan Universal PCR Master Mix and Gene Expression Assay mixes containing sequence-specific primers for glutathione synthase (GSS), glutamate-cysteine ligase catalytic subunit (Gclc), or modifier subunit (Gclcr) and 6-FAM dye-labeled TaqMan MGB sequence-specific probe (Applied Biosystems). As a control, amplification with 18S endogenous control assay mix was used. PCR was carried out in triplicate for each sample using ABI Prism 7900HT instrument. Data quantitation was done using the relative standard curve method. Expression levels of *GSS*, *Gclc*, and *Gclcr* genes were normalized to 18S mRNA level measured concurrently.

Murine glutathione synthetase promoter cloning. Murine genomic DNA was extracted using standard protocols, digested with *Hind*III (Invitrogen), and purified by phenol/chloroform extraction before PCR. The proximal promoter region of GSS gene was amplified using the following primers: forward, 5'-ATGGGTGCCGAGGATAGACTTC-3'; reverse, 5'-GGATGCTCGAGACAGCAATGGCATTACTC-3'. PCR was done using native Pfu DNA polymerase according to the manufacturer's instructions (Stratagene). PCR used an initial denaturation step of 95°C for 1 min, followed by 32 cycles of denaturation at 95°C for 45 s, annealing of primers at 60°C for 45 s, and polymerization at 72°C for 6 min. A final extension of 10 min at 72°C was done. The PCR products were subcloned into pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). The promoter inserts were removed from the pCR2.1-TOPO vector by digesting with *Kpn*I and *Xho*I (both from New England Biolabs) and ligated into *Kpn*I and *Xho*I sites of the pGL3-basic vector. The presence of insert in pGL3-basic-GSS was confirmed by sequencing.

Luciferase assay. Vector pGL3-RARE-luciferase carrying RARE was purchased from Addgene. 32D cells were transfected with either pGL3-RARE-luciferase, pGL3-basic, or pGL3-basic-GSS and cotransfected with pRL-TK (Promega) using Amaxa nucleofector device, buffer V, and program E32. Cells were cultured for overnight and then treated with 80 μmol/L ATRA or vehicle control for 8 h. After that time, cells were collected, lysed with Passive lysis buffer (Promega), and frozen at -80°C. Firefly and renilla luciferase activities were measured using Dual-Luciferase reporter assay system (Promega) and 20/20n luminometer (Turner Biosystems). For each sample, firefly luciferase activity was normalized to renilla luciferase activity.

Chromatin immunoprecipitation assay. 32D cells were treated with 80 μmol/L ATRA for 18 h. Preparation of chromatin-DNA and chromatin immunoprecipitation assay were done as described by the protocol supplied by Upstate Biotechnology. Antibodies against RAR-α, RXR-α, and normal rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology and protein A agarose/salmon sperm DNA was from Upstate (Millipore). Sonication was done by using Branson Sonifier (model 450, VWR Scientific). Purified DNA was subjected to real-time PCR with the following primers spanning the potential ATRA binding site in the GSS promoter: forward,

5'-AGTAATTTGGCTCCTGGTGA-3'; reverse, 5'-AGTTATGAGCCTCCGTG-TGG-3'. Real-time PCR was done on ABI Prism 7900HT instrument using Power SYBR Green PCR master mix (Applied Biosystems).

Gene array analysis. MDSC were isolated from spleens of CT-26 tumor-bearing mice using magnetic separation technique as described above. Cells were plated with 10 ng/mL GM-CSF and CT-26 TCCM and treated with 1.5 $\mu\text{mol/L}$ ATRA or vehicle control (ethanol) for 12 h. After that time, cells were collected and RNA was isolated and cDNA was prepared. cRNA was synthesized and labeled with biotin by *in vitro* transcription using the Enzo Bioarray high-yield RNA transcript labeling kit, followed by hybridization with Affymetrix GeneChip mouse430 2.0 array for 14 to 16 h. After hybridization, the GeneChip arrays were washed and stained with phycoerythrin-conjugated biotin. Chips were subsequently scanned at 570 nm with a GeneChip System confocal scanner. Scanned output files were visually inspected for hybridization artifacts and then analyzed by Affymetrix Microarray Suite 5.0 software.

Results

ATRA induces expression of GSS and accumulation of GSH in MDSC. To investigate the potential mechanisms of ATRA effect on MDSC, Gr-1⁺CD11b⁺ cells isolated from CT-26 tumor-bearing mice were cultured for 18 h in the presence of CT-26 TCCM and GM-CSF with or without 1.5 $\mu\text{mol/L}$ ATRA. RNA was extracted and a genome-wide analysis of gene expression was done using Affymetrix GeneChips. ATRA down-regulated a cluster of IFN-inducible genes in MDSC (Supplementary Table S1). This was not surprising because activation of IFN-associated genes was a dis-

tinct characteristic of these cells in tumor-bearing mice (20). Among genes up-regulated by ATRA, it was noticeable that there was an increase of several genes with antioxidant activity (Supplementary Table S2). Specifically, ATRA substantially increased the expression of GSS. GSS is directly involved in the synthesis of glutathione (L- γ -glutamyl-L-cysteinylglycine; GSH). Because increased level of ROS plays a critical role in MDSC differentiation and function (5, 6), we investigated further the potential effect of ATRA on GSH metabolism in MDSC.

GSH is synthesized from its constituent amino acids by the sequential action of glutamate cysteine ligase (GCL; also known as γ -glutamylcysteine synthetase, referred to below as GCS) and GSS. To verify our preliminary observations, we evaluated the effect of ATRA on the expression of GCS and GSS using quantitative real-time PCR. MDSC were isolated from spleens of CT-26 tumor-bearing (BALB/c), MC38 tumor-bearing (C57BL/6), or EL-4 tumor-bearing (C57BL/6) mice. Cells were cultured overnight in the presence of corresponding TCCM and GM-CSF with or without 1.5 $\mu\text{mol/L}$ ATRA. RNA was extracted and the levels of gene expression were measured using quantitative real-time PCR. ATRA dramatically (7–40-fold) up-regulated the expression of GSS in MDSC from all tumor models. In contrast, expression of two subunits of GCS, catalytic subunit of glutamate cysteine ligase (*Gclc*) and regulatory subunit (*Gclr*), was not affected (Fig. 1A). Increased expression of GSS gene was accompanied by substantial increase in the level of this protein (Fig. 1B). Increase in GSS gene

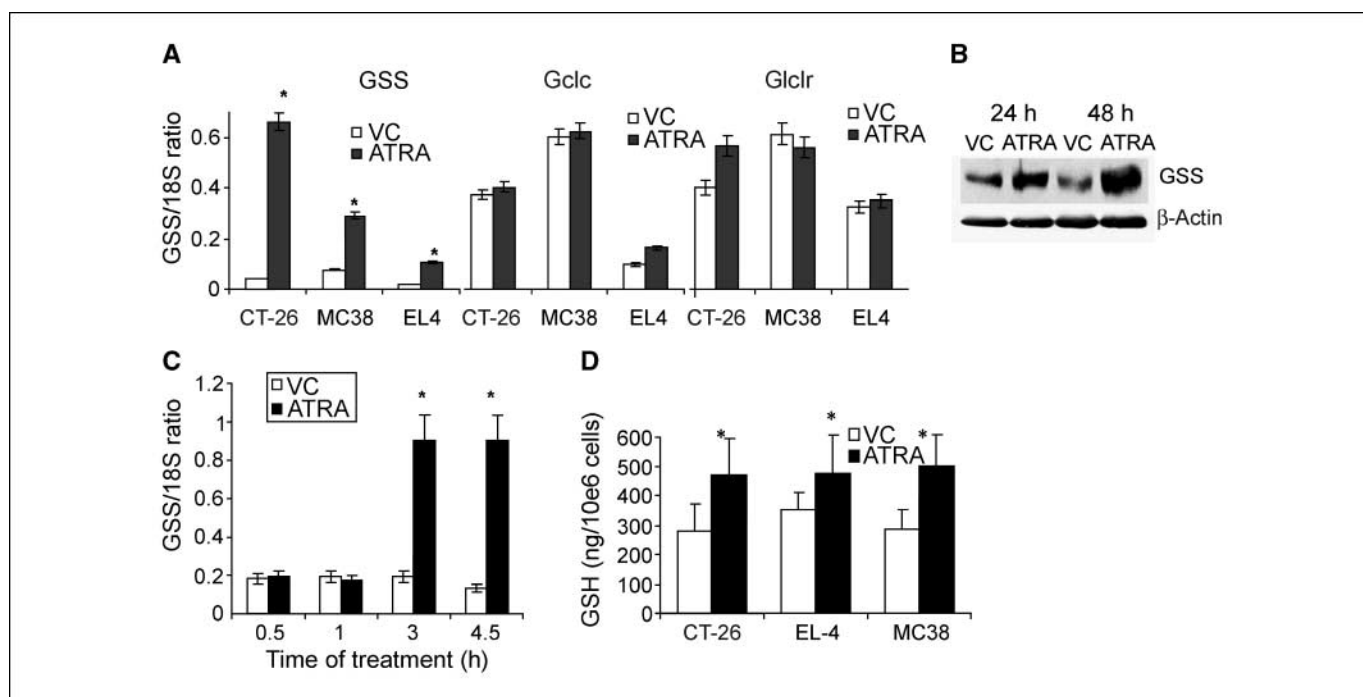


Figure 1. ATRA up-regulates the expression of GSS and GSH in MDSC from tumor-bearing mice. MDSC were isolated from spleens of tumor-bearing mice and cultured in complete culture medium supplemented with corresponding tumor cell condition medium and 1.5 $\mu\text{mol/L}$ ATRA. CT-26, BALB/c mice bearing CT-26 tumor; MC38, C57BL/6 mice bearing MC38 tumor; EL-4, C57BL/6 mice with EL-4 tumor. *, $P < 0.05$, versus cells treated with vehicle alone. A, MDSC cultured overnight with ATRA or vehicle control (VC). RNA was extracted and expression of indicated genes was evaluated by quantitative real-time PCR as described in Materials and Methods. Results were adjusted to the level of 18S mRNA expression. Each experiment was done in triplicate. At least three mice composed each group. *, $P < 0.05$, between ATRA and vehicle control (paired two-sided *t* test). B, MDSC from CT-26 tumor-bearing mice were treated for 24 or 48 h as described above. Whole-cell lysates were obtained and the levels of specific proteins were evaluated in Western blotting as described in Materials and Methods. C, experiments with MDSC from CT-26 tumor-bearing mice were done as described in A. RNA was extracted at indicated time points and the level of GSS mRNA was measured by quantitative real-time PCR. *, $P < 0.05$, between ATRA and vehicle control (paired two-sided *t* test). D, MDSC from different tumor-bearing mice were treated with ATRA for 24 h and the level of GSH was measured with colorimetric GSH detection kit. *, $P < 0.05$, between ATRA and vehicle control (paired two-sided *t* test). The number of mice per group was as follows: CT-26, 5; EL-4, 6; MC38, 4.

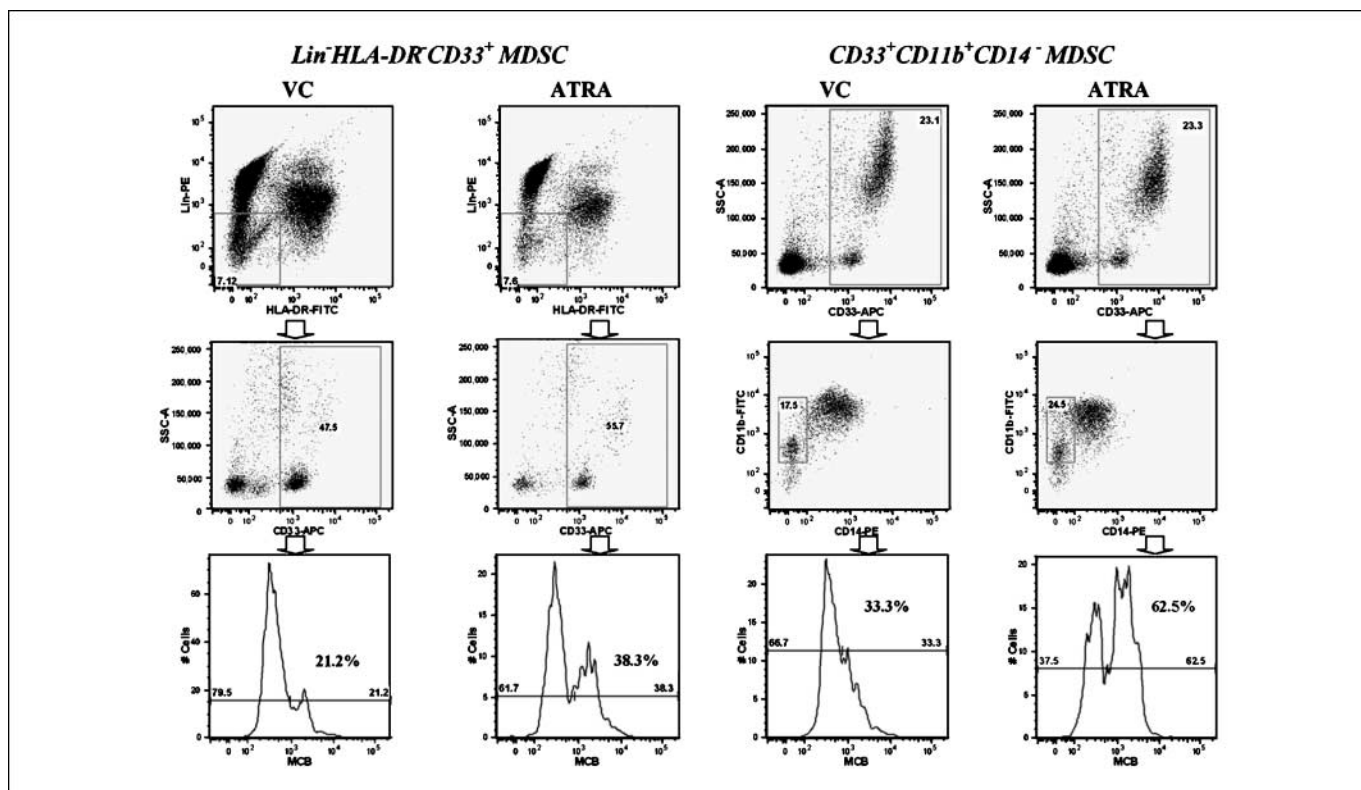


Figure 2. ATRA up-regulates the expression of GSH in MDSC from cancer patients. Peripheral blood was collected from patients with metastatic renal cell cancer. Mononuclear cells were isolated using Ficoll-Paque gradient and cultured for 48 h with 1.5 $\mu\text{mol/L}$ ATRA or vehicle control. Cells were then stained with different antibodies and monochlorobimane to detect intracellular GSH. Lineage cocktail (*Lin*) included phycoerythrin-conjugated antibody against CD3, CD14, CD19, and CD56. Cells were analyzed on LSRII flow cytometer. Three experiments showed similar results.

expression was observed as early as 3 h after start of the treatment with ATRA (Fig. 1C). Up-regulation of GSS was associated with increased level of GSH. Using enzymatic assay, we found that treatment of MDSC with ATRA *in vitro* significantly increased GSH level in these cells (Fig. 1D). This increase was seen after overnight incubation with ATRA and was detectable for at least 72 h (Fig. 1D and data not shown).

To test *in vivo* the effect of ATRA on GSH level in MDSC, CT-26 tumor-bearing mice were treated with daily i.p. injections of ATRA for 3 days and then the level of GSH was measured in MDSC from spleens by flow cytometry. This time frame was selected because longer exposure to ATRA resulted in a substantial decrease in the presence of MDSC in tumor-bearing mice (ref. 9 and data not shown). ATRA induced a statistically significant increase in the level of GSH in MDSC. Mean fluorescence intensity was increased from $5,800 \pm 128.7$ in mice treated with vehicle control to $6,675 \pm 45.2$ in mice treated with ATRA ($P = 0.012$).

To evaluate the effect of ATRA on GSH levels in cancer patients, mononuclear cells were isolated from patients with metastatic renal cell carcinoma and treated with ATRA for 48 h. GSH was measured by flow cytometry. MDSC in peripheral blood of cancer patients were identified either as $\text{Lin}^- \text{HLA-DR}^+ \text{CD33}^+$ or $\text{CD33}^+ \text{CD11b}^+ \text{CD14}^-$ cells. ATRA substantially increased GSH level in both these cell populations MDSC (Fig. 2). Thus, ATRA increased the level of GSH in MDSC from mice and cancer patients.

GSH synthesis is responsible for ATRA effects on MDSC. Next, we investigated whether increased GSH level was responsible for the MDSC differentiation caused by ATRA. BSO is a known

potent inhibitor of GSH synthesis. Twenty-four-hour treatment of bone marrow cells with BSO dramatically reduced the level of GSH in these cells (data not shown), which resulted in accumulation of ROS (Fig. 3A). To evaluate the potential effect of GSH down-regulation and ROS accumulation on differentiation of myeloid cells, bone marrow cells isolated from tumor-free naïve mice were cultured with GM-CSF and IL-4 for 5 days. Different concentrations of BSO were added at days 0 and 3. BSO prevented differentiation of $\text{Gr-1}^+ \text{CD11b}^+$ immature myeloid cells. The presence of BSO in cultures resulted in >3-fold increase in the proportion of these cells (Fig. 3B). To verify these findings, Gr-1^+ cells were isolated from bone marrow of naïve tumor-free mice and cultured for 7 days with GM-CSF. BSO (1 mmol/L) was added on days 0, 2, and 5. BSO significantly decreased differentiation of immature myeloid cells into macrophages and dendritic cells ($P < 0.05$; Fig. 3C). Thus, down-regulation of GSH and accumulation of ROS in immature myeloid cells prevented their differentiation into mature cells, similar to the situation with MDSC from tumor-bearing mice.

To investigate whether BSO might interfere with the effect of ATRA on MDSC differentiation, MDSC were isolated from CT-26 tumor-bearing mice and cultured for 5 days with GM-CSF in the presence of TCCM. Consistent with previous observations, ATRA substantially reduced the proportion of MDSC and increased the presence of macrophages and dendritic cells. However, in the presence of BSO, ATRA did not change the proportion of MDSC, F4/80^+ macrophages, and $\text{CD11c}^+ \text{IA}^{\text{d}+}$ dendritic cells (Fig. 3D). Thus, these data indicated that ATRA-induced differentiation of MDSC could be mediated by increased GSH.

We asked whether increased level of GSH by itself was sufficient to promote differentiation of MDSC. A common limiting factor in GSH synthesis is the bioavailability of cysteine inside the cell. Cysteine is highly unstable in its reduced form. In the N-acetylated form (NAC), cysteine is markedly stabilized. After free NAC enters a cell, it is rapidly hydrolyzed to release cysteine, which results in accumulation of GSH. Overnight treatment of MDSC with NAC increased GSH level in these cells almost 2-fold (data not shown), which was similar to the effect of ATRA. MDSC isolated from tumor-bearing mice were cultured with TCCM and GM-CSF for 5 days. During the first 24 h of culture, cells were treated with 10 mmol/L NAC. NAC significantly reduced the proportion of MDSC and induced their differentiation into macrophages and dendritic cells (Fig. 4).

Mechanism of ATRA effect on GSS. What could be the mechanism of ATRA effect on GSS? Because the promoter region of GSS contains a partial retinoic acid responsive sequence (21, 22), we tested the possibility that ATRA could directly regulate the expression on GSS gene. To determine possible binding of RARs to

GSS promoter, chromatin immunoprecipitation assay was done in ATRA-treated 32D cells or MDSC. In both cases, experiments showed negative results (data not shown). Proximal region of GSS promoter containing potential retinoic acid binding sites was cloned into luciferase reporter plasmid and transfected into 32D cells. Expression of luciferase was measured in these cells after treatment with ATRA. No ATRA effect on luciferase expression was detected (Fig. 5A). Thus, it was highly unlikely that ATRA regulated GSS transcription via direct binding of retinoic receptors with promoter.

Up-regulation of GSS expression did not require a protein synthesis because treatment of murine MDSC with cycloheximide did not cancel the effect of ATRA on GSS expression (Fig. 5B). Our previous studies have shown a critical role of STAT3 activation in accumulation of MDSC (15, 23). Therefore, we hypothesized that ATRA could mediate its effect on GSS via down-regulation of STAT3. However, experiments with DNA binding (Supplementary Fig. S1) and phospho-STAT3 (data not shown) showed that ATRA did not affect STAT3 activity in MDSC. No effect of ATRA was also

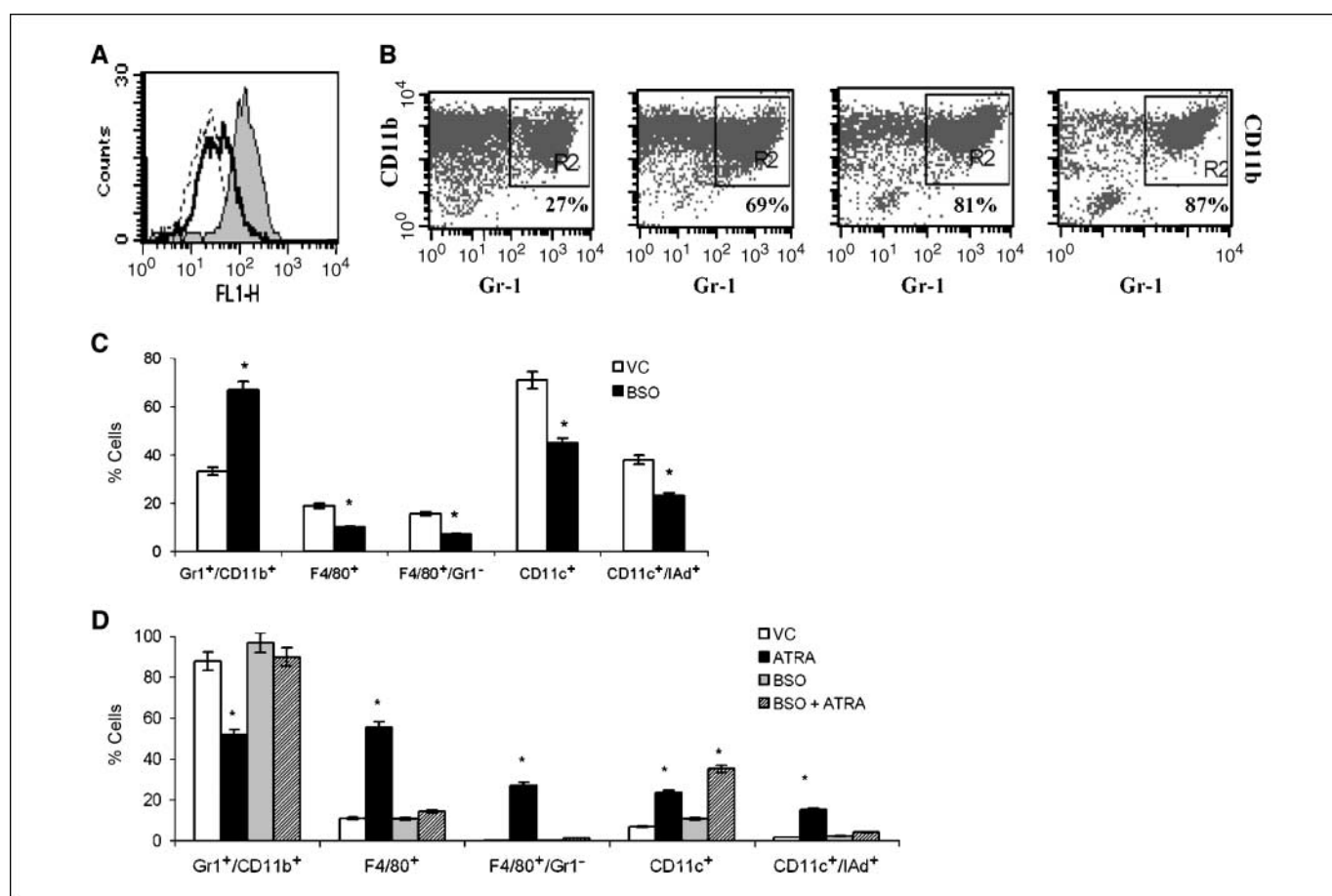


Figure 3. Effect of GSH inhibition on differentiation of myeloid cells. *A*, level of ROS in bone marrow cells treated with GSH inhibitor. Bone marrow cells isolated from naïve BALB/c mice were cultured for 24 h in complete culture medium supplemented with GM-CSF in the presence of control vehicle (dashed line), 1 mmol/L BSO (solid line), or 2 mmol/L BSO (shaded histogram). Cell viability was >80%. Cells were then stained with 1 μ mol/L DCFDA and analyzed on FACSCalibur flow cytometer. Three experiments with similar results were done. *B*, effect of BSO on differentiation of myeloid cells. Bone marrow cells from naïve mice were cultured with GM-CSF and IL-4 for 5 d. Indicated concentrations of BSO were added at days 0 and 3. Proportion of Gr-1⁺CD11b⁺ immature myeloid cells was measured by flow cytometry. Two experiments with the same results were done. *C*, effect of BSO on differentiation of MDSC. MDSC were isolated from CT-26 tumor-bearing mice and cultured for 7 d with GM-CSF and TCCM. BSO (1 mmol/L) or vehicle control was added on days 0, 2, and 5. The presence of different cell populations was evaluated by flow cytometry. *, $P < 0.05$, between BSO and vehicle control (paired two-sided t test). *D*, effect of BSO combination with ATRA on differentiation of MDSC. MDSC were isolated from CT-26 tumor-bearing mice and cultured for 5 d with GM-CSF in the presence of TCCM. ATRA or/and BSO were added on day 0 and cell phenotype was evaluated by flow cytometry. *, $P < 0.05$, versus cells treated with vehicle alone.

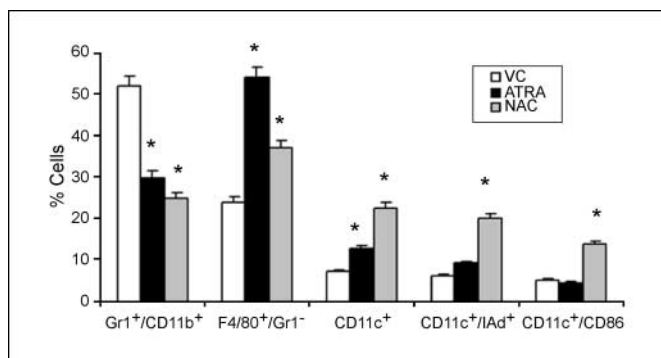


Figure 4. Effect of NAC on differentiation of MDSC. MDSC were isolated from spleens of CT-26 tumor-bearing mice. They were cultured with GM-CSF and TCCM for 5 d in the presence of ATRA or NAC. Cell phenotype was evaluated by flow cytometry. *, $P < 0.05$, versus cells treated with vehicle alone.

found on activation of nuclear factor κ B (NF- κ B) and Nrf-2, the major regulators of antioxidant proteins (Fig. S1).

ATRA did not affect activation of Akt, p38, or JNK (Fig. 6A). In contrast, a substantial activation of ERK1/2 was observed as early as 3 h after start of the treatment with ATRA (Fig. 6A). This coincided with activation of *GSS* transcription. To investigate the link between activation of ERK and *GSS* expression, MDSC were incubated with U0126, a specific inhibitor of MEK1/2, which blocks downstream phosphorylation of ERK1/2 (Fig. 6B). Inhibition of ERK1/2 prevented ATRA-inducible up-regulation of *GSS* (Fig. 6C). This effect was absent when MDSC were cultured with selective JNK/stress-activated protein kinase (SAPK) inhibitor SP 600125 (Fig. 6C). To evaluate the potential role of ERK1/2 in ATRA-mediated effect on MDSC differentiation, these cells were cultured for 5 days with TCCM, GM-CSF, and ATRA with or without U0126. Inhibition of ERK1/2 completely abrogated the effect of ATRA on MDSC differentiation (Fig. 6D).

Discussion

MDSC are one of the major factors mediating tumor-associated immune suppression, and their removal results in improvement of

immune responses in cancer, which could be potentially beneficial for various immunotherapeutic strategies. ATRA substantially reduces the presence of these cells in cancer patients and tumor-bearing mice (9, 10) and now is being tested in clinical trials in combination with different cancer vaccines. Therefore, it would be important to understand the mechanism of ATRA effects on MDSC. Because ATRA has pleiotropic effects on different cell functions, we used a genome-wide analysis of gene expression to identify potential ATRA-affected target genes in MDSC. ATRA dramatically up-regulated the expression of *GSS*, one of the enzymes involved in synthesis of GSH. Importantly, in MDSC, ATRA did not affect the expression of the other rate-limiting enzyme involved in GSH synthesis, GCS. We report here the first demonstration of *GSS* protein regulation by ATRA in any experimental system. Increased expression of *GSS* had, as a consequence, the accumulation of GSH in MDSC *in vivo* and *in vitro*. Several groups have previously reported the contradictory effects of retinoids on GSH in several different cell lines. Retinoic acid decreased the intracellular levels of GSH in chondrocyte (24). In contrast, ATRA increased GSH content in mesangial cells in a dose- and time-dependent fashion. In these cells, ATRA up-regulated the levels of GCS (25). A similar effect of ATRA was described in several leukemia cell lines (26, 27). Thus, up-regulation of GSH synthesis by ATRA is not unique for MDSC. However, an increased level of GSH is especially important for MDSC differentiation. In contrast to their normal counterparts, MDSC generated in tumor-bearing hosts and during various infections had high levels of ROS (5, 28, 29). Inhibition of ROS with different scavengers promoted MDSC differentiation (6). Therefore, increased level of the most potent antioxidant GSH would have a positive effect on MDSC differentiation.

Inhibition of GSH with BSO resulted in an increase of ROS content in normal bone marrow cells and accumulation of Gr-1⁺CD11b⁺ immature myeloid cells. In tumor-bearing mice, BSO abrogated the effect of ATRA on MDSC. In the other set of experiments, high level of GSH was created in MDSC by using the cysteine donor NAC. This resulted in differentiation of MDSC similar to the effect of ATRA. Taken together, these data indicated that ATRA exerted its effect on MDSC via up-regulation of *GSS* and

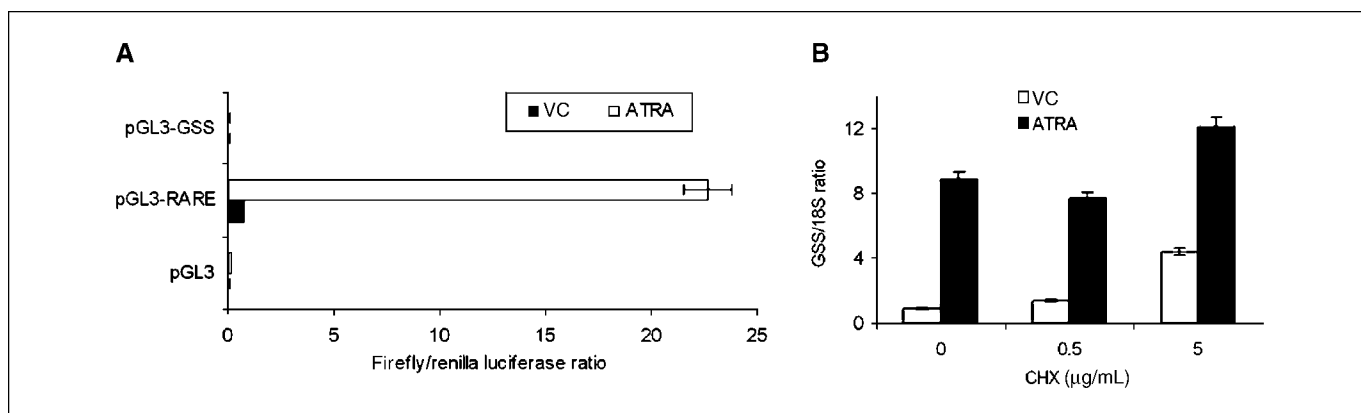


Figure 5. Regulation of *GSS* expression by ATRA in MDSC. **A**, 32D cells were transfected with either pGL3 plasmid (negative control), pGL3 plasmid containing ATRA responsive element (pGL3-RARE; positive control), or pGL3 plasmid containing *GSS* promoter region with putative ATRA binding sites (pGL3-GSS) and cotransfected with pRL-TK (Promega). Cells were cultured overnight and then treated with 80 μ mol/L ATRA or vehicle control for 8 h. Cells were collected, lysed, and frozen at -80°C . Firefly and renilla luciferase activities were measured using Dual-Luciferase reporter assay system (Promega) and luminometer. For each sample, firefly luciferase activity was normalized to renilla luciferase activity. All measurements were done in triplicate. **B**, MDSC were isolated from spleens of CT-26 tumor-bearing mice and then cultured in the presence of GM-CSF and TCCM with 1.5 μ mol/L ATRA or vehicle control. Cycloheximide at indicated concentrations was added together with ATRA and *GSS* expression was measured in MDSC after overnight incubation by quantitative real-time PCR.

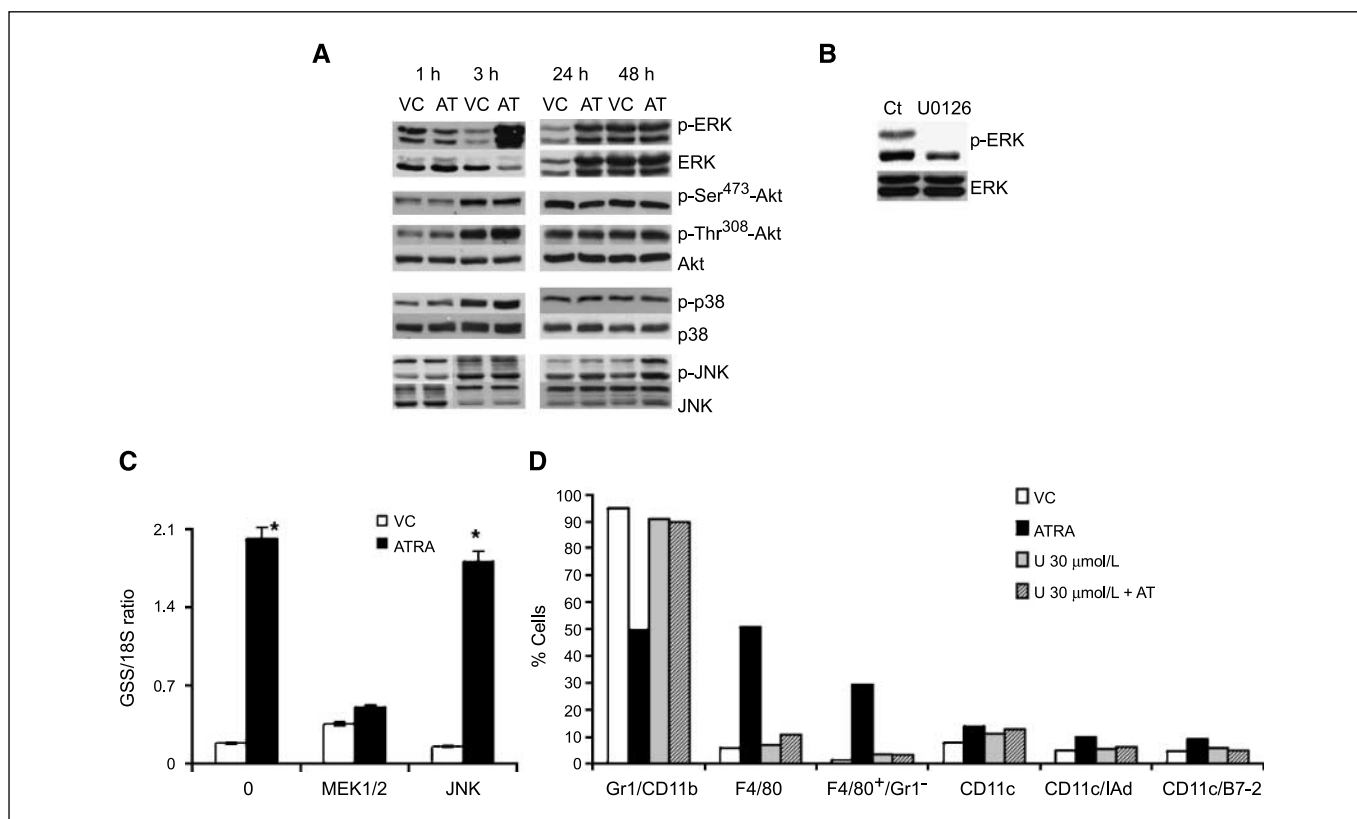


Figure 6. Role of ERK1/2 in ATRA-inducible GSS expression and MDSC differentiation. *A*, Western blot analysis of different proteins in MDSC after treatment with ATRA. *B*, MDSC were isolated from CT-26 tumor-bearing mice and cultured for 3 h with GM-CSF, TCCM, and 1.5 $\mu\text{mol/L}$ ATRA in the presence of 30 $\mu\text{mol/L}$ of specific MEK1/2 inhibitor U0126. Cell lysates were prepared and subjected to Western blot analysis. *Ct*, control (no inhibitor). *C*, MDSC were isolated from CT-26 tumor-bearing mice and cultured for 24 h with GM-CSF, TCCM, and 1.5 $\mu\text{mol/L}$ ATRA in the presence of 30 $\mu\text{mol/L}$ of specific MEK1/2 inhibitor U0126 or 100 nmol/L of JNK inhibitor SP 600125. These doses were selected based on previously reported observations and were maximally tolerated by MDSC (>80% viability) after 24 h. Expression of GSS was measured by quantitative real-time PCR as described in Materials and Methods. *D*, MDSC obtained from CT-26 tumor-bearing mice were cultured for 5 d with GM-CSF and TCCM. ATRA (1.5 $\mu\text{mol/L}$) and/or U0126 (30 $\mu\text{mol/L}$) was added on day 0. Cell phenotype was evaluated by flow cytometry. *, $P < 0.05$, versus cells treated with vehicle alone.

increased synthesis of GSH. It is important to point out that although it is most likely that GSH affects differentiation of MDSC via neutralization of ROS, the direct effect of GSH on myeloid cells cannot be ruled out and required further testing.

How could ATRA affect GSS expression? One of the possible mechanisms is via direct regulation of gene expression. Binding of ATRA to retinoic nuclear receptors induces allosteric changes that result in generation of a novel interaction surface for coactivators, which include histone acetyltransferase, thyroid hormone receptor-associated proteins, vitamin D receptor-interacting protein, and mediator protein-containing complex. Transcription is activated by either derepression, caused by chromatin decondensation, or a receptor-dependent increase in the frequency of transcription initiation via binding of RAR-RXR to RAREs in gene promoters. Our data indicated that a direct binding of nuclear receptors to RARE in *GSS* promoter is unlikely. There was only a partial match of the retinoic binding sequence in the *GSS* promoter. More importantly, we could not detect specific binding in chromatin immunoprecipitation assay and transcriptional activity of *GSS* promoter in the luciferase reporter assay. This suggested that ATRA might affect GSS expression indirectly.

A first candidate for the indirect mechanism of ATRA effect on GSS was STAT3. Hyperactivation of STAT3 was implicated into MDSC accumulation in cancer (15, 23). ATRA could affect phosphorylation or acetylation of STAT3 and thus down-regulate

its activity. However, our data showed no effect of ATRA on STAT3 binding to specific DNA sequences and on STAT3 phosphorylation, and thus strongly argue against this mechanism. ATRA did not induce activation of NF- κ B or Nrf-2, two factors that play an important role in transcriptional activation of antioxidant genes (30, 31).

Next, we evaluated possible effect of ATRA on signal transduction in MDSC. ATRA did not activate the phosphatidylinositol 3-kinase pathway, p38, or JNK kinases. However, it dramatically increased phosphorylation of ERK1/2. Inhibition of ERK1/2 completely abrogated the effect of ATRA on GSS expression and MDSC differentiation, indicating that ATRA indeed mediates its effect on GSS via ERK activation. Activation of ERK by ATRA has previously been described in several studies. ERK2, but not JNK/SAPK or p38, was activated by ATRA in HL-60 human myeloblastic leukemia cells (32, 33). This activation happened before ATRA caused myeloid differentiation and cell cycle arrest. ERK2 activation was necessary for ATRA-induced differentiation, and activation of ERK2 occurred within hours and persisted until the onset of differentiation and arrest (32). Treatment of embryonic stem cell-derived bodies with ATRA resulted in a prolonged activation of the ERK pathway, but not of the JNK, p38 MAPK, or phosphatidylinositol 3-kinase pathway (34). ATRA induced an early stimulation of ERK1/2 in neuronal cells, which was required for transcriptional activity (35). Thus, these results of ATRA effect in

HL-60 cells and in embryonic stem cell-derived bodies are strikingly similar to the effect of ATRA on MDSC and indicate that ATRA may specifically target ERK1/2 in several different types of cells.

The exact mechanism of ERK regulation of GSS expression is not clear. However, recent studies have suggested some potential mechanisms of ERK-mediated regulation of gene transcription. ERK is involved in phosphorylation of transcription factors, transcriptional regulation, nuclear chromatin remodeling, immediate gene induction, and cytokine production, as well as regulation of apoptosis and cell cycle progression. The ERK pathway mediates primarily cell growth and survival signals, but, under certain circumstances, also promotes induction of cell differentiation (36). Extensive studies over the last few years have identified mechanisms that may account for MAPK specificity. It is likely that the events that define such specificity involve physical interactions of MAPK with other proteins (37).

Thus, induction of MDSC differentiation by ATRA involves activation of ERK1/2 MAPK, which leads to up-regulation of GSS and accumulation of GSH in these cells. GSH reduces the level of ROS and thus promotes differentiation of MDSC into mature myeloid cells. These results not only suggest a novel biological mechanism of ATRA effect on nontransformed myeloid cells but also can be used in the monitoring of the potential effects of ATRA in cancer patients.

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