

Myeloid-Derived Suppressor Cells Inhibit T-Cell Activation by Depleting Cystine and Cysteine

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Abstract

Myeloid-derived suppressor cells (MDSC) are present in most cancer patients and are potent inhibitors of T-cell-mediated antitumor immunity. Their inhibitory activity is attributed to production of arginase, reactive oxygen species, inducible nitric oxide synthase, and interleukin-10. Here we show that MDSCs also block T-cell activation by sequestering cystine and limiting the availability of cysteine. Cysteine is an essential amino acid for T-cell activation because T cells lack cystathionase, which converts methionine to cysteine, and because they do not have an intact x_c^- transporter and therefore cannot import cystine and reduce it intracellularly to cysteine. T cells depend on antigen-presenting cells (APC), such as macrophages and dendritic cells, to export cysteine, which is imported by T cells via their ASC neutral amino acid transporter. MDSCs express the x_c^- transporter and import cystine; however, they do not express the ASC transporter and do not export cysteine. MDSCs compete with APC for extracellular cystine, and in the presence of MDSCs, APC release of cysteine is reduced, thereby limiting the extracellular pool of cysteine. In summary, MDSCs consume cystine and do not return cysteine to their microenvironment, thereby depriving T cells of the cysteine they require for activation and function. *Cancer Res*; 70(1): 68–77. ©2010 AACR.

Introduction

Many patients and experimental animals with cancer are immune suppressed because they contain cell populations that inhibit antitumor immunity (1). Suppressive populations from both the lymphoid and myeloid compartments have been identified. Myeloid-derived suppressor cells (MDSC) are found in most patients with advanced cancers (2–5) and are potent inhibitors of innate and adaptive immunity. MDSCs are a heterogeneous population of cells that impair immunity by inhibiting the activation of CD4⁺ (6) and CD8⁺ (2, 7, 8) T cells, blocking natural killer cell cytotoxicity (9), blocking T-cell expression of L-selectin (CD62L) that is needed for T cells to home to lymph nodes (10), and polarizing immunity toward a tumor-promoting type 2 phenotype through the downregulation of interleukin (IL)-12 and production of IL-10 (11). Studies with inhibitors of arginase, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS) showed that both arginase and nitric oxide

contribute to MDSC inhibition of T-cell activation (6, 12–14). However, inhibitors of these molecules do not completely reverse suppression of all MDSC populations, suggesting that MDSCs may use additional mechanisms to block T-cell activation.

Mammalian cells require the amino acid cysteine for protein synthesis and proliferation. Cysteine can be generated by cells through two pathways. If cells express the plasma membrane cystine transporter x_c^- , which consists of the xCT and 4F2 light and heavy chains, respectively, they import disulfide-bonded cystine from the oxidizing extracellular environment (15). Within the reducing intracellular environment, imported cystine is reduced to cysteine (16). Alternatively, if cells synthesize cystathionase, they can convert intracellular methionine to cysteine (17, 18). However, T cells do not contain cystathionase or the xCT chain of the x_c^- transporter (19–21), so they are dependent on other cells to produce cysteine, which is then imported by T cells through the plasma membrane ASC neutral amino acid transporter. T cells require cysteine during antigen presentation and subsequent T-cell activation and typically obtain it from macrophages and/or dendritic cells (DC), which provide it through one of two mechanisms. These cells import cystine, convert it to cysteine, and then export the cysteine through their plasma membrane ASC transporter (22–24). Additionally, DCs and macrophages secrete thioredoxin that converts extracellular cystine to cysteine, which is then available for uptake by T cells (25, 26).

The dependence of T cells on exogenously generated cysteine led us to hypothesize that MDSCs inhibit T-cell activation by limiting extracellular cysteine. We now report that MDSCs express the xCT and 4F2 heterodimeric cystine

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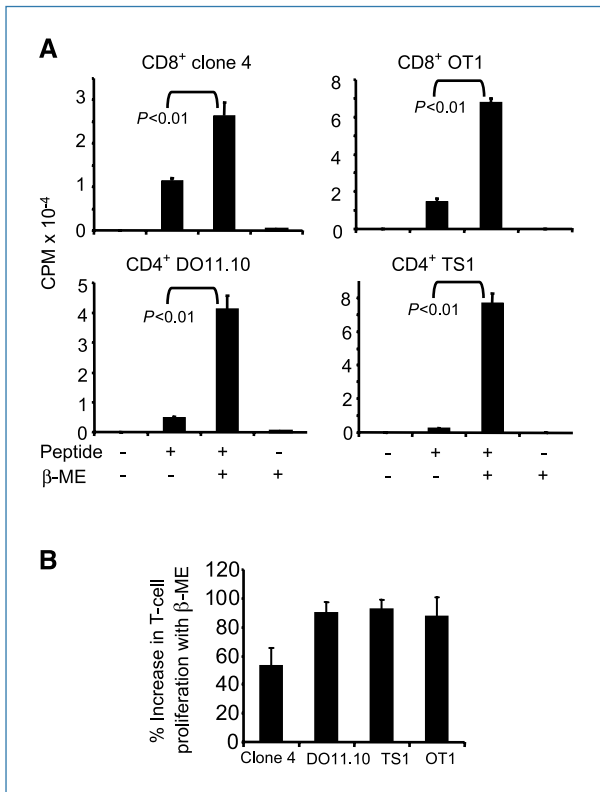


Figure 1. β -ME facilitates antigen-driven T-cell activation. **A**, transgenic CD4⁺ DO11.10 and TS1, and CD8⁺ clone 4 and OT-I splenocytes, were cocultured with their respective peptides in the presence or absence of 5×10^{-5} mol/L β -ME. Data are from one of four (TS1 and clone 4) and one of five (DO11.10 and OT-I) independent experiments. Results of the pooled experiments for each transgenic population are significant at $P < 0.01$ (Wilcoxon paired-sample test). **B**, columns, average percent increase in T-cell proliferation for DO11.10, TS1, clone 4, and OT-I splenocytes activated in the presence of β -ME shown in **A**; bars, SD.

transporter x_c^- , so they can acquire cystine from their environment. However, MDSCs do not express the ASC neutral amino acid transporter, so they do not export cysteine. Furthermore, MDSCs do not express cystathionase so their requirement for cysteine must be fulfilled by their uptake and reduction of cystine. Consequently, MDSCs limit the amount of cysteine in their extracellular environment by consuming cystine and not exporting cysteine and by sequestering cystine that would normally be imported, reduced, and exported as cysteine by macrophages and DCs or converted in the local environment to cysteine by thioredoxin. Therefore, in the presence of MDSCs, DCs and macrophages cannot support T-cell proliferation so tumor-specific T cells are not activated and antitumor immunity is suppressed.

Materials and Methods

Mice and cells. BALB/c, BALB/c DO11.10 transgenic [specific for chicken ovalbumin (OVA) peptide_{323–339} restricted to I-A^d], and C57BL/6 OT-I transgenic (specific for OVA peptide_{257–264} restricted to H-2K^b) mice were obtained from

The Jackson Laboratory. Mating pairs of transgenic BALB/c clone 4 and TS1 [T-cell receptors (TCR) specific for influenza hemagglutinin (HA) peptide 518–526 restricted to H-2K^d and 110–119 restricted to I-E^d, respectively] were provided by Dr. E. Fuchs (Johns Hopkins). Mice were bred and maintained in the University of Maryland Baltimore County (UMBC) animal facility according to NIH guidelines. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee. 4T1 mouse mammary carcinoma cells were maintained as described (6).

Reverse transcription-PCR. Total RNA was isolated from 5×10^6 to 10×10^6 cells and treated with RNase-free DNase I (Qiagen). First-strand cDNA synthesis was performed using an RNeasy Mini kit (Qiagen) and an iScript cDNA synthesis kit (Bio-Rad). Reverse transcription-PCR (RT-PCR) mixture contained 1 to 1.5 μ g of cDNA combined with one pellet of PuReTaq Ready-To-Go PCR beads (GE Healthcare) containing stabilizers, bovine serum albumin, deoxynucleotide triphosphates, 2.5 units of PuReTaq DNA polymerase, reaction buffer, and 1 μ L of a 10 μ mol/L stock of each of the following upstream and downstream primers, respectively: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 421 bp, 5'-AGTATGATGACATCAAGAAGG-3' and 5'-ATGGTATTCAAGAGAGTAGGG-3'), xCT (213 bp, 5'-TCTCCATCATCA-TCGGCACC-3' and 5'-AAGGACCAAAGACCTCCAGAATG-3'), cystathionase (342 bp, 5'-GCCTAGTTTCCAGCATTTTCG-3' and 5'-GATGCCACCCTCCTGAAGTA-3'), 4F2 heavy chain (402 bp, 5'-CGACCTTCAGGCCTTTGTAG-3' and 5'-GCCAAGTACAAGGGTGCATT-3'), and ASC transporter (385 bp, 5'-AGGGGTAGTAGCGGTGGATT-3' and 5'-GGTGACCAG-GAAAAAGAGCA-3'). cDNA was amplified in a PTC-200 Peltier Thermal Cycler (MJ Research) using the following conditions: denature at 94°C for 15 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final incubation at 72°C. PCR products were analyzed on 1.2% to 1.4% agarose gels.

Reagents, peptides, antibodies, and flow cytometry. OVA_{323–339} (ISQAVHAAHAEINEAGR), OVA_{257–264} (SIINFEKL), HA_{110–119} (SFERFEIFPK), and HA_{518–526} (IYSTVASSL) peptides were synthesized in the University of Maryland, Baltimore, Biopolymer Core Facility. Gr1-FITC, CD11b-phycoerythrin (PE), F4/80-FITC, CD11c-PerCP, CD11c-PE, CD86-PE, MHC II-FITC, c-kit-PE, FITC-rat IgG2a, PE-rat IgG2a, and goat anti-rabbit FITC were from BD Pharmingen; rabbit anti-mouse xCT was from Abcam; rabbit anti-mouse ASC was from Transgenic, Inc.; rabbit anti-mouse CD98-FITC (4F2hc) was from MBL International; and β -mercaptoethanol (β -ME) was from Ultrapure Bioreagent. Sca-1-APC was from R&D Systems. Cells were stained with monoclonal antibodies (mAb) and analyzed on an Epics XL or Cyan ADP flow cytometer (6) using FCS express or Summit v4.3 software.

Macrophages, MDSCs, splenocytes, and T cells. Peritoneal macrophages were prepared by injecting mice i.p. with 3% sodium thioglycolate. Peritoneal exudate cells were removed 4 to 5 d later using a 10 mL syringe with an 18-gauge needle. RBCs were lysed with Gey's solution (11). The remaining macrophages were activated with lipopolysaccharide (LPS; 10 ng/mL) for 16 to 18 h and were

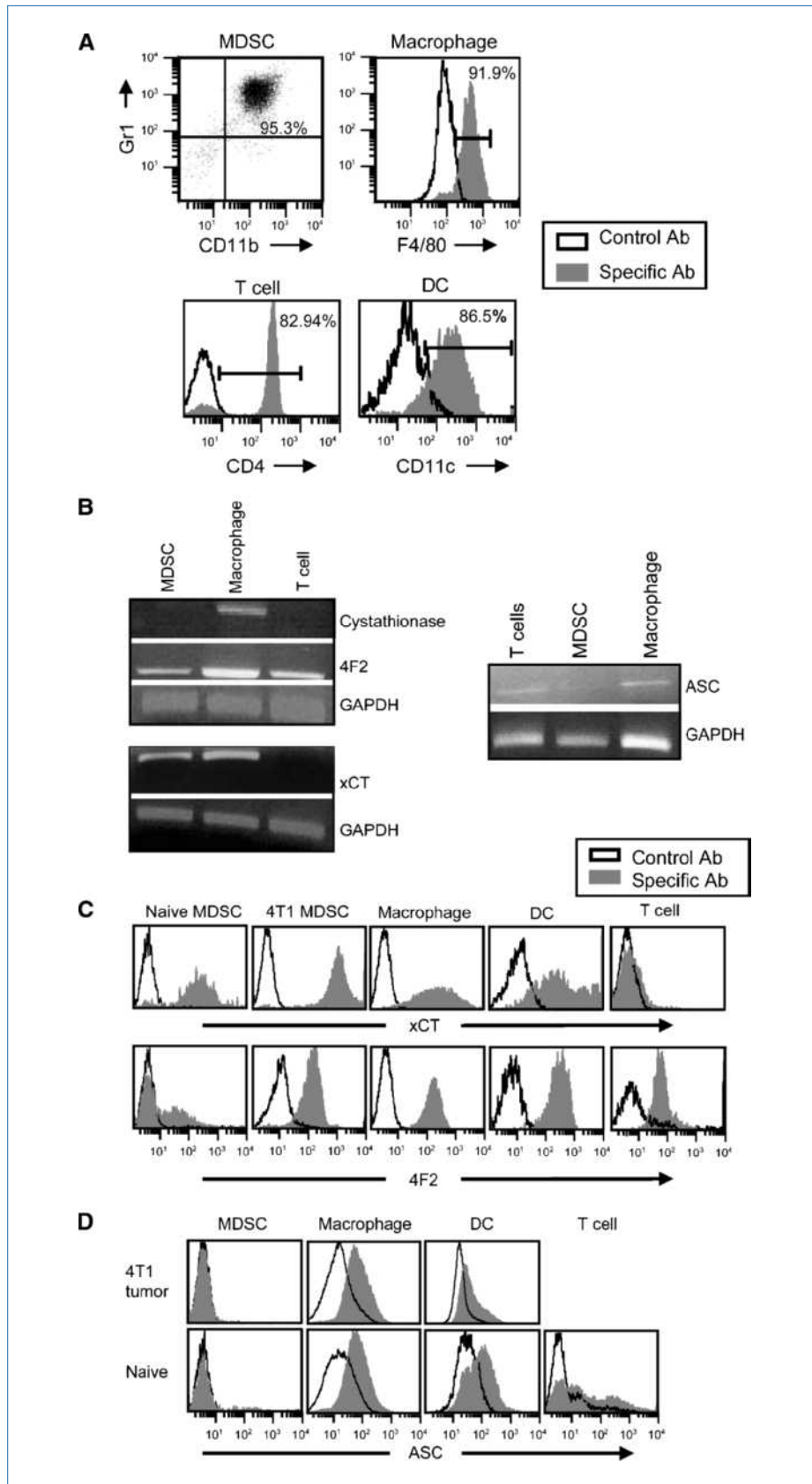


Figure 2. MDSCs express the heterodimeric x_c^- transporter ($xCT + 4F2$) and do not express cystathionase or the ASC neutral amino acid transporter. **A**, peritoneal macrophages, purified $CD4^+$ splenic T cells, and DCs (all from naive BALB/c mice) and 4T1-induced blood MDSCs were stained with mAbs to Gr1, CD11b, F4/80, CD4, and/or CD11c and analyzed by flow cytometry. Purity of each population is shown. **B**, total RNA was isolated from the MDSCs, macrophages, and $CD4^+$ T cells shown in **A**, reverse transcribed, and PCR amplified for the x_c^- (xCT and $4F2$) and ASC transporters, cystathionase, and GAPDH. PCR for cystathionase, $4F2$, xCT , and ASC was performed on two, three, four, and three independent cell preparations, respectively, for each cell type. **C**, peritoneal macrophages, bone marrow DCs, purified splenic $CD4^+$ T cells, and blood $Gr1^+CD11b^+$ cells (all from naive mice) and 4T1-induced blood MDSCs were stained with antibodies to xCT , $4F2$, Gr1, CD11b, F4/80, CD11c, and CD4. Gated populations (MDSCs: $Gr1^+CD11b^+$; macrophages: $F4/80^+$; DCs: $CD11c^+$; and T cells: $CD4^+$) were analyzed for expression of xCT or $4F2$. Data are from one of three independent cell preparations. **D**, splenic macrophages, DCs, purified T cells, and blood MDSCs from tumor-free (naive) or 4T1 tumor-bearing mice were stained with antibodies to Gr1, CD11b, F4/80, CD11c, CD4, and ASC, and the gated $Gr1^+CD11b^+$, $F4/80^+$, $CD4^+$, or $CD11c^+CD11b^+$ populations were analyzed by flow cytometry.

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>87% F4/80⁺ by flow cytometry. Macrophages were identified by gating on F4/80⁺ splenocytes. For Gr1⁺CD11b⁺ MDSCs from tumor-free mice, 14 to 15 naive BALB/c mice were bled from the retro-orbital sinus and isolated by Miltenyi Biotech purification as described (6). Purified naive MDSCs were 85% to 87% Gr1⁺CD11b⁺. For Gr1⁺CD11b⁺ MDSCs from tumor-bearing mice, female BALB/c mice were inoculated in the abdominal mammary gland with 7×10^3 to 1×10^4 4T1 cells. Three to four weeks later when primary tumors were 11.57 ± 0.96 mm in diameter and metastatic disease was established, mice were bled from the retro-orbital sinus or by cardiac puncture, and RBCs were lysed. The resulting leukocytes were 85% to 98% Gr1⁺CD11b⁺ as measured by flow cytometry. Splenocytes from DO11.10, clone 4, TS1, and OT-1 mice were purified using a CD4⁺ T-cell isolation kit (Miltenyi Biotech).

DC isolation. Bone marrow DCs were prepared according to the procedure of Son and colleagues (27). Briefly, bone marrow was flushed from the femurs of two to three 4- to 6-mo-old male mice and depleted of RBCs, and the remaining cells were cultured at 37°C and 5% CO₂ in DC medium (RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, 1% nonessential amino acids, 1% amino acids, and 1% gentamicin). Cells (10×10^6 to 20×10^6 /10 mL) were cultured in 25 cm² T flasks for 8 to 9 d with 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL recombinant IL-4 (Fitzgerald). Medium (5 mL) was removed on days 4 and 7 and replaced with 5 mL DC medium containing 10 ng/mL recombinant GM-CSF and 5 ng/mL recombinant IL-4. On day 8, cells were harvested and positively selected with CD11c magnetic beads (Miltenyi Biotech). DCs were matured by overnight incubation in DC medium supplemented with 1 μg/mL LPS. Nonmatured and LPS-matured DCs were 80% to 90% CD11c⁺ and expressed MHC II and CD86 as analyzed by flow cytometry.

Glutamate uptake. Glutamate uptake was measured using a modified procedure of (28). All reagents were from Sigma unless stated otherwise. Macrophages, purified CD4⁺ T cells, and DCs (all from tumor-free mice) and 4T1-induced blood MDSCs were preincubated for 20 min at 37°C at 6×10^5 cells/200 μL in 2 mL microfuge tubes or in 96-well flat-bottomed plates with 200 to 500 μL of Buffer A [140 mmol/L *N*-methyl-D-glucamine, 5.4 mmol/L KCl, 0.4 mmol/L KH₂PO₄, 10 mmol/L HEPES, 5 mmol/L D-glucose, 1.8 mmol/L CaCl₂, 0.8 mmol/L MgSO₄ (pH 7.4)]. Cells were pelleted and resuspended in 200 to 300 μL of Buffer A containing 170 nmol/L L-[³H]glutamate (52 Ci/mmol; GE Healthcare) with or without 2.5 mmol/L of unlabeled amino acid competitor (L-cystine) or noncompetitor (L-leucine) and incubated for 20 min at 37°C. Glutamate uptake was terminated by washing with excess cold Buffer A three to four times. Washed cells were lysed with 200 μL of 0.5% Triton X-100 in 0.1 mol/L potassium phosphate buffer (pH 7.0). Cell lysate (50 μL) was mixed with 500 μL of Opti-phase Supermix (Perkin-Elmer) and counted using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). Percent inhibition of glutamate transport = $100\% \times [(cpm \text{ without cystine} - cpm \text{ with cystine}) / (cpm \text{ without cystine})]$.

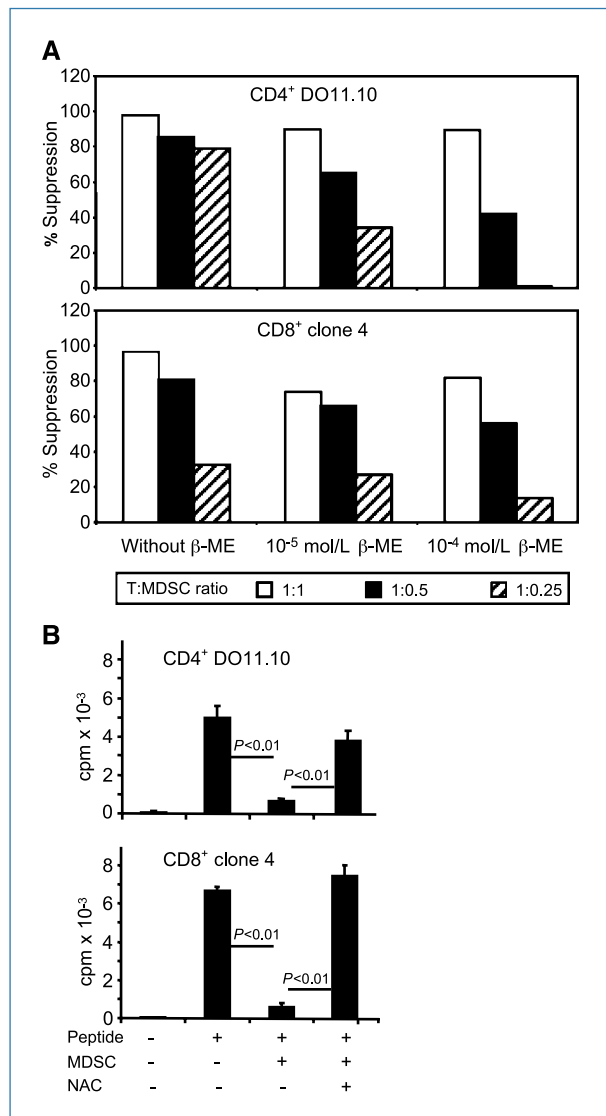
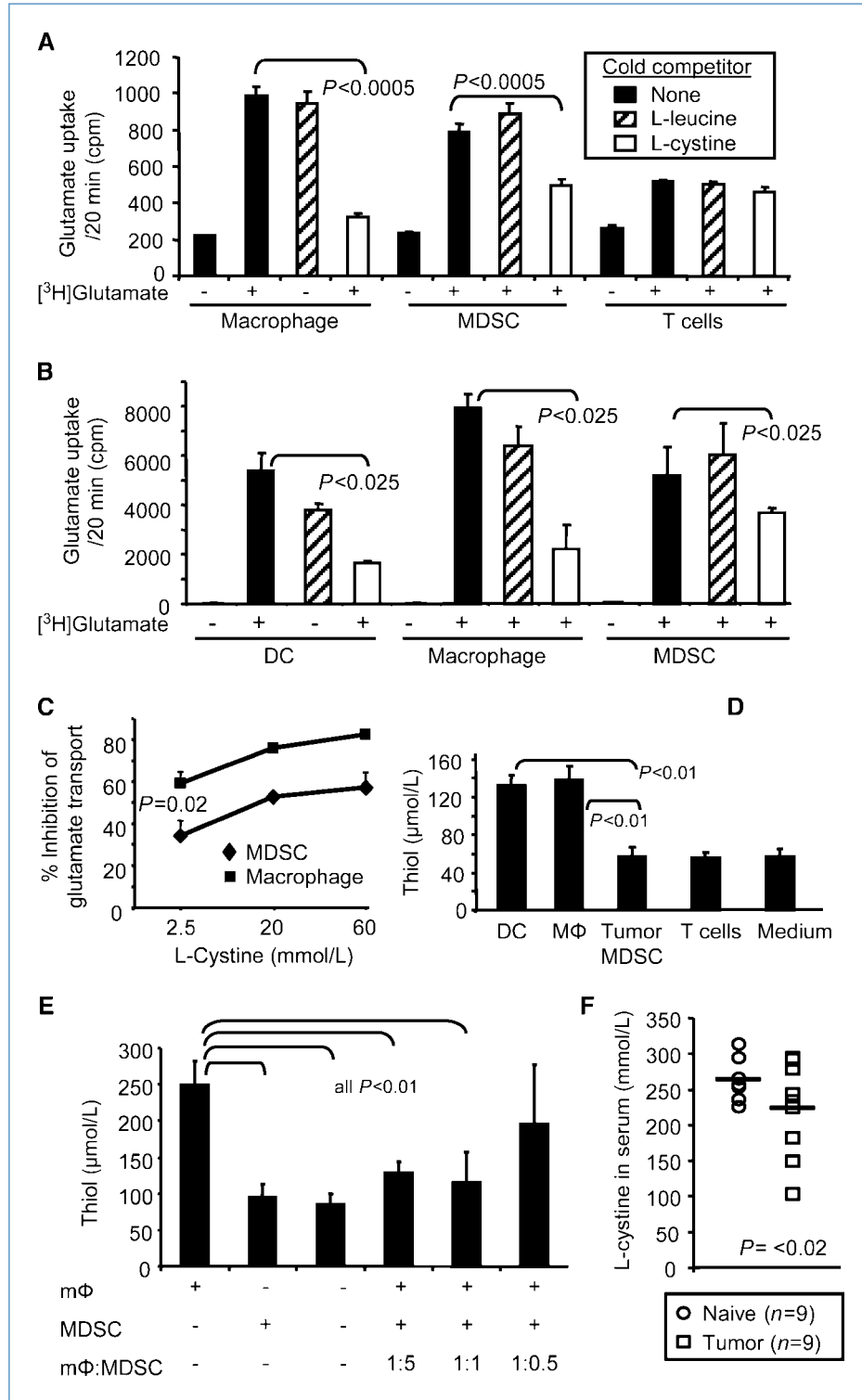


Figure 3. MDSC suppression is reversed by β-ME or NAC. **A**, transgenic splenocytes and their respective peptides were cocultured at varying ratios with 4T1-induced blood MDSCs (90% Gr1⁺CD11b⁺ cells) with or without β-ME, and T-cell activation was measured by [³H]thymidine uptake. Data are plotted as percent suppression relative to T cells plus peptide without MDSCs and are from one of six independent experiments. Values for percent suppression in the presence of β-ME versus no β-ME for the pooled experiments are significant at $P < 0.0005$ (Wilcoxon paired-sample test). **B**, transgenic splenocytes were cocultured as in **A** in the presence of 0.5 mmol/L NAC and without β-ME. Ratio of splenocytes to MDSCs for **B** was 1:0.5. Data are from one of three independent experiments. Pooled experiments are significant at $P < 0.005$ (Wilcoxon paired-sample test).

Cysteine release. Free thiols were measured according to Angelini and colleagues (25) with the following modifications: Purified CD4⁺ T cells, DCs, and peritoneal macrophages (all from tumor-free mice) and 4T1-induced blood MDSCs were incubated for 6 h at 37°C in HL-1 or RPMI 1640 serum-free medium in 96-well plates (6×10^5 cells/200 μL/well). Each supernatant (100 μL) was combined

with 100 μ L of dilution buffer [30 mmol/L Tris-HCl, 3 mmol/L EDTA (pH 8.2)] and 25 μ L of Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); Sigma] solution (29.7 mg DTNB in 25 mL methanol) and incubated at room temperature for 10 to 15 min, and absorbance

was measured at 412 nm. *N*-acetylcysteine (NAC) was used as a standard. For experiments containing a mixture of MDSCs and macrophages, 6×10^5 to 6×10^6 blood MDSCs were plated in 200 μ L/well of Buffer A in 96-well flat-bottomed plates. After a 20-min incubation at 37°C, MDSCs



were washed twice with serum-free HL-1 medium, and 6×10^5 BALB/c macrophages were added per well in a total volume of 200 μ L/well of serum-free HL-1 medium. Cocultures were incubated for 20 min at 37°C, after which thiol release was measured using the DTNB cysteine release assay. Percent inhibition = $100\% - \{[(\text{macrophages} + \text{MDSCs}) - \text{HL-1 medium}] / (\text{macrophages} - \text{HL-1 medium})\}$.

T-cell activation, MDSC suppression, and cystine levels. T-cell proliferation was measured as described (6). For experiments including NAC, transgenic splenocytes (1×10^5 cells/200 μ L/well) were cocultured with their cognate peptide (14 μ mol/L OVA, 28 μ mol/L HA peptide) in HL-1 medium (BioWhittaker) containing 1% penicillin, 1% streptomycin, and 1% Glutamax (Invitrogen Life Technologies). T-cell suppression assays were done as described (6) using transgenic splenocytes (DO11.10, clone 4, OT-1, and TS1; 2×10^5 per well) and their cognate peptides (14 μ mol/L OVA, 28 μ mol/L HA, 12 μ mol/L OVA, and 10 μ mol/L HA peptide, respectively) in the presence of 25-Gy irradiated blood MDSCs from 4T1 tumor-bearing mice in a total volume of 200 μ L of serum-free HL-1 medium. β -ME was added to some wells. Percent suppression = $100\% \times \{[(\text{T cells} + \text{peptide}) - (\text{T cells} + \text{peptide} + \text{MDSCs})] / (\text{T cells} + \text{peptide})\}$. Serum cystine levels were measured using high-performance liquid chromatography (HPLC) as described (29).

Statistical analysis. Data were analyzed using Student's two-tailed *t* test for unequal variances or the Wilcoxon paired rank test.

Results

Exogenous β -ME facilitates T-cell proliferation. Because T cells cannot generate cysteine from cystine or methionine, they must import this essential amino acid. However, extracellular spaces are oxidizing environments, so cystine, the disulfide-bonded form, rather than the free amino acid cysteine, is present extracellularly. Therefore, the reducing agent, β -ME, is included in T-cell cultures to reduce cystine to cysteine (19–21). To confirm that a reducing environment facilitates activation of the transgenic T cells in the present study, splenocytes from TCR-transgenic CD4⁺ DO11.10 and TS1, and CD8⁺ clone 4 and OT-1 mice were cocultured with cognate peptide in the presence or

absence of β -ME. T-cell proliferation in the presence of β -ME was greater than proliferation without β -ME for all transgenic populations (Fig. 1).

MDSCs express the cystine transporter but lack cystathionase and the ASC neutral amino acid transporter. If MDSCs limit T-cell activation by sequestering cystine, then they may import cystine and not export cysteine. To determine if MDSCs acquire, generate, and/or export cystine and/or cysteine, 4T1-induced blood MDSCs (95.3% Gr1⁺CD11b⁺; Fig. 2A, top left) were assayed by RT-PCR for the cystine importer x_c^- (xCT and 4F2), the ASC neutral amino acid transporter, and cystathionase. Activated macrophages, which express xCT, 4F2, ASC, and cystathionase (Fig. 2A, top right; refs. 22–24) and T cells (Fig. 2A, bottom left), which lack cystathionase and xCT, served as positive and negative controls, respectively. MDSCs contain mRNA encoding the x_c^- transporter (xCT and 4F2) but do not contain mRNA encoding cystathionase or the ASC transporter (Fig. 2B). Protein expression of xCT, 4F2, and ASC in macrophages, T cells, and MDSCs from tumor-bearing mice was confirmed by flow cytometry. Bone marrow DCs (Fig. 2A, bottom right) were included in this experiment because they also produce cysteine. Consistent with the RT-PCR and published results (30), macrophages and DCs contain a complete x_c^- cystine transporter, whereas T cells lack xCT. MDSCs from tumor-free (naive) and from mice with 4T1 tumors also contain a complete x_c^- complex (Fig. 2C). Macrophages (splenic and peritoneal), DCs (splenic and bone marrow), and T cells express cell surface ASC; however, Gr1⁺CD11b⁺ cells from tumor-free or tumor-bearing mice do not express the ASC transporter (Fig. 2D; Supplementary Fig. S1). c-kit⁺Sca-1⁻ myeloid progenitor cells express low levels of ASC (Supplementary Fig. S2). Therefore, macrophages, DCs, and MDSCs can import cystine; however, unlike macrophages and DCs, MDSCs are unable to export cysteine. Because there are relatively few myeloid progenitor cells and because they do not localize to sites of T-cell activation, they are unlikely to affect extracellular cystine levels.

Extracellular cysteine partially reverses T-cell suppression. If cysteine deprivation contributes to the immunosuppressive effects of MDSCs, then providing exogenous cysteine may reverse suppression. To test this hypothesis, CD4⁺

Figure 4. MDSCs import cystine and do not export cysteine. A and B, peritoneal macrophages (92% F4/80⁺), T cells (85% CD4⁺), and bone marrow DCs (85% CD11c⁺; all from tumor-free BALB/c mice) and 4T1-induced blood MDSCs (92% Gr1⁺CD11b⁺) were cultured in sodium-free buffer for 20 min followed by incubation in buffer with [³H]glutamate in the presence or absence of cold competitors L-cystine or L-leucine. Data for A and B are from one of five and two independent experiments, respectively. Results of the pooled experiments are significant at $P < 0.0005$ and $P < 0.025$, respectively. C, higher concentrations of cold competitor L-cystine are required to inhibit glutamate uptake by MDSCs relative to peritoneal macrophages. Peritoneal macrophages from tumor-free mice and 4T1-induced MDSCs were cultured with [³H]glutamate as in A and B in the presence of cold L-cystine. Data are from one of four independent experiments. D, macrophages and DCs, but not MDSCs, export cysteine. Peritoneal macrophages (85% F4/80⁺), DCs (82% CD11c⁺), and purified CD4⁺ T cells (95% CD4⁺; all from tumor-free mice) and 4T1-induced blood MDSCs (97% Gr1⁺CD11b⁺) were incubated in serum-free HL-1 medium and cysteine content of the culture supernatants was measured by the DTNB colorimetric assay. Data are from one of five independent experiments. Results of the pooled experiments are significant at $P < 0.0005$. E, MDSCs prevent macrophages from releasing cysteine. Macrophages (86.7% F4/80⁺) and titered numbers of 4T1-induced MDSCs (90.6% Gr1⁺CD11b⁺) were cocultured in serum-free HL-1 medium, and cysteine release was measured by the DTNB colorimetric assay. Data are from one of two independent experiments and are significant at $P < 0.005$ for the pooled experiments. F, serum was obtained from tumor-free BALB/c mice or from BALB/c mice with 4T1 tumors (primary mammary tumors of >5 mm in diameter and >60% Gr1⁺CD11b⁺ leukocytes). Serum levels of cysteine were determined by HPLC. Each symbol represents an individual mouse. Horizontal bars, mean. The two groups are significantly different at $P < 0$. Statistics for all pooled results used the Wilcoxon paired-sample test.

DO11.10 and CD8⁺ clone 4 transgenic splenocytes were cocultured with their respective peptides and tumor-induced MDSCs in the presence or absence of β -ME. T-cell activation was measured by [³H]thymidine uptake (Fig. 3A). Inclusion of MDSCs suppressed T-cell activation regardless of the presence of β -ME; however, β -ME partially restored activation. Therefore, β -ME reverses some, but not all, MDSC-mediated suppression, consistent with the concept that cysteine deprivation contributes to MDSC-mediated suppression.

β -ME is a general reducing agent and may affect T-cell proliferation by acting on many molecules. To determine if extracellular cysteine is specifically involved, transgenic splenocytes were cocultured with their respective peptides in the presence or absence of NAC, which is stable in the extracellular oxidizing environment (31). NAC enters cells via the ASC neutral amino acid transporter and is rapidly hydrolyzed intracellularly to the free amino acid cysteine. Inclusion of increasing amounts of NAC increases T-cell proliferation similar to that seen for β -ME (Supplementary Fig. S3). To determine if NAC overcomes MDSC-mediated suppression, NAC was added to cultures of MDSCs with transgenic T cells and their cognate peptides (Fig. 3B). NAC restored proliferation of both CD4⁺ and CD8⁺ T cells. As shown by Nefedova and colleagues (31), 5 days of culture of MDSCs with NAC, GM-CSF, and tumor cell-conditioned medium differentiates MDSCs into nonsuppressive macrophages and/or DCs, raising the possibility that the results of Fig. 3B are due to MDSC differentiation. However, 1-day-old cultures of MDSCs with NAC under the conditions of Fig. 3B contained <0.5% macrophages or DCs as measured by percent F4/80⁺Gr1⁻ or CD11c⁺Gr1⁻ cells (Supplementary Table S1), and 97.5% of the cells were dead by day 3, indicating that the MDSCs had not differentiated during the course of the experiment. Therefore, excess cysteine partially reverses MDSC suppressive activity, consistent with the hypothesis that MDSCs inhibit T-cell proliferation by depleting the environment of cysteine.

MDSCs import cystine through the x_c^- transporter and do not export cysteine. If MDSCs suppress T-cell activation by competing with macrophages and/or DCs for the uptake of cystine, then their rate of cystine uptake should be similar to that of macrophages and DCs. In macrophages and DCs, the x_c^- transporter imports cystine and exports glutamate due to the higher concentration of cystine and glutamate outside and inside cells, respectively. Because radioactive cystine is not commercially available, and the cystine transporter is an antiporter, radioactive glutamate is used to measure cystine uptake (28, 32, 33). Equal numbers of blood MDSCs, purified splenic T cells, peritoneal macrophages (Fig. 4A), and DCs (Fig. 4B) were incubated with [³H]glutamate. To ascertain the specificity of the x_c^- transporter, cold competitor (L-cystine) or noncompetitor (L-leucine) amino acids were included in some wells. MDSCs, DCs, and macrophages incorporated glutamate at approximately the same rate. Cold L-cystine, but not cold L-leucine, competed for uptake in MDSCs, macrophages, and DCs. The same concentration of L-cystine competed glutamate binding for macrophages and DCs (Fig. 4B), whereas a higher concentration of

L-cystine was required to block glutamate uptake by MDSCs (Fig. 4C), showing that MDSCs have a higher intracellular concentration of cystine than macrophages and DCs. In agreement with published data, T cells had a minimally functional x_c^- transporter. Gr1⁺CD11b⁺ cells from tumor-free mice had similar uptake kinetics of glutamate as Gr1⁺CD11b⁺ MDSCs from tumor-bearing mice (Supplementary Fig. S4). Therefore, MDSCs have a cystine transporter that imports cystine at a rate similar to that of macrophages and DCs.

MDSCs do not export cysteine. Because MDSCs do not express the ASC neutral amino acid transporter, they are unlikely to export cysteine. To measure cysteine export, supernatants from MDSCs, macrophages, DCs, and purified T cells were tested using the DTNB colorimetric assay that detects free thiols (-SH group) cysteine and glutathione (GSH; Fig. 4D). Because GSH is retained intracellularly, it will not be present in supernatants and the only thiol detected in this assay will be cysteine (34). Supernatants from activated macrophages and DCs but not from MDSCs or T cells contain thiols, showing that MDSCs do not export cysteine.

If MDSCs sequester cystine and limit the amount of extracellular cysteine, then cocultures of MDSCs with antigen-presenting cells (APC) will contain less extracellular cysteine than cultures containing only APC. To test this hypothesis, macrophages were cocultured with varying numbers of MDSCs and the amount of extracellular cysteine was measured (Fig. 4E). At ratios of 1:5 and 1:1 macrophages to MDSCs, macrophage release of cysteine was inhibited by >73%, and at a ratio of 1:0.5, cysteine release was inhibited by 32%, consistent with the concept that MDSCs sequester cystine so that APCs are limited in the amount of cystine they import and cysteine they export.

Tumor-bearing mice have lower serum levels of cystine than tumor-free mice. If MDSCs are scavenging cystine, then tumor-bearing mice with elevated levels of blood MDSCs should have less cystine in their blood as compared with tumor-free mice. To test this possibility, tumor-free and 4T1 tumor-bearing BALB/c mice (primary tumors >6 mm in diameter and blood levels of >50% Gr1⁺CD11b⁺ MDSCs) were bled and their serum was tested for cystine by HPLC (Fig. 4F). Tumor-bearing mice have on average less cystine in their serum than tumor-free mice, consistent with the concept that MDSCs sequester cystine, which results in reduced extracellular cysteine.

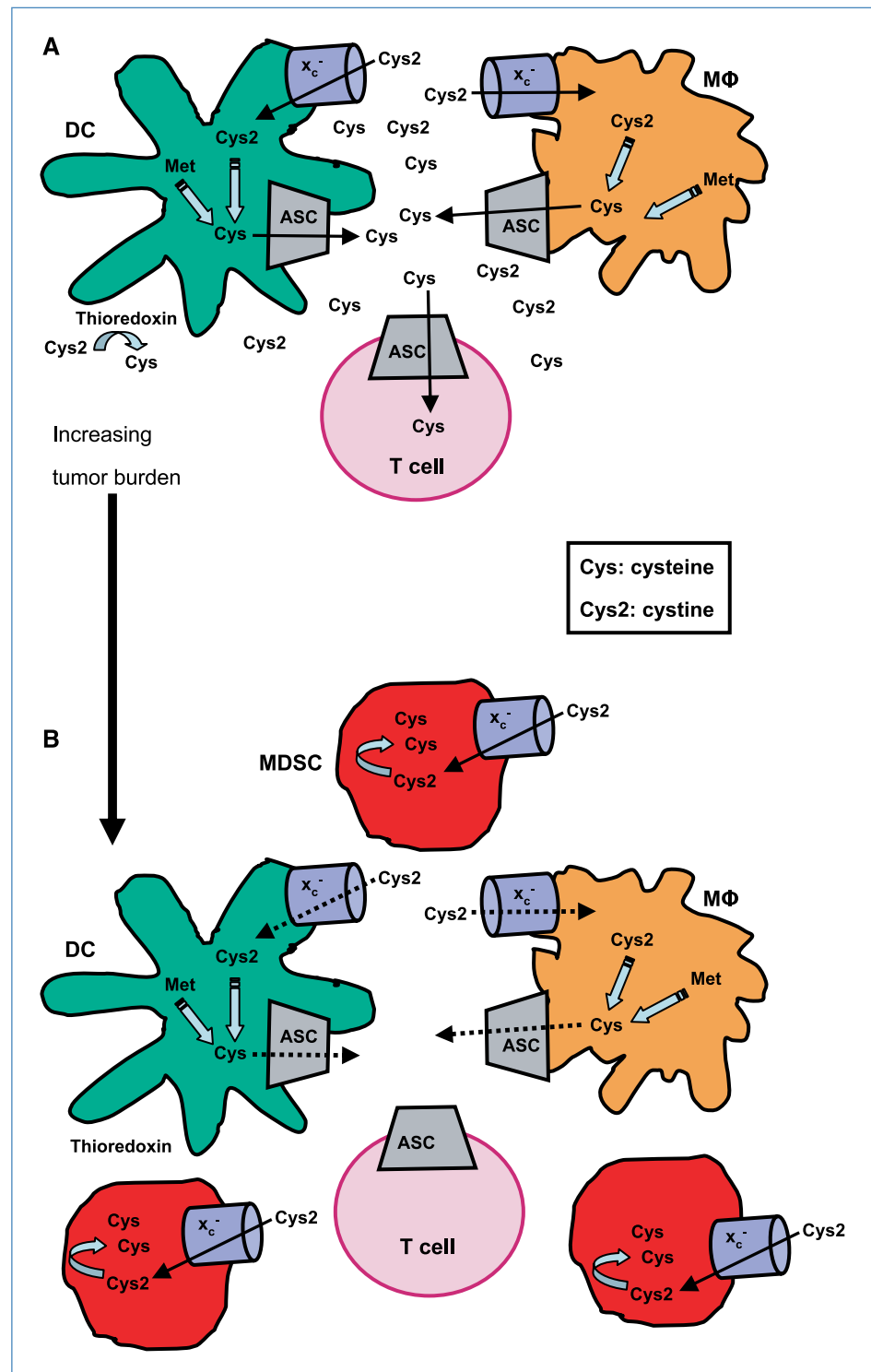
Discussion

MDSCs contribute to tumor progression by facilitating neoangiogenesis (35) and by inhibiting innate (9, 11) and adaptive antitumor immunity (6). MDSC immune effects have been attributed to the production of arginase, iNOS (36), and ROS (37) and to the nitration of TCRs (38). We now report that MDSCs also block T-cell activation by limiting the extracellular pool of cysteine, which is required for T-cell proliferation. Most cells synthesize cysteine from methionine using the enzyme cystathionase, or they use their x_c^- transporter to import cystine, which is converted to cysteine in the intracellular reducing environment. However,

T cells lack cystathionase and the x_c^- transporter and therefore must import extracellular cysteine through their ASC neutral amino acid transporter. Cysteine is essential for the protein synthesis that supports antigen-driven T-cell activation, and the required extracellular cysteine is pro-

vided locally by macrophages and/or DCs during antigen presentation (25, 30). If MDSCs are present during T-cell activation, they perturb these processes. MDSCs do not synthesize cystathionase or the ASC neutral amino acid transporter and therefore must generate all of their cysteine

Figure 5. MDSCs suppress T-cell activation by sequestering cystine and cysteine. **A**, DCs and macrophages generate cysteine by reducing cystine imported through their x_c^- transporter or by the conversion of methionine (*Met*) to cysteine through the action of cystathionase. Excess intracellular cysteine is exported by DCs and macrophages through the ASC neutral amino acid transporter. Extracellular cysteine is also generated by the thioredoxin-mediated reduction of cystine to cysteine. During antigen presentation and T-cell activation, T cells, which are unable to produce their own cysteine because they lack cystathionase and the x_c^- transporter, import cysteine through their ASC neutral amino acid transporter. **B**, MDSCs must obtain all of their cysteine by importing cystine and reducing it to cysteine because they do not synthesize cystathionase and do not express the ASC neutral amino acid transporter. Therefore, as tumor burden and MDSC levels increase, MDSCs consume increasing quantities of cystine. Because MDSCs do not contain the ASC neutral amino acid transporter, they do not return cysteine to their surroundings. The competition between macrophages, DCs, and MDSCs for cystine leads to reduced uptake of cystine by macrophages and DCs, and the concomitant decrease in cysteine released by these cells. Because of the lower levels of extracellular cysteine, thioredoxin-mediated generation of extracellular cysteine is also reduced. Collectively, these effects result in the local depletion of cysteine and the inhibition of T-cell activation.



from cystine that is imported through their x_c^- transporter. Because MDSCs lack the ASC neutral amino acid transporter, they do not recycle cystine and return it to their surroundings as cysteine. Consequently, whereas T cells are being activated by antigen, MDSCs consume cystine that would otherwise be taken up by macrophages and DCs and returned to the local environment as cysteine. Furthermore, increasing levels of MDSCs that accumulate with progressive tumor growth limit the extracellular pool of cystine that could be converted to cysteine by thioredoxin produced by DCs and macrophages (25, 26). Consequently, the extracellular cysteine pool is reduced, and T-cell activation is limited (Fig. 5).

The rate of uptake of cystine by MDSCs is very similar to that of macrophages and DCs, indicating that MDSCs compete equally on a per cell basis with these APCs. Because MDSCs require higher concentrations of cold L-cystine to compete glutamate uptake, they have higher intracellular stores of cystine as compared with macrophages, consistent with the concept that MDSCs sequester much of their imported cystine. Therefore, as tumor growth drives MDSC levels, increasing numbers of MDSCs directly compete with APC for cystine and act as a cystine "sink" to deprive macrophages and DCs of cystine.

In addition to blocking T-cell proliferation, limiting quantities of cysteine may also make T cells more susceptible to oxidative stress. GSH, a major intracellular redox molecule that protects cells from oxidative stress, is essential for optimal T-cell proliferation and activation (39), and its synthesis is limited by cysteine (33, 40). Therefore, cysteine deprivation by MDSCs is also likely to reduce intracellular T-cell levels of GSH and thereby make T cells more susceptible to oxidative stress and its detrimental consequences.

Provision of cysteine in the form of NAC to MDSCs cultured with GM-CSF and tumor cell-conditioned medium induces MDSCs to differentiate into macrophages and DCs, showing that NAC also modulates MDSC function by converting MDSCs to nonsuppressive cells (31). However, the MDSCs in our studies are not differentiating, indicating that the benefits of NAC are potentially 2-fold: limiting the quantity of MDSCs by promoting their differentiation and providing cysteine for T-cell activation. An epidemiologic study showing that high levels of serum cysteine are associated with reduced risk of breast cancer (41) provides clinical support for the harmful effects of cysteine deprivation and the potential benefits of NAC.

As *in vivo* MDSC levels increase, free cystine/cysteine will decrease, and the extent of immune suppression by amino acid deprivation will vary depending on the quantity of

MDSCs at the site of antigen presentation and initial T-cell activation. MDSCs are present in the blood, lymph nodes, and at tumor sites of cancer patients (2) and additionally in the spleen of mice with tumors (6). Because antigen presentation and T-cell priming occur in lymph nodes and spleen, but not in the blood, cystine/cysteine deprivation by MDSCs is likely to be most pronounced in these secondary lymphoid organs. If antigen presentation and T-cell activation occur within tumors, then cystine/cysteine deprivation by MDSCs will also impair T-cell activation in tumor.

NAC has been proposed as an antitumorigenic agent because it reduces the oxidative stress that promotes genetic instability. *In vivo* experiments using mice with progressively growing tumors have shown therapeutic efficacy (42, 43). Our studies show that NAC may have the additional benefit of facilitating T-cell activation by increasing extracellular cysteine levels. However, MDSCs also suppress T-cell activation through their production of arginase and nitric oxide (12, 44), so that supplemental cysteine alone may not significantly reduce the suppressive effects of MDSCs. We have observed that peptide activation of transgenic T cells is increased in tumor-bearing mice maintained on NAC-supplemented water, but the increase was not statistically significant.³ However, administration of NAC, an already Food and Drug Administration–approved drug (45, 46), in combination with other agents that block MDSCs, and combined with active immunotherapy, may limit suppression by MDSCs and thereby facilitate the treatment of established metastatic cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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³ Unpublished data.

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