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STAT1 Signaling Regulates Tumor-Associated Macrophage-Mediated T Cell Deletion[1]

Sergei Kusmartsev[2] and Dmitry I. Gabrilovich[2]

It is well established that tumor progression is associated with the accumulation of myeloid suppressive cells, which in mice include Gr-1$^+$ immature myeloid cells and F4/80$^+$ macrophages. The paradox is that with the exception of terminal stages of the disease or chemotherapy treatment, tumor-bearing mice or cancer patients do not display a profound systemic immune suppression. We therefore raised the question as to whether myeloid cell-mediated T cell suppression is controlled at a local level at the site of the tumor. We have demonstrated that after adoptive transfer to tumor-bearing recipients, Gr-1$^+$ (immature myeloid cells) freshly isolated from spleens of tumor-bearing mice become F4/80$^+$ tumor-associated macrophages (TAM). These TAM, but not F4/80$^+$ macrophages or Gr-1$^+$ cells freshly isolated from spleens of tumor-bearing or naive mice were able to inhibit T cell-mediated immune response in vitro via induction of T cell apoptosis. Arginase and NO were both responsible for the apoptotic mechanism, and were seen only in TAM, but not in freshly isolated Gr1$^+$ cells. Using the analysis of STAT activity in combination with STAT knockout mice, we have determined that STAT1, but not STAT3 or STAT6, was responsible for TAM-suppressive activity. ***The Journal of Immunology,*** **2005, 174: 4880–4891.**

Inadequate function of host immune system is considered as one of the major mechanisms of tumor escape. The presence of immunosuppressive myeloid cell in spleens of tumor-bearing mice is well-established phenomenon first described almost three decades ago (1, 2). These cells share common features such as myeloid origin, macrophage-like morphology, and phenotype of surface receptors, and the ability to suppress CD4$^+$ and CD8$^+$ T cells after culture in vitro (3–9). Initial studies have been focused on immunosuppressive activity of macrophages. More recently, the population of immature myeloid cells (IMC)[3] in spleens of tumor-bearing mice was more precisely identified as Gr-1$^+$CD11b$^+$ cells (reviewed in Refs. 10 and 11). This population is comprised of immature macrophages, granulocytes, dendritic cells (DCs), and myeloid cells at earlier stages of differentiation. IMC are present in bone marrow and spleens of healthy mice, and under normal conditions differentiate into mature myeloid cells (12). However, they accumulate in large numbers in tumor-bearing mice probably due to the effect of various tumor-derived factors (13–18). Gr-1$^+$ IMC freshly isolated from spleen of tumor-bearing mice could not inhibit CD4-mediated T cell response, but were able to suppress CD8$^+$ T cells in Ag-dependent manner (16). This inhibition required direct cell-cell contact and was mediated by reactive oxygen species (19). Interestingly, when Gr-1$^+$ cells were incubated for several days in vitro, they acquired ability to block CD4-mediated T cell responses (20).

Gr-1$^+$ IMC can be found not only in spleens, but also in tumor tissues (12). Because Gr-1$^+$ IMC could be differentiated into macrophages in tumor-free mice (12), it would be interesting and important to trace the fate of these cells in tumor tissues and compare immunosuppressive activity of macrophages localized in the vicinity of tumor and in lymphoid organs. No such direct study has been performed until now. These experiments may help to clarify the role of myeloid cells in cancer-associated nonresponsiveness. Despite the presence of large number of different immunosuppressive myeloid cells, tumor-bearing mice or cancer patients usually do not display significant systemic immunodeficiency (reviewed in Ref. 21). In a recent detailed study, Radoja et al. (22) investigated T cell function in multiple murine tumor models, including different transplantable tumors and also a transgenic model of spontaneous breast carcinoma. They demonstrated that in mice bearing sizable tumors, T cell functions (proliferation, cytokine production, induction of CD8$^+$ alloreactive CTL, development of anti-keyhole limpet hemocyanin CD4$^+$ T cells, rejection of allogeneic or syngeneic regressor tumors) were not systemically reduced (22). However, significant number of studies have reported profound deficiency in T cell function in tumor tissues (reviewed in Refs. 23 and 24). Some authors have reported increased apoptosis of tumor-infiltrating lymphocytes (25), although contrary findings have also been shown (26). We hypothesized that myeloid cells localized in tumor tissues, and ones localized in peripheral lymphoid organs (spleen) may differ in their ability to suppress T cell responses. To test this hypothesis, we, for the first time, have performed a direct comparative analysis of myeloid cells freshly isolated from spleens and tumor tissues of tumor-bearing mice. We have identified the mechanisms, which are used by tumor-associated macrophages (TAM) to inhibit T cell function in vitro, and for the first time have determined that STAT1 transcription factor could be directly responsible for the observed TAM-mediated T cell apoptosis.

Materials and Methods

Mice and tumor models

Female BALB/c and C57BL/6 mice (6–8 wk of age) were obtained from the National Cancer Institute. H-2K^b congenic (CD45.1$^+$) B6.SJL-PtrcaPep3b/BoyJ mice, TCR transgenic for OVA-derived peptide SIINFEKL on C57BL/6 background, and C.129S2-*Stat6^{tm1Gru}* STAT6 knockout mice on BALB/c

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[3] Abbreviations used in this paper: IMC, immature myeloid cell; 7-AAD, 7-amino-actinomycin D; DC, dendritic cell; HA, hemagglutinin; iNOS, inducible NO synthase; L-NMMA, N^G-monomethyl-L-arginine; nor-NOHA, N^W-hydroxyl-nor-L-arginine; TAM, tumor-associated macrophage.

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background were obtained from The Jackson Laboratory. STAT1 knockout mice were obtained from Y. Durbin (Children Research Institute, Columbus, OH) and were described in details elsewhere (27). TCR transgenic mice on BALB/c background expressing an $\alpha\beta$ TCR specific for MHC class II-restricted SFERFEIFPKE peptide, derived from influenza hemagglutinin (HA), were kindly provided by E. Sotomayor (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). The following tumor models were used in this study: CT-26 colon carcinoma (s.c.) on BALB/c background, C3 fibrosarcoma (s.c.) on C57BL/6 background, MethA sarcoma (s.c. and i.p.) on BALB/c background, and EL4 (s.c. and i.p.) lymphoma on C57BL/6 background. All tumor cells were maintained in vitro at 37°C in a 5% CO_2 humidified atmosphere in complete culture medium. To establish s.c. tumors, mice were injected into shaved right flank with 10^5 C3 and CT-26 tumors or 2×10^5 MethA sarcoma or EL-4 cells. To establish i.p. tumors, mice were injected i.p. with 2×10^6 EL4 or MethA sarcoma cells.

Reagents

Arginase inhibitor N^W-hydroxyl-nor-L-arginine (nor-NOHA) and inducible NO synthase (iNOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) were from Calbiochem. Anti-TGF-β-neutralizing Ab was obtained from R&D Systems. HA-derived peptide (I-A^d restricted, aa 110–120, SFERFEIFPKE) and OVA-derived peptide (H-$2K^b$ restricted, aa 257–264, SIINFEKL) were purchased from SynPep. Anti-CD45.2, Gr-1, CD8, CD4, CD11b, and isotype control IgG2a, IgG2b Abs were obtained from BD Pharmingen, and anti-F4/80 Ab from Serotec. Abs against mouse arginase I were obtained from BD Pharmingen, and anti-iNOS from Upstate Biotechnology. Anti-STAT3, phospho-STAT3, STAT-1, and phospho-STAT1 Abs were purchased from Cell Signaling Technology.

Cell isolation

Tumor-bearing or naive mice were sacrificed, and their spleens and tumors were harvested under sterile conditions. Single cell suspensions were prepared, and red cells were removed from spleens using ammonium chloride lysing buffer. Solid tumors were dissected and chopped into small pieces using a scissors before incubation with a mixture of enzymes dissolved in RPMI 1640 (400 U/ml collagenase type IV, 0.05 mg/ml collagenase type I, 0.025 mg/ml hyaluronidase, all from Sigma-Aldrich; 0.01 mg/ml DNase I and 0.2 trypsin inhibitor unit/ml soybean trypsin inhibitor, both from Boehringer Mannheim) for 30 min at 37°C. Cells were recovered by centrifugation and resuspended in PBS, containing 1% of FBS. To harvest ascitic tumors or peritoneal cells from naive mice, mice were injected i.p. with 5 ml of ice-cold PBS and then aspirated. F4/80$^+$ and Gr-1$^+$ cells were isolated from tumors, spleen, or peritoneal cell suspension using magnetic microbeads and MiniMACS columns (Miltenyi Biotec). Briefly, cells were resuspended in MACS buffer, and $5–6 \times 10^6$ cells were incubated with 5 μg of biotinylated anti-Gr-1 or F4/80-PE mAbs for 10 min on ice. Cells were washed with cold buffer to remove unbound Abs, and then incubated with streptavidin or PE microbeads for 15 min at 4°C. Gr-1$^+$ and F4/80$^+$ cell populations were isolated on MiniMACS columns, according to the manufacturer's instructions. Purity of cell populations was evaluated by flow cytometry and exceeded 90%.

Flow cytometry

One million cells were incubated for 30 min on ice in 100 μl of PBS with 1 μg of relevant Abs, and then washed twice with cold PBS. Flow cytometry data were acquired using a FACSCalibur flow cytometer (BD Biosciences), and were analyzed with CellQuest software (BD Biosciences).

Ag-specific and CD3/CD28-induced T cell proliferation

Ag-specific T cell proliferation. Ag-specific proliferation of CD4 T cells was evaluated using HA-TCR transgenic mice, whereas CD8 T cell-mediated response was measured using OT-1 transgenic mice. Splenocytes from transgenic mice were depleted of RBC, washed with PBS, resuspended in complete culture medium, and placed in triplicates into U-bottom 96-well plate (2×10^5/well) in presence of cognate Ags (HA-derived peptide SFERFEIFPKE for TCR-HA transgenic mice or OVA-derived peptide SIINFEKL for OT-1 transgenic mice) and cultured for 4 days. Eighteen hours before harvesting, cells were pulsed with [^{3}H]thymidine (1 μCi/well; Amersham Biosciences). [^{3}H]Thymidine uptake was counted using a liquid scintillation counter and expressed as cpm.

CD3/CD28-induced T cell proliferation. Splenocytes were seeded in triplicates at concentration of 2×10^5 per well into U-bottom 96-well plates in presence of 1 μg/ml anti-CD3 Ab and 5 μg/ml anti-CD28 Ab for 72 h. Eighteen hours before harvesting, cells were pulsed with [^{3}H]thymidine. Cell proliferation was evaluated, as described above.

Apoptosis

Apoptosis was evaluated by flow cytometry using annexin V-PE apoptosis detection kit (BD Pharmingen), according to manufacturer's protocol.

Western blotting

Freshly isolated F4/80$^+$ or Gr-1$^+$ cells were lysed with radioimmunoprecipitation assay buffer in the presence of protease and phosphatase inhibitors. Samples (30 μg protein/lane) were subjected to electrophoresis in 10% SDS-polyacrylamide gels, and then blotted onto 0.45-μm nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% dry skimmed milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl plus 0.1% (v/v) Tween 20) and then probed with rabbit Abs with appropriate specificity overnight at 4°C. Membranes were washed and incubated for 2 h at room temperature with secondary Ab (goat anti-rabbit Ab conjugated with HRP). Results were visualized by chemiluminescence detection using a commercial kit (Amersham Biosciences).

Arginase activity

Arginase activity was measured in cell lysates, as previously described by Corraliza et al. (28). Briefly, cells were lysed for 30 min with 100 μl of 0.1% Triton X-100. Subsequently, 100 μl of 25 mM Tris-HCl and 10 μl of 10 mM $MnCl_2$ were added, and the enzyme was activated by heating for 10 min at 56°C. Arginine hydrolysis was conducted by incubating the lysate with 100 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 15–120 min. The reaction was stopped with 900 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol), followed by heating at 95°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea per min.

Confocal microscopy

Freshly purified F4/80$^+$ or Gr-1$^+$ cells were washed in cold PBS with 1% FBS, fixed in 1% paraformaldehyde, and then stained overnight with anti-phospho-STAT6 (Tyr^{641}) or rabbit IgG, followed by FITC goat F(ab′)$_2$ anti-rabbit Ab (Southern Biotechnology Associates) in a solution containing PBS, 1% FBS, 0.2% saponin, and 3% cold fish gelatin (Sigma-Aldrich). After the final wash, the cells were resuspended in a minimal volume of Vectashield (Vector Laboratories), placed on a slide, and covered with a coverslip, and the edges were sealed with nail polish. Cells were observed on a Leica DM IRBC confocal microscope (Leica Microsystems).

NO production

Equal volumes of culture supernatants (100 μl) were mixed with Greiss reagent. After 10-min incubation at room temperature, the absorbance at 550 nm was measured using microplate plate reader (Bio-Rad). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

Statistical analysis

The statistical significance between values was determined by Student's t test. All data were expressed as the mean ± SD. Probability values >0.05 were considered nonsignificant.

Results

Gr-1$^+$ immature myeloid cells differentiate into suppressive TAM

Previous studies have demonstrated that Gr-1$^+$ cells accumulate in practically all tested mouse tumor models and contain immature myeloid cells at different stages of cell differentiation (10, 11). Adoptive transfer of these cells into naive tumor-free recipients or tumor-bearing mice results in their differentiation into mature myeloid cells, although in tumor-bearing mice significant proportion of donor cells retained their immature phenotype (Gr-1$^+$CD11b$^+$) (12). In this study, we investigated the fate and suppressive activity of myeloid cells localized in tumor tissues. Gr-1$^+$ cells were isolated from spleens of C3 tumor-bearing C57BL/6 mice (CD45.2$^+$). The phenotype of these cells is shown in Fig. 1*A*. Five million of these Gr-1$^+$ cells were injected i.v. into each congenic CD45.1$^+$ mouse-bearing C3 tumor (1–1.5 cm in diameter).

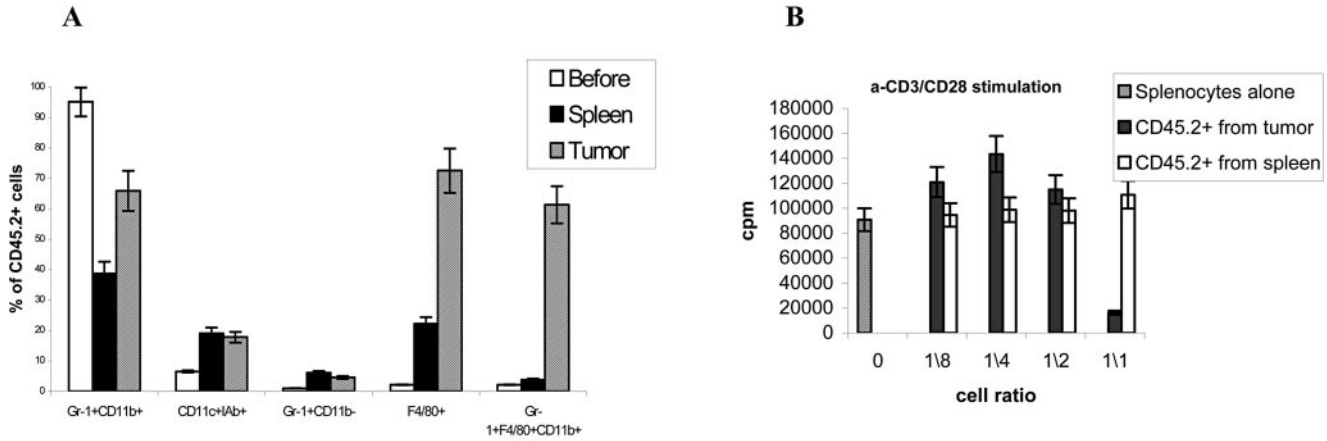

FIGURE 1. Differentiation and immune suppressive activity of myeloid cells in tumor-bearing mice. *A*, Gr-1$^+$ cells transferred into tumor host predominantly become F4/80$^+$ macrophages. Gr-1$^+$CD45.2$^+$ cells were purified from spleens of C3 tumor-bearing C57BL/6 mice using magnetic beads and transferred i.v. into congenic (CD45.1$^+$) C3 tumor-bearing mice (5×10^6 cells/mouse). On day 3 after cell transfer, recipient mice were sacrificed, spleens and tumors were excised, connective tissue was digested, and single cell suspension had been prepared. Presence of myeloid cells within CD45.2$^+$ donor cell population in spleens and tumor site was evaluated by multicolor flow cytometry. Each group included three mice. Average ± SD are shown. Before, cells before adoptive transfer; Spleen, cells isolated from spleens; Tumor, cells isolated from tumor site. *B*, Inhibition of CD3/CD28-induced T cell proliferation by transferred Gr-1$^+$ cells. Donor CD45.2$^+$ cells were purified from spleen or tumors of congenic recipient mice on day 4 after adoptive transfer of Gr-1$^+$ myeloid cells using biotinylated anti-CD45.2 Ab and streptavidin beads. Isolation was performed on MiniMACS columns. CD45.2$^+$ cells were added at different ratios to naive splenocytes (2×10^5/well) activated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAbs. Cells were cocultured for 72 h, and incorporation of [^{3}H]thymidine was measured in triplicates. Results are presented as average ± SD.

Spleens and tumors were collected 3 days after the transfer, and the phenotype of donor's CD45.2$^+$ cells was evaluated using multicolor flow cytometry. Significant proportion of donor's cells isolated from tumor site retained an immature phenotype (Gr-1$^+$CD11b$^+$). This population was substantially smaller in donors' cells isolated from spleens (Fig. 1*A*). In spleen, equal proportion (20%) of donors' cells had the phenotype of DCs (CD11c$^+$IA^{b+}) and macrophages (Gr-1$^-$F4/80$^+$) (Fig. 1*A*). In contrast, >70% of the donors' cells isolated from tumors expressed F4/80 marker specific for macrophages. Practically all F4/80$^+$ donors' cells isolated from tumors were also Gr-1$^+$ and CD11b$^+$ (Fig. 1*A* and data not shown). The percentage of these cells was not changed if cells were collected 5 days after the transfer (data not shown). These results indicate that Gr-1$^+$ myeloid cells are precursors of F4/80 TAM.

Next, we asked whether myeloid cells differentiated from IMC in vivo were able to suppress T cell response. Gr-1$^+$ cells were isolated from spleens of C3 tumor-bearing mice and injected i.v. into tumor-bearing congenic mice, as described above. Four days after the transfer, CD45.2$^+$ donor's cells were isolated using magnetic beads and added at different ratios to splenocytes from control syngeneic C57BL/6 mice stimulated with anti-CD3 and anti-CD28 Abs. Donor's cells isolated from spleens did not affect T cell proliferation, whereas CD45.2$^+$ donor's cells isolated from tumors significantly inhibited T cell response (Fig. 1*B*).

What population of myeloid cells isolated from tumor tissue could be responsible for inhibition of T cells? Because F4/80$^+$ macrophages are the major components of myeloid cells inside the tumors, we have investigated the ability of these cells to induce T cell suppression. F4/80$^+$ cells were isolated from tumor or spleens of CT-26 tumor-bearing BALB/c mice using magnetic beads, and then added at different ratios to splenocytes from HA transgenic BALB/c mice stimulated with control or specific MHC class II-matched HA-derived peptide. Neither Gr-1$^+$ nor F4/80$^+$ cells isolated from spleens of tumor-bearing mice were able to significantly affect CD4-mediated Ag-specific T cell proliferation (Fig. 2*A*). Gr-1$^+$ cells from tumor also failed to suppress T cell response. However, F4/80$^+$ cells isolated from tumor significantly inhibited T cell proliferation at 1:4 ratio and almost completely blocked it at 1:2 ratio (Fig. 2*B*).

We asked whether the observed effects of tumor-associated F4/80$^+$ macrophages were restricted to only one tumor model or only one type of T cell stimuli. F4/80$^+$ macrophages were isolated from spleens of naive and C3 tumor-bearing C57BL/6 mice as well as from tumor of C3 tumor-bearing mice. These cells were incubated together with splenocytes isolated from OT-1 transgenic mice in the presence of control or specific peptide, as described above. F4/80$^+$ cells isolated from tumor, but not from spleen, of tumor-bearing mice significantly inhibited T cell proliferation (Fig. 2*C*) and CTL activity against peptide-loaded targets (Fig. 2*D*). Similar experiments were performed on the model of MethA sarcoma. MethA sarcoma cells were injected i.p. into BALB/c mice. Two weeks later, F4/80$^+$ cells were isolated from peritoneum of tumor-bearing or control mice and added to splenocytes from control BALB/c mice stimulated with anti-CD3 and anti-CD28 Abs. F4/80$^+$ macrophages from tumor-bearing, but not from control mice dramatically reduced T cell proliferation (data not shown).

We evaluated the phenotype of F4/80$^+$ isolated from control and tumor-bearing mice. Typical results of these experiments are shown in Fig. 2*E*. F4/80$^+$ macrophages isolated from spleens of naive tumor-free BALB/c and C57BL/6 mice contained equal proportion of Gr-1$^+$ CD11b$^+$ double-positive cells (36–39%). The proportion of these cells among F4/80$^+$ macrophages isolated from spleens of tumor-bearing mice was slightly increased (46–51%). In contrast, >93% of F4/80$^+$ cells isolated from tumor sites expressed both Gr-1 and CD11b markers (Fig. 2*E*). All tested tumor models demonstrated similar results (Fig. 2*E* and data not shown). Importantly, <30% of Gr-1$^+$ cells isolated from tumor tissues expressed F4/80 marker (Fig. 2*F*). Among all cells in tumor tissues, the proportion of Gr-1$^-$F4/80$^+$ cells was 2–4%, and Gr-1$^+$F4/80$^-$ cells 5–7%. These data indicate that substantial proportion of Gr-1$^+$ cells in tumor tissues does not express F4/80 marker and probably represents myeloid cells at different stage of differentiation. This may explain different ability of F4/80$^+$ and Gr-1$^+$ cells to suppress T cell response.

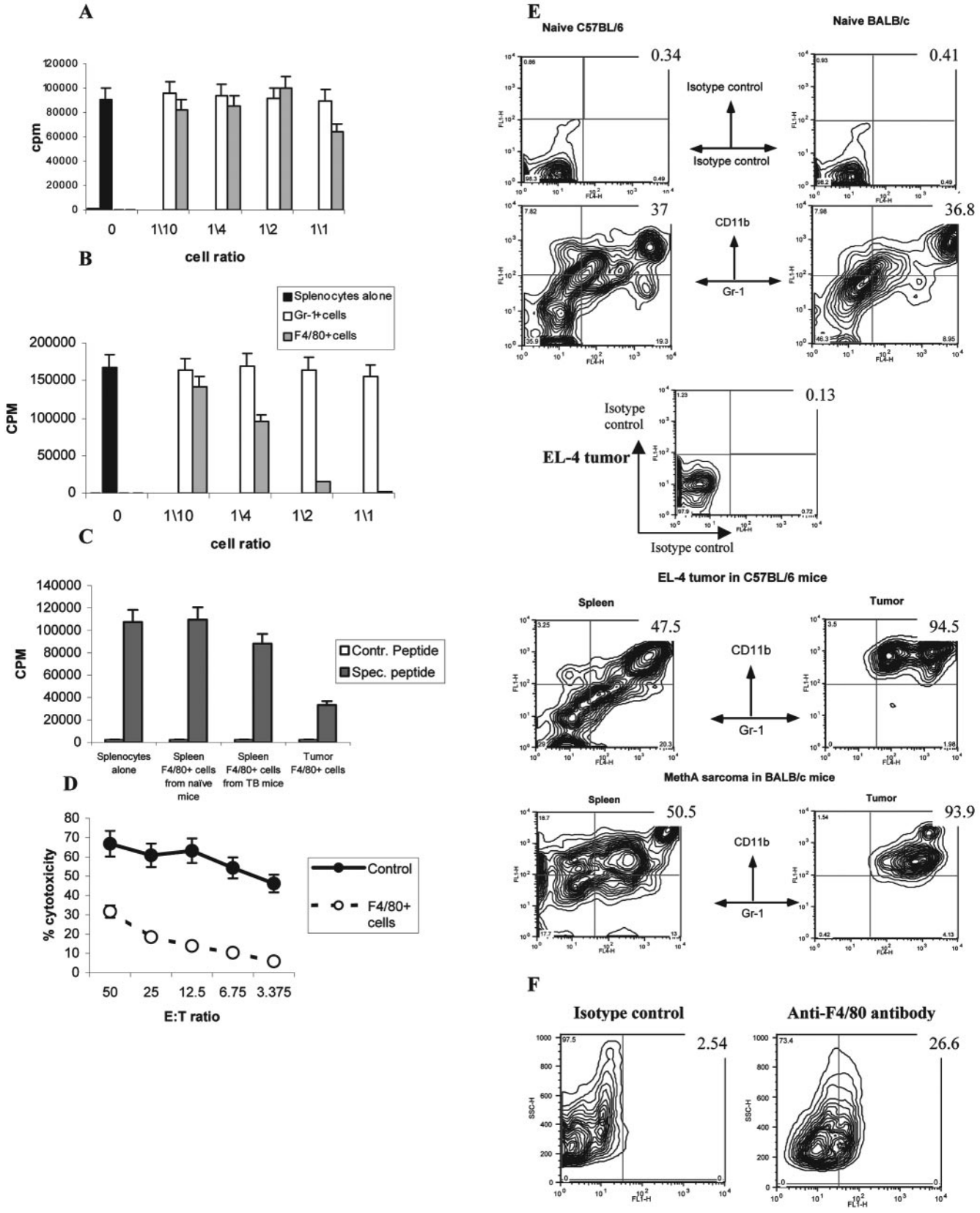

FIGURE 2. Inhibition of T cell immune response by F4/80$^+$ TAM. *A*, Both Gr-1$^+$ and F4/80$^+$ cells from spleens of tumor-bearing mice failed to alter Ag-induced T cell proliferation. Gr-1$^+$ or F4/80$^+$ cells freshly isolated from spleens of CT-26 tumor-bearing BALB/c mice were added at different ratios to splenocytes from HA transgenic BALB/c mice stimulated with 10 μg/ml MHC class II-restricted control or HA-derived peptides. Cells were cocultured for 72 h, and incorporation of [^{3}H]thymidine was measured in triplicates. Results are shown as average ± SD. Thymidine uptake in cells stimulated with control peptide was <1000 cpm. *B*, F4/80$^+$, but not Gr-1$^+$ cells derived from tumor are inhibitory to T cell response. Gr-1$^+$ or F4/80$^+$ cells were purified from CT-26 tumors and immediately added to splenocytes from HA transgenic mice stimulated with control or specific peptides. Incorporation of [^{3}H]thymidine was measured in triplicates 72 h later, as described above. Results are presented as average ± SD. *C*, F4/80$^+$ macrophages were purified (*Figure legend continues*)

Thus, these data indicate that Gr-1$^+$ IMC in spleen or in the vicinity of tumors do not suppress CD4-mediated T cell response, which is consistent with our previous observations (19). In tumor-bearing mice, these cells differentiate into F4/80$^+$ macrophages. However, in the vicinity of tumor and in spleens, the phenotype of these cells and their ability to suppress T cell response were profoundly different. If in spleens only one-half of F4/80$^+$ cells were Gr-1$^+$ and lacked the ability to suppress T cell response, practically all F4/80$^+$ cells inside the tumors expressed Gr-1 marker and were highly immunosuppressive. These results were reproduced in at least four different tumor models on two different mouse strains.

Tumor-associated F4/80$^+$ macrophages induce apoptosis of T cells, which is mediated by increased production of NO and arginase activity

Previous studies have demonstrated that macrophages isolated from tumor-bearing mice may induce apoptosis of T cells (33). To test this possibility, F4/80$^+$ cells were isolated from spleens and tumors of C3 tumor-bearing mice and incubated with splenocytes from control syngeneic C57BL/6 mice stimulated with anti-CD3 and anti-CD28 Abs. Twenty-four and 48 h later, cells were collected and labeled with allophycocyanin-conjugated anti-CD4 Ab, FITC-conjugated anti-CD8 Ab, PE-conjugated annexin V, and 7-aminoactinomycin D (7-AAD). To maintain consistency with experiments described above, we used the same F4/80$^+$ cells:splenocytes ratio, 1:4. The proportion of annexin V-positive, 7-AAD-negative apoptotic cells within populations of CD4$^+$ or CD8$^+$ T cells was calculated. Tumor-associated, but not spleen-derived F4/80$^+$ cells induced significant level of apoptosis in both populations of T cells (Fig. 3*A*). The presence of F4/80$^+$ TAM, however, did not induce apoptosis of nonstimulated T cells (data not shown). Similar effect was seen in a different tumor model in EL-4 tumor-bearing C57BL/6 mice. F4/80$^+$ cells were isolated from spleens and peritoneal exudates of tumor-bearing mice and incubated with splenocytes from transgenic OT-1 C57BL/6 mice in the presence of specific OVA-derived peptide SIINFEKL. Twenty-four and 48 h later, cells were collected and labeled with anti-CD8 Ab, annexin V, and 7-AAD. F4/80$^+$ macrophages isolated from the vicinity of tumor (peritoneum), but not from spleens of tumor-bearing mice induced apoptosis of CD8$^+$ T cells (Fig. 3*B*). After 48 h in culture, this practically resulted in complete elimination of CD8$^+$ cells (Fig. 3*C*). As in experiments described above, no induction of apoptosis was seen if T cells were not stimulated with specific peptide (data not shown). The presence of tumor-derived F4/80$^+$ cells decreased the proportion of alive cells after 48 h of culture >5-fold (Fig. 3*D*). Previous studies implicated IFN-γ and TNF-α in macrophage-induced apoptosis of T cells. In our experimental model, neutralizing anti-IFN-γ Ab partially and anti-TNF-α completely abrogated the effect of tumor-derived F4/80$^+$ macrophages on T cell deletion (Fig. 3*D*). However, these Abs only partially reversed the inhibitory effect of F4/80$^+$ macrophages on T cell proliferation (Fig. 3*E*). Overall, these data are consistent with previous publications (33) and indicate that IFN-γ and TNF-α are important factors mediating the effect of tumor-derived F4/80$^+$ macrophages.

In addition, several factors produced by TAM could contribute to T cell apoptosis. NO and arginase were previously implicated in T cell inhibition associated with myeloid cells in cancer (29–31). We have confirmed previously reported data about increased production of NO by F4/80$^+$ TAM incubated with activated T cells (33). It was produced more than 3-fold higher than by F4/80$^+$ cells isolated from spleen and >5-fold than by Gr-1$^+$ IMC isolated from spleens or tumor (Fig. 4*A*). F4/80$^+$ TAM had significantly higher levels of iNOS than Gr-1$^+$ IMC or F4/80$^+$ cells isolated from spleens of tumor-bearing mice (Fig. 4, *B* and *C*). Gr-1$^+$ IMC from tumor-bearing mice had elevated amount and activity of arginase I (Fig. 4*D*) consistent with previous observations (19, 20). Similar level of arginase was observed in F4/80$^+$ macrophage isolated from spleens of tumor-bearing mice. The total amount of this protein and its activity was much higher in F4/80$^+$ cells isolated from tumor (Fig. 4*E*). Thus, in contrast to myeloid cells isolated from spleen of tumor-bearing mice, tumor-associated F4/80$^+$ macrophages had significantly higher level of iNOS, arginase I, NO production, and arginase activity.

Next, we investigated whether these factors were indeed responsible for macrophage-mediated T cell inhibition. F4/80$^+$ cells were isolated from spleens or tumor of C3 tumor-bearing mice and cultured at 1:4 ratio with splenocytes obtained from OT-1 transgenic mice stimulated with specific peptide (SIINFEKL). Tumor-associated, but not spleen-derived F4/80$^+$ macrophages significantly inhibited CD8$^+$ T cell proliferation (Fig. 4*F*). Inhibitor of iNOS L-NMMA and inhibitor of arginase nor-NOHA were added to the mixture either separately or together. Effective concentrations that do not affect macrophage viability were selected after preliminary testing and matched those reported in previous studies. L-NMMA or nor-NOHA alone did not affect F4/80$^+$ cell-mediated inhibition of T cell proliferation. However, the combination of these two inhibitors completely reversed that inhibition (Fig. 4*F*). These results were verified in a different experimental system in which F4/80$^+$ macrophages were isolated from ascites of MethA sarcoma-bearing mice. Similar results were obtained. Dramatic inhibition of T cell proliferation by tumor-associated F4/80$^+$ macrophages was completely reversed only by the combination of L-NMMA and nor-NOHA, but not these two inhibitors separately (data not shown).

Thus, these results indicate that T cell inhibition induced by TAM is mediated by the combined effect of two major factors: increased production of NO and high arginase activity.

from tumor tissues or from spleens of C3 tumor-bearing mice, or from spleens of naive mice and added at different ratios to splenocytes from OT-1 transgenic mice stimulated with specific MHC class I-restricted OVA-derived peptide SIINFEKL. Incorporation of [^{3}H]thymidine was measured 72 h later, as described above. Results are presented as average ± SD. *D*, F4/80$^+$ TAM suppress CTL development. Splenocytes from OT-1 transgenic mice were mixed at 1:10 ratio together with syngeneic splenocytes from naive C57BL/6 mice and incubated in 24-well plates (4×10^6/well) for 5 days (control group). Cells were stimulated with 10 μg/ml OVA-derived peptide SIINFEKL. To evaluate the effect of TAM on CTL activity, F4/80$^+$ macrophages freshly isolated from C3 tumor were added to the culture on day 0. TAM:splenocytes ratio was 1:10 (F4/80 group). Cytotoxicity against specific peptide-loaded EL-4 tumor cells was measured using ^{51}Cr release assay. The results are presented as average ± SD. *E*, F4/80$^+$ cells were isolated from spleens of naive tumor-free C57BL/6 or BALB/c mice using magnetic beads separation technique and labeled with either isotype control IgG or allophycocyanin-conjugated anti-Gr-1 Ab and FITC-conjugated anti-CD11b Ab. Typical result of three experiments is shown. F4/80$^+$ cells were also isolated from spleens or ascites of EL-4 tumor-bearing C57BL/6 mice or MethA sarcoma-bearing BALB/c mice using magnetic beads separation technique and labeled with allophycocyanin-conjugated anti-Gr-1 Ab and FITC-conjugated anti-CD11b Ab. Typical result of three experiments is shown. *F*, Gr-1$^+$ cells were isolated from tumor of EL-4 tumor-bearing mice using magnetic beads separation and labeled with either isotype control or FITC-conjugated anti-F4/80 Ab. The result of typical experiment is shown.

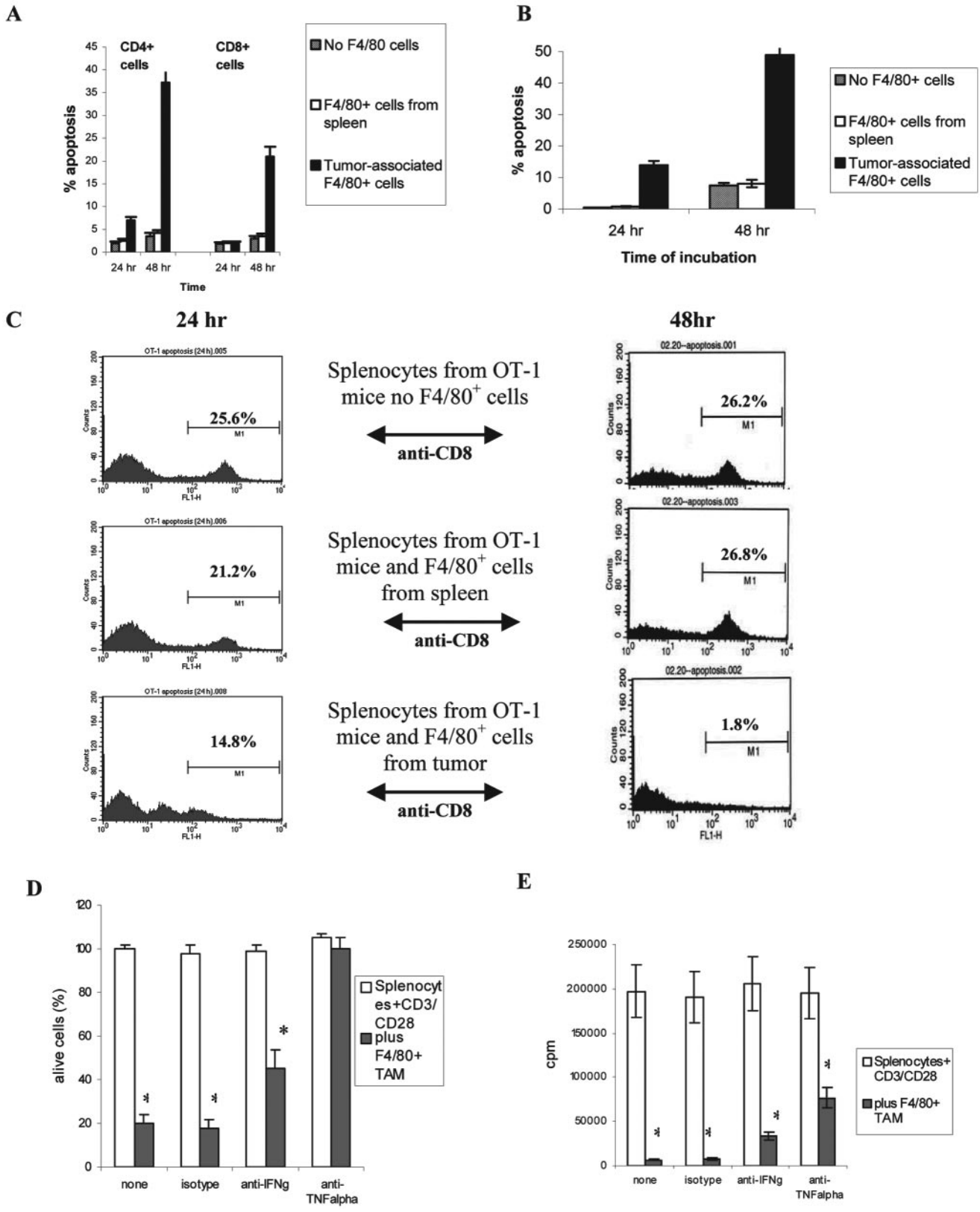

FIGURE 3. Tumor-associated F4/80$^+$ macrophages cause deletion of T cells through induction of apoptosis. *A*, Stimulation of splenocytes with CD3/CD28 mAb in the presence of F4/80$^+$ TAM induces apoptosis of CD4 and CD8 T cells. F4/80$^+$ macrophages were purified from tumors or spleens of C3 tumor-bearing mice and mixed at 1:4 cell ratio with naive syngeneic splenocytes stimulated with anti-CD3 and anti-CD28 mAbs. After 24 and 48 h of incubation, cells were collected, washed in PBS, and labeled with anti-CD4 allophycocyanin, anti-CD8 FITC Abs, PE-conjugated annexin V, and 7-AAD. The proportion of annexin V-positive 7-AAD-negative apoptotic cells within the CD4$^+$ or CD8$^+$ T cell populations was measured by flow cytometry. Combined results of three performed experiments are shown. *B*, Stimulation of splenic cells from OT-1 transgenic mice with specific OVA-derived peptide in presence of F4/80$^+$ TAM induces apoptosis of Ag-specific CD8 T cells. F4/80$^+$ macrophages were purified from ascites or spleens of EL-4 tumor-bearing mice (ascitic tumor), mixed at 1:4 cell ratio with OT-1 transgenic splenocytes stimulated with specific (*Figure legend continues*)

Role of STAT transcription factors in TAM-mediated T cell inhibition

We asked which signaling pathway in myeloid cells could be responsible for the observed phenomena. Recent study implicated STAT6 as one of the possible factors mediating immunosuppressive function of myeloid cells (14). Therefore, we first compared the level of STAT6 expression in TAM and F4/80$^+$ macrophages isolated from naive mice. F4/80$^+$ TAM had substantially higher level of phospho-STAT6 than their control counterparts (Fig. 5*A*). To clarify the role of STAT6 in F4/80$^+$ cell-mediated T cell suppression, EL-4 tumors were established in wild-type STAT6$^{+/+}$ and STAT6$^{-/-}$ knockout mice. F4/80$^+$ cells were isolated from tumor tissues and analyzed. No differences in T cell-suppressive activity between TAM isolated from STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were found (Fig. 5*B*). TAM isolated from STAT6$^{+/+}$ and STAT6$^{-/-}$ tumor-bearing mice had also the same level of arginase activity (Fig. 5*C*). These data indicate that although TAM had increased level of STAT6 activity, it was not directly responsible for the increased level of arginase activity and T cell immunosuppression.

Hyperactivation of Jak2-STAT3 pathway was recently implicated in abnormal myeloid cell differentiation and function in cancer (13, 32). Elevated level of STAT3 activity was observed in different myeloid cells isolated from tumor-bearing mice (13). We evaluated the level of STAT3 activation in F4/80$^+$ cells isolated from control and tumor-bearing mice. F4/80$^+$ macrophages isolated from spleens of tumor-bearing mice had elevated level of phospho-STAT3 compared with F4/80$^+$ cells isolated from spleens of control mice (Fig. 5*D*). However, the level of phospho-STAT3 was practically undetectable in F4/80$^+$ TAM (Fig. 5*D*). STAT1 is another member of STAT family involved in regulation of macrophage function. F4/80$^+$ macrophages isolated from spleens or peritoneal cavity of control mice as well as from spleens of tumor-bearing mice had undetectable level of activated (phosphorylated) STAT1 (Fig. 5*D*). In contrast, F4/80$^+$ TAM demonstrated increased level of phospho-STAT1. This suggests that STAT1 can be involved in the observed TAM-mediated T cell inhibition. To test this hypothesis, we established EL-4 tumor in STAT1 knockout mice. F4/80$^+$ cells were isolated from tumor site of wild-type STAT1$^{+/+}$ and knockout STAT1$^{-/-}$ mice, and their ability to suppress CD3/CD28-stimulated T cells was evaluated. Consistent with our data described above, F4/80$^+$ TAM isolated from STAT1$^{+/+}$ mice dramatically inhibited T cell proliferation at ratio as low as 1:16. However, F4/80$^+$ TAM from STAT1$^{-/-}$ mice failed to inhibit T cell response even at 1:2 ratio (Fig. 5*E*). F4/80$^+$ TAM from STAT1-deficient mice had no detectable level of iNOS or arginase I (Fig. 5*F*). Coculture of F4/80$^+$ TAM from wild-type mice with activated T cells resulted in significant level of NO production and arginase activity (Fig. 5, *G* and *H*). However, F4/80$^+$ TAM from STAT1$^{-/-}$ mice demonstrated no increase in NO production and had minimal arginase activity (Fig. 5, *G* and *H*).

Most of previous studies that described immunosuppressive myeloid cells in spleen of tumor-bearing mice used not freshly isolated splenocytes, but cells cultured for several days in vitro. To clarify the effect of culture on immunosuppressive activity of myeloid cells, we have isolated Gr-1$^+$ cells from spleens of CT-26 tumor-bearing BALB/c mice and cultured them for 5 days in the presence of GM-CSF. The presence of GM-CSF was necessary to preserve viability of IMC. Resulting population of cells was represented mostly by F4/80$^+$ macrophages (data not shown). In contrast to Gr-1$^+$ IMC or F4/80$^+$ macrophages freshly isolated from spleens of tumor-bearing mice, cultured cells had a profound inhibitory effect on T cell proliferation (Fig. 6*A*). This effect was associated with increased arginase activity and NO production (Fig. 6, *B* and *C*). STAT1 was not detected in freshly isolated Gr-1$^+$ cells, but was clearly seen in cells after 5 days in culture (Fig. 6*D*). These data indicate that during in vitro culture, IMC differentiate into immunosuppressive macrophages and that process is associated with up-regulation of STAT1. We asked what condition could convert splenic F4/80$^+$ macrophage into immunosuppressive cells? F4/80$^+$ cells were isolated from spleens of EL-4 tumor-bearing mice and cultured either in complete medium alone or with EL-4 tumor cells. Because F4/80$^+$ cells are mature macrophages, we used short incubation period without presence of growth factors. Two days later, F4/80$^+$ cells were reisolated from cultures using magnetic beads separation technique. Neither freshly isolated F4/80$^+$ cells, nor cells cultured in medium alone were able to suppress Ag-specific T cell proliferation or produce NO (Fig. 6, *E* and *F*). In contrast, macrophages cultured for 48 h with tumor cells acquired the ability to inhibit T cell and produce large amount of NO (Fig. 6, *E* and *F*). These data indicate that tumor environment may support conversion of mature macrophages to immunosuppressive cells, and this process is associated with increased NO production.

Discussion

In this study, we have clarified the role of myeloid cells in inadequate function of immune system in cancer. In tumor-bearing mice, Gr-1$^+$ myeloid cells represent the majority of myeloid cells. Gr-1 marker is normally expressed on <5% of splenocytes and associated with mature granulocytes, small populations of macrophages, and relatively immature myeloid cells. Previous studies have demonstrated that in vitro or after adoptive transfer into naive mice in vivo, Gr-1$^+$ cells quickly differentiate into mature DCs, macrophages, and granulocytes (12). The proportion of Gr-1$^+$CD11b$^+$ cells in spleens of tumor-bearing mice increases dramatically in every tested tumor model (reviewed in Refs. 10 and

OVA-derived peptide SIINFEKL. After 24 and 48 h of incubation, cells were collected, washed in PBS, and labeled, as described above. The proportion of annexin V-positive 7-AAD-negative apoptotic cells within the population of CD8$^+$ T cells was measured by flow cytometry. Combined results of three performed experiments are shown. *C*, F4/80$^+$ TAM cause in vitro deletion of CD8 T cells. F4/80$^+$ macrophages were purified from ascites or spleens of EL-4 tumor-bearing mice (ascitic tumor) and mixed at 1:4 cell ratio with OT-1 transgenic splenocytes stimulated with specific OVA-derived peptide SIINFEKL. After 24 and 48 h of incubation, cells were collected, washed in PBS, and labeled with anti-CD8 FITC Ab. The proportion of CD8$^+$ cells within whole cell population was measured by flow cytometry. *D*, F4/80$^+$ macrophages were purified from ascites or spleens of EL-4 tumor-bearing mice and mixed at 1:4 cell ratio with naive syngeneic splenocytes stimulated with anti-CD3 and anti-CD28 mAbs. Neutralizing anti-IFN-γ, anti-TNF-α Abs, or control rabbit IgG (all from R&D Systems) were added at concentration of 10 μg/ml. After 48 h of incubation, cells were collected and the percentage of viable cells was counted using trypan blue exclusion. Experiments were performed in triplicates. Results are presented as average $\pm$ SD. $*$, Statistically significant differences from cells cultured without F4/80$^+$ cells ($p < 0.05$). *E*, F4/80$^+$ macrophages were purified from ascites or spleens of EL-4 tumor-bearing mice and mixed at 1:4 cell ratio with naive syngeneic splenocytes stimulated with anti-CD3 and anti-CD28 mAbs. Neutralizing anti-IFN-γ, anti-TNF-α Abs, or control rabbit IgG (all from R&D Systems) were added at concentration of 10 μg/ml. Incorporation of [^{3}H]thymidine was measured in triplicates 72 h later, as described above. Results are presented as average $\pm$ SD. $*$, Statistically significant differences from cells cultured without F4/80$^+$ cells ($p < 0.05$).

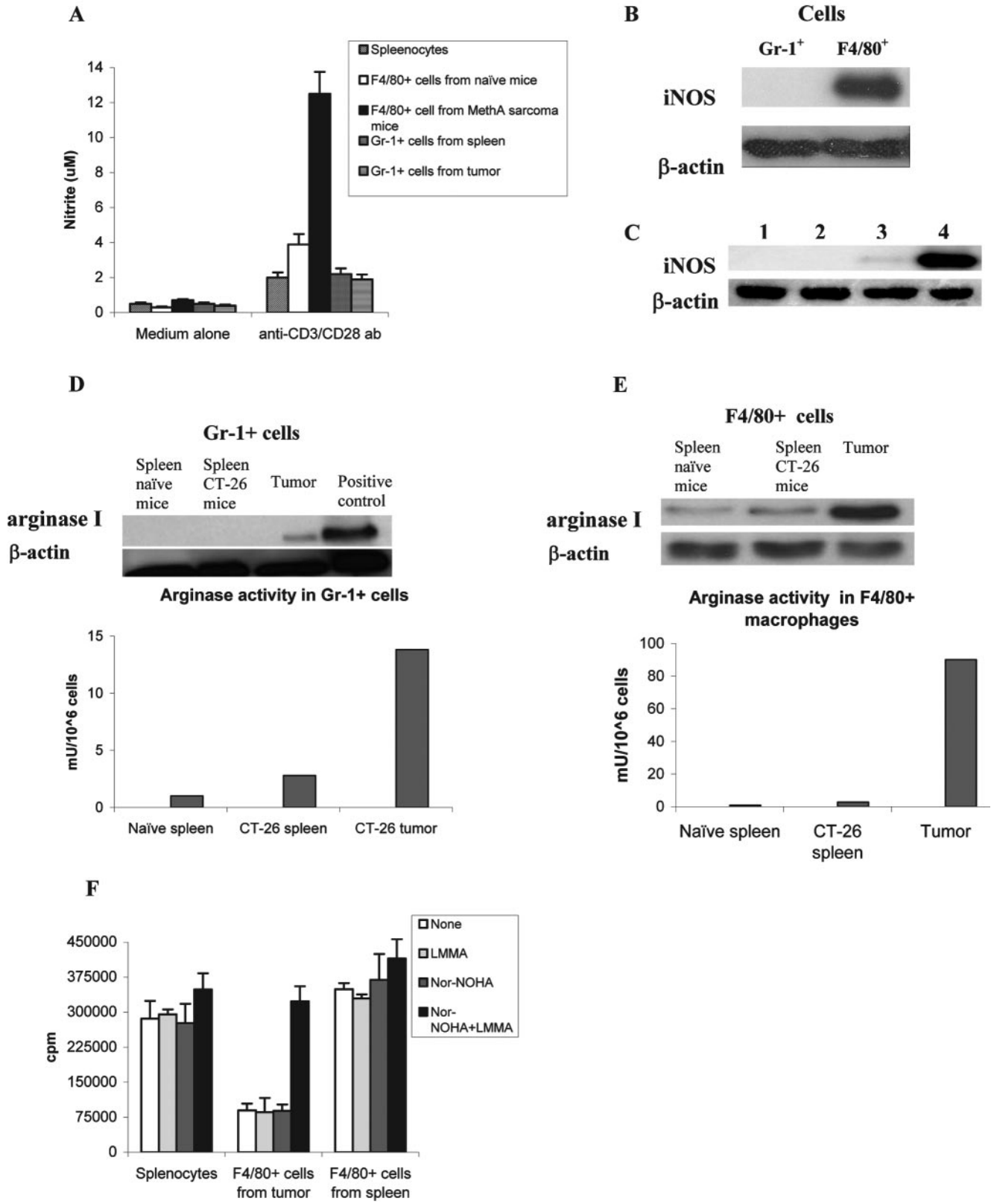

FIGURE 4. Arginase and iNOS are dramatically up-regulated in TAM. *A*, TAM are able to produce high amounts of NO. F4/80$^+$ or Gr-1$^+$ cells were purified from tumors or spleens of tumor-bearing mice and mixed at 1:16 cell ratio with 2×10^5 naive syngeneic splenocytes stimulated with anti-CD3 and anti-CD28 mAbs. After 48-h incubation, cell supernatants were collected, filtered, and assayed for nitrites, as described in *Materials and Methods*. *B*, Freshly isolated F4/80$^+$ TAM, but not Gr-1$^+$ splenic myeloid cells from tumor-bearing mice express iNOS. Whole cell lysates were prepared from purified TAM or Gr-1$^+$ splenocytes (C57BL/6 mice). Samples (30 μg of protein) were subjected to electrophoresis in 10% SDS-polyacrylamide gels, blotted onto 0.45-μm nitrocellulose membranes, and probed with anti-iNOS Ab. Western blotting has been done, as described in *Materials and Methods*. *C*, Comparative analysis of iNOS expression by F4/80$^+$ macrophages derived from C57BL/6 mice: *lane 1*, F4/80$^+$ cells were purified from naïve spleen; *lane 2*, from peritoneum of naïve mice; *lane 3*, from spleens of EL-4 tumor-bearing mice; and *lane 4*, from ascites of EL-4 tumor-bearing mice. Cells were lysed, and expression of iNOS was determined by Western blotting. *D*, Arginase I expression and activity in freshly derived Gr-1$^+$ cells. (*Figure legend continues*)

11). In tumor-bearing mice, the majority of these cells are represented by immature macrophages, DCs, granulocytes, or cells at earlier stages of myeloid cell differentiation (reviewed in Ref. 11). Our previous studies have demonstrated that these cells suppress $CD8^+$ T cells in an Ag-specific manner via direct cell-cell contact that involves reactive oxygen species (16, 19). However, these cells did not affect $CD4^+$ T cells. In contrast, Gr-1^+ cells isolated from spleens or bone marrow of naive or tumor-bearing mice showed strong immunosuppressive ability against $CD4^+$ T cells after in vitro culture (20). In this study, we evaluated differentiation of Gr-1^+ IMC in tumor-bearing mice using adoptive transfer experiments. Significant proportion of IMC localized after adoptive transfer in spleen differentiated into macrophages, granulocytes, and DC. Importantly, when donor's cells were purified from spleens of recipient using congenic marker CD45.2, they lacked the ability to suppress CD4-mediated T cell responses. In contrast, Gr-1^+CD11b$^+$F4/80$^+$ cells represented the majority of donor's cells localized in tumor tissues. F4/80 marker is usually expressed on mature macrophages and is used to distinguish macrophages from granulocytes and DC. These triple-positive cells are likely to be immature macrophages. Currently, we are in the process of clarifying this issue. Donor's cells isolated from tumor tissues significantly inhibited T cell responses in vitro. Thus, in tumor-bearing mice, the fate of Gr-1^+ IMC depends on the site of their differentiation. In spleen, they became primarily DC and macrophages and do not display immunosuppressive features against T cells (at least at 1:1 ratio), whereas in the vicinity of tumor they become immunosuppressive immature macrophages. It is important to note that research design in this study did not allow us to make a clear distinction between the possibility that Gr-1^+ IMC differentiated into triple-positive macrophages within the tumor tissue and the possibility that triple-positive macrophages differentiated from IMC elsewhere and migrated into tumor tissue. However, the fact that very little triple-positive cells were found in spleen of tumor-bearing mice suggested that these cells most likely differentiate from IMC in the vicinity of tumor.

Because F4/80$^+$ cells represented a great majority of myeloid cells in tumor tissues, we focused further analysis on these cells. F4/80$^+$ macrophages isolated from spleens of tumor-bearing mice failed to suppress T cell responses, whereas F4/80$^+$ cells isolated from tumor tissues demonstrated profound inhibition of T cell proliferation in response to different stimuli. These findings were reproduced in two different mouse strains and four different tumor models. It is known that TAM may inhibit T cell responses by inducing apoptosis of activated T cells via up-regulation of NO production and arginase activity. Previous studies have implicated NO, PGs, and TNF-α in macrophage-mediated T cell suppression (33–35). L-Arginine plays a central role in the normal function of the immune system. It is metabolized in macrophages by inducible NO synthase to produce NO, important in the cytotoxic mechanisms, and by arginase I and arginase II to synthesize L-ornithine and urea. Previous studies from Ochoa and colleagues (36, 37) have demonstrated that activated macrophages can modulate extracellular levels of L-arginine and alter T cell function. Human T cells stimulated and cultured in the absence of L-arginine lose the expression of the TCR ζ-chain and have an impaired proliferation and a decreased cytokine production. Very recently, they have shown up-regulation of arginase I in tumor-associated myeloid cells (38). Consistent with our observations, they have found very little arginase I activity in Gr-1^+CD11b$^+$ cells, whereas the bulk of activity was associated with Gr-1^-CD11b$^+$ macrophages (38). Bronte and colleagues (20, 30) have recently demonstrated that induction of arginase and iNOS together causes activated T lymphocytes to undergo apoptosis. In this study, we for the first time demonstrated that this process of simultaneous activation of iNOS and arginase I indeed takes place in TAM. Arginase I and iNOS are both competing for the substrate. Previous studies demonstrated that overexpression of arginase in murine macrophage cell line J774A.1 enhanced L-ornithine and putrescine production and attenuated NO production by the LPS-activated macrophages (39). This suggests that there should be some powerful signaling to simultaneously activate both these enzymes. We suggested that it is likely to be mediated by STAT family of transcription factors.

STAT is a critical component of diverse signal transduction pathway that is actively involved in cellular survival, proliferation, and differentiation. Following ligand binding to the receptor, the activated Janus family of tyrosine kinases (JAK) phosphorylates receptors on target tyrosine sites, which generates docking sites for STATs. Subsequently, recruited STATs are phosphorylated and dimerized, followed by their translocation into the nucleus, where they modulate expression of target genes. The STAT family of transcription factors consists of seven members (reviewed in Ref. 40). Previous studies implicated STAT6 as possible factor involved in immune suppression associated with myeloid cells (14, 41, 42). We have demonstrated important role of STAT3 in accumulation of Gr-1^+ IMC in cancer (13), and a number of groups demonstrated important role of STAT1 in regulation of iNOS activity (43, 44). Present study demonstrated that increased STAT6 activity in TAM was not responsible for their immunosuppressive activity. In line with our previous observation, myeloid cells in spleen of tumor-bearing mice had increased level of STAT3 activity and undetectable STAT1 activity. However, surprisingly opposite results were obtained in TAM. These cells had no detectable STAT3 activity, but increased STAT1. These data are consistent with recent observation that STAT3 inhibits transcription of the iNOS by interacting with NF-κB (45). These data are also consistent with recent observation that pharmacological inhibition of STAT3 activity in macrophages with AG490 did not affect the level of NO production (E. Sotomayor, unpublished observation). TAM isolated from STAT1 knockout mice failed to suppress T cell responses. Importantly, these cells lacked arginase I activity and had very low iNOS expression and NO production. Taken together, these data indicate that STAT1 activation in TAM may be responsible for up-regulation of iNOS and arginase I activity in these cells that in turn result in T cell suppression. At this time, it is not clear what factor or factors of the tumor microenvironment

Gr-1^+ cells were purified from naïve spleens, spleens of CT-26 tumor-bearing mice, or tumor tissues. Cells were immediately lysed, and expression of arginase I was determined by Western blotting. The same cell lysates were used to determine arginase activity, as described in *Materials and Methods*. Two experiments with the same results were performed. Positive control: cell lysates of murine hepatocytes are known to express high arginase I activity. *E*, Arginase I expression and activity in freshly derived F4/80$^+$ cells. Experiments were performed exactly as described in *E* and *F*, with the exception that instead of Gr-1^+ cells F4/80$^+$ macrophages were isolated from naive spleens, spleens, or tumor tissues of CT-26-bearing mice. Two experiments with the same results were performed. *F*, Block of arginase and iNOS activity prevents T cell inhibition mediated by TAM. F4/80$^+$ macrophage isolated from spleens or tumor tissues of C3 tumor-bearing mice was incubated at 1:4 ratio with naive syngeneic splenocytes stimulated with anti-CD3 (1 μg/ml) and anti-CD28 mAbs (5 μg/ml) in the presence of iNOS (0.5 mM L-NNMA) or arginase (0.5 mM nor-NOHA) inhibitors. Cells were cocultured in triplicates for 72 h, and incorporation of [^{3}H]thymidine was measured in triplicates. Results are shown as average $\pm$ SD.

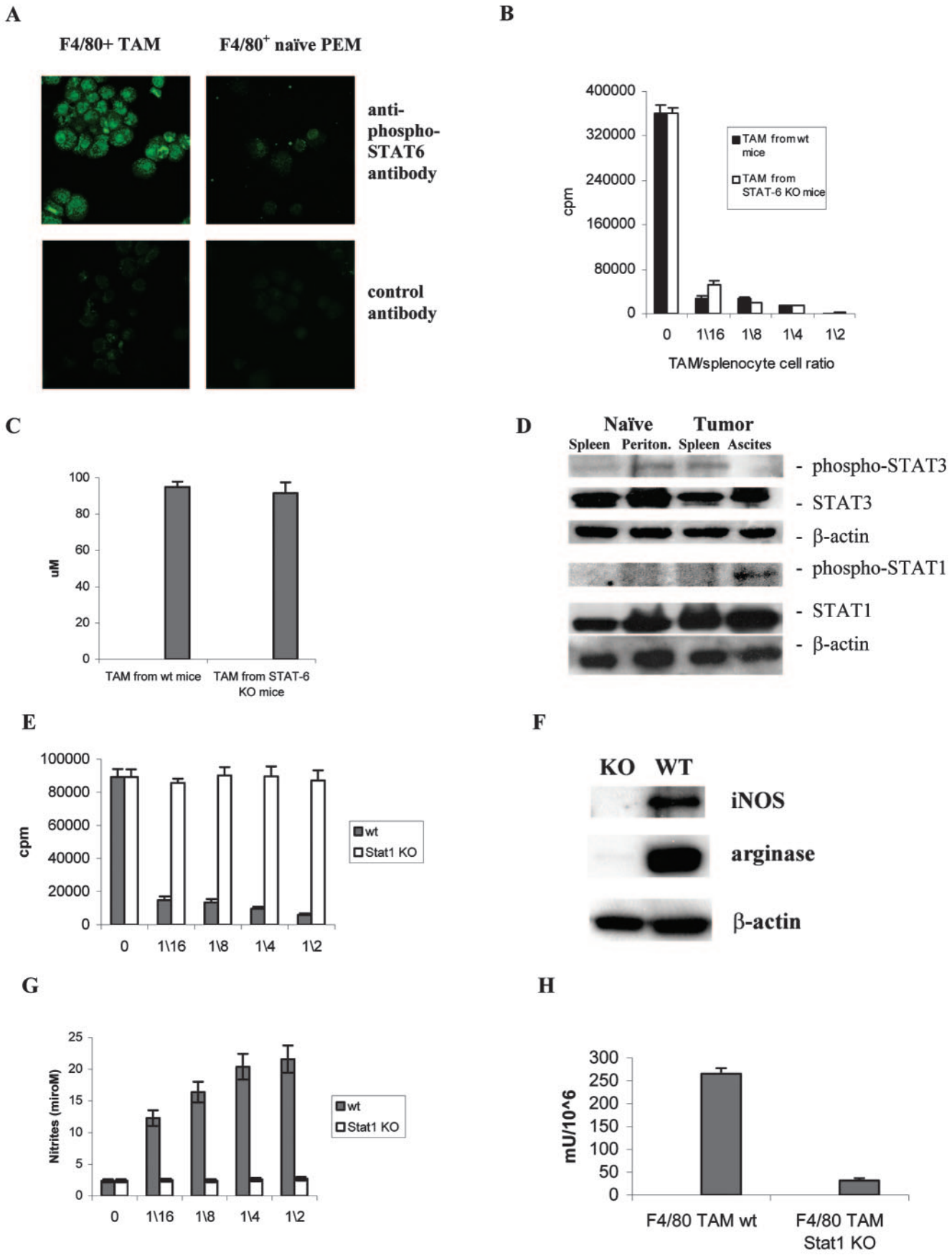

FIGURE 5. The role of STAT signaling in TAM-mediated suppression of T cell response. *A*, Intracellular expression of phospho-STAT6 in F4/80$^+$ macrophages. F4/80$^+$ macrophages were isolated from ascites of EL-4 tumor-bearing mice (TAM) or from peritoneum of naive control mice (PEM). Cells were stained with anti-phospho-STAT6 Ab, as described in *Materials and Methods*. Control Ab: cells stained with isotype control (*Figure legend continues*)

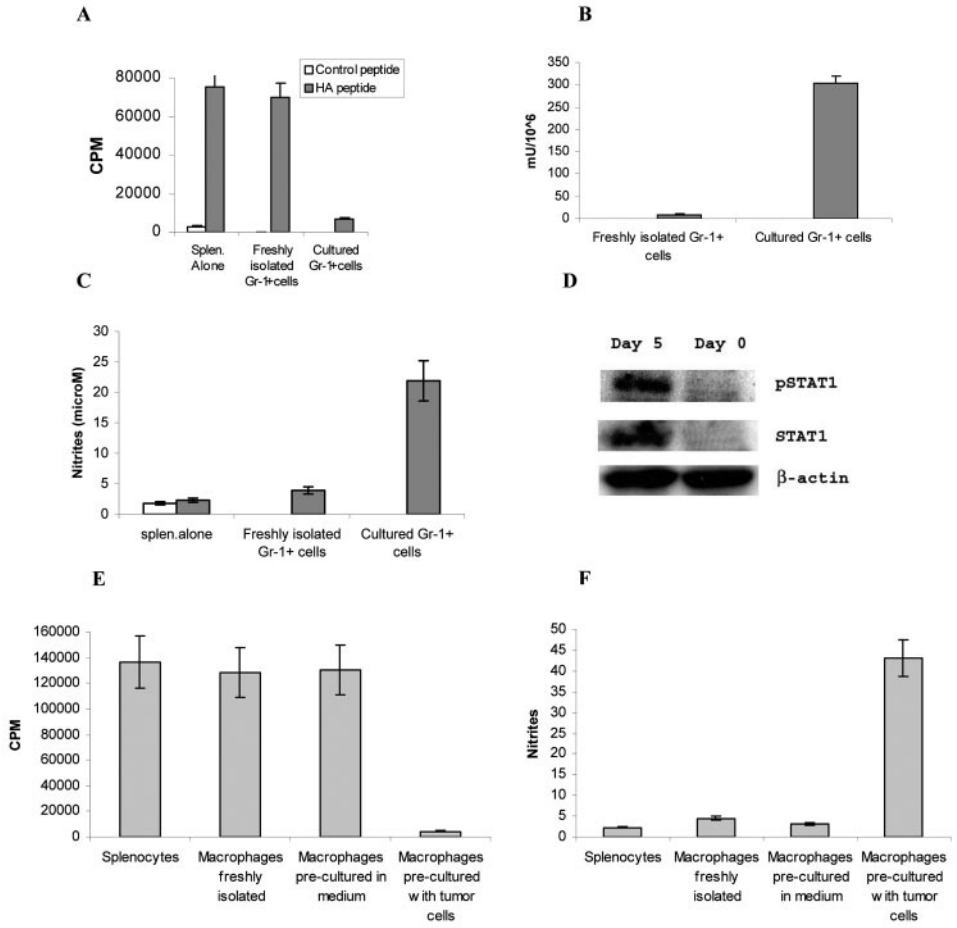

FIGURE 6. Cultured Gr-1$^+$ cells acquire suppressive activity via up-regulation of STAT1 arginase and NO. *A*, Cultured Gr-1$^+$ myeloid cells acquire T cell-inhibitory features. Gr-1$^+$ myeloid cells were isolated from spleen of CT-26 tumor-bearing BALB/c mice and either used freshly isolated or cultured in complete RPMI 1640 medium in the presence of GM-CSF (10 ng/ml). On day 5, cultured cells were collected and added at 1:4 ratio to syngeneic splenocytes from HA transgenic mice. Cells were incubated in triplicates with control or specific HA-derived peptide (10 μg/ml), and incorporation of [^{3}H]thymidine was measured 72 h later, as described above. Results are presented as average $\pm$ SD. Three experiments with the same results were performed. Arginase activity (*B*) and NO production (*C*) were measured in Gr-1$^+$ cells freshly isolated from CT-26 tumor-bearing mice or in cells generated from Gr-1$^+$ cells after 5-day culture with GM-CSF, as described above. Two experiments with the same results were performed. *D*, Whole cell lysates were prepared from Gr-1$^+$ cells freshly isolated from CT-26 tumor-bearing mice (day 0) and cells generated from Gr-1$^+$ cells after 5 days in culture with GM-CSF (day 5). The level of phospho-STAT1, total STAT1, and β-actin was evaluated using Western blotting, as described in *Materials and Methods*. Two experiments with the same results were performed. *E* and *F*, Tumor cells convert F4/80$^+$ macrophages into immune suppressive cells. F4/80$^+$ cells were isolated from spleens of EL-4 tumor-bearing mice using magnetic beads, as described above. These cells were cultured at 37°C in 24-well plates either in complete culture medium alone or with EL-4 tumor cells. Each well contained 0.5 $\times$ 10^6 F4/80$^+$ cells and 0.5 $\times$ 10^6 EL-4 cells. After 48-h culture, F4/80$^+$ cells were reisolated using magnetic beads, and cell viability was verified. F4/80$^+$ macrophages were cultured in triplicates in round-bottom 96-well plates with 2 $\times$ 10^5 splenocytes isolated from OT-1 transgenic mice in the presence of control or OT-1-specific peptide (SIINFEKL). A 1:4 ratio of macrophages/splenocytes was used. *E*, Cell proliferation was measured by incorporation of [^{3}H]thymidine after 72 h, as described above. Results are presented as average $\pm$ SD. Two experiments with the same results were performed. *F*, NO production was measured after 72 h, as described in *Materials and Methods*. Results are presented as average $\pm$ SD. Two experiments with the same results were performed.

IgG and secondary Abs. TAM derived from tumor-bearing mice display high intracellular phospho-STAT-6 expression. *B*, Immunosuppressive activity of TAM from STAT-6 knockout (KO) tumor-bearing mice was not affected. EL-4 tumor was established i.p. in control wild-type (wt) and STAT-6 (KO) mice. F4/80$^+$ cells were isolated from ascites and cultured in triplicates together with control syngeneic splenocytes stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) Abs. Cell proliferation was measured in triplicates using [^{3}H]thymidine uptake. Cumulative results of three performed experiments are shown. The level of [^{3}H]thymidine uptake in cells not stimulated with Abs was $<$1000 cpm. *C*, Arginase activity in TAM from STAT-6 KO mice was not impaired. F4/80$^+$ TAM from EL-4 tumor-bearing wild-type (wt) and STAT-6 KO mice were cultured at 1:16 ratio with syngeneic splenocytes activated with anti-CD3 and anti-CD28 Abs, as described above. NO production was measured, as described in *Materials and Methods*. *D*, Expression of STAT3 and STAT1 in F4/80$^+$ macrophages. F4/80$^+$ cells were isolated from spleens (Spleen) or peritoneum (Periton.) of naive C57BL/6 mice (Naive), or from spleen or ascites (Ascites) of EL-4 tumor-bearing mice (Tumor). Whole cell lysates were prepared and subjected to SDS-PAGE electrophoresis and Western blotting. Membranes were probed with Abs, as indicated. *E*, Experiments were performed exactly as described in *B*. F4/80$^+$ TAM from wild-type (wt) and STAT1 KO EL-4 tumor-bearing mice were used. *F*, F4/80$^+$ TAM were isolated from ascites of EL-4 tumor-bearing wild-type (WT) and STAT1 knockout (KO) mice. Whole cell lysates were prepared, and the level of arginase and iNOS was evaluated using Western blotting, as described in *Materials and Methods*. *G*, NO production by wild-type or STAT1 KO TAM was measured, as described in *C*. Two experiments with the same results were performed. *H*, Arginase activity by TAM from wild-type or STAT1 KO EL-4 tumor-bearing mice was evaluated, as described in *Materials and Methods*. Two experiments with the same results were performed.

are responsible for up-regulation of STAT1 in TAM. It is possible that interaction between TAM and tumor cells or tumor-associated fibroblasts or endothelial cells may contribute to this effect. In our experiments, tumor cells were able to convert nonsuppressive splenic F4/80$^+$ macrophage to highly immunosuppressive cells.

Disclosures

The authors have no financial conflict of interest.

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