**CD11b<sup>+</sup>/Gr-1<sup>+</sup> Myeloid Suppressor Cells Cause T Cell Dysfunction after Traumatic Stress**

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CD11b⁺/Gr-1⁺ Myeloid Suppressor Cells Cause T Cell Dysfunction after Traumatic Stress

Valeriya P. Makarenkova,* Vishal Bansal,* Benjamin M. Matta,* Lori Ann Perez, † and Juan B. Ochoa2*

T cell dysfunction that occurs after surgery or trauma is associated with a poor clinical outcome. We describe that myeloid suppressor cells expressing CD11b⁺/Gr-1⁺ markers invade the spleen after traumatic stress and suppress T cell function through the production of arginase 1. We created a consistent model of traumatic stress in C57BL/6 mice to perform this work. A significant number of CD11b⁺/Gr-1⁺ cells expressing arginase 1 accumulated in T cell zones around the germinal centers of the white pulp of the spleen within 6 h of trauma and lasted for at least 72 h. Increased arginase activity and arginase 1 expression, along with increased ³H]arginine uptake, L-arginine depletion, and L-ornithine accumulation in the culture medium, were observed exclusively in CD11b⁺/Gr-1⁺ cells after traumatic stress. Flow cytometry revealed CD11b⁺/Gr-1⁺ as a heterogeneous myeloid suppressor cell also expressing low levels of MHC class I and II, CD80, CD86, and others. When compared with controls, trauma-induced CD11b⁺/Gr-1⁺ cells significantly inhibited CD3/CD28-mediated T cell proliferation, TCR ζ-chain expression, and IL-2 production. The suppressive effects by trauma CD11b⁺/Gr-1⁺ cells were overcome with the arginase antagonist N-hydroxy-nor-L-arginine or extrasupplementation of medium with L-arginine. Poor Ag-presenting capacity of control and trauma-induced CD11b⁺/Gr-1⁺ cells was detected in allogeneic murine leukocyte reaction. This study demonstrates that CD11b⁺/Gr-1⁺ cells invade the spleen following traumatic stress and cause T cell dysfunction by an arginase-mediated mechanism, probably that of arginine depletion. Understanding the mechanism of immune suppression by these cells has important clinical implications in the treatment of immune dysfunction after trauma or surgery. *The Journal of Immunology, 2006, 176: 2085–2094.

Patients who suffer severe trauma or have undergone major surgery (from here on called traumatic stress) frequently develop sepsis. Despite significant progress, sepsis still occurs after traumatic stress, being an important contributing factor in up to 14% of the in-hospital deaths of trauma patients (1). Impaired host defenses, frequently observed as T cell dysfunction, are central to the development of infections after traumatic stress (2, 3). Despite its known importance, strategies aimed at restoring immune function are limited, due in part to poor understanding of its causes.

T cell dysfunction after traumatic stress is characterized by decreased T cell proliferation, production of cytokines (IL-2 and IFN-γ), and a decreased expression of the TCR due to loss of the ζ-chain peptide (4, 5). Withholding the amino acid arginine from the culture medium can partially reproduce these changes (6–8). We have described previously that arginine levels are dramatically decreased after traumatic stress (9). Arginine levels recover only with its supplementation in the diet at supraphysiologic quantities (10). Not surprisingly, the use of arginine is now shown to restore T cell function after surgery and to decrease infection rates in these patients (11).

Expression of arginase 1 (ARG1),3 an enzyme that catabolizes arginine to ornithine and urea, is increased in peripheral mononuclear cells in humans and in splenic cells in mice after traumatic stress (9, 12). ARG1 expressed in myeloid cells in the immune tissues is associated with increased destruction of arginine (13, 14). Thus, we hypothesized that arginine depletion by myeloid cells expressing ARG1 could explain at least some elements of T cell dysfunction after traumatic stress.

We report here that ARG1 expressed in immune tissues after trauma is observed exclusively in myeloid cells. Soon after trauma, an “invasion” of CD11b⁺/Gr-1⁺ cells is observed in splenic tissues. These cells express very high levels of ARG1 and arginase activity and exhibit increased arginine uptake (as measured by ³H]arginine incorporation in vitro) as well as increased L-arginine depletion from the culture medium. In trauma, CD11b⁺/Gr-1⁺ co-localize with T cells around the germinal centers of the white pulp of the spleen. Trauma-induced CD11b⁺/Gr-1⁺ cells also express MHC class I molecules but express low MHC class II, CD80, CD86, CD34, CD16/32, F4/80, and CD31. CD11b⁺/Gr-1⁺/ARG1⁺ cells placed in the upper chamber of a Transwell and cocultured in vitro with CD3/CD28-stimulated naïve (nontrauma) T cells severely impair T cell proliferation, production of IL-2, and decrease TCR ζ-chain. These changes can be reversed through pharmacologic blockade of ARG1 by N-hydroxy-nor-L-arginine (nor-NOHA) or by extrasupplementation of medium with 1.2 mM L-arginine. CD11b⁺/Gr-1⁺ cells poorly stimulate proliferation of naïve allogeneic T cells.

To our knowledge, this is the first report demonstrating that ARG1-expressing myeloid cells can be a cause of T cell dysfunction after traumatic stress. Furthermore, our work is important in

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3 Abbreviations used in this paper: ARG1, arginase 1; nor-NOHA, N-hydroxy-nor-L-arginine; DC, dendritic cell.
that it demonstrates an otherwise easy and reproducible murine model of traumatic stress that will allow us to better understand immune dysfunction in this disease process.

**Materials and Methods**

**Mice**

Male C57BL/6 mice were obtained from Charles River Laboratories. Four mice per cage were housed and maintained under a 12-h light/dark cycle at a temperature of 20–22°C in a pathogen-free facility. Food and water were available ad libitum. The mice were allowed an acclimation period of 2 wk and used at 6–8 wk of age.

**Mouse traumatic stress model**

The experimental protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and Division of Laboratory Animal Research. Mice were randomized into two groups: a control group, receiving anesthesia alone, and an experimental group of mice undergoing traumatic stress. After administering the anesthetic (Nembutal, 50 mg/kg; Abbott Laboratories), a midline laparotomy incision was made. The intra-abdominal contents were teased for 15 s, taking care not to create injury to the viscera. The incision was closed in two layers, and animals were maintained under a heat lamp until fully recovered from the anesthetic. Animals were sacrificed at 6, 12, 24, 48, or 72 h following laparotomy, and a spleenectomy was performed for cell harvest.

**Isolation of cells**

A single-cell suspension was prepared from the spleens of control and mice subjected to traumatic stress. Erythrocytes were depleted using RBC lysing buffer (Sigma-Aldrich), and splenocytes were washed in MACS buffer (1× PBS supplemented with 2 mM EDTA and 0.5% BSA). CD11b+, CD11c+, NK, and T cells were isolated using corresponding MACS magnetic micro-beads (Miltenyi Biotec). The purity of cells separation ranged between 89 and 95%.

**Protein extracts**

Total cell protein extracts were prepared by lysing washed cell pellets in 10 μl of lysing buffer/10^6 cells (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM NaVO₃, and 0.5% Triton X-100) containing 50 μg/ml aprotinin, 50 μg/ml leupeptin (Roche), 100 μg/ml trypsin-chymotrypsin inhibitor, and 2 mM PMSF (Sigma-Aldrich). Cell pellets were incubated on ice for 7 min with RBC lysing buffer and then centrifuged at 3,000 × g (12,000 rpm) for 15 min at 4°C. Total protein concentration was determined by the Bradford method (15). Prepared protein lysates were aliquoted and stored at −80°C until used for Western blot analysis or arginase activity assay.

**Western blot analysis of ARG1**

Total protein from CD11b⁺, CD11c⁺, NK, and T cells were separated by 12.5% SDS-acrylamide gel for 45 min at 200 V and electrophoretically transferred to a nitrocellulose membrane (Invitrogen Life Technologies). The protein extract from the mouse liver served as a positive control. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST (2.42 g of Tris, 8 g of NaCl, and 0.5 ml of Tween 20 in 1 l of MQO/H₂O) at 4°C overnight. The membrane was incubated with chicken IgG1 anti-mouse ARG1 primary Ab (donated by Dr. S. Morris, Jr., University of Pittsburgh, Pittsburgh, PA), diluted at 1/50,000 in 1% nonfat dried milk, 5% BSA in PBS, for 1 h at room temperature (16). The membrane was washed and the secondary peroxidase-conjugated rabbit anti-chicken IgG Ab (Jackson ImmunoResearch Laboratories) was diluted 1/5,000 and applied for 1 h at room temperature. As an internal control for total protein concentration, the membrane was stripped and washed, and goat anti-mouse β-actin Ab (Santa Cruz Biotechnology) was diluted 1/500 and applied for 1 h at room temperature. After washing, the membrane was incubated with goat anti-donkey IgG Ab (Santa Cruz Biotechnology), diluted 1/5000, and applied for 1 h at room temperature. For both ARG1 and β-actin, immuneoreactive protein was visualized using enhanced lumino reagent and oxidizing reagent (Pierce). Prestained Kaleidoscope molecular mass marker (Bio-Rad) was used to determine the m.w. of immunoreactive bands.

**Arginase activity assay**

Arginase activity in protein lysates of splenocyte subsets was measured from the conversion of l-arginine to l-ornithine according to the technique described by Kornarska and Tomaszewski (17). Available arginase was activated by the addition of 10 mmol/L MnCl₂ (25 μl) to cell protein lysate (25 μl) and incubation at 55°C for 20 min. Carbonate buffer (150 μl, 100 mmol/L, pH 10) was then added along with 100 mmol/L l-arginine (50 μl) to initiate the reaction, and incubated at 37°C. Arginase activity was stopped after 10 min by adding glacial acetic acid (750 μl). Nihinhydrin solution (250 μl) (2.5 g of ninhydrin, 40 ml of 6M phosphoric acid, and 60 ml of glacial acetic acid) was added, and the samples and standards were boiled at 90–100°C for 1 h. Standards were created by using known amounts of l-ornithine from 8 to 250 nmol, and all reagents were added to standards as a control. Standards were cooled and colorimetric reaction measured with a spectrophotometer at 515 nm (Spectramax 340; Molecular Devices). Arginase assay was linear with time and is presented as nanomoles of ornithine per minute per milligram of protein.

**HPLC determination of l-arginine and l-ornithine in culture medium**

The l-arginine and l-ornithine concentration in tissue culture medium was measured by HPLC with electron capture detection using an ESA-Cou lArray Model 540 (ESA) with an 80 × 3.2 column with 120 A pore size. Briefly, supernatants were deproteinized in methanol. After centrifugation at 6000 × g for 10 min at 4°C, the supernatant was derivatized with 0.2 M o-phthalaldehyde containing 7 mM β-ME. Fifty microliters of the sample were injected into the column. Standards of l-arginine and l-ornithine in methanol were run with each experiment.

**Flow cytometry analysis**

Harvested cells were washed in FACS medium (1× PBS supplemented with 0.1% BSA and 0.1% NaN₃) and stained with appropriately diluted Abs directly conjugated with FITC or PE according to the standard procedure, and followed by fixation in 2% paraformaldehyde. Abs used for FACS staining were the following: FITC-labeled anti-mouse CD11b, NK1.1, CD14, CD31, MHC class I, CD40, B220, CD40, CD80, CD36, CD34, CD131, CD13, CD68, F4/80, CD16/32, DEC-205, and PE-labeled GR1, CD6, CD11c, CD16, CD19, CD3, MHC class II, CD8α (BD Pharmingen). All staining procedures were conducted on ice. Phagocytosis was measured using a FACScan flow cytometer (BD Biosciences), and data analysis was performed using CellQuest software (BD Biosciences). Cell sorting was performed on a MoFlo cell sorter (DakoCytomation).

**Morphological analysis**

One hundred microliters of control and traumatic stress-induced CD11b⁺/Gr-1⁺ cell suspensions was loaded into a cytopsin chamber and spun for 5 min at 500 rpm. Slides were air-dried at room temperature for 5 min and stained in a three-step procedure using the LeukoStat stain (Fisher Scientific).

**T cell proliferation assay**

A total of 1 × 10⁵ to 1 × 10⁶ CD11b⁺/Gr-1⁺ cells isolated from control or 24-h traumatic stress mice were cultured in the upper chamber of a Transwell system at 37°C in a humidified 5% CO₂ incubator. The concentration of L-arginine and L-ornithine in tissue culture medium was measured by HPLC with electron capture detection using an ESA-CoulArray Model 540 (ESA) with an 80 × 3.2 column using 120 A pore size. Briefly, supernatants were deproteinized in methanol. After centrifugation at 6000 × g for 10 min at 4°C, the supernatant was derivatized with 0.2 M o-phthalaldehyde containing 7 mM β-ME. Fifty microliters of the sample were injected into the column. Standards of l-arginine and l-ornithine in methanol were run with each experiment.

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**IL-2 production (ELISA)**

To evaluate the effect of CD11b⁺/Gr-1⁺ cells on IL-2 production by Th1 cells, the level of IL-2 in cell-free supernatants was measured by ELISA.
using the Mouse IL-2 Immunossay Quantikine kit (R&D Systems). T cells (10^5 T cells per 0.5 ml of medium per well) were placed in the bottom chamber of a Transwell (24-well plates) and stimulated with 1 μg/ml plate-bound anti-CD3 Ab and 1 μg/ml soluble anti-CD28 Ab (BD Pharmingen). Control or traumatic stress-induced CD11b^+Gr-1^+ cells (1 × 10^6) were placed in 0.5 ml of medium in the upper chamber. Cell-free supernatants were collected in 12 h and stored at ~80°C. Ten micromoles of nor-NOHA (Calbiochem) was added to block arginase. Extrapolimation of the medium with 1.2 mM t-arginine also was used in selected experiments when appropriate. The sensitivity of the ELISA was 6 pg/ml.

**Measurement of TCR ζ-chain**

Naive T cells isolated from control mouse spleen (10^6 T cells per ml of medium per well) were placed in the bottom chamber of a Transwell (24-well plates) and 10^6 control or traumatic stress-induced CD11b^+Gr-1^+ cells were placed in 1 ml of medium in the upper chamber. Cells were cocultured for 24 h in RPMI 1640 medium containing 150 μM of t-arginine (physiologic level), 1 μg/ml plate-bound anti-CD3 Ab, and 1 μg/ml soluble anti-CD28 Ab (BD Pharmingen) at 37°C in a humidified 5% CO2 atmosphere. Nontreated T cells cultured without CD11b^+Gr-1^+ cells served as a control. Intracellular TCR ζ-chain expression in T cells was measured by flow cytometry using anti-mouse-CD3ζ-PE Ab (Santa Cruz Biotechnology). Rat-IgG2-PE Ab (BD Pharmingen) was used as isotype control. Ten micromoles of nor-NOHA were added to block arginase. Medium containing 1.2 mM t-arginine was used in additional experiments when appropriate.

**Mixed lymphocyte reaction**

To study the Ag-presenting capacity of immature myeloid CD11b^+Gr-1^-cells, MLR was performed using naive allogeneic T cell as target. Allogeneic BALB/c T cells (H-2K^b^) were isolated from the spleen by purification on a nylon wool column. Control or traumatic stress-induced CD11b^+Gr-1^- cells were irradiated (3000 rad) and used as stimulator cells in the MLR assay. Dendritic cells (DCs) were isolated from control spleens using CD11c MACS beads and served as a control. Stimulator cells (10^2–10^5) were added to 2 × 10^5 T cells per well in triplicate in 96-well round-bottom culture plates (Falcon) and incubated for 72 h in 200 μl of 150 μM t-arginine medium at 37°C in a humidified 5% CO2 atmosphere. T cell proliferation was tested by [H]thymidine incorporation.

**Immunohistochemical staining**

Immunohistochemistry was performed on mice sacrificed 6, 12, 24, or 48 h after laparotomy, and intact mice were served as controls. Spleens were frozen in the presence of OCT (Sakura) and stored until used at ~70°C. Cryostat sections (4 μm) were used for immunohistochemical evaluation using rat IgG2a, anti-mouse Ly-6G (Gr-1) mAb diluted 1/500 (BD Pharmingen) and goat polyclonal anti-mouse integrin αM (CD11b) diluted 1/50 (Santa Cruz Biotechnology). Nonspecific binding was blocked with Super Block (ScyTek Laboratories) for 8 min at room temperature, and sections were washed three times in 1% Tween 20/PBS buffer for 3 min. Sections were then incubated overnight in a humidified chamber at 4°C with primary rat goat polyclonal anti-mouse Ly-6G (Gr-1) mAb (1.25 μg/ml). After three washes in Tween 20/PBS buffer for 5 min, the sections were incubated for 30 min with the alkaline phosphatase-conjugated appropriated secondary Ab 1/200 or 3 μg/ml (Jackson ImmunoResearch Laboratories). The color reaction was developed for 8 min using alkaline phosphatase substrate kit III (Vector Laboratories). To stain the tissue for CD11b, sections were incubated over-night at 4°C with the goat polyclonal anti-mouse integrin αM Ab (4 μg/ml) followed by quenching in 0.3% H2O2 solution for 10 min and blocking by avidin-biotin kit (Vector Laboratories). After washing in Tween 20/PBS buffer, the sections were incubated with the secondary biotinylated anti-goat Ab at dilution 1/200. Then, the sections were washed and incubated with avidin-biotin-HRP macromolecule complex (Vectastain, Elite ABC kit; Vector Laboratories), and CD11b-positive cells were visualized for peroxidase with the DAB substrate kit (Vector Laboratories). After the final wash in Tween 20/PBS buffer, all the sections were air-dried, dehydrated, cleared, coverslipped, and studied under the microscope with bright field illumination. Control slides included an irrelevant isotype-matched Ab in place of the primary Ab: purified rat IgG1, isotype standard at a 1/500 dilution (BD Pharmingen), goat IgG1, isotype standard at a 1/500 dilution (BD Pharmingen), or PBS in place of the primary Ab to evaluate nonspecific staining. Control sections generally demonstrated negligible levels of endogenous background. Images were acquired from the double-labeled sections using a confocal laser-scanning microscope (Olympus) at magnification ×20.

**Medium and reagents**

RPMI 1640 medium (custom t-arginine free), t-arginine, t-glutamine, gentamicin, HEPES, FBS, nonessential amino acids, sodium pyruvate, and rabbit complement were purchased from Invitrogen Life Technologies; RBC lysing buffer, indomethacin, paraformaldehyde, and 2-ME were obtained from Sigma-Aldrich.

**Statistical analysis**

One-way ANOVA (SigmaStat software; Jandel) was performed to evaluate the significance of differences between the experimental groups. For a single comparison of two groups, Student’s t test was used after evaluation of normality. For all analyses, a probability level of 0.05 was considered significant. Data are presented as the mean ± SEM. All experiments were performed at least three times.

**Results**

**ARG1 expression after trauma is observed exclusively in CD11b^+ cells**

We have reported previously an up-regulation of arginase activity in murine splenic immune cells within 24 h of traumatic stress, although the exact cell type expressing ARG1 was unknown (12, 18). To answer this question, we measured arginase activity in different cell subpopulations isolated through positive and/or negative selection using immunomagnetic beads for CD11b^+, DCs (CD11c^+CD11b^-), NK (DX5^+CD11b^-), and T cells (CD3^+). Fig. 1 demonstrates that minimal baseline arginase activity was detected in all splenocytes. However, we detected an increase in arginase activity in CD11b^+ cells, which was 6-fold greater than that of DCs and 40-fold higher than in NK and T cells (p < 0.05). In fact, as can be seen in Fig. 1, arginase activity was 13-fold higher in splenic CD11b^+ cells obtained from animals after traumatic stress, compared with control CD11b^- cells (412 ± 49 nmol/min/mg vs 17 ± 12 nmol/min/mg; p < 0.05). Thus, traumatic stress specifically induces arginase activity in CD11b^+ cells.

We further determined that increased arginase activity was due to the induction of ARG1 protein expression as determined by Western blot (Fig. 2). ARG1 expression was observed exclusively in splenic CD11b^+ cells isolated from mice undergoing laparotomy. This is in accordance with previous results (Fig. 1). These results demonstrate that, between all tested splenocytes, only CD11b^+ cells express ARG1 after traumatic stress. This is in contrast with CD11b^- cells from control spleens, which do not express significant amount of ARG1. Thus, traumatic stress induces ARG1 in a specific subpopulation of myeloid cells. Arginase activity is proportional to ARG1 protein expression, supporting prior observations that arginase activity is transcriptionally controlled (16).
ARG1 expression is observed only in CD11b^+/Gr-1^+ splenocytes after traumatic stress

Increased arginase activity has been described in immature myeloid cells expressing Gr-1 markers in murine models of cancer (19). We performed the following experiments to determine whether CD11b^+ cells expressing ARG1 after traumatic stress also were immature cells. Arginase activity was measured in CD11b^+/Gr-1^+ and CD11b^+/Gr-1^- cell subpopulations sorted by flow cytometry from CD11b^+ splenocytes. Increased arginase activity (113 ± 9 nmol/min/mg) was observed only in CD11b^+ /Gr-1^- cells harvested from animals subjected to traumatic stress, compared with control CD11b^+/Gr-1^- cells or CD11b^+ /Gr-1^- cells harvested from mice subjected to traumatic stress (Fig. 3).

These results reveal that, among all tested splenocyte subpopulations, only double-positive CD11b^+ /Gr-1^- cells exhibited an induction in ARG1 protein expression, and then only after traumatic stress.

ARG1 activity in CD11b^+ /Gr-1^- splenocytes increases linearly with time up to 72 h

To examine the changes of arginase activity across time after traumatic stress, we examined arginase activity in CD11b^+ /Gr-1^- cells at time intervals of 6, 12, 24, 48, and 72 h after laparotomy. CD11b^+ /Gr-1^- cells isolated from mice undergoing anesthesia alone were used as controls.

Fig. 4A demonstrates that arginase activity in trauma-induced immature CD11b^+ /Gr-1^- cells increased linearly with time. An early increase in CD11b^+ /Gr-1^- arginase activity occurred in the first 6 h, compared with control group (50 ± 14 nmol/min/mg vs 15 ± 7 nmol/min/mg). A significant increase in mean arginase activity was maintained throughout 24 h after traumatic stress (396 ± 55 nmol/min/mg) and increased to 732 ± 87 nmol/min/mg 72 h later. Fig. 4B demonstrates that there also was significant, linear time-dependent increase in ARG1 protein expression in CD11b^+ /Gr-1^- cells induced by traumatic stress, as measured by Western blot. Therefore, we suggest that traumatic stress induces...
an early and long-lasting arginase activity and ARG1 expression in myeloid splenic CD11b$^+$/Gr-1$^+$ cells.

**l-Arginine uptake in trauma-induced CD11b$^+$/Gr-1$^+$ cells is increased, compared with control CD11b$^+$/Gr-1$^+$ cells**

Induction of ARG1 with the use of IL-4 and IL-13 in mouse peritoneal macrophages is associated with increased $[^3]H$arginine uptake and incorporation. This also is associated with increased cat-ionic amino acid transporter expression. To determine whether trauma-induced CD11b$^+$/Gr-1$^+$ cells exhibited increased arginine incorporation, we measured $[^3]H$arginine uptake and incorporation in control and trauma CD11b$^+$/Gr-1$^+$ cells. The results show that CD11b$^+$/Gr-1$^+$ cells harvested from mice 24 h after laparotomy had a significantly increased $[^3]H$arginine uptake and incorporation, which was already present at 10 min but more clearly evident after 30 and 60 min of incubation (Fig. 5). For example, after 60 min in culture, the uptake of L-arginine by traumatic stress-induced CD11b$^+$/Gr-1$^+$ cells was two times higher than in the same cells isolated from control mice ($129,365 \pm 3,786$ and $69,219 \pm 4,191$ cpm, respectively; $p < 0.05$). This finding confirms that traumatic stress induces an increase in arginine uptake by CD11b$^+$/Gr-1$^+$ cells expressing ARG1.

**CD11b$^+$/Gr-1$^+$ cells induced by traumatic stress deplete l-arginine from the culture medium by high arginase activity**

We then determined whether CD11b$^+$/Gr-1$^+$ myeloid cells were able to deplete arginine. To answer this question, we measured l-arginine concentration in culture medium. Control and traumatic stress-induced CD11b$^+$/Gr-1$^+$ cells were cultured for 48 h in 150 μM l-arginine medium, and concentrations of l-arginine and l-ornithine were measured in cell-free supernatants by HPLC analysis. As shown in Fig. 6A, 2 x 10$^6$ CD11b$^+$/Gr-1$^+$ cells induced by traumatic stress deplete arginine from 150 μM down to 37 ± 7 μM l-arginine, whereas the control cells exhibit negligible use of arginine. Importantly, the concentration of l-ornithine, a product of metabolism of l-arginine by ARG1, increased in culture medium of CD11b$^+$/Gr-1$^+$ trauma cells, corresponding to the decrease of l-arginine (Fig. 6B). Thus, our results are in agreement with other findings that prove that traumatic stress-induced CD11b$^+$/Gr-1$^+$ myeloid cells are able to deplete local l-arginine.

**Localization of CD11b$^+$/Gr-1$^+$ cells in mouse spleen detected by immunohistochemistry**

Several investigators, including us, have suggested that ARG1 regulates T cell function through arginine depletion in local microenvironments (6, 13, 14). For this hypothesis to be true, CD11b$^+$/Gr-1$^+$ cells should localize at or near T cell-rich zones of the spleen. To test this hypothesis, we performed serial immunohistochemistry sections of splenic tissues after traumatic stress.

Under resting conditions (controls), few Gr-1$^+$ cells were observed in splenic tissues and were mainly localized to the red pulp (Fig. 7). A dramatic increase in Gr-1$^+$ cells was seen within 6 and especially 12 h after traumatic stress and were tightly localized to the marginal zones and periarteriolar lymphatic sheaths of the spleen. By 24 and 48 h, the number of cells expressing Gr-1$^+$
by 12 h (Fig. 9). Thus, at one point, one CD11b progenitor CD131 marker was not detected in CD11b/H11001 cells. Therefore, one point, one CD11b/H11001 marker was not detected in CD11b/H11001 cells.

markers appeared to slowly decrease and migrate toward the red pulp of the spleen. There was a virtual absence of Gr-1+ cells in the germinal centers (B cell-rich zones). These data render support to the hypothesis that trauma-induced CD11b+/Gr-1+ cells could be playing a regulatory role on T cell function.

Morphological characterization of CD11b+/Gr-1+ cells

As shown in Fig. 8, CD11b+/Gr-1+ cells represented a mixture of myeloid cells in varying stages of differentiation. Trauma-induced immature CD11b+/Gr-1+ cells exhibited a neutrophil-like morphology, suggesting a granulopoietic response. This type of morphology appears similar to that described previously for immature myeloid cells in murine models of cancer and halogenated aromatic hydrocarbon toxicity (20). The morphological characteristics of the cells were similar in CD11b+/Gr-1+ cells from control mice and mice subjected to traumatic stress.

CD11b+/Gr-1+ splenocytes are myeloid precursor cells by phenotype

We then further determined the phenotypical characteristics of trauma-induced CD11b+/Gr-1+ cells. CD11b+/Gr-1+ cells harvested from controls were compared with CD11b+/Gr-1+ cells harvested from mice undergoing traumatic stress. We also compared changes in phenotypical characteristics of trauma-induced CD11b+/Gr-1+ cells across time.

There was a significant accumulation of CD11b+/Gr-1+ cells after traumatic stress, increasing from 2 ± 1% of all splenocytes at baseline to 7.8% of cells by 6 h and reaching a peak of 15 ± 4% by 12 h (Fig. 9). Thus, at one point, one CD11b+/Gr-1+ cells constituted one of seven splenic cells.

We then studied the expression of several cell surface molecules, comparing control and trauma-induced CD11b+/Gr-1+ cells. Neither control nor trauma-induced CD11b+/Gr-1+ cells significantly expressed markers of lymphocytes or mature macrophages, DCs, granulocytes, or NK cells such as CD3, CD19, B220, CD8α, NK1.1, CD14, CD11c, CD40, DEC-205, CD36, CD68, CD13, or CD31. Trauma-induced CD11b+/Gr-1+ cells expressed molecules of MHC class I (89 ± 7%), MHC class II (33 ± 9%), and myeloid hematopoietic precursor marker CD34 (38 ± 5%) (Table I). These cells also were CD16/32 positive (82 ± 5%) and express low levels of F4/80 marker (27 ± 9%). The myeloid cell progenitor CD131 marker was not detected in CD11b+/Gr-1+ cells harvested from controls or in mice subjected to traumatic stress. Other markers studied were inconsistently expressed and included costimulatory molecules CD80 (17 ± 7%), CD86 (23 ± 9%).

Thus, these data demonstrate that trauma-induced CD11b+/Gr-1+ cells are closely related phenotypically to control CD11b+/Gr-1+ cells. CD11b+/Gr-1+ cells induced by traumatic stress are not lymphoid cells because they do not express any significant levels of lymphoid markers (Table I). We conclude that trauma-induced CD11b+/Gr-1+ cells are a heterogeneous population of immature myeloid precursor cells in as much as they express CD34, CD16/32, F4/80, CD80, and CD86 markers. Trauma-induced CD11b+/Gr-1+ cells do not express markers of mature myeloid cells such as DEC 205, NK 1.1, CD36, CD13, and CD68. Interestingly, control CD11b+/Gr-1+ cells that are phenotypically related to trauma-induced CD11b+/Gr-1+ cells do not express ARG1. To our knowledge, these CD11b+/Gr-1+ cells have not been described previously in models of traumatic stress.

CD11b+/Gr-1+ cells suppress T cell proliferation

We performed cocultures of naive T cells and CD11b+/Gr-1+ cells obtained from animals subjected to traumatic stress or controls using Transwell chambers to determine the inhibitory potential of trauma-induced CD11b+/Gr-1+ cells. T cells from control syngeneic mice stimulated with anti-CD3 and anti-CD28 Abs and cocultured in the presence of trauma-induced CD11b+/Gr-1+ cells

FIGURE 8. Morphology of CD11b+/Gr-1+ cells isolated from the spleen of mice undergoing laparotomy (traumatic stress) or anesthesia only (control). CD11b+/Gr-1+ cells were enriched from the spleen (see Materials and Methods, purity = 98%). Cytospin slides were prepared and stained using the LeukoStat Stain kit. Cells were identified as a mixture of mature neutrophils with characteristic lobular-shaped nuclei, as well as immature myeloid cells with a ring-shaped nucleus. Magnification ×100. CD11b+/Gr-1+ cells morphologically show ringed-shaped nuclei with lobulations.

FIGURE 9. Coexpression of Gr-1 and CD11b markers by splenocytes from control and trauma spleen. At different time points after surgical stress, splenocytes were harvested and stained with anti-CD11b (FITC) and anti-Gr-1 (PE) Abs and examined by FACS. At time 0, there is a paucity of CD11b+/Gr-1+ cells (2%). At time intervals of 6, 12, 24, 48, and 72 h, the percentage of CD11b+/Gr-1+ cells increase to 8, 15, 5, 12, and 11%, respectively (p < 0.05).
exhibited a significant decrease in proliferation as measured by \(^{3}\text{H}\)thymidine incorporation, compared with cells cocultured with control CD11b\(^+\)/Gr-1\(^+\) cells or cultured alone (Fig. 10A). The most significant suppressive effect of trauma-induced CD11b\(^+\)/Gr-1\(^+\) cells on T cell proliferation was observed at 1:16 effector:target (E:T) ratio with a two-fold inhibition of T cell proliferation (30,944 ± 3443 for controls vs 13,078 ± 794 cpm for trauma; \(p < 0.05\)). The arginase antagonist nor-NOHA restored T cell proliferation caused by trauma-induced CD11b\(^+\)/Gr-1\(^+\) cells (Fig. 10B). Equally, supplementation of culture medium with an additional 1.2 mM L-arginine abrogated the suppressive effect of CD11b\(^+\)/Gr-1\(^+\) cells on T cell proliferation (data not shown).

Taken together, these data demonstrate that trauma-induced CD11b\(^+\)/Gr-1\(^+\) cells are capable of exerting a T cell suppressive effect, which is mediated by ARG1.

Trauma-induced CD11b\(^+\)/Gr-1\(^+\) cells suppress IL-2 production by T cells

IL-2 is a well-studied cytokine that is essential for T cell activation. Decreased IL-2 production is characteristic of traumatic stress. To determine whether CD11b\(^+\)/Gr-1\(^+\) cells isolated from mice subjected to traumatic stress were suppressing IL-2 production, we measured IL-2 accumulation in culture medium of T cell:CD11b\(^+\)/Gr-1\(^+\) cell cocultures. Appropriate controls were performed using CD11b\(^+\)/Gr-1\(^+\) cells harvested from nontraumatized mice.
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mice. T cells (obtained from nontraumatized mice) were stimulated with anti-CD3 and anti-CD28 Abs and cocultured with CD11b+/Gr-1+ cells harvested after 24 h from control mice and mice subjected to traumatic stress. CD11b+/Gr-1+ cells obtained from mice undergoing laparotomy inhibited TCR \( \zeta \)-chain expression in 4-fold \((p < 0.05)\). The use of nor-NOHA or adding of 1.2 mM L-arginine was associated with restoration of TCR \( \zeta \)-chain expression. No effects of control CD11b+/Gr-1+ cells on \( \zeta \)-chain expression by T cells were observed. Data represent one of three independent experiments.

**FIGURE 12.** Traumatic stress-induced CD11b+/Gr-1+ cells suppress the TCR \( \zeta \)-chain expression by T cells. Naive T cells were stimulated with anti-CD3 plus anti-CD28 Abs and cocultured with CD11b+/Gr-1+ cells harvested after 24 h from control mice and mice subjected to traumatic stress. CD11b+/Gr-1+ cells obtained from mice undergoing laparotomy inhibited TCR \( \zeta \)-chain expression in 4-fold \((p < 0.05)\). The use of nor-NOHA or adding of 1.2 mM L-arginine was associated with restoration of TCR \( \zeta \)-chain expression. No effects of control CD11b+/Gr-1+ cells on \( \zeta \)-chain expression by T cells were observed. Data represent one of three independent experiments.

**FIGURE 13.** Control and trauma CD11b+/Gr-1+ cells poorly stimulate naive allogeneic T cell proliferation. 10^5–10^6 control or trauma-induced CD11b+/Gr-1+ cells (effectors) were added to 2 x 10^5 naive allogeneic T cells per well in triplicate in 96-well round-bottom culture plates and incubated for 96 h in 200 \( \mu \)l of 150 \( \mu \)M L-arginine medium at 37°C in a humidified 5% CO_2 atmosphere. T cell proliferation was measured in triplicates by \( ^{3}H \) thymidine incorporation after 16-h pulse and expressed in cpm. Results are given as mean ± SEM from two independent experiments. At E:T ratios 1:1 and 1:4, both control and traumatic stress-induced CD11b+/Gr-1+ cells stimulate T cell proliferation, although not as strongly as DCs. Importantly, control CD11b+/Gr-1+ cells at 1:1 ratios are better at stimulating T cell proliferation compared to trauma-induced CD11b+/Gr-1+ cells.

**CD11b+/Gr-1+ cells slightly stimulate proliferation of allogeneic naive T cells**

We then investigated the potential Ag-presenting capacity of CD11b+/Gr-1+ cells. To answer this question, we performed a MLR using naive allogeneic T cells as targets. DCs were used as positive controls. Pilot experiments demonstrated that CD11b+/Gr-1+ cells slightly stimulate naive T cells proliferation and this effect is ratio dependent (Fig. 13). The highest T cell proliferation (29208 ± 1355 and 26422 ± 1325 cpm) was observed at 1:1 and 1:4 E:T ratios, respectively, and then linearly decreased down to 1:64 E:T ratio. No significant stimulatory effect was observed at lower E:T ratios. This finding suggests that immature myeloid suppressor CD11b+/Gr-1+ cells can stimulate naive T cells through the expression of MHC class I and II molecules, CD80, and CD86. However, CD11b+/Gr-1+ cells are three times less potent than DCs.
Discussion
T cell dysfunction after trauma appears to be the result of T cell-monocyte interactions being responsible for increased susceptibility to infections (21, 22). T cell dysfunction is characterized by decreased production of IL-2, IFN-γ, and loss of TCR β-chain expression (5, 23, 24). Preventing or reversing immune dysfunction after trauma should result in better patient outcomes and decreased cost.

Trauma is associated with a significant reduction in circulating arginine that reflects a well-known state of arginine deficiency (9, 25). The mechanisms and biological consequences of arginine deficiency have been poorly understood. In an effort to gain further understanding of the changes in arginine metabolism that occur after trauma, our group has developed a mouse model of moderate surgical trauma, whose alterations closely mimic those observed in human surgical patients and trauma victims.

Increased destruction of arginine by the enzyme ARG1 has been recently described as a mechanism of T cell regulation in diseases such as cancer (26). ARG1 is not expressed in immune tissues under resting conditions, although it is induced within hours of a physical injury. Several years ago, our group detected increased arginase activity and ARG1 expression in human peripheral mononuclear cells after trauma or surgery and also in a mouse model of surgical trauma (9, 12). This study identifies the CD11b+/Gr-1− immature myeloid cells that exclusively express ARG1 after traumatic stress (Figs. 1–3). It is interesting that ARG1 is not induced in other cell types by trauma and clearly not expressed in lymphoid cells (Figs. 1 and 2). Similar to reports presented in models of cancer, trauma-induced cells expressing ARG1 are of myeloid origin and express CD11b and Gr-1 markers (27 and Table I). These cells exhibit lobular and ring-shaped nuclei that are characteristic of neutrophils and immature myeloid cells (Fig. 8). However, as confirmed by phenotype analysis, these cells are not mature neutrophils or granulocytes, because they do not express any significant levels of CD68 or CD13 markers. In addition, in our preliminary experiments, we did not detect significant levels of reactive oxygen species production, again suggesting that these cells are not granulocytes (data not shown). Interestingly, ARG1-expressing myeloid cells have varied morphological characteristics, depending on the various models described. For example, Choi et al. (20), depicts a cell that is very similar to the one described by us. However, Rodriguez et al. (28) reports the virtual invasion of an arginase-expressing CD11b+/Gr-1− cell into tumors, which exhibit characteristics of more mature myeloid cells. In contrast, CD11b+/Gr-1− cell subpopulations observed in trauma did not express any significant arginase activity (Fig. 3). Trauma-induced CD11b+/-Gr-1+ cells exhibit significant differences from other myeloid cells reported. For example, they express only low levels F4/80 or CD31 (Table I). There also are dramatic temporal differences in the appearance of trauma-induced immature CD11b+/-Gr-1+ cells (which are observed within hours of a trauma, Figs. 7 and 9) and those observed in cancer, which only occurs after days or weeks of tumor implantation. Finally, the magnitude of the increase in ARG1 expression appears to be far higher after trauma, compared with cancer models. These observations imply significant differences in the type of cell and mechanisms of induction of ARG1 among the different disease processes.

The mechanisms behind the induction of ARG1 and the appearance and accumulation of CD11b+-Gr-1+ cells have been only partially studied. We reported previously that arginase activity could be induced by catecholamines and demonstrated a significant “blunting” of arginase induction in splenic tissue with the systemic use of the β-adrenergic blocker propranolol in a model of trauma (29). Trauma also induces the expression of cytokines such as IL-4, IL-6, IL-10, and IL-13, all known to induce arginase in the RAW 264.7 myeloid cell line. Prostaglandins also are released after trauma and also may be responsible for the induction of arginase after trauma. Sorting out the roles played by these possible candidate substances in the induction of ARG1 in CD11b+/Gr-1− cells after trauma will be an important goal for future work. It is interesting that we observed a second peak of CD11b+/Gr-1− cells occurring 48 h after trauma. Incidentally, we also have observed a secondary peak in arginase activity 4 days after severe trauma in humans. The mechanisms behind this second peak are currently unknown, though we speculate that delayed production of substances that induce the proliferation of CD11b+ cells may be implicated.

Dietary arginine supplementation is associated with the restoration of T cell counts in surgical patients and has been associated with a decrease in postoperative infection rates (30). These clinical observations suggested to us that impaired T cell function after trauma could be caused by arginine deficiency. We have thoroughly tested this hypothesis in vitro using mouse T lymphocytes and T cell lines, identifying key molecular effects of arginine deficiency (6). Withholding L-arginine from the culture medium leads to a significant decrease in the expression of the TCR and the TCR β-chain peptide (7). Loss of β-chain has been described in cancer and also in trauma. We demonstrate in this study that we can reproduce the loss of the TCR β-chain, suppression of T cell proliferation, and IL-2 production through the coculture of T lymphocytes and trauma-induced CD11b+/Gr-1+ cells (Fig. 12). Furthermore, we demonstrate that trauma-induced immature CD11b+/Gr-1− cells exhibit increased L-arginine uptake (Fig. 5) and significantly deplete L-arginine from the culture medium (Fig. 6A). Trauma-induced immature CD11b+/Gr-1− cells rapidly accumulate in the spleen within hours of induction of traumatic stress and colocalize with T cells in specific zones of the spleen, thus suggesting an important interaction with them (Fig. 7).

These findings support the hypothesis that arginine depletion by myeloid cells is a novel mechanism of T cell regulation. The work presented in this study provides new avenues for the development of effective mechanisms of overcoming T cell dysfunction after trauma. These include dietary strategies for arginine replacement, prevention of ARG1 induction, and pharmacologic arginase blockade. Our work presents a reliable trauma model in which to test these possible strategies.

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Disclosures
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