Blockade of Myeloid-Derived Suppressor Cells after Induction of Lymphopenia Improves Adoptive T Cell Therapy in a Murine Model of Melanoma

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*J Immunol* published online 24 October 2012
http://www.jimmunol.org/content/early/2012/10/24/jimmunol.1200274
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Administration of nonmyeloablative chemotherapeutic agents or total body irradiation (TBI) prior to adoptive transfer of tumor-specific T cells may reduce or eliminate immunosuppressive populations such as T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSC). Little is known about these populations during immune reconstitution. This study was designed to understand the reconstitution rate and function of these populations post TBI in melanoma tumor-bearing mice. Reconstitution rate and suppressive activity of CD4+CD25+Foxp3+ Tregs and CD11b+Gr1+ MDSC following TBI-induced lymphopenia was measured in B16 melanoma tumor-bearing mice. To ablate the rapid reconstitution of suppressive populations, we treated mice with docetaxel, a known chemotherapeutic agent that targets MDSC, in combination with adoptive T cell transfer and dendritic cell immunotherapy. Both Treg and MDSC populations exhibited rapid reconstitution after TBI-induced lymphopenia. Although reconstituted Tregs were just as suppressive as Tregs from untreated mice, MDSC demonstrated enhanced suppressive activity of CD8+ T cell proliferation compared with endogenous MDSC from tumor-bearing mice. TBI-induced lymphopenia followed by docetaxel treatment improved the efficacy of adoptive T cell transfer and dendritic cell immunotherapy in melanoma-bearing mice, inducing a significant reduction in tumor growth and enhancing survival. Tumor regression correlated with increased CTL activity and persistence of adoptively transferred T cells. Overall, these findings suggest that TBI-induced MDSC are highly immunosuppressive and blocking their rapid reconstitution may improve the efficacy of vaccination strategies and adoptive immunotherapy.

The Journal of Immunology, 2012, 189: 000–000.

Melanoma is a leading cause of cancer mortality in the United States, resulting in one American dying every 60 min. Currently, there are few nonsurgical options for the treatment of metastatic melanoma (1, 2). Cancer immunotherapy, which focuses on the induction of immunity against tumor cells, is a promising approach for the treatment of melanoma. A major hurdle in the development of effective immunotherapy is tumor-induced suppression, including factors such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) that can limit the effectiveness of tumor-specific T cells (3, 4). Tregs have been shown to suppress antitumor activity and ablation of this population has led to tumor eradication (5). In preclinical melanoma models as well as in cancer patients, depletion of Tregs followed by dendritic cell (DC)-based vaccination has led to enhanced vaccine-mediated tumor-specific T cell responses (3, 6, 7). MDSC are implicated in the maintenance of immune tolerance to tumors (4, 8, 9). MDSC are a phenotypically heterogeneous cell population consisting of myeloid progenitor cells and immature myeloid cells. In mice, these cells are broadly defined as CD11b+ Gr-1+ cells. Recent reports have shown that MDSC by themselves can induce tumor-specific Tregs (10). Increased levels of circulating MDSC have been correlated with advanced disease stage and extensive metastatic tumor burden in cancer patients (11).

There have been several reports showing that lymphopenia induced by nonmyeloablative chemotherapeutic treatments or total body irradiation (TBI) can modulate immune responses and increase antitumor immunity (12–14). These treatments induce a severe lymphopenic condition in the host and leads to the subsequent expansion of T cells in the periphery in a process known as homeostatic proliferation. Homeostatic proliferation can lead to the proliferation and activation of T cells specific for self-Ags (15, 16). Studies have shown increased antitumor responses following irradiation and adoptive T cell transfer in preclinical murine models (17, 18). Adoptive transfer of autologous tumor-infiltrating lymphocytes after lymphodepleting chemotherapy has resulted in objective responses in melanoma patients (12, 19). However, only one-fifth of the treated patients demonstrated complete tumor regressions. Although it has been shown that administration of cyclophosphamide and fludarabine or TBI in mice before adoptive transfer reduces or eliminates immunosuppressive populations (13), little is known about the effects of TBI on reconstitution of Tregs and MDSC. Hence, it is important to understand the role of these suppressor populations after the induction of lymphopenia to design effective immunotherapeutic treatments to achieve complete tumor regression.

Conventional anticancer agents have been explored for their ability to target suppressor populations (20–24). Gemcitabine, a nucleoside analog, has been shown to reduce splenic MDSC in mice bearing large tumors without affecting T cells, NK cells, macrophages, or B cells (25). Serafini et al. (26) showed that sildenafil downregulates arginase activity and NO synthase expression, thereby
reducing the suppressive function of MDSC. Antimicrotubule agents, such as docetaxel (DTX) and paclitaxel, have immune-enhancing properties when used alone or in combination with immunotherapy (22, 27, 28). A recent study has shown that paclitaxel promotes differentiation of MDSC into DC in vitro in a TLR4-independent manner (29). Paclitaxel has also been shown to reduce Tregs and their inhibitory function (28). DTX has been shown to decrease splenic MDSC in mice bearing mammary tumors, leading to an increased antitumor response (22). Although recent reports have shown the effect of DTX on MDSC, it has not been used for specific inhibition of MDSC in the setting of lymphopenia for the treatment of melanoma. Hence, in this study, we investigated the reconstitution of suppressor populations (Tregs and MDSC) after lymphodepletion. Furthermore, we investigated whether blockade of reconstituting MDSC leads to persistence of adoptively transferred T cells and enhances the efficacy of adoptive T cell transfer and vaccination in a murine model of melanoma.

Materials and Methods

Mice
Six- to 8-week-old C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). OT-I mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Cell lines
B16 melanoma was maintained by serial in vitro passages in complete medium (CM) consisting of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 50 mg/ml gentamicin, 0.5 mg/ml fungizone (all from Life Technologies, Rockville, MD), and 0.05 mM 2-ME (Sigma–Aldrich, St. Louis, MO). B16 expressing OVA (M05) cells were maintained in serial in vitro passages in CM supplemented with 0.8 mg/ml G418.

Reagents
For analysis of immune cell populations, the following anti-mouse Abs were purchased from BD Biosciences (San Diego, CA): anti-Ly6G and -Ly6C biotinylated Abs, anti-CD11b PerCP-Cy5.5, anti-CD11b allophycocyanin, anti-Gr-1 PE, anti–Gr-1 PE Cy7, anti-CD4 Alexa Fluor 780, and anti-CD3 FITC. The CD4+CD25+ Treg isolation kit and streptavidin microbeads were purchased from Miltenyi Biotec (Auburn, CA). The anti-mouse Foxp3 T regulatory staining kit was purchased from eBioscience (San Diego, CA). Clinical grade DTX (Taxotere; Sanofi-Aventis) for 20 min at 4˚C. Labeled splenocytes were then incubated with 100 μl biotinylated anti-Ly6C and Ly6G (Gr-1) Abs (Miltenyi Biotec) for 20 min at 4˚C. Labeled cells were then incubated at 4˚C with 100 μl streptavidin microbeads (Miltenyi Biotec) for 15 min. Cells were washed, resuspended in 5 mM CM buffer, and applied to a MACS column for positive selection, according to the manufacturer’s instructions (Miltenyi Biotec). The purity of cell populations as analyzed by flow cytometry was >95%. For functional assays, splenocytes from OT-I mice were used as responder cells. CD8+ T cells from these mice have a transgenic TCR that recognizes the OVA 257–264 peptide. MDSC from naive, tumor-bearing mice with or with out TBI treatment were cultured at different ratios with 2 × 105 splenocytes from OT-I mice in the presence of control or specific peptides. Cell proliferation was measured by [3H]thymidine uptake. All experiments were performed in triplicate.

Functional assays
To test the function of Tregs from TBI-treated or untreated tumor-bearing mice, a CD4+CD25+ regulatory T cell isolation kit was used to isolate Tregs on day 21 after B16 injection. For stimulator cells, T cell-depleted splenic cells from C57BL/6 mice were used and cultured at 5 × 105 cells/well in a 96 well plate coated with 0.25 μg anti-CD3. Naive CD4+CD25+ T cells were cocultured at 5 × 105 cells/well. CD4+CD25+ T cells from B16-bearing mice treated with or without TBI were added at 5 × 105 cells/well. Naive T cells alone, stimulators alone, and inhibitory cells alone served as controls. After 72 h of culture, proliferation was analyzed by the incorporation of [3H]thymidine during the final 6 h of culture. All experiments were performed in triplicate.

Reactive oxygen species production
The oxidation-sensitive dye 2’,7’-dichlorofluorescein diacetate (DCFDA) (Molecular Probes/Invitrogen, Eugene, OR) was used for the measurement of reactive oxygen species production by MDSC (30). Cells were incubated at room temperature in serum-free RPMI 1640 medium in the presence of 3 μmol/l DCFDA with or without 300 nmol/l PMA for 30 min, washed with PBS, and then labeled with anti-CD11b and Gr-1 Abs. After incubation on ice for 20 min, cells were washed with PBS and analyzed using flow cytometry.

Arginase activity
Arginase activity was measured in cell lysates as described previously (31). Briefly, cells were lysed for 30 min with 100 μl 0.1% Triton X-100. Subsequently, 100 μl 25 mM Tris–HCl and 10 μl 10 mM MCA 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 0.5 M L-arginine (pH 9.7) at 37˚C for 2 h. The reaction was stopped with 900 μl H2SO4 (96%) and the absorbance was measured at 540 nm after addition of 40 μl of a-isotiocyanosopropiophenone (dissolved in 100% ethanol), followed by heating at 95˚C for 30 min.

NO production
Equal volumes of culture supernatants (100 μl) were mixed with Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in double-distilled water). After 10 min of incubation at room temperature, the absorbance at 550 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by a serial dilution of 0.25 mM sodium nitrite.

Flow cytometry
Spleens were harvested under sterile conditions. Single-cell suspensions were prepared, and red cells were removed using ammonium-chloride-potassium chloride lysing buffer. One million cells (splenocytes or MDSC) were incubated for 30 min on ice in staining medium with relevant Abs for the surface expression analysis according to the standard protocols. After washing, the samples were analyzed using an LSRII (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star).

Generation of bone marrow-derived DC and Ag pulsing
Erythrocyte-depleted mouse bone marrow cells were cultured in CM supplemented with 20 mg/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) as described previously (18). On day 5, cells were harvested by gentle pipetting and layered onto an Optiprep gradient (Axis-Shield, Oslo, Norway). The low-density cell interface was collected and washed twice. Resulting DC were washed one time and resuspended at 1 × 106 cells/ml CM containing 20 ng/ml GM-CSF and 20 ng/ml IL-4. DC were pulsed overnight with 10 μg/ml of OVA 257–264 peptide (Invitrogen, Carlsbad, CA).

Immunization schedule
MDSC blockade in TBI-treated mice in combination immunotherapy. A total of 3 × 106 M05 tumor cells were injected s.c. into the left flank of C57BL/6 mice. Three days later, mice received a sublethal dose (600 cGy) of TBI administered by a [137Cs] gamma radiation source. Mice received a total of 3 injections of DC with or without OVA 257–264 peptide (1 × 105 cells/mouse/vaccination) on days 4 and 10. Four doses of DTX at 16 mg/kg were given i.p., (i.p), every 3 d starting on day 4.

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Control groups received T cells alone, DC alone, DTX alone, or PBS. Tumor size was measured and recorded every 2 d. In another set of experiments, splenocytes were harvested from these mice 1 wk after the final immunization for in vitro assays.

[^51Cr] release cytotoxicity assay

A[^51Cr] release assay was done as described previously (18). M05 cells were used as targets. Spleens of treated mice were harvested on day 21, and purified T cells were used as effector cells. T cell purity was measured by flow cytometry and cells were 95% positive for CD90 (data not shown). Briefly, target M05 cells were labeled with 100 μCi[^51Cr] (Amersham Biosciences) at 37°C in a 5% CO2 atmosphere for 1.5 h. The labeled tumor cells were washed three times and added to the effector cells in triplicate wells of 96-well round-bottom microplates at 100:1, 50:1, and 25:1 E:T ratios. After 5 h, the percentage of specific [^51Cr] release was determined by the following equation: ([experimental cpm − spontaneous cpm]/[total cpm incorporated − spontaneous cpm]) × 100. All determinations were done in triplicate, and the SE of all assays was calculated and was typically 5% of the mean or less.

**Cytometric bead array**

Briefly, splenocytes were plated at 2 × 10^6, cocultured with 2 × 10^5 irradiated M05 melanoma tumor cells, and incubated for 48 h. Culture supernatants were analyzed for IFN-γ production using commercially available cytometry bead array kit (BD Biosciences). Briefly, 25 μl mixed capture beads were incubated with 25 μl culture supernatant and 25 μl PE detection reagent for 2 h at room temperature. The immunocomplexes were then washed and analyzed using a FACScan®labor equipped with a 488-nm laser (BD Biosciences), according to the manufacturer’s protocol.

**Statistical analysis**

A Mann–Whitney U test (unpaired) or a Student t test was used to compare differences between two treatment groups. All statistical evaluations of data were performed using GraphPad Prism software. Statistical significance was achieved at p < 0.05.

**Results**

**Reconstitution of immune subsets after lymphopenia**

To evaluate the reconstitution of immune subsets after induction of lymphopenia, naive and B16 tumor-bearing mice received 600 rad TBI. We observed lymphopenia and leukopenia in both naive and B16 tumor-bearing mice, evidenced by total splenocyte counts post-TBI (data not shown). As shown in Fig. 1A, CD4^+ and CD8^+ T cells were detected on day 7 in the wild-type mice, with 60% of CD4^+ T cells reconstituted (percentage of normal) and 40% of CD8^+ T cells reconstituted by day 21. In B16 tumor-bearing mice, 30% reconstitution of CD4^+ T cells and 20% CD8^+ T cells was observed (Fig. 1B). CD4^+CD25^+Foxp3^+ Tregs were reconstituted to 70% in wild-type and 100% in B16 tumor-bearing mice by day 10. Although, CD11b^+Gr-1^ T cells were used as targets. Spleens of treated mice were harvested on day 21, and purified T cells were used as effector cells. T cell purity was measured by flow cytometry and cells were 95% positive for CD90 (data not shown). Briefly, target M05 cells were labeled with 100 μCi[^51Cr] (Amersham Biosciences) at 37°C in a 5% CO2 atmosphere for 1.5 h. The labeled tumor cells were washed three times and added to the effector cells in triplicate wells of 96-well round-bottom microplates at 100:1, 50:1, and 25:1 E:T ratios. After 5 h, the percentage of specific [^51Cr] release was determined by the following equation: ([experimental cpm − spontaneous cpm]/[total cpm incorporated − spontaneous cpm]) × 100. All determinations were done in triplicate, and the SE of all assays was calculated and was typically 5% of the mean or less.

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**Repopulating Tregs are suppressive after TBI**

Because Tregs and MDSC undergo rapid reconstitution after TBI, we sought to determine the suppressive function on T cell proliferation. Tregs were purified from the spleens of B16 tumor-bearing mice with or without TBI on day 21 posttumor injection. Inhibitor alone, stimulator alone or responder alone served as controls. As shown in Fig. 2A, CD4^+CD25^+ Tregs from the spleens of TBI-treated B16 tumor-bearing mice were as suppressive as the CD4^+CD25^+ Tregs from tumor-bearing mice with no TBI treatment, whereas CD4^+CD25^- cells in the absence of CD4^+CD25^+ Tregs had significant proliferation (p < 0.01). This result shows that reconstituting Tregs post-TBI are suppressive and behave similarly to endogenous Tregs from tumor-bearing mice. MDSC from non–TBI-treated tumor-bearing mice (not bearing tumor) did not show any suppressive activity compared with control OT-I cells pulsed with peptide alone. To further analyze suppressive activity, MDSC were added at various ratios to OT-I cells in the presence of OVA257–264 peptide, and T cell proliferation was evaluated. OT-I cells alone and MDSC alone served as controls. We observed that TBI-induced MDSC from tumor-bearing mice were significantly more suppressive compared to the endogenous MDSC from tumor-bearing mice that did not receive TBI treatment (Fig. 2B). Even at the lowest ratio of 1 MDSC:12 T cells, there was significant (p < 0.01) suppressive activity, whereas the endogenous MDSC from tumor-bearing mice were not able to inhibit T cell proliferation. MDSC derived from TBI-treated naive mice (not bearing tumor) did show any suppressive activity compared with control OT-I cells pulsed with peptide alone. To further analyze suppressive activity, MDSC were added at various ratios to OT-I splenocytes in the presence of OVA257–264 peptide, and IFN-γ production was evaluated. We observed significantly suppressed IFN-γ production by OT-I cells when cocultured with MDSC from tumor-bearing mice with or without TBI at 1:3 ratios (p < 0.01; Fig. 2C). However, at both 1:6 and 1:12 ratios, no suppressive activity was observed in MDSC from non–TBI-treated tumor-bearing mice, whereas a significant reduction of IFN-γ production was observed in OT-I cells when cocultured with MDSC from TBI-treated tumor-bearing mice. Collectively, these data show that repopulating MDSC post-TBI has enhanced immunosuppressive activity of MDSC on a per cell basis.
Next, we sought to understand the mechanism for the increased suppressive activity. Several factors have been implicated in MDSC-mediated immune suppression, including the production of arginase, NO, and ROS (30–32). We compared the production of these factors in MDSC from B16 tumor-bearing mice treated with or without TBI. Gr-1+ cells were purified from the spleens of TBI-treated or untreated tumor-bearing mice on day 21 posttumor injection. OT-I splenocytes were cultured at different ratios of MDSC in the presence of OVA257–264 peptide for 48 h. OT-I splenocytes alone and MDSC alone served as controls. OT-I splenocytes cultured with different ratios of MDSC in the presence of OVA257–264 peptide had a significant increase in the NO production compared with the endogenous MDSC from the tumor bearing mice (p < 0.05; Fig. 3A). The oxidation-sensitive dye DCFDA was used for the measurement of ROS production by MDSC and analyzed by flow cytometry. As shown in Fig. 3B, there was an increased ROS production in TBI-induced MDSC compared with endogenous MDSC from tumor-bearing mice. Next, arginase activity in cell lysates was also measured. We observed a similar trend as that seen in the NO and ROS levels, with increased arginase activity in the TBI-induced MDSC compared with the endogenous MDSC (p < 0.01; Fig. 3C).

**DTX reduces reconstitution rate of MDSC post-TBI**

Because MDSC from TBI-treated tumor-bearing mice demonstrated enhanced suppressive activity, we wanted to evaluate whether we could abrogate their reconstitution with DTX treatment. To evaluate the effect of DTX on reconstituting MDSC post-TBI, C57BL/6 mice were injected s.c. with B16 cells on day 0 and received 600 rad TBI on day 3. Mice received four doses of DTX injected i.p. at 3 d or weekly intervals. Spleens were harvested on days 3, 7, 10, 14, and 21 posttumor injection, and immune subsets were analyzed by flow cytometry. As shown in Fig. 4A, B16 tumor-bearing mice that received DTX had a significant reduction in TBI-induced MDSC, regardless of whether they received the treatment on a weekly basis or every 3 d (p < 0.05). By day 10, we observed a reduction in TBI-induced MDSC reconstitution in DTX-treated mice compared with the TBI-treated tumor-bearing mice that did not receive DTX. By day 21, mice that received DTX every 3 d had a significant reduction in their MDSC reconstitution, up to 50% (p < 0.002) compared with the untreated mice (250%), while we observed 100% reconstitution in mice receiving the treatment on a weekly interval (p < 0.01).

Previous studies have shown that DTX treatment can increase the number of CD4+ and CD8+ T cells (22, 27). To determine whether DTX, given every 3 d, had an effect on reconstituting T cells, we analyzed the CD4+ and CD8+ T cell reconstitution in mice receiving the treatment on a weekly interval (Fig. 4B, 4C). We did not see any significant difference in reconstitution of CD4+ and CD8+ on days 7, 10, 14, and 21 in mice that received DTX. These data show that DTX given at 3-d intervals significantly reduced the MDSC reconstitution post-TBI without modulating T cell populations.

**FIGURE 2.** The suppressive activity of reconstituted Tregs/MDSC (A) CD4+CD25+ cells were purified on day 20 from B16 tumor-bearing mice treated with or without TBI. T cell-depleted splenic cells from C57BL/6 mice were used as stimulators and were cultured at 5 × 10^4 cells/well in a 96-well plate coated with 0.25 μg anti-CD3. Naïve CD4+CD25+ T cells were cocultured at 5 × 10^4 cells/well. CD4+CD25+ T cells were added at 5 × 10^4 cells/well. After 72 h of culture, proliferation was analyzed by the addition of [3H]thymidine during the final 6 h of culture. All experiments were performed in triplicate. (B) CD11b+Gr-1+ MDSC were purified using a MACS column and were cultured at various ratios with 2 × 10^5 splenocytes from OT-I mice in the presence of OVA257–264 peptide. After 72 h of culture, proliferation was analyzed by the addition of [3H]thymidine during the final 6 h of culture. (C) IFN-γ production was measured in the supernatants after 48 h of coculture with a cytometric bead array assay as described in Materials and Methods. *p < 0.01.

### Table I. Cell number (×10^7) per million of splenocytes

<table>
<thead>
<tr>
<th>CD4</th>
<th>CD8</th>
<th>CD11bGr-1</th>
<th>CD4CD25Foxp3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Naive</td>
<td>B16</td>
</tr>
<tr>
<td>Pre-TBI</td>
<td>Day 3</td>
<td>25 ± 2.3</td>
<td>16.3 ± 1.52</td>
</tr>
<tr>
<td>Post-TBI</td>
<td>Day 3</td>
<td>0.5 ± 0.01</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>2.2 ± 0.03</td>
<td>2.3 ± 0.12</td>
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<tr>
<td></td>
<td>Day 10</td>
<td>9.4 ± 0.80</td>
<td>7.8 ± 0.61</td>
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<td></td>
<td>Day 14</td>
<td>10.1 ± 1.21</td>
<td>11.3 ± 1.10</td>
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<tr>
<td></td>
<td>Day 21</td>
<td>16.5 ± 0.34</td>
<td>12.5 ± 0.20</td>
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n = 4/group. Data compiled from three independent experiments.
Next, we evaluated whether blocking TBI-induced MDSC with DTX would improve the efficacy of adoptive T cell transfer and DC immunotherapy. C57BL/6 mice were injected s.c. with M05 cells on day 0 and received 600 rad TBI on day 3. Mice received $5 \times 10^6$ OT-I T cells on day 4 and were vaccinated with DC pulsed with OVA257–264 peptide (DCOVA) on days 4 and 10. DTX was given every 3 d starting on day 4 for a total of four doses. As shown in Fig. 5A, after four doses of DTX treatment, a significant reduction in tumor size was observed in mice that received OT-I T cells and DCOVA immunotherapy compared with mice that received only T cells and DCOVA vaccination ($p < 0.001$). However, mice that received DTX treatment with DCOVA or T cells alone had a significant reduction in tumor growth compared with the untreated groups ($p < 0.001$; Fig. 5B). These data suggest that blockade of TBI-induced MDSC can improve the efficacy of adoptive T cell transfer and DC vaccination.

### Blockade of TBI-induced MDSC enhances the persistence of adoptively transferred T cells and improves CTL function

We next examined whether adoptively transferred T cells persisted for a longer duration in mice treated with DTX. Ly5.2 mice were injected with $3 \times 10^5$ M05 cells followed by TBI on day 3. Mice received $5 \times 10^6$ OT-I (Ly5.1 mice) T cells on day 4. DCOVA vaccinations were given on days 4 and 10. Four doses of DTX were given every 2–3 d starting on day 4. Splenocytes were collected at...
various time points, and the percentage of OT-I T cells was measured by flow cytometry. Mice that received therapy with OT-I T cells and DCOVA in combination with DTX had a higher percentage of OVA tetramer specific T cells on days 8, 15, and 20. As shown in Fig. 6A, a higher percentage of CD8+ OVA-specific T cells were observed in mice that received DTX treatment compared with the mice that received immunotherapy alone. We next evaluated IFN-γ production as a measure of T cell function. As shown in Fig. 6B, restimulation with OVA peptide for 48 h resulted in higher IFN-γ production by splenocytes from mice that received DTX treatment in combination with OT-I T cells and DCOVA vaccination (980 ± 35.4 pg/ml) compared with splenocytes from mice that received OT-I T cells and DCOVA vaccination alone (597 ± 30.4 pg/ml; p < 0.01). These data suggest that blockade of TBI-induced MDSC
with DTX enhanced the persistence and activity of adoptively transferred T cells.

Next, we examined whether reduction of TBI-induced MDSC by DTX enhances the function of CD8\(^+\) T cells. Splenic T cells from mice receiving T cells and DC\(_{\text{CWA}}\), treated with or without DTX, were purified on day 21 and cocultured with \(^{51}\)Cr-labeled M05 melanoma tumor cells at E:T ratios of 100:1, 50:1, or 25:1. T cells from mice that received DC\(_{\text{CWA}}\) and OT-I T cells in combination with DTX treatment exhibited higher cytotoxicity against M05 melanoma tumor cells at a 100:1 ratio compared with mice that did not receive DTX treatment (\(p < 0.05; \text{Fig. 6C})\). However, no significant differences were measured at lower ratios. These data suggest that blockade of TBI-induced MDSC with DTX improves the CTL function in vaccinated mice against M05 melanoma tumor cells.

Discussion

Impaired immune function is often associated with negative regulatory factors such as Tregs and MDSC (3, 9, 33). These populations can inhibit T cell activation and may limit the efficacy of immunotherapies (34). Induction of lymphopenia by TBI or chemotherapy can eliminate these immunosuppressive populations. Our laboratory and others have shown that adoptive T cell therapy and DC vaccination after the induction of lymphopenia enhances T cell expansion and activation and leads to improved antitumor immune responses against melanoma (13, 17, 18). Adoptive transfer of T cells following the induction of lymphopenia has led to enhanced antitumor immunity in animal models and significant clinical responses in patients with melanoma (14, 17, 19, 35). However, there are few reports on the reconstitution and function of suppressor populations after the induction of lymphopenia.

In this study, we demonstrate that the setting of lymphopenia, MDSC and Tregs reconstitute faster than CD4\(^+\) and CD8\(^+\) T cells. TBI-induced Tregs were as suppressive as the endogenous Tregs. Depletion of TBI-induced Tregs with anti-CD25 Abs enhanced the antitumor efficacy of adoptive T cell transfer and DC immunotherapy in melanoma-bearing mice (data not shown). Our findings suggest that depletion of TBI-induced Tregs improves the antitumor efficacy of adoptive T cell transfer and DC immunotherapy. These data are supported by a recent report showing an increase in the frequency of Tregs in B16 tumor-bearing mice after temozolamide-induced lymphopenia. Systemic Treg depletion during lymphopenia resulted in enhanced antitumor immunity (36). It has been shown that lymphopenia induces expansion of Tregs during immune reconstitution in individuals with cancer (36, 37). A study has shown that Denileukin diftitox (Ontak) administration to stage IV melanoma patients depletes peripheral blood Tregs and causes the regression of metastatic tumors in a subset of patients (38). CD25 depletion in combination with lymphopenia has been shown to result in a potent tumor rejection in a B16 melanoma model and may enhance immunity in lymphopenic patients with metastatic melanoma (39, 40).

Our findings demonstrate that TBI-induced MDSC are more immunosuppressive on a per cell basis compared with endogenous MDSC isolated from B16 tumor-bearing mice. This is a novel finding that repopulating MDSC after the induction of lymphopenia are highly immunosuppressive. On the basis of these initial findings, this study focused on evaluating the immunosuppressive function of TBI-induced MDSC. We tested NO, arginase, and ROS production as possible mechanisms for the observed increase in suppressive function and found increased production of all three suppressive factors in the reconstituted MDSC following lymphodepletion. It has been reported that granulocytic subsets of MDSC express a high level of ROS and very little NO, whereas the monocytic subsets have very little ROS but high levels of NO (31). These suppressive factors have been shown to have a direct effect on the inhibition of T cell function (41–44). Although it has been shown in several studies that MDSC use multiple factors such as NO, ROS, and arginase for their suppressive function on T cells, this study shows that after the induction of lymphopenia, MDSC have increased production of these factors compared with the endogenous MDSC from non–TBI-treated tumor-bearing mice. It has been shown that various tumor-derived factors define the expansion of these MDSC subsets. However, in our study, we observed elevated levels of all of these suppressive factors. It is possible that both monocytic and granulocytic MDSC are being reconstituted following lymphodepletion and that each population contributes to increased immunosuppressive function. Studies to define the factors responsible for the rapid expansion of these MDSC subsets in a lymphopenic environment are ongoing.

MDSC frequency is increased in melanoma patients and is associated with disease progression (45, 46). Strategies to target MDSC include inhibition of expansion or promotion of differentiation into mature cells that no longer possess suppressive activity (21, 47). All-trans-retinoic acid has been shown to induce MDSC differentiation that leads to neutralization of ROS production in both mice and cancer patients (23, 48). Several studies have also shown that MDSC can be directly eliminated using chemotherapeutic drugs such as gemcitabine, DTX, sunitinib, or flurouracil (20, 22, 23, 26, 28). Treatment of mice bearing large tumors with chemotherapeutic drugs has been shown to result in dramatic reductions in the number of splenic MDSC and a marked improvement in immunotherapeutic efficacy (24, 25). To block rapidly reconstituting MDSC in our model, we used DTX, a chemotherapeutic drug previously shown to block MDSC expansion (22). In melanoma-bearing lymphopenic mice, DTX treatment decreased MDSC reconstitution and increased the persistence of adoptively transferred T cells, resulting in delayed tumor growth and enhanced survival. These are the key findings that can potentially improve the design of clinical trials in patients with melanoma.

Although lymphodepletion strategies are an attractive tool for adoptive immunotherapy protocols, our results demonstrate that MDSC and Tregs are rapidly reconstituted and are highly immunosuppressive after the induction of lymphopenia. It is possible that the rapid reconstitution and suppressive functions of TBI-induced MDSC and Tregs may limit the effectiveness of immunotherapy to induce antitumor T cell responses, which in turn contribute to failure at inducing complete tumor regressions. Blocking the reconstitution of TBI-induced MDSC or Tregs enhanced antitumor immunity in a murine model and may improve current adoptive therapy strategies for the treatment of metastatic melanoma.

Acknowledgments

We thank Dr. DmitryGabrilovich for valuable comments during the preparation of this manuscript. We also thank the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center and Research Institute for contributions to this work.

Disclosures

The authors have no financial conflicts of interest.

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