Thioredoxin induces Tregs to generate an immunotolerant tumor microenvironment in metastatic melanoma

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Abbreviations: OE, overexpression; KD, knockdown; Teff, effector T; Treg, regulatory T cell; TRX, thioredoxin; ROS, reactive oxygen species

Metastatic melanoma is a highly aggressive cancer that is very difficult to treat. Additionally, the antitumor immune reaction of melanoma is still unclear. Here we demonstrate an association between the expression and secretion of the antioxidant protein thioredoxin (TRX) and increasing tumor stage and metastasis in melanoma. To elucidate the role of TRX in melanoma, we assessed the correlation of TRX expression with different disease parameters in melanoma. We also examined the in vitro and in vivo effects of modulating TRX levels in melanoma cells using various methods of TRX depletion and augmentation. We further explored the effects of TRX on the cytokine milieu and the ability of TRX to regulate the proportion and specific activities of T-cell populations. We demonstrate that TRX expression correlates with Treg representation in clinical samples and, that modulation of TRX in influences the induction of Tregs and the generation of an immunotolerant cytokine profile in mouse serum. Using a murine metastatic melanoma model, we identified a tumor immune evasion mechanism whereby melanoma cell-secreted TRX enhances Treg infiltration. TRX displays chemotactic effects in recruiting Tregs, stimulates the conversion of conventional T cells to Tregs, and confers survival advantage to Tregs in the tumor microenvironment. In turn, this increase of Tregs generates immunotolerance in tissues and therefore decreases antitumor immune reactions. These results elucidate a mechanism by which TRX promotes metastatic melanoma in part through Treg recruitment to inhibit T-cell antitumor effects and suggest that TRX antibody may be useful in the clinic as a therapy against melanoma.

Introduction

The incidence of melanoma has risen faster than that of any other cancer over the past 4 decades, and it is now reaching epidemic proportions in Caucasian populations worldwide.1 Late-stage metastatic disease is notoriously difficult to treat, resulting in the high mortality rate of melanoma. Hence, the 3-year overall survival of patients with unresealtable melanoma is very poor.2

Immunotherapy studies of melanoma patients have reported success in the expansion of tumor-specific effector T cells, but even in the presence of substantial numbers of circulating functional T cells favorable clinical outcomes are scarce.1,3 Immunosuppression in the tumor microenvironment mediated by regulatory T cells (Tregs) is a dominant mechanism of tumor immune escape and presents a major hurdle for tumor immunotherapy.4 Consequently, the depletion of Tregs may serve as a promising strategy to enhance melanoma-specific immunity. Indeed, murine studies have shown that Treg depletion increases the efficacy of immunotherapy. However, the successful depletion of Tregs in patients has not yet provided a major advance in improving overall clinical efficacy of immunotherapy,5 perhaps due to a lack of specificity in the available strategies for Treg depletion. Thus, a better understanding of the mechanism of Treg function and accumulation is critically needed.

Antioxidants act as electron donors that neutralize reactive oxygen species (ROS) and other free radicals that may otherwise damage DNA and promote tumorigenesis. Conventional wisdom supported by numerous cellular and preclinical studies holds that antioxidants protect against cancer. However, large randomized clinical trials have produced inconsistent results, with some studies indicating that antioxidants may actually increase cancer risk.6 Thioredoxin (TRX) is a low molecular weight redox protein found in both prokaryotic and eukaryotic cells that participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide, which catalyzes dithiol-disulfide exchange reactions.7 TRX is over-expressed in many human cancers including lung, colon, cervical, gastric, and pancreatic cancer.8 However, the role of...
TRX in melanoma is largely unknown. To elucidate the role of TRX in melanoma, we assessed the correlation of TRX expression with different disease parameters in melanoma. We also examined the in vitro and in vivo effects of modulating TRX levels in melanoma cells using various methods of TRX depletion and augmentation. We further explored the effects of TRX on the cytokine milieu and the ability of TRX to regulate the proportion and specific activities of T-cell populations. Our results suggest that the antioxidant TRX may have a specific role in cancer progression by creating an immunotolerant microenvironment and suppressing antitumor immune responses.

**Results**

TRX is overexpressed in human melanoma and is positively associated with metastasis

To examine the expression of TRX in human melanoma, we first performed immunohistochemistry of TRX using a tissue microarray containing 10 human primary melanoma samples, 10 human metastatic melanoma samples and 10 control nevus tissues (Table S1). Tissue staining was scored on the basis of the intensity of TRX labeling. TRX protein was weakly detected in most normal nevus tissues but was readily detectable in most primary melanoma and metastatic melanoma tissues (Fig. 1A). The TRX staining score increased in a stage-dependent manner in melanoma, with T4 stage tissues having significantly higher scores than T0 stage tissues (Fig. 1B). The TRX scores were also significantly higher in metastatic tumors compared to non-metastatic tumors (Fig. 1C and D). Consistently, serum TRX levels were significant higher in metastatic melanoma patients than in both non-metastatic melanoma patients and control patients with nevi (Fig. 1E). Further examination suggested that TRX is also expressed by human and murine melanoma cell lines (Fig. S1A) and can be detected in concentrated serum-free media from tumor cell lines by ELISA and Western blotting (Fig. S1B and 1C). These results suggest that TRX expression is correlated with melanoma stage and that human and mouse melanoma cells express and secrete TRX protein into the tumor microenvironment.

TRX generates an immunotolerant tumor microenvironment via Treg recruitment

To investigate the possibility that secretion of TRX may confer immune privilege to melanoma cells, we transfected mouse B16 melanoma cells with TRX plasmid (overexpression, OE) or TRX shRNA plasmid (knockdown, KD). We then selected representative clones according to TRX expression levels (Fig. 2A). ELISA analysis of supernatants verified the altered levels of TRX secretion in the corresponding cell lines (Fig. 2B). Furthermore, 25 to 30 d after tail vein injection of 5 × 10⁶ B16 cells into C57/B6J mice, the serum TRX protein levels were elevated in mice bearing OE cells and reduced in mice bearing KD cells as compared to mice bearing mock-transfected cells (Mock) (Fig. 2C). These results validate the use of the OE and KD B16 melanoma cells as a stable model for assessing the effects of TRX modulation in mice. The growth of the tumors correlated with TRX expression levels such that the tumors were larger in OE mice and smaller in KD mice relative to the mock controls (Fig. 2D). Representative images of lungs with melanoma metastatic lesions from each group are shown in Fig. 2E. To investigate the antitumor immune responses in each group, tumor-bearing mice were sacrificed at day 30, and serum was collected for ELISA analysis. The OE mice had reduced levels of the pro-inflammatory mediator interferon γ (IFNγ), indicating a weak inflammatory response of OE mice relative to the mock control mice, while the KD mice displayed the converse pattern (Fig. 2F). Moreover, levels of the anti-inflammatory mediators interleukin 10 (IL-10; Fig. 2G) and transforming growth factor β (TGFβ; Fig. 2H) were significantly higher in sera from OE mice and lower in sera from KD mice as compared to sera from mock control mice. These results suggest that TRX mediates a shift to a less inflammatory tumor microenvironment.

To test the hypothesis that TRX mediates a corresponding shift in the balance of Tregs, we performed FACS analysis of tumor-infiltrating lymphocytes from mice bearing mock, OE or KD tumors (Fig. S2). The number of CD4⁺CD25⁺Foxp3⁺ Tregs were increased in the tumors of OE mice and reduced in those of KD mice (Fig. 2I). These results further verify that TRX mediates the establishment of an immunotolerant tumor microenvironment.

**CD4⁺CD25⁺Foxp3⁺ Tregs are enriched in the melanoma patient tumor microenvironment correlating with TRX levels**

Given that tumor specimens from melanoma patients express high levels of TRX, we postulated that Tregs may also be enriched in the melanoma environment. To test this hypothesis, we collected clinical specimens from melanoma patients at various stages as well as control nevi samples. A small population of cells stained positive for both CD25 and FOXP3 as assayed by immunofluorescence staining and FACS analysis (Fig. 3A). Furthermore, CD4⁺CD25⁺FOXP3⁺ Tregs were observed in all melanoma cases, but were hardly detected in control nevi samples, and the number of Tregs was greater for metastatic tumors than for primary tumors (Fig. 3B). Among the melanoma samples, the CD4⁺CD25⁺FOXP3⁺ Treg number increased in a stage-dependent manner (Fig. 3C and D). Significantly, the number of infiltrating Tregs was positively correlated with the immunohistochemistry score of TRX in the human melanoma samples tested in this study (Fig. 3E). These results provide clinical evidence to support the role of TRX in determining the number of Tregs in the melanoma microenvironment.

**Chemotactic effect of TRX on Tregs in vitro and in vivo**

To understand the potential mechanism of TRX in promoting Treg accumulation in melanoma in situ, we test the chemotactic effect of TRX on Treg migration in vitro. Purified Tregs were much more highly attracted to melanoma B16 cell conditioned medium (CM) compared to standard medium (control) (Fig. 4A). Moreover, the migration of Tregs was significantly enhanced when a reduced form of TRX (rTRX) was added to the
However, when a neutralizing antibody to TRX was added in the CM, the Treg migration rate was notably reduced (Fig. 4C). These results suggest that TRX serves as a chemoattractant of Tregs.

To verify these observations in an *in vivo* model, we tested the effects on B16 melanoma migration in B16 tumor-bearing mice. When TRX protein was tail vein injected into tumors for up to 8 h, the number of Treg cells at the tumor site was markedly increased (Fig. 4D). In contrast, when TRX antibody was tail injected, the number of tumor-infiltrating Tregs was reduced relative to control IgG-injected mice (Fig. 4E). To further verify the findings, we repeated migration assays for Tregs isolated from TRX overexpressing (OE) and TRX knockdown (KD) B16 tumor-bearing mice. The migration activity was much higher in the OE group compared to the mock and KD groups (Fig. 4F). These results verify that when administered *in vivo*, TRX increases the migration activity of Tregs.

**TRX induces conversion from conventional T cells (Teffs) to Tregs in vitro**

Secondly, as an additional potential mechanism of increasing Treg levels, we tested whether TRX may mediate the conversion of conventional CD4⁺CD25⁻ effector T (Teff) cells to CD4⁺CD25⁺Foxp3⁺ Tregs. CD4⁺CD25⁻ Teff cells cultured for 4 d in regular conditioned medium (CM) from B16 melanoma cells displayed an increase in both the number of CD4⁺CD25⁺Foxp3⁺ T cells (Fig. 5A) and the ratio of CD4⁺CD25⁺Foxp3⁺ cells (Fig. 5B). As a confirmation of the Treg phenotype, ELISA analysis demonstrated that these converted T cells secreted more IL-10 and TGFβ and less IFNγ (Fig. 5C). Moreover, the addition of rTRX into the CM significantly accelerated the increase of...
CD4+CD25+Foxp3+ cells after treatment (Fig. 5D and Fig. S4), whereas the addition of TRX antibody had the opposite effect (Fig. 5E). These results suggest that TRX may further increase the Treg population by inducing Teff to Treg conversion.

TRX confers enhanced tolerance of Tregs to oxidative stress in melanoma

To assess the ability of TRX-induced Tregs to tolerate environmental stress, we measured the content of reactive oxygen species (ROS) in tumor interstitial fluid from B16 tumor-bearing mice. The tumor interstitial fluid accumulated ROS (Fig. 6A), which confirms that B16 tumor produces an environment of oxidative stress. To further examine the physiological effects of the oxidative environment, we exposed freshly isolated Tregs and Teff cells to interstitial fluid. Our results show that Tregs are more resilient than Teffs to cell apoptosis induced by tumor interstitial fluid (Fig. 6B). Moreover, rTRX significantly lowered the Treg apoptosis rate (Fig. 6C and Fig. S5), whereas TRX neutralizing antibody had the reverse effect (Fig. 6D).

To test the effect of TRX on cell apoptosis in vivo, we assessed the levels of Teff and Treg cell apoptosis in B16 tumor-bearing mice. FACS data suggest that Teff cell apoptosis increased significantly and Treg cell apoptosis increased slightly in tumor-bearing mice over time (Fig. 6E). Furthermore, the apoptosis rate of Tregs was much lower in mice bearing TRX-overexpressing B16 tumor (OE) and much higher in mice bearing TRX knockdown B16 tumor (KD) (Fig. 6F). These results verify that TRX confers enhanced tolerance of Tregs to oxidative stress.

Therapeutic effect of TRX antibody on metastatic melanoma

Next, to examine the potential therapeutic effect of inhibiting TRX in the metastatic melanoma mouse model we exposed B16-injected mice to anti-TRX antibody. The melanoma lung metastatic burden was clearly decreased when TRX was blocked by antibody (Fig. 7A). Additionally, the average size of the metastatic tumors in the lungs was significantly decreased for mice treated with anti-TRX antibody compared with control IgG (Fig. 7B). As expected, the Treg cell number also decreased in lung lesions after anti-TRX antibody treatment (Fig. 7C). Furthermore, serum IFN-γ levels increased and serum IL-10 and TGFβ levels decreased after TRX...
blockade at day 20 and day 30 (Fig. 7D-F), and the Treg migration index and Treg conversion incidence decreased after anti-TRX antibody treatment (Fig. S6A and B), whereas the apoptosis rate of Tregs increased after anti-TRX antibody treatment (Fig. S6C). Collectively, these data support a model whereby TRX benefits intratumoral Treg accumulation by inducing chemotaxis, conversion and survival under oxidative stress (Fig. S7).

**Discussion**

Antioxidants have emerged as regulatory molecules that control fundamental biological processes. TRX is one such antioxidant that is involved in multiple key intracellular and extracellular processes, including redox regulation of gene expression and signal transduction, protection against oxidative stress, anti-apoptotic functions, growth factor and co-cytokine effects, and regulation of the redox state in the extracellular environment.9 TRX has also been linked to the development and expression of tumor phenotypes.10-13 In vivo expression analyses of breast, lung and colorectal cancer support the conclusion that aggressive tumors robustly over-express TRX.14 Such tumors are associated with a high proliferative capacity, a low rate of apoptosis and an elevated metastatic potential, strongly implicating TRX in the processes of oncogenesis and tumorigenesis, and further, providing rationale to target TRX for anticancer therapy against a wide range of human tumors.14 In the current study, we extensively investigated the function of TRX in the tumor microenvironment as a mechanism to explain its tumorigenic effects in melanoma. Our results demonstrate that TRX induces Tregs to generate an immunotolerant tumor microenvironment in metastatic melanoma.

Research studies and clinical trials have shown that melanoma immunological therapeutics are successful in a subset of patients.15-21 However, only a minor fraction of patients have exhibited immune responses against this particular malignancy. CD25+CD4+FOXP3+ Tregs are physiologically present in the immune system and are actively engaged in the maintenance of immunological self-tolerance by suppressing reactive T cells.22,23 Tregs hamper effective antitumor immune responses in cancer patients and can serve as a cellular target to evoke or augment
Preliminary work showed that tumor hypoxia promotes the recruitment of Tregs through induction of expression of the chemokine CCL28, which, in turn, promotes tumor tolerance and angiogenesis. Another study suggested that CCR4 antagonists, an emergent class of Treg inhibitor, block recruitment of Tregs mediated by the chemokines CCL22 and CCL17. In this study we found that TRX induces an immunosuppressive microenvironment in melanoma resulting in an abundance of Tregs. As a likely mechanism by which Tregs become abundant in tumor tissues, tumor cells produce TRX, which chemo-attracts and recruits Tregs to the lesion. This mechanism of TRX is similar to that of CXCL12, which is secreted by ovarian cancer cells in the tumor microenvironment and induces selective migration of Tregs. We also found that TRX has a TGFβ-like effect on Teff cells in stimulating the conversion of conventional T cells to Tregs. Finally, we demonstrated that a survival advantage accompanied by tumor-infiltrating Tregs occurs when TRX is abundant in the tumor microenvironment. Thus, these different mechanisms of TRX are likely to function together to create a tumor-permissive environment under the direction of Tregs (Fig. S7).

One limitation of our study is that we focused on the impact of TRX on the tumor microenvironment but we have no data regarding TRX function on tumor cells. In previous studies, antioxidants have been shown to provide protective effects against chemically-induced lung cancer, and it is possible that high amounts of ROS are required for tumor development. However, experimental studies and large clinical trials quite convincingly suggest that certain antioxidants, including carotenoids, vitamins, and NAC, are contraindicated for the prevention of antitumor immunity. Preliminary work showed that tumor hypoxia promotes the recruitment of Tregs through induction of expression of the chemokine CCL28, which, in turn, promotes tumor tolerance and angiogenesis. Another study suggested that CCR4 antagonists, an emergent class of Treg inhibitor, block recruitment of Tregs mediated by the chemokines CCL22 and CCL17. In this study we found that TRX induces an immunosuppressive microenvironment in melanoma resulting in an abundance of Tregs. As a likely mechanism by which Tregs become abundant in tumor tissues, tumor cells produce TRX, which chemo-attracts and recruits Tregs to the lesion. This mechanism of TRX is similar to that of CXCL12, which is secreted by ovarian cancer cells in the tumor microenvironment and induces selective migration of Tregs. We also found that TRX has a TGFβ-like effect on Teff cells in stimulating the conversion of conventional T cells to Tregs. Finally, we demonstrated that a survival advantage accompanied by tumor-infiltrating Tregs occurs when TRX is abundant in the tumor microenvironment. Thus, these different mechanisms of TRX are likely to function together to create a tumor-permissive environment under the direction of Tregs (Fig. S7).

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lung cancer, with their use associated instead with tumor growth. Importantly, our work suggests that the antioxidant factor TRX induces Tregs to generate an immunotolerant tumor microenvironment to promote melanoma metastasis. Thus TRX provides an attractive target for therapy based on multiple criteria, and its association with tumor progression in human melanoma suggests that it might serve as an effective serum biomarker for selecting patients for immunotherapy in the future.

Materials and Methods

Patient material
The melanoma tissue microarray (contains 10 cases of primary melanoma, 10 case of malignant melanoma and 10 cases of nevi, respectively; US Biomax Inc., Rockville, MD) was used to assess potential differences in TRX and FOXP3 expression between samples. Melanoma biopsies and serum were obtained from patients with informed consent. All the study was approved by the institutional review board of The First affiliated Hospital of Jinan University, Guangzhou, China.

Animals
All animal work was approved by the appropriate Ethics and Animal Care and Use Committee of Jinan University, Guangzhou, China. 8-week-old C57BL/6 mice were obtained from the Vital River Laboratories experimental animal technical Co., LTD (Beijing, China). Mice were kept in a 12-hour light/dark cycle with food and water. All efforts were made to minimize animal suffering. To establish a melanoma metastasis model, 5 x 10^5 B16-F10 cells were tail vein injected into C57/B6J mice. After 15 d, mice were sacrificed, their lungs excised and the number of metastases on the lung surface counted. For antibody treatment, at day 15 following tumor cell injection mice were administered 200 μg anti-TRX antibody (clone C63C6, Cell Signaling Technology, USA) or IgG control intravenously. For each of assays, results are expressed as mean ± standard error of mean (SEM).

Cell culture, transfection and stable cell line generation
B16-F10 and A375 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified essential medium (DMEM) containing 10% FBS. Cells were incubated at 37°C and maintained at 5% CO₂. Prior to transfection, B16-F10 cells were seeded in 25 cm² culture dishes. After 24 h, the cells were transfected with pcDNA3.1-eGFP-TRX vector or pcDNA3.1-eGFP-TRX-shRNA vector.

Figure 5. TRX induces conversion from conventional T cells (Teff) to Treg. (A) Immunofluorescence staining and cytofluorimetric analysis to determine the number of CD4^+CD25^+Foxp3^+ T cells arising upon culture of isolated CD4^+CD25^- T cells with B16 cell conditioned media (CM) in the presence of anti-CD3/CD28 antibodies and 500 U/mL interleukin (IL)-2 for 1–4 d. (B) FACS analysis of CD4^+CD25^-Foxp3^+ and CD4^-CD25^-Foxp3^- T cell ratio when cultured with B16 cell CM in the presence of anti-CD3/CD28 antibodies and 500 U/mL IL-2 for 1–4 d. (C) ELISA measurement of interferon γ (IFNγ), IL-10 and transforming growth factor β (TGFβ) protein levels secreted by isolated CD4^-CD25^- T cells cultured with B16 cell CM for 4 d. (D) Ratio of Tregs arising in isolated CD4^-CD25^- T cells cultured with B16 cell CM in the presence of reduced thioredoxin (rTRX) as determined by FACS (n = 3). (E) Ratio of Tregs arising from isolated CD4^-CD25^- T cells cultured with B16 cell CM in the presence of 1 mg/mL anti-TRX antibody or control IgG antibody (n = 3).
using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. The cells were then selected in DMEM at selected with 800 µg/mL G418 for a week before being screened by flow cytometry for GFP expression. GFP positive cells was isolated and selected in DMEM with 200 µg/mL G418 for months until clone formation. Monoclonal populations were selected by limiting serial dilution and gene stability was verified for at least 5 passages. After selection, stable cell lines were screened by TRX Western blot analysis.

**Treg isolation from tumor and FACS analyses**

Tregs were isolated by Dynabeads FlowComp Mouse CD4^+CD25^+ Treg Cells Kit (Invitrogen) following manufacturer’s instructions. Briefly, after removal of fat, blood, or necrotic areas, tumor tissues were washed in RPMI 1640 containing 50 µg/mL gentamicin (Invitrogen) and cut into 0.5 mm³ pieces in a Petri dish while covered with RPMI 1640 plus antibiotics. Tumor fragments were transferred to flasks and dissociated using 0.05% collagenase (Type IV; Sigma) and 0.02% DNase (Type I; Sigma) in the same medium supplemented with 5% (v/v) fetal bovine serum (Invitrogen). The digest was passed through 90 µm and 50 µm nylon mesh to remove clumps, and the filtrate was washed 2 to 3 times in medium followed by centrifugation at 400 g for 10 min. To separate tumor cells from T lymphocytes, the cell suspension was layered onto a discontinuous Ficoll-Paque gradient of 75% over 100% Ficoll-Paque in medium and centrifuged for 800 g for 20 min at room temperature. Immunofluorescence staining was performed using anti-CD4, anti-FoxP3 and anti-CD25 antibodies. The analysis and sort gates were restricted to the small lymphocyte gate as determined by their characteristic forward and side scatter properties. Cytofluorimetric analysis and cell sorting was performed using an EPICS XL flow cytometer (Beckman Coulter).

Monoclonal antibodies to CD4 (clone L3T4, 1: 100 dilution), CD25 (clone 7D4, 1: 100 dilution) and Foxp3 (clone MF23, 1: 100 dilution) were purchased from BD Pharmin-gen (San Jose, CA). FACS analysis was performed by using FACSCalibur and LSR II flow cytometers (Becton Dickinson, Palo Alto, CA) and were analyzed with FlowJo software. For flow cytometric analysis of CD4^+CD25^+Foxp3^+ subsets, cell populations were stained with antibodies against the indicated markers, followed by permeabilization in Fix/Perm buffer, and intracellular staining in Perm/Wash buffer (BD Pharmingen).
Immunohistochemistry

The slides were equilibrated in PBS prior to blocking peroxidase activity with Peroxidase Suppressor (Thermo Scientific). Non-specific antibody binding was blocked by incubating sections with 2.5% horse serum (Invitrogen, Carlsbad, CA). TRX was detected using rabbit anti-TRX (CST, C63C6, 1:100 dilution, USA). Followed by anti-rat Immunpress and visualized using the 3,3′-Diaminobenzidine kit (Sigma-Aldrich, St. Louis, MO, USA). The scoring for each marker was undertaken by an experienced histopathologist who was blinded to results of other markers or case identity. All the slides were imaged on an Aperio Scanscope CS imager, generating 0.4-μm/pixel whole slide images. These images were compiled and analyzed using the Aperio Spectrum software with a pixel count algorithm.

Cell migration assay

To measure the cell migration activity, Transwell assays were done using a modified Boyden chamber (tissue culture treated, 6.5 mm in diameter, 10-μm thick, 8-μm pores; Transwell, Costar, Cambridge, MA). The lower surface of filters was coated with 10 μg/mL collagen. Tregs suspended in serum-free RPMI 1640 containing 0.1% bovine serum albumin were applied to the upper chamber and B16 tumor cell supernatants were added to the lower chamber. Antibody against TRX and control IgG were obtained from CST Company and the recombinant mouse TRX was obtained from Sino Biological Inc. The chambers were incubated at 37°C for 4 h and then fixed and stained. The cells on the lower surface of the filter were counted (2 fields/well) under a microscope using a 10X objective. Each condition was assayed in triplicate, and each experiment was repeated at least 3 times.

Cell apoptosis assessment

Cell apoptosis was detected by the percentage of propidium iodide (PI) and AnnexinV positive cells. Briefly, before induction of apoptosis, cells were plated with an initial cell number of 2 × 10^5/mL in 6-well plates in RPMI containing 10% fetal bovine serum and incubated 24 h. The cells were then collected by centrifugation, fixed, and permeabilized in 70% cold ethanol and incubated at −20°C overnight. Samples were washed with cold PBS and incubated with the DNA-binding dye PI (5 μg/mL) at room temperature 30 min before analysis. Apoptosis was also evaluated by the binding of fluorescein isothiocyanate (FITC) conjugated Annexin V to phosphatidylserine exposed on the cell surface, according to the manufacturer’s instruction. Moreover, a “supravital” PI staining assay was also done (exposure of unfixed cells to PI) together with Annexin V staining to simultaneously detect living, apoptotic, and necrotic cells. Fluorescence resulting from FITC and PI was measured at 530 and 620 nm,
respectively. All the samples were analyzed by EPICS XL flow cytometer (Beckman) equipped with dedicated software. Histograms were then analyzed with EXPO software.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis**

Total RNA was isolated with TRIzol and further purified with the RNeasy Mini kit (Qiagen Ltd., Germany) in accordance with the manufacturer’s instructions. The A260/A280 ratio of total RNA used was typically ≥1.8, and its quality was assessed using the Agilent 2100 Bioanalyzer. qRT-PCR analyses for Foxp3 were performed by using One Step SYBR® PrimeScript™ RT-PCR Kit II according to manufacturer’s instructions. Primers used for this study have been previously reported. The results were analyzed by using AB7700 system software. The level of 18S rRNA was used as an internal standard.

**In vitro Treg suppression assay**

The Treg suppression assay was evaluated by cell proliferation, measured by [3H]-thymidine incorporation. Briefly, for anti-CD3 stimulated cultures, 50,000 freshly isolated T eff cells (used as responder cells), 0.5 mg/mL anti-CD3 and anti-CD28, and up to 10,000 expanded Tregs were put in 96-well plates in triplicate. Then one µCi 3 H thymidine was added for the last 24 h of a 6 d culture. Percentage of suppression was calculated according to: 100 × [1 – (CPM of Treg culture/CPM of non-Treg culture)].

**Enzyme-linked immunosorbent assay (ELISA)**

IL-10, TGFβ and IFNγ secretion was determined using an ELISA kit (eBioscience, North America) following the manufacturer’s instructions. The concentration of TRX in culture supernatants and serum were determined using a TRX ELISA kit (eBioscience, North America) following the manufacturer’s instructions. The A260/A280 ratio of total RNA used was typically ≥1.8, and its quality was assessed using Agilent 2100 Bioanalyzer. qRT-PCR analyses for Foxp3 were performed by using One Step SYBR® PrimeScript™ RT-PCR Kit II according to manufacturer’s instructions. Primers used for this study have been previously reported. The results were analyzed by using AB7700 system software. The level of 18S rRNA was used as an internal standard.

**Reactive Oxygen Species detection**

ROS Reactive Oxygen Species in B16 cell-bearing mice tumor interstitial fluid was determined by Acidram Lumigen PS-3 reagent as previously reported. Briefly, to measure ROS in the medium, the Acidram Lumigen PS-3 assay was developed in a 96-well plate format using the Acidram Lumigen PS-3 reagent, provided as a chemiluminescent substrate for HRP (in the Amersham ECL Plus kit, GE Healthcare). For the purpose of measuring ROS in the culture media, we mixed Reagent A (H2O2 in Tris buffer, Amersham ECL Plus kit, GE Healthcare) and Reagent B (acidran solution in dioxane and ethanol, Amersham ECL Plus kit, GE Healthcare) in a 40:1 ratio; this mixture was named the ALPS-3 substrate.

**Statistical analysis**

All the results are expressed as mean ± standard error of mean (SEM). Statistical differences among groups were analyzed by one-way analysis of variance (ANOVA) with a post-hoc test (after normalization to baseline in the OVX study and the hindlimb-unloading study) to determine group differences in the study parameters. All statistical analyses were performed with SPSS software, version 13.0. The statistical differences between the 2 groups were determined by the Student’s t-test. P < 0.05 was considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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