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Full Length Article

Cathepsin S inhibition changes regulatory T-cell activity in regulating bladder cancer and immune cell proliferation and apoptosis

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ABSTRACT

Regulatory T cells (Tregs) are immune suppressive cells, but their roles in tumor growth have been elusive, depending on tumor type or site. Our prior study demonstrated a role of cathepsin S (CatS) in reducing Treg immunosuppressive activity. Therefore, CatS inhibition in Tregs may exacerbate tumor growth. Using mouse bladder carcinoma MB49 cell subcutaneous implant tumor model, we detected no difference in tumor growth, whether mice were given saline- or CatS inhibitor-treated Tregs. However, mice that received inhibitor-treated Tregs had fewer splenic and tumor Tregs, and lower levels of tumor and splenic cell proliferation than mice that received saline-treated Tregs. *In vitro*, inhibitor-treated Tregs showed lower proliferation and higher apoptosis than saline-treated Tregs when cells were exposed to MB49. In contrast, both types of Tregs showed no difference in proliferation when they were co-cultured with normal splenocytes. Inhibitor-treated Tregs had less apoptosis in splenocytes, but more apoptosis in splenocytes with MB49 conditioned media than saline-treated Tregs. In turn, we detected less proliferation and more apoptosis of MB94 cells after co-culture with inhibitor-treated Tregs, compared with saline-treated Tregs. $B220^+$ B-cell, $CD4^+$ T-cell, and $CD8^+$ T-cell proliferation and apoptosis were also lower in splenocytes co-cultured with inhibitor-treated Tregs than with saline-treated Tregs. Under the same conditions, the addition of cancer cell-conditioned media greatly increased $CD8^+$ T-cell proliferation and reduced $CD8^+$ T-cell apoptosis. These observations suggest that CatS inhibition of Tregs may reduce overall T-cell immunity under normal conditions, but enhance $CD8^+$ T-cell immunity in the presence of cancer cells.

1. Introduction

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are immunosuppressive cells that play a protective role in inflammatory diseases, such as atherosclerosis, abdominal aortic aneurysms, obesity, and diabetes (Ait-Oufella and Tedgui, 2016; Yildiz and Gur, 2007; Meng et al., 2016; Yin et al., 2010; Zhou et al., 2015; Eller et al., 2011; Feuerer et al., 2009). In contrast, Tregs suppress cytotoxic $CD8^+$ T cells in many solid tumors, thereby having a significant adverse effect on tumor-associated overall survival (Shang et al., 2015). In patients with ovarian cancers, tumor Tregs exhibited more potent suppression of $CD8^+$ T cells than those in the peripheral (Bu et al., 2016). Reduced tumor Treg contents were associated with improved overall survival of these patients (Knutson et al., 2015). Tregs in gastric tumors (Kindlund et al., 2016), in the peripheral blood from patients with B cell lymphoma (Wu et al., 2015), or acute B lymphoblastic leukemia (Ateyah et al., 2016) were all elevated. In patients with breast cancer, tumor Treg contents and expression of PD-L1 (programmed death ligand 1) were positively correlated (Li et al., 2016). In a mouse model of lung adenocarcinoma, Tregs in the

Abbreviations: Treg, regulatory T-cell; CatS, cathepsin S; CatK, cathepsin K; CatL, cathepsin L; PD-L1, programmed death ligand 1; PTEN, phosphatase and tensin homolog; TLR7, toll-like receptor-7; WT, wild-type; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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advanced lung tumors suppressed the anti-tumor T-cell responses. Depletion of Tregs caused immune-mediated tumor destruction (Joshi et al., 2015). In a mouse melanoma cell tumor model, Treg-specific depletion of PTEN (phosphatase and tensin homolog), which stabilizes Tregs, reduced tumor growth and inflammation (Saito et al., 2016). In B-cell acute lymphoblastic leukemia or 4T1 mammary carcinoma mice, Treg ablation led to $CD8^+$ T-cell generation, tumor regression, and extended survival (Manlove et al., 2015; Goudin et al., 2016). All these studies point to a detrimental role of Tregs in cancers. However, Tregs also exert no role or even an opposite role in different types of tumors. For example, in patients with multiple myeloma, the effect of treatment with the anti-tumor drug bortezomib was associated with Treg expansion. Treg *ex vivo* expansion decreased multiple myeloma viability (Ercetin et al., 2016). In colorectal cancers, Treg infiltration indicated better prognosis (deLeeuw et al., 2012; Sinicrope et al., 2009; Salama et al., 2009; Frey et al., 2010). In head and neck or oesophageal cancers, the progonostic role of Tregs was highly influenced by tumor site, and correlated with the molecular subtype and tumor stage (Shang et al., 2015). Therefore, the role of Tregs in tumors can be complicated and may depend on the types of the tumors.

Bladder carcinoma is the fifth most common cancer with increased incidence worldwide (Watts et al., 2011; Dighe et al., 2011). Tumor specimens and peripheral blood mononuclear cells from patients with bladder cancer contain elevated numbers of Tregs (Loskog et al., 2007; Chi et al., 2010; Parodi et al., 2016). Patients with high content of tumor infiltration of Tregs may have elevated incidence of recurrence (Parodi et al., 2016), although a direct role of Tregs in bladder cancer has not been tested. Of note, the role of Tregs in inflammatory diseases or in cancers may vary depending on the subtypes of Tregs. A recent study reported a detrimental role of fat-resident Tregs that contribute to age-associated insulin resistance. Selective depletion of this Treg population increased adipose tissue insulin sensitivity (Bapat et al., 2015). In colorectal cancers, tumor infiltration of non-immunosuppressive $Foxp3^{lo}$ Tregs with no expression of the naive T cell marker CD45RA and instability of Foxp3 showed better prognosis than immunosuppression-competent $Foxp3^{hi}$ Tregs (Saito et al., 2016). Therefore, either depletion of $Foxp3^{hi}$ Tregs or local increase of $Foxp3^{lo}$ Tregs suppressed or prevented tumor formation.

We recently found that Tregs had increased immunosuppressive activity after a brief treatment with a small molecule inhibitor of cathepsin S (CatS), a lysosomal cysteine protease that mediates lysosomal protein proteolysis. CatS participates in toll-like receptor-7 (TLR7) processing in Tregs, thereby changing the TLR7 downstream signaling and cytokine profile leading to elevated immunosuppressive activity (Shi unpublished data). Here we test whether Tregs with or without CatS inhibition affect bladder tumor growth in a mouse bladder cancer MB49 cell subcutaneous implantation model.

2. Materials and methods

2.1. Mice, tumor cell culture, and tumor model

Wild-type (WT) C57BL/6 mice and CD45.1 transgenic mice (C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, ME). MB49 cells are chemically induced murine bladder carcinoma cells derived from C57BL/6 male mice (Summerhayes and Franks, 1979) (American Type Culture Collection, ATCC, Manassas, VA). MB49 cells were maintained in RPMI 1640 medium (Gibco, Big Cabin, OK) supplemented with 10% fetal calf serum, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), penicillin (100 IU/mL), and streptomycin (100 μg/mL), 5×10^{-5} M 2-mecaptoethnal, and 2 mM L-glutamine. Each 9-week-old WT recipient mouse received subcutaneous implantation of 2×10^6 MB49 cells on the right flank. Tumor growth was measured every other day and recorded as length × depth × width in cubic millimeters (mm^3).

2.2. Treg purification and adoptive transfer

To perform Treg adoptive transfer in mice with tumors, we purified splenic $CD4^+CD25^+$ Treg cells from C57BL/6 WT or C57BL/6 CD45.1 transgenic mice according to the manufacturer's instructions (Miltenyi Biotec, Inc., Auburn, CA). The resulting $CD4^+CD25^+$ Treg cells were also further purified with cell sorter (The BD FACSAira™ Cell Sorter, BD Biosciences, San Jose, CA). Treg purity was confirmed by FACS and anti-Foxp3 antibody-mediated immunofluorescent staining. Tregs from WT and CD45.1 transgenic mice were treated with a CatS inhibitor at 10 μg/mL or phosphate-buffered saline (PBS) overnight before adoptive transfer. This inhibitor is a derivative of LY3000328 from a recently completed phase I trial (clinicaltrials.gov/show/NCT01515358, Payne et al., 2014). It selectively inhibited CatS activity at 10 μg/mL (Eli Lilly and Company unpublished data). Each 9-week-old male C57BL/6 WT mouse received intravenous injection of 5×10^6 donor Treg cells three days after mice received MB49 cell subcutaneous implantation. We measured tumor sizes on days 1, 3, 5, and 7 after the adoptive transfer. On day 7, mice were sacrificed, and splenocytes and tumor tissue single cell preparations were analyzed for CD4, CD25 and Foxp3 by FACS analysis. Spleen and tumor tissue were also collected to prepare 5 μm frozen sections for immunohistochemical analysis.

2.3. Immunohistochemistry

Frozen tumor and spleen sections were prepared for immunohistochemical staining using FITC-conjugated anti-mouse CD45.1 monoclonal antibody (1:1000, Abcam, Cambridge, MA), anti-mouse Ki67 monoclonal antibody (1:400, Thermo Fisher Scientific, Waltham, MA), and anti-mouse CD31 monoclonal antibody (1:1500, BD Biosciences). Frozen tumor and spleen sections were also prepared for histological detection of apoptotic cells using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit according to the manufacturer (EMD Millipore, Billerica, MA, USA).

2.4. Treg cell co-cultures

$CD4^+CD25^+$ Tregs were isolated from total $CD4^+$ T cells in spleens from $CD45.1^+$ transgenic mice using a $CD4^+CD25^+$ Treg isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Cambridge, MA). FACS analysis confirmed the purity of each preparation of greater than 93%. $CD4^+CD25^+$ Tregs (1×10^6) were treated with a CatS inhibitor (10 μg/mL) or PBS for 24 h. Mouse bladder carcinoma MB49 cells were cultured in RPMI 1640 complete medium to 90% confluence. Splenocytes were isolated from C57BL/6 WT mice. To assess the interactions between Tregs and MB49 tumor cells or with WT splenocytes, we collected tumor cells and WT splenocytes and added to Tregs on a Costar® 6-well plate at a 1:1 ratio. For splenocyte and Treg co-cultures, we also added MB49 tumor cell-conditioned media. After 24 h of co-culture, Tregs or MB49 tumor cells were collected, washed, and used for FACS analysis.

2.5. Flow cytometry analysis of Tregs in tumors and spleens from MB49-implanted mice

Mouse splenocytes were prepared by removing red blood cells as we described previously (Shi et al., 1999). Tumor single