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Tim3/Gal9 interactions between T cells and monocytes result in an immunosuppressive feedback loop that inhibits Th1 responses in osteosarcoma patients

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ABSTRACT

The Tim3/Gal9 pathway is associated with immunosuppression and worse clinical outcome in multiple cancers. To illustrate the specific mechanism of Tim3/Gal9 interaction in osteosarcoma, we examined expression, function, and regulation of Tim3/Gal9 in various cells from osteosarcoma patients. Data showed that $CD4^+$ T cells, $CD8^+$ T cells, and monocytes from both peripheral blood and tumor of osteosarcoma patients contained high frequencies of $Tim3^+$ cells, while the Gal9 expression was primarily found in regulatory T cells (Tregs) from osteosarcoma patients and was elevated compared to that in non-cancer controls. The $Tim3^+$ $CD4^+$ and $CD8^+$ T cells presented lower proliferation capacity compared to their $Tim3^-$ counterparts, which could be reverted by blocking Tim3 or Gal9. Interestingly, purified $Tim3^+$ $CD4^+$ T cells secreted more interferon gamma (IFNγ) than purified $Tim3^-$ $CD4^+$ T cells, but IFNγ production by $Tim3^+$ $CD4^+$ T cells was vulnerable to Gal9-mediated suppression. In monocytes, Tim3 expression was associated with high interleukin (IL)-10 and low IL-12 cytokine secretion profile. Exogenous recombinant Gal9, as well as $CD4^+CD25^+$ Treg supernatant, further decreased IL-12 expression in monocytes. In $CD4^+$ T cell-monocyte coculture experiments, $Tim3^+$ monocytes inhibited IFNγ expression from total $CD4^+$ T cells and the development of IFNγ response in naive $CD4^+$ T cells. Blocking the Tim3/Gal9 pathway reverted these effects. Together, these results suggested that in osteosarcoma patients, Tim3 expression did not directly mediate immune suppression, but the interaction between $Tim3^+$ T cells and monocytes, naive $CD4^+$ T cells, and Gal9-expressing $CD4^+CD25^+$ Tregs could resulting in progressive suppression of Th1 responses.

1. Introduction

Osteosarcoma is a common primary malignant bone tumor with bimodal age distribution in adolescent teens and older adults [1]. Current treatment typically consists of multi-agent pre-operative chemotherapy, surgical removal of the tumor, and then post-operative chemotherapy. However, this strategy has not been very effective in patients with metastasized cancer, despite the continuing improvements in chemotherapy. Furthermore, most osteosarcomas exhibit a high degree of genetic instability but do not carry recurrent mutations that could serve as specific treatment targets [2]. Therefore, it is critical to develop novel therapeutic strategies for the treatment of osteosarcoma.

The metabolism of the bone shares many common factors and pathways with immune processes [3]. The addition of Liposomal muramyl tripeptide phosphatidylethanolamine, an activator of monocyte and macrophage responses, in the chemotherapy of osteosarcoma has shown signs of improvement in overall survival and disease-free survival [4]. Furthermore, blockade of the immune checkpoint molecules PD-1/PD-L1, which supposedly enhances the antitumor immune responses, has generated significant clinical benefits in the treatment of multiple solid tumors [5]. These lines of evidence suggest that the patients' immune system could be employed in the treatment of osteosarcoma.

T cell immunoglobulin and mucin domain-containing molecule 3 (Tim3) was first discovered as an identifier of Th1 effectors that expressed interferon gamma (IFNγ) [6]. Later on, expression of Tim3 was found on other mononuclear cell (MNC) subsets, including $CD8^+$ T cells, natural killer cells, dendritic cells, monocytes, and macrophages with variable specific functions. However, all these functions shared a common theme of immune suppression. Anti-Tim3 antibody in mice exacerbated experimental autoimmune encephalomyelitis (EAE) [6], while the Tim3 ligand galectin 9 (Gal9) ameliorated EAE [7] Tim3 is also required for inducing peripheral tolerance, as Tim3-deficiency and Tim3-Ig fusion protein both resulted in defects in the generation

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of antigen-specific tolerance in mice [8,9]. In cancer, Tim3 and/or Gal9 expression have been identified on tumor cells as well as immune cells. For example, in melanoma patients, approximately 30% of tumor-antigen NY-ESO-1-specific $CD8^+$ T cells expressed Tim3, and approximately one-third tumor-infiltrating T cells expressed Tim3 in non-small cell lung cancer patients [10,11]. In both cases, co-expression of Tim3 and PD-1 was associated with impairment in proliferation and effector cytokine secretion. In HBV-related hepatocellular carcinoma, Tim3/Gal9 on T cells and antigen-presenting cells mediated T cell dysfunction and predicted poor clinical outcomes. These deleterious effects of the Tim3/Gal9 pathway in cancer immunity suggested that it might serve as an effective target in osteosarcoma immunotherapy.

In osteosarcoma, it has been demonstrated that Tim3 was expressed by tumor cells and T cells [12,13]. The expression level of Tim3 on T cells was inversely correlated with IFNγ production and predicted poor clinical outcomes [13]. The specific mechanism of action of the Tim3/Gal9 pathway in osteosarcoma is still unclear. Furthermore, the monocytes/macrophages are known to express high levels of Tim3 [14], and their role in osteosarcoma has not been explored. In this study, we demonstrated that in osteosarcoma patients, the Tim3/Gal9 interactions involved actions of both monocytes/macrophages and T cells, which resulted in a feedback loop that progressively inhibited proinflammatory immune responses.

2. Methods

2.1. Patients

This study recruited 13 patients with osteosarcoma at the long bones of upper and lower limbs and 13 matching controls, consisting of 4 females and 9 males in each group. All patients and controls were between 12 and 25 years of age. Diagnosis, sample collection, and treatment were all performed in the No.401 Hospital of PLA. Clinical data were further analyzed by DICAT (Vancouver, Canada). Written informed consent was obtained from each patient or patient's guardian and control. The ethics board of No.401 Hospital of PLA approved all study procedures. Patients were excluded from this study if one or more of the following conditions were present: other malignancies, clinically significant autoimmunity, acute or chronic infections by HBV, HCV, or HIV, and older age.

2.2. Isolation of MNCs and MNC subsets

After harvesting, heparinized PB was immediately processed by standard Ficoll (GE Healthcare Life Sciences) density gradient centrifugation to harvest MNCs. Tumors were transferred directly from the operation room to sterile biosafety cabinet in PBS with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin [Invitrogen]), and then minced into small fragments with scissors. The pieces were then placed in enzymatic digestion media (RPMI 1640 [Invitrogen] supplemented with 2 mg/mL collagenase and 50 U/mL hyaluronidase [Sigma]) for 2 × 1 h incubation at 37 °C with shaking. The resulting cell suspension was then filtered through a 70-μm strainer and then processed by standard Ficoll density gradient centrifugation to harvest MNCs. All cell cultures were maintained in RPMI 1640 supplemented with L-glutamine (Gibco), antibiotics and 10% autologous sera in a 37 °C CO_2 humidifier.

All purification of MNC subsets was performed with magnetic beads. Pure $CD4^+$ T cells, $CD8^+$ T cells, monocytes/TAMs, and naive T cells were obtained by negative selection using EasySep Human $CD4^+$ T Cell, $CD8^+$ T Cell, Monocyte, and Naive $CD4^+$ T Cell Enrichment Kits (Stemcell Technologies) following manufacturer's instructions. The $Tim3^+$, $Tim3^-$, $CD25^+$, $CD25^-$, $CXCR3^+$, and $CCR6^+$ $CD4^+$ T cell subsets were purified by first labeling $CD4^+$ T cells using each of the PE-conjugated anti-human Tim3, CD25, CXCR3, or CCR6 mAbs, and then separating labeled and unlabeled cells by EasySep Human PE-positive selection kit. To prevent blocking effects of PE-conjugated anti-Tim3 mAb (clone 2E2) during labeling and isolation, the amount of PE-conjugated anti-Tim3 mAb used in each experiment was determined by serial dilution such that the lowest level of anti-Tim3 mAb without diminishing the frequency of labeled cells was used (0.25 μg/mL for labeling $CD4^+$ T cells. 0.5 μg/mL for labeling monocytes).

2.3. Flow cytometry

Surface staining of fluorescent anti-human CD3, CD4, CD8, CD14, CD25, CXCR3, CCR6 and Gal9 mAbs (BioLegend) was performed using concentrations recommended by the manufacturer, while the concentration of anti-human Tim3 (clone 2E2) was determined by serial dilution to the lowest level without diminishing the frequency of labeled cells (0.25 μg/mL for labeling $CD4^+$ T cells. 0.5 μg/mL for labeling monocytes). The frequency of dividing cells was determined using the CellTrace CFSE Cell Proliferation Kit (Thermo Fisher) following manufacturer's instructions.

2.4. Quantitative real time PCR (RT-PCR)

Quantitative RT-PCR of Gal9 in each sample was performed using the SuperScript IV VILO Master Mix Kit and the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) in a 25 μL reaction, in triplicates. Ct value was measured in the ABI PRISM 7000 Sequence Detection System (Thermo Fisher). The amount of RNA was normalized by 18S rRNA. The primer sets included the following: Galectin-9: forward primer 5′-ctttcatcaccaccattctg-3′, reverse primer 5′-atgtggaacctctgagcactg-3′; GAPDH: forward primer 5′-acagtcagccgcatcttctt-3′, reverse primer 5′-tggaagatggtgatgggatg-3′; and 18S: forward primer 5′-cccagtaagtgcgggtcataa-3′, reverse primer 5′-ccgagggcctcactaaacc-3′.

2.5. ELISAs

Commercially available ELISA kits were used to detect secreted cytokine levels in each sample, following instructions provided by the manufacturers. These kits included Human IFN gamma ELISA Ready-Set-Go (eBioscience), Human IL-10 ELISA Ready-Set-Go (eBioscience), Human IL-12 (p70) ELISA MAX Deluxe (BioLegend), and Human Galectin-9 Quantikine ELISA Kit (R&D Systems). The cell supernatant was serial diluted such that the level of cytokines was well within the provided detection range in each kit. All experiments were performed in triplicates. In some experiments, carrier-free recombinant human Gal9 (R&D Systems) was added into the cell culture.

2.6. Statistics

Comparisons between two groups were examined by Mann-Whitney test. Comparisons between three or more groups were examined by Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparisons test. The two-tailed P value needed to be <0.05 to be considered significant. The software Prism 6.0c (GraphPad) was used for statistical tests.

3. Results

3.1. Osteosarcoma patients presented high Tim3/Gal9 expression on $CD4^+$ T cells and high Tim-3^+ on $CD8^+$ T cells and monocytes/TAMs

We first examined the Tim3 and Gal9 expression in mononuclear cells (MNCs) from non-cancer controls and osteosarcoma patients. Tim3 is a cell surface molecule specifically expressed by IFNγ-differentiating Th1 cells, functionally impaired $CD8^+$ T cells, and resting state macrophages [15]. In peripheral blood (PB), a higher percentage of $CD4^+$ T cells, $CD8^+$ T cells, and monocytes from osteosarcoma patients expressed Tim3, than those from non-cancer controls (Fig. 1A). Gal9 is a secreted ligand of Tim3; its translocation intermediate can also be found on the cell surface [16]. In PB, we found that only $CD4^+$ T cells presented