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*Original Article*

# Orbital rhabdomyosarcoma tumor-associated antigen-specific T cells had a Th1-to-Treg ratio than hemaglutinin- and tetanus toxin-specific T cells

Xiuming Liu[1*], Weidong Feng[2*]

[1]*Department of Ophthalmology, Huai'an First People's Hospital, Huai'an 223300, Jiangsu, China;* [2]*Department of Ophthalmology, Huai'an Women and Children's Hospital, Huai'an 223002, Jiangsu, China.* [*]*Equal contributors.*

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**Abstract:** Orbital rhabdomyosarcoma (RMS) is a malignant tumor most frequently seen in children and teens, with a poor 5-year survival rate. To improve our understanding of anti-tumor immunity to orbital RMS and aid T cell-based immunotherapy research, we analyzed the phenotype and functions of preexisting RMS-specific T cells in orbital RMS patients. We first identified RMS-specific $CD4^+$ and $CD8^+$ T cells as proliferating cells in response to RMS antigen. Compared to hemaglutinin- and tetanus toxin-specific T cells, RMS-specific $CD4^+$ T cells had fewer rounds of proliferation and lower Th1-to-Treg ratio, while RMS-specific $CD8^+$ T cells were lower in frequency, had fewer rounds of proliferation, and presented less CD107a and IFN-gamma production. Together, we discovered a novel and tumor-specific suppression of RMS-specific T cells.

**Keywords:** Orbital rhabdomyosarcoma, T cell

## Introduction

Rhabdomyosarcoma (RMS) is a malignant tumor most frequently seen in children and teens, and represents approximately 8% of all childhood tumors [1, 2]. The head and neck region and in particular, the orbit, represent a major anatomic site for RMS. Orbital RMS is the most common primary orbital malignancy in children. With current treatment options, including multistage chemotherapy, radiation therapy and/or resection of the primary tumor, the 5-year survival rates of the most common RMS subtypes, the embryonal RMS and alveolar RMS, are only approximately 66% and 54%, respectively [3]. Recurrence can happen in nearly 30% of children, and the 5-year survival rate of recurrent RMS is at a meager 17% approximately [4]. Moreover, substantial adverse effects resulting from current standard treatment regimen are observed in adult survivors of childhood cancer, with a cumulative incidence of chronic health conditions at 73% and a cumulative incidence of severe, life-threatening conditions or death at 42% [5]. Therefore, research and development of new treatment strategies against RMS for improved efficacy and safety is urgently needed.

In recent years, T cell-based immunotherapies for tumor rejection have shown promising results [6], and have been investigated for potential uses in treating RMS. A murine model of RMS was confirmed immunogenic and could respond to T cell inflammation [7]. $CD4^+$ T helper cell responses toward RMS antigen could also be induced in vitro by stimulating naive $CD4^+$ T cells with RMS tumor lysate-pulsed dendritic cells (DCs) [8]. These proof-of-concept studies have demonstrated the feasibility of using T cell-based immunotherapy in treating RMS, but the question of whether such therapies can be effective in vivo remains an unresolved issue.

T cell exhaustion and anergy are frequently observed in various cancers [9-11]. Inhibitory receptors, such as TIM-3, LAG-3, PD-1 and CTLA-4, were upregulated on circulating and/or tumor-infiltrating T cells in cancer patients [12-15]. An overrepresentation of regulatory T cells (Tregs) in cancers was shown to impair proin-

flammatory T helper 1 cell (Th1) responses, deplete T cell development factor interleukin (IL-2), induce apoptosis of activated $CD8^+$ cytotoxic T cells, and mediate suppression in the tumor microenvironment [16-20]. On the other hand, lower Treg frequencies and more robust Th1 and $CD8^+$ cytotoxic T cell responses were associated with better prognosis [21, 22]. In RMS patients, the presence, frequency, phenotypes and functions of RMS antigen-specific T cells have not been examined. Answer to these questions will provide insight into the inflammatory status in RMS patients, as well as the feasibility of applying immunotherapy in treating RMS. In this study, we examined RMS antigen-specific T cells in orbital RMS patients.

## Materials and methods

### *Generation of DCs*

A total of 34 orbital RMS patients were recruited in the study. The diagnoses were confirmed by histopathological examinations. Patients did not receive other treatments before surgery. The review board of Huai'an First People's Hospital approved the use of blood samples in this research. Written informed consent was obtained from the guardians of all participants. PBMCs from RMS patients were collected using Ficoll gradient centrifugation from venous blood before surgery. Monocytes were isolated and cultured with medium containing 280 U/mL granulocyte macrophage colony stimulating factor (GM-CSF) and 50 ng/mL IL-4 for 6 days. Medium was replenished every 2 days. DCs were harvested on Day 6.

### *Pulsing DCs with tumor lysates, hemaglutinin, and tetanus toxin*

Primary resected tumors were washed in PBS, cut into 2-mm cubes and gently ground in a tube with medium containing 0.3 mg/mL collagenase (Worthington Biochemical) for 1 h at 37°C with shaking. The suspension was filtered by a 70-μm cell strainer to remove undigested connective tissues. The resulting solution containing single primary tumor cell suspensions were counted and resuspended in serum-free RPMI 1640 at $10^8$ cells per mL, and were then subjected to three freeze-thaw cycles alternating between -80°C and 37°C. Cell debris was removed by centrifuging for 5 min at 12,000 rpm. Supernatant proteins were collected and added to the DC cultures at 1-to-1 tumor cell-to-DC ratio. For the loading of hemaglutinin and tetanus toxin, unadjuvated seasonal influenza vaccine (Sanofi Pasteur) containing three hemaglutinin strains and tetanus toxin (Sigma) were added to DCs at 1 μg per $10^6$ cells. The antigen-loaded DCs were then cultured in RPMI 1640 supplemented with 5% human male AB serum, 0.1 mmol/L MEM nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 50 μg/mL gentamicin for 18 hours at 37°C. Right before use, DCs were washed twice with complete culture medium to remove excess antigen.

### *Isolation and stimulation of primary T cells*

Primary $CD4^+$ and $CD8^+$ T cells were isolated from patient PBMCs using human $CD4^+$ and $CD8^+$ Isolation Kits (Miltenyi) using manufacturer's protocols, and then treated with Cell Trace CFSE Cell Proliferation Kit (Life Technologies) following manufacturer's protocol. The isolated T cells were then incubated with antigen-pulsed DCs at $10^5$ T cells-to-$10^4$ DCs (10-to-1 ratio) per 200 μL culture medium per well in a 96-well plate, for 6 days at 37°C. For intracellular cytokine measurement, anti-CD107a antibody and brefeldin A was added to the cells 12 hours before the end of the co-incubation. Cells were then harvested to prepare for flow cytometry.

### *Flow cytometry*

Cells were first incubated with Fixable Violet Dead Cell Stain (Invitrogen, USA) and 3 μg/mL surface antibodies, including anti-human CD3, CD4, CD8 and CD25 for 30 min at 4°C, after which cells were washed twice and treated with Fixation/Permeabilization Kit (eBioscience) following the manufacturer's protocol. Cells were then stained with anti-human IFN-g and Foxp3 antibodies (BD, USA) for 30 min at 4°C, washed twice and prepared for sample acquisition on a Becton Dickinson FACSCanto cytometer. Data were analyzed using FlowJo (TreeStar).

### *Statistical analysis*

Differences between three groups were compared by ordinary or Kruskal-Wallis one-way ANOVA, followed by Tukey's or Dunn's multiple comparisons test, depending on whether the data distribution passed the D'Agostino and

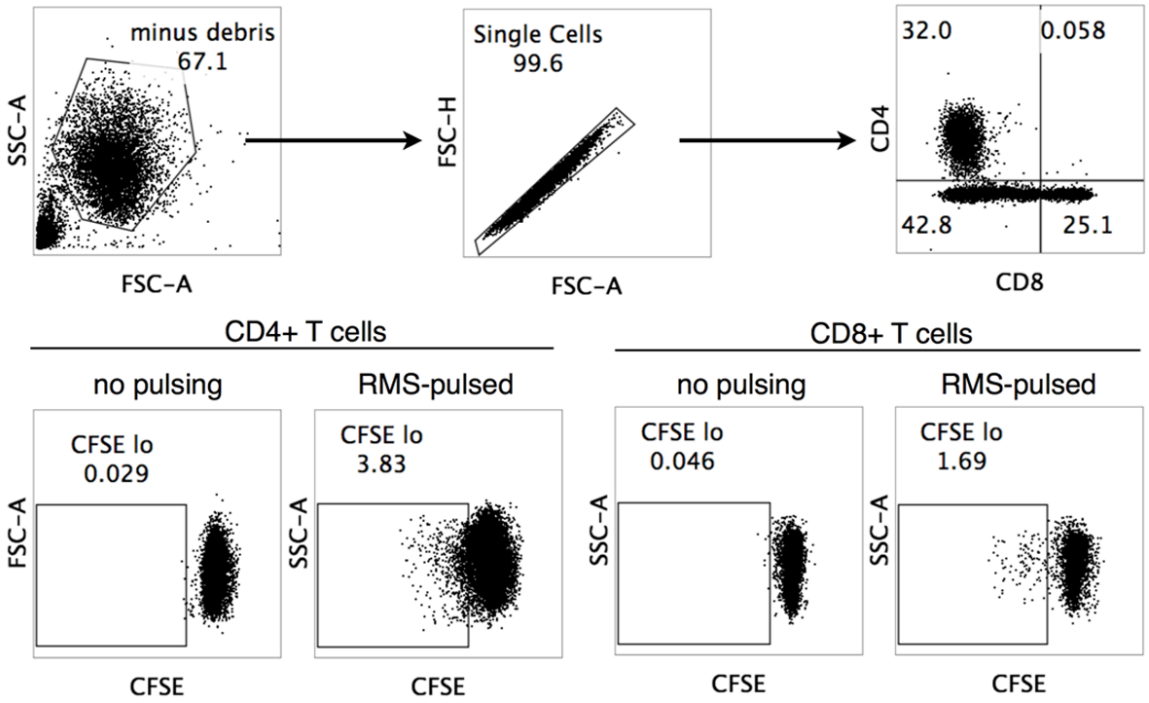


**Figure 1.** Identification of RMS-specific cells by CFSE$^{lo}$ staining. CD4$^+$ and CD8$^+$ T cells were negatively purified and processed with CSFE staining kit, and were then cocultured with RMS tumor antigen-pulsed DCs for 6 days, after which the cells were stained with anti-human CD4 and CD8 antibodies and were examined by flow cytometry. The gating strategy in FlowJo software is shown, and the CFSE$^{lo}$ cells were considered specific for RMS antigen.

Pearson normality test or not. All data were represented as mean ± standard deviation. *P < 0.05. **P < 0.01. ***P < 0.001.

## Results

*Identification of RMS antigen-specific T cells*

T cell receptor (TCR) recognizes peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells. The engagement of TCR-MHC then leads to the assemblage and phosphorylation of the CD3 complex and subsequent signal transduction and T cell proliferation. To allow identification of circulating RMS tumor antigen-specific memory T cells in the peripheral blood of RMS patients, we first pre-loaded RMS antigen obtained from resected primary RMS tumor on autologous DCs based on a previously described method [8]. The patient's memory CD4$^+$ and CD8$^+$ T cells were then negatively selected from peripheral blood mononuclear cells (PBMCs) and were incubated together with antigen-pulsed DCs. We found that T cells incubated with antigen-pulsed DCs demonstrated significantly higher frequencies of CFSE$^{lo}$ cells, compared to T cells incubated with un-pulsed DCs, suggesting antigen-recognition and proliferation in a subset of T cells (**Figure 1**). Both CD4$^+$ and CD8$^+$ T cells with specificity to RMS-tumor-antigen were found.

*RMS-specific CD4$^+$ T cells had lower proliferation than hemaglutinin and tetanus toxin-specific CD4$^+$ T cells*

To examine the composition and functions of RMS-specific CD4$^+$ T cells, we examined the surface marker, intracellular cytokine, and transcription factor expressions in these cells by flow cytometry. Since all patients in our study cohort were previously vaccinated against tetanus toxin, while 10 out of 12 were vaccinated against the seasonal influenza, for comparison, hemaglutinin and tetanus toxin-specific T cells were identified by the same method in these patients. By the percentage of CFSE$^{lo}$ CD4$^+$ T cells, the frequencies of RMS-specific CD4$^+$ T cells were comparable with that of hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells, and all three groups showed high patient-to-patient variability (**Figure 2A**). Interestingly, the CFSE$^{lo}$ cells in RMS-specific CD4$^+$ T cells had signifi-

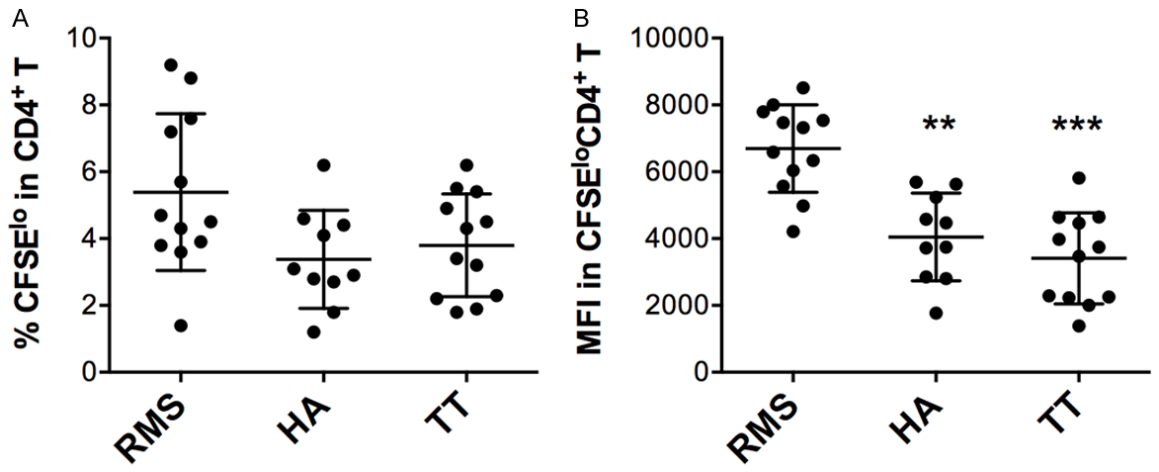


**Figure 2.** Frequency and proliferation of RMS-, hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells in RMS patients. The RMS- and tetanus toxin (TT)-specific CD4$^+$ T cells were examined in all 12 subjects, while the hemaglutinin (HA)-specific CD4$^+$ T cells were examined in 10 subjects with clear seasonal influenza vaccination records out of 12 total subjects. The frequency was shown as the percentage of CFSE$^{lo}$ cells in CD4$^+$ T cells in (A), and the proliferative capacity was shown as the mean fluorescence intensity (MFI) in (B).

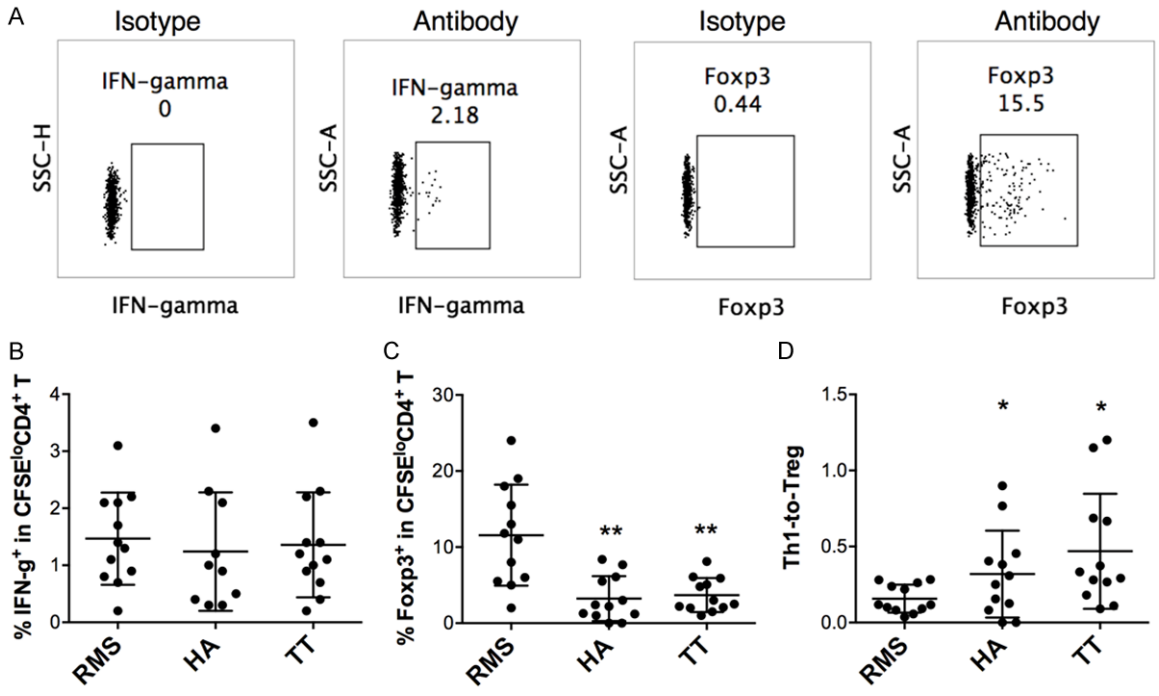


**Figure 3.** Composition of RMS-, hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells in RMS patients. The antigen presentation, CFSE treatment and surface staining were done as previously described. IFN-gamma and Foxp3 expression were subsequently stained intracellularly. (A) shows the gating strategy of IFN-gamma (IFN-g)- and Foxp3-expressing cells in CFSE$^{lo}$ CD4$^+$ T cells responding to RMS-pulsed DCs in one representative individual. The frequencies of IFN-gamma- and Foxp3-expressing cells in CSFE$^{lo}$ CD4$^+$ T cells were shown in (B and C), respectively, and (D) shows the ratios of Th1-to-Treg cells in CFSE$^{lo}$ CD4$^+$ T cells, which were calculated by the frequency of IFN-gamma-expressing cells divided by the frequency of Foxp3-expressing cells.

cantly higher mean fluorescence intensity than hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells, suggesting fewer rounds of duplication in RMS-specific CD4$^+$ T cells (**Figure 2B**).

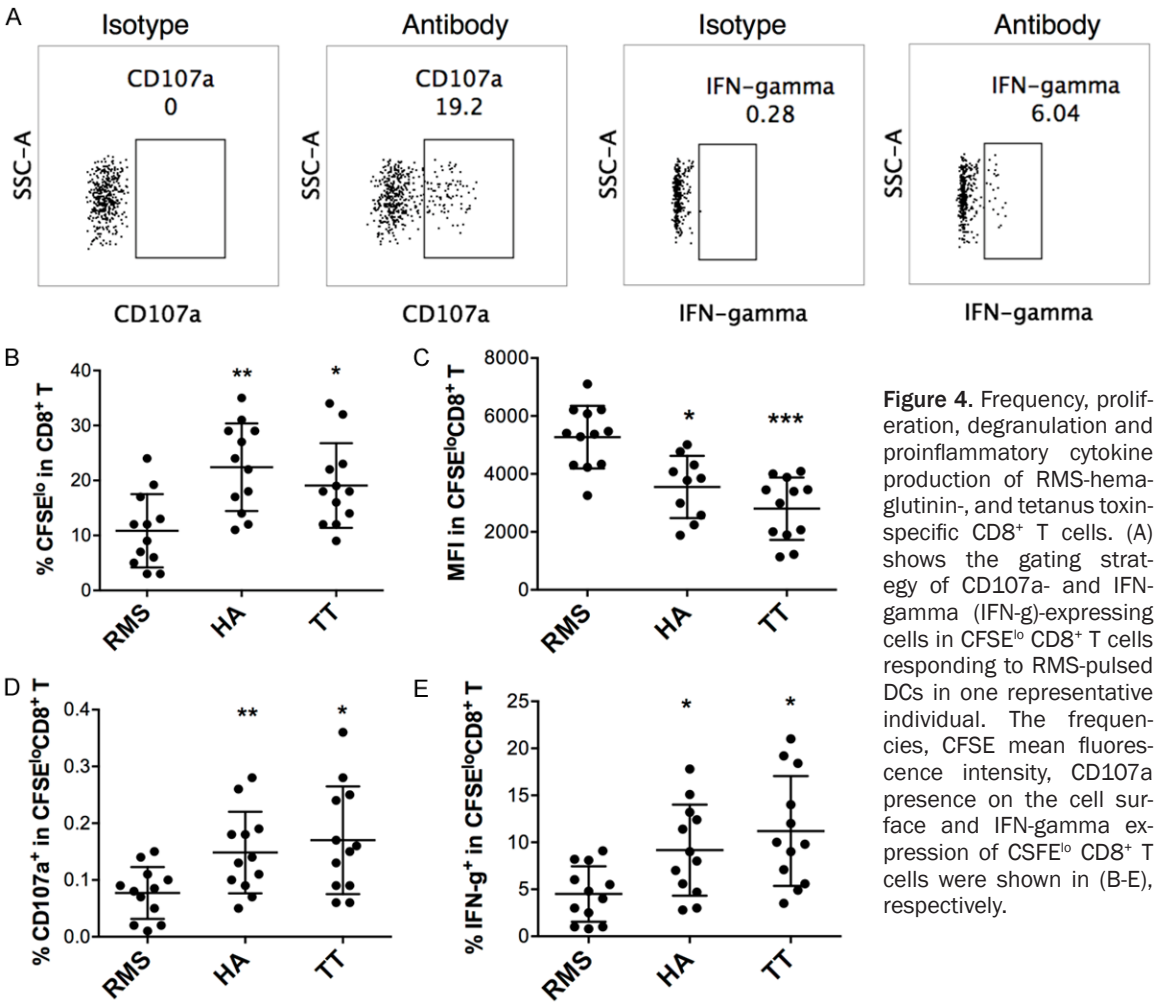


**Figure 4.** Frequency, proliferation, degranulation and proinflammatory cytokine production of RMS-hemaglutinin-, and tetanus toxin-specific CD8$^+$ T cells. (A) shows the gating strategy of CD107a- and IFN-gamma (IFN-g)-expressing cells in CFSE$^{lo}$ CD8$^+$ T cells responding to RMS-pulsed DCs in one representative individual. The frequencies, CFSE mean fluorescence intensity, CD107a presence on the cell surface and IFN-gamma expression of CSFE$^{lo}$ CD8$^+$ T cells were shown in (B-E), respectively.

*RMS-specific CD4$^+$ T cells had lower Th1-to-Treg ratio than hemaglutinin and tetanus toxin-specific CD4$^+$ T cells*

CD4$^+$ T cells are composed of multiple subsets with distinctive functions and cytokine production. The IFN-gamma-producing pro-inflammatory Th1 and CD25$^{hi}$Foxp3$^+$ anti-inflammatory Treg types are among the best studied and were shown to associate with tumor prognosis [23]. We therefore examined the Th1 and Treg frequency in the RMS-specific CD4$^+$ T cell population. We found that the frequency of IFN-gamma-producing cells in RMS-specific CD4$^+$ T cells were comparable to that in hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells, but significantly higher Foxp3-expressing cells were seen in RMS-specific CD4$^+$ T cells (**Figure 3B** and **3C**). Using IFN-gamma and Foxp3 as markers for Th1 and Treg, respectively, we found that RMS-specific CD4$^+$ T cells presented significantly lower Th1-to-Treg ratio than hemaglutinin- and tetanus toxin-specific CD4$^+$ T cells (**Figure 3D**).

*RMS-specific CD8$^+$ T cells were lower in frequency and had lower proliferation than hemaglutinin and tetanus toxin-specific CD8$^+$ T cells*

The cytotoxicity mediated by CD8$^+$ T cells is investigated in various immunotherapy strategies for their direct tumor cell elimination effects [24]. Here, we found that the frequencies of RMS-specific CD8$^+$ T cells were significantly lower than hemaglutinin- and tetanus toxin-specific CD8$^+$ T cells (**Figure 4B**). Similar to the case in CD4$^+$ T cells, the CFSE$^{lo}$ cells in RMS-specific CD8$^+$ T cells had significantly higher mean fluorescence intensity than hemaglutinin- and tetanus toxin-specific CD8$^+$ T cells, suggesting fewer rounds of proliferation in RMS-specific CD8$^+$ T cells (**Figure 4C**). RMS-

specific $CD8^+$ T cells had lower degranulation marker CD107a expression and less IFN-gamma production than hemaglutinin and tetanus toxin-specific $CD8^+$ T cells, suggesting reduced effector activity in the RMS-specific $CD8^+$ T cells (**Figure 4D** and **4E**).

## Discussion

The antitumor adaptive T cell responses have become one of the most active topics in cancer research because a strong and robust antitumor immunity is correlated with better prognosis [25], and in particular, adaptive T cell-based immunotherapies have shown promising results. In this study, we focused on the RMS-specific T cells instead of total T cells, which enabled us to examine tumor-specific characteristics. Also, different patients may exhibit different immune characteristics, with some patients having a more active immune system while others more tolerant. We attempted to avoid the background discrepancies by comparing responses to RMS with responses to hemaglutinin and tetanus toxin. Based on the idea that proliferating T cells responding to a particular antigen presented on DC surface is specific for that antigen, we identified RMS-, hemaglutinin- and tetanus toxin-specific T cells in RMS patients. We found that RMS-specific $CD4^+$ and $CD8^+$ T cells were not present at a significantly elevated frequency compared to hemaglutinin- and tetanus toxin-specific T cells, which was unexpected since RMS was present as an active disease while the patients' exposure to hemaglutinin and tetanus toxin was from vaccines. Moreover, the mean fluorescence intensity of CFSE was higher in RMS-specific $CD4^+$ and $CD8^+$ T cells, demonstrating reduced proliferation capacity. We also found that the RMS-specific $CD4^+$ T cells contained lower Th1-to-Treg ratio, while RMS-specific $CD8^+$ T cells expressed lower levels of degranulation marker CD107a and IFN-gamma, compared to the hemaglutinin- and tetanus toxin-specific T cells. Therefore, we observed that a tumor-specific regulation/inhibition of proinflammatory adaptive T cell response was present in RMS patients.

This study was limited by the fact that the specific RMS antigens recognized by the T cells were unknown. Unlike the case with hemaglutinin and tetanus toxin, where the antigen was known, easily quantifiable, and could be added to the DCs in its pure form, the RMS antigens were obtained from primary tumors and were added at a one tumor cell-to-one DC ratio as a mixture. The lack of better control of antigen concentration in RMS may result in more variations among different patients. Also, it is possible that RMS-specific T cells recognized self-antigens and were autoreactive, which might explain the low proliferative capacity.

The relative suppression of RMS-specific T cells, compared to hemaglutintin- and tetanus toxin-specific T cells, is strongly suggesting that interactions with tumor antigens, likely inside the tumor microenvironment, can be inhibitory to adaptive T cell inflammation. The tumor microenvironment is a critical component in regulating tumor initiation, progression and response to therapy [26]. Multiple immunosuppression mechanisms are present in the intratumoral environment and are found to suppress tumorigenic T cell inflammation and limit the efficacy of T cell immunotherapies [6, 19, 27, 28]. The particular intratumoral environment in RMS has not been extensively studied but is thought to share the inhibitory signatures of many other tumors, such as the presence of M2-type tumor-associated macrophages [29], defective dendritic cells [30], regulatory T cells [31], myeloid-derived suppressor cells [30], suppressive cytokines and inhibitory checkpoint molecules [32, 33]. Results from this study on the preexisting antitumor responses might present an additional challenge of future immunotherapies, but the precise mechanisms of action in RMS require further study.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Xiuming Liu, Department of Ophthalmology, Huai'an First People's Hospital, 6 Beijing West Road, Huai'an 223300, Jiangsu, China. E-mail: xiumingliu_ha@sina.com

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