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IL-15 induces strong but short-lived tumor-infiltrating CD8 T cell responses through the regulation of Tim-3 in breast cancer

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ABSTRACT

IL-15 has pivotal roles in the control of CD8$^+$ memory T cells and has been investigated as a therapeutic option in cancer therapy. Although IL-15 and IL-2 share many functions together, including the stimulation of CD8 T cell proliferation and IFN-γ production, the different in vivo roles of IL-15 and IL-2 have been increasingly recognized. Here, we explored the different effects of IL-15 and IL-2 on tumor-infiltrating (TI) T cells from resected breast tumors. We found that neither IL-2 nor IL-15 induced intratumoral CD8 T cell proliferation by itself, but after CD3/CD28-stimulation, IL-15 induced significantly higher proliferation than IL-2 during early time points, at day 2, day 3 and day 6. However, the IL-15-induced proliferation leveled off at day 9 and day 12, whereas IL-2 induced lower but progressive proliferation at each time point. Furthermore, IL-15 caused an early and robust increase of IFN-γ in the supernatant of TI cell cultures, which diminished at later time points, while the IL-2-induced IFN-γ production remained constant over time. In addition, the IL-15-costimulated CD8 T cells presented higher frequencies of apoptotic cells. The diminishing IL-15-induced response was possibly due to regulatory and/or exhaustion mechanisms. We did not observe increased IL-10 or PD-1 upregulation, but we have found an increase of Tim-3 upregulation on IL-15-, but not IL-2-stimulated cells. Blocking Tim-3 function using anti-Tim-3 antibodies resulted in increased IL-15-induced proliferation and IFN-γ production for a prolonged period of time, whereas adding Tim-3 ligand galectin 9 led to reduced proliferation and IFN-γ production. Our results suggest that IL-15 in combination of Tim-3 blocking antibodies could potentially act as an IL-2 alternative in tumor CD8 T cell expansion in vitro, a crucial step in adoptive T cell therapy.

1. Introduction

CD8 T cells represent a major adaptive T cell subset in the immune infiltrates of breast carcinomas, and are associated with improved clinical outcomes and better prognosis [1,2]. Adoptive transfer of antitumor CD8 T cells into breast cancer patients is being investigated as a promising option in cancer treatment [3–6]. In

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adoptive transfer, T cells are extracted from the peripheral blood of the cancer patient, stimulated and expanded in vitro with costimulatory molecules [7,8]. The expanded cells are then transferred back to the patient to carry out effector function, with the prospect of suppressing tumor growth and eliminating tumor cells. During the stimulation and expansion step, most current protocols chose interleukin 2 (IL-2) to be the costimulatory molecule to support in vitro T survival and to amplify the antitumor responses [6,8–10]. This choice comes with the caveat that IL-2 signaling is also critical to regulatory T (Treg) cell differentiation and maintenance [11–13]. Since T cells in breast tumors are predisposed to immunesuppressive breast tumor microenvironment [14], whether the addition of IL-2 may amplify Treg cell responses instead is currently unclear.

Besides IL-2, a member of the four α-helix bundle family of cytokines IL-15, has recently emerged as a candidate immunomodulator for the treatment of cancer [15]. IL-15 signals through a trimeric receptor composed of the common cytokine receptor gamma chain (γc, CD132), the IL-2/15 receptor beta (IL-15Rβ), and the IL-15 receptor alpha (IL-15Rα), of which the first two are shared with IL-2 [16]. Though many in vivo roles of IL-15 and IL-2 are different [17–19], they also share many functions together, including the stimulation of CD8 T cell proliferation and IFN-γ production [20–23]. IL-15 is also more potent than IL-2 in stimulating the persistence of memory phenotype in CD8 T cells [24]. Whether IL-15 can act as an IL-2 alternative in in vitro CD8 T cell expansion and how it compares to IL-2 remains an interesting area of research.

In this study, we examined the effect of IL-15 on tumor-infiltrating (TI) CD8 T cells obtained from resected breast tumors. We found that compared to IL-2-costimulated cells, IL-15-costimulated cells exhibited markedly stronger early proliferation and IFN-γ production, which were faded in later stages, possibly due to increased upregulation of T cell immunoglobulin- and mucin-domain-containing molecule 3 (Tim-3) in later stages in IL-15-costimulated cells. Addition of Tim-3 ligand galectin 9 had significantly suppressed IL-15 costimulation, while blocking Tim-3 enhanced it. Together, our results demonstrated the kinetic differences between IL-2 and IL-15 costimulation, which is associated with Tim-3 signaling.

2. Methods

2.1. Patients and samples

Tumor samples were obtained from breast cancer patients who undergone surgical tumor resection at PA University Hospital. All the patients were diagnosed invasive ductal carcinoma. Peripheral blood samples were obtained by venupuncture 1 week prior to surgery. Demographic and clinical patient information were also collected for analysis (Table 1). All patients provided written informed consent for enrolment. All study procedures were approved by Institutional Review Board of PA University Hospital.

TI cells were isolated using a published protocol [25]. Briefly, fresh resected tumors were dissected and transferred into 3 ml X-VIVO 20 (Lonza) and was mechanically dissociated with the GentleMACS Dissociator (Program A.01; Miltenyi Biotec). The resulting cell suspension was filtered through a 40-μm cell strainer (BD Falcon) and washed twice in X-VIVO 20. Peripheral blood mononuclear cells (PBMCs) were isolated using the standard Ficoll centrifugation protocol. The resulting cells were then cryopreserved at −80 °C for maximum 1 month.

2.2. Flow cytometry

10^5 cells per 200 μl media were labeled with CFSE Kit (Life Technologies) and incubated at 37 °C with 5% CO_2 concentration. Culture media was RPMI-1640 (Gibco) supplemented with FBS, L-glutamine and Pen Strep (Life Technologies). 1 μg/ml each of anti-CD3 (clone OKT3) and anti-CD28 (clone CD28.2) antibodies (BD) was added to stimulate T cells. Costimulatory cytokines IL-2 and IL-15 (R&D) were used at 20 ng/ml. In some experiments, each culturing condition was replicated in 6 wells of a 96-well round-bottom plate, and was harvested one-by-one at Days 1, 2, 3, 6, 9, and 12 post-incubation. In the Day 12 well, 100 μl supernatant was collected every three days and was replenished with fresh respective media. In other experiments, 2 μg/ml recombinant galectin 9 protein (Abcam) or 5 μg/ml Tim-3 blocking antibody (clone 2E2, Abcam) was added at the beginning of incubation.

The harvested cells were incubated with combinations of fluorescence labeled CD3, CD8, PD-1, and Tim-3 antibodies (BD) for 45 min in 4 °C, washed twice with PBS (Gibco), and were immediately analyzed in a FACSCalibur machine (BD). Apoptotic cells were identified using the Annexin V-FITC Apoptosis Detection Kit (BD), following the manufacturer's instructions.

2.3. ELISA assay

IFN-γ and IL-10 concentrations in the supernatant were measured using Human IFN gamma ELISA Kit and Human IL-10 ELISA kit (Life Technologies), following manufacturer's protocols.

2.4. ELISpot assay

Multiscreen IP 96-well plates (Millipore) were coated with 5 μg/ml primary anti-human IFN-γ antibody (clone 1-D1K, Mabtech) overnight. The next day, the plates were washed and the Day 12 stimulated cells were harvested and enumerated. CD8 T cells were isolated with Human CD8 Negative Selection Kit (Stemcell). 5×10^4 cells per 100 μl media were added to each well and were incubated at 37 °C and 5% CO_2 for 24 h. The plates were then washed and

Table 1
Demographic and clinical information of study subjects.

Parameter	Patient
N	16
Sex (% female)	100
Age (y)	
<60	10
≥60	6
Tumor stage	
I + II	5
III + IV	11
Tumor size	
<2.1	7
≥2.1	9
Nodal status	
Negative	12
Positive	4
ER status	
Negative	3
Positive	13
HER2	
High	2
Low	11
Negative	3

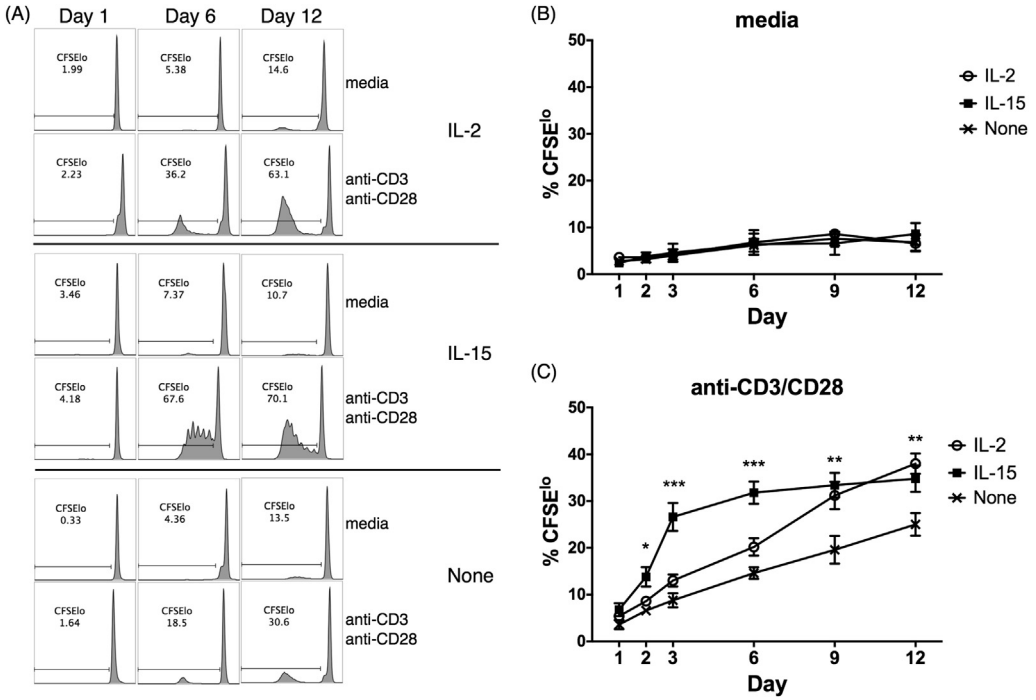

Fig. 1. TI CD8 T cell proliferation following IL-2/IL-15 costimulation. 10^5 TI cells were labeled with CFSE and incubated in 200 μl unstimulated media or with 1 μg/ml anti-CD3 and anti-CD28 antibodies, without costimulatory cytokines or with 20 ng/ml IL-2 or IL-15. Each culturing condition was replicated in 6 wells of a 96-well plate, and was harvested one-by-one at Days 1, 2, 3, 6, 9, and 12 post-incubation. In the Day 12 well, 100 μl supernatant was collected every three days and was replenished with fresh respective media. (A) Representative CFSE expression at Day 1, Day 6 and Day 12 on TI CD8 T cells from one patient. Data shown were gated on $CD3^+CD8^+$ cells. (B) Summary of the frequencies of $CFSE^{lo}$ cells in TI CD8 T cells cultured in unstimulated media from all patients. N = 16. Mean ± SD. (C) Summary of the frequencies of $CFSE^{lo}$ cells in TI CD8 T cells after anti-CD3/CD28 stimulation from all patients. N = 16. Mean ± SD. One-way ANOVA. *P < 0.05. **P < 0.01. ***P < 0.001.

biotinylated secondary anti-IFN-γ antibody (clone 7-B6-1, Mabtech) was added, followed by Streptavidin-alkaline phosphatase (Mabtech). Development of color was done using Alkaline Phosphatase Substrate Kit (Vector Labs) following manufacturer's protocols.

2.5. Statistical tests

All statistical analyses were done using Prism 5 software (GraphPad). P < 0.05 is considered statistically significant.

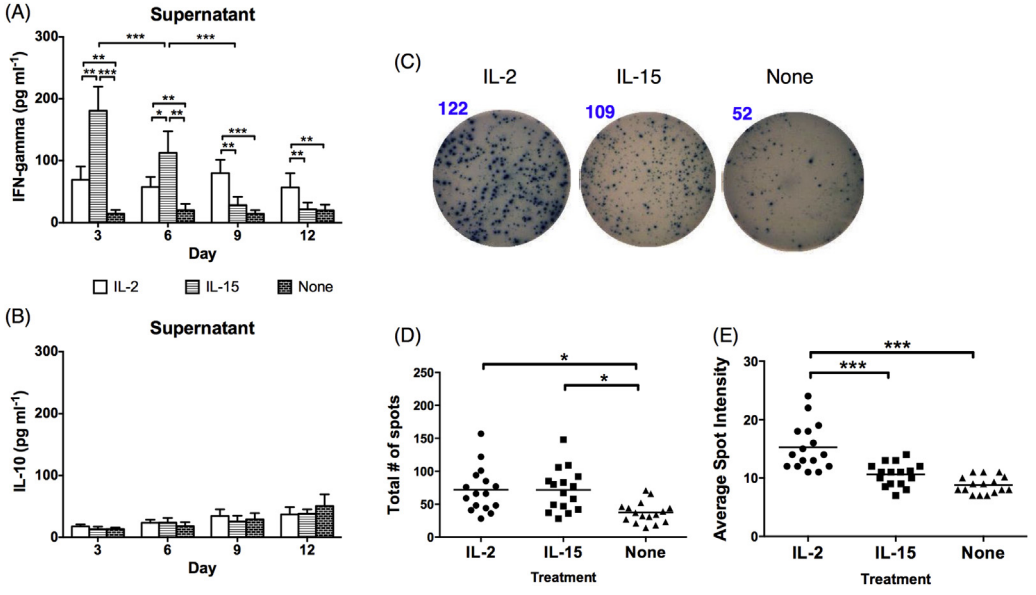

Fig. 2. IFN-γ and IL-10 production from TI cells following IL-2/IL-15 costimulation. The concentrations of (A) IFN-γ and (B) IL-10 from the culture supernatant, collected after every three day period, were measured by ELISA assays. Data shown were calculated by the concentrations from the anti-CD3/CD28-stimulated culture minus the concentration from the unstimulated media culture. N = 16. Mean ± SD. Two-way ANOVA followed by Tukey's test. The production of IFN-γ by Day 12 CD3/CD28-stimulated TI CD8 T cells was also measured by ELISpot. (C) Representative IFN-γ spots from one patient. (D) Summary of the total numbers of IFN-γ spots by Day 12 CD3/CD28-stimulated TI CD8 T cells from all patients. (E) Average IFN-γ spot intensity from all patients. One-way ANOVA followed by Tukey's test. *P < 0.05. **P < 0.01. ***P < 0.001.

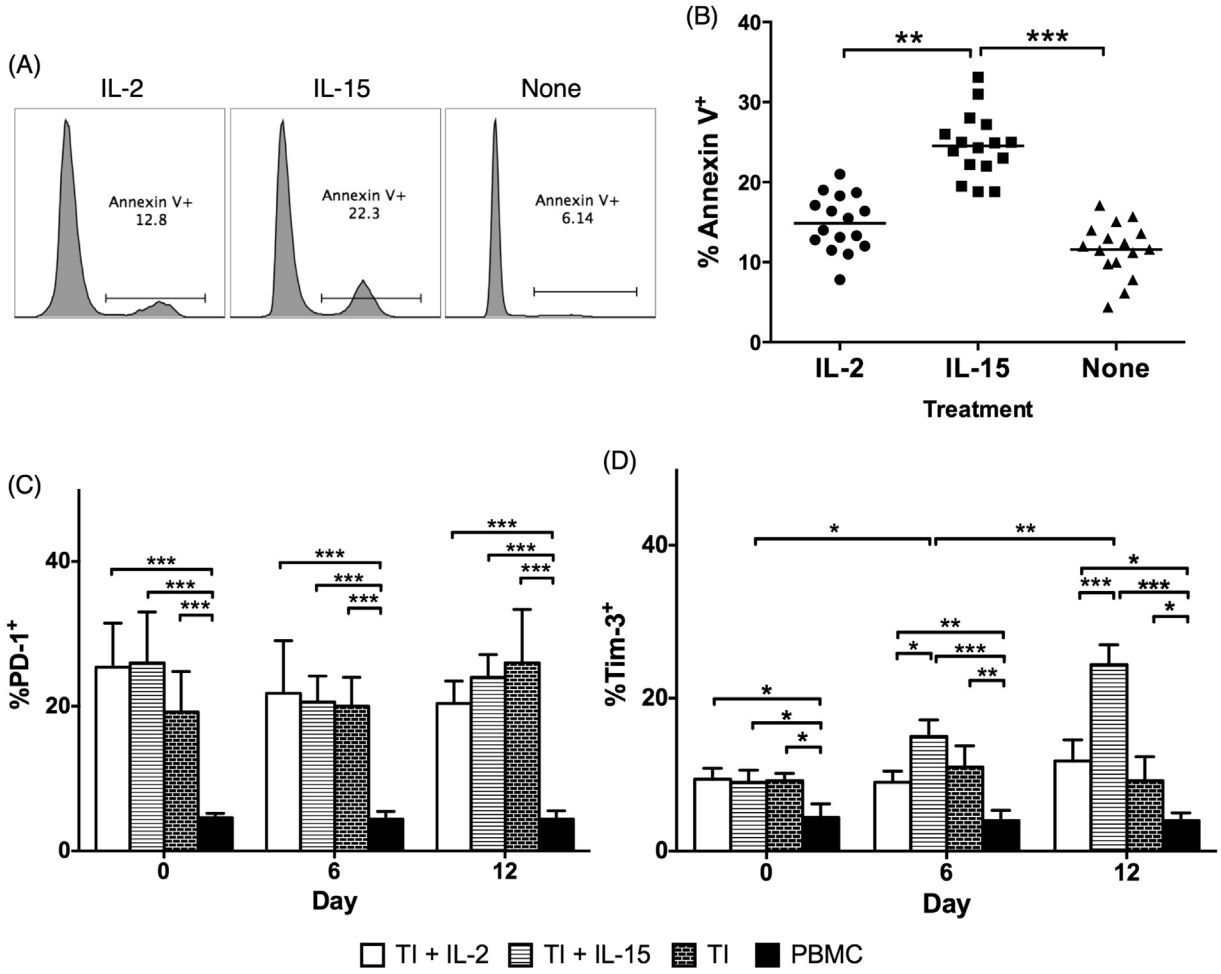

Fig. 3. Apoptosis and exhaustion marker expression by TI CD8 T cells and PBMCs. (A) Representative Annexin V expression on Day 12-stimulated TI CD8 T cells from one patient. (B) Summary of the frequencies of Annexin V^+ cells in TI CD8 T cells from all patients. One-way ANOVA followed by Tukey's test. $^{**}P < 0.01$. $^{***}P < 0.001$. 5×10^4 TI cells and autologous PBMCs were stimulated with anti-CD3/CD28 antibodies in the presence of IL-2, IL-15, or neither (none). The frequencies of (C) PD-1- and (D) Tim-3-expressing CD8 T cells prior to stimulation (Day 0) and at Day 6 and Day 12 post-stimulation were measured by flow cytometry. N = 16. Mean ± SD. Two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

3. Results

3.1. Different TI CD8 T cell proliferation kinetics in IL-2- and IL-15-induced proliferation

TI CD8 T cell-mediated inflammation play a major role in regulating breast tumor growth and are currently explored for cancer treatment [1,2,26–28]. To measure the stimulation of IL-2 and IL-15 on TI CD8 T cells, TI cells from resected tumor were labeled with CFSE and cultured in 200 μl media for 12 days, with or without CD3/CD28 stimulation. Costimulatory cytokine IL-2 and IL-15 were also added respectively. 100 μl supernatant was taken at 3-day intervals. The cell cultures were then replenished with 100 μl fresh respective media every 3 days. Cell proliferation at Day 1, 2, 3, 6, 9 and 12 were examined by flow cytometry (Fig. 1A). We found that IL-2 and IL-15 by themselves do not directly stimulate TI CD8 T cell proliferation (Fig. 1B). After anti-CD3/CD28 stimulation, IL-2 and IL-15 significantly enhanced TI CD8 T cell proliferation (Fig. 1C). Notably, CD8 T cells with IL-15 supplement had significantly higher levels of proliferation at Day 2, Day 3, and Day 6 than those with IL-2 supplement, but later on at Day 9 and Day 12, no significant differences between IL-2 and IL-15 supplements were found (Fig. 1A and C).

3.2. IFN-γ production with IL-15 supplement was initially strong but faded at later time points

Both IL-2 and IL-15 could enhance IFN-γ production in CD8 T cells [29]. We found that IL-15-supplemented TI cell culture supernatant had significantly higher IFN-γ concentration at Day 3 and Day 6, than the IL-2 supplement cell culture (Fig. 2A). But unlike the IL-2-supplemented culture, which contained stable levels of IFN-γ concentration over the entire incubation period, the cultures with IL-15 had significantly downregulated IFN-γ concentration from Day 3 to Day 6, and from Day 6 to Day 9. No significantly differences between different groups were found in terms of IL-10 production (Fig. 2B). Day 12 CD8 T cells were isolated by negative selection and the numbers of IFN-γ-producing cells were enumerated by ELISpot (Fig. 2C). Both IL-2- and IL-15-supplemented cultures had increased numbers of IFN-γ-producing cells compared to the culture with no supplement (Fig. 2D). The average spot intensity, however, was significantly lower in cultures with IL-15 than those with IL-2 (Fig. 2E).

3.3. TI CD8 T cells cultured with IL-15 had elevated Tim-3 and were more prone to apoptosis

The data from both proliferation and IFN-γ production assays suggested a strong early costimulatory effect by IL-15, which faded

away at later time points. To examine the underlying mechanism of this diminishing effect of IL-15 but not IL-2, we examined TI CD8 T cell survival by Annexin V staining (Fig. 3A). We found that TI CD8 T cells cultured with IL-15 had significantly higher frequencies of Annexin V^+ cells than those cultured with IL-2 or no cytokine supplement (Fig. 3B). Programmed death 1 (PD-1) and Tim-3 signaling pathways were shown to induce T cell apoptosis in chronic inflammations [30–33]. In a separate experiment, we found that PD-1 and Tim-3 expressions in TI CD8 T cells were significantly upregulated compared to their autologous counterparts in PBMCs, regardless of culture conditions and culture length (Fig. 3C and D). Addition of IL-2 and IL-15 did not affect PD-1 expression. Interestingly, the addition of IL-15, but not IL-2, had significantly increased Tim-3 expression over time, from Day 0 to Day 6, and from Day 6 to Day 12 (Fig. 3D).

3.4. Tim-3 ligand significantly blocked the costimulatory effect of IL-15, while Tim-3 blockade enhanced it

The contemporaneous upregulation of Tim-3 and reduction of IL-15 costimulation led to the hypothesis that Tim-3 directly contributed to the diminished IL-15 costimulatory effects at later time points. In another experiment, TI cells were stimulated with anti-CD3/CD28 for 12 days, together with IL-2, IL-15, IL-15 and recombinant soluble Tim-3 ligand galectin 9 (IL-15 + Gal9), or IL-15 and Tim-3 blocking antibody (IL-15 + anti-Tim-3). The addition of galectin 9 and Tim-3 blocking antibody did not significantly change Tim-3 expression on the cell surface (Fig. 4A). We found that the addition of galectin 9 significantly downregulated the frequencies of $CFSE^{lo}$ cells, the numbers of IFN-γ-producing cells and the IFN-γ concentration in the supernatant at Day 12 (Fig. 4B–D), while the frequencies of Annexin V^+ cells were upregulated (Fig. 4E). In contrast, blocking Tim-3 in IL-15-supplemented cultures had significantly increased the frequencies of $CFSE^{lo}$ cells and the numbers of IFN-γ-producing cells, as well as supernatant IFN-γ concentration, and had reduced the frequencies of Annexin V^+ cells, compared to culturing with IL-15 alone. Moreover, IL-15 + anti-Tim-3 resulted in significantly higher TI CD8 T cell proliferation and IFN-γ production, compared to IL-2. These data suggest that Tim-3 upregulation contributed to the reduction of IL-15 costimulatory effects at later time points; blocking Tim-3 signal transduction reverted this reduction.

4. Discussion

The data from this study demonstrated both IL-2 and IL-15 could act as a potent costimulatory signal to enhance TI CD8 T cell proliferation and IFN-γ production, with marked kinetic differences. Proliferation induced by IL-15 was stronger initially but leveled off at later times, while that by IL-2 was consistently increasing. When examining IFN-γ production at three-day intervals, the IL-15-supplemented culture contained significantly higher IFN-γ at Day 3 and Day 6 than the IL-2-supplemented culture. But by Day 9 and Day 12, IFN-γ from IL-15-supplemented culture was significantly lower. Comparison between different time points revealed that the IFN-γ production was consistent throughout the incubation period in the IL-2-supplemented culture but was gradually reducing in the IL-15-supplemented culture. By Day 12, the total numbers of IFN-γ-producing TI cells were comparable in IL-2- and IL-15-supplemented cultures, but the average spot intensity in the IL-15-supplemented culture was reduced, which suggested lower IFN-γ production per cell. This type of diminishing inflammation over

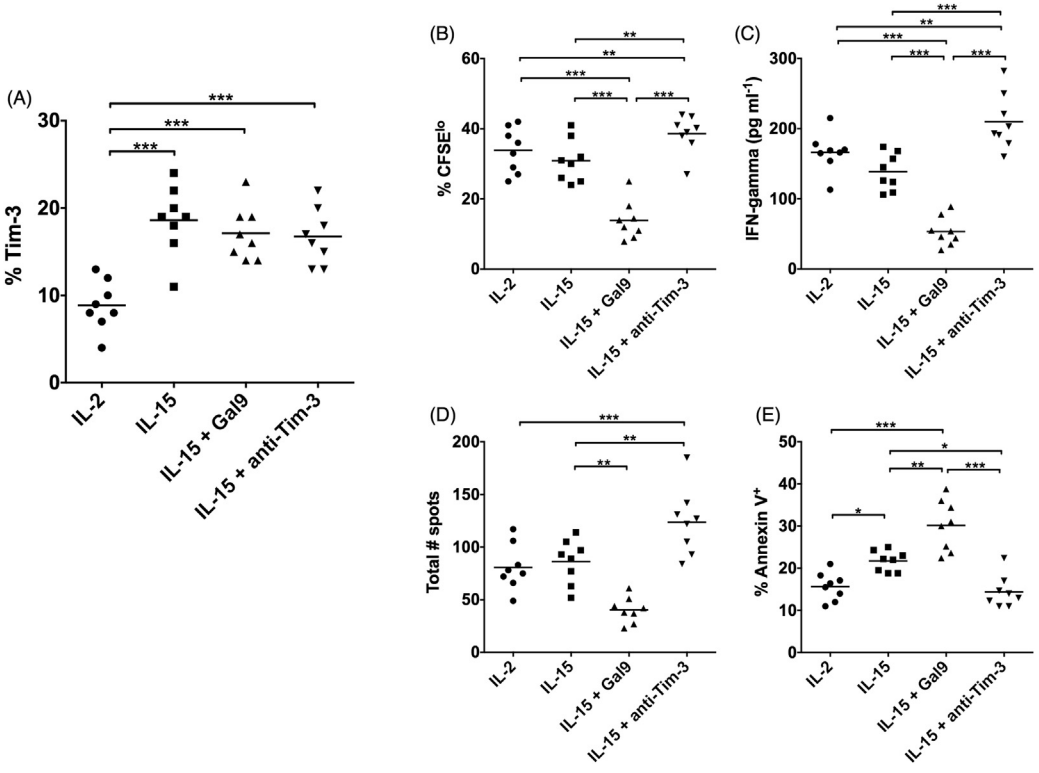

Fig. 4. Effects of Gal9 and Tim-3 blocking antibody on TI CD8 T cells following IL-15 costimulation. 10^5 TI cells were stimulated with anti-CD3/CD28 antibodies in the presence of IL-2, IL-15, IL-15 + Gal9, and IL-15 + anti-Tim-3 blocking antibody for twelve days. The Day 12 frequencies of (A) Tim-3-expressing, (B) $CFSE^{lo}$, and (E) Annexin V-expressing TI CD8 T cells, as well as (C) the supernatant concentration of IFN-γ and (D) the numbers of IFN-γ spots were measured. One-way ANOVA followed by Tukey's test. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

time is consistent with increased apoptosis and T cell exhaustion [31,34,35]. We found that compared to IL-2-costimulated cells, IL-15-costimulated TI CD8 T cells expressed higher apoptosis marker Annexin V at Day 12. Furthermore, incubation with IL-15 had significantly increased Tim-3 expression on TI CD8 T cell surface over time. To examine whether Tim-3 upregulation directly contribute to apoptosis and diminishing inflammation, Tim-3 ligand galectin 9 and Tim-3 blocking antibodies were added into the cell culture. Interestingly, the addition of galectin 9 had significantly decreased TI CD8 T cell proliferation and IFN-γ production, and had significantly increased TI CD8 T cell apoptosis, while blocking Tim-3 had reverted these effects. Moreover, IL-15 in combination of Tim-3 blocking antibodies resulted in significantly higher TI CD8 T cell proliferation and IFN-γ production. Together, these experiments demonstrated the involvement of Tim-3 signaling pathway in IL-15 costimulation of TI CD8 T cells.

Previously, IL-15 was found to preferentially stimulate the persistence of memory type CD8 T cells with only moderate effector functions [21,36,37], but these previous studies did not examine the expression of T cell exhaustion markers. Although γc cytokines, including IL-2, IL-7, IL-15 and IL-21, were generally considered proinflammatory, IL-15 could upregulate Tim-3 expression on circulating T cells in an antigen-independent fashion [38], while IL-2 could stimulate Treg differentiation [12,39]. Indeed, depletion of Treg cells with an IL-2 immunotoxin in a mouse model of breast cancer had improved antitumor immunity and enhanced tumor regression [9]. Here, we found that blocking Tim-3 in vitro had significantly increased IL-15-induced CD8 effector functions in vitro. Also, it should be noted that the TI CD8 T cells in this study were predisposed to the immunosuppressive tumor microenvironment, and were expressing higher levels of PD-1 and Tim-3 than their autologous peripheral blood counterparts. Our results suggest that IL-15 in combination of Tim-3 blocking antibodies could potentially act as an IL-2 alternative in tumor CD8 T cell expansion in vitro, a crucial step in adoptive T cell therapy. Whether IL-15 and Tim-3 blocking antibodies are effective at inducing in vivo tumor regression requires further studies.

Conflict of interests

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.162.

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