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Original Article

Possible therapeutic applicability of galectin-9 in cutaneous T-cell lymphoma

Rina Nakajima[a], Tomomitsu Miyagaki[a,*], Hiroaki Kamijo[a], Tomonori Oka[a], Naomi Shishido-Takahashi[a,b], Hiraku Suga[a], Makoto Sugaya[a,b], Shinichi Sato[a]

[a] Department of Dermatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[b] Department of Dermatology, International University of Health and Welfare, Chiba, Japan

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ABSTRACT

Background: Galectin-9, a member of the galectin family, can promote tumor growth through inducing apoptosis in anti-tumor immune cells *via* T cell immunoglobulin and mucin domain 3 (TIM-3). On the other hand, galectin-9 also induces tumor cell apoptosis in many malignancies and thought to have potential as an anti-cancer agent.
Objective: To examine the expression and therapeutic applicability of galectin-9 in cutaneous T-cell lymphoma (CTCL).
Methods: Galectin-9 expression in lesional skin and sera was measured using CTCL samples. The effect of galectin-9 on CTCL cell lines was investigated *in vitro*. We also examined effect of galectin-9 on tumor growth of CTCL cells in immune-deficient mice. Moreover, we examined the efficacy of galectin-9, anti-TIM-3 blocking antibody, or their combination on tumor growth of EL-4 cells in wild-type mice.
Results: Galectin-9 was expressed on tumor cells in lesional skin of CTCL and the expression levels were associated with decreased $CD8^+$ T-cell infiltration. Serum galectin-9 levels were correlated with disease severity markers. High-dose galectin-9 induced cell death of CTCL cell lines through activation of caspase-3 and caspase-9, independently of TIM-3. High-dose galectin-9 suppressed the growth of CTCL cells and EL-4 cells *in vivo*. Furthermore, additional anti-TIM-3 blocking antibody administration to galectin-9 achieved greater inhibition of tumor growth compared to single administration.
Conclusion: Galectin-9 expression on tumor cells may be associated with CTCL progression through attenuating anti-tumor immunity. On the other hand, exogenous high-dose galectin-9 administration can be a therapeutic strategy for CTCL and anti-TIM-3 blocking antibody can augment the efficacy of galectin-9.
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1. Introduction

Mycosis fungoides (MF) and Sézary syndrome (SS), the most common types of cutaneous T-cell lymphoma (CTCL), are characterized by proliferation of neoplastic skin-homing $CD4^+$ T cells [1]. Generally, MF has a classically prolonged clinical course that progresses over years through patch, plaque, and tumor stages, followed by lymph node, peripheral blood, and visceral involvement. SS is an aggressive form of CTCL, showing erythroderma and blood and lymph node involvement [1]. Patients with advanced stage of MF and SS have poor prognosis [2]. In addition, although the disease course is indolent in early stages of MF, many patients have uncomfortable symptoms, such as pruritus and skin infections [3,4], leading to low quality of life.

Nevertheless, currently, there is no treatment that can cure these diseases [5].

Galectin-9 is a member of the tandem-repeat galectin family, which is widely distributed in human tissues and has a wide spectrum of biological functions [6,7]. Unlike other galectins, galectin-9 plays a role in both promoting and inhibiting tumor growth, depending on interactions with its receptors on T cells, antigen-presenting cells, or tumor cells [8]. Galectin-9 can promote the differentiation of naive T cells into regulatory T cells by enhancing Foxp3 expression [9], resulting in tumor escape from immune surveillance. Furthermore, galectin-9 has the capacity to induce apoptosis in a number of immune cell types such as $CD4^+$ T-

Abbreviations: CTCL, cutaneous T-cell lymphoma; FBS, fetal bovine serum; mRNA, messenger RNA; MF, mycosis fungoides; SS, Sézary syndrome; Th1, T-helper 1; TIM-3, T cell immunoglobulin and mucin domain 3; WT, wild-type.

* Corresponding author at: Department of Dermatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan.
E-mail address: asahikari1979@gmail.com (T. Miyagaki).

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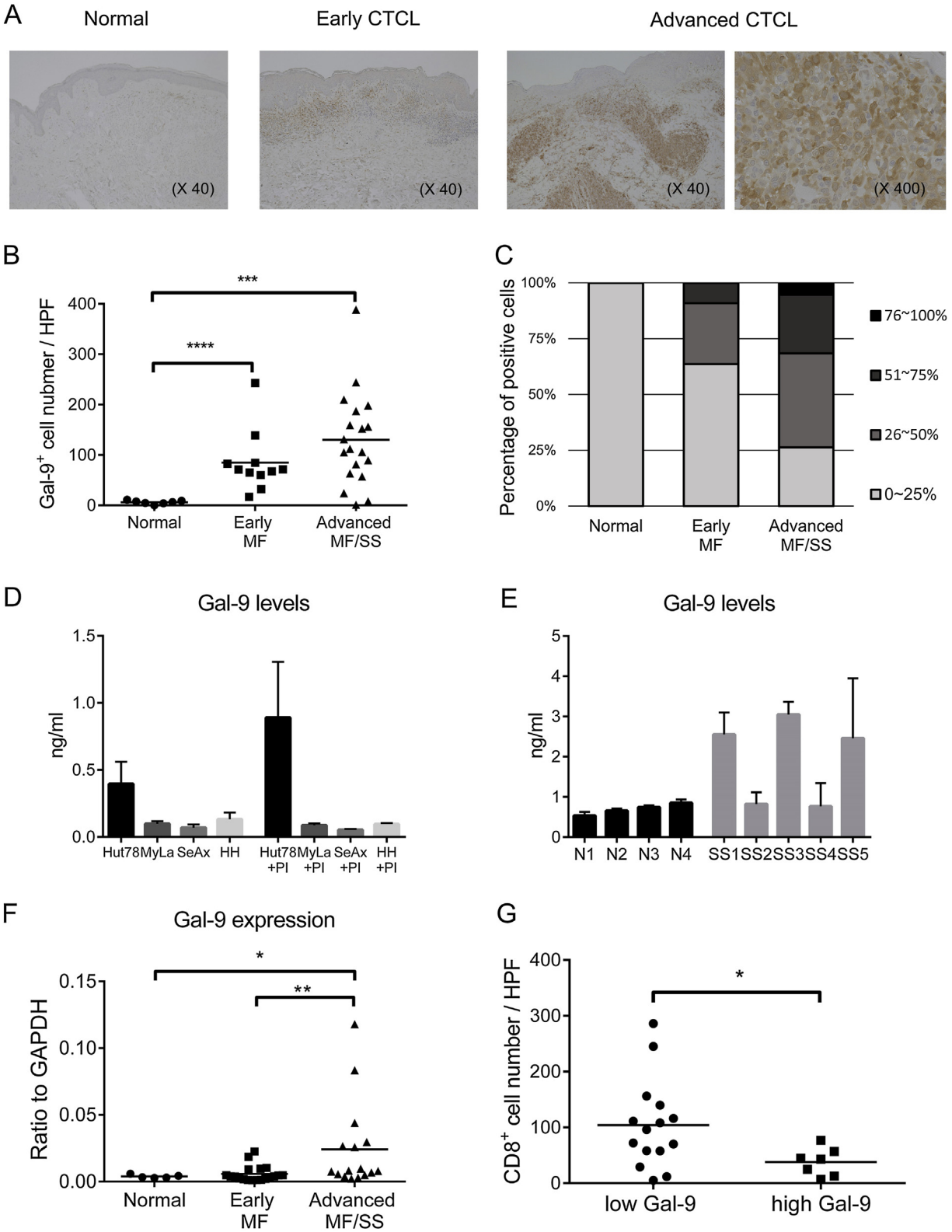

Fig. 1. Galectin-9 expression in lesional skin of CTCL. (A) Galectin-9 expression in lesional skin of CTCL was determined by immunohistochemistry. Representative picture of healthy control, early MF, and advanced MF. Original magnification: x 40 and x 400. (B) The number of galectin-9 positive cells per high power field (HPF) in lesional skin of early MF (n = 11), advanced MF/SS (n = 19), and healthy skin (n = 7). The measured values from individual patients were plotted by dots. The horizontal bars indicate the mean value in each group. ***P < 0.001, ****P < 0.0001. (C) Immunohistochemical scoring of percentage of positive cells ; weak (0 ~ 25%), moderate (26 ~ 50%), strong (51 ~75%), and very strong (76 ~ 100%).
(D) Galectin-9 expression in supernatant of CTCL cell lines after 48 h culture with or without PMA and ionomycin (PI). n = 3. Data are presented as mean ± SD. (E) Galectin-9 levels in supernatant of $CD4^+$ T cells in peripheral blood from five SS patients (SS1 ~ SS5) and four healthy controls (N1 ~ N4) after 48 h culture with PI. n = 3. Data are presented as mean ± SD. (F) Galectin-9 mRNA expression in lesional skin of early MF (n = 19), advanced MF/SS (n = 16), and healthy skin (n = 5). The measured values from individual patients were plotted by dots. The horizontal bars indicate the mean value in each group. *P < 0.05, **P < 0.01. (G) High expression of galectin-9 mRNA is associated with decreased $CD8^+$ cell numbers. The measured values from individual patients were plotted by dots. The horizontal bars indicate the mean value in each group. *P < 0.05.

helper 1 (Th1) cells, Th17 cells, and $CD8^+$ cytotoxic T cells *via* T cell immunoglobulin and mucin domain 3 (TIM-3) [10–12], one of the receptors of galectin-9 [10], leading to attenuation of anti-tumor immunity. Actually, anti-TIM-3 blocking antibody administration suppresses *in vivo* growth of several tumor cell lines, such as ovarian, colon, and liver cancer by enhancing anti-tumor immunity [13–15]. Several human clinical trials using an anti-TIM-3 blocking antibody in advanced solid tumors have been conducted [8]. Despite these tumor-promoting roles of galectin-9, galectin-9 has also been thought to have potential as an anti-cancer agent [16]. Many reports using mouse models *in vivo* and *in vitro* reveal that galectin-9 also induces tumor cell apoptosis in a variety of malignancies, including chronic myelogenous leukemia, malignant melanoma, hepatic cell carcinoma, and gastric cancer [17–20]. Concerning T-cell malignancies, little is known about galectin-9 involvement in their development, although T cells are known to express and release galectin-9 [21]. To explore the clinical application of galectin-9 in CTCL, in this study, we investigated the galectin-9 expression in lesional skin and sera of CTCL patients and the effect of galectin-9 on tumor cells using cell lines. We also examined the efficacy of combination of galectin-9 and anti-TIM-3 blocking antibody.

2. Materials and methods

2.1. Patients and samples

All patients with CTCL were given diagnoses according to the current WHO classification [22]. Skin samples were collected from 59 patients with CTCL and 12 healthy control subjects. Clinical features of patients were summarized in Supplemental Table 1. Serum samples were collected from 50 patients with CTCL (30 male and 20 female patients; mean ± SD age: 58.8 ± 12.9 years; 10 cases of stage IA MF, 15 cases of stage IB MF, 1 case of stage IIA MF, 10 cases of stage IIB MF, 1 case of stage IIIA MF, 2 cased of stage IVA1 MF, 1 case of stage IVA2 MF, 8 cases of stage IVA1 SS, 1 case of stage IVA2 SS, and 1 case of stage IVB SS), and 16 healthy control subjects (8 male and 8 female patients; mean ± SD age: 57.4 ± 8.2 years). Peripheral blood mononuclear cells were harvested from 5 patients with SS and 4 healthy controls. All samples were collected after informed consent during daily clinical practice. The medical ethics committee of the University of Tokyo approved all described studies and the study was conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained to use blood and skin samples from patients and healthy controls. The healthy controls had no history of allergy, CTCL, or any skin diseases. Patients with MF were classified into two groups: early stage of MF (clinical stages IA-IIA) and advanced stage of MF (stages IIB-IVB).

2.2. Analysis of galectin-9 expression in lesional skin, supernatant of CTCL cells and sera of CTCL patients

Quantitative RT-PCR and immunohistochemistry for galectin-9 were performed using lesional skin of CTCL and normal skin. A specific ELISA for galectin-9 was performed using sera of CTCL patients and healthy controls. Galectin-9 levels in supernatant of CTCL cell lines and CD4 + T cells in peripheral blood from SS patients and healthy controls were also measured by a specific ELISA for galectin-9. Detailed methods are given in Supplemental Materials and Methods.

2.3. Analysis of the effects of galectin-9 on CTCL cell lines, a mouse T-cell lymphoma cell line, and normal T cells

After cells were treated with recombinant galectin-9 (R&D systems, Minneapolis, MN, USA), proliferation assays, apoptosis assays, cell cycle analysis, colorimetric assay for caspase-3 and caspase-9, and *in vivo* animal experiments were performed. TIM-3 expression on cells was also examined by flow cytometric analysis. Detailed methods are given in Supplemental Methods.

2.4. Statistical analysis

All *in vitro* experiments were repeated at least 3 times and mean ± SD or SEM was determined. Statistical analysis between 2 groups was performed using the Welch's *t* test. Mann-Whitney's *U* test was used to determine statistical significance of quantitative real time RT-PCR differences and *in vivo* experimental differences. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Galectin-9 expression in lesional skin of CTCL

To investigate the possible involvement of galectin-9 in CTCL, we first examined galectin-9 expression in lesional skin by immunohistochemistry. In normal skin, we barely found galectin-9-positive cells. In lesional skin of CTCL, dermal infiltrating lymphocytes with atypical nuclei were positive for galectin-9 (Fig. 1A). The number and percentage of galectin-9-positive cells were increased as the disease progressed (Fig. 1A–C). To confirm that CTCL cells express galectin-9, we measured gelactin-9 expression in supernatant of CTCL cell lines. Among four CTCL cell lines, Hut78, MyLa, SeAx, and HH cells, Hut78 cells produced galectin-9 and stimulation with PMA and ionomycin tended to augment the production (Fig. 1D). We also determined galectin-9 levels in supernatant of $CD4^+$ T cells in peripheral blood from five SS patients and four healthy controls by ELISA. In three out of five SS patients, $CD4^+$ T cells in peripheral blood expressed galectin-9 (Fig. 1E). We next assessed galectin-9 mRNA expression in lesional skin of CTCL and in normal skin. Galectin-9 mRNA expression was significantly increased in lesional skin of advanced MF/SS, while the expression was not elevated in lesional skin of early MF (Fig. 1F). We also found that infiltrating $CD8^+$ T cells in lesional skin were significantly decreased in patients with high galectin-9 mRNA levels (0.01 or more; Fig. 1G), similar to previous reports describing that galectin-9 increases apoptosis of tumor-infiltrating $CD8^+$ T cells in colon cancer [14]. Thus, tumor cells of some CTCL cases expressed galectin-9 and high galectin-9 expression in lesional skin was associated with decreased infiltrating $CD8^+$ T cells.

3.2. Galectin-9 expression in sera of patients with CTCL

We also measured serum galectin-9 levels in patients with CTCL and in healthy controls. Although serum galectin-9 levels in CTCL patients (11.4 ± 6.92 ng/ml) tended to be higher than those of healthy controls (8.71 ± 2.64 ng/ml), there was no significant difference. In patients with early MF (n = 26), advanced MF (n = 14), and SS (n = 10), serum galectin-9 levels were 7.64 ± 3.35, 14.4 ± 6.73, and 17.0 ± 5.29 ng/ml, respectively. Serum levels of galectin-9 in advanced MF or SS were significantly higher than those in early MF and healthy controls (Fig. 2A). In addition, we found that serum galectin-9 levels significantly correlated with serum levels of lactate dehydrogenase (Fig. 2B, $r = 0.52$, $P < 0.0001$), soluble IL-2 receptor (Fig. 2C, $r = 0.64$, $P < 0.0001$), and thymus and activation-regulated chemokine (Fig. 2D, $r = 0.37$, $P < 0.05$), all of which are regarded as disease severity markers. Thus, serum galectin-9 levels were increased and correlated with disease severity markers in CTCL.

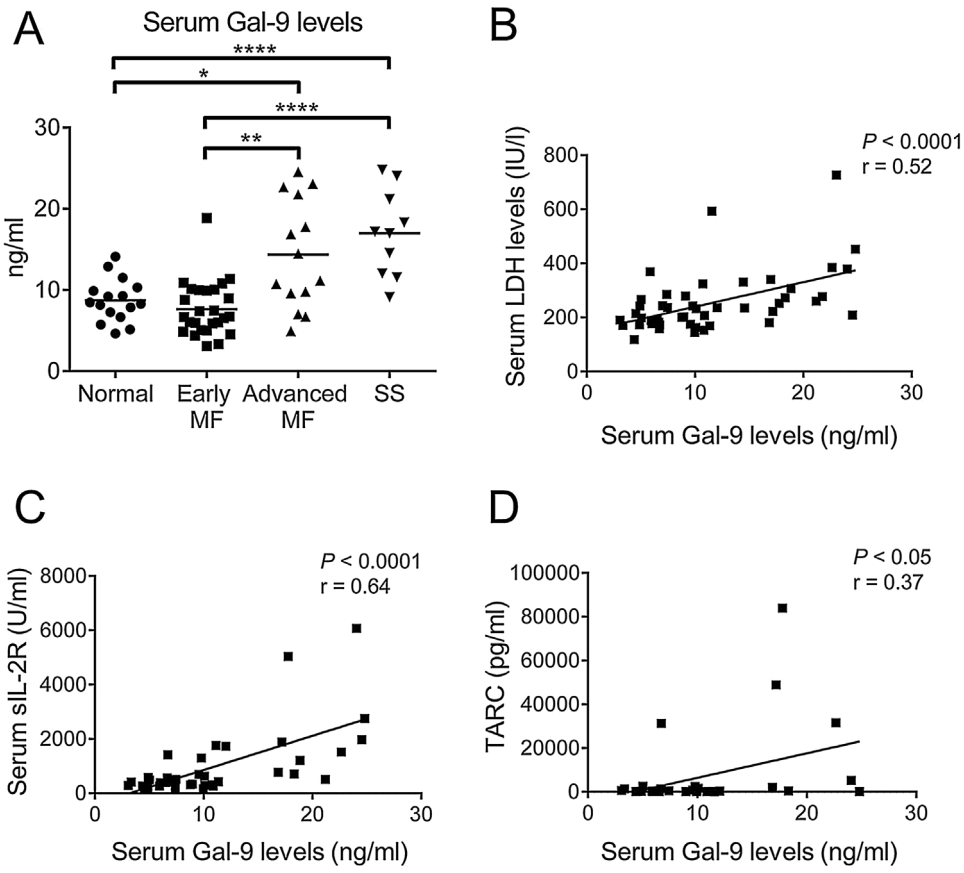

Fig. 2. Serum galectin-9 levels in CTCL patients. (A) Serum galectin-9 levels in early MF (n = 26), advanced MF (n = 14), SS (n = 10) and normal controls (n = 16). The measured values from individual patients were plotted by dots. The horizontal bars indicate the mean value in each group. *P < 0.05, **P < 0.01, ****P < 0.0001. (B–D) Correlations between serum galectin-9 levels and lactate dehydrogenase (LDH) (B), soluble IL-2 receptor levels (sIL-2R) (C), and thymus and activation-regulated chemokine levels (TARC) (D) in CTCL. The measured values from individual patients were plotted by dots.

3.3. Galectin-9 inhibits cell proliferation and survival of CTCL cell lines in vitro

As galectin-9 is known to induce apoptosis in various cancers including various hematopoietic malignancies [16–18,23], we next investigated the direct effect of galectin-9 on CTCL cell lines. High-dose galectin-9 treatment for 24 h significantly suppressed the proliferation of Hut78, MyLa, SeAx, and HH cells in a dose-dependent manner (Fig. 3A). In addition, galectin-9 promoted cell death assessed by Annexin V staining in CTCL cell lines (Fig. 3B and C). We next investigated whether galectin-9 induced cell cycle arrest in CTCL cell lines, only to find that cell cycle arrest was not observed by galectin-9 treatment (Fig. 3D and E), consistent with previous reports on hepatocellular carcinoma and esophageal adenocarcinoma cells [19,24]. Thus, galectin-9 suppressed *in vitro* proliferation of CTCL cell lines without affecting cell cycle.

3.4. Galectin-9 induces cell death in CTCL cell lines through the caspase pathway

To understand the precise mechanism of cytotoxic effect of galectin-9 on CTCL cells, we further investigated the downstream of galectin-9. As caspase activation is reported to be required for galectin-9-induced apoptosis in many types of cells [17,18,24,25], we examined the effects of caspase inhibitors on galectin-9-induced apoptosis in CTCL cell lines. CTCL cell lines were incubated with Z-YVAD-FMK (caspase-1 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), or Z-LEHD-FMK (caspase-9 inhibitor), followed by treatment with galectin-9. Caspase-3 inhibitor and caspase-9 inhibitor suppressed apoptosis of all CTCL cell lines, Hut78, MyLa, SeAx, and HH cells, while caspase-1 inhibitor could not impair galectin-9-induced apoptosis (Fig. 4A). The effect of caspase-8 inhibitor was different depending on CTCL cell lines: the reagent inhibited galectin-9-induced apoptosis in SeAx and HH cells but not in Hut78 and MyLa cells (Fig. 4A). We also confirmed that activation of caspase-3 and caspase-9 was increased by galectin-9 administration in all CTCL cell lines (Fig. 4B and C). Thus, galectin-9 induced cell death of CTCL cell lines through caspase-3 and caspase-9 activation.

3.5. Galectin-9 induces cell death in CTCL cell lines in a TIM-3-independent manner

Galectin-9 induced apoptosis in Th1 cells, Th17 cells, and $CD8^+$ cytotoxic T cells *via* TIM-3 [10–12], while in some malignancies, galectin-9 caused cell death in a TIM-3-independent manner [17–19]. Thus, we next focused on whether galectin-9 induced cell death *via* TIM-3 in CTCL cell lines or not. Flowcytometric analysis revealed that Hut78, MyLa, SeAx, and HH cells did not express TIM-3 on the cell surface (Fig. 5A). Expectedly, anti-TIM-3 antibody did not augment nor suppress galectin-9-induced cell death in CTCL cell lines (Fig. 5B). Thus, galectin-9 induced cell death in CTCL cell lines independently of TIM-3.

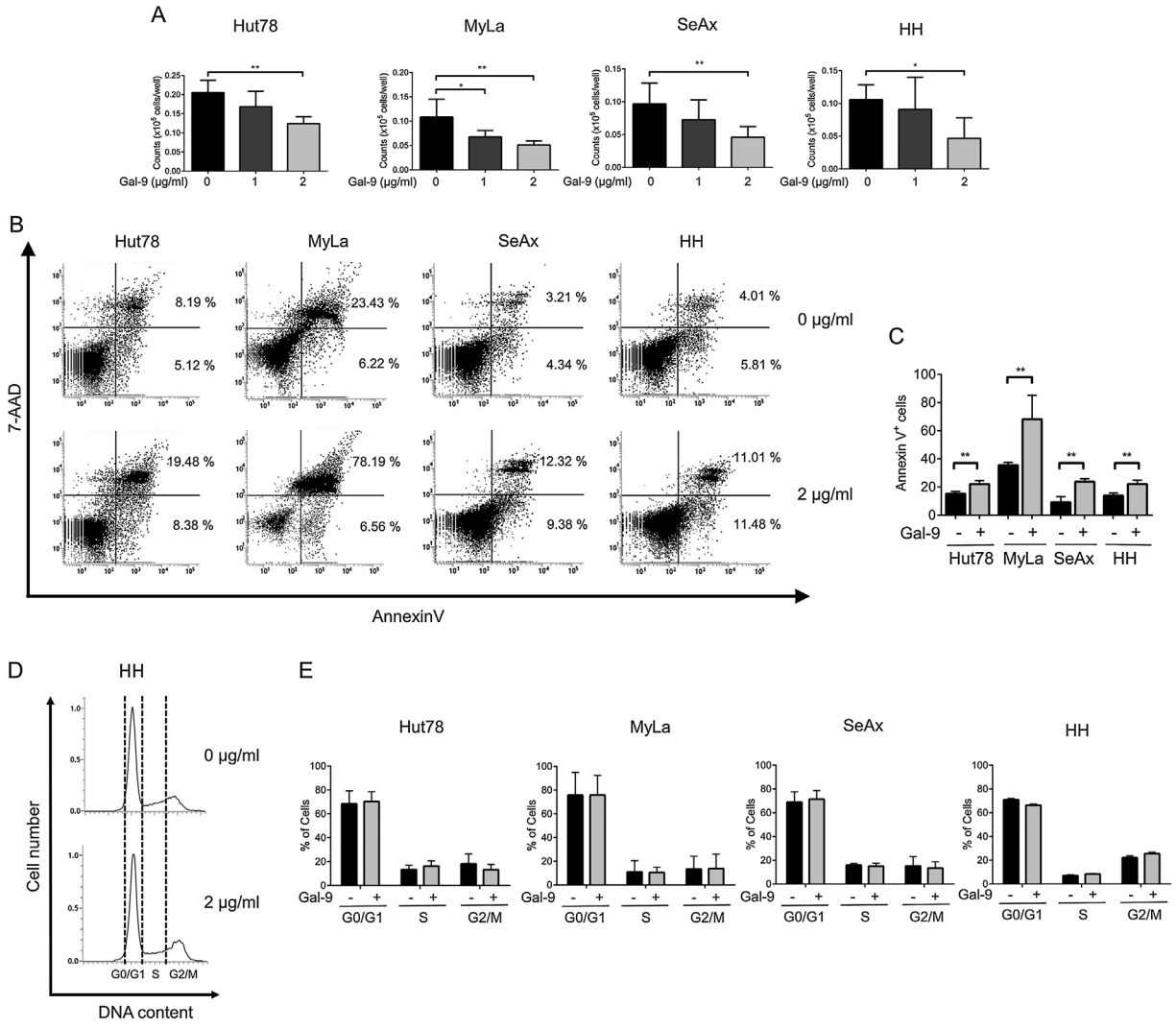

Fig. 3. Galectin-9 inhibits the cell proliferation of CTCL cell lines *in vitro* by inducing apoptosis. (A) CTCL cell lines were cultured with medium only or recombinant human galectin-9 (1, 2 μg/ml) for 24 h. Viable cells were counted by Trypan blue exclusion. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) and (C), CTCL cell lines were cultured with or without 2 μg/ml galectin-9 for 24 h, and Annexin V and 7-amino-actinomycin D (7-AAD) staining for flow cytometry was performed. Representative dot-plot graphs of three independent experiments giving similar results (B). Quantification of Annexin V^{+} cells (C). $^{**}P < 0.01$. (D) and (E), CTCL cell lines were cultured with or without 2 μg/ml galectin-9 for 24 h and cell cycle analysis was performed by flow cytometry. Representative result of HH cells of three independent experiments (D). The percentages of CTCL cell lines in G0/G1, S, G2/M (e).

3.6. Galectin-9 does not induces cell death in TIM-3-negative normal T cells

We next examined whether galectin-9 could cause TIM-3-independent cell death in normal T cells. As TIM-3 expression is induced in activated T cells, we used CD4^{+} T cells and CD8^{+} T cells in peripheral blood from healthy controls. We first confirmed that such T cells barely expressed TIM-3 on their surface (Supplementary Fig. 1A). We also found that galectin-9 failed to induce cell death in those T cells (Supplementary Fig. 1B–D). Thus, galectin-9 did not induce cell death in TIM-3-negative normal T cells.

3.7. Galectin-9 inhibits the tumor growth of T-cell lymphoma cell lines in vivo

To assess the *in vivo* effects of high-dose galectin-9, we used a xenograft model [26]. HH cells were injected subcutaneously into the left flank of SCID-beige mice, followed by repeated treatment with or without galectin-9. Significantly smaller tumors formed in the mice treated with galectin-9 than in those treated with PBS only (Fig. 6A). As immune system is severely impaired in SCID-beige mice, ameliorating effects of galectin-9 on anti-tumor T cell response are ignored in this experimental system. Therefore, we also determined effects of galectin-9 on EL-4 cells, whose proliferation was also suppressed by galectin-9 in a TIM-3 independent manner (Fig. 6B and data not shown), using C57BL/6 mice, in which T cells and B cells are functionally normal. Treatment with galectin-9 significantly decreased tumor formation by EL-4 cells in C57BL/6 mice *in vivo* (Fig. 6C). Thus, galectin-9 inhibited tumor growth of T-cell lymphoma cell lines in both immune-deficient mice and WT mice, suggesting that galectin-9 administration can be a therapeutic tool for CTCL.

3.8. TIM-3 blockade enhances therapeutic efficacy of galectin-9 in vivo

We further examined the effects of galectin-9 on anti-tumor T cell response *in vivo*. Interestingly, CD8^{+} tumor-infiltrating T cells were decreased in galectin-9-treated mice (Fig. 6D, E), although galectin-9 suppressed tumor growth of EL-4 cells in C57BL/6 mice. These results indicated that galectin-9 induced apoptosis in not

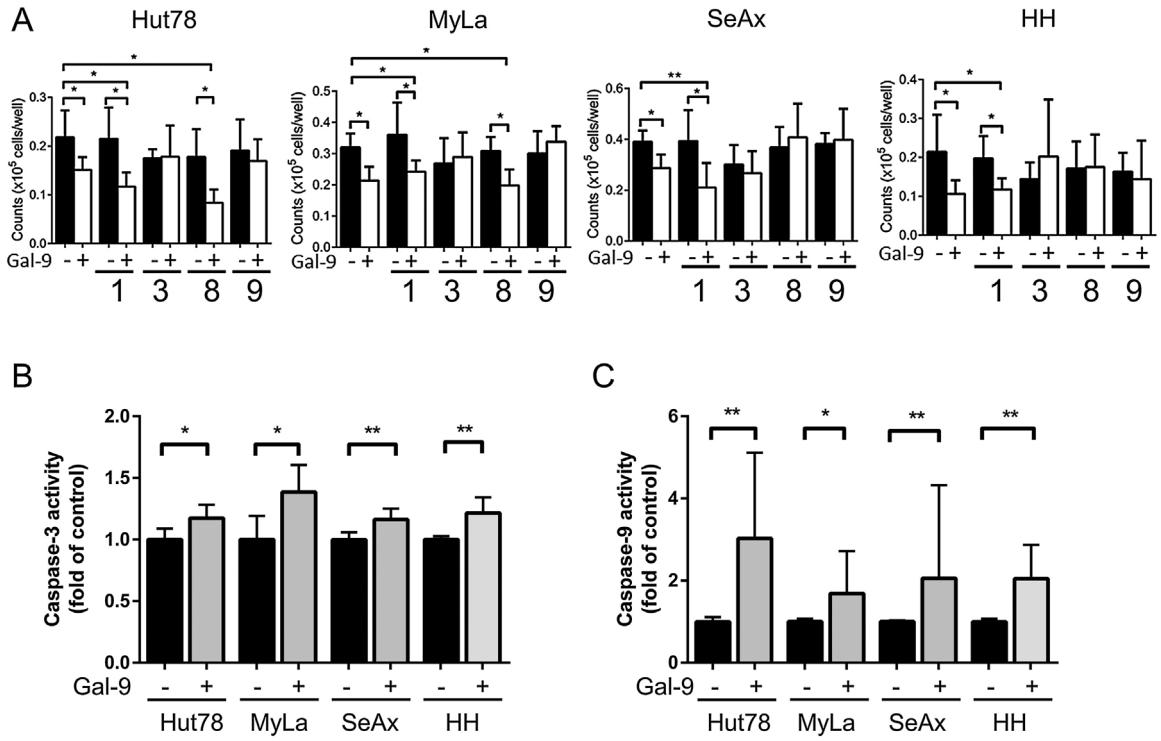

Fig. 4. Galectin-9 induces CTCL cell lines apoptosis through activation of caspase pathways. (A) CTCL cell lines were preincubated with Z-YVAD-FMK (caspase-1 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) for 1 h, followed by 2 μg/ml galectin-9 for 24 h. Data are presented as mean ± SD. * $P < 0.05$, **$P < 0.01$. (B) and (C) Caspase-3 (B) and caspase-9 (C) activity was determined in cell supernatants using caspase-3 and caspase-9 colorimetric assay kit. * $P < 0.05$, **$P < 0.01$.

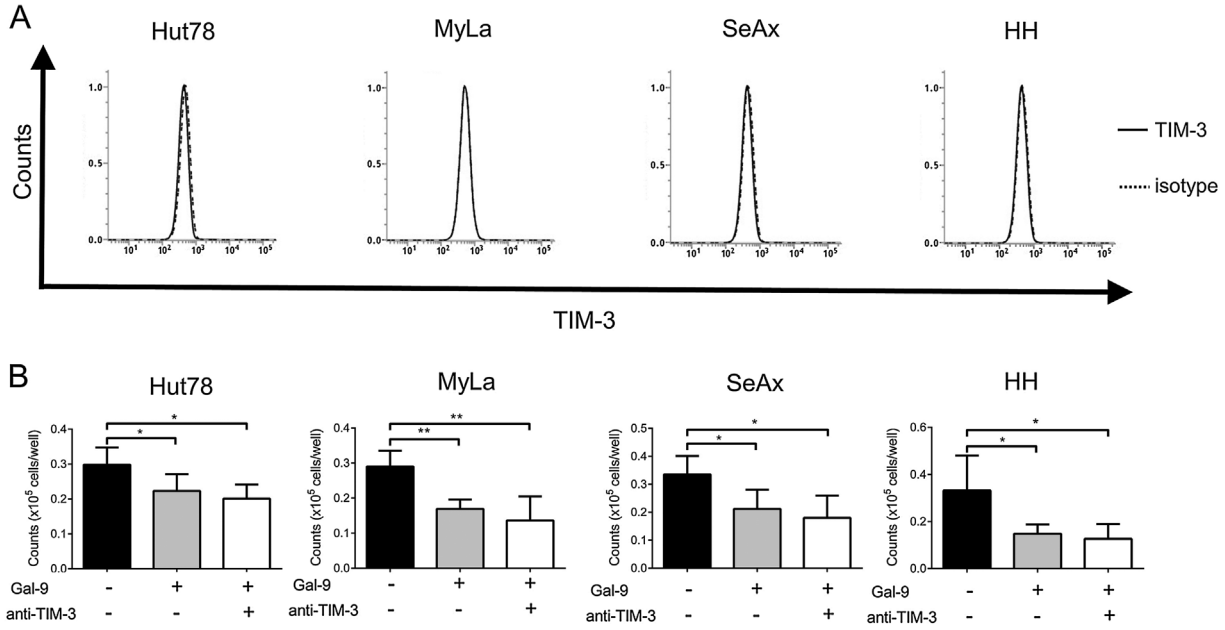

Fig. 5. TIM-3 does not act as a receptor for galectin-9 on CTCL cell lines. (A) TIM-3 surface expression was analyzed by flow cytometry in CTCL cell lines. The histogram shows the overlay of TIM-3 specific staining (solid lines) and isotype controls (dotted lines). Shown is a representative example of three independent experiments giving similar results. (B) CTCL cell lines were cultured with medium or recombinant human galectin-9 (2 μg/ml) after preincubation with anti-TIM-3 antibody for 1 h. Viable cells were counted. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$.

only EL-4 cells but also $CD8^+$ T cells *in vivo*. Consequently, we hypothesized that additional anti-TIM-3 blocking antibody administration can inhibit galectin-9-induced apoptosis of $CD8^+$ T cells but not of EL-4 cells, resulting in augmentation of anti-tumor effect of galectin-9. Thus, we next assessed the effect of combination therapy of galectin-9 and anti-TIM-3 blocking antibody on tumor formation. The inhibition of tumor growth was much more significant with combination therapy compared to single use of either galectin-9 or anti-TIM-3 blocking antibody (Fig. 6C). The number of tumor-infiltrating $CD8^+$ T cells in mice

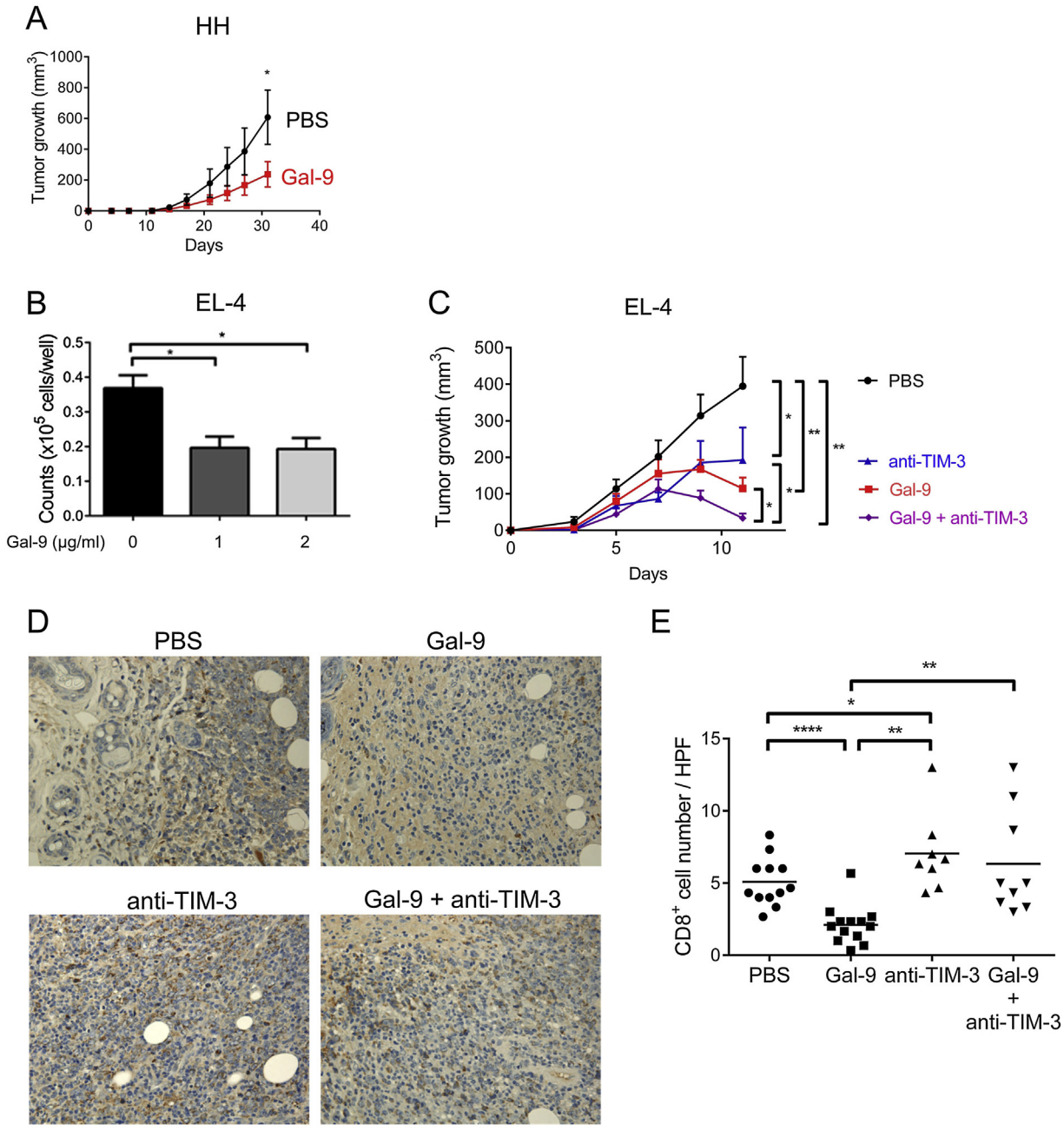

Fig. 6. Additional anti-TIM-3 blocking antibody administration augments galectin-9-induced inhibition of tumor growth of T-cell lymphoma cells *in vivo*. (A) HH cells were injected into SCID-beige mice with or without galectin-9 (1 μg/mouse). Each reagent was injected 3 times in 10 days. The tumor volume was calculated using the equation: $V = \pi$ (length × width × height)/6, where V = volume (mm^3). n = 10. * $P < 0.05$. (B) Galectin-9 inhibited proliferation in EL-4 cells (a mouse T-cell lymphoma cell line). EL-4 cells were cultured with recombinant human galectin-9 (1, 2 μg/ml) or medium for 24 h. Viable cells were counted by Trypan blue exclusion. (C) EL-4 cells were injected into WT mice. Mice were treated with PBS alone, recombinant human galectin-9, anti-TIM-3 blocking antibody, or combination of them. Recombinant human galectin-9 (1 μg/mouse) were injected subcutaneously on days 0, 4, and 7. Anti-TIM-3 blocking antibody (50 μg/mouse) were injected subcutaneously on days 4, and 7. The tumor volume was calculated using the equation: $V = \pi$ (length × width × height)/6, where V = volume (mm^3). n = 8-13. (D) Representative histological characteristics showing $CD8^+$ cell infiltration in the skin of EL-4 injected WT mice. Original magnification: x 400. (E) Numbers of $CD8^+$ cells per high power field were determined by counting respectively. The horizontal bars indicate the mean value in each group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

treated with combination therapy was not decreased and the level was similar to those in mice treated with anti-TIM-3 blocking antibody (Fig. 6D, E), suggesting that anti-tumor immunity is not impaired in those mice. Thus, TIM-3 blockade enhanced therapeutic efficacy of galectin-9 in EL-4 cell growth *in vivo*.

4. Discussion

Galectin-9 expression in cancer has been widely investigated and it is now evident that galectin-9 levels in tumor cells or tumor tissue vary compared to normal counterparts. Thus far, in many types of tumor cells including breast, lung, renal, adrenal, prostate, and melanoma cancer cells, low or decreased galectin-9 expression was detected compared to healthy counterparts [27]. On the other hand, in some malignancies, such as glioma, gastric cancer, colon cancer, Hodgkin's lymphoma, and leukemia, tumor cells or tumor tissue contained high or increased levels of galectin-9 [6,27–30]. Likewise, we found that CTCL tumor cells expressed galectin-9 and the expression levels in lesional skin were increased compared to normal tissue (Fig. 1A–E). In addition, high galectin-9 mRNA

expression was associated with decreased number of infiltrating $CD8^+$ T cells (Fig. 1F and G), consistent with the previous report on colon cancer [14]. We also found that serum galectin-9 levels were increased in advanced CTCL and correlated with disease severity markers (Fig. 2A–D). These results suggest that galectin-9 expression in tumor cells may be associated with disease progression through attenuating anti-tumor immunity in CTCL.

We next examined tumor-suppressive effect of galectin-9 in CTCL, as galectin-9 has been known to induce apoptosis in some hematologic malignancies [17,18]. Although CTCL tumor cells expressed galectin-9, exogenous high-dose galectin-9 administration inhibited *in vitro* proliferation and survival of CTCL cell lines and *in vivo* tumor growth of HH cells (Fig. 3A–C, Fig. 6A), suggesting that high-dose galectin-9 can be a therapeutic tool for CTCL.

Cell cycle arrest was not induced by galectin-9 (Fig. 3D and E) like other malignancies such as hepatocellular carcinoma and esophageal adenocarcinoma [19,24], suggesting that the main role of anti-tumor effects of galcetin-9 depends on the induction of the apoptosis of CTCL cells but not the impairment in cell cycle progression. We also found that activation of caspase-3 and caspase-9 was induced by galectin-9 in all CTCL cell lines (Fig. 4A–C). Caspase-3 plays an important role in both pathways and caspase-9 is deeply associated with the intrinsic pathway [31]. Our results suggest that the intrinsic pathway could be related to apoptosis induced by galectin-9 in CTCL cell lines. Similarly, the intrinsic pathway plays an important role in galectin-9-induced apoptosis in other hematologic malignancies including chronic myelogenous leukemia and multiple myeloma [17,18].

Recently, immune checkpoint therapy, which targets regulatory pathways in T cells to enhance anti-tumor immune responses, has led to important clinical advances and provides a new weapon against cancer. TIM-3 is the most well-known binding receptor for galectin-9, and its expression is upregulated on the surface of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes [32–34]. Galectin-9-TIM-3 interactions induce apoptosis in such cells [10–12], dampening T-cell immune responses. Blockade of galectin-9-TIM-3 interactions by anti-TIM-3 blocking antibody increases proliferation and cytokine production from TIM-3-positive T cells both in human and mouse [32,34]. In addition, anti-TIM-3 blocking antibody shows anti-cancer effect in murine tumor models [13–15]. Thus, the blockade of galectin-9-TIM-3 interactions is thought to be a promising therapy also for human malignancies. On the other hand, a recent study shows that TIM-3-deficient mice does not exhibit autoimmunity [35], unlike CTLA-4-deficient or PD-1-deficient mice [36,37]. In addition, tumor-bearing mice treated with anti-TIM-3 blocking antibody does not exhibit autoimmunity [38]. These studies indicate that targeting TIM-3 can be a safe treatment, while the exhaustion of T-cell response by anti-TIM-3 blocking antibody might be limited. Actually, in most of the clinical trials of anti-TIM-3 blocking antibody, the antibody is used in combination with other drugs, such as anti-PD-1 and anti-PD-L1 antibody [8]. In this study, we found that galectin-9-induced apoptosis in CTCL cell lines was independent of TIM-3 (Fig. 5B), similar to B-cell malignancies and hepatocellular carcinoma [17–19], suggesting that anti-TIM-3 blocking antibody administration does not interrupt galectin-9-induced apoptosis in CTCL cells. We hypothesized that combination of high-dose galectin-9 and anti-TIM-3 blocking antibody could be a superior treatment to either one alone for CTCL. As a matter of fact, we demonstrated that the combination therapy achieved greater inhibition of tumor growth of murine T-cell lymphoma cells in mice with normal immune system, compared to single administrations (Fig. 6C). Moreover, as expected, the additional administration of anti-TIM-3 blocking antibody to galectin-9 restored decreased $CD8^+$ tumor-infiltrating lymphocytes caused by galectin-9 alone (Fig. 6D, E). These results suggest that combination of anti-TIM-3 blocking antibody and galectin-9 can be a new promising therapeutic strategy for CTCL through not only inducing direct cell death of CTCL cells by galectin-9 but also augmenting anti-tumor immunity by anti-TIM-3 blocking antibody. In this regard, we started galectin-9 administration before the tumor formation. Considering that EL-4 cells are sensitive to immune systems, much higher dose of galectin-9 might be needed in clinical use. In addition, drugs are usually used after the disease development in clinical settings. Actually, 30 or 90 μg of galectin-9, which is much higher than the dose we used, was treated subcutaneously 3 times a week to suppress the growth of established cholangiocarcinoma cell tumor in mice [39].

In conclusion, CTCL tumor cells expressed galectin-9 and high galectin-9 expression was related to decreased $CD8^+$ T-cell infiltration. Serum galectin-9 levels were elevated in advanced CTCL patients and correlated with disease severity markers. These results suggest that galectin-9 expression in tumor cells may be associated with disease progression in CTCL. On the other hand, exogenous high-dose galectin-9 administration induced apoptosis in CTCL cell lines through activation of caspase-3 and caspase-9 independently of TIM-3. High-dose galectin-9 also suppressed tumor growth of a CTCL cell line and a murine T-cell lymphoma cell line *in vivo*. Furthermore, the addition of anti-TIM-3 blocking antibody to galectin-9 showed a synergic effect on tumor suppression *in vivo* by augmenting anti-tumor immunity. High-dose galectin-9/anti-TIM-3 blocking antibody combination therapy could be a new promising treatment option for CTCL.

Author contributions

R.N. performed research and analyzed data. T.M. designed the research and wrote the paper. H.K., T.O., N.ST., H.S., and M.S. collected clinical samples and data. S.S. financially supported and helped design the research.

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Declaration of Competing Interest

The authors state no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.09.004.

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