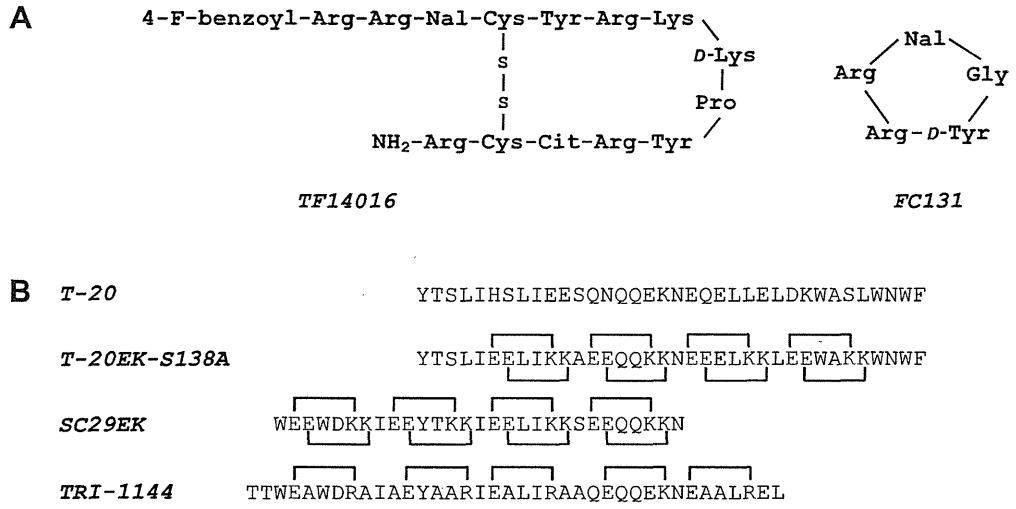

Fig. 1. Amino acid sequences of peptide-based drugs. (A) CXCR4 antagonists used in this study are shown. Nal: L-3-(2-naphthyl)alanine; Cit: L-citrulline. (B) Fusion inhibitors used are shown. T-20 is original sequenced of gp41 C-HR region. Electrostatic interactions are indicated by the linker. SC29EK and T-20EK/S138A, contain EExxKKx motif, while TRI-1144 does ExxxRxx motif. x indicates original and/or modified amino acids. Each motif creates 2 and 1 interaction(s) in each helical turn. T-20 resistance associated mutation, S138A, is introduced into T-20EK sequence (T-20EK/S138A). All peptides are N-terminally acetylated and C-terminally amidated.

with a combined rational design by the introduction of the EK motif for enhancement of α-helicity and increased affinity to mutated gp41 by S138A, a T-20 resistant associated mutation [19]. Dwyer et al. developed another fusion inhibitor, TRI-1144 (T-2635) that also exerted potent activity against T-20 resistant variants [20,21]. The amino acid sequence of TRI-1144 is also modified by substitutions with E and arginine (R), similar to the EK motif introduced into T-20EK and SC29EK.

Analyses of the efficacy and adverse effects of new drugs in animal models are important prior to their clinical application. Indeed, generally, the toxic effects, kinetics, and efficacy of new drugs are expected to be obtained by animal experiments. In the case of anti-HIV-1 drugs, the toxic effects of drug candidates can be determined by animal experiments. Furthermore, the kinetics of some drugs may be examined by some analytical methods such as liquid chromatography–mass spectrometry (LC–MS) [22] or bioimaging with labeled compounds. Unfortunately, these results may not be well-correlated with *in vivo* efficacy due to degradation and/or modification of drugs, and the detection of false positives of similar component(s) *in vivo* [23]. The efficacy of anti-HIV-1 drugs is, so far, hard to examine *in vivo* due to the lack of convenient animal infection models with low cost. One of the main obstacles to establish appropriate animal models is restricted infection of small animals with HIV-1, such as for mice, rats, and ferrets. An HIV-1 receptor-transgenic rat model has been developed for the analysis of HIV-1 infection *in vivo*; however, the levels of plasma viremia in infected rats were modest and not sustained [24,25]. Monkeys infected with simian immunodeficiency virus-HIV chimeric virus (SHIV) is the only model for the evaluation of HIV-1 replication [26], but comes at a high cost, especially for animal infection facilities. Taken together, novel rapid, simple, and sensitive HIV-1 infection models with low cost, such as those in small animals, are urgently needed to be established.

Here, we established a new system to evaluate the anti-HIV-1 activity of drugs and its kinetics in rats in addition to their toxic effects. The bioavailability of anti-HIV-1 drugs in sera was determined for the assessment of antiviral activity *in vitro*. The *in vivo* efficacy of various peptide-based entry inhibitors, such as TF14016, FC131, T-20EK/S138A, SC29EK, and TRI-1144, were assessed using this model and may be useful for the *in vivo* assessment of novel entry inhibitors.

2. Materials and methods

2.1. Drugs and cells

CXCR4 antagonists, TF14016 and FC131, and fusion inhibitors, T-20, T-20EK/S138A, SC29EK and TRI1144, were synthesized as previously described [7,9,16–18,20]. For *in vitro* drug susceptibility assays and *in vivo* administration, the test drugs were dissolved in 50% dimethyl sulfoxide (DMSO; 2 mM) and sterile water (3 or 10 mg/1.5 mL), respectively. MAGI CCR5 cells (HeLa CD4/CCR5/LTR-β-galactosidase cells) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: from Dr. Julie Overbaugh [27–29] and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum [30].

2.2. Administration of drugs

Animal experiments were performed in the Biotechnical Center of the Japan SLC, in accordance with the institutional ethical guidelines. To examine the pharmacological kinetics in sera, rats were used for collection of sera. Drugs were used at 3 mg/1.5 mL/kg of T-20, 3 mg/1.5 mL/kg of TF14016, 10 mg/1.5 mL/kg of FC131, 10 mg/1.5 mL/kg of SC29EK, 10 mg/1.5 mL/kg of T-20EK/S138A, and 3 mg/1.5 mL/kg of TRI1144, and were intraperitoneally administrated to six groups of six male SD rats (7 weeks). Sera were then harvested 0.5, 1, 2, 4, 8, and 12 h from the administrated rat, and stored at −80 °C.

2.3. MAGI assay

The anti-HIV-1 activity of drugs in rat sera after drug administration was detected by the MAGI assay, as previously described [31]. Briefly, MAGI CCR5 cells were transferred to 96-well plates at 1×10^4 cells per well. On the following day, serially-diluted drugs or rat sera were added to cells in triplicate with HIV-1 preparations ($HIV\text{-}1_{IIIB}$ or $HIV\text{-}1_{BaL}$). After 48 h, cultured cells were fixed with 1% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde in phosphate-buffered saline (PBS), and were stained with 0.4 mg/mL 5-bromo-4-chloro-3-indolyl-2-D-galactopyranoside (X-gal). Blue

cells were counted by observation under a light microscope. The 50% effective concentration was defined as the serum dilution fold or drug concentration that inhibited virus infection in 50% of the wells.

3. Results

3.1. Anti-HIV-1 activity of drugs in vitro

Prior to animal experiments, the anti-HIV-1 activity of test drugs *in vitro* was determined by the MAGI assay. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4- and R5-tropic HIV-1 strains, respectively. TF14016 exerted most potent anti-HIV-1 activity *in vitro* compared to other inhibitors as shown in Table 1. As expected, the two CXCR4 antagonists, TF14016 and FC131, inhibited replication of only HIV-1_{IIIB}, but not HIV-1_{BaL}, which uses CCR5 for its entry. All four fusion inhibitors, T-20EK/S138A, SC29EK, TRI-1144 and T-20, comparably inhibited replication of both HIV-1_{IIIB} and HIV-1_{BaL}. Among newly developed fusion inhibitors, T-20EK/S138A showed the strongest inhibitory effect both on HIV-1_{IIIB} and HIV-1_{BaL}. Our antiviral data are similar to previous observations for TF14016 and FC131 [7–10,32], T-20EK/S138A [16], SC29EK [17], and TRI-1144 [20,21].

3.2. Anti-HIV-1 activity of CXCR4 antagonists in rat

First, we examined background anti-HIV-1 activity in four PBS-injected rat sera as negative controls. In the control rat sera, anti-HIV-1_{IIIB} and HIV-1_{BaL} activities were detected (Fig. 2). Rat sera showed antiviral activity up to the 90- and 160-fold dilution for HIV-1_{IIIB} and HIV-1_{BaL} (Fig. 2; shown as a baseline activity).

The two CXCR4 antagonists, TF14016 and FC131, were intraperitoneally injected into six rats and sera were withdrawn at the indicated time as shown in Fig. 2. Drug activities were detected up to 4 h, with peak time point at 1 h after the administration. Surprisingly, sera from two rats injected with TF14016 and four rats with FC131 also weakly showed anti-HIV-1_{BaL} activity (data not shown). However, both CXCR4 antagonists were generally effective only against HIV-1_{IIIB}.

3.3. Anti-HIV-1 activity of fusion inhibitors in rat

Anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities were detected in four rat sera and all six rat sera, respectively, that were administered T-20. Anti-HIV-1 activity of T-20 in rats was detected up to 8 h with a peak time point 1–2 h after administration. Anti-HIV-1_{IIIB} activities were detected in sera of six rats injected with SC29EK, T-20EK/S138A, and TRI-1144, which were detected up to 3, 8, and 8 h, respectively, with serum peak levels at 1–2 h after administration. Anti-HIV-1_{BaL} activities were detected in sera with SC29EK, T-20EK/S138A, and TRI-1144 with similar extent with these for HIV-1_{IIIB}. These results indicate that in rats, intraperitoneally injected drug activities were present in sera and may exert anti-HIV-1 activity *in vivo*. Among these, TRI-1144 showed stable and relatively sustained activity.

3.4. Effect of heat inactivation

To identify component(s) for baseline anti-HIV-1 activity in rat sera, we examined heat inactivation. As expected, non-specific anti-HIV-1 activity in sera decreased in a time-dependent manner. At 1000-fold dilution of sera, non-specific activity was completely abolished (Fig. 3); unfortunately the drugs tested in the study were not heat stable and irreversible even at 56 °C (data not shown). However, when administered a physiological dose, anti-HIV-1 activity was detectable even without heat inactivation (Fig. 2). Therefore, the rat model system proved to be adequate to evaluate the efficacy of drugs.

3.5. Toxic effect of drugs in rats

All peptides tested showed no apparent lethal effect at the administered dosages, except for FC131, where one rat succumbed from unknown causes at a dose of 30 mg/kg.

4. Discussion

To develop effective and safe antiviral agents, *in vitro* screening systems are established for some viruses, while *in vivo* evaluation systems using small animals are hampered by limited infection efficiency and the need for specialized facilities. In the case of animal models for HIV-1, animal models are largely restricted [33]. In the present study, we describe the establishment of a novel evaluation system of anti-HIV-1 drugs through *in vitro* detection of anti-HIV-1 activity in the sera of rats administrated drugs using the MAGI assay. The *in vivo* efficacies of five potential entry inhibitors were evaluated. In this system, only TRI-1144 consistently showed potent and sustained activity compared with T-20. The glutamic acid–arginine (ER) modification, but not the glutamic acid–lysine (EK) modification and/or alanine substitutions to the peptide (Fig. 1), may have beneficial effects on stability and efficacy, resulting in sustained anti-HIV-1 activities. The simple and convenient *in vivo* efficacy evaluation system established in this study not only reveals whether drugs exert anti-HIV-1 activity *in vivo*, but also provides *in vivo* kinetics without the need for infectious animal facilities. Moreover, this system can be used for the evaluation of not only anti-HIV-1 drugs *in vivo*, but also of drugs against other viruses *in vivo*. Nonetheless, the sera produced by the rats can be also applied to resistant virus variants and clinical isolates resulting in a reduction of the number animal experiments required.

Other methods, such as a high performance liquid chromatography (HPLC), may provide accurate measurement of the drug concentration in sera and was performed in this study. Even after administration of FC131 at 30 mg/kg, we could only detect FC131 at the peak concentration (data not shown). In a case of small amount of agents with extremely high activity, it is possible to fail to detect by HPLC. For more sensitive detection by HPLC, further labeling, such as with radioisotopes, may be needed. In addition, HPLC analysis can detect drugs that have been modified and/or degraded by *in vivo* metabolism when they are spectrometrically indistinguishable. However, our system detects only the active

Table 1
Anti-HIV-1 activity of drugs *in vitro*.

Virus	EC_{50}[a] (nM)					
	TF14016	FC131	T-20	T-20EK/S138A	SC29EK	TRI-1144
HIV-1_{IIIB}	0.3 ± 0.0	17.4 ± 5.7	42.3 ± 7.6	2.0 ± 0.5	8.3 ± 1.3	4.6 ± 0.6
HIV-1_{BaL}	>10,000	>10,000	16.2 ± 4.9	0.4 ± 0.2	1.4 ± 0.2	0.4 ± 0.2

[a] Antiviral activity, shown as EC_{50}, was determined using the MAGI assay. Each EC_{50} represents the mean ± SD obtained from at least three independent experiments. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4 and R5 HIV-1 strains, respectively.

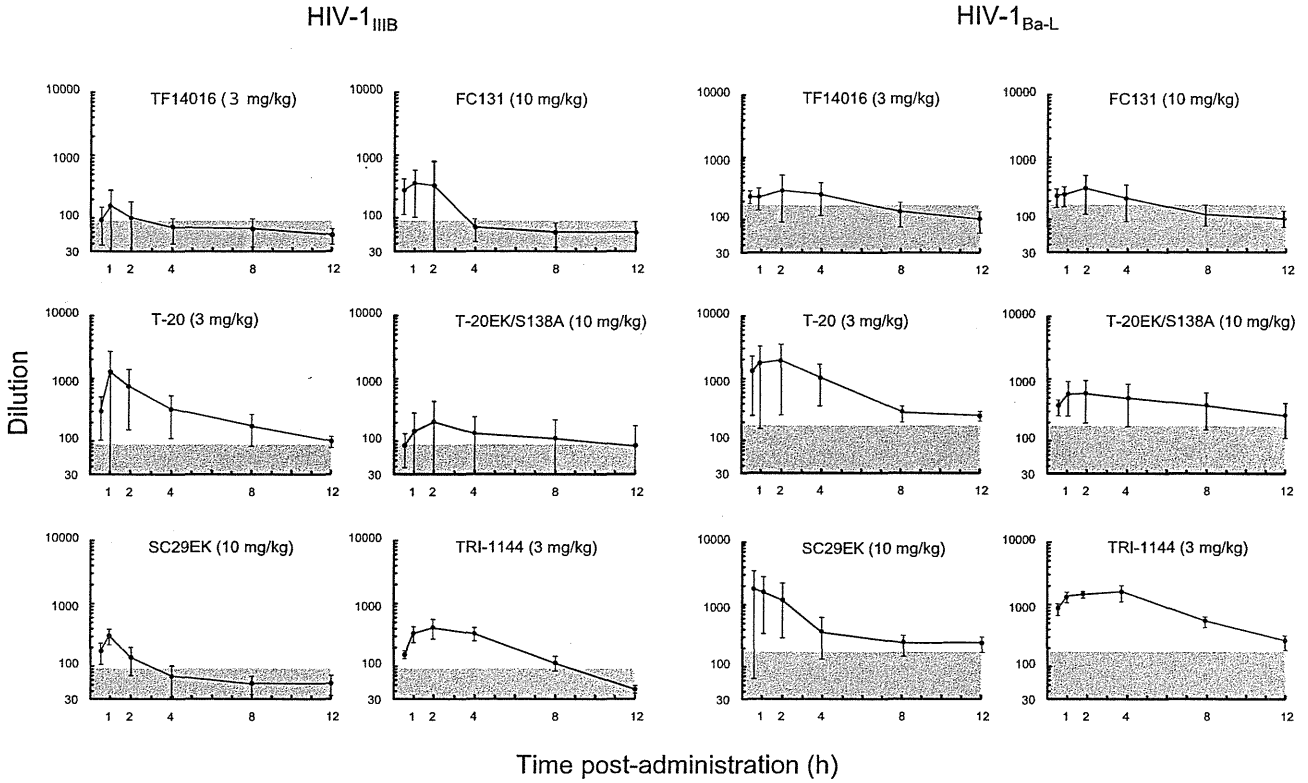

Fig. 2. Anti-HIV-1 activity of drugs *in vivo*. Six groups of six rats were administered each drug by intra-peritoneal injection and rat sera were harvested at different time points post-administration. All serum samples were analyzed by MAGI assay for 50% inhibition of infections of HIV-1_{IIIB} and HIV-1_{BaL}. This experiment was performed in triplicate for each rat. Data represent mean ± SD of from six rats. Gray shade indicates average results of age-matched rat sera as negative controls.

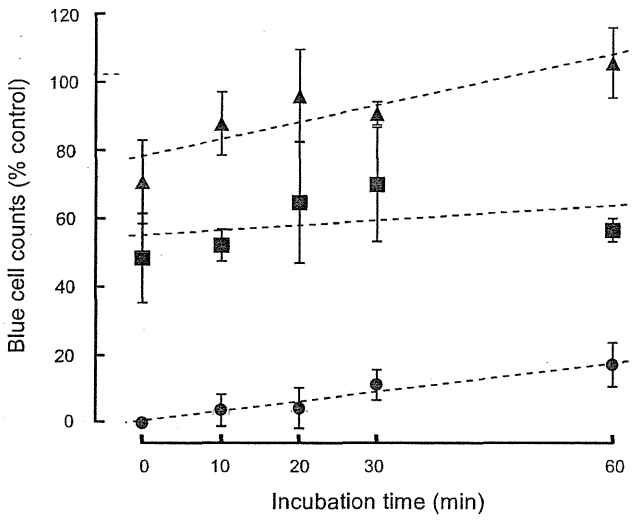

Fig. 3. Heat inactivation of sera. Rat sera without heat activation were examined using the MAGI assay. Heat inactivation was performed at 56 °C. Ten-fold dilutions of sera were resistant to heat inactivation even after 1 h inactivation (~20%). At the 1000-fold dilution, most of the inherent inhibitory effect was removed.

form of the agents, and in addition provides direct comparison of the tested drugs *in vitro* and *in vivo*, since the assay utilizes identical evaluation by the MAGI assay. In comparison, the rat *in vitro* system revealed that TRI-1144 showed strong and sustained activity compared with T-20EK/S138A and SC29EK. In this study, we only performed intraperitoneal injection that may have an effect on drug metabolism(s). Further experiments, such as subcutaneous injection, for which TF14016 shows greater efficacy [34,35], should be performed and compared with other administration roots.

The two CXCR4 antagonists analyzed in this study, TF14016 and FC131, showed moderate anti-HIV-1_{BaL} activity *in vivo*, and sera from two rats administered T-20 inhibited HIV-1 infection less efficiently (data not shown). These unexpected data might result from the relatively high background caused by non-specific inhibitory component(s) in sera. As shown in Fig. 2, sera from rats not administered drugs also showed moderate anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities. Therefore, the development of a reagent or method for removal of background activity in rat sera may improve the accuracy and sensitivity of this *in vivo* drug efficacy evaluation method. For instance, serum albumin [36], lactoferrin [37,38], and transferrin [39] may influence HIV replication. Unfortunately, the drugs used in this study were all peptide-derived agents, therefore, heat-inactivation may reduce antiviral activity. Therefore, administration of relatively high doses of drug may be required to overcome this inhibition.

In conclusion, we established a novel, simple and rapid system for the phenotypic evaluation of anti-HIV-1 drugs in a rat model. This system may also be applicable for the analysis of other antiviral drugs for viruses that do not have an appropriate infection model in rodents, and/or useful for the initial screening, such for dosing, administration root decision and other factors, prior to actual animal infection experiments. In this system for HIV infection, TRI-1144 displayed the most potent anti-HIV-1 activity *in vivo* of the six drugs analyzed.

Acknowledgments

This work was supported by a Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Grant

for the Promotion of AIDS Research from the Ministry of Health, Labour and Welfare, a Grant for Research for Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation and the Science and Technology Incubation Program in Advanced Regions from the Japan Science and Technology Agency.

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Tohoku J. Exp. Med., 2012, **228**, 157-161

Rapid Decrease of Plasma Galectin-9 Levels in Patients with Acute HIV Infection after Therapy

Hiroki Saitoh,[1] Yugo Ashino,[1] Haorile Chagan-Yasutan,[2] Toshiro Niki,[3] Mitsuomi Hirashima[3] and Toshio Hattori[1,2]

[1]Division of Emerging Infectious Diseases, Department of Internal Medicine, Graduate School of Medicine, Tohoku University, Sendai, Japan
[2]Laboratory of Disaster-related Infectious Disease, International Research Institute of Disaster Science, Tohoku University, Sendai, Japan
[3]Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Acute HIV-1 infection is often diagnosed as infectious mononucleosis and the symptoms resolve spontaneously after varying periods of time. After the infection of HIV-1 through the mucosa, the characteristic clinical symptoms and laboratory markers of acute HIV-1 infection appear in each patient through a complicated virus-host interaction. To understand the host responses, we measured two unique proinflammatory cytokines, galectin-9 (Gal-9) and osteopontin (OPN). A β-galactoside-binding mammalian lectin, Gal-9, reduces pro-inflammatory type-1 helper T (Th1) cells and Th17 cells and increases anti-inflammatory regulatory T cells. The plasma level of Gal-9 is known to be associated with HIV-1 viral load in chronic HIV-1 infection. On the contrary, osteopontin induces Th1/Th17 cells and promotes tissue inflammation. OPN is synthesized by variety of cells in the body, and dendritic cells are known to synthesize OPN in HIV-1 infected individuals. It was hypothesized that Gal-9 and/or OPN could be not only immune-modulators but also novel biomarkers of acute HIV-1 infection. We experienced 3 patients with acute HIV-1 and measured the levels of Gal-9 and OPN periodically before and after antiretroviral treatment. The results showed that the plasma levels of Gal-9 were extremely elevated [more than 2,300 pg/ml (normal range < 46 pg/ml)] in all three acute HIV-1 infected individuals and decreased rapidly after treatment. The changes in the OPN levels were less marked. In conclusion, the plasma levels of Gal-9 may be predictive of a severe inflammation status during the acute phase of HIV-1 infection and could be a potential biomarker during acute infection.

Keywords: acute HIV-1; CMV; galectin-9; HBV; osteopontin
Tohoku J. Exp. Med., 2012 Oct, **228** (2), 157-161.

It is estimated that 50-70% of individuals with HIV infection experience acute clinical syndrome 3-6 weeks after primary infection. The syndrome is typical of an acute viral syndrome and has been linked to acute infectious mononucleosis. Symptoms usually persist for one to several weeks and gradually subside as an immune response to HIV develops and the levels of plasma viremia decrease. Opportunistic infections has been reported during this stage of infection, reflecting the immune-deficiency that results from reduced numbers of $CD4^+$ T cells and likely also from the dysfunction of $CD4^+$ T cells owing to infection. (Fauci and Lane 2008). The phenotype of most productively infected cells appears to be the resting $CD4^+$ T cell lacking activation markers and expressing low levels of the chemokine receptor CCR5, which is co-receptor of R5 viruses. Many of these cells express $\alpha 4\beta 7$ integrin receptors and type-17 helper T (Th17)-cell surface markers (Cohen et al. 2011). We previously reported a persistent elevation of the plasma levels of osteopontin (OPN) in acquired immunodeficiency syndrome (AIDS) patients after antiretroviral therapy (ART), though the levels of other inflammatory markers decreased rapidly (Chagan-Yasutan et al. 2009). OPN is a multifunctional protein with known roles in bone remodeling, wound healing, and normal and pathological immune responses. It is known that OPN gene expression is increased in HIV-1 infected lymphoid tissues after treatment. It is also shown that OPN is expressed in follicular dendritic cells (Li et al. 2005). OPN is known to induce Th1/Th17 cells and promote tissue inflammation (Buback et al. 2009; Chen et al. 2010). We also noted the extreme elevation of the galectin-9 (Gal-9) level in one patient with acute human immunodeficiency virus (HIV-1) infection (Chagan-Yasutan et al. 2009). Gal-9 is a β-galactoside-binding mammalian lectin that is known to regulate immu-

Received May 18, 2012; accepted September 11, 2012. Published online October 2, 2012; doi: 10.1620/tjem.228.157.
Correspondence: Toshio Hattori, M.D., Ph.D., Division of Emerging Infectious Diseases, Department of Internal Medicine, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan.
e-mail: hatori286@yahoo.co.jp

Table 1. Patients profile.

Patients	Gender	Age (yr)	Diagnosis	Complications before ART	CD4 count (/ul)	HIV RNA (copies/ml)
1	Male	27	Acute HIV	Syphilis, Fever	67	3.1×10^5
2	Male	25	Acute HIV	Amebic liver abscess	385	2.0×10^5
3	Male	26	Acute HIV	CMV, HBV, Syphilis	207	2.3×10^6

HIV, human immunodeficiency virus; CMV, cytomegalovirus; HBV, hepatitis B virus; ART, antiretroviral therapy.

nity by reducing pro-inflammatory Th1/Th17 cells (Seki et al. 2008). Recently it was found that plasma Gal-9 levels are correlated with HIV-viral load in chronic HIV-1 infection (Elahi 2012). Therefore these two molecules may play important roles in pathogenesis of acute HIV-1 infection and could be good bio-markers of acute HIV-1 infection. Here, two additional patients of acute HIV-1 infection with various opportunistic infections are described (Table 1). Those patients, including patient 3, did not receive any antiretroviral treatment before hospitalization because those patients were diagnosed as acute HIV-1 for the first time in our hospital.

Patient Report

Patient 1: The patient was a 27-year-old male who was described previously (Chagan-Yasutan et al. 2009). He was diagnosed as acute HIV-1 infection on July 2007 one month after the development of acute febrile illness. Upon admission, he had no remarkable clinical symptoms except for a skin rash. Acute HIV-1 infection was diagnosed by confirming seroconversion and western blot profile. ART was introduced due to low CD4 count (67/μl) and his symptoms resolved immediately after ART.

Patient 2: A 25-year-old homosexual man had fever and lymphadenopathy on November in 2008 and was hospitalized in a nearby hospital. He was suspected of hemophagocytic syndrome due to low platelet and white blood cell counts, and steroid was given under the diagnosis of fever of unknown origin. The symptoms temporarily resolved and the patient was discharged, but fever and diarrhea developed two weeks later. CT scan revealed liver abscess and he was diagnosed as amebiasis and was treated by metronidazole (Dec 20, 2008). He developed a skin rash and was found to be HIV-1 positive and was transferred to our hospital (Dec 22, 2008). He was diagnosed as a suspected patient of acute HIV-1 due to the clinical symptoms and metronidazole was continuously given until Dec 31. The clinical symptoms resolved and the virus titers decreased from 2×10^5 copies/ml (Dec 24, 2008) to 8×10^4 (Jan 7, 2009) with a marked increase of $CD8^+$ cells (1,132 to 2,336/μl). ART was not initiated in this patient due to the improvements of patients' conditions.

Patient 3: A 26-year-old homosexual man had been suffering from a high-grade fever (as high as 40 degrees) and headache for a week and was admitted the neurology department of a general hospital (Dec 2010). Brain CT revealed no abnormal signals. Since HIV infection was suspected with ELISA, the patient was transferred to our hospital. He had been treated under a diagnosis of syphilis 4 months before admission and cervical lymph node swelling was noted. Laboratory analysis revealed HIV-RNA: 2.3×10^6 copies/ml, $CD4^+$ cell count: 207/μl and HIV antibody titer: 11.9, but western blot analysis gave no definitive band. From these findings, we diagnosed the patient as acute HIV infection. Furthermore, HBsAg, HBeAg, IgM-HBcAb, and HBcAb were positive and HBsAb and HbeAb were negative. The hepatitis B virus (HBV) DNA level was more than 9.1 log copies/ml and the genotype was confirmed to be Ae. The transaminase levels were also elevated (AST 123 IU/l and ALT 132 IU/l). Therefore, the patient diagnosed as mild acute hepatitis B. The plasma contained both cytomegalovirus (CMV) IgM and IgG antibodies and CMV-DNA (1,077 copies/0.2 μg DNA). The anti-CMV IgG antibody titers increased from 11 to 58 (EIA cut off < 3) during 5 months. Moreover, recent reinfection by syphilis was also suspected because of the high titers of rapid plasma regain (RPR) and treponema pallidum haemagglutination assay (TPHA) (32, 16,470 times each), though the patient had no apparent symptoms of syphilis.

He was initially treated with oral valganciclovir and azithromycin for the CMV and syphilis. Because we measured CMV-DNA once, CMV pp65 antigen was followed to analyze CMV infection. After starting the valganciclovir treatment, the number of CMV pp65 antigen-positive cells decreased after one week (24/33 to 0/0 of duplicated positive cell count per 150,000 of WBC). Azithromycin treatment caused the titers of both RPR and TPHA to gradually become lower during 6 months (RPR, TPHA: 1.0, 413.2 times). The patient complained of urinary pain and had bacteruria due to *Neisseria gonorrhoeae* after admission, which was cured by ceftriaxone infusion. Since, as is well known, both HIV-1 and HBV are sensitive to reverse transcriptase inhibitors, we started antiretroviral therapy (emtricitabine, tenofovir and raltegravir), after which the HIV-1-RNA and HBV-DNA decreased rapidly to less than 40 copies/ml of HIV-1-RNA after 3 months and less than 3.5 log copies/ml of HBV-DNA after 5 months (Fig. 1). The doses of antiretroviral drugs are shown in Fig. 1. Three weeks after the initiation of HAART, the number of CMV pp65 antigen-positive cells mildly increased again (14/8 of duplicated positive cell count per 150,000 of WBC), but decreased with re-treatment by valganciclovir (Fig. 1).

The levels of Gal-9 and OPN in the plasma were measured using ELISA to determine the inflammation status, as

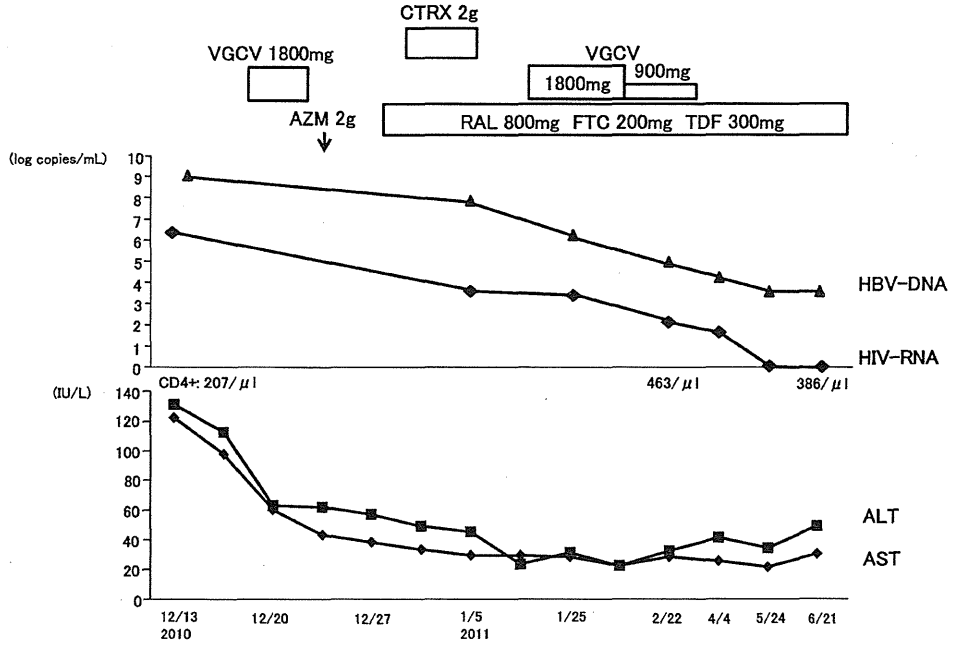

Fig. 1. Treatment and clinical course of patient 3 in acute HBV, HIV and CMV coinfection with syphilis and urinary *Neisseria gonorrhoeae* infection. The patient was treated with oral valganciclovir (VGCV) and azithromycin (AZM) for the viremia of CMV and syphilis before HAART. The viral titers of both HBV and HIV decreased and the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) gradually normalized after the initiation of emtricitabine (FTC), tenofovir (TDF) and raltegravir (RAL). The $CD4^+$ cell count was increased to about twice that before the treatment. Urinary *Neisseria gonorrhoeae* infection was cured by ceftriaxone (CTRX) infusion. A recurrence of mild viremia of CMV was observed and retreatment with VGCV was performed.

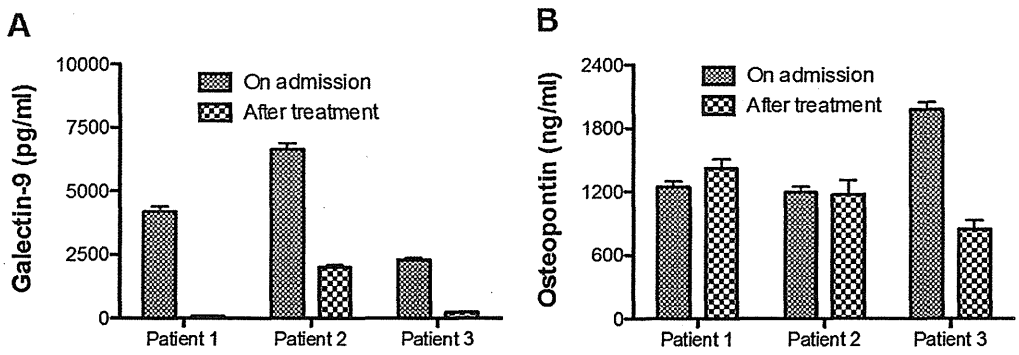

Fig. 2. Plasma levels of galectin-9 and osteopontin in acute HIV patients before and after treatment.

described previously (Chagan-Yasutan et al. 2009). The research on inflammatory markers of febrile patients was approved by the ethics committee of Tohoku University Hospital (No. 2008-135). The levels were measured before and after treatment. All 3 acute HIV-1 patients showed high levels of Gal-9 and the levels were decreased after ART in patient 1 and 3, and after therapy for amebiasis in patient 2 (Fig. 2). Furthermore, periodically collected samples were available in patient 3 and we compared the levels of Gal-9 and OPN with other inflammatory markers such as C-reactive protein (CRP) and serum amyloid acid (SAA) (Table 2). The levels of CRP and SAA were not remarkable. The plasma levels of Gal-9 and OPN were the highest on admission (Dec 12, 2010, 2,304 pg/ml and 1,972 ng/ml) but the peaks of CRP and SAA were delayed. The changes of Gal-9 (−89%) were greater than those of OPN (−58%) (Fig. 2).

Discussion

The levels of Gal-9 and OPN were extremely and moderately elevated, respectively in 3 patients of acute HIV-1 infection, and the changes in the CRP and SAA levels were not remarkable in patients 3, suggesting that the increases of Gal-9 and OPN would be better markers of HIV-1 infection compared to other inflammatory markers. Also, a marked decrease of the Gal-9 levels was noted after

Table 2. The changes of inflammatory markers during the clinical course of patient 3.

	Ref. range	12/14/2010	12/24/2010	1/5/2011	1/25/2011
CRP(mg/dl)	< 0.2	0.2	0.6	0.2	0.1
SAA(μg/ml)	< 7.9	24.2	40.4	11.5	3.3
OPN(ng/ml)	< 820*	1972	1265	840	NA
Gal-9(pg/ml)	< 46*	2304	1132	315	257

CRP, C-reactive protein; SAA, serum amyloid acid; OPN, osteopontin; Gal-9, galectin-9; NA, not available.
*detailed information see reference (Chagan-Yasutan et al, 2009).

therapy, suggesting it could reflect the severity of the disease or response to therapy in acute HIV-1 infection. Furthermore, therapy for co-infected amebiasis without anti-HIV-1 therapy also decreased the Gal-9 levels as well as the HIV virus load in patient 2. It should be clarified whether parasite or other bacterial infections also induce elevations of Gal-9. More extensive and larger numbers of studies are necessary to draw a conclusion, though the induction of Gal-9 by a variety of pathogens in mice was reported (Reddy et al. 2011; Qi et al. 2012). Gal-9 is known to bind to T-cell immunoglobulin-and mucin-domain-containing molecule-3 (Tim-3), which is induced on T cells by HIV-1, HBV, and hepatitis C virus (HCV) (Jones et al, 2008; Mengshol et al. 2010; Li et al. 2012), though the levels of Gal-9 in other virus-induced diseases are not known. In the present patient 3, the plasma from the patient contained CMV-DNA, and the CMV IgM titer was elevated while the CMV IgG titer was very low. Additionally, the CMV IgG titer increased more than 4 fold during 5 months. The patient was diagnosed as both acute HIV-1 and CMV infection, while the recurrence of CMV in an immunosuppressive state was less likely because the CD4 count was more than 200/μl.

The Gal-9 levels were decreased by 50% with valganciclovir alone for the treatment of CMV. CMV is also known to induce Tim-3 on $CD8^+$ cells, though Gal-9 induction by CMV infection has not been reported yet (Wu et al. 2012). The Gal-9 levels were further decreased after ART. Since antiretroviral therapy is known to be successful for both HIV-1 and HBV viruses (Bansal et al. 2010), it is not clear whether the decreased Gal-9 levels can be attributed to the effect of the ART on HBV or HIV-1 in this specific patient. Nevertheless, the amelioration of these virus-induced diseases was reflected in the levels of Gal-9 and OPN, though the changes of Gal-9 were more marked.

Antiretroviral therapy for acute HIV-1 retroviral syndrome is still controversial. However, we treated two of three patients based on the low CD4 count and the persistently high copy numbers of HIV-RNA. It was also suspected that the lower $CD4^+$ cell count due to primary HIV-1 infection might have induced the prolonged hepatitis and inadequate elimination of HBV and CMV. In such patients with multiple virus infections, reliable inflammatory markers are necessary to evaluate the efficacy of the treatment. Analysis of various biomarkers in HIV-1 patients showed that the plasma level of OPN was the one most specifically associated with HIV-1 infection (Siddiqi et al. 2012). In this connection, we already reported the persistent elevation in the OPN levels after HAART (Chagan-Yasutan et al. 2009). It was also shown that macrophages, not malignant cells, are the main producers of OPN in HTLV-1 infected lymphoma (Chagan-Yasutan et al. 2011). Therefore, the elevation of OPN may reflect the activation of macrophages, or stress caused by microorganisms, neoplastic cells and drugs.

Though Gal-9 is also secreted by variety of cells, the marked decline of the Gal-9 levels after treatment in our patients suggest that Gal-9 may be associated with clinical amelioration in acute HIV-1 infection, since Gal-9 has been reported to diminish the susceptibilities against HIV-1 infection by interacting with Tim-3 on $CD4^+$ T cells (Elahi et al. 2012) and Gal-9 is known to regulates immunity by reducing pro-inflammatory Th1/Th17 cells. In addition, Gal-9 also activates macrophages and facilitates the elimination of intracellular mycobacterium, which may render resistance against mycobacterium tuberculosis in acute HIV-1 infection (Jayaraman et al. 2010). The analysis of Gal-9-producing cells in viral-infected individuals is in progress.

Taken together we present the successful treatment of patients with multiple-pathogen infection and found that Gal-9 reflects the therapeutic efficacy.

Acknowledgments

This work was supported by collaborative funding from the Research Centre for Zoonosis Control, Hokkaido University.

Conflict of Interest

The authors declare no conflict of interest.

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Cell Surface Galectin-9 Expressing Th Cells Regulate Th17 and Foxp3$^+$ Treg Development by Galectin-9 Secretion

Souichi Oomizu[1☯], Tomohiro Arikawa[2☯], Toshiro Niki[1,3]*, Takeshi Kadowaki[4], Masaki Ueno[5], Nozomu Nishi[6], Akira Yamauchi[7], Toshio Hattori[8], Tsutomu Masaki[9], Mitsuomi Hirashima[1,3]

1 Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa University, Kagawa, Japan, **2** Department of Biology, Kanazawa Medical University, Ishikawa, Japan, **3** GalPharma Co., Ltd., Kagawa, Japan, **4** Department of Holistic Immunology, Kagawa University, Kagawa, Japan, **5** Department of Inflammation Pathology, Faculty of Medicine, Kagawa University, Kagawa, Japan, **6** Life Science Research Center, Kagawa University, Kagawa, Japan, **7** Department of Breast Surgery, Kitano Hospital, Osaka, Japan, **8** Laboratory of Disaster-related Infectious Disease, International Research Institute of Disaster Science, Tohoku University, Sendai, Japan, **9** Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Abstract

Galectin-9 (Gal-9), a β-galactoside binding mammalian lectin, regulates immune responses by reducing pro-inflammatory IL-17-producing Th cells (Th17) and increasing anti-inflammatory Foxp3$^+$ regulatory T cells (Treg) *in vitro* and *in vivo*. These functions of Gal-9 are thought to be exerted by binding to receptor molecules on the cell surface. However, Gal-9 lacks a signal peptide for secretion and is predominantly located in the cytoplasm, which raises questions regarding how and which cells secrete Gal-9 *in vivo*. Since Gal-9 expression does not necessarily correlate with its secretion, Gal-9-secreting cells *in vivo* have been elusive. We report here that CD4 T cells expressing Gal-9 on the cell surface (Gal-9$^+$ Th cells) secrete Gal-9 upon T cell receptor (TCR) stimulation, but other CD4 T cells do not, although they express an equivalent amount of intracellular Gal-9. Gal-9$^+$ Th cells expressed interleukin (IL)-10 and transforming growth factor (TGF)-β but did not express Foxp3. In a co-culture experiment, Gal-9$^+$ Th cells regulated Th17/Treg development in a manner similar to that by exogenous Gal-9, during which the regulation by Gal-9$^+$ Th cells was shown to be sensitive to a Gal-9 antagonist but insensitive to IL-10 and TGF-β blockades. Further elucidation of Gal-9$^+$ Th cells in humans indicates a conserved role of these cells through evolution and implies the possible utility of these cells for diagnosis or treatment of immunological diseases.

Citation: Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, et al. (2012) Cell Surface Galectin-9 Expressing Th Cells Regulate Th17 and Foxp3$^+$ Treg Development by Galectin-9 Secretion. PLoS ONE 7(11): e48574. doi:10.1371/journal.pone.0048574

Editor: Alexandre Salgado Basso, Escola Paulista de Medicina - UNIFESP, Brazil

Received May 18, 2012; **Accepted** September 26, 2012; **Published** November 7, 2012

Funding: This work was supported by a Grant-In-Aid for young scientists (B) 2008–2009 (20790570) from the Japan Society for Promotion of Science (JSPS), Scientific Research (C) 2010- (22590360) from JSPS, Scientific Research (C) 2011- (23591438) from JSPS, Scientific Research (A) 2011- (23256004) from JSPS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. (JSPS HP: http://www.jsps.go.jp/). No additional external funding was received for this study.

Competing Interests: The authors have read the journal's policy and have the following conflicts: Drs. Niki and Hirashima are board members of GalPharma Co., Ltd. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: niki.t@galpharma.com

☯ These authors contributed equally to this work.

Introduction

Galectin-9 (Gal-9) is a member of the galectin family of mammalian lectins and is characterized by its ability to bind β-galactoside. Gal-9 is expressed by the epithelium of the gastrointestinal tract, endothelial cells and several types of immune cells including T cells, B cells, macrophages and mast cells [1,2]. Recently, the regulatory role of Gal-9 in excessive immunity has become evident. Gal-9 suppresses interleukin (IL)-17-producing effector T helper cells (Th)17 and Th1 [3]; these cells play an exacerbating role in the pathogenesis of various autoimmune diseases, whereas Gal-9 augments Foxp3$^+$ regulatory T cells (Treg), an essential suppressor of excessive immunity [3]. In addition to increasing Treg, Gal-9 expands the population of monocytic myeloid-derived suppressor cells (MDSCs) [4], granulocytic MDSCs [5,6], and plasmacytoid dendritic cell-like macrophages [7–9]. Induction of these regulatory cells appears to be a critical regulatory function of Gal-9.

The function of Gal-9 is thought to be exerted by binding to particular sets of carbohydrate moieties in receptor molecules expressed on the surface of target cells. Among several identified receptors of Gal-9, the T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) has been studied most extensively. Binding of Gal-9 to Tim-3 expressed by activated Th1 and/or Th17 triggers cellular apoptosis and terminates Th1/Th17-skewed immunity [10,11]. Gal-9 must be secreted by some types of cells to initiate this response. However, Gal-9 lacks a signal sequence essential for secretion via the canonical endoplasmic reticulum (ER)-Golgi pathway and is located predominantly in the cytoplasm where it plays roles in protein sorting and transcriptional regulation of cytokine genes [12,13]. Gal-9 secretion has been demonstrated in several cell lines [14,15], but the mechanism of Gal-9 translocation through the lipid bilayer, as well as the identification of Gal-9-secreting cells *in vivo* has yet to be elucidated. Gal-9 expression and secretion are not always correlated. Recently, Wang *et. al.* suggested through indirect observation that Treg secretes Gal-9 and ameliorates Th1 responses [16]. From a functional perspective of the protein, Gal-9 may be secreted by regulatory cells, including Treg.

We hypothesize that Gal-9-secreting cells might express Gal-9 on the cell surface as a translocation intermediate and may be identified by staining the cells with specific antibodies. The goal of this study was to identify Gal-9-secreting cells. We identified CD4 T cells expressing Gal-9 on their surfaces (Gal-9$^+$ Th cells). Gal-9$^+$ Th cells secrete Gal-9 upon T cell receptor (TCR) stimulation, but other CD4 T cells lacking Gal-9 on the surface do not, although they express indistinguishable amounts of Gal-9 intracellularly. The characteristics of Gal-9$^+$ Th cells and the significance of these cells in immunoregulation are discussed.

Results

Gal-9-secreting Cells Emerge from Naïve CD4 T cells by TCR Stimulation

We recently found that exogenous Gal-9 suppresses Th17 development and simultaneously enhances Treg development *in vitro*, even under Th17-skewing conditions, in an IL-2-dependent but Tim-3-independent manner [11]. These results suggest that endogenous Gal-9 plays a role in Th17/Treg development and that Gal-9 production and/or secretion may be suppressed under Th17-skewing conditions. To confirm this, we examined Gal-9 secretion from naïve CD4 T cells cultured under neutral and Th17-skewing conditions. As expected, Gal-9 was secreted under neutral conditions, whereas secretion was suppressed under Th17-skewing conditions (**Figure 1A**) largely because of the presence of IL-6 in the culture (**Figure 1B**). Despite differences in secretion capability, Gal-9 mRNA expression level did not differ between neutral and Th17-skewing conditions (**Figure 1C**). We further examined whether exogenous Gal-9 stimulates endogenous Gal-9 secretion. For this experiment, 30 nM of recombinant human stable Gal-9 was added to the culture, and the secretion of mouse Gal-9 was monitored using a specific enzyme-linked immunosorbent assay (ELISA) for mouse Gal-9. Human recombinant Gal-9 cross-reacts with mouse cells biologically and has been used in various rodent studies, but it was not detected in our mouse Gal-9 ELISA (**Figure S1**). As seen in **Figure 1D**, exogenous Gal-9 enhanced Gal-9 secretion even under Th17-skewing conditions. These results suggest that (1) Gal-9-secreting cells are present under neutral conditions, but the secretion capability and/or the number of Gal-9-secreting cells is reduced under Th17-skewing conditions, largely by IL-6, and (2) exogenous Gal-9 counteracts the inhibition of Gal-9-secretion even under Th17-skewing conditions.

Surface Gal-9-expressing Th cells Secrete Gal-9

We hypothesized that Gal-9 could be detected on the surface of Gal-9-secreting cells as an intermediate during translocation through the lipid bilayer. We thus performed flow cytometry to measure surface Gal-9 expression using cells stimulated as described in **Figure 1A**. During secretion, Gal-9 likely binds to adjacent cells via their cell surface glycoproteins or glycolipids, which may complicate the identification of Gal-9-secreting cells. Therefore, Gal-9 staining was performed in the presence of 30 mM lactose, because this concentration of lactose is sufficient for removing exogenously added Gal-9 bound on the cell surface without affecting Gal-9 staining (**Figure S2A**).

Gal-9 staining revealed the existence of surface Gal-9-expressing CD4 T cells (Gal-9$^+$ Th cells). The frequency of Gal-9$^+$ Th cells was approximately 1.5% without TCR stimulation and was increased to approximately 4% after TCR stimulation under neutral conditions (**Figure 2A**). Interestingly, the frequency of Gal-9$^+$ Th cells as well as CD25$^+$ CD4 T cells was reduced under Th17-skewing conditions (**Figure 2A**). Surface Gal-9 stably adhered to the cell surface during staining at 4°C even in 100 mM lactose (**Figure S2B**). Gal-9 secretion (**Figure 1A**) correlates well with the emergence of Gal-9$^+$ Th cells (**Figure 2A**). To confirm our hypothesis that these Gal-9$^+$ Th cells are the primary source of secreted Gal-9, Gal-9$^+$ and Gal-9$^-$ naïve Th cells were isolated using a cell sorter (**Figure S3**), cultured under neutral conditions and examined for Gal-9 secretion by ELISA. Consistent with our hypothesis, Gal-9$^+$ Th cells, but not Gal-9$^-$ Th cells, secreted Gal-9 upon TCR stimulation (**Figure 2B**). This is the first report of identification of Gal-9-secreting Th cells and demonstrates a useful technique for detecting cells with Gal-9 secretion capability. Enigmatically, expression levels of Gal-9 mRNA and intracellular Gal-9 protein did not show any apparent differences between Gal-9$^+$ and Gal-9$^-$ Th cells stimulated under neutral conditions (**Figure 2C**). Cytokine mRNA measurement demonstrated that compared to Gal-9$^-$ Th cells Gal-9$^+$ Th cells induced significantly higher levels of IL-10 and TGF-β upon TCR stimulation (**Figure 2D**). Induction of IFN-γ was similar to Gal-9$^-$ Th cells, while IL-2, IL-4 and IL-17 were not induced by Gal-9$^+$ Th cells (**Figure 2D**).

The frequency of Gal-9$^+$ CD25$^-$ Th cells in various lymphoid organs of normal mice was determined using flow cytometry. Approximately 25% of CD4 single-positive cells in thymus cells expressed surface Gal-9. Among CD25$^-$ CD4 T cells, approximately 4% in lymph nodes, 7% in spleen and peripheral blood mononuclear cells and 15% in Peyer's patches expressed surface Gal-9 (**Table 1 and Figure S5**).

Gal-9$^+$ Th cells are Different from Treg

Recently, Treg was suggested to secrete Gal-9 to suppress Th1 immunity [16]. As shown in **Figure 2D**, the expression of IL-10 and TGF-β by Gal-9$^+$ Th cells appears to further support the identity between Treg and Gal-9$^+$ Th cells. However, Gal-9$^+$ Th cells were devoid of Foxp3 expression while Foxp3$^+$ Th cells were devoid of surface Gal-9 expression (**Figure 3A**). Therefore, Gal-9$^+$ Th cells were clearly a different population from Foxp3$^+$ Treg and rarely co-expressed Tim-3 (**Figure 3B**). In Gal-9 knockout mice, the frequency of IL-10$^+$ cells was significantly lower in CD4 T cells compared to those from wild type mice (**Figure 3C**). As demonstrated in **Figure 2A**, the number of Gal-9$^+$ Th cells increased under neutral conditions but decreased under Th17-skewing conditions. Similarly, IL-10 expression decreased under Th17-skewing conditions and was further decreased by Gal-9 deficiency (**Figure 3D**). These observations suggest a close relationship between Gal-9$^+$ Th cells and IL-10-producing CD4 T cells.

Gal-9+ Th cells Regulates Th17/Treg Development by Gal-9

When naïve CD4 T cells committed to Th17 development were co-cultured with Gal-9$^+$ Th cells (1:1), IL-17A production was significantly suppressed, whereas Foxp3 expression was reciprocally induced (**Figure 4A**). Suppression of IL-17A production by Gal-9$^+$ Th cells was abrogated by lactose, an antagonist of Gal-9, but not by sucrose (**Figure 4B**). The induction and function of Th17 are known to be regulated by cytokines secreted by other major T cell subsets, including IFN-γ, IL-4, IL-10, and TGF-β [17]. Since IL-10 and TGF-β are highly expressed by Gal-9$^+$ Th cells, we examined the effect of blocking antibodies against IL-10 and TGF-β. However, these cytokine blockades did not affect the regulatory activity of Gal-9$^+$ Th cells (**Figure 4C**). Furthermore, the addition of recombinant IL-10 did not suppress IL-17A production in our assay system (**Figure 4D**).

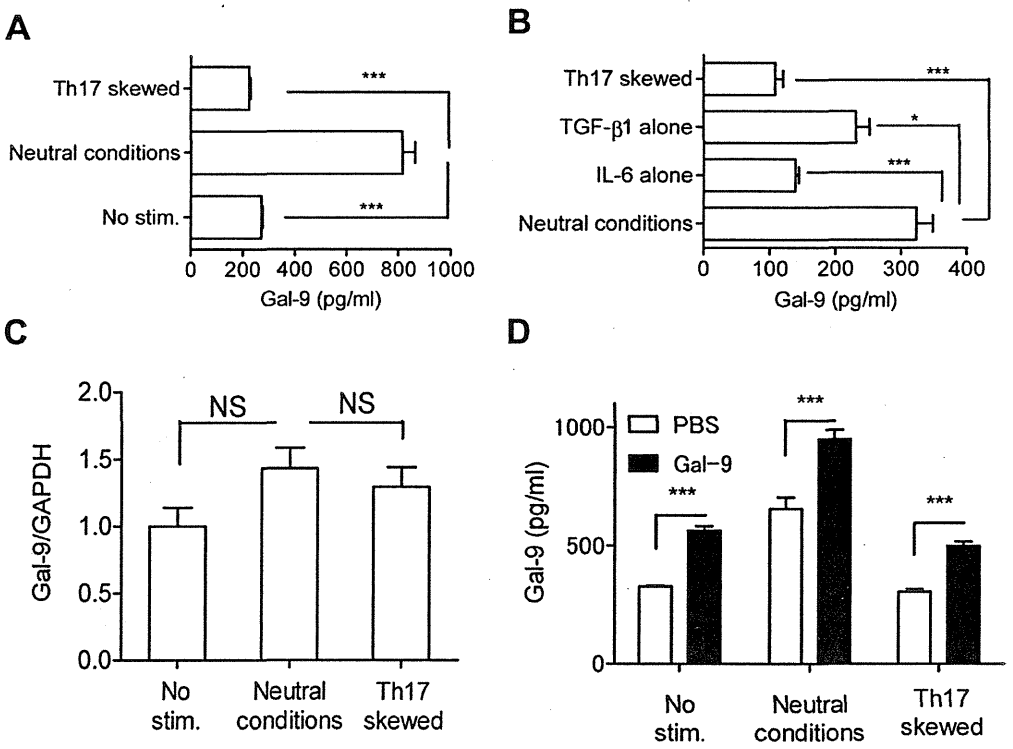


Figure 1. Gal-9 secretion in naïve CD4 T cell culture upon TCR stimulation. (**A**) Naïve CD4 T cells were cultured in Th17-skewing conditions, neutral conditions (TCR stimulation), or without stimulation for 96 h before released Gal-9 was measured using ELISA. (**B**) Same as (**A**), except that the cells were cultured under Th17-skewing conditions, neutral conditions, neutral conditions plus TGF-β1 (TGF-β1 alone), or neutral conditions plus IL-6 (IL-6 alone). (**C**) Gal-9 mRNA was quantified using real-time RT-PCR. (**D**) Gal-9 secretion in the presence of human stable Gal-9 (30 nM) was measured. The human protein was not detected by mouse Gal-9 ELISA (see text for details). All results are shown as the mean ± SEM of quadruplicates. $p<0.001$ (***), $p<0.05$ (*), not significant (NS). Representative data out of at least 2 experiments are shown.
doi:10.1371/journal.pone.0048574.g001

Expansion of Gal-9$^+$ Th cells by Exogenous Gal-9

As demonstrated in **Figure 1D**, the addition of recombinant Gal-9 to naïve CD4 T cell cultures augmented Gal-9 secretion. This observation indicates that exogenous Gal-9 either activates or increases Gal-9$^+$ Th cells (or both). To address the question, naïve CD4 T cells were cultured for 4 days with no stimulation, or under neutral- or Th17-skewing conditions in the presence or absence of recombinant human stable Gal-9, and the frequency of Gal-9$^+$ Th cells was monitored using flow cytometry. As shown in **Figure S1**, our anti-mouse Gal-9 antibody did not cross-react with the recombinant human protein and could detect Gal-9$^+$ Th cells. Exogenous Gal-9 increased the numbers of Gal-9$^+$ CD25$^-$ Th cells irrespective of TCR stimulation (**Figure 5A**). Upon TCR stimulation, exogenous Gal-9 increased the numbers of both Gal-9$^+$ CD25$^+$ and Gal-9$^-$ CD25$^+$ Th cells under neutral and Th17-skewing conditions (**Figure 5A**).

Two major cytokines expressed by Gal-9$^+$ Th cells, IL-10 and TGF-β, were tested to determine whether they affect the expansion of Gal-9$^+$ Th cells. Blocking antibodies specific against IL-10 and TGF-β added to naïve CD4 T cell cultures under neutral conditions for 4 days did not affect the number of Gal-9$^+$ Th cells (**Figure 5B**). IL-10 has been shown to expand type-1 regulatory T cells (Tr1), which are known to strongly express IL-10 and may therefore be related to Gal-9$^+$ Th cells [18–20]. However, recombinant IL-10 at 10 ng/mL did not increase Gal-9$^+$ Th cells in 4-day cultures, whereas exogenous Gal-9 increased the number of cells (**Figure 5C**).

Gal-9$^+$ Th cells in Humans

Recombinant Gal-9 functions in human peripheral blood CD4 T cells to augment Foxp3$^+$ Treg development while suppressing Th17 development (**Figure S4**). These observations demonstrate that the function of Gal-9 in terms of Th17/Treg development is equivalent between humans and mice, and implies the existence of Gal-9$^+$ Th cells in humans. We examined peripheral CD4 T cells from normal subjects to determine whether a surface Gal-9-expressing population could be observed. In accordance with the findings in mouse studies, flow cytometric analysis revealed the presence of Gal-9$^+$ Th cells in peripheral blood mononuclear cells (PBMC) and the expansion of the cells by TCR stimulation (**Figure 6A**). To examine the characteristics of human Gal-9$^+$ Th cells, peripheral CD4 T cells were cultured under neutral conditions for 4 days to allow the expansion of Gal-9$^+$ Th cells, were sorted into Gal-9$^+$ and Gal-9$^-$ Th cells according to surface Gal-9 expression, and were then cultured for another 4 days under neutral conditions before analysis. Human Gal-9$^+$ Th cells secreted higher amounts of Gal-9 and expressed higher levels of IL-10 and TGF-β mRNA compared to Gal-9$^-$ Th cells. Expression of IL-2 and IFN-γ did not differ between the 2 populations, whereas the levels of IL-4 and IL-17 were significantly lower in Gal-9$^+$ Th cells (**Figure 6B**). These characteristics of human Gal-9$^+$ Th cells are identical to those observed in mouse Gal-9$^+$ Th cells (**Figure 2**).

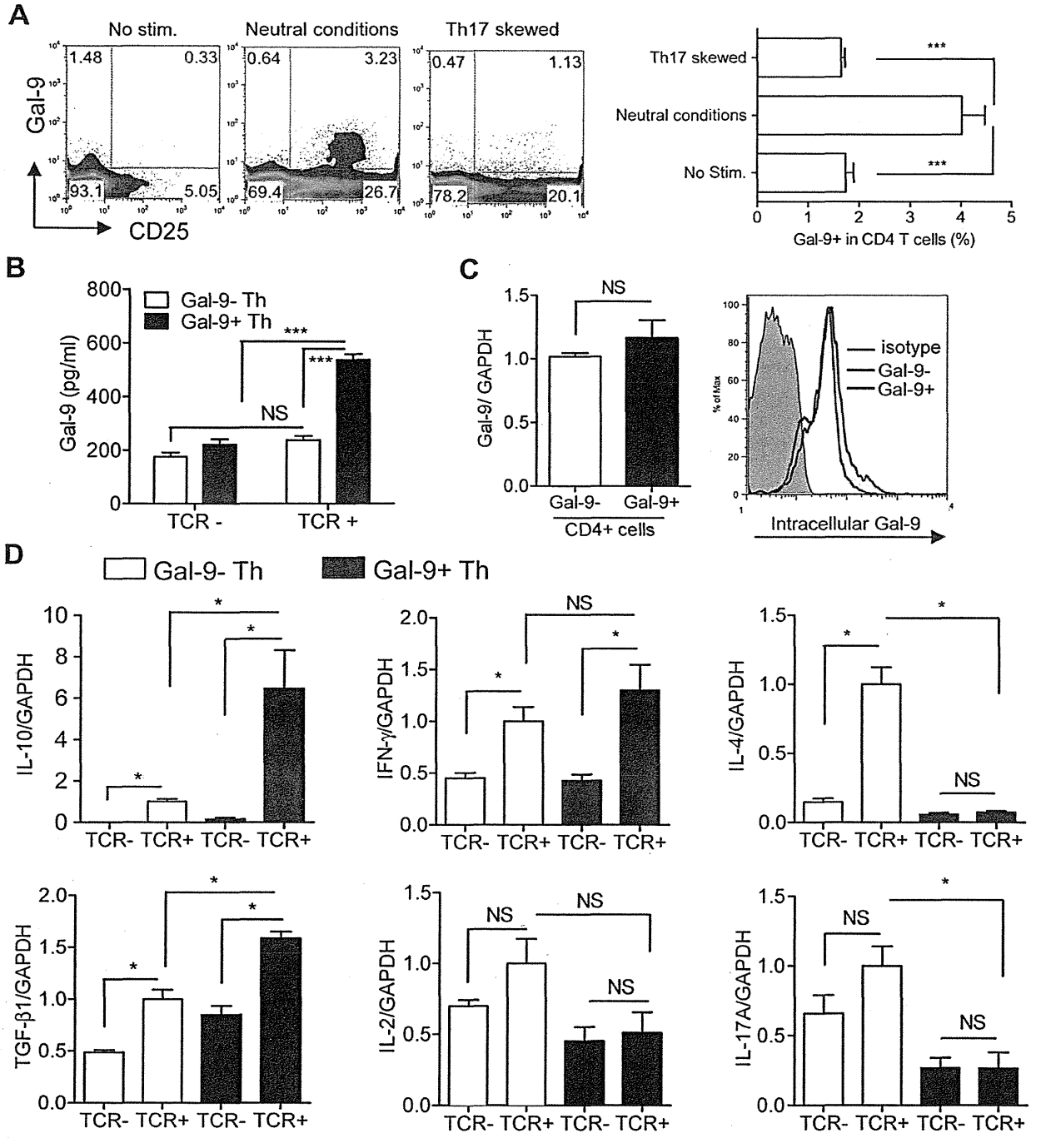


Figure 2. Identification of Gal-9$^+$ Th cells. (**A**) Naïve CD4 T cells were cultured as in **Figure 1A**, and cell-surface Gal-9 expression was monitored using flow cytometry. (**B**) Naïve CD4 T cells were sorted into Gal-9$^+$ Th and Gal-9$^-$ Th cells with a cell sorter and cultured under TCR stimulation or left unstimulated for 4 days before Gal-9 secretion into the culture media was measured. (**C**) Gal-9$^+$ Th and Gal-9$^-$ Th cells cultured under TCR stimulation for 4 days were examined for Gal-9 mRNA expression (left) and intracellular Gal-9 protein expression (right). (**D**) Cytokine mRNA expression in Gal-9$^+$ Th and Gal-9$^-$ Th cells cultured with or without TCR stimulation for 4 days. All the results are shown as the mean ± SEM of quadruplicates. $p<0.001$ (***), $p<0.05$ (*), not significant (NS). Representative data out of at least 2 experiments are shown.
doi:10.1371/journal.pone.0048574.g002

Table 1. Frequency of Gal-9$^+$ Th cells in various organs in mice.

Organs	Phenotype	% in Gal-9$^+$ CD25$^-$ cells
		Mean ± SD
Thymus	in CD4 SP T cells	24.6±0.4
LN	in CD4 T cells	3.6±0.4
Spleen	in CD4 T cells	6.5±0.8
Peyer's patches	in CD4 T cells	14.8±0.7
PBMC	in CD4 T cells	7.2±0.9

Lymphocytes from indicated organs (n = 3) were stained with anti-CD3, anti-CD4, anti-CD25, and anti-Gal-9 antibodies and analyzed using flow cytometry. SP: single positive, LN: lymph node.
doi:10.1371/journal.pone.0048574.t001

Discussion

Gal-9 has been demonstrated to suppress hyper immune reactions through several modes of action, including the induction of apoptosis in Tim-3$^+$ Th1 and Th17, and suppression of Th17 development with concomitant induction of Treg [3,10,11]. These regulatory functions of Gal-9 have been elucidated primarily by pharmacological studies *in vitro* and *in vivo* during which recombinant Gal-9 was administered. However, the cells responsible for Gal-9 secretion have not been resolved. Here, we identified Gal-9$^+$ Th cells that express Gal-9 on their surfaces secrete Gal-9 upon TCR stimulation, and regulate Th17/Treg development. Recently, Treg was reported to secrete Gal-9 and suppress Th1. We clarified that Gal-9$^+$ Th cells are devoid of Foxp3 expression and are the predominant CD4 T cells secreting Gal-9. The discovery of Gal-9$^+$ Th cells will improve the understanding of complex immunoregulation by Gal-9, provide a tool to study expression control and secretion mechanisms of Gal-9, and may provide clinical utilities for diagnosis or cell-based therapy in the future.

It is known that the administration of recombinant Gal-9 modulates immunity bidirectionally, not only suppressing excessive immunity and inflammation but also enhancing these functions in the context of compromised immunity [21,22]. It is not clear whether Gal-9$^+$ Th cells boost immunity or further suppresses it under hypo immune conditions; this can be examined using animal models such as tumor-bearing mice.

We showed that Gal-9 derived from Gal-9$^+$ Th cells play a vital role in regulating Th17/Treg development because the effect of Gal-9$^+$ Th cells was abrogated by a Gal-9 antagonist. However, the concentration of Gal-9 secreted into the culture media was 500–800 pg/mL, which was significantly lower than the effective concentration for recombinant Gal-9 to elicit the same effect (30 nM: approximately 1 μg/mL). We hypothesize that Gal-9$^+$ Th cells secrete Gal-9 in close proximity to target cells via a paracrine mechanism that may involve cell-cell contact to identify and confirm the target cells and achieve efficient and secure regulation by Gal-9. It cannot be ruled out that cell-surface Gal-9 may also be involved for the regulation by directly interacting with target molecules.

Contrary to expectation, both Gal-9$^+$ Th cells and other CD4 T cells express Gal-9 in the cytoplasm at comparable levels. Therefore, it is plausible that Gal-9$^+$ Th cells possess secretion machinery that is absent in Gal-9$^-$ Th cells. Gal-9, like other galectins, does not contain a signal sequence, and the secretion mechanism has remained obscure. In one study examining Gal-1, secretion was found to require a counter-receptor, and translocation through the plasma membrane was found to be energy-independent [23]. In this case, a membrane pore must be present to enable Gal-1 translocation. Conversely, Gal-9 is secreted as a component of the exosome in the case of Epstein-Barr virus–infected nasopharyngeal carcinoma [24]. Whether this mechanism is true for Gal-9 secretion from Gal-9$^+$ Th cells or whether there is a third mechanism remains to be determined.

Using human peripheral T cells, we demonstrated that Gal-9$^+$ Th cells are present in humans, and the immunoregulatory function of Gal-9 on Th17/Treg appears to be equivalent between humans and mice. These findings suggest clinical applications of recombinant Gal-9 and Gal-9$^+$ Th cells. We found that IL-6 abrogates the increase of Gal-9$^+$ Th cells *in vitro*. Thus, neutralization of IL-6 may be a strategy for increasing Gal-9$^+$ Th cells in order to ameliorate Th1/Th17-skewed immunity. An anti-IL-6 receptor-neutralizing antibody, tocilizumab, has been used to treat rheumatoid arthritis. Currently, the primary mechanism is explained by the suppression of Th17 cell development by IL-6 [25]; however, an additional cryptic mechanism of the antibody may involve efficient induction of Gal-9$^+$ Th cells. It would be interesting to measure Gal-9$^+$ Th cells in patients receiving IL-6 blockades, because this may demonstrate the utility of Gal-9$^+$ Th cells as surrogate markers to judge the effectiveness of the therapy.

Gal-9$^+$ Th cells must be further characterized to assess clinical utilities, including cell-based therapies as have been attempted for Tr1 and Treg, for the treatment of refractory autoimmune diseases. Gal-9$^+$ Th cells expand the population by exogenously applied recombinant Gal-9, and the cells can be purified using Gal-9 expressed on the cellular surface. These findings provide useful techniques for obtaining a large number of Gal-9$^+$ Th cells and will help facilitate the study of Gal-9$^+$ Th cells.

Materials and Methods

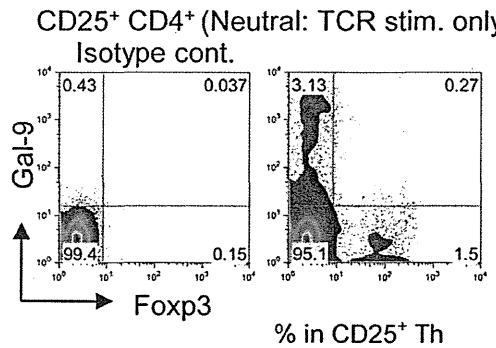
Ethics Statement

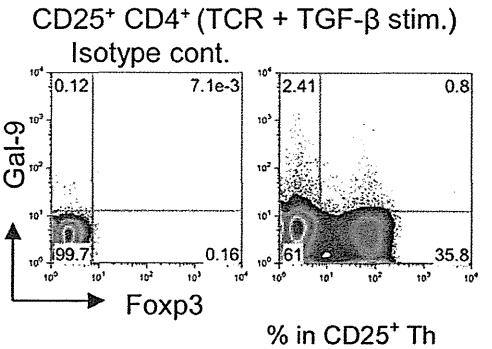
Human PBMCs were obtained from healthy adult volunteers with the approval of the ethical committee at Kagawa University, Faculty of Medicine. Written informed consent was obtained from all participants. Mice used in this research received humane care in accordance with international guidelines and national law. The study protocol was approved by the Animal Care and Use Committee of Kagawa University.

Isolation and Culture of Mouse CD4$^+$ CD62L$^+$ Naïve T cells

C57BL/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Gal-9 knockout (Gal-9 KO) mice were obtained from GalPharma (Takamatsu, Japan). All animals were maintained under standard conditions with a 12-h day/night rhythm and with *ad libitum* access to food and water. CD4$^+$ CD62L$^+$ naïve T cells were isolated from splenocytes of 8–10 week-old mice using a CD4$^+$CD62L$^+$ T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD4$^+$ CD62L$^+$ purity was >94%. Expression and purification of recombinant human stable Gal-9 has been previously described [26]. The recombinant protein was >95% pure on SDS-PAGE with an endotoxin level of <0.001 endotoxin units/μg. Isolated naïve T cells were cultured in 96-well plates at 2 × 10^5 cells/well in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, penicillin G (10 IU/mL, Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (100 μg/mL, Sigma-Aldrich) for 96 h. For stimulation under neutral conditions, cells were cultured in anti-CD3-coated plates (BD Biosciences, Franklin

A

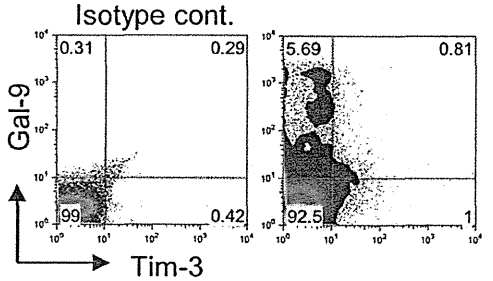




		% in CD25+ Th Mean ± SEM
Gal-9+	Foxp3+	0.28 ± 0.01
Gal-9+	Foxp3-	3.59 ± 0.27
Gal-9-	Foxp3+	1.34 ± 0.07
Gal-9-	Foxp3-	94.78 ± 0.25

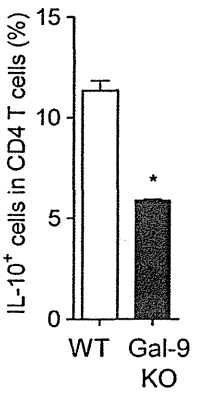
		% in CD25+ Th Mean ± SEM
Gal-9+	Foxp3+	0.86 ± 0.03
Gal-9+	Foxp3-	2.76 ± 0.11
Gal-9-	Foxp3+	34.25 ± 0.54
Gal-9-	Foxp3-	62.15 ± 0.40

B



		% in CD25+ Th Mean ± SEM
Gal-9+	Tim-3+	0.78 ± 0.02
Gal-9+	Tim-3-	6.08 ± 0.28
Gal-9-	Tim-3+	0.90 ± 0.04
Gal-9-	Tim-3-	92.23 ± 0.24

C



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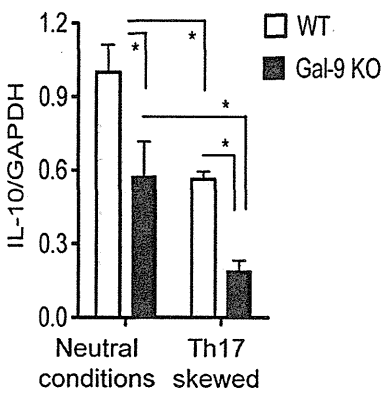

Figure 3. Phenotype of Gal-9+ Th cells. To clearly demonstrate the co-expression of cell-surface Gal-9 with either Foxp3 or Tim-3, highly sensitive Gal-9 staining with biotinylated-anti-Gal-9 antibody plus streptavidin-APC was employed. (**A**) Naïve CD4 T cells cultured under neutral conditions (left) or Treg-skewing conditions (right) for 4 days were examined for Foxp3 (Treg marker) and cell-surface Gal-9 expressions using flow cytometry. (**B**) Co-expression analysis of cell-surface Gal-9 and Tim-3 in naïve CD4 T cells cultured under neutral conditions for 4 days. (**C**) Intracellular IL-10 in naïve CD4 T cells purified from wild-type (WT) or Gal-9 knockout (Gal-9 KO) mice. (**D**) Naïve CD4 T cells from WT and Gal-9 KO mice were cultured under neutral or Th17-skewing conditions, and IL-10 mRNA expression was measured. Results are shown as the mean ± SEM of triplicate or quadruplicate. Symbol (*) represents significant ($p<0.05$) differences from the indicated counterparts. Representative data out of at least 2 experiments are shown.
doi:10.1371/journal.pone.0048574.g003

Lakes, NJ, USA) in the presence of anti-CD28 (2 μg/mL, BD Biosciences) and mouse IL-2 (5 ng/mL, R&D Systems, Minneapolis, MN, USA). For Th17 skewing, cells were cultured in human TGF-β1 (3 ng/mL, R&D Systems) and mouse IL-6 (20 ng/mL, R&D Systems) under neutral conditions. Treg skewing was conducted under the same culture conditions for Th17-skewing but IL-6 was omitted. For some experiments, human stable Gal-9 (30 nM), lactose (30 or 100 mM), sucrose (30 mM), anti-mouse IL-10 blocking antibody (10 μg/mL, BioLegend, San Diego, CA, USA), anti-mouse IL-10 receptor blocking antibody (10 μg/mL, BioLegend), anti-mouse TGF-β blocking antibody (10 μg/mL, Abcam, Cambridge, MA, USA), or mouse IL-10 (1, 3 or 10 ng/mL, R&D Systems) was included in the culture.

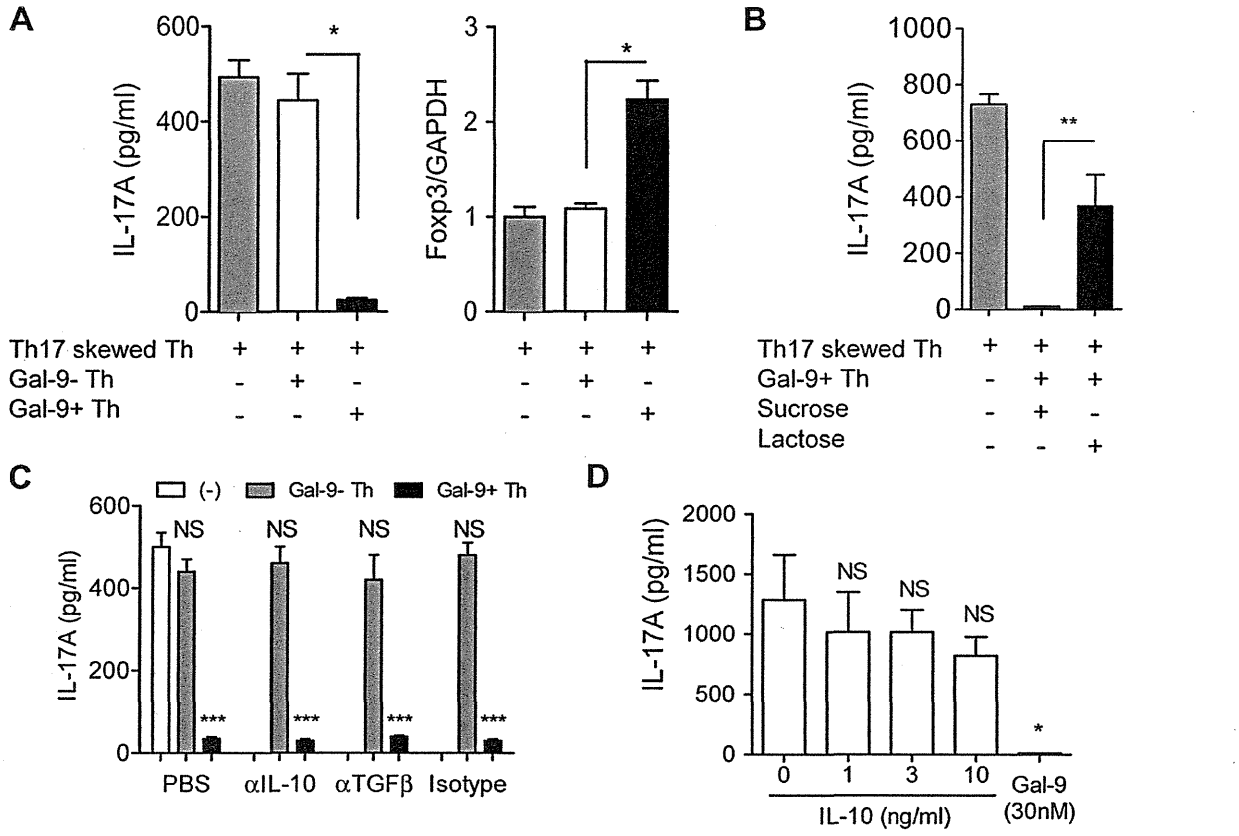

Figure 4. Regulatory function of Gal-9$^+$ Th cells on Th17/Treg development. (**A–C**) Gal-9$^+$ Th and Gal-9$^-$ Th cells were obtained using a cell sorter from naïve CD4 T cells cultured under neutral conditions for 4 days. These cells were co-cultured with Th17-skewed cells at 1:1 ratio and cultured for 90 h before analysis. (**A**) IL-17A and Foxp3 expression measured using ELISA and real-time RT-PCR, respectively. (**B**) IL-17A production in the Gal-9$^+$ Th cell co-culture was examined in the presence of Gal-9 antagonist lactose or irrelevant sugar sucrose. (**C**) IL-17A production was examined in the presence of anti-IL-10 or anti-TGF-β blocking antibodies. (**D**) Naïve CD4 T cells were cultured under Th17-skewing conditions for 4 days in the presence of indicated concentration of IL-10 or human stable Gal-9 before IL-17A was measured. Results are shown as the mean ± SEM of quadruplicate experiments. $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*), not significant (NS). Representative data out of at least 2 experiments are shown.
doi:10.1371/journal.pone.0048574.g004

Isolation of Gal-9$^+$ Th cells and Co-culture with Th17-skewed T cells

Naïve CD4$^+$ T cells were isolated from splenocytes as described above. Gal-9$^+$ and Gal-9$^-$ Th cells were sorted by positive or negative surface Gal-9 expression, respectively, using an anti-mouse Gal-9 antibody (clone 108A2, BioLegend) and a FACSAria cell sorter (BD Biosciences). Cell purity was >97%. For co-culture experiments, naïve T cells (5×10^4) were cultured under Th17-skewing conditions for 6 h, and then co-cultured with Gal-9$^+$ or Gal-9$^-$ Th cells (5×10^4) for an additional 90 h.

ELISA

Quantification of mouse Gal-9 was carried out using ELISA as described previously with minor modifications [27]. Briefly, 96-well plates were coated with an anti-mouse Gal-9 antibody (Clone 108A2), blocked with 3% fetal bovine serum in phosphate-buffered saline, and then incubated with culture supernatant. Gal-9 was detected using polyclonal anti-mouse Gal-9 antibody conjugated with biotin (GalPharma) and streptavidin-conjugated horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA). After color development with tetramethyl benzidine (KPL, Gaithersburg, MD, USA), Gal-9 was quantified using a standard curve constructed with a recombinant mouse Gal-9. Mouse Gal-9 ELISA cannot be used to detect human stable Gal-9. Human Gal-9 ELISA was reported previously [27]. IL-17 was measured using a specific ELISA kit from R&D Systems according to the manufacturer's instructions.

Flow Cytometric Analysis

CD4 T cells were evaluated by flow cytometry using the following antibodies: anti-mouse CD3-PerCP (BD Biosciences), anti-mouse CD4-FITC (BD Biosciences or eBioscience, San Diego, CA, USA), anti-mouse Tim-3-PE (eBioscience), anti-mouse Gal-9-PE (clone 108A2, BioLegend) or biotinylated anti-mouse Gal-9 (clone 108A2, GalPharma), anti-mouse CD25-APC (BioLegend), anti-mouse IL-10-PE (BioLegend), anti-human Gal-9-Alexa488 (clone 9M1-3, GalPharma), anti-human CD3-PerCP (BD Biosciences), anti-human CD4-FITC (BioLegend), anti-human CD4-PE (BioLegend), anti-human CD25-APC (BioLegend), and anti-human/mouse Foxp3-PE (BioLegend). All data were acquired using a FACSCalibur cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

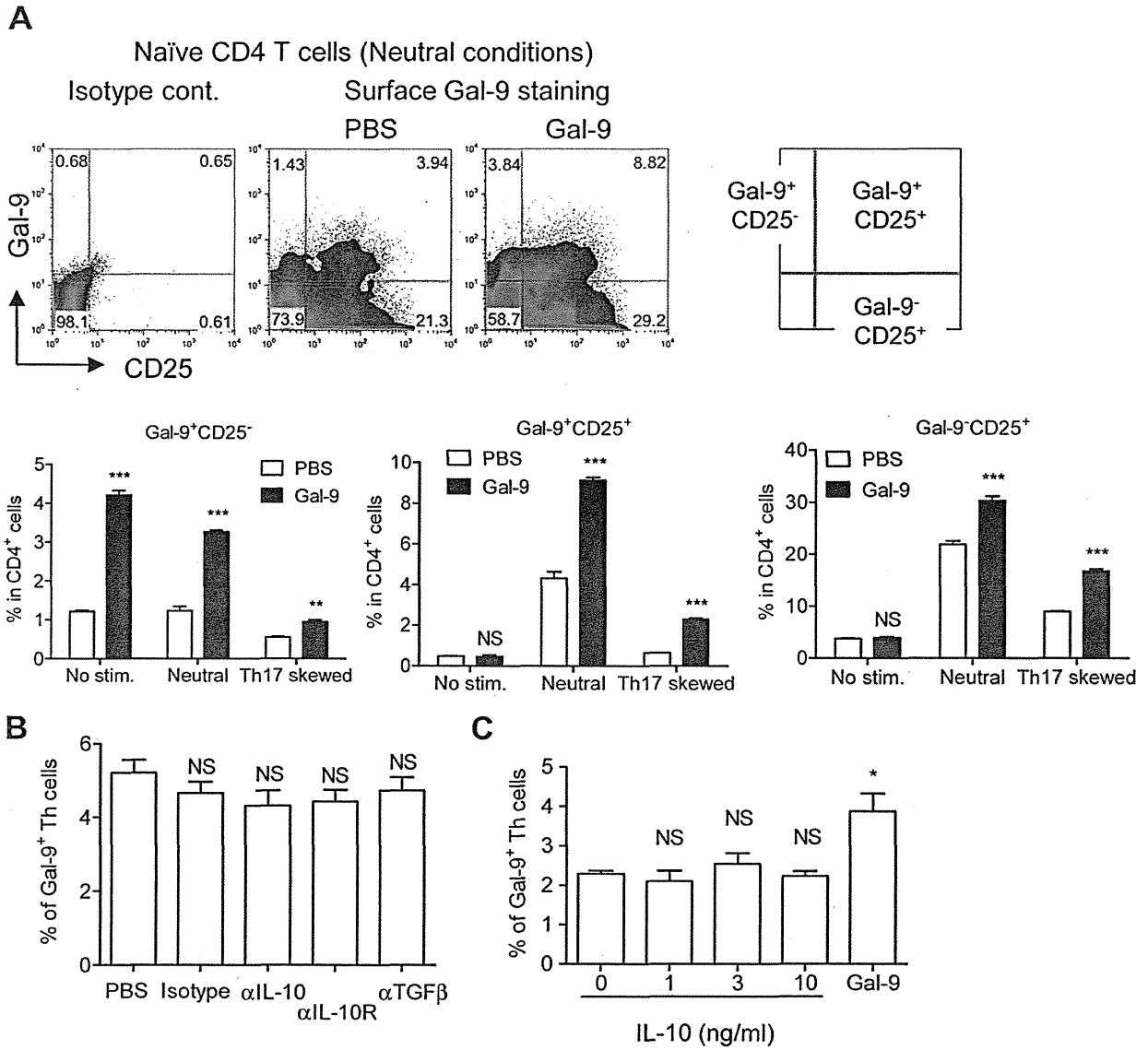

Figure 5. Expansion of Gal-9$^+$ Th cells by exogenous Gal-9. (**A**) Naïve CD4 T cells were cultured under unstimulated, neutral, or Th17-skewing conditions for 4 days in the presence or absence of 30 nM human stable Gal-9 before surface Gal-9 expression was monitored by flow cytometry using an anti-mouse Gal-9 antibody. The antibody does not cross-react with the added human Gal-9. Dot plots are representative results obtained from neutral conditions in the presence or absence of exogenous Gal-9. (**B and C**) Flow cytometric analysis of Gal-9$^+$ Th cell frequency after 4-day culture of naïve CD4 T cells under neutral conditions in the presence of blocking anti-IL-10 or anti-TGF-β antibody (**B**) or in the presence of the indicated concentration of IL-10 or 30 nM human stable Gal-9 (**C**). Results are means ± SEMs of quadruplicates. $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*), not significant (NS). Representative data out of 2 experiments are shown.
doi:10.1371/journal.pone.0048574.g005

Isolation and Culture of Human Peripheral Blood CD4 T cells

PBMCs from healthy donors were prepared using a Lymphocyte Separation Kit (Nakalai, Kyoto, Japan). CD4 T cells were isolated using a CD4 T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The CD4 T cells (2×10^5 cells/well) were cultured under TCR stimulation (anti-CD28 (2 μg/mL, BD Biosciences) and IL-2 (5 ng/mL, R&D Systems) in anti-CD3-coated plates) or left unstimulated for 96 h, and cell-surface Gal-9 expression or released Gal-9 in the culture media was measured. To determine cytokine mRNA expression, CD4 T cells cultured under TCR stimulation for 4 days were sorted into Gal-9$^+$ CD25$^+$ cells and Gal-9$^-$ CD25$^+$ cells using a FACSAria cell sorter at a purity of >97%. Sorted cells were further cultured for 4 days under TCR stimulation. Human Th17 cell development was performed as reported previously [28]. Briefly, CD4 T cells were cultured under TCR stimulation as described above in the presence or absence of IL-1β (50 ng/mL, R&D Systems), IL-6 (20 ng/mL, R&D Systems) and IL-23 (50 ng/mL, R&D Systems) for 9 days.

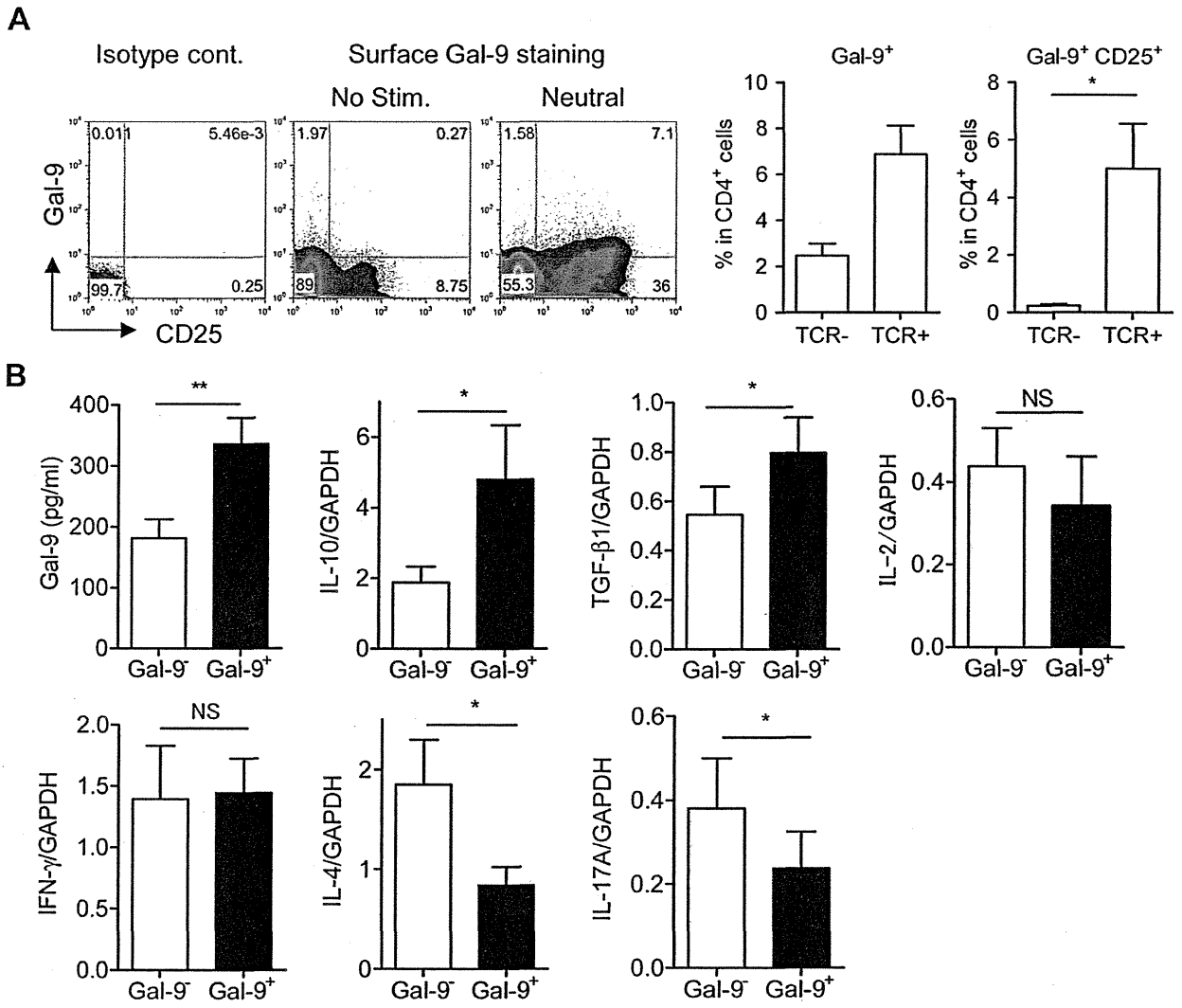

Figure 6. Gal-9+ Th cells in humans. (**A**) CD4 T cells from human peripheral blood were cultured with or without TCR stimulation for 4 days. Surface Gal-9 and CD25 expression was analyzed using flow cytometry. Results are shown as the mean ± SEM from 4 healthy donors. (**B**) CD4 T cells were cultured under neutral conditions for 4 days and then sorted into Gal-9+ CD25+ Th cells and Gal-9− CD25+ Th cells. Sorted cells were cultured under neutral conditions for 4 days before the measurement of secreted Gal-9 by ELISA or cytokine mRNA using real-time RT-PCR. Results are shown as the mean ± SEM from 8 healthy donors. * $p<0.05$. Data from 2 representative experiments are shown.
doi:10.1371/journal.pone.0048574.g006

Real-time RT-PCR

mRNA levels were evaluated using the SYBR Green I-based real-time RT-PCR with an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA, USA) as previously described [3]. All gene primers were obtained from Takara Bio (Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal standard for calibration.

Statistical Analysis

For statistical comparisons, non-parametric two-tailed Mann-Whitney U-tests and one- or two-way analysis of variance were used. All statistical analyses were performed with Prism 5 software (Graphpad Software). A p-value of <0.05 was considered significant.

Supporting Information

Figure S1 Specificity of anti-Gal-9 antibody. (**A**) Schematic drawing of wild-type Gal-9 and stable Gal-9. Gal-9 consists of 2 carbohydrate-recognition domains (CRD) at the N- and C-termini, tethered by a linker peptide. Stable Gal-9 is a gene-engineered linker-less Gal-9, which retains biological activity of wild-type Gal-9. (**B**) Anti-mouse Gal-9 antibody 108A2 recognizes linker peptide of mouse Gal-9 and does not cross-react with stable Gal-9. The indicated proteins were coated in ELISA plates and detected using the 108A2 antibody. Mean ± SD (n = 3). (**C**) Mouse Gal-9 ELISA is constructed by 108A2 antibody as the coating antibody and polyclonal anti-mouse Gal-9 antibody as the detection antibody. The ELISA is highly specific to mouse Gal-9 and does not cross-react to human stable Gal-9 at 0.37 μg/mL.

When mouse Gal-9 is quantified in the presence of human stable Gal-9, the samples were diluted accordingly. Mean values (n = 2).
(TIF)

Figure S2 Elimination of exogenously added Gal-9 bound on the cell surface by 30 mM lactose. (**A**) Naïve CD4 T cells were incubated with biotinylated human stable Gal-9 (30 nM) for 30 min on ice followed by incubation with lactose or sucrose (30 mM) for 30 min on ice. Human stable Gal-9 bound on the cells was stained with streptavidin- APC and analyzed using flow cytometry. (**B**) Naïve CD4 T cells were cultured under neutral conditions for 4 days to allow expansion of Gal-9$^+$ CD25$^+$ Th cells. The cells were incubated in the presence or absence of 100 mM lactose for 30 min on ice before staining of surface Gal-9 and analysis by flow cytometry.
(TIF)

Figure S3 Sorting of Gal-9$^+$ and Gal-9$^-$ Th cells. Naïve CD4 T cells were sorted into Gal-9$^+$ and Gal-9$^-$ Th cells using a FACSAria. The purities of Gal-9$^+$ and Gal-9$^-$ Th cells were more than 97%.
(TIF)

Figure S4 Regulation of human Th17/Treg development by Gal-9. (**A and B**) CD4 T cells were isolated from human peripheral blood (4 healthy donors) by magnetic sorting and were cultured with or without 4 days of TCR stimulation in the presence or absence of 30 nM human stable Gal-9. CD25$^+$ CD4 T cells (**A**) or CD25$^+$ Foxp3$^+$ CD4 T cells (**B**) were determined using flow cytometry. (**C**) Human CD4 T cells from 4 healthy donors were cultured under TCR stimulation in the presence of indicated cytokines and in the presence or absence of 30 nM human stable Gal-9 for 9 days before IL-17 secretion was measured by ELISA. Results are shown as the mean ± SEM of quadruplicate experiments. ***, $p<0.001$. Data representative of 2 experiments are shown.
(TIF)

Figure S5 Gal-9$^+$ Th cells in various organs in mice. The representative dot plots showing the existence of Gal-9$^+$ Th cells in the indicated organs of Table 1 are shown. Events in the gate are cell-surface Gal-9-positive populations.
(TIF)

Acknowledgments

We thank Drs. Yokota and Iwata (Faculty of Pharmaceutical Sciences, Kagawa Campus, Tokushima Bunri University, Japan) for technical assistance with cell sorting.

Author Contributions

Conceived and designed the experiments: SO TA TN TH TM MH. Performed the experiments: SO TA TN TK MU NN. Analyzed the data: TK MU AY MH. Contributed reagents/materials/analysis tools: TN NN AY MH. Wrote the paper: TN TH TM MH.

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Tohoku J. Exp. Med., 2013, **229**, 137-142

Acyclovir Reduces the Duration of Fever in Patients with Infectious Mononucleosis-like Illness

Osamu Usami,[1] Hiroki Saitoh,[1] Yugo Ashino,[2] and Toshio Hattori[3]

[1]Department of Comprehensive Infection, Tohoku University Hospital, Sendai, Miyagi, Japan
[2]Department of Emerging Infectious Diseases, Tohoku University School of Medicine, Sendai, Miyagi, Japan
[3]Laboratory of Disaster-related Infectious Disease, International Research Institute of Disaster Science (IRIDeS), Tohoku University, Sendai, Miyagi, Japan

Acyclovir is known for its antiviral activity against some pathogenic viruses such as the Epstein-Barr virus (EBV) that causes infectious mononucleosis (IM) and IM-like illness. Therefore, we empirically administered acyclovir to patients with suspected EBV-IM and IM like-illness, upon their admission to our hospital. We admitted 25 patients, who were hospitalized for fever and lymphadenopathy, to the Tohoku University Hospital Infectious Disease Ward. As part of treatment, 8 of these patients were given acyclovir (750 mg/day) with their consent and were assigned to the acyclovir group; the remaining 17 patients were assigned to the control group. The mean age of acyclovir patients (all men) was 42 ± 5.2 years, and that of control patients (13 men and 4 women) was 31 ± 3.0 years. The cause of illness was confirmed as EBV-IM in 6 patients (1, acyclovir; 5, control), and remained unknown for the other 19 IM-like illness patients (7, acyclovir; 12, control). A shorter duration of hospitalization and fever was observed in the acyclovir compared to that in the control patients (hospitalization duration: 16 ± 3.7 vs. 27 ± 7.7 days, $P = 0.36$; fever duration: 4.5 ± 1.8 vs. 18 ± 6.5 days, $P = 0.04$). Additionally, serum amyloid A (SAA) levels were lower in acyclovir than that in control patients (98 ± 37 vs. 505 ± 204 μg/mL, $P = 0.02$). Therefore, we propose that acyclovir is a potential therapeutic agent for both EBV-IM and IM like-illnesses. Future studies should further examine its mechanism of action.

Keywords: acyclovir; Epstein-Barr virus; fever; infectious mononucleosis like illness; lymphadenopathy
Tohoku J. Exp. Med., 2013 Feb, **229** (2), 137-142.

Infectious mononucleosis (IM) is specifically caused by the Epstein-Barr virus (EBV), and is characterized by lymphadenopathy, prolonged fever, cervical lymph node swelling, and increased inflammatory reaction (Luzuriaga and Sullivan 2010). IM-like illness, which also has non-EBV etiologies, exhibits similar characteristic symptoms as IM. In the US, adult IM and IM-like illness are caused by EBV in 50-90% of the cases, human herpes virus type 6 (HHV6) in 9%, cytomegalovirus (CMV/HHV5) in 5-7%, herpes simplex virus (HSV)-1 in 6%, human immunodeficiency virus (HIV) in < 2%, and adenovirus in < 1% of the cases (Hurt and Tammaro 2007). However, IM-like illness due to CMV infection appears to be more common in Japan than in the US, and has been reportedly associated with 27.5% of IM-like illness cases in Japan (Naito et al. 2006). An evaluation and treatment algorithm involving patient history, physical examination, symptoms, and size of neck mass has been proposed (Schwetschenau and Kelley 2002).

The outcome of antiviral therapy largely depends on the pathogen. However, it would be impractical to test for all possible viruses due to time and cost constraints. Consequently, EBV-IM cases have been the ones most extensively studied with the aim of improving diagnosis and management, which resulted in the identification of RT-PCR as a useful tool for early diagnosis (Vouloumanou et al. 2012). Unfortunately, no anti-viral drug has been approved yet for the treatment of EBV-IM, and clinical trials involving anti-viral drugs have also yielded controversial results. On the other hand, administration of acyclovir to EBV patients reportedly gave beneficial results in immunocompetent patients (Torre and Tambini 1999; Rafailidis et al. 2010). One study showed that acyclovir pharmacokinetic parameters did not correlate with the virologic or clinical response in young adults with EBV-IM (Vezina et al. 2010). However, another study reported that valacyclovir therapy led to reduced EBV excretion and was clinically beneficial (Balfour et al. 2007). Other studies also showed that high-dose acyclovir and valacyclovir prophylaxis reduced the risk of CMV infection and disease following bone marrow transplantation, although it did not improve overall survival (Prentice et al. 1994); a more recent report

Received November 7, 2012; accepted January 4, 2013. Published online January 19, 2013; doi: 10.1620/tjem.229.137.
Correspondence: Yugo Ashino, M.D., Ph.D., Department of Emerging Infectious Diseases, Tohoku University School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan.
e-mail: ya82@yahoo.co.jp