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Increased Levels of Plasma Galectin-9 in Patients with Influenza Virus Infection

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Galectin-9 (Gal-9) is a β-galactoside-binding protein involved in various biologic processes, including cell aggregation, adhesion, chemoattraction, and apoptosis. Little is known, however, about the regulation mechanisms of Gal-9 production. Recent studies reported high plasma Gal-9 levels in humans infected with human immunodeficiency virus-1 and dengue virus. Viral respiratory infections such as influenza are common human illnesses. A synthetic double-stranded RNA, polyinosinic-polycytidylic acid (PolyIC), mimics the effects of viruses in various cell types and induces the expression of Gal-9 in endothelial cells. To examine the potential link between viral infection and Gal-9 expression, we measured plasma Gal-9 concentrations in patients with influenza. Subjects were 43 patients with influenza virus infection, 20 with pneumococcal pneumonia, and 20 healthy adults. Gal-9 concentrations in the plasma and in culture supernatants of human airway epithelial cells were measured using an enzyme-linked immunosorbent assay. Plasma Gal-9 concentrations were higher in patients with influenza infection than in patients with pneumococcal pneumonia and healthy subjects ($p < 0.05$). Patients with influenza were effectively differentiated from those with pneumococcal pneumonia or healthy subjects, based on the plasma levels of Gal-9 ($p < 0.0001$). Furthermore, using a human airway epithelial cell line, we showed that the presence of PolyIC but not lipopolysaccharides increased the Gal-9 concentration in the culture medium ($p < 0.05$), suggesting that PolyIC enhanced Gal-9 production. These findings support our proposal that Gal-9 production is induced by influenza virus infection in humans. In conclusion, plasma Gal-9 could be a new biomarker for patients with influenza infection.

Keywords: airway epithelial cells; double-stranded ribonucleic acid; galectin-9; human; influenza
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Introduction

Galectin-9 (Gal-9) is a β-galactoside binding protein involved in various biologic processes, including cell aggregation, adhesion, chemoattraction, and apoptosis (Hirashima et al. 2004). Recently, Gal-9 was characterized as an immunomodulator involved in excessive immunity by its effect to reduce pro-inflammatory helper T type 1 (Th1) and Th17 cells in a Tim-3 dependent manner, and expand regulatory T cells and immunosuppressive macrophages (Zhu et al. 2005; Seki et al. 2008; Arikawa et al. 2010). Little is known, however, about the regulation mechanisms of Gal-9 production in vivo. A synthetic double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (PolyIC), induces the expression of Gal-9 in endothelial cells (Ishikawa et al. 2004). Recent studies revealed that humans infected with acute human immunodeficiency virus (HIV)-1 have extremely high plasma Gal-9 levels that rapidly decrease after treatment (Chagan-Yasutan et al. 2009; Saitoh et al. 2012). Furthermore, plasma Gal-9 levels were markedly elevated during the critical phase of acute dengue virus infection (Chagan-Yasutan et al. 2013).

Viral respiratory infections such as influenza are common illnesses in humans. Replication of most respiratory viruses requires the generation of dsRNA that can be recognized by Toll-like receptor 3, resulting in the activation of host immune responses (Guillot et al. 2005). In a recent study, we found elevated Gal-9 levels in the bronchoalveolar lavage fluid of an acute exacerbation model of murine chronic asthma using the *Dermatophagoides farinae*-induced murine model of chronic asthma and dsRNA (PolyIC) (Katoh et al. 2013). Based on previous studies,

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including ours, viral infection may induce Gal-9 production in vivo (Saitoh et al. 2012; Katoh et al. 2013; Chagan-Yasutan et al. 2013). In the present study, to examine the effect of influenza virus infection on Gal-9 production in humans, we estimated plasma Gal-9 concentrations using enzyme-linked immunosorbent assay (ELISA) in patients with influenza infection compared with patients with pneumococcal pneumonia and healthy volunteers. Further, we examined the effect of PolyIC on Gal-9 production in human airway epithelial cells.

Methods

Characteristics of the study patients

Included in this study were 43 patients with influenza virus infection (21 women and 22 men; age, 49.1 ± 3.3 years), 20 with pneumococcal pneumonia (13 women and 7 men; age, 66.6 ± 4.5 years), and 20 healthy adults (10 women and 10 men; age, 61.5 ± 3.8 years). The diagnosis of influenza was made using a rapid flu kit screening test from nasopharyngeal swabs. None of the patients with influenza included in the present study showed pulmonary infiltration on the x-ray. The diagnosis of pneumococcal pneumonia was made based on a urinary antigen test to *Streptococcus pneumoniae* and/or respiratory cultures with pulmonary infiltrates on the x-ray. Patients with both influenza and pneumococcal pneumonia were excluded. Baseline characteristics of the patients, including blood examination data, are shown in Table 1. White blood cells (WBC), percent of neutrophils, and C-reactive protein (CRP) were significantly higher in patients with pneumococcal pneumonia than in patients with influenza. The study was approved by the human ethics committee of Kawasaki Medical School and Japanese Red Cross Nagasaki Genbaku Isahaya Hospital, and written informed consent was obtained from all study participants.

Galectin-9 measurements

Blood samples of patients with influenza and pneumococcal pneumonia were collected for a few days after the onset, and samples of influenza patients were also collected at the improved phase after treatment (on days 10.8 ± 2.6). Gal-9 concentrations in the plasma and culture supernatants were measured using ELISA, as previously described with slight modification (Seki et al. 2007; Katoh et al. 2010). Briefly, 96-well plates (Nunc, Rockild, Denmark) were coated with an anti-human Gal-9 monoclonal antibody (clone 9S2-3; GalPharma, Takamatsu, Japan), and then samples were incubated for 1 hour at 37°C after blocking. Gal-9 remaining in the wells was recognized by biotin conjugated polyclonal anti-human Gal-9 antibody (GalPharma). Quantification was performed using streptavidin-conjugated to a polymer of horseradish peroxidase (Pierce, Rockford, IL) and the colorimetric substrate tetramethylbenzidine (KPL, Gaithersburg, MD). Gal-9 concentrations were quantified using recombinant human stable Gal-9 (G9NC null; GalPharma) as a standard. The detection limit was 15.6 pg/ml. Concentrations below the detection limits were assumed to be zero for statistical analysis.

Culture of human airway epithelial cells

BEAS-2B human airway epithelial cells (Sigma-Aldrich, St. Louis, MO; 1×10^5/well) were cultured with medium alone (RPMI containing 5% heat-inactivated fetal bovine serum), synthetic dsRNA, polyinosinic-polycytidylic acid:PolyIC (InvivoGen, San Diego, CA) (1 μg/ml); PolyIC (3 μg/ml); lipopolysaccharide (LPS) from *Klebsiella pneumoniae* (Sigma-Aldrich; 1 μg/ml); or LPS (3 μg/ml) for 18 h at 37°C. The supernatant was stored at −80°C until analysis.

Statistical analysis

All data are expressed as mean ± standard error (SEM). For statistical comparisons, non-parametric two-tailed Mann-Whitney U test, Wilcoxon signed ranks test, and one-way analysis of variance were used. To investigate the optimum threshold, receiver-operating curves (ROC) were prepared. All statistical analyses were performed with Graphpad Prism 6 software. Differences with probability values of less than 0.05 were considered significant.

Results

Plasma Gal-9 levels increase in influenza infection

To examine the contribution of Gal-9 in influenza virus infection, plasma Gal-9 concentrations were estimated using ELISA. Gal-9 levels were significantly higher in patients with influenza (183.7 ± 26.6 pg/ml) than in patients with pneumococcal pneumonia (45.7 ± 13.5 pg/ml) and healthy volunteers (14.0 ± 3.6 pg/ml) ($p < 0.05$, Fig. 1A). Plasma Gal-9 levels in patients with pneumococcal pneumonia did not differ significantly from those in healthy volunteers ($p = 0.92$, Fig. 1A). Further, Gal-9 levels in patients with influenza were significantly decreased after treatment with anti-influenza drugs ($p < 0.0001$, Fig. 1B). Patients with influenza were younger than patients with pneumococcal pneumonia and healthy subjects ($p < 0.05$). There was no correlation between plasma Gal-9 levels and age in patients with influenza ($r = -0.026$, $p = 0.867$), pneumococcal pneumonia ($r = 0.003$, $p = 0.989$), or healthy sub-

Table 1. Characteristics of the study patients.

	Influenza ($n = 43$)	Pneumococcal pneumonia ($n = 20$)
Age (years)	49.1 ± 3.3	66.6 ± 4.5*
Sex (male, %)	22 (51.2)	7 (35.0)
WBC (/μL)	5,750 ± 261.7	12,469 ± 948.3*
Neutrophil (/μL)	4,206 ± 267.4	10,901 ± 887.3*
CRP (mg/mL)	2.84 ± 0.40	13.08 ± 2.53*

*Represents significant difference between patients with influenza and Pneumococcal pneumonia ($p < 0.05$). Significance was evaluated by the Mann-Whitney *U* test.

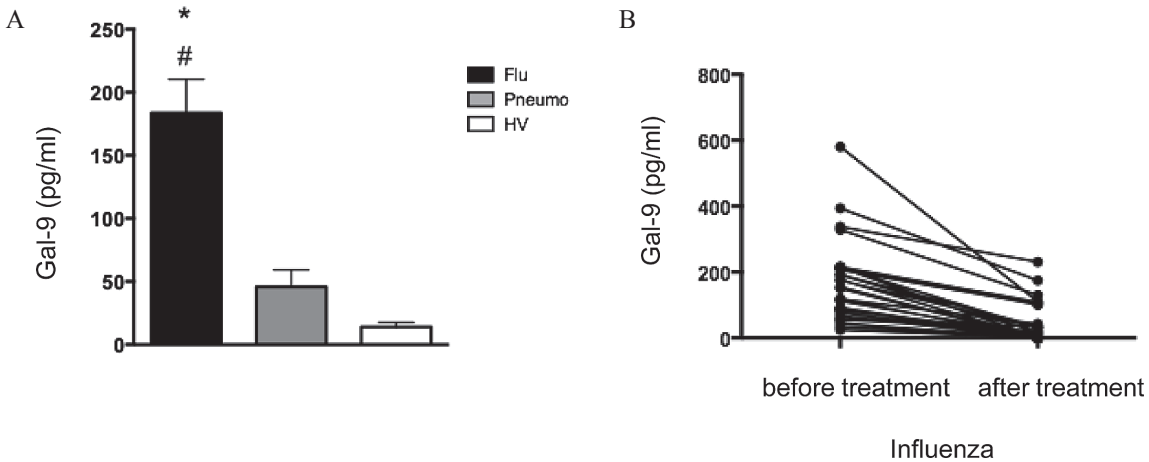

Fig. 1. Plasma levels of galectin-9. (A) Significantly different plasma galectin-9 levels in patients with influenza (Flu), pneumococcal pneumonia (Pneumo), and healthy volunteers (HV). Data represent means ± SEM. *$p < 0.05$ compared with healthy volunteers; #$p < 0.05$ compared with pneumococcal pneumonia (one-way analysis of variance). (B) Comparison of the plasma galectin-9 levels between before and after treatment with anti-influenza drugs (Wilcoxon signed ranks test, $p < 0.0001$).

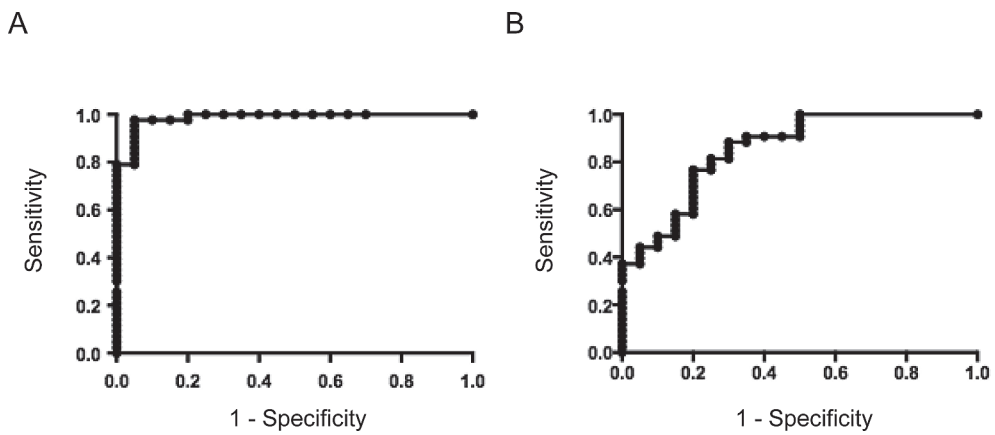

Fig. 2. Sensitivity of galectin-9 plasma levels for influenza infection. Receiver-operating characteristic curve analysis of the sensitivity and specificity of plasma galectin-9 levels for composite influenza infection. Confidence Interval: 95%. AUC, Area under the curve. (A) Control values: Healthy volunteer. (B) Control values: Pnumococcal pneumonia.

jects ($r = 0.317$, $p = 0.173$). Next, an ROC curve was prepared to calculate the optimum plasma Gal-9 threshold to identify influenza patients using healthy subject as a negative control (Fig. 2A), or pneumococcal pneumonia patients as a negative control (Fig. 2B). When healthy volunteers were used as a negative control, with 32.5 pg/ml plasma Gal-9 as the arbitrary cutoff, the sensitivity and specificity were 97.7% and 95.0%, respectively (AUC: 0.986, $p < 0.0001$). When pneumococcal pneumonia patients were used as a negative control, plasma Gal-9 levels > 64.5 pg/ml had 81.4% sensitivity and 75.0% specificity (AUC: 0.854, $p < 0.0001$). Patients with influenza were effectively differentiated from healthy volunteers and pneumococcal pneumonia based on the Gal-9 plasma levels.

PolyIC induces Gal-9 production in human airway epithelial cells

Primary sites for influenza virus infections are airway epithelial cells. To investigate the effect of influenza virus infections on Gal-9 production in airway epithelial cells, PolyIC was used to mimic the viral infection in cultured airway epithelial cells. Gal-9 concentrations were increased in the culture supernatant of airway epithelial cells in the presence of PolyIC, but not LPS ($p < 0.05$, Fig. 3).

Discussion

The findings of the present study demonstrated that patients with influenza infection have elevated plasma levels of Gal-9. Plasma Gal-9 levels could effectively differentiate influenza patients from those with pneumococcal pneumonia and healthy volunteers. Human airway epithelial cells produced Gal-9, and Gal-9 production was upregulated in the presence of PolyIC but not LPS.

Previous studies demonstrated Gal-9 expression in human vascular endothelial cells stimulated with interferon-γ, and the generation of Gal-9 by these cells in

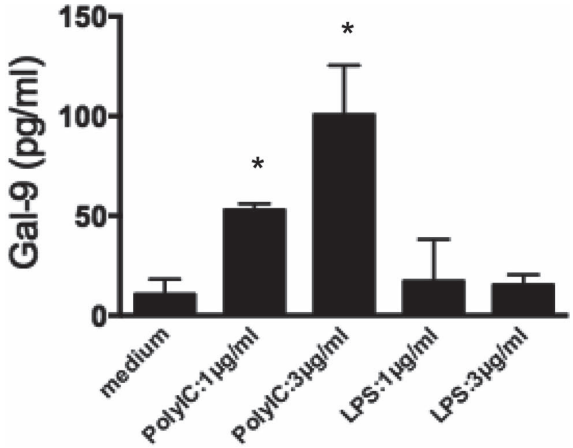

Fig. 3. Effects of PolyIC and lipopolysaccharides (LPS) on galectin-9 production in human airway epithelial cells. BEAS-2B human airway epithelial cells (1×10^5/well) were cultured with medium alone, PolyIC (1 μg/ml), PolyIC (3 μg/ml), LPS (1 μg/ml), or LPS (3 μg/ml) for 18 h at 37°C and Gal-9 concentrations in the culture supernatants were measured by ELISA as described in the Materials and Methods. Data represent means ± SEM. Mean values from 4 independent experiments. *$p < 0.05$ compared with medium alone.

response to dsRNA, an experimental model of viral infection (Imaizumi et al. 2002; Ishikawa et al. 2004). Recently, the role of Gal-9 in influenza A virus (IAV) and Herpes Simplex virus (HSV) infection was examined in Gal-9 deficient mice. An elevated and higher-quality CD8 T cell response to IAV and HSV was observed in Gal-9 deficient animals. Increased endogenous production of Gal-9 occurred after IAV-infection in wild-type mice. Furthermore, exogenous Gal-9 administration reduced the efficiency of CD8 T cell-mediated immunity to HSV infection. IAV-specific CD8 T cells from IAV-infected mice undergo apoptosis upon exposure to recombinant Gal-9 in vitro (Sehrawat et al. 2009, 2010; Sharma et al. 2011). We first demonstrated elevated levels of plasma Gal-9 with influenza virus infection in humans. Taken together with findings from previous studies in mice, Gal-9 might play an important role in viral infection to downregulate antiviral responses.

Previous studies revealed elevated plasma levels of Gal-9 in acute HIV-1 and dengue virus infected individuals (Chagan-Yasutan et al. 2009, 2013; Saitoh et al. 2012) and no changes in Gal-9 protein levels in experimental pneumococcal meningitis (Bellac et al. 2007). We found that Gal-9 production was upregulated by viral, but not bacterial, infection, in vivo. We also demonstrated that PolyIC but not LPS induces Gal-9 production in vitro. Further, influenza patients were effectively differentiated from pneumococcal pneumonia by plasma levels of Gal-9. Gal-9 may thus be a useful biomarker to differentiate viral and bacterial infections. Future studies should be performed to examine the plasma Gal-9 concentrations in patients with other types of viral infection compared with other biomarkers such as procalcitonin.

In the present study, the Gal-9 levels were significantly elevated, but the changes in the numbers of WBC and neutrophils were not remarkable, in patients with influenza. The CRP levels were significantly lower in patients with influenza than in patients with pneumococcal pneumonia. Furthermore, a marked decrease of the Gal-9 levels was observed after treatment in patients with influenza, which could be associated with disease activity. These data suggest that the increases in Gal-9 could be better marker for patients with influenza infection compared to other inflammatory markers.

There was no correlation between plasma Gal-9 levels and age in patients with influenza and pneumococcal pneumonia, and healthy volunteers. Although patients with influenza were younger than those with pneumococcal pneumonia, there was little effect of age on Gal-9 production in vivo.

In conclusion, our findings indicate that Gal-9 production is induced by influenza virus infection. Further, plasma levels of Gal-9 could be a new biomarker for patients with influenza infection.

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Conflict of Interest

The authors declare no conflict of interest.

References

Arikawa, T., Saita, N., Oomizu, S., Ueno, M., Matsukawa, A., Katoh, S., Kojima, K., Nagahara, K., Miyake, M., Yamauchi, A., Kohrogi, H. & Hirashima, M. (2010) Galectin-9 expands immunosuppressive macrophages to ameliorate T-cell-mediated lung inflammation. *Eur. J. Immunol.*, **40**, 548-558.

Bellac, C.L., Coimbra, R.S., Simon, F., Imboden, H. & Leib, S.L. (2007) Gene and protein expression of galectin-3 and galectin-9 in experimental pneumococcal meningitis. *Neurobiol. Dis.*, **28**,175-183.

Chagan-Yasutan, H., Ndhlovu, L.C., Lacuesta, T.L., Kubo, T., Leano, P.S.A., Niki, T., Oguma, S., Morita, K., Chew, G.M., Barbour, J.D., Telan, E.F.O., Hirashima, M., Hattori, T. & Dimaano, E.M. (2013) Galectin-9 plasma levels reflect adverse hematological and immunological features in acute dengue virus infection. *J. Clin. Virol.*, **58**, 635-640.

Chagan-Yasutan, H., Saitoh, H., Ashino, Y., Arikawa, T., Hirashima, M., Li, S., Usuzawa, M., Oguma, S., Telan, E.F.O., Obi, C.L. & Hattori, T. (2009) Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. *Tohoku J. Exp. Med.*, **218**, 285-292.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M. & Si-Tahar, M. (2005) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.*, **280**, 5571-5580.

Hirashima, M., Kashio, Y., Nishi, N., Yamauchi, A., Imaizumi, T.A., Kageshita, T., Saita, N. & Nakamura, T. (2004) Galectin-9 in physiological and pathological conditions.

Glycoconj. J., **19**, 593-600.

Imaizumi, T., Kumagai, M., Sasaki, N., Kurotaki, H., Mori, F., Seki, M., Nishi, N., Fujimoto, K., Tanji, K., Shibata, T., Tamo, W., Matsumiya, T., Yoshida, H., Cui, X.F., Takanashi, S., et al. (2002) Interferon-gamma stimulates the expression of galectin-9 in cultured human endothelial cells. *J. Leukoc. Biol.*, **72**, 486-491.

Ishikawa, A., Imaizumi, T., Yoshida, H., Nishi, N., Nakamura, T., Hirashima, M. & Satoh, K. (2004) Double-stranded RNA enhances the expression of galectin-9 in vascular endothelial cells. *Immunol. Cell Biol.*, **82**, 410-414.

Katoh, S., Nobumoto, A., Matsumoto, N., Matsumoto, K., Ehara, N., Niki, T., Inada, H., Nishi, N., Yamauchi, A., Fukushima, K. & Hirashima, M. (2010) Involvement of galectin-9 in lung eosinophilia in patients with eosinophilic pneumonia. *Int. Arch. Allergy Immunol.*, **153**, 294-302.

Katoh, S., Shimizu, H., Obase, Y., Oomizu, S., Niki, T., Ikeda, M., Mouri, K., Kobashi, Y., Hirashima, M. & Oka, M. (2013) Preventive effect of galectin-9 on double-stranded RNA-induced airway hyperresponsiveness in an exacerbation model of mite antigen-induced asthma in mice. *Exp. Lung Res.*, **39**, 453-462.

Saitoh, H., Ashino, Y., Chagan-Yasutan, H., Niki, T., Hirashima, M. & Hattori, T. (2012) Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J. Exp. Med.*, **228**, 157-161.

Sehrawat, S., Reddy, P.B.J., Rajasagi, N., Suryawanshi, A., Hirashima, M. & Rouse, B.T. (2010) Galectin-9/TIM-3 interaction regulates virus-specific primary and memory $CD8^+$ T cell response. *PLoS Pathog.*, **6**, e1000882.

Sehrawat, S., Suryawanshi, A., Hirashima, M. & Rouse, B.T. (2009) Role of Tim-3/Galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. *J. Immunol.*, **182**, 3191-3201.

Seki, M., Oomizu, S., Sakata, K., Sakata, A., Arikawa, T., Watanabe, K., Ito, K., Takeshita, K., Niki, T., Saita, N., Nishi, N., Yamauchi, A., Katoh, S., Matsukawa, A., Kuchroo, V. & Hirashima, M. (2008) Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin. Immunol.*, **127**, 78-88.

Seki, M., Sakata, K.M., Oomizu, S., Arikawa, T., Sakata, A., Ueno, M., Nobumoto, A., Niki, T., Saita, N., Ito, K., Dai, S.Y., Katoh, S., Nishi, N., Tsukano, M., Ishikawa, K., Yamauchi, A., Kuchroo, V. & Hirashima, M. (2007) Beneficial effect of galectin 9 on rheumatoid arthritis by induction of apoptosis of synovial fibroblasts. *Arthritis Rheum.*, **56**, 3968-3976.

Sharma, S., Sundararajan, A., Suryawanshi, A., Kumar, N., Veiga-Parga, T., Kuchroo, V.K., Thomas, P.G., Sangster, M.Y. & Rouse, B.T. (2011) T cell immunoglobulin and mucin protein-3 (Tim-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. *Proc. Natl. Acad. Sci. USA*, **108**, 19001-19006.

Zhu, C., Anderson, A.C., Schubart, A., Xiong, H., Imitola, J., Khoury, S.J., Zheng, X.X., Strom, T.B. & Kuchroo, V.K. (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.*, **6**, 1245-1252.