主 論 文

Role of *Lgals9* Deficiency in Attenuating Nephritis and Arthritis in BALB/c Mice in a Pristane- Induced Lupus Model

[プリスタン誘導ループスモデルBALB/c マウスの *Lgals9*欠損 による腎炎および関節炎の保護効果]

[Introduction]

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease characterized by a wide array of clinical manifestations and multifactorial pathogenic pathways. Kidney involvements in SLE are still associated with poor clinical outcomes, and lupus arthritis with altered quality of life in lupus patients.

Galectin-9 (Gal-9), an ubiquitously expressed β-galactoside binding lectin, characterized by the presence of two distinct carbohydrate recognition domains joined by a link peptide, Gal-9 induces apoptosis in various cell lines such as human melanoma cell line, activated Th1 and Th17 cells, B cells (BALL-1), monocytes (THP-1) and myelocytes (HL-60).

Recombinant Gal-9 has been successfully used in various disease models such as, collagen induced arthritis (CIA) model, asthma, anti-glomerular basement membrane (GBM) disease, diabetic nephropathy, and autoimmune encephalitis. The role of Gal-9 in SLE is still unknown so we investigated whether total loss of Gal-9 ameliorates or aggravates the disease activity of lupus mice models using the pristane (2, 6, 10, 14-tetramethylpentadecane) (TMPD) induced lupus models of BALB/c, as well as the spontaneously induced SLE model, MRL/MpJ-Fas^{lpr}/J mice

[Materials and methods]

Pristane-induced lupus model in BALB/c mice

Female Gal-9 -/- BALB/c mice (n=12) and age/sex matched control Gal-9 +/+ mice (n=12) were injected intraperitoneally with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane, Sigma-Aldrich, St. Louis, MO) at 7 weeks of age. Severity of arthritis in paws was evaluated every two weeks, serum and urine samples were collected monthly. Peritoneal lavage, lipogranulomas in the peritoneal cavity, spleen, kidney, and paws were obtained at 7 months after pristane injection.

Generation of Gal-9 -/- MRL/MpJ-Fas^{lpr}/J mice (MRL/lpr)

We generated Gal-9 -/- MRL/lpr female Gal-9 -/- (n=15) and Gal-9 +/+ (n=15) MRL/lpr littermates. They were monitored until 16 weeks and the tissues including spleen, lymph nodes, and kidneys were obtained at the end point. All the experimental procedures were approved by the Animal Care and Use Committee of the Department of Animal Resources, Advanced Science Research Center of Okayama University.

Tissues Histology and scoring

kidney sections were stained with hematoxylin and eosin (HE), Periodic acid-Schiff (PAS), Periodic acid-methenamine-silver (PAM), and Masson's trichrome (MT) stain. For immunofluorescence 4-μm cryostat sections were stained with FITC-conjugated goat anti-mouse IgG or rabbit anti-mouse C3, and goat anti-mouse IgG subclasses: IgG1, IgG2a, IgG2b or IgG3.

The most severely affected hind paw of each mouse was removed and fixed in 10% formalin. After decalcification in 10% EDTA, the joints were embedded in paraffin and 4-μm-thick sections were stained with HE and toluidine blue.
Four-μm paraffin embedded lipogranuloma, spleen and lymph node sections were deparaffinized, rehydrated, subjected to inhibition of endogenous peroxidase, and incubated with purified anti-mouse Galectin-9 antibody, followed by Goat anti rat IgG conjugated with horseradish peroxidase, developed with DAB and counterstained with Mayer's Hematoxylin.

Quantitative real-time PCR

Paws and lipogranuloma were disrupted and homogenized in Trizol reagents. Total RNAs were extracted from paws, lipogranuloma and peritoneal macrophage using RNeasy Mini kit. Quantitative real-time PCR was performed to evaluate the gene expression of tumor necrosis factor (*Tnf*), interleukin 1β (*Il1b*), *Il6*, galectin-1 (*Lgals1*), galectin-9 (*Lgals9*), galectin- 3 (*Lgals3*), toll-like receptor 3 (*Tlr3*), *Tlr4*, *Tlr7*, *Tlr9*, *Ccl2*, *Ccl7*, *Ccl12*, *Mx1*, *Cxcl10*, *Irf7*, and *Gapdh* mRNA as the invariant control.

Flow cytometry analysis

Single cell suspensions were prepared from spleen, peritoneal lavage of PI mice, and lymph nodes of MRL/lpr mice. The cells ($1x10^6$) derived from spleen, peritoneum and lymph nodes were incubated at 4° C for 30 min in staining buffer (Biolegend) with the relevant optimized amount of fluorochrome conjugated antibodies or the appropriate isotype controls. All data were acquired with FACSAria I flow cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar, Ashland, OR, USA).

Peritoneal macrophages in vitro assays

Peritoneal macrophages were harvested 24 hours after injection of either pristane or PBS and seeded at the concentration of $1x10^6$ /well. The adherent macrophages were then cultured in the presence or absence of TLRs ligands or underwent protein extraction using Cell Lytic M reagent (Sigma-Aldrich) following the manufacture's instruction. The supernatants were collected after 24 hours, RNA was extracted, and stored at -80° C.

ELISA

Serum levels of total IgG (abcam), anti-double stranded (ds)-DNA (Shibayagi, Japan), anti-nRNP (Alpha Diagnostic International), TNF-α (R&D systems), were measured using commercially available ELISA kits according to the manufacturer's instructions. The cut off values of the assays were 7.8 ng/ml, 15.6 mU/ml, 50 U/ml, and 10.9 ng/ml, respectively.

Western blotting

Tissues were homogenized in ice-cold RIPA buffer. Mini-PROTEAN precast gel (Bio-Rad, Hercules, CA) was equally loaded, electrophoresed, and electrotransferred to PVDF membranes. After blocking membranes were probed with primary antibody rat anti-mouse Galectin-9 clone 108A2 (Biolegend) or rabbit anti-mouse GAPDH (Cell Signaling Technologies). They were incubated with anti-rat IgG conjugated with horseradish peroxidase (abcam), and Donkey anti-Rabbit IgG conjugated with horseradish peroxidase (Santa-Cruz Biotechnology), respectively. Blots were developed by Pierce™ ECL Plus Western Blotting Substrate and visualized using ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences).

Statistical analysis

Data are expressed as the mean ± standard deviation. The normal distribution was assessed by Shapiro-Wilk test, and statistical significances were determined using two-tailed Student t-test and Mann-Whitney U test as appropriate. The incidence of arthritis was analysed using Fisher' s exact test. Data were analysed using JMP 13 software (SAS). *P* values<0.05 were considered as significant. *P* values are indicated by *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

[Results]

Pristane-induced lupus nephritis is attenuated by Lgals9 deficiency

Gal-9 -/- PI mice developed only a modest proteinuria, kidney damage and IC deposits compared to Gal-9 +/+ PI mice. In contrast, there were no differences regarding kidney involvement between Gal-9 -/- MRL/lpr and Gal-9+/+ MRL/lpr, suggesting that Gal-9 deficiency is capable to suppress kidney damages induced by pristane injection, but it is not sufficient to prevent severe forms of kidney disease in MRL/lpr mice model.

Pristane-induced production of IgG and autoantibodies in Gal-9 -/- BALB/c mice

Serum levels of total IgG were significantly lower in Gal-9 -/- compared to Gal-9 +/+ PI mice, wherareas serum levels of anti-ds-DNA and anti-nRNP antibodies were comparable in both groups. In MRL/lpr Gal-9 -/- and MRL/lpr Gal-9 +/+ mice, there were no statistical differences in the levels of ds-DNA antibodies. Although the deficiency of Gal-9 suppressed overall production of IgG, it failed to delete pathogenic plasma cells producing antibodies against autoantigens.

Arthritis is ameliorated in Gal-9 -/- PI mice

Incidence of arthritis, the mean clinical score as well as the histological features of arthritis were significantly reduced in Gal-9 -/- compared to Gal-9 +/+, in addition *Lgals9* deficiency reduced proinflammatory cytokine mRNA expressions in the joints, such as *Il1b*, *Il6* and *Tnfa* in Gal-9 -/- PI mice compared to Gal-9 +/+ PI mice.

Altered chronic peritoneal inflammatory response in Gal-9 -/- PI mice

7 months after pristane injection the number and size of lipogranuloma were markedly reduced in Gal-9 -/- PI mice with absence of follicle-like structures, reduced cellularity, and number of high endothelial venules (HEVs) compared with Gal-9 +/+ PI mice suggesting that Gal-9 is essentially required for the neogenesis of tertiary lymphoid tissue in response to pristane *in vivo*.

Effects of Lgals9 deficiency in innate immunity response by peritoneal macrophages

We observed no alterations in the frequency of numbers of T cells, B cells, effector/memory CD4+ T cells, and CD11b cells in Gal-9 -/- compared to Gal-9+/+ PI mice. At 15 days after pristane injection, the recruitment of CD11b+Ly6C^{hi} inflammatory monocytes into the peritoneal cavity was not impaired in Gal-9 -/- PI mice.

Further analysis of peritoneal macrophages response to various TLRs ligands for 24 hours, unexpectedly demonstrated the positive effects of TRL3, TRL4, and TRL9 ligands on *Tnfa* gene expression in Gal-9 -/- PI mice with statistical significance, while TRL7 ligand did not

reveal stimulatory effect on *Tnfa* mRNA expression. These results suggest that *Lgals9* deficiency did not alter the pristane-induced recruitment of inflammatory leucocytes into the peritoneal cavity nor impair the sensitivities of cytokine productions stimulated by TLR ligands.

Lgals9 deficiency protects BALB/c mice from pristane-induced lupus independent of TLR7-IFN-I pathway

The expression of ISG (*Mx1, Cxcl10* and *Irf7*) and IFN induced chemokines (*Ccl2, Ccl7,* and *Ccl12*) were comparable in Gal-9 -/- PI and Gal-9 +/+ PI peritoneal macrophages. In cultured peritoneal macrophages, there was no difference in ISG and IFN induced gene expression stimulated with various TLR ligands in Gal-9 -/- PI and Gal-9 +/+ PI mice. These results suggest that *Lgals9* deficiency did not alter TLR7-IFN-I pathway in peritoneal macrophages of pristane injected mice.

[Discussion]

Lgals9 deficiency significantly ameliorated glomerulonephritis, arthritis and lipogranuloma formation in pristane injected BALB/c, while glomerulonephritis and lymphadenopathy were not altered in MRL/lpr mice. *Lgals9* deficiency did not alter the production of autoantibodies in both pristane injected BALB/c and MRL/lpr mice, suggesting that Gal-9 is not required for autoantibody production. Although Gal-9 induces the differentiation of naïve T cells to Treg cells and it suppresses differentiation to Th1 and Th17 cells *in vitro* experiments, there are no alterations of cell populations in lymphocytes from spleen and peritoneal lavage between Gal-9 -/- PI and Gal-9 +/+ PI mice.

The exact mechanism of lipogranuloma formation remains largely unknown, but presumably the recruitment of inflammatory cells in response to different chemokines and cytokines triggered by pristane injection plays a crucial role in the development of this unique structures. There was no impairment in leucocytes recruitment into the peritoneal cavity of Gal-9 -/- PI mice; however, the formation and development of lipogranuloma was prominently diminished. Although the initial formation and development of lipogranuloma was not altered in Gal-9 -/- PI mice, they might undergo resolution by the reduction of cellular maintenance and supply due to reduced number of HEVs, or a reduced cytokine production induced by pristane overtime in the peritoneal cavity.

In current investigation, we found no defects and reductions in the expression of several ISG and IFN induced chemokines, accumulation of $Ly6C^{hi}$ inflammatory monocytes into the peritoneal cavity, and the production of anti-nRNP. Thus, *Lgals9* deficiency protected mice from pristane induced lupus independently from TLR7-IFN-I pathway in peritoneal macrophage.

we speculate the reasons why pristane-induced lupus model is protected, while MRL/lpr mice are not. Gal-9 is essentially required for the neogenesis of tertiary lymphoid tissue characterized by lymphoid follicle-like structures and HEVs in response to pristane *in vivo*, HEVs being the major entry port for immune cells secondary lymphoid organs and the reduced cellularity observed in Gal-9 -/- PI lipogranuloma suggests that inflammatory cells could be recruited via these newly formed vessels, and Gal-9 deficiency could have hampered their recruitment by inhibiting the formation of HEVs. Furthermore, Lipogranuloma consists of immune cells clusters and Gal-9 deficiency may impair cell adhesion resulting in a reduced cell aggregation and lipogranuloma formation. Formation of tertiary lymphoid like structures also requires signals provided by the local environment together with an appropriate stromal response, where Gal-9 is highly expressed.

[Conclusion]

In summary, the current investigation in Gal-9 -/- PI mice suggests that the antagonism of Gal-9 is beneficial for the treatment of nephritis and arthritis in SLE. The current therapy for SLE with steroids and immunosuppressants are associated with severe infections, which impair the quality of life in SLE patients. Since *Lgals9* deficient mice mount a more robust and vigorous virus-specific immune response in acute and chronic viral infections resulting in rapid viral clearance. Thus, antagonism of Gal-9 signaling may be beneficial to prevent the occurrence of various infections including viral infection in the patients with SLE.