IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Galectin-9 Is Critical for Mucosal Adaptive Immunity through the T Helper 17—IgA Axis

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Impairment of the intestinal mucosal immunity significantly increases the risk of acute and chronic diseases. IgA plays a major role in humoral mucosal immunity to provide protection against pathogens and toxins in the gut. Here, we investigated the role of endogenous galectin-9, a tandem repeat-type β -galactoside-binding protein, in intestinal mucosal immunity. By mucosal immunization of *Lgals9*^{−/−} and littermate control mice, it was found that lack of galectin-9 impaired mucosal antigen-specific IgA response in the gut. Moreover, *Lgals9*^{−/−} mice were more susceptible to developing watery diarrhea and more prone to death in response to high-dose cholera toxin. The results indicate the importance of galectin-9 in modulating intestinal adaptive immunity. Furthermore, bone marrow chimera mice were established, and galectin-9 in hematopoietic cells was found to be critical for adaptive IgA response. In addition, immunized *Lgals9*^{−/−} mice exhibited lower expression of *Il17* and fewer T helper 17 (Th17) cells in the lamina propria, implying that the Th17—IgA axis is involved in this mechanism. Taken together, these findings suggest that galectin-9 plays a role in mucosal adaptive immunity through the Th17—IgA axis. By manipulating the expression or activity of galectin-9, intestinal mucosal immune response can be altered and may benefit the development of mucosal vaccination. (*Am J Pathol* 2018, 188: 1225–1235; <https://doi.org/10.1016/j.ajpath.2018.01.017>)

Galectin-9, a tandem repeat-type β -galactoside-binding protein, is expressed in a variety of immune cells and exerts biological functions, including cell aggregation, adhesion, apoptosis, and differentiation.^{1–3} Galectin-9 modulates immune functions by altering cell signaling through the formation of galectin–glycoprotein lattices on the surface of immune cells.^{1–3}

The known functions of galectin-9 include induction of T helper 1 (Th1) cell apoptosis by binding to T-cell immunoglobulin and mucin domain-containing protein 3,⁴ suppression of allergic asthma by inhibiting CD44–hyaluronan interaction,⁵ and boosting the function of adaptive regulatory T cells through its interaction with CD44.⁶ However, intracellular galectin-9 can function as a uric acid transporter in renal epithelial cells by inserting itself into membrane lipid bilayers⁷; furthermore, intracellular galectin-9 regulates apical–basal polarity by interaction with Forssman glycosphingolipid.⁸ These results demonstrate that galectin-9 can

exert biological functions in both intracellular and extracellular compartments.

IgA, the most abundant Ig isotype in the intestinal lumen, plays a crucial role in adaptive immunity to prevent pathogenic infections and toxins.^{9–11} Intestinal low-affinity IgA maintains gut homeostasis by shaping a balanced community of commensal microbiota; of more importance, high-affinity IgA derived from T-cell–dependent responses binds to pathogenic bacteria to protect against communicable diseases.^{12–14} Of the T-cell subsets, Th17 cells have recently emerged as an important regulator of T-cell–dependent IgA responses.^{15–17} Although the mechanisms involved are not fully understood, mice deficient in IL-17 exhibit impaired antigen-specific IgA responses for protection against foreign

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pathogens.^{17,18} Instead of a direct role in IgA class switching, Th17 cells secrete IL-17A and IL-21 to support germinal center formation and plasma cell differentiation.^{15–20}

Considering that the impairment of mucosal immunity in the gut significantly increases the risk of acute and chronic diseases,^{21,22} it is of great importance to understand the mechanistic basis of regulation of intestinal immune responses. Previous studies have described that galectin-9 is expressed in intestinal epithelial cells²³ and immune cells,^{2,3} thereby raising the question whether galectin-9 is involved in the modulation of mucosal immunity. In the present study, we investigated the role of endogenous galectin-9 in mucosal immunity of the gut. Our results showed that lack of galectin-9 impairs antigen-specific IgA response in the gut and compromises mucosal adaptive immunity. Furthermore, we found that *Lgals9*^{−/−} mice had fewer number of Th17 cells under the condition of cholera toxin (CT) stimulation, suggesting that the Th17–IgA axis may be implicated in this mechanism.

Materials and Methods

Mice

Lgals9^{−/−} and *Lgals9*^{+/+} mice (B6.CD45.2 background) were obtained from the Consortium of Functional Glycomics and have been described previously.^{4–6,24} Dr. Fang Liao (Academia Sinica, Taipei, Taiwan) kindly provided B6.SJL-*Ptprc*^a *Pepc*^{b/l} BoyJ congenic mice (B6.CD45.1 background), which were intercrossed with *Lgals9*^{−/−} mice (B6.CD45.2 background) to generate CD45.1 *Lgals9*^{−/−} mice. All mice were maintained at Academia Sinica under a specific pathogen-free condition. Eight- to 12-week-old *Lgals9*^{−/−} and *Lgals9*^{+/+} littermates were used for all experiments, unless indicated otherwise. The Institutional Animal Care and Use Committee at Academia Sinica approved all animal experiments.

Immunization and Challenge Protocols

As previously described,^{24,25} mice were deprived of food for 4 hours and subsequently provided with 300 μ L of alkalized buffer [two parts 7% NaHCO₃ diluted in eight parts Hanks' balanced salt solution (HBSS)] to neutralize gastric acid 30 minutes before mucosal immunization with oral gavage. All drugs for mucosal immunization were diluted in 300 μ L of phosphate-buffered saline (PBS). Two oral immunizations with 10 μ g of CT (List Biological Laboratories, Campbell, CA) were administered on day 0 and 10. In some groups, mice were intraperitoneally administered 100 μ g of chicken γ -globulin (CGG; Biosearch Technologies, Novato, CA) on day 0 and were then orally fed with both 500 μ g of CGG and 10 μ g of CT on day 1, 17, and 27. Ten days after the final immunization, mice were sacrificed by carbon dioxide asphyxiation for subsequent experiments. For *in vivo* fluid accumulation test, naive or immunized mice were orally administered 150 μ g of CT

to examine protection against CT-induced diarrhea/fluid loss. To determine death because of massive doses of CT, 150, 300, and 600 μ g of CT was administered to these immunized mice.

Fecal Sample Preparation

Fecal pellets were homogenized in PBS that contained 2 mmol/L EDTA (Merck, Darmstadt, Germany), 2 mmol/L phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO), 0.04 mg/mL trypsin inhibitor (Invitrogen, Carlsbad CA), and protease inhibitor cocktail (Merck) with Tissue-lyzer II (Qiagen, Valencia CA). After centrifugation for 20 minutes, the supernatants were collected for further experiments.

Cell Isolation

Bone marrow (BM) cells were harvested by flushing femurs and tibias with RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Carlsbad, CA) and removed the debris by passing through 40- μ m cell strainers (BD Biosciences, San Jose, CA). To isolate mononuclear cells from Peyer's patch (PP), tissues were shaken for 30 minutes at 37°C in the digestion medium, that is, Ca²⁺/Mg²⁺-containing HBSS (Life Technologies) supplemented with 2% FBS, 10 mg/mL collagenase D (Roche, Indianapolis, IN), 1 U/mL Dispase (Roche), and 5 mg/mL DNase I (Sigma-Aldrich). To isolate intestinal lamina propria (iLP) cells, intestinal tissues were incubated for 20 minutes at 37°C with gentle shaking for three times in Ca²⁺/Mg²⁺-free HBSS (Invitrogen) that contained 2% FBS, 1 mmol/L dithiothreitol (Sigma-Aldrich), and 5 mmol/L EDTA to remove epithelial cells and intraepithelial lymphocytes. Intestinal tissues were then cut into small pieces and incubated in the digestion medium by shaking for 30 minutes at 37°C. Digested cells of PP and iLP tissues, after passing through 40- μ m cell strainers, were resuspended in RPMI 1640 medium supplemented with 10% FBS and carefully loaded between discontinuous 30% and 100% Percoll density gradient (GE Healthcare, Little Chalfont, UK). After centrifugation at 670 $\times$ g for 20 minutes at room temperature, mononuclear cells at the interface were collected.

Enzyme-Linked Immunosorbent Assay

Galectin-9 levels, total IgA amounts, and antigen-specific IgA titers were determined in serum and fecal samples by enzyme-linked immunosorbent assay as previously described.^{23,25–27} In brief, 96-well microplates were coated with 1 μ g/mL goat anti-galectin-9 antibody (R&D Systems, Minneapolis, MN), 2 μ g/mL goat anti-mouse IgA (Southern Biotech, Birmingham, AL), 20 μ g/mL CGG, or 10 μ g/mL CT and were incubated at 4°C overnight. To block the monosialotetrahexosylganglioside (GM1) binding sites on CT, 10 μ g/mL CT and 10 nmol/mL GM1 (Avanti Polar Lipids, Alabaster, AL) were incubated

together for 1 hour at room temperature before coating the microplates. After blocking with 3% bovine serum albumin (Sigma-Aldrich) in PBS for 2 hours at room temperature, serial sample dilutions were incubated at room temperature for 3 hours. Diluted horseradish peroxidase-conjugated goat anti-mouse IgA (Southern Biotech) was added at room temperature for 1 hour. To measure galectin-9 levels, plates were incubated with 0.5 µg/mL biotinylated goat anti-galectin-9 antibody (R&D Systems), followed by adding horseradish peroxidase-conjugated streptavidin (R&D Systems). The reaction was developed with 3,3',5,5'-tetramethylbenzidine (eBioscience, San Diego, CA), followed by adding 1 N HCl, and optical density values at 450 nm were measured with the Infinite Multimode Reader (Tecan, Männedorf, Switzerland). Serial dilutions of purified mouse IgA (BD Pharmingen, San Diego, CA) or recombinant galectin-9 (R&D Systems) were used to establish standard curves. To calibrate the variation in fecal pellets, fecal IgA values were normalized to total protein concentrations in feces.

In Vitro IgA Class Switching

To prepare naive IgD⁺ B cells, mononuclear cells from PP were labeled with anti-CD93 microbeads and biotin-conjugated antibodies against CD43 (Ly-48), CD4 (L3T4), Ter-119 (all from Miltenyi Biotec, Bergisch Gladbach, Germany), and CD138 (281-2; BD Pharmingen). Anti-biotin microbeads (Miltenyi Biotec) were used to collect the negative fraction, which was labeled with phosphatidylethanolamine-conjugated anti-IgD antibody (11-26c; eBioscience) and subsequently labeled with anti-phosphatidylethanolamine microbeads (Miltenyi Biotec). IgD⁺ B cells were isolated with the Quad-roMACS Separator (Miltenyi Biotec) and were used as native B cells (>98% IgD⁺ by flow cytometry; data not shown). Naive IgD⁺ B cells were plated in round bottom 96-well plates at a density of 2×10^5 per well and were directly stimulated with lipopolysaccharide (LPS; InvivoGen, San Diego, CA), transforming growth factor-β1 (TGF-β1; R&D Systems), B-cell-activating factor (Alexis Biochemicals, Lausen, Switzerland), or a proliferation-inducing ligand (Alexis Biochemicals) at indicated concentration for 7 days. The level of α germline transcripts (α-GT) expression was used to examine ongoing IgA class switching in LPS-stimulated B cells.

ELISpot Assay

Enzyme-linked immunospot (ELISpot) assays were performed to enumerate CT-specific IgA-secreting cells. CT was incubated with GM1 before coating on 96-well polyvinylidene difluoride membrane plates (MAIPS4510; Millipore, Billerica, MA) at 4°C overnight. After washing and blocking the plates, various concentrations of mononuclear cells were cultured at 37°C in 5% CO₂ for 4 hours. RPMI 1640 medium supplemented with 5% bovine serum albumin was used for all dilutions. Anti-CT IgA-secreting cells were visualized by adding horseradish peroxidase-conjugated

goat anti-mouse IgA antibody at 1:5000 dilution, followed by adding 3-amino-9-ethylcarbazole substrate (BD Biosciences). The size and number of spots were analyzed with the AID EliSpot Reader System (Autoimmun Diagnostika, Strassbourg, Germany).

Flow Cytometric Analysis

Isolated mononuclear cells from PP and iLP tissues were suspended in PBS supplemented with 2% bovine serum albumin, followed by incubation with CD16/CD32 antibody (2.4G2; BD Pharmingen) for 15 minutes to block Fc γ receptor. For surface staining, fluorophore-labeled antibodies for CD4 (GK1.5), CD45.1 (A20), or CD45.2 (104) were obtained from BioLegend (San Diego, CA). The fixable viability dye (eBioscience) was used to exclude dead cells, followed by fixation with 4% formaldehyde. To detect Th1 and Th17 cells, mononuclear cells of iLP were reactivated by leukocyte activation cocktail that contained phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A (BD Biosciences) for 4 hours at 37°C. Intracellular cytokine staining for IL-17A (TC11-18H10.1; BioLegend) and interferon-γ (XMG1.2; BioLegend) was performed with Cytofix/Cytoperm buffer (BD Pharmingen) according to the manufacturer's instructions. Flow acquisition was performed on LSRII flow cytometer (BD Biosciences), and results were analyzed by the FlowJo software version 10 (Tree Star, Ashland, OR).

Immunofluorescence Staining

Small intestinal tissues from sacrificed mice were fixed in 10% formalin and embedded in paraffin. Tissue sections (5 µm) were deparaffinized, followed by heat-induced epitope retrieval with citrate buffer, pH 6.0 (Thermo Fisher Scientific, Waltham, MA) and then blocked with 5% normal goat serum. For detection of Th17 cells, the sections were incubated overnight with rat anti-CD4 monoclonal antibody (4SM95; eBioscience) and rabbit anti-IL-17 polyclonal antibody (Abcam, Cambridge, MA). Slides were washed and then incubated with fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies (Invitrogen) for 2 hours at room temperature. Finally, coverslips were mounted with the mounting medium that contained DAPI (Invitrogen) and visualized Th17 cells with the use of an LCM 700 confocal microscope (Carl Zeiss, Gottingen, Germany).

Quantitative RT-PCR

RNA was extracted with the use of RNeasy mini kits (Qiagen), followed by removal of contaminating DNA by DNase I treatment (Sigma-Aldrich). Purified RNA was reverse transcribed into cDNA with the use of iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR analyses were conducted in the CFX real-time PCR detection system (Bio-Rad) with the use of specific primers (synthesis by Genomics Taiwan) (Table 1) and corresponding Universal

Table 1 Primer Sequences for Quantitative RT-PCR

Gene	Forward primer	Reverse primer
<i>Il17a</i>	5'-CAGGGAGAGCTTCATCTGTGT-3'	5'-GCTGAGCTTTGAGGGATGAT-3'
<i>Il22</i>	5'-TGCCTTTCTCTGACCAAACTC-3'	5'-CTGGATGTTCTGGTCGTCAC-3'
<i>Il23a</i>	5'-TCCCTACTAGGACTCAGCCAAC-3'	5'-AGAACTCAGGCTGGGCATC-3'
<i>Ifng</i>	5'-ATCTGGAGGAACTGGCAAAA-3'	5'-TTCAAGACTTCAAAGAGTCTGAGGTA-3'
<i>α-GT</i>	5'-CCAGGCTAGACAGAGGCAAG-3'	5'-CGGAAGGGAAGTAATCGTGA-3'
<i>Gapdh</i>	5'-GTATGACTCCACTCACGGCAAATT-3'	5'-GATGGGCTTCCCGTTGATGA-3'
<i>Tgfb1</i>	5'-TGGAGCAACATGTGGAATC-3'	5'-GTCAGCAGCCGGTTACCA-3'
<i>Aldh1a2</i>	5'-CATGGTATCCTCCGCAATG-3'	5'-GCGCATTTAAGGCATTGTAAC-3'
<i>Tnfsf13b</i>	5'-CGCCGACTATACGAAAAGGA-3'	5'-CCTGTTTGCCTCACCACTATT-3'
<i>Tnfsf13</i>	5'-GAAGGATGGGGCGAAATC-3'	5'-TGTCACGTCAGAGTCCTTGG-3'

ProbeLibrary probes (Roche). The mRNA levels were normalized to those of *Gapdh*.

BM Chimera Mice

To establish BM chimera mice, 8- to 12-week-old recipient mice (CD45.1 background) were deprived of food for 2 hours and then exposed to 600 rad γ -irradiation twice at an interval of 4 hours (total 1200 rad). On the subsequent day, 5×10^6 BM donor cells (CD45.2 background) were injected through the tail vein, and mice were fed with antibiotic water (2 g/L neomycin; Sigma-Aldrich) for 2 weeks. Engraftment efficiency was examined by flow cytometry after 8 weeks, and these chimera mice were used for further experiments after 12 weeks.

In Vivo IL-17 Neutralization

For IL-17 neutralization, anti-IL-17 antibody (MAB421; R&D Systems) or rat IgG2a antibody (MAB006; R&D Systems), 100 μ g in 200 μ L of PBS, was injected intraperitoneally every other day for five injections since the time of the second CT dose (ie, day 10) as previously described.²⁸ On day 20, mice were sacrificed for further analysis.

Statistical Analysis

Two-tailed *t*-test or *U*-test was performed as appropriate to examine statistical significances of differences between the groups. Mortality rates were compared between the groups with the log-rank test. Statistical analyses were performed with Prism software version 5 (GraphPad Software, Inc., La Jolla, CA), and *P* values <0.05 were considered statistically significant.

Results

Lack of Galectin-9 Specifically Impairs Mucosal Antigen-Specific IgA Response

To investigate the role of galectin-9 in IgA production, serum and fecal IgA levels were measured in naive *Lgals9^{+/+}* and

Lgals9^{-/-} mice. Under the nonimmunized condition, lack of galectin-9 did not affect IgA levels (Supplemental Figure S1, A and B). Mucosal immunization was performed in mice, and subsequently systemic and mucosal antigen-specific IgA titers were measured. Figure 1A depicts the experimental protocol of mucosal immunization with both CGG and CT. No differences were found in serum CGG-specific IgA titers (Figure 1B), serum CT-specific IgA titers (data not shown), and total fecal IgA levels between the two genotypes (Supplemental Figure S1C). However, *Lgals9^{-/-}* mice had significantly lower fecal CGG-specific and CT-specific IgA titers than *Lgals9^{+/+}* mice (Figure 1, C and D), suggesting that lack of galectin-9 impaired mucosal antigen-specific IgA response.

An alternative protocol (with CT alone) to induce antigen-specific IgA response was tested (Figure 1E). Impaired mucosal antigen-specific IgA response was also found in *Lgals9^{-/-}* mice under this condition (Supplemental Figure S1D). Although previous studies have revealed obvious increased serum and tissue galectin-9 levels in inflammatory diseases,^{29,30} this study showed that the CT-induced immune response did not lead to an increase in serum or fecal galectin-9 levels (Supplemental Figure S1E). The number of CT-specific IgA-secreting cells was next investigated in intestinal lymphoid tissues by ELISpot assay. The numbers of CT-specific IgA-secreting cells in both iLP and PP tissues were fewer in mice that lacked galectin-9 (Figure 1F). Taken together, these data suggested that lack of galectin-9 specifically impaired mucosal antigen-specific IgA response.

Protective Immunity Is Compromised against CT in Mice That Lack Galectin-9

Mice were orally immunized with CT, and then an *in vivo* CT challenge was performed to examine whether the lack of galectin-9 impaired mucosal adaptive immunity (Figure 2A). Consistent with previous studies,^{12,31} this study revealed that nonimmunized mice that received 150 μ g of CT developed severe diarrhea. Both immunized *Lgals9^{+/+}* and *Lgals9^{-/-}* mice had less intestinal water volume than nonimmunized mice, indicating that immunization enabled mice to tolerate

Figure 1 Lack of galectin-9 impairs antigen-specific IgA production. **A:** The experimental protocol of mucosal immunization. Initially, mice were intraperitoneally injected with chicken γ -globulin (CGG) on day 0 and subsequently orally immunized with CGG and cholera toxin (CT) on day 7, 17, and 27. **B–D:** Serum and fecal samples were collected on indicated days to detect CGG- and CT-specific IgA by enzyme-linked immunosorbent assay (ELISA). **E** and **F:** Another approach to mucosal immunization. Mice were administered oral gavage with CT on day 0 and 10. On day 20, mice were sacrificed to quantify CT-specific IgA-forming cells in intestinal lamina propria and Peyer's patch by enzyme-linked immunospot assay (ELISpot). Symbols represent data from individual mice (**F**). Data are expressed as means $\pm$ SEM (**B–D**); horizontal lines indicate means (**F**). Three independent experiments; $n = 6$ to 10 mice per group (**B–D**); $n = 6$ mice per group (**E** and **F**). * $P < 0.05$ and ** $P < 0.01$. Statistical analyses were performed with t -test (**B–D**) or U -test (**F**). ASC, antibody-secreting cell; O.D., optical density; PBS, phosphate-buffered saline.

CT (Figure 2, B and C). Of importance, immunized $Lgals9^{-/-}$ mice developed more severe watery diarrhea than immunized $Lgals9^{+/+}$ mice, demonstrating the defective protection against CT-induced watery diarrhea in mice that lacked galectin-9 (Figure 2C).

To investigate the defect in protective immunity in $Lgals9^{-/-}$ mice, a series of high CT doses were administered to examine the mortality rate (Figure 3A). At a dose of 150 or 300 μ g, most immunized mice were alive irrespective of the galectin-9 expression (Figure 3B). Mice that lacked galectin-9 showed a higher mortality rate than wild-type (WT) littermates at a dose of 600 μ g ($P = 0.03$ by the log-rank test) (Figure 3B). These results indicated that mice lacking galectin-9 developed characteristics of impaired mucosal adaptive immunity against antigens.

Galectin-9 Expression by BM-Derived Cells Is Required for Adaptive IgA Response

To identify which kind of cells were responsible for the functional relevance of galectin-9 to adaptive IgA response, congenic CD45.1 $Lgals9^{+/+}$ or $Lgals9^{-/-}$ host mice were reconstituted with either CD45.2 $Lgals9^{+/+}$ or $Lgals9^{-/-}$ BM. Eight weeks after reconstitution, the engraftment efficiency was analyzed in PP and iLP tissues, and both were found to exhibit $>90\%$ efficiency (Figure 4A). These BM chimera mice received mucosal immunization with CT alone 12 weeks

after reconstitution, and subsequently ELISpot assay was performed to enumerate antigen-specific IgA cells. Fewer antigen-specific IgA cells were observed in the intestinal lymphoid tissues of $Lgals9^{+/+}$ or $Lgals9^{-/-}$ mice that received $Lgals9^{-/-}$ donor cells than those in mice that received $Lgals9^{+/+}$ donor cells (Figure 4, B and C). However, the number of antigen-specific IgA cells was comparable between $Lgals9^{+/+}$ and $Lgals9^{-/-}$ mice that received $Lgals9^{+/+}$ donor cells. These results suggested that galectin-9 expression by BM-derived cells was critical for adaptive IgA response.

B Cells Lacking Galectin-9 Paradoxically Exhibit Higher IgA Production *in Vitro*

Because IgA-secreting B lymphocytes are essential for mucosal adaptive immunity, it was examined whether galectin-9 deficiency in B lymphocytes altered normal IgA response. Our unpublished data revealed that there were no differences in B-cell differentiation, apoptosis, activation, and proliferation between $Lgals9^{+/+}$ and $Lgals9^{-/-}$ mice. However, galectin-9-deficient IgD $^{+}$ B cells stimulated with TGF- β and LPS *in vitro* produced more IgA than that produced by WT IgD $^{+}$ B cells (Supplemental Figure S2A). The gene expression of α -GT, which has been shown to be correlated with IgA synthesis, was further analyzed.^{32,33} $Lgals9^{-/-}$ IgD $^{+}$ B cells had a higher level of α -GT than $Lgals9^{+/+}$ IgD $^{+}$ B cells in the presence of TGF- β and LPS (Supplemental Figure S2B). In

A **B** **C**

Figure 2 *In vivo* fluid accumulation test. **A:** Experimental protocol. Mice were orally immunized with cholera toxin (CT; immunized group) or without CT (naive group) on day 0 and 10. On day 20, mice were orally fed with 150 µg of CT, and intestinal fluid volume was measured after 16 hours. **B:** Representative images of the intestine after feeding with 150 µg of CT. Boxed areas in the lower panels indicate the levels of intestinal water, which are magnified for the upper panels. **C:** Symbols represent data from individual mice. Horizontal lines indicate medians (C). Three independent experiments; $n = 6$ mice in the naive group; $n = 7$ mice in the immunized group. * $P < 0.05$ (statistical analyses were performed by *U*-test). Original magnification, $\times 2.1$ (B, upper panels). KO, knockout; PBS, phosphate-buffered saline; WT, wild-type.

addition, cell numbers were determined with an automated cell counter to investigate if the cell number affected IgA synthesis. The *Lgals9*^{-/-} B-cell density was much lower than WT B-cell density (Supplemental Figure S2C). The results indicated that TGF- β augmented the inhibitory effect on LPS-simulated *Lgals9*^{-/-} B-cell growth. In summary, these data suggested that B cells lacking galectin-9 exhibited increased IgA production in the presence of TGF- β .

Galectin-9—Mediated Mucosal Adaptive Immunity May Involve the Th17—IgA Axis

Previous studies have shown that a sufficient antigen-specific IgA response required the cooperation of mucosal B and T cells.^{9–11} These results led us to consider that galectin-9 expression by T lymphocytes modulated adaptive IgA response. To elucidate which subset of T lymphocytes was involved in this phenotype, mononuclear cells were isolated from iLP to analyze IgA-related cytokine genes. The mRNA levels of T-cell-independent cytokines (*Tgfb*, *Tnfsf13*, *Tnfsf13b*, and *Aldh1a2*) crucial for IgA production were similar in both immunized mononuclear cells regardless of the galectin-9 expression (data not shown). Of note, *Lgals9*^{-/-} cells expressed lower levels of *Il17a*, *Il22*, and *Ifng* but higher levels of *Il23a* than *Lgals9*^{+/+} cells (Figure 5A), indicating that IL-17-producing cells may

participate in this mechanism. Although IL-17 is secreted by multiple cell types, such as innate lymphoid cells type 3, Th17 cells, $\gamma\delta$ T cells, and natural killer cells,³⁴ Th17 cells were found to be primarily involved in adaptive IgA production.^{15–18} Hirota et al¹⁶ reported that intestinal Th17 cells acquired the characteristics of T follicular helper cells (signature genes *Il21* and *Bcl6*) in PP. However, our results showed that there were no differences in mRNA levels of *Il21* and *Bcl6* between *Lgals9*^{+/+} and *Lgals9*^{-/-} PP cells. Moreover, similar proportions of germinal center B cells (CD19⁺PNA⁺CD95⁺) were observed in PP of *Lgals9*^{+/+} and *Lgals9*^{-/-} mice (data not shown).

To investigate the role of Th17 cells in this phenotype, flow cytometric analysis was performed to detect Th17 cells in iLP. Intracellular cytokine staining showed that immunized *Lgals9*^{-/-} mice had a smaller number of CD4⁺IL-17⁺ (Th17) cells in iLP, in comparison with WT mice (Figure 5, B and C). Tissue immunofluorescence staining also revealed the same finding that *Lgals9*^{-/-} mice had fewer Th17 cells than *Lgals9*^{+/+} mice (Supplemental Figure S3). To establish galectin-9—mediated mucosal adaptive immunity through the Th17—IgA axis, *in vivo* IL-17 blockade was performed to investigate the effect of IL-17 on galectin-9—mediated adaptive IgA generation. The difference in mucosal antigen-specific IgA response between immunized *Lgals9*^{+/+} and *Lgals9*^{-/-} mice disappeared after mice were subjected to *in vivo* IL-17

Figure 3 Cholera toxin (CT) mortality test. **A:** Experimental schedule. Groups of mice were orally immunized with 10 µg of CT on day 0 and 10 and orally challenged with different doses of CT on day 20. Mice were monitored daily to record the mortality rate. **B:** Graphs show survival curves for *Lgals9*^{+/+} and *Lgals9*^{-/-} mice. Two independent experiments; *n* = 6 to 8 mice per group (**B**). **P* < 0.05. Statistical analyses were performed by the log-rank test (*P* values for 150, 300, and 600 µg were 1.00, 0.32, and 0.03, respectively). KO, knockout; PBS, phosphate-buffered saline; WT, wild-type.

neutralization, thereby providing robust evidence of the involvement of Th17 cells in galectin-9-mediated IgA response (Figure 5, D and E). From previous studies that have established the role of Th17 cells in antigen-specific IgA production,^{15–18} our results suggested that galectin-9-mediated adaptive immunity may involve the Th17–IgA axis.

Discussion

Although exogenous administration of galectin-9 was described to modulate intestinal and pulmonary allergic

diseases,^{23,35} little is known about the role of endogenous galectin-9 in the context of mucosal immunity. In the present study, it was found that the lack of galectin-9 impaired mucosal antigen-specific IgA response in the gut. Furthermore, *Lgals9*^{-/-} mice developed more severe watery diarrhea and exhibited higher mortality rates when administered high doses of CT, indicating the importance of galectin-9 in regulating intestinal adaptive immunity. Considering that galectin-9 is expressed by T-cell subsets,⁶ B-cell subsets (our unpublished data), dendritic cell subsets,³⁶ fibroblasts,⁵ and intestinal epithelial cells,²³ BM chimera mice were generated, and it was found that galectin-9 in hematopoietic cells is required for competent

Figure 4 Galectin-9 expression by bone marrow (BM)-derived cells is required for adaptive immunity and host protection. **A:** Representative flow plots of BM engraftment efficiency in Peyer's patch (PP) and intestinal lamina propria (iLP) cells. Experiments were performed 8 weeks after BM cells of CD45.2 mice were transferred into CD45.1 hosts. Flow plots gate on viable CD45⁺ cells and numbers in plots indicate the percentage of the cells. **B** and **C:** Twelve weeks after BM transplantation, mononuclear cells were isolated from PP and iLP cells of mice that received mucosal immunization to determine cholera toxin (CT)-specific IgA-forming cells by enzyme-linked immunospot assay. Symbols represent data from individual mice (**B** and **C**). Horizontal lines indicate means (**B** and **C**). Three independent experiments; *n* = 6 mice per group (**B** and **C**). **P* < 0.05 (*U*-test). ASC, antibody-secreting cell; KO, knockout; WT, wild-type.

Figure 5 Galectin-9-mediated mucosal adaptive immunity may involve the T helper 17 (Th17)–IgA axis. **A:** Gene expression of IL-17–associated cytokines in mononuclear cells of intestinal lamina propria (iLP) was determined in mice that received mucosal immunization. Values are normalized to those of *Gapdh*. **B:** Representative flow cytometric plot of CD4⁺ T cells stained for intracellular IL-17 and interferon (IFN)-γ. **C:** Percentages of Th17 cells among CD4⁺ cells in the small intestines of immunized mice. **D and E:** Mice were orally immunized with 10 μg of cholera toxin (CT) on day 0 and 10 and were intraperitoneally injected with 100 μg of anti-IL-17 monoclonal (open symbols) or isotype control antibodies (rat IgG2a; closed symbols) every other day for five consecutive doses since day 10. Feces and intestinal tissues were collected on day 20 to measure the fecal CT-specific IgA titer and IgA-secreting cells in anti-IL-17 or isotype control–treated mice. Data are expressed as means ± SEM (**A** and **C**); symbols represent individual mice and horizontal lines indicate the means (**D** and **E**). Three independent experiments (**A**); two independent experiments (**B**–**E**); *n* = 6 mice per group (**A** and **C**–**E**). **P* < 0.05 (*t*-test). ASC, antibody-secreting cell.

adaptive IgA production. The results indicate that intestinal B cells may not contribute to this phenotype because *Lgals9*^{-/-} B cells paradoxically underwent IgA class switching to a greater extent than *Lgals9*^{+/+} B cells. Moreover, the experiments of *in vivo* IL-17 blockade and flow cytometric analysis of Th17 cells demonstrated the involvement of Th17 cells in galectin-9–mediated mucosal adaptive IgA response. Taken together, these findings indicate that endogenous galectin-9 plays a critical role in mucosal adaptive immunity through the Th17–IgA axis.

Contrary to the study of Seki et al,³⁷ which used recombinant galectin-9 to demonstrate its extracellular activities, including its suppressive effect on Th17-cell differentiation, the present study reveals that immunized *Lgals9*^{-/-} mice exhibited fewer Th17 cells in the iLP under CT stimulation. Furthermore, Kadowaki et al³⁸ described that exogenous galectin-9 prolongs the survival of septic mice by expanding natural killer cells and plasmacytoid dendritic cell antigen-1⁺ CD11c⁺ macrophages and that galectin-9 increases the levels of IL-15 and IL-17 but

decreases tumor necrosis factor α , IL-5, IL-10, and high mobility group box 1. Unlike most cytokines, galectin-9 lacks a classic signal sequence for protein secretion and is predominantly localized within cells, although it can be secreted through a nonclassic secretory pathway.^{1–3} In the present study, the function of endogenous galectin-9 was addressed by comparing *Lgals9*^{+/+} and *Lgals9*^{-/-} mice, rather than using exogenously added recombinant protein, to delineate the relationship between galectin-9 and Th17 cells. Regarding the function of galectin-9 that was demonstrated, it is likely that the protein functions intracellularly, although the possibility that it exerts its function extracellularly cannot be excluded.

Here, it was shown that this regulatory phenomenon primarily occurs at the site of iLP rather than PP, because lack of galectin-9 did not disturb the signature gene expression of T follicular helper cells and percentages of germinal center B cells in PP. It has been suggested that IgA generation with somatic hypermutation could occur outside PPs, for instance in iLP.^{9,39} Not surprisingly, high numbers of eosinophils, innate lymphoid cells, dendritic cells, regulatory T cells, and Th17 cells are present in iLP and secrete IgA-promoting cytokines (such as TGF- β , IL-17, B-cell-activating factor, and a proliferation-inducing ligand) to facilitate class switching recombination for T-cell-dependent IgA induction.^{9,16,40–43} Despite these remarkable observations, whether galectin-9 modulates IgA generation by directly affecting Th17 cells or by indirectly disturbing Th17-cell differentiation through other cell types remains to be elucidated.

This study revealed that immunized galectin-9-deficient mice had higher *Il23a* levels but lower *Il17a* and *Il22* levels in iLP, suggesting that lack of galectin-9 may impair the IL-23–Th17 cell axis as well. It has been well established that IL-23 produced by dendritic cells binds to IL-23 receptors on Th17 cells and subsequently provokes IL-17 and IL-22 production.^{16,44,45} Both IL-17 and IL-22 trigger the up-regulation of antimicrobial proteins and promote epithelial barrier function, which maintains the adequate defense mechanism against pathogens and toxins.^{34,46} Therefore, it is reasonable to assume that impairment of the IL-23–Th17 cell axis partly contributes to a higher mortality rate in *Lgals9*^{-/-} mice that receive high CT doses.

Notably, Sharma et al²⁷ reported that *Lgals9*^{-/-} mice exhibited higher virus-specific serum IgG and lower virus-specific IgA titers in bronchoalveolar lavage fluid, implying that galectin-9 has different roles in humoral immunity between systemic and mucosal immune systems. Our results indicating impairment of the antigen-specific IgA response confined to the gut mucosa further support this observation. The reasons why this phenotype is specifically restricted to the gut mucosal system may originate from the differences in the characteristics of immune systems. In contrast to peripheral lymph nodes, most of the B cells undergoing class switching recombination convert to IgA-secreting plasma cells in PP and iLP.^{9–11} Of more importance, Th2-cell-dominant microenvironment, abundance of Foxp3⁺ T cells, and IgA immune

compartmentalization uniquely exist in the gut mucosa but not in the peripheral lymph nodes.^{9–11}

One striking finding of our study was that the lack of galectin-9 attenuated antigen-specific IgA response *in vivo*, but *Lgals9*^{-/-} B cells exhibited higher IgA generation *in vitro* (Supplemental Figure S2). This contrasting result may be explained because *in vitro* culture conditions do not correspond to the complex circumstances observed in living organisms.⁴⁷ In addition, this discrepancy may be attributed to the differences in the unique combination of molecules and spatial organization between *in vitro* and *in vivo* studies.⁴⁷ Another interesting discovery was that lack of galectin-9 enhanced the inhibitory effect of TGF- β on B-cell proliferation. Beyond the scope of this study, it remains undetermined whether this phenomenon can be observed in non-B cells. It is also worthy to investigate how TGF- β interacts with galectin-9 to affect B-cell proliferation.

This study highlights the role of galectin-9 in coordinating mucosal immunity, which is important for developing strategies for mucosal vaccination. CT is widely known as an effective adjuvant owing to its ability to yield a potent IgA immune response through the oral route^{24,48} and to trigger a robust Th17 response *in vivo* and *in vitro*.^{48,49} Because these results revealed that CT fails to produce sufficient antigen-specific IgA production and Th17 response in the condition of galectin-9 deficiency, manipulating galectin-9 may alter intestinal mucosal immune response to CT and, most importantly, tailor the efficiency of adjuvant and vaccine delivery systems.⁴⁸ Further studies that use conditional knockout mice are warranted to confirm our observations and to dissect the mechanism.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.01.017>.

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