

TLR9 activation induces aberrant IgA glycosylation *via* APRIL- and IL-6-mediated pathways in IgA nephropathy

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Running title

APRIL and IL-6 mediated aberrant IgA glycosylation

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Abstract

Galactose-deficient IgA1 (Gd-IgA1) plays a crucial role in the development of IgA nephropathy (IgAN). However, the pathogenic mechanisms driving Gd-IgA1 production have not been fully elucidated. Innate-immune activation *via* Toll-like receptor 9 (TLR9) is known to be involved in Gd-IgA1 production. A proliferation-inducing ligand (APRIL) and IL-6 are also known to enhance Gd-IgA1 synthesis in IgAN. Here, we investigated how TLR9 activation in IgA-secreting cells results in overproduction of nephritogenic IgA in the IgAN-prone ddY mouse model and in human IgA1-secreting cells. Injection of CpG-ODN (TLR9 ligand) increased production of aberrantly glycosylated IgA and IgG-IgA immune complexes (IC) in ddY mice that, in turn, exacerbated kidney injury. CpG-ODN-stimulated mice had elevated serum levels of APRIL that correlated with those of aberrantly glycosylated IgA and IgG-IgA IC. *In vitro*, TLR9 activation enhanced production of the nephritogenic IgA as well as APRIL and IL-6 in splenocytes of ddY mice and in human IgA1-secreting cells. However, siRNA knock-down of *APRIL* completely suppressed overproduction of Gd-IgA1 induced by IL-6. Neutralization of IL-6 reduced CpG-ODN-induced overproduction of Gd-IgA1.

Furthermore, APRIL and IL-6 pathways each independently mediated TLR9-induced overproduction of Gd-IgA1. In summary, TLR9 activation enhanced synthesis of aberrantly glycosylated IgA that, in a mouse model of IgAN, further enhanced kidney injury. These findings indicate that APRIL and IL-6 synergistically, as well as independently, enhance synthesis of Gd-IgA1.

Key words: IgA nephropathy, APRIL, IL-6, galactose deficient IgA1, immune complex

Translational Statement

Disease-specific therapy targeting galactose-deficient IgA1 can constitute a future treatment of IgA nephropathy. Results from this study, that demonstrated the involvement of APRIL and IL-6 in overproduction of aberrantly glycosylated IgA, support the rationale for targeting APRIL and IL-6 in IgA nephropathy. In fact, a recent study demonstrated positive effects of anti-APRIL monoclonal antibody in murine IgAN,¹ and a clinical study with neutralizing APRIL antibody is currently ongoing in patients with IgAN.

Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis,² and causes end-stage renal disease (ESRD) in 20–40% of patients within 20 years after onset of this disease.³ Although the pathogenesis of this disease remains to be fully elucidated, episodic macroscopic hematuria concurrent with an infection of upper-respiratory tract suggests that the mucosal immune system plays an important role in the clinical manifestations of IgAN.⁴

Aberrant glycosylation of IgA is central in the pathogenesis of IgAN. Glomerular IgA in IgAN patients is restricted to the IgA1 subclass and is enriched for molecules that have some hinge-region O-glycans deficient in galactose (galactose-deficient IgA1; Gd-IgA1).^{5,6} Furthermore, serum levels of Gd-IgA1 and IgA1-containing immune complexes (IC) with Gd-IgA1-specific autoantibodies are elevated in patients with IgAN.⁷⁻⁹ However, the mechanisms for production of Gd-IgA1 are still not fully understood.

We used the IgAN-prone ddY mouse model in this study. ddY mice develop glomerular injuries mimicking those of human IgAN.¹⁰ Although mice do not have IgA molecules with O-glycans typical for human IgA1, human IgAN and

this IgAN-prone ddY mouse model share some disease-specific features, such as serum elevation of aberrantly glycosylated IgA and IgG-IgA IC.^{6,11} In fact, aberrant IgA glycosylation due to deficiency of β 1,4-galactosylation of *N*-glycans induces murine IgAN due to elevation of serum levels of high-molecular-weight IgA and IgA-containing IC with their subsequent renal deposition and glomerular injury.¹² ddY mice show proteinuria and a mesangial proliferative glomerulonephritis with co-deposition of IgA and C3 in the glomeruli. A genome-wide association study (GWAS) identified candidate loci linked with progression of IgAN in ddY mouse.¹³ The loci include genes functionally similar to those associated with human IgAN.^{14,15} These findings suggest that IgAN in ddY mice and in humans may be affected at least in part by the same susceptibility genes. Although the molecular features of murine IgA and human IgA1 differ, aberrantly glycosylated IgA tends to form polymeric IgA and IgG-IgA IC that subsequently drive progression of renal injury in the ddY murine model of IgAN as well as in human IgAN. Thus, the ddY mice model has some common features with human IgAN and can be a useful tool for analysis of various pathogenetic features of IgAN.

Toll like receptors (TLRs) are key molecules in the innate immune system and have been implicated in the pathogenesis of IgAN.¹⁶⁻²⁰ Exogenous antigens derived from pathogens activate the TLR9-MyD88 signaling pathway.²¹ This process leads to significantly increased synthesis of inflammatory cytokines, such as type 1 IFN and IL-6.²² Recently, we demonstrated that TLR9 activation aggravated kidney injury in IgAN-prone ddY mice, with elevation of serum levels of IgA and IgG-IgA IC.¹⁶ Moreover, specific *TLR9* polymorphisms associate with disease progression in patients with IgAN.¹⁶

Serum levels of TNF- α and IL-6 are elevated in patients with IgAN.²³ Moreover, IL-6 and IL-4 increase production of IgA1 as well as accentuate the degree of galactose deficiency, increasing Gd-IgA1 synthesis by IgA1-secreting cell lines from IgAN patients.²⁴ These findings suggest that IL-6 may be a key mediator of this process.^{24,25}

The gene of tumor necrosis factor ligand superfamily member 13 (*TNFSF13*) encodes a proliferation inducing ligand (APRIL) that is a central cytokine for the maturation and survival of B cells.²⁶ APRIL shares some signaling receptors important for B cell development with another TNF super family ligand,

B cell activating factor (BAFF). BAFF is also involved in affinity maturation of B cells. A GWAS identified *TNFSF13* as one of the candidate genes associated with IgAN.²⁷ Indeed, in patients with IgAN, serum levels of APRIL are elevated²⁸ and the levels are associated with renal prognosis.²⁸

To gain a better understanding of the underlying mechanisms involved in the overproduction of aberrantly glycosylated IgA *via* TLR9 activation by the ligand, CpG oligonucleotides (ODN), we used IgAN-prone ddY mice and human IgA1-secreting cells.

Results

Activation of TLR9 aggravated renal injury in ddY mice *via* increased production of nephritogenic IgA

TLR9 can be activated by the corresponding ligands, such as CpG-ODN. To test the effect of TLR9 activation in a murine model of IgAN, we used injection of the ligand in IgAN-prone ddY mice. CpG-ODN-injected mice developed mesangial proliferation and extracellular matrix expansion (Figure 1A). Renal histological scores based on mesangial proliferation and mesangial matrix expansion in CpG-ODN-injected mice were significantly higher than those in control mice ($P<0.01$) (Figure 1A). Urinary albumin levels in the CpG-ODN-injected mice were significantly elevated compared with those of the control mice ($P<0.01$) (Figure 1A). Only the CpG-ODN-injected mice developed mesangial deposits of IgA, IgG, and complement C3 (Figure 1B). CpG-ODN-injected mice showed significantly higher serum levels of IgA ($P<0.01$) and IgG ($P<0.01$) than control mice (Figure 1C). Serum IgA from CpG-ODN-injected mice had lower reactivity with RCA-I and SNA lectins than that from control mice (Figure 1C), indicating a lower content of galactose and sialic acid on *N*-glycans of murine IgA.

Furthermore, serum level of IgG-IgA IC in CpG-ODN-injected mice was significantly higher than that in control mice ($P<0.05$) (Figure 1C). These findings indicated that TLR9 activation by CpG ODN resulted in the overproduction of aberrantly glycosylated IgA and IgG-IgA IC, leading to immune-complex deposition and renal injury.

B cells and dendritic cells were involved in the CpG-ODN responses

We investigated which cell types are involved in the induction of overproduction of aberrantly glycosylated IgA in response to CpG-ODN. B cells and dendritic cells (DCs) were isolated from the spleens of ddY mice using anti-mouse CD19 and CD11c antibodies by FACS, respectively. CpG-ODN stimulation of isolated B cells induced overproduction of IgA. In addition, CpG-ODN significantly increased IgA production when B cells were co-cultured with DCs (Supplementary Figure). These results suggested that CpG-ODN directly activated B cells, and DCs further enhanced IgA production upon TLR9 activation.

Overproduction of APRIL enhanced synthesis of aberrantly glycosylated

IgA and IgG-IgA IC

We assessed whether APRIL and/or BAFF was involved in the synthesis of nephritogenic IgA induced by TLR9 activation. Injection of CpG-ODN in ddY mice significantly elevated serum levels of APRIL and BAFF (Figure 2A). Expression levels of *APRIL* in splenocytes correlated with production of aberrantly glycosylated IgA and formation of IgG-IgA IC (Figure 2B). Expression levels of *BAFF* in splenocytes correlated with production of aberrantly glycosylated IgA, but not with formation of IgG-IgA IC (Figure 2B). Furthermore, serum levels of APRIL but not BAFF significantly correlated with elevated serum levels of aberrantly glycosylated IgA and IgG-IgA IC in the CpG-ODN-injected mice (Figure 2C). These findings suggest that both APRIL and BAFF may be involved in production of aberrantly glycosylated IgA, although APRIL may be more involved in the production of IgG-IgA IC.

IL-6 induced production of aberrantly glycosylated IgA

Serum levels of IL-6 in CpG-ODN-injected mice were significantly higher compared with control mice ($P < 0.05$) (Figure 3A). Using splenocytes of IgAN-

prone ddY mice, we examined whether TLR9-induced cellular activation and overproduction of aberrantly glycosylated IgA was mediated by IL-6. CpG-ODN stimulation enhanced production of IL-6 by splenocytes *in vitro*. Addition of IL-6 to the culture medium of splenocytes increased IgA production. Moreover, IL-6 enhanced synthesis of aberrantly glycosylated IgA and formation of IgG-IgA IC, and increased APRIL production (Figure 3A). IL-6-neutralizing antibody reduced CpG-ODN-induced total IgA, aberrantly glycosylated IgA production, and IgG-IgA IC formation (Figure 3B). These findings indicate that TLR9 activation was mediated, at least in part, by IL-6. Furthermore, there may be another TLR9-mediated pathway(s) involved in production of aberrantly glycosylated IgA.

APRIL and IL-6 enhanced synthesis of Gd-IgA1 in human IgA1-secreting cells

We further tested whether APRIL and IL-6 are common major mediators that enhance production of nephritogenic IgA in human IgA1-secreting cells. CpG-ODN supplementation enhanced increase of IL-6, expression of *APRIL*, production of APRIL protein, and production of IgA and Gd-IgA1 (Figure 4A).

Stimulation with IL-6 as well as with APRIL enhanced production of IgA and Gd-IgA1 (Figure 4B, C). The effects of CpG-ODN stimulation on production of IgA and Gd-IgA1 were partly reduced by IL-6-neutralizing antibody (Figure 4D). Moreover, *APRIL* siRNA knock-down also reduced production of IgA and Gd-IgA1 induced by IL-6 stimulation (Figure 4B), indicating that APRIL and IL-6 synergistically promote the generation of Gd-IgA1.

Discussion

Mesangial IgA in human IgAN is exclusively of the IgA1 subclass that displays abnormal O-glycosylation.^{29,30} Human IgA1 has a hinge region with O-glycans that is absent from murine IgA.^{6,31,32} However, aberrant glycosylation of N-glycans is involved in the pathogenesis of a murine model of IgAN in ddY mice, with elevation of aberrantly glycosylated IgA and IgA-containing IC.^{11,12} Aberrant modifications of IgA carbohydrates in either O- or N-glycans are characteristic properties of nephritogenic IgA-containing IC.

Several studies have shown TLR9 activation in progression of IgAN.¹⁷⁻¹⁹

In the present study, we have shown that activation of TLR9 by intra-peritoneal injection of CpG-ODN in ddY mice aggravated renal injuries accompanied by glomerular IgA and IgG deposits, likely due to increased serum levels of aberrantly glycosylated IgA and IgG-IgA IC. Moreover, we showed that TLR9 activation was involved in synthesis of Gd-IgA1 in human IgA1-secreting cell lines.

We assessed the mechanism by which TLR9 activation mediated enhanced production of aberrantly glycosylated IgA. BAFF and APRIL are known for their roles in B-cell survival and differentiation. McCarthy *et al.* demonstrated

that BAFF-overexpressing transgenic mice develop mesangial IgA deposits with renal injury and urinary abnormalities.³³ Recently, we demonstrated that gene expression of *APRIL* is elevated in tonsillar germinal centers of patients with IgAN. Furthermore, overexpression of *APRIL* in tonsillar germinal centers correlated with serum levels of Gd-IgA1 and disease severity in patients with IgAN.³⁴ These findings suggest that both BAFF and APRIL may be involved in the pathogenesis of IgAN. However, the association between BAFF/APRIL and TLR9 activation is unclear. In the present study, TLR9 activation increased gene expression and serum levels of APRIL. Serum levels of aberrantly glycosylated IgA correlated with expression levels of *BAFF* and *APRIL* in splenocytes. However, serum levels of IgG-IgA IC correlated with expression levels of *APRIL* but not *BAFF*. Furthermore, serum levels of APRIL were associated with overproduction of aberrantly glycosylated IgA and IgG-IgA IC in the TLR9-activated mice. In IgA1-secreting cells, TLR9 activation also induced overproduction of Gd-IgA1 *via* APRIL. These results suggested that APRIL plays an important role in TLR9-induced overproduction of nephritogenic IgA and formation of IgG-IgA IC.

The response by cell type to determine the role of the B and T cells and

dendritic cells (DCs) should be clarified. In the present study, we assessed the role of B cells and plasmacytoid DCs. TLR9 is expressed by DCs, B cells, and macrophages, but not by T cells.³⁵⁻³⁷ The contribution of BAFF/APRIL to T cell-independent antibody response is known.^{38,39} Here, we tested whether TLR9 activation can enhance synthesis of aberrantly glycosylated IgA and formation of IgG-IgA IC *via* APRIL in a T cell-independent manner. We also used cloned human IgA1-secreting cell lines, and showed that TLR9 activation augmented production of Gd-IgA1 *via* APRIL and IL-6, suggesting the process is T cell-independent. Furthermore, we evaluated the roles of B cells and DCs. We showed that CpG-ODN specific for B cells and DCs increased production of aberrantly glycosylated IgA in whole splenocytes. Furthermore, we investigated whether CpG-ODN stimulation *in vitro* can enhance IgA production. CpG-ODN stimulation of isolated B cells induced overproduction of IgA. In addition, CpG-ODN significantly increased IgA production when B cells were co-cultured with DCs (Supplementary Figure S1). These results suggest that CpG-ODN activation resulting in greater IgA production and aberrant glycosylation is mediated by TLR9 in a T cell-independent manner.

IL-6 is known to accentuate proliferation of immunoglobulin-producing cells and the terminal differentiation of plasma cells.⁴⁰⁻⁴² Furthermore, IL-6 may also be a primary-candidate cytokine for T follicular helper (Tfh) cell differentiation.^{43,44} Tfh cells are essential for B cell affinity maturation, class switch recombination, and memory B cell and plasma cell generation within the germinal center.^{43,44} In a lupus mouse model, IL-6 deficiency prevented Tfh cell differentiation and germinal-center B cell expansion, resulting in reduced autoantibody production.⁴⁵ The present study clarified that TLR9 activation by CpG-ODN enhanced IL-6 production in splenocytes of murine IgAN. IL-6 induced overproduction of aberrantly glycosylated IgA and IgG-IgA IC in a murine model of IgAN. In human IgA1-secreting cells, we confirmed that IL-6 enhanced production of Gd-IgA1.²⁴ Our findings suggest that IL-6 may be one of the major molecules inducing overproduction of nephritogenic IgA mediated by TLR9 activation.

APRIL plays an important role in IgA-class switching and plasmacytoid differentiation.^{46,47} IL-6 promotes the terminal differentiation of B cells to IgA-secreting plasma cells.^{41,42} The present study demonstrated that IL-6 stimulation

induced production of APRIL. Then, we hypothesized there was an association between APRIL and IL-6 mediated by TLR9 activation inducing production of aberrantly glycosylated IgA. We found that IL-6-neutralizing antibody did not completely block CpG-ODN-induced overproduction of Gd-IgA1, and siRNA knock-down of *APRIL* reduced overproduction of Gd-IgA1 induced by IL-6. These data suggested that APRIL and IL-6 synergistically promoted the production of Gd-IgA1 mediated by TLR9 activation. Furthermore, both IL-6-neutralizing antibody and siRNA knock-down of *APRIL* reduced production of IgA and Gd-IgA1 more than did IL-6-neutralizing antibody alone. Thus, the TLR9 activation pathway involves IL-6 and APRIL synergistic effect on antibody production and glycosylation (Figure 5). Recent studies have also indicated that IL-6, in combination with APRIL, induced generation and survival of PCs.^{48,49} However, future studies are required to clarify intercellular signaling pathways between APRIL and IL-6 in the production of nephritogenic IgA.

In conclusion, TLR9 activation exacerbated renal injury in a murine model of IgAN by enhancing production of aberrantly glycosylated IgA and formation of IgG-IgA IC. In this process, the overproduction of APRIL and IL-6 induced by

TLR9 activation enhanced production nephritogenic IgA. In addition, the present study clarified that APRIL and IL-6 function synergistically, as well as independently, to produce aberrantly glycosylated IgA. These findings may inform future development of new therapeutic approaches for IgAN.

Materials and Methods

Animals and experimental protocols

The IgAN-prone ddY mouse is a known model of spontaneous IgAN with variable incidence and extent of glomerular injury mimicking human IgAN.¹⁰ ddY mice are divided into three groups based on manifestation of kidney damage: mice with early or late onset and quiescent mice. Early-onset disease in ddY mice presents with proteinuria and IgA deposits in glomerular after 20 weeks of age.¹³ Therefore, we used ddY mice within 18 weeks of age to evaluate whether TLR9 activation aggravated IgAN in this model. A total of 30 female ddY mice (SLC Japan, Shizuoka, Japan) were maintained at the animal facility of Juntendo University, Tokyo, Japan. Mice were fed regular chow (Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum* and were housed in a specific pathogen-free room. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University Faculty of Medicine. At 6 weeks of age the mice were randomly divided into two groups. One group received CpG-ODN injected intra-peritoneal three times per week for 12 weeks; the second group (control) was injected with non-CpG-ODN. CpG-ODN and non-

CpG-ODN were chemically synthesized (Invitrogen; Thermo Fisher Scientific, Kanagawa, Japan). Sequences of the ODN are TCGTCGTTTTCGGCGCGCGCCG or TGCTGCTTTTGGGGGGCCCCC (5'→3') for CpG or non-CpG ODNs, respectively.

Determination of IgA, IgG, IgG-IgA IC, and aberrantly glycosylated IgA in murine serum

Blood samples were collected from the buccal vein. Serum IgA, IgG, and IgG-IgA IC were measured by sandwich ELISA (Bethyl Laboratories, Montgomery, TX), using the modified method based on our previous report.⁵⁰ Lectin-binding assays were used to access the glycoform of IgA. Biotinylated *Ricinus communis* agglutinin I (RCA-I; Vector Laboratories, Burlingame, CA) and *Sambucus nigra* bark lectin (SNA; Vector Laboratories) that recognized galactose and sialic acid attached to galactose (predominantly in α 2,6 linkage), respectively, were used in this assay.⁵¹ Diluted sera were added at 100 ng of IgA per well. Microtiter plates coated with 1 mg/ml goat anti-mouse IgA (100 μ l/well) for quantification of serum IgA were incubated with these samples, and biotinylated SNA or RCA-I was then

added. Avidin–horseradish peroxidase conjugate (ExtrAvidin; Sigma-Aldrich, St. Louis, MO) was applied. Serum levels of aberrantly glycosylated bound IgA were expressed in Units (1 unit as OD 1.0 measured at 490 nm). Serum level of APRIL and BAFF were measured using mouse APRIL ELISA kit (MyBioSource, San Diego, CA) and mouse BAFF ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocols.

Assay for Gd-IgA1 produced by human IgA1-secreting cells

A sandwich ELISA for Gd-IgA1 was constructed using Gd-IgA1-specific antibody (IBL, Japan).⁵² Gd-IgA1-specific antibody (7.5 µg/mL) diluted in PBS was coated onto an ELISA plate for 18 h at room temperature. Samples were applied and incubated for 2 h at room temperature. Plates were washed and incubated for 2 h at room temperature with a 1:1,000-diluted HRP-conjugated mouse anti-human IgA1 α1-chain-specific monoclonal antibody (Southern Biotech, Birmingham, AL). After washing, plates were developed with SIGMAFAST o-phenylenediamine (OPD) solution (Sigma-Aldrich) and the reaction was stopped by addition of 1 M sulfuric acid (Wako, Osaka, Japan). The

levels of Gd-IgA1 were extrapolated by referring to a standard curve (4-parameter logistic curve fitting) of OD at 490 nm and expressed in units. Enzymatically generated Gd-IgA1 from human plasma IgA1⁵² was serially diluted (1.37–1,000 ng/mL) to generate a standard curve. In this study, 1 unit was defined as 1 ng/mL of the standard Gd-IgA1.⁵²

Evaluation of renal injury in ddY mice

Kidneys were removed after perfusion with normal saline. Renal tissue specimens for microscopic examination were fixed in 20% formaldehyde, embedded in paraffin, cut into 3- μ m-thick sections, and then stained with PAS. Specimens were quantitatively analyzed to determine the percentage of glomeruli with segmental and global sclerosis and/or mesangial cell proliferation and/or increase in mesangial matrix. Each section was scored semi-quantitatively for percentages of glomeruli with aforementioned lesions (0, 0%; 1, 1 to 24%; 2, 25 to 49%; and 3, >50% of 20 glomeruli).^{13,53,54} Total maximal score for each section was 9. Renal specimens for immunofluorescence were immersed in OCT compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan) and stored at -80°C.

The specimens were cut into 3- μ m thick sections, fixed with acetone at -20°C for 5 min, washed with PBS, blocked with a blocking agent (DS Pharma Biomedical Co. Ltd., Osaka, Japan) at room temperature for 30 min, and then incubated at room temperature for 2 h with the following primary antibodies: goat anti-murine IgA, Alexa conjugated goat anti-murine IgG and rat-anti-C3 (Bethyl Laboratories). After three more washes with PBS, the slides were mounted with mounting medium (Dako, Tokyo, Japan). Samples were analyzed and imaged using confocal laser microscopy (Olympus Corporation, Tokyo, Japan).

Real-Time Quantitative RT-PCR

RNA from spleen cells was extracted using Torizol solution (Invitrogen, Tokyo, Japan) and purified with RNeasy Mini Kit (74106; Qiagen, Valencia, CA). Real-time RT-PCR was performed using the Applied Biosystems 7500 Real-Time PCR System using SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan) and specific primers: mouse TLR9 forward primer TCTCCCAACATGGTTCTCCGTCG, reverse primer TGCAGTCCAGGCCATGA; mouse MyD88 forward primer

GCACCTGTGTCTGGTCCATT, reverse primer CTGTTGGACACCTGGAGACA; and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer CATTGTGGAAGGGCTCATGA, reverse primer TCTTCTGGGTGGCAGTGATG. The amplification conditions were as follows: preheating at 95°C for 20 s and then 40 cycles of denaturation at 95°C for 3 s, and annealing and extension at 60°C for 30 s. *Homo sapiens*-specific TaqMan gene expression assays (Life Technologies, Carlsbad, CA) were purchased for the primer of *APRIL*, Hs00601664_g1 and Mm03809849-s1, and *BAFF*, Mm01168134-m1. The TaqMan PCR cycling conditions were as follows: preheating at 95°C for 20 s and then 40 cycles of denaturation at 95°C for 3 s, and annealing and extension at 60°C for 30 s.

***In vitro* studies with splenocytes of ddY mice**

Splenocytes were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum, 100 µg/ml streptomycin, and 100 U/ml penicillin. Red blood cells were lysed in Red Blood Cell Lysing Buffer (R7757; Sigma-Aldrich) at room temperature for 1 min, followed by washing in RPMI-1640 medium.⁵⁰ Cell cultures

were kept in a humidified incubator at 37°C with 5% CO₂. Class C CpG-ODN (TCGTCGTTTTCGGCGCGCGCCG) was used as the TLR9 ligand. Recombinant IL-6, anti-IL-6 antibody, and rat IgG1 isotype control were obtained from R&D Systems. Cells were cultured with either medium or recommended concentrations of CpG-ODN, 10 ng/ml recombinant IL-6, and 100 ng/ml anti-IL6 antibody. IgA, aberrantly glycosylated IgA, IgG-IgA IC and APRIL produced by splenocytes were measured by ELISA after 72 h incubation *in vitro*.

***In vitro* studies with human IgA1-secreting cell lines**

B cells from the blood of a healthy person were immortalized with Epstein-Barr virus, subcloned to yield IgA1-secreting cell lines, and cultured as described.⁹ Furthermore, we confirmed expression of receptors for APRIL/BAFF and IL-6. Cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum, streptomycin and penicillin in a humidified incubator at 37°C with 5% CO₂. Class B CpG-ODN (TCGTCGTTTTGTCGTTTTGTCGTT) was used as the TLR9 ligand. Recombinant IL-6, recombinant APRIL, anti-IL-6 antibody and goat IgG control antibody were obtained from R&D Systems. Cells were cultured with

either medium or recommended concentrations of CpG-ODN, 50 ng/ml recombinant IL-6, 40 ng/ml recombinant APRIL, and 100 ng/ml anti-IL-6 antibody for 72 h.

Western blotting

Supernatants from IgA1-secreting cells were separated by SDS-PAGE under reducing conditions using 5%-15% gradient slab gels. The gels were transferred to PVDF membranes and incubated with antibodies specific for APRIL (Abcam Inc., Cambridge, MA) and GAPDH (Abcam Inc). Blots were visualized by chemiluminescence using ECL (GE Healthcare, Tokyo, Japan) and luminescence detector (Konica Minolta Healthcare, Tokyo, Japan). Results were evaluated densitometrically.

***APRIL* siRNA knock-down**

Human IgA1-secreting cells were cultured in 24-well culture plates and transfected with *APRIL*-specific siRNA (GS8741, Qiagen) or siRNA non-targeting control (GS10673, Qiagen) using the transfection protocol according to the

manufacturer's instructions. After 24 h incubation, the cells were incubated with 50 ng/ml recombinant IL-6 (R&D Systems) for 72 h. After incubation, cells and supernatants were harvested for measurement of IgA and Gd-IgA1.

Statistical Analysis

The correlation between different parameters was analyzed using analysis of variance. Data were expressed as the mean \pm standard deviation or median values. *P* values <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA).

Disclosure

All authors declared no competing interests.

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Supplementary material

<Supplementary figure legend>

CpG-ODN-activated B cells and DCs further enhance IgA production.

B cells and DCs were isolated from the spleens of ddY mice. CpG-ODN stimulation increased IgA production in the isolated B cells. This increase was further enhanced by co-culturing B cells with DCs. * $P < 0.05$.

<Supplementary Methods>

Cell sorting

Spleen cells of ddY mice were stained for 30 min at 4°C with the following mouse-specific monoclonal antibodies: PE Rat Anti-Mouse CD19 Clone 1D3 (BD Biosciences, Franklin Lakes, NJ) for B cells and APC Hamster Anti-Mouse CD11c Clone HL3 (BD Biosciences) for DCs. Gating was done using Summit software. Cells were sorted using Moflo Astrios EQs cell sorter (Beckman Coulter, CA) and collected directly into 50 ml tubes containing 10 ml of staining medium.

Supplementary information is available at Kidney International's website.

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Figure legends

Figure 1. CpG-ODN stimulation induced production of nephritogenic IgA. Injection with CpG-ODN increased blood levels of aberrantly glycosylated IgA and IgG-IgA IC and exacerbated kidney injury in ddY mice. (A) ddY mice before development of IgAN were intra-peritoneally injected with CpG-ODN thrice weekly for 12 weeks. In histological analysis of glomerular lesions, CpG-ODN-injected mice showed mesangial cellular proliferation and extracellular matrix expansion. Right panel shows evaluation of renal injuries by semi-quantitative scoring. Renal histological scores, evaluated by percentages of glomeruli with aforementioned lesions, showed that renal injury in the mice injected with CpG-ODN were exacerbated. Urinary albumin levels in the CpG-ODN-injected mice were significantly elevated compared with those of control mice. Bars represent the mean \pm SEM. **** P <0.01.** (B) Immunohistochemical analysis of glomerular IgA, IgG, and C3 deposits. Only CpG-ODN-injected mice developed mesangial deposits of IgA, IgG, and C3. (C) Serum levels of IgA, IgG, and IgG-IgA IC were significantly increased after CpG-ODN treatment for 12 weeks. *** P <0.05, **** P <0.01.** Serum IgA in mice injected with CpG-ODN showed significantly lower**

reactivity with lectin RCA-I and SNA lectins than the IgA in control mice. $**P<0.01$.

These results indicate that injection of CpG-ODN increased serum levels of aberrantly glycosylated IgA. Individual points are shown. $*P<0.05$, $**P<0.01$.

Figure 2. Correlation between overproduction of APRIL and aberrantly

glycosylated IgA in mice injected with CpG-ODN. (A) Injection of ddY mice

with CpG-ODN increased serum levels of BAFF and APRIL. Bars represent the

mean \pm SEM. $*P<0.05$. (B) Expression levels of *BAFF* in whole splenocytes

correlated with reduced reactivity with RCA-I lectin, but did not correlate with

formation of IgG-IgA IC. Meanwhile, expression levels of *APRIL* were associated

with production of aberrantly glycosylated IgA and formation of IgG-IgA IC. (C) In

mice injected with CpG-ODN, serum levels of APRIL were associated with

reduced reactivity of IgA with RCA-I lectin and elevated levels of IgG-IgA IC.

Serum levels of BAFF did not correlate with production of glycosylated IgA and

formation of IgG-IgA IC.

Figure 3. Effect of CpG-ODN and IL-6 on cultured splenocytes of ddY mice.

(A) Serum levels of IL-6 in CpG-ODN-injected mice were significantly higher compared with control mice. * $P < 0.05$. CpG-ODN stimulation increased production of IL-6 of splenocytes of ddY mice in culture. Incubation of splenocytes of ddY mice with recombinant IL-6 increased production of total IgA and aberrantly glycosylated IgA, resulting in the formation of IgG-IgA IC. Recombinant IL-6 increased overproduction of APRIL. Bars represent the mean \pm SEM. ** $P < 0.01$. (B) Splenocytes in culture were stimulated with CpG-ODN in the presence or absence of IL-6-neutralizing antibody. Anti-IL-6 antibody reduced production of total IgA and aberrantly glycosylated IgA, and, consequently, reduced formation of IgG-IgA IC induced by CpG-ODN. ** $P < 0.01$. PBS, Phosphate-buffered saline.

Figure 4. Effect of CpG-ODN or APRIL supplementation on human IgA1-secreting cell lines on IgA1, Gd-IgA1, and IL-6 production. (A) CpG-ODN supplementation enhanced production of IL-6. CpG-ODN enhanced gene expression of *APRIL*. Western-blot analysis demonstrated that CpG-ODN increased protein levels of APRIL. Results were evaluated densitometrically.

Stimulation with CpG-ODN induced production of IgA and Gd-IgA1. Bars represent the mean \pm SEM. * P <0.05, ** P <0.01. (B) *APRIL* siRNA knock-down reduced IL-6-induced overproduction of IgA and Gd-IgA1. * P <0.05, ** P <0.01. (C) IgA1-producing cells stimulated with recombinant APRIL produced more IgA and Gd-IgA1. (D) IgA1-secreting cells were incubated with anti-IL-6 antibody after CpG-ODN stimulation. The enhanced production of IgA and Gd-IgA1 induced by CpG-ODN was reduced by anti-IL-6 antibody and by siRNA knock-down of *APRIL*. PBS, Phosphate Buffered Saline.

Figure 5. Synergistic role between TLR9, APRIL, and IL-6 in IgA-producing cells in IgAN. TLR9 activation induced production of APRIL and IL-6, resulting in overproduction of aberrantly glycosylated IgA. APRIL and IL-6 function synergistically to promote the generation of aberrantly glycosylated IgA. In addition to APRIL inducing production of IL-6, as previously reported, IL-6 can also enhance synthesis of APRIL. Furthermore, APRIL and IL-6 independently promote production of aberrantly glycosylated IgA.

Figure 1.

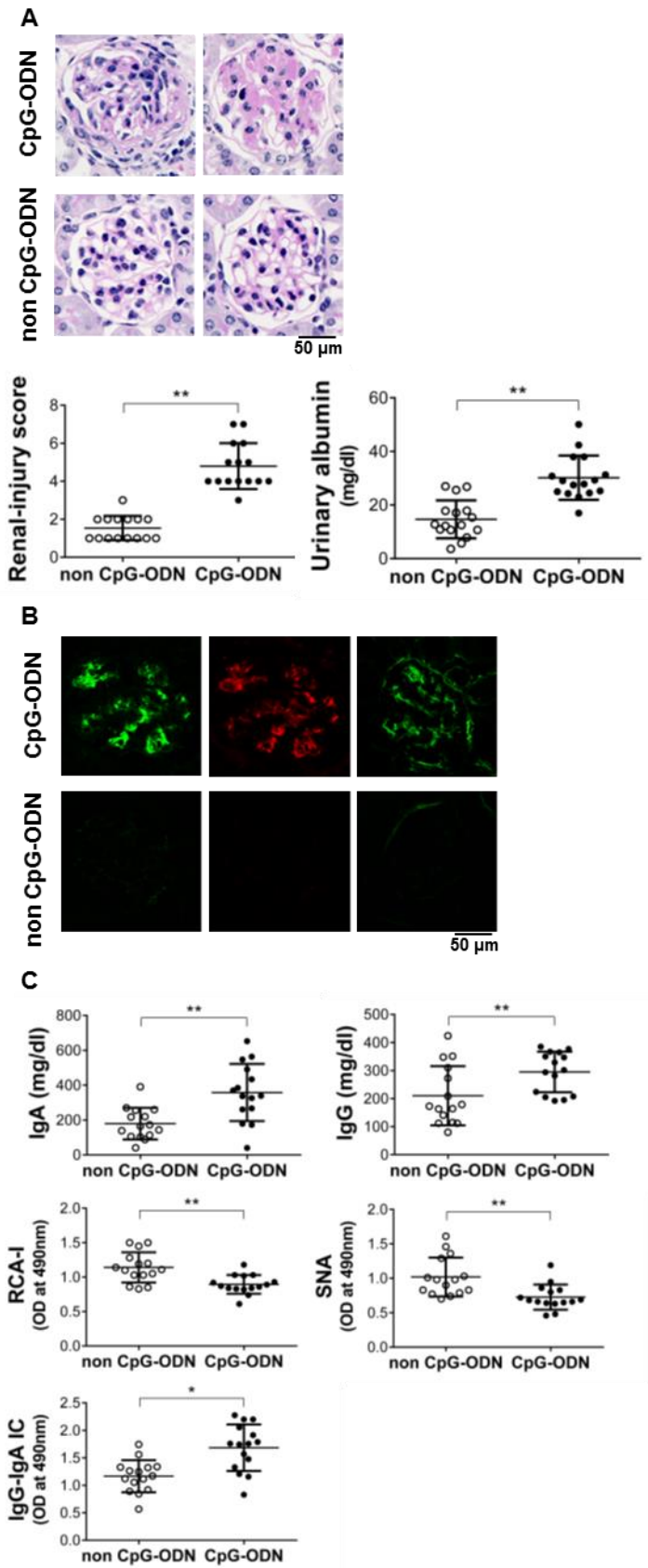


Figure 2.

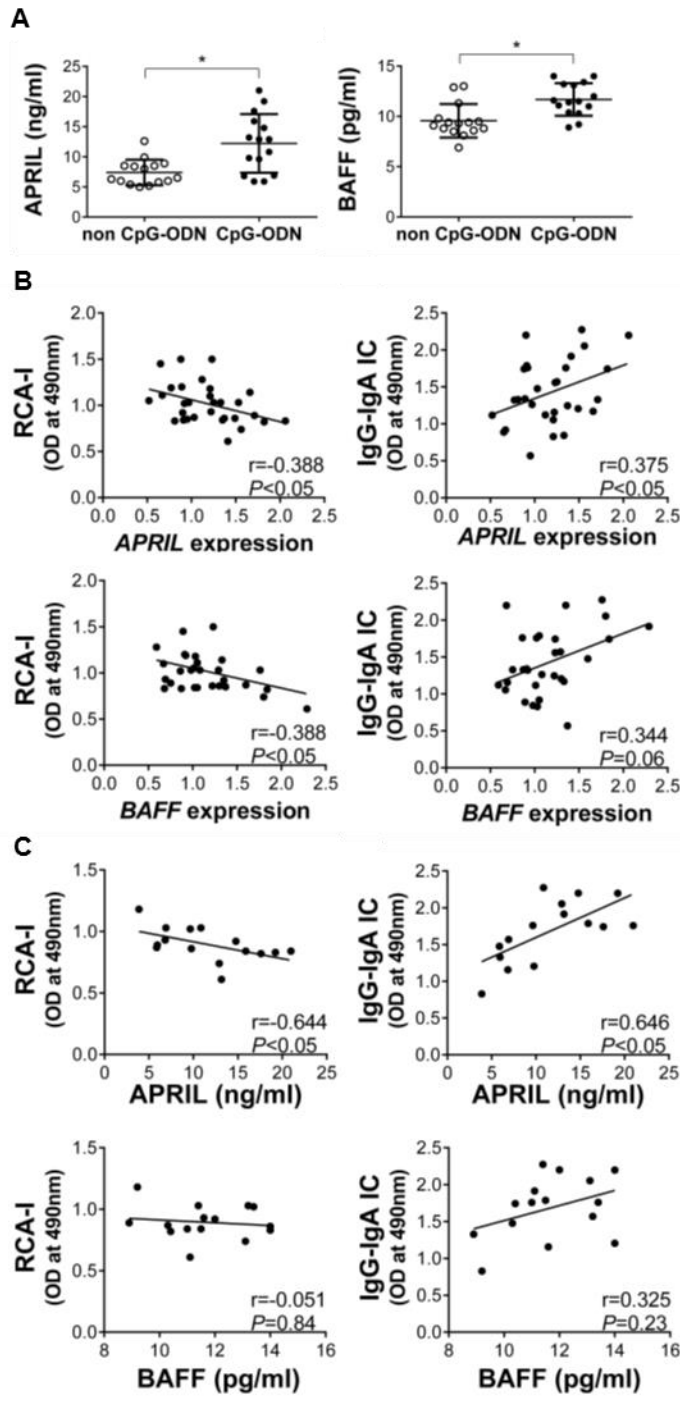
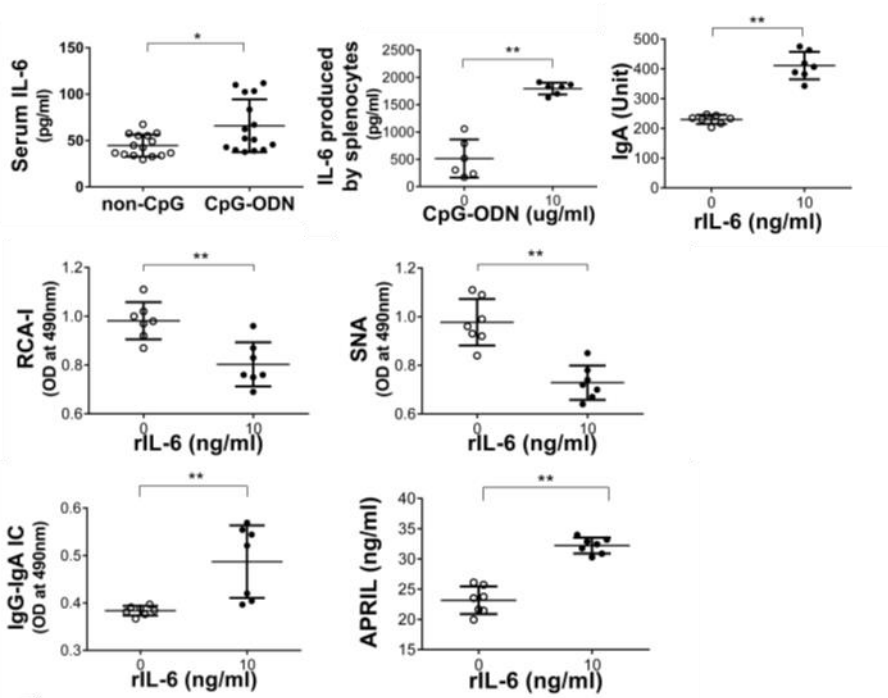


Figure 3.

A



B

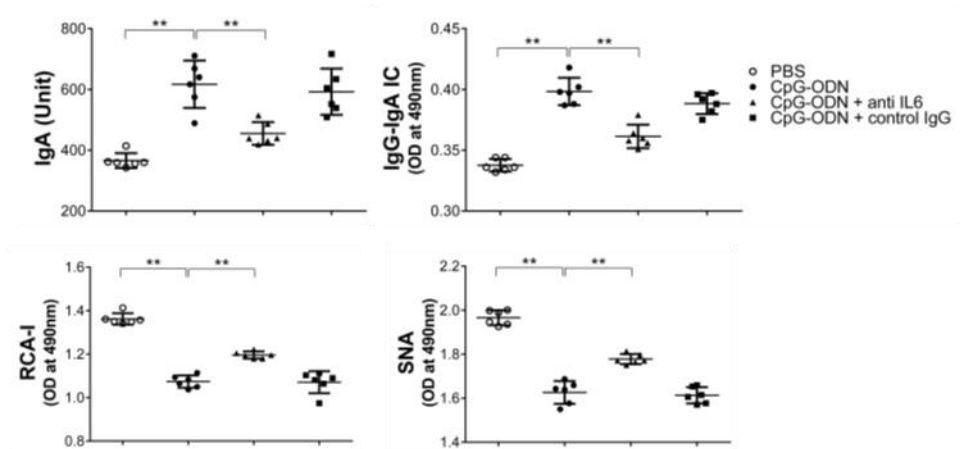
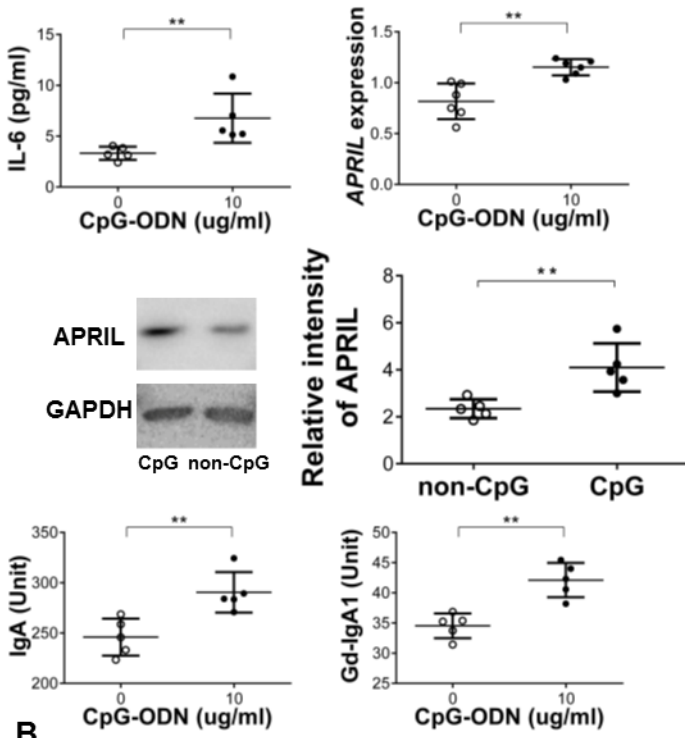
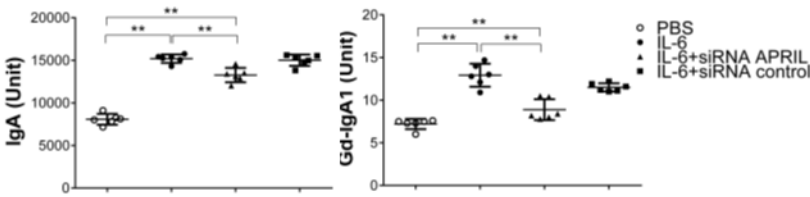


Figure 4.

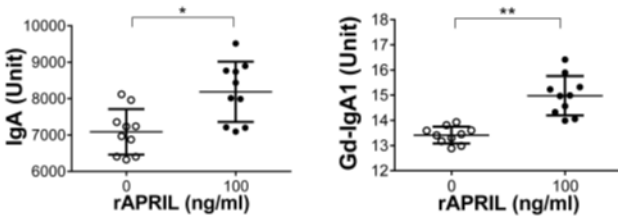
A



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C



D

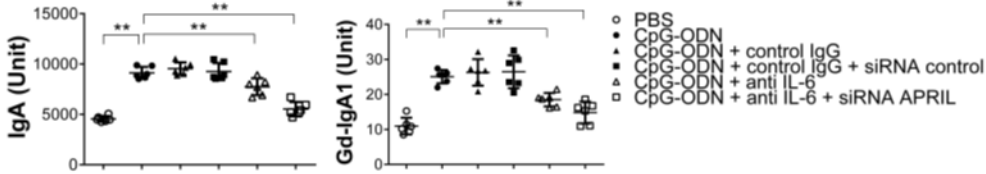
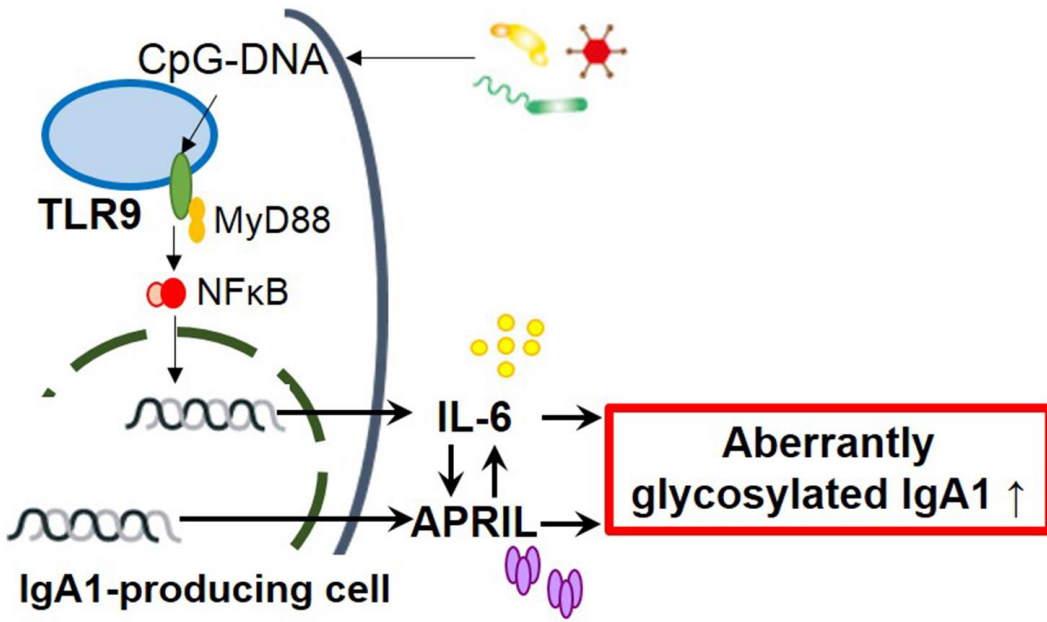
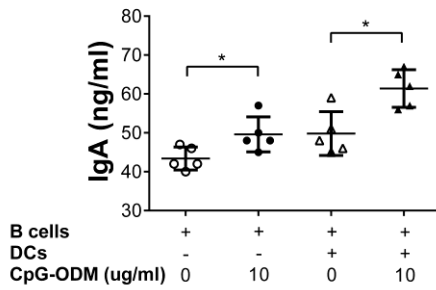


Figure 5.



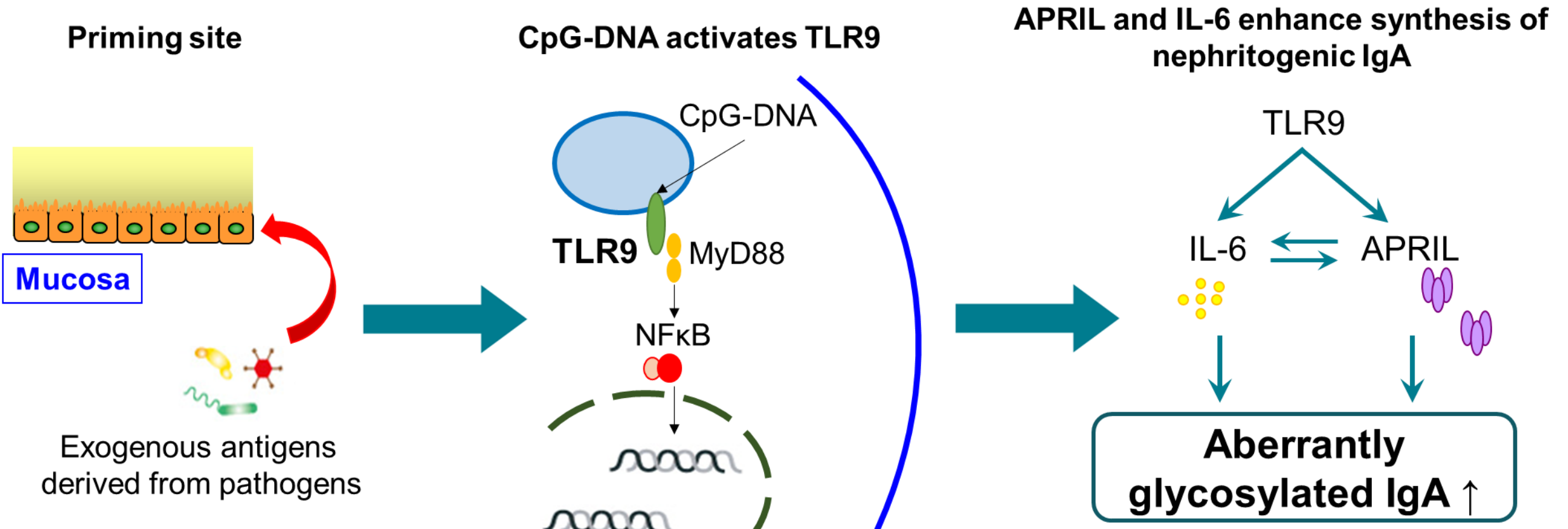
Supplementary Figure.



CpG-ODN-activated B cells and DCs further enhance IgA production.

B cells and DCs were isolated from the spleens of ddY mice. CpG-ODN stimulation increased IgA production in the isolated B cells. This increase was further enhanced by co-culturing B cells with DCs. * $P < 0.05$.

TLR9 activation induces aberrant IgA glycosylation *via* APRIL- and IL-6-mediated pathways in IgA nephropathy



CONCLUSION:
TLR9 activation enhanced synthesis of aberrantly glycosylated IgA resulting in aggravation of kidney injury in murine IgAN. APRIL/IL-6 synergistically induce aberrant glycosylation of IgA.