The nucleotide-sensing Toll-Like Receptor 9/Toll-Like Receptor 7 system is a potential therapeutic target for IgA nephropathy

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Mingfeng Lee[1], Hitoshi Suzuki[1,2], Kei Ogiwara[1], Ryosuke Aoki[1], Rina Kato[1], Maiko Nakayama[1], Yusuke Fukao[1], Yoshihito Nihei[1], Toshiki Kano[1], Yuko Makita[1], Masahiro Muto[1], Koshi Yamada[1] and Yusuke Suzuki[1]

[1]*Department of Nephrology, Juntendo University Faculty of Medicine, Tokyo, Japan; and* [2]*Department of Nephrology, Juntendo University Urayasu Hospital, Chiba, Japan*

The progression determinants of IgA nephropathy (IgAN) are still not fully elucidated. We have previously demonstrated that the mucosal activation of toll-like receptor (TLR) 9, which senses microbial unmethylated CpG DNA, influences progression by producing aberrantly glycosylated IgA. However, numerous recent reports of patients with IgAN presenting with gross hematuria after the mRNA vaccination for coronavirus disease 2019 suggest that the RNA-sensing system also exacerbates IgAN. Here, we investigated whether TLR7, which recognizes microbial RNA, is also involved in IgAN progression using a murine model and tonsil tissue from 53 patients with IgAN compared to samples from 40 patients with chronic tonsillitis and 12 patients with sleep apnea syndrome as controls. We nasally administered imiquimod, the ligand of TLR7, to IgAN-prone ddY mice and found that TLR7 stimulation elevated the serum levels of aberrantly glycosylated IgA and induced glomerular IgA depositions and proteinuria. Co-administered hydroxychloroquine, which inhibits TLRs, canceled the kidney injuries. *In vitro*, stimulating splenocytes from ddY mice with imiquimod increased interleukin-6 and aberrantly glycosylated IgA levels. The expression of TLR7 in the tonsils was elevated in patients with IgAN and positively correlated with that of a proliferation-inducing ligand (APRIL) involved in the production of aberrantly glycosylated IgA. Mechanistically, TLR7 stimulation enhanced the synthesis of aberrantly glycosylated IgA through the modulation of enzymes involved in the glycosylation of IgA. Thus, our findings suggest that nucleotide-sensing TLR9 and TLR7 play a crucial role in the pathogenesis of IgAN. Hence, nucleotide-sensing TLRs could be reasonably strong candidates for disease-specific therapeutic targets in IgAN.

Correspondence: *Hitoshi Suzuki or Yusuke Suzuki, Department of Nephrology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: shitoshi@juntendo.ac.jp or yusuke@juntendo.ac.jp*

Received 21 December 2022; revised 26 July 2023; accepted 7 August 2023; published online 28 August 2023

Kidney International (2023) **104,** 943–955; https://doi.org/10.1016/j.kint.2023.08.013

KEYWORDS: APRIL; hydroxychloroquine; IgA nephropathy; IL-6; TLR7; TLR9

Translational Statement

Currently, there is no established treatment for IgA nephropathy (IgAN), and many patients still progress to kidney failure worldwide. Exploring new therapeutic targets is imperative. Previously, the involvement of toll-like receptor (TLR) 9, which senses DNA, in the pathogenesis of IgAN has been well investigated. However, gross hematuria in patients with IgAN after mRNA vaccination suggests that the RNA-sensing system might be another aggravation trigger. In this study, we revealed that TLR7, which recognizes RNA, is also involved in the progression of IgAN by producing aberrantly glycosylated IgA. Controlling these nucleotide-sensing TLRs might be a reasonable therapeutic strategy for IgAN.

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. Although the disease progression is relatively slow, ~40% of patients progress to kidney failure over 20 years if left untreated. Currently, there is no established disease-specific treatment.

Galactose-deficient IgA1 (Gd-IgA1) is the key effector molecule in the pathogenesis of IgAN.[1] Gross hematuria after upper respiratory tract infections is a hallmark manifestation of IgAN.[2] Mucosal immune dysregulation in nasal-associated lymphoid tissue (NALT), especially the tonsils, is thought to be involved in the production of Gd-IgA1.[2] Anti-glycan antibodies combine with Gd-IgA1 and form IgA1-containing immune complexes (ICs), accumulating in the mesangium and activating mesangial cell proliferation and matrix increase, resulting in glomerular injury. The Gd-IgA1 multihit mechanism is considered the most probable pathogenesis of IgAN.[1]

There is mounting evidence suggesting that the activation of toll-like receptor (TLR) signals is involved in the

pathogenesis of IgAN. TLRs belong to the family of pattern recognition receptors that recognize a wide range of pathogen-associated molecular patterns. TLRs play an essential role in the pathogenesis of various autoimmune diseases.[3] Given that IgAN also has certain characteristics of autoimmune diseases,[4] TLRs are likely important in its pathogenesis.

Our group has reported that the activation of TLR9, which detects microbial unmethylated cytosine-phosphate-guanine (CpG) DNA, induces aberrantly glycosylated IgA via interleukin-6 (IL-6) and a proliferation-inducing ligand (APRIL)–mediated pathways.[5] IL-6 downregulates core 1 β1,3-galactosyltransferase (C1GaLT1) and core 1 β3-Gal-T–specific molecular chaperone, the enzyme and coenzyme involved in the O-glycosylation of IgA1, and promotes the production of Gd-IgA1.[6] APRIL, which belongs to the tumor necrosis factor family, participates in B-cell maturation and mucosal IgA class switch recombination.[7,8] Previously, we reported that single nucleotide polymorphisms in *TLR9* were significantly associated with the pathological severity of IgAN.[9] Furthermore, tonsillar expression of TLR9 and APRIL is well-correlated with the treatment response to tonsillectomy, which is widely performed in some Asian countries.[10–12]

Zheng *et al.* recently reported that TLR7, which recognizes microbial single-stranded RNA, might also promote Gd-IgA1 synthesis and affect disease progression in IgAN.[13] However, the precise role of TLR7 in the pathogenesis of IgAN needs to be further investigated. Both TLR9 and TLR7 belong to the nucleotide-sensing TLR family and are mainly expressed on the endosomes of B cells, dendritic cells, and macrophages.[14]

Several clinical trials recently suggested that hydroxychloroquine (HCQ) might effectively reduce proteinuria in patients with IgAN.[15] HCQ is widely used in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, and its immunomodulatory effects include inflammatory cell suppression, autoantigen presentation inhibition, and the inhibition of TLRs and related cytokines.[16] Although the exact pharmacological mechanism of HCQ in treating IgAN remains unclear, TLR inhibition might play a central role.

Herein, we aimed to verify whether and how TLR7 is involved in the progression of IgAN by using a murine model and mucosal tissue samples from patients with IgAN. Moreover, we tried to examine the potential of nucleotide-sensing TLRs, including TLR9 and TLR7, as therapeutic targets for IgAN.

METHODS

Animals

The ddY mouse is a spontaneous murine model of IgAN.[17] Genetic association studies revealed that IgAN in ddY mice and humans could be affected, at least in part, by the same susceptibility genes.[18,19] Moreover, the cellular transcriptomic signatures in glomeruli are similar between ddY mice and patients with IgAN.[20] IgAN in ddY mice is also considered to follow the multihit pathogenesis.[17] In addition, the primary induction site for nephritogenic IgA in ddY mice is NALT.[21] Usually, ddY mice present with glomerular IgA deposits and proteinuria after 20 weeks of age.[18]

In vivo experimental protocols

We purchased female ddY mice (Japan SLC) and maintained them in a specific pathogen-free condition. At 6 weeks of age, mice were randomly divided into 6 groups of 5 mice that were given the following: (i) control group: drinking water; (ii) HCQ group: HCQ (Selleck Chemicals) at a dose of 30 mg/kg/d in drinking water; (iii) CpG-ODN group: nasally administered class c CpG-oligodeoxynucleotide (ODN: InvivoGen) as a ligand of TLR9 for 50 μg 3 times per week; (iv) CpG-ODN + HCQ group: CpG-ODN + HCQ; (v) imiquimod group: nasally administered imiquimod (InvivoGen) as a ligand of TLR7 for 50 μg 3 times per week; and (vi) imiquimod + HCQ group: imiquimod + HCQ. We killed mice at 10 weeks of age. We set the dose of HCQ as 30 mg/kg/d, a dose in the middle of the range used in previous *in vivo* studies.[22,23] This dose corresponds to ∼5 times the human dose. We preliminarily confirmed that administering 30 mg/kg/d of HCQ for 4 weeks did not affect the murine body weight, lymphocyte counts, and serum immunoglobulin levels (Supplementary Figure S1). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University Faculty of Medicine.

Measurement of IgA, IgG-IgA IC, aberrantly glycosylated IgA, and urine albumin-to-creatinine ratio

The serum levels of IgA and IgG-IgA IC were measured via enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories), and the glycoform of IgA was assessed using lectin-binding assays as per our previous reports.[5,21,24,25] *Ricinus communis* agglutinin I (Vector Laboratories) and *Sambucus nigra* agglutinin (Vector Laboratories), which recognize galactose residues and terminal sialic acid, respectively, were used in these lectin assays. The levels of IgG-IgA IC and aberrantly glycosylated IgA were expressed in optical density at 450 and 490 nm, respectively. Lower optical density with *Ricinus communis* agglutinin I or *Sambucus nigra* agglutinin lectin assays indicates that IgA molecules have lower galactose or sialic acid contents, that is, higher aberrantly glycosylated IgA levels. The degree of urine albumin was assessed as spot urine albumin-to-creatinine ratio. The urinary albumin and creatinine levels were measured using ELISA kits Albuwell M (Exocell) and LabAssay Creatinine (FUJIFILM Wako Chemicals), respectively.

Histological analyses of renal tissues

Renal specimens for microscopic evaluation were stained with periodic acid–Schiff reagent. Specimens were quantitatively analyzed to determine the percentage of glomeruli with segmental and global sclerosis and/or mesangial cell proliferation and/or mesangial matrix increase as per our previous reports.[5,18,21] Each section was scored semiquantitatively for percentages of glomeruli with the lesions mentioned above (0, 0%; 1, 1%–24%; 2, 25%–49%; and 3, >50% of 30 glomeruli). The total points for each section were scored as a renal histological score, and the maximum number of points was 9. Renal specimens for immunofluorescence were stained for IgA, IgG, and C3 with the following antibodies: goat anti-mouse IgA FITC (Bio-Rad), goat anti-mouse IgG TRITC (SouthernBiotech), and rat anti-mouse C3 FITC (Santa Cruz Biotechnology).[5,18,21] Images were taken using the All-in-One Fluorescence Microscope BZ-X710 (KEYENCE). Renal specimens for electron microscopy were

prepared as previously described.[19] Images were taken using the transmission electron microscope HT7700 (Hitachi).

In vitro experiments with the splenocytes of ddY mice

We have previously demonstrated that the splenocytes of ddY mice were useful in analyzing the mechanism of aberrantly glycosylated IgA production *in vitro*.[5] The splenocytes of ddY mice were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 20% fetal calf serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Roswell Park Memorial Institute 1640 medium + fetal calf serum + penicillin + streptomycin). Red blood cells were lysed in a red blood cell lysing buffer (Sigma-Aldrich). Cell cultures were kept at 37 °C with 5% CO_2. Splenocytes were cultured in the medium only or cultured with the stimulation of CpG-ODN (5 μM), imiquimod (5 μg/ml), recombinant IL-6 (r-IL-6; BioLegend; 10 ng/ml), recombinant interferon (IFN)-α (BioLegend; 10 ng/ml), or recombinant IFN-β (BioLegend; 10 ng/ml). We also evaluated whether HCQ, ODN2088 (Miltenyi Biotec), and dehydroxymethylepoxyquinomicin (MedChemExpress) can inhibit the effect of CpG-ODN or imiquimod. ODN2088 is an inhibitory oligonucleotide that competitively binds to TLR7/TLR9 and impairs TLR7/TLR9-induced signaling.[26] Dehydroxymethylepoxyquinomicin is a specific nuclear factor κB (NF-κB) inhibitor.[27] The levels of supernatant IgA, aberrantly glycosylated IgA, IgG-IgA IC, and IL-6, were measured after 72 hours of incubation. IL-6 levels were measured using the ELISA kit (R&D Systems).

Evaluation of the cytokine production with TLR activation and the inhibitory effect of HCQ

The splenocytes from ddY mice were cultured in either the medium only or the above-mentioned concentrations of CpG-ODN or imiquimod for 24 hours. The levels of supernatant cytokines, IL-6, IFN-α, and IFN-β, were measured. In addition, to investigate the effect of the inhibition of TLR signals by HCQ, we added HCQ at different concentrations of 15, 30, and 50 μM to evaluate the changes in the rate of production of each cytokine. Supernatant cytokines were measured using the ELISA kit (R&D Systems).

Evaluation of the cellular apoptotic effect of HCQ

The splenocytes from ddY mice were cultured with HCQ of varying concentrations for 72 hours to evaluate the degree of cellular apoptosis induced by HCQ. The FITC Annexin V Apoptosis Detection Kit with 7-amino-actinomycin D (BioLegend) was used to analyze cellular apoptosis. Annexin V labels early apoptotic cells, while 7-amino-actinomycin D marks necrotic cells. The study data were analyzed using FACSVerse (BD Biosciences).

Analysis of the transcriptional levels of TLR9/TLR7/APRIL in tonsils

We obtained tonsil samples from 53 patients with IgAN. Their clinical information is provided in Supplementary Table S1. Tonsil samples from 40 patients with chronic tonsillitis and 12 patients with sleep apnea syndrome served as disease controls. All these patients had undergone tonsillectomy at Juntendo University Hospital and had given their written informed consent for the use of their tonsil samples. None of the patients with IgAN had received steroids or other immunosuppressants before undergoing tonsillectomy. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Juntendo University Hospital. RNA from tonsillar tissues was extracted using TRIzol Reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). Real-time reverse transcription–polymerase chain reaction was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). TaqMan probes were used to measure expression of TLR9 (Hs00152973_m1), TLR7 (Hs00152971_m1), and APRIL (Hs00601664_g1). Transcriptional levels were normalized by expression of glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1). The quantitative evaluation of mRNA was performed via the ΔΔCT method using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific).

In vitro experiments with tonsillar mononuclear cells

We obtained fresh tonsil samples just after tonsillectomy. Tonsil samples were dissected into small pieces, treated with 2 mg/ml of collagenase D (Roche Diagnostics), and filtered on 70-μm cell strainers (Falcon). Tonsillar mononuclear cells (TMCs) were isolated via the gradient centrifugation method using Ficoll Paque Plus (Cytiva) and cultured in Roswell Park Memorial Institute 1640 medium + fetal calf serum + penicillin + streptomycin. TMCs were cultured in the medium only or cultured with the stimulation of CpG-ODN (5 μM), imiquimod (5 μg/ml), or r-IL-6 (R&D Systems; 10 ng/ml). The supernatant levels of IgA, Gd-IgA1, and IL-6 were measured via ELISA after 72 hours of incubation using Gd-IgA1 (Immuno-Biological Laboratories) and IL-6 (R&D Systems) ELISA kits. We also analyzed the transcriptional levels of APRIL and C1GALT1 in cultured TMCs via reverse transcription–polymerase chain reaction using TaqMan probes of APRIL and C1GALT1 (Hs00750511_s1). Transcriptional levels were normalized via expression of glyceraldehyde-3-phosphate dehydrogenase.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software). Comparisons between groups were analyzed using 1-way analysis of variance followed by Tukey *post hoc* test. The Spearman correlation analysis was used to analyze the correlation between 2 variables. Data are expressed as mean ± SD. P values of <0.05 were considered statistically significant.

RESULTS

Activation of TLR9 and TLR7 in NALT elevated the serum levels of aberrantly glycosylated IgA and induced glomerular injuries in ddY mice

ddY mice nasally administered with either CpG-ODN or imiquimod developed mesangial proliferation accompanied by significant mesangial IgA/IgG/C3 deposition. Electron dense deposits in the mesangial area were also observed (Figure 1a). Renal histological scores and the levels of urine albumin in mice administered with either CpG-ODN or imiquimod were higher than those in control mice (Figure 1b and c). The levels of serum IgA, aberrantly glycosylated IgA, and IgG-IgA IC in mice that were nasally administered with either CpG-ODN or imiquimod were higher than those in control mice (Figure 1d).

HCQ prevented glomerular injuries in ddY mice induced by TLR9 or TLR7 activation

ddY mice treated with HCQ, combined with nasal administration of CpG-ODN or imiquimod, showed neither mesangial proliferative changes nor mesangial IgA/IgG/C3 deposition (Figure 1a). In addition, renal histological scores and the levels of urine albumin did not show significant aggravation

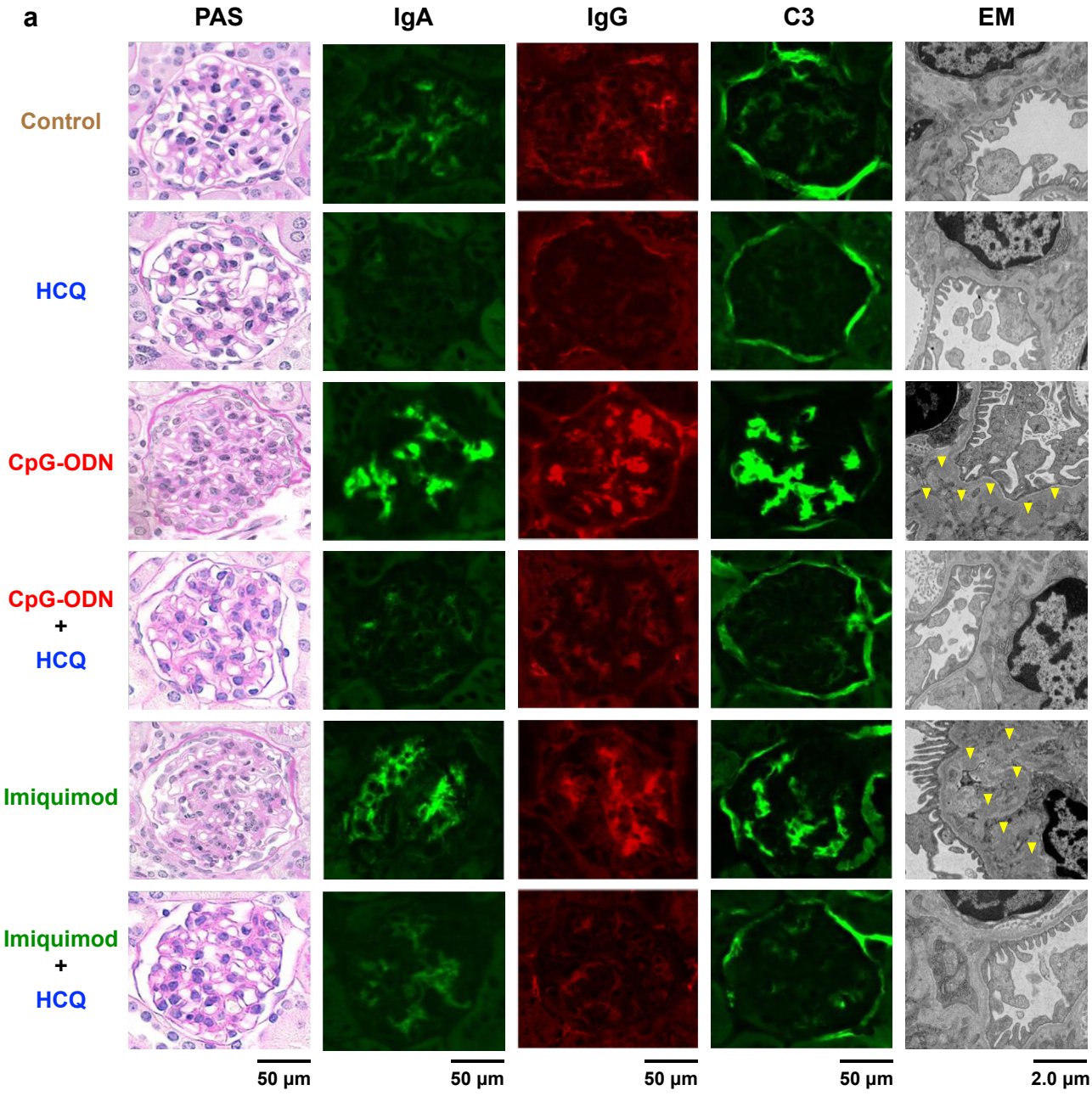

Figure 1 | Nasal administration of either cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN) or imiquimod induced IgA nephropathy in ddY mice while coadministered hydroxychloroquine (HCQ) prevented it. (**a**) ddY mice nasally administered with CpG-ODN or imiquimod developed mesangial proliferation and extracellular matrix expansion accompanied by significant mesangial IgA, IgG, and C3 deposition. Those mice also showed electron dense deposits in the mesangial or paramesangial regions (yellow arrows). Coadministered HCQ prevented these pathological manifestations. (Continued)

compared with control mice (Figure 1b and c). Coadministered HCQ prevented the elevation of serum IgA, aberrantly glycosylated IgA, and IgG-IgA IC levels induced by the nasal administration of CpG-ODN or imiquimod (Figure 1d).

Activation of TLR9 and TLR7 promoted the synthesis of aberrantly glycosylated IgA via IL-6 production

Stimulation with either CpG-ODN or imiquimod enhanced IgA production from the cultured splenocytes of ddY mice. Both CpG-ODN and imiquimod induced significant IL-6 secretion and augmented the production of aberrantly glycosylated IgA and IgG-IgA IC (Figure 2a). HCQ and NF-κB inhibitor (dehydroxymethylepoxyquinomicin) completely suppressed IL-6 secretion and inhibited the elevation of aberrantly glycosylated IgA and IgG-IgA IC. Conversely, TLR9/TLR7 antagonist (ODN2088) suppressed IL-6 secretion to some extent (Supplementary Figure S2) but failed to prevent the increase of aberrantly glycosylated IgA and IgG-IgA

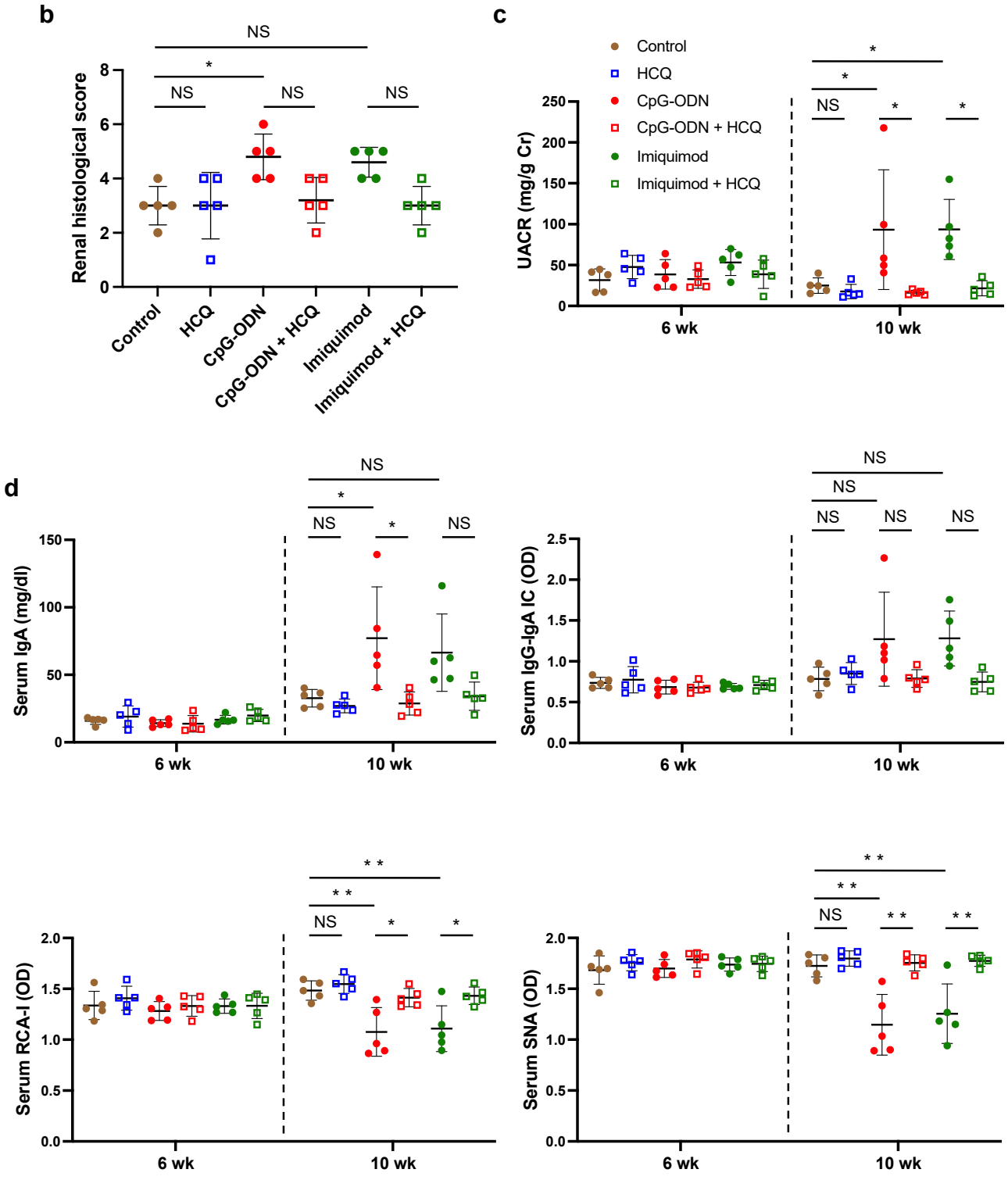

Figure 1 | (Continued) (**b**) Renal histological scores of mice administered with CpG-ODN or imiquimod were higher than those of control mice. Coadministered HCQ prevented the worsening of renal histological scores induced by CpG-ODN or imiquimod. (**c**) Mice administered with CpG-ODN or imiquimod showed higher urinary albumin levels than did control mice. Coadministered HCQ prevented the worsening of urine albumin induced by CpG-ODN or imiquimod. (**d**) Mice administered with CpG-ODN or imiquimod showed higher serum IgA levels than did control mice. In addition, serum IgA obtained from these mice showed lower reactivity with *Ricinus communis* agglutinin I (RCA-I) and *Sambucus nigra* agglutinin (SNA) lectins than did serum IgA from control mice, indicating a lower content of galactose and sialic acid in the murine IgA model. Furthermore, mice administered with CpG-ODN or imiquimod showed higher serum IgG-IgA immune complex (IC) levels. Coadministered HCQ prevented the increase in aberrantly glycosylated IgA and IgG-IgA ICs induced by CpG-ODN or imiquimod. The bars represent mean ± SD. *$P < 0.05$, **$P < 0.01$. EM, electron microscopy; NS, not significant; OD, optical density; PAS, periodic acid–Schiff; UACR, urine albumin-to-creatinine ratio. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

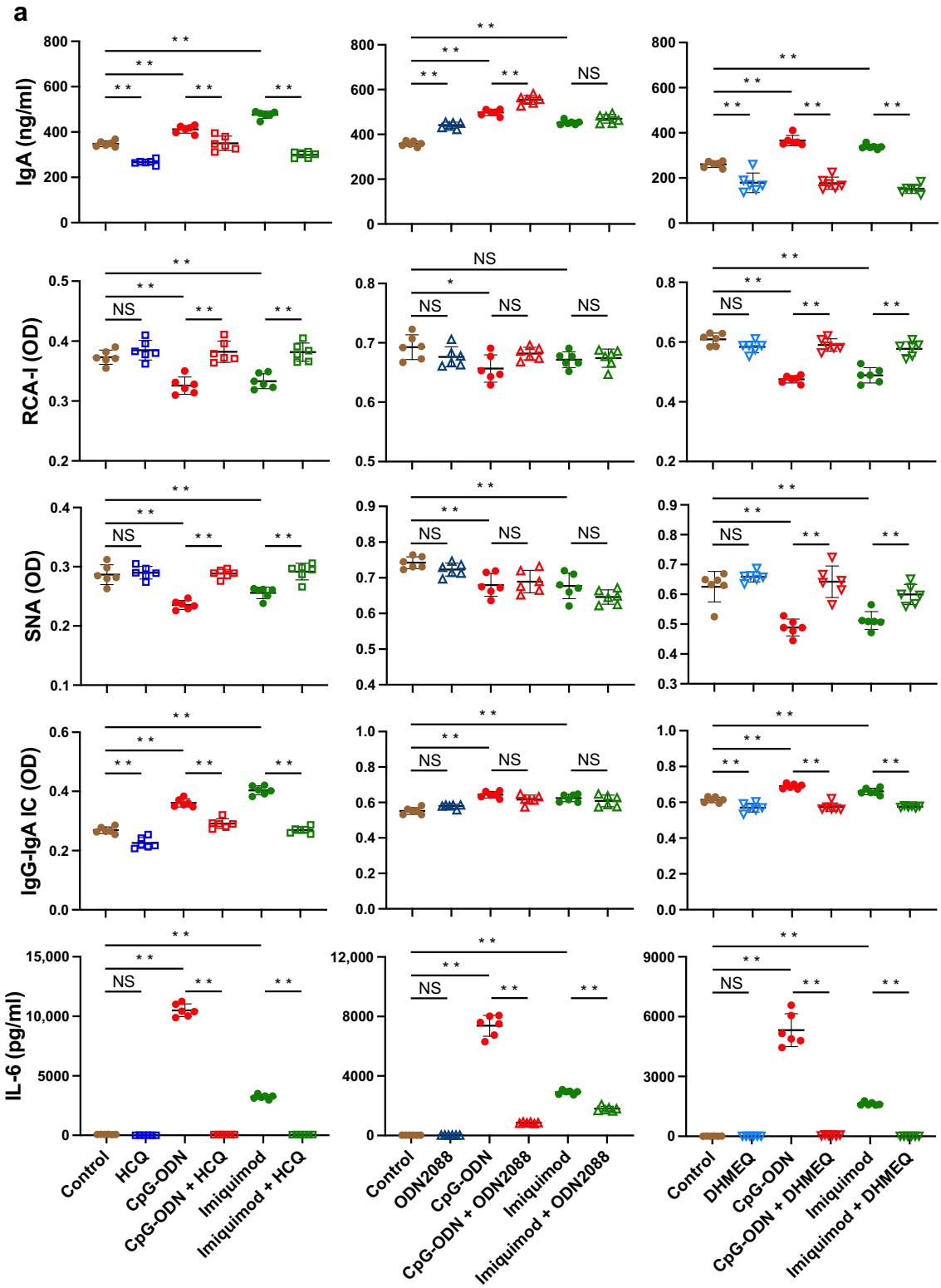

Figure 2| Activation of either toll-like receptor (TLR) 9 or TLR7 promoted the synthesis of aberrantly glycosylated IgA and IgG-IgA immune complexes (ICs) in the cultured splenocytes of ddY mice via interleukin-6 (IL-6) production. (**a**) Stimulation with either cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN; 5 μM) or imiquimod (5 μg/ml) promoted IgA secretion from the splenocytes of ddY mice. Furthermore, the levels of aberrantly glycosylated IgA and IgG-IgA IC were elevated. Both CpG-ODN and imiquimod significantly elevated supernatant IL-6 levels. Hydroxychloroquine (HCQ; 30 μM) and dehydroxymethylepoxyquinomicin (DHMEQ; 10 μM) completely suppressed IL-6 secretion and prevented the elevation of aberrantly glycosylated IgA that was induced by CpG-ODN or imiquimod. Conversely, ODN2088 (10 μM) reduced but did not completely suppress IL-6 secretion induced by CpG-ODN or imiquimod. Accordingly, ODN2088 failed to inhibit the elevation of aberrantly glycosylated IgA with CpG-ODN or imiquimod. (Continued)

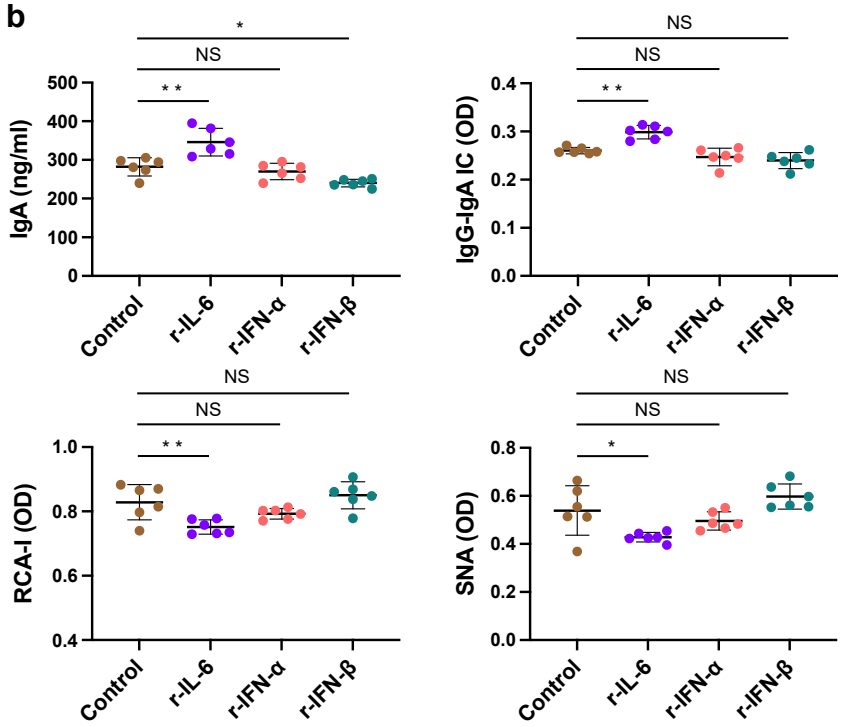

Figure 2 | (Continued) (**b**) Stimulation with recombinant IL-6 (r-IL-6; 10 ng/ml) elevated the supernatant level of IgA, aberrantly glycosylated IgA, and IgG-IgA ICs. On the contrary, recombinant interferon-α (r-IFN-α; 10 ng/ml) and recombinant interferon-β (r-IFN-β; 10 ng/ml) did not increase total IgA levels and did not significantly affect the level of aberrantly glycosylated IgA. The bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. NS, not significant; OD, optical density; RCA-I, *Ricinus communis* agglutinin I; SNA, *Sambucus nigra* agglutinin.

IC. Stimulation with r-IL-6 induced splenocytes to produce more IgA, aberrantly glycosylated IgA, and IgG-IgA IC. On the contrary, stimulation with recombinant IFN-α or recombinant IFN-β did not affect the synthesis of aberrantly glycosylated IgA and IgG-IgA IC (Figure 2b).

HCQ effectively suppressed cytokine production induced by TLR9 or TLR7 activation and also induced cellular apoptosis in a dose-dependent manner *in vitro*

Stimulation with CpG-ODN induced the splenocytes of ddY mice to produce IL-6, IFN-α, and IFN-β. Stimulation with imiquimod produced only IL-6. HCQ (30 μM) almost completely suppressed the cytokines induced by CpG-ODN or imiquimod (Figure 3a). We also assessed the cellular apoptotic effect of HCQ and revealed that HCQ dose dependently induces cellular apoptosis *in vitro* (Figure 3b).

Tonsillar expression of TLR9 and TLR7 was elevated and positively correlated with that of APRIL in patients with IgAN

We investigated the transcriptional levels of TLR9 and TLR7 in the tonsil tissues of patients with IgAN and disease controls, including chronic tonsillitis and sleep apnea syndrome. Expression of TLR9 and TLR7 in the tonsils was higher in patients with IgAN than in disease controls (Figure 4a). Furthermore, tonsillar expression of both TLR9 and TLR7 are positively correlated with that of APRIL in patients with IgAN (Figure 4b).

Activation of either TLR9 or TLR7 upregulated IL-6/APRIL and downregulated C1GALT1, consequently promoting Gd-IgA1 synthesis in TMCs

To further investigate whether the activation of TLRs induces Gd-IgA1 overproduction by human NALT, TMCs from patients with IgAN was cultured with the stimulation of either CpG-ODN or imiquimod. The total IgA and Gd-IgA1 levels in the culture supernatant were elevated. Furthermore, the percentage of Gd-IgA1 in total IgA was elevated. We also cultured TMCs with the stimulation of r-IL-6 and demonstrated similar changes in supernatant IgA and Gd-IgA1 levels (Figure 5a). The supernatant IL-6 levels were significantly elevated with either CpG-ODN or imiquimod. Moreover, the transcriptional levels of APRIL were elevated while those of C1GALT1 were suppressed in TMCs with either CpG-ODN or imiquimod. In addition, r-IL-6 elevated the expression levels of APRIL and suppressed those of C1GALT1 in TMCs (Figure 5b).

DISCUSSION

Both genetic factors and environmental triggers are essential in the pathogenesis of IgAN. Several genome-wide association studies that have been performed since 2010 have identified common variants within several genetic loci associated with the risk of developing IgAN. The susceptibility genes include *DEFA*, *VAV3*, *CARD9*, *LIF*, *OSM*, and *TNFSF13*. *DEFA* encodes α-defensin, which is involved in mucosal innate

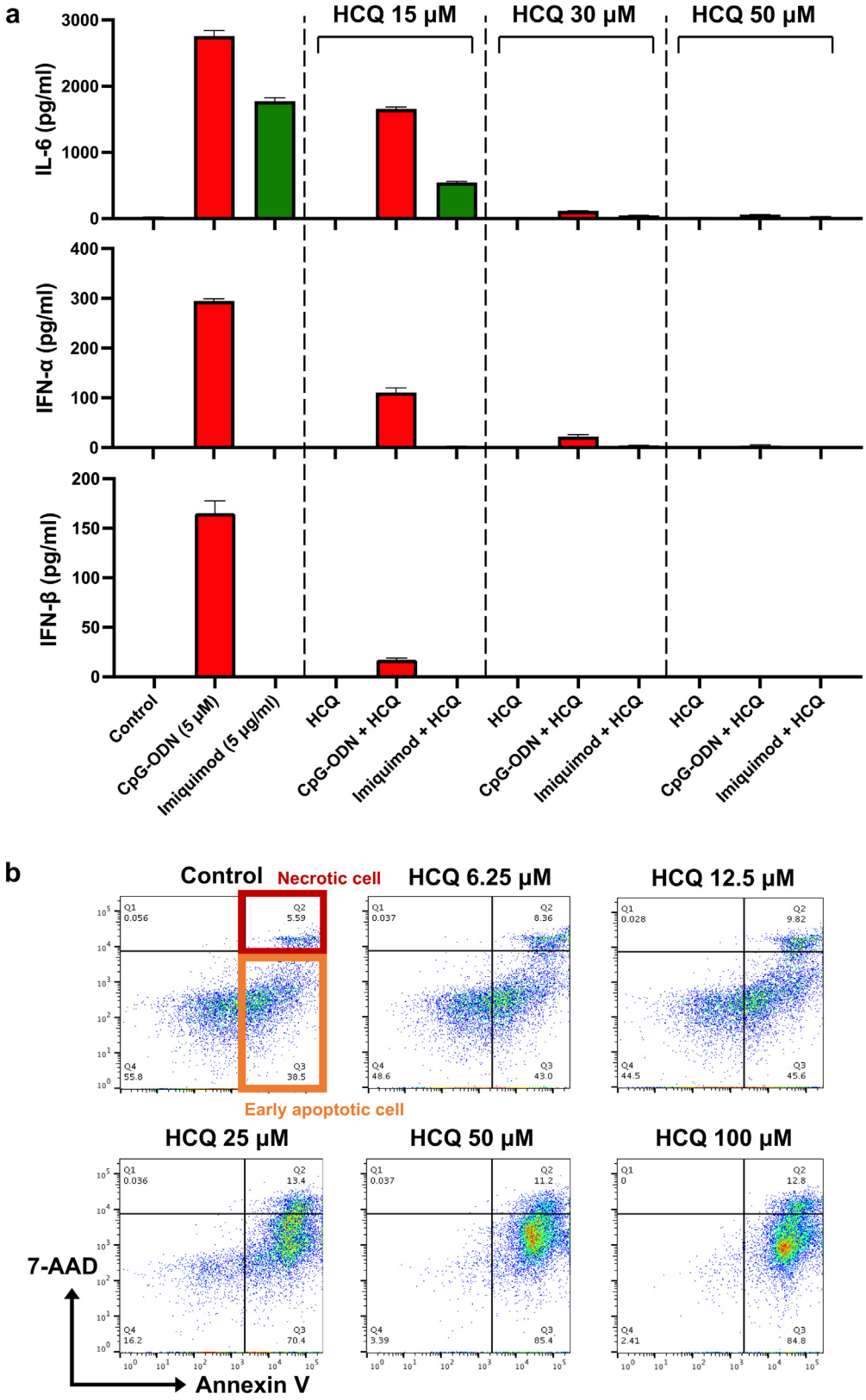

Figure 3 | Hydroxychloroquine (HCQ) effectively inhibited the cytokine production induced by toll-like receptor (TLR) 9 or TLR7 activation and also dose dependently induced cellular apoptosis *in vitro*. (**a**) Stimulation with class C cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN) induced the cultured splenocytes of ddY mice to produce interleukin-6 (IL-6), interferon-α (IFN-α), and interferon-β (IFN-β). Stimulation with imiquimod produced only IL-6. HCQ (30 μM) almost completely suppressed the cytokines induced by CpG-ODN or imiquimod. The bars represent mean ± SD. (**b**) The ratio of early and late apoptotic cells of the splenocytes from ddY mice, which were cultured with variant concentrations of HCQ (continued)

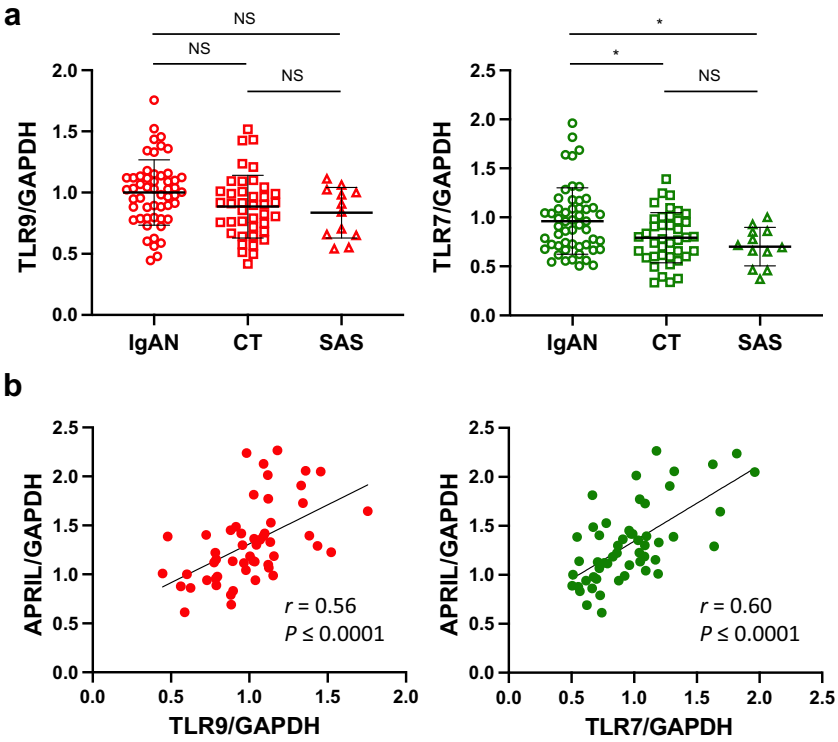

Figure 4 | Tonsils from patients with IgA nephropathy (IgAN) expressed higher levels of toll-like receptor (TLR) 9 and TLR7, which was positively correlated with a proliferation-inducing ligand (APRIL) expression. (**a**) TLR9 and TLR7 expression in the tonsils was higher in patients with IgAN than in disease controls, including chronic tonsillitis (CT) and sleep apnea syndrome (SAS). (**b**) Tonsillar expression of both TLR9 and TLR7 is positively correlated with that of APRIL. *$P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

immunity.[28] *VAV3* and *CARD9* encode proteins related to NF-κB activation.[29,30] Leukemia inhibitory factor and oncostatin M belong to the IL-6 family of cytokines, which are defined as cytokines that use the common signaling receptor subunit glycoprotein 130 kDa.[31] *TNFSF13* encodes APRIL, a powerful B cell–stimulating cytokine that promotes mucosal IgA class switching.[7,8] These findings indicated that IL-6–related cytokines induced by NF-κB activation in the process of mucosal immune response could be involved in the pathogenesis of IgAN. Furthermore, 2 genome-wide association studies related to serum Gd-IgA1 levels revealed 2 genome-wide significant loci, *C1GALT1* and *C1GALT1C1*,[32,33] which encode C1GaLT1 and core 1 β3-Gal-T–specific molecular chaperone, respectively. The expression levels of C1GaLT1 and core 1 β3-Gal-T–specific molecular chaperone in both peripheral and tonsillar B cells are downregulated in patients with IgAN and correlated with surrogate markers, including serum Gd-IgA1 levels.[34–36]

There has been increasing evidence suggesting that TLRs which are strongly related to NF-κB activation play essential roles in the pathogenesis of IgAN. Previously, we focused on TLR9 involvement in the pathogenesis of IgAN.[5,9–12] However, recent clinical reports have alerted gross hematuria after coronavirus disease 2019 vaccination in patients with IgAN.[37,38] These are particularly caused by mRNA vaccine.[38,39] Thus, the RNA-sensing system is also suspected to be involved in the exacerbation of IgAN. Moreover, it is worth noting that postvaccine hematuria occurs dominantly in women (∼70%), despite the absence of sex differences in the incidence of IgAN.[37,39] TLR7, which is encoded by an X chromosome locus, is reported to cause a stronger immune response in females.[40] Thus, the gender difference in the sensitivity of TLR7 might be the reason for the female predominance of postvaccine hematuria. However, several reports described that mRNA molecules used as the vaccine component possess modification of incorporating N1-methylpseudouridine in place of uridine, which is the binding target for TLR7 and TLR8,[41,42] to not trigger TLRs to avoid excessive inflammation.[43] Hence, it is uncertain whether TLR7 is involved in postvaccine hematuria in IgAN. In this study, we tried to clarify whether TLR7 is involved in the progression of IgAN.

Figure 3 | (continued) for 72 hours, was assessed by Annexin V (which labels early apoptotic cells) and 7-amino-actinomycin D (7-AAD; which marks necrotic cells). The splenocytes of ddY mice cultured with 6.25 and 12.5 μM HCQ did not show an apparent increase in the proportion of apoptotic cells compared with the control. However, concerning 25, 50, and 100 μM HCQ, the proportions of apoptotic cells increased dose dependently.

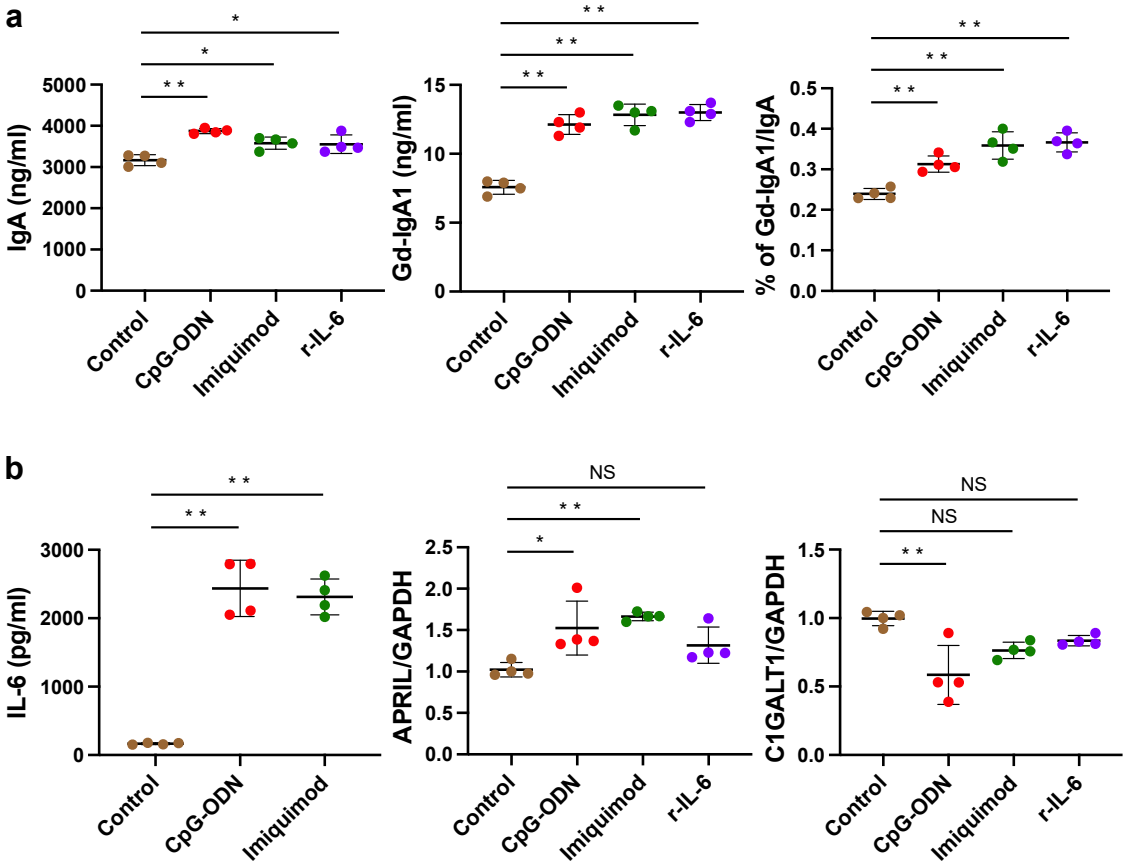

Figure 5 | Activation of either toll-like receptor (TLR) 9 or TLR7 upregulated interleukin-6 (IL-6)/a proliferation-inducing ligand (APRIL) and downregulated core 1 β1,3-galactosyltransferase (C1GALT1), consequently promoting galactose-deficient IgA1 (Gd-IgA1) synthesis from tonsillar mononuclear cells (TMCs). (**a**) The levels of total IgA and Gd-IgA1 in the culture supernatant were significantly elevated with either cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN; 5 μM) or imiquimod (5 μg/ml). Furthermore, the percentage of Gd-IgA1 in total IgA was elevated with either CpG-ODN or imiquimod. Recombinant interleukin-6 (r-IL-6; 10 ng/ml) promoted TMCs to increase Gd-IgA1 production and elevated the percentage of Gd-IgA1 in total IgA. (**b**) The supernatant levels of IL-6 were significantly elevated with the stimulation of CpG-ODN or imiquimod. Moreover, the transcriptional levels of APRIL were elevated whereas those of C1GALT1 were suppressed in TMCs with the stimulation of CpG-ODN or imiquimod. r-IL-6 also elevated APRIL and suppressed C1GALT1 in TMCs. The bars represent mean ± SD. *$P < 0.05$, **$P < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

Our group has reported that TLR9 activation, especially in NALT, would increase the serum levels of aberrantly glycosylated IgA and IgG-IgA IC, consequently inducing glomerular IgA deposition and increasing the prevalence of proteinuria in the murine IgAN model.[5,9,21] In this study, we demonstrated that the activation of TLR7 also accelerates the disease progression of IgAN in the same manner (Figure 1). Moreover, the activation of either TLR9 or TLR7 promotes the synthesis of aberrantly glycosylated IgA in both mice and humans (Figures 2a and 5a). This could be the primary mechanism by which TLR9 and TLR7 activation in the mucosa results in mesangial IgA deposition and renal injuries. Coadministered HCQ, which suppresses TLR9 and TLR7,[16,22] prevented the above pathogenic manifestations (Figure 1).

TLRs activate the myeloid differentiation factor 88 (MyD88)–dependent pathway and the TIR-domain–containing adaptor protein-inducing interferon-β–dependent pathway. TLR9 and TLR7 specifically activate the MyD88-dependent pathway.[3] The MyD88-NF-κB pathway induces pro-inflammatory cytokines such as IL-6; the MyD88–interferon regulatory factor 7 pathway induces type I IFN, including IFN-α and IFN-β.[44] TLR9 and TLR7 basically share common downstream pathways; however, the patterns of cytokines vary depending on the ligand types.[44] In our study using splenocytes from ddY mice, class c CpG-ODN activated both the TLR9-MyD88-NF-κB pathway and the TLR9-MyD88–interferon regulatory factor 7 pathway to produce IL-6, IFN-α, and IFN-β. Imiquimod activated only the TLR7-MyD88-NF-κB pathway to produce IL-6 (Figure 3a).

Based on the previous studies reporting IL-6's involvement in the production of aberrantly glycosylated IgA,[5,6,45,46] IL-6 inducibility might be the factor whose synthesis TLR activation promotes. In addition to IL-6, our group reported that leukemia inhibitory factor induces aberrantly glycosylated IgA.[47] Although type I IFN is another chief cytokine induced by TLR activation, we demonstrated that IL-6 and not type I IFN involves the synthesis of aberrantly glycosylated IgA (Figures 2b and 5a). Indeed, IL-6 upregulates APRIL and

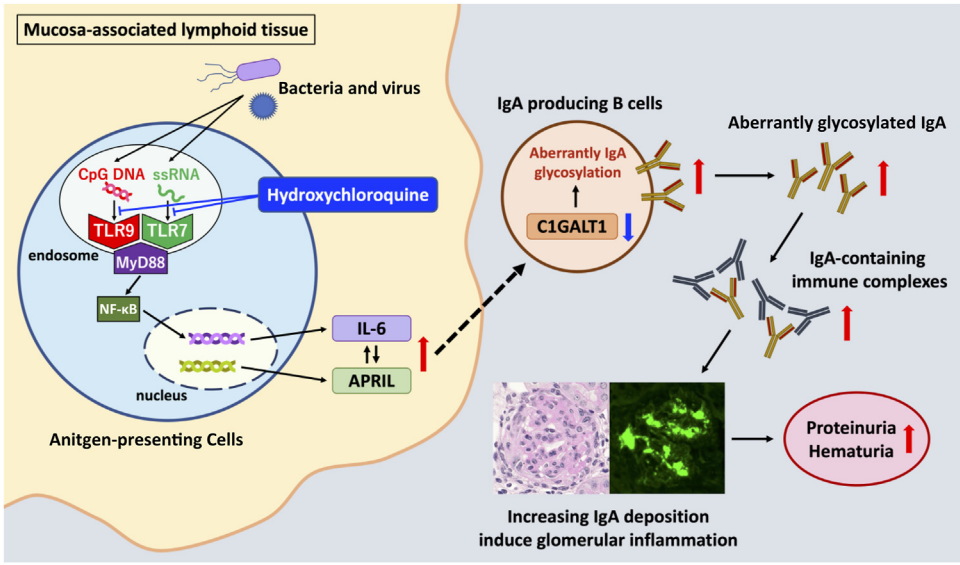

Figure 6| Toll-like receptor (TLR) 9/TLR7–myeloid differentiation factor 88 (MyD88)–nuclear factor-κB (NF-κB) pathway plays a crucial role in the production of aberrantly glycosylated IgA and could be a therapeutic target for IgA nephropathy. The TLR9/TLR7-MyD88-NF-κB pathway induces pro-inflammatory cytokines, including interleukin-6 (IL-6). IL-6 upregulates a proliferation-inducing ligand (APRIL), downregulates core 1 β1,3-galactosyltransferase (C1GALT1), and promotes the synthesis of aberrantly glycosylated IgA. These IgA forms IgA-containing immune complexes that accumulate in the glomeruli, finally inducing glomerular inflammation. Hydroxychloroquine might prevent the synthesis of aberrantly glycosylated IgA by preventing TLR9/TLR7 from binding to their ligands in endosomes and inhibiting downstream signals. CpG DNA, cytosine-phosphate-guanine DNA; ssRNA, single-stranded RNA.

downregulates C1GALT1 in human TMCs (Figure 5b). We also confirmed that NF-κB inhibition could cancel the acceleration of aberrantly glycosylated IgA synthesis by IL-6 under the activation of TLRs (Figure 2a).

The exact mechanism of postvaccine hematuria in IgAN is not fully understood. Several murine studies have demonstrated that mRNA vaccination induces the elevation of serum IL-6 levels and even elevated IL-6 expression in distant organs.[48,49] These results indicate that injecting even a small amount of exogenous RNA can induce a surge in systemic IL-6 and potentially affect lymphoid tissues, accelerating Gd-IgA1 synthesis. Regarding the production site of Gd-IgA1, we previously advocated the concept of the mucosa–bone marrow axis in IgAN.[50] A disruption of mucosal tolerance, which results in abnormal priming and the dissemination of cells, is responsible for the synthesis of Gd-IgA1 at sites such as systemic lymphoid organs and even the bone marrow. Moreover, systemic circulating Gd-IgA1+ B cells are potentially home to mucosal sites.[2,51] In the present study, we demonstrated that splenocytes respond to the ligand of TLR9/TLR7 and increase aberrantly glycosylated IgA production *in vitro* (Figure 2a). Moreover, we confirmed that NALT cells react with the ligand of TLR9/TLR7 similarly (Supplementary Figure S3). Further *in vivo*/*in vitro* studies using mRNA vaccine are required to clarify the exact mechanism for inducing macrohematuria associated with coronavirus disease 2019 vaccination.

The effectiveness of HCQ in treating IgAN has been reported in China since 2017.[15] The mechanism of TLR9/TLR7 inhibition by HCQ is the alteration of the pH of endosomes where TLR9/TLR7 is located and the prevention of the binding of their ligands.[16,22] We confirmed that HCQ effectively inhibits the production of TLR9/TLR7-related cytokines (Figure 3a). However, HCQ is also known to induce cellular apoptosis, which is due to the inhibition of cellular autophagy via the suppression of the cellular lysosomal degradation step.[52] The complex pharmacological actions of HCQ make it challenging to validate the effect of TLR inhibition alone *in vitro* (Figure 3). The clinical use of HCQ seems to be an efficient alternative therapy for patients with IgAN who insufficiently respond to conventional treatment.[15] Further randomized controlled trials with long-term follow-up are required to clarify the effectiveness of HCQ in treating IgAN.

Our group previously reported that the transcriptional levels of TLR9 and APRIL in the tonsils were positively correlated and higher in patients with IgAN.[12] Tonsillar B cells in these patients aberrantly produce APRIL and contribute to the production of Gd-IgA1.[12] Clinically, APRIL expression levels in tonsils are associated with the severity of IgAN and treatment responses to tonsillectomy.[12] In the present study, we found that tonsillar expression of TLR7 also positively correlated with APRIL and was significantly elevated in patients with IgAN (Figure 4). Surprisingly, several reports mentioned that tonsillar B cells express TLR9 and TLR7 but lack other nucleotide-sensing TLRs, including TLR3 and TLR8,[53,54] which could be why TLR9 and TLR7 are important in the pathogenesis of IgAN. In addition, gain-of-function mutations in TLR7 were recently shown to contribute to the pathogenesis of systemic lupus erythematosus.[55] Because IgAN also has the aspects of autoimmune

diseases,[4] a similar mechanism could be involved in its pathogenesis. Investigating the genetic characteristics of TLR9 and TLR7 in IgAN will be helpful in further understanding its pathogenesis and developing novel therapeutic targets.

In conclusion, nucleotide-sensing TLR9 and TLR7 play a crucial role in the production of aberrantly glycosylated IgA and affect the disease progression of IgAN via IL-6/APRIL-mediated pathways. The inhibition of TLR9/TLR7 signals and associated cytokines might be a reasonable strategy for treating IgAN (Figure 6).

DISCLOSURE

All the authors declared no competing interests.

DATA STATEMENT

Regarding animal studies, the raw data were generated at Juntendo University. Derived data supporting the findings of this study are available from the corresponding author on request. Regarding human data, the data are not publicly available due to ethical restrictions approved by the ethics committee of Juntendo University Hospital.

ACKNOWLEDGMENTS

This study was supported in part by Japan Society for the Promotion of Science (JSPS) KAKENHI (grant number: 21K08285). We thank the members of the Laboratory of Molecular and Biochemical Research, Laboratory of Morphology and Image Analysis, Laboratory of Biomedical Research Resources, Laboratory of Molecular Biochemical Research, and Research Support Center, Juntendo University Graduate School of Medicine for their technical assistance. We also thank Terumi Shibata, Department of Nephrology, Juntendo University Faculty of Medicine, for her excellent technical assistance.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Figure S1. We administered different doses of hydroxychloroquine (HCQ; 10, 30, and 60 mg/kg/d) to ddY mice for 4 weeks to evaluate the body weight changes, plasma lymphocyte counts, and serum immunoglobulin (IgA, IgG, and IgM) levels. The groups treated with 10 and 30 mg/kg/d showed no significant changes in body weight, lymphocyte counts, and immunoglobulin levels. However, in the 60 mg/kg/d group, immunoglobulin levels were maintained but lymphocyte counts decreased significantly. The bars represent mean ± SD. *$P < 0.05$. ns, not significant.

Supplementary Figure S2. We assessed the inhibitory effects of ODN2088 and dehydroxymethylepoxyquinomicin (DHMEQ) on interleukin-6 (IL-6) production with cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN) or imiquimod stimulation in cultures of the splenocytes from ddY mice. Although DHMEQ can completely inhibit IL-6 secretion induced by CpG-ODN or imiquimod, ODN2088 can only reduce and cannot completely suppress the supernatant IL-6 levels. The bars represent mean ± SD.

Supplementary Figure S3. We assessed whether nasal-associated lymphoid tissue (NALT) cells respond to cytosine-phosphate-guanine–oligodeoxynucleotide (CpG-ODN) or imiquimod and increase aberrantly glycosylated IgA production. Briefly, NALT cells were purified from NALTs of ddY mice and cultured with the stimulation of CpG-ODN or imiquimod. We analyzed IgA, interleukin-6 (IL-6), and aberrantly glycosylated IgA levels in the culture supernatant. CpG-ODN or imiquimod enhanced the production of IgA, IL-6, and aberrantly glycosylated IgA in NALT cells. The bars represent mean ± SD. **$P < 0.01$. OD, optical density; RCA-I, *Ricinus communis* agglutinin I; SNA, *Sambucus nigra* agglutinin.

Supplementary Table S1. Clinical information at the time of renal biopsy and renal histological scores of 53 patients with IgA nephropathy who had undergone tonsillectomy.

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