TLR9 stimulation induces aberrant APRIL expression by tonsillar germinal center B cells in IgA nephropathy

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ABSTRACT

The tumor necrosis factor (TNF) family member, a proliferation-inducing ligand (APRIL, TNFSF13), produced by myeloid cells, participates in the generation and survival of antibody-producing plasma cells. We studied the potential role of APRIL in the pathogenesis of IgA nephropathy (IgAN). We found that a significant proportion of germinal centers (GC) in tonsils from IgAN patients contained cells aberrantly producing APRIL, conducting to an overall upregulation of APRIL expression compared to control tonsillitis patients. In IgAN GC, antigen-experienced IgD/CD38+/CD19+ B cells expressing a switched IgG/IgA BCR produced APRIL. Notably, these GC B cells expressed the common cleavable APRIL-α but also the less frequent uncleavable APRIL-δ and –zeta, which lacks a furin cleavage site, implying expression as a membrane-bound form. Significant correlation between TLR9 and APRIL expressions existed in tonsils from IgAN patients. In vitro, repeated TLR9 stimulation reproduced APRIL expression in tonsillar B cells from control tonsillitis patients. Clinically, aberrant APRIL expression in tonsillar GC correlated with high urinary protein levels, and IgAN patients harboring aberrant APRIL overexpression in tonsillar GC responded well to tonsillectomy, in parallel to decreases in serum levels of galactose-deficient IgA1. Taken together, our data indicate that antibody disorders in IgAN are associated with TLR9 induced aberrant expression of APRIL in tonsillar GC B cells.
Introduction

Immunoglobulin A nephropathy (IgAN) is the most common form of glomerulonephritis (GN), accounting for 25%-50% of patients with primary GN. Accumulating evidences now suggest that 30%-40% of IgAN patients progress to end-stage renal disease (ESRD) within 20 years from the estimated time of disease onset\textsuperscript{1, 2}. The lack of comprehensive understandings of IgAN development impairs the design of a specific treatment for this disease. The pathogenesis of IgAN may be associated with systemic immune dysregulation from a mucosa-bone marrow axis rather than an abnormality intrinsic to the renal resident cells\textsuperscript{3, 4}. At the mucosal level, infections, particularly in the upper respiratory tract, exacerbate clinical manifestations in IgAN patients. Recent studies report that aberrantly $\alpha$-glycosylated IgA1, i.e. galactose-deficient IgA1 (Gd-IgA1) and the subsequently formed IgA immune complexes (IC) with glycan-specific autoantibodies are pivotal to the development of IgAN\textsuperscript{5-7}.

A proliferation inducing ligand (APRIL) is a member of TNF superfamily of ligands expressed as a type II transmembrane protein\textsuperscript{8}. APRIL is usually cleaved in the Golgi apparatus by a furin convertase, and then secreted as a soluble ligand\textsuperscript{9}. Myeloid and mucosal epithelial cells produced APRIL\textsuperscript{10-12}. APRIL binds to two members of the TNF receptor family: the B-cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)\textsuperscript{13}. Functionally, APRIL mediates class switch, mostly for IgA\textsuperscript{10, 14}. APRIL is also crucial for long-term survival of plasma cells in the bone marrow and mucosa\textsuperscript{11, 12, 14-17}. Recently, high serum level of APRIL in IgAN patients correlating with urinary proteins was reported\textsuperscript{18, 19}. In addition, a genome-wide association study of IgAN patients suggested APRIL ($TNFSF13$) to be a susceptibility gene\textsuperscript{20}. However, the cellular source of APRIL production and the pathway by which this molecule is upregulated in IgAN remains ill defined.

Toll-like receptors (TLRs) are a family of germ-line encoded receptors that recognize a diverse range of conserved molecular motifs commonly found in microbial pathogens. TLR9 recognizes unmethylated DNA sequences in bacterial and viral DNA, and is involved in innate immune responses by providing protective immune responses against invading viral and bacterial pathogens\textsuperscript{21, 22}. Although TLR9 has been shown to
be implicated in the development of kidney diseases including IgAN, the mechanisms by which TLR9 activation contribute to the development of IgAN are poorly understood.
Results

Up-regulation of APRIL expression in tonsillar germinal center (GC) of IgAN patients

Real-time quantitative PCR (RT qPCR) analyses in IgAN \((n = 24)\) and chronic tonsillitis (CT) \((n = 6)\) patients revealed that tonsillar APRIL mRNA expression was significantly higher in IgAN than that in CT (Figure 1A; \(P < 0.01\)). Immunohistochemistry with Stalk-1, an anti-APRIL antibody that detect APRIL-producing cells, further showed that APRIL-producing cells were present in the tonsillar epithelium and outside, in cells identified as neutrophils, of both IgAN \((n=55)\) and CT \((n = 12)\) patients (Figure 1B), consistent with our previous report in CT patients\(^\text{11}\). Stalk-1 and elastase (neutrophil specific) co-staining did not reveal a difference in the number of infiltrating neutrophils between IgAN and CT patients (Figure 1C). The epithelial staining by Stalk-1 was also not different. One obvious difference was the presence of a substantial number of Stalk-1\(^+\) APRIL-producing cells in GC in IgAN patients. We observed that the percentage of Stalk-1\(^+\) GC \((27.4 \pm 21.3)\) in IgAN patients was significantly higher than that in CT patients \((7.2 \pm 6.8; P<0.01)\) (Figure 1D).

Ig-switched GC B cells produce APRIL in tonsils of IgAN patients

Stalk-1\(^+\) cells in tonsillar GC of IgAN patients expressed CD19, identifying them as B cells (Figure 2A). We further observed that Stalk-1\(^+\) APRIL-producing cells in tonsillar GC had a non-switched IgM and switched IgG or IgA phenotypes. Flow cytometric analyses revealed that the Stalk-1\(^+\) CD19\(^+\) cell population had lost IgD surface expression and variably expressing the GC activation marker CD38 in IgAN patients \((n = 13)\) (Figure 2B, upper left panel). We also detected this Stalk-1\(^+\) GC B-cell population in CT \((n = 4)\) (Figure 2B, lower left panel), but this cell population was clearly increased in some IgAN patients (Figure 2B, right panel; \(P<0.05\)).

GC B cells express an uncleavable form of APRIL in IgAN patients.
The Stalk-1 staining pattern obtained in GC B cells from IgAN patients was clearly different from the one in PMN cells described previously\(^\text{11}\) (Figure 3A). We additionally performed immunohistochemical analysis with Aprily-2 antibody, which recognizes the secreted part of APRIL\(^\text{29}\). We observed that the Aprily-2 staining colocalized with Stalk-1 in tonsillar GC from IgAN patients (Figure 3B). Such colocalization of Stalk-1 and Aprily-2 has never been observed in several healthy and pathological tissues\(^\text{30}\). A recent study reported a possible expression of APRIL-\(\delta\) and -zeta, APRIL isoforms lacking the consensus motif for the furin convertase, in B-cell precursor acute lymphoblastic leukemia (B-ALL)\(^\text{31}\), and such isoforms could be stained with both Stalk-1 and Aprily-2. RT-PCR analyses revealed that tonsillar B cells from IgAN and CT patients indeed expressed not only APRIL-\(\delta\) and –zeta in addition to the common furin-cleavable APRIL-\(\alpha\) (Figure 3C). RT qPCR further showed that the abundances of APRIL-\(\alpha\) and APRIL-\(\delta/zeta\) mRNAs in tonsillar B cells of IgAN patients were significantly higher than those in CT patients (Figure 3D).

**TLR9 stimulation induces APRIL expression in GC B cells**

Levels of TLR9 mRNA in whole tonsils and tonsillar B cells of IgAN patients were significantly higher than those of CT patients (Figure 4A), and an increase of TLR9 mRNA well correlated with an increase of APRIL-\(\alpha\) and \(\delta/zeta\) mRNA in tonsillar B cells of IgAN patients (Figure 4B).

We next stimulated whole tonsillar cells from CT patients with the TLR9 ligand CpG-oligodeoxynucleotides (CpG-ODN), and analyzed APRIL expression on CD19\(^+\) B cells. A daily stimulation induced a reactivity of CD19\(^+\) cells with Stalk-1 and Aprily-2 antibodies, starting at day 3, with a maximum seen at day 7, in CD19\(^+\) cells. (Figure 5A). The APRIL reactivity was observed intracellularly with a limited signal at the cells surface. The weak surface APRIL expression on CpG-stimulated B cells was consistent with the absence of surface staining observed ex vivo. Repeated TLR9 stimulation weakly but clearly upregulated expression of the APRIL receptors TACI and BCMA as well (Figure 5B). Consistent with analyses in Figure 5A, same APRIL reactivities were observed in studies with purified tonsillar B cells at day 7 (Figure 5C). Taken together,
these indicate a putative autocrine APRIL signaling in CpG-stimulated B cells.

APRIL expression in tonsillar GC is associated with the severity of IgAN and treatment responses to tonsillectomy

We assessed whether the extent of Stalk-1⁺GC in tonsils may affect the severity of IgAN and therapeutic responses to tonsillectomy. The percentage of Stalk-1⁺GC was significantly higher in IgAN patients with an elevated proteinuria (Figure 6A; \(P<0.01\)). Figure 1D showed that even CT patients showed Stalk-1⁺GC, but less than 10%, suggesting that Stalk-1⁺GC≥10% in tonsils may be more characteristic to IgAN. Therefore, we next focused on IgAN patients with Stalk-1⁺GC more than 10%. Urinary protein level in IgAN patients with Stalk-1⁺GC≥10% was significantly higher than that in IgAN patients with Stalk-1⁺GC<10% (Figure 6B; \(P<0.05\)). Percentage of IgAN patients whose proteinuria decreased more than 50% after the tonsillectomy alone was significantly higher in patients with Stalk-1⁺GC≥10% (62.8%) than those with Stalk-1⁺GC<10% (25.0%; \(P=0.0201\)) (Table 2). Notably, the efficacy of tonsillectomy was similarly observed in severe cases of IgAN who showed proteinuria more than 0.5g/gCr (\(P=0.048\)), independently of basal levels of proteinuria before the treatment (Supplement Table 1). Change of serum levels of whole IgA and Gd-IgA1 before and after tonsillectomy was then evaluated. Significantly higher decrease of serum Gd-IgA1 (\(P<0.05\)), but not whole IgA, was observed in IgAN patients with Stalk-1⁺GC≥10% (Figure 6C and 6D). We also compared proteinuria levels before and after tonsillectomy in whole IgAN patients (Figure 6E), those with Stalk-1⁺GC ≥ 10% (Figure 6F) and those with Stalk-1⁺GC<10% (Figure 6G). There were significant differences between before and after tonsillectomy in the preceding two groups (*\(P<0.01\)), despite no significance in IgAN patients with Stalk-1⁺GC<10%.
Discussion

Mucosal immune dysregulation has already been reported in the pathogenesis of IgAN. However, the underlying mechanism remains unclear. APRIL and B cell activating factor belonging to the tumor necrosis factor family (BAFF) derived from myeloid cells such as neutrophils, monocytes and dendritic cells are TNF super family members best known for their roles in the survival and maturation of B cells. Recent studies revealed that mature B-cell neoplasms, including chronic lymphocytic leukemia\textsuperscript{32}, follicular lymphoma and diffuse large B cell lymphoma may start to produce APRIL\textsuperscript{33, 34} by themselves. This aberrant production of APRIL was also observed in B cells from patients suffering from autoimmune diseases such as systemic lupus erythematos (SLE)\textsuperscript{35}. In fact, repeated stimulation of B cells induced their expression of APRIL\textsuperscript{36}. The present study shows, for the first time, an aberrant up-regulation of APRIL in tonsillar GC B cells from IgAN patients.

In GC B cells from IgAN, we observed that the reactivity of the two APRIL-specific antibodies, Stalk-1 and Aprily-2, was clearly different from the one obtained in other organs. Indeed, we observed an uncommon co-staining of Stalk-1 and Aprily-2 revealing the presence of a full-length form of APRIL. The detection of the two furin uncleavable isoforms of APRIL, \(\delta\) and zeta mRNAs, is consistent with this observation. This uncleavable full-length APRIL was detected intracellularly, stored most likely in vesicles warranting further investigations (see Figure 3A).

Exacerbation of IgAN upon upper respiratory infections allows speculation on the participation of exogenous antigens in disease progression. The palatine tonsils have a unique cellular composition in the reticulated subepithelium, which is ideal for productive antigen sampling for rapid and broad defense against microorganisms at the gate of the respiratory and digestive tracts. Transient mucosal activation of a pattern recognition receptor, such as TLR, by pathogen-associated molecular patterns in IgAN-prone mice is sufficient to exacerbate this disease, with rapid serum elevation of IgA and ICs\textsuperscript{23}. We recently demonstrated that tonsillar levels of TLR9 expression, but not that of other TLRs, were associated with the disease activity of IgAN and clinical outcome of tonsillectomy\textsuperscript{24-28}. Furthermore, the TLR9 genotype was strongly associated with histological severity of IgAN\textsuperscript{23}. Genome-wide scan identifies a copy number
variable region at 3p21.1 that influences the TLR9 expression levels in IgA nephropathy patients. Accordingly, these findings suggest that tonsillar TLR9 signaling pathways may be involved in the pathogenesis of human IgAN. TLR9-ligand CpG-ODN increased the expressions of the APRIL receptors BCMA and TACI on B cells, and enhance B-cell activation and Ig secretion. We are reporting here that chronic CpG-ODN stimulation induced APRIL production by tonsillar B cells. The findings of the present study provide a rationale of tonsillectomy and indicate that TLR9-APRIL axis is a promising specific target for the future treatment, apart from non-specific immunosuppressants or tonsillectomy. Besides, although the possible contribution of specific exogenous antigens to the pathogenesis of IgAN including Haemophilus parainfluenzae has been discussed, there are no consistent antigens yet. Our previous and recent studies regarding the involvement of TLR9 in the pathogenesis of human and murine IgAN indicate that specific antigens are not required for the development of IgAN. However, it seems that there is a biased bacterial flora in tonsil of human IgAN, suggesting that actual exogenous antigens in the pathogenesis may be limited.

We observed that tonsillar levels of APRIL correlated with disease activity and treatment responses to tonsillectomy, indicating that tonsillar GC B cells may be involved in the pathogenesis via their production of APRIL. It is now widely accepted that Gd-IgA1 and related IC are essential effector molecules to induce glomerular damages in IgAN. Serum levels of these molecules, indeed, have clinical diagnostic potential for the assessment of prognosis and disease activity for IgAN, independent of information from renal biopsy. Reports showed abnormal glycosylation of tonsillar IgA and aberrant cytokine profiles in tonsillar B cells, leading to the underglycosylation of IgA1 in IgAN patients. Because total IgA is decreased by approximately 10% on an average after tonsillectomy alone in IgAN patients, and patients who showed a large decrease of serum IgA after the tonsillectomy had better clinical outcome, the palatine tonsil was hypothesized to be a major delivery sources of nephritogenic IgA. Indeed, we recently showed that Gd-IgA1 was significantly decreased after tonsillectomy in IgAN patients who also showed a significant improvement in urinalysis just after tonsillectomy. Present study further demonstrated that IgAN patients with abundant expression of Stalk-1 in tonsillar GC showed more...
proteinuria and better clinical outcomes after the tonsillectomy including improvement of proteinuria and decrease of serum Gd-IgA1 levels. These findings suggest that palatine tonsils with overexpression of APRIL may be one of the major delivery source of nephritogenic IgA. However, some patients notably showed improved hematuria and serum GdIgA1 levels following steroid pulse therapy after the tonsillectomy as compared with just after tonsillectomy, suggesting that GdIgA1-producing cells may also be localized outside the tonsils. Recent data have revealed that some of the NALT-derived and activated B cells, and even tonsillar B cells, can migrate from inductive mucosal sites to systemic effector sites including bone marrow through guiding adhesion molecules and chemokine/chemokine receptors. In addition, our recent study revealed that IgA1 secreted by Epstein–Barr virus (EBV)-immortalized B cells from the peripheral blood of IgAN patients was mostly polymeric with galactose-deficient sialylated O-glycans. These findings support the hypothesis that GdIgA1-producing B cells may travel between the tonsils and systemic lymphoid organs and produce the nephritogenic IgA outside of mucosal sites. Moreover, bone marrow transplantation with an IgAN donor reconstitutes IgAN in humans and mice, suggesting that GdIgA1-producing B cells could localize in the bone marrow. Indeed, it has been reported that IgA plasma cells containing subclass IgA1 were increased in IgAN patients compared to controls. It has also been shown that there was an increase in the proportion of IgA+ cells which express J chain mRNA, which is essential for the production of dimeric IgA, in the bone marrow of IgAN patients. These evidences emphasize the possibility that the bone marrow could be the production site of the IgA1 found in the circulation and mesangial deposits in IgAN patients. Thus, we can speculate that mucosally-primed/activated GdIgA1-producing B cells are disseminated to systemic organs such as lymph nodes and tonsils, or the bone marrow.

In conclusion, APRIL+ GC B cells in tonsils may determine the disease activity of IgAN patients, presumably via production of Gd-IgA1 prior to IC formation. Aberrant TLR9 activation in tonsillar B cells may be involved in the underlying mechanisms of the tonsillar overexpression of APRIL, partly with APRIL variants, in IgAN (Figure 7).
Methods

Patients and treatment protocol
Fifty-five biopsy-proven IgAN (26 males) and 12 CT (6 males) patients, who had undergone tonsillectomy at the Department of Otorhinolaryngology of Juntendo University Hospital, Narita Memorial Hospital and Tokyo Metropolitan Health and Medical Treatment Corporation Okubo Hospital, were included in this study. Patient demographics and clinical characteristics are summarized in Table 1. Both before and after tonsillectomy, IgAN patients were evaluated for the following clinical outcomes: proteinuria [g/g creatinine (Cr); g/gCr] and serum levels of IgA and Gd-IgA1. The average duration from tonsillectomy to quantification of these clinical parameters was 69.2±47.2 days. This study was conducted in accordance with the principles of the Declaration of Helsinki and the study protocol approved by the Institutional Review Boards of each hospital. Informed consents were obtained from all patients before inclusion in the study.

Real-time quantitative reverse transcription PCR and sequencing
qPCR on RNA isolated from tonsillar tissues and cells was performed as described previously. A Homo sapiens-specific Taqman gene expression assay (Life Technologies Co, Carlsbad, CA) was purchased for the TNF (ligand) superfamily, member 13 (APRIL) (Hs00601664_g1) and TLR9 (Hs00370913_s1) as well as for an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1).

A SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA) and a 7500 Real-Time PCR System (Applied Biosystems) were also used for qPCR for APRIL-α and δ/zeta expression. Transcript levels were normalized by GAPDH. The following primers synthesized by Life Technologies Co were used: APRIL-α, 5’-AGGAGAGCAGTGCTCACCC-3’ and 5’-CTCCAGCATCCTGGATTCG-3’; APRIL-δ, 5’-AGAGTCTCCGGAGCAGCAG-3’ and 5’-CTCCAGCATCCTGGATTCG-3’; and GAPDH, 5’-TGACTCCGACCTCCACCTTC-3’ and 5’-CTCTGCTCCTCCTGTCGAC-3’.
For sequencing purposes, total RNA from tonsillar B cells was extracted using the QIA shredder (Qiagen, Valencia, CA) and the RNeasy Mini Kit (Qiagen). The PCR was performed with Takara Ex Taq DNA polymerase (Takara, Shiga, Japan). PCR fragments were isolated from 2% agarose gels using a gel extraction kit (Qiagen). PCR products were ligated into the pMD20 vector (Takara). All standard cloning and plasmid propagation was performed in *Escherichia coli* strain INVαF’ (Invitrogen AG, Basel, Switzerland). Clones were first screened by restriction enzyme digestion (EcoRI and SphI, Takara) and subsequent RT-PCR were subjected to sequence analysis with primers Seq Forward M13 Primer RV (5’-CAGGAAACAGCTATGAC-3’) and Seq Reverse M13 Primer M4 (5’-GTTTTCCCAGTCAGCAG-3’). The REH cell line was obtained from the American Tissue and Cell Collection (Manassas, VA).

**Immunohistochemical analysis**

For immunohistochemistry, the paraformaldehyde-fixed paraffin-embedded tonsillar samples from IgAN and CT patients were cut at a thickness of 3 μm. All sections were deparaffinized in xylene followed by 100% ethanol, and then placed in a freshly prepared methanol/0.3% H₂O₂ solution for 10 min. Microwave antigen retrieval was performed with a hot 0.01 mol/L citrate buffer for 20 min. The sections were cooled to room temperature, and then blocked with a blocking solution (DS Pharma Biomedical, Osaka, Japan). The primary antibody Stalk-1 was used at 5µg/ml. The polyclonal rabbit antiserum Stalk-1 was raised against a peptide in the membrane-proximal part of APRIL extracellular domain remaining associated to the cell membrane after furin cleavage 29. The secondary antibody was a horseradish peroxidase (HRP)-labeled anti-rabbit antibody (1:50, Dako, Tokyo, Japan). Sections were washed with phosphate-buffered saline (PBS; pH 7.4) three times after each incubation. An individual GC was considered a Stalk-1⁺ GC when 25% of its area was covered by Stalk-1⁺ cells. Image acquisition was performed with a ×40 objective. Cell numeration was performed in a tissue area of 30mm². Stainings were evaluated by two nephrologists who were blinded to patients’ clinical data.

For immunofluorescence staining, tonsillar tissues were mounted in optimal cutting
temperature (OCT) compound (Sakura Finetek, Tokyo, Japan), immersed in liquid nitrogen, and stored at -80°C. These frozen specimens were cut into 3 mm sections and fixed with 4% paraformaldehyde at -20°C for 10 min. Paraformaldehyde-fixed frozen tonsillar tissue sections were then stained with Stalk-1, Aprily-2 (mouse IgG, 2 µg/ml), anti-elastase (NP57, mouse IgG, 1:200, Dako), anti-CD19 (LE-CD19, mouse IgG, 1:100, Dako), anti-human IgG (3E8, mouse IgG, 0.5 µg/ml, Santa Cruz, Dallas, TX), anti-human IgA (47C12, mouse IgG, 1:200, Santa Cruz), and anti-human IgM (R1/69, mouse IgG, 1 µg/ml, Santa Cruz). The mAb Aprily-2 was raised against the C-terminal TNF homology domain of APRIL, secreted on furin cleavage 29. Slides were incubated with the following secondary reagents: Alexa 488-conjugated anti-rabbit Ig (Invitrogen AG) and Alexa 555-conjugated anti-mouse Ig (Invitrogen AG). Nuclei were visualized with 4′,6-diamidine-2′phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Indianapolis, IN). Images were acquired with a confocal laser-scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan). The number of Stalk-1^+ elastase^+ positive cells was quantified with KS400 Image Analysis System (Carl Zeiss, Oberkochen, Germany) by two nephrologists.

ELISA for Gd-IgA1

The serum level of Gd-IgA1 was measured by lectin ELISA using GalNAc-specific lectin from Helix aspersa (HAA; Sigma, St. Louis, MO) as previously reported45, 54, 60, 61. Diluted sera were added 100 ng per well of serum IgA. The captured IgA was treated with 10 mU/ml neuraminidase (Roche Diagnostics Corp. Indianapolis, IN) to remove terminal sialic acid residues45, 60. The desialylated IgA1 was then reacted with biotin-labeled HAA and subsequently developed absorbance was measured at 490 nm. The HAA reactivity of IgA1 in each sample was then calculated as OD units/100 ng of serum IgA. Naturally galactose-deficient IgA1 (Ale) myeloma protein60 treated with neuraminidase and was used as the standard. Serum level of total Gd-IgA1 was expressed in relative Units, calculated by multiplying the normalized HAA reactivity by the amount of IgA in the serum sample (mg/ml).
**Tonsillar cell preparation**

After surgery, tonsil samples were dissected into small pieces in 2 mg/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ), and filtered on a 100 μm cell strainer. Tonsil cell stimulation was performed daily with 10 μg/ml of CpG ODN 1826 and control ODN 1982 (Microsynth, Balgach, Switzerland). Tonsillar B cells were purified using a Dynabeads untouched Human B cells Kit (Invitrogen AG) according to the manufacturer’s instructions.

**Flow cytometric analysis**

1×10^6 tonsillar cells were stained for flow cytometry. The cells were preincubated with Fc receptor blocking reagent (MBL, Aichi, Japan), and incubated for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD19 antibody (Biolegend, San Diego, CA), APC mouse anti-human CD38 (BD Pharmingen, San Jose, CA), PE mouse anti-human IgD (BD Pharmingen). Biotinylated anti-TACI and BCMA have been previously described^62_. PE-conjugated streptavidin was from BD Pharmingen. Total staining was performed after permeabilization with a Cytofix/Cytoperm solution (BD Pharmingen) for 30 min at 4°C. After washing twice in Perm/Wash solution, cells were incubated for 30 min at 4°C with Stalk-1 (5 μg/ml) and Aprily-2 (5 μg/ml). Brilliant Violet 421 donkey anti-rabbit or mouse IgG (Biolegend) was used as secondary antibodies. After washing the cells twice in BD Perm/Wash solution, labeled cells were analyzed by flow cytometry using a FACSVerse flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and the FlowJo program (Tree Star, Ashland, OR).

**Statistical Analysis**

Statistical analyses were performed using Graph Pad PRISM software, version 6.0 (GraphPad, La Jolla, CA). Comparisons between groups were analyzed by the Mann-Whitney *U* test. Spearman’s regression analysis was used to analyze the correlation between two variables. Differences at *P*<0.05 were considered significant.
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Authorship

Contribution: M.M. H.S. B.M. and Y.S. performed the experiments and analyzed the data. Y.S. S.I. and B.H. designed the study. Y.S., M.M., S.I. and B.H. wrote the paper. K.J., M.N., S.W., C.R., and M.Maiguma provided patients materials and information. Y.S. and Y.T. designed the research protocol.

DISCLOSURES

None.
Increased APRIL expression in tonsils from IgAN patients.

A) Tonsillar APRIL mRNA expressions in IgAN patients (n = 24) were significantly higher than those in chronic tonsillitis patients (CT; n = 6) (*P<0.01). Bars represent the mean ± SEM. B) Immunohistochemistry with Stalk-1 (specific for APRIL-producing cells) in IgAN (upper panels) and CT (lower panels) patients. Representative germinal centers (GC) are shown on the right panels. Scale bars=250µm and 100µm for left and right panels, respectively. Arrow and arrowhead mark a Stalk-1 stained neutrophil and epithelial cells, respectively. Pictures shown are representative of 56 tonsils from IgAN patients and 12 tonsils from CT patients. C) Quantification of Stalk-1⁺ elastase⁺ neutrophils showed no significant difference. D) However, percentage of GC containing APRIL-producing cells (Stalk-1⁺GC) was significantly different in total tonsillar GC from IgAN patients and CT patients (*P<0.01).

CD19⁺ B cells produce APRIL in tonsillar GC of IgAN patients.

A) IgAN tonsils showed co-staining for Stalk-1 (green) and CD19, IgM, IgG and IgA (red). A representative GC is shown. Pictures shown are representative of tonsils from IgAN patients. Scale bars = 20µm. B) A cell suspension from IgAN and CT tonsils was surface stained for CD19, CD38, IgD and after cell permeabilization for Stalk-1 (left panel). Plots for cells gated on CD19 are representative of 13 IgAN and 4 CT patients. The percentage of Stalk-1⁺ cells among CD19⁺IgD⁻CD38⁻/⁻ cells is also shown (right panel) (**P<0.05).

Tonsillar GC B cells of IgAN express cleavable and uncleavable APRIL.

A) IgAN tonsils were stained for Stalk-1. A representative GC B cells (right panel) are shown. Pictures shown are representative of 56 IgAN patients. B) IgAN tonsils were co-stained for Stalk-1 (green) and Aprily-2 (red). A representative GC is shown. Scale
bars = 20µm. C) Predicted amino-acid sequences of different isoforms of APRIL. The GenBank accession numbers for APRIL-α, APRIL-δ and APRIL-zeta are NM_003808, NM_001198622 and NM_001198623.1, respectively. The furin cleavable site lacking in APRIL-δ and APRIL-zeta is highlighted in grey. Identities are indicated by dashes and deletions by dots. Numbers indicate amino-acid positions. D) Correlation between APRIL-α and APRIL-δ/zeta mRNA expression in purified tonsillar B cells from IgAN (n = 20) and CT (n = 6) patients. Both APRIL-α and APRIL-δ/zeta mRNA expression in tonsillar B cells were significantly higher in IgAN patients (**P<0.05). Bars represent the mean ± SEM.

Figure 4

Correlation between TLR9 and APRIL mRNA expression in IgAN patients

A) TLR9 mRNA expressions in whole tonsils (*P<0.01) (left panel) and purified tonsillar B cells (right panel) (**P<0.05) were significantly higher in IgAN. Bars represent the mean ± SEM. B) TLR9 and APRIL-α (left panel) or δ/zeta (right panel) mRNA expressions in tonsillar B cells were well correlated in IgAN patients.

Figure 5

TLR9 activation induces APRIL expression in tonsillar B cells.

A) Tonsillar B cells isolated from CT patients were stimulated daily with 10 µg/ml of CpG. APRIL expression is shown on viable (upper panel) and permeabilized (middle panel) gated CD19+ B cells. B) Surface expressions of TACI and BCMA are also shown (lower panel). C) CD19+ B cells from CT patients were purified on a FACS ARIA (BD Pharmingen) by positive selection. Purified CD19+ B cells were stimulated daily with 10 µg/ml of CpG. APRIL expression is shown on permeabilized cells. Shaded histograms represent control isotype-matched reactivity. Dotted and straight lines represent indicated antibody reactivities on control and CpG ODN stimulated cells, respectively, at day 7. Histogram plots are representative of at least three experiments performed with tonsils from independent patients.
Correlation between APRIL expression in tonsillar GC and disease activity in IgAN patients.

A) Percentage of Stalk-1⁺GC in tonsils of IgAN patients and their proteinuria level. The percentage of Stalk-1⁺GC in IgAN patients with proteinuria more than 1g/gCr was significantly higher than that in those less than 1g/gCr (*P<0.01). B-D) Comparison of proteinuria level (B), % decrease of the serum IgA (C) and the serum Gd-IgA1 levels (D) between IgAN patients with the percentage of Stalk-1⁺GC ≥ 10%, and those with the percentage of Stalk-1⁺GC < 10%. IgAN patients with Stalk-1⁺GC ≥ 10% showed significantly higher proteinuria before tonsillectomy (**P<0.05) and larger decrease of serum levels of Gd-IgA1 after tonsillectomy (**P<0.05) than those with Stalk-1⁺GC < 10%. E-G) Comparison of proteinuria levels before and after tonsillectomy in whole IgAN patients (E), those with Stalk-1⁺GC ≥ 10% (F) and those with Stalk-1⁺GC<10% (G). There were significant differences between before and after tonsillectomy in the preceding two groups (*P<0.01), despite no significance in IgAN patients with Stalk-1⁺GC<10%. The average duration from tonsillectomy to quantification of these clinical parameters was 69.2±47.2 days.

Cross-talk between APRIL and TLR9 on B cells in tonsillar GC of IgAN patients.

Present study revealed aberrant APRIL expression in tonsillar GC B cells from IgAN patients. Based on our findings, we hypothesize that activation of intracellular TLR9 through exogenous antigens may be involved in this overexpression consisting not only APRIL-α but also uncleaved APRIL, such as APRIL-δ/zeta in tonsillar GC B cells of IgAN patients. This TLR9 activation also upregulates expression of TACI and BCMA, and increases both BCR signaling and APRIL sensitivity. This aberrant APRIL expression may induce long-term survival of GC B cells responsible for the production of aberrant antibodies including Gd-IgA1, and thereby, contribute to subsequent
progression of IgAN.


6. Tomana M, Matousovic K, Julian BA, Radl J, Konecny K, Mestecky J:

Galactose-deficient IgA1 in sera of IgA nephropathy patients is present in complexes with IgG. *Kidney Int* 52: 509-516, 1997


the bone marrow and poorly expressed by early-life bone marrow stromal cells.


TNF superfamily member 13 in the progression of IgA nephropathy. *J Am Soc Nephrol* 2016


identifies a copy number variable region at 3p21.1 that influences the TLR9 expression levels in IgA nephropathy patients. *Eur J Hum Genet* 23: 940-948, 2015


58. van den Wall Bake AW, Daha MR, Evers-Schouten J, van Es LA: Serum IgA and the production of IgA by peripheral blood and bone marrow lymphocytes in patients with primary IgA nephropathy: Evidence for the bone marrow as the source of mesangial IgA. *Am J Kidney Dis* 12: 410-414, 1988


Table 1: Profiles of patients with IgA nephropathy (IgAN) and chronic tonsillitis (CT) just before tonsillectomy

<table>
<thead>
<tr>
<th></th>
<th>IgAN</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>55</td>
<td>12</td>
</tr>
<tr>
<td>age</td>
<td>35.1</td>
<td>31.4</td>
</tr>
<tr>
<td>Male (%)</td>
<td>47.3 (26: 29)</td>
<td>50 (6: 6)</td>
</tr>
<tr>
<td>Duration from onset to tonsillectomy (year)</td>
<td>8.5±8.8</td>
<td>—</td>
</tr>
<tr>
<td>sCr (mg/dl)</td>
<td>0.86±0.3</td>
<td>0.6±0.14</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>13.1±3.2</td>
<td>11.5±3.6</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m2)</td>
<td>79.5±28.1</td>
<td>116.4±26.7</td>
</tr>
<tr>
<td>Proteinuria/urine Cr (g/gCr)</td>
<td>1.22±0.86</td>
<td>—</td>
</tr>
<tr>
<td>Hematuria (RBC/HPF)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1-4/HPF</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>5-9/HPF</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>10-15/HPF</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>16-20/HPF</td>
<td>11</td>
<td>—</td>
</tr>
<tr>
<td>21-25/HPF</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>26-30/HPF</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>&gt;30HPF</td>
<td>16</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ±SD.

eGFR, estimated glomerular filtration rate.

Hematuria: Assessed by assigning scores according to number of red blood cells per high-power field (RBC/HPF).

Table 2: The correlation between Stalk-1+ GC and the severity of the efficacy of tonsillectomy in IgAN patients

<table>
<thead>
<tr>
<th></th>
<th>Stalk-1+GC&lt;10% (n=12)</th>
<th>Stalk-1+GC≥10% (n=43)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (g/gCr) before treatment</td>
<td>0.69±0.48</td>
<td>1.35±0.9</td>
<td>0.0298</td>
</tr>
<tr>
<td>Patients whose proteinuria decrease more that 50% after tonsillectomy (%)</td>
<td>25.0 (3/12)</td>
<td>62.8 (27/43)</td>
<td>0.0201</td>
</tr>
</tbody>
</table>
Figure 1

A

APRIL Ratio/GAPDH

IgAN CT

0 2 4 6 8 10

★

Percentage of GC stained with Stalk-1

B

IgAN

CT

C

Stalk-1 + elastase + cell/mm²

NS

IgAN CT

D

Percentage of GC stained with Stalk-1

★

IgAN CT
**Figure 2**

A

[Images of fluorescence microscopy showing merge, Stalk-1, CD19, IgM, IgG, and IgA staining patterns.]

B

[Scatter plots and histograms illustrating FSC and SSC, CD19 and CD38 expression, and cell counts.]

★★

**CTIgAN**

<table>
<thead>
<tr>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
</table>

Stalk-1+ GC B cells/GC B cells

[Graph showing comparison between CTIgAN and IgAN groups.]
Figure 3

A

B

C

D

Tonsillar B cells

| APRIL α     | 1 mpaspsflapkpoppmpgpvyrepalsva1a1s1gavacommattgtelqelrr |
| APRIL delta  | 1 ---------------------------------------------------------- |
| APRIL zeta   | 1 ---------------------------------------------------------- |
| APRIL α     | 61 avozlqpttgppxnpsoyqpe1qpeqoa1avewqepvse1qepqorpretqepqorpret |
| APRIL delta  | 61 ---------------------------------------------------------- |
| APRIL zeta   | 61 ---------------------------------------------------------- |
| APRIL α     | 121 vpinatskddsdvtevmwqpalrrgrglqaqgygvriqdagvyllysqvlfqvdmgq |
| APRIL delta  | 94 ---------------------------------------------------------- |
| APRIL zeta   | 93 ---------------------------------------------------------- |
| APRIL α     | 181 vrozegqgrqetlfrcirsmpshpdravynscysagvfhlhqgdilsviipraknlsp |
| APRIL delta  | 154 ---------------------------------------------------------- |
| APRIL zeta   | 153 ---------------------------------------------------------- |
| APRIL α     | 241 hqtfiqlfvl |
| APRIL delta  | 214 ---------------------------------------------------------- |
| APRIL zeta   | 213 ---------------------------------------------------------- |

D

**

Tonsillar B cells

<table>
<thead>
<tr>
<th>APRIL δ/zeta Ratio/GAPDH</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgAN</td>
<td>1</td>
</tr>
<tr>
<td>CT</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>APRIL δ/zeta Ratio/GAPDH</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgAN</td>
<td>0</td>
</tr>
<tr>
<td>CT</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 4

A Tonsils

Tonsillar B cells

B

TLR9 Ratio/GAPDH

APRILα Ratio/GAPDH

APRIL δ/zeta Ratio/GAPDH

R²=0.7611

P<0.0001

R²=0.7079

P<0.0001
Figure 5

A

Cell count

Stalk-1

Aprily-2

B

Cell count

Stalk-1

Aprily-2

BCMA

TACI

C

Cell count

Stalk-1

Aprily-2

TACI

BCMA
Figure 6

A. Percentage of GC stained with Stalk-1

B. Proteinuria (g/gCr)

C. % decrease of serum levels of IgA

D. % decrease of serum levels of Gd-IgA1
Figure 6

Proteinuria (g/gCr)

E Total

before tonsillectomy after tonsillectomy

F GC ≥ 10%

before tonsillectomy after tonsillectomy

G GC < 10%

before tonsillectomy after tonsillectomy

NS

Figure 6
Figure 7

B cells in tonsillar GC

- TACI
- BCMA
- TLR9
- endosome
- nuclei
- exogenous antigens
- CpG

Self reproduction

- sAPRIL (APRILα)
- mAPRIL (APRILδ/zeta)

γ: Gd-IgA1
**Supplement Table.1**
The correlation between Stalk-1*GC and the severity or the efficacy of tonsillectomy in severe IgAN patients (proteinuria >0.5 g/gCr)

<table>
<thead>
<tr>
<th></th>
<th>Stalk-1+GC&lt;10% (n=8)</th>
<th>Stalk-1+GC≧10% (n=39)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (g/gCr) before treatment</td>
<td>0.99±0.36</td>
<td>1.46±0.86</td>
<td>0.22</td>
</tr>
<tr>
<td>Patients whose proteinuria decrease more that 50% after tonsillectomy (%)</td>
<td>28.6(1/8)</td>
<td>71.9 (26/39)</td>
<td>0.048</td>
</tr>
</tbody>
</table>

**Supplement Table.2**
Characteristics of IgAN patients before tonsillectomy and after tonsillectomy

<table>
<thead>
<tr>
<th></th>
<th>before tonsillectomy</th>
<th>after tonsillectomy</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>106.3±9.4</td>
<td>109.7±11.7</td>
<td>0.1553</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>64.1±7.0</td>
<td>63.3±8.9</td>
<td>0.1983</td>
</tr>
<tr>
<td>Proteinuria/urine Cr (g/gCr)</td>
<td>1.22±0.86</td>
<td>0.60±0.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Steroid therapy</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>The use of ACEIs and ARBs</td>
<td>19</td>
<td>21</td>
<td>0.6918</td>
</tr>
</tbody>
</table>

ACEI, Angiotensin Converting Enzyme Inhibitor
ARB, Angiotensin II Receptor Blocker