Immunoglobulin A nephropathy is characterized by anti-commensal humoral immune responses

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IgA nephropathy (IgAN) is a leading cause of kidney failure, yet little is known about the immunopathogenesis of this disease. IgAN is characterized by deposition of IgA in the kidney glomeruli, but the source and stimulus for IgA production is not known. Clinical and experimental data suggest a role for aberrant immune responses to mucosal microbiota in IgAN, and in some countries of high disease prevalence tonsillectomy is regarded as standard-of-care therapy. To evaluate the relationship between microbiota and mucosal immune responses we characterized the tonsil microbiota in patients with IgAN versus non-related household-matched control subjects and identified increased carriage of the genus Neisseria and elevated Neisseria-targeted serum IgA in IgAN cases. We reverse-translated these findings in experimental IgAN driven by BAFF overexpression in BAFF-transgenic mice, rendered susceptible to Neisseria infection by introduction of a humanized CEACAM-1 transgene (B x hC-Tg). Colonization of B x hC-Tg mice with Neisseria yielded augmented levels of systemic Neisseria-specific IgA. Using a custom ELISPOT assay, we discovered anti-Neisseria-specific IgA-secreting cells within the kidneys of these mice. These findings suggest a role for cytokine-driven aberrant mucosal immune responses to oropharyngeal pathobionts such as Neisseria in the immunopathogenesis of IgAN. Furthermore, in the presence of excess BAFF, pathobiont-specific IgA can be produced in situ within the kidney.

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Conflict of interest:

Dr. Reich is a national coordinating investigator for the Calliditas NEFIGARD study, and is a site investigator for therapeutic trials in IgAN funded by Alnylam and Omeros. She has provided consultation outside of the submitted work for Novartis, Chinook, and Travere. She is a site investigator for a study in FSGS sponsored by Pfizer. The GN Fellowship at University Health Network is supported by the Louise Fast Foundation.

Dr. Hladunewich reports participation as a site lead for studies in IgAN funded by Ionis, Calliditas, Pfizer, and Alnylam, with consultation outside of the submitted work. She also is a site lead for a Chemocentryx study of FSGS. She is the section editor for the kidney disease and pregnancy section of Uptodate. She is the medical lead for glomerulonephritis and pregnancy for the Ontario Renal Network.

Dr. Novak is a co-founder and co-owner of and consultant for Reliant Glycosciences, LLC and a co-inventor on US patent application 14/318,082 (assigned to UAB Research Foundation) and has a sponsored research agreement with Travere.

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Abstract

IgA nephropathy (IgAN) is a leading cause of kidney failure, yet little is known about the immunopathogenesis of this disease. IgAN is characterized by deposition of IgA in the kidney glomeruli, but the source and stimulus for IgA production is not known. Clinical and experimental data suggest a role for aberrant immune responses to mucosal microbiota in IgAN, and in some countries of high disease prevalence tonsillectomy is regarded as standard-of-care therapy. To evaluate the relationship between microbiota and mucosal immune responses we characterized the tonsil microbiota in patients with IgAN versus non-related household-matched control subjects and identified increased carriage of the genus *Neisseria* and elevated *Neisseria*-targeted serum IgA in IgAN cases. We reverse-translated these findings in experimental IgAN driven by BAFF overexpression in BAFF-transgenic mice, rendered susceptible to *Neisseria* infection by introduction of a humanized CEACAM-1 transgene (B x hC-Tg). Colonization of B x hC-Tg mice with *Neisseria* yielded augmented levels of systemic *Neisseria*-specific IgA. Using a custom ELISPOT assay, we discovered anti-*Neisseria*-specific IgA-secreting cells within the kidneys of these mice. These findings suggest a role for cytokine-driven aberrant mucosal immune responses to oropharyngeal pathobionts such as *Neisseria* in the immunopathogenesis of IgAN. Furthermore, in the presence of excess BAFF, pathobiont-specific IgA can be produced *in situ* within the kidney.
Introduction

The mechanisms underlying the pathogenesis of immunoglobulin A nephropathy (IgAN) are poorly understood, yet it is one of the most common causes of kidney failure. The widely accepted “multi-hit hypothesis” for development of IgAN focuses on the production of pathogenic galactose-deficient IgA1-containing immune complexes in the circulation and deposited within kidney glomeruli of patients with IgAN(1). The source (ie. mucosal-associated lymphoid tissues and/or bone marrow) and stimuli for immune complex production are not known. We have recently demonstrated that IgA-producing plasma cells (PC) from the gut can migrate to non-immune tissues such as the inflamed brain in the experimental encephalitis model of multiple sclerosis, radically shifting prevailing views on the properties of PC(2). It is therefore tempting to speculate that mucosal-derived IgA-secreting cells could also be recruited to other organs such as the kidney.

Clinical and experimental evidence suggest a link between IgAN and mucosal microbial exposures. Patients with IgAN experience macroscopic hematuria concurrently with pharyngitis(3) and tonsillectomy is considered standard-of-care for patients with IgAN in Japan (4-6). Our previous studies of a murine IgAN model suggest that commensal microbial colonization is essential for the development of IgAN in mice overexpressing the TNF-family BAFF(7, 8). This model shares many features with human IgAN, including under-glycosylated IgA, clinical and histologic measures of kidney injury such as proteinuria and IgA-dominant glomerulonephritis(7, 8). The TNFSF13 (APRIL) locus was subsequently linked to IgAN in genome-wide association studies(9). Since APRIL and BAFF share similar receptors, the BAFF/APRIL axis has been implicated in IgAN disease pathogenesis.

We therefore hypothesized that in the setting of high levels of BAFF/APRIL, mucosal-derived IgA-secreting cells are recruited to the kidney and contribute to development of IgAN. To test this hypothesis we characterized the tonsil and stool microbiome profiles of a large cohort of patients with IgAN compared to household-matched non-related control subjects. We identified increased tonsil Neisseria carriage and enhanced production of anti-Neisseria-targeted IgA in the blood of our patient cohort. We reverse translated these observations into an experimental IgAN model and found that elevated levels of BAFF provoke an enhanced IgA-biased systemic immune response to mucosal Neisseria exposure. We noted increased expression of RNA encoding the secreted-spliced variant of IgA (typically mucosal origin) in the kidneys of our experimental mice, suggesting local IgA transcription. Using a custom ELISPOT, we discovered that mucosal-derived anti-Neisseria-specific IgA secreting cells are detectable in the kidneys of these mice. Our findings are the first to show that a maladaptive host response to a commensal organism is associated with IgAN disease and recruitment of mucosal-derived IgA-secreting cells to the kidney.
Results

16s rRNA analysis reveals alterations in the tonsillar microbiome of IgAN patients compared to healthy controls

Given the association between pharyngitis and hematuria in IgAN(4), we compared the tonsillar and stool microbiota of cohort of IgAN patients (n=93) with that of healthy non-related household-matched subjects (n=58) using 16S rRNA V4 sequencing. Clinical characteristics are provided in Table 1. We did not observe any statistically significant differences in Shannon diversity or taxonomic richness between cases and healthy control tonsil samples (Supplementary Figure 1). The dominant tonsillar genus differed between cases and controls (Figure 1A), with a trend towards differences in Neisseria as the dominant genus in IgAN tonsil samples (chi-squared p=0.15). We therefore evaluated the difference in relative abundance of Neisseria (Figure 1b) and confirmed the presence of significantly greater abundance of tonsil Neisseria in IgAN compared to controls (2-tailed unadjusted t test p value 0.002). No differences were noted in diversity or taxonomic richness of stool microbiota in IgAN compared to controls (Supplementary Figure 2A).

Humoral immune responses to Neisseria are biased towards IgA versus IgG in IgAN patients

Based on the observed differences in tonsillar microbial abundance, we hypothesized that patients may exhibit altered titres of anti-commensal antibodies in their circulation. As Neisseria represented the most abundant genus in patients, we measured anti-Neisseria humoral responses evaluating the ratio of anti-Neisseria IgA to anti-Neisseria IgG in serum. This ratio is reported as an average IgA/IgG response against a panel of 4 commensal Neisseria species (anti-N. lactamica, N. sicca, N. cinerea, and N. flavescens) and 4 pathogenic N. meningitidis strains (90/18311, H4476, 208 and 860800). As illustrated in Figure 2, IgAN patients exhibit an increase in the ratio of plasma IgA/IgG anti-Neisseria antibody titers. Increased IgA titres against Neisseria were observed in response to both pathogenic N. meningitidis (Nme) and commensal strains (Supplementary Figure 3). Therefore, IgAN patients exhibit a bias towards generating an enhanced IgA response to a variety of commensal Neisseria species and Nme strains compared to healthy controls.

Effect of BAFF overexpression on the immune response to N. meningitidis in BAFF-Transgenic mice

We previously reported that the cytokine APRIL (TNFSF13) was elevated in the serum of IgAN patients compared to controls in two independent cohorts(8). We confirmed that APRIL is elevated in patients with IgAN in the current cohort (median 1.98 vs. 1.55, IQR 1.75, 0.38, p<0.01). Moreover, we observed a positive correlation between serum APRIL levels and proteinuria (Spearman Rho 0.28, p=0.01). As in our previous study we did not observe differences in serum BAFF levels.
APRIL binds to TACI, a TNF receptor that promotes class switch to IgA(10). TACI is also stimulated by higher order multimers of BAFF, and over-expression of BAFF in BAFF-Tg mice is sufficient to stimulate TACI, thus mimicking an APRIL/TACI signal(11). Importantly, as they age, BAFF-Tg mice exhibit an IgAN-like disease characterized by under-glycosylated IgA in the serum, IgA deposition in the kidney, elevated proteinuria and kidney pathology(8). We previously showed that the microbiota are an essential co-factor promoting the deposition of IgA immune complexes in the kidneys of BAFF over-expressing BAFF-Tg mice(8). This implies that a host-microbiome interaction may play a role in the etiology of IgAN, at least in mice. However in our previous studies we did not assess whether specific microbial candidates could accelerate the pathogenesis of IgAN-like disease in BAFF-Tg mice.

Therefore, we next explored the possibility that *Neisseira*-targeted IgA-biased immune responses may be observed in BAFF-overexpressing mice. *Nme* infects epithelium via the CEACAM-1 receptor. Because *Nme* will bind to human and not mouse CEACAM-1, we crossed BAFF-Tg mice to mice that express the human form of CEACAM-1 (hC-Tg) to generate double transgenic mice (B x hC-Tg). Transgenic mice and their littermate controls were nasally infected with *Nme*, and serum and nasal lavages were subsequently collected for analysis. As illustrated in Figure 3A, although hC-Tg<sup>neg</sup> (WT) mice are not appreciably colonized by *Nme*, hC-Tg and B x hC-Tg mice exhibit similar rates of *Nme* colonization (80% vs 100%, p=NS) and similar bacterial burdens (192.9 vs 116.7, p=NS) at 5 days post infection. These data indicate that B x hC-Tg mice do not have altered rates of *Nme* colonization in comparison to WT mice.

We have previously shown that two nasal infections are required to induce immunity against a subsequent third nasal infection in hC-Tg mice(12). To determine whether B x hC-Tg mice exhibited improved clearance of *Nme* compared to hC-Tg controls, we nasally infected mice twice, on days 1 and 14. At 24 hours post-secondary infection, the rate of infection of hC-Tg mice and B x hC-Tg mice was similar (85% vs 80%), demonstrating a lack of neutralizing immunity induced by 1 previous exposure of *Nme* (Figure 3B). This implies that overexpression of BAFF does not eliminate the need for two infections to induce protection against colonization in our mouse model.

Given that there was no obvious impact of BAFF overexpression on nasal susceptibility to *Nme*, we looked at the mouse antibody response to *Nme* colonization. First, we assessed the local IgA and IgG response to *Nme*. Nasal *Nme*-specific IgG, as evaluated in nasopharyngeal lavage fluid, was not detected in any mice, and nasal anti-*Nme* IgA titers were similar for both B x hC-Tg and hC-Tg mice (Figure 3C). We next measured the systemic antibody response to *Nme* infection. Following the second nasal infection, both hC-Tg and B x hC-Tg mice exhibited an *Nme*-specific IgG response in serum above baseline, with hC-Tg mice exhibiting a mean anti-*Nme* IgG titre 2-fold greater than that in B x hC-Tg mice (164ng/mL vs 54.6ng/mL) (Figure 3D). In contrast, B x hC-Tg mice exhibited a mean anti-*Nme* IgA titer in serum that was 10-fold higher than observed in hC-Tg controls (321.7ng/mL vs 33.09ng/mL) (Figure 3E). Taken together our findings demonstrate that whereas the local IgA response to *Nme* is comparable between hC-Tg and B x hC-Tg mice, the systemic humoral immune response to nasal *Nme* exposure is IgA-biased in B x hC-Tg mice (Figure 3F).
**Effect of N. meningitidis colonization on IgA in the kidneys of BAFF-Tg mice**

Given that systemic Nme-specific IgA levels were significantly elevated in BAFF-Tg mice, we hypothesized that a combination of the BAFF transgene and Nme infection may accelerate kidney pathology compared to uninfected BAFF-Tg mice. We therefore assessed glomerular mesangial expansion by staining kidney sections with periodic acid Schiff (PAS). Using a semiquantitative score of mesangial expansion, we observed a non-significant trend towards increased mesangial expansion in the B x hC-Tg mice (Figure 4A-B).

We next examined the impact of Nme infection on IgA deposition in the kidney, the diagnostic hallmark of IgAN. Using immunohistochemistry, deposition of mesangial IgG was not detected in any mice (data not shown). Moreover, we did not observe mesangial IgA in the kidneys of WT or hC-Tg mice. In contrast, B x hC-Tg and BAFF-Tg mice exhibited IgA deposition in areas of PAS+ mesangial expansion (representative section Figure 4C). For a more quantitative analysis, expression of mRNA encoding the secreted splice form of IgA was evaluated by qPCR in the kidneys of all groups of mice (Figure 4D). There was a significant difference in the expression of the secreted splice form of IgA across groups (Kruskal-Wallis ANOVA p = 0.01), with double transgenic mice (B x hC-Tg) demonstrating significantly higher levels of IgA expression compared to other experimental groups (Dunn’s multiple-testing adjusted p<0.05 for all comparisons). These results show that colonization of BAFF-Tg mice with Nme results in augmented IgA production within the kidney itself.

Having recently discovered that mucosal-derived IgA-secreting cells migrate to brain tissue in mice with experimental encephalitis(2), we hypothesized that the intrarenal expression of IgA mRNA may reflect the presence of anti-commensal IgA-producing cells that have migrated from the site of initial pathobiont exposure. We therefore designed a custom ELISPOT assay to detect Nme-specific antibody secreting cells. High affinity ELISPOT plates were coated with heat-killed N. meningitidis and incubated with a single cell suspension of kidney-derived immune cells from our experimental mouse; HRP-conjugated IgA detection antibodies were subsequently added. We discovered that Nme-specific IgA-secreting cells are identified in the kidney parenchyma of Nme-infected mice predominantly in the setting of increased BAFF levels (B x hC-Tg mice, Figure 5).
Discussion

The diagnostic hallmark of IgAN is the deposition of nephritogenic galactose-deficient IgA-containing immune complexes in the glomerular mesangium, however the origin of and trigger for pathogenic Gd-IgA1 are not known. Genome-wide association studies demonstrate a TNFSF13 (APRIL) variant is associated with IgAN susceptibility (9) and contributes to IgAN pathology in an experimental model, and elevated APRIL levels are documented in patients with IgAN. Our work suggests that in the setting of high levels of BAFF/APRIL, pathobiont-specific IgA is produced in situ within the kidney by mucosal-derived antibody secreting cells that may contribute to development of IgAN.

Given the clinical and experimental association between mucosal microbial exposure and IgAN, we initially hypothesized that there would be differences in commensal mucosal microbiota in patients with IgAN. We observed a significantly higher rates of tonsil colonization with Neisseria in a large cohort of patients with IgAN, compared to healthy non-related household-matched controls. Moreover, patients with IgAN demonstrate increased levels of serum IgA specifically targeted against both commensal and pathogenic Neisseria species. These findings led us to evaluate Nme exposure as a trigger for IgAN in our experimental IgAN model.

We speculated that an augmented IgA response to Neisseria may contribute to IgAN pathogenesis. While it is unlikely that Neisseria are the only microbial trigger to contribute to IgAN pathogenesis, this finding served as a valuable opportunity to explore how a mucosal immune response to a mucosal pathobiont may contribute to the immunopathogenesis of IgAN. We turned to our experimental IgAN model where we previously demonstrated that BAFF overexpression promotes an IgAN-type kidney disease that is dependent on the presence of commensal microbiota(8).

It is not likely that a single pathobiont will be implicated in development of IgAN. However given the requirement of the hCEACAM receptor to colonize our B-Tg mice with Neisseria, our model provided a unique opportunity to evaluate the impact of colonization by a selected organism on mucosal immune responses and antibody-secreting cell migration. Our findings in this model confirm our observations in the human that the host response to the an oropharyngeal pathobiont such as Neisseria is associated with kidney pathology, and that the BAFF/APRIL axis is a key co-factor in this response.

We did not observe significant differences in the renal histopathology following Nme infection, likely due to the limitations of our model. Generation of marked differences in renal pathology would likely require more time. However, the time-frame that we can maintain Nme colonization safely without compromising the health of the mice is relatively short and this limits our ability to assess long term kidney injury.

There is emerging evidence that IgA-producing cells reactive against antigens present at mucosal surfaces can be found beyond mucosal associated lymphoid tissues. For example, commensal-targeted IgA-producing plasma cells can be detected in the blood and bone marrow(13). We
have recently discovered that IgA producing plasma cells and/or plasma blasts egress from the gut and migrate to the central nervous system in response to induction of experimental encephalitis(2). Our current work suggests that IgA-producing cells originating from the site of *Nme* exposure migrate to the kidney. A recent paper suggests the presence of CD19 positive B cells that co-localize with IgA human IgAN biopsies (14). Further work is required to determine the phenotype of the anti-*Nme* IgA-producing cells localized in the kidney, and the mechanisms responsible for facilitating egress of cells to remote sites.

The relative contribution of the tonsillar versus gut-associated lymphoid tissue to the development of IgAN is a topic of ongoing debate in the nephrology community and has implications for clinical care(15, 16). Tonsillectomy is routinely performed to treat IgAN in Japan, supported by clinical trials demonstrating efficacy (4, 17). Production of APRIL within tonsillectomy samples is enhanced in patients with IgAN compared to non-IgAN samples(18).

Given the important role of gut associated lymphoid tissue in IgA production, it is surprising that we did not observe differences in stool microbiota in patients with IgAN, particularly since this finding is in contrast to previous reports(19). There are several possible explanations. Our study may not have been adequately powered to detect these differences. As demonstrated in inflammatory bowel disease, a more sensitive approach may be required, such as targeted sequencing of IgA-coated bacteria, to identify disease-causing species(20). It is also possible that in mice or in individuals that hyper-produce IgA, the transport of IgA into the lumen via the polymeric IgA receptor may be a rate-limiting step, and saturation of this transport mechanism could result in a limited or negligible effect on the microbiome. Despite these potential limitations it is tempting to speculate that our data support the possibility that tonsil microbial exposures play a more dominant role in production of nephritogenic IgA.

Despite the longstanding suspected relationship between microbial exposures and IgAN, studies of systemic microbe-specific IgA responses are limited. Our study, for the first time, demonstrates that anti-commensal IgA responses to an upper airway commensal microbe are augmented in IgAN. Our mouse model demonstrates that the BAFF/APRIL axis can provoke potentially maladaptive IgA responses to upper airway bacterial challenges. Moreover, organism-specific IgA-producing cells are detectable within the kidney tissue. Thus, in the case of genetically susceptible individuals, a normally harmless commensal organism may assume the role of a pathobiont insofar as its ability, in concert with the host immune system, to provoke a pathogenic response that causes IgA-dominant kidney disease.
Methods

Clinical Cohort
A total of 93 subjects with biopsy-proven IgAN and 58, primarily household-matched non-related healthy control volunteers were recruited. Adult patients with biopsy-proven IgAN were eligible for study if they had no history of concurrent systemic illness or infection and had an estimated glomerular filtration rate (eGFR) > 30 mL/min/1.73 m². Control subjects had no history of kidney disease or systemic illness. Whenever possible these consisted of non-genetically-related household-matched subjects to minimize differences in dietary habits and environment. Study participants could not have received antibiotics within 6 months of microbiota sampling.

The characteristics of patients enrolled in the study are provided in Supplementary Table 1. Patients enrolled in the study had a spectrum of disease severity and were comparable with controls with respect to age and self-reported race. Patients did not have severely impaired renal function (ie. eGFR >30 mL/min/1.73 m²).

The study received approval from the research ethics boards of University Health Network and Sunnybrook (Toronto), University of Toronto, and University of British Columbia.

Microbiota characterization
Tonsil bacteria were sampled using 2 sterile specimen collection swabs, which were placed immediately into cryovials and stored at -80°C. The samples were suspended in PBS and proteinase K, and disrupted by vortex. Bacterial DNA was extracted using the DNeasy Blood and Tissue column-based kit (Qiagen). The DNA integrity and quantity was evaluated using a Nanodrop spectrophotometer (Thermo Scientific). Subjects were instructed to collect their stool using a standard sterile specimen collection container, and then placed in the home freezer. All stool samples were snap frozen upon receipt at the research lab. Following sample homogenization with 0.1 mm glass beads (MoBio), stool bacterial DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacture's protocol.

The V4 hypervariable region of the 16S rRNA gene was amplified using a universal forward sequencing primer and a uniquely barcoded reverse sequencing primer to allow for multiplexing(21). Amplification reactions were performed using KAPA2G Robust HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) following the manufacturer’s protocol. For saliva samples, the V4 region was amplified by an initial denaturation at 95°C for 3 minutes, followed by 24 cycles of 95°C for 15 seconds, 50°C for 15 seconds and 72°C for 15 seconds, ending with a 5-minute extension at 72°C. For stool samples the amplification reactions were carried out for 17 cycles instead of 22 cycles. All amplification reactions were performed in triplicate, checked on a 1% agarose TBE gel and then pooled at even concentrations. The final library was purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The purified library was quantified and sequenced on the Illumina MiSeq System (Illumina, San Diego, CA), according to the manufacturer’s instructions, using the 300 cycle V2 sequencing chemistry to generate 150X2 paired end reads.
Sequencing data were demultiplexed using QIIME2 standard paired-end demultiplexing protocol or directly on the Illumina MiSeq. The UNOISE pipeline, available through USEARCH version 10.0.240, was used for sequence analysis(22-24). The last base, typically error-prone, was removed from all the sequences. Sequences were assembled and quality trimmed using –fastq_mergepairs and –fastq_filter, with a –fastq_maxee set at 1.0 and 0.5, respectively. Assembled sequences less than 233bp were removed. Following the UNOISE pipeline, unique sequences were identified from the merged pairs and sorted. Sequences were denoised and chimeras were removed using the unoise3 command in USEARCH. Assembled sequences were then mapped back to the chimera-free denoised sequences at 97% identity operational taxonomic units (OTUs) using the –usearch_global command. Taxonomy assignment was executed using SINTAX(25), available through USEARCH, and the SINTAX compatible Ribosomal Database Project (RDP) database version 16, with the default minimum confidence cut-off of 0.8(26). OTU sequences were aligned using PyNast accessed through QIIME(27). Sequences that did not align were removed from the dataset and a phylogenetic tree of the filtered aligned sequence data was made using FastTree(28). Low abundance OTUs (<0.005% RA) were removed from the OTU table(29).

Beta diversity was calculated using QIIME(27). The data was rarefied to an even depth of 10,000 sequences per sample. Principal Coordinate Analysis plots were of the rarefied data using QIIME and plotted using EMPeror(30).

Preparation of Neisseria cultures

*Neisseria* strains were cultured on GC agar supplemented with IsoVitalex, and in the context of mouse experiments, VCNT inhibitor (Becton Dickinson) at 37°C with 5% CO2. Log phase growth was achieved by transferring overnight cultures from GC agar into 10mL’s of Brain Heart Infusion (BHI) broth (Becton Dickinson) supplemented with 1% IsoVitalex and incubated at 37°C with agitation for 4hr’s. Broth cultures were diluted to an OD600=0.2 prior to heat killing at 65°C for 30 minutes.

Serum procedures

To measure APRIL in serum samples, we used a commercially available kit (eBioscience, CAT# BMS2008). The kit protocol was used with the following modifications: Human serum samples were diluted 1:3 in nuclease-free water and subsequently heat inactivated in a 60°C water bath for 5 minutes. Heat inactivated samples were then loaded neat onto the microwell strips without using sample diluent. Standard was diluted in the plate, in duplicate, as indicated in the protocol but extended for a total of 13 dilutions followed by a sample diluent blank, a water blank and an internal serum control sample from a non IgAN female, heat inactivated in the same manner as the other samples. Samples were incubated without the anti-APRIL detection antibody for 2 hours, as per the protocol. After washing the plates, 50μl/well of the anti-APRIL-biotin antibody was added for 1 hour using, shaking at RT, as per the protocol. Lastly, the plates were developed for 12-13 minutes before stopping the reaction and reading on a photospectrometer at 450nm.
Serum *Neisseria*-specific IgA and IgG were measured by a custom whole bacteria ELISA as previously described(12). Briefly, multi-well plates (Nunc Maxisorp, Nalgene) were coated with heat-killed *Neisseria* strains and left to dry overnight, then washed and blocked with 5% BSA. Patient plasma samples were diluted 1:1000 and assayed for *Neisseria*-specific antibodies across a panel of four *N. meningitidis* (*Nme*) strains (90/18311, H4476, 208 and 860800) and four commensal *Neisseria* strains (*N. lactamica*, *N. flavescens*, *N. sicca* and *N. cinerea*). Patient samples and standards were applied to the wells in duplicate and incubated overnight. A standard curve was created using human plasma IgA (Calbiochem). Following incubation plates were washed again and the detection antibody, an alkaline phosphatase-conjugated goat targeting human IgA (Jackson ImmunoResearch Laboratories), was applied. Absorbance at 600nm wavelength was measured following 40 minute incubation with BluePhos AP detection substrate (SeraCare, KPL).

**Mice**

BAFF-Transgenic (BAFF-Tg) mice (obtained from Drs. Ann Ranger and Jeff Browning, Biogen Inc) were backcrossed with WT mice (Charles River) and subsequently interbred as BAFF-Tg<sup>+/+</sup> or BAFF-Tg<sup>+-</sup> mice(7, 8). Mice that express transgenic human CEACAM-1 (hC-Tg) have been previously described(12). These mice were crossed with BAFF-Tg mice to generate BAFFxhCEACAM-Tg progeny (B x hC-Tg) for infection experiments.

**Tissue mRNA expression**

At the time of sacrifice a section of kidney tissue was preserved in formalin and paraffin embedded to be used for morphologic assessments and RNA extraction. Tissue was sectioned and collected in 100% xylene for deparaffinization and washed with 75% ethanol. Tissue disruption and protease digestion were performed as previously described(31) The Recoverall kit (Invitrogen) was used for remaining steps of column-based RNA extraction. Reverse transcription was performed using cDNA was generated using SuperScript® IV Reverse Transcriptase (Invitrogen). Abundance of the secreted-spliced variant of IgA mRNA was quantitated using RealTime PCR with custom primers and normalized to GAPDH expression. IgA: Forward 5’-GCC TTG CCC ATG AAC TTC AC, reverse 5’- CGC TGA CAT TGG TGT TA, GAPDH: Forward 5’- CAT GGC CTT CCG TGT TCC TA, reverse 5’- GCG GCA CGT AG ATC CA. Samples were diluted with RNase free water and reactions contained 5μL 2x Maxima SYBR Green (Thermo Scientific), 0.4μl each of forward and reverse primers, 2.2μl nuclease-free water and 2μl cDNA. All reactions were run in duplicate on a 384-well plate using the Bio-Rad CFX384 TouchTM Real-Time PCR Detection System.

**In vivo Neisseria infections**

Wild type (hC-Tgneg), C-Tg and littermate B x hC-Tg mice were nasally infected with 1x10<sup>5</sup> colony forming units (CFU) of *Nme* strain 90/18311, resuspended in 10µL of PBS. Mice were infected at day 1 (primary infection) and, where indicated, day 14 (secondary infection). 5 days post-primary infection or 1-4 days following the secondary infection, mice were euthanized via CO<sub>2</sub> inhalation. Serum, used to measure systemic responses to *Nme*, was collected via cardiac puncture while mucosal samples were obtained via tracheal/nasal lavages. Bacterial burden per mouse was enumerated by swabbing nasal cavities and plating re-suspended fluid overnight at 37°C.
Commensal-specific ELISPOT
Membrane plates (0.45µm Hydrophobic High Protein Binding Immobilon-P Membrane, Millipore) were coated with 5mg/ml of heat-killed inactivated *Neisseria meningitidis* and placed at 4°C overnight. The plates were blocked the next day with 10% FBS/complete RPMI for at least 2 hours at 37°C. Single cell suspensions were loaded onto the plate at serial 2-fold dilutions in 10%FBS/complete RPMI and incubated overnight at 37°C. Cells were removed the next day and washed with 0.1% TWEEN-20/PBS 5x. HRP-conjugated IgA and AP-conjugated IgG (in case of two-colour ELISPOT) detection antibodies were subsequently added for 2 hours at 37°C.

Plates were then washed with 0.1% TWEEN-20/PBS 3x, and with PBS 3x. The plates were developed while covered with aluminum foil until spots were visible using AEC Peroxidase (for HRP-conjugated antibodies, Vector Laboratories) and Vector Blue (for AP-conjugated antibodies, Vector Laboratories) substrates. After the development was done the plates were washed with distilled water and were left to dry overnight. The spots were counted based on the original cell dilution.

Preparation of single cell suspensions from kidney
Mouse Kidneys were collected in 10%FBS/complete RPMI and kept on ice until digestion was performed. The kidneys were perfused with PBS for the ELISPOT tests. Multi Tissue Dissociation Kit 1 enzymes (Miltenyi) were pre-warmed to 37°C and in accordance with manufacturer recommendations. Both mouse kidneys were incubated with enzymes in a gentleMACS C tube using MACSmixTM Tube Rotator for 10 mins at 37°C in a CO2 incubator. Next, the mixture was briefly transferred to a 6 well plate and mechanically dissociated with tweezers into ~1mm x 1mm pieces, then returned to C tube for an additional 10 mins incubation as above. After incubation, gentleMACSTM dissociator was used for final mechanical digestion step prior to filtering through 70µm nylon mesh and inactivation of enzymes using ice cold RPMI with 10%FBS.

A Percoll gradient (30% Percoll for 20 minutes at 2000 RPM) was performed on the cells obtained from the digested kidneys to enrich for immune cells. The pellet was then resuspended in 1mL of RBC lysis buffer (155mM NH4Cl, 12mM NaHCO3, 0.1mM EDTA) for 5 minutes on ice. The cells were washed with PBS 1x, and with 10%FBS/complete RPMI 1x and were then ready to be plated.

Statistics
Data distribution was evaluated, and group comparisons were performed using unpaired parametric or non-parametric tests as appropriate. A p value of <0.05 was considered statistically significant.

Study Approval
All clinical investigation was conducted according to Declaration of Helsinki principles. The clinical study was approved by the research ethics boards of all clinical centres (coordinating centre UHN REB 11-0748). All animal experiments were conducted with ethical approval from the University of Toronto, Faculty of Medicine animal care committee (protocols 2001 1363, 1365).
Acknowledgements
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Author Contributions
Patient study design, enrollment, clinical characterization: LP, JN, RC, MH, SB, DC, RP, CL, JG, HR
Mouse nephropathology: RJ
Manuscript drafting, critical review: All authors
Senior responsible authors: JG, HR, contributed equally
References


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Table 1. Characteristics of the cohort. Healthy control subjects had no history of kidney disease and were largely household-paired non-genetically related individuals. A higher proportion of IgAN subjects are male (chi-squared 6.34, p=0.012). Differences in race distribution are not statistically significant.
Figure 1. Tonsil microbiota. A. The most abundant genus (> 2x the relative abundance of the next most abundant genus) in tonsil swabs in IgAN subjects is *Neisseria*. Each colour represents an individual genus, and y axis indicates the proportion of samples with that genus as the most abundant organism (Chi-square p=0.18). B. The relative abundance of *Neisseria* genus is significantly higher in IgAN compared to non-related household-matched healthy control subjects (2-tailed unadjusted t-test p value 0.002).
Figure 2. The anti-Neisseria response in plasma of patients with IgA nephropathy. Patients with IgAN exhibit exaggerated IgA-biased anti-Neisseria responses to both pathogenic and non-pathogenic Neisseria species. Neisseria-specific antibodies were evaluated across a panel of four *N. meningitidis* (*Nme*) strains (90/18311, H4476, 208 and 860800) and four commensal strains (*N. lactamica*, *N. flavescens*, *N. sicca* and *N. cinerea*). Mean and SD shown, Mann-Whitney test 2-tailed-p value.)
Figure 3. The effect of BAFF overexpression on sterilizing immunity against *N. meningitidis* (*Nme*) 90/18311 A. Rate of *Nme* colonization according to genotype during naïve infection. B. Bacterial burden 24 hours following secondary infection in littermate controls. Naïve hC-Tg mice were used as a comparison for bacterial burden. C. At 24 hours post nasal infection B x hC-Tg mice do not exhibit an exaggerated anti-*Neisseria* IgA response in the nasopharynx. No nasal IgG was detected in mice (not shown). D. Following nasal infection, hC-Tg mice exhibit enhanced systemic anti-*Nme* IgG response, but B x hC-Tg mice do not (ANOVA F 5.0, p<0.01, adjusted p<0.05 for indicated comparisons). E. B x hC-Tg mice exhibit an enhanced systemic anti-*Nme* IgA response with 10-fold anti-*Nme* IgA production (ANOVA F 2.6, p=0.07). F. Ratio of Anti-*Nme* IgA/IgG reveals IgA-biased systemic response in the B x hC-Tg mice (ANOVA F 2.3, p=0.09, unadjusted p value as shown). All tests ANOVA with Tukey-adjusted p value, mean and SD shown.
Figure 3.
Figure 4. Kidney pathology and IgA expression in experimental IgA nephropathy. A. Semi-quantitative scoring reveals mesangial expansion in mice that overexpress BAFF. B. Representative images corresponding to mesangial matrix scoring (PAS stain, 40x magnification) in BAFF-Tg x hCEACAM+ (a), BAFF-tg x hCEACAM+ (B x hC-Tg) b) BAFF-wt x hCEACAM1-/- (c) and BAFF-wt x hCEACAM1-/- (d). Red arrow shows cellular proliferation, blue arrow shows mesangial expansion. C. Representative sections showing IgA staining by immunohistochemistry (same order as panel B), confirming mesangial deposition of IgA. D. Quantitative evaluation of IgA RNA expression (secreted splice form) in kidney tissue. Kidneys obtained from double transgenic mice (B x hC-Tg) demonstrate the highest degree of IgA mRNA expression (Dunn’s adjusted p<0.05 for comparisons with other groups by Kruskal-Wallis ANOVA test). Mean and SD shown.
Figure 5. Detection of anti-\textit{N. meningitidis}-IgA-secreting cells in the kidneys of BAFF-transgenic mice nasally infected with \textit{N. meningitidis} via hCEACAM-1. A. Representative photos of an ELISPOT assay developed for detection of \textit{Nme}-reactive IgA-producing cells. B. Anti-\textit{Nme}-specific IgA-ACS are detected predominantly in kidneys of BAFF-Tg x hCEACAM\textsuperscript{+} (B x hC-Tg\textsuperscript{+}) mice (Mean and SD shown, ANOVA with Tukey-adjusted p-value = 0.01 for all comparisons indicated).