Distinct microbota communities exist in the gut, lungs, skin, and at the ocular surfaces. Many studies have examined how resident commensal presence frames immune responses at different locations; however, much remains to be discovered about how these communities affect immunity at a distance. Toward this end, a recent study by de Paiva and colleagues has shown that when gut microbiota is perturbed are significantly influenced in a mouse model of Sjögren’s syndrome. The trend is reversed in untreated controls, suggesting that gut microbiota perturbations can alter immunity at mucosal surfaces. Additional evidence of the impact of host to gut commensal species may serve as a priming signal to generate B-cell repertoires at sites different from the gut, such as EALT, thereby ensuring broad protection. With mounting evidence to suggest that gut microbiome perturbations are a causative factor in disease, the question of the contribution of an ocular microbiome to disease pathology also requires further examination. A study of changes in ocular microbiome constituents after infection with Chlamydia trachomatis, by Zhou and colleagues, reveals that age, environment, and exposure to disease are factors in altering the ocular microbiome. Dong and colleagues define a core ocular microbiome, using culturing and 16S sequencing methods. They note that the microbiome is made up of five phyla with 59 bacterial genera, of which 12 genera make up a core microbiome (with potential transient members) relative to the surrounding skin and buccal mucosa. Of note, Dong et al. have observed that germ-free (GF) rats have 5- to 8-fold lower IgA and IgM plasma cells in their lacrimal glands (LGs) than normal controls, before introduction into a
Microbiota Induced Ocular Surface Immunity by sIgA

Our recent studies prompted us to evaluate how and which commensal bacteria regulated SIgA in the ocular surface and mucosal immunity. We have pioneered the expression of SIgA in eye-associated lymphoid tissues (LGs) and its role in maintaining local immune homeostasis. Further, we hypothesized that memory B cells created in response to initial exposure migrate throughout the body and take up residence in the LGs, subsequently responding to commensal-mediated challenges to prevent pathogenic bacteria from causing disease.

Here, we characterized baseline mouse strain differences in LG IgA expression by quantitative real time PCR (qRT-PCR), IgA ELISA, and quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in mice that underwent gut microbiome profiling by 16S rDNA Sequencing.

Materials and Methods

Ethics Statement
All animal experiments were performed according to the National Institutes of Health guidelines for housing and care of laboratory animals. All the experiments complied with institutional regulations after protocol review and approval by the Brigham and Women’s Hospital Institutional Animal Care and Use Committee (BWH IACUC) Committee and were consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice

GF SW mice were purchased from the Gnotobiotic Core Facility (BWH). Age- and sex-matched SPF SW mice and SPF C57BL/6N GF SW mice were purchased from the Gnotobiotic Core Facility. Four-week-old SPF SW mice were treated with an antibiotic cocktail in the drinking water as previously described. Mass spectra were processed by using the MaxQuant computational platform version 1.5.5.5. The spectra were searched by the Andromeda search engine against the Mus musculus Uniprot sequence databases (acquired April 27, 2016). Quantification in MaxQuant was performed by using the label-free quantification (LFQ) algorithm, and “match between runs” was selected.

LC-MS/MS Data Analysis

DNA was extracted from the fecal pellets with QIAamp DNA Stool Mini Kit (catalog No. 51504; Hilden, Germany). Quality of the DNA was checked by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Libraries were created by targeting the V4 region of the 16S rRNA gene, using qPCR according to the protocol available at www.earthmicrobiome.org/protocols-and-standards/16s/. Purified and size-selected libraries were subjected to pair end 2 × 150 bp cycle run on Illumina MiSeq (Zymo Research Corp., Irvine, CA, USA). The sequencing and analysis were performed by SeqMatic (Fremont, CA, USA). Illumina BaseSpace’s 16S metagenomic application was used to analyze the FASTQ data. All reads obtained from pair end 2 × 150 bp cycle runs on Illumina MiSeq passed the quality check. Green genes database was used to classify reads with species level sensitivity. The Ribosomal Database Project (RD)-naive Bayesian algorithm was used for classification. Illumina BaseSpace’s 16S metagenomic application that used the sequences generated from pair end sequencing (longer reads compared to single end) did not require any upfront operational taxonomic unit clustering for the taxonomic classification. On average 97.5% of the reads were classified at least at a genus level. The unclassified reads constituted 2.4% with the standard deviation of 1.21%.

Monoclonization With Bacteroides acidifaciens

Five-week-old female GF-SW mice (n = 5) were orally gavaged with 10⁶ B. acidifaciens organisms. Fecal pellets and eyewashes were collected on days 0, 7, 14, and 21. On day 21 post colonization, RNA was extracted from LGs, small intestine, and colon.

IL-1β Blocking

SPF SW mice were treated with 100 μg anti-IL-1β antibody (clone B122, No. BE0246; BioXCell, West Lebanon, NH, USA).
or isotype control (Armenian Hamster IgG BioXCell, No. BE0091) IP. After 24 hours, mice were euthanized and cervical lymph nodes (CLNs), LGs, serum, and eyewashes were collected for analysis.

Tissue and Stool Sample Preparation

Tissue samples were mechanically homogenized in Trizol (Thermo Fisher) before processing for RNA by using the Direct-zol RNA mini Prep (Catalog No: R2050; USA) according to manufacturer’s guidelines. Stool samples were homogenized in PBS and centrifuged at 10,000g for 5 minutes at 4°C. Supernatants were collected, protein quantified by Bradford assay, and stored at −80°C supplemented with protease inhibitors (Roche, Indianapolis, IN, USA).

Quantitative RT-PCR

One-step RT-PCR was performed by using the Power SYBR Green RNA to Cq 1-Step Kit (Applied Biosystems, Foster City, CA, USA) in CFX Connect Real Time PCR Detection System (BioRad, Hercules, CA, USA). Relative fold changes in the mRNA expression levels were calculated according to Livak and Schmittgen. The following primers were used to quantify transcripts in the tissue samples: immunoglobulin A (IgA) F: 5’CCTAGTGTGTGGCAGCCCTGA3’ and IgAR: 5’GGAAATGTCGGGATACCTTG3’; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5’GATTCCACCACCATGGCAAATC3’ and GAPDH_R: 5’TGGGATTCCATTGATGACAG3’.

IgA ELISA

SIgA was quantified by using Mouse IgA ELISA Ready-SET-Go (eBioscience, Vienna, Austria) per manufacturer’s instructions.

Statistical and Bioinformatics Analysis

Bioinformatics analysis of the LC-MS/MS data was performed in the Perseus software environment version 1.5.5.5. Data were filtered for common contaminants, log2 transformed; only those proteins which were present in duplicate within at least one sample set were used for further statistical analysis (valid-value filter of 2 in at least one group), and missing values were imputed from a normal distribution. Two-sample Student’s t-tests were performed to identify proteins with a significant differential expression ($P$ value < 0.05) in SPF C57BL6/N and SW-derived samples, using a 5% permutation-based false discovery rate (FDR) filter. Normally distributed data were analyzed by Student’s $t$-test or 1-way ANOVA analysis followed by Dunnett’s multiple comparison (ANOVA). $P < 0.05$ was considered significant.

RESULTS

Ocular SIgA Levels Differ in Genetically Distinct Strains of Mice

We compared the ocular surface proteomes of SPF SW and SPF C57BL/6N mice by using quantitative mass spectrometry–based proteomics. Following stringent filtering, in which each protein must be identified in duplicate in at least one sample set, and imputation of missing values from a normal distribution, 793 unique proteins were identified in SPF SW and SPF C57BL/6N mice and used for the subsequent analyses (Supplementary Table S1). We next used a Student’s $t$-test ($P$ value < 0.05) adjusted for multiple hypothesis testing (FDR < 0.05) to identify
Microbiota Promotes Generation of Ocular SlgA Levels

To evaluate the relative contribution of commensal presence on ocular SlgA, we treated SPF SW mice with an antibiotic cocktail in the drinking water, shown to significantly reduce gut bacterial commensal presence, while preserving microbiota at other sites such as the skin and conjunctiva. Upon completion of treatment, LGs were harvested and IgA transcript levels were quantified. It was noted that antibiotic (ABX)-treated mice had significantly lower levels of LG IgA transcripts than untreated controls (P = 0.001), illustrating the impact of gut microbiota on the abundance of IgA transcripts (Fig. 3A).

75 proteins with significant changes in protein abundance between the different genetic backgrounds (Supplementary Table S1). A significant increase in abundance was observed for 67 proteins in the SPF SW samples as compared to 8 proteins in the SPF C57BL6/N (Fig. 1). A functional overview of the significantly different proteins, based on gene-ontology biological processes classification, highlights the diversity of proteins detected in both genetic backgrounds (Fig. 1). Specifically, proteins associated with cellular, metabolic, and catabolic processes, as well as respiration, response to stimulus, and transport, showed an increase in representation in the SPF SW mice when compared to the SPF C57BL6/N. Among the SPF SW significant outliers, SlgA showed a significant increase in abundance when compared to the abundance of SlgA in the ocular surface washes of SPF C57BL6/N mice.

To further support the LC-MS/MS data, qPCR analysis of EALT, including LGs and CLNs derived from SPF SW and SPF C57BL6/N mice, was performed. Levels of IgA transcripts in SPF SW mice were significantly greater than in C57BL6/N mice with a 6.25- (P < 0.0001) and 142-fold (P = 0.001) increase of IgA transcripts in the CLNs and LGs, respectively (Fig. 2). Taken together, these data demonstrated that different mouse strains have distinct relative basal abundance of SlgA, which is likely due to either strain-specific genetic differences or distinct commensal species.
production of IgA transcripts in EALT and SlgA in the colon. Monocolonization with *B. acidifaciens*, a strain that does not inhabit ocular mucosal sites, upregulated IgA transcript levels in LGs but did not affect surface SIgA, indicative of the need for additional stimulation.

**Microbiota-Induced IL-1β Promotes SIgA Levels**

IL-1β ELISA on serum from GF and SPF SW mice showed that the GF SW mice had approximately 50% less baseline serum IL-1β (7.5 pg/mL GF versus 15.5 pg/mL SPF SW, *P* < 0.0045, Student’s *t*-test) when compared to the SPF SW control (Fig. 6). To determine if systemic IL-1β was a significant factor in the modulation of IgA transcription in EALT, IL-1β-blocking antibody was administered to SPF SW mice. Quantitative PCR analysis of the CLNs and LGs for IgA transcripts showed significantly lower levels of IgA than for isotype controls (CLNs: 1.6-fold reduction, *P* = 0.0329; LGs: 2-fold reduction, *P* = 0.0002, Student’s *t*-test) (Fig. 6). Further analysis of eyewashes for SIgA confirmed the LG IgA data (control IgA concentration: 0.92 ng/mL, IL-1β block concentration: 0.15 ng/mL, *P* = 0.0454, Student’s *t*-test), suggesting that IL-1β is required for maintaining LG IgA mRNA transcript and surface protein levels in SPF SW mice. Taken together, these data suggest that IL-1β is a crucial component in regulating B-cell IgA production in LGs.

**DISCUSSION**

Here, we provided experimental evidence that ocular SlgA is influenced by the genetic background and microbiota. Mass spectrometry analysis of SPF C57BL/6N and SPF SW mice demonstrated lower abundance of surface SlgA in C57BL/6N mice than in SPF SW mice (Fig. 1). Consistently, a comparison of IgA transcripts in EALT by qRT-PCR confirmed the higher levels of IgA transcripts in the SPF SW mice. Further, we found a correlation between gut commensal diversity and SlgA levels, indicative of a mechanism. The SPF SW mice presented with richer gut commensal diversity than the SPF C57BL/6N mice and this correlated with elevated levels of IgA transcripts and SlgA.

Our findings are reminiscent of, but extend, the recent observations that BALB/c mice have a richer microbial diversity and higher IgA expression than C57BL/6J mice in the gut. Fecal transplant studies indicate that C57BL/6J mice are genetically predisposed to having less diverse microbiome. Fransen et al. have demonstrated a correlation between commensal diversity and SIgA levels in colon. However, SIgA levels at distant mucosal sites were not evaluated. Further, since subsequent experiments have demonstrated niche dependence for the generation of SIgA, we questioned whether LG IgA transcript levels, and potentially ocular surface SlgA, changed depending on gut microbiota.

We observed that when GF SW mice were engrafted with SPF SW- or SPF C57BL/6N-derived microbiota, the rise in relative IgA transcripts was more prominent in the SPF SW-engrafted recipients (Fig. 3). These data support the conclusion that gut commensal diversity correlates with IgA transcript levels, as GF SW engrafted with SPF C57BL/6N-derived microbiota had significantly lower transcript levels and lower diversity.

As expected, 16S bacterial metagenomic profiling demonstrated different gut commensal diversity in the two genotypes of mice, with the *Bacteroides* genus being more prominent in the SPF SW mice (Fig. 3). Among the identified commensal
species in the SPF SW mice, *B. acidifaciens* was highly abundant in SPF SW mice. *B. acidifaciens* is a gut commensal and an obligate anaerobe, which has been linked to colon IgA production. When GF SW mice were monocolonized with *B. acidifaciens*, qRT-PCR analysis for colon IgA transcripts demonstrated a significant rise in the IgA transcripts, which translated into elevated SIgA (Fig. 5). No increases in the small-intestine IgA transcripts were noted in agreement with data by Yanagibashi et al. These data illustrate a significant role of *B. acidifaciens* in promoting SIgA synthesis. Of note, not all commensal species that belong to the *Bacteroides* genus induce SIgA expression in the gut. Recent monocolonization experiments have revealed that colonization with *B. fragilis* promotes SIgA, whereas *B. ovatus* does not, while *B. uniformis* inhibits SIgA, illustrating commensal-specific responses, rather than genus-specific responses in the gut.

Interestingly, analysis of LG IgA transcripts at day 21 showed a significant increase in IgA transcript levels when compared to GF controls. Moreover, since the analysis of eyewash protein levels did not yield a significant increase of SIgA in the monocolonized mice when compared to GF SW control, we concluded that a secondary priming signal from the ocular surface may be required for IgA secretion. Consistently, the monocolonized mice did not present recoverable commensal species from the ocular surface upon swabbing (data not shown).
Microbiota Induced Ocular Surface Immunity by sIgA

Our observations suggest a model where naïve B cells are exposed to commensal-derived signals in the gut, class-switch, and initiate IgA production in the colonic compartment. Thereafter, a subset of effector or memory cells may traffic from the colon and enter the LGs. The idea of potential trafficking of B cells from the gut to the LGs is intriguing and is supported by the observation that B cells traffic between the gut and LGs is intriguing and is supported by the observation that B cells traffic between the gut and LGs. Oral administration of a nitrophenylated type III pneumococcal vaccine results in consistent IgA secretion in tears as a response to continued GI vaccine administration, hinting at the possibility of "continuous but variable" populations of IgA-secreting cells flowing in and out of the LGs.

Our data also suggest that generation of SlgA is IL-1β dependent in SW mice. IL-1β-induced expression of lymphotixin 2 and β induced nitric oxide synthase and maintained populations of retinoic acid-related orphan receptor γ T-positive innate lymphoid cells (RORγT+ ILCs), which are known contributors to IgA class switching. Consistently, IL-1β knockout mice showed a breakdown in IgA-mediated gut immune homeostasis and a significant decline in Bacteroides genus.30,31 Cumulatively, data suggest a contribution for IL-1β signaling in regulating IgA production, a finding that has significant implications for ocular immune homeostasis in health and disease.

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