Hypersensitivity toward bacterial stimuli in patients with age-related macular degeneration

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Although the pathogenesis of age-related macular degeneration (AMD) is unclear, genetic screening has revealed that polymorphisms in the complement system may be associated with AMD development. Production of autoantibodies was also found in AMD patients. In this study, we analyzed the antibody response in AMD patients. We found that purified B cells from AMD patients tended to respond to lower concentrations of bacterial antigen stimulation, and produced higher amounts of antibodies, especially in IgM and IgA secretions. When examining clinical symptoms, patients with more severe wet-form AMD tended to exhibit higher sensitivity to bacterial antigens and secreted more IgM and IgA antibodies than those with less severe dry-form cases. In conclusion, our study discovered an altered B-cell antibody production in response to bacterial antigens in AMD patients, which potentially contributes to AMD pathogenesis.

Key words: B cell; age-related macular degeneration; antibody.

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Age-related macular degeneration (AMD) is the most prevalent cause of blindness in otherwise healthy senile populations, characterized by drusen deposits in the retina of the eye (1). Despite decades of extensive research, little is known about the pathogenesis of this disease. The eye is an immune privileged site at which the immune activation is suppressed to avoid tissue damage caused by excessive inflammation in response to foreign antigens (2). Interestingly, factors in the immune system are closely implicated in AMD pathogenesis (3). Genetic screenings have revealed that mutations in the complement system, an important participant in initiating immune responses and linking the innate and adaptive immune systems, are most closely related to AMD generation (4). Polymorphism in Complement Factor H, an inhibitory molecule that accelerates C3 convertase degradation, was found to greatly increase the risk for AMD development (5–7). Indeed, examination of patient tissues revealed that drusen are composed of various immunologically active molecules, together with lipids and other substances that can potentially activate the immune system (8, 9). Little is known about the precise role of the immune system in AMD development.

The complement system is made up of a large number of plasma proteins that can be activated sequentially to induce a series of inflammatory immune responses to fight infections. The classical complement pathway can be activated when complement component C1q binds to antibody–antigen complexes. Similarly, antibody–antigen complexes can also activate the alternative pathway. These initial activation events then lead to the deposition of C3 convertase in the pathogen surface, which lead to the recruitment of phagocytes, lysis of pathogens, release of peptide mediators of inflammation, and initiation of further immune responses. Three major antibody subtypes, including IgM, IgG and...
IgA, are involved in antigen recognition (10). IgM is present in blood and gastrointestinal tract as pentameric high avidity antibody that can interact with a variety of antigens, even without prior antigen encounter. This makes IgM the ideal subtype for initial antigen recognition. IgG is the high affinity monomeric antibody that can be produced during the adaptive immune response and exhibits highly specific antigen binding, which is crucial for antigen clearance. IgA can be found in both monomeric and dimeric forms. Gastrointestinal IgA is resistant to bacterial protease degradation and is essential in limiting excessive microbial growth and promoting stable symbiosis. It can also be found in tear and lung mucus and is thought to have similar functions in these regions. The importance of antibody—antigen interaction in activating the complement system, together with the discovery that autoantibodies specific to the retinal tissue were present in AMD patients (3, 11–13), lead to our suspicion that excessive antibody response might contribute to AMD pathogenesis.

In this study, we examined the antibody response toward microbial antigens in late AMD patients. We found that in B cells in late AMD patients secreted higher levels of antibodies in response to bacterial antigens, and were responding to more diluted concentrations of bacterial antigens, than those in Non-AMD subjects. Moreover, more severe wet-form AMD patients showed higher IgM and IgA expression and higher sensitivity to lipopolysaccharide (LPS) + CpG stimulation, than less severe dry-form AMD patients. Altogether, our study suggested that B cell antibody production in response to bacterial antigens potentially contributes to AMD pathogenesis.

METHODS

Ethics statement

A written informed consent for this study was obtained from all participants. A structured questionnaire was applied to gather all participants’ past medical history, drug history, and smoking status. This study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

Study subjects

A total of 30 AMD patients and 10 Non-AMD controls were recruited from the First Affiliated Hospital of Zhengzhou University. Of the 30 AMD patients, 10 subjects were at the early stage, 10 subjects were at the intermediate stage, and 10 subjects were at the late stage, whereas in the late-stage AMD patients, five presented the dry-type and five presented the wet-type AMD. Demographic information of all participants are shown in Table 1. All AMD patients and Non-AMD controls were between in ages of 50–65 years old. No significant differences in age, sex, and ethnicity between AMD and non-AMD groups were observed. Participants underwent standard ophthalmic examination including measurement of vision acuity, slit lamp examination and fundoscopy through dilated pupils. Optical coherence tomography (OCT), fluorescein angiography, and ICG angiography were also performed in some patients. Diagnosis and classification of AMD were based on the International AMD Epidemiological Study Group criteria. All the patients were newly diagnosed and had the same stage of AMD in both eyes. Cases with hypertension, diabetes, cardiovascular diseases, autoimmune diseases, cancers, and other eye diseases were excluded. Genotyping was conducted in all subjects using a previously published method (14). Briefly, forward primer (sequence 5’-CCATGCTCA GAAAATGTATTTGCTT-3’), reverse primer (sequence 5’-GAGCGAGTCAAGTCTATAGATTACC-3’), 1277 C probe (sequence VIC-TTCTTCATCATGATTTT G-MGB), and 1277 T probe (sequence FAM-TT CTTCCATAATTTGG-MGB) were purchased from Applied Biosystems, Foster City, CA, USA, and were used in combination with the supplied Q-PCR buffer, probe mix and patients’ genomic DNA in duplicate wells, in a thermocycler with 10 min at 95 °C, 40 cycles of 15 s at 92 °C, and 1 min at 60 °C. The allelic level genotyping from fluorescence were then measured in ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA). To exclude potentially confounding effects of the complement factor H (CFH) Y402H polymorphism on the pathogenesis of AMD, only subjects with TT genotype were included in our study.

Cell preparation and culture conditions

A total of 60–100 mL peripheral blood was collected from each subject by venous puncture. PBMCs were obtained standard Ficoll-Hypaque density centrifugation.

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Table 1. Demographic information of study participants. Mean ± standard deviation (range). Student’s t test or chi-square test was applied where applicable

<table>
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<th></th>
<th>Non-AMD (N = 10)</th>
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<th>Intermediate (N = 10)</th>
<th>Late (N = 10)</th>
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<td>Age (year)</td>
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<td>Ethnicity (%)</td>
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tion. Cells were frozen immediately at −80 °C until use. Culture medium is made with RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin + streptomycin (Invitrogen, Carlsbad, CA, USA). For LPS + CpG stimulation, 2 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) + 5 μg/mL CpG-B (sequence TCGTCGGTTTTGTCGTTTTGTCGTT) (InvoGen, San Diego, CA, USA) in culture medium were serially diluted at a factor of 4 with culture medium. Cells were cultured at 5% CO2 concentration and 37 °C, for 72 h with LPS and CpG.

**B-cell purification and flow cytometry**

B cells were isolated from PBMCs with a magnetic bead-based method using Human B cell Negative Selection Kit (Stemcell, Vancouver, BC, Canada). Purity of B cells was confirmed by staining with anti-human CD19 antibody (BioLegend, San Diego, CA, USA) and then running samples through flow cytometry. For flow cytometry staining, cells were first collected in 2% FBS PBS, then stained with anti-human CD19 antibody and Fixable Violet Dead Cell Stain (Life Technologies, Grand Island, NY, USA) for 30 min at 4 °C. Cells were then washed in PBS and fixed with 2% formalin in PBS. Gating of lymphocytes and B cells were shown in Fig. 1A.

**Antibody ELISA**

Antibody ELISAs were performed using IgM, IgG, or IgA Human ELISA Kit (Abcam, Cambridge, UK). The ELISA procedure was then run according to manufac-

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**Fig. 1.** B cells in Non-AMD, early, intermediate, and late AMD patients. (A) B-cell isolation efficiency. B cells were isolated from PBMCs with magnetic bead-based negative selection. Figure shows the gating of lymphocytes and B cells, and the staining of anti-human CD19 B-cell marker before and after purification. (B) Percentage of B cells in PBMCs, as determined by flow cytometry before purification. (C) Secretion of antibodies from each study group after LPS and CpG stimulation. 40,000 purified B cells per well were cultured in medium for 72 h in the presence of 0.5 μg/mL LPS and 1.25 μg/mL CpG. Supernatants were collected and antibody concentration were obtained by total human antibody ELISA. ns: not significant. One-way ANOVA and Dunn’s test. p < 0.05 is considered significant.
ter’s protocols. Detection ranges were 1.5 ng/mL – 100 ng/mL for IgM, 0.02 ng/mL – 15 ng/mL for IgG, and 1.5 ng/mL – 100 ng/mL for IgA. For total human antibody ELISA, 40,000 purified B cells per well were cultured with LPS + CpG for 72 h, after which culture supernatant were collected and placed in a Nunc MaxiSorp ELISA plate (eBioscience, San Diego, CA, USA) pre-coated with 5 μg/mL of anti-human kappa/anti-human lambda antibodies (Rockland, Limerick, PA, USA) overnight. The antibodies were then detected by combining secondary antibody solutions in the IgM, IgG and IgA Human ELISA Kits (Abcam). The rest of the procedures were carried out according to manufacturer’s protocol.

Statistical analysis
Kruskal–Wallis one-way analysis of variance (ANOVA) was used for comparisons between multiple groups and then Dunn’s test was used if there is a statistical difference between multiple groups. Student’s t test or Mann–Whitney U test was used for comparison between two groups. All statistical analyses were done using Prism (GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered significant.

RESULTS

B-cell antibody response toward bacterial antigens is elevated in late AMD patients

The eye is an exposed area that is in frequent contact with external antigens. Danger signal receptors, such as Toll-like receptors (TLRs), can directly activate antibody response in B cells upon sensing the presence of foreign antigens (15). We first examined the B-cell antibody response in patients affected by early, intermediate, and late AMD. To study de novo antibody response in AMD patients, we first collected peripheral blood mononuclear cells (PBMCs) from study subjects, and then washed and purified B cells to avoid contamination from serum. We were able to obtain B cells with purity >95% (Fig. 1A). No significant differences in the percentages of B cells in lymphocytes, as determined by flow cytometry, were seen between different study groups (Fig. 1B). Bacterial infection is a common cause of inflammation in the eye area. Bacterial components, such as LPS and CpG motifs, can interact with danger signal receptors, such as TLRs, and lead to B-cell antibody production. We therefore measured total antibody production in response to LPS and CpG from all AMD patients and Non-AMD controls. As shown in Fig. 1C, late AMD patients secreted significantly higher amounts of antibodies than Non-AMD subjects. No significant differences were seen between early, intermediate, and Non-AMD subjects.

The IgM response in late AMD patients could be activated by much lower concentrations of LPS and CpG

Immunoglobulin M (IgM) is abundantly present in human serum and mucosal secretions, and is essential in recognizing novel antigens and initiating inflammations. The constant region of IgM binds to C1q with high affinity. Binding of IgM on the pathogen surface can directly lead to C1q deposits on the pathogen surface and further activation of C3 convertase though the classical pathway activation. Therefore, we first sought to determine the IgM response in AMD patients. As shown in Fig. 2, at 0.48 ng/mL LPS + 1.22 ng/mL CpG, IgM secretion became detectable in one AMD patient while no Non-AMD subjects had detectable IgM level. At 1.95 ng/mL LPS + 4.88 ng/mL CpG, IgM was detected in seven AMD patients, while only three Non-AMD subjects responded to this concentration. All ten AMD subjects exhibited detectable levels of IgM secretion at 7.81 ng/mL LPS + 19.5 ng/mL CpG, while 31.2 ng/mL LPS + 78.1 ng/mL CpG was required for all ten Non-AMD subjects to respond.

We also examined the level of IgM secretion in both groups. Late AMD patients secreted significantly higher levels of IgM at 7.81 ng/mL LPS + 19.5 ng/mL CpG, 31.2 ng/mL LPS + 78.1 ng/mL CpG, and 500 ng/mL LPS + 1250 ng/mL CpG than Non-AMD subjects. Together, we found that late AMD patients secreted significantly higher levels of IgM than Non-AMD subjects.

Late AMD patients had higher levels of IgG response at intermediate or high LPS and CpG concentrations

Immunoglobulin G (IgG) is the most abundant antibody subtype in the serum and extracellular fluid, and is also present in respiratory and urogenital secretions (10). Coating of IgG at the pathogen surface and binding of C1q to IgG constant region activates the classical pathway in a similar fashion to IgM (16). We examined the IgG response in AMD patients. As shown in Fig. 3, IgG secretion becomes detectable in two AMD patients at 0.48 ng/mL LPS + 1.22 ng/mL CpG, while in Non-AMD subjects, at least 1.95 ng/mL LPS + 4.88 ng/mL CpG was required. All Non-AMD and late AMD subjects exhibited detectable levels of IgG at 7.81 ng/mL LPS + 19.5 ng/mL CpG. When examining the IgG production from the group, AMD subjects secreted significantly higher amounts of IgG at 125 ng/mL LPS + 312 ng/mL CpG, and 500 ng/mL LPS + 1250 ng/mL CpG.
Late AMD patients had higher IgA response toward LPS and CpG stimulation

Immunoglobulin A is an important subtype of antibodies prevalent in mucosal secretions, such as tear, saliva, and gut mucosa, and are crucial in protecting mucosal surfaces and restricting microbial growth (10, 17). Moreover, IgA aggregates either with antigen or in response to danger signals, such as heat, can activate the alternative pathway, which is heavily implicated in late AMD pathogenesis (18, 19). We analyzed B-cell IgA response toward bacterial antigens in late AMD patients. As shown in Fig. 4, IgA secretion becomes detectable in late AMD patients at much lower LPS + CpG dilutions than that in Non-AMD subjects. One late AMD patient had detectable IgA at 0.12 ng/mL LPS + 0.31 ng/mL CpG, 3 at 0.48 ng/mL LPS + 1.22 ng/mL CpG, and eight at 1.95 ng/mL LPS + 4.88 ng/mL CpG, while in Non-AMD subjects, IgA became detectable in one subject at 0.48 ng/mL LPS + 1.22 ng/mL CpG and four at 1.95 ng/mL LPS + 4.88 ng/mL CpG. Late AMD subjects as a group also had secreted significantly higher levels of IgA at 7.81 ng/mL LPS + 19.5 ng/mL CpG, 31.2 ng/mL LPS + 78.1 ng/mL CpG, 125 ng/mL LPS + 312 ng/mL CpG, and 500 ng/mL LPS + 1250 ng/mL CpG.

Combining previous results, we have found that B cells in late AMD patients are hypersensitive to bacterial stimulation, were able to respond to much lower concentrations of bacterial products such as LPS and CpG, and were secreting higher amounts of IgA.
levels of antibodies than those in Non-AMD subjects.

**Antibody production is correlated with AMD severity**

Based on diagnosis of the affected patients, we grouped our late AMD study group into the dry and wet types. Patients suffering from geographic atrophy with loss of vision caused by the breakdown of the light-sensitive cells in the macula and the underneath supporting tissues were classified as dry form. Wet-form AMD, on the other hand, described patients with abnormal blood vessels growing underneath the retina, which can leak blood to the eye and cause more rapid damage to the macula. The wet type is the more severe form of AMD (20). We found that the amount of IgM and IgA production is positively correlated with disease severity. Patients with wet-form secreted higher amounts of IgM and IgA at 125 ng/mL LPS + 312 ng/mL CpG (Fig. 5A). On the other hand, the concentration of LPS and CpG at the first detectable IgA level (i.e., the amount of LPS and CpG required for antibody production to be detected) was negatively correlated with disease severity (Fig. 5B). This revealed the link between antibody production, B-cell hypersensitivity, and late AMD clinical development.

**DISCUSSION**

Multiple factors that impact the human immune system were found to associate with elevated risk of AMD development, including polymorphism in Complement Factor H, presence of immune molecules and potential immune regulators in drusen, and low-grade immune activation. Despite these associations, very little is known about the precise involvement of the immune system in AMD pathogenesis, and the role of components in the immune system. Due to the strong association between Complement Factor H polymorphism with increased risk for AMD, and the central role of antibody–antigen interaction in complement activation, we decided to examine the B-cell antibody response and its role in AMD. We found that B cells in late AMD secreted higher levels of IgM, IgG, and IgA in response to bacterial antigens than those in Non-AMD subjects, indicating elevated antibody response. In addition, several late AMD patients responded to much lower concentrations of bacterial antigens than Non-AMD individuals, demonstrating that B cells from AMD patients are more sensitive to bacterial stimulation. Together, these data demonstrated that late AMD subjects have increased antibody response toward bacterial stimulation. Furthermore, the finding that IgM and IgA secretion level is associated with the more severe wet-type AMD indicated that the hyperactive and hypersensitive antibody response is involved in AMD pathogenesis. The eye is an exposed area in frequent contact with bacterial antigens. The relationship between previous eye bacterial infections and overly active B-cell antibody responses would require further study. To avoid potentially confounding effects by Y402H (1277 T-to-C) polymorphism in complement factor H, we only included subjects with TT genotypes, which reduced the numbers of available participants to a small number and limited the extendibility of our study. In the future, the interactions between overly active B-cell antibody responses and complement activation in individuals with or without Y402H polymorphism would be examined.

To prevent tissue damage from excessive inflammation by consistent external stimulation, in Non-AMD individuals, the eye is protected by ocular
immune tolerance. Indeed, the most prevalent antibody class in the tear is IgA, which was found to downregulate inflammation in the intestinal tract by binding and neutralizing bacterial toxins and allergens, and limiting the growth of pathogenic bacteria. On the other hand, aggregated IgA can lead to an alternative complement pathway activation, which has been heavily implicated in AMD. The role of IgA in ocular immunity and immune tolerance is currently unknown. In this study, IgA sensitivity and production was increased in late AMD patients and further elevated in wet-type AMD. Whether this contributes to immune activation or tolerance requires further study.

Lipopolysaccharide and CpG activate B cells through the TLR4 and the TLR9 pathway, respectively (21, 22). TLR4 and TLR9 then signal through MyD88 and activate a series of downstream phosphorylation events leading to the activation of NF-κB and AP-1 transcription factors, and initiate an inflammatory immune response (23). TLR4 may also signal through TRIF and lead to the activation of NF-κB and IRF-3 (24). Currently, it is unknown how B cells become more active in late stage AMD. Further studies focusing on the expression patterns of TLR4 and TLR9 in the B cells in late AMD patients, as well as downstream signaling events, may help elucidate the mechanism.

Fig. 5. The antibody response in dry-type and wet-type AMD patients. (A) Secretion of IgM, IgG, or IgA from light and severe AMD patients, when B cells were cultured with 125 ng/mL LPS + 312 ng/mL CpG in medium for 72 h. (B) The lowest concentrations of LPS and CpG required for IgM, IgG and IgA to be detected in each patient. The y-axis shows the inverted concentration of LPS + CpG on a log scale. Mann-Whitney test. p < 0.05 is considered statistically significant.
Activation of B cells through TLR4 and TLR9 not only lead to antibody production but can also lead to a series of cytokine productions (25). The cytokine expression profile in late AMD patients is unknown. Since cytokines are important modulators in activating or suppressing lymphocyte activation, as well as promoting cell maturation and localization, the cytokine expression in response to bacterial antigen will likely affect AMD pathogenesis as well. In the future, studies focusing on the cytokine production following bacterial antigen stimulation may help understanding the pathogenesis of AMD.

CONFLICT OF INTERESTS

None.

REFERENCES
