B Cell Anomalies in Autoimmune Retinopathy (AIR)

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Purpose. Autoimmune retinopathy (AIR) is a retinopathy associated with unexplained vision loss presumably linked to circulating antiretinal antibodies; currently, however, there are no standardized criteria regarding the diagnosis, treatment strategy, or pathogenesis of this disease. The importance of B-lymphocyte immunophenotyping in the classification of AIR is unknown.

Methods. We utilized 15-color multiparametric flow cytometry to identify aberrations in B cell subsets that may contribute to the pathophysiology of AIR. Luminex cytokine analysis was also performed on plasma samples from AIR patients.

Results. Significant differences in AIR patients compared to individuals with other inflammatory conditions or healthy donors were found in the B cell memory compartment, including an increase in naïve B cells and a decrease in switched and unswitched memory B cells, which correlated with alterations in immunoglobulin secretion.

Conclusions. These findings suggest that the maturation process of B cells may be impaired and that B cell immunophenotyping may help in understanding disease process in AIR.

Keywords: B cells, flow cytometry, luminex

Autoimmune retinopathy (AIR) is a rare disorder characterized by progressive visual acuity loss and visual field defects without any obvious retinal lesions in the presence of antiretinal antibodies.1,2 Although the precise mechanism of AIR is unknown, circulating antiretinal antibodies are believed to cause photoreceptor dysfunction and vision loss by targeting retinal antigens.3 Currently, the diagnosis of nonparaneoplastic AIR is based on (1) the demonstration of serum antiretinal antibodies and (2) suggestive clinical features, such as abnormal electroretinograms and visual field defects in the absence of a detectable malignancy or retinal pathology (e.g., retinitis pigmentosa).3 However, multiple retinal proteins have been found to be antigenic,1,4-7 and the presence of antiretinal antibodies alone is not sufficient to establish a diagnosis of AIR, as these antibodies are found in other retinal diseases, systemic autoimmune diseases, and well-known paraneoplastic diseases.8,9 Moreover, detection of these autoantibodies is not standardized.1 Clinical manifestations may show minimal correlations to electrophysiology, and there is often considerable overlap with other degenerative retinal disorders.3 The lack of definitive diagnostic criteria for AIR also means that there are no clear indicators for diagnosis; thus the diagnosis of AIR is ultimately a clinical diagnosis of exclusion.1

Ambiguity in diagnosis poses an additional challenge regarding the management of AIR. Treatment options vary considerably. Immunomodulatory approaches tend to be the most widely accepted strategy due to the “presumptive” autoimmune nature of the disease; however, there are no clear guidelines on how to institute and manage immunosuppressive therapy in AIR.1 Additionally, there is reported evidence for the use of targeted B cell therapy, specifically the anti-CD20 monoclonal rituximab because it targets the CD20+ B cells that are the precursors of antibody-secreting plasma cells.10,11 Alternatively, physicians frequently extrapolate from treatment guidelines established for uveitis to manage AIR patients.3 Regardless of the modality, responses to therapy are wide-ranging, and it is unclear if treatment correlates with clinical improvement, especially once extensive retinal degeneration occurs.3,5,8,12 Therefore, there is a need to develop definitive parameters to help in diagnosis and to indicate prognosis and guide treatment decisions in AIR.

We have previously developed and standardized a multiparametric flow cytometry panel for the evaluation of B-lymphocyte populations from peripheral blood in healthy donors.13 This staining utilizes 15 markers to provide detailed characterization in the immunophenotyping of B cells. Here, we use it to study B cell subsets in the peripheral blood of three groups of participants—healthy donors, those with known diagnosis of noninfectious inflammatory posterior/panuveitis, and those with AIR. Additionally, plasma cytokine levels were measured in another cohort including healthy donors, uveitis patients, and AIR patients. To our knowledge, no previous study has investigated the B-lymphocyte lineage in AIR.

Methods

Patients

A first set with a total of 39 subjects (8 AIR, 13 uveitis, and 18 healthy donors) were included in whole blood immunophenotyping, and another set of 40 subjects (10 AIR, 9 uveitis, and 21 healthy donors) was used for the measurement of plasma cytokine levels, with some patients belonging to both sets. All
TABLE 1. Patient Characteristics at Time of Sampling

|                      | Number, n | Median Age, y (Range) | Female, % | Male, % |
|----------------------|-----------|------------------------|-----------|---------|
| Whole Blood Phenotyping |           |                        |           |         |
| AIR                  | 8         | 56 (44–72)             | 75        | 25      |
| Healthy              | 18        | 53.5 (25–62)           | 67        | 33      |
| Uveitis              | 13        | 61 (25–82)             | 61        | 39      |
| Plasma Luminex Cytokines |       |                        |           |         |
| AIR                  | 10        | 56 (52–64)             | 80        | 20      |
| Healthy              | 21        | 39 (24–71)             | 67        | 33      |
| Uveitis              | 9         | 62 (41–82)             | 55        | 45      |

Three of the uveitis patients were treated with rituximab >2 years prior to sampling. None of the uveitis patients had history of being treated with rituximab or other B cell–targeted treatments. All AIR patients had undergone negative workup for cancer and melanoma associated retinopathy and had positive antiretinal antibodies. Median Age, y

FACS buffer (1x phosphate-buffered saline, 0.5% fetal calf serum, 0.5% normal mouse serum, and 0.02% NaN₃) and incubated with fluorochrome-conjugated antibodies from our published panel for surface staining: CD10, CD19, CD20, CD21, CD25, CD27, CD38, CD45, CD80, CD86, IgD, IgA, IgM, and IgG. After incubation with antibodies for 30 minutes, cells were stained with LIVE/DEAD Aqua fixable viability dye (Life Technologies, Carlsbad, CA, USA), washed two times with FACS buffer, and fixed in 1% paraformaldehyde. Cell were acquired on a Fortessa flow cytometer equipped with 405, 488, 532, and 638 laser lines using DIVA 6.1.2 software (Becton Dickinson). Data were analyzed with Flowjo software version 9.7.6 (Treestar, San Carlos, CA, USA). Our gating strategy is presented in Figure 1. All populations are expressed as the percent of parent gate.

Cytokine Luminex Expression

Plasma samples were analyzed for IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-25, IL-31, IL-33, soluble CD40 ligand (sCD40L), IFN-γ, and TNF-α using a 15-plex Human IL-17 Cytokine Kit (Bio-Rad, Hercules, CA, USA). All assays were performed according to the instructions provided by the manufacturer. Briefly, median fluorescence intensities were collected on a Luminex-200 instrument (Bio-Rad) using Bio-Plex Manager software version 6.2 (Bio-Rad). Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits, and cytokine concentrations were determined from the standard curve using a 5-point regression to transform the median fluorescence intensity values into concentrations. Each sample was run in duplicate, and the average of the duplicates was used as the measured concentration. Any value that was below detection level was replaced by the limit of detection (LOD) as reported by Luminex kit. Analyses were performed using Data Pro Manager 1.02 (Bio-Rad) and GraphPad Prism Software version 5.0c (La Jolla, CA, USA).

Statistical Analysis

Data obtained from one donor were considered as one experiment (n). Statistical analysis was performed using a Mann-Whitney (Wilcoxon rank sum) test in GraphPad Prism Software 5.0c.

Whole blood immunophenotyping results were presented as the mean ± standard error of the mean (SEM), and plasma cytokine concentrations were presented as median ± SEM. P values are reported for each experiment, and P values of <0.1 were considered significant for flow cytometry data. P values of <0.05 were considered significant when analyzing Luminex results. P values were adjusted for multiple testing using Benjamini and Hochberg’s false discovery rate (FDR) with Bonferroni corrections.

RESULTS

AIR Patients Display Decreased Frequencies of Total B Cells

B cell populations in the peripheral blood of AIR patients varied significantly compared to healthy donors and patients with uveitis (Fig. 2A). In healthy controls, CD19⁺CD45⁺ B cells represented an average of 11.7 ± 1.2% of the lymphocyte gate. The frequency of total B cells was decreased in both AIR (6.1 ± 1.8%, P = 0.01) and uveitis patients (5.9 ± 1.2%, P < 0.07) compared to healthy. Plasmablasts were identified as CD19⁺CD45⁺CD38⁺CD27++. These are the effector cells of an
antibody response. The frequency of plasmablasts in AIR patients (0.57 ± 0.17%) was significantly lower than in uveitis patients (2.41 ± 0.47%, \( P = 0.008 \)), but similar to that in healthy donors (0.91 ± 0.20%, \( P = 0.43 \)). There were no significant differences within the CD19+CD20+ mature B cell and CD19-CD20+CD10-CD27- transitional B cell compartments among healthy, uveitis, and AIR patients (Fig. 2A).

AIR Patients Have Altered Mature B Cell Memory Subsets

The B cell memory compartment was defined by IgD and CD27 expression on mature B cells: naive B cells (CD27- IgD+), switched memory B cells (CD27+IgD-), unswitched B cells (CD27+IgD+), and double-negative (DN) B cells (CD27- IgD-). The B cell memory subsets differed significantly between healthy, uveitis, and AIR patients (Fig. 2B). We observed an average frequency of 79.6 ± 5.24% naive B cells from AIR patients, which was significantly increased compared to that in uveitis patients (59.91 ± 5.46%, \( P = 0.019 \)) but not healthy donors (71.99 ± 3.46%, \( P = 0.07 \)). Conversely, the average frequency of switched memory B cells in AIR patients (6.78 ± 2.29%) was significantly decreased compared to uveitis patients (21.65 ± 3.15%, \( P = 0.019 \)) but not healthy donors (13.04 ± 2.21%, \( P = 0.11 \)). Further, we observed an average frequency of 3.81 ± 0.95% DN memory B cells in AIR patients, which was significantly decreased compared to both uveitis patients (7.75 ± 1.54%, \( P = 0.045 \)) and healthy donors (7.61 ± 1.39%, \( P = 0.04 \)). There were no significant differences in the unswitched memory compartment.

**FIGURE 1.** Flow gating strategy. Representative gating strategy used in B cell immunophenotyping. We accurately identify and quantify eight distinct known B cell subsets. These subsets were based on the surface expression of CD19, CD20, IgD, IgG, IgA, CD10, CD27, and CD38. IgD and CD27 expression on all mature B cells (CD19+CD20+) allowed us to identify naive (IgD-CD27-), unswitched memory (IgD-CD27+), double-negative memory (IgD-CD27-), and switched memory (IgD-CD27+). A transitional subset was identified with the additional expression characterized by lower level of expression of CD10. The plasmablast subset could be delineated based on the high level of both CD27 and CD38 expression, and they were predominantly CD20-CD10-CD38+.
Elevated CD23 MFI on Naïve Memory B Cells in AIR Patients

Activation of mature B cell memory subsets was measured using the mean fluorescence intensity (MFI) of CD23, CD38, CD80, and CD86. We reported MFIs as the ratio of the positive and negative expressions for each individual antibody. The CD23 MFI ratio of naïve B cells in AIR patients (43.86 ± 14.3%) was significantly higher compared to patients with uveitis (16.25 ± 4.12%, \( P = 0.003 \)), but not healthy controls (21.03 ± 5.22%, \( P = 0.10 \)) (Fig. 3). There were no significant differences for CD38, CD80, and CD86 among any of the groups.

Impaired Immunoglobulin Expression on Mature B Cell Memory Subsets in AIR Patients

In human physiology, B cells evolve progressively from an early-activated IgD⁺IgM⁺ phenotype, and may eventually differentiate through immunoglobulin class switching to express either IgG or IgA. \(^{14,15}\) Immunoglobulin expression from three representative patients from each study group is represented in Figure 4. On average, IgD⁺IgM⁺ accounted for 71.11 ± 3.01% of the mature B cell population in healthy individuals, and 69.09 ± 4.16% in uveitis patients. In AIR patients, 79.28 ± 4.34% of mature B cells were IgD⁺IgM⁺; although the frequency was higher in AIR compared to both healthy and uveitis, it did not reach statistical significance (\( P = 0.14 \) and \( P = 0.21 \) respectively). In contrast, the frequency of both IgG⁺ and IgA⁺ cells in AIR patients was significantly decreased compared to both healthy and uveitis patients. IgA⁺ cells represented 3.90 ± 0.58% of mature B cells in AIR compared to 9.09 ± 1.39% in uveitis (\( P = 0.003 \)) and 6.86 ± 0.75% in healthy controls (\( P = 0.01 \)). Similarly, IgG⁺ cells represented 2.93 ± 0.86% of mature B cells in AIR compared to 9.55 ± 1.93% in uveitis (\( P = 0.016 \)) and 5.93 ± 0.95% in healthy controls (\( P = 0.061 \)). These data indicate that AIR patients not only have lower B cells frequency, but also showed a different isotype profile.
Differential Expression of Plasma Cytokine Concentrations

Plasma cytokine concentrations were assessed by Luminex analysis and are presented in Table 2. AIR patients differed significantly from healthy controls in 6 out of the 15 cytokines tested. Notably, IFN-γ and TNF-α were increased in AIR, whereas IL-22, IL-23, IL-33, and sCD40L decreased in AIR patients compared to the healthy cohort. Compared to uveitis, AIR had significantly lower IL-1β and sCD40L expression.

**Figure 4.** Immunoglobulin expression of IgM, IgD, IgG, and IgA on the CD19⁺CD20⁺ mature B cell population. (A) Representative flow cytometry dot plots and histograms highlighting the IgM IgD double-positive cells and IgA and IgG expression. (B) Cumulative data. (C) Pie chart representation of Ig expression on mature B cells.
**DISCUSSION**

We utilized multiparametric flow cytometry to demonstrate that B-lymphocyte populations may influence the disease pathophysiology of AIR. Using this approach, we have (1) immunophenotypically previously uncharacterized immune cell populations in AIR, and (2) utilized a combination of surface and cytokine analysis to identify markers that can aid in differential diagnosis in the clinical setting. We believe that these findings take a critical step toward better understanding AIR.

Our findings suggest that a primary defect in AIR is the failure of B-lymphocytes to differentiate into switched memory B cells and plasma cells, the frequency of naive B cells was significantly higher in AIR patients, while the frequencies of switched memory B cells and plasmablasts were significantly lower. During the process of maturation, B-lymphocytes arise from the bone marrow and circulate between the peripheral blood, lymph nodes, and splenic follicles until they undergo apoptosis or encounter an antigen. Upon activation by an antigen, immature B cells may differentiate into either plasma cells or memory B cells. The altered frequencies of these populations in AIR, and (2) utilized a combination of surface and cytokine analysis to identify markers that can aid in differential diagnosis, and we included uveitis patients in attempts to elucidate differences in B cell populations, as noninfectious and infectious uveitis syndromes may masquerade as AIR. We found distinct differences in the immunophenotype of B cell memory subsets between uveitis and AIR, only some patients with documented autoantibodies suggest that antiretinal antibodies play a pathogenic role in AIR, and intimately involved in the immunopathogenesis of the disease.

The presence of circulating antiretinal antibodies is the conventional hallmark of AIR. Unexpectedly, we found that the frequency of plasmablasts was significantly decreased in AIR patients. Indeed, it is curious that this population was present at such low frequencies; plasmablasts are transient, proliferating precursors to plasma cells, which are essential for antibody response as they alone synthesize large amounts of immunoglobulins that are antigen specific. Plasmablasts have been seen to be elevated in some stages of rheumatoid arthritis and ulcerative colitis and reported to fluctuate with treatment. How this correlates to AIR and the stages of the disease is yet to be clearly defined. While multiple in vitro and in vivo experiments suggest that antiretinal antibodies play a pathogenic role in AIR, only some patients with documented autoantibodies develop retinopathy, while others do not. Moreover, antiretinal antibodies are detectable in an array of systemic autoimmune disorders and retinal degenerations, as well as in healthy controls, suggesting that their presence is not necessarily diagnostic. It is possible that the antiretinal antibodies detectable in AIR may not be pathogenic, but rather a result of retinal damage untied to the underlying etiology. We found no significant differences in transitional B cells, the peripheral immature B-lymphocytes believed to serve as a checkpoint for autoreactive B cells, further suggesting that central B cell tolerance is properly established in AIR.

The second objective of our study involved the issue of differential diagnosis, and we included uveitis patients in attempts to elucidate differences in B cell populations, as noninfectious and infectious uveitis syndromes may masquerade as AIR. We found distinct differences in the immunophenotyping of B cell memory subsets between uveitis and AIR individuals, particularly in immunoglobulin expression. Further, Luminex cytokine analysis revealed decreased plasma concentrations of the inflammatory markers IL-1β, IFN-γ, IL-6, sCD40L, and TNF-α in AIR patients compared to individuals with uveitis, though only IL-1β and sCD40L reached statistical significance. Some of the inflammatory markers were higher in AIR patients, reinforcing the concept of an inability of immature B-lymphocytes to differentiate into more mature, isotype-switched memory B cells in vivo. It is tempting to hypothesize that alterations in T lymphocytes and/or germinal centers may be responsible for the maturation failures in AIR and intimately involved in the immunopathogenesis of the disease.

**Table 2. Plasma Cytokine Concentrations (pg/mL) Represented as Median ± Standard Error of the Mean for Each Respective Marker in the Bioplex Th-17 (15-Plex) Kit**

| Analyte, pg/mL | AIR, n = 10 | Healthy, n = 15 | Uveitis, n = 9 | AIR vs. Healthy, P Value | AIR vs. Uveitis, P Value |
|----------------|-------------|----------------|---------------|------------------------|------------------------|
| IFN-γ          | 435.40 ± 116.90 | 0.43 ± 108.30 | 734.50 ± 53.59 | 0.013                  | 0.0789                 |
| IL-10          | 14.74 ± 5.55   | 4.92 ± 5.57   | 25.22 ± 24.44 | 0.142                  | 0.1564                 |
| IL-1β          | 1.70 ± 0.87    | 0.76 ± 0.95   | 7.01 ± 0.95   | 0.975                  | 0.003                  |
| IL-4           | 15.41 ± 5.52   | 4.72 ± 4.53   | 16.79 ± 0.98  | 0.066                  | 1.0000                 |
| IL-6           | 27.02 ± 6.88   | 10.67 ± 4.70  | 40.72 ± 6.98  | 0.096                  | 0.2428                 |
| IL-21          | 74.93 ± 37.52  | 8.91 ± 3.78   | 125.20 ± 24.28| 0.021                  | 0.156                  |
| IL-17A         | 1.90 ± 1.52    | 3.70 ± 5.88   | 0.49 ± 0.15   | 0.149                  | 0.056                  |
| IL-17F         | 1.28 ± 1.07    | 30.48 ± 9.01  | 1.28 ± 0.00   | 0.258                  | 0.087                  |
| IL-23          | 2.13 ± 13.05   | 6.29 ± 27.15  | 2.13 ± 0.00   | 0.604                  | 0.087                  |
| IL-22          | 0.30 ± 0.00    | 30.23 ± 11.42 | 0.30 ± 0.00   | <0.0001                | 1                      |
| IL-25          | 1.55 ± 0.00    | 161.20 ± 37.59| 1.55 ± 0.00   | 0.0002                 | 1                      |
| IL-31          | 31.81 ± 11.87  | 57.42 ± 8.23  | 38.44 ± 10.14 | 0.41                   | 0.647                  |
| IL-33          | 0.58 ± 9.24    | 53.34 ± 12.94 | 0.58 ± 1.25   | 0.0025                 | 0.768                  |
| sCD40L         | 284.80 ± 52.20 | 859.60 ± 189.00| 532.20 ± 84.50| 0.005                  | 0.0220                 |

Significance was set at the *P* ≤ 0.05 level and significant *P* values are bolded. SEM less than 0.01 are rounded to 0.00.
than normal, and this could be a reflection of the interactions between B cell– and T cell–mediated inflammation. Of note, there is a high variability in the measurement of IFN-γ and TNF-α (20–30% intertest variability). Previously published work on 14 healthy individuals showed much higher levels of IFN-γ (range: 136–822). These findings are in agreement with previous studies demonstrating that AIR patients have little to no intraocular inflammatory cells and likely do not respond to conventional anti-inflammatory treatment. The levels of circulating cytokines between AIR and uveitis appear to be remarkably different; screening for inflammatory plasma markers or immunoglobulins may suggest differences between the two disease states and combat differential diagnoses.

In summary, high-parameter flow cytometry revealed informative differences in B-lymphocyte subsets in AIR patients. We acknowledge that this study had certain limitations, such as a single center study design and small sample size, though we believe our patient cohort size was reasonable due to the rarity of the disease. The results presented here indicate a failure of B cell differentiation, leading to deficient immunoglobulin secretion in AIR patients; the processes underlying the B cell memory compartment and immunoglobulin class switching may be key aspects in the pathogenesis of AIR. Our data support the notion that the immune cells involved in AIR do not stem from an inflammatory origin. However, we did not find substantial compelling evidence confirming the presumed role of anti-retinal antibodies in AIR; the significance of this is unclear.

Further insights into the central mechanisms involved in AIR may be gained by looking at germinal center physiology and T and B cell interactions, as well as assessing plasma immunoglobulin expression and subclasses. Assessing the phenotypic profile of B cells in AIR is a promising approach that may be utilized to facilitate improved diagnosis and monitoring of patients.

Acknowledgments

Supported by the National Institutes of Health, National Eye Institute, National Heart, Lung, and Blood Institute, and Center for Human Immunology intramural research programs. The authors alone are responsible for the content and writing of the paper.

Disclosure: E. Stansky, None; A. Biancotto, None; P.K. Dagur, None; S. Gangaputra, None; B. Chaigne-Delalande, None; R.B. Nussenblatt, None; H.N. Sen, None; J.P. McCoy Jr, None

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