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Correlation of Immunological Markers with Disease and Clinical Outcome Measures in Patients with Autoimmune Retinopathy

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Purpose: To determine if immunological markers (1) are significantly different between autoimmune retinopathy (AIR) patients and controls and (2) correlate with disease progression in AIR patients.

Methods: We enrolled patients with a possible AIR diagnosis, as well as control participants without eye disease, autoimmunity, or cancer. Immunological markers were tested in all participants. In addition, AIR patients had up to three blood draws for testing over their disease course. For AIR patients, clinical measures, including visual acuity (VA) and Goldmann visual field (GVF) area, were recorded at each draw. We used the Mann–Whitney *U* test to compare the immunological markers between AIR patients and controls. We used multilevel mixed-effect regression to investigate the correlation between markers and clinical parameters over time in AIR patients.

Results: Seventeen patients with AIR and 14 controls were included. AIR patients had a higher percent of monocytes ($Z = 3.076$, $P = 0.002$). An increase in immunoglobulin G against recoverin was correlated with a VA decrease ($\beta = 0.0044$, $P < 0.0001$). An increase in monocyte proportion was correlated with a decrease in GVF area ($\beta = -7.27$, $P = 0.0021$). Several markers of B-cell depletion were correlated with GVF improvement.

Conclusions: Monocytes may play a role in AIR pathophysiology and be a disease activity marker. B-cell depletion markers correlated with clinical parameter improvement, particularly GVF.

Translational Relevance: This work elucidates immunologic markers that may improve the accuracy of diagnosis and treatment of AIR.

Introduction

Autoimmune retinopathy (AIR) is a rare blinding retinal disorder. The spectrum of AIR includes nonparaneoplastic AIR (npAIR) and cancer-associated retinopathy (CAR). The diagnosis is based mainly on clinical features including abnormal electroretinogram (ERG) findings (with or without visual field abnormality), absence of overt intraocular inflammation, and exclusion of inherited retinal degeneration.[1] Although the exact pathogenesis of AIR is unknown, AIR is thought to result from an immunologic attack on the retina by antiretinal antibodies (ARAs).[2–5]

To date, the presence of ARAs is the only immunological marker available for clinical use to potentially guide the diagnosis of AIR; however, ARA testing has a low specificity, as ARAs can also be present in patients with other autoimmune disorders and

normal controls.[6] There is a need to discover additional biomarkers that could help distinguish AIR from other diseases that mimic AIR. Because autoimmune diseases, such as AIR, often involve the dysregulation of the immune system leading to inflammation,[7–9] additional immunological and inflammatory markers have been investigated in AIR. Some studies have found B-cell anomalies.[10,11] Abnormalities in lymphocyte cells and cytokines have been found in AIR patients when compared with controls.[11,12] AIR patient peripheral blood mononuclear cells (PBMCs) have also been shown to preferentially respond to recoverin with a high production of the T helper 1-associated cytokine interferon gamma (IFN-γ) compared to patients with retinitis pigmentosa or controls.[13]

Although there are case reports that have demonstrated ARA titer decreases in patients who recovered visual function following treatment for CAR,[14,15] ARAs as biomarkers of treatment response and disease progression have not been consistent. To the best of our knowledge, immunological markers other than ARAs have not been investigated in relation to clinical outcomes. The purposes of this study were to determine if immunological markers (1) are significantly different between AIR patients and non-AIR patients and (2) correlate with disease progression in AIR patients.

Methods

This study was approved by the University of Michigan and Massachusetts Eye and Ear Infirmary (MEEI) institutional review boards. The study conformed to the Tenets of the Declaration of Helsinki and Health Insurance Portability and Accountability Act regulations. Informed consent was obtained from participants.

Patient Recruitment

We enrolled patients referred to the MEEI Retina/Uveitis Services with a possible diagnosis of AIR from February 2016 to February 2018. The diagnosis was based on characteristic symptoms (sudden or subacute painless loss of vision, photopsias, scotomas, photoaversion, nyctalopia) and findings (abnormal ERG findings included abnormal rod and/or cone responses on full-field or multifocal testing), exclusion of other diagnoses, absence of overt inflammation, and no history of hereditary retinal disease. Exclusion of alternative diagnoses included genetic testing for inherited retinal degenerations and fluorescein and indocyanine angiography to detect occult retinal vasculitis and/or posterior uveitis. Indeed, some patients initially enrolled were eventually diagnosed with alternative diagnoses, including inherited retinal degenerations confirmed by genetic testing and occult posterior uveitis or retinal vasculitis.[16,17] These patients are referred to as patients with AIR-like pathologies from this point forward. There is some overlap in the presenting symptoms and ERG, VF, and OCT findings for AIR, inherited retinal degenerations, and occult posterior uveitis. It is a common clinical scenario that patients can present with ambiguous clinical features that do not allow physicians to easily distinguish between AIR versus inherited retinal degeneration versus occult posterior uveitis at the first encounter. In these cases, the diagnosis may not become clear until results from additional testing including ophthalmic imaging and genetic testing become available, and such testing and results are not always immediately available when the patient is first seen. Control subjects were included if they did not have any eye disease, autoimmune disease, or cancer and if they did not have a family history of retinal degeneration. The controls were enrolled at the University of Michigan.

For AIR patients, to distinguish between npAIR and CAR, an evaluation for malignancy was done for all patients unless the patient already had a known cancer diagnosis. If a cancer diagnosis existed, the association of AIR with cancer diagnosis, and thus the designation of CAR, was established primarily based on the temporal relationship between the cancer onset or recurrence and the onset of visual symptoms and/or the presence of other paraneoplastic phenomena close to the time of AIR diagnosis. It is not always possible to know with complete certainty if the AIR is truly related to a patient's underlying cancer. If we were in doubt as to whether the cancer was truly associated with the AIR, we were conservative and designated the patient as having CAR.

Immunohistochemistry Markers

For each participant, we collected at least one blood sample that was sent for immunological marker testing at the University of Michigan. AIR patients had the option to give a blood sample up to three times during their clinical course. Repeat blood draws were spaced approximately 6 months apart. Testing included ARAs by western blotting, testing by flow cytometry for lymphocyte subsets, recoverin antibody enzyme-linked immunosorbent assay (ELISA) testing, and in vitro stimulation of PBMCs with recoverin followed by evaluation of cytokine release.[13] More

Table 1. Immunologic Markers Examined

Marker	Abbreviation
PBMC response to recoverin	
Tumor necrosis factor alpha	TNF-α
Interferon gamma	INF-γ
Interleukin 10	IL-10
Interferon gamma/interleukin 10 ratio	INF-γ/IL-10
Recoverin antibodies (in response to recoverin)	
Immunoglobulin G response to recoverin	ELISA IgG recoverin
Immunoglobulin M response to recoverin	ELISA IgM recoverin
PBMC subsets	
Monocytes	—
Lymphocytes	—
$CD3^+$ T lymphocytes	$CD3^+$ T cells
$CD4^+$ T lymphocytes	$CD4^+$ T cells
$CD8^+$ T lymphocytes	$CD8^+$ T cells
Non-T-cell, non-B-cell lymphocytes	NonT, NonB
$CD56^+$ natural killer cells	NK cells
$CD56^+CD3^+$ natural killer T cells	NKT cells
$CD19^+$ B lymphocytes	$CD19^+$ B cells
B-cell lymphocyte subsets	
$CD24^{hi}CD38^{hi}$ regulatory B cells	T2-MZP
$CD43^+CD27^+$ B-1 cells	B-1 cells
$CD27^+$ memory B cells	$CD27^+$ memory B cells
$CD24^{hi}CD27^+$ memory B cells	$CD24^{hi}$ memory B cells
$CD24^{lo}CD27^+$ plasmacytes	Plasmacytes

PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation, MZP, marginal zone precursor.

detailed methods have been described in a previously published protocol.[13] The list of markers examined is in Table 1.

Clinical Outcomes

For AIR patients, several measures of visual function or anatomy were recorded at each draw, including best-corrected Snellen visual acuity (VA), ERG parameters, Goldman visual fields (GVFs), and central retinal thickness (CRT) measured by optical coherence tomography (OCT). Full-field ERGs were performed with Burian–Allen electrodes (Hansen Labs, Coralville, IA) according to a previously described protocol.[18] For the full-field ERG, dim scotopic, bright scotopic, 30-Hz flicker amplitudes, and 30-Hz flicker implicit times were recorded. Full-field ERG results were compared to age-matched control normative data. GVF testing with I2e, I4e, and V4e test lights was performed on the ERG Service at MEEI. OCT imaging was performed with a spectral-domain instrument (Spectralis; Heidelberg Engineering, Heidelberg, Germany). Fovea-centered images were acquired (25 lines within a 20° horizontal scan and 25 lines within a 20° vertical scan). CRT was recorded from each visit from a horizontal foveal scan image. It was measured within the central 1-mm-diameter circle surrounding the fovea. The presence of macular cystic changes, as well as treatment status, age, race, and sex, were recorded.

The parameters analyzed were best-corrected VA, dim scotopic amplitudes (rod; blue, 0.5 Hz), bright scotopic amplitudes (maximal combined; white, 0.5 Hz), cone amplitudes and implicit times (white, 30-Hz flicker), GVF I4e isopter area, GVF V4e isopter area, and CRT. Snellen VA was converted to the logMAR scale, where a one-line decrease in Snellen VA corresponds to a 0.1 increase in logMAR for statistical analysis.[19] The area seen by the patient on GVFs was quantified digitally with ImageJ (National Institutes of Health, Bethesda, MD).[20] A 10° radius on the GVF was set to correspond to 12 mm. Two independent measurements were taken for each GVF and averaged. Measurements were redone if the areas differed by more than 10%. CRT was measured in micrometers (μm).

Association of Immunological Markers to AIR Diagnosis

We first used the Mann–Whitney *U* Test to compare the immune markers among different patient groups. These analyses were done using the first blood draw of each participant, which was nearest to the time of first diagnosis for AIR patients. The primary analysis comparison was between all AIR patients versus healthy controls without eye disease. Two sensitivity analyses were executed examining (1) AIR patients who were untreated at the first draw versus controls and (2) AIR patients without any systemic autoimmune diseases at the first draw versus controls. These sensitivity analyses were meant to remove any alterations of immune markers that might have occurred with treatment or from systemic autoimmune diseases. Secondary analyses were executed for the following comparisons: npAIR patients versus normal controls, CAR patients versus normal controls, and AIR patients versus patients with AIR-like pathologies. A significance threshold of $P < 0.0031$ (0.05/16) was determined using a Bonferroni correction for the four independent categories of immune markers examined (see Table 1) and four distinct comparisons (AIR vs. controls, two AIR subtype secondary analyses, and AIR vs. AIR-like pathologies secondary analysis).

Association of Immunological Markers to Clinical Outcomes in AIR Patients

For the linear regression models examining the correlation between markers and clinical outcomes, we included only AIR patients. Although AIR is usually bilateral, it can manifest asymmetrically.[21] Therefore, we considered both eyes of each patient separately and used multilevel mixed-effects regression to account for lack of independence between the eyes. To be included in this longitudinal analysis, patients had to have at least two blood draws (n = 14). All blood draws that were available for each patient were used. We included an additional level to account for the additional draws for some patients. All clinical outcomes and immune markers were considered as continuous variables.

We started with univariate analyses between a change in each clinical outcome and a change in markers. Covariates tested in the models included age, sex, race, baseline value of the clinical outcome measure at the first visit, treatment status (yes/no), and macular cystic changes (for CRT analysis only). All covariates with $P \leq 0.250$ when tested individually with each clinical outcome measure were included in the initial multivariate models. A backward elimination procedure was then used to develop the multivariate models. Age, sex, and race were included in all multivariate models regardless of significance. A significance threshold of $P < 0.0031$ (0.05/16) was determined using a Bonferroni correction for the four categories of immune markers examined and the four visual outcome types (VA, ERG, GVF, and CRT). All statistical analyses were performed in Stata 12.1 (StataCorp, College Station, TX).

Results

Seventeen patients with AIR (12 npAIR, 5 CAR) with a median age of 64 years were enrolled. This included four men and 13 women. The demographic and clinical characteristics of these patients are detailed in Supplementary Table S1. Fourteen normal controls and five patients with AIR-like pathologies were also included. Their characteristics are detailed in Supplementary Tables S2 and S3, respectively. For the five AIR patients, the final diagnoses and supporting evidence for these are detailed in Supplementary Table S3. Both AIR patients and patients with AIR-like pathologies had detectable ARAs. The detection of ARAs in patients who ultimately had diagnoses of inherited retinal degeneration and posterior uveitis is not unexpected and has been previously reported.[22–24] There was some overlap in the molecular weights of the ARAs between the two groups and no discernable difference in the ARA patterns between the two groups. There were also patients in both groups who did not have detectable ARAs. It is also not unexpected that some AIR patients would not have detectable ARAs, as up to one-third of AIR patients may not have detectable ARAs.[25] Of the patients with AIR, six were untreated at their first blood draw. Of those who were treated, treatments within the 6 months before the first blood draw included rituximab (n = 10), prednisone (n = 7), cyclophosphamide (n = 5), intravenous immunoglobulin (n = 3), mycophenolate (n = 1), and methotrexate (n = 1). We used multiple systemic immunosuppressive therapies in some patients because their AIR was difficult to treat and failed successive therapies.

Association of Immunological Markers to AIR Diagnosis

Table 2 shows the median and interquartile range (IQR) of the markers for all AIR patients, treated AIR patients, untreated AIR patients, npAIR patients, CAR patients, patients with AIR-like pathologies, and

Table 2. Median and Interquartile Range of Immune Markers in Different Patient Populations and Subsets of Populations at First Draw

	Patients with AIR (n = 17)					Patients without AIR	
Immune Marker	All (n = 17)	Treated (n = 11)	Untreated (n = 6)	npAIR (n = 12)	CAR (n = 5)	AIR-Like Pathologies (n = 5)	Normal Controls (n = 14)
PBMC response to recoverin, pg/ml							
TNF-α	452.9 (521.8)	457.8 (527.9)	416.9 (564.7)	455.4 (645.4)	380.9 (413.6)	364.3 (172.8)	132.5 (174.6)
INF-γ	95.4 (354.8)	127.3 (1610.5)	63.6 (80.1)	155.8 (994.2)	15.5 (30.1)	160.5 (69.5)	63.1 (173.9)
IL-10	214.4 (359.2)	177.4 (407.1)	302.4 (369.9)	168.8 (395.6)	246.3 (181.1)	109.9 (53.6)	49.0 (324.4)
INF-γ/IL-10 ratio	59.4 (148.9)	59.4 (1111.1)	41.5 (104.9)	112.7 (667.5)	8.5 (5.7)	235.1 (192.3)	46.0 (1050.8)
Recoverin antibodies, ODV							
ELISA IgG recoverin 1:40	0.330 (0.321)	0.264 (0.352)	0.473 (0.154)	0.309 (0.270)	0.483 (0.104)	0.423 (0.155)	0.420 (0.155)
ELISA IgM recoverin 1:40	0.219 (0.145)	0.199 (0.145)	0.262 (0.323)	0.223 (0.240)	0.149 (0.136)	0.208 (0.134)	0.160 (0.115)
Lymphocyte subsets, %							
Monocytes	6.4 (8.5)	6.4 (7.8)	8.4 (21.0)	6.0 (14.1)	12.3 (0.9)	3.7 (8.4)	0.6 (4.0)
Lymphocytes	72.3 (33.3)	77.4 (14.3)	52.0 (35.8)	74.7 (24.8)	57.6 (40.3)	74.9 (11.8)	85.1 (18.4)
$CD3^+$ T cells	72.0 (17.3)	75.9 (21.0)	64.0 (58.7)	73.0 (15.0)	63.0 (59.4)	54.8 (5.2)	69.9 (10.0)
$CD4^+$ T cells	51.3 (20.0)	56.5 (25.2)	48.3 (45.8)	53.9 (11.2)	24.4 (56.8)	44.4 (2.9)	53.1 (18.6)
$CD8^+$ T cells	16.7 (8.7)	19.8 (14.7)	9.18 (14.0)	17.0 (7.2)	4.0 (9.6)	18.1 (15.2)	14.1 (13.3)
NonT, NonB	22.9 (9.5)	22.9 (15.0)	21.6 (29.1)	21.8 (8.6)	24.4 (30.3)	34.6 (5.6)	21.1 (5.2)
NK	12.0 (5.8)	10.1 (9.6)	12.6 (7.5)	12.0 (7.6)	13.4 (11.5)	15.5 (16.1)	14.0 (8.3)
NKT	1.9 (1.4)	2.2 (2.5)	1.3 (1.2)	1.8 (2.1)	1.9 (1.2)	2.0 (2.6)	1.6 (2.8)
$CD19^+$ B cells	3.6 (8.0)	0.4 (3.3)	13.1 (12.3)	0.7 (5.5)	8.4 (15.8)	11.1 (3.6)	6.9 (4.0)
B-cell lymphocyte subsets, %							
T2-MZP	2.9 (8.9)	2.6 (4.9)	9.1 (17.2)	2.3 (2.8)	18.9 (9.0)	7.4 (13.5)	6.9 (3.2)
B-1	14.2 (69.5)	57.4 (66.3)	3.6 (0.8)	43.9 (70.3)	4.1 (10.3)	3.7 (6.0)	3.3 (2.8)
$CD27^+$ memory B cells	46.8 (44.4)	58.3 (42.3)	19.0 (34.3)	50.0 (41.3)	28.4 (39.7)	40.0 (8.9)	30.7 (13.8)
$CD24^{hi}$ memory B cells	11.5 (13.3)	10.3 (10.2)	16.4 (36.5)	10.9 (11.3)	16.0 (13.1)	30.6 (12.8)	26.3 (12.3)
Plasmacytes	10.5 (51.9)	52.2 (51.2)	4.2 (2.2)	30.0 (50.9)	7.0 (6.1)	3.4 (2.8)	3.3 (2.6)

TNF-α, tumor necrosis alpha; ODV, optical density value.

Table 3. Results for the Mann–Whitney *U* Test Comparing Immunologic Markers Among Different Patient Groups

	All AIR (n =17) vs. Normal Controls (n = 14)[a]		Untreated AIR (n = 6) vs. Normal Controls (n = 14)[b]		npAIR (n = 12) vs. Normal Controls (n = 14)		CAR (n = 5) vs. Normal Controls (n = 14)		All AIR (n =17) vs. AIR-Like Pathologies (n = 5)	
Immune Marker	*Z* Score	*P*	*Z* Score	*P*	*Z* Score	*P*	*Z* Score	*P*	*Z* Score	*P*
PBMC response to recoverin										
TNF-α	2.365	0.018	1.491	0.136	2.448	0.014	1.134	0.257	0.196	0.845
INF-γ	0.565	0.572	−0.351	0.726	1.741	0.082	−1.824	0.068	−0.588	0.557
IL-10	1.904	0.057	1.754	0.079	1.686	0.092	1.429	0.153	1.919	0.055
INF-γ/IL-10	−0.544	0.586	−1.097	0.273	0.544	0.587	−2.268	0.023	−1.606	0.108
Recoverin antibodies										
ELISA IgG recoverin 1:40	−0.774	0.439	0.825	0.410	−1.466	0.143	0.833	0.405	−0.744	0.457
ELISA IgM recoverin 1:40	1.449	0.147	1.650	0.099	1.646	0.100	0.417	0.677	−0.588	0.557
PBMC subsets										
Lymphocytes	−2.660	0.008	−2.722	0.007	−2.263	0.024	−2.129	0.033	−0.274	0.784
Monocytes	**3.076**	**0.002**	2.193	0.028	2.611	0.009	2.514	0.012	1.371	0.170
$CD3^+$ T cells	0.437	0.662	−0.990	0.322	0.926	0.355	−0.648	0.517	1.449	0.147
$CD4^+$ T cells	−0.357	0.721	−0.660	0.509	−0.103	0.918	−0.648	0.517	0.823	0.411
$CD8^+$ T cells	0.318	0.751	−1.402	0.161	1.183	0.237	−1.389	0.165	−0.196	0.845
NonT, NonB	0.337	0.736	0.577	0.564	0.077	0.939	0.648	0.517	−1.176	0.240
NK cells	−0.921	0.357	0.395	0.693	−1.360	0.174	0.296	0.767	−0.666	0.505
NKT cells	−0.377	0.706	−0.922	0.357	−0.300	0.765	−0.345	0.730	−0.275	0.783
$CD19^+$ B cells	−2.025	0.043	1.980	0.048	−2.624	0.009	0.000	1.000	−2.077	0.038
B-cell lymphocyte subsets										
T2MZP	−1.449	0.147	0.165	0.869	−2.752	0.006	1.574	0.116	−1.371	0.170
B-1	2.699	0.007	−0.330	0.741	**2.984**	**0.003**	0.926	0.354	1.606	0.108
$CD27^+$ memory B cells	1.548	0.122	−0.907	0.364	2.109	0.035	−0.185	0.853	0.823	0.411
$CD24^{hi}$ memory B cells	**−3.017**	**0.003**	−1.155	0.248	−2.880	0.004	−1.852	0.064	−1.998	0.046
Plasmacytes	**3.235**	**0.001**	0.619	0.536	**3.498**	**0.001**	1.250	0.211	1.919	0.055

Results that were statistically significant after Bonferonni correction of the p value are bolded.
[a]Primary analysis.
[b]Sensitivity analysis.

normal controls. The Mann–Whitney *U* test results are shown in Table 3.

Primary Analysis: Comparing All AIR Patients to Normal Controls

In the primary analysis, which compared normal controls to all AIR patients, the percent of monocytes was significantly higher in AIR patients ($Z = 3.076$, $P = 0.002$). $CD24^{hi}CD27^+$ regulatory B cells were decreased ($Z = -3.017$, $P = 0.003$), and $CD24^{lo}CD27^+$ plasmacytes were increased ($Z = 3.235$, $P = 0.001$) in AIR patients; these two changes in B-cell lymphocyte subsets likely reflect the high proportion of AIR patients treated with rituximab at their first draw. Rituximab selectively targets $CD20^+$ B cells and alters the B-cell subset profile. In the sensitivity analysis comparing untreated AIR patients to normal controls, there were no significant differences between the groups (Table 3).

Two npAIR patients had systemic autoimmune diseases at the time of AIR diagnosis, and one CAR patient had an autoimmune small fiber peripheral neuropathy, thought to be a paraneoplastic phenomenon (Supplementary Table S1). In the sensitivity analysis comparing the 14 AIR patients without any systemic autoimmune disease to normal controls, the results remained largely unchanged, except that the *P* values were slightly larger due to the decrease in power from the smaller sample size.

The percent of monocytes was higher in AIR patients ($Z = 2.912$, $P = 0.0036$), $CD24^{hi}CD27^{+}$ regulatory B cells were decreased ($Z = -2.619$, $P = 0.0088$), and $CD24^{lo}CD27^{+}$ plasmacytes were increased ($Z = 2.826$, $P = 0.0047$) in AIR patients.

Secondary Analyses

In the secondary analysis comparing npAIR patients to controls, the only differences detected were in the B-cell subsets. There were increased proportions of $CD43^{+}CD27^{+}$ B-1 cells ($Z = 2.984$, $P = 0.003$) and $CD24^{lo}CD27^{+}$ plasmacytes ($Z = 3.498$, $P = 0.001$) in npAIR patients. Again, this is likely explained by the high proportion of npAIR patients treated with rituximab. We did not find any differences for the other two secondary analyses. CAR patients compared to controls and AIR-like pathologies compared to all AIR patients (Table 3).

Association of Immunological Markers with Clinical Outcomes in AIR Patients

Next, we examined the correlation between the markers and VA, ERG, GVF, and CRT (Table 4).

Visual Acuity

An increase in immunoglobulin G (IgG) against recoverin was significantly correlated with a higher logMAR visual acuity (corresponding to a decrease in Snellen VA) in both the univariate ($\beta = 0.0041$, $P = 0.0001$) and multivariate ($\beta = 0.0044$, $P < 0.0001$) analyses. An increase in $CD24^{hi}$ memory B cells was correlated with a decrease in logMAR VA (increase in Snellen VA) in both the univariate ($\beta = -0.0067$, $P = 0.0004$) and multivariate ($\beta = -0.0063$, $P = 0.0011$) analyses (Table 4).

Electroretinography

Only one immunologic marker was associated with a change in ERG. An increase in $CD27^{+}$ memory B cells corresponded to a decrease in ERG blue 0.5-Hz (scotopic) amplitudes in the multivariate analysis ($\beta = -0.4245$, $P = 0.0008$). It was not significant in the univariate analysis ($\beta = -0.1708$, $P = 0.0614$) (Table 4).

Goldmann Visual Fields

A change in the monocyte proportion was significantly correlated with the GVF I4e area in the multivariate analysis ($\beta = -7.27$, $P = 0.0021$). This means that for every 0.1% increase in monocytes, the I4e area constricted by 7.27 mm^2. The proportion of natural killer T (NKT) cells was significantly correlated with the GVF I4e area in both univariate ($\beta = 21.99$, $P < 0.0001$) and multivariate ($\beta = 21.11$, $P < 0.0001$) analyses. This means that for every 0.1% increase in NKT cells, the I4e isopter expanded by 21.11 mm^2.

The percent of $CD19^{+}$ B cells was correlated with the GVF V4e area in the multivariate analysis ($\beta = -74.40$, $P < 0.0001$). This means that for every 0.1% increase in $CD19^{+}$ B cells, the I4e isopter constricted by 74.40 mm^2. An increase in the proportion of B-1 cells was associated with an increase in the GVF V4e area ($\beta = 22.62$, $P = 0.0024$). An increase in the proportion of $CD24^{hi}$ memory B cells was correlated with a decrease in the GVF I4e area ($\beta = -69.10$, $P = 0.0018$) (Table 4).

Optical Coherence Tomography

A change in the proportion of lymphocytes was significantly correlated with CRT in the multivariate analysis ($\beta = 0.1077$, $P = 0.0017$). This means that for every 1% increase in lymphocytes, CRT increased by approximately 1 µm. The proportion of NK cells was significantly correlated with CRT in both univariate ($\beta = -0.2549$, $P = 0.0001$) and multivariate ($\beta = -0.2406$, $P = 0.0002$) analyses. This means that for every 1% increase in NK cells, the CRT decreased by approximately 0.24 µm. The proportion of NKT cells was significantly correlated with CRT in both the univariate ($\beta = -0.0648$, $P = 0.0008$) and multivariate ($\beta = -0.0650$, $P = 0.0005$) analyses. This means that for every 1% increase in NKT cells, the CRT decreased by 0.065 µm (Table 4).

Discussion

To the best of our knowledge, this is the first study to evaluate immunological markers longitudinally and correlate them with multiple objective measures of AIR disease activity, including VA, GVF, and ERG. Our primary finding is that several markers of B-cell depletion are correlated with improvements in GVF. We found some evidence that the percentage of monocytes in peripheral blood correlates both with AIR diagnosis and with the clinical course of AIR. We also observed a correlation between immune reactivity to recoverin and AIR progression, whereas, previously, immune reactivity to recoverin had only been correlated to initial diagnosis.

The first question we investigated was whether any markers were associated with AIR diagnosis. We found that differences in the percentage of monocytes and some B-cell subsets were correlated with diagnosis.

Table 4. Results of Univariate and Multivariate Analyses Assessing the Association Between Immune Markers and Clinical Outcomes

		Univariate Analysis								Multivariate Analysis							
		VA	ERG Dim Scotopic (Rod) Amplitude	ERG Bright Scotopic (Maximal Combined) Amplitude	ERG Cone 30-Hz Amplitude	ERG Cone 30-Hz Implicit Time	GVF I4e Area	GVF V4e Area	CRT	VA	ERG Dim Scotopic (Rod) Amplitude	ERG Bright Scotopic (Maximal Combined) Amplitude	ERG Cone 30-Hz Amplitude	ERG Cone 30-Hz Implicit Time	GVF I4e Area (mm^2)	GVF V4e Area (mm^2)	CRT (μm)
Covariates Adjusted for		NA	NA	NA	NA	NA	NA	NA	NA	Age, Sex, Race, Baseline, Treatment	Age, Sex, Race, Baseline	Age, Sex, Race, Baseline	Age, Sex, Race, Baseline	Age, Sex, Race, Baseline	Age, Sex, Race, Baseline, Treatment	Age, Sex, race, Baseline, Treatment	Age, Sex, Race, Baseline
PBMC response to recoverin																	
TNF-α	β	-4.25×10^{-5}	0.0011	−0.0002	0.0009	−0.0003	−0.0324	0.0772	−0.0002	-1.88×10^{-5}	−0.0003	−0.0020	0.0006	−0.0004	0.0256	0.1358	0.0010
	P	0.2377	0.7148	0.9748	0.3810	0.0460	0.9266	0.6902	0.8491	0.6143	0.9306	0.7720	0.5984	0.0315	0.9313	0.4498	0.4514
INF-γ	β	-6.41×10^{-6}	0.0012	0.0033	0.0006	-9.69×10^{-6}	−0.1275	−0.6225	0.0006	-6.77×10^{-6}	0.0016	0.0034	0.0005	-3.46×10^{-5}	−0.1056	−0.3512	0.0007
	P	0.5788	0.2085	0.1025	0.1331	0.8532	0.7892	0.0617	0.1306	0.5407	0.1743	0.1130	0.2214	0.5241	0.8294	0.2302	0.0719
IL-10	β	8.08×10^{-5}	−0.0033	−0.3903	−0.0002	3.52×10^{-5}	0.4633	−0.2514	0.0002	7.45×10^{-5}	−0.0028	−0.0053	0.0002	1.79×10^{-5}	0.8111	−0.1295	−0.0003
	P	0.0034	0.1667	0.1297	0.8096	0.7759	0.3711	0.3996	0.8620	0.0065	0.2956	0.2938	0.8093	0.8929	0.0316	0.6259	0.7642
INF-γ/IL-10, %	β	-1.21×10^{-5}	0.0012	0.0025	0.0002	1.52×10^{-5}	−0.5434	−0.2442	0.0004	-1.12×10^{-5}	0.0014	0.0024	0.0002	1.30×10^{-6}	−0.6738	−0.4020	0.0006
	P	0.3134	0.3085	0.2254	0.5224	0.7702	0.2284	0.3625	0.2864	0.3422	0.3360	0.2545	0.5708	0.9808	0.0464	0.0633	0.1142
Recoverin antibodies																	
ELISA IgG recoverin[a]	β	0.0041	−0.0161	−0.1553	−0.0779	0.0025	−14.46	−9.55	−0.0239	0.0044	0.0558	−0.1296	−0.0655	0.0040	30.15	7.40	−0.0108
	P	0.0001	0.9067	0.5151	0.0423	0.6659	0.3559	0.3402	0.5063	<0.0001	0.7368	0.6012	0.0998	0.5077	0.0241	0.4406	0.7595
ELISA IgM recoverin[a]	β	0.0024	−0.0451	−0.002154	−0.0773	−0.0005	−55.66	−6.3741	−0.0092	0.0026	−0.1239	−0.3301	−0.1177	0.0013	−75.87	13.77	0.0007
	P	0.0840	0.8248	0.4300	0.0732	0.9450	0.0009	0.5027	0.8434	0.0459	0.6227	0.2254	0.0065	0.8494	<0.0001	0.2941	0.9870
PBMC subsets, %																	
Lymphocytes	β	-9.24×10^{-5}	0.0188	−0.0621	0.0136	−0.0087	21.52	−8.38	0.0862	-5.71×10^{-5}	0.0030	−0.1361	−0.0073	−0.0098	27.63	7.50	0.1077
	P	0.9349	0.8899	0.8174	0.7594	0.1745	0.2510	0.4419	0.0177	0.9570	0.9832	0.6374	0.8686	0.1349	0.0470	0.4429	0.0017
Monocytes[b]	β	-3.0×10^{-5}	0.0287	0.0165	−0.0109	0.0022	−5.25	1.77	0.0081	−0.0001	0.0432	0.0278	−0.0122	0.0029	−7.27	−4.49	0.0024
	P	0.9002	0.2958	0.7599	0.2299	0.0885	0.1260	0.4165	0.3583	0.5881	0.1886	0.6588	0.2452	0.0534	0.0021	0.0454	0.8075
$CD3^+$ T cells	β	0.0025	−0.1094	−0.3567	−0.0184	0.0006	27.03	4.82	0.0812	0.0020	−0.1765	−0.4262	−0.0241	−0.0007	16.26	3.80	0.0853
	P	0.0271	0.3333	0.0851	0.6064	0.9114	0.0465	0.5580	0.0417	0.0608	0.1742	0.0490	0.4907	0.9008	0.1815	0.6077	0.0247
$CD4^+$ T cells	β	0.0036	−0.1646	−0.3903	−0.0476	0.0032	21.22	3.98	0.0994	0.0030	−0.1859	−0.4789	−0.0548	1.42×10^{-5}	12.56	8.02	0.1141
	P	0.0055	0.2355	0.1297	0.2819	0.6397	0.2807	0.7296	0.0249	0.0182	0.2105	0.0842	0.2252	0.9984	0.4418	0.4214	0.0084
$CD8^+$ T cells	β	−0.0004	−0.0877	−0.6242	−0.0256	−0.0041	100.82	32.45	0.0665	−0.0015	−0.3033	−0.7950	−0.0483	−0.0003	48.18	6.87	0.0523
	P	0.8975	0.7339	0.1998	0.7482	0.7231	0.0215	0.1594	0.4960	0.6167	0.3363	0.1166	0.5247	0.9795	0.3125	0.8195	0.5831
NonT, NonB lymph	β	−0.0017	0.2124	0.5237	0.0257	0.0017	−35.85	−7.82	−0.0860	−0.0018	0.2852	0.5716	0.0242	0.0026	-2.07×10^{6}	−4.47	−0.0960
	P	0.2212	0.1792	0.0446	0.5659	0.8004	0.0186	0.4200	0.0643	0.1490	0.0980	0.0314	0.5766	0.6867	0.1515	0.6097	0.0321
NK cells	β	−0.0033	−0.1486	0.2002	0.0478	−0.0045	−36.92	−1.49	−0.2549	−0.0029	−0.1915	0.2921	0.0629	−0.0031	−12.54	5.76	−0.2406
	P	0.0955	0.4281	0.6000	0.4597	0.6312	0.2121	0.9288	0.0001	0.1246	0.4084	0.4742	0.3308	0.7532	0.6318	0.7254	0.0002
NKT cells[b]	β	0.0015	−0.0447	−0.0850	0.0184	−0.0006	21.99	10.19	−0.0648	0.0011	−0.0520	−0.0856	0.0186	−0.0002	21.11	4.68	−0.0650
	P	0.0194	0.4284	0.4555	0.3498	0.8488	<0.0001	0.0063	0.0008	0.0673	0.3661	0.4555	0.3260	0.9501	<0.0001	0.3489	0.0005
$CD19^+$ B cells[b]	β	−0.0009	0.0107	0.0647	0.0067	−0.0016	32.21	4.17	0.0097	−0.0008	0.0442	0.1221	0.0202	−0.0021	−69.92	−74.40	0.0104
	P	0.0262	0.7471	0.3368	0.5642	0.3309	0.0724	0.6897	0.4980	0.0586	0.2937	0.1295	0.1458	0.2967	0.0462	<0.0001	0.4865
B-cell lymphocyte subsets, %																	
T2-MZP	β	−0.0014	0.2699	0.2485	−0.0165	0.0037	101.63	96.29	0.0137	−0.0004	0.4920	0.3360	0.0159	0.0053	253.35	34.57	0.0596
	P	0.4996	0.4650	0.5239	0.7969	0.6927	0.6454	0.4273	0.8485	0.8325	0.2509	0.4242	0.8196	0.6106	0.1909	0.7926	0.4047
B-1	β	0.0009	−0.0243	−0.0305	0.0269	−0.0022	7.52	11.59	−0.0236	−0.0001	−0.2182	−0.1346	0.0154	−0.0067	20.27	22.62	−0.0271
	P	0.3797	0.8068	0.8721	0.3712	0.6226	0.6315	0.1725	0.4665	0.8976	0.1569	0.5680	0.6850	0.2140	0.1271	0.0024	0.4327
$CD27^+$ memory B cells	β	0.0006	−0.1708	−0.1934	0.0131	−0.0042	12.68	16.34	−0.0301	−0.0001	−0.4245	−0.3526	−0.0070	−0.0069	6.48	14.68	−0.0498
	P	0.5415	0.0614	0.3297	0.6939	0.3821	0.4675	0.0912	0.3988	0.9247	0.0008	0.1252	0.8517	0.1951	0.6327	0.0717	0.1609
$CD24^{hi}$ memory B cells	β	−0.0067	0.0485	0.2961	0.0147	−0.0092	16.65	10.95	0.0323	−0.0063	0.0578	0.2593	−0.0028	−0.0077	−69.10	−34.19	0.0210
	P	0.0004	0.8508	0.4455	0.8304	0.3456	0.4742	0.3731	0.6285	0.0011	0.8299	0.5138	0.9673	0.4326	0.0018	0.0282	0.7580
Plasmacytes	β	0.0023	−0.1151	−0.1000	0.0312	−0.0051	7.70	12.29	−0.0183	0.0013	−0.3070	0.2002	0.0190	−0.0089	18.87	18.98	−0.0316
	P	0.0424	0.3293	0.6480	0.3801	0.3254	0.6411	0.1944	0.6334	0.2766	0.0422	0.4267	0.6388	0.1285	0.1602	0.0146	0.4181

Baseline, adjusted for baseline value for parameter being tested; treatment, adjusted for whether patient was receiving treatment.
NA = not applicable.
[a]Calculated per 0.01 change in ODV.
[b]Calculated per 0.1% change; all other betas were calculated per 1 unit change in value.

The association with B-cell subsets could be attributed to the fact that a large subset of AIR patients were already treated with rituximab at their first blood draw. When we excluded treated AIR patients in the sensitivity analysis, the association with B-cell subsets was no longer present. The association with monocytes indicates a potential role for this leukocyte subset in AIR. Monocytes are primarily identified as a member of the mononuclear phagocyte system, a component of innate immunity. They differentiate into macrophages when recruited into tissues and generally have proinflammatory functions.[26] An increased release of monocytes into the peripheral blood in response to ongoing retinal injury may serve as a marker for AIR.

One previous study found a decreased number of $CD19^+CD45^+$ B cells in AIR patients (n = 8) compared to healthy controls (n = 18).[10] We did not find a decrease in $CD19^+$ B cells in our untreated AIR patients (n = 6) versus controls (n = 14). One possible reason for the inconsistency between studies is that the previous study included only npAIR patients, whereas we also included CAR patients. Another reason is that our study was also slightly less powered to find this difference.

Another manuscript, authored by a subgroup of the investigators on the current study and using the same detection methods and set of normal controls as the current paper, found that npAIR patients (n = 15) had higher production of IFN-γ in response to recoverin than controls (n = 14).[13] Our npAIR patients (n = 12) did have a higher IFN-γ response compared to controls but it did not reach statistical significance ($P = 0.082$). This may be due to the fact that several of our patients were treated with rituximab, which significantly alters T-cell IFN-γ response. The previous study also found an increase in TNF-α response to recoverin in npAIR (n = 11) compared with controls ($P = 0.04$), and the current study also found this result when comparing npAIR patients versus controls ($P = 0.014$), although this P value did not achieve significance after Bonferroni correction. $CD4^+$ cells were decreased in npAIR in the prior study, but, although the $CD4^+$ cells were decreased in the entire AIR group and all of the AIR subsets compared to normal controls in the current study, these differences did not achieve statistical significance. In the current study, we found an increased percentage of monocytes comparing all AIR patients versus controls, which the prior study did not find.[13] Of note, the prior study examined only npAIR patients, and they had not received treatment within the 90 days prior to blood draw, in contrast to our study, which also included CAR patients and treated patients.

The second question we investigated was whether any of the immunological markers were correlated to clinical outcomes in AIR patients. The main findings were as follows: (1) an increase in IgG levels in response to recoverin was associated with a VA decrease, (2) markers of B-cell depletion were associated with GVF improvement, and (3) changes in the proportions of monocytes and NKT cells were associated with GVF changes.

The association of increased IgG in response to recoverin with worsening vision suggests that a greater immunologic reaction to retinal antigen is associated with decreased vision, potentially through greater immunologically mediated destruction of the retina. This is the first study to examine a correlation between immunologic markers and AIR clinical outcomes, but there is evidence from prior studies of diagnostic markers for AIR that immunologic response to recoverin is an important part of disease pathophysiology.[13] We note that the change in visual acuity is relatively small—approximately 2 Snellen visual acuity letters are lost for every 0.1 ODV increase in the IgG recoverin assay.

To interpret the results related to B-cell markers and GVF, it is important to understand the changes to B-cell subsets with rituximab. Rituximab is a monoclonal antibody that targets CD20 on B cells but also destroys >98% of all $CD19^+$ B cells.[27] Apart from a decline in $CD20^+$ and $CD19^+$ B cells with rituximab, the population of $CD43^+CD27^+$ B-1 cells increases proportionally among B-cell subtypes. Plasmacytes, which are $CD43^+CD24^{lo}CD27^+$, overlap greatly with the B-1 cells. B-1 cells are somewhat resistant to rituximab, and this has implications for treatment outcome because B-1 cells have immune regulatory properties.[28,29] The resistance to B-1 cells to rituximab has also been seen in mice.[30] These B-1 cells are almost always negative for CD24 and thus distinct from the memory B cells. When rituximab therapy is beginning to wear off, the $CD24^+CD27^+$ memory B cells (almost all CD43 negative) are among the first B-cell subsets to repopulate. Increases in the ratio of memory versus naïve/immature B cells after rituximab have been linked to relapses of rheumatoid arthritis.[27]

One of the main findings is that markers of B-cell depletion (decreased percentage of $CD19^+$ B cells, increased percentage of $CD43^+CD27^+$ B-1 cells, and decreased percentage of $CD24^{hi}CD27^+$ B memory cells) were significantly correlated with GVF improvement. For the $CD19^+$ B cells and $CD43^+CD27^+$ B-1 cells, the correlation was significant for the V4e isopter; for the I4e isopter, the results showed the same directions of effect but trended only toward

significance for the $CD19^+$ B cells ($P = 0.0462$). For the $CD24^{hi}CD27^+$ B memory cells, the correlation was significant for the I4e isopter, and the correlation with the V4e isopter had a consistent direction of effect with $P = 0.0282$. In addition, a decreased percentage of $CD27^+$ memory B cells was also associated with improvement in the ERG dim scotopic (isolated rod) amplitudes. The correlations with the other ERG amplitudes had the same direction of effect but were not significant. There were several AIR patients in whom the rods were more affected than the cones at baseline. It may be that there is a greater chance to detect an improvement for the isolated rod amplitude, because for several patients rod function was more severely reduced to begin with and therefore had a greater chance to show an improvement. Other studies have documented a role for B cells in the diagnosis in AIR.[10] Our findings suggest that B cells may also play a role in disease progression, given that B-cell depletion specifically is correlated with improvement in outcome measures in multivariate analyses that include covariates for systemic treatment. These observations are also consistent with clinical improvements reported in AIR patients with rituximab.[31,32]

Apart from B cells, two other cell types were also associated with changes in GVF area: monocytes and NKT cells. The correlation we see between increased peripheral monocytes and worsening of the GVF may indicate that there is increased release of monocytes into the peripheral blood in response to ongoing retinal injury from AIR, which leads to worsening of the visual field. The role of infiltrating macrophages into the outer retina has been documented in models of other retinal diseases, such as age-related macular degeneration.[33] Increased NKT cells were associated with improvement in the GVF. In human autoimmune diseases, NKT cell numbers are decreased in peripheral blood, and it is unclear whether this is a cause or effect of the disease.[34] Therefore, the correlation between increased NKT cells and GVF improvement may indicate decreased activity of the underlying autoimmune component. We note that these correlations between monocytes and NKT cells and GVF met the significance threshold that was corrected for multiple hypothesis testing for the I4e isopter but not the V4e isopter. However, associations with the V4e were in the same direction with $P = 0.0454$ for the monocytes (multivariate analysis) and $P = 0.0063$ for the NKT cells (univariate analysis). It may be that, because the I4e stimulus is smaller than the V4e stimulus, it may be more sensitive to change in disease and thus it may be easier to detect changes in the GVF for the I4e isopter than the V4e isopter.

Higher immunoglobulin M (IgM) in response to recoverin was also associated with decreased GVF area. This, along with the correlation between IgG recoverin and visual acuity, suggests that greater immune reactivity to recoverin not only is a marker for the disease itself as noted by prior studies[11,13] but may also be a marker for ongoing disease activity. The heterogeneity of these results, with IgM recoverin correlating with GVF and IgG recoverin correlating with visual acuity, could reflect underlying pathophysiology, with peripheral visual field loss reflecting an earlier stage of retinal damage, mediated by IgM, and central visual acuity reflecting later retinal damage, mediated by IgG. We cannot, however, exclude that these are chance findings, despite the fact that correction for the multiple testing was done.

There were three markers that were associated with a change in CRT on OCT: lymphocytes, NK cells, and NKT cells. The effect sizes, however, were quite small and not clinically meaningful. There was one finding that was difficult to reconcile with our other results. A decrease in $CD24^{hi}CD27^+$ cells (an indication of B cell depletion) was associated with an increase in logMAR vision, which corresponds to a worsening of vision. This conflicts with decreased percentage of $CD24^{hi}CD27^+$ B memory cells being significantly correlated with GVF improvement.

Other findings with clinical implications include the presence of ARAs in our normal controls and similar patterns of ARAs in our AIR and AIR-like pathology patients. Our study found at least one ARA in 43% of the normal controls. This is similar to other studies that have found rates of ARA detection as high as 48% in controls.[25,35] These figures translate into a specificity in the range of 52% to 57%. Circulating autoantibodies that do not seem to be pathogenic are often observed in healthy human subjects and have been postulated to result from normal processes of cell degradation and turnover that expose self-antigens to the immune system. In studies not requiring the presence of ARA as part of the diagnostic criteria, ARA sensitivity is similarly low at 63%.[25] With the additional observations that the ARA patterns are similar among AIR patients and patients with AIR-like pathologies, these findings emphasize the limited utility of ARA testing in the diagnosis of AIR and the need to find new biomarkers for this disease. It has been suggested that ARAs are detected in patients with AIR-like pathologies (e.g., inherited retinal degenerations, posterior uveitis) because damage to the blood–retinal barrier as part of these disease processes may release retinal antigens into the circulation, and the pathogenicity of these ARAs is not clear.[36]

Of note, we used western blot as the ARA detection method. Western blot involves separating retinal proteins on a membrane using electrophoresis, incubating the membrane with patient sera, and then detecting binding of the patient sera to the proteins using a secondary antibody. When a band is seen on a western blot suggesting antibodies against a specific retinal protein, then a confirmatory blot must be performed using that specific protein, but this is not always done, resulting in lower specificity for this method. Immunohistochemistry (IHC), another detection method, involves incubating patient sera with a section of normal retina and using a secondary antibody to detect the binding of patient sera to the normal retina section. IHC has the advantage of localizing the specific site of binding within the retina. One limitation of our study is that we did not perform IHC for ARA detection and thus are missing that complementary information.

There are other limitations to this study. The sample sizes are limited, in part because of the rare nature of the disease. For the evaluation of markers of disease, a significant number of patients were already being treated with immunosuppressive therapy at baseline which alters their immunological markers; thus, we were further limited in power for identifying differences in markers for disease in treatment-naïve patients. Of note, CAR patients in this study were treated similarly to npAIR patients with systemic immunosuppressive therapy. This therapeutic approach has been used in the literature by various centers successfully,[31,32,37–40] but there is a risk with this approach of unmasking immunological control of their cancer. Therefore, we always discuss this risk with the patient and their oncologist before beginning systemic immunosuppressive therapy. However, to our knowledge, this is the first study to look longitudinally at immunological markers and correlate them with objective disease measures. Our primary finding is that several markers of B-cell depletion are correlated with GVF improvement. We also found a potential role for monocytes in the diagnosis and progression of disease which is novel. Finally, we also extended the prior finding of the correlation between AIR diagnosis and immune reactivity to recoverin to an additional correlation between AIR progression and immune reactivity to recoverin. Additional studies are necessary to further validate these findings in larger sample sizes.

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