INTRODUCTION

Age-related macular degeneration (AMD), which refers to the progressive degeneration of the retinal pigment epithelium (RPE), retina, and choriocapillaris, is the leading cause of legal blindness among the elderly in developed countries. Early and intermediate AMD are characterized by drusen, subretinal drusenoid deposits, or focal hyperpigmentation; and late AMD includes neovascular AMD (nAMD) with choroidal neovascularization (CNV) or geographic atrophy. Although the exact mechanism of AMD is not fully understood, evidence of an immune system disturbance has accumulated over time. Single nucleotide polymorphism analysis has revealed that intronic and common variants in the...
piment factor H gene increases the risk of AMD.3,4 Alternative pathway activity in the complement system is increased in AMD patients.5 The inflammasome, which matures the cytokine interleukin (IL)-1β in response to foreign or endogenous danger signals, can be activated by drusen components.5,6 The expression of chemokines and chemokine receptors is altered in RPE, choroidal endothelium, retinal microglial cells, granulocytes, and monocytes in AMD animal models and patients.6,10

Chemokines are primarily chemotactic cytokines, which regulate cellular migration and homeostasis, especially in immune cells.10 Chemokines orchestrate cellular and humoral immune responses by inducing immune cell trafficking according to each specific situation. In addition, chemokines interact with the receptors expressed in the endothelium and leukocytes and modulate angiogenesis or angiostasis.11 Approximately 50 human endogenous chemokines are currently known, and they are classified into four subgroups based on the common motif sequence: CXC, CC, C, or CX3C.12

Interestingly, the expression of some chemokine receptors on T cells is altered in AMD patients.13,14 Generally, chemokine receptors are differentially expressed in each T cell subset, determining their functions and trafficking to tissues. T-cell subsets are divided according to the cytokines they produce, and each subset has a different repertoire of chemokine receptors.15 CD4+ T helper type 1 (Th1) cells, which produce interferon-gamma (IFN-γ) exclusively, express CXCR3 and CCR5, while Th2 cells produce IL-4 and preferentially express CCR3 and CCR4. Th17 cells, which produce IL-17A, have increased expression of CCR4 and CCR6.16 In the case of cytotoxic CD8+ T cells, chemokine receptors, such as CXCR3, CCR5, and CX3CR1, are associated with cytotoxicity and memory generation.17,18 In cases of AMD, Falk, et al.19 reported a decreased percentage of both CD8+ CXCR3+ T cells and CD8+ CX3CR1+ T cells in nAMD patients compared to age-matched controls.20 However, they assessed limited numbers of chemokine receptors on T cells and evaluated the associated chemokines only in the plasma, not in the ocular specific specimen such as aqueous humor. We supposed that subtle chemokine changes, which are undetectable in plasma, could be found in aqueous humor of AMD patients as the pathology of AMD is primarily confined to the eye.

Based on these differences in the T cells of AMD patients, we hypothesized that the expression of chemokine receptors associated with T cell subtypes differ in nAMD patients compared to healthy control subjects and analyzed the expression of multiple chemokine receptors on peripheral blood T cells from nAMD patients and healthy controls. We also assessed the chemokines and cytokines in the aqueous humor of nAMD patients to find associations with the phenotype changes of T cells.

MATERIALS AND METHODS

Study population

Patients with nAMD and healthy controls were recruited from the Department of Ophthalmology, Konkuk University Medical Center, Seoul, Korea, from August 2014 to December 2015. Treatment-naïve nAMD patients >50 years of age were included. Existence of CNV was confirmed with dilated fundus examination, fluorescein angiography, and spectral domain optical coherence tomography (Spectralis HRA+OCT2 device; Spectralis, Heidelberg Engineering, Heidelberg, Germany). Patients with malignancy, allergic disorder, hematological disorder, recent viral or bacterial infections, previous ocular surgery, or other combined retinal pathologies except AMD were excluded. All of the enrolled subjects underwent a medical interview including previous medical history, current medication, smoking history, height, and body weight. Smoking history was categorized as current smoker, former smoker, or never smoker; and body mass index (BMI) was calculated using height and body weight. Obesity was defined as a BMI of 25 or higher according to the criteria of the Korean Society for the Study of Obesity.

The study protocol was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of at Konkuk University Medical Center, Seoul, Korea (IRB No. KUH 1100025). Written informed consent was obtained from all participants.

Isolation of peripheral blood mononucleated cells and flow cytometry

Peripheral blood samples were drawn from all participants on the day of inclusion, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using lymphocyte separation medium (Corning, Manassas, VA, USA). The isolated PBMCs were cryopreserved in liquid nitrogen until use.

Cryopreserved PBMCs were thawed and stained with Live/Dead Fixable Red Stain dye (Life Technologies, Gaithersburg, MD, USA) and fluorochrome-conjugated monoclonal antibodies for cell surface proteins. The stained cells were analyzed using an LSR II instrument (BD Biosciences, San Jose, CA, USA) and FlowJo v10 software (FlowJo, Ashland, OR, USA). The following antibodies were used: anti-CD3-V500 (BD Biosciences), anti-CD4-BV711 (BD Biosciences), anti-CD8-APC-eFluor780 (eBioscience, San Diego, CA, USA), anti-CCR5-BV650 (BD Biosciences), anti-CXCR3-PE (R&D Systems, Minneapolis, MN, USA), anti-CCR6-BV786 (BD Biosciences), anti-CCR8-PE (R&D Systems), anti-CRTh2-PerCP/Cy5.5 (Biolegend, San Diego, CA, USA), anti-CCR4-PE-Cy7 (BD Biosciences), anti-CCR3-APC (R&D systems), anti-CCR10-PE (R&D systems), anti-CCR9-PerCP/Cy5.5 (Biolegend), and anti-CX3CR1-APC (Biolegend).
Aqueous humor sampling and multiplex bead-based immunoassay

All aqueous humor samples were obtained immediately before the first intravitreal injection of anti-vascular endothelial growth factor in nAMD patients or cataract surgery in control subjects for visual rehabilitation. The collection of all samples was performed using standard sterile procedures, and aqueous samples were obtained via anterior chamber paracentesis using 30-gauge needle. Aqueous samples (100 μL) were placed in safe-lock microcentrifuge tubes (1.5 mL), immediately frozen at -80°C, and stored until analysis.

The aqueous humor concentrations of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F; IL-21, IL-22, IFN-γ, tumor necrosis factor-α, C-C motif chemokine ligand 2 [CCL2, also known as monocyte chemoattractant protein (MCP)-1], CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), CCL20 (MIP-3α), C-X-C motif chemokine ligand 1 (CXCL1, also known as GROα), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) were determined using commercially available bead-based sandwich immunoassay kits (LEGENDplex TM, Biologend) according to the manufacturer’s instructions. In brief, diluted aqueous humor samples (25 μL/tube) or standards (25 μL/tube) were incubated with pre-mixed beads and detection antibodies (75 μL/tube) for 2 hours at room temperature with shaking. Next, 25 μL of streptavidin-PE was added to each tube for an additional 30 min at room temperature with shaking. The range of standard proteins was 2.4–10000 pg/mL, and the stained tubes were analyzed using an LSR II instrument. The concentrations were calculated using LEGENDplex data analysis software provided by the manufacturer.

Statistical analysis

Differences between the two groups were analyzed by either t-test or non-parametric t-test (Mann-Whitney U-test) or Pearson χ² test according to the characteristics of each variable. Two-sided p-values were determined in all analyses. A p-value less than 0.05 was considered significant.

RESULTS

Characteristics of the study population

A total of 24 patients with nAMD and 24 age- and sex-matched controls were enrolled in this study. The mean age of the nAMD group and the control group was 66.5 years and 66.4 years, respectively, and there was no significant difference in gender ratios, past medical history, smoking history and obesity between the two groups. However, BMI was higher in the control group. Additional characteristics of the two groups are provided in Table 1.

Expression of chemokine receptors on T cells

The expression of several chemokine receptors was assessed in CD4+ and CD8+ T cells by multi-color flow cytometry, as presented in Figs. 1 and 2. In CD4+ T cells, the nAMD group revealed decreased expression of CXCXR3 and increased expression of CCR4 (Fig. 2A). In CD8+ T cells, the nAMD group had decreased expression of CCR5, CXCXR3, and CXCR1 and increased expression of CRTh2 (Fig. 2B).

Chemokines and cytokines in aqueous humor

Next, we evaluated the levels of chemokines and cytokines in the aqueous humor from nAMD patients to determine if differences exist in reference to the altered T cell phenotypes. The characteristics of patients in the aqueous humor analysis are shown in Table 2, and have similar tendencies as the whole cohort except BMI. Multiple chemokines and cytokines were measured in the aqueous humor by multiplex bead-based immunoassays (Table 3). In nAMD patients, chemokine MCP-1 was increased, and cytokines IL-4 and IL-22 were decreased significantly.

DISCUSSION

This study aimed to comprehensively evaluate the chemokine receptor expression profiles of CD4+ and CD8+ T cells in peripheral blood and assess the levels of chemokines and cytokines in the aqueous humor of nAMD patients. AMD has been thought to be an ocular-specific, age-related degenerative disease. However, recent advances, especially immunological findings in AMD, have suggested that the etiology of AMD should be considered at the systemic level. Genome-wide association studies on AMD patients revealed that the multiple variants are located in the immune related genes, such as CFH, CFI, C9, C2-

Table 1. Baseline Characteristics of AMD Patients and Healthy Controls Enrolled in the Study

| Characteristics          | AMD (n=24) | Control (n=24) | p value | Test |
|--------------------------|------------|----------------|---------|------|
| Age, yr                  | 66.5 (56–75) | 66.4 (62–69) | 0.972   | T-test |
| Male                     | 19 (79.2) | 19 (79.2) | >0.999 | Pearson χ² |
| Medical history           |            |                |         |      |
| Hypertension              | 6 (25.0) | 4 (16.7) | 0.724 | Pearson χ² |
| Diabetes mellitus         | 5 (20.8) | 6 (25.0) | >0.999 | Pearson χ² |
| Smoking history           |            |                | 0.055   | Pearson χ² |
| Current smokers           | 9 (37.5) | 2 (8.3) |       |      |
| Quit smokers              | 7 (29.2) | 11 (45.8) |       |      |
| Never smokers             | 8 (33.3) | 11 (45.8) |       |      |
| Obesity                   | 4 (16.7) | 10 (41.7) | 0.111 | Pearson χ² |
| BMI, kg/m²                | 23.2 (20.8–24.5) | 25.7 (23.8–27.3) | 0.003 | T-test |

AMD, age-related macular degeneration; BMI, body mass index. Data are presented as n (%) or median (interquartile range).

Young Joon Choi, et al.

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Chemokine Receptors of T Cells in AMD

Lorés-Motta, et al. recently reported the systemic complement activation by assessing the C3d-to-C3 ratio. Alterations in chemokine receptor levels on T cells support this perspective.

Sørensen, et al. reported multiple phenotype changes in the peripheral blood T cells of nAMD patients. The authors reported a decreased frequency of CXCR3+ CD8+ T cells, CXCR1+ CD8+ T cells, CXCR3+IL12RB2+ CD4+ T cells, CXCR3+ T cells, CRTh2+ CD4+ T cells, and CXCR3+CCL6+ CD4+ T cells in the peripheral blood of nAMD patients. This altered expression

**Fig. 1.** Gating strategy and representative figures for chemokine receptor phenotyping of T cells. Peripheral blood mononuclear cells were isolated from neovascular age-related macular degeneration patients and healthy controls. (A) Gating strategy for live CD4+ T cells and CD8+ T cells. (B and C) Representative figures for multiple chemokine receptor evaluation using multi-color flow cytometry on CD4+ T cells (B) and CD8+ T cells (C).

**CFB-SKIV2L, TNFRSF10A, TGFBR1, C3, PILRB, and MMP9.**
of chemokine receptors on T cells correlate well with our results, despite the lack of significant findings for CCR6 expressed on CD4+ T cells. Interestingly, the associated chemokine level did not differ in the plasma of controls and nAMD patients.19

We postulated that chemokine levels in plasma do not manifest ocular-specific changes, and measured the chemokine and cytokine levels in the aqueous humor of both controls and AMD patients. As shown in Table 3, monocyte chemoattractant protein-1 (MCP-1, CCL2; ligand for CCR2) was increased only in the aqueous humor of AMD patients. A previous report showed that MCP-1, a well-known chemokine that is elevated in the aqueous humor of AMD patients, was associated with disease severity.29,31 The ligands of CCR4, CCR5, and CXCR3 showed no significant change in our results (CCR4 ligand: TARC; CCR5 ligand: MIP-1α, MIP-1β, RANTES; CXCR3 ligand: IP10, MIG, I-TAC) (Table 3). When we reviewed the published articles focusing on ligands of CCR4, CCR5, and CXCR3, some authors reported elevated MIP-1α,32 MIP-1β,33 IP10,31,33,35 and MIG36 in the aqueous humor of nAMD patients, whereas others reported no significant difference compared to controls.32,36 Further studies in larger cohorts are needed to clarify whether a relationship exists between the chemokine receptor expression on T cells and intraocular chemokine levels.

The expression of chemokine receptors can be used to characterize T-cell subsets.16,21 CD4+ T cells are divided into multiple subsets by the major cytokine they produce, such as Th1 cells, Th2 cells, Th17 cells, regulatory T cells, and follicular helper T cells, among others.33 CD8+ T cells are called cytotoxic T cells.

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Table 2. Characteristics of AMD Patients and Healthy Controls Utilized in the Aqueous Humor Analysis

| Characteristics | AMD (n=12) | Control (n=6) | p value | Test |
|-----------------|-----------|---------------|---------|------|
| Age, yr         | 64.5      | 67.2          | 0.808   | Mann-Whitney U test |
| Male            | 10 (83.3) | 4 (66.7)      | 0.569   | Pearson χ² |
| Medical history |           |               |         |       |
| Hypertension    | 2 (16.7)  | 1 (16.7)      | >0.999  | Pearson χ² |
| Diabetes mellitus| 2 (16.7) | 2 (33.3)      | 0.569   | Pearson χ² |
| Smoking history |           |               |         |       |
| Current smokers | 6 (50.0)  | 1 (16.7)      |         |       |
| Quit smokers    | 4 (33.3)  | 0 (0)         |         |       |
| Never smokers   | 2 (16.7)  | 5 (83.3)      |         |       |
| Obesity         | 0 (0.0)   | 1 (16.7)      | 0.333   | Pearson χ² |
| BMI, kg/m²      | 22.7      | 24.3          | 0.149   | Mann-Whitney U test |

AMD, age-related macular degeneration; BMI, body mass index. Data are presented as n (%) or median (interquartile range).

Table 3. Concentrations of Chemokines and Cytokines in the Aqueous Humor of AMD Patients and Healthy Controls

| Chemokine, pg/mL | AMD (n=12) | Control (n=6) | p value | Test |
|------------------|-----------|---------------|---------|------|
| IL-8             | 15.3      | 12.8          | 0.180   | Mann-Whitney U test |
| IP10             | 15.0      | 12.6          | 0.102   | Mann-Whitney U test |
| CCL11            | 19.7      | 23.2          | 0.553   | Mann-Whitney U test |
| TARC             | 35.3      | 41.4          | >0.999  | Mann-Whitney U test |
| MCP-1            | 765.3     | 454.5         | 0.018   | Mann-Whitney U test |
| RANTES           | ND        | ND            | >0.999  | Mann-Whitney U test |
| MIP-1α           | 13.9      | 13.8          | 0.820   | Mann-Whitney U test |
| MIG              | 0.0       | 0.0           | 0.130   | T-test |
| ENA-78           | 8.2       | 9.0           | 0.882   | Mann-Whitney U test |
| MIP-3α           | 23.7      | 28.2          | 0.750   | Mann-Whitney U test |
| GROα             | 60.4      | 78.7          | 0.494   | Mann-Whitney U test |
| I-TAC            | 6.7       | 9.2           | 0.494   | Mann-Whitney U test |
| MIP-1β           | 11.3      | 9.8           | 0.180   | Mann-Whitney U test |

| Cytokine, pg/mL | AMD (n=12) | Control (n=6) | p value | Test |
|-----------------|-----------|---------------|---------|------|
| IL-5            | 8.8       | 8.4           | 0.125   | Mann-Whitney U test |
| IL-13           | 16.1      | 22.4          | 0.180   | Mann-Whitney U test |
| IL-2            | 18.2      | 27.6          | 0.250   | Mann-Whitney U test |
| IL-6            | 12.3      | 25.0          | 0.437   | Mann-Whitney U test |
| IL-9            | 4.9       | 6.2           | 0.053   | Mann-Whitney U test |
| IL-10           | 4.2       | 6.8           | 0.053   | Mann-Whitney U test |
| IFN-γ           | 42.2      | 56.0          | 0.125   | Mann-Whitney U test |
| TNF-α           | 5.3       | 15.6          | 0.067   | Mann-Whitney U test |
| IL-17α          | 3.8       | 4.2           | 0.437   | Mann-Whitney U test |
| IL-17f          | 5.2       | 4.8           | 0.964   | Mann-Whitney U test |
| IL-4            | 0.0       | 15.4          | 0.018   | Mann-Whitney U test |
| IL-21           | 3.1       | 3.3           | 0.820   | Mann-Whitney U test |
| IL-22           | 44.7      | 102.3         | 0.041   | Mann-Whitney U test |

ND, not determined; IP10, interferon gamma-induced protein 10; CCL, C-C motif chemokine ligand; TARC, thymus and activation regulated chemokine; MCP, monocyte chemoattractant protein; RANTES, regulated on activation, normal T cell expressed and secreted; MIP-1α, macrophage inflammatory protein 1-alpha; MIG, monokine induced by gamma interferon; ENA-78, epithelial-derived neutrophil-activating peptide 78; GRO, growth-regulated oncogene; I-TAC, interferon-inducible T-cell alpha chemoattractant; AMD, age-related macular degeneration; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

Data are presented as median (±interquartile range).
increased Th1 and Th17 responses in nAMD patients. Additional studies regarding these discrepancies should be performed to verify the exact T cell status in nAMD patients and its biological significance in the pathophysiology of nAMD.

Our study had several limitations. First, we could not determine the causal relationship between AMD and T-cell phenotype based on the observational character of this study design. Therefore, we could not deduce whether the change in T-cell phenotype is a contributing factor in AMD generation or a consequence of AMD and its related conditions. Detailed mechanistic studies using experimental animal models and a longitudinal cohort should be conducted in the future. Another limitation is that the sample size for the aqueous humor study was small. The results should be confirmed by analyzing the association between T-cell phenotypes and aqueous humor in a larger cohort with corresponding blood and ocular samples. Second, the BMI significantly differed between the AMD patients and the age-matched cohort. In previous large cohorts, both low and high BMI were associated with AMD development, and others have reported no definite associations in a Korean cohort. When we analyzed the correlation between BMI and chemokine receptor expression, only CCR4 in CD8+ T cells and CCR10 in CD4+ T cells exhibited a correlation (data not shown), and these chemokine receptors did not differ between AMD patients and controls. It is widely accepted that obesity is a state of low-grade chronic inflammation. There was no significant difference in obesity between the two groups, but it is possible that subtle differences in BMI may have affected the T cell surface phenotype. The detailed control of BMI and obesity should be considered in future study design.

In conclusion, we demonstrated that the expression of multiple chemokine receptors is altered in peripheral blood T cells from AMD patients compared to healthy controls. CCR4 in CD4+ T cells and CCR5 in CD8+ T cells were increased, and CXCR3 in CD4+ T cells, CXCR3, CCR5, and CX3CR1 in CD8+ T cells were decreased. However, chemokine levels in the aqueous humor of AMD patients demonstrated no consequential associations with the chemokine receptor phenotype of T cells. Our results suggest that AMD patients have altered chemokine receptor profiles on T cells and may have different cellular immune responses that could be associated with the development of AMD.

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AUTHOR CONTRIBUTIONS

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Chemokine Receptors of T Cells in AMD

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