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RESEARCH

Impact of intravitreal bevacizumab on immune cells in neovascular age related macular degeneration

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Abstract

Purpose Age-related macular degeneration (AMD) is a multifactorial disease that leads to progressive central vision loss. Bevacizumab (Avastin), a therapeutic agent used in the treatment of AMD, exerts its effect by binding to all isoforms of vascular endothelial growth factor (VEGF), thereby inhibiting angiogenesis. This study aimed to investigate the effects of Bevacizumab on immune system cells in the context of neovascular AMD (nAMD) treatment.

Methods Patients with nAMD receiving Bevacizumab treatment ($n=18$) and a control group of individuals undergoing cataract surgery ($n=19$) were included in the study. Peripheral blood samples were collected from nAMD patients before Bevacizumab treatment (baseline, 0 month) and after three monthly intravitreal injections. Following staining with monoclonal antibodies, innate immune cell, T cell, and B cell subsets were analyzed using flow cytometry panels.

Results In the innate immune cell subsets of patients with neovascular AMD (nAMD), immature and suppressive neutrophils were significantly increased, whereas intermediate and non-classical monocytes were significantly decreased. Following intravitreal bevacizumab (IVB) treatment, these cell populations tended to return toward baseline levels. In the adaptive immune compartment, effector memory (EM) T cell subsets, particularly EM1 (effector memory 1) and EM3 (effector memory 3), exhibited opposing dynamics, showing significant changes both before and after IVB treatment.

Conclusion In conclusion, the findings from this study provide preliminary evidence that various immune cell subsets in both innate and adaptive compartments show associations with AMD pathogenesis and with IVB treatment.

Key messages

What is known

- Neovascular age-related macular degeneration (nAMD) is driven by pathological vascular endothelial growth factor (VEGF)-mediated angiogenesis and is effectively treated with intravitreal anti-VEGF agents such as bevacizumab to control neovascular growth and preserve vision.
- Increasing evidence suggests that chronic inflammation and immune system dysregulation contribute to AMD pathogenesis, but the systemic immune consequences of intravitreal anti-VEGF therapy remain incompletely understood.

What is new

- nAMD is associated with a distinct systemic immune signature characterized by increased immature and suppressive neutrophils and reduced intermediate and non-classical monocytes, consistent with chronic innate immune dysregulation.
- Intravitreal bevacizumab was associated with partial normalization of both innate and adaptive immune profiles, including opposing shifts in effector memory T cell subsets (EM1 and EM3/EM4), suggesting a systemic immunological association that extends beyond direct VEGF inhibition.
- Several immune cell subsets — including suppressive and immature neutrophils, effector memory T cell populations, PD-1^{+} B cells, and CD21^{low} B cells — emerged as candidate circulating biomarkers of disease status and treatment response in nAMD.

Keywords Age related macular degeneration · Neovascular degeneration · AMD · nAMD · Bevacizumab · IVB

Extended author information available on the last page of the article

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Introduction

Age-related macular degeneration (AMD) is a multifactorial disease that causes significant vision loss in individuals over 55 years of age. Globally, AMD accounts for approximately 6–9% of cases of blindness [1]. It is estimated that by 2040, this disease will profoundly affect around 288 million people worldwide [2]. Modifiable risk factors such as physical activity, diet, and smoking play a role in the development of AMD. AMD is classified into two forms: dry (non-neovascular/non-exudative, dAMD) and wet (neovascular/exudative, nAMD) Fleckenstein et al., [4]. Early dry AMD is characterized by changes in Bruch's membrane, photoreceptors, the presence of soft drusen, the choriocapillaris, and the retinal pigment epithelium (RPE). Distinguishing features of late dry AMD include subretinal neovascularization, geographic atrophy, hemorrhage, epithelial pigment detachment, and fibrous scarring [3].

nAMD is a condition that arises from angiogenesis, the formation of new blood vessels. It can lead to visual impairment, intraocular fluid accumulation, hemorrhage, and extensive fibrosis [4]. One of the key mediators involved in the pathogenesis of wet AMD is vascular endothelial growth factor (VEGF), which promotes abnormal neovascularization and facilitates the growth of new blood vessels [5]. Anti-angiogenic agents can slow the growth of ocular blood vessels by blocking VEGF and prevent damage caused by abnormal vasculature [6]. Bevacizumab (Avastin®, Genentech Inc., San Francisco, CA, USA) is a humanized IgG1 monoclonal antibody with a molecular weight of 149 kDa that binds to active forms of VEGF, thereby inhibiting its activity. While FDA-approved for the treatment of lung cancer, breast cancer, and metastatic colorectal cancer, bevacizumab was first reported for use in wet AMD in 2006 [7].

The immune system comprises innate and adaptive components that play a critical role in maintaining tissue homeostasis and responding to stress or injury. The innate immune response provides the first line of defense, offering rapid but non-specific protection through phagocytic cells such as monocytes, neutrophils and macrophages. Dysregulation of the innate immune response in the retina can lead to chronic inflammation and tissue damage, thereby increasing susceptibility to AMD. The adaptive immune response, on the other hand, is characterized by its ability to distinguish self from non-self and to establish immunological memory. It is primarily mediated by B and T lymphocytes. While B cells are responsible for humoral immunity, T cells orchestrate cellular immune responses; both cell types can be further subdivided into distinct subsets based on cytokine production and surface marker expression [8]. In AMD, dysregulated interactions among these immune cell populations may contribute to chronic inflammation, tissue degeneration, and disease progression. This underscores the importance of understanding both innate and adaptive immune mechanisms in the pathogenesis of the disease.

The classification of adaptive immune cells is based on the association of surface markers with their functional properties. T cells are classified into four primary subsets based on CD45RA and CCR7 expression: naïve ($CD45RA^{+}CCR7^{+}$), central memory (CM; $CD45RA^{-}CCR7^{+}$), effector memory (EM; $CD45RA^{-}CCR7^{-}$), and terminally differentiated effector memory (TEMRA; $CD45RA^{+}CCR7^{-}$). These populations exhibit distinct activation and migratory characteristics. Additionally, the expression of the co-stimulatory molecules CD27 and CD28 allows for a more detailed subclassification of memory T cells [9, 10]. Similarly, the classification of B cell subsets is defined by the expression of surface markers which correspond to distinct developmental stages and functional roles. The subsets include naive (IgD^{+} $CD27^{-}$), mature($CD24^{int}$ $CD38^{int}$) class-switched memory (IgD^{-} $CD27^{+}$), and non-switched memory (IgD^{+} $CD27^{+}$) cells [11]. A key regulatory population is the B10 cells ($CD24^{high}$ $CD27^{+}$), defined by its IL-10 production [12]. Expanded populations of atypical B cells ($CD27^{-}$ $CD21^{-}$), PD-1 (programmed cell death protein 1)$^{+}$ B cells, and aberrantly activated plasmablasts are frequently associated with states of chronic immune activation and are hallmarks of immune dysregulation in settings such as autoimmunity, chronic viral infection, and age-related immunosenescence [13].

The impact of the inflammatory state associated with nAMD on the immune system remains incompletely understood. To address this, our study investigated changes in immune system components in peripheral blood samples collected from individuals diagnosed with nAMD before and after intravitreal bevacizumab (IVB) treatment, thereby elucidating the effects of this inflammation-related condition on immune responses.

Methods

Participants

A total of 18 patients aged 50 years and older, diagnosed with nAMD and scheduled to receive bevacizumab treatment, were enrolled from the Department of Ophthalmology at Bursa Uludağ University Faculty of Medicine. Additionally, 19 individuals scheduled for cataract surgery were included as the control group. nAMD patients were diagnosed according to the Age-Related Eye Disease Study (AREDS) classification system for AMD. To minimize

potential confounding effects on systemic immune profiles, strict exclusion criteria were applied to both groups. Individuals with diabetes mellitus, autoimmune or rheumatologic diseases, immunodeficiency, active infections, inflammatory ocular conditions, or known cardiovascular disease were excluded. All participants reported current non-smoking status at the time of enrollment; however, a formal lifetime smoking history was not systematically obtained, and prior tobacco exposure in earlier life stages cannot be excluded. Additionally, a history of previous intravitreal injection was an exclusion criterion specific to the nAMD group. These criteria were implemented to reduce the influence of systemic inflammatory and immunological alterations unrelated to nAMD.

Peripheral blood samples were collected from nAMD patients in EDTA tubes at two predefined time points: first, at baseline on the day of the initial IVB injection immediately before treatment, and second, one month after the third IVB injection, before administration of the fourth dose. (Supp. Figure 1). Similarly, peripheral blood samples were obtained from control group participants in EDTA tubes prior to cataract surgery. Treatment response was defined according to OCT (optical coherence tomography) findings obtained after the three loading IVB injections. Eyes showing complete resolution of both intraretinal fluid (IRF) and subretinal fluid (SRF) were classified as having a good treatment response, whereas eyes with persistent IRF and/or SRF were classified as having a poor treatment response, consistent with a previously reported approach [14].

Flow cytometry

In this study, flow cytometry was employed for immunophenotyping of immune cell populations in peripheral blood collected in EDTA tubes, following standardized whole-blood staining protocols [15, 16]. For each panel, 100 µL of whole blood was incubated with the relevant monoclonal antibody cocktail for 15–20 min in the dark at room temperature. Erythrocytes were then lysed with VersaLyse (Immunotech, Beckman Coulter, USA) or OptiLyse C (Beckman Coulter, USA), depending on the panel, after which samples were washed with IsoFlow Sheath Fluid (Beckman Coulter, USA) and acquired on a Navios EX flow cytometer (Beckman Coulter, USA). An isotype control tube was included for each panel, and data were analyzed using predefined gating strategies with Kaluza software (Beckman Coulter). Monocytes were classified based on CD14 and CD16 expression into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) subsets. Neutrophils were categorized into suppressor ($CD11b^{+}CD11c^{+}CD16^{+}CD62L^{-}$), immature ($CD10^{-}CD16^{low}$), and mature ($CD10^{+}CD16^{hi}$) subsets according to their CD10, CD11b, CD11c, CD16, and CD62L expression profiles. Dendritic cells were identified as lineage-negative (lin^{-}) HLA-DR^{+} cells and subsequently categorized into myeloid (mDC) and plasmacytoid (pDC) subsets based on the differential expression of CD11c and CD123. For T cell subset analysis, CD3, CD4, CD8, CD45RA, CD197 (CCR7), CD57, CD279 (PD-1), CD28, and CD27 markers were used to identify naïve, effector, memory, and exhausted/senescent subsets. Regulatory T cells (Tregs) were identified by CD25 expression in combination with intracellular FoxP3 staining. Natural killer (NK) cells were characterized by the $CD3^{-}CD16/CD56^{+}$ phenotype. B cell subsets were defined using CD19, CD38, CD24, IgD, IgM, CD27, CD21, and CD279 (PD-1) markers, including mature ($CD19^{+}CD38^{-}CD24^{+}$), immature ($CD19^{+}CD38^{+}CD24^{high}$), memory ($CD19^{+}IgD^{-}CD27^{+}$), plasmablast ($CD19^{+}CD38^{high}CD24^{-}$), B10 ($CD24^{high}CD27^{+}$), and atypical ($CD27^{-}CD21^{-}$) populations. All incubation, centrifugation, and washing steps were performed according to standard protocols. The gating strategy used for flow cytometry analysis is illustrated in Supp. Figure 2.

Statistical analysis

All statistical analyses were performed using SPSS software (version 21.0; IBM Corp., Chicago, IL, USA). The normality of the data was evaluated both visually using histograms and analytically with the Kolmogorov–Smirnov and Shapiro–Wilk tests. Continuous variables are expressed as mean ± standard deviation. Homogeneity of variances was assessed using Levene's test. Group comparisons were performed as follows: paired pre- and post-IVB measurements within the nAMD group were analyzed using the paired-samples t-test, while comparisons between the nAMD and control groups were conducted using the independent-samples t-test. Although data from the pre-IVB nAMD, control, and post-IVB nAMD groups are collated in the figures for presentation, the statistical comparisons were intentionally performed as specific paired and independent two-group tests, rather than an omnibus multiple-group comparison. A p value of < 0.05 was defined as statistically significant.

Given the paired within-subject design of the pre- versus post-IVB comparisons, formal multiple-comparison correction was not applied as the primary analytical strategy. Nonetheless, to evaluate the potential risk of Type I error and to assess the robustness of the findings, effect size estimates (Cohen's d) and Benjamini–Hochberg false discovery rate (FDR) correction were applied post hoc within each family of comparisons, with pre- versus post-IVB and pre-IVB versus control analyses evaluated as separate families.

FDR-adjusted p-values for nominally significant comparisons are provided in the Supplementary Materials (Supp. Tables 2, 3).

To further evaluate the clinical relevance of the observed immune changes, an exploratory post-hoc correlation analysis was performed between treatment-induced changes in immune-cell subsets (Δ post–pre) and clinical treatment response. Pearson correlation coefficients were computed for all immune subsets included in the analysis. Correlation dot plots were generated using Python (version 3.14) with the NumPy (version 2.4.3), SciPy (version 1.17.1), and Matplotlib (version 3.10.8) libraries.

Results

Demographic characteristics

This study, conducted with 18 nAMD patients and 19 control subjects with cataracts, found a mean age of 69.44 ± 10.16 years in the control group and 72.72 ± 7.78 years in the nAMD group, with no significant difference between them ($p = 0.285$). The study included a total of 37 participants, with the control group consisting of 13 males and 6 females, and the nAMD group comprising 12 males and 6 females. No significant difference in sex distribution was observed between the groups ($p = 0.337$) (Supp. Table 1).

Based on these OCT-defined criteria after the three loading IVB injections, 13 patients were classified as responders (good response) and 5 as non-responders (poor response). Treatment response was defined according to OCT findings obtained after the three loading IVB injections. Eyes showing complete resolution of both intraretinal fluid (IRF) and subretinal fluid (SRF) were classified as having a good treatment response, whereas eyes with persistent IRF and/or SRF were classified as having a poor treatment response, consistent with a previously reported approach.

Innate immune cells and subsets in nAMD

The distribution and frequency of innate immune cells and their subsets were evaluated in nAMD. Among the major cell populations, an increase in natural killer T (NKT) cells was observed in the nAMD group compared to the control, with a tendency to return to baseline levels following IVB treatment. In contrast, basophil frequency was decreased in nAMD patients and showed an increase after IVB administration. Subset analysis revealed a significant reduction in intermediate and non-classical monocytes in nAMD, with a non-significant tendency toward normalization after IVB therapy. Among neutrophil subsets, immature neutrophils were elevated in nAMD patients compared to control and exhibited a slight decrease post-treatment. In addition, suppressive neutrophils were significantly elevated in AMD and rapidly returned to baseline levels following IVB treatment (Figs. 1 and 2). No significant changes were observed in the other analyzed cell populations, including dendritic cells (Supp. Figure 3).

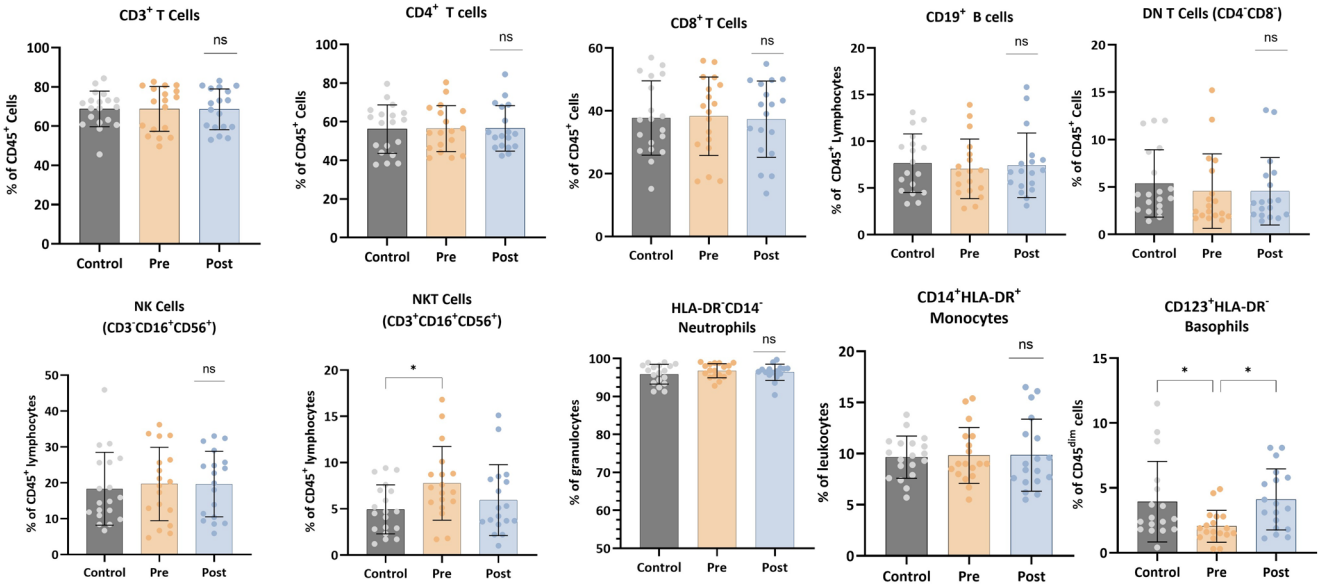


Fig. 1 Peripheral immune cell profile in AMD and after IVB treatment.The overall peripheral immune cell profile remained largely unchanged, with statistically significant differences observed mainly in NKT cells and basophils. Statistical comparisons were performed between control and nAMD, and between pre- and post-IVB measurements within the nAMD group. Symbols indicate statistical significance as follows: *$p < 0.05$ and **$p < 0.01$

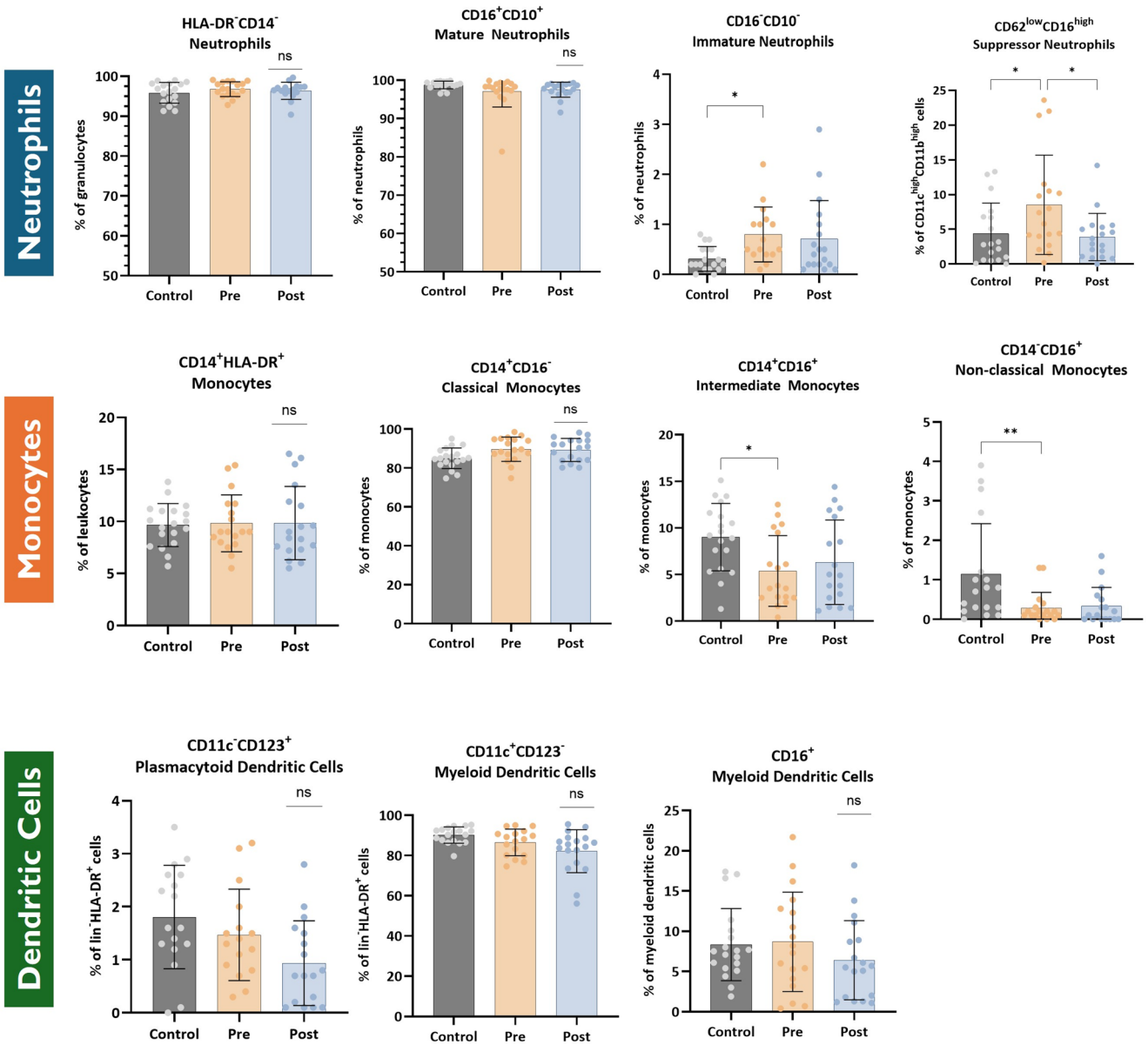


Fig. 2 Analysis of Innate Immune Cell Subsets in AMD and after IVB treatment. An increase in immature and suppressive neutrophils was observed in nAMD, with a non-significant tendency toward baseline levels following IVB treatment. A similar pattern was noted for intermediate and non-classical monocytes. Statistical comparisons were performed between control and nAMD, and between pre- and post-IVB measurements within the nAMD group. Symbols indicate statistical significance as follows: *$p<0.05$ and **$p<0.01$

T Cells and subsets in nAMD

When evaluating T cell subsets in nAMD patients before and after bevacizumab treatment, no significant differences were observed in total $CD3^+$ T cells, $CD3^+CD4^+$, $CD3^+CD8^+$, DN (double-negative, $CD3^+CD4^-CD8^-$) T cells, the CD4/CD8 ratio, or NK cells between pre- and post-IVB measurements. Tregs were also evaluated within the T cell panel, but no statistically significant differences or notable exploratory patterns were observed across the study groups. However, significant changes were observed when examining T cell subsets (Fig. 1). In $CD4^+$ T cell subsets, naïve and EM T cells were significantly reduced in nAMD compared to control, and this reduction further increased after IVB treatment. PD-1^+ ($CD279^+$) T cells were significantly decreased in nAMD, with a non-significant increase following IVB. Conversely, $CD57^+$ T cells were significantly elevated in nAMD compared to control, with a non-significant reduction after treatment. Analysis of EM subsets revealed that EM1 cells were decreased in nAMD but showed a non-significant increase after IVB, whereas EM3 and EM4 (effector

memory 4) cells were increased in nAMD and tended to decrease post-treatment. No significant differences were observed in other CD4⁺ T cell subsets. In CD8⁺ T cells, a profile similar to CD4⁺ T cells was observed. Comparable changes were detected in EM, PD-1⁺, and CD57⁺ subsets, while the effects of IVB were more pronounced in EM1 and EM3 cells. Specifically, EM1 cells were significantly reduced in nAMD but returned to baseline levels following IVB, and EM3 cells exhibited the opposite trend, being increased in nAMD and returning to baseline after treatment. EM4 cells displayed a profile similar to that of CD4⁺ T cells. Finally, in DN T cells, statistically significant changes were observed only in the EM4 subset (Figs. 3 and 4). The overall distribution of DN T cells resembled that of CD4⁺ and CD8⁺ T cells, with no other statistically significant differences detected across subsets, including Tregs. (Supp. Figure 4–6).

B cells and substes in nAMD

B cell subsets, including regulatory B (Breg) cells, were evaluated in nAMD patients. No significant changes were observed in total CD19⁺ or CD27⁺ B cells (Supp Fig. 7). Mature B cells were significantly reduced in nAMD compared to control, with a non-significant increase following IVB treatment. Memory B cells were significantly elevated in nAMD, but showed a non-significant reduction after IVB. CD21^{low}CD38^{-} exhausted B cells were significantly decreased in nAMD, returning rapidly to baseline levels and increasing following IVB. Interestingly, PD-1⁺ B cells displayed an opposite pattern, being significantly elevated in AMD and significantly reduced post-treatment. Plasmablasts (CD27^{high}CD38^{high}) and plasmablast-like B cells (CD24^{-}CD38^{high}) did not differ from control in nAMD but decreased significantly after IVB. Notably, B10 cells were

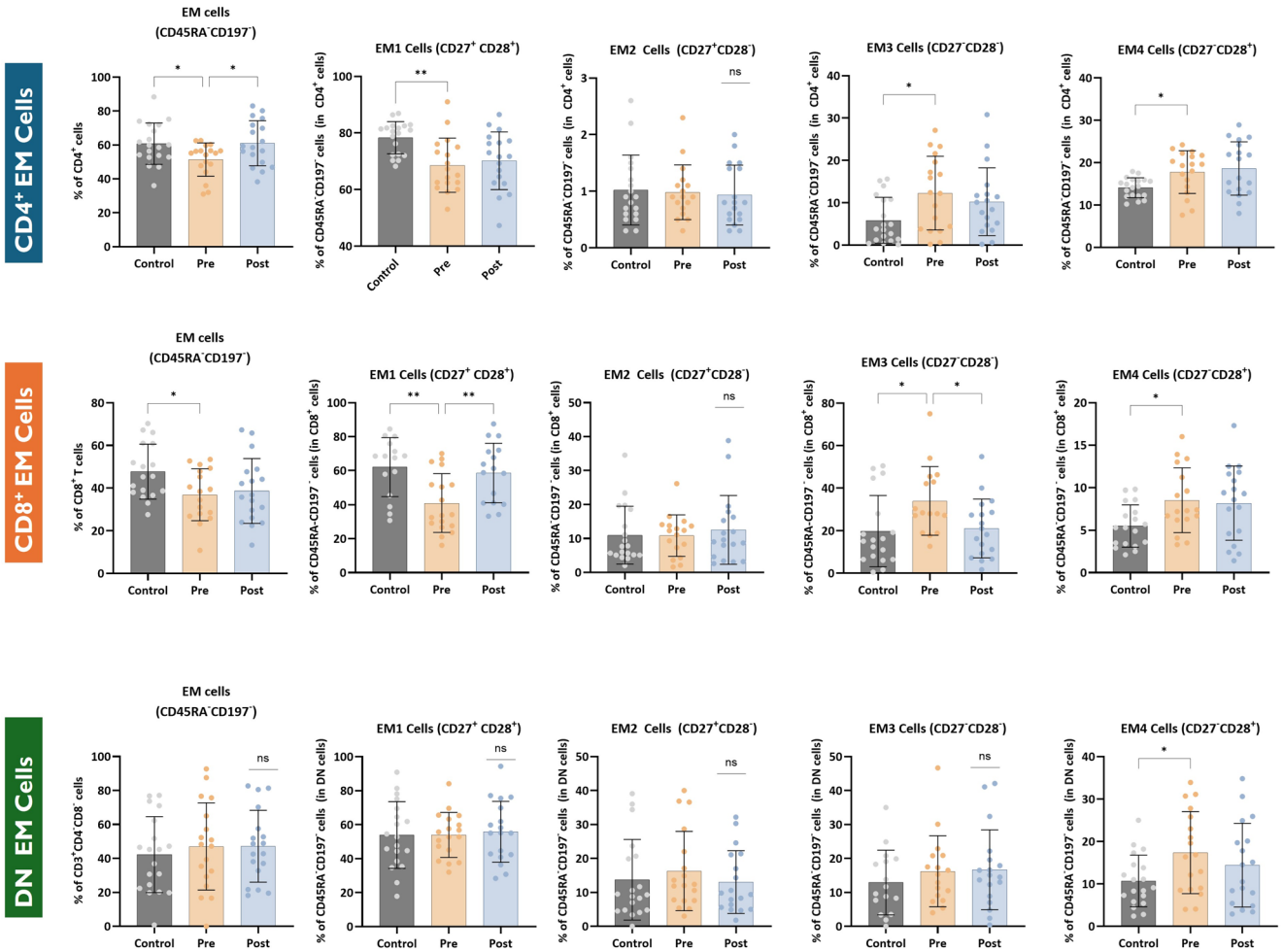


Fig. 3 Status of effector memory T (EM) cells in AMD and following IVB treatment. EM T cells were categorized into four subsets based on CD27 and CD28 expression. In nAMD, CD4⁺ and CD8⁺ T cells exhibited statistically significant and opposing dynamics in the EM1 and EM3/EM4 subsets. Statistical comparisons were performed between control and nAMD, and between pre- and post-IVB measurements within the nAMD group. Symbols indicate statistical significance as follows: *$p<0.05$ and **$p<0.01$

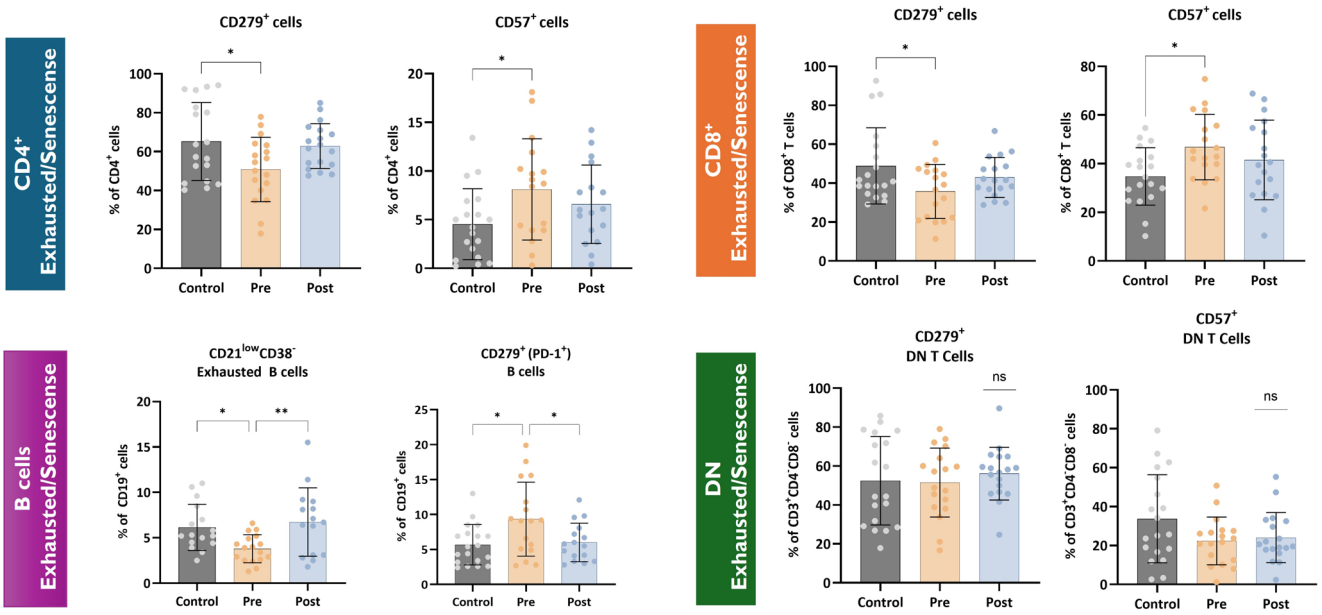


Fig. 4 Status of senescence- and exhaustion-associated adaptive immune cells in AMD and after IVB treatment. CD279 (PD-1) and CD57 expression on $CD4^+$ and $CD8^+$ T cells was evaluated to assess senescence and exhaustion. CD279 expression was decreased in nAMD, whereas CD57 expression was increased; both showed a non-significant tendency toward baseline levels following IVB treatment. In contrast, the CD279-expressing B cell population was increased in nAMD, while $CD21^{low}CD38^-$ exhausted B cells were decreased. Statistical comparisons were performed between control and nAMD, and between pre- and post-IVB measurements within the nAMD group. Symbols indicate statistical significance as follows: $*p<0.05$ and $**p<0.01$

elevated in nAMD and continued to show an upward trend following IVB. $IgM^{high}CD38^{high}$ transitional B cells were significantly increased in nAMD compared to control, with a non-significant decrease following treatment. Finally, atypical or exhausted B cells ($CD21^-CD27^-$) were decreased in AMD, showing minimal increase after IVB (Fig. 5).

Exploratory correlation analysis

We further assessed the clinical relevance of the observed immune changes through an exploratory post-hoc correlation analysis between treatment-induced changes in immune cell populations (Δ post–pre) and clinical treatment response (good = 1, poor = 0) across all analyzed subsets ($n = 18$; 13 responders, 5 non-responders; Supp. Figure 8.). The strongest positive correlation with a good treatment response was observed for effector memory $CD4^+$ T cells ($r = +0.657$, $p = 0.006$), followed by EM1 $CD8^+$ T cells ($r = +0.641$, $p = 0.008$). Significant positive correlations were also found for basophils ($r = +0.594$, $p = 0.019$) and PD-1^+ B cells ($r = +0.347$, $p = 0.034$). Effector memory $CD8^+$ T cells showed a positive but non-significant correlation ($r = +0.390$, $p = 0.108$). In contrast, EM3 $CD8^+$ T cells ($r = -0.422$, $p = 0.078$) and plasmablast B cells ($r = -0.436$, $p = 0.077$) showed negative correlations that approached significance, indicating that the persistence of these populations tended to be associated with a poorer response. Negative correlations of smaller magnitude were also observed for EM3 $CD4^+$ T cells ($r = -0.359$, $p = 0.106$) and EM4 $CD4^+$ T cells, although these did not reach significance.

Discussion

AMD is one of the leading causes of blindness in developed countries. It is a multifactorial disease associated with the complex interaction of environmental and genetic factors [17]. The progression of AMD is characterized by various pathological changes. For nAMD, several anti-VEGF agents have been approved. Studies have reported that Bevacizumab, which is used off-label, is effective in the treatment of nAMD. Bevacizumab is a human monoclonal antibody that binds to all isoforms of VEGF [18]. In a study investigating the pharmacokinetics of Bevacizumab, Han and colleagues reported that the drug has a terminal half-life of approximately 20 days [19].

A study by Matsuyama and colleagues on patients with diabetic retinopathy reported that a single intravitreal injection of Bevacizumab induced a significant and sustained reduction in systemic plasma VEGF levels, measured at day 1, day 7, and 1 month post-IVB. The authors postulated that this phenomenon occurs because intravitreally administered Bevacizumab not only penetrates the intraocular blood

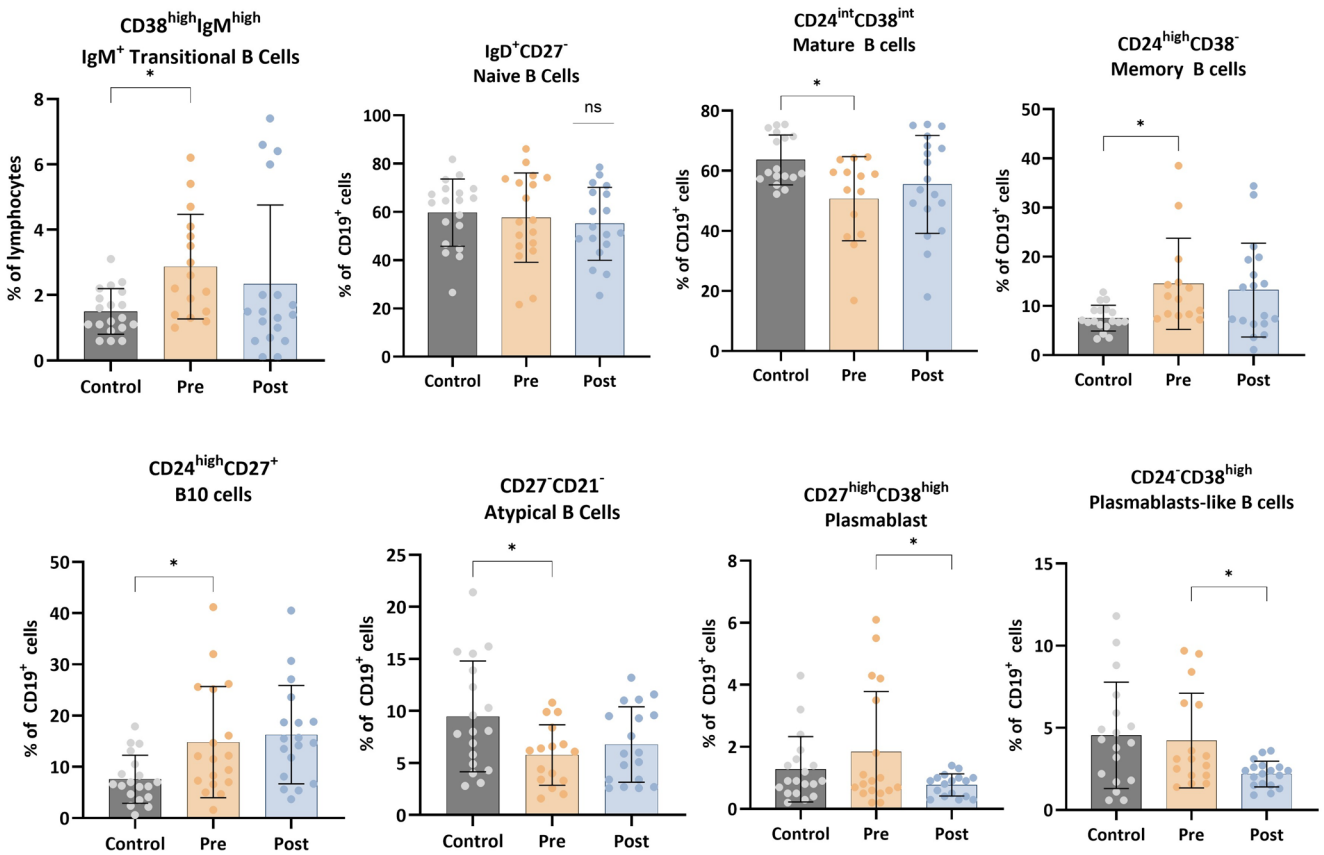


Fig. 5 Status of B cell subsets in AMD and following IVB treatment. Plasmablasts, plasmablast-like cells, B10, memory, and transitional B cells were elevated in nAMD. With the exception of B10 cells, these populations showed a non-significant tendency toward baseline levels after IVB treatment; however, B10 cells maintained their elevated frequency. A decrease in atypical B cells was also observed in nAMD. Statistical comparisons were performed between control and nAMD, and between pre- and post-IVB measurements within the nAMD group. Symbols indicate statistical significance as follows: $*p<0.05$ and $**p<0.01$

vessels, choroid, and retina but also enters the systemic circulation [20]. This finding of systemic exposure is further supported by research from Bakri and colleagues. Their study detected Bevacizumab in the aqueous humor of the uninjected, contralateral eye, with concentrations found to be higher than those in the vitreous. They concluded that the drug reached the anterior segment of the fellow eye via the systemic circulation. Consequently, this study underscores the systemic presence of Bevacizumab following intravitreal administration [21].

This study aimed to evaluate the effects of three doses of Bevacizumab on immune system cells and to identify potential biomarkers. The principal cell populations involved in the innate immune response include monocytes, neutrophils, dendritic cells, NK cells, NKT cells, and basophils, whereas the adaptive immune system is primarily represented by T and B lymphocytes. A preliminary assessment of the overall immunological profile may suggest a limited association between nAMD and immune system cells. This interpretation arises from the observation that, with the exception of less prevalent populations such as NKT cells and basophils, no statistically significant differences were detected—either when compared to the cataract control group or within the nAMD group before and after Bevacizumab treatment. However, as the saying goes, one should not judge a book by its cover; therefore, broad conclusions should be avoided until more detailed analyses are conducted.

To gain a detailed understanding of the innate immune system in nAMD, this study investigated the subsets of neutrophils, monocytes, and dendritic cells. According to a study by Singh and colleagues, the proportion of CD11b-expressing monocytes was found to be increased in patients with nAMD [22]. While some studies have reported an increase in monocytes during the first month following the onset of nAMD, they also indicate that the three monocyte subsets (classified as classical, intermediate, and non-classical) remain unchanged in late-stage nAMD [23, 24]. Results from various studies have shown that the proportion of non-classical monocytes, particularly those expressing markers such as CCR2, is elevated in nAMD. This finding suggests an upregulation in the expression of a range of scavenger receptors and inflammatory mediators [25, 26]. Classical

monocytes are a subset capable of migrating to sites of inflammation during an infection, where they recognize and phagocytose inflammatory agents. Furthermore, this monocyte subset secretes various pro-inflammatory cytokines and can recruit other immune cells to regulate the inflammatory response [27]. According to our findings, the frequency of classical monocytes was elevated in patients with nAMD and remained high after IVB injection; however, this level was statistically similar to that of the control group. This finding appears consistent with the course of chronic inflammation. Intermediate monocytes are the monocyte subset with the highest antigen-presenting capacity and are capable of producing pro-inflammatory cytokines. In contrast, non-classical monocytes are widely regarded as an anti-inflammatory subset due to their role in maintaining vascular homeostasis [28, 29]. Our results indicated that the frequencies of non-classical and intermediate monocytes were lower in nAMD patients, with a tendency to increase after IVB treatment. Intermediate monocytes are often associated with chronic inflammation, and their reduced circulation may suggest potential recruitment and migration to retinal/choroidal tissues. Similarly, given the presence of pathological neovascularization in AMD, non-classical monocytes appear to be consumed locally.

Suppressive neutrophils function in T cell suppression in a hydrogen peroxidase and Mac-1-dependent manner. Compared to other neutrophil subsets, they exhibit a lower adhesion capacity due to insufficient L-selectin (CD62L) expression [30]. However, their hypersegmented nuclear morphology indicates a fully mature state. Furthermore, these cells highly express ICAM-1, a key marker associated with reverse transendothelial migration (rTEM) [31]. Despite their maturity, these neutrophils possess a high capacity for reactive oxygen species (ROS) production. Through endothelial interactions, they can exacerbate tissue damage and exhibit a senescent profile. This suggests they may represent an activated subset that has migrated into tissue, subsequently returned to the circulation via rTEM, and accumulated in the periphery. Their low adhesion capacity facilitates this peripheral mobilization. On the other hand, immature neutrophils have recently emerged as a neutrophil subset of interest due to their potential as biomarkers. Initial findings indicated an increase in these cell populations following post-operative stress or acute physiological challenges, which was subsequently corroborated by reports of their elevation in conditions such as COVID-19, sepsis, systemic lupus erythematosus (SLE), and cancer [32–36]. Although this neutrophil subset demonstrates functional properties that can support T-cell proliferation, its precise relationship with disease pathogenesis remains incompletely understood [37]. Taken together, our findings indicate an association between elevated $CD62L^{low}CD16^{high}$ neutrophil levels and nAMD. Likewise, immature neutrophils exhibited a similar increase in AMD and, despite a slight reduction following IVB treatment, remained persistently elevated. The opposing dynamics of these two neutrophil subsets likely reflect roles in responding to tissue damage and preserving immune homeostasis. This pattern indicates potential utility as biomarkers of cellular senescence and chronic inflammation in nAMD.

Circulating basophils and mast cells are key immune populations that express the tetrameric high-affinity receptor for IgE (FcεRI) and synthesize a wide array of immunoregulatory and proinflammatory molecules [38]. A study by Shima and colleagues reported that IVB injection can lead to both ocular complications—such as ocular inflammation, chemosis, corneal abrasion, lens injury, acute vision loss, and retinal pigment epithelial tears—and systemic complications, including menstrual irregularities, elevated systolic blood pressure, facial skin flushing, cerebral infarction, and pruritic rash [39]. In line with the potential for systemic immune effects, our findings demonstrated an increase in basophil levels following a three-dose regimen of IVB therapy in patients with nAMD. This observation suggests that IVB, which modulates immune system activation, may potentially induce allergic-type reactions and enhance the activation of immune cells such as basophils.

To comprehensively characterize the adaptive immune response in nAMD, a detailed analysis of T cell and B cell subsets was performed. This investigation constitutes one of the most extensive immunophenotyping studies in nAMD to date, encompassing subset-specific assessments of $CD4^+$, $CD8^+$, double-negative (DN; $CD4^-CD8^-$), and $CD19^+$ populations. Within the adaptive immune compartment, available evidence remains limited but suggests a potential contribution of Th1, Th2, and notably Th17 cell responses to nAMD pathogenesis [40, 41]. Similarly, the detection of anti-retinal autoantibodies in nAMD patients suggests a potential role for humoral immunity and their utility as biomarkers; however, definitive evidence directly elucidating B cell involvement remains lacking [42].

In our study, $CD4^+$ and $CD8^+$ T cells showed a similar distribution pattern, with the most pronounced changes occurring among effector memory populations, the memory cells that mediate rapid responses to previously encountered antigens. These cells span a continuum from early, less differentiated forms with high proliferative capacity to terminally differentiated subsets that produce strong inflammatory mediators and carry greater tissue-damaging potential. In nAMD, this balance was shifted toward the more terminally differentiated, inflammatory end of the spectrum, an effect most apparent within the $CD8^+$ compartment. Following IVB treatment, several of these populations moved back toward control levels, indicating

a partial normalization of the adaptive immune profile. In both populations, EM cell frequencies were lower in the patient group compared with control but increased following IVB treatment. The differentiation of EM cells follows the sequence N > CM > EM1 > EM2 (effector memory 2) > pE1 > pE2 > EM4 > EM3 > E, with a progressive decline in proliferative capacity toward terminal differentiation [43]. EM1 cells are highly proliferative but have limited cytotoxic activity, whereas EM3 cells produce large amounts of inflammatory mediators such as IFN-γ and are capable of causing tissue damage. The functional role of EM4 cells is not yet fully defined, though they are hypothesized to be potent cytokine producers [44, 45]. This aligns with findings in other inflammatory conditions. Our group previously reported an increase in highly cytotoxic EM3 cells in severe COVID-19, while Kudryavtsev and colleagues described a similar expansion of EM3 and EM4 cells, accompanied by a reduction in EM1 cells, in primary Sjögren's syndrome [46, 47]. When analyzing EM T cell subsets in $CD4^+$ and $CD8^+$ T cells, an increase in the more cytotoxic and terminally differentiated EM3 cells, as well as in EM4 cells, which represent an intermediate form closer to terminal differentiation, was observed in nAMD. Conversely, EM1 cells, which have higher proliferative capacity but limited cytotoxic potential, were decreased in nAMD. Following IVB treatment, EM3 levels in $CD8^+$ T cells significantly decreased, whereas EM1 cells increased. This suggests that $CD8^+$ EM cells may be more closely associated with IVB treatment and may have potential as candidate biomarkers of treatment response. Furthermore, the co-expression of CD57 on T cells, which predominantly occurred in EM3 and EM4 (in $CD8^+$) subsets, indicates a high level of CD57 expression in these cells, highlighting their distinct cellular phenotype. Normally, CD57 expression is absent in $CD4^+$ T cells, with EM3 cells being the only exception [43]. In contrast, PD-1^+ T cells were observed to decrease relative to the control group, which may reflect the low PD-1 expression of EM3 and EM4 cells. The reduction of EM3 and EM4 cells after IVB treatment appears to be compensated by an increase in PD-1-expressing T cells. In the context of T cells in nAMD, PD-1-expressing T cells appear to be rapidly depleted within the T cell pool, whereas $CD57^+$ EM3 and EM4 cells represent a more cytotoxic profile, potentially contributing to tissue damage. Similar trends were observed in DN T cells, with a particularly significant increase in the EM4 subset.

Analysis of B cell subsets in our study revealed several notable findings. Considering the chronic inflammation and age-related immunosenescence associated with nAMD, the control group was selected from age-matched individuals Among the conventional B cell compartments, no statistically significant differences were observed in naïve or mature B cells, indicating that the principal changes were confined to more functionally specialized subsets. Foremost among these are the so-called "exhausted B cells," a functionally impaired subset that arises under conditions of chronic antigenic stimulation or persistent inflammation. Analogous to T cell exhaustion, these cells develop in response to repetitive or sustained antigen exposure and are characterized by reduced proliferative capacity, impaired antibody production, upregulation of inhibitory receptors such as PD-1, and downregulation of CD21. $CD21^{low}$ B cells represent an early-to-intermediate stage along this exhaustion spectrum and retain some functional capacity, including antibody and cytokine production, albeit at lower levels, whereas PD-1^+ B cells are closer to terminal exhaustion and carry the highest potential for clonal deletion. However, $CD21^{low}CD38^-$ exhausted B cells were reduced in nAMD patients and increased following IVB treatment. Interestingly, PD-1^+ B cells were elevated in nAMD but showed a non-significant tendency toward reduction after treatment. These findings suggest that the degree of B cell exhaustion may have potential as a biomarker in nAMD. $CD21^{low}$ exhausted B cells represent an early stage of the exhaustion spectrum and retain some functional capacity, including antibody and cytokine production, albeit at lower levels. In contrast, PD-1^+ B cells are closer to terminal exhaustion and have the highest potential for deletion. This distribution raises the possibility that $CD21^{low}$ exhausted B cells reside primarily in tissue, whereas PD-1^+ B cells are found in circulation. This peripheral $CD21^{low}$ B cell profile differs from what has been reported in certain autoimmune diseases [48]. Additionally, B10 cells, which have the capacity to produce IL-10, were notably increased in nAMD and maintained their elevated levels following IVB treatment. Similar regulatory effects of B10 cells have been reported in other degenerative conditions, such as Multiple sclerosis and myasthenia gravis [49, 50], suggesting a critical role in balancing the immune response against chronic inflammation. Furthermore, evidence from our study indicates that plasmablast and plasmablast-like B cells may also serve as potential indicators following IVB therapy. Overall, B cell subsets in nAMD not only reflect markers of chronic antigenic stimulation but also reveal components of compensatory immune regulation, highlighting the dual role of B cells in disease pathophysiology.

To further explore the clinical relevance of these immune changes, an exploratory post-hoc analysis correlated treatment-induced changes in immune cell populations (Δ post–pre) with clinical response. Favorable outcomes were associated with effector memory $CD4^+$ T cells, the proliferative EM1 $CD8^+$ subset, basophils, and PD-1^+ B cells, whereas the more terminally differentiated EM3 $CD8^+$ T cells and plasmablasts tended to correlate negatively. This pattern, in which proliferative and regulatory subsets favor

responsiveness while terminally differentiated effector populations track with poorer outcomes, mirrors the overall immune signature described above. Given the post-hoc design and the small number of poor responders, however, these associations remain exploratory and hypothesis-generating, and require confirmation in larger cohorts.

We are fully aware of several limitations inherent in our study. Firstly, the inability to clearly delineate the tissue and circulatory cell distributions constitutes a significant limitation; however, this was due to our inability to obtain retinal tissue samples. As the retina represents an immune-privileged site primarily regulated by resident microglia and complement pathways, peripheral immune findings should be interpreted as systemic associations rather than direct reflections of intraocular immune activity. Secondly, functional analyses of effector immune cells and an assessment of their cytokine production capacity would have provided a valuable addition to our work. Thirdly, with the aim of establishing a reference immune system compatible with age-matching and immunosenescence, we preferentially enrolled patients scheduled for cataract surgery. We considered the recruitment of a healthy control group with matched age and adherence to the inclusion criteria to be a particularly challenging factor. Finally, the absence of systemic VEGF level measurements and bevacizumab pharmacokinetic data precludes direct confirmation of systemic drug exposure and limits causal interpretation of the observed immune changes.

Overall, with aging, the immune system's ability to control inflammation and maintain tissue homeostasis declines, increasing retinal susceptibility to prolonged antigen exposure and thereby promoting the development of nAMD. In this study, both innate and adaptive immune responses were comprehensively examined in nAMD. Within the innate immune compartment, an increase in suppressive neutrophils in nAMD followed by a non-significant tendency toward baseline levels after IVB treatment suggests their potential candidacy as circulating biomarkers for disease monitoring, pending further validation. In the adaptive immune compartment, EM3 and EM1 T cell subsets exhibited opposing dynamics in nAMD and during post-IVB follow-up, providing complementary associative profiles that may be relevant to disease progression and therapeutic response. Furthermore, an exploratory correlation analysis indicated that treatment-induced changes in effector memory CD4 T cells, EM1 CD8 T cells, basophils, and plasmablast B cells were associated with clinical treatment response, offering preliminary support for their candidacy as predictive biomarkers in nAMD. In conclusion, the findings of this study provide preliminary insights into how age-related immunosenescence and chronic inflammation may be reflected in the systemic immune architecture of nAMD. Future studies systematically investigating the functional capacity and tissue versus circulatory distribution of these candidate cell populations will be essential to establish their clinical utility and to further elucidate the pathogenesis of nAMD.

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Data availability The datasets utilized and/or generated in the present study can be obtained from the corresponding author upon request.

Declarations

Ethical approval The procedures and protocols utilized in this study were approved by the Clinical Research Ethics Committee of Bursa Uludag University, in accordance with guidelines (Decision Number: 2022-16/50).

Consent for publication All authors have reviewed the manuscript and agreed to its publication.

Consent to participate Informed consent forms were obtained from all participants.

Competing interests The authors declare no competing interests.

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