

Interleukin-17 production and T helper 17 cells in peripheral blood mononuclear cells in response to ocular lysate in patients with birdshot chorioretinopathy

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Purpose: To determine the cytokine response to ocular lysates of peripheral blood mononuclear cells (PBMCs) from patients with birdshot chorioretinopathy (BSCR).

Methods: In the PBMCs of 19 patients with BSCR, T cell cytokine production in response to human retina and choroid lysates was analyzed with flow cytometry and compared to the responses against skin lysates. Five patients had active disease and had not yet been treated (naïve to systemic therapy); 14 patients had either immunomodulatory therapy (IMT) or inactive disease (referred as inactive/IMT). The PBMCs of 11 HLA-A29-positive healthy individuals were used as controls.

Results: The levels of interleukin-17 (IL-17) in supernatant of cultures stimulated with retina lysate were higher in patients with active BSCR compared to the HLA-A29 positive controls. The levels of other T cell cytokines (IL-10 and interferon- γ [IFN- γ]) in PBMC cultures did not change significantly after stimulation with ocular lysate. The frequency of CD4⁺ IL-17⁺ (T helper 17 [Th17]) T cells but not of CD4⁺ IFN- γ (Th1) T cells was elevated in the PBMCs of patients with active BSCR stimulated by retina lysates compared to skin lysates.

Conclusions: Our data demonstrate that PBMCs exhibit an IL-17-mediated immune response to retina lysate in patients with active disease naïve to systemic therapy. This is accompanied by the enrichment of IL-17-producing CD4⁺ T cells. These findings support the current concept of chronic Th17-cell mediated inflammation and provide evidence that links the Th17 signatures to ocular-specific immune responses in BSCR.

Birdshot chorioretinopathy (BSCR) is an organ-specific and potentially blinding chronic inflammation of the posterior eye segment [1]. Disease hallmarks manifest as distinct multiple hypopigmented chorioretinal lesions [2]. BSCR affects predominantly middle-aged and elderly individuals of European descent [2] who almost all, if not all, carry the HLA-A29 allele [3,4]. The pathogenesis of the disease is poorly understood. The increased peripheral immune response to the retinal S-antigen [3] and the presence of T cell infiltration of the chorioretinal lesions [5,6] support the current view of T cell-mediated autoimmune inflammation in BSCR. T helper 17 (Th17) cells, a subset of CD4⁺ T lymphocytes that typically produce interleukin (IL)-17, play an important role in the pathogenesis of noninfectious uveitis and autoimmune diseases [7-10]. Previously, we reported on elevated levels of IL-17 in the aqueous humor of patients with BSCR [11], indicating a role for Th17 cells in BSCR pathogenesis. Yang and associates [12] recently showed elevated serum

levels of immune mediators that promote Th17 responses in patients with active BSCR. To better understand the potential role of Th17 cells in BSCR, we investigated the cytokine secretion profile in response to human ocular lysate in the peripheral blood of patients with BSCR and healthy controls.

METHODS

Subjects: This case-control study included 19 patients with BSCR, diagnosed based on research criteria established by an international consensus conference [13]. All patients were HLA-A29 positive and had bilateral chronic uveitis with the characteristic hypopigmented chorioretinal lesions and associated vitritis. Whole blood was drawn into Vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density centrifugation and cryopreserved. The isolation conditions were identical for all blood samples. Our group consisted of five patients with active disease that were naïve to systemic therapy and 14 patients with inactive disease and/or a current or past history (<3 months) of immunomodulatory therapy (see Table 1 for more information). Peripheral blood mononuclear cells (PBMCs) from 11 HLA-A29 positive anonymous donors who had no history of intraocular

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inflammation and/or vitreoretinal disease were isolated from buffy coats obtained from the national blood bank (Sanquin, Amsterdam, the Netherlands). Informed consent for the post-mortem donation of ocular tissue was received before the authors acquired the tissue under the auspices of the head of the department of Anatomy, University Medical Center Utrecht, the Netherlands. All tissues were acquired in compliance with Dutch law (“Wet op de lijkbezorging,” Art 18, lid 1/ geldigheidsdatum_18-06-2013) and the institutional guidelines of the University Medical Center Utrecht. Blood samples from patients were collected after written informed consent and were obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University Medical Centre Utrecht, the Netherlands.

Retina, choroid, and skin extracts: Retina and choroid were prepared from post-mortem tissue within 6–9 h. Retina extracts and choroid extracts were prepared from eyes from two unrelated Caucasian donors who had no history of intra-ocular inflammation or vitreoretinal disease. Retinas were carefully dissected from the underlying pigment epithelium and choroid by rinsing with 0.5 ml PBS (140 mM NaCl, 21 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, pH 7.4, with 0.02% NaN₃). Retinas were placed in the same 0.5 mL PBS (0.02% NaN₃) to preserve soluble antigens in the protein lysate. The choroid/pigment epithelium was dissected from the sclera and kept in 0.5 ml PBS (0.02% NaN₃). Skin obtained during reduction mammoplasty in two unrelated anonymous healthy Caucasian patients served as the control and was prepared within 6 h after surgery. The skin was briefly washed in PBS (0.02% NaN₃) to remove blood contamination. Fatty tissue was removed, and the sample was cut into small pieces (0.3 cm²) using a scalpel. About 0.5 ml PBS (0.02% NaN₃) was added to the dissected retina, choroid, and skin and homogenized with 1.4 mm ceramic beads in a Bench Top bead-based Homogenizer (MO BIO, Solana Beach, CA). After centrifugation at 10,000 ×g for 30 min (4 °C), supernatant was carefully collected and heat-inactivated at 100 °C for 7 min to neutralize endogenous tissue protease activity. Protein yields were assessed with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Extracts were aliquoted and stored at –80 °C. All extracts were used at a final concentration of 100 µg/ml to stimulate the PBMCs.

Cytokine analysis of peripheral blood mononuclear cells: The T cell-associated cytokine production by PBMCs was analyzed after they had been exposed to the retina and choroid lysate (100 µg/ml final concentration). Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (1 µg/ml; Sigma-Aldrich, St. Louis, MO) served as a positive

control, and lysate derived from a skin biopsy (100 µg/ml) served as an irrelevant lysate control because the cytokine production of PBMCs in response to skin lysate was similar to cytokine release in medium only (data not shown). About 2.5×10⁵ PBMCs were cultured in serum-free AIM-V medium (Invitrogen, Carlsbad, CA) in 96-well flat-bottom culture plates for 6 days in the presence of lysate. Cell-free supernatant was collected and centrifuged at 1,000 ×g for 5 min. Cytokines (IL-10, IFN-γ, and IL-17A) in the supernatant were measured using the DIAplex multiplex fluorescent bead-based immunoassay (Diaclone, Besançon, France) according to the manufacturer’s specifications. The limit for detection of cytokines (pg/ml) was >1.7 for IL-10, >8.7 for IL-17, and >0.8 for IFN-γ.

Intracellular staining of interleukin-17 and interferon-γ in CD4⁺ T cells: PBMCs were stimulated as described above. Intracellular staining of cytokines in PBMCs was done after the cells were treated with phorbol myristate acetate (12.5 ng/ml) and ionomycin (0.5 µg/ml) for 5 h, the last 4 of which were in the presence of 5 µg/ml brefeldin A (all from Sigma-Aldrich). Cells were then stained for anti-CD3-allophycocyanin, CD4-PerCP, IL-17-PE(SCPL1362); IFN-γ- fluorescein isothiocyanate (4S.B3; BD, Franklin Lakes, NJ; Beckman Coulter, Fullerton, CA) and analyzed with a FACSCanto II flow cytometer (BD). Acquired data were analyzed with FACSDiva software.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego CA). We used the Kruskal–Wallis test with Dunn’s multiple-comparison post-hoc test in GraphPad Prism 5 software to test group differences. Statistical significance was accepted at a significance level of adjusted p<0.05.

RESULTS

The mean age of the patients with BSCR was 61.4 years, ranging from 50 to 74 years, and the female-to-male ratio was 17:2 (Table 1). The mean age of the five patients with active BSCR naïve to systemic treatment was 63.2 (range 54–74 years), the mean age of the 14 patients with inactive BSCR and/or current therapy was 60.7 (range 51–70), and the mean age of the 11 HLA-A29 positive controls was 49.7 (range 28–63 years).

The production of all cytokines to SEB, a positive control antigen, was statistically similar for all groups (Table 2). The PBMCs of patients with active BSCR displayed an enhanced yet not significant IFN-γ response to all lysates compared to patients with inactive BSCR and the healthy controls. In patients with inactive BSCR, the IFN-γ levels in response to retina and choroid lysates were higher, but the levels were

TABLE 1. DEMOGRAPHIC INFORMATION OF PATIENTS IN THIS STUDY.

Patient	Age	Gender	Year of diagnosis	Disease status	Prior treatment	Current treatment	Cataract or aphakic/pseudophakic	Comorbidity
Patients with active disease naïve to immunomodulatory therapy								
1	63	f	2011	active	-	-	-	
2	68	f	2009	active	-	-	-	hypothyroidism
3	54	f	2011	active	-	-	-	
4	74	m	2009	active	-	-	-	Diabetes
5	57	f	2011	active	-	-	-	
Patients with inactive disease and/or current or past history (<6 months) of immunomodulatory therapy								
6	68	f	2004	inactive	Corticosteroids Methotrexate	Methotrexate	+	
7	63	f	2000	inactive	Corticosteroids Methotrexate	Methotrexate	+	hypothyroidism
8	56	f	2006	active	Corticosteroids Methotrexate	Mycophenolate mofetil	-	hypothyroidism
9	61	f	1994	active	Corticosteroids Methotrexate Cyclosporine	Mycophenolate mofetil	+	
10	52	f	2008	inactive	Methotrexate	Methotrexate	+	
11	61	m	2010	inactive	Methotrexate	Methotrexate	-	
12	71	f	1993	inactive	Corticosteroids Methotrexate	Methotrexate	+	
13	53	f	2000	inactive	Methotrexate	-	+	
14	68	f	2004	inactive	Corticosteroids Methotrexate azathioprine	Methotrexate	+	
15	66	f	1992	inactive	Corticosteroids	Methotrexate	+	Multiple-sclerosis
16	58	f	2006	inactive	Corticosteroids Methotrexate	Methotrexate	+	
17	58	f	2007	inactive	Methotrexate	Adalimumab Corticosteroids	-	
18	50	f	2007	inactive	Corticosteroids Methotrexate Mycophenolate mofetil	Methotrexate	+	
19	66	f	2011	inactive	Mycophenolate mofetil	-	+	Mammary carcinoma hypothyroidism

not significantly different from the response to skin lysate. In contrast, the PBMCs of the patients with active BSCR secreted significantly increased levels of IL-17 in response to retina lysate compared to skin lysate (Figure 1). The levels of IL-17 in cultures exposed to retina lysate were significantly higher compared to those of the healthy controls (Figure 1 and Table 2). IL-17 and IFN- γ production in response to choroid lysate increased in patients with active BSCR and patients with inactive BSCR/IMT, but did not reach statistical significance (Figure 1). Next, we investigated the induction of CD17⁺, CD4⁺, CD3⁺ (Th17) T cells and IFN- γ ⁺ CD4⁺, CD3⁺ (Th1) in PBMCs stimulated under the same conditions in five patients with active BSCR, seven patients with inactive BSCR/IMT, and eight controls. Only the PBMCs from the patients with active BSCR demonstrated induction of CD17⁺, CD4⁺, CD3⁺ T cells after stimulation with retina lysate (Figure 2), but not IFN- γ ⁺ CD4⁺ CD3⁺ (Th1). The levels of IL-10 in the PBMC cultures were heterogeneous and did not change significantly after stimulation with SEB or ocular lysates (Figure 1).

DISCUSSION

The current study demonstrates that PBMCs from patients with active BSCR naïve to systemic therapy specifically produce IL-17 in response to retina lysate. This was accompanied by the observation of a modest, but significant, induction of CD17⁺, CD4⁺, CD3⁺ T cells (Th17), but not IFN- γ ⁺ CD4⁺, CD3⁺ T cells (Th1).

BSCR is an autoimmune disease [2], strongly associated with the HLA-A29 antigen [3] and mediated by autoreactive T lymphocytes [11] that specifically target the retina and/or choroid. Although the number of available samples of this rare disease is limited, we investigated differences in cytokine production in response to human retina and choroid lysate in 19 patients with BSCR and 11 HLA A-29-positive controls, and the induction of Th1 and Th17 cells in 12 patients and eight controls. IL-17 can be produced by various cell types, including natural killer (NK) cells, neutrophils, and other innate cells [7,14,15]. However, the predominant source of IL-17 is Th17 cells. In the last decade, experimental and clinical evidence has revealed unequivocal evidence for a common mechanism involving a pathogenic T helper 17 cell subset that predominantly produces the cytokine IL-17, which sustains chronic inflammation in T cell-associated autoimmune diseases, including rheumatoid arthritis [16], Crohn's disease [8], ankylosing spondylitis [17], and noninfectious uveitis [10]. Evidence for a prominent role for Th17 cells in BSCR was previously demonstrated by the presence of increased intraocular levels of IL-17 and elevated intraocular

[11] and serum levels [12] of immune mediators that promote Th17 responses in patients with BSCR. Interestingly, both studies suggested organ-specific Th17 cell-mediated inflammation [11,12]. Our data in patients with BSCR with active disease are consistent with and extend this hypothesis, since we observed IL-17 secretion and the induction of CD17⁺, CD4⁺, CD3⁺ (Th17) cells only after the PBMCs of patients with BSCR were stimulated with ocular lysates. Thus, we provide evidence that retina lysate contains antigens that initiate IL-17 production and link the Th17 signatures to ocular-specific immune responses rather than, for example, nonspecific constitutive Th17 activation. Identification of the exact antigen is highly valuable for understanding the exact disease mechanism of BSCR. Patients with BSCR frequently demonstrate positive proliferation *in vitro* after stimulation with the retinal S-antigen [3] or interphotoreceptor retinoid-binding protein (IRBP) [18,19]. These highly uveitogenic proteins cause uveitis in animal models via Th1 (IFN- γ) and Th17 (IL-17) responses [20,21]. In agreement with these observations, patients with BSCR display Th17 responses when stimulated with retina lysate. However, since many highly immunogenic autoantigens are present in the retina [20,22], a systematic strategy for identifying BSCR-related antigens using biochemical fractionation of the crude extracts, followed by testing overlapping peptides that span the entire amino acid sequence of each potential antigen is necessary to identify the exact antigens recognized in the crude extracts. Although we did not detect significant IL-17 responses to choroid lysates in all patients, several clinical [23] and experimental [5,6] observations suggest the choroid is the primary affected site. Thus, the responses against choroid antigens may still be relevant and should be addressed in a larger group of patients.

Several remarks must be made regarding the results of this study. For instance, some patients with inactive BSCR/IMT appeared to produce IL-17 and IFN- γ in response to the lysates, while others did not produce any cytokine. This heterogeneity may be due to the effectiveness of the treatment. However, probably due to the small size of our cohort, we were not able to link this heterogeneity to any therapy-related data. Therefore, this issue needs further investigation in larger cohorts. In addition, it remains difficult to determine if the observed cytokine responses are the cause or result of BSCR. Retina-specific CD4⁺ T cells in noninfectious uveitis may initiate the disease or may just be the result of the loss of the blood-retinal barrier and exposure of the highly uveitogenic retinal antigens to circulating peripheral retina-specific T cells. In any case, this "secondary" autoimmune response may be essential for significantly amplifying inflammation in the eye and could explain why BSCR shares

TABLE 2. COMPARISON OF THE CYTOKINES LEVELS SECRETED BY THE DIFFERENT GROUPS.

Cytokines	IL-10	IL-17	IFN γ
Differences in Skin response?			
Skin Active versus Skin inactive	ns	ns	ns
Skin Active versus Skin control	ns	ns	ns
Skin inactive versus Skin control	ns	ns	ns
Differences in SEB response?			
SEB Active versus SEB inactive	ns	ns	ns
SEB Active versus SEB control	ns	ns	ns
SEB inactive versus SEB control	ns	ns	ns
Antigen specific responses?			
Skin Active versus SEB Active	ns	***	ns
Skin inactive versus SEB inactive	ns	***	***
Skin control versus SEB control	ns	***	***
Ocular specific response?			
Skin Active versus Retina Active	ns	**	ns
Skin Active versus Choroid Active	ns	ns	ns
Skin inactive versus Retina inactive	ns	ns	ns
Skin inactive versus Choroid inactive	ns	ns	ns
Skin control versus Retina control	ns	ns	ns
Skin control versus Choroid control	ns	ns	ns
Differences in Retinal responses?			
Retina Active versus Retina inactive	ns	ns	ns
Retina Active versus Retina control	ns	**	ns
Retina inactive versus Retina control	ns	ns	ns
Differences in Choroid responses?			
Choroid Active versus Choroid inactive	ns	ns	ns
Choroid Active versus Choroid control	ns	ns	ns
Choroid inactive versus Choroid control	ns	ns	ns

P values were calculated by Kruskal–Wallis with post-hoc Dunn's multiple comparison test in Graphpad Prism v5 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

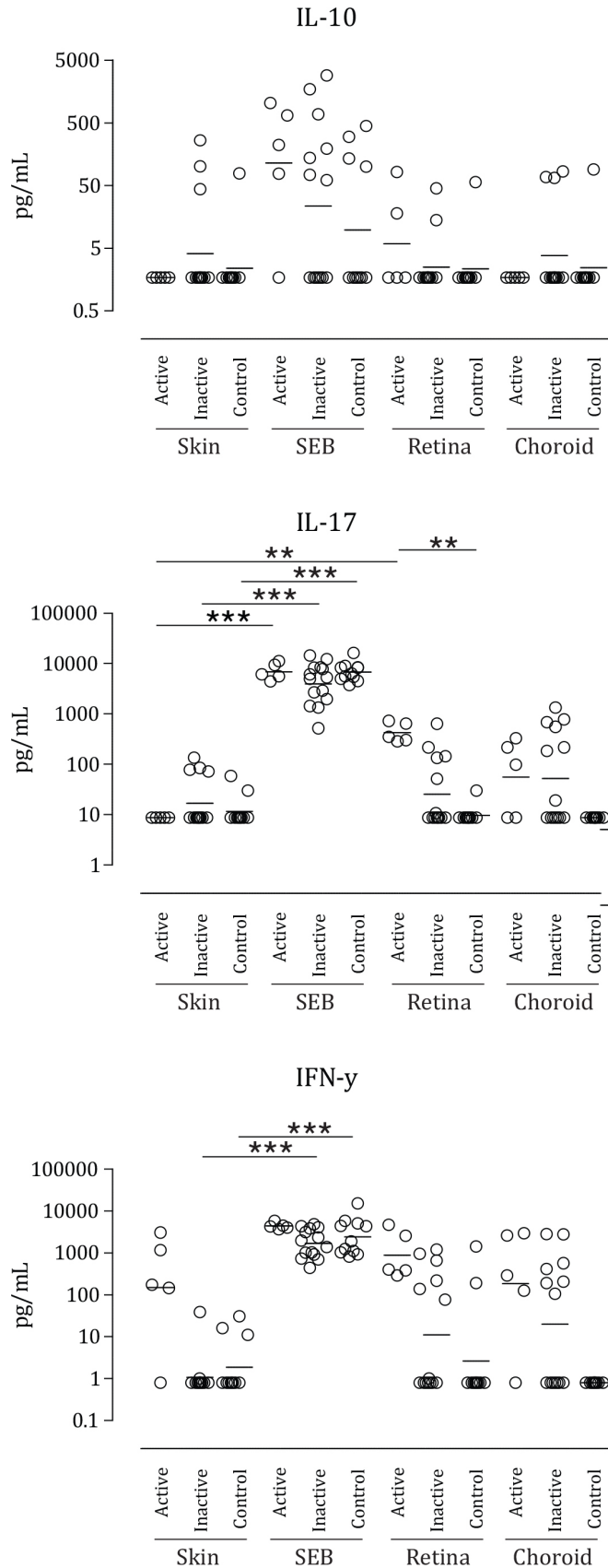


Figure 1. Scatterplots showing the levels of interleukin (IL)-10, IL-17, and interferon (IFN)- γ in supernatant (pg/ml) of peripheral blood mononuclear cell cultures stimulated for 6 days with ocular and control lysates, for each bird-shot chorioretinopathy subgroup and the control group. Each circle represents a patient or control as indicated in the upper right legend. Horizontal lines indicate the geometric mean per group. Active=patients with active disease naïve to immunomodulatory therapy (IMT). Inactive=patients with a current or past history of IMT or inactive disease. Skin=protein lysate derived from a skin biopsy of two healthy controls. SEB=Staphylococcal enterotoxin B. Retina=protein lysate derived from retinas of two healthy controls. Choroid=protein lysate derived from choroid of two healthy controls. Limit for detection of cytokines (pg/ml); interleukin (IL)-10, >1.7; IL-17, >8.7; interferon (IFN)- γ , >0.8. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple-comparison post-hoc test. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

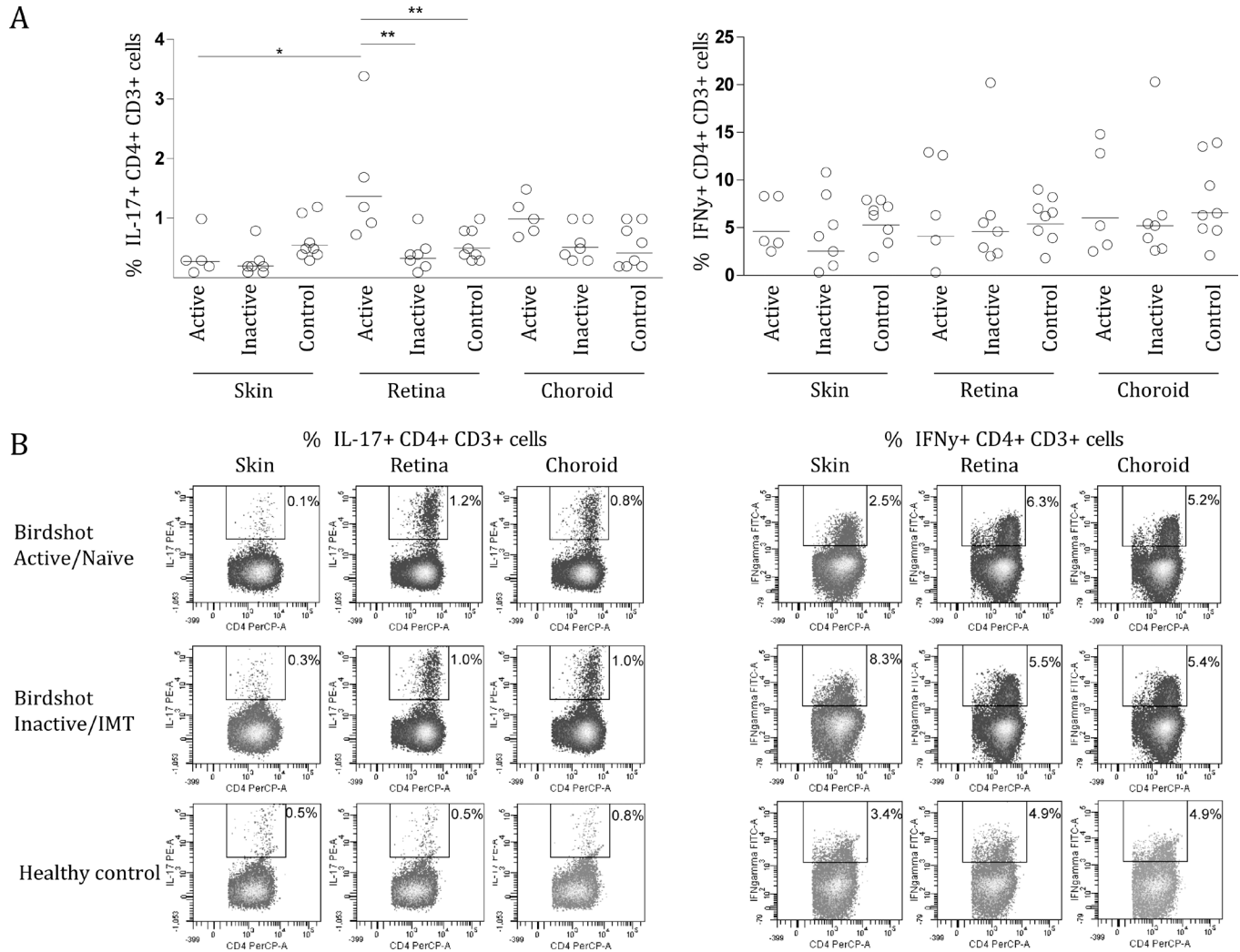


Figure 2. Th1 and Th17 cells in peripheral blood mononuclear cells stimulated with ocular and control lysates. **A:** Scatterplots showing the percentages of interleukin (IL)-17 CD4⁺, CD3⁺, and interferon (IFN)- γ ⁺ CD4⁺, CD3⁺ T cells in peripheral blood monocytes cultures stimulated for 6 days with ocular and control lysates, for each birdshot chorioretinopathy (BSCR) subgroup and the control group. Each circle represents a patient or control as indicated in the upper right legend. Horizontal lines indicate the geometric mean. **B:** Representative flow cytometric plots of a healthy control, a patient with a current or past history of immunomodulatory therapy (IMT), or inactive disease (birdshot inactive/IMT) and a patient naïve to immunomodulatory therapy (birdshot active/naïve). Cells were gated on CD3⁺, CD4⁺ lymphocytes. Skin=protein lysate derived from a skin biopsy of two healthy unrelated controls. Retina=protein lysate derived from retinas of two healthy unrelated controls. Choroid=protein lysate derived from choroid of two healthy unrelated controls. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple-comparison post-hoc test. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

Th17 signatures [11,12] and peripheral immune response to the retinal S-antigen [3] with other clinically and genetically distinct forms of uveitis [10,18]. Other cell types such as dendritic cells have been shown to play an important role in many ocular pathologies [24,25]. Furthermore, we observed that the PBMCs of the patients with active BSCR displayed an overall enhanced IFN- γ response to all lysates compared to the patients with inactive BSCR and the healthy controls. It is tempting to speculate that these results reflect overall enhanced T cell activity in patients with active BSCR [19]

as similar observations have been reported in experimental autoimmune uveitis [23]. The exact mechanisms and contribution of this overall elevated IFN- γ require further investigation, however. Finally, the control group was younger than both groups of patients. Age-dependent changes in the innate and adaptive immune system were reported to affect the individual susceptibility to autoimmunity [26-28]. However, the difference in the mean age of the patient groups was only about 10 years. Moreover, antigen-specific cytokine responses by T cells (including IL-17) do not differ between

various age groups [29]. Therefore, it is unlikely that the ocular lysate-specific IL-17 production in our study is associated with age-related differences.

In conclusion, our results underscore the importance of IL-17 in BSCR and suggest that targeting Th17 cells may be of particular interest in controlling inflammation in BSCR. Recently, anti-IL17 therapy (secukinumab) [30] did not demonstrate significant efficacy in noninfectious uveitis [31]. However, the authors noted that the results may have been confounded by the use of other relatively high doses of immunosuppressive medications in the studied patients [31]. Although the efficacy of anti-IL-17 therapy has not been evaluated in patients with BSCR, a better strategy could be targeting IL-23, because this cytokine is crucial for maintaining Th17 cells and can be targeted by the human monoclonal antibody ustekinumab [32]. Thus, our results warrant further studies on the manipulation of the Th17 cell axis, which hopefully reduces ocular inflammation and preserves visual function in patients with BSCR.

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