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Inflammatory cytokine and chemokine expression in sympathetic ophthalmia: a pilot study

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Summary. Sympathetic ophthalmia is a bilateral uveitis that develops after penetrating injury to one eye. This study aimed to identify the inflammatory cellular subphenotypes and expression of pertinent inflammatory cytokines/chemokines in sympathetic ophthalmia (SO). Dalen-Fuchs nodules (DFN), granulomas, and non-granulomatous foci of inflammation were micro-dissected from 15 cases. RNA was extracted, and quantitative PCR was performed to measure *IL-17, IL-18, IL-23, IFN-γ, CCL19, CXCL11, CCL17,* and *CCL22* transcripts. Immunohistochemical methods were used to characterize CD3, CD4, CD8, CD20, CD68, and CD163 expression. Non-granulomatous lymphocytes were predominantly CD3-positive and expressed more *IFN-γ* than cells within granulomas, consistent with Th1 cells. In contrast, granulomas and DFN contained mainly CD68+, CD163+/- and expressed more *IL-17, IL-18, IL-23, CCL19*, and *CXCL11* than non-granulomatous cells. Our data indicate for the first time that M1 macrophages are the predominant inflammatory cells within granulomas and DFN of SO. We further observed high levels of *IL-17* within granulomas and the presence of Th1 and M1 cells.

Key words: Chemokines, Cytokines, Macrophages, Sympathetic ophthalmia, Uveitis

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Introduction

Sympathetic ophthalmia (SO) is a bilateral granulomatous uveitis that develops following surgery or penetrating trauma to one eye (Sen and Nussenblatt, 2009). Antiretinal autoantibodies have been detected in SO serum (Chan et al., 1985c) and immunologenetical studies show evidence of specific HLA haplotypes (e.g., HLA-DR4, DRw53 and DQw3) and cytokine single nucleotide polymorphisms (SNP) (Davis et al., 1990; Atan et al., 2005).

The cause of SO appears to be a delayed hypersensitivity reaction to antigens localized on photoreceptors, retinal pigment epithelium (RPE), or uveal melanocytes exposed by the traumatic event (Jakobiec et al., 1983; Castiblanco and Adelman, 2009). A leading hypothesis is that perforating injury permits drainage of uveitogenic antigen from the eye and small amounts of adjuvant-like molecules to enter the eye and exacerbate ocular inflammation (Nussenblatt and Whitcup, 2010).

Uveitis may start as early as 5 days or as late as 50 years after injury; however, over 90 % of cases occur 2 weeks to 1 year (Lubin et al., 1980; Chan et al., 1986; Goto and Rao, 1990; Bakri and Peters, 2005). In the past, removal of the injured eye was undertaken to protect against subsequent inflammation of the uninjured eye (Marak, 1979; Winter, 1955). However, experience prior to the use of prophylactic corticosteroids, and later by our retrospective study (Chan et al., 1995), demonstrated that enucleation does not prevent the disease or improve vision (Winter, 1955). Currently, systemic corticosteroids and immunomodulators are recommended for the treatment of this entity (Castiblanco and Adelman, 2009).

Blurred vision and photophobia typically herald the involvement of the uninjured eye, while similar symptoms progress in the injured eye. Granulomatous uveitis ensues with moderate to severe vitritis and multiple white-yellow lesions in the choroid and deep retina. Under the microscope, both diffuse and focal infiltrates are seen in the choroid. Dalen-Fuchs nodules (DFN) represent granulomatous inflammation localized between the RPE and Bruch's membrane (Jakobiec et al., 1983; Chan et al., 1985, 1986; Shah et al., 1993). In severe cases, swelling of the optic nerve head and characteristic "mutton-fat" keratic precipitates on the posterior surface of the cornea can be observed (Chan et al., 1986). Granulomas are composed primarily of macrophages (Marak, 1979; Lubin et al., 1980; Chan et al., 1986), and may also be seen within the retina. These activated macrophages resemble epithelial cells, and are therefore sometimes referred to as "epithelioid histiocytes". During the final stages of the disease, B cells can be found within non-granulomatous infiltrates (Shah et al., 1993; Abu El-Asrar et al., 2007).

Recently, attention has been directed at understanding the functional properties of the cytokine milieu in this inflammatory condition. Cytokines are secreted proteins that mediate and control immune and inflammatory responses. More specifically, cytokines guide the differentiation of naïve T cells into effector subtypes that constitute the inflammatory reaction. T helper (Th) lymphocytes are a distinctive subset of T cells that secrete cytokines that regulate the immune response. Based on their cytokine or interleukin phenotype, Th cells are subdivided mainly into Th1 (signature cytokines, IL-2 and IFN-γ), Th2 (IL-4, IL-5, and IL-13), and Th17 (IL-17) subsets, which possess unique functions (Trinchieri, 2007; Mills, 2008). IL-10 was originally described as a cytokine produced specifically by Th2 cells, but later studies showed that Th1 cells produce at least similar amounts of IL-10 as Th2 cells (Fitzgerald et al., 2007; Trinchieri, 2007).

IFN-γ is considered the hallmark cytokine of Th1 cells, whereas Th2 cells produce IL-4 and Th17 cells elaborate IL-17. From a functional perspective, IFN-γ has antiviral, immunoregulatory, and anti-tumor properties (Schroder et al., 2004). IFN-γ also enhances antigen presentation to macrophages, activates and increases lysosome activity, and suppresses Th2 cell activity. Th1 cells produce IL-2, IFN-γ, and tumor necrosis factor (TNF)-α. In addition, they activate macrophages responsible for cell-mediated immunity, and are implicated in the pathogenesis of organ-specific autoimmune disorders.

Similar to their T-cell counterparts, macrophage populations are diverse and express distinctive repertoires of chemokine and chemokine receptors. Macrophages can be induced to polarize into classically activated M1 macrophages by IFN-γ treatment (Mantovani et al., 2004). M1 macrophages express high levels of IL-12, IL-23 and low levels of IL-10, and CCL19 and CXCL11 chemokines.

CCL19 binds to the chemokine receptor CCR7 and recruits macrophages. In contrast, CXCL11, a small chemotactic protein for activated T cells, sometimes referred to as the interferon-inducible T-cell alpha chemoattractant (I-TAC) interferon-gamma-inducible protein 9 (IP-9), binds to its receptor, CXCR3 (Erdel et al., 1998; O'Donovan et al., 1999). The gene expression of CXCL11 is strongly induced by IFN-γ and IFN-ß and weakly induced by IFN-α (Rani et al., 1996).

M2 macrophages are involved in the Th2 response and express CCL17 and CCL22 (Benoit et al., 2008). Unlike defined subtypes of Th lymphocytes, the plasticity of macrophages has resulted in alteration of their phenotypes over time (Mosser and Edwards, 2008).

IL-23 is a heterodimeric cytokine and an important component of the inflammatory response against infectious agents that promotes upregulation of matrix metalloprotease 9, increases angiogenesis, and reduces CD8+ T-cell infiltration. In conjunction with IL-6 and transforming growth factor-ß (TGF-ß), it stimulates naïve CD4+ cells to differentiate into Th17 cells, which are distinct from classical Th1 and Th2 cells. According to previous investigators, IL-23 is necessary to elicit experimental autoimmune uveoretinitis by immunization of mice with retinal antigen (Luger et al., 2008). Classic M1 macrophage activation is elicited by IFN-γ and selected cytokines. In addition, M1 macrophages produce abundant reactive oxygen and nitrogen intermediates and inflammatory cytokines and are components of the afferent and efferent limbs of polarized Th1 responses (Mantovani, 2006).

The objective of the present study was to explore the role of inflammatory cells, especially T cells and macrophages, in the pathogenesis of SO. In addition, we aimed to further characterize the cytokine milieu of this disease.

Materials and methods

Specimens

This study was conducted in compliance with the Declaration of Helsinki and was approved by the National Eye Institute Institutional Review Board for human subjects. Archival tissue specimens from 15 cases of SO from the National Eye Institute/National Institutes of Health and the Armed Forces Institute of Pathology were collected for this study. The clinical records were reviewed, and the pathological diagnosis was confirmed by at least two ophthalmic pathologists.

Microdissection, RNA extraction, and real-time quantitative PCR

Microdissection was performed manually using hematoxylin and eosin (H&E)-stained samples on glass slides, prepared from formalin-fixed, paraffin-embedded tissue, as described previously (Zhuang et al., 1995a,b; Zhuang et al., 1995; Shen et al., 1998). The slides were

cut at 6 μm and stored in room temperature.

To obtain reasonably pure populations of inflammatory cells, efforts were made to avoid sampling regions with overlapping features. Accordingly, inflammatory cells that comprised the DFN (mainly macrophages), granulomas (mainly macrophages), and non-granulomatous areas (mainly lymphocytes) were microdissected and collected separately. Subsequently, macrophage samples collected from the same case were combined and analyzed. Two to three areas of tissue were microdissected and studied from each case.

Total RNA was isolated using the Paradise Sample Quality Assessment Kit per the manufacturer's instructions (Arcturus, Mountain View, CA). The Paradise Sample Quality Assessment Kit is well known for RNA isolation from the cells on formalin fixed, archived slides. Total RNA was reverse-transcribed with Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Samples were DNase 1-treated (Fermentas, Glen Burnie, MD), and cDNAs were synthesized. The resulting cDNA from the SO tissue specimens was used to measure the relative expression of *IFN-γ, IL-17, IL-18, IL-23, CCL17, CCL19, CCL22*, and *CXCL11* (SA Biosciences, Frederick, MD). These chemokines/cytokines were selected because they represent the characteristic molecules produced by Th1, Th2, Th17, M1 and M2 cells. cDNA amplification was performed using the MX3000P QPCR system (Agilent, La Jolla, CA) with Green/Rox PCR master mix (SA Biosciences, Frederick, MD) and a QPCR kit (SA Biosciences, Frederick, MD). SYBR Green fluorescence (PCR product formation) was monitored in real time. The threshold for detection of PCR products was set in the log-linear phase of amplification, and the threshold cycle (Ct, number of cycles required to reach the threshold of detection) was determined for each reaction. Target mRNA levels were quantified using the manufacturer's instructions and were reported relative to the level of the housekeeping gene beta-actin by the comparative using ΔΔCT method. In the event that an individual mRNA level was more than two standard deviations above those of the group when not included, it was considered an outlier and excluded.

Immunohistochemistry

The avidin-biotin-immunoperoxidase complex (ABC) technique was applied as described previously (Chan et al., 1985b; Chan and Li, 1998). Briefly, the slides were deparaffinized and hydrated. Primary antibodies included monoclonal mouse anti-human CD3 (Dako North America Inc, Carpinteria, California), 1:40 dilution; CD4 (Novocastra, United Kingdom), 1:20 dilution; CD8 (Dako North America Inc, Carpinteria, California), 1:50 dilution; CD20 (Dako North America Inc, Carpinteria, California), 1:40 dilution; CD68 (Dako North America Inc, Carpinteria, California), 1:100 dilution; and CD163 (Novocastra, United Longdom), 1:200 dilution antibodies with biotin-conjugated horse anti-mouse IgG as the secondary antibody.

Statistical analysis

The Mann-Whitney U-test was used to evaluate the statistical differences. SPSS version 17.0 (SPSS Inc., IL) was used for all data analyses. Given the large differences among the measurements in archival tissue, the median value, which is more appropriate for skewed data, was calculated. A p value of ≤ 0.05 was considered statistically significant.

Results

The clinicopathological features of SO are summarized in Table 1. Among the 15 cases, 9 had ocular penetrating injury and 6 were operated previously. The cardinal pathological feature of uveal granulomatous inflammation (Fig. 1) was observed in all

Table 1. The clinical features of SO in 15 cases.

Case#	Age	Sex	Type of Injury or Surgery
1	46	F	Multiple intraocular surgery, catract extraction, vitrectomy with repare RD 2times
2	38	M	Ruptured globe
3	6	M	Penetrating corneal injury
4	NA	F	RD
5	NA	M	Perforated corneoscleral injury
6	82	F	Vitrectomy for endophthalmitis following catract extraction
7	30	F	Trauma
8	72	NA	Pars planau vitrectomy and scleral buckling procedure for RD, extracapsular catract extraction
9	59	F	Intracapsular catract extraction and blunt trauma
10	32	F	Ruptured globe, perforating limbal cornea
11	11	F	Lacerated corneal injury with prolapsed iris
12	24	F	Perforating limbal corneal injury
13	34	M	Perforating limbal injury
14	44	F	Pals plana Vitrectomy RD, DM
15	36	F	Corneal perforation

Footnote: NA, not applicable due to missing data.

15 cases. The inflammatory population outside the granulomas expressed higher *IFN-γ* mRNA levels (1.0-34.2) than cells within the granuloma (1.5-7.3), indicating that they were Th1 cells (Fig. 2). Within granulomas, the relative expression of *IL-18* transcripts in granulomas and DFN was much greater (0-8190.4) than in non-granulomatous infiltrates (T lymphocytes, 0-1746.0) (Fig. 2), reflecting their origin from bone marrow-derived macrophages. In addition, two M1 macrophage chemokines (*CXCL11, CCL19*) and two cytokines (*IL-18, and IL-23*) were elevated in granulomatous inflammatory cells (1.3-29.8, 2.1-134722.0, 0.0-8190.4, and 1.9-670.8; respectively) (Fig. 3). In particular, *IL-23* and *CXCL11* showed significantly greater expression in granulomatous (macrophages, 1.9-670.8 and 1.3-29.8, respectively) as compared to non-granulomatous (0.0-245.5 and 0.1-3.7, respectively) inflammation ($p<0.05$). The relative expression of the *CCL17,* transcript, a M2 macrophage chemokine, was greater in non-granulomatous (1.1-95.7) infiltrates. Alternatively, the relative expression of *CCL22,* a M0 and M2 chemokine, was significantly greater in granuloma-associated T lymphocytes (granuloma 0.9-

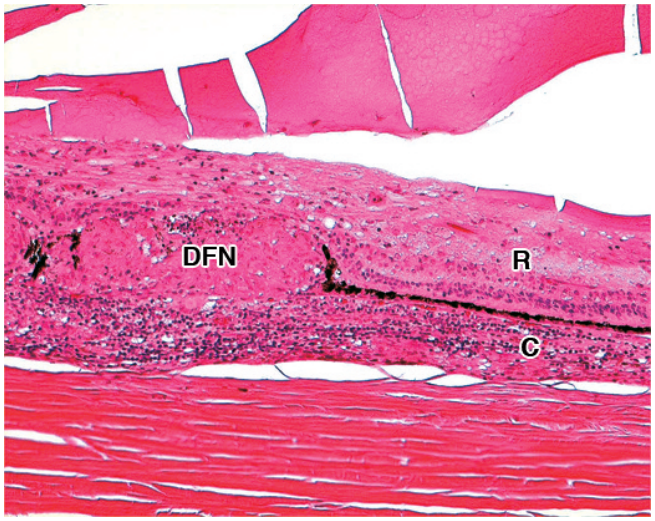

Fig. 1. Histopathology of SO. A large Dalen-Fuchs nodule (DFN) composed predominantly of macrophages is located between the retina (R) and choroid (C), which is infiltrated by many lymphocytes. H&E. x 100.

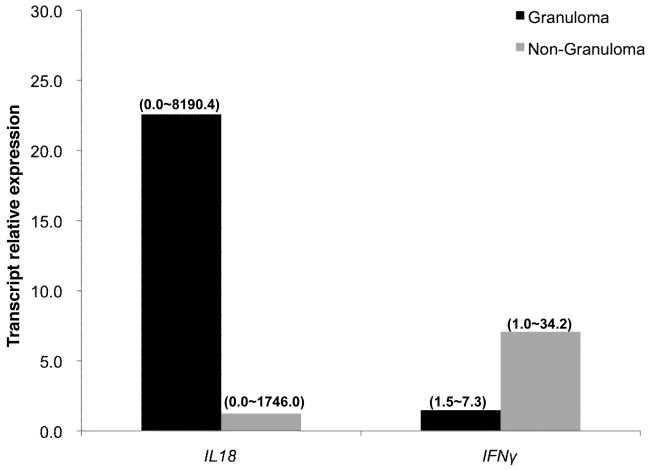

Fig. 2. Cytokine gene (*IL18* and *IFNγ*) relative expression in SO. *IL18* is highly elevated in the granuloma, and higher levels of *IFNγ* are expressed by lymphocytes.

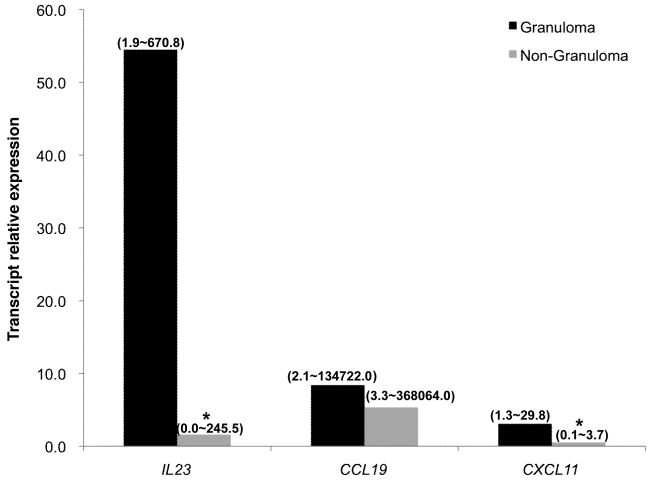

Fig. 3. M1 macrophage chemokines (*CXCL11, CCL19*) and cytokines (*IL23*) in SO. Chemokines/cytokines are higher in the granulomatous inflammation (M1 macrophages) as compared to the non-granulomatous inflammation (T lymphocytes). *: $P<0.05$

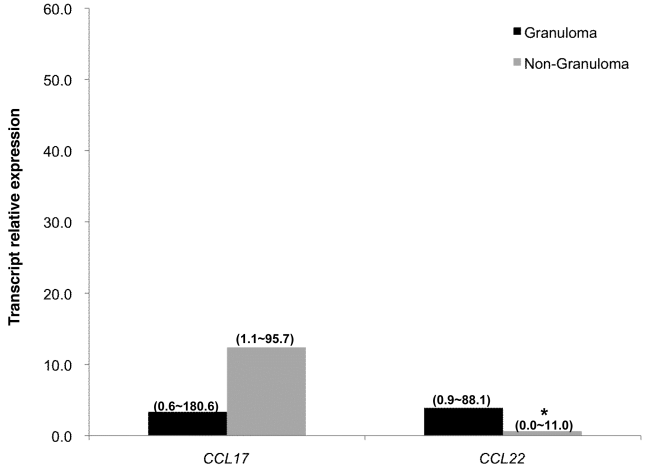

Fig. 4. M2 macrophage chemokines (*CCL17* and *CCL22*) in SO. M2 macrophage chemokines are higher within granulomatous (macrophages) as compared to non-granulomatous inflammation (T lymphocytes). *: $P<0.05$.

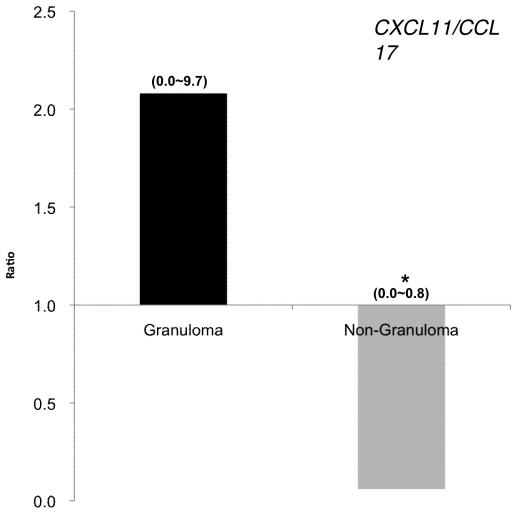

Fig. 5. The ratio of M1 (*CXCL11*) to M2 (*CCL17*) macrophage chemokines in SO. The ratio of *CXCL11* to *CCL17* was greater than 1.00 in macrophages, indicating that more M1 than M2 cells were found in granulomas and Dalen-Fuchs nodules. *: P<0.05.

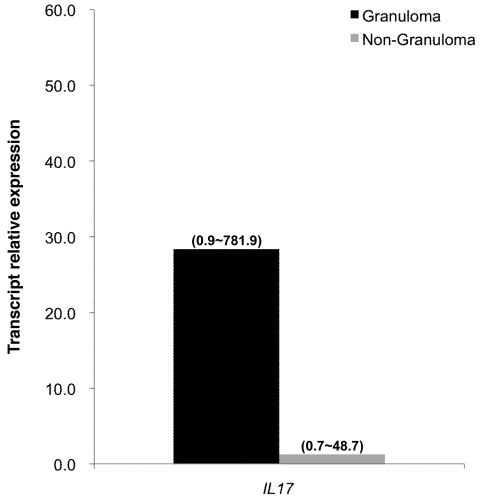

Fig. 6. Cytokine gene relative expression (*IL17*) in SO. *IL17* expression is higher within granulomatous as compared to non-granulomatous inflammation.

88.1 vs. non-granuloma 0.0-11.0, p<0.05) (Fig. 4).

CXCL11 and *CCL17* were used to analyze the relative distribution of M1 and M2 macrophages. The ratio of *CXCL11 to CCL17* was greater than 1.00 in macrophages, indicating that more M1 than M2 cells were recruited in granulomas and DFN (p<0.05). Taken together, M1 macrophages comprised a greater proportion of the granulomatous population, and M2 macrophages were mixed with T lymphocytes in non-granulomatous areas. The relative expression of *IL-17* transcripts in granulomatous inflammatory cells (0.9-781.9) was much greater than in the non-granulomatous inflammatory cells (T lymphocytes, 0.7-48.7) (Fig. 6).

Discussion

In the present study, our results are the first to provide preliminary evidence that selected M1 macrophage cytokine and chemokines, IL-23, CCL19, and CXCL11, predominate within the granulomatous infiltrates of SO. Additionally, we documented relatively high levels of *IL-17* in granulomatous areas and confirmed that IFN-γ-producing Th1 cells are key components of the non-granulomatous inflammation.

The source of the high levels of *IL-17* within granulomas in this condition remains enigmatic. Interestingly, Gu et al. reported that IL-10-deficient or IL-10R-deficient macrophages produce high levels of IL-17 and its transcription factor RORγt following LPS stimulation (Gu et al., 2008). Since macrophages represent the main inflammatory cell comprising granulomas and DFN, it is possible that macrophages are responsible for the elaboration of IL-17. In support of this hypothesis, other studies have documented IL-17 production by alveolar macrophages in asthmatic patients (Song et al., 2008), bone marrow-derived monocytes in Crohn's disease (Fujino et al., 2003), and in Langerhans cell histiocytosis (Coury et al., 2008). However, we cannot exclude the possibility that small numbers of infiltrating Th17 cells may have contributed to the elaboration of IL17 within granulomas in our samples.

Our results demonstrate that IFN-γ-producing Th1 cells represent the key component of non-granulomatous inflammation. As reported previously (Chan and Li, 1998; Shen et al., 1998), T-cells in non-granulomatous areas are mainly CD3-positive (CD4 > CD8) and express *IFN-γ* and *IL-2* but lack *IL-4*. In our samples, high levels of IFN-γ mRNA in non-granulomatous areas documented the presence of active Th1 cells, as observed in an earlier publication (Chan and Li, 1998). The precise role of IFN-γ in the development of the disease remains uncertain, with questions persisting as to whether *IFN-γ* has a protective or detrimental function with respect to the host eye. One possible explanation is that CXCL11, a ligand of CXCR3, tends to be expressed on Th1 and CD8 cells, both of which are detected in SO and both are known to produce IFN-γ. Although we did not quantify CXCL11 levels, it is likely that CXCL11 production recruits inflammatory cells into the eye that subsequently damage normal tissue. Future studies will be needed to clarify this hypothesis.

The present study shows that mainly T-lymphocytes and a few macrophages within non-granulomatous infiltrates not only produce IFN-γ, but also small amounts of CCL17, a M2 chemokine. We also found that *CCL17* transcripts were greater in non-granulomatous infiltrates.

CCL22 expression was low in granulomas,

compared to M1 macrophage chemokines (*CCL19* and *CXCL11*) and *IL-23*. It has been hypothesized that monocyte-derived dendritic cells are capable of elaborating CCL22 (Yamashita and Kuroda, 2002). In contrast, cells from the granulomas and DFN, mainly CD68+, CD163+/- cells, expressed higher levels of *IL-17, IL-18, IL-23, CCL19*, and *CXCL11* transcripts. These observations are consistent with previous reports showing that granulomas and Dalen-Fuchs nodules are composed mainly of macrophages (CD68+ cells) (Jakobiec et al., 1983; Chan et al., 1986). This investigation also established that *IL-23* and *CXCL11* transcripts dominate within granulomas and DFN, indicating that they are M1 macrophage-rich.

Functional polarization of macrophages into M1 or M2 cells is a useful concept that describes the plasticity of mononuclear phagocytes. More precisely, the phenotype of a macrophage population can change over time and may exist in a diverse range of phenotypes (Mosser and Edwards, 2008). Accordingly, our findings may only reflect a snapshot of the changing inflammatory milieu of SO in an immune privileged organ, the eye.

Compared to studies of pulmonary granulomatous diseases, mainly M1 macrophages are found in *Mycobacterium tuberculosis*-induced granulomas, whereas M2 macrophages predominate in *Schistosoma mansoni*-induced granuloma (Joshi et al., 2008; Redente et al., 2010). The former is a Th1 initiated disease and the latter is a Th2 initiated disease. Since SO is considered a Th1 disease, it follows that M1 macrophages are seen in choroidal granulomas and DFN.

In summary, this is the first investigation to demonstrate that M1 macrophages represent the predominant cellular element within granulomas and DFN in SO. In addition, we documented high levels of *IL-17* within granulomas and the presence of Th1 and M1 cells. Although methodological limitations exist with manually isolating specific cell populations, our findings provide evidence for the participation of dominant inflammatory cell subsets within the granulomatous and nongranulomatous areas. Further analysis is warranted to better define the mechanisms of cytokines/chemokines in the pathogenesis of SO, and whether targeting Th1 lymphocytes, M1 macrophages, and their cytokines/chemokines plus IL-17, may represent effective therapy.

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