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Recurrent ocular toxoplasmosis is associated with interferon-gamma deficiency possibly due to genetic origin

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ABSTRACT

Objective Ocular toxoplasmosis (OT) can cause posterior uveitis; causes of recurrent OT are not well understood. We explored clinical, immunological and genetic properties associated with recurrent OT.

Methods and analysis A recurrent OT patient population (n=9) was identified. Clinical history, ophthalmological findings and immunological properties were assessed. B and T cell immunophenotyping including interferon-gamma (IFN-γ) responses were analysed. An analysis of 592 immunodeficiency genes was performed.

Results The patients experienced 2–7 OT episodes (average 3.7). The first episode occurred at an average of 23.8 (SD 10.1) years of age. All patients had anterior uveitis, vitritis and various fundus lesions of OT. The patients had lymphocyte maturation abnormalities; the proportion of naive $CD4^+CD45RA^+CCR7^+$ T cells was high in 5/9 cases, and the percentage of $CD4^+CD45RA^-CCR7^-$ T effector memory cells was reduced in 7/9 cases. An increased percentage of $CD19^+CD38^{low}CD21^{low}$ activated B cells was observed in 5/9 cases. IFN-γ response was reduced in $CD4^+$ (8.45±4.17 vs 21.27±11.0, p=0.025) and $CD8^+$ (39.0±9.9 vs 18.1±18.1, p=0.017) T cells. Genetic analysis revealed several potentially harmful variants in immunologically active *ERCC3, MANBA, IRF4, HAVCR2, CARMIL2, CD247, MPO, C2* and *CD40* genes.

Conclusion Our recurrent OT cases had deviations in lymphocyte maturation and IFN-γ responses possibly caused by genetic reasons. However, limitations of our study include failure to identify uniform genetic mechanisms. In addition, we cannot rule out the possibility that the immunological abnormalities can be triggered by chronic toxoplasmosis. Despite the limitations, our findings contribute to the understanding of ocular immunity and development of recurrent OT.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Ocular toxoplasmosis (OT) is a common infectious cause of posterior uveitis which can affect immunocompromised patients such as those suffering from advanced HIV infection. Immunological or genetic causes of recurrent OT among HIV-negative populations, however, are not well understood.

WHAT THIS STUDY ADDS

⇒ We found that recurrent OT is associated with deviation in T cell maturation and IFN-γ responses. While we failed to demonstrate a monogenic mechanism, several potentially harmful immunologically significant gene variants were found.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings support the possibility that genetic and immunological properties may predispose to recurrent OT. These preliminary observations may open novel approaches for OT treatment and prevention.

INTRODUCTION

Ocular toxoplasmosis (OT) caused by *Toxoplasma gondii* is a common infectious posterior uveitis presenting with focal necrotising retinitis, overlying vitritis and hyperpigmented retinochoroidal scarring.[1] Macular involvement and large scars may cause permanent vision loss or negatively affect vision.[2 3] OT diagnosis depends on the characteristic clinical features in patients with positive serological test results. In recurring cases, new active retinochoroiditis foci typically develop around the scars of previous lesions.[4] The hyperpigmented scar harbours cysts that remain inactive until the cyst ruptures and releases the parasites to the surrounding retina. Although management guidelines of OT have been generated, understanding of predisposing factors of recurrent OT especially among immunocompetent patients remains incomplete.

Parasite properties and genetic regulation of host immune responses are believed to influence the OT clinical outcome and risk of recurrence.[4–6] For example, a recent study in a rodent model highlights the importance of interferon-gamma (IFN-γ) dependent *Toxoplasma* genes.[7] While evaluation of toxoplasma virulence remains challenging, disease caused by genotype I may be related to more invasive disease when compared with infection by genotype II.[8] Although OT may

recur in immunocompetent patients, compromised T cell-mediated immunity caused by advanced HIV infection may explain the OT activation.[9] Proper immune responses, including IFN-γ activity, are needed to control the toxoplasmosis.[5 10] Genetic polymorphism, such as IFN-γ +874 T/A, for example, can be associated with OT recurrence.[11] Recurrence risk can also be elevated among patients older than 40 years and those who develop new lesions within 1 year after the first episode or have macular involvement, chorioretinal lesions greater than 1 disc diameter, congenital toxoplasmosis or bilateral disease.[4] Although clinical studies and previous association studies of genetic polymorphisms in small patient cohorts have been completed,[1 5] immunological and genetic mechanisms of OT recurrence are not well understood.

In this study, we identified recurring HIV-negative OT patients in a population with low toxoplasmosis prevalence and genotype II *T. gondii* predominance.[12] We analysed their clinical presentations and explored their peripheral blood immune cell populations first with mass cytometry, followed by extensive immunophenotyping according to EuroFlow guidelines developed to identify inborn error in immunity (IEI) conditions.[13] We also stimulated peripheral blood T cells in vitro to test their IFN-γ response capacity. Further, the presence of deleterious mutations in the immunologically relevant genes was analysed with whole exome sequencing (WES) to search for potential genetic causes of the immunological abnormalities.[14] Our findings support the view that defective T cell maturation and IFN-γ response possibly due to genetic properties can be associated with OT recurrence.

PATIENT POPULATION AND METHODS

Patients and clinical characteristics

All patients with posterior uveitis or retinitis (n=238) at the Oulu University Hospital Ophthalmology clinic responsible for tertiary care for a population of approximately 410 000 inhabitants were identified from the hospital's electronic patient database during a 9-year period. The International Classification of Diseases (ICD-10) diagnostic codes for posterior uveitis (H30.2), toxoplasma retinitis (H32.0*B58.0) and retinitis (H30) were used. The patients with serologically identified toxoplasmosis (n=24) were further evaluated. Recurrent OT (n=9) cases, who are negative for HIV infection or any other immunosuppressive condition, were recruited for clinical, immunological and genetic evaluation. Clinical data were gathered from comprehensive ophthalmic examination (best-corrected visual acuity, intraocular pressure and biomicroscopy) completed during the study, and retrospectively from the hospital's electronic patient records. In addition to clinical evaluation, fundus imaging was performed during active OT. Information about family history was obtained by interviewing the participants. The patients did not have evidence of active toxoplasmosis and they did not have antimicrobial treatment when their blood samples were collected. For IFN-γ in vitro response analysis, healthy age-matched controls were recruited from the personnel of the University of Oulu and Oulu University Hospital. Although toxoplasma antibodies were not analysed, results of comprehensive ophthalmic examinations showed no evidence of toxoplasma retinal lesions or any other ocular diseases.

Immune cell profiling with mass cytometer

Immune cell populations were first screened and compared with healthy age-matched and sex-matched controls with the Maxpar Human Immune Monitoring Panel Kit (Cat. No. 201324, Fluidigm Corporation, San Francisco, California, USA) and analysed with Helios mass cytometer (Fluidigm) according to the manufacturer's instructions. Mass cytometry data files were analysed using Cytobank (Beckman Coulter, Indiana, USA). The results obtained from each OT patient were compared with those obtained from healthy age-matched and sex-matched control cases. Statistical significance was analysed with a paired t-test (IBM SPSS software version 29).

B and T cell immunophenotyping

B and T cell immunophenotyping at the individual level was analysed according to EuroFlow methods[13] and diagnostic protocols developed to identify immunodeficiencies.[15] The analysis was completed from fresh heparin-blood samples. Four or 10-colour flow cytometry panel with monoclonal antibodies against the surface antigens IgM, IgD, CD3, CD4, CD8, CD16\56, CD19, CD21, CD27, CD33, CD34, CD38, CD45, CD56, CD57, CD133, HLA-DR, CD62L, CD45RA and CD45RO (BD Biosciences). T cell immunophenotyping was studied with the antibody panel including anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-CD45RA and anti-CCR7 (R&D Systems).

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (lithium heparin tubes). The cells were aliquoted in 90% FBS (ThermoScientific; SV301800.03) and 10% dimethyl sulfoxide (Applichem; A3672,0250) and stored at −140°C. Cells were cultured in RPMI 1640 (Sigma Aldrich; R0883), supplemented with 100U penicillin and 100 µg/mL streptomycin, 10 mM HEPES, 2 mM L-glutamine and 10% FBS (ThermoScientific; SV301800.03) at 37°C with 5% CO_2 in a humidified incubator.

IFN-γ response in vitro

IFN-γ response capacity of stimulated $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was tested in vitro by comparing each OT case to their age-matched and sex-matched control. PBMCs were plated at 3×10^6/mL density and allowed to rest overnight. Protein transport inhibitors (Invitrogen; 00–4980) were added to unstimulated cells, or the cells were stimulated for 5 hours with Phorbol-12-myristate-13-acetate/Cell Stimulation Cocktail (PMA/ionomycin) with protein transport inhibitors (Invitrogen; 00-4975-93). After stimulation, the cells were collected, washed

twice, permeabilised with Cytofix/Cytoperm (BD Biosciences; 554714) for 20 min at +4°C, washed twice with cold Perm-Wash (BD Biosciences; 554714) and stained with IFN-γ antibody for 35 min at +4°C and analysed by flow cytometry (BD LSRFortessa, Becton Dickinson). The results obtained from each OT patient were compared with the results obtained from age-matched and sex-matched control cases. Statistical significance was evaluated with a paired t-test (IBM SPSS software).

Genetic analysis

Sequencing was performed at the Institute for Molecular Medicine Finland Technology Centre. Libraries were prepared from DNA samples extracted from blood with NimbleGen SeqCap EZ MedExome kit (Roche Nimblegen, Madison, Wisconsin, USA) or Agilent SureSelect ClinicalResearchExome kit (Agilent, Santa Clara, California, USA) according to the manufacturer's instructions. WES libraries were sequenced with Illumina HiSeq1500 system, with paired-end reads 2×100 bp. Bioinformatic analysis of raw data was performed with a variant calling pipeline. Reads were trimmed and thereafter aligned to the GRCh37 reference genome with BWA-MEM. GATK Base Recalibrator was used to clean the alignment and duplicates were removed using Picard MarkDuplicates and GATK IndelRealigner for indel sites. Variants were called with the use of Mpileup from the SAMTOOLS package7.

We aimed to explain the observed immunological deficiencies by analysing 592 genes (online supplemental material) involved in IEI.[14] Variants identified in selected genes were filtered using VariantInterpreter software (V.2.17.0.60, Illumina) and alleles absent from or with minor allele frequency <0.01 in GnomAD (V.2.1.1) were included. Remaining variants were interpreted based on allele frequencies in the reference population (GnomAD V.2.1.1, https://gnomad.broadinstitute.org/; SISu, https://sisuproject.fi/), in silico predictions (Polyphen, SIFT, MutationTaster, CADD, REVEL) and ACMG guidelines.[16] Public disease databases (Clinvar, OMIM) were used in the analysis and known non-pathogenic variants were excluded. All remaining variants were visualised using Integrative Genomics Viewer (V.2.16.2). For the observed rare genetic variants with an in silico prediction of being potentially harmful or deleterious, any associations with immune deficiencies were evaluated using FinnGen data (FinnGen Freeze 9 database, https://r9.finngen.fi/).

RESULTS

Clinical parameters

The patients or their family members had no history of secondary immunodeficiency, consanguinity, generalised infection susceptibility or any other evidence of IEI conditions.[14] The average age of the patients with recurrent OT was 23.8 (SD 10.1) years at the time of the first OT episode. The average number of recurrences was 3.7 (SD 2.2, range 2–7). All patients had anterior uveitis, vitritis, and 4 (44%) had toxoplasma lesions in the macula, 4 (44%) had lesions next to the optic disc, and 4 (44%) in the peripheral retina. Three patients (33%) had papillitis or retinal haemorrhages. Only two out of nine patients (22%) had a permanent decrease in visual acuity after OT episodes, but none was visually impaired. Ophthalmic findings of OT are summarised in online supplemental table 1.

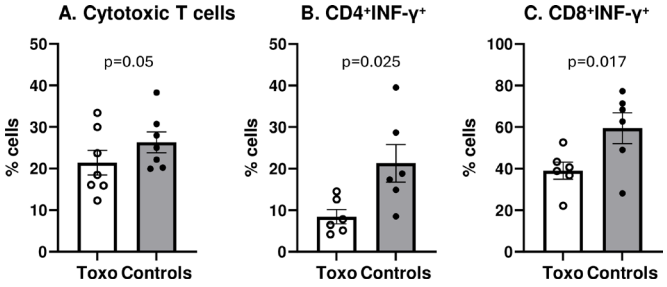

Figure 1 (A) Peripheral blood cytotoxic T cells ($CD3^+CD8^+CD45^+TCRgd^-$) in recurrent toxoplasmosis (Toxo) patients and healthy controls. (B) Interferon-gamma (IFN-γ^+) response in $CD3^+CD4^+$ (8.45±4.17 vs 21.27±11.0, p=0.025) and (C) $CD3^+CD8^+$ (39.0±9.9 vs 18.1±18.1, p=0.017) T cells obtained from the recurrent OT patients and their healthy age-matched controls after PMA/ionomycin stimulation in vitro. The statistical significance of the recurrent OT patient cohort compared with control cohort was calculated with paired t-tests (IBM SPSS software). OT, ocular toxoplasmosis; PMA, phorbol-12-myristate-13-acetate.

Lymphocyte immunophenotyping

PBMCs were first screened and compared with healthy age-matched controls using the mass cytometer method. The results of screening demonstrated that total B and T cell counts were comparable in OT and control cases. Comparison of proportions of cytotoxic T cells ($CD3^+CD8^+CD45^+TCRgd^-$) among the recurrent OT patients and healthy age-matched and sex-matched controls did not reach the level of confirmed statistical significance (p=0.05, figure 1A). PBMCs were further evaluated for specific subpopulations in each individual patient with flow cytometric analysis according to protocols developed to identify primary or secondary immunodeficiencies (online supplemental table 2).[15] In $CD19^+$ B cell compartment, two cases (patients 1 and 5) presented with reduced total $CD19^+$ count (online supplemental table 2). Activated $CD38^{low}CD21^{low}$ B cell population was elevated in six cases indicating B cell activation possibly caused by abnormalities in genetic regulation of the immune system or chronic infection.[17] Within $CD3^+$ T cell compartment, reduced $CD4^+$ and $CD8^+$ counts were observed in two cases (patients 3 and 6). Evidence of T cell maturation abnormalities was found especially in $CD3^+CD4^+$ populations; the percentage of naive $CD3^+CD4^+CD45RA^+CCR7^+$ T cells was high in five cases (5/9) and $CD3^+CD4^+CD45RA^-CCR7^-$ T effector memory cells were reduced in seven cases (7/9) (online supplemental

table 2). Natural killer cell count was below normal level in three individuals.

IFN-γ response in vitro

The proportion of IFN-γ-positive T cells after PMA/ionomycin stimulation in vitro was lower in the patient's $CD3^+CD4^+$ T cells when each OT case was compared with their own age-matched and sex-matched control cases (8.45±4.17 vs 21.27±11.0, p=0.025) (figure 1B). Proportions of IFN-γ-positive cells were also reduced in $CD3^+CD8^+$ T cell populations when each OT case was compared with age-matched and sex-matched control cases (39.0±9.9 vs 59.5±18.1, p=0.017) (figure 1C). Statistical significance was tested with a paired t-test (IBM SPSS software).

Genetic analysis

Consistent with a limited history of infection susceptibility, confirmed pathogenic IEI mutations were not found.[14] However, most patients were positive for rare, damaging or possibly damaging heterozygous variants in immunologically relevant genes (table 1). Potentially harmful variants in immunologically active genes *C2, CARMIL2*, *CD247*, *CD40, ERCC3, HAVCR2*, *IRF4*, *MANBA* and *MPO* were found. Only one rare genetic variant c.580G>T, p.(Asp194Tyr) in *HAVCR2* was shared by two patients (P5 and P6). *HAVCR2* encodes a T cell immunoglobulin and mucin-domain containing-3 (TIM-3), which is involved in regulation of T cell activation and cytokine signalling in *T. gondii* infection.[18]

As some of the variants were too common to cause a dominantly inherited IEI, or the phenotype was not consistent with the previously described condition, we continued to test the role of the variants at the population level by using the FinnGen project data (https://r9.finngen.fi/). Interestingly, three of the variants observed in OT patients were associated with immune disorders: the *IRF4* c.263C>A variant is associated with autoimmune diseases and allergic contact dermatitis ($p=1.34\times10^{-6}$ and $p=5.68\times10^{-5}$), the *HAVCR2* c.580G>T with immunodeficiencies (p=0.006), and the *CD247* c.162+2T>G variant with toxoplasmosis (p=0.0067), suggesting a putative predisposing role.

DISCUSSION

The immune system in most healthy individuals can control the primary *T. gondii* infection.[19] However, clinical history of recurrent OT in our patient cohort in the absence of HIV infection or any other known immunosuppressive condition may be suggestive of deficient host immunity as indicated by our findings of immune cell phenotyping and T cell IFN-γ responses. These patients or their families had no consanguinity, they had no history of unusual presentations of infections or known IEI conditions suggesting that OT recurrence susceptibility can be highly selective. Toxoplasmosis activity in these patients is evident only in their eyes demonstrating the anatomical and immunological vulnerability of the eye.

Results of our immune cell phenotyping as well as in vitro analysis of stimulated T cells demonstrate that the capacity for producing IFN-γ responses is reduced in patients with recurrent OT. Importantly, our findings are based on protocols designed to recognise IEI conditions.[13 15] We found, for example, low proportions of $CD4^+CD45RA^-CCR7^-$ T lymphocytes, a T cell population active in IFN-γ responses, when compared with healthy age-matched controls. Previously, similar findings have been described in association with susceptibility to mycobacterial infections, for example.[20] Such immunological properties can also share features with advanced HIV-infected patients among whom poor $CD3^+CD4^+$ immunity and frequent activation of toxoplasmosis is observed.[9] IFN-γ response in vitro was compromised not only in $CD3^+CD4^+$ but also in $CD3^+CD8^+$ T cells; this observation is also consistent with previous findings of toxoplasma immunity.[21] Although our immunological findings can be caused by genetics, we cannot rule out the possibility that chronic toxoplasmosis may trigger similar T cell abnormalities by transcription factor BLIMP-1 or PD-1-mediated mechanisms.[21 22] Further, we found that the proportion of activated $CD38^{low}CD21^{low}$ B cells is elevated in recurrent OT patients consistent with, for example, chronic infection or genetically defective B cell maturation as observed among common variable immunodeficiency (CVID) patients.[17] Our patients do not, however, have a low percentage of switched memory B cells and their serum immunoglobulin concentrations are normal suggesting that CVID phenotype may not explain the OT recurrences.[23 24]

Despite the genetically isolated study population, our analysis did not identify a uniform genetic cause for the deviation in B or T cell maturation, the low IFN-γ production or the recurrent OT. Two patients, however, shared a rare and potentially deleterious *HAVCR2* c.580G>T variant. *HAVCR2* gene encodes for T cell immunoglobulin and mucin-domain containing-3 (TIM-3), a type I transmembrane receptor that was initially identified as a marker for IFN-γ secreting T cells.[25] While homozygous TIM-3 deficiency has previously been connected with subcutaneous panniculitis-like T cell lymphoma, haemophagocytic lymphohistiocytosis, lupus nephritis and autoinflammatory myocarditis,[26–28] recent findings support disease-causing potential also for a heterozygous *HAVCR2* mutation.[29] Additionally, the deleterious-predicted *IRF4* c.263C>A missense variant is associated with autoimmune diseases and allergic contact dermatitis ($p=1.34\times10^{-6}$ and $p=5.68\times10^{-5}$) in the FinnGen cohort. Intriguingly, both *HAVCR2* and *IRF4* gene expressions are in response to toxoplasmosis.[18 30] Further, potentially harmful genetic variants in immunologically active CD247, CARMIL2, *C2, CD40* and *ERCC3* genes identified in our study are known to regulate immune responses or to participate in development of toxoplasma immunity.[31–35]

Table 1 Rare and potentially harmful or deleterious genetic variants in immunologically relevant genes

ID	Variant*	Biological function association†	Genomic position (Grch37/hg19)	MAF, All GnomAD‡	MAF, Fin GnomAD‡	Predicted effect§	ACMG¶
P2	*ERCC3* c.1856A>T p.(Glu619Val)	DNA repair, associated with toxoplasmosis**	Chr2:128029001	0.000008, 2/251476	0.00009, 2/21648	Deleterious	VUS
	MANBA c.673+1G>A	Innate immune system	Chr4:103635594	0.00002, 5/251166	0.00014, 3/21646	Deleterious, splice donor	Pathogenic
P4	*IRF4* c.263C>A p.(Pro88Gln)	Interferon-gamma signalling	Chr6:394867	0.00053, 151/282736	0.0040, 100/25122	Deleterious	VUS
P5	*HAVCR2* c.580G>T p.(Asp194Tyr)	Cytokine signalling in immune system	Chr5:156522413	0.00046, 130/282680	0.0050, 125/25114	Possibly damaging/ conflicting	VUS
P6	*C2* c.377C>T p.(Ser126Leu)	Complement cascade	Chr6:31896629	0.000008, 2/251444	0.0, 0/21644	Possibly damaging/ conflicting	VUS
	HAVCR2 c.580G>T p.(Asp194Tyr)	Cytokine signalling in immune system	Chr5:156522413	0.00046, 130/282680	0.0050, 125/25114	Possibly damaging/ conflicting	VUS
P7	*CARMIL2* c.1063G>A p.(Asp355Asn)	Immunodeficiency, maturation of T-memory cells	Chr16:67681853	0.0001, 30/269572	0.00090, 22/24232	Possibly damaging/ conflicting	VUS
	*CD247*c.162+2T>G	Immunodeficiency, immune response NFAT	Chr1:167409899	0.00001, 3/282090	0.0001, 3/24864	Deleterious, splice donor	Pathogenic
	MPO c.238C>T p.(Arg80Trp)	Innate immune system	Chr17:56357737	0.00004, 12/282400	0.00008, 2/25058	Possibly damaging/ conflicting	VUS
P8	*CD40* c.256+4C>T	Immunodeficiency, toxoplasmosis infection	Chr20:44751001	0.00003, 8/250970	0.0, 0/21632	Splice region	VUS

*All heterozygous.
†Genecards GeneCards - Human Genes | Gene Database | Gene Search.
‡GnomAD V.2.1.1 https://gnomad.broadinstitute.org/.
§PolyPhen, SIFT, MutationTaster, CADD, REVEL.
¶Richards *et al,* 2015.[16]
**Smolarz *et al,* 2014.[31]
††VanGrol *et al,* 2013.[32]
ACMG, American College of Medical Genetics; MAF, mean allele frequency; VUS, genetic variant of unknown significance.

In summary, we found evidence supporting the view that defective T cell immunity and IFN-γ responses possibly caused by genetic mechanisms may at least partly predispose to recurrent OT among HIV-negative individuals without additional features of infection susceptibility. Although we failed to show a common monogenic causative mechanism of OT susceptibility, several genetic variants with a potential of disturbing the T cell maturation or IFN-γ production were identified. Despite the advances in the understanding of OT, large gaps still exist in the knowledge concerning the host biological properties of this common and potentially blinding disease. Although our findings are highly suggestive of impaired T cell-mediated immunity, research in larger patient cohorts is needed to elucidate the biological mechanisms and clinical significance of our observations. Further scientific questions may also arise from the potential of IFN-γ treatment in sight-threatening OT cases.[36]

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval The study followed the tenets of the Declaration of Helsinki, and it was conducted with the approval of the Oulu University Hospital Research Committee (89/2017). All patients were informed of the study protocol and a written consent of participation was obtained from each patient. Complete anonymity was adhered to, and all study information was anonymised.

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