

## CLINICAL STUDY

# Detection of thyroid peroxidase mRNA and protein in orbital tissue

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## Abstract

**Objective:** We have previously reported that the absence of thyroid peroxidase antibodies (TPOAb) in Graves' disease (GD) was associated with an increased risk of Graves' ophthalmopathy (GO). This observation raised the possibility that TPOAb could act as a protective factor. However, the presence of thyroid peroxidase (TPO) in the orbit has not been previously reported. The aim of this study was to confirm or exclude the presence of orbital TPO.

**Methods and design:** Relative TPO mRNA expression from GO ( $n = 6$ ) and normal ( $n = 5$ ) orbital fat tissue was determined using real-time PCR technique. Orbital fat in the normal group from blepharoplasty represents extraconal (anterior) fat. mRNA expression in fibroblasts grown from these tissues before and after adipocyte differentiation was also documented. Finally, Western blotting was carried out to verify translation of TPO mRNA transcripts.

**Results and discussion:** TPO transcripts were detected in the orbital fat tissue obtained from normal and GO subjects using the real-time PCR technique. TPO expression was increased in GO compared to normal (N) tissues. However, TPO expression in cultured fibroblasts was similar in both groups and adipogenesis did not appear to alter TPO expression. Protein was detected by Western blot analysis using the TPO MAB 47 (mAb 47). The predicted 110-kDa band was detected in orbital fat as well as in orbital fibroblasts. Our results suggest the presence of TPO in GO and N orbital tissues. We hypothesise that immune responses directed against orbital TPO might play a role in modulating the clinical expression of GO.

*European Journal of Endocrinology* 155 213–218

## Introduction

The close association between Graves' disease (GD) and Graves' ophthalmopathy (GO) has led to the assumption that a shared autoantigen(s) in the eye and thyroid is likely to exist. The leading candidate is the thyrotrophin receptor (TSH-R) (1). Numerous groups using different methodologies have detected TSH-R mRNA in orbital tissue. Orbital inflammation has been observed after genetic immunisation of NMRI outbred mice treated with full-length TSH-R in an eukaryotic expression plasmid and after passive transfer of TSH-R sensitised T cells in BALB/c mice (2–4). The hallmark of GD is the production of TSH-R antibodies (TRAb) particularly thyroid-stimulating immunoglobulin (TSI) antibodies (5). Quantitative associations between these antibodies and GO have been reported. Furthermore, TRAb are detectable in more than 90% of patients with euthyroid and hypothyroid GO. The epidemiological data therefore suggest links between TRAb and GO (6).

In a cohort of 100 newly diagnosed non-smoking cases of GD, we previously reported that the absence of thyroid peroxidase antibodies (TPOAb) was associated with a five-fold increase in the risk of GO. We hypothesized that these antibodies might modulate TRAb effects on GO (7). How TPOAb might affect the orbit is unknown and the presence of TPO at that site has not been previously described. In view of our epidemiological data suggesting a possible protective role of TPOAb and the previous detection of thyroid-specific proteins at extrathyroidal sites, we sought to confirm or exclude the presence of TPO in orbital tissue.

## Materials and methods

### Tissue collection and RNA extraction

Human thyroid tissue (T) was obtained from patients undergoing thyroid lobectomy and immediately frozen.

For the purpose of comparison, prostate and brain tissues were also obtained for the study. Prostate tissue was obtained from patients undergoing surgery for prostate enlargement, while brain tissue was harvested from brain autopsies. The orbital fat tissue collected, comprised normal (N) or Graves' Ophthalmopathy (GO) subjects. GO tissue (extraconal-posterior) was obtained from patients undergoing orbital decompression. GO patients were euthyroid at the time of orbital surgery. N tissue (extraconal-anterior) was obtained from patients undergoing blepharoplasty. Informed consent from patients was obtained before tissue collection. Tissue collected were placed in RNAlater (Ambion, Austin, TX, USA) and stored at  $-20^{\circ}\text{C}$ . Total RNA was prepared from tissue using Trizol (Gibco) as described in manufacturer's protocol. The Singapore General Hospital Ethics Committee approved these studies and ethical codes are in compliance with the Helsinki Declaration 2000.

### Cell culture and adipocyte differentiation

GO or N orbital fat were minced and placed in plastic culture flasks allowing the orbital fibroblasts to emerge from the tissue. Cells were grown to confluence in medium 199 containing 10% foetal bovine serum (JRH Biosciences, Lenexa, TX, USA), penicillin (100 U/ml) and gentamicin (20  $\mu\text{g}/\text{ml}$ ) in a humidified  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$ .

To initiate differentiation, orbital cells were grown to confluence in 60 mm petri dishes. Differentiation was carried out as reported previously (8). Differentiation occurred over a 3-week period. Fibroblasts were harvested at various time points  $T_0$  (untreated),  $T_{11}$  (treatment day 11) and  $T_{22}$  (treatment day 22) for quantitation of TPO mRNA. To visualise the lipid accumulated in orbital fibroblasts, cultures were stained with Oil Red O (9). When required, orbital fibroblasts were harvested immediately for RNA extraction.

### Real-time PCR

Primers for real-time PCR were designed to amplify the region recognised by mAb 47 using Primer 3 software (Whitehead Institute/MIT Centre for Genome Research, Cambridge, MA, USA).

First-strand cDNA was synthesised using 1  $\mu\text{g}$  total RNA with oligo dT and Superscript II (Invitrogen). Following the first strand synthesis, a 294 bp TPO cDNA fragment (amplicon) was generated using forward primer position (82 525) 5'-GGAAGCAGAT-GAAGGCTCTG-3', reverse primer position (85 165) 5'-AGTGCACAAAGTCCCCATTC-3' and visualised with SBYR Green fluorogenic dye (manufacturer's conditions as described in Qiagen Quantitect kit, Qiagen). Results were normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using forward primer position (1067) 5'-GAAGATGGTGATGGGATTTC-3' and reverse primer position (1302) 5'-GAAGGTGAAGGTCGGAGT-3'.

The PCR cycling conditions used were 15 min at  $95^{\circ}\text{C}$  for initial denaturation, 45 cycles of 15 s at  $95^{\circ}\text{C}$ , 30 s at  $56^{\circ}\text{C}$  and 30 s at  $72^{\circ}\text{C}$ . Prostate/brain RNA was used as a negative control (NC), while thyroid RNA was the positive control. Serial dilution of cDNA (generated from thyroid mRNA) was included in every experiment. Specificity of the amplicon was verified by running a melting curve. Amplicons from the samples with the same melting curve as the positive control were considered positive. Real-time PCR reactions were carried out on a Rotor Gene 3000 (Corbett Research, NSW, Australia).

Each real-time PCR was carried out in duplicate and the threshold cycle values averaged. Calculations of relative gene expression were based on the differences in the threshold cycles. The fold change in expression between samples was calculated by:

$$\text{Fold change} = 2^{-\Delta\Delta C_t},$$

where

$$\Delta\Delta C_t = (C_{t \text{ TPO}} - C_{t \text{ GAPDH}})_{\text{sample}} - (C_{t \text{ TPO}} - C_{t \text{ GAPDH}})_{\text{prostate}}$$

### Protein extraction

The organic phase from Trizol extraction was saved for protein extraction. Extraction was carried out as described in manufacturer's protocol.

### SDS-PAGE and Western blot

Extracted proteins (15  $\mu\text{g}$ ) were heat denatured in electrophoresis sample buffer (62 mM Tris-Cl, pH 6.8, 0.2% glycerol, 2% SDS, 0.04%  $\beta$ -mercaptoethanol, 0.04% bromophenol blue) and loaded onto 8% Tris-glycine SDS-polyacrylamide gel. After electrophoresis on the gel, separated protein bands were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% Blotting Grade Blocker non-fat dry milk powder (BioRad) for 2 h at room temperature, on an orbital shaker. Western blot was carried out by incubating with TPO mAb47 diluted (1:2000) with 0.1% BSA overnight at  $4^{\circ}\text{C}$ , on a shaker. The membrane was washed three times with  $1\times\text{TBST}$  (Tris-buffered saline, 0.1% Tween 20) on an orbital shaker. After washing, the membrane was incubated with peroxidase-labelled anti-mouse IgG (1:3000) for 2 h on an orbital shaker at room temperature. Membrane was washed with  $1\times\text{TBST}$  three times for 5 min on a shaker. The blot was developed with ECL reagent (Amersham) according to manufacturer's instructions. mAb 47 was a kind gift from Dr Esteinne V, Faculte de Medecine Timone U555 INSERM, 27 av. Jean Moulin, Marseille, France.

## Statistical analysis

Statistical analysis of real-time quantitative PCR results was performed using Mann–Whitney test.

## Results

### Real-time PCR

Figure 1 illustrates the results of relative mRNA quantitation of various tissue samples.  $\log_{10}$  values used in the figure allowed graphical representation of spread of results obtained for fat tissue samples. Thyroid tissue was used as a positive control and it has the highest amount of TPO mRNA amongst the tissue studied. In contrast, prostate/brain tissue contains the least and serves as a NC. TPO primers chosen were approximately 2600 bp apart. The 294 bp amplicons detected were therefore mRNA transcripts and not amplification of genomic DNA. TPO mRNA was found in both GO and N orbital fat tissue. The median for TPO mRNA was higher in GO orbital fat tissue compared to tissue from normal subject. The TPO content in GO tissue was found to be significantly higher than in N tissue ( $P=0.009$ , Table 1). In order to determine the validity of the  $P$ -value statistics generated from our small sample group, *post hoc* sample size calculation was carried out. To detect the difference between the GO and N groups taking the estimate of median and percentage of s.d. from our data, with  $\alpha=0.05$  and power=80%, the sample size calculated was 1 in each arm. The levels of TPO mRNA found in all orbital fibroblast cultures, grown out from various primary tissue sources, were much lower than those levels found in fat tissue. Unlike fat tissue, no difference in TPO mRNA content was found between GO and N fibroblasts (Table 2).

We next investigated the relative abundance of TPO mRNA when fibroblasts were induced to accumulate lipid. Figure 1B illustrates orbital fibroblasts at time point  $T_0$ . Orbital fibroblasts are spindle shaped and do not stain red with Oil Red O. At time point  $T_{22}$ , orbital fibroblasts have been induced to accumulate lipid (Fig. 1C). mRNA expression at various time points,  $T_0$ ,  $T_{11}$  and  $T_{22}$  were compared against prostate tissue which served as NC. TPO mRNA expression in treated fibroblasts remained in the range observed for untreated GO and N fibroblasts throughout the treatment period (Table 3). From our experiments, TPO mRNA was not up-regulated when orbital fibroblasts were induced to accumulate lipid.

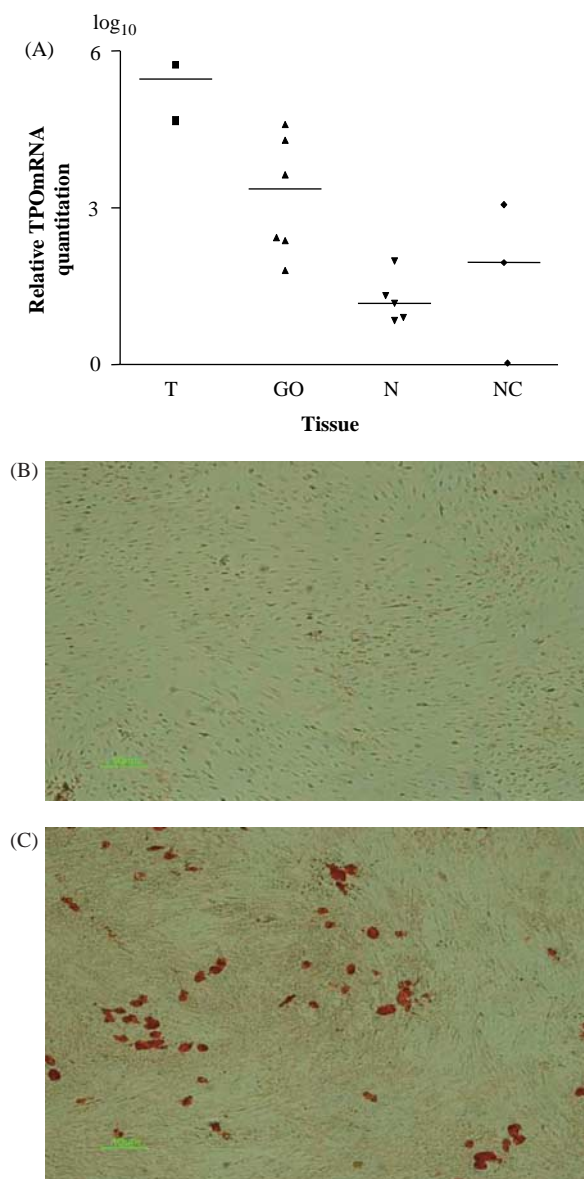
### Western blotting

Presence of TPO protein would validate our real-time results for the presence of TPO mRNA. By using the organic phase method of extraction, a 110-kDa band corresponding to TPO can be seen in T, N and GO specimens. Figure 2A is a Western blot carried out on

orbital fat tissue. Figures 2B and 2C are Western blots carried out on orbital fibroblast samples. The Western blots indicate the presence of translated TPO proteins in both tissue and fibroblasts.

## Discussion

The primary autoantigen in GO has long been sought and to date, the mouse model provides the strongest



**Figure 1** (A) Relative quantitation of TPO mRNA from various tissue sources. ■ thyroid tissue (T), ▲ Graves's ophthalmopathy orbital fat (GO), ◆ negative control (NC) and ▼ normal orbital fat (N). The horizontal line represents the median of the scattergram values for each group. (B) Oil Red O stain of orbital fibroblasts at  $T_0$  (day 0). Magnification  $4\times$ . (C) Oil Red O stain of orbital fibroblasts at  $T_{22}$  (day 22). Magnification  $4\times$ .

**Table 1** Statistical analysis of real time TPO mRNA quantitation data from various tissue sources.

Group	<i>n</i>	Median	Range (min, max.)	Comparison of groups	<i>P</i> -value*
T	2	308,868.6	(47,975.2, 569,762.0)	T vs. GO	0.046
GO	6	2,394.9	(66.7, 40,342.0)	GO vs. N	0.009
N	5	15.7	(7.0, 97.7)	T vs. N	0.053
NC	3	91.78	(1.0, 1226)	N vs. NC	0.786

T, thyroid tissue; GO, Graves' ophthalmopathy tissue; N, normal tissue; NC, normal control. \*Mann-Whitney test.

evidence that the TSH-R is the likely candidate (3, 4). Interactions between TSI and orbital TSH-R as the inciting event would be an attractive hypothesis. A significant proportion of patients with high TSI levels, however, do not have clinically evident disease. This suggests that co-factors may be necessary or that protective elements exist. While antibodies directed against TSH-R may be the first reaction in GO (10), there is accumulating evidence that immune reactions against other antigens play a significant role.

In a previous study, we reported that the absence of TPOAb was associated with a five-fold increased risk of GO (7). A high percentage of TPOAb negativity has also been reported in Dutch (11) and German patients with GO (12). These antibodies are frequently absent among patients with euthyroid GO as well as in patients in whom GO precedes hyperthyroidism. How TPOAb might confer protection is unknown. TPOAb have been associated with a diverse range of non-thyroidal conditions including depression (13), infertility (14), Hashimoto's encephalopathy (15) and improved prognosis in breast cancer (16). Autoantibodies to TPO are polyclonal and are largely directed to a region on the native antigen, termed the immunodominant region (17, 18). Because of its ability to fix complement, it remains worthwhile to study the role TPO plays in complement-mediated damage in autoimmune thyroid diseases. In the eye compartment, B cell may exhibit phenotype function and cell dynamics, distinct from B cells in other compartments (e.g. bone marrow, thyroid) (19, 20). Understanding the process responsible for autoimmune thyroid diseases will enable the development of immunospecific forms of therapy. While numerous studies have examined the role TPO autoantibodies play in the pathogenesis of autoimmune thyroid disease, its role is debated (21–23).

The human TPO gene is about 150 kb long and consists of 17 exons and 16 introns. The full-length transcript is 3048 bp codes for a molecule, which

consists of 933 amino acid residues. Increasing diversity of human thyroperoxidase generated by alternate splicing has been reported in normal thyroid tissues (24–26). The functional significance of the newly described spliced mRNA variants still remains to be elucidated. TPO's primary role is the coupling of iodine to tyrosine molecules in thyroglobulin. It is a membrane-bound enzyme located at the apical pole of thyrocytes.

Using standard real-time PCR amplification, we were able to detect a 294 bp amplicon in orbital fat and fibroblasts using TPO-specific primers. No evidence of TPO expression was found in prostate tissue.

We therefore sought to confirm our real-time PCR findings using Western blot analysis. The TPO mAb 47 recognizes a linear epitope (amino acid 713–721) in TPO as it has been shown to be reactive with most published TPO sequences. Our Western blots indicate the TPO mRNA transcripts are translated to TPO. While it may be tempting to use band intensity as an estimate of protein quantity, protein bands on Western blots are not indicative of quantity, mainly due to incomplete transfer of protein from polyacrylamide gel to membrane during the transfer from gel to membrane. Studies to quantitate the amount of TPO protein in orbital fat were not carried out, as the amount of tissue available was inadequate.

The extrathyroidal manifestations remain the most enigmatic aspect of GD. While we found significantly higher levels of TPO mRNA in orbital fat tissue of GO compared to N subjects, the pathogenesis is still unclear.

It should also be pointed out that fat obtained from blepharoplasty cases represents extraconal (anterior) orbital fat, while the fat from decompression surgery patients was obtained from the intraconal (posterior) space. While it is best to compare tissue from the same compartments, there was no observed difference in fat tissue accumulation between the two sites. To date, there are no publications to suggest differences in fat

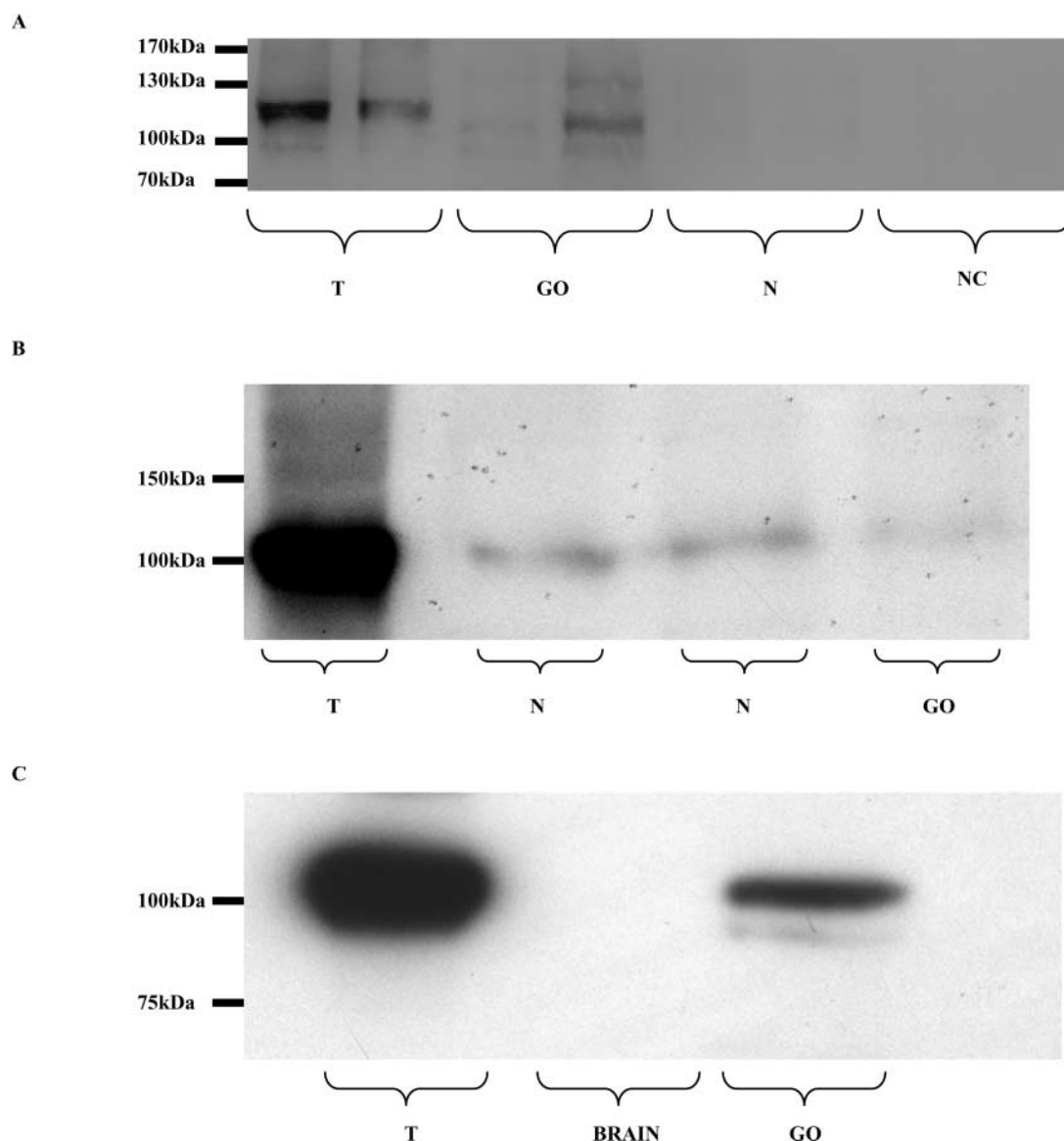
**Table 2** Relative quantitation of TPO mRNA in untreated orbital fibroblasts.

Group	<i>n</i>	Median	Range (min., max.)
GO fibroblasts	5	0.074	(0.01, 0.25)
N fibroblasts	5	0.078	(0.01, 0.21)

GO, Graves' ophthalmopathy tissue; N, normal.

**Table 3** Relative quantitation of TPO mRNA in orbital fibroblasts induced to accumulate lipid at time points; T<sub>0</sub> (day 0), T<sub>11</sub> (day 11) and T<sub>22</sub> (day 22).

Group	<i>n</i>	Median	Range (min., max.)
T <sub>0</sub>	4	0.025	(0.010, 0.060)
T <sub>11</sub>	4	0.015	(0.009, 0.04)
T <sub>22</sub>	4	0.015	(0.010, 0.03)



**Figure 2** (A) Presence of thyroid peroxidase protein in orbital fat tissue. Western blot for TPO protein (100–110 kDa) was performed as described with protein extracted from thyroid (T), Graves' ophthalmopathy (GO) tissue, normal (N) tissue and negative controls (NC). (B) Presence of thyroid peroxidase in orbital fibroblast. Western blot for TPO protein was performed as described with equal amounts of protein from thyroid (T), normal (N) and GO fibroblasts. (C) Presence of thyroid peroxidase in orbital fibroblast. Western blot for TPO protein was performed as described with equal amounts of protein from Graves' ophthalmopathy (GO) fibroblasts.

tissue accumulation at the intraconal (posterior) and extraconal (anterior) sites. A publication by Wolfram-Gabel R *et al.* found no septum between intra- and extraconal fat and histologic sections demonstrated that the adipose body of the orbit is a single anatomic entity (27). It was clear, however, from our work that in cultured fibroblasts, the degree of TPO mRNA expression was similar in the two groups.

From our real-time *in vitro* work with orbital fibroblasts, inducement of lipid accumulation alone did not affect TPO mRNA levels in both GO and N fibroblasts. This implies that the observed elevated

expression of TPO mRNA in GO fat is not linked with adipogenesis unlike the case with TSH-R (28, 29). When TSH secreted by the pituitary moves into the thyroid cell, it binds the TSH-R, which in turn signals the production of enzymes needed for thyroid hormone production such as TPO. It is certainly intriguing to observe an increase in TPO mRNA expression in GO eye fat. TPO has no obvious functional role in the eye orbit. Whether activation of TSH-R in the GO eye is a stimulus for increased TPO expression and the translated TPO protein function are questions that need to be addressed.

Although many studies have shown associations of immunoglobulins (particularly TSH-R) with thyroid eye disease, there is lack of evidence for a pathogenic role. We have shown previously that the presence of TPOAb is protective against GO. Much work has been carried out to map TPO autoantibody epitopes using B cells from thyroid biopsies. Given the data we have accumulated, perhaps, it would be worthwhile to examine the role TPO plays in modulating B and T cell response in the eye compartment.

## Acknowledgements

This work was supported by funds from National Medical Research Council grant no 0500/2000. Primer 3 software address is [www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi)

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Received 20 April 2006

Accepted 5 May 2006