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**THE NEONATAL Fc RECEPTOR IS EXPRESSED BY HUMAN RETINAL
PIGMENT EPITHELIAL CELLS AND IS DOWNREGULATED BY TUMOR
NECROSIS FACTOR-ALPHA**

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STATEMENTS

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Competing Interest: None declared

ABSTRACT

Background/aims: The neonatal Fc receptor (FcRn) protects IgG from catabolism, controls its transport between cell layers and extends its serum half-life. In the human vitreous IgG can be found but how vitreous IgG is processed or transported is currently unknown. The FcRn is a candidate molecule to regulate these processes. In this study we examined FcRn expression and regulation in human retinal pigment epithelium (RPE) cells.

Methods: In three primary RPE cell cultures (from three donor eyes) and in ARPE-19 cells FcRn and beta-2-microglobulin (β 2M) mRNA levels were determined by RQ-PCR. FcRn protein expression was analyzed by Western blot studies. Stimulation experiments were performed with recombinant human TNF- α and IFN- γ . HT-29, THP-1 and HeLa cell lines were used as FcRn positive and negative non-ocular controls, respectively.

Results: Expression of FcRn mRNA and protein was demonstrated in all three RPE cultures. After stimulation with TNF- α FcRn expression is down regulated in RPE cells and upregulated in HT-29 and THP-1 cells. IFN- γ has no effect on FcRn expression in RPE cells.

Conclusions: Human RPE cells express FcRn. The proinflammatory cytokine TNF- α downregulates FcRn expression. We speculate that the FcRn may play a pivotal role in the immune privilege of the human eye.

INTRODUCTION

The neonatal Fc receptor (FcRn) structurally resembles major histocompatibility complex (MHC) class I molecules: it comprises a heavy (α) chain - the glycosylated FcRn (40-45 kD) - and a beta-2-microglobulin (β 2M) light chain.¹ Unlike the other Fc γ receptors the FcRn does not act as a signaling receptor. Instead it plays a pivotal role in mediating transplacental immunoglobulin G (IgG) transport from mother to fetus and IgG transport from mothermilk to breast-fed infants, hence its name. Additionally, the FcRn mediates recycling and transport of albumin and IgG throughout life.² In vascular endothelial cells, the FcRn is mainly located within endosomes where it binds and subsequently directs IgG and albumin out of the lysosomal pathway. This prevents degradation of the molecules and promotes their recycling into the circulation, thereby prolonging the half-life of both proteins to approximately three weeks.^{3, 4} In line with this, β 2M- and FcRn-deficient mice have low levels of circulating IgG and albumin due to a shortened serum half-life. So far, only one case concerning a human counterpart of FcRn deficiency has been reported.^{3, 5, 6} FcRn can also direct IgG from apical to basal cell surfaces, thus transporting IgG through tissue.⁷ This is the main function of the receptor in lung epithelium and brain microvasculature.^{8, 9} FcRn expression is shown to be enhanced through activation of NF- κ B signaling by cytokines as tumor necrosis factor (TNF)- α and interferon (IFN)- γ ¹⁰, suggesting that its expression may be altered under inflammatory conditions.

Data on FcRn expression in the eye are rare, but has been studied in rat eye.^{11, 12} Kim *et al.* demonstrated abundant FcRn expression in various tissues of the rat eye but not retinal pigment epithelium (RPE) tissue and showed a role for FcRn in elimination of intravitreally administered IgG. Understanding of ocular IgG

metabolism is extremely important because of interpretation of ocular serology and its role in immune privilege. In addition, ocular FcRn expression and its regulation under inflammatory conditions could be of importance for determining outcome of local ocular monoclonal antibody therapies.

Currently there are no data concerning FcRn expression in the human eye.

Therefore, we conducted the present study to determine whether FcRn is expressed in human RPE as well as the regulation of its expression by TNF- α and IFN- γ and compared this to its expression and regulation in other non-ocular cell types.

MATERIALS AND METHODS:

Cell lines and culture conditions

The human RPE cell line ARPE-19 was obtained from ATCC (Manassas, VA, USA) and grown in a 1:1 medium mixture of DMEM (BioWhittaker, Verviers, Belgium) and HAM's F-12 with L-Glutamine (Gibco, Paisley, UK), 10% heat inactivated fetal calf serum (FCS; BioWhittaker) and penicillin/streptomycin (BioWhittaker), hereafter referred to as RPE medium. Three primary RPE cell lines (designated OZR1, OZR5 and RPE172 (the latter kindly provided by dr. Verjans, Erasmus MC, Rotterdam, The Netherlands)) were established from three donor eyes harvested for cornea transplantation and grown in RPE medium. The cultured RPE cells were tested positive by PCR on cDNA for CRALBP (cellular retinaldehyde binding protein, an RPE specific marker), positive for keratin (epithelial marker) and negative for vimentin (mesenchymal marker) and Von Willebrand factor (endothelial marker). All primary RPE cells used for this studies were between passage 6 and 10. ARPE-19 cells used for studies were between passage 25 and 39. The human colon adenocarcinoma grade II (intestinal epithelial) cell line HT-29 (kindly provided by dr. Ron Smits, Erasmus MC) and human cervical carcinoma (epithelial) HeLa cell line (ATCC) were maintained in DMEM (BioWhittaker) supplemented with FCS (10%) and penicillin/streptomycin. The human acute monocytic leukemia THP-1 cell line was maintained in RPMI (Gibco) supplemented with FCS (10%) and penicillin/streptomycin. All cell cultures were grown in a humidified atmosphere of 5.0% CO₂ at 37°C.

Transfection RPE cells

Cells were seeded at 5×10^5 cells/well into six-well plates in RPE medium containing 10% FCS and allowed to adhere overnight. Cells were transfected with a construct that contains a short hairpin sequence that specifically targets FCGRT mRNA (pSM2-shRNAmir; Open Biosystems, Huntsville, AL, USA). Heretoo, both polyethylenimine (PEI:Polysciences Inc, Warrington, PA, USA) and the short hairpin construct each were diluted in 25 μ l PBS, subsequently added together in a PEI:DNA ratio of 3.5:1 and incubated for 30 minutes at RT. The PEI:DNA mixture was gently added to the cells and gently mixed. After overnight incubation in RPE medium containing 10% FCS the cells were washed twice with PBS to remove dead cells. Approximately 25% of the cells survived the transfection procedure. In order to establish specific selection of transfected cells the medium was changed into RPE medium containing 10% FCS and 1 ng/ml puromycin (Enzo Life Sciences BVBA, Zandhoven, Belgium). Every 2-3 days cells were provided with fresh medium containing 1 ng/ml puromycin.

Stimulation Assay

Cells were seeded at 5×10^5 cells/well into six-well plates in designated medium containing 1% FCS and allowed to adhere overnight. They were then incubated in the presence or absence of 50 ng/ml recombinant human TNF- α (R&D Systems, Oxfordshire, UK) or 25 ng/ml recombinant human IFN- γ (R&D Systems) for the indicated time points, in medium containing 1% FCS. After stimulation supernatants were removed and the cells were used for further analyses. The cytokine concentrations used affected neither cell viability nor cell adherence.

Real-time quantitative PCR (RQ-PCR)

RQ-PCR was performed as previously described.¹³ In brief, RNA was isolated using Isol-RNA Lysis Reagent (5 Prime; Hamburg, Germany) according to the manufacturer's instructions. Subsequently RNA was reversed transcribed into cDNA. *FCGRT* (encoding FcRn) transcript levels were determined using a commercial available Taqman Gene Expression assay (Applied Biosystems; Foster City, CA, USA). *B2M* (encoding β 2M) transcript levels were determined using the following primer-probe combination, forward primer; TGCTCGCGCTACTCTCTCTTT, reverse primer; TTCAATGTCCGATGGATGAAAC, probe; TCAGGTTTACTCACGTCATCCAGCAGAGAAT. *IL-8* transcript levels were determined using the following primer-probe combination, forward primer; GGCCGTGGCTCTCTTGG, reverse primer; GGGTGGAAAGGTTTGGAGTATGT, probe; TGTGTGAAGGTGCAGTTTTGCCAAGGA. Transcript levels were determined using an Applied Biosystems 7900 PCR machine (Applied Biosystems) and quantified by normalization to the control gene Abelson as described previously¹³.

Western blotting analysis

Western blotting analysis was performed as previously.¹⁴ In brief, samples were blotted using standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) procedures. Subsequently, Western blots were stained using rabbit anti-human FcRn antiserum (kindly provided by prof. Zhu University of Maryland, USA) and secondary swine anti-rabbit immunoglobulines/HRP (Dako, Heverlee, Belgium). As loading control, the blots were stained using mouse monoclonal

antibodies against β -Actin (Abcam, Cambridge, UK) and secondary mouse anti-rabbit immunoglobulins/HRP (Dako).

Statistical analysis

Analysis of relative FcRn mRNA expression was performed using the paired *t*-test. A value of $p < 0.05$ was considered significant. In the figures a single asterix indicates a statistically significant difference at $p < 0.05$.

RESULTS

FcRn mRNA expression

All primary RPE cells as well as the cell line ARPE-19 expressed FcRn mRNA to the same extent (Figure 1A). In accordance with literature¹⁰ abundant FcRn mRNA was detected in the cell lines HT-29 and THP-1, which was higher than that observed in the tested human RPE cells. HeLa cells did not express FcRn mRNA, again conform literature¹⁰. All tested cells did express β 2M mRNA, albeit at highly variable levels (Figure 1B).

FcRn protein expression

A clear FcRn protein band of ~45 kDa was detected in all primary RPE cells as well as in the ARPE-19 cells and the HT-29 and THP-1 cells while it was absent in the HeLa cells (Figure 2A). No clear correlation was observed between the FcRn protein levels and the FcRn mRNA transcript levels. Nevertheless, these data indicate that human RPE cells do express FcRn protein. This is further confirmed by the fact that FcRn protein expression was reduced in primary RPE cells transfected with a FCGRT mRNA targeting shRNA construct (Figure 2B).

The effect of TNF- α and IFN- γ on FcRn mRNA expression

TNF- α downregulated FcRn mRNA expression in ARPE-19 cells. There was a trend towards a dose dependent manner with maximal downregulation at 50 ng/ml TNF- α (Figure 3A). Also TNF- α concentrations below 10 ng/ml downregulated FcRn mRNA expression (data not shown), however this was not as robust as the higher concentrations tested. Therefore, a TNF- α concentration of 50 ng/ml was used for further studies. TNF- α (50 ng/ml) downregulated FcRn mRNA expression in ARPE-

ARPE-19 cells significantly in a time-dependent manner over 24 hours (measured at 0,1,3,6 and 24 hours; Figure 3B). TNF- α treated OZR1 and OZR5 showed a similar trend towards downregulation of FcRn mRNA after 24 hours, although this was not significant (Figure 4A). IL-8 is a cytokine that increases upon TNF- α stimulation. In contrast to FcRn mRNA expression, TNF- α significantly enhanced IL-8 mRNA expression in ARPE-19 cells (Figure 3B) as well as in OZR1 and OZR5 cells (Figure 4A). IFN- γ (25 ng/ml), another key mediator in the inflammatory response, did not affect FcRn mRNA expression in ARPE-19 cells while it modestly but non-significantly downregulated IL-8 mRNA expression (Figure 3B).

The effect of TNF- α on FcRn protein expression

Whole cell lysates obtained from ARPE-19, OZR1, OZR5 and HT-29 cells after 24 hr TNF- α (50 ng/ml) stimulation revealed a reduction of FcRn protein expression in ARPE-19, OZR1 and OZR5 cells. In contrast, TNF- α enhanced FcRn protein expression in HT-29 cells (Figures 4B, C).

DISCUSSION

FcRn plays a crucial role in promoting IgG transport in various organs as well as preventing its degradation within the endosome. FcRn is highly expressed in vascular endothelium and is supposed to play a major role in prolonging the serum half-life of IgG. Other cell types in which FcRn is expressed are antigen presenting cells, monocytes, epithelia of skin, gut, kidney, brain and lung tissue.^{8, 9, 15-17}

In this study we demonstrate for the first time that FcRn is expressed in human RPE cells and that its expression is unexpectedly downregulated by the pro-inflammatory cytokine TNF- α .

FcRn expression in human RPE cells is discordant with the results obtained from rat eyes by Kim *et al.* They showed FcRn expression in many rat ocular tissues, including the retina, but not in the retinal pigment epithelial cells.¹¹ Possibly this discrepancy is due to species differences. Alternatively, methodological differences may account for the opposite results. We performed RQ-PCR analysis on RPE cell cultures instead of total RNA obtained from a mixture of RPE and choroid tissue. It has been recognized previously that impurity of cell populations, i.e. the presence of non-target cells within the target cell population, highly influences the reliability of gene expression levels.¹⁸ RQ-PCR, protein analysis and micro-array (unpublished data) expression profiles clearly show the presence of FcRn in human RPE cells, however modest. Therefore, the FcRn protein level in RPE might be too low for detection by immunohistochemical staining. The fact that Kim *et al.* did find FcRn expression in various rat-ocular tissues, including ciliary body, iris and optic nerve bundle, calls for similar immunohistochemical analysis and in situ hybridization

studies in primary human ocular tissue as well as physiologic studies using various ocular cell types.

Stimulation tests with the proinflammatory cytokine TNF- α showed a significant dose and time dependent upregulation of FcRn in the intestinal epithelial cell line HT-29, concordant with the findings of Liu *et al.*¹⁰ They described upregulation of FcRn by TNF- α and IL-1 β in a dose-dependent manner in monocytes (THP-1) and HT-29. It was also shown that - at least in monocytes - FcRn expression is regulated via the NF- κ B signaling pathway. Contrary to the effect of TNF- α on these non-ocular cell types we found that TNF- α induced a significant dose and time dependent downregulation of FcRn expression in ARPE-19 cells both on mRNA and protein level. Moreover, also in primary RPE cultures a trend to suppression of FcRn expression was found upon TNF- α stimulation. In order to rule out possible artifacts we also measured IL-8 mRNA expression in both ARPE-19 and HT-29 cells upon stimulation with TNF- α . IL-8 is a proinflammatory cytokine (neutrophil chemoattractant) known to be upregulated by TNF- α in an NF- κ B dependent manner. In both cell cultures IL-8 mRNA expression was upregulated by TNF- α , as expected. Therefore, the downregulation of FcRn expression in RPE by TNF- α suggests that the mechanism that regulates FcRn expression in RPE cells differs from that in the non-ocular cell types tested.

TNF- α is an important key mediator of intra-ocular inflammation. Increased intra-ocular TNF- α levels are found during infectious and non-infectious ocular inflammatory diseases such as uveitis¹⁹ and TNF- α is also overexpressed in neovascular membranes in ARMD.²⁰ Moreover, TNF- α blocking therapy is a

therapeutic tool in the treatment of non-infectious uveitis.^{21, 22} Based on our findings the level of TNF- α expression may be expected to affect the intraocular immunoglobulin metabolism. Until now, it is unknown if FcRn is responsible for transcellular transport of IgG through the RPE cell to the choroid (like in the gut) or apical secretion of IgG back in the ocular fluid comparable with the action in endothelium. We speculate that downregulation of FcRn contributes to the regulation of TNF- α driven intraocular inflammation by changing the intra-ocular IgG levels. In case of decreased apical secretion (as in endothelium), deterioration of FcRn function may result in lower intraocular IgG levels tapering inflammatory activity and may contribute to the immune privilege in eye.

Clinically, specific IgG antimicrobial antibodies are used in the diagnosis of intra-ocular infections. The so-called Goldmann-Witmer coefficient (GWC) is a diagnostic tool for several ocular infections like toxoplasmosis²³. Changes in intraocular IgG degradation may directly affect the GWC, especially when TNF- α levels are increased. Besides the metabolism of natural antimicrobial antibodies for diagnostics, the metabolism of monoclonal therapeutic antibodies is of increasing importance. Vascular endothelial growth factor (VEGF) blocking antibodies are nowadays the first choice of treatment in neovascular ARMD. It is supposed that the ocular pharmacology of these therapeutic antibodies depends also on the FcRn expression. This may be of special importance in clinically refractory patients.²⁴ Non-responsiveness may be related to the level of FcRn expression.

In this study we show that human primary RPE cultures and ARPE-19 express FcRn on protein level. Unexpectedly, FcRn in RPE is downregulated by TNF- α which is

contrary to earlier findings in other cell types suggesting an other regulatory pathway in RPE cells. We speculate that FcRn is involved in the regulation of intraocular inflammation, however, more studies are warranted to determine the exact role of FcRn in ocular inflammatory diseases.

ACKNOWLEDGEMENTS

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FIGURES AND LEGENDS

Figure 1

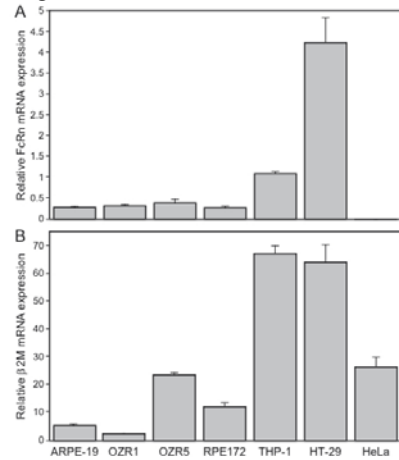


Figure 1.

Cells were cultured in medium supplemented with 10% FCS. FcRn and β 2M mRNA transcript levels were determined using RQ-PCR and their expression levels were expressed relative to the household gene ABL. A. The primary human RPE cells OZR1, OZR5 and RPE172 as well as the human RPE cell line ARPE-19 expressed FcRn mRNA to the same extend. FcRn mRNA was abundantly expressed by the human intestinal epithelial cell line HT-29 and the human acute leukemia cell line THP-1. The human cervical cancer cell line HeLa did not express FcRn mRNA. B. All tested cells expressed β 2M mRNA, albeit at highly variable levels.

Figure 2

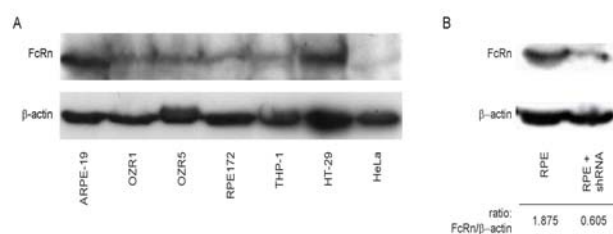


Figure 2

A. Cells were cultured in medium supplemented with 10% FCS. FcRn protein expression was determined by Western blot using anti-human FcRn antiserum (kindly provided by prof. Zhu University of Maryland, USA). As loading control, the blots were stained using a mouse monoclonal antibody against β -Actin. A clear FcRn protein band of ~45 kDa was detected in all primary RPE cells and ARPE-19 cells as well as the HT-29 and THP-1 cells, while it was absent in the HeLa cells. B. Primary RPE cells (OZR5) were transfected with a construct that contains a short hairpin sequence that specifically targets FCGRT mRNA (indicated as shRNA). In order to establish specific selection of transfected cells the medium was changed into RPE medium containing 10% FCS and 1 ng/ml puromycin. FcRn and β -Actin protein expression was determined and densitometric analysis was performed. FcRn protein expression was substantially decreased in the transfected primary RPE cells.

Figure 3

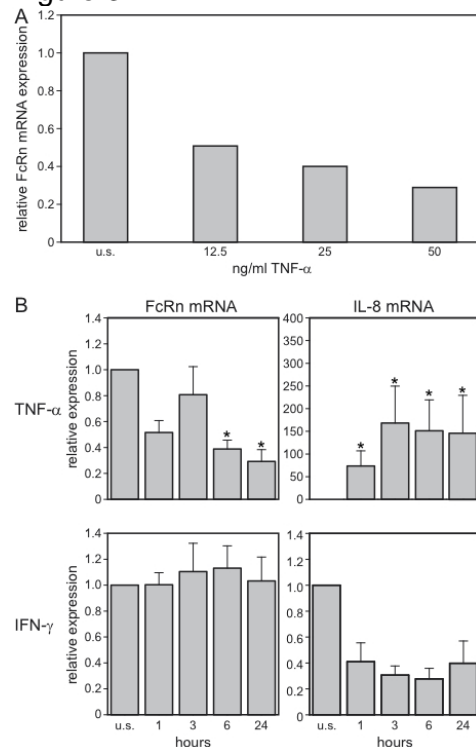


Figure 3.

ARPE-19 cells were seeded at 5×10^5 cells/well into six-well plates in medium containing 1% FCS and allowed to adhere overnight. A. The cells were stimulated with indicated concentrations of TNF- α for 24 hrs. FcRn mRNA transcript levels were determined using RQ-PCR and the expression level was expressed relative to the household gene ABL. TNF- α downregulated FcRn mRNA expression in ARPE-19 cells and there was a trend towards a dose dependent manner with maximal downregulation at 50 ng/ml. B. The cells were stimulated with TNF- α (50 ng/ml) or IFN- γ (25 ng/ml) for indicated time points. FcRn and IL-8 mRNA transcript levels were determined using RQ-PCR and their expression levels were expressed relative to the household gene ABL. TNF- α (50 ng/ml) downregulated FcRn mRNA expression in ARPE-19 cells significantly in a time-dependent manner over 24 hours (measured at

0,1,3,6 and 24 hours. In contrast, TNF- α increased IL-8 mRNA expression in ARPE-19 cells. IFN- γ (25 ng/ml), another key mediator in the inflammatory response, did not affect FcRn mRNA expression in ARPE-19 cells while it modestly but non-significantly downregulated IL-8 mRNA expression. * Indicates statistical significance with a p-value <0.05. U.S. means unstimulated.

Figure 4

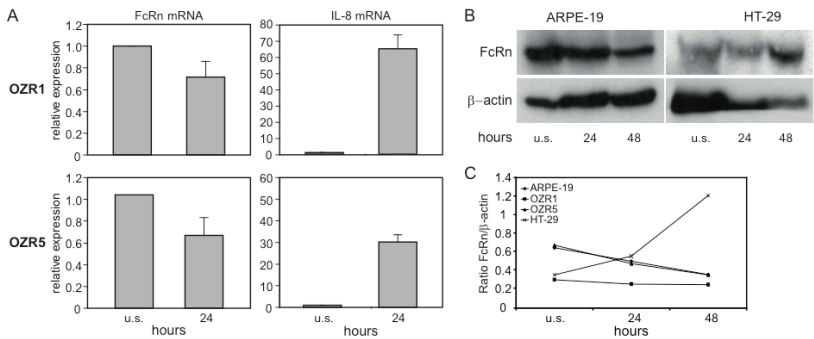
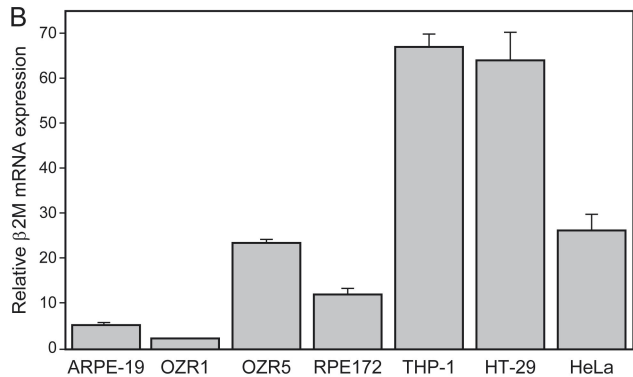
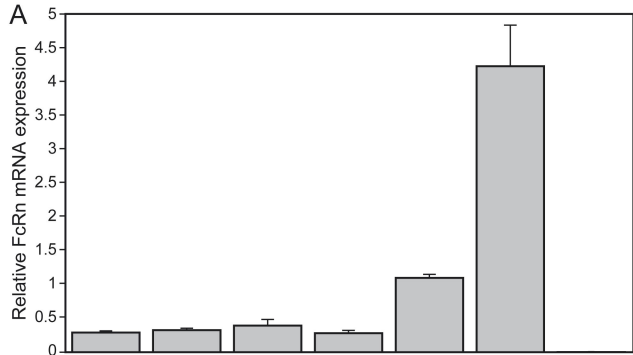


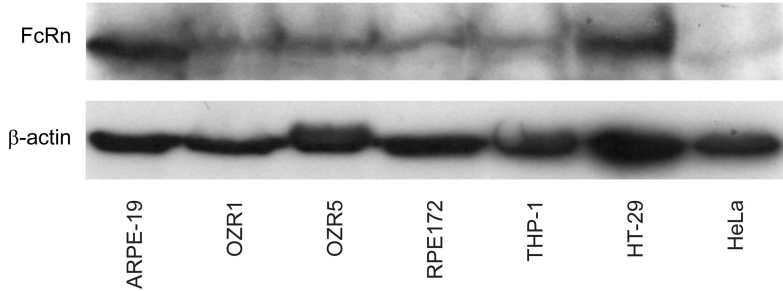
Figure 4

A. The primary human RPE cell lines OZR1 and OZR5 were stimulated for 24 hrs with TNF- α (50 ng/ml) in medium supplemented with 1% FCS. FcRn and IL-8 mRNA expression levels were determined by RQ-PCR and their expression levels were expressed relative to the household gene ABL. TNF- α down regulated FcRn mRNA expression while it increased IL-8 mRNA expression levels. The primary human RPE cells lines OZR1, OZR5, the human RPE cell line ARPE-19 and the human intestinal epithelial cell line HT-29 were stimulated for 24 and 48 hrs with TNF- α (50 ng/ml) in medium supplemented with 1% FCS. FcRn protein expression was determined by Western blot using anti-human FcRn antiserum (kindly provided by prof. Zhu University of Maryland, USA). As loading control, the blots were stained using a

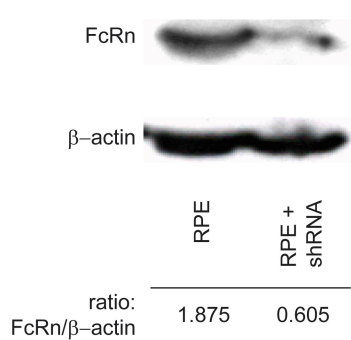
mouse monoclonal antibody against β -Actin. B. Shows representative Western blots for ARPE-19 and HT-29. C. Densitometric analysis was performed and the ratio of FcRn/ β -Actin was determined for OZR1, OZR5, ARPE-19 and HT-29 and demonstrates that TNF- α consistently down regulates FcRn protein expression by human RPE while it enhances the FcRn protein expression by HT-29. U.S. means unstimulated.

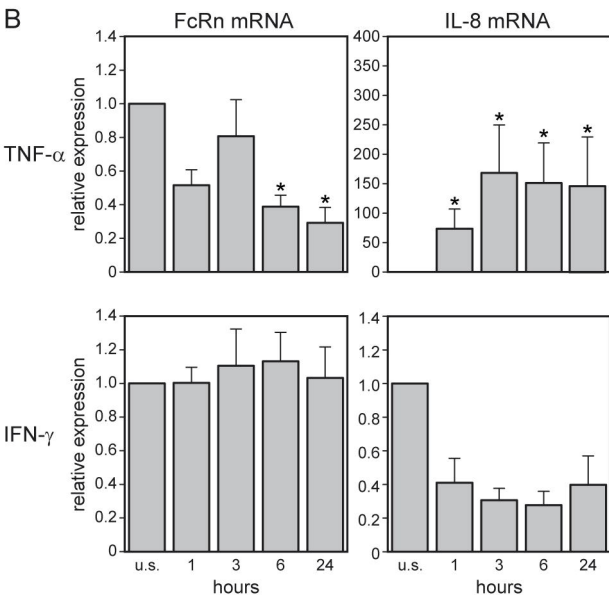
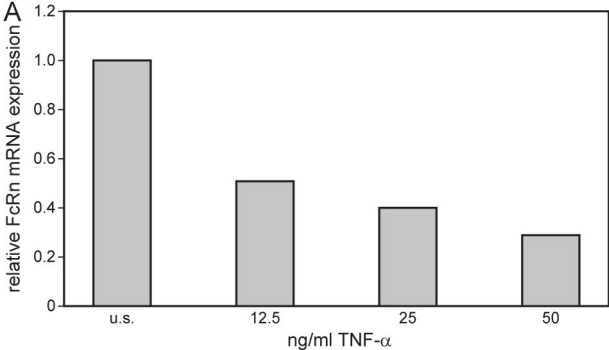


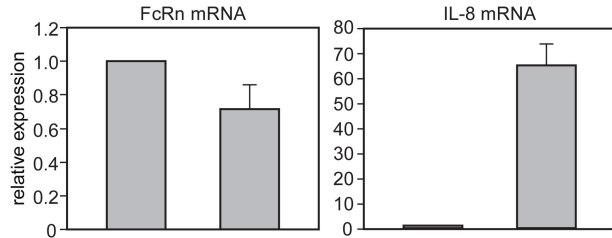
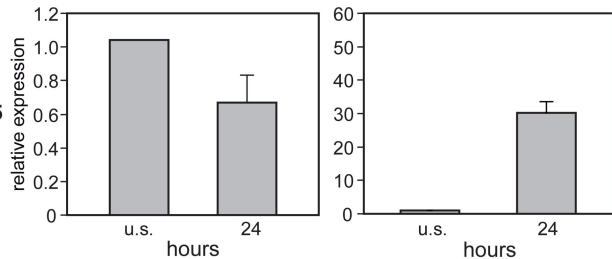
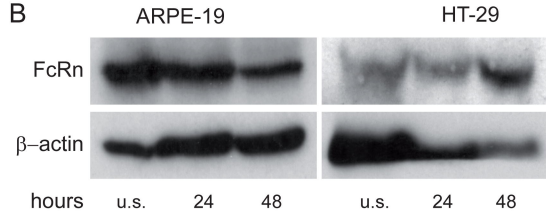
A



B





A**OZR1****OZR5****B****C**