Interplay between IDO1 and iNOS in human retinal pigment epithelial cells

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Abstract

Human retinal pigment epithelial (hRPE) cells form a selectively permeable monolayer between the neural retina and the highly permeable choroidal vessels. Thus, hRPE cells bear important regulatory functions and are potential targets of pathogens in vivo. Endogenous bacterial endophthalmitis (EBE) is frequently caused by infections with the Gram-positive bacterium Staphylococcus aureus (S. aureus). Upon microbial infection, interferon gamma (IFN-γ), a major cytokine of the adaptive immune response, induces a broad spectrum of effector molecules, such as the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase-1 (IDO1). We stimulated human RPE (hRPE) cells in vitro with proinflammatory cytokines and analyzed the expression levels and enzymatic activities of IDO1 and inducible nitric oxide synthase (iNOS), another antimicrobial effector molecule. The antimicrobial capacity was analyzed in infection experiments using S. aureus and Toxoplasma gondii (T. gondii). Our aim was to characterize the particular importance of IDO1 and iNOS during EBE. We found that an IFN-γ stimulation of hPRE cells induced the expression of IDO1, which inhibited the growth of T. gondii and S. aureus. A co-stimulation with IFN-γ, interleukin-1 beta, and tumor necrosis factor alpha induced a strong expression of iNOS. The iNOS-derived nitric oxide production was dependent on cell-culture conditions; however, it could not cause antimicrobial effects. iNOS did not act synergistically with IDO1. Instead, iNOS activity inhibited IDO1-mediated tryptophan degradation and bacteriostasis. This effect was reversible by the addition of the iNOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine.

In conclusion, iNOS mediates anti-inflammatory effects in hRPE cells stimulated with high amounts of IFN-γ together with tumor necrosis factor alpha and Interleukin-1 beta and prevents potential IDO1-dependent tissue damage.

Keywords Endogenous bacterial endophthalmitis · Indoleamine 2,3-dioxygenase-1 · IDO1 · Inducible nitric oxide synthase · iNOS · Retinal pigment epithelial cells · Staphylococcus aureus · Toxoplasma gondii

Introduction

Endophthalmitis and infectious retinitis are sight-threatening diseases, which are characterized by infections and subsequent proinflammatory immune responses. The strict control of proinflammatory responses is required to minimize tissue damage [1, 2]. The immune response against pathogens is dependent on the production of interferon gamma (IFN-γ), which is mainly derived from NK cells and T cells [3]. However, the eye is an immune-privileged organ and the vulnerable neurosensory cells have to be protected from damage by proinflammatory effector cells. Retinal pigment epithelial (RPE) cells are able to regulate T cell responses in the eye [2, 4] and are well known effector cells against Toxoplasma gondii (T. gondii) [5, 6] and cytomegalovirus (CMV) [7, 8].
Both cause severe diseases in immunocompromised patients, for example, retinitis, pneumonia, and encephalitis.

An exogenous endophthalmitis frequently occurs after penetrating ocular traumata and is a serious complication after eye surgery. In this case, environmental pathogens such as bacteria or fungi invade the eye and cause an infection. Despite the fact that exogenous endophthalmitis is a harmful infection, it is usually no source of bacteremia [1]. Post-surgery infections mostly occur due to coagulase negative staphylococci and propionibacteria, while the majority of post-traumatic exogenous endophthalmitis cases are caused by Bacillus cereus [1].

In contrast to an exogenous endophthalmitis, endogenous bacterial endophthalmitis (EBE) is a rare infection, but is often associated with poor visual outcome and can be life threatening. EBE is usually a complication of a systemic infection. During a systemic infection, characterized by sepsis (positive blood culture in 58%) and fever (in 74%), bacteria are distributed via the blood stream throughout the body. Bacteria that reach the eye can cross the blood–retinal barrier and establish EBE. Staphylococcus aureus (S. aureus), streptococci, and enterobacteria are the most frequently found causative agents of EBE [9, 10]. Infection of indwelling catheters or prosthetic devices with S. aureus is associated with bacterial endocarditis and is the major cause of EBE in Europe and the United States of America. Diabetes mellitus and immunosuppression are additional risk factors for a systemic bacterial infection and thus EBE [9, 10].

Pathogens causing EBE have to pass the blood–ocular barrier, namely, the blood–aqueous barrier and the blood–retinal barrier. The blood–aqueous barrier consists of endothelial cells of the retina capillary vessels and is in direct contact with pericapillary glial cells. The outer blood–retinal barrier consists of the RPE cells and the underlying Bruch’s membrane and separates the neuronal retina from the fenestrated choriocapillaris. The barrier function of RPE cells is accomplished by tight junctions. The flow of nutrients and macromolecules from the blood to the retina is thereby regulated and the entry of pathogens and immune cells to the eye is restricted [11]. In addition, RPE cells are immune competent. They produce several cytokines including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα), can activate T cells and are able to restrict the growth of pathogens [12]. The antimicrobial capacity of the RPE has been analyzed by several groups. For example, RPE cells are susceptible to different viruses such as Herpes simplex virus, cytomegalovirus, adenovirus types 1 and 7, and measles virus [13]. Furthermore, IFN-γ activated RPE cells can restrict the growth of T. gondii and CMV, which are both capable to induce retinitis. In general, indoleamine 2,3-dioxygenase-1 (IDO1) and inducible nitric oxide synthase (iNOS) were identified as prominent IFN-γ induced effector mechanisms against CMV [7].

In contrast to the antiviral effects mediated by iNOS in murine cells, Bodagi et al. found that iNOS is not important in the defense of hRPE cells against CMV [7]. Instead, IFN-γ induced IDO1 served as an antiviral defense mechanism in native hRPE cells. In accordance Nagineni et al. described, the IFN-γ stimulation of primary hRPE cells induced IDO1, which inhibited the growth of T. gondii [6].

Thus, IDO1- and iNOS-mediated effects against T. gondii and CMV have been studied intensively. However, relatively few information is available regarding the RPE cell-mediated antimicrobial defense against bacteria, especially with the focus on S. aureus causing EBE. Healthy and diabetic C57BL/6 mice develop EBE after intravenous infection with S. aureus [23]. An in vitro infection with S. aureus results in disruption of the tight junctions of hRPE cells [10]. Furthermore, hRPE cells can phagocytose S. aureus and present staphylococcal superantigens to T cells [24, 25]. The defense mechanisms against S. aureus mediated by the RPE are unclear; however, respiratory burst is likely not involved [24]. Therefore, we aimed to analyze the role of iNOS and IDO1 in the defense against S. aureus in hRPE cells.

**Materials and methods**

**Cells, media, and reagents**

Human retinal pigment epithelial (hRPE) cells (ARPE-19, ATCC, Wesel, Germany), as well as human foreskin fibroblasts (HFF) (ATCC, Wesel, Germany) and human glioblastoma cells (86HG39) were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Grand Island, USA), supplemented with 5% heat-inactivated fetal calf serum (FCS). Cells were cultured in culture flasks (Costar, Cambridge, USA) in a humidified incubator (37 °C, 5% CO₂). Cells were passaged weekly in 1:10 ratios using trypsin/EDTA (Gibco, Grand Island, USA). Hypoxia growth experiments were carried out using a HERAcell 150 I CO₂ incubator (Thermo Fisher Scientific, Carlsbad, USA) or the Anoxomat™ system (Mart Microbiology B. V., Drachten, Netherlands) with 1–10% O₂ and 5% CO₂. Mycoplasma
contamination was regularly excluded by culture methods and PCR.

A tryptophan-auxotrophic *Staphylococcus aureus* isolate was used. This *S. aureus* isolate was obtained from a routine diagnostic specimen [26]. *S. aureus* was grown on brain heart infusion agar containing 5% sheep blood (Difco, Hamburg, Germany) at 37 °C in 5% CO₂-enriched atmosphere overnight.

*Toxoplasma gondii* tachyzoites (ME49 strain, ATCC, Wesel, Germany) were maintained in HFF in IMDM containing 5% FCS. Extracellular tachyzoites were harvested from culture supernatants by centrifugation, resuspended in tryptophan free RPMI, counted and used as indicated for infection experiments.

Stimulation of hRPE cells

For infection experiments as well as for the determination of IDO1 and iNOS activity, hRPE cells were seeded in 96-well plates (3 × 10⁴ cells per well) or 24-well plates (3 × 10⁴, 1 × 10⁵, 3 × 10⁵, or 5 × 10⁵ cells per well) and stimulated with recombinant human IFN-γ (0–2000 U/ml) (R&D Systems, Minneapolis, USA) and/or recombinant human IL-1β (100 U/ml) (R&D Systems, Minneapolis, USA) and/or recombinant human TNFα (100 U/ml) (R&D systems, Minneapolis, USA) for 72 h in a humidified incubator (37 °C, 5% CO₂). In the iNOS activity kinetic experiments, supernatants were harvested after 0, 24, 48, 72, or 96 h and analyzed with the nitric oxide assay. Subsequent infection experiments as well as IDO1 and iNOS activity measurements were performed with pre-stimulated cells.

In some experimental groups, the NOS inhibitor N⁵-G-monomethyl-L-arginine (N⁵MMMA) (Merck, Darmstadt, Germany) (100 µg/ml) or the competitive IDO inhibitor 1-L-methyl-tryptophan (1-MT) (Sigma-Aldrich, St. Louis, USA) (1.5 mM) were used.

Table 1  Real-time PCR primers for the detection of human beta-actin, nitric oxide synthase, and indoleamine 2,3-dioxygenase-1 transcripts

| Gene of interest | Gene ID | Probe | Sequence [5′→3′] | Length |
|------------------|---------|-------|-----------------|--------|
| β-Actin          | ENSG000000075624 | #64   | ccacccgagtagatga | 18     |
| iNOS (inducible, NOS2) | ENSG00000007171 | #16   | ttctcttgctgtctt | 23     |
| eNOS (endothelial, NOS3) | ENSG00000164867 | #67   | gcagtcacaaggcatctg | 19     |
| nNOS (neuronal, NOS1) | ENSG00000089250 | #39   | ttggaatgctgctcctc | 20     |
| IDO1             | ENSG00000131203 | #9    | tgcctgctgctgctg | 21     |
| IDO2             | ENSG00000188676 | #4    | ttgcctgctgctgctg | 21     |

Kynurenine assay

The enzymatic activity of IDO1 directly correlates with the concentration of kynurenine in supernatants of tissue culture cells, and therefore, the measurement of kynurenine can be used to determine IDO1 activity [27]. The kynurenine content of supernatants from unstimulated or stimulated cells was analyzed using 4-(dimethylamino) benzaldehyde (Ehrlich’s reagent) as described before [27].

Nitric oxide assay

Nitrite accumulation in the supernatant of cultured cells was used as an indicator of nitric oxide production and was determined by the Griess reaction [28]. In brief, 100 µl cell-culture supernatant of pre-stimulated hRPE cells (for 72 h) was mixed with 100 µl Griess Reagent (0.1% N-(1-naphthyl) ethylenediamine in purified water and 2.5% sulfonamide in 15% hydrochloric acid in a 1:1 ratio; Merck, Darmstadt, Germany). After an incubation time of 15 min, the absorbance was measured at 540 nm (TECAN Sunrise microplate reader, Cailsheim, Germany). The amount of nitrite accumulation was determined using a calibration curve of graded concentrations of sodium nitrite.

Determination of bacterial growth

A 24-h-old *S. aureus* colony was picked, resuspended in PBS (Gibco, Grand Island, USA), and serial diluted. After pre-stimulation with cytokines for 72 h, hRPE cells (cultured in 96 flat-bottom culture plates) were infected with 10 µl of the bacterial dilution containing 10–100 colony forming units (cfu). Alternatively, conditioned medium from stimulated hRPE cultures (harvested 72 h after cytokine stimulation) was inoculated with the same amount of bacteria. Infected cultures were incubated in a humidified incubator (37 °C, 5% CO₂).
CO₂) for 16 h. Bacterial growth was monitored by measuring the optical density of resuspended cultures at 620 nm (TECAN Sunrise microplate reader, Crailsheim, Germany) [26].

**Determination of parasite growth**

After pre-stimulation for 72 h, hRPE cells were infected with 2 × 10⁴ *T. gondii* tachyzoites per well. *T. gondii* growth was determined by the ³H-uracil incorporation method as described before [26, 29]. In brief, ³H-uracil (0.33 μCi per
Activity of indoleamine 2,3-dioxygenase (IDO) in human retinal pigment epithelial (hRPE) cells. a Relative expression of IDO1 and IDO2 in unstimulated (medium control) or IFN-γ (500 U/ml for 24 h) stimulated hRPE cells, detected by real-time PCR. b Exemplary western blot analysis showing IDO1 negative and positive control (human glioblastoma lysates and IDO1 and β-actin protein in unstimulated and IFN-γ (500 U/ml for 24 h) stimulated hRPE cells. c 3 × 10⁴ hRPE cells were stimulated in 96-well plates with indicated concentrations of human IFN-γ (0–2000 U/ml) in the presence of 1-tryptophan (L-tryp; 100 µg/ml). After 72 h, the cell-culture supernatants were harvested and the kynurenine content was determined by the use of Ehrlich’s reagent. (d + e) 3 × 10⁴ hRPE cells pre-stimulated with IFN-γ for 72 h. d Pre-stimulated hRPE cell cultures were infected with Toxoplasma gondii (1 × 10⁵ ME49 tachyzoites/well) or (e) Staphylococcus aureus (10–100 cfu/well) without additional 1-tryptophan (medium control) or with 1-tryptophan (100 µg/ml) supplementation. Parasite growth was determined via the 3H-Uracil method and the bacterial growth was detected by the optical density at 620 nm (OD₆₂₀ₙₚ). f 3 × 10⁴ hRPE cells were stimulated with indicated IFN-γ concentrations under atmospheric oxygen concentration (normoxic) or hypoxic (8% O₂, 5% O₂, or 1% O₂) conditions in the presence of 1-tryptophan (100 µg/ml). Ehrlich’s reagent was used to measure the kynurenine content. Data are given as mean ± SEM of two (a), one (b), three (c), five (d), four (e), or three (f) experiments, each performed in triplicate. Significant differences to the unstimulated or normoxic group are indicated with asterisks (n.s. not significant, *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.0001). The unpaired, two-tailed student’s t test was used.

Real-time PCR

hRPE cells were seeded into 6-well plates (10⁶ hRPE cells per well) and stimulated with combinations of the respective cytokines IFN-γ (500 U/ml), IL-1β (100 U/ml), TNFα (100 U/ml), and NGMMA (100 µg/ml) or left untreated for 24 h in a humidified incubator at 37 °C and 5% CO₂ [8]. For sample collection, the medium was aspirated and cells were detached by scraping. Total RNA was extracted according to the TRI Reagent protocol (Merck, Darmstadt, Germany). RNA was dissolved in UltraPure™ distilled water (Thermo Fisher Scientific, Carlsbad, USA) and RNA concentration was determined via NanoDrop (Thermo Fisher Scientific, Carlsbad, USA). Reverse transcription of 2 µg total RNA to cDNA was performed with M-MLV reverse transcriptase and oligo(dT)₁₂₋₁₈ primers (Thermo Fisher Scientific, Carlsbad, USA). PCR primers to amplify the genes of interest are listed in Table 1. Real-time PCR was performed with the Takyon NoRox Probe MasterMix dTTP (Eurogentec, Lüttich, Belgium) on a Bio-Rad CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, USA). All reactions, including non-template contamination controls, were performed in duplicates. Each well of a multiplate 96-well PCR plate contained 5 µl cDNA template, 12.5 µl Takyon NoRox Probe Master Mix dTTP, 0.3 µl primer (20 µM each), 0.5 µl probe (10 µM), and 6.4 µl H₂O for a total reaction volume of 25 µl (Table 1). The PCR conditions were 7 min at 95 °C and 40 cycles of each 94 °C for 20 s and 60 °C for 1 min.

Western blot analysis

6.75 x 10⁶ hRPE cells were incubated in cell-culture flasks (Costar, Cambridge, USA) in the absence or presence of IFN-γ (500 U/ml), IL-1β (100 U/ml), and TNFα (100 U/ml) for 24 h. The supernatant was discarded and the cell monolayer was washed three times with cold PBS. The cells were detached by scraping in 200 µl PBS containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Thereafter, the cells were lysed by three freeze/thaw cycles, centrifuged and the cell extract was stored at −80 °C. IFN-γ (500 U/ml) stimulated human glioblastoma cell extracts served as a control. The protein amount was determined via Bradford assay (Bio-Rad Laboratories, Hercules, USA). Separated proteins were semi-dry blotted on nitrocellulose membranes (CarboGlas, Schleicher & Schüll, Dassel, Germany). After blocking the membranes in 5% (w/v) skim milk powder in PBS for 1 h at room temperature, the nitrocellulose membranes were incubated for 1.5 h at room temperature in the respective primary antibodies diluted in 0.5% (w/v) skim milk powder in TBS: anti-β-actin antibody (1:5000, Sigma, St. Louis, USA) or anti-human-IDO1 antibody (1:500, Merck, Darmstadt, Germany). Thereafter, the membranes were washed with PBS for three times (5 min) and incubated for 2 h at room temperature with goat anti-mouse HRP-conjugated (IDO1) or goat anti-rabbit HRP-conjugated (β-actin) IgG (1:10,000-70,000, Jackson Immuno Research Laboratories, Dianova, Hamburg, Germany), diluted in 0.5% (w/v) skim milk powder in PBS. After additional washes with PBS, bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

Statistical analysis

All experiments were performed in duplicates or triplicates (as indicated in the figure legends) and data are given as mean ± SEM of a minimum of three independent experiments. For statistical analysis the unpaired two-tailed students t test was used and significant differences were marked with asterisks (*p < 0.05; **p < 0.001; ***p < 0.0001). The statistical analysis was performed with GraphPad Prism software.
Fig. 2 Influence of the inducible NO synthase (iNOS) on indoleamine 2,3-dioxygenase-1 (IDO1) activity in human retinal pigment epithelial (hRPE) cells. a $3 \times 10^4$ hRPE cells were stimulated in 96-well plates with indicated concentrations of human IFN-γ (0–1000 U/ml) in the presence of 100 µg/ml l-tryptophan and in the absence (−) or presence (+) of IL-1β (100 U/ml) and TNFα (100 U/ml). After 72 h, the cell-culture supernatants were harvested and the kynurenine content was determined by the use of Ehrlich’s reagent. b Same experimental setting with additional supplementation of the iNOS inhibitor NGMMA (100 µg/ml). c Relative expression of IDO1, IDO2, nNOS, iNOS, and eNOS detected by real-time PCR 24 h after stimulation. Data are given as mean ± SEM of five (a, b) or two (c) experiments, each performed in triplicate. Significant differences to the unstimulated group were marked with asterisks (n.s. not significant, n.d. not detected, *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$). The unpaired, two-tailed student’s t test was used.
Results

Indoleamine 2,3-dioxygenase-1 mediates antimicrobial functions in hRPE cells

Our aim was to analyze antibacterial effectors in IDO-positive human retinal pigment epithelial (hRPE) cells, since IDO-mediated antibacterial effects have been described in human cells before [6, 7]. Hence, we stimulated hRPE cells with IFN-γ and found a strong expression of IDO1 mRNA (10^6 fold higher than in unstimulated cells), whereas IDO2 mRNA was only marginally induced (32-fold) and thus might be neglectable (Fig. 1a, b).

IDO1 activity was assayed by the measurement of kynurenine, the product of IDO1-mediated tryptophan degradation. For this purpose, hRPE cells were stimulated in a tryptophan-enriched cell-culture medium with different amounts of IFN-γ for 72 h and the accumulated amount of kynurenine in the cell-culture supernatants was quantified by the use of Ehrlich’s reagent. IDO1 activity was clearly dependent on the IFN-γ dose used (Fig. 1c) and even 4 U/ml IFN-γ were sufficient to induce a significant increase (p > 0.001) of kynurenine production. To further prove that IDO1 is the responsible antimicrobial effector molecule in stimulated hRPE cells, we performed additional experiments using the IDO1-specific inhibitor 1-methyl-l-tryptophan (1-MT).

These data are included in the electronic supplementary material (ESM 1). 1-MT treatment of IFN-γ stimulated hRPE cells inhibits IDO1 activity, as detected by kynurenine measurement (ESM 1a).

Next, we tested whether IDO1 could sufficiently degrade the conventional tryptophan amounts in the IMDM cell-culture medium to inhibit the growth of pathogens. It has been shown before that IDO1 inhibits RH (type I) strain Toxoplasma gondii in hRPE cells [6]. Herein, we tested the efficiency of IDO1 activity against ME49 parasites, since type II strains are the most frequently found T. gondii strains in patients [30]. IFN-γ pre-stimulated hRPE cells were infected with T. gondii and the intracellular growth was quantified after 3 days using the ^3H-uracil method. T. gondii tachyzoites grew in unstimulated hRPE cells, but the presence of 30 U/ml IFN-γ was sufficient to inhibit the parasite growth significantly (Fig. 1d). This antiparasitic effect was due to IDO1-mediated tryptophan degradation, since the supplementation of l-tryptophan at the timepoint of infection abrogated the effect (Fig. 1d). Interestingly, there was no full recovery of T. gondii growth in hRPE cells that were pre-treated with high doses of IFN-γ.

Since stimulated hRPE cells displayed an effective IDO1 activity, we then checked their ability to inhibit S. aureus growth as well. We used a patient-derived tryptophan auxotroph S. aureus strain to infect hRPE cells that were pre-stimulated with different amounts of IFN-γ for 72 h. The bacterial growth was analyzed 16 h postinfection by measuring the optical density. Bacteria grew in the presence of unstimulated cells. S. aureus growth was significantly inhibited by hRPE cells pre-stimulated with 30 U/ml IFN-γ or more (p < 0.0001, Fig. 1e). The antibacterial effect could be ascribed to IDO1-mediated tryptophan degradation, since the supplementation of L-tryptophan at the timepoint of infection allowed an unrestricted bacterial growth (Fig. 1e). Furthermore, the IDO-specific inhibitor 1-MT abrogated the antibacterial effect as well (ESM 1b).

hRPE cells are localized in a region of the eye with a physiologic oxygen concentration of approx. 8% O₂ [35]. Since IDO1 activity is dependent on the presence of oxygen, we analyzed the kynurenine production in hRPE cells under different oxygen conditions. hRPE cells were incubated under normoxia, 8% O₂, 5% O₂, or 1% O₂ in the presence of different IFN-γ concentrations. IDO1 activity was again measured on the basis of the kynurenine content in the cell-culture supernatants. At 8% O₂, IDO1 activity in hRPE cells is slightly reduced at lower IFN-γ concentrations (60 and 125 U/ml). However, there was no significant impact on IDO1 activity when the hRPE cells were treated with higher amounts of IFN-γ (250 U/ml) (Fig. 1f). In contrast to this, the hypoxic environment of only 1% O₂ resulted in a significant decrease of IDO1 activity, even in the presence of higher IFN-γ concentrations (Fig. 1f). In addition, the presence of 1% O₂ results in a strong inhibition of IFN-γ induced antibacterial effect, while in the presence of 8% O₂, a more or less complete antibacterial effect of IFN-γ was detected (ESM 1b).

Inducible NO synthase is induced in hRPE cells by cytokine co-stimulation

The antiparasitic effect against RH strain T. gondii tachyzoites is not only provoked by IFN-γ, but also by other pro-inflammatory cytokines and synergistic effects have been described [26]. Therefore, we co-stimulated 3 × 10⁴ hRPE cells with IFN-γ and IL-1β or TNFα, respectively, in 96-well plates. This co-stimulation leads to a slight increase of IDO1 activity, as observed by the formation of kynurenine in the cell-culture supernatants obtained after stimulation with 60–500 U/ml IFN-γ (Fig. 2a). Therefore, we expected a synergistic effect of all three cytokines. However, unexpectedly, the co-stimulation of IFN-γ, IL-1β, and TNFα resulted in a significant reduction of the kynurenine production at IFN-γ concentrations above 125 U/ml. Such an inhibition of IDO1 activity could be the result of the induction of iNOS. To find out whether iNOS is involved in the inhibition of IDO1 activity, we repeated the experiment in the presence of N⁰-monomethyl-l-arginine (N⁰MMA), a competitive inhibitor that reduces the iNOS-mediated conversion of arginine...
to nitric oxide (NO). $N^G$MMA had no effect on IFN-γ-induced IDO1 activity (Fig. 2b). In addition, the presence of $N^G$MMA did not influence IDO1, which was co-stimulated by IFN-γ and IL-1β or IFN-γ and TNFα (Fig. 2b). Interestingly, $N^G$MMA supplementation restored IDO1 activity after co-stimulation with all three cytokines (Fig. 2b). Therefore, it seems probable that the co-stimulation of hRPE cells with IFN-γ, IL-1β, and TNFα indeed induced iNOS in hRPE cells, which was inhibited by $N^G$MMA. Furthermore, NO, the product of iNOS activity must have been the causing agent for the IDO1 inhibition.
were stimulated in the presence (+) or absence (−) of IFN-γ (500 U/cell-culture supernatants of 1 × 10^5 up to 5 × 10^5 co-stimulated hRPE cells; not shown). However, higher levels of NO accumulated in the presence of the competitive inhibitor NGMMA as expected (Fig. 2c). Due to the absence of the competitive inhibitor NGMMA, the inhibition of IDO1 in hRPE (0.1% N-(1-naphthyl)ethylendiamine [in aqua dest. plus 2.5% sulfanilamid (in 15% HCl) in equal parts] and the nitrite was quantified by measuring the optical density at 540 nm. c 1 × 10^5 or 3 × 10^5 hRPE cells were stimulated in the presence (+) or absence (−) of IFN-γ (500 U/mL), IL-1β (100 U/mL), TNFα (100 U/mL), or NGMMA (100 µg/ml). After 72 h, 100 µl cell-culture supernatant was added to 100 µl Griess Reagent (0.1% N-(1-naphthyl)ethylendiamine [in aqua dest. plus 2.5% sulfanilamid (in 15% HCl) in equal parts] and the nitrite was quantified by measuring the optical density at 540 nm. c 1 × 10^5 or 3 × 10^5 hRPE cells were stimulated in the presence (+) or absence (−) of IFN-γ (500 U/mL), IL-1β (100 U/mL), TNFα (100 U/mL), or NGMMA (100 µg/ml). After 72 h, the cell-culture supernatants were harvested and the kynurenine content was determined by the use of Ehrlich’s reagent. Data are given as mean ± SEM of two experiments, each performed in triplicate.

Nevertheless, we wanted to confirm that indeed, iNOS and not the other NO-synthases (endothelial or neuronal NOS, eNOS, and nNOS, respectively) were responsible for the formation of NO. Real-time PCR studies revealed a highly significant increase of iNOS in hRPE cells after co-stimulation with IFN-γ, IL-1β, and TNFα (Fig. 2c), which was not affected by the addition of the competitive inhibitor NGMMA as expected (Fig. 2c). Due to the low mRNA expression of the other NO-synthases, we assume that nNOS and eNOS do not play a major role in the inhibition of IDO1 in hRPE cells (Fig. 2c). In sum, the co-stimulation of IFN-γ, IL-1β, and TNFα results in the induction of NO producing iNOS, which inhibits the IDO1-mediated degradation of tryptophan to kynurenine.

Next, we verified nitric oxide production indirectly by detection of nitrite in the supernatant of IFN-γ, IL-1β, and TNFα co-stimulated hRPE cells by the use of Griess Reagent. We could not detect significant amounts of NO in 3 × 10^4 co-stimulated hRPE cells in 96-well plates (data not shown). However, higher levels of NO accumulated in cell-culture supernatants of 1 × 10^5 up to 5 × 10^5 co-stimulated hRPE cells in 24-well plates over time. Therefore, the amount of NO is dependent on the cell number used in the experiments (Fig. 3a).

We continued with the 24-well-plate experimental setting and analyzed the NO production in 1 × 10^5 differently stimulated hRPE cells. IFN-γ stimulation did not exceed the NO production of the negative control, whereas a combinatorial treatment of hRPE cells with IFN-γ and IL-1β leads to iNOS activity (Fig. 3b). In contrast, NO was detectable after the combinatorial treatment with IFN-γ and TNFα (Fig. 3b). Interestingly, as expected, the stimulation with all three cytokines resulted in an intense formation of NO and the addition of NGMMA prevented the generation of NO (Fig. 3b). All observations were enhanced by the usage of 1 × 10^5 hRPE cells (Fig. 3b).

Since not only the co-stimulation with all three cytokines resulted in a significant NO production in the modified experimental system, but also the combination of IFN-γ and IL-1β alone, we were interested in whether the latter stimulation had an influence on IDO activity. Although IDO activity has not been influenced by IFN-γ and IL-1β stimulation in 3 × 10^5 hRPE cells (Fig. 2a) and in 1 × 10^5 hRPE cells (Fig. 3c), IDO activity was clearly inhibited by IFN-γ and IL-1β-induced NO production in 3 × 10^5 stimulated hRPE cells (Fig. 3c). These results indicate that a sufficient NO concentration is indispensable for a significant inhibition of IDO activity.

**Influence of iNOS activity on indoleamine 2,3-dioxygenase-1 in human retinal pigment epithelial cells**

Next, we elucidated whether iNOS activity inhibits IDO1 on transcriptional, translational, or post-translational level. In real-time analyses, IDO1 mRNA induction was detected upon IFN-γ stimulation (Fig. 4a). Neither combinatorial stimulation with IFN-γ/IL-1β, nor IFN-γ/TNFα, nor IFN-γ/IL-1β/TNFα lead to a reduced IDO1 mRNA expression (Fig. 4a).

Western blot analyses revealed no reduction of IDO1 protein levels upon IFN-γ/IL-1β, IFN-γ/TNFα or IFN-γ/IL-1β/TNFα stimulation (Fig. 4b). As expected, NGMMA had no effect on IDO1 protein levels as well.

Finally, we wanted to elucidate whether iNOS-generated NO is sufficient to have a potential role during EBE. Since NO inhibits the IDO1-mediated kynurenine production, we wanted to find out whether IDO1-mediated antibacterial effects are also abolished. Therefore, IFN-γ or IFN-γ/IL-1β/TNFα pre-stimulated hRPE cells were infected with S. aureus and the bacterial growth was determined after additional 16 h by measurement of the optical density. Bacterial growth was inhibited in IFN-γ pre-stimulated cells as before (Fig. 3c). Interestingly, the co-stimulation with IFN-γ, IL-1β, and TNFα impeded the IDO1 antibacterial activity, which allowed an unhindered S. aureus growth (Fig. 4c). The presence of NGMMA during the stimulation period sufficiently neutralized this effect (Fig. 4c).

**Discussion**

The eye is an immune-privileged site. This privilege is established by the RPE. The RPE cells play a crucial role in retinal physiology, in the induction of immunosuppression and in antimicrobial defense [4]. Eye infections cause harm to vision. The work of several research groups focusses...
especially on CMV and T. gondii-caused infections, since both pathogens induce numerous eye infections.

For example, Noguereia et al. described barrier dysfunction of human RPE cells due to altered tight junctions after in vitro infection with T. gondii RH strain tachyzoites [31]. Delaire et al. found that IFN-γ activated native Lewis rat RPE cells are able to inhibit the growth of T. gondii (RH strain) tachyzoites [5]. Furthermore, stimulation with TNF-α alone inhibited T. gondii growth slightly. A combined treatment of native Lewis rat RPE cells with IFN-γ and TNF-α resulted in a synergistic effect. Further data from this group suggest that tryptophan supplementation abrogates this antibacterial effect. These data indicate that hRPE cells are antimicrobial effector cells that might influence EBE. The cleavage of tryptophan by IDO1 restricts the growth of S. aureus and that tryptophan supplementation abrogates this antibacterial effect. These data indicate that hRPE cells are antimicrobial effector cells that might influence EBE. We found that IDO1 activity significantly. However, a combined stimulation with IFN-γ, IL-1β, and TNF-α resulted in a strong inhibition of IFN-γ-induced IDO1 activity, especially at higher IFN-γ doses. Since nitric oxide is capable of inhibiting IDO1 activity [37], we assumed that iNOS induction in hRPE cells by this cytokine cocktail might be responsible for the observed IDO1 inhibition.

Earlier publications described iNOS transcription, expression, and activity in human native RPE cells as well as in the RPE cell line ARPE19 [7, 17, 22]. In this context, iNOS activity was induced by stimulation with different cytokines (for example, with IFN-γ, TNF-α, and/or IL-1β). In addition to the cytokine-dependent iNOS activation, also non-immune iNOS activation has been observed in hRPE cells. Here, iNOS was induced by all-trans retinal, an intermediate in the vision cycle, or by cultivation under high-glucose conditions [22, 38]. In addition, iNOS is detectable in RPE cells from other species, including pigs, cows, mice, and rats [18, 39]. Interestingly, iNOS has potent antimicrobial activities and inhibits the spread of murine CMV, but overexpression of iNOS can also damage RPE cells, change neovascularization in ischemic retinopathy, and is associated with the severity of diabetic retinopathy in mice and humans [16, 19, 40].

Here, we found that hRPE cells do not express iNOS activity after IFN-γ stimulation, but after co-stimulation of hRPE cells with IFN-γ/IL-1β as well as IFN-γ/TNF-α/IL-1β. The magnitude of nitric oxide production is dependent on several factors, including cell number, cytokine concentration, and cultivation time. iNOS activity in hRPE cells...
Fig. 4 Influence of iNOS activity on indoleamine 2,3-dioxygenase-1 in human retinal pigment epithelial cells. a Relative expression of IDO1, IDO2, neuronal (nNOS), inducible (iNOS), and endothelial nitric oxide synthase (eNOS) 24 h poststimulation as determined by real-time PCR. hRPE cells were untreated or stimulated with combinations of IFN-γ (500 U/ml), IL-1β (100 U/ml), TNFα (100 U/ml), and NGMMA (100 µg/ml) as indicated. b Western blot analysis of IDO1 and β-actin protein expression by hRPE cells in the presence (+) or absence (−) of IFN-γ (500 U/ml), IL-1β (100 U/ml), or TNFα (100 U/ml). Unstimulated and IFN-γ (500 U/ml) stimulated human glioblastoma cell lysates were used as controls, respectively. c 3 × 10^4 hRPE cells pre-stimulated with IFN-γ and/or IL-1β and TNFα (100 U/ml each) for 72 h were infected with Staphylococcus aureus (10–100 cfu/well). The bacterial growth was detected by measurement of the optical density at 620 nm after 16 h. Data are given as mean ± SEM of two (a), one (b) or three (c) experiments, each performed in triplicate. Significant inhibition of bacterial growth in IFN-γ stimulated hRPE cells is marked with asterisks (*p ≤ 0.05, **p ≤ 0.001, and ***p ≤ 0.0001). Significant inhibition of the antibacterial effect by co-stimulation with IL-1β and TNFα is marked with triangles. Significant recovery of the antibacterial effector mechanism via NGMMA supplementation is marked with diamonds. The unpaired, two-tailed student’s t test was used.
could be blocked by addition of the NOS antagonist N\textsuperscript{G}-monomethyl-l-arginine (N\textsuperscript{G}MMA). We confirmed via real-time PCR analyses that the inducible form of NO-synthases (iNOS, also known as NOS2) was indeed upregulated after co-stimulation with IFN-γ, TNFα, and IL-1β, whereas no noteworthy mRNA amounts of endothelial (eNOS also known as NOS 3) or neuronal NO-synthases (nNOS, also known as NOS 1) were detectable. IDO1 as well as iNOS are described as potent antimicrobial effector mechanisms [41]. However, we could not find a synergistic antimicrobial effect of both IFN-γ-induced effector mechanisms directed against \textit{S. aureus} or \textit{T. gondii}. Instead, iNOS activity generated sufficient NO concentrations that were capable to inhibit IDO1.

The intensity of IDO induction directly correlates with the amount of IFN-γ used for stimulation. Since IDO is the rate-limiting enzyme in the kynurenine pathway, an excessive IDO1 induction results in tryptophan starvation, which is an unfavorable situation for the host cells. Furthermore, the production of toxic metabolites along the kynurenine pathway such as kynurenine and 3OH-kynurenine might be harmful for immune and tissue cells [45, 46] or induce a plethora of effects via aryl hydrocarbon receptor signaling [47]. Therefore, control of IDO1 activity by NO might be beneficial to reduce detrimental IDO1-mediated cell damage.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human and/or animal participants** This article does not contain any studies with human participants or animals performed by any of the authors.

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