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Protein Kinase Cζ (PKCζ) Regulates Ocular Inflammation and Apoptosis in Endotoxin-Induced Uveitis (EIU)

Signaling Molecules Involved in EIU Resolution by PKCζ Inhibitor and Interleukin-13

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We show that inhibitory effect of interleukin-13 on endotoxin-induced uveitis in the Lewis rat is dependent on signaling activity of protein kinase Cζ (PKCζ). To understand the effect of interleukin-13 or PKCζ inhibitor treatment, the activation status of rat bone marrow-derived macrophages was studied *in vitro*. At 6 hours, lipopolysaccharide-stimulated macrophages produced tumor necrosis factor-α (TNF-α) with nuclear factor κB (NF-κB)/p65 expression. Treatment led to absence of NF-κB/p65 expression and low levels of TNF-α, suggesting accelerated inactivation of macrophages. At 24 hours after lipopolysaccharide stimulation, nuclear NF-κB/p65 decreased and nuclear NF-κB/p50 increased, associated with nuclear BCL-3 and a low level of TNF-α, indicating onset of spontaneous resolution. Treatment limited PKCζ cleavage, with expression of nuclear NF-κB/p50 and BCL-3 and low nuclear NF-κB/p65 promoting macrophage survival, as evidenced by Bcl-2 expression. At 24 hours, intraocular treatment decreased membranous expression of PKCζ by ocular cells, reduced vascular leakage with low nitric-oxide synthase-2 expression in vascular endothelial cells, and limited inflammatory cell infiltration with decreased intraocular TNF-α, interleukin-6, and nitric-oxide synthase-2 mRNA. Importantly, treatment decreased nuclear NF-κB/p65, increased transforming growth factor-β2, and reduced caspase 3 expression in infiltrating macrophages, implying a change of their phenotype within ocular microenvironment. Treatment accelerated endotoxin-induced uveitis resolution through premature apoptosis of neutrophils related to high expression of toll-like receptor 4 and caspase 3. ***(Am J Pathol 2007, 170:1241–1257; DOI: 10.2353/ajpath.2007.060236)***

Clinical and experimental studies have implicated gram-negative bacteria as a triggering factor for acute anterior uveitis.[1] Indeed, the systemic injection of lipopolysaccharide (LPS) induces an acute, nonspecific inflammation of the anterior segment of the eye[1–3] with chorioretinal involvement[4] in the Lewis rat. This ocular inflammation, named endotoxin-induced uveitis (EIU), serves as an animal model for human ocular inflammations, a group of diseases that may have blinding complications and that collectively are referred to as uveitis.[2] Uveitis occurs after the breakdown of blood-ocular barriers allows inflammatory cells, including macrophages and polymorphonuclear leukocytes (PMNs), to invade the ocular tissues.[5] Inflammatory cells, together with ocular resident cells such as vascular endothelium, retinal Müller glial cells, microglia, and retinal pigment epithelium, have been shown to produce inflammatory cytokines,[6–8] chemokines,[9] and nitric oxide (NO).[10,11] These inflammatory mediators are all involved in the initiation of EIU. Although the inflammation induced by injection of LPS resolves

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spontaneously after several days, it induces apoptosis in inflammatory and resident cells within the eye.[12]

Many stimuli, including LPS and inflammatory cytokines, induce a cellular stress that results in the activation of the atypical protein kinase Cζ (PKCζ) isoform, which controls downstream signaling pathways involving the transcription factor nuclear factor κB (NF-κB).[13] Lipopolysaccharide also activates the caspase pathway, including caspase 3, which is responsible for a critical step in apoptosis, namely, the cleavage of PKCζ.[14,15] More recently, LPS was reported to activate the member of the class of pattern recognition receptors or toll-like receptors (TLRs) known as TLR4.[16–18] Activation of TLRs initiates a signaling cascade that involves a number of proteins including PKCs, leading to the activation of NF-κB, which induces the secretion of cytokines that direct the innate immune response.

NF-κB is present in the cytoplasm of all cells in a resting state,[19] consisting mainly of a heterodimer of p50 and p65 (RelA) sequestrated by IκB. Following phosphorylation, ubiquitination, and degradation of IκB, the p50-p65 component is released and translocates into the nucleus, where it activates transcription of genes involved in innate immunity, inflammation, or cell survival.[20–22] NF-κB has been reported to play an important role in the induction of various inflammatory molecules including tumor necrosis factor-α (TNF-α) and nitric-oxide synthase-2 (NOS-2).[20,23] However, recent studies reported that NF-κB activation during the early and late phases of inflammation independently activates expression of proinflammatory and anti-inflammatory genes, respectively.[22,24] In addition, it has been also reported that human monocytes and macrophages stimulated with LPS initially transcribe TNF-α mRNA in response to increased nuclear NF-κB/p65/50, but subsequently, TNF-α mRNA levels decrease as NF-κB/p50/p50 increases[25] in parallel with BCL-3 expression.[26,27]

PKC isoforms constitute a family of at least 12 serine/threonine kinases. The atypical PKCζ was recently identified as an important secondary messenger, mediating a number of cellular responses to various exogenous stimuli and stress agents. It is required for the regulation of NF-κB pathway by phosphorylation and activation of IκK kinase, resulting in phosphorylation of IκB, and direct phosphorylation of NF-κB/p65. Stress leads to cleavage of PKCζ at its hinge region, generating a fragment of 50 kd via caspase 3 activation.[15,28] The 50-kd fragment is an independent catalytic domain that represents the activated form of this enzyme.[13,29,30] Targeted disruption of the PKCζ gene in mice results in the impairment of the NF-κB pathway,[31] suggesting a role for PKCζ in NF-κB signaling.[32] This PKCζ gene disruption results also in increased B-cell apoptosis.[33] Interestingly, NF-κB $p50^{-/-}p65^{+/+}$ mice are extremely susceptible to develop LPS-induced shock compared with wild-type mice.[34] Recently, we have reported that protection against PKCζ cleavage and nuclear translocation of PKCζ prevented *N*-methyl-D-aspartate-induced neuronal cell death, strongly suggesting that PKCζ is acting as a key molecule in the survival pathway.[14]

B-cell lymphoma 3 (BCL-3) is a member of the IκB subfamily of signaling inhibitors. The relative levels of the various family members control apoptosis versus survival of activated cells. BCL-3 interacts with NF-κB/p50 and is recruited to the TNF-α promoter, facilitating NF-κB/p50-mediated inhibition of TNF-α expression.[35]

Infection and stress lead to activation of TLRs, which induce a signaling cascade resulting in the activation of NF-κB/Rel family transcription factors.[36] TLR4 is a signal transduction receptor for LPS that is known to respond to gram-negative bacteria and to be involved in the production of inflammatory cytokines.[37] Expression of TLR4 mRNA has been previously detected in normal human ocular cells and tissues,[37,38] suggesting an important role for these molecules in the pathogenesis of uveitis. Because LPS-induced activation of cells occurs through TLR4, we investigated the expression of this receptor by infiltrating cells during EIU and in the presence of PKCζ inhibitor (PKCζi) or interleukin (IL)-13.

We have previously shown that a single systemic or intraocular injection of IL-13 reduces the severity of EIU, with decreased expression of inflammatory cytokine and chemokine mRNA and reduced production of nitrite within the eye.[39,40] IL-13, an anti-inflammatory cytokine produced by Th2 lymphocytes,[41] has been found to inhibit TNF-dependent *in vitro* activation of NF-κB and a second transcription factor, activation protein-1, and to block apoptosis of a number of human cell lines, activities that are presumably responsible for its immunosuppressive and anti-inflammatory effects.[15] These effects are blocked by the PKC-specific inhibitor H-7, implicating PKC in IL-13 signaling. IL-13 has been shown to inhibit the PKC-triggered respiratory burst and to suppress NO release from macrophages.[15] PKCζ appears to be essential for *in vitro* LPS-induced macrophage activation,[42] and inhibition of the enzyme was associated with reduced TNF-α production.

Macrophages play a critical role in ocular inflammation.[43,44] These nonspecific effector cells have a complex role, participating both in the process of tissue damage and in the resolution of inflammation.[45] This prompted us to investigate the effect of IL-13 or PKCζi treatment on the expression of PKCζ and downstream signaling molecules responsible for macrophage activation in LPS-stimulated rat bone marrow-derived macrophages. We also analyzed the role of PKCζ in EIU and in the inhibition of this uveitis that is achieved by the administration of IL-13. We show that EIU treatment with IL-13 or PKCζi, induced the activation of signaling pathways involving PKCζ and NF-κB that regulate apoptosis or survival of resident ocular cells and infiltrating inflammatory cells.

Materials and Methods

Induction of EIU in Lewis Rats

Male Lewis rats (Charles River, Saint-Aubin-les-Elbeuf, France), aged 8 weeks, were injected in one hind footpad with 500 μg/kg LPS from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO) in 0.1 ml of sterile pyrogen-

free saline.[39] This dose of LPS takes into account the weight of the animals and corresponds to 100 to 200 μg of LPS. Animals were housed in a 12-hour light and 12-hour dark cycle and fed water and dried ration ad libitum.

A total of six separate experiments were performed, including a total of 59 rats. Rat tissues were separately processed for histopathological and immunohistochemical studies, reverse transcription-polymerase chain reaction analysis, and Western blot and other protein quantifications. Experimental protocols were developed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Intraocular Injection of IL-13 or PKCζ Inhibitor PKCζi in Rats with EIU

Anterior chamber injection was performed as previously described.[40] In brief, rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg Nembutal; Abbot, Saint-Remy sur Avre, France). Pupils were dilated by instillation of 1 drop of 5% tropicamide (Ciba Vision, Toulouse, France) and 1 drop of 1% tetracaine (Ciba Vision) was administered for local anesthesia. Four microliters of sterile pyrogen-free saline, PKCζi (PKCζ-specific inhibitory peptide, myr-SIYRRGARRWRKL, no. 539624, lot B42131; Calbiochem, San Diego, CA) and/or IL-13 (R&D, Abingdon, UK) was injected through the cornea into the anterior chamber under a surgical microscope, using a sterile syringe and 30-gauge needle (Microfine; Becton Dickinson, Meylan, France). The needle was left in the eye for 10 seconds to allow aqueous humor to leave the eye via the trabecular meshwork rather than by reflux along the needle track. The injection was performed near the apex of the cornea, taking care not to damage the iris or the lens.

To investigate the role of PKCζ in transduction signals induced by LPS and to determine whether the inhibitory effect of IL-13 on EIU originated from an interaction with this signaling, different protocols were tested: intracameral injection of 4 μl of selected doses of PKCζi (3, 2, 1, 0.5 μg); intracameral injection of recombinant human IL-13 (2 ng); intracameral injection of PKCζi (1 μg) with IL-13 (1 ng); and saline as a negative control. All these injections were given simultaneously with the systemic injection of LPS. However, different time intervals of the PKCζi injection (3 hours before or 6 hours after LPS injection) were also tested.

Clinical Examination

Intensity of the ocular inflammation was scored on a scale of 0 to 5. Animals were examined at a biomicroscope (slit lamp) by a masked investigator, and the degree of inflammation was scored at 24 hours after LPS injection, as previously described.[12,40] The level of protection was also expressed as percentage of protection, which was defined as: (EIU grade in saline-injected animals − EIU grade in PKCζi-injected animals)/(EIU grade in saline-injected animals).

Protein Determination in Aqueous Humor/Vitreous Body

At the time of death, 24 hours after administration of PKCζi (3 μg in 4 μl) or saline and the LPS injection, aqueous humor and vitreous body were collected from both eyes of each animal and pooled. Protein concentration was determined in 1 μl of each sample using a Bradford assay with γ globulin as the standard (Bio-Rad Laboratories, Les Ulis, France).

Immunohistochemistry

Enucleated eyes were fixed in 2% paraformaldehyde, embedded in OCT (Tissue-Tek; Miles Inc., Diagnostic Division, Elkhart, IN), and 10-μm anteroposterior cryostat sections were cut at the level of the optic nerve and stained using the following antibodies diluted 1:100, as described previously[43]: mouse monoclonal anti-rat ED1 (macrophages and dendritic cells) (Serotec, Oxford, UK); rabbit polyclonal antibodies directed against PKCζ, NF-κB/p65, Bcl-2, and caspase 3 active form (described as detecting with a 50-fold preference for the active form compared with the inactive form), transforming growth factor (TGF)-β2 (SC-90) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal antibody directed against inducible NOS (NOS-2; Transduction Laboratories, Lexington, KY); and goat polyclonal antibody anti-TLR4 antibody (SC-12511; Santa Cruz Biotechnology).

Double immunostaining was performed as follows. Tissue sections were incubated overnight at 4°C with rabbit or goat polyclonal antibodies directed against the molecule of interest, followed the next day by a 1-hour incubation at room temperature with Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) or rabbit anti-goat IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), as appropriate, diluted 1:250. Subsequently, mouse monoclonal anti-rat ED1 was applied for 1 hour at room temperature followed by Alexa 594-conjugated goat anti-mouse IgG (H+L) (Molecular Probes), diluted 1:250. Control sections incubated without primary antibodies, or with addition of normal serum Ig in place of rabbit and goat polyclonal antibodies or mouse Ig of corresponding isotype in place of monoclonal mouse antibodies, were included in every staining run. Sections were mounted with an anti-fade medium with 4,6-diamidino-2-phenylindole (DAPI) or with propidium iodide (Vectashield; Vector laboratories, Burlingame, CA) and observed by fluorescence photomicroscopy (FXA, Microphot; Nikon, Melville, NY). Digitized micrographs were obtained using a digital camera (Spot; BFI Optilas, Evry, France). Apoptosis of cells was assessed with a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (Roche, Indianapolis, IN) in strict accordance with the manufacturer's instructions.

Quantification of Positively Stained Cells

To quantify EIU and the effect of PKCζI treatment, all DAPI-positive inflammatory cells were counted across the entire ocular cross section, and cell number was expressed as the mean ± SEM of total cell number/animal as previously described.[43] To determine the effect in EIU rats of the PKCζi treatment on the expression of specific signaling molecules by infiltrating inflammatory cells, dual immunostaining was performed as described above, using anti-PKCζ, anti-NF-κB, and anti-caspase 3 antibodies, as well as anti-ED1 antibody. ED1-negative inflammatory cells showed the characteristic morphological appearance of PMNs, with trilobed nuclei, after DAPI staining.

Infiltrating inflammatory cells ($ED1^+$ cells and PMNs) showing cytoplasmic or nuclear localization of PKCζ, NF-κB, and caspase 3 were counted in ocular tissues on cryostat sections from rats injected with LPS and treated with saline or with PKCζi (500 cells were counted per treatment). The percentage of nuclear or cytoplasmic staining was expressed as follows: 1) (number of $ED1^+$ cells expressing specific marker in nucleus or cytoplasm)/(total number of $ED1^+$ cells expressing the marker in the nucleus + $ED1^+$ cells expressing the marker in the cytoplasm); 2) (number of PMNs expressing specific marker in nucleus or cytoplasm)/(total number of PMNs expressing the marker in the nucleus + PMNs expressing the marker in the cytoplasm). Some cells expressed the specific markers simultaneously in the nucleus and in the cytoplasm, and their percentage has been specified in Results.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from enucleated eyes using the acid guanidinium thiocyanate-phenol-chloroform method, as described previously.[46] TNF-α, NOS-2, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense and anti-sense primers were obtained from GENSET SA (Paris, France). Primers were as follows: TNF-α sense primer, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′; TNF-α anti-sense primer, 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′; NOS-2 sense primer, 5′-TTTCTCTTCAAAGTCAAATCCTACCA-3′, NOS-2 anti-sense primer, 5′-TGTGTCTGCAGATGTGCTGAAAC-3′; IL-6 sense primer, 5′-GACTGATGTTGTTGACAGCCACTGC-3′, IL-6 anti-sense primer, 5′-TAGCCACTCCTTCTGTGACTCTAACT-3′; GAPDH sense primer: 5′-ATGCCCCCATGTTTGTGATG-3′, GAPDH anti-sense primer: 5′-ATGGCATGGACTGTGGTCAT-3′. These primers were designed specifically to amplify cDNA fragments representing mature mRNA transcripts of 295 bp for TNF-α, 657 bp for NOS-2, 508 bp for IL-6, and 162 bp for GAPDH. The polymerase chain reaction fragments were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The GAPDH gene was used to assess the amount and integrity of RNA samples. The quantities of cytokine mRNA present were expressed relative to the quantity of the housekeeping gene GAPDH. These data were obtained by determining the ratio of intensity of signals obtained by scanning the bands of the cytokine and GAPDH amplified fragments in agarose gel, using the NIH Image 1.57 software package (Bethesda, MD).

Preparation of Bone Marrow-Derived Macrophages

Uncommitted bone marrow-derived macrophages were prepared as previously described.[47,48] In brief, bone marrow cells were flushed aseptically with RPMI 1640 medium from the dissected femurs of male Lewis rats using a 21-gauge needle, yielding a single-cell suspension. The cells were cultured in a 75-mm tissue culture flask and adhered to plastic in Dulbecco's modified Eagle's medium containing 20% L929-conditioned medium as a source of macrophage colony-stimulating factor, 10% fetal calf serum, and 5% horse serum (all obtained from Gibco, Grand Island, NY). After 5 days in culture, macrophages were removed using trypsin and recultured in 24-well plates at the concentration of 5×10^5 per well in Dulbecco's modified Eagle's medium containing 2% fetal calf serum. Cells were then stimulated with LPS from *S. typhimurium* (100 ng/ml) alone or in combination with IL-13 (20 ng/ml) or with PKCζi (20 and 40 μg/ml). Cells and cell-free supernatants were taken at 6 and at 24 hours for Western blotting. Nuclear and cytoplasmic PKCζ and NF-κB/p65, NF-κB/p50, and BCL-3 present under these different conditions were also isolated for the Western blot experiments (see above).

Because nitrite and nitrate are stable end products of NO metabolism, NO synthesis was determined with nitrite release using a spectophotometric assay based on the Griess reaction using the Griess Reagent kit for nitrite determination (Molecular Probes).[39] Nitrite levels were determined in stimulated-macrophage supernatants. Briefly, 50 μl of cell free supernatant was mixed with 50 μl of Griess reaction solution. After 20 minutes, the absorbance was read at 550 nm and compared with nitrite standards.

TNF-α production was measured with enzyme-linked immunosorbent assay kits,[8] according to the manufacturer's instructions (BioSource International, Inc., Camarillo, CA) (data representative of two independent experiments). The absorbance was read at 450 nm and compared with standard concentrations of TNF-α diluted from 1000 to 31.2 pg/ml. The limit of sensitivity of the enzyme-linked immunosorbent assay test was 4 pg/ml.

Western Blots

Western blots were performed on cell lysates made from normal rat bone marrow macrophages, stimulated *in vitro* with LPS coincubated or not with IL-13 or PKCζi. Total protein extracts (50 μg per lane) were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond-ECL nitrocellulose membrane, as previously described.[49] For these experiments, specific rabbit polyclonal antibodies directed

against PKCζ (SC-216 raised against the C-terminal region of PKCζ), anti-NF-κB [p65, SC-7151, p50 (SC-7178)], Bcl-2 (SC-492), Bax (SC-493), and BCL-3 (SC-185) were obtained from Santa Cruz Biotechnology, and specific rabbit polyclonal antibodies directed against caspase 3 active form (67341A), described as having a 50-fold greater sensitivity for the active form compared with the inactive form, was obtained from PharMingen (Becton Dickinson). All antibodies were diluted 1:200 in phosphate-buffered saline containing 1% skim milk and 0.5:1000 Triton X-100 and 0.5:1000 Tween 20. Horseradish peroxidase-conjugated goat anti-rabbit F(ab′)2 IgG fragment from Caltag Laboratories (Burlingame, CA) was used as a secondary antibody, diluted 1:2000 in phosphate-buffered saline containing 1% skim milk, 0.5:1000 Triton X-100, and 0.5:1000 Tween 20, and a chemiluminescence (ECL) kit system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was used for detection of antigen-antibody complexes. To compare the different lanes, quantities of protein of interest for each set of experimental conditions were expressed relative to the quantity of tubulin in the sample. Data presented in the graphs represent ratios of intensities of signals obtained by scanning the bands of the proteins of interest and the bands of tubulin in each agarose gel, using the NIH Image 1.57 software package.

To clarify the significance of NF-κB activity, nuclear and cytoplasmic PKCζ and NF-κB/p65 were isolated from macrophages that had been stimulated *in vitro* under different conditions. To determine subcellular PKCζ and NF-κB/p65, NF-κB/p50, BCL-3 locations, we used a ProteoExtract subcellular proteome extraction kit (S-PEK; Calbiochem), which takes advantage of the differential solubility of cellular compartments in special reagent mixtures, thus preserving the integrity of the subcellular structures before and during extraction. The S-PEK yields the total proteome, fractionated into four subproteomes of decreased complexity: cytosolic fraction, membrane organelles, nucleic protein, and cytoskeleton. Here, we analyzed cytosolic and nuclear fractions. Cytosolic and nuclear fractions were subjected to Western blot analysis. Comparison of transferred proteins, stained by ponceau, indicated that cytosolic or nuclear fractions were equally loaded.

Statistical Analysis

Clinical and histological scores of EIU were presented as mean ± SE of mean. Severity of EIU was compared using the nonparametric Mann-Whitney *U*-test or the Kruskal-Wallis nonparametric analysis of variance test followed by the Bonferroni multiple comparison test, as appropriate. A *P* value of less than 0.05 was considered statistically significant.

Results

During ocular inflammation, macrophages are exposed to a range of molecular stimuli that are integral to the ocular microenvironment.[48] These cells are the principal source of cytokines and molecules, including TNF-α and nitrite, which are produced in response to inflammatory stimuli. Because macrophages play a key role in the induction and down-regulation of inflammation, we cultured rat bone marrow-derived macrophages in the presence of LPS, with or without an inhibitor (ie, IL-13 or PKCζi), to understand better the effect of the inhibitor on the expression of signaling molecules in early and late phases of the inflammatory response.

Signaling Molecules Involved in Initiation and Resolution of Inflammation in LPS-Stimulated Bone Marrow-Derived Macrophages

Western blots of bone marrow-derived macrophage lysates demonstrated that incubation of macrophages with LPS for 6 hours induced increased NF-κB/p65 expression (Figure 1, B2) compared with control cells (Figure 1, B1) with high levels of TNF-α in supernatants (336.5 ± 9.5 pg/ml) (Figure 2). However, coincubation with LPS and IL-13 suppressed NF-κB/p65 expression (Figure 1, B3) and significantly decreased production of TNF-α (197.3 ± 39 pg/ml, $P = 0.02$) (Figure 2), suggesting an inhibitory effect of IL-13 on macrophage activation. In addition, our results suggested that macrophage activation status varied according to the treatment. At 6 hours, LPS-activated macrophages are in a proinflammatory phase, whereas IL-13-treated macrophages are already shifted into the resolution phase, as indicated by the decrease in TNF-α production.

A prolonged LPS exposure (24 hours) induced cleavage of PKCζ at its hinge region, generating a fragment of 50 kd (Figure 1, A2) compared with control (Figure 1, A1) that correspond to the PKCζ catalytic subunit, in association with low expression of NF-κB/p65 and Bcl-2 (Figure 1, B2 and C2, respectively) and expression of Bax (Figure 1, C2). Analysis of nuclear extracts from LPS-exposed cells showed that the PKCζ catalytic subunit translocated into the nucleus together with the native form of 72 kd (Figure 3A). At this time point, we observed a depletion of nuclear NF-κB/p65 (Figure 3B), whereas NF-κB/p50 and BCL-3 were clearly detectable in the nucleus (Figure 3, B and C). Lower levels of TNF-α (170.4 ± 19 ng/ml) were secreted at this time point (24 hours) compared with TNF-α production at 6 hours (336.5 ± 9.5 pg/ml) (Figure 2). These results are consistent with previous observations[26,27] showing that accumulation of NF-κB/p50 and BCL-3 decreases TNF-α production. These results suggest that, at 24 hours, LPS-stimulated macrophages are entering a resolution phase.

The addition of IL-13 or PKCζi to LPS impeded the cleavage of PKCζ at 24 hours (Figure 1, A3 and A4), with increased expression of NF-κB/p65 (Figure 1, B3 and B4) and Bcl-2 (Figure 1, C3 and C4) and no Bax expression (Figure 1, C3, and C4), consistent with activation of a survival pathway. To demonstrate further the activation of this pathway during IL-13 or PKCζi treatment, we analyzed nuclear extracts of macrophages. We observed the

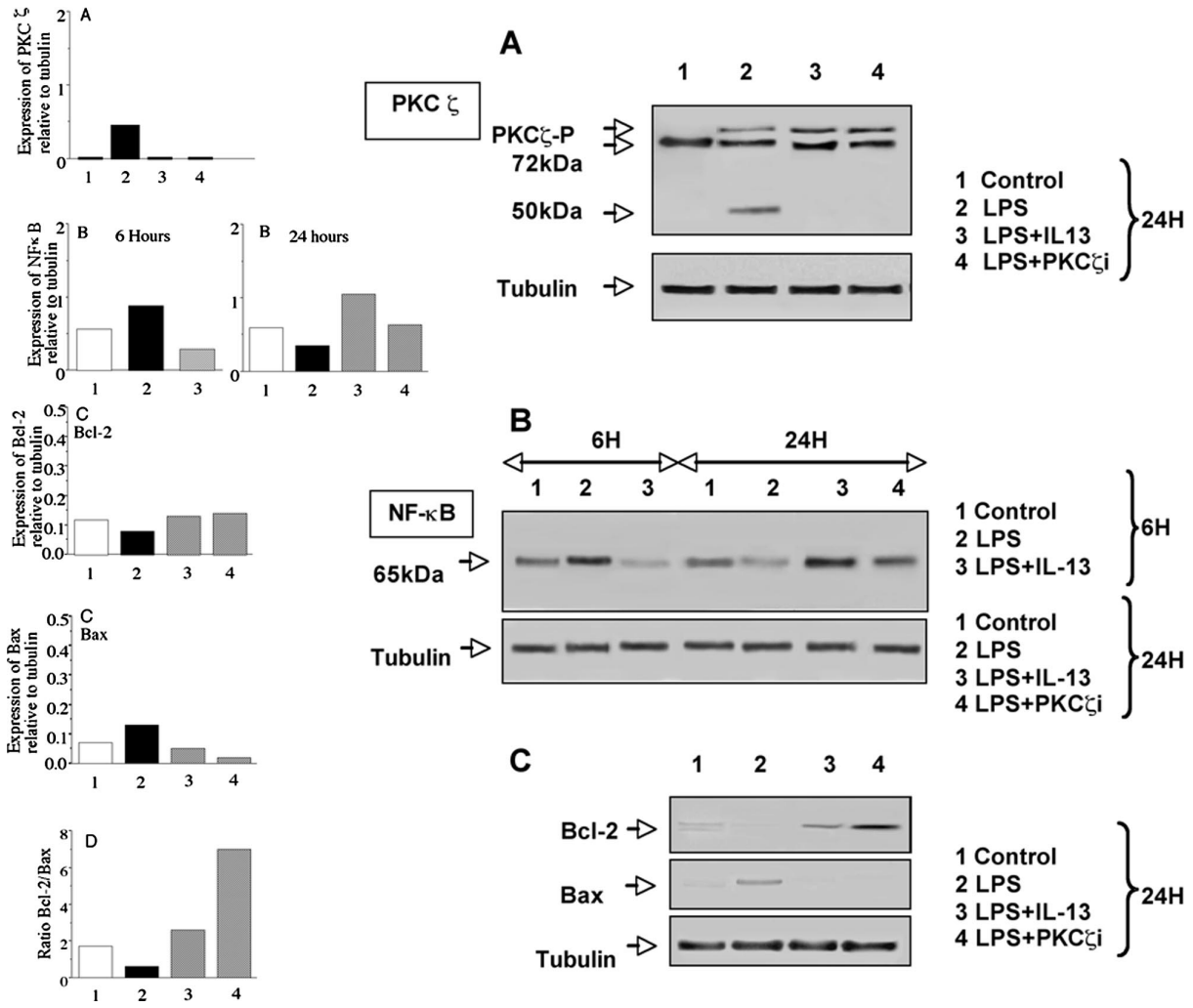


Figure 1. Western blots (**right**) of bone marrow-derived macrophages stimulated *in vitro* with LPS, LPS + PKCζi, and LPS + IL-13; expression of PKCζ, NF-κB, Bcl-2, and Bax. Constitutive expression of PKCζ, NF-κB, and Bcl-2 and Bax (lanes 1 of **A**, **B**, and **C**). Expression of NF-κB (**lane 2**, **B**) after incubation of macrophages with LPS (100 ng/ml) or with LPS + IL-13 (20 ng/ml) (**lane 3**, **B**) for 6 hours. Expression of PKCζ (**lane 2**, **A**), NF-κB (**lane 2**, **B**) Bcl-2 and Bax (**lane 2**, **C**) after incubation of macrophages with LPS (100 ng/ml) for 24 hours or with LPS + IL-13 (20 ng/ml) (lanes 3 of **A**, **B**, and **C**) or with LPS + PKCζi (20 μg/ml) (lanes 4 of **A**, **B**, and **C**). A high ratio of Bcl2/Bax was observed after incubation with LPS + PKCζi (**graph D**, bar 4). Results from two separate experiments. Data presented in the graphs represent ratios of intensities of signals obtained by scanning the bands of the proteins of interest and the bands of tubulin in each agarose gel. **Graph A** represents 50-kd PKCζ expression.

nuclear expression of the NF-κB/p50 subunit with a low level of nuclear phosphorylated NF-κB/p65, very faint BCL-3 expression (Figure 3, B and C, respectively), and very low levels of TNF-α (50.4 ± 11.8 pg/ml, $P = 0.03$) compared with TNF-α production in LPS-stimulated macrophages. In summary, the preservation of intact PKCζ by treatment with IL-13 or PKCζi induced a signaling cascade that led to a high ratio of Bcl-2 to Bax and very low levels of TNF-α, indicating that these cells were in the postresolution period and were committed to survival. In agreement with our findings, predominance of NF-κB/p50/p50 homodimers have been shown to be responsible for suppression of inflammatory mediators, and suppression of NF-κB during this phase is known to impede resolution.[27]

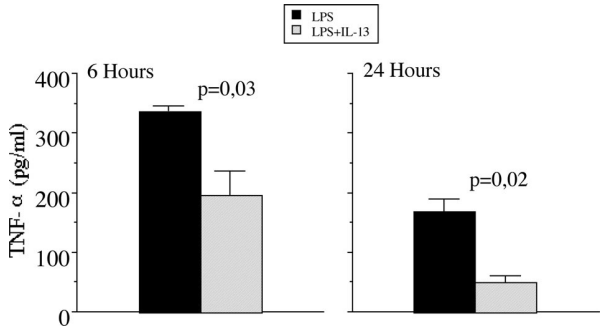


Figure 2. TNF-α levels at 6 hours and 24 hours in supernatants from bone marrow-derived rat macrophages after stimulation with LPS (100 ng/ml) in combination with or without IL-13 (20 ng/ml).

Nitrite Production in Macrophage Supernatants

Nitrite levels in supernatants from unstimulated macrophages were below the limit of detection. At 6 hours, low levels of nitrite were detected in supernatants from LPS-stimulated cells and cells incubated with LPS combined with IL-13 and PKCζi. At 24 hours, LPS

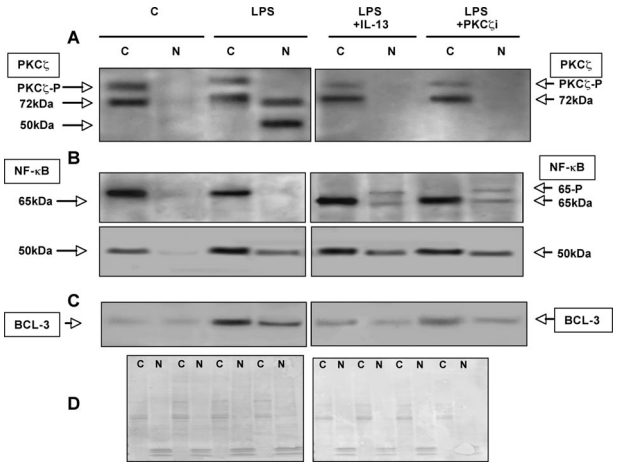


Figure 3. Western blot analysis of subcellular location of PKCζ (**A**), NF-κB/p65 (**B**), NF-κB/p50 (**B**), and BCL-3 (**C**) in bone marrow-derived macrophages. Cytosolic (C) and nuclear (N) fractions from macrophages cultured in the presence of LPS (100 ng/ml), LPS + IL-13 (20 ng/ml), and LPS + PKCζi (40 μg/ml) were compared with the untreated control. Comparison of transferred proteins, stained by ponceau, indicated that cytosolic or nuclear fractions were equally loaded (**D**). Data are representative of two separate experiments.

induced the release of nitrite (57.7 ± 2.9 μmol/L) by macrophages. The incubation of cells with LPS combined with IL-13 (20 ng/ml) or with PKCζi (20 μg/ml) significantly decreased nitrite levels (17.7 ± 1.2 μmol/L, $P = 0.03$, and 36.7 ± 7.1 μmol/L, $P = 0.03$, respectively) demonstrating the deactivation of macrophages by IL-13 or PKCζi treatment (data representative of two independent experiments).

Injection of PKCζ Inhibitor PKCζi into the Anterior Chamber of the Eye Reduces LPS-Induced Ocular Inflammation

Injection of LPS into the footpads was shown to induce an inflammatory ocular response that reached its maximum at 16 to 24 hours.[43] To demonstrate the role of PKCζ in the development of ocular inflammation, the specific inhibitory peptide of PKCζ, PKCζi, was injected into the anterior chamber of rats with EIU and its inhibitory effect on ocular inflammation was checked at 24 hours.

Clinical Examination

A significant reduction ($P = 0.007$) in the severity of the ocular inflammation was detected by biomicroscopic examination at 24 hours after induction of EIU by injection of LPS and treatment with PKCζi (2 μg/eye) (mean EIU score: 3.4 ± 0.3, $n = 6$ rats), corresponding to 24% of inhibition in EIU score compared with the score observed in saline-injected controls (mean EIU score: 4.5 ± 0.2, $n = 5$ rats). These results are representative of three separate experiments. Injection of 3 μg of PKCζi/eye did not result in an increased inhibitory effect (mean EIU score in treated rats: 3.8 ± 0.2, $n = 7$ rats versus mean EIU score in saline-injected controls: 5 ± 0.001, $n = 7$ rats, $P = 0.0001$, corresponding to 25% inhibition in severity of EIU). These results suggest that the reduction of clinical EIU induced by the modulation of this signaling pathway reaches a maximum, beyond which the degree of inflammation cannot be further reduced. A trend to dose dependence of this inhibitory effect was suggested since injections of 0.5 and 1 μg of PKCζi induced a reduction in the severity of EIU ($n = 4$ rats/group) (data not shown).

The inhibitory effect of PKCζi was compared with the effect of IL-13. Injection of 2 ng of IL-13/eye significantly reduced the severity of EIU (mean EIU score: 2.1 ± 0.3, $n = 8$ rats) compared with the EIU score in saline-injected controls (mean EIU score: 3.2 ± 0.3, $n = 8$ rats) by 35% ($P = 0.05$). These results are representative of three separate experiments. In a previous work,[40] we reported 63% inhibition in EIU as a result of the injection of 6 ng of IL-13/eye, suggesting a dose dependence of IL-13 inhibitory effect.

The result of injecting PKCζi at different time points was also tested. When PKCζi was injected 3 hours before LPS injection, a significant reduction in clinical score was recorded for PKCζi-injected rats (mean score: 2.2 ± 0.3, $n = 4$ rats) compared with saline-injected rats (mean score: 4 ± 0, $n = 4$ rats), $P = 0.005$ (data representative of two independent experiments). However, in this experiment, corneal edema was detected by slit lamp examination in two of four rats in the PKCζi-injected group. When PKCζi was injected 6 hours after LPS injection, although there was reduction of EIU, the difference observed between treated and saline-injected control rats ($n = 4$ rats/group) was not statistically significant.

Quantification of Cell Number

The inhibitory effect on clinical EIU of injection of PKCζi into the anterior chamber was confirmed by cellular counts of inflammatory cells on cryostat sections. Twenty-four hours after systemic injection of LPS and intraocular injection of PKCζi (2 μg/eye) or saline, the number of DAPI-positive cells was counted for each group. A significantly lower number of DAPI-positive cells was found in ocular tissues of treated rats (208 ± 26 cells) compared with saline-injected rats (400 ± 39 cells) ($P = 0.005$). This inhibitory effect of the PKCζi injection on inflammatory cell infiltration was also found when it was performed 3 hours before LPS injection (data not shown). Data are expressed as mean ± SEM of two sections/rat/group in which control and treatment groups contained four rats. Data are representative of two independent experiments.

Protein Concentration in Aqueous Humor/Vitreous Body

PKCζi treatment induced a trend to reduced protein exudation into the aqueous humor/vitreous body compared with levels measured for control rats (mean protein value

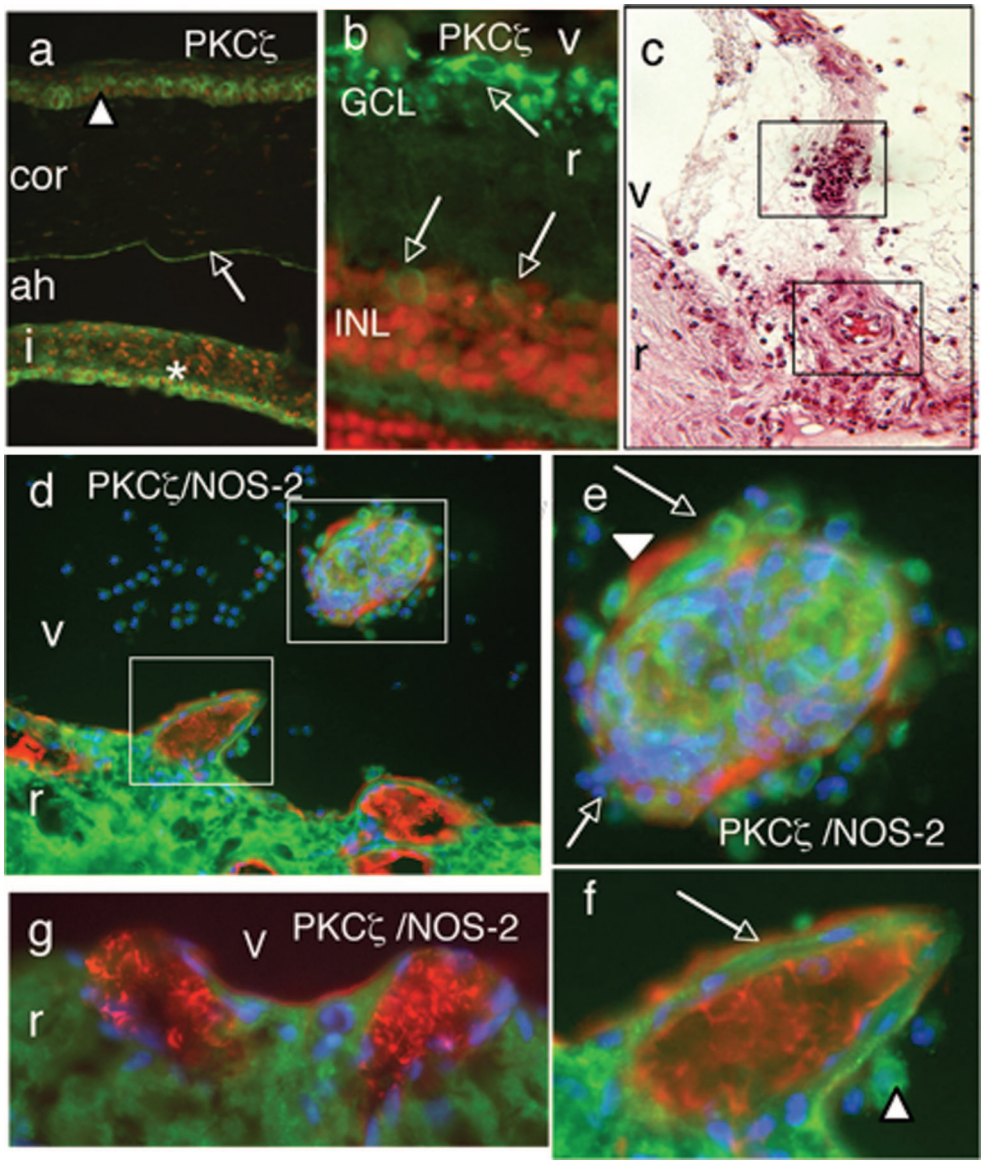


Figure 4. Immunohistochemical expression of PKCζ in the normal rat eye. **a:** Expression of PKCζ (green) detected in corneal epithelium (**arrowhead**), corneal endothelium (**arrow**), and iris (**asterisk**) [nuclear staining with propidium iodide (red)]. **b:** Ganglion cells and the inner nuclear cell layer the retina (**arrows**) [nuclear staining with DAPI (blue)]. **c:** Hematoxylin and eosin-stained section of a large vessel in the vitreous in a rat with EIU. **d–g:** Immunohistochemical expression of PKCζ and NOS-2 in retinal vascular endothelial cells from saline- (**d–f**) or PKCζi- (**g**) injected rats at 24 hours after LPS injection [PKCζ (green), NOS-2 (red), nuclear staining with DAPI (blue)]. cor, cornea; ah, aqueous humor; i, iris; GCL, ganglion cell layer; INL, inner nuclear layer; v, vitreous; r, retina. Photomicrographs were obtained from one eye of one rat and are representative of similar sections stained from five rats per experiment in two separate experiments. Original magnifications: ×120 (**a**); ×430 (**b**); ×200 (**c**); ×260 (**d**); ×680 (**e**); ×680 (**f**); ×340 (**g**).

in treated, 30.2 ± 4.4, $n = 7$ rats, compared with mean protein value in controls, 45.8 ± 7.4, $n = 7$ rats, $P = 0.2$).

PKCζ, NF-κB, Caspase 3, and TLR4 Expression by Resident Ocular Cells and by Infiltrating Inflammatory Cells: Immunohistochemical Study

To better understand the mechanisms of the inhibitory effect of the intraocular injection of PKCζi or IL-13 during EIU, we determined the intracellular localization of the signaling molecules in ocular tissues. Cytoplasmic versus nuclear expression of NF-κB, PKCζ, and activated caspase 3 were quantified on immunostained ocular sections from control and PKCζi- or IL-13-treated rats.

Expression of PKCζ Is Detected in Normal Ocular Tissues

Expression of PKCζ was detected in normal ocular tissues, ie, in corneal epithelium, iris epithelium, ciliary body epithelium (Figure 4a), and in the ganglion cell and inner nuclear layers of the retina (Figure 4b).

Expression of PKCζ and NOS-2 in Vascular Endothelial Cells from Rats with EIU and IL-13- or PKCζi-Treated Rats

Vascular endothelial cells regulate leukocyte trafficking out of the blood vessels during ocular inflammation.[50–52] To understand better the role of PKCζ in the regulation of tight junction activity at the site of leukocyte extravasation, we checked the expression of PKCζ in vessels from rats with EIU, as well as PKCζi-treated rats. In rats with EIU, vessels protruded into the vitreous at the level of the papilla of the optic nerve (Figure 4, c–f). In the portion of the vessel close to the internal limiting membrane of the retina, PKCζ was expressed and translocated to the vascular endothelial cell membrane. Limited numbers of inflammatory cells were detected in the vitreous adjacent to this site (Figure 4, d and f). In contrast, where the vessel passed into the vitreous, PKCζ could not be detected in vascular endothelial cells (Figure 4e) and inflammatory cells were observed within the vessel and extravasating into the vitreous through the vessel wall (Figure 4, d and e). Lipopolysaccharide is a potent inducer of NOS-2 in retinal capillary endothelial cells.[53] It was not unexpected, therefore, that expression of NOS-2 was found in the retinal vascular endothelial cells and the adjacent pericytes (Figure 4, d–f). Treatment with PKCζi at the same time as the LPS injection impeded activation of PKCζ in vascular endothelial cells of the papilla. In this setting, vessels did not demonstrate membranous expression of PKCζ, and there was also no expression of NOS-2 (Figure 4g). Correlating with this, no inflammation was visible at this location. These results strongly suggest the participation of PKCζ in the regulation of the permeability of the retinal vascular endothelial cell junction, in NOS-2 expression in the vessel walls, and in the movement of inflammatory cell from ocular vessels into eye tissues.

Difference in Expression of PKCζ, NF-κB, Caspase 3, and NOS-2 by Macrophages and PMNs

Injection of LPS induced an infiltration of the ocular tissues by numerous inflammatory cells (Figure 5a), and this was reduced by treatment with PKCζi (Figure 5b). To study the mechanisms responsible for the PKCζi effect, we investigated the signaling pathways responsible for survival or apoptosis of intraocular macrophages and PMNs.

Expression of PKCζ, NF-κB, Caspase 3 (Active Form), TGF-β2, and NOS-2 in Macrophages

At 24 hours, in EIU rats injected with saline alone, numerous macrophages expressed cytoplasmic and nuclear PKCζ (Figure 5c) and NF-κB (Figure 5d) with low numbers of ED1-positive macrophages expressing caspase 3 (active form) (Figure 5e). We counted immunostained sections to evaluate the proportion of ED1-positive macrophages and PMNs expressing nuclear NF-κB/p65 and caspase 3. Macrophages showed nuclear expression of NF-κB/p65 in 39% of ED1-positive cells (Figure 6). In addition, nuclear expression of caspase 3 (active form) was detected in only 16% of cells, suggesting that most macrophages would survive.

In rats treated with PKCζi at the same time they received the LPS, the number of macrophages expressing nuclear NF-κB/p65 fell to 27% of cells, with a reduced expression of nuclear caspase 3 (6.1% of cells) compared with the saline-injected controls (Figure 6), suggesting that ED1-positive macrophages that had infiltrated the ocular tissues were resistant to apoptosis. No change in the percentage of cells expressing nuclear versus cytoplasmic localization of PKCζ was observed in LPS versus PKCζi-treated rats, further suggesting that macrophages were destined for survival.

To further analyze the activation state of macrophages, we measured NOS-2 expression at 24 hours. Cytoplasmic coexpression of NOS-2 and PKCζ was detected in rats with EIU (Figure 5f). Treatment with PKCζi inhibited NOS-2 expression in ED1-positive macrophages (Figure 5g), suggesting that expression of NOS-2 was dependent on PKCζ signaling.

It is important to note that TGF-β2 is released during the resolution of ocular inflammation.[54] We investigated the expression of TGF-β2 in IL-13-treated rats. At 16 hours, TGF-β2 was expressed by macrophages from rats injected with LPS and treated with IL-13, but not in macrophages from control LPS-injected rats (Figure 5, h and i), indicating that the resolution of inflammation is accelerated in treated versus control animals.[24,55]

It is known that infiltrating macrophages adapt their response to the local microenvironment.[56] Considered together, our results are consistent with previous observations[49] reporting that macrophages remain resistant to apoptosis, showing different phenotypes, ie, either an effector phenotype, producing TNF-α and nitrite, or an alternatively activated phenotype with low TNF-α and NO, and high TGF-β as a result of IL-13 or PKCζi treatment.

Expression of PKCζ, NF-κB, Caspase 3, and NOS-2 in PMNs

At 24 hours, in LPS-injected and PKCζi-treated rats, only 9 and 7.6% of PMNs and only 1.9 and 2.1%, respectively, expressed exclusively nuclear NF-κB/p65 and PKCζ (Figure 6). Nuclear expression of caspase 3 was detected in 24.4% of PMNs in LPS-injected rats (Figures 5e and 6) with fragmented nuclei observed in limited number of cells (Figure 7a) and high level of Bax expression (Figure 7b), whereas 49.8% of cells were stained in treated rats (Figure 6). The treatment with PKCζi induced a marked increase in the number of cells expressing caspase 3 in the nucleus (49.8% of cells) (Figure 6). This is consistent with the dramatic increase of the number of apoptotic PMNs with fragmented nuclei present in the vitreous detected in IL-13-treated rats (Figure 7c).

Altogether, the low number of PMNs showing nuclear and cytoplasmic expression of NF-κB and PKCζ and the

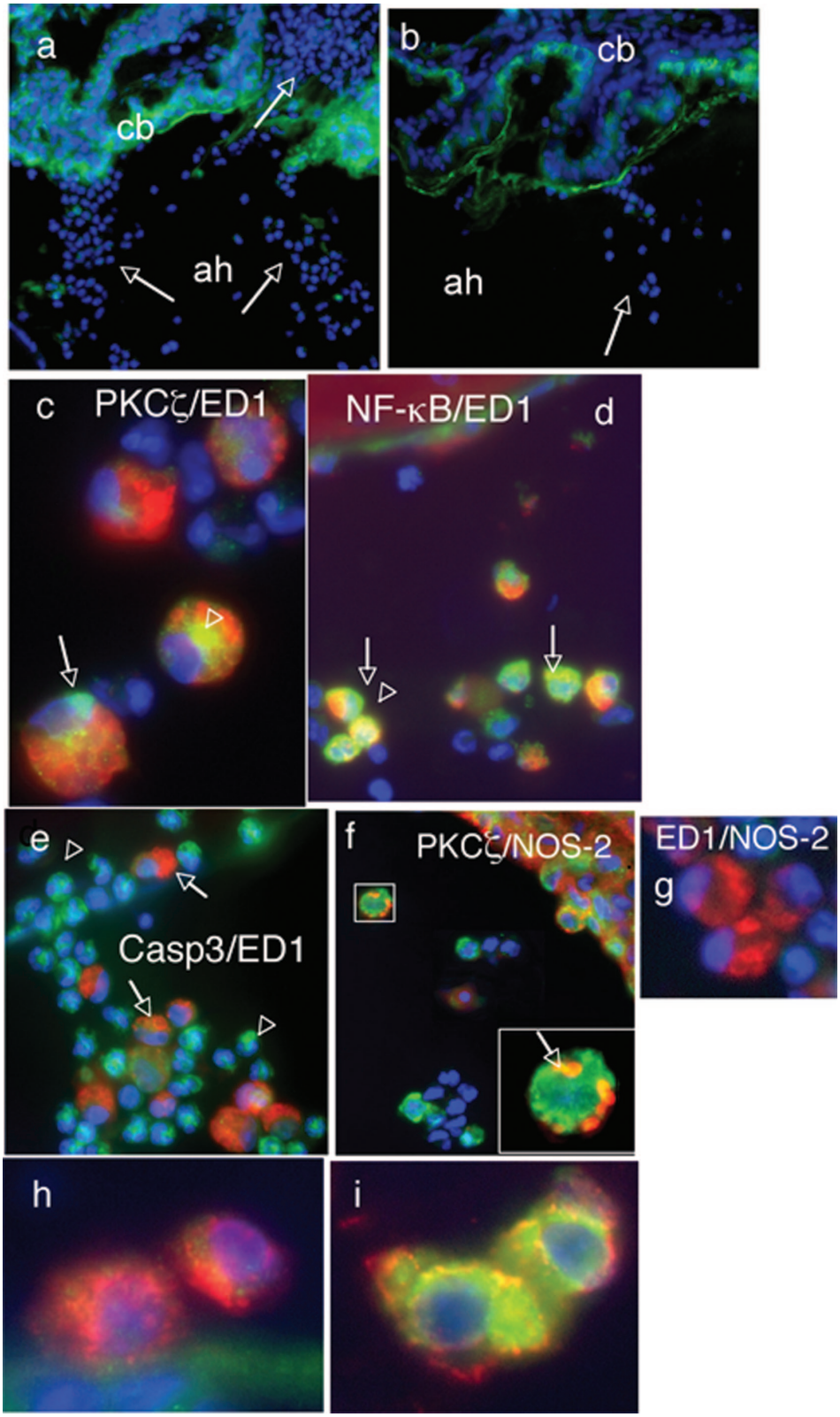


Figure 5. PKC*ζ*, NF-κB, activated caspase 3, and NOS-2 in ED1-positive macrophages and PMNs from saline- or PKC*ζ*i-injected rats with EIU at 24 hours after LPS injection. **a:** Numerous inflammatory cells in EIU rats (**arrows**). **b:** Low numbers of cells (**arrow**) in PKC*ζ*i-treated rats (nuclei staining blue with DAPI; PKC*ζ*, green). **c** and **d:** In EIU rats, numerous ED1-positive macrophages (red) expressing nuclear PKC*ζ* (turquoise) (**c**) or NF-κB (turquoise) (**arrows**) (**d**) and cytoplasmic PKC*ζ* (yellow) (**c**) or NF-κB (yellow) (**arrowhead**) (**d**). **e:** Low number of ED1-positive macrophages (red) expressing caspase 3 (green) (**arrows**) and high nuclear expression of caspase 3 (green) in PMNs (**arrowheads**). **f:** Cytoplasmic coexpression (yellow) of NOS-2 (red) and PKC*ζ* (green) (**arrow**) in ED1-positive macrophages. **g:** No expression of NOS-2 (green) in ED1-positive macrophages (red) in PKC*ζ*I-treated rats. **h** and **i:** TGF-*β* expression by ED1+ macrophages in controls (**h**) and IL-13-treated rats (**i**). cb, ciliary body; ah: aqueous humor. Photomicrographs are representative of similar sections stained from five rats per experiment in two separate experiments. Original magnifications: ×150 (**a, b**); ×2250 (**c, h, i**); ×750 (**d**); ×850 (**e, f**); ×950 (**g**).

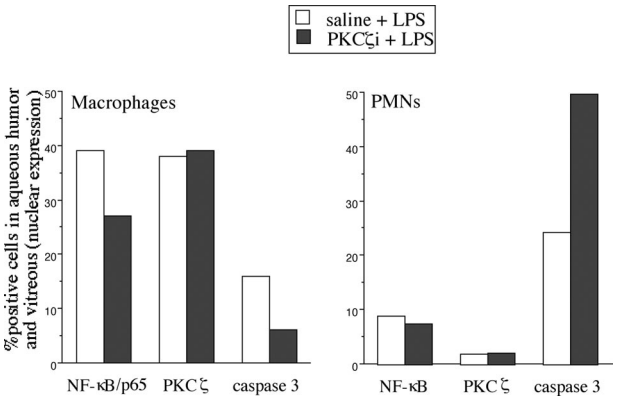


Figure 6. Effect of saline or PKCζi treatment at 24 hours after LPS injection on the percentage of ED1-positive macrophages and PMNs expressing nuclear NF-κB/p65, PKCζ, and activated caspase 3 within the aqueous humor and vitreous body of rats with EIU. Infiltrating inflammatory cells (ED1-positive cells and PMNs) showing nuclear localization of PKCζ, NF-κB/p65, and caspase 3 were counted on cryostat sections from rats with EIU that were treated with saline ($n = 5$ rats) or with PKCζi ($n = 5$ rats). A total of 500 cells were counted per treatment, representing approximately 100 cells/rat. Percentages of nuclear staining were expressed as described in Materials and Methods. Results show one experiment that was representative of two independent experiments.

high number expressing caspase 3 in the nucleus suggested that PMNs were undergoing apoptosis during EIU and that this was further increased when treatment with PKCζi was given. Altogether, these results suggest that different pathways of activation, survival, and apoptosis are operating, depending on the subtype of the infiltrating leukocyte.

Expression of PKCζ, NF-κB, and Caspase 3 in Resident Ocular Cells

Because uveitis causes damage to intraocular structures and because nuclear translocation of PKCζ was shown to play a role in events crucial for the initiation of apoptosis,[33] we analyzed the expression of PKCζ, NF-κB, and caspase 3 in ocular resident cells from rats with EIU, including rats treated with IL-13 or PKCζi. In the anterior segment of the eye during EIU, corneal endothelial cells showed nuclear translocation of PKCζ (Figure 7d) and caspase 3 (Figure 7e) in the vicinity of an ED1-positive macrophage, suggesting that endothelial cells were undergoing apoptosis. Iris and ciliary body epithelium showed translocation of PKCζ from the cytosol to the cell membrane, indicating an activation of cells (Figure 8a) that was inhibited by treatment with IL-13 (Figure 8d). In the posterior segment of the eye, in the ganglion cell layer of the retina, the nuclear translocation of caspase 3 (Figure 7f), PKCζ (Figure 8b), and NF-κB (Figure 8, f and g) suggested ongoing cell death. In bipolar cells, translocation of PKCζ from the cytosol to the cell membranes was noted (Figure 8b); this was reduced in treated animals (Figure 8e), suggesting a protection of these cells by IL-13 treatment. In the external nuclear layer, the detection of some TUNEL-positive nuclei of retinal photoreceptors suggested apoptosis of these cells during EIU (Figure 7g). Taken together, our findings suggest that LPS induces activation of PKCζ and nuclear translocation of activated caspase 3 in resident ocular cells, leading to cell death that may be prevented by treatment with IL-13 or PKCζi.

TLR4 Signaling by Infiltrating Inflammatory Cells

Because TLR4 has been reported as a member of pattern-recognition receptors essential in the recognition of LPS, we analyzed its expression in inflammatory cells infiltrating the eyes of rats with EIU (Figure 9a). In LPS-injected rats, in the anterior segment of the eye, expression of TLR4 was detected in the epithelium of the iris and in several ED1-positive cells adjacent to this epithelial layer (Figure 9b). In addition, most PMNs stained positively for TLR4, with a cytoplasmic perinuclear distribution (Figure 9c). IL-13 treatment led to a down-regulation of TLR4 expression by inflammatory cells (Figure 9d). These results further suggested that PKCζ, together with TLR4, were participating in the control of the movement of inflammatory cells into the tissues and to their activation.

Analysis of Cytokine mRNA Expression in the Eye

The expression of cytokine mRNA was investigated by semiquantitative reverse transcription-polymerase chain reaction analysis of eyes from rats taken at 24 hours after injection with LPS and PKCζi or saline treatment. Results in two PKCζi-treated and two saline-injected control rats are shown in Figure 10. When compared with controls, PKCζi treatment significantly reduced the mRNA levels of TNF-α ($P = 0.0001$), NOS-2 ($P = 0.0001$), and IL-6 ($P = 0.06$).

Discussion

Inflammation is a pathological condition in which different signaling mechanisms control a complex network of cellular and molecular interactions. The crosstalk between apparently independent biochemical cascades results in the activation of signaling pathways that lead to expression of cytokine genes. Nuclear factor-κB transcription factor complexes are critically involved in the control of a number of cellular responses during inflammation as well as in the innate and adaptive immunity and in repression of apoptosis.[22,57,58] In the canonical pathway, NF-κB is retained in the cytosol of unstimulated cells by IκB, the inhibitor protein that is degraded on cell activation by a number of stimuli, including TNF-α and LPS. During EIU, modulation of NF-κB signaling by the administration of antioxidants has been reported to reduce ocular inflammation.[59,60] In our study, we show that the development of inflammation and the preventive effect of IL-13 or PKCζi on ocular inflammation and injury are dependent on the signaling activity of PKCζ, regulating the activity of different subunits of NF-κB and modulating genes involved in cell survival and apoptosis.

To understand the effect of IL-13 or PKCζi treatment on

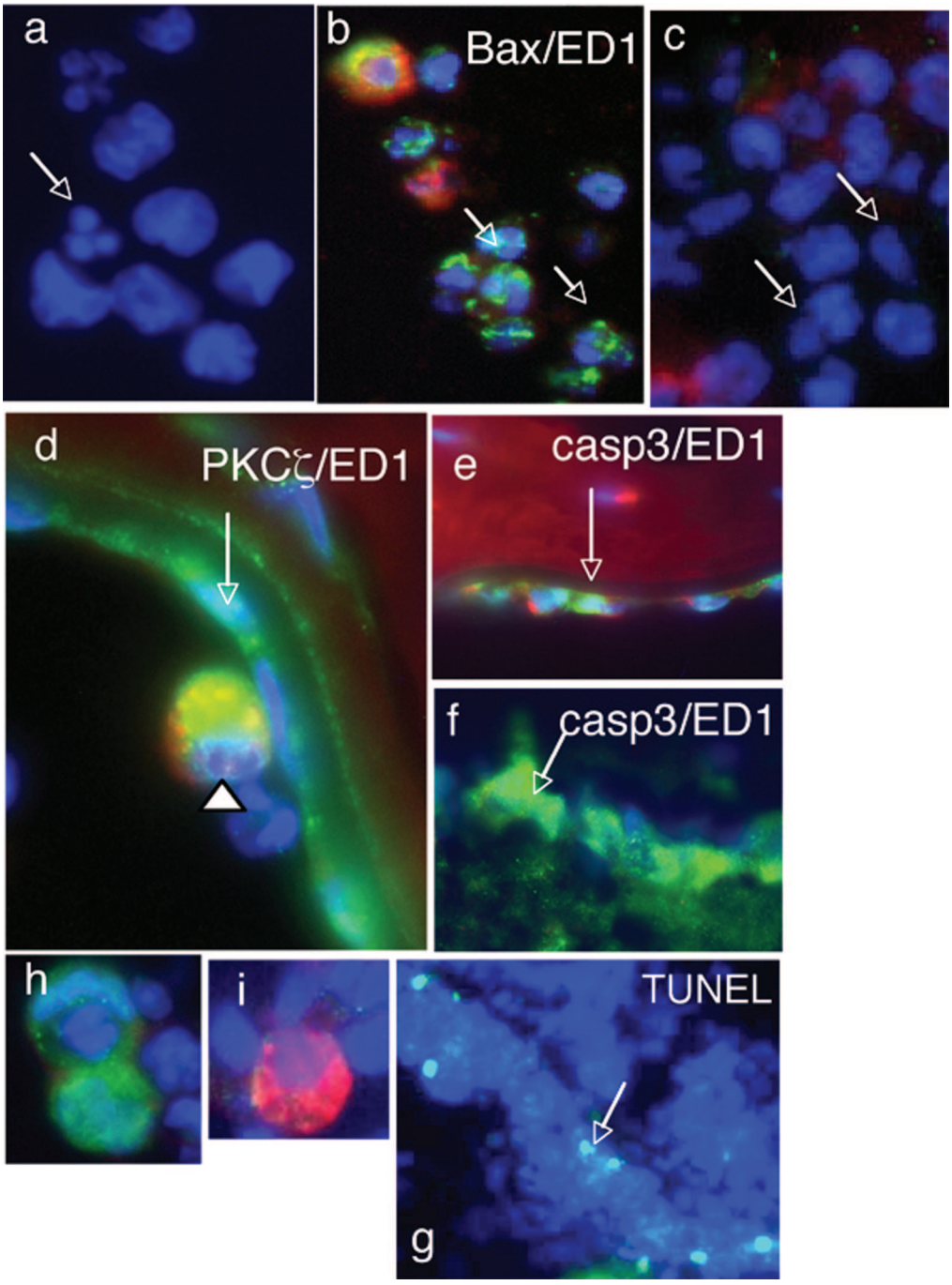


Figure 7. Apoptosis in ocular tissues and in infiltrating inflammatory cells in saline-, IL-13-, and PKCζi-treated EIU rats, at 24 hours after LPS injection. PKCζ, green; Bax, green; caspase 3, green; ED1, red; nuclear staining with DAPI, blue. **a:** In EIU rats, 24 hours after LPS injection, PMNs showing nuclear condensation (**arrow**). **b:** Bax expression at 24 hours in PMNs (**arrows**). **c:** At 24 hours after IL-13 treatment, apoptotic PMNs (nuclei DAPI staining). **d:** In EIU rats, corneal endothelial cells with nuclear translocation of PKCζ (**arrow**) in the vicinity of an ED1-positive macrophage showing cytoplasmic and nuclear PKCζ expression (**arrowhead**). **e:** Nuclear translocation of activated caspase 3 in corneal endothelial cell (**arrow**). **f:** Nuclear translocation of caspase 3 in retinal ganglion cells (**arrow**). **g:** TUNEL-positive retinal photoreceptors (**arrow**). **h:** Negative control with the replacement of mouse anti-ED1 monoclonal antibody by an isotype-matched control mouse monoclonal antibody, showing no ED1 staining but staining for PKCζ (green). **i:** Negative control with the replacement of rabbit anti-PKCζ polyclonal antibody by normal rabbit serum Ig, showing no PKCζ staining but staining for ED1 (red). Photomicrographs were obtained in one eye in each group and are representative of similar sections stained from five rats per group in two separate experiments. Original magnifications: ×1950 (**a, c**); ×1500 (**b**); ×2250 (**d**); ×500 (**e**); ×1500 (**f**); ×300 (**g**); ×2250 (**h, i**).

the expression of signaling molecules, the state of activation of rat bone marrow-derived macrophages was first studied *in vitro*. We demonstrated that stimulation of macrophages with LPS induced NF-κB/p65 expression at an early time point (ie, 6 hours), resulting in high TNF-α production. Coincubation of cells with LPS and IL-13 reduced NF-κB/p65 expression and lowered TNF-α levels, suggesting an accelerated reduction in the production of inflammatory mediators by macrophages as well as accelerated inactivation of these cells.

After a prolonged LPS stimulation of 24 hours, cleavage of PKCζ resulted in depletion of nuclear NF-κB/p65, activating the alternative NF-κB pathway involving NF-κB/p50 and BCL-3, consistent with the onset of spontaneous resolution of inflammation with a low ratio of Bcl-2 to Bax. At this time point, coincubation of cells with LPS and IL-13 or PKCζi impeded PKCζ cleavage, with reduced nuclear phosphorylation of NF-κB/p65, expression of nuclear NF-κB/p50, and low BCL-3. At the same time, the amount of TNF-α was dramatically decreased and the ratio of Bcl-2 to Bax was increased. These results suggested that IL-13 or PKCζi treatment accelerated the repression of transcription of genes encoding proinflammatory molecules in comparison to the effect of LPS alone. Resultant macrophage deactivation was manifest as shown by the reduction of nitrite and TNF-α production. In keeping with our findings, BCL-3 has been reported to inhibit NF-κB/p65 binding during the resolution time in acute experimental pleuritis by forming ternary complexes with DNA-bound NF-κB/p50/p50.[27] This inhibited proinflammatory cytokine expression-directed cells into a survival pathway by induction of Bcl-2 expression via NF-κB/p65/50 with restoration of intracellular homeostasis.[61] Taken together, these data suggest that a DNA site that binds both NF-κB/p65/p50 and NF-κB/p50/p50 might function as an initial transcriptional activator for proinflammatory genes but as a repressor of inflammation in the later phase of the response.[26] Because nitrite and TNF-α play a key role in pathogenesis of inflammatory diseases including uveitis,[56,62] these regulatory mechanisms could be of major importance in inflammatory eye disease.

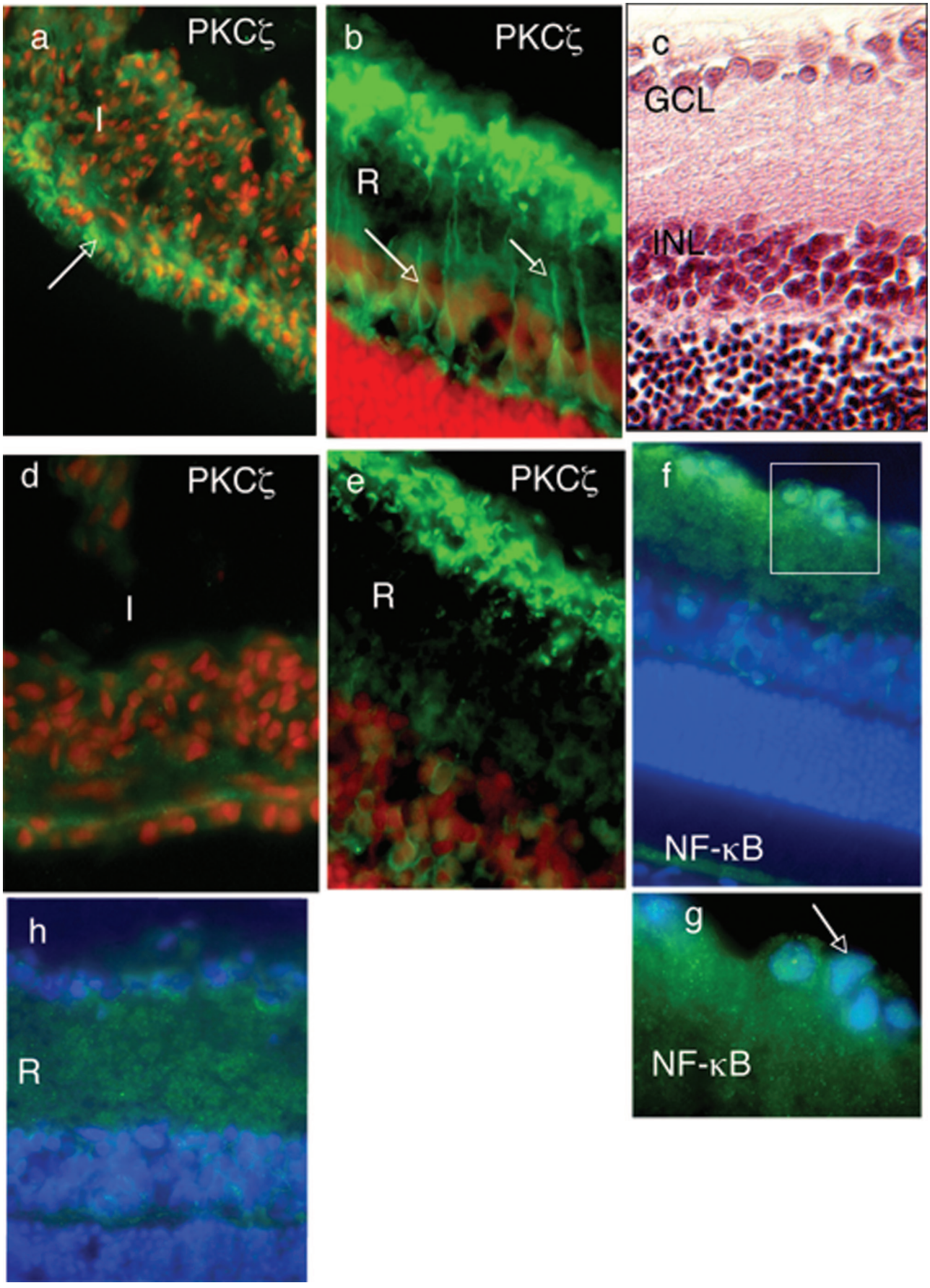
We then tested the *in vivo* effect of IL-13 or PKCζi on LPS-induced ocular inflammation. LPS injection induced a clinically apparent ocular inflammation that was downregulated when IL-13 or PKCζi were injected simultaneously with the LPS injection. A similar effect was found when the PKCζi injection was performed 6 hours after LPS injection, suggesting that this effect also operated during ongoing inflammation (data not shown). The clinical results were confirmed by histopathology; infiltrating cell counts were significantly decreased in ocular tissues and media in the context of treatment with IL-13 or PKCζi.

Endotoxin-induced ocular inflammation is associated with increased vascular permeability responsible for the early inflammatory cell infiltration into the eye.[63] Indeed, leukocyte trafficking out of the ocular vessels is regulated at the level of the vascular endothelial cells, which contain tight junctions.[50–52] Here, we observed that systemic injection of LPS in Lewis rats induced the translocation of PKCζ to the endothelial cell membrane in retinal vessels. This was not observed in PKCζi-injected rats, suggesting a protection of the endothelial cell tight junctions in that situation. Indeed, PKCζ has been reported to co-localize with the ZO-1 protein at the tight junction with a pattern of distribution similar to ZO-1, and thus it may regulate formation and permeability of tight junctions.[64,65] Our results suggest that PKCζ cleavage observed during EIU could be involved in the alterations of the retinal endothelial cell junctions and egress of inflammatory cells. In addition, IL-13 and PKCζi treatments were associated with a trend toward decreased protein exudation in aqueous humor/vitreous body of PKCζi-injected rats compared with controls. This is consistent with the observation that it takes the blood-aqueous barrier several days to be restored after inflammation.[66]

Endotoxin is a potent inducer of NOS in retinal capillary endothelial cells,[63] and NO has been shown to regulate vasodilation and permeability in the vessel walls.[63,67] Because inhibition of NOS-2 by an inhibitor of nitric oxide synthesis, NG-nitro-L-arginine methyl ester, was reported to reduce EIU,[11,67] we considered the effect of IL-13 or PKCζi on NOS-2 expression in vascular endothelial cells during the inflammation. We showed that PKCζi injection inhibited NOS-2 expression in vascular endothelium and pericytes, suggesting that PKCζ might also maintain tight junctions through the inhibition of NOS-2. The anti-inflammatory effect of IL-13[39,40] or PKCζi (the present study) was associated with decreased intraocular levels of inflammatory mediators, including TNF-α and NOS-2. During EIU, these mediators are produced locally by resident cells before the onset of inflammatory cell infiltration.[11,62,68] It is of interest that PKCζ has been implicated in TNF-α-induced ICAM-1 gene transcription in endothelial cells, inducing adhesion of PMNs.[62,69] These results suggest that IL-13 and PKCζi suppressed the induction of NO by vascular endothelial cells, thus limiting vascular leakage, reduced infiltration of the eye by inflammatory cells, and promoted the re-establishment of the immunosuppressive microenvironment.

Immunohistochemistry showed that LPS injection induced activation of PKCζ in different ocular cell types with a nuclear and/or membrane translocation of PKCζ detected in iris and ciliary body epithelium and in retinal ganglion and bipolar cells. Importantly, nuclear activated caspase 3 expression was detected in corneal endothelium and retinal ganglion cells, together with TUNEL-positive cells in the photoreceptor cell layer, suggesting that these cells were undergoing apoptosis. IL-13 or PKCζi injection decreased PKCζ activation in ocular tissues of rats with EIU. The absence of nuclear caspase 3 and TUNEL-positive cells suggested that the treatment protected ocular cells against cell death. These results were well in keeping with our previous observation that when cultured PC12 cells were activated with *N*-methyl-D-aspartate, translocation of PKCζ into the nucleus was followed by apoptosis that could be blocked by caspase 3 inhibition. This suggested that the proapoptotic effect of nuclear PKCζ was dependent on caspase 3 activation.[33]

Lipopolysaccharide is reported to induce apoptosis in inflammatory cells.[12,70–73] However, the molecular mechanisms that regulate the survival or apoptosis of inflammatory cells are not fully understood. In our study, 24 hours after LPS injection, very low expression of PKCζ and NF-κB was found in PMNs, with expression of nuclear caspase 3 in 24% of cells and expression of TLR4 in the majority of cells. Whereas the PKCζi treatment did not alter the percentage of cells expressing PKCζ or NF-κB, it increased considerably the percentage of cells that expressed caspase 3 (50% of cells). Consistent with this finding, IL-13 treatment resulted in a substantial increase in apoptosis of PMNs in the vitreous. These results fit with the delay in apoptosis observed in LPS-activated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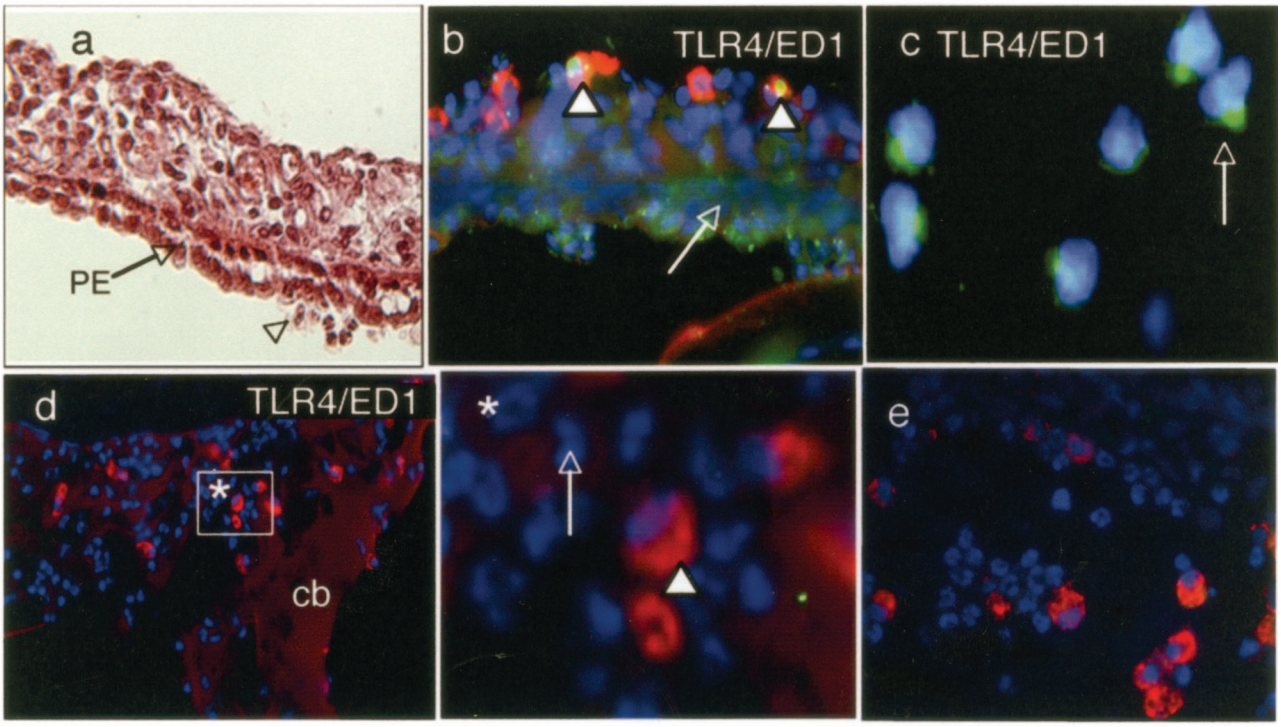

Figure 9. Immunostaining for TLR4 (green) and ED1 (red), and nuclear staining with DAPI (blue) in ocular tissues from rats with EIU, untreated (**a**, **c**) or treated with IL-13 (**d**, **e**). **a:** Hematoxylin-eosin stained section of iris [iris epithelium (**arrow**), inflammatory cells (**arrowhead**)]. **b:** Expression of TLR4 in the iris epithelium (**arrow**) and in ED1-positive macrophages (**arrowhead**). **c:** Expression of TLR4 in PMNs (**arrow**). **d:** In IL-13-treated rats, there is no expression of TLR4 in cells infiltrating the ciliary body. **Inset** provides high-magnification view of ED1-positive macrophages (**arrowhead**) and PMNs (**arrow**). **e:** Negative control with the replacement of goat anti-TLR4 polyclonal antibody by normal goat serum Ig, verifying the specificity of TLR4 staining. cb, ciliary body; PE, pigment epithelium. Photomicrographs were obtained in one eye in each group and are representative of similar sections stained from five rats per group in two separate experiments. Original magnifications: ×270 (**a**); ×300 (**b**); ×1500 (**c**); ×120 (**d**); ×1500 (**inset**, *); and ×360 (**e**).

human PMNs that is believed to relate to inhibition of mitochondrial depolarization with a subsequent reduction of caspase 3 processing.[74] Human uveitis may occur as a chronic inflammation or as an acute recurrent condition. Endotoxin-induced uveitis in rats has been reported to be a self-limited inflammation, with the number of neutrophils present in aqueous humor returning to basal levels by 1 week after intraperitoneal injection of LPS.[75] We have previously reported that 24 hours after footpad injection of LPS, the majority of PMNs present in anterior uvea are TUNEL-negative.[72] Our present results suggest that IL-13 treatment induces a premature apoptosis in PMNs, thus inducing an early resolution of EIU.

Immunostaining showed that during EIU, treatment with PKCζi decreased nuclear expression of NF-κB/p65 and caspase 3 in infiltrating ED1-positive macrophages, indicating that macrophages from PKCζi-treated rats would survive. This *in vivo* reactivity of macrophages is consistent with the deactivation of macrophages observed *in vitro* after PKCζi or IL-13 treatment. Moreover, TGF-β2 expression was detected in macrophages from LPS + IL-13-treated rats but not in macrophages from LPS-injected control rats. Interestingly, TGF-β is known to be released by macrophages in the presence of apoptotic PMNs during the resolution of inflammation.[27,55] In keeping with our experimental results, in uveitis in humans, TGF-β2 levels are reduced in aqueous humor and vitreous body during ocular inflammation.[54] In addition, in our study, decreased expression of TNF-α, IL-6, and NOS-2 mRNA was observed in ocular tissues from PKCζi-treated rats compared with saline-injected EIU rats. Furthermore, decreased production of TNF-α and nitrite was observed in supernatants from IL-13- or PKCζi-treated macrophages. This effect of IL-13 or PKCζi on infiltrating macrophages during EIU is consistent with reports on the generation of an "alternatively activated" phenotype in macrophages during IL-4 and IL-13 treatment.[76]

In conclusion, preservation of PKCζ by injection of IL-13 or PKCζi during EIU led to a protection of ocular tissues, and, presumably by effects on the permeability of ocular vascular endothelium, to a decrease in inflammatory cell migration into the eye. In addition, treatment controlled the fate of cells, leading to PMN apoptosis and deactivation of macrophages with resultant decrease in

Figure 8. Effect of LPS and treatment with IL-13 on expression of PKCζ and NF-κB expression in resident ocular cells. In rats with EIU, PKCζ expression in the iris epithelium (**a**) and retinal bipolar and ganglion (**b**) (**arrows**). **c:** Hematoxylin and eosin-stained section of retina showing bipolar cells (INL) and ganglion cells (GCL). After treatment with IL-13, expression of PKCζ (green) in iris epithelium (**d**) and bipolar and ganglion cells (**e**). Nuclear staining with propidium iodide. **f** and **g:** NF-κB expression (green) in ganglion cell nuclei (**arrow**). Nuclear counterstain with DAPI (blue). **h:** Negative control with the replacement of rabbit anti-PKCζ polyclonal antibody by normal rabbit serum Ig, showing no PKCζ staining in retina. Photomicrographs were obtained in one eye in each group and are representative of similar sections stained from five rats per group in two separate experiments. i, iris; GCL, ganglion cell layer; INL, inner nuclear layer; r, retina. Original magnifications: ×600 (**a, b, d, e**); ×1000 (**c**); ×1000 (**f**); ×1500 (**g**); and ×1000 (**h**).

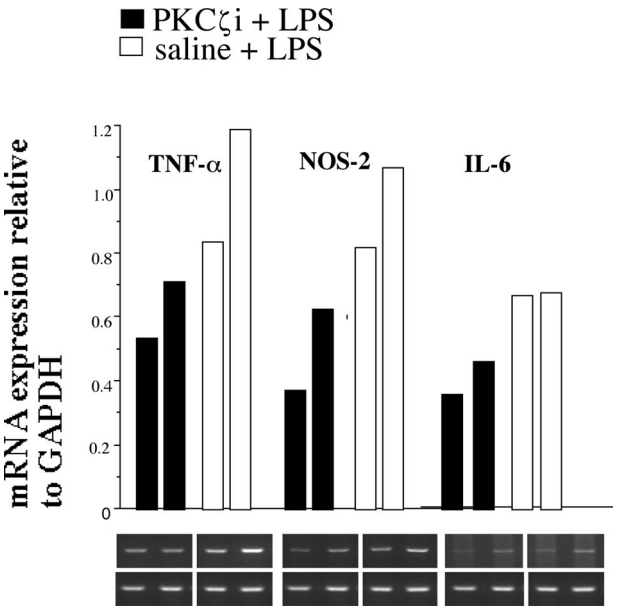

Figure 10. Effect of PKCζi treatment on cytokine mRNA expression in rat eyes. mRNA expression was evaluated by semiquantitative reverse transcription-polymerase chain reaction. Total mRNA was isolated from whole eyes at 24 hours after simultaneous injection of LPS in the footpad and saline or PKCζi into the anterior chamber, and reverse-transcribed into cDNA. The polymerase chain reaction fragments were analyzed by agarose gel electrophoresis. Results obtained for two rats from each group are shown and are representative of results found in all PKCζi- ($n = 4$ rats) or saline- ($n = 5$ rats) injected rats. PKCζi treatment significantly reduced TNF-α ($P = 0.0001$), NOS-2 ($P = 0.000$), and IL-6 ($P = 0.06$) mRNAs compared with levels measured for saline-injected rats. Agarose gel electrophoresis of the polymerase chain reaction products is presented: TNF-α, 295 bp; NOS-2, 657 bp; IL-6, 508 bp; GAPDH, 162 bp.

inflammation and promotion of wound healing as described by others.[24,55,77] In this study, our results show the importance of modulating PKCζ activity in the treatment of intraocular inflammation of short duration and could represent a novel approach for the control of chronic uveitis.

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