Restoring Conjunctival Tolerance by Topical Nuclear Factor–κB Inhibitors Reduces Preservative-Facilitated Allergic Conjunctivitis in Mice

Mauricio Guzmán,[1] Florencia Sabbione,[1] María Laura Gabelloni,[1] Silvia Vanzulli,[2] Analía Silvina Trevani,[1] Mirta Nilda Giordano,[1] and Jeremías Gastón Galletti[1]

[1]Institute of Experimental Medicine, National Academy of Medicine/CONICET, Buenos Aires, Argentina
[2]Institute of Oncologic Research, National Academy of Medicine, Buenos Aires, Argentina

Correspondence: Jeremías Gastón Galletti, Pacheco de Melo 3081, Immunology Laboratory, Institute of Experimental Medicine, National Academy of Medicine/CONICET, (1425) Buenos Aires, Argentina; jeremiasg@gmx.net.

Submitted: February 1, 2014
Accepted: August 22, 2014

Citation: Guzmán M, Sabbione F, Gabelloni ML, et al. Restoring conjunctival tolerance by topical nuclear factor–κB inhibitors reduces preservative-facilitated allergic conjunctivitis in mice. *Invest Ophthalmol Vis Sci*. 2014;55:6116–6126. DOI:10.1167/iovs.14-14075

Purpose. To evaluate the role of nuclear factor–κB (NF-κB) activation in eye drop preservative toxicity and the effect of topical NF-κB inhibitors on preservative-facilitated allergic conjunctivitis.

Methods. Balb/c mice were instilled ovalbumin (OVA) combined with benzalkonium chloride (BAK) and/or NF-κB inhibitors in both eyes. After immunization, T-cell responses and antigen-induced ocular inflammation were evaluated. Nuclear factor–κB activation and associated inflammatory changes also were assessed in murine eyes and in an epithelial cell line after BAK exposure.

Results. Benzalkonium chloride promoted allergic inflammation and leukocyte infiltration of the conjunctiva. Topical NF-κB inhibitors blocked the disruptive effect of BAK on conjunctival immunological tolerance and ameliorated subsequent ocular allergic reactions. In line with these findings, BAK induced NF-κB activation and the secretion of IL-6 and granulocyte-monocyte colony-stimulating factor in an epithelial cell line and in the conjunctiva of instilled mice. In addition, BAK favored major histocompatibility complex (MHC) II expression in cultured epithelial cells in an NF-κB–dependent fashion after interaction with T cells.

Conclusions. Benzalkonium chloride triggers conjunctival epithelial NF-κB activation, which seems to mediate some of its immune side effects, such as proinflammatory cytokine release and increased MHC II expression. Breakdown of conjunctival tolerance by BAK favors allergic inflammation, and this effect can be prevented in mice by topical NF-κB inhibitors. These results suggest a new pharmacological target for preservative toxicity and highlight the importance of conjunctival tolerance in ocular surface homeostasis.

Keywords: conjunctival tolerance, benzalkonium chloride, allergic conjunctivitis, NF-κB, conjunctival epithelium, preservative

Local side effects of medical glaucoma treatment represent a significant challenge in clinical practice because of their negative impact on patients' quality of life and compliance.[1] Allergic conjunctivitis and dry eye can arise or worsen after initiating topical treatment with hypotensive eye drops,[2–4] and a significant body of evidence suggests that the microbicidal agents in glaucoma formulations are responsible for this phenomenon.[1] The most common among the preservatives approved for human use, benzalkonium chloride (BAK), is found at concentrations ranging from 0.004% (4×10^{-3}%) to 0.025% (2.5×10^{-2}%).[1] The toxic effects of BAK on the ocular surface epithelium have been extensively described, encompassing apoptosis and/or necrosis induction in vitro[5–7] and the release of proinflammatory cytokines and increased expression of epithelial activation markers both in vivo and in vitro.[8] In contrast, our understanding of how topical exposure to BAK facilitates allergic conjunctivitis and dry eye is quite limited, and there are few reports on the functional impact of the preservative on ocular surface immunity. We have previously demonstrated that BAK can profoundly disrupt conjunctival immunological tolerance to a harmless antigen in mice,[9] but the clinical implications of these findings and the underlying molecular mechanisms remained unexplored.

The conjunctiva, as we and others have shown,[9–11] actively regulates the immune response when an innocuous antigen reaches its confines. Under physiological conditions, conjunctival immunological tolerance sets in and requires the migration of antigen-presenting cells to the draining lymph nodes. The development of a tolerogenic response involves the expansion of antigen-specific regulatory T cells[12] and prevents unwanted inflammation on subsequent encounters with the same antigen.[13] This specific function of the conjunctiva has been proposed as an efficient vaccine delivery route,[11] but mucosal tolerance is not exclusive to the eye. Respiratory immune tolerance can successfully dampen allergic airway inflammation and constitutes one of the bases of specific immunotherapy for asthma.[14] Remarkably, and despite extensive research on bronchial and nasal tolerance, the implications of conjunctival tolerance (and lack thereof) have not been addressed in the clinical approach to immune-mediated ocular surface disorders.

In any mucosa, the epithelium plays a key role in the immune decision-making process by either exerting a tolero-

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genic or an immunogenic conditioning on dendritic cells,[15] which in turn drives the expansion of either regulatory or effector T cells. Activation of the nuclear factor–κB (NF-κB) pathway in epithelial cells is paramount to this process, and whereas a low threshold of activity is required for epithelial homeostasis, chronic engagement of this signaling pathway leads to T-cell–mediated mucosal inflammation.[16,17] On the other hand, there are numerous examples of how environmental exposure of the skin or mucosal linings to toxic agents can facilitate an antigen-specific immune response.[18–20] Some of these substances are capable of triggering NF-κB in epithelial cells and, more importantly, mucosal inhibition of this signaling pathway can ameliorate disease in animal models.[21–23]

Based on preliminary findings that suggested a potential involvement in the immune effects of BAK on cultured epithelial cells,[9] we directly explored the role of the NF-κB pathway in both BAK toxicity and epithelial–T-cell interaction. In addition, we hypothesized that exposure to BAK might facilitate allergic conjunctivitis by the disruptive immune effect already described.[9] To this aim, we designed a murine model of BAK-facilitated allergic conjunctivitis and evaluated the effect of topical NF-κB modulation as a therapeutic strategy.

Methods

Mice

Female Balb/c mice (8–12 weeks old), which were bred and maintained in our conventional animal facility, were used for the in vivo experiments. All experiments were approved by the Institute of Experimental Medicine Animal Ethics Committee (approval number 20130610) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Lines and Cultures

Cell cultures were performed in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol in a humidified incubator with 5% CO_2 at 37°C. The Pam212 cell line, which was originally derived from murine epidermal keratinocytes,[24] was a kind gift from Adriana Casas, PhD (Hospital de Clínicas, University of Buenos Aires, Buenos Aires, Argentina), and was maintained in the same medium.

Reagents and Antibodies

All reagents were from Sigma-Aldrich (Buenos Aires, Argentina) unless otherwise specified. Fluorochrome-tagged antibodies were from BioLegend (San Diego, CA, USA). Grade V ovalbumin (OVA) was used in all experiments.

Benzalkonium Chloride Treatment of Epithelial Cells for Supernatant Collection and T-Cell Cocultures

Confluent Pam212 cells were exposed to fresh medium alone or with the specified BAK concentration for 15 minutes at 37°C, washed twice, and finally cultured in fresh medium without serum. Nuclear factor–κB inhibitors were added to some cultures 30 minutes before BAK. After overnight culture, supernatants were collected and the viability of the remaining cells was assessed by crystal violet staining. For coculture experiments, Pam212 cells were grown in 48-well plates and treated likewise before adding 2×10^5 T cells obtained from Balb/c mouse lymph nodes. After 4 days of culture, nonadherent cells were removed by gentle washing and then the epithelial cells were detached by mild trypsin treatment.

Immunostaining and Flow Cytometry

For surface antigen staining (major histocompatibility complex II [MHC II], CD40, and CD86), Pam212 cells were washed in PBS with 0.5% BSA and then incubated with fluorochrome-conjugated antibodies at previously titrated concentrations for 30 minutes at 4°C. For intracellular antigen staining (IκBα), Pam212 cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed with PBS with 50 mM glycine, permeabilized, and blocked with 0.1% saponin in PBS with 5% goat serum for 30 minutes, and then anti-IκBα antibody (1:100 dilution; Abcam ab32158; Abcam, Cambridge, MA, USA) was added for another 30 minutes. After thorough washing, the cells were labeled with DyLight 488 goat anti-rabbit IgG (1:500 dilution; Abcam ab96899) for 30 minutes. For flow cytometry analysis, cells were thoroughly washed in PBS with 1 mM EDTA before acquisition on a FACScalibur cytometer (Becton Dickinson, Buenos Aires, Argentina). Data were analyzed with Flowing Software (Perttu Terho, Turku Centre for Biotechnology, Turku, Finland; www.flowingsoftware.com). Optimal compensation and gain settings, as well as viable cell gating, were determined as previously described.[25] Overlayed histogram graphs are plotted in normalized form as percentage of maximum for each histogram.

Western Blotting

After BAK and/or NF-κB inhibitor treatment, Laemmli buffer (with 5% 2-mercaptoethanol) was added to Pam212 monolayers, and the resulting extracts were then heated at 95°C for 5 minutes and subjected to SDS-PAGE in 10% gel. Proteins were later transferred from the gel to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for 2 hours and then blocked in PBS containing 0.05% Tween 20 and 5% nonfat milk for 1 hour at room temperature. Membranes were incubated overnight with anti-IκBα antibody (1:1000 dilution; Abcam ab32158) and the bound antibodies were visualized with horseradish peroxidase–conjugated anti-rabbit IgG Abs and the ECL Western Blotting System (Amersham Biosciences, Amersham, UK). Signal intensity in digital images was quantified with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Enzyme-Linked Immunosorbent Assay

Interleukin-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations in culture and explant supernatants were determined with commercial ELISA kits according to the manufacturer's instructions (Peprotech, Rocky Hill, NJ, USA).

Confocal Microscopy

Pam212 cells grown on coverslips were treated with BAK and/or NF-κB inhibitors, rinsed with PBS at the specified time points, fixed for 30 minutes in 4% paraformaldehyde, permeabilized with ice-cold methanol for 10 minutes, and washed with 50 mM glycine in PBS. The coverslips were then incubated with 1 μg/mL polyclonal rabbit anti–NF-κB p65 antibody (sc372; Santa Cruz Biotechnology, Dallas, TX, USA) or the corresponding isotype control for 2 hours, blocked with 5% goat serum in PBS for 2 hours and then incubated with DyLight 488 conjugated goat anti-rabbit IgG (1:100 dilution) for

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2 hours. Coverslips were finally mounted with 1 μg/mL propidium iodide using antifade medium. Images were acquired with a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon ×60/1.42 objective and analyzed with Olympus FV10-ASW software.

Conjunctival Instillation of BAK, OVA, and NF-κB Inhibitors and Parenteral Immunization

Phosphate-buffered saline, 10^{-2}% BAK, 20 μg OVA (2 mg/mL), 0.1 mM pyrrolidine dithiocarbamate (PDTC), and 0.5 mM sulfasalazine (SSZ) or different combinations of these compounds in PBS were instilled daily in a volume of 5 μL to the conjunctiva of both eyes for 5 days. On day 7, mice were injected subcutaneously in the flank with 0.1 mL of 1:1 complete Freund' s adjuvant (CFA) emulsion in PBS containing 100 μg OVA.

Conjunctival Explants

Mice were instilled PBS or BAK alone or combined with 0.1 mM PDTC and 0.5 mM SSZ in both eyes once daily for 3 days before euthanasia. The tarsal conjunctivas were excised under aseptic conditions with the aid of a dissection microscope, as described elsewhere.[9] The conjunctival explants from each animal (approximately 30 mg tissue) were pooled, washed three times with PBS, and then cultured in 1 mL medium without serum. Supernatants were collected after 24 hours for further analysis.

Myeloperoxidase Colorimetric Quantification Assay

Conjunctival explants from each mouse were rendered into a suspension with a tissue homogenizer in 0.3 mL PBS and then 0.5% Triton X-100 was added. Suspensions were then centrifuged at 2000*g* for 5 minutes and the supernatants collected for enzymatic assay. In brief, 50 μL 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific, Waltham, MA, USA) was added to 50 μL of each sample in duplicate in microtiter plates and incubated at room temperature for 15 minutes, after which the reaction was stopped by adding 50 μL 1 N sulfuric acid. Absorbance was measured at 450 nm with the reference filter set at 570 nm.

Measurement of Delayed-Type Hypersensitivity Responses

Mice were immunized after conjunctival instillation as described above. A group of untreated mice served as a negative control. On day 15, OVA (100 μg in PBS) and PBS alone were injected in a volume of 50 μL into the right and left foot pads, respectively. Antigen-induced swelling was measured 48 hours later as the mean difference in thickness between the right and left foot pads of each mouse.

T-Cell Proliferation Assays

Single-cell suspensions were obtained by mechanical dissociation of spleens and sieving through wire mesh. For antigen proliferation assays, 2×10^5 cells were cultured for 3 days in 96-well plates containing 100 μg/mL OVA, and 1 μCi per well of [^{3}H]-thymidine was added for the final 18 hours. Thymidine incorporation was measured by liquid scintillation counting and the results of triplicate cultures are expressed as the mean stimulation index ± SEM, calculated as the ratio of counts per minute in antigen-containing cultures to the counts in control cultures.

Ocular Antigenic Challenge and Assessment of Allergic Response

Groups of three mice were treated as detailed in the figure legends and 2 weeks after parenteral immunization, they were challenged with 5 μL of 250 mg/mL OVA in each eye. Twenty-four hours later, each eye was photographed before euthanasia. Clinical inflammation was graded by a masked observer according to a previously validated scale[26] that considers lid edema, chemosis, conjunctival redness, and tearing and discharge. The eye globes with the attached lids were excised and immediately fixed in 10% buffered neutral formalin.

Histopathological Study

Formalin-fixed eye specimens were embedded in paraffin, serially cut into vertical 5-μm-thick sections, and stained with hematoxylin and eosin. Six to eight sections from each sample were examined for evaluating the conjunctival tissue and inflammatory cells.

Immunohistochemistry

Immunohistochemical detection of NF-κB p65 was performed on formalin-fixed, paraffin-embedded sections using the ABC technique. Briefly, after endogenous peroxidase activity was inhibited by 3% H_2O_2 and microwave antigen retrieval was performed, sections were blocked with 2% normal goat serum, then incubated overnight with polyclonal rabbit anti-NF-κB p65 antibody (sc372, dilution 1:100; Santa Cruz Biotechnology), rinsed three times with PBS, incubated with a biotin-conjugated secondary antibody (1:200 in PBS, 3% normal goat serum; Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature, rinsed three times with PBS and finally incubated with ABC peroxidase (horseradish peroxidase) complex (Vector Laboratories) for 30 minutes at room temperature. The reaction was developed with 3,3′-diaminobenzidine (3 μg/mL in PBS with 0.5% H_2O_2) and controlled under the microscope. Specimens were lightly counterstained with hematoxylin before mounting. Primary antibody was omitted in the negative control.

Statistical Analysis

Student's *t*-test and ANOVA with Dunnett's post hoc test were used to compare means of two and three or more samples, respectively. Significance was set at $P < 0.05$ (two-tailed tests) and calculations were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Results

Benzalkonium Chloride Induces NF-κB Pathway Activation in Cultured Epithelial Cells

As the epithelial NF-κB pathway plays a key role in determining the immune outcome in other mucosal sites, we reasoned that it could be involved in the disruptive effect of BAK on conjunctival tolerance that we had previously reported.[9] Therefore, we directly assessed by different approaches the activation status of the NF-κB pathway in epithelial cells on BAK treatment. As primary murine conjunctival epithelial cells could not be obtained in sufficient number by standard culture techniques,[27] we used the Pam212 epithelial cell line, which we had previously used for exploring the immune effects of BAK.[9] To model the instillation of a BAK-containing eye drop onto the ocular surface, we exposed Pam212 monolayers for 15 minutes (the

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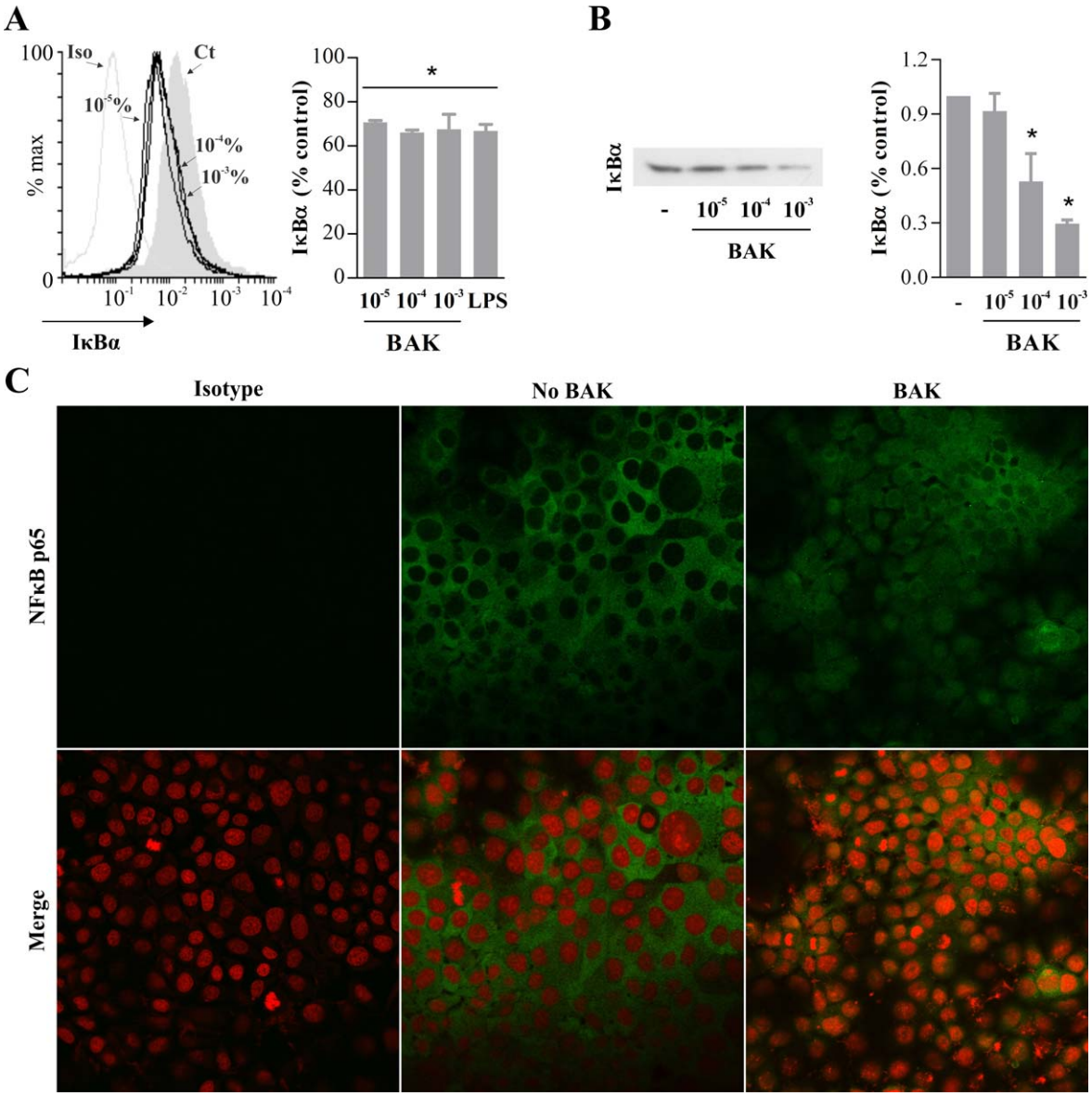

Figure 1. Benzalkonium chloride triggers NF-κB activation in epithelial cells. (**A**) Confluent Pam212 monolayers were exposed for 15 minutes to different BAK concentrations. Then cells were trypsinized, fixed, and permeabilized and the intracellular expression of IkBa protein was analyzed by FACS. Representative histograms are shown: isotype control (*gray histogram*), basal expression (*filled histogram*), and after exposure to BAK (*black histogram*). *Bar graph* ($n = 3$) of IκBα expression relative to unstimulated cells under different BAK concentrations and 1 μg/mL LPS stimulation. *Asterisk* indicates a statistically significant difference with unstimulated cells (ANOVA with Dunnett's post hoc test). (**B**) Pam212 cells were treated as described in (**A**) and then whole cell extracts were prepared to evaluate IκBα levels by Western blot. Shown are representative immunoblots of three independent experiments (*left*) and the relative IκBα expression after BAK treatment (*right*). *Asterisk* indicates a statistically significant difference with unstimulated cells (ANOVA with Dunnett's post hoc test). (**C**) Pam121 cells were exposed for 15 minutes to 10^{-3}% BAK, washed twice, and cultured for an additional hour before fixation and NF-κB p65 staining. Representative micrographs ($n = 3$) show NF-κB p65 in *green* and cell nuclei in *red* (propidium iodide staining).

accepted tear film clearance time[28,29]) to a BAK concentration curve starting at 10^{-2}% (0.01%, the most frequently used concentration in medical formulations), and extending up to 1000-fold dilution (10^{-5}%). We have previously shown that 10^{-4}% and 10^{-3}% BAK treatment for 15 minutes does not affect Pam212 cell viability on overnight culture, whereas comparable exposure to 10^{-2}% BAK readily induces cell death.[9] First we quantified the expression of the IκBα protein, an inhibitory cytoplasmic protein that binds the NF-κB complex and prevents its translocation to the nucleus. Benzalkonium chloride decreased IκBα protein levels in epithelial cells, as did lipopolysaccharide (LPS), a well-characterized activator of the NF-κB pathway (Fig. 1A). These results, obtained by FACS, were confirmed by Western blot under the same conditions (Fig. 1B). We also determined by immunofluorescence microscopy the actual intracellular location of the NF-κB transcriptional complex in Pam212 cells. Strict cytoplasmic localization of the NF-κB p65 subunit was detected in resting cells, whereas significant translocation to the nucleus was observed 1 h after BAK treatment (Fig. 1C). Altogether these results show that a brief exposure to BAK is sufficient to trigger the NF-κB pathway in cultured epithelial cells.

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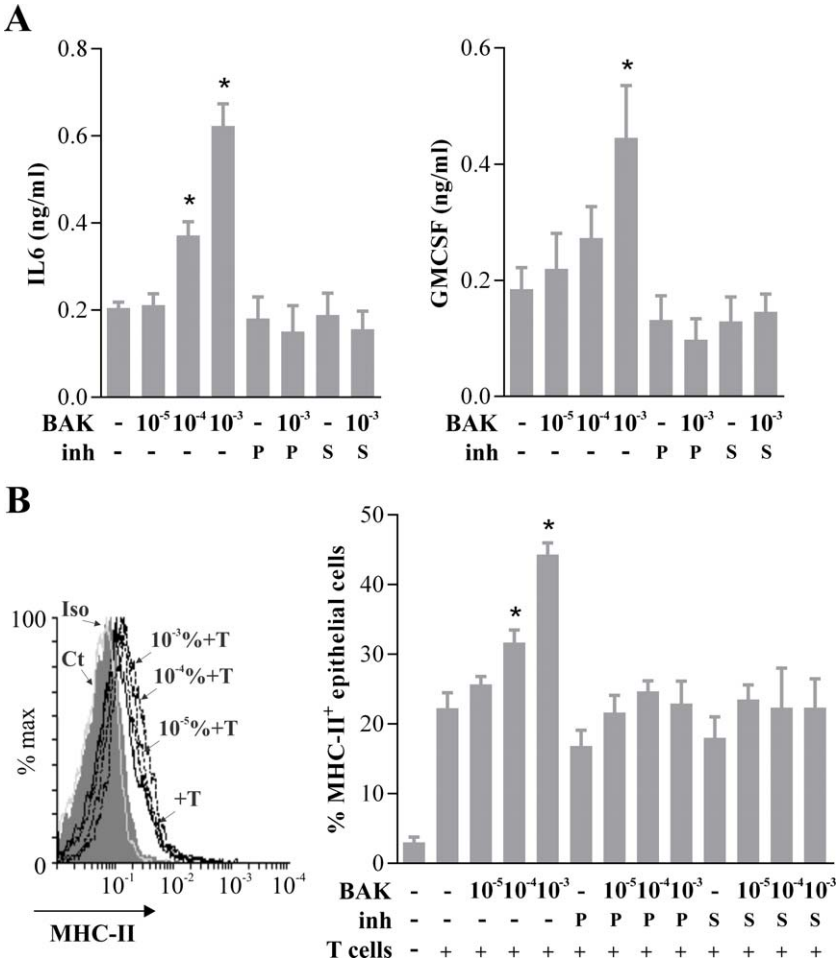

Figure 2. Benzalkonium chloride induces IL-6 and GM-CSF release and favors MHC II expression by epithelial cells. Pam212 cells were exposed to different BAK concentrations for 15 minutes, thoroughly washed, and cultured further. For some experiments, NF-κB inhibitors (inh) were added to the cultures 30 minutes before BAK treatment. Either 10 μM PDTC (P) or 0.5 mM sulfasalazine (S) at were used. (**A**) *Bar graph* ($n = 4$) of IL-6 and GM-CSF concentrations in 18- h supernatants as assayed by ELISA. *Asterisk* indicates a statistically significant difference compared with control cultures, according to ANOVA with Dunnett's post hoc test. (**B**) Major histocompatibility complex II expression in Pam212 cells after 96 hours of culture with and without T cells. (*Left*) Representative experiment with isotype control (*light gray histogram*), untreated epithelial cells alone (*filled histogram*), untreated epithelial cells with T cells (*black histogram*), and BAK-treated epithelial cells with T cells (*dashed histogram*). (*Right*) *Bar graph* ($n = 6$) of pooled data. *Asterisk* indicates a statistically significant difference compared with control Pam212–T-cell cocultures, according to ANOVA with Dunnett's post hoc test.

Benzalkonium Chloride–Exposed Epithelial Cells Release Proinflammatory Cytokines and Express MHC II in an NF-κB–dependent Fashion

To assess whether NF-κB activation has functional immune consequences in epithelial cells, we collected culture supernatants of Pam212 cell monolayers that were briefly exposed to BAK. Two well-characterized NF-κB activation inhibitors, SSZ and PDTC, were added to some cultures before BAK treatment. The SSZ inhibits IκB kinase by competing for the adenosine triphosphate binding site,[30] whereas PDTC acts downstream in the NF-κB activation cascade by blocking the ubiquitylation of IκB protein.[31] Thus, both inhibitors act independently and prevent the degradation of IκB, which in turn inhibits the nuclear translocation of NF-κB dimers. On BAK exposure, we found that Pam212 cells released increased levels of IL-6 and GM-CSF, two cytokines whose transcription is enhanced by NF-κB, and more importantly, that this release could be reversed by pretreating the cells with either PDTC or SSZ (Fig. 2A). To evaluate additional functional effects of BAK on epithelial cells, we focused on MHC II expression, a widely accepted marker of epithelial activation that is associated with ocular surface damage in glaucoma patients.[32] We assessed by FACS the levels of MHC II and CD40 and CD86 costimulatory molecules in Pam212 cells, but could not detect the expression of any of them under basal conditions or after BAK treatment. As MHC II expression in epithelial cells is dependent on interferon γ stimulation,[33,34] we reasoned that this in vitro setup lacked the T cells usually found in the conjunctiva as a potential source for this cytokine. Therefore, we added syngeneic T cells to the epithelial cell cultures and found a significant increase in the MHC II$^+$ epithelial cell fraction after 4 days of coculture (Fig. 2B). Major histocompatibility complex II expression was further enhanced in a dose-dependent fashion when the epithelial cells were previously exposed to BAK, and remarkably, the increase was completely inhibited by pretreating the epithelial cells with either PDTC or SSZ. By contrast, coculture with T cells induced neither CD40 nor CD86 expression in epithelial cells (data not shown). Altogether, these results show that BAK-induced NF-κB activation in Pam212 cells is sufficient to increase proinflammatory cytokine secretion, such as IL-6 and GM-CSF, and indirectly augments MHC II expression by affecting their interaction with T cells.

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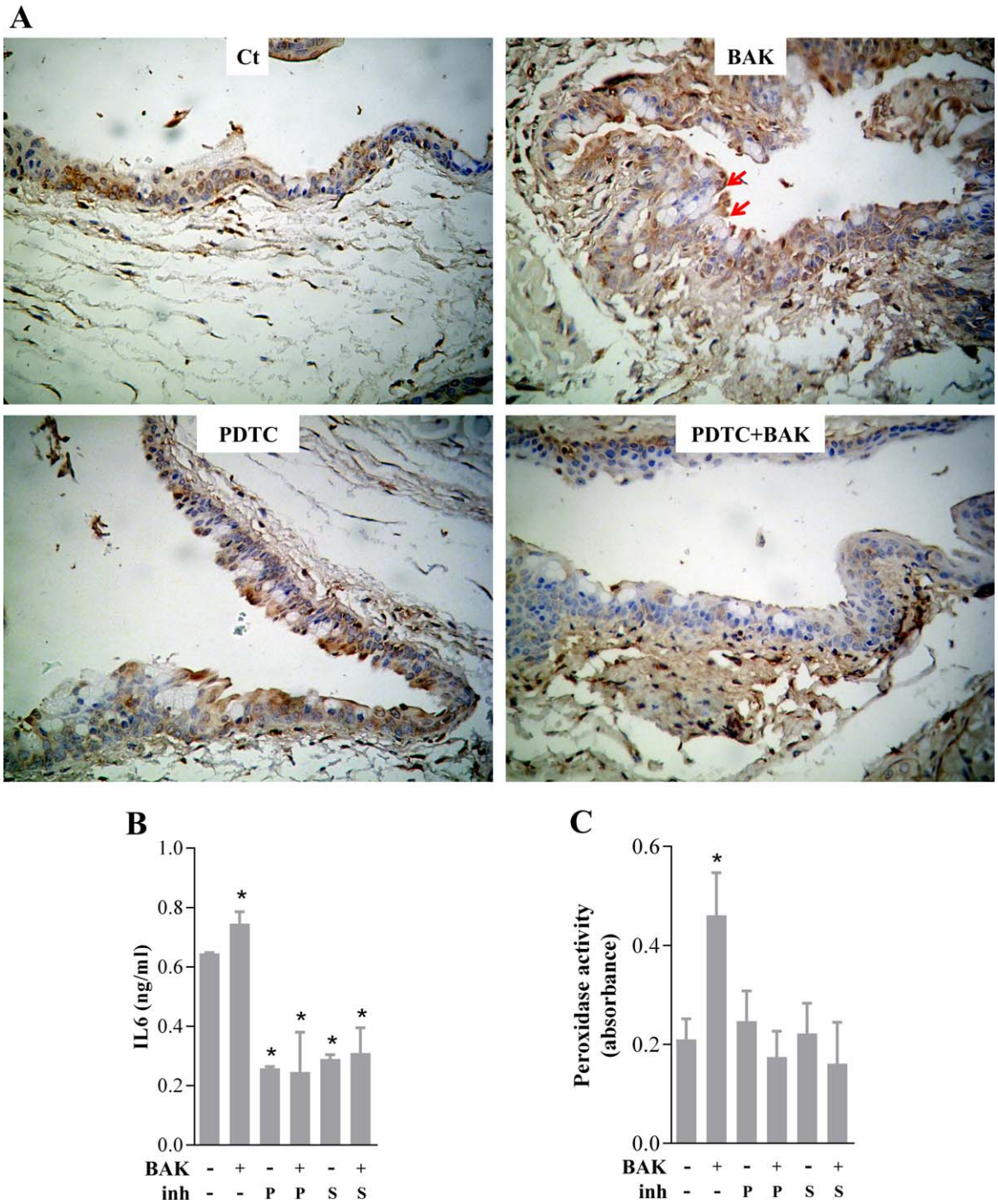

Figure 3. Benzalkonium chloride induces NF-κB activation in the conjunctival epithelium in vivo and proinflammatory changes. Groups of three mice were instilled daily for 3 days with saline or 10^{-2}% BAK combined or not with NF-κB inhibitors (inh): 0.1 mM PDTC (P) or 0.5 mM sulfasalazine (S), and then the eyes were surgically excised for analysis. (**A**) Representative immunohistochemical images of conjunctival tissue with NF-κB p65-specific staining. Increased epithelial p65 expression is evident only in BAK-treated eyes, with nuclear staining in the superficial epithelial cells (*red arrows*) (IHC DAB staining, ×200). (**B**) *Bar graph* ($n = 3$) of IL-6 concentration in 18-hour culture supernatants from ocular explants. *Asterisk* indicates a statistically significant difference compared with saline-treated mice, according to ANOVA with Dunnett's post hoc test. (**C**) *Bar graph* ($n = 3$) of peroxidase enzymatic activity in whole-eyelid homogenates. *Asterisk* indicates a statistically significant difference compared with saline-treated mice, according to ANOVA with Dunnett's post hoc test.

Benzalkonium Chloride Induces Conjunctival Epithelial NF-κB Activation and Proinflammatory Effects In Vivo

To validate our in vitro findings, we explored ocular surface changes in mice exposed daily to 10^{-2}% BAK in both eyes. We assayed NF-κB activation in the conjunctival epithelium by immunohistochemistry after 3 days of treatment (Fig. 3A), observing increased expression of NF-κB p65, and more importantly, nuclear p65 localization throughout the epithelial layer in BAK-treated but not in control mice. These changes were abrogated by adding 0.1 mM PDTC (Fig. 3A) or 0.5 mM

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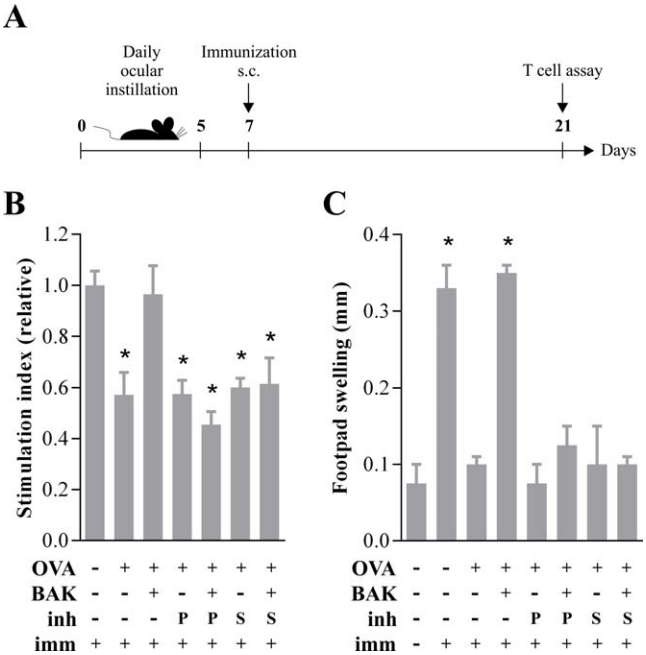

Figure 4. Topical NF-κB inhibitors prevent conjunctival tolerance disruption by BAK. (**A**) In vivo model of conjunctival tolerance under BAK exposure. Groups of three mice were instilled in both eyes daily for 5 days with 2 mg/mL OVA with and without 10^{-2}% BAK in different combinations with NF-κB inhibitors (inh): 0.1 mM PDTC (P) or 0.5 mM sulfasalazine (S). On day 7, mice were subcutaneously immunized (imm) with OVA in CFA, and 2 weeks later, the T-cell response was assayed. (**B**) *Bar graph* ($n = 6$) of OVA-specific proliferation in splenocyte cultures as assayed by [^{3}H]-thymidine incorporation. Proliferation is expressed as stimulation index (counts per minute in the presence of antigen/counts per minute without antigen) of each treatment group relative to untreated immunized mice. *Asterisk* indicates a statistically significant difference compared with saline-treated mice, according to ANOVA with Dunnett's post hoc test. (**C**) *Bar graph* ($n = 3$) of OVA-induced delayed hypersensitivity response, expressed as the difference in swelling between the OVA-injected and the saline-injected footpad of each mice. *Asterisk* indicates a statistically significant difference compared with untreated, unimmunized mice according to ANOVA with Dunnett's post hoc test.

SSZ (data not shown) to the BAK formulation. We also detected increased production of IL-6 (Fig. 3B), but not of GM-CSF (data not shown), in ocular explants of mice that were topically treated with 10^{-2}% BAK. Again, this effect was completely inhibited by concomitant administration of either of the two NF-κB inhibitors. As BAK favored conjunctival neutrophil infiltration in other models,[35] we quantified the number of conjunctival inflammatory cells in whole eyelid homogenates by a peroxidase enzymatic assay (Fig. 3C). We detected a significant increase in peroxidase levels in BAK-treated mice that was blocked by simultaneous delivery of NF-κB inhibitors. Altogether these results show that ocular exposure to BAK triggers epithelial NF-κB activation and other inflammatory changes in mice.

Topical NF-κB Inhibitors Restore Conjunctival Tolerance in a Murine Model of BAK Toxicity

We have previously shown that BAK skews the ocular surface milieu in such a way that mice fail to develop mucosal tolerance to a harmless antigen (OVA) that is topically given at the same time. Instead, the mice develop a Th2-like proinflammatory response.[9] To assess if topical NF-κB inhibitors could affect the conjunctival immune outcome, we instilled preparations containing OVA, 10^{-2}% BAK, and inhibitors in different combinations once daily for 5 days and then evaluated the systemic immune response, as represented in Figure 4A. On subsequent challenge (Fig. 4B), we detected reduced antigen-specific cellular responses in OVA-treated but not in BAK+OVA-treated mice, confirming the disruptive effect of the preservative on conjunctival tolerance. When either PDTC or SSZ were included in the antigen preparations, conjunctival tolerance was not affected. However, the addition of these inhibitors to the BAK+OVA preparations reversed the systemic effect induced by BAK. We also obtained comparable results in vivo by evaluating the delayed-type hypersensitivity response to OVA (Fig. 4C). Our findings show that topical NF-κB inhibitors can restore conjunctival tolerance in BAK-treated mice.

Exposure to BAK Facilitates a Conjunctival Allergic Response in a NF-κB–Dependent Fashion

To investigate the potential clinical implications of breakdown of conjunctival tolerance to harmless antigens, we evaluated the magnitude of ocular allergic responses in a widely accepted murine model that involves active immunization. As depicted in Figure 5A, mice were instilled OVA with and without BAK and/or NF-κB inhibitors daily for 5 days to induce a conjunctival T-cell response,[9] and then they were immunized with the same antigen in adjuvant to elicit an effector T-cell response. Two weeks later, the pathogenic potential of the effector T-cell response was assayed by topical challenge of both eyes with OVA. Tolerized (OVA-treated) mice developed less conjunctival inflammation than their nontolerized (untreated) and BAK-exposed cage mates (Figs. 5B, 5C), highlighting the protective role of this mucosal function. Addition of an NF-κB inhibitor to the conditioning phase resulted in reduced allergic responses in both OVA and BAK+OVA mice. These results indicate that conjunctival tolerance is capable of protecting from subsequent antigen challenge, and that topical NF-κB inhibitors favor its development even under BAK exposure. Histopathological analysis of enucleated eye samples was consistent with the clinical assessment of allergic changes (Fig. 6). Congestive vessels, edema, and inflammatory cells (predominantly eosinophils) were observed in the lamina propria mucosae of the palpebral and bulbar conjunctiva of immunized-only and OVA- and BAK+OVA-instilled mice. Compared with OVA-instilled mice, eosinophil infiltration was slightly more abundant in the conjunctiva of BAK+OVA-instilled mice but it was similar in OVA+PDTC- and OVA+SSZ-instilled mice. Interestingly, samples from mice receiving BAK+OVA+PDTC or BAK+OVA+SSZ showed fewer, if any, eosinophils. Altogether these results suggest that conjunctival tolerance can protect from an allergic reaction and that topical NF-κB inhibitors favor tolerance even under BAK exposure.

Discussion

Immune tolerance has become a powerful ally in the management of asthma[13] and chronic inflammatory bowel disease,[36] but its role in ophthalmological practice has not yet been addressed. In this work we show that immune tolerance is involved in ocular surface homeostasis and that its disruption could explain the exacerbation of ocular allergy that frequently affects patients treated with BAK-containing eye drops. In addition, we demonstrate that in vitro exposure of epithelial cells to this preservative leads to proinflammatory changes that resemble those already described in patients, and more importantly, that these changes can be reverted by well-characterized NF-κB inhibitors. From these results, we suggest

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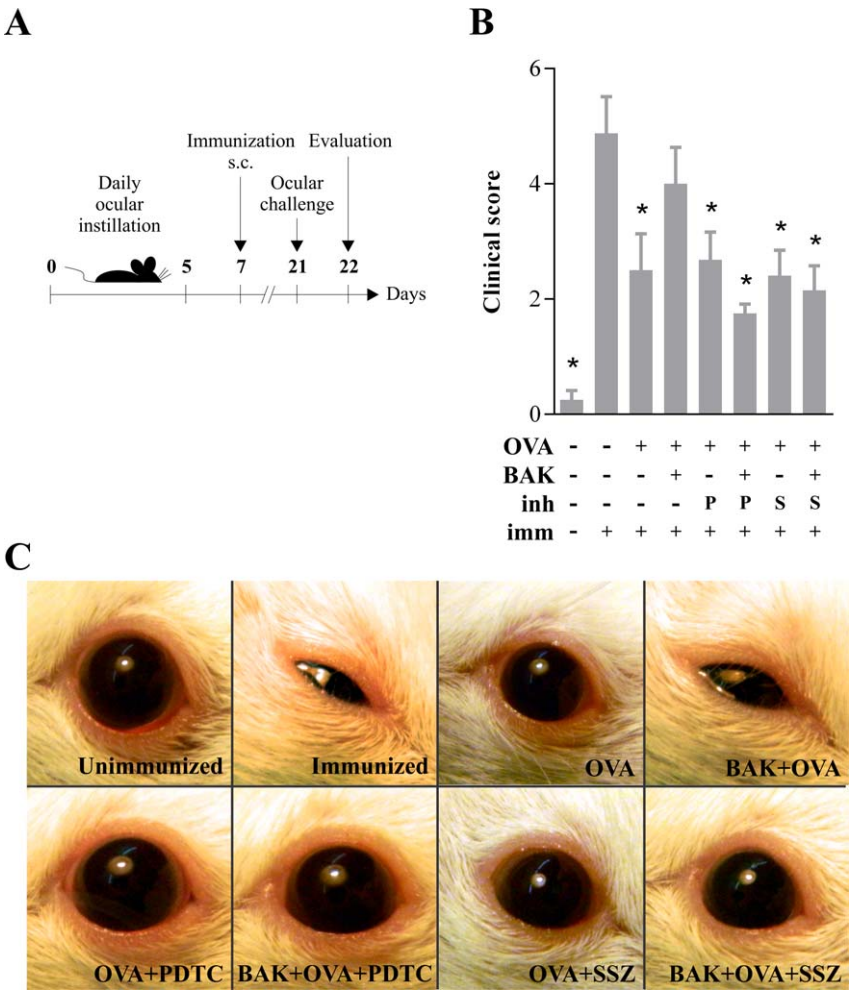

Figure 5. Topical NF-κB inhibitors protect from BAK-facilitated allergic conjunctivitis. (**A**) In vivo model of allergic conjunctivitis after BAK exposure. Groups of three mice were instilled in both eyes daily for 5 days with 2 mg/mL OVA with and without 10^{-2}% BAK in different combinations with NF-κB inhibitors (inh): 0.1 mM PDTC (P) or 0.5 mM sulfasalazine (S). On day 7, mice were subcutaneously immunized (imm) with OVA in CFA, and 2 weeks later, both eyes were challenged with OVA alone. Clinical assessment was performed 24 hours later. (**B**) Clinical score of ocular inflammation as detailed in the Methods section. Shown are mean ± SD of 3 animals/group. *Asterisk* indicates a statistically significant difference compared with OVA-challenged immunized mice, according to ANOVA with Dunnett's post hoc test. (**C**) Representative photographs from one experiment showing the effect of PDTC and sulfasalazine (SSZ) instillation in OVA- and BAK+OVA-treated mice.

that modulation of this pathway could have a role in the management of ocular surface disease.

Benzalkonium chloride exerts deleterious effects on conjunctival epithelial cells, in part by producing reactive oxygen species and inducing apoptosis.[6] Here we demonstrate that BAK also triggers NF-κB activation in epithelial cells in vitro, even at concentrations 100- and 1000-fold lower than those used in glaucoma formulations (Fig. 1). At such reduced levels of BAK, we observed little impact on cell viability after 15 minutes of exposure. Nevertheless, we still detected functional consequences of NF-κB activation, such as increased production of IL-6 and GM-CSF by epithelial cells (Fig. 2A). Pretreatment with NF-κB inhibitors completely prevented this effect, and validating these in vitro findings, we observed the same pattern in IL-6 production by ex vivo ocular surface explants (Fig. 2B). Our results are in line with previous reports on BAK[32] and other related preservatives[37] and probably reflect a common epithelial response to injury that involves NF-κB activation. A similar effect has been described for cigarette smoke, another inducer of oxidative stress, on primary cultures of bronchial epithelial cells and immortalized cell lines.[38]

In addition to increased IL-6 levels in tear samples, expression of MHC II in conjunctival epithelial cells constitutes another marker of ocular surface inflammation in patients treated with BAK-containing eye drops.[32] In our in vitro system, BAK alone did not induce expression of this inflammatory marker, a finding consistent with the already described dependence of epithelial cells on exogenous interferon γ for MHC II expression.[33,34] As T lymphocytes and NK cells are the major producers of interferon γ, we added T cells to the cultures to model the conjunctival lymphoid population. Under these conditions, we observed that exposure to BAK increased epithelial MHC II expression, in agreement with the clinical reports. Moreover, prior NF-κB inhibition completely abolished this effect, suggesting that activation of this signaling pathway in epithelial cells somehow conditions neighboring T cells to favor interferon γ production. It remains to be established whether augmented MHC II expression in conjunctival epithelial cells represents simply a bystander effect of increased T-cell activation or if it plays any role in potentiating ocular surface inflammation. A considerable limitation of in vitro systems involving epithelial cell monolayers to mimic mucosal linings is that these cells

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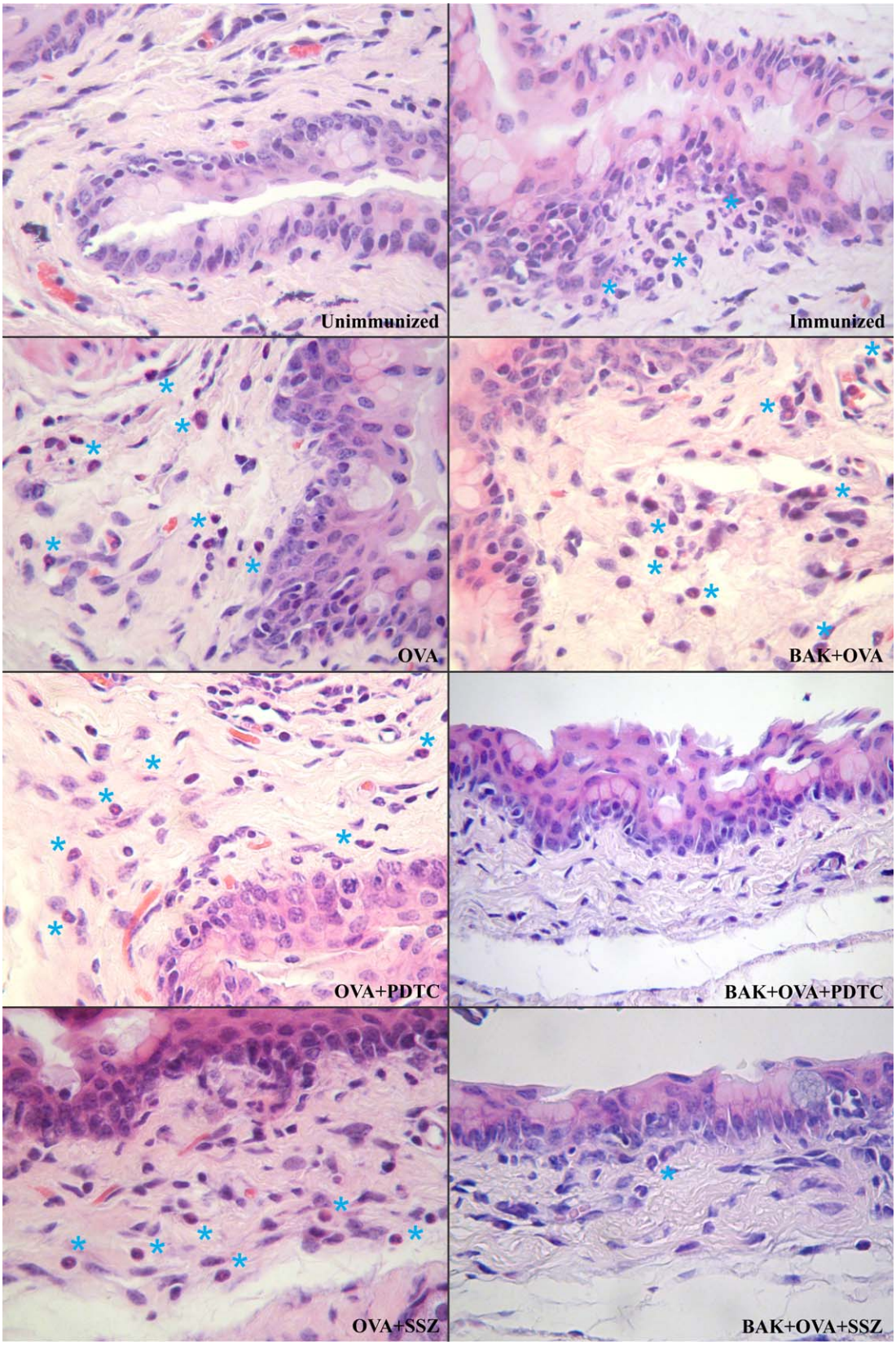

Figure 6. Topical NF-κB inhibitors protect from BAK-facilitated allergic conjunctivitis. Histopathological analysis of eyes obtained by enucleation 48 hours after antigen challenge, as described in Figure 5. Representative micrographs from conjunctival sections from one experiment ($n = 3$) are shown. Infiltrating eosinophils are marked by a *blue asterisk* . Note the absence/paucity of eosinophils in BAK+OVA+PDTC and BAK+OVA+SSZ mice (hematoxylin-eosin staining, ×400).

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produce high levels of TGF-β on contact with T cells, which in turns leads to late inhibition of T-cell activation.[39–42] In any case, our results suggest that the increased MHC II expression observed in treated glaucoma patients could be the end result of BAK promoting epithelial–T-cell interaction and not a direct effect of BAK on the ocular surface epithelium.

We have previously reported that topical BAK prevents the induction of conjunctival tolerance in mice,[9] and in this work we show that concomitant administration of NF-κB inhibitors abolishes the proinflammatory effect of the preservative. Remarkably, both inhibitors when instilled alone had no effect on tolerance induction (Fig. 4), which involves the generation of antigen-specific regulatory T cells.[9] The NF-κB pathway contributes to epithelial homeostasis,[16] and its complete inhibition in epithelial cells results in severe mucosal and/or skin inflammation in genetically modified mice.[43,44] In line with these reports, we observed reduced epithelial cell viability in vitro after exposure to BAK in the presence of NF-κB inhibitors.[9] On the other hand, excessive NF-κB activation in epithelial cells promotes mucosal inflammation, and there are numerous reports of disease improvement by local administration of NF-κB inhibitors in murine models.[21,22] Pharmacological inhibition does not differentiate whether the effect is limited to either the epithelial lining or mucosal immune cells or if it encompasses both cell populations. More importantly, it does not imply complete epithelial inhibition of the NF-κB pathway, which seems to be proinflammatory by itself.[43,44] In agreement with this hypothesis, we did not observe inflammatory changes in the conjunctiva of mice treated with NF-κB inhibitors alone.

Until recently, conjunctival tolerance had not been considered in the pathophysiology of ocular surface inflammation. Stern et al.[45] proposed that dry eye could be conceived as a mucosal autoimmune disease in which breakdown of immunological tolerance leads to ocular surface inflammation. To the best of our knowledge, there are no reports on the possible role of conjunctival tolerance in immune exacerbations of ocular surface disease that frequently affect medically treated glaucoma patients.[2–4] In this work, we show that prior ocular exposure to an antigen markedly hampers allergic conjunctivitis in systemically immunized mice. It is noteworthy that ocular challenge was performed with an antigen dose 125-fold higher than that used for tolerization, highlighting the powerful protection afforded by conjunctival tolerance. Conversely, exposure to BAK abrogated this beneficial effect, suggesting a possible mechanism by which local allergic reaction might be facilitated in patients. Finally, topical NF-κB inhibitors did not affect conjunctival tolerance, but were able to prevent its breakdown by BAK in our model. Altogether, these results suggest that mucosal tolerance plays a key role in ocular surface immune homeostasis, and that NF-κB modulation could be a pharmacological target to reduce BAK adverse effects while at the same time maintaining its microbicidal effect and pharmacokinetic advantages.

Acknowledgments

Supported by Subsidio para Investigación Biomédica 2013, Fundación Allende, Córdoba, Argentina.

Disclosure: **M. Guzmán**, None; **F. Sabbione**, None; **M.L. Gabelloni**, None; **S. Vanzulli**, None; **A.S. Trevani**, None; **M.N. Giordano**, None; **J.G. Galletti**, None

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