In vivo confocal microscopy and ex vivo flow cytometry: new tools for assessing ocular inflammation applied to rabbit lipopolysaccharide-induced conjunctivitis

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Purpose: Lipopolysaccharide (LPS) may act as a key stimulatory agent in ocular surface diseases (OSDs) through TNF-α release. We used in vivo confocal microscopy (CM) and ex vivo flow cytometry, two new tools for assessing ocular inflammation induced by LPS.

Methods: We investigated a model of acute inflammation in rabbits by subconjunctival injection of LPS and developed new evaluation techniques for animal models: CM, to observe inflammatory infiltrates, and conjunctival impression cytology (IC) specimens processed with in vitro CM and flow cytometry for assessing TNF-α and TNF receptor-1 (TNFR-1) expression. A neutralizing anti-TNF-α antibody was used to assess the role of TNF-α.

Results: In vivo CM provided high-resolution images of inflammatory infiltrates and leukocyte rolling in blood vessels. It showed that the LPS group presented strong conjunctival inflammation, reaching its maximum level 4 h after injection. Flow cytometry and immunostaining in IC specimens showed an increased expression of TNF-α and TNFR-1 in the epithelium. Immunohistology confirmed these results and showed infiltration of vimentin+, CD4+, and CD8+ cells in the conjunctiva. TUNEL-positive cells were found 4 h after injection. Neutralizing anti-TNF-α significantly inhibited LPS-induced inflammation and apoptosis evaluated by in vivo CM; and inhibited LPS-induced TNF-α and TNFR-1 expression by ex vivo conjunctival IC specimens evaluated by flow cytometry.

Conclusions: IC specimens and new-generation in vivo CM were thus in good agreement with immunohistology and appeared to be reliable, effective, and nonharmful methods to investigate experimental models of OSDs. The two new tools applied here evaluate the animal models in vivo on the cellular lever. This study is consistent with the experimental research’s strategy by reducing the number of experimental animals used.

Gram-negative (GN) bacterial infections may lead to serious ocular surface inflammation with a high risk of visual impairment and even blindness [1]. Lipopolysaccharide (LPS), a component of the GN bacteria membrane, is directly involved in bacterial ocular diseases, mainly keratitis and conjunctivitis, and a sight-threatening intraocular inflammation, uveitis. It has a major role in ocular inflammation because of its potent proinflammatory and proapoptotic effects, mediated through membrane receptors expressed on host cells. Toll-like receptor (TLR)-4 mediates LPS-induced cellular signaling activation [1-3]. In the respiratory tract, TLRs also mediate the local immune responses, inducing a switch from T helper (Th) 2 to Th1 in children [4], with a differential type of activation depending on the level of LPS exposure [5]. In the ocular epithelia, they may participate in the defense against microbial agents in contact with the ocular surface [6]. Human cornea, conjunctiva, and uvea were shown to express functional LPS receptor proteins such as CD14, LPS-binding protein (LBP), and TLR-4 [7,8]. LPS was demonstrated to induce proinflammatory mediators in cultured human corneal fibroblasts and to promote the breakdown of corneal epithelial tight junctions [9,10]. Altogether, these studies point to a direct role of LPS and its receptors in ocular surface disorders (OSDs), not only in bacterial infections, but also through a potentially important relationship between bacterial components and inflammatory and/or allergic reactions.

A variety of LPS-induced models of ocular diseases have been established in animals. LPS-induced acute corneal ulcers associated with prominent neutrophil, lymphocyte, and monocytic cell infiltration [11,12]. LPS also induced the activation of NF-κB, promoting overexpression of TNF-α mRNA [13]. LPS has been an important research topic in intraocular inflammation mainly through a model of endotoxin-induced uveitis because of its ability to stimulate the release of various inflammatory mediators [14,15], particularly TNF-α [16,17]. Compared with these widely used models, scant published information is available regarding local effects of LPS in the conjunctiva. In an LPS-injected rat conjunctivitis model, the vascular permeability of conjunctiva was found significantly increased 4 h after injection with upregulation of cyclooxygenase-2 [18]. However, our research and other studies over the past few years have convinced us that conjuncti-
val cells directly contribute to the pathogenesis of human OSDs by producing proinflammatory mediators [19-21]. It seemed of interest to extend knowledge on LPS-induced inflammatory infiltrates, cytokine release, and epithelial involvement in the conjunctiva, a highly immunologically active tissue that plays major roles in lacrimal secretion, ocular inflammatory reactions, and host defenses to environmental injuries.

The evaluation of impression cytology (IC) specimens by flow cytometry (FCM), a procedure developed by our group, has been widely used in patients suffering from various OSDs to detect inflammation, apoptosis, or Th1/Th2 profiles [19,20,22,23]. To our knowledge, the use of FCM to evaluate IC specimens has never been utilized in experimental animals. We have also noninvasively explored the human ocular surface with the new-generation in vivo confocal microscope and the Heidelberg Retina Tomograph II/Rostock Cornea Module (HRT II/RCM), and found that histologic-like images can be useful for examining OSDs. We have already used the HRT II/RCM for assessing wound healing after filtration surgery in glaucoma patients [24] and in different laboratory normal animal corneas [25]. We hope to use this system to explore the pathological processes of ocular surface (cornea and conjunctiva) in experimental models.

The development of in vivo research tools could help us to reduce and refine experimental models. With the aim of investigating the effects of LPS on ocular surface tissues, we undertook a series of experiments in rabbits, adapting the aforementioned techniques to animal models. We paid particular attention to LPS proinflammatory actions, TNF-α activation in epithelium, and possible inhibition of LPS-induced inflammation by anti-TNF-α blocking antibodies to meet the double objective of improving our knowledge on LPS effects on the ocular surface and developing new tools for assessing OSD models.

METHODS
Experimental animals and treatments: New Zealand White adult rabbits weighing 2.5-3.0 kg were purchased from CEGAV (les Hautes Noës, St Mars d’Egrenne, France). All procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research under the supervision of a health authority accredited staff member for animal care and management.

Rabbits were anesthetized by subcutaneous injection of a 1:5 mixture of 100 mg/ml xylazine (Bayer, Puteau, France) and 100 mg/mL ketamine HCL (Imalgène 500, Merial, Lyon, France). A first set of in vivo and ex vivo experiments was conducted over 24 h in three groups of four rabbits each, injected with endotoxin-free balanced salt solution (BSS), LPS, or LPS+neutralizing anti-TNF-α, for clinical observation using a modified Draize test, in vivo confocal microscopy (CM), and ex vivo IC. A single 10 µl subconjunctival injection of endotoxin-free BSS or LPS from Escherichia coli (Sigma Aldrich, St. Louis, MO) at 200 ng/µl (2 µg/eye) was administered to the superior bulbar conjunctiva of the right eye, with the left eye remaining intact. A neutralizing antibody (MAB210, R&D Systems, Lille, France) was injected with LPS to inhibit TNF-α activity. In this group, 5 µl of LPS at 400 ng/µl and 5 µl of neutralizing TNF-α at 20 µg/ml were injected together in the subconjunctival space. Thus, the final LPS concentration was 200 ng/µl (2 µg/eye) and the neutralizing anti-TNF-α was used at a final concentration of 10 µg/ml, as previously described in retinal cell cultures [26]. Once the maximal reaction time was determined, a second set of three groups of four rabbits each were used in a similar way and sacrificed at the maximal reaction time for assessing the correlation between in vivo CM and immunohistological patterns. Twenty-four rabbits were used for the experiments.

Clinical findings and Draize test: Conjunctival inflammation criteria were scored according to a modified Draize test at 0, 1, 2, 4, 18, and 24 h: chemosis, watering, and redness in conjunctiva. Chemosis: 0, no chemosis; 1, little chemosis; 2, obvious chemosis; 3, obvious chemosis with more than half of the eyelid closed. Watering: 0, no watering; 1, little watering; 2, watering with moist eyelid and eyelash; 3, watering covering entire eyeball. Redness: 0, normal blood vessels; 1, definitely injected vessels; 2, diffuse crimson red, individual vessels not easily discernible; 3, diffuse beefy red. The final scores were presented as the score of chemosis plus watering plus redness.

In vivo confocal microscopy: The HRT II/RCM (Heidelberg Engineering GmbH, Heidelberg, Germany) in vivo confocal microscope for animal study was used based on a procedure reported previously [25]. After general anesthesia, the rabbits were positioned sideways under the objective. Several confocal microscopic images of the superficial epithelium and the subepithelial tissue were taken of each eye. Images consisted of 384x384 pixels covering an area of 400x400 µm, with lateral and vertical resolutions of 1 µm and 2 µm, respectively. In selected cases, short videos were recorded to analyze the rolling phenomenon of inflammatory cells in blood vessels. The image acquisition time was 0.024 s with two dimensional 384x384 pixel digitized images. As shown in Figure 1, inflammatory cell infiltrations were analyzed separately in the conjunctival epithelium (Figure 1A) or substantia propria (Figure 1B) near the injection area with Cell Count® software (Heidelberg Engineering GmbH) in manual mode and expressed as density ±SD (cells/mm²). At least 10 images were counted for each experimental animal. Later the results of infiltration cells were expressed as the average of four rabbits. The whole examination required 3-5 min for each eye. No side effects related to confocal microscopy examination were noted.

Impression cytology collection: At baseline, 4 h, and 24 h after subconjunctival injection, IC specimens were collected by following techniques previously used in humans [19]. Polyethersulfone filters (Supor®; Gelman Sciences, Ann Arbor, MI) were applied to the superior bulbar conjunctiva. At least four filters were used for each eye, two for further immunocytochemistry, and two immediately dipped in tubes containing 1.5 ml of cold PBS (pH 7.4) with 0.05% paraformaldehyde (PFA) and kept at 4 °C until FCM procedures.
**Flow cytometry analysis from rabbit impression cytology specimens:** Conjunctival cells were extracted by implementing a procedure that was described in human specimens [19,20,22,23]. Cells were extracted by gentle agitation for 30 min and centrifuged (1600 rpm, 5 min), then counted in a Malassez cell before processing for flow cytometry. Direct immunofluorescence procedures were employed to study, respectively, the expressions of cytoplasmic TNF-α (cTNF-α, clone 6402; R&D Systems) and membrane TNFR-1 (mTNFR-1, clone 16803; R&D Systems). For cTNF-α, cells were first permeabilized with 0.1% saponin for 5 min before incubation with anti-TNF-α at a 1:10 dilution for 1 h in the dark at room temperature. Samples were washed in PBS and centrifuged 5 min at 1,600 rpm, then suspended in PBS before FCM reading. For mTNFR-1, cells were directly stained with anti-TNFR-1 antibody at a 1:40 dilution. FITC-conjugated IgG1 (Beckman Coulter, Miami, FL) was used as negative isotypic control. For each antibody, a minimum of 1,000 conjunctival cells were acquired on an EPICS XL flow cytometer (Beckman Coulter) equipped with the System II software. Analytic gates were set around the conjunctival epithelial cell population to exclude cellular debris and inflammatory cells, a cell population smaller in size and easily distinguishable from larger epithelial cells according to our previous research conducted on IC specimens [20,22,23]. This pattern of identification, already validated in humans, was confirmed, however, in a preliminary study conducted in rabbit conjunctiva using double immunostaining with anti-CD45 antibodies (data not shown). Results were expressed as percentages of positive cells.

**Immunohistology on cryosections:** According to the Draize test score and in vivo CM in the first experiment, the maximal reaction time for LPS-injected rabbits was observed 4 h after injections, consistent with previous reports [14]. Therefore, in the second experiment, rabbits of another three groups were euthanized 4 h after injections by lethal overdose of pentobarbital. Enucleated eyes were fixed in 4% PFA, embedded in optimal cutting-temperature (OCT) compound (Tissue-Tek®; Miles Inc., Bayer Diagnostic, Puteaux, France), and kept in liquid nitrogen. The 8-µm cryosections were incubated overnight at 4 °C with monoclonal antibodies to TNF-α (1:50), TNFR-1 (1:200), vimentin (clone V9, 1:50; DakoCytomation, Glostrup, Denmark), CD4 (clone W3/25, 1:50; Serotec, Oxford, UK), CD8 (clone MRC OX-8, 1:50; Serotec), or with IgG1 (Immunotech, Marseilles, France) as negative control. Sections were stained with immunoglobulins Alexa Fluor®488 (1:250) for 1 h and with propidium iodide (PI). Cells were counted in at least five areas of 100x100 µm for substantia propria and 100-µm long for conjunctival epithelium, in a masked manner. At least nine cryosections of different conjunctivas were analyzed. Images were digitized using an Olympus BX-UCB (Olympus, Melville, NY) fluorescence microscope equipped with DP70 Olympus digital camera and image analysis software, to determine the total number of cells positive to the different markers.

**Apoptosis evaluation:** A terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay (Roche Diagnostics, Meylan, France) was used according to the manufacturer’s instructions. The cryosections were permeabilized with a 0.1% Triton X-100-0.1% sodium citrate (2V:1V) solution for 2 min and then incubated with an apoptosis detection kit including the 10 µl TUNEL enzymes and 90 µl TUNEL label at 37 °C for 1 h. After three washes in PBS, the slides were stained with PI and examined under the fluorescence microscope.

**Statistical analysis:** Results were expressed as means±SD of positive cells. Comparisons among the three groups were performed by factorial analysis of variance (ANOVA) and the Bonferroni/Dunnet method for pair-wise comparisons (Statview V; SAS Institute Inc., Cary, NC).

**RESULTS**

**Clinical appearance and Draize test:** Figure 2A summarizes the evaluation of the conjunctival modified Draize test score at 0, 1, 2, 4, 18, and 24 h after the injections. The 4 h score was maximal among all the observation times. Clinically, the conjunctiva injected with BSS presented little redness 4 h after injection (Figure 2B), which disappeared 18 h after injection. The rabbits injected with LPS presented typical acute conjunctivitis characterized by redness, swelling, chemosis, watering, and purulent secretion (Figure 2C) at a maximum level 4 h after injection (Draize score p<0.05, compared to the BSS-injected eyes). When anti-TNF-α was coinjected with LPS, the conjunctivas consistently presented less inflammation than those injected with LPS alone (Figure 2D; Draize score: p<0.05 compared to the LPS injected eyes at 1, 2, 4 and 18 h after injection), with no significant difference with the BSS-injected group at all time points.

**Confocal microscopy observation of lipopolysaccharide-induced conjunctival inflammation:** In vivo CM technique showed conjunctival epithelium (Figure 3A) and subepithelial space (Figure 3B) with almost normal aspects and no obvious infiltration of inflammatory cells 1, 4, 8, and 24 h after injection of BSS. Few hyperactive or dendritic-shaped cells were observed.

One hour after injection of LPS, the conjunctival epithelium also presented normal aspects. The infiltration by inflammatory cells started at 2 h and reached a maximum level at 4 h (Figure 3C), then progressively decreased. At 24 h after injection, the density of infiltrating cells remained; however, at levels above those of normal eyes. At 4 h, in vivo CM showed major infiltration by inflammatory cells in LPS-injected eyes, composed in part of dendritic-shaped cells (Figure 3C, arrows) surrounded by many round cells of various sizes, most likely corresponding to lymphocytes and/or polymorphonuclear cells (Figure 3C, arrowheads). Conjunctival substantia propria was infiltrated by inflammatory cells as early as 1 h after injection, and showed maximal levels (Figure 3D) 2 and 4 h after injection, and continued until 24 h. Anti-TNF-α partially inhibited LPS-induced infiltration cells in the epithelium (Figure 3E) and subepithelial space of conjunctiva (Figure 3F).

Cell counts using the HRT II/RCM Cell Count® software (Figure 3G,H) showed a highly significant increase in inflammatory cells in the LPS group compared to the BSS and LPS+anti-TNF-α groups (p<0.001 and p<0.01, respectively).
at all time points. However, the inflammatory cell density in the LPS+anti-TNF-α group remained higher than that of the BSS-injected group (p<0.001).

In vivo confocal images and short videos also allowed us to observe blood vessels in the conjunctiva. Compared with BSS-injected conjunctiva (Figure 3I), LPS-injected conjunctivas presented the characteristic rolling and leukocyte margination, typical phenomena of inflammatory cells (Figure 3J), as early events in leukocyte recruitment in the margin of blood vessels, especially visible 2 and 4 h after injections. Moreover, cornea adjacent to conjunctival injection sites showed, in the LPS group only, inflammatory infiltrates composed of dendritiform cells (mostly located in the corneal epithelium and anterior stroma), large round polymorphonuclear cells, and smaller lymphocyte resembling cells in the corneal stroma. Central cornea and peripheral cornea far from the injection site showed no abnormalities (data not shown).

Based on clinical evaluation and in vivo CM observations, we chose 4 h as a maximal inflammatory reaction time for further histological analysis.

**Figure 1.** Inflammatory cell infiltrations were analyzed separately in the conjunctival epithelium (A) or substantia propria (B) near the injection area with Cell Count® software in manual mode. The data were expressed as density ±SD (cells/mm²).

**TNF-α and TNFR-1 expression in impression cytology specimens:** Results of flow cytometry in IC specimens on TNF-α and TNFR-1 expressions are shown in Figure 4A,B. The rabbits injected with LPS, considered as a whole, at 4 h and 24 h, showed significantly higher levels of TNF-α and TNFR-1 compared to those injected with BSS. LPS-injected conjunctivas showed 68±4% (mean±SD) of epithelial cells positive for TNF-α and 76±8% for TNFR-1 (Figure 4C; p<0.001, compared to BSS-injected eyes). When neutralizing anti-TNF-α was coinjected 4 h after injection, only 30±5.5% of epithelial cells were positive for TNF-α and 48±6% for TNFR-1 (p<0.001 for both markers, compared to the LPS-injected group at the same time point), but with levels still higher than in the BSS group (p<0.001 for both markers). In BSS-injected eyes, there were only 9.5±4% positive cells for TNF-α and 27.5±4.5% for TNFR-1. The expression in the LPS group decreased 24 h after injection, with 51±3% of conjunctival cells positive for TNF-α and 45±4% for TNFR-1, but still presented a higher level than did BSS-injected conjunctiva with 9±1% cells positive for TNF-α and 14±4% for TNFR-
1 (p < 0.001, for both markers). In the LPS + anti-TNF-α group, at 24 h, 17 ± 3% of TNF-α positive cells and 16 ± 3% for TNFR-1 positive cells were found. This level was lower than in the LPS group (p < 0.001) but was not significantly different from the BSS group. At 24 h, BSS-injected conjunctivae presented almost the same levels of TNF-α or TNFR-1 positive cells as those found at baseline, with 5-15% of cells positive for each marker in normal healthy rabbits.

**TNF-α and TNFR-1 expression in lipopolysaccharide-injected conjunctiva cryosections:** Histologically, BSS-injected rabbit eyes remained almost negative for TNF-α (Figure 5A) and TNFR-1 (Figure 5B). LPS induced important TNF-α (Figure 5C) and TNFR-1 (Figure 5D) stainings, 4 h after injection, in the conjunctival epithelium and in the substantia propria. Two patterns of positivity were found, with cells strongly positive for TNF-α or TNFR-1 infiltrating the conjunctiva both at the epithelial and subepithelial levels, most likely infiltrating leukocytes, and a weaker, diffuse staining of conjunctival epithelial cells that were positively stained for both TNF-α and TNFR-1 compared to BSS-injected eyes in which the epithelium remained almost negative in all eyes. When neutralizing anti-TNF-α antibody was injected, consistent decreases in TNF-α (Figure 5E) and TNFR-1 (Figure 5F) were observed in both infiltrating leukocytes and conjunctival epithelial cells. Quantitative comparisons are given in Table 1 and confirm that anti-TNF-α antibodies significantly decreased cell counts compared to those of LPS-injected eyes, although the levels remained higher than those found in the BSS-injected eyes.

**Infiltration of vimentin positive and CD4+ or CD8+ T-cells in lipopolysaccharide-stimulated conjunctiva:** Subconjunctival LPS administration caused dramatic infiltration by vimentin-positive, dendritiform cells (Figure 6A,B), mostly in the substantia propria and at the junction of the conjunctival epithelium and subepithelial space (Figure 6A), and to a lesser extent in the conjunctival epithelium (Figure 6B). Upon examination at higher magnification, it was found that these cells joined together and seemed to move toward the conjunctival epithelium. Subconjunctival BSS showed few, sparse dendritiform cells, and only in the substantia propria (Figure 6C). Similarly, there was little staining of CD4+ (Figure 6D) or CD8+ (Figure 6E) in BSS-injected eyes. In LPS-injected conjunctivae, both CD4+ and CD8+ cells infiltrated the epithelium and substantia propria. CD4+ cells were found in abundance, especially located in the substantia propria (Figure 6F), whereas CD8+ cells were mainly located in the basal layers of the epithelium and the conjunctival substantia propria (Figure 6G). Compared to LPS injected eyes, anti-TNF-α induced significantly lower infiltration by CD4+ (Figure 6H) or CD8+ cells (Figure 6I and Table 1).

**Apoptosis in the conjunctival epithelium and substantia propria:** In BSS-injected conjunctivae (Figure 7A) under CM observation, we observed only faint and rare TUNEL positive staining in the epithelium, and never in the substantia propria. Cells undergoing an apoptotic process, which showed colocalized nuclear staining by both PI and the TUNEL reaction, were observed in the substantia propria (Figure 7B) of LPS-injected conjunctivae. No apoptotic cells were detected at any level in the conjunctiva injected by LPS plus anti-TNF-α.

Table 1 summarizes counts of TNF-α-, TNFR-1-, CD4-, CD8-, and TUNEL positive cells in the three groups. Neutralizing anti-TNF-α significantly decreased the LPS-induced density of all markers in the conjunctiva compared to the LPS-injected group (p < 0.005 for all markers). However, counts of cells positive to TNF-α, TNFR-1, CD4, and CD8 remained significantly higher than those found in BSS-injected eyes (p < 0.01), with only densities of TUNEL positive cells not significantly different from those of BSS-injected conjunctivae.

As found with confocal microscopy, corneal areas adjacent to the conjunctival injection site showed significant infiltration in the LPS group by cells positive to TNF-α, TNFR-1, vimentin, or CD4, but not CD8 or TUNEL, with densities higher in the peripheral cornea, progressively decreasing as distance from injection increased. In the two other groups, no significant infiltration could be seen in the cornea, except for a few vimentin-positive cells in the stroma and the peripheral corneal epithelium.

**DISCUSSION**

This study is the first to combine two minimally invasive techniques, which has been successfully used in humans, for assessing OSDs in an animal model: flow cytometry in IC specimens and high resolution in vivo confocal microscopy using a new-generation device. Our study demonstrates their feas-

![Figure 2](http://www.molvis.org/molvis/v12/a157/)
bility and their usefulness in experiments and promotes their use for refining ocular irritation assays in agreement with animal laboratory guidelines [27]. We developed these techniques in experimental animals both in vivo and ex vivo and used them to evaluate LPS-induced conjunctival inflammation in rabbits.

We found that LPS induced pathological effects in rabbit conjunctiva consisting of substantial and rapid inflammatory reactions, release of TNF-α and TNFR-1 in the epithelium, and apoptosis, as identified by the TUNEL technique. Despite the known limitations of the TUNEL technique, which only detects one stage of the apoptotic cascade, the overall effects

Figure 3. Heidelberg Retina Tomograph II in vivo confocal microscopy images of conjunctival epithelium (A, C, and E) and substantia propria (B, D, and F) 4 h after injections of BSS (A and B), LPS (C and D), and LPS+anti-TNF-α (E and F; 400x400 µm). C: The dendritic-shaped cells (arrows) were surrounded by many smaller round cells suggestive of lymphocytes (arrowheads). Cell counts of inflammatory cell infiltration in the epithelium/mm² (G) and substantia propria/mm² (H). The asterisk indicates a p<0.001 compared to BSS-injected conjunctivias and the sharp (hash mark) denotes a p<0.01 compared to LPS+anti-TNF-α-injected conjunctivias. Images of a blood vessel in a BSS-injected conjunctiva I and in a LPS-injected conjunctiva (J) show in vivo rolling and leukocyte migration of inflammatory cells. The scale bar indicates 100 µm.
we found on inflammation and apoptosis were consistent with those described in other studies in ocular tissues, such as mouse corneas, which were capable of expressing and releasing TNF-α under LPS stimulation [28], or in LPS-induced uveitis models, in which there were significant increases in leukocyte rolling, adhesion, and inflammatory cell infiltrates, and increased levels of apoptosis in the vascular endothelium [29]. In this uveitis model, anti-TNF-α treatment was shown to reduce LPS-

Table 1.

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Positive cell counts per mm² assessed by immunostaining. The asterisk indicates a p<0.001 compared to BSS-injected conjunctivas, the sharp (hash mark) denotes a p<0.005 compared to LPS+anti-TNF-α-injected conjunctivas, and the double asterisk indicates a p<0.01 compared to BSS-injected conjunctivas.

Figure 4.  TNF-α and TNFR-1 expression evaluated by flow cytometry in rabbit conjunctival IC specimens. A,B: Flow cytometry analysis of IC specimens. Percentages (mean±SD) of positive cells in rabbit eyes injected with BSS, LPS, or LPS+anti-TNF-α at 4 h and 24 h. The asterisk indicates a p<0.001 compared to BSS-injected conjunctivas and the sharp (hash mark) denotes a p<0.001 compared to LPS+anti-TNF-α-injected conjunctivas. C: Flow cytometric fluorescence histograms of TNF-α and TNFR-1 expression overlaid with isotypic negative controls in IC specimens from rabbits injected with LPS at 4 h.

Figure 5. Immunofluorescence staining of TNF-α (A,C,E) and TNFR-1 (B,D,F) in conjunctiva (A,B) injected with BSS, injected with LPS (C,D), or injected with LPS+anti-TNF-α (E,F). Immunostaining of TNF-α and TNFR-1 appears in green and nuclei are in red following propidium iodide staining. The scale bar indicates 100 µm.
neutralizing antibody, expression levels of TNF-α and TNFR-1 in the conjunctival epithelium were significantly reduced, as were vimentin-positive, CD4+, and CD8+ inflammatory infiltrates, and TUNEL-positive cells in deeper layers of the ocular surface.

The high resolution confocal images offered by the HRT II/RCM clearly revealed the infiltration by inflammatory cells after subconjunctival injection of LPS. Histological analyses confirmed the results of HRT II observation, showing inflammatory cells, at least partly lymphocytes (CD4+ and CD8+) and dendritic cells (dendritic-shaped, vimentin-positive cells). Although immunohistology remains the classic method to distinguish between different cells using various antibodies, the HRT II provided an in vivo histologic-like assessment of conjunctival inflammation, with clearly identified infiltration by inflammatory cells of various patterns, blood vessels, and even the appearance of the rolling phenomenon of leukocytes along the vascular walls. In vivo confocal microscopy has already been used for corneal observation in humans [24,25] and, using first-generation devices, in animals [30,31]. This new gen-

Figure 6. A, B, and C: Immunofluorescence staining of vimentin (green) and propidium iodide (red) showing dendritiform cells. Subconjunctival LPS administration increased dendritiform cell density at the junction of the conjunctival epithelium and the substantia propria (A), and also within the conjunctival epithelium (B). C: Subconjunctival BSS injections showed weak infiltration of vimentin-positive cells. D-I: Immunofluorescence staining of CD4 (D, F, and H) or CD8 (E, G, and I) in conjunctivas injected with BSS (D and E), LPS (F and G), or LPS+anti-TNF-α (H and I). The scale bars indicate 100 µm.
eration of in vivo confocal microscopy offers a much higher resolution and allows the investigation of peripheral ocular tissues and quantification of conjunctival inflammatory processes. To our knowledge, relatively few studies concerning in vivo confocal microscopy have been done in conjunctiva pathological process. Compared to the cornea, the conjunctiva is more difficult to examine due to its position and a hyperreflectivity background of the nontransparent sclera.

We also developed and used a flow cytometry analysis technique based on animal IC for the first time and showed the feasibility of this technique in rabbits, despite the potential difficulties of collecting conjunctival cells and the specific issues of immunolabeling procedures in the rabbit. IC specimens allowed the analysis of the most superficial layers of the conjunctival epithelium, and flow cytometry found numerous applications in human ocular surface diseases. Although this method provides less information on deep epithelial layers than does histology, it has the major advantage of being almost painless and minimally invasive and allows analyses of the superficial conjunctival layers. IC, therefore, can investigate inflammatory cells infiltrating the conjunctiva, goblet cells, and expression of various markers by epithelial cells, which are known to overexpress inflammatory antigens, adhesion molecules, interleukins, chemokine receptors, or cytokines, including TNF-α [19-22]. We also used these two new methods to demonstrate the key role of TNF-α in this model by showing the direct antiinflammatory effects of a neutralizing anti-TNF-α antibody.

LPS and TNF-α have been shown to have similar effects on the maturation of dendritic cells, apoptosis, and inflammatory cascades. Our results showed that TNF-α and TNFR-1 could be abundantly expressed in all conjunctival layers, both by infiltrating cells and in the epithelium, after subconjunctival injection of LPS. By analyzing all superficial cells collected by IC, flow cytometry confirmed that TNF-α and its main receptor were not restricted to infiltrating inflammatory cells but were also found in a high percentage of epithelial cells. Histology confirmed this dual expression in the substantia propria and the epithelium, both in inflammatory and epithelial cells. The expression of TNF-α in the conjunctiva seemed to be the same as that found in a LPS-stimulated central cornea culture system [28] and in the rat iris and ciliary body after injection of LPS [29,32,33]. In conjunctival tissue, our studies using neutralizing TNF-α showed that many pathophysiological effects of LPS were at least in part promoted by this proinflammatory cytokine, which had consistently been found to be an essential mediator during LPS-induced cascade reactions in previous studies [17].

Our study provided us with a model in which inflammation and apoptosis occurred at the same time. LPS was previously found to induce apoptotic changes including internucleosomal DNA fragmentation and activation of caspases [34,35]. Injection of LPS thus induced an apoptotic response in endothelial cells of intestinal crypts, the lung, and thymus after 6 h, mediated by TNF-dependent mechanisms [36]. As a key effector candidate for LPS actions, TNF-α possibly acted via its major receptor TNFR-1 [37]. In our model, both TNF-α and its main receptor were overexpressed under LPS stimulation and were found not only in inflammatory infiltrating cells, but also in conjunctival epithelial cells, thus

Figure 7. TUNEL evaluation in the conjunctival substantia propria in (A) BSS- and (B) LPS-injected conjunctivas. Apoptotic cells, appearing as rounded cells with bright yellow or green nuclei, were assessed in the conjunctivas by TUNEL staining (green) and propidium iodide counterstaining (red). The scale bar indicates 100 µm.
confirming that ocular surface epithelial cells may play a direct amplifying role in inflammatory responses to bacterial, toxic, or chemical injuries [38]. The apoptotic cells observed in this model also showed that LPS could induce apoptosis in the conjunctiva. The abundant release of TNF-α may have participated in inducing apoptosis and when TNF-α was inhibited, apoptosis was blocked, at least according to the TUNEL assay. The inflammatory and apoptotic phenomena mediated by LPS seemed to occur in large part, but not exclusively, via the release of TNF-α in our model, confirming the close relationship between LPS and TNF-α in conjunctiva tissues. TNF-α alone was thus shown to induce the same inflammatory and apoptotic phenomena as did LPS in other models, such as keratitis and uveitis [31-33].

LPS has also been shown to lead to the maturation process of dendritic cells in vitro and in vivo [39,40]. Immature human dendritic cells exposed to LPS triggered an early and sustained caspase-like activity, which is required for the survival of these cells [41]. Intravenous LPS stimulated rapid and abundant dendritic cell accumulation in T cell areas of conjunctiva tis-

In conclusion, LPS-injected conjunctivas presented inflammatory cell infiltration and apoptosis in the conjunctival epithelium and substantia propria, with high levels of TNF-α and TNFR-1 expression, both in inflammatory cells and in epithelial cells. Neutralizing anti-TNF-α reduced these effects and demonstrated the central, although not exclusive, role of TNF-α in LPS-induced inflammation and apoptosis. The HRT II/RCM high-definition in vivo confocal microscope and FCM analysis of IC specimens were useful, reliable, and nonharmful methods to evaluate OSDs in experimental animal models. These two methods follow the current guidelines for in vivo experimentation that recommend reducing, refining, and replacing the use of laboratory animals as much as possible [43]. Future investigations of other models of OSDs, e.g., allergic conjunctivitis, dry eye, blepharitis, or toxicological assessments of ocular drugs, may thus be usefully evaluated using these two promising new methods.

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