EXTENDED REPORT

TLRs and NODs mRNA expression pattern in healthy mouse eye

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Br J Ophthalmol 2005;**89**:904–910. doi: 10.1136/bjo.2004.056218

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Accepted for publication 1 December 2004

Aims: To look for TLR and NOD mRNA expression in the healthy eye and in other immune privileged and non-immune privileged mouse organs.
Methods: Semiquantitative RT-PCR was performed to look for TLR1–9 and NOD1 and NOD2 mRNA expressions in the whole eye, in the anterior (AP) and posterior (PP) portions of the eye, in corneal fibroblasts (CF) and in ovary, brain, testis, heart, lung, and spleen.
Results: All the TLR mRNAs were expressed in the whole eye of Balb/c mice. NIH and C57BL/6 did not express TLR9 and TLR8, respectively. NIH expressed higher levels of TLR1, 2, 3, and 6 than the other strains. C57BL/6 expressed the lowest levels of all TLRs. TLR9, 5, and 4 were the less expressed in all strains. All TLRs were expressed in Balb/c PP and TLR1 was not expressed in AP. In NIH and Balb/c CF the majority of TLRs were overexpressed with LPS. In testis, expression of most TLRs was absent. Non-immune privileged organs expressed most of the TLRs. All the organs expressed NOD1 and NOD2. In PP NOD2 was not expressed.
Conclusion: TLRs and NODs are expressed in the eye, and could have an important role in the innate immunity.

The eye is considered an immunologically privileged organ because it can accept corneal transplantation and by the presence of immunosuppressors. This means that intraocular inflammation, which could interfere with the transparency needed to preserve the vision, is self limited. The immune privilege is in part the result of what has been called anterior chamber immune deviation (ACAID).[1] It has been demonstrated that the aqueous humour contains immunosuppressors and immunomodulators[2 3] that inhibit T cell activation. It has been suggested that transforming growth factor (TGF) β2 is the main agent in this process, with interleukin 6 (IL-6) as an antagonist and modulator.[4–6] In experimental ocular inflammation induced by LPS, the immune suppressive properties of the aqueous humour are lost.[7]

The innate immunity is the first line of defence in the host and limits the infection during the first hours after the exposure to micro-organisms. Innate immunity recognises molecular structures of the micro-organism called "pathogen associated molecular patterns" (PAMPs) through the pattern recognition receptors (PRRs). The PRRs are expressed in cells of the innate immune system, including those that function as antigen presenting cells (APC) in the adaptive immunity.[8] The toll-like receptors (TLRs) and the nucleotide binding oligomerisation domain (NOD) proteins are PRRs involved in the recognition of multiple microbial products.[9 10] The TLRs are transmembrane receptors with an extracellular domain, involved in the recognition of the PAMP ligand and with an intracellular domain called TLR/IL-1 receptor (TIR), essential for the signal transduction that drives the activation of the nuclear factor kappa B (NF-κB).[11 12] The NOD proteins is a family of cytosolic proteins that also has been implicated in the recognition of bacteria and in the induction of inflammatory response.[10 13]

The expression of the TLRs and NODs in the eye has been scarcely studied. Recent reports only indicate that TLR4 is expressed in human cornea[14] and in APC of uvea,[15] and that TLR5 binds *Pseudomonas aeruginosa* flagellin in corneal epithelium.[16] We propose that these molecules could be expressed in the eye and could have an important role in its innate immunity. In this work, we studied the expression of the TLR and NOD molecules in the healthy eye and other organs from three different mouse strains.

METHODS

Animals

Balb/c, C57BL/6, and NIH mice were used. All the mice were healthy, 4–6 weeks old, and did not present any ophthalmological alterations. The experimental protocols were performed in accordance to the Instituto de Oftalmología, Fundación Conde de Valenciana statements for the use of animals in ophthalmic and vision research.

Organs

Mice were killed by cervical dislocation. Eyes, testis, ovary, brain (immune privileged organs), and spleen, lung, and heart (non-immune privileged organs) were obtained from each mouse.

To analyse different portions of ocular tissues we obtained the eyes by enucleation and the eye was cut in two portions. The anterior portion (AP) contained corneal tissues and the posterior portion (PP) contained all the other tissues of the eye.

Culture of primary corneal fibroblasts

NIH and Balb/c corneas were digested with 24 U of dispase solution (Invitrogen, Carlsbad, CA, USA) at 37℃ for 4 hours, followed by 10 minutes of digestion with trypsin-EDTA (Invitrogen) at 37℃. After being washed with DMEM, the cells were resuspended in DMEM (150 000 cells/ml) containing 5% of FBS (Gibco, Rockville, MD, USA), gentamicin, and

Abbreviations: ACAID, anterior chamber immune deviation; AP, anterior portion; APC, antigen presenting cells; CF, corneal fibroblasts; IL, interleukin; LPS, lipopolysaccharide; NOD, nucleotide binding oligomerisation domain protein; PAMPs, pathogen associated molecular patterns; PP, posterior portion; PRRs, pattern recognition receptors; RT-PCR, reverse transcriptase polymerase chain reaction; TGF, transforming growth factor; TIR, TLR/IL-1 receptor; TLRs, toll-like receptors

Table 1 Primer sequence

	Primer sequence	Amplified segment (pb)
TLR1	Sense: 5′-GGACTTCCACATGTCTCCACTATCC-3′	596
	Anti-sense: 5′-TCCATGCTTGTTCTTCTCTGTGG-3′	
TLR2	Sense: 5′-GTGGTACCTGAGAATGATGTGGG-3′	541
	Anti-sense: 5′-GTTAAGGAAGTCAGGAACTGGGTG-3′	
TLR3	Sense: 5′-AGGTACCTGAGTTTGAAGCGAGC-3′	489
	Anti-sense: 5′-GAGCATCAGTCTTTGAAGGCTGG-3′	
TLR4	Sense: 5′-CTGGGTGAGAAATGAGCTGG-3′	249
	Anti-sense: 5′-GATACAATTCCACCTGCTGCC-3′	
TLR5	Sense: 5′-TATCTCCCTGTTCTTCAGACGGC-3′	410
	Anti-sense: 5′-TGGTTGCCAGATAGGTCTAAGCG-3′	
TLR6	Sense: 5′-TTAACTGACCTTCCTGGGTGTGG-3′	308
	Anti-sense: 5′-GCAGAACAGTATCACAGGACAGTGG-3′	
TLR7	Sense: 5′-CAAACTTCTGTAGACCGTCATGGG-3′	300
	Anti-sense 5′-AAGTACCGCAACTCTCTCAACGG-3′	
TLR8	Sense: 5′-GTTATGTTGGCTGCTCTGGTTCAC-3′	203
	Anti-sense: 5′-TCACTCTCTTCAAGGTGGTAGC-3′	
TLR9	Sense: 5′-GACTTACTGTTGGAGGTGCAGACC-3′	313
	Anti-sense: 5′-GAACACCACGAAGGCATCATAGG-3′	
NOD1	Sense: 5′-AGCTGCAGCCTTGCTTTAGCC-3′	547
	Anti-sense: 5′-TCAGCCATAAATGCCGTAGCG-3′	
NOD2	Sense: 5′-CCGAAGCCCTAGCACTGATGC-3′	476
	Anti-sense: 5′-CAACCATCACGACTCCTCGGG-3′	
β actin	Sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′	324
	Anti-sense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′	

fungizone. The corneal fibroblasts (CF) were cultured overnight in 25 cm^2 flasks; the medium with non-attached cells was discarded and the incubation was continued in new medium, which was changed every 3 days, until 90% of confluence was achieved.[17] The CF were treated with LPS (10 μg/ml) in fresh DMEM medium for 3 or 6 hours. The total RNA was obtained with TRIzol reagent (Invitrogen).

RNA isolation and RT-PCR analysis

All the organs were washed in D-PBS to eliminate blood contamination. Total RNA extraction was performed with TRIzol reagent. Total RNA was treated with free RNAse-DNAse I and RNA was re-extracted with TRIzol reagent. For the reverse transcriptase (RT) reaction, total RNA (3 μg) with 0.5 μg of oligo-$(dT)_{15-18}$ (Invitrogen) was denatured at 70℃ for 10 minutes. Then, 1X single strand buffer, 0.5 mM DTT, 500 μM of each dNTPs and 200U of MMLV reverse transcriptase (Invitrogen) were added. The RT reactions were performed at 42℃ for 1 hour. The polymerase chain reactions (PCR) were performed with 1 μl of the cDNA, 1X buffer, 1 mM $MgCl_2$, 200 μM of each dNTPs, and 0.2 μM of each TLR, NOD, and β actin specific primers (table 1). Optimal PCR conditions were 30 cycles of 30 seconds at 92℃, 30 seconds at 60℃, and 30 seconds at 72℃.

Semiquantitative PCR

The intensity of the amplified bands was analysed with the Alpha Imager software. The band intensities were normalised with the corresponding β actin signal (TLR/β actin or NOD/β actin rate). These results were analysed by the Kruskal-Wallis statistical test.

RESULTS

Expression of TLRs mRNA in the eye of Balb/c, C57BL/6, and NIH mice

Expression analysis of each TLR (TLR1–9) mRNA in the healthy eye of the studied mouse strains was performed. Balb/c mice expressed all the TLRs, whereas NIH and C57BL/6 mice did not express the TLR9 and TLR8, respectively, and TLR5 was only expressed in 20% of the NIH mice (fig 1). In order to quantify the expression of the TLRs, we performed a semiquantitative analysis of the RT-PCR products using the β actin as housekeeping gene. Figure 2 shows the median (SD) of the TLRs expression levels (TLR/β actin expression) in the three mouse strains studied. We found that in the eye of the NIH mice, levels of TLR1, 2, 3, and 6 were higher than in Balb/c and C57BL/6 ($p<0.05$). C57BL/6 mice always presented the lowest expression levels of TLRs. Among all the TLRs, TLR9, 5, and 4, were the less expressed, and TLR2, 6, 1, and 3 were highly expressed in the eye of the three mouse strains studied.

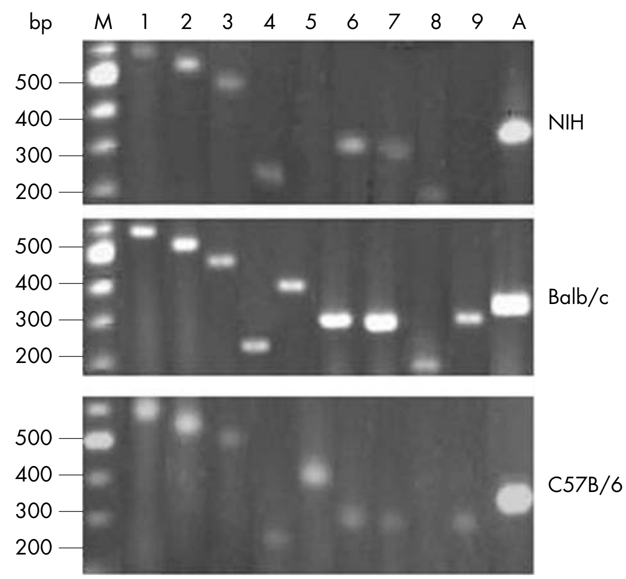

Figure 1 Expression of TLR mRNAs in the healthy eye of three mouse strains. RT-PCRs were performed in the eyes of NIH, Balb/c and C57BL/6 mice. Lane M corresponds to 100 bp molecular ladder; lanes 1–9 correspond to mRNA expression of TLRs1-9, respectively, and lane A to β actin expression.

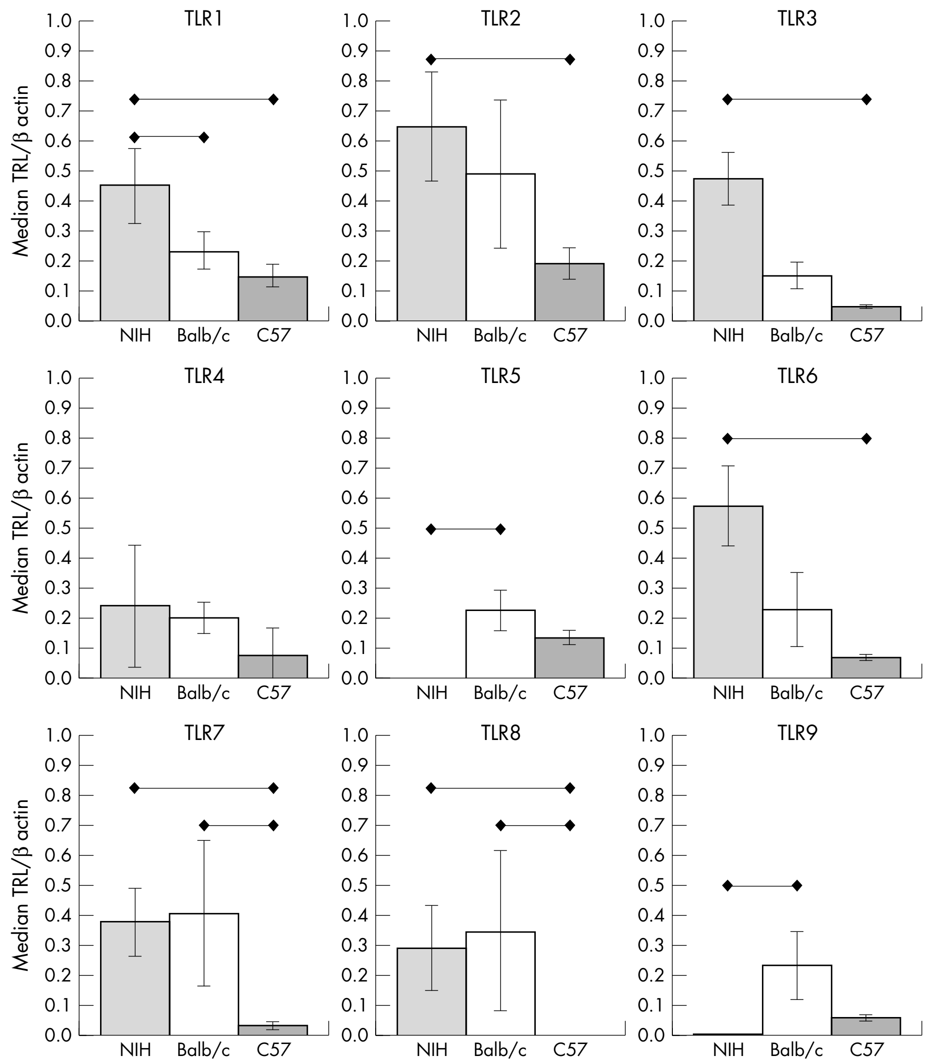

Figure 2 Intensity of mRNA expression of TLRs in the eye of mice. The intensity of each band of figure 1 was measured in an Alpha Imager system. Each bar corresponds to the median value (SD) of the relation TLR/β actin mRNA expression in the whole eye of 10 NIH mice, five Balb/c mice, and five C57BL/6 mice, respectively. Horizontal lines show statistical difference ($p<0.05$) according to the Kruskal-Wallis test.

To explore the TLR expression in the eye regions that could be considered with and without immune privilege we worked with the AP and PP of Balb/c mice, respectively. All the TLRs were expressed in both portions, except for TLR1 in the AP (fig 3A).

Expression of TLRs mRNA in corneal fibroblast from Balb/c and NIH mice

In CF from Balb/c mice only TLR1 and TLR3 were not expressed and in CF from NIH mice no TLR was expressed, but the LPS induced the overexpression of all TLRs in CF

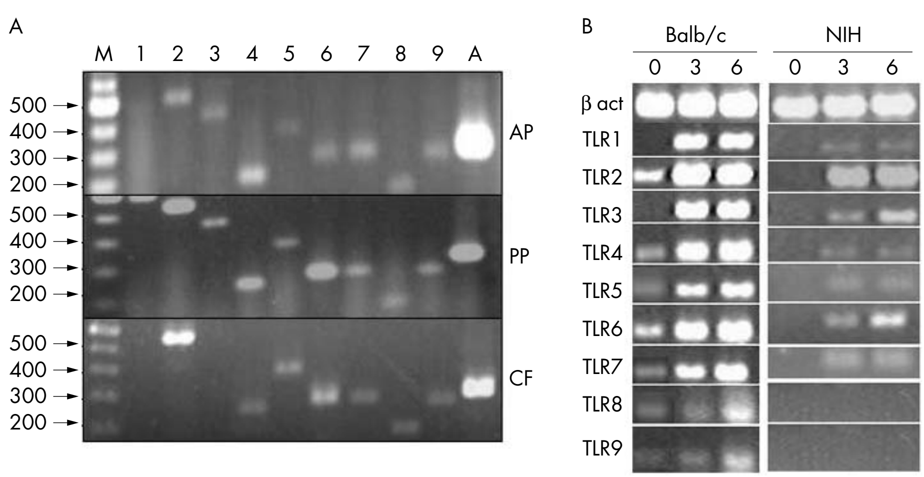

Figure 3 Expression of TLR mRNAs in different compartments of the eye. (A) RT-PCRs were performed in anterior portion (AP), posterior portion (PP), and corneal fibroblasts (CF) from a pool of 16 Balb/c mice eyes. In each gel image, lane M corresponds to 100 bp molecular ladder, lanes 1–9 correspond to mRNA expression of TLRs1-9, respectively, and lane A to β actin expression. (B) RT-PCRs were performed on TLR1-9 in corneal fibroblast of Balb/c and NIH mice treated at 0, 3, and 6 hours with LPS (10 μg/ml).

from Balb/c and TLR1, 2, 3, 4, 5, 6, and 7 in NIH mice. C57BL/6 CF were not assayed (fig 3B).

Expression of TLRs mRNA in immune privileged and non-immune privileged organs

TLR expression was analysed in other organs: immune privileged (brain, testis, and ovary) and in non-immune privileged (spleen, lung, and heart). As can be seen in figure 4, all the TLRs were expressed in the ovary of the three strains studied. In contrast, in the testis of the three strains studied; only TLR2 and 8 were found. TLR2, 3, 6, and 9 were expressed in Balb/c mice brain; C57BL/6 mice brain also expressed TLR2, 3, 6 and 9, as well as TLR5. NIH mice brain expressed most of the TLRs except TLR3, TLR8, and TLR9.

In the non-immune privileged organs, all the TLRs were expressed in at least one of the strains, except TLR1 which was absent in the heart of the three strains (fig 4).

Expression of NODs mRNA

The mRNAs of the NOD1 and NOD2 molecules were found in the whole eye of the three studied strains, but NOD1 expression was higher than NOD2 in NIH and Balb/c mice. In C57BL/6 mice, NOD1 and NOD2 were expressed with similar intensity (fig 5A and 5B). In the AP and in the CF from Balb/c mice, NOD1, and NOD2 were expressed, and again, NOD1 with higher intensity. However in the PP, NOD2 was not expressed (fig 5C). NOD1 and NOD2 were expressed in all the immune privileged and non-immune privileged organs assayed, and as in the eye, NOD1 expression was higher than NOD2 (fig 6).

DISCUSSION

In this work, we studied the mRNA expression of TLRs and NODs in the eyes of three different mouse strains, as we suppose that the innate immunity molecules could be playing an important part in the ocular transparency, providing protection against pathogenic agents, thus avoiding inflammation.

We found all the TLRs were expressed in the healthy eye of Balb/c mice; only TLR9 and TLR8 were not expressed in NIH and C57BL/6 mice, respectively. These results suggest that these molecules may have a protecting role in the healthy eye by limiting a possible ocular inflammation through the recognition of different PAMPs. Although it is well known that TLRs can induce the expression of endogenous signals, as inflammatory cytokines and chemokines,[11] Lemaitre *et al* have reported that the activation of Toll leads to the production of antimicrobial peptides in *Drosophila*, a phenomenon that has also been demonstrated in mammalian cells.[18] Paulsen *et al* detected, by RNA expression of the antimicrobial molecules bactericidal-permeability-increasing protein (BPI), heparin binding protein (CAP37), and β defensin 1, in samples of healthy nasolacrimal duct epithelium.[19] The expression of β defensin 1 was also detected constitutively in human corneal epithelial cell cultures and the expression of β defensin 2 was induced by IL-1β in the same cells type.[20 21] Human β defensin 1 and the inducible β defensin 2 have been detected also in cilliary body and retinal pigment epithelial cells.[22] We suggest that these antimicrobial peptides could also be induced by PAMPs through the TLRs that we have found in both portions of the eye. Human corneal and conjunctiva epithelial cells express β defensin 2 mRNA, and this expression is upregulated by heat killed *Pseudomonas aeruginosa* and its LPS.[23 24] LPS, a PAMP binding to TLR4, has been involved in the induction of β defensin 2 in human tracheobronchial epithelium and astrocytes.[25 26] This could occur in the eye, as in our study we found expression of TLR4 in the eye of the three studied strains. Saint *et al*, have recently demonstrated, in a murine model, that river blindness is the result of a TLR4 dependent inflammatory response against the endosymbiotic bacterium *Wolbachia*.[27]

In this work we observed that in NIH, a non-syngenic strain of mice, the expression of TLR and NOD mRNA molecules was higher than in the syngenic strains Balb/c and C57BL/6 mice, ($p<0.05$). The growth environment for NIH mice is less strict than that required for syngenic mice. Then, these results could suggest that the exposure to an agent induces an overexpression of the TLR in this strain. Song *et al* reported that human corneal epithelial cells expressed CD14 and TLR4, and that these molecules were overexpressed after the induction

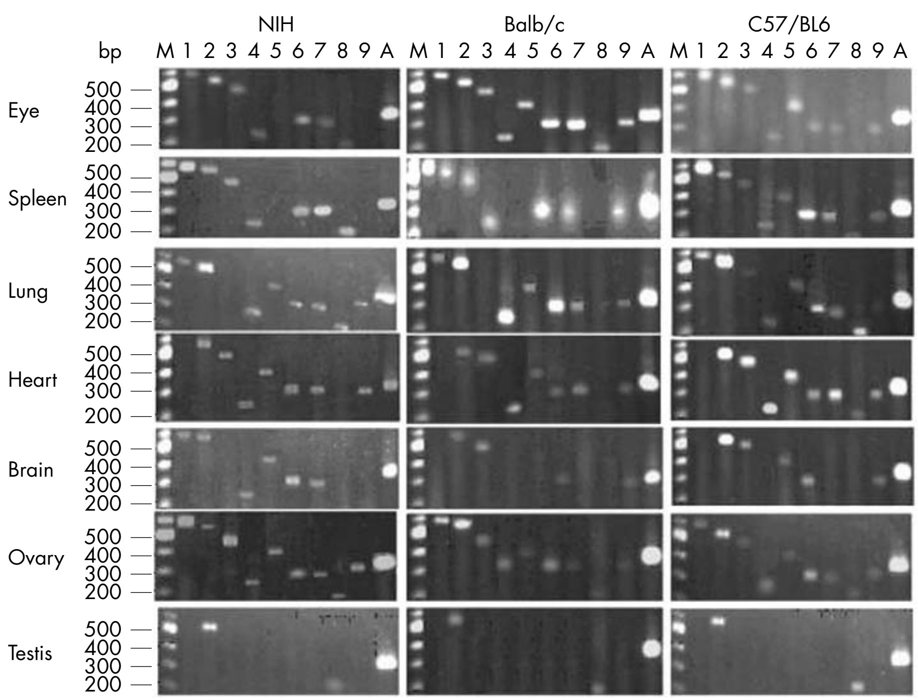

Figure 4 Expression of TLR mRNAs in non-immune privileged and immune privileged organs. RT-PCR was performed on whole eye, brain, testis, ovary, spleen, lung, and heart of NIH, Balb/c and C57BL/6 mice. In every gel image, lane M corresponds to 100 bp molecular ladder, lanes 1–9 correspond to mRNA expression of TLRs1-9, respectively, and lane A to β actin expression.

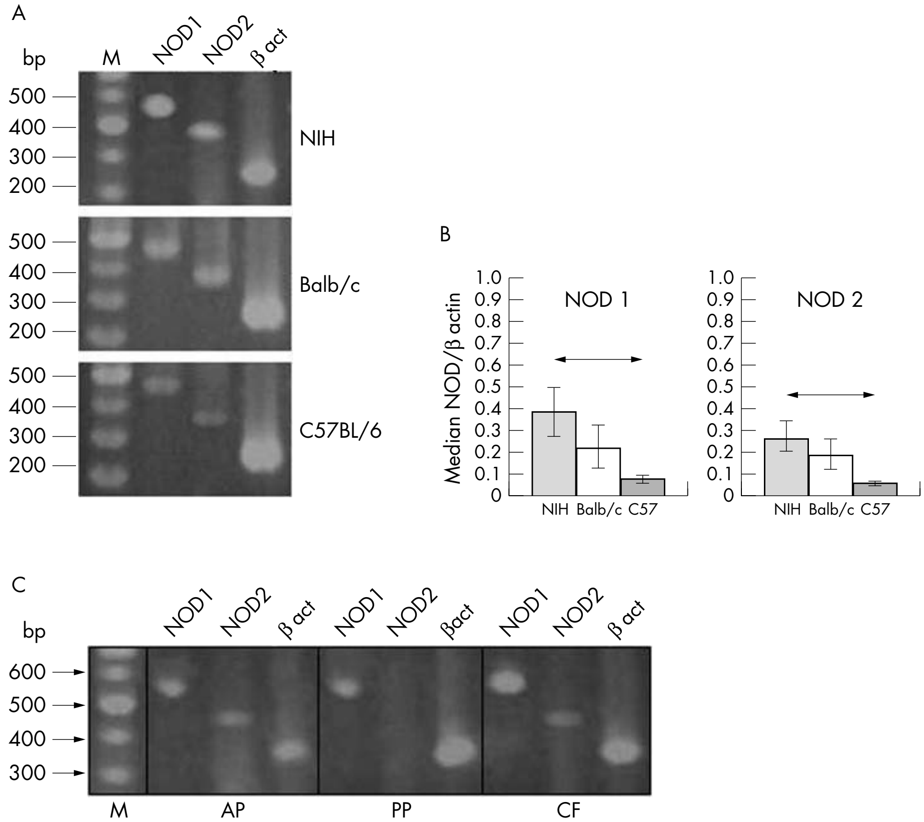

Figure 5 Expression of NOD1 and NOD2 mRNAs in the eye. (A) RT-PCR in whole eye of NIH, Balb/c, and C57BL/6 mice. Lane M corresponds to 100 bp molecular ladder. (B) Intensity of mRNA expression of NODs in the eye. Each bar shows the median (SD) of the relation NOD/β actin mRNA expression in the whole eye of 10 NIH, five Balb/c, and five C57BL/6 mice, respectively. Horizontal lines show statistical difference ($p<0.05$) by Kruskal-Wallis test. (C) mRNA expression of NODs in different eye compartments of Balb/c mice. AP, anterior portion; PP, posterior portion; CF, corneal fibroblasts.

with *Pseudomonas aeruginosa* LPS.[14] Moreover, Jan-Michel *et al* reported the overexpression of TLR2, 3, 4, 6, and 7 in intestinal myofibroblast cells after LPS stimulation.[28] We found similar results in the treatment of CF with LPS. Nevertheless, the response of the CF to LPS was different in each strain.

Our results show that TLR2, TRL1, and TLR6 expression are the strongest in the eye of the three strains of mice studied. These results are in accordance with several reports, which described the required co-expression of TLR2 and TLR6.[29] Besides, it is known that TLR2 forms heterodimeric complexes with TLR1.[30] The co-function of these TLRs increases the ability to recognise a wide variety of PAMPs and could explain the highest expression of these three TLRs in the eye. The co-function TLR1-TLR2 cannot occur in the AP, as TLR1 was not detected in this compartment.

TLR3 and TLR7 were also expressed in the three strains. These results suggest that the healthy eye is able to respond to viral infections because the TLR3 recognises viral double stranded RNA,[11] and TLR7 and TLR8 can bind to the antiviral agent R-848.[31]

TLR5 is involved in the recognition of the bacterial flagellin of pathogenic bacteria like *Salmonella*.[32] In the eye of the studied mice we found a low expression of TLR5. However, in the report by Zhang *et al*, flagellin of *Pseudomonas aeruginosa* was shown to contribute to the inflammatory responses of corneal epithelium in a TLR-5-NF-κB signalling pathway dependent manner, suggesting that the expression of TLR5 can be induced.[16] We found that TLR5 was induced by LPS in CFs of both strains.

There are no reports about TLR9 in the eye. We found low expression of TLR9, that recognises the non-methylated

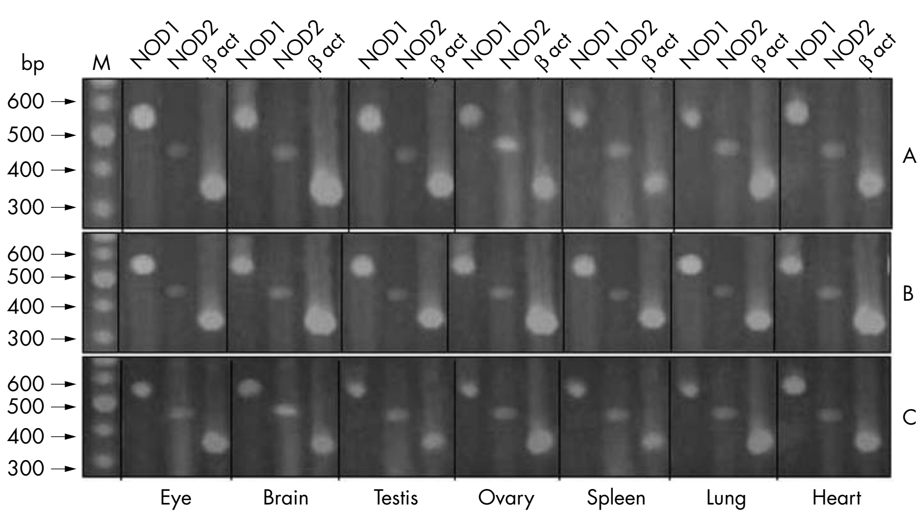

Figure 6 Expression of NOD mRNA in non-immune privileged and immune privileged organs. RT-PCR from the whole eye, brain, testis, ovary, spleen, lung, and heart of NIH (A), Balb/c (B), and C57BL/6 (C) mice. Lane M: 100 bp molecular ladder.

sequence CpG of bacterial DNA,[33] in the eye of Balb/c and C57BL/6 mice, and was absent in the eye of NIH mice, even in its CF treated with the LPS.

In the eye, the TLRs could have an important role in the rapid elimination of microbial pathogens through antimicrobial peptide induction. When the microbial pathogens invade ocular tissues the TLRs also can induce pro-inflammatory cytokines and chemokines and trigger the adaptive immune response. Song *et al* reported that the human corneal epithelial and stromal cells secreted IL-6 and IL-8 when treated with LPS.[14] Oakes *et al* reported that the IL-8 gene expression is associated with herpes simplex virus infection of human keratocytes but not in human corneal epithelial cells.[34] We found expression of TLR7 and TLR8, which are associated with the recognition of antiviral products in CF of Balb/c mice.

We found a differential expression of TLRs between the eye and the other immune privileged organs, such as testis and brain, where some TLRs were absent. According to our results, the eye has a TLRs expression pattern as that seen in the non-immune privileged organs.

NOD1 and NOD2 are intracellular molecules, which have a role in the regulation of pro-inflammatory pathways through NF-κB induced by bacterial ligands.[10] NOD2 recognises a specific peptidoglycan motif from bacteria, the muramyl dipeptide,[35] and NOD1 is able to detect a bacterial motif brought into the cytosolic compartment, as gamma-D-glutamylmesodiaminopimelate.[36] So far, reports about the expression of NOD1 and NOD2 in the eye do not exist.

We found expression of NOD2 in the AP and in the Balb/c CF, but not in the PP. NOD1 is present in both portions and in Balb/c CF. The expression of NOD molecules in the eye suggests that the eye is also able to respond to intracellular PAMPs. Recently, mutations in NOD2 have been associated with inflammatory diseases as Blau syndrome, a rare autosomic dominant disorder, in which the patient presents diverse ailments, such as arthritis, uveitis, skin rashes, and granuloma.[37]

Several reports indicate that there are different susceptibilities to infections in the three strains studied. For example, C57BL/6 is resistant to *Toxoplasma gondii*,[38] to herpes simplex virus 1,[39] and to *Trypanosoma congolense*,[40] but is susceptible to *Salmonella typhimurium*,[41] to hepatitis virus type 3,[42] whereas Balb/c is resistant to P*seudomona aeruginosa*,[43] to adenovirus type 1,[44] and susceptible to cytomegalovirus,[45] to *Yersinia*,[46] and to *Mycobacterium*.[47] We think that in these differences could be involved the different expression patterns of TLRs and NODs seen in the strains.

ACKNOWLEDGEMENTS

This work was supported by the Instituto Politécnico Nacional (CGPI20040430) and by the Patronato del Instituto de Oftalmología Fundación Conde de Valenciana. Sandra Rodríguez-Martínez received scholarships from CONACYT and PIFI-IPN for her postgraduate studies in CQB. Luis Jiménez-Zamudio, Ethel García-Latorre, and Mario E Cancino-Díaz are fellows of COFAA-IPN, EDI-IPN, and SNI-CONACYT. Juan C Cancino-Díaz is a fellow of EDI-IPN and SNI-CONACYT.

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