Diverse Expression of Toll-Like Receptor-9 and β-Defensin-2 in Corneal Cells during Herpes Simplex Virus-1 Keratitis

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Abstract — Involvement of the innate immunity, especially toll-like receptors (TLR) and β-defensins (BD) in the pathogenesis of various ocular disorders has been intensively studied in recent years. However, a role for TLR and BD in keratitis induced by HSV-1 is not fully elucidated. In our study, we examined TLR9 and BD2 expression in HSV-1 keratitis in the murine model and infected children. TLR9 and BD2 mRNA expression was detected by real-time PCR in corneal cells obtained from C57BL/6 and BALB/c mice during 7 days after HSV-1 application on scarified cornea as well as in corneal and conjunctival cells from children with HSV-1 keratitis. We observed elevated frequency of HSV-1-infected mice in both mouse strains, reaching a peak 3 days post infection when 86% C57BL/6 and 50% BALB/c mice were found to be infected. Concurrently, in C57BL/6 mice, we observed a robust decline in TLR9 expression, especially on the 1st and 7th days post topical infection. BALB/c mice revealed a modest reduction in TLR9 expression on the 1st and 7th days post infection. Contrary to TLR9, the expression of murine BD2 (mBD2) was not changed post topical HSV-1 infection in both mouse strains. We also demonstrated a constitutive expression of TLR9 and human BD2 (hBD2) in corneal cells obtained from healthy children with no difference between 2-5 and 6-16 age groups. The expression of TLR9 was found to be more than 30-fold elevated in children with HSV-1 keratitis as compared to healthy individuals, with no difference in hBD2 expression. Our data demonstrate diverse changes in TLR9 and BD2 expression in both murine and children corneal cells suggesting their involvement in HSV-1 keratitis. However, further study is required to elucidate the role of TLR9 and BD2 in the pathogenesis of this disorder.

Keywords — innate immunity, herpes simplex virus type 1, keratitis, toll-like receptor-9, β-defensin-2

I. INTRODUCTION

HERPES simplex virus type 1 (HSV-1) infection is associated with ocular diseases, and keratitis is a major cause of human infectious blindness (1-4). The ability of the ocular surface to respond to pathogens is in part attributed to involvement of the innate immunity (5-9). Corneal epithelial cells can initiate the innate immune response to HSV-1 through activation of their toll-like receptors (TLR) leading to rapid up-regulation of variety of chemokines and proinflammatory cytokines in the cornea detectable at the protein level within hours of HSV-1 infection (10). Among the TLR, TLR9 was shown to induce a strong immune response in the cornea by ligating HSV-1 unmethylated CpG-rich motifs that are found in high abundance in HSV-1 DNA (11), and its expression was detected in murine eyes(12, 13).

In addition to TLR, a number of cationic antimicrobial peptides were also identified in corneal and conjunctival epithelial cells such as β-defensins (BD) (14). Among them, BD2 was found to be constitutively expressed in corneal cells(15-18). An association between TLR and BD expression has been established in a number of tissues including ocular surface(7, 9, 14). However, possible physiological role for such link in HSV-1 keratitis is largely unknown. Recent data demonstrated the changes in TLR9 and BD2 expression in murine corneal infection induced by Pseudomonas aeruginosa (17, 19) and human dry eye disease (18), suggesting their involvement in these disorders. In this study, we evaluated TLR9 and BD2 expression at the mRNA levels in HSV-1 keratitis in the murine model and also in HSV-1 infected children.

II. MATERIALS AND METHODS

Murine model of corneal HSV-1 infection

C57BL/6 and BALB/c male and female mice (6-10 weeks old) were purchased from the Experimental Biomedical Technology Center “Andreevka” of the Russian Academy of Medical Sciences. All procedures were approved by the animal experimentation ethics committee of the I. I. Metchnikov Institute of Vaccines and Sera, Russian Academy of Medical Sciences, Moscow, Russian Federation. For HSV-1 infection,
we applied the protocol described by Wuest et al. (20). Briefly, both murine eyes were scarified on their corneas with a 25-gauge needle, and HSV-1 at a titer of 10^7 cytopathic doses (causing death of 50% target cells in preliminary experiments) was applied in a volume of 3.0 μl in RPMI-1640 medium. On days 0, 1, 3 and 7 post scarification/infection, the mice were euthanized, both eyes were swabbed and assayed for infectious virus as well as for TLR9 and mBD2 mRNA expression by RT-PCR (see below). The mice have been divided in 3 groups: uninfected naive mice, scarified uninfected mice, and scarified and topically (as described above) HSV-1 infected mice.

**HSV-1 infected children**

All procedures involving human subjects have been performed in accordance with the Declaration of Helsinki and were approved by the ethics committee of Morozov’s Children’s Hospital, Moscow, Russian Federation. The details of the study were explained to all children parents and written informed consents were obtained.

Children were divided into two main groups: 2-5 and 6-14 years old, each divided into two additional groups: healthy uninfected children and children suffered from HSV-1 keratitis, 25 children in each group, total 100 participants. All children underwent ophthalmic examinations including ophthalmoscopy, biomicroscopy, and autorefractometry. Clinical diagnosis of HSV-1 infection was confirmed by applying HSV-1 serology. Before cell collections, a drop of 0.4% inokain (Promed Exports, India) was instilled on the eye, and conjunctival cells were collected by sterile swabs from both eyelids and then assayed for TLR9 and hBD2 mRNA expression by RT-PCR.

**Real time PCR for detection of TLR9, BD and HSV-1**

Total RNA was isolated from corneal cells using RNeasy Mini Kit Qiagen and RIBO-sorb kit (ILS, Russia) according to the manufacturer's protocol. 1 microgram of each RNA sample was reverse transcribed with Qiagen OneStep RT-PCR Kit and reverse transcriptase (SibEnzyme, Russia). Real-time PCR was performed with an RT-PCR ANC-32 machine (Institute for Analytical Instrumentation, Russian Federation) using SYBER Green PCR master mix (Synthol, Russia) and the following primers:

- hTLR9 sense 5’- tgt-ttg-ttg-tga-agg-aca-gtt-ctc-tc -3’
- hTLR9 antisense 5’- cac-tcg-gag-gtt-tcc-cag-c -3’
- hBD-2 sense 5’- tgt-atc-tcc-tct-tct-cgt-tc-3’
- hBD-2 antisense 5’- tgc-caa-ttt-gtt-tat-acc-ttc-tc -3’
- mTLR9 sense 5’- cgg-ctc-tcc-ttg-atc-tcc-aa -3’
- mTLR9 antisense 5’- ctc-agg-ctg-aca-ttc-acc-ag -3’
- mBD-2 sense 5’- agt-gcc-ctt-tct-acc-ace-cg-3’
- mBD-2 antisense 5’- cac-agt-acc-ctc-cat-tgg-tg -3’

Raw data from reactions using TLR9 and BD-2 primers were normalized to the corresponding data from the same reactions using control β-actin primers:

- hβ-actin sense 5’- ggg-gga-aat-cct-ggg-tga-cat-t -3’
- hβ-actin antisense5’- gat-gga-gtt-gaa-ggt-agt-ttc-tgt -3’
- mβ-actin sense 5’- tac-cac-agg-cat-tgt-gat-gg -3’
- mβ-actin antisense 5’- ctt-tga-tgt-cac-gca-cga-tt -3’

The reaction was carried out by the following protocol: 5 min at 500C, 5 min at 950C, and 40 cycles of 50 sec at 640C and 20 sec at 950C. The data were calculated using software supplied with ANK-32 amplifier. The development of infection was verified by HSV-1 detection by real-time PCR kit (HERPOL, Litech, Moscow, Russia) according to the manufacturer’s instruction.

**Statistical analysis.** Significance levels of the data were determined by nonparametric Mann Whitney test. P-values lower than 0.05 were considered significant.

### III. RESULTS

**Murine model.**

Various mouse strains differently respond to HSV-1 infection, but the reasons for such diversity remain unclear (21). We studied kinetics of TLR9 and mBD2 expression, both proposed to be associated with ocular pathology (7, 14), in C57BL/6 and BALB/c mice shown previously to respond differently to HSV-1-induced encephalitis (5, 22). Since HSV replication in the eye occurs almost exclusively in corneal epithelial cells(23), corneal swabbed material obtained from uninfected intact (control), scarified and scarified and HSV-1 infected eyes was examined by RT-PCR for the presence of HSV-1 as well as for TLR9 and mBD2. As shown in Fig. 1, a significant elevation in the percentage of infected C57BL/6 mice, as compared with scarified mice only, was determined in a bell-shape manner: 32%, 86% and 55% at the 1st, 3rd and 7th days post infection, respectively, whereas BALB/c mice displayed different infection profile, namely, 50%, 50% and 20% infected mice have been detected at the similar time points (Fig. 1). Excessive frequency and extended infection observed in C57BL/6 mice suggest that, at least at our experimental settings, these mice are more susceptible to HSV-1-induced keratitis than BALB/c mice.

![Figure 1. Kinetics of murine HSV-1 infection. HSV-1 was inoculated into the conjunctival sac after scarification of the cornea, and the infection in corneal cells was determined by RT-PCR. The data are representative of 2-3 separate experiments, total of 10-15 mice per each time point. Each circle represents the percentage of infected mice identified in the](http://www.researchpub.org/journal/iti/iti.html)
To study possible involvement of the innate immunity in HSV-1-induced keratitis, we measured TLR9 and mBD2 expression by RT-PCR in the same mice. The log10 of the transcript numbers were calculated and normalized to β-actin expression, and the data are presented as relative fold changes between scarified vs scarified and HSV-1 infected mice as well as between naïve vs scarified mice. We found that topical HSV-1 infection of C57BL/6 mice was accompanied by a striking reduction in TLR9 expression in corneal cells obtained from scarified and infected mice as compared to scarified mice only at all time points post infection (8.317-fold, 91.2-fold and 131,825-fold changes at the 1st, 3rd and 7th days post infection, respectively) (Fig. 2a).

As calculated by log10 transcript number normalized to β-actin transcripts, all these differences were found to be highly statistically significant, namely, 1st day: 2.25±0.14 vs 6.17±0.21, P<0.001; 3rd day: 3.45±0.22 vs 5.41±0.16, P<0.01; 7th day: 2.68±0.22 vs 7.8±0.19, P<0.001). Of note, in C57BL/6 mice, scarification by itself lead to reduction in TLR9 expression on the 1st and 3rd days post infection (4.2-fold and 24.5-fold, respectively; in transcription levels: 6.8±0.23 vs 5.41±0.16, P=0.027, respectively), and to a 10-fold elevation in its expression on the 7th day (in transcription levels: 6.8±0.23 vs 7.8±0.19, P=0.21) (Fig. 2b).

In BALB/c mice, the expression of TLR9 was also found to be decreased but much less pronounced as compared to C57BL/6 mice and only on the 1st and 3rd days post infection (4.1-fold and 5.7-fold, respectively; in the transcript levels: 5.76±0.29 vs 6.38±0.16, P=0.032, and 5.6±0.32 vs 6.36±0.18, P=0.035, respectively) (Fig. 2c) with no difference between naïve and scarified mice.

There was a trend in the reduction of mBD2 expression in C57BL/6 on the 3rd and 7th days post infection (6.3-fold and 3.2-fold in scarified and infected cornea as compared to scarified cornea, respectively) but the difference did not reach significance due to the high variability of mBD2 expression in these mice (Fig. 2d). Likewise, we did not observe the differences in mBD2 expression in BALB/c mice.

Altogether, these data demonstrate different prevalence of HSV-1-induced keratitis in C57BL/6 and BALB/c mice accompanied by reduction in TLR9 expression in corneal cells (much more significantly in C57BL/6 mice as compared to BALB/c mice) with no differences in mBD2 expression.

Children model.

Although the innate immunity components such as TLR and BD are associated with the pathogenesis of HSV-1 keratitis in adult individuals (15, 24), the contribution of TLR and BD in its pathogenesis in children has not been addressed yet. In our study, we demonstrated a constitutive expression of TLR9 in corneal and conjunctival epithelial cells obtained from healthy children with no difference between 2-5 and 6-14 age groups (in the transcript levels: 3.67±0.27 and 4.97±1.33, P=0.051).

Remarkably, corneal and conjunctival epithelial cells obtained from children with HSV-1 keratitis revealed significantly elevated levels of TLR9 expression as compared to uninfected children: in 2-5 age group there was a 151-fold increase (in the transcript levels: 5.85±1.57 vs 3.67±0.27, P<0.01), in the 6-14 age group there was a 9.5-fold increase (in...
the transcript levels: 5.95±2.03 vs 4.97±1.33, P=0.043) and after combing all the data there was a 39.8-fold increase (in the transcript levels: 5.97±1.71 vs 4.37±1.27, P<0.01) (Fig. 2e). Similarly to TLR9, we observed a constitutive expression of hBD2 in corneal and conjunctival epithelial cells obtained from healthy children also with no difference between 2-5 and 9-14 age groups: 6.61±0.22 and 7.47±1.09, P=0.057. However, contrary to TLR9, the levels of hBD2 expression were found to be similar in uninfected and HSV-1 infected children (6.89±1.04 vs 6.82±1.25, P=0.49).

In the group of healthy children in a tear determined cytokines: IL-β is defined in 40-45 % of cases in the concentration of 1.8+ 0.7pg/ml TNF alpha is determined in 30% of cases in the concentration of 1.4 to+0.8 pg/ml, IL-6 concentration of 0.8+0.6 pg/ml, IL-8 in a concentration of 2.0 to+0.8 pg/ml. In the tears of children with disorders cytokines are defined in 100% of cases, while concentrations of IL-1 beta - 3,18 pg/ml, IL-6 - 4,3±1.2 pg/ml TNF - 4,65±2,2 pg/ml; IL-8 - 8,7±1,5 pg/ml. Thus we can conclude that the levels of proinflammatory cytokines in the tears was significantly sozdateli when we have studied the pathology. This pattern is likely to be associated with activation of TLRs, in particular with TLR9. We have shown changes in the expression of TLR9 cells of the cornea. Many scientists have shown that the protein level of this receptor is also changed (10, 18).

Collectively, these data demonstrate significant elevation in TLR9 expression during HSV-1 keratitis suggesting its involvement in the pathogenesis of this disorder in children.

IV. DISCUSSION

It has been previously shown that C57BL/6 mice seem to be more resistant to HSV-1 infection than BALB/c mice (5, 22), which was attributed to TLR-mediated different ability of these mouse strains to develop a rapid type 1 interferon (IFN-α/β) response leading to a transient delay in HSV-1 replication at the inoculation site (5, 21). Unexpectedly, our data suggest that C57BL/6 mice are more susceptible to topically applied HSV-1 than BALB/c mice (Fig. 1). Although the reasons for the discrepancy are not clear, they may be attributed to differences in protocols of topical HSV-1 infection, applied viral strains and dosage, infection determination assays, kinetics of the infection and the read-out evaluation.

It is well established that TLR activation during HSV-1-induced infection results in the production of BD and vice versa (13, 19, 25, 26). However, the role of TLR9 and BD2 in HSV-1 keratitis remains poorly understood. We examined TLR9 and BD2 expression in HSV-1-infected corneal cells and found their diverse expression both in mouse and children models underling a complexity in the possible involvement of these innate immunity components in the pathogenesis and outcome of HSV-1 keratitis. It is believed that triggering of multiple innate immune protective and inflammatory responses, including TLR9 and BD2 expression along with production of interferons, cytokines and chemokines that attract a range of immune cells, all contribute to the clearance of HSV-1 from the cornea during primary infection (7-10, 14, 21, 27). However, accumulating evidence indicates that although activation of the innate immunity mainly succeeded in clearing the virus from the cornea, it can leave the tissue with immune cell-orchestrated chronic inflammatory lesion thus culminating the infection in immunopathology and impaired vision. Indeed, it has been shown that the absence of TLR9 resulted in significantly diminished ocular lesions (13). Likewise, mice lacking the adapter molecule MyD88 that is downstream to TLR signaling pathway and is essential for the production of inflammatory cytokines, were resistant to ocular lesion development, but such animals were also unable to control infection, and most succumbed to lethal encephalitis (13, 28-31). Similarly, human corneal endothelial cells were shown to transcriptionally initiate inflammatory programs in response to HSV-1 infection related to NF-κB and some other transcriptional factors and express arrays of inflammatory cytokine induction by TLR9, but, on the other hand, it seems that HSV-1 exploits TLR9-mediated NF-κB activation for its own replication (10). These findings suggest that the TLR9 ligand activity of HSV-1 may be a major mechanism by which the virus, usually confined to the epithelium of the infected cornea (23), induces early events necessary for the development of the inflammatory disease. Thus, while the precise role of TLR9 in the innate response to HSV-1-induced keratitis is not fully understood, it appears to have a role in mediating both immune protection and immune pathology.

As for a link between TLR and BD, our results confirm previously described constitutive expression of TLR9 and BD2 in corneal and conjunctival epithelial cells (13, 15-18). Moreover, an increased TLR9 and mBD2 corneal expression has been described in topically HSV-1-infected C57BL/6 mice (13). In our study, however, we observed decreased or unchanged corneal expression of TLR9 and mBD2, respectively, in C57BL/6 mice with no difference in their expression in BALB/c mice (Fig. 2). The differences in the results are probably due to different protocols including viral strains, primers and types of RT-PCR applied in these experiments.

TLR9 and hBD2 expression in ocular children pathology has not been previously investigated. Recently, down- and up-regulated TLR9 and hBD2, expression was observed in adult dry eye patients (18). According to Redfern et al., in the conjunctiva of mice with EDE (experimental dry eye), TLR 9 was found to be upregulated by 6.7 ± 4.1-fold, and in the lacrimal gland TLR9 was downregulated by 1.3 ± 0.5-fold, while mBD-4 (hBD-2 orthologue) were upregulated although this did not reach statistical significance (18). In human, according to Takeda et al., DTS subjects had significant decrease in TLR9 mRNA to 0.13 ± 0.22, while hBD-2 mRNA was significantly increased by 19.8 ± 13.2-fold compared with age- and sex-matched healthy subjects (10). Based on this data, further research on this topic was conducted by us.

In our study, we demonstrated a constitutive expression of TLR9 and hBD2 in corneal and conjunctival epithelial cells. Noteworthy, TLR9 expression was found to be dramatically elevated in children with HSV-1 keratitis (Fig. 2e) with no
changes in hBD2 expression. To our knowledge, these findings are the first to illustrate a possible link between TLR9, hBD2 and HIV-1 keratitis in children.

V. CONCLUSIONS

In conclusion, our findings demonstrate that TLR9 and BD2 are diversely expressed in the healthy and HIV-1-infected murine and children cornea, suggesting involvement of these innate immunity components in HSV-1 keratitis. Further studies of TLR9 and BD2 expression and functional activity may provide insight into the mechanisms of innate immunity control of ocular HSV-1 infection.

Declaration of interest: The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

REFERENCES AND FOOTNOTES


