Polymorphisms in Toll-like receptor 3 confer natural resistance to human herpes simplex virus type 2 infection

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Lack of Toll-like receptor 3 (TLR3) functional activity predisposes children to human herpesvirus 1 (HSV-1) encephalitis. In this study, we have investigated whether there is any link between TLR3 and adult HSV-2 infection by studying genetic variations in TLR3. The frequency of four single-nucleotide polymorphisms (SNPs) in the TLR3 gene in 239 patients with genital HSV-2 infection and 162 healthy controls, as well as the impact of these variants on TLR3 gene-expression levels, were compared. Two SNPs in the TLR3 gene (rs13126816 and rs3775291) were associated with a reduced incidence of HSV-2 infection. The minor allelic variants at both rs13126816 and rs3775291 were more common among healthy HSV-2-seronegative subjects than among HSV-2-infected individuals. This was even more apparent in HSV-1-seronegative individuals. There was, however, no association between any of the four TLR3 SNPs and HSV-2 disease severity, as they were expressed at similar proportions in asymptomatic and symptomatic HSV-2-infected patients alike. Furthermore, when assessing TLR3 mRNA expression in a limited number of HSV-2-infected individuals, we found that individuals carrying the homozygous genotypes for the minor alleles had significantly higher levels of TLR3 mRNA expression in peripheral blood mononuclear cells in response to HSV-2 stimulation than individuals that were homozygous for the major allele variants. Taken together, these results suggest that genetic variations in TLR3 may affect the susceptibility to HSV-2 infection in humans.

INTRODUCTION

In recent years, Toll-like receptor 3 (TLR3) signalling has evolved as a key factor in the immune-mediated control of human herpesvirus 1 (HSV-1) infections. Several different genetic deficiencies/single-nucleotide polymorphisms (SNPs) in the human TLR3 gene or in the genes of other molecules involved in the TLR3 signalling pathway (UNC93B and TRAF3) have been identified in children suffering from HSV-1 encephalitis (HSE). These defects lead to a diminished production of type I and type III interferons (IFN) by fibroblasts, and thus an increased virus replication in vitro (Casrouge et al., 2006; Guo et al., 2011; Pérez de Diego et al., 2010; Zhang et al., 2007). Furthermore, mice that lack TLR3 also show impaired virus clearance after HSV-1 challenge, which is probably due to impaired generation of virus-specific CD8+ T-cells (Davey et al., 2010). Little is known about the impact of TLR3 SNPs/deficiency on HSV-2 susceptibility and disease severity in man. One case report has been published where a homozygous polymorphic TLR3 allele was identified in a patient suffering from severe and recurrent episodes of HSV-2 meningitis (Mollaret’s meningitis) (Willmann et al., 2010). However, in mice it has been conclusively proven that TLR3 is required to protect the central nervous system (CNS) from exacerbated HSV-2 infection, mainly by protecting astrocytes from infection through the induction of endocrine type I IFN responses (Reinert et al., 2012).

TLR3 is an innate pattern-recognition receptor that recognizes viral dsRNA as well as synthetic dsRNA analogues such as poly(I:C). Immune cells that express TLR3 include dendritic cells, macrophages, NK cells and mast cells (Vercammen et al., 2008). The expression of TLR3 is strongly induced in a variety of cells by type I IFN, viral infections or exposure to dsRNA (Miettinen et al., 2001; Vercammen et al., 2008). Signalling through TLR3 potently activates IRF3 and NF-κB, leading to the expression of IFN-β and other pro-inflammatory cytokines (Matsumoto et al., 2002; Oshiumi et al., 2003; Yamamoto et al., 2002).

†These authors contributed equally to this work.
HSV-2 is the causative agent of genital herpes infection, which is one of the most common sexually transmitted infections in the world. Up to one-quarter of the Swedish population is seropositive for HSV-2 (Berntsson et al., 2009; Forsgren et al., 1994; Persson et al., 1995), whilst the prevalence in some sub-Saharan African countries is as high as 80% (Weiss, 2004). The clinical effects of HSV-2 infection range from no symptoms (asymptomatic infection) to severe and recurrent episodes of genital lesions and ulcers. The mechanisms underlying the susceptibility and severity of HSV-2 infection are not fully known, but appear to involve both innate and adaptive immune responses. Several genes have been implicated in disease severity, such as TLR2 (Bochud et al., 2007), mannan-binding lectin 2 (Seppänen et al., 2009) and HLA (Lekstrom-Himes et al., 1999), whereas TBX21 has been associated with susceptibility to infection (Svensson et al., 2008). It is, however, not yet known whether TLR3 affects HSV-2 infection in humans.

This study was undertaken to evaluate whether genetic variations in the TLR3 gene could affect the incidence and/or severity of HSV-2 infection. For this purpose, we selected four common SNPs in the human TLR3 gene that were analysed with respect to allele frequency in 239 HSV-2-infected individuals and in 162 healthy HSV-2-seronegative control subjects. Our data show that two of these four SNPs in the TLR3 gene are associated with the incidence of HSV-2 infection. In addition, we show that patients homozygous for both of the TLR3 minor alleles express higher levels of TLR3 mRNA in response to HSV-2 stimulation than do patients homozygous for the major alleles.

### RESULTS

**SNPs at rs13126816 and rs3775291 are associated with a reduced incidence of genital HSV-2 infection**

To study the association of gene polymorphisms in TLR3 and the incidence of genital HSV-2 infection, we compared the frequency of four different TLR3 SNPs – rs13126816, rs13108688, rs3775292 and rs3775291 – in 239 HSV-2-infected individuals and in 162 healthy HSV-2-seronegative individuals.

We found that two of the four SNPs were correlated with the incidence of HSV-2 infection. Substitution with an adenine (A) allele instead of a guanine (G) at rs13126816 in an intron region of the TLR3 gene was correlated with reduced incidence of HSV-2 infection. The A allele variant was found in 30% of HSV-2-seronegative subjects, whereas its frequency among HSV-2-infected individuals was 23% (\(P = 0.0158\)) (Table 1). Moreover, 13% of HSV-2-seronegative individuals were found to be homozygous for the A allele, while only 4% of HSV-2-infected individuals had a homozygous genotype (\(P = 0.008\)) (Table 2).

The SNP at rs3775291 in the TLR3 gene is a missense mutation that leads to the replacement of a leucine by a phenylalanine at amino acid position 412. In our study

### Table 1. Allele frequencies in HSV-2-infected and -seronegative individuals for four SNPs in the TLR3 gene

Bold type indicates SNPs that are associated with a reduced incidence of HSV-2 infection.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor allele</th>
<th>Allele frequency [% (absolute no.)] Uninfected</th>
<th>Infected</th>
<th>(P)-value ((\chi^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13126816</td>
<td>A</td>
<td>30 (94)</td>
<td>23 (99)</td>
<td>0.0158</td>
</tr>
<tr>
<td>rs13108688</td>
<td>A</td>
<td>17 (53)</td>
<td>21 (88)</td>
<td>0.1527</td>
</tr>
<tr>
<td>rs3775292</td>
<td>A</td>
<td>16 (49)</td>
<td>16 (69)</td>
<td>0.8842</td>
</tr>
<tr>
<td>rs3775291</td>
<td>T</td>
<td>35 (109)</td>
<td>28 (120)</td>
<td>0.0272</td>
</tr>
</tbody>
</table>

### Table 2. Genotype frequencies in HSV-2-infected and HSV-2-seronegative individuals for rs13126816 and rs3775291

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Genotype frequency [% (absolute no.)] Uninfected</th>
<th>Infected</th>
<th>(P)-value ((\chi^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13126816</td>
<td>GG</td>
<td>52 (82)</td>
<td>59 (129)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>35 (56)</td>
<td>37 (81)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>13 (20)</td>
<td>4 (9)</td>
<td>0.008</td>
</tr>
<tr>
<td>rs3775291</td>
<td>CC</td>
<td>44 (67)</td>
<td>51 (110)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>42 (65)</td>
<td>43 (92)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>14 (22)</td>
<td>6 (14)</td>
<td>0.036</td>
</tr>
</tbody>
</table>
The minor allele variant, thymine (T), was associated with reduced incidence of HSV-2 infection. The replacement with a T instead of a C allele at rs3775291 was significantly more common among HSV-2-seronegative individuals than among HSV-2-infected subjects; the T allele was found in 35% of HSV-2-seronegative and in 28% of HSV-2-infected individuals (P = 0.0272) (Table 1). Similarly to rs13126816, the homozygous variant of the minor allele at rs3775291 was also correlated with reduced incidence of disease.

These differences became even more apparent when we excluded all HSV-1-infected individuals. Fifteen per cent of HSV-1-/HSV-2-seronegative individuals were found to be homozygous for the A allele at position rs13126816, while none of the HSV-2-infected but HSV-1-seronegative individuals had a homozygous genotype (P=0.0005) (Table 3). Twenty per cent of HSV-2-seronegative individuals had the homozygous variant of the T allele at position rs3775291, compared with 2% of HSV-2-infected individuals (P = 0.036) (Table 3).

We also performed a haplotype analysis on the four selected TLR3 SNPs. The combination ATGT was correlated significantly with the incidence of HSV-2 infection (P=0.0063) (Table 4). This combination was found in 25% of HSV-2-seronegative individuals compared with 17% of HSV-2-infected individuals (Table 4).

No correlation between polymorphisms in TLR3 and HSV-2 disease severity

We also assessed the impact of the four different SNPs in the TLR3 gene on HSV-2 disease severity in patients with recurrent disease and in asymptptomatically HSV-2-infected individuals. However, none of the four SNPs could be correlated with the severity of HSV-2 infection. At rs13126816, the frequency of the minor A allele was 22% for asymptomatic individuals compared with 23% among the symptomatically infected individuals (P=0.8764). At rs3775291, the allelic frequency of the minor allele (T) was 24% compared with 30% for asymptomatic and symptomatic HSV-2-infected individuals, respectively (P=0.1616) (Table 5).

TLR3 mRNA levels are increased in HSV-2 patients carrying the minor allele variants at both rs13126816 and rs3775291

To assess possible functional effects of the SNPs in TLR3, we recruited HSV-2-infected individuals who were homozygous for the minor alleles at both rs13126816 and rs3775291, as well as HSV-2-infected individuals who were homozygous for the major alleles at both rs sites. Only seven of the HSV-2-infected patients were homozygous for both minor alleles, and three of these seven patients volunteered to provide an additional blood sample. Peripheral blood mononuclear cells (PBMCs) from these individuals were then stimulated with HSV-2 or recombinant (r)IFN-α and assessed for TLR3 mRNA expression. We found that the levels of TLR3 mRNA were increased significantly in HSV-2-stimulated PBMCs from HSV-2-infected individuals carrying the minor alleles at both rs13126816 and rs3775291, compared with HSV-2-infected individuals carrying the major alleles at these sites (P=0.011) (Fig. 1a). The mean relative mRNA levels were 61.31 and 13.16 for the groups with the minor and major allele variants, respectively. After stimulation with rIFN-α, the relative levels of TLR3 mRNA from HSV-2-infected individuals

Table 3. Genotype frequencies for rs13126816 and rs3775291 in HSV-1-seronegative individuals

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>HSV-2 frequency [% (absolute no.)]</th>
<th>P-value (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HSV-2-negative</td>
<td>HSV-2-positive</td>
</tr>
<tr>
<td>rs13126816</td>
<td>GG</td>
<td>54 (28)</td>
<td>60 (55)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>31 (16)</td>
<td>40 (37)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>15 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>rs3775291</td>
<td>CC</td>
<td>42 (21)</td>
<td>52 (48)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>38 (19)</td>
<td>46 (42)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>20 (10)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

Table 4. Haplotype frequencies in HSV-2-infected and -seronegative individuals for four SNPs in the TLR3 gene

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency (%)</th>
<th>P-value (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>GTGC</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>ATGT</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>GACC</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>GTGT</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GAGC</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>ATGC</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>GTCC</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Estimated by Haploview 4.2 in the order rs13126816, rs13108688, rs3775292 and rs3775291.
carrying both minor alleles were somewhat higher than those from individuals with both major allele variants (Fig. 1b). Even though there was a 2.75-fold difference between the groups, the difference was not statistically significant ($P = 0.078$), probably reflecting the limited number of patients included.

Additionally, we measured the secretion of type I and type II IFN after HSV-2 and poly(I:C) stimulation of PBMCs from patients homozygous for the minor and major allele variants at rs13126816 and rs3775291. No notable differences were found in HSV-2-mediated IFN secretion, either in type I IFN ($\text{IFN-}\alpha$ and $\text{IFN-}\beta$) or in type II IFN ($\text{IFN-}\gamma$) secretion (Fig. 2). We were not able to detect any measurable levels of type I or type II IFN after poly(I:C) stimulation (data not shown).

**DISCUSSION**

We have shown previously that variations in host genetic factors may influence susceptibility to HSV-2 infection. In a prior paper, we reported that a relatively rare SNP in the T-bet-encoding gene, $\text{TBX21}$, was associated with increased incidence of genital HSV-2 infection (Svensson et al., 2008). In the current paper, we show that genetic variations in the $\text{TLR3}$ gene are also associated with HSV-2 infection. SNPs at rs13126816 and rs3775291 in the $\text{TLR3}$ gene were correlated with reduced incidence of genital HSV-2 infection, but did not affect disease severity in the infected hosts.

SNP variations in $\text{TLR3}$ appear to affect susceptibility to HSV-2, rather than being involved in the control of recurrent peripheral disease. The allelic substitution C→T at rs3775291 and the replacement G→A at rs13126816 were both less frequent among individuals with HSV-2 infection than in HSV-2-seronegative individuals. These correlations became even more obvious in the HSV-1-negative population. The ability of the minor allele variant at rs3775291 to confer natural resistance to virus infection is substantiated by other studies showing that the protective allele is less common both in patients infected by tick-borne encephalitis virus and in human immunodeficiency virus type 1 (HIV-1)-infected individuals (Kindberg et al., 2011; Sironi et al., 2012). There was, however, no difference in the allele distribution of the $\text{TLR3}$ SNPs between asymptomatic HSV-2 carriers and patients with recurrent genital HSV-2 disease, indicating that it is the innate rather than the acquired immune response that is affected by polymorphisms in $\text{TLR3}$. This is in line with the situation in children lacking a functional $\text{TLR3}$ gene, as HSE in these children is not associated with either herpes labialis or viraemia (Guo et al., 2011). $\text{TLR3}$ thus appears to be dispensable for the control of peripheral symptoms.

Willmann et al. (2010) recently published a case study of a man, homozygous for the $\text{TLR3}$ rs3775291 mutation, who suffered from frequent and recurrent HSV-2-caused Mollaret’s meningitis. It is not known whether the $\text{TLR3}$ SNP contributed to the development of Mollaret’s meningitis in this man, or
SNPs in TLR3 affect human HSV-2 infection

whether this or other TLR3 SNPs are overrepresented in HSV-2 meningitis patients. None of the patients included in our study suffered from any recognized CNS complications, precluding any such analysis in our study population. That TLR3 plays an important role in protecting the CNS from disseminated spread of HSV-1 and HSV-2 is, however, known from studies in mice lacking TLR3 (Andersen et al., 2004; Langenberg et al., 1999; Lekstrom-Himes et al., 1999; Singh et al., 2003). To assess possible functional effects of our two identified TLR3 SNPs, we measured TLR3 mRNA expression and IFN secretion in PBMCs from HSV-2-infected individuals after various stimuli. We found that HSV-2-infected individuals homozygous for the minor alleles at both rs13126816 and rs3775291 had higher relative levels of TLR3 mRNA expression in response to HSV-2 stimulation than individuals carrying both of the major alleles at these sites. These data should, however, be treated with caution, given the limited number of patients investigated in this part of the study. Taking this into account, the data still indicate that SNPs in the TLR3 gene may alter the expression of TLR3. Others have shown that the rs3775291 SNP reduces the expression of cell-surface, but not intracellular, TLR3 (Ranjith-Kumar et al., 2007). However, this study was carried out in a cell line. Furthermore, it would be of interest to assess TLR3 expression in epithelial cells of the vaginal mucosa in these patients, as epithelial cells of the human genital tract have been shown to express functional TLR3 (Andersen et al., 2006; Herbst-Kralovetz & Pyles, 2006).

Stimulation of TLR3 leads to the secretion of type I IFN (Kawai & Akira, 2006), yet we were not able to correlate the TLR3 SNPs to HSV-2- or poly(I:C)-induced cytokine secretion (i.e. IFN-α, -β or -γ). This is similar to previous observations where PBMCs from HSE patients with TLR3 deficiency responded with normal type I IFN production to HSV-1 and poly(I:C) stimulation, even though fibroblasts from these patients had a defective response to the same stimuli (Zhang et al., 2007). The most plausible explanation for the responsiveness to poly(I:C) in TLR3-deficient children is of course that other intracellular receptors present in PBMCs can compensate for the lack of TLR3. One such receptor is MDA-5, which also mediates the interferon response to poly(I:C) in mononuclear cells (Miyake et al., 2009).

In conclusion, we show that genetic variations in the TLR3 gene affect the incidence of genital HSV-2 infection.

Fig. 2. SNPs at rs13126816 and rs3775291 do not affect IFN production in PBMCs from HSV-2-infected patients. PBMCs from HSV-2-infected individuals with genotypes homozygous for the minor alleles (filled bars) or for the major alleles (empty bars) at rs13126816 and rs3775291 were stimulated with HSV-2 for 48 h and analysed for IFN-α (a), IFN-β (b) and IFN-γ (c) secretion. Data represent the mean ± SEM cytokine secretion from three HSV-2-infected individuals per group.
indicates that small individual genetic variations in immune-regulating genes may affect the function of antiviral receptors and, consequently, perhaps the susceptibility to genital HSV-2 infection.

METHODS

Human sample collection. The genetic material consisted of genomic DNA from 239 HSV-2-infected individuals and DNA from 162 HSV-2-seronegative healthy controls. The HSV-2-infected individuals consisted of 57 % males and 43 % females recruited from the sexually transmitted infection (STI) clinics at Sahlgrenska University Hospital, Borås Hospital and Uddevalla Hospital, Sweden. The mean age was 39 years for men (range, 21–70 years) and 37 years for women (range, 20–68 years). HSV-2 infection was confirmed serologically by using an HSV-2 ELISA kit (HerpesSelect2 ELISA IgG; Focus Technologies). Co-infection with HSV-1 was assessed by using an HSV-1 ELISA kit (HerpesSelect1 ELISA IgG; Focus Technologies). To assess the role of TLR3 SNP in disease severity, the patients were divided into two groups based on their clinical status, as follows.

Symptomatic HSV-2 infection. This group contained 149 patients [53 % males (mean age, 38 years; range, 23–70 years) and 47 % females (mean age, 38 years; range, 23–68 years)] with a typical history of recurrent genital herpes. Fifty percent (n=75) of the symptomatic patients were seropositive for HSV-1 and 44 % (n=65) were seronegative. HSV-1 data were missing for 6 % (n=9). Symptomatic HSV-2 infection was, in addition to the serology and clinical recurrences, also confirmed by PCR. The aim was to recruit individuals with more than six relapses per year to assess clinical disease. Of the 149 symptomatically infected individuals, 112 fulfilled this criterion, whereas 22 of the symptomatic individuals had fewer than six relapses per year. Relapse data were missing for 15 HSV-2-infected individuals. Fifty-six of the symptomatic individuals were on antiviral treatment at the time of sampling.

Asymptomatic HSV-2 infection. This group contained 90 patients [63 % males (mean age, 40 years; range, 21–66 years) and 37 % females (mean age, 34 years; range, 20–52 years)] who were seropositive for HSV-2 without any signs of clinical disease. Fifty-eight percent (n=52) of the asymptomatic individuals were seropositive for HSV-1 and 36 % (n=32) were seronegative. HSV-1 data were missing for 7 % (n=6). The asymptomatic patients were recruited from an ongoing screening study of HSV-2 infection in visitors to the STI clinics and among partners of HSV-2-infected patients. All had been given detailed information about the clinical spectrum of herpes and interviewed about genital symptoms. Presumably asymptomatic HSV-2-seropositive patients who, after being given information, admitted to having genital symptoms were excluded from the study.

Three individuals (all symptomatic) were on immunosuppressive treatment (against rheumatoid arthritis, Bechterew’s disease and CNS vasculitis) at the time of sampling. Other diseases that were present among the study population were hepatitis B (one symptomatic individual), diabetes (two symptomatic), allergic asthma (one symptomatic), multiple sclerosis (one asymptomatic) and lichen sclerosis (one asymptomatic individual).

Control individuals. For the control group, 162 healthy HSV-2-seronegative adult blood donors were recruited from the Blood Bank at Sahlgrenska University Hospital. This anonymous group consisted of 54 % males and 46 % females with mean ages of 41 and 40 years, respectively (range, 20–65 and 20–65 years, respectively). Eighty-eight of the healthy controls were positive for HSV-1 and 63 were HSV-1-negative. HSV-1 data were missing for 11 individuals. All individuals were routinely screened (and found to be negative) for blood-derived contaminating diseases, including hepatitis A and B, HIV-1 and -2 and human T-lymphotropic virus 1 and 2.

Permission for this study was granted by the Ethics Committee of the University of Gothenburg, and all patients gave informed consent.

Genotyping. DNA used for genotyping was extracted from heparinized venous blood using the salting-out method (Alden-Cannavà & Olerup, 1996). Genotyping was performed for 239 HSV-2-infected individuals and 162 HSV-2-seronegative control subjects for four TLR3 SNPs – rs13126816 (G→A), rs13108688 (T→A), rs3775292 (C→G) and rs3775291 (C→T) – using TaqMan allelic discrimination (pre-made or customized Applieda assays) at the Core facility at the Sahlgrenska Academy, Gothenburg University, Sweden.

Cell culturing. PBMCs were extracted from heparinized blood using a Ficoll gradient (Pharmacia). Cells (3×10^6 cells per well for mRNA extraction or 2×10^5 cells per well for cytokine determination) were stimulated with 7×10^5 p.f.u. HSV-2 ml⁻¹, 10 ng IFN-α ml⁻¹, 10 μg poly(1:C) ml⁻¹ or medium alone for 16 or 48 h at 37 °C in a humid atmosphere with 7.5 % CO₂. For mRNA extraction, cells were harvested after 16 h and lysed using an RNeasy kit (Qiagen) according to the manufacturer’s instructions. For cytokine determination, supernatants were collected after 48 h culture and stored at −20 °C until further use.

RNA isolation and cDNA synthesis. Total RNA was isolated from cultured PBMCs using an RNeasy kit (Qiagen) according to the manufacturer’s instructions. Total RNA was used for cDNA synthesis using 0.5 μg random hexamer ml⁻¹ and 0.01 mM dNTPs in a total volume of 12 μl. This was followed by heating to 65 °C for 5 min, a quick chill on ice and brief centrifugation (500 r.p.m. for 10 s). RNAse inhibitor (20–40 U ml⁻¹), 0.2 mM DTT, 1× First-Strand buffer and 200 U SuperScript reverse transcriptase ml⁻¹ (Invitrogen) were then added and incubated at 37 °C for 50 min, followed by termination of the reaction at 70 °C for 15 min.

Relative quantification of mRNA. TLR3 mRNA expression levels were determined using a TaqMan Gene Expression Assay (Applied Biosystems). Primers and probes for TLR3 and housekeeping gene GAPDH were purchased from Applied Biosystems (cat. no. Hs00152933_m1 and Hs99999905_m1, respectively). Each qPCR was carried out in a total volume of 20 μl containing 10 ng cDNA, 0.9 μM each primer and 0.25 μM probe under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min in 40 cycles. A cDNA standard was generated by measuring amplification in a mix of cDNA from 10 different blood donors. Data were analysed using the 7500 system software for the 7500 real-time PCR system (Applied Biosystems).

Cytokine determination. IFN-α and IFN-β levels were determined using Verikine human IFN-α and IFN-β ELISA kits from PBL InterferonSource according to the manufacturer’s instructions. IFN-γ levels were determined using a human IFN-γ DuoSet ELISA kit according to the manufacturer’s instructions (R&D Systems).

Statistical analysis. Genotype and allele frequencies were compared using the χ² test. Differences were considered significant at P<0.05. Calculations were made by using an online device (http://www.quantitativeskills.com/sisa/index.htm). A Hardy–Weinberg test and a haplotype reconstruction with association test were performed using Haplovie (Barrett et al., 2005). All allele and genotype frequencies were found to be in Hardy–Weinberg equilibrium. Student’s t-test was used for the statistical analysis of TLR3 mRNA expression and cytokine secretion.
ACKNOWLEDGEMENTS

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