Human cytomegalovirus induces a distinct innate immune response in the maternal–fetal interface

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Human cytomegalovirus (HCMV) is the leading cause of congenital infection, affecting 0.5–2% of newborns. Congenital HCMV disease develops in ~25% of infected children, and is associated with a wide spectrum of neurodevelopmental disabilities, intrauterine growth retardation (IUGR), and placental insufficiency (Cannon, 2009; Ross and Boppana, 2005). Our current understanding of HCMV transmission and pathogenesis is limited by the absence of animal models for congenital HCMV infection.

HCMV is transmitted from the mother to the fetus via the placenta (Pereira et al., 2005), which, in addition to serving in viral transmission, is actively injured by HCMV, contributing to the observed IUGR and placental (fetal) villi. Our studies in a clinically-relevant surrogate human model, provide a novel insight into the first-line decidual tissue response which could affect the outcome of congenital infection.

Introduction

The initial interplay between human cytomegalovirus (HCMV) and innate tissue response in the human maternal–fetal interface, though crucial for determining the outcome of congenital HCMV infection, has remained unknown. We studied the innate response to HCMV within the milieu of the human decidua, the maternal aspect of the maternal–fetal interface, maintained ex vivo as an integral tissue. HCMV infection triggered a rapid and robust decidual-tissue innate immune response predominated by interferon (IFN)γ and IP-10 induction, dysregulating the decidual cytokine/chemokine environment in a distinctive fashion. The decidual-tissue response was already elicited during viral-tissue contact, and was not affected by neutralizing HCMV antibodies. Of note, IFNγ induction, reflecting immune-cell activation, was distinctive to the maternal decidua, and was not observed in concomitantly-infected placental (fetal) villi. Our studies in a clinically-relevant surrogate human model, provide a novel insight into the first-line decidual tissue response which could affect the outcome of congenital infection.

Both immune and non-immune cells are known to be rapidly activated upon HCMV infection, with induction of innate immune response signaling cascades and inflammatory cytokines upon viral sensing (Mocarski et al., 2007; Rossini et al., 2012). Thus far, HCMV-induced innate responses have been studied mainly in single-cell-type cultures, such as fibroblasts and myeloid-lineage cells (Chaudhuri et al., 2009; Compton et al., 2003; Juckem et al., 2008; Mezger et al., 2009; Varani et al., 2007; Yurochko and Huang, 1999). In the context of congenital HCMV infection, adaptive immunity has clearly been more extensively evaluated than innate immune responses (Schleiss, 2013). While the potential contribution of innate responses to antiviral defense as well as to adverse tissue damage is increasingly recognized for diverse viral infections (Iwasaki and Medzhitov, 2013; Kobasa et al., 2007; Monroe et al., 2014; Rossini et al., 2012), their role in congenital HCMV transmission and outcome has remained elusive, in the absence of clinically-relevant model systems.

We have recently established a novel ex vivo model of HCMV infection in human decidual tissues maintained as multi-cell-type
organ cultures, and demonstrated the spread and the broad target-cell range of the virus in the decidua (Weisblum et al., 2011). These findings reflected the ability of the ex vivo infected decidual culture to address the complexity of HCMV interactions with its diverse cellular targets within the natural tissue milieu. In the present work, we have employed the decidual culture model to study the decidual-tissue innate response to HCMV infection. Using both laboratory-adapted and clinically-pathogenic strains from congenital HCMV cases, we have identified a distinctive decidual tissue innate immune response pattern to HCMV infection.

Results

**HCMV induces a robust decidual-tissue innate immune response**

The decidual tissue is composed of multiple cell types in addition to the extracellular milieu. Its innate response to HCMV, as an integral tissue, is therefore likely to recapitulate the in vivo responses more accurately than individual cell types grown in culture. To evaluate the effect of HCMV infection on decidual cytokines/chemokines profile, we first analyzed the levels of representative immune and angiogenic cytokines/chemokines in cleared supernatants of HCMV-infected and mock-infected decidual organ cultures at 7 days post infection (dpi), using an ELISA immune assay (Table 1). To account for the tissue-to-tissue variability, mean values were calculated for 5 independent tissue cultures from different lots. While the majority of the analyzed cytokines were not altered by more than ~2-fold following infection, HCMV substantially induced decidual secretion of interferon γ (IFNγ) (~35 fold; p = 0.03), the T-cell attracting chemokine Interferon Inducible Protein-10 (IP-10; 4.7 fold; p = 0.008) and IFNβ (5.5 fold, not statistically significant). Similar induction of IFNγ and IP-10 were observed at 24 h post infection (hpi; data not shown).

In further comparative analyses, we quantified the effect of HCMV infection on decidual-tissue cytokines/chemokines mRNA levels, using quantitative real-time RT-PCR. In accordance with the secretion pattern, HCMV infection significantly induced decidual transcription of IFNγ and IP-10 as well as the interferon stimulated gene MxA (Fig. 1A) in all the decidual tissues examined (> 100), despite the expected tissue-to-tissue variations. Induction was similarly observed in tissues obtained from HCMV seropositive (64% of the total) and HCMV seronegative (36%) donors. No significant alterations of IL-10 and VEGF mRNA levels were observed (Fig. 1A).

To evaluate whether HCMV infection affects the tissue distribution of IP-10, histological sections of infected and mock-infected decidua were examined at 8 dpi (Fig. 1B). Immunohistochemical analysis revealed that in addition to the upregulation of IP-10 expression, HCMV infection drastically altered the distribution of IP-10 from distinct focal expression (in mock-infected tissues) to widely-distributed expression (in infected decidual tissues), detected in both infected and uninfected cells throughout the decidua.

**Decidual tissue innate response kinetics**

We quantified the HCMV immediate early (IE)-1 gene and the tissue cytokine mRNA levels over time. MxA, IFNγ, and IP-10 expression was already induced at 6 hpi, with continued increase reflecting the viral spread (Fig. 2).

Together, these findings reveal the rapid immune activation with creation of a proinflammatory environment in the decidual-tissue upon HCMV infection.

**Induction of decidual innate response by viral particles**

When decidual tissues were infected with 10-fold escalating doses of purified viral particles, a dose-proportional increase in IFNγ and IP-10 induction was demonstrated (Fig. 3A). The rapid and dose-dependent innate response of the decidual-tissue suggested that it was triggered by the viral particles immediately upon infection. We have therefore compared the induction of IFNγ and IP-10 expression following exposure to infectious versus UV-inactivated virions. Whereas no viral gene expression was detected following decidual exposure to UV-inactivated virions (Fig. 3B), a similar induction of IFNγ and IP-10 expression was observed, compared to infectious virions (Fig. 3B). This finding indicated that the induced decidual response does not require viral gene expression, but is rather triggered by virion structural component/s.

Furthermore, a similar innate response pattern was observed after infection with HCMV laboratory-derived strain AD169, the clinically-derived strain TB40/E, and the low-passage clinical isolates CI851, CI893, and CI943 (Fig. 3C), suggesting the potential triggering by conserved viral structural protein/s.

HCMV glycoprotein B (gB) is a major viral-envelope glycoprotein, implicated in the induction of cellular innate responses (Compton et al., 2003; Rossini et al., 2012). Incubation of decidual cultures with purified soluble gB resulted in a significant induction of IFNγ and IP-10 expression (Fig. 3D). This significant induction was similarly observed for each of 2 independent lots of mammalian cell-expressed gB (results are shown for one lot) and an unrelated preparation of baculovirus-insect-cell expressed and purified soluble gB, whereas no induction was observed following parallel incubation of decidual cultures with control glycosylated bovine serum albumin (BSA; Fig. 3D). These results identify the contribution of HCMV gB to activation of the decidual-tissue innate response.

**Decidual-tissue innate response is already induced upon virus-cell contact**

Neutralizing HCMV hyperimmune globulins (HIG) do not inhibit the decidual-tissue innate response

Clinical studies have suggested that treatment with HCMV HIG can reduce maternal–fetal transmission and congenital disease, although more recent controlled studies have not confirmed the effect on transmission (Nigro et al., 2005; Revello et al., 2014). We have shown that HCMV HIG efficiently neutralize HCMV infection of decidual organ culture (Weisblum et al., 2011). Here we sought to examine whether blocking the first steps of the virus replication cycle by neutralizing antibodies could also inhibit the viral-induced decidual innate response. Interestingly, despite the complete inhibition of infection (as revealed by the abrogated viral IE-1 expression), preincubation of purified viral particles with HCMV HIG had no effect on the decidual innate response to HCMV (Fig. 4A). As HCMV neutralizing antibodies have been shown to block the viral fusion and internalization step, but do not interfere with the initial virus binding at the cell surface (Compton, 2004; Potzsch et al., 2011)—our finding indicates that the decidual-tissue innate response is already activated upon the earliest stages of infection involving viral glycoprotein binding to cell surface receptor/s.

Heparin partially abolishes the decidual-tissue innate response. Initial viral attachment involves binding of viral envelope glycoproteins to cell surface heparan sulfate proteoglycans (HSPG), a process known to be inhibited by heparin. To investigate the contribution of virion—HSPG binding to the decidual-tissue innate response, purified HCMV was incubated with heparin prior to decidual infection. Heparin completely inhibited HCMV decidual infection, which resulted in a significant (p < 0.05) albeit partial abolishment of IP-10 and IFNγ induction (Fig 4B). No non-specific cytokine effects of heparin on the examined parameters were observed (Fig. 4B). This differential effect, identifies the relative contribution of viral recognition by cell surface
Fig. 1. Effect of HCMV infection on decidual cytokines/chemokines expression. (A) HCMV ($5 \times 10^4$ PFU/well, strain TB40/E) and mock-infected decidual tissue RNA was extracted at 7 dpi and the indicated mRNA levels were analyzed by quantitative RT-PCR and normalized by the housekeeping gene $\beta$-actin. Results are shown for 3 independent tissues. UD: undetectable levels. (B) Decidual cultures were infected with $\beta$-galactosidase-encoding HCMV strain RC256 ($5 \times 10^4$ PFU/well). Mock and infected cultures were subjected to immunohistochemistry analysis for IP-10 antigen (red staining) at 8 dpi. HCMV infected cells were detected by X-gal staining (blue). The insert shows a cell co-stained for IP-10 and X-gal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
HSPG, yet suggests the involvement of additional viral cell-receptor interactions in activation of the decidual-tissue innate response.

HCMV-induced innate response pattern is distinctive to the decidual tissue

The maternal-decidua constitutes a unique site, containing both immune and non-immune cells. To determine whether the observed innate response pattern is specific to the decidual tissue, we compared the response between the decidual tissues and the adjacent placental chorionic villi (representing the fetal aspect of the maternal–fetal interface). Interestingly, induction of IFNγ was unique to the decidual-tissue, and was not observed in concomitantly-infected villi from the same donors. In contrast, MxA and IP-10 expression was induced in both tissues (Fig. 5). Since IFNγ is known to be produced exclusively by cells of the immune system (Rossini et al., 2012), the specific predominance of IFNγ response in the decidual tissue clearly indicates active and distinctive decidual immune-cell response to HCMV infection.

Discussion

Whereas the role of adaptive immunity in the context of congenital HCMV infection has been extensively evaluated, the initial interplay between the invading virus and the innate tissue response in the human maternal–fetal interface, though crucial for determining the outcome of congenital HCMV infection, has remained unknown. Our studies of HCMV infection in structurally-integral human decidual tissues are the first to reveal the earliest innate response to the virus within the whole-tissue decidual milieu.

We demonstrated that HCMV infection induced a robust tissue innate immune response, significantly altering the decidual cytokine and chemokine environment. A consistent innate response pattern was
reextracted at 12 hpi, and the indicated mRNA levels were analyzed by quantitative RT-PCR and normalized by the house keeping gene β-actin. The data shown are representative of at least three independent experiments, each tested in 5 replicates. UD; undetectable levels.

Fig. 4. Effect of early viral inhibitors on the decidual tissue response to HCMV. HCMV strain TB40/E (purified virus; 5 × 10^4 PFU/ml = 5 × 10^5 PFU/well) was incubated with HCMV hyperimmune globulins (HIG; Megalotect 10 mg/ml) (A), or heparin (HEP; 500 IU/ml) (B), for 1 h prior to infection. Decidual tissue RNA was extracted at 12 hpi, and the indicated mRNA levels were analyzed by quantitative RT-PCR and normalized by the house keeping gene β-actin. The data shown are representative of at least three independent experiments, each tested in 5 replicates. UD; undetectable levels.

Fig. 5. Comparison of decidual and placental response to HCMV infection. Decidual and placental chorionic villi cultures from the same donors were concomitantly infected with HCMV strain TB40/E (5 × 10^4 PFU/well). Decidual and placental tissue RNA was extracted at 7 dpi, and the indicated mRNA levels were analyzed by quantitative RT-PCR and normalized by the house keeping gene β-actin. The data shown are representative of at least three independent experiments, each tested in 5 replicates. UD; undetectable levels. DEC; decidua, PLA; placenta.

noted in all the decidual tissues examined (over 100), regardless of the HCMV serological status of the donors. This observation (in tissues from seronegative donors) indicates a primary innate immune response to infection that is independent of adaptive immunity. While we found predominant induction of IFNγ and IP-10 expression, reflecting immune activation within the decidua upon HCMV infection, no significant induction of the anti-inflammatory cytokines IL-10 and IL-4 was observed (Table 1, Fig. 1). Of note, a genome-wide transcriptome analysis of infected and mock-infected decidual tissues confirmed this pattern, with the highest induction found in IFNγ, CXCL11, and CXCL10 (IP-10) expression (data not shown). This overall shift towards a proinflammatory environment is in agreement with the proinflammatory bias exerted by HCMV in clinical settings of systemic infection, and with the increased expression of Th1 cytokines, as reported in infected cells and term-placental cultures and in amniotic fluid specimens from a few cases of congenital HCMV (Chaudhuri et al., 2009; Hamilton et al., 2012; Mocarski et al., 2007; Rossi et al., 2012; Scott et al., 2012). However, the decidual innate response followed a distinctive pattern: unlike the findings in cultured cells, we did not observe a predominant induction of type I interferons and an increase of proinflammatory cytokines TNFα, IL-6, and IL-1β. Furthermore, the decidual-tissue response pattern differed from the reported response of isolated dNK, characterized by IL-6 induction, with no upregulation of IFNγ, and rather downregulation of IP-10 (Siewiera et al., 2013). These discordances could result from, first, the difference between studies in cultured single-type cells versus our study in a multi-cell-type solid tissue, containing a variety of maternal and fetal cells (which more closely mirrors infection in vivo; (Weisblum et al., 2014) and, second, the particular composition of the decidua, cohabited by both immune and non-immune cell types, and geared for protection and immune-tolerance of the developing fetus.

Interestingly, and further attesting to the specific nature of the decidual-tissue response, we found that the HCMV-induced expression of IFNγ was exclusive to the maternal-decidua, and was not observed in concomitantly-infected placental villi, whereas MxA and IP-10 were similarly-induced in both tissues (Fig. 5). The predominance of IFNγ—known to be produced exclusively by cells of the immune system—clearly indicates active ex vivo decidual immune-cell response to infection. In this regard, decidual immune cells are predominated by a unique population of dNK, accounting for ~70% of the decidual lymphocytes (which are absent in placental villi), and we and others have shown that HCMV infection modulates dNK and NK responses (Aron et al., 2005; Le Bouteiller, 2013; Markel et al., 2002; Stern-Ginossar et al., 2007). Indeed, we could detect a multitude of CD56bright dNK cells co-mingling with HCMV infected cells in the decidual organ culture (data not shown). Yet we cannot exclude the additional contribution of adaptive ex vivo lymphocyte response in tissues obtained from seropositive women. Further studies directly focusing on the responding cell populations within the infected decidual tissue are currently underway in our laboratory.

The decidual tissue innate immune response was already elicited upon initial interaction with virion particles, as revealed by the rapid kinetics (Fig. 2), the viral particle-dose-dependent manner, and the similar induction of IFNγ, IP10 and MxA by infectious and UV-inactivated virions (Fig. 3). The triggering role of a conserved surface glycoprotein was further suggested by the finding that low-passage clinical strains derived from congenital HCMV cases and laboratory-adapted strains elicited similar decidual response (Fig. 3). In accordance with previous studies in cell cultures (Compton et al., 2003; Rossi et al., 2012), we directly demonstrated the induction of decidual-tissue innate response by soluble viral gB (Fig. 3D). Of note, the tissue response was only partially abolished by heparin (Fig. 4) - known to inhibit binding of viral envelope glycoproteins to cell surface HSPG (Compton et al., 1993). This suggested the potential contribution of additional viral interactions with pattern recognition receptors, as indeed shown in infected cell cultures for Toll-like receptor 2, CD14, and surface integrins (Compton et al., 2003; Rossi et al., 2012). We further showed that neutralizing HCMV HIG demonstrated a differential effect, completely inhibiting viral infection, yet leaving the decidual-tissue response unaffected (Fig. 4). As neutralizing antibodies have been shown to block viral-cell membrane fusion without affecting
the preceding events of viral glycoproteins’ binding to cell surface receptors (Compton, 2004; Potzsch et al., 2011), their disparate effect corroborates the immediate activation of decidual innate immune response by an initial viral glycoprotein binding step. In view of the known key-roles of IFNγ and IP-10 in both innate and adaptive immune responses, and their increasingly recognized non-immune functions during healthy placentation (Hanna et al., 2006; Iwasaki and Medzhitov, 2013; Murphy et al., 2009; Sadler and Williams, 2008), it is conceivable that their local induction by HCMV as identified herein, could have consequences on virus transmission and placental development. Furthermore, an over-exuberant response has been shown to be associated with poorer disease outcomes following diverse viral infections (Gross et al., 2011; Iwasaki and Medzhitov, 2013; Kobasa et al., 2007; Monroe et al., 2001, 1995). The profound dysregulation of the decidual chemokine homeostasis following HCMV infection, as demonstrated by the significant induction of IP-10 and CXCL11 along with the modified tissue distribution of IP-10 (Fig. 1), is expected to lead to increased lymphocyte access to the maternal–fetal interface, hence impeding the specialized immune-privileged environment in the decidua. In this regard, our data could provide a mechanistic basis for the characteristic pathological features of naturally-infected placenta, namely, increased lymphocyte infiltration (forming the characteristic villitis and deciduitis), necrosis, edema, and fibrosis (Gabrielli et al., 2012; Pereira et al., 2014). The predominance of IFNγ and adaptive immune responses, and their increasingly recognized roles in the complex tissue response in the pathogenesis of congenital infection. Application of the decidual organ culture system for the study of innate immune responses to HCMV has obvious limitations in terms of analyzing the distinct molecular mechanisms underlining the observed findings at the cellular level. Yet, the complex structure of the decidual-tissue, which is composed of a multitude of cell types and extracellular matrix components, better reflects the in vivo clinical setting of HCMV infection, spread, and pathogenesis within the maternal–fetal interface. Our studies in a clinically-relevant surrogate human model, provide a novel insight into the first-line decidual tissue response which could affect the outcome of congenital infection.

Materials and methods

Cells and viruses

Primary HFF were used for HCMV propagation (Wolf et al., 2001, 1995). The HCMV strains used were AD169 (obtained from the American Type Culture Collection), TB40/E expressing IE2-fused EYFP (generously provided by M. Winkler, Germany) (Dal Pozzo et al., 2008), and Towne-RC-256, containing the LacZ gene (Spaete and Mocarski, 1987). In addition, we used the low-passage clinical isolates C1B51, C1B93, and C1B43, recovered at the Hadassah Clinical Virology Laboratory from urines of congenitally-infected newborns, and propagated in HFF for 3–5 passages. Viral UV inactivation was performed with UV Stratalinker 2400 (Stratagene) at 0.99 J. For viral purification, ultracentrifugation (at 24,000 rpm) of cleared virus supernatants through a 10% sucrose cushion was performed.

Preparation and infection of decidual and placental chorionic villi organ cultures

Decidual organ cultures were prepared and infected as previously described (Weisblum et al., 2011). Similar procedures were followed for preparation and infection of cultures derived from adjacent placental chorionic villi. The study was approved by the Hadassah Medical Center Institutional Review Board (0138-08-HMO), and performed according to the Declaration of Helsinki, Good Clinical Practice guidelines, and the Human-Experimentation Guidelines of the Israeli Ministry of Health. All participants signed written informed consent. In brief, first-trimester decidual tissues were cut by a microtome into thin slices and incubated in decidual medium: DMEM with 25% Ham’s F12, 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Biological industries), in 37 °C, 5% CO2. Tissue viability was monitored as described previously (Weisblum et al., 2011). For infection of decidual organ cultures, the tissues were placed in 48-well plates and inoculated with the indicated virus (5 × 104 plaque forming units (PFU)/well unless otherwise stated) for 12 h to allow effective viral adsorption. Following viral adsorption, the cultures were washed extensively and further incubated for the duration of the experiment with replacement of the culture medium every 2–3 days.

Specific treatments

HCMV glycoprotein B (gB): decidual organ cultures were incubated with 2 μg/ml soluble recombinant HCMV gB (Sino biological Inc. and an additional unrelated baculovirus-insect cell expressed and purified soluble gB, kindly provided by Yarden Opatowsky, Bar-Ilan University), or control glycosylated bovine serum albumin (BSA; Promega).

HCMV hyperimmune globulins (HIG) and heparin: HCMV (5 × 106 PFU/ml of purified virus) was pre-incubated with 10 mg/ml HCMV HIG (Megalotype; Biotest) or 500 IU/ml heparin (Sigma-Aldrich) for 1 h, prior to inoculation.

At 12 hpi, cultures were washed extensively and stored at −70 °C until RNA purification.

Multiplex-ELISA immunoassay

 Supernatants of mock- and HCMV-infected decidual cultures (and placental chorionic villi cultures, when indicated) were collected at 1 d and 7 d post infection (dpi) and stored at −70 °C. Samples thawed on ice were subjected to analysis by a

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Infected/Mock ± SEM</th>
<th>p Valueabc</th>
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<tr>
<td>IFNγ</td>
<td>35.2 ± 18.3</td>
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<tr>
<td>IFNβ</td>
<td>5.5 ± 3.8</td>
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<tr>
<td>IP-10</td>
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<td>IL-2</td>
<td>0.6 ± 0.1</td>
<td>0.002</td>
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a Ratios between the levels of cytokines secreted from HCMV-infected and mock-infected decidual cultures at 7 dpi were tested in 4 replicates for 5 independent tissues obtained from different individuals. Results are shown as mean ± standard error (SEM).

b All data (mean ± SEM) were analyzed using unpaired, two-tailed t tests for comparisons between two groups.

Significant differences are highlighted in bold and marked with an asterisk (*).
microplate-based immunoassay, measuring 16 analytes (Table 1) in a single well (Q-plex™ array, Quansys Biosciences).

Histological and immunohistochemistry analysis

Mock- and HCMV-(strain RC-256) infected decidual tissues were stained for β-galactosidase [Braun et al., 2006], formaldehyde-fixed, paraffin-embedded, and sectioned (5 μm-thickness). Sections were placed in 0.01 M citrate buffer and warmed in a pressure-cooker to 110 °C for 5 min, cooled to room temperature, incubated with IP-10 mouse monoclonal antibodies (1:20 dilution; R&D systems), washed, and incubated with HRP-conjugated goat anti-mouse antibodies (Biocare Medical).

RNA purification and quantification

Infected decidual tissues and HFF were washed and stored at –70 °C until assayed. RNA was extracted (NucleoSpin RNA isolation kit; Macherey-Nagel), and subjected to reverse-transcription (GoScript™; Promega), followed by quantitative real-time PCR (7900HT; Applied Biosystems). Primers and probes are listed in Table S1.

Statistical analysis

All data (mean ± SEM) were analyzed using unpaired, two-tailed t tests for comparisons between two groups: p values of <0.05 were considered significant.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.06.023.

References


