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## Balance between herpes viruses and immunosuppression after lung transplantation

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*Document Version*

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*Publication date:*

2006

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*Citation for published version (APA):*

Verschuuren, E. A. M. (2006). *Balance between herpes viruses and immunosuppression after lung transplantation*. s.n.

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## Chapter 5

### *Expression Dynamics of Human Cytomegalovirus Immune Evasion Genes US3, US6 and US11 in the Blood of Lung Transplant Recipients.*

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*Journal of infectious diseases 2001;184(3):247-255*

## Abstract

Delayed elimination of human Cytomegalovirus (HCMV)-infected cells by the host immune system may contribute to viral dissemination and pathogenesis of HCMV infection. The mRNA expression dynamics of HCMV-encoded immune evasion genes US3, US6 and US11 expressed after active HCMV infection were analyzed in blood samples of lung transplant recipients by means of quantitative nucleic acid sequence-based amplification. The results were compared with the expression dynamics of IE1 mRNA and pp67 late mRNA, levels of pp65-antigenemia, and antiviral treatment. During acute infection, high levels of US3 and US6 RNA were detected before antigenemia, which were detected simultaneous with IE1 RNA. US11 RNA was detected simultaneous with antigenemia but before late pp67 RNA. These data suggest an active role of viral immune evasion during HCMV infection in vivo. Interestingly, immune evasion RNA remained detectable after clinical recovery, often independently of IE1 RNA expression, indicating persistent viral activity, which may have implications for long-term control of HCMV.

## Introduction

Human Cytomegalovirus (HCMV) is a widespread herpesvirus with a prevalence of  $\geq 60\%$ , depending on the socio-economic structure of the population (1). Herpesviruses are rarely pathogenic and mainly cause disease in immunocompromised persons. HCMV establishes a lifelong latent infection, which is characterized by periodic reactivation from latency (2). The predominant site of HCMV latency may be the myeloid lineage, including CD34+ myeloid precursor cells, monocytes, and macrophages (3, 4). These cells play a key role in antigen processing and presentation, and are essential for inducing immune reactivity. During latency, HCMV expresses several genes in these cells (5) yet fails to be eliminated by the immune system.

Recent studies have revealed that the HCMV genome encodes a number of genes, expressed at different stages of the HCMV replication cycle, which can actively interfere with distinct steps in the antigen presentation pathway and thus contribute to viral persistence in vivo in the face of a primed host immunity (6). For several years, it has been known that major histocompatibility complex (MHC) class I molecules are rapidly degraded in HCMV- infected cells (7, 8). Four genes are identified that are responsible for this interference: US2 (9), US3 (10, 11), US6 (12, 13) and US11 (8, 14).

US2 and US11 bind to MHC class I molecules in the early phase of infection and redirect the heavy chains into the cytosol, where the complexes are degraded by the proteasome (8, 9). Further studies revealed that the US3 encoded protein down-regulates MHC class I antigen presentation by forming stable complexes with mature peptide-loaded MHC class I molecules and  $\beta 2$ -microglobulin, thereby retaining the MHC class I molecules in the endoplasmic reticulum (10, 11). The US3 gene is the first expressed immune evasion gene during the immediate-early phase of infection and also may be expressed selectively in cells non-lytically infected with HCMV (15, 16, 17). The protein encoded by US6 is a type I membrane protein expressed during the delayed-early phase of infection, interacting with transporter-associated protein inside the endoplasmic reticulum lumen. US6 prevents translocation of proteasome-digested peptides into the endoplasmic reticulum (12, 13).

The combined action of these US-encoded genes results in significant down-regulation of MHC class I presentation on the cell (7). However, loss of MHC class I molecules on the cell surface may trigger NK cells to destroy the cell (18). To compensate for this, HCMV encodes a late protein, UL18, which is expressed on

the cell surface and has structural and functional homology with MHC class I (19,20).

The complex and diverse ways used by HCMV to elude the host immune system suggest that expression of immune evasion genes might be related to the pathogenesis of HCMV infection and progression to HCMV disease.

Our main hypothesis was that the profile of the expression of immune evasion RNA might be informative to identify patients at risk for developing HCMV disease. To address this hypothesis, we used quantitative nucleic acid sequence-based amplification (Qt NASBA) assays (21) to measure the level of expression of the US3, US6 and US11 genes in the blood of lung transplant patients during active primary or secondary HCMV infection and after recovery. The results were compared with those of the routinely performed pp65 antigenemia assay and IgM and IgG serology. In addition, Qt NASBA results for the immune evasion genes were compared with expression levels of IE1 (UL123) and late pp67 (UL65) RNA, which were determined in parallel (22).

## Materials and Methods

### *Patients and samples*

During 1997-1998, whole blood samples were prospectively collected from 17 patients undergoing lung transplantation. The samples were taken weekly during admission and at every out patient visit. The duration of sampling varied between 33 and 72 weeks after transplantation. Four patients had an incomplete follow-up because of early death not due to HCMV infection. Thirteen patients with a complete follow-up were included in this study. Of these, 2 patients had a seronegative organ match and were used as control subjects (53 samples). Three seronegative patients received a seropositive donor organ and developed a primary HCMV infection, and 8 patients were seropositive before transplantation (275 samples). Fresh whole blood samples from 50 healthy blood donors were obtained from the blood bank (Eindhoven, The Netherlands). For NASBA, 1 ml of EDTA-treated whole blood was mixed thoroughly with 9 ml of NASBA lysis buffer within 4 h after collection and was stored at -80°C until use. For routine pp65 antigenemia testing, 1.8 ml of EDTA-treated blood was obtained and processed, as described below.

### *Treatment*

All patients received immunosuppressive treatment with rabbit antithymocyte globulin (3 mg/kg, 2-5 times postoperatively; Thymoglobulin®,

Merieux, France), azathioprine (1.5-3 mg/kg/day), cyclosporine A (dose-adjusted to whole blood trough levels of 400 µg/l within 3 weeks tapered to levels of 150 µg/l), and prednisolone (3 x125 mg on day 1, 0.2 mg/kg/day from day 2 to the month 3, and 0.1 mg/kg/day thereafter), pneumocystis carinii prophylaxis with cotrimoxazole (960 mg on alternate days day). For prophylaxis against Pneumocystitis carinii, cotrimoxazole (960 mg on alternate days) was given, and, for prophylaxis against herpesvirus, acyclovir (200 mg 4 times daily for 6 months) was used.

Acute rejection was treated with pulse therapy with methylprednisolone (500-1000 mg iv daily for 3 days). Recurrent rejection was treated by replacement of cyclosporine A by FK506 (Prograf; Fujisawa) and, subsequently, was changed from azathioprine to methylmycophenolate mofetil (cellcept, Roche). Patients who were seronegative before transplantation and who developed primary infection received antiviral therapy on confirmed positive results of the antigenemia assay. Patients who were seropositive before transplantation received antiviral therapy when clinical symptoms became apparent, which was confirmed by a pp65 antigenemia result of >10 positive cells per 50.000 leukocytes.

Cytomegalovirus-related disease was treated with ganciclovir iv. (Cymevene, Roche) or Foscarnet (Foscavir) until pp65-antigenemia levels dropped below the limit of detection (23). One patient received hyperimmune globulin (Megalotect, Biotest, Dreieich, Germany) in addition to ganciclovir and foscarnet.

### *HCMV antigenemia and routine serology*

HCMV antigenemia was determined as described previously (22, 23). In brief, peripheral blood leukocytes were isolated by dextran sedimentation and were cytocentrifuged onto glass slides. These slides were air-dried, were fixed and permeabilized with Nonidet P-40, and were stained for pp65 (UL83) with a mixture of monoclonal antibodies (C10 and C11), by means of an indirect immunoperoxidase technique. The total number of HCMV antigen- positive cells was scored and expressed per 50,000 leukocytes screened. IgG and IgM antibodies to HCMV were determined by means of a semiquantitative ELISA with alkaline glycine-extracted HCMV antigens obtained from HCMV AD169-infected fetal fibroblasts and, in parallel, on an extract of mock-infected fibroblasts, as described elsewhere (25).

## Nucleic acid isolation

Total nucleic acid was isolated from a 0.1 ml blood equivalent, essentially as described by Boom et al. (26). Before isolation, a fixed amount of internal calibrator RNA (Q RNA) was spiked into the sample (US3  $3 \times 10^4$  copies, US6  $5 \times 10^4$  copies, and US11  $3 \times 10^4$  copies of calibrator RNA), which allows optimal quantification of the amplified target RNA (21). The Q-RNA had a sequence identical to that of the wild-type target RNA, except for a stretch of 23 randomized bases in the region of the detection probe. Nucleic acids were bound to activated silica and were washed 2 times with wash buffer (5.3 M guanidinium thiocyanate and 50 mM Tris [pH 6.4]), 2 times with 70% ethanol, and 1 time with acetone, after which the silica was dried. Nucleic acids were eluted with 50  $\mu$ l elution buffer (1mM Tris [pH 8.5]) at 56°C for 10 min, and 5  $\mu$ l of eluate was used as input for the Qt NASBA reactions.

## Qt NASBA

NASBA amplification was done with 2 primers, each of which were designed to amplify part of the mRNA encoded by US3, US6 and US11 (Table 1). For quantification, wt RNA was co-amplified simultaneously with Q-RNA. NASBA reactions were carried out in a 20- $\mu$ l reaction mixture in detail for quantification of HCMV IE1 and pp67 RNA, as described elsewhere (22).

**Table 1:** Sequences of primers and probes used in this study to analyse immune evasion genes encoded by human Cytomegalovirus.

	Primer 1*	Primer 2	Capture probe	ECL probe
US3	GCTGAAGGTACCA GTTGA	TCTTACATGGACA GACTGCA	GGTGACGCACACC GTGGAT	TCGAGTGGAAACAT CGGTGGGCA
US3 Q	GCTGAAGGTACCA GTTGA	TCTTACATGGACA GACTGCA	GGTGACGCACACC GTGGAT	GTTGGCTGCCGGAG AGACTCAAG
US6	AGTAGCCGACGGA CTCGTTGACA	TCGCACAGACCCG TTTGTTA	GGGTGAGGACTTT GCGCACCAA	CAGATGATAGCTG GAAACA
US6 Q	AGTAGCCGACGGA CTCGTTGACA	TCGCACAGACCCG TTTGTTA	GGGTGAGGACTTT GCGCACCAA	AGGGAACGAGTAC AACTTAG
US11	AAAATGTCGGTGC AGCCA	CCTGCCACCAATG CCAA	CTGTGGAGCTCGT TAGAGGA	GGTGCCCTCGTGCC TTGGGTT
US11 Q	AAAATGTCGGTGC AGCCA	CCTGCCACCAATG CCAA	CTGTGGAGCTCGT TAGAGGA	TTTGTTCGGTGC GGGCCGC

\* Primer 1 has a generic extension at the 5' end: AATTCTAATACGACTCACTATAGGG

For optimal detection, the amplification products of US3 and US11 Qt NASBA were diluted 6 times in detection reagent (1 mM Tris [pH 8.5] and 0.3 mM of 2-methylisothiazolone HCL/L), the amplification products of US6 Qt NASBA were diluted 11 times, and 5  $\mu$ l of diluted amplification product was incubated with a specific biotine-labeled capture probe and a ruthenium-labeled detection probe

for 30 min. at 41 °C. Assay buffer (100 mM tripropylamine, [pH 7.5]) was added, and the tubes were placed in an electrochemiluminescence (ECL) instrument (NASBA QR System, Organon Teknika). The number of wild type RNA copies per 100 µl of blood was calculated by the ratio between the wild-type signal and the calibrator signal, as described elsewhere (21, 26).

## Results

### *Performance of the Qt NASBA for US3, US6 and US11*

As determined in a separate study, the analytical sensitivity of Qt NASBA amplification of US6 and US 11 was 100 RNA molecules, with use of RNA isolated in a whole blood background by means of silica extraction. For Qt NASBA for US3, the sensitivity was 1000 RNA molecules. The dynamic range of the Qt NASBA amplification of US6 and US11 was linear from  $3 \times 10^3$  to  $1 \times 10^6$  copies. The quantification of the Qt NASBA for US3 was reproducible over a range of  $1 \times 10^4$  to  $1 \times 10^6$  copies of RNA (21).

RNA from a variety of different human cell lines (fibroblasts, smooth muscle cells, aortic endothelial cells, and umbilical vein endothelial cells) and cells infected with other human herpesviruses (Epstein-Barr virus, herpes simplex virus type I and II, and human herpesvirus 8) was analyzed by QT NASBA for immune evasion genes, which gave negative results. The specificity of Qt NASBA assays for US3, US6 and US11 was further analyzed in blood samples of 50 random healthy persons, 28 of whom were HCMV seropositive. Qt NASBA assays for US3 and US6 each revealed a single weak positive result in blood samples from HCMV-seronegative donors. Therefore, the positive results were most likely caused by contamination. The specificity of the Qt NASBA assays was 98% for US3, 98% for US6, and 100% for US11.

Blood samples obtained from 2 patients who had undergone lung transplantation without HCMV infection (negative donor and negative recipient) were tested for 36 and 60 weeks after transplantation (25 and 28 samples, respectively). All samples were negative, indicating that the Qt NASBA assays for immune evasion genes are highly specific.

### *Immune evasion gene expression during active HCMV infection*

To detect the expression of immune evasion RNA and to determine the sensitivity of the Qt NASBA assays for US3, US6 and US11, blood samples (n=275) from patients who had undergone lung transplantation with HCMV infection were analyzed. The HCMV antigenemia assay was used as a standard for defining active



## Expression of US3, US6 and US11

HCMV infection. The results (Table 2) were compared with those of pp65 antigenemia. Additionally, the results were compared with results of Qt NASBA assays for IE1 and pp67, obtained in a previous study of the same samples (22). The sensitivity of the Qt NASBA assays for immune evasion genes among patients with a primary infection was 82.4 % for US3 RNA, 88,2% for US6 RNA, and 82.4% for US11 RNA. In patients with a secondary infection, the percentages were 79.7%, 79.7% and 76.8%, respectively. At clinically relevant levels of antigenemia, RNA of the 3 immune evasion genes could be detected in 100% of the samples. The presence of RNA encoded by the immune evasion genes was detected more frequently than pp67 RNA, which is considered to be a diagnostic marker for disseminating late stage of infection closely correlating with viraemia. Late pp67 RNA was detected in 60.8% and 54.5% of the samples of patients with primary and secondary infection, whereas IE1 RNA was detected in 92,2% and 87,3%, respectively.

**Table 2:** Comparison of results quantitative nucleic acid sequence-based amplification for human Cytomegalovirus (HCMV) immune evasion genes US3, US6 and US11 with IE1 and pp67 RNA levels and antigenemia results.

Type of infection, Result of assay for antigenemia	No. of samples	US3	US6	US11	IE1 <sup>a</sup>	pp67 <sup>a</sup>
Primary						
Positive	51	42 (82.4)	45 (88.2)	42 (82.4)	47 (92.2)	31 (60.8)
Negative	26	12 (46.2)	12 (46.2)	8 (30.8)	18 (69.2)	6 (23.1)
No result <sup>b</sup>	10	1 (10.0)	1 (10.0)	1 (10.0)	2 (20.0)	0 (0)
Secondary						
Positive	69	55 (79.7)	55 (79.7)	53 (76.8)	69 (87.3)	17 (21.5)
>10 Positive cells <sup>b</sup>	11	11 (100)	11 (100)	11 (100)	11 (100)	6 (54.5)
Negative	53	16 (30.2)	20 (37.7)	7 (13.2)	23 (43.4)	1 (1.89)
No result <sup>b</sup>	66	23 (34.8)	31 (47.0)	12 (18.2)	29 (43.9)	5 (7.58)

NOTE. Data are no. (%) of samples

a As described in (21)

b Because no clinical signs indicated active HCMV infection, antigenemia was not determined.

c Clinically significant threshold values.

In Table 3, the time to the first positive result obtained by the different assays is shown. For 2 of the 3 patients with a primary infection, the first positive result of antigenemia assay and Qt NASBA assay for IE1, US3 and US6 RNA was obtained simultaneously. In 1 transplant recipient with primary infection, Qt NASBA assays for US3 and IE1 yielded positive results 6 days before antigenemia or Qt NASBA tests for US6 and US11. In blood samples of patients with a secondary infection, US3 and US6 RNA were detected ~3 days earlier than

antigenemia and even prior IE1 RNA. US11 RNA was detected with a mean delay of 1.5 days, compared with that of antigenemia, but 2 weeks before Qt NASBA for pp67 became positive. The immune evasion gene expression appeared early in the course of infection, except for US11, with a mean detection time of 3.2 weeks for US3, 3.3 weeks for US6, and 3.7 weeks for US11, compared with 3.5 weeks for antigenemia. The immune evasion gene expression closely paralleled IE1 mRNA expression, except for US11, which was intermediate between IE1 and pp67.

**Table 3:** Time to first human Cytomegalovirus (HCMV) in blood samples obtained from patients with active primary or recurrent infection by quantitative nucleic acid sequence-based amplification (Qt NASBA) for US3, US6 and US11, compared with antigenemia assay and Qt NASBA for IE1 and pp67.

	HCMV assay	No. of patients	Mean weeks post transplantation of first HCMV appearance (first - last result)
Primary	Antigenemia	3	3.4 (1.7-4.4)
	US3 Qt NASBA	3	3.1 (1.7-4.1)
	US6 Qt NASBA	3	3.4 (1.7-4.4)
	US11 Qt NASBA	3	3.4 (1.7-4.4)
	IE1 Qt NASBA	3	3.4 (1.7-4.4)
	pp67 Qt NASBA	3	4.1 (2.7-5.4)
Secondary	Antigenemia	5	3.6 (2.1-6.0)
	US3 Qt NASBA	5	3.2 (1.3-5.0)
	US6 Qt NASBA	5	3.2 (1.6-5.0)
	US11 Qt NASBA	5	3.8 (1.3-7.0)
	IE1 Qt NASBA	5	3.3 (1.6-6.0)
	Pp67 Qt NASBA	5	5.8 (3.3-8.0)

### *HCMV US3, US6 and US11 mRNA levels in transplant recipients*

To determine the potential diagnostic value of quantification of expression levels of immune evasion RNA in lung transplant recipients, expression levels of US3, US6 and US11 RNA were compared with levels of antigenemia (Table 4). In patients with a primary HCMV infection, a parallel trend was observed between the increasing numbers of pp65-positive cells and the mean RNA levels of the immune evasion genes. The highest levels of RNA expression were detected for US3 and US6 (log 6.1, and log 6.4, respectively) in patients with primary HCMV infection. These levels are lower than those found for IE1 RNA (log 7.3), whereas US11 levels peaked at ~1 log lower (log 5.1). The mean levels of US3 and US6 mRNA were comparable (log 5.3 and log 5.0, respectively) and higher than the mean level of US11 RNA was lower (log 4.5).

**Table 4: Mean RNA levels (log) in 100  $\mu$ l whole blood, compared with those of antigenemia assay, in lung transplant recipients with a primary and secondary human Cytomegalovirus infection**

Infection	Antigenemia	0		US3 Qt NASBA		US6 Qt NASBA		US11 Qt NASBA		IE1 Qt NASBA		Pp67 Qt NASBA	
		No. positive cellsa	No.	No. Positives	RNA copies	No. Positives	RNA copies	No. Positives	RNA copies	No. Positives	RNA copies	No. positives	RNA copies
Primary	0	24	10	3.6	18	3.9	6	4.1	17	3.8	6	3.6	
0	1	13	5	2.9	9	4	2	2.9	10	4.1	7	2.7	
0	38758	14	13	4.5	13	4.8	7	4.2	12	4.3	13	3.2	
0	>10	24	24	5.4	23	6	22	4.7	23	5.4	23	4.6	
Secondary	0	52	16	3.5	24	3.6	1	0.7	21	3.7	8	2.5	
0	1	23	13	4.1	20	4.2	3	3.3	16	3.6	13	3.7	
0	38758	35	31	4.3	35	4.6	8	4	28	4.1	29	2.9	
0	>10	11	11	4.8	11	4.7	6	4.6	11	4.9	11	3.6	

<sup>a</sup> No. of positive cells per 50,000 cells.

In patients with secondary HCMV infection, similar RNA expression patterns were observed. However, at the peak of antigenemia, the levels of immune evasion RNA expression did not reach the high levels observed in the patients with primary infections. Mean levels detected in the blood samples of patients with a secondary infection were log 4.4, log 4.3 and log 3.3 for US3, US6 and US11 RNA, respectively. The mean levels of RNA encoded by the immune evasion genes US3 and US6 was similar to the levels of pp67 RNA (log 4.9). IE1 RNA achieved the highest expression at a mean level of log 5.6. The results indicate that immune evasion RNA levels peaked simultaneously with IE1 RNA and antigenemia and earlier than pp67 RNA. Monitoring of immune evasion gene expression did not add value for early diagnosis of active HCMV infection in these patients, compared with that of IE1 RNA, which was shown recently to be the most sensitive diagnostic marker for active HCMV infection (28,29).

### *Kinetics of immune evasion mRNA expression in transplant recipients*

The dynamics of the immune evasion RNA expression were analyzed in samples collected weekly in terms of time after transplantation. Fig. 1 shows an example of a lung transplantation recipient with a complicated primary HCMV infection that could not be suppressed by ganciclovir because of the emergence of a resistant strain. In the early period of infection, immune evasion gene expression closely followed IE1 and pp67 RNA levels, paralleling the level of

pp65-positive cells. However, during the final recovery phase, when HCMV pp65-antigenemia and late pp67 RNA became undetectable, both IE1 and immune evasion RNA remained detectable for prolonged periods in the absence of any HCMV-associated disease symptoms. Similar findings of rapidly increasing RNA levels for all HCMV genes tested during the acute phase and persistent but fluctuating low-level expression of IE1 and immune evasion RNA after recovery were observed in the other 2 patients with primary HCMV infection.

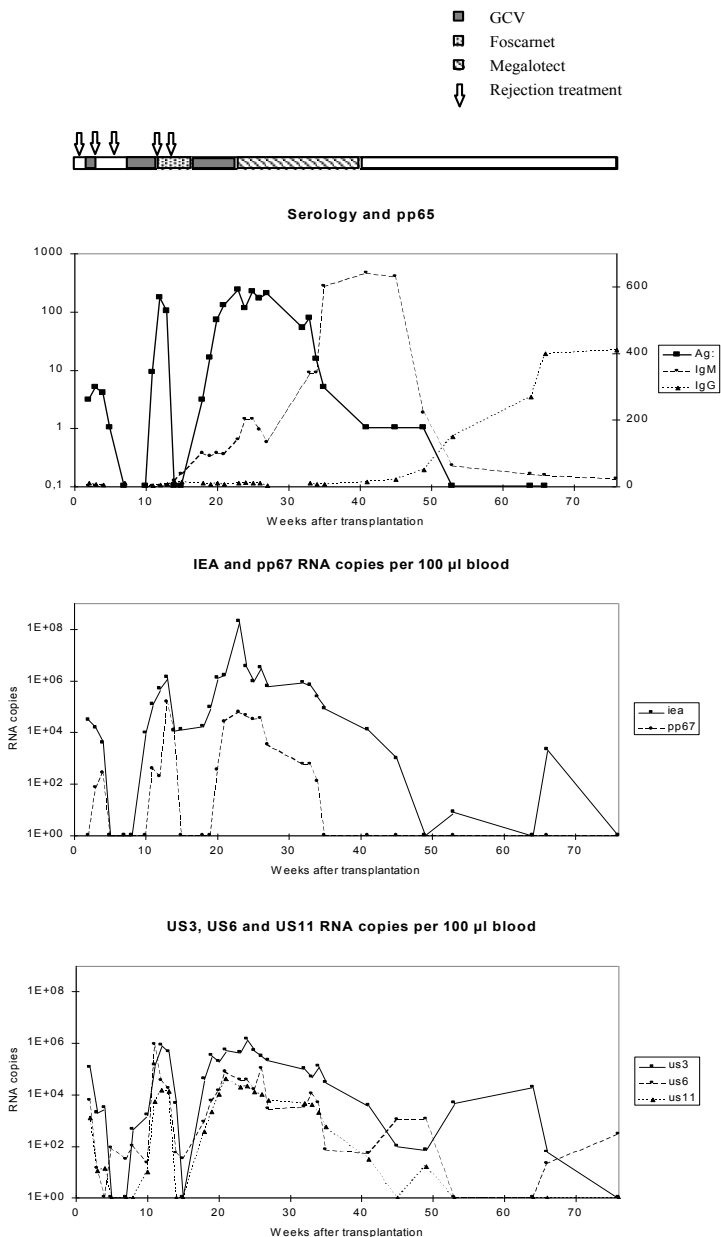
In contrast to the acute phase of primary infection, when most genes were switched on simultaneously, more interesting data were obtained from patients with a secondary HCMV infection. Therefore, the results of 3 representative patients are shown in detail in Fig. 2.

The patient shown in Fig. 2A had 3 periods of active infection. During the early acute phase of infection, immune evasion RNA levels paralleled levels of antigenemia, IE1 and pp67 RNA, as observed in the patients with primary infection. The onset of active HCMV infection was detected by antigenemia, US11, and pp67 RNA at 4 weeks, whereas US3 and US6 RNA were present in a previous sample, simultaneously with IE1 RNA. Two courses of ganciclovir were given to control HCMV replication. All immune evasion genes showed a considerable reduction upon resolution of the active phase of infection but remained detectable, occasionally even in the absence of IE1 RNA. No clinical HCMV-related symptoms were recorded in this patient after week 25.

The patient presented in figure 2B had persistent low antigenemia levels and therefore received no antiviral therapy. All 3 immune evasion genes actively were expressed during the period of low antigenemia and showed an initial peak simultaneously with positive signals for IE1 RNA. Late mRNA was not detected throughout, except at 2 isolated time points (weeks 9 and 51), confirming the non-productive nature of the infection in this patient. The first positive result for antigenemia coincided with the detection of US3 and US11 RNA, whereas US6 RNA was already present together with IE1 RNA at 2 weeks after transplantation. The US6 and US11 RNA levels remained at low and relatively stable levels, which were comparable to those of antigenemia.

Expression of US3, US6 and US11

**Figure 1:** Analysis of course of infection in a lung transplant recipient with a primary HCMV infection, Top antigenemia (Ag) and serology, middle IE1 and pp67 RNA expression, bottom immune evasion US3, US6 and US11, as determined by quantitative nucleic acid sequence-based amplification. RNA copies are expressed per 100  $\mu$ l of whole blood. Time course of treatment is shown



However, US3 was detected in 2 peaks. The second peak of US3 gene expression appeared shortly before rejection therapy was given and was followed by the second weak signal for pp67 RNA.

The patient shown in Fig. 2C had a brief period of active HCMV infection shortly after transplantation, as defined by serology (data not shown). The first samples were positive for IE1 RNA, and all immune evasion genes were expressed, whereas neither the antigenemia assay nor the Qt NASBA assay for pp67 showed any positive result. Antiviral therapy was given because of clinical symptoms suggestive for HCMV infection during a period of increased immunosuppression for treatment of acute rejection. Although all routine parameters became negative after a short period of HCMV activity, immune evasion genes continued to be expressed independently of IE1 expression, reflecting a persistent low viral activity.

Similar low but detectable expression of immune evasion RNA, independent of IE1 RNA expression, was observed in the majority of patients after clinical recovery from either primary or secondary HCMV infection. These data indicate a persistent but fluctuating HCMV activity in the infected host in periods of undetectable clinical disease and suggest controlled latency that follows episodes of active viral gene expression disease.

## Discussion

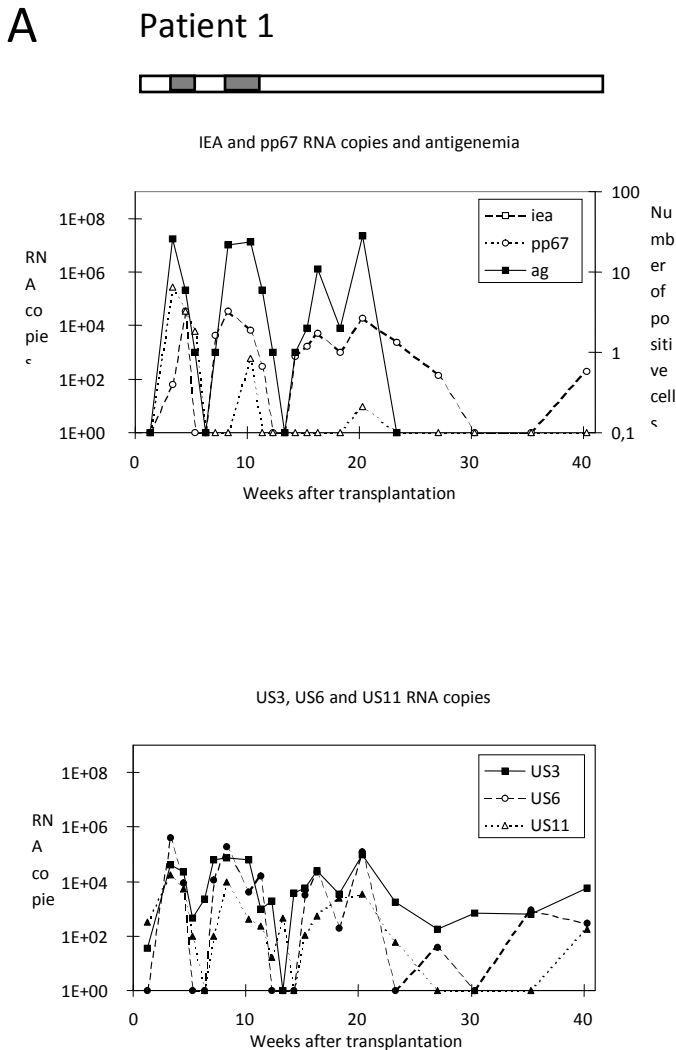
The immune evasion gene products encoded by HCMV are considered to modulate host immune surveillance, thereby allowing HCMV to replicate without being noticed by the immune system (6). A concerted action of all immune evasion genes leads to a significant reduction of MHC class I peptide complexes on the cell surface, as defined by *in vitro* experiments (7,8,10,30). The expression of the immune evasion genes has not been studied *in vivo*, except for UL18, which was detected only at the symptomatic stages of HCMV infection (20). The level of expression of the immune evasion genes may determine the ability of HCMV to disseminate without being eliminated by the host's immune system, thereby contributing to pathogenesis.

For a better understanding of the possible role of immediate early and early immune evasion gene expression *in vivo*, the RNA levels of US3, US6 and US11 were studied by Qt NASBA of samples from HCMV-infected lung transplant recipients. Qt NASBA proved to be highly specific and allowed for the detection of both spliced (US3) and nonspliced mRNA molecules (US6 and US11). RNA encoded by US3 and US6 was detected earlier in the infection than antigenemia,

Expression of US3, US6 and US11

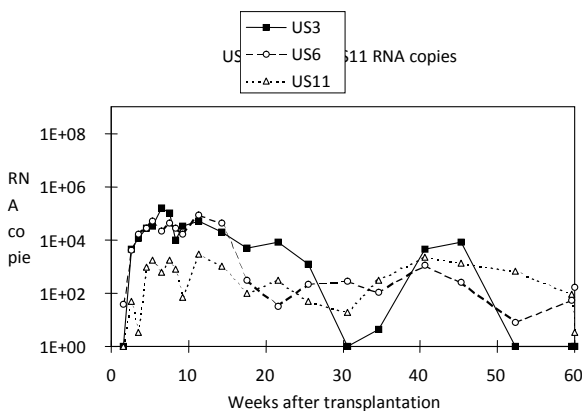
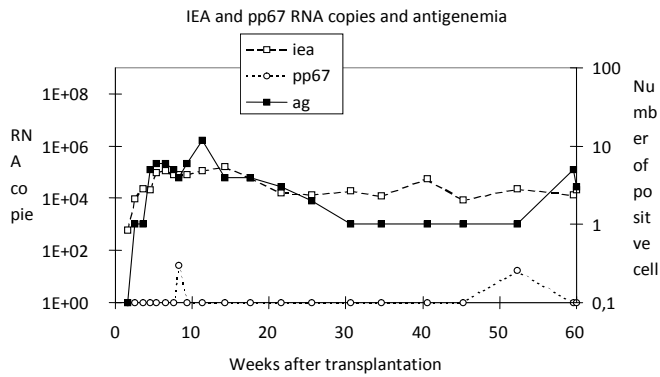
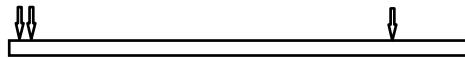
a mean of 2 and 1,5 days, respectively, whereas US11 RNA was detected ~1,5 days later than antigenemia.

**Figure 2:** Analysis of the course of infection in 3 lung transplant recipients with a secondary human Cytomegalovirus infection: patient 1, active infection leading to clinical symptoms; patient 2, infection with low activity; patient 3, inactive infection. Top, Antigenemia (Ag) and IE1 and pp67 RNA; bottom, immune evasion US3, US6 and US11, as determined by quantitative nucleic acid sequence-based amplification. RNA copies are expressed per 100  $\mu$ l of whole blood. Time course of treatment is shown at top (Fig. 1 for key).



Expression of US3 RNA was occasionally detected before IE1 RNA, despite the reduced sensitivity of Qt NASBA assay for US3, indicating the strong expression dynamics of this gene. This may be of direct biological relevance. US3 interferes with antigen processing at very early stages of infection and therefore may prevent immunodominant immediate-early protein-derived peptides from being presented at the cell surface, thus providing a selective advantage to the infected cell.

## B Patient 2





## Expression of US3, US6 and US11

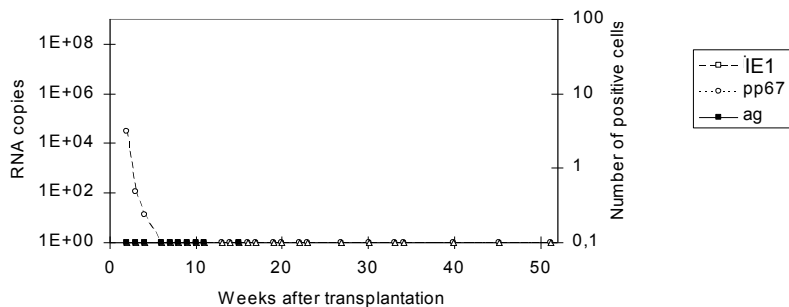
IE1 RNA (UL123) distinctly precedes the appearance of pp65-positive granulocytes and late pp67 mRNA, which are more closely linked to disseminating productive infection (22, 28, 29, 31). On the other hand, IE1 (and US3) gene expression may be associated with abortive or restricted HCMV activity not leading to disease (31,32). During the early stages of active infection *in vivo*, the immune evasion RNA levels progress in parallel with IE1 and late pp67 gene expression.

### C

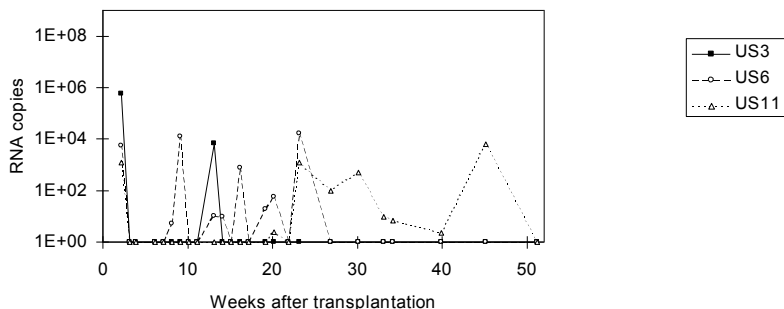
#### Patient 3



IEA and pp67 RNA copies and antigenemia



US3, US6 and US11 RNA copies



RNA of the immune evasion genes was often detected in blood samples at early times when antigenemia assay results were still negative or below the clinical relevant number of pp65-positive cells (US3 RNA was detected in 56%, US6 RNA in 61%, and US11 RNA in 47% of the samples with negative results for antigenemia during early times after transplantation). Although monitoring of immune evasion gene expression had no added value for early diagnosis relative to IE1 RNA (28,29,31), the results suggest that low-level immune evasion RNA expression during early stages of HCMV infection plays a role in supporting subclinical viral activity and possible non-symptomatic dissemination from the original site of HCMV infection. Immune evasion gene expression may reflect more subtle activity of HCMV with its host, because in some patients immune evasion gene expression was still detected independently of IE1 RNA expression after the infection was resolved, as indicated by the disappearance of all standard diagnostic parameters of viral activity. The expression of the immune evasion genes in absence of IE1 RNA indicates independent viral activity, which may have implications for understanding of long-term control of HCMV. This may be of clinical relevance, because active HCMV in the post transplantation period is related to chronic vascular disease, the pathogenesis of which remains to be defined (33).

In conclusion, immune evasion gene expression may contribute to (subclinical) progression of HCMV infection and viral dissemination, at both early and late stages of HCMV infection *in vivo*. In blood samples of lung transplant recipients, the US11 expression was generally lower (mean, log. 4.1) than US3 and US6 expression (mean, log 4.9 and log 4.8, respectively). In contrast, the expression level of US6 in infected cells *in vitro* is lower than that of US3 and US11 (21). The observed differences in expression levels in infected cells *in vitro* and infected cells circulating in the blood *in vivo* may be explained by the fact that the mRNA levels in blood represent an average of the expression profiles in different circulating cells: monocytes, dendritic cells, and occasional detached endothelial cells (32,34). The late appearance of US11 in the circulation of transplant recipients might be due to a lower expression level of the US11 gene in circulating cells *in vivo* compared with US3 and US6. Cell sorting experiments combined with Q<sub>t</sub> NASBA may provide more detailed insight about the *in vivo* expression dynamics in individual HCMV-infected cell population.

The actual contribution of the expression of individual immune evasion genes to pathogenesis of HCMV infection remains to be defined, as is the case for the recently described cytokine and cytokine receptor genes that are encoded in the HCMV genome (35). These newly defined HCMV-encoded functions may provide new targets for antiviral intervention at early stages of infection. A more

## Expression of US3, US6 and US11

detailed understanding of the pathogenic, diagnostic, and prognostic consequences of immune evasion and cytokine and cytokine receptor gene expression in different cell types in the human host to be achieved. Methods for quantification of specific mRNA populations, as described in this study, may contribute to increased knowledge about the subtle interactions between HCMV and the host immune surveillance during convalescence and establishment of persistent latency.

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