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Balance between herpes viruses and immunosuppression after lung transplantation

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2006

[Link to publication in University of Groningen/UMCG research database](#)

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 7

Dynamics and function of anti-Epstein-Barr virus and anti-Cytomegalovirus immune responses in the transplant patient: implications for immunosuppression

Running head: Immune responses against EBV and CMV after transplantation

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Abstract

We studied the development of primary immune responses to Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in a CMV and EBV naive lung transplant patient in relation to virus parameters and clinical symptoms. Primary CMV infection readily resolved after treatment with ganciclovir and after seroconversion CMV antigenemia remained negative during follow-up. Despite treatment with ganciclovir primary EBV infection presented with post-transplantation lymphoproliferative disease (PTLD). PTLD was reversed by lowering immunosuppression. However, even after seroconversion PTLD relapsed twice with high levels of EBV DNA. EBV specific CD3⁺, CD8⁺, CD69⁺, Interferon-Gamma producing-T-cells became detectable at 10 weeks after lung transplantation (Ltx) and remained positive at significant levels. "Ex vivo" EBV specific lysis of autologous PTLD-derived lymphoblastoid cell-line could be shown by ⁵¹Cr release assay. "In vivo" function was suggested by rapid remission of PTLD after reduction of the immunosuppression.

The EBV DNA load in peripheral blood as determined by quantitative-competitive-PCR fluctuated with immunosuppression and not with the use of antiviral drugs.

We here show the development of immune responses to CMV and EBV despite a high level of immunosuppression early after Ltx. After seroconversion primary CMV infection resolved but EBV infection resulted in relapsing PTLD in spite of substantial numbers of EBV specific CTL's. We suggest that EBV activated CTL's were not restricted in number but in function due to immunosuppression, reflecting differences in immune control against CMV and EBV infected cells.

Introduction

Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are ubiquitous human herpesviruses that persist lifelong after primary infection. While major advances in the monitoring and treatment of CMV have been made, EBV remains a source of morbidity and mortality after solid organ transplantation(1).

Since monitoring of CMV became possible several strategies to control CMV after solid organ transplantation have been described. These range from treatment of clinical disease to pre-emptive and prophylactic antiviral treatment. With the availability of antiviral drugs effective against CMV such as ganciclovir and foscarnet these treatment strategies have been proven to be highly successful (2). Antiviral drugs, however, are only effective against the productive replication cycle of herpesviruses. Because CMV only has a productive way of replication, CMV is susceptible to these drugs. Additionally, the productive replication cycle entails that upon host cell lysis new virus particles are exposed to the humoral immune system and CMV neutralising antibodies have been described (3).

EBV infected cells, however, have both a productive and a non-productive replication cycle. The latter functions by EBV-mediated proliferation of infected B-cells, whereas EBV lytic replication is mainly restricted to mucosal epithelia (4). In the healthy virus carrier EBV-neutralizing antibodies are readily detectable but EBV-specific-cytotoxic T-cells (EBV-CTLs) are thought to be predominantly responsible for the lifelong control of non-productive EBV replication (5,6). If the balance between EBV-driven proliferation and EBV specific immunity is disturbed, e.g. due to the use of immunosuppression, uncontrolled B-cell proliferation may result, ultimately leading to post transplant lymphoproliferative disease (PTLD) and outgrowth of malignant lymphoma (7).

Because T-cells are responsible for transplant rejection they are the major targets for immunosuppression. This immunosuppression is both directed at the function of the T-cells, e.g. by using calcineurin inhibitors (cyclosporine A, tacrolimus), as well as at the numbers of T-cells, e.g. by using anti T-cell antibodies (ATG, OKT-3) and proliferation inhibitors (azathioprine and Mycophenolate Mofetil). Consequently, the intensity and spectrum of the T-cell directed immunosuppression correlates with development of PTLD (8).

While antiviral drugs have been shown to be effective against the productive replication cycle of EBV (9), the effect on the proliferative replication cycle is controversial (10,11). Treatment strategies for EBV should thus be

focussed on restoring the balance between EBV driven B-cell proliferation and T-cell mediated EBV-specific immunity.

In allogeneic bone marrow transplantation (BMT), with rapid tapering of iatrogenic immunosuppression, the impairment of EBV control lies in the limited numbers of EBV-CTLs present at early stages post BMT. In this situation adoptive transfer of EBV-CTLs, either by bulk donor lymphocyte infusion or infusion of in vitro expanded EBV-CTLs, has been demonstrated to be effective (12-16). After solid organ transplantation, especially after induction therapy with anti-T-cell antibodies, EBV-CTLs will initially be absent in the circulation. Because most EBV-seropositive patients do not develop PTLD, they must be able to restore functional EBV-CTLs in time. In solid organ transplant patients who develop PTLD however, it is not directly clear whether the impairment in EBV control is a consequence of impaired function or decreased numbers of EBV-CTLs, because the immunosuppression used in these patients afflicts both. Also, the time necessary for EBV-CTLs to develop after primary infection or to restore their function in EBV-seropositive patients during immunosuppression is not known and the selective effects of different immunosuppressive regimens on cellular immune responses are not known. This, however, has implications for the choice between the therapeutic strategies for PTLD. On the one hand, when the numbers of EBV-CTLs are too low or absent as suggested by Haque et al (16,17), this would be a strong argument for adoptive transfer of EBV-CTLs. On the other hand, in case of functional impairment of adequate numbers of EBV-CTLs, the therapeutic goal should be to improve EBV-CTL function by reduction or modification of immunosuppression. Adoptive transfer of EBV-CTLs would then be a less logical treatment strategy.

To explore these options we studied the development of the humoral and cellular immune responses against CMV and EBV in a CMV and EBV seronegative lung transplant recipient who was transplanted with a transplant from a CMV and EBV positive donor.

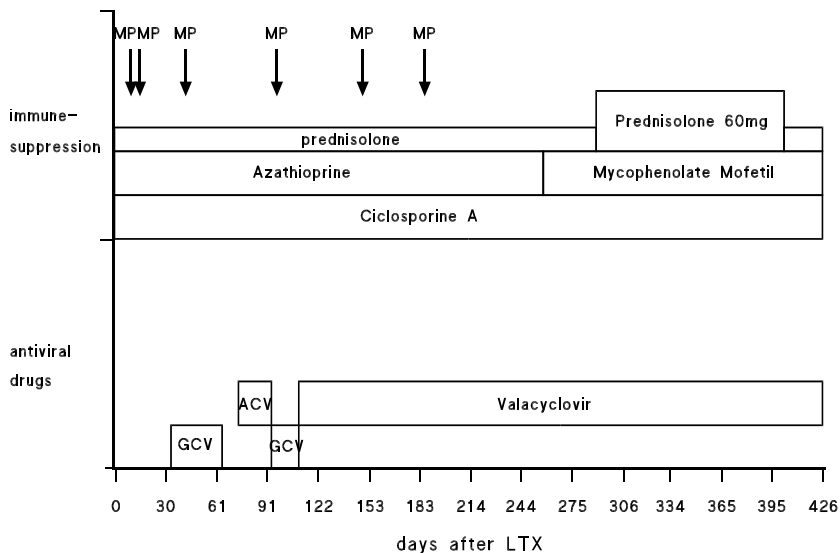
This patient had many known risk factors for PTLD, that is: primary EBV- and primary CMV infection and use of r-ATG induction therapy (18). We hypothesized that, in case adequate numbers of EBV CTL's could be demonstrated in this patient, the development of relapsing PTLD must have been due to the impaired function of EBV CTL's.(19,20).

Patient, Materials and Methods

Patient and Treatment

A 24 years old CMV-seronegative and EBV-seronegative patient with cystic fibrosis received a bilateral lung transplant from a CMV and EBV seropositive donor. He received standard immunosuppression (Fig. 1A) including rabbit-Anti-Thymocyte Globulin (r-ATG, 3 mg/kg, 3 times postoperatively)(Merieux, France), azathioprine (1.5-3 mg/kg/day), cyclosporine-A (Neoral, Novartis)(dose adjusted to trough levels of 400 µg/l within 3 weeks tapered to trough levels of 150 µg/l), prednisolone (3 times 125 mg the first day, 0.2 mg/kg/day from day 2 to the third month and 0.1 mg/kg/day thereafter). Co-trimoxazole was given as *Pneumocystis Jerovici* (previously *Carinii*) prophylaxis (960 mg eod) and Acyclovir (200 mg qd) was given as prophylaxis for alphaherpesviruses. Acute rejection episodes were diagnosed on clinical signs and symptoms and chest x-ray findings with histology if clinically indicated, and treated with pulse therapy methylprednisolone (500 or 1000 mg iv for 3 days). Ongoing transplant dysfunction was treated by replacement of azathioprine with Mycophenolate Mofetil (cellcept, Roche) from week 37 onwards.

Figure 1A: Immunosuppression and antiviral therapy. CsA = cyclosporine-A, MP= pulse therapy methylprednisolone, GCV= ganciclovir, ACV= acyclovir



Cytomegalovirus-related disease was treated with ganciclovir I.V. (Cymevene, Roche) until pp65-antigenemia levels dropped below limit of detection (21). PTLD episodes were treated by decreasing cyclosporine A trough levels to 50% (75-100 µg/l) combined with high-dose aciclovir administration (800 mg, 5 doses/day) or Valaciclovir (1000 mg tid).

CMV antigenemia and CMV serology

CMV antigenemia was determined as described previously (22,23). IgM and IgG antibodies against HCMV were determined weekly by a semi-quantitative ELISA using alkaline glycine-extracted HCMV antigens obtained from HCMV AD169 infected fetal fibroblasts and in parallel on an extract of mock-infected fibroblasts (24).

EBV serology

Serum samples were obtained twice weekly and at every visit to the outpatient clinic and stored at -20°C until use. One serum sample before transplantation and all sera until week 25 were examined in one assay. EBV-specific IgG and IgM antibodies were determined as described before (25). In short the test procedure was as follows. Polystyrene micro titer plates were incubated during 48 hours at 4° C with the peptide solution (1 µg/ml in 0.1mM carbonate buffered solution, pH 9.6) and subsequently blocked with bovine serum albumin (BSA 3%) and sucrose (5%) in phosphate buffered saline, dried at room temperature and stored at 4° C until use. Plates were washed and sera diluted in incubation buffer containing 0.01 M Tris, 0.3 M NaCl, 0.05% Tween-20 and 2% BSA (pH7.5). Sera were diluted 1:100, 1:200, 1:400 and 1:800 for IgG and IgM. Sera were incubated on an ELISA shaker for 45 minutes at room temperature. Plates were washed and conjugates were added. (IgG conjugate, Goat-anti-human IgG-Peroxidase labeled (De Beer, the Netherlands), IgM conjugate, Goat-anti-human IgM-peroxidase labeled (Pasteur, France)) Plates with conjugates were incubated for 30 minutes at room temperature and washed. Substrate, 100 micro liter of 0,3g/l OPD in citrate buffered solution (pH=5.05) with 0,0002% H₂O₂, was added and after 20 minutes 100 µl of 1M H₂SO₄ was added to stop the reaction. Plates were read at 490nm.

In every assay four EBV-negative control sera were tested to determine cutoff values. The amount of antibody present in the patients' serum was expressed in (arbitrary) Elisa Units (EU)(24).

DNA isolation

Whole blood samples for EBV DNA detection were taken weekly after transplantation. One ml of fresh unfractionated whole blood was lysed in 9 ml of NASBA lysis buffer (5 M guanidine isothiocyanate, 1.2 % Triton X-100, 20 mM EDTA, 0.1 M Tris-HCl. pH 6.4; Organon Teknika, Boxtel, the Netherlands) and stored at -80°C until use. DNA was isolated from 1 ml of lysate by silica-based extraction as described previously (26). One negative control (water) was included for each run of ten whole blood samples.

Quantitative Competitive EBV-DNA PCR

The experimental approach for determination of EBV DNA load in clinical specimens has been described previously (27). Briefly, the DNA equivalent of 5 µl whole blood or serum was amplified in a qualitative EBNA-1 PCR. EBV-DNA load in PCR positive samples was subsequently determined by quantitative competitive EBNA-1 PCR (Q-PCR) as described (27). DNA quality of whole blood samples was checked by β-globin PCR according to Roda Husman et al (28). To ensure the validity of the results, several precautions were taken to avoid contamination of the PCR (29). As control for accuracy and reproducibility of quantification, a fixed amount of WT plasmid DNA was quantified in each experiment in duplicate. In addition, all samples were screened blindly and appropriate negative and positive controls for DNA isolation, preparation of PCR master mix and EIA detection were included (one negative control for each ten tested samples and one positive control per experiment).

Isolation of PBMCs, expansion of tumor cells ex vivo and production of LCL

Blood samples for isolation of peripheral blood mononuclear cells (PBMC) were taken before transplantation and at regular time intervals after transplantation. PBMC were isolated using Lymphoprep density gradients (Nycomed, Oslo, Norway) and cryopreserved in liquid nitrogen until further use. Upon use, PBMC were thawed and resuspended in RPMI supplemented with 10% heat-inactivated FCS (RPMI/FCS). Previous testing showed no loss of functionality with this procedure (data not shown).

The patient-derived EBV-transformed lymphoblastoid cell-line (LCL) was expanded from (tumor cell-positive) biopsies by culturing a small sample of PTL D biopsy material, obtained during the diagnostic open lung biopsy, in RPMI/FCS containing 0.5 µg cyclosporine A per ml. Growth of cells was observed within 3 weeks of culturing and a stable cell culture was obtained. HLA-typing

demonstrated that the outgrowing tumor cells were of recipient origin. Control LCLs were obtained by infection and transformation of B-cells from healthy controls with the B95-8 EBV strain *in vitro* using standard procedures.

Measurement of EBV activated T-cells by flowcytometry

Recipient PBMC 2×10^6 were added to polystyrene tubes (Greiner) containing 4 ml RPMI/FCS only (unstimulated samples) or 2×10^6 irradiated biopsy-derived autologous LCL in RPMI/FCS. If sufficient recipient PBMC were available, the PBMC were also stimulated with 25 ng/ml Phorbol Myristate Acetate (PMA, Sigma) and 1 μ g/ml ionomycin (Sigma) to serve as a positive control. Unstimulated and LCL-stimulated samples were incubated at 37°C and 5% CO₂ overnight. Brefeldin A (Sigma; 10 μ g/ml) was added to these cultures only after the initial 6 hr of culture. PMA/ionomycin-stimulated samples were incubated for 4 hr in the immediate presence of Brefeldin A (10 μ g/ml). After stimulation, samples were washed with PBS containing 1% BSA, and resuspended in a small volume PBS containing 1% BSA and 5% human AB serum. Then, surface marker-specific monoclonals were added (aCD3-CyQ; IQ Products, Groningen, The Netherlands, and aCD8-APC; Pharmingen) and cells were incubated for 15' at room temperature. Next, FACS lysing solution (Becton Dickinson) was added and cells were incubated for 10' at room temperature. They were then spun down, resuspended in FACS Permeabilizing Solution (Becton Dickinson) and incubated for 10' at room temperature. After washing, CD69-specific PE-labeled antibody (Coulter), Gamma-IFN-specific FITC-labeled antibody (Becton Dickinson) or FITC-labeled isotype-matched control antibody (IQ Products) was added and cells were incubated for 30'. After washing, stained cells were analyzed on a flowcytometer (Coulter EPICS Elite) and data were analyzed by WinList 3D (Verity Software House inc, Maine USA). Usually, over 20.000 counts (CD8 T cells) were collected for the analysis. As controls, EBV-seronegative and EBV seropositive healthy volunteers were tested.

Calculation of the absolute numbers of EBV-specific CD3+, CD8+, IFN- γ + T-cells

Total numbers of lymphocytes were determined in peripheral blood by routine methods. The percentage of CD3+, CD8+ T-cells within the lymphocyte population was analyzed by flowcytometry. Also the percentage CD3+,CD8+,CD69+, IFN- γ + EBV specific T-cells within the CD3+,CD8+ T cell population was analyzed by flowcytometry as described above. Absolute numbers of EBV-CTLs were then calculated by multiplying total number of lymphocytes

with the percentage of CD3+, CD8+ T-cells, and the percentage of CD3+, CD8+, CD69+, INF- γ + T-cells, respectively (Table 1).

Table 1: Calculation of absolute numbers of EBV-activated-CD8+ T-Cells. White blood cell counts (WBC) and lymphocyte counts were determined in peripheral blood. Percentages of CD3+, CD8+ T-cells were analyzed by flowcytometry. Of these CD3+, CD8+ T-cells percentages of EBV-activated T-cells were analyzed by determining CD69-positive and Interferon- γ (IFN- γ) positive cells after stimulation with cultured autologous EBV-LCLs. Absolute numbers of EBV-activated CD3+, CD8+ T-cells were then calculated by multiplying total number of lymphocytes with CD3+, CD8+ percentage and CD69+, IFN- γ + percentage. ND= not done

Period after Ltx	WBC 10 ⁹ /L	Lymphocytes 10 ⁹ /L	% CD3+ & CD8+	% CD69+ & IFN- γ +	Absolute numbers of CD3+, CD8+, CD69+, IFN- γ + T-cells/L blood	Chromium Release assay
Pre Ltx	11.8	ND	19	0.025	ND	7 %
5 weeks	2.2	0.198	12	0.02	0.05 x 10 ⁷ /L	ND
10 weeks PTLD	5.2	0.364	13	1.12	5.3 x 10 ⁷ /L	30%
17 weeks	6.1	1.15	33	0.91	34.5 x 10 ⁷ /L	55%
24 weeks	5.7	1.23	45	0.54	29.9 x 10 ⁷ /L	50%
35 weeks	8.7	0.94	50	0.30	14.1 x 10 ⁷ /L	ND
39 weeks	7.1	1.19	51	0.71	43.1 x 10 ⁷ /L	43%
60 weeks	14.9	1.25	40	0.25	12.5 x 10 ⁷ /L	ND

Cytotoxicity assay

The capacity of the T cells to lyse the EBV-positive tumor cells was measured after in vitro prestimulation. Briefly, recipient T cells were isolated from cryopreserved and thawed PBMC using Lymphokwik-T (One Lambda, Montpellier, France). Then 2x10⁶ T cells, together with 1x10⁶ irradiated (20 Gy) PBMCs as feeder cells, were added to 1x10⁵ autologous irradiated biopsy-derived LCL cells in a total volume of 2 ml RPMI/FCS. On days 3, 6 and 9, 0.5 ml of medium was replaced with fresh RPMI/FCS medium containing 10 U IL-2/ml. At day 12, the expanded T cells were harvested and added to ⁵¹Cr-labeled autologous and allogeneic control LCL cells (see before) at effector: target ratio's of 50:1, 25:1 and 12.5:1. After 4 hr of incubation, supernatants were harvested and the released ⁵¹Cr release was measured in a Packard gamma-counter. ⁵¹Cr release was measured in six individual wells per condition. Spontaneous release and maximum release were determined by incubating the labeled cells in medium alone or 5% Triton X-100 in PBS, respectively. Specific release was calculated

using the following formula: % specific lysis = (experimental release - spontaneous release) / (maximal release - spontaneous release). No lysis of the allogeneic control LCL cells was observed in these assays.

Results

Clinical course of the patient and diagnosis of PTLD episodes

Shortly after transplantation 2 rejection episodes were treated with pulse Methyl Prednisolone (Fig. 1A, Fig. 1B). The first treatment was given because of low-grade fever and persistent pleural effusion and the second also because of low-grade fever without signs of infection. CMV infection was diagnosed at week 3 and successfully treated with ganciclovir (see below). At 10 weeks after lung transplantation the patient presented with multiple nodules of 1-2 cm in size on the routinely obtained chest X-ray. Lung function was still improving (Fig. 1B) and there were no clinical symptoms of infection or rejection. An open lung biopsy revealed a PTLD by demonstrating a proliferation of EBER positive, CD20 positive and IgM kappa-positive large blastoid cells. After reduction of immunosuppression (trough level of cyclosporine to 75ng/L) all nodules disappeared within 2 weeks. Lung function decreased however gradually thereafter, and 23 weeks after lung transplantation pulse methylprednisolone was given because of suspected rejection. Cyclosporine A trough level was increased to standard maintenance level (150 ng/L). Lung function initially stabilized but then decreased further and at 27 weeks after transplantation a bronchoscopy was performed. Transbronchial biopsies showed active bronchiolitis obliterans and, to a minor extent, small aggregates of small and large lymphocytes. The large lymphocytes were EBER-positive, CD20 positive B-cells, histologically considered as residual PTLD. Presumptive diagnosis was ongoing rejection and some residual PTLD.

The active bronchiolitis was treated with pulse methylprednisolone. Lung function, however, did not improve and FEV1 values decreased gradually. Azathioprine was converted to Mycophenolate Mofetil (cellcept, Roche), but FEV1 only stabilized after another pulse methylprednisolone at 42 weeks and increased maintenance dose of prednisolone. Patient then withdrew from control until week 58. At this time all blood samples were retrospectively analyzed for EBV-DNA load. An increase in EBV-DNA load in the peripheral blood (Fig. 2A, see below) raised suspicion of ongoing PTLD.

Figure 1B: Lung function depicted as FEV1 in absolute volume and as % of baseline. Baseline lung function is calculated according to ISHLT criteria: the average of the 2 best FEV1 results with at least 3 weeks interval. BOS= Bronchiolitis Obliterans Syndrome IS= Immunosuppression, MP= pulse therapy methylprednisolone

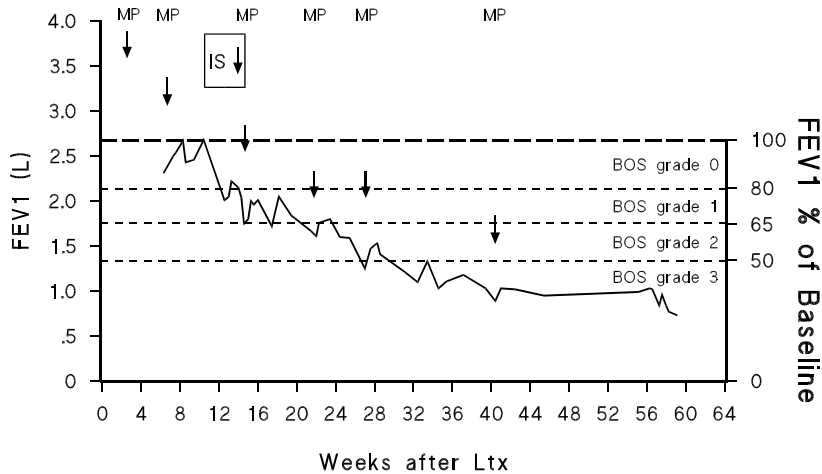
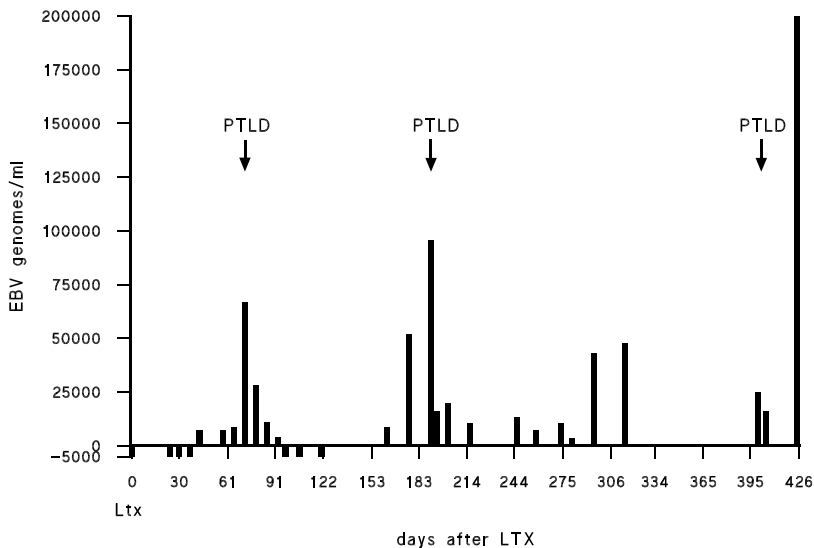


Figure 2A: Results of EBV-qPCR. Results are expressed as genomes/ml.



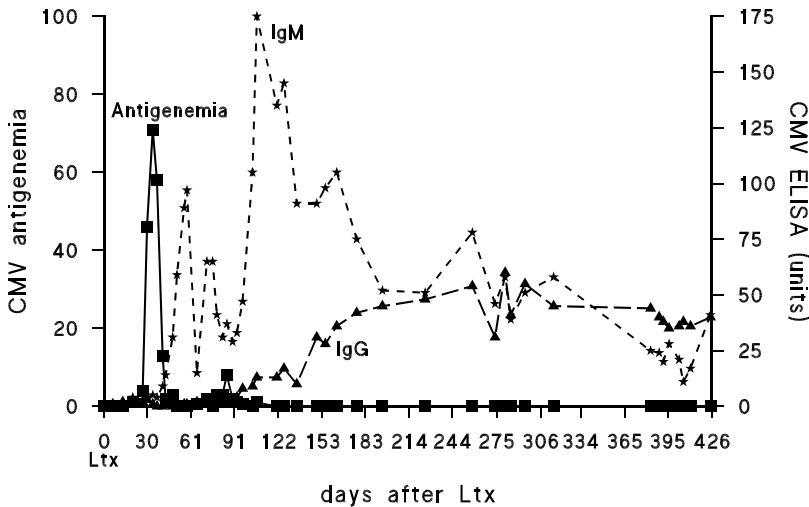
At 58 weeks after lung transplantation a third episode of PTLD was diagnosed. In the transbronchial biopsies aggregates of lymphoid cells were seen again. In these aggregates CD20+, EBER positive cells were demonstrated. Liver function tests deteriorated and the cyclosporine level rose to toxic levels. All

hepatotoxic medication was stopped and patient died soon thereafter due to respiratory failure. Permission for postmortem was not obtained.

Cytomegalovirus pp65-antigenemia and serology (Fig. 3)

In the immediate post-transplant period the patient developed a primary CMV infection. CMV-antigenemia became positive 3 weeks after Ltx and ganciclovir iv was started (5 mg/kg bd). There were no clinical signs or symptoms of CMV disease except for a minor rise in body temperature (37,4°C). CMV-antigenemia reached a peak of 76 pp65 positive cells /50,000 and became negative 7 weeks after Ltx.

Figure 3: CMV antigenemia and Serology. After anti-CMV IgM and IgG became positive CMV antigenemia remained negative.



Ganciclovir was continued until CMV-antigenemia was negative for 2 weeks and then stopped (week 9). Ganciclovir was also given from week 13 to 16 because of a positive CMV antigenemia result during rejection treatment. CMV antigenemia remained negative thereafter. IgM anti-CMV antibodies were detectable from week 3 on. IgG anti-CMV antibodies became positive 13 weeks after Ltx.

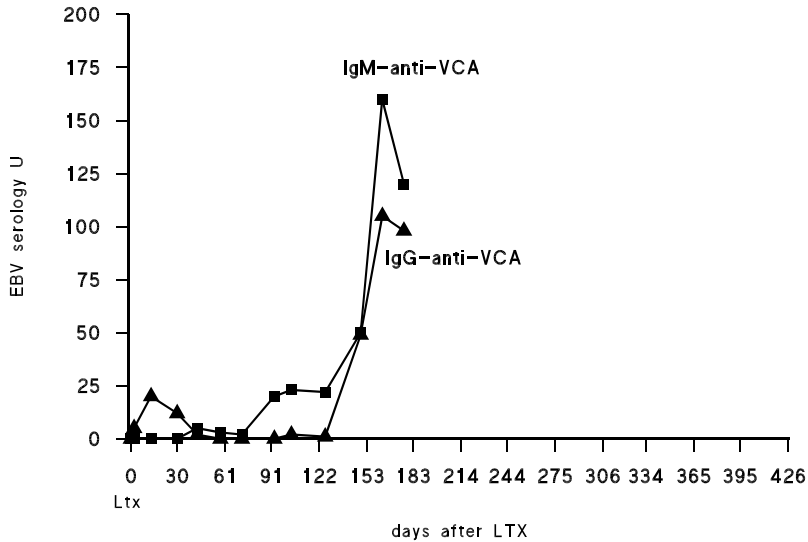
EBV-DNA load dynamics in peripheral blood (Fig. 2B)

The patient also developed a primary EBV infection as confirmed by EBV-serology (Fig. 2B). EBV-DNA became detectable in week 6 at 7,000 EBV DNA copies/ml blood during treatment with ganciclovir iv. It remained detectable in week 8 and 9 at this level (7,200 and 8,600 EBV DNA copies/ml blood), until

ganciclovir was stopped when CMV antigenemia was negative for 2 weeks. In week 10 the patient presented with PTLD, accompanied by a high viral load of 66,600 EBV-DNA copies/ml blood and was admitted in our hospital.

Upon admission high dose aciclovir was started (1000 mg qd) because of suspected PTLD. One week later this diagnosis was confirmed by open lung biopsy and subsequently immunosuppression was decreased. Clinical course and EBV DNA load is described above.

Figure 2B: Results of EBV serology. Only anti-VCA antibodies were detected.



EBV-activated CD3+, CD8+ T-cells, Cytotoxicity assay and EBV antibody response (Table 1, Fig. 2B, Fig. 2C)

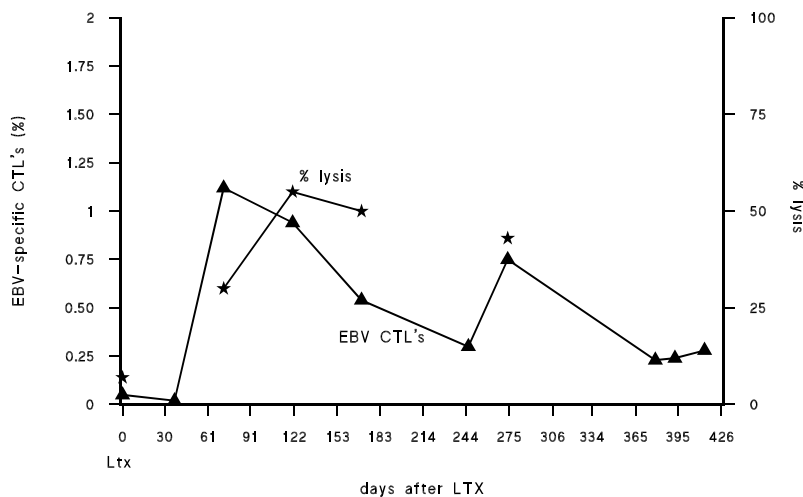
In order to detect T-cell-mediated EBV-immunity, PBMC from an EBV seronegative healthy volunteer and 2 EBV seropositive healthy volunteers were stimulated with autologous and allogeneic EBV LCL's and subsequently tested for CD69 expression and IFN- γ production in CD3+, CD8+ T-cells as measured by flowcytometry. Using this test no CD3+, CD8+, CD69+, IFN- γ + T-cells could be demonstrated in the EBV seronegative healthy control whereas 1.10% and 1.17% of the CD3+, CD8+ T-cells, obtained from the two EBV seropositive healthy controls, expressed CD69 and produced IFN- γ after stimulation with autologous EBV-LCL's. This corresponds with results of T-cell mediated EBV immunity described by Kuzushima et al (30).

In the blood of the patient, 0.025% and 0.02% CD3+, CD8+, CD69+, IFN- γ + T-cells were detected, at the time of transplantation and 5 weeks after

transplantation respectively, upon ex vivo stimulation with autologous EBV-LCLs. These negative results were consistent with the EBV-seronegative status of the patient before transplantation. Ten weeks after Ltx, 1.12 % CD3+, CD8+, CD69+, IFN- γ + T-cells were detected, which co-occurred with the time the patient presented with PTLD. After 17 weeks 0.94 % and thereafter between 0.71 and 0.25 % CD3+, CD8+, CD69+, IFN- γ + T-cells were detected.

Absolute numbers of CD3+, CD8+, CD69+, IFN- γ + T-cells were calculated per liter of peripheral blood. Before Ltx and after 5 weeks EBV-activated-CD3+, CD8+ T-cells were considered negative. At presentation of PTLD at 10 weeks 5.3×10^7 /L CD3+, CD8+, CD69+, IFN- γ + T-cells further increasing to 34.5×10^7 /L at 17 weeks after Ltx were detected, paralleled by the resolution of the first PTLD episode. During follow-up CD3+, CD8+, CD69+, IFN- γ + T-cells remained detectable but decreased gradually corresponding with the increase in immunosuppression, rise of EBV DNA load and relapses of PTLD.

Figure 2C: Ex vivo EBV-specific cytotoxicity and absolute numbers of EBV activated-CD3+, CD8+, CD69+, IFN- γ + T-cells as calculated from peripheral blood lymphocyte count. In vitro EBV-specific lysis of autologous EBV LCL's was 25% at 10 weeks (presentation of PTLD) and rose to 55% at 17 weeks (after reduction of immunosuppression and disappearance of PTLD). It was 50 and 43% at week 24 and 39 respectively



The pattern observed in the development of CD3+, CD8+, CD69+, IFN- γ + T-cells corresponded with the pattern in EBV-specific lysis as measured by chromium release assay. Before transplantation the EBV specific lysis was, as expected, very low (7 % lysis). Specific lysis increased to 30 % in week 10, when the patient presented with PTLD and increased further to 55 % in week 17 and remained high with 43 % in week 40. This shows that functional EBV-specific T-

cells remained present throughout the patient follow-up, despite the relapse of PTLD and rise in EBV DNA load.

EBV-VCA specific IgM and IgG antibodies were negative prior to Ltx and became detectable in week 12 and 21 respectively, confirming a primary EBV-infection and reflecting the patients immune responses to EBV antigens *in vivo*. No antibodies were detected against EA(d) or EBNA-1 during the follow-up period showing only a limited serological response to EBV antigens.

Discussion

In this case study we noticed a clear dichotomy between the development of immunity against CMV and EBV.

Our patient was seronegative for both CMV and EBV prior to transplantation and received an organ from a CMV and EBV seropositive donor. He subsequently developed a primary infection for both CMV and EBV during the initial phase of high immunosuppression, given after lung transplantation. While CMV was easily controlled with a pre-emptive strategy using ganciclovir, EBV was never controlled in this patient and ultimately might have caused his death. This difference might be explained by the ways of replication for both viruses. CMV replicates in a lytic productive fashion exposing both structural and infected cell-associated antigens to cellular and humoral immune system, leading to the rapid induction of cytolytic and virus neutralizing effector mechanisms. EBV however associates with latent replication by inducing proliferation of infected B-cells with only limited lytic virus replication. This latent lymphoproliferation enables EBV to multiply its genome with minimal exposure to a virus-neutralizing humoral immune response. This may in part explain why in our patient EBV escaped from immune control. Analysis of the source of EBV-DNA in this patient revealed an exclusive cellular origin, as no cell-free EBV-DNA was detected in plasma or serum, not even at the highest peaks of EBV DNA-emia (14). Although VCA-IgM and VCA-IgG responses were detected, these developed rather late and their contribution to effective immune control remains undefined. The absence of anti-EBNA1 antibody responses may be a reflection of poor cellular immunity as suggested by previous publications (31,32). This also illustrates the importance of functional cellular immune responses for the control of EBV.

When analysing the cellular immune response against EBV we noticed that EBV-responsive CD3+, CD8+ T-cells developed within 10 weeks and that these EBV-activated-CTLs were functional upon further stimulation *ex vivo*, as reflected by their capacity to lyse autologous PTLD-derived LCL. Clinically their functionality *in vivo* was suggested by the rapid regression of all PTLD lesions

when immunosuppression was decreased. So we conclude that EBV-specific cytotoxic T-cells (EBV-CTLs) developed already 10 weeks after transplantation in spite of high level of induction therapy with r-ATG and maintenance immunosuppression. However, despite the primed cellular immune status, subsequent PTLD episodes developed, as reflected by increasing EBV-DNA levels and presence of multiple EBV-positive cells in biopsy specimens. This suggests a functional deficiency in the circulating T-cells to counteract the growth potential of EBV-infected B-cells, which may reach a doubling time of 56 hours in vivo (14). Although we found between 5.3 and 43.1×10^7 EBV-activated-CTLs/L (30), these numbers are considerably higher than reached by the adoptive transfer of ex vivo cultured EBV-CTLs (12,16,33). This raises the question whether the detected EBV-specific T cells were truly functional in vivo. To explain this paradox, it may be assumed that the EBV-specific CTLs we detected by flow cytometric detection of intracellular IFN- γ production display a 'functional diversity'. This 'functional diversity' has previously been illustrated for EBV-specific T cell lines which showed an inverse relationship between IFN- γ production and cytotoxic potential (34,35). This could mean that the EBV-specific cytotoxic T cells, as detected in the 51-chromium release assay, arise from a different subpopulation of CD8 T cells than EBV specific T cells with IFN- γ producing capacity, which we demonstrated by flow cytometry. On the other hand, effector CD8 T cells usually display both perforin/granzymes and IFN- γ (36). It is of interest to note that Van Baarle et al found that increasing numbers of IFN- γ producing EBV specific T-cells rather than increasing numbers of EBV tetramer-positive T cells predicts good antiviral responses and reduction of EBV viral load in immunocompromised patients, such as HIV carriers (37). Finally, it can be argued that a combined low proliferative and functional capacity of the EBV-reactive T-cells, caused by the immunosuppressive therapy, contributed to the failure to control EBV. Summarizing, we think that the EBV-CTLs in our patient were not impaired in numbers, but in their in vivo function, most likely due to the effects of maintenance immunosuppression.

Apparently this dysfunction of T-cells is still adequate to control CMV infection, once acute replication is suppressed by antiviral drug treatment.

An alternative explanation for the impaired EBV control might be that recurrent PTLD were due to a variant EBV strain with mutations in important CD8-Tcell epitopes, thus escaping immune control (38). Additionally, chronic (low-grade) CMV infection with accumulation of large clones of replicative senescent T-cells may have hampered the development and maintenance of an effective T-cell mediated EBV specific immune response (39,40)

In the studies on adoptive transfer of EBV-CTLs for PTLD Khanna et al (33) describe a lung transplant recipient with primary EBV infection and PTLD. All PTLD lesions disappeared after infusion of in-vitro (in absence of immunosuppressive) expanded EBV-CTLs. In this study PTLD also relapsed (33), indicating that the restoration of the number of EBV CTLs alone was not enough and led only to a temporary control of EBV as observed in our patient. Thereafter maintenance immunosuppression again impaired their in vivo function. This indicates that, in the long term, not only restoration of numbers but also restoration of function should be the therapeutic objective. Reduction of immunosuppression should thus be part of any treatment strategy for EBV after solid organ transplantation.

The results of the EBV-DNA Q-PCR clearly reflect the fragile balance between EBV and the impaired immune system after solid organ transplantation. These observations also point to a crucial difference in the long term control of CMV and EBV infection respectively, the latter being more dependent on T-cell mediated functions.

Initially, the patient developed a primary EBV infection associated with low levels of circulating EBV-DNA that developed into a PTLD 10 weeks after Ltx. Reduction of CsA led to a complete remission and EBV-DNA disappeared from the peripheral blood. The acute rejection that was triggered by this decrease in immunosuppression, was treated with pulsed methylprednisolone (MP) and an increase of CsA. Immediately EBV became detectable again (Fig 2A), suggesting a profound effect of immunosuppressive drugs on the EBV-CTL control of EBV. Because all samples were retrospectively analyzed this was not known at that time and the subsequent transplant dysfunction was interpreted as ongoing rejection and therefore treated again with pulse MP. This clearly demonstrates the need for careful and parallel monitoring EBV-DNA levels.

This patient also illustrates the contrast in the effects of antiviral medication between EBV and CMV infection. The primary CMV infection was rapidly controlled by ganciclovir therapy which effectively blocks lytic viral replication and viral spread. After CMV-specific antibodies became detectable in the peripheral blood CMV-antigenemia remained negative and no CMV related morbidity was seen. This is in contrast to the EBV primary infection. Initial rise in EBV-DNA levels occurred during ganciclovir treatment, and EBV-specific antibodies became detectable only after the first episode of PTLD. Thereafter two relapses of PTLD were seen and EBV-DNA remained detectable in the circulation despite continued high dose (val-)aciclovir. The explanation of this contrast between the two herpesviruses is believed to be twofold. First, CMV mainly produces a lytic viral infection after which the virus has to spread

systemically to infect distant organs and cells. EBV on the other hand remains latent while driving infected cells (B-cells) into proliferation. This way, the EBV multiplication is relatively shielded from the humoral immune response. Secondly, the pre-emptive use of ganciclovir reduces CMV replication levels which may allow even a suboptimal T-cell system to maintain control, whereas EBV replication is relatively resistant to antiviral drugs because EBV uses the cellular DNA polymerase to multiply the latent genome (41) and lytic cycle associated phosphokinases are not induced. It is suggested that EBV infection require a more optimal T-cell system for effective short- and long-term control (42).

In conclusion we suggest that not the lack in EBV-CTL numbers but a lack in function induced by immunosuppression is responsible for PTLD after solid organ transplantation. Under the same therapeutic regimen, CMV infection can be controlled effectively. Regarding the fragile balance between EBV viral load and levels of immunosuppression we suggest that pre-emptive treatment guided by EBV-DNA levels should be the strategy of choice. This approach should include reduction of immunosuppression in relation to EBV-CTL function. Furthermore, studies are required to clarify the mechanistic differences in long-term control of CMV and EBV infection, including TCR-diversity and effector functions of humoral immunity.

Acknowledgements

We thank the laboratory of transplant Immunology of the University Hospital Groningen for the collection of the whole blood samples.

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