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ORIGINAL ARTICLE

Mireille T.M. Vossen
Mi-Ran Gent
Jean-Claude Davin
Paul A. Baars
Pauline M.E. Wertheim-van Dillen
Jan F.L. Weel
Marijke T.L. Roos
Debbie van Baarle
Jaap Groothoff
René A.W. van Lier
Taco W. Kuijpers

Spontaneous outgrowth of EBV-transformed B-cells reflects EBV-specific immunity in vivo; a useful tool in the follow-up of EBV-driven immunoproliferative disorders in allograft recipients

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M.T.M. Vossen (✉) · M.-R. Gent
P. A. Baars · R. A. W. Baars
R. A. W. van Lier
Department of Experimental Immunology,
Academic Medical Center,
Meibergdreef 9, 1105 AZ Amsterdam,
The Netherlands
E-mail: m.t.vossen@amc.uva.nl
Tel.: +31-20-5667688
Fax: +31-20-5669756

M.T.M. Vossen · M.-R. Gent · J.-C. Davin
J. Groothoff · T. W. Kuijpers
Emma Children's Hospital,
Academic Medical Center,
Amsterdam, The Netherlands

P.M.E. Wertheim-van Dillen · J.F.L. Weel
Department of Clinical Virology,
Academic Medical Center,
Amsterdam, The Netherlands

M.T.L. Roos · D. van Barrle
Department of Clinical Viro-Immunology,
Sanquin Research at CLB, Amsterdam,
The Netherlands

Abstract During immunosuppressive medication, Epstein-Barr virus (EBV) infection is associated with a risk of developing posttransplant lymphoproliferative disease (PTLD). The appropriateness of a spontaneous EBV B-cell transformation (SET) assay as a monitor of EBV-specific immunity was evaluated to investigate if it safely allows reducing immunosuppressive medication, thereby decreasing the risk of developing PTLD. PBMC were isolated longitudinally from 20 pediatric renal allograft recipients treated with prednisone and cyclosporine combined with either azathioprine or mycophenolate mofetil. Most significantly, EBV-peptide-specific $CD8^+$ T cells were detectable in the blood of patients with negative SET assays, coinciding with significantly lower EBV loads, whereas these cells were less frequent in the blood of patients with positive SET assays. Reducing the levels of immunosuppression resulted in normalization of the SET assays. Therefore, the SET assay is a reflection of the interaction between viral replication, transformation of B cells, and EBV-specific immunity in vivo and hence a valuable screening test for EBV-driven lymphoproliferative phenomena in allograft recipients.

Keywords Epstein-Barr virus · Transplantation · Immunosuppression · $CD8^+$ T cells · Posttransplant lymphoproliferative disorder

Abbreviations *ALP* Alkaline phosphatase · *AZA* Azathioprine · *B-LCL* B cell lymphoblasts · *CMV* Cytomegalovirus · *CTLs* Cytotoxic T lymphocytes · *EA* Early antigen · *EBNA* Epstein-Barr virus-encoded nuclear antigen · *EBV* Epstein-Barr virus · *ELIspot* Enzyme-linked immunospot assay · *HSV* Herpes simplex virus · *MMF* Mycophenolate mofetil · *PHA* Phytohemagglutinin · *PTLD* Posttransplant lymphoproliferative disease · *SFU* Spot forming units · *SET* Spontaneous EBV B-cell transformation · *VCA* Viral capsid antigen · *VZV* Varicella-zoster virus

Introduction

The remarkable improvement in the survival rates of solid organ transplant patients is to a large extent due to the increased use of immunosuppressive medication. Most significantly, the introduction of the immunosuppressive agent cyclosporine has led to the increased success of allograft function over longer time ranges. Intensified use of immunosuppression has however concomitantly resulted in an increase of the compli-

cations from infectious disease in allograft recipients during primary infections and in reactivation of latent viruses of the *Herpesviridae*.

In the setting of immunosuppressive medication, both primary Epstein-Barr virus (EBV) infection and, to a lesser extent, EBV reactivation, are associated with the risk of developing posttransplant lymphoproliferative disease (PTLD) [1]. PTLD is a constellation of various potentially fatal diseases, ranging from polyclonal B-cell proliferation, which regresses when immunosuppression is stopped, to the aggressive monoclonal B-cell lymphomas that usually appear to be resistant to therapy [2, 3].

Insight into the mechanism of viral reactivation is rudimentary. While reactivation of the *herpes simplex* virus (HSV)-type 1 and the *varicella-zoster* virus (VZV) may occur as oro-labial disease or as shingles during an acute bacterial infection or during moments of severe physical or emotional stress, reactivation of EBV and cytomegalovirus (CMV) occur only rarely, if ever, in healthy individuals [4]. Cytotoxic $CD8^+$ T lymphocytes (CTLs) are thought to be essential for suppression of the viral replication and dissemination of viral agents [5]. Without these CTLs, EBV-infected B cells would proliferate in an unlimited fashion, inducing the B cells to produce their own growth factors among which are IL-6 or (viral) IL-10 [6].

The interaction between EBV and host immune factors can be evaluated by assessing spontaneous EBV transformation of peripheral blood mononuclear cells (PBMC) [7]. We designed a prospective study in a cohort of renal transplant patients under various immunosuppressive regimens to evaluate spontaneous EBV B-cell transformation (SET)-assay as a monitor of EBV-specific immunity in pediatric recipients of kidney allografts.

Materials and methods

Study design

From January 1998 onward, we enrolled 20 patients with a renal allograft. Informed consent for a 2-monthly blood check for immune parameters was obtained from the parents of the children enrolled in the studies, as is required by the institutional medical ethical committee, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

The patients were all under immunosuppressive regimens of prednisone (7.5 mg/m^2) and cyclosporine (plasma trough levels range: 100–150 ng/ml); or tacrolimus, when cyclosporine-related side-effects were found), and either azathioprine (AZA: 1–2 mg/kg), or mycophenolate mofetil (MMF: 1.2 g/m^2 per day in two doses). The choice among the regimes was arbitrary and non-randomized. EBV-reactivation was defined by serology: i.e. at least a fourfold increase in titer of anti-early antigen (EA) in the absence or disappearance of anti-Epstein-Barr virus-encoded nuclear antigen (EBNA)-1 antibodies in immune [anti-viral capsid antigen (VCA) IgM negative, anti-EA antibody negative, and anti-EBNA1 antibody positive] individuals [8].

Laboratory tests for EBV infection

Specific IgM and IgG antibodies against EBV-VCA and EBV-EA were determined by indirect immunofluorescence. Antibodies to EBNA1 were determined by anticomplement immunofluorescence (Gull Laboratories, Salt Lake City, USA) ELISA.

Quantification of EBV load

In a limited number of samples, their was sufficient material to retrospectively quantify the virus concentration of EBV in blood, using an adaptation of the previously reported quantitative PCR for EBV-DNA [9], combined with the electrochemiluminescence assay as described before by R. Boom and coworkers [10]. Viral loads were determined as the number of copies per ml blood.

Lymphocyte subsets

Absolute numbers of B cells ($CD19^+$) and T-cell ($CD3^+$, $CD4^+$, $CD8^+$) subsets were determined longitudinally by standard FACScan procedures on a FACScan (Becton Dickinson (BD), San Jose, Calif.); the MoAbs were produced by Sanquin Research (Amsterdam, The Netherlands).

Spontaneous EBV B-cell transformation (SET)-assay

After standard purification steps and several washes, PBMC were cultured in two 96-well plates at 1×10^5 PBMC per well for 4 to 6 weeks. At least 60 wells were plated per patient (range 60–248, median 114). By the use of this low cell number per well, spontaneous transformation of PBMC in healthy seropositive controls cannot be observed, as described before [7]. Proliferation of B cells was in no cases observed in PBMC from individuals with acute EBV (IgM+, EBV-DNA load >10,000 copies/ml blood; $n=5$), healthy EBV-seropositive (VCA IgG+/EBNA1 IgG+; $n=20$), and seronegative donors (VCA IgG−/EBNA1 IgG−, $n=20$). The outgrowth of B cell blasts of these healthy individuals was only observed when infected in vitro by exogenous EBV, prior to the addition of PHA. In the standard SET assay, positive controls (exogenous EBV added) were included in each experiment. Culture medium containing phytohemagglutinin (PHA) (1 μg/ml) was refreshed weekly. PHA was added to the cultures to exhaust T cells in these long-term cultures and thereby exclude the influence of cytotoxic T cells on the lymphoproliferation in vitro to ensure that the outcome of the SET assay is purely a reflection of the capacity of EBV to transform B cells in vivo ([11]). As shown in Fig. 1, B-cell numbers remained constant over time, with no difference between the MMF- or AZA-treated patients. The number of positive wells was scored by light microscopy after 4–8 weeks of culture and expressed as a percentage of total wells. The proliferation of B cells in vitro was confirmed by spontaneous outgrowth after transfer to another plate and by flow cytometric analysis of B cell markers. Resulting B cell lines were tested for clonality using IgH DNA rearrangement [12]. Toxic effects of AZA or MMF per se on B-cell transformation and outgrowth capacity was excluded in this assay by the positive EBV transformation result upon addition of exogenous infectious virus.

Enumeration of cytotoxic effector cells

HLA-typing information of the allograft recipients as performed prior to transplantation was used to define the HLA-A*0201-positive ($n=8$) and HLA-B8 positive ($n=1$) patients in our cohort. None of the HLA-A*0201 individuals were HLA-B8 positive and

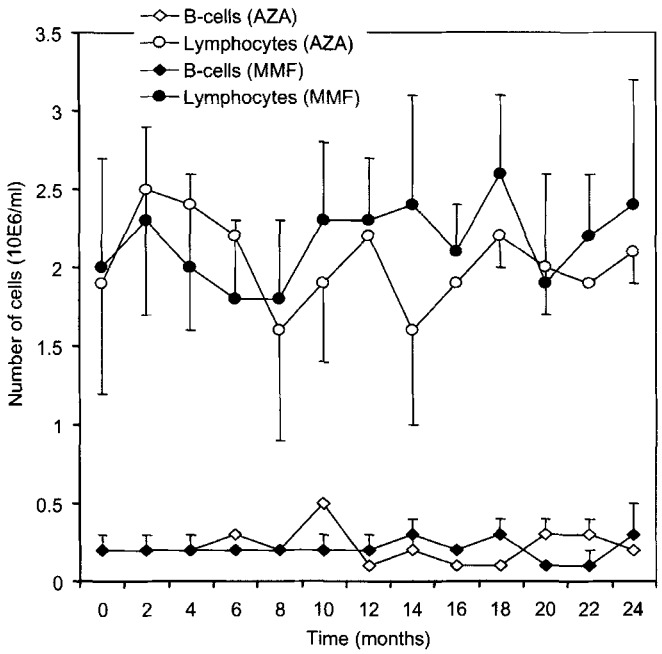

Fig. 1 The absolute number of total lymphocytes and $CD19^+$ B cells in pediatric renal transplant patients. There was no significant difference, neither in absolute $CD4^+$ nor in $CD8^+$ T-cell subpopulations between the AZA- and MMF-subgroups. Furthermore, B-cell numbers remained constant over time. Numbers are expressed as mean ± SD (10^6 cells/ml) over time

vice-versa. In HLA-A*0201 positive individuals, EBV-specific $CD8^+$ T cells were quantified with an APC-labeled tetramer (Sanquin) containing HLA-A*0201, β2-microglobulin and the peptide GLCTLVAML, which is derived from the lytic protein BMLF-1. BMLF-1-specific $CD8^+$ T cells can be detected with this tetramer during acute infection as well as during latency [13]. In the same manner, the EBV-specific $CD8^+$ T cells of the HLA-B8-positive participant were quantified by use of a tetramer containing the peptide RAKFKQLL, derived from the lytic protein BZLF1. PBMC from an HLA-A*0201$^+$/HLA-B8$^+$ seropositive healthy donor were included in each experiment to standardize the assay. To identify $CD8^+$ T cell subsets, cells were subtyped by use of a combination of CD45RA and CCR7 MoAbs. For staining with the mouse anti human CCR7 mAb, a three-step staining protocol was performed, comprising incubation with the CCR7 antibody (Pharmingen, San Diego, Calif.) for 30 min, washing, incubation with biotinylated goat anti mouse IgM (Pharmingen) for 30 min, followed by incubation with streptavidin-PE (BD). Next, PBMC were incubated with 10% (v/v) normal mouse serum (Sanquin), directly conjugated mAbs and tetrameric complexes for 30 min.

Determination of functionality of the EBV-specific $CD8^+$ T cells

Functionality of EBV-specific $CD8^+$ T cells as defined by IFN-γ production, was determined by enzyme-linked immunospot assay (ELIspot) (Mabtech, Nacka, Sweden). Thawed PBMC of HLA-A*0201 positive donors were resuspended in RPMI, containing 10% FCS and antibiotics. For the EBV-specific ELIspot, resuspended PBMC isolated from HLA-A*0201 positive donors were stimulated with the HLA-A*0201 restricted peptide GLCTLVAML (10 μg/ml), as well as with the HLA-B8 restricted peptide RAKFKQLL (negative controls) (10 μg/ml) at 37 °C and 5% CO_2 in α-huIFN-γ precoated silent screen 96-wells plates (Nalge Nunc, Rochester, N.Y.). In the same manner, resuspended PBMC isolated from the HLA-B8 positive donor were stimulated with the HLA-B8 restricted peptide RAKFKQLL (10 μg/ml), as well as with the HLA-A*0201 restricted peptide GLCTLVAML (negative controls) (10 μg/ml). PBMC were cultured in final amounts of 250000, 125000 and 62500 PBMC/well. After 18 h, cells were removed by washing with PBS and the wells were incubated with biotin-labeled α-huIFN-γ for 3 h at room temperature. Thereafter, the wells were incubated with streptavidin conjugated alkaline phosphatase (ALP) for 2 h at room temperature. Finally, spots were visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma Chemical, St. Louis, Mo.). Spots were quantified by the AELVIS ELIspot reader and software (AELVIS, Hannover, Germany). Results are expressed as spot forming units (SFU) per 10^6 $CD8^+$ T cells (mean of duplicate cultures) after correction for background staining. All peptides used in this study were purchased from the IHB-LUMC peptide synthesis library facility (Leiden, The Netherlands). The peptides were generated by standard Fmoc techniques and purified by ether precipitation and HPLC techniques. The peptides were dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) in a concentration of 5 mg/ml. PBMC from an HLA-A*0201$^+$/HLA-B8$^+$ seropositive healthy donor were included in each experiment to standardize the assay.

Statistics

Student's *t*-test and ANOVA were applied for normally distributed data, and the Wilcoxon two-sample test and Kruskal-Wallis test for non-normally distributed data. Fisher's exact test was used for statistical analysis of symptoms in the patient cohorts.

Results

Clinical description of the study groups

The study groups were formed according to the immunosuppressive regimen of the participants. In addition to the formerly standard corticosteroids with cyclosporine [or tacrolimus (FK506) in the case of cyclosporine toxicity], patients received either (1) azathioprine (AZA), or (2) mycophenolate mofetil (MMF). Both groups' members were of similar age and received the same duration of follow-up (Table 1). Although five patients had a primary EBV infection after undergoing transplantation, episodes of symptomatic EBV infection during the period of sequential monitoring occurred only once. Symptoms were acute fever, hepatitis, and EBV-related

Table 1 Study population. [1] EBV reactivation is defined as at least a fourfold increase in titer of anti-EA in the absence or disappearance of anti-EBNA1 antibodies in immune individuals

	n	Mean age (months)	Mean duration of follow-up (months)	Primary EBV infection	EBV reactivation[1]
AZA	10	160 ± 33.2	54 ± 21.5	3	5
MMF	10	177 ± 29.8	56 ± 34.4	2	2

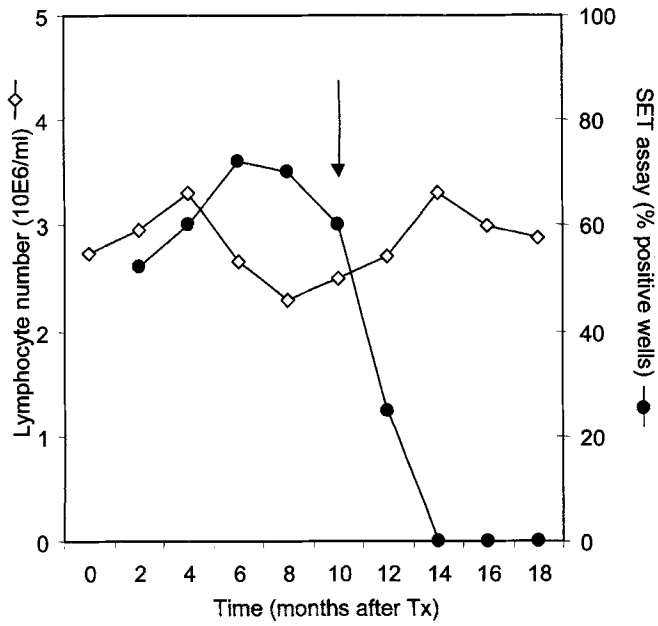

Fig. 2 Spontaneous outgrowth of endogenously infected EBV-transformed B cells (SET assay) in relation to immunosuppression. After a long period of positive SET assays in a patient (AZA#1), AZA treatment was stopped and the dose of cyclosporin was reduced (*arrow*). Lymphocyte counts (*open diamonds*) are expressed as absolute counts in 10^6/ml; results of the SET assay (*closed circles*) are expressed as % of positive wells. *Tx* transplantation

biopsy-proven acute rejection of the allograft (AZA#2). The number of patients suspected of EBV-reactivation in our cohort by serological criteria was seven in total (35%), of whom five were in the AZA group and two in the MMF group (Table 1).

Detection of EBV-specific immunity by the SET assay

The use of the SET assay was evaluated as a measurement of the potential infectious activity of EBV in direct relation to the cytotoxic potential of PBMC to suppress EBV-induced B cell infection and transformation in vivo. The SET assay is both sensitive and specific. The SET assays remained negative in healthy seropositive controls and with acute EBV infection ([14], P.A. Baars, unpublished). All individuals turned positive after in vitro infection by exogenous EBV, prior to the addition of PHA. Furthermore, reproducibility of the SET assay is high. For example, PBMC from three donors, each drawn 2 days apart, resulted in SET positivities of 6.9 and 7.6%, 1.2 and 1.4% and twice 0%, respectively. An illustration of the value of this assay in the follow-up of immunosuppressive medication is shown in Fig. 2. In this patient (AZA#1) with the highest positive SET assay results measured over 6 consecutive months (outgrowth of PBMC was detected in up to 74% of the wells), a decrease in positive wells only occurred after reduction of the immunosuppressive medication (Fig. 2).

In the AZA group, five patients were found with outgrowth of B cell lymphoblasts (B-LCL), versus two in the MMF group (Table 2). Some remained positive over several months, while others were positive for short periods of 2–4 months. The B-LCL of these six patients showed an oligoclonal pattern that changed over time, thus making early development and detection of persistent PTLD less likely (not shown). A weak correlation between positive SET assay and negative EBNA1-serology (and vice-versa) in EBV-positive patients was observed in our cohort ($r = -0.40$) (Table 2). Also, when anti-EBNA1 and anti-EA serology versus outcome of the SET assays were analyzed longitudinally after EBV infection, no definite correlation was observed (Fig. 3). This is best illustrated in the same patient with the strongly positive SET assays (AZA#1, see also Fig. 2); anti-EBNA1 remained negative, anti-EA became positive at the moment the SET assays normalized (Fig. 3a). Another patient with negative anti-EBNA1 serology and rising titers of anti-EA (AZA#9)—who could be serologically expected to have incomplete EBV clearance and potential EBV reactivation ([8]; see Materials & Methods)—, remained negative in the SET assays over time (Fig. 3b). The results of the SET assay seemed to correspond to the plasma levels of cyclosporine. In four patients, the spontaneous outgrowth disappeared when lower plasma levels of cyclosporine were accepted. However, no correlation could be observed between the

Table 2 Correlation of EBV reactivation defined by serology and defined by spontaneous EBV-B cell transformation. [1] = no positive wells detected in SET assay; + positive wells detected in SET assay

AZA #	SET[1]	Anti-EA	Anti-EBNA1	MMF #	SET[1]	Anti-EA	Anti-EBNA1
1	+	+	–	1	+	–	+
2	–			2	–	–	+
3	+	–	+	3	–	–	+
4	+	–	+	4	–		
5	–	–	+	5	–	–	+
6	–	+	±	6	+	+	±
7	+	+	–	7	–	+	+
8	+	+	–	8	–	–	+
9	–	+	–	9	–	–	+
10	–	–	+	10	–	–	+

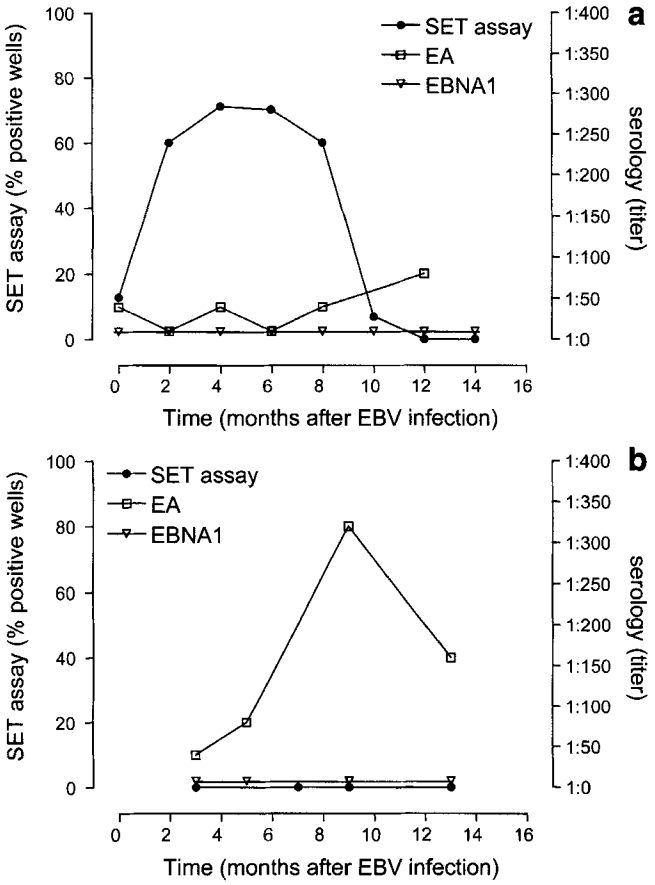

Fig. 3a, b Anti-EBNA1 and anti-EA serology versus outcome of the SET assays were measured longitudinally after EBV infection, to study a possible correlation between the outcome of the SET assays and EBV reactivation as defined by classical serology. **a** In a patient with strongly positive SET assays (AZA#1), anti-EBNA1 remained negative and anti-EA became positive at the moment the SET assays normalized. **b** Another patient with negative anti-EBNA1 serology and rising titers of anti-EA (AZA#9), who could be serologically expected to have incomplete EBV clearance and potential EBV reactivation, remained negative in the SET assays over time

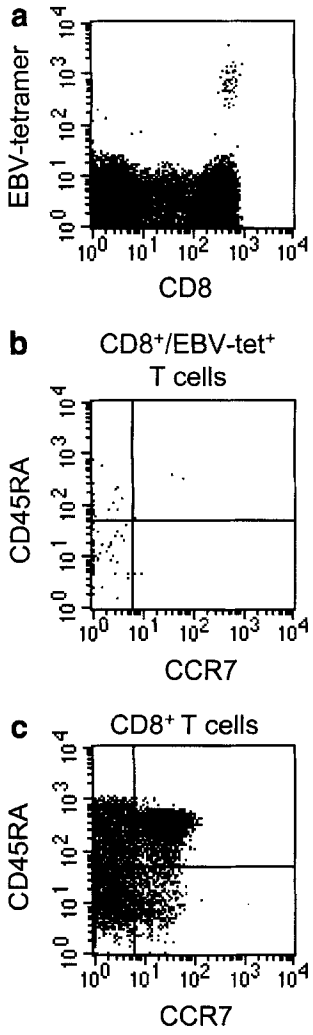

Fig. 4a–c Characterization of EBV-specific $CD8^+$ T cells. **a** Approximately 0.45% of the $CD8^+$ T cells were specific for the EBV GLC-epitope derived from BMLF-1, as shown by tetramer staining gated on lymphocytes. **b** The EBV-specific $CD8^+$ T cells of this patient were $CCR7^-$, and mainly of the effector memory phenotype (i.e. $CCR7^-/CD45RA^-$). The summarized data of the characterization of the EBV-specific $CD8^+$ T cells of the HLA-A*0201 positive patients are shown in Table 3. **c** Both $CCR7^+$ and $CCR7^-$ populations were detected in total $CD8^+$ T cells from the patient

use of ATG as induction therapy and the positivity of SET assays (data not shown).

EBV-specific T-cell immunity

Since reactivation defined by serology correlated only weakly to the outcome of the SET assay, EBV-specific $CD8^+$ T cell immunity was determined by both tetramer staining and by production of IFN-γ after stimulation with virus-specific peptides. An example of the enumeration of EBV-specific T cells by tetramer staining is shown in Fig. 4. EBV-specific $CD8^+$ T cells could be detected in only one of three HLA-A*0201 positive patients with positive SET assays, whereas these EBV-specific $CD8^+$ T cells could be detected in all of the HLA-A*0201 or HLA-B8 patients with negative SET assays (Table 3). The frequencies of EBV-specific $CD8^+$ T cells were significantly different between the two groups (0.12 ± 0.21 for SET+ vs. 0.42 ± 0.13 for SET−; $P = 0.04$). The detection of EBV-specific $CD8^+$ T cells in the patient with a positive SET assay appeared to be of minor significance in terms of immunity, since EBV-specific $CD8^+$ T cells were detected only at one single moment in a series of 7 time points studied (data not shown). Representative time curves of patients with negative and positive SET assays are shown in Fig. 5. Data of all patients analyzed are summarized in Table 3.

Table 3 Parameters of EBV immunity in SET− vs. SET+ patients. [1] after seroconversion. *n.a.* not applicable; *n.d.* not done; patient was HLA-A*0201$^-$/B8$^-$

	Patient	Peak EBV load (copies/ml blood)[1]	Peak % tetramer pos. CD8$^+$ T cells	Peak ELIspot (SFU/ 10^6CD8$^+$ T cells)
SET−	AZA#2	16000	0.45	1316
	AZA#5	3274	0.59	888
	AZA#6	340	n.d.	n.d.
	AZA#9	8700	0.44	233
	AZA#10	6600	n.d.	n.d.
	MMF#2	5302	n.d.	n.d.
	MMF#3	3704	n.d.	n.d.
	MMF#4	0	0.47	4796
	MMF#5	0	0.21	328
	MMF#7	0	0.38	937
	MMF#8	115	n.d.	n.d.
	MMF#9	0	n.d.	n.d.
	MMF#10	0	n.d.	n.d.
SET+	AZA#1	92580	0.00	83
	AZA#3	6305	n.d.	n.d.
	AZA#4	2119	0.36	79
	AZA#7	721	n.d.	n.d.
	AZA#8	29954	n.d.	n.d.
	MMF#1	20286	0.00	113
SET−(mean ± SD)		3387 ± 4820	0.42 ± 0.13	1416 ± 1704
SET+ (mean ± SD)		25620 ± 34859	0.12 ± 0.21	92 ± 19
P-value		0.03	0.04	0.02

After EBV infection, EBV-peptide-specific CD8$^+$ T cells were found in the blood of the patients with negative SET assays up to a frequency of 0.6% of total CD8$^+$ T cells. These EBV-specific T cells were mainly CCR7$^-$. Furthermore, IFN-γ producing EBV-specific CD8$^+$ T cells could be detected in all patients with tetramer-positive cells. The numbers of IFN-γ producing EBV-specific CD8$^+$ T cells as measured by ELIspot analysis were significantly different between the two groups (92 ± 19 for SET+ vs. 1416 ± 1704 for SET−; $P = 0.02$).

Finally, significantly higher EBV viral loads (up to 92,580 copies/ml blood) were detected in patients with positive outcomes of the SET assays than in patients with negative outcomes of the SET assays (25,620 ± 34,859 vs. 3,387 ± 4,820 resp, $P = 0.03$; calculated as mean of highest EBV viral load detected in patients during follow-up) (Table 3).

Discussion

The development and evaluation of techniques for early detection and prevention of life-threatening lymphoproliferative diseases has become indispensable with the increased risk of PTLD associated with EBV infection due to the use of more potent immunosuppression. The effect of immunosuppressive medication on immune parameters relevant for EBV reactivation was prospectively studied in a cohort of pediatric renal allograft recipients. Serologic definition of EBV-reactivation was compared with an assay system in which spontaneous proliferation and outgrowth of EBV-transformed B cell lines were determined. We presume that in this SET assay, the test result is a reflection of the in vivo balance between infectious EBV, B cells, and cytotoxic lymphocytes (consisting of natural killer cells and CTL function) in the blood. The relevance of the SET assay was demonstrated by the fact that immunosuppression seemed to be directly linked to B-LCL generation in vitro, as illustrated by a patient in whom reduction of medication resulted in a complete normalization of the subsequent SET assays (Fig. 2).

As shown in this study, EBV-specific CD8$^+$ T cell immunity could be detected more frequently in the patients who did not show spontaneous outgrowth of B cells in the SET assays in comparison to the patients with positive SET assays (Table 3), which correlated with IFN-γ production upon virus-specific stimulation and significantly lower EBV loads in the blood, reflecting the ability of these CD8$^+$ T cells to control the virus. This correlation of the outcome of the SET assay with EBV-specific T-cell immunity (Fig. 5) is stronger than the correlation—if any—with EBV reactivation as defined by classical serology (Fig. 3). In the latent phase of viral infection, at least three different virus-specific memory populations can be discriminated:(1) CD45RO$^+$ CD27$^+$ CCR7$^+$; (2) CD45RO$^+$ CD27$^+$ CCR7$^-$; and (3) CD45RA$^+$ CD27$^-$ CCR7$^-$ [15, 16]. These subsets have important characteristics. Cytotoxic potential increases with loss of CD27 and the expression of CD27 appears to differ between CD8$^+$ T cells directed to different viruses [17]. The EBV-specific CD8$^+$ T cells detected in the patients were mainly CCR7$^-$, which typifies them as effector-memory and effector-like CD8$^+$

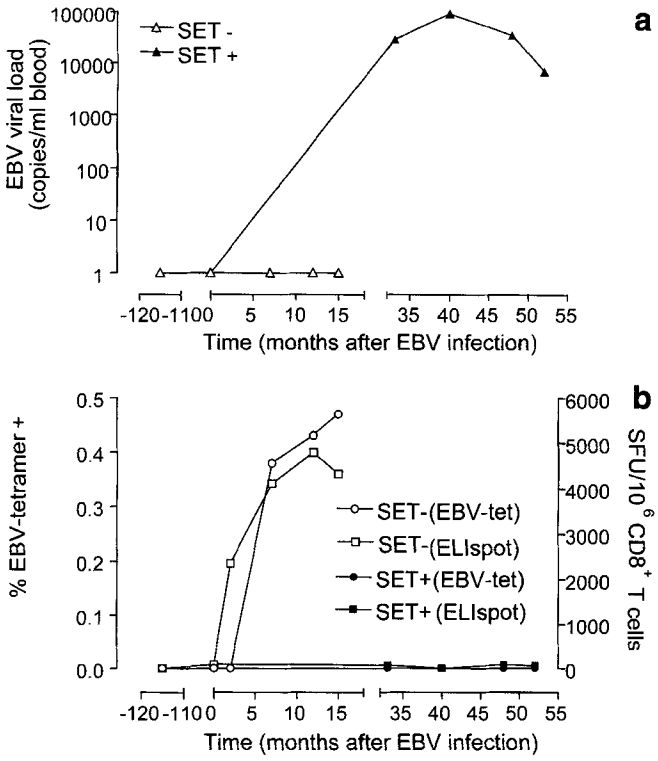

Fig. 5a, b EBV-specific $CD8^+$ T cell immunity in relation to spontaneous EBV-transformed B cell outgrowth (SET). **a** Significantly higher EBV viral loads were present in the blood of patients with positive SET assays (SET+), compared to EBV viral loads in the blood of patients with negative SET assays (SET−) ($25{,}620 \pm 34{,}859$ vs. $3{,}387 \pm 4{,}820$ resp., $P < 0.05$). **b** Accordingly, significantly higher amounts of EBV-specific $CD8^+$ T-cells (up to 0.6% of $CD8^+$ T cells) (*circles*) could be detected in the patients with negative SET assays, reflecting the capability of these cells to control viral replication. These cells exhibited effector functions, demonstrated by IFN-γ release in the EBV-peptide specific ELIspot assay (*open squares*). Data on time curves are derived from two representative patients. $t = 0$ time of EBV infection

T cells [16]. EBV is a lymphocytotropic virus that resides largely in the lymph nodes. Although the loss of CCR7 correlates with high cytotoxic potential and increased IFN-γ production [16, 18] these $CCR7^-$ cells have lost the capacity to re-enter secondary lymphoid organs by ligation to the ligands MIP3β (CCL19) and SLC (CCL21). This loss would impede their ability to cope with EBV-infected cells. However, it cannot be excluded that the virus-specific lymphocytes, e.g. EBV-specific T cells, detected in blood are part of different subsets than the virus-specific cells within the secondary lymphoid organs. Furthermore, during EBV reactivation, effector-type T cells regain expression of CCR7 [19] and are thereby facilitated to re-enter secondary lymphoid organs such as lymph nodes and tonsillar tissue, which are the target sites of these EBV-specific cells [20].

MMF has been reported to be very potent in its immunomodulating effects to prevent acute renal, lung and heart allograft rejection without many side-effects [21, 22, 23]. Although its use may imply stronger immunosuppression, its in-vivo effects can be positively judged, as is also reflected by our findings related to EBV. A difference in the incidence of reactivation of EBV, either defined by serology or SET assay, was established between the two subgroups in our cohort, which differed only in the immunosuppressive medication that was prescribed (AZA versus MMF) (Table 2). The SET assays remained largely negative in the MMF group. Moreover, the effect of MMF on EBV reactivation does not seem to be specific for EBV, since the number of CMV reactivations was also reduced in the MMF group (data not shown). It has been presumed that MMF inhibits antibody production [24] and this could influence the determination of EBV reactivation by classical serology. However, anti-EBNA1 antibodies as well as anti-VCA antibodies could be detected in the patients treated with MMF without significant differences in titers (Table 2). Some reports even suggest potentiating anti-viral effects of MMF [25, 26]. Yet, since dissemination of reactivated VZV and CMV have been reported in patients treated with MMF [27, 28], we do not think that MMF has a *general* anti-viral effect. In our cohort an almost fatal primary VZV infection occurred in an MMF-treated patient resulting in multi-organ failure and acute allograft rejection (MMF#6). Thus, a profound explanation for this phenomenon is still lacking. Larger study cohorts are needed to determine the significance of the differences in lymphoproliferative disorders between patients treated with MMF or AZA.

In conclusion, the SET assay is a valuable overall screening test for lymphoproliferative phenomena driven by EBV in allograft recipients. The absence of EBV-specific $CD8^+$ T cells strongly correlated with spontaneous outgrowth of EBV-transformed B cells and high EBV loads, and vice versa. Furthermore, reducing the levels of immunosuppression resulted in normalization of the SET assays. Therefore, the SET assay indeed appears to reflect the interplay between viral replication and transformation of B cells, and EBV-specific immunity of the host in vivo. PCR-based quantification of EBV concentrations in blood must be evaluated in larger studies to fully understand its prognostic potential [29, 30, 31]. Determination of EBV viral load to predict the risk of development of PTLD is probably only indicative in combination with data on EBV-specific T-cell immunity [31]. After all, the final goal is to find applicable parameters to reduce immunosuppressive medication in a patient-tailored approach. The present study is a step toward such an approach.

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