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TRANSFER OF HUMORAL AND CELLULAR HEPATITIS B IMMUNITY BY ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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Background. **Previous data indicate that a transfer of specific humoral and cellular immunity by way of allogeneic hematopoietic cell transplantation (HCT) should, in principle, be possible.**

Methods. **In the HCT setting with a follow-up of up to 55 months, we studied the transfer of hepatitis B virus (HBV) specific immunity from electively immunized donors into HLA compatible recipients suffering from chronic myeloid leukemia (CML). After excluding preexisting HBV specific immunity in donor–recipient pairs, 27 prospective donors were vaccinated against HBV. In addition, on an average of 22 months postHCT, 8 of the 19 recipients were immunized once for HBV.**

Results. **Donor vaccination resulted in detectable hepatitis B surface (HBs) antibodies in 85% of donors and specific cellular in vitro responses in 77%. Two weeks postHCT, 86 and 67% of the recipients displayed positive humoral and cellular HBV reactions, respectively, which then decreased. Afterwards, HBV immunity reappeared in 83% of the recipients without revaccination. Following a single vaccination in recipients, seven of eight displayed a typical memory response. An HBV specific response was already detectable 1 week after vaccination, approximately 1,300-fold (humoral) and 60-fold (cellular) higher than observed in the corresponding donors after a single immunization.**

Conclusions. **The "spontaneous" recurrence of HBV immunity and the memory response in recipients give evidence for an elective immune transfer (e.g., for viral antigens) by way of allogeneic HCT.**

Allogeneic hematopoietic cell transplantation (HCT) results in a transfer of "naive" and specific memory cells. Our former studies in a preclinical model of the dog showed a successful transfer of functionally active B and T cells with memory toward recall antigens as facilitated by allogeneic HCT (*1*). In men, the feasibility of a transfer of humoral immunity (e.g., against tetanus toxoid or hepatitis B virus [HBV]) by stem-cell grafting or peripheral blood lymphocyte injection has also been demonstrated (*2–5*). In addition, a recent report on five allogeneic bone marrow recipients 2 to 3 years postgraft indicated the adoptive transfer of specific cellular HBV immunity (*6*). Furthermore, case reports about the clearance of chronic HBV infection (seroconversion to nondetectable HBV surface antigen [HBsAg]) in patients receiving bone marrow from an anti-HBs positive donor demonstrated that a transfer of hepatitis B immunity by way of HCT should, in principle, be possible (*4*, *7*, *8*). Conversely, Dhedin et al. (*9*) showed that in 4 of 30 bone marrow recipients, a previously cured HBV infection was reactivated when the recipient received a transplant from a nonimmune donor, whereas reactivation was observed in 0 of 7 patients who received grafts from an HBV immune donor.

In the present study, HBV immunity served as a model to follow the transfer of antibodies and cellular immunity from immunized donors to recipients through allogeneic HCT. Considering the concept that HCT recipients could be protected against infectious diseases when receiving grafts from an immune donor, we advised related donors of chronic myeloid leukemia (CML) patients to receive vaccinations recommended by the German advisory board for vaccination (STIKO) against tetanus, diphtheria, and poliomyelitis (*10*) and additionally against HBV. Because transplant recipients are at risk of acquiring a transfusion-related HBV infection and of reactivating a cleared HBV infection (*9*), an adoptive immunity against HBV could be beneficial to these patients.

Although HBs antibodies are generally determined with commercial enzyme-linked immunosorbent assay (ELISA), only a few studies describe the measurement of T–helper-cell responses against HBV (*6*, *11–16*). In those studies, recipients at least 2 years after bone-marrow transplantation (BMT) (*6*), patients after HBV infection (*11*), or HBV vaccinated probands (*12–16*) were investigated using various antigen preparations (recombinant HBsAg, preS2-S protein, and three protein monomers [S, preS1-S, preS2-S], or purified preS1-preS2-S protein). To our knowledge, cellular HBV immunity was never measured within the first 2 years after BMT (i.e., during a period when patients are highly immunosuppressed because of myeloablative chemoradiotherapy and prophylactic treatment against graft-versus-host disease [GvHD]), and it was not at all analyzed in grafted patients after peripheral blood hematopoietic cell transplantation (PBCT).

Here, we present data on 27 prospective donors and 19 CML graft recipients after BMT or PBCT, which indicate that a transfer of HBs antibodies and HBV specific T–helper-

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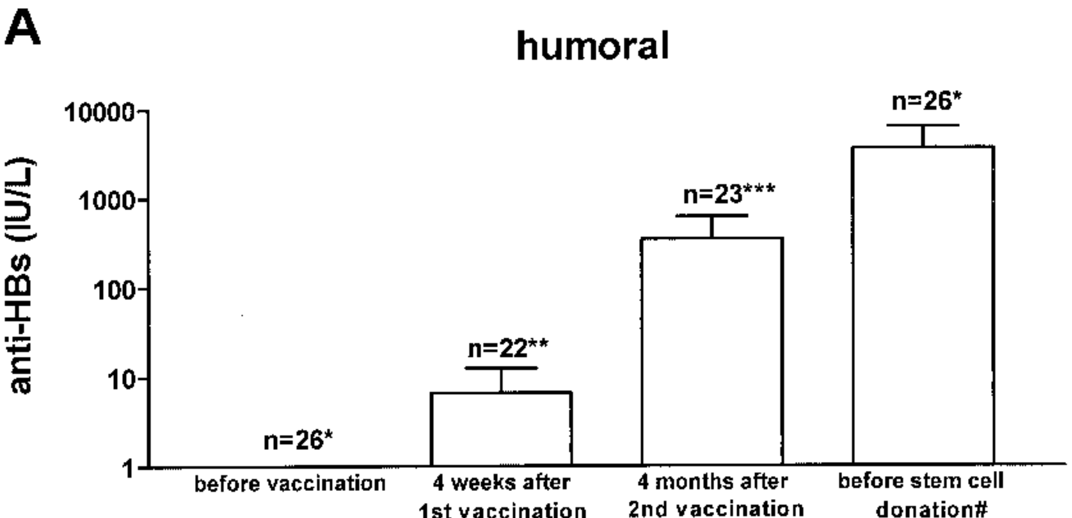
cell responses occurred immediately and in the long-term follow-up after HCT. On an average of 22 months postHCT, a typical memory response after a single HBV vaccination served as final proof for long-lasting adoptive immunity.

MATERIALS AND METHODS

Study Subjects

Tenty-seven HLA-compatible (*17*) related stem-cell donors tested before and after each HBV vaccination were included in this study, of whom 17 donors were HLA-identical siblings and 10 partially mismatched family donors. Mean age of donors (10 male, 17 female) was 39 (range 17–64) years. Donor candidates received three vaccinations at 0, 1, and 3 to 7 (mean 5) months (10 μg recombinant HBs antigen per dose, Gen H-B-Vax, Chiron Behring, Marburg, Germany). The corresponding 19 CML patients (8 male, 11 female, mean age 34 [21–57] years) were also tested for their HBV immunity before and after unmanipulated BMT and PBCT (n=6 and n=13, respectively). Follow-up of patients started 2 weeks after HCT and continued at monthly intervals. Eight of the transplanted patients were "booster" vaccinated once at a mean of 22 (15–31) months postHCT, followed by determination of HBV specific immunity 1 week after this single vaccination. In addition, immunity against tetanus and diphtheria was tested in pairs of vaccinated donors (Td-Rix, GlaxoSmithKline, München, Germany) and recipients before HCT and in recipients 2 to 4 weeks after HCT; immunity against poliomyelitis was not analyzed. For all investigations, we procured 10 mL serum and 20 mL heparinized blood from each donor and patient after informed consent.

Fourteen of the 19 CML patients were conditioned for HCT by total-body irradiation (10 Gy in four fractions) and cyclophosphamide, in 1 patient, combined with thiotepa. The remaining patients received a polychemotherapy regimen consisting of variable combinations of busulfan, cyclophosphamide, fludarabine, thiotepa, and



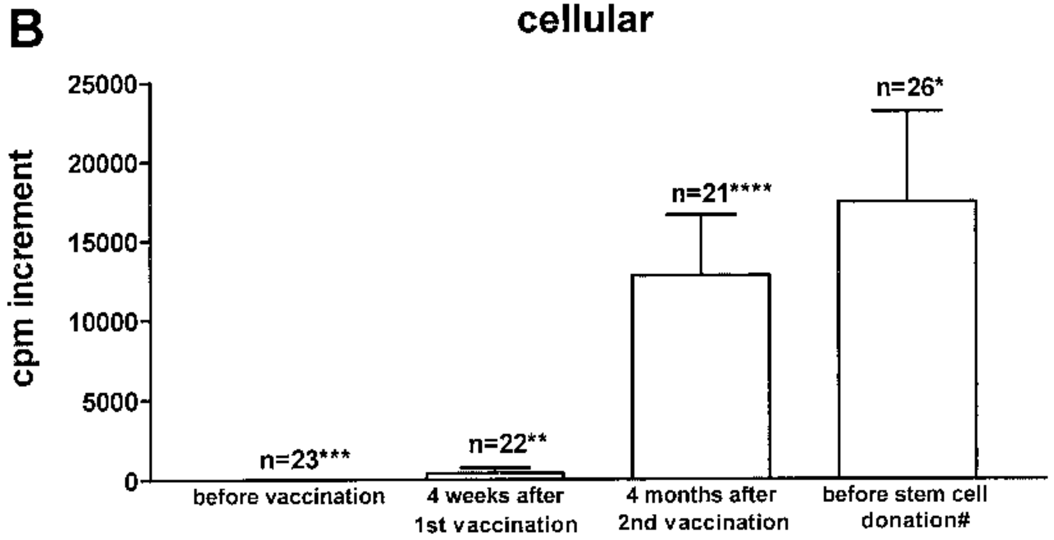


FIGURE 1. Development of humoral (A) and cellular (B) hepatitis B virus (HBV) immunity in stem-cell donors (n=27). Anti-hepatitis B surface (HBs) and HBV-specific counts per minute (cpm) increment values were determined before and after HBV vaccinations and given as mean±SEM. #Before stem-cell donation n=4 donors were vaccinated two, n=22 three, and n=1 four times against HBV. Not tested: *n=1, **n=5, ***n=4, and ****n=6.

antithymocyte globulin. As GvHD prophylaxis, all patients received cyclosporin A and methotrexate; 14 also received prednisone. They were treated with cyclosporin A (3 mg/kg body weight) immediately after HCT, and afterwards the dose was adapted to receive a serum trough level of 50 to 70 μg/L. Methotrexate was applied on day 1 (15 mg/m^2 body surface) and on days 3, 6, and 11 (10 mg/m^2 body surface); prednisone was administered initially at 2 mg/kg body weight.

Three CML patients died 2, 6, and 7 months postHCT, respectively, from multiorgan failure caused by acute GvHD, one patient from relapse 13 months postHCT. They were therefore lost from follow-up.

Determination of Humoral and Cellular HBV Immunity

For immune monitoring of stem-cell donors and recipients, the humoral HBV immunity was determined by an HBs antibody ELISA (Enzygnost Anti-HBs II, Dade Behring, Marburg, Germany), and positive reactions were defined as greater than or equal to 5 IU/L. In addition, before starting vaccination, the patients and donors were also tested for HBc antibodies and HBs antigen by ELISA (Enzygnost Anti-HBc monoclonal, and Enzygnost HBsAg 5.0, Dade Behring, Marburg, Germany) to exclude an acute or chronic HBV infection. The ELISA tests were performed according to the manufacturer's instructions.

Cellular HBV immunity was assessed by antigen-specific lymphocyte in vitro transformation using 50,000, 100,000, and 250,000 peripheral blood mononuclear cells (PBMC) per microtiter culture (0.2 mL). The HBV antigen was purified from the serum of an HBV infected donor (adw subtype) and contained all three surface proteins (L, M, and S) encoded by the preS1-preS2-S sequences at concentrations of 0.25, 0.5, and 1.0 μg/mL. The quality of purified HBs particles was analyzed by spectralanalysis and by immunoblotting with binding of specific monoclonal antibodies (MA18/7 for the preS1 epitope and Q19/10 for the preS2 epitope) as described previously (*18*). Cultures were set up as triplicates for 6 days. Separation of PBMC, cell culture conditions, and measurement of H3-TdR uptake

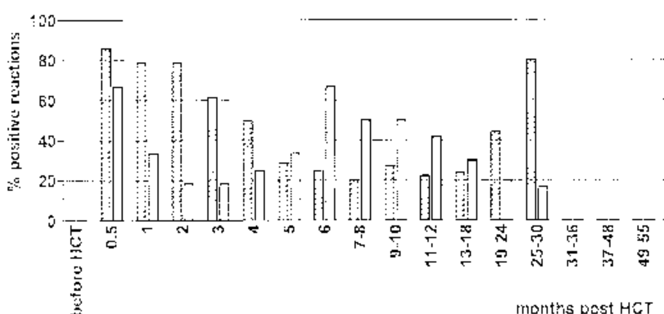


FIGURE 2. HBV immunity in 19 CML patients following allogeneic hematopoietic cell transplantation (HCT). (*bars*) Percentages of patients with positive humoral and cellular reactions (i.e., with an antibody titer ≥5 IU/L (*hatched*) or with a stimulation index ≥2.5 (*open*), respectively). Data after booster vaccination were excluded from this analysis and given separately.

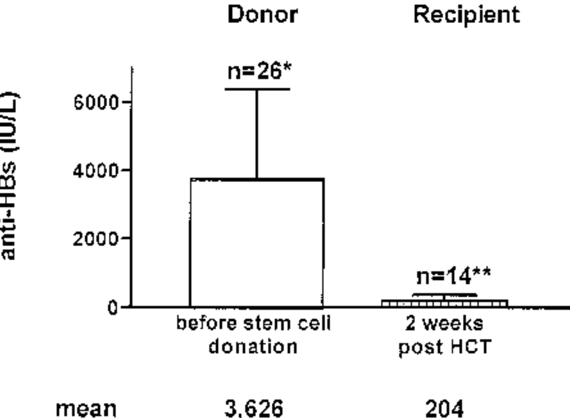
TABLE 1. Humoral and cellular hepatitus B virus (HBV) immunity in 19 stem-cell recipients

Months postHCT	Humoral[a] mean	SEM	Cellular[b] mean	SEM
0.5	204	169	3,724	1,093
1	164	105	7,329	5,988
2	145	90	5,905	5,249
3	23	13	812	545
4	12	6	3,118	2,082
5	6	4	25,742	16,947
6	6	6	33,861	28,772
7–8	3	2	25,399	16,511
9–10	15	13	17,048	9,189
11–12	17	12	12,284	7,685
13–18	5	3	11,274	8,233
19–24	93	59	2,644	1,843
25–30	132	77	136	136
31–36	0	0	0	0
37–48	0	0	0	0
49–55	0	0	0	0

[a] In 19 chronic myeloid leukemia (CML) patients receiving grafts from HBV-immunized donors, humoral HBV immunity is given as HBs antibody titer (IU/L).

[b] Cellular immunity as HBV specific lymphocyte proliferation (counts per minute [cpm] increment).

Values after HBV "booster" vaccination were excluded.



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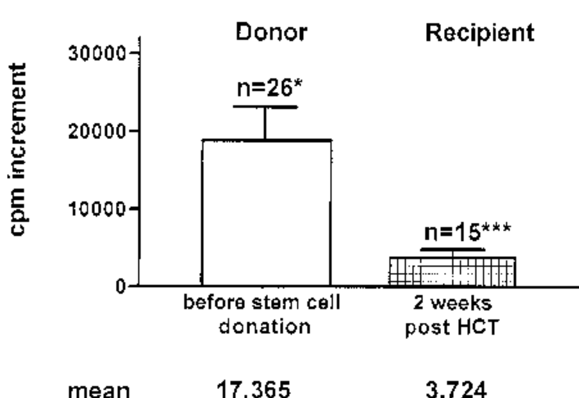
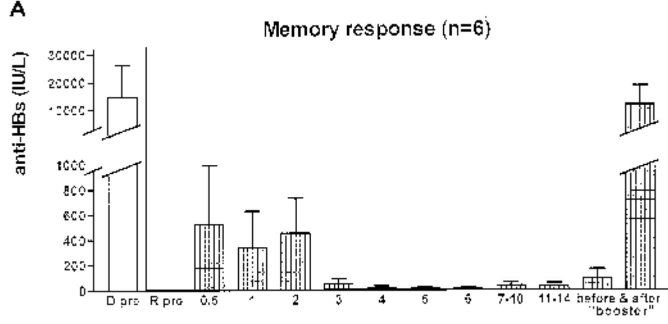
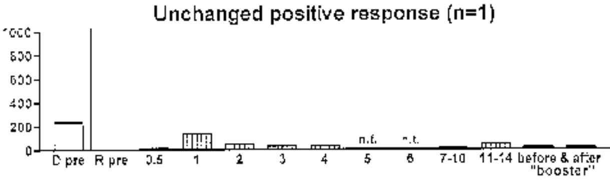
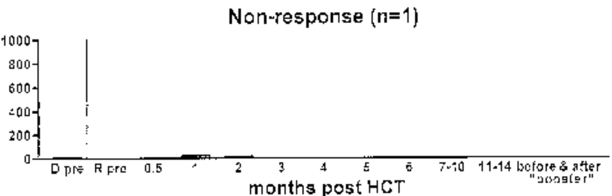


FIGURE 3. Transfer of humoral (A) and cellular (B) HBV immunity from electively immunized donors to stem cell recipients. Data are given as mean±SEM. Not tested: *n=1, **n=5, and ***n=4.

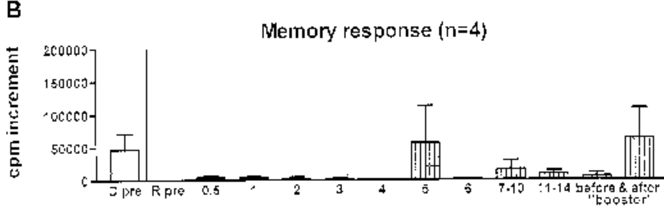
A

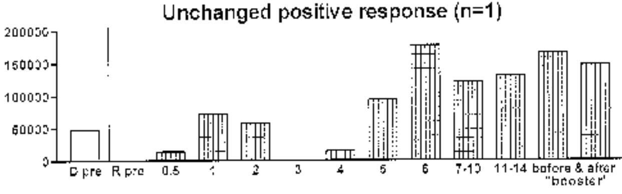






B





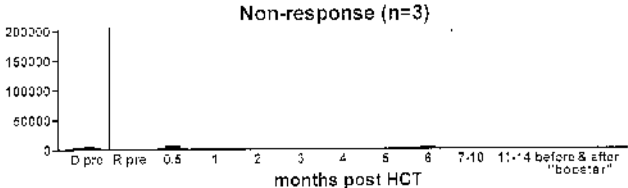


FIGURE 4. Humoral (A) and cellular (B) HBV immunity in eight CML patients booster vaccinated after HCT, with data for corresponding donors. Courses were grouped into those displaying typical memory response, an unchanged positive response before and after booster vaccination, and nonresponse following booster vaccination. Data represent mean±SEM. D pre, donor preHCT; R pre, recipient preHCT; n.t., not tested.

followed a protocol described previously (*19*). For evaluation, different amounts of PBMC and antigen concentrations were first studied in healthy HBV-immune probands. Median values of antigen stimulated cultures (triplicates) were divided by the median autologous values (stimulation index [SI]). The maximum of the SI values generated with different PBMC numbers and antigen concentrations was considered for the classification of positive or negative (SI<2.5) cellular response against HBV. In addition, for the quantitative analysis of cellular reactions, the second highest H3-TdR uptake value for HBV specific and autologous cultures was chosen. Increment counts per minute (cpm) were generated as HBV-specific minus autologous (unstimulated) proliferation.

Determination of Humoral and Cellular Immunity Against Tetanus and Diphtheria

In donor–recipient pairs, humoral immunity against tetanus and diphtheria was determined by ELISA (Tetanus-Toxoid IgG and Diphtheria-Toxin IgG, IBL Hamburg, Germany), and cellular immunity was determined using standardized assay formats (LT2-antigen, No. 7051, BAG, Lich, Germany) as described previously (*19*).

RESULTS

After the exclusion of preexisting humoral or cellular HBV immunity in donor-recipients pairs, the stem-cell donors were analyzed 4 weeks after their first vaccination, 4 (2–6) months after the second vaccination, and before stem-cell donation (in four donors after two HBV immunizations, in 22 after three, and one after four HBV immunizations). As shown in Figure 1, anti-HBs titers following vaccinations were 7±6 IU/L (mean±SEM), 346±271 IU/L, and 3,626±2,755 IU/L, respectively. Humoral reactions were considered as positive in 2 of 22 (9%), 18 of 23 (78%), and 22 of 26 (85%) donors after vaccination. Following immunizations, cellular reactions quantified as cpm increment values in donors were 426±307, 12,757±3,803, and 17,365±5,748, corresponding to SI values of 2.1±0.3, 5.6±1.5, and 8.8±2.8, respectively (mean±SEM). Specific proliferative in vitro responses could be detected in 3 of 22 (14%), 12 of 21 (57%), and 20 of 26 (77%) measurements.

Interestingly, among the four donors with humoral nonresponse, one displayed the extended HLA haplotype B8, DRB1*0301, DQB1*0201 and another B44, DRB1*0701, DQB1*0202—both known to be associated with a poor or nonresponse to HBV vaccines (*20, 21*). In contrast, we did not observe any obvious association of HLA haplotypes with cellular donor immunity or recipient immunity toward HBV.

In the follow-up of 19 CML patients for up to 55 months postHCT, we observed an initial (passive) transfer of humoral and cellular HBV immunity with detectable HBs antibodies in 12 of 14 (86%) and specific proliferative responses in 10 of 15 (67%) of the recipients 2 weeks postHCT, respectively (Fig. 2). In consideration of only those patients grafted from donors with positive HBV status, similar data were obtained for humoral and slightly increased values for cellular HBV immunity (82 and 73% of recipients displayed positive HBV reactions, respectively). Although a minimum percentage of recipients with positive HBs antibody titers was reached 8 months postHCT, the percentage of positive T–helper-cell responses against HBV declined up to month 2. Thereafter, starting 4 months postHCT and ending a maximum 6 months postHCT, a recurrence of cellular immunity (67% of patients showed specific proliferative responses at

month 6) could be observed without vaccination. Altogether, humoral or cellular HBV immunity "spontaneously" reappeared in 83% of recipients. Six months postHCT, the dose of prednisone was reduced to 19% of the starting level (166±6–32±7 mg/d) and of cyclosporin A to 26% (250±9–64±11 mg/d [mean±SEM]). Absolute levels of HBV immunity in the postHCT period displayed similar kinetics as observed for the percentage of positive reactions (Table 1). Comparing CML patients after BMT and PBCT, we did not observe a significant difference in humoral or cellular HBV immunity. Furthermore, courses of humoral and cellular HBV immunity were comparable in patients grafted from HLA-identical sibling donors (n=10) and from partially mismatched family donors (n=9). In addition, we could not detect a significant influence of the conditioning regimen on HBV immunity in stem-cell recipients (data not shown).

Analyzing mean anti-HBs titers and cpm increment values in donors and recipients, those in recipients at 2 weeks postHCT were 6% and 21% of donors, respectively (Fig. 3). A single vaccination against HBV in 8 of 19 patients on an average of 22 months postHCT resulted in a humoral or cellular immune response as is characteristic for a booster vaccination in 7 patients (Fig. 4). In greater detail, humoral memory response was observed in 6 of 8 (Fig. 4A) and cellular response in 4 of 8 recipients (Fig. 4B). In addition, 1 of 8 of the responses were positive before and after booster vaccinations (different individuals displayed unchanged humoral and cellular HBV immunity), whereas a nonresponse was observed in 1 of 8 and 3 of 8 recipients (humoral and cellular, respectively). HBV memory responses in recipients were already detectable 1 week after vaccination. Mean values for the eight recipients were approximately 1,300-fold (humoral) and 60-fold (cellular) higher than observed in the corresponding healthy donors after a single vaccination.

Humoral and cellular HBV immunity was positively correlated in donors preHCT (Spearman test $r=0.4$, $P=0.04$). But, in contrast, no significant results were obtained for recipients, presumably because the cohort was too small. Furthermore, correlation analysis was performed to compare the level of HBV immunity in donors and corresponding recipients 2 weeks postHCT. Although HBs antibody titers were significantly correlated ($r=0.6$, $P=0.03$), we did not observe similar results for cellular HBV immunity.

In extension of the findings on HBV, in some cases, an immune transfer could also be observed for tetanus and diphtheria: with humoral immunity, 2 and 4 of 11 tested donor-recipient pairs were informative, respectively. Here, donors displayed good immunity (>0.5 IU/mL for tetanus and >0.1 IU/mL for diphtheria) (*22*, *23*), whereas recipient antibody titers were below these limits. In all of those cases, a transfer of immunity (defined as an increase in recipient antibody titers) was shown (mean antibody titers [IU/mL] against tetanus and diphtheria: donors before HCT 4.5/0.7, recipients before HCT 0.25/0.03, and recipients 2 weeks after HCT 3.2/0.6, respectively]. Furthermore, cellular immunity against tetanus and diphtheria was informative in 3 and 2 of 15 tested pairs (tetanus–diphtheria: cpm increment value in donors >20,000/4,000, in recipients negative [SI<2.5]). In 2 of 3 pairs, a transfer of cellular immunity against tetanus was found, and in 1 of 2, a transfer of immunity against diphtheria was found (mean cpm increment value for tetanus–diphtheria: donors before HCT 87,065/7,256; recipients before HCT 0/0; and recipients 2–4 weeks after HCT 12,654/625, respectively).

DISCUSSION

Our data clearly demonstrate the successful transfer of humoral and cellular HBV immunity from vaccinated donors to recipients. Furthermore, some preliminary data indicate a transfer of immunity against tetanus and diphtheria by way of HCT. We investigated a homogeneous group of 19 CML graft recipients and started the monitoring of cellular HBV immunity 2 weeks postHCT. Compared with previous data (*6*), our study gives additional information on the early postHCT phase (i.e., the initial passive transfer of immunity and the recurrence of cellular immunity after the reduction of immunosuppressive therapy starting from month 4 and displaying a maximum value at month 6 postHCT). It cannot be concluded from our data to what extent the decline in HBV responses after initial detection is attributable to the disappearance of transferred antibodies and HBV specific T-helper cells or to an altered immune function caused by heavy immunosuppression. Furthermore, this study includes 13 patients after grafting by PBCT, a condition not previously studied for cellular HBV immunity, and shows that transferred humoral and cellular HBV immunity was comparable with that of BMT patients.

The currently presented results indicate the establishment of a highly sensitive assay for HBV-specific cellular immunity even in patients receiving grafts that were severely immunocompromised because of conditioning therapy (total body irradiation with 10 Gy, polychemotherapy) and GvHD prophylaxis (cyclosporin A, methotrexate, prednisone). In our study, cellular HBV immunity in recipients 2 to 3 years postHCT was comparable with data published by Ilan et al. (*6*), who analyzed five BMT patients. No previous data on cellular immunity are available for an earlier phase of postHCT. Furthermore, in 27 stem-cell donors, cpm increment or SI values were comparable with previously published data (*12–16*).

Some groups studied the correlation of humoral and cellular HBV immunity. Min et al. (*15*) described a weak correlation between T-cell responses to HBsAg and antibody titers following HBV vaccination, and Leroux-Rouels et al. (*12*) observed a significant correlation when analyzing humoral and cellular immunity dichotomously (i.e., high vs. low responder and proliferation vs. non-proliferation, respectively). In contrast, no correlation was reported by McDermott et al. (*16*), in agreement with Degrassi et al. (*24*), because HBsAg-responsive T and B cells display different kinetics of recirculation in the peripheral blood. Our own results are in accordance with Min et al. (*15*) and Leroux-Rouels et al. (*12*) because we obtained a weak but significant correlation between humoral and cellular HBV specific immune responses in donors.

As a final proof of a functioning HBV immunity in patients receiving grafts from an HBV vaccinated donor, we immunized 8 of 19 recipients once on an average of 22 (15–31) months postHCT. This interval after transplantation was chosen because a previous study focusing on tetanus immunity showed that vaccinations could induce protective antibody levels 6 and 18 months postBMT (*25*). When comparing HBV immunity in the recipients and donors after their first HBV immunization, the results demonstrate a much stronger humoral and cellular HBV immunity in the patients. The

appearance of HBs antibodies and the specific recognition of HBV antigen just 1 week after a single immunization and the strength of this immune response both demonstrate the adoptive transfer of humoral and cellular immunity. In principle, stem-cell donor immunization offers the opportunity to perform an elective immune transfer (for other viral antigens as well) by way of allogeneic HCT.

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Adoptive Immune Transfer of Hepatitis B Virus Specific Immunity From Immunized Living Liver Donors to Liver Recipients

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Background. Liver transplantation is often the ultimate option of therapy for chronically hepatitis B virus (HBV) infected patients. Prevention of reinfection is therapy intensive and cost-effective. Adoptive transfer of HBV-specific immunity with the liver from an immune living liver donor (LLD) could be a new approach to prevent reinfection.
Methods. Forty-six potential LLDs were vaccinated against HBV. Humoral (antibodies to hepatitis B virus surface antigen [anti-HBs]-titer) and cellular (IFN-γ-ELISpot and proliferation-assay) immune responses were examined in donors after immunization and in recipients before and after transplantation.
Results. Anti-HBs-titers of up to 50,000 IU/L were detected in LLDs. Fourteen recipients received livers from these donors. We detected humoral immunity in one HBV-naïve recipient and in one chronically HBV-infected recipient after transplantation. A transfer of cellular immunity (SI>3) was seen in three recipients. These patients received livers from donors with high anti-HBs-titers of more than 9000 IU/L. Cellular immunity was also detected in the corresponding donors (SI >3 and spots >22).
Conclusions. Our study demonstrates that HBV-specific humoral and cellular immunity can be transferred by liver transplantation after vaccination of the donors. The transfer of B-cell and T-cell immunity correlates with the magnitude of immune responses in the donor.

Keywords: Living liver transplantation, HBV, Adoptive immune transfer.

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Approximately 350 million people are chronically hepatitis B virus (HBV)-infected worldwide. Two thirds of them will probably develop liver cirrhosis resulting in liver failure or hepatocellular carcinoma (HCC) (*1*, *2*). In those patients liver transplantation (LTX) is the last therapeutic option. Reinfection of the transplanted liver, however, is associated with poor long-term survival (*3*). The implementation of long-term, high-dose, passive immunoprophylaxis using HBV immunoglobulin (HBIg) has reduced the reinfection rates (*3–5*). Combination of HBIg with nucleoside analogues is even more effective (*6*, *7*). This therapy, however, has to be applied life-long and is cost-intensive. The transfer of HBV-specific immunity by transplantation of liver grafts from immunized or immune donors would be a promising way to prevent HBV reinfection after LTX.

Adoptive transfer of HBV immunity has already been observed in humans after bone marrow transplantation (*8–11*). The transfer of HBV-specific immunity by LTX has been already shown in animal models. After transplantation of the liver, heart, or kidney of immunized rats to naïve recipients, HBV-specific humoral immunity was transferred (*12*). Further, a protective immune transfer was shown after LTX from immunized donors to chronically hepadnavirus-infected recipients in woodchucks (*13*). Transplanting the livers of immune donors resulted in a delay of reinfection and a reduction of viral load. Recently, it has been shown that humoral immunity can be transferred to chronically HBV-infected patients by transplantation of the liver from immune donors (*14*, *15*). These promising results encouraged us to examine the HBV-specific adoptive immune transfer in the clinical setting of LTX from living liver donors (LLDs) vaccinated against HBV.

Hepatitis B virus vaccines of the second generation are produced in yeast and contain only the recombinant small surface (S) protein of HBV (*16*). These vaccines have been shown to be immunogenic. Approximately 5% to 10% of vaccines, however, are so called "non-responders" (*17*). In such persons protective immunity can often be induced with vaccines containing a new immunostimulating adjuvant or third generation vaccines (*18–20*). The third generation vaccines contain not only the S protein, but also the middle (M, PreS2) and the large (L, PreS1) surface proteins, and have been shown to induce earlier and stronger humoral and cel-

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lular immunity. Recently, we have established a short-term immunization protocol to be used in LLDs (*21*).

Livers from LLDs immunized against HBV were transplanted to 14 patients with chronic liver disease. Transfer of humoral and, for the first time, of cellular HBV immunity was observed in 3 of 14 donor/recipient pairs.

MATERIALS AND METHODS

Human Subjects

This study was approved by the Institutional Review Board. All donors and recipients provided written informed consent.

Donors

Potential LLDs (n=46) received immunizations with Sci-B-Vac (n=39), a third generation vaccine (kindly provided by SciGen Ltd., Singapore, Singapore) or booster immunizations with HBVAXPRO (n=7) (Sanofi Pasteur MSD, Frankfurt, Germany). The immunization protocol for HBV-naïve patients (hepatitis B virus surface antigen [HBsAg], antibodies to hepatitis B virus core antigen [anti-HBc], and antibodies to hepatitis B virus surface antigen [anti-HBs] negative) included four biweekly injections of Sci-B-Vac (20 μg). This protocol was established in healthy volunteers previously (*21*). In 29 potential donors, immunization was not completed because they were excluded from donation or the liver had been transplanted before the course was finished. In one recipient the date of the surgery allowed a fifth immunization to increase a so far poor immune response. Six recipients who had already been immunized earlier received booster immunizations of HBVAXPRO at the recommended dosage of 10 μg. Donor 3 presented with a history of immunization against HBV, however, showed no anti-HBs-titer. Therefore, we decided to start a new immunization course with Sci-B-Vac.

Humoral and cellular immunity in donors were determined by anti-HBs-titer, proliferation-assay, and IFN-γ-ELISpot before each and 4 weeks after the last immunization and in 14 LLDs (Table 1) additionally pretransplant.

Recipients

In recipients (n=14) (Table 1), the HBV-specific humoral and cellular immune responses were measured pre- and posttransplant at least monthly. They were immunized with HBVAXPRO at month 12 posttransplant. To prevent rejection, recipients were treated with various combinations of cyclosporin A, tacrolimus, mycophenolate mofetil, and prednisone. Eleven recipients were HBV-naïve pretransplant (HBsAg, anti-HBc, and anti-HBs negative). Three chronically HBV-infected patients (HBsAg and anti-HBc positive, and anti-HBs negative pretransplant) received HBIg prophylaxis (Hepatect, Biotest, Dreieich, Germany). Hepatitis B immunoglobulin was administered intravenously using a previously described protocol (*22*). The combination of this medication with the nucleoside analogue lamivudine was indicated, if HBV-DNA showed high levels pretransplant or was detectable at all posttransplant.

The data of one exemplary chronically HBV-infected patient who received the liver from an HBV-naïve donor were included to compare the course of the anti-HBs-titer of this patient posttransplant with that of recipient 3.

Hepatitis B Virus Specific Serology

Hepatitis B virus surface antigen, anti-HBc, hepatitis B virus e-antigen (HBeAg), and anti-HBs were measured by Chemiluminescent-Microparticle-Immunoassay (Architect-System, Abbott Laboratories, Wiesbaden, Germany) following the manufacturer's instructions. The detection limit of the anti-HBs-titer is 10 IU/L.

Transferred Antibodies to Hepatitis B Virus Surface Antigen

Recipient 3 received 1000 IU anti-HBs (HBIg) per day intravenously for 21 days. These antibodies were diluted in approximately 3.5 L plasma of a recipient weighing 75 kg and resulted in an anti-HBs-titer of 286 IU/L. Additionally, the recipients required fresh frozen plasma (FFP) during the surgery that contained small amounts of anti-HBs. The portion of the HBIg- and FFP-derived antibody titer was calculated considering an anti-HBs half-life of 21 days (*23*) and using generally approved formulas (*24*). This calculation seems to be accurate enough to obtain the expected HBIg- and FFP-derived anti-HBs-titers.

Hepatitis B Virus Specific Cellular Immune Responses

Peripheral blood mononuclear cells were separated by density gradient centrifugation. Cell culture conditions and measurement of lymphocyte proliferation (proliferation-assay) and IFN-γ production (ELISpot) followed a protocol described previously (*21*). Values of SI more than 2.5 were defined as positive. IFN-γ production in the ELISpot was analyzed by automated image recognition and a value of more than or equal to 10 spots (HBV-specific minus autologous response) was considered as positive.

Qualitative and Quantitative Detection of Hepatitis B Virus DNA

Hepatitis B virus-DNA was extracted from 200 μL of serum using QIAamp-blood-kit (Qiagen GmbH, Hilden, Germany). The qualitative HBV-DNA detection was performed with the Artus-HBV-LC-PCR-Kit (Qiagen GmbH) using the LightCycler 1.2 instrument (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. Here, the detection limit is 100 IU/mL. The quantification was performed with the VERSANT-HBV-DNA-3.0-Assay (bDNA) (Siemens, Fernwald, Germany) on the BAYER-VERSANT-440-MOLECULAR-SYSTEM (Siemens) following the manufacturer's instructions. The detection limit of HBV-DNA is 357 IU/mL.

Determination of Hepatitis B Virus Subtype by Polymerase Chain Reaction

Hepatitis B virus-DNA was extracted from serum and subjected to polymerase chain reaction (PCR) amplification for the HBV genome region comprising the S gene (nt 131-845, numbering according to the sequence of AY220698) as described (*25*).

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TABLE 1. Donor and recipient characteristics in living donor liver transplantation

No.	Donor age[a] (yrs)	Gender	Race	HBV status[a]	Anti-HBs (IU/L) pre-LDLT	Immunizations	Recipient age[a] (yrs)	Gender	Race	HBV status	Anti-HBs (IU/L) pre-LDLT and week 2 post-LDLT		HBIg	Underlying disease	Outcome
1	25	M	White	Immunized	57,500	1	55	F	White	Naïve	0	1800	−	Cryptogenic liver cirrhosis	Alive
2	48	F	White	Naïve	9831	4	49	F	White	Naïve	0	10	−	Multiple liver abscesses	Alive
3	30	F	White	Immunized	22,200	3	58	M	White	Chronic	0	43,040	+	Chronic HBV infection and HCC	Dead
4	32	M	White	Naïve	410	3	58	M	White	Naïve	0	21	−	Cryptogenic liver cirrhosis	Alive
5	52	M	White	Naïve	56	4	28	M	White	Naïve	0	0	−	Primary sclerosing cholangitis	Alive
6	43	F	White	Naïve	44	4	44	M	White	Naïve	0	20	−	Secondary biliary cirrhosis	Alive
7	38	F	White	Naïve	928	3	64	F	White	Naïve	0	13	−	Primary biliary cirrhosis	Alive
8	22	M	White	Naïve	6275	5	52	M	White	Naïve	0	26	−	Secondary biliary cirrhosis	Alive
9	28	M	White	Naïve	445	3	63	M	White	Naïve	0	0	−	Ethyl toxic liver cirrhosis	Alive
10	25	M	White	Naïve	169	3	51	M	White	Naïve	0	0	−	Multifocal HCC (no HBV infection)	Alive
11	42	F	White	Naïve	79	4	42	F	White	Naïve	0	11	−	Multifocal hemangioendothelioma	Dead
12	27	M	White	Naïve	32	1	50	M	White	Naïve	0	0	−	Chronic HCV infection and HCC	Alive
13	21	F	White	Naïve	615	3	39	F	White	Chronic	0	153	+	Chronic HBV infection	Alive
14	23	M	White	Naïve	578	4	59	M	White	Chronic	0	680	+	Chronic HBV infection	Alive

Recipient 1–3: presenting adoptive immune transfer. Recipient 4–14: no adoptive immune transfer detected.
[a] Status before donor immunization.
LDLT, living donor liver transplantation; Anti-HBs, antibodies to hepatitis B virus surface antigen; HBIg, hepatitis B immunoglobulin; HCC, hepatocellular carcinoma.

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Microchimerism Analysis

In donor/recipient pair 1 microchimerism posttransplant was analyzed using quantitative real-time–PCR of Y-chromosome-specific sequences as published (*26*).

RESULTS

Donors

In total, 46 potential LLDs were immunized with HBV vaccines (Sci-B-Vac or HBVAXPRO). Humoral and cellular immune responses were measured in all donors after the last immunization (Fig. 1). The number of immunizations differed from 1 to 5 because of the time available before transplantation or the time-point of exclusion from donation. Seven donors received one booster immunization with HBVAXPRO because of pre-existing vaccine-derived HBV immunity. One donor received three booster immunizations using Sci-B-Vac before donation because no anti-HBs antibodies were detectable despite two earlier immunizations.

The humoral immunity was measured in all potential donors 2 to 3 weeks after immunization (Fig. 1A). In the group of LLDs, who received only one immunization with Sci-B-Vac (n=11), four showed detectable anti-HBs-titers of up to 189 IU/L. In LLDs immunized twice anti-HBs antibodies were detected in four of the seven donors with up to 825 IU/L. After three immunizations, anti-HBs antibodies were present in all LLDs (n=11) with a peak of 2069 IU/L. Seven of the fourfold immunized donors (n=8) displayed anti-HBs-titers between 44 and 9831 IU/L. A single donor, who received five immunizations, showed an anti-HBs-titer of 6275 IU/L. A single booster immunization with HBVAXPRO (n=7) resulted in higher anti-HBs-titers of up to 61,265 IU/L. The Sci-B-Vac boosted donor displayed an anti-HBs-titer of 22,203 IU/L. As expected, the strength of humoral immunity increased with the number of vaccinations. Groups of donors who received booster immunizations showed higher immune responses compared with those without previous immunizations.

The cellular immune responses were detected using proliferation-assay and IFN-γ ELISpot. In donors (n=11), who received one immunization with Sci-B-Vac, three displayed cellular immunity in the proliferation-assay (SI of up to 11.9) and one in the ELISpot (70 spots). Seven donors who were immunized twice displayed comparable results. Two showed lymphoproliferative responses (SI of up to 44.6) and

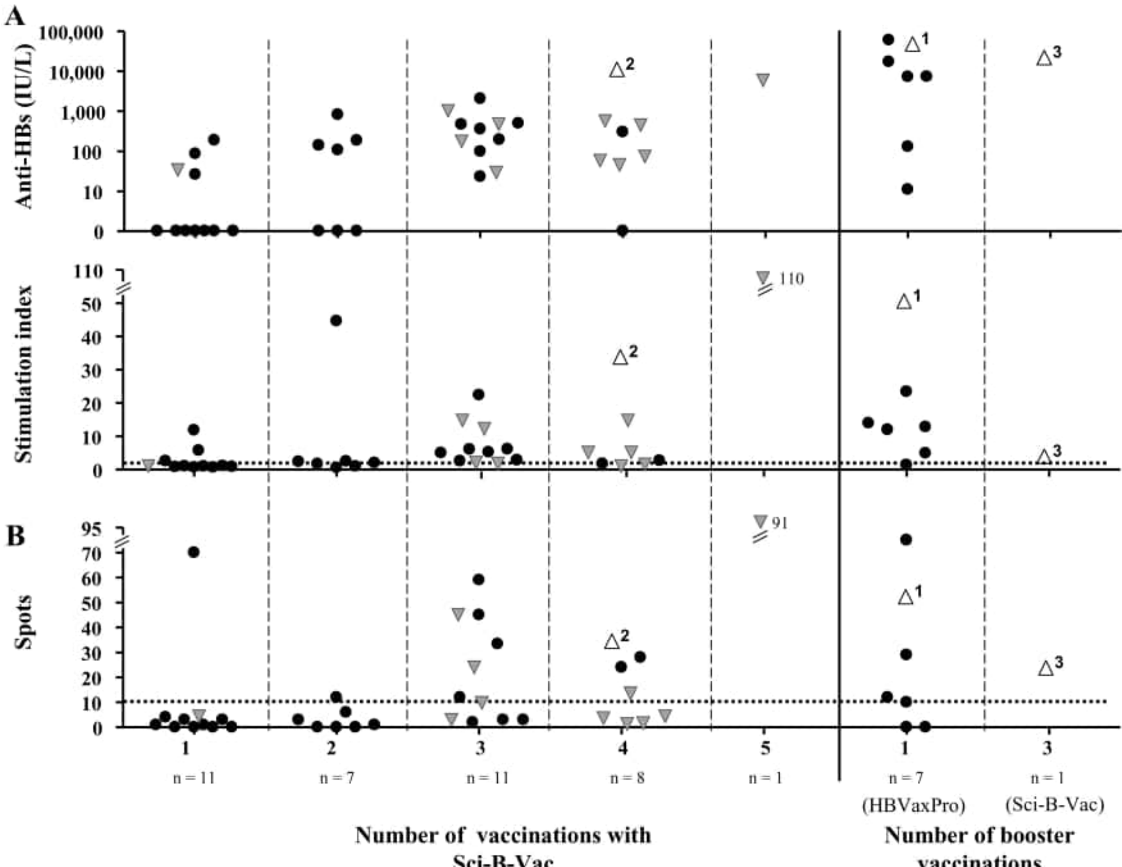

FIGURE 1. Humoral and cellular immune responses of 46 potential living liver donors (LLDs). The x-axis indicates the number of vaccinations or booster-vaccinations. Thirty-two donors (●) were excluded from transplantation because of medical or psychological reasons. Fourteen donors (△ ▼) donated a part of their liver to the corresponding recipients. Values in donors one to three who transferred their hepatitis B virus (HBV)-specific immunity are depicted as *hollow triangles* with numbers 1–3. (A) Antibodies to hepatitis B virus surface antigen (anti-HBs)-titers in the LLDs before transplantation or after the last immunization, (B) cellular immune response in the proliferation-assay, and (C) in the IFN-γ-ELISpot. The horizontal lines indicate the cut-off (stimulation index of 2.5 or 10 spots, respectively).

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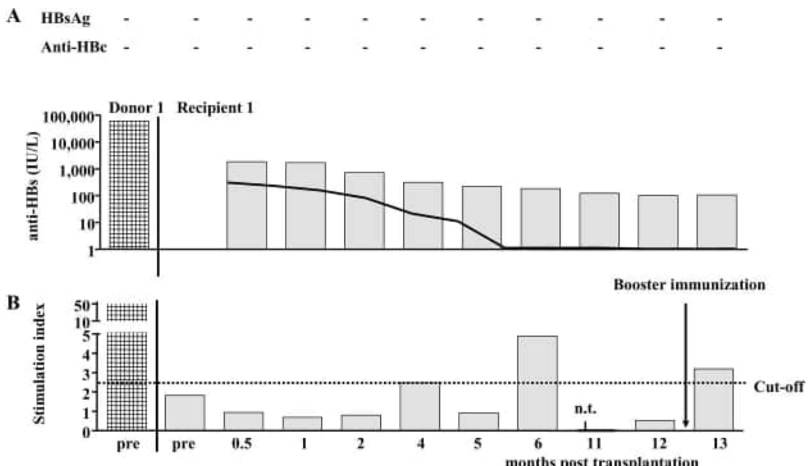

FIGURE 2. Donor/recipient pair 1: Hepatitis B virus (HBV)-specific immunity of the donor pretransplantation and the recipient pre- and posttransplantation. The x-axis indicates time points of analysis. (A) The y-axis shows anti-HBs titers in IU/L of the donor (▦) and the HBV negative recipient (▭). The curve indicates the half-life value time of antibodies to hepatitis B virus surface antigen (anti-HBs) derived from blood products during the transplantation. Negative (−) values for hepatitis B virus surface antigen (HBsAg) and antibodies to hepatitis B virus core antigen (anti-HBc) detection are indicated at the top. (B) The y-axis shows the ratio between stimulated and unstimulated proliferations (stimulation index) of the donor and the HBV negative recipient using L-HBsAg as stimulus. The horizontal line indicates the cut-off (stimulation index of 2.5). n.t.: not tested.

one displayed 12 spots. After three immunizations the cellular immune response increased: 10 of 11 donors showed cellular immunity in the proliferation-assay (SI of up to 22.4) and seven in the ELISpot (up to 103 spots). In fourfold immunized donors (n=8) cellular immune responses were detected in five in the proliferation-assay (SI of up to 32.5) and four displayed up to 34 spots in the ELISpot. A single donor who received five immunizations displayed the highest cellular immune response (SI=110.2 and 91 spots). Seven donors who received one booster immunization with HBVAXPRO also displayed high SI values of up to 23.4 and 75 spots. The 3-fold booster vaccinated (Sci-B-Vac) donor showed lower cellular immunity with an SI of 3.3 and 22 spots. To summarize, the magnitude of cellular immune responses correlated with the number of administered vaccinations.

Fourteen of the potential LLDs donated a part of their liver, whereas 32 were excluded from transplantation because of medical or psychological reasons. Three donors showing anti-HBs-titers more than 9000 IU/L (Fig. 1A) transferred their HBV-specific immunity to the recipients, although donors showing lower anti-HBs-titers did not. All three donors with high antibody titer showed cellular immunity (Fig. 1B and C). Pretransplant SIs of 3.3, 32.5, and 50 were detected in proliferation-assays and 22, 34, and 52 spots in the ELISpot. For three donor/recipient pairs presenting with an adoptive immune transfer the course is described below.

Donor/Recipient Pair 1

In January 2006, a male donor (25 years) was evaluated for living liver donation for his HBV-naive mother (55 years). She received the liver because of cryptogenic liver cirrhosis. The donor had already been immunized against HBV and showed an anti-HBs-titer of 10,413 IU/L. After one booster immunization with HBVAXPRO an increase of the anti-HBs-titer upto 57,500 IU/L was detected pretransplant (Fig. 2A).

Two weeks after transplantation an anti-HBs-titer of 1800 IU/L was detected in the recipient. This could partly be due to low amounts of antibodies in the FFPs received during surgical treatment. To demonstrate that antibodies were transferred with the liver and produced by B cells of the donor we calculated the amount of anti-HBs that was passively transferred (380 IU/L). The decrease of these antibodies based on the half-life time of 21 days is visualized in Figure 2(A). The amount of antibodies measured in the recipient clearly exceeds this value and can, therefore, be attributed to anti-HBs antibodies transferred to or produced by B cells of the recipient. An anti-HBs-titer of 100 IU/L in the recipient 1 year after transplantation suggests that anti-HBs was actively produced in this patient because passively transferred anti-HBs antibodies are unable to persist over such a long period. A booster immunization with HBVAXPRO at month 12 after transplantation induced no increase in the antibody titer so far.

In addition, the recipient showed cell proliferation after stimulation with L-HBsAg (SI of 4.9) at month 6 after transplantation, at month 12 proliferation was no longer detectable. A booster immunization at this time-point induced again an HBsAg-specific cell proliferation (SI of 3.1, month 13) (Fig. 2B). ELISpot results were negative in the recipient after transplantation (data not shown).

To clarify the origin of HBV-specific cells, we investigated the presence of microchimerism. In donor/recipient pair 1, cells of the male donor were not measurable by Y-chromosome-specific PCR in the peripheral blood mononuclear cells of the female recipient 1, 1-year after transplantation.

To summarize, in this HBV-naïve recipient who received a part of the liver from an immunized donor we de-

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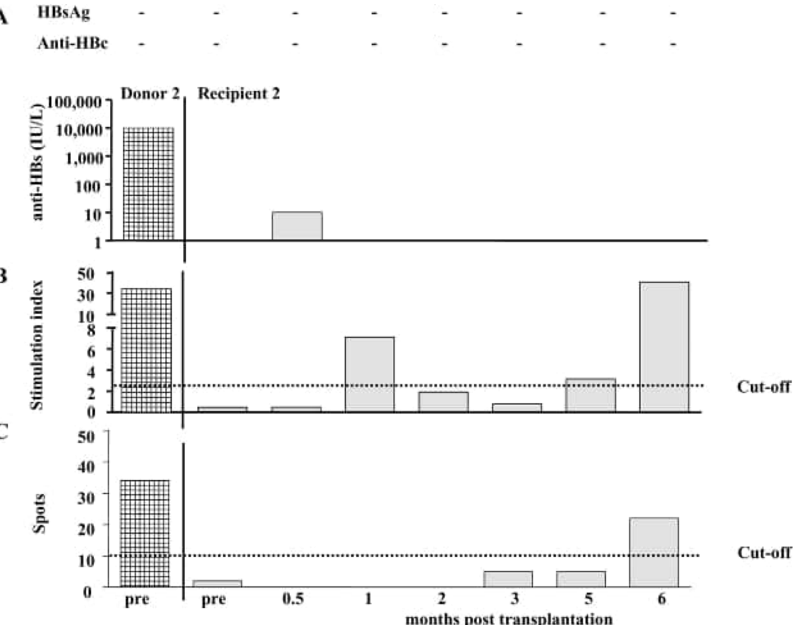

FIGURE 3. Donor/recipient pair 2: Hepatitis B virus (HBV)-specific immunity of the donor pretransplantation and the recipient pre- and posttransplantation. The x-axis indicates time points of analysis. (A) The y-axis shows antibodies to hepatitis B virus surface antigen (anti-HBs) titers in IU/L of the donor (▦) and the HBV negative recipient (▭). Negative (−) values for hepatitis B virus surface antigen (HBsAg) and antibodies to hepatitis B virus core antigen (anti-HBc) are indicated at the top. (B) The y-axis shows the ratio between stimulated and unstimulated proliferation (stimulation index) of the donor and the HBV negative recipient using L-HBsAg as stimulus. The horizontal line indicates the cut-off. (C) The y-axis shows the production of IFN-γ (spots) measured by ELISpot in donor and recipient after incubation with the L-HBsAg. The horizontal line indicates the cut-off (10 spots).

tected the transfer of HBV-specific humoral and, for the first time, of cellular immune responses.

Donor/Recipient Pair 2

In September 2006, a female HBV-naïve LLD (48 years) was evaluated for living liver donation for her HBV-naïve sister (49 years). She received the graft because of multiple liver abscesses. The donor was immunized with Sci-B-Vac in a short-time immunization protocol (four injections in 2 weeks intervals) and an anti-HBs-titer of 9831 IU/L was observed pretransplant (Fig. 3A).

The anti-HBs-titer measured at week 2 after transplantation (10 IU/L) was likely due to low amounts of antibodies in blood products given during transplantation. Subsequent samples of this patient were anti-HBs negative (Fig. 3A). Already 1 month after transplantation, however, the recipient showed lympho-proliferation after stimulation with the L-HBsAg (SI of 7.1) (Fig. 3B). Again, positive results were observed at 5 months (SI of 3.1) and 6 months (SI of 40) after transplantation. Further, a transfer of cellular immunity was demonstrated by IFN-γ-ELISpot at month 6 (22 spots) (Fig. 3C).

Despite high anti-HBs-titer in the donor, only cellular immunity was measurable in the recipient because of the transfer of HBV-specific T cells with the liver. The absence of the humoral immunity could be caused by a medication with rituximab pretransplant and several plasmapheresis. This therapy was necessary to overcome blood group incompatibility of the donor and recipient. Several examinations by flow cytometry have shown that no or extremely low numbers of B cells persisted in this recipient (data not shown).

Donor/Recipient Pair 3

In January 2006, a female HBV-naïve LLD (30 years) was evaluated for liver donation for her chronically HBV-infected father (58 years). He was transplanted because of HBV-caused liver cirrhosis and HCC. The donor did not show a measurable anti-HBs-titer at the time of enrolment despite previous documented vaccinations in 2000 and 2001 with 10 μg HBVAXPRO. This donor was immunized three times with 20 μg of Sci-B-Vac at biweekly intervals. An anti-HBs-titer of 22,200 IU/L was detected pretransplant (Fig. 4A).

During transplantation the recipient received blood products and HBIg (286 IU/L daily) was administered for the following 21 days to prevent reinfection of the new liver (Fig. 4A). On the one hand, the anti-HBs-titer measured on day 6 (120 IU/L) after transplantation could be due to low amounts of antibodies in blood products given during transplantation, or on the other hand to HBIg administration. To demonstrate that anti-HBs antibodies were transferred with the liver and produced by B cells of the donor or/and the patient, we calculated the amount of HBIg-derived antibodies. The antibodies that were passively transferred achieved the highest concentration at day 21 with 4681 IU/L. At day 14 after transplantation an unexpected high antibody titer of 43,040 IU/L was detected in the recipient. At day 21 and week 5 after transplantation the anti-HBs-titer rose up to 47,000 and 57,993 IU/L, respectively, and decreased at weeks 8 (49,965 IU/L), 14 (8,397 IU/L), 19 (226 IU/L), and 26 (no anti-HBs detected). To summarize, the anti-HBs-titer peak of 57,993 IU/L at week 5 after transplantation cannot be attributed to HBIg medication. A transfer of the humoral immunity with the liver graft is assumed.

To show the difference in the course of anti-HBs-titers between recipient 3 and a chronically HBV-infected recipient who received the liver from a donor without HBV immunity we compiled the serological results of one exemplary patient (Fig. 5). Antibodies to hepatitis B virus surface antigen-titers of up to 2300 IU/L (week 26) could be observed after transplantation. Hepatitis B immunoglobulin was administered to this recipient for 11 days (286 IU/L per day) after the surgery. In addition, the same HBIg dose (286 IU/L daily) was administered for 10 days at weeks 8 and 9 and at weeks 19 and 20

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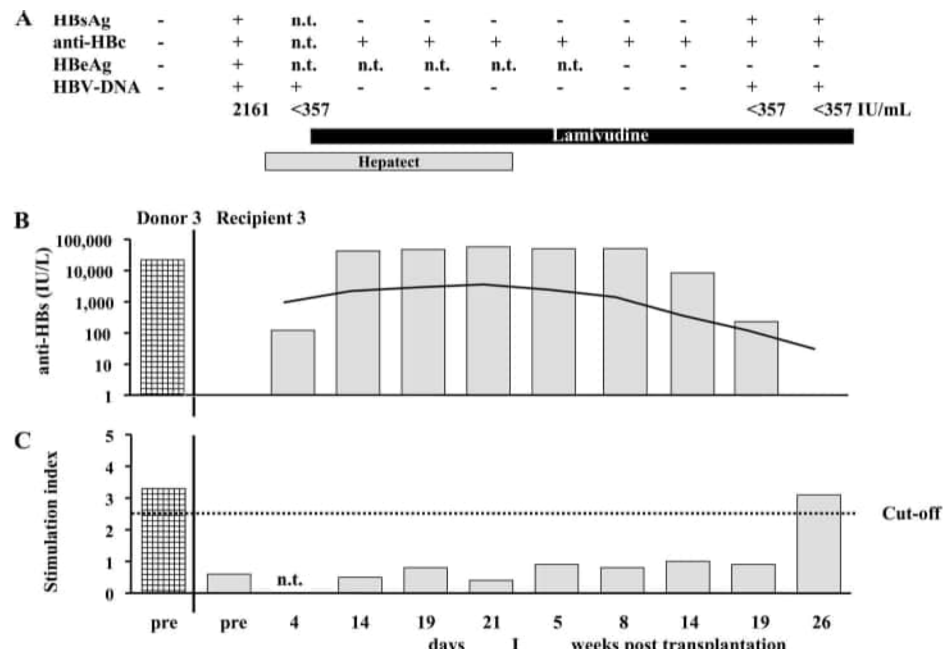

FIGURE 4. Donor/recipient pair 3: Hepatitis B virus (HBV)-specific immunity of the donor pretransplantation and the chronically HBV-infected recipient pre- and posttransplantation. The x-axis indicates time points of analysis. (A) The y-axis shows antibodies to hepatitis B virus surface antigen (anti-HBs) titers in IU/L of the donor (▦) and the chronically HBV-infected recipient (▭). Antiviral therapy with lamivudine is visualized at the top with a horizontal black bar. The curve shows the level of anti-HBs because of hepatitis B immunoglobulin (HBIg) medication (286 IU/L daily) considering the half-life value of the antibodies. Positive (+) or negative (−) results of antibodies to hepatitis B virus core antigen (anti-HBc), hepatitis B virus e-antigen (HBeAg), hepatitis B virus surface antigen (HBsAg), and HBV-specific qualitative and quantitative polymerase chain reaction (PCR) are indicated at the top. (B) The y-axis shows the ratio between stimulated and unstimulated proliferations (stimulation index) of the donor and the chronically HBV-infected recipient after incubation with the L-HBsAg. The horizontal line indicates the cut-off (stimulation index of 2.5). n.t.: not tested.

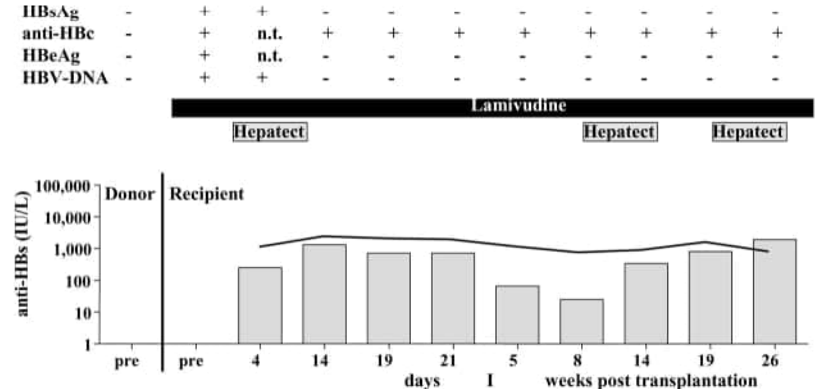

FIGURE 5. Humoral immune response of a chronically hepatitis B virus (HBV)-infected recipient, who received the liver from a donor without HBV immunity. The x-axis indicates time points of analysis. Positive (+) or negative (−) result of antibodies to hepatitis B virus core antigen (anti-HBc), hepatitis B virus e-antigen (HBeAg), hepatitis B virus surface antigen (HBsAg), and HBV-specific qualitative polymerase chain reaction (PCR) are indicated at the top. The y-axis shows anti-HBs titers in IU/L of the donor and the chronically HBV-infected recipient (▦). The administration of hepatitis B immunoglobulin (HBIg) (Hepatect) for 11 days after transplantation and for 10 days at weeks 8 and 9 (286 IU/L daily) as well as at weeks 19 and 20 are visualized as *gray bars*. Before and after transplantation the recipient received lamivudine therapy as indicated with a black bar.

after transplantation, respectively. The half-life time of HBIg-derived antibodies was calculated and a peak of 2988 IU/L (week 8) is visualized in Figure 5. The amounts of anti-HBs antibodies measured in this patient can usually be observed in recipients because of HBIg medication (data not shown). The anti-HBs antibodies in recipient 3 were more than 1 log higher and, therefore, not solely because of HBIg administration.

To show that the vaccine that was used for the immunization of the donor is suitable to overcome chronic infec-

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tion in the recipient, we examined the HBV-genotype and HBV-subtype persisting in the recipient. The vaccine, Sci-B-Vac, which was used for the immunization of the donor, is based on A2/HBsAg-genotype and subtype adw2. The recipient was infected with an HBV-isolate of the same genotype and subtype (A2/HBsAg adw2). Therefore, the vaccine was suitable to overcome the infection in the recipient. The specificity of anti-HBs antibodies in the recipient was tested. These antibodies were neutralized by all the three different HBsAg subtypes (adw, adr, and ayw) indicating that the major portion of anti-HBs was directed to the a-determinant (data not shown). Thus, anti-HBs antibodies present in the patient posttransplant were likely to possess the ability to neutralize HBV viral particles. However, a part of the circulating anti-HBs antibodies were derived from HBIg administration.

In addition to the humoral immunity to HBsAg cellular immune responses were measured in proliferation-assays. Pretransplant L-HBsAg protein specific immunity (SI of 3.3) was detected in the donor and no immune response in the recipient (Fig. 4B). At week 26 after transplantation, the recipient developed L-HBsAg protein-specific cellular immunity (SI of 3.1). No immune responses could be detected in the recipient before and after transplantation by ELISpot (data not shown).

We measured HBsAg, HBeAg, and HBV-DNA 1 week before and at several time-points after transplantation in patient 3. The three parameters were positive pretransplant. At day 4 after transplantation a low level of HBV-DNA was detected despite the presence of anti-HBs. Therefore, antiviral therapy with lamivudine was started on day 5 after transplantation. Afterward, HBV-DNA was no longer detectable. From week 19 onward low levels of HBV-DNA (<357 IU/mL) and HBsAg were detected again in the serum. However, no HBeAg could be measured. Unfortunately, further observation was not possible, because the patient died at week 28 after transplantation because of perforating lung metastases.

DISCUSSION

Liver transplantation is mostly the ultimate therapeutic option for patients with liver failure because of liver cirrhosis and HCC. The lack of organs from multiorgan donors led to an increased number of partial liver donations from LLDs (*27*). The transfer of HBV-specific immunity with the graft of vaccinated donors is an option to prevent reinfection in chronically HBV-infected recipients. In countries with a high prevalence of HBV, the chance to transplant a liver from an immune donor is high and subsequent adoptive immune transfer has been described (*14*, *15*). In low-prevalence areas like Europe, however, immune donors are rare. Therefore, vaccination of LLDs for adoptive immune transfer could be an alternative.

In this ongoing study, HBV-specific adoptive transfer of humoral, and for the first time, of cellular immunity with the human liver was demonstrated. An immunogenic HBV vaccine was used to induce high anti-HBs-titers in donors during the short-time (approximately 2 months) between screening of donors and performing LTX. We have previously shown that vaccination with this vaccine induces strong immune responses in a short-time immunization protocol (*21*). The analysis of the immune responses in HBV-naïve recipients who received a liver of an immunized LLD is the best opportunity to demonstrate proof of principle for HBV-specific adoptive immune transfer. In chronically HBV-infected recipients, the adoptive transfer of the humoral immune response is masked by high anti-HBs-titers because of regular HBIg prophylaxis. Transfer of humoral or cellular immunity was detected in 3 of 14 recipients. Two recipients were HBV-naïve (1 and 2) and one was chronically HBV-infected (3). In the corresponding donors anti-HBs-titers more that 9000 IU/L were measured. Probably, because of the induction of low anti-HBs-titers less than 1000 IU/L in 10 of 14 donors we were not able to show adoptive immune transfer to their corresponding recipients. The transfer of antibodies seems to depend on the level of the HBV-specific humoral immunity in the donor before transplantation. Luo et al. (*15*) have shown that particularly the humoral immunity (anti-HBs-titers >1000 U/L) in the donor is essential for the adoptive immune transfer. These donors achieved immunity to HBV after infection. In our study, however, one donor with a high anti-HBs-titer of 6000 IU/L and additionally the highest cellular immune response did not transfer HBV-specific immunity to the corresponding recipient. Possibly, immunity after infection is associated with a higher amount of HBV-specific cells in the liver compared with that in livers of immunized donors. Therefore, immunized donors probably require higher anti-HBs-titers to transfer their immunity than donors after resolved HBV-infection. A final statement on the minimal anti-HBs-titer that is required for adoptive immune transfer from immunized donors is pending.

Over all, in 2 of 11 HBV-naïve recipients transfer of adoptive immunity was demonstrated. Vaccination of cirrhotic patients pretransplant (weeks 0, 2, and 6 with 40 μg of HBVPRO, respectively) induced low anti-HBs-titers ($\geq$10 IU/L) in 7 of 16 cirrhotic patients in a pilot study (M. Fiedler, unpublished data). Pretransplant immunization is not successful up to now.

In the chronically HBV-infected recipient 3, an anti-HBs-titer of 57,993 IU/L was measured after transplantation. This titer is more than 1 log higher than that detected in a similarly HBIg-treated control recipient who received the liver from an HBV-naïve donor. The approximate anti-HBs-titer due to HBIg was calculated to be approximately 4700 IU/L in recipient 3. In addition, the treatment with lamivudine starting on day 4 after transplantation could have influenced the level of anti-HBs after transplantation. The remarkable amount of anti-HBs, however, is unlikely to be due to effects of the lamivudine therapy alone. In week 19 after transplantation, HBV-DNA reappeared and anti-HBs was no longer measurable. This possibly indicates that adoptive transfer of HBV-specific immunity was not sufficient in this patient to prevent graft reinfection. Probably, immunization in combination with immunogenic adjuvant-like MPL/QS21 could be able to improve the immune response and the transfer of immunity. Recently, it was shown that HBV/MLP/QS21 vaccine-induced significant anti-HBs-titers in liver recipients. These recipients were chronically HBV-infected (HBsAg positive pretransplant) and remained on HBIg prophylaxis (*18*). The state of health of the chronically HBV-infected recipient in our study rapidly worsened because of tumor metastases. The patient died 6 months posttransplant. Probably, this

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course impaired the immune function. This aspect needs to be examined in a larger cohort of patients.

Chimerism could be a potential explanation for the detection of HBV-specific immune responses after LTX. In donor/recipient pair 1 microchimerism could not be detected 1 year posttransplant. In other studies, it was shown that the frequency of transferred donor cells is mostly below 1%, 1-month after transplantation and even lower thereafter (*28*, *29*). The sensitivity of the methods established so far is probably not sufficient to detect microchimerism in all cases. Flow cytometry could be another sensitive method to detect donor and HBV-specific T-cells or B-cells in the recipient (*30*). We were not able to detect HBV-specific cells in the recipient after 1 year. This could be due to a low frequency of HBV-specific cells and findings that the detection of antigen-specific T cells by flow cytometry is less sensitive than by ELISpot (*31*).

In conclusion, HBV-specific adoptive immune transfer to chronically HBV-infected patients in the setting of living LTX is feasible. Our results clearly indicate that only high anti-HBs-titers in the donor result in immune transfer. It could be beneficial to extend the time for donor vaccination if possible. Hopefully, in the future most possible donors shall be already immune because in many countries children are now vaccinated against HBV. Further, a promising way to overcome the reinfection of the new graft in chronically HBV-infected liver recipients could be the combination of adoptive immune transfer followed by booster immunizations. Both vaccines with immunogenic adjuvants-like MPL/QS21 (*18*, *32*) or third generation vaccines are promising (*33*).

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LETTER TO THE EDITOR

Control of hepatitis B virus infection in hematopoietic stem cell recipients after receiving grafts from vaccinated donors

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Protection of hematopoietic stem cell recipients from infection remains challenging because of severe immunosuppression after transplantation. In patients with hepatitis B virus (HBV) infection prior to transplantation, the risk of HBV reactivation 5 years after transplantation was 70–86%.[1,2] To overcome HBV reactivation, active immunization of donors and early post-transplant vaccination of recipients has been suggested.[3,4] This recommendation is based on the fact that adoptive immune transfer from HBV-vaccinated donors was detected after hematopoietic stem cell transplantation.[3,5–9] However, protection from reactivation depended on vigorous HBV immunity in the donor.[3,10] Therefore, donors should optimally receive more than one immunization, a rather high Ag dose and a highly immunogenic vaccine. As clearance from HBV infection is dependent on effective T-cell immunity, the protection of recipients can be estimated better if their HBV-specific cellular immune response is known. However, cellular HBV immunity had not been determined in a previous study showing that donor vaccination can control HBV infection in hematopoietic stem cell recipients.[3]

We here present two effective strategies to select the optimal donor for hematopoietic stem cell recipients at risk of HBV reactivation or persistence of chronic HBV infection. Donors could either be electively vaccinated with highly immunogenic vaccines (for example, third generation vaccines containing PreS1, PreS2 and S HBV Ags[11]) or donors with strong HBV immunity after vaccination could be selected. Our study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects.

Humoral and cellular HBV immunity were measured as described previously.[8,11,12] To assess HBV-specific T-cell proliferation and interferon-γ production, peripheral blood mononuclear cells were incubated with the PreS1, PreS2 and S HBV Ags (unformulated bulk). A stimulation index (SI, quotient of HBV-specific and non-stimulated proliferation) $\geqslant 2.5$ was considered positive.[12] ELISpot results were generated as HBV-specific minus non-stimulated interferon-γ production.[11] The cutoff value was defined as an HBV-specific value that was threefold higher than the non-stimulated control.

Patient #1, a 43-year-old male with Non-Hodgkin lymphoma, suffered from reactivation of HBV infection 5 months prior to PBSC transplantation (PBSCT) during chemotherapy. Chemotherapy was started 8 months prior to PBSCT and led to PR. Owing to HBV reactivation, he received lamivudine and was transplanted after HBV DNA became undetectable. The first stem cell donor was his HLA-identical, 40-year-old sister. As she was HBV-naïve, we actively immunized her four times (day 0, week 2, week 4 and month 5) with 20 μg of a vaccine containing PreS1, PreS2 and S HBV Ags (Bio-Hep-B, Berna Biotech, Bern, Switzerland). She developed humoral and cellular HBV immunity prior to PBSCT (Figure 1, patient #1). The corresponding recipient displayed humoral HBV immunity prior to PBSCT (90 IU/L anti-HBs); but cellular immunity was absent (SI of 0.5). HBV DNA was undetectable prior to transplantation (Figure 2, patient #1). One month after PBSCT, humoral HBV immunity increased in the recipient (633 IU/L anti-HBs). A maximum was reached at month 19 after transplantation (1483 IU/L anti-HBs). At the same time, the recipient had detectable cellular HBV immunity (SI of 4.8 and 8.5 spots increment); indicating that HBV infection was controlled by donor T cells. HBV DNA remained negative until month 27 post transplantation. However, the patient relapsed at month 20. Thereafter, HBV immunity declined. Twenty-seven months after the initial transplantation, the patient received a second graft from his HLA-haploidentical, 49-year-old sister (CD3/CD19-depleted graft). This sister had resolved HBV infection (17 IU/L anti-HBs, anti-HBc-positive, HBsAg- and HBV DNA-negative). After the second transplantation, HBV DNA became intermittently positive. The recipient showed a short-term, minor increase in anti-HBs antibodies (88 IU/L). In the follow-up, antibodies became undetectable. Fifteen months after the second transplantation, the patient relapsed again. Four months thereafter, he died from pneumonia.

Patient #2, a 49-year-old male, suffered from myelodysplastic syndrome and acute HBV infection 4 months prior to PBSCT. He was also treated with lamivudine and was transplanted after HBV DNA became undetectable. His HLA-identical, HBV-naïve sister was vaccinated four times (day 0, day 10, day 24 and month 3) by her general practitioner with 20 μg of a German standard vaccine containing the HBV S Ag (Engerix-B, GlaxoSmithKline, Munich, Germany). He had detectable anti-HBs antibodies prior to and post transplantation (Figure 1, patient #2). By means of Ab titers, it cannot be determined whether anti-HBs had been transferred with the graft. After stem cell transplantation, the recipient remained HBV DNA-negative and cleared his HBV infection (for example, HBsAg-negative, 19 IU/L anti-HBs, anti-HBc-positive at month 5) (Figure 2, patient #2). Anti-HBs antibodies in the recipient declined until month 5 after PBSCT. From month 5 to month 9, anti-HBs antibodies slightly increased. The patient suffered from acute and thereafter from limited chronic GvHD and was treated by cyclosporine A. At month 9, GvHD was well controlled and low-dose treatment with cyclosporine was sufficient. After month 9, lamivudine treatment was stopped. At month 10, there was a strong increase to an anti-HBs titer exceeding 1000 IU/L, HBV DNA in the peripheral blood remained negative. Most likely, a combination of omitting antiviral treatment—leading to a 'booster' by HBAg persisting outside the peripheral blood—and reconstitution of immune function led to the increased anti-HBs titer. At year 9 (month 112) after transplantation, HBsAg remained negative and humoral HBV immunity was detectable (18 IU/L anti-HBs- and anti-HBc-positive). Cellular HBV immunity could also be measured (SI of 5.5 and 6 spots increment); indicating long-term control of HBV infection.

Patient #3, a 51-year-old female with secondary acute myeloid leukemia after myelodysplastic syndrome, was chronically infected with HBV. Her donor was an unrelated female, previously immunized with an HBV vaccine containing S Ag. The donor was selected based on her high anti-HBs titer (265 647 IU/L) and was phenotypically HLA-identical (Figure 1, patient #3). The patient was furthermore treated by entecavir from month 3 pre-transplantation until month 3 post transplantation. Prior to PBSCT,

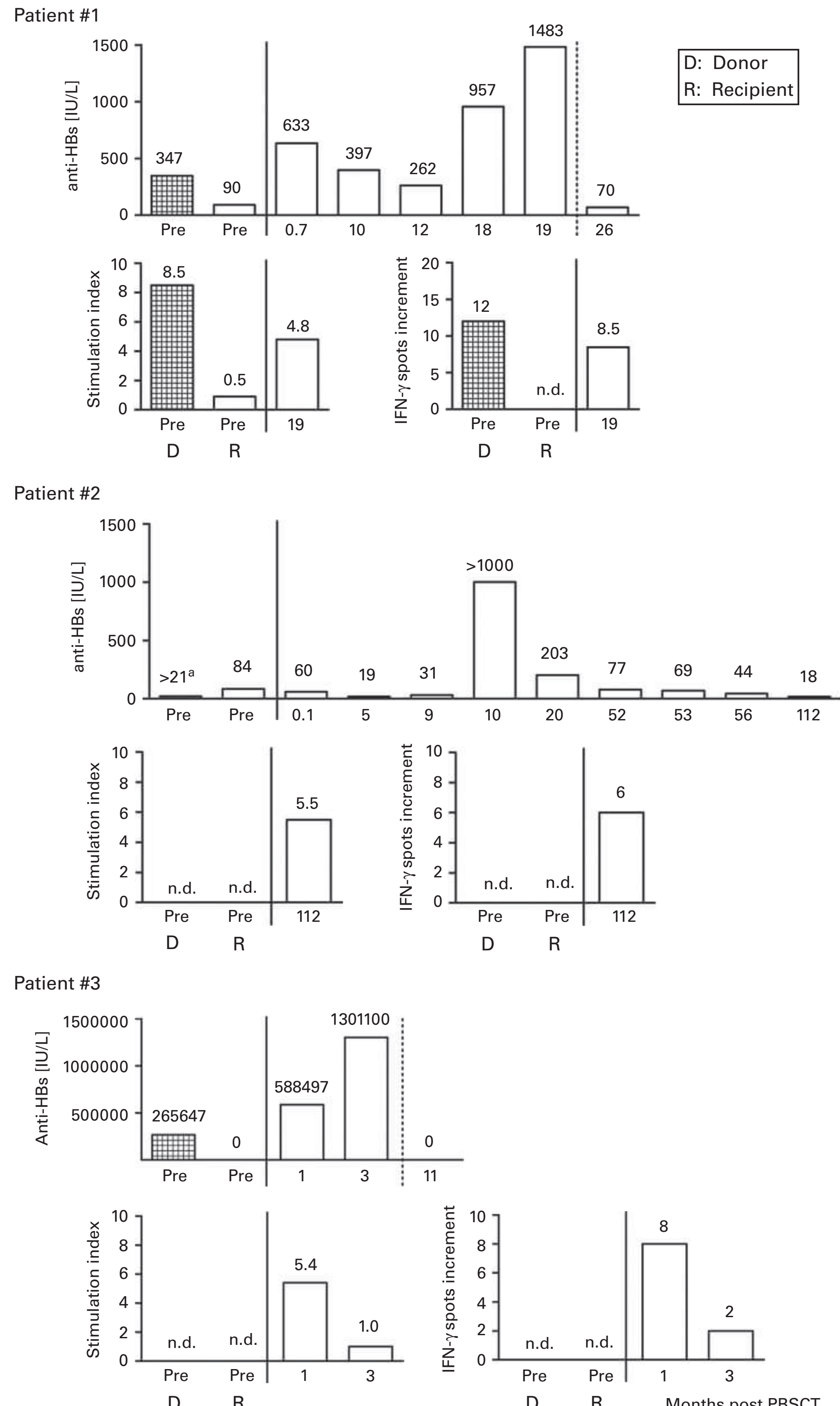

Figure 1. HBV-specific immunity of vaccinated donors and recipients with reactivated, acute HBV infection (patient #1), acute HBV infection (patient #2) and chronic HBV infection (patient #3) pre- and post PBSCT. The donor was either an HLA-identical sister (patients #1 and #2) or an HLA-matched unrelated donor. The *x* axes indicate time points of analysis (pre-transplantation or months post transplantation); the *y* axes show humoral HBV immunity (anti-HBs titers), HBV-specific cell proliferation (SI) or HBV-specific ELISpot results (interferon-γ production). Donor immunity is shown as hatched bars (first bar in each panel), recipient immunity as open bars. PBSCT is indicated by vertical continuous lines, relapse by vertical dotted lines. n.d., not determined. [a]The anti-HBs titer in this donor was determined 1 year after the fourth HBV vaccination. Owing to antibody kinetics, the titer was most likely higher than 21 IU/L at the time of PBSCT, that is, at day 26 after the fourth vaccination.

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Patient #1

HBV reactivation — 1st PBSCT — Month 20: 1st relapse

	-8	-5	-4.5	-4	-3	-2	-1	-0.2	0.5	1	4	8	10	12	18	19	26
HBV-DNA qualitative	Ø	+	+	+	+	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
HBV-DNA quantitative		2,E+07	1,E+06	5293	<357												
HBsAg	Ø	+				Ø	Ø	Ø		Ø			Ø	Ø		Ø	Ø
Anti-HBs						21	89	90		633			397	262	957	1483	70
Anti-HBc	Ø	Ø				+	+	+		+			+	+		+	
Anti-HBc IgM		Ø				+	Ø	Ø		Ø						Ø	
HBeAg		+						Ø		Ø						Ø	
Anti-HBe		Ø						Ø		Ø						+	
Lamivudine																	

2nd PBSCT — Month 15: 2nd relapse

	0.1	0.5	2	8	11	15	16
HBV-DNA qualitative	Ø	+	Ø				
HBV-DNA quantitative		<357					
HBsAg		Ø	Ø				
Anti-HBs		24	88	Ø	Ø	Ø	Ø
Anti-HBc			Ø				
Anti-HBc IgM							
HBeAg							
Anti-HBe							
Lamivudine							

Months pre and post PBSCT

Patient #2

Acute HBV infection — PBSCT

	-4	-3	-2	-1	-0.5	-0.2	0.1	0.5	0.7	1	2	5	7	9
HBV-DNA qualitative	+	Ø	Ø	Ø		Ø	Ø			Ø	Ø	Ø	Ø	Ø
HBV-DNA quantitative	590700													
HBsAg	+	+	Ø	Ø	Ø		Ø				Ø	Ø		Ø
Anti-HBs	Ø	12	Ø	39	84		60					19		31
Anti-HBc	Ø	+	+	+	+		+				+	+		+
Anti-HBc IgM	Ø	+	+	+	+			Ø	Ø	Ø				
HBeAg	Ø	Ø	Ø	Ø			Ø							
Anti-HBe	Ø	+	+	+			+							
Lamivudine														

	10	20	46	52	53	56	112
HBV-DNA qualitative	Ø		Ø				
HBV-DNA quantitative							
HBsAg				Ø	Ø		Ø
Anti-HBs	>1000	203		77	69	44	18
Anti-HBc				+	+		+
Anti-HBc IgM							
HBeAg							Ø
Anti-HBe							+
Lamivudine							

Months pre and post PBSCT

Patient #3

Chronic HBV infection — PBSCT — Month 3: Relapse

	-0.7	-0.4	-0.2	0	0.1	0.5	0.9	1	3	11
HBV-DNA qualitative	+		Ø	Ø	Ø	Ø	Ø	Ø		
HBV-DNA quantitative	<357		<357							
HBsAg	+	+	+					Ø	Ø	+
HBsAg quantitative	>250	>250	>250							
Anti-HBc	Ø		Ø					6E+05	1E+06	Ø
Anti-HBc	+	+	+					+	+	+
Anti-HBc IgM	Ø		Ø					Ø	Ø	
HBeAg	Ø		Ø					Ø	Ø	Ø
Anti-HBe	+		+					+	+	+
Entecavir										

Months pre and post PBSCT

Figure 2. Course of HBV DNA and HBV-specific humoral immunity in PBSC recipients with HBV infection pre-transplantation. Patient #1: The first hematopoietic stem cell donor was an HLA-identical, HBV-vaccinated sister, the second one an HLA-haploidentical sister (CD3/CD19-depleted graft) with resolved HBV infection. The second PBSCT was performed 27 months after the first PBSCT. Patient #2: The donor was an HLA-identical, HBV-vaccinated sister. Day 3 was indicated as month 0.1. Patient #3: The donor was a vaccinated unrelated donor with an anti-HBs titer of 265 647 IU/L. HBV-DNA quantitative and anti-HBs are both given in IU/L. The detection limit for HBV-DNA (qualitative and quantitative test) was 100 and 357 IU/mL, respectively. Antiviral prophylaxis with either lamivudine or entecavir is indicated by black horizontal bars. Ø, negative; +, positive.

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no anti-HBs antibodies could be detected in the patient. At month 1 after PBSCT, however, she displayed humoral and cellular HBV immunity (588 497 IU/L anti-HBs, SI of 5.4 and 8 spots increment). She cleared her chronic HBV infection and was HBV DNA-negative (Figure 2, patient #3). At month 3, humoral HBV immunity further increased (1 301 100 IU/L anti-HBs). Cellular HBV immunity, however, was undetectable (SI of 1.0 and 2 spots increment) and HBV DNA remained negative. Most likely, after PBSCT, HBsAg had persisted outside the peripheral blood and 'boosted' anti-HBs production. Unfortunately, the patient relapsed at month 3 after transplantation. She was treated with three courses of donor leukocyte infusions (month 8–11). At month 11, she developed GvHD of the liver, humoral HBV immunity was lost and HBsAg re-appeared. Treatment with entecavir was started again. At month 12, the patient presented with cachexia and died from relapse.

In summary, HBV infection could be controlled after patients received grafts from HBV-vaccinated donors. But following relapse, HBV immunity was lost. The study indicates that the optimal donor for an HBV-infected recipient is a vaccinated person with humoral and cellular HBV immunity. Third generation HBV vaccines are especially useful when time is short to *de novo* induce sufficient HBV immunity. As compared with conventional (second generation) HBV vaccines, HBV immunity develops earlier and immunity is stronger after application of the third generation vaccines.[11,13,14] In the current study, anti-HBs clearly increased in patient 1 after receiving a graft from the donor vaccinated with a third generation vaccine. On the contrary, even a decline in anti-HBs titers was observed in patient 2 receiving a graft from the donor vaccinated with a conventional HBV vaccine. Nevertheless, patient 2 remained HBsAg-negative for 9 years. Donor vaccination may have helped to control HBV infection. If time is very short, it would be optimal to select a donor with high anti-HBs titer and to apply one 'booster' immunization. One week after 'booster' immunization, peak HBV immunity is expected and harvesting of hematopoietic stem cells would be most promising to achieve an HBV-specific immune transfer. However, if *de novo* or 'booster' vaccination of the donor is not possible, long-term antiviral prophylaxis and vaccination of the recipient would be an alternative.[4]

Extending current literature,[3] we were able to show that after transplantation cellular HBV immunity was detectable in previously HBsAg-positive stem cell recipients. This is important because HBV-specific T cells are a prerequisite to control HBV infection. As hematopoietic stem cell recipients usually have impaired T-cell function for many months,[15] the detection of HBV-specific cellular immunity only 1 month after transplantation is remarkable.

In conclusion, we recommend vaccinating donors for HBV-infected stem cell recipients with highly immunogenic vaccines or to apply a single 'booster' immunization in pre-vaccinated donors. Donor vaccination can help to control HBV infection in the recipient and should be considered as a highly efficient therapeutic option.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

ML, MR, MF, MK and DWB were involved in the conception and design of the study. MK, HDO and HDB provided patient samples. ML, MK, MF, AS and FMH were involved in the collection and assembly of data. ML and AS were involved in data analysis and interpretation. ML and PAH wrote the manuscript. All the authors gave final approval of the manuscript.

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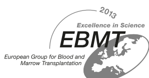

CORRESPONDENCE

Adoptive transfer of cellular immunity against cytomegalovirus by virus-specific lymphocytes from a third-party family donor

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Infection remains a major challenge after hematopoietic stem cell transplantation [1]. In transplant recipients, reactivation of herpes viruses, especially of the human cytomegalovirus (CMV), is associated with severe and fatal complications. CMV is a life-long persisting virus with a seroprevalence between 45 and 100% in the general adult population [2]. Whereas symptomatic CMV infection of healthy individuals is rare, in immunodeficient patients, such as transplant recipients, CMV infection or reactivation commonly manifests as a life-threatening disease affecting different organ systems. Transplant recipients with CMV viremia are usually treated with antiviral drugs [3]. However, myelotoxicity as well as mutations of the viral DNA sequence may limit the use of these drugs [3–5]. Thus, in selected cases alternative therapeutic options are mandatory.

We here present the follow-up of a 21-year-old female with acute myeloid leukemia, who was treated with CMV-specific T cells (virus-specific T cells, VSTs). Written informed patient consent has been obtained. Viral load and cellular CMV immunity were closely monitored for almost 3 years. The patient was transplanted in cytomorphologic complete remission but with cytogenetically minimal residual disease. She received allogeneic peripheral blood stem cells from a 9/10 HLA-matched, unrelated male donor in May 2015. Myeloablative conditioning consisted of fludarabin, carmustine, antithymocyte globulin, and melphalan, and the immunosuppressive regimen included cyclosporine A and budesonide. Anti-infective prophylaxis/therapy included amoxicillin, metronidazole, itraconazole, valganciclovir, cotrimoxazol, calciumfolinate, and pentamidine. She received 9.4×10^6 CD34+ cells/kg body weight. Three weeks after peripheral blood stem cell transplantation (PBSCT) the patient suffered from acute graft vs. host disease (GvHD) of the skin (stage 1, grade 1), which did not require systemic treatment. The unrelated donor was CMV IgM- and IgG-negative, and the patient was CMV IgM-negative but IgG-positive. Thus, the recipient was at high risk of CMV reactivation. In July 2015 anti-CMV IgM and IgG became positive in the recipient and the viral load reached up to 1,022,908 copies (1,595,800 IU)/ml (day 35) as shown in Fig. 1. Despite antiviral therapy (sequentially with valganciclovir, ganciclovir, and foscarnet) and CMV-specific immunoglobulins no adequate decrease of the viral load was observed. At the peak of CMV reactivation, the patient suffered from a generalized seizure, which was considered cyclosporine-related. Treatment with cyclosporine and budesonide was switched to mycophenolate mofetil and prednisone. The patient received levetirazepam and no further seizure occurred.

It was then decided to treat the patient with CMV-specific lymphocytes of her HLA haploidentical sister who was CMV IgG-positive. Manufacturing of CMV-specific T cells was carried out at Hannover Medical School with the CliniMACS® Plus Instrument and GMP PepTivator® HCMVpp65 for antigenic restimulation, as described previously [6, 7]. Enrichment of IFN-γ-secreting CMV-specific T cells was performed by immunomagnetic separation using the CliniMACS Cytokine Capture System (Miltenyi Biotec,

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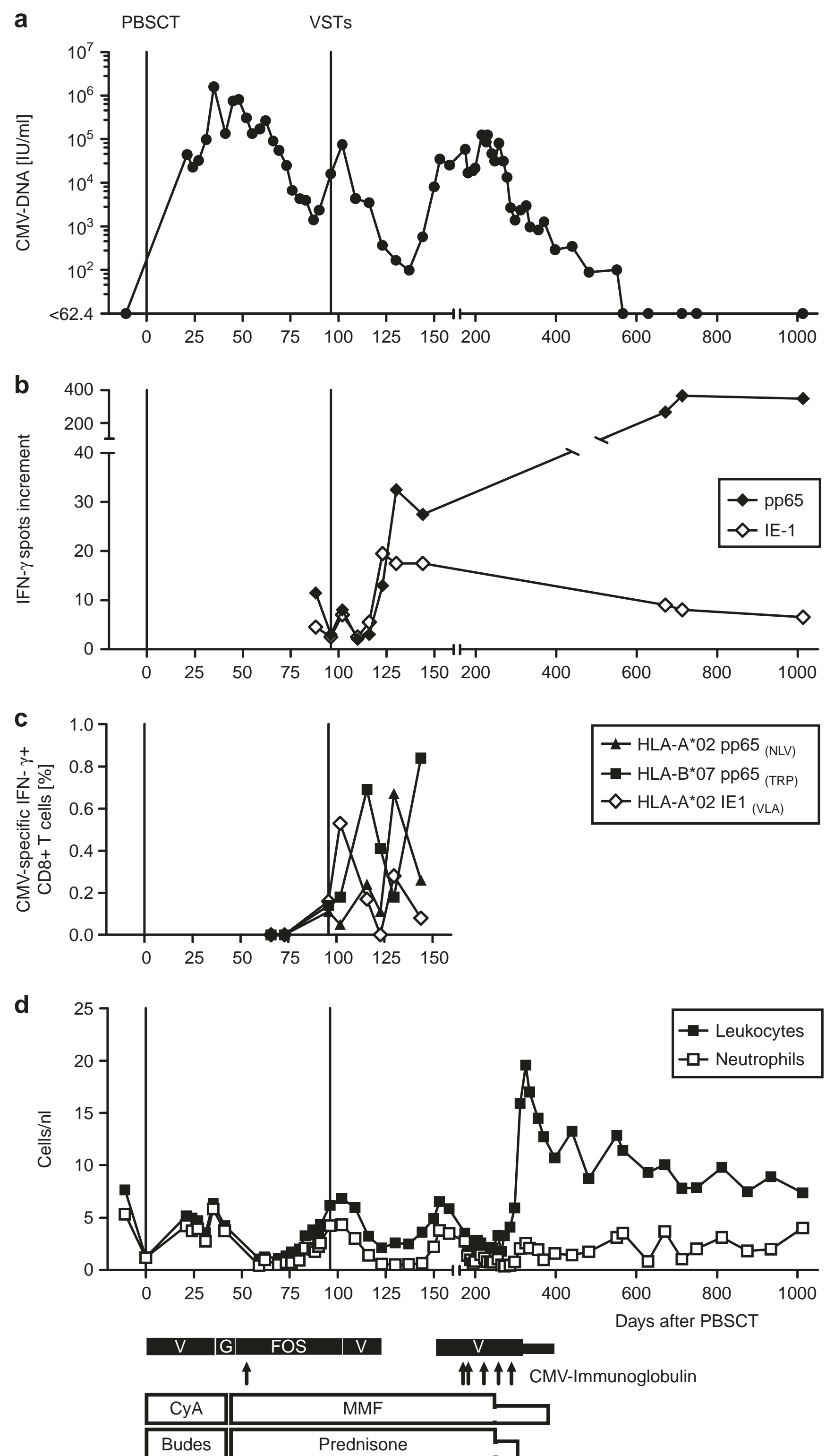

Fig. 1 Course of cytomegalovirus (CMV) DNA, of cellular CMV-specific immunity, and of leukocytes in a transplant recipient receiving CMV-directed virus-specific T cells (VSTs). The 21-year-old female suffered from acute myeloid leukemia (AML) and received peripheral blood stem cell transplantation (PBSCT) from an unrelated donor. The recipient was CMV IgG-positive, whereas the unrelated donor was negative. Despite antiviral treatment and CMV-specific immunoglobulins CMV reactivation after transplantation could not be adequately controlled. Therefore, the patient was treated with VSTs of her HLA-haploidentical sister, who was CMV IgG-positive. Panel (**a**) shows the course of the viral load, panel (**b**) results to a CMV-specific IFN-γ ELISpot, panel (**c**) results of CMV-specific multimer analysis, and panel (**d**) numbers of leukocytes and neutrophils. PBSCT and VSTs are indicated by vertical continuous lines, antiviral prophylaxis with either valganciclovir (V), ganciclovir (G), or foscarnet (FOS) by black horizontal boxes and immunosuppression with cyclosporine A (CyA), mycophenolate mofetil (MMF), budesonide (Budes), or prednisone by open boxes. Arrows indicate treatment with CMV-specific immunoglobulins

Bergisch Gladbach, Germany), consisting of the CliniMACS Catchmatrix and IFN-γ Enrichment Reagents. The final T-cell product had a viability of 80% with a purity of 41.8% CMV-specific IFN-γ+ T cells. Selection of the donor was based on the CMV serostatus and the presence of CMV-specific T cells as specified in Table 1. The patient

Table 1 Cytomegalovirus (CMV)-specific donor T cells

Ex vivo donor cells	
Frequency of CMV-specific, IFN-γ+ T cells[a]	0.29% of CD3+ T cells
	0.15% of CD4+ T cells
	0.44% of CD8+ T cells
Preselected T cells (prior to enrichment)	
Viability	96%
Frequency of CMV-specific, IFN-γ+ T cells	0.43% of CD3+ T cells
	0.39% of CD4+ T cells
	0.47% of CD8+ T cells
Final T cell product (positive fraction after enrichment)	
Viability	70%
Lymphocytes (in a volume of 38 ml)	4.0×10^6 of viable CD45+ lymphocytes
	97.9% viable CD3+ T cells
	3.9×10^6 of viable CD3+ T cells
	5.0 CD4/CD8 ratio (viable CD3)
Lymphocytes per kg body weight	8.1×10^4 of viable CD45 +lymphocytes
	7.9×10^4 of CD3+T cells
	3.3×10^4 of viable CD3+/IFN-γ +T cells
	2.3×10^4 of viable CD4+/IFN-γ+ T cells
	1.0×10^4 of viable CD8+/IFN-γ+ T cells
Frequency of CMV-specific, IFN-γ+ T cells	41.8% of CD3+ T cells
	35.0% of CD4+ T cells
	76.4% of CD8+ T cells
Frequency of contaminating lymphocytes	1.73% CD3−CD19+ B cells
	1.54% CD3−CD56+ NK cells

[a]The frequency was determined by cytokine secretion assay in response to a CMVpp65-overlapping peptide pool. The respective negative control has been subtracted from the CMVpp65-specific response

received 2.5×10^4 CD3+ cells/kg body weight (12.3 ml) of CMV-VSTs on day 96 after PBSCT. CMV DNA, cellular CMV immunity, and leukocyte numbers were monitored before and after transfer of CMV-specific T cells. To determine viral load, CMV DNA was purified from blood samples using the Abbott m2000sp automated nucleic acid extraction system (Abbott, Wiesbaden, Germany). The viral load was quantified with the Abbott m2000rt real-time PCR system using the Abbott RealTime CMV amplification reagent kit according to the manufacturer's instructions. The detection limit of this assay is 40 copies (62.4 IU)/ml.

After infusion of VSTs CMV viral load decreased until day 137 after transplantation (day 41 after VSTs) and reached a minimum of 63 copies (98 IU)/ml (Fig. 1a). On day 141 after PBSCT the viral load increased again. Without further VSTs, however, it started to decrease on day 269. From day 566 onwards, CMV viral load remained undetectable.

Cellular CMV immunity was determined by IFN-γ ELISpot, based on the stimulation of 200,000 peripheral blood mononuclear cells (PBMCs) with pp65 and IE-1 CMV proteins (T-Track® CMV-Assay, Lophius Biosciences GmbH, Regensburg, Germany), as described recently [8]. This CE-approved assay measures CMV-specific responses of both CD4+ and CD8+ T lymphocytes within 19 h. Spot numbers were determined by an ELISpot plate reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). The median spot number of duplicate cultures was considered for further analysis and values of negative controls were subtracted from CMV-specific values (spots increment). The sister's PBMC showed strong CMV immunity (423 CMV pp65 and 16 CMV IE-1 spots increment), whereas CMV immunity was very weak in the patient prior to VSTs (12 CMV pp65 and 5 CMV IE-1 spots increment; Fig. 1b). After VSTs, CMV pp65-specific spots increased until day 713 (367 spots increment). However, a minimum on day 110 and day 144 could be observed. CMV IE-1-specific spots reached a maximum on day 123 (19.5 spots increment) and then started to decrease. Taken together, viral control by VSTs could be assumed for 41 days after VSTs.

Moreover, CMV-specific T cells were determined by multimer analysis as previously described [6, 9], using HLA-A*02- and HLA-B*07-restricted peptides (Fig. 1c). After VSTs, the percentage of CMV pp65- and IE-1-specific IFN-γ+ CD8+ T cells increased up to 0.84 and 0.53%, respectively.

Increased viral replication was always followed by low numbers of leukocytes and neutrophils (Fig. 1d), which may be due to myelotoxic antiviral drugs (valganciclovir and foscarnet).

Finally, we analyzed the origin of the CMV-specific cells that provided long-term control of the virus. On day 713 after transplantation, patient PBMCs were stimulated with the CMV pp65 protein, using the same conditions as for the ELISpot. Patient PBMC without CMV stimulation served as control. Hematopoietic chimerism testing was performed using the KMRtype® and KMRtrack® Chimerism Monitoring Reagents (GenDx, Utrecht, the Netherlands) according to the manufacturer's recommendations, determining bi-allelic insertion–deletion polymorphism [10]. The input DNA for each reaction was 100 ng, resulting in a detection limit of 0.06%. We found 100% donor chimerism in the CMV-specific cells as well as in the cells without CMV stimulation. Thus, all cells displayed the genetic markers of the unrelated donor. No genetic markers of the sister who donated the VSTs or of patient origin were observed. Moreover, XY-FISH analysis [11] was performed nearly every month after PBSCT and showed 100% donor

chimerism in the peripheral blood of the patient, e.g., immediately prior to VSTs, on day 48 and day 917 after VSTs. On day 54 after VSTs also CD3+ T cells (without CMV stimulation) were tested by XY-FISH analysis, yielding 100% donor cells. A disease-specific marker, a mutation of nucleophosmin 1 (NPM1) [12], which was present prior to transplantation, remained undetectable throughout the whole follow-up after PBSCT. Most likely, CMV disease was controlled early by the VSTs and later by CMV-specific immunity that was established from the unrelated donor-derived immune system.

Overall, side effects of VSTs were moderate. On day 144 after PBSCT (day 48 after VSTs) the patient suffered from acute GvHD of the skin (stage 2) and of enteral GvHD (stage 2), i.e., grade 2. On day 199 after PBSCT she presented with moderate, histologically confirmed chronic GvHD with sclerodermic areas, exudative enteropathy, and malabsorption with hypoproteinemia edema, which improved after increasing the dose of prednisone. Starting on day 214 after PBSCT the dose of mycophenolate mofetil and prednisone could be tapered. Since day 287 the treatment with prednisone could be stopped and since day 370 the patient is without any immunosuppression. Moreover, at day 356 after PBSCT, lymphocyte subpopulations reached the level of healthy controls. Currently, 3 years after VSTs, the patient is in good health and is able to work. Thus, alloreactivity that could have been induced by VSTs was treated successfully.

In summary, our data indicate that CMV replication could be intermittently controlled by VSTs from a CMV-positive donor. Thus, if antiviral therapy fails VSTs may represent a suitable treatment alternative. Presumably, a second infusion of VSTs ~1 month after the first could have prevented the subsequent re-appearance of the virus. The course of the viral load (Fig. 1a) indicates that it took more than 6 days until the VSTs led to a decrease of viral replication. Since the patient and her sister who donated the VSTs were HLA-haploidentical, alloresponses toward the foreign HLA haplotype may have limited the lifespan of the VSTs. However, in a different solid organ transplant recipient who received EBV-VSTs from a 5/10 HLA-matched donor, these donor T cells could be detected for 1 year, i.e., the end of the current follow-up. According to our previous data [13], the transfer of antiviral immunity may last longer if donor and recipient were HLA-identical. In HLA-identical donor/recipient pairs donor-derived hepatitis B virus-specific immunity was detectable for up to 5 years after hematopoietic stem cell transplantation.

In conclusion, in selected cases the infusion of VSTs should be considered to control CMV replication. The optimal frequency of VSTs' application may depend on the histocompatibility of donor and recipient.

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Author contributions D.W.B., B.E.V., M.L., R.B., and M.K. were involved in the conception and design of the study. M.K., N.K.S., V. K., and D.W.B. provided the samples. M.L., M.K., B.M.K., M.F., and A.H. were involved in the collection and assembly of data. B.E.V., B. M.K., and R.B.L. were involved in the donor selection, quality control of the CMV-VSTs, and the final release of the T-cell product. M.L. and M.K. were involved in data analysis and interpretation. M.L., M. K., and P.H.O. wrote the manuscript. All the authors gave final approval of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Long-Term Follow-Up after Adoptive Transfer of BK-Virus-Specific T Cells in Hematopoietic Stem Cell Transplant Recipients

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Abstract: The BK virus (BKV) causes severe hemorrhagic cystitis in hematopoietic stem cell transplant (HSCT) recipients. To eliminate reactivated BKV, symptomatic patients can be treated with a reduction of the immunosuppressive therapy, with the antiviral drug cidofovir, or with virus-specific T cells (VSTs). In the current study, we compared the effect of VSTs to other treatment options, following up specific T cells using interferon-gamma ELISpot assay. We observed BKV large T-specific cellular responses in 12 out of 17 HSCT recipients with BKV-related cystitis (71%). In recipients treated with VSTs, 6 out of 7 showed specific T-cell responses, and that number in those without VSTs was 6 out of 10. In comparison, 27 out of 50 healthy controls (54%) responded. In HSCT recipients treated for BKV-related cystitis, absolute CD4+ T-cell numbers and renal function correlated with BKV-specific cellular responses (p = 0.03 and 0.01, respectively). In one patient, BKV-specific cellular immunity could already be detected at baseline, on day 35 after HSCT and prior to VSTs, and remained increased until day 226 after VSTs (78 vs. 7 spots increment). In conclusion, the ELISpot appears to be suitable to sensitively monitor BKV-specific cellular immunity in HSCT recipients, even early after transplantation or in the long term after VSTs.

Keywords: BK virus; hematopoietic stem cell transplantation; treatment with virus-specific T cells; immunosuppression; cidofovir; JC virus; CMV; EBV; monitoring of T-cell immunity; ELISpot

1. Introduction

Protection of hematopoietic stem cell transplant (HSCT) recipients from infection remains challenging owing to severe immunosuppression after transplantation. The BK virus (BKV), a DNA virus also called Betapolyomavirus hominis, is a cause of severe hemorrhagic cystitis and of nephropathy in patients treated with allogeneic HSCT [1]. BKV was first isolated in 1971 from the urine of a kidney transplant recipient with the initials B.K. The virus can be transmitted via smear infections with urine, droplet infection, or contaminated drinking water and, in the adult population, the infestation rate with BKV is greater than 80% [2]. In HSCT patients, the gastrointestinal tract could be identified

as a major persistence site, apart from the uroepithelium [3]. Fecal BKV excretion was detectable in 40% of these patients [3].

BKV remains persistent after primary infection and may reactivate during immunosuppression. In immunocompetent individuals, however, BKV infection is usually asymptomatic. Nevertheless, a study in 400 healthy blood donors showed that 7% shed the virus into the urine [4], indicating that BKV transiently escapes from immune control even in immunocompetent individuals [5]. In immunocompromised individuals, BKV replication increases in rate and magnitude, progressing to hemorrhagic cystitis and nephropathy in 5–50% of allogeneic HSCT recipients [6–8]. The incidence of BKV-related hemorrhagic cystitis after allogeneic HSCT is highly variable among adult (up to 50%) and pediatric recipients (up to 25%), especially in the setting of graft versus host disease (GvHD) [7,8]. Other studies reported a profound increase in the incidence of BKV-related hemorrhagic cystitis in the setting of allogeneic haploidentical HSCT and after utilizing posttransplant cyclophosphamide (PT/Cy) as GvHD prophylaxis. On the one hand, PT/Cy leads to direct damage of the bladder mucosa and, on the other hand, to delayed T-cell reconstitution with a deficiency of BKV-specific T cells in the circulation, resulting in BKV replication due to a lack of immune surveillance [9–12].

Several therapeutic approaches have been used for the treatment of BKV-related hemorrhagic cystitis. Reduction or cessation of immunosuppression has been considered an attempt to enhance anti-BKV immunity, but a favorable risk–benefit ratio in allogeneic HSCT recipients must be weighed against the risk of donor alloreactivity to the host, and the severity of GvHD must be considered. Alternatively, patients can be treated with the antiviral drug cidofovir, a cytosine derivative of an acyclic nucleoside-phosphonate analogue, which has broad-spectrum activity against many DNA viruses including BKV [13]. Of note, BKV does not have a DNA polymerase. However, treatment with cidofovir can lead to nephrotoxicity and neutropenia. Finally, virus-specific T cells (VSTs), which play a key role in the elimination of reactivated BKV infection, are a promising treatment option [14–21]. Whereas the generation and clinical impact of BKV-specific VSTs in the HSCT setting is now described by several groups [16–22], data on the monitoring of BKV-specific T cells in HSCT recipients are still scarce [17,19]. The majority of studies reported excellent clinical responses, with a decrease in viral load and symptomatic improvement in 74–100% of patients [16,17,19]. However, Holland et al. described one patient with severe cytokine release syndrome following BKV-specific VSTs [22]. Tzannou et al. and Olsen et al. determined BKV-specific cellular immunity either by ELISpot [17] or by intracellular cytokine assays [19] and presented follow-up data until month 3 after VSTs.

In the current study, we sequentially analyzed HSCT recipients with BKV-related cystitis, who were treated by a reduction of immunosuppressive drugs, with cidofovir and/or with VSTs. As compared with the previous studies on cellular responses in HSCT recipients after infusion of BKV-specific VSTs [17,19], follow-up was longer (up to 910 days, i.e., 30 months, after VSTs) and we show in parallel the time courses of BKV-specific T-cell immunity, viral load, and immunosuppressive medication. In contrast to the two previous studies [17,19], we monitored cellular BKV-specific immunity using IFN-γ ELISpot assays, where we compared responses to various BKV antigens. As five HSCT recipients received VSTs also directed against cytomegalovirus (CMV) and/or Epstein–Barr virus (EBV), we monitored cellular immunity against CMV and EBV in parallel.

It was our aim to compare the effect of VSTs and other treatment options especially on antiviral T-cell immunity, which was followed up over the long term. Moreover, we analyzed whether co-variates such as leukocyte subpopulations, immunoglobulins, or renal function correlated with BKV-specific cellular immunity. Finally, we assessed whether BKV-specific immunity differed between 17 HSCT recipients with dysuria and BKV-related cystitis, 5 HSCT recipients with dysuria but without BKV-related cystitis, and 50 healthy controls.

2. Materials and Methods

2.1. Volunteers

The study comprises 22 adults treated with allogeneic HSCT (median age 52 years, range 21–77), of whom 4 were female (Table 1). Two patients received their stem cell graft from a matched sibling donor, 4 from a human leukocyte antigen (HLA) haploidentical related donor, and 16 from an unrelated donor. All patients were recruited at the University Hospital Essen (Essen, Germany) when they presented with current or previous dysuria and agreed to participate in the study. Enrollment in the study—which comprised the monitoring of virus-specific cellular immunity—was offered to all patients in our outpatient clinic. In addition to measuring immunity to BKV, we also included JC virus (JCV) as a control because it can also cause cystitis or nephropathy and is structurally related to BKV [23,24]. The median interval between HSCT and BKV-related cystitis was 35 days (range 1–3359 days, i.e., 9.2 years), that between the onset of cystitis and the first ELISpot analysis was 28 days (range 0–973), and that between HSCT and study inclusion was 85 days (range 26 days–12 years). As a control group, we tested 50 age-matched, related HSCT donors (median age 49 years, range 21–66). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (19-9039-BO). Informed consent was obtained from all subjects involved in the study.

2.2. Determination of Antiviral Cellular Immunity by ELISpot

Nine milliliters of heparinized blood were collected and peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient centrifugation. The numbers of PBMC were determined by an automated hematology analyzer (XP-300, Sysmex, Norderstett, Germany). Duplicate cultures of 400,000 freshly isolated PBMCs were grown with and without three peptide mixtures of BKV (large T (LT) peptides from AID, Autoimmun Diagnostika GmbH, Strassberg, Germany; LT peptides from JPT Peptide Technologies GmbH, Berlin, Germany; peptides of viral protein 1 (VP1) from AID).

Table 1. Characteristics of 22 hematopoietic stem cell transplant recipients with cystitis.

ID	Sex	Age	MAC	ATG	Onset Cystitis	Days after Cystitis	BKV Cystitis	i.v. CIDO	Intravesical CIDO	VSTs
1	M	72	N	Y	18	66	Y	N	N	N
2	F	56	Y	Y	3359	973	Y	N	N	N
3	M	49	N	Y	44	212	Y	80 (2)	N	N
4	M	54	N	Y	18	67	Y	320 (1)	N	N
5	M	41	N	N	32	21	Y	110 (2)	N	N
6	M	70	Y	N	58	10	Y	240 (4)	N	N
7	F	24	N	N	677	15	Y	180 (4)	N	N
8	M	39	N	N	35	50	Y	230 (4)	N	N
9	F	27	Y	Y	57	67	Y	150 (3)	(1)	N
10	M	29	Y	N	22	93	Y	230 (5)	(4)	N
11	M	21	N	Y	13	63	Y	N	N	BCE (1)
12	M	37	N	N	78	19	Y	N	N	BCE (1)
13	M	50	N	N	10	47	Y	400 (3)	N	BCE (1)
14	M	77	N	Y	1	34	Y	240 (2)	N	B (3)

Table 1. *Cont.*

ID	Sex	Age	MAC	ATG	Onset Cystitis	Days after Cystitis	BKV Cystitis	i.v. CIDO	Intravesical CIDO	VSTs
15	M	65	N	N	12	14	Y	N	(1)	B (2)
16	M	62	Y	Y	96	5	Y	360 (1)	N	BC (1)
17	M	74	Y	Y	53	1	Y	160 (4)	(1)	BC (1)
18	M	57	N	Y	929	7	N	N	N	N
19	M	50	Y	Y	65	1	N	N	N	N
20	M	48	N	N	628	0	N	N	N	N
21	M	63	Y	Y	27	57	N	N	N	N
22	F	71	Y	N	50	7	N	N	N	N

Age—patient age at the time of the first ELISpot (inclusion into the study), MAC—myeloablative regimen, ATG—anti-thymocyte globulin, onset cystitis—start of (last) cystitis (days after HSCT), days after cystitis—first timepoint of ELISpot analysis (days after onset of cystitis), M—male, F—female, Y—yes, N—no. The dose of cidofovir (CIDO) that was applied intravenously (i.v.) for the first cycle of treatment is given in mg (cycles). In one patient, the dose for the subsequent cycles was reduced because of impaired renal function (#13). Intravesical treatment contained 375 mg cidofovir and the number of cycles is indicated in brackets. Virus-specific T cells (VSTs) were applied at a dose of 25,000 CD3+ T cells per cycle (1–3), either directed against BKV only (B), against BKV and CMV (BC), or against BKV, CMV, and EBV (BCE).

The LT peptide mix from AID contains 75 peptides and covers the complete LT antigen. It was used at a concentration of 1.3 µg/mL per 20 mer peptide. The LT peptides mix from JPT contains 170 peptides and also spans the complete antigen. Each of the 15 mer peptides, which overlap by 11 amino acids, was used at a concentration of 1 µg/mL. The VP1 antigen consists of 40 peptides and covers the complete antigen. Like the LT antigen from AID, it was used at a concentration of 1.3 µg/mL per 20 mer peptide.

For comparison, cells were stimulated with 1.3 µg/mL per 20 mer peptide of JCV (AID), which consists of six peptides. Of note, the JCV peptides, partly derived from VP1 of JCV, were selected using several tools predicting T-cell epitopes, e.g., http://tools.iedb.org/main/ accessed on 20 February 2023, and they are considered as highly specific. Nevertheless, owing to their localization, a cross-reaction with BKV cannot be ruled out completely [25]. The production of IFN-γ was determined using pre-coated ELISpot plates and a standardized detection system (T-Track® ELISpot kit, Mikrogen GmbH, Neuried, Germany). PBMCs were incubated in 150 µL AIMV medium (Gibco, Grand Island, NE, USA) at 37 °C for 1 day. Stimulation with the T-cell mitogen phytohemagglutinin (PHA, 4 µg/mL) served as a positive control. These conditions could be defined as optimal and were used if not stated otherwise. In order to optimize the ELISpot conditions, we used 250,000 and 400,000 freshly isolated PBMC per culture, titrated viral antigens (BKV LT peptide mix, 0.8–1.7 µg/mL per peptide, AID; BKV LT peptide mix, 0.1–10 µg/mL peptide, JPT), and performed the cell cultures for 1–3 days. BKV VP1 was not titrated, but used at the concentration recommended by the manufacturer, which was the same as for BKV LT (1.3 µg/mL).

In a subset of six HSCT recipients (of which five received also CMV- and/or EBV-specific VSTs), cellular responses to CMV and/or EBV were determined in parallel, with 200,000 PBMCs and an ELISpot protocol described previously [26]. We either used duplicates with two T-activated® CMV proteins, immediate early antigen-1 (IE-1) and phosphoprotein 65 (pp65), according to the manufacturer's instructions (T-Track® CMV, Mikrogen, Neuried, Germany), or an EBV lysate (whole virus, R02100, Meridian Bioscience, Cincinnati, OH, USA) plus the T-Track® ELISpot Basic Kit strip (Mikrogen, Neuried, Germany).

Colorimetric detection of cytokine secreting cells was performed according to the manufacturer's instructions (Mikrogen). Spot numbers were analyzed by an ELISpot reader (Fluorospot, AID, Autoimmun Diagnostika GmbH, Strassberg, Germany). Apart from considering individual concentrations of viral antigens, we determined the spots increment,

i.e., we determined median values of virus-specific responses and subtracted the median of negative (unstimulated) controls. The cut-off definition for positive responses was based on negative control values and on the consideration that threefold higher values for virus-stimulated versus unstimulated cells are frequently considered as a positive response in cellular assays. In HSCT recipients, the negative controls for IFN-γ had a median of 0.5 spots (range 0–8) and its threefold standard deviation was 3 $\times$ 1.57 spots = 4.71 spots (which we considered as the background of the negative controls). In healthy individuals, the negative controls for IFN-γ had a median of 0.5 spots (range 0–5) and its threefold standard deviation was 3 $\times$ 0.91 spots = 2.73 spots. Based on these numbers, we chose a value of at least five spots increment as the criterion for positivity. In all patients with at least a five spots increment, the virus-stimulated responses were more than threefold higher than the unstimulated controls.

2.3. Quantitative Real-Time PCR

Viral load for BKV was determined as described previously, using the RealStar BKV PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany) and the Light Cycler 96 system (Roche, Basel, Switzerland) [27]. CMV- or EBV-DNA was quantified with the fully automated Abbott m2000rt real-time PCR system using the Abbott RealTime CMV or EBV amplification reagent kit according to the manufacturer's instructions (Abbott, Wiesbaden, Germany). The manufacturer reported the lower limit of quantification (LLQ) as 360 IU/mL (BKV), 65 IU/mL (CMV), and 150 IU/mL (EBV).

2.4. Flow Cytometry

We collected whole peripheral blood samples from patients with BKV viremia and the samples were analyzed at the BMT Flow Cytometry Laboratory, University Hospital Essen. PBMCs were isolated using an automatic red blood cell lysing system (TQ-Prep, Beckman Coulter, Brea, CA, USA), washed with fluorescence-activated cell sorting buffer, and stained for surface markers. No intracellular staining was performed. Flow cytometric analysis of the patient's immune status was performed on a NAVIOS flow cytometer (Beckman Coulter) using the manufacturer's software. The following cell subsets within the CD45+ lymphocyte gate were characterized: T cells, CD3+; CD4+ T cells, CD3+/CD4+; activated T cells, CD3+/HLA-DR+; CD8+ T cells, CD3+/CD8+; naïve CD4+ T cells, CD3+/CD4+/CD45RA+; memory CD4+ T cells, CD3+/CD4+/CD45RO+; B cells, CD19+; NK cells, CD16+/CD56+; T-cell receptor α/β, TCR α/β; T-cell receptor γ/δ, TCR γ/δ; regulatory ($Treg^{low}$) CD4+ T cells, CD3+/CD4+/CD25+/CD127+low; effector ($Treg^{high}$) CD4+ T cells, CD3+/CD4+/CD25-/CD127+high. All antibodies were obtained from Beckmann Coulter (Krefeld, Germany), except for antibodies against TCR α/β and TCR γ/δ, which were from Miltenyi Biotec (Bergisch Gladbach, Germany). For the discrimination of live and dead cells, samples were incubated with 7-aminoactinomycin D (7-AAD, BD Biosciences, Heidelberg, Germany) directly prior to analysis.

2.5. Detection of Immunoglobulins and Soluble Interleukin 2 Receptor (sIL2R)

In order to quantify immunoglobulins of the classes IgA, IgE, IgG, and IgM and the level of soluble interleukin 2 receptor (sIL2R) in patients after HSCT, Immunoglobin Isotyping and IMMULITE® 2000 IL2R System (Siemens Healthcare Diagnostics GmbH, Erlangen, Germany) was used according to the manufacturer's instructions.

2.6. Preparation of Virus-Specific Donor T Cells for Adoptive Transfer

We selected partially HLA compatible third-party donors (8/10 and 6/10 HLA low resolution (single-field resolution); 6/10 and 6/10 high resolution (two-field resolution)) from the pre-examined T-cell donor registry alloCELL (www.allocell.org accessed on 20 February 2023). In addition to the HLA type of the patient, we considered the HLA type of the donor and excluded the mismatched HLA antigens to avoid patient-specific immunization. Donor pretesting was performed using IFN-γ Cytokine Secretion Assay

(CSA) as described, with overlapping peptide pools covering LT and VP1 proteins of BKV as well as CMVpp65 and with EBV EBNA-1/EBV-Select (all Miltenyi Biotech, Bergisch Gladbach, Germany) [28,29]. Manufacturing of clinical-grade BKV-specific CD4+ and CD8+ T cells (VSTs) was performed on a CliniMACS Prodigy device using MACS GMP Peptivators LT and VP1 in combination and the IFN-γ Cytokine Capture System (Miltenyi Biotech, Bergisch Gladbach, Germany). For multivirus-specific T-cell products, the GMP peptide pools CMVpp65 and EBV EBNA-1/EBV-Select were additionally used (all Miltenyi Biotech). Sterile (aerobic and anaerobic) and quality controls such as the measurement of viability and determination of CD3+/IFN-γ+/− T-cell counts were performed on both the starting material and the final products, as described [28–31]. The patients received fresh and cryopreserved VSTs from a single manufacturing process each. The median viability of total CD3+ T cells prior to treatment was 90% (range 63–96%), as determined by 7-AAD staining. Every cycle of VSTs contained 25,000 CD3+ T cells/kg body weight, which were monospecific in two patients (against BKV), bi-specific in two patients (against BKV and CMV), and tri-specific in three patients (against BKV, CMV, and EBV).

2.7. Statistical Analysis

Data were analyzed using GraphPad Prism 8.4.2.679 (San Diego, CA, USA). ELISpot responses at the first time point (first dataset) and maximum responses of HSCT patients were compared by Wilcoxon matched pairs test. Spearman test was used to correlate ELISpot results with numerical variables and the Mann–Whitney test was used to analyze the impact of categorical variables. If not otherwise stated, median values are indicated. Two-sided p-values <0.05 were considered significant.

3. Results

3.1. ELISpot Responses in Patients with BKV-Related Cystitis and Control Patients

Titration experiments and time kinetics could define 400,000 PBMCs per 1-day cell culture, 1.3 μg/mL per peptide of BKV LT (AID), or 1 μg/mL per peptide of BKV LT (JPT) as optimal for clinical application (Appendix A). BKV VP1 and JCV were used as recommended by the manufacturer, without titration. Applying these conditions, we compared 17 HSCT patients with BKV-related cystitis to five HSCT patients with cystitis that was not caused by BKV, who served as controls. In patients with and without BKV-related cystitis, the responses to the BKV LT peptides were overall at a similar level (Appendix B, Figure A2). However, in HSCT recipients with BKV-related cystitis, responses to BKV VP1 tended to be higher than in the controls without BKV. Vice versa, in recipients with BKV-related cystitis, responses to JCV tended to be lower than in patients without BKV-related cystitis (median of 0 vs. 5 spots increment). Two patients without BKV-related cystitis had 11 and 90 spots increment after stimulation with JCV peptides, indicating that cystitis could have been caused by another polyomavirus like JCV.

3.2. Assessment of Treatment Responses

We divided the 17 HSCT patients with BKV-related cystitis by treatment group (with reduction of immunosuppression only, with cidofovir only, with VSTs only, and with cidofovir and VSTs; Table 2) and assessed whether viral load decreased and/or T-cell responses were detectable. As BKV LT from JPT yielded overall the most robust T-cell response and was available throughout the whole study, the further evaluation of T-cell responses is related to this antigen. In the two patients with reduction of immunosuppression only, we detected a decrease in viral load in serum and/or urine and measured BKV-specific T cells. All eight patients treated with cidofovir only showed a reduction in the viral load. Half of them displayed specific T-cell responses. In the two patients treated with VSTs only, we observed a reduction in the viral load and could detect specific T-cell immunity. Five patients were treated with cidofovir and VSTs. Three of them showed a reduction in the viral load and four showed a BKV-specific T-cell response. Thus, six out of seven patients receiving VSTs had a detectable T-cell response, as did 6 out of 10 who did not receive VSTs.

Table 2. Assessment of treatment responses in 17 hematopoietic stem cell transplant recipients with BKV-related cystitis.

ID	Immuno-suppression ↓	CIDO	VSTs	CIDO + VSTs	VL ↓	T-Cell Response	Spots Increment	Day after HSCT
1	x				✓	+	31	183
2	x				✓	+	33	4332
3		x			✓	+	13	266
4		x			✓	∅	4	316
5		x			✓	∅	3	73
6		x			✓	∅	0	68
7		x			✓	∅	4	692
8		x			✓	+	239	85
9		x			✓	+	18	124
10		x			✓	+	32	115
11			x		✓	+	95	76
12			x		✓	+	5	106
13				x	✓	+	7	98
14				x	✓	+	184	167
15				x	∅	∅	1	48
16				x	✓	+	5	101
17				x	∅	+ *	10 *	54

CIDO–cidofovir, VSTs—virus-specific T cells, VL—viral load, spots increment—BKV-specific spots minus negative control, maximum of virus-specific IFN-γ spots after stimulation with BK virus (BKV) large T (LT) peptides (from JPT). The last column (day after HSCT—hematopoietic stem cell transplantation) indicates the timepoint at which T-cell responses were measured. With the exception of recipient #17, this T-cell response was measured after treatment. In recipient #17, VL remained above the upper limit of detection of 5 million IU/mL at day 5 and 11 after VSTs. * Spots prior to treatment. As spot numbers usually increased after treatment, it is assumed that the response after treatment should also be positive. Further information on patient treatment is given in Table 1 (ID #1–17) and more details on individual treatment responses are depicted in Figure 1. ↓—Reduction of immunosuppression or VL, x—This treatment was applied, ✓—BK viral load decreased, ∅—BK viral load did not decrease or BKV-specific T cells were undetectable after treatment, +—BKV-specific T cells were detectable after treatment.

3.3. Individual Time Courses of BKV-Specific ELISpot Responses and BK Viral Load

We present here individual courses of HSCT patients with BKV-related cystitis, including BKV-specific T-cell responses, BK viral load, antiviral treatment, immunosuppressive drugs, and time of transplantation and cystitis. The maximum follow-up was 1890 days (63 months) after the last reduction of immunosuppressive drugs, 1083 days (36 months) after treatment with cidofovir, 380 days (13 months) after VSTs, and 910 days (30 months) after VSTs plus cidofovir.

In two patients, only immunosuppression was reduced. In one of these HSCT patients (#1), in whom cystitis occurred at day 18 after transplantation (shown by a dotted vertical line), BKV-specific cellular immune responses increased to 31 spots increment after immunosuppression was reduced, at day 183 after HSCT (Figure 1a). Immunosuppressive treatment was tapered at day 355 after HSCT, when BKV-specific cellular responses could be detected. BK viral load in the urine decreased at the time when we observed the maximum cellular response. In the serum, BK viral load was always below the LLQ of 360 IU/mL. The second patient (#2) suffered from BKV-related cystitis on day 210 and 3359 (9.2 years) after HSCT (Figure 1b). After the first BKV infection, immunosuppression was reduced and, at the time of the second BKV infection, the patient did not receive any immunosuppressive therapy. On day 4332 after HSCT (i.e., 32 months after the last infection), we could detect strong BKV-specific cellular immunity (33 spots increment) and, at month 32 and 63 after the last infection, we detected the absence of BK viral load in urine and serum. Of note, as this patient suffered from late-onset cystitis, the cellular immune function cannot be compared to those patients with typical early-onset cystitis. The rather high spot number could thus also reflect the reconstitution of cellular immune function. As expected, his T-cell response was higher than the median of the total cohort.

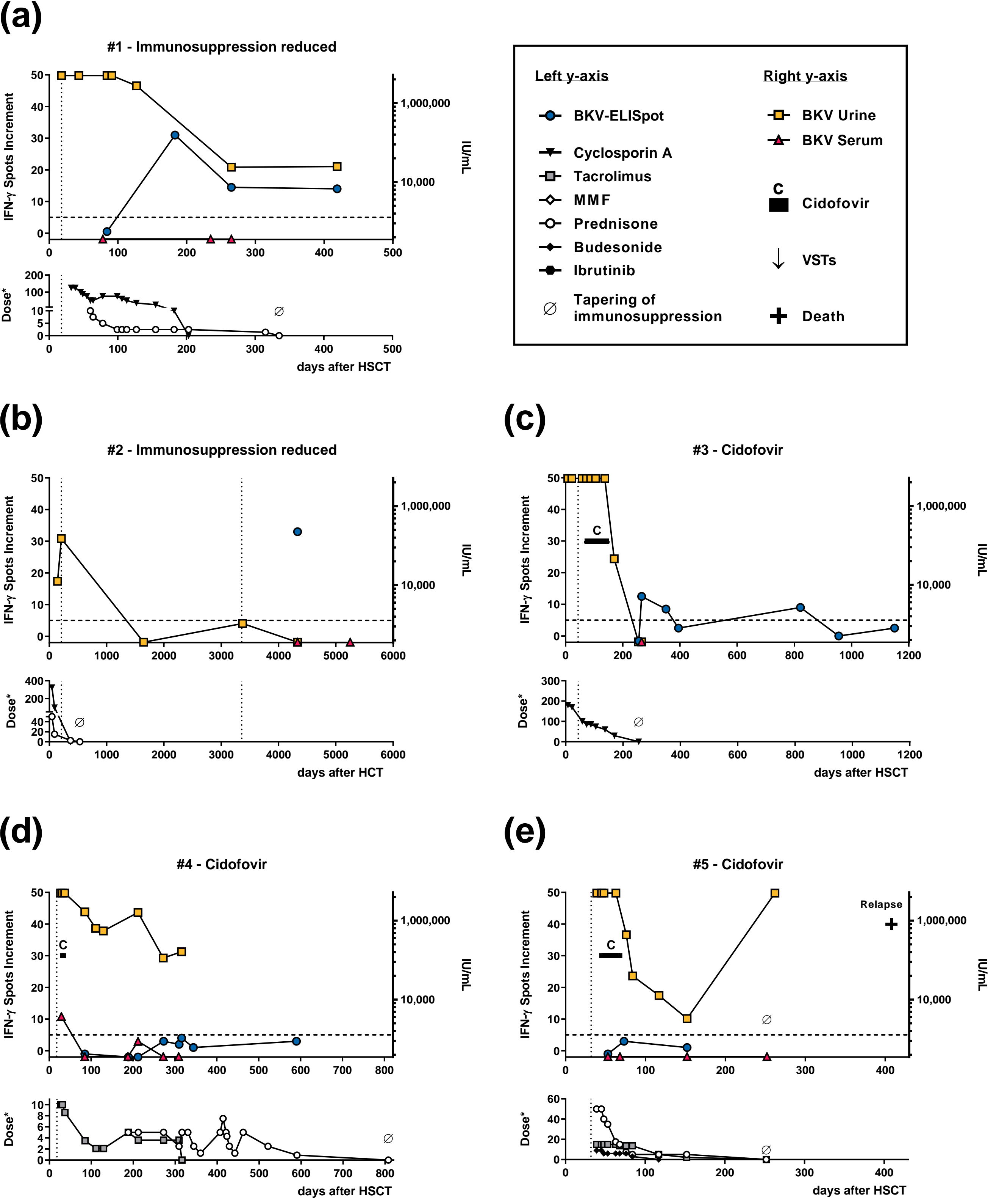
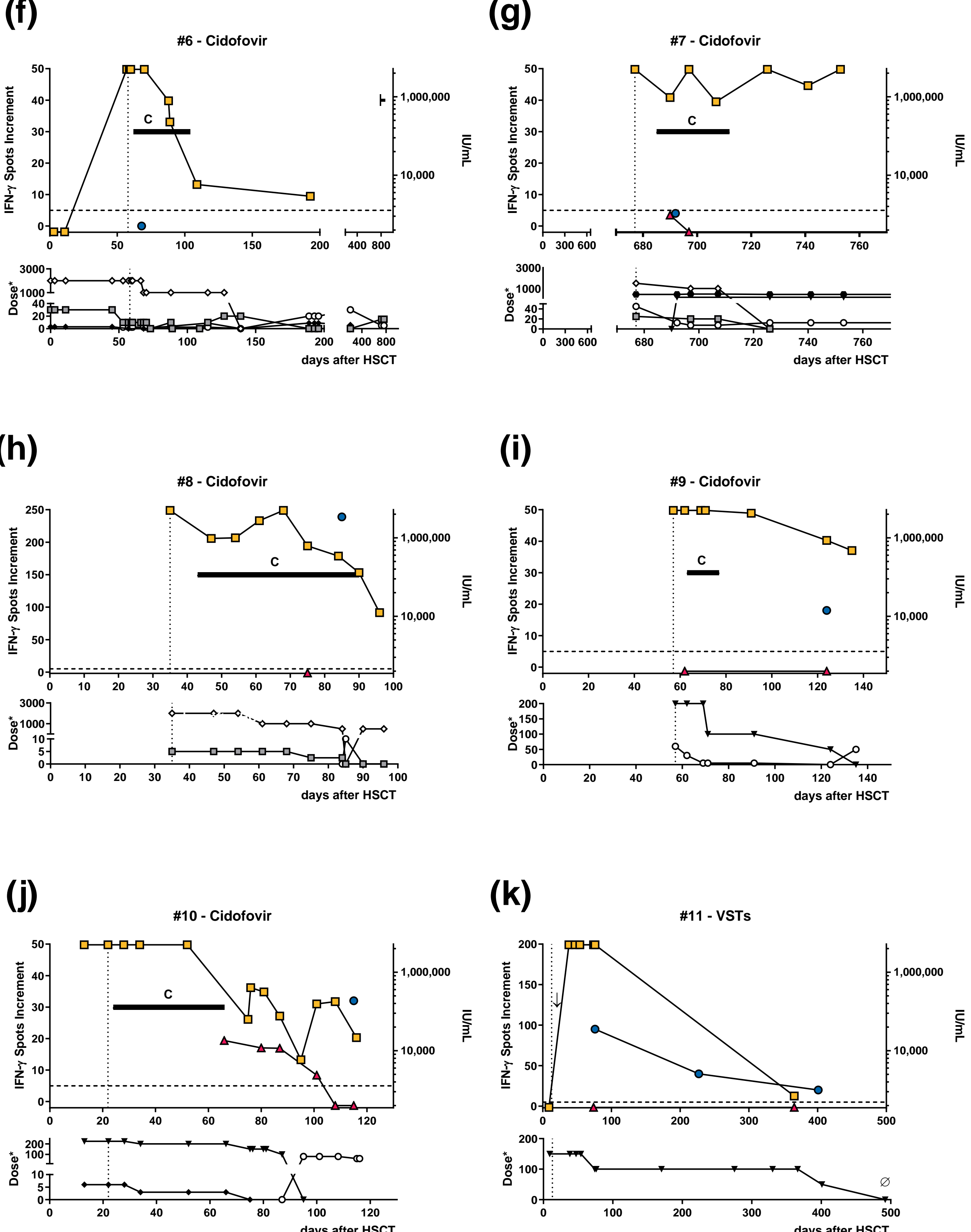


Figure 1. *Cont.*



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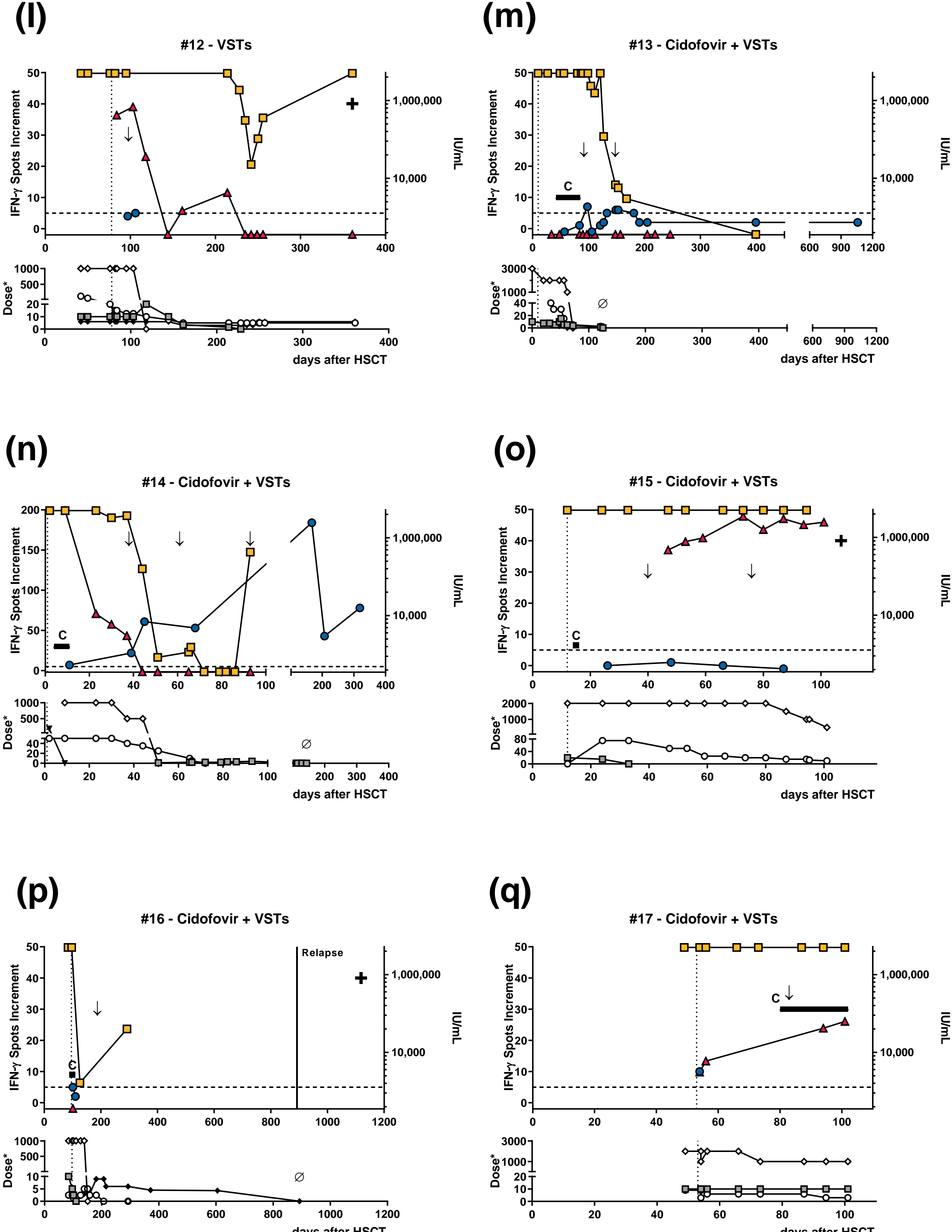


Figure 1. Follow-up data of hematopoietic stem cell transplant (HSCT) recipients who suffered from BK virus (BKV)-related cystitis. The dotted vertical line indicates the onset of BKV-related

cystitis. The patients were either treated only with a reduction of their immunosuppressive drugs (**a**,**b**); with cidofovir (**c**–**j**); with BK virus-specific T cells (VSTs), as indicated by an arrow (↓) (**k**,**l**); or with a combination of cidofovir and VSTs (**m**–**q**). Cellular responses towards BKV large T (LT) peptides (from JPT) are indicated on the upper left *y*-axis as spots increment, i.e., BKV-specific spots minus negative control. The dotted horizontal line indicates the cut-off for positive responses (five spots increment). The upper right *y*-axis shows the viral load in urine and serum. With one exception, the lower left *y*-axis indicates the dose of immunosuppressive drugs as mg per day (*). To be visible, the dose of tacrolimus is given as mg * 10 per day. MMF, mycophenolate mofetil.

Eight patients (#3–10) were treated with cidofovir and we detected BKV LT-specific cellular immunity (at least 5 spots increment) in four of these patients after treatment (#3 and #8–10) (Table 2, Figure 1c,h–j). Four of these eight patients received a graft from a haploidentical donor (#3, 6, 8, and 10). Of note, the patients with detectable BKV-specific immunity were the youngest patients and two of them were female. However, immunosuppression was usually reduced in parallel, and could thus also have augmented specific cellular immunity. In the first patient (#3), BKV-specific ELISpot responses (13 and 9 spots increment) were detectable on day 115 and day 670 (month 22) after treatment with cidofovir, i.e., on day 266 and 821 after HSCT (Figure 1c). The cellular responses were below the cut-off (maximum of three and four spots increment) in the other two patients (#4 and #5) (Figure 1d,e). BK viral load in the urine became undetectable (Figure 1c) or declined (Figure 1d,e) in the follow-up after treatment with cidofovir. BK viral load in the serum was either undetectable (Figure 1c,e) or could be observed only at a low level (Figure 1d). Unfortunately, one patient (#5) died as a result of relapse at day 408 after HSCT (Figure 1e). The remaining five patients (Figure 1f–j) were also tested for BKV-specific cellular immunity after having received cidofovir treatment for BKV-related cystitis. Whereas the patients tested at day 6 and 7 after the initiation of treatment, i.e., at day 68 and 692 after HSCT (#6 and #7, respectively), did not show BKV-specific cellular immunity (zero and four spots increment), those tested on days 42, 61, and 91 after the initiation of treatment (#8–10) displayed strong positive responses (239, 18, and 32 spots increment, respectively). The latter three patients were tested on days 85, 124, and 115 after HSCT.

One out of two patients who received VSTs but not cidofovir (#11) showed up to 95 spots increment to the ELISpot on day 55 after VSTs, which is on day 76 after HSCT (Figure 1k). In this patient, follow-up until day 401 after HSCT (day 380 after VSTs) indicated that BKV-specific cellular immunity declined, but was still rather strong (20 spots increment), although the patient received cyclosporin A. In the other patient receiving VSTs only (#12), cellular immunity was weakly positive on day 8 after receiving VSTs, i.e., on day 106 after HSCT (five spots increment) (Figure 1l). Whereas BK viral load decreased thereafter in the serum, the viral load in the urine remained high for more than 100 days. After a minimum on day 242 after HSCT, BK viral load in the urine increased again and the patient finally died on day 361 from septic shock due to *Pseudomonas* infection.

Five patients received cidofovir plus one to three doses of VSTs (#13–17). The first patient (#13) showed an increase in BKV-specific cellular immunity after he was treated with cidofovir and the first dose of VSTs, on day six after VSTs, i.e., day 98 after HSCT (Figure 1m). The maximum response was seven spots increment. After the second dose of VSTs, however, the increase in cellular immunity was only minor. Nevertheless, the BKV-specific response on day 910 after VSTs was still slightly higher that prior to VSTs (2 vs. −1 spot increment). In parallel, BK viral load in the urine decreased after VSTs. The second patient (#14) already showed BKV-specific cellular immunity at baseline, on day 35 after HSCT, and prior to VSTs (seven spots increment), which increased on day 35 after initiation of treatment with cidofovir (22 spots increment) and further increased after receiving the first and third dose of VSTs (61 and 184 spots increment, respectively) (Figure 1n). The data after the second dose, however, indicate no clear change. As compared with the baseline prior to VSTs, BKV-specific cellular responses were still increased on day 226 (month 7.5) after the third dose of VSTs, which was day 319 after HSCT (78 vs.

7 spots increment). Parallel to the increase in specific immunity, BK viral load in urine and serum declined. The third patient (#15) did not show BKV-specific immunity (maximum of one spot increment on day 48 after HSCT) (Figure 1o). Thus, despite treatment with cidofovir and two doses of VSTs, cellular immunity was absent and the BK viral load was very high. The patient finally died on day 107 after HSCT, also from septic shock due to *Pseudomonas* infection. The fourth patient (#16) showed only a weak effect of cidofovir treatment on BKV-specific cellular immunity (five and two spots increment on day 2 and 11 after cidofovir, respectively, i.e., on day 101 and 110 after HSCT) (Figure 1p). However, BK viral load in the urine was lower after treatment. On day 1119 after HSCT, the patient died from relapse. The fifth patient (#17, Figure 1q) suffered from BKV-related cystitis on day 53 after HSCT. He already showed BKV-specific cellular immunity prior to treatment with cidofovir and VSTs, on day 54 after HSCT (10 spots increment). BK viral load in the urine also exceeded the upper limit of detection of 5 million IU/mL on day 88 and 94 after HSCT, i.e., 8 and 14 days after initiation of treatment with cidofovir, respectively, and 5 and 11 days after VSTs, respectively. Unfortunately, we could not test BKV-specific cellular immunity after treatment.

Taken together, BKV-specific cellular immunity could be observed until month 32 after the last BKV-related cystitis in a patient with reduction of immunosuppressive drugs (patient #2), until month 22 after treatment with cidofovir (the last follow-up of patient #3 was month 36), until month 13 after VSTs (patient #11,) and until day 226 (month 7.5) after VSTs plus cidofovir (patient #14). In 12 out of 17 HSCT patients, BKV LT-specific cellular immunity was detectable before and/or after treatment for BKV-related cystitis. Two further patients showed increasing cellular responses after treatment with cidofovir (maximum of three and four spots increment). In the majority of cases, an increase in specific cellular immunity was observed parallel to a decrease in BK viral load. The patients who finally died (#5, #12, #15, and #16) all showed rather weak cellular responses to BKV LT (one to five spots increment). Two patients with poor responses to BKV LT (#7 and #15) showed a positive response to BKV VP1 peptides, either after treatment with cidofovir or with cidofovir and VSTs (14 and 210 spots increment, respectively). Patient #7 suffered from severe acute/chronic GvHD grade 3, respectively, and severe hemorrhagic BKV-associated cystitis. Under combined systemic immunosuppression with ibrutinib and biweekly extracorporeal photopheresis therapy, GvHD stabilized or showed an improvement, with concomitant regression of cystitis and an improvement in immune reconstitution. The patient with acute GvHD grade 3 who received PT/Cy (#15) developed severe BKV-associated cystitis with acute renal failure, having previously suffered from chronic kidney disease. Switching immunosuppression to a tacrolimus-free regimen and intravesical cidofovir therapy and VSTs resulted in symptom relief, but persistent dysuria.

3.4. Time Course of CMV- and EBV-Specific ELISpot Responses

Three patients (#11–13) received tri-specific VSTs and were followed up not only for their BKV-specific immunity, but also for their CMV- and EBV-specific immunity (Figure 2). In patient #11 (Figure 2a), the CMV viral load in whole blood intermittently increased and was undetectable since day 44 after VSTs. EBV viral load increased after VSTs and declined approximately three weeks thereafter. In the long term, we observed strong T-cell responses towards CMV pp65, CMV IE-1, and EBV lysate. In patient #12 (Figure 2b), CMV and EBV viral load in the blood showed median values below the lower limit of quantification (65 IU/mL for CMV and 150 IU/mL for EBV). However, both viruses were detectable intermittently throughout the whole follow-up period. Cellular responses to CMV pp65 and CMV IE-1 and to EBV were clearly detectable prior to and post VSTs. In patient #13 (Figure 2c), the CMV viral load in the blood became undetectable after VSTs' infusion and EBV was (nearly) undetectable throughout the study. After the infusion of two cycles of VSTs, we observed an increase in CMV-specific cellular immunity. Moreover, we observed strong cellular immunity towards EBV after both cycles. Thus, the three patients who received tri-specific VSTs all showed cellular responses against CMV and EBV. Two further

patients received bi-specific VSTs (against BKV and CMV), but were unfortunately not followed up for CMV-specific cellular immunity.

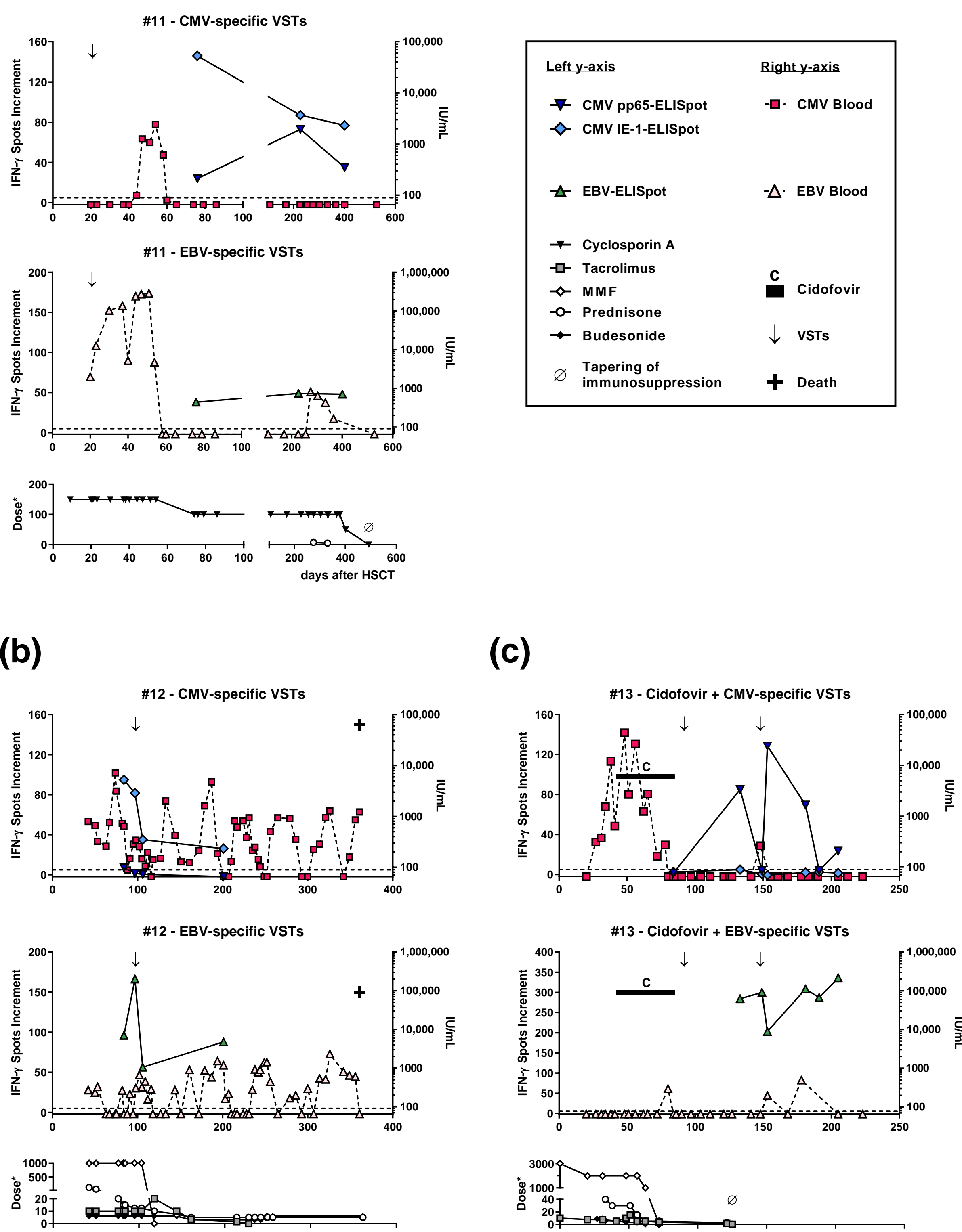


Figure 2. Follow-up data of hematopoietic stem cell transplant (HSCT) recipients who received tri-specific virus-specific T cells (VSTs), directed against BK virus, cytomegalovirus (CMV), and

Epstein–Barr virus (EBV). The graphs show cellular in vitro responses against CMV phosphoprotein 65 (pp65) and immediate early-1 (IE-1) and EBV and viral load of CMV and EBV. Panel (**a**) corresponds to the patient shown in Figure 1k, panel (**b**) corresponds to Figure 1l, and panel (**c**) corresponds to Figure 1m, where immunity against BKV is displayed. Infusion of VSTs is indicated by an arrow (↓). The dotted horizontal line indicates the cut-off for positive responses (five spots increment). Cellular responses are shown on the upper left *y*-axis as spots increment, i.e., CMV- or EBV-specific spots minus negative control. The upper right *y*-axis shows the viral load in whole blood. With one exception, the lower left *y*-axis indicates the dose of immunosuppressive drugs as mg per day (*). To be visible, the dose of tacrolimus is given as mg * 10 per day. MMF, mycophenolate mofetil.

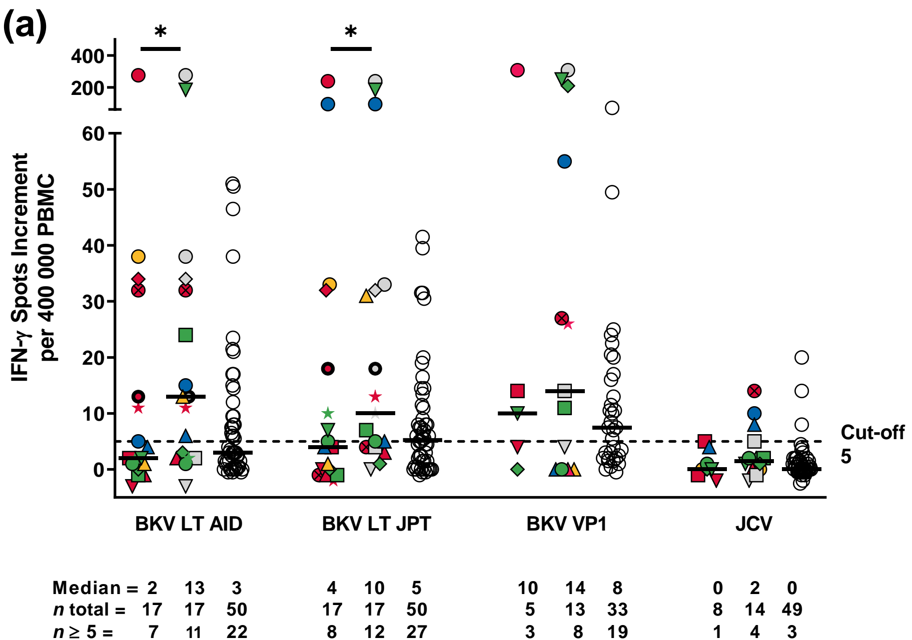
Moreover, we compared the strength of BKV-specific cellular responses in three patients who received tri-specific VSTs (against BKV, CMV, and EBV, patients #11–13), in one patient who received bi-specific VSTs (against BKV and CMV, patient #16), and in two patients who received monospecific VSTs (only against BKV, patients #14–15). We here considered the maximum response as displayed in Table 2, which was 95, 5, and 7 spots increment after tri-specific VSTs; five after bi-specific VSTs; and 184 and 1 after monospecific VSTs.

3.5. Correlation of BK-Virus-Specific Cellular Immunity with Co-Variates

Using the first dataset in each patient with BKV-related cystitis (first timepoint as indicated in Table 1), we analyzed whether patient covariates correlated with ELISpot responses towards BKV LT peptide (JPT), using 400,000 PBMC in a 1-day cell culture (Appendix B, Table A1). As expected, leukocyte numbers showed a wide range (Appendix B, Figure A3) and cell counts were dependent on the time to HSCT (Appendix B, Figure A4). Spearman analysis yielded a positive correlation with the absolute number of CD4+ T cells ($r = 0.52$, $p = 0.03$) and with the absolute number of effector CD4+ T cells ($r = 0.51$, $p = 0.04$). Moreover, the glomerular filtration rate correlated positively with ELISpot responses ($r = 0.64$, $p = 0.01$) and serum creatinine and urea correlated negatively ($r = -0.51$, $p = 0.04$ and $r = -0.52$, $p = 0.03$, respectively). However, the time to HSCT only by trend correlated with BKV-specific ELISpot responses ($r = 0.28$, $p = 0.3$).

3.6. Comparison of BKV-Specific ELISpot Responses in Hematopoietic Stem Cell Transplant Recipients with BKV-Related Cystitis and Healthy Controls

Using BKV LT and VP1 peptides as well as JCV peptides as stimuli, median responses in HSCT patients with BKV-related cystitis and healthy controls were similar, when considering the first dataset each (Figure 3a). In 8 out of 17 patients with BKV-related cystitis (47%) and in 27 out of 50 controls (54%), cellular responses to BKV LT (JPT) were detectable. The respective numbers for BKV VP1 were 3 out of 5 (60%) and 19 out of 33 (58%). Considering the maximum response each, usually after treatment for BKV cystitis, these numbers increased in the HSCT recipients to 12 out of 17 (71%) for BKV LT (JPT) ($p < 0.05$, compared with the first dataset) and to 8 out of 13 (62%) for BKV VP1. The median spots increment in the first dataset were 4 for BKV LT (JPT) and 10 for VP1, at maximum the respective numbers were 10 and 14.



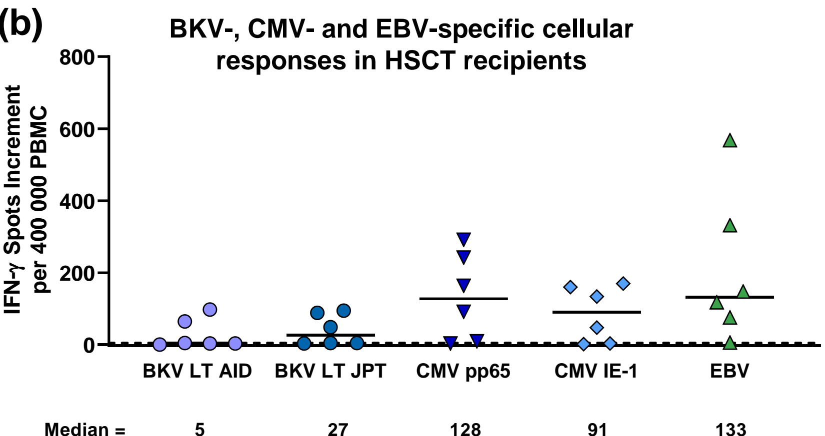


Figure 3. Specific cellular immunity against peptides of the BK virus (BKV). (**a**) Responses against large T (LT) and viral protein (VP) 1 peptides of BKV and against a structurally related polyomavirus, the JC virus (JCV), in 17 hematopoietic stem cell transplant (HSCT) recipients with BK virus-related cystitis (coloured symbols) and in 50 healthy controls (HC, white circles). The first dataset in each patient is shown as the left panel, with the maximum response as the middle panel. Each patient is depicted by an individual symbol. Treatment groups are colour-coded (yellow: with reduction of immunosuppression only, red: with cidofovir only, blue: with VSTs only, and green: with cidofovir and VSTs). For patients tested only once, the first set of data is equal to the maximum response, which is shown as a grey symbol (and which is not considered for comparison of the first and maximum response). Please note that only part of the datasets contained VP1 and JCV. One-day cell cultures were performed with 400,000 peripheral blood mononuclear cells and the antigen concentrations defined as optimal. (**b**) Specific cellular immunity against LT peptides of BKV, cytomegalovirus (CMV) phosphoprotein 65 (pp65), CMV immediate early-1 (IE-1), and Epstein–Barr virus (EBV), analyzed in parallel in six HSCT recipients with BKV-related cystitis. Each viral antigen is indicated by a specific symbol and color. Median values are indicated with solid horizontal lines. The dotted horizontal line indicates the cut-off for positive responses (five spots increment). * $p < 0.05$ (Wilcoxon matched pairs test). BKV LT AID—BKV large T peptides (from AID), BKV LT JPT—BKV large T peptides (from JPT), BKV VP1—BKV viral protein 1 peptides.

3.7. Correlation of ELISpot Responses to Various BK Virus and JC Virus Peptide Pools

Spearman analysis indicated that, in 17 HSCT recipients with BKV-related cystitis and in 50 healthy controls, responses to the BKV LT and VP1 peptide pools showed a highly significant correlation ($r > 0.7$, $p < 0.0001$) (Appendix B, Table A2). However, in both cohorts, we could not observe correlation between responses to BKV and JCV (r = 0.12–0.25 and p = 0.2–0.6, depending on the BKV peptide mix), arguing against major cross-reactivity. Similarly, the responses to BKV and to the positive control showed no correlation in the HSCT recipients (r = −0.15 to −0.08), but weak positive correlation in the healthy controls (r = 0.15 to 0.40, p = 0.03 to 0.03). Thus, in HSCT patients, higher cellular responses against BKV did not correlate with a generally stronger T-cell function (response against the T-cell mitogen PHA).

3.8. Comparison of ELISpot Responses to BK Virus and Herpes Viruses

To further assess the strength of cellular responses, we stimulated PBMCs from six HSCT recipients in parallel with BKV, CMV pp65, CMV IE-1, and EBV antigens. To allow a direct comparison, we determined spot numbers per 400,000 PBMCs. As compared with responses to BKV LT (JPT), responses to CMV pp65, CMV IE-1, and EBV were approximately five-, three-, and fivefold higher, respectively (Figure 3b).

4. Discussion

The current study indicates that more than two-thirds of the HSCT recipients treated for BKV-related cystitis displayed detectable BKV-specific cellular responses, as determined by IFN-γ ELISpot. In recipients treated with VSTs, 6 out of 7 displayed specific T-cell responses to BKV LT and that number in those without VSTs was 6 out of 10. As shown in patient #14, BKV-specific cellular immunity could be detected as early as day 35 after HSCT (before VSTs) and remained elevated (78 vs. 7 spots increment) until day 226 after VSTs, indicating the high sensitivity of the ELISpot assay in the HSCT setting too.

Two previous studies on HSCT recipients treated with BKV-specific VSTs reported monitoring of specific cellular immunity [17,19]. Tzannou et al. [17] performed BKV-specific ELISpot assays until week 12 after infusion of BKV-specific VSTs and observed that 7 out of 16 HSCT patients (44%) showed an increase in specific T-cell responses. If we change our cut-off for positive reactions and take 10 spots increment instead of 5, four patients with weak positive responses would have been classified as negative, which results in a positivity rate of three out of seven, similar to the previous data [17]. However, two of these four patients (#12 and # 13) showed a decrease in viral load after VSTs, indicating BKV-specific T-cell immunity. In four of the patients from the previous study [17], follow-up data until week 3, 4, 6, and 12 after VSTs were presented, respectively, indicating an overall declining frequency of VSTs. After stimulation with epitope-specific peptides, Tzannou et al. [17] detected BKV-specific T cells derived from the VSTs in three patients at the latest time point of follow-up, at week 3, 4, and 12. However, in the fourth patient, VSTs were no longer detectable at week 6. Moreover, Olsen et al. [19] measured the frequency of BKV-reactive T cells by intracellular cytokine assays in 32 patients and presented data until month 3 after VSTs. They showed a peak in spot numbers at day 28. Using flow chimerism assays in three patients, they could further detect T-cell responses derived from the infused BKV-VSTs for up to three months. Extending these previous data, the current study presents a longer follow-up in a subset of patients, where BKV-specific cellular immunity was monitored up to day 910 after VSTs (patient #13). In this patient, at least five spots increment were found until day 30 after VSTs. At day 43, however, numbers declined to two spots increment. This number remained constant until day 910 and was higher than the baseline, prior to VSTs (−1 spot increment). In patient #11, we could still detect BKV-specific T cells on day 380 (week 54) after VSTs (20 spots increment). Moreover, in patient #14, we detected BKV-specific T cells at day 226 (week 32) after VSTs (78 spots increment), when spot numbers were still 11-fold higher than at baseline prior to VSTs. Thus, our data show that, after infusion of VSTs, the frequency of BKV-specific T cells increased for more than eight months

(patient #14), possibly even for more than 2.5 years (patient #13). However, unlike the previous studies [17,19], we did not determine if cellular BKV immunity was derived from the VSTs. Nevertheless, long-term BKV-specific T-cell immunity—either adoptively transferred or by the patient's own immune system—should control viral replication.

The BKV-related cystitis often occurs between 2 and 12 weeks (up to months) after HSCT and typically starts in the peri-engraftment period, when cystitis is caused by the toxicity of the conditioning regimen, e.g., with cyclophosphamide or total body irradiation [14]. Other viruses such as CMV, herpes simplex virus, adenovirus, and JCV, as well as bacterial infections and non-infectious etiologies (especially hemorrhage, catheter injury, and so on), should be considered as additional causes. Moreover, subclinical urotoxic exposure may damage the urothelial cell layer, causing local inflammation, favoring BKV replication owing to impaired antiviral immune control by cytotoxic T cells [32]. In particular, unrelated donor and haploidentical transplants, as well as transplant-associated complications such as GvHD, contribute to BKV pathogenesis, because of altered allogeneic immunity [33]. At many centers, the current management of BKV-related cystitis includes testing for viral reactivation only in symptomatic cases and in the presence of risk factors of BKV-related cystitis. Owing to the limited prophylactic and therapeutic options and the lack of an effective, clinically validated antiviral drug for the treatment of BKV-associated cystitis, a reliable diagnosis of specific antiviral immunity is very relevant. Of note, the two patients who were only treated with a reduction of immunosuppression both showed specific cellular responses thereafter. Although BKV viremia can predict cystitis, the positive predictive value of viremia remains low or uncertain. Recently, Laskin and coworker showed that screening for BKV viremia in children and young adults after HSCT identifies asymptomatic recipients at risk for kidney disease and reduced survival. Their data suggest potential changes to clinical practice, including prospective monitoring for BKV viremia, and to test for virus-specific T cells, in order to identify early BKV replications [15].

ELISpot was suitable to detect BKV-specific immunity already on day 35 after HSCT (patient #14), when T-cell immunity is usually considered as severely impaired. In the HSCT patients, median T-cell responses towards BKV were similar to responses in healthy controls, considering the first dataset. Of note, cellular responses towards BKV LT and VP1 peptides in 50 healthy controls in the current study were in a similar range to previous reports including 10 healthy individuals [34] (5 and 8 spots increment vs. 10 and 10 spots increment per 400,000 PBMCs, respectively). After treatment for BKV-related cystitis, the rate of positive cellular responses increased in the HSCT recipients, most likely because a decreased dose of immunosuppressive drugs enhanced specific T-cell responses and infused VSTs persisted. Interestingly, in HSCT patients, the strength of T-cell responses against BKV did not correlate with a generally stronger T-cell response. The cellular response to BKV LT peptides (JPT) and to the T cell mitogen PHA even showed a negative correlation coefficient ($r = -0.08$). It may be assumed that BKV persistence is favored by generally weaker T-cell function and that BKV-related cystitis could have induced BKV-specific T-cell responses. In accordance with this assumption, we previously observed a similar phenomenon for CMV-specific cellular immunity [35]. The percentage of positive CMV-specific cellular responses was higher in HSCT recipients as compared with healthy controls. However, there is a second factor that influences cellular immune function in the opposite direction—the reconstitution of T-cell numbers and function after HSCT, which includes BKV-specific cellular immunity. Stervbo and coworker demonstrated in kidney transplanted patients an association between the resolution of BKV reactivation and reconstitution of BKV-specific CD4+ T cells. They followed T-cell receptor (TCR) single clone levels with multi-parameter flow cytometry and next-generation sequencing (NGS)-based CDR3 beta chain receptor repertoire analysis, and showed that the TCR repertoire diversity and exhaustion status of BKV-specific T cells affected the duration of viral clearance [36]. In line with these data, we found that the number of CD4+ T cells and effector CD4+ T cells significantly correlated with the magnitude of BKV-specific cellular responses, indicating their direct involvement in the antiviral cellular responses that we measured by ELISpot. Of note, CD4+ T cells are

a major source of IFN-γ production and, as we used 15 and 20 mer peptides, it is most likely that CD4+ T cells were detected by our ELISpot assay. Supporting this assumption, Wilhelm et al. [37] showed an expansion of IFN-γ CD4+ T cells after stimulation with 15 and 27 mer BKV peptides. However, as the ELISpot assay used PBMCs and not T-cell subpopulations, we cannot prove this hypothesis. As expected, T-cell immunity in patients with better kidney function was stronger, as has been observed previously in kidney transplant recipients after vaccination [38]. Adoptively transferred VSTs generated from eligible donors could provide broad antiviral protection to recipients of HSCT, including infections from BKV or other virus-related pathogens with low side effects, and appears to be an effective approach to treat severe viral infection [17].

Of note, although BKV-specific immunity was clearly detectable in a subset of HSCT recipients, it was approximately three- to fivefold lower than CMV- or EBV-specific cellular immunity. In parallel to an increase in BKV-specific immunity, the BK viral load usually declined, which indicates that the T cells should be functionally active. In two out of three patients, a similar phenomenon was observed for CMV- and EBV-specific T-cell immunity and viral load (Figure 2a,c).

According to previous data, BKV viruria is detected in approximately 40% of patients after allogeneic HSCT [14,15,39]. In our current cohort from Essen, however, we observed BKV-related cystitis in less than 10% of the patients. Cyclosporin A, which may cause or aggravate cystitis, is rarely used as an immunosuppressive treatment at our center. It is possible that additional patients suffered from BKV infection, but not from BKV-related cystitis. Supporting this consideration, 6 out of 17 patients with BKV-related cystitis were treated with cyclosporin A.

The rather low patient number and the variation in the time points of measurement are a clear limitation of this study. Nevertheless, we believe that the data are of interest as we present a long-term follow-up and comprehensively describe not only ELISpot data, but also viral load, antiviral treatment, immunosuppressive drugs, and timing of transplantation and cystitis.

The current study indicates that a peptide pool of the BKV LT protein is a suitable stimulus for cellular in vitro assays in patients after HSCT. However, it may be reasonable to use various BKV antigens (LT and VP1) when assessing cellular immunity. In our cohort, a combined evaluation would have resulted in an increased frequency of positive BKV-specific cellular responses. VP1 appears as immunodominant in a subset of HSCT patients with BKV-related hemorrhagic cystitis.

5. Conclusions

Using an IFN-γ ELISpot assay, we detected BKV-specific cellular immunity in six out of seven HSCT patients with BKV-related cystitis treated with specific VSTs. ELISpot was highly sensitive. It was able to detect BKV-specific cellular immunity even early after transplantation and to measure increased specific immunity for more than seven months after VSTs. We thus propose ELISpot as a response marker in this patient cohort, in which we observed the median onset of BKV-related cystitis on day 35 after HSCT. BKV-specific cellular responses correlated with the absolute number of CD4+ T cells and effector CD4+ T cells, renal function, and general clinical condition.

Author Contributions: Conceptualization, M.K., P.A.H. and M.L.; Data curation, M.L.; Formal analysis, M.K. and M.L.; Funding acquisition, M.K. and P.A.H.; Methodology, B.E.-V., B.M.-K., U.D. and M.L.; Project administration, M.K. and M.L.; Resources, M.K., B.E.-V., B.M.-K. and N.K.S.; Supervision, M.K. and M.L.; Visualization, M.L.; Writing—original draft, M.K. and M.L.; Writing—review and editing, M.K., B.E.-V., B.M.-K., N.K.S., U.D., P.A.H. and M.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (19-9039-BO).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available owing to privacy restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Initial titration experiments were performed with 250,000 PBMCs from HSCT recipients at 0.8–1.7 µg/mL per peptide of BKV large T (LT) from AID in 1-day cell cultures and showed a maximum response at a concentration of 1.3 µg/mL; this is the manufacturer's recommended concentration (Figure ??a). Titration of 250,000 and 400,000 PBMCs with 0.1–10 µg/mL per peptide of BKV LT (JPT) in 1–3-day cell cultures indicated the strongest response for 400,000 PBMCs, a concentration of 1 µg/mL and 1–2-day cell cultures (Figure ??b). Again, the titration experiments were able to reconfirm the manufacturer's recommended peptide concentration. Using the optimal peptide concentrations, we found that 1-day and 2-day cell cultures with 400,000 PBMCs gave similar results (Figure ??c,d), and we decided to use 1-day cultures for further experiments, which is more convenient for clinical use.

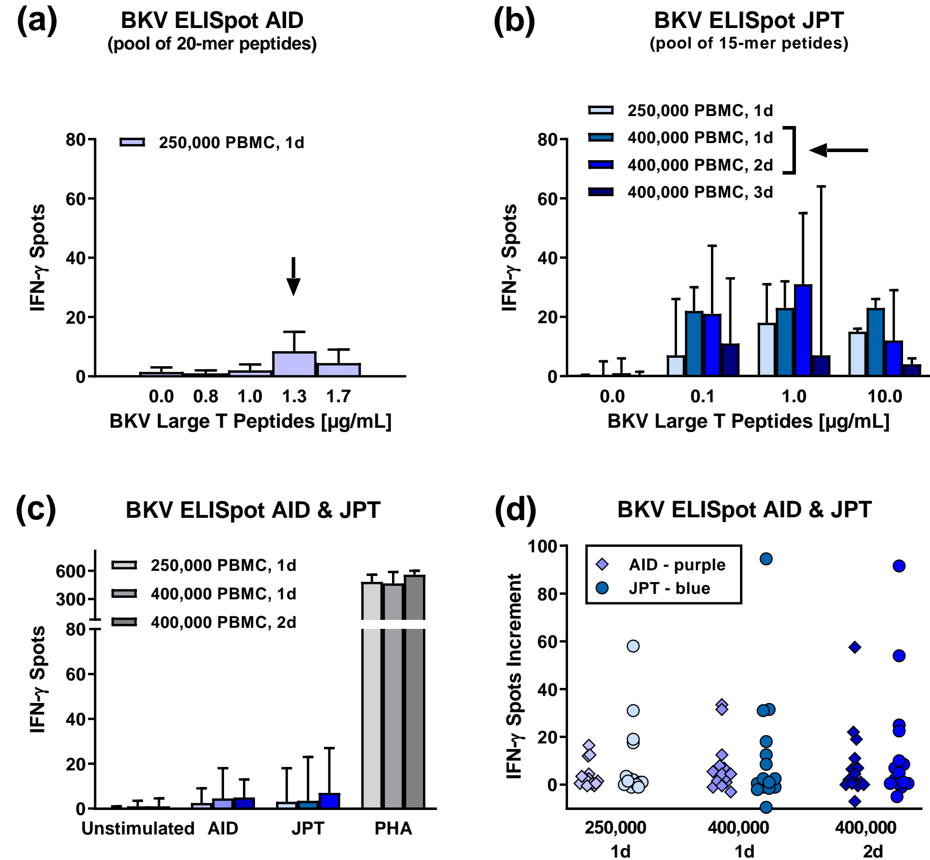

Figure A1. Optimization of ELISpot conditions to determine specific cellular immunity against BK virus (BKV) in patients after hematopoietic stem cell transplantation. Panel (**a**) shows the titration of 20 mer peptides of BKV large T (AID), using 250,000 peripheral blood mononuclear cells (PBMCs) in

1-day cell cultures (n = 2); panel (**b**) shows the titration of a 15 mer peptide of BKV large T (JPT) with 250,000 to 400,000 PBMCs in 1–3-day cell cultures (n = 3); and panel (**c**) shows data from 250,000 and 400,000 PBMCs and various culture durations with both BKV peptides used at optimal concentrations (1.3 and 1 µg/mL per peptide, respectively, indicated by an arrow) (n = 15). Each left column indicates data on 250,000 PBMCs and 1-day cell cultures, each middle column on 400,000 PBMCs and 1-day cell cultures, and each right column on 400,000 PBMCs and 2-day cell cultures. The graphs (**a**–**c**) show the median and 75% percentile. Panel (**d**) is based on the data as displayed in panel (**c**). It indicates individual values, given as spots increment, i.e., response towards BK virus (BKV) large T peptides (from AID or JPT) minus unstimulated controls (n = 15). PHA, phytohemagglutinin (positive control). In panel (**c**) and (**d**) the brightest color (either grey, blue or purple) indicates 250,000 PBMC and 1d culture, the intermediate color 400,000 PBMC and 1d culture and the darkest color 400,000 PBMC and 2d culture.

Appendix B

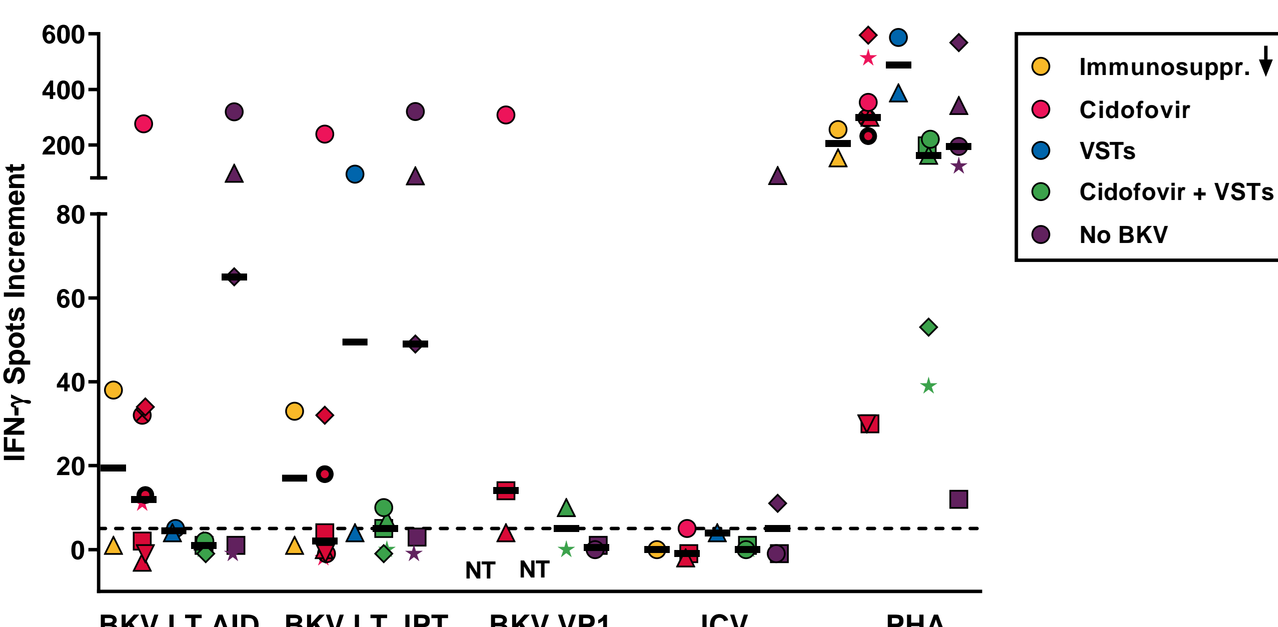

Figure A2. Cellular responses towards BK virus (BKV) large T (LT) peptides (from AID or JPT), as well as towards BKV viral protein 1 (VP1) and JC virus (JCV) peptides. Cell cultures stimulated with phytohemagglutinin (PHA) served as a positive control. The graph shows data on the first timepoint in hematopoietic stem cell transplant recipients with BKV infection, in whom only immunosuppression was reduced (n = 2), who was treated by cidofovir (n = 8), who received virus-specific T cells (VSTs) (n = 2), and who received cidofovir plus VSTs (n = 5). For comparison, we present the results of transplant recipients who suffered from cystitis that was not related to BKV (control group, n = 5). Each patient is depicted by an individual symbol. The values correspond to the first timepoint, as shown in Figure 1, which is a median of 28 days after the onset of cystitis. Data are indicated as spots increment, i.e., BKV-specific spots minus negative control. Solid horizontal lines indicate median values. The dotted horizontal line indicates the cut-off for positive responses (five spots increment). NT, not tested. BKV LT AID—BKV large T peptides (from AID), BKV LT JPT—BKV large T peptides (from JPT), BKV VP1—BKV viral protein 1 peptides.

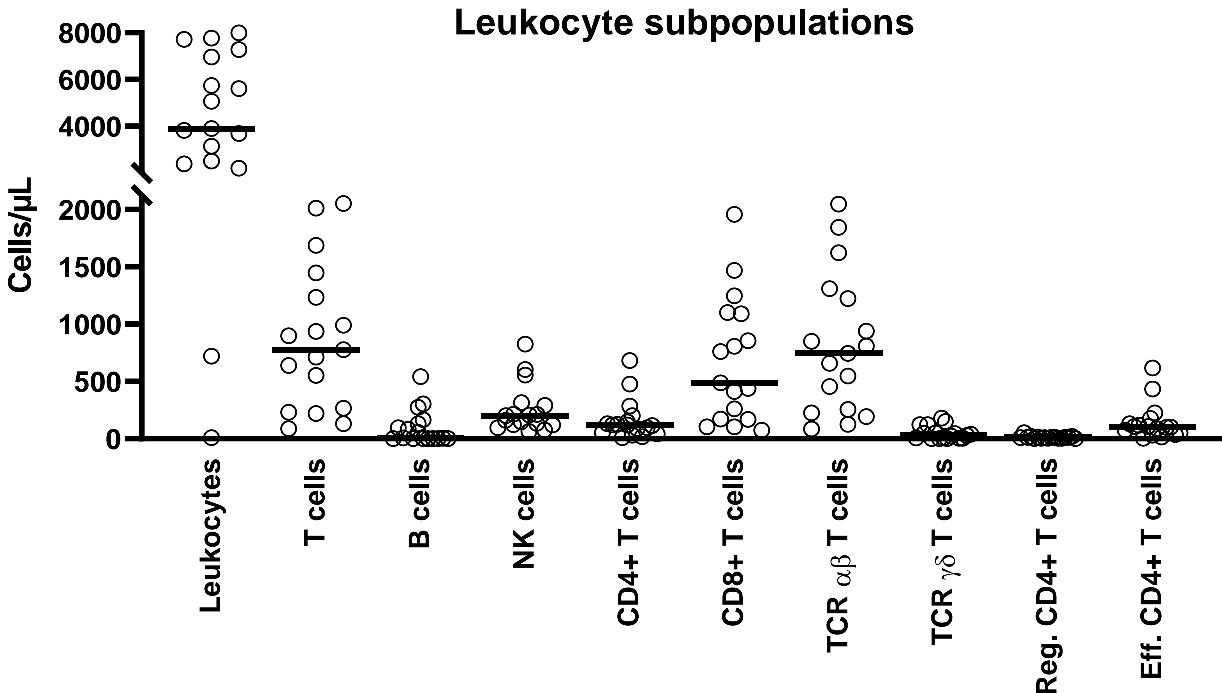

Figure A3. Distribution of leukocyte subpopulations in the patient cohort shown in Table A1. This figure contains the first dataset of each HSCT recipient with BKV-related cystitis. The median interval between HSCT and study inclusion (first dataset) was 85 days (range 26 days–12 years). Median values are indicated by horizontal lines. Reg.—regulatory, Eff.—effector.

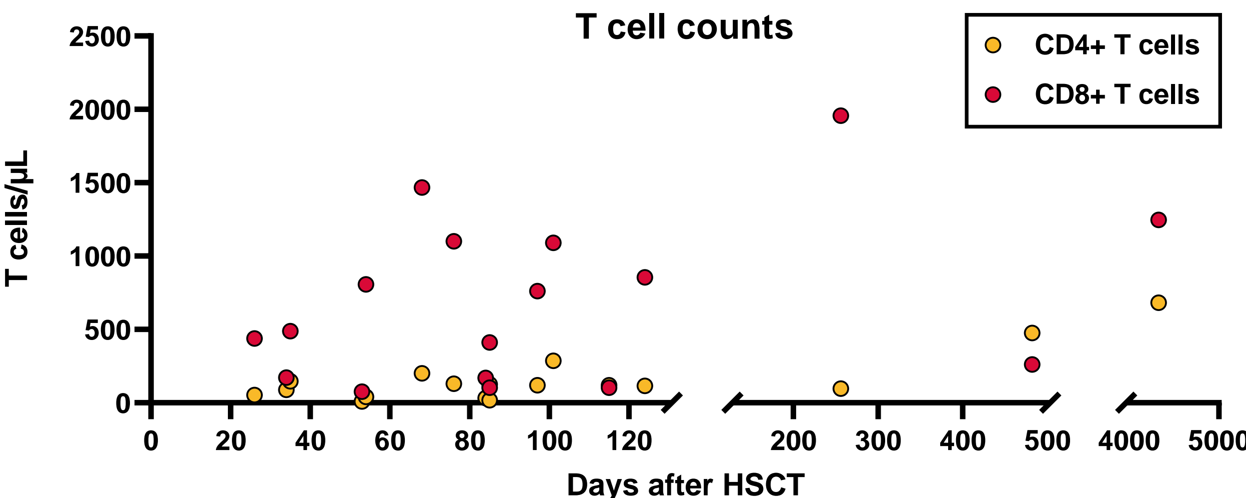

Figure A4. Correlation of the interval between HSCT and study inclusion (days after HSCT) and CD4+ and CD8+ T-cell counts in the patient cohort shown in Table A1. Spearman analysis indicated that CD4+ T-cell counts showed a stronger correlation with days after HSCT ($r = 0.46$, $p = 0.06$) than CD8+ T-cell counts ($r = 0.28$, $p = 0.3$).

Table A1. Spearman correlation analysis of ELISpot responses towards BKV large T peptides (JPT) in 17 hematopoietic stem cell transplant recipients with BKV-related cystitis.

Parameter	*r*	*p*
Age	−0.25	0.3
Interval to HSCT	0.28	0.3
Hemoglobin	−0.11	0.7
IgA	−0.06	0.8
IgE	−0.22	0.4
IgG	−0.25	0.3
IgM	0.06	0.8
sIL-2R	−0.19	0.5
Leukocytes	−0.22	0.4
T cells	0.22	0.4
B cells	0.25	0.8
NK cells	−0.06	0.8
CD4+ T cells	0.52	0.03
CD8+ T cells	0.19	0.5
TCRaβ T cells	0.24	0.4
TCRγδ T cells	0.26	0.3
Regulatory CD4+ T cells	0.40	0.1
Effector CD4+ T cells	0.51	0.04
GFR	0.64	0.01
Creatinine	−0.51	0.04
Urea	−0.52	0.03

This analysis considered the first dataset of each patient. Age—patient age at the time of the first ELISpot (inclusion into study), interval to HSCT—interval between hematopoietic stem cell transplantation and first ELISpot, sIL-2R—soluble interleukin 2 receptor, GFR—glomerular filtration rate.

Table A2. Spearman correlation analysis of ELISpot responses towards peptide pools of large T (LT) from AID and JPT, of BKV viral protein (VP) 1, and of JC virus (JCV).

		HSCT [a]		HC [b]	
Antigen 1	**Antigen 2**	*r*	*p*	*r*	*p*
BKV LT1 AID	BKV LT1 JPT	0.76	<0.0001	0.85	<0.0001
BKV LT1 AID	BKV VP1	0.74	<0.0001	0.73	<0.0001
BKV LT1 JPT	BKV VP1	0.75	<0.0001	0.84	<0.0001
BKV LT1 AID	JCV	0.12	0.5	0.12	0.4
BKV LT1 JPT	JCV	0.15	0.4	0.13	0.4
BKV VP1	JCV	0.13	0.6	0.25	0.2
BKV LT1 AID	PHA	−0.11	0.4	0.15	0.3
BKV LT1 JPT	PHA	−0.08	0.5	0.25	0.08
BKV VP1	PHA	−0.15	0.5	0.40	0.03
JCV	PHA	0.25	0.14	−0.10	0.5

[a] Hematopoietic stem cell transplant recipients with BKV-related cystitis (n = 17 patients with 61 samples), [b] healthy controls (n = 50), PHA—phytohemagglutinin (positive control).

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