T and NK cell immunity after hematopoietic stem cell transplantation

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Chapter 4

The effect of cidofovir on adenovirus plasma DNA levels in stem cell transplantation recipients without T cell reconstitution

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Abstract

Background: Cidofovir is frequently used to treat life-threatening human adenovirus (HAdV) infections in immunocompromised children after hematopoietic stem cell transplantation (HSCT). However, the anti-viral effect irrespective of T cell reconstitution remains unresolved.

Methods: Plasma HAdV DNA levels were monitored by real-time quantitative PCR during 42 cidofovir treatment episodes for HAdV viremia in 36 pediatric allogeneic HSCT recipients. HAdV load dynamics was related to T and NK cell reconstitution measured by flow cytometry.

Results: To evaluate the *in vivo* anti-adenoviral effect of cidofovir, we focused on 20 cidofovir treatment episodes lacking concurrent T cell reconstitution. During 2-10 weeks of follow-up in the absence of T cells, HAdV load reduction (n=7) or stabilization (n=8) were observed in 15/20 treatments. Although HAdV load reduction was always accompanied by NK cell expansion, HAdV load stabilization was measured in two children lacking both T and NK cell reconstitution. In cases with T cell reconstitution, rapid HAdV load reduction (n=14) or stabilization (n=6) was observed in 20/22 treatments.

Conclusion: In the absence of T cells, cidofovir treatment was associated with HAdV viremia control in the majority of cases. Although the contribution of NK cells cannot be excluded, cidofovir has the potential to mediate HAdV load stabilization in the time pending T cell reconstitution.

Highlights

- Cidofovir is used to treat human adenovirus viremia in pediatric HSCT recipients
- Evaluation of the antiviral effect of cidofovir is biased by T cell reconstitution
- In the absence of T cells, cidofovir can mediate HAdV load stabilization
- NK cell expansion during HAdV load reduction in the absence of T cells

Introduction

Human adenoviruses (HAdV) are non-enveloped double-stranded DNA viruses. Currently, more than 50 serotypes have been described. In healthy individuals, HAdV infections cause self-limiting infections such as conjunctivitis, upper respiratory tract-, urinary tract- or gastrointestinal infections.[50] In pediatric hematopoietic stem cell transplantation (HSCT) recipients, HAdV reactivations or primary infections can progress to viremia and disseminated disease. It is broadly accepted that T cells are essential for the protection from and clearance of HAdV viremia.[38;204;205] However, in the absence of T cell surveillance, the mortality of HAdV viremia is high because of progression to HAdV related multi-organ failure (HAdV/MOF).[38;41;51;206-208]

The adoptive transfer of donor-derived adenovirus specific T cells is a promising treatment,[205;209] but is not available in all transplant centers. Therefore, pre-emptive pharmacological treatment is of great importance for the majority of patients with HAdV viremia. To this end, ribavirin and cidofovir have been explored. The evidence for a beneficial effect of ribavirin is limited to case reports and ribavirin could not prevent the progression of HAdV viremia in the absence of lymphocyte reconstitution.[52] Cidofovir (Vistide®), a monophosphate nucleotide analogue with *in vitro* anti-viral activity against different HAdV strains,[210-213] is a widely used anti-viral agent for HAdV infections post HSCT.

A number of studies have addressed the anti-adenoviral effect of cidofovir, but results are highly variable with treatment successes ranging from 24-98%.[55-59;214;215] The discrepancies in cidofovir effectiveness might be related to the fact that HAdV viremia and cidofovir treatment generally occur during the critical phase of lymphocyte reconstitution after HSCT.[38;41;206;207] When T cell reconstitution is not taken into account, this will result in a biased evaluation of the effect of cidofovir. An unbiased evaluation of the *in vivo* anti-adenoviral effect of cidofovir is required because the use of cidofovir is associated with considerable nephrotoxicity.[53-56]

In this study, we aimed to evaluate the *in vivo* anti-viral effect of cidofovir in patients with HAdV viremia after pediatric HSCT without the confounding effect of concomitant T cell reconstitution. Hereto, we monitored the change of plasma HAdV DNA levels during cidofovir treatment, focusing on cidofovir treatments in the absence of T cell reconstitution.

Methods

Ethics statement

Transplantations were performed according to European society for Blood and Marrow Transplantation (EBMT) guidelines. Peripheral blood samples were routinely obtained. Data were analyzed after approval by the institutional review board (protocol P01.028 and P02.099). Informed consent was provided by the patient and/or a parent or guardian.

Patients and cidofovir treatment

Between January 2003 and December 2012, 321 children received 363 transplantations at the pediatric HSCT unit of the Leiden University Medical Center (LUMC). Thirty-nine HSCT recipients were treated with cidofovir for HAdV viremia. One patient was not evaluable as she died from pre-existent neurodegenerative disease within the first two weeks of treatment. From two other patients, no follow-up of the plasma HAdV DNA levels (HAdV load) was available, leaving 36 evaluable HSCT recipients. Six patients received two separate cidofovir treatment episodes for the same HAdV viremia and were analyzed twice. In total, 42 cidofovir treatment episodes were analyzed (Table 4.1).

In general, cidofovir treatment was initiated pre-emptively when the HAdV load was 3 log (1000) viral DNA copies/ml (c/ml) at two consecutive time points.[41] Reasons to stop treatment were: HAdV load reduction, HAdV load stabilization below <3 log c/ml, nephrotoxicity or treatment failure. Cidofovir (Vistide®, Gilead Sciences Inc., Foster City, CA, US) was administered intravenously thrice weekly at 1 mg/kg body weight.[214] Supportive care consisted of hyperhydration with intravenous saline (3L/m^2 body surface/24h) and oral probenecid (25 mg/kg) at -3, +1 and +8 hours from the start of cidofovir infusion.[53] Other anti-viral drugs were discontinued during cidofovir treatment. See supplemental methods and Table 4.S1 for a detailed description of patient inclusion and HSCT characteristics.

Monitoring of HAdV plasma DNA levels and lymphocyte reconstitution

HAdV reactivations were routinely monitored through twice-weekly plasma screening for viral DNA by real time quantitative PCR as described previously.[159] The lower level of detection of this assay was 1.7 log viral DNA c/ml. Monitoring was initiated at day +3 post HSCT and continued until T cells reached 300 cells/ µl of peripheral blood. To monitor immune reconstitution, peripheral blood white blood cell counts including full leukocyte differentiations were performed 2-3x/week. The lower level of detection of lymphocytes was 10-20 cells/µl. Flow cytometric analysis was performed weekly to quantify NK and T cell reconstitution as described in the supplemental methods and Table 4.S3. T cells were defined as $CD3^+$ cells in the $CD45^+$ $CD33/CD235a/CD14^-$ lymphocyte gate and NK cells were defined as $CD3^-$ $CD56^+$ cells in the lymphocyte gate.

Definitions of HAdV load dynamics and lymphocyte reconstitution during cidofovir treatment

HAdV load dynamics was evaluated during cidofovir treatment. Reduction and increase were defined as a ≥1 log (tenfold) change of the HAdV load. Stabilization was defined as a <1 log change in HAdV load. Both reduction and stabilization of the HAdV load were regarded as viremia control. To discriminate between the (potential) effect of T cell reconstitution and cidofovir treatment on HAdV load dynamics, we applied the low threshold of 50 T cells/µl of peripheral blood.

We first analyzed HAdV load dynamics in 42 cidofovir treatments without (n=20) and with (n=22) T cell reconstitution in the first two weeks after treatment initiation. Subsequently, we

focused on the 20 cidofovir treatments in the absence of concomitant T cell reconstitution. In this group, HAdV load change was evaluated between treatment initiation and one week after the last dose of cidofovir. In cases with T cell reconstitution before the end of treatment (n=6), follow-up was stopped at 1 week before T cell numbers reached 50 cells/µl to exclude the confounding effect of T cells on the HAdV load.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 20 (IBM SPSS Inc., Chicago, IL, US). GraphPad Prism 6.00 (GraphPad Software, San Diego, CA, US) was used to construct figures. Because data did not follow Gaussian distribution, the Mann-Whitney U test was used for the analysis of numerical parameters. Pearson's Chi-square tests were used for analysis of categorical parameters.

Patient characteristics (n=36)		n / median	% / range
Age (year)		**4.5**	0.5 - 18
HSCT indication	*Primary Immunodeficiency*	**8**	22%
	Benign Hematological Disorder	**12**	33%
	Hematological Malignancy	**16**	44%
Conditioning	*Reduced Intensity*	**6**	17%
	Myeloablative	**30**	83%
Donor Type	*Identical Related Donor*	**2**	6%
	Other Related Donor	**7**	19%
	Matched Unrelated Donor	**27**	75%
Graft Source	*Bone Marrow, T cell replete*	**16**	44%
	Bone Marrow, T cell depleted	**1**	3%
	PBSC, T cell replete	**2**	6%
	PBSC, T cell depleted	**8**	22%
	Cord Blood	**9**	25%
Serotherapy	*Anti-thymocyte globulin*	**23**	64%
	Alemtuzumab	**13**	36%
GvHD prophylaxis	*None*	**4**	11%
	CsA	**3**	8%
	CsA + Methotrexate	**18**	50%
	CsA + Methylprednisolone	**9**	25%
	CsA + MMF	**2**	6%
Acute Graft versus Host Disease ≥ grade II		**5**	14%
Cidofovir treatment	*One episode*	**30**	83%
	Two episodes	**6**	17%
First day plasma HAdV DNA level >1.7 log c/mL[1]		**20**	3 - 96
First day plasma HAdV DNA level 2x >3 log c/mL[1]		**28**	8 - 117
First day of first cidofovir treatment episode[1]		**29**	7 - 121
Final outcome	*HAdV clearance*	**27**	75%
	Death from HAdV / MOF	**6**	17%
	Death from other cause	**3**	8%

Cidofovir treatment episodes (n=42)	n / median	range
Day start cidofovir[1]	**31**	7 - 214
Plasma HAdV DNA level at start (*Log c / mL)*	**4.1**	1.7 - 6.5
Treatment duration (days)	**16**	1 - 99

Table 4.1. Patient and cidofovir treatment characteristics.

Abbreviations: HAdV: human adenovirus, HSCT: hematopoietic stem cell transplantation, PBSC: peripheral blood stem cells, GvHD: graft-versus-host disease, CsA: Cyclosporin A, MMF: mycophenolate mofetil, HAdV/MOF: HAdV related multi-organ failure. 1: day post HSCT. Categorical data: number (percentage). Numerical data: median (range).

Results

Characteristics of cidofovir treated patients

The effect of cidofovir treatment on the HAdV load was evaluated in 36 HSCT recipients, of whom the characteristics are summarized in Tables 1 and S1. Six patients received a second cidofovir treatment episode (Figure 4.S1), resulting in 42 evaluable cidofovir treatments. Cidofovir treatment was initiated at a median of 31 (range 7-214) days after HSCT. At treatment initiation, the HAdV load was median 4.1 (range 1.7-6.5) log c/ml and median treatment duration was 16 (range 1-99) days. 40/42 cases received ≥1 week (3 doses) of cidofovir (Tables 1 and 2).

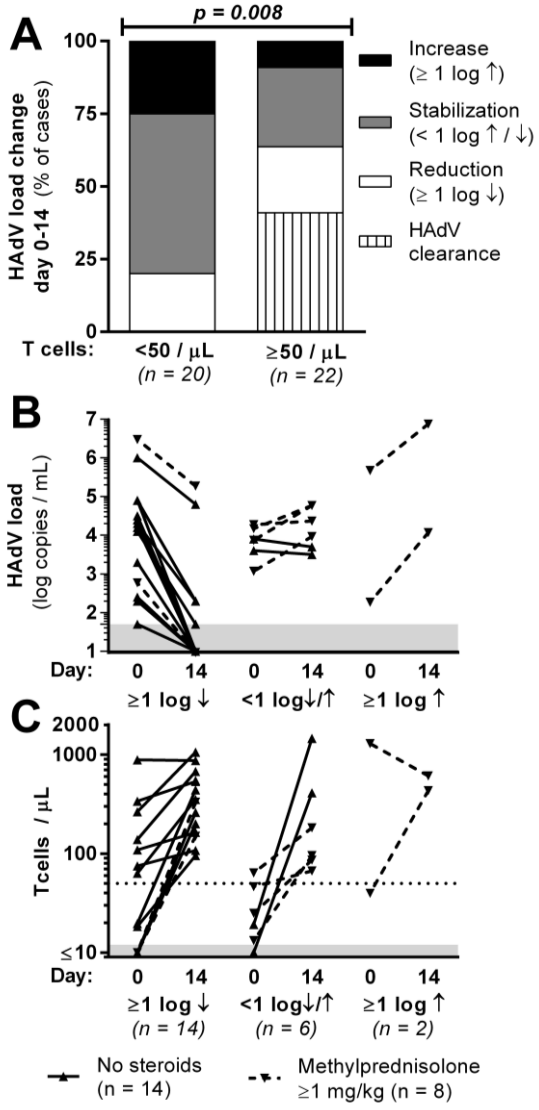

Figure 4.1. HAdV load dynamics in relation to T cell reconstitution.

(A) Change of plasma human adenovirus DNA levels (HAdV load) between the start of treatment (day 0) and day 14 of cidofovir treatment in cases with T cell numbers <50 cells/µl (n=20, left bar) and T cell numbers ≥50 cells/µl (n=22, right bar) within this time period. HAdV load change: clearance: dashed, ≥1 log reduction: white, stabilization: gray, ≥1 log increase: black. *p*-value: Pearson's Chi Square test.

(B) Change of HAdV load between day 0 and 14 in 22 cidofovir treatments with T cell numbers ≥50 cells/µl. Solid lines: cases without steroid treatment. Interrupted lines: methylprednisolone >1 mg/kg of body weight. Shaded area: HAdV load below limit of detection (1.7 log c/ml).

(C) Change of T cell numbers between day 0 and 14 in 22 cidofovir treatments with T cell reconstitution**.** Solid lines: cases without steroid treatment. Interrupted lines: methylprednisolone >1 mg/kg of body weight. Shaded area: T cells below limit of detection (10 cells/µl).

HAdV load dynamics in relation to T cell reconstitution

T cells have been demonstrated to play a crucial role in viral control and could form a major confounder in the analysis of the anti-viral effect of cidofovir. To test this hypothesis, we first divided the 42 cidofovir treatments in cases without (n=20) and with (n=22) T cell reconstitution in the first two weeks after treatment initiation (Table 4.2). The groups did not differ with respect to HSCT related parameters and HAdV viremia characteristics (Table 4.S2).

In 11/20 cidofovir treatments (55%) with T cell numbers below 50 cells/µl, plasma HAdV DNA levels were stable in the first two weeks after treatment initiation. The HAdV load increased ≥1 log c/ml in 5/20 treatments (25%) and HAdV load reduction –but no clearance– was measured in 4/20 treatments (20%) without T cell reconstitution (Figure 4.1A).

In contrast, in 14/22 cidofovir treatments (64%) with concomitant T cell reconstitution, HAdV load reduction or HAdV clearance was observed within two weeks after treatment initiation. The HAdV load was stable in 6/22 treatments (27%) with T cell reconstitution and increased ≥1 log in only 2/22 cases (9%), who simultaneously received high dose (>1mg/kg methylprednisolone) steroid treatment (Figure 4.1 B-C). Hence, it can be concluded that the evaluation of the antiviral effect of cidofovir treatment is strongly influenced by T cell reconstitution (*p=0.008*, Figure 4.1A).

HAdV load dynamics in the absence of T cell reconstitution

To exclude the confounding effect of T cells, HAdV load dynamics was further analyzed in the 20 cidofovir treatments with <50 T cells/µl of peripheral blood (Table 4.2 & Figure 4.2). In these cases, the evaluation period was extended –from 14 days used in the previous section– to the time frame between treatment initiation and one week after the last dose of cidofovir (n=14) or 1 week before T cell numbers reached 50 cells/µl (n=6).

A ≥1 log HAdV load reduction was observed in 7/20 treatments (35%) during a median 28 day evaluation period in the absence of T cells (Figure 4.2A). In 8/20 cidofovir treatments (40%), the HAdV load did not change significantly between the start and end of the evaluation period (median 18 days, Figure 4.2B). In the 5 remaining cases (25%), the HAdV load increased ≥1 log despite cidofovir treatment during median 21 days of follow up in the absence of T cells (Figure 4.2C).

NK cell expansion during HAdV control in the absence of T cell reconstitution

In the majority of the cases without T cell reconstitution, $CD3^{-}CD56^{+}$ NK cells were already present at the start of cidofovir treatment (Figure 4.2 D-F). Reduction of the HAdV load during cidofovir treatment coincided with a dynamic increase in NK cell numbers in all 7 cases (8-29 fold NK cell expansion, Figure 4.2D). In comparison, NK cell expansion was observed in 4/8 cidofovir treatments with HAdV load stabilization (Figure 4.2E) and 2/5 treatments with HAdV load increase (Figure 4.2F, *p=0.05*).

HAdV load stabilization in the absence of both T and NK cells

We finally analyzed the anti-viral effect of cidofovir on the HAdV load in HSCT recipients lacking both T- and NK cell reconstitution to exclude the possible contribution of NK cells to HAdV control as well. Two HSCT recipients fulfilled these criteria. In patient A (UPN 600.1a), no engraftment occurred and the HAdV viremia was stable during 3 weeks of cidofovir treatment in the absence of any lymphocytes (Figure 4.2 A, D, open diamonds & Figure 4.S1 D). The viremia was ultimately cleared after lymphocyte reconstitution following a re-transplantation. In patient B (UPN 545.1b), a graft rejection was treated with the lymphocyte depleting antibody alemtuzumab. The HAdV load was stable in the absence of lymphocytes over a 4 weeks period, after which HAdV dissemination occurred under cidofovir treatment (Figure 4.2 C, F, open diamonds & Figure 4.S1 B).

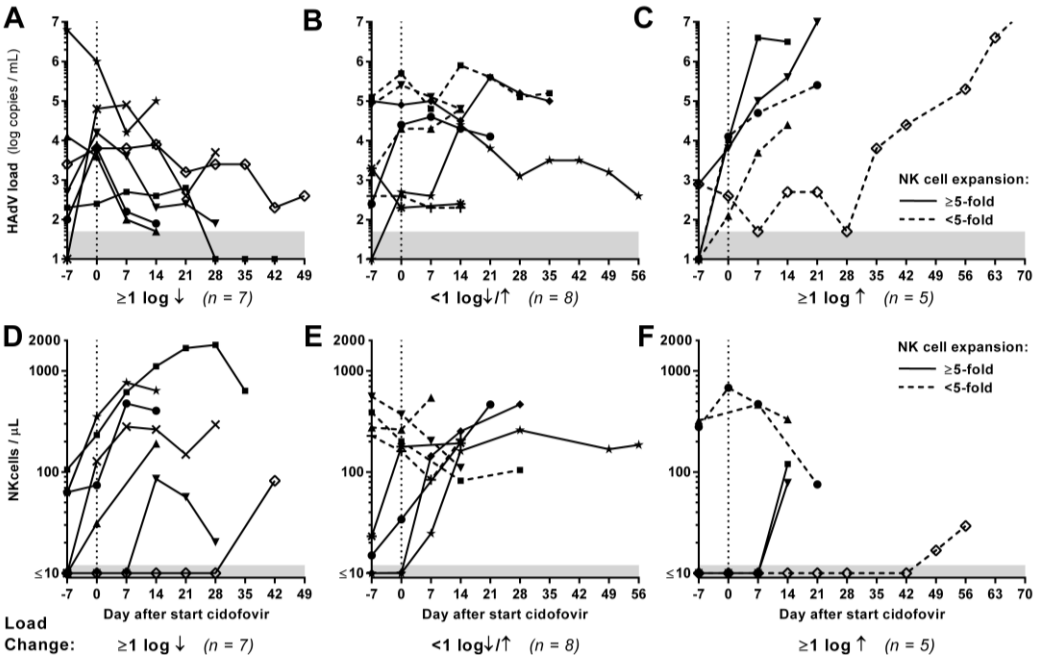

Figure 4.2. HAdV control in the absence of T cells.
(A-C) Plasma human Adenovirus (HAdV) DNA levels in 20 cidofovir treatments with T cell numbers <50 cells/µl. Follow-up was stopped at 1 week after the last dose of cidofovir or 1 week before T cell numbers reached 50 cells/µl. HAdV load change: ≥1 log reduction (A, n=7), stabilization (B, n=8) or ≥1 log increase (C, n=5). Solid lines: cases with ≥5 fold NK cell expansion, interrupted lines: cases without NK cell expansion. Vertical dotted line: start of treatment. Shaded area: HAdV load below limit of detection (1.7 log c/ml).
(D-F) Absolute numbers of NK cells in peripheral blood in cases with HAdV load reduction (D), stabilization (E) and increase (F). Solid lines: cases with ≥5 fold NK cell expansion, interrupted lines: cases without NK cell expansion. Identical symbols in corresponding figures represent individual cases. Vertical dotted line: start of treatment. Shaded area: NK cells below limit of detection (10 cells/µl).

Discussion

Here, we report a systematic analysis of the effect of cidofovir treatment on HAdV viremia after HSCT, using plasma viral DNA levels as an objective parameter while taking concomitant lymphocyte reconstitution into account. In line with our hypothesis, rapid HAdV load reduction and HAdV clearance during cidofovir treatment were associated with concurrent T cell reconstitution. In the absence of T cell reconstitution, the HAdV viremia was controlled in 75% of cidofovir treatments, which can be attributed to cidofovir although a role of NK cell reconstitution cannot be excluded. Of note, in 2 cases, HAdV load stabilization was observed during a >3 week cidofovir treatment in the absence of both NK and T cells.

Because of the strong correlation between T cell reconstitution and HAdV load reduction as reported earlier [38;204;205] and supported by our data, we focused our analysis on the 20 cidofovir treatment episodes in HSCT recipients lacking T cell reconstitution. In view of the often fatal outcome of a progressing HAdV viremia,[51] HAdV load reduction or stabilization can be regarded as a beneficial result. In line with the *in vitro* virostatic capacity of cidofovir,[210-213] control of the HAdV viremia was observed in 15 out of 20 cidofovir treatments in the absence of T cell reconstitution. However, reduction of the HAdV load (n=7) always coincided with a ≥5 fold increase of NK cell numbers in the peripheral blood. Consequently, the contribution of NK cell reconstitution to HAdV control in HSCT recipients without T cell reconstitution cannot be excluded. Usually, NK cells are the first lymphocytes to reach normal levels after HSCT.[216;217] Although the NK cell response to HAdV is not as well described as the NK cell mediated control of other viruses like influenza, cytomegalovirus and hepatitis C virus,[218-220] a limited number of studies reported NK cell activity against HAdV infected cells.[221-223] Our data further support a role for NK cells in the initial HAdV control in patients with a delayed T cell reconstitution. At the same time, NK cell expansion did not always coincide with HAdV load reduction and the presence of NK cells was no guarantee for HAdV load stabilization.

In many studies, final outcome of HAdV viremia is used as a primary endpoint of cidofovir effectiveness, and concomitant lymphocyte reconstitution is often ignored. This bears the risk to overestimate the *in vivo* anti-viral effect of cidofovir. Indeed, Walls *et al.* reported the clearance of HAdV viremia in 8 out of 9 pediatric HSCT recipients with HAdV loads >3 log c/ml in the absence of anti-viral therapy.[224] For this reason, an unbiased evaluation of the anti-viral capacity of cidofovir –and other pharmacological interventions– for human adenoviral infections after HSCT is only possible by monitoring HAdV load dynamics in patients that lack concomitant lymphocyte reconstitution. Only two patients in our cohort met this condition. In both cases, the HAdV viremia remained stable over a 3-4 weeks period. Consequently, cidofovir may also have contributed to the HAdV load stabilization observed in HSCT recipients lacking T cell reconstitution or with suppressed anti-viral T cell responses due to high dose systemic steroids.[37;225;226]

Of note, cidofovir treatment could not prevent the progression of HAdV viremia in 7/42 cidofovir treatments (17%), all in the absence of T cell reconstitution or during high dose steroid treatment. With respect to the cause of these treatment failures, no firm conclusions can be drawn. *In vitro* studies reported comparable cidofovir susceptibility between different HAdV species.[210-213] In our

		HAdV viremia					CDV treatment			Lymphocyte reconst.			HAdV load change[7]		
	UPN	First day PCR+[1]	Day PCR 2x >3 log[1]	Load at start CDV[2]	Sero-type	Species	Treat-ment #	Day Start[1]	Dura-tion[3]	T cell[4]	NK cell[5]	Ster-oids[6]	Day 14[3]	Last day follow-up[3,8]	Reason stop follow-up[8]
T cells <50 cells/µL 14 days after start cidofovir	543.1a	14	19	5.9	31	A	1	28	9	-	+	-	↓	↓ (14)	Stop CDV
	549.1	19	30	3.8	2	C	1	30	7	-	+	-	↓	↓ (14)	Stop CDV
	555.1	8	23	3.6	31	A	1	28	7	-	+	-	↓	↓ (14)	Stop CDV
	808.1	12	23	4.2	?	?	1	23	21	-	+	-	↓	↓ (28)	Stop CDV
	600.1a	26	29	3.8	1	C	1	30	49	-	+	+	=	↓ (56)	Stop CDV
	616.1	13	20	4.4	3,31	B,C	1	17	27	-	+	+	=	↓ (28)	T cell ≥50
	721.1	39	-	2.4	?	?	1	44	37	-	+	+	=	↓ (42)	Stop CDV
	514.3	20	27	4.4	2	C	1	31	14	-	+	-	=	= (21)	Stop CDV
	543.1b	n.a.	n.a.	5.7	2,31	A,C	2	55	41	-	-	-	=	= (35)	T cell ≥50
	554.2	17	53	5.4	1	C	1	53	8	-	-	-	=	= (14)	Stop CDV
	560.1	27	48	4.9	5	C	1	48	42	-	+	+	=	= (35)	T cell ≥50
	625.2	43	-	2.3	1	C	1	59	9	-	+	-	=	= (14)	Stop CDV
	648.2	70	75	4.3	31	A	1	78	50	-	-	-	=	= (14)	T cell ≥50
	681.1a	24	34	2.9	?	?	1	40	7	-	-	-	=	= (14)	Stop CDV
	545.1b	n.a.	n.a.	2.6	5,31	A,C	2	39	64	-	-	+	=	↑ (70)	Stop CDV
	551.1	19	33	2.7	31	A	1	21	47	-	+	-	↑	= (56)	Stop CDV
	613.1	40	46	4.1	5	C	1	44	17	-	-	+	↑	↑ (21)	Stop CDV
	719.1	5	8	4.0	31	A	1	7	28	-	+	-	↑	↑ (14)	T cell ≥50
	799.1	25	39	2.1	31	A	1	28	40	-	-	-	↑	↑ (14)	T cell ≥50
	804.1	7	16	3.8	31	A	1	17	11	-	+	-	↑	↑ (21)	Stop CDV
T cells ≥50 cells/µL 14 days after start cidofovir	558.1	14	19	1.7	31	A	1	23	7	+	+	-	**c**	n.a.	T cell ≥50
	586.1	61	75	4.5	1	C	1	77	12	+	-	-	**c**	n.a.	T cell ≥50
	648.1	6	-	2.8	31	A	1	17	14	+	+	+	**c**	n.a.	T cell ≥50
	681.1b	n.a.	n.a.	2.3	?	?	2	95	3	+	-	-	**c**	n.a.	T cell ≥50
	697.1	5	23	4.3	?	?	1	21	12	+	-	-	**c**	n.a.	T cell ≥50
	732.1	10	20	4.4	?	?	1	19	1	+	+	-	**c**	n.a.	T cell ≥50
	787.1b	n.a.	n.a.	2.4	?	?	2	112	21	+	-	-	**c**	n.a.	T cell ≥50
	816.1	5	22	4.2	31	A	1	23	11	+	+	-	**c**	n.a.	T cell ≥50
	817.1	34	37	3.3	?	?	1	38	10	+	-	-	**c**	n.a.	T cell ≥50
	515.2	12	16	6.5	1,2	C	1	16	24	+	+	+	↓	n.a.	T cell ≥50
	587.1	20	-	4.1	31	A	1	31	7	+	+	-	↓	n.a.	T cell ≥50
	594.1b	n.a.	n.a.	6.0	1	C	2	214	73	+	-	-	↓	n.a.	T cell ≥50
	600.1b	n.a.	n.a.	4.9	1	C	2	100	10	+	-	-	↓	n.a.	T cell ≥50
	823.1	20	23	4.9	?	?	1	22	12	+	+	-	↓	n.a.	T cell ≥50
	515.1	65	72	4.3	2	C	1	79	48	+	-	+	=	n.a.	T cell ≥50
	545.1a	3	10	3.9	5	C	1	10	15	+	-	-	=	n.a.	T cell ≥50
	594.1a	21	28	3.9	1	C	1	32	80	+	+	+	=	n.a.	T cell ≥50
	597.1	5	12	4.2	31	A	1	8	99	+	-	+	=	n.a.	T cell ≥50
	686.2	87	101	3.6	2	C	1	107	15	+	+	-	=	n.a.	T cell ≥50
	787.1a	18	20	3.1	1	C	1	20	28	+	-	+	=	n.a.	T cell ≥50
	634.1	96	117	5.4	2	C	1	121	36	+	-	+	↑	n.a.	T cell ≥50
	671.1	52	66	2.3	1	C	1	48	70	+	-	+	↑	n.a.	T cell ≥50

cohort, cidofovir treatment failures were observed both in patients with HAdV species A as well as species C. Whereas cidofovir resistant HAdV strains have been generated *in vitro*,[227] no resistance was reported in clinical HAdV isolates from cidofovir treated HSCT recipients.[210-213] Possibly, inter-patient variations in cidofovir pharmacokinetics may have contributed to failure of cidofovir treatment as well. In view of the nephrotoxicity of cidofovir,[53-56] the observed treatment failures emphasize the need for new and less toxic pharmacological interventions like brincidofovir (CMX001), the orally bioavailable lipid conjugate of cidofovir [61] as well as adoptive immunotherapy based interventions [205;209] for patients with a delayed T cell reconstitution.

The sensitive PCR methods used for the virological monitoring post HSCT carry the risk of overtreatment which might lead to avoidable toxicity.[224;228] Longitudinal monitoring of lymphocyte reconstitution can identify patients with better odds on a favorable outcome of HAdV viremia. Indeed, the presence of even a low number of T cells (≥50 cells/µl) in patients without steroid treatment was associated with a rapid HAdV load reduction. The frequent monitoring of T cell reconstitution can be a valuable tool to prevent the unnecessary installment or continuation of cidofovir treatment. A comparable approach has already been applied successfully in the management of cytomegalovirus and Epstein-Barr virus infections post HSCT.[39;229]

Altogether, a subgroup of patients with HAdV viremia post HSCT might benefit from cidofovir treatment through a stabilization of the HAdV load pending lymphocyte reconstitution. Nevertheless, T cell reconstitution remains essential for viral clearance. For clinical decision making, the combined monitoring of plasma HAdV DNA levels and lymphocyte reconstitution provides an objective tool for the guidance of personalized antiviral treatment and to prevent the unnecessary exposure to cidofovir.

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Table 4.2 (previous page). Details of human adenovirus viremia, cidofovir treatment, lymphocyte reconstitution and HAdV load dynamics.

Treatments are ordered based on T cell reconstitution and HAdV load change. Abbreviations: UPN: Unique Patient Number, decimal: 1st, 2nd or 3th HSCT, letter: 1st (a) or 2nd (b): cidofovir treatment episode. HAdV: Human Adenovirus, n.a.: not applicable, ? : not analyzed, CDV: cidofovir. [1]Days after hematopoietic stem cell transplantation (HSCT). [2]Plasma HAdV DNA levels in log copies/ml. [3]Days from start cidofovir treatment. [4]T cell numbers in peripheral blood ≥50 cells/µl within 14 days after treatment initiation. [5] ≥5 fold expansion of NK cell number in peripheral blood. [6]Prednisone ≥1 mg/kg body weight during cidofovir treatment. [7]Reduction (↓) / Increase (↑): plasma HAdV DNA levels changed ≥1 log c/ml after initiation of cidofovir treatment. Stabilization (=): <1 log c/ml change of HAdV load. Clearance (c): plasma HAdV DNA levels below lower limit of detection at 2 consecutive time points. [8]Follow-up was stopped at 1 week after the last dose of cidofovir or 1 week before T cell numbers reached 50 cells/µl.

Supplemental Data

Supplemental Methods

Patient inclusion

During post-transplant follow up, HAdV DNA was detected in plasma at least once in 111 out of 363 transplantations (31%) and the HAdV load reached 3 log c/ml at two consecutive time points after 40 transplantations (11%, disseminated HAdV viremia). In general, cidofovir was initiated when the HAdV load reached 3 log c/ml at two consecutive time points.[41] Patients treated with cidofovir after two different HSCT procedures (n=2, UPN 515 and 648) were regarded as separate HSCT recipients. Of 40 patients with a disseminated HAdV viremia, five were not treated because of a palliative setting (n=3) or a stable HAdV load (n=2).

Thirty-two out of 35 cidofovir treated disseminated HAdV viremias were evaluable: one patient died of pre-existent neurodegenerative disease within the first two weeks of treatment and from two patients, no follow up of HAdV load was available. Additionally, in 4 patients, cidofovir was initiated for HAdV viremia with a HAdV load <3 log c/ml, adding up to 36 patients. Six patients received two separate cidofovir treatment episodes for the same HAdV viremia and were analyzed twice. Cidofovir treatment was discontinued for at least 2 weeks between two treatment episodes. In total, 42 cidofovir treatment episodes were analyzed (Table 4.2 and S1).

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were separated using ficoll-isopaque density gradient centrifugation (LUMC Pharmacy, Leiden, NL), washed twice and resuspended in RPMI cell culture medium (PAA Laboratories, Pasching, AT), supplemented with Human Serum Albumin (HSA, 0.8 mg/ml, Sanquin, Amsterdam, NL) and penicillin/streptomycin (P/S, 100 U/ml and 100 μg/ml, Lonza, Verviers, BE). Red cell lysis buffer (LUMC Pharmacy) was added to the cells and after 10 minutes incubation at room temperature, PBMC were washed and resuspended in FACS buffer (Phosphate Buffered Saline (Braun, Melsungen, GE) containing 10 mg/ml Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, US) and 3 mM Ethylenediaminetetraacetic Acid (Merck, Darmstadt, GE). Cells were stained for 30 minutes at 4°C with the antibodies listed in Table 4.S3 at their optimal concentration. Four-color flow cytometry was performed on a BD FACS Calibur II flow cytometer (Becton Dickinson Biosciences (BD), Franklin Lakes, NJ, US) and data were analyzed using BD Cellquest software.

Patient Characteristics			HSCT characteristics								
UPN	Age (year)	Sex	HSCT indication	Donor Type	Graft Source	Graft Manip.	Condi-tioning	Serotherapy	aGVHD prophylaxis	aGvHD	Cidofovir treatments
514.3	13.7	M	SAA	IRD	BM	-	RIC	Alemtuzumab	CSA/MTX	-	1
515.1	3.6	M	WAS	MUD	PBSC	TCD	RIC	Alemtuzumab	CSA	-	1
515.2	4.5	M	WAS	MUD	PBSC	-	RIC	Alemtuzumab	CSA/MTX	-	1
543.1	4.7	F	AML	MUD	PBSC	TCD	MA	Alemtuzumab	CSA/MTX	-	2
545.1	2.3	M	JMML	MUD	BM	-	MA	ATG	CSA/MTX	-	2
549.1	1.2	F	MDS	MUD	BM	TCD	MA	ATG	CSA	-	1
551.1	17.8	M	ALL	ORD	PBSC	TCD	MA	Alemtuzumab	-	-	1
554.2	10.6	F	ALL	MUD	BM	-	MA	ATG	CSA/MTX	-	1
555.1	0.5	M	SCID-OS	ORD	BM	-	RIC	Alemtuzumab	CSA/MTX	-	1
558.1	13.2	M	β-Thalass.	MUD	BM	-	MA	Alemtuzumab	CSA/MTX	-	1
560.1	7.6	M	FA	MUD	CB	-	MA	Alemtuzumab	CSA/Pred	-	1
586.1	15.1	M	AML	MUD	BM	-	MA	ATG	CSA/MTX	Grade II	1
587.1	13.2	F	LAD-1/var.	MUD	PBSC	-	RIC	Alemtuzumab	CSA/MMF	-	1
594.1	1.7	M	WAS	MUD	CB	-	MA	ATG	CSA/Pred	-	2
597.1	1.3	F	JMML	MUD	CB	-	MA	ATG	CSA/Pred	Grade III	1
600.1	6.3	F	AML	MUD	CB	-	MA	ATG	CSA/Pred	-	2
613.1	4.1	F	MDS	MUD	CB	-	MA	ATG	CSA/Pred	Grade III	1
616.1	3.4	M	β-Thalass.	ORD	PBSC	TCD	MA	Alemtuzumab	CSA	-	1
625.2	2.2	F	β-Thalass.	IRD	BM	-	MA	ATG	CSA/MTX	-	1
634.1	16.6	F	β-Thalass.	ORD	PBSC	TCD	MA	Alemtuzumab	CSA/MMF	-	1
648.1	1.9	M	HLH	MUD	CB	-	MA	ATG	CSA/Pred	-	1
648.2	2.4	M	HLH	ORD	PBSC	TCD	MA	ATG	-	-	1
671.1	10.2	F	CID	MUD	CB	-	MA	ATG	CSA/Pred	Grade III	1
681.1	3.5	F	β-Thalass.	MUD	BM	-	MA	ATG	CSA/MTX	-	2
686.2	7.3	M	AML	ORD	PBSC	TCD	MA	ATG	-	-	1
697.1	3.1	M	AML	MUD	BM	-	MA	ATG	CSA/MTX	-	1
719.1	2.1	M	ALL	MUD	BM	-	MA	ATG	CSA/MTX	-	1
721.1	4.4	M	SAA/DKC	MUD	CB	-	MA	ATG	CSA/Pred	-	1
732.1	3.9	M	β-Thalass.	MUD	BM	-	MA	ATG	CSA/MTX	Grade II	1
787.1	4.5	M	ALL	MUD	CB	-	MA	ATG	CSA/Pred	-	2
799.1	8.1	F	SAA	ORD	PBSC	TCD	MA	Alemtuzumab	-	-	1
804.1	1.1	M	β-Thalass.	MUD	BM	-	MA	ATG	CSA/MTX	-	1
808.1	17.8	M	MDS	MUD	BM	-	MA	Alemtuzumab	CSA/MTX	-	1
816.1	7.6	M	AML	MUD	BM	-	MA	ATG	CSA/MTX	-	1
817.1	14.7	M	AML	MUD	BM	-	MA	ATG	CSA/MTX	-	1
823.1	7.3	F	BMF	MUD	BM	-	RIC	ATG	CSA/MTX	-	1

Table 4.S1. Patient characteristics

Abbreviations: UPN: Unique Patient Number, decimal: 1st, 2nd or 3th transplantation M: Male, F: Female. ALL: Acute Lymphoid Leukemia, AML: Acute Myeloid Leukemia, β-Thalass.: β-Thalassemia major, BMF: Bone Marrow Failure, CID: Combined Immunodeficiency, DKC: Dyskeratosis Congenita, FA: Fanconi Anemia, HLH: Hemophagocytic Lymphohystiocytosis, JMML: Juvenile Myelomonocytic Leukemia, LAD-1/Var.: Leukocyte Adhesion Deficiency 1 / Variant, MDS: Myelodysplastic Syndrome, OS: Omenn Syndrome, SAA: Severe Aplastic Anemia, SCID: Severe Combined Immunodeficiency, WAS: Wiskott-Aldrich Syndrome. HSCT: Hematopoietic Stem Cell Transplantation, MUD: Matched Unrelated Donor, IRD: Identical Related Donor, ORD: Other Related Donor, BM: Bone marrow, CB: Cord blood, PBSC: Peripheral Blood Stem Cells, TCD: T cell depletion of the graft. RIC: Reduced Intensity Conditioning, MA: Myeloablative conditioning. ATG: Anti-Thymocyte Globulin, CSA: Cyclosporin A, MMF: Mycophenolate Mofetil, MTX: Methotrexate, Pred: Prednisone, aGvHD: acute Graft-versus-Host-Disease.

		T cells < 50 (n=20)		T cells ≥ 50 (n=22)		
		n / median	% / range	n / median	% / range	*p- value*
Age (year)		**4.3**	0.5 - 18	**4.5**	1.1 - 17	*0.55*
HSCT indication	*Primary Immunodeficiency*	**2**	10%	**7**	32%	*0.18*
	Benign Hematological Disorder	**8**	40%	**5**	23%	
	Hematological Malignancy	**10**	50%	**10**	46%	
Conditioning	*Reduced Intensity*	**2**	10%	**4**	18%	*0.45*
	Myeloablative	**18**	90%	**18**	82%	
Donor Type	*Identical Related Donor*	**2**	10%	**0**	0%	*0.26*
	Other Related Donor	**5**	25%	**2**	9%	
	Matched Unrelated Donor	**13**	65%	**20**	91%	
Graft Source	*Bone Marrow, T cell replete*	**9**	45%	**9**	41%	*0.26*
	Bone Marrow, T cell depleted	**1**	5%	**0**	0%	
	PBSC, T cell replete	**0**	0%	**2**	9%	
	PBSC, T cell depleted	**6**	30%	**3**	14%	
	Cord Blood	**4**	20%	**8**	36%	
Serotherapy	*Anti-thymocyte globulin*	**11**	55%	**17**	77%	*0.13*
	Alemtuzumab	**9**	45%	**5**	23%	
GvHD prophylaxis	*None*	**3**	15%	**1**	5%	*0.33*
	CyclosporinA	**2**	10%	**1**	5%	
	CyclosporinA + Methotrexate	**11**	55%	**10**	46%	
	CyclosporinA + Methylprednisolone	**4**	20%	**8**	36%	
	CyclosporinA + MMF	**0**	0%	**2**	9%	
Acute Graft versus Host Disease ≥ grade II		**1**	5%	**4**	18%	*0.19*
Cidofovir treatment	*First episode*	**18**	90%	**18**	82%	*0.45*
	Second episode	**2**	10%	**4**	18%	
First day HAdV load >1.7 log c/mL[1] (n=36 1st episodes)		**20**	5 - 70	**19**	3 - 96	*0.73*
First day HAdV load 2x>3 log c/mL[1] (n=36 1st episodes)		**30**	8 - 75	**23**	10 - 117	*0.84*
First day of first cidofovir treatment[1] (n=36 1st episodes)		**30**	7 - 78	**23**	8 - 121	*0.61*
Day start cidofovir[1]		**31**	7 - 78	**32**	8 - 214	*0.71*
HAdV load at start (*Log c / mL)*		**3.9**	2.1 - 5.9	**4.2**	1.7 - 6.5	*0.72*
Peak HAdV load in period day -7 to +14 from start treatment		**4.6**	2.7 - 6.8	**4.7**	2.4 - 6.9	*0.68*
Treatment duration (days)		**24**	7 - 64	**15**	1 - 99	*0.78*

Table 4.S2. Patient characteristics of treatments with <50 and ≥ 50 T cells/ µl 14 days after start cidofovir
Abbreviations: HAdV: human adenovirus, HSCT: hematopoietic stem cell transplantation, PBSC: peripheral blood stem cells, GvHD: graft-versus-host disease, MMF: mycophenolate mofetil, HAdV/MOF: HAdV related multi-organ failure. HAdV load: plasma HAdV DNA level. [1]: day post HSCT. Categorical data: number (percentage). Numerical data: median (range). *p*-values: Pearson's Chi Square test (categorical data) or Mann-Whitney U test (numerical data) comparing the two groups.

Type	Antibody	Fluorochrome	Clone	Supplier
Lymphocyte gate	**CD45**	FITC	2D1	BD
	CD14	PE	MOP9	BD
	CD33	PE	P67.6	BD
	CD235a	PE	11E4B-7-6	BC
Lymphocyte subsets	**CD3**	PerCP-Cy5.5	SK7	BD
	CD19	APC	J4.119	BC
	CD56	APC	N901	BC

Table 4.S3. Antibodies used for flowcytometry

FITC: Fluorescein isothiocyanate, PE: phycoerythrin, PERCP: Peridinin chlorophyll, Cy: Cyanine, APC: Allophycocyanin, BD: Becton Dickinson Biosciences, Franklin Lakes, NJ, US, BC: Beckman Coulter, Brea, CA, US,

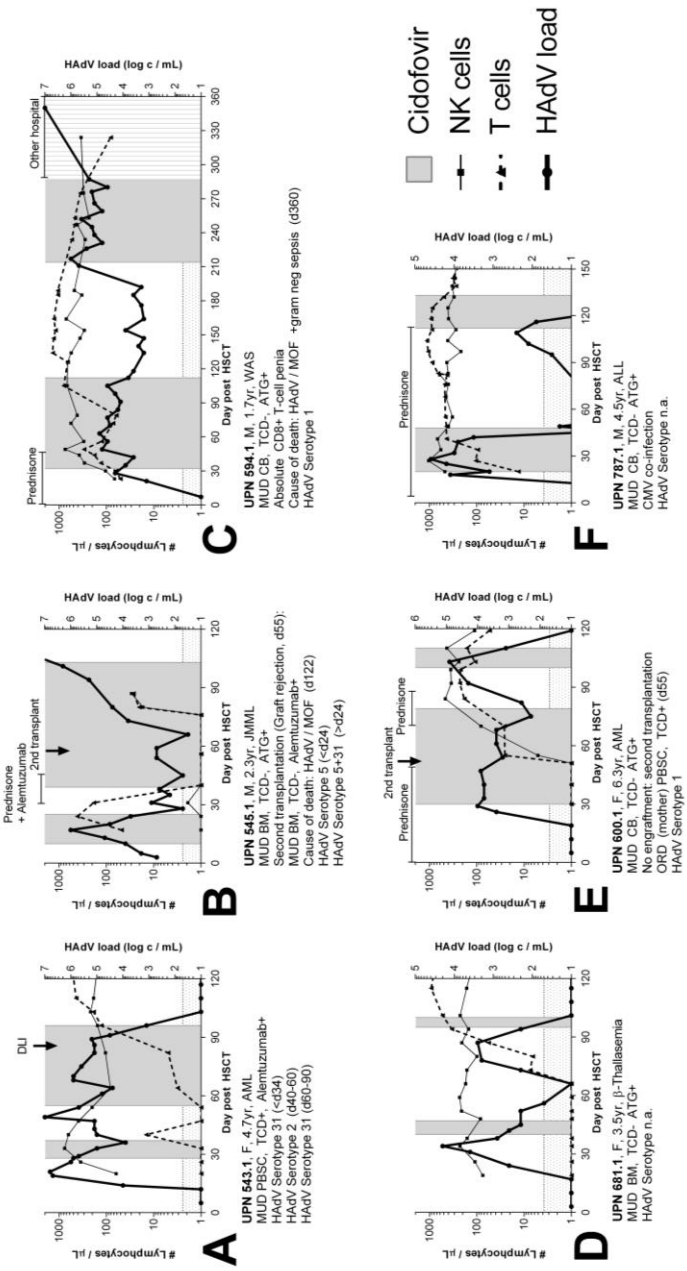

Figure 4.S1. HAdV load dynamics in 6 patients who received two cidofovir treatment episodes.

Plasma human adenovirus DNA levels (HAdV load, bold lines, right Y-axis) are plotted as a function of time post HSCT (days, X-axis). T cell numbers (interrupted lines) and NK cell numbers (solid lines) are plotted on the left Y-axis. Gray shaded area: Cidofovir treatment episodes. Abbreviations: UPN: Unique Patient Number, decimal: 1st, 2nd or 3th transplantation M: Male, F: Female. Yr: Year. ALL: Acute Lymphoid Leukemia, AML: Acute Myeloid Leukemia, β-Thalass.: β-Thalassemia major, JMML: Juvenile Myelomonocytic Leukemia, WAS: Wiskott-Aldrich Syndrome. MUD: Matched Unrelated Donor, BM: Bone marrow, CB: Cord blood, PBSC: Peripheral Blood Stem Cells, TCD: T cell depletion of the graft. ATG: Anti-Thymocyte Globulin, DLI: donor lymphocyte infusion. N.A.: not available.