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Ex vivo detection of adenovirus specific CD4$^+$ T-cell responses to HLA-DR-epitopes of the Hexon protein show a contracted specificity of T_{HELPER} cells following stem cell transplantation

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ABSTRACT

Human adenovirus (HAdV) is a cause of significant morbidity and mortality in immunocompromised patients, especially after stem cell transplantation (SCT). Viral clearance has been attributed to CD4$^+$ T-cell responses against the Hexon-protein, but the frequency of specific T_{HELPER} cells is extremely low or not detectable *ex vivo* and preference for different CD4$^+$ T-cell epitopes is variable among individuals. We therefore analyzed 44 healthy donors and 6 SCT-recipients for Hexon-specific CD4$^+$-responses *ex vivo*, to identify epitopes which would be broadly applicable. We selected 19 candidate epitopes with predicted restriction to HLA-DR1/DR3/DR4/DR7; 16 were located within the highly conserved regions, indicating cross-reactivity of T cells among HAdV-subspecies. Ten epitopes induced CD4$^+$-proliferation in >50% of individuals, confirmed by intracellular IFN-γ detection. Three SCT recipients who recovered from an infection with HAdV displayed reactivity towards only a single hexon epitope, whereas healthy individuals were responsive to two to eight epitopes (median 3). The *ex vivo* detection of Hexon-specific CD4$^+$ T-cells, without any long-term culture *in vitro*, enables the detection and generation of HAdV-specific CD4$^+$ T cells for adoptive T-cell transfer against HAdV-infection post SCT.

Introduction

Adenoviral (HAdV) infections are relevant causes of morbidity and mortality in immunocompromised patients, especially in recipients of an allogeneic hematopoietic stem cell transplantation (SCT) (Handgretinger et al., 2001; Lang et al., 2003; Mohty et al., 2003). In children post SCT, adenovirus has become the most common viral pathogen, responsible for systemic infections or localized infections of the gastrointestinal tract, upper and lower airway and urinary tract (Lion et al., 2003; Walls et al., 2003; Flomenberg et al., 1994). Increased frequencies of severe HAdV infections have also been detected in solid organ transplant recipients (Shirali et al., 2001) and in human immune-deficiency virus (HIV)-positive patients (Kojaoghlanian et al., 2003). An increased risk of adenovirus infection correlates with the lack of corresponding endogenous T-cell immunity (Chakrabarti et al., 2002; Feuchtinger et al., 2005; Heemskerk et al., 2005). Drug therapy could be efficient to limit, but not cure the infection (Chakrabarti et al., 2002; Heemskerk et al., 2005; Feuchtinger et al., 2005; Feuchtinger et al., 2006; Yusuf et al., 2006). This has led to efforts to improve the detection and isolation of HAdV-specific T cells by the characterization of their T-cell epitopes. The detection of HAdV-specific T cells may improve the risk assessment of patients with HAdV infection and the isolation may facilitate adoptive transfer of HAdV-specific T cells from the donor into the recipient to restore protective immunity. The T-cell response against HAdV has been described predominantly specific for capsid proteins (Molinier-Frenkel et al., 2000), adenoviral Hexon protein being the immunodominant region (Molinier-Frenkel et al., 2002). Among adenovirus species and subtypes, the amino acid sequence of the Hexon protein can be subdivided into hypervariable and conserved regions (Rux et al., 2003). MHC-I restricted peptides have been identified, demonstrating that adenovirus-specific T cells can be either broadly cross-reactive or reactive to a restricted spectrum of viral strains (Leen et al., 2004; Tang et al., 2006). MHC-II restricted peptide epitopes within the Hexon protein have been described, with one HLA-DP (Tang et al., 2004; Leen et al., 2008), six with undefined HLA restriction pattern (Olive et al., 2002; Veltrop-Duits et al., 2006), five HLA-DR17 and DP4 restricted Hexon epitopes (Heemskerk et al., 2006), and three HLA-DRB1 restricted epitopes (Onion et al., 2007). In a recent study, five CD4 epitopes were identified in the Hexon protein

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with undefined HLA restriction showing a reactivity among six out of 26 HAdV-specific T-cell lines (Leen et al., 2008). All these epitopes were either identified or characterized using HAdV-specific T-cell lines, which have been repetitively stimulated *in vitro*. Diagnostic tools for the detection of HAdV specific T cells and an adoptive T-cell transfer require (i) epitopes with known HLA-binding pattern, (ii) dominant epitopes that are recognized by the majority of healthy donors and by patients recovering from HAdV infection and (iii) epitopes with an antigenicity that enables an *ex vivo* detection of a specific T-cell response without long-term culture steps *in vitro*.

In this paper we therefore selected 19 candidate CD4 epitopes with predicted restriction to HLA-DR1, -DR3, -DR4 and -DR7 using the SYFPEITHI database. With these peptides 44 healthy donors and 5 patients post SCT were tested for the presence of Hexon-specific $CD4^+$ responses. Ten epitopes showed a proliferation response in >50% of individuals with major differences in the response of healthy donors and patients post SCT.

Table 1
Amino acid sequences of predicted MHC-II restricted peptides from the Hexon protein (swissprot database entry P04133).

No.	Amino acid sequence	Mass	Position	Score	Predicted HLA restriction
3545	GTAYNALAPKGAPNP	1440.7	117–131	36	DR1
3534	QWSYMHISGQDASEY	1440.7	8–22	32	DR1
3542	TETLTKVKPKTGQEN	1672.9	422–436	24	DR1
3540	TGNMGVLAGQASQLN	1459.7	341–355	32	DR1
3526	VDCYINLGARWSLDY	1786.8	513–527	36	DR1
3547	EWNFRKDVNMVLQSS	1851.9	581–595	33	DR3
3549	GASIKFDSICLYATF	1634.8	604–618	31	DR3
3590	THDVTTDRSQRLTLR	1797.9	52–66	30	DR3
3592	PGSYTYEWNFRKDVN	1874.9	575–589	28	DR4
3621	TLRFIPVDREDTAYS	1781.9	64–78	28	DR4
3594	SQWYETEINHAAGRV	1759.8	208–222	28	DR4
3595	ENGWEKDATEFSDKN	1768.8	435–449	28	DR4
3597	GNNFAMEINLNANLW	1719.8	454–468	28	DR4
3551	ATFFPMAHNTASTLE	1636.8	616–630	28	DR4
3619	GWAFTRLKTKETPSL	1733.9	676–690	28	DR4
40002	LMYYNSTGNMGVLAG	1589.7	335–349	32	DR7
3625	FKKVAITFDSSVSWP	1709.9	717–731	30	DR7
3623	DPYYTYSGSIPYLDG	1709.7	695–709	30	DR7
3596	PQKFFAIKNLLLLPG	1698	562–576	30	DR4, DR7

Results

Alignment of Hexon protein sequences of different adenovirus strains/subtypes

An alignment of the sequences of 51 different adenovirus subtypes shows the known conserved and hypervariable regions of the adenoviral Hexon protein (Fig. 1). The localization of the defined CD4 T-cell epitopes is in the highly conserved regions for 16 peptides and in variable regions for 3 peptides. Localization of an epitope in a conserved region means, that the corresponding T_{HELPER}-cell response is most probably cross-reactive against different adenovirus subtypes.

Analysis of T_{HELPER}-cell responses ex vivo

We investigated the response of $CD4^+$ T cells in healthy donors against the candidate Hexon epitopes. All out of 19 predicted epitopes listed in Table 1 showed a specific T-cell response in at least one donor, but the number of responding donors was variable. An epitope was considered immunodominant, if a reactivity was found in more than 50% of tested individuals. Among 19 tested epitopes, 10 were found immunodominant (Fig. 2): one epitope predicted for promiscuous DR4/DR7 presentation (3596), three epitopes predicted for HLA-DR1 presentation (3534, 3545, 3540), one epitope for HLA-DR3 (3549), four epitopes for HLA-DR4 (3592, 3596, 3619, 3621) and three epitopes predicted for HLA-DR7 (3596, 3623, 40002). The antigen specific proliferation response was mainly within the $CD4^+$ T-cell population (Fig. 2).

The antigen specific proliferation response of $CD4^+$ T cells was confirmed by intracellular IFN-γ detection after *ex vivo* stimulation with peptides. This method allows a quantification of specific T cells in peripheral blood and is important for antigen detection and selection of Ag-specific T cells. The frequency of peptide-specific T cells in peripheral blood is rather low. Sixteen out of 19 peptides showed a response above the detection threshold of 0.01% (% of viable T cells) (Fig. 3). All *ex vivo* responses to peptide epitopes represented only a fraction of the response to complete Hexon protein, which means in all donors the response to Hexon protein was a multi-epitope response.

Individual response pattern to different Hexon epitopes

All 44 healthy donors showed an individual response pattern. The combination as well as the quantity of the $CD4^+$ T-cell response was different among donors. All healthy donors had T_{HELPER}-cell responses against two or more Hexon epitopes, with a median reactivity against three (2–8) epitopes (mean 3.5 ± 1.4 SD). Some donors showed a strong response to epitopes, which are recognized only by a minority of donors. As an example for this phenomenon serves peptide 3597, which is recognized by only 20% of donors (Fig. 2), but induced a strong response comparable to the immunodominant epitope 3592 (Fig. 3). Three peptides (3540, 3592 and 3621) showed a proliferation response almost as high as the percentage of donors responding to Hexon protein (Fig. 2).

T_{HELPER}-cell response to HLA-DR epitopes in patients after recovery from HAdV infection post allogeneic SCT

Analysis of specific $CD4^+$ T-cell response to HLA-DR binding Hexon peptides was performed in six patients post allogeneic SCT.

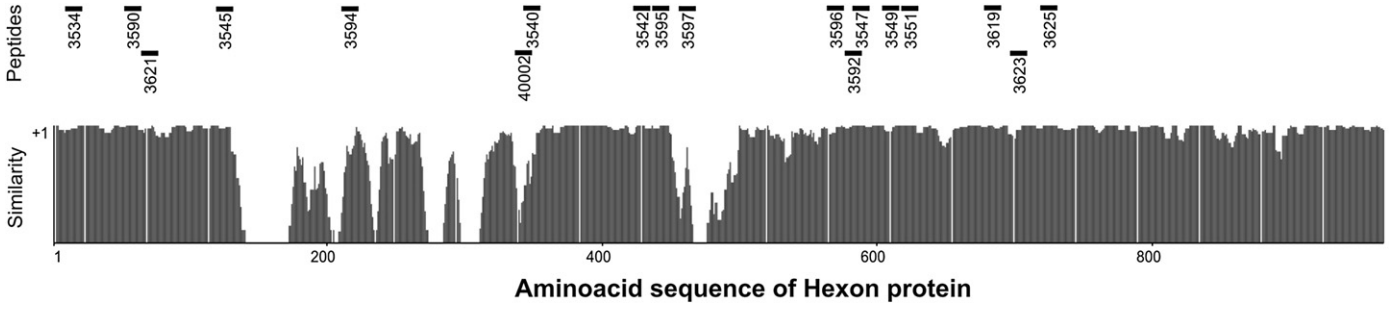

Fig. 1. Alignment of the sequences of Hexon proteins from the 51 different adenovirus subtypes: The alignment shows conserved regions with high similarity and hypervariable regions with low similarity. In the upper panel the localization of the identified Hexon peptides is shown. In the conserved regions are 16 out of 19 epitopes, which suggests cross-reactivity of the corresponding T-cell responses to several adenovirus subtypes. Three epitopes (40002, 3540 and 3597) are in variable regions.

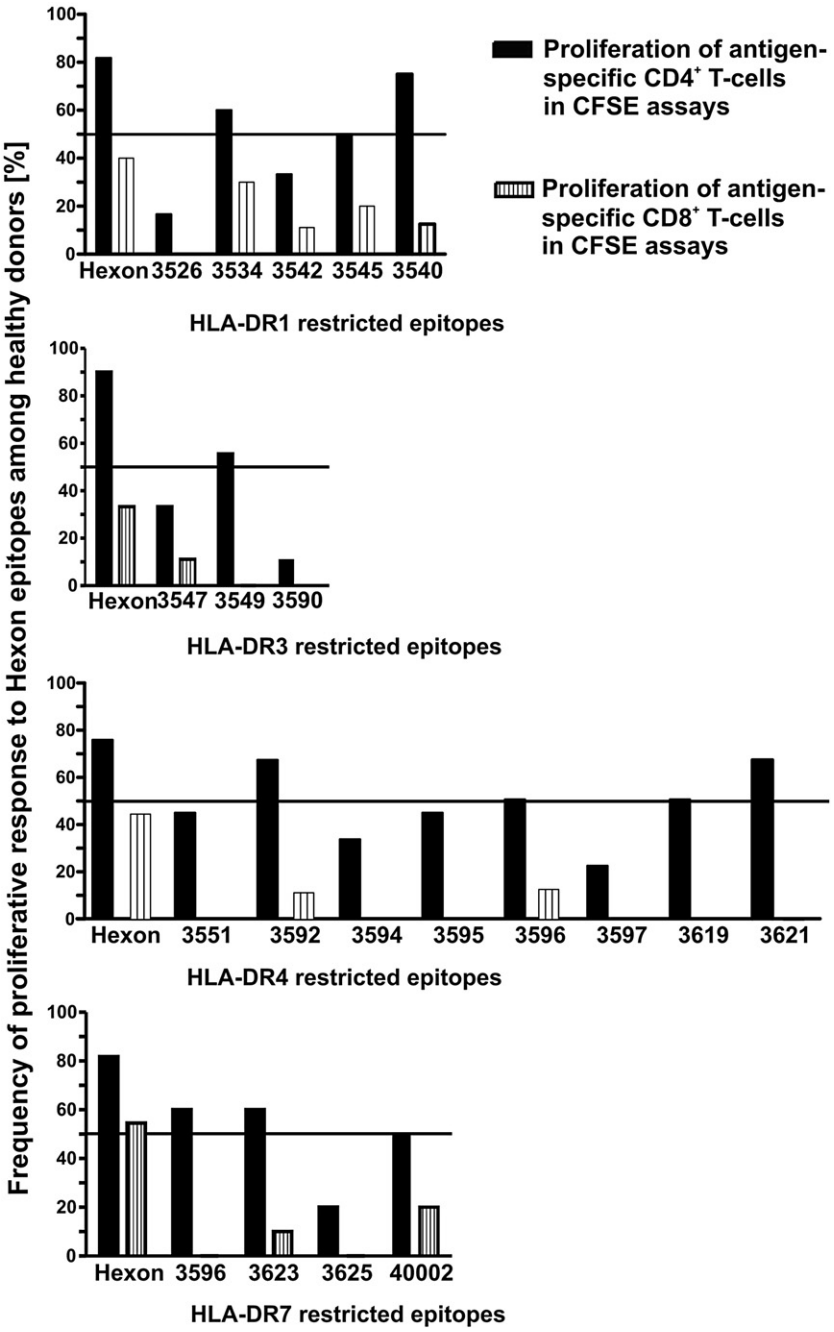

Fig. 2. Frequency of $CD4^+$ T-cell responses to Hexon epitopes among 44 healthy donors. T-cell proliferation was assessed *ex vivo* by CFSE and is shown in response to recombinant protein and to HLA-DR restricted peptides. Every panel represents a different group of donors ($n=11$ each panel) with an HLA type corresponding to the predicted HLA-DR restriction of the epitopes. If more than 50% of healthy donors responded to peptides, the epitope was termed immunodominant, since only 70–90% of healthy donors have detectable cellular response *ex vivo* to the adenoviral Hexon protein.

Patient characteristics are listed in Table 2. A HAdV-specific T-cell response was detectable in patients only after recovery from an HAdV infection (patient nos. 1–3). In patients without a preceding infection, no HAdV-specific T-cell response was detectable (patient nos. 4–6). In all samples with a Hexon-specific T-cell response, a reactivity of $CD4^+$ T cells to HLA-DR-restricted peptides was found. A reactivity to a maximum of one epitope per patient could be detected.

$CD4^+$ T cells showed strong proliferation response to single HLA-DR epitopes. In contrast to healthy donors, the reactivity in patients post SCT was focused to single epitopes (Fig. 4), whereas healthy donors demonstrated always multi-epitope responses. The restriction after SCT of the T_{HELPER} cell response to single epitopes was confirmed in these three patients by detection of intracellular IFNγ (Fig. 4B).

HLA-DR restriction pattern of Hexon-epitopes

The HLA-DR restriction of Hexon epitopes was assigned by their binding probability according to SYFPEITHI. This algorithm-based prediction of HLA allele-specific presentation was re-examined *in vitro* with Elispot assays. All peptides were grouped according to their HLA-binding prediction and used as an antigen cocktail in Elispot assay. In donors expressing HLA-DR1, -DR3, or -DR4 the strongest response is seen to the cocktail matching the HLA restriction (Fig. 5). In donors with a mismatched HLA type, the response was negative or

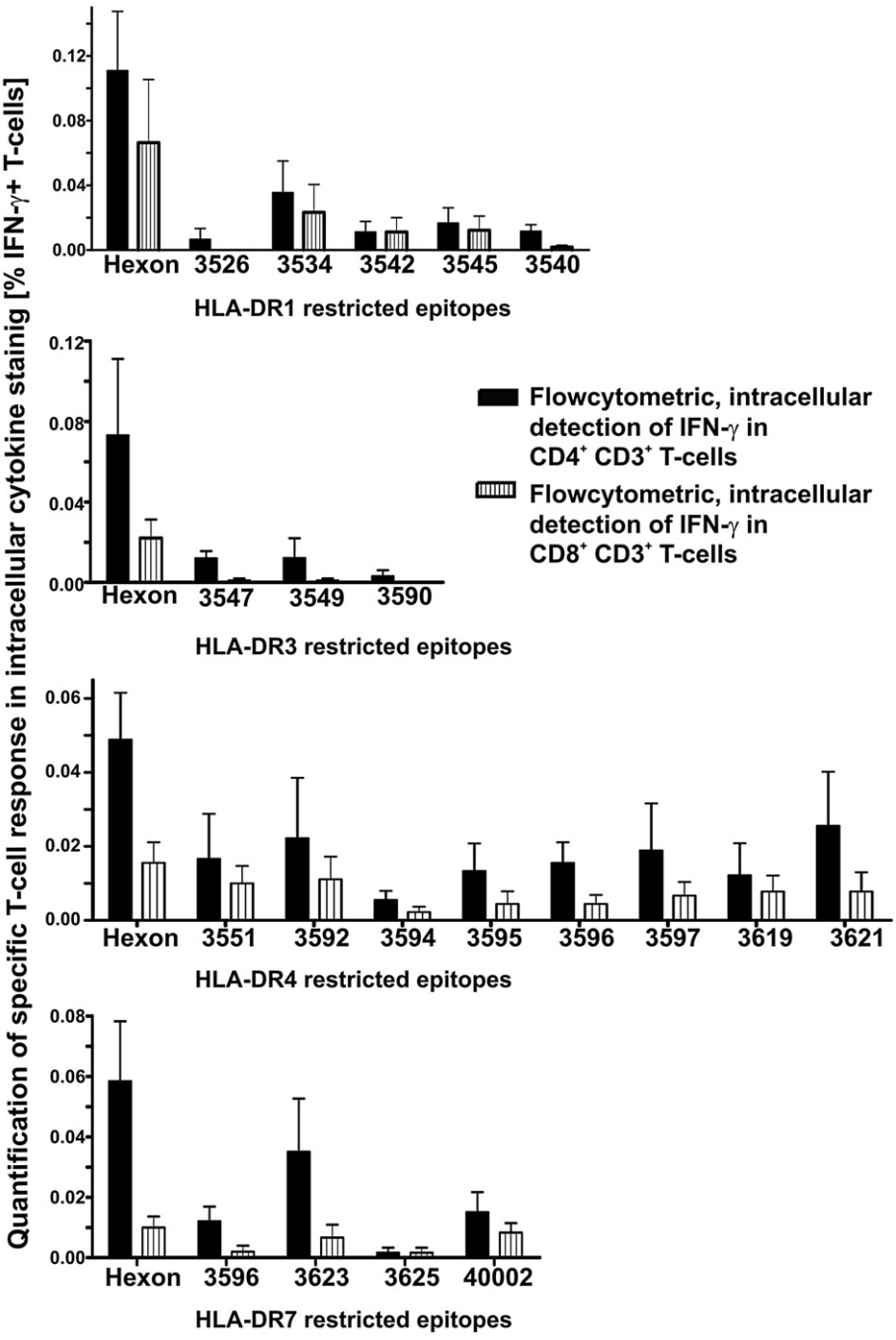

Fig. 3. Quantification of Hexon-specific T-cell response in intracellular cytokine staining: The frequency of peptide-specific T cells was measured by intracellular IFN-γ staining after stimulation with antigen *ex vivo*. The quantitative analysis of the IFN-γ response to HLA-DR binding peptides is shown in comparison to complete protein (mean ± SD). Every panel represents a different group of donors with an HLA type corresponding to the HLA-DR restriction of the epitopes. A detectable cytokine response is seen even in non-immunodominant donors. The frequency of peptide-specific T cells in peripheral blood is rather low. Sixteen out of 19 peptides showed a response above the detection threshold of 0.01% (of viable T cells).

weak (Fig. 5). In HLA-DR7 donors the response did not agree with the prediction.

One peptide was found to be a promiscuous HLA-DR epitope, recognized by HLA-DR1, -DR3, -DR4 and -DR7 donors. Promiscuous recognition of peptide 3596 was confirmed by two different methods, intracellular IFN-γ detection and CFSE proliferation assays. PBMC responses were tested from four donors with mutual exclusive HLA-DR typing regarding HLA-DR1, -DR3, -DR4 and -DR7. Intracellular IFN-γ detection by flow cytometry in response to 3596 stimulation and proliferation response in CFSE assays have shown equivalent responses to stimulation with 3596. The response of $CD4^+$ T cells to this epitope in different HLA background confirmed the promiscuous nature of this epitope (data not shown).

Discussion

Characterization of the specific T-cell immune response to adenovirus is a precondition for immunotherapy approaches that target the prevention of infection related complications in the immunocompromised host. The presence of a detectable CD4 T-cell immunity has been shown essential to overcome potential life threatening HAdV infection/reactivations post allogeneic SCT

Table 2
Patient characteristics of SCT recipients after recovery from HAdV infection (nos. 1–3) and patients without HAdV infection (nos. 4–6) post allogeneic SCT.

Patient no.	Clinical data								Immune reconstitution at time of HAdV specific CD4+ T cell analysis					
	Age [years]	Diagnosis	Graft	Conditioning regimen	GvHD prophylaxis	Day of ADV infection post SCT	Site of HAdV infection/ detection	Symptoms of HAdV infection	Day post SCT	Immunosuppression	Lymphs/μl	CD3+ [%]	CD3+ CD4+ [%]	CD3+ CD8+ [%]
1	6	SCID/ Artemis	haplo	Flud, TT, Mel, OKT3	MMF	194–210	stool	diarrhea	260	MMF	3750	19,6	16,7	3,3
2	16	ALL	haplo	Flu, TT, TLI, OKT3, ATG	MMF	78–174	stool	diarrhea	244	–	2520	69,3	53	27,4
3	9	ALL	haplo	Flu, TT, Mel, OKT3	MMF	minus 11–171	blood, stool	fatigue, diarrhea	257	–	7540	78,3	13,8	67,2
4	8	Ewing sarcoma	haplo	Flud, TT, Mel, OKT3	MMF	–	–	–	84	–	1800	16,6	14,6	1,4
5	14	ALL	MUD	TBI, VP16, ATG	CSA, MTX	–	–	–	105	–	1130	71,3	30,6	58,1
6	6	β-Thalassemia major	haplo	Flud, TT, Mel, OKT3	MMF	–	–	–	100	–	1440	84,9	35,2	51,7

Abbreviations: ALL (acute lymphoblastic leucemia), SCID (severe combined immune deficiency), MUD (matched unrelated donor), haplo (the SCT donor is the father or mother), Flu (Fludarabin), TT (Thiotepa), Mel (Melphalan), OKT3 (Muronomab), MMF (mycophenolate mofetil), CSA (Cyclosporin A), ATG (anti thymocyte globulin), TLI (total lymphoid irradiation), GvHD (graft versus host disease.
Patient nos. 1–3 had a detectable HAdV specific T-cells response in contrast to patient nos. 4–6.

(Flomenberg et al., 1995; Feuchtinger et al., 2005). Several CD4 and CD8 T-cell epitopes have been recently identified (Olive et al., 2002; Tang et al., 2004; Leen et al., 2004). Application of these data to clinical diagnostic and therapeutic procedures is hampered by different hurdles. The frequency of the T-cell response in healthy donors (as a source for immunotherapy) is extremely low or unknown with often uncertain immunodominance and HLA-binding pattern. We have stimulated T cells from healthy donors *ex vivo* with 19 HLA-DR binding epitopes using proliferation and IFN-γ secretion of CD4 T cells as a readout to identify immunodominant epitopes, HLA-restriction and differences between healthy donors and the immunocompromised host.

The T-cell response to hexon protein is driven towards a predominance of T_{HELPER} cells. Flomenberg et al. described already in 1995, that the T-cell response to adenovirus is predominantly CD4. Here we show that the same is true for the hexon protein. However, the T-cell response in *in vitro* assays may also depend on the nature of the antigen. Using protein antigens, the *in vitro* response may be driven towards T_{HELPER} cells.

The percentage of donors responding to a Hexon epitope showed a wide range between 10% and 80% of donors. In addition, every donor showed an individual response pattern to Hexon epitopes. These findings underline the importance of analyzing immunodominance, since a response of a single donor or T-cell line to a single epitope could hardly predict a relevance of this epitope in other healthy donors or even an immunocompromised host. The quantity of response detected by IFN-γ secretion *ex vivo* was independent of the response prevalence among healthy donors. Above a threshold of 0.01% an isolation of virus specific T cells has been possible for adoptive T-cell therapy (Feuchtinger et al., 2004, 2006; Feuchtinger et al., 2008; Leen et al., 2008). According to this threshold 16 peptides would be eligible for an adoptive T-cell transfer, since the *ex vivo* response is strong enough in healthy donors.

The analysis of the acute immune response in immunocompetent humans is difficult, because primary infections are mainly during infancy and early childhood. These infections are normally mild and do not require blood analysis. Therefore the analysis of the acute immune response is restricted to the immunologically unusual situation post SCT in which T-cell activation, proliferation and the T-cell spectrum is markedly altered (Eyrich et al., 2001). We show in three examples post SCT, that the polyclonal response in healthy donors in narrowed to single epitopes during the response to HAdV post SCT. These responses to single epitopes are involved in the acute response to a HAdV infection. Additionally they are likely to be involved in the clearance of HAdV infection in these three patients, since patients without a HAdV infection post SCT had no detectable T-cell response. T-cell depletion during SCT is one possible explanation for the absent T-cell response in patients without a HAdV-infection. T-cell depletion leaves SCT recipients with a naive immune system and protective immunity has to be reestablished through infections or vaccinations.

The clinical applicability of T-cell epitopes is additionally influenced by the HLA-binding pattern of identified epitopes and the potential cross-reactivity among different HAdV subtypes. HLA-binding could be clarified in 15 out of 19 peptides (except DR7). Restriction of epitopes to a certain HLA-type reduces the availability of possible immunotherapeutic applications with this antigen, since even frequent HLA-types represent minorities in the transplant population.

In conclusion, immunodominance, quantification of CD4 T-cell response and HLA-restriction has been characterized in 19 HLA-DR binding peptides of the adenoviral Hexon protein. Single epitopes appear to be involved in the recovery from HAdV infection post SCT. These data may provide information to translate data on T-cell epitopes into diagnostic and therapeutic clinical application. The current study indicates that in adoptive T-cell transfer strategies, the use of entire hexon (or a mixture of capsid proteins) provides a broader spectrum and hence an inductive response is more likely, compared to single epitope approaches.

Material and methods

Healthy donors and patients post SCT

Blood samples from healthy donors were received from the local Transfusion Medicine. Patient samples were from children with HAdV infection post allogeneic SCT within the first 6 months after SCT. Patients were treated in the Pediatric Stem Cell Transplant Program of the University Children's Hospital Tübingen. The patient characteristics and transplant data are summarized in Table 2. None of the patients received immunosuppressive treatment at the time of T-cell analysis. All donors and patients gave informed consent. The analysis of blood samples was reviewed by the ethical institutional review

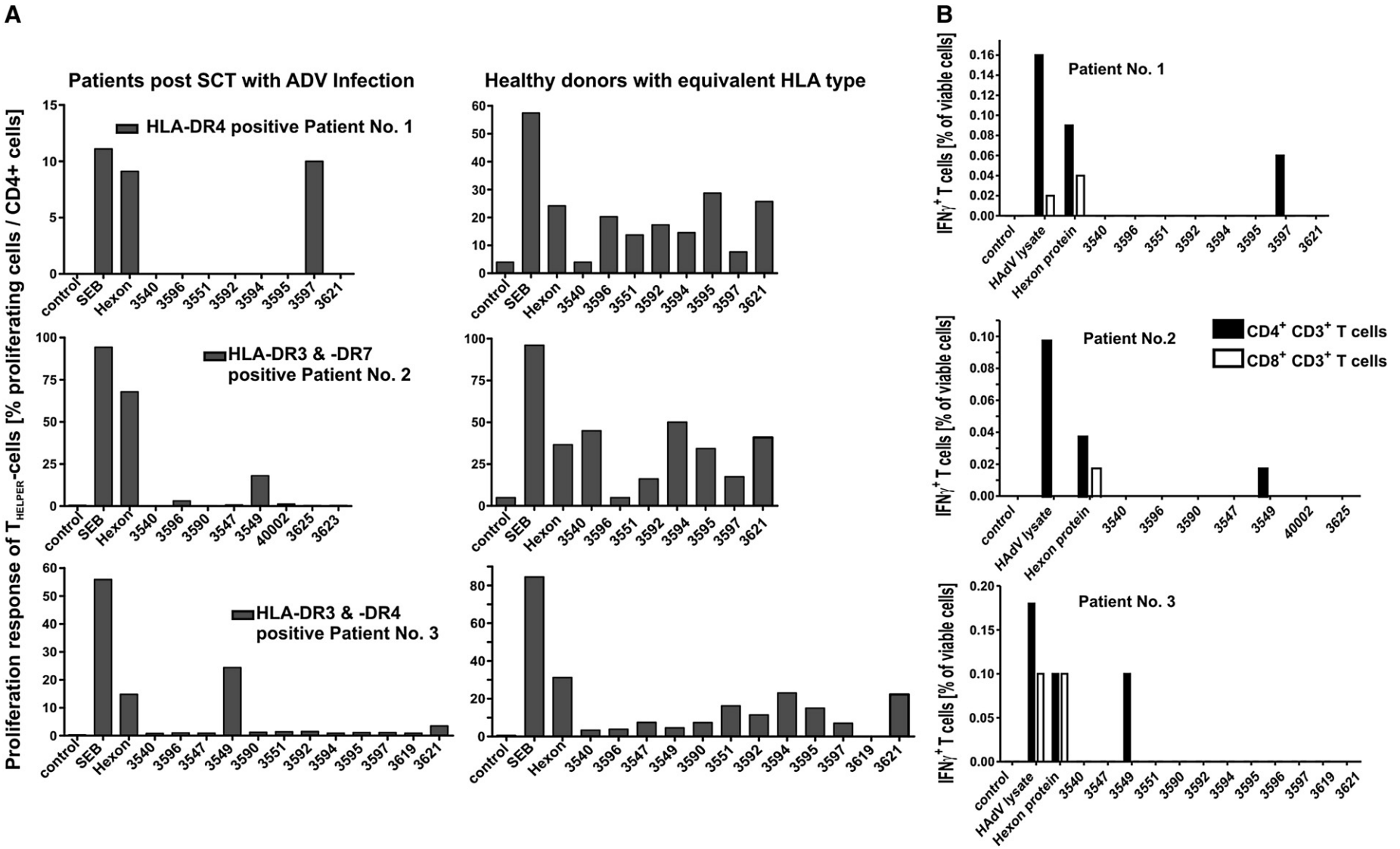

Fig. 4. Patients after recovery from HAdV infection post allogeneic stem cell transplantation show a narrowed T-cell response against single epitopes in comparison to a multi-epitope response in healthy donors. Proliferation responses are shown in three children with HAdV infection during the first year post allogeneic SCT (panel A, left column of graphs) and representative proliferation responses in three healthy donors with the same HLA type (panel A, right column of graphs). The intensity of the response was similar to responses seen in healthy donors, but patients responded solely to single epitopes. In contrast, healthy donors always showed a response to multiple epitopes. The proliferation results shown in Panel A are confirmed with the detection of intracellular IFN-γ by flow cytometry (panel B). These responses to single epitopes are likely to be involved in the acute response to a HAdV infection. Additionally they are likely to be involved in the clearance of HAdV infection in these three patients, since patients without a HAdV infection post SCT had no detectable T-cell response. The patient numbers correspond to the patient numbers in Table 2.

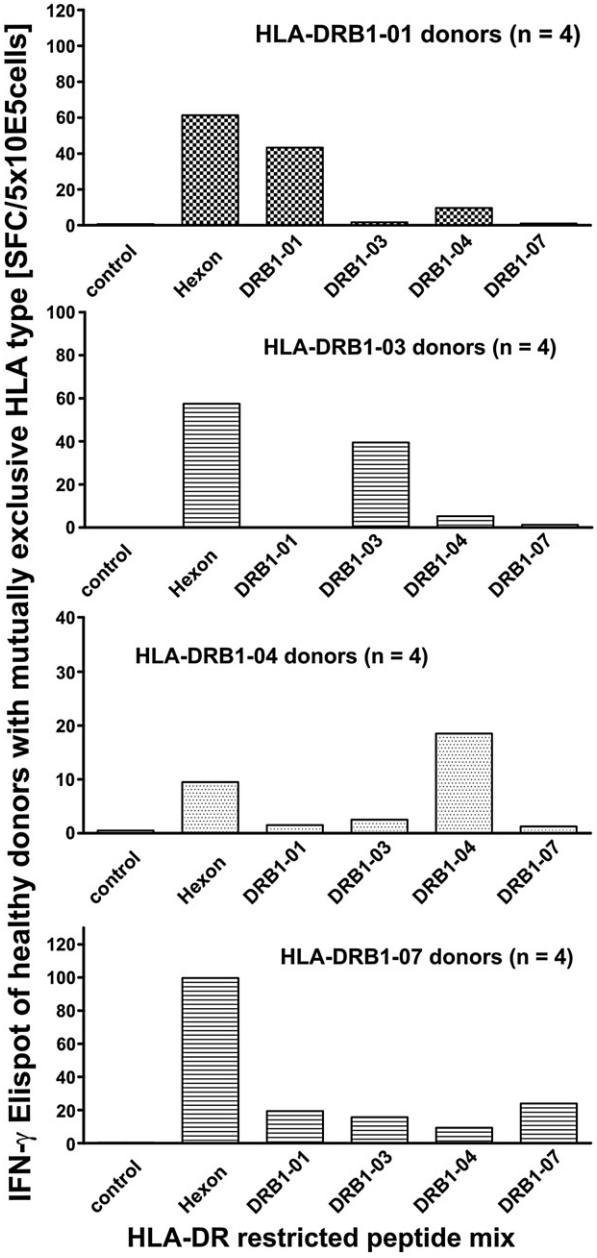

Fig. 5. HLA-DR restriction of Hexon peptides in Elispot assays performed with PBMC of healthy donors. *Ex vivo* IFN-γ response against mixed peptides pools was tested in healthy donors. PBMCs were stimulated *ex vivo* with HLA-DR restricted peptide mixes (HLA-DR1 mix: 3534, 3542, 3545, 3526, 3540; HLA-DR3 mix: 3547, 3549, 3590; HLA-DR4: 3551, 3592, 3594, 3595, 3597, 3619, 3621; HLA-DR7: 3623, 3625, 40002). IFN-γ expression was then measured by IFN-gamma ELISPOT (SFC = Spot Forming Cells/5×10^5 PBMCs). Donors expressed each one of the four HLA-DR types (HLA-DR1, -DR3, -DR4, -DR7) and were negative for the three other ones. The response to the HLA-DR1, -DR3 and -DR4 restricted peptides showed a pattern according to the predicted HLA restriction while the response to HLA-DR7 restricted peptides did not follow the prediction (mean ± SD).

board of the University Hospital Tübingen and complied with the Declaration of Helsinki.

Prediction of CD4$^+$ T-cell epitopes

The sequence of Hexon protein from human adenovirus serotype 5 (sequence obtained from the swissprot database at http://www.expasy.ch, Accession No. P04133) was screened for candidate epitopes fitting to HLA-DR peptide motifs using the SYFPEITHI database (www.syfpeithi.de). From all possible 15mer sequences, the top scoring peptides were selected as follows: From the DR1, DR3, and DR7 predictions, all peptides with scores higher than 30 were chosen for synthesis (11 peptides in total). Since 28 is the maximal score in HLA-DR4 predictions, all nine peptides scoring 28 in the DR4 prediction were also selected. Since peptide 3596 ranked high in two different DR predictions, the total number of synthesized peptides was 19 (see Table 1).

Proliferation assays

Proliferation was detected with CFSE (Carboxyfluorescein succinimidyl ester, Molecular Probes, Eugene, OR, USA) according to a recently published protocol (Feuchtinger et al., 2008; De Rosa et al., 2003). PBMCs were enriched by Ficoll® separation and washed with PBS. CFSE was then added to the cells to a final concentration of 1.6 μM in PBS and cells were incubated for 9 min at 37 °C. CFSE was then neutralized by Fetal Bovine Serum and washed off with RPMI 1640®. PBMCs were resuspended in RPMI 1640 with 10% AB serum to a final concentration of 2.5×10^6 cells/ml. After stimulation by addition of the respective peptides at a concentration of 10 μg/ml, the stained cells were incubated in a 96 well plate, cell proliferation was finally assessed by flow cytometry. A positive response was defined as >10% above background proliferation in the unstimulated negative controls. Positive controls were carried out with Staphylococcal enterotoxin B (SEB, Sigma Chemical, St. Louis, USA).

Flow cytometry

Ag-specific T cells were detected as described recently (Feuchtinger et al., 2005). In brief, peripheral blood mononuclear cells (PBMC) were stimulated *ex vivo* with either 10 μg/ml Hexon peptides or 6 μg/ml Hexon protein from type 2 adenovirus (Virion-Serion GmbH, Würzburg, Germany), or viral lysate. The lysate was derived from a HAdV strain isolated from peripheral blood of a pediatric patient after SCT, who suffered from systemic HAdV infection type C. The viral particles were inactivated with 65 °C for 30 min. Positive responses were confirmed with a concentration of 1 μg/ml peptides for stimulation. T cells with antigen-specific secretion of IFN-γ were detected on the following day. Flow cytometric assessment of IFN-γ secretion of viable T cells was carried out by intracellular cytokine staining. Surface staining was performed using saturating conditions of the following antibodies: anti-CD4 or anti-CD8 (clones SK3 or SK1), anti-IFN-γ (clone 25723.11), anti-CD3 (clone SK7), all from Becton Dickinson (BD) or BD Pharmingen (Heidelberg, Germany). At least 100,000 lymphoid cells were analyzed on a FACS-Calibur with Cellquest software (both from BD). Negative and positive controls were carried out with unstimulated samples and staphylococcal enterotoxin B (SEB, Sigma Chemical). Experiments were evaluated only when unstimulated controls were negative and response to SEB was positive. Although control antigens did not usually stimulate any IFN-γ production, the percentage of specific T cells was calculated by subtraction of the frequency obtained by the respective negative control. Negative controls were carried out with isotype staining, unstimulated controls and irrelevant peptides.

Elispot assays

The Elispot assay was performed using Multiscreen-HA® plates (Millipore SA., Molsheim, France) coated with monoclonal anti-human IFN-γ antibody (Mabtech AB, Clone 1-D1K, Hamburg, Germany). After incubation of 5×10^6 cells/ml (100 μl/well) for 20 h at 37 °C with peptides/antigen, positive controls, biotinylated anti-human IFN-γ (both Mabtech AB, Clone 7B 6-1) was added. Thereafter, staining was performed with an Avidin-Peroxidase-Komplex, and Vectastain Elite ABC-Kit (Vector Laboratories, Burlingame, CA, USA) according to the

manufacturer's instructions with 3-Amino-9-Ethylcarbazole-substrate (Sigma-Aldrich, Steinheim, Germany) and Dimethylformamide (Merck, Darmstadt, Germany). Results were analyzed in triplicate on an automated Elispot reader (Aelvis, Hannover, Germany).

Alignment of Hexon protein sequence

The alignment was done, using *Vector-NTI Advance 10* software. The complete Hexon amino acid sequences of all 51 different adenovirus subtypes were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez). The NCBI database entry were (HAdV1–HAdV51): AP_000512, P03277, AAO24079, AAD03654, P04133, AAZ99997, AAC35275, AAZ99998, BAE66671, AAZ99999, AAP49209, P19900, ABA00000, AAZ99996, ABA00001, P36854, NP_597869, AAZ99994, ABA00002, ABA00003, AAG21823, ABA00004, ABA00005, ABA00006, ABA00007, ABA00008, ABA00009, ABA00010, ABA00011, ABA00012, AAZ99995, ABA00013, ABA00014, BAB20014, BAB20015, ABA00015, ABA00016, ABA00017, ABA00018, CAA36077, P11820, ABA00019, ABA00020, ABA00021, ABA00022, ABA00023, ABA00024, ABO61301, ABA00025, ABA00027 and ABA00026.

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References

Chakrabarti, S., Mautner, V., Osman, H., Collingham, K.E., Fegan, C.D., Klapper, P.E., Moss, P.A., Milligan, D.W., 2002. Adenovirus infections following allogeneic stem cell transplantation: incidence and outcome in relation to graft manipulation, immunosuppression, and immune recovery. Blood 100, 1619–1627.

De Rosa, S.C., Brenchley, J.M., Roederer, M., 2003. Beyond six colors: a new era in flow cytometry. Nat. Med. 9, 112–117.

Eyrich, M., Lang, P., Lal, S., Bader, P., Handgretinger, R., Klingebiel, T., Niethammer, D., Schlegel, P.G., 2001. A prospective analysis of the pattern of immune reconstitution in a paediatric cohort following transplantation of positively selected human leucocyte antigen-disparate haematopoietic stem cells from parental donors. Br. J. Haematol. 114, 422–432.

Feuchtinger, T., Lang, P., Hamprecht, K., Schumm, M., Greil, J., Jahn, G., Niethammer, D., Einsele, H., 2004. Isolation and expansion of human adenovirus specific CD4+ and CD8+ T-cells according to IFN-gamma secretion for adjuvant immunotherapy. Exp. Hematol. 32, 282–289.

Feuchtinger, T., Lucke, J., Hamprecht, K., Richard, C., Handgretinger, R., Schumm, M., Greil, J., Bock, T., Niethammer, D., Lang, P., 2005. Detection of adenovirus-specific T cells in children with adenovirus infection after allogeneic stem cell transplantation. Br. J. Haematol. 128, 503–509.

Feuchtinger, T., Matthes-Martin, S., Richard, C., Lion, T., Fuhrer, M., Hamprecht, K., Handgretinger, R., Peters, C., Schuster, F.R., Beck, R., Schumm, M., Lotfi, R., Jahn, G., Lang, P., 2006. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br. J. Haematol. 134, 64–76.

Feuchtinger, T., Richard, C., Joachim, S., Scheible, M., Schumm, M., Hamprecht, K., Martin, D., Jahn, G., Handgretinger, R., Lang, P., 2008. Clinical grade generation of Hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J. Immunother. (1997.) 31, 199–206.

Flomenberg, P., Babbitt, J., Drobyski, W.R., Ash, R.C., Carrigan, D.R., Sedmak, G.V., McAuliffe, T., Camitta, B., Horowitz, M.M., Bunin, N., 1994. Increasing incidence of adenovirus disease in bone marrow transplant recipients. J. Infect. Dis. 169, 775–781.

Flomenberg, P., Piaskowski, V., Truitt, R.L., Casper, J.T., 1995. Characterization of human proliferative T cell responses to adenovirus. J. Infect. Dis. 171, 1090–1096.

Handgretinger, R., Lang, P., Schumm, M., Pfeiffer, M., Gottschling, S., Demirdelen, B., Bader, P., Kuci, S., Klingebiel, T., Niethammer, D., 2001. Immunological aspects of haploidentical stem cell transplantation in children. Ann. N.Y. Acad. Sci. 938, 340–357.

Heemskerk, B., Lankester, A.C., van Vreeswijk, T., Beersma, M.F., Claas, E.C., Veltrop-Duits, L.A., Kroes, A.C., Vossen, J.M., Schilham, M.W., van Tol, M.J., 2005. Immune reconstitution and clearance of human adenovirus viremia in pediatric stem-cell recipients. J. Infect. Dis. 191, 520–530.

Heemskerk, B., van Vreeswijk, T., Veltrop-Duits, L.A., Sombroek, C.C., Franken, K., Verhoosel, R.M., Hiemstra, P.S., van Leeuwen, D., Ressing, M.E., Toes, R.E., van Tol, M.J., Schilham, M.W., 2006. Adenovirus-specific CD4+ T cell clones recognizing endogenous antigen inhibit viral replication in vitro through cognate interaction. J. Immunol. 177, 8851–8859.

Kojaoghlanian, T., Flomenberg, P., Horwitz, M.S., 2003. The impact of adenovirus infection on the immunocompromised host. Rev. Med. Virol. 13, 155–171.

Lang, P., Handgretinger, R., Niethammer, D., Schlegel, P.G., Schumm, M., Greil, J., Bader, P., Engel, C., Scheel-Walter, H., Eyrich, M., Klingebiel, T., 2003. Transplantation of highly purified CD34+ progenitor cells from unrelated donors in pediatric leukemia. Blood 101, 1630–1636.

Leen, A.M., Sili, U., Vanin, E.F., Jewell, A.M., Xie, W., Vignali, D., Piedra, P.A., Brenner, M.K., Rooney, C.M., 2004. Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific CD8+ T cells. Blood 104, 2432–2440.

Leen, A.M., Christin, A., Khalil, M., Weiss, H., Gee, A.P., Brenner, M.K., Heslop, H.E., Rooney, C.M., Bollard, C.M., 2008. Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy. J. Virol. 82, 546–554.

Lion, T., Baumgartinger, R., Watzinger, F., Matthes-Martin, S., Suda, M., Preuner, S., Futterknecht, B., Lawitschka, A., Peters, C., Potschger, U., Gadner, H., 2003. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood 102, 1114–1120.

Mohty, M., Jacot, W., Faucher, C., Bay, J.O., Zandotti, C., Collet, L., Choufi, B., Bilger, K., Tournilhac, O., Vey, N., Stoppa, A.M., Coso, D., Gastaut, J.A., Viens, P., Maraninchi, D., Olive, D., Blaise, D., 2003. Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin-based reduced intensity preparative regimen. Leukemia 17, 2168–2177.

Molinier-Frenkel, V., Gahery-Segard, H., Mehtali, M., Le Boulaire, C., Ribault, S., Boulanger, P., Tursz, T., Guillet, J.G., Farace, F., 2000. Immune response to recombinant adenovirus in humans: capsid components from viral input are targets for vector-specific cytotoxic T lymphocytes. J. Virol. 74, 7678–7682.

Molinier-Frenkel, V., Lengagne, R., Gaden, F., Hong, S.S., Choppin, J., Gahery-Segard, H., Boulanger, P., Guillet, J.G., 2002. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. J. Virol. 76, 127–135.

Olive, M., Eisenlohr, L., Flomenberg, N., Hsu, S., Flomenberg, P., 2002. The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. Hum. Gene Ther. 13, 1167–1178.

Onion, D., Crompton, L.J., Milligan, D.W., Moss, P.A., Lee, S.P., Mautner, V., 2007. The CD4+ T-cell response to adenovirus is focused against conserved residues within the hexon protein. J. Gen. Virol. 88, 2417–2425.

Rux, J.J., Kuser, P.R., Burnett, R.M., 2003. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. J. Virol. 77, 9553–9566.

Shirali, G.S., Ni, J., Chinnock, R.E., Johnston, J.K., Rosenthal, G.L., Bowles, N.E., Towbin, J.A., 2001. Association of viral genome with graft loss in children after cardiac transplantation. N. Engl. J. Med. 344, 1498–1503.

Tang, J., Olive, M., Champagne, K., Flomenberg, N., Eisenlohr, L., Hsu, S., Flomenberg, P., 2004. Adenovirus hexon T-cell epitope is recognized by most adults and is restricted by HLA DP4, the most common class II allele. Gene Ther 11, 1408–1415.

Tang, J., Olive, M., Pulmanausahakul, R., Schnell, M., Flomenberg, N., Eisenlohr, L., Flomenberg, P., 2006. Human CD8+ cytotoxic T cell responses to adenovirus capsid proteins. Virology 350, 312–322.

Veltrop-Duits, L.A., Heemskerk, B., Sombroek, C.C., van Vreeswijk, T., Gubbels, S., Toes, R.E., Melief, C.J., Franken, K.L., Havenga, M., van Tol, M.J., Schilham, M.W., 2006. Human CD4+ T cells stimulated by conserved adenovirus 5 hexon peptides recognize cells infected with different species of human adenovirus. Eur. J. Immunol. 36, 2410–2423.

Walls, T., Shankar, A.G., Shingadia, D., 2003. Adenovirus: an increasingly important pathogen in paediatric bone marrow transplant patients. Lancet Infect. Dis. 3, 79–86.

Yusuf, U., Hale, G.A., Carr, J., Gu, Z., Benaim, E., Woodard, P., Kasow, K.A., Horwitz, E.M., Leung, W., Srivastava, D.K., Handgretinger, R., Hayden, R.T., 2006. Cidofovir for the treatment of adenoviral infection in pediatric hematopoietic stem cell transplant patients. Transplantation 81, 1398–1404.