Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Human adenovirus can cause morbidity and mortality in immunocompromised patients after allogeneic stem cell transplantation. Reconstitution of adenovirus-specific CD4+ T cells has been reported to be associated with sustained protection from adenovirus disease, but epitope specificity of these responses has not been characterized. Since mainly CD4+ T cells and no CD8+ T cells specific for adenovirus have been detected after allogeneic stem cell transplantation, the relative contribution of adenovirus-specific CD4+ and CD8+ T cells in protection from adenovirus disease remains to be elucidated.

Design and Methods

The presence of human adenovirus hexon-specific T cells was investigated in peripheral blood of pediatric and adult allogeneic stem cell transplant recipients, who showed spontaneous resolution of disseminated adenovirus infection. Subsequently, a clinical grade method was developed for rapid generation of adenovirus-specific T-cell lines for adoptive immunotherapy.

Results

Clearance of human adenovirus viremia coincided with emergence of a coordinated CD8+ and CD4+ T-cell response against adenovirus hexon epitopes in patients after allogeneic stem cell transplantation. Activation of adenovirus hexon-specific CD8+ and CD4+ T cells with a hexon protein-spanning peptide pool followed by interferon-γ-based isolation allowed rapid expansion of highly specific T-cell lines from healthy adults, including donors with very low frequencies of adenovirus hexon-specific T cells. Adenovirus-specific T-cell lines recognized multiple MHC class I and II restricted epitopes, including known and novel epitopes, and efficiently lysed human adenovirus-infected target cells.

Conclusions

This study provides a rationale and strategy for the adoptive transfer of donor-derived human adenovirus hexon-specific CD8+ and CD4+ T cells for the treatment of disseminated adenovirus infection after allogeneic stem cell transplantation.

Key words: allogeneic stem cell transplantation, adoptive immunotherapy, adenovirus infection, adenovirus-specific T cells.

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Introduction

Human adenovirus (HAdV) can cause serious morbidity in immunocompromised patients, in particular in pediatric patients after allogeneic stem cell transplantation (SCT), and progression to disseminated HAdV disease is associated with high mortality.[1-5] The efficacy of antiviral agents such as ribavirin and cidofovir has not been proven in controlled trials yet, and their administration is limited by toxicity. It has been demonstrated that reconstitution of HAdV-specific T cells is essential to control HAdV infection after allogeneic SCT.[5-9] Manipulation of immune reconstitution by adoptive transfer of donor-derived HAdV-specific T cells may, therefore, be an effective strategy to provide short- and long-term antiviral protection.

In healthy individuals, low frequencies of T cells recognizing target cells infected with HAdV or loaded with HAdV lysate have been reported.[10-12] Most HAdV-specific T cells were $CD4^+$, although HAdV-specific $CD8^+$ T cells could also be detected. Further characterization demonstrated that most HAdV-specific T cells recognized major capsid proteins, predominantly the abundant HAdV hexon protein.[13-15] Only recently, a number of immunodominant $CD8^+$ and $CD4^+$ epitopes of HAdV hexon have been defined.[14-19] Since these epitopes are largely conserved, specific T cells were shown to be cross-reactive towards HAdV serotypes from different HAdV subgroups and may, therefore, provide protection against a wide range of HAdV serotypes. HAdV hexon-specific T cells have been reported to be detectable in 72% of healthy donors, but may be present at very low frequencies in the remaining donors.[20]

In allogeneic SCT recipients, higher frequencies of HAdV-specific T cells have been detected after clearance of HAdV infection, but the epitope specificity of these responses has not been further characterized.[7-9] Furthermore, mainly $CD4^+$ T cells and no $CD8^+$ T cells specific for HAdV were detected. Since HAdV-specific $CD4^+$ T cells are capable of directly lysing HAdV-infected target cells, the relative contribution of HAdV-specific $CD4^+$ and $CD8^+$ T cells in protection against HAdV disease after allogeneic SCT remains to be elucidated.[21,22]

For the generation of donor-derived HAdV-specific T-cell lines for clinical application, peripheral blood has been stimulated with HAdV antigens or HAdV-transduced antigen-presenting cells, resulting in enrichment of either $CD4^+$ or $CD8^+$ T cells specific for HAdV.[20,23-29] Although alloreactivity was reduced using these strategies, the frequency of HAdV-specific T cells in the cell lines was not determined or was limited, even when interferon (IFN)γ-based isolation steps were included.

In this study, we demonstrate that $CD8^+$ and $CD4^+$ T-cell responses against HAdV hexon epitopes were associated with clearance of HAdV infection in pediatric and adult patients after allogeneic SCT, providing a rationale for HAdV hexon-specific adoptive immunotherapy. For the generation of clinical grade combined $CD8^+$ and $CD4^+$ HAdV-specific T-cell lines, efficient activation of both $CD8^+$ and $CD4^+$ HAdV-specific T cells by stimulation with an HAdV hexon protein-spanning peptide pool was followed by IFNγ-based isolation. Cultured T-cell lines derived from all healthy donors tested contained high frequencies of $CD8^+$ and $CD4^+$ T cells specific for multiple HAdV hexon epitopes, and efficiently lysed HAdV-infected target cells. The adoptive transfer of these donor-derived HAdV hexon-specific T-cell lines may be used for the treatment of disseminated HAdV disease after allogeneic SCT.

Design and Methods

Donors' and patients' cells

Cells from donors and patients were obtained after informed consent, with approval from the local institutional review board. Peripheral blood mononuclear cells (PBMC) were obtained after Ficoll-Isopaque separation. Measurement of HAdV DNA in the patients' plasma was performed by real time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) as previously described.[30] Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Lonza, Basel, Switzerland).

Human adenovirus antigens

Peptides were derived from the HAdV serotype 5 hexon protein (AP_000211). MHC I restricted peptides were TDLGQNLLY (HLA-A*0101), YVLFEVFDVV (HLA-A*0201), LLYANSAHAL (HLA-A*0201), TYFSLNNKF (HLA-A*2401), MPNRPNYIAF (HLA-B*0702/HLA-B*3501), KPYSGTAYNAL (HLA-B*0702), and IPYLDGTFY (HLA-B*3501).[15,18,19] The 30-mer peptides used corresponded to amino acids 571-600, 691-720, 796-825, 856-885, and 901-930.[16,17] Protein-spanning pools of 30-mer peptides overlapping with 15 amino acids or 15-mer peptides overlapping with 11 amino acids (Miltenyi Biotec, Bergisch Gladbach, Germany) were used.[17] A titrated stock of HAdV serotype 5 was produced as previously described.[8]

Flow cytometry

Cells were stained with FITC-labeled CD3, CD4, CD27, CD28 (BD Biosciences, San Jose, CA, USA), CD45RO (Caltag, Burlingame, CA, USA), CD62L (BenderMedSystems, Vienna, Austria), CCR7 (R&D Systems, Minneapolis, MN, USA), PE-labeled CD28 (BD), CD45RA, CD40L (Beckman Coulter, Fullerton, CA, USA), CCR7 (R&D Systems), PerCP-labeled CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD45RA, CD45RO, and IFNγ (BD) monoclonal antibodies. PE- and APC-labeled HAdV 5 hexon peptide-MHC tetramers were produced as described previously.[31] Fluorescent events were analyzed for each sample using a FACSCalibur and Cellquest software (BD). The limit of detection was defined as a cluster of at least ten specific events, after correction for background events in negative control samples.

Intracellular interferon-γ staining

PBMC were stimulated with 10^{-6} M HAdV 5 hexon peptides for 6 h. During the last 5 h, 10 μg/mL brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. For detection of low frequencies of HAdV-specific T cells, PBMC were cultured for 1 week with 10^{-7} M HAdV 5 hexon peptides and interleukin (IL)-2 100 IU/mL (Chiron, Amsterdam, The Netherlands) prior to analysis. For the analysis of T-cell lines, we used as stimulator cells autologous Epstein-Barr virus-transformed B-cell lines (EBV-LCL) labeled with CFSE (Molecular Probes, Leiden, The Netherlands) for distinction in FACS analysis as previously described.[32] Stimulator cells were loaded overnight with 10^{-6} M HAdV 5 hexon peptides and the T cells were incubated with stimulator cells for 4 h with 10 μg/mL BFA. After stimulation, cell-surface staining with monoclonal antibodies was performed, followed by intracellular staining as previously described.[33]

Isolation and culture of interferon-γ-secreting cells

PBMC (25×10^6) were stimulated with 10^{-6} M HAdV 5 hexon pro-

tein-spanning 15-mer peptide pool for 4 hours. Cells were thoroughly washed in phosphate-buffered saline and IFNγ-secreting cells were labeled using the IFNγ capture assay (Miltenyi Biotec) according to the manufacturer's instructions, and isolated using the midi-MACS system (Miltenyi Biotec). The IFNγ-enriched and IFNγ–depleted cell fractions were both cultured with 50 IU/mL IL-2 and 30 Gy-irradiated feeder cells derived from the IFNγ-depleted fraction. Fresh medium and IL-2 were supplemented every 3 to 4 days.

Cytotoxicity assay

A standard ^{51}Cr release assay was performed as described previously.[34] Briefly, fibroblasts incubated with 200 U/mL IFNγ (Boehringer Ingelheim, Alkmaar, The Netherlands) for 5 days, were incubated overnight with 10^{-6} M HAdV 5 hexon peptides or HAdV 5 virus particles (MOI 500). After washing, fibroblasts were labeled with $Na_2{}^{51}CrO_4$ (GE Healthcare, London, UK) for 1 h at 37°C, and incubated with effector T-cell lines. After 10 h of incubation, supernatant was harvested for ^{51}Cr analysis.

Proliferation assay

Uncultured donor PBMC or HAdV-specific T-cell lines were incubated with allogeneic 30Gy-irradiated PBMC to analyze residual alloreactivity. At day 6 of incubation, ^{3}H-thymidine (Amersham International, Amersham, UK) was added for a further 16 h of incubation and ^{3}H-thymidine uptake was subsequently measured. The residual alloreactivity was calculated by comparing the ^{3}H-thymidine uptake of the HAdV-specific T-cell lines with that of the uncultured PBMC.

Interferon-γ enzyme-linked immunosorbent assay

As stimulator cells, EBV-LCL were loaded with the relevant HAdV hexon peptide at 10^{-6} M for 2 h. After washing, stimulator cells were incubated with the HAdV-specific T-cell line. After overnight incubation, supernatant was harvested and the IFNγ concentration was analyzed by enzyme-linked immunosorbent assay (ELISA, CLB, Amsterdam, The Netherlands).

Results

Human adenovirus hexon-specific T cells in healthy donors

To analyze the HAdV hexon-specific T-cell response in PBMC from healthy adult donors, we determined frequencies of $CD8^+$ and $CD4^+$ T cells specific for HAdV serotype 5 hexon epitopes. Analysis of the kinetics of IFNγ production after stimulation showed maximal IFNγ production 4 h after stimulation with the HAdV 5 hexon protein-spanning 15-mer peptide pool, which was similar for cytomegalovirus-specific T cells as described recently.[33] Therefore, cumulative intracellular IFNγ staining was measured 6 h after stimulation with the hexon 15-mer peptide pool. Intracellular IFNγ staining showed hexon-specific $CD8^+IFN\gamma^+$ T-cell populations in 6/16 healthy donors (Figure 1A and 1B). Stimulation with minimal peptides corresponding to known hexon $CD8^+$ epitopes induced responses in 3/16 donors (Figure 1B). Addition of minimal peptides to the hexon 15-mer pool did not increase the number of donors responding, indicating that the hexon 15-mer pool efficiently induced activation of hexon-specific $CD8^+$ T cells. Protein-spanning 15-mer peptide pools derived from cytomegalovirus or Epstein-Barr virus were used as irrele-

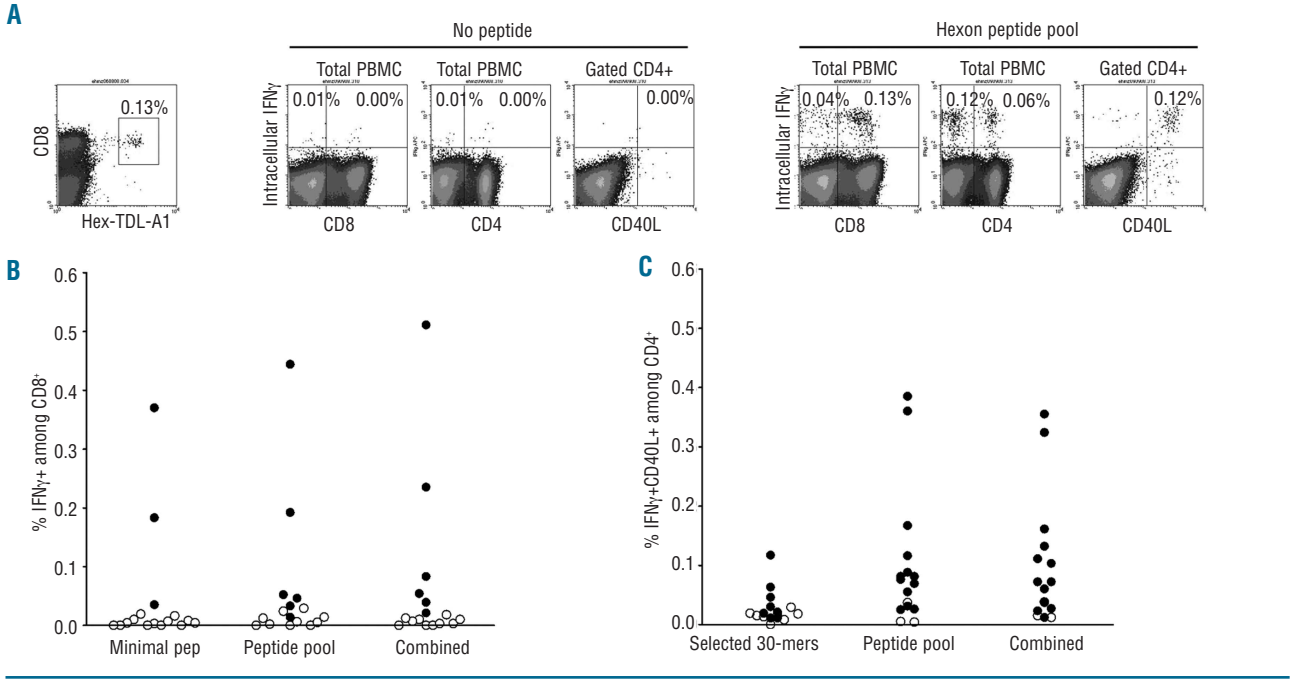

Figure 1. HAdV hexon-specific T cells in healthy donors. (A) Example (donor 4) of detection of HAdV hexon-specific $CD8^+$ T cells by peptide-MHC tetramer staining and HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells by intracellular IFNγ and CD40L staining upon stimulation with the HAdV hexon 15-mer peptide pool. (B) Frequencies of IFNγ-producing cells among $CD8^+$ T cells in PBMC of 16 healthy donors after stimulation with HAdV hexon minimal peptides, 15-mer peptide pool, or these two sets of peptides combined. (C) Frequencies of IFNγ-producing $CD40L^+$ cells among $CD4^+$ T cells in PBMC of 16 healthy donors after stimulation with HAdV hexon selected 30-mers, 15-mer peptide pool, or these two sets of peptides combined. Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection.

vant control peptides in cytomegalovirus and/or Epstein-Barr virus seronegative individuals. No T-cell responses were observed using these control peptides (*data not shown*), confirming the specificity of HAdV hexon-specific IFNγ production. The presence of hexon-specific CD8+ T cells was confirmed in five of the six positive donors by staining with peptide-MHC tetramers corresponding to known hexon CD8+ epitopes, with frequencies of 0.06-0.46% of CD8+ T cells (*data not shown*).

CD4+ T cells specific for HAdV hexon were detected by intracellular IFNγ and CD40L staining upon stimulation of PBMC with a HAdV hexon peptide pool (Figure 1A). Hexon-specific CD4+ IFNγ+CD40L+ T-cell populations could be detected in 13/16 donors (Figure 1C, range 0.03-0.39% of CD4+ T cells). Using five selected hexon 30-mer peptides, which have been described to contain immunodominant hexon CD4+ epitopes,[17] lower frequencies of hexon-specific CD4+ T cells were activated. These data indicate that significantly more CD4+ T cells, presumably specific for other hexon CD4+ epitopes, were activated using the hexon protein-spanning 15-mer peptide pool than with the five selected 30-mer peptides.

Addition of the five selected 30-mer peptides to the hexon 15-mer pool did not result in increased frequencies of IFNγ-producing CD4+ T cells, indicating that the hexon 15-mer pool also efficiently induced activation of CD4+ T cells specific for previously described hexon CD4+ epitopes.

These data show that low frequencies of HAdV hexon-specific CD4+ T cells were detected directly *ex vivo* in peripheral blood from 81% of healthy adults, while hexon-specific CD8+ T cells could be detected directly *ex vivo* in 38% of donors. To determine whether the HAdV hexon-specific T-cell response was below the threshold of detection in the donors for whom no HAdV hexon-specific responses could be detected directly *ex vivo*, PBMC were stimulated for 1 week with the HAdV hexon peptide pool and IL-2 for specific expansion. Subsequently, re-stimulation with the HAdV hexon peptide pool followed by intracellular IFNγ staining showed hexon-specific CD8+ and CD4+ T cells in PBMC from all healthy adults tested (*data not shown*), indicating that low frequencies of HAdV hexon-specific T cells were present in PBMC from all healthy adults.

Human adenovirus-specific T-cell responses in allogeneic stem cell transplant recipients

We next analyzed whether HAdV hexon-specific T-cell responses were associated with clearance of HAdV infection in allogeneic SCT recipients, using the same method as for the healthy individuals. From previously described cohorts of patients, we selected seven pediatric and six adult patients who developed HAdV viremia, as determined by positive HAdV DNA plasma load, and subsequently showed spontaneous resolution of disseminated infection.[4,8] HAdV-specific T-cell responses could not be analyzed in patients who had died from HAdV viremia, since peripheral blood samples were available for only a few patients and the lymphocyte numbers in these samples were too low for analysis. Peripheral blood samples obtained during the period of positive HAdV plasma load were available for ten patients, and peripheral blood samples obtained after resolution of HAdV viremia were available for all 13 patients. During HAdV viremia hexon-specific CD8+ T cells could not be detected directly *ex vivo* and hexon-specific CD4+ T cells were detected in two patients (Figure 2A). After 1 week of culture with the hexon peptide pool and IL-2 for specific expansion, low frequencies of hexon-specific CD4+ T cells were observed in five patients, while no hexon-specific CD8+ T cells could be detected. Following resolution of HAdV viremia, direct intracellular staining showed hexon-specific CD8+ T cells in seven patients, and hexon-specific CD4+ T cells in all patients (Figure 2B). After 1 week of culture of these cells with hexon peptides and IL-2, both hexon-specific CD8+ and CD4+ T cells were shown to be present in PBMC from 12 of the 13 patients. In four patients, some of the hexon-specific CD8+ T cells were directed against previously described epitopes, as detected by peptide-MHC tetramer staining after expansion (*data not shown*).

A more detailed analysis of the development of HAdV-specific T-cell responses is shown for five patients, from whom multiple peripheral blood samples were available (Figure 3). During HAdV viremia, direct intracellular IFNγ staining upon hexon peptide pool stimulation showed no or low frequencies of hexon-specific CD8+ or CD4+ T cells in these patients. After clearance of the HAdV plasma load, high frequencies of up to 30% of CD8+ T cells and 16% of

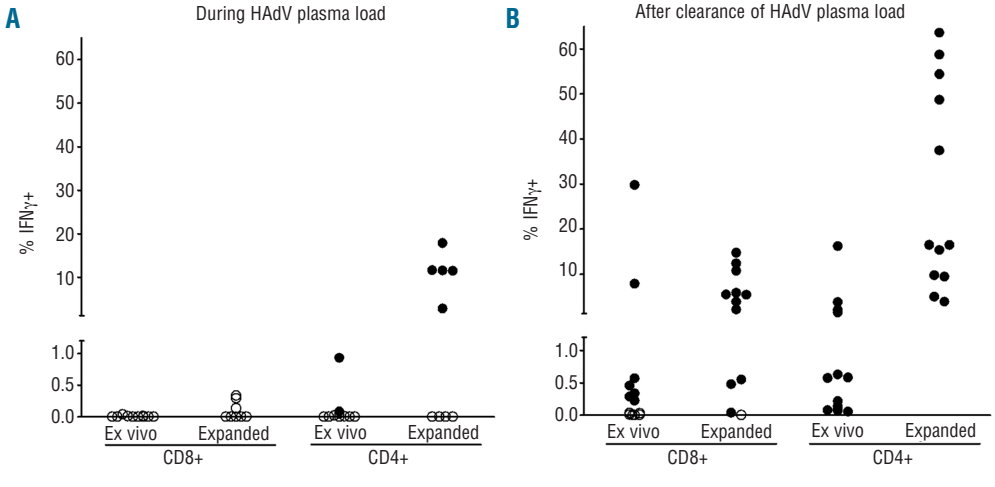

Figure 2. HAdV-specific T-cell responses in allogeneic SCT recipients. Frequencies of CD8+ and CD4+ T cells producing IFNγ upon HAdV hexon peptide pool stimulation of PBMC derived from seven pediatric and six adult patients (A) during or (B) after HAdV viremia as detected by HAdV DNA plasma load. HAdV-specific IFNγ production was analyzed directly in PBMC (*ex vivo*) or after 1 week culture of PBMC with the HAdV hexon peptide pool and IL-2 (expanded). Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection.

CD4+ T cells producing IFNγ upon hexon peptide pool stimulation appeared in peripheral blood. The hexon-specific T cells emerged directly after resolution of HAdV viremia in most patients, although proliferation of high frequencies of hexon-specific T cells was shown to continue for several months after clearance of the HAdV plasma load in pediatric patient 1 (Figure 3A). The relation between the development of hexon-specific T cells and control of HAdV infection was illustrated by the minor HAdV reactivation at day 152 after transplantation in pediatric patient 2, which was followed by an increase in frequencies of HAdV hexon-specific CD8+ and CD4+ T cells and concomitant viral clearance (Figure 3C). These results demonstrate that a coordinated CD8+ and CD4+ T-cell response specific for HAdV hexon epitopes was associated with clearance of HAdV infection in both pediatric and adult patients following allogeneic SCT.

Generation of human adenovirus hexon-specific CD8+ and CD4+ T-cell lines

As the presence of CD4+ as well as CD8+ HAdV hexon-specific T cells was associated with viral clearance after allogeneic SCT, a clinical grade protocol was developed for the generation of HAdV hexon-specific T-cell lines containing both T-cell subsets. PBMC from healthy adult donors were stimulated with the hexon peptide pool, and IFNγ-secreting cells were isolated and cultured with autologous feeders and IL-2. The data for a representative T-cell line generated are shown (Figure 4A-C). Before enrichment, low frequencies of HAdV-specific T cells were detected in PBMC (Figure 4A). In the T-cell line generated from the enriched cell population (Figure 4B), 74% of CD8+ T cells (58% of total cells) were directed against the MPN/HLA-B35 epitope.[19] Re-stimulation of the T-cell line with autologous EBV-LCL loaded with the hexon peptide pool induced spe-

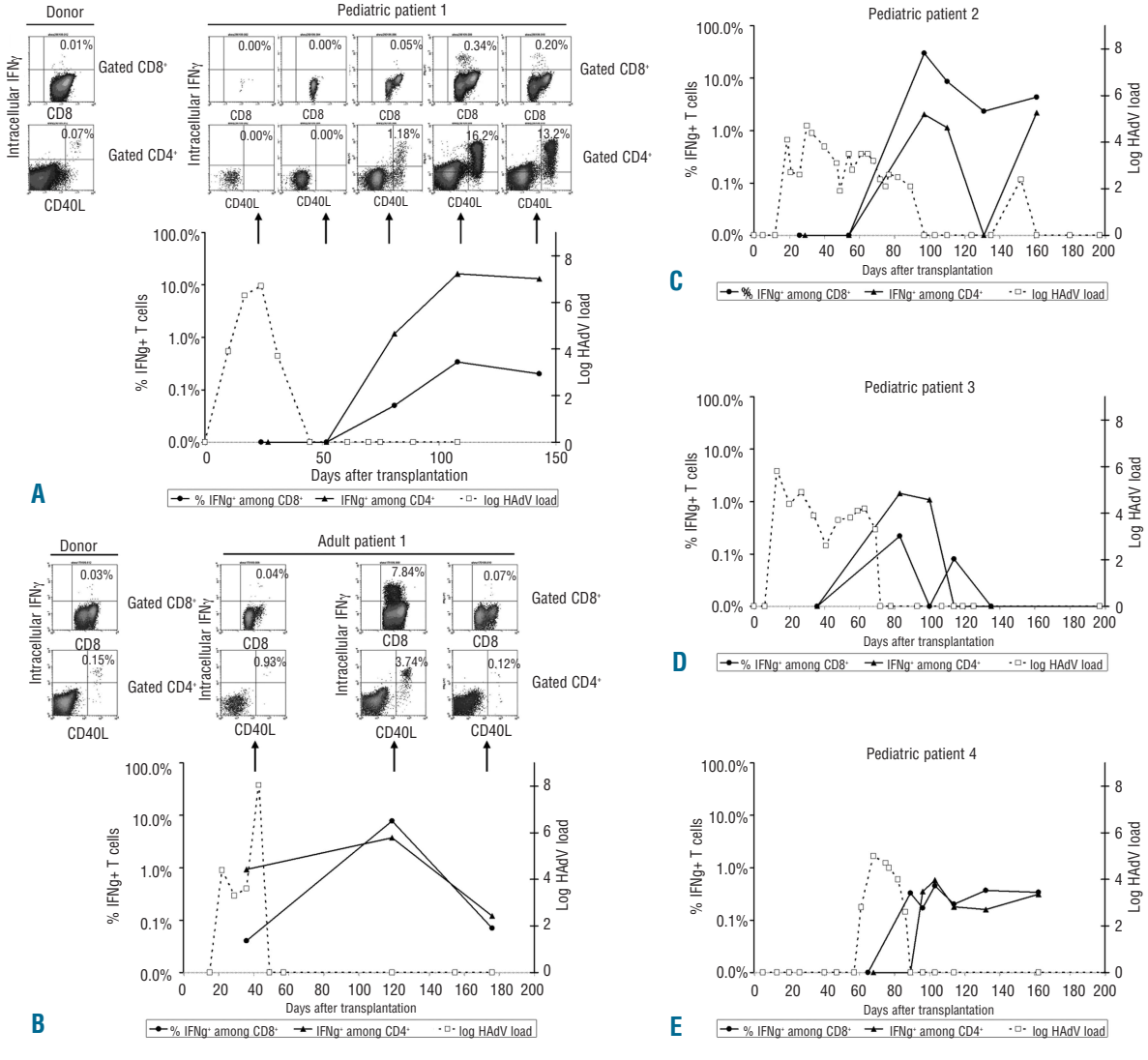

Figure 3. Detailed analysis of HAdV-specific T-cell responses in allogeneic SCT recipients. The dot plots show direct intracellular IFNγ and CD40L staining following HAdV hexon peptide pool stimulation of (A) PBMC from pediatric patient 1 (= patient 16)[8], and (B) PBMC from adult patient 1 (= patient 3)[4], obtained during and after resolution of HAdV viremia. The graphs show frequencies of IFNγ-producing cells among CD8+ T cells (black circles) and among CD4+ T cells (black triangles), and the HAdV DNA plasma load (open squares). Similar graphs are presented in (C), (D), and (E) for pediatric patients 2, 3, and 4.

cific IFNγ production by 94% of CD8+ T cells, indicating that additional hexon CD8+ epitopes were recognized. Furthermore, hexon-specific IFNγ production upon re-stimulation was observed by 91% of CD4+ T cells. Only low frequencies of hexon-specific T cells could be detected in the IFNγ-depleted fraction (Figure 4C). Results from all six donors are summarized in Table 1. HAdV-specific T-cell lines could be generated from donors with relatively high frequencies as well as from donors with low or undetectable frequencies of hexon-specific CD8+ and CD4+ T cells. To analyze residual alloreactivity, the HAdV-specific T-cell lines derived from donors 1, 2, 3, and 5 were incubated with PBMC from three different allogeneic donors. The proliferation of the HAdV-specific T-cell lines compared with the uncultured PBMC was only 0.0-6.8%,indicating absent to very low levels of alloreactive capacity (*data not shown*).

To investigate whether HAdV-specific T-cell lines recognized HAdV-infected target cells which endogenously processed the synthesized HAdV antigen, we determined

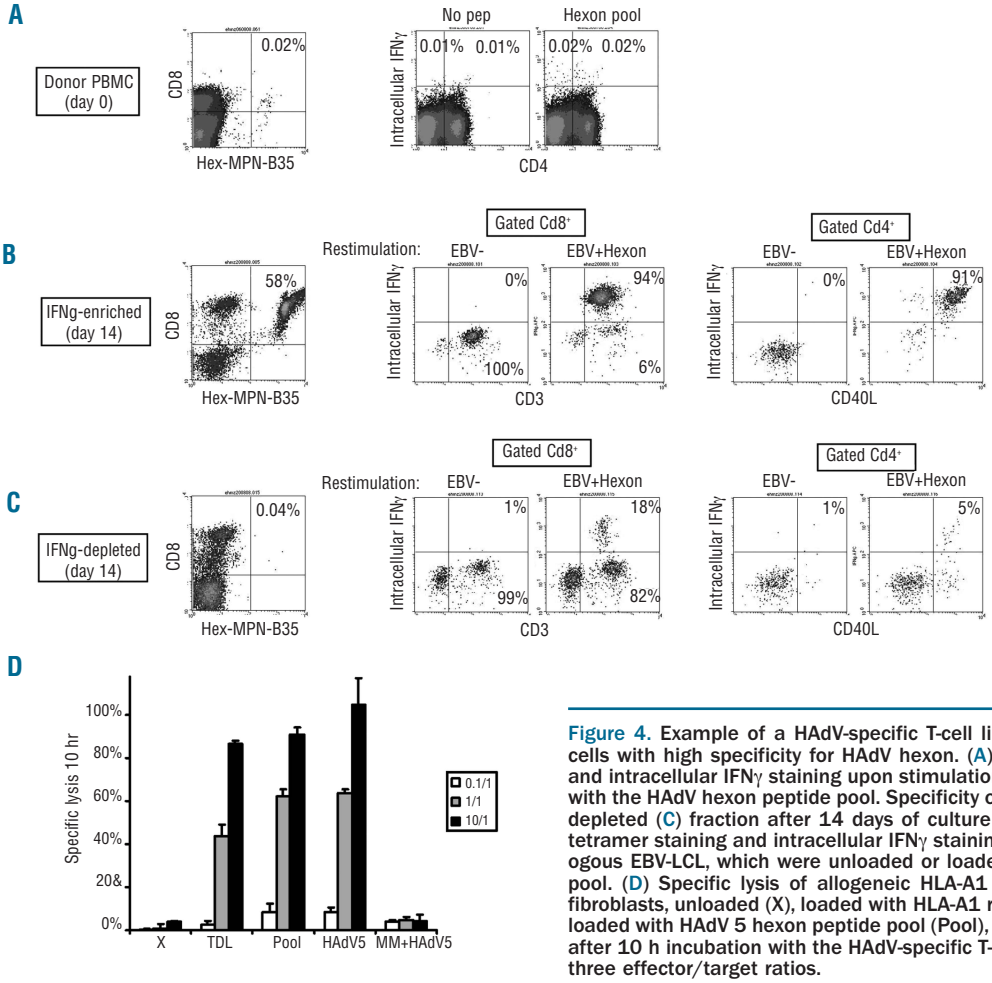

Figure 4. Example of a HAdV-specific T-cell line containing CD8+ and CD4+ T cells with high specificity for HAdV hexon. (A) Peptide-MHC tetramer staining and intracellular IFNγ staining upon stimulation of PBMC from healthy donor 1 with the HAdV hexon peptide pool. Specificity of the IFNγ-enriched (B) and IFNγ-depleted (C) fraction after 14 days of culture as determined by peptide-MHC tetramer staining and intracellular IFNγ staining upon restimulation with autologous EBV-LCL, which were unloaded or loaded with the HAdV hexon peptide pool. (D) Specific lysis of allogeneic HLA-A1 matched or mismatched (MM) fibroblasts, unloaded (X), loaded with HLA-A1 restricted minimal peptide (TDL), loaded with HAdV 5 hexon peptide pool (Pool), or infected with HAdV 5 (HAdV5) after 10 h incubation with the HAdV-specific T-cell line derived from donor 4 at three effector/target ratios.

Table 1. Generation of T-cell lines containing CD8+ and CD4+ T cells with high specificity for HAdV hexon derived from six healthy adult donors.

Donor	Donor	PBMC	Day 1	Day 14/15						
			Cell n.	Cell n.	Composition			Specificity		
	% IFNγ+ / CD8+*	% IFNγ+ / CD4+*	Isolated	Cultured	% CD8+	% CD4+	% NK	% tetramer+ / CD8+*	% IFNγ+ / CD8+*	% IFNγ+ / CD4+*
1	0.04	0.06	8.4x104	2.9x106	77	20	3	74	94	91
2	0.00	0.01	1.2x105	4.3x106	36	10	48	49	89	61
3	0.02	0.02	2.3x105	3.0x106	53	24	23	10	49	13
4	0.51	0.12	5.2x105	2.0x107	74	15	8	47	88	45
5	0.00	0.04	1.6x105	1.2x106	3	92	5	24	25	92
6	0.24	0.11	2.4x105	1.9x106	38	35	20	86	87	79

**To analyze the frequency of HAdV hexon-specific T cells in donor PBMC and in generated T-cell lines, the percentage of tetramer-positive T cells was determined after gating on CD8+ T cells, and the percentage of IFNγ+ T cells was determined after gating on either CD8+ T cells or CD4+ T cells.*

specific lysis of partially matched allogeneic fibroblasts that were loaded with the minimal hexon peptide, with the hexon 15-mer peptide pool, or infected with HAdV serotype 5. HAdV-specific T-cell lines very efficiently lysed both HAdV peptide-loaded and HAdV-infected fibroblasts, while fully mismatched HAdV-infected fibroblasts were not recognized (Figure 4D). Specific lysis of MHC class I restricted peptide-loaded fibroblasts demonstrated the cytotoxic capacity of HAdV hexon-specific $CD8^+$ T cells. Specific lysis by HAdV hexon-specific $CD4^+$ T cells was shown in the T-cell line derived from donor 5, containing predominantly $CD4^+$ T cells, which specifically lysed autologous EBV-LCL loaded with the hexon 15-mer peptide pool (67% specific lysis). Lysis by this T-cell line could be blocked using anti-MHC class II antibodies (95% blockade).

The phenotype of HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells in donor PBMC and the T-cell lines was investigated. In donor PBMC, HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells were $CD56^-$, $CD45RO^+$, $CD27^+$, $CD28^+$, and $CD62L^{+/-}$. After isolation and culture, the phenotype remained identical except for the expression of CD62L, which first increased and subsequently decreased on HAdV-specific $CD8^+$ T cells and increased on HAdV-specific $CD4^+$ T cells. This phenotypic analysis demonstrated that HAdV-specific T cells did not differentiate into end-stage effector T cells during *in vitro* activation and culture.

These results show that IFNγ-based isolation after stimulation with hexon 15-mer peptide pool allowed rapid generation of T-cell lines with high frequencies of both HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells from all healthy adult donors tested, including donors with very low frequencies of circulating HAdV hexon-specific T cells. Furthermore, the HAdV hexon-specific T-cell lines induced specific and efficient lysis of HAdV-infected target cells.

Characterization of the human adenovirus hexon epitopes recognized

To determine the repertoire of HAdV hexon epitopes recognized by HAdV-specific T-cell lines, staining was performed with peptide-MHC tetramers covering all known HAdV hexon $CD8^+$ epitopes restricted by prevalent MHC class I molecules. $CD8^+$ T-cell populations specific for seven previously described hexon epitopes were detected in the HAdV-specific T-cell lines derived from six healthy donors (*Online Supplementary Table S1*). Analysis of specific IFNγ production by $CD8^+$ T cells upon re-stimulation with separate hexon overlapping 30-mer peptides showed recognition of four additional hexon peptides by the HAdV-specific T-cell lines, of which two were recognized by $CD8^+$ T cells from two different donors (*Online Supplementary Table S1*). To investigate HLA-restriction of these epitopes, $CD8^+$ T-cell recognition of a panel of EBV-LCL sharing MHC class I molecules with the donor, loaded with specific hexon 30-mer peptide, was determined. Subsequently, minimal peptides were predicted to bind by algorithms, and were shown to be recognized by specific $CD8^+$ T cells (*Online Supplementary Figure S1*). Specific $CD8^+$ T cells were shown to be stained with IPFSSNFMSM/HLA-B*3501 tetramers, which confirmed HLA-restriction of this peptide. The HLA-restriction of the other peptides (FRKDVNMVL/HLA-B7, ETYFSLNNKF/HLA-B52, and YSYKARFTL/HLA-B63) was confirmed by specific recognition of peptide-loaded EBV-LCL that expressed these HLA molecules (*Online Supplementary Figure S1*). Despite the recognition of FRKDVNMVL peptide in HLA-B7 by specific $CD8^+$ T cells, production of fluorescently labeled FRKDVNMVL/HLA-B*0702 tetramers was not successful. The HAdV hexon sequences recognized by $CD8^+$ T cells in HAdV-specific T-cell lines derived from all six donors are presented in *Online Supplementary Table S1*. Both previously described and newly identified hexon $CD8^+$ epitopes were largely conserved between different HAdV species (*Online Supplementary Table 2*). Furthermore, $CD4^+$ T cells in all T-cell lines recognized multiple hexon 30-mer peptides, indicating a broad specificity which included conserved HAdV hexon regions. These data demonstrate that HAdV-specific T-cell lines generated by IFNγ-based isolation after hexon peptide pool stimulation recognized multiple $CD8^+$ and $CD4^+$ HAdV hexon epitopes, including both known and novel epitopes, thereby exploiting the full donor HLA repertoire.

Discussion

Based on our observation that resolution of HAdV viremia coincided with the appearance of a coordinated $CD8^+$ and $CD4^+$ HAdV hexon-specific T-cell response in patients following allogeneic SCT, we developed a method for generation of $CD8^+$ and $CD4^+$ T-cell lines with high and defined specificity for HAdV hexon for adoptive immunotherapy. HAdV-specific $CD8^+$ T cells have been detected before in healthy adults and in patients following allogeneic SCT.[7,15,18-20,27] Our study further shows a kinetic association between HAdV-specific $CD8^+$ T-cell responses and clearance of HAdV infection in allogeneic SCT recipients.

Since in previous studies predominantly HAdV-specific $CD4^+$ T cells were detected in peripheral blood from healthy individuals, and since these $CD4^+$ T cells showed direct cytotoxic capacity, the role of $CD8^+$ T cells in protection from HAdV disease has been questioned.[1,10,12,17,21,22] In this study, HAdV hexon-specific T cells were detected with high sensitivity using peptide-MHC tetramer staining and intracellular cytokine staining following stimulation with a HAdV hexon peptide pool. Using these methods, low frequencies of HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells were detected directly *ex vivo* in peripheral blood from 38% and 81% of healthy adults, respectively. Subsequently, activation of HAdV hexon-specific T cells with the hexon peptide pool followed by IFNγ-based isolation allowed rapid expansion of both $CD8^+$ and $CD4^+$ T cells specific for HAdV hexon epitopes from all healthy donors tested. These data suggest that coordinated responses of $CD8^+$ and $CD4^+$ T cells specific for HAdV hexon epitopes contribute to the control of HAdV infection in healthy individuals, and persist as memory T cells afterwards, which is similar to T-cell responses described against most other viral infections.

While the development of HAdV-specific T-cell responses in patients after allogeneic SCT was previously shown to be associated with protection from HAdV disease, only HAdV-specific $CD4^+$ T cells, and no HAdV-specific $CD8^+$ T cells were detected.[7-9] To further investigate this, we analyzed T-cell responses specific for the immunodominant HAdV hexon protein in patients who showed spontaneous resolution of disseminated HAdV infection. Using the same sensitive methods of detection as for the healthy donors, combined $CD8^+$ and $CD4^+$ T-cell responses specific for HAdV hexon epitopes were demonstrated to be associated with clearance of HAdV infection in patients after allogeneic

SCT. The HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells were shown to develop synchronously upon clearance of the HAdV plasma DNA load, supporting the coordinated action of HAdV-specific $CD8^+$ and $CD4^+$ T cells in viral control. It is unlikely that immunosuppressive drugs hampered the detection of HAdV hexon-specific T cells directly *ex vivo*, since only some of the patients were using immunosuppressive drugs at the time points of analysis, and expansion of HAdV hexon-specific T cells was observed in PBMC from all patients. These data indicate that both $CD8^+$ and $CD4^+$ T cells specific for HAdV hexon epitopes contribute to the resolution of HAdV infection in allogeneic SCT recipients, and provide a rationale for the adoptive transfer of donor-derived HAdV hexon-specific $CD8^+$ and $CD4^+$ T cells for treatment of HAdV infection after allogeneic SCT.

Despite the low to undetectable frequencies of HAdV hexon-specific T cells in healthy individuals, using HAdV hexon peptide pool stimulation followed by IFNγ-based isolation, highly specific T-cell lines could be generated from all donors tested. In contrast to previous studies, we demonstrated high and defined specificity of the T-cell lines for HAdV hexon epitopes (median 87% of $CD8^+$ T cells and 70% of $CD4^+$ T cells). The risk of graft-*versus*-host disease may be determined by the repertoire of non-HAdV-specific T cells and, therefore, by the absolute number of non-HAdV-specific T cells isolated from donor PBMC. The very low numbers of non-HAdV-specific T cells isolated using this method are assumed to result in a minimal risk of graft-*versus*-host disease. This assumption was supported by the absence or very low levels of residual alloreactive capacity of the HAdV-specific T-cell lines generated. Furthermore, no adverse events associated with administration of HAdV- or cytomegalovirus-specific T-cell lines generated by IFNγ-based isolation have been reported.[23,35,36]

The vigorous expansion and central memory phenotype ($CD45RO^+$, $CD27^+$, $CD28^+$, $CD62L^{+/-}$) of HAdV hexon-specific T cells indicate a HAdV-specific memory T-cell response present in most healthy individuals, which rapidly reacts upon specific activation. While expression of CD27 and CD28 was reported to be transiently up-regulated upon activation and subsequently down-regulated on cytomegalovirus-specific T cells during culture,[33,37] we here demonstrated that HAdV-specific T cells retained expression of CD27 and CD28. This phenotypic analysis indicated that cells did not differentiate to the end-stage effector cell type that might show impaired proliferative capacity *in vivo*.

Stimulation of donor PBMC with the HAdV hexon protein-spanning pool of 15-mer peptides (11-mer overlapping) efficiently induced activation of HAdV-specific $CD8^+$ and $CD4^+$ T cells. Although the frequencies of HAdV hexon tetramer-positive $CD8^+$ T cells were not sufficiently high to perform co-staining with intracellular IFNγ directly *ex vivo*, the activation of cytomegalovirus-specific $CD8^+$ T-cell populations upon stimulation with 15-mer peptide pools has recently been confirmed by tetramer and IFNγ co-staining.[38] Although the mechanism of processing and presentation of these 15-mer peptides has not been investigated, the data suggested that stimulation with 15-mer peptides results in efficient presentation in both MHC class I and II, in contrast to endogenously synthesized antigen being predominantly presented in MHC class I, and exogenous antigens such as full-length protein or lysate being mainly presented in MHC class II.[20,23-29] The use of synthetic peptides, which can readily be produced under GMP regulations, may contribute to the general applicability of generating clinical grade virus-specific T-cell lines. Furthermore, the flexibility of peptide synthesis allows inclusion of peptides derived from multiple viral proteins and viruses.

T cells recognizing multiple MHC class I and II restricted epitopes derived from HAdV serotype 5 hexon protein were isolated from all healthy adults. Like the HAdV hexon epitopes described previously, the HAdV hexon epitopes identified in this study are largely conserved between different HAdV serotypes.[14,17-19,26] The newly identified epitopes increase the repertoire of known immunodominant HAdV hexon epitopes, which may improve the analysis of HAdV-specific T-cell lines generated for adoptive transfer, and the monitoring of HAdV-specific T-cell responses in patients after allogeneic SCT. T cells specific for conserved HAdV epitopes have been demonstrated to be cross-reactive to a wide range of HAdV serotypes.[14,17,18,21,26] The T-cell lines generated in this study may, therefore, provide protection from infection with all prevalent HAdV serotypes.

The presence of both $CD8^+$ and $CD4^+$ HAdV hexon-specific T cells in healthy individuals and in allogeneic SCT recipients after clearance of HAdV viremia suggests that both subsets are required for adequate antiviral protection. This study provides a strategy for the adoptive transfer of donor-derived $CD8^+$ and $CD4^+$ T cells specific for multiple known and unknown HAdV hexon epitopes, which allows treatment of all patients irrespective of their HLA type.

Authorship and Disclosures

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