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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Abstract

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

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

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

Conclusion. This study provides a rationale and strategy for the adoptive transfer of donor-derived HAdV hexon-specific CD8+ and CD4+ T cells for treatment of disseminated HAdV infection after alloSCT.

Introduction

Human adenovirus (HAdV) can cause serious morbidity in immunocompromised patients, in particular in pediatric patients after allogeneic stem cell transplantation (alloSCT), and progression to disseminated HAdV disease is associated with high mortality [1-5]. Efficacy of antiviral agents such as ribavirin and cidofovir has not been proven in controlled trials yet, and administration is limited by toxicity. It has been demonstrated that reconstitution of HAdV-specific T cells is essential to control HAdV infection after alloSCT [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]. The majority of HAdV-specific T cells was 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 alloSCT recipients, higher frequencies of HAdV-specific T cells have been detected after clearance of HAdV infection, but 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 to directly lyse HAdV infected target cells, the relative contribution of HAdV-specific CD4+ and CD8+ T cells in protection from HAdV disease after alloSCT 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 IFNγ-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 alloSCT, 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 IFN-γ-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 treatment of disseminated HAdV disease after alloSCT.

Design and Methods

Donor and patient cells
Donor and patient cells 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 patient plasma was performed by RQ-PCR as previously described [30]. Fibroblasts were cultured from skin biopsies in DMEM with 10% fetal calf serum (Lonza, Basel, Switzerland).

HAdV 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&DSystems, Minneapolis, MN, USA), PE-labeled CD28 (BD), CD45RA, CD40L (Beckman Coulter, Fullerton, CA, USA), CCR7 (R&DSystems), PerCP-labeled CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD45RA, CD45RO, and IFNγ (BD) monoclonal antibodies (mAbs). 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 10 specific events, after correction for background events in negative control samples.

Intracellular IFNγ staining
PBMC were stimulated with 10⁻⁶ M HAdV 5 hexon peptides for 6 hours. During the last 5 hours, 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 1 week with 10⁻⁷ M HAdV 5 hexon peptides and 100 IU IL-2/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 hours with 10 µg/ml BFA. After stimulation, cell-surface staining with mAbs was performed, followed by intracellular staining as previously described [33].

Isolation and culture of IFNγ-secreting cells
25x10⁶ PBMC were stimulated with 10⁻⁶ M HAdV 5 hexon protein-spanning 15-mer peptide pool for 4 hours. Cells were thoroughly washed in PBS 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 3-4 days.

Cytotoxicity assay
Standard ⁵¹Cr 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 Na¹⁰⁵CrO₄ (GE Healthcare, London, UK) for 1 hour at 37°C, and incubated with effector T cell lines. After 10 hours of incubation, supernatant was harvested for ⁵¹Cr 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, \(^{3}\text{H}\)-thymidine (Amersham International, Amersham, UK) was added for a further 16 hours of incubation and \(^{3}\text{H}\)-thymidine uptake was measured subsequently. The residual alloreactivity was calculated by comparing the \(^{3}\text{H}\)-thymidine uptake of the HAdV-specific T cell lines with the uncultured PBMC.

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

Results

HAdV 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 IFN\(_{\gamma}\) production after stimulation showed maximal IFN\(_{\gamma}\) production 4 hours after stimulation with the HAdV 5 hexon protein-spanning 15-mer peptide pool, which was similar for CMV-specific T cells as described recently [33]. Therefore, cumulative intracellular IFN\(_{\gamma}\) staining was measured 6 hours after stimulation with the hexon 15-mer peptide pool. Intracellular IFN\(_{\gamma}\) staining showed hexon-specific CD8\(^{+}\)IFN\(_{\gamma}\)^{+} T cell populations in 5/16 healthy donors (Figure 1A). Stimulation with minimal peptides corresponding to known hexon CD8\(^{+}\) epitopes induced responses in 3/16 donors (Figure 1B). Protein-spanning 15-mer peptide pools derived from cytomegalovirus or Epstein-Barr virus were used as irrelevant 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 IFN\(_{\gamma}\) production. The presence of hexon-specific CD8\(^{+}\) T cells was confirmed in 5 of the 6 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 IFN\(_{\gamma}\) and CD40L staining upon stimulation of PBMC with HAdV hexon peptide pool (Figure 1A). Hexon-specific CD4\(^{+}\)IFN\(_{\gamma}\)^{+}CD40L\(^{+}\) T cell populations could be detected in 13/16 donors (Figure 1C, range 0.03-0.39% of CD4\(^{+}\) T cells). Using 5 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 5 selected 30-mer peptides. Addition of the 5 selected 30-mer peptides to the hexon 15-mer pool did not result in increased frequencies of IFN\(_{\gamma}\)-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.

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 IFN\(_{\gamma}\) and CD40L staining upon stimulation with the HAdV hexon 15-mer peptide pool. B) Frequencies of IFN\(_{\gamma}\)-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 2 sets of peptides combined. C) Frequencies of IFN\(_{\gamma}\)-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 2 sets of peptides combined. Filled symbols represent specific IFN\(_{\gamma}\)-producing populations, and open symbols represent frequencies below the limit of detection.
These data show that low frequencies of HAdV hexon-specific CD8+ 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 where no HAdV hexon-specific responses could be detected directly ex vivo, PBMC were stimulated for 1 week with HAdV hexon peptide pool and IL-2 for specific expansion. Subsequently, restimulation with 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.

HAdV-specific T cell response in alloSCT recipients

We next analyzed whether HAdV hexon-specific T cell responses were associated with clearance of HAdV infection in alloSCT recipients, using the same method as for the healthy individuals. From previously described cohorts of patients, we selected 7 pediatric and 6 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 deceased from HAdV viremia, since only for a few patients peripheral blood samples were available and the lymphocyte numbers in these samples were too low for analysis. Peripheral blood samples obtained during positive HAdV plasma load were available for 10 patients, and peripheral blood samples obtained after resolution of HAdV viremia were available for all 13 patients. During HAdV viremia hexon-specific CD8+ T cell could not be detected directly ex vivo and hexon-specific CD4+ T cells were detected in 2 patients (Figure 2A). After 1 week of culture with the hexon peptide pool and IL-2 for specific expansion, the presence of low frequencies of hexon-specific CD4+ T cells was observed in 5 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 7 patients, and hexon-specific CD4+ T cells in all patients (Figure 2B). After 1 week 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 4 patients, part of hexon-specific CD8+ T cells were targeted against previously described epitopes, as detected by peptide-MHC tetramer staining after expansion (data not shown).

A more detailed analysis of development of HAdV-specific T cell responses is shown for 5 patients, of 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 up to 30% of CD8+ T cells and 16% of 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 alloSCT.
Chapter 4

Figure 3. Detailed analysis of HAdV-specific T cell responses in alloSCT recipients. The dot plots show direct intracellular IFN\(\gamma\) and CD40L staining following HAdV hexon peptide pool stimulation of A) PBMC from pediatric patient 1 (patient 16 reference [8]), and B) PBMC from adult patient 1 (patient 3 reference [4]), obtained during and after resolution of HAdV viremia. The graphs show frequencies of IFN\(\gamma\)-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 patient 2, 3, and 4.

Generation of HAdV 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 alloSCT, 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 IFN\(\gamma\)-secreting cells were isolated and cultured with autologous feeders and IL-2. The data of 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]. Restimulation of the T cell line with autologous EBV-LCL loaded with the hexon peptide pool induced specific IFN\(\gamma\) production by 94% of CD8+ T cells, indicating that additional hexon CD8+ epitopes were recognized. Furthermore, hexon-specific IFN\(\gamma\) production upon restimulation was observed by 91% of CD4+ T cells. Only low frequencies of hexon-specific T cells could be detected in the IFN\(\gamma\)-depleted fraction (Figure 4C). Results from all 6 donors are summarized (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 donor 1, 2, 3, and 5 were incubated with PBMC from 3 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).

Table 1. Generation of T cell lines containing CD8+ and CD4+ T cells with high specificity for HAdV hexon derived from 6 healthy adult donors.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Day 1</th>
<th>Day 14/15</th>
</tr>
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<tbody>
<tr>
<td>PBMC</td>
<td>Cell nr.</td>
<td>Cell nr.</td>
</tr>
<tr>
<td>% IFN(\gamma)/ CD8+</td>
<td>% IFN(\gamma)/ CD4+</td>
<td>Isolated</td>
</tr>
<tr>
<td>1</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>0.12</td>
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<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>0.24</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*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 IFN\(\gamma\)-positive T cells was determined after gating on either CD8+ T cells or CD4+ T cells.*
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 HAdV hexon peptide pool. Specificity of 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 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 hours incubation with the HAdV-specific T cell line derived from donor 4 at 3 effector/target ratios. 

To investigate whether HAdV-specific T cell lines recognized HAdV-infected target cells which endogenously processed the synthesized HAdV antigen, specific lysis was determined 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 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% block).

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 HAdV 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 7 previously described hexon epitopes were detected in the HAdV-specific T cell lines derived from 6 healthy donors (Table 2). Analysis of specific IFNγ production by CD8+ T cells upon restimulation with separate hexon overlapping 30-mer peptides showed recognition of 4 additional hexon peptides by the HAdV-specific T cell lines, of which 2 were recognized by CD8+ T cells from 2 different donors (Table 2). 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 (Figure 5). 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-B85, and YSYKARFTL/HLA-B63) was confirmed by specific recognition of peptide-loaded EBV-LCL that expressed these HLA molecules (Figure 5). 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 6 donors are presented (Table 2). Both previously described and newly identified hexon CD8+ epitopes were largely conserved between different HAdV species (Table 3). 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.

Table 2. Characterization of HAdV hexon CD8+ epitopes recognized by T cell lines as determined by peptide-MHC I tetramer staining or recognition of specific hexon peptides.

<table>
<thead>
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<th>Donor</th>
<th>Peptide</th>
<th>AA HLA restriction</th>
<th>Peptide</th>
<th>AA HLA restriction</th>
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<td>1</td>
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<td>320-329 B35</td>
<td>IPF</td>
<td>873-882 B35</td>
</tr>
<tr>
<td></td>
<td>IPF</td>
<td>715-713 B35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>YVL</td>
<td>917-926 A2</td>
<td>IPF</td>
<td>873-882 B35</td>
</tr>
<tr>
<td></td>
<td>MPN</td>
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<td></td>
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<td></td>
<td>IPF</td>
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<tr>
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<td>YSY</td>
<td>78-86 B63</td>
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<td>37-45 A24</td>
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<td>6</td>
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<td></td>
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<td>YSY</td>
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Figure 5. Confirmation of newly identified HAdV hexon CD8+ T cell epitopes. Intracellular IFNγ staining of HAdV-specific T cell lines after restimulation with minimal MHC class I restricted HAdV hexon peptides. In addition, the HAdV-specific T cell line derived from donor 2 was stained with IPF/HLA-B35 tetramers. To confirm HLA-restriction of the other epitopes, IFNγ release was determined by ELISA upon incubation with autologous or partially HLA-matched allogeneic EBV-LCL without peptide (white bars), or loaded with the relevant minimal HAdV hexon peptide (black bars).
In this study, HAdV hexon-specific T cells were detected with cytotoxic capacity, the role of CD8+ T cells in protection from HAdV disease has been questioned. In previous studies, predominantly HAdV-specific CD4+ T cells were detected in healthy adults and in patients following alloSCT, we demonstrated high and defined specificity of the T cell lines 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 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 alloSCT has previously been shown to be associated with protection from HAdV disease, only HAdV-specific CD4+ T cells, and no HAdV-specific CD8+ T cells have been detected. To further investigate this, we analyzed T cell responses specific for the immunodominant HAdV hexon protein in patients that 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 alloSCT. 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 part of the patients used immunosuppressive drugs during the time points of analysis, and expansion of HAdV hexon-specific T cells was observed in PBMC from all patients. These data indicated that both CD8+ and CD4+ T cells specific for HAdV hexon epitopes contribute to resolution of HAdV infection in alloSCT 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 alloSCT.

Despite the low to undetectable frequencies of HAdV hexon-specific T cells in healthy individuals, using HAdV hexon peptide pool stimulation followed by IFN-γ-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 highly sensitive 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 IFN-γ-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 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.

### Table 3. Conservation of HAdV hexon CD8+ epitopes between different HAdV species.

<table>
<thead>
<tr>
<th>HAdV species</th>
<th>HAdV serotype</th>
<th>Previously described HAdV hexon CD8+ epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAdV 12</td>
<td>C 5</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td>HAdV 35</td>
<td>A 12</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td></td>
<td>B 35</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td>HAdV 40</td>
<td>C 2</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td></td>
<td>D 30</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td>HAdV 4</td>
<td>E 4</td>
<td>VYFSLNNKF</td>
</tr>
<tr>
<td></td>
<td>F 40</td>
<td>VYFSLNNKF</td>
</tr>
</tbody>
</table>

HAdV hexon sequences were compared between serotype 5 (AP_000211), serotype 12 (AP_000121), serotype 35 (AP_000958), serotype 2 (AP_000175), serotype 30 (ABA00012), serotype 4 (AA024097), and serotype 40 (NP_040862).

The sequence derived from serotype 5 is depicted for each HAdV hexon CD8+ epitope, and amino acids that differ are shown for the other serotypes.

### Discussion

Based on our observation that resolution of HAdV viremia coincided with appearance of a coordinated CD8+ and CD4+ HAdV hexon-specific T cell response in patients following alloSCT, we developed a method for generation of CD8+ and CD4+ T cell lines 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 IFN-γ-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 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.

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Despite the low to undetectable frequencies of HAdV hexon-specific T cells in healthy individuals, using HAdV hexon peptide pool stimulation followed by IFN-γ-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, that rapidly reacts upon specific activation. While expression of CD27 and CD28 was reported to be transiently upregulated upon activation and subsequently downregulated on CMV-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.

From all healthy adults, T cells recognizing multiple MHC class I and II restricted epitopes derived from HAdV serotype 5 hexon protein were isolated. 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 alloSCT. 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 alloSCT 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.

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References


Chapter 4

HAdV-specific T cell lines after alloSCT
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