Adenovirus-Specific CD4+ T Cell Clones Recognizing Endogenous Antigen Inhibit Viral Replication In Vitro through Cognate Interaction

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Adenovirus-Specific CD4+ T Cell Clones Recognizing Endogenous Antigen Inhibit Viral Replication In Vitro through Cognate Interaction

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Human adenovirus (HAdV) infection is a frequent and potentially severe complication following allogeneic stem cell transplantation in children. Because treatment with antiviral drugs is often ineffective, adoptive transfer of donor-derived HAdV-specific T cells able to control viral replication of HAdV of multiple serotypes may be an option for therapy. In healthy donors, predominantly HAdV-specific T cells expressing CD4 are detected. In this study, a preclinical in vitro model was used to measure the antiviral effect of HAdV-specific CD4+ T cells. CD4+ HAdV-specific T cell clones restricted by HLA class II molecules were generated and most of these clones recognized conserved peptides derived from the hexon protein. These cross-reactive T cell clones were able to control viral replication of multiple serotypes of HAdV in EBV-transformed B cells (B-LCL), melanoma cells (MJS) and primary bronchial epithelial cells through cognate interaction. The HAdV-specific CD4+ T cell clones were able to specifically lyse infected target cells using a perforin-dependent mechanism. Antigenic peptides were also presented to the CD4+ T cell clones when derived from endogenously produced hexon protein. Together, these results show that cross-reactive HAdV-specific CD4+ T cells can control replication of HAdV in vitro and provide a rationale for the use of HAdV-specific T cells in adoptive immunotherapy protocols for control of life-threatening HAdV-infections in immunocompromised patients. The Journal of Immunology, 2006, 177: 8851–8859.

H

uman adenoviruses (HAdV) rarely cause severe clinical symptoms in healthy children and adults, because infections in immunocompetent individuals are usually self-limiting. However, HAdV may cause life-threatening complications in immunocompromised patients (1, 2). In recent years, the incidence of HAdV infections in pediatric stem cell transplant (SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7). Recipients of a T cell-depleted or CD34(SCT) recipients has increased remarkably (3–7).
Effects of retrovirus were used to transduce MJS cells, and the cells were supplemented (504–743 aa) and hexon D fragment (761–952 aa), respectively, as a retroviral constructs were produced that transcribed HAdV hexon C fragment. These CD4^+ T cells are a promising tool for immunotherapy in immunocompromised patients at risk of developing severe HAdV infection.

Materials and Methods

Cells and viruses

The human EC line HeP2 was used to propagate HAdV. Cells were maintained in RPMI 1640 medium with glutamax (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI 1640 medium/10% FCS). HAdV strains of serotypes 2 and 5 (species C), 12 (species A), and 35 (species B) from the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) were grown on HeP2 cells and harvested when cytopathological effect was present. Virus was released from the cells by two freeze-thaw cycles and purified by CsCl density-gradient centrifugation. Virus stocks were titrated using the plaque assay on 293 cells at the Department of Molecular Cell Biology (Leiden, The Netherlands). Inactivation of HAdV was performed by incubation with virus with 1.3 μM methylene blue (MB) followed by irradiation with visible light for 1 h (31, 34). B-LCL were generated from PBMC of HLA- typed donors by incubation with supernatant of the marmoset B cell line B95-8 in the presence of cyclosporin A. Cell lines were cultured in supplemented RPMI 1640 medium with 10% FCS.

MJS (Meljuso, HLA class II-positive melanoma) cells were cultured in RPMI 1640 medium/10% FCS. To generate MS cells expressing fragments of the hexon protein, the Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells Φ-NX-A were used (35). Two retroviral constructs were produced that transcribed HAdV hexon C fragment (504–743 aa) and hexon D fragment (761–952 aa), respectively, as a bicistronic messenger RNA with enhanced GFP (eGFP). Replication-defective retrovirus was used to transduce MJS cells, and the cells were isolated for high eGFP expression by FACS. The two cell lines were called MJS-Hex-C and MJS-Hex-D, respectively.

PBEC were obtained from resected lung tissue obtained from patients who underwent surgery for lung cancer by enzymatic digestion as described previously (36). Cells were subcultured in a 1/1 mixture of DMEM (Invitrogen Life Technologies) and bronchial epithelial growth medium (Clonetics) supplemented with 0.4% (w/v) bovine pituitary extract, 0.5 mg/ml epidermal growth factor, 5 μg/ml insulin, 0.1 mg/ml retinoic acid, 10 μg/ml forskolin, 1 μM hydrocortisone, 6.5 mg/ml T3, 0.5 μg/ml L-thyronine (all from Clonetics), 1.5 mg/ml BSA (Sigma-Aldrich), 1 mM HEPES (Invitrogen Life Technologies), 20 μM penicillin, and 20 μg/ml streptomycin (BioWhittaker).

HAdV-specific T cell clones

PBMC from healthy blood bank donors (Sanquin) were isolated using Ficoll gradient centrifugation. Monocytes were depleted by adherence to plastic for 2 h, after which the nonadherent peripheral blood lymphocytes were collected and used as responder cells. Peripheral blood lymphocytes (2 × 10^6 per well) were added in a 24-well plate (Corning) and stimulated with 1 × 10^6 irradiated (30 Gy) autologous PBMC preincubated with MB- inactivated HAdV5 at a multiplicity of infection (MOI) of 10. Cells were cultured for 12 days in RPMI 1640 medium/10% human AB medium, harvested, seeded at 2.5 × 10^6 cells per well, and restimulated with 1 × 10^6 HAdV5-infected stimulator PBMC as before. IL-2 (50 IU/ml; Chiron) was added at day 15 and replenished two or three times a week thereafter.

At day 28, a limiting dilution assay was performed in which cells were plated at a density of 10, 1, or 0.3 cells per well in 96-well round-bottom plates (Corning) and stimulated with 1 μg/ml PHA (Murex Biotech), 150 IU/ml IL-2, and 1 × 10^6/ml irradiated allogeneic PBMC. After 2 weeks, wells with expanding cells were restimulated as before with PHA, IL-2, and allogeneic PBMC. Specificity of clones was determined by proliferation as described below, and the phenotype of the clones was assessed by flow cytometry (see below).

HAdV-specific proliferation

T cell clones (2 × 10^6 cells per well) were plated in 96-well round-bottom plates in triplicate. As stimulator cells, autologous EBV-transformed B cells (B-LCL) were resuspended in RPMI 1640 medium with 0.5% BSA at 5 × 10^5 B-LCL/ml, irradiated at 40 Gy, and infected for 1 h at 37°C with HAdV5 at an MOI of 100, or uninfected as control. Hereafter, a total of 5 × 10^5 stimulator cells was added per well in RPMI 1640/10% AB medium. After 3 days at 37°C and 5% CO_2, cultures were pulsed with 1 μCi 3H-thymidine (Amersham Biosciences) per well for 18 h. Plates were harvested (Skatron), and filters were subsequently counted in a Betaplate counter (Wallac).

To determine which protein was recognized by a T cell clone, B-LCL were loaded with recombinant hexon protein (protein II, generated in four parts as IIA (1–273 aa), IIB (245–509 aa), IIC (479–743 aa), and IID (749–952 aa) (37), the MHC class II peptide pool for HLA-A2, and E1A, at a final concentration of 5 μg/ml. For peptide recognition, the hexon protein was synthesized in overlapping peptides of 30 aa with a 15-aa overlap (37). Proliferation was determined against B-LCL loaded with pools of five to six peptides (5 μg/ml), and specific peptides were identified from positive peptide pools by loading single peptides on B-LCL.

HAdV replication inhibition assay

HAdV-specific T cell clones were tested for cytokine production by intracellular staining and flow cytometry. T cell clones (2 × 10^5) were pulsed with 1 × 10^5 infected autologous B-LCL either uninfected, infected with HAdV5 at an MOI of 100, or loaded with the specific peptide in round-bottom polystyrene 5-mL tubes in a total volume of 400 μL. After 1 h, brefeldin A (Sigma-Aldrich) was added for 18 h to block exocytosis. Cells were stained on ice and analyzed using flow cytometry. Cells were washed with PBS containing 0.2% w/v NaCl, fixed with freshly made 4% paraformaldehyde (Sigma-Aldrich), washed in PBS containing 1% BSA, and permeabilized with PBS/NaCl. Cells were permeabilized by washing with PBS/NaCl containing 0.1% saponin and 0.5% BSA, and nonspecific binding was blocked with PBS/NaCl/saponin/BSA/10% FCS for 10 min. After washing with PBS/NaCl/saponin/BSA, Abs were added: anti-CD3 PerCP-Cy5.5, anti-CD4 FITC, anti-IFN-γ PE/anti-TNF-a-PE/anti-IL-10-PE/anti-IL-4-PE (BD Biosciences), and anti-CD8-PE (BD Biosciences), and anti-CD4-FITC, anti-CD25-PE (BD Pharmingen), and anti-CD107a-PE (BD Pharmingen). Plates were washed three times with 10 mL of RPMI 1640 medium/10% FCS in tubes, and MJS/PBEC three times with 1 mL per well to remove free virus. For B-LCL, cells were plated at 1 × 10^5 in 500 μL of RPMI 1640 medium/10% FCS per well of a 24-well plate (Corning). HAdV-specific T cell clones (at an E:T ratio of 10:1 unless indicated otherwise) were added to the three types of target cells in 500 μL of RPMI 1640 medium/10% FCS, or medium alone as a control for viral replication. In preliminary experiments, cells were harvested daily from days 0 through 6 to determine the kinetics of viral replication in the B-LCL. In later experiments, cells were harvested at day 3.
after infection, which was found to be optimal. Cells were washed three times with RPMI 1640 medium/10% FCS to remove free virus, and virus was released from the cells by two freeze-thaw cycles. The virus titers of cell lysates were determined with the tissue culture-infective dose of 50% (TCID50) assay. For this, lysates were diluted 10-2 to 10-5-fold, and each dilution was plated in 10-fold in 96-well flat-bottom plates. HEP2 cells were added to the wells at 5 × 104 cells per well. After 7 days of incubation at 37°C and 5% CO2, wells were scored for the presence of cytopathological effect, which indicates the presence of infectious virus in those wells. The viral titer of each lysate was calculated via the method of Reed and Munch, which determines the dilution at which 50% of wells are infected as described elsewhere (31). For detection of HAdV12 and HAdV35 viral replication, A549 cells were used to determine the viral titer, as these viruses do not grow optimally on HEP2 cells.

In some experiments, a coculture of autologous B-LCL and HLA-DR-mismatched B-LCL was performed. To stimulate the HAdV-specific T cell clone, autologous B-LCL were loaded with the specific peptide, while the mismatched B-LCL were infected with HAdV5. Viral titers were determined in cell lysates to investigate the bystander effect of soluble factors or non-HLA-restricted recognition on inhibition of viral replication.

Chromium release assay (CRA)

To determine HAdV-specific cytotoxicity, CRA were performed on B-LCL or MJS cells. Target cells were infected with HAdV5 (infectious or MB-inactivated) at an MOI of 100 as described above or loaded with peptide at 5 μg/ml and cultured for 16 h unless otherwise indicated. Target cells were labeled with 51Cr for 1.5 h at 37°C and washed three times with RPMI 1640 medium. Various numbers of effector cells were cocultured with 2.5 × 104 B-LCL or 2.5 × 105 MJS per well for 4 h at 37°C and 5% CO2, and 51Cr release was determined in the supernatant. Maximal release was determined by the addition of 2 N HCl to target cells, and spontaneous release was determined by the addition of medium alone. Counts were measured in an automated counter (Wallac). The percentage of specific release was calculated as: (cpm experimental release − cpm spontaneous) / (cpm maximal − cpm spontaneous) × 100.

In some experiments, inhibitors of the perforin-mediated lysis were added. Concanaamycin A (CMA; 50 nM; a gift from Dr. J. P. Medema, Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands) or EGTA (at 2 mM; Boehringer Mannheim) was added to the effector cells 1 h before coculture with target cells, and CMA or EGTA were added during cocultivation.

To determine whether antigenic peptides were transferred between cells during culture, MJS cells were mixed with MJS-Hex-D cells in a 1:1 ratio and cultured for 4–5 days. Then, the two cell populations were separated by FACs sorting based on eGFP expression, incubated with peptide or medium during labeling with 51Cr, and used as target cells.

Results

Characterization of HAdV-specific CD4+ T cell clones

HAdV-specific CD4+ T cell clones, generated against HAdV5 (species C), have been obtained from several healthy donors as described recently (31). These clones exhibited a restricted or a broad (most clones) cross-reactivity pattern, recognizing serotypes from species C only or from A, B, and C, respectively. Because such broadly cross-reactive T cell clones are ideal candidates to boost HAdV-specific immunity in immunocompromised patients, the nature of the epitopes recognized by these clones was characterized. First, it was investigated which protein of HAdV was recognized using proliferation assays with recombinant hexon protein (generated in four parts, IIA–IIID), recombinant penton base, and early gene product E1A (37). Three of eight HAdV-specific T cell clones were unresponsive to any of these proteins, but the remaining five recognized the C-terminal part of the hexon protein (II-C or IIID) (Table I and example in Fig. 1A), which is highly conserved among different species (38, 39) and might explain the broad cross-reactivity that we observed previously (31). To further investigate which peptides of the hexon protein were recognized, 63 overlapping peptides of 30 aa with a 15-aa overlap of the hexon protein (peptides IIH–II66) were synthesized and tested in proliferation assays. An example is shown in Fig. 1B, and results are summarized in Table I. Two clones recognized II64, which contains a conserved epitope II910–924 that has recently been described (29, 40). Proliferation was also observed against this minimal epitope, indicating that these two HAdV-specific clones specifically recognized this II910–924 epitope contained within peptide II64. The other clones recognized three different hexon peptides. HLA restriction of these clones was determined by stimulation with HLA-DR- or HLA-DP-matched B-LCLs or the addition of blocking Abs. The T cell clones of which the protein specificity could not be determined were all HLA-DR7 restricted, while the other five clones that recognized various peptides in the hexon were HLA-DR17 or HLA-DP4 restricted (Table I).

![FIGURE 1. Most HAdV-specific CD4+ T cell clones recognize peptides in hexon protein (protein II). A. T cells from clone A5 1.32 were tested in a 3-day proliferation assay against autologous B-LCL infected with HAdV5 or loaded with recombinant proteins from hexon (IIA–IIID), the penton base protein (II), or the early E1A protein. Proliferation, as measured by [3H]thymidine incorporation, was only observed against HAdV and the recombinant IID protein. B. Clone A5 1.32 was further tested for proliferation against autologous B-LCL loaded with 30-aa peptides from the IID protein, and was found to proliferate exclusively against peptide II64. This peptide contains a minimal epitope 910–924 as described recently (29), which was found to be the epitope for this clone. Results are representative for five clones.](http://www.jimmunol.org/lookup/image/8853f1)

<table>
<thead>
<tr>
<th>T Cell Clone</th>
<th>Donor</th>
<th>Hexon Protein</th>
<th>Hexon Peptide</th>
<th>HLA Restriction</th>
</tr>
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<tr>
<td>A1.1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>DR7</td>
</tr>
<tr>
<td>D1.23</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>DR7</td>
</tr>
<tr>
<td>C30.4</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>DR7</td>
</tr>
<tr>
<td>A4 0.3–4</td>
<td>3</td>
<td>IIC</td>
<td>II44 (43)</td>
<td>DR17</td>
</tr>
<tr>
<td>A5 1.32</td>
<td>3</td>
<td>IIID</td>
<td>II64, 910–924</td>
<td>DP4</td>
</tr>
<tr>
<td>M2.11</td>
<td>4</td>
<td>IIID</td>
<td>II57</td>
<td>DR17</td>
</tr>
<tr>
<td>K1.2</td>
<td>5</td>
<td>IIID</td>
<td>II64, 910–924</td>
<td>DP4</td>
</tr>
<tr>
<td>K3.1</td>
<td>5</td>
<td>IIID</td>
<td>II61</td>
<td>DR17</td>
</tr>
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</table>

- Indicates none of the hexon, penton base, or E1A proteins were recognized.
- Indicates that peptide II44 induced the highest proliferation; proliferation to II43 was observed to a lesser extent, suggesting that the minimal epitope resides in the N-terminal part of peptide II44.
- Peptide II64 was recognized as well as the minimal epitope that is present in II64, the II910–924 epitope described in Ref. 29.
When the clones were stimulated with HAdV5-infected B-LCL, ~40% of the T cells produced IFN-γ and TNF-α as determined by intracellular cytokine staining (Fig. 2), despite the fact that HAdV infection of B-LCL is inefficient and results only in ~5–10% infected cells as visualized by immunohistochemistry (Ref. 41 and data not shown). IL-2 was produced in few cells (Fig. 2), whereas IL-4, IL-5, and IL-10 were not detected (Fig. 2 and data not shown). When T cell clones were stimulated with peptide-loaded B-LCL, >90% of T cells coexpressed IFN-γ and TNF-α (data not shown).

Inhibition of HAdV replication in vitro

If HAdV-specific CD4+ T cells are to be administered to patients as adoptive immunotherapy, these T cells should have antiviral functions to combat the infection in vivo. To investigate whether HAdV-specific CD4+ T cell clones can exert antiviral activity, an in vitro inhibition assay was developed to determine the effect of these T cell clones on HAdV replication. HAdV replication in B-LCL was determined by measuring the virus titers in cell lysates daily from days 0 through 3 after infection. HAdV virus titers increased 100- to 1000-fold in 3 days (Fig. 3A). When an HAdV-specific T cell clone, A1.1, was added to autologous infected B-LCL at an E:T ratio of 10:1, this increase in viral titers was almost completely inhibited (Fig. 3A). The inhibitory effect of the CD4+ T cell clones was dependent on the number of T cells present. At an E:T ratio of 1:1, viral replication was still inhibited significantly, whereas inhibition could no longer be observed at an E:T ratio of 0.1:1 (Fig. 3B). Strong reduction in viral titers was observed not only when autologous B-LCL were used, but also with HLA-DR- or HLA-DP-matched B-LCL as targets (Fig. 3C). However, viral titers were not reduced when mismatched B-LCL were applied as target, indicating that inhibition of viral replication is HLA restricted (Fig. 3C).

B cells are not likely to be the physiological target cells for infection with HAdV. To test the ability of the HAdV-specific T cells to inhibit virus production in a more appropriate cell type, PBEC with the relevant HLA-DR genotype were used. To induce HLA-DR on the surface of the EC, PBEC were treated with IFN-γ for 2 days before infection. Virus production in infected EC was inhibited by the T cells at a ratio of 50:1 and less well at a ratio of 10:1 (Fig. 3D). The higher E:T ratio needed to inhibit virus production in the primary EC, compared with when B-LCL were used as targets, may partly be due to the fact that the fraction of infected cells in the case of B-LCL (5–10%) is lower than that of the EC (>50%) (data not shown). The results indicated that the HAdV-specific T cells also inhibited virus production in physiological target cells infected with HAdV.

Cognate interaction required for inhibition of viral replication

To elucidate potential mechanisms by which these HAdV-specific CD4+ T cell clones exert their antiviral effect, we investigated whether cognate interaction between T cells and target cells was required or whether the inhibition of viral replication could be achieved by soluble factors produced by the T cells. Therefore, a coculture experiment was performed with autologous B-LCL loaded with the specific peptide together with HLA-mismatched B-LCL infected with HAdV5. In this setting, T cell clones were stimulated by the peptide-loaded autologous B-LCL, while the viral replication was assessed in the HLA-mismatched B-LCL. Any factor secreted by the peptide-stimulated T cells may then directly influence the infected cells. PBEC were cocultured for 1 day with IFN-γ, then trypsinized and replated in the presence of IFN-γ at 1 × 10^6 cells per well in 24-well plates. The next day, IFN-γ was removed, and the cells were infected with HAdV for 1 h. Virus was removed and T cells were added at E:T ratios of 50:1 and 10:1. Three days later, cell lysates were harvested and viral titers determined as described above (left panel, clone A.1.1; right panel, clone K3.1).

When the clones were stimulated with HAdV5-infected B-LCL, or uninfected (medium) as control, T cells from clone M2.11 were added for 16 h, and cytokine production was determined with intracellular cytokine staining using anti-IFN-γ-PE, anti-TNF-α-PE, anti-IL-2-PE, or anti-IL-4-PE in combination with anti-CD4-FITC. The percentage of CD4+ T cells producing a specific cytokine is shown in the upper right quadrant. Results are also representative for the other clones tested (A1.1 and K3.1).

In FIGURE 2, HAdV-specific CD4+ T cell clones secrete IFN-γ and TNF-α. Autologous B-LCL were infected with HAdV5 at an MOI of 100 or uninfected (medium) as control. T cells from clone M2.11 were added for 16 h, and cytokine production was determined with intracellular cytokine staining using anti-IFN-γ-PE, anti-TNF-α-PE, anti-IL-2-PE, or anti-IL-4-PE in combination with anti-CD4-FITC. The percentage of CD4+ T cells producing a specific cytokine is shown in the upper right quadrant.

In FIGURE 3, inhibition of viral replication in B-LCL and PBEC by HAdV-specific CD4+ T cell clones. The antiviral activity of T cell clones was tested in a 3-day in vitro assay in which viral replication was assessed. A, Autologous B-LCL were infected with HAdV5 at an MOI of 100 and cultured for 3 days in the presence (●) or absence (○) of T cell clone A1.1 at an E:T ratio of 10:1. Cell lysates were obtained on days 0, 1, 2, and 3, and the titer of infectious virus was determined with the TCID50 assay on HEP2 cells. B, Autologous B-LCL were infected with HAdV5 at an MOI of 100 and cultured for 3 days in the presence of the T cell clone K3.1 at different E:T ratios of 10:1, 1:1, and 0.1:1. At day 3, cell lysates were harvested and viral titers were determined. C, Autologous B-LCL, HLA-DR- or HLA-DP-matched B-LCL, or HLA-mismatched B-LCL were infected with HAdV5 at an MOI of 100 and used in the in vitro assay at an E:T ratio of 10:1. At day 3, cell lysates were harvested and viral titers were determined. Two representative examples (left panel, clone A1.1; right panel, clone K3.1) are shown of four clones (A1.1, M2.11, K3.1, A51.32) tested. D, PBEC cells were incubated for 1 day with IFN-γ, then trypsinized and replated in the presence of IFN-γ at 1 × 10^6 cells per well in 24-well plates. The next day, IFN-γ was removed, and the cells were infected with HAdV for 1 h. Virus was removed and T cells were added at E:T ratios of 50:1 and 10:1. Three days later, cell lysates were harvested and viral titers determined as described above (left panel, clone A.1.1; right panel, clone K3.1).
reduced only marginally (Fig. 4). This lack of inhibition was observed despite the fact that the T cells were strongly stimulated by the peptide-loaded autologous B-LCL as measured by IFN-γ production in the supernatant (~50 ng/ml IFN-γ). Together, these results indicate that the antiviral effect of HAdV-specific CD4+ T cell clones does not seem to be mediated by soluble factors (such as IFN-γ) or non-HLA-restricted cell-cell contact. Instead, cognate interaction between the T cell and the target cell appears to be required to achieve an antiviral effect.

Lysis of HAdV-infected target cells

As HLA-restricted recognition of target cells as well as cell-cell contact appeared to be essential for the antiviral function of the T cell clones, we investigated whether these HAdV-specific CD4+ T cell clones had cytolytic activity against infected target cells. Maximal lysis of HAdV5-infected B-LCL was usually low (<30%) (Fig. 5A), but this observation is probably due to the fact that HAdV infection of B-LCL is rather inefficient as mentioned above. However, when B-LCL were loaded with the specific 30-aa peptide, lysis increased to ~80% or more (Fig. 5B). A melanoma cell line, MJS, that is more easily infected (>90% infected cells; data not shown) and expresses HLA-DR17, was used to further investigate lysis of HAdV-infected cells. Using these targets, lysis of HAdV5-infected MJS by the CD4+ HAdV-specific T cell clones was as efficient as lysis obtained with the peptide-loaded B-LCL (Fig. 5C).

Mechanisms of contact-dependent T cell-induced death of target cells include release of preformed vacuolar perforin and granzymes into the intercellular space and up-regulation of cell surface molecules such as FAS ligand and TRAIL (42–44). The presence of both perforin and granzyme B in the cytoplasm of HAdV-specific T cell clones was confirmed by intracellular staining, supporting their role in HAdV-specific lysis (Fig. 6). The perforin effector pathway can be inhibited by the action of CMA, which raises the pH in intracellular vacuoles and inactivates perforin, as well as by EGTA which binds intracellular calcium that is required for release of perforin (45). Lysis was almost completely abrogated by either of these inhibitors, indicating that these CD4+ T cells lyse predominantly using the perforin pathway (Fig. 5D and data not shown).

Loading of hexon peptide on MHC class II molecules

To address the question whether recognition of infected cells requires protein synthesis, we compared T cell recognition of cells exposed to infectious virus or MB-inactivated virus. MJS cells pulsed with inactivated HAdV were recognized as well as cells infected with infectious virus (Fig. 7A). MB inactivation prohibits gene transcription (34), indicating that viral protein synthesis is not required, and that processing of input virions can be sufficient for presentation of T cell epitopes on HLA class II.

Subsequently, it was investigated whether the antigenic peptides recognized by HAdV-specific cytotoxic CD4+ T cell clones could also be processed from proteins synthesized in the cytosol. Two MJS-derived cell lines were generated, each containing a different fragment of ~700-bp coding sequence of the hexon gene, MJS-Hex-C and MJS-Hex-D. The antigenic peptides recognized by the CD4+ T cell clones M2.11 and K3.1 are encoded by fragment D and not by fragment C. The T cell clones lysed the MJS-Hex-D, whereas the MJS-Hex-C cells were not lysed, indicating that the

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**FIGURE 4.** Inhibition of viral replication is not mediated via soluble factors. In a coculture experiment, autologous B-LCL were loaded with the specific peptide (+P) to stimulate the HAdV-specific T cell clone K3.1. In the same well, HLA-DR-mismatched B-LCL were added, which were infected with HAdV5 at an MOI of 100. In control wells, HAdV-infected autologous B-LCL were added that could directly be recognized by the T cell clone. T cells were added at an E:T ratio of 10:1. Viral titers were determined in cell lysates at day 3. Similar results were obtained with clone M2.11.

**FIGURE 5.** HAdV-specific CD4+ T cell clones lyse HAdV-infected or peptide-loaded targets using the perforin-pathway. A, HLA-DR-matched (DR7/DR17-positive) B-LCL were infected with HAdV5 at an MOI of 100 (●), loaded with peptides I61 (▼) and I64 (▲) or uninfected (◆) as controls (clone A1.1 does not recognize any peptide in the hexon protein) and used as targets in a 4-h CRA. T cells from clone A1.1 were added at different E:T ratios, and the percentage of specific lysis is shown. B, The same targets as in A were used in a CRA with T cells from clone K3.1 at different E:T ratios. C, MJS cells (HLA-DR17) were infected with HAdV5 at an MOI of 10 (●), loaded with specific peptide II57 (●), or uninfected as control (◆) and used as targets in a CRA with T cells from clone M2.11 as effectors. D, T cells from clone M2.11 were preincubated with 50 nM CMA for 1 h before the addition of T cells to MJS target cells in medium containing CMA. Lysis results in A are representative of eight T cell clones tested against HAdV5, and in B of five clones that were tested against the specific peptide. CMA results (D) are representative for three T cell clones tested.
epitope was processed from cytosolic protein sources (Fig. 7B and data not shown). As a control, MJS-Hex-C cells pulsed with the relevant peptides were lysed, indicating that this cell line still expressed all molecules necessary for presentation of Ag.

A possible explanation for the observed recognition of MJS-Hex-D cells could be that endogenously produced protein is released from the cells during culture and is taken up by neighboring cells and presented as if it is exogenously derived. To test this hypothesis, untransduced MJS cells were mixed in a 1:1 ratio with the MJS-Hex-D cells (which are eGFP\textsuperscript{+}). After 5 days of coculture, the two cell populations were separated by FACS on the basis of eGFP expression and used as target cells in a cytotoxicity assay. Clones M2.11 and K3.1 did not recognize the eGFP\textsuperscript{-} MJS cells, indicating that processing of hexon protein fragment released in any form from neighboring MJS-Hex-D cells had not occurred at a sufficiently high level to lead to recognition (Fig. 7C and data not shown). The same (untransduced and sorted MJS) cells pulsed with the relevant peptide were lysed, indicating that the experimental procedures had not rendered the cells resistant to cytotoxicity. These results indicate that recognition of the transduced MJS-Hex-D cells by the T cells is not due to endocytosis of secreted protein or cellular fragments from neighboring cells, sug-

gesting that endogenously produced hexon protein is also presented directly on HLA class II molecules in infected MJS cells.

Inhibition of viral replication of different serotypes by HAdV-specific T cell clones

After SCT, patients can be infected with HAdV belonging to various serotypes. If adoptive immunotherapy is to be effective, T cells should be cross-reactive to multiple serotypes. The peptide recognized by the broadly cross-reactive HAdV-specific T cell clone, K3.1, is reasonably well conserved between several serotypes of HAdV (Fig. 8). To analyze whether the T cell clones are also able to inhibit viral replication of different serotypes, viral replication inhibition assays with multiple serotypes from different HAdV species were performed. As not all serotypes tested were able to replicate in B-LCL, the MJS melanoma cell line was used in these experiments. Clone K3.1 was able to inhibit viral replication of HAdV5 as well as HAdV2 (belonging to the same species C), HAdV12 (species A) and HAdV35 (species B) (all >1000-fold reduction of titer), confirming that T cell clones showing cross-reactivity in proliferation assays can exert cross-reactive antiviral activity as well (Ref. 31 and Fig. 8). These results are promising for adoptive immunotherapy, as T cells generated against HAdV5 could also be effective in patients infected with different serotypes of HAdV.

Discussion

To develop clinical protocols for adoptive immunotherapy with T cells for children suffering from disseminated HAdV infections, we and others have studied the human immune response to HAdV (19, 28, 31, 46, 47). In healthy donors the majority of T cells reactive against HAdV is CD4\textsuperscript{+}, although CD8\textsuperscript{+} T cells are also observed. In general, CD4\textsuperscript{+} T cells can mediate antiviral effects indirectly by providing help to B cells in the production of neutralizing Abs or by initiating and maintaining an HLA class I-restricted CD8\textsuperscript{+} T cell response through the activation of dendritic cells (48). More directly, CD4\textsuperscript{+} T cells can restrict viral growth by producing cytokines, e.g., IFN-γ, or by lysing infected target cells (49, 50). For HAdV, the relative contribution of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and of Ab-producing B cells to in vivo protection against
infection are largely unknown. Murine models using HAdV provide only limited information because HAdV does not produce a permissive infection in murine cells. Studies in mice using murine adenovirus reveal that B cells are involved in protection from early disease, whereas T cells, either CD4+ or CD8+, are responsible for late viral clearance, even in the absence of perforin (51, 52). However, there are significant differences with adenovirus infections in humans; the main target cells in mice are endothelial cells vs EC in humans, T cells can cause immunopathology, and the course of infection is dependent on the genetic background of the mice (52). Therefore, conclusions about protective immune responses in murine models of adenovirus infection are of limited relevance to HAdV infections in humans.

Because it has not been investigated whether and by which mechanism CD4+ HAdV-specific T cells are capable of mediating antiviral activity, several CD4+ HAdV-specific T cell clones were generated. The clones recognize peptides derived from structural viral proteins and appeared to have a Th1-like phenotype based on the secreted cytokines. Whether directly ex vivo isolated memory T cells have the same cytokine secretion pattern remains to be investigated. These clones were then tested in a newly developed in vitro assay in which the effect of T cells on viral replication was measured. In this assay, viral replication was determined in infected B-LCL, which have the advantage that they can be generated from PBMC of a large panel of donors expressing almost all HLA alleles. Although B cells may not be the main physiological target of HAdV, they do facilitate a productive infection in which a fraction (5–10%) of the cells is infected (53). The addition of a T cell clone at a ratio of 10:1 or 1:1 reduced viral replication by a factor of 1000 in 3 days. Although the addition of the T cell clone did not result in a lower number of B-LCL, the number of infected cells, as shown by intense staining with an anti-hexon Ab, was strongly decreased (data not shown). Virus production in melanoma cells and also in PBEC, which constitute a more physiological host cell type for HAdV, was also inhibited by the T cell clones.

Because HAdV infects primarily EC, which under normal conditions do not express high levels of HLA class II molecules, the question arises how HAdV-specific CD4+ T cells would be able to recognize their infected targets. It could be that the levels of HLA class II expression are high enough for activated T cells to be able to recognize the infected EC. In experiments with peptide-pulsed PBEC that were not pretreated with IFN-γ, low levels of IFN-γ were produced by T cells indicating that at least some recognition occurred (data not shown). Subsequently, the IFN-γ produced by the activated T cells could then induce class II up-regulation in the infected cells and the surrounding uninfected cells (54). Alternatively, class II expression on these cells might be up-regulated by the HAdV infection itself via production of IFN-α or IFN-β and other stress proteins or by interaction with IFN-γ-producing NK cells. Preliminary data from our group obtained in postmortem biopsies from patients who succumbed to HAdV infection showed that, in some cases, HAdV-infected cells were strongly positive for HLA class II (B. Heemskerk, unpublished data).

For other viruses such as CMV and EBV, numerous reports on virus-specific CD8+ T cells have been published (reviewed in Ref. 55). Nevertheless, in recent years, a growing number of reports on virus-specific CD4+ T cells with lytic activity have been published for EBV, CMV, and HSV (49, 56, 57), which in some reports have been shown to inhibit viral outgrowth in vitro (49, 58, 59). Most reports indicate that perforin-mediated lysis is the main mechanism of human cytotoxic CD4+ T cells, either directly ex vivo (60) or in cell lines or clones (49, 56, 57, 61), whereas some CD4+ T cell clones have been shown to use the FAS/FAS-L pathway (58). In this respect, it is noteworthy that HAdV has developed immune evasion mechanisms. The early protein E3-gp19K has been reported to down-regulate MHC class I expression by retention of MHC molecules in the endoplasmic reticulum, which might result in reduced recognition of the infected cell by CD8+ T cells and, conversely, in increased NK cell recognition (reviewed in Ref. 62). Another early E3 protein complex, the RIDoβ (receptor internalization and degradation) complex, has been reported to internalize FAS, TNF-R1, and TRAIL-R and induce their degradation, thereby circumventing FAS- or TRAIL-mediated lysis of infected cells (63). In view of these immune evasion strategies of HAdV, it is not surprising that HAdV-specific, HLA class II-restricted CD4+ T cells are present that have antiviral activity against HAdV by mediating lysis of infected cells using the perforin pathway. Nevertheless, HAdV-specific CD8+ T cells have been described (32, 33), indicating that these viral evasion mechanisms may not be absolute.

The origin of the peptides presented in the HLA class II molecule and recognized by the CD4+ T cells is a point of interest. When cells are infected with HAdV, hexon protein is available from the input virions, but hexon will also be produced intracellularly for the production of new virions. Classically, it is assumed that antigenic determinants presented on HLA class II originate from endocytosed Ags and are processed via the endosomal-lysosomal pathway. However, a growing number of recent studies describe exceptions to this assumption (64–69). The hexon epitopes described in this study were shown to be presented from exogenous sources, as illustrated by the recognition of target cells pulsed with inactivated virus or hexon protein fragments. In addition, cells retrovirally transduced with the relevant fragment of the hexon gene were recognized, indicating that processing and presentation of endogenously produced hexon protein can occur as well, although expression of full-length hexon protein could have given different results, for example, because of its localization to different cellular compartments. In cultures of the transduced cells, exogenous loading of the HLA class II molecules by peptides taken up from neighboring cells was not very likely as demonstrated in the 5-day coculture experiments, although it cannot be excluded that longer periods of coculture would have been necessary to observe such events. It was reported recently that, in cultures of B-LCL, transfer of BHRF1 Ag derived from the few cells entering lytic cycle to latently infected cells could take as long as 21 days (70). Thus, despite these limitations, our experiments suggest that the same functional HAdV epitope can be presented via both an exogenous as well as an endogenous pathway. During an in vivo infection, cells are most likely infected by only few virus particles. If CD4+, HLA class II-restricted T cells are to be effective in the response to HAdV-infected cells, antigenic peptides presented directly after intracellular synthesis of hexon protein on HLA class II molecules should also be recognized.

It is tempting to speculate that, if the reactivity of HLA class I-restricted CD8+ T cells is hampered due to immune evasion strategies of a virus, HLA class II-restricted CD4+ T cells can and will fulfill this function by expressing perforin-mediated cytotoxicity (71). The fact that the epitopes recognized by the cytotoxic CD4+ T cells are presented on class II MHC molecules when derived from endogenous sources would be in agreement with this role-switching and raises an interesting question. Could there be a correlation between processing of epitopes from endogenous sources and cytotoxicity of CD4+ T cells? The cross-reactive inhibition of viral replication of serotypes from different species of HAdV by the CD4+ T cell clones, which were generated against HAdV5, is encouraging for the clinical
application of these cells in immunocompromised patients suffering from disseminated HAdV infection of various serotypes. However, the observed cytolytic activity and cross-reactivity of HAdV-specific CD4+ T cell clones may have implications for gene therapy trials with HAdV-based vectors as well. The longevity of gene expression might be reduced when HAdV-specific, cytotoxic CD4+ T cells are present, and the cross-reactivity of HAdV-specific T cells may also reduce the applicability of switching to vectors based on different serotypes of HAdV. In conclusion, HAdV-specific CD4+ T cell clones have antiviral activity, as they were able to inhibit viral replication in vitro, most likely dependent on perforin-mediated lysis. It is to be expected that, in the near future, generation of HAdV-specific T cell lines with antiviral activity may become a feasible option for immunotherapy in immunocompromised patients suffering from disseminated HAdV infections.

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