Adenovirus DNA polymerase is recognized by human CD8+ T cells

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Donor lymphocytes have potential as a treatment for adenovirus (Ad) disease in haematopoietic stem cell transplant (SCT) recipients, but better understanding of Ad-specific T-cell responses is required. Most healthy adults exhibit memory T-cell responses to hexon, a capsid protein synthesized late after infection. However, since the Ad E3-19k downregulates major histocompatibility complex (MHC) class I molecules, cytotoxic T cells (CTLs) targeted to early viral proteins may be more effective in eliminating Ad-infected cells in vivo. Here we show that Ad-specific CTLs recognize the early region 2 proteins DNA polymerase (Pol) and DNA-binding protein (DBP). Firstly, memory Ad-specific CD8+ T cells were amplified from healthy donors by in vitro stimulation with Ad-infected dendritic cells and found to exhibit MHC-restricted cytotoxicity to targets expressing Pol and DBP. Secondly, gamma interferon responses to HLA A2-binding motif peptides from Pol and DBP were directly detected in peripheral blood mononuclear cells (PBMCs) from a recently infected normal donor. Peptide-specific CTLs generated to Pol and DBP epitopes were confirmed to exhibit HLA A2-restricted killing of targets expressing Pol or DBP. Lastly, Pol-epitope-specific T cells were detected at similar or higher frequencies than hexon and DBP in three of three SCT recipients recovering from invasive Ad disease. Pol epitopes were well conserved among different Ad serotypes. Therefore, Pol is a promising target for immunotherapy of Ad disease.

INTRODUCTION

Adenoviruses (Ads) can cause life-threatening infections in haematopoietic stem cell transplant (SCT) recipients, including hepatitis, pneumonitis, colitis, nephritis and encephalitis (Flomenberg et al., 1994; La Rosa et al., 2001). The common group C serotypes (Ad1, A2 and Ad5) and the uncommon group B serotypes (Ad11 and 35) have most frequently been associated with disease post-SCT (Ison, 2006). Therapy options are limited. Although recent data suggest that prompt treatment with cidofovir may limit Ad replication (Ljungman et al., 2003; Muller et al., 2005; Neofytos et al., 2007; Yusuf et al., 2006), patients need to develop a cell-mediated immune response in order to recover (Chakrabarti et al., 2002; Heemskerk et al., 2005). Therefore, immunotherapy with donor lymphocyte infusions may improve survival in allogeneic SCT recipients with Ad disease who have delayed immune reconstitution or graft versus host disease (GVHD). As a precedent, infusions of donor lymphocytes have been beneficial in the treatment of Epstein–Barr virus (EBV)-associated lymphoproliferative disease and cytomegalovirus infections in SCT recipients (Einsele et al., 2002; Papadopoulos et al., 1994; Rooney et al., 1998; Walter et al., 1995).

Most healthy adults exhibit memory T-cell responses to Ads, but little is known about their specificities. Prior studies of Ad-specific T-cell targets have focused on the viral capsid proteins (Flomenberg et al., 1995, 1996; Smith et al., 1998). The capsid protein hexon is the dominant T-cell target and is recognized by nearly all individuals (Olive et al., 2002; Tang et al., 2006). Hexon represents the most abundant capsid protein and is synthesized during late infection following viral DNA replication. Several CD8+ and CD4+ T-cell epitopes from hexon have been identified that are highly conserved among different Ad serotypes, such as the HLA A2-restricted CD8+ T-cell epitope H892-901 (Leen et al., 2004; Tang et al., 2006). The CD4+ T-cell epitope H910-924, restricted by the common HLA DP4 allele, is recognized by 70 % of healthy adults (Tang et al., 2004).

We propose that T cells targeted to non-structural Ad proteins synthesized early in infection, prior to onset of
viral replication, may be more efficient than hexon-specific T cells in limiting Ad replication in vivo. Elimination of infected cells prior to viral replication will reduce virus production. Moreover, targeting cells soon after infection may help avoid the effects of Ad proteins that inhibit the immune response (Lichtenstein et al., 2004). In particular, the Ad early region 3 E3-19k specifically downregulates cell surface expression of HLA class I molecules and inhibits recognition of infected cells by CD8+ T cells (Burget & Kvist, 1985). In support of this hypothesis, we have previously shown that human Ad-specific CTLs cannot kill autologous fibroblasts infected with wild-type Ad5 but kill fibroblasts infected with an E3 deletion mutant (Flomenberg et al., 1996).

Two early region 2 proteins (E2) were selected for initial evaluation, DNA polymerase (Pol) and the single-stranded DNA-binding protein (DBP). Pol is a 140 kDa (1000 aa) protein that contains seven regions that are well conserved among different Ad serotypes (overall homology between 70 and 80%; Liu et al., 2000). The Ad Pol is a member of a large class of family B DNA polymerases that includes human DNA polymerase and other viral DNA polymerases (Knopf, 1998). Notably, polymerases from other DNA and RNA viruses, such as influenza, HIV and hepatitis B, have been identified as T-cell targets (Bennink et al., 1982; Mizukoshi et al., 2004; Tsomides et al., 1994). The 72 kDa (500 aa) DBP is the early Ad protein expressed at highest levels in infected cells. However, it is less well conserved among different Ad groups (overall homology ranges from 55 to 75%; Kitchingman, 1985; Vos et al., 1988).

The goal of this study was to determine whether Ad early proteins Pol and DBP represent significant human CTL targets. High frequencies of memory CD8+ T cells specific for herpes viruses such as EBV can be directly detected in peripheral blood mononuclear cells (PBMCs) (Catalina et al., 2001). In contrast, Ad-specific memory CD8+ T cells are present at low frequencies in most donors and need to be amplified in vitro for study (Tang et al., 2006). Therefore, purified CD8+ T cells from healthy donors were briefly stimulated in vitro with Ad5-infected dendritic cells (DC) to prepare Ad-specific CD8+ T cell lines (TCLs). MHC-restricted cytotoxic responses to Pol and DBP were identified using targets expressing Pol and DBP from recombinant vaccinia virus (VACV) vectors. T-cell epitopes from Pol and DBP were identified by screening PBMCs from a recently infected healthy donor for responses to HLA A2-binding motif peptides and confirmed by analysis of peptide-specific TCLs. Finally, T-cell responses to Pol and DBP epitopes were directly detected in PBMCs from SCT recipients recovering from invasive Ad disease.

METHODS

Study participants. Buffy coat collections were obtained from six healthy adults. Additionally, one or two blood specimens (50 ml each) were obtained from four SCT recipients with documented invasive Ad infections. The research protocol was approved by the Institutional Review Board, and written informed consent was obtained from participants. Donors were HLA-typed by DNA typing using the sequence-specific priming method.

Viruses. Group C Ad5 and Group B Ad35 ATCC prototype strains were obtained from Marshall Horowitz (Albert Einstein College of Medicine, Yeshiva University, NY, USA). UV-inactivated Ad5 antigen was prepared from infected A549 lysates (Flomenberg et al., 1995). Ad virions were purified by anion-exchange chromatography (Clonetech) and titrated by plaque assay (Green & Wold, 1979). Clinical Ad isolates were typed by Focus Technologies. VACV vectors expressing the Ad2 Pol and Ad5 DBP under the control of the T7 RNA polymerase promoter (VACV/Pol and VACV/DBP) were gifts from Jeffrey Engler (University of Alabama) and Geoffrey Kitchingham (St Jude Children’s Research Hospital, TN, USA), respectively (Joung & Engler, 1992). A VACV vector expressing T7 RNA polymerase (VACV/T7) was provided by Laurence Eisenlohr, Thomas Jefferson University (Fuerst et al., 1986). VACV recombinants were grown and titrated in 143 TK– cells (Wysocka et al., 1994).

Western blot assay. Expression of Pol and DBP from the VACV recombinants was confirmed by Western blot analysis of infected cell lysates, as previously described (data not shown) (Tang et al., 2006). Pol- and DBP-specific mAbs were provided by Jeffrey Engler and Douglas Brough (GenVec), respectively (Cleghon et al., 1993).

Cell lines. A549 and MRC-5 lines were obtained from the ATCC. The HLA homozygous B cell lymphoblastoid cell lines (LCLs) Mou and Boleth were a gift from David Eckels (Medical College of Wisconsin) (Yang et al., 1989). LCLs were prepared from donor PBMCs, as previously described (Olive et al., 2002).

Synthetic peptides. HLA-A2-binding motif 9- and 10-mer peptides were identified from the Ad5 Pol and DBP sequences using the search programs http://thr.cit.nih.gov/molbio/hla_bind (Parker et al., 1994) and http://www.syfpeithi.de/ (Rammensee et al., 1995). The hexon epitopes H892–901 (LLYANSAHAL) and H910–924 (DEPTLLYLFVF) were utilized as positive controls. An Ad5 EIA peptide (SPMNLYPEV) was utilized as a non-specific control peptide (Olive et al., 2002). HLA A2-restricted epitopes from EBV BMLMF1 (GLCTLVAML) and HIV reverse transcriptase (RT 476–484 ILKPVHVG) were used as additional controls (Catalina et al., 2001; Tsomides et al., 1994). Crude peptides were synthesized by Research Genetics or Sigma-Genosys. 10 mM stock solutions were prepared in 90% DMSO and aliquots stored at −80 °C.

Ex vivo ELISPOT assay. The gamma interferon (IFN-γ) spot ELISA (ELISPOT) assay was performed as previously described (Olive et al., 2001). Briefly, either PBMCs (2.5 × 10^6) were incubated with 10 μM peptide or TCLs (5 × 10^6) were incubated with 50 000 LCL targets loaded with 10 μM peptide overnight, for 18 h. Cells were suspended in 100 μl RPMI supplemented with 10% pooled human AB sera (Atlanta Biologicals), 10 mM HEPES, 2 mM glutamine, 100 U penicillin ml−1 and 100 μg streptomycin ml−1 (P/S) (T cell media) in duplicate wells. Spot-forming cells (SFCs) were detected with a biotin–avidin alkaline phosphatase conjugate, using an immunospot analyser (Cellular Technology).

Statistical analysis. ELISPOT data were analysed using a paired t-test to compare the mean number of spots in duplicate control and experimental microwells. Error bars indicate SD.

Preparation of Ad-infected dendritic cells. CD14+ cells were isolated from PBMCs by positive immunomagnetic separation using CD14 Microbeads (Miltenyi Biotec). Cells (1 × 10^6 ml−1) were placed in RPMI 1640 supplemented with 5% human AB sera, HEPES,
glutamine and penicillin/streptomycin containing granulocyte/macrophage colony stimulating factor (800 U ml\(^{-1}\)) (Berlex) and IL-4 (1000 U ml\(^{-1}\)) (BD Pharmingen) in a six-well plate. Fresh cytokines were added on day 3. Non-adherent cells were harvested on day 5 and infected with Ad5 (200 p.f.u. per cell) in 200 \(\mu\)l media for 1.5 h. DC maturation was induced with 10 ng interleukin (IL)-1\(\beta\) ml\(^{-1}\) (BD Pharmingen), 1000 U IL-6 ml\(^{-1}\), 10 ng tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) ml\(^{-1}\), and 1 \(\mu\)g prostaglandin E2 (PGE2; Sigma) ml\(^{-1}\) for 48 h.

**Isolation of CD8\(^+\)-enriched T cells.** CD8\(^+\) T cells were purified from PBMCs by immunomagnetic depletion of non-CD8\(^+\) T cells (Miltenyi Biotec) and purity confirmed by flow cytometry.

**Generation of Ad-specific T cell lines (TCLs).** For preparation of bulk Ad-specific CTLs, \(3 \times 10^6\) purified CD8\(^+\) T cells were suspended in T cell media in 24-well plates. Ad5-infected DC were added at a T cell:DC ratio of 5:1 or 10:1. TCLs were tested for cytotoxicity on day 7. In some cases, cells were restimulated with Ad-infected DC (irradiated with 30 Gy). For preparation of peptide-specific TCLs, \(3 \times 10^6\) PBMCs were incubated with 10 \(\mu\)M peptide and 25 ng IL-7 ml\(^{-1}\) (R&D Systems). Cells were restimulated with peptide-loaded, irradiated PBMCs on day 7. IL-2 (20 U ml\(^{-1}\)) was added to each TCL on day 8, and every 3 days thereafter. TCLs were assayed for cytotoxicity around day 14.

**Cytotoxic T-cell assay.** Cytotoxicity was measured by calcein release assay, as previously described (Olive et al., 2002). LCL targets were co-infected for 5 h with either VACV/PoI or VACV/DBP and VACV/T7 (10 p.f.u. per cell each). MRC-5 fibroblast targets were pretreated for 24 h with 200 U human IFN-\(\gamma\) ml\(^{-1}\) and infected overnight with either Ad5 or Ad35 (200 p.f.u. per cell).

**Cytokine flow cytometry assay (CFC).** The CFC assay for IFN-\(\gamma\) was performed using reagents from BD Sciences, as previously described (Tang et al., 2006).

**RESULTS**

**Healthy donors exhibit memory CD8\(^+\) cytotoxic T-cell responses to Pol and DBP**

PBMCs from three healthy adults (donors 1–3) were tested for memory cytotoxic T-cell responses to Pol and DBP. Purified CD8\(^+\) T cells from each donor were stimulated \textit{in vitro} with Ad5-infected autologous DC for 1–2 weeks. TCLs were tested for cytotoxicity against autologous LCL targets infected with recombinant VACV expressing either Ad Pol (VACV/PoI) or DBP (VACV/DBP) under the control of the T7 RNA polymerase promoter. Targets were co-infected with a recombinant VACV that expressed T7 RNA polymerase (VACV/T7). As a control, targets were infected with VACV/T7 alone. Ad-specific CD8\(^+\) TCLs from all three donors specifically killed autologous targets expressing Pol and DBP (Fig. 1). There was minimal cytotoxicity against HLA-mismatched targets expressing Pol or DBP. Therefore, all donors exhibited MHC-restricted cytotoxicity against both Pol and DBP. The cytotoxic responses against Pol were stronger than against DBP from each donor, suggesting that Pol-specific CTLs were present at higher frequencies.

![Fig. 1. Comparison of CD8\(^+\) T-cell responses to Pol and DBP in healthy donors. Purified CD8\(^+\) T cells from donors 1–3 (a–c) were stimulated with Ad5-infected DC for 7–14 days. Cytotoxicity was measured against targets expressing Pol or DBP from recombinant VACV vectors under the control of the T7 RNA polymerase promoter by calcein release assay, Auto, Autologous LCL; Allo, HLA-mismatched LCL. E:T, effector:target.](attachment:image.png)

**Pol contains HLA A2-restricted T-cell epitopes**

Based on analysis of the Ad5 Pol sequence, five 9–10 aa HLA A2-binding motif peptides were synthesized to screen PBMCs from HLA A2\(^+\) donors 4–6. PBMCs from all donors (1–6) were tested for Ad-specific CD4\(^+\) T-cell
responses to inactivated Ad antigen and the HLA DP4-restricted hexon epitope H910–924 by IFN-γ ELISPOT assay (Fig. 2a). All six donors exhibited responses to Ad antigen and H910–924. Notably, the HLA A2+ donor 4 exhibited 10-fold higher frequency CD4+ T-cell responses to both Ad antigen and H910–924, suggestive of a recent infection. Donor 4 also exhibited high level IFN-γ responses to four of five Pol peptides, as well as the HLA A2-restricted hexon epitope H892–901 (Fig. 2b). In contrast, PBMCs from the HLA A2+ donors 5 and 6 did not directly recognize the HLA A2-restricted hexon or Pol epitopes. As a positive control, all three HLA A2+ donors recognized an HLA A2-restricted EBV epitope (data not shown).

The three Pol epitopes with the highest frequency T-cell responses from donor 4 (Pol 608, 779 and 977) were further evaluated. Donor 4 PBMCs were stimulated in vitro with each peptide for 2 weeks. A Pol 608-specific TCL exhibited peptide-specific responses shown to be mediated by CD8+ T cells by CFC assay (Fig. 3a–c). Additionally, the Pol 608-specific TCL killed both autologous and HLA A2-matched-only targets expressing Pol (co-infected with VACV/Pol and VACV/T7), but did not kill targets infected with VACV/T7 alone or HLA-mismatched targets expressing Pol. Similarly, Pol 977- and Pol 779-specific TCLs exhibited HLA A2-restricted cytotoxicity against Pol (Fig. 3d–f and g–h, respectively). Both TCLs exhibited MHC-restricted killing of targets expressing Pol, and cytotoxicity was specifically blocked by a monoclonal antibody to HLA class I antigens. These data confirm that all three Pol epitopes are naturally processed from the intact protein and presented via HLA A2.

**HLA A2+ donors exhibit memory responses to Pol epitopes**

PBMCs from HLA A2+ donors 5 and 6 did not directly respond to either hexon or Pol HLA A2-restricted epitopes. Therefore, Ad-specific memory CD8+ T cells were amplified in vitro with Ad-infected autologous DC for 2 weeks and screened by ELISPOT assay. Ad-specific TCLs from both donors exhibited specific IFN-γ responses to the HLA A2-restricted hexon epitope H892–901 in comparison with the HLA A2-restricted HIV peptide (negative control) (Fig. 4). Both Ad-specific TCLs responded to Pol 977. The donor 5 TCL also responded to Pol 779 and Pol 608. In contrast, the donor 6 TCL responded to Pol 608, but not to Pol 779.

**Identification of HLA A2-restricted DBP epitopes**

Recently infected donor 4 PBMCs were tested for IFN-γ responses to three HLA A2-binding motif peptides from the Ad5 DBP. Donor 4 responded directly to DBP 243 and DBP 206, but not to DBP 407 (Fig. 5a). Precursor T-cell frequencies specific for DBP were lower than for Pol (shown in Fig. 2b), however, and attempts to directly amplify peptide-specific TCLs using DBP 243 and DBP 206 were unsuccessful. The Ad-specific CD8+ TCL from HLA A2+ donor 5, prepared as described above, was screened against DBP peptides in the ELISPOT assay. As shown in Fig. 5(b), IFN-γ responses were detected to DBP 407, but not to DBP 243 (DBP 206 was not tested). The TCL was then stimulated with DBP 407 for 2 weeks and further analysed. The DBP 407-peptide-specific TCL exhibited HLA A2-restricted recognition of peptide-loaded LCL targets (Fig. 5c). Moreover, the TCL exhibited MHC-restricted killing of targets expressing DBP (Fig. 5d), confirming that DBP 407 is naturally processed from the intact DBP.

**Detection of T-cell responses to Pol and DBP in SCT recipients recovering from Ad disease**

PBMCs from three HLA A2-positive related matched SCT recipients who recovered from invasive Ad infections were
Fig. 3. Pol peptide-specific T cell lines exhibit HLA A2-restricted cytotoxicity. Donor 4 PBMCs were stimulated with each Pol peptide for 2 weeks. (a–c) Pol 608. (d–f) Pol 977. (g, h) Pol 779. (a, d, g) Each TCL was tested against autologous (Auto) LCL loaded with Pol peptide by IFN-γ ELISPOT assay. (b) Pol 608-specific T cells were incubated with peptide for 6 h, and intracellular IFN-γ expression and CD69 activation were measured by flow cytometry after gating on CD3+CD8+ cells. (c, e, h) Each line was tested for cytotoxicity against targets expressing Pol by calcein release assay. Auto, autologous LCL (HLA A2, A3, B57, B60); Allo, HLA-mismatched Mou (HLA A29, B44); A2 match, HLA A2-matched only Bolet (HLA A2, B75). Targets were co-infected with VAC/Pol and VACV/T7 or infected with VACV/T7 alone. Responses to VACV/T7-infected allo targets were negative (data not shown). (f) Pol 977-specific T cells were tested for cytotoxicity against auto LCL expressing Pol after pre-incubation of targets with mAbs (40 μg ml⁻¹) against HLA-A, B, C or HLA DR (E : T ratio 10 : 1) for 30 min. SFC, Spot-forming cells. Error bars, SD.
Patient 1, a 33-year-old man with acute myelogenous leukaemia (AML), developed severe Ad haemorrhagic cystitis and nephritis 7 months post-SCT following immunosuppressive therapy for GVHD. Although the patient’s Ad isolate was not typable, urinary tract disease is exclusively caused by Group B Ad types 11, 34 and 35. His case was described as part of a small series of patients with invasive Ad disease successfully treated with cidofovir (Neofytos et al., 2007). PBMCs collected 2 and 6 weeks after clinical improvement were directly tested for IFN-γ responses to the Pol and DBP peptides by IFN-γ ELISPOT assay (Fig. 6a). Week 2 PBMCs exhibited detectable T-cell responses to the hexon peptide H892–901 and Pol 977 only. At 6 weeks, PBMCs exhibited significantly higher responses to all three Pol peptides 977, 779 and 608 (242 Pol 977-specific T cells per 10⁶ PBMCs) compared with H892–901. Additionally, responses were detected to all three DBP peptides 407, 243 and 206 (mean frequency 95 per 10⁶ PBMCs). From 1 to 10 weeks, his CD4 count remained between 50 and 100, but his CD8 count rose from 132 to 274.

Patient 2 is a 59-year-old woman with non-Hodgkins lymphoma who developed severe haemorrhagic cystitis and nephritis five weeks post-SCT. She exhibited a rising Ad viraemia over 2 weeks (maximum 1.8×10⁷ copies ml⁻¹), and Group B Ad11 was isolated from her urine. She responded to cidofovir with resolution of viraemia and symptoms. PBMCs collected 6 weeks after treatment exhibited higher responses to Pol 977, Pol 779 and DBP 407 (average 360 per 10⁶ PBMCs) compared with Hex 892 (296 per 10⁶ PBMCs) (Fig. 6b). Lower frequency responses were detected to DBP 206 and DBP 243 but not to Pol 608. At week 10, her CD4 and CD8 counts were 121 and 393, respectively.

**Fig. 4.** Memory T-cell responses to Pol peptides in healthy donors. Purified CD8⁺ T cells from HLA A2⁺ donors were stimulated for 2 weeks with Ad5-infected DC. Ad-specific TCLs were tested against autologous LCL loaded with peptides by IFN-γ ELISPOT assay. H892, HLA A2-restricted hexon epitope. HIV pep, HLA A2-restricted HIV epitope. *P<0.024; **P<0.0018. SFC, spot-forming cells. Error bars, SD.

**Fig. 5.** CD8⁺ T-cell responses to DBP epitopes. (a) PBMCs from recently infected donor 4 were tested against DBP peptides by IFN-γ ELISPOT assay. (b) Donor 5 Ad-specific CD8⁺ TCL was tested against peptide-loaded autologous (Auto) LCL by IFN-γ ELISPOT assay. *P<0.005. (c, d) Donor 5 Ad-specific T cells were restimulated after 2 weeks with DBP 407 and tested for specificity. (c) Cells were tested against a panel of targets loaded with DBP 407 by IFN-γ ELISPOT assay. Auto, HLA A1, A2, B49, B57; Allo, HLA-mismatched Mou HLA A29, B44. A2 match, HLA A2-matched only Boleth (HLA A2, B75). (d) Cells were tested for cytotoxicity against targets expressing DBP by calcein release assay. LCL were co-infected with VACV/Pol and VACV/T7 or infected with VACV/T7 alone. E:T, effector : target. SFC, spot-forming cells.
Patient 3, a 43-year-old man with AML, developed severe group C Ad1 hepatitis and pneumonitis 6 months post-SCT, while receiving immunosuppressive therapy for GVHD (Neofytos et al., 2007). He responded promptly to cidofovir, while his CD4 and CD8 counts were both >50. PBMCs collected 6 weeks later revealed a distinctive pattern (Fig. 6c). This patient exhibited an over threefold higher frequency response to a single epitope Pol 608 (800 per 10⁶ PBMCs), compared with Pol 977, Pol 779 and Hex 892. Additionally, he did not exhibit detectable responses to any of the DBP epitopes. At week 10, his CD4 and CD8 counts rose to 62 and 300, respectively.

As a control, a HLA 2-positive SCT recipient who died from invasive Ad disease was studied. Patient 4, a 72-year-old man with AML, developed invasive Ad colitis and hepatitis 10 weeks after a non-myeloablative matched unrelated SCT, in the setting of active GVHD (Neofytos et al., 2007). He was treated with cidofovir, but died 2 weeks later. His CD4 and CD8 counts were both under 20. PBMCs collected before his death did not exhibit detectable responses to any of the above HLA A2-restricted Ad epitopes (data not shown).

**Pol epitopes are well conserved among different Ad serotypes**

The sequences of the above HLA A2-restricted Pol and DBP epitopes were compared among different Ad serotypes. The N-terminal portion of Ad Pol contains three domains involved in its exonuclease activity (EXO I–III), whereas the C-terminal portion contains seven conserved domains involved in its polymerase activity (Pol I–VII) (Liu et al., 2000). Overall, Pol sequences are well conserved between Ad serotypes. For instance, the group C Ad5 and group B Ad35 protein sequences exhibit 76% identity and 94% similarity. The Pol epitopes are well conserved among representative serotypes from Ad groups A–F (Table 1). The HLA A2-binding motif includes major hydrophobic amino acid anchors at positions 2 and 9 (P2 and P9) (Parker et al., 1994). Pol 977, which is located between the Pol III and Pol I domains, is completely conserved among serotypes. Pol 779, which is part of the Pol VI domain, is well conserved, with minor differences among groups (group F has one major difference in P9). Pol 608, which lies between the Exo III and Pol II domains, is well conserved among group C and F Ads, but exhibits moderate differences among other Ad groups.

In contrast to Pol, the HLA A2-binding motif DBP epitopes are less well conserved. The heavily phosphorylated DBP contains an N-terminal domain (1/3) involved in host-range determination, which exhibits significant sequence heterogeneity among different serotypes. However, the C-terminal domain (2/3), which functions in DNA replication and DNA binding, has several conserved regions (Vos et al., 1988). For example, the Ad5 and Ad35 DBP protein sequences exhibit only 58% identity and 67% similarity, respectively. As shown in Table 1, all three DBP epitopes are well conserved among group C Ads, but exhibit moderate sequence differences among other Ad groups.

**Pol 977-specific TCL kills fibroblast targets infected with diverse Ad serotypes**

The Pol 977-specific TCL from donor 4 was tested for the ability to kill HLA A2⁺ MRC-5 fibroblasts infected with...
either the group C Ad5 or the distantly related group B Ad35. The Pol 977-specific TCL killed both group C Ad5- and group B Ad35-infected fibroblast targets (Fig. 7). These data confirm that Pol 977-specific CTLs kill cells infected with diverse wild-type adenoviruses representing the most common causes of invasive disease in SCT recipients.

DISCUSSION

This is the first study, to our knowledge, to document that Ad early proteins Pol and DBP are human CD8+ T-cell targets. The E2 Ad Pol was identified as the dominant target and found to contain HLA A2-restricted epitopes that are conserved among different serotypes. CD8+ T-cell responses to the E2 DBP were lower in frequency, and DBP epitopes were not as well conserved. Moreover, both Pol- and DBP-specific T-cell responses were directly detected in PBMCs from one recently infected normal donor and three SCT recipients recovering from invasive Ad infections. Pol- and DBP-specific CD8+ T-cell responses were directly identified in PBMCs from a healthy donor utilizing HLA A2-binding motif peptides. This unique donor exhibited 10-fold higher Ad-specific T-cell frequencies compared with over 30 healthy donors tested in our present and previous studies. Although clinical data are not available, we propose that this donor had a recent Ad infection. TCLs prepared to three Pol peptides and one DBP peptide each exhibited HLA A2-restricted cytotoxicity against targets expressing Pol or DBP, confirming that the epitopes were processed from the intact proteins. Following in vitro amplification, CD8+ T-cell responses to Pol and DBP proteins were identified in three of three additional healthy donors, and responses to Pol and DBP epitopes were confirmed in two other HLA A2-positive donors.

The HLA A2-restricted Pol and DBP epitopes were utilized to analyse Ad-specific CD8+ T-cell responses in four SCT recipients with invasive Ad disease. Notably, all three patients who recovered developed CD8+ T-cell responses to one or more Pol epitopes at higher or comparable frequencies compared with a HLA A2-restricted hexon epitope. Although these patients initially required cidofovir treatment to control their Ad disease, each patient completely recovered without recurrence of Ad infection at 6 months follow up. Therefore, antiviral treatment helped control the patients’ Ad disease until they were able to mount a virus-specific cellular immune response.

Differences in response patterns to Pol and DBP epitopes among SCT patients correlated with Ad serotypes. The patient with group C Ad1 disease developed the highest frequency of response to Pol 608 and did not recognize the DBP epitopes tested. The fact that all epitope sequences are completely matched with Ad1 suggests that Pol 608 is a major epitope for group C Ads. In contrast, both patients with group B Ad disease developed a broader response pattern to both Pol and DBP epitopes, with strongest responses to Pol 977 and Pol 779. This probably reflects the fact that Pol 977 and Pol 779 are the most well-conserved epitopes for the group B Ads.

Notably, Pol 977 was recognized by six of six HLA A2+ donors, and comparison of Pol sequences determined that this epitope is highly conserved among all serotypes. A Pol

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Fig. 7. Comparison of Pol 977-specific T-cell cytotoxicity against Ad5- and Ad35-infected fibroblasts. The Pol 977-specific TCL prepared from donor 4 was tested against HLA A2+ fibroblast cell line MRC-5 targets infected with Ad5 or Ad35 or mock-infected. Cytotoxicity was measured by calcein release assay. E:T, effector : target.
977-specific TCL killed fibroblast targets infected with either the group C Ad5 or the distantly related group B Ad35, confirming its cross-reactivity. In contrast, Pol epitope 608 is less well conserved among different serotypes, and there is also significant heterogeneity among the DBP epitopes.

Ad-specific T-cell responses to early proteins have been previously identified in murine models only. These data are difficult to extrapolate to human infections because Ad replication is restricted in mice. Early region 1 E1A was a CTL target in rodent tumours infected with either wild-type Ad or E1-transformed tumour cells (Kast et al., 1989; Routes et al., 1991). One study using wild-type Ad identified T-cell responses to the early proteins E1B and DBP (Sparer et al., 1997). CD8+ T-cell IFN-γ responses were detected to hexon and DBP peptides in mice infected with wild-type Ad (McKelvey et al., 2004). In another study using E1-deleted Ad, CTL responses were identified to hexon, and to a lesser extent, the capsid proteins fibre and penton, but not to the early proteins Pol, DBP, or pre-terminal protein (Joos et al., 1998). However, in the absence of the E1 transactivating region, expression of early proteins from E1-deleted Ads is limited.

Prior data regarding targets of Ad-specific T-cell responses in humans have been limited to the viral capsid proteins. We previously documented that Ad-specific CD8+ T cells from healthy donors kill hexon, but exhibit weaker and variable responses to both penton base and fibre (Tang et al., 2006). Leen et al., (2004) also found that most Ad-specific CD8+ T-cell clones prepared were targeted to hexon. In contrast to the present study using wild-type Ad, this group stimulated PBMCs with a replication-deficient E1 deletion mutant that expresses little or no early viral proteins. Therefore, CD8+ T cells specific for early proteins would not be efficiently amplified by their method. We previously identified a highly conserved CD4+ T-cell hexon epitope, and hexon was confirmed to be a major CD4+ T-cell target by analysis of Ad-specific CD4+ T-cell clones (Heemskerk et al., 2006). One study identified CD4+ T-cell responses to Ad E1, E3, Pol and fibre peptides, but their specificities were not confirmed (Haveman et al., 2006).

We propose that Ad Pol-specific CTLs play an important role in clearing Ad infections in vivo. There are several theoretical advantages of CTLs targeted to early non-structural Ad proteins. Firstly, CTLs will eliminate Ad-infected cells before viral replication occurs. Secondly, since Ads express a number of immunomodulatory proteins that interfere with the host immune response, CTLs targeted to early proteins will kill infected cells before these proteins exert their effects. In particular, the E3-19k glycoprotein prevents cell surface expression of MHC class I molecules via two different mechanisms. E3-19k specifically binds to class I molecules in the endoplasmic reticulum and prevents their transport to the cell surface (Burgert & Kvist, 1985). Additionally, E3-19k binds to the transporter associated with antigen processing (TAP) and blocks peptide transport and association with class I molecules (Bennett et al., 1999). Therefore, CTLs targeted to late viral proteins may not be able to recognize infected cells in which class I-associated antigen presentation is blocked by E3 proteins. Other E3 early proteins inhibit TNF- and FAS-induced apoptosis (Horton et al., 1991; Shisler et al., 1997).

In support of the importance of CTLs targeted to early viral proteins, protection from cytomegalovirus infection after SCT correlates with immediate early 1-specific CD8+ T cells (Bunde et al., 2005). Additionally, following a second exposure to VACV, CD8+ T cells targeted to early viral proteins strongly predominate over responses to late viral proteins (Kastenmüller et al., 2007).

The limitations of this study include analysis of CTL responses to only two early Ad proteins and the small number of study participants. Unfortunately, it is difficult to identify and study adults with acute Ad infections. We have subsequently amplified Pol-specific CD8+ CTLs from three consecutive additional healthy donors (data not shown). The role of CTLs targeted to early Ad proteins could be more readily defined in an animal model. However, a suitable animal model for human Ad infection is not available, and animal Ads, including mouse Ads, have significantly different properties (Kring & Spindler, 1996).

There is a need to identify new strategies for the treatment of Ad disease in SCT recipients. Cidofovir has in vitro and in vivo activity, but treatment is limited by its nephrotoxicity (Izzedine et al., 2005). Despite the use of cidofovir, Ad infections have caused fatalities in SCT recipients who have profound immune dysfunction (such as patient 4 in this study) (Ljungman et al., 2003). Several cases of Ad infections treated with donor Ad-specific T cells have been published (Bordignon et al., 2001; Chakrabarti et al., 2000; Hromas et al., 1994). Most recently, SCT recipients were treated with donor lymphocytes stimulated in vitro with autologous LCL infected with an E1-deleted Ad (Leen et al., 2006). Reductions in Ad viral loads were documented in three paediatric patients with active infection and clinical improvement noted in one patient with Ad pneumonia. In another study, Ad-specific T cells were directly isolated from donor PBMCs and infused into eight paediatric SCT recipients with Ad infections (Feuchttinger et al., 2006). All patients were also treated with cidofovir. Ad-specific T-cell responses were documented in five patients. However, there was no definite impact on survival. Four of five patients with diarrhoea alone or no symptoms resolved their infections, whereas all three patients with disseminated Ad disease died.

The presence of CD8+ T-cell responses to Pol and DBP following acute Ad infection suggests that CTLs targeted to early viral proteins play a role in limiting Ad replication in vivo. Therefore, it may be important to include T cells targeted to early Ad proteins, such as Pol, in donor lymphocyte infusions. In particular, Pol contains T-cell epitopes that were well conserved among different...
serotypes. Further investigation is needed, however, to fully characterize the specificities of Ad-specific T cells and their roles in recovery from Ad infections.

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