ADENOVIRUS INFECTION AND IMMUNITY IN CHILDREN AFTER STEM CELL TRANSPLANTATION

ADENOVIRUS INFECTION AND IMMUNITY IN CHILDREN AFTER STEM CELL TRANSPLANTATION

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The beginning of knowledge is
the discovery of something
we do not understand
(Frank Herbert)

Voor mijn ouders
Voor Sasja
To Andrew

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ABBREVIATIONS

- ALL: acute lymphoblastic leukemia
- AML: acute myeloid leukemia
- APC: antigen presenting cell
- ATG: anti-thymocyte globulin
- B-LCL: EBV transformed B-lymphoblastoid cell line
- BM: bone marrow
- CAR: coxsackie adenovirus receptor
- CML: chronic myeloid leukemia
- CMV: cytomegalovirus
- CPE: cytopathological effect
- CsA: cyclosporin A
- CTL: cytotoxic T lymphocyte
- DC: dendritic cell
- EBV: Epstein-Barr virus
- G-CSF: granulocye-colony stimulating factor
- GvHD: graft versus host disease
- HAdV: human adenovirus
- HLA: human leukocyte antigen
- HSV: herpes simplex virus
- HvGD: host versus graft disease
- IFN: interferon
- IL: interleukin
- IVIG: intravenous immunoglobulin
- MACS: magnetic cell sorting
- MB: methylene blue
- MFD: matched family donor
- MHC: major histocompatibility complex
- MMFD: mismatched family donor
- MUD: matched unrelated donor
- NAb: neutralizing antibody
- NK: natural killer cell
- PBSC: peripheral blood stem cell
- PCR: polymerase chain reaction
- PTLD: post-transplant lymphoproliferative disorder
- RID: receptor internalization and degradation
- RQ-PCR: real-time quantitative PCR
- SAA: severe aplastic anemia
- SCID: severe combined immunodeficiency
- SCT: stem cell transplantation
- TBI: total body irradiation
- TCID50: tissue culture infectious dose 50%
- TCR: T cell receptor
- TNF: tumor necrosis factor
- UCB: umbilical cord blood
- VZV: varicella zoster virus

Chapter 1

General Introduction

1. IMMUNITY

The main function of the immune system is to combat invading pathogens. Two lines of defense are activated upon infection with a microorganism. The first one is the innate immune response, which is mediated by certain cell populations and soluble factors. These cells include neutrophils and macrophages that can recognize constituents of cell membranes of e.g. bacteria and engulf them, as well as natural killer (NK) cells which can directly or indirectly exert antiviral activity. Upon activation, neutrophils and macrophages secrete cytokines and chemokines such as type I interferons (IFN-α/β), interleukins (IL-1, IL-6, IL-8) and tumor necrosis factor (TNF-α) that attract other cells to the site of infection and may elicit the specific immune response (1,2). Other soluble factors for opsonization of microorganisms, as well as elimination of cell debris and immune complexes, are acute phase proteins such as mannose binding lectin (MBL) and complement factors (3).
The second line of immunity, the adaptive immune response, is initiated in the lymph nodes after presentation of specific epitopes to T and B lymphocytes. Antigens from the microorganisms, taken up in the infected tissues by specialized antigen-presenting cells (APC) - the dendritic cells (DC) - are processed to peptides and presented by major histocompatibility complex (MHC) molecules to T cells in the lymph nodes. These T cells can in turn provide help to B cells that recognize the tertiary structure of the antigen. Upon encounter with the specific antigen in the context of appropiate co-stimulation, both T and B cells become activated leading to extensive proliferation and differentiation by which cells gain effector functions (4). For B cells, this involves immunoglobulin class switching and affinity maturation in the germinal center reaction, resulting in the production of high affinity antibodies against extracellular pathogens such as the majority of bacteria and parasites. Antiviral antibodies also contribute to viral clearance mainly by blocking virus entry into susceptible cells, thereby preventing extracellular viral spread. Effector T cells produce cytokines and in some cases gain cytolytic activity which will lead to eradication of intracellular pathogens such as viruses or intracellular bacteria (1,4). After the infection is cleared, the size of the effector cell pool is reduced as most cells die, while some cells differentiate to long-living B and T memory cells which can confer protection of a host upon re-infection by more rapid and enhanced secondary responses.

2. ALLOGENEIC STEM CELL TRANSPLANTATION

2.1 Introduction

Allogeneic stem cell transplantation (SCT) is a remedy to cure a number of inherited diseases of hematopoietic precursor cells, and can also be applied to rescue deficient hematopoiesis following intensive radio/chemotherapy for hematologic malignancies. Defects in one or more components of the immune system may result in immunodeficiency,

either primary or secondary. Primary immunodeficiencies are caused by a germ-line mutation affecting one of several genes that control the expression and activities of immune responses (5). Secondary immunodeficiencies are acquired as a consequence of environmental factors or other diseases, such as viral infections (e.g. HIV), malnutrion, or hematopoietic neoplastic tumors which suppress normal immune cell function or are treated with myeloablative medication (1).

A great number of gene defects inducing primary immunodeficiency have been discovered, resulting in an absence of $CD8^+$ or $CD4^+$ T cells in for example MHC class I or II deficiency, respectively, or a lack of the total T cell population (sometimes accompanied by an absence of NK cells, and/or B cells) in severe combined immunodeficiency (SCID). In SCID, at least 8 entities can be distinguished based on the genetic defect and according to their phenotype and inheritance pattern (5). Patients with one of these deficiencies have a clinical presentation which is characterized by an early onset of infections, mainly of the respiratory and intestinal tract. Persistent diarrhoea with growth impairment and/or interstitial pneumonia are the most frequent infectious manifestations, besides superficial Candida infections of the skin and mucous membranes. Opportunistic microorganisms as *Pneumocystis jirovecii,* intracellular microorganisms as *Listeria* and *Legionella*, as well as viruses such as those from the Herpes family can also lead to devastating disease in these patients. SCT with stem cells from bone marrow of an allogeneic healthy donor is in most cases the only option for curing these severely immunocompromised patients. Important properties of stem cells include self-renewal potential as well as pluripotency, implicating that all lineages of recipient hematopoiesis can be reconstituted following SCT.

Immunodeficiencies were the first hematological disorders eligible for SCT in the late sixties. In the next decades, SCT has also proven to be an effective treatment for hematological malignancies and nonmalignant hematological disorders. Pediatric hematological malignancies for which SCT is part of a possible therapy are acute lymphoblastic leukemia (ALL, the most common form of leukemia in children), acute myelogenous leukemia (AML), and chronic myelogeneous leukemia (CML). When these patients are in first (for the high-risk type) or second remission after chemotherapy, SCT is a good therapeutic option for treatment which results in an increased disease-free survival as compared to conventional chemotherapy (6). Nonmalignant disorders curable by SCT are e.g. severe aplastic anemia (SAA), Fanconi anemia and β-thalassemia, in which SCT is given to correct the underlying hematological defect (7). More recently, SCT has been performed as experimental therapy for metabolic disorders and autoimmune diseases.

Depending on the disease for which the SCT is performed, as well as the age of the patient, different conditioning regimens are used. Most myeloablative conditioning regimens are based either on total body irradiation (TBI) for older children, or busulfan for children under the age of 3 years. Non-myeloablative conditioning regimens (also indicated as reduced

intensity conditioning regimens) are not meant to eradicate malignant or normal hematopoietic precursor cells, but to suppress more mature cells together with the adaptive immunity of the host. Reduced intensity regimens consist mostly of cyclophosphamide-based regimens in combination with anti-thymocyte globulins (ATG) or monoclonal anti-T cell antibodies, and may include moderate amounts of busulfan or TBI.

2.2 HLA compatibility in SCT

The degree of human leucocyte antigen (HLA)-matching between donor and recipient determines the feasibility and outcome of an allogeneic SCT to a large extent. The genes for the HLA antigens are located in the major histocompatibility complex (MHC) on chromosome 6. The HLA antigens are heterodimers grouped in 2 classes according to their chemical structure and function. For HLA class I, three loci are relevant in this respect, HLA-A, B and C. In 2001, 220 HLA-A, 460 HLA-B and 110 HLA-C alleles were described. Similarly, three loci are present for HLA class II, of which 360 alleles for DR, 70 for DQ, and 116 for DP, respectively, have been described (8). The enormous polymorphism of the HLA antigens makes it difficult to find a fully HLA-matched donor outside of the close family. The donor should share at least some HLA molecules with the graft recipient in order to assure proper positive and negative selection of precursor T cells in the thymus. In the case of an HLA-mismatched transplant, transfer of mature alloreactive donor T cells into the host may induce a severe complication, called graft-versus-host disease (GvHD) where T cells react against host HLA molecules, and attack tissues of the recipient. Alternatively, residual host hematopoietic cells may recognize donor cells as foreign and induce a host-versus-graft disease (HvGD), resulting in failure of engraftment or graft rejection.

In 1968, the first successful stem cell transplantations were performed for SCID patients with an HLA-identical sibling (matched family donor, MFD) (9,10). In this setting, patients did not require any conditioning prior to SCT because they were not at risk of graft rejection as a result of their intrinsic immune incompetence. Improved knowledge and experience has led to the use of SCT for the treatment of other diseases, such as other inborn errors and leukemia in the late 1970’s (11). To successfully engraft these patients, pretransplant conditioning therapy, usually consisting of chemotherapy with either TBI or busulfan, is needed in order to create space in the bone marrow, to suppress the allo-immune reactivity of the host, and eradicate malignant cells, e.g. in the case of leukemia. Because an HLA-identical sibling donor is only available for approximately one third of all patients, alternative donors are required. These donors include partially HLA-mismatched (or haplo-identical) family donors (MMFD) as well as (mis)-matched unrelated donors ((M)MUD). In these cases, one or more HLA mismatches (up to 6 out of 12 HLA loci for a haplo-identical parent) may be present between donor and recipient. In early studies, SCT with unrelated donors resulted in higher frequencies of graft rejection, an increased risk of GvHD and of

infections post-SCT, and a lower disease-free survival (12-17). To circumvent these transplant-related complications, T-cell depletion of the graft and additional immunosupression prior to SCT (with ATG or Campath) and after SCT (with cyclosporin A (CsA) or tacrolimus) were applied in order to enhance engraftment and prevent GvHD (18,19). In this respect, suppression of the gut microflora by gastro-intestinal decontamination (GID) of pediatric recipients who were kept in a strict protective environment significantly reduced the incidence and severity of acute GvHD (20), also in MUD-SCT for children (Donker *et al.*, personal communication).

Further development of SCT-technology in recent years is the result of improved HLA matching by high-resolution DNA typing (21,22), and the use of alternative sources of stem cells. These stem cells can be obtained from umbilical cord blood (UCB) or $CD34^+$ peripheral blood stem cells (PBSC), mobilized from the bone marrow by cytokines such as granulocyte colony stimulating factor (G-CSF). These strategies have comparable rates of engraftment and acute GvHD as following bone marrow transplantation, but a somewhat increased frequency of chronic GvHD and a delayed lymphocyte recovery, especially in the case of PBSCT (23-26).

2.3 Immune reconstitution after SCT

In the first months post-SCT, patients are immunocompromised because stem cells from the graft need to develop into all myeloid and lymphoid lineages. After SCT with a T-cell depleted graft, e.g. in the case of MMUD or MMFD transplants, immunological recovery is more delayed than in patients receiving a full graft of an HLA-identical MFD donor. Leukocyte recovery (usually assessed as granulocyte counts $\geq 0.5\times10^9$/L) following a T-cell depleted SCT is achieved around week 2-3 post-SCT, whereas lymphocyte recovery ($\geq 0.5\times10^9$/L) can be observed at ≥ 1-2 months after SCT. This recovery (merely based on cell counts and not on cell function) is not statistically different between unrelated or related donors (MMUD vs MMFD) (27). Repopulation with different lymphocyte subsets after SCT from patients in our clinic is shown in Figure 1 (Jol-van der Zijde, unpublished data).

NK cells constitute the major proportion of lymphocytes that repopulate the peripheral blood early after SCT, followed by $CD8^+$ T cells and B cells. $CD4^+$ T cell recovery is further delayed, which is most pronounced following a SCT from a T-cell depleted graft (17,28). These $CD4^+$ T cells consist mainly of $CD4^+CD45RO^+$ cells characteristic for memory cells. As a consequence of the delayed $CD4^+$ T cell recovery, the CD4/CD8 ratio is inverted compared to the situation in healthy persons. Naive $CD4^+$ and $CD8^+$ T cells only appear after 6-9 months or later (29-31). This is also accompanied by a severely skewed T cell repertoire in the first 6-12 months following SCT as assessed by TCR-BV spectratyping, with normalization only after naive T cells have repopulated the blood (29,32,33). This reflects the 2 ways of T cell regeneration that contribute to the reconstitution of the T cellular

compartment after SCT. The first regeneration develops from mature T cells in the graft that may expand in the host under antigenic stimulation (referred to as peripheral expansion) (34). The second regeneration consists of *de novo* maturation of naive T cells from the pluripotent stem cells via passage through the thymus (27). Furthermore, acquisition of full T cell function requires time and may be hampered by the prolonged use of immune suppressive drugs such as CsA administered to minimize the severity of GvHD (35).

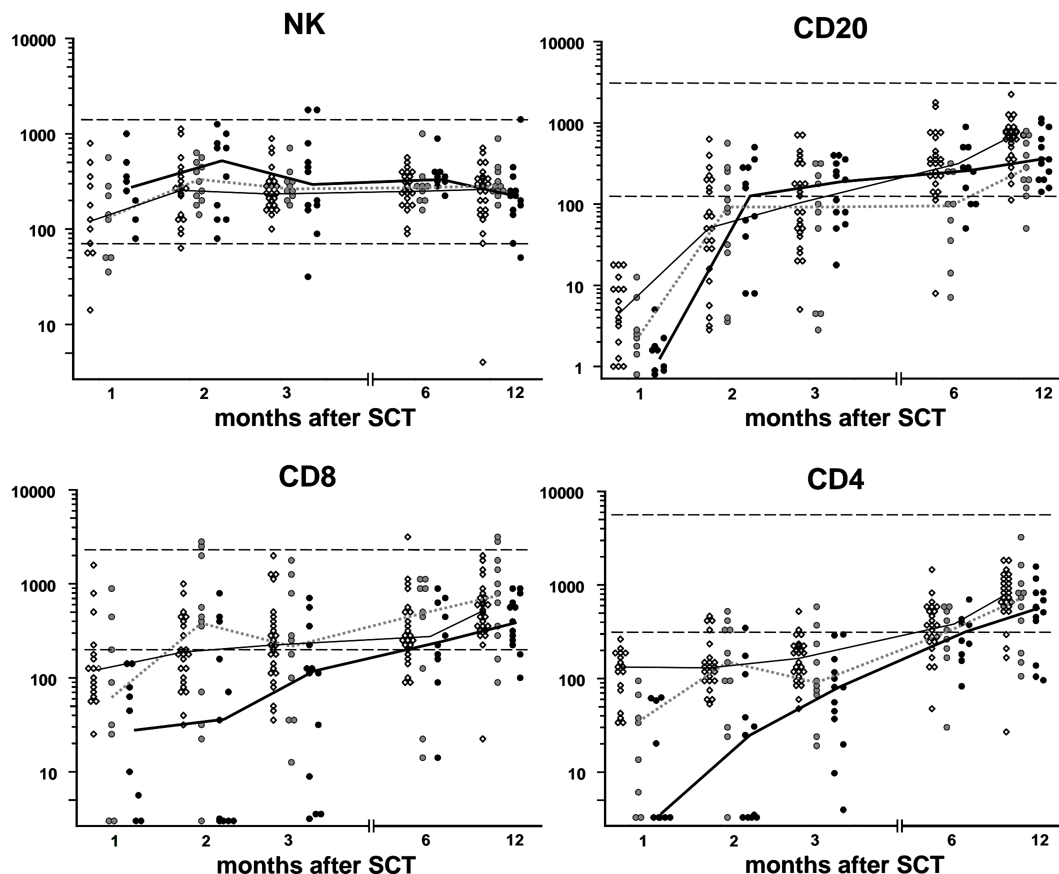

Figure 1. Recovery of NK cells, CD20$^+$ B cells, CD8$^+$ T cells and CD4$^+$ T cells in the first 12 months after SCT. Open symbols represent MFD recipients (geometrical mean (GM): thin black line), gray symbols represent MUD transplant without T cell depletion (GM: gray dotted line), and black symbols represent MUD transplants with T cell depletion (GM: thick black line). The dotted lines indicate the 95% confidence interval (GM ± 2×SD) of healthy controls (including children and adults) *(Jol-van der Zijde, unpublished data).*

Immunoglobulin levels of IgM and IgG in serum of graft recipients are usually normal compared to healthy controls, whereas IgA levels are markedly reduced (26,31). However, intravenous immunoglobulin (IVIG) support is often applied after SCT, which could account for the normal levels of IgG in serum. Specific antibody production, such as following infection or vaccination, is severely delayed after MMFD/MMUD SCT in comparison to MFD SCT (36-38).

The occurrence of GvHD and cytomegalovirus (CMV) reactivation has been reported to have an impact on immune recovery (31,39). For GvHD, this might be due to the fact that GvHD targets the lymphoid system besides the epithelial system of the skin, liver and gut, causing lymphoid hypocellularity. As a result of the prolonged immunodeficiency state of the patient after SCT, especially following MMFD or MMUD SCT, the risk of opportunistic infections is high (27,40,41).

2.4 Infectious complications after SCT

Infections are a major cause of morbidity and mortality after allogeneic SCT (42). Usually, allograft recipients are treated in a protective environment and receive (general or intestinal) antimicrobial prophylaxis to prevent infections with bacteria, fungi and *P. jirovecii* during the early post-transplant period, when the recipient is unable to mount an effective immune response to pathogenic microorganisms. By this means, *de novo* infections with these microorganisms and respiratory viruses are minimalized. On the other hand, the recipient might have encountered many DNA viruses prior to SCT, which persist in the recipient in a latent form and may reactivate after SCT.

The sequential occurrence of infections is linked with the changing immunologic state of the graft recipient and is depicted for viral infections in Figure 2.

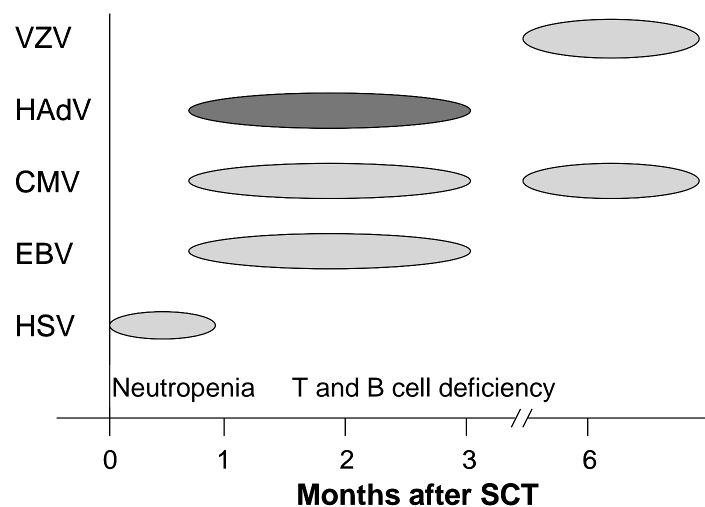

Figure 2. Viral complications in the first months after SCT. Patients are neutropenic during the first month, in which HSV reactivations occur. In the absence of T and B cellular immunity, DNA viruses such as EBV, CMV and HAdV are prominent. In a later phase after SCT, late CMV infections are reported as well as VZV infections *(Adapted from refs (42,43))*.

Three phases of immune recovery can be observed: the first phase, up to 30 days post-SCT, is the neutropenic phase in which bacterial infections are the major concern, as well as clinically evident herpes simplex virus (HSV) reactivation due to disruption of the mucosal integrity by the preparative regimen (43). Appropriate antibiotics and acyclovir, respectively,

are usually effective. The second phase is the early post-transplant phase (30-100 days post-SCT) that is characterized by impaired cell-mediated immunity in which mainly viral reactivations occur that may result in severe infections. Some of these viruses, such as varicella zoster virus (VZV), are rapidly diagnosed clinically in an early phase of infection and treatment with (val)acyclovir is adequate. However, other herpes viruses such as CMV and Epstein-Barr virus (EBV), which are most common in the population, can cause severe problems because signs and symptoms of a beginning infection are not evident (44). CMV is latently present in leukocytes and can be reactivated in host cells or it can be transferred by cells from a seropositive donor to a seronegative patient, inducing a primary CMV infection in the host. EBV latency is installed in B cells, which upon reactivation can cause the severe post-transplant lymphoproliferative disease (PTLD) (45). Morbidity and mortality due to CMV or EBV infections were high in MUD transplants (up to 85% and 100%, respectively) in the era prior to the development of sensitive laboratory detection methods and treatment (45,46). Recently, early detection of viral DNA by polymerase chain reaction (PCR) (47,48) as well as improved treatment with ganciclovir (given either prophylactically or pre-emptively) or α-CD20 antibodies, for CMV and EBV infections, respectively, have resulted in a significant decrease in morbidity and mortality due to these infections (46,49-51). The third phase is the late post-transplant phase (>100 days) in which VZV infections frequently occur (43), as well as late CMV infection which has recently been described as causing late morbidity and mortality (52).

In recent years, the human adenovirus (HAdV) has emerged as an important opportunistic pathogen in recipients of SCT, especially in pediatric recipients of a (M)MUD or MMFD donor (53-55). This virus is known to induce mild upper respiratory tract infections in immunocompetent individuals. However, in immunocompromised patients, HAdV infections are increasingly detected during the early phase after SCT and may cause severe and even lethal infections in viremic patients. HAdV infections, its detection, and HAdV-specific immune responses are the focus of this thesis.

3. HUMAN ADENOVIRUS (HADV)

3.1 Structure of the virus

In 1953, the virus was first identified by Rowes and colleagues who observed spontaneous degeneration of primary cell cultures derived from human adenoids (56). In 1956, Enders proposed to name the virus adenovirus, after the original tissue where the prototype strain was discovered (57).

The adenoviruses belong to the family of Adenoviridae, which is divided into four genera: two genera were found in mammals and birds only, the Mastadenoviruses and the Aviadenovirus, respectively, while the other two genera, Atadenovirus and Sadenovirus, have a broader range of hosts, including reptiles and ruminants. Mastadenoviruses contain

human, simian, murine and many other adenoviruses (58). Within each genus, viruses are grouped into species, which are named from the host and supplemented with letters from the alphabet. For human adenoviruses (HAdV), 51 serotypes have been described to date that can be distinguished on the basis of neutralization by antisera. These serotypes are grouped into 6 species (A-F) based on their ability to agglutinate red blood cells of different animals, the percentage of DNA homology and their oncogenic potential in rodents (59). Within species, DNA homology ranges from 48% for species A to 99% for species C, allowing intra-species recombinations, whereas homology between species is less than 20%. Human serotypes and species are shown in Table I (59,60).

Table I. HAdV species and serotypes[a]

Species	Serotypes	Hemagglutination type
A	12, 18, 31	IV (none)
B [b]	*3*, *7*, 11, 14, *16*, *21*, 34, 35, *50*	I (monkey)
C	1, 2, 5, 6	III (partial rat)
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	II (complete rat)
E	4	III
F	40, 41	III

[a] Adapted from refs (54,61,62).
[b] Serotypes in italics represent B1 serotypes, the other serotypes belong to the B2 species (54,63).

The virus is non-enveloped and contains double-stranded DNA. The virus particles are ~70-100 nm in diameter and have an icosahedral shape. The capsid, which is the protein shell surrounding the DNA, is composed of 252 subunits that consist of 240 hexon proteins and 12 penton proteins. The penton protein is formed by a penton base that resides in the capsid surface, and a fiber which projects outwards. This fiber is the first to make contact with a receptor present on a cell to ensue cell-entry by the virus. In total, 11 virion proteins have been discovered and numbered II, III, IIIa, IV, V, VI, VII, VIII, IX, mu and terminal protein (59). The capsid contains 7 of these proteins, of which the hexon protein is the most abundant and is composed of three tightly associated polypeptides II. The second most abundant protein is the penton that is built up of five units of polypeptide III, while the projecting fiber is composed of three polypeptides IV. The hexon is further supported by three proteins, VI, VIII and IX to form a stable structure, as well as IIIa which is found adjacent to the penton base (59). The other 4 proteins are found in the core of the virus together with the viral DNA. Three proteins are in contact with the viral DNA, V, VII and mu, of which VII is the major core protein and serves as a histone-like center for the DNA. Polypeptide V can also bind to the penton to stabilize the core and capsid. Finally, the

terminal protein is covalently attached to the ends of the viral DNA, and serves as a primer for DNA replication (59). A schematic representation of the virion is shown in Figure 3.

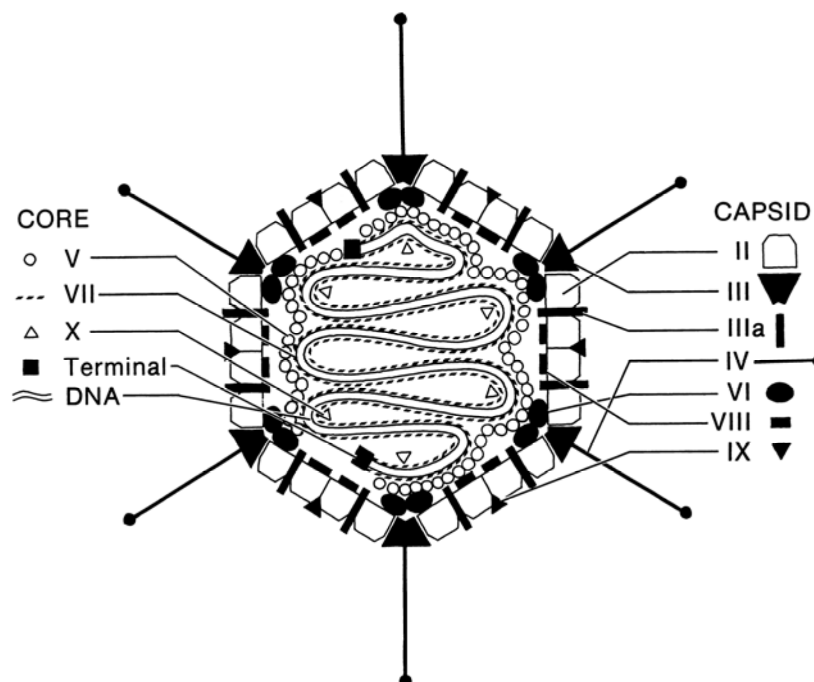

Figure 3. Representation of an HAdV virion. Viral constituents are designated by their polypeptide numbers with the exeption of the terminal protein (TP) *(Picture from ref (64), with permission).*

3.2 Adenoviral replicative cycle

HAdV requires infection of a host cell for its replication and production of progeny. The initial step is attachment of the knob of the trimeric fiber molecule to the receptor for HAdV. This receptor was discovered in 1997 and found to be shared with the Coxsackie virus; hence the receptor has been named the Coxsackie-Adenovirus receptor (CAR) as no other function of the receptor has been found (65,66). CAR belongs to the immunoglobulin superfamily and serves as a high-affinity receptor for HAdV from species A, C, D, E and F, but not species B (67). Recently, species B has been shown to bind and internalize via the CD46 molecule, which is a ubiquitously expressed complement regulatory protein (68-70). The distribution of CAR in rodents is more restricted to epithelial cells of several organs such as lung, liver, intestine, heart, pancreas and nervous system (71). In a study of human hematopoietic cells, CAR expression was only detectable on cells from myeloid origin (66), whereas CD46 is present on all hematopoietic cells. A second interaction between HAdV and the host cell, besides binding to CAR, is required for efficient entry, i.e. the binding of penton-base to integrins, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$, through an arginin-glycin-asparagin (RGD) motif in the penton-base (72,73). After adsorption, the virus is internalized by receptor-mediated endocytosis via clathrin-coated pits in endosomes (74). HAdV can escape the early endosome prior to the formation of a lysosome, which is induced by the low pH in the

endosome and most likely occurs via conformational changes in the penton-base. The virion is subsequently stripped from some of its proteins in a selective manner while the virion is transported via microtubules towards the nucleus (75). At the nuclear membrane the DNA is released from the remaining virion and introduced into the nucleus (76) where replication of the virus can occur.

This replicative cycle is by convention divided into 2 phases that are separated by the onset of viral DNA replication. The early phase contains adsorption, penetration and movement of viral DNA into the nucleus, and expression of an early set of genes. The function of these early genes is to facilitate DNA replication, to induce cell cycle progression, to block apoptosis and to antagonize the host immune response (see below) (59). In the human epithelial cell line HeLa, the early phase lasts about 5-6 h, whereas the cycle is complete after 20-24 h.

The viral chromosome carries 5 early transcription units (E1A, E1B, E2, E3 and E4), 2 delayed early units (IX and IVa2) and 1 late unit (major late), which is processed to generate 5 families of late mRNAs, L1-L5. The early gene E1A is the first transcription unit to be expressed, followed by E1B, E4 and E2, which sets the stage for DNA replication. All these mRNAs are transcribed by RNA polymerase II, whereas the 1 or 2 virus-associated genes VA (depending on the serotype) are transcribed by RNA polymerase III (59). After DNA replication is initiated, late genes begin to be expressed in a single large transcription unit. After transcription by RNA polymerase II, multiple mRNAs are formed by alternative splicing, a phenomenon that was first discovered in adenovirus (77). When late mRNAs are synthesized, cytoplasmic accumulation of cellular mRNA is blocked, thereby shutting off the production of cellular proteins. Trimeric hexon capsomeres are rapidly assembled from monomers, followed by a slower assembly of penton capsomeres consisting of pentameric penton base and trimeric fiber in the cytoplasm. After transport to the nucleus, an empty capsid is formed which is subsequently entered by a viral DNA molecule. Release from the cell is in part mediated by the E3 11.6-kD protein, also called the adenovirus death protein, as it is able to lyse cells by an unknown mechanism (78). In HeLa cells, one virus infecting a cell may produce a progeny of 10^4 viruses (59).

3.3 Immune evasion by HAdV

HAdV has developed multiple mechanisms to escape from the antiviral defense of the host. These mechanisms aim at protecting the infected cell from apoptosis induced by the infection itself, or from cellular immune responses, such as reduced recognition of the infected cell by $CD8^+$ T cells or reduced susceptibility of the infected cell to lysis induced by these cytotoxic T cells. These mechanisms are described below in more detail.

The E1A protein

The E1A protein has several functions in establishing the balance of survival or death of the infected cell. On one hand, it affords protection from the antiviral effects of IFN-α, IFN-β and IFN-γ by blocking the activation of interferon response genes, partly by binding to the transcription factor STAT1 and interfering with the formation of IFN specific transcription factors, IFN-stimulated gene factor 3 for type 1 IFNs and γ-stimulated factor for IFN-γ (79,80). Also, E1A downregulates the expression of the transforming growth factor β1 receptor on infected cells (81). On the other hand, E1A stabilizes the tumor suppressor protein p53, which could mediate cell cycle arrest or apoptosis upon DNA damage, which is unfavorable for survival of the virus (59).

The E1B-55kD and E1B-19kD proteins

As E1A induces the stabilization of p53, the virus has provided the proteins E1B-55kD and E1B-19kD to circumvent the subsequent intrinsic apoptosis. The 55kD protein forms a complex with p53 and thereby suppresses p53-mediated transcription (82). The E1B-19kD protein is a functional homolog of BCL-2 which can bind the pro-apoptotic members of the BCL-2 family such as BAX and BAK and prevent apoptosis through caspases (83,84).

The E3-gp19kD protein

The E3 transcription unit is responsible for maintaining cell viability by preventing cytolysis by $CD8^+$ T cells. For recognition of an infected cell by $CD8^+$ T cells, viral peptides need to be displayed on the surface in a complex with class I MHC molecules. E3-19kD is a transmembrane protein that resides in the endoplasmic reticulum where the luminal portion can bind the MHC class I α1 and α2 domains. The cytoplasmic tail of the protein contains an endoplasmic reticulum retention signal, which prevents the transport of the MHC molecules from the endoplasmic reticulum to the Golgi and the plasma membrane (85). As a consequence, MHC class I expression on the cell surface is greatly reduced. E3-19kD binds to different class I molecules with varying affinity (86). However, the protein is also involved in an alternative mechanism to delay overall class I presentation on the cell surface by binding to the TAP transporter, thereby preventing proper peptide-loading of MHC class I molecules (87). The E3-19kD gene is present in HAdV species B, C, D and E, but not in species A or F (88). Species A has developed an alternative mechanism of MHC class I reduction via the E1A protein, as has been shown for HAdV12 (89,90). The E1A protein of species A interferes with the transcription of class I genes via inhibition of precursors of NFκB, which in an active state binds to an element in the MHC class I promoter (91).

The E3-RID complex (10.4kD and 14.5kD)

When a T cell recognizes a viral peptide in the context of the right MHC molecule, this T cell can exert antiviral activity by inducing death signals to the virally infected cell. The E3-RID (receptor internalization and degradation) complex inhibits one pathway of cytolysis by targeting receptors of the TNF receptor superfamily that contain Death Domains, such as TNF-RI, FAS and TRAIL-RI (92). These receptors are internalized from the cell membrane by RID and subsequently degraded in lysosomes (93,94). The ligands for these receptors are expressed on activated cytotoxic T lymphocytes (CTLs); upon ligation and oligomerization of the receptors, the death-inducing signaling complex is formed which initiates cleavage of caspases to induce apoptosis. Reduction in expression levels of FAS has been shown to partially protect HAdV-infected lymphocytes from FAS-induced apoptosis (95).

It has been suggested that this resistance to lysis of HAdV-infected lymphocytes is a mechanism of viral persistence (93) since lymphocytes have been identified in the tonsils and adenoids as a source of adenoviral DNA (96). The question remains whether HAdV is capable of transforming into a true latent state in those infected lymphocytes as has been suggested by some authors (95,96), since no molecular mechanism of latency has been discovered. In EBV infection, latency is induced in infected B cells and results in expression of only a few early proteins to escape detection by the immune system of the host. For HAdV, evidence has been presented that HAdV viral DNA is present in lymphocytes of the tonsil and in the blood, but attempts to grow live virus out of these cells have failed (96,97). Nevertheless, even though the virus may not go into a latency state, it could be possible that some virus particles remain after most of the infection is cleared by the host’s immune response, as has been shown by shedding of the virus in feces for months after an infection (98). These viral particles might then cause a reactivation and severe infection when immunity is lacking in an immunocompromised host.

3.4 Immune response to HAdV

Upon HAdV infection, a variety of intracellular processes can be triggered in a target cell or resident macrophage that will lead to the production of cytokines and chemokines such as IFN-α, IL-6, IL-8, TNF-α and RANTES as has been observed both *in vivo* and *in vitro* (99,100). The early innate response that is initiated results in recruitment of effector cells, including neutrophils, monocytes/macrophages and NK cells, to the site of infection. These cells in turn will limit the infection directly by killing infected cells or indirectly by secreting antiviral cytokines (2). In addition, the recruitment and activation of antigen-presenting cells to the site of infection is essential for the development of an adaptive immune response. These adaptive responses have scarcely been studied in healthy donors experiencing an HAdV infection, simply because - due to the mild course - infection is often not diagnosed in

immune competent individuals. Adaptive immune responses in patients receiving HAdV-based vectors for gene therapy trials, on the other hand, have been studied extensively in order to improve vector design for prolonged transgene expression. Results from studies in both settings (natural infection versus exposure to gene therapy vectors) will be discussed in this section.

When B cells recognize a protein from HAdV and receive costimulatory signals from T cells, they will produce antibodies that can be divided into non-neutralizing antibodies (Ab) and neutralizing antibodies (NAb) (101). Non-neutralizing antibodies are not serotype specific and, therefore, bind to different serotypes. These antibodies were measured classically by the complement fixation test and more recently by ELISA in which the plate is coated with whole virus (irrespective of the serotype). NAb, on the other hand, are serotype-specific and induce neutralization predominantly via binding to epitopes on the hexon protein and terminal knob portion of the fiber protein. Hypervariable regions have been identified in the hexon protein that make up serotype-specific loops on the surface of the protein which are recognized by these NAb (102,103). To determine the titer of the serotype-specific NAb, a serum neutralization assay is performed in which addition of serum prevents infection of a permissive cell line by that specific serotype. The incidence and prevalence of several serotypes have been studied in healthy individuals by investigating the presence of NAb against those serotypes, indicative of exposure to that serotype in the past. NAb against HAdV1, 2, 3 and 5 are most common in the population; the majority of individuals (>85%) carry NAb against several of these serotypes when they reach adulthood. In an extensive epidemiological study, species A and C infections were mostly detected in infants, species B in infants and young children, while species D and E were mainly present in adults (104). A study on children with cystic fibrosis as well as healthy adult donors tested for NAb against HAdV1-7 showed the highest incidence (almost 100%) of HAdV3, followed by HAdV2, 1 and 5 (105). In more recent studies, NAb against HAdV5 have been shown to be present in 40-80% of healthy individuals (106-108). An exceptionally high risk for HAdV infections with serotypes HAdV4 and 7 is present in military recruits in the US army, due to the crowding and numerous stressors (109,110). A live attenuated vaccine containing HAdV4 and 7 has been applied from 1971 until 1997, when the manufacturer ceased production. Promptly, an increase in infections with HAdV4 and 7 has been reported in military recruits in the US (110).

The kinetics of humoral responses are mainly obtained from gene therapy trials, as the exact HAdV infection date in healthy persons is usually not known. In the latter case, a fourfold rise in Ab (either total Ab or NAb) in sera obtained several weeks apart is generally accepted as indicative of a recent exposure to HAdV. In one study on healthy children attending day care, HAdV infections as well as NAb were investigated and results showed that in 76% of HAdV-infected children a fourfold rise in NAb was observed (111). In gene therapy studies,

humoral responses are usually observed within 2-4 weeks after vector administration (112). However, variability between responses is observed, as delivery in the airway resulted in hardly any rise in titer, even following repetitive administration, whereas a single injection intradermally induced sustained responses (113). In this study, a striking positive correlation was found between the peak NAb titer that was evoked against the HAdV5 backbone of the vector and the level of pre-existing NAb against this serotype, i.e. high pre-existing titers resulted in the highest peaks after vector delivery.

At the initiation of the work described in the current PhD thesis, reports on cellular immune responses against HAdV upon natural infection have also been limited. Studies on HAdV-specific T cells in healthy individuals have been initiated with the aim to improve gene therapy strategies or alternatively, with the aim to eventually expand these T cells for adoptive immunotherapy in immunocompromised patients with severe HAdV infection. This strategy has already been pursued successfully for CMV and EBV infections (114,115). Proliferative responses by HAdV-specific $CD4^+$ T cells were observed in a few reports (106,116), whereas another report also showed lytic activity against HAdV-infected targets, which was probably due to $CD8^+$ T cells (117). After HAdV-vector delivery in gene therapy trials, proliferative responses have been observed against the vector itself (as well as against the transgene product) (112). In an additional report, the authors showed lysis of HAdV-infected cells by $CD8^+$ T cell lines obtained from patients after a single injection with replication-deficient HAdV5 (118).

However, all of these vector studies are based on $E1^-$ (with or without $E3^-$) deleted recombinant viruses in order to render them replication deficient, which might alter the immune response *in vivo* since these molecules are important for immune evasion properties of the virus as described above. In addition, many studies on gene transfer efficacy and host response to the viral vector have been performed in animal models, mainly mice and other rodents (119,120). However, these animals do not represent the natural host of the human AdV resulting in abortive HAdV replication upon infection of these animals. For example, since the full viral replicative cycle can not be completed in these animals, main immune targets for murine (C57B6) $CD8^+$ T cells are peptides located in the E1A or E2A proteins which do get expressed by infected cells (121-123). In contrast, the T cellular immune response in humans seems to be mainly directed against the capsid proteins as *de novo* gene expression is not required (117,118). These differences in results cast a doubt on the validity of using human AdV in mice as a model system for the interaction between HAdV and the human immune system.

3.5 Clinical symptoms, monitoring of HAdV infection and treatment

HAdV is endemic in the human population and is known to infect humans via the respiratory, the fecal-oral or ocular conjunctival routes. The illness is generally acute and

nonfatal in immune competent individuals, and recovery is associated with development of specific immunity. HAdV infections are associated with a wide spectrum of disease symptoms. Respiratory tract disease is the most frequently reported condition associated with HAdV infection, although the gastrointestinal tract, urinary tract, central nervous system and the ocular conjunctiva may also be affected in HAdV infection (reviewed in (54,61,62)). Young children attending day care centers have a high risk of acquiring HAdV infections because the virus can be shed from the feces for prolonged periods of time (98). Nevertheless, HAdV is not the etiological agent of the common cold, as it is only responsible for 5-10% of respiratory illness in children (55).
In immunocompromised patients, on the other hand, HAdV infections can progress towards severe disseminated disease, resulting in multi-organ failure and death. In these patients, mild symptoms include fever and diarrhea, which can progress to hemorrhagic cystitis, pneumonia and hepatitis and eventually death. Table II summarizes the main symptoms per HAdV species as well as the immune status of the infected person.

Table II. **Clinical symptoms associated with HAdV infection of the different species**[a]

Species	Symptoms	Immune status of patients
A	Respiratory syndrome, gastroenteritis	Competent and compromised
B1	Respiratory syndrome, pneumonia	Competent and compromised
B2	Acute hemorrhagic cystitis	Compromised
C	Respiratory syndrome, gastroenteritis, hepatitis	Competent and compromised
D	Epidemic keratoconjunctivitis	Competent[b]
E	Respiratory syndrome, conjunctivitis	Competent
F	Gastroenteritis	Competent

[a] Adapted from (54,61,62).
[b] Species D serotypes are also commonly found in patients with AIDS, which are immunocompromised (61,124).

Conventional tissue culture has for a long time been the most reliable diagnostic method for the detection of HAdV in clinical specimens. For definitive proof of HAdV as the cause of the infection, the presence of cytopathological effect (CPE) has to be combined with virus-specific immunostaining. Specimens that are usually investigated include throat swabs, fecal and urine samples. Reports using this technique have shown incidences of HAdV infection in SCT patients between 3-21% (53,125-129). When the infected patients are divided according to age, the infection frequency differs markedly. The incidence is 3-14% in adults, whereas it is 23-31% in children (53,130,131). Reported risk factors for HAdV infection include SCT performed with grafts from mismatched or unrelated donors, T cell depletion of the graft or ATG in the conditioning, and the occurrence of GvHD (131,132).

As the conventional culture technique is moderately sensitive, laborious and time-consuming, delivering results at ~2 weeks, alternative detection methods for earlier detection as well as increased sensitivity have been developed in recent years. PCR strategies have been described to detect the presence of HAdV viral DNA in plasma, leukocytes or feces, as has been done for CMV and EBV (46,47). Due to the genetic variability within the group of HAdV, several strategies have been developed to detect all HAdV serotypes using degenerate (133,134) or non-degenerate primer pairs (135-137). Other approaches use species-specific (138) or serotype-specific (139) primer combinations. A preliminary report showed a correlation between the presence of viral DNA in serum and subsequent death of HAdV-infected patients (140).
Several antiviral drugs for the treatment of HAdV infection have been applied, but results have not been unequivocally promising. Ribavirin is a synthetic guanosine analogue with *in vitro* inhibitory activity against a broad range of RNA and DNA viruses, including HAdV (141). Successful treatment with ribavirin has been described (142), but failures have also been reported (130,143-145). Cidofovir, a nucleotide homologue with potent reactivity against several DNA viruses, has also been applied in HAdV infection with varying success (132,146,147). However, treatment was usually initiated solely on the basis of HAdV infection with or without clinical symptoms, whereas data on the course of viral load in plasma (viremia) or immune recovery were lacking in these studies. Therefore, firm conclusions on the effectiveness of these antiviral drugs could not be drawn. These results as well as the shortcoming of contemporary antiviral drugs to combat HAdV are a stimulation to investigate the natural defense processes, e.g. neutralizing antibodies or T cell responses to HAdV, in order to explore the use of these natural tools to control the dissemination and fatality of HAdV in SCT recipients.

4 SCOPE OF THE THESIS

HAdV infection in pediatric SCT recipients is the main focus of this thesis. HAdV has become increasingly apparent as a severe infectious pathogen following SCT, with high morbidity and mortality in recent years. Nevertheless, at the initiation of this study only limited data on the optimal detection of the virus, the immune response that is invoked by HAdV, as well as successful treatment strategies using antiviral drugs or alternative treatment such as adoptive immunotherapy were available.
Therefore, a semi-quantitative PCR has been developed based on a primer set published by Echavarria *et al.* (136) in order to quantify the viral DNA load that is present in patients with HAdV infection. In a retrospective cohort of pediatric SCT recipients, we were able to correlate the presence of high DNA loads in serum of HAdV-infected patients with a fatal outcome (**Chapter 2**). In collaboration with the Department of Medical Microbiology of the LUMC, this PCR strategy has been optimized resulting in a real-time quantitative PCR (RQ-

PCR) in which viral loads were quantified with a quantitated HAdV5 stock. In a prospective study, pediatric patients receiving SCT have been monitored for HAdV infection on a weekly basis both by conventional culture of feces, throat swabs and urine samples as well as by RQ-PCR on plasma samples. Risk factors for HAdV infection and HAdV viremia, i.e. a sign of dissemination of the infection, were determined as well as the correlation between these parameters and immune reconstitution (**Chapter 3**). Detection of viral DNA in plasma as an early sign of dissemination permits early initiation of treatment. The efficacy of ribavirin treatment initiated upon two consecutive positive RQ-PCR signals above 1000 copies/mL in the absence of immune recovery has been described in **Chapter 4**.

Immune responses against HAdV have been extensively studied in patients as well as healthy donors. In patients surviving HAdV infection, NAb and HAdV-specific T cell responses were investigated (**Chapter 3**). Adoptive immunotherapy might be a promising therapy for patients with HAdV infection without immune recovery, who are at high risk of a fatal course of the infection. In healthy donors, a protocol to culture and expand HAdV-specific $CD4^+$ T cells with inactivated HAdV5 was developed and resulting T cell lines and clones were investigated for cross-reactivity against different species and serotypes (**Chapter 5**). To further dissect the immune response against HAdV in healthy donors, several recombinant proteins of HAdV5 were generated and tested as targets for recognition by T cells. The hexon protein was most often recognized and synthesized in overlapping peptides in order to select a peptide pool which is recognized by the majority of healthy individuals (**Chapter 6**). Finally, as the majority of HAdV-specific T cells were $CD4^+$, the antiviral effect of HAdV-specific $CD4^+$ T cell clones on infected target cells was investigated (**Chapter 7**).

The results described in this thesis indicate that adoptive immunotherapy with HAdV-specific $CD4^+$ T cells, generated using a set of 5 hexon peptides, is feasible and should be considered for patients with HAdV viremia without immune reconstitution.

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Chapter 2

High levels of adenovirus DNA in serum correlate with fatal outcome of adenovirus infection in children after allogeneic stem cell transplantation

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ABSTRACT

An increasing incidence of adenovirus (HAdV) infection leading to mortality in children after allogeneic stem cell transplantation has called for new ways to monitor HAdV infection. In this retrospective study, levels of HAdV DNA in serum from 36 HAdV feces culture positive transplant recipients were measured by PCR in a semi-quantitative way by making dilutions of the DNA template. Six out of 7 (86%) children with a fatal HAdV related outcome versus only 2 out of 29 (7%) of the remaining patients, had high levels of HAdV DNA (≥ 100-fold) in their sera ($p < 0.0001$). High serum levels of HAdV DNA were reached 18 days before death (range: 6-29 days). These results indicate that quantification of adenoviral DNA in serum may prove to be a valuable tool in clinical practice to diagnose and monitor HAdV infection and disease in these immunocompromised children.

INTRODUCTION

During the first months after transplantation, recipients of allogeneic stem cells are severely immunocompromised and, as a consequence, susceptible to viral infections and reactivations. Since about 1995 the frequency of adenovirus (HAdV) infections in pediatric stem cell recipients has increased remarkably (1-12) (van Tol *et al.*, manuscript in preparation) leading to clinical manifestations like hemorrhagic cystitis, enteritis, hepatitis, encephalitis, pneumonitis and multi-organ failure (1-3,5,7,11,13,14). Disseminated infections frequently result in fatal outcome. In contrast, adenoviral infections in healthy children are usually not associated with serious clinical symptoms, indicating that effective immune responses to HAdV in humans contain the infection. During the first years of life, children develop neutralizing antibodies and T cell responses against various strains of HAdV (14,15). As a result, most adults have strong preexisting immunity to HAdV, which is a complicating factor in the use of adenoviral vectors for gene therapy (16).

At present, 51 serotypes of HAdV able to infect human cells have been identified that are distinguished from each other on the basis of antigenic determinants recognized by neutralizing antibodies. The different serotypes are grouped into six species, A to F, according to their ability to agglutinate red blood cells as well as to their DNA homology (17,18). At the DNA level, homology within a species varies from 50% to almost 100%. Even strains belonging to the same serotype can still differ with respect to their DNA sequence (18). Between species the homology can be as little as 4% (18). Clinically, HAdV infections are detected and diagnosed using various techniques (14). Culture of virus and subsequent identification using immunofluorescence with HAdV-specific antibodies is the most common approach but takes several days depending on the viral load of the sample. Alternatives are direct immunofluorescence or detection of viral antigen by agglutination of antibody-coated latex beads, dot blot hybridization, and more recently detection of viral

DNA by PCR. Several PCR based strategies have been developed. Due to the genetic variability within the group of human HAdV, the choice of primers varies with the application. Some of the techniques detect HAdV strains belonging to almost all serotypes using degenerate (19-22) or non-degenerate (20,23-27) primer pairs. Sequences conserved in most of the 51 serotypes can be found in the hexon gene (19,20,22,26,27), E1A (20) and VA-RNA (21). Analysis of the PCR products by restriction enzyme analysis or sequencing can supply sufficient information to assign a species or serotype to an unknown isolate (19,21,22,27-30). Other approaches employ species (28,31) or serotype-specific (32) primer combinations.

In the present study we have performed a retrospective analysis of 36 pediatric patients with adenoviral infection after allogeneic stem cell transplantation. A generic PCR amplifying a conserved part of the HAdV genome, allowing detection of all disease causing HAdV strains was employed (25,33) in a qualitative as well as in a semi-quantitative fashion. The presence of adenoviral DNA in serum as well as its level were determined at different time points after transplantation. Because high levels of HAdV DNA correlated with fatal outcome, quantification of the HAdV DNA load in serum may be a valuable tool to diagnose the dissemination of HAdV infection in immunocompromised patients.

MATERIALS & METHODS

Patients

Thirty-six patients from a cohort of 328 stem cell transplant recipients treated at the pediatric transplantation unit of the Leiden University Medical Center between 1985 and 1998 had at least one HAdV positive feces culture. Feces were tested by culture on Hep-2 cells followed by immunofluorescence (Imagen HAdV, DAKO Diagnostics, Ely, UK). The 36 patients were classified into 3 groups based on clinical symptoms (Table 1). Group I consisted of 17 patients who did not show any clinical symptoms of HAdV infection. Group II consisted of 12 patients who developed local clinical symptoms that were most likely associated with HAdV infection. The localized clinical symptoms were enteritis (n=11), hemorrhagic cystitis (n=2) and hepatitis (n=1). Group III consisted of 7 patients who died of fatal disseminated disease caused by HAdV. Clinical symptoms in group III were enteritis (n=5), hemorrhagic cystitis (n=1), hepatitis (n=3), encephalitis (n=1) and pneumonia (n=1) and HAdV was cultured from multiple sites confirming dissemination. In 5 of the 7 cases dissemination was also confirmed after autopsy. Eight of the 36 HAdV isolates were not serotyped (due to the lack of available antisera or lack of original isolate). The remaining 28 were serotyped using standard techniques; 19 (68%) belonged to species C (HAdV1, 2, 5 and 6), 5 (18%) belonged to species A (HAdV12, 31), 2 (7%) belonged to species B (HAdV7) and 2 (7%) to F (HAdV41). No significant differences in serotypes were observed between patients with high viral load compared to those with low viral load. Patients were transplanted for immunodeficiencies, hemopoietic defects and leukemia (Familial Hemophagocytic Lymphohistiocytosis, Severe Combined Immunodeficiency, Wiskott-Aldrich Syndrome, Metachromatic Leukodystrophy, β-Thalasemia, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Myelodysplastic Syndrome, Fanconi, Severe Aplastic Anemia). The sources of stem cells were HLA-identical matched family donors (MFD), mismatched family donors (MMFD) and matched unrelated donors (MUD). T cell depletion, if necessary, was performed by sheep erythrocyte rosetting and albumin gradient centrifugation, immuno-rosetting using anti-CD2 and anti-CD3 (sometimes combined with anti-CD19 and anti-CD22) monoclonal antibodies (34) (< 3-log T cell depletion), Campath-1G "in the bag" or $CD34^+$ precursor cell enrichment on a CliniMACS (≥ 3-log T cell depletion). Of the 36 recipients, only 2 patients

developed a graft-versus-host disease (GvHD) grade ≥ II. For comparison, serum samples from 17 healthy stem cell donors, 10 children and 7 adults, were screened as well.

Table 1. Characteristics of adenovirus infected patients.

	Patient group		
Variable	I (n = 17)	II (n = 12)	III (n =)7
Median age in years (min-max)	3.7 (0.6-15.8)	7.1 (0.9-17.0)	4.4 (0.6-11.6)
Donor type (n)			
Matched Family Donor	4	2	-
Mismatched Family Donor	6	3	5
(Matched) Unrelated Donor	7	7	2
T cell depletion			
No depletion	9	7	3
< 3 log[a]	4	3	-
≥ 3 log[b]	4	2	4
AGvHD[c](n)			
0	14	7	5
I	3	4	1
II-IV	-	1	1
Adenovirus related parameters			
Feces culture	+	+	+
Clinical symptoms	-	+	+
Death	-	-	+

[a] E-Rosetting plus albumin gradient centrifugation or immuno-rosetting using anti-CD2 and anti-CD3 (sometimes including anti-CD19 and anti-CD22) monoclonal antibodies (34).
[b] Campath-1G “in the bag” or enrichment of $CD34^+$ precursor cells on a CliniMACS.
[c] Acute Graft-versus-Host Disease (39).

Isolation of DNA
Serum samples had been stored at -20°C during a period of 1 to 14 years. DNA was isolated from 200 µl of serum using QIAamp columns (QIAamp DNA blood mini kit, Qiagen, Hilden, Germany) according to the manufacturer’s directions. Negative controls (water) were included in each run of extractions. DNA was eluted in 200 µl water and stored at –20°C until further use.

PCR
Twenty µl of template DNA was added to a final volume of 50 µl containing 1x Taq PCR Master Mix (Qiagen, Hilden, Germany) and 0.2 µM of the primers Hex3: GACATGACTTTCGAGGTCGA-TCCCATGGA and Hex4: CCGGCTGAGAAGGGTGTGCGCAGGTA (23,25). The primer pair amplified DNA from HAdV strains belonging to the serotypes 1, 2, 3, 4, 5, 7, 7A, 8, 11, 12, 16, 19, 30, 34, 35, 37, 48, 49 (25), 31, 41 (this study) and 6, 9, 40, 50 and 51 (unpublished data).
PCR amplification was carried out on a Perkin Elmer 2400 thermocycler. An initial denaturation of 3 min at 94°C was followed by 40 cycles of denaturation of 15 sec 94°C, annealing of 15 sec at 52°C and elongation of 30 sec at 72°C. Finally, a 7 min elongation step completed the program. To obtain

a semi-quantitative measure of the load of HAdV DNA in serum, the extracted template DNA was diluted (10-, 100- and 1000-fold) in a 10mM Tris solution prior to PCR analysis. The highest dilution still yielding a PCR product was considered to be the load of DNA in the serum sample.

Detection and identification of PCR products
PCR products were detected by electrophoresis of 10 µl of the amplified product on a 1.5% agarose gel containing ethidiumbromide. A molecular size marker (SmartladderSF, Eurogentec, Seraing, Belgium) and an aliquot of the HAdV positive PCR control were mounted on the gel as well. UV light visualized a positive PCR as a 139 bp amplified product. In addition, the results were confirmed by an EIA hybridization using a digoxigenin labeled probe (Hex-30, GACCCCACCCTTCTTT-ATGTTCTGT) (25) detected with anti-digoxigenin antibody labeled with horse radish peroxidase.

RESULTS

Qualitative analysis of sera for the presence of adenoviral DNA

Sera from all 36 patients were analyzed for the presence of HAdV DNA by PCR. Serum samples, as far as available due to the retrospective character of this study, were taken from time points just before transplantation (if available), around the first positive feces (or urine, throat or sputum) culture and just before HAdV-related disease or death.

A total of 156 samples was investigated (group I: 62 samples, group II: 45 samples and group III: 49 samples) by PCR. Forty-two of these 156 serum samples were HAdV positive by PCR. The 42 positive serum samples were from 17 patients: 7 of 17 (41%) patients of group I, i.e. asymptomatic patients, 4 of 12 (33%) patients of group II with localized disease and 6 of 7 (86%) patients of group III with fatal disseminated disease. This indicates that the presence of DNA did not correlate with the presence of clinical symptoms. However, it did correlate with fatal outcome, when compared with all 29 non-fatal cases, but the statistical significance was low ($P = 0.04$, Fisher exact) due to the occurrence of DNA in serum of 11 out of 29 patients with a non-fatal course.

Semi-quantitative determination of viral DNA load

To investigate whether a quantitative measure of the DNA load in serum would be more informative about the course of an HAdV infection in these patients than the qualitative detection of DNA, a semi-quantitative PCR was performed by analyzing serial dilutions of serum template DNA. In the serum of 7 (of 17) asymptomatic patients of group I and of 4 (of 12) patients with localized disease of group II, HAdV DNA was detected (Figure 1). In 9 of these 11 patients the level of HAdV DNA was low. The two remaining patients (numbers 168 and 286) demonstrated transiently high loads of HAdV DNA, i.e. a PCR product still detectable after 1000-fold dilution of template DNA. In contrast, the HAdV DNA load was high (100- or 1000-fold dilution) in the sera of 6 of the 7 patients with disseminated infection and fatal disease (group III). These high loads of HAdV DNA were first detected 18 days (range between 6 and 29 days) before death, remained high until death and were always accompanied and sometimes preceded by positive feces cultures.

Figure 1. Results of the semi-quantitative HAdV PCR analysis. Pediatric SCT recipients are grouped according to the absence or presence of clinical symptoms (Group I-III). Culture results on feces, throat and urine samples (if performed), HAdV PCR results on serum samples and mortality (HAdV related or otherwise) are indicated in relation to the time of (first) SCT (dotted vertical line). Data in the first column (-30/-7) represent data obtained between 30 and 7 days prior to SCT. The different symbols for PCR results represent the highest dilution of template DNA (up to 1000-fold) after which a positive result was still obtained. All feces cultures are shown, but only positive urine and throat cultures are indicated. UPN = Unique Patient Number.

The only patient with fatal outcome who remained PCR negative had serum samples obtained until 3 weeks before death. No further serum samples were available.
In general, there was a poor correlation between HAdV DNA positivity in serum and HAdV positive feces cultures. For example, no HAdV DNA was detected in the sera of some patients from groups I and II despite prolonged periods of HAdV positivity in feces.
Although a transient presence of HAdV DNA can occur in patients without clinical symptoms of HAdV infection, a persistently high level of HAdV DNA correlated with fatal outcome. A high level of HAdV DNA in serum (100-1000 fold dilution) was observed in 6 of 7 fatal cases and in only 2 of 29 non-fatal cases (P <0.0001; Fisher exact, Table 2).

Table 2. Levels of HAdV DNA in serum from HAdV-infected stem cell recipients, as determined by semi-quantitative PCR.

		PCR result				
			Positive, according to dilution[a]			
Group	n	Negative	None	10-fold	100-fold	1000-fold
I	17	10	3	2	0	2
II	12	8	1	3	0	0
III	7	1	0	0	2	4
Relative virus load		Negative	Low	Low	High	High

[a] The highest dilution of template DNA at which a specific PCR product was obtained is indicated ($P < 0.0001$, for groups I and II versus III, threshold between 10- and 100-fold dilution).

HAdV DNA in healthy subjects

To determine whether HAdV DNA is detectable in serum of healthy individuals, serum samples from 17 stem cell donors were tested by PCR for the presence of HAdV DNA. This group consisted of 10 children (between 3 and 13 years old) and 7 adults. DNA template was analyzed by PCR, either undiluted or after 10-fold dilution. All but one of the tested stem cell donors were negative when undiluted samples were tested. The positive sample belonged to a pediatric case (13 years old) and this was positive, but only undiluted. Thus HAdV DNA may be detected occasionally in serum of healthy subjects, but only at very low levels.

DISCUSSION

The data presented indicate that a high level of HAdV DNA (i.e. a PCR-product after 100-fold or more dilution of template DNA) in sera of pediatric stem cell transplant recipients infected with HAdV after transplantation correlates with fatal outcome due to disseminated HAdV disease. In 86% of the patients who developed fatal disease, a high HAdV DNA load was measured, whereas only 7% of the patients without fatal outcome had similar high loads

of HAdV DNA ($P < 0.0001$). It has been recently reported that the detection of HAdV DNA in serum by PCR was associated with fatal outcome (33). However, in the present extended retrospective analysis HAdV DNA was also detected in a significant proportion of patients without fatal outcome albeit at low levels. Thus, quantitative determination of HAdV DNA in serum contributes significantly to the identification of patients with disseminated fatal HAdV infection.

Only two patients without HAdV related clinical symptoms (numbers 168 and 286) had high levels of HAdV DNA in their sera although only transiently. Patient UPN 168 was a SCID patient who was infected with HAdV serotype 31. Prior to transplantation, cultures from feces and urine were already HAdV positive and a high load of HAdV DNA was detected in serum. After transplantation, the DNA load in serum remained high until day 20 and then declined to undetectable levels at day 34. Feces cultures became also negative two months after transplantation. The other patient (UPN 286) with a high level of HAdV DNA in serum was transplanted for acute lymphoblastic leukemia. Feces was negative before SCT but became HAdV positive at day 6, which was typed as serotype 2. At day 21 post-transplantation serum was strongly PCR positive (1000-fold dilution). Serum samples from day 35 and 42 were positive but lower (10- and 100-fold dilution, respectively) indicating a decline in load. At day 70 no HAdV DNA was detected any longer in serum despite the continued presence of HAdV in feces. The patient died at day 107 post SCT due to a relapse of the leukemia. The reason why both patients transiently had high loads of HAdV DNA in their serum, without any accompanying clinical symptoms, is unclear at present. Interestingly, it appears that some of the patients who carried high loads of HAdV DNA in their serum were able to resolve this situation, in the absence of any anti-HAdV pharmacological treatment.

The group of 36 patients whose serum was analyzed by PCR was originally identified by the presence of HAdV in feces cultures. Positive cultures of throat swabs, in the absence of positive feces cultures, were observed in another four cases in the period between 1985 and 1999, but these cases were all asymptomatic (data not shown). Although it is difficult to be certain in this retrospective study, screening of feces therefore appears to include all patients who may develop HAdV related clinical symptoms. However, not all patients with a positive feces culture developed symptoms or disease and therefore there is a need for an additional tool to differentiate between transient asymptomatic infections and disease caused by HAdV. The results of this study demonstrate that semi-quantitative data on the HAdV load obtained from serum provide clinically relevant information in relation to the outcome of HAdV infection, especially to fatal outcome. The less severe clinical symptoms in group II did not lead to increased DNA loads in serum. However, this could be due to the lack of appropriate serum samples in this retrospective analysis. More precise quantitative data from frequent

serum samples in a prospective setting will demonstrate whether serum load of HAdV DNA can provide information that is relevant to predict the course of infection more accurately.
Because quantitative data on HAdV DNA load in serum appear to be important, we have started to develop a real-time PCR approach to detect HAdV DNA in an automated and more quantitative fashion (35). Detection of viral infections by PCR also has several other advantages. Results are obtained faster than with conventional culture techniques. Cultures may require 3-4 days until results are known, while PCR detection can be performed in 1 day. Eventually, multiplex PCR reactions may lead to simultaneous detection of multiple viruses in one reaction tube.
In this study a generic PCR amplifying a conserved region of the hexon gene was used (23,25,33). The advantage of this strategy is that all clinically relevant HAdV strains are detected. An additional possibility might be, that the availability of a segment of HAdV DNA amplified from a clinical sample provides the opportunity to type the infecting virus by sequencing the PCR product without being dependent on time-consuming virus neutralization tests. To investigate whether virus strains could be typed based on this limited sequence information, PCR products of a number of serotyped clinical isolates were sequenced. Comparison of the sequences from the PCR fragments with published sequences revealed that sequence analysis, in this limited number of cases, could provide sufficient information to assign an unknown strain to a certain species of human HAdV, but not to a specific serotype (data not shown). This single primer pair, therefore, seems suitable for detection and, potentially, for partial typing of all clinically relevant HAdV strains.
Although the diagnosis of HAdV infections may be improved by a quantitative PCR approach, treatment options to reduce the number of fatal cases are still very limited. Ribavirin and cidofovir have been used in recent years with variable success (9,10,36-38). No systematic study on the effectiveness of these drugs has been published. Pre-emptive treatment based on an increase in HAdV DNA load in serum, followed by careful quantitative monitoring of DNA loads in patients with adenoviral infection could provide objective information on the antiviral efficacy of these drugs in immunocompromised patients. The time period between high-level HAdV DNA detection and death should be long enough to install treatment at a stage when the disease has not yet progressed to a terminal course. In this retrospective study, this time period varied between 6 and 29 days. It is possible that more frequent sampling may have an additional benefit of detecting HAdV DNA in blood earlier for the rapid installation of treatment. Since quantification of HAdV DNA load during infection in this patient group appears to be a valuable tool both in diagnosis and in clinical management of this condition we have initiated a prospective study to address these issues more systematically.

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Chapter 3

Immune reconstitution and clearance of human adenovirus viremia in pediatric stem cell recipients

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ABSTRACT

Adenovirus (HAdV) infections are increasingly frequent complications after allogeneic stem cell transplantation (SCT), especially in children. Only few data are available on the correlation between immune recovery and the course of HAdV infection, and data on HAdV-specific responses are lacking. In a prospective study, we determined the correlation between the load of HAdV DNA in plasma and lymphocyte reconstitution in 48 children after allogeneic SCT. Additionally, HAdV-specific humoral and cellular immune responses were investigated. HAdV infection occurred in 21 patients (44%) and in 6 of these patients the infection progressed to viremia as demonstrated by the presence of HAdV DNA in plasma. Low lymphocyte counts at the onset of infection were predictive for HAdV viremia. Survival of patients with viremia was associated with a rise in lymphocyte counts during the first weeks after infection. In these patients, HAdV-specific $CD4^+$ T cell responses as well as rises in neutralizing antibody titers were detected following clearance of the viral DNA from plasma. Lymphocyte reconstitution appears to play a crucial role in clearance and survival of HAdV viremia, warranting further development of therapeutic interventions aimed at improving immune recovery.

INTRODUCTION

In recent years, adenovirus (HAdV) infections are observed with increasing frequency after allogeneic SCT, especially in young children, resulting in high mortality rates when the virus disseminates (1-8). Currently, 51 serotypes of adenovirus have been identified, classified in six species (A-F) (9,10). Species A, B and C serotypes are most frequently isolated from pediatric immunocompromised hosts and are the major cause of HAdV-related disease (1,9,11). Unfortunately, HAdV infections are usually not recognized until a late stage of disease is reached, due to non-specific clinical symptoms of infection in an immunocompromised host. Diagnostic virological culture techniques are time consuming and moderately sensitive for progression to viremia (12,13). With the development of real-time quantitative PCR (RQ-PCR) techniques, patients can be diagnosed at an early phase of viremia and the course of infection can be monitored by the quantification of the HAdV DNA load in plasma (14-17).

Immunological recovery after SCT is of great importance to clear virus infections, as has been shown for other viral reactivations such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (18-24). So far, only few data are available on the correlation between the recovery of lymphocytes and HAdV infection (25) and no data have been published on HAdV-specific cellular and humoral immune reconstitution in relation to the course of HAdV infection in SCT recipients. Better understanding of the role of immune recovery in

adenovirus infections might be relevant to improve therapeutic options as medication with ribavirin or cidofovir is not unequivocally effective (26-32).

We conducted a prospective study in which all pediatric allogeneic SCT recipients transplanted between 2001 and 2003 were monitored weekly for HAdV infection by culture of feces, throat swabs and urine samples as well as by RQ-PCR of plasma samples. Lymphocyte recovery as well as HAdV-specific T and B cell responses were investigated in HAdV-infected patients at the onset and during the course of the infection.

PATIENTS & METHODS

Patient cohort

In 2001 and 2002, 53 pediatric patients received an allogeneic SCT in the pediatric bone marrow transplant unit of the Leiden University Medical Center. The study has been approved by the local Institutional Review Board and all patients were included in the study following informed consent. Five patients died within 2 months after SCT due to transplant related mortality or relapse. Characteristics, donor types and graft manipulations of 48 remaining patients are summarized in Table 1. Pre-treatment of the graft recipient was according to disease-specific protocols of the relevant working parties of the European Group for Blood and Marrow Transplantation (EBMT).

Rabbit-ATG (IMTIX, 10 mg/kg in 4 days) or Campath-1H (1 mg/kg in 5 days) was given shortly before the transplant date in all HLA non-identical SCT. GvHD prophylaxis consisted of cyclosporin A (CsA, trough level 100-200 μg/L). All patients were kept in protective isolation for at least 4 weeks after SCT, and received total gut decontamination. Neither systemic antibacterial nor antiviral prophylaxis was given; pre-emptive treatment was administered for proven (i.e. RQ-PCR-based) CMV (ganciclovir) and EBV reactivation (rituximab® administration for depletion of B cells to prevent post transplant lymphoproliferative disease). For HAdV viremia, ribavirin (60 mg/kg/day, starting dose 30 mg/kg) or cidofovir (5 mg/kg/week) was given (32). Intravenous immunoglobulin (IVIG) substitution was given until 3-6 months after SCT to patients with grafts from unrelated donors.

Virological monitoring

Patients were screened for 6 months after SCT, with weekly sampling during the first 8-12 weeks and every 2-4 weeks thereafter. In this cohort, retransplantation was considered (n=3) as endpoint for follow-up. Feces, urine and throat swab samples were inoculated on human A549 cells and scored for cytopathological effect (CPE). Plasma samples were tested by RQ-PCR for the presence of adenoviral DNA. DNA was extracted from plasma by standard procedures and 10 μL of DNA containing extract was amplified by real-time PCR using the oligonucleotide primers previously described (33). The amplification protocol was 15 min at 95 °C followed by 50 cycles of 95°C, 55°C and 72°C (each 30 sec). To detect these amplified products, a molecular beacon (GAGCCCACCCTTCTTTATGT) with a 5' FAM label and 3' dabcyl label was applied (Biolego, Malden, the Netherlands) using an iCycler IQ system (Biorad laboratories, Hercules, CA). Sensitivity of the assay was 50-250 copies/mL (Claas, submitted). A quantitated HAdV5 stock was a kind gift of M. Havenga (Crucell, the Netherlands).

HAdV infection was defined as ≥ 2 consecutive positive viral cultures. Likewise, HAdV viremia was defined as the presence of >1000 copies/mL HAdV DNA in ≥ 2 consecutive plasma samples. Serotyping of HAdV isolates was performed by virus neutralization using a panel of serotype-specific antisera (RIVM, Bilthoven, the Netherlands).

Viruses

HAdV serotypes 1, 2, 5 and 11 (RIVM) were grown on human Hep2 cells. 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. Purified HAdV serotypes 6 and 31 were a kind gift of M. Havenga. For the antibody neutralization assay, crude lysates of HAdV-infected cells (Hep2 or A549 cells) were generated as above. The 50% tissue culture infective dose (TCID50) of each lysate

was determined by diluting the lysate in tenfold followed by addition of Hep2 or A549 cells. After 7 days, the TCID50 was calculated as the dilution at which 50% of 10 wells showed CPE.

Table 1. Characteristics of all patients and those with HAdV infection and viremia

	Total (n = 48)	HAdV infection no (n=27) / yes (n=21)	HAdV viremia no (n=12) / yes (n=6 [a])
Male	36	22 / 14	10 / 1
Female	12	5 / 7	2 / 5
Age median	7.8	8.5 / 7.6	7.5 / 7.7
(range)	(0.2-17.3)	(0.2-17.3) / (1.4-14.7)	(2.7-14.7) / (1.4-13.3)
Diagnosis:			
Hematol malignancies	25	15 / 10	3 / 4
AL[b]	14	9 / 5	2 / 2
MDS[c]	5	2 / 3	1 / 2
other	6	4 / 2	0 / 0
Hematol disorders	13	7 / 6	5 / 1
Inborn errors	10	5 / 5	4 / 1
Donor type:			
MFD[d]	17	11 / 6	5 / 0
MMFD	7	4 / 3	2 / 0
MUD	24	12 / 12	5 / 6
HLA-matching:			
10/10 match	33	22 / 11	7 / 2
≤ 9/10 match	15	5 / 10	5 / 4
Graft:			
BM unmanipulated[e]	32	18 / 14	9 / 4
BM T cell depleted[f]	7	5 / 2	0 / 1
PBSC[g]	9	4 / 5	3 / 1
Conditioning host:			
Myeloablative[h]	40	21 / 19	11 / 5
Reduced intensity	8	6 / 2	1 / 1
Immunosuppression:			
no ATG/Campath	13	11 / 2	2 / 0
ATG/Campath	35	16 / 19	10 / 6

[a] 3 patients excluded due to loss to follow-up shortly after culture positivity.
[b] AL acute leukemia : ALL and AML.
[c] MDS: myelodysplastic syndrome including JMML.
[d] MFD: matched family donor, MMFD: mismatched family donor, MUD: matched unrelated donor.
[e] bone marrow unmanipulated including 1 cord blood.
[f] immunorosetting T ± B cell depletion or Campath in the bag.
[g] peripheral blood stem cells $CD34^+$ selected (CliniMACS).
[h] either TBI ≥7 Gray or ≥16 mg/kg busulfan or ≥140 mg/kg melphalan.

Immunological reconstitution
Leukocyte and lymphocyte counts were determined every one to three days during the first 2 months after SCT. Subset analysis of peripheral blood mononuclear cells (PBMC), i.e. $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells, was performed every 4-8 weeks when sufficient lymphocytes were present (usually from 4-8 weeks after SCT onwards) using flow cytometry.

HAdV-specific antibodies
To determine neutralizing antibody (NAb) titers, sera were heat inactivated at 56°C for 30 min and diluted in twofold from 1:4 to 1:2048 in a 96-well plate in duplicate. HAdV lysate was diluted to 100 × TCID50 and added to each well, followed by 1 h incubation at 37°C. Hep2 or A549 cells were added and wells were scored for CPE after 7 days of culture. The neutralizing titer was the highest serum dilution at which CPE was no longer observed. Rises in titers (>4-fold increase) against the serotype that infected the patient while NAb against other serotypes remained stable were considered specific responses.

HAdV-specific proliferation
Autologous irradiated stimulator PBMC (30 Gray) were infected at a multiplicity of infection (MOI) of 100 with the purified HAdV serotype infecting the patient. In uninfected patients, infection was performed with the common serotype HAdV5, as HAdV-specific T cells are usually cross-reactive (34). Infection was performed in RPMI/0.5% BSA (Sigma-Aldrich, St. Louis, MO) at 10^7 cells/mL and after 1 h PBMC concentration was adjusted to 10^6 cells/mL with RPMI/10% human AB serum. Stimulator cells (1×10^5, infected or uninfected as control) were co-cultured with 1×10^5 responder PBMC per well in a 96-well round-bottom plate for 4 days, after which [^{3}H] thymidine (0.5 μCi/well, Amersham International, Amersham, UK) was added for 16 h. A stimulation index (SI) >3 was considered a specific response. As a control, PBMC (4×10^4/well) were stimulated for 4 days in a 96-well flat-bottom plate with α-CD3 antibodies (Janssen-Cilag BV, the Netherlands), α-CD3 + IL-2 (50 IU/mL, Chiron, Emeryville, CA) or 5 μg/mL phytohemagglutinin (PHA, Murex Biotech Ltd., Dartford, UK).

HAdV-specific cytokine production
For IFN-γ ELIspot, PBMC (2×10^5/well) in RPMI/10% human AB serum were stimulated with the HAdV serotype infecting the patient at MOI 10 in a 96-well round-bottom plate, or unstimulated as control. After 4 days, IFN-γ ELIspot was performed according to instructions of the manufacturer (Mabtech, Nacka, Sweden) (35). More than 25 specific spots (spots $_{\text{HAdV}}$ – spots $_{\text{control}}$) were considered a specific response.
For intracellular cytokine staining, cells were incubated with Brefeldin A (BFA, 5 μg/mL, Sigma-Aldrich) for 5 h at day 5, and stained for IFN-γ or TNF-α as described previously (36). Antibodies were: αCD3-PerCP-Cy5.5, αCD4-FITC, αIFN-γ-PE or αTNF-α-PE (Becton Dickinson) and αCD8-APC (Beckman Coulter, Miami, FL). Cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson).

Statistical analysis
Transplant-related variables and immune reconstitution were evaluated in relation to HAdV infection and viremia by univariate analyses in binary logistic regression models using SPSS software. Multivariate analysis was not performed due to small patient numbers.

RESULTS

Incidence of HAdV infection and viremia

HAdV infection was present in 21 of 48 patients (44%). Detailed information on the 21 infected cases is given in Table 2. Three of the 21 patients were lost to follow-up (patients 1 - 3 in Table 2) due to retransplantation or transplant related mortality. In 6 of the remaining 18 patients, the infection progressed to viremia as defined by the presence of HAdV DNA in plasma (33%, patients 16-21).

Table 2. Transplant and infection characteristics of HAdV-infected and HAdV-viremic patients

Patient	Sex	Age	Diagn[a]	Donor	Source	Condit[b]	T depl	sero-type	Sites[c]	Culture$^+$ (day)	Duration culture$^+$ (days)	PCR$^+$ (day)	Peak PCR load	Duration PCR$^+$ (days)	Censored (day)	Status at 6 months[e]
1	M	10.9	AML	MUD	BM	C T	no	3	F	21	12[d]				death (34)	died asp
2	M	12.1	NHL	MMFD	BM	A E M	yes	1	F	34	17[d]				death (51)	relapse
3	M	7.4	Ph$^+$CML	MMFD	PBSC	C T	CD34$^+$	1	F	29	7[d]				2nd SCT (36)	died MF
4	M	7.6	THAL	MFD	BM	C M T	no	2	F	41	7					A/W
5	F	7.4	OP	MUD	BM	C B	no	2	FT	13	254					A/W
6	M	6.3	Amegak	MFD	BM	C B	no	31	FT	20	56					A/W
7	M	10.9	ALL	MMFD	PBSC	C E T	CD34$^+$	2	FT	23	145					A/W
8	F	5	THAL	MFD	BM	C B M	no	6	FTU	27	168					A/W
9	M	2.8	JMML	MUD	PBSC	C B M	CD34$^+$	2	F	27	9					A/W
10	M	14.8	ALL	MUD	BM	C E T	no	5	FT	91	47					A/W
11	M	7.7	X-LPD	MUD	BM	C B	no	31	F	82	15					A/W
12	M	3.2	X-LPD	MUD	BM	C B	no	1	FT	47	70					A/W
13	M	6.5	CA-EBV	MFD	BM	C B	no	2	F	34	79					A/W
14	M	10.5	THAL	MFD	BM	C F M	no		F	62	14					A/W
15	M	8.9	Fanconi	MMFD	PBSC	C F T	CD34$^+$	31	F	19	21					A/W
16	F	1.4	ALL	MUD	BM	C E B	yes	1	F	18	6	10	6.7	21		A/W
17	M	13.3	dyskerat	MUD	BM	C F	no	5	F	27	113	49	5.7	19		A/W
18	F	4.5	AML	MUD	BM	C B M	no	6	FTU	12	71	19	7.9	37		A/W
19	F	1.4	SAA	MUD	PBSC	C F B	CD34$^+$	31	FTU	12	50[f]	0	11.3	69[f]		died HAdV[f]
20	F	11.0	MDS	MUD	BM	C B M	no	5	FTU	26	28[d]	33	7.8	23[d]		died HAdV
21	F	13.1	MDS	MUD	BM	C B M	no	2	FU	42	12[d]	47	4.6	26[d]		died MF

[a] Amegak: amegakaryocytosis congenita, ALL: acute lymphoblastic leukemia, AML: acute myelocytic leukemia, CA-EBV: chronic active EBV-infection, Ph$^+$CML: Ph positive chronic myelocytic leukemia, dyskerat: dyskeratosis congenita (autos. recessive), Fanconi: Fanconi anemia, JMML: juvenile myelomonocytic leukemia, MDS: myelodysplastic syndrome, NHL: non-Hodgkin lymphoma, OP: osteopetrosis (autos. recessive), SAA: severe aplastic anemia, THAL: β- thalassemia major, X-LPD: X-linked lymphoproliferative disease

[b] conditioning C:cyclophosphamide, M:melphalan, T:TBI, B:busulfan, E:etoposide, A:ARA-C, F:fludarabine ; all patients received ATG or Campath in vivo except pat 5 and 13

[c] F: feces, T: throat, U: urine

[d] duration of infection/viremia until death/retransplantation

[e] asp: aspergillosis, MF: multifactorial cause of death including HAdV, A/W: alive and well

[f] 2nd transplantation at day 49

Of these patients, 3 survived the infection, 2 died due to HAdV (1 after a second SCT) and 1 patient died with HAdV as a probable cofactor in combination with GvHD.

The Kaplan-Meier curve for infection in correlation with the type of donor shows that patients with grafts from matched and mismatched family donors (MFD and MMFD, respectively) as well as patients with grafts from matched unrelated donors (MUD) were infected with HAdV in similar frequencies (Figure 1). However, only in patients with unrelated donors the infection progressed to viremia (data not shown).

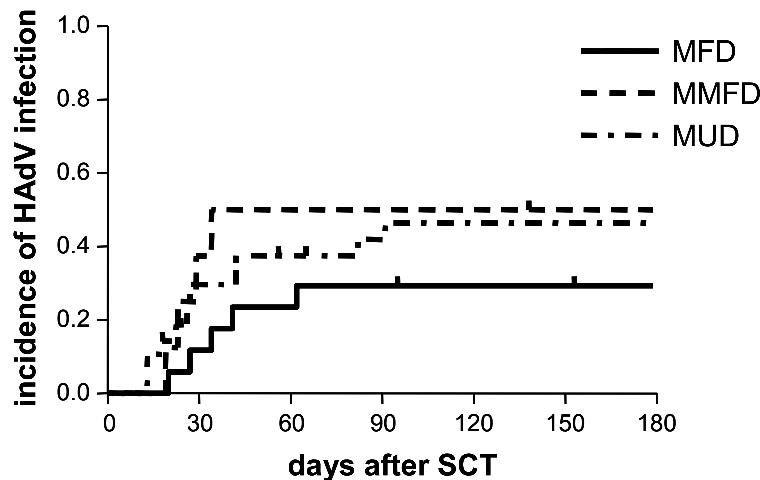

Figure 1. HAdV infection occurs in recipients of grafts from all donor types. Kaplan-Meier curves for HAdV infection in recipients of matched family donors (MFD), mismatched family donors (MMFD) and matched unrelated donors (MUD) are shown in relation to the time of infection after SCT.

The onset of infection in patients who remained PCR negative (median 31 days, range 13-91 days after SCT) was not significantly different from the patients with HAdV viremia (median 22 days, range 12-42 days after SCT) (P = .129) (Table 2). PCR in plasma samples became positive at a median of 26 days (range 0-49). PCR positivity followed 1-3 weeks after the first positive culture in 4 patients, and preceded the first positive culture by 1-2 weeks in 2 patients. Duration of the infection was in some cases prolonged, both in patients with and without viremia, indicating that sustained infection can occur without progression to viremia (Table 2).

At the time of first detection of HAdV DNA in plasma of patients, clinical symptoms that could exclusively be attributed to HAdV were not observed; in patients who died due to infection, severe symptoms as liver failure were evident only at the endstage of the disease.

All 21 infected patients were HAdV positive in feces; in 11 patients no other site became culture positive. Four of the 5 patients who were positive at multiple sites including urine also had HAdV viremia as defined by the presence of HAdV DNA in plasma (Table 2).

Risk factors for HAdV infection and viremia

Risk factors for HAdV infection were HLA mismatched donors (≥ 1 mismatch, *P* = .017), serotherapy with ATG or Campath *in vivo* (*P* = .026) and melphalan in the conditioning regimen (*P* = .018). To determine the risk factors for viremia (PCR positivity), patients with HAdV viremia were compared with HAdV-infected patients without viremia. A MUD donor was a risk factor for viremia (*P* = .005), as well as the female gender of the recipient (*P* = .016). All other variables tested in univariate analyses, such as *ex vivo* manipulation of the graft, either by T cell depletion or $CD34^+$ enrichment, underlying disease, use of cyclosporin A, occurrence of GvHD and age of the recipient were not significantly correlated with HAdV infection or viremia.

Immune reconstitution and course of HAdV infection

As delayed recovery of the immune system might be of importance for the occurrence and progression of HAdV infection, absolute leukocyte and lymphocyte counts were compared between uninfected patients, HAdV-infected patients and patients with HAdV viremia. The time period to leukocyte reconstitution (set at 1×10^9/L) was identical in all groups (data not shown), whereas lymphocyte reconstitution (set at 0.2×10^9/L) was significantly delayed in patients who developed viremia (*P* = .027) (Figure 2A).

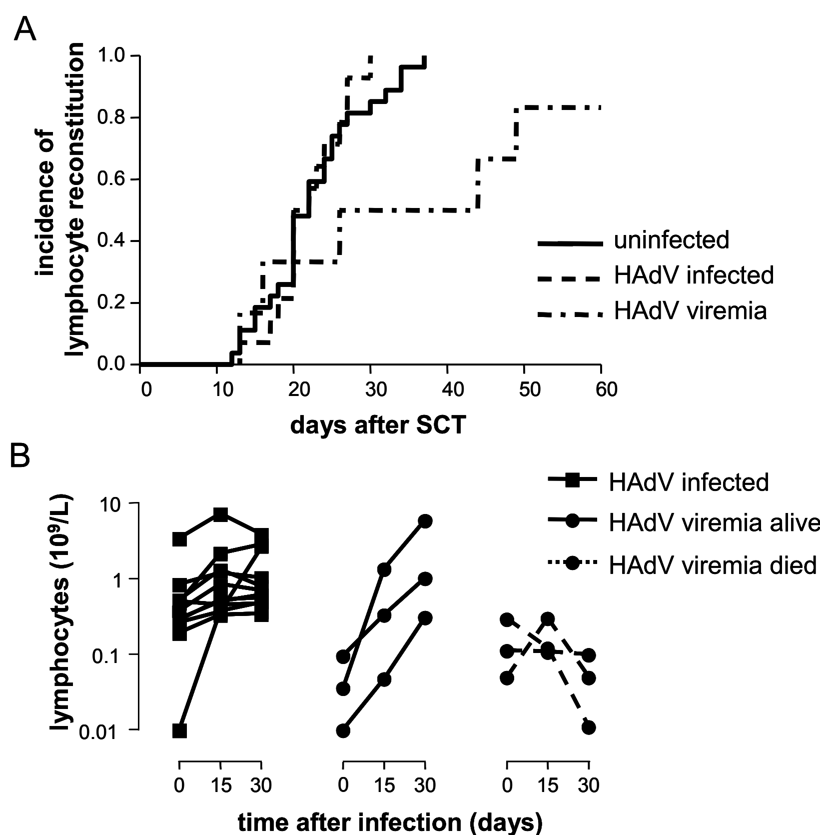

Figure 2. Lymphocyte recovery is correlated with development and outcome of HAdV viremia. A) The time to lymphocyte recovery (set at 0.2×10^9/L) is shown for uninfected patients (solid line), HAdV-infected patients (dotted line) and patients with HAdV viremia (combined line). B) Lymphocyte counts are shown at the onset of infection and within the first 30 days after onset of infection for HAdV-infected patients (■), patients surviving HAdV viremia (●, solid line) and patients succumbing to HAdV viremia (●, dotted line). At t=0, patients with viremia had significantly lower lymphocyte counts than HAdV-infected patients (*P* = .025), and at t=30 patients surviving HAdV viremia had higher lymphocyte counts than patients succumbing to viremia (*P* = .067).

Furthermore, absolute lymphocyte counts were determined at the onset of infection and during the first 30 days thereafter. Patients with HAdV viremia had lower lymphocyte numbers (geometrical mean 62 per µl) at the onset of infection (t=0) than those without viremia (geometrical mean 310 per µl) (P = .025) (Figure 2B). Furthermore, in the six patients with HAdV viremia an increase in lymphocyte counts during the next 30 days correlated with clearance of the infection and survival (t=30, geometrical mean 926 lymphocytes per ul in patients surviving viremia versus 28 lymphocytes per µl in patients succumbing to viremia, P = .067). Patients succumbing to viremia had continuously increasing HAdV DNA loads in their plasma without lymphocyte recovery (Figure 3A and (32)), whereas in patients surviving viremia, lymphocyte recovery coincided with the decrease in HAdV DNA viral load (Figure 4A).

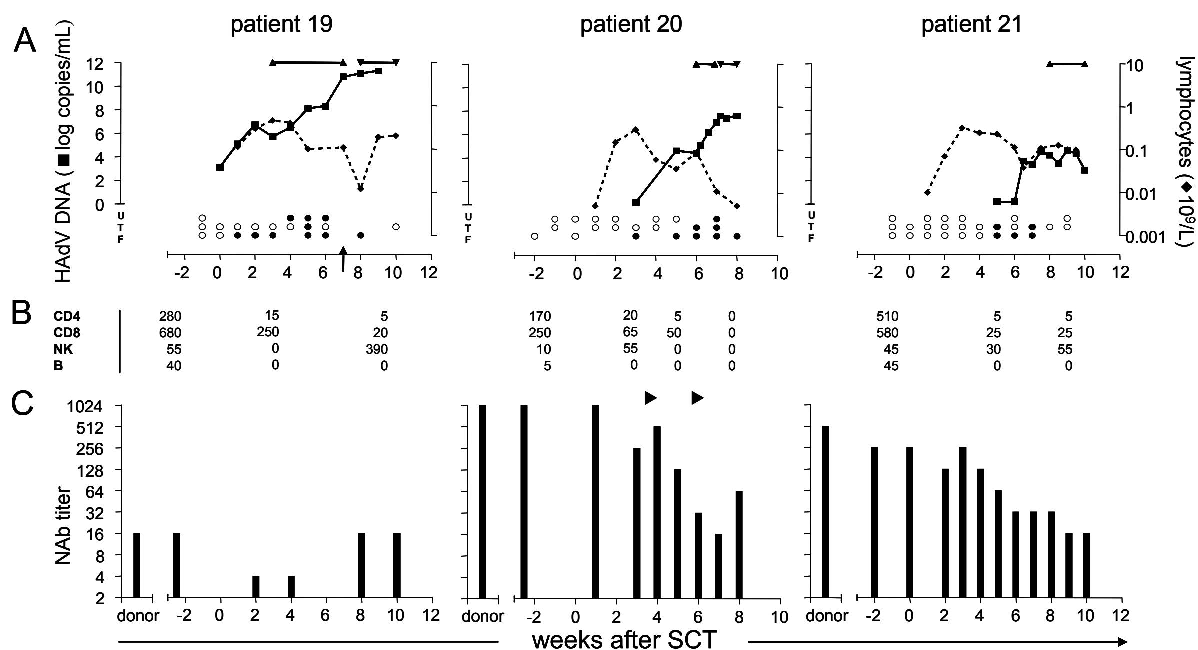

Figure 3. Patients succumbing to HAdV viremia have no cellular immune recovery and no increase in HAdV-specific NAb. Patients with HAdV viremia who succumbed to the infection are shown for viral and immunological parameters pre- and post-SCT. A) Viral parameters are shown as follows: HAdV culture on feces (F), throat (T) and urine (U) (○: culture negative, ●: culture positive); HAdV DNA load by RQ-PCR on plasma (solid line). Lymphocyte recovery is depicted as a dotted line. Treatment with ribavirin (▲) and cidofovir (▼) are indicated. The time point of second transplantation is indicated by an arrow. Patient 20 had grade IV acute GvHD and an EBV infection, and patient 21 had grade II acute GvHD and a CMV infection (not shown). B) Absolute $CD4^+$ and $CD8^+$ T cell counts, as well as NK and B cell numbers per µl blood are shown at several time points pre- and post-SCT. C) HAdV-specific NAb against the serotype infecting the patient are shown. Treatment with α-CD20 antibodies (rituximab) for EBV reactivation is indicated (►). The HAdV DNA loads of these 3 patients have been described for evaluation of ribavirin treatment in (32).

All lymphocyte subsets, e.g. T cells, B cells and NK cells, were reconstituting in patients surviving viremia, whereas only few lymphocytes recovered in patients succumbing to

HAdV viremia (Figure 4B and 3B, respectively). Viral infections other than HAdV occurred in patients from both groups and were successfully treated with ganciclovir and rituximab. Of the 3 patients that survived HAdV viremia, one received no antiviral medication aiming at HAdV, one patient received a short course of cidofovir and one patient a short treatment with ribavirin. However, HAdV clearance coincided with rising lympocyte counts in all 3 patients. Moreover, inefficacy of ribavirin treatment was documented in the 3 patients who died due to HAdV viremia without lymphocyte recovery (32).

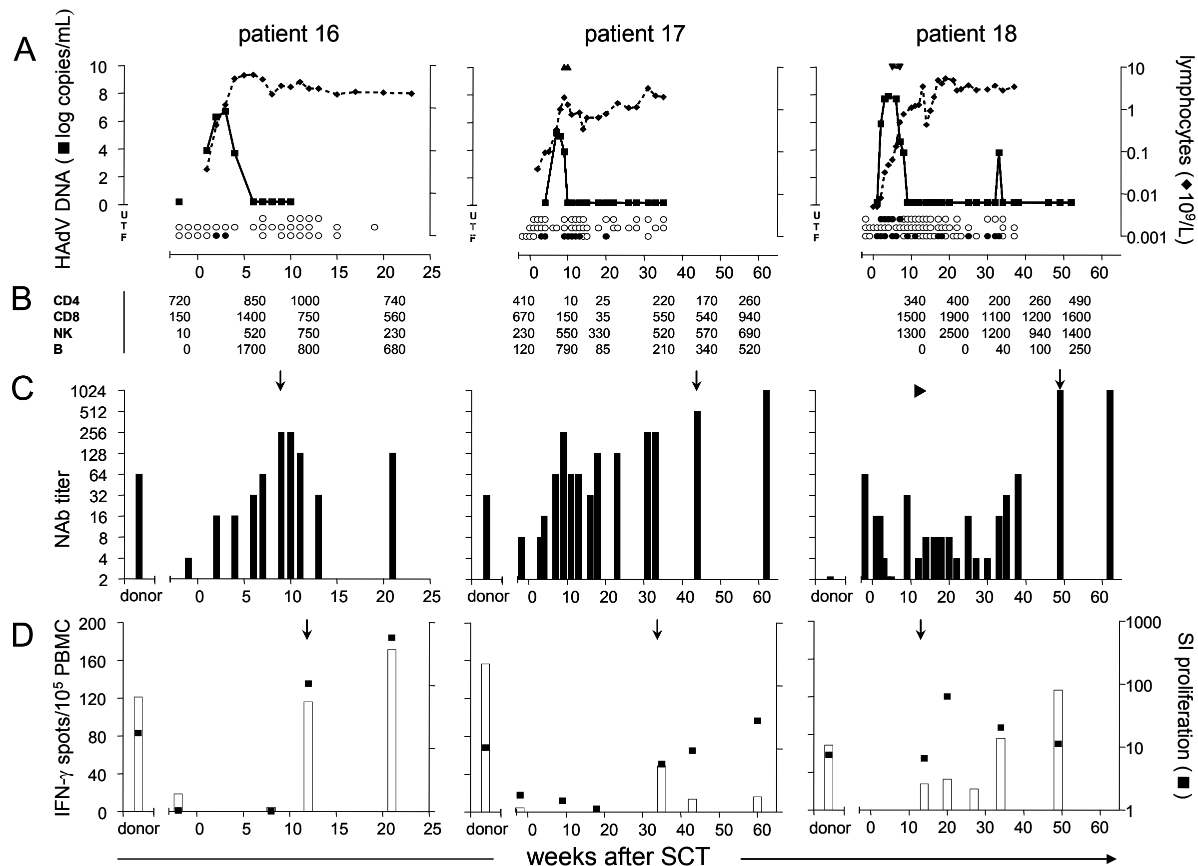

Figure 4. Patients surviving HAdV viremia have cellular immune recovery, as well as an increase in HAdV-specific NAb and HAdV-specific T cells. Patients with HAdV viremia who survived the infection are shown for viral and immunological parameters pre- and post-SCT. A) Viral parameters are shown as follows: HAdV culture on feces (F), throat (T) and urine (U) (○: culture negative, ●: culture positive); HAdV DNA load by RQ-PCR on plasma (solid line). Lymphocyte recovery is depicted as a dotted line. Treatment with ribavirin (▲) and cidofovir (▼) are indicated. Patient 17 had a CMV infection and patient 18 had an EBV and CMV infection (not shown). B) Absolute $CD4^+$ and $CD8^+$ T cell counts, as well as NK and B cell numbers per μl blood are shown at several time points pre- and post-SCT. C) HAdV-specific NAb against the serotype infecting the patient are shown. The specific NAb response (>4-fold increase, see Patients and Methods) is indicated with an arrow. Treatment with α-CD20 antibodies (rituximab) for EBV reactivation is indicated (►). D) HAdV-specific T cell responses are shown in relation to the time after SCT. PBMC from donors and patients were stimulated with the HAdV serotype that infected the patient and tested for proliferation (shown as stimulation index (SI), ■) and IFN-γ production by ELIspot (bars: HAdV-specific spots after subtraction of background spots). The first detection of HAdV-specific T cell responses is indicated with an arrow.

HAdV-specific immune recovery

In patients who died with HAdV viremia, serotype-specific neutralizing antibodies (NAb) were present in serum prior to infection, in some cases even at high titers (Figure 3C, patient 20 and 21). Nevertheless, infection as well as progression to viremia occurred in these patients. During the infection, titers gradually declined to low levels.

In patients with HAdV viremia who cleared the infection, humoral immune responses were observed as serotype-specific NAb increased 8- to 16-fold (indicated in Figure 4C), while NAb titers against other serotypes remained unchanged (data not shown). Responses were observed 1-9 months after clearance of HAdV from the plasma. Patient 18 received rituximab treatment because of EBV reactivation, which might explain the delayed humoral response to HAdV. The early rises in NAb shortly after SCT in patient 16 and 17 were most likely due to IVIG administration since NAb against other serotypes increased simultaneously (data not shown).

As PBMC of patients who died from HAdV viremia were not available due to poor lymphocyte recovery, we were unable to test for HAdV-specific T cells in these patients. In patients surviving viremia, HAdV-specific responses were observed around 5-6 weeks after clearance of the viremia in two patients (patients 16 and 18), and 6 months after clearance in one patient (patient 17) (Figure 4D). Accordingly, T cell recovery was fast in patients 16 and 18, whereas T cell recovery in patient 17 was delayed (Figure 4B). The latter observation was reflected by severely impaired responses to α-CD3 stimulation in this patient prior to detection of HAdV-specific T cells (data not shown).

HAdV-specific T-cell responses were also investigated at 3 and 6 months after SCT in HAdV-infected patients without viremia and in some patients without HAdV infection (Figure 5). At 3 months, responses to the infecting serotype were observed in 3 out of 7 (43%) of HAdV-infected patients and 2 out of 6 (33%) uninfected patients responded to HAdV5. At 6 months, the number of HAdV-infected patients with specific T cell responses increased to 75% (6 of 8 patients), whereas the proportion of responders in uninfected patients remained 33% (Figure 5).

Intracellular cytokine stainings after *in vitro* stimulation of PBMC with HAdV revealed that most IFN-γ (and TNF-α) producing cells were $CD4^+$ T cells, both in donors as well as in patients (Figure 6 and data not shown). These data suggest that the HAdV-specific T cells detected after clearance of infection are $CD4^+$ T cells with a Th1-like phenotype.

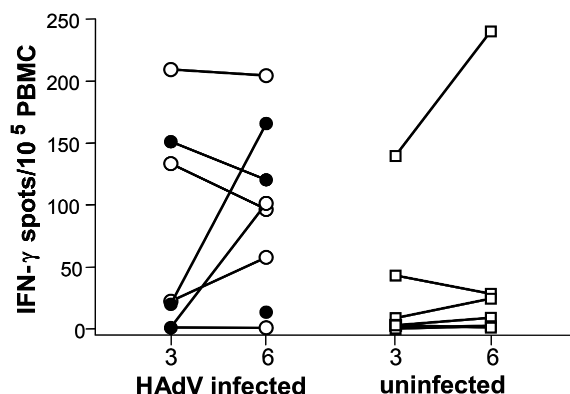

Figure 5. HAdV-specific T cell responses can be detected in most HAdV-infected patients without viremia and in some uninfected patients at 6 months after SCT. HAdV-infected patients without viremia were tested for HAdV-specific responses at 3 months (n = 7) and 6 months after SCT (n = 8), as well as some uninfected patients (n = 6 at both time points). The number of HAdV-specific IFN-γ producing cells by ELIspot is shown (>25 spots is considered as a response). ○: HAdV-infected patients in which infection was cleared from the feces before that time point, ●: HAdV-infected patients in which feces was still HAdV positive at that time point, □: uninfected patients.

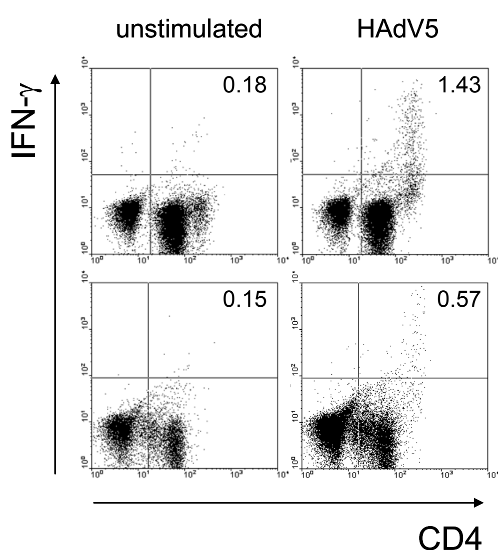

Figure 6. HAdV-specific T cells are IFN-γ producing CD4^{+} T cells, both in donors as well as in patients surviving HAdV infection. PBMC from donors and patients were tested by intracellular cytokine stainings for production of IFN-γ after a 5-day stimulation with the HAdV serotype that infected the patient, or unstimulated as control. An example of donor (upper panel) and patient (lower panel; patient number 17 at week 60 after SCT) CD3^{+} cells producing IFN-γ is shown. These IFN-γ producing cells are large CD3^{+}CD4^{+} cells based on their forward and side scatter plot. Results are representative for 7 donors and 11 patients of which 9 patients had HAdV infection with or without viremia.

DISCUSSION

In this prospective study, 48 pediatric SCT patients were monitored for the occurrence of adenovirus infection and viremia in relation to HAdV-specific immune reconstitution. The value of detection of viral DNA in plasma has been well documented for the diagnosis and management of virus infections, such as EBV and CMV and more recently also for HAdV (15-17,33,37-39). Quantification of the HAdV DNA load is essential as only persistent and rising loads have been shown to correlate with progressive disease and eventually death (12,13). In this study, infection occurred in 44% of patients, of whom 33% developed viremia as documented by the presence of viral DNA in plasma. The risk factors identified in this study, HLA mismatched transplants and immunosuppression with ATG or Campath *in vivo* are likely to have an impact on lymphocyte recovery (5,6,25,40). A low lymphocyte count at the time of the first virus isolation was a strong predictor of HAdV viremia. Furthermore, increasing lymphocyte counts during the first weeks of HAdV viremia were correlated with survival. In patients succumbing to HAdV viremia, the lack of increase in

lymphocyte numbers could be due to a variety of factors such as the occurrence and treatment of GvHD or retransplantation. This strong correlation between lymphocyte recovery and clearance of HAdV confirms and extends a previous report (25) and is in accordance with results obtained with respect to CMV infection (23,24,41).
This is, to our knowledge, the first report in which HAdV-specific immunity is investigated longitudinally in SCT recipients with documented infection. Serotype-specific humoral immune responses were detected in patients recovering from HAdV viremia several weeks to months after clearance of the viral DNA from the circulation. In comparison, peak titers of HAdV-specific antibodies in healthy individuals were observed 2-4 weeks after adenoviral vector administration for gene therapy purposes (42). This difference in kinetics is probably due to the slowly recovering immune system in allogeneic SCT recipients. Interestingly, the presence of pre-existing high HAdV-specific NAb titers in serum of 2 out of 6 patients did not prevent progression to viremia. Viremia did not occur in 9 HAdV-infected patients, despite the fact that 5 patients had only low NAb titers (≤ 32) and 3 patients had intermediate titers (between 32 and 256) at the time of infection. Together, these results suggest that the role of pre-existing NAb (as measured *in vitro*) in protection against progression to viremia may be limited. As the number of patients is small, more patients should be evaluated to confirm these findings.
In patients who had cleared the HAdV viremia, predominantly $CD4^+$ HAdV-specific T cells producing IFN-γ were detected weeks to months after clearance of viremia. In healthy blood bank donors, HAdV-specific $CD4^+$ T cells producing IFN-γ upon HAdV stimulation *in vitro* have also been detected (34,43). In individual patients, the increase in total lymphocyte numbers coincided with a decrease of viral DNA from plasma. As this result suggests a causal relationship, the question remains why specific humoral or cellular responses were only detected at later time-points. At this stage, we can only speculate about the reasons for this observation. The decrease in viral DNA load strongly suggests that a specific immune response has been initiated at the site of infection, but it might be that responses are undetectable in the circulation because the frequency of specific cells is below detection limit. In CMV and varicella zoster virus infections, specific $CD4^+$ cells were also observed only after clearance of the virus in patients with symptomatic disease (44) (RAW van Lier, personal communication), possibly reflecting the redistribution characteristics of $CD4^+$ T cells after viral infection. In other viral infections such as EBV, the presence of specific $CD8^+$ cells at the time of clearance of the virus has recently been demonstrated using MHC-antigen tetramers, making detection independent of *in vitro* functionality (45,46). Defining HAdV-specific immunodominant epitopes, as has been described by Olive at al (47), may be instrumental in the detection of HAdV-specific T cells at earlier time points using tetramer technology. Another possibility is that the initial control of infection is established by other

(innate) immune mechanisms, for instance NK cells, while the adaptive immune response is initiated at a later time point. Further research is needed to address these questions.
Taken together, weekly RQ-PCR based screening of plasma for HAdV during the first months following allogeneic SCT, especially in recipients of grafts from MMFD or MUD donors, identifies high-risk patients at an early stage of viremia. If viremia is accompanied by a rise in lymphocyte counts, the viral infection will most likely be controlled. When lymphocyte counts remain low, alternative measures should be taken. As the antiviral effect of ribavirin appears limited and cidofovir is not proven to be unequivocally effective (26,27,29-32), other interventions aimed at improving immune reconstitution should be considered. Tapering of the immunosuppression post-SCT has been suggested to improve the outcome of the infection (25). However, this strategy bears a high risk of concurrent severe GvHD, especially in a non-HLA-identical transplant. Adoptive immunotherapy with HAdV-specific T cells, as has been performed for EBV and CMV (48-50), or infusion of donor cells that are depleted of alloreactive T cells (51,52) might be options for treatment in immunocompromised graft recipients at early signs of HAdV viremia in the near future.

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Chapter 4

Effect of ribavirin on the plasma viral DNA load in patients with disseminating adenovirus infection

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ABSTRACT

Adenovirus infections are an increasingly frequent and potentially fatal complication in allogeneic stem cell recipients. To determine the antiviral potential of ribavirin in an unbiased way, four cases without immune recovery were prospectively analyzed by quantitative measurement of HAdV DNA in plasma. Administration of ribavirin at the first signs of HAdV dissemination was not accompanied by a reduction of viral DNA load in plasma in any of these cases, and even a further increase of the viral load was documented in three of them. These observations question the potential of ribavirin to improve outcome in disseminating HAdV infections, and support a critical evaluation of antiviral therapies in HAdV infections by using kinetics of viral DNA load as an objective parameter of viral replication.

INTRODUCTION

Adenovirus (HAdV) infections are increasingly recognized and frequently result in a fatal complication in allogeneic stem cell transplant (SCT) and solid organ transplant recipients (1). At present, 51 HAdV serotypes have been identified, which are classified into six species (A-F). In pediatric immunocompromised patients, HAdV species A-C are most frequently isolated. In the majority of cases HAdV infections are restricted to one anatomical site, mostly the intestinal or urinary tract (2,3). In these cases the localized HAdV infections may present with clinical symptoms e.g. diarrhea or hemorrhagic cystitis, but frequently an asymptomatic course is seen. Progression to disseminated HAdV infection may initially occur in the absence of typical clinical symptoms, but it usually results in clinical disease which is fatal in the majority of the patients. In pediatric patients disseminated HAdV infections are largely restricted to SCT recipients using other than HLA-identical family donors. Similar to what has been reported for Epstein-Barr virus (EBV) (4) and cytomegalovirus (CMV) (5,6), absent or inadequate immune recovery in the early posttransplant period is probably a major determinant for the occurrence, dynamics and outcome of the HAdV infection (7). Until recently, monitoring of HAdV infections was hampered by the lack of a reliable parameter of viral dissemination. Recently, HAdV DNA load measurements in plasma have become available for monitoring infections (8-11). In these studies, evidence has been provided that viral DNA detection and quantification in plasma is an informative and objective parameter to identify HAdV-infected patients at high risk of disseminating disease.

Treatment of HAdV infections has been hindered by the lack of antiviral medication, whose efficacy has been demonstrated unequivocally. Ribavirin and cidofovir are the two antiviral drugs that are being used in most transplant centers. Conflicting results on the presumed efficacy of these drugs have been reported in several studies (12-17). The relatively small

number of systematically analyzed cases of ribavirin treatment has not provided convincing evidence for a beneficial effect of this antiviral drug compared to the natural course of the infection in these patients. It should be noted that the presumed therapeutic success in some cases might well be biased by a concomitant immune recovery.

Viral DNA load assays have been found to be extremely valuable not only to study the natural dynamics of infection but also to assess the therapeutic benefits of antiviral regimens. This has been convincingly shown in the field of HIV and hepatitis B virus (18-19) and more recently also for CMV (reviewed in (20)) and EBV (21). In the current prospective setting, we have applied the quantitative measurement of HAdV DNA in plasma by real-time quantitative PCR (RQ-PCR) to study the antiviral potential of ribavirin in SCT recipients at the first signs of dissemination of the infection.

PATIENTS & METHODS

From 2001 onwards, all pediatric SCT recipients in the pediatric transplant unit of the Leiden University Medical Center have been prospectively analysed for the occurrence of HAdV infection (Heemskerk *et al.* manuscript in preparation). Viral screening for HAdV infection included weekly cultures of feces, throat swabs and urine. HAdV isolates were serotyped by classical viral neutralization assays on A549 cells using type-specific antisera (10). Real-time quantitative PCR for HAdV DNA was performed on weekly plasma samples as recently described (9). In summary, DNA was extracted from plasma samples by standard procedures and 10 µL of DNA containing extract was amplified by real-time PCR using the oligonucleotide primers described by Echavarria *et al.* (22). To detect these amplified products, a molecular beacon with a 5' FAM label and 3' dabcyl label was used (Biolegio, Malden, The Netherlands). A quantitated adenovirus type 5 stock was obtained as a kind gift from dr. M. Havenga (Crucell, Leiden, The Netherlands). Using an iCycler IQ system (BioRad laboratories, Hercules, CA), real-time amplification of a dilution series of this stock resulted in an external standard line based on the threshold cycles (Ct). This standard was used to quantitate HAdV DNA in the plasma samples by its Ct value.

Four recipients of an HLA non-identical SCT who presented with HAdV infection received treatment with ribavirin. Clinical characteristics are summarized in Table 1. These patients fulfilled the following criteria: 1) HAdV DNA load in plasma > 1000 copies (cp)/mL as measured by RQ-PCR at two consecutive time points within a period of one week at the start of treatment. Clinical and additional laboratory parameters that might be related to HAdV infection were not considered for decision making about ribavirin treatment. 2) Intravenous ribavirin was administered as first line treatment during the period of positive HAdV DNA load in plasma. 3) Follow-up of HAdV DNA load was performed weekly. 4) Signs of immune recovery, defined as lymphocyte counts exceeding 100 cells/µL, were absent during the entire treatment period. Stable engraftment finally occurred in all cases. Engraftment (> 500 neutrophils/µl) occurred on day + 19 (patient 1), day + 19 (patient 2), day +18 (patient 3, following 3rd SCT), and day + 14 (patient 4, following 2nd SCT), respectively.

Graft-versus-host disease (GvHD) grade II was documented in patient 1 and 2, starting on day +14 and +20 respectively, requiring prolonged steroid treatment. None of the patients received any form of antiviral prophylaxis. Ribavirin (ICN Pharmaceuticals, Zoetermeer, The Netherlands) was administered as a loading dose of 30 mg/kg, followed by a maintenance dose of 60 mg/kg q.i.d.. Cidofovir (Gilead Sciences Int. Ltd, Cambridge, UK) was administered at a dose of 5 mg/kg once a week, combined with Probenecid and hyperhydration to prevent nephrotoxicity.

RESULTS

Ribavirin was administered as first line therapy for 1-4 weeks in all four patients (Figure 1). In none of the patients a decrease in viral DNA load was documented during ribavirin

treatment. A continuously increasing level of HAdV DNA load was measured in three of the four cases. In two patients (patient 3 and 4) symptoms that could be attributed to HAdV infection were absent at the initiation of treatment. In the other two cases (patient 1 and 2) symptoms possibly related to HAdV infection (i.e. fever, diarrhea and slightly elevated transaminases and bilirubin) were documented at the initiation of treatment. However, in both cases concurrent, histologically confirmed, intestinal GvHD was probably mainly responsible for these symptoms. HAdV was cultured from stool prior to the detection of HAdV DNA in plasma in all cases except for patient 4. HAdV was also isolated from urine and/or throat swabs in all cases during the infection episode. In three cases, the positive HAdV plasma PCR preceded or coincided with HAdV isolation by culture from extra-intestinal sites. HAdV serotypes 1, 2, 5 (all species C), and 18 (species A) were isolated in these four cases. Because of clinical deterioration, sampling of culture specimens was discontinued in the final stages of clinical disease.

Table 1. Patient characteristics

Patient	Age (yr)	Disease[a]	Donor	Conditioning	Graft manipulation	Serotype
1	14	MDS	MUD	B/C/M/ATG	None	2
2	8	MDS	MUD	B/C/M/ATG	None	5
3	6	CML	MUD[b]	T/F/ATG	$CD34^+$ selection[c]	1
4	3	Schwachman	MUD[b]	B/F/C/ATG	$CD34^+$ selection[c]	18

[a] MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; MUD, matched unrelated donor; B, Busulfex; C, Cyclophosphamide; M, Melphalan; T, Thiotepa; ATG, anti-thymocyte globulins.
[b] 3rd transplant following two previous rejections of haplo-identical SCTs in patient 3; 2nd transplant following non-engraftment of the first SCT from the same donor in patient 4.
[c] $CD34^+$-selection of peripheral blood stem cells performed using Clinimacs®.

Due to the apparent lack of antiviral effect of ribavirin in these patients, a switch was made to cidofovir in two cases (patient 2 and 4). Some stabilization of HAdV DNA levels in plasma was subsequently observed, although cidofovir was only initiated at very high viral load levels > 10^6 cp/mL and > 10^{10} cp/mL, respectively). However, follow-up was too limited in these cases to draw firm conclusions about the antiviral effect of cidofovir, as both patients succumbed from their disease. Disseminated HAdV infection was the major determinant of mortality in two of the patients (patient 2 and 4). In the other two cases (patient 1 and 3) HAdV infection and GvHD were considered jointly responsible for the fatal outcome.

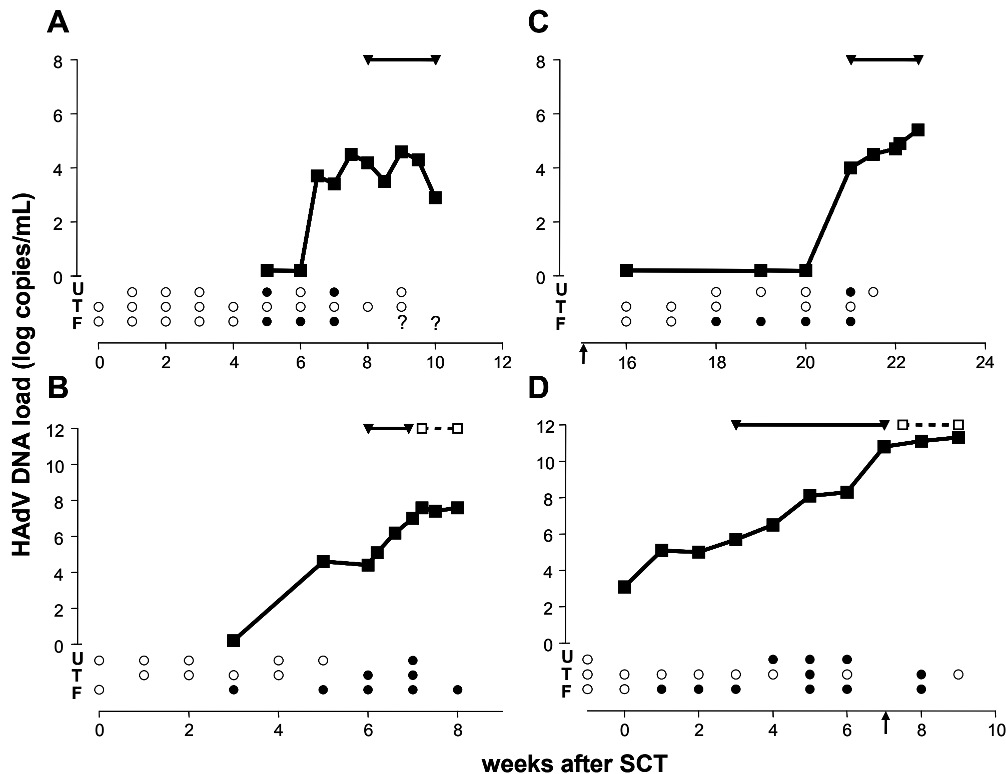

Figure 1. Culture results and viral DNA loads in 4 patients treated with ribavirin. Patient 1-4 are depicted in Figure 1A-D, respectively. HAdV was cultured in feces (F), urine (U) and throat swabs (T) and results are shown as positive (closed symbols) or negative (open symbols). Viral DNA load is shown in logarithmic values of copies/mL. The period of ribavirin and cidofovir administration is indicated by (▼) and (□), respectively. The arrow at week 15 in Figure 1C indicates the third SCT, the arrow at week 7 in Figure 1D indicates the second SCT. The results of feces cultures of patient 1 (Figure 1A) at week 9 and 10 were inconclusive ('?') because of the presence of clostridium toxin in the samples.

In contrast to HAdV infection, in 3 out of 4 patients concurrent infections with other DNA viruses responded well to specific antiviral treatment (data not shown). In patient 1 CMV reactivation was first documented on day +19 upon which viral DNA load further increased to a maximum of 10^6 copies/mL on day +35. A firm and persisting decrease in CMV DNA load (1000-fold) was observed within 3 weeks following treatment with ganciclovir (5 mg/kg b.i.d.) and subsequently foscarnet (60 mg/kg t.i.d.). In patient 2 EBV reactivation was diagnosed based on an increase in EBV DNA load that responded rapidly upon a single infusion of Rituximab (375 mg/m^2) on day +30. Finally, patient 3 was treated with intravenous aciclovir (10 mg/kg t.i.d.) and topical cidofovir emulsion (1% w/w) (23) because of persisting labial herpes virus type 1 infection resulting in regression of the mucosal lesions.

DISCUSSION

We have previously reported that HAdV DNA measurement and quantification in plasma of HAdV-infected SCT recipients are instrumental in defining individuals at risk for fatal disseminated infections (8,10). These observations have provided the rationale for pre-emptive treatment of HAdV infections guided by viral DNA levels in plasma. Recently, HAdV DNA load quantification by RQ-PCR has become available and this approach has now been introduced in the clinical management of pediatric SCT recipients in our center. As has been suggested before, this diagnostic tool might also be of great value to document any changes in viral replication upon the administration of proposed antiviral therapies (9-11).

So far, ribavirin and cidofovir are the drugs most often used to treat HAdV infections. Ribavirin is a synthetic guanosine analogue with *in vitro* inhibitory activity against a broad range of DNA and RNA viruses. *In vivo* efficacy has been demonstrated mainly in infections with respiratory syncytial virus and chronic hepatitis C, both RNA viruses (24-26). The mechanism of action is still unclear, but a potentiating effect on treatment with type I IFN has been reported in chronic hepatitis C infections.

It is commonly accepted that effective antiviral therapy by a certain therapeutic concentration of a drug in a certain period of time should result in a consistent and proportional reduction in the level of viral replication. This means that a drug should result in a reduction in viral replication by a constant factor, which in a steady state situation would be measurable by a reduction in the plasma load of the viral nucleic acid, if a systemic and disseminated infection is present.

In the present study the efficacy of ribavirin to inhibit HAdV replication and progressive disease was analyzed using a pre-emptive treatment strategy in four patients without evident immune reconstitution at the first signs of disseminating HAdV infection and during the period of treatment. In none of the cases a reduction in viral DNA load was observed during the period in which ribavirin was administered. Strikingly, even an apparently unaffected rise in viral DNA load was documented in three of the cases.

From our present study, it may be concluded that ribavirin lacks significant antiviral activity *in vivo* against at least various HAdV strains. Our data provide evidence that ribavirin is not the drug of choice to treat HAdV infection in these severely immunocompromised patients. Within the framework of an international collaborative study, investigations on the pharmacokinetics of ribavirin and *in vitro* susceptibility of clinical HAdV isolates towards ribavirin are currently being performed in order to further document these findings. Studies along this line will bear relevance also to the question whether ribavirin could have a role in approaches of prophylactic treatment.

Because an apparent effect on viral DNA load kinetics following ribavirin therapy was lacking, therapy was switched to cidofovir in two cases. Cidofovir is a nucleotide analogue with potent reactivity against several DNA viruses and therapeutic efficacy *in vivo* has been reported in CMV infection (27). It should be noted that in our patients cidofovir therapy was initiated at a very high viral DNA load, which is compatible with serious disseminated infection. Although a further increase of the viral DNA load was tempered, it is not justified to draw firm conclusions with respect to antiviral efficacy of cidofovir based on the observations in these end-stage patients. However, together with several recently published studies in which the anti-HAdV efficacy of cidofovir was reported in patients without significant T-cell reconstitution (12,13,16), our observations support that further study of this drug as first line pre-emptive treatment in less deteriorated patients in earlier stages of infection is warranted.

In accordance to what is already regarded common practice in most other systemic viral infections, like EBV and CMV, this case series does emphasize the value of HAdV DNA load as an objective endpoint, also in the assessment of the therapeutic efficacy of antiviral therapies in cases of disseminated HAdV infections.

Note added in proof:

Upon revision of the manuscript, the report by Leruez-Ville *et al.* (CID, 2004, 38:45) was published, which essentially confirmed the need for adenoviral DNA load determination in the evaluation of the treatment responses and in this way found evidence for the potential use of cidofovir in the treatment of this condition.

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Chapter 5

Extensive cross-reactivity of $CD4^+$ adenovirus-specific T cells: implications for immunotherapy and gene therapy

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ABSTRACT

Adenovirus (HAdV)-specific T cell responses in healthy adult donors were investigated. HAdV5, inactivated by Methylene Blue plus visible light, induced proliferation and IFN-γ production in PBMC of the majority of donors. Responding T cells were $CD4^+$ and produced IFN-γ upon restimulation with infectious HAdV5 and HAdV of different species. T cell clones showed distinct cross-reactivity patterns recognizing HAdV serotypes from either one species (C), two species (B and C) or three species (A, B, and C). This cross-reactivity of HAdV-specific T cells has relevance both for adenovirus-based gene therapy protocols as well as for the feasibility of T-cell mediated adoptive immunotherapy in recipients of an allogeneic stem cell transplantation.

INTRODUCTION

Adenoviruses (HAdV) rarely cause severe clinical symptoms in healthy children and adults as infections in immunocompetent individuals are usually self-limited. For this reason, adenovirus has been considered to be a safe vector for gene delivery and vaccination strategies, although pre-existing immunity is a major limitation when vectors are being administered repeatedly (16,47). However, HAdV may cause life-threatening complications in immunocompromised children (20,29). In recent years, the incidence of HAdV infections in pediatric recipients of an allogeneic stem cell transplant (SCT) has increased remarkably (2,8,14,17,22,39) (and van Tol *et al.*, manuscript in preparation). Recipients of a T-cell depleted allogeneic graft, i.e. patients with a non-HLA-identical donor, have a higher risk of developing HAdV infection, probably due to the delayed immune reconstitution in these children after SCT (17) (and van Tol *et al.*, manuscript in preparation). Dissemination of the infection often leads to a fatal outcome (14,17,20,21,32).
Currently, 51 serotypes of adenovirus have been identified, distributed among six species (A-F) (11,20). Species A, B and C serotypes are most frequently isolated from pediatric immunocompromised hosts and are the major cause of disease (4,14,20). Treatment of adenoviral infections with antiviral medication using drugs such as cidofovir and ribavirin has not been unequivocally effective (3,5,23,25,28,31,38). A novel therapeutic approach may be immunotherapy by means of HAdV-specific lymphocytes as case reports have suggested that donor lymphocyte infusions (DLI) may contribute to clearance of an HAdV infection (6,24). This study focuses on the feasibility of generating HAdV-specific T cells from graft donor origin with the final goal of infusing these cells into the infected SCT patient. This strategy has already successfully been pursued for other viral infections or reactivations such as CMV or EBV (13,19,46).

RESULTS & DISCUSSION

Inactivation of adenovirus

Stimulation of HAdV-specific T cells using wild-type or $E1^{-}E3^{-}$ recombinant HAdV-vectors has been reported previously (9,14,43,44). In clinical practice, however, biosafety constraints require a validated inactivation procedure of the HAdV used for T cell stimulation in order to circumvent infusion of infectious or genetically modified virus into the patient. Our strategy has therefore focused on complete inactivation of purified wild-type virus using the photosensitizer Methylene Blue (MB) and visible light (40). MB is already in use for routine treatment of fresh frozen plasma prior to infusion to inactivate viruses such as hepatitis C virus (33,35,42).

MB inactivation of HAdV was previously shown to reduce viral infectivity by at least 4 log after illumination for 10 min (40). Prolonging the illumination period revealed that infectious particles could no longer be detected after 30 minutes of inactivation as determined by the lack of cytopathological effect in human epithelial cells (Hep-2) cells, indicating that MB can inactivate HAdV5 at least 7 log (data not shown).

Frequency of T cell responses to MB-inactivated HAdV5

As the use of MB-inactivated HAdV5 as antigen has not been reported previously, the frequency of donors reacting against MB-inactivated HAdV5 was determined in a panel of healthy adults by proliferation and IFN-γ ELIspot assays. In total, 19 out of 25 (76%) donors responded to MB-inactivated HAdV5 by proliferation, with stimulation indices (SI) ranging from 4.5 to 234 (Figure 1A). Analyzing this panel of healthy adults simultaneously for IFN-γ producing cells by ELIspot revealed that 80% of donors responded after 4 days of stimulation with MB-inactivated HAdV5 (Figure 1B). Calculated SI for the ELIspot results (in responding donors ranging from 4.1 to 109) correlated significantly with the SI from the proliferation assay (Pearson correlation coefficient 0.757, $P < 0.001$) (Figure 1C). Donors not reacting against MB-inactivated virus were also tested by proliferation against UV- or heat-inactivated HAdV5 as well as non-inactivated wild-type HAdV5 to determine whether the non-responsiveness was due to the nature of the viral antigen. However, these donors were unresponsive to any type of adenoviral stimulation, indicating that the MB inactivation of the virus was not responsible for the lack of response (data not shown). Unfortunately, sera from these donors were unavailable to test for previous HAdV infections by serology. As MB-inactivated HAdV induced good proliferative responses of PBMC, our results confirm previous data which suggested that T cell responses are directed to structural proteins (14,34,43,45). The frequency of responders obtained with MB-inactivated HAdV5 was comparable to frequencies that have been described using purified $E1^{-}E3^{-}$ HAdV5 (64%) (9) or using HAdV2-lysate from Hep-2 cells (>90%) (14), indicating that MB-inactivated HAdV

is a valid source for antigenic stimulation (36). This high response rate in adult donors indicates that the generation of HAdV-specific T cells would be a feasible option for the majority of transplant recipients at risk for developing a disseminated HAdV infection.

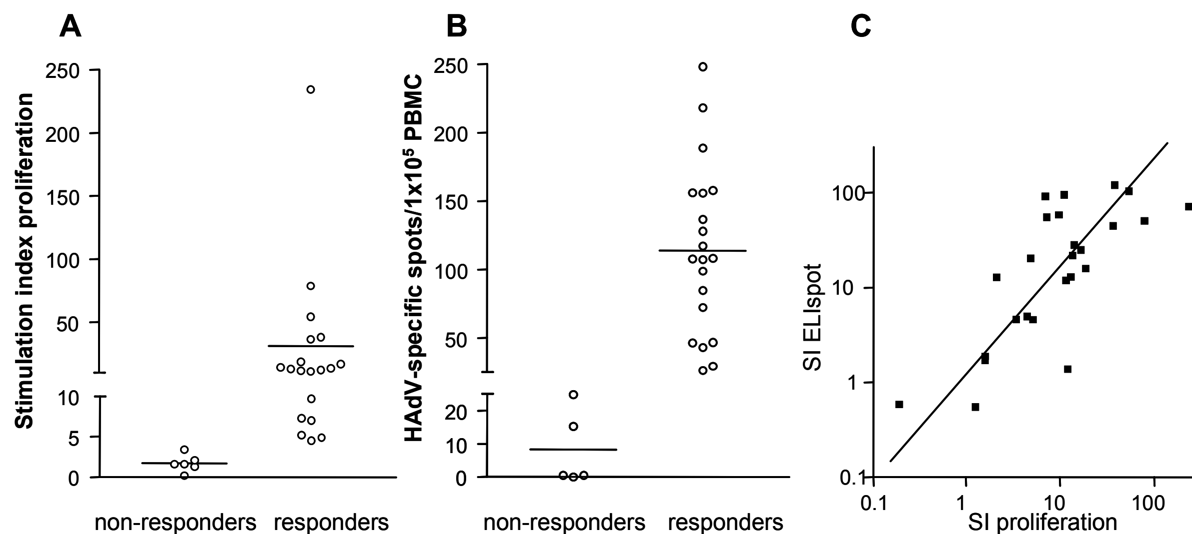

Figure 1. Proliferation and IFN-γ responses against MB-inactivated HAdV5 in healthy adults. PBMC from 25 healthy adult donors were stimulated with MB-inactivated HAdV5 or not stimulated as control. A) Proliferation against MB-inactivated HAdV5 is shown as the stimulation index (SI) for each donor. The SI is defined as proliferation (cpm of [^{3}H] thymidine incorporation) against MB-inactivated HAdV5 divided by the proliferation against PBMC without virus. Donors with SI >3 and $mean_{HAdV}$ > ($mean_{control}$ +3×SD) were considered responding donors (19 out of 25). All donors responded to PHA stimulation (not shown). B) IFN-γ production was determined by ELIspot in the same donors. Specific spots (background spots are substracted) are shown for each donor. Donors with at least 25 specific spots/100,000 PBMC and mean $spots_{HAdV}$ > (mean $spots_{control}$ +3×SD) were considered responding donors (20 out of 25). C) SI were calculated for both assays for comparison. The Pearson correlation coefficient is 0.757 (*P* <0.001); the orthogonal regression line is shown (y = 0.04 + 1.14x).

Increased HAdV-specific IFN-γ production and reduced alloreactivity after restimulation

If HAdV-specific T cell cultures are to be infused in patients, alloreactive, potentially GvHD-causing T cells should be eliminated. One way to reach this goal is a prolonged period of repetitive stimulation with specific antigen in culture. Furthermore, cells generated against MB-inactivated virus should be reactive against cells infected with infectious virus to be able to combat an ongoing infection in a patient. To this end, bulk cultures were initiated with PBMC from donors responding against MB-inactivated HAdV5 and restimulated at day 12; IL-2 was added from day 15. At day 28, T cells proliferated specifically against autologous PBMC with MB-inactivated HAdV5 as well as with active HAdV5 (Figure 2A) showing that T cells specific for structural proteins do indeed respond to cells with infectious HAdV.

Intracellular cytokine staining combined with immunophenotyping revealed a strong enrichment of $CD4^+$ T cells producing IFN-γ upon restimulation with HAdV5 (median 5% IFN-γ^+ cells in the $CD3^+CD4^+$ subset, range 1.2% – 30.1%, n=8) (Figure 2B). $CD8^+$ cells specifically producing IFN-γ were not detected using intracellular stainings, neither using MB-inactivated HAdV nor infectious HAdV for stimulation (data not shown). Accordingly, other groups have demonstrated $CD4^+$ T cell responses against HAdV, either in proliferation assays (9,14,18) or in IFN-γ ELIspot assays (36). In the same cultures alloreactive responses tested after 28 days of culture were strongly reduced when compared with the alloreactive responses at the initiation of culture (Figure 2C).

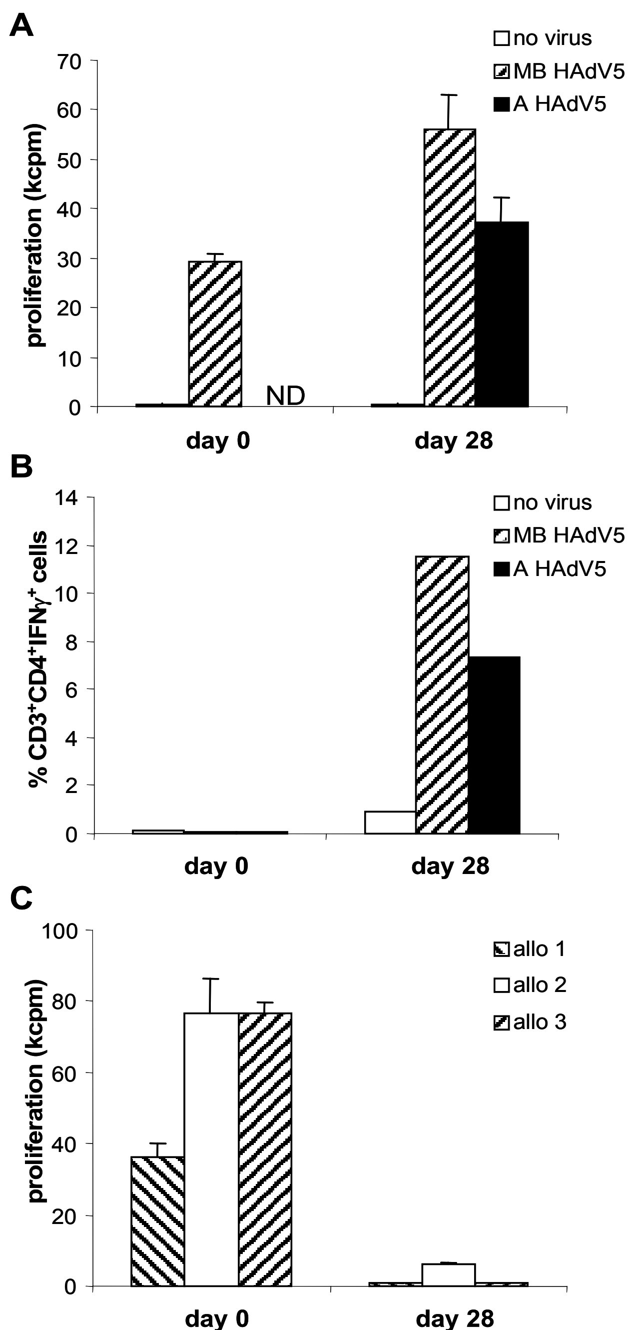

Figure 2. Proliferation of T cells, frequency of IFN-γ producing $CD4^+$ T cells and alloreactivity after 28 days of culture. PBMC of healthy adult donors were stimulated with MB-inactivated HAdV5 (MOI 10) at day 0 and restimulated at day 12. At day 28, specificity of cultures was assessed and compared to the response at initiation of culture. A) Proliferation against irradiated autologous PBMC with MB-inactivated HAdV5 (MB HAdV5) and active HAdV5 (A HAdV5) at MOI 100 or without virus was measured in a 6-day proliferation assay. The mean and SEM of triplicate wells are shown (ND = not done). B) The percentage of IFN-γ producing T cells at day 28 was assessed by intracellular FACS staining following an 18 h stimulation with irradiated autologous PBMC with MB-inactivated HAdV5 and active HAdV5 at MOI 100 or without virus. Brefeldin A was added after 1 h of co-culture. The percentages IFN-γ^+ cells within the $CD3^+CD4^+$ subset prior to culture (day 0) and following 28 days of culture are shown. IFN-γ^+ cells were not detected in the $CD3^+CD8^+$ subset (data not shown). C) Alloreactivity was tested in a 6-day proliferation assay against irradiated allogeneic PBMC of 3 donors. The mean and SEM of triplicate wells are shown. All data are representative for experiments with 6 different healthy adults.

Cross-reactivity of HAdV5-specific bulk cultures and T cell clones

A complicating factor in generating T cells for immunotherapy of HAdV infection is that 51 serotypes of HAdV are described to date. It is currently not possible to predict which serotype will lead to infection in individual patients. Therefore, it is of interest to know whether T cells generated by stimulation with HAdV5 are also able to recognize other serotypes of HAdV, since HAdV strains belonging to species A, B and C have been reported to cause severe infections in immunocompromised hosts (4,14,20).

Cells from 28-day T cell cultures generated against MB-inactivated HAdV5 were tested against a panel of HAdV serotypes, including strains belonging to species C (HAdV1, HAdV5 and HAdV6), species B (HAdV3, HAdV7, HAdV11, HAdV34 and HAdV35) and species A (HAdV12, HAdV18 and HAdV31). Cells proliferated against HAdV5 as well as against other serotypes belonging to the same species (C), which have extensive homology with HAdV5. However, proliferation was also observed against the less homologous serotypes from species B and species A in several bulk cultures (example shown in Figure 3A). These results suggest that HAdV serotypes belonging to different species share T cell epitopes.

The recognition pattern of different HAdV serotypes was further investigated at the clonal level. To this end, T cell clones were generated by limiting dilution from 28-day bulk cultures and resulting clones were tested for specificity using uninfected and HAdV5-infected autologous EBV-transformed B cells (B-LCL). FACS analysis showed that HAdV-specific T cell clones were $CD4^+$, IFN-γ producing cells which is in agreement with the phenotype of the responding cells from bulk cultures (data not shown).

In total, 11 independent T cell clones from 4 different donors were tested for cross-reactivity against HAdV belonging to species C (HAdV1, HAdV5 and HAdV6), species B (HAdV3, HAdV7, HAdV11, HAdV34 and HAdV35) and species A (HAdV12, HAdV18 and HAdV31). Proliferation assays showed three different recognition patterns. Two clones were reactive against species C viruses only (Figure 3B). Four clones recognized both species C and species B viruses (Figure 3C). Finally, five clones recognized HAdV from all three species tested, showing the broadest reactivity profile (Figure 3D). HAdV5-specific clones used either HLA-DR or HLA-DP as restriction element for antigen recognition, as assessed by addition of blocking antibodies to HLA-DR and HLA-DP (Figure 3B-D). There was no correlation between the restriction element (HLA-DR or -DP) and the cross-reactivity pattern. Although reactivity of HAdV-specific T cell cultures to other serotypes has been reported previously (14,44), this is the first report in which extensive cross-reactivity of HAdV-specific $CD4^+$ T cells is described at the clonal level indicating that different HAdV strains harbour shared antigenic epitopes.

In general, human T cell recognition of epitopes that are conserved between different but related subtypes of viruses has been described for enteroviruses, herpes viruses, flaviviruses and influenza A viruses (1,7,26,30) and is not unique for HAdV.

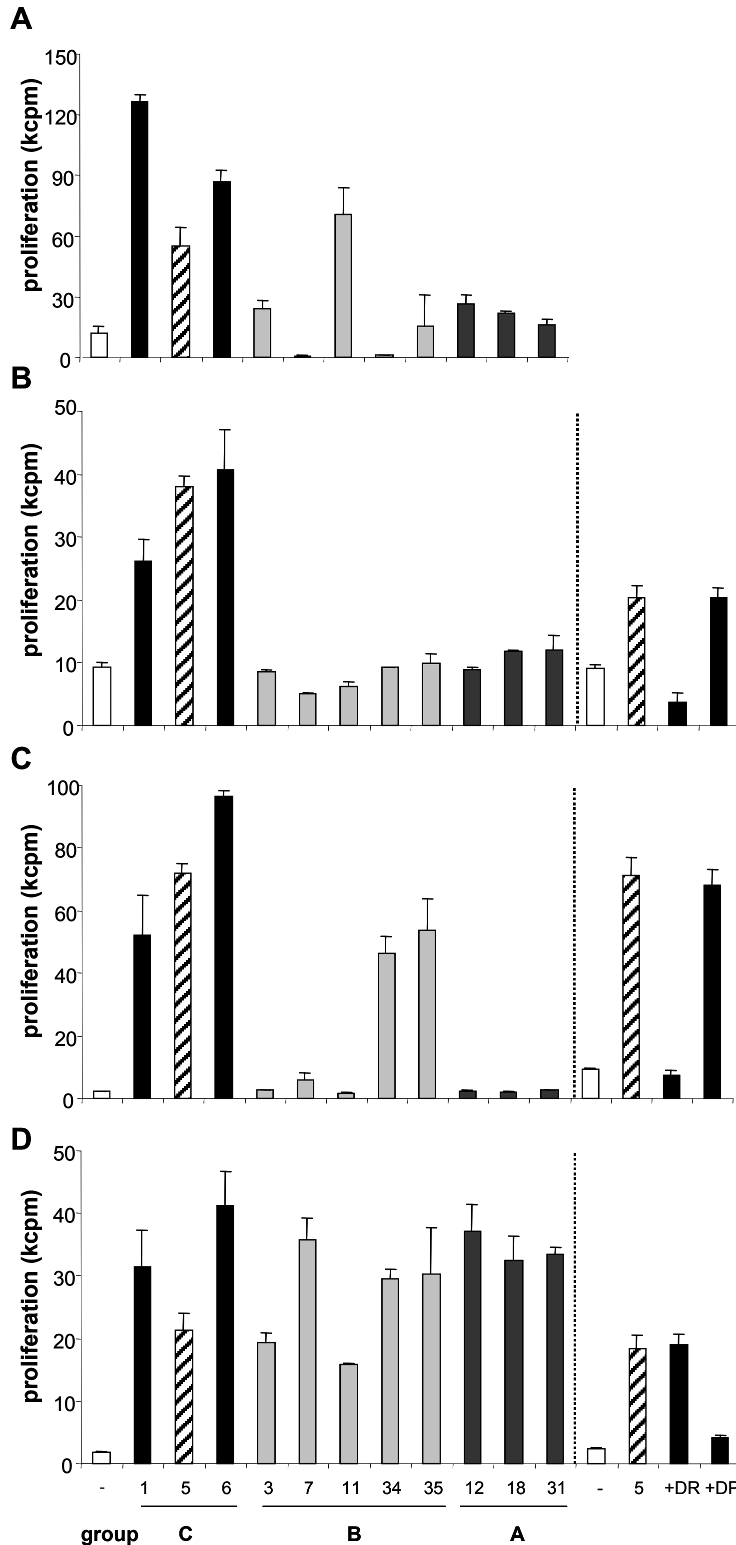

Figure 3. Cross-reactivity of T cells from HAdV5-induced bulk cultures and CD4+ T cell clones. A) Twenty-eight day T cell cultures were tested in a 6-day proliferation assay for reactivity against irradiated autologous PBMC without virus (-) or infected with HAdV strains from species C (HAdV1, 5, 6), B (HAdV3, 7, 11, 34, 35) and A (HAdV12, 18, 31). The response pattern of a representative donor out of 4 donors tested is shown. B-D) CD4+ T cell clones, obtained by limiting dilution, were stimulated with irradiated autologous B-LCL without virus (-) or infected with HAdV strains from species C (HAdV1, 5, 6), B (HAdV3, 7, 11, 34, 35) and A (HAdV12, 18, 31) and tested in a 4-day proliferation assay. B) represents a clone with restricted recognition (only species C). C) represents a clone with a broader reactivity against both species C and B serotypes. D) depicts a clone which recognizes HAdV from all species tested (species A, B and C). Blocking antibodies to HLA-DR and HLA-DP were added to B-LCL infected with HAdV5 (hatched bars) to determine the restriction element (separate experiment, dashed line; anti-HLA-DQ and anti-class I was also added and did not block responses).

Recently, it has been proposed that exposure of individuals to consecutive infections with different strains of a virus will result in repeated cycles of stimulation and expansion of those T cells that recognize shared epitopes (15). In this way, consecutive infections with different strains of HAdV could explain the observed cross-reactivity. Such cross-reactivity implies the presence of sequence homology in structural proteins between the HAdV serotypes from different species. For instance, the hexon protein which is the major structural protein and comprises about 95% of the capsid, is approximately 80% homologous between species A, B and C (10). Further characterization of the cross-reactive T cell epitopes will demonstrate whether these shared epitopes are completely identical between HAdV strains or whether amino acid differences exist that have no crucial effect on HLA-binding or T cell receptor recognition.

Pre-existing immunity limits the application of HAdV5 both in gene therapy protocols as well as in vaccine delivery. The neutralizing antibody response to the HAdV-based vector may be circumvented efficaciously by subsequent use of vectors containing hexons from different serotypes (37,48). Pre-existing cellular immunity, however, may not be as easily circumvented by this hexon gene switch strategy due to the extensive cross-reactivity of T cells described in this study.

In conclusion, our results show that HAdV-reactive T cells can be cultured after stimulation with MB-inactivated HAdV5. In order to control HAdV infections in patients, these T cells should probably be infused pre-emptively when HAdV DNA is detected for the first time in plasma by PCR, indicating dissemination of the infection (12,27,41). The high degree of cross-reactivity enables the use of these cells in immunocompromised patients irrespective of the serotype of the HAdV-strain that infects a particular patient and may thus be a valuable tool to decrease the mortality rate in this pediatric patient group.

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Chapter 6

Human CD4$^+$ T cells preferentially recognize conserved peptides of the adenovirus hexon protein: consequences for immunotherapy and gene therapy

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Submitted

ABSTRACT

The immune response against human adenovirus (HAdV) has gained interest because of the high incidence of infections in pediatric recipients of an allogeneic stem cell graft and because of gene therapy with HAdV-based vectors. Since antiviral medication is frequently ineffective, the option of adoptive transfer of HAdV-specific donor-derived T cells receives much attention. Responses against HAdV5 in PBMC of healthy adults are primarily found in $CD4^+$ T cells suggesting that a large proportion of the response is directed against structural viral proteins. Here we compare T cell recognition of HAdV5 hexon and penton capsid proteins with that of E1A, one of the early proteins. Most donors responded to the conserved C-terminal part of the hexon protein. By screening a panel of overlapping peptides of the hexon protein, five peptides were identified, that were recognized by the majority of donors. $CD4^+$ T cells from long-term cultures of PBMC stimulated with this panel of five peptides recognized not only HAdV5, but also other HAdV serotypes belonging to different species. These data demonstrate that conserved peptides are present in HAdV, which have consequences for gene therapy, but also may represent a useful tool for the generation of HAdV-specific T cells for adoptive immunotherapy.

INTRODUCTION

Human adenovirus (HAdV) infections are usually mild and self-limiting in the immunocompetent host. However, recently it has been recognized that HAdV may cause life-threatening complications in severely immunocompromised patients, such as pediatric recipients of allogeneic stem cell transplants (1-5). In recent years, the incidence of HAdV infection increased in these recipients (6-8) (and van Tol, submitted). Risk factors for HAdV infection included HLA-mismatched transplants, the use of immunosuppression with ATG and Campath and delayed immune reconstitution (8), indicating a role for the adaptive immune system. Treatment of HAdV infection in immunocompromised patients with ribavirin and cidofovir are being administered, but these two drugs have not been shown to be unequivocally efficacious and closely monitoring of patients indicates that immune recovery is essential to eliminate the virus (9-14). Therefore, immunotherapy by infusion of HAdV-specific donor-derived T cells might be an option for treatment. Case reports have already reported that infusion of donor lymphocytes contributes to the clearance of HAdV infection (15,16). Since infusion of unselected donor lymphocytes bears the risk of inducing graft-versus-host disease (15), it would be more attractive to infuse only HAdV-specific donor cells. In peripheral blood mononuclear cells (PBMC) of healthy donors the response to HAdV is dominated by $CD4^+$ T cells recognizing structural viral proteins (17-21).

To date, 51 serotypes of HAdV have been described, which are classified into 6 species (A to F). Species A, B and C are responsible for most of the infections in pediatric recipients

(8,13,22). The protein capsid of the virion is composed of seven polypeptides. Polypeptide II is the most abundant virion component forming the hexon protein. The penton base protein, present at each vertex of the icosahedral particle, is formed by five polypeptides III (reviewed by (23)). Since HAdV-specific T cells show cross-reactivity between the different serotypes and species, the epitopes recognized by the HAdV-specific T cells are probably derived from conserved parts of the structural proteins (17,19,20). Until now, only one epitope for $CD4^+$ T cells has been reported, which is restricted by HLA-DP4 (24,25). For CD8+ T cells, 5 epitopes have been identified that are restricted by HLA-A1, -A2, -A24 and -B7 (26). For both studies, peptides were selected by a predictive algorithm. Although 50% or more of the population has an HLA-A1, -A2, -B7 or -DP4 allele, these data do not guarantee that the selected peptides are widely recognized in the population. To perform a comprehensive unbiased study of T cell epitopes of HAdV, we investigated the recognition of several HAdV5 proteins, i.e. the structural polypeptide II, and polypeptide III and the early protein E1A. Since polypeptide II was recognized more frequently, this protein was synthesized as a set of overlapping peptides. Screening of PBMC from a panel of healthy donors revealed that a relatively limited set of peptides was able to evoke responses in almost all donors, apparently independently of HLA-typing. $CD4^+$ T cells specific for these peptides recognized target cells pulsed with HAdV of different serotypes, thus avoiding the use of complete virus or recombinant virus for the production of T cells for clinical application in immunotherapy.

MATERIALS & METHODS

PBMC

PBMC were isolated from blood of anonymous healthy blood bank donors; these donors were typed for HLA-A, B, C and HLA-DR. The HLA-types of the selected donors represented the normal distribution of HLA-alleles in the Dutch population.

Antigens

The HAdV5 polypeptide II gene in plasmid pJM17 (kindly provided by Prof. R. Hoeben, Department of Molecular Cell Biology, Leiden) was amplified by PCR in four overlapping parts (Table 1). The PCR products were cloned by Gateway technology (Invitrogen, San Diego, CA) in a bacterial expression vector containing an N-terminal histidine tag. The proteins were overexpressed in *Escherichia coli* BL21 (DE3), purified, dissolved in DMSO and diluted in PBS (27). The four overlapping parts are indicated by the numbers of the first and last amino acid in the polypeptide (IIA_{1-273}, $IIB_{245-509}$, $IIC_{479-743}$, $IID_{720-952}$). Recombinant HAdV5 polypeptide III and E1A protein were gifts from A. den Boer and Dr. S.H. van den Burg (Department of Immunohematology and Bloodtransfusion, Leiden).

Peptides spanning the HAdV5 polypeptide II consisted of 63 peptides of 30 amino acids with an overlap of 15 amino acids and are indicated as II4 to II66. They were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS22, Langenfeld, Germany). Peptides were analyzed by reverse phase HPLC, dissolved in DMSO and diluted in PBS. The 63 peptides were grouped in 12 pools; each pool containing 5-6 adjacent peptides (e.g. peptide pool P1 consisted of peptide II4 to II9, pool P2 of peptides II10 to II15, etc).

Table 1. Primers for recombinant polypeptide II proteins

Recomb. protein	Primer	Nucleotide of polypepide II gene	Specific part for cloning system	Specific polypeptide II sequence
$IIA_{1\text{-}273}$	IIA-sense	225 - 244	5'-ggggacaagtttgtacaaaaaagcaggctta-	atggctaccccttcgat
	IIA-antisense	1043 - 1026	5'-ggggaccactttgtacaagaaagctgggtca-	ggtcgcctcagtagttg
$IIB_{245\text{-}509}$	IIB-sense	960 - 977	5'-ggggacaagtttgtacaaaaaagcaggctta-	gggcaaggcattcttgt
	IIB-antisense	1751 - 1732	5'-ggggaccactttgtacaagaaagctgggtca-	caccactcgcttgttca
$IIC_{479\text{-}743}$	IIC-sense	1659 - 1676	5'-ggggacaagtttgtacaaaaaagcaggctta-	ctgtatttgcccgacaag
	IIC-antisense	2453 - 2436	5'-ggggaccactttgtacaagaaagctgggtca-	aaactcgttgggggtaag
$IID_{720\text{-}952}$	IID-sense	2382 - 2400	5'-ggggacaagtttgtacaaaaaagcaggctta-	aaggtggccattaccttt
	IID-antisense	3083 - 3066	5'-ggggaccactttgtacaagaaagctgggtca-	ttatgttgtggcgttgcc

HAdV
Wildtype HAdV2, HAdV5, HAdV6, HAdV12, and HAdV35 (RIVM, Bilthoven, the Netherlands) were grown on Hep2 cells and purified (8). Methylene blue (MB) inactivation of virus was performed as previously described (28).

T cell stimulation
PBMC were cultured in RPMI 1640 (GibcoBRL, Grand Island, NY) supplemented with 10% human AB serum (AB) at 2×10^6 cells/mL. Cells were stimulated with 5 µg/mL recombinant protein or peptide, HAdV5 at a multiplicity of infection (MOI) of 10, or with medium alone as negative control. Cells were cultured for 4 or 5 days either in a round-bottom 96-wells plate (Corning Incorporated, Life Sciences, Acton, MA) in order to measure the amount of secreted IFN-γ by ELISA or in a 48-wells plate (Corning Incorporated) to determine the IFN-γ spot forming units (SFU) by ELIspot or by intracellular cytokine staining and flow cytometry.

IFN-γ levels in culture supernatant
To determine which peptide pools were recognized by PBMC of healthy donors, we measured the level of IFN-γ secreted by PBMC in culture supernatants after 4 days. Secreted IFN-γ was determined using an ELISA kit (Sanquin, Amsterdam, the Netherlands). The response to a distinct peptide pool was considered positive if the IFN-γ production to this pool was at least 10% of the sum of IFN-γ production in response to all 12 peptide pools.

IFN-γ ELIspot
Recognition of the recombinant proteins or polypeptide II peptides by T cells was analyzed by ELIspot as described (29). After 4 days of culture the cells were transferred to an αIFN-γ IgG antibody (MabTech, Nacha, Sweden) coated Multiscreen 96-wells plate (Millipore, Etten-Leur, the Netherlands), which was blocked for aspecific binding by 10% FCS (GibcoBRL) in RPMI at a concentration of 1×10^5 cells per well. After 16 h of incubation the spot forming units (SFU) were visualized using αIFN-γ IgG antibody MAb 7-B6-1-biotin (Mabtech), Avidin-AP (Sigma-Aldrich, St. Louis, MO), followed by the substrate NBT-BCIP (NitroBlue tetrazolium and 5-Bromo-4-Chloro-3-indolyl phosphate (Sigma-Aldrich) in 0.1 M Tris-HCl pH 9.5 and 5 mM $MgCl_2$. In between each detection step the plate was washed with PBS/0.05%Tween-20. Spots with a diameter > 35 µm were counted by computer-assisted video image analysis using KS ELIspot software release 4.1 (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). The response was considered as positive if the mean of the number of spots was > 25 spots and exceeded the mean of the negative control + 3 times SD.

Intracellular cytokine staining
To determine the phenotype of the IFN-γ-producing cells, cells were incubated at day 5 for 5 h with 5 µg/mL Brefeldin A (BFA, Sigma-Aldrich) for intracellular IFN-γ staining. The procedure was performed as described (30). In short, cells were washed with PBS containing 0.2% w/v NaN_3 and fixed with freshly made 4% paraformaldehyde (Sigma Aldrich). Cells were permeabilized with PBS/NaN_3 containing 0.1% saponin and 0.5% BSA, and non-specific binding was blocked by incubating with PBS/NaN_3/saponin/BSA/10% FCS. Cells were stained with αCD3-PerCP-Cy5.5,

αCD4-FITC, αIFN-γ-PE (Becton Dickinson Biosciences, San Jose, CA) and αCD8-APC (Beckman Coulter, Miami, FL). Cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson).

DC generation
PBMC were cultured at a concentration of 2-3×10^6 cells/mL in a T75 flask in RPMI enriched with 10% FCS. After 2 h the cells were washed and the non-adherent fraction (peripheral blood lymphocytes, PBL) was removed. The adherent fraction was cultured in RPMI with 10% FCS supplemented with 800 U/mL recombinant GM-CSF (rGM-CSF, Leucomax, Novartis Pharma AG, Basel, Switzerland) and 40 ng/mL recombinant IL-4 (Peprotech, Rocky Hill, NJ) for 5 days. These cells were referred to as immature DC (iDC) and were CD14 negative and CD1a positive as measured by FACS analysis. The iDC were transferred to a round-bottom 96-wells plate at a concentration of 1×10^4 DC/well in RPMI enriched with 10% AB supplemented with 800 U/mL rGM-CSF and 40 ng/mL IL-4. After 24 h, the iDC were matured by adding 0.25 µg/mL αCD40 (Mab89, Immunotech, Beckman-Coulter, Miami, FL) and 500 U/mL IFN-γ for an additional 24 h. Mature DC were typically CD14 negative and CD83 positive, as assessed by FACS analysis (31).

T cell culture
Mature DC were loaded with peptides in a volume of 25 µl/well of RPMI containing 1 mM of Ca^{2+}/Mg^{2+} at 37°C. After 2 h of incubation, autologous PBL were added at a DC:PBL ratio of 1:20 in 200 µl RPMI/10% AB supplemented with 10 IU/mL of IL-2 (Proleukin, Chiron, Emeryville, CA) and 2 ng/mL of IL-7 (Peprotech).
At day 12, responding cells were restimulated using autologous irradiated PBMC (30 Gray) loaded with the same concentration of peptides as used for loading the DC at the initiation of the culture in RPMI/0.5% BSA at 1×10^7 cells/mL for 2 h. The T cells were co-cultured with the stimulator cells in a T cell: stimulator ratio of 1:5 in a 24-wells plate in RPMI/10% AB in the presence of 0.1 µg/mL anti-CD40 and 5 ng/mL IL-7. At day 19, 10 U/mL IL-2 and 5 ng/mL IL-7 were added. At day 12 and 26 cells were tested for specificity.

Proliferation and IFN-γ production by peptide-specific T cells
To test T cell specificity, autologous irradiated stimulator PBMC were infected at day -1 with HAdV5 at a MOI of 100 or loaded with 10 µg/mL peptide, or medium alone as a negative control in RPMI/0.5% BSA at 1×10^7 cells/mL for two h and left overnight at 1×10^6 cells/mL in RPMI/10% AB. For intracellular IFN-γ production, 2×10^5 responder cells were co-cultured with 2×10^5 stimulator cells in 400 µl in a polystyrene round-bottom tube (5 mL Falcon, Becton Dickinson, Franklin Lakes, NJ). After 1 h, 5 µg/mL BFA was added, and 16 h later intracellular staining was performed as described above.
To determine the specific proliferative response, 2×10^4 responder cells were co-cultured with 1×10^5 stimulator cells in triplicate in a 96-well round-bottom plate. At day 5, [^{3}H]-thymidine (0.5 µCi/well, Amersham International, Amersham, UK) was added for 16 h.

RESULTS

Selecting HAdV5 peptides

We have shown that MB-inactivated HAdV induces $CD4^+$ T cells that can recognize cells with infectious HAdV, suggesting that epitopes for these T cells are derived from structural polypeptides (19). Because of the abundance of polypeptide II in the virion capsid, we compared the recognition of the recombinant proteins IIA_{1-273}, $IIB_{245-509}$, $IIC_{479-743}$, $IID_{720-952}$, III and, for comparison, E1A in cultures of PBMC from healthy donors and measured the IFN-γ SFU. A representative reaction profile is shown in Figure 1A, where PBMC of donor A responded to recombinant protein $IIC_{479-743}$, $IID_{720-952}$ and III (Figure 1A). The responses to IIA_{1-273}, $IIB_{245-509}$ and E1A were below 25 spots and were considered negative.

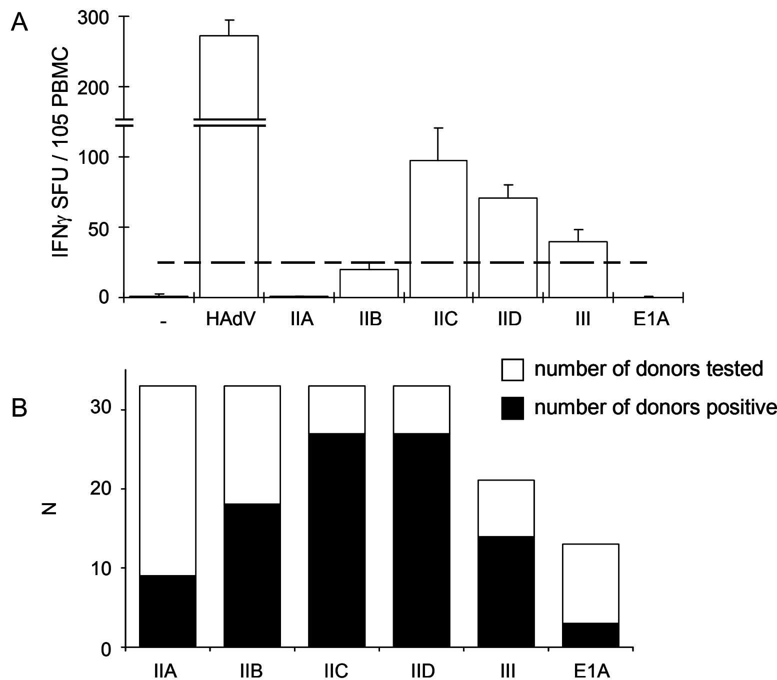

Figure 1. Recognition of HAdV5 recombinant polypeptides II and III. (A) PBMC of healthy donor A were cultured for 4 days with HAdV5 or recombinant polypeptides, after which IFN-γ SFU per 1×10^5 PBMC was measured. Dotted line represents threshold (25 spots) above which the response is considered positive. Results are representative for the results obtained from 33 donors. (B) Overview of healthy donors tested for their recognition of the recombinant proteins IIA-IID, III and E1A. White bar represents the number of donors tested, black bar the number of donors responding to the recombinant protein.

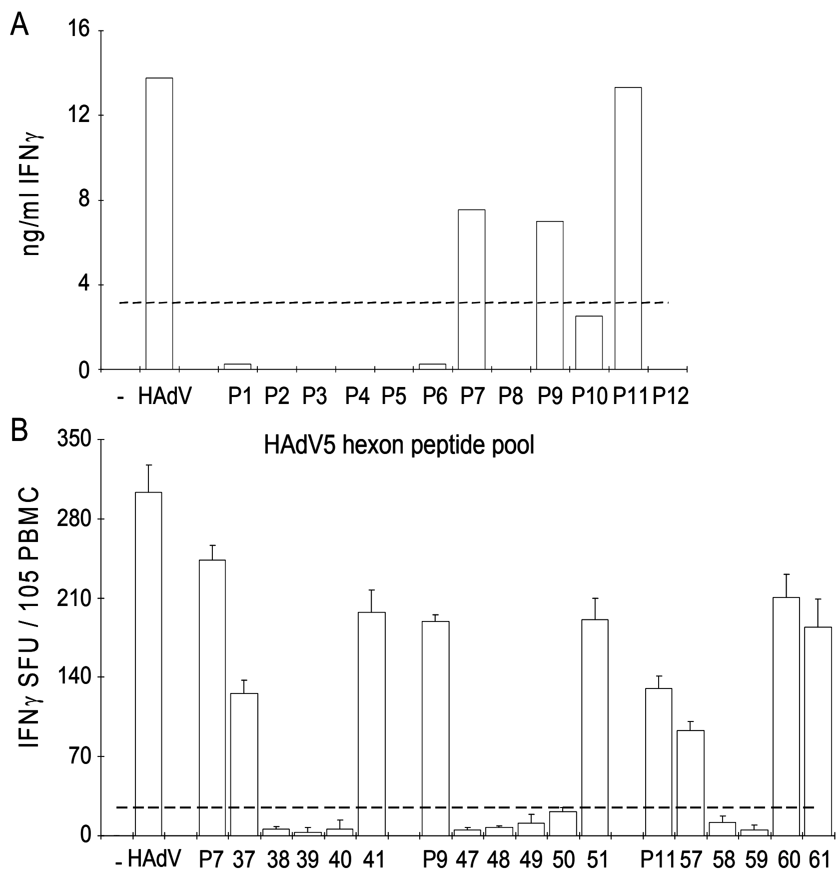

Figure 2. Recognition of HAdV5 polypeptide II peptides. PBMC of healthy donor B were cultured for 4 days (A) with peptide pools (P1-P12), after which the amount of IFN-γ secreted in culture supernatant was determined or (B) with the single peptides of the selected peptide pools, after which the IFN-γ SFU per $1\text{x}10^5$ PBMC was measured. Dotted line represents threshold above which the response is considered positive. Results are from one donor and representative for the results obtained from 21 donors.

In total, PBMC from 33 healthy donors, recognizing HAdV5, were tested. The C-terminal part of polypeptide II, which includes recombinant proteins $IIC_{479-743}$ (82%) and $IID_{720-952}$ (82%), was recognized by more donors than the N-terminal part of polypeptide II, IIA_{1-273} (27%), $IIB_{245-509}$ (55%), or recombinant polypeptide III (67%) (Figure 1B). The recognition of the early gene product E1A (20%) was low. These data indicate that the conserved part of polypeptide II contains widely recognized T cell epitopes.

To further elucidate recognition of the hexon protein, overlapping peptides of 30 amino acids with an overlap of 15 amino acids of the complete polypeptide II (peptide II4 to II66) were synthesized and tested. PBMC of donor B contained T cells that recognized peptide pools P7, P9 and P11 (Figure 2A). Next, PBMC of donor B were cultured with the single peptides of each of the positive peptide pools, i.e. peptides II37-II41 of peptide pool P7, peptide II47-

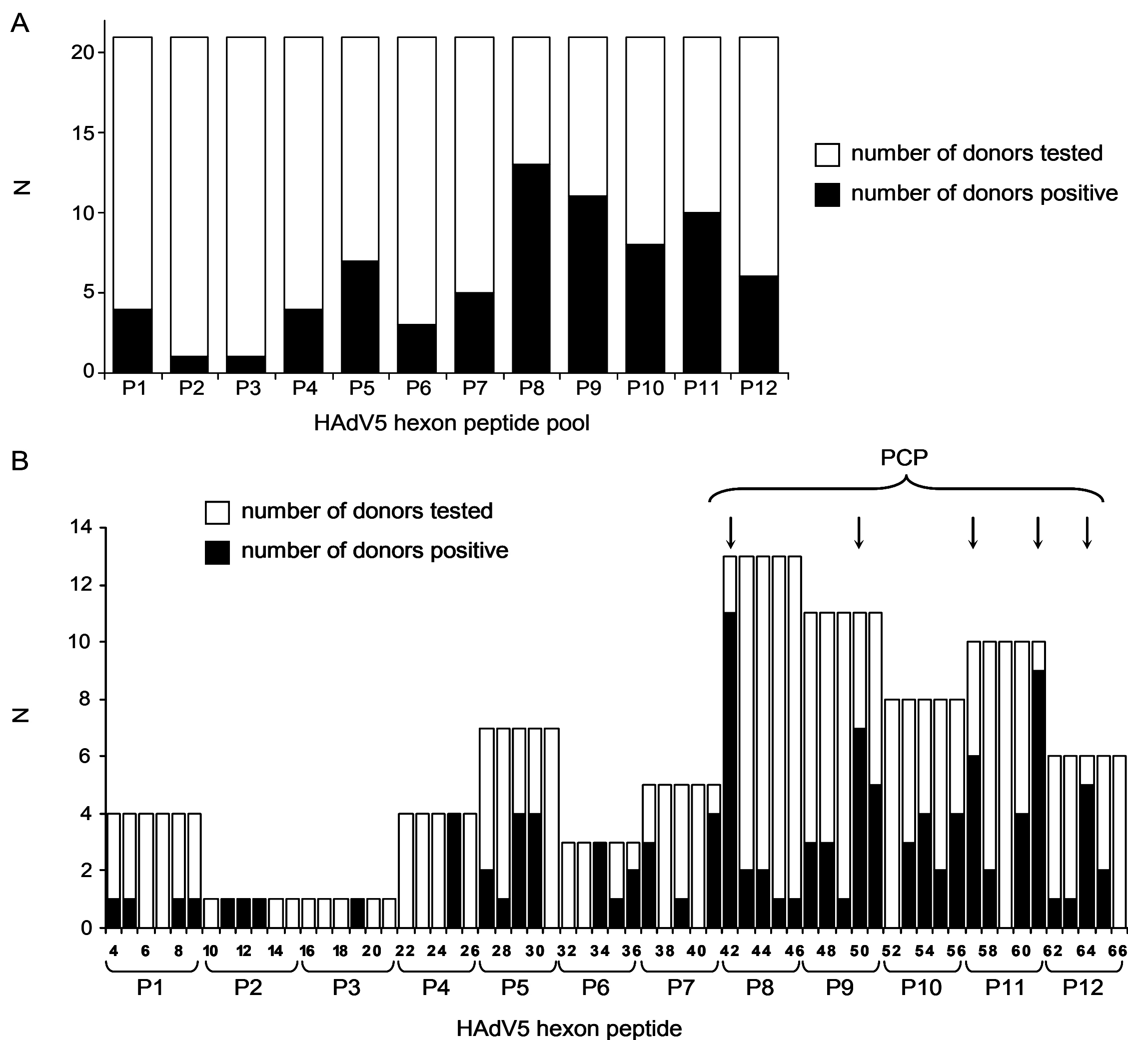

Figure 3. Overview of recognition of HAdV5 polypeptide II peptides. (A) PBMC of 21 donors were tested for their recognition of polypeptide II peptide pools (P1-P12) (IFN-γ secretion in culture supernatant). (B) Donors with a positive response to the peptide pool were tested for their recognition of the single peptides from that pool (IFN-γ SFU). White bar represents the number of donors tested, black bar the number of donors recognizing the indicated (A) peptide pool or (B) the indicated single peptide. Peptides selected to constitute the "peptide culture pool" (PCP) are indicated by an arrow.

II51 of peptide pool P9 and peptides II57-II61 of peptide pool P11, and the response was measured by ELIspot assay. Responses against HAdV5 or medium alone were used as positive and negative control, respectively. Positive responses were observed against peptides II37, II41, II51, II57, II60 and II61 (Figure 2B).

A total of 21 donors was tested accordingly. Most responses were observed against peptides from peptide pools at the C-terminal part of polypeptide II, which is in agreement with the results obtained using the recombinant polypeptides (Figure 3A). Peptides from pools P8 (13 donors), P9 (11 donors) and P11 (10 donors) were recognized most frequently. At the level of single peptides most donors responded to peptide II42 (11 out of the 13 peptide pool P8 positive donors), peptide II61, peptide II50 and peptide II57 (Figure 3B). Peptide II64 of peptide pool P12, containing epitope $II_{910\text{-}924}$ was recognized by 5 of the 6 peptide pool positive donors (24). This screening resulted in 5 peptides of polypeptide II, which are frequently recognized by PBMC of healthy donors.

Since it would be attractive for adoptive immunotherapy to culture PBMC with HAdV peptides instead of complete virus, these five most recognized peptides (peptides II42, 50, 57 61 and 64 (Figure 3B)) were selected and combined to a HAdV peptide culture pool (PCP). To test whether these five single peptides and the PCP were able to induce responses in the majority of donors, 14 different healthy blood bank donors, responding to HAdV5, were tested. Thirteen (92%) of these donors recognized one or more of the 5 peptides as well as PCP, whereas only one donor did not recognize any of the peptides (Table 2).

Table 2. Recognition of 5 selected HAdV5 hexon peptides by 14 donors

	peptides of HAdV PCP					
donor	II42	II50	II57	II61	II64	PCP
1	+	+	+	+	+	+
2	+	-	+	+	+	+
3	+	+	-	-	-	+
4	-	-	+	+	+	+
5	-	-	-	-	-	-
6	-	-	-	+	-	+
7	-	+	-	-	+	+
8	+	-	+	+	+	+
9	+	-	-	-	-	+
10	-	-	+	+	+	+
11	+	-	+	+	+	+
12	-	-	-	+	-	+
13	+	+	-	-	-	+
14	+	+	+	+	+	+
positive	8	5	7	9	8	14

IFN-γ SFU / 10^5 PBMC: +: ≥ 25 spots and exceeding the mean of the negative control + 3 times SD

The differences in percentages of responders during screening for the identification of the reactive peptide (Figure 3B) and during testing the selected peptides (Table 2) can be

explained by the use of different techniques. The IFN-γ ELISA used to determine the positive peptide pools in a first screening is not as sensitive as the ELIspot technique used for identification and recognition of the single peptides.

HAdV5 PCP almost exclusively induced IFN-γ production in the $CD4^+$ T cell population, which was also observed after culture with wild type HAdV5 (data not shown). Thus, with the set of these 5 peptides, we are able to stimulate T cells of most donors, and these reactive T cells show the same phenotype as T cells cultured with intact virus.

Peptide-generated T cells recognize HAdV

To investigate whether PCP reactive T cells also recognize cells infected with HAdV, PBL of healthy donors were cultured for 28 days with HAdV5 PCP in different concentrations (range 1 µg/mL to 0.08 ng/mL). T cells stimulated with low concentrations of PCP (optimal concentration 0.4 ng/mL) recognized MB-inactivated HAdV5 better than T-cells stimulated with high concentrations of PCP, although at all concentrations T cells were peptide specific (data not shown). At day 12, 3.0% of the $CD4^+$ T cells recognized autologous PBMC loaded with MB-inactivated HAdV5, which increased to 8.6% at day 26 (Figure 4A). The recognition of HAdV PCP itself increased from 7.4% of the $CD4^+$ T cells at day 12 to 21.9% at day 26 (Figure 4A). Besides IFN-γ production, these peptide reactive T cells also showed proliferative capacity specifically to cells pulsed with MB-inactivated HAdV5 at day 12 and 26 (Figure 4B). These data show that T cells cultured with HAdV peptides are able to recognize MB-inactivated HAdV5.
To investigate whether peptide-cultured T cells also recognize other HAdV serotypes, PBL cultured with 0.4 ng/mL HAdV5 PCP were tested at day 26 against autologous PBMC pulsed with one of 8 different MB-inactivated HAdV serotypes from 4 different species. The $CD4^+$ T cells from this culture responded to all 8 MB-inactivated HAdV from species A, B, C and D in IFN-γ production and proliferation (Figure 5A and B), indicating that these peptide-cultured T cells are indeed broadly reactive against different HAdV species.
Furthermore, it is important for adoptive immunotherapy that the cultured cells also recognize autologous PBMC infected with infectious HAdV. Ten percent of the $CD4^+$ T cells produced IFN-γ in response to infectious HAdV5 stimulation, confirming that these T cells not only recognized MB-inactivated virus but also infectious HAdV (Figure 6). In addition, these peptide-cultured T cells also recognized infectious HAdV35 infected stimulator cells, but did not show reactivity towards allogeneic PBMC (data not shown). Because the peptide generated T cells recognized infectious HAdV but not allogeneic PBMC, they may be good candidates for adoptive transfer.

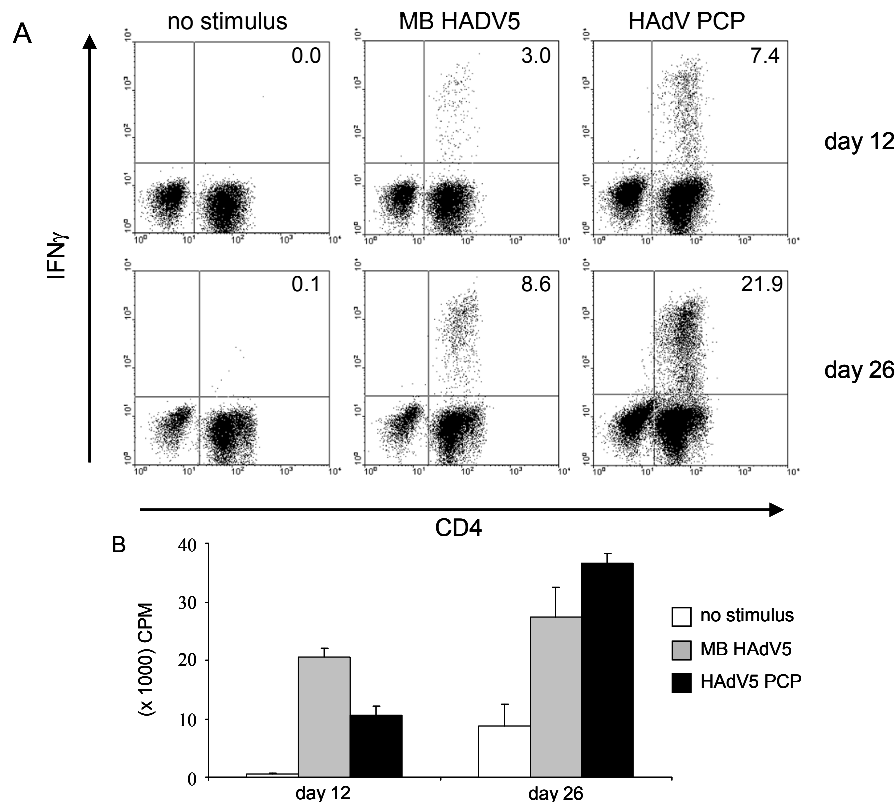

Figure 4. T cells cultured with HAdV5 PCP recognize MB-inactivated HAdV5. PBL were cultured for 26 days with 0.4 ng/mL PCP and tested at day 12 and 26 for recognition of autologous PBMC pulsed with MB-inactivated HAdV5 (MOI 100) (gray bars) in (A) IFN-γ production and (B) proliferation. Medium alone (white bars), referred to as no stimulus, and PBMC pulsed with HAdV5 PCP (black bars) were used as negative and positive control. Results are from one donor and representative for the results obtained from three different donors tested.

DISCUSSION

The main conclusion from this study is that HAdV polypeptide II, forming the hexon protein, contains multiple long peptides, that a) are recognized by $CD4^+$ T cells of a majority of the population, b) are conserved between HAdV serotypes, and c) induce T cells that recognize HAdV-infected target cells. Thereby, these peptides are good candidates for culture of HAdV-specific T cells for adoptive immunotherapy. It has already been reported that the responses of human peripheral blood ($CD4^+$) T cells to HAdV were directed (at least partly) against structural proteins (17,32). So far, no comparison in recognition of different HAdV proteins has been performed in the healthy human population. Comparison of T cell responses against the two structural proteins II (hexon) and III (penton) and one early gene product, E1A, of HAdV5 in the present study revealed that most T cell responses were directed against the structural polypeptides and the C-terminal part of polypeptide II in particular. This part represents the section that is most conserved between different serotypes (33,34), offering an explanation for the cross-reactivity found in cultured HAdV-specific T cells (17,19,20). In contrast, the epitopes recognized by serotype-specific neutralizing

antibodies are mainly located in the N-terminal part of the hexon protein (35,36). The phenomenon of recognition by T cells of epitopes conserved between different but related viral subtypes is also described for other viruses (37-40). An explanation for this observation could be that consecutive exposure of individuals to multiple infections with different viral subtypes will probably result in preferential expansion of those T cells, that recognize shared epitopes (41).

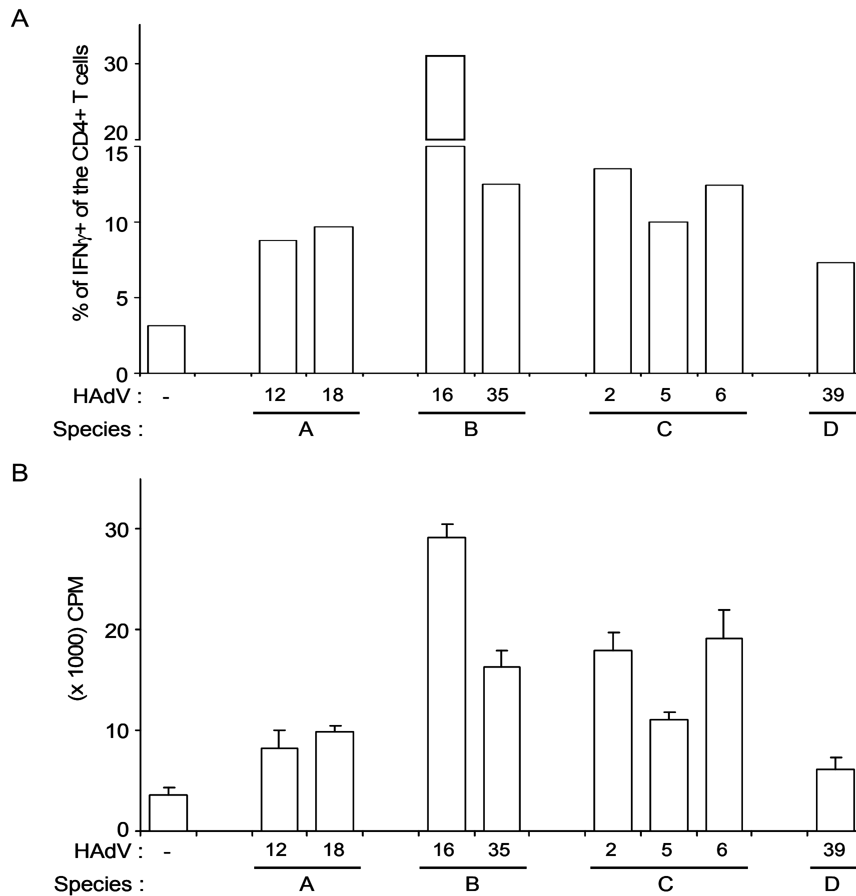

Figure 5. T cells cultured with HAdV5 PCP recognize HAdV from different species. PBL were cultured for 26 days with 0.4 ng/mL PCP and tested for recognition of autologous PBMC pulsed with MB-inactivated HAdV from different species (MOI 100) in (A) IFN-γ production (intracellular staining and flow cytometry) and (B) proliferation. Medium alone, referred to as no stimulus, was used as negative control. Results shown are of one donor and representative for the results obtained from two different donors tested.

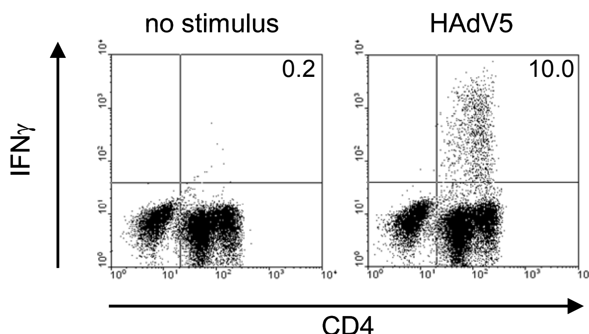

Figure 6. T cells cultured with HAdV5 PCP recognized active HAdV5. PBL were cultured for 26 days with 0.4 ng/mL HAdV5 PCP and tested for their recognition of autologous PBMC infected with HAdV5 (MOI 100) by measuring IFN-γ producing cells after intracellular staining. Medium alone, referred to as no stimulus, was used as negative control. Results are from one donor and representative for the results obtained from three different donors.

This is the first comprehensive study comparing T cell responses to HAdV hexon peptides in the healthy population. The five most frequently recognized peptides identified in this study are derived from the conserved part of polypeptide II. The amino acid sequences of these peptides were compared between different HAdV serotypes from various species. Peptide II42, 50, 61 and 64 show minimal differences between species (Table 3). Peptide II64 contains the described HLA-DP restricted, epitope $II_{910-924}$, which is conserved between many human and other mammalian adenoviruses (24). Peptides II42, 57 and 61 have not been described before as containing major T cell epitopes but are recognized at a high frequency (57%, 50% and 64%, respectively). Peptide 50 is recognized at slightly lower frequency (36%). No correlation between recognition of peptides and expression of HLA-DR alleles could be observed. It might be possible that, because 30-mers were used, the peptides contain two or more T cell epitopes (42). Another reason for this lack of correlation may be due to the fact that the hexon peptides are presented by certain HLA-DP alleles that occur very frequently in the population, as described for the $II_{910-924}$ peptide presented by HLA-DP4 (25). Establishment of correlations between HLA class II alleles and peptide recognition would require a much larger cohort of donors. However, for this study it was not our aim to determine the exact T cell epitopes of polypeptide II, but to select peptides in HAdV with broad recognition for culturing HAdV-specific T cell applicable in adoptive immunotherapy.

The results from this study might also have important implications for the use of recombinant adenoviral vectors for gene therapy and vaccination. It has been well established that pre-existing immunity, especially humoral, against the adenoviral vector can limit the application of HAdV5 for both gene therapy protocols and vaccine delivery. To circumvent the neutralizing antibody response to the HAdV-based vector different strategies are being pursued including the use of other human serotypes (43) and non-human adenoviruses (44). Recent studies suggest that T cells may also have a role in limiting the potency of HAdV5 gene transfer and vaccine potency (45). Our results indicate that human HAdV-specific T cells recognize conserved peptides of the hexon protein and therefore will also recognize vectors derived from other serotypes. In a C57/BL6 mouse model, no evidence for T cells cross-reactive between HAdV serotypes 5 and 35 was found using artificial induction of immunity against HAdV (46). Nevertheless, detailed studies on the interaction of HAdV and the human immune system are warranted to better understand the influence of cross-reactive T cells on gene therapy procedures and vaccination potency.

Table 3. Comparing amino acid composition of the peptides of PCP within different species

II42$_{571-600}$ HAdV5	n	l	l	l	l	p	g	s	y	t	y	e	w	n	f	r	k	d	v	n	m	v	l	q	s	s	l	g	n	d
Species A																						i				t				
B																														
C																														
E	s																					i								
F																						i								

II50$_{691-720}$ HAdV5	l	g	s	g	y	d	p	y	y	t	y	s	g	s	i	p	y	l	d	g	t	f	y	l	n	h	t	f	k	k
Species A					f				f	v				t			y,c													
B					f				f	v																				
C																														
E					f				f	v																				
F					f				f						v															

II57$_{796-825}$ HAdV5	r	n	f	q	p	m	s	r	q	v	v	d	d	t	k	y	k	d	y	q	q	v	g	i	l	h	q	h	n	n
Species A													t		e			n,e		k	k		t	v	e	f				
B													e	v,i	n		k,t		y,f	k	a		t,a	l,y	p	y				
C																		d,e												
E													e	v	n						a		t	l	a	y				
F												d,n	t		t		k,t	e			n		t	l	p	f				

II61$_{856-885}$ HAdV5	v	d	s	i	t	q	k	k	f	l	c	d	r	t	l	w	r	i	p	f	s	s	n	f	m	s	m	g	a	l
Species A		e,q		i,v										v	m															
B		k,n,t		v											m															
C																														
E		t		v						i				v	m															
F		p		l											m															

II64$_{901-930}$ HAdV5	l	d	m	t	f	e	v	d	p	m	d	e	p	t	l	l	y	v	l	f	e	v	f	d	v	v	r	v	h	r
Species A											d,e																	i		q
B																		l												q
C																														q
E				n															v											q
F																												i		q

As reference the amino acid sequence of HAdV5 is given (NCBI AAO24091). Species A: HAdV12 and 18; species B: HAdV3, 7, 11, 21, 34 and 35; species C: HAdV1 and 2, species E: HAdV4; species F: HAdV 40 and 41. No sequence data on HAdV species D are available. The indicated sequence in II64 is the hexon epitope described by M. Olive, *et al*, Hum. Gene Ther. 2002, 13:1167-1178

In allogeneic stem cell graft recipients, HAdV infection or reactivation can be a serious clinical problem (1-5). Since medication for HAdV infection is usually not effective, the option of transferring HAdV-specific T cells to the infected immunocompromised recipient is an attractive option for treatment. A number of studies suggested that it might be possible to generate HAdV-specific T cells for adoptive immunotherapy (19,20,47). The disadvantage of generating HAdV-specific T cells using infectious wild type HAdV might be the transfer of the virus strain to the patient. Introduction of replication-deficient virus harbors the risk of recombination with the wild-type virus in the patient. For these various reasons, it is very attractive to culture HAdV-specific T cells with HAdV peptides. In the present study we demonstrated that HAdV-specific T cells, recognizing wild-type HAdV-infected cells, can be generated using a panel of only 5 HAdV5 hexon peptides. Thirteen out of 14 (93%) healthy adult donors responded to this panel. Since 4 out of 5 selected peptides are conserved between HAdV species, the peptide-cultured $CD4^+$ T cells recognized HAdV of various species. This is of utmost importance because it is usually unknown in advance which serotype will infect a certain patient.

HAdV peptide-cultured T cells of healthy adults and pediatric patients are $CD4^+$ T cells, as is also mainly found for HAdV-stimulated T cells (8,19). The function of these $CD4^+$ T cells might be indirect, by cytokine (i.e. IFN-γ) secretion, providing help to B cells or inducing maturation of dendritic cells, or direct, by lysis of infected target cells (Heemskerk, in preparation).

In conclusion, a set of five HAdV5 peptides derived from the conserved region of the hexon forming protein polypeptide II were recognized by T cells of most adult donors. With this peptide pool, HAdV-specific T cells were generated that not only respond to HAdV5 but also to other HAdV serotypes belonging to various species. These data suggest that these peptides are promising candidates for generating HAdV-specific T cells for adoptive immunotherapy, as peptides can easily be generated under GMP conditions. Infusion of these HAdV-specific T cells may be a valuable tool to decrease the high mortality rate due to HAdV viremia in immunocompromised pediatric patients after allogeneic stem cell transplantation.

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Chapter 7

Adenovirus-specific $CD4^+$ T cell clones inhibit viral replication *in vitro* through cognate interaction by perforin-mediated lysis

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Submitted

ABSTRACT

Adenovirus (HAdV) infection is a frequent and potentially severe complication following allogeneic stem cell transplantation in children. Since treatment with the antiviral drugs ribavirin or cidofovir is often ineffective, adoptive transfer of donor-derived HAdV-specific T cells may be an option for therapy of HAdV viremic immunocompromised patients. In healthy donors, predominantly $CD4^+$ HAdV-specific T cells are detected. In this study, a pre-clinical *in vitro* model was used to analyze whether HAdV-specific human $CD4^+$ T cells have an antiviral effect. $CD4^+$ HAdV-specific T cell clones from 5 different donors were generated. These clones all produced IFN-γ and most clones recognized peptides that were derived from the conserved part of the hexon protein. HAdV-specific T cell clones were able to control viral replication as viral titers in HAdV-infected EBV transformed B cells (B-LCL) were reduced 100- to 1000-fold in the presence of HAdV-specific T cells. In a co-culture of T cells with peptide-loaded autologous B-LCL as well as HAdV-infected HLA-mismatched B-LCL no inhibition of viral outgrowth was observed, suggesting that cognate interaction between T cells and target cells was necessary to control viral replication. The HAdV-specific $CD4^+$ T cell clones expressed perforin and granzyme B and were able to specifically lyse infected target cells using a perforin-dependent mechanism. Together, these results show that 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.

INTRODUCTION

Adenoviruses (HAdV) rarely cause severe clinical symptoms in healthy children and adults as 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 $CD34^+$ enriched allogeneic stem cell graft, i.e. patients with a non-HLA-identical donor, have a higher risk of developing HAdV infection and dissemination, due to the delayed immune reconstitution in these children after SCT (7,8), and van Tol, manuscript in preparation). Clinical symptoms include gastroenteritis, hemorrhagic cystitis, hepatitis, pneumonia, encephalitis and multi-organ failure. Dissemination of the infection, documented by a rise of HAdV DNA loads in plasma, is associated with a fatal outcome (7,9-13).

Currently, 51 serotypes of adenovirus have been identified, distributed among six species (A-F) based on their differential hemagglutination with erythrocytes and their DNA homology (14,15). Species A, B and C serotypes are most frequently isolated from pediatric immunocompromised hosts and are the major cause of disease (1,7,16-18).

Treatment of adenoviral infections with antiviral medication such as cidofovir and ribavirin has not been unequivocally effective (reviewed in (19)). Therefore, new strategies to treat HAdV infections are needed. A potential approach is adoptive immunotherapy by infusion of HAdV-specific lymphocytes. This strategy has already been successfully pursued for other viral infections or reactivations such as CMV or EBV (20-22). Furthermore, case reports have suggested that donor lymphocyte infusions (DLI) or tapering of immunosuppression may contribute to clearance of an HAdV infection, illustrating the potential role of T cells in the immune response to HAdV (8,23,24).

Few reports on HAdV-specific immune responses in healthy donors have been published until recently (18,25,26). Current reports on the occurrence and frequency of HAdV-specific T cells have shown that predominantly HAdV-specific CD4$^+$ T cells are detected, although HAdV-specific CD8$^+$ T cells have been described (27-31). Furthermore, in a previous report we have shown the presence of CD4$^+$ HAdV-specific T cells in the blood of patients recovering from HAdV infection or viremia (7), suggesting that these cells may be functionally involved in clearance of the virus.

As mainly CD4$^+$ HAdV-specific T cells can be detected in healthy donors and patients after SCT, the question arises whether these CD4$^+$ T cells can exert direct antiviral functions and if so, by which mechanisms. To study this, we generated HAdV-specific CD4$^+$ T cell clones as described previously (30), and determined the HLA restriction, protein and peptide specificity of these clones. To investigate the antiviral activity of these T cells, inhibition of viral replication in HAdV-infected EBV transformed B cells (B-LCL) or melanoma cells was determined in a newly developed *in vitro* inhibition assay. In this assay, the viral titer was determined in cell lysates of HAdV-infected B-LCL after 3 days of culture in the presence or absence of an HAdV-specific CD4$^+$ T cell clone. These CD4$^+$ T cells were able to inhibit viral replication, which required cognate MHC-peptide interaction. T cell clones lysed target cells in a perforin-mediated manner, which could be the mechanism of inhibition. Together, these results imply that HAdV-specific CD4$^+$ T cells are a promising tool for immunotherapy in immunocompromised patients at risk of developing severe HAdV infection. On the other hand, HAdV-specific CD4$^+$ T cells might hamper the efficacy or longevity of gene transfer with HAdV-based vectors in gene therapy trials.

MATERIALS & METHODS

Cells and viruses

The human epithelial cell line Hep2 was used to propagate HAdV. Cells were maintained in RPMI 1640 with glutamax (GIBCO-BRL, Paisley, Scotland, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO-BRL), 100 U/mL penicillin and 100 µg/mL streptomycin. HAdV strains of serotypes 2 and 5 (species C), 12 (species A) and 35 (species B) (RIVM, Bilthoven, the Netherlands) were grown on Hep2 cells and harvested when cytopathological effect (CPE) 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.

HAdV-specific T cell clones

PBMC from healthy blood bank donors (Sanquin, Leiden, the Netherlands) were isolated using Ficoll gradient centrifugation. Monocytes were depleted by adherence to plastic for 2 h, after which the non-adherent peripheral blood lymphocytes (PBL) were collected and used as responder cells. PBL (2×10^6/well) were added in a 24-well plate (Corning Incorporated, Acton, MA) and stimulated with 1×10^5 irradiated (30 Gy) autologous PBMC pre-incubated with methylene blue (MB)-inactivated HAdV5 at a multiplicity of infection (MOI) of 10. Cells were cultured for 12 days in RPMI/10% human AB medium, harvested, seeded at 2.5×10^5 cells per well and restimulated with 1×10^5 HAdV5-infected stimulator PBMC as before. IL-2 (50 IU/mL, Chiron, Emeryville, CA) was added at day 15 and replenished twice 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/well in 96-well round-bottomed plates (Corning) and stimulated with 1 µg/mL PHA (Murex Biotech Ltd., Dartford, UK), 150 IU/mL IL-2 and 1×10^6/mL irradiated allogeneic PBMC. After two 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 cells (2×10^4 per well) were plated in 96-well round-bottomed plates in triplicate. As stimulator cells, autologous EBV transformed B cells (B-LCL) were resuspended in RPMI with 0.5% BSA at 5×10^6 B-LCL/mL, irradiated at 40 Gy and infected for 1 hour (h) at 37°C with HAdV5 at an MOI 100, or uninfected as control. Hereafter, 5×10^4 stimulator cells were added per well in RPMI/10% AB medium. After 3 days at 37°C and 5% CO_2, cultures were pulsed with 0.5 µCi [^{3}H]-thymidine (Amersham International, Amersham, UK) per well for 18 h. Plates were harvested (Skatron, Sterling, VA) and filters were subsequently counted in a betaplate counter (Wallac, Turku, Finland).

To determine which protein was recognized by a T cell clone, B-LCL were loaded with recombinant hexon protein (protein II, generated in 4 parts as IIA (amino acids (aa) 1-273), IIB (aa 245-509), IIC (aa 479-743) and IID (aa 720-952)) (Veltrop-Duits, manuscript in preparation), the penton protein or an early protein, E1A, at a final concentration of 5 ug/mL. For peptide recognition, the hexon protein was synthesized in overlapping peptides of 30 aa with a 15-aa overlap (Veltrop-Duits, manuscript in preparation). Proliferation was determined against B-LCL loaded with pools of 5-6 peptides (5 ug/mL), and specific peptides were determined from positive peptide pools by loading single peptides on B-LCL.

The HLA restriction element was determined by addition of blocking monoclonal antibodies against HLA class I (W6/32), HLA-DR (B811.2), HLA-DQ (SPVL3), and HLA-DP (B7.21) in the proliferation assay (antibodies were a kind gift of Dr. A. Mulder, Dept. of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands) and using partially HLA-matched B-LCL as stimulators.

Detection of cytokine production and mediators of apoptosis

HAdV-specific T cell clones were tested for cytokine production by intracellular staining and flow cytometry. T cells (2×10^5) were stimulated with 1×10^5 irradiated autologous B-LCL either uninfected, infected with HAdV5 at MOI 100, or loaded with the specific peptide in round-bottomed polystyrene 5 mL tubes in a total volume of 400 µl. After 1 h, brefeldin A (Sigma-Aldrich, St. Louis, MI, USA) 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 NaN_3, fixed with freshly made 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 4 min, and washed twice with PBS/NaN_3. Cells were permeabilized by washing with PBS/NaN_3 containing 0.1% saponin and 0.5% BSA, and non-specific binding was blocked with PBS/NaN_3/saponin/BSA/10% FCS for 10 min. After washing with PBS/NaN_3/saponin/BSA, antibodies were added: αCD3-PerCP-Cy5.5, αCD4-FITC, αIFN-γ-PE / αTNF-α-PE / α-IL-10-PE / αIL-4-PE (Becton Dickinson, Mountainview, CA, USA) and αCD8-APC (Immunotech, Prague, Czech Republic) and incubated for 30 min. For detection of perforin and granzyme B intracellularly, cells were stained with α-perforin-FITC (Hölzel Diagnostika, Köln, Germany) or with α-granzyme B-PE (Sanquin, Amsterdam, the Netherlands) in combination with αCD3-PerCP-Cy5.5 and αCD4-APC as above. After washing, cells were either fixed in 0.5% PFA or analyzed immediately on a FACSCalibur flow cytometer using the CellQuest software (Becton Dickinson).

The level of secreted IFN-γ in culture supernatant was measured with an ELISA kit (Sanquin, Amsterdam, the Netherlands) according to manufacturer's guidelines.

Chemokine receptor expression on HAdV-specific T cell clones

Primary antibodies used for chemokine receptor stainings were PE-labeled αCCR3 and unlabeled αCXCR6 (R&D Systems, Minneapolis, MN), and unlabeled αCXCR3 (BD Pharmingen, San Diego, CA). For detection of the unlabeled primary antibodies, cells were stained with the relevant PE-conjugated isotype-specific secondary antibody (Southern Biotechnology Associates Inc., Birmingham, Alabama), followed by αCD3-PerCP-Cy5.5 and αCD4-APC staining. Cells were analyzed on a FACSCalibur flow cytometer using the CellQuest software.

HAdV replication inhibition assay

To test whether HAdV-specific T cell clones were able to inhibit viral replication, we developed an *in vitro* inhibition assay. B-LCL or a melanoma cell line expressing HLA-DR17, mel juso (MJS, a kind gift from Dr. M. Ressing, Dept. of Virology, Leiden University Medical Center, Leiden, the Netherlands), were infected with HAdV5 at MOI 100 for B-LCL (5×10^6/mL) or MOI 10 for MJS as described above. MJS cells (1×10^5) were plated one day prior to infection in the wells of a 24-well plate. Infected B-LCL were washed three times with 10 mL RPMI/10% FCS, and MJS three times with 1 mL per well to remove free virus. For B-LCL, cells were plated at 1×10^5 in 500 μl RPMI/10% FCS per well of a 24-well plate (Corning). HAdV-specific T cell clone (at an effector to target (E:T) ratio of 10:1 unless indicated otherwise) was added in 500 μl RPMI/10% FCS, or medium alone as a control for viral replication. In preliminary experiments, cells were harvested daily from day 0 till day 6 to determine the kinetics of viral replication in the B-LCL. In later experiments, cells were harvested at day 3 after infection. Cells were washed three times with RPMI/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 10^0 till 10^8-fold and each dilution was plated in tenfold in 96-well flat-bottomed plates. Hep2 cells were added to the wells at 5×10^3 cells/well. After 7 days of incubation at 37°C and 5% CO_2, wells were scored for the presence of CPE, which indicates 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 (30). 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 co-culture 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, ^{51}Cr release assays were performed on B-LCL or MJS cells. Target cells were infected with HAdV5 at MOI 100 or 10 as described before or loaded with peptide at 5 μg/mL and cultured for 16 h. Target cells were labeled with ^{51}Cr for 1.5 h at 37°C and washed 3 times with RPMI. Various numbers of effector cells were co-cultured with 2.5×10^3 B-LCL or 1.25×10^3 MJS/well for 4 h at 37°C and 5% CO_2 and ^{51}Cr release was determined in the supernatant. Maximal release was determined by addition of 2 N HCl to target cells, and spontaneous release was obtained by addition of medium alone. Counts were measured in an automatic gamma counter (Wallac). 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. Concanamycin A (CMA at 50 nM, a kind gift from Dr. J.P. Medema, Dept. of Clinical Oncology, Leiden University Medical Center, Leiden, the Netherlands) or EGTA (at 2 mM, Boehringer Mannheim GmbH, Mannheim, Germany) were added to the effector cells 1 h prior to co-culture with target cells and CMA or EGTA were also present in the medium during co-cultivation.

RESULTS

Most HAdV-specific CD4+ T cell clones recognize peptides derived from the hexon protein

HAdV-specific CD4+ T cell clones, generated against HAdV5 (species C), have been obtained from several healthy donors as recently described (30). These clones exhibited a restricted to broad cross-reactivity pattern against serotypes from different species, most clones recognizing serotypes from species A, B and C. To further characterize these clones, we investigated which protein of HAdV was recognized using proliferation assays with recombinant hexon protein (generated in 4 parts, IIA-D), recombinant penton, and an early gene product E1A (Veltrop-Duits, in preparation). Three out of 8 HAdV-specific T cell clones were unresponsive to any of these proteins, but the majority of clones (n=5) recognized the C-terminal part of the hexon protein (IIC or IID) (Table I and example in Figure 1A), which is highly conserved between different species (32,33) and might explain the broad cross-reactivity that we observed previously (30).

Table I. HAdV-specific CD4+ T cell clones with hexon peptides and HLA restriction

T cell clone	Donor	Hexon protein	Hexon peptide	HLA restriction
A1.1	1	-[a]	-	DR7
D1.23	1	-	-	DR7
C30.4	2	-	-	DR7
A4 0.3-4	3	IIC	II44(43)[b]	DR17
A5 1.32	3	IID	II64, 910-924[c]	DP4
M2.11	4	IID	II57	DR17
K1.2	5	IID	II64, 910-924	DP4
K3.1	5	IID	II61	DR17

[a] indicates none of the hexon, penton or E1A proteins were recognized.
[b] indicates that peptide II44 induced the highest poliferation; proliferation to II43 was observed to a lesser extent suggesting that the minimal epitope resides in the N-terminal part of peptide II44.
[c] peptide II64 was recognized as well as the minimal epitope which is present in II64, the $II_{910-924}$ epitope (described in (28)).

To further investigate which peptides of the hexon protein were recognized, 64 overlapping peptides of 30 aa with 15 aa overlap of the hexon protein (peptides II4-II68) were synthesized and tested in proliferation assays. An example is shown in Figure 1B, and results

are summarized in Table I. Two clones recognized II64, which contains a conserved epitope $II_{910-924}$ that has recently been described by Olive *et al* (28,34). Proliferation was also observed against this minimal epitope, indicating that these two HAdV-specific clones specifically recognized this $II_{910-924}$ epitope in peptide II64. HLA restriction of these clones was determined by stimulation with HLA-DR or -DP matched B-LCLs or addition of blocking antibodies. The T cell clones of which the peptide specificity could not be determined were all HLA-DR7 restricted, while the other 5 clones which recognized various peptides in the hexon were HLA-DR17 or HLA-DP4 restricted (Table I).

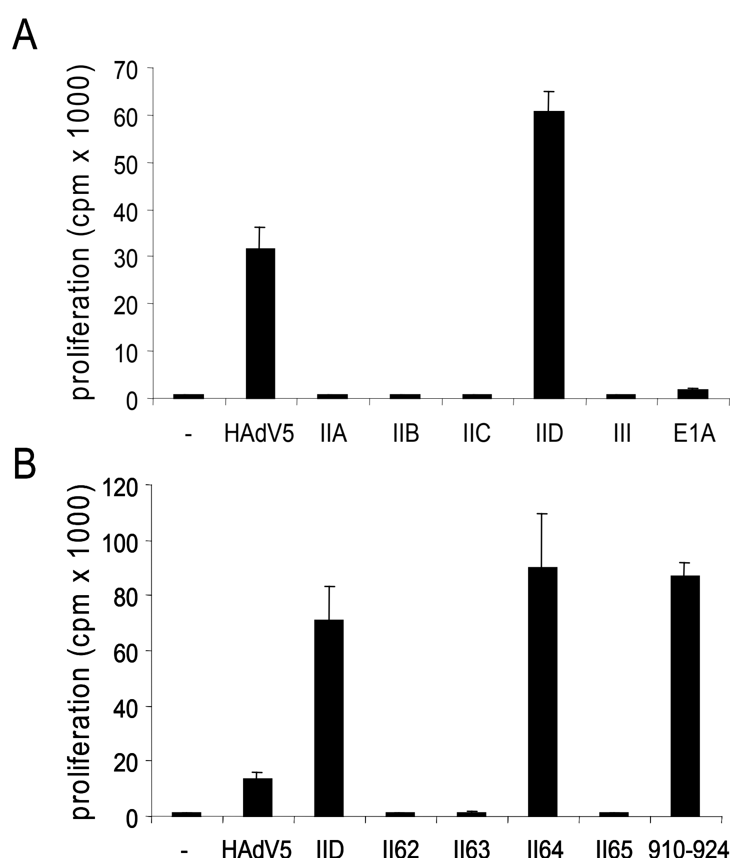

Figure 1. Most HAdV-specific CD4+ T cell clones recognize peptides in hexon protein (protein II). A) T cells from clone A5 1.32 were tested in a three-day proliferation assay against autologous B-LCL infected with HAdV5 or loaded with recombinant proteins from hexon (IIA-D), the penton protein (III), 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 recently described (28), which was found to be the epitope for this clone. Results are representative for 5 clones.

Cytokine production and chemokine receptor expression of HAdV-specific CD4+ T cell clones

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 (Figure 2A). IL-2 was produced in few cells (Figure 2A), whereas IL-5 and IL-10 was not detected (data not shown). Upon stimulation with HAdV5-infected B-LCL, no increase in IL-4 production was detectable compared to uninfected BLCL (Figure 2A). When T cell clones were stimulated with peptide-loaded B-LCL, >90% of T cells co-expressed IFN-γ and TNF-α. IL-2 production was strongly increased, while IL-5 and IL-10 remained undetectable (Figure 2B and data not shown).

In agreement with the cytokine production pattern, HAdV-specific T cell clones expressed the chemokine receptors CXCR3 and CXCR6 on their surface, while the chemokine receptor CCR3 was expressed on <10% of cells (Figure 2C and data not shown). CXCR3 is reported to be expressed on $CD4^+$ T cells early after commitment to a Th1 phenotype, and the presence of CXCR6 has also been correlated with $CD4^+$ T cells with a Th1 phenotype, predominantly in the liver (35-37). The other chemokine receptor, CCR3, has been reported on Th2 cells (38,39). Together, the phenotype and cytokine profile of the HAdV-specific T cell clones indicate that these cells are committed to a Th1 effector cell lineage.

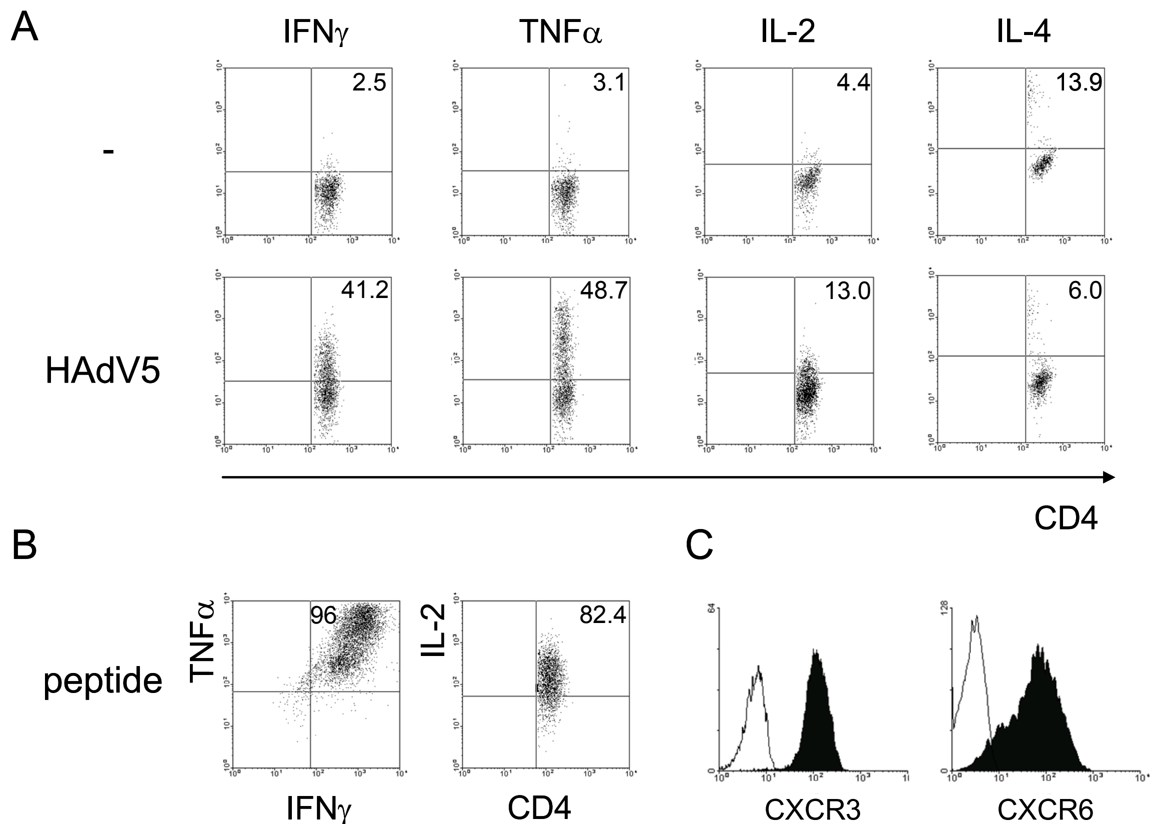

Figure 2. **HAdV-specific $CD4^+$ T cell clones secrete IFN-γ and TNF-α and express the chemokine receptors CXCR3 and CXCR6.** A) Autologous B-LCL were infected with HAdV5 at MOI 100 or uninfected as control. T cells from clone M2.11 were added for 16 hr, and cytokine production was determined with intracellular cytokine staining using α-IFN-γ-PE, α-TNF-α-PE, α-IL-2-PE or α-IL-4-PE in combination with α-CD4-FITC. The percentage of $CD4^+$ T cells producing a specific cytokine is shown in the upper right quadrant. B) T cells were stimulated with B-LCL loaded with the specific peptide II57. Intracellular cytokine staining was performed with α-IFN-γ-FITC in combination with α-TNF-α-PE, and α-IL-2-PE in combination with α-CD4-FITC. C) Chemokine receptor expression was determined on HAdV-specific T cell clones. Shown are CXCR3 and CXCR6 expression (filled histogram). As a control, secondary antibodies were added to unstained cells (open histograms). Results are representative for the investigated clones A 1.1, M2.11 and K3.1.

Inhibition of HAdV replication *in vitro*

If HAdV-specific $CD4^+$ T cells will be administered to patients as adoptive immunotherapy, these T cells are required to have antiviral functions in order 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 which determines 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 day 0 till day 3 after infection. HAdV virus titers increased 100- to 1000-fold in 3 days. When an HAdV-specific T cell clone, A1.1, was added to autologous infected B-LCL at an E:T ratio of 10:1, viral titers were more than 1000-fold reduced (Figure 3A). Prolonging the experiment till 6 days resulted in a similar reduction in titer when T cells were added (data not shown). Therefore, in further experiments cells were harvested at day 3 and viral titers were compared between infected B-LCL cultured alone or in the presence of T cells. The inhibitory effect of the CD4+ T cell clones was dependent on the number of T cells present, as shown with different E:T ratios. At an E:T ratio of 1 T cell per B-LCL, viral replication was inhibited significantly, whereas inhibition could no longer be observed at an E:T ratio of 0.1:1 (Figure 3B).

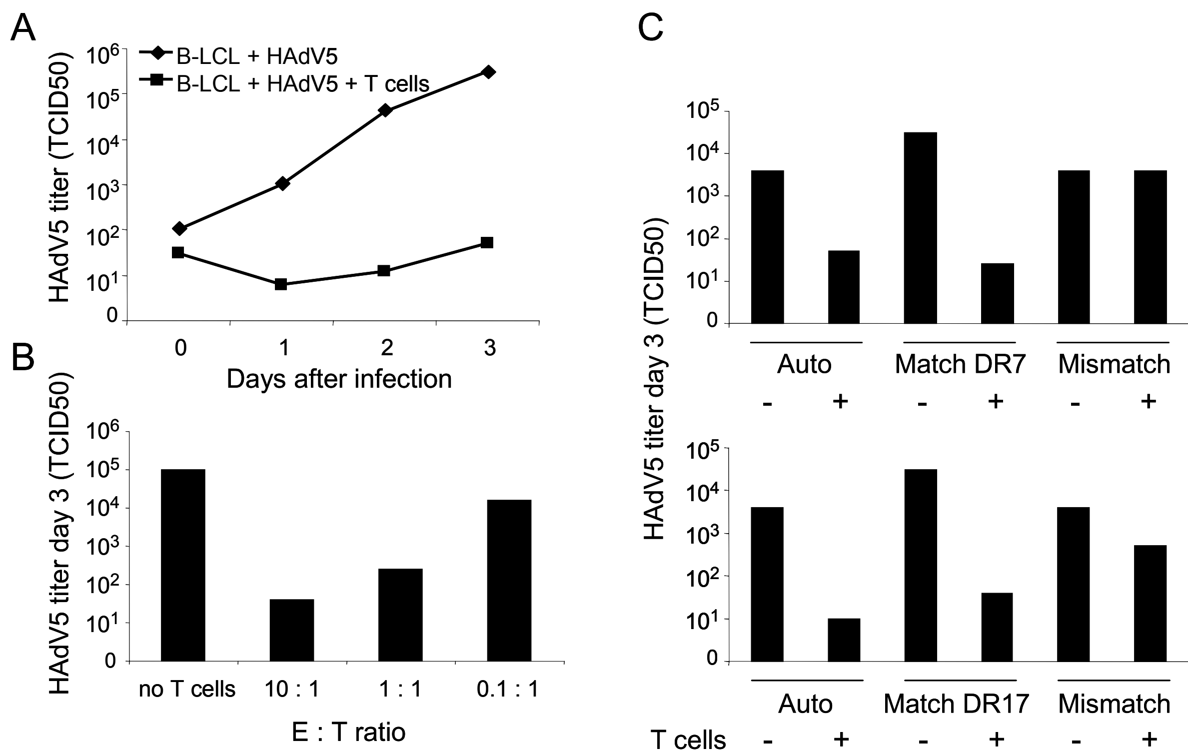

Figure 3. Inhibition of viral replication in B-LCL by HAdV-specific CD4+ T cell clones is HLA-restricted. The antiviral activity of T cell clones was tested in a three-day *in vitro* assay in which viral replication was assessed. A) Autologous B-LCL were infected with HAdV5 at MOI 100 and cultured for three 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 day 0, 1, 2 and 3 and the titer of infectious virus on Hep2 cells was determined with the TCID50 assay. B) Autologous B-LCL were infected with HAdV5 at MOI 100 and cultured for three 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 matched B-LCL or HLA-mismatched B-LCL were infected with HAdV5 at an MOI 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 are shown out of 4 clones (A1.1, M2.11, K3.1, A5 1.32) tested.

Strong reduction in viral titers was not only observed when autologous B-LCL were used, but also when HLA-DR or -DP matched B-LCL (depending on the clone) were used (Figure 3C). However, viral titers were not reduced when mismatched B-LCL were applied as target, indicating that inhibition of viral replication is HLA-restricted (Figure 3C).

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 or via non-specific cell-cell contact. Therefore, a co-culture 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 should then directly exert the potential effect on the virus production in HAdV-infected HLA-mismatched B-LCL. As a control, the virally infected B-LCL was, like the peptide-loaded B-LCL, of autologous origin. As expected, a >1000-fold reduction in titer was seen in the control experiment in which T cells were co-cultured with peptide-loaded as well as infected autologous B-LCL (Figure 4).

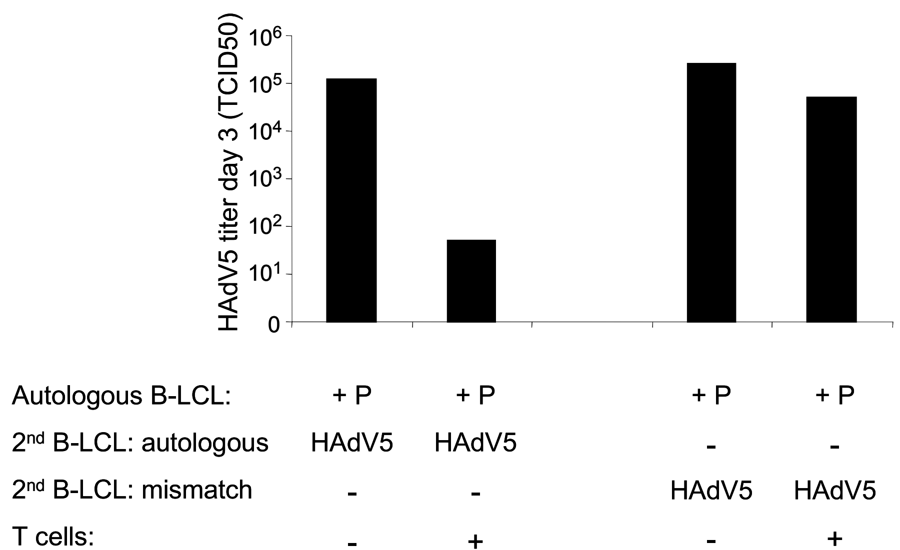

Figure 4. Inhibition of viral replication is not mediated via soluble factors. In a co-culture experiment, autologous B-LCL were loaded with the specific peptide (+P) in order to stimulate the HAdV-specific T cell clone K3.1. In the same well, HLA-DR mismatched B-LCL were added which were infected with HAdV5 at MOI 100. In control wells, HAdV-infected autologous B-LCL were added which 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.

When the virus was present in the mismatched B-LCL, however, only a slight reduction in viral titer was observed (Figure 4), even though T cells were strongly stimulated with the peptide presented by 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. Thus, 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%) (Figure 5A), but this observation is probably due to the fact that HAdV infection of B-LCL is inefficient and results in ~5-10% infected cells as visualized by immunohistochemistry (40) (and data not shown). However, when B-LCL were loaded with the specific 30-aa peptide, lysis increased to ~80% or more (Figure 5B). A melanoma cell line, mel juso (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 (Figure 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 (41-43). The perforin effector pathway can be inhibited by the action of concanamycin A (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 (44). Lysis was almost completely abrogated by either of these inhibitors, indicating that these $CD4^+$ T cells lyse predominantly using the perforin pathway (Figure 5D and data not shown). 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 (Figure 6).

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

Most HAdV-specific T cell clones raised against HAdV5 were highly cross-reactive towards many serotypes in proliferation assays as described recently (30). The peptide specificity found in the present study supports these results since most peptides are located within the conserved region of the hexon protein (Veltrop-Duits, manuscript in preparation).

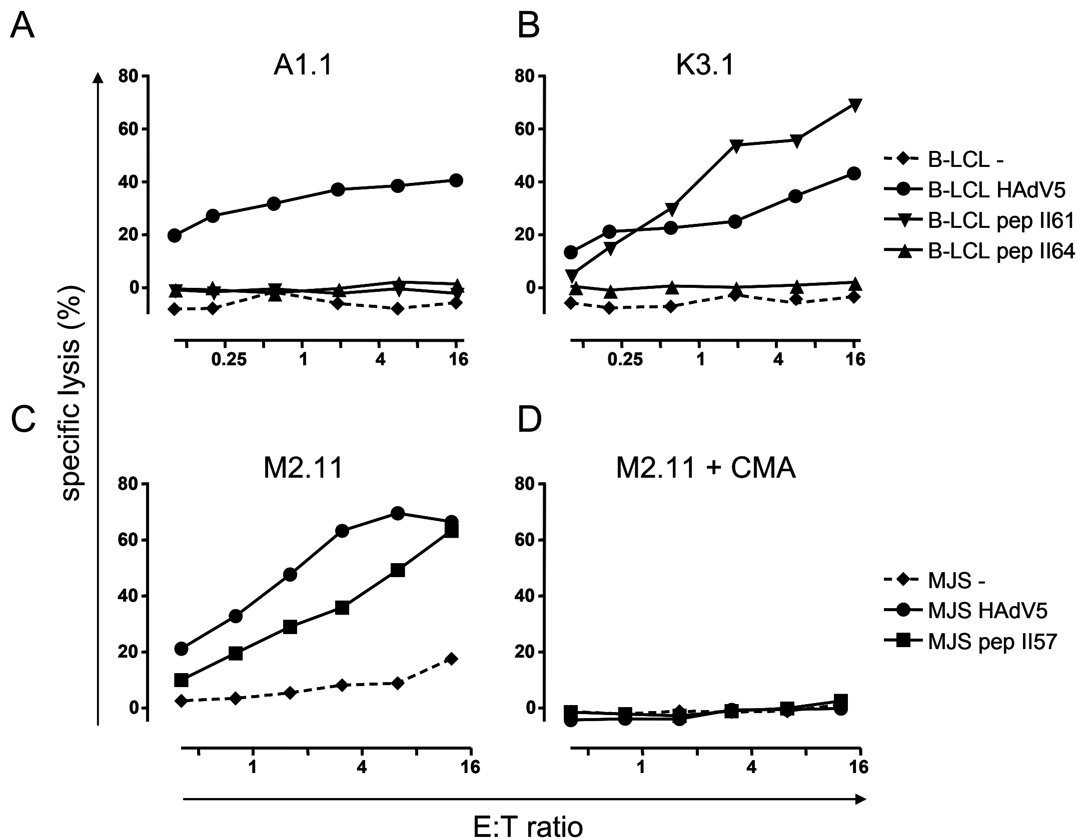

Figure 5. HAdV-specific T cell clones lyse HAdV-infected or peptide-loaded targets using the perforin-pathway. A) HLA-DR matched (DR7/DR17 positive) B-LCL were infected with HAdV5 at MOI 100 (●), loaded with peptides II61 (▼) and II64 (▲) or uninfected (♦) as controls since this clone does not recognize any peptide in the hexon protein, and used these targets in a 4 h ^{51}Cr-release assay (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 MOI 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 pre-incubated with 50 nM CMA for 1 h prior to addition of T cells to MJS target cells in medium containing CMA. Lysis results in (A) are representative of 8 T cell clones tested against HAdV5, and in (B) of 5 clones that were tested against the specific peptide. CMA results (D) are representative of 3 clones.

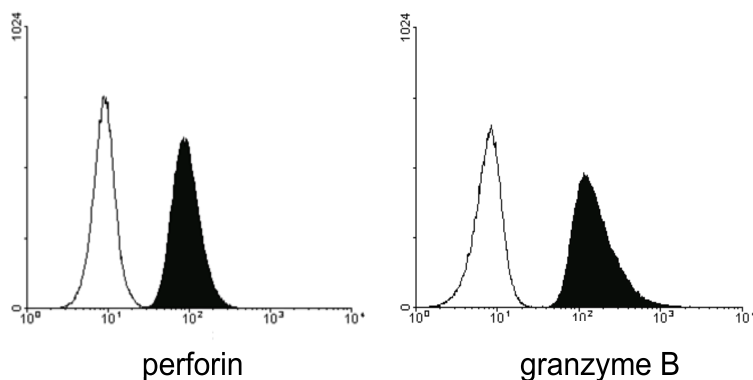

Figure 6. HAdV-specific T cell clones express perforin and granzyme B. Intracellular FACS staining was performed on T cell clones. Cells were stained with α-perforin-FITC or α-granzyme B-PE (filled histograms) or unstained as control (open histograms). Shown is one example out of 3 T cell clones (A1.1, M2.11 and K3.1) tested.

To analyze whether the T cell clones are not only cross-reactive in proliferation assays, but 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. A broadly cross-reactive HAdV-specific T cell 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 (Figure 7). 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.

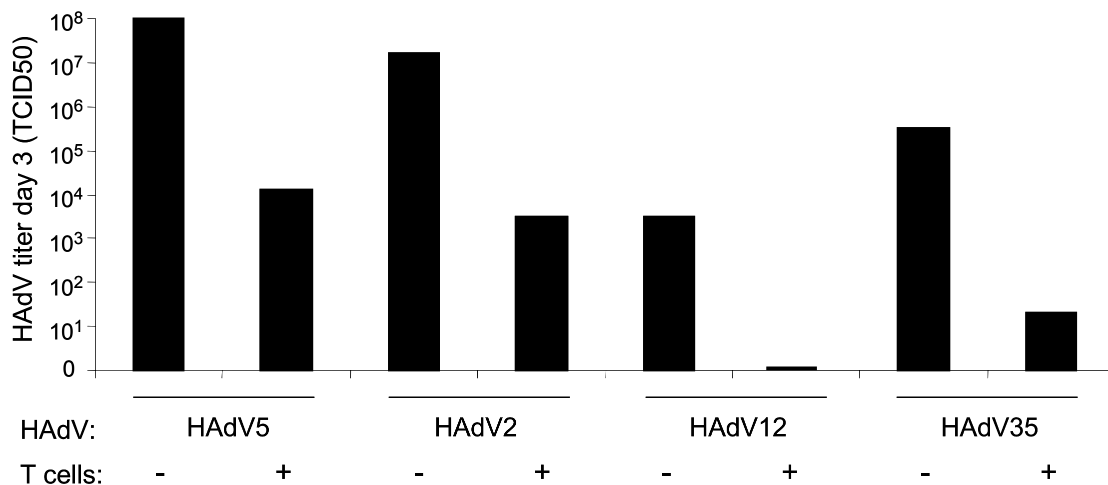

Figure 7. Inhibition of viral replication of serotypes from different HAdV species. MJS were infected at MOI 10 with HAdV5 and HAdV2 (both species C), HAdV12 (species A) and HAdV35 (species B). Inhibition of viral replication of these serotypes was tested using the T cell clone K3.1 at an E:T ratio of 10:1. Viral titers were determined in cell lysates at day 3.

DISCUSSION

In this study, human HAdV-specific $CD4^+$ T cell clones have been extensively characterized for their effector functions and antiviral activity. HAdV infection in immunocompromised patients is a severe complication, especially in pediatric patients following SCT (3-7). As medication with ribavirin or cidofovir is frequently ineffective (reviewed in (19)), immunotherapy might be a promising option for treatment. In healthy donors as well as in patients that cleared HAdV infection, mainly $CD4^+$ HAdV-specific T cells are detected (7,27,30,31). To date, however, it has not been investigated whether and by which mechanism $CD4^+$ HAdV-specific T cells are capable of mediating antiviral activity, which would be required for optimal immunotherapy. Therefore, HAdV-specific $CD4^+$ T cell clones from healthy donors were generated against HAdV5 and characterized for their recognition of specific peptides from HAdV, their phenotype and their antiviral function. Most of our T cell clones reacted against peptides that are in the C terminal part of the hexon

protein, which is in accordance with other reports showing that the hexon protein is recognized by $CD4^+$ T cells (28,45) (Veltrop-Duits, manuscript in preparation).
The cytokine profile of the HAdV-specific $CD4^+$ T cell clones suggested that these T cells have a Th1 phenotype as they produced large amounts of IFN-γ and TNF-α, as well as IL-2 upon peptide stimulation. Furthermore, detailed analysis of chemokine receptor expression indicated a Th1 phenotype as the T cell clones cells expressed CXCR3 and CXCR6 (35,37).
To determine whether $CD4^+$ HAdV-specific T cells were capable of mediating antiviral activity, we developed an *in vitro* assay in which the effect of T cells on viral replication was investigated. Viral titers were determined in cell lysates of infected B-LCLs, which have the advantage that they can be obtained with almost all HLA alleles and have previously been shown to be able to facilitate a productive infection (46). Addition of a T cell clone at a 10:1 or 1:1 ratio had a strong impact on viral replication by reducing replication >1000-fold (99.9% inhibition of viral outgrowth). This antiviral activity by the $CD4^+$ T cell clone was HLA-restricted as viral replication in infected mismatched B-LCL was not inhibited. In the co-culture experiments, peptide-stimulated T cells were in close proximity to HAdV5-infected B-LCL without the proper HLA-restriction element, allowing detection of a possible effect of all short-range factors and/or non-specific cell-cell contact. As viral titers were not reduced in this setting, cognate interaction between the $CD4^+$ T cell clone and the HAdV-infected B-LCL appeared to be required for the antiviral effect. Furthermore, these $CD4^+$ T cell clones were able to lyse infected targets (B-LCL or MJS), which could completely be inhibited by obstructing the perforin pathway, suggesting that lysis of target cells appeared to be the mechanism of the observed *in vitro* inhibition of viral replication.
In other viral infections such as CMV and EBV infection, many reports on virus-specific $CD8^+$ T cells have been published (reviewed in (47)). 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 (48,49), which in some reports have been shown to inhibit viral outgrowth *in vitro* (50,51). The cytolytic pathway used by human $CD4^+$ T cells is less well defined than in mice, where the FAS pathway is most important for $CD4^+$ T cell-induced lysis (42). Most reports indicate that perforin-mediated lysis is the main mechanism of human cytotoxic $CD4^+$ T cells, either directly *ex vivo* (52) or in cell lines or clones (48,49,53), while some $CD4^+$ T cell clones have been shown to utilize the FAS pathway (50). In this respect, it is noteworthy that HAdV has developed immune evading mechanisms. The early protein E3-gp19K has been reported to downregulate 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 (54)). Another early E3 protein complex, the RIDα/β (receptor internalization and degradation) complex, has been reported to internalize FAS, TNF-RI and TRAIL-R and induce their degradation, thereby circumventing FAS or TRAIL mediated

lysis of infected cells (55). In view of these immune evasion strategies of HAdV, it is not surprising that HAdV-specific $CD4^+$ 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 (31), indicating that these viral evasion mechanisms may not be absolute. *In vivo*, the $CD4^+$ T cells described here may provide help to these HAdV-specific $CD8^+$ T cells indirectly by maturing DC via CD40-CD40L interactions (56,57).

As HAdV infects primarily epithelial cells, which under normal conditions do not express HLA class II molecules, the question arises how HAdV-specific $CD4^+$ T cells would be able to recognize their infected targets. Several mechanisms might be induced which result in class II expression on these cells. First, the HAdV infection itself induces the cell to produce IFN-α or -β and other stress proteins, which might also induce the upregulation of class II expression. Preliminary data from our group obtained in post-mortum biopsies from patients which succumbed to HAdV infection showed in some cases that HAdV-infected cells were also HLA class II positive (Heemskerk, unpublished data); however, these findings will need to be confirmed and require further investigation. Second, when $CD4^+$ T cells have been activated by antigen-presenting cells in the draining lymph node and return to the site of infection, the IFN-γ produced by these T cells is able to induce class II upregulation in infected and uninfected epithelial cells (58).

In conclusion, HAdV-specific $CD4^+$ T cell clones have antiviral activity as they were able to inhibit viral replication *in vitro*, which required cognate HLA-peptide interactions and is most likely dependent on perforin-mediated lysis. The cross-reactive inhibition of viral replication with serotypes from different species by the $CD4^+$ T cell clones generated against HAdV5 is encouraging for the clinical application of these cells in immunocompromised patients. The observed cytolytic activity and cross-reactivity of HAdV-specific $CD4^+$ T cell clones have implications for gene therapy trials with HAdV-based vectors as well, as the longevity of gene expression might be reduced when HAdV-specific $CD4^+$ T cells are present that could eliminate the transduced cells. For monitoring HAdV-specific responses *ex vivo* in the future, it might be useful to determine the minimal epitopes present in the 30 aa peptides and to generate HLA class II tetramers. These investigations are currently being addressed in our laboratory so that infusion of HAdV-specific T cell lines with antiviral activity in immunocompromised patients suffering from disseminated HAdV infection post-transplant is a feasible option for immunotherapy in the near future.

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Chapter 8

General Discussion

Stem cell transplantation is the preferable curative therapy for a great number of disorders of the hematopoietic system in children. More diseases may be eligible for SCT in the future, and most likely more transplants will be performed with alternative donors than MFD as these donors are frequently not available. This large number of non-MFD transplantations requiring manipulation of the graft implies that infections will remain a major problem in these prolonged immunocompromised graft recipients. HAdV infections will probably account for a substantial part of morbidity and mortality within the infections or reactivations with viruses post-SCT in the future. Interestingly, HAdV has only recently emerged as an important pathogen, whereas other viruses such as EBV and CMV have been known for many years to cause complications after transplantation (van Tol *et al*., submitted). Several explanations for this apparent increase in HAdV infections can be given. Improved therapy for EBV and CMV infections in recent years might have opened a window for other viruses to cause severe infections (1-3). Also, in recent years more high-risk transplants with other than MFD donors have been performed, resulting in a prolonged immunocompromised state of the recipient, which might be responsible for the increased incidence of HAdV infection. Another possibility could be a change in policy with regard to removing the adenoids and tonsils in children, as T cells in tonsils and adenoids have been reported to contain DNA from HAdV species C in 79% of specimens (4). Therefore, these organs might represent a reservoir of HAdV from which the virus can reactivate. Before the mid-nineties, these lymphoid organs were surgically removed in almost all children with upper respiratory tract problems, while in recent years a more restricted policy of surgical removal has been applied. As a result, many children are being transplanted nowadays with their adenoids and tonsils still present. Unfortunately, we were not able to investigate this hypothesis in our retrospectively or prospectively studied patients, nor have others reported on a possible relationship between the presence of tonsils/adenoids and HAdV infection. In this respect, the question where the virus originates from is relevant. The virus might reactivate in the recipient, be introduced via the graft from the donor, or be introduced from the environment inducing a *de novo* infection. If the virus reactivates from the host, this reactivation may originate from the HAdV reservoir in adenoids and tonsils. However, even though viral DNA was detected in T cells derived from tonsils, the presence of infectious virus was rare (4). The authors hypothesized therefore that these cells might contain HAdV in a quiescent or perhaps latent form. Whether latency exists and by which mechanism the virus might reactivate is not known yet, but the use of TBI and chemotherapy in the conditioning for SCT might possibly damage the lymphoid tissues of tonsils and adenoids and release virus particles. Alternatively, the conditioning - especially melphalan (Chapter 3) - may damage gut tissues which might harbour persistent HAdV particles that are responsible for intermittent secretion of HAdV in the feces in healthy individuals (5). If the virus is derived from the host, this would implicate that the virus is present in the inoculum

of the SCT graft, most likely the lymphocytes. In an international collaborative EU study from 2003 onwards, prospective follow-up of HAdV infection in children is being performed in our center as well as investigation of the presence of HAdV in grafts by quantitative PCR. The presence of viral DNA (in low titers) has been observed in 2 grafts from 41 donors tested. However, these patients did not develop HAdV infections (unpublished data). Therefore, it seems unlikely that the viral DNA is present in the graft or that it is responsible for reactivations in the recipient. The third hypothesis, *i.e.* that the virus is a *de novo* infection from the environment, seems unlikely in the case of an HAdV infection early post-SCT. During this period, patients are kept in strict isolation to prevent infections. As we have observed in almost all patients in whom the virus disseminated, the virus was already detected within the first 4 weeks after SCT (Chapter 3), suggesting that early reactivation of HAdV was present instead of a *de novo* infection. To further differentiate between donor versus recipient origin, results of serological studies may be helpful. A reactivation of HAdV from the recipient is likely if the donor does not possess NAb against the particular serotype causing the infection early post-SCT, indicating that the donor has not been in contact with that serotype recently. Conversely, if the recipient has no NAb to the infecting virus but the donor does, these serological data might suggest that the virus was present in the graft and proceeded to initiate a new infection in the patient. In one study, a correlation was found between the presence of antibodies against HAdV in the donor (measured by ELISA) and the occurrence of HAdV infections in the recipient (6). However, we were not able to confirm this correlation using the same ELISA which detects all antibodies against HAdV (unpublished observations). Furthermore, our data on the presence of NAb in patients and donors have so far not been conclusive as most donors and patients had NAb against the infecting serotype (as well as other serotypes) in their serum pre-SCT (Chapter 3 and unpublished observations). More patients are currently being investigated in the prospective EU study mentioned above.

It is also remarkable that HAdV infection poses such a threat in children after SCT, but appears to be of limited impact in adult recipients of an SCT (7-9). If the hypothesis about the reactivation of HAdV from adenoids is true, then this could also explain why adult patients do not suffer from HAdV reactivations, as these lymphoid organs were probably removed in their youth, or at least reduced in size due to atrophy. An alternative explanation could be that adults have less HAdV present in their lymphoid organs, as Garnett *et al.* observed a lower amount of viral DNA in lymphocytes from older children. This is most likely due to the fact that young children are infected with different serotypes of HAdV more frequently and more recently (5), as attending day care centers has been reported to be a source of HAdV outbreaks among children (10). If the virus is transferred from donor to recipient, it is likely that an adult recipient will have been infected with that serotype at some point during life, in which case the adult recipient has immunity against that serotype,

whereas a pediatric recipient may not, which results in a primary infection with an HAdV serotype and a greater challenge to the immune system. The question is whether any residual immunity against HAdV remains after conditioning treatment of a patient prior to SCT, which will also depend on the type of conditioning (myeloablative or reduced intensity) that is given. In a young child with mixed chimerism after SCT for acute lymphoblastic leukemia, we observed an HAdV-specific T cell response at 12 weeks after SCT. Surprisingly, HAdV-specific T cell clones generated from this time point were derived from T cells originating from the patient instead of the donor (Chapter 3, Figure 4, patient 16 and unpublished data). Immune responses to CMV and *Candida albicans* from recipient origin have also been observed in children after an UCB-SCT, in which mainly naive T cells from donor origin are transferred to the recipient (11). In addition, plasma cells, residing in the bone marrow, may remain present for years after SCT and could be a source of ongoing NAb production after SCT. However, consumption of such antibodies by the infecting virus appeared to occur in the absence of an immune response post-SCT (Chapter 3, Figure 3, patient 20).

The increasing frequency of HAdV infections and the high mortality of disseminated infection, reflected by an increase in viral DNA load in the blood of patients, warrant investigations to improve HAdV-specific antiviral therapies. Several possibilities for treatment will be discussed in the section below.
HAdV infection might be treated with antiviral drugs. So far, two drugs, ribavirin and cidofovir have been tested in relatively large cohorts of patients. Some reports suggested that ribavirin might have an antiviral effect against HAdV (12,13). However, results obtained in this thesis showed that lymphocyte recovery was associated with clearance of the virus (Chapter 3), and data on lymphocyte recovery were lacking in studies on ribavirin efficacy. Using sensitive RQ-PCR techniques to determine viral DNA loads, the use of ribavirin as an antiviral drug turned out to be ineffective as administration of this drug did not result in a decrease of the viral load in the absence of lymphocyte recovery (Chapter 4). In addition, a report on the *in vitro* susceptibility of HAdV serotypes to ribavirin treatment showed that serotypes belonging to species C were more sensitive to ribavirin than other species (14). This might explain the somewhat controversial findings on efficacy of ribavirin *in vivo*, for example in a report that showed inefficacy of ribavirin in patients infected with HAdV11 (species B) or HAdV12 (species A) (15). In the collaborative EU study, additional data on ribavirin efficacy will be obtained via the *in vitro* testing of susceptibility of clinical HAdV isolates to ribavirin, as well as by studying the pharmacokinetics of ribavirin administration *in vivo*. Similar tests are being performed for cidofovir, which has been shown to have antiviral activity *in vitro* and *in vivo* in animal models (16,17). Several studies have recently been published in which an antiviral effect of cidofovir was reported in patients without

significant T cell reconstitution (18,19). So far, only one report on cidofovir treatment has been published in the context of HAdV viral load monitoring during treatment, which reported a success rate of 63% (20). Unfortunately, as data on immune recovery were lacking in this report, the possibility of viral clearance by the immune system cannot yet be ruled out. Therefore, further investigation of this drug as a first-line pre-emptive treatment for patients at risk for HAdV dissemination is needed in which the efficacy of cidofovir is evaluated by monitoring HAdV viral loads in plasma together with the recurrence of lymphocyte counts and subsets. These studies also need to address the occurrence and severity of renal toxicity that has been described for cidofovir treatment (overall renal toxicity of ~26% in pediatric SCT recipients) (19). In our clinic, patients have been prospectively monitored from 2003 onwards in the context of the EU collaborative study of HAdV infections. When patients develop viremia exceeding 1000 copies/mL in two consecutive plasma samples, treatment with cidofovir is initiated. At the time of writing, 6 patients have been treated with cidofovir (until November 2004). Preliminary data show that in 5 HAdV viremic patients with lymphocyte recovery, cidofovir treatment resulted in a decrease of the viral load. However, cidofovir treatment in one patient without any lymphocyte recovery ($<0.1\times10^9$/L) was unsuccessful, suggesting that lymphocyte recovery might be more important for clearance of the disseminated infection than cidofovir treatment (unpublished observations).

The encouraging results with DLI infusions indicate that transfer of immunity from donor to host can be effective as a treatment for disseminated HAdV infection (21,22). However, the risk of inducing GvHD is high in the case of an HLA-mismatched donor-recipient combination ((23) and unpublished observations). To circumvent the transfer of alloreactive T cells, most studies focus on transfer of HAdV-specific T cells (24-26), but passive transfer with NAb specific for the serotype infecting the patient might also be an option for treatment as has been suggested in a case report (27). Our data indicate that high titers of pre-existing NAb are not sufficient to prevent HAdV infection, dissemination or death post-SCT (Chapter 3 and unpublished data), but these NAb will most likely be consumed by the HAdV in the absence of an active humoral immune response of the host. However, it is not yet clear whether high-titered NAb can be obtained, either from serum of healthy donors or generated *in vitro* by the use of, for example, phage libraries. Administration of pooled immunoglobulins from healthy donors (IVIG) was not effective for the prevention of infection and death, but these concentrated stocks of IVIG batches usually contain NAb with a titer of ~256-512 against the common serotypes HAdV1, 2 and 5 (unpublished data). After administration, these IVIG will be diluted *in vivo*. Nevertheless, if it were feasible to generate stocks with very high NAb titers, infusion of these stocks might be effective in preventing spreading of the infection in the patient. An additional problem that arises if this approach would be pursued is the great variability in HAdV serotypes. Currently, 51

serotypes are known, many of which can cause severe infections after SCT, especially serotypes from species A, B and C. Most commonly HAdV1, 2, 5, 6, and 31 are detected, and to a lesser extent HAdV3, 11, and 12. In studies performed in the US, HAdV35 has been reported as a frequently detected pathogen (9), while this pathogen is largely absent in European studies. Furthermore, serotyping of HAdV strains cultured from frequently obtained feces samples of individual HAdV-infected patients has in some cases resulted in the detection of several serotypes within one patient (up to 5 different HAdV serotypes; unpublished observations). Interestingly, patients with sequential infections with multiple serotypes usually survive the infection. Since NAb are only specific for one serotype, it would be very laborious and expensive to have high-titered NAb stocks available for each serotype.

These problems are less pronounced when HAdV-specific T cells will be used for adoptive immunotherapy. $CD4^+$ HAdV-specific T cells, generated against HAdV5, were able to cross-react against HAdV from species A, B, C and D (Chapter 5, 6 and 7), which is in agreement with other reports (26,28). It has been proposed that exposure of individuals to consecutive infections with different strains of a virus will result in repeated cycles of stimulation and expansion of those T cells that recognize epitopes shared between different serotypes (29). On the other hand, T cells recognizing strain-specific epitopes will only be stimulated by infections with that particular strain. This mechanism provides a relative survival and/or growth advantage for T cells that are specific for shared epitopes. In this way, consecutive infections with different strains of HAdV could explain the observed cross-reactivity. This is of great importance as it is not yet possible to predict which HAdV serotype will infect a patient, and serotyping of an infecting strain as well as generation of HAdV-specific T cells requires time. A possibility would be to perform a serotype- or species-specific PCR on feces of the patient pre-SCT in order to investigate which serotypes are present in the feces and thus to predict which serotype might reactivate post-SCT. However, in a report by Lion *et al*., PCR was performed on feces but HAdV DNA could only be detected in feces prior to SCT in 10 out of 132 patients tested (30). The observed cross-reactivity of the HAdV-specific $CD4^+$ T cells might also have important implications for the use of recombinant adenoviral vectors for gene therapy and vaccination. Pre-existing humoral immunity against the adenoviral vector can limit the application of HAdV5 for both gene therapy protocols and vaccine delivery. To circumvent the neutralizing antibody response to the HAdV-based vector, vector backbones of other human serotypes (31-33) and non-human adenovirus serotypes (34) are being developed for gene therapy. As the HAdV-specific T cells recognize conserved peptides of the hexon protein, these cells could limit the potency of HAdV gene transfer and vaccine delivery, irrespective of the serotype of the vector backbone (Veltrop-Duits, unpublished observations). Detailed studies on the interaction of HAdV and the

human immune system are warranted to better understand the influence of cross-reactive T-cells on gene therapy procedures and vaccination potency.

Since a major part of this thesis focuses on adoptive immunotherapy for patients that otherwise succumb to the infection, what can be learned from the patients that survived a disseminated infection? In other words, which immunological factors may have been important in patients that cleared the HAdV infection? In the 5 patients who were included in the current cidofovir study and survived the infection, 8 episodes of viral dissemination were documented. Interestingly, subset analysis of the lymphocytes present during these 8 episodes revealed that T cells were absent in 6 episodes, whereas NK cells were present in all episodes (unpublished observations). In the cases where only NK cells were present, viral DNA was detected for prolonged periods in the plasma, but the viral DNA titers remained either stable or decreased. Likewise, in the patient group described in Chapter 3, one patient cleared disseminated HAdV infection at week 10 after SCT. In this patient, only 2-3% T cells were detected in the blood until week ~30 after transplant, whereas NK cells were rapidly reconstituted (Chapter 3, Figure 4, patient 17). In another patient, the absence of a humoral response until week ~50 after SCT (due to αCD20 treatment for an EBV reactivation) did not hamper the clearance of the virus from the blood at 10 weeks after SCT, although prolonged viral shedding from the feces was observed (Chapter 3, Figure 4, patient 18). Which population of lymphocytes (T cells, NK cells) is required for the initial reduction of a viral titer in plasma, or whether the presence of antibodies is required for the clearance of the virus from the gut remains to be studied in further detail. No data on NK cell reactivity against HAdV in humans has been published yet, although it can be envisaged that the MHC class I reduction induced by the virus will result in an increased recognition by NK cells (35); T cell reactivity could only be detected several weeks after viral clearance from the plasma (Chapter 3). An explanation for the delayed detection of the $CD4^+$ T cell responses could be that $CD4^+$ T cell responses were initiated earlier, but that the detection of these cells *ex vivo* was not optimal, possibly because the frequency of HAdV-specific T cells in the circulation was too low or that cells were unresponsive *in vitro*. This unresponsiveness might be due to the fact that patients received immunosuppressive medication (e.g. CsA to prevent GvHD), which could explain the severely impaired proliferative responses to polyclonal stimuli such as PHA or αCD3 in the first months after SCT. Alternatively, it could be that the HAdV-specific $CD4^+$ T cells were present in the infected tissues at the time of viral clearance, and only redistributed in the circulation after the infection was cleared. The kinetics of the redistribution of virus-specific $CD4^+$ T cells could be different from that of virus-specific $CD8^+$ T cells which have been observed in the blood of patients with CMV and EBV infection at the time of viral clearance (36,37). Another possibility could be that NK cells prove to be essential in the initial control of the viremia shortly after SCT, while

the development of specific T cell immunity only develops in a later phase after SCT. An important role for NK cells in containing viral infections has already been observed for other viruses such as herpesviruses, and is best illustrated by a case-report of a patient with a complete absence of NK cells who succumbed to severe herpesvirus infections at an early age (38-40).

Nevertheless, it remains surprising that the majority of HAdV-specific T cells that were detected consisted of $CD4^+$ T cells, both in patients and in healthy donors. Even the use of different culture protocols, including different cells for presentation such as DCs or monocytes, addition of cytokines or ligands such as low-dose IL-2 and IL-7 or CD40L, or stimulation with an $E1^-E3^-$ recombinant virus in which the MHC class I evasion has been abolished, did not result in significant increases of HAdV-specific $CD8^+$ T cells (Heemskerk and Veltrop-Duits, unpublished observations). Of course, the possibility remains that some factors required for proper stimulation of $CD8^+$ T cells were absent in the cultures and that, therefore, these T cells could not be detected. In agreement with our results, though, many reports have described HAdV-specific $CD4^+$ T cells (25,26,41-44), whereas reports on HAdV-specific $CD8^+$ T cells are scarce (45,46).

The results described in this thesis indicate that HAdV-specific $CD4^+$ T cell clones have antiviral activity *in vitro* (Chapter 7). Cytotoxicity exerted by $CD4^+$ T cell lines and clones has been observed previously (47-49), but much scepticism has been raised whether these findings represent *in vitro* artefacts without having a physiological function *in vivo*. In recent years, reports on *ex vivo* cytotoxic $CD4^+$ T cells have emerged indicating that these effector cells are not merely an artefact but rather a cell type with an advanced stage of cellular differentiation as shown by a loss of CD27 and CCR7 surface expression (50,51). However, whether the antiviral effect of HAdV-specific $CD4^+$ T cells is also present in short-term cultured cells in addition to T cell clones remains to be further investigated. Studies using such short-term cultures *in vitro* require application of HAdV-infected targets other than EBV-transformed B cells since EBV reactivity might still be present in these cultures, confounding the anti-adenoviral effect. Furthermore, the fact that an antiviral effect is observed *in vitro* does not automatically imply relevance for the situation *in vivo*. Whether these $CD4^+$ T cells have effect *in vivo* can only be determined by infusing HAdV-specific $CD4^+$ T cells in patients, and even in that case it might be difficult to determine by which mechanism this effect is induced (either direct lysis, production of IFN-γ, stimulating immune recovery by providing help to $CD8^+$ T cells or B cells, etc.)

What would be the optimal strategy for diagnosis of life threatening HAdV infections in the near future? According to the results described in this thesis, recipients from HLA-identical grafts (MFD) have a similar frequency of HAdV infection as other graft recipients. However, recipients from HLA-identical grafts are not at risk of developing disseminated

and fatal HAdV infection (Chapter 3, van Tol *et al*., submitted, and (7,52)). Therefore, screening of these patients is not required. Recipients of a MUD, MMUD or MMFD graft should be monitored weekly for HAdV infection pre-SCT and during the first 3-4 months post-SCT. An optimal screening program would be to perform RQ-PCR on plasma samples to detect early dissemination of an HAdV infection. Additional weekly culture of feces and urine could be abandoned, as it is labor-intensive and only results in a diagnosis of a higher proportion of HAdV-infected patients without virus-associated complications. In patients with a positive RQ-PCR signal in plasma, feces, throat swab and urine should be obtained for culture. When an HAdV strain is cultured, this clinical isolate can then be used for serotyping and tested for drug susceptibility. Alternatively, a multiplex RQ-PCR could be used that distinguishes the different species or perhaps even the different serotypes when the PCR product is sequenced. In this respect, it should be noted that the RQ-PCR that was used in this study was able to detect all HAdV serotypes, but quantification was optimized for species C and A. More recently, we were able to improve this RQ-PCR technique allowing quantification of species A, B, C and D using species-specific primers and/or probes (Claas *et al*., submitted). More reports on multiplex RQ-PCR have been published in recent years, confirming the need for rapid detection and geno- or serotyping of infecting HAdV strains (20,30,53).

Similarly, when would be the best time to initiate (which) treatment? As has been performed in the ribavirin study (Chapter 4), treatment should be initiated when the presence of viral DNA has been confirmed in a consecutive plasma sample, and reaches levels above 1000 copies/mL (as lower levels have been observed in occasional plasma samples from patients who did not develop disseminated infection, unpublished observations). As cidofovir treatment has *in vitro* activity against all HAdV species, but has not yet been properly investigated in relation to viral load and lymphocyte recovery, this would be the first choice of pharmacological treatment. However, if at the time of initiation of treatment lymphocyte numbers are already increasing, one could speculate that treatment may not even be required as was observed in a few patients that cleared infection without any treatment (Chapter 3). Nevertheless, this referral or withdrawal of treatment would be a high-risk procedure that requires careful retrospective analyses of additional patients before it can be implemented in the clinic. Boosting the immune recovery has also been suggested as treatment for (HAdV) infections in some reports (54). This could be achieved either by tapering the immune suppression (55), or by infusion of DLI cells which have been depleted of the alloreactive T cells (56). In the latter case, donor lymphocytes were mixed with patient lymphocytes for 3-5 days and T cells that had upregulated their IL-2 receptor, *i.e.* $CD25^+$ T cells, were depleted via MACS sorting. Besides antiviral drug treatment, or perhaps even instead of cidofovir treatment as this was unsuccessful in one patient without lymphocyte recovery, we would favor the application of adoptive immunotherapy with HAdV-specific T cells, which has

already been performed for other viral infections such as CMV and EBV post-SCT (57-59). If these T cells were to be infused at the second positive RQ-PCR result, the T cells need to be generated before the patient has actually been diagnosed with HAdV dissemination as it requires several weeks to expand these cells. This would imply that HAdV-specific T cells should be generated for all patients at risk of HAdV dissemination. The main risk factor for dissemination of HAdV is to receive a graft from an other than MFD donor; possible other risk factors are T cell depletion/CD34^{+} enrichment of the graft and ATG/Campath treatment of the patient *in vivo*, although these risk factors need to be confirmed in larger studies. Generating T cells for all these patients is very costly and labor-intensive, and adoptive therapy will probably only be necessary for a small proportion of this group of patients. Alternatively, with the improvement of techniques such as tetramers or artificial liposomal APC for identification or stimulation (60-62), and MACS or FACS sorting of HAdV-specific T cells (44) followed by a short, aspecific expansion period, these T cells might be obtained within 1 to 2 weeks in the future. Another way to obtain more HAdV-specific T cells from the donor could be to vaccinate this donor prior to SCT donation, for example with the 5 peptides selected in Chapter 6. This has two potential benefits which will need to be investigated: the graft may contain more HAdV-specific memory T and B cells, thus conferring immunity to the host, and more HAdV-specific T cells may be isolated from the DLI, which could circumvent the need for prolonged culture to expand these cells when the patient develops a disseminated HAdV infection.

In conclusion, the results described in this thesis have substantially contributed to the knowledge on the occurrence of HAdV infection and dissemination in children after SCT as well as to the unraveling of the human immune response against HAdV. The major goals for the future remain to optimize adoptive immunotherapy, in which short stimulation periods are sufficient. Infusion of these HAdV-specific T cells may then prove to be a valuable tool to decrease the mortality rate due to HAdV infection or reactivation in immunocompromised pediatric patients after allogeneic stem cell transplantation.

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Chapter 9

Summary

Allogeneic stem cell transplantation (SCT) is a curative treatment for many disorders of the hematopoietic system, including primary immune deficiencies such as SCID and inborn errors such as β-thalassemia, as well as for high-risk leukemia patients in whom chemotherapy treatment failed to induce a long-lasting remission. The SCT-procedure is, however, associated with many complications as a result of toxicity of the drugs used and of the severely suppressed hematopoiesis. Notably, infusing stem cells - often depleted of T cells in the case of HLA mismatched transplants – after myeloablative conditioning renders the patients immunocompromised for a prolonged period post-SCT. Infectious complications frequently occur and are a major cause of morbidity and mortality. In recent years, HAdV infections have become increasingly prominent in this respect, especially in pediatric graft recipients (**Chapter 1**). This thesis focuses on the occurrence and treatment of HAdV infections as well as humoral and cellular immune responses against the virus in immunocompromised pediatric recipients of SCT.

Surveillance of patients for HAdV infection with a detection method that allows early recognition of progressive infection with a high sensitivity and specificity is a first requirement to determine whether a patient is at risk of developing HAdV related disease or death. For a long time, culturing of feces or other specimens obtained from the patient has been the standard method for virus detection. However, results obtained with this technique are only available after 1-2 weeks of culture, during which period the infection might progress unnoticed if clinical symptoms are absent. Furthermore, some HAdV strains may grow out (too) slowly in this system, and no information is obtained on the severity of the infection. As for other viruses, a rapid detection method that determines the viral DNA load in serum or plasma was considered useful to detect early dissemination of the virus in order to allow timely initiation of treatment. Therefore, we have set out to develop a semi-quantitative PCR based on a primer set described by Echavarria *et al*, in which a conserved part of the hexon gene was amplified in order to detect all HAdV serotypes (1). DNA was extracted from serum and amplified by PCR at several dilutions of the template ranging from undiluted to 1000-fold diluted. A high load of viral DNA would result in a positive signal in the 1000-fold diluted template. **Chapter 2** describes the results that have been obtained using this semi-quantitative PCR retrospectively on a group of 36 HAdV-infected pediatric SCT patients. A high level of HAdV DNA (*i.e.*, detection of a PCR product after 100-fold or greater dilution of template DNA) in serum samples correlated with fatal disseminated HAdV disease. A high HAdV DNA load was detected in 86% of the patients who developed fatal disease, whereas only 7% of the patients who survived had similar high loads of HAdV DNA ($P < .0001$), indicating a high sensitivity and specificity of the detection method. Previously, it was reported that the detection of HAdV DNA by PCR in serum as such was associated with fatal HAdV disease (2). However, in the present extended retrospective analysis, HAdV DNA - albeit mostly at low levels - was also detected in a significant

proportion of patients who survived. Interestingly, in some patients, it appeared that high loads of HAdV DNA in serum resolved in the absence of any treatment. Together, the results of this retrospective study demonstrated that semi-quantitative data on HAdV DNA load in serum provide clinically relevant information about the outcome of HAdV infection, which warranted more precise quantitative data based on the analysis of serum/plasma samples obtained more frequently in a prospective study. Therefore, we have developed a real-time quantitative (RQ)-PCR to detect HAdV DNA in an automated and more quantitative fashion. Using this RQ-PCR in a pilot study of two patients with a different outcome of the infection, the importance of quantification of viral loads was confirmed as this was the only parameter discriminating between the two patients (*i.e.*, clinical symptoms and culture results were comparable) (3). Thus, we initiated a prospective study to address the presence and course of the plasma DNA load in relation to immune reconstitution more systematically (**Chapter 3**). In this prospective study, 48 pediatric SCT patients were monitored for the occurrence of adenovirus infection and viremia by culture and RQ-PCR, respectively. Infection occurred in 44% of patients, of whom 33% developed HAdV viremia as documented by the presence of viral DNA in plasma. A low lymphocyte count at the time of the first virus isolation was a strong predictor of HAdV viremia, and survival of the viremic infection was correlated with increasing lymphocyte counts during the first weeks of HAdV infection. This strong correlation between lymphocyte recovery and clearance of HAdV infection confirms and extends a previous report (4) and data from a retrospective study of another cohort of pediatric SCT recipients (van Tol *et al.*, submitted). In addition, HAdV-specific immune responses were investigated longitudinally in patients post-SCT. Serotype-specific humoral immune responses as well as $CD4^+$ HAdV-specific T cell responses were detected in patients surviving HAdV viremia several weeks to months after clearance of the viral DNA from the circulation. Interestingly, the presence of pre-existing high HAdV-specific NAb titers in serum of 2 out of 6 patients did not prevent progression to viremia. These results suggest that the role of pre-existing NAb (as measured *in vitro*) in protection against progression to viremia may be limited.

Ribavirin and cidofovir treatment have been used in recent years with variable success, but information on viral load or immune reconstitution was usually lacking in these studies (5-8). Data on immune reconstitution in combination with antiviral therapy is essential as we observed that lymphocyte recovery correlated with clearance of disseminated HAdV in the absence of treatment (Chapter 3). With the development of RQ-PCR techniques, quantitative monitoring of DNA loads in patients treated with antiviral drugs is feasible and may result in objective information on the therapeutic efficacy of these antiviral drugs in immunocompromised patients with disseminated HAdV infection. In **Chapter 4**, we investigated the efficacy of ribavirin treatment to inhibit HAdV replication in 4 patients without lymphocyte recovery, implicating that any observed effect would be due to the

ribavirin treatment. Treatment was initiated at an HAdV DNA load exceeding 1000 copies/mL in two consecutive plasma samples. No reduction in the viral DNA load was observed in any of the patients during the period in which ribavirin was administered. Strikingly, even an apparently unaffected increase in viral DNA load was documented in 3 of the cases, suggesting that ribavirin lacks significant antiviral activity *in vivo* against various HAdV strains. These results provide evidence that ribavirin is not the drug of choice to treat HAdV infection in severely immunocompromised patients.

The lack of effective antiviral drugs for the treatment of severe HAdV infections in pediatric SCT recipients and case reports on the positive effects of DLI on HAdV clearance (9,10) have prompted us to investigate the possibility of adoptively transferring immunity from the donor to the immunocompromised host. This adoptive immunotherapy with T cells from the donor has already been successfully pursued for EBV and CMV reactivations (11,12). Several requirements have to be considered: (i) the T cells need to be specific for HAdV and preferably recognize multiple serotypes from different species (ii) alloreactive T cells should not be present to circumvent GvHD, (iii) sufficient numbers of T cells should be obtained for infusion, (iv) infectious virus, used for stimulation, should not be present in the inoculum, nor $E1^-E3^-$ recombinant virus as homologous recombination with the serotype infecting the patient might occur. To meet these requirements, we studied the possibilities of inactivating HAdV5 completely and to determine the frequency of healthy donors responding to this inactivated HAdV5 (**Chapter 5**). HAdV5 was very efficiently inactivated using the photosensitizer methylene blue (MB, >7 log reduction of infectivity), which is important for the biosafety requirements for adoptive immunotherapy. The proliferative response to MB-inactivated HAdV5 was comparable to that of infectious, wild-type virus or differently inactivated HAdV5. Furthermore, T cells cultured against MB-inactivated HAdV5 also responded to infectious HAdV5, indicating that the antigenic determinants presented by APCs were largely unaltered by MB treatment. After 28 days of culture, alloreactivity in the expanding T cell lines was drastically reduced, probably due to the fact that the alloreactive T cells died by neglect or were diluted out by the expansion of the HAdV-specific T cells. Together, these data suggest that MB-inactivated HAdV5 is a safe source of antigen which can be used to culture T cells that recognize infectious HAdV5. HAdV5-specific T cells that were detected were predominantly $CD4^+$ T cells and secreted IFN-γ upon stimulation in accordance with results of other groups (13,14). To characterize these HAdV-specific $CD4^+$ T cells further, $CD4^+$ T cell clones were obtained to dissect whether the HAdV-specific response was serotype-specific or cross-reactive. All T cell clones that were originally stimulated with HAdV5 recognized not exclusively HAdV5, but also other serotypes from the same species (C) or even serotypes from other species (A and B), indicating that the HAdV-specific T cellular immune response is predominantly cross-reactive (Chapter 5). Other reports have been published describing different methods to expand HAdV-specific T

cells *ex vivo*, which either make use of prolonged culture periods to reduce alloreactivity (15,16), or positive selection of the HAdV-specific, IFN-γ secreting T cells via magnetic bead sorting with the MACS (17).

Another possibility to circumvent the use of (in)active virus would be to culture T cells with antigenic peptides derived from HAdV. To define these peptides, we generated several recombinant structural proteins as well as overlapping hexon peptides from HAdV5 and tested whether these proteins and peptides were recognized by a panel of healthy donors (**Chapter 6**). Comparison of T cell responses against the two structural proteins hexon (protein II) and penton (protein III) and one early gene product, E1A, of HAdV5 revealed that most T cell responses were directed against the structural proteins and the C-terminal part of the hexon protein in particular. The C-terminal part of the hexon protein is the most conserved region between different serotypes (18), offering an explanation for the cross-reactivity found in cultured HAdV-specific T cells that we and others have observed (Chapter 5 and (19-21)). The five most frequently recognized peptides that were identified are derived from the conserved part of the hexon protein. The amino acid sequences of 4 of these peptides were highly conserved between different HAdV serotypes from various species, while one peptide was mainly restricted to serotypes belonging to species C. One of the conserved peptides, peptide II64, contains a previously described HLA-DP-restricted hexon-epitope $II_{910-924}$, which is conserved between many human and other mammalian adenoviruses (22,23). The peptide pool of these 5 hexon-derived peptides was recognized by $CD4^+$ T cells of a majority of the healthy adult population (93%). After culturing with this peptide pool, HAdV-specific $CD4^+$ T cells were detected that recognized target cells infected with wildtype HAdV from different species. Therefore, these data suggest that these peptides are promising candidates for generating HAdV-specific T cells for adoptive immunotherapy as peptides can easily be generated under GMP conditions.

At this stage, however, it remained unknown whether and by which mechanism these $CD4^+$ HAdV-specific T cells are capable of mediating antiviral activity, which would be required for optimal immunotherapy. Therefore, the HAdV-specific $CD4^+$ T cell clones that had been obtained (Chapter 5) were further characterized for their recognition of specific peptides from HAdV, their phenotype and their antiviral function in **Chapter 7**. Most of the T cell clones reacted against peptides that are located in the conserved C terminal part of the hexon protein, and all clones had a predominant Th1 phenotype as they produced IFN-γ and TNF-α upon stimulation with HAdV5 or specific peptide. To determine whether these $CD4^+$ HAdV-specific T cells were capable of mediating antiviral activity, we developed an *in vitro* assay in which the effect of T cells on viral replication was investigated. Viral titers were determined in cell lysates of infected B-LCLs in the presence or absence of a T cell clone. Addition of T cells at a 10:1 or 1:1 ratio had a strong impact on viral replication by inhibiting viral outgrowth more than 99%, suggesting that HAdV-specific $CD4^+$ T cell clones do

indeed have antiviral activity *in vitro*. Cognate interaction between TCR and the MHC II-peptide complex was required for the T cells to inhibit viral replication, which was most likely mediated by perforin-mediated lysis induced by these $CD4^+$ T cells.

Finally, in **Chapter 8** these findings and their implications for future research, both for treatment of disseminated HAdV infection and the use of adenoviral vectors for gene therapy, are discussed.

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NEDERLANDSE SAMENVATTING

Stamceltransplantatie (SCT) is een procedure waarbij stam cellen (= voorloper cellen), die afkomstig zijn van een gezonde donor en waaruit rode en witte bloedcellen kunnen ontstaan, worden overgebracht naar een patiënt. Deze behandeling wordt toegepast bij verschillende aandoeningen van de aanmaak van cellen van het immuunsysteem, primaire immuundeficiënties, en voor andere aangeboren afwijkingen zoals β-thalassemie. Ook wordt SCT uitgevoerd bij patiënten met kanker van cellen van het immuunsysteem, genaamd leukemieën, die niet voldoende reageren op de standaard chemotherapie. De procedure van SCT is echter geassocieerd met veel complicaties als gevolg van de toxiciteit van de behandeling en vanwege de langdurige afwezigheid van een goed functionerend immuunsysteem na de behandeling aangezien de stamcellen moeten uitrijpen tot nieuwe functionele cellen van het immuunsysteem, zoals T en B lymfocyten.

Het zoeken van een juiste donor is erg belangrijk omdat normaliter een transplantaat van een donor door het afweersysteem van de patiënt wordt afgestoten doordat het transplantaat als lichaamsvreemd wordt herkend. Deze afstoting wordt veroorzaakt doordat de patiënt en de donor van elkaar verschillen voor bepaalde weefselantigenen, die gezamenlijk het “major” histocompatibiliteitscomplex (MHC) worden genoemd. Een donor moet dus in het ideale geval genetisch identiek zijn met de patiënt voor deze MHC antigenen, zoals het geval kan zijn bij een broer en zus. Een MHC-identieke familiedonor (MFD) is echter niet altijd aanwezig. In zo’n geval wordt er gezocht naar een vrijwillige beenmergdonor die qua MHC wel volledig overeenkomt met de patiënt, maar die niet tot de familie behoort (matched unrelated donor, MUD), of anders naar familiedonoren of beenmergdonoren die mismatch(es) hebben (mismatched family donor, MMFD of mismatched unrelated donor, MMUD). In het geval dat de donor en de patiënt verschillen van elkaar voor het MHC, worden de T cellen die verantwoordelijk zijn voor de afstoting, uit het transplantaat verwijderd. Dit heeft tot gevolg dat de patiënt na SCT een verminderde immunologische afweer heeft aangezien het meer tijd kost om voldoende nieuwe T en B cellen te genereren. Bij deze patiënten komen zeer frequent virale infecties voor, die normaal gesproken geen problemen zouden veroorzaken, maar in de context van een verlaagde afweer zeer ernstig en soms fataal kunnen zijn.

In de laatste jaren zijn infecties door het adenovirus veelvuldig voorgekomen bij kinderen die een SCT ondergingen. Dit humane adenovirus (HAdV), waarvan 51 typen bekend zijn, geeft in gezonde kinderen slechts een verkoudheid, maar dit virus kan fatale infecties opleveren na een SCT. Dit proefschrift beschrijft het optreden van zulke HAdV infecties na SCT, evenals de mogelijke behandeling met medicijnen die momenteel beschikbaar zijn. Verder zijn in dit proefschrift de verschillende componenten van de specifieke immuunrespons tegen HAdV gekarakteriseerd bij gezonde personen en bij kinderen na SCT.

Deze componenten zijn onder andere de T cellen, die verantwoordelijk zijn voor het herkennen en opruimen van geïnfecteerde cellen (cellulaire immuniteit), en de B cellen, die verantwoordelijk zijn voor de productie van afweerstoffen genaamd antilichamen die aan het virus kunnen binden en zo infectie van cellen kunnen voorkomen (humorale immuniteit).
Patiënten moeten regelmatig vervolgd worden na SCT om een HAdV infectie, die mogelijk fataal zou kunnen verlopen, snel op te sporen. De conventionele techniek waarmee vastgesteld kan worden of een patiënt een HAdV infectie heeft, is gebaseerd op het kweken van feces, urine of een keeluitstrijkje in aanwezigheid van cellen die gevoelig zijn voor HAdV infectie. Deze techniek is echter zeer tijdrovend omdat het virus eerst deze cellen moet infecteren, wat enkele dagen kan duren, voordat de uitslag bekend kan worden. Tevens geeft de kweektechniek weinig informatie over de ernst van de infectie. Om deze redenen hebben we een andere detectie methode opgezet, waarmee een ernstige HAdV infectie beter vast te stellen is. Deze detectietechniek is gebaseerd op het aantonen van het adenovirale DNA door middel van PCR (polymerase chain reaction). Met de PCR wordt viraal DNA vermenigvuldigd zodat het met grote gevoeligheid aantoonbaar wordt in het bloed van een patiënt. Een positief signaal met deze methode is een teken van verspreiding van HAdV via het bloed en dus van een zich door het lichaam verspreidende (gedissemineerde) infectie, die mogelijk een ernstig beloop kan hebben. In **Hoofdstuk 2** hebben we deze PCR techniek toegepast op bloedplasma monsters die bewaard waren van 36 kinderen die in de jaren ervoor na SCT een HAdV infectie hadden doorgemaakt. Bij deze kinderen was de HAdV infectie aangetoond door middel van positieve feces kweken. Tevens werd het te testen materiaal verdund, om een indruk te krijgen van de aanwezige hoeveelheid viraal DNA. Met andere woorden, als het signaal nog steeds meetbaar was na 1000 × verdunning, betekende dit dat er meer viraal DNA aanwezig was in het plasma dan wanneer het alleen onverdund aantoonbaar was en bij hogere verdunningen negatief bleek te worden. De resultaten toonden aan dat een hoge virale load, dus het detecteren van viraal DNA in 100 of 1000 × verdund plasma, correleerde met een fataal beloop van de HAdV infectie. Bij patiënten die alleen een milde infectie hadden gehad werd weliswaar soms een positief PCR signaal in het plasma gevonden, maar dit signaal was hooguit nog aantoonbaar in 10 × verdund plasma. Uit deze resultaten bleek dat deze semi-kwantitatieve methode van het bepalen van de virale load van belang was om de uitkomst van de infectie (overlijden of niet) te kunnen voorspellen.
De studie in hoofdstuk 2 was opgezet met monsters die retrospectief (achteraf) geanalyseerd werden. Daarom wilden we deze bevindingen bevestigen in een patiëntengroep die prospectief gevolgd werd voor HAdV infectie, waarbij mogelijkerwijs meer infecties aantoonbaar zouden zijn omdat in de retrospectieve studie alleen feceskweken afgenomen waren tijdens een verdenking op HAdV infectie. Tevens was inmiddels de techniek van de PCR geoptimaliseerd, zodat er nu in plaats van een verdunningswaarde een echte kwantitatieve virale load bepaald kon worden, die overeenkwam met het aantal kopieën van

het virale DNA (bijvoorbeeld 1000 kopieën, of 1 miljoen kopieën) per milliliter plasma. In **Hoofdstuk 3** wordt deze prospectieve studie beschreven, waarin 48 patiënten die tussen januari 2001 en januari 2003 een SCT hadden ondergaan, gevolgd zijn voor het optreden van HAdV infectie na SCT, zowel door het uitvoeren van kweken op feces materiaal als van kwantitatieve PCR op plasma eenmaal per week. In 44% van de patiënten werd een HAdV infectie aangetoond door middel van feces kweek. In 33% (n=6) van de geïnfecteerde patiënten bleek het virus te dissemineren,wat resulteerde in een virale load in het plasma. Drie van deze 6 patiënten overleden uiteindelijk aan de HAdV infectie, terwijl de andere 3 patiënten in staat waren om het virus te elimineren. Het bleek dat aanwezigheid van een laag aantal lymfocyten in het bloed na SCT samenhing met de verspreiding van het virus door het lichaam. Bij de patiënten met een gedissemineerde infectie die uiteindelijk het virus wisten op te ruimen was bovendien een sterke stijging van het aantal lymfocyten waarneembaar, die niet optrad bij patiënten die overleden aan de infectie. Kortom, herstel van het aantal lymfocyten was cruciaal voor het overleven van een HAdV infectie. In dit hoofdstuk werd tevens onderzocht welke immuunresponsen tegen het HAdV opgebouwd werden door de patiënten die de infectie overleefden. Het bleek dat de T cellen in staat waren om een anti-HAdV respons te ontwikkelen, wat werd aangetoond door het vastsellen van de uitscheiding van antivirale stoffen (genaamd cytokinen zoals interferon gamma, IFN-γ) na stimulatie met HAdV. Tevens was er een B cel respons meetbaar, doordat er een toename zichtbaar was van de hoeveelheid antilichamen in het bloed van de patiënt, die gericht waren tegen het specifieke HAdV type dat de infectie had veroorzaakt. Deze T en B cel responsen waren over het algemeen pas te detecteren nadat het virus was verdwenen uit het bloed, dus nadat de disseminatie was geklaard.

Er zijn niet veel medicijnen beschikbaar om een HAdV infectie mee te behandelen. Twee medicijnen, ribavarine en cidofovir, worden daarvoor gebruikt, maar de resultaten van deze behandelingen zijn niet erg overtuigend. Tevens waren de door andere onderzoeksgroepen gerapporteerde bevindingen alleen gebaseerd op de resultaten van feceskweken, terwijl we inmiddels wisten dat in een groot deel van deze patiënten het virus uiteindelijk niet dissemineert al naar gelang het herstel van het immuunsysteem bij de patiënt. Om deze redenen hebben we in **Hoofdstuk 4** gekeken naar het effect van de behandeling met ribavarine van patiënten waarin het virus kort tevoren aantoonbaar werd in het bloed, dus aan het begin van de disseminatie van de infectie. Aangezien deze patiënten geen lymfocytenherstel hadden, moest elk effect op de virale load in het plasma een gevolg zijn van de ribavirine behandeling en niet het gevolg van het herstel van het immuunsysteem. In de 4 patiënten die beschreven zijn in hoofdstuk 4 bleek dat er geen effect op de virale load aantoonbaar was tijdens ribavarine behandeling. In drie gevallen was er zelfs een toename in de virale load tot hoge aantallen viruskopieën in het plasma. Hieruit kon worden geconcludeerd dat ribavarine geen antiviraal effect op HAdV heeft, en dus niet geschikt is

om patiënten met een HAdV infectie te behandelen in afwezigheid van immuunreactieve cellen.
Het gebrek aan goede antivirale geneesmiddelen voor de behandeling van ernstige HAdV infecties bij kinderen na SCT, alsmede het feit dat enkele patiënten waren beschreven met een gunstig effect van het geven van donorlymfocyten op het klaren van een HAdV infectie, hebben ons ertoe aangezet om de mogelijkheid van adoptieve overdracht van immuniteit (door middel van HAdV-specifieke T cellen) van de stamceldonor naar de patiënt te onderzoeken. Deze benadering van het overdragen van immuniteit is al eerder succesvol gebleken bij de bestrijding van andere virale infecties, zoals infecties met het Epstein Barr virus of het cytomegalovirus, bij patiënten na SCT. Er zijn meerdere eisen waaraan moet worden voldaan om deze benadering te kunnen toepassen: (i) de T cellen die worden gegeven aan de patiënt moeten specifiek zijn voor HAdV, en bij voorkeur ook nog in staat zijn om verschillende serotypen (varianten) van het virus te herkennen; (ii) T cellen die reactief zijn tegen weefselantigenen op cellen van de patiënt (alloreactief genaamd) moeten niet meer aanwezig zijn in de celpopulatie die overgebracht wordt naar de patiënt; (iii) er moeten voldoende T cellen kunnen worden gegenereerd; en (iv) levend infectieus virus mag niet aanwezig zijn in de celpopulatie, omdat dit mogelijk een verergering van de infectie bij de patiënt zou kunnen veroorzaken. Om aan deze eisen te voldoen, hebben we de mogelijkheden onderzocht om HAdV5 (serotype 5) te inactiveren en tevens onderzocht hoe groot het percentage gezonde donoren is dat respondeert tegen dit geïnactiveerde HAdV5 (**Hoofdstuk 5**). HAdV5 bleek gemakkelijk geïnactiveerd te worden met de fotosensitizer methyleen blauw (MB), wat belangrijk is voor de veiligheid van adoptieve immuuntherapie (dus geen overdracht van levend virus). De proliferatieve respons (de deling van T cellen als respons op het virus) was vergelijkbaar na stimulatie met infectieus virus en MB-geïnactiveerd virus. T cellen gekweekt met MB-geïnactiveerd virus waren ook nog in staat om infectieus virus te herkennen, wat aangeeft dat de antigenen (eiwitten van het virus waar de T cellen op reageren) behouden blijven na inactivatie met MB. Na 28 dagen kweken van cellen uit bloed van de donor in aanwezigheid van virus was de alloreactieve T cel respons sterk verminderd, terwijl de HAdV-specifieke respons was toegenomen. Deze resultaten suggereren dat MB-geïnactiveerd HAdV5 een veilige bron voor stimulatie van T cellen is, waarmee T cellen kunnen worden gegenereerd die levend virus kunnen herkennen (wat uiteindelijk aanwezig zal zijn in de patiënt). De HAdV-specifieke T cellen waren voornamelijk cellen van de $CD4^+$ T cel populatie, die IFN-γ uitscheiden (een cytokine met antivirale werking). Om deze cellen verder te kunnen bestuderen werden T cel klonen gegenereerd, wat inhoudt dat de cellen zo ver verdund worden dat de functie van de nakomelingen van één unieke T cel onderzocht kan worden. Met behulp van deze T cel klonen is vastgesteld dat een T cel in staat is om meerdere serotypen van HAdV (er zijn momenteel 51 serotypen bekend) te herkennen, wat aangeeft dat de T cel respons

kruisreactief is (dus T cellen gestimuleerd met HAdV5 kunnen bijvoorbeeld ook HAdV35 herkennen).

Een andere mogelijkheid voor T cel stimulatie, waarbij het gebruik van (in)actief virus wordt vermeden, is het kweken van T cellen in aanwezigheid van bepaalde peptiden (eiwitfragmenten) van het virus. Om vast te stellen welke peptiden van het virus een T cel respons kunnen opwekken, hebben we een paar eiwitten van HAdV gemaakt. Van één van deze eiwitten zijn ook kortere overlappende peptiden gemaakt, elk met een lengte van 30 aminozuren en met een overlap van 15 aminozuren tussen de verschillende peptiden. Deze eiwitten en peptiden zijn vervolgens getest in een panel van gezonde donoren om te bepalen welke peptiden bij de meeste personen een T cel respons opwekken (**Hoofdstuk 6**). Het is gebleken dat één van de structurele eiwitten, het hexoneiwit, door de meeste personen herkend werd, en dan met name het geconserveerde deel van het hexoneiwit (met de grootste overeenkomt tussen de verschillende varianten van het virus). Dit kan ook verklaren waarom de meeste T cel klonen kruisreactief waren, aangezien deze T cellen ook peptiden in het geconserveerde deel herkenden. De 5 peptiden die het meest frequent herkend werden door $CD4^+$ T cellen van gezonde donoren werden vervolgens gebruikt om T cellen te stimuleren in kweek. Na 28 dagen kweken waren de T cellen in staat om infectieus virus van verschillende serotypen te herkennen. Deze peptiden zouden dus goed gebruikt kunnen worden om T cellen te kweken voor adoptieve immunotherapie aangezien deze peptiden relatief eenvoudig te produceren zijn en duidelijke voordelen hebben boven het gebruik van intact virus.

Het was echter nog onbekend of $CD4^+$ HAdV-specifieke T cellen een antivirale functie hebben, met andere woorden of deze cellen daadwerkelijk in staat zijn om het virus op te ruimen. Dit is natuurlijk van groot belang voor het succes van immunotherapie in een patiënt met een ernstige HAdV infectie. De $CD4^+$ T cel klonen, die waren gegenereerd in het onderzoek beschreven in Hoofdstuk 5, hebben we daarom onderzocht op hun antivirale functie zoals weergegeven in **Hoofdstuk 7**. Hiervoor is een test opgezet waarin het effect van het toevoegen van T cellen op de adenovirale replicatie (ontstaan van nieuwe virusdeeltjes) in geïnfecteerde cellen werd bestudeerd. Wanneer HAdV-specifieke T cellen werden toegevoegd aan geïnfecteerde B cellen werd de virale uitgroei sterk verminderd tot minder dan 1% van de uitgroei. Dit suggereert dat deze T cellen inderdaad antivirale activiteit bezitten. De remming van virale replicatie werd hoogstwaarschijnlijk veroorzaakt door het direct vernietigen (lyseren) van de geïnfecteerde B cel. Tenslotte zijn de resultaten van dit proefschrift en het belang hiervan voor de behandeling van patiënten met ernstige HAdV infecties na SCT kritisch besproken in **Hoofdstuk 8**.

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NAWOORD

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Veel liefs,

Bianca

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 22 augustus 1977 te Noordwijkerhout. De middelbare school werd doorlopen op Atheneum College Hageveld in Heemstede van 1989 tot 1995. In datzelfde jaar begon zij de studie Biomedische Wetenschappen in Leiden, waarin zij onderzoekservaring opdeed tijdens stages op de afdelingen Klinische Oncologie (bij Corlien Aarnoudse/ Peter Schrier) en Reumatologie (bij Paulien Goossens/ Tom Huizinga) in het LUMC, Leiden, en in the Department of Respiratory Medicine, Rayne Laboratory, (bij Jean-Michel Sallenave) Edinburgh, Schotland. Haar afstudeerstage deed zij op de afdeling Immunobiologie (bij Kiki Tesselaar/ René van Lier) op het CLB in Amsterdam, waar zij onderzoek deed naar de effecten van CD27-CD70 interactie. Het diploma werd cum laude behaald in september 2000.
Aangezien het vakgebied van de immunologie haar grootste interesse had, begon zij vervolgens aan een AIO onderzoek op het Immunologisch Laboratorium van de afdeling Kindergeneeskunde in het LUMC. Het onderzoek zoals beschreven in dit proefschrift is verricht van oktober 2000 tot en met maart 2005, waarin tevens de opleiding tot Immunoloog (SMBWO) werd voltooid. Vanaf 1 juni 2005 zal zij beginnen als postdoc onder de leiding van Dr. Steven Rosenberg in the National Cancer Institute, wat een onderdeel is van the National Institutes of Health (Bethesda, Washington, USA). Hier zal zij gaan werken aan het optimaliseren van immunotherapie voor patiënten met kanker.