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Low influence of irradiation on the functional activity of in-vitro expanded ADV-specific T cells: A safe and potential therapeutic option for adoptive immunotherapy

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Somewhere, something incredible
is waiting to be known.

Dr. Carl Sagan

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Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) has become a standard procedure in the management of malignant and nonmalignant diseases. Successful HSCT depends on immunosuppression, which is necessary to avoid graft rejection and graft-versus-host disease (GvHD) until the donor-derived immune system reconstitutes. The duration depends on the tissue HLA-type (human leukocyte antigen) between donor and patient, and intensity of chemotherapy or irradiation prior to transplantation. As a result, patients are highly susceptible to a wide range of viral infections, and adenovirus (ADV), for example, has become the most common viral pathogen responsible for significant post-transplantation morbidity and mortality in pediatric patients.

Antiviral drugs seem to control but not cure the infection and exhibit undesired side effects. However, it was shown that recovery of ADV-specific T cells correlates with the clearance of ADV infections. Since the frequency of ADV-specific T cells of healthy ADV-seropositive individuals is very low in fresh blood, we established a very fast method to expand and detect ADV-specific T cells. We repeatedly stimulated peripheral blood mononuclear cells (PBMCs) with ADV-specific antigens within a 12 day culture period. After expansion, the number of ADV-specific T cells was 1-2 log increased and could then easily be detected by HLA-peptide multimers. Furthermore, the expansion protocol was optimized by the addition of interleukin (IL) -15, which was superior (3,5 fold) over the treatment with IL-2 or IL-7.

Independent of the interleukins used, after in vitro expansion, 96% of all cells exhibited an effector immunophenotype. However, about 3% of all cells maintained a central memory phenotype, providing long-lived in vivo protection against viral infections.

Next, we confirmed that expanded ADV-specific T cells are functionally active by showing increased secretion of interferon-gamma (IFN-γ) and expression of activation markers such as CD137 and CD107a after stimulation with the virus-specific antigen. Additionally, cytotoxicity was observed against both autologous and partially mismatched antigen-pulsed target cells but was highly reduced against completely mismatched target cells, indicating a very low likelihood of alloreactivity of expanded cells. However, to completely reduce possible residual alloreactivity without influencing functional activity, expanded cells have been irradiated. Strikingly, irradiated ADV-specific T cells showed similar antigen-specific IFN-γ response and cytotoxicity as compared to non-irradiated cells without alloreactivity. These results demonstrate a milestone in the field of adaptive T cell transfer and could have major implications for patients suffering from viral infections after HSCT.

Zusammenfassung

Allogene hämatopoetische Stammzellentransplantationen (HSZT) haben sich als Standardbehandlung für maligne und nicht-maligne Erkrankungen etabliert. Die Erfolgsrate einer HSZT wird stark vom Grad der Immunsuppression beeinflusst, welche notwendig ist um die Transplantatabstoßung und Transplantat-gegen-Wirt Reaktion (graft-versus-host disease, GvHD) zu verhindern, bis sich das neue (vom Spender stammende) Immunsystem vollständig entwickelt hat. Die dafür erforderliche Zeit hängt vom Gewebe HLA-Typ zwischen Spender und Empfänger und der Intensität der Chemotherapie oder Bestrahlung vor Beginn der Transplantation ab. In diesem Zeitraum sind die Patienten einem erhöhtem Risiko für virale Infektionen ausgesetzt. Der humane Adenovirus (ADV) gilt dabei als der häufigste virale Erreger, der eine signifikant erhöhte Erkrankungswahrscheinlichkeit und Sterblichkeitsrate bei Kindern nach Transplantationen aufweist.

Antivirale Medikamente können die Infektion in den meisten Fällen nur eindämmen, jedoch nicht heilen, zudem weisen diese viele unerwünschte Nebenwirkungen auf. Es konnte jedoch gezeigt werden, dass das Auftreten von ADV-spezifischen T Zellen mit der Beseitigung der ADV Infektion korreliert. Die Frequenz ADV-spezifischer T Zellen von gesunden ADV-seropositiven Personen ist im Blut sehr gering. Aus diesem Grund entwickelten wir ein Protokoll um diese Zellen anzureichern und nachzuweisen. Dafür wurden mononukleare Zellen des peripheren Blutes isoliert und mehrmals innerhalb einer 12 Tage Kultivierung mit einem ADV-spezifischen Antigen stimuliert. Nach der Expansion war die Zahl der ADV-spezifischen T Zellen um 1-2 log Stufen angestiegen und konnte mittels HLA-Peptid Pentamer nachgewiesen werden. Weiters konnten wir die Anreicherung durch eine Interleukin (IL) -15 Zugabe verbessern, was die Expansion um das 3.5 fache im Vergleich zu IL-2 und IL-7 Behandlung erhöhte.

Unabhängig von der Interleukingabe entwickelten 96% aller angereicherten ADV-spezifischen Zellen einen Effektor Phänotyp. Wir konnten jedoch auch zeigen, dass 3% als Zentrale-Gedächnis-T-Zellen (central memory T cells, TCM) erhalten bleiben und somit einen lebenslangen Schutz gegen virale Infektionen gewährleisten.

Im nächsten Schritt analysierten wir die Funktionalität von angereicherten ADV-spezifischen T Zellen durch den Nachweis der gesteigerte Sekretion von Interferon-gamma (IFN-γ) und der Expression von Aktivierungsmarkern wie CD137 und CD107a, nach der Stimulation mit dem Virus-spezifischen Antigen. Die Analyse der Zytotoxizität zeigte, dass autologe, aber auch nur zum Teil übereinstimmenden Antigen-beladenen Zielzellen mit einer hohen Inzidenz eliminiert wurden. Vollständig unpassende und unbeladene Zielzellen wurden dagegen nicht erkannt, was auf eine spezifische Erkennung mit einem geringen Potential für Alloreaktivität schließen lässt. Um die Gefahr einer GvHD vollständig zu reduzieren, ohne dabei die Funktionalität zu beeinflussen, wurden die angereicherten Zellen bestrahlt.

Bemerkenswerterweise zeigten bestrahlte ADV-spezifische T Zellen ähnliche Antigen-spezifische IFN-γ Reaktionen wie unbestrahlte Zellen, ebenso war die Zytotoxizität, welche ein Indiz für Alloreaktivität wäre, im Vergleich nicht verändert.
Diese Ergebnisse liefern wichtige Informationen für ein besseres Verständnis auf dem Gebiet der Adaptiven-T-Zellenübertragung und verbessern die Prognosen bei der Bekämpfung von viralen Infektionen nach HSZT.

Table of contents

ACKNOWLEDGEMENT 5
ABSTRACT 7
ZUSAMMENFASSUNG 9
TABLE OF CONTENTS 11
1 INTRODUCTION 13
1.1 HEMATOPOIETIC STEM CELL TRANSPLANTATION AND ITS POTENTIAL RISKS 13
1.2 ADENOVIRUS 14
1.2.1 Biology of adenovirus 14
1.2.2 Infection 14
1.2.3 Diagnosis and clinical manifestation 15
1.2.4 ADV-specific immunity 16
1.2.5 Treatment 20
1.3 AIMS OF RESEARCH 22
2 MATERIALS AND METHODS 23
2.1 MATERIALS 23
2.1.1 Apparatuses and Instruments 23
2.1.2 Plastic Material 23
2.1.3 Chemicals and Regents 24
2.1.4 Buffer and Media 24
2.1.5 Stimulants reagents 25
2.1.5.1 Adenovirus 25
2.1.5.2 Staphylococcal enterotoxin B (SEB) 25
2.1.5.3 Phytohaemagglutinin (PHA) 25
2.1.6 Cytokine 26
2.1.7 Antibodies 26
2.1.8 Viability staining 26
2.1.9 ADV pentamere 27
2.1.10 Further equipment 27
2.1.11 Patient samples 27
2.2 METHODS 28
2.2.1 Generation of virus-specific T cells 28
2.2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs) 28
2.2.1.2 Freezing and Thawing 28
2.2.1.3 Cell culture 28
2.2.1.4 Expansion of ADV-specific T cells (in vitro cell stimulation) 29
2.2.2 Irradiation 30
2.2.3 Detection of circulating virus-specific T cells 30
2.2.3.1 Column-based isolation of antigen-specific cells 30
2.2.3.1.1 Isolation of CD14+ cells (monocytes) 30
2.2.3.1.2 Isolation of ADV-specific T cells 31
2.2.3.2 Pentamer staining 31

2.2.3.3 Cytokine secretion assay (CSA) 32
2.2.3.4 Phenotype analysis 33
2.2.4 Cytotoxic assay (CTA) 33
2.2.4.1.1 Autolog system 33
2.2.4.1.2 Target cell population 33
2.2.4.1.3 Effector cell population 33
2.2.4.1.4 Assay 35
2.2.4.2 Allogeneic system 35
2.2.5 Flow cytometry 35
2.2.5.1 Cytometric cell sorting 35
2.2.5.2 Flow cytometric analysis 35
2.2.5.3 Data evaluation and gating strategy 36
2.2.6 Statistics 37
3 RESULTS 38
3.1 EXPANSION AND CHARACTERIZATION OF IN VITRO-GENERATED ADV-SPECIFIC T CELLS 38
3.1.1 Expansion of ADV-specific T cells 38
3.1.2 Influence of different interleukins on the expansion of ADV-specific T cells 41
3.1.3 Phenotypic characterization of T cells 41
3.1.3.1 Phenotypic characterization of T cell populations before and after the expansion period 41
3.1.4 The effect of interleukins on the immunophenotype of T cells during in vitro expansion 42
3.2 IRRADIATED VIRUS-SPECIFIC T CELLS FOR ADOPTIVE IMMUNOTHERAPY AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION 45
3.2.1 Viability of expanded ADV-specific T cells after irradiation 45
3.2.2 Effect of irradiation on the functional activity of ADV-specific T cell population 47
3.2.3 Effect of irradiation on the cytotoxic activity of ADV-specific T cells 49
3.2.4 Clinical Applications 52
3.2.4.1 Frozen expanded ADV-specific T cells show no quality reduction 52
3.2.4.2 Analysis of alloreactivity of non-irradiated and irradiated expanded ADV-specific T cells 52
4 DISCUSSION 55
5 REFERENCES 59
6 APPENDIX 63
6.1 LIST OF FIGURES 63
6.2 LIST OF TABLES 64
6.3 LIST OF ABBREVIATIONS 65
6.4 CURRICULUM VITAE 67

1 Introduction

1.1 Hematopoietic stem cell transplantation and its potential risks

Hematopoietic stem cell transplantation (HSCT) has evolved into an effective strategy for the treatment of certain hematological malignancies and inborn errors. Allogeneic transplantation involves the transfer of stem cells from donor to recipient, and as a consequence, an introduction of histoincompatible cells occurs. The degree of histoincompatibility influences the incidence of graft rejection and graft-versus-host disease (GvHD).[1]

In addition to preventing the graft from immunological rejection, the patient has to be immunosuppressed at the time of stem cell transfer.[2,3] A combination of chemotherapy and total body irradiation was the choice of treatment in the past; nowadays, powerful immunosuppressive drugs instead of, or in combination with irradiation are common practice. Under these circumstances stem cells derived from the transplant donor are required to rescue hematopoiesis.[1] The immunosuppression has to be continued for 3-6 months, until the donor-derived immune system reconstitutes.[2,3] The velocity of the immune reconstitution following HSCT depends on donor type, intensity of post-transplant immunosuppression and on the extent of T cell depletion in the graft.[3]

As a result of immunosuppression, recipients are susceptible to a wide range of serious and often lethal opportunistic infections.[1,2] Although bacterial infections are common, in general they can be controlled by the appropriate antibiotics. Viral infections are less well controlled and are the main clinical burdens nowadays.[1,4] New infections play a subordinate role, more often the reactivation of latent viruses, such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Adenovirus (ADV) are common and cause symptomatic disease.[4] For CMV and EBV, antiviral pharmacologic agents are cost intensive but eliminate the infection quite effectively. ADV-infection treatment of immunocompromised patients, on the other hand, is still unsatisfactory and less effective.[5]

In the last years an increasing incidence of ADV-infections has been observed, attributed either to an improvement of diagnostic tools and a more widely spread screening or to an extension of stem cell transplantation to higher risk patients.[2,3,6]

An unmanipulated hematopoietic stem cell (HSC) inoculum from the bone marrow contains a large number of T cells from the donor (1×10^9 - 5×10^9). The amount could increase to 5×10^{10}, if peripheral-blood HSC are used, through donor stimulation with granulocyte colony-stimulating factor (G-CSF).

Although donor T cell populations provide partial protection against infection, unfortunately, these cells are also responsible for the development of GvHD. To minimize this risk, T cell depletion is widely used for unmatched donor transplants, whereas the optimal T cell dose remains a hotly debated issue in the field of clinical transplantation.[1]

1.2 Adenovirus

1.2.1 Biology of adenovirus

Human ADV is a non-enveloped, ubiquitous, lytic double-stranded, up to 36kb DNA virus. More than 50 different human serotypes with varying tissue tropism have been identified and the number of new potential serotypes has increased in the last years. They are divided into 6 subgroups (A-F) according to their oncogenic potential in rats, hemaglutinating properties, morphological and DNA sequence characteristics.[3,7,8]

The morphology of ADV is characterized by an icosahedral protein capsid, comprising three major proteins: hexon, penton base and a fiber (Figure 1).[3,8]

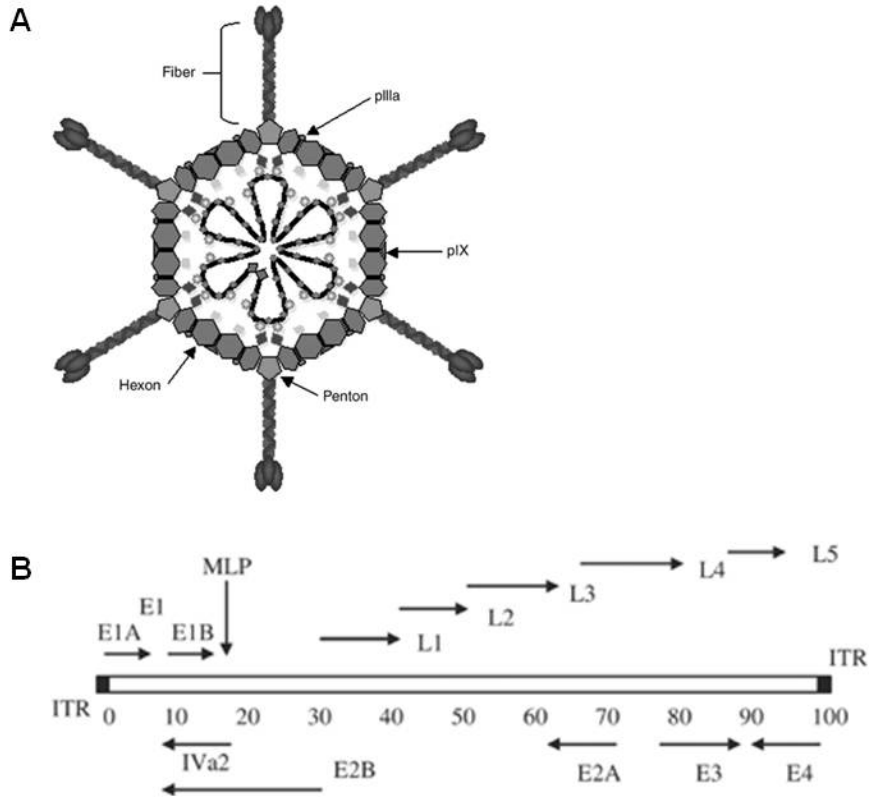


Figure 1. Morphology and genome organization of adenovirus.
(A) Adenovirus structure with the major structural components: fiber, hexon and penton, furthermore pIIIa and pIX stabilize the virus particle, inside a double-stranded DNA is located.[8]
(B) The genome of about 36kb is divided into early (E1-4), intermediate (Iva2) and late (L1-5) genes, flanked by right and inverted terminal repeats (ITR). MLP: major late promoter.[9]

1.2.2 Infection

Transmission occurs through respiratory droplets or the fecal-oral route.[3] Adenoviruses are endemic in all populations throughout the year, 80% of children between the ages of 1 and 5 years have antibodies to one or more serotypes, which indicates a past or current ADV infection.[6]

ADV can infect nearly all human cells,[3,10] although they persist preferably in epithelial cells and lymphoid tissue.[7] Lion at al. suggest that the intestinal tract may be the primary site of ADV reactivation, at least in pediatric transplant recipients.[7]

1.2.3 Diagnosis and clinical manifestation

In the last years real-time PCR assays have evolved as a main diagnostic tool for detection and quantification of ADV in blood, stool, urine and throat.[7] The wide range of serotypes with major genetic differences has become manageable by designing practical primer combinations from the hexon and the VA RNA region.[11,12]
In general, human ADV is not considered to be highly pathogenic in immunocompetent individuals, apart from that, only half of the strains are associated with clinical symptoms.[3,5] Primary infection appears very often during infancy and in most cases it is and remains asymptomatic. Trivial gastrointestinal and respiratory symptoms can sometimes be observed,[3] yet the diseases are typically mild and mostly resolve without sequelae. Only in rare cases, hepatitis, myocarditis, meningoencephalitis or nephritis are observed.[5,7]

In patients with compromised immunity, however, ADV-infections are associated with a high incidence of morbidity and mortality, occurring in 20-30% after HSCT in pediatric patients, whereas only 3-13% of adults are affected.[6] In patients with disseminated disease the mortality rate could be as high as 60%[6] to 70%[13].

Detection and selection of virus-specific T cells by human leukocyte antigen (HLA)-peptide pentamers

The direct measurement of specific lymphocytes with the multimer technology was described the first time 1996 by Altman et al.[14] This tool is based on the fact that T cells recognize peptides, derived from pathogens that are bound to major histocompatibility complex (MHC) molecules on the surface of target cells or antigen-presenting cells (APCs). This recognition is very specific and makes it possible to identify antigen-specific T cells by binding of MHC allele attached to fluorochrome (Figure 2).[15]

However, most tools focus on the analysis of CD8+ T cells (MHC I), although new tools for the analysis of MHC-class-II-restricted T cell responses and CD1-restricted natural-killer T cell responses are being developed.[15]

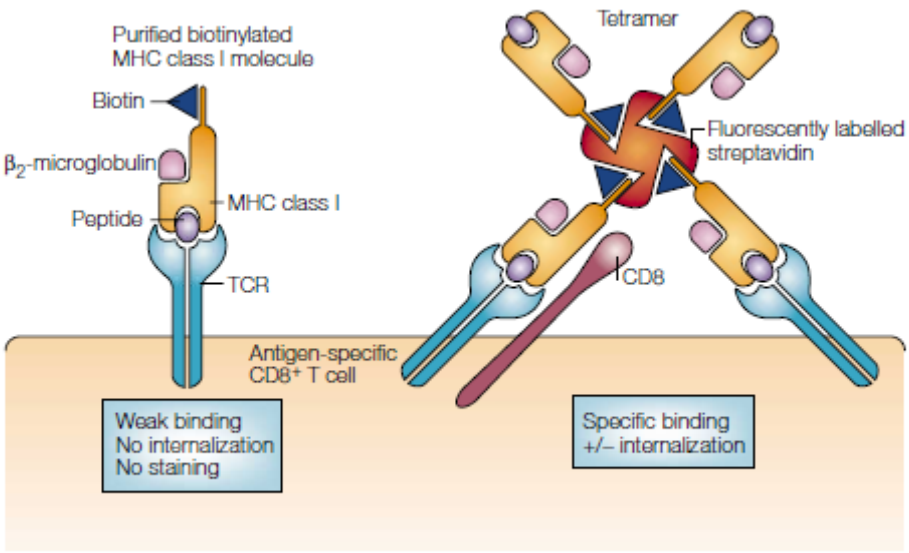


Figure 2. Multimer technology.
MHC class I (yellow) is conjugated via biotin (blue) to a fluorescently labeled straptavidin (red). This complex binds to peptide (violet) pulsed T cell receptor (TCR, blue), whereas CD8 (pink) support the complex binding. A schematic drawing of a tetramer (consisting of 4 equal MHC I-peptide molecules) used for CMV staining is shown. For analysis of ADV, a pentamer (consisting of 5 equal MHC I-peptide molecules) was used to quantify ADV-specific T cells.[15]

1.2.4 ADV-specific immunity

Innate and adaptive immune systems respond to ADV infections, nevertheless, the long-term success rate after HSCT is mainly affected by the acquired immune mechanism.
The frequency of antigen-specific T cells increases in response to an acute infection and normally declines after successful control of the virus. These results correlate with the findings in patients after HSCT, where T cell reconstitution is required for the control of ADV-infections.[3,6] Feuchtinger et al. have shown that the presence of ADV-specific T cells in post-HSCT is associated with a favorable prognosis, whereas their absence could lead to ADV-associated mortality.[3]

In the presence of viral antigen and a high viral load in peripheral blood, virus-specific T cells undergo expansion in vivo and decline to normal values after successful elimination of ADV infection (HAdV DNA) (Figure 3A). For a lifelong protection memory T cells remain behind. In case of reactivation or reinfection, clonal expansion of effector cells from the memory pool is initiated and leads to an increased size of virus-specific T cell population. Furthermore, Feuchtinger et al observed the reconstitution of different cell phenotypes (Figure 3B) after HSCT: The recovery of T cells takes about 100 days and the T_{HELPER} cells reach normal values after 150 days. Natural killer cells (NK cells), which are part of the adaptive immune system, reach their maximum already 1-2 months after transplantation, T cell counts, on the other hand, are only completely normal after 1 year.[3]

The influence of T cell depletion on the T cell reconstitution is shown in Figure 4. T cell reconstitution correlates with the time needed for immunological tolerance between host and donor cells, which is normally established after one year at the latest. In contrast, the transplantation of solid-organ allografts requires a lifelong immunosuppression treatment.[1]

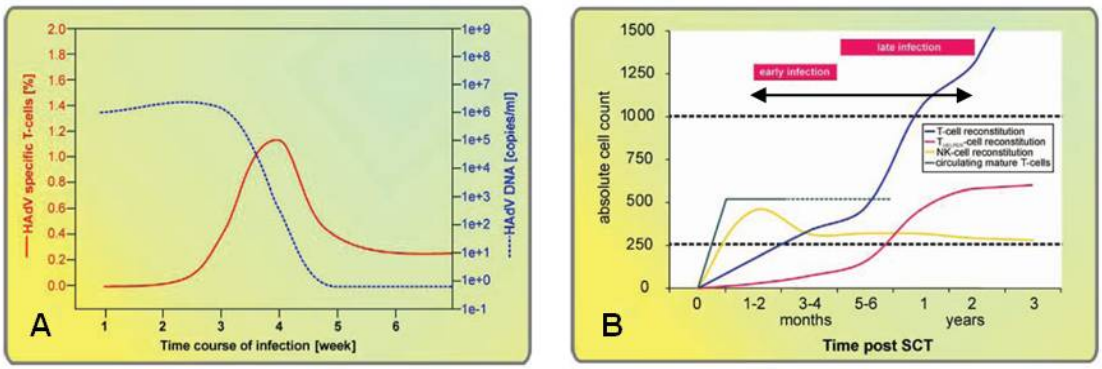


Figure 3. Immune reconstitution after T cell depleted stem cell transplantation.
(A) During an ADV infection the correlation of the viral load (HADV DNA, blue) with the percentage of ADV-specific T cells (HAdV specific T cells, red) is shown. (B) Reconstitution of T cells (blue), T_{HELPER} cells (red), NK-cells (yellow) and circulating mature T cells (green) regarding the time after haploidentical stem cell transplantation (SCT). Arrow indicate the period, where patients are particularly susceptible to ADV infections.
Original image:[3], modified by Christine Freimüller.

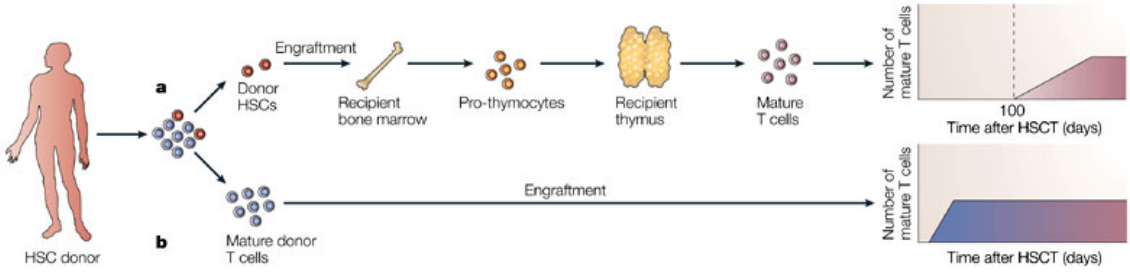


Figure 4. T cell immune reconstitution after allogeneic HSCT.
a) After T cell depletion, T cells mature from the HSCs of the donor and are functionally competent but are fully tolerant of both host and donor tissue. This process takes at least 100 days. b) Without T cell depletion mature T cells are present in the HSC graft, providing a cellular immunity, however bearing the risk to induce GvHD.[1]

It is known that CMV and EBV infections are mainly excluded by CD8+ T cells.[13] Instead, eradication of ADV is assumed to be mediated by CD4+ T cell response,[6,16] although newer findings indicate the importance of CD8+ T cells.[2,5] Leen at al. used a peptide library spanning the entire sequence of the hexon protein, to stimulate ADV-specific T cells. Thereby they discovered new HLA-dependent peptide-sequences for CD8 and CD4 positive T cells, which could be used for the production of multimers.[17] Based on these findings, strategies for new treatments are considered.

Cytotoxic potential

Virus-infected cells are recognized by immune cells with a strong cytolytic response. Immediately after activation, through MHC-mediated recognition of cognate peptide, cytotoxic CD8+ T lymphocytes produce cytokines and chemokines and mediate target cell elimination.[18] Different surface markers for cytotoxic killing, such as CD107a, CD107b and CD63, were identified in recent years.[18,19] These molecules are expressed on the surface immediately after cytotoxic granule release.[18]

Cross reactivity over subtypes and species

In general, human T cell recognition of epitopes is conserved between different but related subtypes of viruses, for ADV, however, this does not always apply.[16] The T cell response is mainly directed against the hexon protein, which is located at the capsid protein. The amino acid sequence of the hexon protein varies between species and subtypes whereas variable and conserved regions were identified.[3] Since these epitopes are largely conserved, specific T cells were shown to be cross-reactive towards ADV serotypes from different ADV subgroups, and may therefore provide protection against a wide range of ADV serotypes, albeit with a few exceptions.[20]

Differentiation of T cells and their ability to remember

"The adaptive immune system has evolved a unique capacity to remember a pathogen through the generation of memory T cells, which rapidly protect the host in the event of reinfection."[21]

We focused on central memory T cells (TCM), which could provide long-lived protection from viral disease after HSCT.

Over the past years, it has become evident that the first contact of a naïve T cells with the priming antigen does not turn irrevocably into memory or effector cells, they can convert into nearly all different subtypes during their lifespan.

If an infection is entirely cleared a small fraction of long-lived memory cells (Figure 5) can persist in the absence of further antigen stimulation. These populations comprise only 5-10% of the initial burst size.[22] These cells have generally been described as specialized cells with the ability to reactivate effector functions in a short time period and expand huge populations of them.[23]

Central memory cells have also been identified by high basal and cytokine-induced STAT5 phosphorylation, reflecting their capacity for slow but constant self-renewal (Figure 5).[22,24]

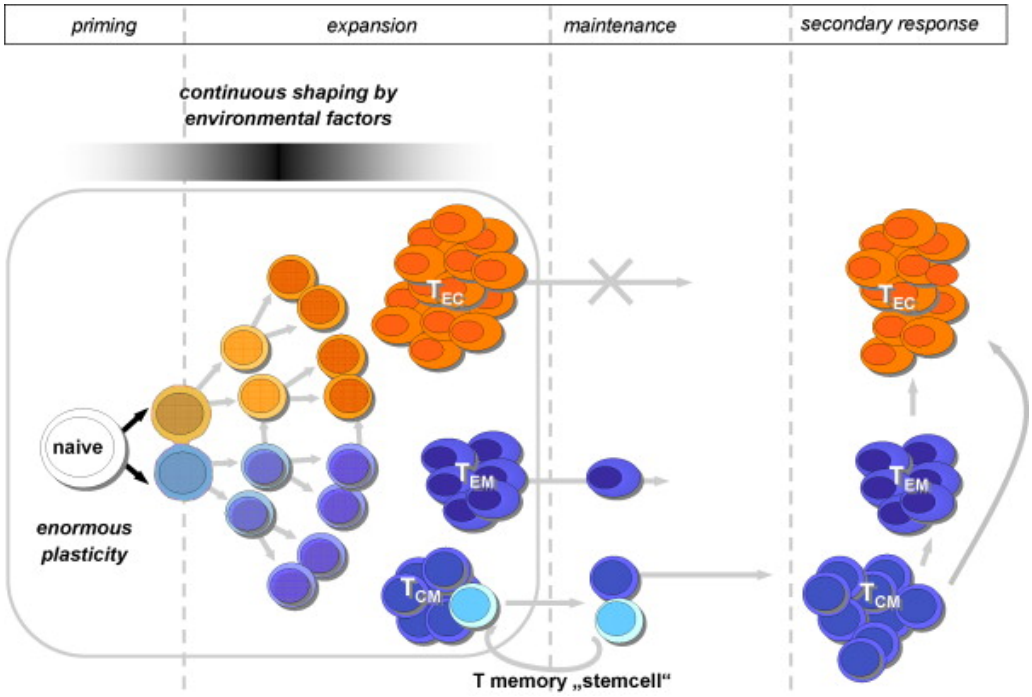


Figure 5. Differentiation capacity of naïve and memory CD8+ T cells.
Priming of a single naïve antigen-specific CD8+ T cell initiates the differentiation into effector T cell (TEC, Teff), effector memory T cells (TEM) and central memory T cell (TCM). A few TCM cells develop a “stem cell-like” differentiation capacity like naïve CD8+ T cells.[22]

The different phenotypes of T cells are characterized by the expression of several surface markers, which can be down or up-regulated during their lifespan. Lymph node homing receptors CD62L and CCR7 (L-selectin) are only expressed on naïve T cells, which have not been exposed to antigens, and on central memory T cells, which can return for a secondary clonal expansion.[25]

Furthermore, CD45 (Protein tyrosine phosphatase, receptor type C ; PTPRC) was selected to distinguish between naive T Tells, expressing the large isoform of CD45 (CD45RA) and activated and memory T cells, showing CD45RO on their surface.[26,27]

Taken together, T cell populations could be separated by their surface marker expression into four distinct sub-populations (Table 1). However, in humans the developmental relationship among TCM, Teff, and Temra is still controversial, Stemberger et al. illustrate an overview of the common findings (Figure 5).

Table 1. T cell populations and there surface marker expression.

	CD45RA	CD62L
TCM (central memory T cell)	-	+
Temra (effector memory RA+ T cell)	+	-
Teff (effector T cell)	-	-
naïve T cell	+	+

The isolation of the "right" T cell subset would decrease the required large number of in vitro-expanded effector T cells, as is currently common for adoptive immunotherapy. If less-differentiated antigen-specific T cells can be isolated and their fate, either to differentiate into an effector or a memory cell can be influenced, only very low numbers of adoptively transferred T cells are necessary.[23]

The process of T cell maturation is continuously shaped by the surrounding environment, whereas cytokines play an important role in vitro and in vivo.
Williams et al. suggest that the presence or absence of Interleukin-2 (IL) signaling determines whether T cells become differentiated effector or memory cells.[28] Further on Pipkin et al. confirmed that the transcriptional program for inflammation after infection is induced by IL-2 signaling and so ensures a balance of effector and memory T cell differentiation.[29] Stemberger et al. noted that IL-15 is more likely to promote homeostatic proliferation and IL-7 signals are mainly needed for survival.[22] Weninger et al. showed the dose-effect of IL-2: if cells were cultured in the presence of ≥5ng/ml IL-2, they developed the phenotype and function of effector T cells. In contrast, ≤5ng/ml IL-2 mediated rapid recall responses - when transferred into naïve animals - which represents a memory type. IL-15 administration turns antigen-primed CD8+ T cells into central memory T cells.[30]
Most reports, however, focus on the observation of CD8+ T cells, but with the addition that CD4+ T cells show similar characteristics.[24,30]

1.2.5 Treatment

Interestingly there is no formally approved effective anti-adenoviral agent available.[5,10] Ribavirin, cidofovir, ganciclovir, vidarabine and ddC are the most widely used antiviral agents, however, their efficacy to provide protection against the adenovirus is limited and associated with significant side effects.[3,5,10,17,31] Furthermore, there is the risk of overtreatment on a large scale if the administration of antiviral drugs in all patients with ADV detectable in stool is to be implemented.[7]

One approach to prevent and treat the viral infection is the administration of donor-leukocyte infusion (DLI), which consists of unmanipulated T cells isolated from the stem cell donor (Figure 6B).[2] Witt et al. published a case report, where a HSCT-treated 2-year old patient was cured of CMV infection after irradiated leukocyte transfusion therapy from a CMV seropositive donor. By that time, CMV-specific T cell analysis was not established, yet Witt et al. assumed that the T cells infused together with the irradiated leukocytes played a decisive role.[32] The mechanism of DLI is not completely understood, but there might be a switch in chimerism of antigen-presenting cells (APCs) from host to donor. In general, this approach is associated with an increase in the incidence and severity of acute GvHD.[1] Nevertheless, this

kind of treatment has long been a favored therapy – not least because there were no better therapy options available so far.

It is now assumed that the degree of immune reconstitution and the resulting efficiency of virus-elimination is mainly influenced by the frequency of virus-specific T cells. Therefore, adoptive transfer of virus-specific T cells from matched or even haploidentical allogeneic donors into patients, was shown to cure viral infections (Figure 6C).[1,2] Pure ADV-specific T cells can be delivered without any concern about generating alloreactive immunity. The transfer will, however, only be successful, if the donor has immunity to the relevant pathogen.[1] There are some promising results for CMV and EBV infections in immunocompromised patients.[4,5]

For ADV several approaches were published in the last years, whereas large-scale clinical applications are still missing. Chatziandreou et al., for example, published a potential method: they isolated interferon-gamma (INF-γ) secreting ADV-specific T cells.[13] Feuchtinger et al. reported the induction of an effective and specific T cell response after the infusion of ADV-specific T cells (CD4+ and CD8+) into pediatric patients undergoing HSCT.[5,33] Leen et al. produced bivirus- (EBV, ADV) and trivirus- (EBV, ADV, CMV) specific T cell lines, through the use of chimeric adenoviral vectors, with a proven record of success.[34,35]

The disadvantages of these tools are the labor-intensive techniques and the resulting high costs, otherwise the specific administration increases the survival rate and reduces the undesired side effects of antiviral drugs.[1]

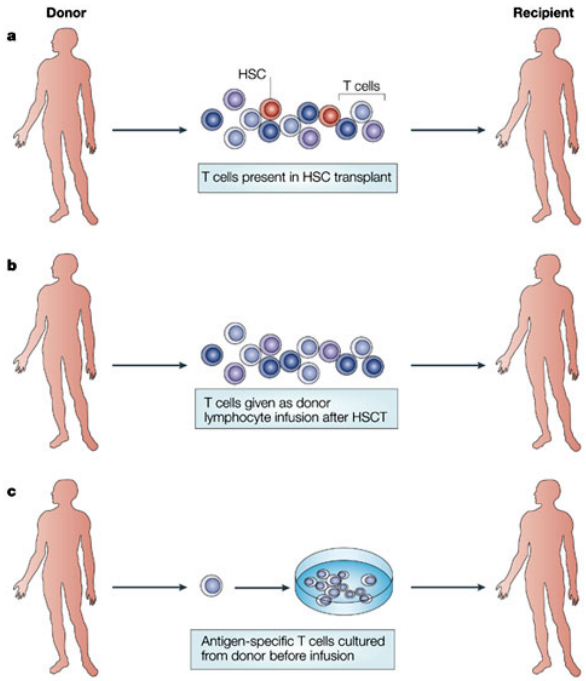


Figure 6. Evolution of adaptive T cell therapy for viral infections after allogeneic HSCT.
(A) Hematopoietic stem cell inoculum (B) Donor-leukocyte infusion (C) Virus-specific T cells isolated and clonally expanded in vitro before adoptive transfer to the host.[1]

1.3 Aims of research

ADV-infection after HSCT is a serious complication, with a high risk of morbidity and mortality, especially in children. Treatment options have remained unsatisfactory, only occasionally adoptive T cell therapy has yielded promising results. In terms of reliability as well as regarding the costs, the application of adenovirus HLA-multimers simplifies the analysis of ADV-specific T cells.

Based on these findings, two main aims for the study were determined:

(1) Expansion and characterization of in vitro-generated ADV-specific T cells.

The first aim was to increase the yield of ADV-specific T cells and to shorten the required expansion time. Therefore optimal cell culture conditions and the influence of several cytokines such as IL-2, IL-7 and IL-15, were approved.

Furthermore, the distribution of effector memory versus central memory cells during the expansion process was studied. In addition the functional activity and alloreactivity of expanded cells were analyzed.

(2) Irradiated virus-specific T cells as therapy after allogeneic hematopoietic stem cell transplantation.

The second aim was to completely exclude the alloreactivity of expanded T cells without influencing their functional activity. Irradiation of T cells is a commonly used method to impede cell proliferation, resulting in induced apoptosis in T cells after several days. However the functional activity of irradiated cells before going into apoptosis was never tested so far. Therefore we analyzed the influence of irradiation, on the functional activity of expanded T cells.

2 Materials and Methods

2.1 Materials

2.1.1 Apparatuses and Instruments

Cell counter (Sysmex Europe, Norderstedt, Germany)
Cell incubator (Heraeus, Newport Pagnell, UK)
Centrifuges with applications for plates and tubes (Heraeus, Newport Pagnell, UK)
Fluorescence-activated cell sorter (FACS Aria™, BD, San Jose, CA, USA)
Freezers (-20° C, -80° C, Nitrogen)
Fridges
Laminar flow (Esco, Hatboro, PA, USA)
LSR II flow cytometer (BD, San Jose, CA, USA)
MACS® Seperation Colums: MS, LS (Miltenyi Biotec, Bergisch Gladbach, Germany)
Mini/MidiMACS™ Separator (Miltenyi Biotec, Bergisch Gladbach, Germany)
Octo/QuadroMACS™ Separator (Miltenyi Biotec, Bergisch Gladbach, Germany)
Optical microscope, Nikon TMS (Nikon Corporation, Tokyo, Japan)
Pipette (Gilson, Middelton, WI, USA)
Radiation apparatus: IBL 437 C (CIS BIO International, Bagnols, France)
Rotator (MACS Mix, Miltenyi Biotec, Bergisch Gladbach, Germany)
SPHEROTM Ultra Rainbow fluorescent particles (Sperotech, Libertyville, US)
Vortex Genie 2 (Lactan, Graz, Austria)

FACSDiVa™ software (Version 6.1.2, BD, San Jose, CA, USA)
GraphPad Prism 5 Software (GraphPad, San Diego, CA, USA)

2.1.2 Plastic Material

Tissue culture plate, 96-well, round bottom (Nunc™, Roskilde, Denmark)
Tissue culture plate, 6-well, 12-well, 24-well, 48-well (IWAKI Europe, Willich, Germany)
FACS tubes (Polystyrene round-bottom tube, B, San Jose, CA, USA)
TruCOUNT™ Tubes (BD, San Jose, CA, USA)
Filter tubes (Polystyrene round-bottom tube with cell-strainer cap, BD, San Jose, CA, USA)
CryoTube vials (Nunc™, Roskilde, Denmark)
Cell scraper (PAA, Pasching, Austria)
Tissue culture flasks, 25cm^2, 75cm^2 (IWAKI Europe, Willich, Germany)
Sterile tips, 10, 20, 100, 200, 1000µl (MβP, San Diego, USA)
Tubes with lid, 15, 50ml (BD, San Jose, CA, USA)
Pipettes, 1, 2, 5, 10, 25ml (SPL Lifesciences, Gyeonggi-do, Korea)

2.1.3 Chemicals and Regents

Human serum type AB, AB-Serum (PAA, Pasching, Austria)
AIM-V® medium (Invitrogen, Lofer, Austria)
Antibodies (2.1.7)
Aqua bidestillata (Mayrhofer, Leonding, Austria)
Bovine serum albumin, BSA (Sigma-Aldrich, St Louis, MO, USA)
CFSE 1mM (Sigma-Aldrich, St Louis, MO, USA)
CliniMACS® PBS/EDTA (Miltenyi Biotec, Bergisch Gladbach, Germany)
Dimethylsulfoxid, DMSO (CryoSuve, Impfstoffwerk Desau-Tornau, Germany)
Ficoll: LSM 1077 Lymphocyte (PAA, Pasching, Austria)
Hepes buffer 1M (Invitrogen, Lofer, Austria)
Human serum albumin, HSA (Octapharma, Wien, Austria)
L-Glutamine (PAA, Pasching, Austria)
Natriumacid 10% (produced by Triimed, Wien, Austria)
Octaplas® AB (Octapharma, Wien, Austria)
Phytohaemagglutinin (PHA, Sigma Aldrich, Austria)
Phosphate-buffered saline, PBS(1x) (PAA, Pasching, Austria)
RPMI 1640, with or without L-Glutamine (PAA, Pasching, Austria)

2.1.4 Buffer and Media

AIM-V+++

AIM-V® supplemented with:	2% Octaplas® AB
	1% L-Glutamine
	1M Hepes buffer

CFSE label buffer

PBS(1x) supplemented with:	0.1% BSA

Freezing medium

20% DMSO
39% Octaplas® AB
41% AIM-V®

MACS buffer CSA

CliniMACS® PBS/EDTA supplemented with: 2.5% Human serum type AB

MACS buffer

CliniMACS® PBS/EDTA (Miltenyi)

Monocyte adherence buffer

AIM-V® supplemented with:	1% Octaplas® AB

Secretion medium CSA

RPMI 1640 supplemented with:	1% L-Glutamine
	5% Human serum type AB

Washbuffer ADV

PBS (1x) supplemented with:	0.1% BSA
	0.1% Natriumacid

2.1.5 Stimulants reagents

All reagents were dissolved and diluted according to manufacturers´ instructions.

2.1.5.1 Adenovirus

PepTivator ADV

PepTivator-AdV5 Hexon consisting of 15mer sequences with 11 amino acid overlap covering all the hexon protein from ADV type 5 (Miltenyi Biotec). Used concentration: 0.5µg/ml.

Peptide ADV

Customized proteins of ADV2 Hexon produced by IBA (Göttingen, Germany).

HLA-A*0101	LTDLGQNLLY
HLA-A*0201	LLDQLIEEV
HLA-A*2401	LLDQLIEEN
HLA-B*0702	KPYSGTAYNAL
HLA-B*3501	MPNRPNYIAF

Used concentration: 2µg/ml.

2.1.5.2 Staphylococcal enterotoxin B (SEB)

SEB stimulates unspecific immune response (Sigma-Aldrich, St Louis, MO, USA). Used concentration: 0.1mg/ml.

2.1.5.3 Phytohaemagglutinin (PHA)

PHA triggers cell division into immature T-lymphocytes. Used concentration: 2.5µl/ml.

2.1.6 Cytokine

All reagents were dissolved and diluted according to manufacturer´s instructions.

	Concentration	Source
IL-2	5ng/ml	PeptroTech, NY, USA
IL-7	5ng/ml	R&D Systems, Minneapolis, MN, USA
IL-15	5ng/ml	R&D Systems, Minneapolis, MN, USA

2.1.7 Antibodies

AB specifity	Conjugate	Clone	Used volume (µl) /2.5 x 10^5 cells	Source
CD3	Pe-TR	UCHT1	1#	BD Bioscience*
CD3	PerCP	SK7	4	BD Bioscience*
CD3	PE	UCHT1	2#	DAKO****
CD4	PerCP-eFlour® 710	SK3	2	eBioscience**
CD4	PE-Cy7	SK3	3#	BD Bioscience*
CD8	V500	RPA-T8	2	BD Bioscience*
CD8	APC-Cy7	SK1	4#	BD Bioscience*
CD8	PerCP	SK1	8#	BD Bioscience*
CD19	APC-Cy7	SJ25C1	1	BD Bioscience*
CD20	APC-Cy7	L27	1	BD Bioscience*
CD27	PE-CyTM7	M-T271	1.5	BD Bioscience*
CD45RA	PE-TR	HI100	0.01	BD Bioscience*
CD57	FITC	HNK-1	2	BD Bioscience*
CD62L	BD Horizon™ V450	DREG-56	0.5	BD Bioscience*
CD107	FITC	H4A3	4#	BD Bioscience*
CD137	PE	1HA2	4#	BD Bioscience*
CCR7	Alexa Fluor® 647	TG8/CCR7	5	BioLegend***
isotype				
	Alexa Fluor® 647	MOPC-173	5	BioLegend***

for 10^6 cells

* BD Bioscience, San Jose, CA, USA

** eBioscience, San Diego, CA, USA

*** BioLegend, San Diego, CA, USA

**** Dako, Glostrup, Denmark

2.1.8 Viability staining

DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Lofer, Austria), 16.5µl/ml

Syto41 (Invitrogen, Lofer, Austria), 3-4µl/ml from 1:200 dilution

2.1.9 ADV pentamere

All reagents were dissolved and diluted according to manufacturers´ instructions.

Pro5® MHC class I pentamer (ProImmune, Oxford, UK):

HLA-Type	Sequence	Epitope origin	Conjugate
A*0101	TDLGQNLLY	Adenovirus 5 Hexon 886-894	PE
A*2402	TYFSLNNKF	Adenovirus 5 Hexon 37-45	PE
B*0702	KPYSGTAYNAL	Adenovirus Hexon 114-124	PE
B*3501	MPNRPNYIAF		PE

2.1.10 Further equipment

Cytokine secretion assay kit

	Conjugate	Used volume[#]	Source
INF-y Catch Reagent	to cell surface (CD45) specific monoclonal antibody	10µl	Miltenyi Biotec*
INF-y Detection Antibody	APC	10µl	Miltenyi Biotec*

[#] for 10^6 cells

* Miltenyi Biotec, Bergisch Gladbach, Germany

Magnetic cell sorting: Isolation of CD14+ cells

CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany)

2.1.11 Patient samples

Blood samples (heparin or EDTA – ethylenediaminetetraacetic acid) were obtained from healthy donors at the Children´s Cancer Research Institute (CCRI) with their full knowledge and permission.

2.2 Methods

All cell culture work was performed under sterile conditions.
A washing step describes the following procedure: adding washing solution, centrifugation at 300g, 4°C for 10min and removing the supernatant.

2.2.1 Generation of virus-specific T cells

2.2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from whole blood of informed healthy volunteers by density gradient centrifugation using ficoll (LSM 1077 Lymphocyte). Whole blood supplemented with EDTA or Heparin was diluted 1+1 to 1+2 with room temperature (RT) PBS. For density gradient centrifugation a layer of diluted blood was pipetted onto 3-15ml of ficoll and was spun for 30min at 1000g without brake. The mononuclear cell layer, which formed a ring in the interphase, was carefully transferred into a new tube and washed twice with cold PBS or RPMI. After the first washing step, cells were resuspended in a defined volume and 65µl were removed for the determination of the cell number by Sysmex.

2.2.1.2 Freezing and Thawing

Mammalian cells can be stored in liquid nitrogen for a long time or for shorter periods at -80°C with minimal loss of viability. For this application, cells were counted and resuspended in cell culture medium AIM-V+++. This suspension was transferred into CryoTube vials and charged with freezing media in a ratio of 1:1. Samples were cooled down at -80°C, and if the cells were intended to be stored for a longer period, they were put into liquid nitrogen after one day.

For thawing, frozen cells were warmed up with pipetting up and down cold PBS or RPMI. The cell suspension was transferred into a new tube and washed twice to remove DMSO. Cell number was determined after the first washing step. After the second washing the pellet was resuspended in cell culture medium.

2.2.1.3 Cell culture

Cells were cultured in AIM-V® medium supplemented with 2% octaplas, 1% L-glutamine and 1M hepesbuffer. Cultivation took place at 37°C supplied with 5% CO_2 and 90% relative humidity in an incubator.

2.2.1.4 Expansion of ADV-specific T cells (in vitro cell stimulation)

Depending on the aim of the experiment 6, 12, 24, 48 well-plates or 25, 75cm^2 flasks were used. Isolated PBMCs were seeded at a density of 5 x 10^6/ml (Table 2).

For ADV cell stimulation PBMCs were incubated with 1µg/ml (20µl/ml) PepTivator ADV or 2µg/ml (2µl/ml) peptide ADV.

After three days 5ng/ml IL-15 was added.

On day 6 expanded cell culture was restimulated: for this purpose, monocytes were isolated from PBMC by means of monocyte adherence. Frozen PBMCs were thawed like described above, resuspended in AIM-V® supplemented with 1% octaplas® AB, and seeded at the same density as on day 0. After 2h monocytes had settled down on the bottom of the flask or plate. To remove unwanted non adherent cells, cells were washed shortly with PBS. In the meantime the expanded ADV-specific T cells were scraped from the flask or plate and washed once with PBS or RPMI. The pellet was resuspended in the same volume of AIM-V+++ as before the washing step. Washed ADV-specific cell culture was given to the adherent monocytes and was treated with PepTivator ADV or Peptide ADV, like on day 0.

On day 9 the cell culture was enriched with 5ng/µl IL-15. After further 3 days, expansion of ADV-specific T cells was completed (Figure 7).

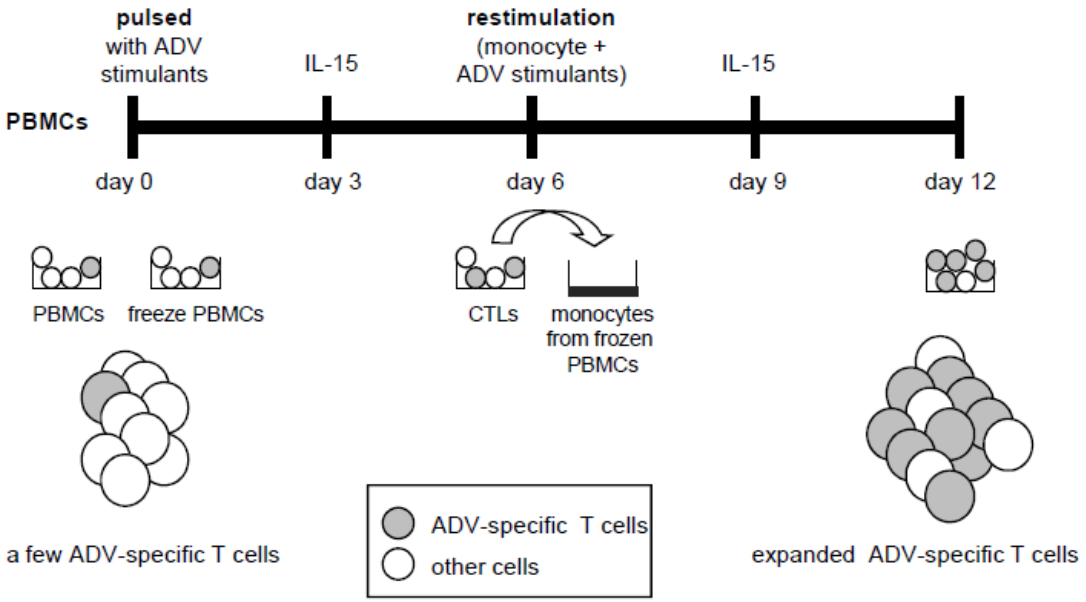


Figure 7. Expansion of ADV-specific T cells.

Table 2. Plate size and absolute cell count.

	Absolute cell count	Volume
6-well plate	10 x 10^6	2ml
12-well plate	5 x 10^6	1ml
24-well plate	2.5 x 10^6	0.5ml
48-well plate	1.25 x 10^6	0.25ml
96-well plate	0,625 x 10^6	0.125ml

2.2.2 Irradiation

The irradiation treatment of cell culture was accomplished with the IBL 437 C (CIS BIO International, Bagnols, France) at the AKH Wien, Transfusionsmedizin. Cells were irradiated with 30Gy (3000rad), which corresponds to the typical irradiation procedure of blood products.

2.2.3 Detection of circulating virus-specific T cells

2.2.3.1 Column-based isolation of antigen-specific cells

Magnetic cell sorting (MACS) is a method for selective enrichment or depletion of cells, expressing a surface marker characteristic for their cell type. Cells can be primary labeled with magnetic antibodies, which are retained in the column, unlabeled cells, on the other hand, pass through.

Pre-cooled solutions were used and cells were kept on ice, unless otherwise mentioned. All used volumes of reagents were calculated for 10^7 cells, when more cells were loaded on the column, the amount was increased in relation to the cell count.

2.2.3.1.1 Isolation of CD14+ cells (monocytes)

Monocytes were positively selected by MACS using the human CD14 MicroBeads (Miltenyi Biotec).

Frozen PBMCs were thawed and washed once with PBS at 300g, 10min, 4°C and 1 x 10^7 cells were resuspended in 80µl of MACS buffer. 20µl CD14 magnetic beads were added and incubated for 15min at 4°C in the dark. Cells were washed by adding 2ml MACS buffer and resuspended up to 10^8 cells in 500µl of MACS buffer. MS column (Miltenyi Biotec) was placed in the magnetic field of the MACS separator and equilibrated with 500µl ml MACS buffer. The sample was applied onto the column and washed three times with 500µl MACS buffer. After removing the column from the separator, the labeled $CD14^+$ cell fraction was

flushed out with 1ml MACS buffer by pushing the plunger into the column. The monocyte-containing fraction was washed twice with PBS and was resuspended in the volume of choice for the further use at the cytokine secretion assay (2.2.3.3 Cytokine secretion assay (CSA)).

2.2.3.1.2 Isolation of ADV-specific T cells

Following ProImmune protocol "Column-based isolation of antigen-specific cells using anti-fluorochrome beads", 30 - 40 x 10^6 expanded cells were used for each approach. The following description is designed for 10^6 cells.
Cells were washed once with 1ml washbuffer ADV and resuspended in 50µl washbuffer. For 20min, at RT, 5µl corresponding PE labeled pentamer was added. After washing, cells were resuspended in 80µl washbuffer ADV and 20µl anti-PE magnetic beads (Miltenyi Biotec) were added for 15min at 4°C. Cell-bead complex was washed once and resuspended in 500µl washbuffer. LS column was prepared as described in the company´s instruction (Miltenyi Biotec) and loaded with the cell-bead complex. For collecting the negative fraction, 3 times 3ml washbuffer ADV were added. Afterwards, the column was replaced from the magnetic field and the ADV-specific T cells (positive fraction) were eluted with 5ml washbuffer ADV through pushing the plunger into the column.
The positive fraction was washed and all cells were stained as described in "2.2.3.4 Phenotype analysis".

2.2.3.2 Pentamer staining

For each staining condition 2.5 x 10^5 cells were used.
Cells were washed once with 1ml washbuffer ADV (0.1% BSA, 0.1% Natriumacid in PBS) and resuspended in 50µl washbuffer ADV. Afterwards cells were incubated with the corresponding pentamer labeled to PE for 20 min, dark, at RT. The used amount of the pentamer is listed in Table 3. Another washing step was done and further surface markers were stained as described in "2.2.3.4 Phenotype analysis".

Table 3. Pentamer staining for ADV.

ADV	Pentamer	Used volume
positive	corresponding pentamer (A*0101, A*2402, B*0702, B*3501)	5µl / 2 x 5*10^5 cells
negative	non corresponding pentamer (A*0101, A*2402, B*0702, B*3501)	5µl / 2 x 5*10^5 cells

2.2.3.3 Cytokine secretion assay (CSA)

This assay is designed for the quantification of live antigen-stimulated INF-γ secreting CD4+ and CD8+ memory and effector T cells (Figure 8).
The assay was carried out with the cytokine secretion assay kit (Miltenyi Biotec) according to the manufacturer´s instructions.

Expanded ADV-specific T cells were washed, counted and adjusted: 50µl = 8 x 10^5 cells. Half of the cells were irradiated, the others served as control.
Monocytes were positively selected by using CD14 MicroBeads (Miltenyi Biotec) as described in 2.2.3.1.1 Isolation of CD14+ cells (monocytes). Selected cells were counted and adjusted: 50µl = 2 x 10^5 cells.
Monocytes and expanded T cells, either irradiated or not, were mixed in a rati of 5:1, (50µl+50µl). Stimulation was performed for 16h or 4h, with either 0.5µg PepTivator ADV, 10µg SEB or, for the negative control, without stimulant.
After the stimulation period cells were washed with 10ml washbuffer MACS CSA (MACS buffer with 0.5% HSA) and resuspended in 80µl cold secretion medium (RPMI 1640 with 1% L-Glutamine and 5% AB-serum). 10µl INF-γ catch reagent were added and incubated for 5min on ice, then 5ml 37°C warm secretion medium were added and under rotation INF-γ were secreted for 45min, at 37°C. Subsequently, cells were washed with washbuffer CSA and resuspended in 80ml cold washbuffer CSA. 10µl detection–antibody conjugated to APC and following antibodies were added for 20min, dark and on ice: CD3-PeTR, CD4-PeCy7, CD8-APC-Cy7, or CD3-Pe, CD4-PeCy7, CD8-PerCP and in a few cases CD137-PE and/or CD107a-FITC. Cells were washed again with 10ml washbuffer CSA, resuspended in 150µl washbuffer CSA and 3-4µl Syto41 was adjoined.

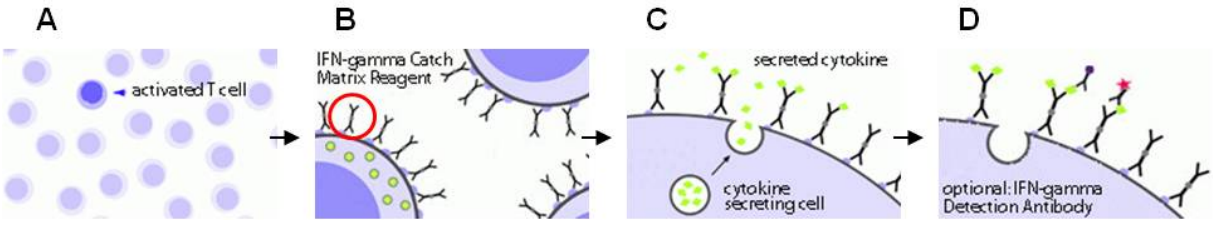


Figure 8. Principle of the INF-γ secretion assay
(A) Via stimulation - T cells get activated. (B) An INF-γ specific Catch Reagent is attached to the cell surface of all leukocytes through CD45 binding. (C) Cells are incubated for 45min at 37°C to allow cytokine secretion. The secreted INF-γ binds to the INF-γ Catch Reagent of the producing cells. (D) An INF-γ detection antibody conjugated to APC is used to visualize the secreting cells by flow cytometry. (INF-γ Secretion Assay- Detection Kit (APC), datasheet Miltenyi Biotec)

2.2.3.4 Phenotype analysis

The washed cell pellet was resuspended in 50µl washbuffer ADV and supplemented with the following surface antibodies: CD3 – PerCP, CD4 – PerCP eFlour710, CD8 – V500, CD19 – APC-Cy7, CD20 – APC-Cy7, CD2 – Pe-Cy7, CD45RA – PeTR, CD57 – FITC, CD62L – Horizon and CCR7 – Alexa Flour 647. Labeling was performed for 15min at 4°C under dark conditions. Cells were washed once with washbuffer ADV, the pellet was resuspended in 100µl PBS, stored cold and dark until measurement.
Control samples were stained with appropriate isotype-matched antibodies.

2.2.4 Cytotoxic assay (CTA)

2.2.4.1.1 Autolog system

2.2.4.1.2 Target cell population

PHA blasts and the target cells were generated from 3 x 10^6/ml PBMCs by adding 2mg/ml PHA on day 0. After 3 days 5ng/ml IL-2 was added and - when necessary - further cell culture medium was added.

CFSE labeling
After 6 days PHA blast were carboxyfluorescein diacetate (CFSE) labeled over night to distinguish between target and effector cells at the assay.
PHA blasts were washed twice with PBS and the cells were resuspended in 0.1% BSA in PBS at a cell density of 10^7/ml. For CFSE staining, 3nM CFSE were added and incubated for 10min at 37°C, for a good dispersion the cell culture was gently shacken every 2 minutes. For stopping the reaction, 1ml octaplas was added at room temperature for 5 minutes. Cells were washed one time with cell culture medium, a cell density of 1 – 2 x 10^6 was suspended and cultured over night.
Cells were sorted for their viability using FACS Aria™.
For the pulsing procedure, 5 x 10^6/ml target cells were stimulated for 2h with 1µg/ml PepTivator or 2 µg/ml peptide ADV at a 96er well plate (round bottom). Additionally, cells were washed once and 50µl = 12.500 cells (2.5 x 10^5/ml) were adjusted. Unpulsed targets cells were treated in the same way but without simulation.

2.2.4.1.3 Effector cell population

Over 12 days expanded ADV-specific T cells were used as effectors, either irradiated or not. Viable cells were sorted by FACS Aria™, washed once and a cell density of 5 x 10^6/ml (50µl=250.000 cells) was adjusted.

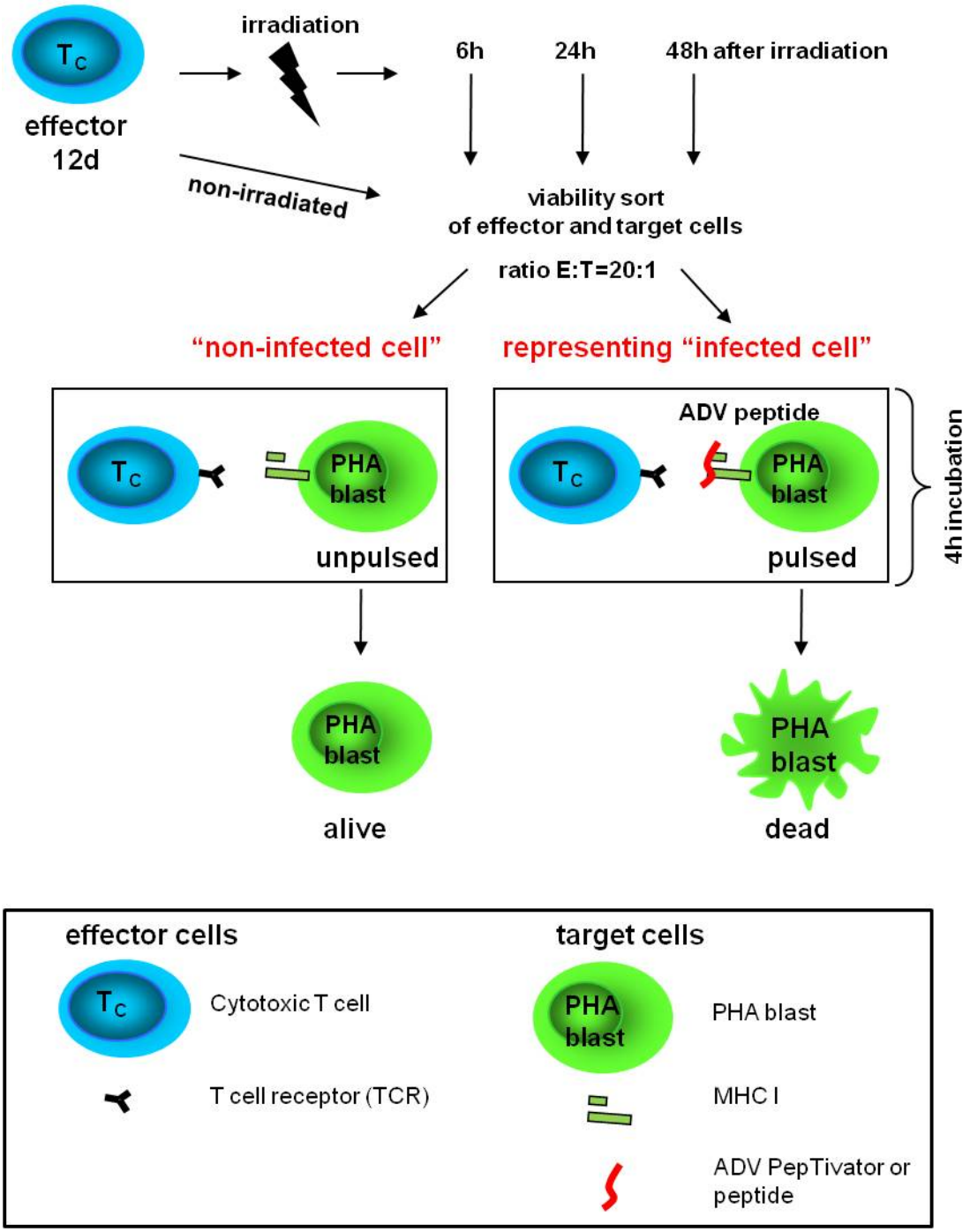


Figure 9. Schematic illustration of Cytotoxic Assay (CTA).
Part of the expanded ADV-specific T cells (effector) were irradiated and after 6, 24 and 48h the assay was performed. A viability sort was performed before incubation for target and effector cells. PHA blasts, which function as target cells, were pulsed for 2h with either peptide or PepTivator ADV (not shown), afterwards effector and targets were mixed together in a ratio of 20:1 and IL-15 were added. After 4h incubation the value of death target cells was evaluated by flow cytometry.

2.2.4.1.4 Assay

An illustration of the CTA is shown in Figure 9. Target cells were placed in 96-well round–bottom plates at 1.25 x 10^4cells/well. Effector cells were added at effector-to-target cell ratio of 20:1 in duplicates or triplicates. To determine the background, target cells were plated alone. Additionally, every well was filled up with cell culture medium to 200µl, further 1ng IL-15 was added. Plate was centrifuged at 1200rpm for 10min at 4°C without brake and subsequently incubated for 4h.
Cells were transferred into TruCOUNT™ tubes, 3.3µl DAPI were added and the value of death target cells was determined by LSRII. The amount of dead target cells is calculated in death target cells/µl.

2.2.4.2 Allogeneic system

ADV-specific T cell cytotoxicity was evaluated against autologous and allogeneic PHA blast target populations. Therefore non-irradiated or irradiated effector T cell population were incubated with the appropriate target cells. CTA was performed under the same conditions as described above.

2.2.5 Flow cytometry

2.2.5.1 Cytometric cell sorting

For comparable results, the value of viable cells plays an important role.
To achieve this, effector cells were sorted before the incubation and target cells were sorted before pulsing with an ADV stimulant. Cells were washed once with PBS, resuspended in 0.5-3ml cell culture medium and filtered through a tube with cell-strainer cap. Viable cells were determined by the forward side scatter area (FSC-A) and side scatter area (SSC-A) and sorted in a separate tube by FACS Aria™ cytometer. After the procedure, cells were washed once with cell culture medium and the used cell density was determined.

2.2.5.2 Flow cytometric analysis

Single cell staining, a non-stained and a full stained sample were performed to determine the perfect compensation between different fluorochromes.
The LSRII cytometer was equipped with three lasers (solid state lasers 405nm and 488nm, He-Ne laser 635nm). The cytometer performance is checked weekly using SPHERO™ Ultra Rainbow fluorescent particles (8-peak-beads; Sperotech, Libertyville, US).

For a precise analysis, cells were transferred into TruCOUNT™ tubes (BD) where the absolute counts of leucocytes were determined with the following formula:

$$\frac{\text{\# of events in region containing cell}}{\text{\# of events in absolute count bead region}} \times \frac{\text{\# of beads per test*}}{\text{test volume}} = \text{absolute count of cell}$$

*This value is found on the package and varies from lot to lot (BD TruCOUNT™ Tubes datasheet)

For leukocytes, an acquisition threshold of 500 was set on FSC. Between 20.000 (CTA) and 100.000 total events and a minimum of 1.000 beads were acquired per analysis.

2.2.5.3 Data evaluation and gating strategy

The BD FACS DiVa™ software was used for data evaluation.
Depending on the used antibodies, different gating strategies were used, the following steps were always the same: First the beads were defined and then this gate was inverted to continue with everything without beads. For discrimination of doublets, cells were gated on SSC-area (SSC-A) versus FSC-width (FSC-W).

For pentamer staining, CD19 and CD20+ cells were excluded, because they could provide a false-positive result for pentamer-positive cells. Further CD3+ cells were gated, dead cells were excluded by FSC-A versus SSC-A and splited in their surface expression of CD4 and CD8. The used pentamers are only able to recognize MHC Class I, therefore only virus-specific CD8+ T cells could be detected with this analysis. CD8+ cells were analyzed in a plot PE versus SSC-A to distinguish between virus-specific T cells (pentamer+) and non-virus-specific T cells (pentamer-). For the memory status CD62L and CD45RA provided important evidence, CCR7, CD57 and CD27 were stained but not appropriated for the results.

For the cytokine secretion assay, first only viable cells have been gated by staining with the dye Syto41. Afterwards, CD3+ population was gated, accordingly CD4+ and CD8+ cells were determined. From both cell populations CD107, CD137 and INF-γ production was analyzed depending on their stimulation duration.

For CTA analysis, CFSE positive cells, which comply with target cells, were gated and separated according to their viability status by differentiating between DAPI+ (dead cells) and DAPI- (viable cells).

2.2.6 Statistics

Data are presented in means ± SEM.

Statistical analysis was conducted using GraphPad Prism 5 and Microsoft Office Excel 2007. A Student´s t test was used to compare two analyzed groups and significance considered when P was less than 0.05.

3 Results

Only donors positive for ADV-specific T cells were eligible for this study.
In all experiments, the unspecific binding of ADV-specific pentamer to CD8+ T cells (background level) was below 0.1%.

3.1 Expansion and characterization of in vitro-generated ADV-specific T cells

3.1.1 Expansion of ADV-specific T cells

The presence of ADV-specific T cells in patients after HSCT was associated with clearance of viral infections. Therefore, the detection of ADV-specific T cells in HSCT donors and/or patients after HSCT could have implications for the onset of clinical symptoms of disseminated viral diseases. Since the frequency of ADV-specific T cells in fresh blood is very low, we developed an in vitro expansion period to be able to clearly detect and generate ADV-specific T cells. PBMCs of healthy donors were stimulated with an ADV-specific peptide pool (ADV-PepTivator) on day 0 and restimulated with autologous monocytes on day 6. On day 3 and 9, IL-15 was added to the culture. After 12 days cells were analyzed by flow cytometry using HLA-type pentamers or the IFN-γ CSA.
As shown in Figure 10A, the frequency of ADV-specific T cells in freshly isolated PBMCs at day 0 is similar to that of cells stained with the unmatched HLA-type pentamer (negative control) and therefore below the limit of detection.
However, after 12 days of expansion, a 1-2 log increase of pentamer positive ADV-specific T cells was observed, independent of whether cells/µl (Figure 10B and C) or the percentage of ADV-specific T cells in CD8+ T cells (Figure 10D and E) were analyzed. The ratios of ADV-specific T cells on day 12 between different donors vary from 3.5 to 198 cells/µl (Figure 10B and C) and 1.9 to 43% of ADV-specific T cells in CD8+ T cells (Figure 10D and E).
Since ADV viremia could disseminate within one or two weeks in patients after HSCT, it is important to develop very fast protocols for detection and expansion of ADV-specific T cells. In order to investigate the kinetics and earliest time points for detection of ADV-specific T cells, samples were harvested and subsequently frozen every day during the 12 days of expansion. Afterwards, all samples were thawed and analyzed by flow cytometry using ADV-specific pentamers. In all three donors the highest number of ADV-specific T cells/µl was found after 12 days of expansion (Figure 10F and G). Strikingly, ADV-specific T cells were already detected after 5-6 days of expansion (Figure 10F and G). Figure 1G shows the average of ADV-pentamer+ T cells in cells/µl and percentage of CD8+ T cells of all 3 donors, implying a correlation between both types of analysis.
These results indicate that our in vitro expansion protocol allows rapid generation and therefore clear detection of ADV-specific T cells in healthy donors.

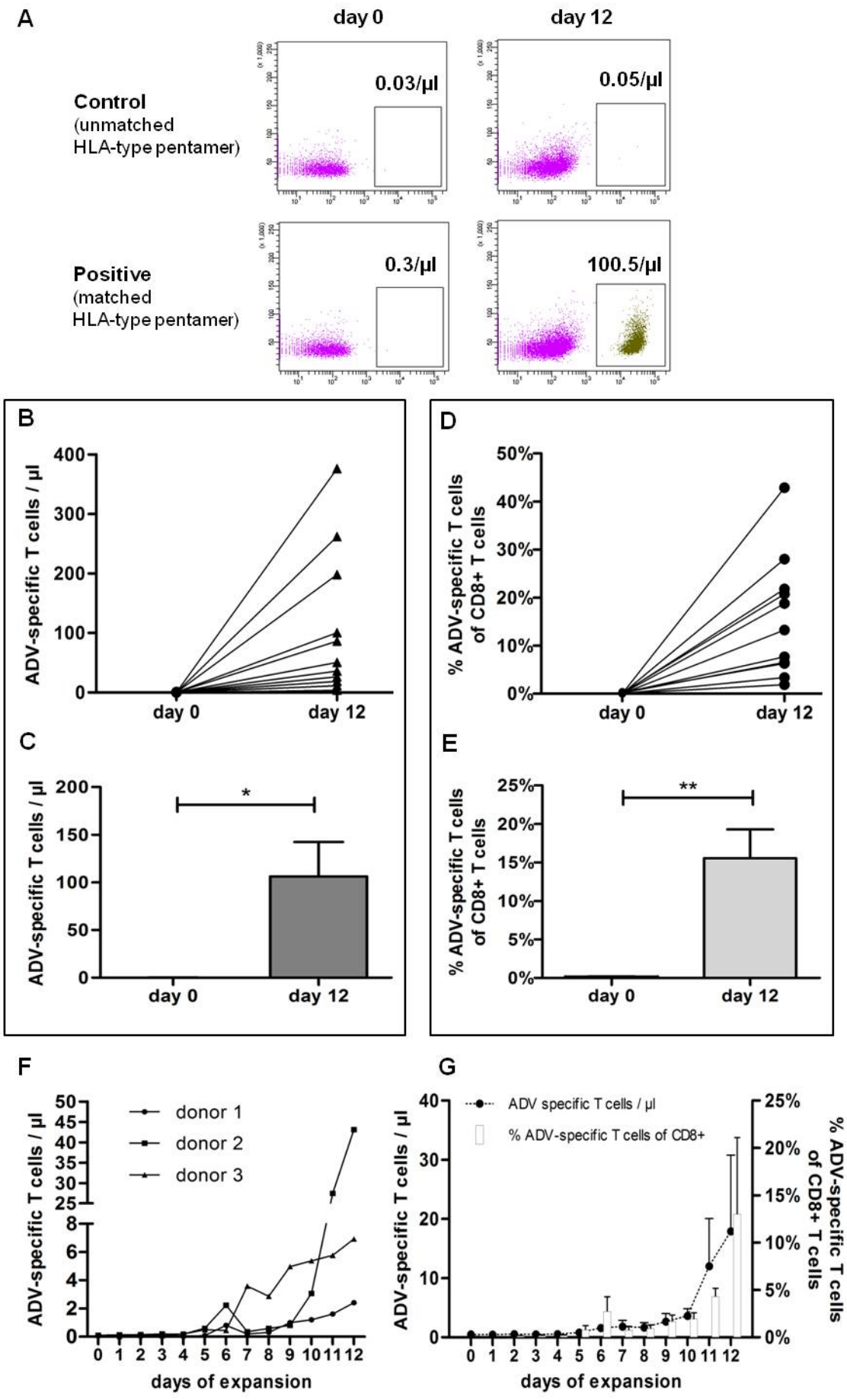
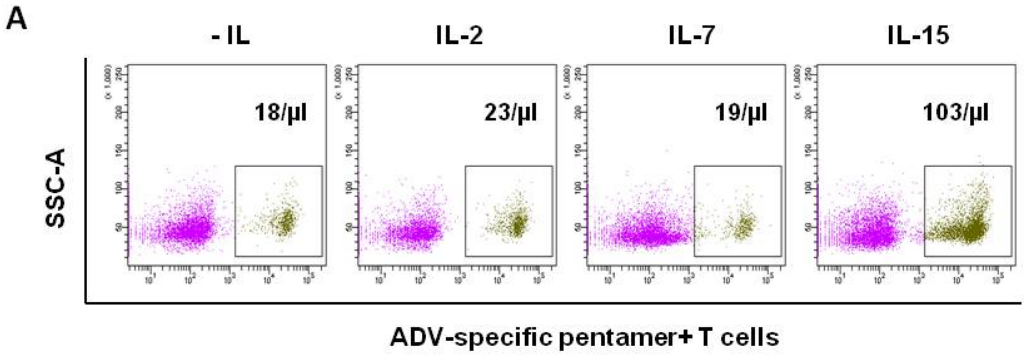

A
day 0
day 12
Control
(unmatched
HLA-type pentamer)
0.03/µl
0.05/µl
Positive
(matched
HLA-type pentamer)
0.3/µl
100.5/µl
B
ADV-specific T cells / µl
day 0
day 12
C
ADV-specific T cells / µl
*
day 0
day 12
D
% ADV-specific T cells of CD8+ T cells
day 0
day 12
E
% ADV-specific T cells of CD8+ T cells
**
day 0
day 12
F
ADV-specific T cells / µl
donor 1
donor 2
donor 3
days of expansion
G
ADV specific T cells / µl
% ADV-specific T cells of CD8+
ADV-specific T cells / µl
% ADV-specific T cells of CD8+ T cells
days of expansion

Figure 10. Generation and detection of ADV-specific T cells.
ADV-specific T cells of donor PBMCs before expansion and after expansion were analyzed by MHC I restricted ADV-specific pentamer. For expansion, PBMCs were stimulated with the ADV-PepTivator on day 0 and restimulated with monocytes on day 6. On day 3 and 9, IL-15 was added to the culture.
(A) Analysis of ADV-specific T cells before (day 0) and after expansion (day 12). One representative experiment is shown. Cells were analyzed with an unmatched MHC I ADV pentamer to determine the background level (first row) and with the matched pentamer (second row). ADV-specific T cells/µl are shown in the gate represented on each dot plot. Analysis of ADV-specific T cells of 11 donors on day 0 and day 12 are shown in cells/µl (B) and in percentages of CD8+ T cells (D). The average of ADV-specific T cells of different donors on day 0 and day 12 analyzed in cells/µl (C) and percentage of CD8+ cells (E) are shown. The summarizing diagrams show means ± SEM of 11 healthy donors on day 0 and day 12. The number of ADV-specific T cells/µl of 3 different donors were analyzed every day during the expansion period (F). (G) A summarizing diagram including the number of ADV-specific T cells/µl (black circles) and percentages of CD8+ T cells (white bars) of all 3 donors are shown in means ± SEM.



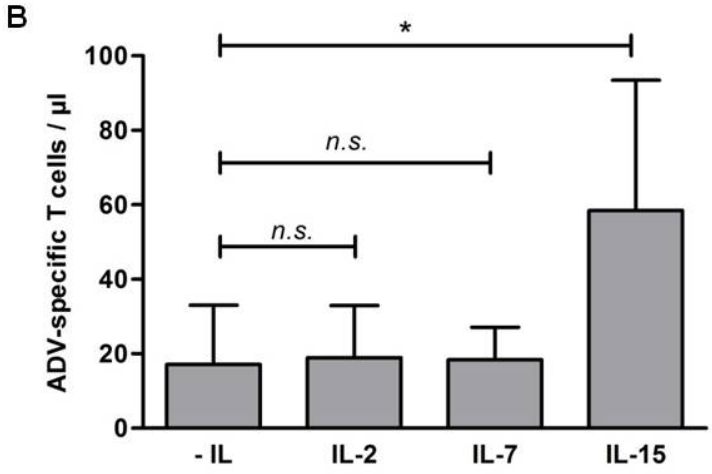


Figure 11. Influence of IL-2, IL-7 and IL-15 on the expansion of ADV-specific T cells.
PBMCs of 8 different donors were cultured for 12 days with PepTivator ADV as described (2.2.1.4 Expansion of ADV-specific T cells (in vitro cell stimulation)). On day 3 and 9 cells were either untreated (-IL) or treated with IL-2, IL-7, or IL-15 as indicated.
(A) One representative blot is shown. ADV-specific pentamer+ T cells/µl are shown in the gate represented on each dot plot. (B) A summarizing diagram shows means ± SEM of ADV-specific T cells after treatment with different interleukins as indicated. Significance versus untreated ADV-specific T cells: n.s., non significant; *, $P \leq 0.05$.

3.1.2 Influence of different interleukins on the expansion of ADV-specific T cells

Interleukins (IL), a subgroup of cytokines, play a major role in vitro and in vivo in the maintenance and development of different cell types.

To evaluate the effect of interleukins on the expansion of 12 days cultured ADV-specific T cells, cells were either untreated or treated with IL-2, IL-7 or IL-15 on day 3 and 9. Surprisingly, neither IL-2 nor IL-7 showed significantly increased expansion of ADV-specific T cells/µl compared to untreated samples. In contrast, treatment with IL-15 significantly increased (3,5 fold) the number of ADV-specific T cells/µl after 12 days of expansion (Figure 11A and B).

These results revealed that IL-15 seems to be the most efficient interleukin for rapid expansion of ADV-specific T cells.

3.1.3 Phenotypic characterization of T cells

3.1.3.1 Phenotypic characterization of T cell populations before and after the expansion period

To further evaluate the influence of the expansion period on the phenotype of different T cell subpopulations, immunophenotype staining was performed by flow cytometry. Previous data have described four different populations of T cells, termed naïve (CD62L+ CD45RA+), central memory (TCM; CD62L+ CD45RA-), effector memory (Teff; CD62L- CD45RA-), and CD45RA+ effector memory T cells (Temra; CD62L- CD45RA+). Particularly central memory T cells are known to mediate long-term immunity and could therefore be highly relevant for adoptive T cell therapies. First we determined the immunophenotype of CD4+ and CD8+ T cells on day 0 and day 12 after expansion. As shown in Figure 12A, CD4+ T cells analyzed on day 0 showed no significant differences in the TCM subpopulation compared to day 12 (24.3% *vs* 27.9%). In contrast, the Teff population was significantly increased (38.7% *vs* 60.6%) whereas the naïve (22.8% *vs* 10.8%) and Temra (14.2% *vs* 0.7%) populations were reduced after 12 days of expansion (Figure 12 A). In CD8+ T cells, the TCM population was significantly increased (9.8% *vs* 22.1%, P=0.0049) after 12 days of expansion compared to day 0. Similar to the results of CD4+ T cells, the mean number of naïve (21.7% *vs* 16.8%) and Temra (29.3% *vs* 4.1%) populations was reduced after 12 days of expansion whereas the mean number of the Teff population (39.2% *vs* 57.1%; P=0.0461) was significantly increased (Figure 12 B).

Next, we analyzed the percentages of different subpopulations of pentamer+ ADV-specific T cells on day 0 and day 12. As the frequency of ADV pentamer+ T cells was very low on day 0 (Figure 10), ADV-specific pentamer+ T cells were enriched by MACS technology as described in materials and methods. The number of pentamer+ T cells/µl after magnetical enrichment was more than 3 log increased compared to that of freshly isolated PBMCs.

Similar results were seen by analyzing the percentage of ADV-specific T cells in CD8+ T cells. Additionally, ADV-specific T cells were analyzed in cells/µl and percentage of CD8+ T cells. In both cases, ADV-specific T cells were 1 to 2 log increased after 12 days of expansion (Table 4).

Table 4. ADV-specific T cells on day 0, on day 0 after enrichment and after expansion on day 12.

	ADV-specific T cells	% ADV-specific T cells of CD8+ T cells
day 0	0.23 (0.1-0.4) /µl	0.6 (0.01-0.37)
day 0 enrichment	752,4(164.6-1628.6)	18.5 (0.11-45.16)
day 12	65.8 (18.6-102.8) /µl	20.6 (6.3-42.9)

As shown in Figure 12C, magnetically enriched ADV-specific pentamer+ T cells analyzed on day 0 showed significantly reduced TCM populations (46.3% *vs* 2.8%) compared to day 12. As expected, naïve and Temra populations were decreased whereas Teff populations (41.2% *vs* 96.3%) were significantly increased after 12 days of expansion (Figure 12C). In one case (date not shown and not included) TCM of ADV-specific T cells increased (59.3% *vs* 73.8% of ADV-specific T cells) after expansion.

Yet despite 12 days of expansion, TCM populations could still be detected, indicating that both Teff and TCM ADV-specific T cells could be generated. Additionally, as is well known, only few TCM cells are necessary for a lifelong protection against the ADV virus in patients after HSCT.

3.1.4 The effect of interleukins on the immunophenotype of T cells during in vitro expansion

Previous studies suggested that interleukins could play a role in the manifestation of virus-specific memory or effector cells. To investigate the influence of different cytokines on the immunophenotype of T cells, addition of IL-2, -7, and -15 was attempted with regard to TCM, which could provide lifelong protection from viral disease as mentioned above. We analyzed the effect of different interleukins on the percentages of TCM, naïve, Teff, and Temra T cell populations in CD8+, CD4+, and ADV-specific pentamer+ T cells after 12 days of expansion. Surprisingly, treatment with different interleukins showed no significant influence on the percentage of TCM, naïve, Teff, and Temra T cells when gated on CD8+ T cells (Figure 13A), CD4+ T cells (Figure 13B), and ADV-pentamer+ T cells (Figure 13C). The T cell subgroups CD8 and CD4, showed few differences in their phenotype analysis: The percentage of TCMs in CD4+ T cells was higher as compared to that of CD8+ T cells (CD4: 31.6%, CD8:19.9%) and the percentage of Temra cells was higher in CD8+ T cells as compared with that of CD4+ T cells (CD4: 1.1%, CD8: 5.8%).

Although the percentage of TCMs in ADV-pentamer+ T cells was again reduced after 12 days of expansion (35% *vs* 7.5%; data not shown), TCM T cells could nonetheless be detected.

Taken together, the effects described in the literature could not be confirmed, interleukin treatment had no influence on the expansion of TCM T cell populations in our experimental setting.

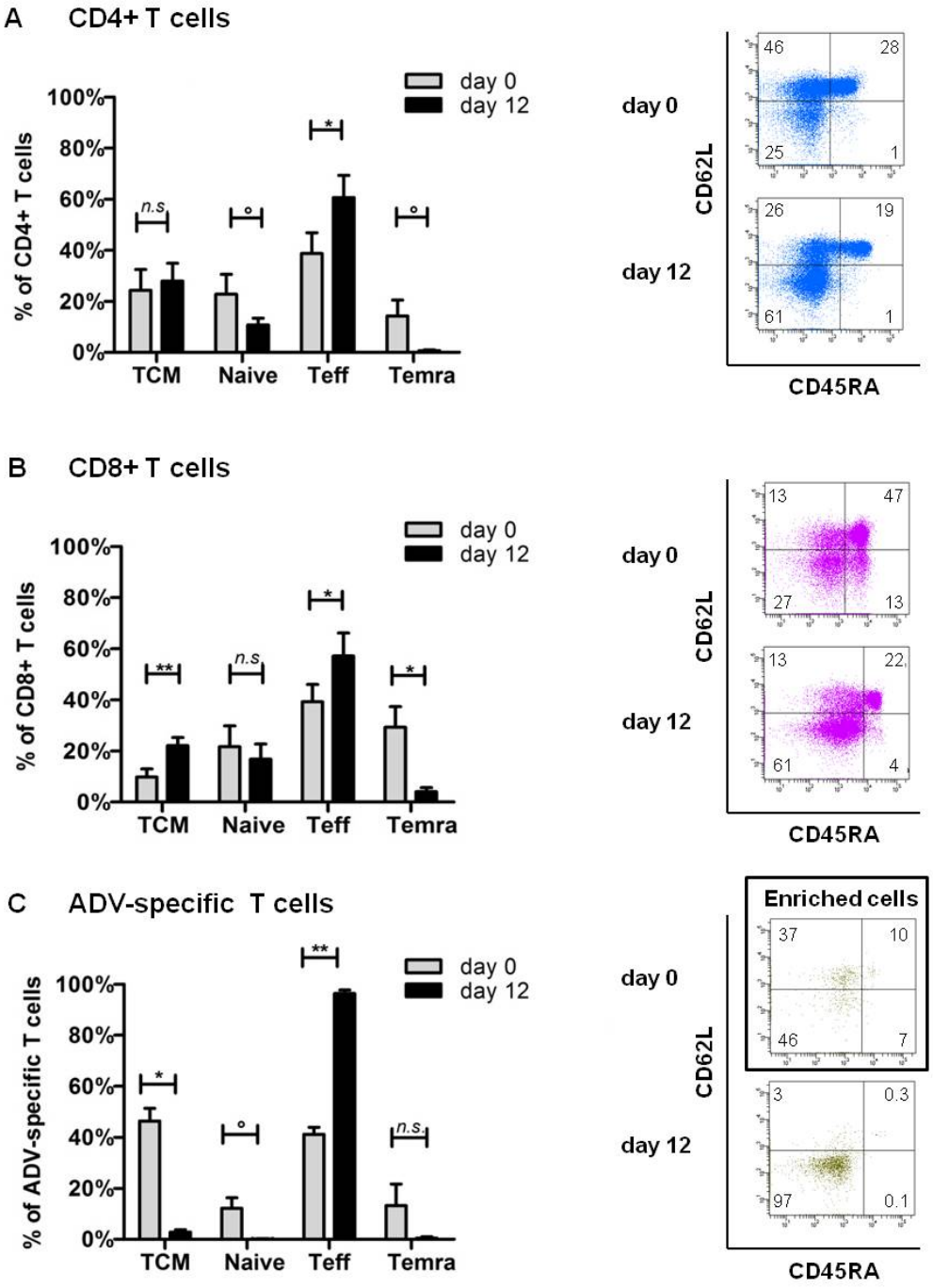


Figure 12. Immunophenotype analyses of different T cell subpopulations before and after in vitro expansion.

PBMCs of different donors were cultured for 12 days with ADV-PepTivator and IL-15 as described in material and methods. Cells were stained by the MHC I pentamer and immunophenotypical markers such as CD3, CD8, CD4, CD62L, and CD45RA were analyzed by flow cytometry. Central memory T cells (TCM) and effector T cells (Teff) were identified by their low or high expression of CD62L, respectively. Furthermore, CD45RA was used to distinguish between TCM and naïve T cells, Teff and effector memory RA+ T cell (Temra). Numbers in blots represent % of CD8, CD4 or ADV-specific T cells of the following phenotypes: TCM (CD45RA-, CD62L+), Naïve T cell (CD45RA+, CD62L+), Teff (CD45RA-, CD62L-), Temra (CD45RA+, CD62L-). For detailed phenotypical analysis, CD8+, CD4+, and pentamer+ T cells were gated.
The percentage of TCM, naïve, Teff, and Temra T cell subpopulations of CD8+ T cells (A) and CD4+ T cells (B) on day 0 (gray bars) and day 12 (black bars) are shown. Data show means and SEM of 6 independent experiments. (C) The percentage of TCM, naïve, Teff, and Temra T cell subpopulations of magnetically enriched ADV-pentamer+ T cells on day 0 (gray bars) and ADV-pentamer+ T cells on day 12 (black bars) are shown. Data show means and SEM of 4 independent experiments.
In some experiments, the gated dots vary as a consequence of their modification during the expansion process. Typical dot blots of CD8+, CD4+, and ADV-pentamer+ gated T cells out of 4 to 6 donors are shown. Significance versus day 0 of different T cell populations (as indicated): n.s., non significant; o, P ≤ 0.11; *, P ≤ 0.05; **, P ≤0.01.

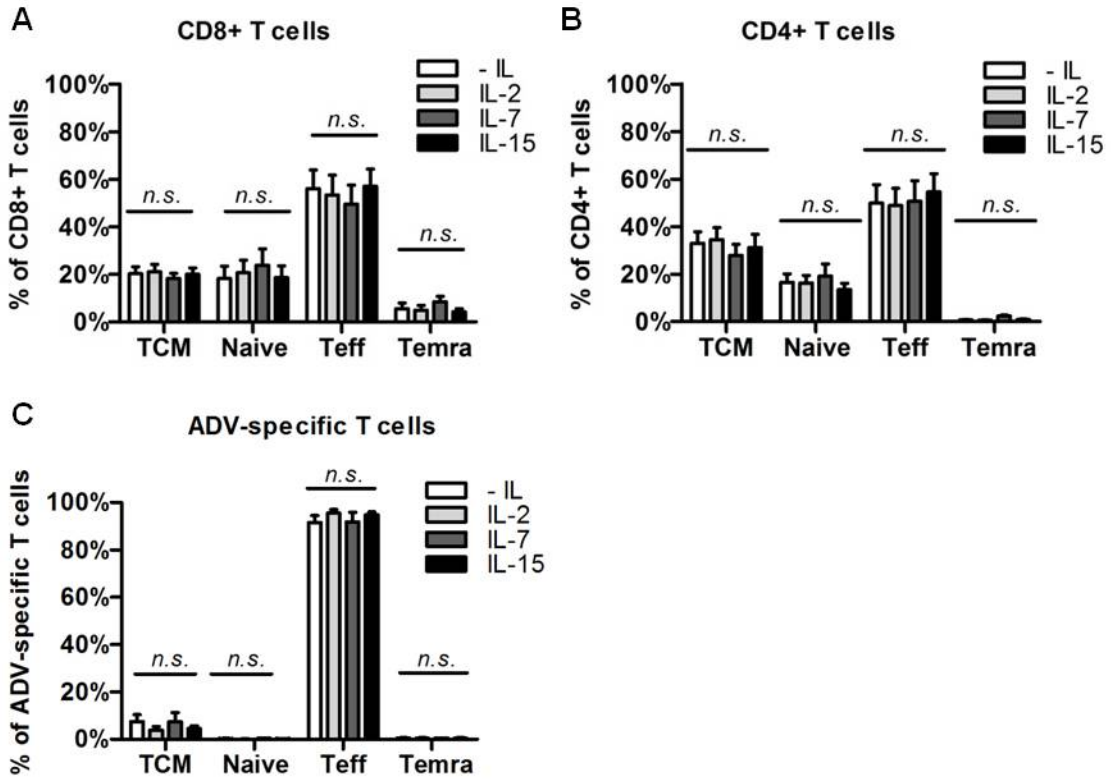


Figure 13. Phenotype analysis of expanded CD8+, CD4+ and ADV-specific T cells under interleukin treatment.
PBMCs were cultured in median for 12 days with PepTivator ADV as described in materials and methods. On day 3 and 9 cells were either untreated (-IL, white bars) or treated with IL-2 (light grey bars), IL-7 (dark grey bars), or IL-15 (black bars). All cell populations together represent

100%. TCM indicates CD45RA-, CD62L+ cells, naïve T cells CD45RA+, CD62L+, Teff CD45RA-, CD62L- and Temra CD45RA+, CD62L- cells.
The percentage of TCM, naïve, Teff, and Temra cell populations of CD8+ T cells (A), CD4+ T cells (B) and ADV-pentamer+ T cells (C) treated with different interleukins (as indicated) are shown. The diagrams give means and SEM of 8 independent experiments. Significance vs. untreated (-IL) cells : n.s., non significant.

3.2 Irradiated virus-specific T cells for adoptive immunotherapy after allogeneic hematopoietic stem cell transplantation

We established a fast and reliable protocol for the expansion of ADV-specific T cells. However, although previous data showed that the alloreactivity of in vitro expanded ADV-specific T cells seems to be significantly reduced as compared to PBMCs, the risk for GvHD after transfer into the patient could not be excluded. So in order to prevent cell proliferation and subsequently GvHD, transfusion products are irradiated in the daily hospital routine. Since little is known about irradiated T cells and their contribution in fighting against viral infections,[32] we analyzed the viability, functional activity, and alloreactivity of ADV-specific T cells after irradiation.

3.2.1 Viability of expanded ADV-specific T cells after irradiation

The viability and functionality of ADV-specific T cells is one of the key points for successful application in the clinical setting. First we expanded ADV-specific T cells for 12 days as described (2.2.1.4 Expansion of ADV-specific T cells (in vitro cell stimulation)). Then the cells were untreated or irradiated with 30Gy and subsequently cultured for 24, 48, and 72 hours. The viability was assessed by flow cytometry using Trucount™ tubes, which enables the analysis of cells/µl. The samples were analyzed directly on day 0, 24h, 48h, and 72h after cell culture. The viability was compared to non-irradiated ADV-specific T cells at every time point of measurement. Generally, the viability of non-irradiated T cells was not affected by additional culture periods.
Viable cells were identified by their size (SSC-A) and granularity (FSC-A) immediately after cell harvesting. A typical dot blot is shown in Figure 14, the yield of viable cells constituted 2460/µl (non-irradiated) and reached 851/µl 72h after irradiation. As expected, the viability was affected by the process of irradiation. Nevertheless, 6h after irradiation treatment, still 92% could be identified as viable cells (data not shown). After 24h, 49% viable cells were identified (non-irradiated: 86% viable cells), the same tendency could be observed after 48h with 24.2% viable cells. In fact, a slowdown could be detected after 72h, when 20.4% of the cells, compared with non-irradiated cells, showed indications of viability. Strikingly, even 24h after irradiation, no significant difference to that of non-irradiated samples was seen

(P=0.1206). After 48h (P=0.0214) and 72h (P=0.0208), however, a significance could be observed.

Taken together, although ADV-specific T cells lose viability after irradiation treatment, at least 20.4% of the cells are viable 72h after irradiation. These findings indicate that even 72h after irradiation, viable cells are detectable.

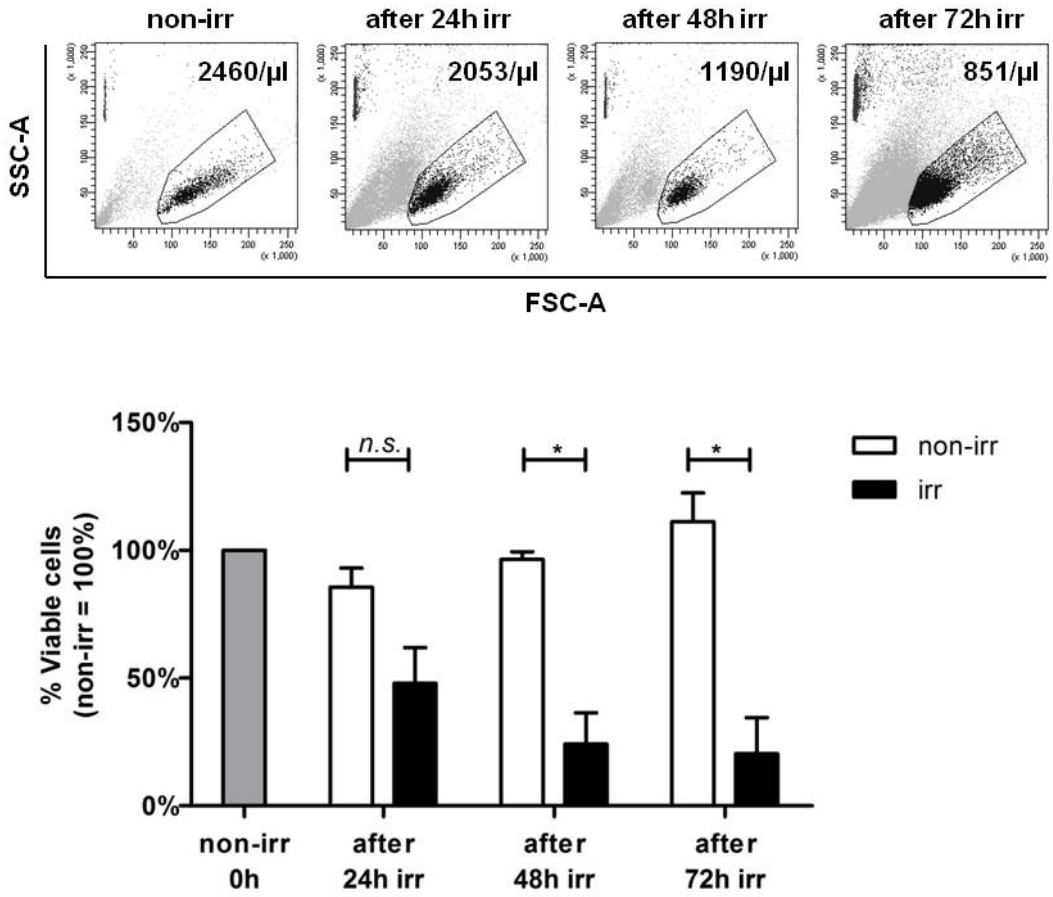


Figure 14. Viability of irradiated ADV-specific T cells after different time points.

PBMCs were stimulated with PepTivator ADV and treated with IL-15 as described in materials and methods. For each approach 5 x 10^5 expanded ADV-specific T cells were placed in a 96-well plate, either non-irradiated (non-irr) or irradiated (irr) with 30Gy (3000rad). Each well was completely harvested after 24h, 48h and 72h, at every time point non-irradiated cells were analyzed as control. Cells were transferred into TruCOUNT™ tubes and analyzed with flow cytometer LSRII.

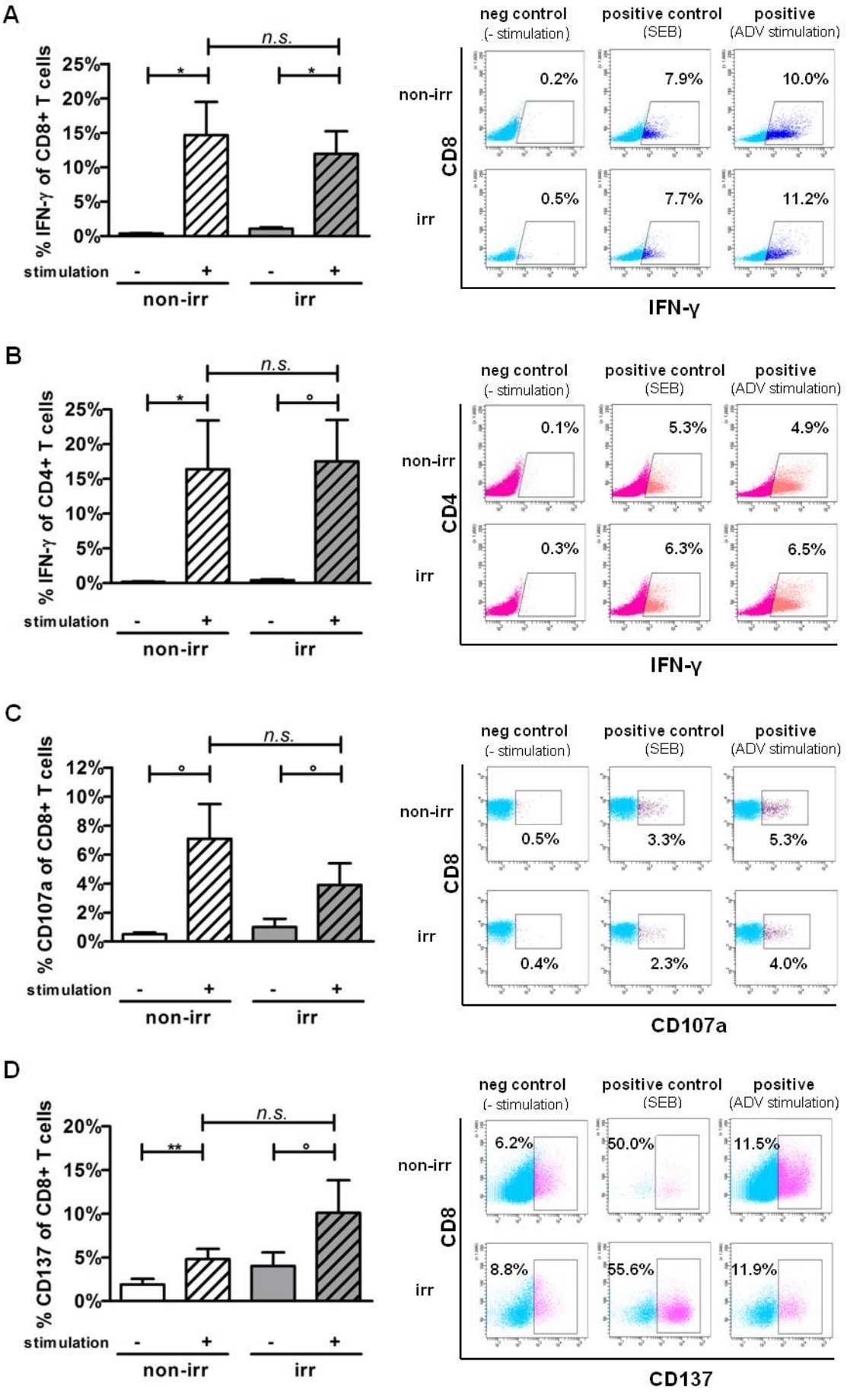
Viable cells were defined by gating forward scatter (FSC) vs side scatter (SSC). After 24h, 48h, and 72h two populations are visible: a vital cell population with unchanged scatter signals and an apoptotic cell population with decreased FSC and increased SSC signals. The percentage of viable cells was calculated as follows: (number of viable irradiated cells/µl) / (number of viable (sham) non-irradiated cells/µl) x 100. In all experiments, results were referred to the non-irradiated cells measured on day 0 (gray bar) which was set to 100%. Non-irradiated cells after 24h, 48h, and 72h are indicated as white bars, irradiated cells as black bars. Data show means ± SEM of 4 independent experiments. Significance versus non-irradiated ADV-specific T cells at the time point of analysis: n.s., non significant; *, P ≤ 0.05. Typical dot blots out of 4 experiments are shown. Values indicate the living cells per µl in the gated area.

3.2.2 Effect of irradiation on the functional activity of ADV-specific T cell population

Next, we analyzed the functional activity of non-irradiated and irradiated ADV-specific T cells. Therefore, after restimulation of already expanded ADV-specific T cells with the ADV-specific antigen (PepTivator), T cell activation markers such as INF-γ, CD137, and CD107a were analyzed by flow cytometry.

To investigate the impact of irradiation on the functional activity of expanded ADV-specific T cells, after 12 days stimulation, cells were irradiated by 30Gy and cultured for 16h over night. As a positive control, non-irradiated cells were also cultured for 16h. On the following day, non-irradiated and irradiated ADV-specific T cells were either unstimulated (negative control) or restimulated for 4h with the superantigen (positive control; data not included in the diagram, showing means ± SEM) or the appropriate ADV-specific antigen (PepTivator). After 4h of restimulation, the secretion of IFN-γ and the expression of CD107a of cells were analyzed at the same time by the IFN-γ cytokine secretion assay (CSA) according to manufacturers´ instructions. In all experiments, additional staining of CD3, CD4, and CD8 were performed in order to be able to gate different T cell subpopulations.
Strikingly, the percentage of IFN-γ secreting CD8+ T cells was significantly increased in both settings, the non-irradiated cells from 0.4% to 14.7% and the irradiated cells from 1.1% to 12.0% after restimulation with the ADV-PepTivator (Figure 15A). Similar results were obtained by analyzing the non-irradiated (0.2% to 16.4%) and irradiated (0.5% to 17.5%) CD4+ T cells (Figure 15B). These results indicate that the IFN-γ secretion of CD4+ as well as CD8+ T cells was not significantly affected 16h after irradiation.
Similarly, we analyzed the expression of CD107a, a marker for the cytotoxic activity of CD8+ T cells, within the same samples (Figure 15C). In accordance with the data shown in Figure 15A, the expression of CD107a was induced in both the non-irradiated - from 0.5% to 7.1% - as well as in the irradiated cells - from 1.0% to 3.9%. However, the reduced expression of CD107a after irradiation from 7.1% to 3.9% was not significant (Figure 15C).
Next we analyzed the expression of CD137, another important T cell activation marker. Since the expression of CD137 was shown to be optimal after 16h of restimulation, cells were directly restimulated after irradiation. Although the background level (negative control, -) was relatively high, an induction of CD137 expression after restimulation was seen in CD8+ (Figure 15D) as well as in CD4+ (Figure 15E) T cells irrespective of irradiation. Again no significant effect of irradiation was seen by comparing the percentage of CD137 expression of non-irradiated with that of irradiated CD8+ T cells (4.8% vs 10.1%) and CD4+ T cells (7.7% vs 11.3%).

These results clearly indicate that the functional activity of CD8+ as well as CD4+ ADV-specific T cells was not affected - at least not 16h after irradiation.


A
% IFN-γ of CD8+ T cells
25%
20%
15%
10%
5%
0%
n.s.
*
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stimulation
-
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irr
neg control
(- stimulation)
positive control
(SEB)
positive
(ADV stimulation)
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CD8
0.2%
7.9%
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11.2%
IFN-γ
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5%
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n.s.
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non-irr
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neg control
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positive control
(SEB)
positive
(ADV stimulation)
non-irr
irr
CD4
0.1%
5.3%
4.9%
0.3%
6.3%
6.5%
IFN-γ
C
% CD107a of CD8+ T cells
12%
10%
8%
6%
4%
2%
0%
n.s.
°
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stimulation
-
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neg control
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(SEB)
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(ADV stimulation)
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CD8
0.5%
3.3%
5.3%
0.4%
2.3%
4.0%
CD107a
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20%
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(SEB)
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CD8
6.2%
50.0%
11.5%
8.8%
55.6%
11.9%
CD137

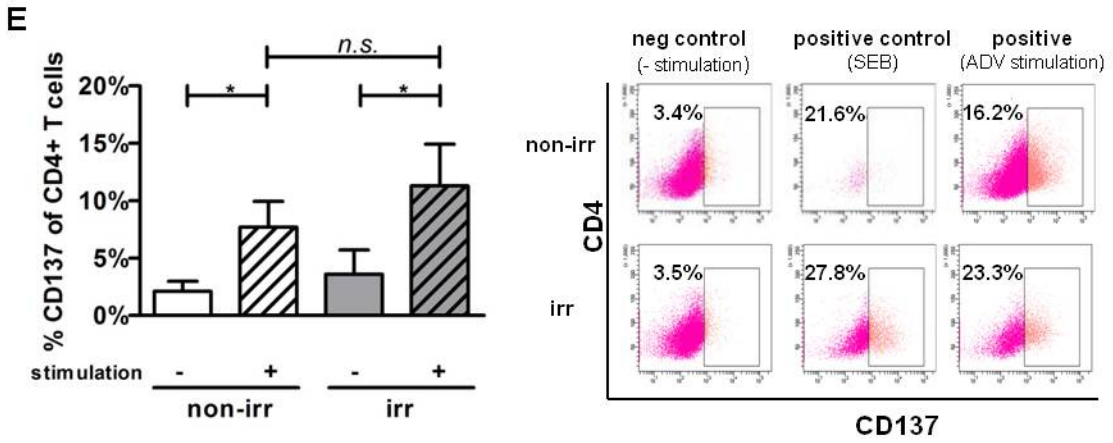

Figure 15. Functionality analysis of irradiated, expanded, ADV-specific T cells.
Over 12 days PepTivator expanded PBMCs were irradiated by 30Gy and cultured for 16h over night. Non-irradiated cells were also cultured for 16h.
Afterwards, non-irradiated and irradiated cell cultures were either unstimulated (negative control, -stimulation) or stimulated for 4h with either superantigen (positive control, SEB) or ADV-specific antigen (positive, ADV-stimulation). Staining was performed in combination with a cytokine secretion assay. For CD137 analysis, 16h stimulation represents the optimal duration. Therefore, stimulation starts at the same time point as irradiation treatment occurs.
Representative dot blots show percentage of CD8 or CD4+ T cells. The first row represents non-irradiated (non-irr), the second row 16h irradiated cells (irr). Diagram shows means ± SEM of 3-8 independent experiments. The percentage of IFN-γ of CD8+ T cells (A) and CD4+ T cells (B); percentage of CD107a of CD8+ T cells (C); percentage of CD137 of CD8+ T cells (D) and CD4+ T cells (E) are shown. Significance: n.s., non significant; o, P ≤ 0,13; *, P ≤ 0.05; **, P ≤ 0.01.

3.2.3 Effect of irradiation on the cytotoxic activity of ADV-specific T cells

Generally, besides the ability of virus-activated T cells to produce IFN-γ and other activation markers, they can be cytotoxic, being able to lyse virus infected cells. In order to investigate the cytotoxicity of non-irradiated and irradiated expanded ADV-specific T cells, a newly developed cytotoxic Assay (CTA), based on flow cytometric analysis, was used to avoid the common radioactive chromium release assay. Therefore, non-irradiated and irradiated expanded ADV-specific T cells, were incubated with unpulsed (negative control), ADV-peptide-pulsed or PepTivator-ADV5 Hexon-pulsed autologous target cells for 4h at a ratio of 20:1 (T cell to target cell) to induce cytotoxic killing. To analyze the impact of irradiation on ADV-specific T cells after several hours, the cytotoxic assay was performed 6h, 24h, and 48h after irradiation. Induced cell death was determined by staining with DAPI and Trucount™ tubes.

As shown in Figure 16, the number of dead cells/µl of unpulsed peptide-pulsed, and PepTivator-pulsed autologous target cells without the incubation of ADV-specific T cells ranged from 4.1-7.7/µl. Non-irradiated ADV-specific T cells showed increased specific lysis of

ADV-peptide (18.2 dead target cells/µl ± 2.9, P=0.0021) and PepTivator – pulsed (13.5 ± 2.2, P=0.0480) target cells compared to unpulsed target cells (5.8 ± 0.7). Also, the peptide-specific (17.6 ± 2.3, P=0.0080) and PepTivator-specific (14.2 ± 2.3, p<0.0740) lysis of ADV-specific T cells, 6h after irradiation was significantly higher as compared to the respective unpulsed target cells (5.2 ± 0.4). ADV-specific T cells 24h after irradiation still showed increased lysis of peptide-pulsed (10.2 ±1.3, P=0.0052) and PepTivator-pulsed (10.1 ± 2.9, P=0.0382) targets as compared to unpulsed targets (4.9 ± 0.5) - even though the peptide-specific lysis was not significant. Even 48h after irradiation, increased lysis of peptide-pulsed targets (11.6 ± 3.8, P=0.2400) as compared to unpulsed targets (6.2 ± 0.9) were seen (Figure 16).

However, the increase of peptide-specific lysis induced by irradiated T cells was similar, or slightly lower, 6h (3.4 fold), 24h (2.1 fold), and 48h (1.9 fold) after irradiation, compared to non-irradiated T cells (3.1 fold). In addition, also the increase of PepTivator-specific lysis induced by irradiated T cells was similar, 6h (2.7 fold) and 24h (2.1 fold) after irradiation, as compared to non-irradiated T cells (2.3 fold). 48h after irradiation, no specific lysis was seen (results of a single experiment are shown). Notably, the corresponding numbers of apoptotic cells/µl of unpulsed target cells were very similar to each other. Taken together, these results clearly indicate that even after 6h, 24h, and 48h after irradiation, ADV-specific T cells are still cytotoxic against ADV-pulsed target cells.

Focusing on the affect of target pulsed with peptide or PepTivator, the former achieved higher success rates, independent of the irradiation status. The incidence of the higher killing frequency for peptide pulsed target cells is still unclear, but there is evidence that ADV-PepTivator needs longer incubation time for a perfect presentation on the MHC I receptor.

Specific lysis was tested using autologous PHA blasts (target) pulsed with either ADV-PepTivator (TP PepTivator) or peptide ADV (TP peptide), as control unpulsed autologous targets (TuP) were used. As effectors, over 12 days PepTivator stimulated ADV-specific T cells, either non-irradiated (non-irr) or irradiated (irr, 6, 24, 48h before the assay) was applied. An effector-target ratio of 20:1 was observed. After 4h incubation, cells were analyzed by flow cytometer, whereas dead target cells/µl were displayed.

(A) Dot blots of one representative donor out of 6 experiments are shown. The first vertical row represents the FSC/SSC from peptide pulsed target cells with effector cells. Horizontal the different time points after irradiation of effector cells is indicated. (B) Summary of 1-6 independent experiments. Dashed bars indicate unpulsed targets, either with effectors or without, black bars represent targets pulsed with peptide ADV and grey bars with ADV-PepTivator. Data show means and SEM. Significance target unpulsed vs pulsed target cells: o, P ≤ 0.1; *, P ≤ 0.05; **, P ≤ 0.01.

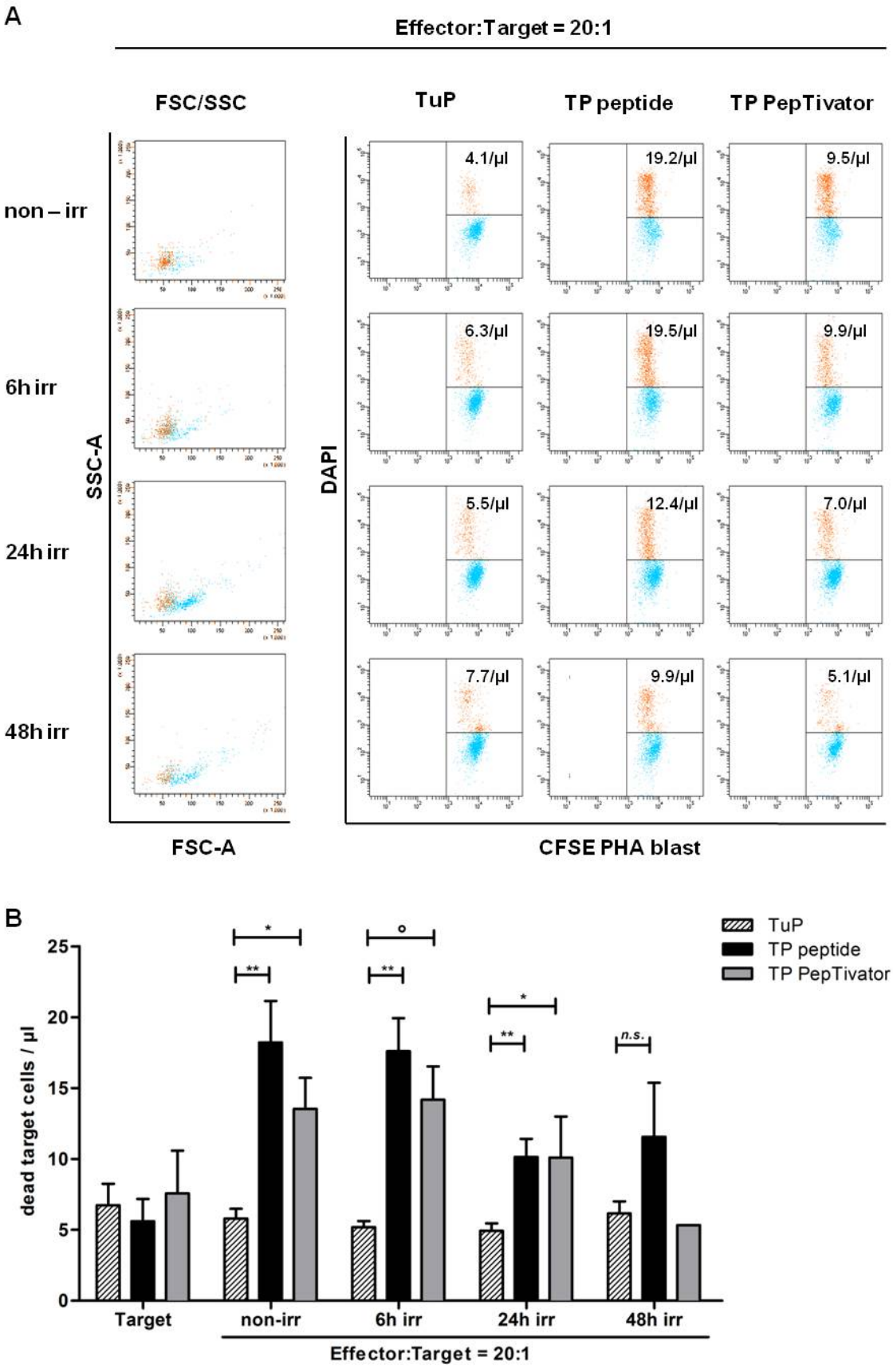

Figure 16. Cytotoxic assay of irradiated ADV-specific T cells.

3.2.4 Clinical Applications

3.2.4.1 Frozen expanded ADV-specific T cells show no quality reduction

Although real-time PCR analysis permits early detection of impending invasive viral-infection in patients after HSCT, the exact time point and need for a T cell immunotherapy in the daily clinical practice is often influenced by unexpected complications and changes in treatment schemas and is mostly unpredictable. Therefore, prophylactic generation and freezing of virus-specific T cells followed by thawing, irradiation and administration of such cells after the occurrence of invasive viral infections in patients could provide an attractive treatment option.

In order to test the effect of freezing and thawing on the cytotoxicity of ADV-specific T cells following irradiation, unfrozen and frozen expanded ADV-specific T cells were analyzed by flow cytometry. After thawing, cells were cultured for one day to optimize their viability performance. Afterwards, a CTA was performed.
Because very high numbers of PBMCs are needed, only one single experiment could have been performed.
The induced specific lysis of peptide-pulsed target cells by non-irradiated unfrozen ADV-specific T cells (11.9 dead target cells/µl) was comparable to that of frozen ADV-specific T cells (10.9 dead target cells/µl). Also, freezing did not have any effect on the cytotoxicity of ADV-specific T cells 24h after irradiation (Figure 17). However, in this experiment, the irradiated ADV-specific T cell population showed only a slightly increased lysis of peptide-pulsed target cells (6.2 dead target cells/µl) compared to unpulsed target cells (4.6 dead target cells/µl), which might be explained by the intra-individual variability of donors.
Thus, in this experiment, a clear conclusion whether freezing is influencing the functional activity of irradiated T cells was not possible and needs to be further investigated.

These results indicate that freezing does not influence the cytotoxicity of non-irradiated expanded ADV-specific T cells.

3.2.4.2 Analysis of alloreactivity of non-irradiated and irradiated expanded ADV-specific T cells

The adoptive transfer of ADV-specific T cells into mismatched recipients increases the risk for GvHD in patients. According to the literature, ADV-specific T cells are described to recognize only ADV-infected cells because of the expression of restricted ADV-specific TCR receptors. Therefore, the cytotoxic assay was performed by incubating non-irradiated or irradiated generated ADV-specific T cells of one donor (a) with that of autologous (A), partially mismatched by including at least 1 matched locus of HLA I (B and C) or completely mismatched (D) target cells.

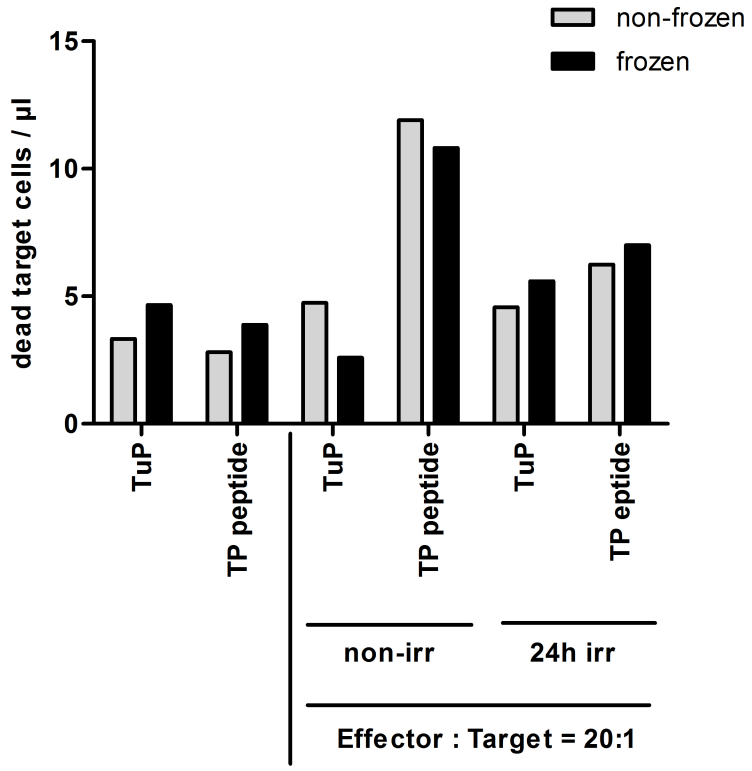

Figure 17. Cytotoxic assay of frozen and non-frozen expanded ADV-specific T cells in comparison.
Specific lysis was tested with autologous peptide ADV pulsed PHA target cells (TP peptide), as control unpulsed autologous targets (TuP) were used. As effector cells, expanded ADV-specific T cells were severed, either non-frozen (grey bars) or frozen (black bars), furthermore half of them were irradiated 24h before the assay (24h irr), the others were used as non-irradiated control (non-irr). Result of one experiment is shown at an effector-target ratio of 20:1, whereas dead target cells/µl are illustrated.

Comparable to the results seen in Figure 16, the specific lysis of autologous peptide-pulsed target cells is similarly induced by non-irradiated (bar 4: 13.4 dead cells/µl) and irradiated (bar 6: 8.2 dead cells/µl) ADV-specific T cells as compared to the respective unpulsed target cells (bar 3: 3.7 dead cells/µl; bar 5: 3.4 dead cells/µl) (Figure 18). Next, we assessed the alloreactivity of donor ADV-specific T cells in the partially (B and C) and completely unmatched (D) setting. Strikingly, the specific lysis of unpulsed autologous control target cells (bar 3: 3.7 dead cells/µl) was only slightly induced or even reduced as compared to the partially mismatched target cells of recipient B (bar 9: 5.4 dead cells/µl) and C (bar 15: 4.3 dead cells/µl) and the completely mismatched target cell of recipient C (bar 21: 2.9 dead cells/µl). These data indicate that the alloreactivity of expanded ADV-specific T cells was highly reduced in most mismatched recipients. As already shown in Figure 16, the background lysis generally ranged from 3 to 7 cells/µl.

However, although generated ADV-specific T cells should not be alloreactive, killing of ADV-infected cells in partially matched recipients, as it is the case after haploidentical HSCT, is desirable. To resemble this in vivo situation, the cytotoxicity of ADV-specific T cells against partially mismatched target cells (B and C) was analyzed. In fact, the specific lysis of ADV-pulsed target cells of recipient B was increased as compared to the unpulsed target cells (bar 10: 12.7 dead cells/µl vs bar 9: 5.4 dead cells/µl, respectively). A slightly increased cytotoxicity was also seen in the ADV-pulsed target cells of recipient C as compared to unpulsed target cells (bar 16: 6.3 dead cells/µl vs bar 15: 4.3 dead cells/µl, respectively). Strikingly, at least in recipient B also irradiated ADV-specific T cells showed specific lysis of ADV-pulsed target cells as compared to unpulsed target cells (bar 12: 8.7 dead cells/µl vs bar 11: 5.2 dead cells/µl, respectively).

Although the alloreactivity of generated ADV-specific T cells was very low or even not existent, alloreactivity can only be completely excluded by additional irradiation. The fact that, despite irradiation, ADV-specific T cells are still able to kill virus-infected target cells also in a partially mismatched setting, opens a new window for adoptive T cell immunotherapies and would even allow the infusion of third party ADV-specific T cells without the risk for GvHD. In the clinical situation, non-irradiated and irradiated cells could be used, depending on the number of mismatched HLA types between donor and recipient, which is known to correlate with the risk for GvHD.

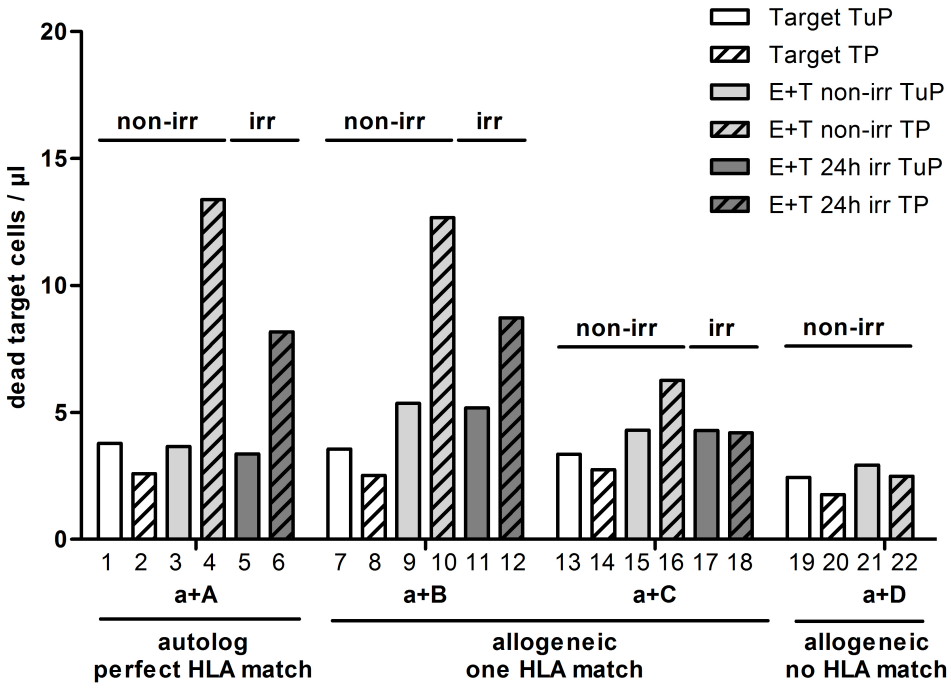

Figure 18. Alloreactivity: specific lysis of allogeneic pulsed target cells.
Expanded ADV-specific T cells, irradiated and non-irradiated serve as effector cells (a). As target cells PHA blast from 4 different donors, autologous (A) and allogeneic (B,C,D) were either non pulsed (dashed bars) or pulsed with peptide ADV (filled bars). Shown are triplicates of 4 different experiments.

4 Discussion

Considerable progress of HSCT techniques has led to significantly higher survival rates of patients who either receive T cell-depleted products or who require more intensive and prolonged post-transplant immune-suppression. On the other hand, however, an increasing number of patients contract severe or lethal adenovirus infections during the post-transplant phase after HSCT. So far, current pharmacological strategies for preemptive and acute therapies are insufficient and limited by significant side effects. Evidence indicating that recovery of ADV-specific T cells correlates with the clearance of ADV infections[36,37] prompted us to develop a protocol for detection and generation of ADV-specific T cells.

Expansion and characterization of in vitro-generated ADV-specific T cells

The frequency of ADV-specific T cells is very low in freshly isolated peripheral blood (0.2 ± 0.03 cells/µl or 0.1% ± 0.03 of CD8+ T cells) and is quite often below the limit of detection. Therefore, to increase the frequency of ADV-specific T cells, additional, fast in vitro expansion for 12 days was performed resulting in a 1 to 2 log increase of ADV-specific T cells. In order to define the earliest time point for detection of ADV-specific T cells, pentamer staining was performed each day. We could demonstrate that a first clear detection of ADV-specific T cells was even possible on day 6 of expansion followed by a decrease on day 8 and 9. This could, however, easily be explained by the fact that after restimulation on day 6, T cell receptor (TCR) internalization occurs, which reduces the binding of multimers to the TCR.[38,39]

Several groups tried different strategies to isolate ADV-specific T cells. Comoli et al. cultured PBMCs for 4 weeks to achieve an 2.5 fold increase of ADV-specific T cells.[40] Another very time consuming and costly expansion protocol (6 weeks), including repeated in vitro stimulation by antigen-pulsed dendritic cells, was developed by Leen et al.[35] In contrast to these time consuming protocols, Feuchtinger et al. and Aïssi-Rothé et al. established a method where IFN-γ secreting ADV-specific T cells could be directly isolated after a magnetic selection process.[6,41] However, since only very low frequencies of ADV-specific T cells are present in fresh blood, large starting blood volumes are necessary for such therapies. Additionally, isolation of virus-unspecific IFN-γ producing T cells cannot be excluded, which bears the risk of GvHD. A combination of both methods, where first IFN-γ producing T cells were magnetically isolated followed by in vitro expansion, showed a 1-2 log increase of ADV-specific T cells within 2 weeks.[13,35] However, again large starting blood volumes, which are often not available, are necessary for such therapies. Therefore, we reasoned that our fast repeated in vitro stimulation without the need of large blood volumes, would fulfill all requirements for a safe and efficient strategy.

Next, on the basis of our encouraging results, we wanted to further optimize our protocol. It is known that different cytokines influence the process of T cell expansion and maturation in vivo and in vitro. Feuchtinger et al., for example, used IL-2 for expansion of isolated ADV-induced INF-γ producing cells.[6] Another group also used IL-2 for expansion of ADV-specific T cells.[40] In contrast, Fujita et al. achieved the best expansion results by either treatment with IL-15 alone or with a combination of IL-7, IL-12 and IL-15 (per 5ng/ml).[42] We therefore tested the influence of IL-2, IL-7, and IL-15 on the expansion and the immunophenotype of ADV-specific T cells. Surprisingly, whereas treatment with IL-15 significantly increased (3.4 fold) the expansion of ADV-specific T cells as compared to untreated cells, treatment of IL-2 and IL-7 showed no influence. This result could be explained by the fact that, in contrast to other groups, PBMCs were treated with cytokine for only a short period of time (day 3 to 6 and day 9 to 12). However, that cytokine treatment is not necessarily needed for T cell expansion, was also shown by Zandvliet et al.[43] Their strategy, by the way, could also help to reduce the costs for immunotherapies.

Beside the effect of cytokines on the expansion of T cells, they are also known to influence the effector/memory surface phenotype of cultured cells.[44,45] Thus, we compared the surface phenotype of PBMCs at day 0 with that of 12 days expanded ADV-specific T cells, which were additionally treated with IL-15. As expected, the number of naïve and central memory T cells was reduced, whereas the number of effector ADV-specific T cells had increased. These results mimic the in vivo situation, where mostly effector T cells are necessary to fight against an acute infection. An increased number of effector T cells during the expansion period was also observed by other groups.[13,43,46] Obar et al. demonstrated that in vivo IL-2–derived signals promote the downregulation of CD62L and formation of the effector memory T cell (TEM) population, whereas IL-15–derived signals promote the expression of CD62L and formation of central memory T cells (TCM) within the culture.[44] We could, however, not observe an increase of TCMs after treatment with IL-15. Nevertheless, about 2,8% of TCMs, providing long-lived immunity, were maintained after the expansion period. Interestingly, treatment with IL-2, IL-7, and IL-15 showed similar results concerning the effector/memory phenotype after the expansion period. These results could again be explained by the short treatment period. However, avoidance of early interleukin treatment is indispensable to exclude the expansion of virus-unspecific T cells, which could result in a higher risk of GvHD. Summarizing these results, we could show that after expansion, high numbers of effector T cells and low numbers of TCMs had been be generated or maintained, respectively.

Irradiated ADV-specific T cells for adoptive immunotherapy after allogeneic hematopoietic stem cell transplantation

Although several groups could show reduced alloreactivity of in vitro expanded ADV-specific T cells,[13,17,40] a risk of GvHD in vivo can never be definitely excluded. To overcome this limitation for immunotherapies, the effect of irradiation of expanded ADV-specific T cells was analyzed.

In the 1990s, it became clear that the lethality of tissue cells after radiation is rather due to activation of apoptosis than to irreversible damage of cells.[47,48] Indeed, lymphocytes have been found to be among the most radiosensitive mammalian cells.[49] However, another study showed that the sensitivity of T cells to irradiation could be reduced, when already stimulated T cells were used.[50,51] A further hint, that irradiation is not completely inhibiting T cells, was shown in a case report by Witt et al.[32] In this work, a clearance of CMV infection in a CMV seronegative patient after receiving irradiated fractions of granulocytes, including T cells of a CMV seropositive donor, suggested for the first time that even irradiated T cells could have been responsible for this effect - although definite evidence was missing.

Therefore we first tested the effect of irradiation on ADV-specific T cells after expansion. Strikingly, although the viability of cells was decreased after irradiation, still about 25% of cells proved highly vital. Since it is suggested that virus-specific T cells need about 1-2 days to find and kill virus-infected cells, even irradiated cells should have enough time to fulfill their task before dying. A hallmark of T cells is their ability to secrete IFN-γ and express activation markers such as CD137 and CD107a after getting stimulated by viral antigens. Strikingly, restimulation of ADV-specific T cells 24h after irradiation showed similar secretion of IFN-γ and expression of CD137 compared to non-irradiated cells. In addition, the expression of CD107a, representing the cytotoxic potential of these cells,[19] was also not decreased after irradiation, indicating that irradiation did not alter their functional activity.

To mimic the cytotoxicity of ADV-specific T cells in vivo, an in vitro cytotoxic assay was performed. Thereby the ability of irradiated ADV-specific T cells to kill antigen-pulsed target cells (PHA blasts) was analyzed. We could clearly demonstrate that adenovirus-specific lysis of target cells was induced 6h, 24h, and even 48h after irradiation. Interestingly, the number of killed target cells was lower when cells had been pulsed with the PepTivator as compared to peptide-pulsed cells. This result was surprising because PepTivator-pulsed target cells can be killed by CD4 and CD8+ T cells, whereas peptide-pulsed target cells can only be killed by the appropriate peptide-recognizing CD8+ T cells. We suggest that the lower concentration of the appropriate peptide within the peptide pools (PepTivator) could be the reason for these differences.

Taken together, these results indicate that, despite irradiation, ADV-specific T cells are still functionally active and able to kill virus-infected cells.

Clinical applications

In the clinical situation, the median time span between the detection of ADV loads in stool probes of patients after HSCT and first observation of lethal symptoms is approximately 21 days.[11] Therefore, depending on either a long culture process[34,35] or fast magnetic isolation,[52] only prophylactic treatment or treatment on demand of patients with ADV-specific T cells have been reported, respectively. Since we could generate ADV-specific T cells within 12 days, both options, prophylactic treatment as well as treatment on demand, are conceivable. However, for some patients, even the generation of ADV-specific T cells within 12 days would take too long. To overcome this problem, preemptive generation and freezing of ADV-specific T cells, which could then be thawed and infused on demand, could be a possible approach. For this reason we analyzed the viability and functional activity of non-irradiated and irradiated ADV-specific T cells, after freezing and thawing. Strikingly, non-irradiated ADV-specific T cells showed similar cytotoxicity against peptide-pulsed target cells, regardless of whether the cells had been frozen or not. Since in this experiment, irradiated T cells showed only a slight induction of specific target cell lysis, a clear statement about the effect of irradiation on thawed T cells was not possible.

Since for most patients, a matched donor is often not available, T cells have to be generated from mismatched donors, bearing, however, the risk of GvHD. To minimize this risk, the efficacy of T cells to kill allogeneic, partially unmatched and completely unmatched ADV-pulsed target cells was analyzed. Indeed, the alloreactivity of non-irradiated ADV-specific T cells was very low and even absent after irradiation. These data are in accordance with studies by Leen et al. and Comoli et al.,[35,40] who showed less alloreactivity of ADV-specific T cells after in vitro expansion. However, apart from the loss of alloreactivity, cells must be able to kill virus-infected target cells even in allogeneic recipients where at least one HLA locus is matched. Consequently, we analyzed whether expanded ADV-specific T cells from one donor are able to kill peptide-pulsed target cells of partially mismatched recipients. Interestingly, the killing of partially mismatched target cells was similar to that of autologous target cells, representing a specific HLA-type dependent lysis in an allogeneic setting. Therefore, we assume that in a mismatched clinical setting, our in vitro expanded non- and irradiated ADV-specific T cells would be able to kill only specifically virus-infected cells. Based on our results, we suggest preemptive generation of ADV-specific T cells followed by freezing, which would allow the establishment of a library of ADV-specific T cells from even third party donors with decreased alloreactivity, which could then be infused on demand. The need for irradiation of ADV-specific T cells would depend on the donor/recipient matching.

We have thus established a rapid, simplified and reliable method for the detection and generation of ADV-specific T cells that can be easily translated into a good manufacturing practice (GMP)-conform therapy as required for manipulation of cells prior to infusion into immunocompromised patients.

5 References

1. Moss P, Rickinson A. Cellular immunotherapy for viral infection after HSC transplantation. Nat Rev Immunol. 2005;5:9-20.
2. Leen AM, Heslop HE. Cytotoxic T lymphocytes as immune-therapy in haematological practice. Br J Haematol. 2008;143:169-179.
3. Feuchtinger T, Lang P, Handgretinger R. Adenovirus infection after allogeneic stem cell transplantation. Leuk Lymphoma. 2007;48:244-255.
4. Fujita Y, Rooney CM, Heslop HE. Adoptive cellular immunotherapy for viral diseases. Bone Marrow Transplant. 2008;41:193-198.
5. Lenaerts L, De Clercq E, Naesens L. Clinical features and treatment of adenovirus infections. Rev Med Virol. 2008;18:357-374.
6. Feuchtinger T, Lang P, Hamprecht K, et al. Isolation and expansion of human adenovirus-specific CD4+ and CD8+ T cells according to IFN-gamma secretion for adjuvant immunotherapy. Exp Hematol. 2004;32:282-289.
7. Lion T, Kosulin K, Landlinger C, et al. Monitoring of adenovirus load in stool by real-time PCR permits early detection of impending invasive infection in patients after allogeneic stem cell transplantation. Leukemia. 2010;24:706-714.
8. Glasgow JN, Everts M, Curiel DT. Transductional targeting of adenovirus vectors for gene therapy. Cancer Gene Ther. 2006;13:830-844.
9. Ferreira TB, Alves PM, Aunins JG, Carrondo MJ. Use of adenoviral vectors as veterinary vaccines. Gene Ther. 2005;12 Suppl 1:S73-83.
10. Hoffman JA. Adenovirus infections in solid organ transplant recipients. Curr Opin Organ Transplant. 2009;14:625-633.
11. Lion T, Baumgartinger R, Watzinger F, et al. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood. 2003;102:1114-1120.
12. Heim A, Ebnet C, Harste G, Pring-Akerblom P. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol. 2003;70:228-239.
13. Chatziandreou I, Gilmour KC, McNicol AM, et al. Capture and generation of adenovirus specific T cells for adoptive immunotherapy. Br J Haematol. 2007;136:117-126.
14. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science. 1996;274:94-96.
15. Klenerman P, Cerundolo V, Dunbar PR. Tracking T cells with tetramers: new tales from new tools. Nat Rev Immunol. 2002;2:263-272.
16. Heemskerk B, Veltrop-Duits LA, van Vreeswijk T, et al. Extensive cross-reactivity of CD4+ adenovirus-specific T cells: implications for immunotherapy and gene therapy. J Virol. 2003;77:6562-6566.

17. Leen AM, Christin A, Khalil M, et al. Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy. J Virol. 2008;82:546-554.
18. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003;281:65-78.
19. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. Cell Immunol. 2009;254:149-154.
20. Zandvliet ML, Falkenburg JH, van Liempt E, et al. Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection. Haematologica. 2010.
21. Ahmed R, Bevan MJ, Reiner SL, Fearon DT. The precursors of memory: models and controversies. Nat Rev Immunol. 2009;9:662-668.
22. Stemberger C, Neuenhahn M, Gebhardt FE, Schiemann M, Buchholz VR, Busch DH. Stem cell-like plasticity of naive and distinct memory CD8+ T cell subsets. Semin Immunol. 2009;21:62-68.
23. Stemberger C, Neuenhahn M, Buchholz VR, Busch DH. Origin of CD8+ effector and memory T cell subsets. Cell Mol Immunol. 2007;4:399-405.
24. Willinger T, Freeman T, Hasegawa H, McMichael AJ, Callan MF. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. J Immunol. 2005;175:5895-5903.
25. Bannard O, Kraman M, Fearon D. Pathways of memory CD8+ T-cell development. Eur J Immunol. 2009;39:2083-2087.
26. Soares MV, Borthwick NJ, Maini MK, Janossy G, Salmon M, Akbar AN. IL-7-dependent extrathymic expansion of CD45RA+ T cells enables preservation of a naive repertoire. J Immunol. 1998;161:5909-5917.
27. Baldwin TA, Gogela-Spehar M, Ostergaard HL. Specific isoforms of the resident endoplasmic reticulum protein glucosidase II associate with the CD45 protein-tyrosine phosphatase via a lectin-like interaction. J Biol Chem. 2000;275:32071-32076.
28. Williams MA, Holmes BJ, Sun JC, Bevan MJ. Developing and maintaining protective CD8+ memory T cells. Immunol Rev. 2006;211:146-153.
29. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity. 2010;32:79-90.
30. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of CD8+ T cells. Immunol Rev. 2002;186:221-233.
31. Neofytos D, Ojha A, Mookerjee B, et al. Treatment of adenovirus disease in stem cell transplant recipients with cidofovir. Biol Blood Marrow Transplant. 2007;13:74-81.

32. Witt V, Fritsch G, Peters C, Matthes-Martin S, Ladenstein R, Gadner H. Resolution of early cytomegalovirus (CMV) infection after leukocyte transfusion therapy from a CMV seropositive donor. Bone Marrow Transplant. 1998;22:289-292.
33. Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol. 2006;134:64-76.
34. Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med. 2006;12:1160-1166.
35. Leen AM, Christin A, Myers GD, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. Blood. 2009;114:4283-4292.
36. Feuchtinger T, Lucke J, Hamprecht K, et al. Detection of adenovirus-specific T cells in children with adenovirus infection after allogeneic stem cell transplantation. Br J Haematol. 2005;128:503-509.
37. Heemskerk B, Lankester AC, van Vreeswijk T, et al. Immune reconstitution and clearance of human adenovirus viremia in pediatric stem-cell recipients. J Infect Dis. 2005;191:520-530.
38. D'Oro U, Munitic I, Chacko G, Karpova T, McNally J, Ashwell JD. Regulation of constitutive TCR internalization by the zeta-chain. J Immunol. 2002;169:6269-6278.
39. Gonzalez PA, Carreno LJ, Coombs D, et al. T cell receptor binding kinetics required for T cell activation depend on the density of cognate ligand on the antigen-presenting cell. Proc Natl Acad Sci U S A. 2005;102:4824-4829.
40. Comoli P, Schilham MW, Basso S, et al. T-cell lines specific for peptides of adenovirus hexon protein and devoid of alloreactivity against recipient cells can be obtained from HLA-haploidentical donors. J Immunother. 2008;31:529-536.
41. Aissi-Rothe L, Decot V, Venard V, et al. Rapid generation of full clinical-grade human antiadenovirus cytotoxic T cells for adoptive immunotherapy. J Immunother. 2010;33:414-424.
42. Fujita Y, Leen AM, Sun J, et al. Exploiting cytokine secretion to rapidly produce multivirus-specific T cells for adoptive immunotherapy. J Immunother. 2008;31:665-674.
43. Zandvliet ML, Falkenburg JH, Jedema I, Willemze R, Guchelaar HJ, Meij P. Detailed analysis of IFNg response upon activation permits efficient isolation of cytomegalovirus-specific CD8+ T cells for adoptive immunotherapy. J Immunother. 2009;32:513-523.

44. Obar JJ, Lefrancois L. Early signals during CD8 T cell priming regulate the generation of central memory cells. J Immunol. 2010;185:263-272.
45. Mitchell DM, Ravkov EV, Williams MA. Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells. J Immunol. 2010;184:6719-6730.
46. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest. 2008;118:294-305.
47. Gudkov AV, Komarova EA. Radioprotection: smart games with death. J Clin Invest. 2010;120:2270-2273.
48. Ross GM. Induction of cell death by radiotherapy. Endocr Relat Cancer. 1999;6:41-44.
49. Moroff G, Leitman SF, Luban NL. Principles of blood irradiation, dose validation, and quality control. Transfusion. 1997;37:1084-1092.
50. Lavin MF, Kidson C. Repair of ionizing radiation induced DNA damage in human lymphocytes. Nucleic Acids Res. 1977;4:4015-4022.
51. Lankinen MH, Vilpo LM, Vilpo JA. UV- and gamma-irradiation-induced DNA single-strand breaks and their repair in human blood granulocytes and lymphocytes. Mutat Res. 1996;352:31-38.
52. Feuchtinger T, Opherk K, Bethge WA, et al. Adoptive transfer of pp-65 specific T-cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood. 2010.

6 Appendix

6.1 List of Figures

Figure 1. Morphology and genome organization of adenovirus. 14
Figure 2. Multimer technology. 16
Figure 3. Immune reconstitution after T cell depleted stem cell transplantation. 17
Figure 4. T cell immune reconstitution after allogeneic HSCT. 17
Figure 5. Differentiation capacity of naïve and memory CD8+ T cells. 19
Figure 6. Evolution of adaptive T cell therapy for viral infections after allogeneic HSCT. 21
Figure 7. Expansion of ADV-specific T cells. 29
Figure 8. Principle of the INF-γ secretion assay 32
Figure 9. Schematic illustration of Cytotoxic Assay (CTA). 34
Figure 10. Generation and detection of ADV-specific T cells. 40
Figure 11. Influence of IL-2, IL-7 and IL-15 on the expansion of ADV-specific T cells. 40
Figure 12. Immunophenotype analyses of different T cell subpopulations before and after in vitro expansion. 43
Figure 13. Phenotype analysis of expanded CD8+, CD4+ and ADV-specific T cells under interleukin treatment. 44
Figure 14. Viability of irradiated ADV-specific T cells after different time points. 46
Figure 15. Functionality analysis of irradiated, expanded, ADV-specific T cells. 49
Figure 16. Cytotoxic assay of irradiated ADV-specific T cells. 51
Figure 17. Cytotoxic assay of frozen and non-frozen expanded ADV-specific T cells in comparison. 53
Figure 18. Alloreactivity: specific lysis of allogeneic pulsed target cells. 54

6.2 List of Tables

Table 1. T cell population and there surface marker expression. _____ 19
Table 2. Plate size and absolute cell count. _____ 30
Table 3. Pentamer staining for ADV._____ 31
Table 4. ADV-specific T cells on day 0, on day 0 after enrichment and after expansion on day 12._____ 42

6.3 List of Abbreviations

ADV, AdV	Adenovirus
APC	Antigen-presenting cell
APC	Allophycocyanin
CCRI	Children´s Cancer Research Institute
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate
CMV	Cytomegalovirus
CSA	Cytokine secretion assay (CSA)
CTA	Cytotoxic assay
DAPI	4',6-diamidino-2-phenylindole
DLI	Donor-leukocyte infusion
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
FSC-A/W	Forward side scatter area/wide
G-CSF	Granulocyte colony-stimulating factor
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IL	Interleukin
INF-γ	Interferon-gamma
Irr	Irradiated
MACS	Magnetic cell sorting
MHC	Major histocompatibility complex
NK	Natural killer cell
Non-irr	Non-irradiated
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
RT	Room temperature
SCT	Stem cell transplantation
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean

SSC-A	Side scatter area
TCM	Central memory T cells
TCR	T cell receptor
Teff	Effector T cell
TEM	Effector memory T cells
Temra	Effector memory RA+ T cell
TP	Target pulsed
TR	Texas red
TuP	Target unpulsed

6.4 Curriculum Vitae

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