Human Adenovirus-Specific γ/δ and CD8+ T Cells Generated by T-Cell Receptor Transfection to Treat Adenovirus Infection after Allogeneic Stem Cell Transplantation

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Abstract

Human adenovirus infection is life threatening after allogeneic haematopoietic stem cell transplantation (HSCT). Immunotherapy with donor-derived adenovirus-specific T cells is promising; however, 20% of all donors lack adenovirus-specific T cells. To overcome this, we transfected α/β T cells with mRNA encoding a T-cell receptor (TCR) specific for the HLA-A*0101-restricted peptide LTDLGQNLLY from the adenovirus hexon protein. Furthermore, since allo-reactive endogenous TCR of donor T lymphocytes would induce graft-versus-host disease (GvHD) in a mismatched patient, we transferred the TCR into γ/δ T cells, which are not allo-reactive. TCR-transfected γ/δ T cells secreted low quantities of cytokines after antigen-specific stimulation, which were increased dramatically after co-transfection of CD8α-encoding mRNA. In direct comparison with TCR-transfected α/β T cells, TCR-CD8α-co-transfected γ/δ T cells produced more tumor necrosis factor (TNF), and lysed peptide-loaded target cells as efficiently. Most importantly, TCR-transfected α/β T cells and TCR-CD8α-co-transfected γ/δ T cells efficiently lysed adenovirus-infected target cells. We show here, for the first time, that not only α/β T cells but also γ/δ T cells can be equipped with an adenovirus specificity by TCR-RNA electroporation. Thus, our strategy offers a new means for the immunotherapy of adenovirus infection after allogeneic HSCT.

Introduction

After allogeneic haematopoietic stem cell transplantation (HSCT) human adenovirus (HAdV) infection is a life threatening complication. The overall HAdV-associated mortality ranges from 18 to 26% [1] and mortality rates of 14 to 100% in infected patients despite virostatic treatment are described [2]. Additionally, treatment with antiviral drugs is associated with substantial nephron- and myelotoxicity [3].

Immunotherapy with either magnetically separated [4] or expanded [5] HAdV-specific T cells represents a promising treatment option to overcome viral infections after allogeneic HSCT. More recent approaches are based on the short-term expansion of HAdV-specific T cells with overlapping 15-mer polypeptides from highly conserved regions of the immunodominant major capsid protein hexon [6,7], to facilitate broad recognition and protection against several HAdV species [8].

However, as a prerequisite for such immunotherapies, the T-cell donor has to have virus-specific T cells. Recent data from our laboratory showed that in 12 out of 50 donors, no HAdV-specific T cells were detectable via MHC class I multimers and/or IFNγ ELIspot (unpublished data). Although the serotype was not analysed, this is in accordance with the generally high prevalence (<80%) of the common species C HAdV infection in the human population [9], with some geographic variations between 40% of adults in America [10], 93% of children in Sub-Saharan Africa [11], and about 77% in southern China [12]. Due to the incomplete match of donor and recipient, the

use of donor T cells is further restricted because they only react in the presence of matching HLA molecules.

One alternative would be the transfer of T-cell receptors (TCR) with defined antigen specificities to peripheral blood T cells [13]. TCR specific for tumor antigens were already effectively transferred in several animal models [14–16] and at least in one clinical phase I/II study [17]. To treat CMV-infections, the use of TCR-redirected CMV-specific T cells was recently discussed [18]. Although several CMV-specific TCR are already known, no HAdV-specific TCR have been identified until now.

In contrast to retroviral transduction, mRNA electroporation avoids potential severe side effects by inducing only transient expression of the exogenous TCR, lasting several days [19]. However, this implies multiple infusions of high cell numbers. Recently, it was shown that despite transient functionality, the TCR electroporated T cells were able to efficiently prevent tumor seeding and suppress tumor growth in a xenograft model of hepatocellular carcinoma [20]. Because the period during which an HSCT recipient suffers complete immunosuppression is temporary, we consider this setting well suitable for the use of mRNA-transfected T cells.

The infusion of donor-derived TCR-redirected α/β T cells would, therefore, be a possible treatment strategy for HLA-matched patients suffering of severe HAdV complications [21]. Nevertheless, the number of donor-derived α/β T cells that can be infused into HLA-mismatched patients post HSCT is limited, as these cells exhibit allo-reactivity via their endogenous TCR.

This could be overcome by using γ/δ T cells, which do not recognize MHC molecules and are hence not allo-reactive [22]. It was shown that γ/δ T cells – retrovirally transfected with α/β TCR against e.g. CMV or a tumor antigen- were highly functional in vitro [23] and in mice [24,25].

In this study we expanded HAdV-specific T cells by stimulation with the HLA-A*0101-restricted, immunodominant, and cross-reactive epitope LTDLGQNLLY (LTD) from the hexon protein of HAdV-species C, the predominant species in patients after HSCT [1]. We identified, for the first time, HAdV-specific TCR α/β chain sequences, which were then cloned and transfected via mRNA electroporation into CD8+ α/β T cells and γ/δ T cells and tested for their capability to induce cytokine secretion and lysis of peptide-loaded, or adenovirus-infected target cells. Therefore, we report here on a new therapeutic possibility for the treatment of HAdV infection after allogeneic stem cell transplantation with HAdV-TCR-transfected CD8+ α/β T cells and γ/δ T cells.

Materials and Methods

Cells and reagents

All human material was obtained following written informed consent and approved by the institutional review board in Erlangen (Ethik-Kommission der Medizinischen Fakultät der Friedrich-Alexander-Universtität Erlangen-Nürnberg, #3928) and the Ethic Committee in Vienna (Ethik-Kommission der Medizinischen Universität Wien, EK Nr. 514/2011), and all investigations were conducted according to the principles expressed in the Declaration of Helsinki. PBMC of healthy volunteers were prepared by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). CD8+ α/β T cells were isolated from PBMC using anti-CD8 MACS beads according to the manufacturer's instructions (Miltenyi Biotec), and were cultured in MLPC medium consisting of RPMI 1640 (Lonza), 10% human serum (Lonza), 2 mM L-glutamine (Lonza), 20 mg/L gentamicin (Sigma-Aldrich), 10 mM HEPES (PAA, GE healthcare), 1 mM sodium pyruvate (Sigma-Aldrich),

and 1% MEM nonessential amino acids (100x; PAA, GE healthcare), supplemented with 1000 IU/ml IL-2 (Proleukin; Novartis) and 10 ng/ml IL-7 (Peprotech). In some experiments expanded CD8+ α/β T cells and γ/δ T cells were used basically as described previously [26]. In short, PBMC were cultured at 1×10^6 cells/ml in R10 medium consisting of RPMI 1640 containing final concentrations of 10% (v/v) heat-inactivated fetal bovine serum (PAA, GE healthcare), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza), 2 mM HEPES, and 20 µM β-mercaptoethanol (Gibco, Life Technologies). A final concentration of 0.1 µg/ml anti-CD3 antibody (Orthoclone OKT-3; Jannsen-Cilag) and 10^3 IU/ml IL-2 were added on day 0. Fresh IL-2 (10^3 IU/ml) was added on day 2. On day 3, the cells were counted and diluted to 0.2×10^6 cells/ml in R10 medium and 10^3 IU/ml IL-2 were added. The same amount of IL-2 was added on day 5 of culture. The total culture volume was doubled by adding fresh medium on day 7, and IL-2 (10^3 IU/ml) was added as well. Cells were harvested after nine days of expansion and subsequently CD8+ α/β T cells and γ/δ T cells were isolated from PBMC using anti-CD8 MACS beads and the TCRγ/δ+ T Cell Isolation Kit, respectively, according to the manufacturer's instructions (Miltenyi Biotec). The cells were cultured in R10 medium overnight. Mature DC were generated as described [27]. The melanoma cell line colo829 (acquired from ATCC) (HLA-A1+) was cultured in R10 medium. The EBV-transformed cell line CCL (HLA-A1+; published before by Felzmann et al. [28]) was cultured in R20, which is R10 medium containing 20% (v/v) heat-inactivated fetal bovine serum instead of 10%. HLA-A1-binding peptides used in this study were HAdV: LTDLGQNLLY, MAGE-A3: EVDPIGHLY, and MAGE-A1: EADPTGHSY (Eurogentec).

Isolation of ADV-specific oligoclonal T cells. In total, 160×10^6 PBMC (10^7/ml) from an HLA-type A*0101-positive donor were cultured in AIM-V (Invitrogen, Carlsbad, CA) supplemented with 2% Octaplas (Octapharma, Vienna, Austria), 2 mM L-Glutamine, and 25 mM HEPES, and were stimulated for 6 days with HAdV (subgroup C-derived Hexon AdV5)-specific peptide pools (Miltenyi Biotec) at a final concentration of 0.6 nmol for each peptide per ml. On day 6, cultured cells were added to adherent monocytes (as described in [6]) and re-stimulated with the peptide pool and IL-2 (R&D Systems) at 5 ng/ml. On day 12, 10^8 cells were washed, stained with 500 µl PE-labeled A*0101-ADV-specific pentamers (Proimmune), incubated with anti-PE MicroBeads (Miltenyi Biotec), and isolated according to the manufacturer's instructions. As a next step, highly pure PE-pentamer+ T cells were sorted by flow cytometry and resuspended in TRIzol (Invitrogen).

Cloning of TCR genes and in vitro transcription of mRNA

The subtypes of TCR α and β chain of the HLA-A1/adenovirus-specific CTL clone, which was most prominent in the isolated oligoclonal T cells were identified as described previously [29]. The TCR α chain was of the AV30S1AT [30]/AV20 (IMGT) subtype, and the TCR β chain was of the BV4SA1T [30]/BV29-1 (IMGT) subtype. The full length TCR chains were cloned into a pGEM4Z-5′UTR-sig-huSurvivin-DC.LAMP-3′UTR vector, replacing the huSurvivin-DC.LAMP. As controls, an HLA-A1/MAGE-A3-specific TCR and an HLA-A1/MAGE-A1-specific TCR were used. In vitro transcriptions of TCR RNA were performed using mMESSAGE mMACHINE T7 ULTRA kits (Life technologies) according to the manufacturer's instructions.

Jurkat T cell/luciferase assay. The Jurkat T cell (acquired from ATCC)/luciferase assay was performed as described previously [29]. As target cells DC, which were loaded for 1 h at 37°C with the indicated peptides (all at 10 µg/ml), or were left unloaded, were used. To determine antigen-specific luciferase production, the luciferase activity was set in relation to the luciferase activity measured with the non-loaded target cells as stimulators.

RNA electroporation of T lymphocytes

CD8⁺ α/β T cells and γ/δ T cells were electroporated with the following settings: square-wave pulse, 500 V, 3 ms or 5 ms, as described previously [31].

Adenoviruses

The replication-deficient Ad5Luc1 virus was amplified in 293 T cells (acquired from ATCC) as described before [32]. Non-replication restricted Ad5wt was amplified in colo829 cells [33].

Cell surface marker and TCR staining. Thawed T cells transfected with either the control TCR specific for MAGE1/A1 or the HAdV/A1 TCR were washed in FACS-buffer and 0.25×10^6 cells per condition were stained using the following antibodies: IgG1-FITC (BD Biosciences), IgG2a-FITC (BD Biosciences), anti-γ/δ pan TCR-FITC (Pierce Antibody products, Thermo Scientific), anti-CD4-FITC (BD Biosciences), anti-CD8-FITC (BD Biosciences), anti-CD14-FITC (BD Biosciences), anti-CD16-FITC (BD Biosciences), anti-CD19-FITC (BD Biosciences) in 50 µl of FACS-buffer for 30 min at 4°C. Cells were then washed once and analyzed on a FACScan (BD Biosciences). For staining of HAdV-TCR-transfected T lymphocytes via MHC I streptamers, in total 0.25×10^6 T cells were washed and then resuspended in 50 µl buffer. Cells were then incubated with streptamers comprising the HLA-A1 (LTDLGQNLLY) MHC class I (1 µl) and Strep-Tactin-PE (1.25 µl) (IBA GmBH, Göttingen, Germany) for 30 min at 4°C according to manufacturer's instructions. After washing, cells were resuspended in buffer and analyzed by flow-cytometry.

Induction and determination of cytokine production by TCR-transfected T lymphocytes. T cells electroporated with TCR-encoding mRNA were co-cultivated with the melanoma cell line colo829, EBV-transformed B cells (CCL), or DC (all HLA-A1⁺) that were either non-loaded or loaded with a control peptide or the peptide recognized by the ADV-specific TCR (LTDLGQNLLY) (all at 10 µg/ml) for 1 h at 37°C. Alternatively, HAdV-infected target cells were used. Therefore, 4×10^6 DC were seeded in 6-well plates (Falcon) followed by transduction with adenovirus at 5000–10000 viral particles/ml (vp/ml) in a final volume of 1 ml R10 medium containing additionally 550 U/ml GM-CSF and 800 U/ml IL-4 (both Miltenyi Biotec). After 1.5 hours of incubation at room temperature on a rocker, 4 ml of growth medium replenished with cytokines as described before was added per well. Cells were incubated for 48 h at 37°C, 5% CO_2, before they were used for further experiments. Transduction efficacy (GFP expression) and percentage of living cells was determined by flow cytometric analysis with a FACScan cell analyzer (BD Biosciences). Cytokine production was determined as described previously [34]. Alternatively, intracellular cytokine staining was performed as described before [35]. In short, a total of 1×10^6 T cells were stimulated with 1×10^6 non-peptide-loaded or HAdV-peptide-loaded CCL cells in 500 µl MLPC with 2.5 µg BrefeldinA (Sigma-Aldrich) and 373 ng monensin (Sigma-Aldrich) for approximately 12 h. Cells were stained with Live/Dead aqua-mix (Invitrogen) and were extracellularly stained

with αCD8-PerCP- (BD Biosciences), αCD4-V450- (BD Biosciences), and αCD14-Pacific Orange antibodies (Invitrogen). Subsequently, cells were fixed and permeabilized with reagents from eBioscience, and intracellularly stained with αCD8-PerCP- (BD Biosciences), αIL-2-APC- (BD Biosciences), αTNF-PE-Cy7- (BD Biosciences), and IFNγ-Alexa Fluor 700 (BD Biosciences) antibodies. Cells were measured with the FACS Canto II (BD Biosciences) and analyzed with FCS Express 4 Software (De Novo Software).

Cytotoxicity assay

Cytotoxicity was tested in standard 4–6 h ^{51}Cr release assays as described previously [31]. Peptide-loaded and HAdV-infected target cells were used. Percentage cytolysis, i.e., ^{51}Cr release, was calculated as follows: [(measured release – background release)]/[(maximum release with 1% triton – background release)] ×100%.

Statistics

Statistical analysis was performed using the Graph Pad Prism software. P-values were calculated by the Mann-Whitney U test.

Results

The newly cloned HAdV/HLA-A1-specific TCR can be functionally transferred to T cells

To investigate the functional transfer of a HAdV/HLA-A1 (HAdV/A1)-specific TCR, we first cloned such a TCR from a CD8⁺ CTL clone, and tested it in a Jurkat T cell/luciferase assay [29]. Jurkat cells were co-electroporated with RNA encoding the TCR and the DNA Transluc vector that encodes luciferase under the control of an NFAT-inducible promoter. As a control mRNA encoding a MAGE-A3/HLA-A1 (M3/A1)-specific TCR was used in parallel. These cells were co-cultured with HLA-A1-positive dendritic cells (DC) loaded with either the adenovirus peptide or the MAGE-A3 peptide. As a negative control, DC were left unloaded. Then luciferase activity induced by an antigen-specific TCR signal was determined (Fig. 1). To examine the TCR's dependence on the co-receptor CD8, a CD8-transgenic Jurkat derivative was used in addition. In these CD8⁺ Jurkat T cells, the transferred HAdV/A1-specific TCR did not recognize the non-loaded DC or MAGE-A3-peptide-loaded DC, while it recognized the adenovirus peptide specifically (Fig. 1). CD8⁺ Jurkat cells transfected with the M3/A1-specific control TCR only recognized the MAGE-A3-peptide-loaded DC (Fig. 1). Even in the CD8-negative parental Jurkat T cells the HAdV/A1-specific TCR recognized adenovirus-peptide-loaded DC specifically, while the M3/A1-specific TCR did not (Fig. 1).

Taken together, these data indicate that the cloned TCR is functional and HAdV/A1-specific, and binds its target in a partially CD8 independent manner.

HAdV/A1-TCR-transfected CD8⁺ T cells recognize target cells antigen-specifically

To study the functionality of the cloned HAdV/A1-specific TCR in primary T cells, we transferred the TCR to CD8⁺ T cells isolated from healthy donor blood by mRNA electroporation. The TCR-transfected CD8⁺ T cells were incubated with peptide-loaded HLA-A1⁺ DC overnight, and cytokine production was determined. Only HAdV/A1-TCR-transfected CD8⁺ T cells recognized the adenovirus-peptide-loaded DC and responded with IL-2, TNF, and IFNγ production (Fig. 2A). Non-loaded DC were not recognized by these T cells, and mock-electroporated
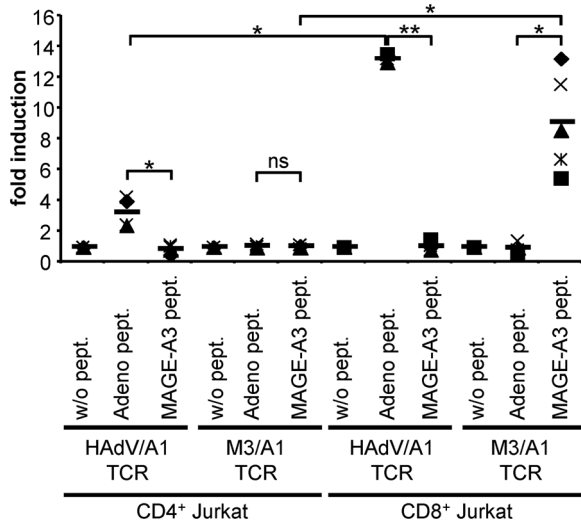
Figure 1. The newly cloned HAdV/HLA-A1-specific TCR is functional in Jurkat T cells. Parental CD4^{+} and transgenic CD8^{+} Jurkat T cells were co-electroporated with RNA encoding the HAdV/A1-specific TCR or MAGE-A3/A1 (M3/A1)-specific TCR and an NFAT-inducible luciferase reporter plasmid. These Jurkat T cells were stimulated with DC either non-loaded (w/o pept.) or loaded with the adenovirus peptide (Adeno pept.) or the MAGE-A3 peptide (MAGE-A3 pept.) (as indicated). The luciferase activity was measured, and the specific activation of the Jurkat T cells was calculated as fold induction by dividing the luciferase activity induced by peptide-loaded DC by that of similarly electroporated Jurkat T cells stimulated with non-loaded DC. Data of 4 (CD4^{+} Jurkat T cells) and 5 (CD8^{+} Jurkat T cells) individual experiments are shown. Bars indicate mean values. P-values were calculated by the Mann-Whitney U test. ns = not significant; ** p≤0.01; * p≤0.05. Raw data are summarized in Table S1 in File S1.
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CD8^{+} T cells did not produce cytokines after incubation with non-loaded or adenovirus-peptide-loaded DC (Fig. 2A).

Furthermore, the response of HAdV/A1-specific TCR-transfected CD8^{+} T cells to adenovirus-infected DC was investigated (Fig. 2B). Again, only the HAdV/A1-TCR-transfected CD8^{+} T cells recognized the adenovirus-infected DC and produced substantial amounts of IL-2, TNF, and IFNγ (Fig. 2B). Non-infected DC were not recognized, and mock-electroporated CD8^{+} T cells did not produce any cytokines after incubation with non-infected DC. Marginal, but significant amounts of IL-2 and IFNγ were produced by mock-electroporated T cells in response to adenovirus-infected DC, probably due to a pre-existing adenoviral activity of the donor blood (Fig. 2B).

Taken together, CD8^{+} T cells transfected with the HAdV/A1-specific TCR by mRNA electroporation antigen-specifically secreted pro-inflammatory cytokines.

HAdV/A1-TCR and CD8α co-transfected γ/δ T cells produce cytokines antigen-specifically

Since the endogenous TCR of the donor T lymphocytes would induce GvHD in a mismatched patient, we investigated whether the HAdV/A1-specific TCR can be functionally transferred into not allo-reactive γ/δ T cells by mRNA electroporation. γ/δ T cells were isolated from expanded PBMC of healthy donors to obtain sufficient numbers for electroporation. TCR-transfected cells were incubated with peptide-loaded DC (Fig. 3A) or colo829, a melanoma cell line (Fig. 3B). The TCR-transfected γ/δ T cells produced minor amounts of IFNγ after stimulation with adenovirus-peptide-
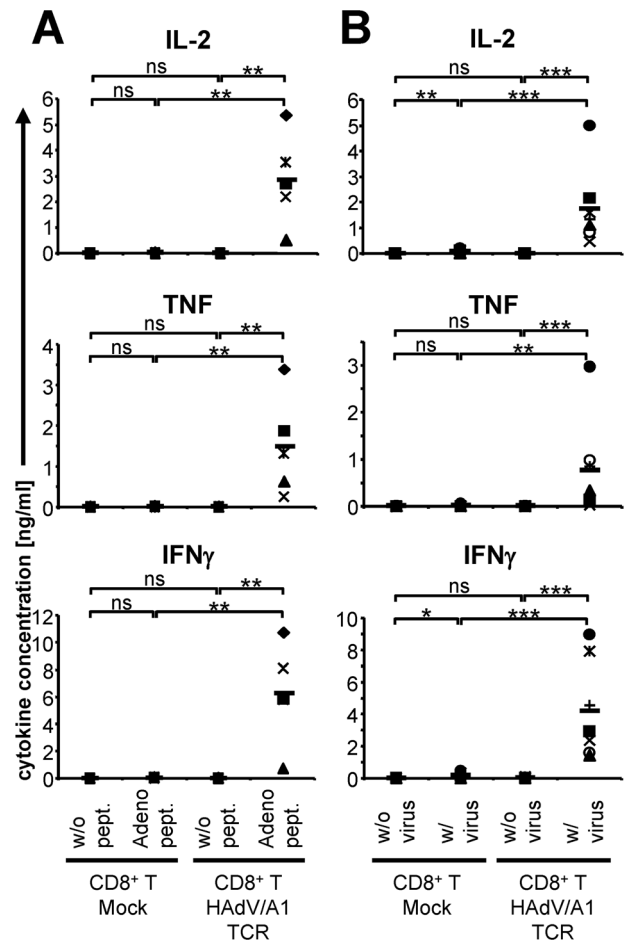

Figure 2. Antigen-specific cytokine production by HAdV/A1-TCR-transfected CD8^{+} T cells in response to peptide-loaded and adenovirus-infected targets. CD8^{+} T cells were either mock electroporated or electroporated with HAdV/A1-TCR-RNA and were stimulated with DC, which were either left unloaded (**A**: w/o pept., **B**: w/o virus), or were loaded with the adenovirus peptide (Adeno pept.) (**A**), or were infected with adenovirus (w/virus) (**B**). Cytokine concentrations (IL-2, TNF, and IFNγ) in the supernatant after over-night co-incubation are depicted. Data of 5 (**A**) and 7 (**B**) individual experiments are shown. Bars indicate mean values. P-values were calculated by the Mann-Whitney U test. ns = not significant, *** p≤0.001; ** p≤0.01; * p≤0.05. Raw data are summarized in Table S2A and B in File S1.
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loaded DC (Fig. 3A). The response to adenovirus-peptide-loaded colo839 cells by TCR-transfected γ/δ T cells was higher; i.e. an antigen-specific secretion of IL-2, TNF, and IFNγ was clearly detected (Fig. 3B). As the HAdV/A1-TCR was cloned from a CD8^{+} T-cell clone, we reasoned that the TCR might require CD8 co-binding for optimal functionality. Hence, we co-transfected γ/δ T cells with the HAdV/A1-TCR and CD8α by mRNA electroporation and tested these cells on the same target cells as described above. This resulted in a clear increase in the antigen-specific cytokine secretion in response to adenovirus-peptide-loaded DC (Fig. 3A) and colo829 cells (Fig. 3B). TCR-transfected γ/δ T cells did not recognize non-loaded target cells, and mock-electroporated γ/δ T cells did not produce any cytokine (Fig. 3).

In summary, we were able to functionally transfer the HAdV/A1-TCR to γ/δ T cells by mRNA electroporation leading to an antigen-specific cytokine secretion. Co-transfection of TCR and
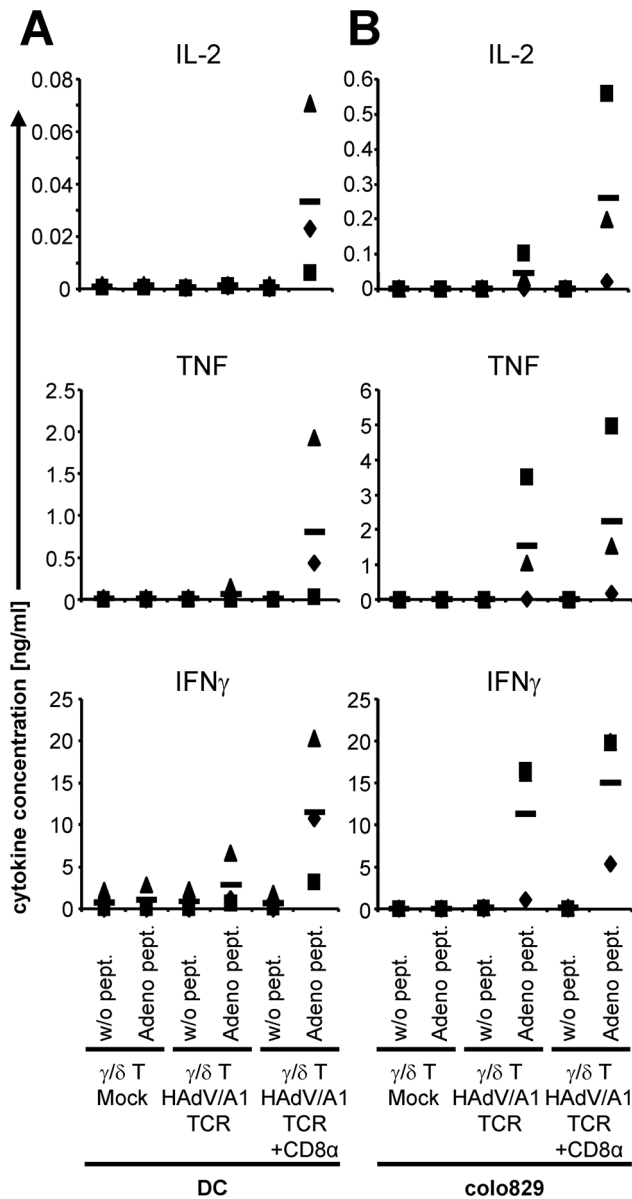
A

B

Figure 3. Antigen-specific cytokine production by HAdV/A1-TCR-transfected γ/δ T cells in response to peptide-loaded target cells. γ/δ T cells were either mock electroporated, electroporated with HAdV/A1-TCR-RNA alone, or electroporated with HAdV/A1-TCR-RNA and CD8α-RNA. These cells were stimulated with DC (**A**) or colo829 cells (**B**), which were either left unloaded (w/o pept.), or were loaded with the adenovirus peptide (Adeno pept.). Cytokine concentrations (IL-2, TNF, and IFNγ) in the supernatant after overnight co-incubation are depicted. Data of 3 individual experiments are shown. Bars indicate mean values. Raw data are summarized in Table S3A and B in in File S1.
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CD8α into γ/δ T cells further increased the functionality of these cells.

TCR-transfected γ/δ T cells appear to have a better functionality than TCR-transfected CD8+ T cells in direct comparison

To determine the reactivity of the TCR-transfected γ/δ T cells in comparison to TCR-transfected CD8+ T cells of the same

donor, we directly compared these cells. Both T-cell populations were expanded and transfected with the HAdV/A1-TCR, and γ/δ T cells were co-transfected with CD8α. To check for purity of the isolated and expanded cells, expression of CD4, CD8, CD14, CD16, CD19, and γ/δ TCR on the cell surface were determined (Fig. 4A). Of the isolated and expanded CD8+ T cells, approximately 90% were CD8 positive, and only 4.5% were γ/δ TCR positive (Fig. 4A). Of the isolated and expanded γ/δ T cells, which were co-transfected with TCR and CD8α,>90% were γ/δ TCR positive, and approximately 50% were CD8 positive (Fig. 4A). To determine TCR-transfection efficiency, the RNA-transfected cells were stained with the HAdV/A1-streptamer (Fig. 4). In average, approximately 21% of the CD8+ T cells and 24% of the γ/δ T cells expressed the TCRα and TCRβ chain of the HAdV/A1-specific TCR efficiently enough to facilitate streptamer-binding (Fig. 4).

Then the TCR-transfected cells were incubated with peptide-loaded colo829 (Fig. 5A) or CCL cells (Fig. 5B and 6). Mock-electroporated CD8+ T cells did not produce any cytokines, or only at background levels (Fig. 5). The γ/δ T cells produced cytokines in response to adenovirus-peptide-loaded target cells, but not to control-peptide-loaded target cells (Fig. 5). The TCR-transfected CD8+ T cells produced lower amounts of IFNγ compared to TCR-transfected γ/δ T cells on colo829 and lower amounts of TNF on both targets (Fig. 5). Intracellular cytokine staining showed that only CD8+ T cells and γ/δ T cells transfected with the HAdV/A1-specific TCR recognized target cells loaded with the HAdV peptide and produced cytokines (Fig. 6). In average 32% of the transfected CD8+ T cells and 31% of the transfected γ/δ T cells specifically produced IFNγ, indicating that a higher percentage of the T cells was functionally transfected with the TCR, than one could have concluded from the streptamer-staining (Fig. 4).

Taken together, the co-transfection of CD8α and the HAdV/A1-specific TCR into γ/δ T cells led to an efficient antigen-specific cytokine production, and suggested that these cells might even have an increased functionality compared to HAdV/A1-TCR-transfected CD8+ T cells.

Both HAdV/A1-TCR-transfected CD8+ T cells and γ/δ T cells lyse adenovirus-infected cells efficiently

In our intended clinical setting, the elimination of virus-infected target cells will be one of the most important functions of the TCR-transfected T cells. Hence, the lytic capacity of HAdV/A1-TCR-transfected CD8+ T cells and HAdV/A1-TCR/CD8α co-transfected γ/δ T cells against adenovirus-infected DC was investigated. CD8+ T cells or γ/δ T cells transfected with a control TCR specific for MAGE-1 presented by HLA-A1 (M1/A1) induced no lysis of untreated DC and weak lysis of adenovirus-peptide-loaded and adenovirus-infected DC (Fig. 7A and B). HAdV/A1-TCR-transfected CD8+ T cells and HAdV/A1-TCR/CD8α co-transfected γ/δ T cells lysed adenovirus-peptide-loaded target cells very efficiently, while untreated target cells were not lysed (Fig. 7A and B). Most importantly, HAdV/A1-TCR-transfected CD8+ T cells and HAdV/A1-TCR/CD8α co-transfected γ/δ T cells were both able to lyse adenovirus-infected target cells antigen-specifically, although to a lesser extent than peptide-loaded target cells (Fig. 7A and B).

To address the point of allo-reactivity, we investigated the lytic capacity of allogeneic TCR-transfected CD8+ T cells and TCR/CD8-co-transfected γ/δ T cells on peptide-loaded and adenovirus-infected DC. As shown in figure 7C and D, the antigen-specific lysis was similar to that of the autologous system, and only a small increase in the background lysis of untreated DC by allogeneic
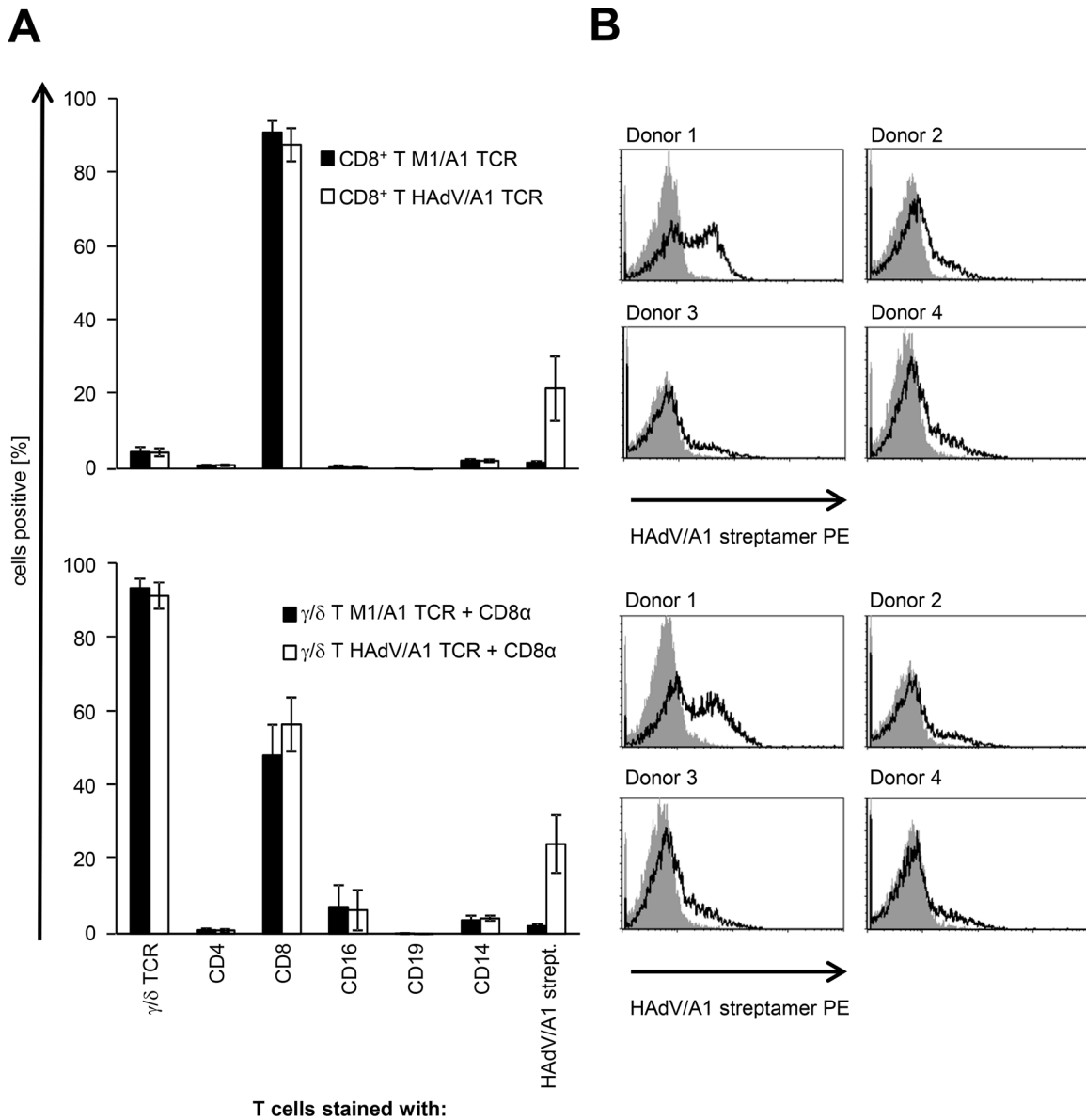
Figure 4. Surface marker and TCR expression on HAdV/A1-TCR-transfected CD8$^+$ and γ/δ T cells. CD8$^+$ T cells (A and B; upper panels) and γ/δ T cells (A and B; lower panels) were either electroporated with M1/A1-TCR RNA (+CD8α RNA in case of γ/δ T cells) or with HAdV/A1-TCR-RNA (+ CD8α RNA in case of γ/δ T cells) and were cryoconserved 4 h after electroporation. Surface marker and TCR expression on thawed T cells was determined by staining with anti-CD4, anti-CD8, anti-CD16, anti-CD19, anti-CD14, and anti-pan γ/δ TCR antibodies, and the expression of the HAdV/A1-specific TCR was determined by staining with HAdV/A1 streptamer. Expression levels were analyzed by flow-cytometry and shown as % positive cells; isotype control stainings were subtracted (**A**; average values of 4 experiments ± SEM) or as histograms (**B**; grey histogram: T cells electroporated with M1/A1-TCR RNA, black line: T cells electroporated with HAdV/A1-TCR-RNA). Raw data are summarized in Table S4A and B in File S1.
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CD8$^+$ T cells compared to autologous CD8$^+$ T cells was observed (compare to Fig. 7A and B). When comparing the background lysis by TCR-transfected CD8$^+$ T cells and TCR/CD8-co-transfected γ/δ T cells in the allogeneic system, a slightly increased lysis induced by the former was seen.

Taken together, here we show, for the first time, that not only α/β T cells but also γ/δ T cells can be equipped with a HAdV specificity by TCR-RNA electroporation and that these cells are able to lyse adenovirus-infected target cells highly significantly.

Discussion

In the current study we developed a novel therapeutic option for patients suffering from HAdV infection after allogeneic HSCT. It is based on the generation of HAdV-specific T cells from HAdV-seronegative donors by TCR-RNA electroporation, and it might present a promising alternative for other recently described procedures, like the cost-intensive and laborious long-term (10–14 weeks) *in vitro* expansion of HAdV-specific T cells from partially HLA-matched third-party donors, which are effective without the induction of severe GvHD as shown in a few clinical trials [36–38]. Another method reported the *in vitro* generation of
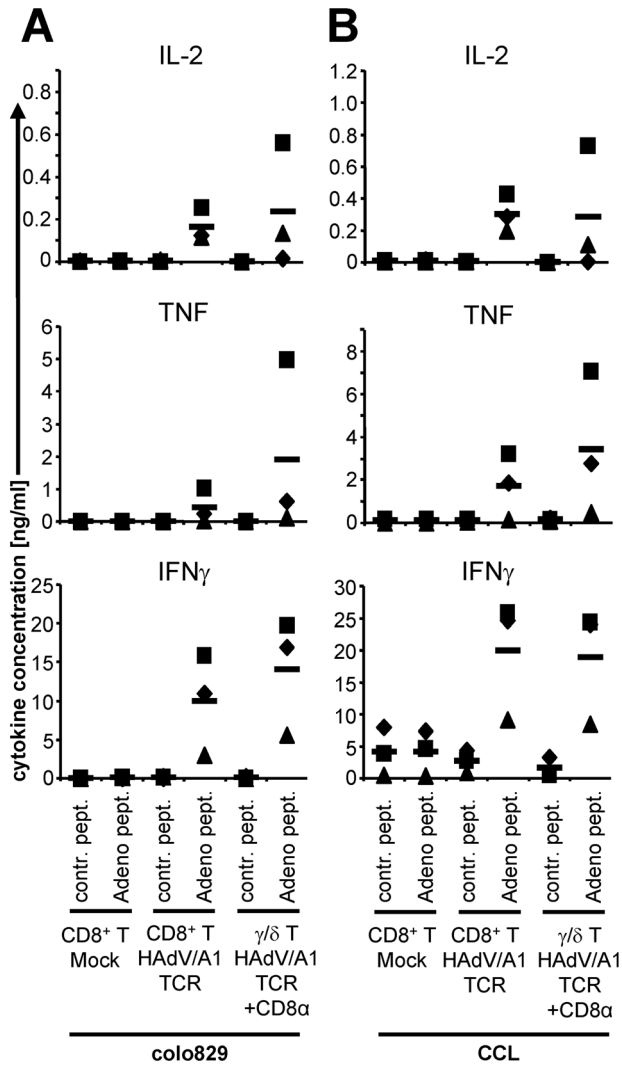
Figure 5. Direct comparison of cytokine production by HAdV/A1-TCR-transfected CD8⁺ T cells and HAdV/A1-TCR-transfected γ/δ T cells. CD8⁺ T cells and γ/δ T cells of the same donors were either mock electroporated, electroporated with HAdV/A1-TCR-RNA alone, or electroporated with HAdV/A1-TCR-RNA and CD8α-RNA. These cells were stimulated with colo829 cells (**A**) or CCL cells (**B**), which were either loaded with a control peptide (contr. pept.) or the adenovirus peptide (Adeno pept.). Cytokine concentrations (IL-2, TNF, and IFNγ) in the supernatant after overnight co-incubation are depicted. Data of 3 individual experiments are shown. Bars indicate mean values. Raw data are summarized in Table S5A and B in File S1.
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Figure 6. Antigen-specific cytokine production by HAdV/A1-TCR-transfected CD8⁺ and γ/δ T cells in response to peptide-loaded target cells. CD8⁺ T cells and γ/δ T cells were either electroporated with M1/A1-TCR RNA (+CD8α RNA in case of γ/δ T cells) or with HAdV/A1-TCR-RNA (+CD8α RNA in case of γ/δ T cells) and were cryoconserved 4 h after electroporation. After thawing, these cells were stimulated with CCL cells, which were either left unloaded (w/o pept.), or were loaded with the adenovirus peptide (w/pept.). Intracellular cytokine stainings for IL-2 (white bars), TNF (grey bars), and IFNγ (black bars) were performed and analyzed by flow-cytometry. The percentages of cytokine containing cells are depicted. Average values of 4 (CD8⁺ T cells) and 3 (γ/δ T cells) individual experiments ± SEM are shown. Raw data are summarized in Table S6 in File S1.
doi:10.1371/journal.pone.0109944.g006

HAdV-specific T cells from naïve T-cell populations in cord blood [39], which is even more costly and time-consuming. This holds also true for the recently described application of third-party "off the shelf" long-term expanded HAdV-specific T-cell lines, which showed impressive clinical results [40].

Quite recently, the use of TCR-transduced CMV-specific T cells has also been discussed to be a viable therapeutic option for patients after HSCT or solid organ transplantation (SOT) in case of sero-negative donors [18]. Although several TCR specific for CMV [23,41–43] and EBV [44] are known, none were known for HAdV. We were the first to clone a HAdV-specific TCR sequence recognizing the immunodominant HLA-type A*0101-restricted viral 10-mer epitope LTDLGQNLLY (LTD) from the hexon
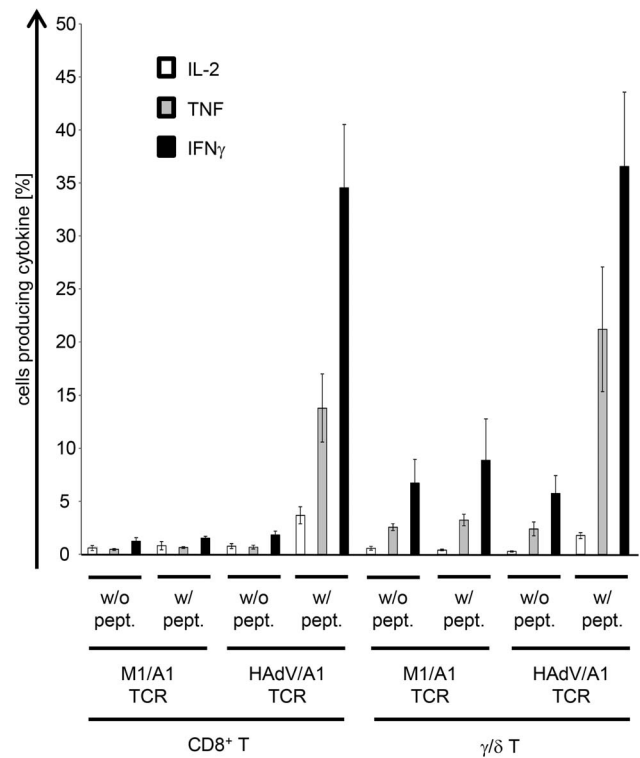
protein of HAdV-species C, the predominant species in patients after HSCT [1]. These LTD-derived HAdV-specific T cells are also cross-reactive against several other HAdV species [6], which would enable a broad coverage of different species. According to data provided by www.allelefrequencies.net the HLA-type A*0101 is highly frequent and covers about 30% of the White population. Therefore, approximately 30% of all pediatric patients could be potential candidates for this kind of treatment. The coverage could be even increased to 72% if three additional TCRs against the already known and immunodominant peptide-MHC class I complexes for the HLA-types A*2401, B*0702, and B*3501 [6] would be identified and used for transfer to T cells.

The feasibility of mRNA electroporation to transfer TCR to bulk T cells has already been described for tumor-specific [31,45], HIV-1-specific [34], and CMV-specific [43] TCR. Although receptor expression via mRNA electroporation is transient, effector functions such as cytokine production and lytic activity can still be measured for at least 3 days post electroporation [19,20,31,46]. Complete loss of receptor expression and redirected specificity was seen 9 days after electroporation [19]. Because the period of complete immunosuppression after HSCT is limited, and only a temporary exogenous defense against the virus is required, we consider the mRNA-electroporation technology well
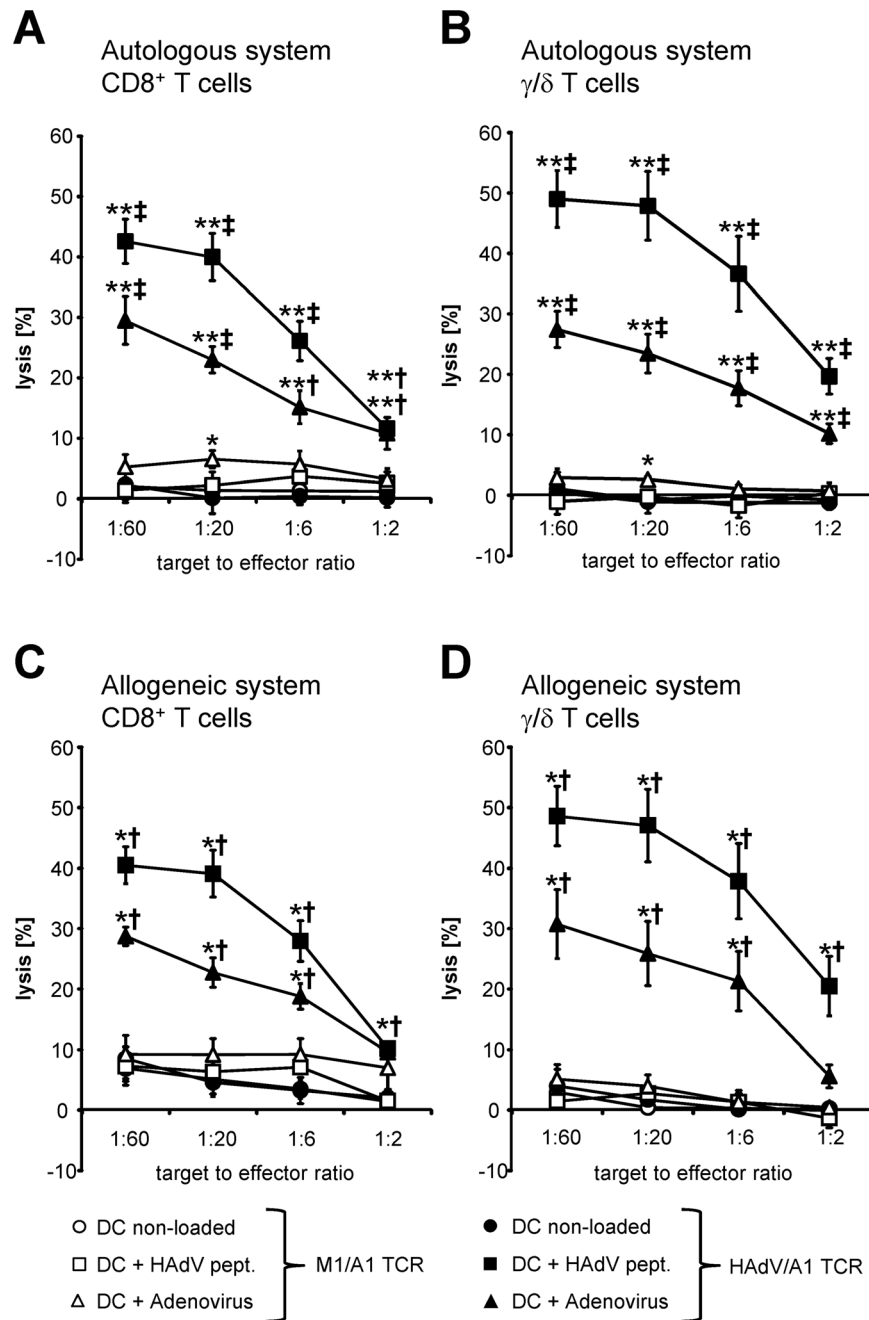
Figure 7. Cytolytic capacity of HAdV/A1-TCR-transfected CD8$^+$ T cells and γ/δ T cells against adenovirus-infected target cells. CD8$^+$ T cells (**A** and **C**) and γ/δ T cells (**B** and **D**) of the same donors were either electroporated with mRNA encoding the MAGE-1/A1-specific TCR (open symbols) or electroporated with mRNA encoding the HAdV/A1-specific TCR (closed symbols) either in combination with CD8α mRNA (γ/δ T cells) or not (CD8$^+$ T cells). These cells were used as effector cells in standard 4–6 h cytotoxicity assays. Autologous (**A** and **B**) and allogenic (**C** and **D**) DC either untreated (non-loaded), loaded with the adenovirus peptide (Adeno pept.), or infected with adenovirus (adenovirus) were used as target cells and the percentage of lysed cells was calculated. The target to effector cell ratios were 1:60, 1:20, 1:6, and 1:2. Average values of 6 (**A** and **B**) or 4 (**C** and **D**) individual experiments (each performed in triplicates) +/− SEM are shown. P-values were calculated by the Mann-Whitney U test. For statistics, conditions were compared to the non-loaded DC condition (* and **) and to T cells transfected with the M1/A1 control TCR (‡ and †). (** or ‡ p≤0.01; *or † p≤0.05). Raw data are summarized in Table S7A to D in File S1.
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suited to be applied here. All together, these results support the assumption that there is sufficient time for TCR-RNA-electroporated T cells to fulfill their task before losing their specificity, provided that multiple injections over the period of complete immune suppression are performed.

Although the time-limited TCR expression is a disadvantage compared to retroviral transduction, mRNA electroporation is a much safer method [31]. In contrast to lentiviral transduction, mRNA electroporation is faster and allows for high numbers of modified T cells due to high transfection rates (>80%) [20]. However, infusion of high doses of mRNA-electroporated T cells,

which still retain their allo-reactive potential, highly increases the risk for GvHD in patients after allogeneic HSCT and is only suitable for HLA-matched related donors. Therefore, we used γ/δ T cells, which are known to be involved in viral defense after HSCT without the potential to induce GvHD in patients [22], instead of α/β T cells. Furthermore, preliminary results of adoptive immunotherapy using γ/δ T cells showed promising results in clinical trials [47,48]. Moreover, γ/δ T cells were isolated and expanded to sufficient numbers for clinical application by several groups [49–52]. An additional risk that is overcome by the use of γ/δ T cells, is the problem of TCR-chain mispairing, as it has been shown that introduced TCR α- and β-chains can mispair with their endogenous counterparts [41]. This could result, in theory, in the formation of new, auto-reactive receptors. Although the resulting danger of autoimmunity is already reduced even in α/β T cells by the transiency of the mRNA transfection, it is further reduced by the use of γ/δ T cells because TCR α/β-chains preferentially pair with each other instead of forming heterodimers with γ/δ-chains [53,54].

Although our isolated γ/δ T cells lacked the co-stimulatory molecule CD8α, HAdV/A1-specific TCR-redirected γ/δ T cells were able to produce cytokines after stimulation with adenovirus-peptide-loaded target cells. Nevertheless, the cytokine production was highly increased after co-transfection with the co-stimulatory molecule CD8α. This is in accordance with the data shown in Figure 1, where the signal strength benefited from the presence of CD8. These data provide evidence that the functionality of the HAdV/A1-specific TCR is still obvious without CD8α but even higher when combined with CD8α transfection. Similar results have been obtained by Van der Veken et al. [24]. Notwithstanding, the combined transfection of the HAdV/A1-specific TCR and CD8α can be easily implemented into a clinical-based protocol.

Strikingly, both HAdV/A1-TCR-transfected primary CD8^{+} α/β T cells and HAdV-TCR/CD8α co-transfected γ/δ T cells lysed adenovirus-infected target cells (Fig. 7). Moreover, co-transfection of γ/δ T cells with the HAdV/A1-TCR and CD8α seems to be a feasible method to generate not allo-reactive T cells with strong antiviral activity. In fact, we expected to see an allo-reactivity of CD8^{+} T cells (independent of the TCR introduced) against allogeneic DC. The observed weakness of this allo-reactivity could have been caused by the limited duration of the assay. Elongating the incubation time in the allogeneic system could show a clearer difference in allo-reactive capacity between γ/δ T cells and CD8^{+} α/β T cells.

Taken together, this is the first report identifying the coding sequence of a HAdV-specific TCR, which we then functionally transferred to α/β T cells and γ/δ T cells by RNA electroporation. Because of the high frequency of HLA-type A*0101 in the White population, about 30% of pediatric patients might benefit from treatment with TCR-transfected γ/δ T cells. Although the use of third-party HAdV-specific T-cell lines is a promising option to treat patients whose original HSCT-donors have no detectable HAdV-specific T-cells, the use of TCR-transfected γ/δ T cells could be a promising alternative. It should further reduce the risk for GvHD, the costs, and laborious expansion protocols (2 weeks instead of 10–14 weeks).

We strongly believe that especially the use of TCR/CD8α-co-transfected γ/δ T cells offers a new means for the immunotherapy of HAdV-infected patients after allogeneic HSCT.

Supporting Information

File S1 **Combined file of supporting tables.** Table S1: raw data to Fig. 1. Table S2A: raw data to Fig. 2A. Table S2B: raw data to Fig. 2B. Table S3A: raw data to Fig. 3A. Table S3B: raw data to Fig. 3B. Table S4: raw data to Fig. 4. Table S5A: raw data to Fig. 5A. Table S5B: raw data to Fig. 5B. Table S6: raw data to Fig. 6. Table S7A: raw data to Fig. 7A. Table S7B: raw data to Fig. 7B. Table S7C: raw data to Fig. 7C. Table S7D: raw data to Fig. 7D.
(PDF)

Author Contributions

Conceived and designed the experiments: JD CK CH IK SS ST GF GS NS RG. Performed the experiments: JD CK CH IM VW IK EZ DP NS RG. Analyzed the data: JD CK CH IM VW IK EZ DP GF NS RG. Contributed reagents/materials/analysis tools: ST EZ DP GF RG IK SS. Wrote the paper: JD IK CK NS RG. Critically revised the manuscript: JD CK CH IM VW IK SS ST EZ DP GF GS NS RG. Approved the version to be published: JD CK CH IM VW IK SS ST EZ DP GF GS NS RG.

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