PD-L1 blockade effectively restores strong graft-versus-leukemia effects without graft-versus-host-disease after delayed adoptive transfer of T cell receptor gene-engineered allogeneic CD8+ T cells

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Running title: T cell receptor transfer in allogeneic HCT
ABSTRACT

Adoptive transfer (AT) of T cells forced to express tumor-reactive T cell receptor (TCR) genes is an attractive strategy to direct autologous T cell immunity against tumor-associated antigens (TAAs). However, clinical effectiveness has been hampered by limited in vivo persistence. We investigated whether the use of MHC-mismatched T cells would prolong the in vivo persistence of tumor-reactive TCR gene expressing T cells by continuous antigen-driven proliferation via the endogenous potentially alloreactive receptor. Donor-derived CD8$^+$ T cells engineered to express a TCR against a leukemia-associated antigen mediated strong graft-versus-leukemia (GVL) effects with reduced graft-versus-host-disease (GVHD) severity when given early post-transplant. AT later post-transplant resulted in a complete loss of GVL. Loss of function was associated with reduced expansion of TCR-transduced T cells as assessed by CDR3 spectratyping analysis and PD-1 upregulation on T cells in leukemia-bearing recipients. PD-L1 blockade in allogeneic transplant recipients largely restored the GVL efficacy without triggering GVHD, whereas no significant anti-leukemia effects of PD-L1 blockade were observed in syngeneic controls. These data suggest a clinical approach in which the AT of gene-modified allogeneic T cells early post-transplant can provide a potent GVL effect without GVHD, while later AT is effective only with concurrent PD-L1 blockade.
INTRODUCTION

Hematopoietic stem cell transplantation (HCT) from HLA-mismatched family donors is a potentially curative option for patients with high-risk hematologic malignancies lacking an HLA-matched donor\textsuperscript{1,2}. For haploidentical HCT, this procedure typically requires rigorous T cell depletion of the graft eliminating the cellular component that can contribute to the curative potential of an allogeneic HCT\textsuperscript{3}. To overcome this limitation donor-derived lymphocytes have been infused later post-transplant to provide a graft-versus-malignancy effect. Although preclinical and clinical studies were initiated to minimize the side effects of such a procedure\textsuperscript{4,5}, the risk of inducing severe graft-versus-host-disease (GVHD) remains substantial and relapse rates continue to be significant in part due to tumor escape mechanisms that evolve over time\textsuperscript{6}.

Enforced expression of T cell receptor (TCR) genes directed against a tumor-associated antigen (TAA) has been explored as a means by which the potency of T cell adoptive transfer (AT) may be augmented. When using allogeneic T cells, such an approach may serve to direct the donor T cell response preferentially to the host leukemia cells instead of the normal host cells, thereby increasing the therapeutic index of T cell AT. Lessons from studies of murine autologous T cell AT models have shown that: 1. TCR gene therapy can be expected to break tolerance against self-antigens such as tumor-associated antigens; 2. with few exceptions TCR gene transfer was associated with an acceptable toxicity profile; and 3. the transfer of TCR-engineered T cells has been shown to impact large tumor burdens\textsuperscript{7}.

However, clinical translation of TCR gene-modified T cell AT has been hampered by the growing evidence that in vivo proliferation and persistence of engineered T cells is more limited than needed for an optimal anti-tumor response\textsuperscript{8,9}. Increasingly, T cell AT is performed in the
context of a lymphodepleted recipient to provide a more favorable environment for their homeostatic expansion. However, while cytokines that accumulate in lymphodepleted recipients can drive T cell expansion until the cytokines are consumed, long-term T cell activation and expansion requires continued TCR engagement. In this study we sought to take advantage of dual specific TCR-transduced T cells obtained from MHC-mismatched donors that would receive allogeneic MHC antigenic signals via the endogenous TCR that may be useful in sustaining the persistence of adoptively transferred T cells. In support of this hypothesis, virus-specific T cells reprogrammed to express a TCR directed against host hematopoietically restricted minor histocompatibility antigens remained responsive against their allo-targets without losing their viral reactivity.

Here, we evaluated the converse concept that the in vivo infusion of T cells forced to express a tumor-specific antigen could be driven to expand and persist as a result of host alloantigen signaling of the endogenous TCR, thereby providing a potent GVL effect. In a fully mismatched murine HCT model T cells were transduced with a TCR directed against a surrogate leukemia-associated antigen, characterized in vitro and evaluated in the transplant setting. Our studies demonstrate that TCR transfer into allogeneic T cells can result in a functionally relevant downregulation of the endogenous TCR that accounts for its capacity for alloresponse. Whereas GVL effects mediated by TCR-engineered CD8+ T cells were achieved after AT early post-HCT, anti-leukemic effects were completely abolished if given later post-HCT. We further show that GVL effects after early AT are associated with prominent in vivo skewing of the Vβ-families within the transferred T cell population. After late AT, markedly reduced oligoclonal expansion was observed and baseline PD-1 expression was higher in allogeneic than syngeneic transplant
recipients. Notably, GVL in allogeneic recipients could be restored without GVHD induction when AT was given combined with PD-L1 blockade.

MATERIALS AND METHODS

Animals and HCT

Animals in the experiments were used under protocols approved by the State Government of Niedersachsen, Germany. C57BL/6 (B6, H-2^b) mice were purchased from Charles River, Germany. B10.A (H-2^a) mice were obtained from Taconic Laboratories (Bornholt, Denmark). DsRed (H-2^b)^13 mice were purchased from Jackson Laboratory (Bar Harbor, MA, USA), and 2C TCR transgenic mice (H-2^b)^14 were kindly provided by M. Sykes (Boston, USA). Fully MHC-mismatched allogeneic HCT or syngeneic HCT was performed using B10.A (H-2^a) → B6 (H-2^b) or B6 (H-2^b) → B6 (H-2^b) strain combinations. B6 recipients received total body irradiation (TBI) of 10.5 Gy from a linear accelerator source. After four hours, BM was reconstituted with TCD 15 × 10^6 B10.A BM cells or with 3 × 10^6 B6 bone marrow (BM) cells. TCD of CD4^+ and CD8^+ T cells from BM was performed in vitro by complement lysis using monoclonal antibodies (GK1.5 and 2.43, respectively) and rabbit complement (Biozol, Eching, Germany). For in vivo blocking experiments rat anti-mouse PD-L1 antibody (clone 10F.9G2) was used from Bio X Cell (West Lebanon, NH, USA). Rat anti-human CD154 mAb was generated by Andrew Flatley.

Cell lines, retroviral constructs, and transduction of T cells

C1498-OVA is a B6-derived myeloid leukemia cell line (H-2^b, C57BL/6 background) expressing the experimental surrogate antigen that was generated and cultured as described previously^15. To induce leukemia, a 100% lethal dose (1.2 × 10^6) of C1498-OVA cells was injected into mice by lateral tail vein injection. The retroviral vector OTI-2A-pMIGII and the GP+E86 (ATCC:
CRL.9642) producer cells were provided by one of us.16 DCs were generated as previously described and pulsed with leukemia cell lysates.4 Single cell suspensions of donor splenocytes were co-cultured with the irradiated producer cells for 2 days in the presence of ConA (2.5 \( \mu \text{g/ml} \)) (Sigma, St. Louis, USA), IL2 (20 U/ml) (Amgen, Munich, Germany) and IL7 (4 ng/ml) (R&D Systems, Minneapolis, USA) in RPMI 1640 complete media (PAA, Pasching, Austria). Controls were co-cultured on the same producer cells not containing the respective viral construct, further referred to as non-transduced controls. After priming on maturated recipient-derived DCs for another two days, T cells were harvested and expanded using \( \alpha \)-CD3/\( \alpha \)-CD28 (BD Biosciences, Heidelberg, Germany) coated microspheres (Invitrogen, Karsluhe, Germany) in the presence of recombinant IL2 (20 U/ml) and IL7 (4 ng/ml).

**Flow cytometry**

The following antibodies were used: CD8\( \alpha \)-(PE/PE-Cy7/APC-H7/PE-Cy5.5), CD4-(PE/APC/PE-Cy7/Pacific Blue), V\( \alpha \)2-biotin, V\( \beta \)3-PE, V\( \beta \)4-biot., V\( \beta \)6-biot., V\( \beta \)8-PE, V\( \beta \)5-PE, PD-1-PE, CD25-PE, CD69-PE, CD44-PE, CD62L-PE, CD95L-APC, CD95-PE-Cy7, CD127 biot., annexin V-APC, (BD Biosciences, Heidelberg, Germany). For intracellular Foxp3 staining cells were labeled with CD4, fixed, permeabilized (Fix/Perm; eBioscience, Frankfurt, Germany), and stained with Foxp3-AF647 (eBioscience). 1B2 antibodies were kindly provided by M. Sykes. For detection of intracellular IFN\( \gamma \) anti-IFN\( \gamma \)-PE (Invitrogen, Darmstadt, Germany) and Golgi Plug (BD Biosciences) were used. Flow cytometry was performed using a LSR-II (Becton Dickinson, Heidelberg, Germany).
Assessment of GVHD

Clinical GVHD assessment was done in a double blinded fashion for eight weeks after AT. We used a clinical scoring system that was described previously grading the animals based on weight loss, skin integrity, posture, fur texture, and activity.

In vitro cytotoxicity and mixed leukocyte reaction (MLR)

The JAM assay was used to assess cytotoxicity against C1498 or C1498-OVA target cells in vitro as described. MLR standard techniques were adjusted to the specific biological needs of pre-activated CD8+ T cells: Responder cells were serially diluted in triplicate wells: 4 × 10^5, 2 × 10^5, 1 × 10^5, and 0.5 × 10^5. Stimulators were irradiated with 3000 cGy and co-cultured for 24h with responder cells (1 × 10^5 stimulator cells per well) in the presence of IL2 (2 U/ml). Each well was pulsed with 1 μCi of [3H]-thymidine (Amersham, MA, USA) 16 h before harvesting with an automated harvester (Perkin Elmer, Rodgau, Germany). Incorporation of [3H]-thymidine was measured by a beta plate counter. The averages of the differences in counts per minute (cpm) were determined according to the following formula: \( \Delta \text{cpm} = \text{average of cpm responder with stimulators} - \text{average of cpm responder w/o stimulators} \).

TCR spectratype analysis and assessment of proliferation

For TCR spectratype analysis total mRNA was transcribed into cDNA with Superscript III reverse transcriptase and oligo-dT12–18 primers (Invitrogen, Karlsruhe, Germany). cDNA was amplified by PCR for 30 Cycles with 21 Vβ-specific primers and one Cβ1-Cβ2 primer as described previously. PCR products were FAM-labeled by nested primer extension and analyzed by capillary electrophoresis (ABI 3110). To determine skewing within the Vβ-family the areas under the peaks representing varying CDR3-size length were compared to the
corresponding peaks from naïve controls. A CDR3-size length was considered skewed if the area under the peak was greater than the mean + 3 SD of the respective control peak\textsuperscript{21}. In vivo proliferation of hepatic and splenic T cells was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation.

**Statistical analysis**

Kaplan-Meier product-limit method was used to calculate survival rates. Differences between the groups were determined using Log-rank statistics. In other cases, statistical analyses were performed by Mann-Whitney test or by Student’s t-test (two-tailed). P values < 0.05 were considered to be significant and were designated by *. P values < 0.01 were designated by **, p values less than 0.001 were designated by ***.
RESULTS

Gene engineering of allogeneic T cells with anti-leukemia reactive TCR genes augments the GVL effect in vitro and vivo

To determine the feasibility and the biological consequences of equipping a T cell of allogeneic origin with a TCR against a recipient leukemia-specific antigen, we transduced naïve MHC-mismatched T cells (B10.A-derived: H-2\textsuperscript{a}) with the ovalbumin-reactive OT-I TCR that would drive donor T cell reactivity toward B6 (H-2\textsuperscript{b})-derived leukemia cells forced to express the nominal and surrogate leukemia-specific antigen, chicken ovalbumin, C1498-OVA. The vector contained the \(\alpha\)- and \(\beta\)- TCR genes of the OT-I TCR, together with an eGFP reporter-cassette, expressed via 2A and IRES sequences (TCR\(\alpha\)-T2A-TCR\(\beta\)-IRES-GFP). After transduction, T cells were primed on B6 recipient-derived DCs that had been pulsed with lysates from C1498-OVA cells. Since all reported clinical studies applying TCR-engineered T cells to date have been conducted with autologous T cells, the respective syngeneic system (B6 \(\rightarrow\) B6) was used as a control.

Priming followed by expansion using \(\alpha\)-CD3/\(\alpha\)-CD28-coated microspheres led to a 50-fold expansion of transduced GFP\(^+\)CD8\(^+\) T cells within seven days of culture. GFP expression was comparable in the allogeneic and syngeneic setting and continuously increased during short time in vitro expansion (Figure 1A). At the end of the generation process GFP expression was detected in 60-75\% of T cells both in the syngeneic and the allogeneic setting. (Figure 1B). Gene-modified T cells were mainly CD8\(^+\) (~80\%) and co-expression of the OT-I-specific V\(\alpha\)2\(^+\)/V\(\beta\)5\(^+\) TCR chains was observed in about 50\% to 60\% (Figure 1B). They displayed an effector phenotype that was comparable between syngeneic and allogeneic T cells: CD127\(^{\text{neg}}\), CD44\(^{\text{hi}}\), CD69\(^{\text{hi}}\), and 80\% of cells being CD62L\(^{-}\) (Figure 1C). Expression of the introduced TCR
translated into OVA-specific cytotoxicity against C1498-OVA target cells and was associated with the upregulation of IFNγ (Figure 1D and suppl. Figure 1). Of note, gene-modified T cells of allogeneic and syngeneic origin demonstrated comparable cytotoxicity in vitro against their respective non-tumor targets. To exclude severe functional deficits induced by TCR gene transfer, in vitro functionality was comparatively assessed with activated transgenic OT-I T cells (suppl. Figure 1). To assess the GVL effects of TCR-transduced T cells in vivo we used the MHC-disparate transplantation model (B10.A [H-2a] → B6 [H-2b]). Eight weeks after HCT (B10.A → B6), mice were challenged with a lethal dose of C1498-OVA leukemia cells (1.2 × 10^6). On the following day, increasing numbers (5, 10, 20 or 40 × 10^6) of TCR-transduced or non-transduced B10.A-derived T cells were transferred into cohorts of leukemia-bearing mice. Untreated animals succumbed to leukemia within 30 to 60 days after leukemia challenge. The highest dose of allogeneic TCR-transduced T cells resulted in survival rate of 60% with lower doses resulting in 10-25% survival. Only recipients receiving the 2 highest doses of transduced T cells had evidence of a significant GVL effect. Treatment with non-transduced, allogeneic T cells produced some GVL effect in the cohort that received the highest cell dose only, although long-term survival was 0% (Figure 1E). Hypothesizing higher apoptosis rates of TCR-engineered T cells after AT, transferred cells were retrieved from the recipients after three days. Freshly isolated naïve CD8+ T cells from donor spleens were given as controls. Unexpectedly, apoptosis rates as defined by annexin V positivity were comparable (suppl. Figure 2).

**TCR gene transfer of allogeneic T cells reduces the expression of the endogenous TCR and diminishes allore sponsiveness in vitro**

To determine whether the retrovirally introduced TCR might impact the expression of endogenous TCRs directed against host alloantigens, we assessed alloreactivity in vitro.
Alloreactivity was significantly reduced in TCR-transduced versus non-transduced T cells as measured by MLR. Reduced alloreactivity of TCR-transduced allogeneic T cells might have been caused by increased apoptosis rates due to TCR gene transfer. Non-labeled T cells from MLR cultures were analyzed for PI and annexin-V by FACS. Interestingly, apoptosis rates were significantly higher (65% versus 16%) in the non-transduced controls as compared to transduced T cells. This suggests that the drop of MLR-activity in the TCR-transduced cells was not due to increased apoptosis (Figure 2A). Since alloresponses are mediated by endogenous TCRs, the expression of endogenous TCRs was assessed by staining of four randomly chosen Vβ chains. Endogenous TCR expression was reduced in retrovirally induced TCRhi T cells vs the TCRlo T cell counterparts (Figure 2B). Results obtained from a polyclonal endogenous TCR repertoire might not reflect the situation where strong alloreactivity is driven by a strongly dominant T cell clone. To simulate the situation of a dominant TCR directed against the host, we used T cells derived from 2C mice, transgeneic for a high-affinity TCR recognizing the Ld MHC class I alloantigen. Endogenous TCR expression decreased with increasing retrovirally induced TCR expression (Figure 2C). Together, these data indicate that the transduced TCR competes with endogenous Vβ proteins for cell surface expression. As a consequence of the expression of the model TCR, there is a reciprocal downmodulation of the endogenous TCR which may contribute to a reduced GVHD observed when transduced T cells expressing high levels of the model TCR are infused in vivo.

**TCR-transduced CD8+ T cells mediate strong GVL effects with decreased GVHD rates when administered early after HCT.**

To assess the GVHD-triggering potential of the TCR-engineered T cell product an inflammatory post-transplant milieu was chosen known to facilitate severe in vivo alloreactivity. Hypothesizing that an inflammatory environment might promote in vivo persistence and as a
consequence support GVL effects we monitored adoptively transferred T cells in vivo. We performed early AT (day 21) of either allogeneic (40 × 10^6 B10.A cells) or syngeneic (40 × 10^6 B6 cells) TCR-transduced T cells into allogeneic (B10.A → B6) or syngeneic transplant recipients (B6 → B6). Seven days after transfer frequencies reached comparable levels after syngeneic and allogeneic transplant (Figure 3A). To evaluate the impact of this finding on GVL, a lethal dose of C1498-OVA leukemia (1.2 × 10^6 C1498-OVA cells) was administered on day seven after AT. Treatment with allogeneic and syngeneic T cells resulted in comparable GVL efficacy with long-term survival rates of 100% and 85% respectively. In contrast, survival was significantly lower after treatment with the same total cell number of naïve DLI illustrating a strong anti-leukemic effect beyond alloreactivity mediated by the introduced TCR (Figure 3B).

To investigate how TCR gene transfer of allogeneic T cells might impact GVHD, we transferred 40 × 10^6 TCR-transduced, non-transduced T cells or naïve DLI (40 × 10^6 total cell number) into established mixed hematopoietic chimeras (MCs) on day 21 after a fully myeloablative transplant (B6/B10.A → B6, 40-60% host hematopoiesis). MCs were chosen because we have previously shown that this environment early after transplant remained highly sensitive for GVHD upon transfer of in vitro primed T cells⁴. Mice treated with non-transduced T cells developed clear signs of GVHD. The degree was not statistically different from scores reached after the application of naïve DLI. In contrast and in accordance to the in vitro results presented in Figure 2, TCR-transduced T cells mediated significantly less GVHD than non-transduced T cells and naïve DLI (Figure 3C). To assess the repertoire of expanding gene-modified CD8⁺ T cells in vivo, TCR CDR3-size spectratyping was performed on transferred T cells re-isolated from the mice 40 days after AT. Cultured TCR-transduced T cells pre-injection and the respective cells retrieved after syngeneic transplants were used as controls. Skewing indicates clonal or
oligoclonal expansion within the Vβ-family. Representative spectratypes of non-skewed and skewed Vβ-families are shown (Figure 3D). Upon AT only 2/21 Vβ-families were skewed indicating that oligoclones must have grown out after transfer. Of note, the Vβ-family harboring the introduced gene (Vβ 5.2) was reproducibly detectable in all samples. Whereas in the syngeneic setting 6 families showed Vβ-shifts, more prominent (13/21) shifts were found in the allogeneic setting (Table 1). In vivo, this is associated with a certain degree of GVHD in these mice suggesting oligoclonal expansion driven by alloreactivity.

**Allogeneic TCR-transduced CD8⁺ T cells fail to induce GVL when given later after HCT.**

Delayed AT of allogeneic T cells has the potential for strong GVL effects and reduced GVHD due to absence of lymphopenia, repair of tissue injury, and presence of donor APCs while still permitting a graft-vs-lymphohematopoietic effect to occur, especially since fewer T cells may be required to mediate GVL than GVHD lethality. To further reduce the risk of GVHD but aiming to maintain strong GVL effects we evaluated the biological consequences of late AT. We transferred either allogeneic (B10.A) or syngeneic (B6) TCR-transduced T cells into full allogeneic chimeras (B10.A → B6) or syngeneic transplant recipients (B6 → B6) and monitored the peripheral blood for GFP-expressing cells. Allogeneic T cells showed a decline from day one to day seven whereas the frequencies of syngeneic TCR-transduced T cells remained similar (Figure 4A). When we administered C1498-OVA 3 days after AT, GVL effects were completely abolished. In contrast, consistent rescue rates of ~60% could be documented after syngeneic AT (Figure 4B). The interval between AT and leukemia challenge, C1498-OVA, was shortened to 1 day. Here, 85% of mice were rescued in the allogeneic setting suggesting that TCR-transduced T cells were functional, at least 1 day after AT (suppl. Figure 3). Clinical monitoring for GVHD did
not reveal any signs of GVHD after treatment with TCR-transduced allogeneic T cells or naïve T cells (Figure 4C).

A high affinity alloreactive endogenous TCR contributes to low frequencies of TCR-transduced CD8⁺ T cells in vivo.

In view of the results depicted in Figures 3 and 4 we investigated whether the degree of alloresponsiveness mediated by the endogenous TCR influences in vivo persistence and anti-leukemic efficacy. On day 56 after HCT, 1 × 10⁶ TCR-transduced 2C-derived T cells together with 10 × 10⁶ TCR-transduced DsRed T cells were given to either allogeneic (B6 → B10.A) or syngeneic transplant recipients (B6 → B6). This allowed us to simultaneously track a CD8⁺ T cell population with a polyclonal alloreactive endogenous TCR repertoire (DsRed) and a high affinity monoclonal alloresponsive population (2C). In non-leukemia-bearing animals AT of a TCR-transduced and polyclonal T cell mixture into allogeneic recipients produced a significantly lower frequency of T cells in the peripheral blood when compared to syngeneic controls on days one and three after AT (Figure 5A). This effect was more pronounced within the CD8⁺ population with the high affinity allo-endogenous TCR on day three after AT.

To evaluate whether reduced numbers in the periphery might reflect a different migration pattern of allogeneic T cells lymph nodes, spleen, and bone marrow were analyzed for the respective T cell population. Allogeneic T cells were reduced in all organs analyzed on day three after AT, which again was more pronounced for 2C than polyclonal T cells (Figure 5A). To determine whether low frequencies of allogeneic T cells were a consequence of increased apoptosis we analyzed the transferred endogenous T cell population for annexin V, CD95, and CD95L expression. Neither differences were observed between low and high affinity allogeneic
populations nor whether the respective cell population was retrieved from allogeneic or syngeneic transplant recipients (Figure 5B). These results suggest that lower numbers of transferred T cells found after AT in vivo inversely correlated with the degree of alloresponsiveness of the endogenous TCR. From these data we deduced that environmental factors rather than intrinsic T cell defects hampered the expansion of allogeneic T cells after delayed AT.

**Loss of GVL effects is associated with increased PD-1 expression and reduced oligoclonal expansion in allogeneic transplant recipients and can be effectively restored by PD-L1 blockade.**

The PD-L1/PD-1 pathway has been reported to be critical in tolerance induction in solid organ transplant recipients and has been even described in the context of tumor escape mechanisms. We therefore examined comparatively the PD-1 expression on gene-modified T cells after delayed (day 56 post-HCT) AT in the allogeneic (B10.A → B6) and syngeneic (B6 → B6) setting. Within the first week after transfer, we did not observe significant differences between allogeneic and syngeneic TCR-transduced T cells in non-tumor-bearing animals (data not shown). In contrast, we found a 4-fold higher frequency of PD-1 expressing CD8+ T cells in the peripheral blood of leukemia-bearing allogeneic (B10.A → B6) vs syngeneic recipients (B6 → B6) (Figure 6A). To determine whether this observation had functional relevance mice received either α-PD-L1 blocking antibodies or isotype control antibodies along with TCR-modified T cells. Consecutively, mice were challenged with C1498-OVA leukemia three days after delayed AT on day 59. Treatment with PD-L1 blocking antibody alone resulted in significantly prolonged survival compared to controls (Figure 6B). The combination of PD-L1 blockade and gene-modified syngeneic T cells showed no significant prolonged survival when compared to isotype
treated controls (Figure 6C). In contrast, PD-L1 blockade restored GVL efficacy of adoptively transferred allogeneic T cells raising leukemia-free survival from 0% to 60% after AT (Figure 6D). Notably, mice treated with PD-L1 blockade and allogeneic TCR-transduced CD8+ T cells did not develop any signs of GVHD (Figure 6E). To evaluate the impact of PD-L1 blockade on the fate of transduced T cells after late AT, cells were retrieved 40 days after transfer. In contrast to the results after early AT (13/21, Table 1), spectratype analysis showed lower numbers of skewed Vβ-families (5/21), independently of PDL-1 blockade (4/21, Table 2). Additionally performed BrdU experiments revealed an increase of the proliferative response after PD-L1 blockade (Figure 6F). Together with the GVHD data these results suggest that oligoclonal expansion driven by alloreactivity does little contribute to the GVL effects seen after PD-L1 blockade. However, the different effect in the allogeneic and syngeneic setting remained striking.

Very recently Zhou et al. demonstrated the synergistic role of regulatory T cells (Tregs) and PD-1/PD-L1 interaction in AML-induced suppression of CD8+ cytotoxic T cells at tumor sites. Therefore, T cells were retrieved from the liver 25 days after leukemia challenge. Whereas the frequency of Tregs was similar after allogeneic and syngeneic transplant, PD-1 expression of CD8+ T cells was significantly higher in allogeneic versus syngeneic non-leukemia-bearing controls. Their ability to produce IFNγ was reduced in both, AML-bearing allogeneic and syngeneic transplant recipients and increased significantly after PD-L1-blockade (Figure 6G). From these results we deduce that in the allogeneic setting an increased baseline of PD-1 expression results in a stronger impact of PD-L1-blockade after leukemia challenge.
DISCUSSION

The genetic transfer of TCRs is a powerful approach to rapidly generate tumor-specific T cells. Clinical studies have focused on the use of TCR-engineered autologous T cells and outcome has been correlated with in vivo persistence of adoptively transferred T cells\textsuperscript{8,27,28}. To our knowledge, our study is the first assessing the potential use of TCR-engineered T cells of allogeneic origin after MHC-mismatched hematopoietic cell transplantation. We demonstrate that donor-derived CD8\(^+\) T cells engineered to express a TCR against a leukemia-associated antigen mediated strong GVL effects at significantly reduced GVHD-rates when transferred early after transplant. Delayed transfer of the same cell type was followed by rapidly decreasing cell numbers and reduced oligoclonal expansion as shown in TCR spectratype analysis. This was associated with a complete loss of GVL effects. This loss of function was accompanied with increasing PD-1 expression on adoptively transferred T cells and PD-L1 blockade widely restored GVL effects without triggering GVHD.

In vivo persistence of transferred T cells seems to be a prerequisite for significant anti-tumor effects. This implies that virus-specific T cells additionally equipped with a TCR directed against a tumor-associated antigen might persist longer in vivo in patients since encounter with relevant viruses would drive homoeostatic proliferation of such T cells over a longer period of time\textsuperscript{29,30}. Although intriguing, this concept, to our knowledge, has not been widely tested in vivo. Modifying this concept for the setting of allogeneic HCT we hypothesized that a largely polyclonal set of alloreactive TCRs on donor-derived T cells would drive continuous in vivo proliferation propagating simultaneously transduced T cells expressing a leukemia-reactive TCR.

The transduced TCR has to compete for cell surface expression with endogenous TCRs for CD3 binding\textsuperscript{29,31}. Indeed, reduced expression of endogenous TCRs was observed in our
studies of TCR gene transduction. In this context it is important to note that the number of TCR molecules being triggered by specific peptide-MHC complexes is crucial for T cell activation and effector function\textsuperscript{32}. Indeed, in our study, the transfer of a high affinity TCR in completely MHC-mismatched T cells decreased alloreactivity in vitro and translated into significantly lowered GVHD-scores. However off target toxicity after TCR gene transfer remains a concern since Schumacher and colleagues have shown that lethal cytokine driven autoimmune pathology can occur due to the formation of self-reactive TCR dimers\textsuperscript{33}. For allogeneic T cells others have revealed that a substantial proportion of non-modified virus-specific T cells can exert allo-HLA reactivity\textsuperscript{34}.

For long-term protection against leukemia, we hypothesized that stimulatory responsiveness via both exogenous and endogenous TCRs would be important. We hypothesized that the AT of T cells in allogeneic transplant recipients should result in continuous exposure to alloantigen, thus leading to selective survival. With a growing burden of minimal residual disease larger amounts of leukemia-associated antigens would engage transferred T cells expressing the introduced TCR. Repetitive stimulation would then restore functionality of the leukemia-reactive TCR herein preventing relapse. This concept did not prevail in our in vivo studies. In the syngeneic setting, 70\% of recipients of syngeneic T cells survived when the leukemia challenge was given 3 days after transfer. Whereas significant numbers of TCR-engineered T cells were found in lymph nodes, spleen, and bone marrow after syngeneic transfer, fewer T cells were present after allogeneic transfer. Thus, the poor GVL effects in recipients of allogeneic but not syngeneic TCR gene-transduced T cells appears to be stressed by environmental factors of the allogeneic transplant milieu. Of note, 180 days after late AT GFP\textsuperscript{+} T cells of central memory type could be re-isolated, although none of the originally injected cells had shown this phenotype.
This is in accordance with observations from the Riddel group, demonstrating central memory development after adoptive T cell transfer (suppl. Figure 4)^35.

Consistent with the findings of others^25 PD-L1 was expressed by the leukemia cell line C1498 and the level was elevated by IFNγ treatment. Upregulation of PD-1 was correlated with the suppression of IFNγ production by antigen-specific CD8^+ T cells in the hepatic microenvironment^36. In both of these studies, the non-OVA bearing C1498 line was used. Earlier studies by Blazar et al.^37 demonstrated that donor T cells upregulated PD-1 early post-BMT during the course of a GVHD reaction. Moreover, Asakura et al. recently published that PD-I was upregulated on donor lymphocytes given at the time of lethal radiation to double chimeras^38, although neither Blazar et al. nor Asakura et al. identified the inciting factors leading to PD-1 upregulation. Interaction of PD-L1 expressed by C1498 leukemia cells with PD-1 on T cells correlated with the suppression of IFNγ production by antigen-specific CD8^+ T cells in the hepatic microenvironment^25,39. Additionally, increased numbers of Tregs were found in the liver of AML-bearing mice 25 days after leukemia challenge^40. In the reported model PD-1/PD-L1 blockade completely abrogated the ability of Tregs to suppress CD8^+ proliferation and IFNγ production. Blockade increased the proliferation of CD8^+ T cells in the liver of AML-bearing animals and rescued their ability to produce IFNγ^26. Since the effect of PD-L1 blockade was more pronounced in the allogeneic setting we comparatively evaluated the effects after syngeneic and allogeneic transplantation. The baseline of PD-1 expression on CD8^+ T cells was already significantly higher in non AML-bearing animals after allogeneic transplant. In contrast, the number of Tregs isolated was comparable in syngeneic and allogeneic transplant recipients, both, in AML-bearing and in non-challenged mice. In either transplant setting PD-L1 blockade restored the ability of retrieved CD8^+ T cells to produce IFNγ after AML-leukemia challenge. One might therefore hypothesize that a higher baseline expression of PD-1 on CD8^+ T cells after allogeneic
transplants renders them more susceptible for C1498-induced functional suppression and therefore PD-L1 blockade after allogeneic transplant had a stronger impact. These data are consistent with those of Asakura et al.\textsuperscript{38} in a minor antigen mismatched double chimera BMT model system in which donor lymphocytes are given at the time of radiation.

Although PD-L1 blockade after late AT restored GVL activity to a large extent, this was not accompanied with clinically apparent signs of GVHD. This was somewhat unexpected since PD-1 blockade has been described to accelerate GVHD development when this occurs at the time of BMT\textsuperscript{37}. TCR-spectratyping analysis performed on TCR-transduced T cells re-isolated after late AT showed little impact of PD-L1 blockade on oligoclonal expansion. The function of TCR-engineered T cells could be restored without evoking GVHD. However, it is probably reasonable to speculate that PD-L1 blockade after early AT (here functional relevant oligoclonal expansion was demonstrated) would lead to increased, potentially lethal GVHD.

These findings are of clinical relevance since first clinical trials have shown the feasibility of AT with TCR-transduced T cells. Carefully chosen concepts of inhibitory blockade could improve efficacy and even widen the therapeutic window between GVL and GVHD after AT with allogeneic T cells later post-HCT.
Acknowledgements and grant support:

We thank R. Schwinzer and W. Baars for providing their radioisotope laboratory and we would like to acknowledge the assistance of the Cell Sorting Core Facility of the Hannover Medical School supported in part by Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft. This research was supported by grants SFB-738-A3 (Deutsche Forschungsgemeinschaft, Bonn, Germany; MGS), NIH R01 CA72669, and P01 AI 056299 BRB)

Contribution:

W.K. designed research, performed experiments, analyzed and interpreted data, drafted and edited the manuscript; M.H., J.H., M.W., and J.F. performed experiments; A.F. contributed vital new reagents; C.K., K.W., E.J., and D.A.V. analyzed and interpreted data; B.R.B. analyzed and interpreted data and edited the manuscript; M.G.S. designed research, analyzed and interpreted data, drafted and edited the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests. Correspondence: Martin G. Sauer, Department of Pediatric Hematology and Oncology, Medizinische Hochschule Hannover, OE 6780, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany; e-mail: sauer.martin@mh-hannover.de.
References


Table 1. Vβ spectratype analysis shows oligoclonal expansion of TCR-transduced allogeneic T cells in contrast to syngeneic TCR-transduced T cells after early AT.

40 × 10^6 TCR gene-transduced B10.A T cells were adoptively transferred early (21 days after HCT) into established allogeneic (B10.A → B6) or syngeneic recipients (B10A → B10A). Mice were sacrificed 40 days after AT. Splenocytes were sorted for CD8+GFP+ T cells and CDR3-size spectratype analysis was performed as described in the Materials and Methods section. TCR-engineered T cells directly from the culture were used as controls (AT). CDR3-size length skewing was determined by comparison of the experimental spectratypes to the respective naive B10.A controls (n=6) as described in the Materials and Methods section. Skewed CDR3-size lengths of individual allogeneic (n = 3) and syngeneic (n = 3) mice are depicted. (-) indicates no skewing.

Table 2. PD-L1 blockade does not induce oligoclonal expansion of allogeneic TCR-transduced T cells after late AT.

40 × 10^6 TCR gene-transduced allogeneic (B10.A) T cells were adoptively transferred late (56 days after HCT) into established allogeneic (B10.A → B6) recipients. Treatment with α-PD-L1 or isotype control was performed as described in the legend of figure 6. 40 days after AT CD8+GFP+ T cells were sorted from spleens and CDR3-size spectratype analysis was performed as described in the Materials and Methods section. Skewed CDR3-size lengths of individual mice after treatment with α-PD-L1 (n = 3) or isotype control (n = 3) are shown. (-) indicates no skewing; n.a., not available.
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Figure 1: Gene engineering of allogeneic T cells with anti-leukemia reactive TCR genes augments the GVL effect in vitro and vivo. TCR-transduced allogeneic or syngeneic T cells were further characterized in vitro. (A) Donor-derived allogeneic (B10.A) or syngeneic (B6) T cells were transduced with the OT-I TCR, primed on tumor lysate-pulsed recipient-type DCs (B6) for two days, stimulated with αCD3/αCD28-coated microspheres for one day, and further expanded for two days with cytokines (IL-2/IL-7). GFP⁺CD8⁺ T cells of allogeneic (-■-) and syngeneic (-□-) origin are depicted in the graph. Results from one representative experiment are shown. Expansion rates of approximately 50-fold were obtained after day seven of culture. Flow cytometric plots depict increasing rates of GFP⁺CD8⁺ expression during the generation process: after transduction (d2), after priming on DCs (d4), and of the final T cell product (d7). (B) Representative plots of allogeneic or syngeneic TCR-transduced T cells from the final T cell product (d7) are shown. The upper two plots were gated on live cells by forward side scatter exclusion and stained for CD8; the bottom plots were gated on GFP⁺CD8⁺ T cells and stained for the introduced OT-I TCR chains Vβ5/Vα2. (C) Further phenotypic features known to impact in vivo function are shown on syngeneic (upper row) or allogeneic GFP⁺CD8⁺ (bottom row) T cells. (D) Gene-modified T cells were assessed for cytotoxicity at the stated E : T ratios in a JAM assay. TCR-transduced allogeneic (-■-) and syngeneic (-□-) T cells displayed a nearly identical cytotoxicity profile against C1498-OVA. Antigen non-expressing C1498 target cells were used as controls (■••••/□••••). Values are shown +/- 1 SE. One representative experiment out of four is shown. (E) Lethally irradiated B6 recipients were reconstituted with 15 × 10⁶ TCD B10.A BM and received 1.2 × 10⁶ C1498-OVA cells intravenously on day 56 after HCT. On day 57, recipients were treated with increasing doses of either TCR-transduced B10.A T cells (left) or non-transduced controls (right). Differences in survival after treatment with TCR-transduced T cells or PBS: p < 0.001 between 40 × 10⁶ T cells versus PBS; p < 0.05 between 20 × 10⁶ versus
PBS, \( p = \) n.s. between \( 10 \times 10^6 \) and \( 5 \times 10^6 \) T cells versus PBS. \( P \)-values for non-transduced T cells versus PBS: \( p < 0.05 \) between \( 40 \times 10^6 \) versus PBS; \( p = \) n.s. for all other groups versus PBS. \( n = 9 \)-10/group

**Figure 2:** TCR gene transfer of allogeneic T cells reduces the expression of the endogenous TCR and diminishes alloresponsiveness in vitro. (A) Alloreactivity after TCR gene transfer was assessed in a MLR adjusted to the specific biological needs of pre-activated CD8\(^+\) T cells (see methods part). TCR-transduced (-■-) or non-transduced (-◆-) B10.A T cells were stimulated either with irradiated allogeneic B6 splenocytes or syngeneic B10.A splenocytes as controls (-□/-◇-). Values are shown +/- 1 SE. In order to assess the viability of transduced versus non-transduced responders at the time of MLR read out, samples were drawn from the wells and stained for annexin V/PI. (B) Either CD8\(^+\)GFP\(^lo\) or CD8\(^+\)GFP\(^hi\) expressing T cells (B10.A) were gated on the V\(\alpha 2/V\beta 5\) double positive population after transduction. Transduced T cells were co-stained for four randomly chosen endogenous TCR V\(\beta\) chains. Bar graphs display the reduction of the respective endogenous V\(\beta\) chains in % as calculated by the ratio of MFI (endogenous V\(\beta\)) on GFP\(^hi\) / MFI (endogenous V\(\beta\)) on GFP\(^lo\). (C) To directly visualize a high affinity alloreactive endogenous TCR, L\(^d\) alloantigen-specific TCR-transgeneic 2C T cells were used for transduction and consecutively gated on either GFP\(^lo\) or GFP\(^hi\). FACS plots depict the respective population expressing both of the introduced TCR chains (V\(\alpha 2\) and V\(\beta 5\), middle plots). Cells were analyzed for endogenous 2C TCR expression (right plots) using the 2C-specific clonotypic marker 1B2.

**Figure 3:** TCR-transduced CD8\(^+\) T cells mediate strong GVL effects with decreased GVHD rates when administered early after HCT. \( 40 \times 10^6 \) TCR gene-transduced allogeneic (B10.A)
or syngeneic (B6) T cells were adoptively transferred early (21 days after HCT) into established allogeneic (B10.A → B6) or syngeneic recipients (B6 → B6). (A) On day one, three, and seven after AT peripheral blood was monitored for GFP expressing allogeneic (●) or syngeneic (○) TCR-transduced T cells (***, p < 0.0001 for d1; **, p < 0.005 for d3; p = n.s. for d7. n = 14-17/group). (B) Mice received early AT with either allogeneic (-■-) or syngeneic (-□-) TCR-transduced T cells. Cohorts treated with naïve DLI (-▲-) or PBS (-•-) were used as controls. Seven days after AT mice were challenged with 1.2 × 10^6 C1498-OVA cells intravenously (n = 8-10/group; p < 0.05 between cohorts, that received DLI or allogeneic TCR-transduced T cells; p = n.s. between cohorts, that received syngeneic or allogeneic TCR-transduced T cells). (C) For a sensitive GVHD set up mixed chimeras (MCs) were established by reconstituting lethally irradiated B6 recipients with a mixture of 15 × 10^6 TCD B10.A BM plus 5 × 10^6 TCD B6 BM. 40 × 10^6 TCR-transduced (-■), non-transduced (-◆-) B10.A T cells, or DLI (-▲-) were adoptively transferred on day 21 after HCT. Clinical GVHD scores were performed weekly for eight weeks after AT. The highest GVHD score/observed individual is graphed (n = 8-10; *** , p < 0.0001 between cohorts that received TCR-transduced T cells or DLI. **, p < 0.005 between cohorts that received TCR-transduced or non-transduced T cells, p = n.s. for cohorts that received non-transduced T cells or DLI). (D) Representative examples of CDR3-size spectratype analysis of TCR-transduced T cells are shown. 40 × 10^6 TCR gene-transduced B10.A T cells were adoptively transferred early (21 days after HCT) into established allogeneic (B10.A → B6) or syngeneic recipients (B10A → B10A). 40 days after AT GFP^+CD8^+ T cells were sorted from recipient spleens and CDR3-size spectratype analysis was performed as described in the Materials and Methods section. Naïve CD8^+ B10.A T cells (used for transduction) and TCR-transduced T cells (used for AT) were used as controls. Spectratype histograms depict an example for a skewed (Vß 16) and a non-skewed (Vß 8.1) Vß-family.
Figure 4: Allogeneic TCR-transduced CD8+ T cells fail to induce GVL when given later after HCT. 40 × 10^6 TCR gene-transduced allogeneic (B10.A) or syngeneic (B6) T cells were adoptively transferred late (56 days) after HCT into either allogeneic (B10.A → B6) or syngeneic (B6 → B6) transplant recipients. (A) On day one, three, and seven after AT peripheral blood was monitored for GFP expressing allogeneic (-●-) or syngeneic (-○-) TCR-transduced T cells (p-values between cohorts that received allogeneic or syngeneic T cells: p = n.s. for d1; ***, p < 0.0001 for d3; *** p < 0.0001 for d7, n = 10-18, pooled data from two independent experiments are shown). (B) Mice received AT with either allogeneic (-■-) or syngeneic (-□-) TCR-transduced T cells or PBS (-●-) 56 days after HCT. Three days after AT mice were challenged with 1.2 × 10^6 C1498-OVA cells intravenously (p < 0.005 between TCR-transduced syngeneic T cells and PBS, p < 0.05 between TCR-transduced allogeneic and syngeneic T cells, p = n.s. between TCR-transduced allogeneic T cells and PBS, n = 8/group). (C) Mice received AT with TCR-transduced allogeneic T cells (-■-) as in (B) or naïve DLI (-▲-) without subsequent leukemia challenge. Clinical GVHD scoring was performed weekly for eight weeks after AT (n = 8-10/group, p = n.s. between cohorts that received TCR-transduced allogeneic T cells or DLI).

Figure 5: A high affinity alloreactive endogenous TCR contributes to low frequencies of TCR-transduced CD8+ T cells in vivo. 1 × 10^6 TCR-transduced transgeneic 2C T cells (B6 background) were mixed with 10 × 10^6 TCR-transduced transgeneic DsRed T cells (B6 background) and adoptively transferred into either established allogeneic (B6 → B10A) or syngeneic (B6 → B6) transplant recipients late after HCT. Frequencies of transferred DsRed+GFP+ T cells (upper row) or 2C+GFP+ T cells (lower row) in peripheral blood (PBL), spleen (SPL), lymph node (LN), and bone marrow (BM) were determined. Black bars indicate
allogeneic recipients and white bars syngeneic recipients (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)). Values are shown as mean +/- 1SD. (B) Splenocytes harvested on day 1 after AT were gated on 2C\(^*\)GFP\(^*\) T cells and consecutively analyzed for annexin V expression (left panel). Equally (middle and right panel), DsRed splenocytes were gated on GFP\(^*\)CD8\(^+\) T cells and analyzed for CD95L and CD95 expression (\( p = \text{n.s. between either group} \)).

**Figure 6: Loss of GVL effects is associated with increased PD-1 expression and reduced oligoclonal expansion in allogeneic transplant recipients and can be effectively restored by PD-L1 blockade.** (A) - (F) Established allogeneic (B10.A \( \rightarrow \) B6) or syngeneic transplant recipients (B6 \( \rightarrow \) B6) received \( 40 \times 10^6 \) allogeneic (B10.A) or syngeneic (B6) TCR-transduced T cells on day 56 after HCT. Three days after AT mice were challenged with \( 1.2 \times 10^6 \) C1498-OVA cells intravenously. (A) PD-1 expression on transferred cells was analyzed in peripheral blood seven days after leukemia challenge on syngeneic (white bar) versus allogeneic (black bars) and GFP\(^*\)CD8\(^+\) T cells. CFSE-labelled naïve DLIs were used as controls (*, \( p < 0.05 \); \( n=4-5 \)/group). In consecutive experiments transplanted mice received either \( \alpha \)-PD-L1 blocking antibodies or an isotype control intraperitoneally starting one day before delayed (day 56) AT. A loading dose of \( 300\mu g \) was used followed by \( 200 \mu g \) on days 57, 59, 62, 65, and 68 post HCT. A lethal dose of leukemia was given on day 59. (B) Survival graphs of cohorts are shown that did not receive AT on day 56 but were injected with PBS instead (**, \( p < 0.01 \) between treatment with \( \alpha \)-PD-L1 antibody (-\( \blacklozenge \) -) and isotype control (-\( \blacklozencherry \) -), \( n = 10 \)/group). (C) Syngeneic transplant recipients (B6 \( \rightarrow \) B6) received gene-modified T cells (B6) on day 56 after HCT. No significant survival differences were seen between the treatment groups (\( \alpha \)-PD-L1 (-\( \blacklozencherry \) -) or isotype control (-\( \blacklozencherry \) -) antibody, \( n = 8 \)/group). (D) PD-L1 blockade resulted in significantly increased survival rates in allogeneic transplant recipients (**, \( p < 0.005 \) between cohorts that received \( \alpha \)-PD-L1
(−−−) and isotype control (−−), n = 8/group). (E) Allogeneic transplant recipients were either treated with α-PD-L1 antibody or isotype control and monitored for GVHD as previously described. (F) Established allogeneic recipients (B10.A → B6) received 2.5 × 10^6 TCR-transduced B10.A T cells on day 56 after HCT and were treated with α-PD-L1 antibody or isotype control on days 55, 57 and 59. Additionally mice were injected with 1mg BrdU i.p. daily from day 57 to day 59. On day 60 splenocytes were sorted for GFP^+ cells and BrdU expression of gated CD8^+ T cells was determined (*, p < 0.05 between groups, n = 3/group). (G) Established allogeneic transplant recipients (B10.A → B6) or B6 mice were challenged with 1.2 × 10^6 C1498-OVA cells and T cells from the liver were analyzed on day 25 post tumor injection by flow cytometry. Cohorts were treated with either α-PD-L1 or isotype control antibody (200μg/dose) from day 10-20 every other day. Cells were either gated on CD4 and analyzed for Foxp3 expression (left bar graph; *, p < 0.05; n = 6/group), gated on CD8 and analyzed for PD-1 expression (middle bar graph; ***, p < 0.001; n = 6/group), or stimulated with α-CD3/α-CD28, gated on CD8, and analyzed for IFNγ expression (right bar graph; *, p < 0.05; n = 6/group). Black bars indicate allogeneic recipients and white bars syngeneic recipients.
Figure 1

A

B

C

D

E
Figure 2

A

![Graph showing Δcpm vs. Responder:stimulator ratio](image)

B

![Flow cytometry plots showing GFP expression](image)

C

![Flow cytometry plots showing GFP expression](image)
Figure 3

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Figure 4

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Percent survival

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<th>10</th>
<th>9</th>
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<th>7</th>
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</thead>
</table>

[d]
Figure 6

A

% PD-1 of GFP CD8+ cells

Syngeneic Allogeneic Naive

B

Percent survival

C

Percent survival

D

Percent survival

E

Lethal

10 9 8 7 6 5 4 3 2

α-PD-L1 - +

F

% BRDU of GFP CD8+

G

% Proapoptotic CD4+ T cells

Naive AML Naive AML

% PD-1 of CD8+

AML w/o PD-L1 AML + PD-L1

% MHC-II of CD8+

AML w/o PD-L1 AML + PD-L1
PD-L1 blockade effectively restores strong graft-versus-leukemia effects without graft-versus-host-disease after delayed adoptive transfer of T cell receptor gene-engineered allogeneic CD8+ T cells

Wolfgang Koestner, Martin Hapke, Jessica Herbst, Christoph Klein, Karl Welte, Joerg Fruehauf, Andrew Flatley, Dario A Vignali, Matthias Hardtke-Wolenski, Elmar Jaeckel, Bruce R Blazar and Martin G Sauer