Expression of a dominant T-cell receptor can reduce toxicity and enhance tumor protection of allogeneic T-cell therapy

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ABSTRACT

Due to the lack of specificity for tumor antigens, allogeneic T-cell therapy is associated with graft-*versus*-host disease. Enhancing the anti-tumor specificity while reducing the graft-*versus*-host disease risk of allogeneic T cells has remained a research focus. In this study, we demonstrate that the introduction of 'dominant' T-cell receptors into primary murine T cells can suppress the expression of endogenous T-cell receptors in a large proportion of the gene-modified T cells. Adoptive transfer of allogeneic T cells expressing a 'dominant' T-cell receptor significantly reduced the graft-*versus*-host toxicity in recipient mice. Using two bone marrow transplant models, enhanced anti-tumor activity was observed in the presence of reduced graft-*versus*-host disease. However, although transfer of T-cell receptor gene-modified allogeneic T cells resulted in the elimination of antigen-positive tumor cells and improved the survival of treated mice, it was associated with accumulation of T cells expressing endogenous T-cell receptors and the development of delayed graft-*versus*-host disease. The *in vivo* deletion of the engineered T cells, mediated by endogenous mouse mammary tumor virus MTV8 and MTV9, abolished graft-*versus*-host disease while retaining significant anti-tumor activity of adoptively transferred T cells. Together, this study shows that the *in vitro* selection of allogeneic T cells expressing high levels of a 'dominant' T-cell receptor can lower acute graft-*versus*-host disease and enhance anti-tumor activity of adoptive cell therapy, while the *in vivo* outgrowth of T cells expressing endogenous T-cell receptors remains a risk factor for the delayed onset of graft-*versus*-host disease.

Introduction

TCR gene transfer is an attractive strategy to direct T-cell specificity towards defined target antigens.[1-6] This approach can readily produce large numbers of antigen-specific T cells for adoptive cell therapy and no longer requires the selection and expansion of rare specificities present in the autologous TCR repertoire. Recent clinical trials have shown the feasibility and efficacy of TCR gene therapy in melanoma and synovial cell carcinoma.[7] However, the safety concerns of TCR gene therapy include the mis-pairing of introduced TCR chains with the endogenous chains expressed in the gene-modified T cells. Recent murine model experiments have indicated that TCR mis-pairing can generate auto-reactive T cells that cause toxicity when adoptively transferred into syngeneic hosts.[8] It is likely that similar toxicities can occur in humans, although it has not yet been described in clinical trials to date.

One of the most effective forms of T-cell therapy in humans is the adoptive transfer of allogeneic donor T cells into patients transplanted with hematopoietic stem

cells derived from the same donor, typically as a T-cell replete allogeneic hematopoietic stem cell transplantation (HSCT) or post allo-HSCT donor lymphocyte infusion (DLI).[9] In this scenario, allo-reactive donor T cells can efficiently attack and eliminate patient leukemia cells, resulting in cure of the malignant disease. However, since the major and minor histocompatibility antigens recognized by allo-reactive T cells are not selectively expressed in leukemia cells, but also in normal tissues, this can lead to normal tissue damage and result in graft-*versus*-host disease (GvHD).[10] Thus, enhancing the leukemia specificity while reducing the 'damaging' allo-reactive potential of donor T-cell therapy has remained the focus of research.

A recent study has demonstrated in a fully MHC-mismatched murine transplant model that TCR gene transfer into donor T cells was able to enhance graft-*versus*-leukemia (GvL) and reduce GvHD. In this study, Koestner *et al*. transferred the OT1-TCR, specific for a peptide epitope of ovalbumin, into donor T cells and achieved 20%-70% reduction of the endogenous TCR repertoire.[11] Here, we explored whether 'dominant' TCR can mediate complete inhibition of endogenous TCR expression in donor T cells *in vivo*. Together with other groups, we had previously shown that 'dominant' TCR can suppress the expression of 'non-dominant' TCR on the surface of gene-modified T cells *in vitro*.[12-14] The mechanism of TCR dominance has not yet been fully defined, but it may involve transcriptional and translational regulation as well as the efficiency of the TCR α and β chain pairing and assembly with CD3 chains prior to the migration of the TCR complex from the endoplasmic reticulum to the cell surface. The observation that modifications improving RNA translation into protein and modifications improving TCR α/β chain pairing both enhanced TCR dominance indicated that efficient protein synthesis and efficient formation of α/β heterodimers have a direct impact on TCR dominance.[12] Here, we have codon optimized the TCR gene sequences and introduced additional disulphide bonds in the constant domains to generate 'dominant' TCR. We tested the ability for these 'dominant' TCR constructs to suppress the surface expression of endogenous TCR in polyclonal primary T cells *in vitro*. We then performed *in vivo* experiments to test the working hypothesis that the introduction of a 'dominant' TCR into allogeneic donor T cells may control graft-*versus*-host toxicity, while enhancing protection against tumors expressing the target antigen recognized by the 'dominant' TCR.

Methods

Mice

Female C57BL/6, C57BL/6 (Thy1.1), BALB/c, BALB/c (Thy1.1) and DBA/J1 mice were purchased from Charles Rivers Laboratories or bred in house by UCL Biological Services. All experiments were conducted in accordance with United Kingdom Home Office regulations.

Cell lines, pentamer and peptide

Phoenix-Ecotropic (PhEco) adherent packaging cells (Nolan Laboratory) were transiently transfected with retroviral vectors for the generation of supernatant containing the recombinant retrovirus required for infection of target cells. EL4 is a murine lymphoma cell line, EL4-NP a variant, stably expressing the influenza A virus nucleoprotein (NP) (a kind gift from Dr B. Stockinger). The pentamer consisted of pNP366 bound to H2-D^b. The influenza A virus NP-derived peptide, pNP366 (ASNENMDAM) is presented by H2-D^b MHC Class-I molecules and was obtained from Proimmune.

Vectors

The retroviral F5-TCR vector pMP71-TCRα-2A-TCRβ was modified from pMX-TCRα-IRES-TCRβ (a kind gift from Prof T. Schumacher). The F5-TCR recognizes the influenza A virus NP ($NP_{366-374}$) peptide in the context of murine H2-D^b MHC Class-I. For tumor challenge experiments, we either used the F5-TCR vector or a version of it encoding for a truncated CD19 downstream of an IRES sequence as a marker of transduction. CD19 staining was used to track adoptively transferred DBA/J1 donor T cells, which lacked a congenic marker. The retroviral OTII-TCR vector pMP71-TCRα-2A-TCRβ was generated in the lab. The OTII-TCR recognizes the OVA ($OVA_{323-339}$-ISQAVHAAHAEINEAGR) peptide in the context of murine H2-A^b MHC Class-II. The control vector pMP71-iCre-IRES-GFP (inverted Cre-recombinase) was generated in the lab. The retroviral pMP71 vectors containing the F5- or OTII-TCR were modified for optimal gene (and surface TCR) expression by codon optimization and addition of an engineered cysteine bond between the TCR chains (Geneart).

Bone marrow transplantation, T-cell transfer and tumor challenge

Recipient mice (Thy1.2) were lethally irradiated (C57BL/6, 11 Gy; BALB/c, 8 Gy X-ray irradiation split into 2 fractions, separated by 24 h) and T-cell depleted bone marrow cells were injected intravenously 4 h later. Vβ11 or NP-pentamer sorted donor T cells (Thy1.1) were adoptively transferred *via* the tail vein the following day. Mock transduced T cells were used as control. On the same day, mice received i.p. injections of C57BL/6 bone marrow derived DCs pulsed with NP peptide. Five doses of 10^5U IL2 (Chiron) i.p. were administered to recipient mice, with the first dose given on the day of T-cell transfer and subsequently twice daily on the following two days.

For tumor challenge experiments, C57BL/6 recipient mice (Thy1.2) were conditioned as described above, but with the addition of subcutaneous inoculation of 10^6 EL4-NP cells on the day of bone marrow transplantation. NP-pentamer sorted donor T cells, either from DBA/J1 (Figure 4) or BALB/c (Figure 5) origin were transduced with the F5-TCR and adoptively transferred *via* the tail vein the following day. GFP sorted or mock transduced T cells were used as a control. Tumors were measured with a calliper in two different dimensions (*a* and *b*) at different intervals and the growth evaluated applying the following formula: *axbx* /4.

Results

Dominant TCR can suppress expression of endogenous TCR

In this study, we have used an MHC Class-I restricted TCR (F5-TCR) specific for a peptide epitope of the influenza virus nucleoprotein presented by H2-D^b and an MHC Class-II restricted TCR (OTII-TCR) specific for an ovalbumin-derived peptide presented by H2-A^b. Both TCR constructs were codon optimized and contained an additional disulphide bond in the constant domain to improve RNA translation and α/β chain pairing. The mod-

ified F5- and OTII-TCR genes were inserted into the retroviral pMP71 vector for gene transfer into primary murine T cells. In order to test the ability of the two TCR constructs to suppress the cell surface expression of the endogenous TCR chains, we used murine splenocytes and purified the T cells expressing Vβ8.1, 8.2 and 8.3 TCR, which represented approximately 16% of the total T cells. This allowed us to use antibodies specific for Vβ8.1,2,3 to measure the expression of endogenous TCR, and antibodies specific for the Vβ11 and Vβ5 chains to assess expression of the introduced F5-TCR and OTII-TCR, respectively. Figure 1 shows the staining profile of purified Vβ8.1,2,3 T cells that were mock transduced, or transduced with the retroviral constructs encoding the F5-TCR or the OTII-TCR. The majority of freshly transduced T cells expressed high levels of the introduced Vβ11 or Vβ5 TCR chains and sharply reduced levels of the endogenous Vβ8.1,2,3 chains. Approximately 30% of the T cells expressed both the introduced as well as the endogenous TCR chains. Less than 10% of T cells expressed the endogenous TCR only, which most likely represents untransduced T cells as the efficiency of retroviral TCR gene transfer does not usually reach 100%. We observed an inverse correlation between the level of expression of the introduced TCR and the expression levels of the endogenous TCR. For example, the mean fluorescent intensity (MFI) of Vβ11 or Vβ5 expression in the 'single-positive' T cells expressing primarily the introduced TCR was higher than the Vβ11 or Vβ5 MFI in the 'double-positive' T cells expressing both introduced as well as endogenous TCR. In the case of the F5-TCR, the MFI for the introduced β chain in the 'single' *versus* 'double-positive' T cells was 16300 *versus* 11300, and for the OTII-TCR the difference was 10300 *versus* 7815. Together, these experiments indicated that 'dominant' TCR can suppress the cell surface expression of endogenous TCR, and that the suppression was most effective in T cells expressing high levels of the 'dominant' TCR.

Reduced toxicity of allo-reactive T cells expressing 'dominant' TCR

We used an MHC-mismatched bone marrow transplantation model to test whether the introduction of the 'dominant' F5-TCR into C57BL/6 T cells reduced the toxicity of donor T-cell infusion into BALB/c mice transplanted with C57BL/6 hematopoietic stem cells. BALB/c mice received myeloablative irradiation and were then transplanted with T-cell depleted C57BL/6 (Thy1.2) bone marrow, followed by adoptive transfer of polyclonal donor T cells (Thy1.1) that were mock transduced or transduced to express the F5-TCR (Figure 2A). A short course of high-dose IL2 was given to promote *in vivo* T-cell expansion.

Following transduction of the donor T cells, we used flow cytometry sorting to purify cells expressing high levels of the introduced Vβ11 chain (Figure 2B). These T cells were adoptively transferred into the transplanted mice, which were then monitored using weight loss and survival as two objectively measurable criteria of potential toxicity caused by the infused T cells. After three weeks the animals were sacrificed to analyze the number and the phenotype of the engrafted T cells. Figure 2C shows that the measured weight of mice treated with F5-TCR transduced T cells was similar to the weight of control mice receiving a bone marrow transplant, but no T cells. In contrast, mice treated with mock transduced donor T cells showed substantial weight loss which was most pronounced in the first week after T-cell transfer (Figure 2C). Comparison of the weight score at day 8 showed that

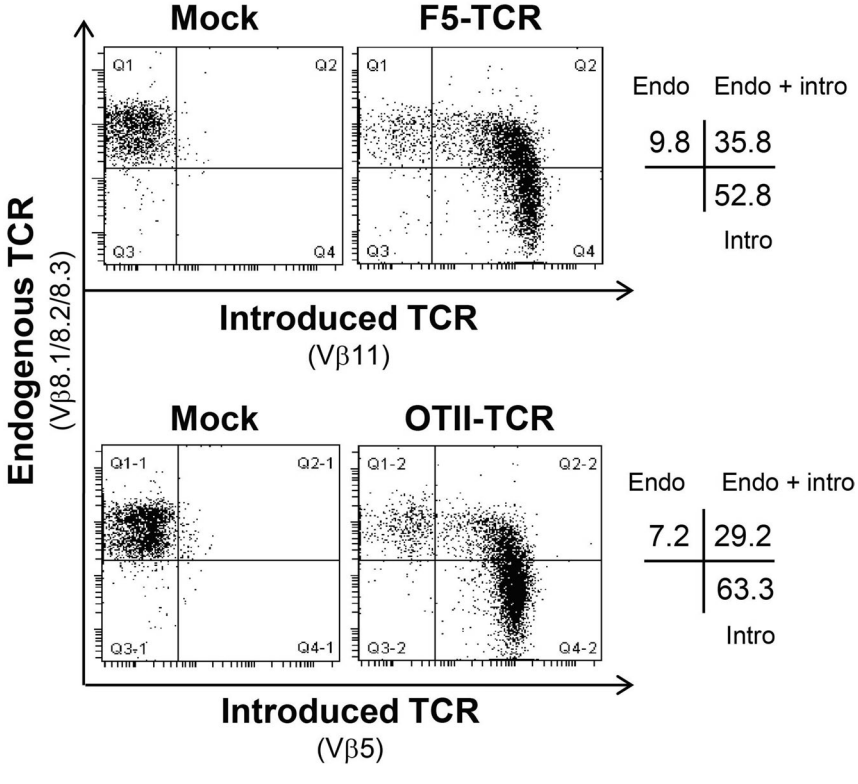

Figure 1. TCR transfer suppresses expression of endogenous TCR chains. BALB/c splenocytes were Vβ8 sorted (representing one endogenous TCR Vβ chain family) followed by mock transduction or transduction with the F5- or OTII-TCR and then stained with antibodies against CD3, Vβ8 (endogenous TCR), Vβ11 (F5-TCR) or Vβ5 (OTII-TCR) to assess the expression levels of endogenous (endo) and introduced (intro) TCR. Plots show live-gated CD3$^+$ T cells.

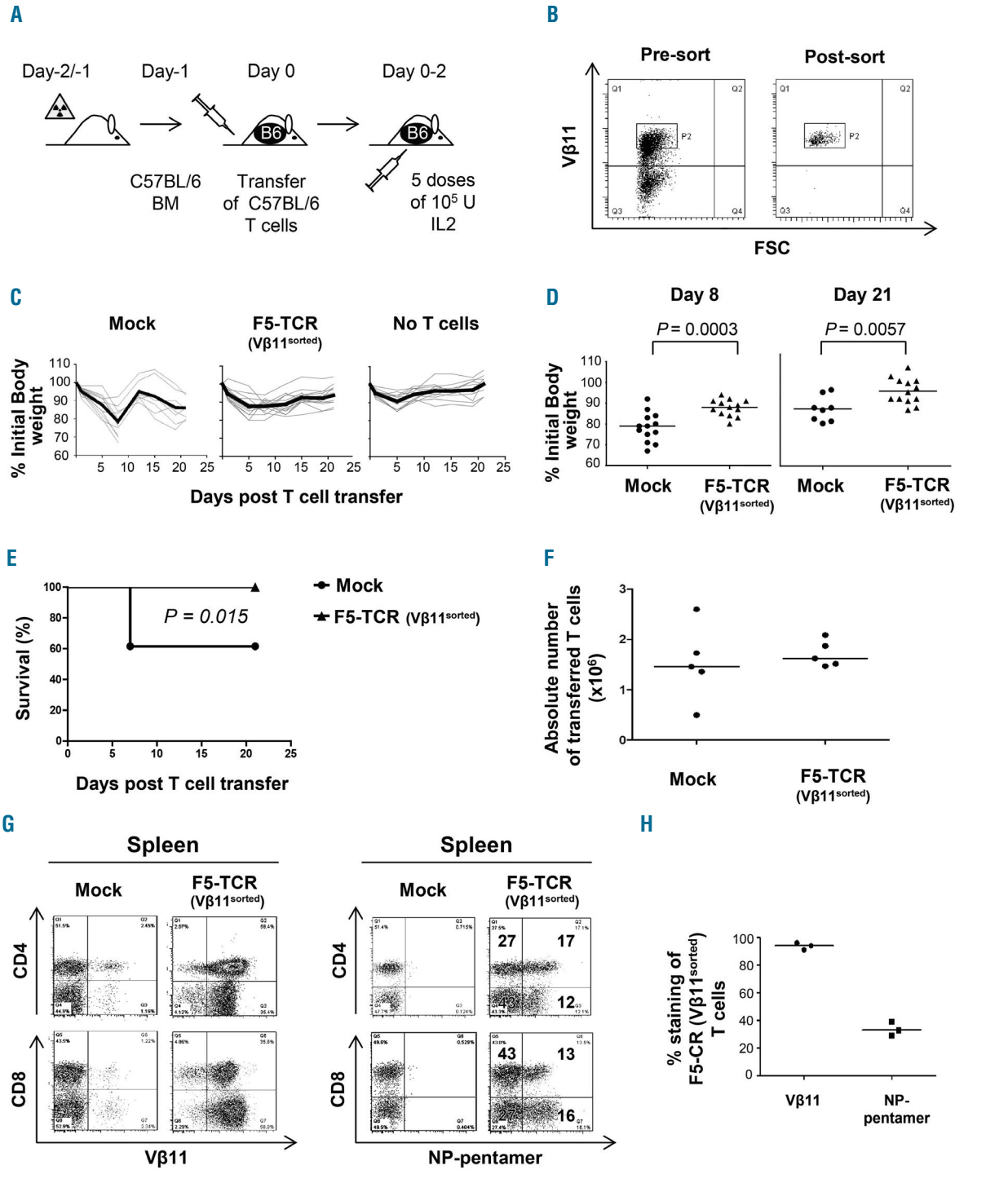

Figure 2. Allogeneic T cells expressing introduced TCR display reduced toxicity in MHC-mismatched bone marrow transplantation (BMT) recipients. (A) Allogeneic chimeras were generated by lethal irradiation of recipient BALB/c mice (Thy1.2) followed by reconstitution with T-cell depleted (TCD) C57BL/6 bone marrow (BM). Mice received 3x10^6 C57BL/6 T cells (Thy1.1) transduced with the F5-TCR sorted to express high levels of the introduced Vβ11 chain. Control animals received 3x10^6 mock transduced C57BL/6 T cells (Thy1.1) or no T cells. To stimulate antigen driven expansion of F5-TCR donor T cells all mice were injected intraperitoneally with 0.5x10^6 C57BL/6 DCs loaded with NP peptide. 10^{5}U of IL2 were administered on the day of T-cell transfer and twice daily for the following two days. (B) Staining profile of F5-TCR transduced T cells before and after Vβ11 sorting. The indicated P2 gate representing the top 33% of Vβ11-expressing T cells was used for FACS sorting. Purity after sorting is shown. Plots show live-gated Thy1.1$^+$ lymphocytes. (C) Kinetics of weight loss observed in the 3 groups of mice [(mock and F5-TCR (Vβ11^{sorted}) T cells: n=13; no T cells: n=12)]. Faint black lines represent individual mice and the bold black line represents the average of weight loss per group. Pooled results of 3 independent experiments are shown. (D) Weight loss on day 8 and day 21 (end point). Symbols represent individual mice, bars show group averages. *P* value for mock *versus* F5-TCR (Vβ11^{sorted}) transduced T cells; *P*=0.0003 (weight loss on day 8: n=13 mice per group) and *P*=0.0057 [(weight loss on day 21, mock transduced T cells: n=8; F5-TCR (Vβ11^{sorted}) T cells: n=13)]. Pooled results of 3 independent experiments are shown. (E) Kaplan-Meier survival plot for mice receiving mock or F5-TCR (Vβ11^{sorted}) transduced T cells. *P*=0.015; n=13 mice per group. Pooled results of 3 independent experiments are shown. (F) Absolute number of transferred Thy1.1 donor T cells in the spleens of recipient mice. Symbols represent individual mice, bars show group averages (n=5 mice per group). One representative experiment of 2 is shown. (G) *Ex vivo* phenotypical analysis of spleens. Splenocytes from mice that had received mock or F5-TCR (Vβ11^{sorted}) transduced T cells were stained with antibodies against Thy1.1, CD4, CD8, Vβ11 and NP-pentamer. Plots show live-gated Thy1.1$^+$ T cells of one representative mouse per group. (H) Summary of percentages of F5-TCR (Vβ11^{sorted}) donor T cells binding Vβ11 antibody and NP-pentamer of all mice (n=3).

mice treated with mock transduced donor T cells lost significantly more weight than mice treated with F5-TCR transduced T cells (Figure 2D). In fact, 5 out of 13 mice succumbed to severe weight loss or had to be sacrificed in accordance with UK Home Office regulations (Figure 2E). The scoring of the surviving mice at week 3 after T-cell transfer showed that the group treated with mock transduced T cells continued to weigh significantly less than the F5-TCR group (Figure 2D). Although these data indicate that the F5-TCR prevented GvHD-associated weight loss in the first three weeks, our experiments did not test whether F5-TCR transduced T cells might cause long-term pathology such as chronic GvHD. At week 3, all mice were sacrificed to analyze the level of T-cell engraftment and TCR expression. The absolute number of engrafted donor Thy1.1 T cell in the spleen of recipient mice was similar for mock and F5-TCR transduced T cells (Figure 2F). Flow cytometric analysis showed that the majority of F5-TCR transduced T cells continued to express high levels of the introduced Vβ11 chain. Surprisingly, staining with H2-Db/NP-peptide pentamer reagents showed that the majority of the Vβ11$^+$ donor T cells were unable to bind the pentamer, indicating that they did not express the correctly paired F5-TCR α/β heterodimer, although they expressed high levels of the F5-TCR β chain (Figure 2G and H).

Pentamer-negative donor T cells expand preferentially in vivo

We explored whether pentamer-negative T cells existed before injection, or whether they arose *in vivo* from T cells expressing F5-TCR α/β heterodimer. Most of the freshly transduced T cells expressing high levels of the Vβ11 chain did bind the pentamer, although a minority of T cells were pentamer-negative (Figure 3A). If the pentamer-negative, potentially allo-reactive T cells expanded preferentially after *in vivo* transfer, then exclusion of these cells before adoptive transfer should avoid the emergence of pentamer-negative T cells. We therefore FACS purified transduced donor T cells to obtain a highly pure Vβ11$^+$/pentamer$^+$ population (Figure 3B). These C57BL/6 donor T cells (Thy1.1) were then adoptively transferred into

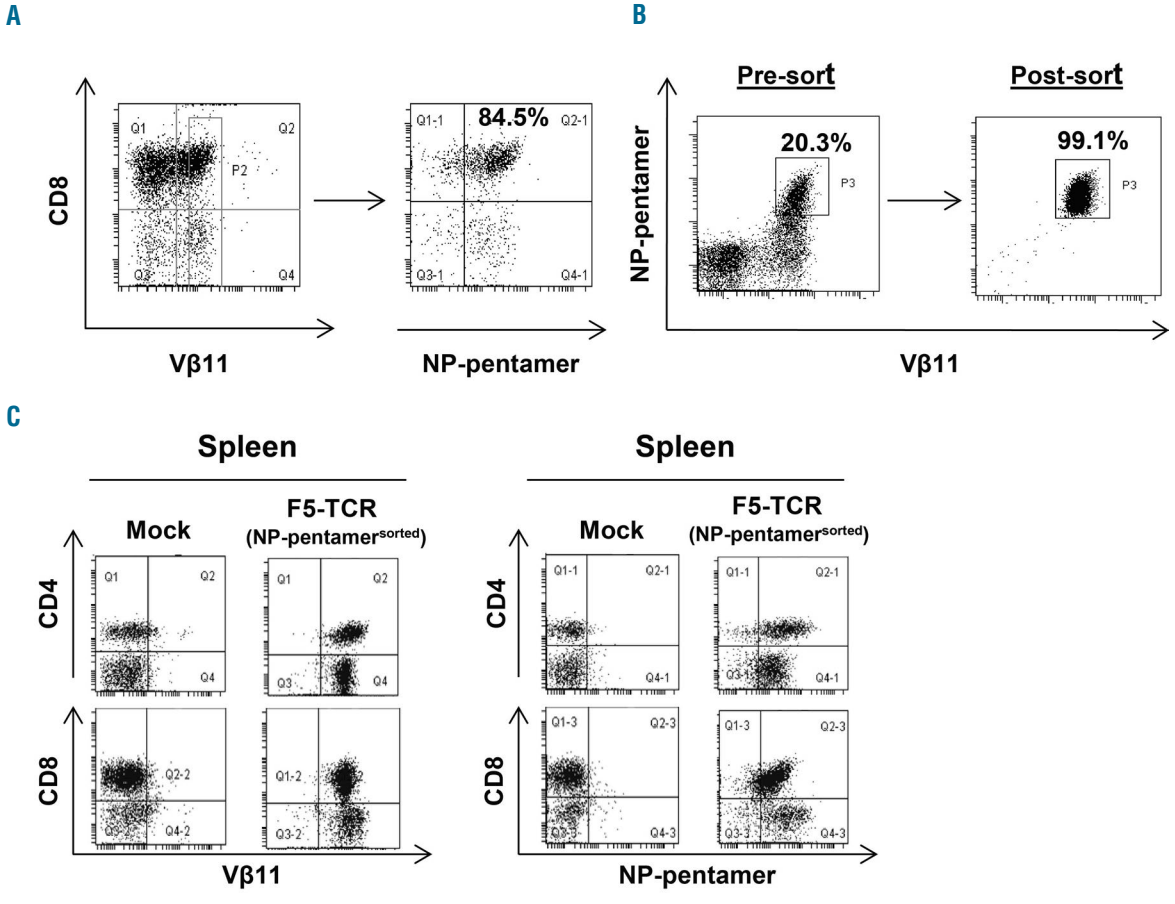

Figure 3. Pentamer sorted T cells retain NP-pentamer binding in MHC-mismatched bone marrow transplantation (BMT) recipients. (A) The pre-injection Vβ11$^+$ T-cell population contains a small number of NP-pentamer negative cells. C57BL/6 splenocytes (depleted of the endogenous Vβ11 T-cell population) were transduced with the F5-TCR and stained with antibodies against CD8 and Vβ11 (left panel). The right panel shows the NP-pentamer binding profile of the gated Vβ11$^+$ T-cell population. (B) FACS sorting of NP-pentamer binding T cells. C57BL/6 T cells were transduced with the F5-TCR and stained with antibodies against Vβ11 and NP-pentamer. The P3 gate was used for FACS sorting. Purity after sorting is shown in the right panel. Plots show live-gated Thy1.1$^+$ lymphocytes. (C) *Ex vivo* phenotypical analysis of C57BL/6 donor T cells (Thy1.1) after adoptive transfer into BALB/c recipient mice previously transplanted with T-cell depleted C57BL/6 bone marrow. Donor T cells were transduced with the F5-TCR and purified as shown in (B); mock transduced T cells were used as a control. Three weeks after T-cell transfer, mice were sacrificed and splenocytes were stained with antibodies against Thy1.1, CD4, CD8, Vβ11 and NP-pentamer. Plots show Vβ11 staining (left panels) and NP-pentamer staining (right panels) of live-gated Thy1.1$^+$ donor T cells of mice that received mock transduced T cells or F5-TCR (NP-pentamer^{sorted}) T cells.

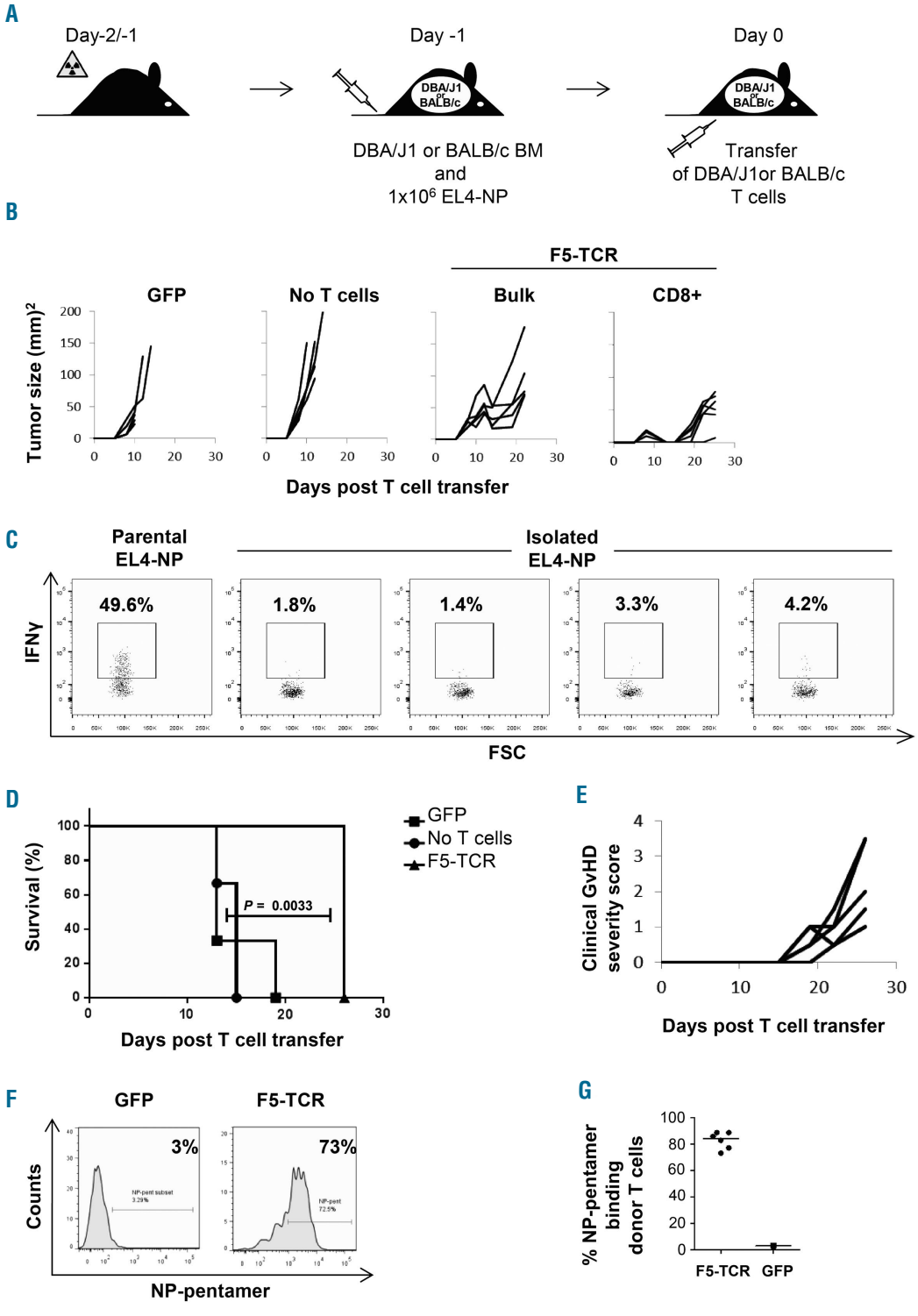

Figure 4. TCR transfer enhanced the anti-tumor effects of allogeneic T-cell therapy. (A) Allogeneic chimeras were generated by lethal irradiation of C57BL/6 mice transplanted with allogeneic T-cell depleted bone marrow followed by EL4-NP tumor challenge and allogeneic T-cell therapy. The allogeneic bone marrow and T cells were either of DBA/J1 origin (see Figure 4) or BALB/c origin (see Figure 5). (B) Tumor-bearing mice were treated with $1x10^6$ F5-TCR-CD19 (NP-pentamer^{sorted}) bulk T cells or purified CD8$^+$ T cells from DBA/J1 donors. Control mice received no T cells or $1x10^6$ GFP transduced and FACS sorted T cells from DBA/J1 donors. Tumor growth observed in the 4 groups of mice is shown (n=5, except for the CD8$^+$ group n=6). *P* values on day 11 post T-cell transfer are: GFP control T cells *versus* bulk F5-TCR-CD19 T cells is non-significant (ns); bone marrow transplantation (BMT) control *versus* bulk F5-TCR-CD19 T cells (*P*=0.02) and GFP control T cells *versus* CD8$^+$ F5-TCR-CD19 T cells (*P*=0.0002). (C) EL4-NP tumors from mice treated with F5-TCR-CD19 CD8$^+$ T cells were re-isolated at the time point when mice were sacrificed due to GvHD toxicity. They were cultured for four days and then mixed with F5-TCR transduced T cells to analyze IFNγ responses. Parental EL4-NP cells were used as a positive control. FACS plots show percentage IFNγ production of transduced CD8$^+$ T cells. (D) Kaplan-Meier survival plot for mice receiving GFP control T cells, F5-TCR-CD19 bulk T cells or no T cells. (E) Clinical graft-*versus*-host disease (GvHD) severity score of mice treated with F5-TCR-CD19 CD8$^+$ T cells. Mice were weighed and assessed for signs of clinical GvHD 2-3 times a week (see Methods). GvHD score is shown. (F) *Ex vivo* phenotypical analysis of mice treated with GFP control T cells or F5-TCR-CD19 CD8$^+$ T cells. Splenocytes were stained with antibodies against CD19, CD4, CD8, and NP-pentamer. Plots show the level of pentamer binding of live-gated GFP$^+$ T cells (left) and live-gated CD19$^+$T cells (right). Combined data of all analyzed mice are shown (G). Data of one representative mouse per group are shown or combined data of all analyzed mice (F5-TCR n=6; GFP T cells n=1).

BALB/c recipients previously transplanted with C57BL/6 bone marrow cells (Thy1.2). As before, recipient mice were sacrificed three weeks after T-cell transfer to determine TCR expression. The majority of Thy1.1 donor T cells expressed high levels of Vβ11 and bound H2-Db/NP-pentamer (Figure 3C). However, some of the re-isolated T cells did not bind pentamer *ex vivo* (Figure 3C). It is difficult to know whether these cells derived from the small number (<1%) of pentamer-negative cells present prior to transfer (Figure 3B) or whether they lost pentamer binding *in vivo*.

TCR-transfer improves tumor protection of allogeneic donor T-cell therapy

We next explored whether transfer of the 'dominant' F5-TCR enhanced the ability of donor T cells to control tumor growth. To address this question, we challenged C57BL/6 mice with syngeneic EL4 lymphoma cells expressing the TCR-recognized NP antigen. Prior to tumor challenge, the C57BL/6 mice ($H2^b$) were transplanted with T-cell depleted bone marrow from MHC-mismatched BALB/c (H2d) or DBA/J1 (H2q) donor mice (Figure 4A). While H2d mice express MHC class-II IE molecules, this is not the case in H2b and H2q mice which lack the gene encoding the alpha chain of the IE alpha/beta heterodimer. Lack of IE molecules results in lack of the presentation of endogenous super-antigens that are encoded by mouse mammary tumor virus MTV8 and MTV9 present in the genome of most inbred mouse strains.[15] The presentation of MTV8 and MTV9 by IE leads to the selective reduction of T cells expressing the Vβ11 chain in the natural repertoire of BALB/c mice.[16] Since the F5 TCR uses Vβ11, this is expected to reduce T-cell engraftment in C57BL/6 mice reconstituted with IE-positive BALB/c bone marrow, while allowing efficient engraftment and persistence in mice reconstituted with IE-negative DBA/J1 bone marrow.

We first investigated allogeneic T-cell therapy of EL4-NP tumor in C57BL/6 mice transplanted with DBA/J1 bone marrow. DBA/J1 donor T cells were transduced with an F5-TCR construct containing a CD19 marker (see Methods) to identify transduced T cells *in vivo*. As control, donor T cells were transduced with GFP. We used flow cytometry to sort donor T cells binding high levels of NP-pentamer (F5-TCR) or for GFP expression (control) prior to adoptive transfer. Compared to mice that did not receive any T cells, the transfer of allogeneic GFP transduced T cells did not reduce tumor growth (Figure 4B). In contrast, treatment with allogeneic bulk T cells transduced with the F5-TCR resulted in substantial reduction in tumor burden. Transfer of purified TCR transduced $CD8^+$ T cells was more effective than transfer of bulk T cells. This is possibly due to our previous observation that $CD8^+$ T cells expressing this TCR recognized approximately 10-fold lower concentration of antigen than $CD4^+$ T cells expressing the same TCR.[17] However, despite the near elimination of tumors after treatment with DBA/J1 $CD8^+$ T cells, protection was incomplete and tumors relapsed in most animals. We explored whether this was due to immune-editing and tumor escape. The analysis of tumors re-isolated from the mice treated with TCR transduced

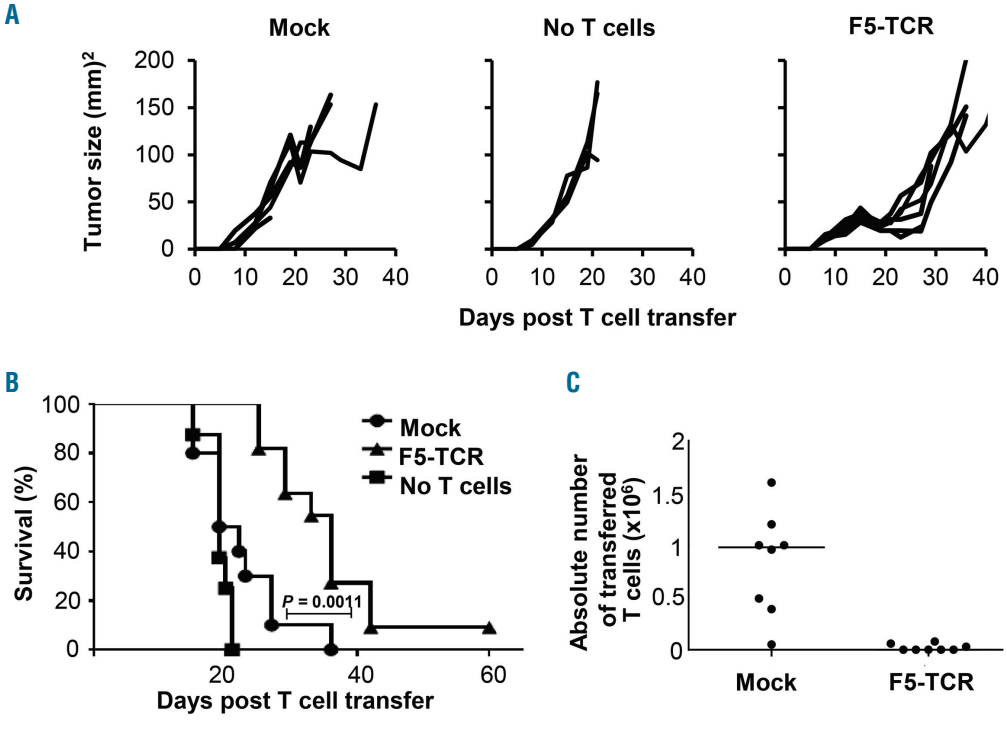

Figure 5. Depletion of TCR transduced T cells reduces toxicity and tumor protection. In these experiments, C57BL/6 mice were transplanted with BALB/c bone marrow and treated with TCR transduced BALB/c donor T cells (see Figure 4A). (A) EL4-NP tumor growth in mice receiving no T cells (n=3) or treated with mock (n=5) or F5-TCR transduced bulk T cells (n=7). One representative experiment of 2 is shown. (B) Kaplan-Meier survival plot for mice receiving mock T cells (n=10), F5-TCR T cells (n=11) or no T cells (n=8). Pooled data from 2 independent experiments are shown. (C) Absolute numbers of transferred mock or F5-TCR transduced T cells in the spleen of treated mice, showing selective depletion of $V\beta11^+$ F5-TCR T cells.

CD8+ T cells revealed that they no longer stimulated antigen-specific cytokine production by responder T cells expressing the F5-TCR (Figure 4C). This indicates that T-cell therapy was effective, and that immune-edited escape variants were responsible for tumor relapse in treated mice.

The impressive anti-tumor activity of F5-TCR transduced DBA/J1 T cells was also associated with improved survival of F5-TCR treated mice. Mice that did not receive any T cells or were treated with GFP control T cells died between day 13-19 with progressive tumor growth (Figure 4D). In contrast, mice treated with F5-TCR transduced CD8+ T cells did not die because of tumor growth, but instead had to be sacrificed at day 26 because of development of GvHD as assessed by clinical and histological GvHD severity scoring (Figure 4E and *Online Supplementary Figure S1*). When mice were sacrificed, we used flow cytometry analysis of splenocytes to detect the transferred DBA/J1 T cells by their expression of GFP (control T cells) or CD19 (TCR transduced T cells). Both, GFP+ and CD19+ T cells were readily detectable in the spleen of treated mice. As expected, the gated GFP+ control T cells did not bind the NP-pentamer, while most of the gated CD19+ T cells were NP-pentamer-positive (Figure 4F). However, the summary data of all mice show that 15%-30% of the CD19+ T cells had lost pentamer staining, suggesting that these T cells no longer expressed high levels of the F5-TCR, but instead potentially allo-reactive endogenous or mis-paired TCR that could account for the observed GvHD toxicity (Figure 4G).

Finally, we explored whether the toxicity that was observed approximately three weeks after T-cell transfer could be reduced by limiting the *in vivo* persistence of the F5-TCR transduced T cells. Therefore, we performed tumor protection experiments in C57BL/6 mice reconstituted with BALB/c bone marrow. In these mice, treatment with F5-TCR transduced BALB/c T cells delayed tumor growth when compared to treatment with mock transduced T cells (Figure 5A). The delay in tumor growth was associated with improved survival of mice treated with F5-TCR transduced BALB/c T cells (Figure 5B). In this setting, the mice did not develop any signs of GvHD toxicity but succumbed to progressive tumor growth. When mice treated with control or F5-TCR T cells reached lethal tumor burden, they were sacrificed and analyzed to determine the number of engrafted BALB/c T cells. As expected, control transduced T cells not bearing the F5-TCR were readily detectable in the spleen of treated mice, while the Vβ11-positive T cells expressing the F5-TCR were selectively depleted (Figure 5C). The data indicated that the depletion of the F5-TCR expressing T cells prevented any detectable GvHD toxicity, but did not abolish an improvement in tumor protection compared to mock transduced T cells.

Discussion

Strategies to reduce graft-*versus*-host disease and enhance anti-tumor activity of allogeneic T cells remain an important area of research.[18] Various approaches have been developed, including the transfer of purified memory T cells[19,20] or the *in vitro* depletion of allo-reactive T cells stimulated with recipient antigen-presenting cells.[21-23] Similarly, *in vitro* stimulation of allo-reactive T cells in the presence of co-stimulation blockade was used to induce T-cell anergy.[24] An elegant alternative to the *in vitro* depletion of allo-reactive T cells is the introduction of 'suicide genes' that can be triggered *in vivo* in patients developing GvHD after T-cell transfer.[25-28] Most recently, the disruption of endogenous TCR genes has been achieved using zinc finger nucleases, generating T cells unable to respond to allo-stimulation *in vitro*.[29,30] The introduction of TCR or single chain antibody constructs has been used to equip these TCR-disrupted T cells with new specificities.

In this study, we explored a simplified strategy to simultaneously suppress endogenous TCR expression while introducing therapeutic TCR. The described approach is based on the working hypothesis that 'dominant' TCR can redirect T-cell specificity towards tumor antigens and also suppress the expression of endogenous TCR that are required for donor T cells to recognize major and minor histocompatibility antigens involved in GvHD.

We have taken advantage of previous reports showing that codon optimization and introduction of additional cysteine bonds can improve TCR expression and reduce unwanted mis-pairing.[31-33] Using modified F5- and OTII-TCR constructs, we found that both were effective in suppressing the expression of endogenous TCR in the majority of transduced primary T cells. We found that *in vitro* selection of transduced T cells for high-level expression of introduced TCR β chain (using antibodies) or for the correctly paired TCR α/β combination (using pentamer) was required to reduce expression of potentially allo-reactive endogenous TCR. Following *in vivo* transfer, we observed the selective expansion of T cells that lost pentamer binding, indicating that they expressed endogenous or mis-paired TCR that might be driven by alloantigen recognition *in vivo*. While pentamer selected T cells remained largely pentamer-positive when they were transferred into tumor-free mice, the transfer into tumor-bearing recipients resulted in the accumulation of pentamer-negative T cells. A major difference between tumor-free and tumor-bearing mice is the absence and presence, respectively, of the F5-TCR recognized target antigen. It is possible that the stimulation *via* the F5-TCR lowers the triggering threshold for endogenous or mis-paired TCR that can recognize allo-antigens. Once triggered, these allo-reactive T cells may preferentially expand in the MHC-mismatched recipient mice. The results described here are similar to the results obtained by Koestner *et al.*, when they showed that allogeneic T cells engineered to express the OT1-TCR displayed increased GvL and reduced GvHD activity.[11] In these studies, the engineered T cells contained some untransduced cells and the OT1-TCR achieved only 20%-70% reduction in the level of endogenous TCR expression. In our case, we purified T cells for high expression of the introduced F5-TCR, and this 'dominant' TCR was capable of suppressing endogenous TCR expression to undetectable levels. Nevertheless, our results show that, despite the use of purified T-cell populations, the *in vivo* exposure to allo-antigens provided a strong selection for reduced F5- and increased endogenous TCR expression. When the F5-TCR transduced allogeneic T cells were able to expand and persist in large numbers, they mediated effective control of EL4 tumors expressing the TCR recognized target antigen. Only immune-edited tumor variants that were no longer recognized by the TCR transduced T cells were able to grow progressively in

these mice. However, persistence of the TCR transduced allogeneic T cells led to the delayed development of severe GvHD toxicity, which was associated with the enrichment of T cells that no longer expressed the F5-TCR α/β combination. The GvHD toxicity was not seen when the *in vivo* persistence of the F5-TCR transduced T cells was limited. This was achieved by the use of recipient mice presenting the Vβ11 depleting MTV8 and MTV9 antigens in the context of MHC class-II IE molecules. In this setting, the F5-TCR was still able to mediate anti-tumor activity, but the lack of T-cell persistence was associated with the lack of GvHD toxicity. These experiments indicated that 'dominant' TCR gene transfer can substantially enhance the anti-tumor activity of allogeneic T-cell therapy, but that long-term persistence of these T cells leads to the expansion of allo-reactive T cells that no longer express high levels of the 'dominant' TCR. Although this relatively simple strategy of using 'dominant' TCR can clearly improve tumor immunity, the complete removal of the risk of GvHD requires the complete depletion of endogenous TCR to avoid *in vivo* expansion of allo-reactive specificities.[29,30]

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