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Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy

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The transfer of T cell receptor (TCR) genes can be used to induce immune reactivity toward defined antigens to which endogenous T cells are insufficiently reactive. This approach, which is called TCR gene therapy, is being developed to target tumors and pathogens, and its clinical testing has commenced in patients with cancer. In this study we show that lethal cytokine-driven autoimmune pathology can occur in mouse models of TCR gene therapy under conditions that closely mimic the clinical setting. We show that the pairing of introduced and endogenous TCR chains in TCR gene-modified T cells leads to the formation of self-reactive TCRs that are responsible for the observed autoimmunity. Furthermore, we demonstrate that adjustments in the design of gene therapy vectors and target T cell populations can be used to reduce the risk of TCR gene therapy–induced autoimmune pathology.

Most human tumor-associated antigens that are shared between individuals consist of nonmutated self antigens[1]. Consequently, the endogenous T cell repertoire that reacts to these antigens will generally be small in size and activity[2,3]. Given these limitations, it has been argued that infusion of the 'missing' T cell repertoire, which can recognize such antigens with high avidity, could be valuable[4,5]. As the antigen specificity of a T cell is controlled by the α and β chains of the TCR, such a tumor-reactive T cell repertoire can be generated by the introduction of genes encoding tumor-reactive TCR αβ chains in peripheral T cells, an approach called TCR gene therapy. In addition to its potential value in targeting tumors, TCR gene therapy could also be used to enhance T cell immunity to pathogens such as HIV[6].

Studies in mice have shown that TCR-transduced T cells can be active against tumors[7–9], and two phase 1 clinical trials have shown that it is feasible to translate TCR gene therapy to the clinic[10,11]. However, a number of potential safety concerns associated with TCR gene therapy have been noted[4]. A particular concern is the possibility that the assembly of endogenous and newly introduced TCR chains would lead to the formation of TCRs with undefined specificity potentially yielding self-reactive T cells. Previous work, in which infusion of TCR-transduced T cells was combined with antigen-specific vaccination in mice, showed no evidence of toxicity[12]. However, it has since become clear that the *in vivo* activity of adoptively transferred T cells can be enhanced when the endogenous T cell pool has largely been removed by lymphodepleting chemotherapy or total body irradiation (TBI)[5,9]. Furthermore, data from other adoptive T cell transfer studies suggest that the use of more highly expressed TCR genes[9], optimized T cell activation conditions during *in vitro* T cell culture[13] and *in vivo* interleukin-2 (IL-2) administration[14,15] can further enhance the *in vivo* function of TCR-transduced T cells. However, it is unclear whether such improved therapeutic conditions can increase the likelihood of TCR gene transfer–induced toxicity.

RESULTS

Lethal autoimmune pathology in mice after TCR gene transfer

To investigate the effect of more efficient engraftment regimens on the potential side effects of TCR-transduced T cells, we used cohorts of C57BL/6J mice that had been rendered lymphopenic by sublethal TBI. We gave the mice small numbers of T cells that had been retrovirally gene modified with the ovalbumin-specific OT-I TCR (1×10^6 TCR-transduced $CD8^+$ T cells, translating to the same dose used in the clinic). T cell infusion was followed by high-dose IL-2 administration between days 10 and 12 after transfer (**Fig. 1a**), when a substantial amount of lymphopenia-induced T cell proliferation has already occurred[9]. Around 14 d after transfer, OT-I TCR–transduced T cell recipients showed marked cachexia, but recipients of nontransduced T cells that had been activated under the same conditions did not (**Fig. 1b**). In most mice, cachexia progressed rapidly, with the mice needing to be killed within 24 h (**Fig. 1c**). At necropsy, the major organs (liver, kidney, spleen etc) and blood appeared severely anemic, consistent with bone marrow failure (data not shown). Histopathological analysis showed a profound loss of hematopoietic activity in the bone marrow (**Fig. 1d**) and spleen (**Supplementary Fig. 1a**), as well as depletion of lymphocytes in lymph nodes, Peyer's patches, thymus and spleen (shown for spleen in **Supplementary Fig. 1a**). Furthermore, 39% of mice had marked

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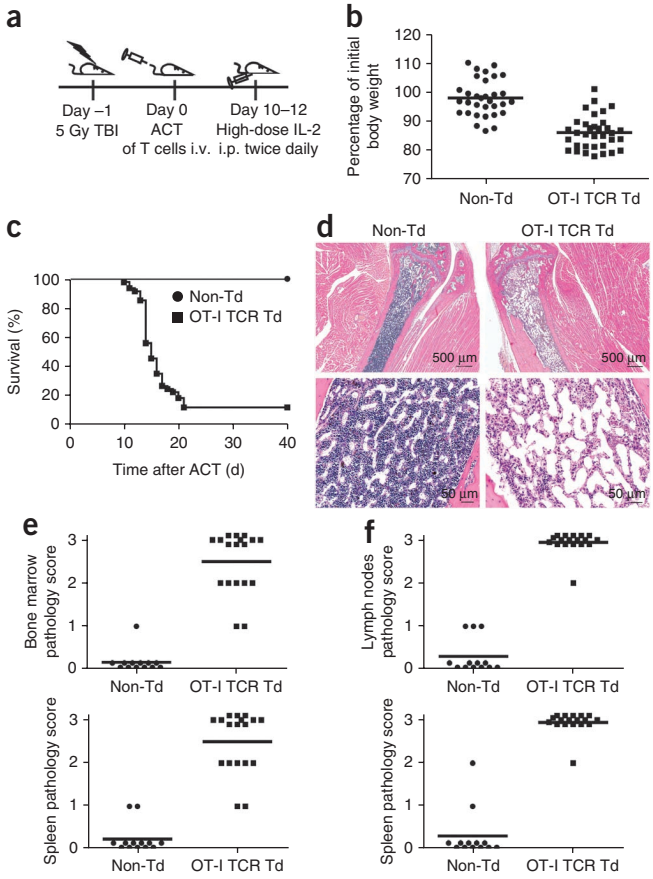


Figure 1 Lethal autoimmune pathology induced by OT-I TCR–modified T cells. (**a**) Experimental setup. (**b**) Cachexia in recipients of OT-I TCR–transduced (Td) T cells. Shown are pooled results of six independent experiments. Symbols represent individual mice; bars indicate group averages. For non-Td versus OT-I TCR-Td, $P < 0.0001$. (**c**) Kaplan-Meyer survival plot for recipients of OT-I TCR-Td T cells ($n = 47$; Td efficiency: 53%–72%) or non-Td T cells ($n = 33$). Shown are pooled results of seven independent experiments. P value of non-Td versus OT-I TCR-Td: $P < 0.0001$. (**d**) Bone marrow sections from recipients of OT-I TCR-Td T cells or non-Td T cells showing the marked disappearance of hematopoietic cells in recipients of OT-I TCR-Td T cells. (**e**) Histopathological scoring showing reduction in hematopoiesis in bone marrow and spleen of recipients of OT-I TCR-Td T cells. P values: OT-I TCR-Td versus non-Td bone marrow: $P < 0.0001$; OT-I TCR-Td versus non-Td spleen: $P < 0.0001$. (**f**) Histopathological scoring showing depletion of lymphocytes in lymph nodes and spleen of OT-I TCR-Td T cell recipients. P values: OT-I TCR-Td versus non-Td lymph nodes: $P < 0.0001$; OT-I TCR-Td versus non-Td spleen: $P < 0.0001$.

pancreatitis (**Supplementary Fig. 1a**,**b**) and 17% of mice had colitis (**Supplementary Fig. 1a**,**b**). The development of this pathology depended on the infusion of TCR-transduced T cells, as recipients of nontransduced T cells that also received TBI and IL-2 administration showed no pathology (**Fig. 1d–f** and **Supplementary Fig. 1**).

On the basis of the incidence of the various pathologies, we considered the depletion of lymphocytes in all lymphoid organs studied (100% of mice analyzed) along with a more general destruction of the hematopoietic compartment (88.2% of mice analyzed) to be the defining features of the pathology (**Fig. 1e**,**f** and **Supplementary Fig. 1b**). We observed this pathology in seven independent experiments using 48 mice that received OT-I TCR–transduced T cells, with an overall mortality of 87.5% (**Fig. 1c**).

Given that we found pathology in both Ovalbumin-transgenic mice (data not shown) and C57BL/6J mice, which do not express the cognate antigen recognized by the OT-I TCR (**Fig. 1**), disease development was evidently not due to on-target activity of the TCR-transduced T cells. However, this did not rule out the possibility of a trivial cross-reactivity of the OT-I TCR with a self antigen expressed in C57BL/6J mice. To address this possibility, we treated cohorts of C57BL/6J mice as before (**Fig. 1a**) and adoptively transferred to them OT-I TCR–transduced T cells, OT-I TCR–transgenic T cells or GFP-transduced T cells (all activated under the same conditions). As in previous experiments, recipients of OT-I TCR–transduced T cells developed fatal autoimmune pathology. By contrast, neither the recipients of OT-I TCR–transgenic T cells nor the recipients of GFP-transduced T cells developed cachexia, and 100% of these mice survived (**Fig. 2a**). Together, these data show that the observed autoimmune pathology is not due to cross-reactivity of the introduced TCR but depends on transduction of the infused T cells with TCR genes. Reflecting this dependency on infusion of a TCR gene–modified cell graft, we have called this pathology TCR gene transfer–induced graft-versus-host disease (TI-GVHD).

TI-GVHD can be explained by mixed TCR dimer formation

The above data are consistent with the possibility that the formation of mixed TCR dimers consisting of endogenous and exogenous TCR chains caused the observed TI-GVHD. To test whether the expression of mixed TCR dimers on TCR-transduced T cells is sufficient to induce TI-GVHD, we treated C57BL/6J mice as before (**Fig. 1a**) and administered an adoptive transfer of cells transduced with either the OT-I TCR α-chain or the OT-I TCR β-chain. Notably, we observed TI-GVHD in recipients of T cells transduced with only the TCR α-chain and also in recipients of T cells transduced with only the TCR β-chain, with slightly different kinetics and incidence (**Fig. 2b**,**c**). As a second test for the involvement of mixed TCR dimer–expressing cells, we investigated the effect of *in vivo* depletion of such cells just before IL-2 administration. We transduced T cells with a modified TCR α-chain that carries an extracellular myc tag that has been shown to render transduced T cells sensitive to *in vivo* depletion by the administration of tag-specific monoclonal antibodies[16]. *In vivo* depletion of T cells transduced with the myc-tagged OT-I TCR α-chain in peripheral blood was almost complete in monoclonal antibody (mAb)-treated mice (**Fig. 2d**), and most of these mice did not develop TI-GVHD (75% survival; **Fig. 2e**). By contrast, recipients of T cells transduced with the myc-tagged OT-I TCR α-chain that did not receive tag-specific mAb and recipients of T cells transduced with the nonmodified OT-I TCR α-chain (with or without mAb administration) all developed fatal TI-GVHD (**Fig. 2e**).

To determine the underlying mechanism behind TI-GVHD, we analyzed serum cytokine concentrations in C57BL/6J mice that were treated as before (**Fig. 1a**) and that had received an adoptive transfer of OT-I TCR–transduced T cells or nontransduced T cells. This revealed a moderate elevation of monocyte chemoattractant protein-1 and tumor necrosis factor concentrations in recipients of T cells transduced with the OT-I TCR (**Supplementary Fig. 2a**) and a substantial increase in interferon-γ (IFN-γ) concentrations (average 4.7-fold increase; **Fig. 3a**). To assess whether increased serum IFN-γ concentrations reflected a causal role of IFN-γ in the pathogenesis of TI-GVHD, we gave cohorts of C57BL/6J mice either OT-I TCR–transduced C57BL/6J cells or OT-I TCR–transduced *Ifng*$^{-/-}$ (lacking the gene encoding IFN-γ) C57BL/6J cells. Notably, the majority of mice that received TCR-transduced

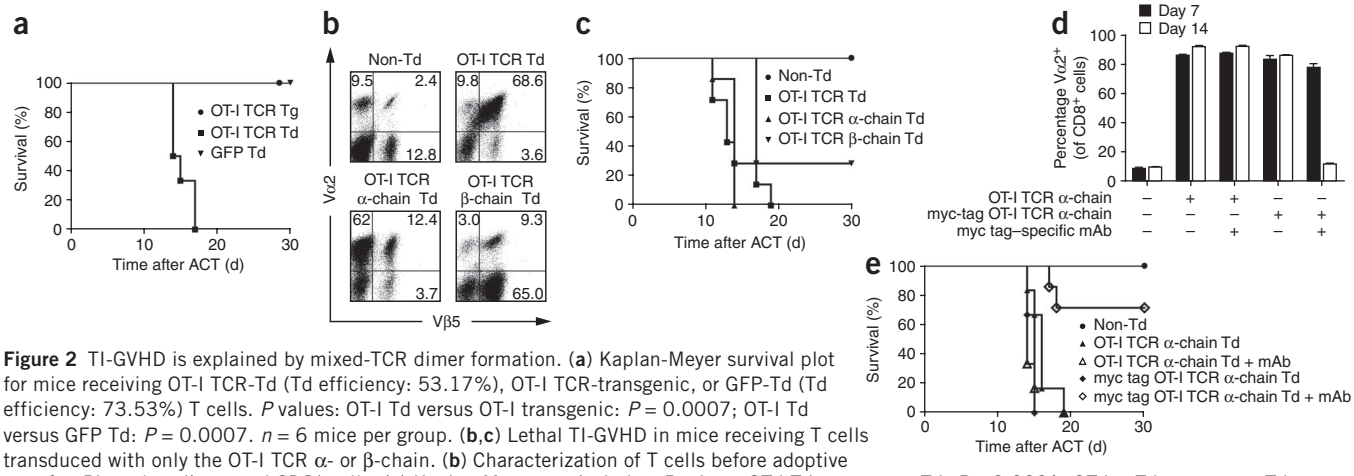


Figure 2 TI-GVHD is explained by mixed-TCR dimer formation. (**a**) Kaplan-Meyer survival plot for mice receiving OT-I TCR-Td (Td efficiency: 53.17%), OT-I TCR-transgenic, or GFP-Td (Td efficiency: 73.53%) T cells. *P* values: OT-I Td versus OT-I transgenic: $P = 0.0007$; OT-I Td versus GFP Td: $P = 0.0007$. $n = 6$ mice per group. (**b**,**c**) Lethal TI-GVHD in mice receiving T cells transduced with only the OT-I TCR α- or β-chain. (**b**) Characterization of T cells before adoptive transfer. Plots show live-gated $CD8^+$ cells. (**c**) Kaplan-Meyer survival plot. *P* values: OT-I Td versus non-Td: $P < 0.0001$; OT-I α-Td versus non-Td: $P = 0.0004$; OT-I β-Td versus non-Td: $P = 0.0072$. $n = 7$ mice per group. (**d**,**e**) Depletion of OT-I TCR α-chain Td T cells limits fatal TI-GVHD. (**d**) Depletion of myc-tagged OT-I TCR α-chain Td T cells in peripheral blood after tag-specific mAb administration. Flow cytometric data represent mean number of $V\alpha2^+CD8^+$ cells per mouse. Error bars indicate s.e.m. *P* values: myc-tagged OT-I α-Td and mAb day 7 versus day 14: $P < 0.0001$; OT-I α-Td and mAb day 7 versus day 14: $P = 0.006$. (**e**) Kaplan-Meyer survival plot for mice receiving OT-I TCR α-chain Td T cells (Td efficiency: 65%) or myc-tagged OT-I TCR α-chain Td T cells (Td efficiency: 58%). *P* values: OT-I α-Td versus non-Td: $P = 0.0007$; myc-tagged OT-I α-Td versus non-Td: $P = 0.0012$; OT-I α-Td and mAb versus non-Td: $P = 0.0004$; myc-tagged OT-I α-Td and mAb versus non-Td: $P = 0.05$; OT-I α-Td versus myc-tagged OT-I α-Td: $P = 0.04$; OT-I α-Td versus OT-I α -Td and mAb: $P > 0.05$; myc-tagged OT-I α-Td versus myc-tagged OT-I α-Td and mAb: $P = 0.0006$. $n = 6$ mice per group except for the myc-tagged OT-I α-Td and mAb treated group ($n = 7$).

T cells that were deficient for IFN-γ production showed no signs of TI-GVHD upon IL-2 administration, and 87.5% survived (**Fig. 3b**). By contrast, induction of TI-GVHD was not significantly reduced when we used TCR-transduced T cells from *Gzmb*$^{-/-}$ mice that are deficient in granzyme B (**Supplementary Fig. 2b**).

TI-GVHD with various TCRs and adjuvant strategies

The above results indicate that the formation of mixed TCR dimers on T cells transduced with the OT-I TCR resulted in TI-GVHD in mice that received TBI and a high dose of IL-2. To understand the role of the observed pathology for the clinical development of TCR gene therapy, it was crucial to establish whether this observation could be extended to other TCRs and other strategies that promote *in vivo* T cell function.

To address whether TI-GVHD is observed with different TCRs, we gave cohorts of C57BL/6J mice T cells that were transduced with one of five mouse TCRs: the melanocyte differentiation antigen gp100-specific pmel-1 TCR[17], the SV40 large T oncogene–specific $SV40_{IV}$ TCR[8], the influenza A nucleoprotein–specific F5 TCR[18], the melanocyte differentiation antigen tyrosinase–related protein-2 (TRP2)-specific TRP2 TCR (described in the Online Methods) or the OT-I TCR. Groups of mice that received cells modified with one of these TCRs, which are either restricted by major histocompatibility complex (MHC) class I molecule H2-K^b or H2-D^b, all showed fatal TI-GVHD (**Fig. 4**). Notably, the incidence of lethal pathology ranged from low (F5 and pmel-1) to very high (OT-I), suggesting that it will be difficult to extrapolate a lack of toxicity seen in clinical trials of one TCR to other TCRs. Macroscopic and histological analysis revealed the same pattern of pathology as observed with OT-I TCR–transduced T cells (data not shown).

Having shown that TI-GVHD occurs in mice receiving high-dose IL-2, we assessed whether it also occurs in mice receiving a different IL-2 dose or regimen. We observed fatal TI-GVHD in mice receiving OT-I TCR–transduced T cells combined with low-dose IL-2 (**Fig. 5a**,**b**), with an overall mortality of 31%. Of note, 58.3% of the surviving mice developed a chronic form of TI-GVHD, characterized by colitis, diarrhea and a reduction in lymphocytes in the spleen (**Supplementary Fig. 3a**,**b** and **Supplementary Table 1**). Furthermore, when we restricted high-dose IL-2 administration to a single day, fatal TI-GVHD still occurred (**Supplementary Fig. 3c**).

To determine whether the development of TI-GVHD depends specifically on IL-2 administration, we evaluated the effect of another strategy for promoting the *in vivo* function of TCR-transduced T cells. Transforming growth factor-β (TGF-β) signaling inhibits the proliferation and effector functions of cytotoxic T cells[19], and blockade of this signaling pathway in adoptively transferred tumor-reactive T cells augments their antitumor function[20]. To assess the effect of blockade of TGF-β signaling on the toxicity of adoptive therapy with TCR-transduced T cells, we transduced these cells with a dominant-negative TGF-β receptor-II (dnTGFβRII)[20] (**Fig. 5c**). Cohorts of C57BL/6J mice were conditioned with TBI and received

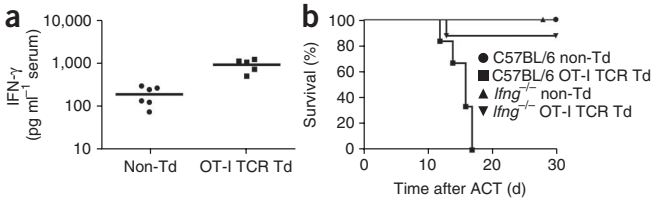


Figure 3 IFN-γ–mediated pathogenesis of TI-GVHD. (**a**) Increased serum IFN-γ levels on d11 after adoptive cell transfer in recipients of OT-I TCR-Td T cells ($n = 5$; Td efficiency: 67%) compared to non-Td T cells ($n = 6$). *P* value of OT-I TCR-Td versus non-Td: $P = 0.0003$. Data are representative of two experiments. (**b**) Kaplan-Meyer survival plot showing reduced mortality in mice receiving OT-I TCR Td T cells from *Ifng*$^{-/-}$ donor mice (Td efficiency: 65%) compared to C57BL/6J donor mice (Td efficiency: 69%). *P* values: OT-I TCR-Td C57BL/6J versus OT-I TCR-Td *Ifng*$^{-/-}$: $P = 0.0027$; OT-I TCR-Td *Ifng*$^{-/-}$ versus non-Td *Ifng*$^{-/-}$: $P = 0.4292$; OT-I TCR-Td C57BL/6J versus non-Td C57BL/6J: $P = 0.0008$. Number of mice: non-Td C57BL/6J, $n = 6$; OT-I TCR-Td C57BL/6J, $n = 6$; non-Td *Ifng*$^{-/-}$, $n = 5$; OT-I TCR-Td *Ifng*$^{-/-}$, $n = 8$.

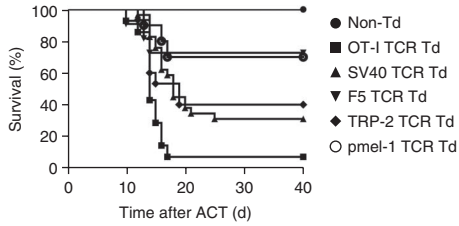


Figure 4 TI-GVHD is observed with multiple TCRs. Kaplan-Meyer survival plot showing that lethal TI-GVHD is observed in mice receiving an adoptive transfer of T cells transduced with OT-I (Td efficiency: 53%–56%), $SV40_{IV}$ (Td efficiency: 30%–62%), TRP2 (Td efficiency: 62%–76%), F5 (Td efficiency: 33%–37%) or pmel-1 TCR (Td efficiency: 15%–36%). *P* values: OT-I Td versus non-Td: $P < 0.0001$; $SV40_{IV}$ Td versus non-Td: $P = 0.0014$; TRP2 Td versus non-Td: $P = 0.0051$; F5 Td versus non-Td: $P > 0.05$; pmel-1 Td versus non-Td: $P > 0.05$. Number of mice: non-Td, $n = 9$; OT-I Td, $n = 14$; F5 Td, $n = 11$; TRP2 Td, $n = 15$; pmel-1 Td, $n = 10$; $SV40_{IV}$ Td, $n = 28$. Data represent cumulative results from four independent experiments.

either T cells transduced with the OT-I TCR, T cells transduced with dnTGFβRII or T cells transduced with both the OT-I TCR and dnTGFβRII, in all cases without IL-2 administration. One hundred percent of recipients of T cells that had been transduced with OT-I TCR and dnTGFβRII developed fatal TI-GVHD (**Fig. 5d**), again characterized by anemia, hematopoietic cell destruction and more general symptoms of GVHD (data not shown). This pathology was not due to an aspecific effect of the introduced dnTGFβRII, as it was not observed upon infusion of dnTGFβRII-modified cells that did not receive the OT-I–encoding transgene (**Fig. 5d**). These data show that TI-GVHD can also occur when a strategy other than IL-2 administration is used to promote the *in vivo* function of infused TCR-transduced T cells.

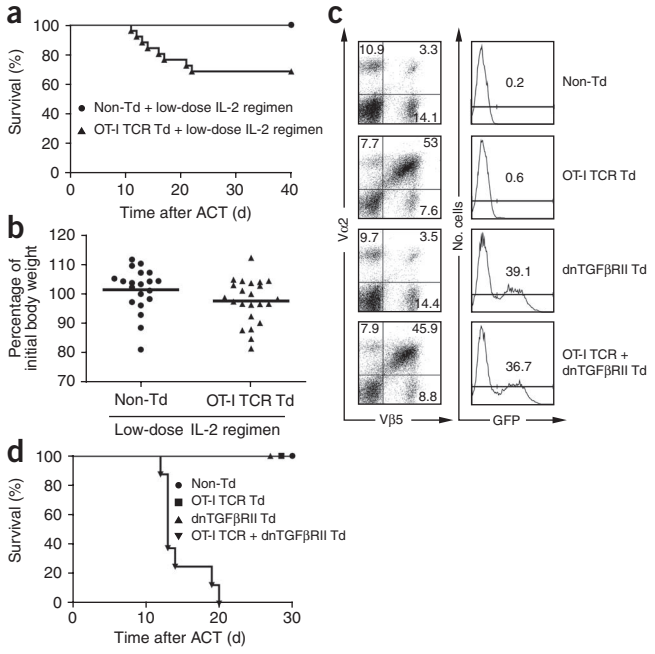
Limiting the occurrence of TI-GVHD

In view of the possible risk that mixed TCR dimer formation has been considered to pose, a number of strategies that aim to reduce the formation of potentially autoaggressive mixed TCR dimers have been developed[21]. One set of strategies is based on the use of engineered TCR genes that have a higher propensity to form the intended TCR αβ dimer[22–27]. A conceptually unrelated strategy that has been proposed is the use of oligoclonal or monoclonal T cell populations as recipient cells for TCR gene transfer[28,29]. In this situation, assembly of mixed TCR dimers is not influenced, but, because the diversity of endogenous TCRs is highly limited, the likelihood of generating autoreactive mixed TCR dimers is reduced.

Figure 5 TI-GVHD is observed with different strategies that promote *in vivo* T cell function. (**a**) Kaplan-Meyer survival plot showing lethal TI-GVHD in mice receiving low-dose IL-2 after adoptive transfer of OT-I TCR-Td T cells (Td efficiency: 42%–53%). *P* value of non-Td versus OT-I TCR-Td: $P = 0.005$. Shown are pooled results of four independent experiments (OT-I TCR-Td, $n = 26$; non-Td, $n = 22$). (**b**) Change in body weight at day 13 after adoptive cell transfer of OT-I TCR-Td T cells or non-Td T cells and treatment with low-dose IL-2. Shown are pooled results of four independent experiments. Symbols represent individual mice; bars indicate group averages. *P* value of non-Td versus OT-I TCR-Td: $P > 0.05$. (**c**,**d**) TI-GVHD is observed when blockade of TGF-β signaling is used to promote *in vivo* T cell function. (**c**) Characterization of OT-I TCR-Td T cells (Td efficiency: 53%), dnTGFβRII Td T cells, OT-I TCR/dnTGFβRII co-Td T cells (Td efficiency: 46%) and non-Td T cells before adoptive transfer. Dot plots and histograms show live-gated $CD8^+$ cells. (**d**) Kaplan-Meyer survival plot. *P* values: OT-I/dnTGFβRII co-Td versus non-Td: $P = 0.0006$; OT-I/dnTGFβRII co-Td versus dnTGFβRII Td: $P < 0.0001$; OT-I/dnTGFβRII co-Td versus OT-I Td: $P = 0.0002$. Number of mice: non-Td, $n = 5$; OT-I Td, $n = 6$; dnTGFβRII Td, $n = 7$; OT-I/dnTGFβRII co-Td, $n = 8$.

To assess whether the use of a largely monoclonal T cell population as recipient cells for TCR gene transfer can reduce mixed dimer–induced toxicity, we treated C57BL/6J mice as before (**Fig. 1a**) and gave them an infusion of either F5 TCR–transgenic T cells transduced with the OT-I TCR or polyclonal C57BL/6J T cells transduced with the OT-I TCR (**Supplementary Fig. 4a**). After IL-2 administration, none of the recipients of F5 TCR–transgenic T cells transduced with the OT-I TCR showed signs of cachexia, and survival was 100% (**Fig. 6a**), showing that the use of a largely monoclonal T cell population for TCR gene transfer forms a viable strategy to prevent TI-GVHD.

To determine whether genetic engineering strategies can also be used to limit or prevent the development of TI-GVHD, we first focused on the introduction of an additional disulfide bond between the TCR α and β constant domains as a means to reduce mixed dimer formation. To this end, we produced T cells expressing cysteine-modified variants (Cys-TCR) of the OT-I and $SV40_{IV}$ TCRs and evaluated them for induction of TI-GVHD in a side-by-side comparison with cells expressing the corresponding unmodified TCRs. In recipients of cells transduced with Cys-TCR variants, we found a reduced incidence of fatal TI-GVHD (**Supplementary Fig. 4b**,**c**; $P = 0.0004$). However, in line with the observation that cysteine modification of TCRs can reduce mixed dimer formation only partially[27,30], a substantial number of mice receiving Cys-TCR–transduced T cells still developed fatal TI-GVHD (23% of all mice that received T cells transduced with a Cys-TCR compared to 73% of mice that received T cells transduced with a nonmodified TCR) (**Supplementary Fig. 4b**,**c**). To further reduce the likelihood of mixed TCR dimer formation, we generated retroviral vectors in which the internal ribosomal entry site (IRES) sequence that links the TCR α- and β-chain genes was replaced with the porcine teschovirus-derived P2A sequence (**Supplementary Fig. 4d**). In contrast to an IRES sequence, a P2A sequence ensures equimolar production of the introduced TCR α- and β-chains, thereby reducing the likelihood of mispairing with endogenous TCR chains. Furthermore, the fact that both TCR chains are translated by the same ribosome may also increase the likelihood of correct pairing[31].This replacement did not affect the antigen sensitivity of these TCRs *in vitro* or *in vivo* (data not shown). For the OT-I TCR,



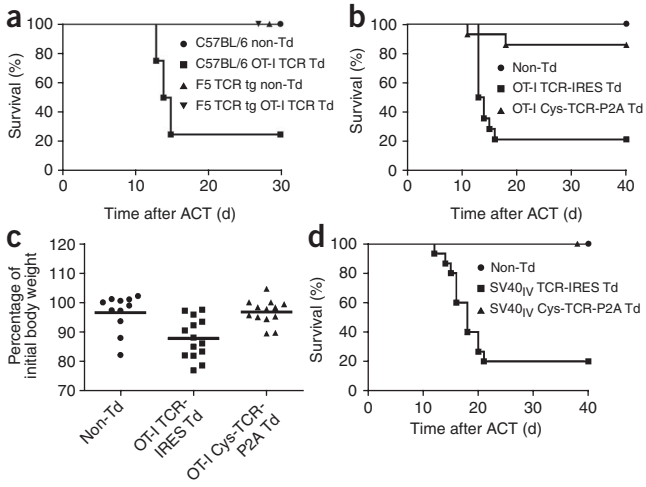


Figure 6 Prevention of TI-GVHD by TCR engineering or by the use of oligoclonal T cell populations. (**a**) Kaplan-Meyer survival plot showing that TI-GVHD is prevented by TCR gene transfer into oligoclonal F5 TCR–transgenic T cells. *P* value: OT-I TCR-Td C57BL/6J versus OT-I TCR-Td F5 Tg: $P = 0.0045$. Number of mice: non-Td C57BL/6J, $n = 4$; OT-I TCR-Td C57BL/6J, $n = 8$ (Td efficiency: 65%); non-Td F5 Tg, $n = 5$; OT-I TCR-Td F5 Tg, $n = 7$ (Td efficiency: 55%). (**b**) Kaplan-Meyer survival plot showing reduced mortality with an OT-I Cys-TCR-P2A gene expression cassette compared to the OT-I TCR-IRES gene expression cassette. *P* values: OT-I-IRES Td versus OT-I Cys_TCR-P2A Td: $P = 0.0006$; OT-I Cys_TCR-P2A Td versus non-Td: $P > 0.05$. Number of mice: non-Td, $n = 11$; OT-I-IRES Td, $n = 14$ (Td efficiency: 42–63%); OT-I Cys_TCR-P2A Td, $n = 14$ (Td efficiency: 44–73%). (**c**) Change in body weight at day 13 after adoptive T cell transfer. Symbols represent individual mice, bars indicate group averages. *P* values: non-Td versus OT-I-IRES Td: $P = 0.0031$; OT-I Cys_TCR-P2A Td versus non-Td: $P > 0.05$; OT-I-IRES Td versus OT-I Cys_TCR-P2A Td: $P = 0.0004$. Data shown in **b** and **c** are cumulative results from three independent experiments. (**d**) Kaplan-Meyer survival plot showing that lethal TI-GVHD is prevented by an $SV40_{IV}$ Cys-TCR -P2A gene expression cassette. *P* value: $SV40_{IV}$ TCR-IRES Td versus $SV40_{IV}$ Cys-TCR-P2A Td: $P < 0.0001$. Number of mice: non-Td, $n = 6$; $SV40_{IV}$ TCR-IRES Td, $n = 15$ (Td efficiency: 30%–51%); $SV40_{IV}$ Cys-TCR-P2A Td, $n = 13$ (Td efficiency: 37%–41%). Data shown are cumulative results from two independent experiments.

which produced the highest incidence of pathology of the five TCRs tested, the incidence of lethal TI-GVHD was reduced from 80% to 14.3% (**Fig. 6b**), with a corresponding reduction in cachexia (**Fig. 6c**). Furthermore, for the $SV40_{IV}$ TCR, which induces an only somewhat lower incidence of fatal TI-GVHD, the same engineering approaches prevented pathology altogether (**Fig. 6d**). These data illustrate how straightforward engineering strategies can prevent an otherwise fatal autoimmune attack.

DISCUSSION

In this study, we show that lethal autoimmune pathology can occur in mouse models of TCR gene therapy under various conditions that promote the *in vivo* function of TCR-transduced T cells. This therapy-induced autoimmune process results in a fatal destruction of the hematopoietic compartment, accompanied by more general aspects of GVHD. The evidence that this pathology is due to the formation of self-antigen–reactive mixed TCR dimers is compelling. First, pathology does not depend on the presence of cognate antigen, as shown for the OT-I, F5 and $SV40_{IV}$ TCRs. Second, pathology depends on the introduction of TCR genes but is not due to the reactivity of the heterodimer itself, as shown by the lack of toxicity seen upon transfer of OT-I–transgenic T cells and by the fact that the introduction of single TCR chains suffices to induce pathology. Third, pathology can be induced not only by $CD8^+$ T cells modified with an MHC class I–restricted TCR but also by $CD4^+$ T cells (**Supplementary Fig. 5**), indicating that the MHC restriction of the parental receptor is not relevant. Fourth, pathology can be prevented by the *in vivo* depletion of T cells expressing mixed TCR dimers or by the provision of cell grafts that lack the capacity to produce IFN-γ. Finally, the most convincing evidence for the involvement of mixed dimers in TI-GVHD comes from the observation that pathology can be limited or prevented through the use of systems in which the pairing of endogenous and exogenous TCR chains is reduced or in which the diversity of the endogenous TCR repertoire of the transduced T cells is highly limited.

We note that the TI-GVHD observed here bears a similarity to transfusion-associated graft-versus-host disease (TA-GVHD)[32–34], a syndrome most commonly observed when immunocompromised individuals receive 'fresh blood' from relatives. Analogous to the toxicity observed here, TA-GVHD is characterized by the destruction of hematopoietic cells by infused T cells, is rapidly progressive and is nearly always fatal. Furthermore, in both this study of TI-GVHD and in mouse models of TA-GVHD, hematopoietic cell destruction is at least partially mediated by excess production of type I cytokines[35,36].

The key question that arises from our data is whether the toxicity seen in these mouse models can be expected to be predictive for the clinical translation of TCR gene therapy. No such toxicity was observed in the two phase 1 trials performed so far[10,11]. However, the protocol for the preparation of TCR-transduced T cell grafts used in these trials and in particular the fact that cells were grown for ~10–20 d could have contributed to the lack of observed toxicity. In support of this idea, we observed that *in vitro* culture of mouse TCR-transduced T cells for 10 d also results in a substantial reduction of TI-GVHD (**Supplementary Figs. 6** and **7**). This observation fits well with evidence from earlier adoptive cell transfer studies that the *in vivo* function of 'older' T cells is highly diminished[37–39]. An alternative explanation might be provided by the observation that in most cases the cell graft infused into patients contained a low frequency of $CD4^+$ T cells[11], and our mouse data indicate that $CD4^+$ T cells might have a more dominant role in the development of TI-GVHD (**Supplementary Fig. 5**).

Further observations suggesting that mixed TCR dimer–dependent toxicity may also become a safety concern in the clinical setting have been made by another group. Their work demonstrates that mixed TCR dimers are readily formed after TCR gene transfer into human T cells, and, crucially, that such mixed TCR dimers can show autoreactive specificities in *in vitro* assays (M. Heemskerk (Leids Universitair Medisch Centrum) personal communication). Thus, with proof for mixed dimer–dependent pathology in mouse models and evidence for the formation of self-reactive human T cells in *in vitro* assays, it can be expected that the pathology seen here will occur within the clinic with the use of more advanced T cell growth and engraftment regimens. The evaluation and implementation of technologies that can prevent TI-GVHD should therefore be a high priority.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

G.M.B. designed experiments, performed experiments, analyzed and interpreted data and wrote the paper. C.L. designed experiments, performed experiments, analyzed and interpreted data and wrote the paper. A.I.H. designed experiments, performed experiments, analyzed and interpreted data. L.B. performed experiments and analyzed data. M.A.d.W. and A.J. made the initial observation of cachexia and rapid death of mice in mouse models of TCR gene therapy. A.D.M.K. performed experiments and interpreted data. N.P. and R.D. generated and provided the TRP2 TCR sequences. E.K. and W.U. provided the Tag-specific mAb and advice on *in vivo* depletion. J.-Y.S. performed experiments and analyzed data. J.B.A.G.H. interpreted data. T.N.M.S. designed experiments, interpreted data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. We obtained C57BL/6J mice from Charles River Laboratories and F5 TCR-transgenic mice and OT-I TCR-transgenic mice from The Experimental Animal Department of The Netherlands Cancer Institute. *Ifng*$^{-/-}$ (B6.129S7-Ifngtm1Ts/J) mice and *Gzmb*$^{-/-}$ (B6.129S2-Gzmbtm1ley/J) mice were obtained from The Jackson Laboratory. We performed all mouse experiments in accordance with institutional and Dutch guidelines, and they were approved by the Experimental Animal Committee of The Netherlands Cancer Institute.

Retroviral constructs, retroviral transduction and adoptive transfer of T cells. We created pMX retroviral vectors in a pMX-TCRα-IRES-TCRβ configuration for OT-I[9], $SV40_{IV}$ (specific for the SV40 large-T oncogene)[8], F5 (specific for the influenza-A-nucleoprotein)[18], pmel-1 (specific for the melanocyte-differentiation antigen gp100)[17], TRP2 (specific for the TRP2-melanocyte differentiation antigen), OT-I Cys, $SV40_{IV}$ Cys and TRP2 Cys TCRs. We created pMX retroviral vectors in a pMX-TCRβ-P2A-TCRα configuration for the OT-I Cys and $SV40_{IV}$ Cys TCRs. The OT-I, $SV40_{IV}$, pmel-1, and TRP2 TCR genes and derivatives used in this study were gene optimized[9,17] by GeneArt (GeneArt). TRP2 TCR cDNA was derived from the cytotoxic T lymphocyte clone LP9[40] (kindly provided by G. Adema, Radboud University). TCR-V encoding DNAs were obtained by progressive 5′ amplification reactions of cDNA ends starting from conserved sequences of TCR-C domains. Full TCR α- and β-chain sequences were cloned into the retroviral vector pBullet and subcloned into the retroviral vector pMX. We validated TRP2 TCR α- and β-chains (sequences available upon request) with respect to surface expression and reactivity by flow cytometry. For experiments involving the sole introduction of the OT-I TCR α-chain, OT-I TCR β-chain or myc-tagged OT-I TCR α-chain, we generated pMX-OT-Iα, pMX-OT-Iβ and pMX-myc–tagged OT-Iα constructs, respectively. We cloned the dnTGFβRII[20] into the pMX retroviral vector to obtain a pMX-dnTGFβRII-IRES-GFP construct. All retroviral constructs were used to transfect Phoenix-E packaging cells to generate retrovirus[41]. We modified mouse splenocytes by retroviral transduction as described[18].

One day before adoptive cell transfer, irradiation-induced host conditioning was achieved by 5 Gy TBI with a radiobiology constant potential X-ray unit (Pantak HF-320; Pantak Limited). We treated mice with an adoptive cell transfer containing 1×10^6 TCR-transduced $CD8^+$ T cells on day 0 and, where indicated, high-dose (7.2×10^5 IU) or low-dose (1.8×10^4 IU) IL-2 (Proleukin, Novartis) intraperitoneally twice daily between 10 and 12 d after adoptive cell transfer. For the depletion of myc-tagged TCR-transduced T cells, we treated mice with 200 μg of myc-specific mAb (9E10) on d7, 9 and 11.

Flow cytometry. We measured surface TCR expression 24 h after transduction by flow cytometry. Cells were stained with the following mAbs (all from BD Biosciences): FITC-conjugated antibody to TCR Vβ5 and phycoerythrin (PE) conjugated antibody to TCR Vα2 for the OT-I TCR; FITC-conjugated antibody to TCR Vβ9 and PE-conjugated antibodies to TCR Vβ2, Vβ3, Vβ4, Vβ5, Vβ8, Vβ11 and Vβ10b (^{SV40}Vβ-pool antibodies) for the $SV40_{IV}$ TCR; PE-conjugated antibody to TCR Vβ3 and FITC-conjugated antibody to TCR Vβ4, Vβ5, Vβ8, Vβ8.3, Vβ9, Vβ10, Vβ11, Vβ13 and Vβ14 (^{TRP2}Vβ-pool antibodies) for the TRP2 TCR PE-conjugated antibody to TCR Vβ11 and FITC-conjugated antibody to TCR Vβ4, Vβ5, Vβ8, Vβ8.3, Vβ9, Vβ10, Vβ13 and Vβ14 (^{F5}Vβ-pool antibodies) for the F5 TCR; FITC-conjugated antibody to TCR Vβ13 and PE-conjugated antibody to TCR Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ8.1, Vβ11 and Vβ10b ($^{pmel\text{-}1}$Vβ-pool antibodies) for the pmel-1 TCR. We stained T cells co-transduced with the OT-I TCR and dnTGFβRII with PE-conjugated mAb to TCR Vα2, biotin-conjugated mAb to TCR Vβ5, PerCP–Cy5-conjugated mAb to CD8α and allophycocyanin-conjugated streptavidin (BD Biosciences). Propidium iodide (Sigma) was used to select for live cells. We acquired and analyzed data on a FacsCalibur (BD Biosciences) with FlowJo software (Tree Star) or CELLQuest-Pro software (BD Biosciences).

Cytokine analysis. We determined IFN-γ, tumor necrosis factor, IL-10, IL-6, monocyte chemoattractant protein-1 and IL-12p70 serum concentrations using the BD Cytometric Bead Array mouse inflammation kit (BD Biosciences) according to the manufacturer's instructions.

Histopathology. We collected organs and tissues including gastrointestinal tract, liver, kidney, lung, heart, spleen, lymph nodes, thymus, Peyer's patches and bone marrow and sampled them in EAF (ethanol–acetic acid–formalin) fixative. Paraffin sections were stained with H&E. We defined pathological alterations (such as lymphocytic or inflammatory infiltrations and reduction or depletion of lymphoid or hematopoietic compartments in various organs) semiquantitatively as mild (grade 1), moderate (grade 2) and severe (grade 3). We reviewed sections using a Zeiss Axioskop2 Plus microscope (Carl Zeiss Microscopy) equipped with Plan-Apochroma (×5/0.16, ×10/0.45, ×20/0.60, and ×40/0.95) and Plan-Neofluar (×2.5/0.075) objectives. Images were captured with a Zeiss AxioCam HRc digital camera and processed with AxioVision 4 software (both Carl Zeiss Vision).

Statistical analysis. We compared survival curves using a log-rank (Mantel-Cox) test. We compared changes in body weight and differences in serum cytokine concentrations using a two-tailed Student's *t* test. We compared histopathological scores using a Cochrane-Armitage trend test and exact two-sided *P* values are reported. *P* values <0.05 were considered significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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