Transplantation of mouse HSCs genetically modified to express a CD4-restricted TCR results in long-term immunity that destroys tumors and initiates spontaneous autoimmunity

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The development of effective cancer immunotherapies has been consistently hampered by several factors, including an inability to instigate long-term effective functional antitumor immunity. This is particularly true for immunotherapies that focus on the adoptive transfer of activated or genetically modified mature CD8+ T cells. In this study, we sought to alter and enhance long-term host immunity by genetically modifying, then transplanting, mouse HSCs. We first cloned a previously identified tumor-reactive HLA-DR4–restricted CD4+ TCR specific for the melanocyte differentiation antigen tyrosinase-related protein 1 (Tyrp1), then constructed both a high-expression lentivirus vector and a TCR-transgenic mouse expressing the genes encoding this TCR. Using these tools, we demonstrated that both mouse and human HSCs established durable, high-efficiency TCR gene transfer following long-term transplantation into lethally irradiated mice transgenic for HLA-DR4. Recipients of genetically modified mouse HSCs developed spontaneous autoimmune vitiligo that was associated with the presence of a Th1-polarized memory effector CD4+ T cell population that expressed the Tyrp1-specific TCR. Most importantly, large numbers of CD4+ T cells expressing the Tyrp1-specific TCR were detected in secondary HLA-DR4–transgenic transplant recipients, and these mice were able to destroy subcutaneously administered melanoma cells without the aid of vaccination, immune modulation, or cytokine administration. These results demonstrate the creation of what we believe to be a novel translational model of durable lentiviral gene transfer that results in long-term effective immunity.

Introduction

A major obstacle in the development of effective cancer immunotherapies has been the inability to instigate long-term effective functional antitumor immunity. Recent clinical studies involving adoptive transfer of T cells into transiently myelodepleted hosts, though starting in their capacity to induce tumor regression, have been consistently hampered by the relative short-term lifespan of tumor-specific T cells (1, 2).

Given these limitations, current immunotherapies have sought to improve T cell durability by shifting the emphasis from demonstrable high-level in vitro reactivity to early-differentiation T cell phenotypes. This conceptual change in the approach to cancer immunotherapy is supported by evidence of a progressive pathway of T cell differentiation. Phenotypic changes in early to late effector T cells are associated with diminished in vivo effector function and persistence (3, 4). Current strategies involving adoptive transfer of naive, central memory, or stem cell memory T cell populations have sought to take advantage of this continuum of functional change (5–8). Despite these advances, however, it remains unclear what the stability and long-term immune reactivities of these populations will be, and how they can be translated to patient care.

While most immunotherapies have emphasized CD8+ T cell responses, there is nearly overwhelming evidence to support the crucial role of CD4+ T helper cells in antitumor immunity (9, 10). Murine studies have consistently demonstrated that CD4+ T cells exert effects through induction and maintenance of B cells and CD8+ T cells, trigger long-term maintenance of antigen-activated memory CD8+ T cells (11, 12), and, when of sufficiently high avidity, overcome host Tregs (13). There is also evidence that combined administration of class I and II epitopes derived from the same tumor antigen can potentiate antitumor immunity (14, 15). Despite these strongly supportive data, very few studies have used CD4+ T cells as the principal driving force of therapy (16, 17).

Given limitations with existing cancer immunotherapies, we sought to establish a model of long-term immunity characterized by a durable, high-frequency population of permanently unmanipulated tumor-reactive CD4+ T cells. New technologies in lentiviral (LV) gene transfer have allowed for the introduction of large genetic elements into mitotically quiescent HSCs (18). To design a model of TCR gene transfer, we utilized a previously identified autoreactive, melanoma-reactive HLA-DRB1*0401–restricted (HLA-DR4–restricted), tyrosinase-related protein 1–specific (Tyrp1-specific or TRP-1–specific) TCR (13, 19). Tyrp1, a melanosomal protein present in normal melanocytes and melanomas, has been exploited as an immunotherapeutic target in several animal models, inducing both autoimmune vitiligo (20–22) and eradication of established tumors (13, 23). We then generated a new lentivector expressing the α and β TCR subunits and a TCR Tg expressing the same TCR genes. Herein, we demonstrate durable, high-efficiency TCR gene transfer in similarly executed iso-

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congenic HSC transplants following 12-month primary and 6-month secondary transplants. We then demonstrate induction of spontaneous autoimmune vitiligo and destruction of subcutaneous B16 melanoma without the aid of vaccination, cytokine administration, or immune modulation. To more fully reveal the translational patient care potential of this model, we also demonstrate long-term TCR gene expression in humanized HSC transplants.

Results

Typr1-specific TCR is highly expressed and functional in vitro. To develop a model of TCR transfer into HSCs, we cloned TCR α/β genes from a previously identified Typr1-specific, tumor reactive HLA-DRB1*0401-restricted (HLA-DR4–restricted) CD4+ T cell from a patient with metastatic melanoma (19) using previously described cloning methodology (24, 25): TCR-α: Vα13-2*01, J22*01; TCR-β: Vß5.4*01, J1-1*01, D1*01. We then constructed 2 separate high-expression, self-inactivating (SIN) lentivectors to deliver TCR genes to HSCs. Two strategies were employed for expressing TCR chains on either a bicistronic or monocistronic message driven by the same heterologous promoter (CMV). For the first strategy, TCR α and β chains were co-expressed using the IRES element (26, 27). The second involved replacing the IRES sequence with a Furin cleavage site fused to a post-translational 2A self-cleaving peptide (28). This strategy has been used to boost expression of the second gene with combined Ig heavy and light chain genes by creating a not affected by control MHC class I– and CD8-specific antibodies. To control for experimental specificity and background (<1%), we used a pentamer specific for gp100 44–59 DR4-restricted T cells (Ultimer-gp100) (31). This multimeric peptide antibody control was used for all subsequent flow cytometry analyses.

LV gene–modified HSCs engraft long-term engraftment and repopulation in transplant recipients. To quantify the engraftment potential, gene transfer efficiency, and long-term stability of transplanted HSCs, we assessed a competitive CD45.1/CD45.2 repopulation. Boy-J (CD45.1) lineage-depleted (Lin−) donor cells (>95% Lin−; data not shown) were transduced with concentrated LV supernatant from LV-TCR, then combined (following an overnight recovery) at a total of 5 × 106 cells with unFractionated isogenic BM (CD45.2, 2.5 × 106 cells) from DR4 Tg mice, before transplantation by tail vein injection into lethally irradiated (900 cGy) DR4 Tg recipients (5–10 mice/group/experiment). Similarly prepared and prestimulated control transplants (of both nontransduced and LV-GFP transduced cells) were also assessed. To investigate TCR gene transfer efficiency, a parallel group involving prestimulated Lin− cells (5 × 104) from TCR Tg mice were combined with unFractionated BM cells (2.5 × 106) from DR4 Tg mice and similarly transplanted into irradiated DR4 Tg recipients (5–10 mice per experiment). An overview of the transplant procedure is shown in Figure 2A.

Beginning at 1 month after transplantation, peripheral blood (PB) was stained (at multiple time points) with fluorochrome-conjugated antibodies specific for murine CD45, CD4, CD8, CD3, and the TRP1-Ultimer. As shown in Figure 2B, LV-TCR gene–specific CD4+ T cells were identified at a level of 53% (CD45/CD3/CD4+/Ultimer+) at 6 months and at a level of 28% at 12 months after transplantation. This compared with a level of 78% (CD45/CD3/CD4+/Ultimer+) at 6 months and 69% at 12 months in the TCR Tg group. As seen in Figure 2C, the majority of CD3+Ultimer+ cells were also found to be CD4+, with less than 5% CD8+ cells, suggesting that co-expression of CD4 was essential for specific TCR selection. In addition, as shown in Figure 2D, 12-month PB samples were found to co-express the endogenous murine TCRβ (mTCRβ) chain (consistently >95%) in both CD4+Ultimer+ and CD4+Ultimer− subpopulations. We did, however, find statistically significant differences in MFI between the 2 populations (224 ± 44 vs. 577 ± 46 MFI; P = 0.0005), suggesting a lower density of mTCR on those cells shared by the TCR transgene (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43274DS1). Lastly, we found no significant difference in frequency of Tregs (CD4+CD25Foxp3+) between control and LV-TCR transplants, including those subpopulations that were Ultimer+ and Ultimer− (see Supplemental Figure 2, A and B). Calculations for all displayed results were based on combined CD45.1 and CD45.2 gates to more accurately quantify TCR gene expression within the entire T cell compartment.

In general, TCR-specific levels were consistently and statistically higher at 1, 2, 4, 6, and 12 months after transplantation in the TCR Tg group compared with the LV-TCR group. These overall findings were observed within the total cellular compartment (Figure 3A; CD45+Ultimer−), the global T cell compartment (Figure 3B; CD4+CD3+Ultimer−), and the CD4+ T cell subpopulation (Figure 3C; CD45+CD3+CD4+Ultimer−). Levels of TCR-specific CD4+ T cells reached their peak (in the LV-TCR group) at 6 months after transplantation, before diminishing in frequency by 12 months.
(52.3% ± 2.0% at 6 months vs. 26.1% ± 2.4% at 12 months; \(P = 0.004\)). These results contrasted with those from the TCR Tg group, in which gene expression was statistically unchanged from 6 to 12 months (74.3% ± 5.5% at 6 months vs. 73.2% ± 5.4% at 12 months; \(P = 0.19\)) and statistically insignificant from levels in the endogenous nontransplanted population (see Figure 1C; 79.3% ± 8.3%; \(P = 0.12\)).

The overall differences between groups were, therefore, contrasted by a long-term rate of genetic stability in the TCR Tg group. Given these findings, and to evaluate overall transplant efficiency (itself a function of LV gene transfer efficiency and LV gene stability), we measured TCR gene expression as a function of TCR expression positivity (% = LV-TCR/TCR-Tg × 100) within the total CD3 and CD4 compartments. Efficiency peaked in both populations at 6 months (CD3, 53%; CD4, 70%) before trailing off by 12 months (CD3, 48%; CD4, 40%) (see Figure 3D).

To more specifically gauge TCR gene expression that developed from LV-TCR–transduced CD45.1+ cells, PB samples from 6- and 12-month transplant samples were analyzed by flow cytometry. As shown in Figure 4, A and B, TCR gene–specific T cells represented a dominant fraction of all CD3+ and CD4+ lymphocytes within the CD45.1 compartment (at 12 months: CD3, 37% ± 7.4%; CD4, 54% ± 7.8%; \(P = 0.04\)). Overall, these results (total CD45 and CD45.1) are consistent with high-frequency, durable repopulation of gene-specific T cells derived from LV-transduced HSCs.

High-level TCR expression is identified within thymic subpopulations 1 year after transplantation. Early lymphoid progenitors travel to the thymus and undergo a complex, multi-step process of differentiation, proliferation, and repertoire selection (32). Although much of this process occurs early in mammalian life, there is increasing clinical and experimental evidence that thymopoietic activity undergoes
Figure 2
LV gene–modified HSCs functionally engraft and repopulate transplant recipients over the long term. (A) Overview of transplant procedure. Lin– cells were isolated and transduced with LV-TCR. Competitive reconstitution was performed using transduced Lin– cells (CD45.1, $5 \times 10^4$) combined with congenic BM cells (CD45.2, $2.5 \times 10^5$) from DR4 Tg mice, then transplanted into irradiated DR4 Tg recipients. A parallel arm involving Lin– cells ($5 \times 10^4$) from TCR Tg mice combined with unfractionated BM ($2.5 \times 10^5$) from DR4 transgenic mice was transplanted into similarly irradiated hosts. (B) Flow cytometry analysis of PB at 6 and 12 months demonstrated high-level TCR gene expression: CD45+CD3+CD4+Tyrc1 Ultimer+ staining of LV-TCR and TCR Tg transplant groups. (C) Flow cytometry analysis of PB at 12 months demonstrated low-level TCR gene expression within CD8 compartment: CD45+CD3+CD8–Tyrc1 Ultimer+ staining of LV-TCR and TCR Tg transplant groups. (D) Flow cytometry analysis of PB at 12 months demonstrated high-level co-expression of the murine TCRβ chain in both CD4+Ultimer+ and CD4–Ultimer– subpopulations. Data are representative of flow cytometry studies done on 4 separate transplant experiments.
substantial re-amplification (or "thymic rebound") well into late adult life (33). These findings are particularly true in patients successfully treated for HIV or following chemotherapy-induced lymphopenia and subsequent transplantation (34). Given our results that both the appropriate restriction element (HLA-DR4) and coreceptor expression (CD4, not CD8) are critical for TCR expression, we evaluated cellular density and compositional makeup of individual thymic subpopulations in 12-month-old transplant recipients (approximately 14 months overall age). In addition, we again sought to determine whether an intact endogenous murine TCR was co-expressed in thymic TCR-specific subpopulations.

From a cohort of 4–6 mice per experiment, 12 months post LV-TCR transplantation, thymocytes were analyzed by multicolor flow cytometry with fluorochrome antibodies to CD45, CD3, CD4, CD8, murine TCRβ, and the Tyrp1 Ultimer. Despite the advanced age of the mice, a high percentage of TCR-specific gene expression was observed in double-negative (DN), double-positive (DP), and single-positive (SP) populations (see Figure 5, A–C), findings consistent with continued HSC production and thymic replenishment of gene-specific T cell progenitors. We also observed a steep and significant drop in TCR gene expression (DP, 45% ± 7.3%; SP CD4, 13.3% ± 3.1%; P = 0.002), with no corresponding change in MFI, as thymocytes transitioned from DP to SP, which was consistent with partial central elimination of autoreactive Tyrp1L277–297–specific DR4-restricted T cells. These findings are in agreement with existing models of self-tolerance, in which autoreactive T cells are eliminated within the thymus by medullary thymic epithelial cells that promiscuously express

Figure 3
Summary results of 12-month primary transplants. (A) Twelve-month transplant summary of TCR gene expression in LV-TCR and TCR Tg groups within the total cellular compartment as measured by evaluation of 10,000 gated events from PB. At 12 months, 8% ± 1.7% versus 22.6% ± 1.5%; P = 0.001. (B) Twelve-month transplant summary of TCR gene expression in LV-TCR and TCR Tg groups within CD3 compartment. At 12 months, 17.8% ± 2.3% versus 36.7% ± 2.4%; P = 0.006. (C) Twelve-month transplant summary of TCR gene expression in LV-TCR and TCR Tg groups within CD4 compartment. At 12 months, 28.1% ± 2.4% versus 65.2% ± 6.3%; P = 0.004. (D) Twelve-month transplant summary of TCR gene transfer efficiency (% = LV-TCR/TCR Tg × 100) within CD3 and CD4 compartments. Data (mean ± SEM with P value; n = 5–10 per group) are representative of 3–4 independent experiments.
cognate antigen, by thymic dendritic cells, or by cross-reactive peptides (35, 36). These finding are also supported in our own antigen model, in which higher-avidity T cells are generated in mice genetically deficient in Tyrp1 (13). Despite low systemic (IHC). As shown in Figure 7, subdermal hair follicle–based melanoctyes were found obliterated, with effacement of normal architecture, based on staining with H&E (Figure 7, A and B) and S-100 (Figure 7, C and D). These findings were further supported by a dense infiltration of CD3 T cells into the subdermal space in and around obliterated hair follicles (Figure 7, E and F). When frozen sections of identical skin samples were stained with antibodies to CD4 and CD8, only CD4 staining was observed (Figure 7, G and H). To determine whether TCR gene-specific T cells were invading the subdermal compartment, sections were stained with an exclusively human TCR β-chain specific antibody (Figure 7I). As shown, staining was identical in both pattern and location to CD3 staining and distribution. To verify antibody specificity, human tonsil and mouse spleen samples were similarly analyzed and found to be positive and negative, respectively (data not shown).

Figure 4
TCR gene-specific T cells represent a dominant fraction of all lymphocytes within the CD45.1 subcompartment. (A) Flow cytometry analysis of PB at 12 months post-transplantation demonstrated high-level TCR gene expression: CD45.1+CD3+Tyrp1 Ultimer+ and CD45.1+CD4+Tyrp1 Ultimer+ staining of LV-TCR transplants. (B) Six- and 12-month transplant summary of TCR gene expression within the CD3 and CD4 subpopulation as a function of CD45.1-gated cells. At 12 months: CD3, 37% ± 7.4%; CD4, 54% ± 7.8%; P = 0.04. Data (mean ± SEM with P value; n = 5–10 per group) and flow cytometry are representative of 2 independent experiments.

Recipients develop spontaneous autoimmune vitiligo after transplant. Between 8 and 12 weeks after transplantation, LV-TCR mice were consistently found to develop progressive skin depigmentation or vitiligo, a finding not observed in control transplanted mice, including those transplanted with LV-GFP–transduced HSCs (see Figure 6). This pattern of depigmented skin bordered by areas with no apparent signs of pigment loss resembled vitiligo development in other models of Tyrp1 autoreactivity (20–23). Vitiligo development was also observed in mice transplanted with HSCs derived from TCR Tg mice, but not in endogenous, nontransplanted TCR Tg mice nor in transplanted mice lacking expression of HLA-DR4 (Table 1). These results suggest that myelodepletion and transplantation combined with specific immune reconstitution (either from LV gene–modified or Tg HSCs in combination with HLA-DR4 expression) provide appropriate conditions for development of autoimmune vitiligo.

To address the pathogenesis of autoimmune vitiligo, we analyzed both paraffin-embedded and liquid nitrogen–frozen skin samples from LV-TCR and control transplants by immunohistochemistry (IHC). As shown in Figure 7, subdermal hair follicle–based mela..
endogenous, nontransplanted TCR Tg mice. The CD4*Ultimer* subpopulation was similar in its expression profile to that of the control transplant population (CD4*CD45RB*CD62L*), suggesting divergence of 2 distinct T cell populations within the same host — the TEM CD4*Ultimer* versus the effector CD4*Ultimer* — independent of effects of transplantation.

Given the spontaneous TEM status of CD4*Ultimer* cells, we then addressed whether or not T cells from LV-TCR transplants would immediately react to Tyrp1-specific targets. To test this, splenocytes from 6-month-old LV-TCR and control transplants were harvested, then stimulated ex vivo for 24 hours with peptide or tumor. As shown Figure 8C, cells obtained from the LV-TCR group responded to Tyrp1-specific targets, secreting IFN-γ only (but not IL-4 or IL-17A; data not shown), in response to Tyrp1_{277–297} at titering peptide concentrations and to B16-DR4, but not to control targets HA_{306–318} or to control tumor MC38-DR4. Control transplants were completely unresponsive to all Tyrp1-specific targets (data not shown). These results appear consistent with specific and high-avidity (recognition observed at 1 nM) Th1-polarized T cell population.

CD4* T cells from secondary transplants exhibit ongoing gene-specific TCR stability and multi-copy integration of the LV gene product. Secondary transplants were prepared to further demonstrate stability of TCR gene expression. Chimeric unfractionated BM was obtained from three 12-month-old primary transplants (per experiment), as shown in Figure 2, and injected without addition of competitive BM into lethally irradiated DR4 Tg mice (5 mice per experimental group). Six months after secondary transplantation, TCR-specific cells were identified at a level of 7.1% ± 4.5% within the total cellular compartment (CD45*Ultimer*+), 47.5% ± 8.3% within the global CD3 compartment (CD45*CD3*Ultimer*+), and 53.1% ± 10.7% within the CD4 subcompartment (CD45*CD3*CD4*Ultimer*+) (see Figure 9, A and B). These results were notable for higher expression levels compared with the 12-month expression levels observed in the parental primary transplant group (12-month primary CD4 vs. 6-month secondary CD4; P = 0.02). Again, high-level co-expression (93%) of the endogenous murine TCRβ was observed in the CD4*Ultimer* subpopulation.

Having demonstrated stable and high-level TCR gene expression 18 months after a primary transplantation, we evaluated genetic stability of 6-month-old secondary transplant recipients. Genomic
lymphocytes (41) enhances antitumor immune responses. Given high-level TCR gene expression and by standard PCR to determine the number of LV integrants. Using a probe specific for the LV vector backbone, 4–6 integration sites and internal control fragment were identified by nested PCR in 3 separate LV-TCR transplant samples, compared with control transplant (0 integrants) (see Figure 9D). Both results demonstrate long-term post-transplant genetic stability and integration.

Secondary TCR transplant recipients reject subcutaneous melanoma. Immune reconstitution after BM transplantation (39), dendritic cell vaccination (40), or adoptive transfer of antigen-specific lymphocytes (41) enhances antitumor immune responses. Given high-level TCR gene expression, spontaneous autoimmune, T_{EM} activation, and in vitro antitumor activity, we asked whether long-term secondary transplant recipients would induce protective tumor immunity. To address this question, transplant recipients (both LV-TCR and control, 5 mice per group) were subcutaneously injected with B16-DR4 tumor cells. Because of the preexisting “activated” T_{EM} status of transplanted animals, no additional cytokines, immune modulators (such as α-CTLA-4), or vaccinations were added to enhance the antitumor effect. Beginning 3 days after injection, and every 3 days thereafter, the product of the perpendicular tumor diameters was recorded. As shown in Figure 10A, secondary LV-TCR transplant recipients significantly rejected tumor growth compared with control transplants over a 20-day period. In addition, tumor size between day 12 and day 20 in the LV-TCR group was not significant (P = 0.09), suggesting cessation of tumor growth. Based on IHC staining of paraffin-embedded specimens, tumors from LV-TCR transplants but not controls were characterized by extensive necrosis and hemorrhagic infiltrates (Figure 10, B and C; H&E staining). Tumors from LV-TCR transplants were also found to contain dense trafficking populations of CD3+ and human TCR-β (gene-specific) tumor-infiltrating lymphocytes (Figure 10, C–E). A similar infiltrative pattern was observed in the autoreactive vitiligo skin specimens.

LV-TCR gene-modified human HSCs repopulate 6-month-old NS2 transplant recipients and develop into TCR gene–specific CD4+ T cells. Having now established herein an animal model of gene-modified HSC transplantation, as well as having previously shown that transduced mature human lymphocytes can specifically recognize tumor targets, we next sought to determine whether human HSCs would engraft and develop into TCR gene–specific CD4+ T cells. NOD-SCID-IL2rcnull (NS2) mice have emerged as a reliable vehicle of human T cell development following transfer of either HSCs or progenitor T cell populations (42). To address this question, CD34+CD38− cells were purified from cord blood of 5 separate donors. Following in vitro prestimulation, LV-TCR–transduced CD34+CD38− cells were transplanted into sublethally irradiated NS2 mice (5–6 per experiment). To ensure co-expression of HLA-DR4, animals were competitively reconstituted with BM from DR4 Tg mice. Six months after transplantation, splenocytes were column enriched for human CD45 expression, then stained with antibodies to human CD3, CD4, and CD8 and the Tyrp1 Ultimer, and analyzed by flow cytometry. As shown in Figure 11, A and B, although human CD45+ cells represented only 10% of all mononuclear cells, human CD3+ T cells were largely CD4+ (45% human CD3+/SP CD4+ vs. 4% human CD3+/SP CD8+). A subpopulation of DP (49% CD3+CD4+CD8−) cells also appeared to have developed, suggesting that NS2 mice may fail in this experimental setting to fully differentiate all T cells. Most importantly, TCR-specific levels were equally well expressed within all T cell populations, suggesting autonomous TCR gene expression regardless of phenotype.

**Table 1**

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Txp, transplant.

**Figure 6**

LV-TCR transplant recipients develop spontaneous autoimmune vitiligo. DR4 Tg mice transplanted with LV-TCR–transduced HPCs (right mouse) developed autoimmune vitiligo at 10 weeks, but the control transplants (left mouse) did not.
Discussion

While most gene therapy strategies have focused on the introduction of defective genes or antigens or used fluorescent gene products to track long-term development, few have introduced TCR genes into HSCs (18). In those examples that do exist, TCR genes introduced with retroviral vectors targeted only experimental antigens (OVA) (43, 44). Herein, we report a new translational model of TCR-based immunotherapy with potential direct applicability to patients with metastatic melanoma. We demonstrate spontaneous autoimmunity and destruction of subcutaneous melanoma that occur following long-term transplantation with gene-modified HSCs. Our results, based on use of a high-expression LV system to deliver to HSCs a potent class II–restricted CD4+ TCR, were not assisted by use of vaccines, cytokines, or immune modulators to either induce a population of autoreactive memory T cells or achieve antitumor effects.

To design a model encompassing features that would address both self-tolerance and tumor immunity while also serving as a potential source of preclinical information, we utilized a previously identified tumor-reactive, self-reactive, Tyrp1-specific, DR4-restricted T cell clone (19). We then engineered both a high-expression LV and a TCR Tg mouse expressing the same TCR genes. We focused on current generation SIN lentivectors not only because of their enhanced capacity to stably transduce metabolically dormant HSCs, but because of their improved biosafety profile compared with standard γ retroviruses (45). Although a case of clonal dominance has been reported to the NIH (but not formally published) following use of a β-globulin LV, overall evidence suggests that lentivectors have lower rates of genotoxicity and an altogether safer integration profile when compared with γ retroviruses (46). These experimental observations have been further supported by clinical trials either ongoing or in preparation for treatment of patients with HIV, β-thalassemia, Parkinson disease, adrenoleukodystrophy, and Wiskott-Aldrich syndrome (47).

Following creation of a dual-functional LV-TCR and TCR Tg model, we demonstrated engraftment, thymic trafficking and differentiation, specific CD4+ T cell selection, long-term TCR gene expression in both murine and humanized transplants, genomic LV integration, spontaneous post-transplant autoimmunity, and hemorrhagic necrosis of subcutaneous melanoma. We also discovered that despite integration of LV-TCR into HSCs, endogenous mouse TCR expression was still fully intact and co-expressed with our transgene. These new findings were based on staining with antibodies specific for murine CD3, an antibody specific for murine TCRβ chain, and the pentamer specific for the combined human TCR chains. These results indicated that co-expression likely results from 2 distinct autonomous sources of TCR gene expression: native TCR genes under the control of endogenous TCR promoter/enhancer elements and LV-TCR genes under control of the heterologous CMV promoter. There also appeared to be little evidence that any significant chain mispairing occurred. We conjecture that human TCR genes were co-expressed in mouse T cells, creating a species-specific human-mouse TCR chain dichotomy, thus minimizing the risk of mixed TCR-dimer formation. Such a hypothesis is supported by results from studies that have introduced murine TCR genes specific for p53 into human T cells (48) or following genetic modification of TCR chains (49).

Given the importance of early effector phenotypes, we designed an experimental translational model focused on long-term expression of unmanipulated CD4+ T cells. A permanently reconfigured immune system of durable high-frequency lymphocytes has long been a goal of immunologists and clinicians in pursuit of treatments for viral pathogens and cancer (50). Concerns persist, however, regarding the feasibility of delivering such a treatment within a sufficiently narrow time frame. Most clinical therapies and translational models have focused on fully actualized treatments that deliver their effect with little delay, such as vaccines (peptides, viruses, dendritic cells, heat shock proteins, gene-modified tumor cells), cytokine infusions (IL-2, IL-7, IL-12, GM-CSF), immune modulators (Treg depletion, anti–CTLA-4), or adoptive immunotherapies (tumor-infiltrating lymphocytes, cloned T cells,
gene-modified T cells) (51, 52). Limitations and delays in immune reconstitution are a well-described physiologic consequence of autologous HSC transfer, and kinetics of individual mononuclear populations favor early return of granulocytes and B cells, with CD4+ T cells often being the last population to return to normal levels (53). In our model, gene-specific CD4+ T cells reached their peak expression after 4–6 months, suggesting a similar problem of transplant T cell recovery. To improve T cell output, IL-7 administration has been successfully used in patients with cancer (54). Nevertheless, the Achilles heel of such a potential treatment in patients pertains to the relatively late emergence of CD4+ T cells.

Despite these delays, timing of maximum (or even normal) T cell recovery may, ironically, not be the most meaningful physiologic endpoint of autologous HSC transfer, and kinetics of individual mononuclear populations favor early return of granulocytes and B cells, with CD4+ T cells often being the last population to return to normal levels (53). In our model, gene-specific CD4+ T cells reached their peak expression after 4–6 months, suggesting a similar problem of transplant T cell recovery. To improve T cell output, IL-7 administration has been successfully used in patients with cancer (54). Nevertheless, the Achilles heel of such a potential treatment in patients pertains to the relatively late emergence of CD4+ T cells.

In our model, vitiligo developed not only in DR4 Tg mice but also in C57BL/6 transplant recipients. These observations were associated with corresponding similar levels of TCR-specific cells (data not shown). It is well known that melanocytes isolated from normal C57BL/6 mice have low class II expression (55). Vitiligo development in C57BL/6 recipients underscores a broader range of effects by gene-specific CD4+ T cells, regardless of their relative magnitude or frequency or the presence of Tregs (13). Polarized

Figure 8
Transplant recipients exhibit a T EM phenotype and react spontaneously to tumor ex vivo. (A) PB samples at 6 months post-transplantation were obtained and stained with antibodies against CD4, Tyrp1 Ultimer, CD44, CD45RB, and CD62L. LV-TCR transplant CD4+Ultimer+ cells exhibited a T EM phenotype (CD45RB LO CD62L LO CD44 HI), while the polyclonal CD4+Ultimer+ and CD4+ control population exhibited a more generalized effector profile (CD45RB HI CD62L LO CD44 HI), in contrast with naive CD4+Ultimer+ cells (CD45RB HI CD62L LO CD44 HI) from endogenous nontransplanted TCR Tg. (B) Summary of activation status of all CD4+ subpopulations. (C) Gene-modified T cells exhibited a Th1-polarized cytokine profile in response to specific peptide and tumor. Splenocytes from 6-month-old LV-TCR and LV-GFP transplants were stimulated ex vivo for 24 hours with peptide (Tyrp1277–297 or HA306–318) or tumor (MC38-DR4 or B16-DR4). Cytokine release (IFN-γ) was measured by ELISA. Data (mean ± SEM; n = 5 per group) and flow cytometry are representative of 3 independent experiments.
The production of IFN-γ by Th1 cells has been shown to produce multiple effects, including direct melanocyte cytotoxicity, upregulation of MHC molecules that enhance target recognition, and enhancement of APC activation, leading to further amplification of T helper response (9). Such functions have been associated with the unique but underutilized properties of CD4⁺ T cells.

While long-term cellular persistence was an initial objective of this model, the amplitude of gene-specific T cell expansion (between 8 and 24 weeks) and T₃₈₄ activation were dramatic and, we propose, associated with both autoimmunity and antitumor activity. Both spontaneous and homeostatic proliferation (HP) represent naturally occurring multi-log expansion of lymphocytes in

Figure 9
CD4⁺ T cells from secondary transplant recipients exhibit high-level gene TCR expression and multi-copy integration of the LV-TCR gene product. PB from 6-month secondary transplants were stained with antibodies against CD4, CD8, CD3, and Tyrp1 Ultimer and analyzed by flow cytometry. (A) TCR-specific CD4⁺ T cells were identified at a level of 61% within the CD4 subcompartment (CD4⁺CD4⁺Ultimer⁺). Co-expression (93%) of endogenous murine TCRβ was also observed in the CD4⁺Ultimer⁺ subpopulation. (B) Summary of 6-month TCR gene expression within secondary transplant. Total, CD3, and CD4 populations: 7.1% ± 4.5% within the total cellular compartment (CD45⁺CD4⁺Ultimer⁺), 47.5% ± 8.3% within the global CD3 compartment (CD45⁺CD3⁺Ultimer⁺), and 53.1% ± 10.7% within the CD4 subcompartment (CD45⁺CD3⁺CD4⁺Ultimer⁺). (C) Relative TCR gene expression was more than 1,000-fold greater than control transplants but 43-fold less than TCR Tg transplant (P = 0.01). gDNA was prepared from BM obtained from 6-month-old secondary LV-TCR and control transplants and 12-month-old primary TCR Tg transplants and analyzed by qPCR for relative TCR gene expression using an α-chain-specific probe. Relative gene expression is displayed; error bars indicate the 95% confidence interval. (D) Multicopy integration of the LV-TCR gene product was found in secondary transplant recipients. Using a vector backbone–specific probe, 4–6 integration sites were identified in 3 separate LV-TCR transplant samples compared with control. Data (mean ± SEM; n = 5 per group) and flow cytometry are representative of 2 independent experiments.
response to depletion or ablation, followed by either BM reconstitution or adoptive transfer (56, 57). Immune responses that occur in response to both forms of proliferation favor the precipitation of autoimmune events (58). These observations have been associated with a number of factors, including increased availability of homeostatic cytokines (IL-7, IL-15, IL-21), depletion or inhibition of Tregs, generalized host inflammation, and availability of self-ligands (56, 59, 60). IL-7 availability, in particular, has been found to be a critical factor in T helper and thymocyte survival along with promoting HP of both naïve and memory T cells (61).

There are a number of possible explanations for the proliferative response of TCR-specific CD4⁺ T cells. Even though the importance of IL-7 has been clearly stated, we found IL-7 receptor expression by T cells, Tyrp1-loaded dendritic cells may be uniquely available to the homeostatic cytokines (IL-7, IL-15, IL-21), depletion or inhibition or Tregs, generalized host inflammation, and availability of self-ligands (56, 59, 60). IL-7 availability, in particular, has been found to be a critical factor in T helper and thymocyte survival along with promoting HP of both naïve and memory T cells (61).

As a consequence of either scenario, T cell clones can give rise to multiple effector and memory T cell populations by means of asymmetric cell division (38, 68). After antigen exposure, memory cell expansion is classically followed by contraction and stable persistence (69). Although we showed activation status at 6 months following primary transplantation, we found a sharp increase in total CD4⁺Ultimer⁺ T cells between 12-month primary and 6-month secondary transplants, consistent with both memory decay and re-expansion. We also found a splitting of 2 distinct T cell populations within the same host irrespective of the effects of transplantation. CD4⁺Ultimer⁺ T cells may have had both a competitive survival advantage and propensity for memory differentiation based on the relatively high availability of Tyrp1 and a corresponding high-frequency, high-affinity cognate TCR (57).

The most clinically significant evidence we present is the near total liquefaction of tumor following subcutaneous implantation in long-term secondary transplants. These results demonstrate not only potent antitumor activity 18 months after initial transplanta-
While many adoptive immunotherapies have either maximized their therapeutic effect or been totally dependent upon cytokines that these freestanding antitumor effects were likely secondary to. We developed a new TCR Tg mouse strain described below. All mice were 6–10 months of age, and all gated mononuclear cells (mouse and human; pre-fractionation sample) and post-fractionation expression of human CD45 within human SP CD8+ T cells. (Invitrogen). mAbs were used to block T cell interactions (at 50 μg/ml) and included L243 (against HLA-DR; IgG2a, eBioscience), W6/32 (against HLA-A, -B, and -C; IgG2a, eBioscience), L3T4 (against CD4; IgG1, eBioscience), and HIT8a (against CD8; IgG1; eBioscience). To evaluate specific T cell reactivity, 1088 EBV-B cells were pulsed for 3 hours with peptide (at various concentrations) or tumor lysates (at 10^6 cell equivalents/ml). To upregulate MHC class II, 624 Mel was pretreated with IFN-γ for 48 hours at 200 U/ml. All targets (10^4) and T cells (10^5) were cocultured in duplicates in U-bottom 96-well plates for 24 hours. Culture supernatants were assayed for IFN-γ using commercially available ELISA kits (BD Biosciences). All assays were performed at least twice, with similar experimental results.

Cloning of TCR. We had previously identified a Trypt-specific, tumor-reactive HLA-DR4–restricted CD4+ T cell from a patient with metastatic melanoma (19). The established line was cloned by limiting dilution (73), then followed by the isolation and identification of full-length TCR gene expression within human SP CD8+ T cell subpopulations. Data (mean ± SEM; n = 5 per experiment) and flow cytometry are representative of 3 independent experiments.

Methods

Animals and cell lines. Murine class II–deficient, DR4-IE transgenic (DR4 Tg) mice fully backcrossed onto a C57BL/6 background were purchased from Taconic Farms. B6.SJL-Ptpc<sup>p</sup>/Pepe<sup>2</sup>/Boy (or Boy/J) and NOD/SCID/IL2<sup>−/−</sup> mice were purchased from The Jackson Laboratory. We developed a new TCR Tg mouse strain described below. All mice were 6–10 weeks old for experiments and were housed and bred at the Indiana University Laboratory Animal Research Center barrier facility under an established animal protocol. Murine tumor lines B16 (ATCC) and MC-38 (ATCC), human melanoma lines 624 and 1102 Mel, human EBV-B cell line 1088 EBV-B (provided by S.A. Rosenberg, NCI/NIH, Bethesda, Maryland, USA), and human lymphoid cell line Sup-T1 (ATCC) were maintained in complete media. HLA-DRB1<sup>+</sup> genotypes of human tumor lines used included 624 Mel (0401,0701), 1102 Mel (0401,1502), and 1088 EBV-B (0301,0401) (72). Human melanoma line 1102 Mel was transduced with retrovirus pLXSN (Clontech) expressing either GFP or Trypt1 (provided by P. Robbins, NCI/NIH, Bethesda, Maryland, USA).

Murine tumors B16 and MC-38 were transduced with lentivirus expressing DRα and DRB1<sup>+</sup>0401 (described below). DR4-expressing tumors were periodically enriched by flow sorting to maintain expression at more than 80%. All reagents and studies reported here were approved by animal, biosafety, and internal review boards at the Indiana University School of Medicine.

In vitro assays. Purified peptides (HAP306-318, PKVVKQNTLK, human Tyrrp1<sub>277-285</sub>, ISPNSVFSQWRVCDSEL-DYD) were purchased from BioSource (Invitrogen). mAbs were used to block T cell interactions (at 50 μg/ml) and included L243 (against HLA-DR; IgG2a, eBioscience), W6/32 (against HLA-A, -B, and -C; IgG2a, eBioscience), L3T4 (against CD4; IgG1, eBioscience), and HIT8a (against CD8; IgG1; eBioscience). To evaluate specific T cell reactivity, 1088 EBV-B cells were pulsed for 3 hours with peptide (at various concentrations) or tumor lysates (at 10<sup>6</sup> cell equivalents/ml). To upregulate MHC class II, 624 Mel was pretreated with IFN-γ for 48 hours at 200 U/ml. All targets (10<sup>4</sup>) and T cells (10<sup>5</sup>) were cocultured in duplicates in U-bottom 96-well plates for 24 hours. Culture supernatants were assayed for IFN-γ using commercially available ELISA kits (BD Biosciences). All assays were performed at least twice, with similar experimental results.

Cloning of TCR. We had previously identified a Trypt-specific, tumor-reactive HLA-DR4–restricted CD4+ T cell from a patient with metastatic melanoma (19). The established line was cloned by limiting dilution (73), then followed by the isolation and identification of full-length TCRαβ and -γδ subunits, as previously described (24, 25). In short, total RNA was extracted from TD Clone-2, and each TCR subunit was amplified by a 5′ RACE method (GeneRacer Kit; Invitrogen). PCR products were TA cloned and sequenced to determine V<sub>α</sub> and V<sub>β</sub> expression within human SP CD8+ T cell subpopulations. Data (mean ± SEM; n = 5 per experiment) and flow cytometry are representative of 3 independent experiments.

Figure 11

LV-TCR gene–modified human CD34+/CD38− HPCs obtained from cord blood functionally engraft and repopulate 6-month-old NOD-SCID-IL2<sup>−/−</sup> (NS2) transplant recipients. (A) Flow cytometry analysis of human CD45 expression within human SP CD8+ T cells. (Invitrogen). mAbs were used to block T cell interactions (at 50 μg/ml) and included L243 (against HLA-DR; IgG2a, eBioscience), W6/32 (against HLA-A, -B, and -C; IgG2a, eBioscience), L3T4 (against CD4; IgG1, eBioscience), and HIT8a (against CD8; IgG1; eBioscience). To evaluate specific T cell reactivity, 1088 EBV-B cells were pulsed for 3 hours with peptide (at various concentrations). Data (mean ± SEM; n = 5 per experiment) and flow cytometry are representative of 3 independent experiments.
amplified from the same full-length sequence (described above) and TA cloned into pCR4TOPO (Invitrogen) validated by sequencing, subcloned into TCR cassette vectors provided by D. Mathis (Harvard Medical School, Boston, Massachusetts, USA; ref. 30), and co-injected into fertilized C57BL/6 embryos yielding 9 TCR Tg founder lines. Founder mice were screened by PCR for presence of transgene and then crossed with founder mice and with DR4 Tg mice to generate experimental colonies. Purity were screened for allelic copies of TCR and DR4 genes by PCR or by flow cytometry using PE-labeled DR4 Ultimer containing the Tyrp1_{277,297} epitope (ProImmune Ltd).

**Construction of lentivectors.** TCRα and β chains were re-amplified with an optimal Kozak initiation sequence and restriction sites using Platinum-Pfx polymerase (Invitrogen). To generate the IRES vector, the α chain was cloned into an IRES-containing derivative of pENTR1a (Invitrogen) from Dral to a SpeI site 5’ to IRES. The β chain was cloned 3’ from an Ncol site at the 13th ATG of IRES to XbaI. Both chains were sequenced and LR-cloned into a modified Gateway (Invitrogen) destination LV plasmid (pRRL-Dest-Wsin) (74). These modifications included a cPPT (central polypurine tract derived from HIV-pol) sequence inserted between the REV response element (RRE), the CMV promoter, and the woodchuck hepatitis virus posttranslational regulatory element (WPRE) element proximal to the 3’ SIN-LTR (75). To generate the F2A vector, Furin-2A peptide sequence was exchanged through a series of intermediates for the IRES element, transferred into the pENTR plasmid, then LR-cloned from the pENTR plasmid into pRRL-Dest-Wsin. Similarly constructed vectors which contain GFP (LV-GFP) and DRA-IRES-DRB1*0401 (LV-DR4) were also prepared.

**Preparation of LV supernatant and determination of viral titer.** LV-TCR-IRES and LV-TCR-F2A LV supernatants were prepared by transient transfection of 293T cells in T-150 flasks with vector DNA (26.4 μg), followed by a 2-hour 37°C incubation, using the Calcium Phosphate Kit (Promega). Culture medium was replaced 16–18 hours after transfection with OptiPro SFM (Invitrogen). Virus supernatants were harvested at 24 and 48 hours after media exchange, centrifuged at 2,000 × g for 30 min. The concentrated viral supernatants were aliquoted and frozen at -80°C before titer calculation. To determine the titer of frozen viral supernatants, SupT1 cells were plated at 10^6 cells/well in 6-well plates, exposed to serial dilutions (10^1, 10^2, 10^3, 10^4) of concentrated vector supernatants in DMEM medium, and incubated for 4 hours in presence of polybrene at 8 μg/ml (5% CO₂, 37°C). Cells were maintained in culture for 72 hours, stained with a PE-conjugated anti-human TCRβ chain antibody (BD Biosciences), and analyzed by flow cytometry. Titters were calculated using the following formula: titer = \( \frac{(F \times C / V)}{D} \), where \( F \) is the frequency of human TCRαβ chain (FITC; BD Bioscience) positive cells determined by flow cytometry, \( C \) is the total number of target cells infected, \( V \) is the volume of the inoculum, and \( D \) is the virus dilution factor.

**Transduction protocol.** Human PBMCs were transduced with either LV-TCR-IRES or LV-TRP1-F2A at titering MOI after overnight prestimulation with IL-7 (29). Surface TCR expression and function were measured by flow cytometry 72 hours after transduction, using the Tyrp1_{277,297}-specific Ultimer (ProImmune), or were measured with a standard cocculture and ELISA assay against targets. Transduction of murine HSCs was performed by first isolating BM from femurs and tibiae of mice. Erythrocytes were removed from BM using ACK lysis buffer, and remaining cells were filtered through a 100-μm filter and washed in PBS, 0.5% BSA, and 2 mM EDTA. Lineage depletion of BM was performed using a MACs lineage cell depletion kit (Miltenyi Biotec), yielding a purity of more than 95%, a viability of more than 99%, and with less than 0.1% staining for CD3 (data not shown).

Lin’ cells were maintained in StemPro SFM-24 medium (Invitrogen) supplemented with SCF 100 ng/ml, TPO 10 ng/ml, and Flt-3L 50 ng/ml and cultured overnight. Lin’ cells were then harvested, counted, assessed for viability, and plated for transduction. Human CD34+/CD38- cells were isolated from cord blood as previously described (76) and similarly prestimulated. All transductions were performed in 24-well plates using a 500-μl reaction volume containing 1 × 10^5 CD34+/CD38- cells, polybrene 8 μg/ml, and concentrated lentivirus at an MOI of 15. Transductions included a 2-hour 25°C spinoculation at 240 g, followed by a 2-hour 37°C incubation. Cells were incubated for an additional 12–24 hours in cytokine media, then either analyzed by flow cytometry (after an additional 48 hours) or transplanted into recipient mice.

**Transplantation protocol.** Lin’ cells were isolated and transduced with LV-TCR-F2A at an MOI of 15. A control transplant arm of similarly prestimulated Lin’ cells was also prepared. Competitive reconstitution was performed using Lin’ cells (CD45.1, 5 × 10^6) combined (LV or control) with unfractionated BM (CD45.2, 2.5 × 10^6) from DR4 Tg, and transplanted by tail vein injection into previously irradiated (990 rads) DR4 Tg recipient mice. A parallel arm involving Lin’ cells (CD45.2, 5 × 10^6) from TCR Tg mice were similarly combined with unfractionated BM (2.5 × 10^6) from DR4 Tg mice and transplanted into similarly irradiated hosts. Secondary transplants were generated by injecting 5 × 10^6 unfractionated BM chimeras from 12-month-old primary transplants into irradiated DR4 Tg mice. For humanized transplants, a similar competitive reconstitution was performed using transduced human CD34+/CD38- cells (hCD45, 5 × 10^6) combined with unfractionated BM cells (mCD45.2, 2.5 × 10^6) from DR4 Tg, and then transplanted by tail vein into previously sublethally (500 cGy) irradiated NS2 mice. Transplanted animals were kept in isolation and treated prophylactically with dexamethasone.

**Flow cytometry sorting and analysis.** At different time points after transplantation, PB cells, splenocytes, or thymocytes were collected and stained with fluorochrome-conjugated antibodies specific for various murine surface molecules and analyzed by multicolor flow cytometry to determine percent frequency. To determine gene-specific T cell frequency, we used a combination of antibodies against CD45 (FITC; eBioscience), CD45.1 (FITC; eBioscience), CD3 (PE Cy5; BD Biosciences), CD4 (APC; BD Biosciences), CD8 (Pacific Blue; eBioscience), CD25 (PE Cy7; eBioscience), Foxp3 (Pacific Blue; eBioscience), murine TCRβ (PE Cy5; BD Biosciences), and the DR4 pentamer containing either the Tyrp1_{277,297} epitope (Ultimer; TCR Tg) or the control gp100_{254,269} epitope (gp100 Ultimer) (31) (both PE-labeled; Promimmune). To determine T cell activation status, a combination of antibodies against CD4 (APC), Tyrp1 Ultimer (PE), CD44 (PE Cy5; eBioscience), CD45RB (FITC; eBioscience), and CD62L (Pacific Blue; eBioscience) was used. To determine frequency of post-transplant human mononuclear cells following transplantation into NS2 mice, splenocytes were column enriched for human CD45 expression (Miltenyi Biotec), then stained with antibodies to human CD45 (PE Cy5; BD Biosciences), human CD3 (FITC; eBioscience), human CD4 (APC; BD Biosciences), human CD8 (Pacific Blue; eBioscience), and TCR Tg (Ultimer PE).

**IHC.** Skin or tumor samples were fixed in formalin and embedded in paraffin or snap frozen in liquid nitrogen. Standard H&E staining was done on all specimens. For skin samples, blocks were stained with S100 (rabbit polyclonal 1:1,000; Dako; Envision Rabbit Polymer DAB), CD3 (rabbit polyclonal 1:200; Dako; Envision Rabbit Polymer DAB), or human TCRβ (mouse IgG1 1:50, Santa Cruz Biotechnology Inc.; Envision Rabbit Polymer DAB). Skin samples were also frozen in liquid nitrogen and stained with antibodies to CD4 (RM4-5 1:500; Serotec; donkey anti-rat DAB) and CD8 (169.4 1:200; Serotec; donkey anti-rat DAB). For tumor samples, blocks were stained with CD3 (Envision Rabbit Polymer NovaRED), or human TCRβ (Envision Rabbit Polymer NovaRed).
TaqMan probe (Applied Biosystems) specific for the LTR-TCR gene. A qPCR analysis using ABI One-Step Real-Time PCR System (Applied Biosystems) was then performed using a custom designed TaqMan probe (Applied Biosystems) specific for the α-variable chain of the LV-TCR vector. A standard curve was generated by making serial dilutions of plasmid DNA from the LV-TCR. Relative TCR gene expression was based on the amplification of each sample against the standard curve. The PCR reactions were set up with TaqMan PCR Core Reagents (Applied Biosystems) using about 100 ng of gDNA (20 μl total reaction volume) and amplified at 50°C for 2 minutes, then 95°C for 10 minutes plus 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Lentivirus integration analysis. gDNA was prepared (Qiagen) from 1 x 10^8 BM cells obtained from 6-month secondary transplant recipients (both LV-TCR and control). Concentration and purity of the gDNA were determined by spectrometry. All samples showed a 260/280 ratio of 1.7 or greater, indicating a high purity. Sample concentrations varied from 0.400 to 0.0500 mg/ml. The gDNA from transplant recipients were individualy digested with Tsp5091 (New England BioLabs) at 65°C for 4 hours. After digestion, gDNA fragments were purified using a MinElute Reaction Cleanup Kit (Qiagen). In short, 150 μl of elution reaction cleanup (ERC) buffer was added to each sample and transferred to spin columns. Columns were centrifuged for 1 minute at 10,000 gDNA was isolated from 1 μl of the bead slurry was added to each PCR reaction tube and a second round of nested PCR was performed. Microbeads with covalently attached streptavidin (Dynabeads M-280; Invitrogen) were washed, and 40 μl of the bead slurry was added to each biotinylated primer. Beads were subsequently washed and blunt-end adaptors ligated with T4 DNA ligase. Integration sites were then identified by nested PCR: master mix consisting of 2x Extensor HiFi PCR mix (Invitrogen) and gDNA fragments were aliquoted into 200-μl PCR reaction tubes. The microbead-primer bead slurry (1 μl) was added and PCR amplified (94°C for 2 minutes, 95°C for 15 seconds, 60°C for 30 seconds, 68°C for 2 minutes for 30 cycles; 68°C for 10 minutes, 4°C hold). After the first round of nested PCR, a 1:50 dilution was done from each PCR reaction tube and a second round of nested PCR was performed. Each PCR sample was analyzed by agarose gel electrophoresis. In Figure 9D, “internal control fragment” refers to amplification of the proviral DNA 3′ to the 5′ LTR; “integration site” refers to amplification of DNA flanking the 3′ LTR (77).

Tumor treatment assays. Six-month-old secondary DR4 Tg transplant recipients in an experimental group (LV-TCR-F2a) and a control group were subcutaneously implanted (5 x 10^6 cell/200 μl HBSS) with B16-DR4 at 5 mice per group. Perpendicular tumor diameters were blindly recorded every 3 days. Mice were sacrificed at 20 days after implantation, and tumors were excised for IHC analysis. Experiments were performed twice in a blinded, randomized fashion, with similar results.

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