

HDAC inhibition prevents transgene expression downregulation and loss-of-function in T-cell-receptor-transduced T cells

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T cells that are gene-modified with tumor-specific T cell receptors are a promising treatment for metastatic melanoma patients. In a clinical trial, we treated seven metastatic melanoma patients with autologous T cells transduced to express a tyrosinase-reactive T cell receptor (TCR) (TIL 1383I) and a truncated CD34 molecule as a selection marker. We followed transgene expression in the TCR-transduced T cells after infusion and observed that both lentiviral- and retroviral-transduced T cells lost transgene expression over time, so that by 4 weeks post-transfer, few T cells expressed either lentiviral or retroviral transgenes. Transgene expression was reactivated by stimulation with anti-CD3/anti-CD28 beads and cytokines. TCR-transduced T cell lentiviral and retroviral transgene expression was also downregulated *in vitro* when T cells were cultured without cytokines. Transduced T cells cultured with interleukin (IL)-15 maintained transgene expression. Culturing gene-modified T cells in the presence of histone deacetylase (HDAC) inhibitors maintained transgene expression and functional TCR-transduced T cell responses to tumor. These results implicate epigenetic processes in the loss of transgene expression in lentiviral- and retroviral-transduced T cells.

INTRODUCTION

Despite increased public health program-induced awareness of the risks of sun exposure, the incidence of melanoma has doubled in the last 40 years.¹ This year alone, approximately 100,000 people will be diagnosed with melanoma in the United States, and 6,800 will die of their disease.² Cancer immunotherapy has become a prevalent modality of treatment for metastatic melanoma. Forms of immunotherapy such as checkpoint inhibitors (e.g., anti-PD-1 and anti-CTLA-4)³ and tumor-infiltrating lymphocyte (TIL) therapy depend on activating and expanding pre-existing tumor-specific T cells.⁴ In contrast, genetic modification of autologous T cells to

target the tumor does not depend on pre-existing anti-tumor T cells and has led to clinical responses in metastatic melanoma patients for over 14 years.^{5,6} Current testing of gene-modified T cell therapy is extensive, with over 100 clinical trials listed on ClinicalTrials.gov.

T cell receptor (TCR)-transduced T cells can induce partial or complete remission and can persist for years after transfer.^{7,8} While promising, many patients fail to respond to TCR-transduced T cells, and efforts are needed to improve the efficacy and durability of TCR-transduced T cell therapies.^{9–16} Studies have found that clinical efficacy correlates with the expansion and persistence of adoptively transferred T cells.¹⁷ Unfortunately, most patients treated with TCR-transduced T cells have few detectable TCR-transduced T cells in the blood beyond a month post-transfer.^{8,9,11} Previous work demonstrated that TCR-transduced T cells may lose transgene transcription over time.¹³ Such loss of transgene transcription may be predicted to lead to loss of expression of the anti-tumor TCR, reducing long-term efficacy of gene-modified T cells.

Despite the importance of transgenic TCR expression for the anti-tumor function of transduced T cells, the processes regulating maintenance of transgene expression and effects of transgene expression on functional responses of transduced T cells are largely unexplored. While conducting a clinical trial, we had an opportunity to study transgene regulation in both lentiviral- and retroviral-transduced T cells. All patients were treated with autologous T cells transduced

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to express the TIL 1383I receptor, which responds to an HLA-A2-restricted tyrosinase peptide in a CD8-independent fashion, permitting treatment with both CD4⁺- and CD8⁺-transduced T cells. The first cohort of three patients were treated with lentiviral-transduced T cells, while the remaining four were treated with retroviral-transduced T cells. In this study, we found that both lentiviral- and retroviral-transduced T cells exhibit reversible downregulation of transgene expression in patients and *in vitro*. During *in vitro* culture, the presence of interleukin (IL)-15, but not antigen or IL-2, prevented downregulation of transgene expression. Culture with sodium butyrate, an HDAC inhibitor, reduced transgene expression downregulation and retained T cell function in the absence of cytokines. Collectively, these results suggest that transgene downregulation in T cells is epigenetically regulated by histone deacetylation. Although further testing is needed, these results suggest the possibility that histone deacetylase (HDAC) inhibitor treatment might enhance the long-term clinical efficacy of transduced T cell therapies in patients.

RESULTS

Seven patients were treated with TIL 1383I TCR-transduced T cells, of which the first three were treated with lentiviral-transduced T cells (lentiviral transduced)⁸ and the remaining four with γ -retroviral-transduced T cells (retroviral transduced).¹⁸ We previously observed that the percentage of transduced T cells in the peripheral blood was highest in the first week post-transfer and then decreased thereafter.⁸ Closer investigation revealed a declining level of expression of the truncated CD34t marker gene (CD34) in CD34⁺ T cells in the weeks following adoptive transfer (Figure 1A). These decreases occurred in both CD34⁺ CD4⁺ (Figure 1B, top) and CD34⁺ CD8⁺ (Figure 1B, bottom) T cells in patients given retroviral- and lentiviral-transduced T cells. Decreasing transgene expression levels were observed in most patients, with the interesting exception of patient 2, who achieved a partial response after adoptive transfer, which eventually became a complete response after further immunotherapies.⁸ By 4 weeks post-transfer, a small percentage of patient T cells expressed low levels of the truncated CD34 marker gene (CD34) encoded in the lentiviral and retroviral vectors. We aimed to investigate whether TCR-transduced T cells might remain present but undetectable due to loss of transgene expression. To induce an activation state similar to that of cells undergoing transduction, T cells from patient samples taken 4 weeks post-adoptive transfer were activated with CD3- and CD28-crosslinking Human T-Activator beads (CD3/CD28 beads) in the presence of IL-2 and IL-15. Following stimulation, both the percent of transgene (CD34)-expressing T cells and the level of transgene expression were determined by flow cytometry (Figure 1C). Representative gating is shown in Figure S1. In all patients, activating the T cells increased the percent of detectable CD8⁺CD34⁺ T cells, although it did not consistently increase the percent of detectable CD4⁺CD34⁺ T cells (Figure 1D). However, the level of CD34 expression (the mean fluorescence intensity [MFI] of CD34 staining) on both CD4⁺CD34⁺ as well as CD8⁺CD34⁺ T cells was significantly increased, suggesting that activation did enhance transgene expression in both CD4⁺ and CD8⁺ T cells. Patients 4–7, treated with retrovirus-transduced T cells, ap-

peared to have greater CD34 upregulation after restimulation than patients 1–3, treated with lentivirus-transduced T cells. This apparent difference might be due to the higher dose of cells in patients treated with retrovirus, to higher transgene expression in retrovirus-transduced T cells, or to differences in epigenetic regulation of the transgene expression between viral constructs. CD34 staining on CD34-negative CD4⁺ and CD8⁺ T cells was, as expected, low, and did not significantly change after restimulation (Figure S2, top). CD34 staining on total T cells from healthy donors also was low and showed only a very slight increase with stimulation, likely due to increased autofluorescence (Figure S2, bottom). These results indicate that retroviral- and lentiviral-transduced CD4⁺ and CD8⁺ T cells reversibly downregulate transgene expression in patients, potentially decreasing efficacy of transduced T cells over time.

Prior to transfer into patients, T cells were maintained with high levels of IL-2 and IL-15 to promote T cell survival and growth. These levels of IL-2 and IL-15 are optimized for *in vitro* culture and are far higher than what has been observed in normal peripheral blood.^{19,20} In our trial, patients received low-dose IL-2 (72,000 IU/kg), for the first 7 days post-transfer, during which the percentage of transgene-expressing (CD34⁺) T cells increased in six of seven patients⁸ (Figure S3). After day seven, the percent of transduced T cells decreased. It was possible that the combination of low-dose IL-2 and increased bioavailability of IL-15 due to lymphopenia^{21,22} contributed to transgene expression in patients. We hypothesized that when levels of these cytokines naturally declined as patients stopped receiving IL-2 and recovered from lymphopenia, transgene expression was also declining. We cultured patient T cells transduced with lentiviral or retroviral vectors without adding IL-2 or IL-15 to determine whether transgene expression would downregulate *in vitro* in the absence of exogenous cytokines. During culture without cytokines, both the marker gene, CD34, and transgenic TCR expression (via V β 12, the β chain of the TIL 1383I TCR) were monitored by flow cytometry over time. Both CD34 and V β 12 expression decreased on transduced CD4⁺ and CD8⁺ T cells cultured without cytokines compared to cells cultured with IL-2 and IL-15 (Figure 2A). By day seven, a significant fraction of transgene expression was lost in T cells without cytokines compared to T cells maintained with IL-2 and IL-15 (Figure 2B). Interestingly, V β 12 expression was more sensitive to downregulation than the CD34 marker, perhaps due to TCR cycling and competition for CD3 with the endogenous TCR.²³ These results suggest that transgene expression is maintained by cytokines, and loss of transgene expression may be induced by cytokine withdrawal.

Culturing T cells without cytokines causes some apoptosis (viability for cells cultured without cytokines for 23 days was 14.9% \pm 4.2%, while viability for cells cultured with cytokines was 33.2% \pm 4.2%). It was therefore possible that transduced T cells were selectively dying without cytokine support. To rule out the possibility that transgene expression loss *in vitro* might be due to selective death of transduced cells, we determined whether cells that had downregulated CD34 and V β 12 expression *in vitro* were able to re-express transgene if

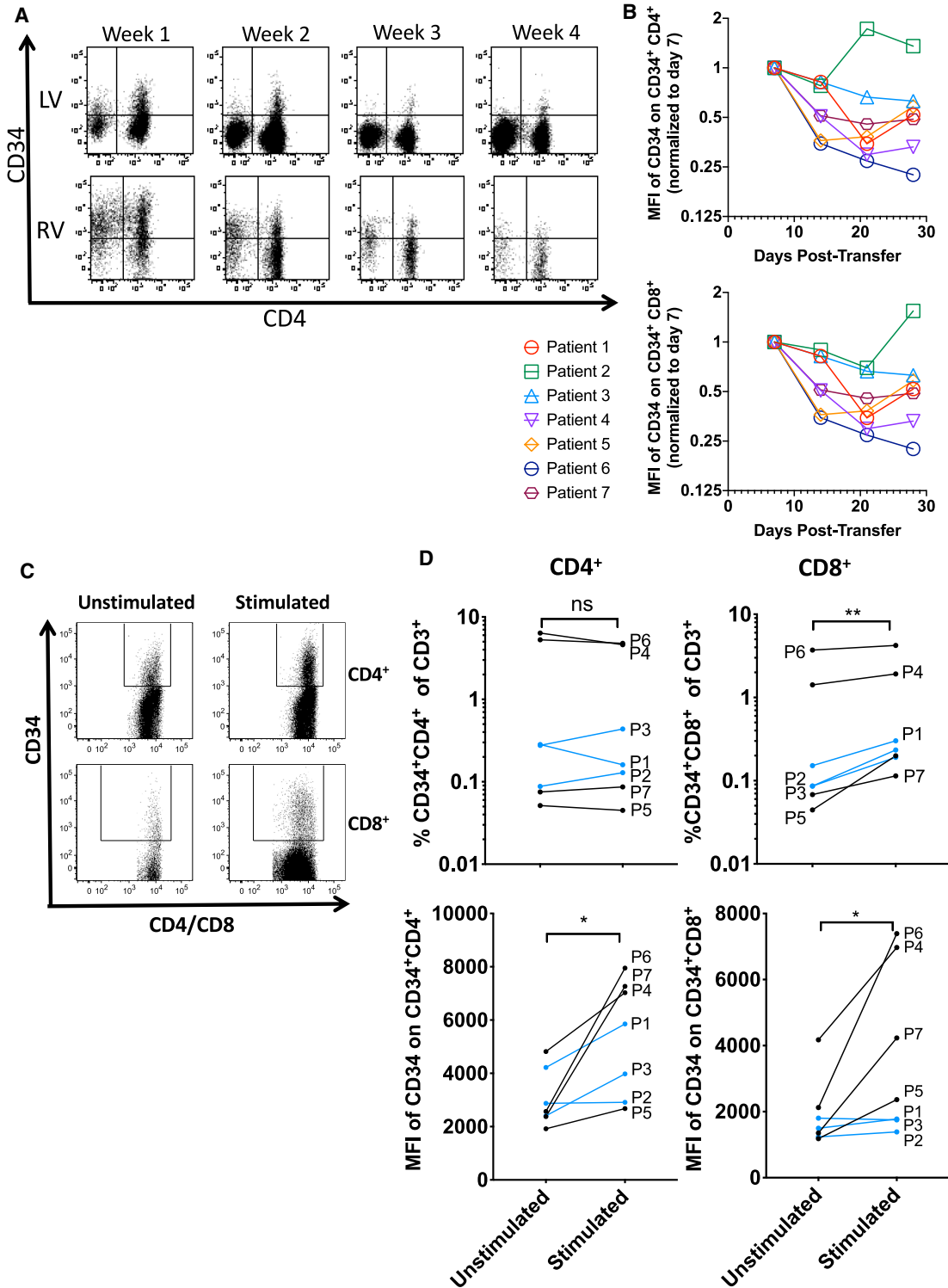


Figure 1. Transgene expression is reversibly downregulated in patients

(A and B) Cryopreserved patient PBMCs were thawed and analyzed by flow cytometry for expression of CD34 on live CD3⁺CD4⁺CD34⁺ or live CD3⁺CD8⁺CD34⁺ T cells. (A) Expression of CD34 versus CD4 on live CD3⁺ T cells for a patient given lentivirally transduced T cells (top, LV) and retrovirally transduced T cells (bottom, RV). (B) MFI of CD34 on live CD34⁺ CD4⁺ (top) and CD34⁺ CD8⁺ (bottom) T cells. (C and D) Patient blood samples were thawed then rested or cultured in complete media with IL-2 (300 IU/mL)

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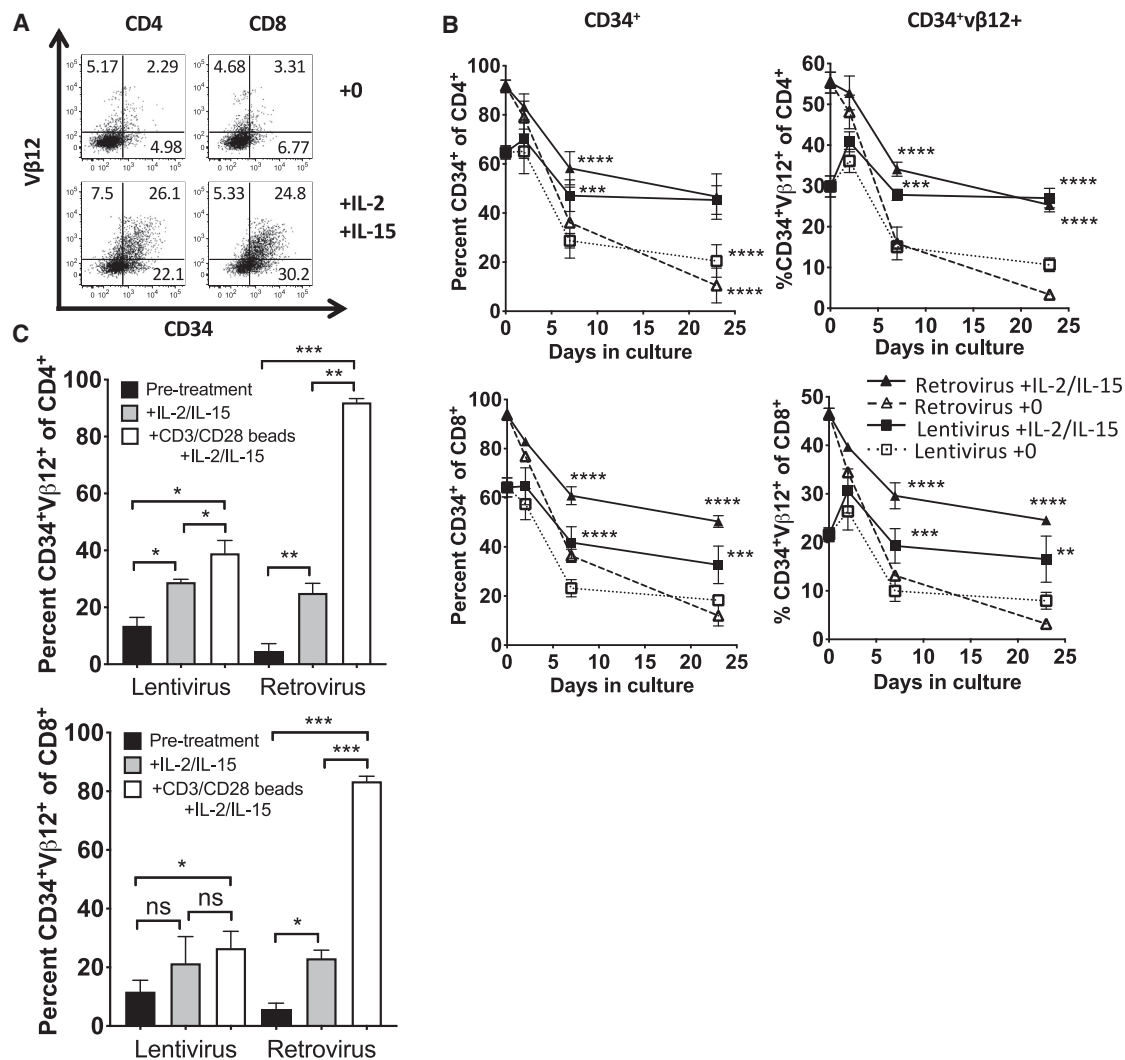


Figure 2. Reversible transgene expression downregulation occurs in the absence of cytokines

(A and B) T cells from the apheresis of patients 1–3 were activated, separated into two groups, and transduced with lentivirus or retrovirus and expanded. Transduced T cells were cultured with and without cytokines for 23 days. CD34 and Vβ12 expression was assessed by flow cytometry. (A) Example of CD34 versus Vβ12 expression on CD4⁺ and CD8⁺ T cells on day 23. (B) Time course of CD34 (left) and Vβ12 (right) expression on CD4⁺ (top) and CD8⁺ (bottom) T cells. Data in (B) were analyzed by 2-way ANOVA and Tukey’s multiple comparisons test. (C) Transduced T cells were cultured for 4 weeks without cytokines, then restimulated. CD34 and Vβ12 expression was assessed on CD4⁺ and CD8⁺ T cells prior to and after restimulation for 2 days with IL-2 and IL-15 or with CD3/CD28 beads as well as IL-2 and IL-15. Data in (C) were analyzed by paired t test. p values are summarized as ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05.

restimulated. Lentiviral- and retroviral-transduced T cells cultured for 4 weeks in the absence of cytokines were restimulated for 2 days with cytokines (IL-2 and IL-15) with or without anti-CD3/anti-CD28 beads. Restimulation with both cytokines and anti-CD3/anti-CD28 significantly increased the percent of CD34⁺Vβ12⁺ cells in

CD4⁺ and CD8⁺ populations for both lentiviral- and retroviral-transduced T cells (Figure 2C). Restimulation with cytokines alone significantly increased transgene expression in retroviral-transduced CD4⁺ and CD8⁺ T cells as well as lentiviral-transduced CD4⁺ T cells. There was a trend toward increase in transgene expression in lentiviral-

and IL-15 (100 ng/mL) for 2 days, then restimulated with Human T-Activator CD3/CD28 beads at a 1:1 cell:bead ratio. (C) Expression of CD34 and gating on CD34⁺CD4⁺ and CD34⁺CD8⁺ are shown. (D, top) The percent CD34⁺CD4⁺ (left) and CD34⁺CD8⁺ (right) are shown for every patient; (bottom) the MFI of CD34 on CD34⁺CD4⁺ (left) and CD34⁺CD8⁺ (right) T cells is shown for every patient. Patients receiving lentiviral-transduced T cells are shown in blue, while those receiving retroviral-transduced T cells are shown in black. p values were calculated by a ratio paired t test and summarized with **p < 0.01 and *p < 0.05.

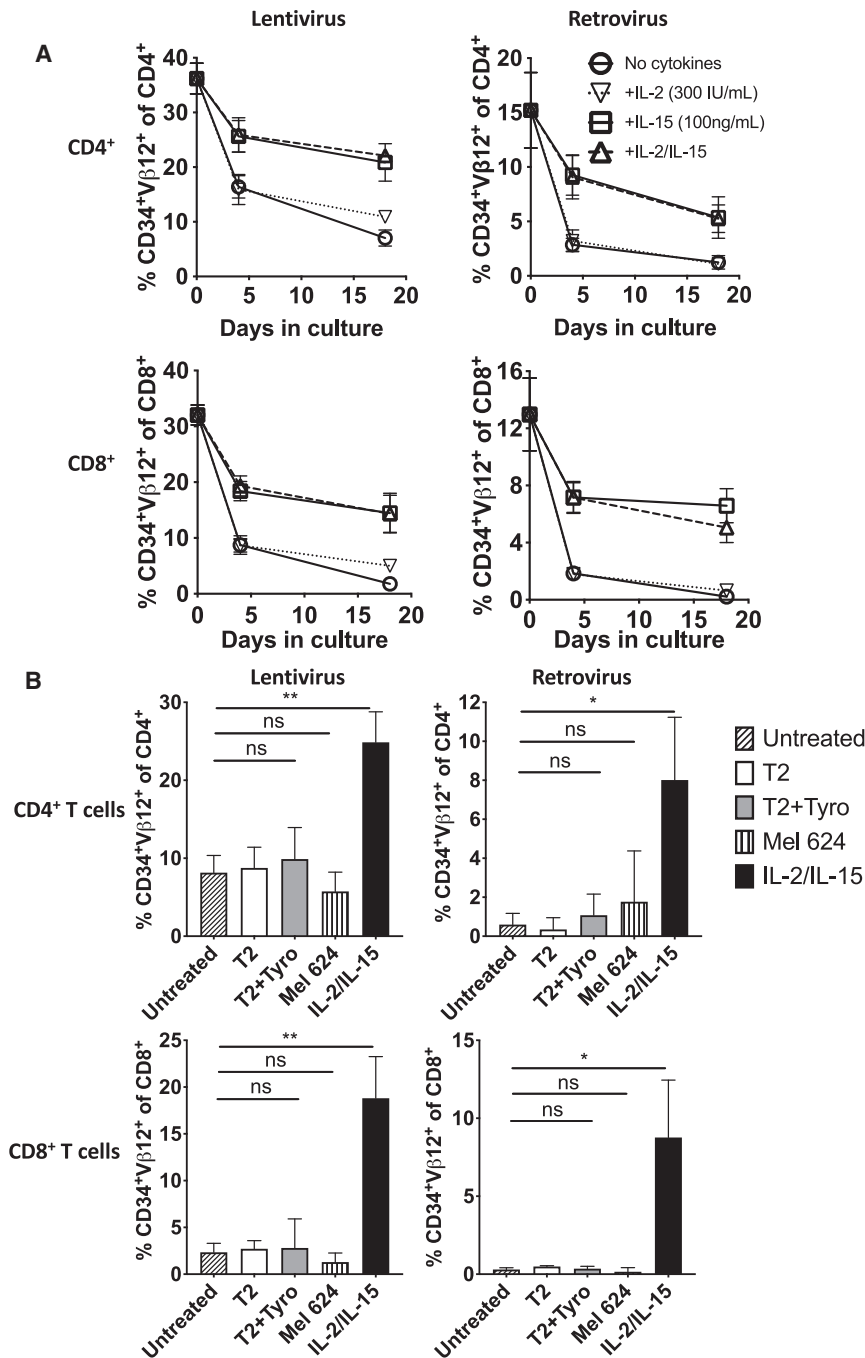


Figure 3. Culture in IL-15 reduces downregulation of transgene expression

Healthy donor T cells were activated, transduced with lentivirus (pLVX-13831, left) or retrovirus (SAMEN-13831, RV, right), then CD34-sorted and rapidly expanded. (A) Lentiviral- and retroviral-transduced T cells were then cultured in the presence or absence of IL-2 and IL-15. Expression of CD34 and Vβ12 was assessed by flow cytometry on CD4⁺ or CD8⁺ CD3⁺ T cells at indicated time points. (B) Lentiviral- and retroviral-transduced T cells were cultured for 14 days in the absence or presence of T2 cells, T2 cells pulsed with tyrosinase, 624 MEL cells, or IL-2 (300 IU/mL) and IL-15 (100 ng/mL). Tumor cells were added at a ratio of 1:4 tumor:T cell. On day 12, tumor cells were no longer detectable, and a second dose of tumor cells was added. Error bars represent standard deviation. Significance is shown by Student's t test with p values summarized as **p < 0.01, and *p < 0.05.

plastic—more susceptible to both downregulation and reversal of downregulation than the lentiviral transgenes. Overall, these results indicate that retroviral and lentiviral transgene expression is lost *in vivo* and during culture without cytokines due to a reversible process.

We next investigated whether IL-2, IL-15, or both were necessary for maintaining transgene expression. Culture of either lentiviral- or retroviral-transduced healthy donor CD4⁺ and CD8⁺ T cells with IL-15 alone maintained transgene expression equivalently to culture with both IL-2 and IL-15 (Figure 3A). Conversely, culture with IL-2 alone only maintained transgene expression slightly above culture with no cytokines. These results demonstrate that IL-15, but not IL-2, contributes to the maintenance of transgene expression over time *in vitro*.

Both TCR and cytokine stimuli were present during initial transduction. To investigate whether culturing with antigen would be sufficient to maintain transgene expression, transduced healthy donor T cells were cultured

transduced CD8⁺ T cells after cytokine restimulation, but it did not reach significance. Interestingly, although the percent of transgene downregulation was greater in retroviral-transduced T cells (Figures 2B and 2C), the retroviral-transduced T cells re-expressed transgene at substantially higher levels than lentiviral-transduced T cells as well. Although there are insufficient data to draw general conclusions about all retroviruses and lentiviruses, our results suggest that for this retrovirus and this lentivirus, the retroviral transgenes are more

with either HLA-A2⁺ T2 cells pulsed with tyrosinase peptide (T2/tyrosinase), or an HLA-A2⁺ tyrosinase-expressing melanoma line, 624 MEL. Neither culture with T2/tyrosinase nor culture with 624 MEL cells prevented loss of transgene expression in T cells, whereas culture with IL-2 and IL-15 maintained significantly higher transgene expression than culture without cytokines (Figure 3B). These *in vitro* results, in addition to the loss of transgene expression in patients who have both tumors and melanocytes bearing antigen, suggest that

antigen exposure alone is insufficient to maintain transgene expression. In the presence of IL-2 and IL-15, culturing T cells with antigen-expressing tumor cells did increase transgene expression on CD8⁺ T cells compared to CD8⁺ T cells cultured with cytokines alone (Figure S4). These results suggested that antigen stimulation of T cells enhances transgene expression in the presence of cytokines but is not sufficient to maintain transgene expression as a single agent.

Most of the DNA in resting T cells is bound up in transcriptionally inactive heterochromatin.^{24,25} T cell activation reduces heterochromatin and promotes upregulation of gene expression of many activation-associated genes.²⁶ T cells were activated prior to transduction to enhance retroviral and lentiviral transgene integration. Most likely, retroviral and lentiviral transgenes then integrated into open chromatin near transcriptionally active sites. It is possible that as T cells enter resting states in patients or *in vitro* without cytokines, there is epigenetic downregulation of transgene expression along with the genes near which the transgenes inserted. Two methods of epigenetic downregulation of gene expression are through DNA methylation or histone deacetylation by histone deacetylases (HDACs). Therefore, we tested whether T cell culture with HDAC inhibitors, sodium butyrate or suberoylanilide hydroxamic acid (SAHA),²⁷ or a DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (5a2d), prevented downregulation of transgene expression. Culture of transduced T cells with HDAC inhibitors prevented downregulation of transgene expression in the absence of cytokines (Figure 4). When cells were cultured with sodium butyrate and cytokines, the addition of both agents acted to maintain higher transgene expression than either alone, although SAHA treatment did not act additively with cytokines. DNA methyltransferase inhibition did not affect transgene expression in our model, a finding consistent with previous work showing that there is little methylation of transgenes in patients.¹³ These results indicate that when T cells are removed from high cytokine environments, histone deacetylation reduces transgene expression, and this process may be prevented by treatment with HDAC inhibitors. Further, T cells cultured with cytokines may also undergo some HDAC-mediated transgene downregulation, as treatment with sodium butyrate enhanced transgene expression in T cells cultured with cytokines.

Culture in HDAC inhibitors prevented downregulation of transgene expression, leading us to question whether culture in HDAC inhibitors would further prevent loss of functional responses of transduced T cells to antigen. Transduced T cells from three donors were cultured with complete medium only, complete medium with cytokines, complete medium with sodium butyrate (1 mM), or complete medium with cytokines and sodium butyrate. After 10 days in culture, the T cells were then cocultured overnight (without cytokines or sodium butyrate) with T2/tyrosinase or with 624 MEL. CD4⁺ and CD8⁺ T cells were assessed for lytic granule production by staining for CD107a expression and for production of inflammatory cytokines IL-2, tumor necrosis factor alpha (TNF- α), and interferon (IFN)- γ by intracellular cytokine staining. The percentage of T cells producing one, two, three, or all four effector molecules is shown (Figure 5B). In both lentiviral- and retro-

viral-transduced T cells cultured without cytokines, culture in sodium butyrate increased the percent of cytokine-producing cells in both CD4⁺ and CD8⁺ T cells. The addition of sodium butyrate to T cells cultured in IL-2 and IL-15 also appeared to increase cytokine production in both CD4⁺ and CD8⁺ T cells, though the difference did not reach significance in some cases due to substantial variability. A breakdown of the exact cytokine-producing profiles (Figure S5) of all groups suggests that culture in sodium butyrate increases the percentage of retroviral- and lentiviral-transduced T cells producing many cytokine profiles in both CD4⁺ and CD8⁺ T cells activated with 624 MEL or T2/tyrosinase. These results indicate that HDAC inhibition can retain antigen-specific T cell effector functions in TCR-transduced T cells cultured without cytokines and enhance effector functions in T cells cultured with cytokines. Further testing is required to determine whether HDAC inhibition can maintain T cell effector functions *in vivo*. If so, HDAC inhibition may permit more effective and long-lived transgenic T cell therapies.

DISCUSSION

We found that transgene expression was downregulated in TCR-transduced CD4⁺ and CD8⁺ T cells adoptively transferred into metastatic melanoma patients. This phenomenon occurred regardless of whether patients received lentiviral- or retroviral-transduced T cells. Lentiviral and retroviral transgene expression downregulation was reproducible by *in vitro* culture without cytokines. *In vitro* culture in IL-15, but not IL-2 or antigen, maintained T cell transgene expression. Culturing T cells in the presence of an HDAC inhibitor prevented T cell transgene downregulation and maintained T cell functional responses in the absence of cytokines. Although we have previously observed that detectable (by flow cytometry) expression of transgene is not required for antigen-specific T cell responses of transduced T cells,²⁸ here we observe that culture of transduced T cells with an HDAC inhibitor increased effector responses as well as transgene expression. Collectively, our results suggest that histone deacetylase activity reduces transgene expression and effector functions of transduced T cells.

Although both γ -retroviruses and lentivirus are being used in clinical trials, these vectors may not have identical epigenetic regulation. Both γ -retroviruses and lentiviruses integrate semi-randomly into the genome at sites associated with histone binding, which are also near transcriptionally active sites. However, γ -retrovirus and lentivirus integration patterns are non-identical, with γ -retroviruses favoring areas 5' of genes, while lentiviruses favor introns.²⁹ We compared transgene expression regulation across both types of viruses and found that both retroviral- and lentiviral-transduced T cells lost transgene expression, and transgene expression was restored after cytokine and TCR stimulation in both retroviral- and lentiviral-transduced T cells. Interestingly, downregulation and stimulation-induced reexpression appeared to be more robust in γ -retroviral-transduced T cells than in lentiviral-transduced T cells (Figures 1, 2, 3, and 4). It is possible that γ -retrovirus integration sites are more sensitive to epigenetic effects (both downregulation and upregulation) than lentiviral integration sites. It is also possible that the specific viruses used in this study

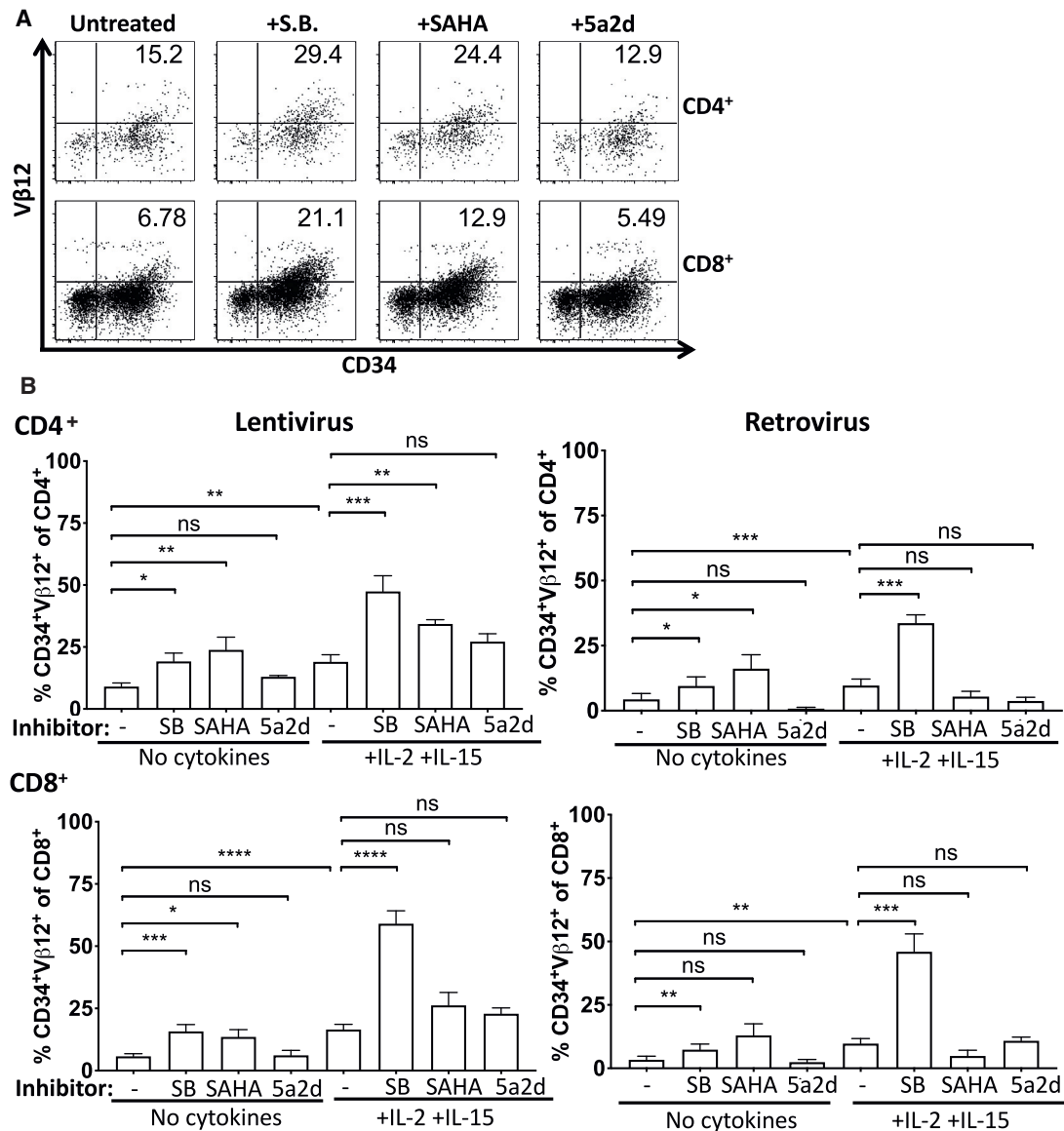


Figure 4. Culture with HDAC inhibitors reduces transgene expression downregulation

Transduced T cells (from healthy donors) were cultured for 12 days with no cytokines or with IL-2 and IL-15 in the absence or presence of HDAC inhibitors, sodium butyrate (SB) or suberoylanilide hydroxamic acid (SAHA), or a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5a2d). (A) Representative fluorescence-activated cell sorting (FACS) plots of CD34 versus vβ12 on CD4⁺ (top) and CD8⁺ (bottom) T cells. (B) Comparison of percentages CD34⁺vβ12⁺ of CD4⁺ (top) and CD8⁺ (bottom) T cells. Results are representative of at least four donors and three independent experiments. Significance was determined by paired t test (pairing on donor and virus type) with p values summarized as ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05

are regulated differently due to uncontrolled factors, such as different promoters, regulatory elements, or copy number. Overall, however, neither retroviral- nor lentiviral-transduced T cells are immune to transgene expression loss. Further, similar mechanisms (IL-15, histone deacetylases) appear to regulate transgene expression in both lentiviral- and retroviral-transduced T cells.

Notably, the effects of HDAC inhibition were greater on expression of the T cell receptor (Vβ12) than on the CD34 gene. Both genes are co-

transcribed as one mRNA, separated by a ribosomal skip sequence, so HDAC inhibition and prevention of chromatin condensation and gene transcription downregulation cannot account for this difference. It is likely that the TCR requires more mRNA for similar expression, as the TCR protein is constantly being cycled away from and back to the cell surface³⁰ and must compete with endogenous TCR for CD3ζ chains.³¹ It is further possible that effects of HDAC inhibitors on other cellular pathways also contribute to the regulation of T cell receptor expression. For example, retroviruses have been shown to reduce

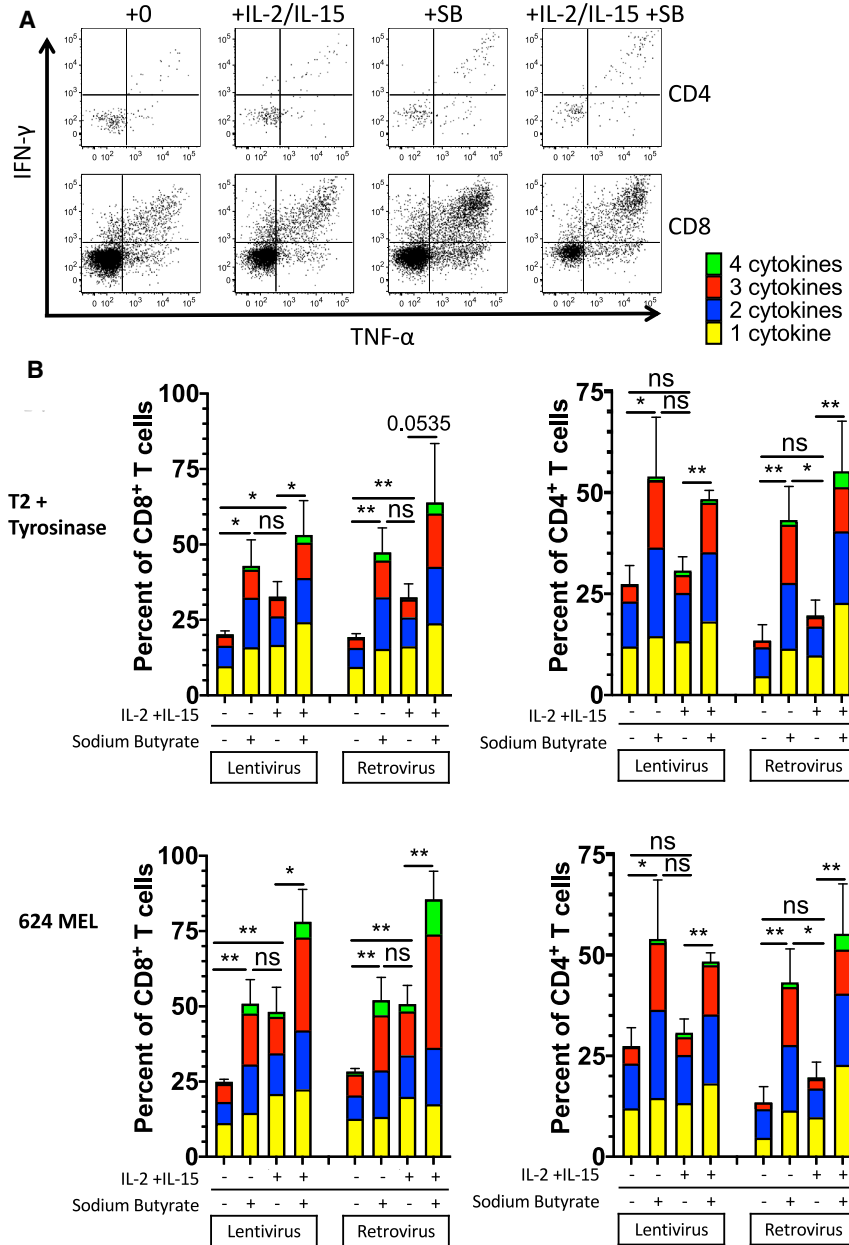


Figure 5. Transduced CD4⁺ and CD8⁺ T cell responses to antigen are protected in the absence of cytokines by sodium butyrate

Transduced T cells (from healthy donors) were cultured for 10 days with and without sodium butyrate and cytokines. After 10 days, cells were washed and cocultured with T2/tyrosinase or 624 MEL in the presence of Golgi blockers and a labeled anti-CD107a antibody. After the coculture, the cells were stained extracellularly for CD3, CD4, CD8, and CD34; stained with a viability dye; then fixed, permeabilized and stained intracellularly for TNF- α , IFN- γ , and IL-2. Statistics shown are Student's t test of differences in total cells expressing 1 or more effector molecules (IL-2, IFN- γ , TNF- α , and CD107a). p values are summarized as **p < 0.01, and *p < 0.05.

tion of CD8⁺ T cells^{35,36} and induce energy in naive T cells.³⁷ However, recent papers have demonstrated that sodium butyrate and other HDAC inhibitors can enhance T helper 1 (Th1) responses,^{38,39} reduce T cell activation-induced cell death (AICD),⁴⁰ and enhance the development of CD8⁺ T cell memory.⁴¹ Collectively, these results suggest that while sodium butyrate and other HDAC inhibitors can prevent or reduce naive cell activation, these inhibitors can also enhance existing effector T cell responses. In our transduced cells, is likely that HDAC inhibition results in maintaining expression of activation and/or growth-associated genes and maintaining expression of transgenes inserted into activation and growth-associated genetic loci as well.

Our results suggest that IL-15 treatment and/or HDAC inhibitors might enhance transduced T cell therapies in the clinic by enhancing TCR transgene expression. Preclinical studies have shown that HDAC inhibitors can synergize with other immunotherapies and enhance anti-tumor responses.^{42–45} HDAC inhibitors may act to change the immune environment of tumors⁴⁶ and are currently being tested in

expression of multiple CD3 components,^{32,33} and HDAC inhibition was shown to rescue CD3 expression. Therefore, HDAC inhibitors may be rescuing transgene expression and function via multiple mechanisms—both directly and by enhancing CD3 expression.

Culture of T cells in the presence of sodium butyrate maintained or increased the ability of TCR-transduced CD4⁺ and CD8⁺ T cells to make cytokines in response to antigen. This appears to be in contrast to past studies in which sodium butyrate was found to be suppressive and drive regulatory T cell (T_{reg}) formation in the gut microenvironment³⁴ and inhibit antigen-presenting cell (APC)-mediated activa-

clinical trials in conjunction with immunotherapies.⁴⁶ One caveat of using HDAC inhibitors (HDACi) that should be noted is that HDAC inhibitors such as vorinostat may be lymphotoxic. We observed a wide variety of toxicity from both sodium butyrate and vorinostat treatment in our assays, with the percent of viable cells reduced approximately 0%–50% with sodium butyrate and 25%–75% with vorinostat in the absence of cytokines. The presence of cytokines reduced the toxicity of the HDACi treatments. Interestingly, the toxicity of vorinostat *in vitro* was more dramatic than that observed in patients, in which less than 30% of patients had demonstrated any decrease in their white blood cell counts.⁴⁷ Although IL-15

did not show dramatic results in the clinic as a single agent, and significant toxicity was observed,⁴⁸ there is an IL-15 conjugate that is well tolerated by patients⁴⁹ and has shown promising anti-tumor results when given with PD-1 blockade in an initial small-scale safety trial.⁵⁰ It is possible that patients given transduced T cells would benefit from concurrent treatment with HDAC inhibitors or an IL-15 conjugate to enhance transgene expression and function. Overall, this study demonstrated that HDAC inhibitors can enhance T cell transgene expression and function *in vitro*. Further *in vivo* studies are necessary to determine the effects of HDAC inhibitors and/or IL-15 conjugates on transduced T cells *in vivo* in the tumor microenvironment, where effects on tumor, the presence of antigen, and suppressive mechanisms may enhance or detract from the effects of HDAC inhibitors or IL-15 treatment.

MATERIALS AND METHODS

Clinical trial design

These studies were performed at Loyola University Medical Center (Maywood, IL, USA) and the Earle A. Chiles Research Institute (Portland, OR, USA). The study was registered with [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT01586403 [lentivirus] and NCT02870244 [retrovirus]). Informed consent was obtained prior to enrolling patients. These studies were approved by the Institutional Review Board (LU 203732 [lentivirus] and LU 203729 [retrovirus]) and the Institutional Biosafety Committee (LU 203729 [retrovirus] and LU 203732 [lentivirus]) at Loyola University Medical Center as well as the Institutional Biosafety Committee and the Institutional Review Board (16-004B) at Providence Portland Medical Center. These studies were also approved by the Recombinant DNA Advisory Committee (RAC Protocol 1101-1086) at Loyola University Medical Center, the Cancer Therapy Evaluation Program (CTEP 9358), and the US Food and Drug Administration (IND 14971 [lentivirus] and 16315 [retrovirus]).

Patients 1–3 received 2.5×10^6 lentivirus-transduced T cells per kilogram. Patients 4–6 received 7.5×10^6 γ -retrovirus-transduced T cells per kilogram. Patient 7 received 2.5×10^7 γ -retrovirus-transduced T cells per kilogram. All patients received non-myeloablative lymphodepletion with intravenous once-daily administration of cyclophosphamide and fludarabine prior to T cell infusion (the day of infusion was designated as day 0). Cyclophosphamide was administered at 60 mg/kg on days –2 and –1 for patients 1–3, 60 mg/kg on days –3 and –2 for patients 4–6, and 1,000 mg/m² on days –3 and –2 for patient 7. Fludarabine was administered at 25 mg/m² on days –5, –4, –3, –2, and –1. All patients were further given low-dose IL-2 (72,000 IU/kg, Aldesleukin, Novartis, East Hanover, NJ, USA) for 1 week following the T cell infusion.

T cells for patient treatment were generated and patients were treated as follows. Briefly, patient peripheral blood mononuclear cells (PBMCs) were activated in AIM-V medium (Thermo Fisher) with 5% human AB serum (Valley Biomedical, Winchester, VA, USA) (AIM-V CM) with 50 ng/mL anti-CD3 antibody (OKT3, Miltenyi Biotec, Somerville, MA, USA), 300 IU/mL rhIL-2 (Aldesleukin, Novartis, East Hanover, NJ, USA) and 100 ng/mL rhIL-15 (Biological Resources Branch, NCI,

Frederick, MD, USA). Three days after activation, T cells were spinoculated with lentivirus- or retrovirus-containing media on retronectin-coated (Takara Bio, Mountain View, CA, USA) plates. Three days after spinoculation, T cells were enriched for CD34-expressing cells (transduced T cells) using biodegradable CD34-binding immunomagnetic iron-dextran particles (GMP-grade, Miltenyi Biotec) and a CliniMACS (Miltenyi Biotec). Enriched CD34⁺ T cells were expanded by a rapid expansion protocol (REP). For the REP, T cells were expanded by culture with irradiated pooled allogeneic PBMCs from at least three donors at a 1:200 T:PBMC ratio in AIM-V CM with 30 ng/mL anti-CD3 antibody, 300 IU/mL rhIL-2, and 100 ng/mL rhIL-15. At various points post-T cell transfer, patient blood samples were taken; white blood cells were isolated by density gradient centrifugation and then cryopreserved and analyzed at a later time point.

Viruses

The SAMEN-1383I γ -retroviral vector has been previously described.⁵¹ Briefly, expression is driven by a hybrid 5' long terminal repeat (LTR), containing the human cytomegalovirus enhancer and promoter fused to the MMLV 5' LTR followed by splice donor, packaging, and splice acceptor sites; then by the expression cassette of the cloned TIL 1383I TCR α gene; a P2A ribosomal skip site; the TIL 1383I TCR β gene; a T2A ribosomal skip site; and the truncated non-signaling CD34t expression marker. The lentiviral vector used to transduce T cells from patients 1–3 was a previously described proprietary vector (Lentigen Technology, Gaithersburg, MD, USA).⁸ For all *in vitro* experiments with healthy donor T cells, the pLVX-1383I lentiviral vector was used. The pLVX-1383I vector was produced from the pLVX-EF1alpha-mCherry-N1 vector (Clontech, Mountain View, CA, USA), which was modified to remove the mCherry and then include the same TIL 1383I TCR and CD34t expression cassette as the retroviral vector. Vector maps are shown in [Figure S6](#).

Patient characteristics

Patients 1–3 were previously described.⁸ Patients 4–7 are described in [Table S1](#).

Cell lines and primary cells

Healthy donor PBMCs were purchased as fully deidentified apheresis products (Key Biologicals, Memphis, TN, USA). Cell lines used for T cell stimulation included T2 cells, a TAP-deficient HLA-A2⁺ cell line⁵² purchased from ATCC, and 624 MEL, a tyrosinase-producing, HLA-A2⁺ human melanoma line obtained from the Surgery Branch of the National Cancer Institute (NCI, Bethesda, MD, USA).⁵³ Viral producer cell lines included GPRTG, a stable lentiviral producer line made from HEK293T cells transfected with the HIV gag-pol, rev, tat, and VSV-G env genes,⁵⁴ provided by the National Gene Vector Biorepository (Indiana University, Indianapolis, IN, USA), and PG-13, a stable retroviral producer line made from NIH 3T3 cells transfected with Gibbon Ape Leukemia Virus (GALV) envelope and the Moloney murine leukemia virus (MMLV) gag-pol genes.⁵⁵ GPRTG cells were transduced with the modified pLVX-1383I construct described above to generate a stable lentiviral producer line. PG-13 cells were transduced with the SAMEN-1383I construct

described above to generate a stable retroviral producer line. T2 cells were maintained in RPMI (Thermo Fisher, Waltham, MA, USA) with 10% fetal calf serum (FCS) (RPMI CM). 624 MEL and GPRTG were maintained in DMEM (Thermo Fisher) with 10% FCS (DMEM CM). PG-13 cells were maintained in Iscove's modified DMEM (Thermo Fisher) with 10% FCS (IMDMEM CM). After transduction, transduced T cells were maintained in culture in RPMI CM with 300 IU/mL rhIL-2 and 100 ng/mL rhIL-15 unless otherwise noted. When cultured without cytokines, T cells were cultured in RPMI CM alone. When cultured with HDAC inhibitors or methyltransferase inhibitors, T cells were cultured with 1 mM sodium butyrate, 1 μ M SAHA, or 130 nM 5-aza-2-deoxycytidine.

Flow cytometry

Extracellular staining

T cells were stained with fluorescently labeled antibodies to human CD3, CD4, CD8, CD34, CD14, CD19 (Biolegend, San Diego, CA, USA), and TCR-V β 12 (Beckman Coulter, Indianapolis, IN, USA), washed three times in PBS with 2% bovine serum albumin (2% PBSA), then resuspended in 2% PBSA for analysis on the flow cytometer. Live/dead differentiation was determined either using Live/Dead Fixable Aqua (Thermo Fisher Scientific, Waltham, MA, USA) after extracellular staining or by adding DAPI (Millipore Sigma, St. Louis, MO, USA) to a final concentration of 2 μ g/mL immediately prior to analyzing samples on the flow cytometer. All samples were analyzed in the Loyola University Medical Center Flow Cytometry Core, on either an LSR Fortessa (BD Biosciences, San Jose, CA, USA) or a FACSCanto (BD Biosciences), and analyzed on FlowJo Software (BD Biosciences).

Intracellular cytokine staining

T cells were stimulated overnight in RPMI CM with either T2 cells pulsed with 10 μ g/mL tyrosinase peptide (T2/tyrosinase) or a tyrosinase-expressing HLA-A2⁺ melanoma line (624 MEL) in the presence of Golgi blockers Brefeldin A (BFA, Biolegend) and Monensin (Biolegend). Fluorescently labeled anti-CD107a antibody (Biolegend) was also added to the coculture to stain for lytic granule release. After the culture, cells were washed and stained extracellularly with labeled antibodies to CD3, CD4, CD8, and CD34, then stained with Live/Dead Fixable Aqua as described above. After extracellular staining, cells were fixed and permeabilized using Fixation Buffer (Biolegend) and Intracellular Staining Permeabilization Wash Buffer (Perm/Wash Buffer, Biolegend) and stained intracellularly with fluorescently labeled antibodies specific for IL-2, IFN γ , and TNF α (Biolegend). Stained cells were then washed twice in Perm/Wash buffer and once in 2% PBSA prior to resuspension in 2% PBSA for analysis. Results of intracellular cytokine staining were assessed on an LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed using FlowJo software (BD Biosciences).

Transduction, CD34-isolation, and rapid expansion

Patient or healthy donor PBMCs were isolated by apheresis, and then red blood cells were removed using Ficoll (Cytiva Life Sciences, Marlborough, MA, USA) density gradient separation. T cells were

activated by culturing PBMCs in RPMI CM with 50 ng/mL anti-CD3 antibody (OKT3, CD3 Pure, Miltenyi Biotec), 300 IU/mL rhIL-2 (Aldesleukin, Novartis), and 100 ng/mL rhIL-15 (Biological Resources Branch, NCI) for 3 days. Activated T cells were then transduced by spinoculation with lentiviral (pLVX-TIL 1383I-CD34t) or retroviral (SAMEN-TIL 1383I-CD34t) supernatants in the presence of 8 μ g/mL polybrene (Millipore Sigma, St. Louis, MO, USA). Post-transduction, T cells were maintained in media containing rhIL-2 and rhIL-15 unless otherwise noted. After transduction, successfully transduced T cells were positively selected by CD34-binding magnetic bead isolation (Miltenyi Biotec) of CD34-expressing cells. Isolated CD34⁺ T cells were expanded using a rapid expansion protocol (REP). For the REP, T cells and pooled irradiated (5,000 Rads) feeder PBMC from at least three donors were cultured in RPMI CM with 30 ng/mL anti-CD3 antibody (OKT3, Miltenyi Biotec), 300 IU/mL rhIL-2 (Aldesleukin, Novartis) and 100 ng/mL rhIL-15 (Biological Resources Branch, NCI) for 10 days, after which T cells were used in experiments or cryopreserved for later use.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omto.2021.01.014>.

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AUTHOR CONTRIBUTIONS

Conceptualization: T.V.M. and M.I.N.; methodology: T.V.M., G.M.S., and M.I.N.; investigation: T.V.M., G.M.S., M.D., S.Y.W., A.V.D., C.R.W., K.A.H., J.J.S., C.F., J.F., T.M., M.T., and D.M.; resources: C.V.G., B.D.C., J.I.C., B.A.F., and M.I.N.; writing – original draft: T.V.M. and M.I.N.; writing – review & editing: T.V.M., A.V.D., S.Y.W., and M.I.N.; supervision: M.I.N.; project administration: T.V.M., G.M.S., and M.I.N.; funding acquisition: M.I.N.

DECLARATION OF INTERESTS

T.V.M., G.M.S., M.D., S.Y.W., A.V.D., C.R.W., K.A.H., J.J.S., C.V.G., C.F., J.F., T.M., M.T., D.M., B.D.C., B.A.F., and M.I.N. have no conflicts of interest to disclose. J.I.C. is on the speakers' bureau for Bristol Meyers Squibb (BMS) and Merck; is a consultant for Clinigen; receives research support (to institution) from BMS, Prometheus, AVEO, and Roche/Genentech; and has a family member employed full time by BMS.

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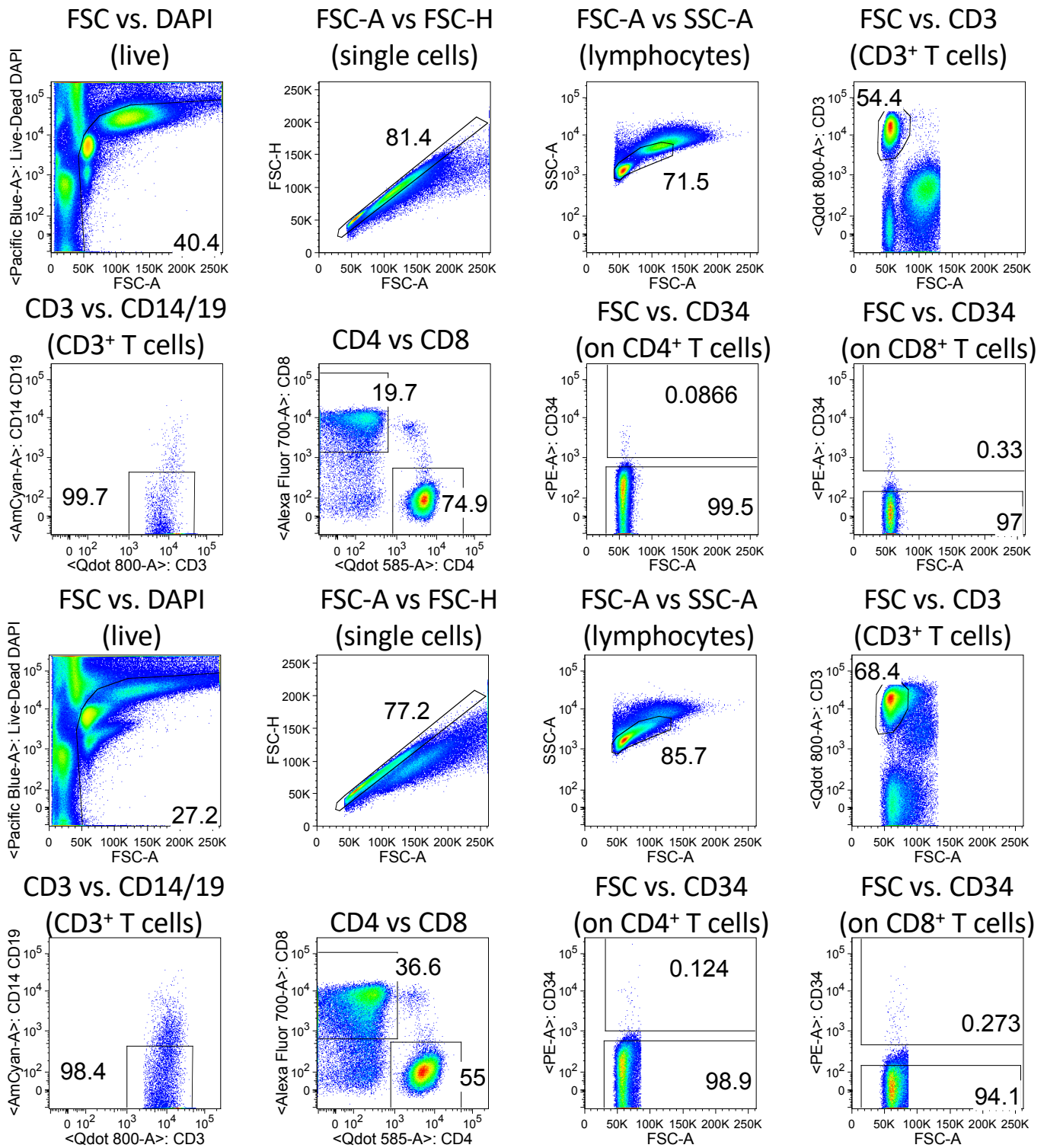
Supplemental Information

HDAC inhibition prevents transgene expression downregulation and loss-of-function in T-cell-receptor-transduced T cells

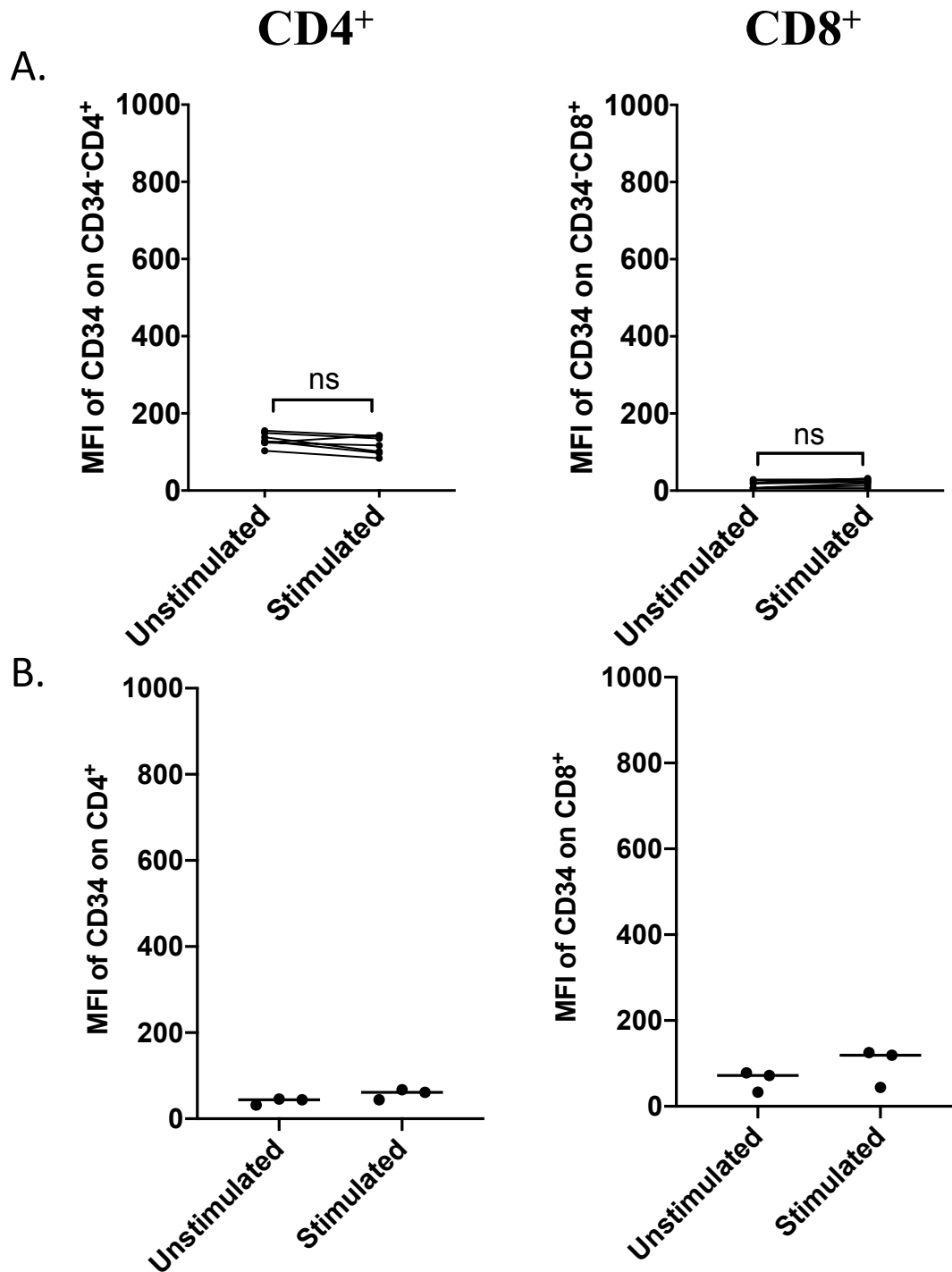
Tamson V. Moore, Gina M. Scurti, Matthew DeJong, Siao-Yi Wang, Annika V. Dalheim, Courtney R. Wagner, Kelli A. Hutchens, Jodi J. Speiser, Constantine V. Godellas, Chris Fountain, Jessica Fleiser, Tarsem Moudgil, Mallory Thomas, David Murray, Brendan D. Curti, Joseph I. Clark, Bernard A. Fox, and Michael I. Nishimura

Supplemental Table S1: Patient and Transduced T cell Treatment Characteristics

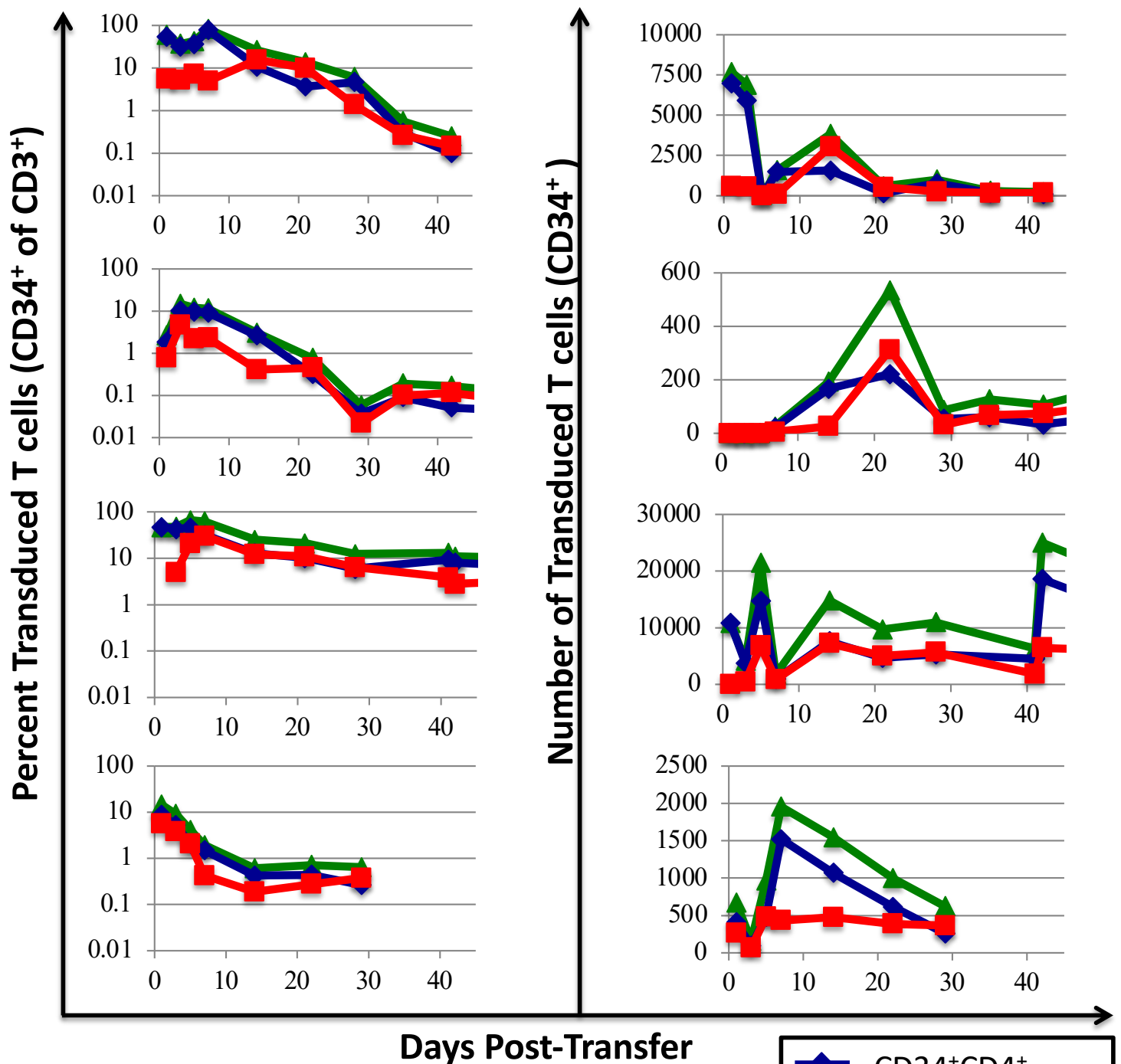
	Patient 4	Patient 5	Patient 6	Patient 7
Age at time of treatment	51	61	63	51
Sex	Female	Female	Male	Male
Stage of Melanoma at Diagnosis	T3aN1aM0, Stage IIIB	T2bN1aM0, Stage IIIB	T3bN2aMx, Stage IIIC	T1N0M0 Stage I
Prior therapies received	Nivolumab + Ipilimumab, Dabrafenib + trametinib, Pembrolizumab	Pembrolizumab, Ipilimumab, Nivolumab, Ipilimumab + Nivolumab	Ipilimumab, Nivolumab	Nivolumab, Ipilimumab
Stage of Melanoma at Treatment	Stage IV	Stage IV	Stage IV	Stage IV
Tissue Involvement	Ovaries, brain, subcutaneous LNs, adrenals, stomach	In transit mets in lower extremities, lung, pelvic LNs	Lung, liver, axillary LNs, spleen, bones	Rectal, liver, bones, lungs
Time (at treatment) since initial diagnosis (months)	50	26	39	16
Largest Decrease in Tumor Size (cm)	R. Adrenal: 6.9 → 6.0 Retroperitoneal: 2.5 x 2.8 → 2.1 x 1.5	No Response	L. axilla: 2.2 x 2.3 x 3.5 → 1.9 x 1.5 x 3.3	No Response
Observed effects on tumors (pre-treatment to four weeks post-treatment)	Mixed	None	Stable Disease	None
Response to therapy (RECIST)	Progressive Disease	Progressive Disease	Progressive Disease	Progressive Disease
Percent CD34 ⁺ CD3 ⁺ pre-sort	30.7	31.3	9.7	15.1
Percent Vβ12 ⁺ CD3 ⁺ pre-sort	7.61	19.4	3.9	7.4
Percent CD34 ⁺ CD3 ⁺ post-sort	99.9	98.5	99.4	93.6
Percent Vβ12 ⁺ CD3 ⁺ post-sort	33.7	67.4	34.8	44.2
Percent CD34 ⁺ CD4 ⁺ post-sort	64.0	74.3	62.4	60.2
Percent CD34 ⁺ CD8 ⁺ post-sort	36.7	25.1	36.5	36.3
Percent Vβ12 ⁺ CD4 ⁺ post-sort	25.1	50.7	34.5	35.1
Percent Vβ12 ⁺ CD8 ⁺ post-sort	8.6	18.5	17.7	16.4
Copy number post-sort	2.8	2.8	1.8	2.1
Pre-Rep Cell number (Cells undergoing REP)	1.7 x 10 ⁸ (8 x 10 ⁶)	2.98 x 10 ⁸ (8 x 10 ⁶)	7.2 x 10 ⁷ (8 x 10 ⁶)	5.75 x 10 ⁷ (8 x 10 ⁶)
Day 4 cell number	2.78 x 10 ⁸	2.68 x 10 ⁸	1.66 x 10 ⁸	1.88 x 10 ⁸
Day 6 cell number	5.98 x 10 ⁸	6.5 x 10 ⁸	4.1 x 10 ⁸	4.39 x 10 ⁸
Doubling time (days/division)	1.81	1.56	1.54	1.63
REP day 5 cell number	3.7 x 10 ⁸	8.45 x 10 ⁸	9.0 x 10 ⁸	1.1 x 10 ⁹
REP day 7 cell number	9.81 x 10 ⁸	4.58 x 10 ⁹	1.8 x 10 ⁹	2.1 x 10 ⁹
REP day 11 cell number (day 10 for patients 6 and 7)	5.1 x 10 ⁹	4.38 x 10 ⁹	5.4 x 10 ⁹	3.90 x 10 ⁹
REP Doubling time (days/division) (day 5 to 7)	1.422	0.820	2.0	2.144
Final %CD34 ⁺ CD3 ⁺	98.2	95.6	86.3	95.2
Final %Vβ12 ⁺ CD3 ⁺	21.2	23.6	26.9	21.3
Final %CD4 ⁺ CD34 ⁺	61.0	42.9	48.0	31.7
Final %CD8 ⁺ CD34 ⁺	36.8	54.2	38.2	63.5
REP d5 IFN-γ: T2+Tyro (T2 alone)	12732 (982)	7611 (12)	15533 (79)	9379 (201)
REP d5 IFN-γ: 624-Mel (624-28 A2- Mel)	10023 (508)	3743 (14)	11941 (91)	13551 (186)
REP d8 IFN-γ: T2+Tyro (T2 alone) [pg/mL]	5614 (12)	3882 (70)	58234 (61)	16088 (67)
REP d8 IFNγ: 624-Mel (624-28 A2-neg) [pg/mL]	2739 (0)	1828 (14)	20592 (48)	23493 (0)
Transduced Treatment Cell Number (total cell number)	5.79 x 10 ⁸ (5.9 x 10 ⁸)	8.42 x 10 ⁸ (8.85 x 10 ⁸)	5.9 x 10 ⁸ (6.95 x 10 ⁸)	1.76 x 10 ⁹ (1.87 x 10 ⁹)



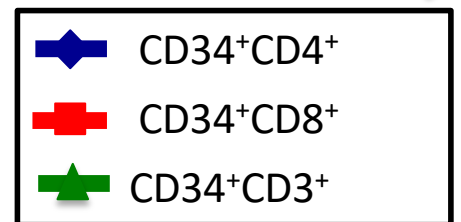
Supplemental Figure S1: Gating strategy for identifying CD34⁺ T cells

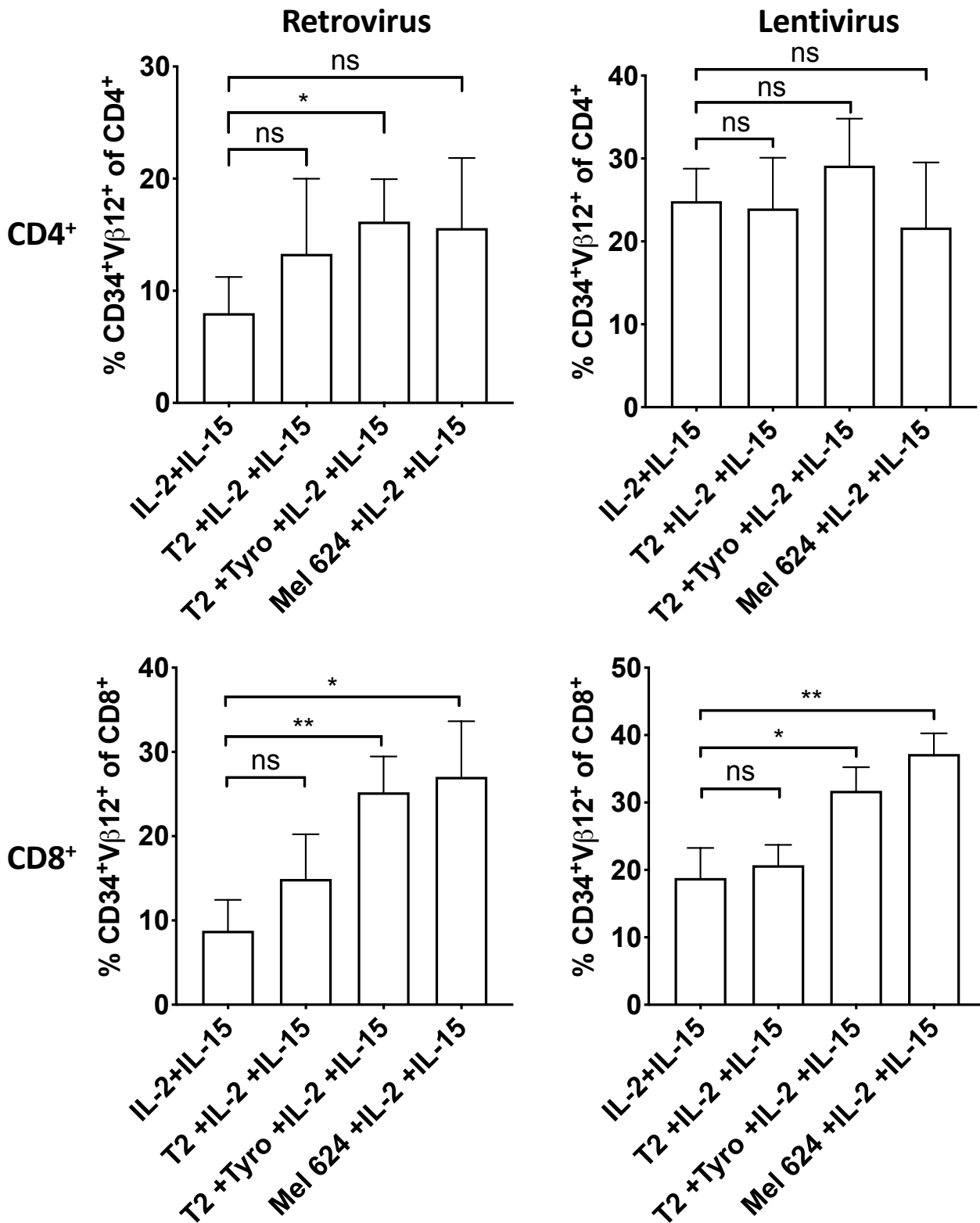


Supplemental Figure S2: Non-transduced T cells have low levels of CD34 with and without stimulation. A. At 28 days post-transfer into patients, patient PBMC were collected via apheresis or blood draw. These cells were depleted of red blood cells and then cryopreserved. In a cohort, the cells were thawed and either left untreated (Unstimulated) or rested in complete media with IL-2 (300IU/mL) and IL-15 (100ng/mL) for 2 days, then stimulated with anti-CD3/anti-CD28 beads (1:1 bead:cell ratio) (Stimulated). Cells were stained for CD3, CD4, CD8, and CD34, and gated as shown in Supplemental Figure 2. The calculated MFI values for CD34 on CD34⁻CD4⁺ and CD34⁻CD8⁺ T cells are shown. B. Cells from the aphereses of three healthy donors were thawed and either left untreated (Unstimulated) or cultured with IL-2 and IL-15 for two days then stimulated with anti-CD3/anti-CD28 beads (1:1 bead:cell ratio) (Stimulated). The calculated MFI values for CD34 on CD4⁺ (left) or CD8⁺ (right) T cells are shown. P values were calculated by a ratio paired t test and summarized with ns indicating not significant (p >0.05).

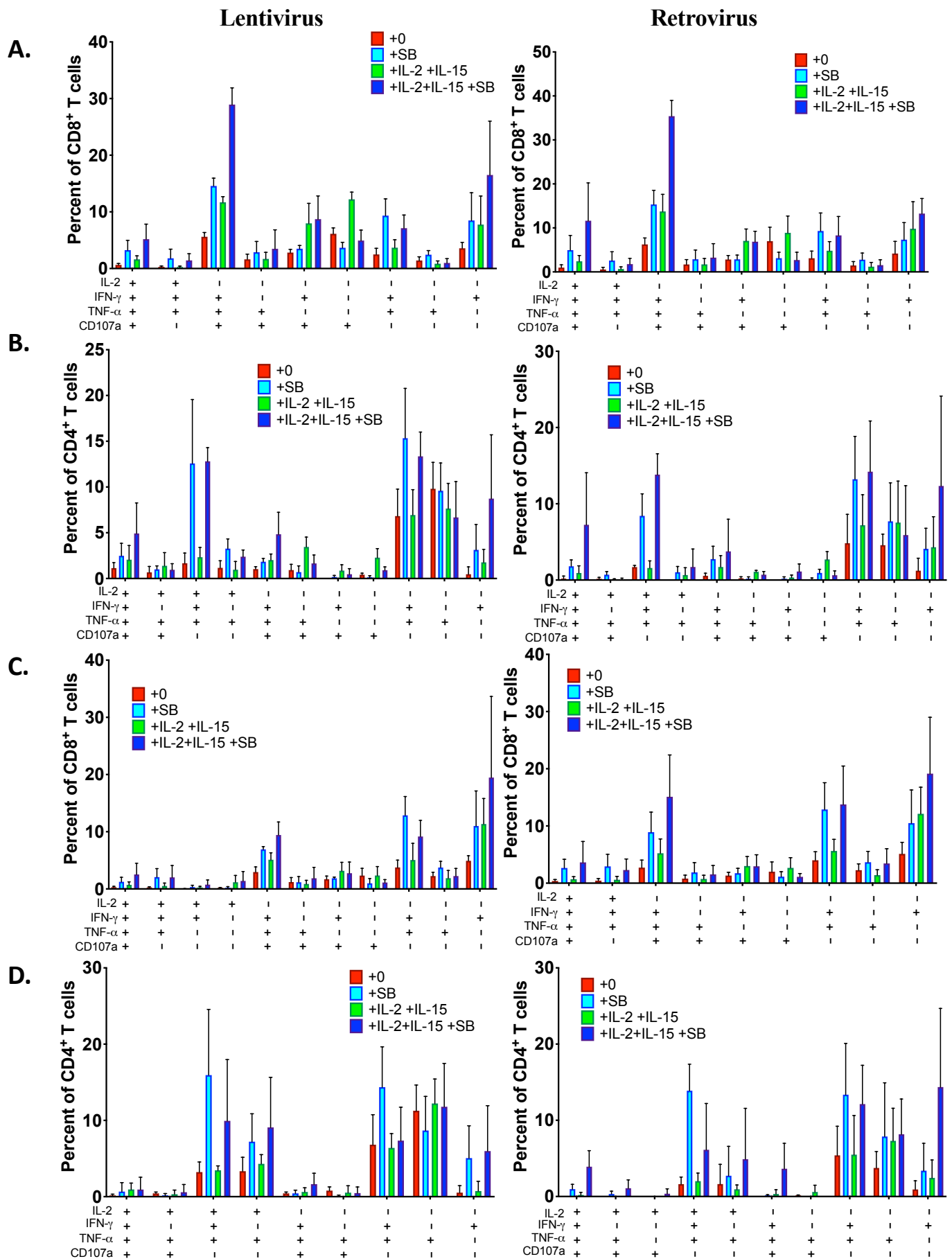


Supplemental Figure S3: The percent of transduced T cells begins declining by seven days post-transfer. At indicated days post-transfer into patients, patient PBMC were collected via apheresis or blood draw. These cells were depleted of red blood cells and then cryopreserved. In a cohort, the samples from each patient were thawed and immediately stained for live/dead, CD3, CD4, CD8, CD11b, CD19, and CD34 expression. Stained samples were analyzed by flow cytometry for the percent of CD34+ of total, CD4+, and CD8+ T cells. The number of CD34+ T cells per milliliter of blood was estimated by multiplying the percent CD34+CD3+ (or CD34+CD4+CD3+ or CD34+CD8+CD3+) of viable cells by the total number of viable white blood cells per milliliter of blood drawn.





Supplemental Figure S4: The presence of antigen appears to maintain higher transgene expression in the presence of cytokines. Healthy donor T cells from three independent donors were activated, transduced with lentivirus (pLVX-1383I, left) or retrovirus (SAMEN-1383I, RV, right), then sorted using magnetic beads (Miltenyi) for CD34⁺ cells, and rapidly expanded. Retroviral and lentiviral transduced T cells were cultured with IL-2 and IL-15 in the presence or absence of T2 cells, T2 cells pulsed with tyrosinase peptide (T2/tyrosinase) and MEL 624 cells. Tumor cells were added at a ratio of 1:4 tumor:T cell. Percentages of CD34⁺ Vβ12⁺ cells within CD4⁺ T cells (top) and CD8⁺ T cells (bottom) are shown. Error bars represent standard deviation. Significance is shown by Student's t-test with p-values summarized as *** < 0.001, ** < 0.01, * ≤ 0.05, and ns > 0.05.



Supplemental Figure S5: CD4⁺ and CD8⁺ T cell responses to antigen in the presence or absence of cytokines and sodium butyrate. T cells were transduced, sorted, expanded, stimulated and stained as described in Figure 5. Shown are the percentages of lentiviral and retroviral transduced cells making each combination of cytokines specified. A. CD8⁺ T cells cocultured with 624 MEL cells. B. CD4⁺ T cells cocultured with 624 MEL cells. C. CD8⁺ T cells cocultured with T2 cells pulsed with tyrosinase peptide. D. CD4⁺ T cells cocultured with T2 cells pulsed with tyrosinase peptide. Error bars represent SD.

SAMEN-1383I



pLVX-1383I



Supplemental Figure S6: Maps of the retroviral and lentiviral constructs used to transduce T cells. LTR are long terminal repeat sequences. The MMLV-CMV LTR is a hybrid LTR containing both essential MMLV LTR components and a CMV promoter sequence. CD34t is the truncated non-signaling human CD34 sequence.