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CD137 Costimulation of CD8+ T Cells Confers Resistance to Suppression by Virus-Induced Regulatory T Cells

Shelly J. Robertson,* Ronald J. Messer,* Aaron B. Carmody,† Robert S. Mittler, † Christopher Burlak,* and Kim J. Hasenkrug2*

Chronic viral infections cause high levels of morbidity and mortality worldwide, making the development of effective therapies a high priority for improving human health. We have used mice infected with Friend virus as a model to study immunotherapeutic approaches to the cure of chronic retroviral infections. In chronic Friend virus infections CD4+ T regulatory (Treg) cells suppress CD8+ T cell effector functions critical for virus clearance. In this study, we demonstrate that immunotherapy with a combination of agonistic anti-CD137 Ab and virus-specific, TCR-transgenic CD8+ T cells produced greater than 99% reductions of virus levels within 2 wk. In vitro studies indicated that the CD137-specific Ab rendered the CD8+ T cells resistant to Treg cell-mediated suppression with no direct effect on the suppressive function of the Treg cells. By 2 weeks after transfer, the adoptively transferred CD8+ T cells were lost, likely due to activation-induced cell death. The highly focused immunological pressure placed on the virus by the single specificity CD8+ T cells led to the appearance of escape variants, indicating that broader epitope specificity will be required for long-term virus control. However, the results demonstrate a potent strategy to potentiate the function of CD8+ T cells in the context of immunosuppressive Treg cells. The Journal of Immunology, 2008, 180: 5267–5274.

Infection of resistant strains of adult mice with Friend virus (FV) results in lifelong low-level infections predominantly harbored in a minute fraction of splenic B cells (1, 2). FV is a natural viral complex isolated from mice in 1957 (3) and contains replication-competent Friend murine leukemia helper virus (F-MuLV), a replication-defective spleen focus-forming virus, and lactate dehydrogenase-elevating virus, which enhances pathogenicity (4). Chronic FV infection is associated with the induction of CD4+ regulatory T (Treg) cells that suppress CD8+ T cell effector functions, thereby allowing the virus to evade CD8+ T cell-mediated cytolysis and persist long term (5). Due to Treg cell-mediated suppression, adoptive transfer of CD8+ T cells bearing transgenic (Tg) TCRs specific for a FV epitope is ineffective as a therapy to eliminate chronic FV infection (6). The virus-specific CD8+ T cells up-regulate activation markers and proliferate in response to the chronic infection, but their differentiation into perforin-positive, granzyme B-positive, IFN-γ-secreting cytolytic effector cells is suppressed (6). This Treg cell-mediated suppression of CD8+ T cells has been recapitulated in an in vitro suppression assay (5). In this assay, FV-induced Treg cells were shown to suppress CD8+ T cells in a cell contact-dependent manner regardless of CD8+ T cell specificity and in the absence of APC. Although an important issue, the Ag specificity of FV-induced Treg cells is currently not known.

In previous experiments, the ability of CD8+ T cells to develop effector function was moderately improved by immunotherapy with Ab specific for glucocorticoid-induced TNFR-related protein, a member of the TNFR superfamily (6, 7). The current study focuses on stimulation of another member of the TNFR superfamily, CD137 (4-1BB), a costimulatory molecule that is transiently up-regulated following TCR engagement accompanied by CD28 costimulation (8, 9). CD137 was of particular interest because it was reported that Ab-mediated signaling through CD137 not only inhibited the suppressive function of activated Treg cells (10), but also stimulated CD8+ T cell proliferation (11, 12), survival (13), and IFN-γ production (14). Furthermore, CD137 costimulation has been shown to be important in antiviral CD8+ T cell responses (15–18). The current study analyzed the effects of CD137 costimulation on the suppressive activity by CD4+CD25+ Treg cells and on the activation, proliferation, and development of effector function of CD8+ T cells in chronically infected mice. Results showed that anti-CD137 rendered CD8+ T cells resistant to Treg cell-mediated suppression and allowed them to develop antiviral activity, resulting in 99% reductions in chronic virus levels. No direct effect of anti-CD137 on CD4+CD25+ Treg cells themselves was observed. The results demonstrate a potent immunotherapy with implications for the treatment of chronic infections.

Materials and Methods

Mice

All mice were bred at the Rocky Mountain Laboratories except BALB/c mice, which were purchased from Harlan Breeders. Infection experiments were performed in female (C57BL/10 × AB.Y)F1 mice 12–24 wk old at onset. The relevant FV resistance genotypes of these mice are: H-2b/b, Fv1b/b, Fv2α/α, and Rfv3α/α. The TCR Tg mice were bred to B6.GFP mice (21). All mice were treated in accordance with the regulations and guidelines of the Animal Care and Use Committee of the Rocky Mountain Laboratories and the National Institutes of Health.

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3 Abbreviations used in this paper: FV, Friend retrovirus complex; F-MuLV, Friend virus; Treg, regulatory T; Tg, transgenic; PD-1, programmed cell death 1.
Virus and infections

All infections were done by i.v. injection of 1500 spleen focus-forming units of uncloned virus stock containing B-tropic F-MuLV and polycythe-mia-inducing spleen focus-forming virus. As previously described, the FV complex also contains lactate dehydrogenase-elevating virus (4, 22). Mice were considered chronically infected at 8 wk postinfection. Infectious center assays were used to measure spleen infection levels as previously described (23). Where noted, 5 × 10⁶ spleen cells were adoptively transferred into BALB/c mice as a highly sensitive method to expand cells infected with the FV complex.

CD8⁺ T cell enrichment, adoptive transfers, and Ab injections

FV-specific CD8⁺ T cells were isolated from Tg mice using anti-CD8⁺ paramagnetic beads and the MidiMACS Separation System (MACS) as recommended by the manufacturer (Miltenyi Biotec). CD8⁺ T cell purity was >95%. A total of 4 × 10⁶ cells in 0.5 ml of PBBS containing 2% FBS and 15 U/ml heparin sodium (SoloPak Laboratories) was transferred per mouse by i.v. injection. For assessment of proliferation, purified CD8⁺ T cells were labeled with CFSE before adoptive transfer as previously described (24). Where indicated, mice were treated with 100 μg of anti-CD28 (clone 37.51; BD Biosciences) or anti-CD137 (clone 3H3) (29) administered by i.p. injection. For experiments done to measure infectious centers at weeks 2 and 4 after treatment, Ab injections were given on days 0, 3, and 6 relative to adoptive transfer of cells.

In vivo activation and cell enrichments for in vitro suppression assays

To obtain in vivo-activated FV-specific GFP⁺ CD8⁺ T cells for coculture suppression assays, CD8⁺ T cells were isolated from (TCR.Tg × B6.GFP)F1 mice using the MACS system as described above and 8 × 10⁶ purified cells were adoptively transferred into mice infected with FV 2 days previously. After 4 days, the CD8⁺ T cells were reisolated using the MACS system, stained with PE-anti-CD44 and allophycocyanin-anti-CD8⁻, and sorted for GFP⁺ CD44⁺ CD8⁺ on a FACSria flow cytometer (BD Biosciences) to obtain purities greater than 95%. In cases where mice were treated with Abs during in vivo activation, 100 μg of Ab (anti-CD28 or anti-CD137) was given by i.p. injection at the time of adoptive transfer of CD8⁺ T cells. CD4⁺ CD25⁺ T cells were enriched using the MACS system. Briefly, after blocking with anti-FcγRII/III receptor (clone 2.4G2), spleen cell suspensions were incubated with biotin-conjugated anti-CD25 (clone PC61.5.3) (Caltag Laboratories) followed by streptavidin paramagnetic microbeads and purification using the MACS system. For experiments in which Treg cells were purified from anti-CD137-treated mice, chronically infected mice were given one injection of 100 μg of Ab and Treg cells were harvested 4 days later.

In vitro suppression assay

For assays in which the CD8⁺ T cells were activated in vitro, cocultures were set up containing enriched Treg cells, naive TCR Tg FV-specific CD8⁺ T cells, and GagL peptide-pulsed APC, as previously described (5). Cells were cultured in IMDM (Cambrex) containing 10% FBS, 2 mM t-glutamine, 50 μM 2-ME, and 100 U/ml each of penicillin and streptomycin at 37°C in 5% CO₂. For assays in which the CD8⁺ T cells were activated in vivo, the cocultures contained only Treg cells and CD8⁺ T cells that were kept stimulated by addition of D⁺GagL, MHc class I tetramer (25). D⁺GagL MHc class I tetramer stock solution (Beckman Coulter) was added to 200 ng/well for cultures containing in vivo-activated CD8⁺ T cells. All cocultures were done with 6–8 × 10⁶ CD4⁺ CD25⁺ T cells and 3–4 × 10⁶ CD8⁺ T cells in flat-bottom 96-well plates. Culture supernatants were collected after 68 h and tested for IFN-γ by ELISA as previously described (5).

Abs, tetramers, intracellular cytokine staining, and flow cytometry

Directly conjugated Abs used in flow cytometric analyses were: PE-anti-CD44 (clone IM7), allophycocyanin-anti-CD8 (clone 53-6.7), and allophycocyanin-anti-IFN-γ (clone XM1G1.2; BD Biosciences) and PE-D⁺GagL MHc class I tetramer (Beckman Coulter). For intracellular IFN-γ staining, 1 × 10⁶ spleen cells were stained as described (6), except no in vitro restimulation with anti-CD3 was performed. Analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo version 6.3.2 software (Tree Star).

Infectious center assay

Serial dilutions of spleen cells were plated onto susceptible Mus dunni cells, cocultured for 3 days, fixed with ethanol, stained with F-MuLV envelope-specific mAb720, and developed with peroxidase-conjugated goat anti-mouse IgG and substrate to detect foci of infected cells (23).

Sequencing of the FV GagL T cell epitope

Total DNA was extracted with WizardPrep (Promega) from spleen cell suspensions and sequencing was performed as described previously (6), with the exception of using Big Dye Terminator Cycle Sequencing version 3.0 (Applied Biosystems) on an Applied Biosystems 3730xl DNA Analyzer. Nucleotide and translated protein sequences were analyzed using MacVector (Accelrys).

Statistical analysis

Differences between groups were evaluated for statistical significance by Student’s two-tailed t test using Prism software (GraphPad). As noted, a Bonferroni correction to the Student’s t test was used for the in vitro suppression data where two comparisons were made with a single group. A value of p < 0.05 was considered significant.

Results

CD137 costimulation enhances virus-specific CD8⁺ T cell proliferation and development of effector function

Previous studies demonstrated that chronic FV infections were associated with virus-induced Treg cells that displayed an activated phenotype in vivo (5) and suppressed CD8⁺ T cell effector functions such as IFN-γ production both in vivo (6) and in vitro (5). This Treg-mediated suppression occurred at a postproliferative stage. Thus, FV-specific TCR Tg CD8⁺ T cells adoptively transferred into chronically infected mice became activated and proliferated in response to chronic infection, but were suppressed in their ability to produce cytolytic granules and IFN-γ (6). We used similar adoptive transfer experiments to determine whether signaling via the CD137 (4-1BB) costimulatory molecule would inhibit Treg cell-mediated suppression of CD8⁺ T cell function in chronically infected mice. Mice were adoptively transferred with virus-specific TCR Tg CD8⁺ T cells with or without agonistic anti-CD137 Ab therapy. To assess proliferation, TCR Tg CD8⁺ T cells were labeled with CFSE before adoptive transfer. Flow cytometric analysis of spleen cells at 4 days after transfer showed Ag-driven activation (CD44high) and proliferation of virus-specific TCR Tg CD8⁺ T cells when adoptively transferred into chronically infected mice compared with naive mice (Fig. 1A, inset). Anti-CD137 therapy significantly enhanced donor cell proliferation. Massive accumulation of TCR Tg CD8⁺ T cells was observed in the chronically infected mice treated with anti-CD137 compared with mice receiving cells without Ab (Fig. 1A, right). The effect of anti-CD137 treatment was dependent on antigenic stimulation since no significant proliferation or accumulation of TCR Tg CD8⁺ T cells was observed in naive mice (Fig. 1A, left). The experiments were repeated using GFP-labeled donor cells to test for effector functions. First, the ability of CD8⁺ T cells to produce IFN-γ was measured directly ex vivo with no in vitro restimulation. Consistent with previous results (6), very few donor CD8⁺ T cells produced IFN-γ in the immunosuppressive setting of chronic FV infection (Fig. 1B, middle). Interestingly, anti-CD137 treatment significantly increased the percentage of donor CD8⁺ T cells producing IFN-γ (Fig. 1B, right).

Analysis of the effects of anti-CD137 on CD8⁺ T cell effector function was extended to include granzyme B, an effector molecule important for CTL-mediated killing of F-MuLV-infected cells (26), and surface expression of CD107a (LAMP-1), an indicator of recent cytolytic activity (27, 28). At 6 days after treatment, anti-CD137-treated, chronically infected mice had significantly greater numbers of endogenous CD8⁺ T cells that expressed granzyme B.
Anti-CD137 Ab renders CD8^+ T cells resistant to in vitro suppression by FV-induced Treg cells

To determine whether in vivo treatment with anti-CD137 was directly inhibiting FV-induced Treg cells or rendering the CD8^+ T cells resistant to the effects of suppression, both cell types were analyzed in an in vitro suppression assay as previously described (5). Unlike assays for natural Treg cells that measure suppression of proliferation and require in vitro stimulation, the virus-induced Treg cells in this assay were tested directly ex vivo with no in vitro stimulation. CD4^+CD25^+ Treg cells obtained from chronically infected mice that had been treated with anti-CD137 suppressed IFN-γ production by TCR Tg CD8^+ T cells as well as Treg cells from untreated mice. For example, in one experiment, the mean IFN-γ production by CD8^+ T cells was reduced from 67.8 ng/ml (±5.2) to 3.6 ng/ml (±2.6) when they were cocultured with Treg from chronically infected mice that had been treated with anti-CD137. Fig. 3A shows the compiled data from two separate experiments. Thus, there was no indication that anti-CD137 Ab abrogated the suppressive activity of FV-induced Treg cells.

To assess the impact of anti-CD137 treatment on the CD8^+ T cells, GFP-expressing TCR Tg CD8^+ T cells were activated in a physiologically relevant manner by adoptive transfer into acutely infected mice (5). Concurrent with adoptive transfer, the mice were left untreated or treated with either anti-CD137 or anti-CD28. After 4 days in vivo, activated (CD44^high^) CD8^+ T cells were purified by cell sorting and tested for IFN-γ production in the presence or absence of Treg cells. As previously shown (5), in vivo-activated CD8^+ T cells were reduced from a mean of 9.1 ng/ml (±2.6) to 3.6 ng/ml (±2.6) when they were cocultured with Treg from chronically infected mice that had been treated with anti-CD137. To test this hypothesis, we performed an in vitro suppression assay as previously described (5).

The most remarkable effect of anti-CD137 treatment was observed in mice that also received adoptive transfer of TCR Tg CD8^+ T cells. Anti-CD137 costimulation enhanced granzyme B and CD107a expression within the donor CD8^+ T cell population and, due to their remarkable expansion, the absolute numbers of these cells far exceeded that of responding host CD8^+ T cells. Thus, CD137 costimulation not only increased proliferation, but also significantly improved development of antiviral effector function in CD8^+ T cells. This increase in CD8^+ T cell effector function suggested that CD137 costimulatory molecules treatment interfered with Treg cell-mediated suppression in some manner.

and CD107a compared with untreated control mice (Fig. 2). The FIGURE 1. Agonist anti-CD137 costimulation enhances CD8^+ T cell proliferation and IFN-γ production. A, CD8^+ T cells from spleens of naive and chronically infected mice were analyzed by flow cytometry 4 days after the first treatment. Adoptive transfers were done with 4 × 10^6 TCR Tg CD8^+ T cells, and mice that received anti-CD137 Ab were injected with 100 μg on the day of adoptive transfer. To assess proliferation, TCR Tg CD8^+ T cells were labeled with CFSE before adoptive transfer. The inset dot plots show representative flow analyses of CD44 and CFSE on CD8-gated cells. Proliferation of donor cells was significantly increased in mice that also received adoptive transfer of TCR Tg CD8^+ T cells. GFP-expressing TCR Tg CD8^+ T cells were distinguished from host cells based on GFP expression. The graph depicts the total numbers of host (□) and donor CD8^+ T cells (■). Treatment with anti-CD137 induced significantly higher percentages (p = 0.0002) and numbers of donor CD8^+ T cells per spleen (p < 0.0001, n = 7). The spleens of mice that received adoptive cell transfer plus anti-CD137 were engorged with CD8^+ T cells (average spleen weight 749 mg compared with 309 mg in mice receiving cells alone, p = 0.0015, n = 4 mice/group). B, Spleen cells from chronically infected mice 4 days after the first treatment were stained for intracellular IFN-γ after 5 h of culture in the presence of brefeldin A without in vitro stimulation. The dot plots show representative flow analyses of CD44 and CFSE on CD8-gated cells. Donor (GFP^+) T cells from chronically infected mice treated with anti-CD137 averaged 10.4% IFN-γ-positive while mice that received cells alone averaged 1.1% IFN-γ-positive (p = 0.0218, n = 3 mice/group).
CD8<sup>+</sup> T cells from anti-CD137-treated mice showed no significant suppression by Treg cells (Fig. 3B, middle). In vivo treatment of mice with anti-CD137 boosted IFN-γ production by CD8<sup>+</sup> T cells to 152.8 ng/ml (±23.6) consistent with previous reports (29), but there was no reduction upon addition of Treg from chronically infected mice (mean IFN-γ production 153.3 ng/ml, ±9.0). Resistance to suppression was not due to general effects of costimulation because CD8<sup>+</sup> T cells activated in vivo in the presence of agonistic anti-CD28 could still be suppressed in vitro (Fig. 3B, right). Taken together, these results indicated that the in vivo antisuppressive effect of agonistic anti-CD137 was predominantly on the CD8<sup>+</sup> T cell effectors rather than on the FV-induced Treg cells. This anti-CD137-dependent change in susceptibility to Treg cell-mediated suppression also indicated that the improved function of adoptively transferred CD8<sup>+</sup> T cells in vivo was due to an intrinsic phenotypic change and not simply due to high numbers of T cells overwhelming the capacity of the Treg cells to suppress them.

**Anti-CD137 treatments enable CD8<sup>+</sup> T cell-mediated attack on chronic virus in vivo**

To determine whether the improved functional phenotype of the adoptively transferred T cells in anti-CD137-treated mice enabled an immunological attack on chronically infected cells, mice were treated three times in 1 wk and then assayed for levels of chronic infection 1 wk later. As shown previously (2), untreated mice chronically infected with FV generally harbored in the range of 10,000 infected cells per spleen (Fig. 4). Ab and adoptive transfer controls, including adoptive transfers of virus-specific CD8<sup>+</sup> T cells combined with anti-CD28 agonistic Abs produced little or no reduction in chronic infection levels. In contrast, the combination of adoptive transfer plus anti-CD137 produced greater than 99% reductions in viral loads. In seven of nine mice from two separate experiments, no infectious centers were detectable in 10,000,000 spleen cells. Furthermore, 50,000,000 spleen cells from each of three mice with apparent virus clearance were adoptively transferred into highly susceptible BALB/c mice without causing FV infection, whereas cells from one mouse with 30 infectious centers/50,000,000 cells caused overt infection (data not shown). Thus, anti-CD137 treatment enabled a highly effective CD8<sup>+</sup> T cell-mediated attack on chronic FV infection that dramatically reduced infectious virus levels within 2 wk of treatment initiation.

**Loss of donor CD8<sup>+</sup> T cells in anti-CD137-treated mice**

At 2 wk after treatment, chronically infected mice that received virus-specific CD8<sup>+</sup> T cells without Ab or with control anti-CD28 Ab still had expanded populations of donor cells (Fig. 5A). Unexpectedly, mice that received CD8<sup>+</sup> T cells with anti-CD137 Ab had lost almost all donor cells by 2 wk after transfer, despite having had enormously expanded populations at 4–6 days after treatment. Analysis at 4 wk after transfer also showed extremely low levels of tetramer-positive (virus-specific) CD8<sup>+</sup> T cells (Fig. 5B). A possible explanation for this cell loss was that anti-CD137...

**FIGURE 3.** Effect of anti-CD137 treatment on the in vitro suppression of CD8<sup>+</sup> T cells by virus-induced Treg cells. Suppression of CD8<sup>+</sup> T cell IFN-γ production by Treg cells (CD4<sup>+</sup>CD25<sup>+</sup> T cells) was measured by in vitro suppression assay. The data are compiled from two independent experiments that gave equivalent results. The graph shows the percent suppression of CD8<sup>+</sup> T cell IFN-γ production in cultures containing Treg cells relative to cultures without Treg cells. Error bars represent SEs of the mean. A, TCR Tg CD8<sup>+</sup> T cells were activated in vitro with peptide-pulsed APC. The cells were cocultured with Treg cells harvested from chronically infected mice that were either untreated or had been treated 4 days previously with 100 μg of anti-CD137. Treg cells from anti-CD137-treated mice significantly suppressed production of IFN-γ in a manner quantitatively equivalent to Treg cells from untreated mice (p < 0.002 by Student’s t test with Bonferroni correction, n = 5 mice in the untreated group; n = 7 mice in the anti-CD137-treated group). B, TCR Tg CD8<sup>+</sup> T cells were activated by adoptive transfer into acutely infected mice that were untreated or treated with 100 μg of either anti-CD137 or anti-CD28 on the day of transfer. In vivo-activated (CD44<sup>high</sup>) TCR Tg CD8<sup>+</sup> T cells were then purified by cell sorting 4 days after transfer and used in the in vitro suppression assay. In vivo-activated TCR Tg CD8<sup>+</sup> T cells from anti-CD137-treated mice were resistant to Treg-mediated suppression in vitro compared with TCR Tg CD8<sup>+</sup> T cells from untreated mice or mice treated with anti-CD28 (p < 0.002 by Student’s t test with Bonferroni correction, n = 4 mice/group).

**FIGURE 4.** Reduction of chronic infection in mice treated with anti-CD137 and virus-specific CD8<sup>+</sup> T cells. Eleven million spleen cells from each mouse were assayed for infectious virus by an infectious center assay. Each dot represents results from a single mouse. The mice treated with both anti-CD137 and virus-specific CD8<sup>+</sup> T cells had significantly lower infectious centers per spleen than mice treated with cells only (p < 0.0001). Spleen weights indicated significantly less FV-induced splenomegaly in the mice receiving dual therapy (mean = 218 mg) compared with mice receiving CD8<sup>+</sup> T cells without anti-CD137 (mean = 305 mg, p = 0.0011). The untreated control and anti-CD137 plus CD8<sup>+</sup> T cell adoptive transfer groups contain results from two separate experiments.
evoked activation-induced cell death. Consistent with this hypothesis, adoptively transferred donor cells from anti-CD137-treated mice had significantly increased expression of Fas and programmed cell death 1 (PD-1) at 1 wk postinfection compared with donor cells in untreated groups (Fig. 5, C and D). Interestingly, analysis of the endogenous population of tetramer-positive CD8\(^+\) T cells in mice that received anti-CD137 without CD8\(^+\) T cell transfers demonstrated a significant increase rather than decrease in percentage at the 4-wk time point (Fig. 5B). Thus, anti-CD137 appeared to have differential effects on the transferred and endogenous populations of virus-specific CD8\(^+\) T cells, possibly reflecting differences in differentiation or activation states. Unfortunately, despite the increased numbers of virus-specific CD8\(^+\) T cells in the anti-CD137-treated mice, as well as increased expression of granzyme B (Fig. 2), the endogenous cells were unable to mount an effective antiviral attack (Fig. 4).

A possible explanation for the failure of anti-CD137 to potentiate the function of the endogenous population of virus-specific CD8\(^+\) T cells was that the endogenous cells were previously suppressed and not as responsive to anti-CD137 treatment as naive cells. To investigate this issue, naive CD8\(^+\) T cells were adoptively transferred into chronically infected mice as previously, but suppressed for 2 wk in vivo before anti-CD137 treatment. Analysis of virus-specific CD8\(^+\) T cells at 2 wk after transfer revealed proliferation to essentially the same levels shown in Fig. 5A (33.2% mean donor CD8\(^+\) T cells) and a suppressed phenotype (Fig. 6A, pretreatment). Two weeks after initiation of anti-CD137 injections (4 wk postadoptive transfer), spleen cells were analyzed for tetramer-positive CD8\(^+\) T cell levels, CD8\(^+\) T cell phenotype, and virus loads. The percentage of CD8\(^+\) T cells that were tetramer positive averaged 15.1%, the same as observed in untreated mice at 4 wk after transfer (Fig. 5B). Thus, there was not the dramatic loss of tetramer-positive T cells as was seen when Ab therapy was concomitant with adoptive transfer. Analyses of the tetramer-positive CD8\(^+\) T cells showed that anti-CD137 treatments were associated with a slight increase in the percentage of IFN-\(\gamma\)-producing cells, a large increase in the percentage of granzyme B-positive cells, but no increase in the percentage of recently cytotoxic cells (CD107a positive) (Fig. 6A). Most importantly, there was no decrease in the number of virus-producing cells in the spleen (Fig. 6B). Thus, anti-CD137 treatment did not restore a fully functional phenotype to CD8\(^+\) T cells previously suppressed in vivo.

Virus mutation and escape at 4 wk after treatment
Since the anti-CD137-costimulated TCR Tg CD8\(^+\) T cells effective in reducing virus loads at 2 wk after therapy were specific for a single F-MuLV epitope, there was a concern that escape via mutation of this single epitope would occur. Experiments were

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**FIGURE 5.** Loss of virus-specific CD8\(^+\) T cells in anti-CD137-treated mice at 2 and 4 wk after therapy. A and B, Spleens cells were analyzed by flow cytometry to determine the percentage of tetramer-positive CD8\(^+\) T cells. The histograms represent tetramer staining in the CD8-gated subpopulation (host and donor) at 2 wk (A) and 4 wk (B) after treatment. The mean percentages from four to five mice per group are indicated above the brackets. At both time points the difference between mice that received anti-CD137 plus virus-specific CD8\(^+\) T cells and mice that received cells alone was statistically significant (\(p < 0.0001\)). *, Indicates that the higher percentage of tetramer-positive CD8\(^+\) T cells in chronically infected mice receiving anti-CD137 without adoptive transfer was significantly higher than that of untreated chronically infected mice (\(p = 0.0016\)). The 2.0% tetramer-positive cells at 4 wk after transfer/anti-CD137 were >97% CD127 negative and therefore did not have a memory phenotype. C and D, Adoptively transferred virus-specific CD8\(^+\) T cells from chronically infected mice without anti-CD137 treatment (shaded histograms) or with anti-CD137 treatment (open histograms) were analyzed by flow cytometry for the expression of Fas (C) and PD-1 (D) at 1 wk after treatment.
levels between the groups. Results from one mouse. There were no significant differences in virus analyzed for virus-producing cells as in Fig. 4. Each dot represents the inferred with 4

CD8\(^{+}\) T cells after anti-CD137 treatment. Mice were adoptively transferred with 4 \(\times 10^6\) TCR Tg CD8\(^{+}\) T cells and the test mice were given three injections of 100 \(\mu\)g of anti-CD137 on days 14, 17, and 20 relative to adoptive transfer. A, Phenotypic analysis of tetramer-positive CD8\(^{+}\) T cells from spleens of chronically infected mice. Cells were analyzed before initiation of therapy (left) and 2 wk following the first treatment with antiCD137 (right). Results are from five mice per group, except for the pre-treatment group, which had only three mice. Granzyme B percentages were significantly higher in mice that received both cells and anti-CD137 compared with mice that received cells only (\(p = 0.0035\)). B, Spleen cells were analyzed for virus-producing cells as in Fig. 4. Each dot represents the results from one mouse. There were no significant differences in virus levels between the groups.

extended to 4 wk after therapy to measure virus levels and to determine whether any rebound had occurred. Indeed, all mice tested at this time point had detectable virus, and three of five had significantly higher levels of virus than the mean at 2 wk after treatment (Fig. 7). To determine whether escape was due to mutation of the CTL epitope recognized by the Tg TCR, provirus was amplified from splenic DNA as previously described (6) and subjected to sequence analysis. The two mice that maintained the greatest control over chronic virus both had only the wild-type sequence detectable in the CTL epitope. In contrast, the three mice with the highest virus levels all had single point mutations in the proviral DNA encoding the CTL epitope (Fig. 7, mice 3–5).

Discussion

Following the original findings from the FV model that a virus can use suppression by Treg cells to evade CD8\(^{+}\) T cell-mediated clearance and establish or maintain chronic infections (30), similar findings were reported for human viruses including HIV (31–35), hepatitis C virus (36–38), and HSV (39). The current results show that Treg cell-mediated suppression can be overcome to enable an effective antiviral response using adoptively transferred CD8\(^{+}\) T cells costimulated with the agonistic anti-CD137 Ab. The Ab-mediated costimulation rendered adoptively transferred CD8\(^{+}\) T cells resistant to FV-induced Treg-mediated suppression in vitro and evidence suggested a similar in vivo effect. In vivo, CD137 co-stimulation enabled expression of IFN-\(\gamma\) and cytolytic activity by CD8\(^{+}\) T cells producing greater than 99% reductions in chronic FV titers within 2 wk. These results provide proof for the principle that given the proper stimulation, virus-specific CD8\(^{+}\) T cells can proliferate and develop effector function even in the context of an immunosuppressive microenvironment.

Interestingly, anti-CD137 had a potent effect on naive CD8\(^{+}\) T cells but only a partial or weak effect on previously suppressed CD8\(^{+}\) T cells. CD137 is a member of the TNFR superfamily (tnf-s9, also known as 4-1BB), which plays key roles in regulating T cell immune responses (reviewed in Ref. 40), especially CD8\(^{+}\) T cell responses (29, 41). CD137 is transiently expressed on activated CD8\(^{+}\) T cells following TCR stimulation with peak in vivo expression at around 24 h (41). One explanation for the failure of anti-CD137 to act on suppressed CD8\(^{+}\) T cells is that such cells fail to up-regulate CD137 sufficiently to receive an optimal co-stimulatory signal from the Ab. If this is the case, discovery of ways to stimulate suppressed T cells vigorously enough to up-regulate CD137 could allow anti-CD137 to be effective on the endogenous population of virus-specific CD8\(^{+}\) T cells. Reactivation of endogenous cells would not only negate the need for adoptive transfers of virus-specific CD8\(^{+}\) T cells as part of the therapy, but might also solve the problem of virus escape via mutation. Clonotypic TCR Tg CD8\(^{+}\) T cells are elegant tools with which to study immune responses, but using them for clinical therapy against a retroviral infection is analogous to using a single antiretroviral drug and escape via mutation is probably inevitable. However, the endogenous population of virus-specific CD8\(^{+}\) T cells should be oligoclonal and virus escape would be much more difficult.

The finding that three of five mice developed escape variants with mutations in the CTL epitope by 4 wk after treatment revealed other important features of the model. First of all it demonstrated that the previous failure to detect mutations in that region of the viral genome in untreated, chronically infected mice (6) was not due to the failure of such mutants to survive. Rather, it was due to the lack of CD8\(^{+}\) T cell-mediated selective pressure. Although there was an expanded population of CD8\(^{+}\) T cells specific for the DbGagL CTL epitope, they provided no selective pressure because they were rendered dysfunctional by Treg cells and lacked antiviral activity (6, 42). Second, the data indicate that strong selective pressure was placed on the virus following combined treatment with anti-CD137 and adoptively transferred CD8\(^{+}\) T cells. Together with the findings of increased expression of cell surface CD107a and decreases in the numbers of infected cells at 2 wk, the finding of escape mutants strongly suggests that in vivo cytolysis

![Image](https://example.com/image1.png)

**FIGURE 6.** Functional status not restored in previously suppressed CD8\(^{+}\) T cells after anti-CD137 treatment. Mice were adoptively transferred with 4 \(\times 10^6\) TCR Tg CD8\(^{+}\) T cells and the test mice were given three injections of 100 \(\mu\)g of anti-CD137 on days 14, 17, and 20 relative to adoptive transfer. A, Phenotypic analysis of tetramer-positive CD8\(^{+}\) T cells from spleens of chronically infected mice. Cells were analyzed before initiation of therapy (left) and 2 wk following the first treatment with antiCD137 (right). Results are from five mice per group, except for the pre-treatment group, which had only three mice. Granzyme B percentages were significantly higher in mice that received both cells and anti-CD137 compared with mice that received cells only (\(p = 0.0035\)). B, Spleen cells were analyzed for virus-producing cells as in Fig. 4. Each dot represents the results from one mouse. There were no significant differences in virus levels between the groups.

![Image](https://example.com/image2.png)

**FIGURE 7.** CTL epitope sequences from dually treated mice at 4 wk post-treatment. Chronically infected mice were treated by adoptive transfers of TCR Tg CD8\(^{+}\) T cells combined with anti-CD137 as described. Four weeks after the initial treatment, DNA was amplified with virus-specific oligomers, and the PCR products were directly sequenced. This figure shows the single-letter amino acid code deduced from the DNA sequence that differed from the wild-type sequence shown at the bottom. Amino acids matching the wild-type sequence are indicated by a dot. Wild-type sequences as previously described (19) were obtained from mice with either acute or chronic infections.
of infected cells was an important mechanism of antiviral activity by the CD137-costimulated CD8+ T cells.

It must be pointed out that CD137 can deliver potent activation signals that can result in adverse effects in naïve mice (43). Depending on the status and type of T cell, CD137 can deliver diametrically opposed effects such as proliferation (29, 44) and survival (13) on one hand vs apoptosis and anergy signals on the other (44–48). Interestingly, it appeared to do both in our present study. After 1 wk of treatment, we observed strong expansion and development of effector function in adoptively transferred CD8+ T cells, although at that point we also observed signs of impending apoptosis such as increased expression of Fas and PD-1. Accordingly, by week 2 the adoptively transferred cells had all but disappeared in the mice treated with anti-CD137. Thus, the activated CD8+ T cells worked very quickly to reduce virus loads, but in the context of chronically infected mice, CD137 signaling was not sufficient to induce a survival signal as has been shown to occur in superantigen (13, 14) and protein/adjuvant-stimulated CD8+ T cells (13, 14). Thus, although CD137 signaling may be important in CD8+ T cell survival, it is not always sufficient and additional signals may be required. It is known that CD137 can work in conjunction with positive stimuli such as OX40, which has been shown to synergize CD137 signaling (49), but there may also be negative regulatory stimuli that influence whether CD137 signaling produces survival or apoptosis. It is also possible that the loss of transferred CD8+ T cells following CD137 stimulation was due to an insufficiency of factors such as IL-15 (50) or IL-2. IL-2 is not necessary for initiation of proliferation, but it is required at later stages for sustained expansion of activated CD8+ T cells (51).

In contrast to previous reports that CD137 signaling in CD4+ Treg cells antagonized their suppressive activity in vitro (10), we found no evidence that the Treg cells from anti-CD137-treated mice had diminished suppressive activity in vitro. These discrepant results are likely due to the use of quite different assays to measure suppression. Our assay measures virus-induced Treg cells, which are tested directly ex vivo without any in vitro stimulation, and the results closely mimic known aspects of in vivo suppression in mice chronically infected with FV (5). In contrast, the Kwon group (10) used naive, natural Treg cells that were stimulated in vitro by anti-CD3 and that stimulation was required to produce suppression. Furthermore, our measurement of suppression is CD8+ T cell function, while the Kwon group (10) measured proliferation of conventional CD4+ T cells. Thus, these studies were quite different in several respects and a number of factors could be responsible for the difference in outcome.

Although the finding that CD137 signaling can render CD8+ T cells resistant to Treg cell-mediated suppression is novel, numerous studies have shown a variety of other effects on CD8+ T cell functions. For example, studies on OVA-specific CD8+ T cells showed that IFN-γ secretion but not the development of cytolytic activity was dependent on CD137 signaling (14). Our results confirm an important role in IFN-γ expression, but also indicate that although CD137 signaling may not be required for the development of cytolytic activity, it certainly was able to promote such activity in vivo as indicated by significantly increased expression of CD107a and granzyme B on CD8+ T cells. It is important to note that mice treated with anti-CD137 without accompanying transfer of virus-specific CD8+ T cells showed a 2- to 3-fold expansion of tetramer-positive CD8+ T cells as well as improved granzyme B production. Like the endogenous population of tetramer-positive CD8+ T cells, adoptively transferred CD8+ T cells that were suppressed before anti-CD137 therapy also up-regulated granzyme B. However, neither type of cell developed high IFN-γ production as a result of anti-CD137 therapy, a cytokine known to be important in control of chronic FV infections (52, 53). Although there was a slight but significant reduction in chronic virus levels in anti-CD137-treated mice without T cell transfers at the 2-wk time point (p = 0.0389), by 4 wk virus levels were back to normal (data not shown). Thus, suppressed virus-specific CD8+ T cells responded partially to anti-CD137 therapy, but their proliferative responses, IFN-γ responses, and ultimately their antiviral responses were quite weak compared with those observed from adoptively transferred cells. At this time it is not known whether the observed effects were from weak or altered signaling by anti-CD137 directly on the CD8+ T cells or whether the effects were indirect, possibly from costimulation of CD4+ T cells. In that regard, we observed significantly increased IFN-γ production by CD4+ T cells (more than double, p = 0.0159) in anti-CD137-treated mice compared with the untreated mice in Fig. 6 (data not shown).

The current results are relevant to numerous situations where it is desirable to activate CD8+ T cell responses in the face of Treg cell-mediated suppression. Such situations include chronic viral infections such as HIV, hepatitis C virus, EBV, as well as chronic infections with other intracellular pathogens that induce Treg cells (reviewed in Refs. 54–57). Of interest, recent in vitro studies on functionally impaired CD8+ T cells from HIV patients indicate that CD137 signaling can help restore CD8+ T cell functions (58). Although there remain obstacles in achieving full cures to chronic infections, the current study achieved virus reductions of several orders of magnitude. It will require much additional research to translate findings from mouse regulatory T cells to treatments for human diseases (59), but this exciting new result indicates that anti-CD137 therapy could be a key component in future therapeutic strategies to reduce or eliminate chronic viruses where Treg cells have suppressed CD8+ T cell responses (reviewed in Refs. 60 and 61).

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Disclosures

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In the Materials and Methods section, there are typographical errors in the fifth sentence of the paragraph under the heading In vitro suppression assay. The sentence should read as follows: “All cocultures were done with 6–8 \(10^5\) CD4\(^+\)CD25\(^+\) T cells and 3–4 \(10^5\) CD8\(^+\) T cells in flat-bottom 96-well plates.


The fourth author’s name should have been published as David R. Curran.