HHS Public Access

Author manuscript

Immunol Cell Biol. Author manuscript; available in PMC 2013 August 01.

Published in final edited form as:

Immunol Cell Biol. 2013 February ; 91(2): 173–183. doi:10.1038/icb.2012.74.

CD134/CD137 Dual Costimulation-Elicited IFN-γ Maximizes Effector T Cell Function but Limits Treg Expansion

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Abstract

T cell tolerance to tumor antigens represents a major hurdle in generating tumor immunity. Combined administration of agonistic monoclonal antibodies to the costimulatory receptors CD134 plus CD137 can program T cells responding to tolerogenic antigen to undergo expansion and effector T cell differentiation, and also elicits tumor immunity. Nevertheless, CD134 and CD137 agonists can also engage inhibitory immune components. To understand how immune stimulatory versus inhibitory components are regulated during CD134 plus CD137 dual costimulation, the current study utilized a model where dual costimulation programs T cells encountering a highly tolerogenic self-antigen to undergo effector differentiation. IFN-γ was found to play a pivotal role in maximizing the function of effector T cells while simultaneously limiting the expansion of $CD4^+CD25^+Foxp3^+$ Tregs. In antigen-responding effector T cells, IFN-γ operates via a direct cell-intrinsic mechanism to cooperate with IL-2 to program maximal expression of granzyme B. Simultaneously, IFN-γ limits expression of the IL-2 receptor alpha chain (CD25) and IL-2 signaling through a mechanism that does not involve T-bet-mediated repression of IL-2. IFN-γ also limited CD25 and Foxp3 expression on bystanding $CD4^+Foxp3^+$ Tregs, and limited the potential of these Tregs to expand. These effects could not be explained by the ability of IFN-γ to limit IL-2 availability. Taken together, during dual costimulation IFN-γ interacts with IL-2 through distinct mechanisms to program maximal expression of effector molecules in antigen-responding T cells while simultaneously limiting Treg expansion.

Keywords

CD134; CD137; CD25; costimulation; IFN-γ; Treg

INTRODUCTION

Tumor antigens are a form of self-antigen, and thus tolerance mechanisms that evolved to inactivate self-reactive T cells have the undesired effect of dampening tumor immunity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Steady state dendritic cells play a central role in this process by presenting antigens deriving from both healthy tissues as well as tumors in a tolerogenic manner due to a lack of inflammation-induced costimulatory molecules and cytokines.[1] Agonistic monoclonal antibodies to costimulatory ligands or receptors that are otherwise not engaged when cognate naïve T cells are primed by steady state dendritic cells have thus been used to break tolerance to tumor antigens.[2,3] Agonists to the TNF/TNFR costimulatory family members CD134 (OX-40) and CD137 (4-1BB) are particularly effective in programming T cells encountering tolerogenic antigen to undergo expansion and effector differentiation rather than anergy and deletion[4–10] and also elicit tumor immunity in mouse models.[9,11,12] Further, humanized agonists to CD134[13] and CD137[14] have produced encouraging results in phase I and II human cancer clinical trials.

Given that individual costimulatory pathways program unique facets of T cell responsiveness,[15,16] and that engaging multiple immune effector arms may increase the likelihood of generating durable anti-tumor immunity, the application of multiple agonists may boost therapeutic efficacy. Indeed, combined administration of CD134 plus CD137 agonists synergistically programs robust effector T cell responses that control tumor growth in a variety of mouse models.[17–22]

Despite their ability to prime robust effector T cell responses, CD134 and CD137 agonists can also elicit immune dampening effects. For instance, depending upon the timing of administration, CD137 agonist can either augment or inhibit specific autoimmune and anti-viral T cell responses.[23,24] Further, a single high dose or multiple dosings of CD137 agonist causes global immune dysfunction.[25] CD137 agonist administered in combination with TLR agonists can also elicit CD8 T cell-mediated suppressor function.[26] CD134 agonist has a complex effect on $CD4^+CD25^+Foxp3^+$ Tregs. It can block naïve CD4 T cells from differentiating into inducible Tregs,[27–29] but expands pre-existing thymically-generated Tregs.[28,29] These CD134-expanded Tregs require IL-2 for maintenance of suppressor function and high-level expression of Foxp3 and IL-2 receptor alpha chain (CD25).[30]

The optimization of CD134 plus CD137 dual costimulation therapy to treat cancer will be aided by a better understanding of how the response of effector and regulatory T cells are controlled. The current study addressed this question using a model where dual costimulation programs self-reactive CD4 and CD8 T cells to undergo expansion and effector differentiation rather than tolerization. Dual costimulation programs self-reactive CD8 T cells to expand, and also to express IFN-γ and the cytotoxic effector molecule granzyme B (GzmB) when corresponding CD4 helper T cells are simultaneously dual-costimulated.[10] Notably, the CD4 T cells themselves differentiate into cytotoxic Th1 effectors that also express IFN-γ and GzmB.[21] Further, IL-2 produced by the specific cytotoxic Th1 effectors programs expanded $Foxp3^+$ Tregs to express GzmB,[21] which has been linked to enhanced suppressive potential in both transplantation[31] and tumor immunity[32] models. This model is thus ideal to analyze how dual costimulation simultaneously impacts the response of both effector and regulatory T cells.

IFN-γ was found to play a central role in regulating the response of both effector and regulatory T cells by both augmenting and dampening distinct IL-2-mediated response

pathways. Thus, IFN-γ cooperates with IL-2 via a cell-intrinsic mechanism to program maximal GzmB expression in CD4 and CD8 effector T cells. Simultaneously, IFN-γ limits CD25 expression and downstream STAT5 phosphorylation through an indirect IL-2-dependent mechanism. During standard T cell priming conditions, IFN-γ induces T-bet[33] that represses IL-2.[34,35] This suggested that IFN-γ was limiting IL-2-supported CD25 expression by inducing T-bet that in turn represses IL-2 production. To the contrary, neutralization of IFN-γ enhanced CD25 expression in dual-costimulated T cells without altering expression of T-bet. Further, although T-bet$^{-/-}$ T cells expressed elevated IL-2, they did not express substantially elevated CD25 unless IFN-γ was neutralized. Importantly, IFN-γ also limited CD25 and Foxp3 expression on bystanding CD4^{+}Foxp3^{+} Tregs, and limited the potential of these Tregs to expand. These effects could not be explained by the ability of IFN-γ to limit IL-2 supplied from the dual-costimulated effector T cells. In sum, IFN-γ plays a pivotal role during dual costimulation in maximizing the function of effector T cells while limiting the expansion of Tregs.

RESULTS

Role of IL-2 and IFN-γ in programming effector function in dual-costimulated T cells

HA-specific TCR transgenic (Tg) CD8 T cells adoptively transferred into Tg self-HA-expressing recipient mice initially divide but ultimately undergo anergy and deletion. Agonistic mAbs to the costimulatory receptors CD134 plus CD137 (dual costimulation or DCo) programs these self-reactive CD8 T cells to expand, although Tc1 differentiation marked by the acquisition of IFN-γ and GzmB expression requires co-transfer of TCR Tg HA-specific CD4 helper T cells[10] that themselves differentiate into IFN-γ and GzmB-expressing cytotoxic Th1 effectors.[21] T cell tolerance induction to self-HA in the absence of costimulatory agonists is mediated by the same steady state dendritic cells[36] (and data not shown) that induce tolerance to tumor antigens.[37,38] Thus, understanding how dual costimulation breaks tolerance and programs self-HA-specific T cells to undergo effector differentiation should provide insight into dual costimulation-elicited anti-tumor therapeutic responses.

To begin dissecting how dual costimulation programs effector differentiation, serum levels of an array of cytokines were compared in DCo-treated self-HA mice that received adoptively transferred HA-specific CD8 T cells either by themselves (Un-Helped) or with co-transferred HA-specific CD4 helper T cells (Helped) (Supplementary Figure 1). The inclusion of CD4 helper T cells resulted in greater concentrations of IFN-γ, TNF, IL-17, IL-1β, IL-10, IL-2, IL-12p70 and MIP-1α with increasing time following adoptive T cell transfer. IL-2 and IFN-γ were chosen for further study in part because they are both produced predominantly by T cells (as opposed to IL-1β, IL-12p70 and MIP-1α that are expressed predominantly by innate cells). Further, both IL-2 and IFN-γ were expressed at >5-fold and statistically greater amounts in “Helped” compared to “Un-Helped” mice as early as 48 h post-transfer (in fact IL-2 was different at 24 h), and were thus candidate drivers of effector T cell differentiation as opposed to simply being products of expanding committed effectors.

To assess the relative contributions of IL-2 and IFN-γ in dual costimulation-mediated programming of effector T cell differentiation, corresponding neutralizing antibodies were administered individually or in combination to DCo-treated self-HA mice receiving TCR Tg CD4 plus CD8 T cells (Figure 1). The expansion of TCR Tg CD8 and CD4 T cells in recipients treated with anti-IL-2, anti-IFN-γ, anti-IL-2 plus anti-IFN-γ and control IgG were comparable (not shown). IL-2 neutralization had no effect on the ability of either CD4 helper or helped CD8 T cells to express IFN-γ and TNF (Figure 1a), but it did reduce GzmB expression (measured by mean fluorescence intensity (MFI)) in both T cell subsets (Figure 1b).

IFN-γ neutralization reduced the percentage of both CD4 and CD8 T cells that could express IFN-γ ($p \leq 0.0004$) (but not TNF) to that observed in un-helped CD8 T cells (Figure 1a). IFN-γ neutralization also reduced GzmB MFI in both CD4 helper and helped CD8 T cells, and simultaneous neutralization of IL-2 plus IFN-γ had an additive effect in further reducing GzmB MFI in both T cell subsets (Figure 1b).

Both IL-2[21,39–41] and IFN-γ[42,43] can program T cells to express GzmB. Our current data indicate that the two cytokines cooperate in dual-costimulated T cells to program maximal GzmB expression (Figure 1b). We next utilized a well-controlled *in vitro* system to assess whether this cooperation occurs via a cell-intrinsic or rather an indirect mechanism (Figure 2). WT T cells and counterparts deficient for the IFN-γR1 (*Ifngr1*$^{-/-}$)[44] cultured separately or admixed at a 1:1 ratio were activated with anti-CD3 mAb +/−CD134 plus CD137 agonists (DCo). Consistent with the *in vivo* DCo response[10,21] (and Figure 1b), DCo augmented GzmB expression in both WT CD4 and CD8 T cells, and DCo-treated *Ifngr1*$^{-/-}$ CD4 and CD8 T cells expressed reduced GzmB compared to WT counterparts (Figure 2). Further, IL-2 neutralization reduced GzmB expression in both WT and *Ifngr1*$^{-/-}$ CD4 and CD8 T cells (Figure 2), confirming that IL-2 and IFN-γ cooperate to program maximal GzmB expression. Importantly, the impact of IFN-γR1 deficiency and IL-2 neutralization on GzmB expression was not influenced by whether the WT and *Ifngr1*$^{-/-}$ CD4 and CD8 T cells were cultured separately or together (Figure 2). This revealed that IFN-γ cooperates with IL-2 to program maximal GzmB expression via a cell-intrinsic mechanism.

IFN-γ controls the IL-2 signaling axis in dual costimulation T cells

The IL-2 receptor alpha chain (CD25) that confers high affinity binding capacity is induced by TCR ligation and sustained by IL-2-induced STAT5 phosphorylation (pSTAT5).[45,46] Consistently, IL-2-neutralization markedly reduced CD25 expression on both CD4 helper (Figure 3b) and helped CD8 T cells (Figure 3a). That CD25 expression on IL-2-neutralized helped CD8 T cells remained higher compared to un-helped CD8 T cells suggested that IL-2 signaling had not been completely blocked (also refer to Figure 4b). This may not have been the result of a sub-saturating dosage of IL-2 neutralizing mAbs per se, but rather an inability of these mAbs to access homotypic T cells synapses through which paracrine IL-2 can be delivered.[47] Nevertheless, IFN-γ neutralization tended to have the opposite effect by augmenting CD25 expression on both CD4 helper (Figure 3b) and helped CD8 T cells (Figure 3a). Further, in both T cell subsets simultaneous neutralization of IL-2 plus IFN-γ rescued CD25 expression from the suppressed levels observed with IL-2 single

neutralization back to an intermediate level comparable to control IgG (Figure 3a & b). Taken together, these results indicated that IFN-γ neutralization restored normal CD25 expression when IL-2 availability was reduced, and augmented CD25 expression when IL-2 availability was un-restricted. Thus, IFN-γ limits IL-2-supported CD25 expression.

Direct *ex vivo* staining indicated that a greater percentage of DCo-treated IFN-γ-neutralized $CD25^+$ T cells contained pSTAT5 compared to IgG-treated counterparts (Figure 4a), consistent with the increased CD25 (Figure 3) and IL-2 expression (Figure 6a, c & e) on the former. Confirming that this pSTAT5 was induced by IL-2 (as opposed to other common gamma chain-associated cytokines that also activate STAT5 such as IL-4, IL-7 and IL-15), IL-2-neutralizing mAbs given to DCo-treated IFN-γ-neutralized mice 2 h immediately prior to analysis substantially reduced the percentage of $CD25^+$ T cells that contained pSTAT5 (p=0.008 and 0.02 for CD4 and CD8 T cell, respectively) (Figure 4b). Thus, augmented CD25 expression on DCo-treated IFN-γ-neutralized T cells was associated with enhanced IL-2 signaling.

IFN-γ limits CD25 expression through an indirect IL-2-dependent, but T-bet-independent, mechanism

We next used the *in vitro* priming assay described in Figure 2 to assess whether IFN-γ controls CD25 expression via a cell-intrinsic or rather an indirect mechanism. Consistent with the *in vivo* DCo response[21] (and Figure 3), DCo dramatically increased CD25 MFI on both WT CD4 and CD8 T cells activated *in vitro*, and IFNγR1 deficiency increased CD25 MFI several-fold on DCo-treated T cells (Figure 5). Importantly, when WT and *Ifngr1*$^{-/-}$ T cells were admixed in equal proportions, CD25 MFI on WT CD4 and CD8 T cells increased ~2-fold while on the co-cultured *Ifngr1*$^{-/-}$ T cells CD25 MFI decreased ~2-fold (Figure 5). This result suggested that *Ifngr1*$^{-/-}$ T cells might be producing greater amounts of a soluble factor that drives CD25 expression, thus explaining why co-culture enhances CD25 expression on WT T cells. Conversely, co-culture would also explain the reduced CD25 expression on *Ifngr1*$^{-/-}$ T cells since WT T cells produce less of this factor. Hence, co-culture would equalize CD25 expression on the two populations. A candidate for this presumptive factor is IL-2 since it induces CD25.[46] Indeed, IL-2-neutralization completely blocked CD25 expression on both WT and *Ifngr1*$^{-/-}$ DCo-treated CD4 and CD8 T cells cultured both separately and admixed (Figure 5).

In standardly primed T cells IFN-γ induces T-bet,[33] which transactivates the *Ifng* gene[34] while repressing the *Il2* gene.[34,35] This suggested that IFN-γ was controlling CD25 expression by first reinforcing expression of T-bet,[33] which then represses *Il2*[35] and hence limits IL-2-supported CD25 expression.[46] This was tested by comparing the response of WT vs T-bet$^{-/-}$ specific CD4 T cells in DCo-treated self-HA recipients. As previously observed,[21] control IgG-treated T-bet$^{-/-}$ specific CD4 T cells expressed less IFN-γ (Figure 6a) but similar GzmB (Figure 6f) compared to WT counterparts. The impact of IFN-γ on T cell expansion and survival is complex,[48] although it appears that IFN-γ promotes expansion during the initial phase of T cell priming.[49] Consistently, T-bet$^{-/-}$ and WT anti-IFN-γ-treated specific CD4 T cells exhibited a trend towards reduced accumulation compared to control IgG-treated WT specific CD4 T cells (Figure 6b). Also consistent with T-bet's

potential to repress *Il2*,[34,35] the percentage of IgG-treated T-bet$^{-/-}$ specific CD4 T cells that expressed IL-2 as well as their IL-2 MFI was greater compared to IgG-treated WT (Figure 6a, c & e). Notably, IFN-γ neutralization also enhanced the percentage of WT specific CD4 T cells that could express IL-2 (Figure 6a & c) as well as IL-2 MFI (Figure 6e), but did so without altering expression of T-bet (p=0.8) (Figure 6g). This indicates that although T-bet can repress IL-2 expression, in dual costimulated CD4 T cells IFN-γ represses IL-2 expression independently of T-bet. Further, CD25 MFI on IgG-treated T-bet$^{-/-}$ specific CD4 T cells was only 1.5-fold higher compared to IgG-treated WT but 3-fold lower than IFN-γ-neutralized WT (Figure 6h). Finally, anti-IFN-γ augmented CD25 MFI 5-fold on T-bet$^{-/-}$ specific CD4 T cells (Figure 6h) despite the fact that IL-2 expression was not significantly elevated in anti-IFN-γ compared to IgG-treated T-bet$^{-/-}$ CD4 T cells (Figure 6a & c–e). Taken together, IFN-γ-mediated control of CD25 expression (and hence IL-2 responsiveness) occurs mainly through a mechanism that involves neither T-bet nor repression of IL-2 expression.

IFN-γ limits expansion of CD4$^+$CD25$^+$Foxp3$^+$ cells during dual costimulation

CD134 agonist induces Treg expansion, although paracrine IL-2 is required for maintenance of suppressor function and high-level expression of Foxp3 and CD25.[30] Consistently, CD134 plus CD137 dual-costimulated specific CD4 T cells that produce robust IL-2 augment CD25, Foxp3 and GzmB expression on expanded Tregs.[21] In DCo-treated self-HA mice that received WT specific CD4 T cells IFN-γ neutralization enhanced expansion of bystanding CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Figure 7a–c) and augmented their CD25 MFI (Figure 7a & d) and Foxp3 MFI (Figure 7a & e). Notably, although IgG-treated T-bet$^{-/-}$ specific CD4 T cells expressed elevated IL-2 (Figure 6a, c & e), they did not augment expansion (Figure 7a–c) or CD25 MFI (Figure 7a & d) and Foxp3 MFI (Figure 7a & e) in bystanding Tregs beyond IgG-treated WT counterparts. Further, IFN-γ neutralization comparably boosted Treg expansion (Figure 7a–c) and CD25 MFI (Figure 7a & d) and Foxp3 MFI (Figure 7a & e) in DCo-treated self-HA mice that received either WT or T-bet$^{-/-}$ specific CD4 T cells. Taken together, these data indicate that similar to its effects on effector T cells (Figure 6), the ability of IFN-γ to limit Treg expansion and expression of CD25 and Foxp3 during dual costimulation cannot be solely ascribed to its ability to induce T-bet or repress IL-2.

Since Tregs constitutively express CD134 and CD137 and directly respond to cognate agonists,[28,30,50] we next assessed the extent to which the effect of anti-IFN-γ on Treg homeostasis during dual costimulation depends on specific CD4 T cells (Figure 8). In naïve mice that received neither DCo nor specific CD4 T cells, anti-IFN-γ did not significantly impact Treg frequency (Figure 8a & b), number (Figure 8c) or Foxp3 MFI (Figure 8e), but did elicit a slight increase in CD25 MFI (Figure 8d). DCo administration to IgG-treated mice that did not receive specific CD4 T cells elicited a modest ~2-fold increase in Treg frequency (Figure 8a & b), number (Figure 8c) and Foxp3 MFI (Figure 8e). Anti-IFN-γ elicited a slight (statistically non-significant) Treg expansion in DCo-treated mice that did not receive specific CD4 T cells (Figure 8a–c). Adoptive transfer of WT specific CD4 T cells into DCo and anti-IFN-γ treated mice further boosted the frequency of CD4$^+$Foxp3$^+$ cells (Figure 8a & b) as well as their CD25 MFI (Figure 8d) and Foxp3 MFI (Figure 8e).

Taken together, IFN-γ minimally impacts Treg homeostasis during the steady state (i.e., in the absence of dual costimulation), but IFN-γ elicited from specific CD4 T cells during dual costimulation controls the expansion of Tregs as well as their expression of CD25 and Foxp3.

Discussion

CD134 plus CD137 dual costimulation synergistically programs robust effector T cell responses that control tumor growth in a variety of mouse models.[17–21] This multi-pronged anti-tumor response involves not only the activation of $CD8^+$ CTL[17,18] and NK cells,[51] but also the induction of cytotoxic CD4 Th1 cells.[21] This powerful effector T cell response is balanced, however, by an expansion in $CD4^+CD25^+Foxp3^+$ Tregs.[21] Tregs often increase in number or function during effector T cell responses elicited under a variety of conditions,[52–54] presumably to prevent excessive inflammation. Tregs constitutively express CD134 and CD137, and respective agonist induces Treg expansion even when antigen-specific effector T cells are not being primed.[28,30,50] Notably, Tregs expanded with CD134 agonist lose CD25 and Foxp3 expression as well as suppressive function unless supplied with IL-2.[30] Dual costimulation-induced cytotoxic CD4 Th1 cells produce robust IL-2 and thus enable expanded Tregs to not only express elevated CD25 and Foxp3, but also GzmB[21] that has been linked to enhanced suppressive potential in both transplantation[31] and tumor immunity[32] models. That dual costimulation can elicit tumor immunity suggests that mechanisms exist to prevent the Treg response from overwhelming the anti-tumor effector T cell response. Understanding how this balance is established could provide insight into how dual costimulation can be optimized to shift the balance more in favor of the effector T cell response. Our current data indicate that during dual costimulation IFN-γ plays a central role in balancing the response of effector T cells and Tregs by simultaneously augmenting and limiting distinct IL-2-mediated response pathways.

IFN-γ operates via a cell-intrinsic mechanism to cooperate with IL-2 to program maximal GzmB expression in CD4 and CD8 effector T cells. Both IL-2[21,39–41] and IFN-γ[42,43] have been shown to individually program GzmB expression in T cells, and our current data extend these findings by demonstrating that these two cytokines can cooperate to program maximal GzmB expression. Somewhat paradoxically, however, IFN-γ controls the responsiveness of these effector T cells to IL-2 by limiting the expression of the IL-2 receptor alpha chain (CD25) that confers high affinity binding. CD25 is induced on effector T cells by TCR ligation and subsequently sustained through IL-2-mediated positive feedback.[46] Consistently, IL-2 must be available for IFN-γ neutralization to augment CD25 expression on dual-costimulated effector T cells. In standardly primed T cells IFN-γ induces T-bet,[33] which transactivates the *Ifng* gene[34] while repressing the *Il2* gene.[34,35] This led us to hypothesize that IFN-γ was controlling CD25 expression by first reinforcing expression of T-bet, which then represses *Il2* and hence limits IL-2-supported CD25 expression. To the contrary, although IFN-γ neutralization and T-bet deficiency both enhanced the potential of dual-costimulated CD4 effector T cells to produce IL-2, IFN-γ neutralization did not diminish expression T-bet. Further, only IFN-γ neutralization (and not T-bet deficiency) substantially increased CD25 expression. Taken together, IFN-γ controls CD25 expression through a mechanism that involves repression of neither T-bet nor IL-2.

Consistent with the potential of IFN-γ to induce T-bet in T cells primed under standard conditions,[33] we have observed that IFN-γ neutralization reduces T-bet expression several-fold in virally-primed CD8 effector T cells (data not shown). Our current observation that IFN-γ neutralization does not diminish T-bet expression in dual-costimulated CD4 effector T cells indicates that dual costimulation induces T-bet via an unknown alternate pathway. This ability of dual costimulation to engage alternate T cell response pathways is not without precedent. For instance, CD134 agonist programs CD4 T cells to express IFN-γ independently of CD28, CD40, IL-12Rβ2 and T-bet.[55] Further, CD134 plus CD137 dual costimulation programs CD4 T cells to differentiate along the non-canonical (but physiologically[56] and therapeutically[57,58] relevant) cytotoxic Th1 lineage.[21] The T-box transcription factor Eomesodermin (that normally programs GzmB, perforin and IFN-γ expression in NK and CD8 T cells[59,60]) enables cytotoxic Th1 CD4 cells to express GzmB[21] and is likely also responsible for enabling CD134 and dual-costimulated CD4 T cells to express IFN-γ independently of T-bet.[21,55]

In addition to limiting CD25 expression on dual-costimulated CD4 and CD8 effector T cells, IFN-γ also limited expansion of bystanding $CD4^+$ Tregs as well as their expression levels of CD25 and Foxp3. Previous studies have been split as to whether IFN-γ augments[61,62] or inhibits[63,64] Treg function, suggesting that the impact of IFN-γ on Treg homeostasis is context-dependent. During dual costimulation, we hypothesized that the ability of IFN-γ to limit Treg expansion was related to its ability to limit CD25 expression (and hence IL-2 responsiveness) on Tregs. This possibility was consistent with the essential role IL-2 plays in Treg homeostasis and function,[65–67] that Tregs are exquisitely sensitive to IL-2[68] and that they can expand when conventional T cells produce IL-2.[69,70] Analysis of T-$bet^{-/-}$ dual-costimulated CD4 effector T cells suggests that IFN-γ-mediated control of Treg homeostasis is not directly linked to the amount of available IL-2. Thus, although T-$bet^{-/-}$ effectors produce elevated IL-2 in comparison to WT counterparts when IFN-γ is not neutralized, they do not elicit enhanced Treg expansion or CD25 and Foxp3 expression. This indicates that IFN-γ elicited during dual costimulation controls Treg homeostasis through a pathway that does not depend upon increasing the supply of IL-2.

Our current findings may provide insight into how dual costimulation therapy might be optimized to further tip the balance of the overall response towards effector T cells. Thus, modifications that enhance production of IFN-γ (rather than suppress IL-2) may further control CD134 agonist-induced Treg expansion. A potential side effect associated with many immune-based cancer therapies is toxicity caused by exposure to large amounts of cytokine. For instance, administration of IL-2 to cancer patients in dosages sufficient to produce tumor regression elicits substantial toxicity.[71] Thus, given that dual-costimulated specific CD4 T cells robustly expand and produce IL-2,[21] their ability to also produce IFN-γ that limits CD25 expression may represent an in-built therapeutic advantage by which dual costimulation limits IL-2-mediated toxicity. Thus, through its ability to control CD25 expression, augmenting IFN-γ during dual costimulation may have the additional benefit of further minimizing IL-2-associated toxicity.

METHODS

Mice, adoptive transfer and cytokine neutralization

6.5 CD4[72] and clone 4 CD8[73] TCR transgenic (Tg) T cells specific for influenza (PR8 strain) hemagglutinin (HA) epitopes restricted to I-E^d and K^d, respectively, on the B10.D2 (H-2^d) Thy1.1$^+$ background were prepared from pooled spleens plus LN. The TCR Tg CD4 and CD8 T cells were then depleted of CD8$^+$ or CD4$^+$ cells using magnetic beads, respectively, and 5×10^5 of each population was adoptively co-transferred into congenic Thy1.2$^+$ self-HA transgenic mice (137 founder line[74]) treated with or without CD134 (50 μg) plus CD137 (25μg) agonists.[10] Spleens were recovered on day 4 to measure TCR Tg T cell frequencies and numbers as well as intracellular cytokine expression following 5 h *in vitro* stimulation with corresponding peptides in the presence of Brefeldin A, or CD25, GzmB, Foxp3, pSTAT5 or T-bet directly *ex vivo* as previously described.[10,21,68,75] Serum cytokine levels were measured at the indicated time points using Q-Plex mouse cytokine arrays (Quansys Biosciences). T-bet$^{-/-}$ Thy1.1$^+$ 6.5 TCR Tg CD4 T cells were previously described.[21]

In vivo cytokine neutralizations were performed using 50 μg each S4B6 plus JES6-1 (anti-IL-2 mAbs) given intraperitoneally (i.p.) 24 and 48 h post-adoptive transfer or 1 mg XMG1.2 (anti-IFN-γ mAb) given i.p. 0 and 48 h post-transfer (eBioscience, BD biosciences or Bio X Cell). Controls received rat IgG (Sigma-Aldrich).

All mouse protocols were approved by The University of Connecticut Health Center's Animal Care and Use Committee.

In vitro cultures

Splenocytes from Thy1.1$^+$ WT and Thy1.2$^+$ *Ifngr1*$^{-/-}$ C57BL/6 mice (Jackson Lab)[44] containing both CD4$^+$ and CD8$^+$ T cells were cultured at 1×10^6 cells/ml in 24-well plates separately or admixed 1:1 and stimulated with 0.5 μg soluble anti-CD3 mAb (eBioscience) with 5 μg CD134 plus 2.5 μg CD137 agonists or 7.5 μg rat IgG. IL-2 was neutralized with 50 μg/ml each S4B6 plus JES6-1, while controls received 100 μg rat IgG. Media and IL-2 neutralizing mAbs were changed at 24 h, and CD25 and GzmB measured at 48 h.

Statistical analysis

Quantitative data are expressed as the mean ± SEM, and *p* values were calculated using an unpaired two-tailed *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants RO1CA109339 and RO1AI094640 to Adam J Adler and Anthony T Vella and T32 AI007080 to Harry Z Qui. We thank Drs. Robert Clark and Antoine Menoret for critical reading of the manuscript.

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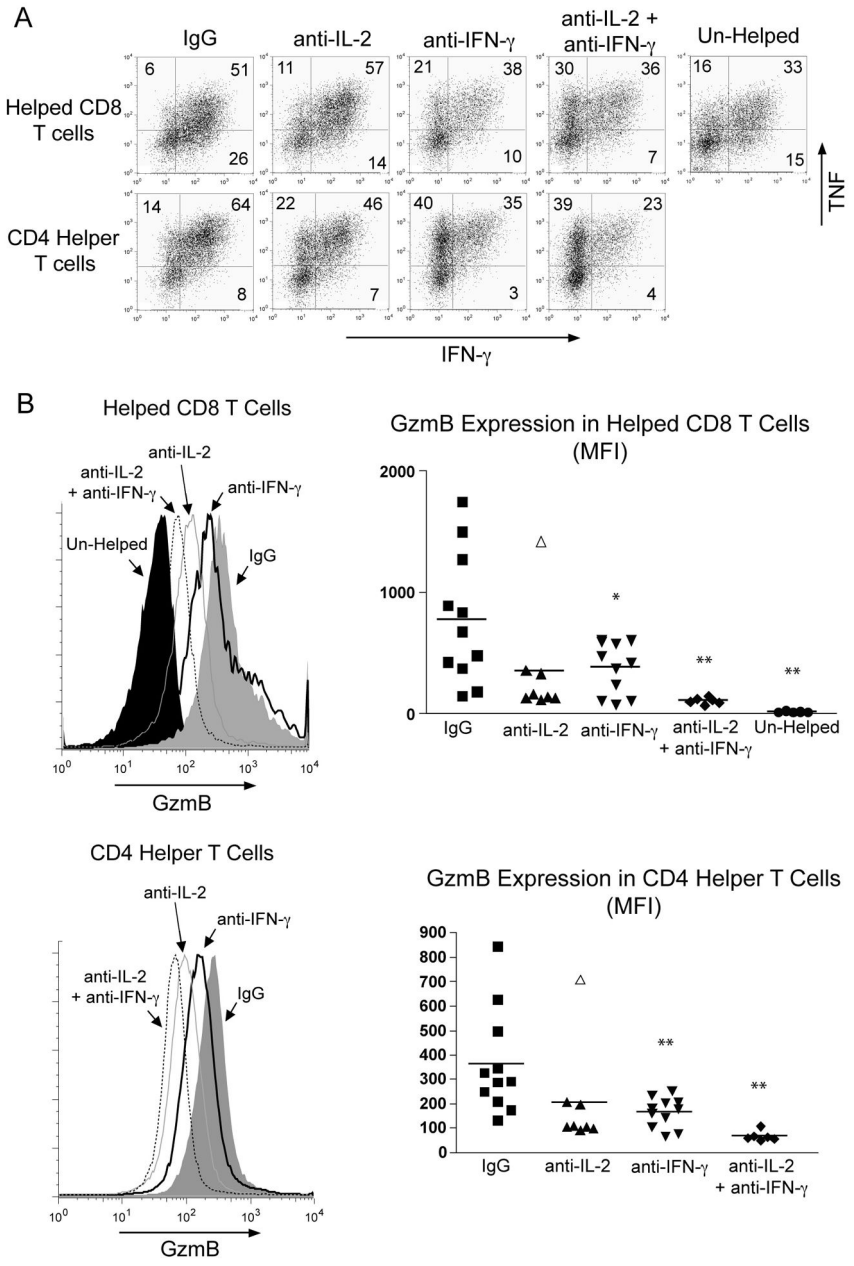

Figure 1.

Impact of IL-2 and IFN-γ neutralization on effector molecule expression in CD4 helper and helped CD8 T cells. Thy1.1$^+$ WT TCR Tg CD8 and CD4 T cells were adoptively co-transferred into DCo-treated Thy1.2$^+$ self-HA mice treated with rat IgG (control), anti-IL-2 mAbs, anti-IFN-γ mAb or anti-IL-2 plus anti-IFN-γ mAbs and recovered from spleens on day 4. Rat IgG-treated Un-Helped CD8 T cells provide a baseline comparison. All FACS plots and histograms are representative of 6 to 11 replicates per group. (**a**) Representative plots of intracellular IFN-γ vs TNF expression following *in vitro* stimulation with cognate peptide. (**b**) *Ex vivo* expression of GzmB. Left, representative FACS histogram overlays. Right, graphs of GzmB mean fluorescence intensity (MFI), * indicates $p<0.05$ and **

$p<0.01$ compared to control IgG. Please note that the graphs are shown as scatter plots to illustrate that the lack of statistical significance between the IgG and anti-IL-2-treated groups was due to a single outlying mouse whose GzmB MFI values were several-fold higher than the other 7 mice (refer to open triangles).

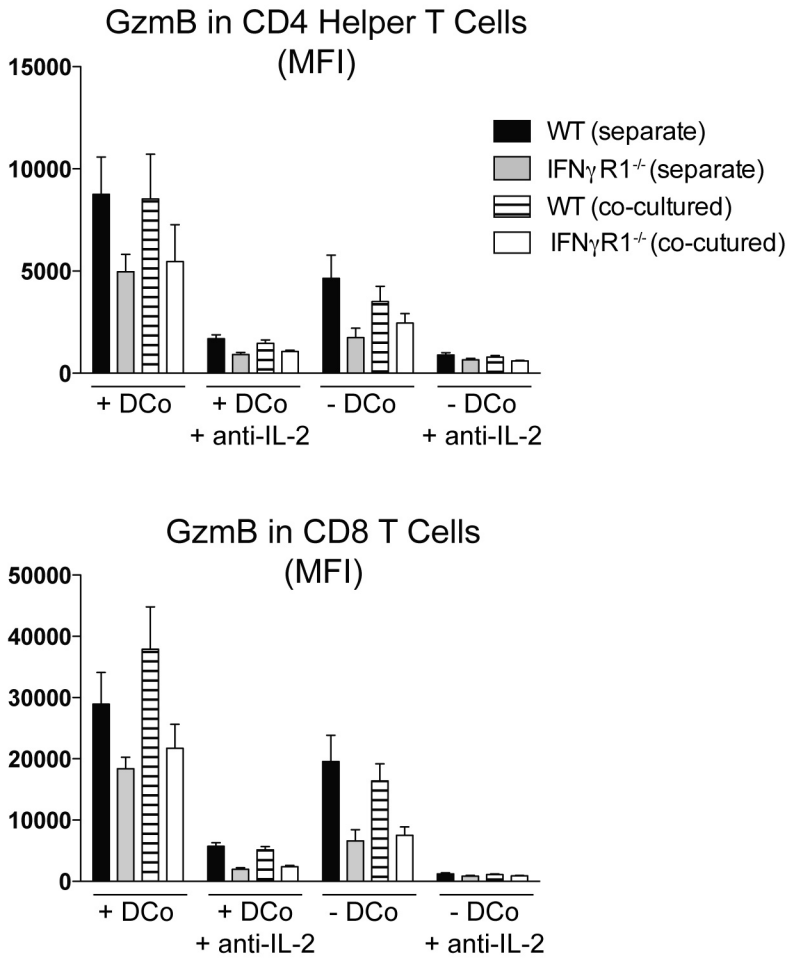

Figure 2.

IFN-γ cooperates with IL-2 to program maximal GzmB expression via a cell-intrinsic mechanism. WT and *Ifngr1*$^{-/-}$ splenocytes containing both CD4 and CD8 T cells were activated *in vitro* with anti-CD3 +/− DCo and +/− anti-IL-2, and GzmB MFI was measured 48 h later (*n*=4 per group).

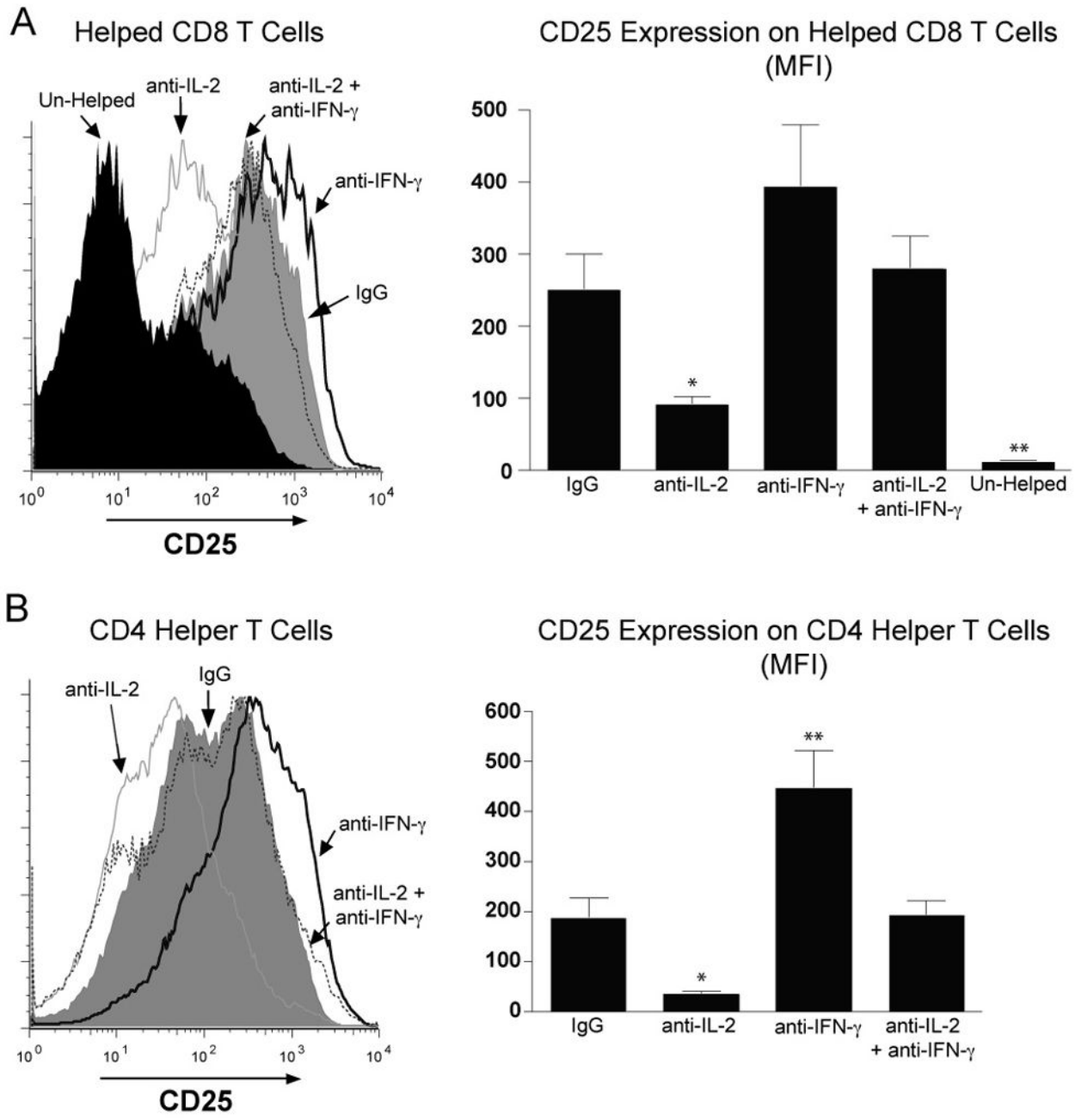

Figure 3.

IFN-γ limits IL-2-supported CD25 expression. CD25 MFI was measured on the CD4 helper (**a**) and helped CD8 (**b**) T cells described in Figure 1. Left, representative histogram overlays. n=5–6 per group in the graphs shown on the right, and * indicates $p<0.05$ and ** $p<0.01$ compared to control IgG.

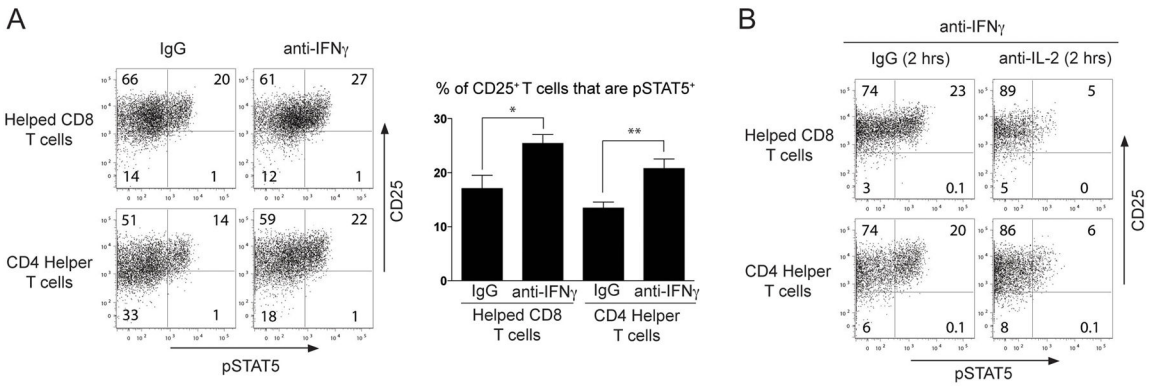

Figure 4.

Augmented CD25 expression on DCo-treated IFN-γ-neutralized T cells is associated with enhanced IL-2 signaling. (**a**) Left, representative direct *ex vivo* staining plots of pSTAT5 vs CD25 on DCo-treated Thy1.1$^+$ CD4 helper and helped CD8 T cells treated with anti-IFN-γ or control IgG. Right, graph showing the percentage of CD25$^+$ T cells that contain pSTAT5. *N*=5–6 per group, and * indicates $p<0.05$ and ** $p<0.01$. (**b**) DCo-treated IFN-γ-neutralized adoptive transfer recipients were treated with control IgG or anti-IL-2 mAbs 2 h immediately prior to harvest and directly stained for pSTAT5 vs CD25. Plots are representative of 2–3 replicates per group.

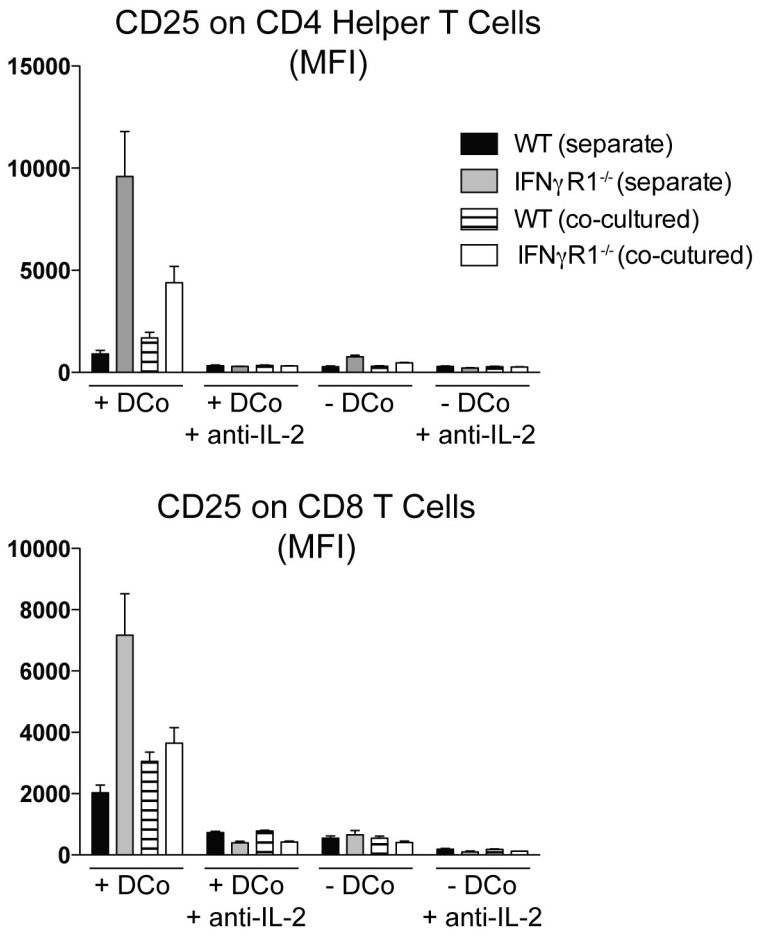

Figure 5.

IFN-γ limits CD25 expression through an indirect IL-2-dependent mechanism. CD25 MFI was measured on the *in vitro*-primed WT and *Ifngr1*$^{-/-}$ CD4 and CD8 T cells described in Figure 2.

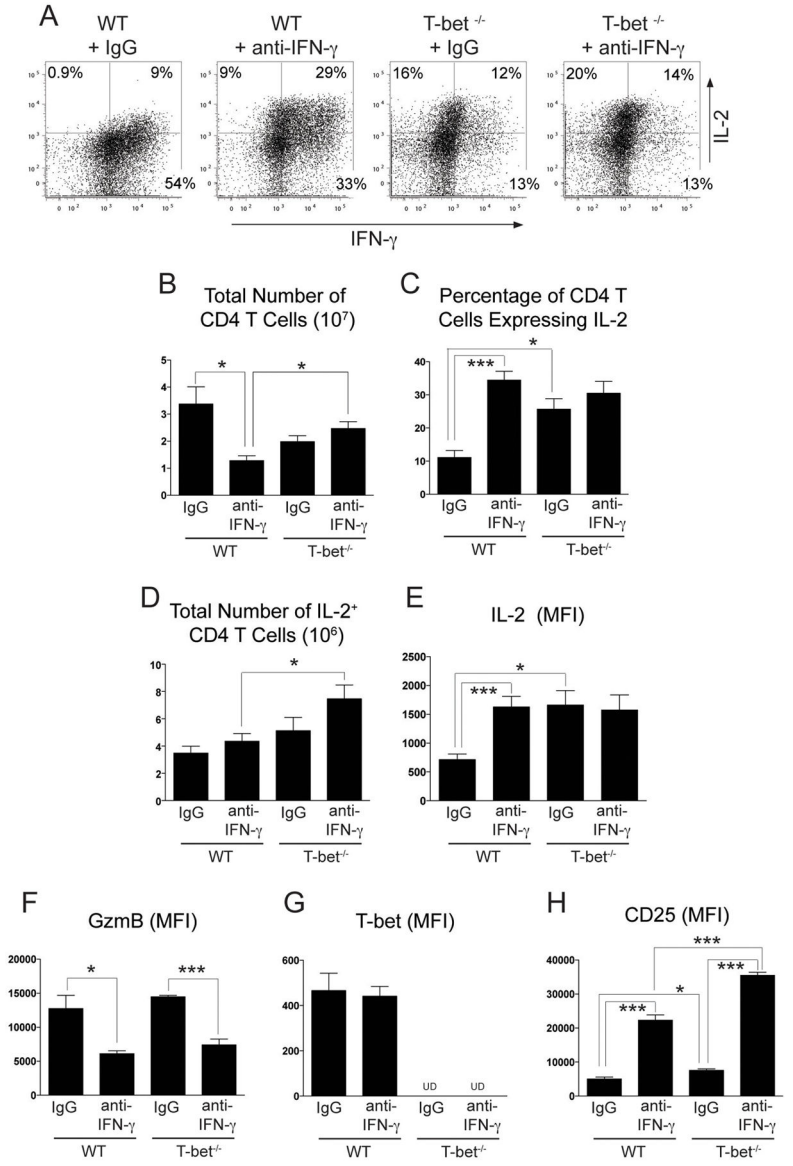

Figure 6.

IFN-γ controls CD25 expression on specific dual-costimulated T cells independently of T-bet. WT or T-bet−/− Thy1.1+ TCR Tg CD4 T cells were transferred into DCo-treated self-HA recipients and recovered from spleens on day 4. (a) Representative plots of IFN-γ vs IL-2 expression following in vitro peptide stimulation. Graphs showing total number of Thy1.1+ CD4 T cells (b), percentage of Thy1.1+ CD4 T cells expressing IL-2 (c), total number of IL-2+ Thy1.1+ CD4 T cells (d) and IL-2 MFI (e) following in vitro peptide stimulation. Direct ex vivo expression of GzmB (f), T-bet (g) and CD25 (h). N=3–4 per group, UD indicates un-detectable, * indicates $p<0.05$ and *** $p<0.005$.

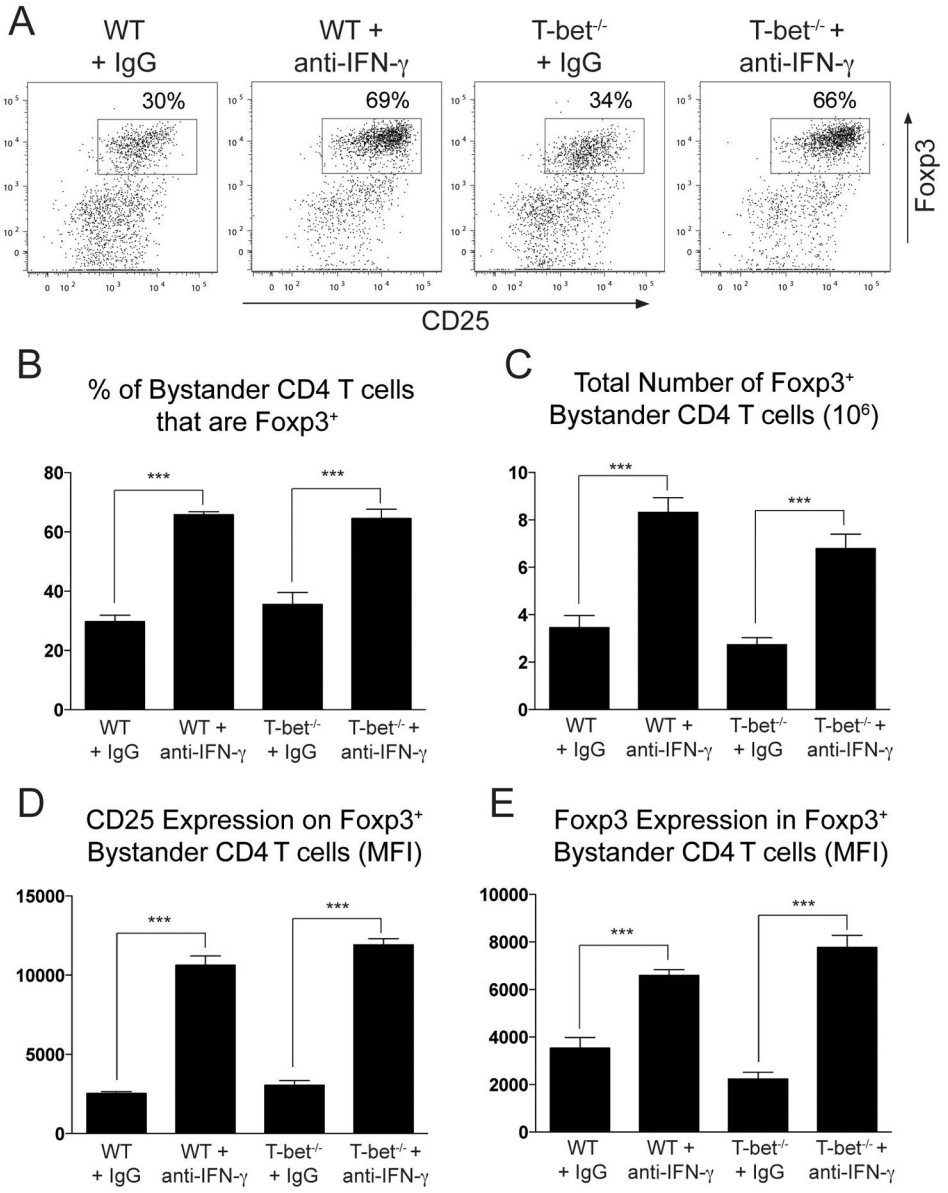

Figure 7.

IFN-γ controls CD25 and Foxp3 expression on and expansion of bystanding Treg cells during dual costimulation. The samples described in Figure 7 were analyzed for CD25 and Foxp3 expression on bystanding $CD4^{+}Thy1.1^{-}$ cells. (**a**) Representative plots of CD25 vs Foxp3 staining on $CD4^{+}Thy1.1^{-}$ cells. Graphs showing the percentage (**b**), total number (**c**), CD25 MFI (**d**) and Foxp3 MFI (**e**) of the $CD4^{+}Thy1.1^{-}$ cells. *** indicates $p<0.005$.

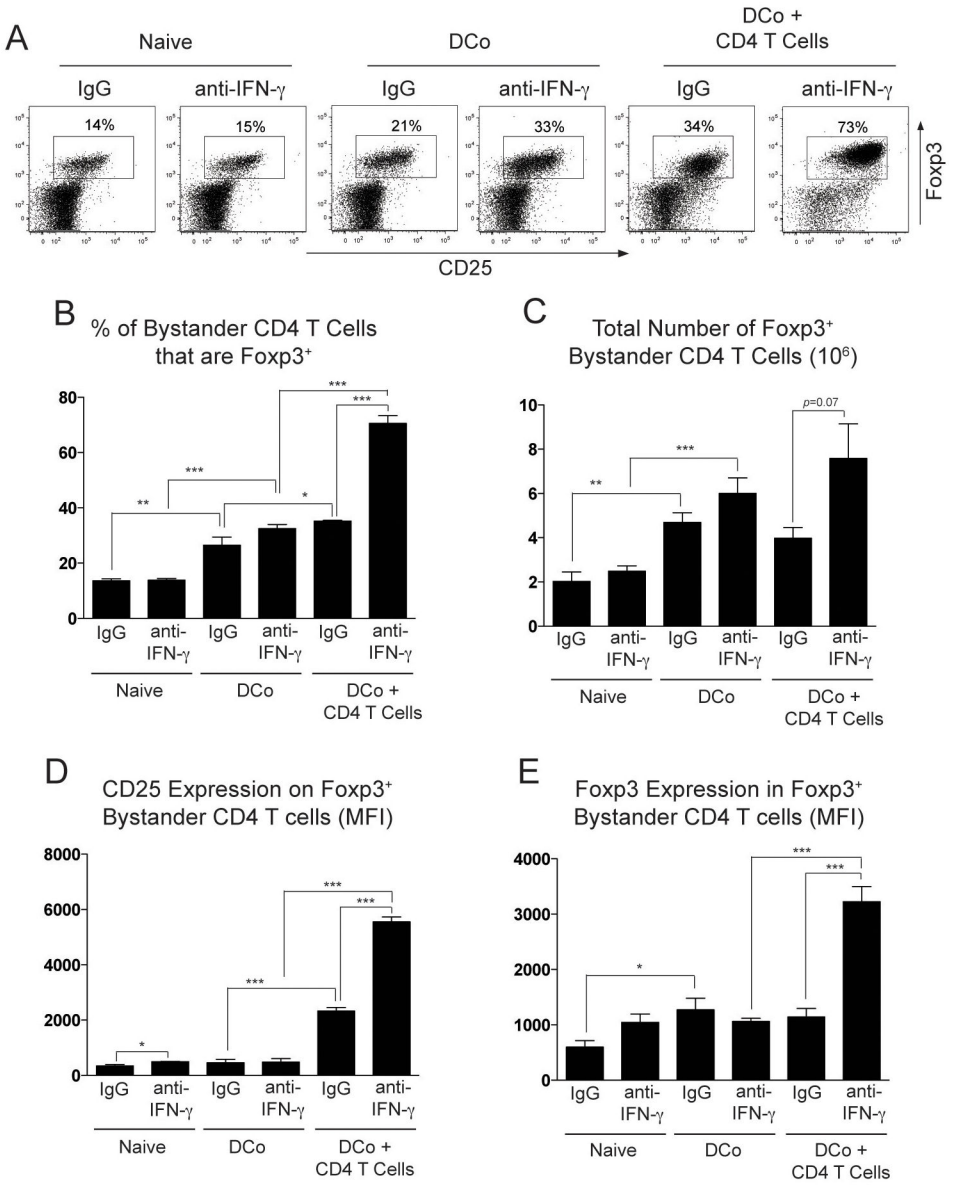

Figure 8.

Anti-IFN-γ-mediated augmentation of Treg expansion and expression of CD25 and Foxp3 during dual costimulation is potentiated by specific CD4 T cells. Spleens were analyzed on day 4 from self-HA mice that (as indicated) did or did not receive adoptively transferred WT specific CD4 T cells and treated with IgG or anti-IFN-γ and with or without (naïve) DCo. (**a**) Representative plots of CD25 vs Foxp3 staining on $CD4^{+}Thy1.1^{-}$ cells. Graphs showing the percentage (**b**), total number (**c**), CD25 MFI (**d**) and Foxp3 MFI (**e**) of the $CD4^{+}Thy1.1^{-}$ cells. N=4 per group, and * indicates $p<0.05$, ** $p<0.01$ and *** indicates $p<0.005$.