CD134 Plus CD137 Dual Costimulation Induces Eomesodermin in CD4 T Cells To Program Cytotoxic Th1 Differentiation


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CD143 Plus CD137 Dual Costimulation Induces Eomesodermin in CD4 T Cells To Program Cytotoxic Th1 Differentiation

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Cytotoxic CD4 Th1 cells are emerging as a therapeutically useful T cell lineage that can effectively target tumors, but until now the pathways that govern their differentiation have been poorly understood. We demonstrate that CD134 (OX40) costimulation programs naive self- and virus-reactive CD4 T cells to undergo in vivo differentiation into cytotoxic Th1 effectors. CD137 (4-1BB) costimulation maximized clonal expansion, and IL-2 was necessary for cytotoxic Th1 differentiation. Importantly, the T-box transcription factor Eomesodermin was critical for inducing the cytotoxic marker granzyme B. CD134 plus CD137 dual costimulation also imprinted a cytotoxic phenotype on bystanding CD4 T cells. Thus, to our knowledge, the current study identifies for the first time a specific costimulatory pathway and an intracellular mechanism relying on Eomesodermin that induces both Ag-specific and bystander cytotoxic CD4 Th1 cells. This mechanism might be therapeutically useful because CD134 plus CD137 dual costimulation induced CD4 T cell-dependent tumoricidal function in a mouse melanoma model. The Journal of Immunology, 2011, 187: 000–000.

Antigen-primed naive CD4 T cells can differentiate into numerous helper subsets that secrete a variety of cytokines specialized to coordinate different facets of adaptive immunity (1, 2). CD8 T cells similarly acquire cytokine potential, but inherently develop cytotoxic capacity (1, 3). CD4 T cells can also develop cytotoxic function during in vitro culture (4, 5), and cytotoxic CD4 T cells are induced in vivo in response to certain infections (6, 7). Thus, cytotoxic CD4 T cells might be useful in rejecting MHC class II+ tumors, in particular melanomas (6, 7). Cytotoxic CD4 T cells are induced in vivo in response to certain infections (6, 7). Thus, cytotoxic CD4 T cells might be useful in rejecting MHC class II+ tumors, in particular melanomas (6, 7).

Given the power of TNF family costimulatory molecules to boost both CD4 and CD8 T cell responses (17, 18), we postulated that the costimulators that greatly impact CD8 T cells may also affect CD4 T cells in a similar manner. In particular, combined treatment with agonists to CD134 and CD137 profoundly boosts CD8 T cell responses (19, 20) and elicits tumor immunity in various models (19, 21, 22). Likewise, CD134 costimulation programs CD4 T cells encountering self-Ag to expand and express Th1-associated cytokines (23, 24). In this study, we show that CD134 costimulation programs CD4 T cells to express lytic molecules and cytotoxic function, whereas CD137 costimulation maximized clonal expansion of the cytotoxic Th1 effector cells. This dual costimulation (DCo) through CD134 and CD137 enhanced IL-2 signaling in CD4 T cells responding to cognate Ag, and expression of granzyme B (GzmB) was entirely IL-2 dependent.

Notably, in dual costimulated CD4 T cells, the Th1 master-regulatory transcription factor T-bet (25) supported two-thirds of the potential to synthesize IFN-γ, but none of the GzmB-producing capacity. Rather, Eomes was responsible for programming cytotoxic function because its expression was induced by DCo, and DCo-induced GzmB expression was impaired in Eomes−/− CD4 T cells. Although, to our knowledge, these studies identify for the first time specific costimulatory pathways (CD134 plus CD137) that can induce and expand Ag-specific cytotoxic CD4 Th1 cells in vivo, perhaps the most unexpected observation was the imprinting of Ag-inexperienced bystanding T cells with similar functional capabilities. Thus, CD134 plus CD137 DCo programs cytotoxic function in both specific and bystanding CD4 T cells through induction of the CD8+ CTL-associated factor Eomes.

Finally, although the potential of CD134 plus CD137 DCo to elicit CD8 T cell-dependent tumor immunity has been established (19, 21, 22), our current study demonstrates the potential of DCo to program CD4 T cells to control a murine melanoma independently of CD8 T cells. CD134 and CD137 costimulatory pathways that govern their differentiation have been poorly understood. We demonstrate that CD134 (OX40) costimulation programs naive self- and virus-reactive CD4 T cells to undergo in vivo differentiation into cytotoxic Th1 effectors. CD137 (4-1BB) costimulation maximized clonal expansion, and IL-2 was necessary for cytotoxic Th1 differentiation. Importantly, the T-box transcription factor Eomesodermin was critical for inducing the cytotoxic marker granzyme B. CD134 plus CD137 dual costimulation also imprinted a cytotoxic phenotype on bystanding CD4 T cells. Thus, to our knowledge, the current study identifies for the first time a specific costimulatory pathway and an intracellular mechanism relying on Eomesodermin that induces both Ag-specific and bystander cytotoxic CD4 Th1 cells. This mechanism might be therapeutically useful because CD134 plus CD137 dual costimulation induced CD4 T cell-dependent tumoricidal function in a mouse melanoma model. The Journal of Immunology, 2011, 187: 000–000.
agonists are currently being developed separately as cancer therapeutics in human clinical trials (26, 27), and our current findings suggest that the application of DCo might be particularly effective in controlling disease progression because it can engage both CD8 and CD4 T cells that directly target tumors.

**Materials and Methods**

**Mice, adoptive transfer, and tumor challenge**

TCR transgenic (Tg) CD4 T cells specific for an I-E<sup>d</sup>-restricted epitope deriving from influenza (PR8 strain) hemagglutinin (HA) (19) or fixed DCo (CD8<sup>+</sup>) prepared from CD8-depleted lymph nodes of 6.5 TCR Tg mice (28) on the B10.D2 (H-2<sup>d</sup>) Thy1.2<sup>+</sup> background were adoptively transferred into congenic Thy1.2<sup>+</sup> recipients. Single-cell suspensions of Thy1.2<sup>+</sup> recipients were prepared and cultured on anti-CD3 mAb-activated splenocytes from Thy1.2<sup>+</sup> donors, and IFN-γ production was measured after 4 h, and the percentage of specific killing was calculated using an unpaired two-tailed Student’s t test.

**Bone marrow (BM) chimeras**

Bone marrow (BM) chimeras were generated, as previously described (30), with the following modifications. Lethally irradiated WT B10.D2 Thy1.2<sup>+</sup> mice were rescued with 2 × 10<sup>5</sup> BM cells from WT B10.D2 Thy<sup>+</sup>1.2<sup>+</sup> plus 8 × 10<sup>5</sup> BM cells from either IL-2<sup>-/-</sup>/Thy1.2<sup>-/-</sup> or IL-2<sup>-/-</sup>/Thy1<sup>-/-</sup>/Thy1.1<sup>/-/-</sup> donors. Recipient spleen samples containing 5 × 10<sup>5</sup> Thy1<sup>-/-</sup>/Thy1<sup>-/-</sup> (bystander) CD4 T cells.

**Statistical analysis**

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

**Results**

**CD134 plus CD137 DCo programs cytotoxic CD4 Th1 cell differentiation**

We asked whether DCo through CD134 and CD137 exerts effects on specific CD4 T cells similar to its known effects on CD8<sup>+</sup> CTL. DCo induced HA-specific TCR Tg CD4 T cells transferred into HA-expressing (self-HA) recipients to express high amounts of GzmB mRNA (Fig. 1A, B). Because the serine protease GzmB is a lytic granule component normally expressed in CD8<sup>+</sup> CTL and NK cells (39), this result suggested that DCo programs cytotoxic CD4 Th1 cell differentiation. Consistent with this effect being genetically programmed, DCo induced mRNA encoding not only GzmB, but also the pore-forming component of lytic granules perforin 1 (40) (Fig. 1C). Also consistent with the epigenetic profile of CD8<sup>+</sup> CTL (41), real-time PCR-based ChIP indicated that DCo significantly increased the amount of AcH3 bound to the Gzm<sup>b</sup> and Prf1<sup>+</sup> gene promoters (Fig. 1D). Thus, DCo programmed these genes to undergo epigenetic remodeling toward a transcriptionally permissive configuration. Confirming the fidelity of the ChIP assay, DCo increased ACh3 bound to the Ifng promoter (Fig. 1D), consistent with the enhanced IFN-γ expression (24).

**Flow cytometry**

TCR<sup>+</sup> CD4<sup>+</sup> T cells (CD4<sup>+</sup>Thy1.1<sup>+</sup>) were analyzed for expansion and intracellular GzmB expression directly ex vivo, as previously described (24). Where indicated, GzmB was measured in polyclonal CD4 T cells following 24-h stimulation with plate-bound anti-CD3 mAb. GzmB expression was quantified by mean fluorescence intensity (MFI). Intracellular cytokine expression was analyzed following restimulation with the I-E<sup>d</sup>-restricted HA peptide or PMA plus ionomycin in the presence of Brefeldin A for 5 h, as previously described (30, 35). Intracellular T-bet staining was performed directly ex vivo (using eBioscience catalog 12-4875) following permeabilization with methanol (36). Eomes staining was also performed directly ex vivo (using eBioscience catalog 12-4875), but following permeabilization with eBioscience FixODN staining buffer. CD25 versus anti-PE<sup>-</sup>anti-Stat5 and Foxp3 versus GzmB staining was performed directly ex vivo, as previously described (36).

**Real-time RT-PCR and chromatin immunoprecipitation**

mRNA samples prepared from MACS-purified TCR<sup>+</sup> CD4 T cells were analyzed by quantitative SYBR Green-based real-time RT-PCR, as previously described (24, 35). Briefly, after reverse transcribing cDNA, T-bet, Eomes, perforin 1, and GzmB mRNAs were quantified by first normalizing for input sample amounts by calculating the difference in threshold cycle (ΔC<sub>T</sub>) values relative to hypoxanthine phosphoribosyltransferase (ΔC<sub>T</sub>), and then using the ΔΔC<sub>T</sub> method to calculate the ratio between experimental samples and a representative naive CD4 T cell sample. Primers to T-bet, Eomes, and hypoxanthine phosphoribosyltransferase were previously described (24, 35). Primers to GzmB were forward, 5′-AATGTAACCC-CGGGAGATTGTTCG-3′ and reverse, 5′-CCGAGAAGGAGACCGCTTTGGTCTT-3′. Primers to perforin 1 were forward, 5′-GACCCCTGGAC-ACATTACTGGA-3′ and reverse, 5′-ACATCTTCAAATCCACTCT-3′.

Real-time quantitative SYBR Green PCR-based chromatin immunoprecipitation (ChIP) to measure acetylated histone H3 (AcH3) within the Ifng, Eomes, Prf1, and GzmB promoters was performed, as previously described (24, 37), with the following modifications. DNA immunoprecipitated from fixed and sheered chromatin samples using anti-AcH3 was amplified using primers specific to the various gene promoters. The amount of AcH3 at each promoter was first normalized for input sample amounts by normalizing to the G6PD promoter (ΔC<sub>T</sub>) and then calculating the fold difference (ΔΔC<sub>T</sub>) relative to naïve TCR<sup>+</sup> CD4 T cells. Primers corresponding to Ifng (35) and G6PD (24) were previously described. Primers to Eomes were forward, 5′-ACCATGGTGCCGACATTCTGA-CCC-3′ and reverse, 5′-TATTAAATGTCGCGGTCGTCCTG-3′. Primers to Prf1 were forward, 5′-CAGGGGCAAGAGTAGTGAATGTAGT-3′ and reverse, 5′-CTTCTCCTCCCTTCTACCATGTAAC-3′. Primers to GzmB were forward, 5′-ACTAGATGGTCATGTTGCTGTCG-3′ and reverse, 5′-ATCCTCCGGGAAAGCCGGCCTAGG-3′.

**Degranulation assay**

Spleen samples were cultured for the indicated times with either 100 μg/ml HA peptide or 5 μg/ml soluble anti-CD3 mAb. FITC-conjugated mAbs to both CD107a and CD107b were present throughout the culture. CD107a/CD107b fluorescence was subsequently analyzed on CD4<sup>+</sup>Thy1.1<sup>+</sup> (specific) and both CD107a and CD107b were present throughout the culture. CD107a/CD107b fluorescence was subsequently analyzed on CD4<sup>+</sup>Thy1.1<sup>+</sup> (specific) and thy1.2<sup>-</sup> (bystander) CD4 T cells.

**Lytic assay**

Recipient spleen samples containing 5 × 10<sup>5</sup> TCR<sup>+</sup> CD4 T cells were cultured with 2.5 × 10<sup>5</sup> A20 cells (38) that had been labeled with 500 nM CFSE and pulsed with cognate HA peptide plus 2.5 × 10<sup>5</sup> peptide-pulsed A20 cells labeled with 50 nM CFSE in 96-well plates. Naïve WT splenocytes were added so that the total number of cells/well was 10<sup>6</sup> while maintaining an E:T ratio of 1:1. The proportion of CFSE<sup>-</sup>A20 cells within the live gate (based on forward and side scatter) was measured after 4 h, and the percentage of specific killing was calculated using the following formula: (1 - [% CFSE<sup>-</sup> peptide-CFSE<sup>-</sup> nonpeptide] × 100%).

**CD134 plus CD137 DCo programs cytotoxic CD4 Th1 cell differentiation**

We asked whether DCo through CD134 and CD137 exerts effects on specific CD4 T cells similar to its known effects on CD8<sup>+</sup> CTL. DCo induced HA-specific TCR<sup>+</sup> CD4<sup>+</sup> T cells transferred into HA-expressing (self-HA) recipients to express high amounts of GzmB protein (Fig. 1A, B). Because the serine protease GzmB is a lytic granule component normally expressed in CD8<sup>+</sup> CTL and NK cells (39), this result suggested that DCo programs cytotoxic CD4 Th1 cell differentiation. Consistent with this effect being genetically programmed, DCo induced mRNA encoding not only GzmB, but also the pore-forming component of lytic granules perforin 1 (40) (Fig. 1C). Also consistent with the epigenetic profile of CD8<sup>+</sup> CTL (41), real-time PCR-based ChIP indicated that DCo significantly increased the amount of AcH3 bound to the GzmB and Prf1<sup>+</sup> gene promoters (Fig. 1D). Thus, DCo programmed these genes to undergo epigenetic remodeling toward a transcriptionally permissive configuration. Confirming the fidelity of the ChIP assay, DCo increased ACh3 bound to the Ifng promoter (Fig. 1D), consistent with the enhanced IFN-γ expression (24).

**Mechanism of cytotoxic Th1 differentiation**

Cytokine expression was analyzed following restimulation with the I-E<sup>d</sup>-restricted epitope [(24)] or following 24-h stimulation with plate-bound anti-CD3 mAb. GzmB expression was quantified by mean fluorescence intensity (MFI). Intracellular cytokine expression was analyzed following restimulation with the I-E<sup>d</sup>-restricted HA peptide or PMA plus ionomycin in the presence of Brefeldin A for 5 h, as previously described (30, 35). Intracellular T-bet staining was performed directly ex vivo (using eBioscience catalog 45-5825) following permeabilization with methanol (36). Eomes staining was also performed directly ex vivo (using eBioscience catalog 12-4875), but following permeabilization with eBioscience FixODN staining buffer. CD25 versus anti-PE<sup>-</sup>anti-Stat5 and Foxp3 versus GzmB staining was performed directly ex vivo, as previously described (36).
To test whether DCo-programmed specific CD4 T cells are cytotoxic, we measured degranulation of lytic granules via cell surface localization of CD107a (LAMP1) and CD107b (LAMP2) (42). Surface CD107a/b increased 6-fold on DCo-treated specific CD4 T cells following 2-h stimulation with cognate peptide. In contrast, peptide-stimulated DCo-nontreated counterparts expressed substantially less surface CD107a/b (Fig. 1E).

Secondly, DCo-treated CD4 T cells induced >50% specific lysis of cognate specific CD4 T cells following 2-h stimulation with cognate peptide. In contrast, peptide-stimulated DCo-nontreated counterparts expressed substantially less surface CD107a/b (Fig. 1E).
FIGURE 3. DCo induces GzmB in both specific and bystander CD4 T cells. A, B6 mice were treated with 1 μg SEA ± DCo (naive mice received neither SEA nor DCo). On day 5, SEA-specific (Vβ3+) and nonreactive bystander (Vβ14+) CD4 T cells in spleens were stained for GzmB directly ex vivo. Histograms are representative of three to four replicates per group that were highly comparable. B, IFN-γ staining following in vitro restimulation with PMA plus ionomycin in the samples corresponding to A. C and D, Self-HA mice that received Thy1.1+-specific CD4 T cells were treated with DCo (+ specific), whereas DCo-treated WT mice that were not given specific CD4 T cells served as the control (+ specific). Spleens were analyzed on day 4. C, Intracellular GzmB in Thy1.2+ bystander CD4 T cells directly ex vivo, representative of two to four replicates per group that were highly comparable. D, Surface CD107a/b on bystander CD4 T cells recovered from self-HA DCo-treated specific CD4 T cell adoptive transfer recipients before (0 h) and 5 h following in vitro restimulation with soluble anti-CD3 mAb. Representative histograms (left) and bar graph (right) are from n = 3 per group. E and F, Either 10⁶ or 10³ Thy1.1+-specific CD4 T cells were transferred into WT B10.D2 recipients infected with viral-HA and treated with DCo, and recovered from spleens on day 5. E, Intracellular IFN-γ was measured after restimulation with HA peptide. Left and middle panels, Representative histograms, with a dashed line indicating the cutoff for positive staining determined using an isotype control. Right panel, Graph of the percentage of IFN-γ+–specific CD4 T cells (n = 4–5 per group). F, Ex vivo GzmB expression is presented as in E, except that the data are expressed as MFI (n = 4, 5, 10, and 10³ DCo, 10³ + DCo, 10³ DCo, and 10³ + DCo, respectively).
peptide-pulsed MHC class II* A20 cells at an E:T ratio of only 1:1 (Fig. 1F). Because DCo increases specific CD4 T cell expansion several-fold (24), this assay was performed in vitro so that lytic activities could be compared at the same E:T ratio. Taken together, our data identify costimulation as a process to induce genetic programming of naive CD4 T cells into cytotoxic Th1 cells.

We next assessed whether CD134, CD137, or both costimulatory signals are required to program cytotoxic CD4 Th1 cell differentiation. CD137 costimulation alone significantly enhanced expansion (Fig. 2A) and expression of IFN-γ, TNF (Fig. 2B), and GzmB (Fig. 2C) in specific CD4 T cells, but CD134 alone induced greater expansion and expression of IFN-γ, TNF, and GzmB. DCo did not boost effector molecule expression beyond CD134 alone (Fig. 2B, 2C), but it did boost clonal expansion (Fig. 2A). CD134 is thus sufficient to program cytotoxic Th1 differentiation, whereas addition of CD137 costimulation maximizes clonal expansion of the cytotoxic CD4 Th1 cells.

To test whether DCo programming of cytotoxic CD4 Th1 cells is a natural phenomenon not limited to artificial TCR Tg adoptive transfer models, we analyzed endogenous CD4 T cell responses to the pathogenic bacterial protein SEA. SEA stimulates TCR Vβ3*, but not Vβ14* CD4 T cells (43). After in vivo stimulation with SEA, DCo programmed specific Vβ3* CD4 T cells to express high amounts of GzmB (Fig. 3A, left) and IFN-γ (Fig. 3B, left). Thus, DCo programming of cytotoxic CD4 Th1 cells is a general phenomenon. Strikingly, SEA plus DCo induced SEA-nonreactive Vβ14* bystander CD4 T cells to express large amounts of GzmB (Fig. 3A, right), but lower amounts of IFN-γ (Fig. 3B, right).

In this SEA model, the initial frequency of specific Vβ3* CD4 T cells was ~5% of the overall CD4 T cell repertoire (data not shown). To test whether GzmB is induced in bystander CD4 T cells when fewer Ag-responsive T cells are initially present, we used the TCR Tg transfer system in which the initial frequency is ~0.1% after transfer of 10^6 cells. Bystander CD4 T cells expressed substantially more GzmB in DCo-treated mice that contained specific CD4 T cells than controls (Fig. 3C). Because bystander CD4 T cells have a diverse TCR repertoire, cytotoxic potential was assessed by degranulation. Importantly, anti-CD3 mAb induced surface CD107a/b expression on these bystander CD4 T cells recovered from DCo-treated recipients (Fig. 3D), albeit at lower magnitude and with slower kinetics compared with specific CD4 T cells (data not shown). Thus, although bystander CD4 T cells may be less potent, they do appear to have cytotoxic potential.

HA-specific CD4 T cells transferred into WT mice infected with a recombinant vaccinia virus expressing HA (viral-HA) difer-

![FIGURE 4](http://www.jimmunol.org/)

DCo enhances IL-2 signaling. A. Specific CD4 T cells transferred into self-HA mice treated ±DCo were recovered from spleens on day 4 and directly stained for pSTAT5 versus CD25 and GzmB versus Foxp3. Plots are gated on both Thy1.1+-specific and Thy1.2 + bystander CD4 T cells. The cutoff for positive CD25, GzmB, and Foxp3 staining was based on isotype controls, whereas the cutoff for positive pSTAT5 staining was based on the −DCo groups. B. Time course of CD25, pSTAT5, and GzmB expression on specific and bystander CD4 T cells between 0 and 48 h posttransfer into self-HA mice treated ±DCo. The arrow in the top left graph indicates that DCo was given at 24 h. n = 3 for each 0- and 24-h point and 4 for each 36- and 48-h point. Asterisks indicate p < 0.05 for ±DCo comparisons.
differentiate into conventional Th1 cells that express IFN-γ (Fig. 3E), but not GzmB (Fig. 3F). The addition of DCo programmed these virally-primed specific CD4 T cells to express GzmB as well as higher amounts of IFN-γ. DCo thus enhanced a Th1-inducing virus to induce the cytotoxic Th1 phenotype. Next, the response 10^6 versus 10^5 specific CD4 T cells transferred into DCo-treated viral-HA–infected mice was compared to explore whether DCo can program the cytotoxic Th1 profile when the initial specific CD4 T cell frequency is within a physiological range. Adoptive transfer of 10^6 TCR Tg CD4 T cells seeds recipient secondary lymphoid organs at numbers (~100) approximating individual specificities within the steady state repertoire (44, 45). As expected, 10^5 specific CD4 T cells transferred into viral-HA–infected DCo-treated recipients accumulated to a lesser degree compared with a 10^6 transfer group (6.6 × 10^5 ± 2.5 × 10^5 versus 6.9 × 10^5 ± 8 × 10^5 at day 5, n = 10 and 5 per group, respectively). Perhaps surprisingly, however, in both the 10^5 and 10^6 groups, DCo significantly boosted expression of IFN-γ (Fig. 3E) and GzmB in specific CD4 T cells (p ≤ 0.03) as well as GzmB in bystander CD4 T cells (p ≤ 0.008) (Fig. 3F). Importantly, GzmB MFI values for specific and bystander CD4 T cell treatments did not differ between the 10^5 and 10^6 DCo-treated groups (p ≥ 0.2). Bystander GzmB MFI values also increased in virus-infected DCo-treated mice that did not receive any transferred HA-specific TCR Tg CD4 T cells (data not shown), an effect that was presumably mediated by endogenous vaccinia-specific CD4 T cells.

The cellular mechanism mediating cytotoxic CD4 Th1 differentiation

CD134 costimulation enhances IL-2 production by CD4 T cells (46), and IL-2 induces both GzmB (12, 47, 48) and IFN-γ (49, 50). We thus postulated that the CD134→IL-2 axis might promote cytotoxic CD4 Th1 differentiation. To explore this possibility, expression of CD25 on specific and bystander CD4 T cells treated ± DCo was examined. Four days following self-HA Ag encounter with DCo, CD25 was expressed on 24.6% ± 1.0% (mean ± SEM, n = 3) of the specific CD4 T cells, but only 1.7% ± 0.2 were CD25^+ without DCo. Even at this later time point, some of the DCo-treated CD25^+–specific CD4 T cells contained phosphorylated STAT5 (17.5% ± 6.8) (Fig. 4A, first and second top panels). CD25 and pSTAT5 expression on specific CD4 T cells peaked 24 h after transfer in the absence of DCo, but expression of both IL-2 signaling molecules increased between 24 and 48 h with DCo. Furthermore, GzmB expression was several-fold higher in DCo-treated compared with nontreated specific CD4 T cells at 48 h (Fig. 4B). DCo thus enhances and prolongs IL-2 signaling, an effect that correlates with the acquisition of GzmB expression. DCo-treated specific CD4 T cells did not express Foxp3 at day 4 (Fig. 4A, third and fourth top panels), consistent with studies in which CD134 costimulation blocked inducible Treg differentiation (51, 52). DCo also increased the proportion of bystander CD4 T cells expressing CD25 and Foxp3 by 1.9- and 1.8-fold, respectively (p ≤ 0.001 in both) (Fig. 4A, bottom panels), and induced GzmB not only in Foxp3^− (11.6-fold, p = 0.006), but also in Foxp3^+ (2.6-fold, p = 0.01) bystander CD4 T cells (Fig. 4A, third and fourth bottom panels). Taken together, DCo augments IL-2 signaling followed by induction of GzmB in specific CD4 T cells, and in both Foxp3^+ and Foxp3^− bystander CD4 T cells.

FIGURE 5. IL-2 facilitates DCo-induced GzmB expression in both specific and bystander CD4 T cells. A, IL-2−/− Thy1.1/2 and IL-2^+/− Thy1.1/2 CD44-depleted specific CD4 T cells from native TCR Tg donors were transferred alone or together into DCo-treated self-HA recipients, recovered from spleens on day 4, and directly stained for intracellular GzmB. B, IL-2−/− and IL-2^+/−–specific CD4 T cells from mixed BM chimeric donors were transferred and analyzed, as in A. These mixed BM chimeric donors were generated by rescuing irradiated WT Thy1.2/1.2 hosts with BM from 6.5 TCR Tg donors that were either IL-2−/− Thy1.1/2 or IL-2^+/− Thy1.1/2 plus WT Thy1.2/1.2 BM that supported normal Treg cell function. In both panels, n = 3 per group for the bar graphs showing specific CD4 T cell expansion, and GzmB expression plots are highly representative for each group.

MECHANISM OF CYTOTOXIC Th1 DIFFERENTIATION
GzmB expression was rescued in the IL-2−/-–specific CD4 T cells when they were cotransferred with IL-2+/- counterparts (Fig. 5A, middle panel). This result was confirmed using a second approach that used IL-2−/- and IL-2+/+–specific CD4 T cells that developed in mixed BM chimeric mice that contained functional Treg cells (Fig. 5B, middle and right panels). Interestingly, IL-2−/-–specific CD4 T cells prepared from native donors expanded less than IL-2+/+ counterparts (Fig. 5A, left panel), whereas the opposite pattern was observed with specific CD4 T cells prepared from mixed BM chimeric donors (Fig. 5B, left panel). Thus, although the expansion of specific CD4 T cells following adoptive transfer was influenced by the environment they developed in, this effect did not impact their ability to undergo cytotoxic Th1 differentiation or the necessity for IL-2 in this process.

Eomes programs cytotoxic CD4 Th1 cell differentiation

To genetically dissect how DCo programs cytotoxic CD4 Th1 differentiation, relevant transcription factors were examined. T-bet drives IFN-γ expression in both CD4 and CD8 T cells, but is not essential in CD8 T cells (32). CD8 T cells express another T-box family member, Eomes, which drives IFN-γ, perforin, and GzmB expression (15, 16). We show that DCo increased T-bet mRNA (Fig. 6A) and protein (Fig. 6C) in specific CD4 T cells. Importantly, DCo also increased Eomes mRNA (Fig. 6A), AchH3 bound to the Eomes promoter (Fig. 6D), and Eomes protein (Fig. 6C).

To test whether T-bet programs cytotoxic CD4 Th1 differentiation, the response of Tbx21−/- (T-bet-deficient)–specific (32) CD4 T cells to DCo was analyzed. WT and T-bet−/-–specific CD4 T cells underwent comparably robust expansion in DCo-treated self-HA recipients (data not shown), but T-bet−/-–specific CD4 T cells expressed one-third the amount of IFN-γ and similar amounts of TNF compared with WT (Fig. 7A). In viral-HA–primed conventional Th1 cells, T-bet deficiency completely eliminated IFN-γ expression and partially reduced TNF potential (Fig. 7A). GzmB production was not altered in T-bet−/-–specific CD4 T cells after DCo (Fig. 7B).

To test whether Eomes was responsible for GzmB expression in DCo-treated specific CD4 T cells, WT, T-bet−/-, or conditionally Eomes−/- (33) polyclonal T cells were adoptively transferred into TCR β−/- × δ−/- recipients, and subsequently treated with SEA ± DCo. As expected, DCo significantly induced GzmB expression in WT and T-bet−/-–SEA-specific Vβ3 CD4 T cells, but, importantly, DCo failed to induce GzmB in Eomes−/-–Vβ3 CD4 T cells (Fig. 8A, 8B). Although T-bet−/-– but not Eomes−/-–specific CD4 T cells were programmed by DCo to express GzmB, DCo boosted the ability of both T-bet−/- and Eomes−/-–specific CD4 T cells to express IFN-γ (Fig. 8C). Taken together, DCo-induced T-bet and Eomes can both contribute to enhancing IFN-γ expression in specific CD4 T cells, but GzmB expression depends on Eomes.

DCo programs CD4 T cell-dependent tumoricidal activity

DCo can induce CD8 T cell-dependent tumor immunity (19, 21, 22). We currently used the B16-F10 (B16) murine melanoma to test whether DCo can program CD4 T cells to mediate tumor immunity. B16 can be induced by IFN-γ to express MHC class II in vivo, thus becoming susceptible to lysis by specific cytotoxic CD4 Th1 cells (10, 11). Nonactivated polyclonal CD4 T cells (depleted of CD8+, NK1.1+, and B220+ cells) transferred into RAG−/- recipients were unable to control the growth of B16 melanoma inoculated intradermally 5 d later (Fig. 9A, 9B). In RAG−/- recipients given polyclonal CD4 T cells, DCo administered either the day of or 4 d prior to tumor inoculation substantially limited tumor growth (p < 0.006) (Fig. 9A, 9B). Importantly, CD4 T cells recovered from DCo-treated mice expressed higher levels of IFN-γ (p = 0.01) (Fig. 9C) and GzmB (p = 0.009) (Fig. 9D) compared with nontreated counterparts. DCo showed a trend toward limiting tumor growth in RAG−/- mice that did not receive CD4 T cells (Fig. 9B), most likely resulting from the activity of NK or other innate immune cells that can respond to CD134 and CD137 costimulation (54, 55). Taken together, DCo can program CD4 T cells to become tumoricidal as part of a multipronged antitumor immune response.

Discussion

Cytotoxic CD4 Th1 cells are emerging as a physiologically relevant (6, 7) and therapeutically useful (10, 11) effector T cell lineage. The differentiation process by which these multifunctional effec-

FIGURE 6. DCo induces T-bet and Eomes in specific CD4 T cells. Specific CD4 T cells transferred into self-HA recipients treated ±DCo were recovered from spleens on day 4. A, T-bet and Eomes mRNAs were measured by real-time RT-PCR using the samples shown in Fig. 1C. B, AchH3 bound to the Eomes promoter measured by ChIP using the samples shown in Fig. 1D. C, Intracellular T-bet and Eomes histograms, representative of three replicates per group that were highly comparable.

FIGURE 7. DCo induces GzmB expression in specific CD4 T cells independently of T-bet. T-bet+/+ (WT)– and T-bet−/-–specific CD4 T cells were transferred into DCo-treated self-HA or DCo-nontreated viral-HA–infected recipients and recovered from spleens on day 4. IFN-γ versus TNF expression following restimulation with HA peptide (A) and GzmB directly ex vivo (B). Plots are representative of three to four replicates per group that were highly comparable.
tor CD4 T cells develop can be facilitated by IL-2 in vitro (48) and lymphopenia in vivo (10, 11), but the relevant costimulatory pathways and transcriptional networks have until now been poorly understood. Our current study identifies a specific costimulatory pathway that programs cytotoxic CD4 Th1 cells in vivo. CD134 costimulation is sufficient to program the cytotoxic Th1 profile in Ag-primed CD4 T cells, consistent with the ability of CD134 to induce GzmB in CD8 T cells (56). The addition of CD137 (dual) costimulation maximized clonal expansion of these cytotoxic CD4 Th1 cells, suggesting that the combination would be a potent therapeutic regimen for eliciting tumor cytotoxicity.

CD134 plus CD137 DCo increased CD4 T cell expression of the T-box transcription factor T-bet that drives IFN-γ expression in conventional Th1 cells (25), consistent with the increased ability of these CD4 T cells to express IFN-γ. Nevertheless, dual costimulated T-bet+specific cytotoxic CD4 Th1 cells exhibited only partially impaired IFN-γ expression. T-bet+ CD8 T cells can express moderate amounts of IFN-γ because they express the related T-box factor Eomes that also drives granzyme and perforin expression (15, 16). Importantly, dual costimulated specific cytotoxic CD4 Th1 cells expressed Eomes, and Eomes+ CD4 T cells were unable to be induced by DCo to express GzmB. Thus, Eomes programs cytotoxic function in dual costimulated CD4 T cells. In addition to Eomes, IL-2 was also required for cytotoxic Th1 differentiation. DCo induced specific CD4 T cells to express higher levels of both CD25 and pSTAT5, consistent with IL-2 acting directly on the specific CD4 T cells. That an IL-2–Eomes axis might be critical for cytotoxic CD4 Th1 cell differentiation is consistent with the recent observation that IL-2 induces Eomes in CD8 T cells (14).

Regardless of whether CD134 and/or CD137 costimulation programs cytotoxic CD4 Th1 cell function under physiological conditions such as during viral infection (6, 7), our current finding is clinically relevant. Thus, CD134 and CD137 costimulatory agonists are being developed separately as cancer therapeutics (26, 27), and, in the case of MHC class II+ tumors such as B cell lymphomas (57, 58) and melanomas (8), the ability of tumor-reactive CD4 T cells to develop cytotoxic function would allow them to directly kill tumor cells. This would expand their role in tumor immunity beyond their traditionally assumed role as helpers that enhance the function of CD8 T cells and other immune cells (59–61). Indeed, DCo effectively programmed purified polyclonal CD4 T cells to control the growth of B16 melanoma in RAG−/− mice.

**FIGURE 8.** DCo induces GzmB expression in specific CD4 T cells via Eomes. Lethally irradiated B6 RAG1−/− mice were reconstituted with B6 BM that was WT, T-bet−/−, or conditionally Eomes−/−. Lymphocytes from reconstituted chimeras containing 1 × 10⁹ CD4 T cells were then adoptively transferred into B6 TCR β−/− × δ−/− recipients that were treated the following day with SEA and DCo (as in Fig. 3A, 3B). Intracellular GzmB was measured directly ex vivo in SEA-specific Vβ3+ CD4 T cells recovered from spleens 5 d following SEA and DCo treatment. A, Representative GzmB histograms. B, Bar graph corresponding to A (n = 4 per group); Asterisk indicates that DCo significantly induced GzmB (p ≤ 0.05) over nontreated. C, DCo-treated and nontreated SEA-specific Vβ3+ T-bet−/− and Eomes−/− CD4 T cells (generated as in A and B) were compared for their abilities to express GzmB directly ex vivo and IFN-γ following restimulation with PMA plus ionomycin. Plots are representative of two to three replicates per group that were highly comparable.

**FIGURE 9.** DCo programs CD4 T cell-dependent tumoricidal function. RAG1−/− mice were intradermally inoculated with 1 × 10⁵ B16 melanoma cells and tumor size measured 13 d later. As indicated, some mice received 1 × 10⁶ polyclonal CD4 T cells (depleted of CD8+, NK1.1+, and B220+ cells) 5 d prior to tumor inoculation and/or DCo the day of (day 0) or 4 d prior to tumor inoculation (day −4). Asterisks indicate p < 0.05 compared with the control group that received neither CD4 T cells nor DCo (open bar). A, n = 4 for each group. B, n = 8 or 7 for the control and all other groups, respectively. C and D, CD4 T cells were recovered from the spleens of mice corresponding to B on day 14, and intracellular IFN-γ was measured following 5 h in vitro stimulation with PMA plus ionomycin (C), and GzmB was measured following 24-h stimulation with anti-CD3 mAb (D).
mice. Consistent with the ability of innate immune cells to respond to CD134 and CD137 costimulation (54, 62–64), DCo elicited moderate control of tumor growth in RAG−/− mice that did not receive T cells (albeit this effect was not statistically significant). Nevertheless, the ability of DCo to engage multiple arms of the immune response (CD8+ CTL, NK cells, and cytotoxic CD4 Th1 cells) that can target distinct tumor determinants should be therapeutically beneficial.

In response to CD134 plus CD137 DCo, Ag-specific cytotoxic CD4 Th1 cells imprint Ag-inexperienced bystander CD4 T cells with similar functional capabilities. Ag-stimulated CD4 T cells can augment proliferation in other Ag-stimulated CD4 T cells (65), but our current result is novel because Ag-stimulated CD4 T cells programmed bystander CD4 T cells to express an effector molecule typically expressed in CD8+ T cells and NK cells. The expression of GzmB in these bystander CD4 T cells is dependent on paracrine IL-2 secreted by Ag-specific CD4 T cells, although other bystander-programming factors may also be involved. An interesting facet of the bystander response is that GzmB is not only induced in conventional bystander CD4 T cells, but also in FoxP3+ Treg cells that express high-affinity IL-2R. Treg cells can exert suppressive function through GzmB (66, 67), which raises the possibility that one function of the bystander response might be to limit collateral damage inflicted by the robust Ag-specific cytotoxic CD4 Th1 cell response.

The DCo-induced bystander response may have a complex effect during tumor immunotherapy. For instance, polyclonal granzyme-expressing bystander T cells may eliminate residual Ag-loss variant tumor cells that lack expression of vaccine-targeted epitopes (68–72), but their polyclonal nature raises the potential for autoimmune toxicity. In this regard, it was previously noted that careful titration of each costimulatory agonist significantly lowered the effective dose of the other required to elicit optimal specific CD8+ T cell responses. This strategy would be of interest in the development of tumor-specific CD4+ T cells differentiated in vivo eradicating established melanoma. J. Exp. Med. 207: 651–667.


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NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. Cell. Immunol. 190: 167–172.


