Repression in CD4 T Cells

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An Immunotherapeutic CD137 Agonist Releases Eomesodermin from ThPOK Repression in CD4 T Cells

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Agonists to the TNF/TNFR costimulatory receptors CD134 (OX40) and CD137 (4-1BB) elicit antitumor immunity. Dual costimulation with anti-CD134 plus anti-CD137 is particularly potent because it programs cytotoxic potential in CD8⁺ and CD4⁺ T cells. Cytotoxicity in dual-costimulated CD4 T cells depends on the T-box transcription factor eomesodermin (Eomes), which we report is induced via a mechanism that does not rely on IL-2, in contrast to CD8⁺ CTL, but rather depends on the CD8 T cell lineage agonist, administered individually, CD137 agonist failed to induce CD134

expression; however, the CD4 T cell lineage commitment factor ThPOK represses transcription of Eomes and other CD8 lineage genes, such as Cd8α. Hence, CD4 T cells can differentiate into Eomes⁺ cytotoxic CD4⁺CD8⁺ double-positive T cells by terminating ThPOK expression. In contrast, dual-costimulated CD4 T cells express Eomes, despite the continued expression of ThPOK and the absence of CD8α, indicating that Eomes is selectively released from ThPOK repression. Finally, although Eomes was induced by CD137 agonist, but not CD134 agonist, administered individually, CD137 agonist failed to induce CD134⁻/⁻ CD4 T cells to express Eomes or Runx3, indicating that both costimulatory pathways are required for cytotoxic ThI programming, even when only CD137 is intentionally engaged with a therapeutic agonist. The Journal of Immunology, 2018, 200: 000–000.
expression in dual-stimulated CD4 T cells (9, 23), and IL-2 induces Eomes in CD8+ CTLs (24). To the contrary, dual costimulation–mediated induction of Eomes did not require IL-2; rather, it depended on the transcription factor Runx3, which directs CD8 T cell lineage commitment during thymic development (25) and subsequently supports Eomes expression in mature CD8+ CTLs (26). Further, both Eomes and Runx3 were indispensable for dual-stimulated CD4 T cells to mediate antitumor activity in an aggressive melanoma model. Runx3 is also expressed in standard CD4 Th1 cells where it promotes IFN-γ expression (27, 28); however, the CD4 T cell lineage transcription factor ThPOK represses Runx3-mediated induction of Eomes, GzMB, and other CD8 T cell lineage markers, such as CD8α (29, 30). Specifically, ThPOK directly represses Eomes transcription by binding sites within the Eomes promoter (31). Hence, a mechanism by which CD4 T cells differentiate into Eomes+ cytotoxic CD4+CD8+ double-positive T cells during normal homeostasis in the intraepithelial compartment is through termination of ThPOK expression (32). In contrast, dual-stimulated CD4 T cells expressed Eomes, despite the continued expression of ThPOK and the absence of CD8α, indicating that Eomes is selectively released from ThPOK repression. Finally, although Eomes was induced by CD137 agonist, but not CD134 agonist, administered individually, CD137 agonist failed to induce CD134+/− CD4 T cells to express Eomes or Runx3. Thus, both costimulatory pathways are required for cytotoxic Th1 programming, even when only one is intentionally engaged with a therapeutic agonist.

Taken together, our current results indicate that CD137 costimulation, which is enabled by CD134, programs CD4 T cells to express Eomes through a novel mechanism that selectively releases Eomes from ThPOK repression.

Materials and Methods

**Mice, adoptive transfers, immunizations, and costimulatory agonist treatment**

CD4 T cells from 6.5 TCR-transgenic (Tg) mice recognize an I-Eβ-restricted epitope derived from influenza (PR8 strain) hemagglutinin (HA) (13). Serum-free recombinant vaccinia virus expressing HA (v-HA) 1 day previously served as recipients of the adoptively transferred 6.5 CD4 T cells. TEa TCR-Tg CD4 T cells specific to an I-Ab–restricted epitope derived from influenza (PR8 strain) hemagglutinin (HA) (13) were used in all experiments. Results were confirmed in multiple experiments, and representative figures are shown. For all experiments, data are presented as mean ± SD.

**Flow cytometry**

Tumor immunotherapy experiments

B16-F10 (B16) melanoma cells (1×10⁶) were injected intradermally into RAG1−/− mice that received 1×10⁶ polyclonal CD4 T cells (depleted of CD8+ and NK1.1+ cells via magnetic beads) that were WT or Eomes−/− (22) [from BM chimeras, as previously described (9)] or were WT or Runx3−/− (from native mice) 4 d earlier and treated with control IgG or dual costimulation or were injected intradermally into B6 mice that subsequently received WT, CD134−/−, or Runx3−/− TEa CD4 T cells and were treated with control rat IgG or dual costimulation. Mice were measured using calipers, and perpendicular diameters were multiplied to calculate surface areas in square millimeters. Tumor growth was quantified using area under the curve (AUC) analysis (40), as previously described (11, 41). All mouse protocols were approved by the UConn Health Institutional Animal Care and Use Committee.

**qRT-PCR analysis**

RNA was first prepared from FACS-sorted T cells using an RNeasy Mini Kit (Qiagen), which was then reverse transcribed using an iScript Reverse Transcription Supermix for qRT-PCR Kit and analyzed by qRT-PCR using SsoAdvanced Universal SYBR Green Supermix on a CFX96 Real-Time System C1000 Touch Thermal Cycler (all from Bio-Rad). Data were normalized to β-actin. The following primers were used: Eomes forward, 5′-TTCTACAGCGGTGAGAGCCGCTGACA-3′ and reverse, 5′-CAATGCATCTGAGGTGCTGC-3′ (39); GzMB forward, 5′-AATGTGAAGCCAGGAGATGTGTGC-3′ and reverse, 5′-GCCAGGGAAGACGGTGTTGAG-3′; CD8α forward, 5′-GCCGGTGCACTACCATGGAA-3′ and reverse, 5′-ACATCTC-CAAAGTCATCTT-3′ (9); ThPOK (Zbtb7b) forward, 5′-CCCGAGGATGACCTGTATT-3′ and reverse, 5′-GGTGCCTGCTATGGT-3′; Il2ra forward, 5′-GGCTTGTCCCTGTTGACTGGA-3′ and reverse, 5′-GCGGTCTCAAGAGTGGAAA-3′; and β-actin forward, 5′-AAGGCACAAGGGTTACAG-3′ and reverse, 5′-GGTGGTTGAAGAAGGTAAC-3′ (41).

**Statistical analyses**

The p values were calculated using an unpaired two-tailed t test. Unless otherwise noted, each figure contains data collected from a single experiment.

**Results**

The role of Eomes in CD4 T cell–dependent tumor immunotherapy

We previously found that dual costimulation with agonists to CD134 plus CD137 induces the differentiation of Eomes+ cytotoxic Th1 CD4 T cells that are capable of reducing the growth of B16 melanoma independently of CD8 T cells (9). Other groups have similarly found that Eomes+ cytotoxic CD4 T cells can be elicited with CD134 agonist plus cyclophosphamide (10) or CD137 agonist plus Flt3 ligand (42) and that Eomes is necessary for CD4 T cell–dependent B16 tumor control (10, 42). Supporting...
a similar requirement for Eomes in CD4 T cell–mediated tumor control during CD134 CD137 dual costimulation immunotherapy, dual costimulation elicited only a slight (statistically nonsignificant) reduction in tumor growth compared with treatment with control rat IgG in B16 tumor–bearing RAG1−/− recipients that received adoptively transferred Eomes−/− polyclonal CD4 T cells (Supplemental Fig. 1A, 1B). This minimal effect was similar to our previous results using dual-costimulated RAG1−/− recipients that had not received any T cells (9), and it may be mediated by recipient-derived Eomes+ CD4+ polyclonal CD4 T cells (Supplemental Fig. 1D) that have been shown to be activated by CD137 agonist (16). In contrast, and consistent with our previous study (9), WT CD4 T cells significantly reduced B16 tumor growth in dual-costimulated recipients compared with rat IgG–treated RAG1−/− recipients (p = 0.04) (Supplemental Fig. 1A, 1B). This dual costimulation–mediated tumor control was associated with greater splenic accumulation of CD4 T cells (Supplemental Fig. 1C), of which ∼20% were Eomes+ (Supplemental Fig. 1D, 1E).

IL-2 is not required for dual costimulation–mediated induction of Eomes

Given the role of Eomes in programming CD4 T cell tumoricidal function, we next sought to understand how its expression is induced by dual costimulation. We initially considered a role for IL-2 because, along with Eomes, is required for dual-costimulated CD4 T cells to express GzmB (9), and it can induce Eomes in CD8 T cells (24); also, dual costimulation boosts IL-2 and CD25 expression and STAT5 phosphorylation in CD4 T cells (9, 23).

To evaluate the role of IL-2 in dual costimulation–mediated Eomes induction, we first used our previous model in which TCR-Tg HA-specific CD4 T cells adoptively transferred into recipient mice that express HA as a self-antigen (35) are induced to express Eomes upon administration of dual costimulation (9) to compare the responses of WT and CD25−/− (lacking the high-affinity IL-2R) specific CD4 T cells (Fig. 1). Because IL-2 is required for Foxp3+ regulatory T cell (Treg) homeostasis (43), specific CD4 T cells (Thy1.1+) were generated in mixed BM chimeras containing congenically marked WT BM (Thy1.2−IL-2−/−) that supports Tregs and, thus, prevents premature activation of specific CD4 T cells prior to adoptive transfer (9). Consistent with our previous study (9), dual costimulation induced WT specific CD4 T cells to express CD25 and Eomes (p < 0.01, compared with control rat IgG [−DcoI] (Fig. 1A, 1B). Notably, dual costimulation also induced CD25−/− specific CD4 T cells to express Eomes (p < 0.0001) (Fig. 1A, 1B). To confirm this initial observation that IL-2 signaling may not be critical for Eomes induction, we performed a second more comprehensive experiment in which the response to dual costimulation was compared among adoptively transferred WT, IL-2−/−, and CD25−/− specific CD4 T cells in recipient mice that had been infected with viral-HA. Consistent with the results from the self-HA model (Fig. 1A, 1B) (9), in viral-HA–infected recipients, dual costimulation induced WT specific CD4 T cells to express Eomes (p < 0.01), as well as GzmB (p < 0.01) (Fig. 1C–E). Also, as predicted from our previous study (9), dual-costimulated IL-2−/− specific CD4 T cells expressed 3-fold lower GzmB levels compared with their WT counterparts (as measured by mean fluorescence intensity [MFI], p = 0.02), although expression was elevated compared with control rat IgG–treated IL-2−/− specific CD4 T cells (p = 0.03) (Fig. 1C, 1D). This modest dual costimulation–mediated induction of GzmB in IL-2−/− specific CD4 T cells may have been due to their modest expression of CD25 (Fig. 1F, 1G) that likely binds paracrine IL-2 produced by recipient-derived IL-2+ vaccinia-specific T cells (44). Indeed, dual costimulation induced negligible GzmB in CD25−/− specific CD4 T cells (Fig. 1C, 1D). Importantly, although dual-costimulated IL-2−/− specific CD4 T cells expressed substantially reduced GzmB compared with WT (Fig. 1C, 1D), they exhibited only a slight (1.4-fold) statistically nonsignificant trend toward decreased Eomes expression (Fig. 1C, 1E). Further, dual costimulation also induced CD25−/− specific CD4 T cells to express Eomes (p = 0.001) at levels similar to their WT (p = 0.08) and IL-2−/− (p = 0.5) counterparts (Fig. 1C, 1E). Taken together, although IL-2 signaling is required for dual costimulation to induce GzmB, Eomes induction can occur independently of IL-2.

Induction of Eomes in dual-costimulated CD4 T cells depends on Runx3

We next investigated whether dual costimulation induces Eomes in CD4 T cells by altering expression of the T cell subset lineage-commitment transcription factors Runx3 and ThPOK (45). Thus, Runx3 directs thymic development of single-positive CD8 T cells (25), and fosters CTL differentiation in mature CD8 T cells, in part by facilitating expression of Eomes (26). Conversely, ThPOK directs development of CD4+CD8+ thymocytes (46, 47) and normally represses CD8 (30), Runx3 (31), and GzmB (48). Hence, CD4+Foxp3+ intestinal intraepithelial lymphocytes (IELs) express Eomes and gain cytotoxic potential as a consequence of terminated ThPOK expression (32, 49). Therefore, we considered the possibility that dual-costimulated CD4 T cells downregulate ThPOK, hence relieving repression of Runx3 and Eomes.

To test this possibility, we used another well-characterized dual-costimulation model in which naive ThPOK+ Runx3+/− Thy1.1+ TCR-Tg TEa CD4 T cells (36) are programmed to differentiate into Eomes+GzmB+ cytotoxic Th1 cells following adoptive transfer into congenic Thy1.2+ B6 recipients and priming with the cognate MHC class II–restricted Eε peptide plus dual costimulation (11). These Eα–specific dual-costimulated CD4 T cells also facilitate GzmB expression and tumoricidal activity in bystander polyclonal CD8 T cells (that are not specific to Eα). This bystander effect depends on IL-2 produced by dual-costimulated specific CD4 T cells (9) and, thus, could potentially be mediated, in part, through IL-2 induction of CD137 on effector/memory CD8 T cells (50).

Importantly, during dual-costimulation immunotherapy, this bystander effect, also referred to as “Ag-nonlinked help,” can boost CD8+ T cell–mediated antitumor activity (11) in lieu of tumor-infiltrating lymphocytes. Notably, ThPOK expression can become CD8α+ (32) due to the loss of ThPOK repression of the Cd8a gene (30), dual-costimulated specific TEa CD4 T cells remained CD8α− (Fig. 2A). This suggested that dual costimulation does not terminate ThPOK expression; indeed, intracellular flow cytometry confirmed that dual-costimulated specific CD4 T cells maintained ThPOK (Fig. 2B). Nevertheless, ∼35% of the dual-costimulated CD4 T cells expressed Runx3 (Figs. 2B–D, 3F, by–

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stander host–derived CD8 T cells that receive nonlinked help are shown as a positive staining control for CD8α, Runx3, and Eomes and a negative control for ThPOK). Importantly, dual costimulation also programmed ∼15% of specific CD4 T cells to express Eomes (Fig. 2C, 2E, 2G), of which ∼75% coexpressed Runx3 (Fig. 2C, 2I). In contrast, only ∼40% of GzmB+ specific CD4 T cells coexpressed Runx3 (although the Runx3+ fraction had a slightly higher GzmB staining intensity) (Fig. 2D, 2I), and only ∼20% of GzmB+ specific CD4 T cells coexpressed Eomes (Fig. 2E, 2J). Thus, the expression of Eomes and Runx3 is closely associated in dual-costimulated CD4 T cells.

To test whether Runx3 is required for Eomes expression, we next compared the response of adoptively transferred TEa CD4 T cells
that were Runx3−/− (Cd4-Cre × Runx3fl/fl) with their control Cre+ littermates (referred to as WT). Notably, the percentage of dual-costimulated Runx3−/− TEa CD4 T cells that expressed Eomes was reduced ~3-fold compared with WT (p < 0.01) (Fig. 3A, 3B). The level of Eomes expression (MFI) in Runx3−/− Eomes+ specific CD4 T cells was also lower than WT (p < 0.01) (Fig. 3A, 3C). Thus, Runx3 is required for robust Eomes expression. Runx3−/− dual-costimulated specific CD4 T cells were also less functional. Specifically, they exhibited reduced phosphorylation of the mTOR downstream target S6 (pS6, p < 0.01)
and also expressed reduced levels of IFN-γ following in vitro restimulation (p, 0.05, Fig. 3F). Finally, they were unable to help bystander CD8 T cells express Eomes (Fig. 3G) or GzmB (Fig. 3H).

Runx3 is required for CD4 T cell–dependent dual costimulation–mediated tumor immunotherapy

Dual-costimulated CD4 T cells can facilitate tumor immunotherapy through multiple mechanisms. First, when primed to tumor-nonspecific Ag, they can provide Ag-nonlinked help to CD8 T cells in TDLNs, as well as intratumorally (11). That Runx3 might be necessary for this therapeutic nonlinked help was suggested by the inability of Runx3−/− TEa CD4 T cells to foster Eomes and GzmB expression in bystander CD8 T cells (Fig. 3G, 3H). To directly test this possibility, the therapeutic helper potentials of WT versus Runx3−/− TEa CD4 T cells were compared in B6 mice harboring established B16 tumors that do not express the Eα Ag recognized by TEa CD4 T cells. In contrast to mice that received WT TEa CD4 T cells in which dual costimulation significantly controlled tumor burden (p < 0.05), dual costimulation elicited minimal and statistically nonsignificant tumor control in mice given Runx3−/− TEa CD4 T cells (Fig. 4A, 4B), similar to the effect previously observed in B6 mice not given TEa CD4 Th cells (11). That the inability of Runx3−/− TEa CD4 T cells to significantly augment tumor control was a consequence of their insufficient capacity to deliver nonlinked help within tumors was suggested by their reduced intratumoral accumulation (Fig. 4C) and corresponding reduction in intratumoral CD8 T cells (Fig. 4D) compared with tumors controlled by WT TEa CD4 T cells. Additionally, although WT dual-costimulated TEa CD4 T cells reduced the frequency of intratumoral Foxp3+ Tregs (Fig. 4E) (11), Runx3−/− counterparts were less able to do so (p < 0.05, Fig. 4E). One potential mechanism by which the dual-costimulated CD4 T cells might facilitate CTL-mediated tumor

**FIGURE 2.** Dual costimulation induces Runx3 in CD4 T cells. Thy1.1+ TEa TCR-Tg (specific) CD4 T cells were adoptively transferred into congenic Thy1.2+ B6 recipients immunized with cognate Eα peptide and control rat IgG (−DCo) or dual costimulation (+DCo) (n = 3 or 4 per group). Spleens were analyzed 5 d posttransfer. (A) Representative FACS of surface CD4 versus CD8α on specific CD4 (Thy1.1+) and nonspecific (Thy1.1neg) T cells. Representative ex vivo intracellular FACS of specific CD4 T cells and nonspecific CD8 T cells showing Runx3 versus ThPOK (B), Runx3 versus Eomes (C), Runx3 versus GzmB (D), and Eomes versus GzmB (E). Scatter plots of the percentage of specific CD4 T cells expressing Runx3 (F), T cells expressing Eomes (G) and GzmB (H), the percentage of Eomes+ and GzmB+ specific dual-costimulated CD4 T cells that also express Runx3 (I), and the percentage of dual-costimulated GzmB+ T cells that also express Eomes (J). **p < 0.01, ***p < 0.001, ****p < 0.0001, costimulatory agonist treatment group versus rat IgG control.

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cell killing is through IFN-γ-induced upregulation of MHC class I and II on tumor cells (8, 41), and the Runx3<sup>−/−</sup> TEa CD4 T cells might be less capable of eliciting this effect given their reduced capacity to express IFN-γ (Fig. 3F). Indeed, although WT TEa CD4 T cells enabled dual costimulation to upregulate MHC class I (p < 0.01, Fig. 4F) and MHC class II (p < 0.01, Fig. 4G) on B16 tumor cells, this effect was diminished in mice given Runx3<sup>−/−</sup> TEa CD4 T cells (p < 0.05 for class I and II). Lastly, the reduced functionality of dual-costimulated Runx3<sup>−/−</sup> TEa CD4 T cells observed earlier in spleen (Fig. 3) was confirmed in B16 TDLNs, in which there was reduced accumulation of total cells (p < 0.01, Fig. 4H) and TEa specific CD4 T cells (p < 0.05, Fig. 4I, 4J), along with reduced expression of Eomes in specific CD8 T cells. *p < 0.05, **p < 0.01, costimulatory agonist treatment group versus rat IgG control. #p < 0.05, ##p < 0.01, gene-targeted treatment group versus WT.

**FIGURE 3.** Runx3 supports Eomes expression in dual-costimulated CD4 T cells. Conditional Runx3<sup>−/−</sup> (Cd4-Cre × Runx3<sup>flox/flox</sup>) or Cre<sup>neg</sup> (WT) Thy1.1<sup>+</sup> TEa CD4 T cells were adoptively transferred into congenic Thy1.2<sup>+</sup> B6 recipients immunized with Ea peptide and rat IgG (−DCo) or dual costimulation (+DCo) (n = 3–5 per group in this experiment, which was representative of two separate trials). Spleens were analyzed on day 5 posttransfer. (A) Representative ex vivo intracellular staining of Runx3 versus Eomes in Thy1.1<sup>+</sup> specific CD4 T cells. Scatter plots of the percentage of Eomes<sup>+</sup> specific CD4 T cells (B) and level of Eomes expression (MFI) (C). Representative line graph overlays of specific CD4 T cell ex vivo staining of phosphorylated S6 (pS6) (D) and scatter plot of the percentage of specific CD4 T cells that were pS6<sup>+</sup> (based on an isotype staining control; data not shown) (E). Scatter plots of intracellular IFN-γ in specific CD4 T cells following in vitro restimulation (F) and ex vivo staining of Eomes (G) and GzmB (H) in host-derived (helped) CD8 T cells. *p < 0.05, **p < 0.01, costimulatory agonist treatment group versus rat IgG control. #p < 0.05, ##p < 0.01, gene-targeted treatment group versus WT.
data reveal a dual costimulation-mediated CD4 T cell–intrinsic role for Runx3 in enabling the delivery of antitumor therapeutic help.

To assess the ability of Runx3 to facilitate CD8-independent dual costimulation–elicited CD4 T cell tumoricidal function, we modified the B16 RAG1$^{-/-}$ model (9) used earlier to examine Eomes$^{-/-}$ CD4 T cells (Supplemental Fig. 1) to compare the potential of adoptively transferred WT and Runx3$^{-/-}$ polyclonal CD4 T cells to control tumor growth. Consistent with Supplemental Fig. 1 and (9), dual-costimulated RAG1$^{-/-}$ recipients

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**FIGURE 4.** Runx3 is required for immunotherapeutic CD4$^{+}$ help. WT mice with day-3 established B16 melanomas received WT or Runx3$^{-/-}$ TEa CD4 T cells and were treated with Ex peptide and rat IgG (−DCo) or dual costimulation (+DCo) ($n = 7$ or 8 per group). Analysis of TDLNs and intratumoral T cells (TILs) was performed on day 12. (A) Tumor growth curves for individual mice. (B) AUC analysis (tumor burden) corresponding to (A). (C) Percentage of TILs that are specific CD4 T cells. (D) Percentage of CD8$^{+}$ TILs. (E) Percentage of Foxp3$^{+}$/CD4$^{+}$ TILs. Percentage of B16 tumor cells that express MHC-I (F) and MHC-II (G). TDLN analyses: total cell numbers (H), percentage of lymphocytes that are specific CD4 T cells (I), total number of specific CD4 T cells (J), percentage of specific CD4 T cells that express Eomes (K), and percentage of CD8 T cells that are Eomes$^{+}$/GzmB$^{+}$ (L). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, costimulatory agonist treatment group versus IgG control. #$p < 0.05$, $$p < 0.01$, $$$p < 0.001$, gene-targeted treatment group versus WT.
receiving WT polyclonal CD4 T cells exhibited significantly reduced tumor growth compared with IgG-treated controls (p < 0.0001, Fig. 5A, 5B). In contrast, dual costimulation elicited only a slight and statistically nonsignificant reduction in tumor growth in RAG1−/− recipients that received Runx3−/− CD4 T cells (Fig. 5A, 5B), which was associated with decreased numbers of dual-costimulated Runx3−/− compared with WT CD4 T cells accumulating in TDLNs (p < 0.05, Fig. 5C) and intratumorally (p < 0.05, Fig. 5D). Additionally, Runx3−/− CD4 T cells failed to reduce the frequencies of intratumoral Foxp3+ Tregs in response to dual costimulation, in contrast to WT CD4 T cells, which markedly reduced intratumoral Tregs (p < 0.01, Fig. 5E). Interestingly, dual costimulation did induce Eomes in Runx3−/− CD4 T cells within TDLNs (p < 0.05, Fig. 5F) and GzmB expression within tumors (p < 0.05, Fig. 5G), suggesting that Eomes can be induced independently of Runx3 when CD4 T cells are dual costimulated under lymphopenic conditions. Nevertheless, Eomes (Supplemental Fig. 1) and Runx3 (Fig. 5) are required for tumor control under these conditions.

CD134 and CD137 are required to induce Eomes and Runx3

Given that dual costimulation–mediated induction of Eomes and Runx3 in CD4 T cells is critical for tumor immunotherapy (Figs. 4, 5, Supplemental Fig. 1), we asked whether these two factors are induced through the combined actions of CD134 and CD137 costimulation or, alternatively, via a single pathway.

**FIGURE 5.** Runx3 is required for CD8 T cell–independent CD4 T cell–dependent antitumor immunotherapy. RAG1−/− mice received WT or Runx3−/− CD8-depleted splenocytes that contained 1 × 10^6 CD4 T cells and 4 d later were injected intradermally with 1 × 10^5 B16 tumor cells and treated with rat IgG (−DCo) or dual costimulation (+DCo) (n = 6 or 7 per group). (A) Tumor growth curves for individual mice. (B) AUC analysis (tumor burden) corresponding to (A). (C) Total number of CD4 T cells in TDLNs. (D) % of CD4 T cells within TILs. (E) % of CD4+ TILs that are Foxp3+. (F) % of CD4 T cells in TDLNs that express Eomes. (G) GzmB expression (MFI) in CD4+ TILs. *p < 0.05, **p < 0.01, ****p < 0.0001, costimulatory agonist treatment group versus rat IgG control. #p < 0.05, ###p < 0.001, gene-targeted treatment group versus WT.
Consistent with a previous study (42), CD137 agonist, but not CD134 agonist, induced Eomes in Ea peptide–specific T Ea CD4 T cells (Fig. 6A). In contrast, individual administration of either agonist induced Runx3 (Fig. 6B).

These results suggested that, although Eomes can only be induced through CD137 costimulation, Runx3 is induced through CD134 or CD137. However, it could not be ruled out that both pathways operate to induce these transcription factors. For instance, although CD137 agonist can act directly on Ag-stimulated T cells (53), dendritic cells also express CD137 (54) and, thus, might be induced by CD137 agonist to express CD134 ligand, which engages CD134 on CD4 T cells. In fact, we previously described a reciprocal scenario in which genetic ablation of CD137 prevents CD134 agonist from augmenting CD8 T cell effector responsiveness (13). Because CD137 agonist alone induces Eomes and Runx3, in a second experiment its effect was compared in WT and CD134−/− T Ea specific CD4 T cells transferred into WT (CD134+/+) recipients. Importantly, although WT T Ea CD4 T cells can potentially receive agonist-induced CD137 costimulation, as well as CD134 costimulation, from
activated CD134L+ dendritic cells, CD134+/− Tera CD4 T cells would only be able to receive CD137 costimulation. Thus, an inability of the CD137 agonist to induce Runx3 or Eomes in CD134+/− Tera CD4 T cells would reveal that both costimulatory pathways must be engaged, whereas unaffected expression would indicate that CD137 costimulation acts independently of CD134. Confirming the initial experiment (Fig. 6A, 6B), WT Tera CD4 T cells were induced by CD134 and CD137 agonists given individually, as well as together, to express Runx3 (Fig. 6E) and by CD137 agonist and dual costimulation to express Eomes (Fig. 6D). Also as expected, Runx3 was not induced in CD134−/− Tera CD4 T cells by CD134 agonist (Fig. 6E). Strikingly, CD137 agonist also failed to induce CD134−/− Tera CD4 T cells to express Runx3, as well as Eomes (Fig. 6D, 6E). Interestingly, dual costimulation programmed CD134−/− Tera CD4 T cells to express Runx3 in two of four mice tested, although Eomes was absent in all four mice (Fig. 6D, 6E). This variable response might be explained by the ability of CD134 and CD137 agonists to alter the function of WT host-derived Tregs (55, 56). Nevertheless, CD134 needed to be expressed on CD4 T cells for CD137 agonist to induce Eomes, suggesting that both costimulatory pathways are essential, even when only CD137 is intentionally engaged with a therapeutic agonist.

To confirm and extend this unanticipated result, in a third experiment, the response of CD134−/− Tera specific CD4 T cells to CD137 agonist was compared with WT Tera CD4 T cells in WT (CD134+/+) recipients that received individual transfers of WT or CD134−/− Tera CD4 T cells or CD134−/− Tera CD4 T cells cotransferred with WT Tera CD4 T cells (Fig. 6G–I). As in the previous two experiments, CD137 agonist induced WT Tera CD4 T cells to express Eomes (p < 0.01, Fig. 6G) and Runx3 (p < 0.01, Fig. 6H), but it failed to induce Runx3 and Eomes in CD134−/− Tera CD4 T cells that had been individually transferred into WT (CD134+/+) recipients (Fig. 6G, 6H). Further, cotransferred WT Tera CD4 T cells did not rescue the ability of CD134−/− Tera CD4 T cells to express Runx3 and Eomes in response to CD137 agonist (fourth column in Fig. 6G, 6H), indicating that CD137 plays a CD4 T cell–intrinsic role in enabling CD137 to engage the Runx3–Eomes axis. This result could not be explained by a simple requirement for CD134 on CD4 T cells to sustain expansion/survival, because the total number of CD134−/− Tera CD4 T cells that accumulated in spleens was actually several-fold greater than WT Tera CD4 T cells in both experiments (Fig. 6F, 6I). Another possibility was that CD134 on CD4 T cells is necessary to support TCR-induced expression of CD137. Because CD137 expression is transient, we compared its induction on WT versus CD134−/− Tera CD4 T cells stimulated in vitro with cognate Eα peptide. As expected (53, 57), WT Tera CD4 T cells were induced to express CD137 by 24 h (Fig. 7A), and CD134 expression became evident at 48 h (Fig. 7B). Importantly, CD134−/− Tera CD4 T cells were similarly induced to express CD137 by 24 h (Fig. 7A). Further, these CD137 expression patterns were comparable when WT and CD134−/− Tera CD4 T cells were cultured separately (Fig. 7A, 7B, upper panels), as well as when they were cultured together (Fig. 7A, 7B, lower panels). Additionally, WT and CD134−/− Tera CD4 T cells exhibited a similar induction of CD25 expression (peaking at 48 h) (Fig. 7C), which, considered along with the robust in vivo expansion of CD134−/− Tera CD4 T cells treated with CD137 agonist (Fig. 6F, 6I), suggested that CD134 deficiency did not globally impact CD4 T cell activation.

Finally, the ability of cognate Eα peptide–stimulated CD134−/− Tera CD4 T cells to express CD137 comparably to WT CD4 T cells was confirmed in a second independent trial (Fig. 7D). Together, these data point toward a more focused CD4 T cell–intrinsic role for CD134 in enabling CD137 agonist to engage the Runx3–Eomes axis.

CD137 agonist selectively releases Eomes from ThPOK repression

Thus far, the data presented in concert with the previous literature suggest that CD137 agonist, enabled by CD134, programs cytotoxic CD4 Th1 cells to express Eomes by overriding the ability of ThPOK to repress the Eomes gene. Thus, similar to CD8 T cells (26), induction of Eomes in CD4 T cells requires Runx3 (Fig. 3). Further, although Runx3 is also expressed in standard Th1 cells, where it fosters IFN-γ expression (27, 28), it is blocked by

**FIGURE 7.** CD134 is not required on CD4 T cells for Ag-induced CD137 expression. WT (Thy1.1/1) and CD134−/− (Thy1.1/1) Tera CD4 T cells were cultured separately (upper panels) or together at a 1:1 ratio (lower panels) in the presence of 1 μg/ml Eα peptide. Percentage of WT and CD134−/− Tera CD4 T cells expressing CD137 (A), CD134 (B), and CD25 (C) that had been cultured for 0, 24, and 48 h. (D) Percentage of WT and CD134−/− Tera CD4 T cells expressing CD137 in a second independent trial that used a similar experimental setup. In both experiments, the 24- and 48-h values represent the mean ± SEM calculated from triplicate or quadruplicate wells.
ThPOK from inducing Eomes and, hence, cytotoxicity (29–32). Further, in dual-costimulated CD4 T cells, Eomes appears to be selectively released from ThPOK repression, because these ThPOK+Eomes+ CD4 T cells do not express CD8α (Fig. 2A), whose gene is also repressed by ThPOK (29–32). Nevertheless, a recent study reported that in vitro–primed CRTAM+ cytotoxic CD4 T cells express CD8α mRNA, but not CD8α protein (58), raising the possibility that CD137 agonist globally blocks ThPOK-mediated transcriptional repression, and the absence of CD8α protein is caused by a post–gene expression event.

We addressed this question using another previously characterized dual-costimulation model, in which CD8-depleted B6 mice are immunized with SEA, which stimulates TCR Vβ3+ T cells (59). Similar to adaptively transferred HA-specific (Fig. 1) and Teα (Fig. 2) CD4 T cells, dual-costimulation programs endogenous Vβ3+ (specific) CD4 T cells to undergo cytotoxic TH1 differentiation (9). Thus, this SEA system provides the opportunity to confirm and extend earlier results generated with adaptively transferred CD4 T cells with regard to the relationship among costimulation, ThPOK, and Eomes. Importantly, the high frequency of SEA-specific Vβ3+ CD4 T cells increases the efficiency of FACS sorting specific CD4 T cells for gene-expression analysis. Similar to the Teα adoptive-transfer model (Fig. 6), CD137 agonist and, to a slightly lesser extent, dual costimulation programmed SEA-specific Vβ3+ CD4 T cells to express Eomes protein, whereas CD134 agonist given alone did not induce Eomes (Fig. 8A, 8B). Interestingly, CD137- and dual-costimulated Eomes+ SEA-specific CD4 T cells expressed slightly reduced ThPOK protein compared with their Eomes− counterparts (~20% reduced MFI, p ≤ 0.05). Nevertheless, ThPOK MFI in these Eomes+ specific CD4 T cells remained 6-fold greater compared with specific CD8 T cells recovered from intact mice treated with SEA plus CD137 agonist (p < 0.0001, Fig. 8A, 8C). Further, CD8α protein remained undetectable on specific CD4 T cells, regardless of costimulation or ThPOK expression (Fig. 8A, 8D). As expected, qRT-PCR analysis of FACS-sorted Vβ3+ SEA-specific CD4 T cells indicated that CD137 agonist and dual costimulation induced greater amounts of Eomes (Fig. 8E), GzmB (Fig. 8F), and perforin (Prf1, Fig. 8G) mRNAs compared with CD134 agonist. Additionally, only CD137 agonist partially reduced ThPOK mRNA (Fig. 8H). Importantly, CD8α mRNA remained undetectable in all CD4 T cell treatment groups (Fig. 8I), indicating that CD137 agonist does not relieve ThPOK repression of CD8α. Taken together, these data support the conclusion that, although CD137 agonist slightly reduces ThPOK expression, it does not globally block ThPOK-mediated transcriptional repression; rather, it selectively releases Eomes from ThPOK repression.

Discussion

Dual costimulation can drive powerful antitumor immunity by eliciting tumoricidal NK cells and CD8+ CTLs (12–16), as well as by programming CD4 T cells to differentiate into cytotoxic Th1 effectors that can directly kill MHC class II+ tumor cells (9) and provide potent help to CD8 T cells in the classical Ag-linked manner (i.e., both cognate epitopes are presented by the same dendritic cell) (9, 23, 39), as well as through a less common Ag-nonlinked pathway (11). This study addressed how dual costimulation programs CD4 T cells to acquire these tumoricidal functions.

We previously found that IL-2 and the T-box transcription factor Eomes are essential for dual-costimulated CD4 T cells to express IFN-γ and the cytotoxic effector molecule GzmB (9, 23). In this study, we found that Eomes was necessary for dual costimulation-mediated, CD8 T cell–independent, and CD4 T cell–dependent therapeutic efficacy in the aggressive B16 melanoma model (Supplemental Fig. 1). Because dual costimulation programs conventional CD4 T cells and Foxp3+ Tregs to express Eomes (11), and it has also recently been shown that CD137 agonist can reprogram Tregs into antitumor cytotoxic effectors (56), it may be possible that Eomes induction in both CD4 T cell compartments contributes to the antitumor effect observed in this study.

Eomes was initially identified as a transcription factor involved in programming cytotoxicity and IFN-γ expression in CD8 T and NK cells (20–22). That Eomes can be induced by IL-2 in CD8 T cells (24), in conjunction with our previous observation that both IL-2 and Eomes are critical for GzmB expression in dual-costimulated CD4 T cells (9, 23), suggested that IL-2 might induce Eomes, which, in turn, induces GzmB. In contrast, it has been reported that partial CD25 deficiency does not impact Eomes induction in flu-specific CD4 T cells (60). Indeed, dual costimulation induced normal Eomes expression in CD4 T cells fully deficient for IL-2 or CD25, despite the loss of GzmB induction (Fig. 1). Taken together, these previous and current data suggest that, although induction of Eomes occurs independently of IL-2, Eomes and IL-2 subsequently act in concert to induce GzmB.

We next asked whether dual costimulation induces Eomes in CD4 T cells by altering the expression of the T cell lineage transcription factors ThPOK and Runx3 (45). Runx3 directs thymic development of single-positive CD8 T cells (25), and it facilitates CTL differentiation in mature CD8 T cells by supporting expression of Eomes (26). Conversely, ThPOK guides the development of CD4+CD8α+ thymocytes (46, 47) and normally represses Runx3 (48), GzmB (29), and Eomes (31). Accordingly, because CD4+ IELs become Eomes+ and cytotoxic upon terminating ThPOK expression (32), we postulated that dual costimulation might terminate ThPOK expression in CD4 T cells, hence relieving repression of Runx3 and Eomes. Dual-costimulated cytotoxic CD4 Th1 cells did upregulate Runx3, and analysis of conditional Runx3−/− CD4 T cells revealed that Runx3 indeed supports expression of Eomes (Figs. 2, 3), as well as antitumor capacity (Figs. 4, 5). It is worth noting that, although Runx3 deficiency can diminish the suppressive capacity of Foxp3+ Tregs (61, 62), this did not appear to be a complicating factor in this study, because dual-costimulated Runx3−/− conventional CD4 T cells failed to gain tumoricidal capacity, despite the potential reduction in Treg suppressor activity. Regardless, dual-costimulated Runx3+Eomes+ CD4 T cells did not terminate ThPOK expression. Importantly, it has previously been shown that standard CD4 Th1 cells express Runx3, which supports IFN-γ expression (27, 28), although ThPOK blocks Runx3-mediated induction of Eomes and cytotoxicity (29, 31). Thus, our current data indicate that dual costimulation programs Runx3+–supported Eomes expression in CD4 T cells via a mechanism that resists ThPOK repression. Comparison of mRNA and protein levels in dual-costimulated CD4 T cells indicated that, although Eomes induction is associated with a slight (~20%) reduction in ThPOK, CD8α, a separate target of ThPOK repression (29–32), is not derepressed (Fig. 8). A potential mechanism for this differential effect is that dual costimulation selectively alters the epigenetic structure of the Eomes locus, but not Cd8a, to resist ThPOK repression. Alternatively, Cd8a may be more sensitive to ThPOK repression, thus explaining why Cd8a, but not Eomes, remains repressed when ThPOK expression is partially diminished. Regardless, our data demonstrate that dual costimulation elicits Eomes+ cytotoxic CD4 Th1 cells via a mechanism that is distinct from how terminated ThPOK expression in CD4+ IELs causes differentiation into Eomes+ cytotoxic CD4+CD8α+ double-positive T cells (32, 49).
FIGURE 8. CD137 agonist induces Eomes in specific CD4 T cells, despite the continued presence of ThPOK and repression of CD8α. CD8-depleted B6 mice were immunized with SEA and treated with rat IgG or CD134 or CD137 agonists administered individually or in combination. SEA-specific Vβ3+ CD4 T cells were analyzed following recovery from spleens 6 d later directly ex vivo for flow cytometry (gated on CD4+Vβ3+ cells) (A–D) or following FACS sorting (CD4+CD8negVβ3+ cells, ≥90% purity) for qRT-PCR (E–I). SEA-specific Vβ3+ CD8 T cells recovered from intact mice immunized with SEA and treated with CD137 agonist served as a positive control for Eomes and CD8α expression and a negative control for ThPOK. *p < 0.05, **p < 0.01, ***p < 0.001.
CD134 and CD137 each contribute to dual costimulation–programmed T cell responses (9, 12, 13). In analyzing the respective roles of CD134 and CD137 agonists in programming cytotoxic CD4 Th1 differentiation, this study confirms a previous report that CD137 agonist, but not CD134 agonist, administered individually is sufficient to induce Eomes in CD4 T cells (42), while further revealing that, nevertheless, CD134 is critical in this process. Thus, that CD134 and CD137 agonists can individually induce Runx3 (Fig. 6), and that Runx3 is necessary for Eomes expression (Fig. 3), but CD134 agonist alone fails to induce Eomes (Fig. 6), indicates that Runx3 is necessary, but not sufficient, for Eomes expression. Further, only CD137 agonist provides the requisite additional signal(s). Nevertheless, CD137 agonist failed to induce Runx3 and Eomes in CD134−/− CD4 T cells (Fig. 6). Notably, CD134 deficiency did not impact Ag induction of CD137 expression; in fact, CD134 deficiency did not impact Ag induction of CD137 expression. Further, only CD137 agonist provides the requisite additional signal(s). Nevertheless, CD137 agonist failed to induce Runx3 and Eomes in CD134−/− CD4 T cells (Fig. 6). Notably, CD134 deficiency did not impact Ag induction of CD137 expression; in fact, CD134 deficiency did not impact Ag induction of CD137 expression. Further, only CD137 agonist provides the requisite additional signal(s). Nevertheless, CD137 agonist failed to induce Runx3 and Eomes in CD134−/− CD4 T cells (Fig. 6).

References