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CD134 Costimulation Couples the CD137 Pathway to Induce Production of Supereffector CD8 T Cells That Become IL-7 Dependent

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The TNFR superfamily members CD137 and OX40 (CD134) are costimulatory molecules that potently boost CD8 and CD4 T cell responses. Concomitant therapeutic administration of agonist anti-CD137 and anti-CD134 mAbs mediates rejection of established tumors and fosters powerful CD8 T cell responses. To reveal the mechanism, the role of CD137 expression by specific CD8 T cells was determined to be essential for optimal clonal expansion and accumulation of effector cells. Nonetheless, dual costimulation induced production of supereffector CD8 T cells when either the specific T cells or the host alone bore CD137. Perhaps surprisingly, the total absence of CD137 prevented anti-CD134 augmentation of supereffector differentiation demonstrating an unappreciated link between these related pathways. Ultimately, it was reasoned that these powerful dual costimulatory responses involved common γ family members, and we show substantial increases of CD25 and IL-7Rα-chain expression by the specific CD8 T cells. To investigate this further, it was shown that IL-7 mediated T cell accumulation, but importantly, a gradual and preferential effect of survival was directed toward supereffector CD8 T cells. In fact, a clear enhancement of effector differentiation was demonstrated in populations of peptide-specific CD8 T cells. Therefore, dual costimulation through CD137 and CD134 drives production and survival of supereffector CD8 T cells through a distinct IL-7-dependent pathway. The Journal of Immunology, 2007, 179: 2203–2214.

The role of TNFR superfamily members for T cell costimulation has received a great deal of attention, especially in their ability to potently stimulate effector T cells, impact tolerance, and deliver long-term survival signals to specific T cells (1). In their own right, signaling through CD137 (4-1BB) and CD134 (OX40) have been shown to support very similar responses although not identical. CD134 is expressed early after CD4 T cell activation and mediates induction of effector T cell differentiation (2–4). Recent reports have also shown that CD134 can costimulate T regulatory cells (5), influence bystander cells, and also facilitate accumulation of effector T cells in pulmonary tissue (6, 7). In much the same way, stimulation of CD137 induces very similar effects in populations of peptide-specific CD8 T cells (1, 8, 9). In particular, CD137-stimulated T cells display enhanced cytotoxicity and cytokine production (1, 8, 10), while at the same time endowing CD8 T cells to persist for long periods of time (9, 11–13), as well as developing suppressor function (14–19). Nevertheless, both costimulatory pathways can overlap in their effects on CD4 and CD8 T cell subpopulations (10, 12, 20–22).

Previously, we demonstrated that certain combinations of dual costimulation such as combined activation through CD137 and CD134 induced a profound effect on the generation and quality of peptide-specific CD8 T cells (23). Stimulation of both costimulators, in a protocol that mimicked immunotherapy (24), induced CD8 T cell clonal expansion and effector T cell generation, which was accentuated in a mouse tumor cell model. Similar data using a wider variety of experimental murine tumor models was also reported (25). Additionally, recent data expanded upon these results by demonstrating that concomitant immunotherapy with anti-DR5, -CD137, and -CD40 mAbs were able to eradicate pre-established tumors (26). Finally, dual costimulation induced by CD80 and CD137L was shown to enhance CD8 T cell responses in lymphocyte populations from long-term infected HIV+ donors (27). Therefore, these data collectively suggest that combined stimulation of costimulatory pathways can lead to efficacious immunotherapy of tumors in humans as well as having the potential for vaccine development.

Although the potency of dual costimulation is notable, the mechanism explaining why two costimulators can be synergistic is unclear. This is particularly interesting for CD137 and CD134, where surface expression on T cells is relatively similar (28, 29), and both molecules use comparable, but perhaps not identical, signaling pathways (1, 30, 31). A significant difference, however, is that in general CD134 predominantly stimulates CD4 over CD8 T cells (2, 32–35), and the opposite is the case for CD137 (8, 35–38). Furthermore, CD137 is widely expressed on many different cell types including dendritic cells (DCs) (36, 39–44), but based on

Abbreviations used in this paper: DC, dendritic cell; γc, common γ; PLN, peripheral lymph node; MLN, mesenteric lymph node; WT, wild type.

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current data, CD134 does not appear to be as pervasively expressed (3, 4, 45). In this report, some of these concepts were reinforced and extended using the OT-I CD8 T cell transfer model interfaced with immunotherapeutic use of agonist anti-CD137 and -CD134 mAbs. It is shown that an optimal dual costimulatory effect through CD137 and CD134 required CD137 expression on the specific CD8 T cells. Specifically, effector differentiation was still retained when T cells were CD137 deficient, but accumulation was substantially reduced in lymphoid and peripheral sites. Second, CD134 costimulation was largely ineffectual at inducing superproduction of effector cytokines in the absence of the CD137 pathway. These results suggested that dual costimulation induced effects on both the specific T cells and host APCs, and revealed an unappreciated interdependent link of CD137 function for CD134 costimulation. Delving deeper, we detected a preferential increase of the common γ (γc) cytokine receptors CD25 and IL-7Rα (CD127) on the dual costimulated CD8 T cells vs the individually costimulated cells. Purified CD8 T cells responded to IL-7 by surviving better in an overnight culture, but more importantly they acquired supereffector function. An in vivo test of this latter notion demonstrated that although IL-7 was important for overall T cell accumulation, it played a crucial role for the survival of supereffector T cells.

Thus, the TNFR members CD137 and CD134 impart conditioning on specific CD8 T cells to use the γc cytokine family members for optimal effector differentiation and survival. These characteristics further support the approach of using the TNFR superfamily members to promote T cell costimulation to eradicate tumors in humans, and for vaccine development.

Materials and Methods

**Mice**

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) or The Jackson Laboratory. IL-7−/− mice were provided by Drs. L. Puddington (46) and L. Lefranc¸ois (47) (University of Connecticut Health Center, Farmington, CT). The OT-I, OT-I RAG−/−, CD137−/−, OT-1, and CD137−/+(48) transgenic mice were bred by our laboratory. All mice were maintained in the animal facility at the University of Connecticut Health Center in accordance with National Institutes of Health guidelines.

**Injection schedules and immunization procedure**

In all experiments, 1–2 × 10⁶ cells from the lymph nodes and spleen of either OT-I, OT-I RAG−/−, or CD137−/− OT-1 transgenic mice were adoptively transferred into C57BL/6 or various kinds of knockout mice by the i.v. route. The following day, 100 μg of SIINFEKL peptide (Invitrogen Life Technologies) in PBS was injected i.p. The anti-CD137 mAb is an anti-mouse serum (Sigma-Aldrich), 10 μg/ml human gamma-globulin (Sigma-Aldrich), and 0.1% sodium azide in culture supernatant from the 2.4.G.2 hybridoma (anti-FcR; Ref. 50) for 30 min on ice and then washed in wash buffer (3% FBS and 0.1% sodium azide in BSS). For CFSE staining, cells were isolated, pelleted, and resuspended in TC solution (MEM, amino acids, and antibiotics). The cells were labeled with 15 μM CFSE (Molecular Probes), incubated at 37°C for 10 min, and labeling was stopped with cold CTM.

For intracellular cytokine staining, 1 × 10⁶ splenocytes, or peripheral blood cells were cultured with 1 μg of brefeldin A (Calbiochem) in the presence of 1 μg of SIINFEKL peptide at 37°C for 5 h. For overnight-cultured cells (Fig. 5), naïve splenocytes were provided into a well at the beginning of restimulation. The cells were stained with either allophycocyanin-conjugated anti-CD45.1, anti-CD8, or anti-CD127 mAbs on ice, and after a couple washes, the cells were fixed with 2% paraformaldehyde in BSS. The cells were placed in permeabilization buffer (0.25% saponin in wash buffer) and then incubated with anti-IFN-γ, anti-TNF, or an isotype control rat IgG1 for 20 min at room temperature.

**Results**

**Effects of dual costimulation on specific CD8 T cells**

To study the robust effect of dual costimulation in vivo, a low dose of agonist anti-CD137 and -CD134 was tested in an OT-I adoptive transfer model. OT-I CD8 T cells were transferred into C57BL/6 mice, immunized as in Materials and Methods, and tracked by CD45.1 congenic marker staining. On days 2.5–10.5, the frequency of donor OT-I CD8 T cells from peripheral blood was analyzed by flow cytometry (Fig. 1A). At the peak of immune response (day 4.5), dual costimulation led to massive clonal expansion of OT-I T cells compared with the single costimulation groups. For example, there was a >2.9-fold increase in the frequency of dual costimulated specific CD8 T cells in blood at this time point. On day 10.5, spleen, PLNs, and MLN were analyzed for the presence of OT-I CD8 T cells (Fig. 1B). Dual costimulation profoundly enhanced the accumulation of OT-I T cells in all tissues compared with the other single costimulation treatments. For instance, dual costimulation induced a >7-fold increase in the percentages and a >12.6-fold increase in the numbers of OT-I CD8 T cells in spleen. Collectively, these data show a remarkable synergy through CD137 and CD134 dual costimulation by enhancing the frequency and number of Ag-specific CD8 T cells without the use of known adjuvants.
Next, we tested effectiveness of effector function in these groups. Day 4.5 peripheral blood cells were restimulated in vitro with SIINFEKL peptide for 5 h and then assayed for intracellular cytokine production. Approximately 65% of dual costimulated OT-I T cells made TNF (Fig. 1C, upper panel), and ~80% synthesized IFN-γ (data not shown). Compared with the single costimulator treatments, there was a 2-fold increase in the percentage of TNF-producing OT-I T cells, and no substantial increase in IFN-γ-producing OT-I T cells in the dual costimulated group. Effector function of day 10.5 dual costimulated OT-I T cells in spleen induced ~96% IFN-γ production and around 85% for TNF (Fig. 1D, upper panel). Although there was little difference in the percentage of OT-I T cells producing effector cytokines, there was, however, a massive increase from spleen, when normalized for the absolute numbers of OT-I effector cells (Fig. 1D, lower panel). Dual costimulation generated an 18- and 16-fold increase in TNF and IFN-γ, respectively, over anti-CD137 alone. The fact that the number of cytokine producers was much greater than the percentage of producers suggests that dual costimulation has a much greater effect on the expansion of effector T cells, rather than just impacting their effector differentiation process. Thus, dual costimulation generates excellent quality of effector T cells that accumulated in huge numbers.

Next, we characterized the role of CD137 signaling directly on T cells during clonal expansion and accumulation of effector cells. To test this notion, one group of C57BL/6 mice received wild-type (WT) OT-I spleen cells, and the other CD137-deficient (CD137−/−) OT-I cells. The following day, both groups were immunized with dual costimulation plus peptide, and on days 2.5 to 10.5, peripheral blood cells were analyzed by flow cytometry (Fig. 2A). The data show a substantial decrease of OT-I T cell clonal expansion in blood in the absence of CD137 expression on CD8 T cells, and the same is true when the host fails to express CD137. On day 4.5, dual costimulation generated ~18% of CD137−/− OT-I T cells, which was a ~2.5-fold decrease compared with WT OT-I. Therefore, clonal...
expansion of specific CD8 T cells was dependent upon direct CD137 signaling, because the WT host possesses CD137 on DCs and other cells of the immune system (39–44). For example, expression of CD137 on the host was also important for expansion because there was a 1.8-fold reduction in blood on day 4.5 when the host did not express CD137 while the OT-I T cells were WT. This was also the case for OT-I T cells in various tissues on day 4.5. The data show reduced accumulation of specific CD137 OT-I CD8 T cells in lymphoid and nonlymphoid sites. For example, after dual costimulation, the lack of CD137 expression on OT-I T cells led to an ~7-fold decrease in the numbers of OT-I T cells in spleen and MLN and ~4-fold decrease in liver. A smaller reduction was also detected when the CD137 OT-I host received WT OT-I cells, 1.7-fold less in spleen and 1.6-fold less in liver (data not shown). In general, these responses are much better than a rat IgG control response in a WT OT-I to WT recipient as we detect about a 10- and 60-fold decrease in spleen on days 4 and 5, respectively, after rat IgG compared with dual costimulation (data not shown). Thus, these results suggest that direct CD137 signaling on CD8 T cells is crucial for the accumulation of specific CD8 T cells and this trend was also observed on day 10.5 (data not shown). Taken together, these data show that CD137 expression on specific CD8 T cells and host is required for optimal T cell clonal expansion, but they may not necessarily relate to effector function.

Next, we asked whether effector function was also affected by the absence of CD137 expression on CD8 T cells. Day 4.5 spleen (Fig. 2, C and D) and liver (Fig. 2E) cells taken from mice that received either WT or CD137 OT-I OT-I (gray-filled circle) splenocytes were adoptively transferred into C57BL/6 mice, and OT-I cells into CD137 OT-I OT-I (light gray-filled triangle). The next day, all recipient mice were immunized with SIINFEKL peptide plus dual costimulation. A, On days 2.5–10.5, blood OT-I CD8 T cells was analyzed by staining for the congenic marker (CD45.1+ for WT OT-I or CD45.2+ for CD137 OT-I) CD8+ double-positive cells. The mean percentages ± SEM of OT-I cells are shown. Each group contained at least 13 mice per group from six different experiments. B, On day 4.5, spleen, MLN, PLNs, and liver cells were isolated, and the presence of OT-I T cells in each tissue was evaluated. Bar graphs represent the mean numbers ± SEM pooled from five separate experiments with a total of at least 13 mice in each group. C–E, Day 4.5 spleen and liver cells were restimulated in vitro and then assayed for intracellular cytokine production. C, Representative dot plots are shown from each group, and the numbers indicate the percentages of spleen OT-I T cells producing IFN-γ and TNF. D, Shown are the mean percentages (upper panel) and numbers (lower panel) ± SEM of spleen OT-I T cells staining positive for IFN-γ (left) or TNF (right). All data are combined from three different experiments for a total of at least 10 mice per group. E, Bar graphs represent the mean percentages (upper) or numbers (lower) ± SEM of liver OT-I T cells producing IFN-γ and TNF. These are pooled from three independent experiments with a total of at least eight mice in each group. The arrows show the fold reduction in the CD137 OT-I transfer group compared with the WT OT-I group.
direct CD137 signaling on T cells. However, when normalized for the actual number of OT-I T cells producing effector cytokine, it was profoundly reduced in CD137−/− OT-I transfer group. There was an 11.3-fold decrease in IFN-γ positive cells and an 11.6-fold decrease in TNF-producing OT-I T cells (Fig. 2D). This trend was similar in the frequency and absolute numbers of OT-I effector cells when liver was examined, a site where effector CD8 T cells are known to reside (Fig. 2E) (51, 52). In slight contrast, a smaller

![Diagram](image1)

**FIGURE 3.** CD134 stimulation induces supereffector T cells through dependency of CD137 expression on either CD8 T cells or host. A, WT OT-I splenocytes were adoptively transferred into CD137−/− mice (group I), and spleen cells from CD137−/− OT-I transgenic mice were transferred into either C57BL/6 (group II) or CD137−/− (group III) mice. On day 0, recipient mice received SIINFEKL peptide plus anti-CD134 Abs, and rat IgG was injected into another portion of recipient mice in each group as a control. After 60 h, spleen cells were restimulated in vitro and then analyzed for IFN-γ production. The dot plot in the lower left panel is representative of IFN-γ producing cells from the anti-CD134-treated group I mice, and these IFN-γ+ CD8 cells are gated. Six dot plots in the lower right panel show representatives of superproducers gated from IFN-γ+ CD8 double-positive cells from each treatment. The superproducers were determined by setting an analysis region of the anti-CD134-treated IFN-γ+CD8+ cells from group I at ~10% of the top IFN-γ-producing cells and then applying this exact region to all the samples. B, Bar graphs show the mean percentages (left) and numbers (right) ± SEM of IFN-γ superproducers within the CD8+ IFN-γ+ population, and these represent combined data from four separate experiments with a total of at least four mice in each group.

![Diagram](image2)

**FIGURE 4.** Dual costimulation increases CD25 and IL-7Rα expression on specific CD8 T cells. OT-I Rag−/− splenocytes were adoptively transferred into C57BL/6 mice. On day 0, recipient mice were given SIINFEKL peptide with anti-CD137 (gray-filled circle); SIINFEKL peptide with anti-CD134 (○); or SIINFEKL peptide with dual costimulation (■). On days 3–5, spleen cells were analyzed for the presence of OT-I cells positive for CD25 (A and B) or IL-7Rα (C and D). Analysis region from the isotype control staining in each mouse was set at ~2% to determine the percentage of CD45.1+ cells expressing CD25 or IL-7Rα-positive cells within CD45.1+ cells. A, Shown are representative zebra plots from day 4 spleen cells examining CD25 expression. The numbers listed indicate the percentages of CD45.1+ cells expressing CD25. B, Shown are the mean percentages ± SEM of CD45.1+ cells positive for CD25 expression. C, Zebra plots are representative of day 4 splenic OT-I T cells to analyze for IL-7Rα expression. The numbers represent CD45.1+ cells expressing IL-7Rα. D, Data show the mean percentages ± SEM of CD45.1+ cells positive for IL-7Rα expression. These data are pooled from four separate experiments for a total of at least four mice per group.
1.7-fold reduction was seen for the number of IFN-γ/H9253 producers for spleen and liver when WT OT-I were transferred into CD137/H11002/H11002 mice (data not shown). Thus, clonal expansion of peptide-specific CD8 T cells through dual costimulation depends on direct CD137 signaling, but differentiation into effector CD8 T cells was independent of direct CD137 activation.

Supereffector function driven by CD134 signaling is dependent on CD137 expression

To test the role of CD134 signaling on the CD8 T cell response, WT OT-I spleen cells were adoptively transferred into CD137−/− mice (group I); CD137−/− OT-I splenocytes into C57BL/6 mice (group II); and CD137−/− OT-I spleen cells into CD137−/− mice (group III) (Fig. 3A). All recipient mice were immunized with SIINFEKL peptide plus either rat IgG control or anti-CD134 mAb. Spleen cells were isolated 2.5 days later, restimulated in vitro, and evaluated for IFN-γ synthesis. Because CD134 endows CD8 T cells with effector function (22, 29, 53, 54), we reasoned that CD134 costimulation would induce the production of supereffectors as we have recently demonstrated using CD4 T cells (55). The proportion of IFN-γ positive cells within the OT-I CD8 population was analyzed for superproducers (Fig. 3A, lower panel). In group I, anti-CD134 treatment boosted the IFN-γ-producing population compared with control IgG (Fig. 3B). This showed that CD137 on accessory cells was not important for CD134 costimulation of CD8 T cells. Group II showed a 3-fold increase indicating that CD137 on the specific CD8 T cells was also indispensable. In group III, neither the accessory cells nor the specific CD8 T cell bore CD137 (Fig. 3A, bottom schematic), which profoundly inhibited the CD134 immune response (Fig. 3B). Also, results from WT OT-I transfer into WT were very similar to the results from groups I and II, although these mice were assayed at day 3 instead of day 2.5 (data not shown). These data show that in a system where CD4 T cell help is unnecessary for CD8 effector production, CD134 costimulation depends on CD137 expression on either the specific CD8 T cells or accessory cells.

We also examined CFSE dilution of the transferred OT-I T cells and detected no substantial differences between the groups regardless of whether the mice received anti-CD134 (data not shown). Based on numbers of OT-I T cells at this time point, we detected an increase in groups I and II, compared with group III.
but these were relatively minor differences as were the effects of anti-CD134 (data not shown). Importantly, day 2.5 is not an optimal time point for measuring clonal expansion so these results were not surprising; however, it is critical to note that later time points such day 5 are difficult to interpret because the CFSE is completely diluted out preventing any distinction between expansion of endogenous T cells with that of the transferred cells.

**Dual costimulation induces higher CD25 and IL-7Rα expression on OT-I T cells**

To study whether any survival cytokine was involved in the clonal expansion through dual costimulation, the expression level of several cytokine receptors in the γc chain cytokine family such as CD25 and IL-7Rα (CD127) was evaluated. Thus, OT-I-recipient C57BL/6 mice were immunized (SIINFEKL peptide with either rat IgG control, anti-CD137, -CD134, or dual costimulation), and on days 3–5, spleen cells were analyzed for the percentages of OT-I T cells positive for CD25 (Fig. 4, A and B) and IL-7Rα (Fig. 4, C and D) expression. In all cases, CD25 expression on OT-I T cells gradually decreased between days 3 to 5 (Fig. 4B), whereas IL-7Rα expression progressively increased (Fig. 4D). However, dual costimulated OT-I T cells reproducibly contained a greater proportion of CD25- and IL-7Rα-expressing cells on days 3 and 4 compared with the single costimulation treatments.

To test the idea that these cytokines were responsible for the accumulation of dual costimulated effector CD8 T cells, OT-I-recipient mice were immunized in vivo as in Fig. 4. CD45.1+ cells were purified from spleen on either days 2.5 or 4, cultured overnight either in the absence or presence of IL-7, and then restimulated with peptide to measure effector cytokine synthesis (Fig. 5A). Fig. 5B shows that dual costimulated OT-I T cells generated more IFN-γ superproducers in the presence of IL-7 compared with the single costimulators, and this is also the case in the absence of IL-7. Importantly, when normalized for the percentage of surviving superproducers (by multiply the percentage of viable OT-I T cells by the percent of superproducers), day 4 dual costimulated OT-I T cells contained a higher frequency of surviving superfactor T cells whether or not IL-7 was present (Fig. 5C). Survival cytokine IL-7 was able to enhance accumulation of the superfactor subpopulation regardless of any kind of costimulation, and this was the case for IL-2 as well (data not shown). Taken together, the data implies that survival cytokines such as IL-7 are likely to be involved in the action of dual costimulation.

Thus, it was hypothesized that IL-7 might play a critical role in the mechanism of dual costimulation. Therefore, OT-I splenocytes were adoptively transferred into either C57BL/6, IL-7−/−, or IL-7−/−, immunized with dual costimulation and peptide, and on days 2.5–10.5, the presence of OT-I CD8 T cells detected in peripheral blood by flow cytometry (Fig. 6A). There was no significant difference in the clonal expansion of OT-I T cells in the absence or presence of IL-7 suggesting that clonal expansion of dual costimulated OT-I CD8 T cells was independent of IL-7. On day 10.5, however, the frequency of OT-I T cells in blood was 1.2-fold lower in the IL-7−/− group compared with control C57BL6 mice. This was consistent with the percentages of OT-I CD8 T cells in spleen, lung, and liver. For example, there was a 2-fold decrease in IL-7−/− mice compared with the control group (Fig. 6B, left panels); however, there were equivalent percentages in peripheral LN (data not shown). The absolute numbers of splenic OT-I T cells were dramatically reduced in the absence of IL-7, where there was a 14-fold reduction (Fig. 6B, right panel), but this may be due to the reduced cellularity in lymphoid tissue in IL-7−/− mice.

Nonetheless, we still observed a >2-fold reduction of the absolute numbers of OT-I T cells in lung and liver (Fig. 6B, right panel), which was comparable to the reduction in percentages (Fig. 6B, left panel). Together, these data suggest that IL-7 did not influence clonal expansion but had impacted accumulation of dual costimulated OT-I T cells in peripheral tissue and spleen.

To answer the question of whether the absence of IL-7 had an effect on effector function of dual costimulated OT-I T cells, day 10.5 spleen and liver cells taken from either control, IL-7−/−, or IL-7−/− mice were restimulated in vitro and analyzed for intracellular cytokine production. Liver (data not shown) and spleen...
In the absence of IL-7, adoptive transfer of OT-I Rag−/− splenocytes into either control (C57BL/6 and IL-7−/−), or IL-7−/− mice was conducted, and the next day, mice were immunized with SIINFEKL peptide plus dual costimulation. On day 10.5, spleen (A and C) or liver (B and D) cells were restimulated with peptide in vitro. A, Splenic CD45.1+ cells were analyzed for intracellular IFN-γ or TNF level. Upper panel, Representative histograms of IFN-γ and TNF production by gated CD45.1+ cells. The gray histogram is the isotype control and the thick black lines represent cytokine staining. The numbers show IFN-γ or TNF superproducers by setting an analysis region on CD45.1+ cells at ~10% of the top cytokine producer and keeping the exact region for all the samples. Lower panel, Individual percentages of IFN-γ or TNF producers and superproducers in splenic CD45.1+ cells are quantitated in scatter plots. Data are combined from six different experiments. B, Liver OT-I effector T cells were examined as in A, and are from five separate experiments. The numbers of liver OT-I superproducers appeared in the lower scatter plot. C and D, Splenic (C) and liver (D) OT-I effector T cells were also analyzed for negative to low (Neg/low) IFN-γ or TNF producers. The negative to low producers were determined by setting an analysis region at ~30% of the lowest cytokine producer and keeping the exact region for all the samples. The lines mark the average values and the arrow indicates the fold reduction in the IL-7−/− group compared with control.

(Fig. 7A, upper panel) OT-I T cells from the IL-7−/− group generated fewer effector T cell producers, and the cells from heterozygous mice showed intermediated responses. Importantly, the percentages of superproducers in both spleen (Fig. 7A, lower panel) and liver (Fig. 7B, upper panel) were profoundly reduced in IL-7−/− mice, where there was a ~3-fold decrease compared with control mice. The absolute numbers of splenic OT-I superproducers were >16-fold lower in the absence of IL-7 (data not shown), which may be more of an outcome as the reduced cellularity in lymphoid tissues in these knockout mice as discussed earlier. In liver where cellularity was comparable to percentage (Fig. 6B), there was an 8-fold reduction in the numbers of IFN-γ superproducers, and a 6-fold decrease in TNF superproducers (Fig. 7B, lower panel). Second, we tested whether the lack of IL-7 augmented an increase of negative to low effector cytokine producers. The IL-7−/− group showed a 2-fold increase in the percentage of negative to low effector cytokine producers in the IL-7−/− group compared with WT (Fig. 7C, spleen, and Fig. 7D, liver). The absolute numbers of IFN-γ or TNF negative to low producers in liver were about the same in the IL-7−/− compared with WT (Fig. 7D, lower panel). Thus, unavailability of IL-7 resulted in accumulation of the negative to low effector OT-I T cell population. Collectively, the data suggest that IL-7-mediated survival after dual costimulation through CD137 and CD134 preferentially acts on superproducing effector CD8 T cells.

To further test the idea that IL-7-mediated survival acts on superproducers, day 10.5 peripheral blood cells were restimulated in vitro and then assayed for intracellular cytokine production (Fig. 8A). IFN-γ TNF double superproducers were reduced in IL-7−/− mice. When compared between day 4.5 and 10.5 blood OT-I T cells, very little decrease in frequency of double superproducers from IL-7−/− mice on day 4.5, but a 3-fold reduction of double superproducers on day 10.5 was observed (Fig. 8B). These data suggest the increasing importance of IL-7 for survival of supereffector CD8 T cells was based on elapsed time, rather than an all-or-nothing effect.
To test whether WT DCs were able to increase effector function in the IL-7−/− group, OT-I T cells on day 12.5 or 14.5 were isolated from either control or IL-7−/− mice, and restimulated in vitro in the presence of WT APCs (Table I). Although the effector cytokine producing ability was comparable between the control and IL-7−/− group, the frequency of double superproducers was substantially reduced in OT-I T cells taken from IL-7−/− mice even in the presence of WT APCs. This implies that after dual costimulation, accumulation of supereffector T cells is defective in the absence of IL-7 or that OT-I T cells are conditioned to differentiate into superproducers in the presence of IL-7.

Moreover, it was examined whether a proportion of IL-7Rα-expressing OT-I T cells had different effector cytokine production profiles. Day 8.5 peripheral blood cells from dual costimulated OT-I recipient C57BL/6 mice were assayed for IL-7Rα expression and IFN-γ production upon restimulation (Fig. 8C). IL-7Rαhigh expressing OT-I T cells synthesized 82% of IFN-γ, whereas the IL-7Rα mixed population generated 26% of IFN-γ producers and the negative population produced very little IFN-γ. We also observed similar results at different time points, where higher IL-7Rα expression tracked with more IFN-γ production (data not shown).

**Discussion**

Previously, it was demonstrated that simultaneous administration of agonist anti-CD137 and -CD134 mAbs substantially enhanced CD8 effector T cell function leading to rapid rejection of murine tumors, but the mechanism of dual costimulation remained unknown (23). Here, it is shown that CD137 was critical for optimal T cell clonal expansion and CD134 triggered supereffector function, which was also dependent upon CD137 signaling. In combination, the CD137 and CD134 costimulatory pathways facilitated effector T cell survival with coincident induction of IL-7Rα, but exogenous IL-7 was essential for accumulation of these supereffector CD8 T cells. Thus, this model shows an interdependent bridge between the TNFR and the γc chain cytokine families.

To characterize the effect of dual costimulation by CD137 and CD134 on CD8 T cells, we examined whether the dose of the anti-CD137 and -CD134 mAbs dictated the synergistic action of dual costimulation on OT-I T cells. Our data persuasively demonstrated that a low dose of dual costimulation synergistically increased clonal expansion of OT-I CD8 T cells. These results showed that dual costimulation induced a bona fide synergistic signal between the two different costimulatory pathways. Second,

**Table I. Added APCs do not rescue recall of OT-I T cells that have been conditioned in IL-7−/− mice**

<table>
<thead>
<tr>
<th>Expt. Group</th>
<th>IFN-γ Producer</th>
<th>TNF Producer</th>
<th>IFN-γ TNF Double Superproducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>94.5 ± 0.4</td>
<td>97.0 ± 0.6</td>
<td>14.7 ± 4.2</td>
</tr>
<tr>
<td>(Day 14.5) IL-7−/−</td>
<td>81.0 ± 2.8</td>
<td>91.6 ± 0.8</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>WT</td>
<td>88.8 ± 1.0</td>
<td>89.2 ± 0.9</td>
<td>9.0 ± 1.2</td>
</tr>
<tr>
<td>(Day 14.5) IL-7−/−</td>
<td>79.7 ± 1.3</td>
<td>85.1 ± 0.1</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>WT</td>
<td>93.4 ± 0.9</td>
<td>94.3 ± 1.0</td>
<td>22.3 ± 3.4</td>
</tr>
<tr>
<td>(Day 12.5) IL-7−/−</td>
<td>76.9 ± 4.4</td>
<td>79.1 ± 6.6</td>
<td>13.4 ± 4.5</td>
</tr>
<tr>
<td>WT</td>
<td>95.0 ± 0.4</td>
<td>87.9 ± 3.7</td>
<td>12.3 ± 1.6</td>
</tr>
<tr>
<td>(Day 12.5) IL-7−/−</td>
<td>93.9 ± 0.4</td>
<td>93.6 ± 2.6</td>
<td>7.7 ± 0.8</td>
</tr>
</tbody>
</table>

*OT-I spleen cells were adoptively transferred into either C57BL/6 (WT) or IL-7−/− mice. The next day, all recipient mice were injected with SIINFEKL peptide plus dual costimulation. On day 14.5 (expt. 1 and 2), OT-I T cells were purified from spleen using MACS columns with anti-CD45.1 Abs. On day 12.5 (expt. 3 and 4), peripheral blood cells were run through a nylon wool column. Afterward, the day 14.5 or 12.5 cells were placed into a well of a 96-well plate containing naive splenocytes from C57BL/6. They were restimulated in vitro with peptide for 5 h and assayed for intracellular cytokine. The data are the mean percentages ± SEM, and each experiment has at least two mice per group.*
dual costimulation profoundly enhanced cumulative CD8 effector function compared with single costimulators (Fig. 1D). Perhaps, this is the reason that dual costimulation effectively retarded tumor growth (23, 25), and permitted expansion of OT-I T cells after transfer of just 1000 cells (23).

Because CD137 is expressed on activated T cells, DCs, and other cells of the immune system (5, 36, 39–44, 56), the role of direct CD137 expression on CD8 T cells was unclear. Therefore, we tested this notion and demonstrated that CD137−/− OT-I T cells expanded poorly compared with WT OT-I T cells, but unexpectedly the effector function of CD137−/− OT-I T cells was commensurate with that of WT OT-I T cells (Fig. 2, C–E). Based on numerous studies that CD137 stimulation enhances CD8 effector function (8, 10, 28, 57), our data implied that CD137 signaling on host accessory cells was able to compensate for the lack of CD137 function (8, 10, 28, 57), our data implied that CD137 signaling on host accessory cells was able to compensate for the lack of CD137 expression on the activated T cells. Second, when CD137−/− mice were used as recipients and immunized, effector function of WT OT-I T cells was also optimal (data not shown). Thus, in the presence of CD137 expression on either CD8 T cells or host, CD8 T cells were able to differentiate into effectors. This suggests that differentiation of effector CD8 T cells may be mediated through factors such as cytokines produced from either CD137 stimulated-CD8 T cells or -APCs. Perhaps the APCs produce important proinflammatory cytokines that can provide help to the responding T cells for effector differentiation. This is borne out in recent work clearly demonstrating that CD137 stimulation on DCs can induce secretion of IL-6 and IL-12 (58, 59).

Second, this idea is consistent with our results showing that the CD137-deficient host supported weaker responses than a WT host (Fig. 2A). Ultimately, however, our data did show that direct CD137 activation on CD8 T cells was critical for cumulative enhancement of effector function rather than improved effector differentiation.

An interesting contrast was recently observed by Lee et al. (29) showing that CD137−/− OT-I effector T cells exceeded expansion by the WT OT-I T cells after in vivo priming with OVA-expressing adenovirus. Thus, different model systems, where the combination of strength/duration of Ag stimulation, cytokine milieu, or engagement of other signaling pathways such as TLR stimulation (60), can dramatically influence the effect of CD137 costimulation. A more specific difference is that our system used enforced costimulation, such as that for tumor immunotherapy (8, 24, 26), with SIINFEKL peptide that limits CD4 T cell help. Therefore, the role of CD134 in our dual costimulation system may be different from that in an infection model, because our system has few CD4 T cell targets compared with CD8. Consistent with this possibility are several studies demonstrating that CD134 can also directly enhance CD8 T cell responses (22, 29, 53, 54). Thus, to clarify the role of CD134 on CD8 T cells, we stimulated production of CD8 effectors through CD134 activation, but surprisingly this was positively regulated by CD137 signaling (Fig. 3). Taken together, our data suggest that CD134 costimulation of CD8 T cells is linked to the function of the CD137 pathway. Thus, dual costimulation may promote CD137-driven expansion with CD134-induced effector function of specific CD8 T cells, suggesting a dichotomous role between CD137 and CD134 as recently proposed (29).

Clonal expansion and effector differentiation are mechanically tied to T cell survival. Previously, dual costimulation was shown to enhance in vitro survival of activated CD8 T cells compared with single costimulators (23), and this evidence suggested that survival was requisite for the action of dual costimulation. Consistent with this notion, dual costimulation induced IL-7Rα expression on OT-I T cells (Fig. 4), a cytokine receptor pathway known to enhance survival of naive and memory T cells (61–63). Exogenous IL-7 enhanced accumulation of supereffector T cells (Fig. 5), suggesting that IL-7 may be involved in the action of dual costimulation. This hypothesis was tested in vivo by transferring OT-I cells into IL-7−/− mice and stimulating them with peptide in the presence of dual costimulation. There was a decrease in survival (Fig. 6), but it appeared to be a subpopulation rather than a general decline. This idea was consistent with earlier results demonstrating a survival dependency of IL-7 for memory T cells (61–63). Nevertheless, upon close examination, we found that effector function of dual costimulated OT-I T cells in IL-7−/− mice was profoundly reduced, especially the superproducers (Fig. 7A).

This new data led us to speculate that IL-7-mediated survival was distinctly critical for accumulation of supereffector cells, which is an important therapeutic outcome by CD137 and CD134 dual costimulation (23). Accumulation of effector cells in nonlymphoid tissues such as liver is pivotal for controlling infection, and is a destination whereby primary memory effector CD8 T cells migrate to and from lymphoid tissues (51, 52). In IL-7−/− mice, there was a substantial decrease in the frequency and numbers of superproducers in liver (Fig. 7B), implying an important role for IL-7 in inducing the accumulation of this subpopulation in nonlymphoid tissue. This evidence was supported by a reciprocal test showing that after transfer and immunization, in IL-7−/− mice, there was an increase in the proportion and numbers of negative to low cytokine producers in liver as well as in spleen (Fig. 7, C and D). Thus, IL-7 deprivation had a preferential effect on diminishing the accumulation of superproducers. Accordingly, the literature has shown that the IL-7Rα is down-regulated after activation, but returns on memory T cells (61, 64), and our idea was that the effect of IL-7 on dual costimulated superproducers would be greater when IL-7Rα expression was restored at a later time. In fact, it was shown that as the amount of IL-7Rα expression increased, the ability to produce IFN-γ in CD8 effector T cells was substantially greater (Fig. 8). These data may explain earlier studies demonstrating that IL-7 can be an effective adjuvant by improving the immune response of CD8 T cells in patients with cancer, AIDS, or old age (65–70).

The intracellular molecular mechanism of how dual costimulation enhanced clonal expansion and increased accumulation of effector CD8 T cells is currently unclear, but is at least predicated on a heightened survival signaling program. Specifically, dual costimulation may increase growth factor or IL-2-driven clonal expansion, followed by intensified IL-7 survival signaling leading to accumulation of supereffector cells. Because both costimulatory pathways function through TRAFs, dual costimulation may ultimately depend on an enhanced loading, or poising of NF-κB components (30, 31, 71).

In summary, our results demonstrate how CD8 T cell supereffector responses develop after dual costimulation. This was based on CD137 and CD134 augmenting IL-7 signaling on CD8 T cells driving accumulation of supereffectors. Ultimately, this study may provide a protocol for rapid induction of CD8 effector responses in the absence of functional T cell help, such as that during AIDS.

Disclosures
The authors have no financial conflict of interest.

References


