Synergy of IL-21 and IL-15 in regulating CD8$^+$ T cell expansion and function

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Interleukin (IL)-21 is the most recently recognized of the cytokines that share the common cytokine receptor γ chain (γ_c), which is mutated in humans with X-linked severe combined immunodeficiency. We now report that IL-21 synergistically acts with IL-15 to potently promote the proliferation of both memory (CD44^{high}) and naive (CD44^{low}) phenotype CD8$^+$ T cells and augment interferon-γ production in vitro. IL-21 also cooperated, albeit more weakly, with IL-7, but not with IL-2. Correspondingly, the expansion and cytotoxicity of CD8$^+$ T cells were impaired in IL-21R$^{-/-}$ mice. Moreover, in vivo administration of IL-21 in combination with IL-15 boosted antigen-specific CD8$^+$ T cell numbers and resulted in a cooperative effect on tumor regression, with apparent cures of large, established B16 melanomas. Thus, our studies reveal that IL-21 potently regulates CD8$^+$ T cell expansion and effector function, primarily in a synergistic context with IL-15.

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Abbreviations used: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; rFPVhgp100, recombinant fowlpox virus encoding hgp100; vPE16, vaccinia virus–expressing HIV gp160.

The common cytokine receptor γ-chain (γ_c) is mutated in X-linked severe combined immunodeficiency (1), a disease with severely impaired T cell and NK cell development and diminished B cell function (2). γ_c is a critical component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (2), which together regulate lymphocyte development and control a broad spectrum of activities that shape innate and acquired immune responses. The IL-21/IL-21 receptor (IL-21R) system is the most recently identified of these cytokine systems (3, 4). IL-21 is produced by activated CD4$^+$ T cells, and its receptor is expressed on T, B, and NK cells. IL-21 is most closely related to IL-2, IL-4, and IL-15, and IL-21R is most similar to the IL-2 receptor β chain (IL-2Rβ), which is a component of the IL-2 and IL-15 receptors. Based on in vitro assays, IL-21 was originally implicated as a regulator of T and B cell proliferation as well as of NK cell maturation (4), whereas another group later reported that IL-21 could inhibit NK cell expansion (5). IL-21R$^{-/-}$ mice have normal lymphocyte compartments, including normal NK cell development (5, 6), indicating that IL-21 is not essential for the development of lymphoid lineages, but leaving open the possibility that it contributes to this process in a potentially redundant fashion. Together with IL-4, IL-21 plays a critical role in regulating Ig production (6). IL-21R$^{-/-}$ mice have diminished IgG1, but greatly elevated IgE levels in response to antigen challenge, whereas IL-21R$^{-/-}$IL-4$^{-/-}$ double knockout mice exhibit a severely impaired IgG response as well as diminished IgE levels, indicating a cooperative role of these two cytokines for Ig production (6).

The size of naive and memory T cell pools is tightly regulated, at least in part, by growth and survival signals conferred by γ_c-dependent cytokines (7, 8). IL-7 is crucial for the survival and homeostatic expansion of naive CD8$^+$ T cells and also can contribute to memory CD8$^+$ T cell homeostasis (9, 10). IL-15 potently promotes proliferation of memory CD8$^+$ T cells (8, 11, 12); the major subset of memory (IL-2Rβ^{high}CD44^{high}) CD8$^+$ T cells depends on IL-15 for survival and turnover, whereas the IL-2Rβ^{low}CD44^{high} CD8$^+$ T cells are IL-15 independent (13). IL-15 may also contribute to the homeostatic proliferation of naive CD8$^+$ T cells (14, 15). In contrast, IL-2 can decrease memory CD8$^+$ T cell function by inducing regulatory T cells (16–18). Late in the proliferative phase of the T cell response, activated T

The online version of this article contains supplemental material.

cells differentiate into effector T cells that produce critical effector molecules. We have now investigated the role of IL-21 in T cell homeostasis and effector functions.

RESULTS

IL-21 synergistically acts with IL-15 to expand CD8$^+$ T cells

IL-21 was initially reported to costimulate anti-CD3–activated murine thymocytes and mature murine T cells in vitro and to enhance the proliferative effects of IL-2, IL-7, and IL-15 even without the addition of anti-CD3 (4). Interestingly, however, a second group reported that the addition of IL-21 blocked the IL-15–dependent, TCR-independent expansion of CD44^{high}CD8$^+$ T cells (5). To further explore the actions of IL-21 and how it integrates its signals with other γ_c-dependent cytokines, we studied the effect of a range of concentrations of IL-15 and IL-21 on normal splenocytes cultured for 7 d. As reported previously (5), IL-21 inhibited IL-15–mediated expansion of resting NK cells; we observed a dose-dependent inhibition by IL-21 that was most evident at 100 ng/ml of IL-15 (Fig. 1 A, lanes 16 and 17 vs. 5 and 15; also, Fig. 1 E, e vs. c). However, in contrast with a previous paper (5), we observed a marked increase, rather than decrease, in the number of T cells after culture with both IL-21 and IL-15 as compared with IL-15 alone (Fig. 1 B, lanes 9–11 vs. 3, 12–14 vs. 4, and 15–17 vs. 5). The majority of these expanded cells were CD8$^+$ T cells (Fig. 1 F, e vs. c). IL-21 by itself had little effect on T cell cellularity (Fig. 1 B, lanes 6–8 vs. 2), but again there was an increase in the percentage of CD8$^+$ T cells (Fig. 1, C, lanes 6–8 vs. 2, and F, d vs. b). Although IL-15 alone induced an increase in the percentage of CD8$^+$ T cells (Fig. 1 F, c vs. b), the absolute number of CD8$^+$ T cells was, if anything, slightly less than the number on day 0 (Fig. 1 C, lane 5 vs. 1). Strikingly, after 7 d of culture with IL-15 plus IL-21, the total numbers of CD8$^+$ T cells markedly increased (Fig. 1 C, lanes 9–17). IL-15 and IL-21 had a synergistic effect on CD8$^+$ T cell proliferation, as a combination of low concentrations of these two cytokines (10 or 50 ng/ml) was more potent than the effect of either cytokine alone at a concentration of 50 or 100 ng/ml (Fig. 1 C, lane 9 vs. 4 and 7, and lane 13 vs. 5 and 8). In

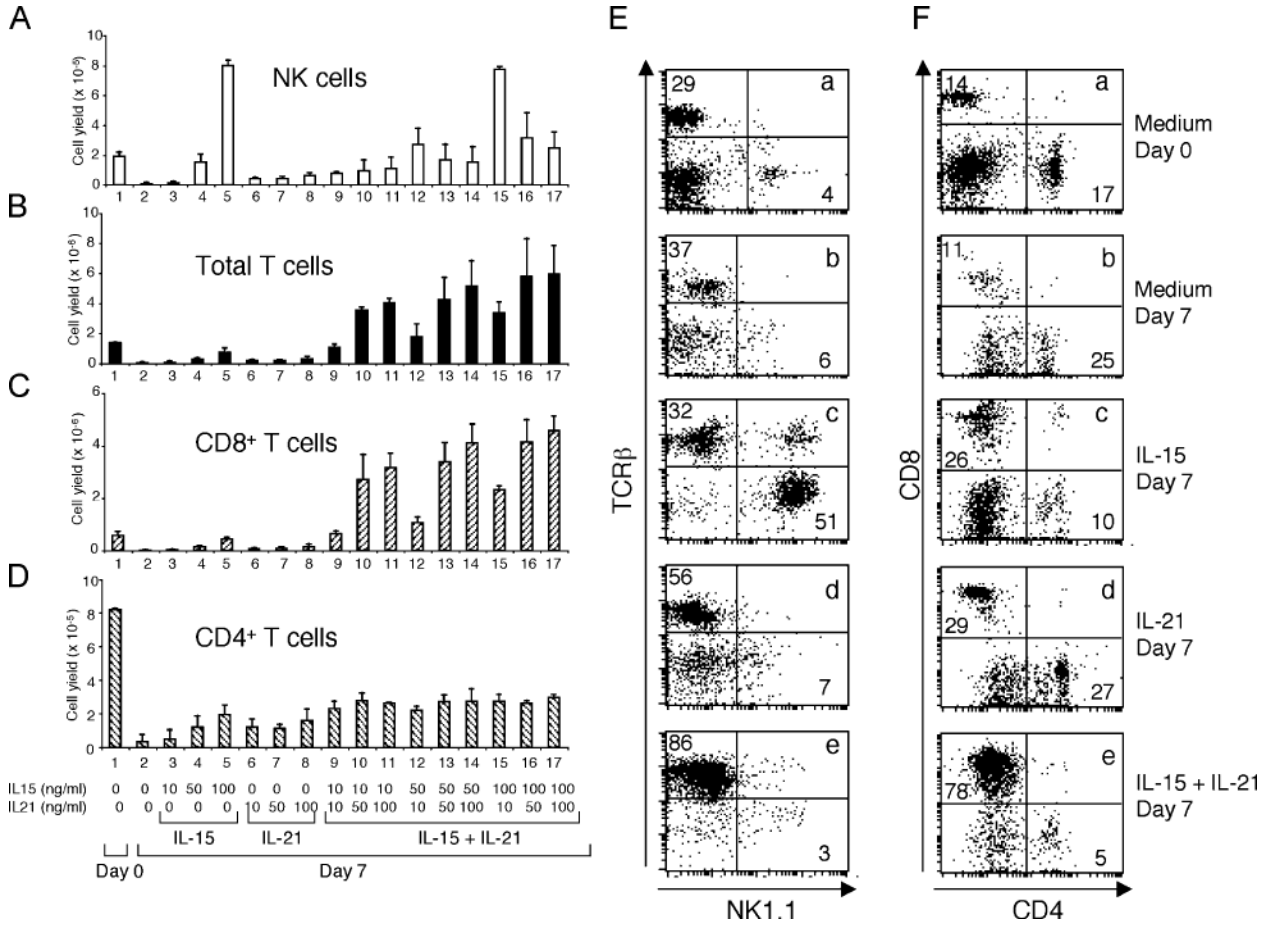

Figure 1. IL-21 acts synergistically with IL-15 to expand CD8$^+$ T cells. 5 × 10^6 splenocytes were pooled from two to three wild-type mice and cultured for 7 d in medium containing IL-15, IL-21, or combinations of these cytokines, as indicated. The number of NK cells (A), T cells (B), CD8$^+$ T cells (C), and CD4$^+$ T cells (D) were determined as NK1.1$^+$TCRβ$^-$, NK1.1$^-$TCRβ$^+$, CD8$^+$CD4$^-$, and CD8$^-$CD4$^+$ cells, respectively. Results shown are means ± SD from three experiments. (E and F) Cells cultured in medium for 0 d (a) or 7 d (b), or in 100 ng/ml of IL-15, IL-21, or both cytokines (c, d, and e, respectively) were analyzed by flow cytometry for 7 d. The percentages of NK1.1$^-$TCRβ$^+$ and NK1.1$^+$TCRβ$^-$ cells (E) and CD8$^-$CD4$^+$ and CD8$^+$CD4$^-$ cells (F) are indicated in the quadrant corners.

contrast with the effect on CD8$^+$ T cells, cooperative effects on CD4$^+$ T cells were not evident (Fig. 1 D).

IL-21 also synergistically acts with IL-7, but not IL-2

Because IL-21 was reported to act in concert with IL-2 or IL-7 to enhance T cell proliferation (4), next we compared the effects of IL-2, IL-7, IL-15, and IL-21 on splenocytes cultured for 3, 5, and 7 d (Fig. 2). Consistent with the results of Fig. 1, IL-21 increased the total T cell and CD8$^+$ T cell expansion mediated by IL-15, but it had no effect on CD4$^+$ T cells (Fig. 2, A and B, lanes 25–27 vs. 13–15, and C, h vs. d, and not depicted). The synergy of IL-15 and IL-21 on CD8$^+$ T cell expansion was evident as early as day 3 (Fig. 2 A, lane 25 vs. 13 and 16). Interestingly, IL-21 did not significantly increase expansion of CD8$^+$ T cells when combined with IL-2 (Fig. 2 A, lanes 19–21 vs. 7–9), even though it increased the percentage of these cells (Fig. 2 C, f vs. b); however, it cooperated with IL-7, although not as potently as it did with IL-15 (Fig. 2, A, lanes 22–24 vs. 10–12, and C, g vs. c; Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041057/DC1). The cooperative effects of IL-15 and IL-21 were confirmed in studies using IL-15$^{-/-}$ and IL-21R$^{-/-}$ mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20041057/DC1). We also evaluated the effect of IL-15, IL-21, and the combination of both cytokines on cell survival by staining with annexin V. CD8$^+$ T cells cultured without any cytokine essentially all died within 7 d; however, the addition of IL-15, IL-21, or combinations of these cytokines resulted in >94% viability in all cases (Fig. 2 D, k–m vs. j). Thus, although the cytokines increased the viability, the synergistic effect on cell expansion seen in response to IL-15 plus IL-21 as compared with either cytokine alone primarily results from increased cell proliferation rather than a synergistic effect on survival.

IL-15 and IL-21 cooperatively enhance the effector function of memory-phenotype CD8$^+$ T cells

Because IL-15 is known to expand memory-phenotype CD44^{high}CD8$^+$ T cells, we examined if IL-21 could en-

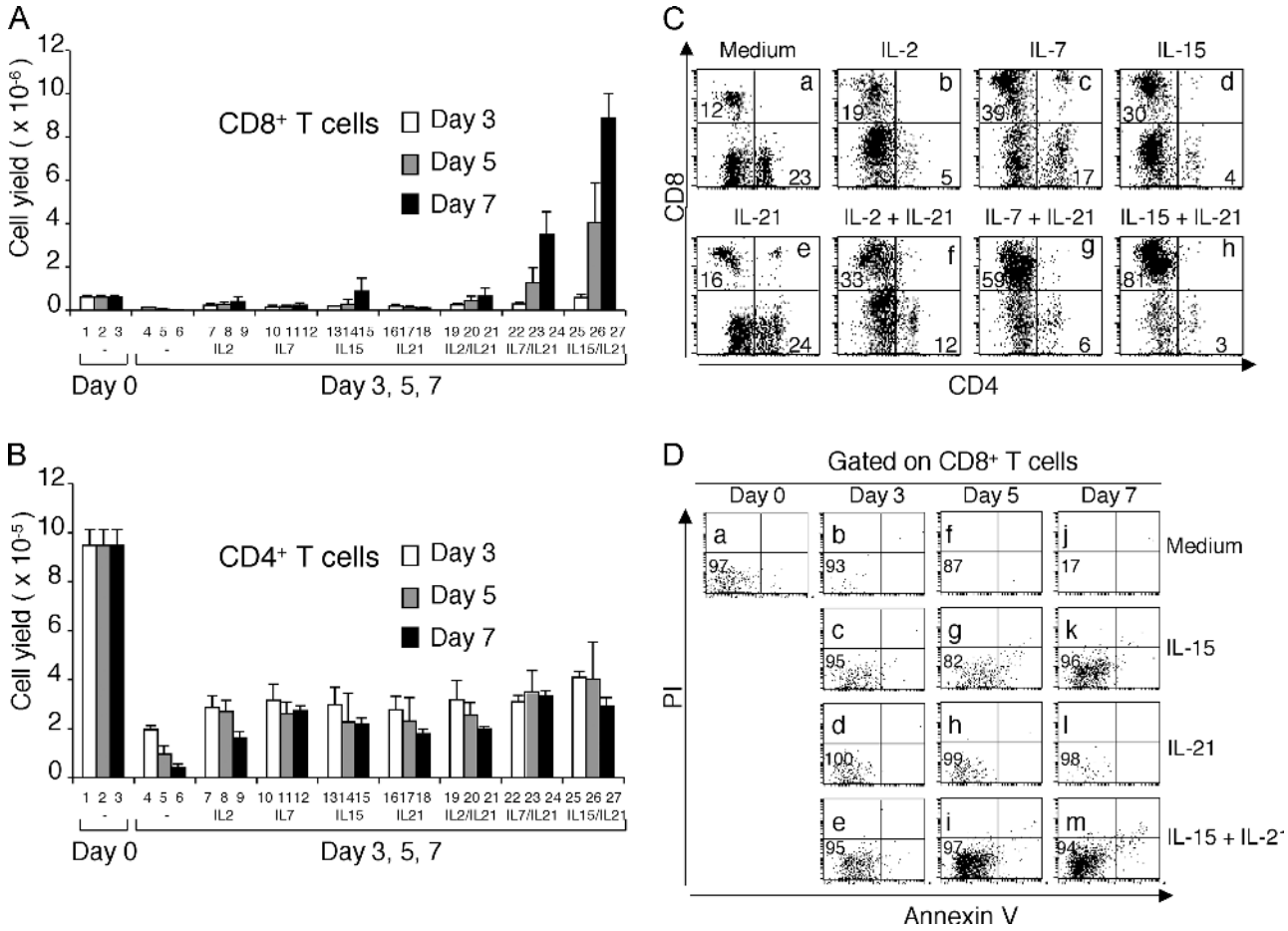

Figure 2. IL-21 acts in concert with IL-15 or IL-7 but not with IL-2 to expand CD8$^+$ T cells. (A and B) 5×10^6 splenocytes pooled from three wild-type mice were cultured for 3, 5, and 7 d in medium containing 100 U/ml IL-2, 100 ng/ml IL-7, 100 ng/ml IL-15, 100 ng/ml IL-21, or combinations of these cytokines, as indicated. CD8$^+$ T (A) and CD4$^+$ T (B) cell subsets were identified as CD8$^+$CD4$^-$ and CD8$^-$CD4$^+$, respectively. Results shown are means ± SD from three experiments. (C) Representative flow cytometric analysis of cells cultured for 7 d as described in A and B. Percentages of selected cell populations are indicated in the quadrant corners. (D) Cells from A were also stained with annexin V and propidium iodide (PI). Percentages of double negative subpopulations corresponding to viable cells are indicated in the quadrant corners. Data representative of three separate experiments are shown.

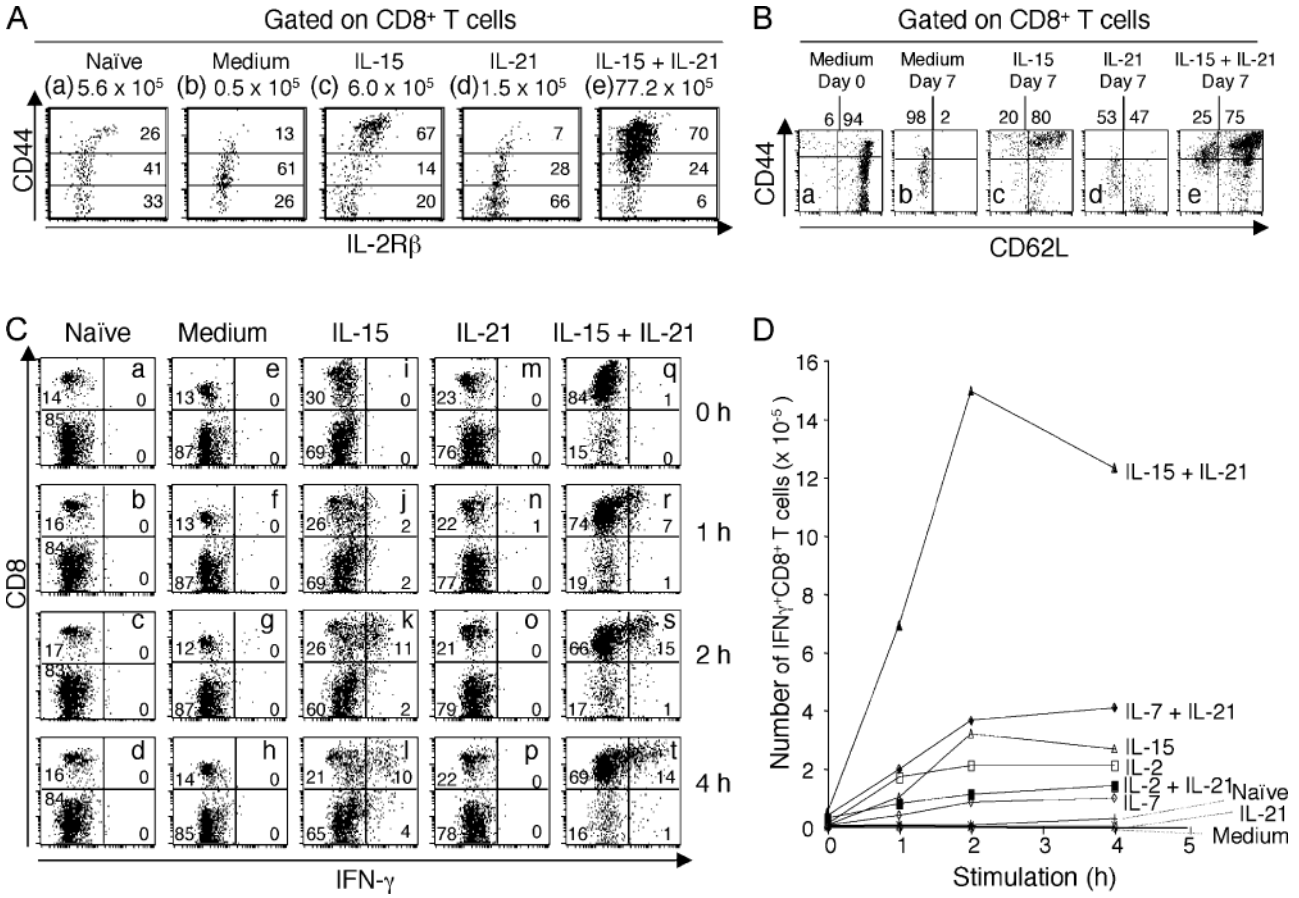

Figure 3. IL-15 and IL-21 cooperatively enhance effector function of memory-phenotype CD8$^+$ T cells. (A and B) 5 × 10^6 splenocytes pooled from two to three wild-type mice were cultured for 7 d in medium containing 100 ng/ml of IL-15, IL-21, or combinations of these cytokines, as indicated (b–e). The naive cells (A, subpanel a) were splenocytes from a littermate that were directly analyzed without culture. Cells were analyzed by flow cytometry after staining with (A) CD8-allophycocyanin, CD4-FITC, CD44-CyChrome, and IL-2Rβ-PE or (B) CD8-allophycocyanin, IL-2Rβ-FITC, CD44-CyChrome, and CD62L-PE, respectively. Data representative of three separate experiments are shown. The absolute numbers of gated CD8$^+$ T cells and the percentages of CD44^{high}, CD44^{intermediate}, and CD44^{low} subpopulations are indicated in A. The percentages of CD62L^{low} and CD62L^{high} subpopulations are indicated in B. (C and D) Freshly isolated splenocytes or cells cultured for 7 d with the indicated cytokines were stimulated with anti-CD3 and anti-CD28 for 0, 1, 2, or 4 h. (C) Representative flow cytometric profiles. (D) The absolute number of IFN-γ–producing CD8$^+$ T cells. Mean values for three similar experiments are shown. The increase in IFN-γ$^+$CD8$^+$ T cells with IL-15 and IL-21 was highly significant.

hance IL-15–mediated expansion and function of these cells. IL-21 alone had little effect on CD44^{high}CD8$^+$ T cells, but the number and percentage of CD44^{low}CD8$^+$ T cells was higher than was found in cultures without cytokines (Fig. 3 A, d vs. a and b). As expected, IL-15 expanded CD44^{high} cells (Fig. 3 A, c vs. a and b), but the combination of IL-15 and IL-21 resulted in a striking further increase in CD44^{high} cells (note percent and total cellularity; Fig. 3 A, e vs. c). To distinguish effector and central memory-phenotype CD8$^+$ T cells (11), we stained cells with anti-CD62L mAb. Cells stimulated with IL-15 were primarily central memory-phenotype (CD62L^{high}CD44^{high}; Fig. 3 B, c vs. a and b), whereas those stimulated with IL-21 lacked high expression of CD44 (Fig. 3 B, d). When both IL-15 and IL-21 were added, CD62L^{high}CD44^{high} cells were expanded, analogous to what was seen with IL-15 (Fig. 3 B, e vs. c); in addition, a prominent CD62L^{low}CD44^{high} population of cells was evident, which may represent effector memory-phenotype cells (Fig. 3 B, panel e). These data suggest that IL-21 contributes to the expansion of both subsets of memory-phenotype CD8$^+$ T cells.

To characterize the effector function of cytokine-expanded CD8$^+$ T cells, we examined intracellular IFN-γ levels after stimulation with anti-CD3 and anti-CD28. The combination of IL-15 and IL-21 resulted in a marked increase in the number of CD8$^+$ T cells, with a modest increase in the percent of IFN-γ–producing cells at 1, 2, and 4 h, as compared with that seen in cells expanded with IL-15 alone (Fig. 3 C, r–t vs. j–l), whereas IL-21 by itself had little effect (Fig. 3 C, n–p). As shown in Fig. 3 D, the combination of IL-15 and IL-21 greatly increased the total number of IFN-γ–producing CD8$^+$ T cells. Thus, IL-21 has a cooperative effect with IL-15 on the expansion of memory-phenotype CD8$^+$ T cells as well as on their effector function.

IL-21 and IL-15 together markedly accelerate cell division of both memory-phenotype and naive-phenotype CD8$^+$ T cells

In the aforementioned in vitro culture assays, we used total splenocytes. Thus, it is possible that CD4$^+$ T cells or other cells might contribute to the expansion of CD8$^+$ T cells via paracrine secretion of cytokines, and that IL-15 and IL-21 might act on other cells to indirectly affect the expansion/survival of CD8$^+$ T cells. To investigate whether IL-15 and IL-21 had a direct synergistic effect on cell cycle progression in CD8$^+$ T cells, we isolated CD8$^+$ T cells from splenocytes and stained them with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). These cells divided faster when cultured with both IL-15 and IL-21 as compared with either cytokine alone (Fig. 4 A, d vs. b and c, and h vs. f and g). On day 4, cells treated with both IL-15 and IL-21 exhibited one to five more divisions than cells in control cultures and the recovered cell number was significantly higher (Fig. 4 A, d vs. a–c). On day 7, these effects were even greater (Fig. 4 A, h vs. e–g). The combination of IL-7 and IL-21 had a similar albeit less potent effect to that seen with IL-15 + IL-21 (unpublished data). To further examine the effect of IL-15 and IL-21 on memory-phenotype or naive-phenotype CD8$^+$ T cells, we purified CD44^{high} and CD44^{low} CD8$^+$ T cells, respectively, and stained with CFSE. IL-15 more potently promoted the division of CD44^{high}CD8$^+$ T cells than did IL-21, but both cytokines together had the greatest effect (Fig. 4 A, l vs. i–k, and p vs. m–o). The cell number was four- to fivefold higher in culture with IL-15 + IL-21 than with IL-15 alone on day 4 (Fig. 4 A, l vs. j). The relative fold increase on day 7 was reproducibly lower than on day 4; this might result from a decrease in the survival rate on day 7 (Fig. 4 B, h vs. f). Neither IL-15 nor IL-21 alone had much of an effect on the division of CD44^{low}CD8$^+$ T cells (Fig. 4 A, r and s vs. q, and v and w vs. u), but each improved the cell survival rate (Fig. 4 B, j and k vs. i, and n and o vs. m). Strikingly, stimulation with both IL-15 and IL-21 potently promoted cell division (Fig. 4 A, t vs. r and s, and x vs. v and w) and cell survival (Fig. 4 B, l vs. j and k, and p vs. n and o) of CD44^{low}CD8$^+$ T cells. Thus, IL-21 and IL-15 synergistically affect cell division of memory-phenotype CD8$^+$ T cells. Moreover, in contrast with IL-15 alone, IL-21 + IL-15 increased the division of naive-phenotype CD8$^+$ T cells as well.

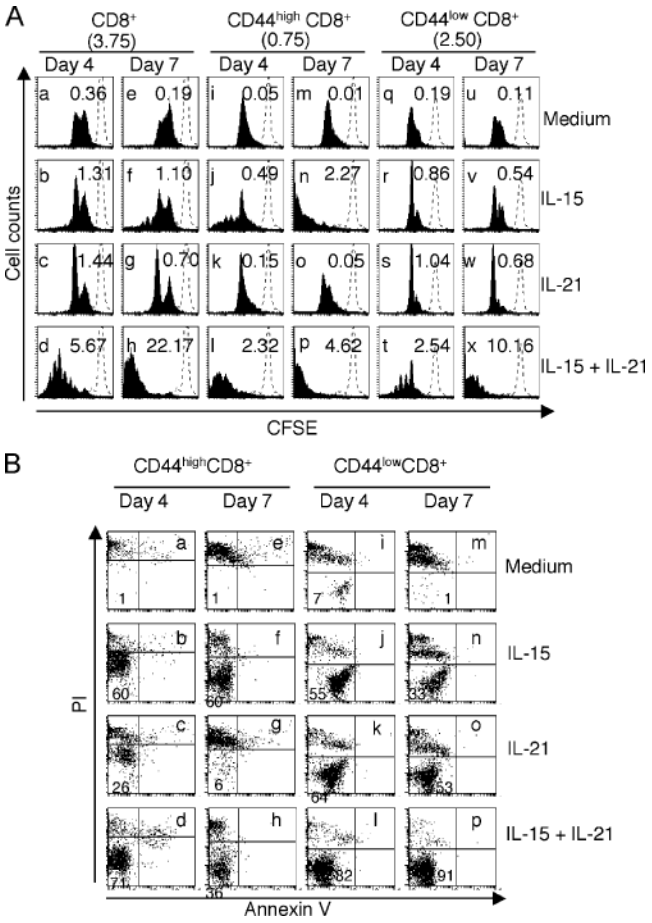

Figure 4. IL-15 and IL-21 cooperatively increase cell cycle progression of both CD44^{high} and CD44^{low} CD8$^+$ T cells. (A) All cell subpopulations were isolated and stained with CFSE as described in Materials and Methods. The "basal" CFSE profile on day 0 is shown as the dashed line. Cells were cultured in complete medium at 2–5 × 10^5 cells/ml without cytokine or with 100 ng/ml of IL-15 and/or IL-21 for 4 or 7 d, as indicated, and analyzed by flow cytometry. The indicated cell numbers (×10^{-6}) are an average of cell numbers from three independent experiments. In each experiment, the starting cell numbers were normalized to 3.75 × 10^6 (CD8$^+$ T cells), 0.75 × 10^6 (CD44^{high}CD8$^+$ T cells), and 2.5 × 10^6 (CD44^{low}CD8$^+$ T cells), respectively, which allowed comparison of the different experiments. Flow cytometric results shown are representative of three experiments. (B) Cells in A were also stained with annexin V and PI. The percentages of double negative subpopulations corresponding to viable cells are indicated in the quadrant corners. Data representative of three separate experiments are shown.

Defective antigen-specific CD8$^+$ T cell responses in IL-21R$^{-/-}$ mice

To further examine the effect of IL-21 on in vivo expansion and effector function of antigen-specific CD8$^+$ T cells, both wild type and IL-21R$^{-/-}$ mice were immunized with vaccinia virus expressing HIV gp160 (vPE16). The cytotoxic activity of CD8$^+$ T cells from IL-21R$^{-/-}$ mice was significantly lower than CD8$^+$ T cells from wild-type mice (Fig. 5, A and B), suggesting that IL-21R signaling contributes to the primary CD8$^+$ cytotoxic T cell response. Correspondingly, the frequencies of tetramer-positive (Fig. 5 C) and IFN-γ–positive (Fig. 5 D) CD8$^+$ T cells, as analyzed immediately ex vivo or after 1 wk of restimulation in vitro, were lower in IL-21R$^{-/-}$ mice than in wild-type mice. The difference in the frequency of antigen-specific cells (Fig. 5, C and D) can at least in part account for the difference in CTL activity. These results demonstrated a role for IL-21 in antigen-specific CD8$^+$ T cell expansion and function.

Cooperative effect of IL-21 and IL-15 on tumor regression, with cures of established B16 melanomas

Given the effects of IL-15 and IL-21 on CD8$^+$ T cell expansion and cytotoxicity in vitro, next we investigated the effects of these cytokines in vivo using a tumor model. IL-

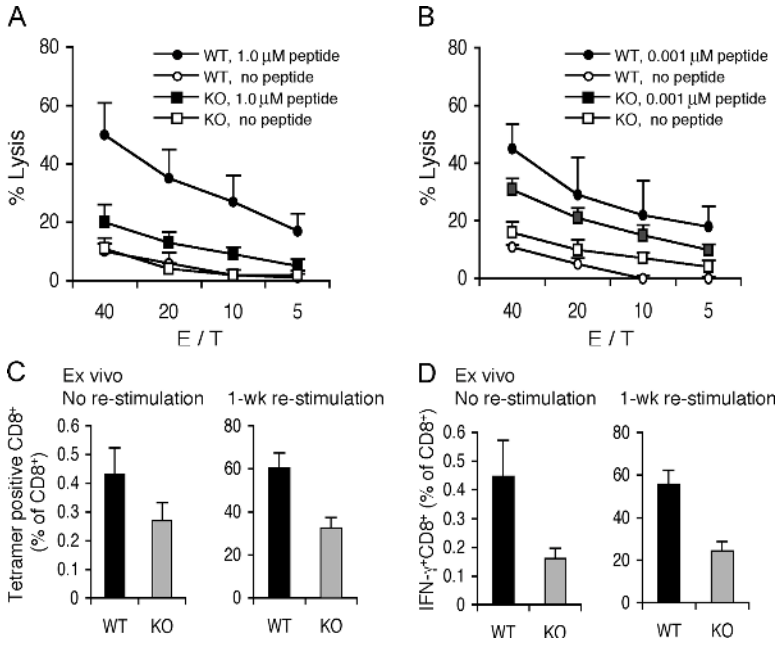

Figure 5. Antigen-specific CD8$^+$ T cell responses are impaired in IL-21R$^{-/-}$ mice. Five mice in each group were immunized i.p. with 5×10^6 PFU of vPE16. (A and B) On day 5, splenocytes of immunized WT and IL-21R$^{-/-}$ (KO) mice were restimulated with 1.0 μM (A) or 0.001 μM (B) P18-I10 for 1 wk, and lytic activity was measured by a 5-h ^{51}Cr release assay. P815 cells pulsed with 1.0 μM (A) or 0.001 μM (B) P18-I10 were used as target cells. Means ± SEM are shown. (C) The frequency of P18-I10–specific splenic CD8$^+$ T cells was measured by H-2D^d–P18-I10 tetramer staining ex vivo without restimulation in vitro (left) or after 1 wk of restimulation with 1.0 μM P18-I10 (right). Shown is the mean percent ± SEM of tetramer-positive CD8$^+$ T cells in total CD8$^+$ T cells for five mice per group. The difference in the two groups was statistically significant ($P < 0.05$). (D) The frequency of IFN-γ$^+$CD8$^+$ T cells in spleens was measured by intracellular staining ex vivo without restimulation in vitro (left) or after 1 wk of restimulation with 1.0 μM P18-I10 (right). Cells were stimulated with 1.0 μM P18-I10 for 10 h in the presence of 1 μg/ml brefeldin A. Shown is the mean percent ± SEM of IFN-γ–positive (IFN-γ$^+$) CD8$^+$ T cells in total CD8$^+$ T cells for five mice per group. The difference in the two groups was statistically significant ($P < 0.05$).

21 has been reported to have antitumor effects (19–22). We used a tumor immunotherapeutic strategy that allowed us to evaluate the effects of cytokines on the regression of large, established solid tumors when cytokines and CD8$^+$ T cells specific for the gp100 self-antigen were administered concomitantly (23–25). pmel-1 transgenic mice on a C57BL/6 background express the Vα1Vβ13 TCR from a cloned T cell, which recognizes an H-2D^b–restricted epitope corresponding to amino acids 25–33 of gp100 (23). More than 95% of the CD8$^+$ T cells in pmel-1 TCR transgenic mice were Vβ13$^+$, amounting to ~20% of all splenocytes (23). In vitro–activated pmel-1 splenocytes were adoptively transferred into sublethally irradiated mice bearing subcutaneous B16 melanomas (an H-2D^b gp100$^+$ spontaneous murine melanoma) established for 8 or 10 d (Fig. 6, A and B). This adoptive transfer was followed by vaccination with recombinant fowlpox virus encoding hgp100 (rFPVhgp100), which encodes the hgp100-altered peptide ligand, in the presence of exogenous cytokines. IL-15 + IL-21 (without pmel-1 cells) had little if any effect on the tumor (Fig. 6 A), indicating that reconstituted endogenous lymphocytes have no significant effect on tumor regression. Pmel-1 cells with IL-15 + IL-21 also had relatively little effect on the tumor unless vaccination with rFPVhgp100 was included (Fig. 6 A). This confirms that antigen-specific cells, vaccination, and cytokine are all required for maximal tumor regression in this model (23). As shown in Fig. 6 B, treatment with either IL-15 or IL-21 induced partial tumor regression, whereas the combination of IL-15 and IL-21 was much more effective. In the experiment shown in Fig. 6 B, all of the mice died of tumor within 32 d of treatment except for those treated with the combination of both IL-15 and IL-21 (not depicted). Moreover, all five mice receiving IL-15 plus IL-21 were alive at day 32; two of these animals had complete regression of their tumors with vitiligo at the former melanoma sites, whereas the other three mice had residual tumor. In an independent experiment, treatment with 5 μg/dose of IL-15 plus 5 μg/dose of IL-21 was more effective than either cytokine alone at 10 μg/dose (unpublished data). Consistent with an enhanced effect of IL-15 plus IL-21 on CD8$^+$ T cells, at 3 and 4 wk of treatment, the absolute number of pmel-1 TCR transgenic CD8$^+$ T cells (Vβ13$^+$ CD8$^+$) in blood was higher in mice treated with both IL-15 and IL-21 than with either cytokine alone (Fig. 6 C, left). These findings were confirmed in a second experiment at day 20; day 28 data are not available for this experiment as it was terminated at day 23, at which point four out of six mice treated with the combination of IL-15 and IL-21 had complete regression of their tumors (unpublished data). Thus, IL-15 and IL-21 synergistically expand CD8$^+$ T cells in vivo and this correlated with marked regression of large, established solid tumors.

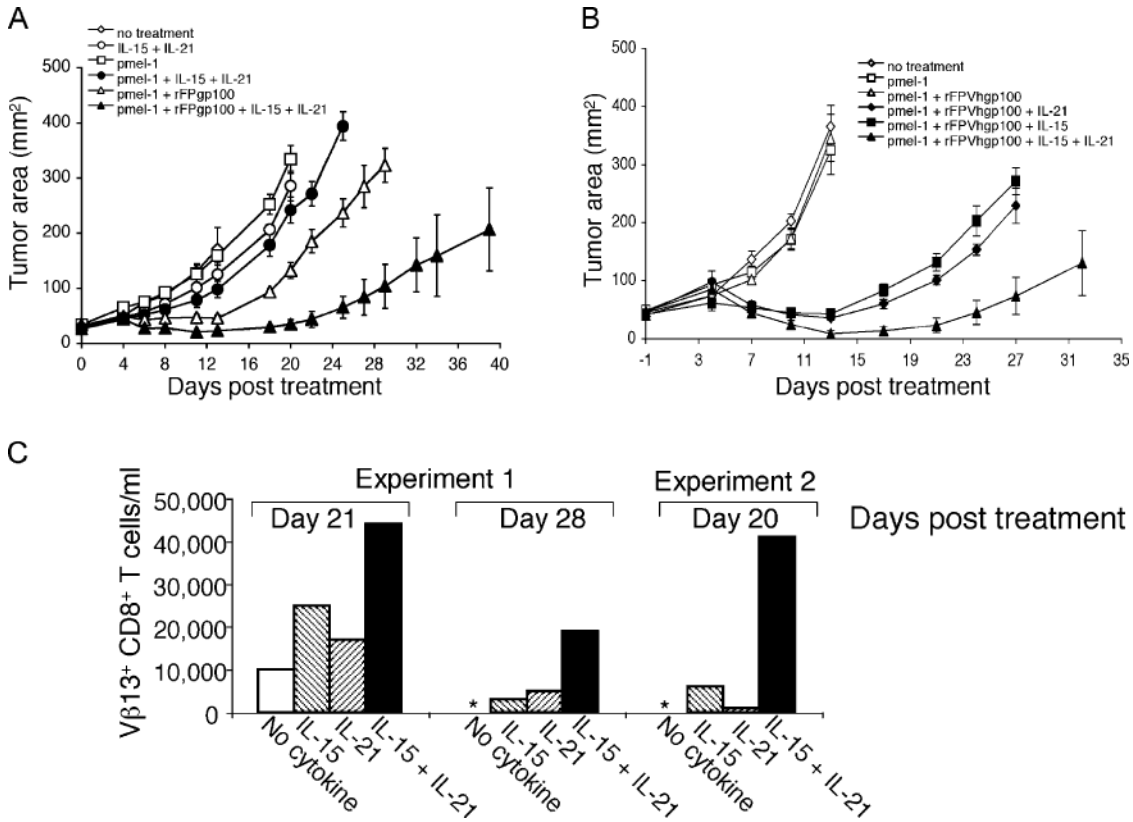

Figure 6. In vivo administration of IL-21 in combination with IL-15 yields tumor regression with cures of large, established B16 melanomas. (A) Sublethally irradiated female C57BL/6 mice were implanted subcutaneously with B16 melanomas. 8 d later, mice were treated by adoptively transferring cultured pmel-1 splenocytes (0.5×10^6 Vβ13$^+$ CD8$^+$ T cells) as indicated and, where indicated, vaccinating with rFPVhgp100. In some conditions, IL-15 and IL-21 were administered twice daily for six doses (5 μg each/dose; five to seven mice in each group). (B) Sublethally irradiated female C57BL/6 mice were implanted subcutaneously with B16 melanomas. 10 d later, mice were treated by adoptively transferring cultured pmel-1 splenocytes (10^6 Vβ13$^+$ CD8$^+$ T cells) and vaccinating with rFPVhgp100. IL-15, IL-21, or both cytokines was administered twice daily for six doses (10 μg each /dose; five to seven mice in each group). A repeat experiment showed similar results. Shown are mean tumor sizes ± SEM. (C) Shown are Vβ13$^+$CD8$^+$ T cell numbers from mice from two experiments (days 21 and 28 from Experiment 1 in B and day 20 from Experiment 2, which was terminated at day 23). *, Mice not receiving cytokine had all died from their tumors, so samples were not available for analysis.

A subset of genes is synergistically regulated by IL-21 and IL-15

To begin to clarify the molecular mechanisms by which IL-21 cooperates with IL-15 on CD8$^+$ T cell expansion and function, next we performed DNA microarray analyses (Affymetrix, Inc.) using mRNAs isolated from naive CD8$^+$ T cells treated for 4 h with cytokines, and we found ~300 genes that were regulated by IL-15 and/or IL-21. The overall expression pattern seen in CD8$^+$ T cells stimulated with both IL-15 and IL-21 is more similar to that stimulated with IL-21 than IL-15 (Fig. 7 A). However, as expected, some genes were regulated by the combination of IL-15 and IL-21 in similar fashion to that seen with either cytokine alone (Fig. 7, B and C), whereas other genes, such as granzyme B and c-Jun, exhibited induction or repression that was greater with IL-15 plus IL-21 than with either cytokine alone (Fig. 7 D and see Discussion).

DISCUSSION

In this paper, we demonstrate that T cells are markedly expanded by IL-21 in synergy with IL-15 or IL-7, and that this expansion is most potent for CD8$^+$ T cells. IL-15 and/or high doses of IL-7 are known to be required for memory (CD44^{high}) CD8$^+$ T cell survival and proliferation (8). Our results indicate that both memory-phenotype and naive-phenotype CD8$^+$ T cells are expanded by IL-21 + IL-15 and that IL-21 is necessary for an optimal CD8$^+$ T cell response to antigen. Kasaian et al. reported an enhancement of the antigen-driven CD8$^+$ T cell response by IL-21 but surprisingly found no effect of IL-21 on IL-15–mediated, TCR-independent T cell expansion (5), which differs from our results. The concentration of IL-21 in their paper is not defined in nanograms per milliliter as they used conditioned medium from transfected COS cells as a source of IL-21 and, thus, we speculate that the amount of IL-21 used in their work may not have been sufficient to achieve the synergistic effect that we observed. At higher levels of IL-15, we observed marked synergy even with 10 ng/ml of IL-21, and importantly this synergistic effect of IL-15 and IL-21 on expansion in vitro of purified CD8$^+$ T cells is consistent with the effect that we observe in vivo.

How do IL-15 and IL-21 regulate CD8 T cell expansion and effector functions? Our results indicate that IL-15 and

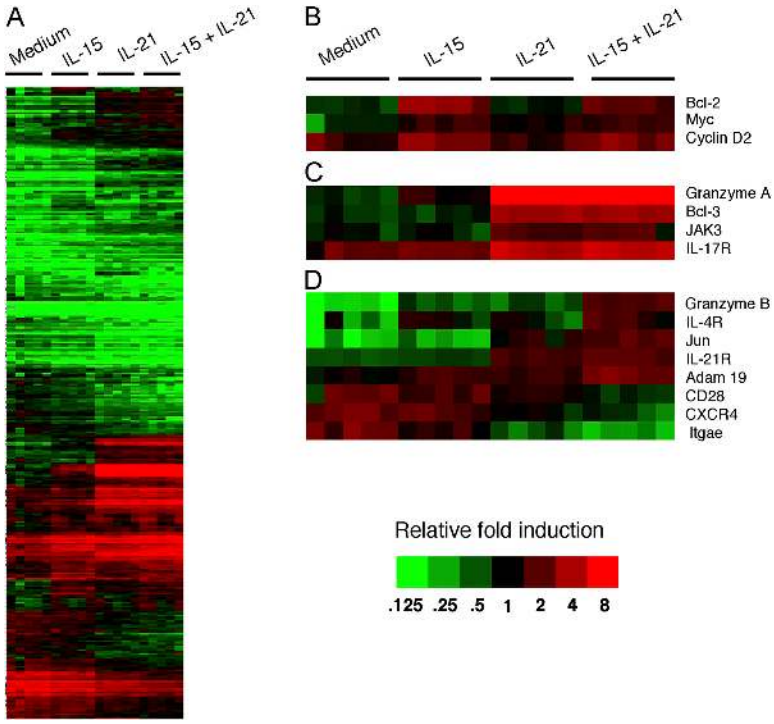

Figure 7. Several genes are synergistically regulated by IL-21 and IL-15 in CD8$^+$ T cells. Naive CD8$^+$ T cells were isolated, treated without cytokine or with 100 ng/ml of IL-15 and/or IL-21 for 4 h, and subjected to RNA isolation and Affymetrix Gene Chip analysis as described in Materials and Methods. Results shown are from five independent experiments. Shown in A are the overall expression patterns of genes that are induced or repressed more than twofold by IL-15, IL-21, or both cytokines. Also shown are selected genes that are preferentially regulated by IL-15 alone (B), IL-21 alone (C), or IL-15 plus IL-21 (D).

IL-21 together accelerate cell division of isolated CD8$^+$ T cells. Although more work is needed to clarify which genes mediate the synergistic actions of IL-15 and IL-21, it is interesting that granzyme B, which plays an important role for cytotoxicity, and c-Jun, which is important for optimal proliferation, are both preferentially induced by the combination of IL-15 and IL-21.

Homeostatic control of CD8$^+$ T cells is essential for defense against infectious pathogens. IL-15 is known to be a critical regulator of memory CD8$^+$ T cell homeostasis and might also contribute to naive CD8$^+$ T cell survival. IL-7 is required for naive CD8$^+$ T cell survival and homeostatic proliferation but also contributes to memory CD8$^+$ T cell homeostasis. We have now identified IL-21 as a new regulator of these cells, suggesting that it is yet another γ_c-dependent cytokine that critically regulates T cell homeostasis. Although IL-21 alone showed little effect on CD8$^+$ T cells, it synergistically promoted the proliferation and survival of both memory and naive CD8$^+$ T cells. Our data are consistent with a cooperative effect of IL-15 and IL-21 on the generation and expansion of cytotoxic T cells, as would occur, for example, after viral infection. Although IL-15 was previously shown to cooperate with IL-21 in preventing the establishment of a murine lymphoma, our data demonstrate that synergistic actions of IL-15 and IL-21 can result in complete regression of large established B16 melanomas, with an associated expansion of tumor-specific CD8$^+$ T cells. This supports the previously suggested role of IL-15 as an antitumor agent (26). A recent paper suggested that IL-21 contributed to the homeostatic expansion of T cells, but that it could not support their survival (27). Our experiments collectively indicate that IL-21 has both proliferative and cell survival effects for CD8$^+$ T cells and that its effects on expansion are greatly augmented when IL-15 is also added. Although CD8$^+$ T cells expanded in vitro using IL-2 or IL-15 can be reintroduced in vivo to augment the killing of tumor cells (28, 29), our results indicate that the combination of IL-21 with IL-15 may be a more powerful method for expanding CD8$^+$ T cells, both in vitro and in vivo, and enhancing CD8$^+$ T cell function.

MATERIALS AND METHODS

Mice. WT mice (C57BL/6 or Balb/c) were obtained from the National Cancer Institute, IL-15$^{-/-}$ mice were purchased from Taconic Laboratory, and IL-21R$^{-/-}$ mice were described previously (6). Mice were analyzed at 8–16 wk of age. All experiments were performed under protocols approved by the appropriate Animal Use and Care Committees and followed the National Institutes of Health (NIH) guidelines entitled "Using Animals in Intramural Research."

In vitro cell culture and survival assay. Single cell suspensions of spleen were prepared by gently pressing the tissues through fine nylon screen. Erythrocytes were depleted with ACK lysis buffer (BioFluids). CD8$^+$ T cells were prepared as described in the next paragraph. Cells were plated at $\sim 5 \times 10^5$/ml in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM β-mercaptoethanol (RPMI 1640 complete medium) with human IL-2 (Roche), mu-

rine IL-7 (PeproTech), human IL-15 (PeproTech), or murine IL-21 (R&D Systems) as indicated. Cells were cultured at 37°C for 3, 4, 5, or 7 d, and a second dose of cytokines was added on day 4. Cells were counted and analyzed by flow cytometry on the indicated day. The cell survival assay was performed using annexin V–FITC or annexin V–Biotin Apoptosis Detection kit according to the manufacturer's protocols (R&D Systems).

CD8$^+$ T cell isolation and labeling with CFSE. CD8$^+$ T cells were positively selected using paramagnetic Microbeads conjugated to anti–mouse CD8α (Ly-2) monoclonal antibody according to the manufacturer's instructions (MACS; Miltenyi Biotec). To purify CD44^{high} and CD44^{low} CD8$^+$ T cells, CD8$^+$ T cells were negatively selected using paramagnetic Microbeads conjugated to anti–mouse CD4 (L3T4) and anti–mouse CD45R (B220) monoclonal antibodies. The resultant cells were labeled with anti-CD8–allophycocyanin, anti-CD44–CyChrome, and anti–IL-2Rβ (CD122)–PE and sorted for CD44^{high} and CD44^{low}CD8$^+$ T cells on a MoFlo Cell Sorter (DakoCytomation). The resulting populations were >95% pure. Isolated CD8$^+$ T cells were labeled with 5 μM CFSE (Molecular Probes) for 15 min at 37°C.

Immunization of mice with HIV-1IIIB gp160 and measurement of cytotoxicity and tetramer$^+$ and IFN-γ$^+$ CD8$^+$ T cells. The recombinant vaccinia virus expressing the full-length HIV-1IIIB gp160 (vPE16) was described previously (30). Wild-type and IL-21R$^{-/-}$ mice were immunized i.p. with 5×10^6 PFU of vPE16. The immunodominant peptide epitope (RGPGRAFVTI; known as the P18-I10 peptide) within HIV-1IIIB gp160 in H-2D^d mice (31, 32) was synthesized (Multiple Peptide Systems). Splenocytes from the immunized mice were cultured at 4×10^6 cells/well in 24-well plates containing 2 ml of RPMI 1640 complete medium supplemented with 10% rat T-stim (Collaborative Biomedical). To stimulate peptide-specific CD8$^+$ T cells in vitro, 1.0 μM or 0.001 μM P18-I10 peptide was added into the cultures. On day 7, CTL activity was measured using a 5-h ^{51}Cr release assay. P815 cells, which were maintained in RPMI 1640 complete medium and pulsed with 1.0 μM or 0.001 μM P18-I10, were used as target cells. The percent specific lysis was calculated as 100 × (experimental release-spontaneous release)/(maximum release-spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 2.5% Triton X-100. For P18-I10 H-2D^d tetramer staining, cells were incubated with FITC-labeled anti-CD8 for 30 min, PE-labeled P18-I10-H-2D^d-tetramer (provided by the NIH Tetramer Core Facility, Atlanta, GA) was added, and the cells were incubated for an additional 30 min on ice. The tetramer was used at dilutions of 1:200 or 1:300 for fresh spleen cells and 1:50 for in vitro–restimulated cells. Background staining was assessed by use of an isotype control antibody. For IFN-γ induction, cells were stimulated with 1.0 μM P18-I10 for 10 h in the presence of 1 μg/ml brefeldin A.

Intracellular IFN-γ staining. Splenocytes were cultured in RPMI 1640 complete medium in 96-well plates at 2×10^5 cells/well containing no cytokine, IL-15, IL-21, or both cytokines. After 7 d, cells were stimulated for 1, 2, or 4 h with 2 μg/ml of soluble anti-CD3ε and 2 μg/ml anti-CD28, and stained for cell surface markers. The cells were fixed and permeabilized using Cytofix/Cytoperm solution, followed by staining with PE-conjugated IFN-γ mAb (BD Biosciences) as described previously (33).

Flow cytometric analyses. Cells were stained and analyzed on a FACSCaliber or FACSort with CellQuest software (BD Biosciences). The following mAbs, all from BD Biosciences, were used: anti-CD4–FITC, anti-CD8–allophycocyanin, anti–mouse CD8-FITC, anti-TCRβ–allophycocyanin, anti–IL-2Rβ (CD122)–FITC, anti–IL-2Rβ (CD122)–PE, anti-CD44–CyChrome, anti-B220–FITC, anti-NK1.1–PE, anti-Vβ13–FITC, and anti-CD44–PE.

Immunotherapy of B16 melanoma. Sublethally irradiated (500 rad) female C57BL/6 mice (The Jackson Laboratory) were injected subcutaneously with 3×10^5 mycoplasma-free B16-F10 melanoma cells. B16 is an H-2^b gp100$^+$ spontaneous murine melanoma and was maintained in RPMI 1640 complete medium. 8–10 d later, animals (n = 5–7 for each group) were treated by intravenous injection of in vitro–cultured splenocytes ($0.5–1 \times 10^6$ Vβ13$^+$CD8$^+$ T cells) from pmel-1 TCR transgenic mice (23). For culturing, fresh splenocytes from pmel-1 mice were depleted of erythrocytes and cultured in RPMI 1640 complete medium containing 2 ng/ml of human IL-2 (Chiron Corp.) and 1 μM hgp100$_{25-33}$. Cells were used for adoptive cell transfer (ACT) 6–7 d later. Where indicated, mice were also immunized with 2×10^7 PFU of rFPVhgp100 (Therion Biologics; reference 23). 5–10 μg IL-15 or IL-21 was freshly reconstituted in PBS and administered by i.p. injection twice daily, beginning the day of adoptive transfer, for 3 d. Tumors were measured in a blinded fashion using calipers, and the products of the perpendicular diameters were calculated. Tumor size (rank sum test) and survival data (Kaplan-Meier) were recorded for over 4 wk after treatment and analyzed. For quantifying pmel-1 T cells, for each group, treated mice were bled by tail vein, samples were pooled, and total lymphocyte numbers and flow cytometric profiles for Vβ13 and CD8 were determined by the NIH Clinical Center Clinical Immunology Laboratory.

RNA purification and Affymetrix Gene Chip analysis. Total RNA was isolated (RNeasy; QIAGEN) from naive CD8$^+$ T cells with 4 h of stimulation of cytokines (100 ng/ml each), processed to cRNA probes for gene chip analysis, and probes were hybridized to U430A GeneChips (Affymetrix, Inc.; these chips contain oligonucleotides corresponding to 22K transcripts per microarray), washed, and scanned (Hewlett Packard Gene Array scanner G2500A) according to procedures outlined by the manufacturer (Affymetrix, Inc.). Data were analyzed with open source clustering software Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/index.html; reference 34).

Online supplemental material. Fig. S1 shows that IL-21 cooperated with IL-7 but not as potently as it does with IL-15 on CD8$^+$ T cell expansion. Fig. S2 shows that synergistic expansion of CD8$^+$ T cells is diminished in both IL-15$^{-/-}$ and IL-21R$^{-/-}$ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041057/DC1.

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