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Materials and Methods

Mice

C57BL/6 (B6), B6.PL (Thy1.1), pmel-1 T-cell receptor (TCR) transgenic (32), β2-microglobulin$^{-/-}$ (β2m$^{-/-}$), and IL7Rα$^{-/-}$ mice were obtained from The Jackson Laboratory. IL15$^{-/-}$ mice were purchased from Taconic. H3T TCR transgenic mice were generated as previously described (33). Pmel-1 mice were maintained by crossing a pmel-1 (male) to a Thy1.1 (female) generating hemizygous offspring. IL7Rα$^{+/-}$ heterozygous mice were generated by crossing a IL7Rα$^{-/-}$ male to either a Thy1.1/1.1 homozygous B6 female (generating B6 IL7Rα$^{+/-}$ Thy1.1/1.2 mice) or a pmel$^{+/+}$ Thy1.1/1.1 homozygous female (generating pmel$^{+/-}$ IL7Rα$^{+/-}$ Thy1.1/Thy1.2 mice). All mice used were between 6 and 16 weeks of age. Mice were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines at the Medical University of South Carolina (MUSC; Charleston, SC).

Cell cultures

B16-F1 tumor cells were obtained from the ATCC and immediately expanded and frozen down into a large number of aliquots. Cells were verified to be *Mycoplasma* free and one aliquot was briefly expanded for each experiment using culture conditions as previously described (18). All T cells were grown in RPMI-1640 complete media as described previously (18). For generation of mouse gp100-reactive T cells, pmel-1 TCR transgenic splenocytes (1 × 10^6 cells/mL) were stimulated with 1 μg/mL H-2D^b-restricted human gp100$_{25–32}$ peptide (KVPRNQDWL; American Peptide Company) for 3 days with or without mIL12 (10 ng/mL; Shenandoah Biotechnology) to generate Tc1 or Tc0 T cells, respectively. For generation of mouse tyrosinase-reactive T cells, h3T TCR transgenic splenocytes were cultured with irradiated T2-A2 cells loaded with 1 μg/mL HLA-A2–restricted human tyrosinase$_{368–376}$ peptide (YMDGTMSQV; American Peptide Company) for 3 days with or without mIL12. Polyclonal stimulations were performed by adding 1 μg/mL soluble anti-CD3 mAb (145-2C11) ± 2 μg/mL anti-CD28 mAb (37.51) directly or by coating a 24-well plate with 1 μg/mL anti-CD3 ± 2 μg/mL anti-CD28 before addition of splenocytes.

Cytokine responsiveness

Cytokine responsiveness was assessed by washing cells three times in PBS, then replating cells at 0.8–1 × 10^6/mL with the indicated cytokine (mouse cytokines from Shenandoah Biotechnology). After overnight incubation, cells were either fixed/permeabilized for phosflow analysis per the manufacturer's instructions (Phosflow; BD Bioscience) or 10 μmol/L bromodeoxyuridine (BrdU) was added for 1 hour at 37°C and cells were processed according to the manufacturer's protocol (BrdU Flow Kit; BD Bioscience) . Note that the percentage of cells that were pSTAT5$^+$ 15 minutes after restimulation was not significantly different from values obtained after overnight incubation (data not shown).

Flow cytometry

For flow-cytometric analysis, cells were processed as previously described (18) and analyzed on either an LSRII or Accuri C6 flow cytometer (BD Bioscience). Data were processed using FlowJo (TreeStar) or C6 software (BD Bioscience). Mouse antibody clones used in this study include: CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61.5), CD62L (MEL-14), CD122 (TM-β1), IL7Rα (SB/199 or A7R34), Eomes (Dan11mag), granzyme B (GB12), IFNγ (XMG1.2), pAKT S473 (D9E), pSTAT5 (47/Stat5 pY694), pS6 (D57.2.2E), Thy1.1 (OX-7 or HIS51), TNFα (TN3-19.12), and Tbet (4B10). Human antibody clones used are CD8 (OKT8 or SK1) and IL7Rα (eBioRDR5 or A019D5). These were purchased from BD Bioscience, BioLegend, Invitrogen, eBioscience, and/or Cell Signaling Technology.

Tumor challenge, lymphodepletion, and adoptive T-cell transfer

For tumor experiments, B6 mice were injected subcutaneously (s.c.) with 2.5 × 10^5 B16-F1 tumor. Tumor growth was measured by an observer blinded to treatment groups with calipers two to three times per week and tumor surface area (mm^2) was calculated as length × width. Mice were sacrificed when tumors reached ≥400 mm^2. Total body irradiation (TBI) was administered at 6 Gy the day before adoptive transfer. Mice were excluded from analysis if they developed i.p. tumor spread within the first 4 weeks after injection.

In vivo cytokine neutralization

All neutralizing antibodies were purchased from BioXCell except for JES6-1A12 (UCSF monoclonal antibody core). Unless otherwise indicated, the following amounts of mAb were injected i.p. on days 0, 2, 5, 8, 12, and 17 following adoptive transfer: αIL7 (M25, 200 μg), αIL7Rα (A7R34, 500 μg), αIL2 (250 μg each of S4B6 and JES6-1A12 injected together), and mIgG2b isotype control (MPC-11, 200 μg).

Measurement of IFNγ

Day 3 culture supernatants were analyzed for mIFNγ via ELISA per the manufacturer's instructions (BioLegend).

Experiments involving human PBMCs

Deidentified human PBMCs were isolated from a leukapheresis pack obtained from Research Blood Components and experiments were performed in accordance with MUSC Institutional Review Board (IRB) guidelines. For *in vitro* stimulation, cells were thawed and rested in 100 IU/mL hIL2 overnight. The next day, 0.5 μg/mL soluble αCD3 (Okt3, NCI repository) was added to culture ± 10 ng/mL hIL12. After 3 days of activation, cytokine responsiveness and phenotype were assessed. In some experiments, activated cells were maintained in cytokines as indicated for 2 weeks. Every 2 to 3 days cells were counted and given fresh cytokine-containing media to maintain a concentration of 0.8 × 10^6 cells/mL. For generation of TCR-modified human T cells, we used a modification of a previously described protocol (34). On day 1, human PBMCs were stimulated with soluble anti-CD3 mAb (OKT3, NCI preclinical repository) for 48 hours. Beginning on day 3, cells were cultured with hIL2 (300 IU/mL) and hIL15 (100 ng/mL), and maintained between 1 and 2 × 10^6 cells/mL. Also on day 3, activated T cells were transduced by coculture with 50% retroviral supernatant from PG13 packaging cells transfected with the TIL1383ITCR/CD34t construct (35). Transduction was done with retronectin-coated plates and spinoculation (2,000 × *g* for 2 hours at 32°C). On day 8, cells underwent a rapid expansion protocol (REP) by incubation in a G-Rex 100 flask (Wilson Wolf Manufacturing) of 1 × 10^6 transduced T cells with 2 × 10^8 irradiated (50 Gy) allogeneic feeder cells from human donors. Soluble anti-CD3 mAb (OKT3, 30 ng/mL)

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was also added to the cultures. On REP day 14, cultures were harvested, washed, and replated for IL7Rα analysis 3 days later.

Statistical analysis

Statistical analysis was done with GraphPad Prism 6 software. One-way ANOVA with a Tukey multiple comparisons correction or a two-sided two-sample *t* test was used to evaluate statistical significance of means between groups. When variances were unequal, the Welch *t* test was used. Data expressed on a ratio scale (e.g., fold change) were first log-transformed to normalize the distribution, then analyzed by the *t* test or one-way ANOVA, as appropriate. For survival data, the log-rank test was used. Unless otherwise indicated, summary statistics in figures are presented as mean ± SEM.

Results

The enhanced initial engraftment of IL12-conditioned effector $CD8^+$ T cells (Tc1) transferred into lymphodepleted hosts is dependent on IL7 but not IL15

We previously demonstrated that the persistence and antitumor abilities of IL12-conditioned pmel-1 $CD8^+$ T (Tc1) cells were enhanced by cyclophosphamide, a lymphodepleting agent (18). Similarly, lymphodepletion with 6-Gy TBI before adoptive transfer of Tc1 significantly delayed the growth of established B16 tumors, while transfer of Tc1 alone or transfer of cells activated without IL12 (Tc0) into irradiated hosts did not (Fig. 1A and B). The persistence of Tc1 cells was also strikingly enhanced relative to Tc0 cells, with the peak of expansion seen about 1 week after transfer (Fig. 1C and D). This enhanced persistence with multiple

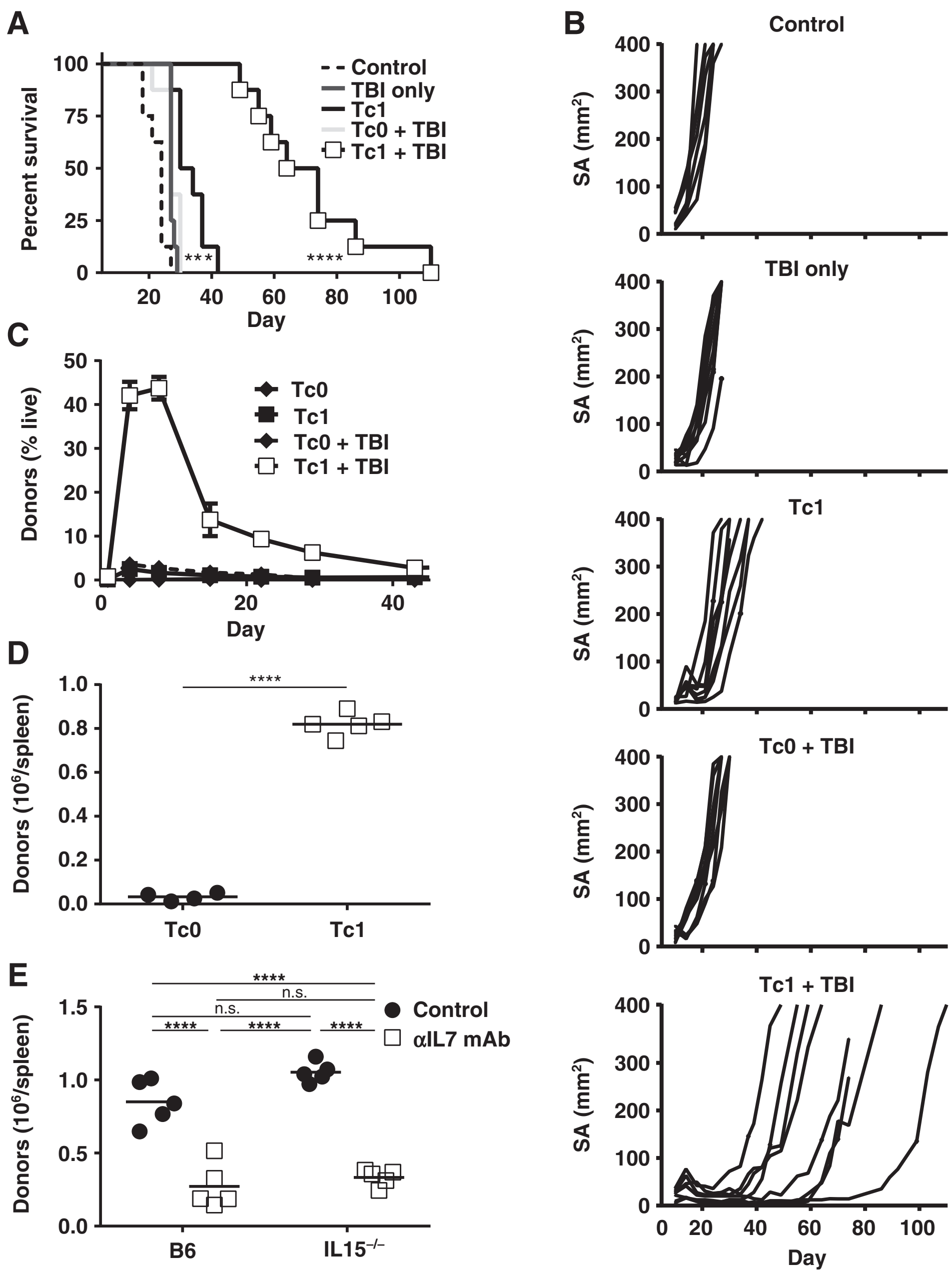

Figure 1.
The enhanced persistence of IL12 conditioned $CD8^+$ T cells (Tc1) in lymphodepleted hosts is dependent on IL7. A and B, B6 mice were injected with B6 melanoma tumor s.c. on day −12 and then irradiated on day −1. On day 0, mice were adoptively transferred with 2×10^6 3-day activated pmel-1 $CD8^+$ T cells with IL12 conditioning (Tc1) or without (Tc0). A, survival curves ($n = 8$; ***, $P = 0.001$ for Tc1 vs. control, $P < 0.0001$ for Tc1 vs. Tc1 + TBI), and B, individual tumor growth curves. C and D, 5×10^6 Tc1 or Tc0 cells were transferred into mice with or without 6 Gy TBI and $Thy1.1^+$ donors were tracked in the (C) peripheral blood over time ($n = 5$) or D, in the spleens 7 days after transfer ($n = 5$; ****, $P < 0.0001$). E, as in D, except cells were transferred into WT B6 or $IL15^{-/-}$ mice with or without αIL7-neutralizing mAb (clone M25; $n = 5$; ****, $P < 0.0001$). All results are representative of at least two independent experiments. n.s., not statistically significant; SA, surface area.

forms of lymphodepletion but without the need for IL2 or vaccination establishes the feasibility of using our Tc1 model to investigate the host cytokine requirements of effector $CD8^+$ T cells.

Because IL7 and IL15 are thought to be the dominant cytokines for T-cell homeostatic expansion (1–3), and they are elevated post-lymphodepletion (21–23), we assessed their importance for the expansion of Tc1 cells. We transferred Tc1 cells into irradiated wild-type (WT) or $IL15^{-/-}$ mice with or without an IL7-neutralizing mAb (clone M25). We then harvested spleens at day 7 after transfer, as this correlated with the peak of their expansion (Fig. 1C). Surprisingly, Tc1 cells exhibited a significant expansion defect at day 7 in WT mice treated with IL7-neutralizing antibodies, but not in $IL15^{-/-}$ mice (Fig. 1E). Removal of both cytokines did not further decrease the engraftment of these cells (Fig. 1E). We confirmed our results by administering a blocking antibody against IL7Rα (A7R34; Supplementary Fig. S1A). Like IL15, IL2 was not critical, as a combination of neutralizing IL2 antibodies (JES6-1A12 and S4B6; ref. 36) did not significantly affect Tc1 cell expansion (Supplementary Fig. S1B). In addition, the absence of host IL2, IL7, and/or IL15 did not significantly impair the ability of Tc1 cells to secrete IFNγ and TNFα after *ex vivo* restimulation (Supplementary Fig. S2). In summary, Tc1 cells are dependent on host IL7 alone for their initial expansion.

Certain T-cell subsets require TCR engagement for homeostatic maintenance (3, 4). Because pmel-1 T cells have engineered specificity against gp100, a self-antigen, we transferred Tc1 cells into $\beta2m^{-/-}$ mice, which are devoid of MHC-I presentation. Tc1 cells persisted equally well in WT B6 and $\beta2m^{-/-}$ B6 mice, indicating that Tc1 did not require TCR engagement for effector expansion (Supplementary Fig. S3A). To confirm our results in a second model, we used the h3T TCR transgenic mouse, whose T cells recognize tyrosinase in an HLA-A2–restricted manner (33). h3T T cells activated in the presence or absence of IL12 showed

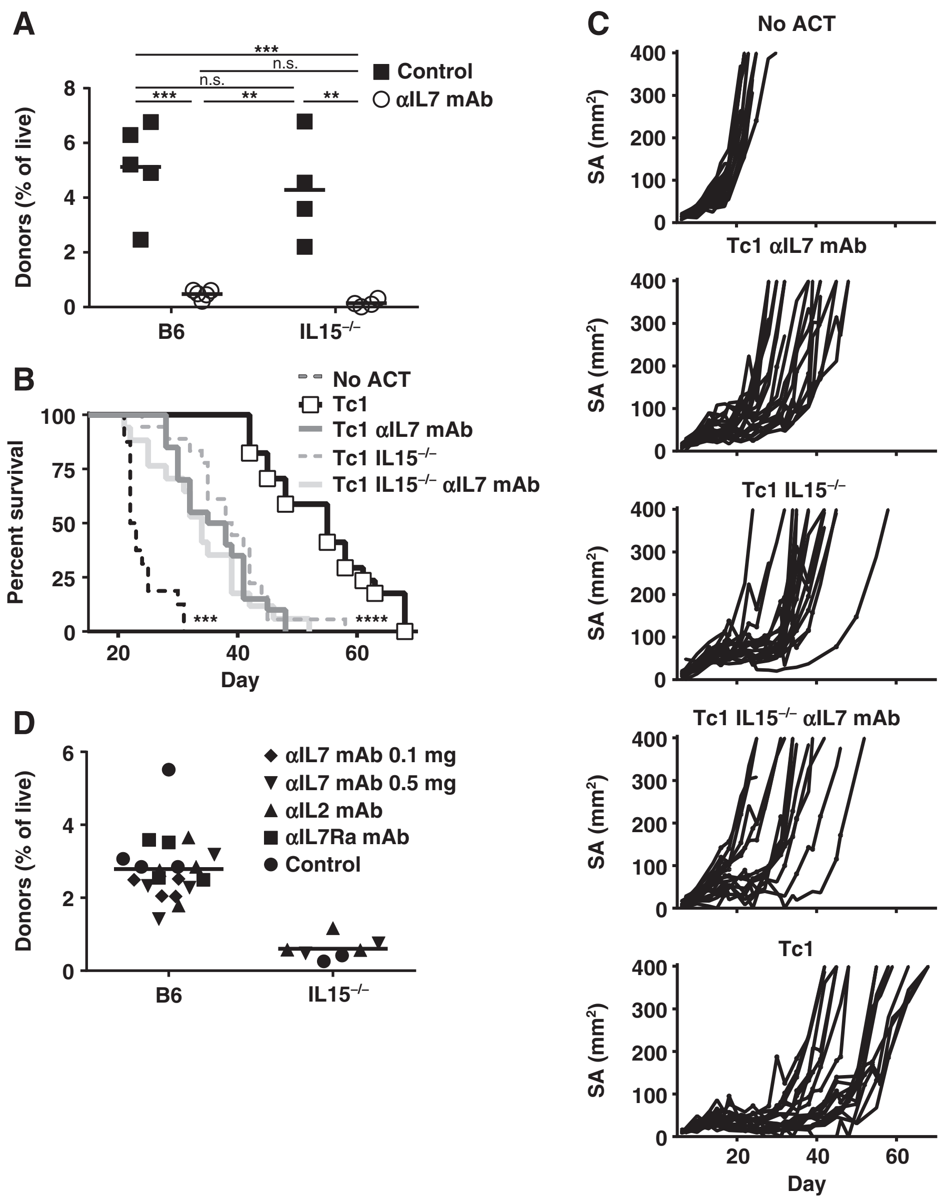

Figure 2.
IL7 and IL15 are required for maximal antitumor efficacy of IL12-conditioned $CD8^+$ (Tc1) T cells. A–C, B6 mice were injected with B16 melanoma tumor s.c. on day −12 and then irradiated (6 Gy) on day −1. On day 0, mice were adoptively transferred with 2×10^6 Tc1 $CD8^+$ effector T cells. A, donor cells in blood on day 5 ($n = 4$–5; **, $P < 0.01$; ***, $P < 0.001$; representative of two independent experiments). B, survival data ($n = 16$–20, ***, $P < 0.001$ for No ACT vs. $IL15^{-/-}$ Tc1 + αIL7 mAb and ****, $P < 0.0001$ for Tc1 $IL15^{-/-}$ vs. Tc1). C, tumor growth curves are pooled from two independent experiments of 8 to 10 mice. D, 5×10^6 Tc1 cells were injected into irradiated WT or $IL15^{-/-}$ mice with or without administration of the indicated antibodies. Anti-IL7 mAb was given at either 100 or 500 μg per injection. After 77 days, the frequency of donor cells in the peripheral blood was measured. Results are representative of two independent experiments. n.s., not statistically significant; SA, surface area.

similar persistence when transferred into irradiated WT B6 or HLA-A2 transgenic mice (Supplementary Fig. S3B). Thus, activated Tc1 cells do not require contact with cognate MHC-I for maximal effector expansion in irradiated hosts.

IL7 and IL15 are required for maximal antitumor efficacy of Tc1 cells

The results above were obtained in tumor-free animals. Therefore, we assessed the cytokine requirements for optimal expansion of effector CD8$^+$ T cells adoptively transferred into B6 mice bearing 12-day established B16 tumors. In a manner similar to tumor-free mice, the initial engraftment of Tc1 cells was dependent on IL7 but not IL15 (Fig. 2A). Consistent with our early expansion data (Fig. 2A), Tc1 cells required IL7 for maximum antitumor efficacy (Fig. 2B and C). In contrast with these data, Tc1 cells also needed IL15 for maximal antitumor efficacy (Fig. 2B and C). This result is likely because IL15 is required for the long-term persistence and memory formation of Tc1 cells (Fig. 2D), although IL15-dependent host cells may be relevant. Thus, Tc1 cells require IL7 for initial expansion but both IL7 and IL15 for maximal antitumor efficacy.

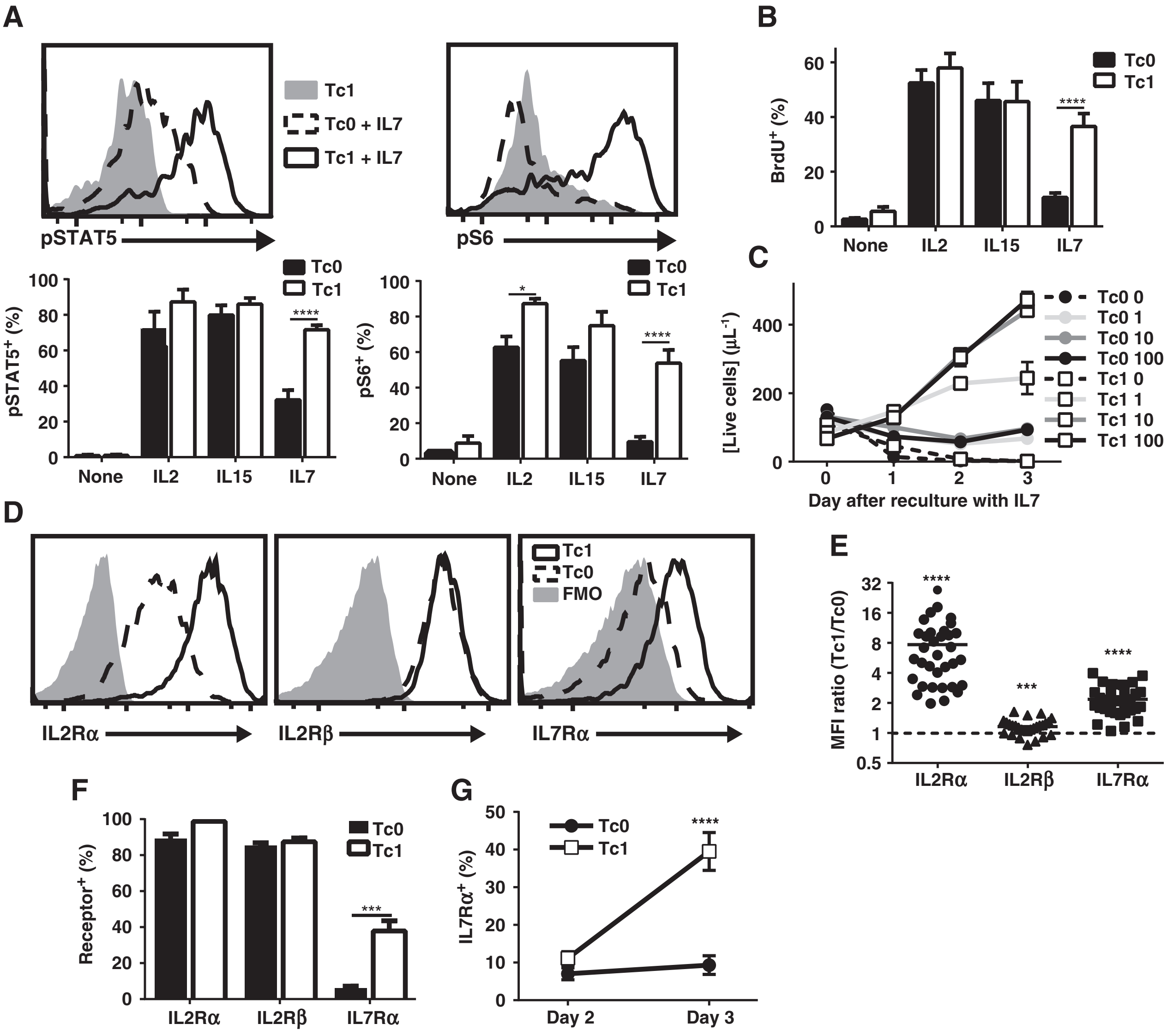

Figure 3.

IL12 conditioning during CD8$^+$ T-cell activation leads to elevated IL7 responsiveness and IL7Rα expression *in vitro*. A–C, Pmel-1 T cells were activated for 3 days with (Tc1) or without (Tc0) IL12, washed and replated in the indicated cytokines (A, top). Representative histograms depicting pSTAT5 and pS6 levels after reculture without cytokine or with IL7 (A, bottom). Mean pSTAT5 and pS6 levels after reculture in 100 ng/mL of the indicated cytokine ($n = 4$; *, $P < 0.05$; ****, $P < 0.0001$). B, BrdU was added for the final hour after overnight culture in the indicated cytokine ($n = 10$; ****, $P < 0.0001$). C, cells were counted on days 0, 1, 2, and 3 after replate in the indicated concentration of IL7 in ng/mL (results are from one experiment with two replicates and are representative of at least three independent experiments). D–F, Tc0 and Tc1 cells were analyzed for the indicated cytokine receptors via flow cytometry. D, representative histograms and E, MFI ratios (***, $P < 0.001$; ****, $P < 0.0001$; P values represent statistically significant difference from Tc0, which is indicated by the dashed line). F, the percentages of cells expressing each cytokine receptor are shown ($n = 11$ independent experiments; ***, $P < 0.001$ via the Welch t test). G, the percentage of cells expressing IL7Rα on days 2 and 3 after stimulation ($n = 7$; ****, $P < 0.0001$ for all comparisons with Tc1 day 3; not statistically for others).

Tc1 cells show superior IL7 responsiveness and elevated IL7Rα levels *in vitro*

Because Tc1 cells exhibited IL7-dependent expansion in irradiated hosts, we assessed the *in vitro* IL7 responsiveness of Tc1 cells compared with Tc0 cells. We also assessed IL2 and IL15 signaling as controls. We first cultured Tc0 cells and Tc1 cells in high doses (100 ng/mL) of IL2, IL15, or IL7 overnight and then assessed phosphorylation of STAT5 and ribosomal S6 (Fig. 3A), both of which are downstream of IL2/7/15 cytokine signaling (4, 36). As expected, IL2 and IL15 led to high levels of phosphorylation in both Tc0 and Tc1 cells. However, when cultured with IL7, only Tc1 cells robustly phosphorylated STAT5 and S6 (Fig. 3A). These enhanced signaling events translated into increased proliferation of Tc1 cells after reculture in IL7 as determined by BrdU incorporation (Fig. 3B). In contrast, Tc0 and Tc1 cells proliferated extensively in IL2 or IL15, as over half of the cells had incorporated BrdU in 1 hour (Fig. 3B). The enhanced proliferation rate after overnight culture led to about a 5-fold expansion of Tc1 over Tc0 cells after 3 days of culture in IL7 (Fig. 3C). Remarkably, even 100-fold lower levels of IL7 (1 ng/mL) led to an increased concentration of Tc1 cells after 3 days, while Tc0 cells at the highest dose barely maintained their numbers (Fig. 3C). These signaling and proliferation events were inhibited by JAK-STAT and PI3K inhibitors, but not mTOR inhibitors (Supplementary Fig. S4), indicating that IL7 was engaging established pathways for cytokine-mediated T-cell proliferation (38–40). In summary, these findings demonstrate the ability of IL12 conditioning to induce IL7 responsiveness in effector CD8$^+$ T cells.

We next sought to delineate the mechanism(s) responsible for the enhanced IL7 responsiveness of Tc1 cells by evaluating IL7Rα as well as IL2Rβ and IL2Rα expression on Tc0 and Tc1 cells. The expression of all three receptors was increased by the addition of IL12 (Fig. 3D and E), although the magnitude of these increases varied (Fig. 3E). When expressed as a proportion of cells staining positive for the receptor rather than the magnitude of expression, a striking difference was seen with IL7Rα. A large proportion of Tc1 cells expressed IL7Rα while Tc0 cells had almost none, in contrast with high levels seen with IL2Rβ and IL2Rα on Tc0 and Tc1 cells

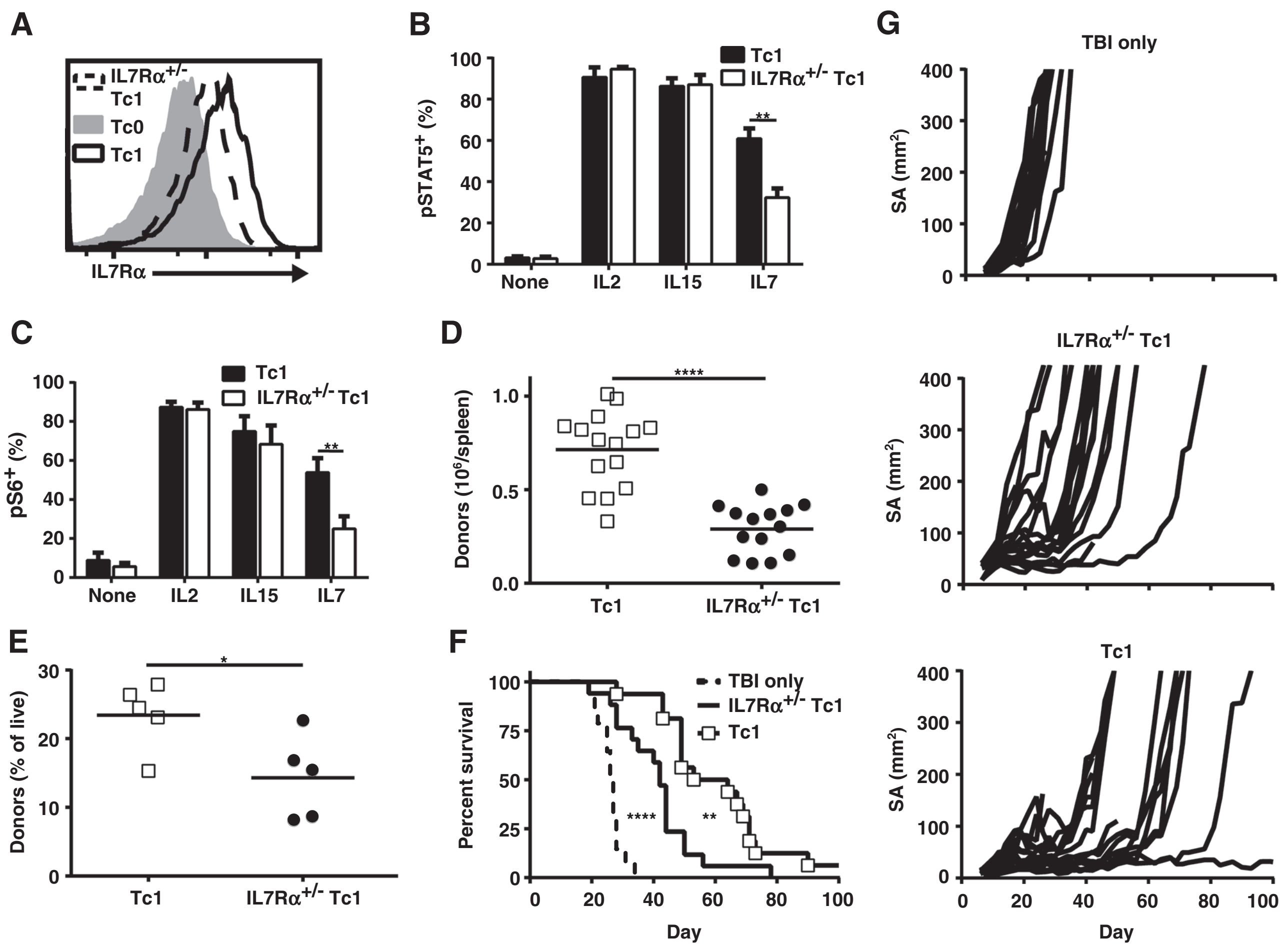

Figure 4.
IL7Rα expression is required for maximal expansion and antitumor efficacy of Tc1 cells. A, representative histogram of IL7Rα levels in Tc0, Tc1, and IL7Rα$^{+/-}$ Tc1 cells. B, pSTAT5 and C, pS6 levels of Tc1 and IL7Rα$^{+/-}$ Tc1 cells after replate in 100 ng/mL of the indicated cytokine ($n = 4$–6; **, $P < 0.01$). D, 3 to 5 × 10^6 pmel Tc1 or IL7Rα$^{+/-}$ Tc1 cells were transferred into irradiated hosts (6 Gy), and the absolute number of donor cells in host spleens 7 days later is displayed (data are combined from three independent experiments; ****, $P < 0.0001$). E–G, on day 12 B16 tumor-bearing mice were injected with 2 × 10^6 T cells the day after irradiation. E, the percentage of donor cells in the peripheral blood on day 8 after transfer (*, $P < 0.05$). F, survival curves (****, $P < 0.0001$ for TBI only vs. IL7Rα$^{+/-}$ Tc1; **, $P < 0.01$ for IL7Rα$^{+/-}$ Tc1 vs. Tc1; combined from two independent experiments for total $n = 14$–17). G, growth curves. SA, surface area.

(Fig. 3F). We next investigated the kinetics of IL7Rα expression. As expected, IL7Rα was initially decreased on both cell types after T-cell activation, but Tc1 cells showed increased expression by 72 hours after stimulation (Fig. 3G). Thus, IL12 promotes IL7Rα reexpression in Tc1 cells, a finding that may explain the enhanced IL7-mediated persistence of effector CD8$^+$ T cells (Tc1) cells after transfer into lymphodepleted hosts.

IL7Rα upregulation is responsible for the enhanced IL7 responsiveness and subsequent *in vivo* persistence of Tc1 cells

To directly test whether IL7Rα was critical for the enhanced IL7 responsiveness of Tc1 cells, we generated pmel-1 IL7Rα$^{+/-}$ mice. As expected, Tc1 cells generated from IL7Rα$^{+/+}$ and IL7Rα$^{+/-}$ pmel-1 mice expressed similar levels of IL2Rβ, IL2Rα, granzyme B (GrzB), Tbet, Eomes, and CD62L (Supplementary Fig. S5A), and produced equivalent levels of IFNγ after 3-day culture (Supplementary Fig. S5B). In contrast, IL7Rα levels in the IL7Rα$^{+/-}$ Tc1 cells were about half that of Tc1 cells (Fig. 4A and B). This decreased IL7Rα expression translated to reduced IL7-induced STAT5 and S6 phosphorylation for IL7Rα$^{+/-}$ Tc1 compared with WT Tc1, despite having similar levels when maintained in IL2 or IL15 (Fig. 4B and C). BrdU incorporation also trended lower with IL7 cultures of IL7Rα$^{+/-}$ Tc1 relative to Tc1 (Supplementary Fig. S5C).

These *in vitro* results indicate that IL7Rα$^{+/-}$ Tc1 cells can be used to evaluate the functional importance of IL7Rα, given that they appeared identical to WT Tc1 in all aspects tested except for IL7Rα expression and IL7 responsiveness. Therefore, we transferred WT and IL7Rα$^{+/-}$ Tc1 cells into irradiated hosts. On day 7 after transfer into irradiated hosts, there were about half as many IL7Rα$^{+/-}$ Tc1 cells as WT Tc1 cells in the spleens of recipient mice (Fig. 4D). Similar results were observed in the peripheral blood of tumor-bearing mice 7 days after transfer (Fig. 4E). Importantly, this decreased initial expansion of Tc1 cells also led to significantly reduced antitumor activity in IL7Rα$^{+/-}$ Tc1 cells relative to WT pmel-1 Tc1 cells (Fig. 4F and G). Together, these results indicate that elevated IL7Rα expression is critical for driving the initial engraftment and subsequent antitumor activity of Tc1 cells.

Host IL7 and donor IL7Rα are required for maximal persistence of polyclonal CD8$^+$ T cells in lymphodepleted hosts

Next, we investigated the importance of IL7Rα for the initial engraftment of effector CD8$^+$ T cells activated without IL12. As shown in Fig. 1C and D, pmel-1 T cells stimulated with hgp100 alone (Tc0) persisted poorly, presumably due to low IL7Rα expression (Fig. 3F). Therefore, we sought IL12-independent activation conditions that would elevate IL7Rα appreciably and thereby generate effector cells capable of persisting in lymphodepleted hosts. Because TCR strength has been shown to modulate IL7Rα levels in human CD4$^+$ T cells (41), we activated pmel-1 T cells over a broad range of hgp100 concentrations. Although

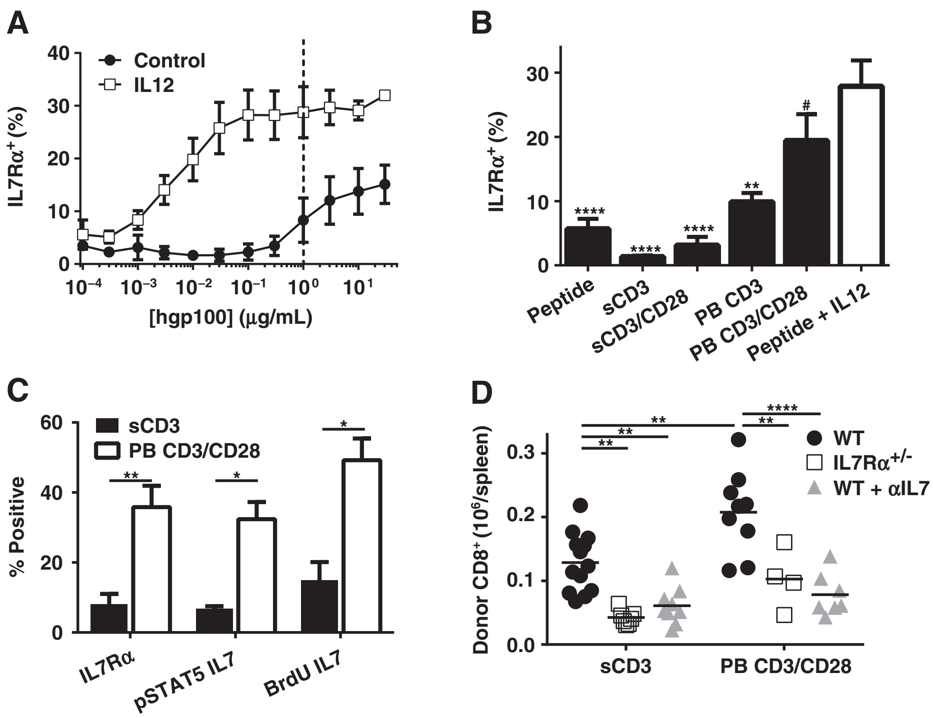

Figure 5.
TCR strength modulates IL7Rα expression, which dictates engraftment of activated CD8$^+$ T cells. A, Pmel-1 CD8$^+$ T cells were stimulated for 3 days ± IL12 with titrated hgp100 peptide. B, Pmel-1 T cells were stimulated with soluble anti-CD3 mAb (sCD3), sCD3 + soluble anti-CD28 mAb (sCD3/CD28), plate-bound anti-CD3 mAb (PB CD3), PB CD3 + plate-bound anti-CD28 mAb (PB CD3/CD28), or hgp100 peptide with or without IL12 for 3 days and assessed for IL7Rα expression (combined data from four to five independent experiments; $^{\#}$, $P > 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ vs. hgp100 + IL12). C, B6 T cells were stimulated as indicated and assessed for IL7Rα expression ($n = 5$; **, $P < 0.01$) or responsiveness to IL7 ($n = 3$ for pSTAT5 and BrdU assays; *, $P < 0.05$). D, WT or IL7Rα$^{+/-}$ mice were stimulated with soluble or plate-bound antibodies and then transferred into irradiated hosts. Where indicated, the IL7-blocking antibody clone M25 was administered on days 0, 2, and 5 after transfer. Shown are absolute numbers of donor CD8$^+$ T cells 7 days after transfer (**, $P < 0.01$; ****, $P < 0.0001$; data are combined from three independent experiments).

higher peptide concentrations increased IL7Rα expression, the receptor levels did not reach those achieved with IL12 (Fig. 5A). To further increase the strength of TCR stimulation, we next activated T cells nonspecifically with soluble or plate-bound anti-CD3 mAb with or without anti-CD28 mAb. Consistent with reports demonstrating elevated TCR signaling with immobilized anti-CD3 mAb (42) and costimulation with anti-CD28 mAb (43), IL7Rα levels were increased in plate-bound conditions and even higher when anti-CD28 mAb was added (Fig. 5B). In fact, plate-bound anti-CD3 mAb and anti-CD28 mAb (PB CD3/CD28) were statistically indistinguishable from Tc1 cells (hgp100 + IL12; Fig. 5B).

Having established that higher TCR signals increase IL7Rα expression in the pmel-1 model, we evaluated this relationship in $CD8^+$ T cells from WT B6 mice. As was the case with pmel-1 T cells, PB CD3/CD28 produced the highest IL7Rα levels in polyclonal T cells, and IL12 further enhanced IL7Rα expression across all TCR stimuli (Supplementary Fig. S6). Next, we characterized the PB CD3/CD28 and soluble αCD3 (sCD3) conditions as they possessed the highest and lowest IL7Rα expression, respectively (Supplementary Fig. S6). As expected, sCD3 stimulated T cells had decreased IL7 responsiveness compared with PB CD3/CD28 (Fig. 5C). When transferred into irradiated hosts, PB CD3/CD28 stimulated $CD8^+$ T cells accumulated at significantly higher levels than cells stimulated with soluble αCD3 alone (Fig. 5D). Importantly, $IL7R\alpha^{+/-}$ cells stimulated with either TCR strength failed to engraft as well as their WT counterparts. Finally, both WT cell types were also dependent on IL7, as IL7 neutralization led to significant reductions in donor $CD8^+$ cell numbers (Fig. 5D). In sum, these data indicate that host IL7 and donor IL7Rα are critical for maximal accumulation of activated $CD8^+$ effector cells transferred into lymphodepleted hosts.

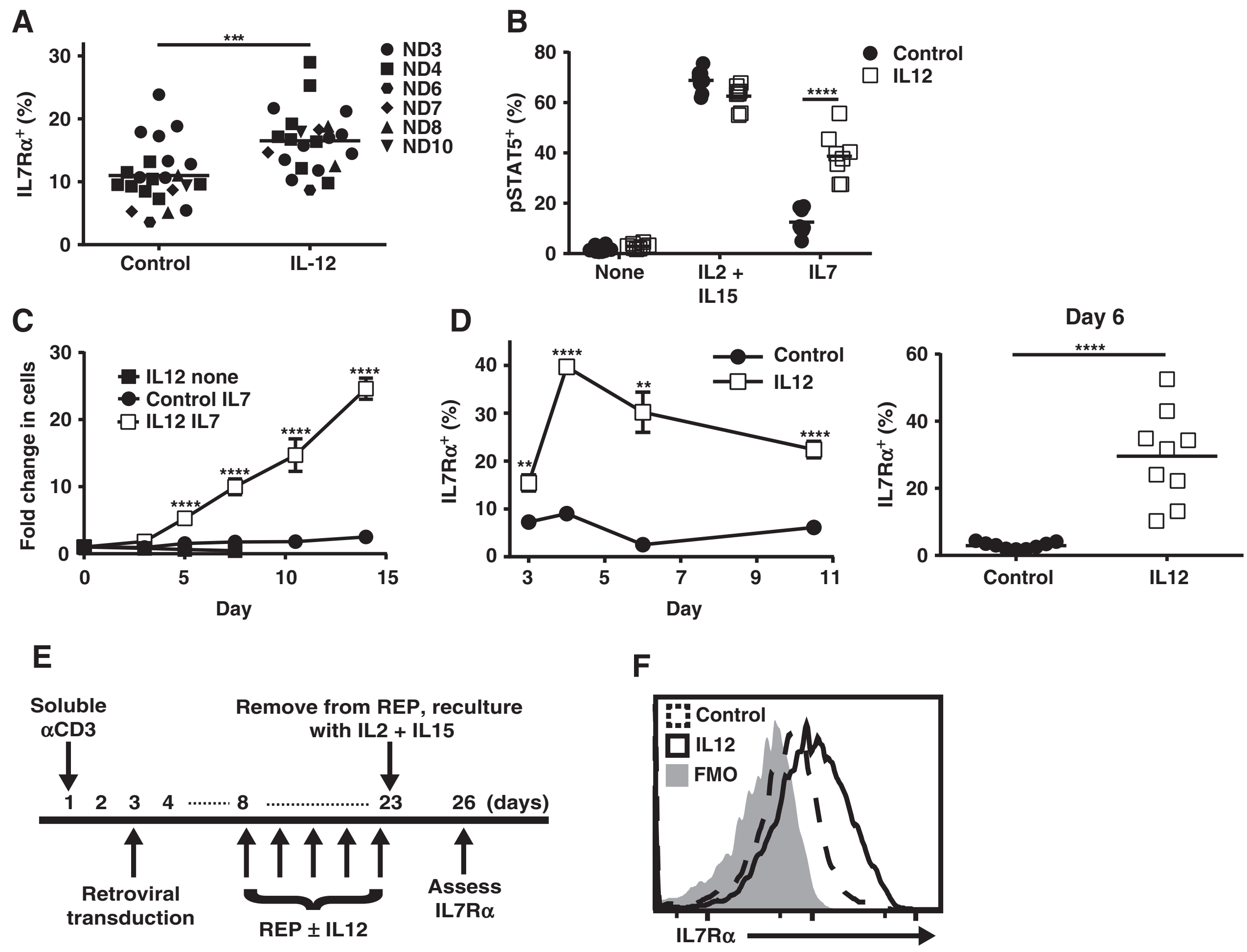

Figure 6.

Human T cells conditioned with IL12 display enhanced IL7Rα expression and IL7 responsiveness. A–D, human PBMCs were activated with soluble anti-CD3 mAb (0.5 μg/mL, Okt3 clone) with or without hIL12 (10 ng/mL) for 3 days. A, IL7Rα expression after 3-day activation (***, $P < 0.001$; "ND" is normal donor). B and C, 3-day activated T cells were washed and then replated in the indicated cytokines (300 IU/mL IL2 + 100 ng/mL IL15; IL7, 100 ng/mL). B, pSTAT5 staining via flow cytometry after overnight culture ($n = 8$ from two independent experiments with four normal donors; ****, $P < 0.0001$). C, cells were counted and given fresh media every 2 to 3 days ($n = 6$ from two independent experiments with three normal donors). D, as in C except activated cells were recultured in IL2 + IL15 on day 3 and then assessed for IL7Rα expression at the indicated time points ($n = 6$–9 from two independent experiments with four normal donors; **, $P < 0.01$; ****, $P < 0.0001$ via the Welch t test). E, overview of the clinical transduction protocol to generate TCR-transduced melanoma-reactive human T cells. Shown is the timing of IL12 addition and 3-day reculture in IL2 (300 IU/mL) + IL15 (100 ng/mL). F, IL7Rα expression at day 26 of above timeline of human T cells initially grown with or without hIL12. This result is representative of two independent experiments.

Human T cells conditioned with IL12 display enhanced IL7Rα expression and IL7 responsiveness

Given the importance of donor IL7Rα and host IL7 for the persistence of effector CD8$^+$ T cells in mice, we next tested the ability of IL12 to enhance IL7Rα expression in activated human CD8$^+$ T cells. CD8$^+$ T cells from day 3 activated human peripheral blood mononuclear cells (PBMC) exhibited higher IL7Rα expression with IL12, although the magnitude of this effect was not as large as our murine data (Fig. 6A compared with mouse data in Fig. 3D). In contrast with this small change in IL7Rα expression, human T cells were only able to phosphorylate STAT5 robustly in response to IL7 if they were activated with IL12 (Fig. 6B). When these activated T cells were washed and recultured *in vitro*, only those activated with IL12 expanded in the presence of IL7 (Fig. 6C). Given the discordance between initial IL7Rα levels (Fig. 6A) and IL7 responsiveness (Fig. 6B and C), we assessed IL7Rα levels after reculture of cells. We speculated that the ability to reexpress IL7Rα after withdrawal of TCR stimulation might explain the observed differences in IL7 responsiveness. Consistent with this hypothesis, the presence of IL12 during the first 3 days of activation led to a striking enhancement in IL7Rα expression that lasted for at least 1 week after reculture (Fig. 6D). Finally, we sought to evaluate the translatability of our findings from 3-day cultures in a clinically relevant scenario by using the retroviral transduction protocol depicted in Fig. 6E, in which IL12 was added or withheld during the REP. We found that the inclusion of IL12 did not significantly increase IL7Rα levels at the end of the REP. As was the case in our 3-day cultures, however, the transduced T cells that underwent the REP in the presence of IL12 possessed higher IL7Rα expression 3 days after reculture (Fig. 6F). These results suggest that the addition of IL12 to human T-cell cultures during the REP is a feasible strategy to augment IL7Rα levels, and this may be applicable in a number of clinically used protocols (44–46).

Discussion

In this study, we evaluated the host cytokines required for the initial engraftment of effector CD8$^+$ T cells transferred into lymphodepleted hosts. Contrary to our expectations, IL7 was initially required, whereas IL15 was not. Because multiple methodologies for the activation of CD8$^+$ T cells, including IL12 conditioning or strong TCR stimulation, demonstrated IL7 and IL7Rα dependence, our results are likely generalizable to a variety of T-cell activation methodologies.

Our results indicate that transferred effector T cells should be IL7 responsive for maximal engraftment in a lymphodepleted host without exogenously provided cytokine. In our murine models, CD8$^+$ T cells required IL7Rα for maximal engraftment after adoptive transfer; however, in a clinical setting, expression of IL7Rα on donor T cells was one of 45 markers that failed to differentiate persisting T-cell clones from those that failed to engraft (47). In this prior study, T cells were not conditioned with IL12. Our results with human T cells suggest that reexpression of IL7Rα after cessation of TCR stimulation and extended culture corresponds most directly with IL7 responsiveness (Fig. 6). We therefore predict that assessing IL7Rα levels after extended reculture may have more clinical utility than determining IL7Rα levels at the predetermined point of infusion.

An intriguing result from this work is that IL15 does not initially play a role in the support of effector Tc1 cells. These data are in contrast with results from prior studies with memory phenotype CD8$^+$ T cells transferred into lymphopenic hosts (10–12). Because IL15 is known to be elevated in the lymphodepleted host (21), these differences are potentially explained by distinct trafficking of activated versus resting T cells.

That *in vitro* IL12 priming increases IL7Rα expression appears to be discordant with the well-described phenomenon that enhanced IL12/inflammation during effector responses *in vivo* leads to more terminally differentiated CD8$^+$ T cells with decreased IL7Rα expression (28, 30, 48). A potential explanation is that the programming for terminal differentiation has not yet occurred after 3 days of activation in the presence of IL12, a theory supported by the increased IL7Rα and CD62L expression observed with IL12 priming on day 3 (25). The kinetics of IL7Rα reexpression we observed further support this idea, as IL7Rα transcription appears to be initiated on day 2 of culture. Given that the expression of IL7Rα is modulated by the transcription factors Gfi-1 and GABPα, the relationship between IL12 and these transcription factors warrants further investigation (49).

In summary, our results suggest a model in which effector CD8$^+$ T cells are dependent on host IL7 for maximal persistence and antitumor efficacy in a lymphodepleted host. This represents a shift in the current paradigm that considers IL15 as the critical cytokine capable of modulating effector CD8$^+$ T-cell durability and efficacy in this increasingly relevant clinical setting. In practical terms, our results demonstrate that a direct and feasible way to produce IL7Rα-expressing, IL7-responsive effector T cells is *ex vivo* IL12 conditioning.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.B. Johnson, D.J. Cole, M.P. Rubinstein
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.B. Johnson, B.P. Riesenberg, B.R. May, S.C. Gilreath, M.P. Rubinstein
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.B. Johnson, B.P. Riesenberg, K.F. Staveley-O'Carroll, E. Garrett-Mayer, D.J. Cole, M.P. Rubinstein
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