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IL-18 Synergizes with IL-7 To Drive Slow Proliferation of Naive CD8 T Cells by Costimulating Self-Peptide–Mediated TCR Signals

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Naive T cell populations are maintained in the periphery at relatively constant levels via mechanisms that control expansion and contraction and are associated with competition for homeostatic cytokines. It has been shown that in a lymphopenic environment naive T cells undergo expansion due, at least in part, to additional availability of IL-7. We have previously found that T cell–intrinsic deletion of TNFR-associated factor (TRAF) 6 (TRAF6ΔT) in mice results in diminished peripheral CD8 T cell numbers. In this study, we report that whereas naive TRAF6ΔT CD8 T cells exhibit normal survival when transferred into a normal T cell pool, proliferation of naive TRAF6ΔT CD8 T cells under lymphopenic conditions is defective. We identified IL-18 as a TRAF6–activating factor capable of enhancing lymphopenia-induced proliferation (LIP) in vivo, and that IL-18 synergizes with high-dose IL-7 in a TRAF6-dependent manner to induce slow, LIP/homeostatic-like proliferation of naive CD8 T cells in vitro. IL-7 and IL-18 act synergistically to upregulate expression of IL-18R genes, thereby enhancing IL-18 activity. In this context, IL-18R signaling increases PI3K activation and was found to sensitize naive CD8 T cells to a model noncognate self-peptide ligand in a way that conventional costimulation via CD28 could not. We propose that synergistic sensitization by IL-7 and IL-18 to self-peptide ligand may represent a novel costimulatory pathway for LIP. ***The Journal of Immunology*****, 2014, 193: 3992–4001.**

CD8 T cells are primary facilitators of adaptive immune killing in response to intracellular infections and tumors, and they undergo vigorous expansion and differentiation in response to cognate Ag (1, 2). For proper immune function, it is critical not only for subsets of responding Ag-specific CD8 T cells to acquire memory cell function, but also to maintain peripheral steady-state homeostasis of the broader CD8 T cell compartment (2–4). With age, thymic involution and chronic viral infections both contribute to diminution of the naive CD8 T cell pool (5, 6). In clinical contexts, the effects of lymphopenia on CD8 T cell homeostasis are significant for anti–retroviral treatment of HIV infection, T cell–ablative therapy associated with bone marrow transplant, and lymphopenia-induced autoimmunity following transplant (7–9). Elsewhere, there is evidence that mimicking lymphopenic conditions may provide therapeutic benefits for enhancing CD8 T cell antitumor responses (10, 11). Therefore, understanding both the extracellular stimuli and the cell-intrinsic mechanisms that enable naive CD8 T cells to adapt to lymphopenic conditions are of considerable interest.

Lymphopenia-induced proliferation (LIP) (sometimes also "homeostatic" or "cognate Ag–independent" proliferation) occurs more slowly than cognate Ag-induced proliferation, and it may be triggered by increased availability of the homeostatic cytokine IL-7 (or possibly IL-15) that occurs in the absence of competing cells (3, 8, 12). LIP also requires below-threshold "tonic" TCR stimulation provided by low-affinity self-peptides, and cells undergoing LIP do not blast or produce significant levels of effector cytokines (3, 13, 14). Interestingly, whereas enhanced IL-7R signaling is known to be essential for LIP in vivo, it is difficult to recapitulate or model this type of proliferation in vitro, suggesting that additional signals may also be required. Emerging use of IL-7 in clinical contexts of lymphopenia involving cancer or after allogeneic stem cell transplant highlights the importance of identifying complementary factors and characterizing their relevant signaling mechanisms (15, 16).

By focusing on cell-intrinsic homeostatic mechanisms in the context of CD8 T cell biology, we previously identified TNFR-associated factor (TRAF) 6–dependent signaling as critical to maintenance of the CD8 T cell pool using T cell–specific TRAF6-deficient mice (TRAF6ΔT) (17, 18). The TRAF6 E3 ubiquitin ligase is activated by TGF-βR, TLR/IL-1R, and TNFR superfamilies and further activates downstream pathways NF-κB, MAPK, and NFAT (19, 20). Although we have previously determined that TRAF6ΔT CD8 T cells stimulated with cognate Ag are hyperresponsive (17, 18), we now show that naive cells exhibit defective LIP.

By focusing on known TRAF6-dependent pathways that may operate in naive CD8 T cells, we identified the IL-1 family member IL-18 (21, 22) as a factor that enhances LIP in vivo, and that synergizes with IL-7 in vitro to sensitize naive CD8 T cells to self-peptide. This mechanism appears distinct from conventional CD28 costimulation and may represent a novel form of costimulation that could enable better understanding of the signals that

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; LIP, lymphopenia-induced proliferation; MHC-I, MHC class I; TRAF, TNFR-associated factor; TRAF6ΔT, T cell–intrinsic deletion of TRAF6.

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control LIP, and possibly improve clinical intervention strategies for boosting (or controlling) peripheral T cell pools.

Materials and Methods

Reagents and Abs

Western blotting Abs specific for p-Akt (S473), Akt, Bcl-x_L, Cdk6, and cyclin D3 were purchased from Cell Signaling Technology (Danvers, MA). For cell culture, anti-CD3 (2C11) and anti-CD28 (37.51) were prepared in-house or purchased from Becton Dickinson (Franklin Lakes, NJ). Anti–MHC class I neutralizing Ab (Y-3) was provided by Philippa Marrack (National Jewish Health, Denver, CO), and mouse IgG2b isotype control Ab was purchased from Becton Dickinson. For flow cytometry, anti–CD90.1-Pacific Blue, anti–CD8-allophycocyanin-Alexa Fluor 750, anti–CD4-Pacific Blue, anti–CD45.1-allophycocyanin-Alexa Fluor 750, anti–CD44-FITC, anti–CD69-FITC, rat IgG2b-allophycocyanin, and Armenian hamster IgG FITC were purchased from eBioscience (San Diego, CA), and anti–CD8-FITC, anti–CD45.2-PerCP-Cy5.5, and anti–CD90.2-allophycocyanin were purchased from Becton Dickinson. For ELISA, anti–IFN-γ capture and detection Abs, as well as detection reagents, were purchase from Becton Dickinson. Recombinant murine IL-12 and IL-7 were purchased from PeproTech (Rocky Hill, NJ). Recombinant murine IL-18 and IL-1β were purchased from R&D Systems (Minneapolis, MN). For CFSE proliferation staining, CFDA-SE was purchased from Invitrogen (Carlsbad, CA). For survival assays, 7-amino-actinomycin D (7-AAD) vital dye was purchased from Becton Dickinson. The PI3K inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). Purified synthetic peptides based on the chicken OVA protein sequence $OVA_{257–264}$ and the OVA E1 mutant were purchased from Anaspec (Fremont, CA).

Mice

Mice conditionally deficient for TRAF6 in the T cell compartment (TRAF6ΔT) were generated by crossing $TRAF6^{flox/flox}$ mice with CD4-Cre transgenic mice obtained from Taconic (Germantown, NY). CD45.1 congenic mice (5–8 wk of age) were purchased from the National Cancer Institute Mouse Repository (Frederick, MD) to be used as recipients for adoptive transfer experiments. STAT5b-CA transgenic mice were obtained from Michael Farrar (University of Minnesota, Minneapolis, MN). OT-I TCR transgenic mice and CD90.1 congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were crossed to C57BL/6 mice at least 10 times. Unless otherwise indicated, tissues were harvested from mice between 4 and 8 wk of age. Mouse care and experimental procedures were performed in accordance with protocols from Institutional Animal Care and Use Committees of the University of Pennsylvania.

In vivo lymphopenia-induced proliferation

For all experiments involving polyclonal donor mice, as well as some experiments involving OT-I TCR transgenic donor mice, spleen and lymph node cells were harvested and sorted by FACS for $CD8^+CD62L^{hi}CD44^{lo}$ T cells. Alternatively, in some experiments involving young OT-I TCR transgenic donor mice, CD8 T cells were purified from lymph nodes using MACS, and the $CD62L^{hi}CD44^{lo}$ phenotype was assayed by flow cytometry to confirm >95% naive phenotype before proceeding. In all cases, donor cells were washed twice with PBS before being labeled with 5 μM CFSE or left unlabeled and injected i.v. into either RAG1 knockout mice or irradiated (700 rads) CD45.1 congenic recipient mice ($1.5–2 \times 10^6$ cells/mouse). Cells were harvested and analyzed 7 d after transfer unless otherwise indicated. For quantitative analysis of $CD8^+$ T cell expansion/survival in a lymphopenic environment, $CD45.2^+CD90.2^+$ naive $CD8^+$ test cells were mixed 1:1 with naive $CD8^+CD45.2^+CD90.1^+$ reference cells prior to either CFSE labeling or transfer into at least three irradiated $CD45.1^+$ recipient mice per genotype per time point. Tissues were harvested, processed, and analyzed as before, with donor cells distinguished from recipient cells by CD45.2/CD45.1, and test (control/TRAF6ΔT) cells distinguished from reference cells by CD90.2/CD90.1. At each time point, ratios of test cells to reference cells were calculated for each mouse, and means and SDs were calculated for ratios across groups of mice receiving the same test genotype. For experiments involving gene expression during LIP, cells were sorted based on CD45.2 by FACS directly into TRIzol reagent. For experiments involving in vivo treatment with IL-18, mice were treated i.v. with PBS or 400 ng rIL-18 daily for the length of the experiment.

In vitro T cell cultures

Naive ($CD62L^{hi}CD44^{lo}$) T cells were obtained by FACS sorting (polyclonal and OT-I TCR transgenic) to yield >99% naive phenotype or by $CD8^+$ MACS (young OT-I or RAG1 knockout.OT-I only [although all OT-I experiments also included at least one replicate using FACS-sorted cells]) to yield >95% naive phenotype. Cells were typically cultured at 40,000 per well in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, and 2-ME (all purchased from Invitrogen) in 96-well round-bottom tissue culture–treated plates. Relative cell counts of culture wells were made by acquiring equal volumes for the same period of time on a FACSCalibur flow cytometer at high flow rate. For CFSE labeling, cells were resuspended in PBS without serum at $5–10 \times 10^6$/ml, then mixed 1:1 with PBS containing 10 μM CFSE. Labeling suspensions were rocked at room temperature for 5 min and then quenched with 20% FBS. Cells were washed twice with serum-containing medium before being prepared for culture or transfer. Cells treated with anti–MHC-I (Y-3; 10 μg/ml) neutralizing Ab or LY294002 PI3K inhibitor were pretreated with these reagents in culture for 30 min prior to addition of activating cytokines. For cell cultures of 6 d or longer, half the culture medium was replaced every 3 d.

Flow cytometry

Purified cultured cells harvested for flow cytometric analysis were stained with 7-AAD vital dye and assayed on a FACSCalibur (Becton Dickinson) for 30 s per tube at high flow rate to attain consistent cell counts. For adoptive transfer analysis, cell suspensions were stained on ice for 30 min in PBS plus 2% serum and 0.1% sodium azide (w/v) with Abs against CD90.2 and CD8 to identify $CD8^+$ T cells CD45.1 and CD45.2 to distinguish recipient and donor cells, respectively, and, in some cases, CD90.2 and CD90.1 to distinguish test cells from reference cells. These cells were analyzed on an LSR II flow cytometer (Becton Dickinson).

ELISA

Cell culture supernatants were harvested after 60 h of culture and stored frozen. Once thawed, multiple dilutions were prepared, along with titrations of IFN-γ standard. Supernatants were incubated on capture Ab–coated ELISA plates for 2 h, followed by incubation with detection Ab and HRP-conjugated secondary Ab for 1 h. Plates were washed repeatedly with PBS plus 0.05% Tween 20. Substrate solution was added for 30 min, followed by 0.1 M phosphoric acid, at which point plates were read at 450 nm with a 570 nm correction.

Western blotting

One to 2×10^6 purified naive T cells per sample were harvested as indicated with ice-cold PBS plus phosphatase inhibitor (Active Motif, Carlsbad, CA), pelleted, and lysed with 100 μl lysis buffer (20 mM HEPES buffer [pH 7.5], 150 mM NaCl, 10% [w/v] glycerol, protease inhibitor mixture [Roche, Basel, Switzerland], 2 mM sodium orthovanadate, 2 mM NaF, 100 ng/ml calyculin A [Cell Signaling Technology], with 1.0% [w/v] Triton X-100). Lysates were incubated on ice for 20 min and clarified by centrifugation at $20,000 \times g$ for 10 min. Five microliters of each lysate was used for determining protein concentration, and lysates were normalized accordingly. Extracts were aliquoted, added to 6× SDS loading buffer, and boiled. Samples were run on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). Blots were probed with primary Abs in 5% milk dissolved in PBS with 0.1% Tween 20, followed by secondary anti-rabbit HRP or anti-mouse HRP (Promega, Madison, WI). Western blots were incubated with ECL substrate (Pierce, Rockford, IL) and exposed to film.

Real-time PCR

Cells (typically $1–2 \times 10^6$/condition) were cultured as indicated, harvested, pelleted, and lysed in 1 ml TRIzol reagent (Invitrogen). RNA was prepared via separation with chloroform, precipitation with isopropanol, and washing with 75% ethanol. RNA was quantified with UV spectrometry. cDNA generation was accomplished by aliquoting 1 μg RNA into 20 μl SuperScript II (Invitrogen) reverse transcriptase reactions for 50 min, and then diluted with double distilled H_2O to 750 μl. Ten microliters of each cDNA stock was used for 25 μl real-time PCR reactions containing 2× Master mix (Applied Biosystems, Norwalk, CT) and FAM dye assay-on-demand primers (Applied Biosystems) specific for indicated targets. Assays were performed in triplicate and normalized to 18S RNA using an Applied Biosystems 7300 real-time PCR machine running Sequence Detection System software version 1.4.

Microarray

Freshly isolated naive OT-I TCR transgenic CD8 T cells were cultured for 24 h with low-dose (0.1 ng/ml) or high-dose (1.0 ng/ml) IL-7 in the presence or absence of 10 ng/ml IL-18, then harvested, pelleted, and lysed with TRIzol reagent, and RNA was prepared as for real-time PCR samples. Total RNA was converted to first-strand cDNA using SuperScript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter.

Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nucleotides or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to five microarrays per group. The microarrays were then washed at low (6× standard saline citrate phosphate/EDTA) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-PE. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-PE stain. A confocal scanner was used to collect fluorescence signal at 3-μm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature.

Statistical analysis

Unless otherwise indicated, all experiments were performed a minimum of three times, with representative data depicted. For comparative in vitro cell count or real-time PCR analysis, triplicate wells were used for all conditions within a given experimental replicate, and differences were determined by p values <0.05, as calculated on Microsoft Excel using the two-tailed equal variance Student t test. For comparative in vivo cell count analysis, three recipient mice were used for all conditions within a given experimental replicate, and differences were determined by p values <0.05, as calculated on Microsoft Excel using the two-tailed equal variance Student t test.

Results

Naive TRAF6-deficient CD8 T cells exhibit defective LIP

We previously reported that TRAF6ΔT mice contain a diminished peripheral naive CD8 T cell pool (17), but we find that sorted naive CD8 T cells from these mice transferred to a normal environment exhibit normal survival dynamics (Supplemental Fig. 1). To investigate the role that T cell–intrinsic TRAF6 might play in other homeostatic mechanisms, we examined the behavior of naive TRAF6ΔT CD8 T cells under lymphopenic conditions. Sorted naive CFSE-labeled control or TRAF6ΔT CD8 T cells were adoptively transferred into irradiated congenic recipient mice for 7 d. CFSE profiles of TRAF6ΔT donor cells harvested from recipient mice revealed a striking defect in the slow form of proliferation associated with lymphopenia (Fig. 1A). In addition to conventional cytokine-dependent LIP, polyclonal CD8 T cells undergo a spontaneous (or "chronic") form of proliferation associated with commensal-derived Ags (23). To exclude the effects of spontaneous proliferation, we generated TRAF6ΔT.OT-I TCR transgenic mice, whose T cells are specific for the chicken $OVA_{257–264}$ peptide in the context of H2-K^b. When naive cells from these mice were transferred into lymphopenic recipients for 7 d, they exhibited a more uniform pattern of proliferation than did the polyclonal cells, but again the TRAF6ΔT cells demonstrated a proliferative defect (Fig. 1B). To determine whether defects in proliferative profiles reflected population defects, control or TRAF6ΔT OT-I CD8 T cells were mixed in equal proportions with CD90.1 OT-I reference cells (cotransferred reference cells were used to minimize recipient variability effects in a given

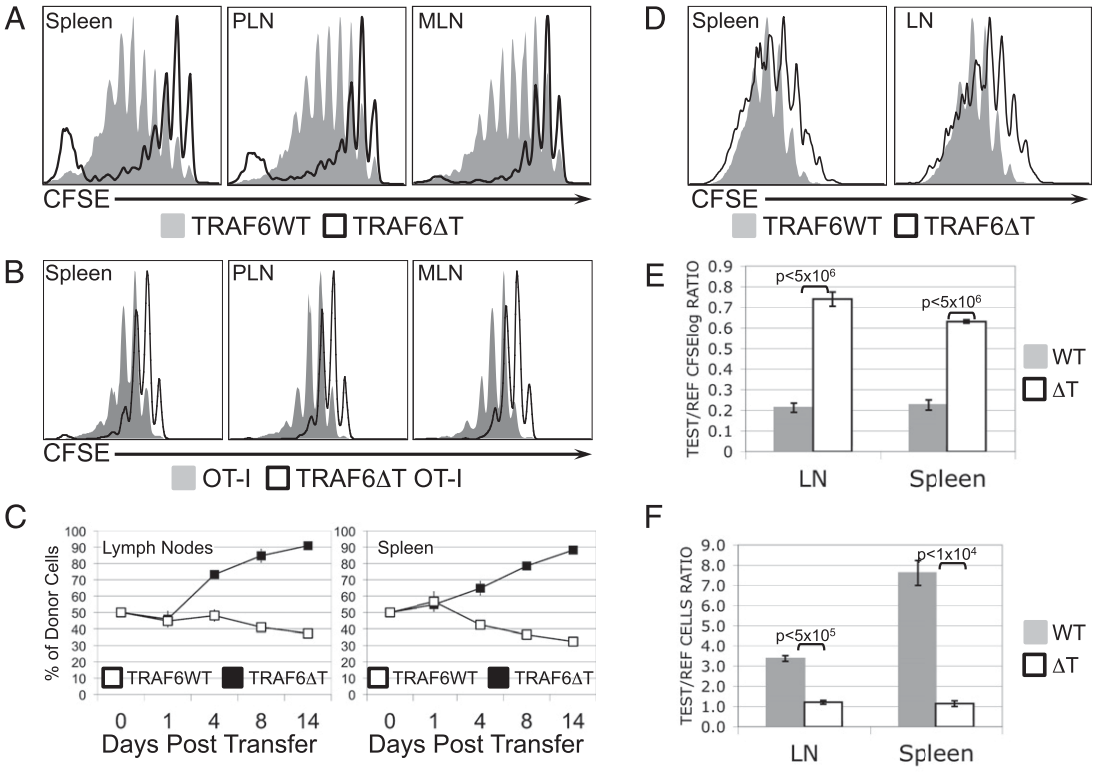

FIGURE 1. Naive TRAF6-deficient CD8 T cells exhibit defective LIP. CFSE profiles of sorted naive polyclonal (**A**) or OT-I (**B**) control (filled gray) or TRAF6-deficient (black line) CD8 T cells transferred for 7 d into lymphopenic RAG1 knockout mice and then harvested from recipient spleens, peripheral lymph nodes (PLN), and mesenteric lymph nodes (MLN). (**C**) Sorted naive control (filled) or TRAF6-deficient (open) OT-I CD8 T cells were mixed 1:1 with sorted naive CD90.1 congenic OT-I CD8 T cells and transferred into lymphopenic recipient mice (three per group per time point). As indicated, mice were sacrificed and cells from spleens and lymph nodes quantified according to the percentage of control (filled) or TRAF6-deficient (open) as a function of congenic reference cells. (**D**) CFSE profiles of sorted naive STAT5bCAtg.OT-I control (filled gray) or TRAF6-deficient (black line) CD8 T cells transferred for 7 d into lymphopenic recipient mice. (**E** and **F**) Sorted naive control (filled) or TRAF6-deficient (open) STAT5bCAtg.OT-I CD8 T cells were mixed 1:1 with sorted naive CD90.1 congenic STAT5bCAtg.OT-I CD8 T cells, CFSE labeled, and transferred into lymphopenic recipient mice (three per group) for 7 d. Ratios of CFSE dilution (calculated as logarithmic ratios) of test cells (wild-type [WT] or TRAF-deficient [ΔT]) over CFSE dilution of congenic reference cells from the same mouse (E) and ratios of recovered relative cells numbers (F) for control or TRAF6-deficient (test) cells over congenic (reference) cells from the same mouse are depicted. Error bars represent SD. A p value <0.05, calculated by a Student t test, indicates statistical differences between control (WT) and TRAF6-deficient (ΔT) experimental conditions.

experiment), transferred to lymphopenic recipient mice, and then relative cell counts were calculated from harvested recipient mouse tissues at various time points. Consistent with our observations of defective TRAF6ΔT LIP, we found that the population of TRAF6ΔT cells also progressively diminished relative to the total donor cell population over time (Fig. 1C). IL-7 signaling is critically important for normal progression of LIP. To address the possibility that defective IL-7 signaling results in reduced TRAF6ΔT LIP, we generated TRAF6ΔT.OT-I.STAT5bCAtg mice, which express a constitutively active form of the IL-7R–activated transcription factor STAT5b (24), and performed additional LIP experiments using donor OT-I.STAT5bCAtg cells. Surprisingly, we found that although constitutively active STAT5b augmented LIP by both control and TRAF6ΔT cells, a relative proliferative defect in the TRAF6ΔT cells persisted (Fig. 1D). Furthermore, when compared with control cells, TRAF6ΔT.OT-I.STAT5bCAtg cells exhibited defects in both proliferation (Fig. 1E) and cell counts (Fig. 1F) relative to cotransferred CD90.1 OT-I.STAT5bCAtg reference cells. Collectively, these findings suggested that an additional, previously unrecognized TRAF6-dependent pathway, nonredundant with STAT5-mediated signaling, might be involved in the induction of LIP.

IL-18 potentiates LIP in vivo and synergizes with high-dose IL-7 to induce proliferation in vitro

To further elucidate the mechanisms driving LIP and their relationship to TRAF6, we focused on examining, in the context of LIP, factors known to signal through TRAF6. Because the cytokine IL-18 signals through the TRAF6-dependent IL-18R complex (21) and has an established capacity to act on naive CD8 T cells by synergizing with IL-12 to induce production of IFN-γ (25, 26), we chose to investigate its effect on LIP first. Therefore, mice harboring lymphopenically proliferating CD8 T cells were treated daily with rIL-18 to reveal that both the CFSE profiles (Fig. 2A) and the percentages of donor cells as a function of total live cells (Fig. 2B) were enhanced by IL-18, but only when the donor cells expressed TRAF6, raising the possibility that IL-18 acts directly on lymphopenically proliferating CD8 T cells. IL-7, whose availability increases under lymphopenic conditions, has been identified as a cytokine critical to naive CD8 T cell LIP, but interestingly, IL-7 alone does not efficiently induce this type of proliferation in vitro (27). However, experiments involving transgenic mice that overexpress IL-7 suggest that in the proper context, high-dose IL-7 promotes LIP (28, 29). To determine whether IL-18 can complement IL-7–dependent proliferation, we designed an in vitro model of lymphopenia, whereby control or TRAF6ΔT naive CD8 T cells were cultured for 2 or 6 d (to account for the possible occurrence, respectively, of faster conventional proliferation or slower LIP-like proliferation) with or without a high dose (1.0 ng/ml, which is >10 times the minimum dose required to maintain cell viability) of rIL-7 in the presence or absence of 10 ng/ml rIL-18. To determine whether any observed effects of IL-18 were specific, we simultaneously tested the related TRAF6-dependent cytokine IL-1β. After normalizing for the increased in vitro cell counts at both days 2 and 6 attributable to the addition of IL-7 alone, we found that neither IL-18 nor IL-1β alone at any time point, nor IL-18 or IL-1β in combination with IL-7 at day 2, had an effect on cell numbers. Surprisingly, however, in the 6-d culture we found that IL-18, but not IL-1β, synergized with IL-7 to increase cell numbers in a TRAF6-dependent manner (Fig. 2C). Using CFSE-labeled cells, we demonstrated that this increase correlated with the induction of proliferation by naive control, but not TRAF6ΔT, CD8 T cells treated with IL-7 in the presence of IL-18 (Fig. 2D).

IL-7/IL-18 synergy is specific to CD8 T cells and functionally distinct from IL-12/IL-18 synergy

To determine the specificity of IL-7/IL-18 synergy on naive T cells, we subjected sorted naive CD4 or CD8 T cells to treatment with different doses of IL-7 in the presence or absence of IL-18 for 2 or 6 d of culture. Interestingly, we found that whereas both CD4 and CD8 T cell counts increased dose-wise with IL-7, only CD8 T

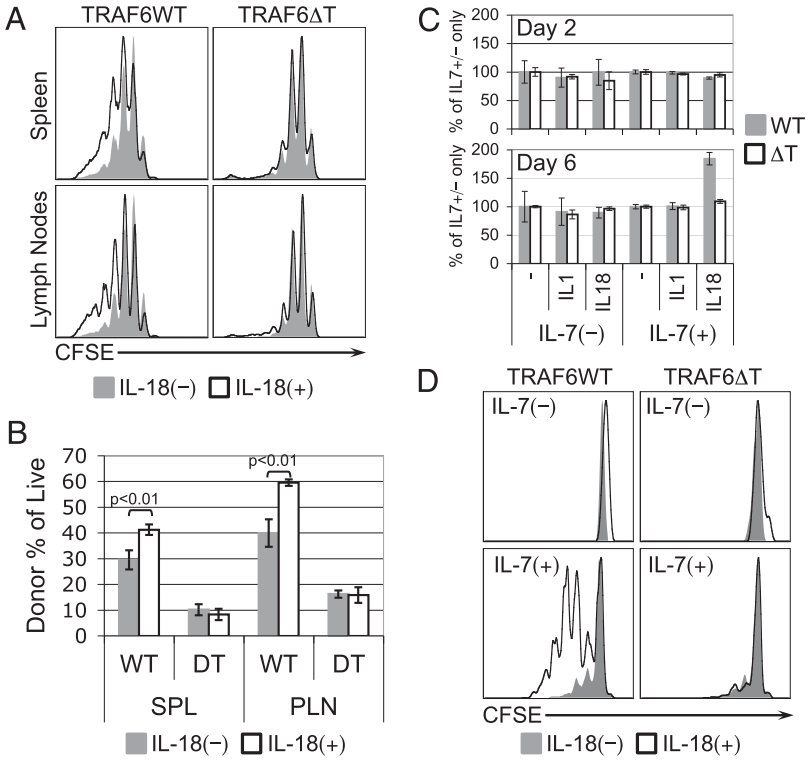

FIGURE 2. TRAF6-dependent LIP of naive CD8 T cells is enhanced in vivo, and induced in vitro, by IL-18. (**A**) CFSE profiles of sorted naive control or TRAF6-deficient OT-I CD8 T cells recovered after having been adoptively transferred for 7 d into lymphopenic recipient mice that were treated daily with vehicle (filled gray) or rIL-18 (black line). (**B**) Percentage of live spleen (SPL) or peripheral lymph node (PLN) cells harvested from vehicle- (gray filled) or rIL-18–treated (black lines) recipient mice derived from sorted naive control (wild-type [WT]) or TRAF6-deficient (ΔT) OT-I CD8 T cells adoptively transferred 7 d earlier. (**C**) Live relative cell counts from in vitro cell cultures of sorted naive control (gray filled) or TRAF6-deficient (black lines) OT-I CD8 T cells cultured for 2 or 6 d with and without 2 ng/ml IL-7 in the presence of no additional cytokine (−) or 10 ng/ml of either IL-1β or IL-18. (**D**) CFSE profiles of sorted naive control or TRAF6-deficient OT-I CD8 T cells cultured in vitro for 6 d with or without IL-7 in the absence (filled gray) or presence (black line) of 10 ng/ml IL-18. Error bars represent calculated SD across three biological replicates. Live relative cell counts were made by collection for equal time at equal flow rate of equal culture volumes on a FACSCalibur flow cytometer.

cells exhibited a synergistic increase in response to the addition of IL-18, and only after 6 d of culture in the presence of the higher dose of IL-7 (Fig. 3A). These increases in cell counts correlated with induction of proliferation specific to CD8 T cells treated with high-dose IL-7 in combination with IL-18 (Supplemental Fig. 2). Because of the specificity of IL-7/IL-18 synergy for CD8 T cells, we conducted subsequent experiments using OT-I TCR transgenic mice as a source of monoclonal, Ag-inexperienced CD8 T cells. Using these cells, we first demonstrated that unlike the synergistic relationship between IL-18 and IL-12, IL-18 does not induce IFN-γ production by naive CD8 T cells when combined with IL-7 for 60 h (Fig. 3B). At the same time, titration of IL-7 in the presence or absence of a constant dose of IL-18 further demonstrated that a minimum threshold of IL-7 signaling, higher than that required for maintaining cell viability, is required for IL-7/IL-18 synergy (Fig. 3C). This threshold concentration of IL-7 could not be overcome by increasing the accompanying dose of IL-18 (Fig. 3D), suggesting that IL-7 has a requisite role in sensitizing cells to IL-18 in this context.

CD8 T cell LIP and IL-7/IL-18 in vitro synergy are associated with upregulation of IL-18R expression

IL-12 has been shown to synergize with IL-18 by upregulating IL-18R gene expression, thereby sensitizing CD8 T cells to IL-18 (26). To determine whether lymphopenic conditions induce IL-18R expression on naive CD8 T cells, we adoptively transferred sorted naive OT-I CD8 T cells into irradiated CD45.1 congenic recipient mice, and harvested donor cells by sorting 8 d later. cDNA was prepared and gene expression compared by real-time PCR to expression levels from untransferred naive cells. It was found that both IL-18R α- and β-chain genes were induced by lymphopenic conditions, whereas the related gene, IL-1Rα, was not induced (Fig. 4A). To gain insight into any specific relationship between IL-7, IL-18, and IL-18R expression in vitro, we performed real-time PCR on cDNA prepared from OT-I CD8 T cells treated for 4 h in the presence or absence of IL-18 with varying concentrations of IL-7. We found that high-dose IL-7 synergized with IL-18 to enhance expression of the IL-18R α- and β-chains (Fig. 4B), possibly explaining the increased sensitivity of these cells to IL-18.

IL-18– and IL-7–dependent in vitro LIP requires PI3K activation

IL-7 induces gene expression primarily through activation of the JAK/STAT pathway and the transcription factor STAT5. Whereas IL-7 has been shown to synergize with IL-6 or IL-21 to augment STAT5 activation (12, 30), we found that, unlike IL-6, IL-18 had no effect on IL-7–dependent phosphorylation of STAT5 (Supplemental Fig. 3). IL-7 has also been shown to activate the PI3K/Akt pathway, although this activation pathway is delayed compared with STAT5 (31). It has also been demonstrated that recent thymic emigrants require PI3K to proliferate in response to IL-7 (32). Interestingly, Akt activation has been shown to depend on functional TRAF6 downstream of various receptor-signaling complexes (19, 33). We demonstrated that treatment of naive CD8 T cells with high-dose IL-7 in combination with IL-18 during a 5-d culture results in strikingly enhanced activation of Akt, which correlated with increased expression of the anti-apoptotic factor Bcl-x_L, and cell cycle–related factors Cdk6 and cyclin D3 (Fig. 5A). Furthermore, pretreatment of IL-7/IL-18–stimulated cultures with the PI3K inhibitor LY294002 revealed that IL-18–dependent increases in cell size and cell numbers are particularly sensitive to PI3K inhibition, whereas the IL-7–mediated cell survival effect is less sensitive (Fig. 5B), suggesting that enhanced PI3K/Akt activation may be the key molecular mechanism of IL-7/IL-18 synergy. Pretreatment of CFSE-labeled IL-7/IL-18–stimulated CD8 T cell cultures with LY294002 further demonstrated that PI3K inhibition prevents IL-7/IL-18–dependent proliferation (Fig. 5C).

Effect of IL-18 on in vitro LIP is linked to TCR-mediated signals

To further elucidate the mechanism of synergy between IL-7 and IL-18 in the context of lymphopenia-induced–like proliferation of

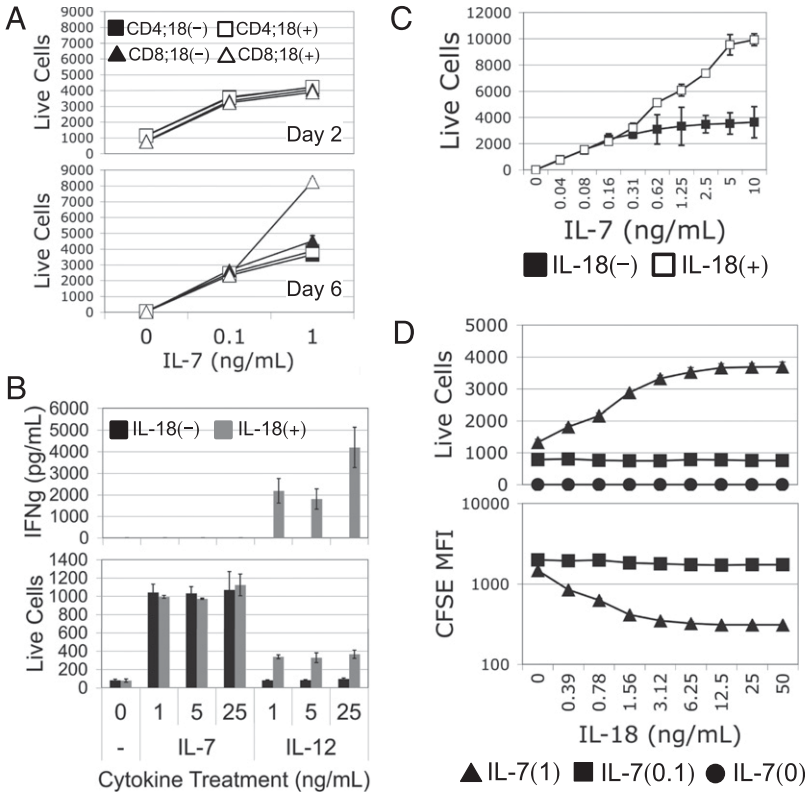

FIGURE 3. IL-7/IL-18 synergy is specific to CD8 T cells and functionally distinct from IL-12/IL18 synergy. (**A**) Live relative cell counts from in vitro cell cultures of sorted naive CD4 (squares) or CD8 (triangles) T cells cultured for 2 or 6 d with either 0, 0.1, or 1.0 ng/ml IL-7 in the presence (open) or absence (filled) of 10 ng/ml IL-18. (**B**) ELISA for IFN-γ secreted by (*top*), and live relative cell counts of (*bottom*), naive OT-I CD8 T cells cultured for 60 h with varying doses of IL-7 or IL-12 in the presence (gray) or absence (black) of 10 ng/ml IL-18. (**C**) Live relative cell counts of naive OT-I CD8 T cells cultured in vitro for 6 d with various doses of IL-7 in the absence (filled) or presence (open) of 10 ng/ml IL-18. (**D**) Live relative cell counts (*top*), and CFSE mean fluorescence intensity (*bottom*), of naive OT-I CD8 T cells cultured in vitro for 6 d with various doses of IL-18 combined with 0 (circles), 0.1 ng/ml (squares), or 1.0 ng/ml (triangles) of IL-7. Error bars represent calculated SD across three biological replicates. Live relative cell counts were made by collection for equal time at equal flow rate of equal culture volumes on a FACSCalibur flow cytometer.

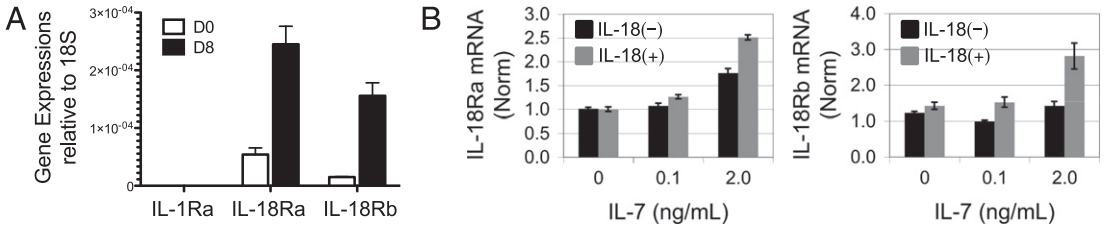

FIGURE 4. CD8 T cell LIP and IL-7/IL-18 in vitro synergy are associated with upregulation of IL-18R expression. (**A**) Real-time PCR for IL-1Rα, IL-18Rα, and IL-18Rβ message expressed by sorted naive OT-I CD8 T cells (D0) or OT-I CD8 T cells recovered by sorting 8 d after being transferred to congenic lymphopenic recipient mice (D8). Errors represent SD calculated from cell samples harvested from three independent recipient mice ($n = 3$). (**B**) Real-time PCR for IL-18Rα (*left panel*) and IL-18Rβ (*right panel*) message expressed by naive OT-I CD8 T cells cultured for 4 h with no, low-dose (0.1 ng/ml), or high-dose (2.0 ng/ml) IL-7 in the presence (gray) or absence (black) of 10 ng/ml IL-18. Error bars represent calculated SD across three biological replicates.

naive CD8 T cells in vitro, we performed microarray analysis of cells treated for 24 h with low- or high-dose IL-7 in the presence or absence of IL-18. We found by Kyoto Encyclopedia of Genes and Genomes pathway analysis that genes whose expression levels are affected by high-dose IL-7 specifically in the presence of IL-18 are most closely linked to the TCR pathway (Fig. 6A). In addition to IL-7, tonic TCR signaling is required for both survival and proliferation of CD8 T cells under lymphopenic conditions (12, 34). We cultured naive CD8 T cells in the presence of IL-7 and IL-18 in the presence of blocking anti–MHC-I (Y3) or isotype control Abs and found that interfering with tonic interaction between MHC-I and TCR complexes within a population of purified CD8 T cells resulted in decreased proliferation (Fig. 6B). Whereas the effect of MHC-I blocking Ab on CFSE dye dilution was significant in the presence or absence of IL-18, its effect on total cell numbers was specific to cultures treated in the presence of IL-18 (Fig. 6C). These data further suggested a role for IL-18 in sensitizing naive CD8 T cells to the tonic signals transmitted through TCR during LIP.

IL-18 synergizes with IL-7 to sensitize naive CD8 T cells to antagonist peptide

To examine the role of IL-7/IL-18 synergy in sensitizing naive CD8 T cells to tonic (i.e., self-peptide) TCR signals, we used the OT-I TCR transgenic system, for which an array of peptides has been characterized with respect to affinity to TCR and capacity to mediate T cell selection (35). Whereas the chicken OVA peptide 257–264 acts as a cognate Ag for OT-I T cells, the E1 mutant version of this peptide is antagonistic and has been shown to be capable of mediating both positive selection and LIP in vivo (13, 14). Because CD8 T cells express MHC-I, they are capable of being activated by the provision of free peptide (36). We assessed upregulation of Bcl-x_L message by IL-7/IL-18–treated naive OT-I CD8 T cells cultured in the presence or absence of either OVA or E1 mutant peptide and determined that although both peptides had a positive effect, OVA peptide acted more quickly and did not require the presence of IL-18, whereas E1 peptide acted more slowly, and only when IL-18 was present (Fig. 7A). Likewise, IL-18 significantly increased surface expression of early (preceding induction of proliferation) activation markers CD69 and CD44 on cells cultured with-high dose IL-7 and E1 peptide by 36 h (Fig. 7B). These findings suggested that IL-7, IL-18, and E1 peptide might synergistically recapitulate slow lymphopenia-induced–like proliferation in vitro. Indeed, we found that during a relatively short (60 h) culture OVA peptide induced the same degree of CFSE dye dilution independently of IL-18 cotreatment, whereas cells treated with E1 peptide and IL-7 were induced to proliferate, but only in the presence of IL-18 (Fig. 7C). Live cell counts in cultures stimulated with various concentrations of OVA or E1 peptides revealed, not surprisingly, that OT-I CD8 T cells accumulated at a much lower concentration of OVA peptide than of E1 mutant peptide when cotreated with IL-7 and IL-18 (Fig. 7D). Interestingly, when treated with IL-18, OVA-stimulated cells responded equally well in the presence or absence of IL-7, whereas cells treated with IL-18 and E1 peptide (regardless of concentration) failed to accumulate, or even survive, in the absence of IL-7. This finding raised the possibility that IL-7 and IL-18 together provide a "costimulatory" signal distinct from conventional CD28 ligation, in a manner specifically suited to sensitizing naive CD8 T cells to low-affinity or antagonist peptide. To test this idea, we stimulated naive OT-I CD8 T cells with IL-7 and either OVA or E1

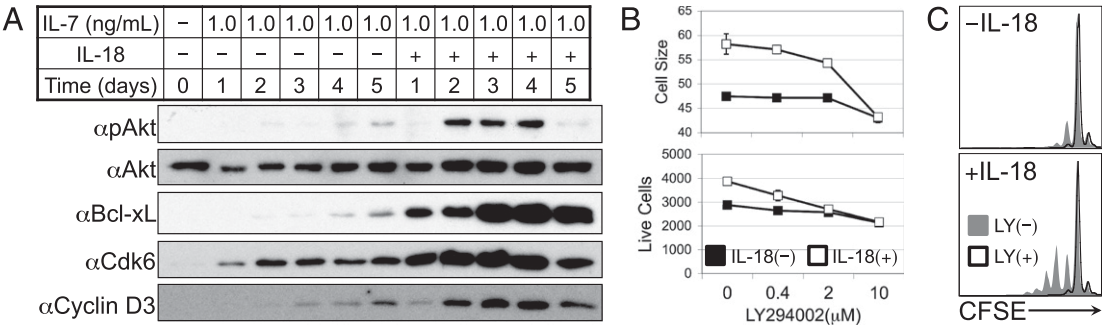

FIGURE 5. PI3K signaling is required for IL-18–dependent in vitro LIP. (**A**) Western blot analysis of naive OT-I CD8 T cells treated with 1 ng/ml IL-7 in the presence or absence of 10 ng/ml IL-18 for the number of days indicated. (**B**) Relative cell size (*top*) and live relative cell counts (*bottom*) from in vitro cell cultures of naive OT-I CD8 T cells treated for 6 d with 1.0 ng/ml IL-7 in the presence (open) or absence (filled) of 10 ng/ml IL-18 and the indicated concentration of LY294002. (**C**) CFSE profiles of sorted naive OT-I CD8 T cells cultured in vitro for 6 d with or without 10 ng/ml IL-18 in the absence (filled gray) or presence (black line) of 10 μM LY294002. Error bars represent calculated SD across three biological replicates. Live relative cell counts were made by collection for equal time at equal flow rate of equal culture volumes on a FACSCalibur flow cytometer.

A

Category	Term	Count	%	P-Value
KEGG_PATHWAY	T cell receptor signaling pathway	9	3.8	4.4E-4
KEGG_PATHWAY	B cell receptor signaling pathway	7	3.0	1.3E-3
KEGG_PATHWAY	Toll-like receptor signaling pathway	8	3.4	3.5E-3
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	11	4.7	1.9E-2

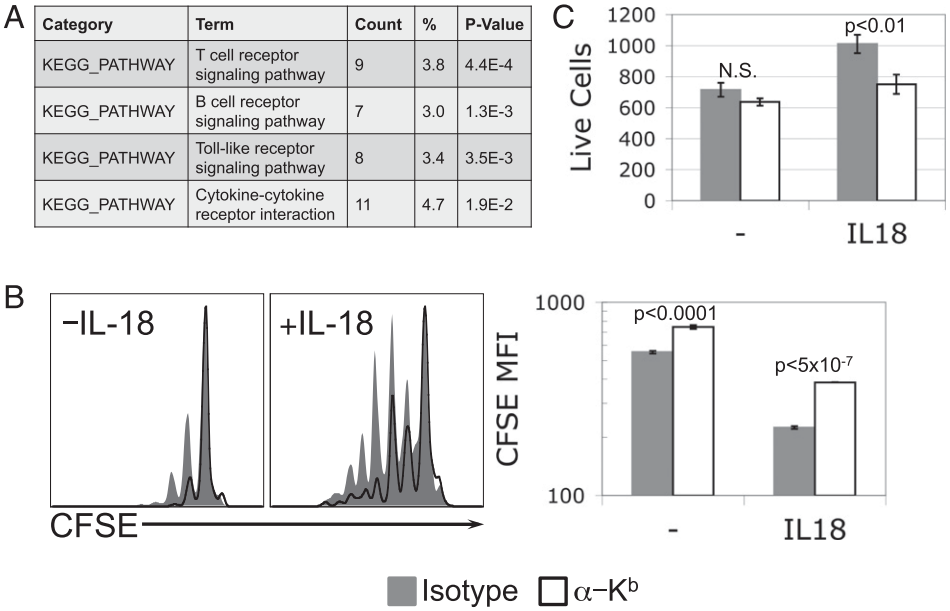

FIGURE 6. Synergy between IL-7 and IL-18 in naive CD8 T cells affects TCR-associated signaling. (**A**) Naive OT-I CD8 T cells were stimulated for 24 h with and without 10 ng/ml IL-18 in the presence of low-dose (0.1 ng/ml) or high-dose (1.0 ng/ml) IL-7, and RNA message was subjected to microarray analysis. Differential gene expression across experimental groups ($n = 5$) was determined by ANOVA, and the resulting gene lists were used for Kyoto Encyclopedia of Genes and Genomes pathway analysis. The relevant pathways are ranked by p value for functional assignment of genes that changed in the presence of IL-18 when IL-7 dose was increased from 0.1 to 1.0 ng/ml. (**B**) Representative CFSE profiles (*left panel*) and mean CFSE fluorescence from triplicate wells (*right panel*) of naive OT-I CD8 T cells cultured in vitro for 6 d with 1.0 ng/ml IL-7 with and without 10 ng/ml IL-18 in the presence of isotype control (gray, filled) or anti–MHC-I (Y3) neutralizing (black, unfilled) Ab. (**C**) Live relative cell counts from triplicate wells (*right panel*) of naive OT-I CD8 T cells cultured in vitro for 6 d with 1.0 ng/ml IL-7 with and without 10 ng/ml IL-18 in the presence of isotype control (gray, filled) or anti–MHC-I (Y3) neutralizing (black, unfilled) Ab. Error bars represent SD. A p value <0.05, calculated by a Student t test, indicates statistical differences between isotype control and anti-MHC neutralizing Ab conditions. Live relative cell counts were made by collection for equal time at equal flow rate of equal culture volumes on a FACSCalibur flow cytometer.

peptide in the presence or absence of IL-18 or anti-CD28. Predictably, cells stimulated with OVA peptide proliferated and accumulated equally well whether costimulated with IL-18 or anti-CD28. However, OT-I cells stimulated with E1 peptide were induced to proliferate (Fig. 7E) and accumulate (Fig. 7F) (although at lower levels than when stimulated with OVA) when costimulated with IL-18, but not with anti-CD28, thereby demonstrating the unique role of IL-7/IL-18 synergy in sensitizing naive CD8 T cells to low-affinity or antagonistic TCR–peptide interactions.

Discussion

In this study, we have identified a novel signaling mechanism in naive CD8 T cells that is mediated by a TRAF6-dependent pathway and required for LIP. We have further shown that this pathway may be directly activated in CD8 T cells by the inflammatory cytokine IL-18 when it acts in synergy with IL-7 at a "lymphopenia-like" concentration. IL-7/IL-18 synergy apparently functions to both enhance expression of the IL-18R complex and to sensitize naive CD8 T cells to noncognate peptides similar to self-peptides required both for positive selection and LIP.

Previous microarray analyses of CD8 T cells undergoing LIP reveal a gene expression program likened to an attenuated cognate Ag-mediated TCR stimulation (though lacking effector gene expression) (37). It has been well established that TCR-mediated activation via cognate peptide Ag requires amplifying costimulatory signals (38), and that this mechanism is important as a check on potentially harmful activation that could lead to autoimmunity (38, 39). However, it has been shown that conventional costimulatory pathways (e.g., CD28) are not required for LIP (40–42), and that blockade of conventional costimulation does not prevent pathology related to LIP following organ transplant (8, 43). Based on our present findings, we speculate that TRAF6-dependent activation of Akt in naive CD8 T cells encountering lymphopenic (or similar) conditions may represent a novel and unique costimulatory signal. As demonstrated, IL-18 synergizes with IL-7 in a manner analogous to CD28 to provide a complementary signal necessary for activation of naive CD8 T cells encountering noncognate Ag or self-peptide. We have provided evidence that these factors together enhance activation of the PI3K/Akt pathway, and expression of prosurvival and cell cycle–related proteins associated with TCR-mediated activation (Fig. 5). Importantly, however, we assert that costimulation provided through IL-7 and IL-18 synergy is mechanistically unique from that provided by CD28, as we found that CD28 costimulation was able to enhance proliferation of naive CD8 T cells stimulated with cognate Ag, but not with the noncognate self-peptide (Fig. 7). Our data suggest that unlike TCR and CD28, which can signal on naive cells at the same time, IL-7 and IL-18 signaling occurs sequentially, as IL-7 appears necessary to sensitize cells to IL-18 via IL-18R upregulation (Fig. 4). This is likely one reason (along with suboptimal TCR signaling) that this form of proliferation occurs more slowly than proliferation by cognate Ag and conventional costimulation. It is possible, however, that similar to conventional costimulation, IL-7/IL-18 synergy may also serve to prevent unnecessary and potentially dangerous immune responses from developing. For instance, it has been shown that excessive IL-7R signaling can lead to systemic autoimmunity, and that lymphopenia-related signals more generally may be implicated in loss of T cell tolerance (12, 44–47). Note also that whereas in vivo provision of IL-18 results in moderately enhanced LIP profiles (Fig. 2A), and CD8 T cells upregulate IL-18R genes under lymphopenic conditions (Fig. 4A), our preliminary work using IL-18–deficient recipient

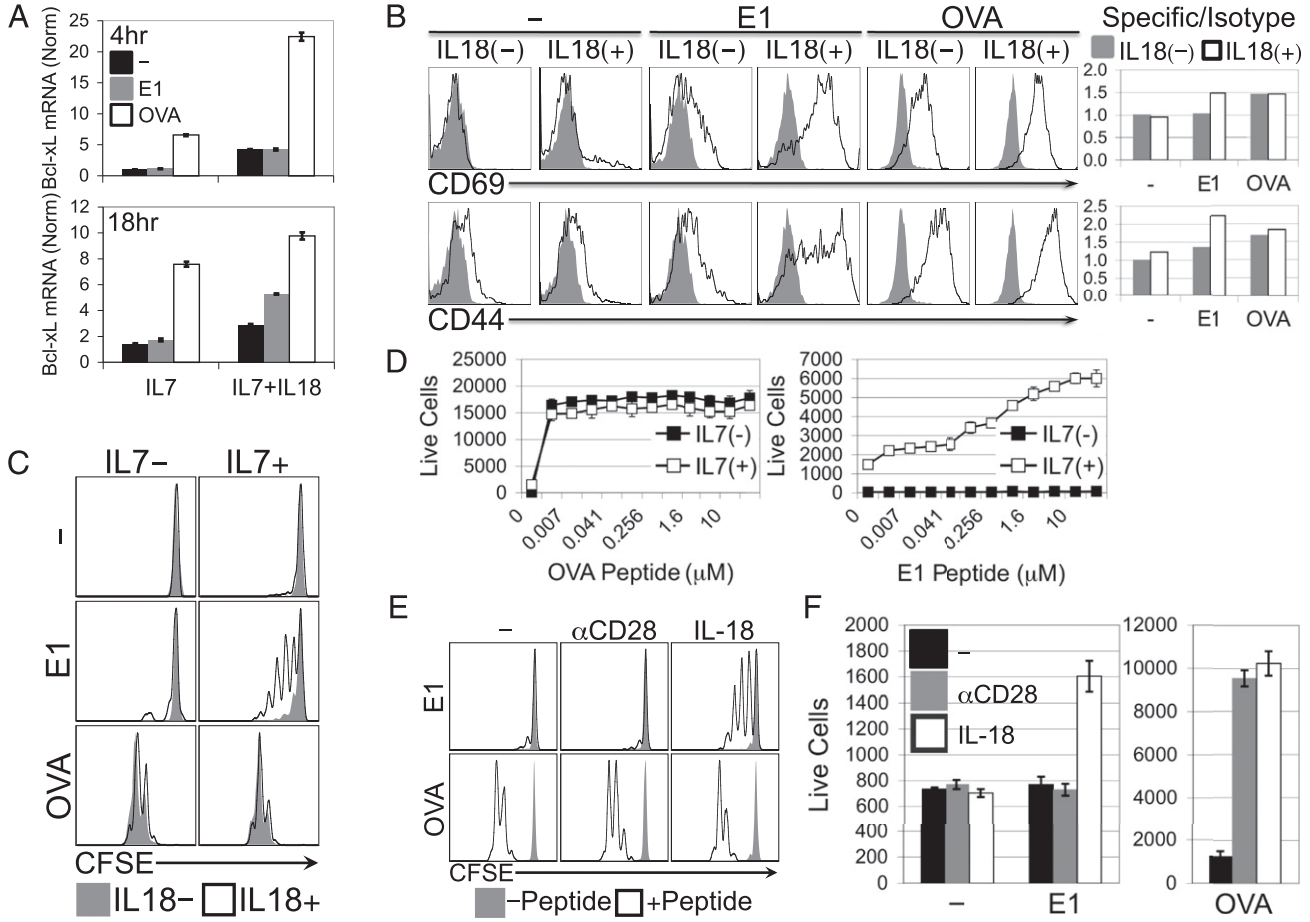

FIGURE 7. IL-18 synergizes with IL-7 to positively sensitize naive CD8 T cells to an antagonistic model peptide. (**A**) Real-time PCR for Bcl-xL message expressed by naive OT-I CD8 T cells cultured for 4 h with 1.0 ng/ml IL-7 alone or 1.0 ng/ml IL-7 plus 10 ng/ml IL-18 in the presence of either no peptide (black), 1 μM OVA (E1) mutant peptide (gray), or 1 μM $OVA_{257–264}$ peptide (open). (**B**) Flow cytometry histograms (*left*) depicting isotype (gray, filled) and specific staining (black, open) and accompanying ratiometric analysis (*right*) of CD69 (*top*) and CD44 (*bottom*) expression on naive OT-I CD8 T cells cultured for 36 h with 1.0 ng/ml IL-7 alone or 1.0 ng/ml IL-7 plus 10 ng/ml IL-18 in the presence of either no peptide, 1 μM OVA (E1) mutant peptide, or 1 μM $OVA_{257–264}$ peptide. (**C**) CFSE profiles of naive OT-I CD8 T cells cultured in vitro for 60 h with and without IL-7 in the presence (black, open) or absence (gray, filled) of IL-18 with either no peptide, 1 μM $OVA_{257–264}$ peptide, or 1 μM OVA (E1) mutant peptide. (**D**) Live relative cell counts of naive OT-I CD8 T cells cultured in vitro for 60 h with 10 ng/ml IL-18 alone (filled) or 10 ng/ml IL-18 plus 1.0 ng/ml IL-7 (open) in combination with various concentrations of either $OVA_{257–264}$ peptide (*top*) or OVA (E1) mutant peptide (*bottom*). (**E**) CFSE profiles and (**F**) live relative cell counts of naive OT-I CD8 T cells cultured in vitro for 60 h with 1.0 ng/ml IL-7 plus either no peptide, low-dose (0.001 nM) $OVA_{257–264}$ peptide, or high-dose (1000 nM) $OVA_{257–264}$ or OVA (E1) mutant peptide, in combination with either no costimulation, 1 μg/ml anti-CD28, or 10 ng/ml IL-18. Error bars represent calculated SD across three biological replicates. Live relative cell counts were made by collection for equal time at equal flow rate of equal culture volumes on a FACSCalibur flow cytometer.

mice or IL-18R–deficient donor T cells suggests that the requirement for IL-18 per se during LIP is either minor or highly context-dependent (data not shown). However, our work demonstrates that the TRAF6-mediated pathway is required for both in vivo LIP and in vitro IL-7/IL-18–mediated synergy. The most obvious explanation for our observations of the modest requirement for IL-18 in vivo is that there may be numerous other receptors expressed by naive CD8 T cells that may be capable of activating TRAF6 signaling. Although in this context we have found that IL-18 possesses unique activity among the IL-1/IL-18/IL-33 family members (Fig. 2C and data not shown), TRAF6 may also be activated through other TLR or TNFR family members, as well as through the TGFβ receptor complex. Interestingly, two recent reports have shown enhanced CD8 T cell LIP, and, in one case, induction of autoimmunity, in mouse models of defective T cell–specific TGF-β signaling (48, 49). These results are particularly intriguing because we have previously demonstrated negative modulation of TGF-β–mediated Smad activation by TRAF6 in T cells (50). Future work might therefore benefit from incorporating the role of TGF-βR signaling into studies of TRAF6-regulated naive CD8 T cell LIP (and even homeostasis more broadly).

Finally, IL-18 is a factor currently being studied in clinical applications, and it may be a promising immunostimulatory anticancer agent (51, 52). Therefore, whereas IL-18 may not itself represent the TRAF6-related factor required to drive LIP of naive CD8 T cells in vivo, it is important to recognize that IL-18 may still have potential as a therapeutic tool in cases where enhanced low-level activation of naive (or semiactivated) T cells is desirable. For instance, techniques have been demonstrated for enhancing antitumor responses by mimicking homeostatic proliferation conditions in vitro, as well as by eliminating sources of depletion of homeostatic cytokines in vivo (10, 53, 54). Although evidence suggests that conventional CD28 costimulation is required to drive lymphopenia-aided antitumor CTL differentiation and effector function in the presence of strong TCR stimulation (55), we present evidence in the present study for IL-18–mediated enhancement of lymphopenia-triggered proliferation

(but not necessarily effector differentiation) in response to "weak" TCR stimulation. We suggest that IL-18 and CD28 have distinct costimulatory functionalities, and that it may be possible to improve or enhance immunotherapeutic protocols (or others) with additional IL-18 treatment, and that, regardless, IL-18 may continue to serve as a useful tool for elucidating the physiologic underpinnings of proliferative mechanisms of naive T cell homeostasis.

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Disclosures

The authors have no financial conflicts of interest.

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