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IL-7 signaling must be intermittent, not continuous, during CD8 T cell homeostasis to promote cell survival instead of cell death

Motoko Y. Kimura, **Leonid A. Pobezinsky**, **Terry Guinter**, **Julien Thomas**, **Anthony Adams**, **Jung-Hyun Park**, **Xuguang Tai**, and **Alfred Singer**[1]

Experimental Immunology Branch, National Cancer Institute, Bethesda, MD 20892, USA

Abstract

Maintenance of naive CD8 T cells is necessary for lifelong immunocompetence but for unknown reasons requires both interleukin-7 (IL-7) and T cell receptor (TCR) signaling. We now report that naive CD8 T cells require IL-7 signaling to be intermittent, not continuous, because prolonged IL-7 signaling induces naive CD8 T cells to proliferate, produce interferon-γ (IFN-γ), and undergo IFN-γ-triggered cell death. Homeostatic TCR engagements interrupt IL-7 signaling and thereby support CD8 T cell survival and quiescence. However, CD8 T cells with insufficient TCR affinity for self-ligands receive prolonged IL-7 signaling and die during homeostasis. This study identifies the regulation of IL-7 signaling duration by homeostatic TCR engagements as the basis for *in vivo* CD8 T cell homeostasis.

Keywords

interferon-γ; apoptosis; cytokine induced cell death

Mature T cells develop in the thymus and then emigrate to the periphery where they must be maintained for the life of the organism in order to avoid the loss of potentially unique and important T cell antigen receptor specificities. Recent thymic emigrants arrive in the periphery as antigen-inexperienced naive cells which possess both the greatest proliferative and differentiative potential of any mature T cell. However, the thymus involutes during puberty and thymic production of new T cells diminishes, increasing the importance of those T cells already resident in the periphery. Indeed, life-long immunocompetence depends on maintaining mature peripheral T cells and, ideally, maintaining them as naive cells with full proliferative and differentiative potential. The process of maintaining mature T cells in the periphery is known as T cell homeostasis and requires signaling by both interleukin-7 (IL-7) and T cell receptor (TCR)[1–5].

[1]Address correspondence to: Alfred Singer Experimental Immunology Branch National Cancer Institute Bethesda, MD 20892, USA. Tel: +1 301 496 5461 Fax: +1 301 496 0887 singera@nih.gov.

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The strict dependence of naive CD8 T cells on IL-7 was first appreciated by their inability to survive in either IL-7 deficient or anti-IL-7α treated mice [6–8]. IL-7 is not produced by T cells but is produced by stromal cells in the thymus and periphery. Binding of IL-7 to its cellular receptor activates STAT5 and phosphatidylinositol-3-OH kinase (PI(3)K) and leads to expression of Bcl-2 and Mcl-1 proteins, both of which promote naive CD8 T cell survival [9–12]. IL-7 signals are transduced by the IL-7 receptor (IL-7R) which is composed of IL-7Rα and common α (αc) chain proteins. Whereas signaling by other αc cytokines such as IL-2 or IL-4 increases cellular expression of their cognate receptors and results in CD8 T cell proliferation and differentiation, signaling by IL-7 transcriptionally reduces expression of its cognate receptor [13–15]. IL-7 induced IL-7R downregulation has the beneficial effect of increasing the number of CD8 T cells that can be supported by limiting amounts of *in vivo* IL-7[13].

Low affinity TCR engagements of self-ligands in the periphery are also necessary for CD8 T cell homeostasis[16–23]. The dependence of naive CD8 T cells on low affinity TCR engagements is curious because IL-7 signaling would seem to be otherwise sufficient for their survival. However, IL-7 and TCR signaling have been shown to affect one another in naive CD8 T cells in a process referred to as `coreceptor tuning'[24]. On the one hand, IL-7 signals dynamically adjust CD8 coreceptor levels on individual CD8 T cells so that their TCRs engage and disengage from self-ligands in the periphery, causing homeostatic TCR signaling to be intermittent. On the other hand, homeostatic TCR signaling blocks IL-7 signal transduction, causing IL-7 signaling of individual CD8 T cells to also be intermittent[24]. But why naive CD8 T cells would require both intermittent homeostatic TCR engagements and intermittent IL-7 signaling for long-term survival remains an unsolved mystery.

The present study was undertaken to examine the effects of uncoupling IL-7 and TCR signaling so that IL-7 signaling of naive CD8 T cells could be continuous. We now report that intermittent IL-7 signaling promotes naive CD8 T cell quiescence and survival, but continuous IL-7 signaling induces naive CD8 T cells to proliferate, produce interferon-γ (IFN-γ), and die. In fact, CD8 T cell death was due to apoptosis triggered by IFN-γ produced by continuously IL-7 signaled CD8 T cells, an outcome we refer to as “cytokine induced cell death” (CICD). Continuous IL-7 signaling of IFN-γ production was prevented by intermittent homeostatic TCR engagements that interrupt IL-7 signaling to limit its duration. Consequently, naive CD8 T cell survival and quiescence requires expression of TCR with sufficient affinity for peripheral ligands to prevent prolonged IL-7 signaling. This study fundamentally alters the understanding of IL-7's function during CD8 T cell homeostasis and reveals the importance of IL-7 signaling interruptions by homeostatic TCR engagements.

RESULTS

Consequences of continuous IL-7 signaling

Maintenance of peripheral naive CD8 T cells requires both IL-7 mediated survival signals and intermittent homeostatic TCR engagements, with the interplay between IL-7 and TCR critical for CD8 T cell homeostasis. Because IL-7 signaling transcriptionally reduces surface

IL-7R expression[13], intermittent blockade of IL-7 signaling by homeostatic TCR engagements is necessary for CD8 T cells to maintain IL-7R expression[24]. The present study was undertaken to determine if disrupting the relationship between TCR signaling and IL-7Rα expression would allow CD8 T cells to be continuously signaled by IL-7 and alter the requirement for homeostatic TCR engagements.

To generate naive CD8 T cells bearing IL-7Rα proteins that would be refractory to IL-7 downregulation, we introduced the hCD2-driven IL-7Rα transgene into *Il7r*$^{-/-}$ mice [13]. We herein refer to transgenic IL-7Rα proteins as 7RTg and to IL-7Rα transgenic *Il7r*$^{-/-}$ mice as 7RTg mice. CD8 T cells in 7RTg mice were modestly reduced in number as previously reported[13] but displayed a predominantly naive CD44^{lo} phenotype that could be isolated with >98% purity by magnetic bead separation (Supplementary Fig. 1a and 1b). Naive CD8 T cells freshly obtained from 7RTg and B6 mice expressed similar levels of surface IL-7Rα and responded similarly to 30 min IL-7 stimulation, with comparable elevations in phosphorylated STAT5 (p-STAT5) (Fig. 1a). In contrast, only naive CD8 T cells from 7RTg mice expressed surface IL-7Rα and maintained p-STAT5 elevation for the 7 day duration of IL-7 stimulation (Fig. 1b, and Fig. 1c). These results indicated that persistent IL-7Rα expression permitted continuous IL-7 signaling of CD8 T cells and prolonged STAT5 activation.

To determine if continuous IL-7 signaling altered the requirement for homeostatic TCR engagements, we examined the ability of naive 7RTg CD8 T cells to undergo *in vivo* lymphopenic proliferation, a response that otherwise requires both IL-7 survival signals and homeostatic TCR engagements. To assess IL-7 driven lymphopenic proliferation in the presence and absence of homeostatic TCR engagements, we adoptively transferred 1×10^6 naive CD8 T cells bearing the major histocompatability class I (MHC-I)-restricted transgenic P14 TCR into either MHC-I-sufficient (B6) or MHC-I-deficient β2m knockout (*B2m*$^{-/-}$) animals that had been rendered lymphopenic by sub-lethal irradiation and examined the reconstituted mice 14 days later (Fig. 1d). In MHC-I-sufficient B6 host mice, donor P14 CD8 T cells bearing endogenous IL-7Rα proteins proliferated (as determined by CFSE dye dilution) and expanded as more donor cells were recovered than the 1×10^6 initially transferred (Fig. 1d); whereas, in MHC-I-deficient *B2m*$^{-/-}$ host mice, donor P14 CD8 T cells down-regulated IL-7Rα expression, did not proliferate, and decreased in number (Fig. 1d), indicating that P14 CD8 T cells neither proliferated nor survived in the absence of *in vivo* homeostatic TCR engagements, (Fig. 1d, compare top two rows). In contrast donor 7RTgP14 CD8 T cells did proliferate in *B2m*$^{-/-}$ host mice and proliferated as extensively as P14 T cells in B6 mice (Fig. 1d, compare bottom two rows). Despite extensive proliferation, fewer donor 7RTgP14 CD8 T cells were recovered from *B2m*$^{-/-}$ host mice than initially transferred (Fig. 1d) and this was due to absent homeostatic TCR engagements in *B2m*$^{-/-}$ host mice as recovery in B6 host mice was much greater (Fig. 1d). These results revealed that continuous signaling by the pro-survival cytokine IL-7 was sufficient to replace homeostatic TCR engagements for *in vivo* lymphopenic proliferation but did not replace homeostatic TCR engagements for *in vivo* cell survival.

The failure of CD8 T cells to survive despite continuous signaling by the pro-survival cytokine IL-7 was paradoxical, so we turned to *in vitro* analyses to determine its molecular

basis. We found that we could replicate this failure of survival *in vitro* by simply placing naive CD8 T cells from 7RTg mice in IL-7 cultures (Fig. 2a). Similar to our *in vivo* observations in *B2m*$^{-/-}$ host mice, IL-7 signaled 7RTg CD8 T cells proliferated extensively but underwent extensive cell death, with >90% of 7RTg CD8 T cells apoptotic after 14 days of continuous IL-7 signaling (Fig. 2a). Because continuous IL-7 signaling prolonged expression of phosphorylated STAT5 which can bind to the IFN-γ gene and activate IFN-γ gene expression [25, 26], we also assessed IL-7 signaled 7RTg CD8 T cells for IFN-γ expression. In fact, we found that IL-7 signaled 7RTg CD8 T cells expressed both IFN-γ mRNA and IFN-γ protein (Fig. 2b).

To rigorously document that these *in vitro* events were the result of continuous IL-7 signaling independently of homeostatic TCR engagements, we also utilized naive CD8 T cells from 8DP4 experimental mice that could not possibly generate homeostatic TCR engagements in IL-7 cultures because they neither expressed MHC-I specific TCR nor MHCI-dependent self-ligands despite being CD8 T cells[27]. CD8 T cells from 8DP4 experimental mice express only MHCII-specific TCRs and are genetically *B2m*$^{-/-}$ so they do not bear self-ligands that can be engaged by either their TCR or CD8 coreceptors[27]. Precisely as observed with 7RTg CD8 T cells, continuous IL-7 signaling induced 7RTg. 8DP4 CD8 T cells to proliferate, express IFN-γ, and die (Fig. 2a, and Fig. 2b), confirming that these *in vitro* results occurred in the absence of homeostatic TCR engagements. Thus continuous IL-7 signaling failed to maintain naive CD8 T cells, but instead induced them to proliferate, produce IFN-γ, and die.

Intermittent versus continuous IL-7 signaling

That continuous IL-7 signaling induced naive CD8 T cell proliferation, IFN-γ expression, and cell death was discordant with IL-7's maintenance of naive CD8 T cells during homeostasis. To explain this discordance, we considered that IL-7 signaling might have different effects on CD8 T cells, depending on its duration. We speculated that intermittent (i.e. short duration) IL-7 signaling might promote CD8 T cell quiescence and survival, whereas uninterrupted (i.e. continuous) IL-7 signaling might induce CD8 T cell proliferation and death. To assess this possibility, we utilized naive 7RTg CD8 T cells and exposed them to IL-7 either intermittently or continuously (Fig. 2c). In this experiment, naive 7RTg CD8 T cells were cultured with IL-7 for the first 14 hours of each day, washed, and then re-cultured for the remaining 10 hours of each day in either IL-7 again or medium (Fig. 2c). This cycle was repeated daily for the 2–3 week duration of the experiment. Continuous exposure to IL-7 for 14 days caused naive 7RTg CD8 T cells to proliferate, express IFN-γ and FasL, and die, in that apoptotic cell frequency increased steadily throughout while viable cell number first increased and then declined (Fig. 2d, and Fig. 2e). Continuous exposure to IL-7 also caused naive 7RTg CD8 T cells to phenotypically convert into CD44^{hi} cells, indicative of an activation/memory phenotype (Fig. 2f). However, in marked contrast to continuous IL-7 exposure, intermittent daily exposure to IL-7 maintained naive 7RTg CD8 T cells as quiescent cells which did not proliferate, die, or express either IFN-γ or FasL (Fig. 2d, and Fig. 2e). Moreover, intermittent IL-7 exposure maintained 7RTg CD8 T cells as naive CD44^{lo} cells even after 3 weeks in IL-7 culture (Fig. 2f). These results document that intermittent IL-7 signaling maintained naive CD8 T cells as viable and quiescent cells even

in the apparent absence of homeostatic TCR engagements, whereas continuous IL-7 signaling induced naive CD8 T cell activation and death. The consequences of continuous IL-7 signaling (i.e. activation and death) were observed even at low doses of IL-7 (Supplementary Fig. 2).

Cytokine induced cell death (CICD)

IL-7 signaled T cell death has not previously been observed and we refer to T cell death resulting from continuous IL-7 signaling as "cytokine induced cell death" or CICD. To determine the mechanism of CICD, we examined if continuous IL-7 signaling either down-regulated expression of pro-survival proteins or up-regulated expression of pro-apoptotic proteins. Continuous IL-7 signaling of 7RTg CD8 T cells did not down-regulate expression of the pro-survival proteins Bcl-2, Mcl-1, and Bcl-xL (Fig. 3a), but instead in some instances even increased their expression. However, we found that continuous IL-7 signaling up-regulated expression of pro-apoptotic proteins. First, we found that continuous IL-7 signaling of 7RTg CD8 T cells induced the appearance of active caspase-3 which is a death effector molecule whose expression in cells invariably results in cell death (Fig. 3b). Second, we found that continuous IL-7 signaling induced expression of intermediates in both of the known pro-apoptotic cellular pathways, in that it upregulated Fas and FasL in the (extrinsic) death receptor pathway (Fig. 3c) and up-regulated Bim mRNA and protein (though not Puma or Noxa) in the mitochondrial (intrinsic) death pathway (Fig. 3d). To examine the death receptor and mitochondrial death pathways individually, we introduced the IL-7Rα transgene into $Fas^{lpr/lpr}$ and into Bim-KO (*Bcl2l11*$^{-/-}$) mice to generate 7RTg.$Fas^{lpr/lpr}$ mice and 7RTg.Bim-KO mice which either lacked Fas death receptor signals or lacked Bim proteins. Continuous IL-7 exposure of naive CD8 T cells from 7RTg.$Fas^{lpr/lpr}$ and 7RTg.Bim-KO mice caused only modest, but statistically significant, reductions in apoptosis and little change in viable cell numbers compared to 7RTg CD8 T cells in which both death pathways were intact (Fig. 3e, and Fig. 3f). These results indicated that continuous IL-7 signaling activated both apoptotic pathways, with neither predominating in CICD.

Because continuous IL-7 signaling induced IFN-γ expression as well as CICD, we wondered if IFN-γ contributed to CICD by examining induction of active caspase-3 in 7RTg CD8 T cells that were unresponsive to IFN-γ because they lacked expression of IFN-γ receptor proteins (IFN-γR) (Fig. 4a). IFN-γR deficiency (*Ifngr1*$^{-/-}$) prevented IL-7 induction of active caspase-3 (Fig. 4a). Moreover, deficiency in either IFN-γ (*Ifng*$^{-/-}$) or IFN-γR reduced the death of IL-7 signaled 7RTg CD8 T cells (Fig. 4b), revealing that IFN-γ from continuously IL-7 signaled CD8 T cells was in fact responsible for triggering CICD. Concordant with IFN-γ triggering of CICD, we also observed that addition of exogenous rIFN-γ significantly increased IL-7 induced apoptosis of IFN-γ-deficient CD8 T cells; and reciprocally, addition of anti-IFN-γ antibody reduced the death induced by IL-7 signaling of IFN-γ-intact CD8 T cells (Fig. 4b). We conclude that continuous IL-7 signaling induces CD8 T cells to produce IFN-γ which triggers CICD.

Anti-IFN-γ blockade promotes cell survival *in vitro*

Identification of IFN-γ as the necessary trigger of CICD made it possible to determine the consequences of continuous IL-7 signaling in the absence of CICD. Continuous IL-7 signaling induced 7RTg CD8 T cells to initially increase in number and then, as a result of CICD, decline (Fig. 4c). However, in the presence of anti-IFN-γ antibody to prevent triggering of CICD, continuous IL-7 signaling induced 7RTg CD8 T cells to undergo continuous exponential growth with cell numbers increasing ~200 fold in 14 days (Fig. 4c). Such continuous exponential growth of CD8 T cells was specifically due to absent IFN-γ signaling because continuous IL-7 signaling similarly induced CD8 T cells with IFN-γR-deficiency to undergo continuous exponential growth in the absence of any blocking mAb (Fig. 4c). Thus, in the absence of IFN-γ triggered CICD, continuous IL-7 signaling induced CD8 T cells to both proliferate and survive.

To prove this point even further, we also examined the effect of continuous IL-7 signaling on naive CD8 T cells from 7RTg.*Ifng*$^{-/-}$ mice. Continuous IL-7 signaling resulted in continuous exponential growth of IFN-γ-deficient CD8 T cells which markedly contrasted with its effect on IFN-γ-sufficient CD8 T cells (Fig. 4c). Moreover, to prove that the continuous exponential growth of IFN-γ-deficient CD8 T cells was specifically due to absent IFN-γ production, we added exogenous rIFN-γ which blocked the exponential growth of IFN-γ-deficient CD8 T cells (Fig. 4c). These results document that CICD is triggered by IFN-γ produced by CD8 T cells in response to continuous IL-7 signaling.

Anti-IFN-γ blockade promotes cell survival *in vivo*

Our results indicated that, in the absence of IFN-γ, continuous IL-7 signaling was sufficient to promote CD8 T cell survival in the apparent absence of *in vitro* homeostatic TCR engagements. To assess this possibility *in vivo* in the defined absence of homeostatic TCR engagements, we examined the effect of *in vivo* anti-IFN-γ antibody injections on the survival of CD8 T cells adoptively transferred into MHC-I-deficient host mice made lymphopenic by sub-lethal irradiation (Fig. 4d). As in Fig. 1d, we transferred 1×10^6 naive 7RTg CD8 T cells bearing the MHC-I-restricted transgenic P14 TCR into irradiated β2m° host mice, but in this experiment each reconstituted mouse was injected every other day with either anti-IFN-γ mAb or saline (Fig. 4d). After 14 days, few donor CD8 T cells were recovered from control saline injected *B2m*$^{-/-}$ host mice indicating that continuous IL-7 signaling did not sustain CD8 T cells in the absence of *in vivo* homeostatic TCR engagements (Fig. 4d). In marked contrast, significantly more donor CD8 T cells were recovered from anti-IFN-γ injected *B2m*$^{-/-}$ host mice than initially transferred (Fig. 4d). In fact, 7-fold more donor CD8 T cells were recovered from anti-IFN-γ injected than saline injected *B2m*$^{-/-}$ host mice (Fig. 4d). These results demonstrate that blockade of IFN-γ signaling permitted IL-7 signaled CD8 T cells to both proliferate and survive *in vivo* in the absence of homeostatic TCR engagements.

Homeostatic TCR engagements inhibit IFN-γ expression

Our experiments have revealed that IL-7 signaling, when continuous, induces expression of IFN-γ and triggers cell death; whereas IL-7 signaling, when intermittent, maintains naive CD8 T cells. Because IL-7 signaling of naive CD8 T cells is interrupted by *in vivo*

homeostatic TCR engagements[24] and other TCR signals[28–30], we thought that *in vivo* homeostatic TCR engagements in intact mice should prevent continuous IL-7 signaling and prevent induction of IFN-γ expression.

To determine if homeostatic TCR engagements prevented *in vivo* CD8 T cells from being continuously signaled by IL-7 to express IFN-γ, we compared peripheral CD8 T cells freshly obtained from intact 7RTg and 7RTg.8DP4 mice for expression of IFN-γ (Fig. 5a). Whereas both 7RTg and 7RTg.8DP4 CD8 T cells expressed transgenic IL-7Rα proteins, *in vivo* homeostatic TCR signaling would be lacking in peripheral 7RTg.8DP4 CD8 T cells which expressed CD8 coreceptors with mismatching MHC-II specific TCR[27]. Indeed, *in vivo* homeostatic TCR signaling was impaired in 7RTg.8DP4 CD8 T cells as revealed by significantly lower CD5 protein expression on CD8 T cells from 7RTg.8DP4 mice compared to 7RTg mice (Fig. 5a). More importantly, expression of IFN-γ, as well as expression of FasL mRNA, was significantly increased in 7RTg.8DP4 CD8 T cells compared to 7RTg CD8 T cells (Fig. 5a), documenting that homeostatic TCR engagements did in fact prevent *in vivo* CD8 T cells from expressing IFN-γ.

If the same were true for normal CD8 T cells bearing endogenous IL-7Rα proteins, preventing IFN-γ expression would explain why *in vivo* homeostatic TCR engagements were necessary for naive CD8 T cell survival. Consequently, to assess the impact of *in vivo* homeostatic TCR engagements on IFN-γ expression by CD8 T cells in intact normal mice, we compared peripheral CD8 T cells expressing endogenous (not transgenic) IL-7Rα proteins from B6 and 8DP4 intact mice (Fig. 5b). Impaired *in vivo* homeostatic TCR signaling was reflected in lower CD5 protein on 8DP4 CD8 T cells (Fig. 5b) and resulted in greater IL-7 signaling *in vivo* as reflected by lower IL-7Rα surface expression on 8DP4 compared to B6 CD8 T cells (Fig. 5b). Greater IL-7 signaling during *in vivo* homeostasis resulted in significantly increased mRNA expression of IFN-γ, as well as FasL, in 8DP4 CD8 T cells (Fig. 5b). Thus, during *in vivo* CD8 T cell homeostasis, weaker homeostatic TCR engagements resulted in uninterrupted IL-7 signaling and increased expression of IFN-γ and FasL.

To further examine the effects of homeostatic TCR engagements on IFN-γ expression during *in vivo* homeostasis in intact mice, we next examined HY.*Rag2*$^{-/-}$ female mice whose CD8 T cells express endogenous IL-7Rα proteins because the transgenic HY TCR is notable for extremely weak homeostatic TCR signaling (Fig. 5c). However, weak homeostatic TCR signaling can be strengthened by replacing CD8α with CD8.4 coreceptor proteins that are encoded by endogenous *Cd8a* genes that had been re-engineered by gene knock-in technology to express the cytosolic tail of CD4 instead of CD8[31]. Even though HY.*Rag2*$^{-/-}$ CD8.4 T cells and HY.*Rag2*$^{-/-}$ CD8 T cells bear TCR and CD8 coreceptors with identical ligand specificities, HY.*Rag2*$^{-/-}$ CD8.4 T cells were more strongly signaled *in vivo* as revealed by higher CD5 levels (Fig. 5c). A consequence of stronger *in vivo* homeostatic TCR signaling in HY.*Rag2*$^{-/-}$ CD8.4 T cells was interruptions to *in vivo* IL-7 signaling as revealed by increased IL-7Rα levels (Fig. 5c). Interruptions to *in vivo* IL-7 signaling caused reduced mRNA expression of IFN-γ and FasL (Fig. 5c). Thus, these results demonstrate that stronger homeostatic TCR engagements interrupt *in vivo* IL-7 signaling and, as a result, reduce IFN-γ and FasL expression.

These findings in intact mice demonstrate that, regardless of whether CD8 T cells express transgenic or endogenous IL-7Rα proteins, homeostatic TCR engagements interrupt IL-7 signaling and reduce expression of IFN-γ and FasL.

Loss of CD5^{lo}CD8 T cells during normal homeostasis

Having demonstrated that TCR-mediated interruptions of IL-7 signaling during normal CD8 T cell homeostasis reduced IFN-γ expression, we wanted to assess the importance of IFN-γ-induced CICD during normal *in vivo* CD8 T cell homeostasis. We reasoned that weakly TCR signaled CD5^{lo}CD8 T cells were the most likely to receive prolonged IL-7 signaling and to express IFN-γ during *in vivo* homeostasis. We further reasoned that, because autocrine signaling is more efficient than paracrine signaling, CD5^{lo}CD8 T cells were the most likely to be triggered by their own IFN-γ to undergo CICD. Based on this reasoning, we hypothesized that CD5^{lo}CD8 T cells failed to survive during *in vivo* homeostasis in intact mice because they received prolonged IL-7 signaling and underwent IFN-γ-triggered CICD.

As a first test of this hypothesis, we examined *Ifng*$^{-/-}$ mice whose CD8 T cells were IFN-γ-deficient and so could not trigger CICD. We predicted that CD5^{lo}CD8 T cells which received prolonged IL-7 signaling would survive in *Ifng*$^{-/-}$ mice, but would be stimulated by prolonged IL-7 signaling to convert into CD44^{hi}CD8 memory T cells (as we observed in Fig. 2f). In fact, examination of *Ifng*$^{-/-}$ mice revealed a striking increase relative to B6 mice in CD44^{hi}CD8 splenic T cells, especially in CD5^{lo}CD44^{hi}CD8 memory splenic T cells (Fig. 6a, top). The increase in CD44^{hi} memory CD8 splenic T cells in *Ifng*$^{-/-}$ mice was a consequence of peripheral homeostasis because it was neither a feature of CD8 T cells in the thymus nor CD4 splenic T cells in the periphery (Fig. 6a, top). Moreover, careful analysis of CD5 expression on naive and memory CD8 splenic T cells in *Ifng*$^{-/-}$ and B6 mice revealed that CD5^{lo} cells were specifically increased in memory CD8 splenic T cell populations in *Ifng*$^{-/-}$ mice (Fig. 6a, bottom). To subject these findings to quantitative analysis over multiple experiments, we defined low CD5 expression as the level of CD5 expressed by the lowest 30% of B6 splenic T cells (Fig. 6b). In such an analysis, it was evident that IFN-γ-deficiency specifically and significantly increased the frequency of CD5^{lo} cells among memory CD44^{hi}CD8 T cells, precisely as hypothesized (Fig. 6b, top). In fact, the increased frequency of CD5^{lo} memory CD8 T cells in *Ifng*$^{-/-}$ mice reduced the CD5 MFI of their overall memory CD8 T cell population (Fig. 6b, bottom). These results demonstrate that IFN-γ deficiency maintained CD5^{lo}CD8 T cells that would otherwise not survive *in vivo* homeostasis.

As another test of our hypothesis, we examined IL-7Tg mice whose peripheral CD8 T cells were exposed *in vivo* to elevated IL-7 concentrations. We reasoned that elevated IL-7 would make it more difficult for weak homeostatic TCR engagements to interrupt IL-7 signaling, resulting in longer duration IL-7 signaling and elimination of CD5^{lo}CD8 T cells in IL-7Tg mice. In fact, consistent with longer duration IL-7 signaling, we found that a majority of CD8 splenic T cells in IL-7Tg mice were memory CD44^{hi} cells, with CD5^{lo}CD8 T cells less skewed toward a memory phenotype than the overall CD8 T cell population (Fig. 6c, top). As we had observed in *Ifng*$^{-/-}$ mice, the marked increase in CD44^{hi} memory CD8 splenic T

cells in IL-7Tg mice was a consequence of peripheral CD8 T cell homeostasis because it was unique to CD8 splenic T cells and was neither a feature of CD8 thymocytes nor CD4 T cells (Fig. 6c, top). Careful analysis of CD5 expression revealed that $CD5^{lo}$ cells were decreased in both naive $CD44^{lo}$ and memory $CD44^{hi}$CD8 splenic T cell populations in IL-7Tg mice (Fig. 6c, bottom), although their TCRβ and CD8 protein expression were unchanged (Supplementary Fig. 3). Quantitative analysis over multiple experiments confirmed that the frequency of $CD5^{lo}$ cells among both naive and memory CD8 T cells was significantly reduced (Fig. 6d, top) and that the overall CD5 protein expression of both naive and memory CD8 T cell populations in IL-7Tg mice was consequently increased (Fig. 6b, bottom). Thus, increased *in vivo* IL-7 signaling in IL-7Tg mice specifically eliminated $CD5^{lo}$CD8 T cells, an effect opposite to that of IFN-γ-deficiency. These results indicate that, by requiring stronger homeostatic TCR engagements to prevent CICD, increased *in vivo* IL-7 signaling alters the CD8 T cell population maintained during peripheral homeostasis.

DISCUSSION

The present study provides insights into CD8 T cell homeostasis by identifying the ability of IL-7 to induce cell death and reveals how the interplay between IL-7 and TCR signaling results in the long-term maintenance of naive CD8 T cells. A key observation in the present study was that intermittent IL-7 signaling was itself sufficient for naive CD8 T cell survival and quiescence, but that continuous IL-7 signaling had the paradoxical effect of inducing naive CD8 T cells to proliferate, acquire memory markers, produce IFN-γ, and undergo IFN-γ-triggered cell death which we refer to as CICD. Consequently, naive CD8 T cell survival requires *in vivo* IL-7 signaling to be intermittent. Prolonged IL-7 signaling was interrupted by homeostatic TCR engagements which prevented IFN-γ expression and induction of CICD. IFN-γ-triggered CICD could not be prevented in CD8 T cells whose homeostatic TCR engagements were of insufficient strength to interrupt IL-7 signaling, with the result that such CD8 T cells were eliminated during homeostasis. Thus, this study identifies the regulation of IL-7 signaling duration by homeostatic TCR engagements as the basis for *in vivo* CD8 T cell homeostasis.

IL-7 signaling dynamically adjusts CD8 coreceptor levels up and down on individual naive CD8 T cells and to cyclically promote TCR engagement and then disengagement of self-ligands in a continuous feedback loop referred to as `coreceptor tuning'[24]. In coreceptor tuning, IL-7 signaling of naive CD8 T cells transcriptionally increases CD8 coreceptor expression to the point that MHC class I-specific TCRs engage peripheral self-ligands to generate homeostatic TCR signals that then disrupt IL-7 signaling; disruption of IL-7 signaling then causes CD8 coreceptor expression levels to decline to the point that MHC class I-specific TCRs disengage from peripheral self-ligands and IL-7 signaling is restored, beginning the cycle anew. In this way, naive CD8 T cells are continually and sequentially signaled by IL-7 and TCR, with IL-7 signaling promoting homeostatic TCR engagements which, in turn, interrupt IL-7 signaling. The present study now reveals that interruptions to IL-7 signaling during coreceptor tuning are not an inadvertent consequence of homeostatic TCR engagements, but rather are critical for the survival of individual naive CD8 T cells during homeostasis. Consequently, interruption of IL-7 signaling and prevention of CICD are a major function of TCR engagements during CD8 T cell homeostasis.

The dynamic interaction between IL-7 and TCR that prevents CICD provides a continual screening of the peripheral TCR repertoire so that only naive CD8 T cells with TCRs of appropriate affinity to intermittently engage and disengage from peripheral self ligands are maintained during homeostasis. The present study indicates that CD8 T cells with TCRs of insufficient affinity to engage peripheral self-ligands fail to survive *in vivo* because they are continuously signaled by IL-7 to express IFN-γ and to preferentially undergo IFN-γ-triggered CICD because autocrine signaling by IFN-γ occurs far more efficiently than paracrine signaling of other cells. Consequently, IL-7 induced CICD provides a quality control mechanism for eliminating CD8 T cells whose TCRs lack specificity or affinity for *in vivo* self-ligands. The net effect of the dynamic interplay between IL-7 and TCR signaling is that only naive CD8 T cells with TCRs possessing sufficient affinity for *in vivo* self-ligands to interrupt IL-7 signaling are maintained during *in vivo* homeostasis.

The molecular basis by which TCR engagements can interrupt signaling by IL-7 and other common γ chain (γc)-dependent cytokines have previously been identified[28–30]. TCR engagements induce rapid degradation of the cytosolic tail of the γc component of the IL-7R complex, preventing IL-7 signal transduction[28]. TCR engagements also block proximal events in γc-dependent cytokine signal transduction by preventing cytokine signaled phosphorylation of Janus kinases and by preventing cytokine induced phosphorylation of STAT5[29, 30]. Additional mechanisms may be operative as well. We speculate that the multiple molecular levels at which TCR engagements interrupt γc-dependent cytokine signaling are indicative of its importance in lymphocyte biology.

While homeostatic TCR engagements interrupt *in vivo* IL-7 signaling, we found that any means of interrupting IL-7 signaling was sufficient to avoid inducing IFN-γ expression and was sufficient to maintain naive CD8 T cell survival. Nevertheless, down-regulation of endogenous IL-7Rα proteins was apparently insufficient, without concurrent homeostatic TCR engagements, to prevent prolonged IL-7 signaling and induction of IFN-γ expression. Indeed, we observed that freshly obtained CD8 T cells with the lowest affinity *in vivo* TCR engagements displayed the lowest surface levels of endogenous IL-7Rα proteins and yet displayed the highest expression levels of IFN-γ Specifically, we observed that 8DP4 CD8 T cells, which could not generate *in vivo* homeostatic TCR signals because of mismatching TCR and coreceptor specificities, displayed low endogenous IL-7Rα surface expression levels yet expressed IFN-γ; and HY.*Rag2*$^{-/-}$ CD8 T cells whose *in vivo* homeostatic TCR engagements were very low affinity and displayed low surface IL-7Rα expression, also expressed IFN-γ. Therefore IL-7Rα down-regulation was itself not sufficient to prevent induction of IFN-γ expression or IFN-γ-triggered CICD. Thus, IL-7 signals continue to be transduced, albeit at low levels, by the relatively few endogenous IL-7Rα proteins remaining on the cell surface after IL-7 induced down-regulation[14].

In our view, IL-7 signaling intensity and IL-7 signaling duration have different consequences in naive CD8 T cells. IL-7 signaling intensity is primarily limited by endogenous IL-7Rα downregulation, whereas IL-7 signaling duration is primarily limited by homeostatic TCR engagements. Consequently, the major importance of IL-7Rα downregulation is to maximize the number of peripheral CD8 T cells that can be supported by limiting amounts of *in vivo* IL-7[13], whereas the major purpose of homeostatic TCR

engagements is to interrupt IL-7 signaling to prevent CICD. Therefore it should be appreciated that intermittent homeostatic TCR engagements do not interrupt IL-7 signaling by inducing IL-7Rα down-regulation since homeostatic TCR engagements of self-ligands, as opposed to antigenic TCR engagements of foreign agonist ligands[6], actually have the opposite effect and maintain IL-7Rα surface expression[24].

IFN-γ has not previously been appreciated to play a role in naive CD8 T cell homeostasis. However, IFN-γ is known to participate in the contraction phase of antigen-specific memory and effector CD8 T cells following infection[32, 33]. During infection, the number of antigen-specific CD8 T cells increases but is then reduced following resolution of the infection, and the reduction in antigen-specific CD8 T cells is dependent on IFN-γ[32, 33]. Indeed, IFN-γ is capable of triggering T cell death[32–37], but the mechanism by which IFN-γ activates intracellular caspases to trigger cell death has not been clearly established. It has been suggested that IFN-γ induces production of reactive oxygen species (ROS) and nitric oxide[36, 38–40], but, in our preliminary experiments, we have not observed IFN-γ-dependent elevations in ROS in CD8 T cells signaled by IL-7 to undergo IFN-γ-triggered CICD.

In conclusion, the present study documents that IL-7 signaling has contradictory effects on naive CD8 T cell maintenance and survival, depending on its duration. Intermittent IL-7 signaling promotes naive CD8 T cell survival and quiescence, but continuous IL-7 signaling promotes CD8 T cell production of IFN-γ which triggers CD8 T cell death. Homeostatic TCR engagements interrupt IL-7 signaling during CD8 T cell homeostasis, thereby limiting IL-7 to providing survival signals and preventing CICD. CD8 T cells with insufficient homeostatic TCR engagements to interrupt IL-7 signaling undergo IFN-γ-triggered CICD.

Online Methods

Animals

C57BL/6 (B6) and B6.Ly5.2 (CD45.1) mice were purchased from Charles River Laboratories (Frederick, MD). *Il7r*$^{-/-}$, *B2m*$^{-/-}$, B6.MRL-Fas^{lpr}/J, *Ifng*$^{-/-}$, *Ifngr1*$^{-/-}$, *Bcl2l11*$^{-/-}$, and Eα (MHC class II) promoter driven IL-7Tg mice[41] were obtained from The Jackson Laboratory (Bar Harbor, ME). Human CD2 (hCD2) minicassette-driven IL-7Rα transgenic mice (7RTg) have been described[42]. CD8.4 mice were generated by replacing the cytosolic tail of CD8α with the cytosolic tail of CD4 by gene knock-in technology as previously described[31]. 8DP4 mice were generated by introducing the E8III-driven CD4 transgene into *Cd4*$^{-/-}$*B2m*$^{-/-}$ mice as described[27]. CD45.1 congenic *B2m*$^{-/-}$, 7RTg.*Il7r*$^{-/-}$ (7RTg), 7RTg.8DP4, 7RTg.P14.*Rag2*$^{-/-}$, 7RTg.HY.*Rag2*$^{-/-}$, HY.CD8.4.*Rag2*$^{-/-}$, 7RTg.B6.MRL-Fas^{lpr}/J (7RTg.Fas$^{lpr/lpr}$), 7RTg.*Bcl2l11*$^{-/-}$ (7RTg.Bim-KO), 7RTg.*Ifng*$^{-/-}$, and 7RTg.*Ifngr1*$^{-/--}$ mice were bred in our own colony. Animal experiments were approved by the National Cancer Institute Animal Care and Use Committee. All mice were maintained in accordance with National Institutes of Health (NIH) guidelines.

Flow cytometry

Monoclonal antibodies with the following specificities were used in this study: CD4 (GK1.5 and RM4.5), CD8α (53-6-7), CD5 (53-7.3), CD45.1 (A20), CD45.2 (104), CD44 (IM7),

CD95 (Jo2), IL-7Rα (A7R34), TCRβ (H57-597), Vα2 (B20.1), Vβ8 (F23.1), HY-TCR (T3.70) and phospho-Stat5 (47). For cell surface staining of fresh cells, cells were incubated first with 2.4G2 and then fluorochrome-conjugated antibodies, with dead cells excluded by forward light-scatter gating and propidium iodide staining. For detection of apoptotic cells, cells were incubated with EtBr (1 μg/ml) solution. For intracellular staining, cells were fixed, permeabilized, and then stained with fluorochrome-conjugated antibodies. For intracellular IFN-γ staining, cells were cultured with Golgi stop (BD Pharmingen) for 6 hours and then fixed, permeabilized, and stained with anti-IFN-γ Ab (XMG1.2). Samples were analyzed on a FACSVantage SE, FACSCalibur, or LSRII flow cytometer (Becton Dickinson). Data were analyzed using software developed at the US National Institutes of Health.

Cell purification and culture

For purification of naive CD8 T cells, lymph node (LN) cells were stained with both anti-CD44 and anti-CD4 mAbs and then exposed to BioMag beads (goat anti-mouse and goat anti-rat IgG (Qiagen)) to eliminate $CD44^+$, $CD4^+$, and Ig^+ cells. For CD8 T cells from HY and 8DP4 mice, LN cells were incubated with anti-CD8 microbeads and then purified on a MACS column (Miltenyi Biotec). For CFSE labeling, cells were incubated with 1μM CFSE (Carboxyfluorescein diacetate succinimidyl ester, Molecular Probes).

For *in vitro* cell cultures, cells were cultured at $1–2\times10^6$/ml in RPMI with 10% charcoal stripped fetal calf serum at 37°C in 7.5% CO_2. Where indicated, IL-7 (Peprotech, 10ng/ml or as otherwise stated), recombinant IFN-γ (Peprotech, 10ng/ml), or anti-IFN-γ (XMG1, BD-Pharmingen, 5μg/ml,) were added to culture. For intermittent IL-7 exposure, cells were cultured in IL-7 (10ng/ml) for 14 hours, washed twice with medium and then re-cultured in either medium or IL-7 for the remaining 10 hours, with the cycle repeated daily for the duration of the experiment.

In vivo adoptive transfer

1×10^6 purified CD8 T cells were injected i.v. into host mice that had been sub-lethally irradiated (6 Gray) the previous day. Where indicated, anti-IFN-γ Ab (XMG1, 500μg/injection) was injected i.p. every other day starting on day 1.

Quantitative RT-PCR

Total RNA was isolated with Trizol (Invitrogen) and treated with DNase I (Invitrogen) to eliminate possible genomic DNA comtamination. cDNA synthesis was done by superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) with oligo dT primers. Quantitative RT-PCR was done with QuantiTect SYBR green PCR system (Qiagen) or TaqMan PCR system (Applied Biosystems) as indicated. The primer sequences for SYBR green PCR system are as follows. β-actin; forward(gAg Agg gAA ATC gTg CgT gA), reverse(ACA TCT gCT ggA Agg Tgg AC), IL-7Rα; forward(ggA Tgg AgA CCT AgA AgA Tg), reverse(gAg TTA ggC ATT TCA CTC gT), Bim; forward(ggA gAC gAg TTC AAC gAA ACT T), reverse(AAC AgT TgT AAg ATA ACC ATT TgA gg), FasL; forward(CAG CTC TTC CAC CTG CAG AAG G), reverse(GCG GTT CCA TAT GTG TCT TCC C). TaqMan probes for the detection of IFN-γ, Puma, Noxa, β-actin, were

purchased from Applied Biosystems. Gene expression values were normalized to those of *Actb* (β-actin gene) in the same sample.

Immunoblotting

Cells were solubilized with RIPA buffer and lysates were resolved by SDS-PAGE and, where indicated, were immunoblotted anti-Bim (BD-Pharmingen), anti-Bcl-2 (3F11, BD-Pharmingen), anti-Mcl-1 (Rockland), anti-Bcl-xL (4/Bcl-x, BD-Pharmingen), anti-Caspase-3 (Cell Signaling) and anti-actin (C4, Chemicon) and were visualized by enhanced chemoluminescence (Perkin Elmer) using HRP-conjugated anti-mouse IgG (Amersham Biosciences) or anti-rabbit IgG (Amersham Biosciences).

Statistical analysis

Student's t-test with two-tailed distributions was used for statistical analyses. P values of 0.05 or less were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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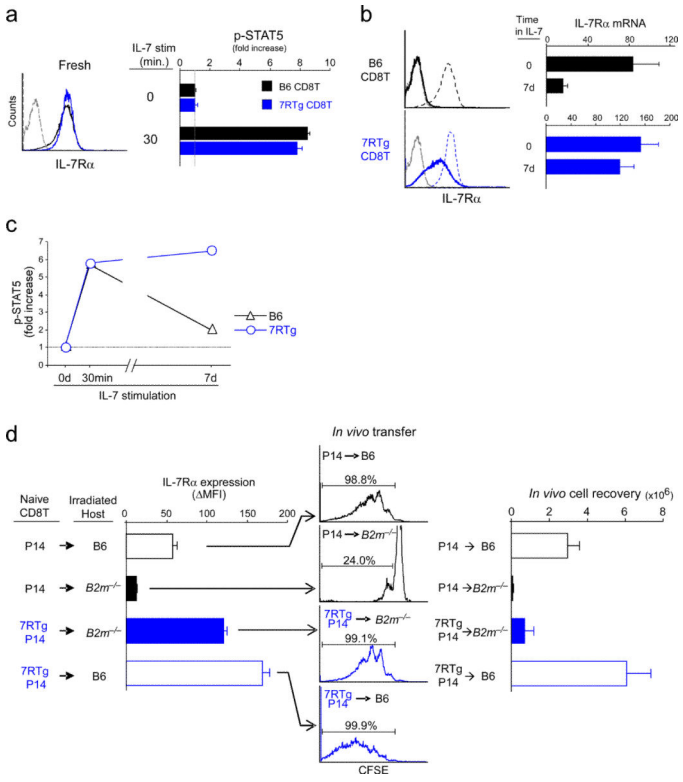

Fig. 1. Effect of continuous IL-7 signaling on naive CD8 T cell proliferation and survival

(**a**) Expression and signaling by IL-7Rα proteins on naive CD8 T cells from B6 and 7RTg mice. Freshly obtained lymph node (LN) CD8 T cells from B6 (black lines) and 7RTg mice (blue lines) were assessed for IL-7Rα surface expression (left) and then were assessed for p-STAT5 content after stimulation with IL-7 (1 ng/ml) for 30 minutes (right). Data indicate mean + s.e.m. of replicate cultures and are representative of more than five independent experiments.

(**b**) IL-7Rα surface protein expression (left) was assessed prior to (dashed curves) and after 7 days in IL-7 culture (solid lines); gray dashed lines indicate negative control staining. IL-7Rα mRNA expression (relative to β-actin) was assessed by quantitative RT-PCR prior to (day 0) and after 7 days in IL-7 culture (right). CD8 T cells from B6 (black line) and 7RTg (blue line) mice are indicated. Data indicate mean + s.e.m. of replicate cultures and are representative of more than three independent experiments.

(**c**) p-STAT5 levels in purified naive CD8 T cells after stimulation with IL-7 for varying times. Data are representative of two independent experiments. CD8 T cells from B6 (black triangles) and 7RTg (blue circles) mice are indicated.

(**d**) Adoptively transferred CD8 T cells after recovery from host mice. 1×10^6 naive CD8 T cells from P14.*Rag2*$^{-/-}$ mice and 7RTg.P14.*Rag2*$^{-/-}$ mice were CFSE labeled and adoptively transferred into either B6 or MHC-I-deficient (*B2m*$^{-/-}$) host mice that had been made lymphopenic by sub-lethal irradiation (6 Gray) and assessed 14 days after transfer. Recovered donor CD8 T cells were assessed for IL-7Rα surface expression (left), proliferation (middle) and cell recovery (right). Data indicate mean + s.e.m of 3 mice per experimental group and are representative of more than 3 independent experiments.

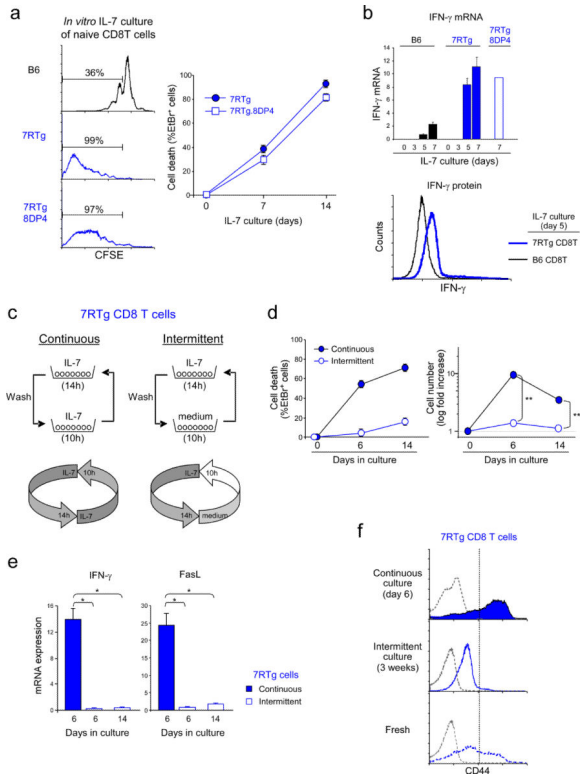

Fig. 2. Continuous IL-7 signaling promotes CD8 T cell death *in vitro*

(**a**) Proliferation (left) and cell death (right) of naive CD8 T cells in IL-7 cultures. Naive CD8 T cells from the indicated mice were cultured in IL-7 for 14 days. Proliferation was assessed on day 7 by CFSE dye dilution and cell death was determined by ethidium bromide (EtBr) staining. Data are representative of more than five independent experiments.

(**b**) Expression of IFN-γ mRNA and protein during IL-7 culture. IFN-γ mRNA and IFN-γ protein in naive CD8 T cells after varying times in IL-7 was determined by quantitative RT-PCR or intracellular staining. IFN-γ mRNA is expressed relative to β-actin. Data indicate mean + s.e.m. of replicate cultures and are representative of two independent experiments.

(**c**) Experimental design in which naive CD8 T cells were exposed to IL-7 either continuously or intermittently. Purified naive 7RTg CD8 T cells ($1–2\times10^6$/2ml) were cultured in IL-7 for 14 hours of each day, washed, and then re-cultured in either medium or IL-7 for the remaining 10 hours of each day, with the cycle repeated daily for 2–3 weeks.

(**d**) CD8 T cell viability. Viable cell number (top) and apoptotic cell frequency (bottom) of naive 7RTg CD8 T cells subjected to continuous (closed symbols) versus intermittent (open symbols) exposure to IL-7. Data indicate mean + s.e.m. of replicate cultures and are representative of two independent experiments.

(**e**) Expression of IFN-γ and FasL mRNA. mRNA expression of the indicated genes in 7RTg CD8 T cells as determined relative to β-actin by quantitative RT-PCR that were subjected to either continuous (closed bars) or intermittent (open bars) IL-7 exposure. Data indicate mean + s.e.m. of replicate samples and are representative of two independent experiments.

(**f**) Continuous IL-7 exposure stimulates naive CD8 T cells to acquire a memory phenotype. CD44 expression was assessed on purified 7RTg CD8 T cells that were subjected to continuous (top) versus intermittent (middle) IL-7 exposure for the indicated times. Naive

CD8 T cells subjected to continuous IL-7 exposure became $CD44^{hi}$ by day 6, whereas naive CD8 T cells subjected to intermittent IL-7 exposure remained $CD44^{lo}$ even by day 21. Gray dotted line indicates negative control staining. Freshly prepared total 7RTg LN CD8 T cells were included as a CD44 staining control (bottom).

*, $p<0.01$; **, $p<0.001$.

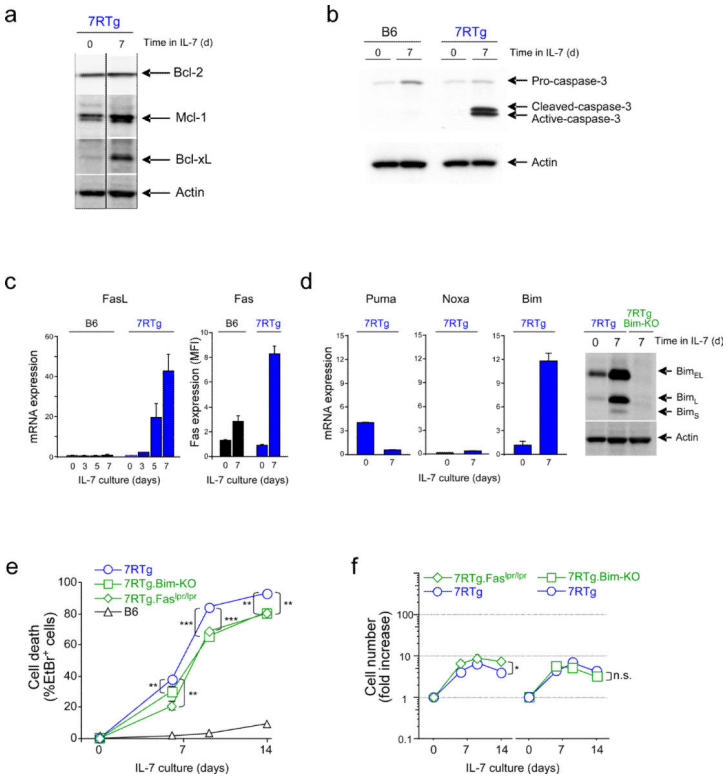

Figure 3. Continuous IL-7 signaling causes cytokine induced cell death (CICD)

(**a,b**) Effect of continuous IL-7 signaling on expression of pro-survival proteins and active caspase-3. Cell lysates from purified naive CD8 T cells that had been cultured in IL-7 for 7d were resolved by SDS-PAGE and immunoblotted with mAbs specific for the pro-survival proteins Bcl-2, Mcl-1, and Bcl-xL (**a**) or for the death effector molecule caspase-3 (**b**). Data are repesentative of 2 independent experiments.

(**c,d**) Expression of death receptor and mitchondria mediated apoptotic molecules in IL-7 cultured CD8 T cells. mRNA expression of genes encoding FasL, Puma, Noxa, and Bim were determined by quantitative RT-PCR, surface expression of Fas protein was determined by flow cytometry, and Bim protein expression was determined by immunoblotting. Data indicate mean + s.e.m. of replicate samples and are representative of 2 independent experiments.

(**e,f**) Viability of IL-7 cultured CD8 T cells deficient in either death receptor or mitochondria mediated death pathways. Apoptotic cell frequency (**e**) and viable cell number (**f**) were determined in IL-7 cultured CD8 T cells that were B6 (black triangles), 7RTg (blue circles), 7RTg.Bim-KO (green squares), and 7RTg.Fas$^{lpr/lpr}$ (green diamonds). Data are representative of more than 3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not significant.

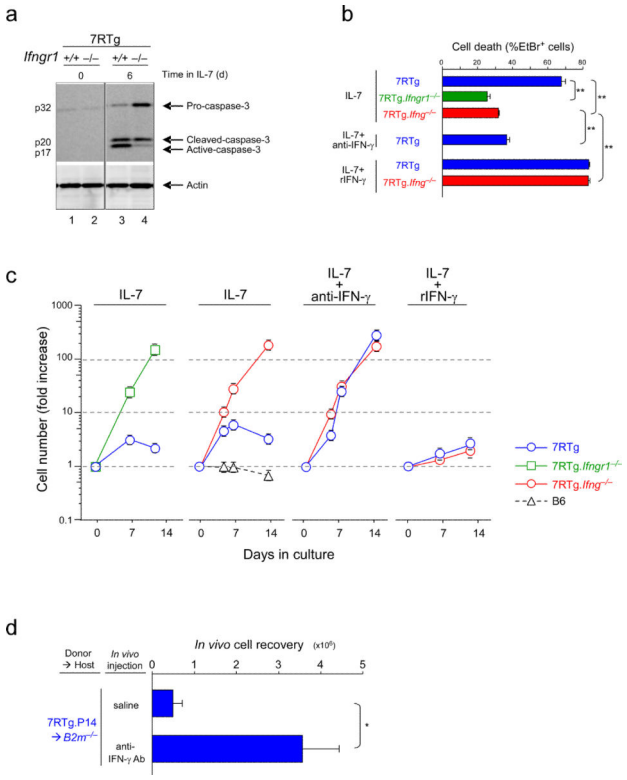

Figure 4. IFN-γ signaling is responsible for triggering CICD

(**a**) Induction of active caspase-3 requires IFN-γ signaling. Cell lysates from purified naive 7RTg CD8 T cells that were *Ifngr1*$^{+/+}$ or *Ifngr1*$^{-/-}$ and had been cultured in IL-7 for 6d were resolved by SDS-PAGE and immunoblotted with mAbs specific for caspase-3. Data are repesentative of two independent experiments.

(**b,c**) Effect of IFN-γ signaling on viability of IL-7 cultured CD8 T cells. Apoptotic cell frequencies on day 7 (**b**) and viable cell numbers at varying times (**c**) were assessed in IL-7 cultures of CD8 T cells that were 7RTg (blue circles), 7RTg.*Ifngr1*$^{-/-}$ (green squares), 7RTg *Ifng*$^{-/-}$ (red circles), and B6 (black triangles). Where indicated, anti-IFN-γ (5μg/ml) and rIFN-γ (10ng/ml) were added to IL-7 cultures. Data indicate mean + s.e.m. of replicate cultures and are representative of 2–5 independent experiments.

(**d**) Anti-IFN-γ treatment increases *in vivo* cell recovery of donor CD8 T cells adoptively transferred into MHC-I-deficient (*B2m*$^{-/-}$) host mice. Naive CD8 T cells from 7RTg.P14.*Rag2*$^{-/-}$ mice were adoptively transferred into irradiated (6 Gray) *B2m*$^{-/-}$ host mice and then recovered from LNs and spleens 14 days later. Where indicated, host mice were injected either with saline or anti-IFN-γ (500μg) every other day beginning on day 1. Data indicate mean + s.e.m. of 3 mice per experimental group and are representative of 2 experiments. *, $p<0.05$; **, $p<0.001$.

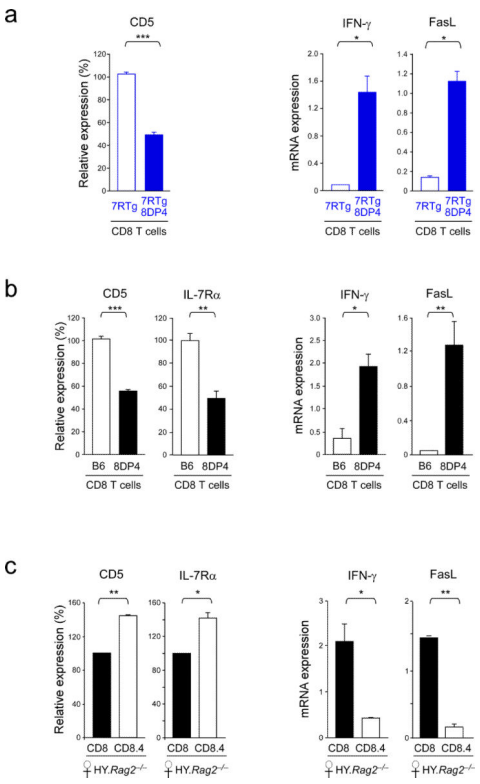

Figure 5. Homeostatic TCR engagements prevent IFN-γ induction during *in vivo* homeostasis

(**a,b**) *In vivo* homeostatic TCR signaling diminishes IL-7 signaling and prevents expression of IFN-γ and FasL. Unlike CD8 T cells in wildtype mice, CD8 T cells in 8DP4 mice are unable to receive homeostatic TCR signals *in vivo* because their TCRs and coreceptors are mis-matched[27]. Freshly obtained CD8 T cells from B6 and 8DP4 mice expressing either transgenic IL-7Rα proteins (**a**) or endogenous IL-7Rα proteins (**b**) were stained for CD5 and IL-7Rα surface expression (left), and were assessed for both IFN-γ and FasL mRNA by quantitative RT-PCR (right). Surface CD5 and IL-7Rα expression are expressed relative to that on B6 CD8 T cells. IFN-γ and FasL mRNA are expressed relative to β-actin. Data indicate mean + s.e.m. of more than 5 independent experiments or indicate mean + s.e.m. of replicate samples in two independent experiments.

(**c**) Strengthened homeostatic TCR engagements diminish *in vivo* IL-7 signaling in intact HY.*Rag2*$^{-/-}$ mice. Freshly obtained CD8 T cells from intact HY.*Rag2*$^{-/-}$ mice that expressed either endogenous CD8α coreceptor proteins or re-engineered endogenous CD8.4 coreceptor proteins were stained for CD5 and IL-7Rα surface expression (left), and were assessed for both IFN-γ and FasL mRNA by quantitative RT-PCR (right). Surface CD5 and IL-7Rα expression are expressed relative to that on B6 CD8 T cells. IFN-γ and FasL mRNA are expressed relative to β-actin. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

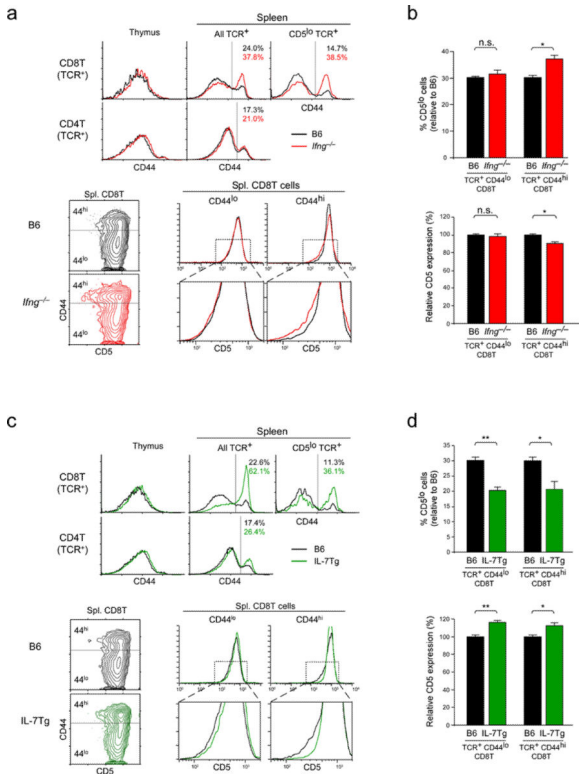

Figure 6. Effect of *in vivo* homeostasis on weakly signaled CD5^{lo}CD8 T cells in normal mice expressing endogenous IL-7Rs

(**a**) Naive and memory CD8 T cells in IFN-γ-deficient mice. CD44 expression on T cells from thymus and spleen of *Ifng*$^{-/-}$ (red) and B6 (black) mice (top panels). Numbers in histograms (top panels) indicate frequencies of CD44^{hi} cells. CD5 expression on naive (CD44^{lo}) and memory (CD44^{hi}) TCR^{+}CD8^{+} splenic T cells are displayed in two color histograms (bottom left) and in expanded one color histograms (bottom right). Data are representative of at least three independent experiments with a total of at least 6 individual mice of each strain.

(**b**) Effect of IFN-γ-deficiency on CD5^{lo}CD8 T cells. Low CD5 expression was defined for naive and memory cells as the level of CD5 expressed by the lowest 30% of B6 splenic CD8 T cells. Bar graphs in the top panel display the percentage of naive (CD44^{lo}) and memory (CD44^{hi}) TCR^{+}CD8^{+} splenic T cells in B6 (black) and *Ifng*$^{-/-}$ mice (red) that were CD5^{lo} (top). Bar graphs in the bottom panel display the relative CD5 MFI of naive (CD44^{lo}) and memory (CD44^{hi}) TCR^{+}CD8^{+} splenic T cells compared to B6 which was set equal to 100 (bottom). Data indicate mean + s.e.m. of at least three independent experiments with a total of at least 6 individual mice of each strain.

(**c**) CD8 T cells in IL-7Tg mice. Naive and memory CD8 T cells in IL-7Tg mice. CD44 expression on T cells from thymus and spleen of IL-7Tg (green) and B6 (black) mice (top panels). Numbers in histograms (top panels) indicate frequencies of CD44^{hi} cells. CD5 expression on naive (CD44^{lo}) and memory (CD44^{hi}) TCR^{+}CD8^{+} splenic T cells are displayed in two color histograms (bottom left) and in expanded one color histograms (bottom right). Data are representative of at least three independent experiments with a total of at least 6 individual mice of each strain.

(**d**) Effect of increased IL-7 on $CD5^{lo}CD8$ T cells. Low CD5 expression was defined for naive and memory cells as the level of CD5 expressed by the lowest 30% of B6 splenic CD8 T cells. Bar graphs in the top panel display the percentage of naive ($CD44^{lo}$) and memory ($CD44^{hi}$) $TCR^{+}CD8^{+}$ splenic T cells in B6 (black) and IL-7Tg mice (green) that were $CD5^{lo}$ (top). Bar graphs in the bottom panel display the relative CD5 MFI of naive ($CD44^{lo}$) and memory ($CD44^{hi}$) $TCR^{+}CD8^{+}$ splenic T cells compared to B6 which was set equal to 100 (bottom). Data indicate mean + s.e.m. of at least three independent experiments with a total of at least 6 individual mice of each strain. *, $p<0.01$; **, $p<0.001$; n.s., not significant.