IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo

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TCR ligation and co-stimulation induce cellular division; however, optimal accumulation of effector CD8 T cells requires direct inflammatory signaling by signal 3 cytokines, such as IL-12 or type I IFNs. Although in vitro studies suggest that IL-12/type I IFN may enhance T cell survival or early proliferation, the mechanisms underlying optimal accumulation of CD8 T cells in vivo are unknown. In particular, it is unclear if disparate signal 3 cytokines optimize effector CD8 T cell accumulation by the same mechanism and how these inflammatory cytokines, which are transiently produced early after infection, affect T cell accumulation many days later at the peak of the immune response. Here, we show that transient exposure of CD8 T cells to IL-12 or type I IFN does not promote survival or confer an early proliferative advantage in vivo, but rather sustains surface expression of CD25, the high-affinity IL-2 receptor. This prolongs division of CD8 T cells in response to basal IL-2, through activation of the PI3K pathway and expression of FoxM1, a positive regulator of cell cycle progression genes. Thus, signal 3 cytokines use a common pathway to optimize effector CD8 T cell accumulation through a temporally orchestrated sequence of cytokine signals that sustain division rather than survival.

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Abbreviations used: Bad, BCL-XL/BCL-2-associated death promoter; Bcl2, B cell CLL/lymphoma 2; Bcl2l1, B cell CLL/lymphoma-like 1; Bcl2l11, B cell CLL/lymphoma Like 1; Bid, BH3-interacting domain death agonist; DAVID, database for annotation, visualization and integrated discovery; FLT3L, FMS-related tyrosine kinase 3 Ligand; GSEA, gene set enrichment analysis; KLRG-1, killer cell lectin-like receptor G1; LCMV Arm, lymphocytic choriomeningitis virus, Armstrong clone; PI3K, phosphoinositide 3-kinase; virLM-OVA, virulent *Listeria monocytogenes* strain expressing OVA_{257}.

The magnitude of the effector CD8 T cell response is critical for eliminating intracellular pathogens and for regulating the size of the memory pool after resolution of infection or vaccination (Hou et al., 1994; Badovinac and Harty, 2006; Schmidt et al., 2008). TCR stimulation by mature DCs expressing cognate antigen in the context of MHC I initiates activation of naive, pathogen-specific CD8 T cells. Additional signals from co-stimulatory molecules amplify the magnitude and/or duration of the TCR signaling, thereby enhancing activation and functionality (Cronin and Penninger, 2007). Although these two signals are sufficient to induce the division of naive CD8 T cells, pathogen-, or adjuvant-induced inflammatory cytokines act as third signals to promote optimal accumulation of effector CD8 T cells (Curtsinger and Mescher, 2010). Because the clearance of intracellular pathogens is often dependent on the total number of responding effector CD8 T cells (Badovinac and Harty, 2006; Hikono et al., 2006; Lefrançois, 2006), it is important to understand how the magnitude of CD8 T cell responses are regulated to effectively control pathogen burden.

Using short-term (~3 d) in vitro experiments, an early study by Curtsinger et al. (1999) clearly established that addition of a specific inflammatory cytokine (IL-12) during T cell activation in response to artificial APCs expressing signal 1 and signal 2 and with exogenous addition of IL-2 increased the accumulation of responding CD8 T cells. The importance of signal 3 cytokines for the optimal accumulation of effector CD8 T cells has also been established in vivo (Gately et al., 1992; Trinchieri, 1998). For example, direct IL-12 signaling is essential for optimal accumulation of antigen-specific CD8 T cells after *Listeria monocytogenes* (LM) infection (Keppler et al., 2009; Xiao et al., 2009; Keppler et al., 2012). Direct IFN-α/β receptor

The Rockefeller University Press $30.00
J. Exp. Med. 2014 Vol. 211 No. 1 105-120
www.jem.org/cgi/doi/10.1084/jem.20130901

The Journal of Experimental Medicine

signaling has also been shown to be critical for the optimal accumulation of CD8 T cells in some in vitro studies (Curtsinger et al., 2005) and for P14 TCR-transgenic CD8 T cells responding to lymphocytic choriomeningitis virus (LCMV) infection (Aichele et al., 2006; Kolumam et al., 2005). Together, these studies highlighted the impact of IL-12 and IFN α/β on the accumulation of activated CD8 T cells. However, a mechanistic understanding of how inflammatory cytokines such as IL-12 and IFN α/β regulate accumulation of effector CD8 T cells in vivo has yet to be determined.

Results from short-term in vitro studies provide two models to explain how the signal 3 cytokine IL-12 promotes the optimal accumulation of activated CD8 T cells. The first model suggests that IL-12 stimulation during activation promotes increased accumulation by conferring a survival advantage to responding CD8 T cells (Mitchell et al., 2001; Valenzuela et al., 2005; Curtsinger and Mescher, 2010). This conclusion was drawn from experiments where IL-12 enhanced accumulation of CD8 T cells over the 3-d culture period, without detectable impact on cellular division. However, validated survival pathways regulated by signal 3 cytokines in vivo have not been identified to date. Alternatively, other data suggest that IL-12 increases the accumulation of activated CD8 T cells by transiently increasing the expression of the high-affinity IL-2 receptor subunit (IL-2Rα; CD25; Pipkin et al., 2010; Valenzuela et al., 2002) and IL-2Rβ (CD122; Valenzuela et al., 2002), providing an early proliferative advantage leading to increased accumulation in short-term in vitro studies (Valenzuela et al., 2002; Curtsinger and Mescher, 2010; Pipkin et al., 2010). Consistent with this notion, the absence of CD25 prevented optimal accumulation of CD8 T cells after LM infection (Obar et al., 2010) or LCMV infection (Williams et al., 2006). However, the IL-12–stimulated increase in CD25 expression in vitro was transient, peaking 2 d after cognate-antigen stimulation (Valenzuela et al., 2002). Thus, mechanistic assessment of signal 3 activities to date are limited to short-term in vitro studies focused on IL-12 and the mechanisms by which IL-12 or other signal 3 cytokines (e.g., type I IFN) regulate CD8 T cell accumulation in vivo are not established. For example, it remains unknown if signal 3 cytokines function by common or distinct mechanisms, if these mechanisms regulate survival pathways in vivo or confer an early proliferative advantage, or if both mechanisms account for signal 3-dependent optimal accumulation of effector CD8 T cells in vivo.

In addition, the temporal disconnection between early and transient production of signal 3 cytokines (Pham et al., 2009; Keppler et al., 2012) and optimal accumulation of effector CD8 T cells at the peak of the response, many days later, has not been addressed (Harty and Badovinac, 2008). For example, most in vivo experiments used gene KO mouse strains or TCR-transgenic T cells (Kolumam et al., 2005; Aichele et al., 2006; Keppler et al., 2009; Pham et al., 2011; Keppler et al., 2012) that constitutively lack the receptors for inflammatory cytokines, and most in vitro studies were analyzed within a short window (~3 d) after CD8 T cell activation (Curtsinger et al., 1999, 2003a, 2005; Valenzuela et al., 2002, 2005). These are important considerations given that acute infections (as well as adjuvant-coupled immunizations) induce transient elevations of inflammatory cytokines, often peaking within hours of stimulation, and then returning to baseline within 1–2 d (Pham et al., 2009; Keppler et al., 2012), whereas corresponding CD8 T cell responses generally peak in numbers between day 7 and 9 after immunization/infection (Harty and Badovinac, 2008). Here, we address this temporal conundrum and-dissect the mechanisms by which signal 3 cytokines IL-12 and type I IFN guide the optimal accumulation of CD8 T cells in response to in vivo activation. To address these issues, we use an immunization model with mature, peptide-loaded DCs, wherein antigen concentrations are fixed but the inflammatory milieu can be manipulated by co-administration of Toll-like receptor ligands (Badovinac et al., 2005; Boyman et al., 2006; Pham et al., 2009). Using this model, we uncover a molecular pathway whereby signal 3 cytokines (both IL-12 and IFN-α/β) promote optimal CD8 T cell accumulation after in vivo activation, not by improving survival or early proliferation, but rather by a common mechanism regulating cytokine signaling pathways that maintain cellular division at late time points.

RESULTS

Molecular profiling of CD8 T cells activated in the presence or absence of inflammation

To address the impact of signal 3 cytokines on CD8 T cell accumulation in vivo, a low number (~600) of naive Thy1.1$^+$ TCR-transgenic OT-I CD8 T cells (specific for H-2K^b/$OVA_{257–264}$ peptide) were adoptively transferred into naive C57BL/6 (Thy1.2$^+$) recipient mice. 1 d after transfer, hosts were immunized with LPS-matured DCs coated with $OVA_{257–264}$ peptide in the absence (DC) or presence of the TLR 9 agonist CpG ODN (DC+CpG) to induce systemic signal 3/inflammatory cytokine production, which includes IL12p40 and many other proinflammatory cytokines (Pham et al., 2009). By day 7 after immunization, mice receiving co-administration of CpG during DC immunization have significantly ($P < 0.01$) higher frequencies of OT-I CD8 T cells in their PBL relative to mice receiving DC immunization alone (Fig. 1 A), demonstrating the enhancing effect of signal 3 on effector CD8 T cell accumulation. It should be noted that ability for CpG administration to enhance the accumulation of activated OT-I CD8 T cells in response to DC immunization is dependent on the host TLR9 expression, indicating that CpG treatment does not increase CD8 T cell accumulation by direct interaction with adoptively transferred T cells or peptide-coated DCs (Pham et al., 2009). These results indicate that CpG-induced inflammation enhances the accumulation activated CD8 T cells in mice given DC immunization.

To address the impact of inflammation on activated CD8 T cells, we compared genome-wide mRNA expression in OT-I CD8 T cells purified from mice 7 d after DC and DC+CpG immunization. Inflammation, elicited by CpG

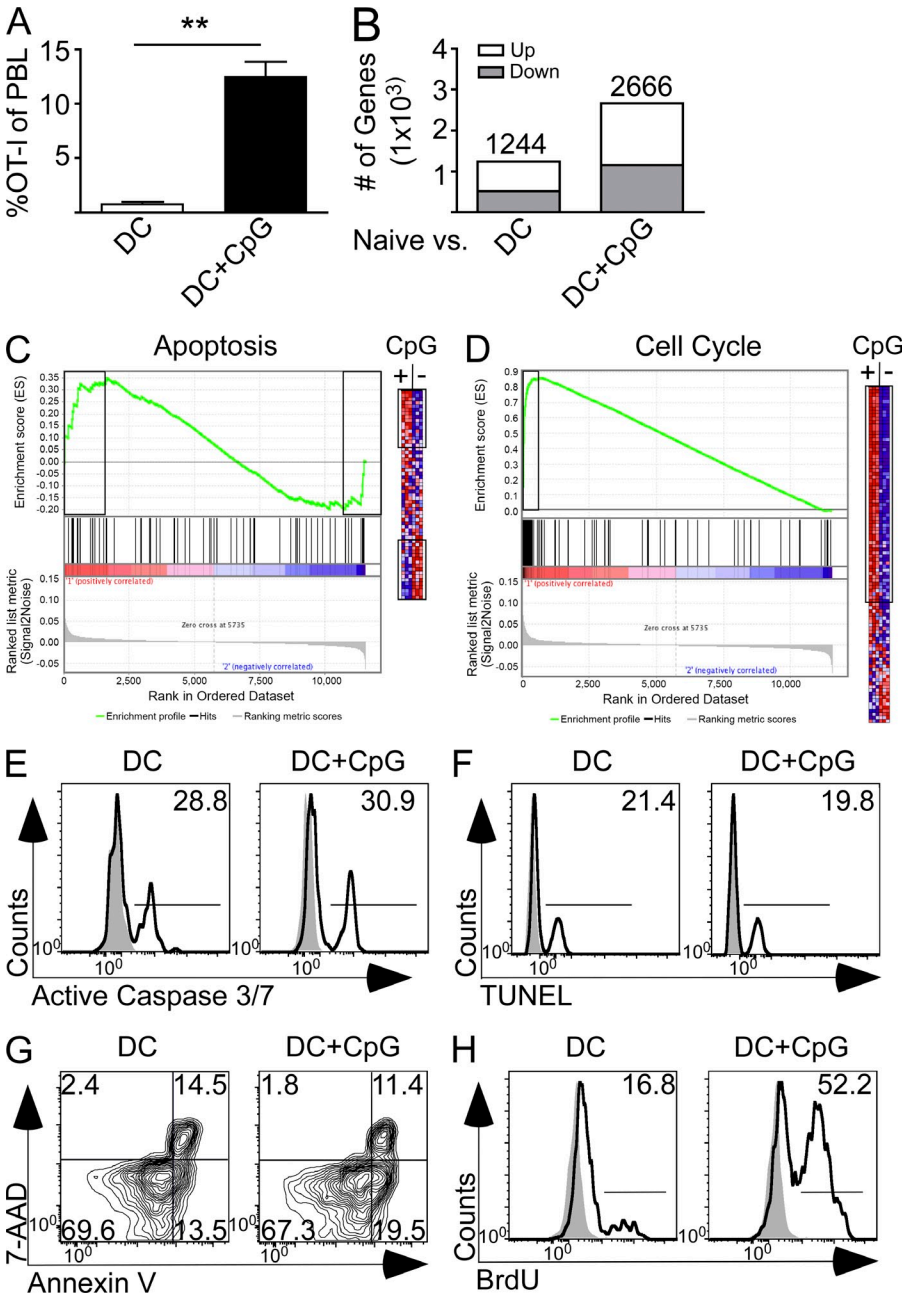

Figure 1. Inflammatory immunization promotes the expression of division-associated genes during the expansion of CD8 T cells. ~600 OT-I CD8 T cells (Thy1.1) were adoptively transferred into naive (Thy1.2) hosts and, 1 d later, recipient mice were injected with 5×10^5 DC-OVA (DC) alone or combined with CpG ODN (DC+CpG). (A) Frequencies of OT-I CD8 T cells were measured in the blood at day 7 after immunization. Represented as mean ± SEM. Data are representative of more than three independent experiments. Statistical analysis used Student's *t* test ($n = 3$ from group). *, $P < 0.05$; **, $P < 0.01$. (B–D) OT-I CD8 T cells were isolated from the spleen from immunized mice at d7 after immunization, RNA was isolated and subjected to microarray analysis. Data were analyzed by GSEA. (B) Number of significantly (FDR < 0.01) up- or down-regulated genes in OT-I cells from DC alone or DC+CpG-immunized mice. Representative GSEA enrichment plots of Apoptosis (C) or Cell Cycle (D) gene sets are described for DC+CpG versus DC comparison. Mice adoptively transferred with OT-I cells were immunized with DC or DC+CpG, and BrdU was injected for 15 h starting on day 6 after immunization. OT-I cells were isolated at day 7 and analyzed for active Caspase 3/7 (E), apoptosis by TUNEL (F), Annexin V/7-AAD (G), and BrdU incorporation (H). Data in E–H are representative of at least two experiments. Numbers indicate in each panel are of percentage positive within the displayed gate.

administration, robustly increased the number of differentially regulated transcripts in activated OT-I CD8 T cells relative to cells isolated from DC alone–immunized hosts by day 7 after immunization (Fig. 1 B). Because previous studies have suggested that signal 3 cytokines enhance the survival of responding T cells (Curtsinger et al., 1999, 2003b; Mitchell et al., 2001; Valenzuela et al., 2002, 2005; Kolumam et al., 2005; Aichele et al., 2006; Keppler et al., 2009), we hypothesized that CpG administration significantly altered the expression of apoptosis-associated gene pathways between OT-I CD8 T cells from DC+CpG and DC alone–immunized mice. Surprisingly, gene set enrichment analysis (GSEA) did not identify significant enrichment for survival-associated gene sets in OT-I CD8 T cells from DC+CpG-immunized hosts compared with those from DC alone–immunized mice, nor were apoptosis-associated gene sets enriched in OT-I CD8 T cells from DC alone–immunized hosts compared with those from mice given DC+CpG immunization (Fig. 1 C and Table 1). In sharp contrast, GSEA analysis revealed that cell cycle/division-associated gene sets were significantly enriched (FDR < 0.001) in OT-I CD8 T cells from mice given DC+CpG immunization compared with those from DC alone-immunized hosts (Fig. 1 D and Table 1).

For example, in a gene set titled Cell Cycle, 60 of 98 associated genes were significantly (FDR < 0.001) up-regulated in OT-I CD8 T cells from DC+CpG-immunized recipients,

Table 1. GSEA reveals that signal 3 cytokines during priming promote the expression of cell cycle–associated transcripts

Gene set	NES	FDR	Group	No. of enriched genes	Genes (ordered by rank)
Apoptosis	1.12	0.601	DC	9/59	*Irf7, Tnfrsf10b, Lta, Jun, Ikbkb, Traf1, Myc, Bcl2, Tnf*
Apoptosis	1.12	0.601	DC+CpG	15/59	*Birc5, Gzmb, Tnfsf10, Casp7, Casp4, Prf1, Casp3, Bad, Apaf1, Casp1, Hrk, Fas, Tnfrsf1a, Bnip3l, Dffb*
Cell cycle	2.97	<0.001	DC	0/98	*Not applicable*
Cell cycle	2.97	<0.001	DC+CpG	60/98	*Hmmr, Bub1, Tpx2, Ckap2l, Pbk, Ccna2, Esco2, Cdc25c, Ckap2, Anln, Cdkn3, Uhrf1, Cccnb2, Cdca8, Ube2c, Asf1b, Melk, Ncaph, Spag5, Top2a, Cdca5, Dhfr, Foxm1, Rad51ap1, Aurka, Bard1, Cdc6, Kif23, Tacc3, Cks1b, Fam83d, Mad2l1, Rrm1, Gmnn, Depdc1b, Exo1, Gins2, Fam64a, Prim1, Wdr51a, Fam72a, Mcm5, Gtse1, Cenpa, Tipin, Mnd1, Cccnf, Mcm8, H2afx, Hells, Rfc4, Fen1, Mcm4, Cdca7, Ube2t, Tyms, Anp32e, Atad2, Mlf1ip*
G1 to S cell cycle reactome	2.27	<0.001	DC	2/59	*Atm, E2f5*
G1 to S cell cycle reactome	2.27	<0.001	DC+CpG	21/59	*Ccnb1, Mcm7, Cdc45l, Ccne2, Rpa3, Cdkn2c, Orc1l, Prim1, Mcm5, Pole, Mcm3, Ccnd3, E2f2, Cdkn1a, Cdk2, Mcm2, Rb1, Mcm4, Pole2, Wee1, Orc6l*

GSEA gene sets in OT-I CD8 T cells responding to DC alone or DC+CpG immunization at day 7 after immunization.

whereas none of these genes were up-regulated in OT-I CD8 T cells from DC alone–immunized hosts (Fig. 1 D and Table 1). To extend our analyses, we used an independent bioinformatic approach (Database for Annotation, Visualization, and Integrated Discovery [DAVID]) and again found that OT-I CD8 T cells from DC+CpG-immunized mice did not significantly (FDR = 0.67) regulate the expression of apoptosis-associated transcripts compared with those from DC alone–immunized mice (Table 2). However, CpG administration during priming significantly (FDR < 0.001) increased the expression of genes associated with M phase and DNA replication in responding OT-I CD8 T cells (Table 2). According to DAVID, >20% of all significantly (FDR < 0.01) up-regulated genes in OT-I CD8 T cells responding to DC+CpG immunization (relative to those from DC alone–immunized mice) were associated with cellular division (Table 2). These data indicate that CpG-induced inflammation during CD8 T cell activation enhances the transcription of division-associated genes, rather than those associated with cell survival/death.

Our transcriptome data do not support the model wherein signal 3 cytokines optimize activated CD8 T cell accumulation by enhancing survival, but rather suggests that division may be altered by signal 3 cytokine stimulation. Division, as detected by a short pulse of BrdU incorporation between days 6 and 7 after immunization, was increased in OT-I CD8 T cells from DC+CpG-immunized mice when compared with OT-I CD8 T cells from mice immunized with DC alone (Fig. 1 H). Additionally, markers of apoptosis, including the percentage of OT-I CD8 T cells positive for active Caspase 3/7, TUNEL staining, and Annexin V^+/7-AAD^- staining, did not significantly differ between OT-I CD8 T cells responding to DC alone or DC+CpG immunization (Fig. 1, E–G). Together these data suggest that, in vivo, the presence

Table 2. DAVID reveals that signal 3 cytokines promote the expression of cell cycle–associated transcripts but not apoptosis-related transcripts during priming

Functional clustering	No. of genes	% of input	FDR	Genes
Regulation of apoptosis	7	1.92	0.670	*Pycard, Rab27a, Dapk2, Gzmb, Itm2b, Muc20, Plekhf1*
M phase	59	16.25	<0.001	*Fbxo5,Ndc80, Nek2, Nsl1, Ra21, Rad51, F6360043A0Rik, Spc24, Spc25, Tpx2, Zwilch, Anln, Mki67, Aspm, Aurkb, Birc5, Bub1, Bub1b, Cdc25c, Cdc6, Cdca2, Cdca3, Cdca5, Cdca8, Cenpe, Cenph, Cep55, Cit, Ccna2, Ccnb2, Dlgap5, Dctn3, Ercc6l, Exo1, C79407, Espl1, Hells, Incenp, Kif11, Kif18a, Kif20b, Ncapd2, Ncaph, Ncapg2, Nusap1, Plk1, Ccnb1, Psmd13, Spgol1, Spgol2, Nuf2, Mad2l1, Spag5, Smc2, Suv39h2, Timeless, Tacc3, Tubb5, Ube2c*
DNA replication	20	5.52	<0.001	*Prim1, Prim2, Dna2, Gins1, Gins2, Cdc6, Chaf1a, Dscc1, Dtl, Mcm10, Mcm3, Mcm4, Mcm5, Mcm7, Mcm8, Orc1l, Pole, Polh, Rfc3, Rrm1*

DAVID analysis for functional clustering of genes that were up-regulated in DC+CpG-immunized OT-I CD8 T cells compared to those immunized with DC alone at day 7 after immunization.

of inflammation optimizes accumulation of CD8 T cells by increasing division rather than enhancing survival.

Direct IL-12 and type I IFN signaling in vivo prolongs the expression of cell cycle–associated genes and enhances division, while not altering survival

Our results thus far have indicated that CpG-induced inflammation promotes optimal accumulation by enhancing division of activated CD8 T cells. Based on the transient nature of CpG-induced signal 3 cytokine (IL-12/type I IFN) expression (Pham et al., 2009), we hypothesized that DC+CpG immunization would increase division by activated CD8 T cells beginning at time points proximal to CpG administration. In contrast to this hypothesis, cellular division and numbers of activated OT-I CD8 T cells were similar after DC+CpG and DC immunization at day 5 after immunization (Fig. 2, A and B). Interestingly, OT-I CD8 T cells in hosts given DC+CpG immunization maintained higher rates of division and had markedly enhanced accumulation at later time points (day 6–7) after immunization relative to OT-I CD8 T cells primed with DC alone (Fig. 2, A and B). Similar results were obtained when examining endogenous OVA_{257}-specific CD8 T cell responses in C57BL/6 hosts (Fig. 2, C and D). These data indicate that, in contrast to prior in vitro results (Valenzuela et al., 2002; Curtsinger and Mescher, 2010; Pipkin et al., 2010), inflammation does not enhance division at early time points after antigen exposure in vivo but rather sustains the division of activated CD8 T cells, and that this effect is not restricted to OT-I CD8 T cells.

Signal 3 cytokines, such as IL-12, peak in the serum within 12 h after CpG administration and return to baseline by 24–48 h (Pham et al., 2009). Thus, we predicted that inflammation would rapidly increase the transcription of cell cycle-associated genes in activated CD8 T cells and maintain their expression throughout expansion. In contrast to this notion, we observed similarly high expression of cell cycle–associated transcripts (*Ccna2*, *Ccnb1*, *Ccnb2*, *Foxm1*, and *Ccne2*) between OT-I CD8 T cells from DC+CpG and DC alone–immunized hosts at day 5 after immunization (Fig. 2 E). However, OT-I CD8 T cells from DC+CpG-immunized hosts maintain higher expression of these cell cycle-associated gene transcripts at day 7 after immunization compared with those from DC alone–immunized mice (Fig. 2 F). Also, many target genes of the FoxM1 transcription factor (a critical master regulator of T cell division; Costa et al., 2005; Laoukili et al., 2005; Wang et al., 2008; Xue et al., 2010) were significantly upregulated in OT-I CD8 T cells from DC+CpG-immunized mice compared with those from DC alone–immunized mice (Fig. 2 G). Importantly, changes in transcription were associated with concurrent changes in protein for select cell-cycle-associated genes. Cyclin A, Cyclin B1, and FoxM1 protein were similarly abundant at day 5 after immunization in both groups but expression of these proteins was maintained only in OT-I CD8 T cells from DC+CpG-immunized hosts at day 7 after immunization (Fig. 2 H). These findings indicate that inflammation does not promote immediate changes in the expression of cell cycle–associated gene products in activated CD8 T cells, but instead prolong their mRNA and protein expression, thereby extending the duration of activated CD8 T cell division to enhance accumulation.

To verify that the presence of CpG-induced inflammation during CD8 T cell activation did not alter survival, we measured the expression of select genes known to effect T cell survival in OT-I CD8 T cells from hosts given DC or DC+CpG immunization (Veis et al., 1993; Hildeman et al., 2002, 2007; Masson et al., 2011). Prosurvival factors expression (*Bcl2* and *Bcl1l1/Bclxl*) were not significantly increased in OT-I CD8 T cells from DC+CpG-immunized hosts compared with those given DC alone immunization at any time point (Fig. 3, A–C). Antisurvival/proapoptotic factors (*Bcl2l11/Bim*, *Bid*, and *Bad*) were not significantly increased in OT-I CD8 T cells from DC alone–immunized hosts compared with those from DC+CpG-immunized hosts at any time point (Fig. 3, A–C). Expression of *Bcl3*, previously suggested to control signal 3–mediated survival of activated CD8 T cells (Mitchell et al., 2001; Valenzuela et al., 2005), was also not different between groups at these time points (Fig. 3, A–C). However, *Bcl2* expression is elevated in OT-I CD8 T cells from DC alone–immunized hosts relative to those from DC+CpG-immunized hosts between days 6 and 7 after immunization (Fig. 3 A). Although the data do not support the role for signal 3 cytokines in promoting survival, the percentages of OT-I CD8 T cells expressing active caspase 3/7, TUNEL, and Annexin V^+/7-AAD^- was not significantly altered in DC alone or DC+CpG immunization at these time points (Fig. 3, D–F). Thus, the presence of CpG-induced inflammation during CD8 T cell priming does not affect the survival during the window of extended division.

IL-12 cytokine signaling is dependent on both IL-12Rβ1 and IL-12Rβ2 expression; the absence of either β-chain abrogates IL-12 signaling (Chua et al., 1995; Vignali and Kuchroo, 2012). To address a role for direct IL-12 signaling in extending CD8 T cell division, we compared the expression of the transcription factor FoxM1 between WT and IL-12Rβ1 KO OT-I CD8 T cells after adoptive transfer and DC alone or DC+CpG immunization. Similar to Fig. 2 H, differences in FoxM1 expression did not occur between WT and IL-12Rβ1 KO OT-I CD8 T cells on day 5 after immunization, regardless of immunization (Fig. 4 A). However, only WT OT-I CD8 T cells from DC+CpG-immunized mice maintained FoxM1 expression at day 7 after immunization, whereas expression of FoxM1 was decreased in IL-12Rβ1–deficient OT-1 (Fig. 4 A). To determine whether this was a general effect of signal 3–associated cytokines, we also examined the expression of FoxM1 in type I IFN receptor (IFNAR) KO OT-I CD8 T cells during DC+CpG immunization. Similarly to IL-12Rβ1 KO, IFNAR KO OT-I CD8 T cells in DC+CpG-immunized mice also expressed reduced amounts of FoxM1 at day 7 after immunization, relative to WT OT-I CD8 T cells (Fig. 4 A).

To understand whether the expression of FoxM1 was concordant with the size of the CD8 T cell response, we also measured the division and accumulation of WT, IL-12Rβ1 KO, and IFNAR KO OT-I CD8 T cells in DC alone or DC+CpG-immunized hosts. The absence of either IL-12 or type I IFN signaling on OT-I CD8 T cells did not affect accumulation or division at day 5 or 7 after DC alone immunization (Fig. 4, B–E). Similarly, IL-12Rβ1 or IFNAR-deficiency did not affect the accumulation or division of OT-I CD8 T cells at day 5 after DC+CpG immunization, relative to WT

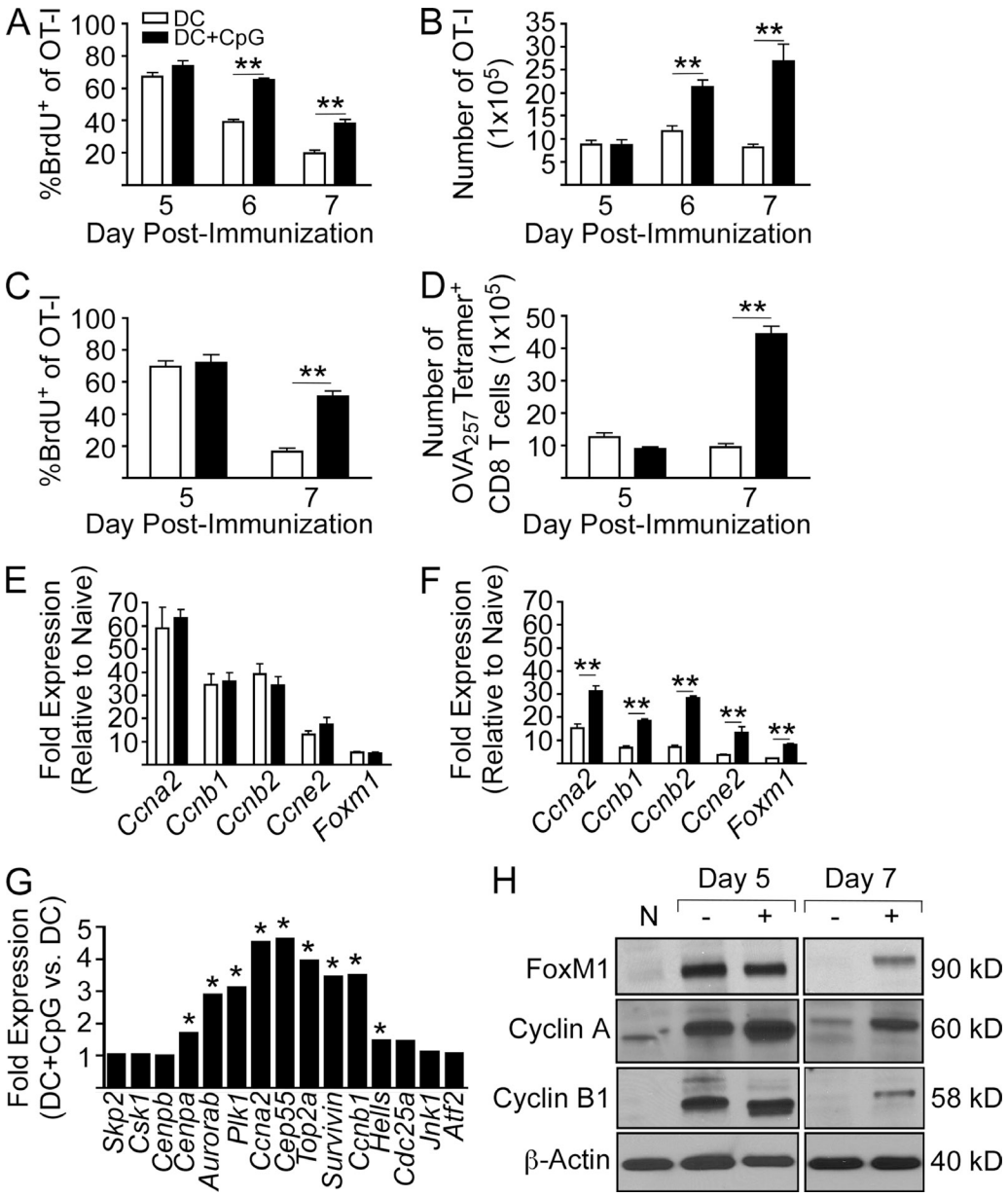

Figure 2. Expression of cell cycle–associated genes and division are sustained in CD8 T cells when activated in the presence of inflammation. Mice were injected with OT-I CD8 T cells and immunized with DC or DC+CpG as in Fig. 1. BrdU was injected in an ∼15-h pulse. The frequency (A and C) and absolute number (B and D) of OT-I CD8 T cells (A and B) or $OVA_{257-264}$ tetramer+ CD8 T cells (C and D) in spleen was measured by flow cytometry. Data are represented as mean ± SEM. Statistical analysis used Student's *t* test ($n = 3$ from each group). **, $P < 0.01$; NS = $P > 0.05$. Data are representative of two independent experiments. (E–H) OT-I T cells were isolated from the spleen at day 5 (E and H) or day 7 (F–H) after immunization, and mRNA levels of *Ccna2*, *Ccnb1*, *Ccnb2*, *Ccne2*, *Foxm1* (E and F) were analyzed by RT-PCR and the indicated FoxM1 target genes (G) were analyzed using microarray data previously described in Fig. 1. mRNA levels in (E and F) are expressed relative to naive OT-I T cells; mRNA levels in G are displayed as fold expression in cells from DC+CpG- versus DC alone-immunized mice. Protein expression of Cyclin A, Cyclin B1, and FoxM1 was analyzed by Western blot (H). Data in E and F are represented as mean ± SEM. Statistical analysis used Student's *t* test ($n = 3$ from each group). **, $P < 0.01$; NS = $P > 0.05$. Data in E, F, and H are representative of two independent experiments. *, FDR < 0.01.

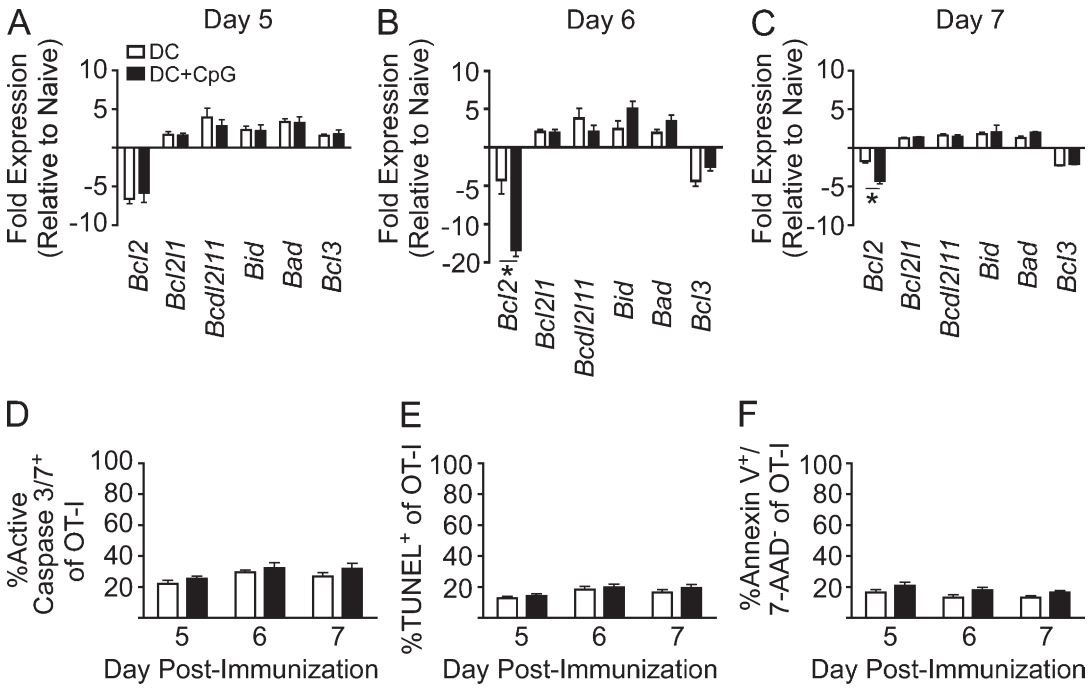

Figure 3. Expression of survival-associated mRNA transcripts by CD8 T cells activated in the presence or absence of inflammation. Mice were injected with OT-I CD8 T cells and were immunized with DC or DC+CpG immunization. mRNA expression of prosurvival-associated genes (*Bcl2* and *Bcl2l1*), pro-apoptosis-associated genes (*Bcl2l11*, *Bid*, and *Bad*), and *Bcl3* were analyzed in OT-I CD8 T cells harvested from the spleens of DC alone or DC+CpG-immunized hosts on days 5 (A) , 6 (B), or 7 (C) after immunization. Data are represented as mean ± SEM. Statistical analysis used Student's *t* test ($n = 3$ from each group). **, $P < 0.01$; *, $P < 0.05$; NS = $P > 0.05$. Data are representative of two independent experiments. Frequencies of active caspase 3/7$^+$ (D), TUNEL$^+$ (E), and Annexin V$^+$/7-AAD$^-$ OT-I CD8 T cells (F) were measured in the spleens of DC or DC+CpG-immunized hosts between 5 and 7 d after immunization. Data are represented as mean ± SEM. Statistical analysis used Student's *t* test ($n = 3$ from each group). NS = $P > 0.05$. Data are representative of two independent experiments.

OT-I CD8 T cells (Fig. 4, B–E). In contrast, both IL-12Rβ1 and IFNAR KO deficiencies significantly reduced accumulation and division of OT-I CD8 T cells at 7 d after DC+CpG immunization (Fig. 4, B–E), while not altering apoptosis-phenotype as measured by active Caspase 3/7 (Fig. 4, F and G). Similar results were seen with IL-12Rβ2 KO OT-I CD8 T cells (not depicted). Together, these results indicate that classical signal 3 cytokines directly interact with activated CD8 T cells to maintain the expression of cell cycle progression gene products and extend the division of activated CD8 T cells. Additionally, these data raise a temporal conundrum—how can an early (~6–12 h after CpG administration) and transient peak of signal 3-cytokines (IL-12/type I IFN; Pham et al., 2009) sustain the division of activated CD8 T cells at days 6–7 after immunization?

IL-2 stimulation acts secondarily to inflammation to extend division of activated CD8 T cells in vivo

We next determined when inflammation instructs responding CD8 T cells to extend cellular division and enhance accumulation. To do so, we isolated OT-I CD8 T cells from DC+CpG and DC alone immunized hosts at day 4 after immunization, CFSE-labeled, and then adoptively transferred cells into naive recipients or incubated cells in vitro wherein subsequent division was evaluated. OT-I CD8 T cells from DC+CpG-immunized hosts underwent more divisions and accumulated in greater number after transfer into naive recipients than those primed with DC alone (Fig. 5, A and B). However, neither OT-I CD8 T cell population (from DC+CpG or DC alone–immunized mice) was able to divide after in vitro incubation (Fig. 5 A). These results indicate that early inflammation instills an extended proliferative program in activated CD8 T cells before their manifestation of optimal accumulation. Because exposure to inflammation during priming did not extend the division of activated CD8 T cells in vitro, we predicted that the maintenance of division was dependent on a secondary signal provided by an in vivo environment.

To determine the secondary signal required for the extended division of activated CD8 T cells exposed to inflammation, we evaluated the activity of molecular pathways known to direct the functionality and production of cell cycle–associated gene products. The PI3K–Akt pathway regulates many aspects of cell cycle progression including the prevention of Cyclin D1 degradation (Diehl et al., 1998) and inhibition of cyclin-dependent kinase inhibitors p27 Kip (Gesbert et al., 2000) and p21 Waf1–CIP1 (Zhou et al., 2001). The PI3K–Akt pathway also enhances the expression (Wang et al.,

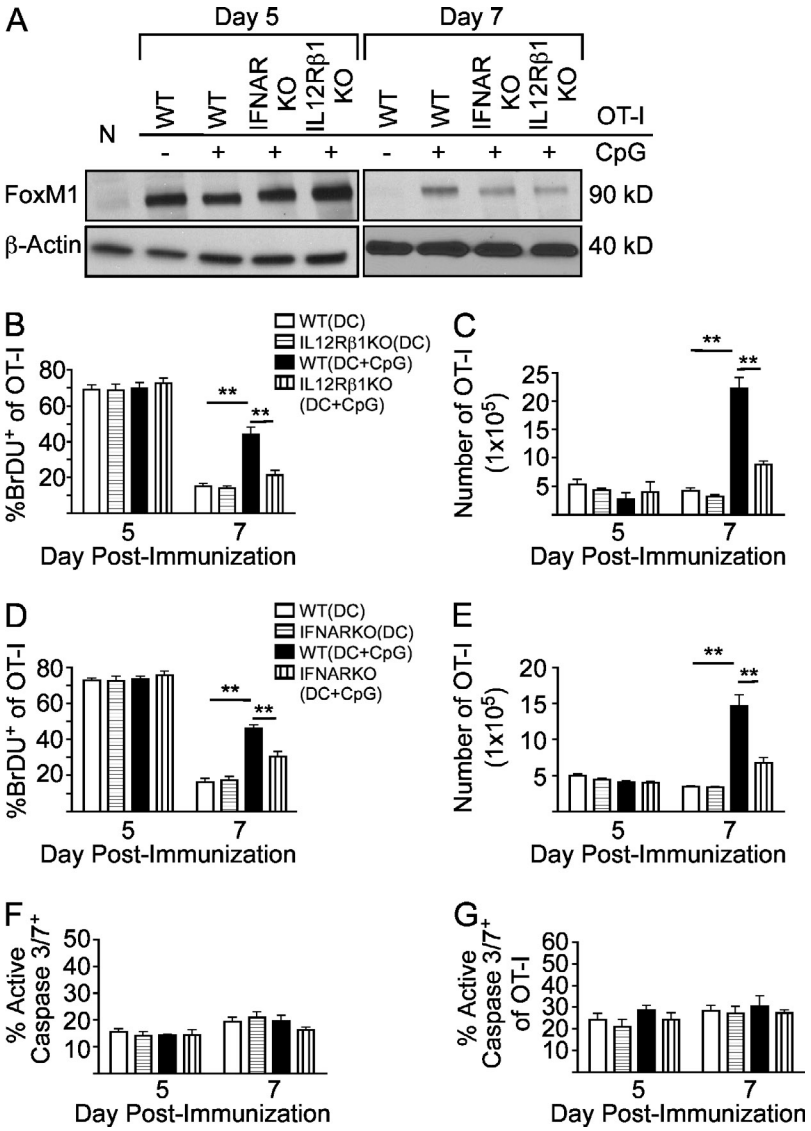

Figure 4. IL-12 and type I IFN contribute to prolonged division of activated CD8 T cells after inflammatory immunization. (A–E) 1:1 ratio of WT and cytokine receptor KO (i.e., IL-12Rβ1 KO or IFNAR KO) OT-I CD8 T cells were transferred into allelic disparate hosts. Recipient mice were subsequently given DC alone or DC+CpG immunization. WT and cytokine receptor KO OT-I were purified from the spleens of immunized mice at 5 or 7 d after DC immunization or naive hosts (N), and then cell lysates were analyzed for FoxM1 and β-actin protein expression (A). In similarly immunized mice, BrdU incorporation after ∼15-h pulse (B and D) and accumulation of OT-I in the spleen (C and E) were analyzed on days 5 and 7 after immunization. The percentage of WT and IL-12Rβ1 (F) or IFNAR KO OT-I CD8 T cells (G) that were active caspase 3/7+ in the spleens of DC alone or DC+CpG-immunized hosts were also measured on days 5 and 7 after immunization. All data represented as mean ± SEM. Experiments in B–G were analyzed by ANOVA ($n = 3$ from each group) comparing either WT (DC+CpG) group to WT (DC) group or WT (DC+CpG) group to IFNARKO or IL12Rβ1KO group. **, $P < 0.01$. All panels are representative of at least two to three independent experiments.

2010) and activity of FoxM1 (Major et al., 2004; Park et al., 2009). Because FoxM1 and many of its known targets are up-regulated at both the mRNA and protein level in DC+CpG-immunized OT-I CD8 T cells (Fig. 2, E–H; and Fig. 4 A), we analyzed PI3K–Akt activity (as indicated by phosphorylation of Akt) in OT-I CD8 T cells from DC+CpG or DC alone–immunized hosts directly ex vivo. Interestingly, OT-I CD8 T cells from DC+CpG-immunized mice exhibited higher amounts of activated Akt (Akt-pS473) measured directly ex vivo from day 5 after immunization compared with OT-I CD8 T cells from mice given DC immunization alone (Fig. 5, C). However, differences in Akt-pS473 were decreased when OT-I CD8 T cells from DCs alone or DC+CpG-immunized mice were harvested at day 4 after immunization, and then incubated in vitro without stimulation for 48 h (Fig. 5 D). These results suggest that maintenance of PI3K–Akt pathway signaling enables continued division of OT-I CD8 T cells from DC+CpG-immunized hosts in vivo.

IL-2 is an important cytokine in driving T cell accumulation and is known to directly activate the PI3K–Akt pathway (Taniguchi and Minami, 1993; Bensinger et al., 2004; Lali et al., 2004; Waldmann, 2006; Malek, 2008). Interestingly, low concentrations (5 ng/ml) of exogenous IL-2 sustained in vitro division of OT-I CD8 T cells obtained at day 4 after DC+CpG (but not DC alone) immunization, and these cells exhibited high levels of Akt-pS473 (Fig. 5, E and F). In contrast, the same concentration of exogenous IL-2 did not sustain in vitro division or Akt-pS473 of OT-I harvested from DC alone–immunized mice at day 4 after immunization (Fig. 5, E and F). Inhibition of PI3K activity by Wortmannin or Ly294002 during in vitro IL-2 stimulation reduced the extent of division and Akt-pS473 exhibited by OT-I CD8 T cells harvested from DC+CpG-immunized hosts (Fig. 5, E and F). These results indicate that, at least in vitro, IL-2 stimulation can act secondarily to in vivo signal 3 cytokines to sustain PI3K-dependent division of activated CD8 T cells.

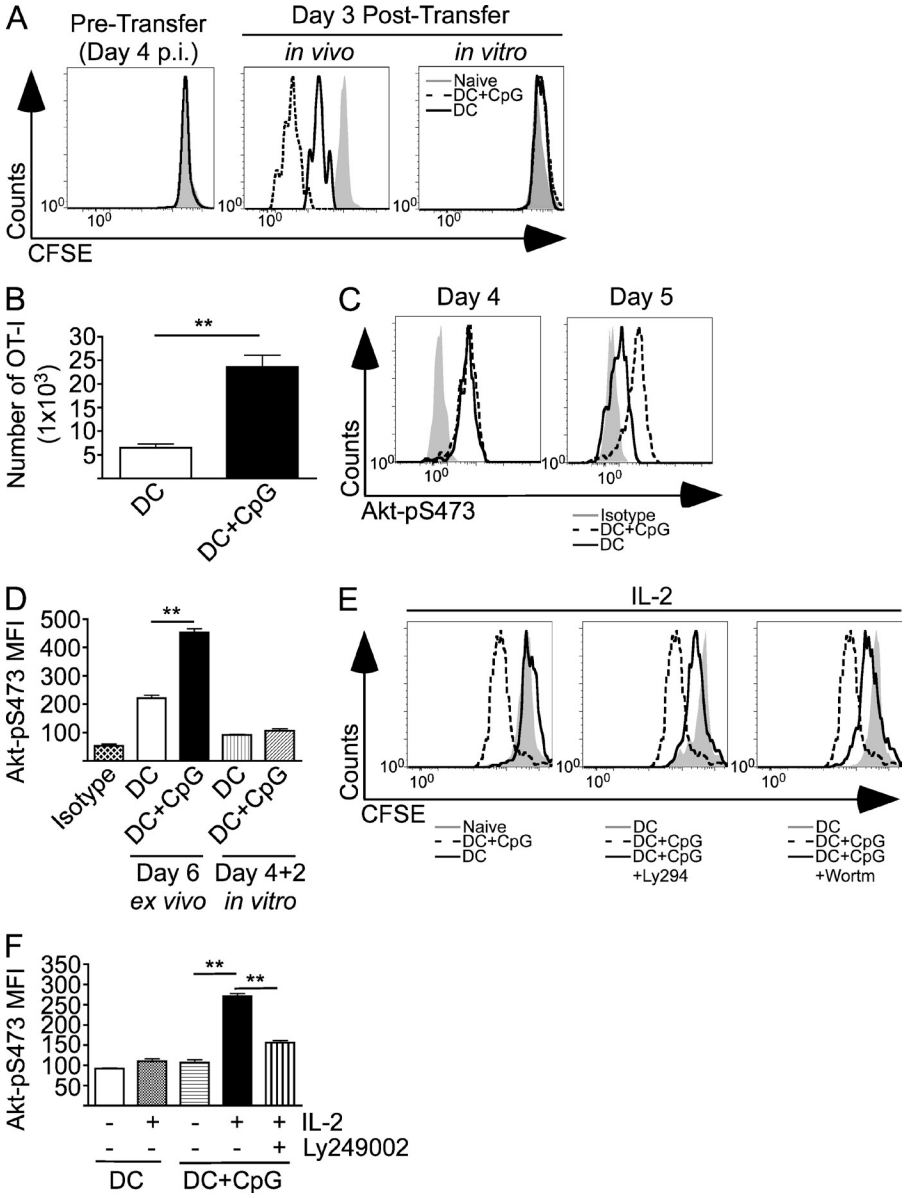

Figure 5. IL-2 stimulation acts secondarily to inflammation to permit extended division in a PI3K-dependent manner. ~600 OT-I CD8 T cells (Thy1.1/1.1 or Thy1.1/1.2) were adoptively transferred into naive (Thy1.2) hosts and, 1 d later, recipient mice were injected with 5×10^5 DC alone or DC+CpG. At d4 after DC or DC+CpG immunization, splenic OT-I cells (Thy1.1/1.1 or Thy1.1/1.2, respectively) and WT OT-I cells from naive mice (Thy1.2/1.2) were purified, CFSE-labeled, mixed into a 1:1:1: ratio, and then injected into naive (CD45.1) hosts or plated in vitro. (A) CFSE dilution by OT-I cells 3 d after in vivo transfer or plating. (B) Accumulation of transferred OT-I cells in the spleen 3d after transfer into naive hosts. (C) Splenic OT-I cells were stained with anti–Akt-pS473 directly after harvest at 4 and 5 d after DC or DC+CpG immunization. (D) After anti–Akt-pS473 staining, geometric MFI was compared between splenic OT-I CD8 T cells from mice given DC+CpG and DC alone–immunized mice directly ex vivo at day 6 after immunization, or when splenic OT-I cells were harvested at day 4 after immunization and plated in vitro for 48 h. (E and F) Naive OT-I cells and OT-I cells purified from hosts 4 d after DC alone or DC+CpG immunization were CFSE-labeled, and stimulated with 0.5 ng/ml murine IL-2 with or without Ly2940002 or Wortmannin treatment (PI3K inhibitors) in vitro. At 72 h, CFSE dilution (E) and Akt-pS473 MFI (F) were analyzed. All data are represented as mean ± SEM. In B, D, and F, statistical analysis used Student's *t* test ($n = 3$ from each group). **, $P < 0.01$. All experiments are representative of two to three independent experiments.

Both IL-12 and type I IFN sustain IL-2Rα expression on activated CD8 T cells during inflammatory immunization

IL-2 signaling is dependent on both IL-2Rβ (CD122) and common cytokine receptor-γ chain surface expression (CD132; Boyman and Sprent, 2012). However, IL-2 sensitivity can be increased by the expression of IL-2Rα (CD25), the receptor subunit that creates the trimeric high-affinity IL-2R (Taniguchi and Minami, 1993). Because low concentrations of exogenous IL-2 promote the division of DC+CpG-primed (but not DC alone–primed) OT-I CD8 T cells in vitro, we asked if signal 3 cytokine regulation of CD25 expression could account for extended division in vivo. Surface CD25 expression was similar between OT-I CD8 T cells from DCs and DC+CpG-immunized hosts from days 2–3 after immunization; however, surface expression of CD25 remained retained at a markedly higher level on OT-I CD8 T cells responding to DC+CpG immunization from 4–6 d after immunization when compared with OT-I CD8 T cells from mice given DC alone immunization (Fig. 6, A and B). Expression of the other IL-2 receptor subunits, CD132 and CD122 on the surface of OT-I CD8 T cells were not significantly altered by the presence of CpG-induced inflammation during immunization (Fig. 6 B and not depicted). Importantly, sustained CD25 expression at day 5 after immunization was decreased by OT-I CD8 T cells from DC+CpG-immunized hosts when either IL-12 and/or type I IFN signaling was absent (Fig. 6 C), or when IL-12Rβ2 was absent on OT-I CD8 T cells responding virLM-OVA infection (Fig. 6 D). These results indicate that the presence of IL-12 and/or type I IFN signaling during CD8 T cell priming extends CD25 expression by responding T cells and, potentially, their sensitivity to IL-2–driven division.

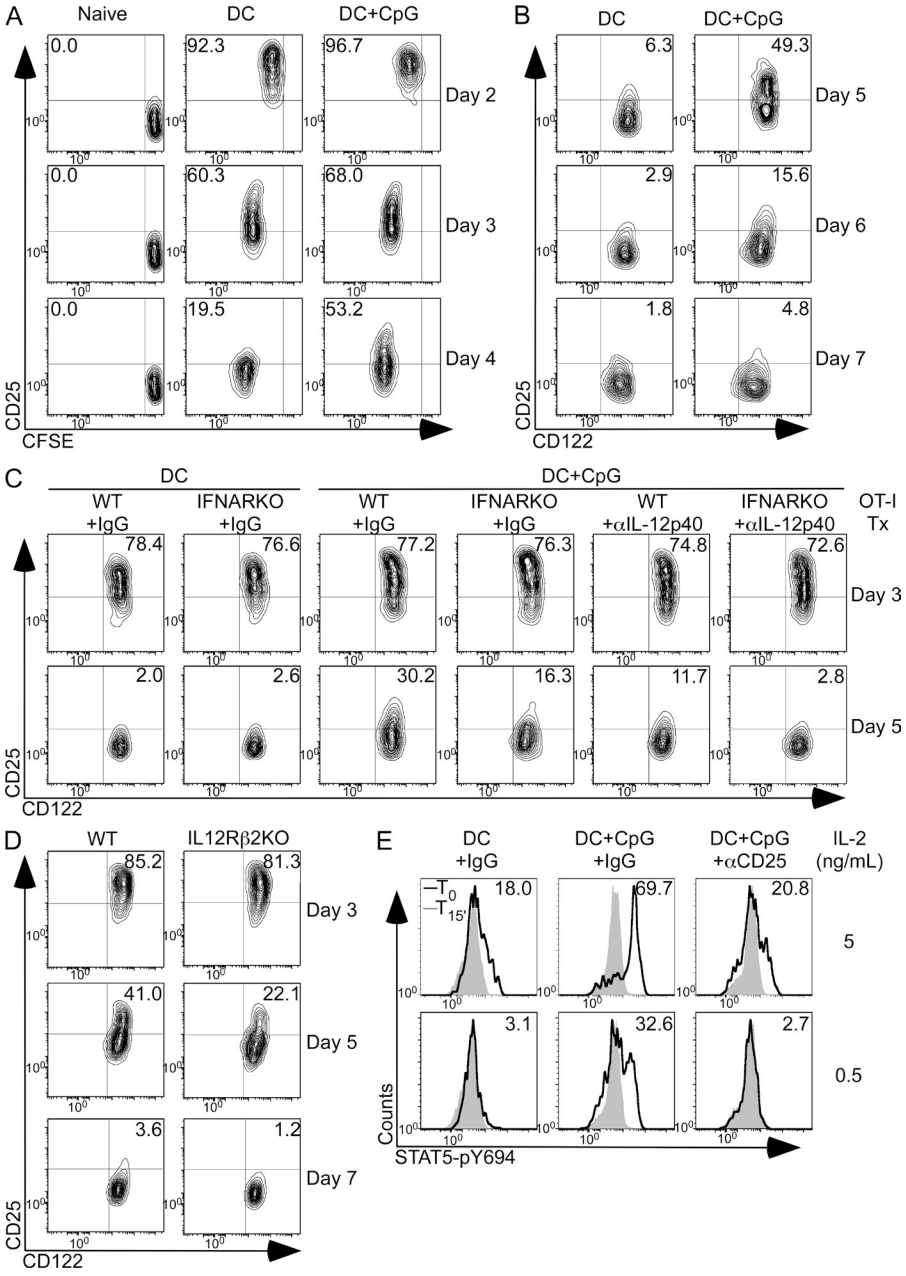

Figure 6. Both IL-12 and type I IFN promote IL-2 sensitivity by sustaining expression of IL-2Rα by activated CD8 T cells. OT-I CD8 T cells were adoptively transferred into naive hosts and, 1 d later, recipient mice were given DC or DC+CpG immunization. (A) Surface expression of IL-2Rα (CD25) and CFSE dilution by splenic OT-I cells from unimmunized hosts or from hosts given DC alone or DC+CpG immunization 2–4 d prior. (B) Surface CD25 and IL-2Rβ (CD122) expression by splenic OT-I cells from mice immunized with DC alone or DC+CpG d5-7 d before. (C) Surface CD25 and CD122 expression by splenic WT or IFNAR KO OT-I CD8 T cells from DC or DC+CpG-immunized hosts at days 3 or 5 after immunization after being previously treated with control IgG (IgG) or anti–IL-12p40 (C17.8) at day 0 after immunization. (D) Surface expression of CD25 and CD122 by splenic WT or IL-12Rβ2 KO OT-I CD8 T cell from d3, 5, and 7 after virLM-OVA–infected hosts. (E) At day 4 after DC or DC+CpG immunization, splenic OT-I cells were purified from mice given DC+CpG or DC alone immunization, and then left unstimulated (T_0; solid gray histogram) or given IL-2 stimulation for 15 min ($T_{15'}$; hollow black histogram) at indicated concentrations in the presence of control IgG or PC61 (CD25 blocking antibody) prepretreatment. All experiments are representative of two to three independent experiments.

To determine whether the presence of inflammation during CD8 T cell activation maintains IL-2 sensitivity, we harvested OT-I CD8 T cells from DC+CpG- or DC alone–immunized mice at day 4 after immunization and measured STAT5 activation (STAT5-pY694) in response to titrated IL-2 stimulation in vitro. Consistent with their CD25 expression, OT-I CD8 T cells from DC+CpG-immunized hosts exhibit more robust STAT5-pY694 in response to at lower concentrations of IL-2 than those from DC alone–immunized mice (Fig. 6 E). When CD25 was blocked on the surface of OT-I cells at day 4 after immunization from DC+CpG-immunized mice, IL-2 sensitivity (as measured by STAT5-pY694) was reduced to levels exhibited by OT-I CD8 T cells from mice immunized with DC alone (Fig. 6 E). These data indicate that both IL-12 and type I IFN increase the duration of CD25 surface expression by activated CD8 T cells, and suggests that maintenance of high-affinity IL-2 signaling may act secondarily to inflammatory cytokines to promote extended division and accumulation of activated CD8 T cells.

Sustained IL-2 signaling is important secondarily to inflammation for extended division and optimal accumulation of activated CD8 T cells in vivo

To assess the contribution of extended IL-2 signaling on the accumulation of CD8 T cells activated in the presence of inflammation in vivo, we neutralized endogenous IL-2 with blocking antibody (JES6-1A12; Boyman et al., 2006) after

DC alone or DC+CpG immunization at time points corresponding to the window of extended CD25 expression (4–6 d after immunization). Although the frequencies of OT-I CD8 T cells in DC alone–immunized mice were unaffected by in vivo IL-2 blockade at day 5 after immunization, this treatment significantly decreased the division and accumulation of DC+CpG primed OT-I CD8 T cells at both day 6 and 7 after immunization (Fig. 7, A and B). Importantly, IL-2 blockade reduced the expression of FoxM1 protein in OT-I CD8 T cells from DC+CpG-immunized hosts at day 6–7 after immunization compared with IgG control treatment (Fig. 7 G). These results directly show that the ability of inflammation to maintain high levels of FoxM1 expression and to extend the division/accumulation of activated CD8 T cells depends on continued IL-2 signaling throughout later phases (day 4–7) of expansion.

To test whether continued IL-2 signaling was sufficient to extend the division and accumulation of OT-I CD8 T cells primed in the absence of inflammation, we treated DC alone–immunized mice with stimulatory IL-2/S4B6 antibody complexes (Boyman et al., 2006) from day 4 to 6 after immunization. IL-2–S4B6 antibody complexes substantially increases the proliferation of CD122$^+$ CD8 T cells (independently of CD25) by inducing high-affinity IL-2 signaling similarly to that elicited by IL-2Rα (Boyman et al., 2006; Boyman and Sprent, 2012). Interestingly, administration of IL-2/S4B6 complexes enhanced the accumulation and division of OT-I CD8 T cells from DC alone–immunized mice when compared with those treated with IgG control (Fig. 7, C and D). Not only was IL-2–S4B6 complex treatment able to extend the division of WT OT-I CD8 T cells in DC alone–immunized mice, but this treatment also increased the

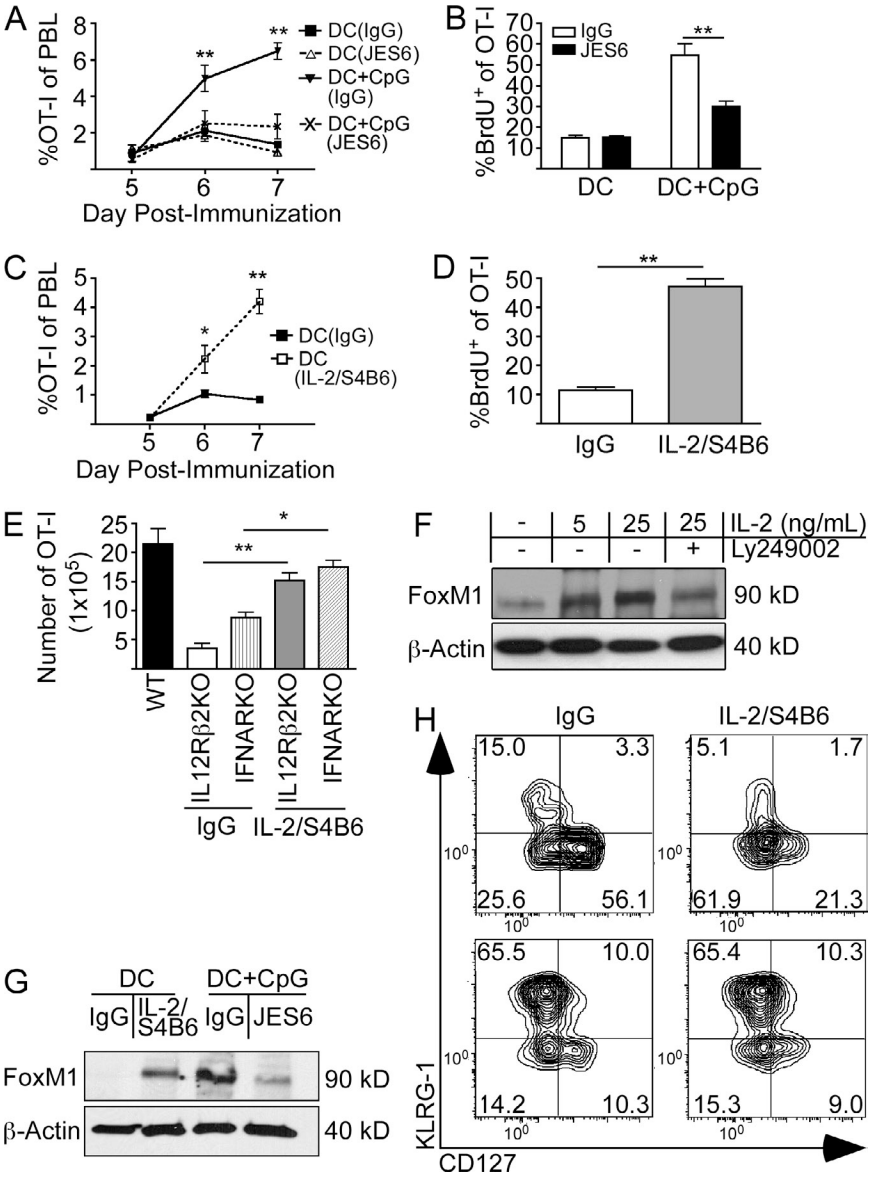

Figure 7. Curtailing IL-2 signaling during expansion reduces the division of CD8 T cells responding to inflammatory immunization. OT-I CD8 T cells were adoptively transferred into naive hosts and one day later recipient mice were given DC or DC+CpG immunization. At day 4 after immunization, immunized mice were treated with control IgG, IL-2 blockade (JES6-1A2A) or high-affinity IL-2 receptor stimulating complex (0.3 µg IL-2/10 µg S4B6 mAb) from days 4-6 after immunization. (A and C) Frequency of OT-I CD8 T cells in the blood and (B and D) frequency of BrdU$^+$ OT-I CD8 T cells in the spleen after DC alone or DC+CpG immunization and specified treatments. (E) Number of WT, IFNARKO, or IL-12rβ2KO OT-I cells in the spleen of mice given DC+CpG immunization and control or IL-2/S4B6 treatment at day 7 after immunization. (F) At day 5 after DC+CpG immunization, OT-I CD8 T cells were purified and plated in vitro and left unstimulated or stimulated with 5 ng/ml of IL-2, or 25 ng/ml IL-2+/−PI3K inhibitor (Ly249002). After 48 h, FoxM1 and β-actin expression was evaluated from whole-cell lysates using Western blot analysis. (G) FoxM1 and β-actin Western blot analysis and (H) KLRG-1/CD127 protein expression were analyzed in OT-I CD8 T cells from mice that had received DC alone or DC+CpG immunization 7 d before. All data represented as mean ± SEM. Statistical analysis used Student's *t* test in A–E ($n = 3$ from each group). **, $P < 0.01$. All panels are representative of two to three independent experiments.

number of IL-12β2R KO and IFNAR KO OT-I CD8 T cells at day 7 after DC+CpG immunization (Fig. 7 E). Importantly, in vitro stimulation of DC+CpG primed OT-I CD8 T cells with IL-2 was able to maintain FoxM1 expression in a PI3K-dependent manner (Fig. 7 F). Concordantly, extending high-affinity IL-2 signaling with IL-2–S4B6 complexes also enhanced FoxM1 protein expression in vivo by OT-I CD8 T cells in from DC alone–immunized hosts (Fig. 7 G). Although it is already known that robust IL-2 signaling will decrease the expression of CD127 by activated CD8 T cells (Xue et al., 2002), the reduction of IL-2 signaling during later phases of the CD8 T cell response did not substantially alter differentiation according to CD127/KLRG-1 expression (Fig. 7 H). Together our data indicate that the presence of CpG-induced inflammation early in the response permits continued CD8 T cell division and accumulation by extending the window of high-affinity IL-2 signaling.

DISCUSSION

Optimal accumulation of effector CD8 T cells is critically dependent on the integration of multiple signals, including signal 3 cytokine stimulation (Curtsinger and Mescher, 2010). In vivo, signal 3 cytokines such as IL-12 and IFN-α/β are known to be important for the optimal accumulation of effector CD8 T cells after LM (Keppler et al., 2009, 2012; Xiao et al., 2009) or LCMV infection (Kolumam et al., 2005; Aichele et al., 2006). Most studies to date have relied on short-term in vitro T cell stimulations to address the underlying mechanisms to account for the ability of inflammatory cytokines to optimize CD8 T cell accumulation (Curtsinger et al. ., 1999, 2003a; Valenzuela et al., 2002, 2005; Mescher et al., 2006; Xiao et al., 2009). From this work, two models have emerged to explain the mechanism of signal 3 cytokines; however, their physiological relevance to the in vivo accumulation of effector CD8 T cells remained undefined.

The first signal 3 model suggested that inflammatory cytokine stimulation increases the accumulation of CD8 T cells after activation by enhancing survival during proliferation (Valenzuela et al., 2005). In contrast to the survival model, CpG-induced inflammation during immunization in vivo did not substantially alter mRNA expression of genes with described apoptotic/survival roles in responding CD8 T cells, nor did we observe decreased expression of apoptosis markers in this T cell population. Previous in vitro work has attributed signal 3 cytokine-enhanced survival to increased Bcl-3 expression by activated CD8 T cells (Mitchell et al., 2001; Valenzuela et al., 2005); however, we did not detect any difference in *Bcl3* transcript between OT-I CD8 T cells from DC or DC+CpG-immunized mice at 5 or 7 d after immunization in our in vivo study. Thus, it appears that the current signal 3 survival model is insufficient to explain how inflammatory cytokines increase activated CD8 T cell accumulation in vivo. Instead, our study indicates that signal 3 cytokines induce the optimal accumulation of activated CD8 T cell by guiding the extent of their division.

The second model proposes that signal 3 cytokines (i.e., IL-12) rapidly and transiently increased both IL-2Rα (Pipkin et al., 2010; Valenzuela et al., 2002) and IL-2Rβ (Valenzuela et al., 2002) expression. Importantly, enhanced expression of these receptors in CD8 T cells was transient, peaking at 36 h after in vitro cognate antigen stimulation, and then declining (Valenzuela et al., 2002; Curtsinger and Mescher, 2010; Pipkin et al., 2010). Thus, these data suggest that signal 3 cytokines provide a proliferative advantage to responding CD8 T cells at early time points after activation. However, we demonstrate that the transient expression of signal 3 cytokines during in vivo activation does not immediately enhance CD25 expression or CD8 T cell accumulation/division at early time points. Instead, the presence of signal 3 cytokines during immunization substantially increased activated CD8 T cell accumulation only at relatively late time points (i.e., from days 6–7 after immunization). Our in vivo data also indicate that rather than transiently increasing the overall expression of IL-2Rα and IL-2Rβ expression at early time points, signal 3 cytokine stimulation in vivo promotes optimal accumulation of activated CD8 T cells by specifically increasing the duration of IL-2Rα expression (but not modulating IL-2Rβ expression), and thus maintaining sensitivity to IL-2 signaling at relatively late time points (days 4–6 after immunization) in the response. Not only were we able to verify that in vivo production of IL-12 is capable of prolonging IL-2Rα expression, but this study is also the first to indicate that type I IFN signaling is also important for sustained expression of the high-affinity IL-2 receptor in DC+CpG-immunized mice. Interestingly, Gil et al. (2012) have indicated that type I IFN stimulation can activate STAT4 signaling (typically associated with IL-12 stimulation) in CD8 T cells during infection. Whether IL-12 and type I IFN signal by parallel or synergistic pathways to sustain IL-2rα expression by activated CD8 T cells remains to be explored by future studies. In turn, these data may resolve a long standing conundrum in the field, whereby the critical signal 3 cytokine depends on the pathogen, and specific pathogen inflammatory milieu, used to elicit the T cell response (Haring et al., 2006).

Consistent with this mechanism, sustained IL-2 signaling (days 4–6 after immunization) was critical for continued expression of cell cycle progression genes and proteins, extended division, and enhanced accumulation of OT-I CD8 T cells in DC+CpG-immunized hosts. Because OT-I CD8 T cells from DC+CpG undergo extended division when transferred into naive hosts and activated OT-I effector CD8 T cells produce almost no IL-2 additional in vitro stimulation (Badovinac et al., 2005), we predict that continued division is in response to basal levels of IL-2 produced by the recipient host (similarly described for regulatory T cells; Malek et al., 2008) rather than autocrine IL-2 (Feau et al., 2011). Thus, we propose that signal 3 cytokine stimulation enhances the accumulation of activated CD8 T cells, not by increasing early survival or proliferation, but rather by temporally orchestrating responsiveness to secondary cytokine signals that permit continued division late during the expansion phase.

Data from our study also sheds light on the complex involvement of IL-2 on the differentiation of activated CD8

T cells. Early CD25 expression and IL-2 signaling have previously been shown to promote effector CD8 T cell differentiation (Kalia et al., 2010; Pipkin et al., 2010). Another study, by Williams et al. (2006), indicates that the acquisition of memory characteristics (e.g., CD127 expression) by CD8 T cells is accelerated in CD25-deficient CD8 T cells. In contrast, in vivo blockade of IL-2 from days 4–6 after immunization was not coupled to changes in CD127 and KLRG-1 expression by responding CD8 T cells at day 7 after infection. Thus, the impact of IL-2 signaling on effector differentiation and accumulation can be separated. Together, these data suggest that early IL-2 signaling has a profound and indelible impact in promoting effector CD8 T cell differentiation, whereas late high-affinity IL-2 signaling extends the division and accumulation of effector CD8 T cells. Interestingly, serum IL-2 levels also peak within 24 h after CpG treatment (unpublished data), thus, the strength of IL-2 signaling at early and late time points may also determine the impact on differentiation and proliferation.

Currently, the role of IL-2 during the primary expansion of activated CD8 T cells is controversial. In a previous study, Obar et al. (2010) used WT/CD25 KO bone marrow chimeric mice to indicate that the absence of CD25 decreases the expansion of LM-specific CD8 T cells by ∼4-5 fold relative to their WT counterpart. However, Williams et al. used adoptive transfer study with P14 TCR-transgenic cells to indicate that the expansion of antigen-specific CD8 T cells during LCMV Arm infection is only modestly (approximately twofold) decreased in the absence of CD25 (Williams et al., 2006), whereas our study indicates that the importance of late (day 4–6 after immunization) IL-2 signaling is more substantial (∼5 fold). However, this discrepancy may be explained by differences in the duration of antigen presentation. Antigen presentation during DC immunization is not detectable 48 h after immunization (Badovinac et al., 2005). In contrast, antigen presentation during LCMV Arm infection persists as late as 5 d after infection (unpublished data; Ahmed, R., personal communication). As a result, we speculate that the maintenance of high-affinity IL-2 signaling is important for guiding the optimal division and accumulation of activated CD8 T cells when antigen presentation is limiting. However, future work is required to verify this notion.

Expression of transcription factors such as T-bet, Id2, Eomes, and Blimp-1 can be affected by cytokine signaling and are also known to dictate the differentiation of CD8 T cells (Intlekofer et al., 2005; Cannarile et al., 2006; Kallies et al., 2006, 2009; Joshi et al., 2007; Yang et al., 2011). However, a transcription factor that is regulated by signal 3 cytokines and is specifically responsible for the magnitude of CD8 T cell responses has yet to be identified. In this study, we are the first to demonstrate that sustained mRNA and protein expression of forkhead family transcription factor FoxM1 in activated CD8 T cells depended on early IL-12/type I IFN signaling and, thereafter, continued IL-2 signaling in vivo. Not only was FoxM1 expression concordant with extended division of activated CD8 T cells in response to inflammatory immunization, but we also found that the presence of inflammation during priming extends the transcript expression of many FoxM1 targets. Because FoxM1 has been previously shown to be critical for the division of naive CD8 T cells after activation via anti-CD3/CD28 stimulation (Xue et al., 2010), it is likely to be an important transcription factor that works to extend the division of CD8 T cells when activated in the presence of inflammation.

In conclusion, our data indicate that IL-12 and type I IFN work together to promote the accumulation of activated CD8 T cells by extending the duration of cellular division, rather than by enhancing survival of responding cells or providing an early proliferative advantage. Early induction of inflammatory cytokines, such as IL-12 and type I IFN, work together to extend division of activated CD8 T cells by sustaining the window of high-affinity IL-2 signaling into late time points during expansion. Thoroughly understanding the sequence of events, which ultimately guide the magnitude of CD8 T cell responses, would be of benefit for the design of future therapies that critically depend on appropriately sized CD8 T cell responses.

MATERIALS AND METHODS

Mice, bacteria, and DCs. C57BL/6 mice (Thy1.2 and CD45.1) were obtained from the National Cancer Institute and used for experiments at 6–10 wk of age. T cell receptor transgenic OT-I (Thy1.1) mice have been previously described (Hogquist et al., 1994). IL-12Rβ1 KO OT-I CD8 T cells and IFNAR KO mice were kindly provided by M. Mescher (University of Minnesota, Minneapolis, MN). IFNAR KO OT-I mice were generated by appropriate breeding. IL-12Rβ2 KO mice were purchased from Jackson Laboratory. All KO mice were backcrossed at least 10 times onto the C57BL/6 background. Mice were used in compliance with the United States Department of Health and Human Services Guide for Use of Laboratory Animals, as documented in writing and approved by the University of Iowa Animal Care and Use Committee. Virulent LM strain expressing OVA_{257} (virLM-OVA) were grown, quantified, and injected at 2×10^4 cfu/mouse as previously described (Pope et al., 2001; Pham et al., 2009; Wirth et al., 2010). Splenic DCs were isolated after subcutaneous injection of C57BL/6 mice with 5×10^6 B16 cells expressing Flt3L as described previously (Pham et al., 2009).

Antibodies, MHC class I tetramers, PI3K inhibitors. The FACS antibodies were used with the indicated specificity and the appropriate combinations of fluorochromes. The following antibodies were purchased from BioLegend: Thy1.1 (OX-7), Thy1.2 (30-H12), CD45.2 (104), CD8α (53–6.7), BrdU (Bu20a), CD132 (TUGm2), and KLRG-1 (2F1). The following antibodies were purchased from eBioscience: CD127 (A7R34), CD25 (PC61.5), CD122 (5H4), CD8 (53–6.7), Syrian Hamster, rat IgG1 (eBFG1), rat IgG2b (eB149), and IgG2a (eBRa). Vβ5.1 (MR9-4) was purchased from BD. Anti-STAT5 (pY694; 47/STAT5) was purchased from BD. Anti-Akt (pS473) was obtained from Cell Signaling Technology. Western mAb for Cyclin A (CY-A1; mouse) was purchased from Sigma-Aldrich, FoxM1 (rabbit) and Cyclin B1 (rabbit) were purchased from Cell Signaling Technology, and β-actin (mouse) was purchased from Santa Cruz Biotechnology. S. Varga (University of Iowa, Iowa City, IA) provided PC61.5 mAb used for CD25-blocking experiments. Anti–IL-2 mAb were purified from S4B6 and JES6-1A12 hybridomas were purchased from American Type Culture Collection. IL-12p40 neutralizing antibody was provided by G. Trinchieri (The Wistar Institute, Philadelphia, PA). MHC class I tetramers (K^b) specific for $OVA_{257–264}$ were prepared using published protocols (Altman et al., 1996; Busch et al., 1998). PI3K inhibitors (Wortmannin and Ly249002) were kindly provided by J. Houtman (University of Iowa, Iowa City, IA).

Microarray. At day 7 after immunization, OT-I CD8 T cells were FACS sorted from separate pools of naive DC+CpG or DC alone–immunized mice. OT-I CD8 T cell populations were ∼99% pure and vital. Absolute $CD8^+$ T cell yields ranged from 6×10^5 to 2×10^6 T cells per sorted sample. RNA from three independent pools of purified CD8 T cells from each immunization was extracted using RNEasy kit (QIAGEN), and 5–50 ng of total RNA was used for microarray analysis. RNA quality was assessed using the Agilent Model 2100 Bioanalyzer. RNA for the microarray was processed using the NuGEN WT-Ovation Pico RNA Amplification System along with the NuGEN WT-Ovation Exon Module. Samples were hybridized and loaded onto Affymetrix GeneChip Mouse GENE 1.0 ST arrays. Arrays were scanned with the Affymetrix Model 7G upgraded scanner, and data were collected using the GeneChip Operating Software. Significant regulation scored a false-discovery rate (FDR) < 0.01. GSEA (Subramanian et al., 2005), and DAVID (Huang et al., 2009) were performed as previously described. The microarray data are available in the Gene Expression Omnibus (GEO) database under the accession no. GSE53200.

Adoptive transfer experiments and BrdU incorporation. Thy1.1 and Thy1.2 OT-I CD8 T cells were obtained from the blood or spleens of naive donors, and the number of input OT-I was calculated as previously described (Badovinac et al., 2007). Approximately 600 Thy1.1 or Thy1.2 OT-I CD8 T cells were transferred into each naive Thy1.2 or CD45.1 C57BL/6 recipient mouse, respectively. For early time points (e.g., days 2−4 after immunization), $\sim 5 \times 10^4$ OT-I CD8 T cells were transferred unless otherwise stated. To induce systemic inflammation/signal 3 cytokine production during priming, 200 µg of CpG oligonucleotide 1826 (Integrated DNA Technology) was injected i.p. at time of DC immunization (Pham et al., 2009). All mice given DC immunizations were injected i.v. with $\sim 5 \times 10^5$ matured/peptide-coated DCs at day 0 after immunization. To measure division, BrdU (Sigma-Aldrich) was injected i.p. (2 mg/mouse) for ∼15 h before spleen harvest. Detection of BrdU incorporation was performed according to manufacturer's protocol (BrdU Flow kits; BD).

Quantification and phenotypic analysis of Ag-specific T cells. The magnitude of epitope-specific CD8 T cell response was determined by either tetramer staining as previously described (Badovinac et al., 2002) or by staining for Thy1.1 marker exclusively expressed on transferred TCR-transgenic cells (Badovinac et al., 2007).

Analysis of apoptosis. Detection of active caspase-3/7 was determined using the Vybrant FAM Caspase-3 and -7 Assay kit (Invitrogen) according to manufacturer's protocol. In brief, $\sim 2 \times 10^6$ total splenocytes were incubated for 1 h in 300 µl of RPMI containing 10% (vol/vol) FCS and 1× FLICA reagent at 37°C. Cells were thoroughly washed, and then stained with additional antibodies against T cell surface antigens. Detection of Annexin V^+ cells was determined using the Annexin V Apoptosis Detection kit (BD) according to manufacturer's protocol.

Cell purification, CFSE staining, and in vitro IL-2 stimulation. To purify responding OT-I CD8 T cells and naive OT-I CD8 T cell populations, spleens were harvested from allele-disparate hosts that had received naive OT-I CD8 T cells and DC^+ alone or DC^+ CpG immunization at indicated time points or from naive OT-I mice (Thy1.2/Thy1.2). Single-cell suspensions of total splenocytes from immunized mice were stained with phycoerythrin (PE)-anti-Thy1.1 mAb (OX-7; BD) or naive splenocytes were stained with PE-anti-Thy1.2 mAb (30-H12; BioLegend) in PBS containing 5% (vol/vol) FCS. OT-I CD8 T cells were then purified with anti-PE bead sorting using standard AutoMACS protocols (Miltenyi Biotec). After AutoMACS purification, cells were mixed into 1:1:1 ratio (DC: DC+CpG:Naive) and then labeled with 1 µM CFSE for 15 min and thoroughly washed with RPMI containing 10% (vol/vol) FCS. Then $\sim 2 \times 10^6$ of each OT-I CD8 T cell population were injected i.v. into naive CD45.1 recipients or incubated in vitro in the presence or absence of murine IL-2 (0.5 ng/ml unless otherwise noted). PI3K inhibition or CD25 blockade were initiated by pretreating cells with indicated drugs (1 µM Wortmannin or 10 µM of Ly249002) or PC61.5 mAb (200 µg/ml), respectively, for 30 min at room temperature before murine IL-2 stimulation. Akt-pS473 and STAT5-pY694 activation in purified responding OT-I CD8 T cells was measured directly ex vivo or after murine IL-2 stimulation at indicated time points and murine IL-2 concentrations using manufacturer's protocol (Phosflow; BD). When looking at Akt-pS473 after in vitro IL-2 stimulation, cells were incubated in serum-free media for 1 h before ex vivo stimulation.

Immunoblot analysis. At indicated time points, cells were isolated with AutoMACS purification, and then then immediately lysed in NP-40 lysis buffer (20 mM Hepes, pH 7.9, 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl, 1% NP-40, 1 mM PMSF, and 10 µM MG-132) for 15 min on ice. Whole-cell lysates were clarified by centrifugation at ∼20,000 *g* for 5 min at 4°C. Extracts were then resolved by SDS-PAGE (Bio-Rad Laboratories). Primary antibodies were detected with goat anti–mouse IgG or goat anti–rabbit IgG coupled to HRP (Santa Cruz Biotechnology) and SuperSignal West Pico Chemiluminescence (Thermo Fisher Scientific).

RT-PCR analysis. Approximately 50–100 ng of RNA template from FACS-sorted cells was converted to cDNA and amplified using Script One-Step RT-PCR kit with SYBR Green according to manufacturer's protocol (BIO RAD). The following oligonucleotides were used to analyze expression of indicated transcripts; 5′-CTTGGCTGCACCAACAGTAA-3′ and 5′-ATGACTCAGGCCAGCTCTGT-3′ for *Ccna2*; 5′-TGGACTACGACATGGTGCAT-3′ and 5′-CTTTGTGAGGCCACAGTTCA-3′ for *Ccnb1*; 5′-TGACGCTCGTCGACTATGAC-3′ and 5′-TGCTGCTGGCATACTTGTTC-3′ for *Ccnb2*; 5′-TCTGTGCATTCTAGCCATCG-3′ and 5′-CAAAAGGCACCATCCAGTCT-3′ for *Ccne2*; 5′-TGCATGAAGAAATCCTGCTG-3′ and 5′-CTAAGGAAGCCAGGCAAGTG-3′ for *Foxm1*; 5′-TTACTCTACCCCGACGATGG-3′ and 5′-CCAAGCTTGAAAAGGCTGAG-3′ for *Bcl3*; and 5′-CCTCATGGACTGATTATGGACA-3′ and 5′-TATGTCCCCGTTGACTGAT-3′ for *Hprt*; 5′-AGGAGCAGGTGCCTACAAGA-3′ and 5′-GCATTTTCCCACCACTGTCT-3′ for *Bcl2*; 5′-GCTGGTGGGACCTGTTTCTA-3′ and 5′-TTCAGTGAGCCATCTTGACG-3′ for *Bim* (*Bcl2l11*); 5′-AGGACTTATCAGCCGAAGCA-3′ and 5′-GCTCAAACTCTGGGATCTGG-3′ for *Bad* (*Bbc2*); 5′-CTCTGCGTTCAGCTTGAGTG-3′ and 5′-CAGAAGCCCACCTACATGGT-3′ for *Bid*; and 5′-CCTTCAGGCCTCTCTCTCCT-3′ and 5′-CCAGCAGCTCCTCACACATA-3′ for *Bclxl* (*Bcl2l1*). PCR reaction was performed using ABI PRISM 7700 Sequence System. Expression of transcripts was made relative *Hprt* and to controls groups as indicated.

In vivo cytokine neutralization/stimulation. IL-12 neutralization in DC+CpG-primed mice was done by injecting 500 µg of C17.8 i.p. twice; once at time of immunization and a second 6-h later. IL-2 neutralization during DC alone or DC^+ immunization was accomplished by injecting 250 µg JES6-1A2 daily from day 4–6 after immunization. IL-2/mAb complexes were generated by incubating murine IL-2 (PeproTech) with S4B6 anti–IL-2 mAb at a 2:1 molar ratio (1.5 µg/ml IL-2: 50 µg/ml S4B6) for 15 min at room temperature. 300 ng IL-2–10 µg S4B6 complex were injected into DC alone–immunized mice. For controls, equal amounts of rat IgG (Sigma-Aldrich) were used.

The authors would like to thank members of the Harty lab for helpful discussions and V. Badovinac for critical review of the manuscript.

The authors wish to acknowledge support from the National Institutes of Health (NIH; T32 AI 007511 to G.R. Starbeck-Miller), American Cancer Society (RSG-11-161-01-MPC to H.-H. Xue), NIH (HL095540 and AI080966 to H.-H. Xue H.-H. Xue), and NIH (AI42676, AI85515, AI95178, AI96850, AI100527 to J.T. Harty).

The authors have no conflicting financial interests.

Author contributions: G.R. Starbeck-Miller designed and performed experiments, analyzed data, and wrote the paper. H-HX analyzed data and wrote the paper. J.T. Harty designed experiments, analyzed data, and wrote the paper.

Submitted: 1 May 2013
Accepted: 18 November 2013

REFERENCES

Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke, and S. Vucikuja. 2006. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. *J. Immunol.* 176:4525–4529.

Altman, J.D., P.A. Moss, P.J. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. 274:94–96. http://dx.doi.org/10.1126/science.274.5284.94

Badovinac, V.P., and J.T. Harty. 2006. Programming, demarcating, and manipulating CD8+ T-cell memory. *Immunol. Rev.* 211:67–80. http://dx.doi.org/10.1111/j.0105-2896.2006.00384.x

Badovinac, V.P., B.B. Porter, and J.T. Harty. 2002. Programmed contraction of CD8(+) T cells after infection. *Nat. Immunol.* 3:619–626.

Badovinac, V.P., K.A. Messingham, A. Jabbari, J.S. Haring, and J.T. Harty. 2005. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat. Med.* 11:748–756. http://dx.doi.org/10.1038/nm1257

Badovinac, V.P., J.S. Haring, and J.T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity*. 26:827–841. http://dx.doi.org/10.1016/j.immuni.2007.04.013

Bensinger, S.J., P.T. Walsh, J. Zhang, M. Carroll, R. Parsons, J.C. Rathmell, C.B. Thompson, M.A. Burchill, M.A. Farrar, and L.A. Turka. 2004. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J. Immunol.* 172:5287–5296.

Boyman, O., and J. Sprent. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol.* 12:180–190.

Boyman, O., M. Kovar, M.P. Rubinstein, C.D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science*. 311:1924–1927. http://dx.doi.org/10.1126/science.1122927

Busch, D.H., I.M. Pilip, S. Vijh, and E.G. Pamer. 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity*. 8:353–362. http://dx.doi.org/10.1016/S1074-7613(00)80540-3

Cannarile, M.A., N.A. Lind, R. Rivera, A.D. Sheridan, K.A. Camfield, B.B. Wu, K.P. Cheung, Z. Ding, and A.W. Goldrath. 2006. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat. Immunol.* 7:1317–1325. http://dx.doi.org/10.1038/ni1403

Chua, A.O., V.L. Wilkinson, D.H. Presky, and U. Gubler. 1995. Cloning and characterization of a mouse IL-12 receptor-beta component. *J. Immunol.* 155:4286–4294.

Costa, R.H., V.V. Kalinichenko, M.L. Major, and P. Raychaudhuri. 2005. New and unexpected: forkhead meets ARF. *Curr. Opin. Genet. Dev.* 15:42–48. http://dx.doi.org/10.1016/j.gde.2004.12.007

Cronin, S.J., and J.M. Penninger. 2007. From T-cell activation signals to signaling control of anti-cancer immunity. *Immunol. Rev.* 220:151–168. http://dx.doi.org/10.1111/j.1600-065X.2007.00570.x

Curtsinger, J.M., and M.F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* 22:333–340. http://dx.doi.org/10.1016/j.coi.2010.02.013

Curtsinger, J.M., C.S. Schmidt, A. Mondino, D.C. Lins, R.M. Kedl, M.K. Jenkins, and M.F. Mescher. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* 162:3256–3262.

Curtsinger, J.M., C.M. Johnson, and M.F. Mescher. 2003a. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J. Immunol.* 171:5165–5171.

Curtsinger, J.M., D.C. Lins, and M.F. Mescher. 2003b. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J. Exp. Med.* 197:1141–1151. http://dx.doi.org/10.1084/jem.20021910

Curtsinger, J.M., J.O. Valenzuela, P. Agarwal, D. Lins, and M.F. Mescher. 2005. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J. Immunol.* 174:4465–4469.

Diehl, J.A., M. Cheng, M.F. Roussel, and C.J. Sherr. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* 12:3499–3511. http://dx.doi.org/10.1101/gad.12.22.3499

Feau, S., R. Arens, S. Togher, and S.P. Schoenberger. 2011. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat. Immunol.* 12:908–913. http://dx.doi.org/10.1038/ni.2079

Gately, M.K., A.G. Wolitzky, P.M. Quinn, and R. Chizzonite. 1992. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell. Immunol.* 143:127–142. http://dx.doi.org/10.1016/0008-8749(92)90011-D

Gesbert, F., W.R. Sellers, S. Signoretti, M. Loda, and J.D. Griffin. 2000. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. *J. Biol. Chem.* 275:39223–39230. http://dx.doi.org/10.1074/jbc.M007291200

Gil, M.P., M.J. Ploquin, W.T. Watford, S.H. Lee, K. Kim, X. Wang, Y. Kanno, J.J. O'Shea, and C.A. Biron. 2012. Regulating type 1 IFN effects in CD8 T cells during viral infections: changing STAT4 and STAT1 expression for function. *Blood*. 120:3718–3728. http://dx.doi.org/10.1182/blood-2012-05-428672

Harty, J.T., and V.P. Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. *Nat. Rev. Immunol.* 8:107–119. http://dx.doi.org/10.1038/nri2251

Haring, J.S., V.P. Badovinac, and J.T. Harty. 2006. Inflaming the CD8+ T cell response. *Immunity*. 25:19–29. http://dx.doi.org/10.1016/j.immuni.2006.07.001

Hikono, H., J.E. Kohlmeier, K.H. Ely, I. Scott, A.D. Roberts, M.A. Blackman, and D.L. Woodland. 2006. T-cell memory and recall responses to respiratory virus infections. *Immunol. Rev.* 211:119–132. http://dx.doi.org/10.1111/j.0105-2896.2006.00385.x

Hildeman, D.A., Y. Zhu, T.C. Mitchell, P. Bouillet, A. Strasser, J. Kappler, and P. Marrack. 2002. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity*. 16:759–767. http://dx.doi.org/10.1016/S1074-7613(02)00322-9

Hildeman, D., T. Jorgensen, J. Kappler, and P. Marrack. 2007. Apoptosis and the homeostatic control of immune responses. *Curr. Opin. Immunol.* 19:516–521. http://dx.doi.org/10.1016/j.coi.2007.05.005

Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell*. 76:17–27. http://dx.doi.org/10.1016/0092-8674(94)90169-4

Hou, S., L. Hyland, K.W. Ryan, A. Portner, and P.C. Doherty. 1994. Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature*. 369:652–654. http://dx.doi.org/10.1038/369652a0

Huang, W., B.T. Sherman, and R.A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4:44–57. http://dx.doi.org/10.1038/nprot.2008.211

Intlekofer, A.M., N. Takemoto, E.J. Wherry, S.A. Longworth, J.T. Northrup, V.R. Palanivel, A.C. Mullen, C.R. Gasink, S.M. Kaech, J.D. Miller, et al. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6:1236–1244. http://dx.doi.org/10.1038/ni1268

Joshi, N.S., W. Cui, A. Chandele, H.K. Lee, D.R. Urso, J. Hagman, L. Gapin, and S.M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity*. 27:281–295. http://dx.doi.org/10.1016/j.immuni.2007.07.010

Kalia, V., S. Sarkar, S. Subramaniam, W.N. Haining, K.A. Smith, and R. Ahmed. 2010. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity*. 32:91–103. http://dx.doi.org/10.1016/j.immuni.2009.11.010

Kallies, A., E.D. Hawkins, G.T. Belz, D. Metcalf, M. Hommel, L.M. Corcoran, P.D. Hodgkin, and S.L. Nutt. 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat. Immunol.* 7:466–474. http://dx.doi.org/10.1038/ni1321

Kallies, A., A. Xin, G.T. Belz, and S.L. Nutt. 2009. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity*. 31:283–295. http://dx.doi.org/10.1016/j.immuni.2009.06.021

Keppler, S.J., K. Theil, S. Vucikuja, and P. Aichele. 2009. Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8(+) T cells. *Eur. J. Immunol.* 39:1774–1783. http://dx.doi.org/10.1002/eji.200839093

Keppler, S.J., K. Rosenits, T. Koegl, S. Vucikuja, and P. Aichele. 2012. Signal 3 cytokines as modulators of primary immune responses during infections:

the interplay of type I IFN and IL-12 in CD8 T cell responses. *PLoS ONE*. 7:e40865. http://dx.doi.org/10.1371/journal.pone.0040865

Kolumam, G.A., S. Thomas, L.J. Thompson, J. Sprent, and K. Murali-Krishna. 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J. Exp. Med.* 202:637–650. http://dx.doi.org/10.1084/jem.20050821

Lali, F.V., J. Crawley, D.A. McCulloch, and B.M. Foxwell. 2004. A late, prolonged activation of the phosphatidylinositol 3-kinase pathway is required for T cell proliferation. *J. Immunol.* 172:3527–3534.

Laoukili, J., M.R. Kooistra, A. Brás, J. Kauw, R.M. Kerkhoven, A. Morrison, H. Clevers, and R.H. Medema. 2005. FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat. Cell Biol.* 7:126–136. http://dx.doi.org/10.1038/ncb1217

Lefrançois, L. 2006. Development, trafficking, and function of memory T-cell subsets. *Immunol. Rev.* 211:93–103. http://dx.doi.org/10.1111/j.0105-2896.2006.00393.x

Major, M.L., R. Lepe, and R.H. Costa. 2004. Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. *Mol. Cell. Biol.* 24:2649–2661. http://dx.doi.org/10.1128/MCB.24.7.2649-2661.2004

Malek, T.R. 2008. The biology of interleukin-2. *Annu. Rev. Immunol.* 26:453–479. http://dx.doi.org/10.1146/annurev.immunol.26.021607.090357

Malek, T.R., A. Yu, L. Zhu, T. Matsutani, D. Adeegbe, and A.L. Bayer. 2008. IL-2 family of cytokines in T regulatory cell development and homeostasis. *J. Clin. Immunol.* 28:635–639. http://dx.doi.org/10.1007/s10875-008-9235-y

Masson, F., F. Kupresanin, A. Mount, A. Strasser, and G.T. Belz. 2011. Bid and Bim collaborate during induction of T cell death in persistent infection. *J. Immunol.* 186:4059–4066. http://dx.doi.org/10.4049/jimmunol.1001918

Mescher, M.F., J.M. Curtsinger, P. Agarwal, K.A. Casey, M. Gerner, C.D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by CD8+ T cells. *Immunol. Rev.* 211:81–92. http://dx.doi.org/10.1111/j.0105-2896.2006.00382.x

Mitchell, T.C., D. Hildeman, R.M. Kedl, T.K. Teague, B.C. Schaefer, J. White, Y. Zhu, J. Kappler, and P. Marrack. 2001. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat. Immunol.* 2:397–402.

Obar, J.J., M.J. Molloy, E.R. Jellison, T.A. Stoklasek, W. Zhang, E.J. Usherwood, and L. Lefrançois. 2010. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc. Natl. Acad. Sci. USA*. 107:193–198. http://dx.doi.org/10.1073/pnas.0909945107

Park, H.J., J.R. Carr, Z. Wang, V. Nogueira, N. Hay, A.L. Tyner, L.F. Lau, R.H. Costa, and P. Raychaudhuri. 2009. FoxM1, a critical regulator of oxidative stress during oncogenesis. *EMBO J.* 28:2908–2918. http://dx.doi.org/10.1038/emboj.2009.239

Pham, N.-L., V.P. Badovinac, and J.T. Harty. 2011. Differential role of "Signal 3" inflammatory cytokines in regulating CD8 T cell expansion and differentiation in vivo. *Front Immunol.* 2:4.

Pham, N.L., V.P. Badovinac, and J.T. Harty. 2009. A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation. *J. Immunol.* 183:2337–2348. http://dx.doi.org/10.4049/jimmunol.0901203

Pipkin, M.E., J.A. Sacks, F. Cruz-Guilloty, M.G. Lichtenheld, M.J. Bevan, and A. Rao. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity*. 32:79–90. http://dx.doi.org/10.1016/j.immuni.2009.11.012

Pope, C., S.K. Kim, A. Marzo, D. Masopust, K. Williams, J. Jiang, H. Shen, and L. Lefrançois. 2001. Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. *J. Immunol.* 166:3402–3409.

Schmidt, N.W., R.L. Podyminogin, N.S. Butler, V.P. Badovinac, B.J. Tucker, K.S. Bahjat, P. Lauer, A. Reyes-Sandoval, C.L. Hutchings, A.C. Moore, et al. 2008. Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc. Natl. Acad. Sci. USA*. 105:14017–14022. http://dx.doi.org/10.1073/pnas.0805452105

Subramanian, A., P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, and J.P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA*. 102:15545–15550. http://dx.doi.org/10.1073/pnas.0506580102

Taniguchi, T., and Y. Minami. 1993. The IL-2/IL-2 receptor system: a current overview. *Cell*. 73:5–8. http://dx.doi.org/10.1016/0092-8674(93)90152-G

Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* 70:83–243.

Valenzuela, J., C. Schmidt, and M. Mescher. 2002. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *J. Immunol.* 169:6842–6849.

Valenzuela, J.O., C.D. Hammerbeck, and M.F. Mescher. 2005. Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. *J. Immunol.* 174:600–604.

Veis, D.J., C.M. Sorenson, J.R. Shutter, and S.J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*. 75:229–240. http://dx.doi.org/10.1016/0092-8674(93)80065-M

Vignali, D.A., and V.K. Kuchroo. 2012. IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* 13:722–728. http://dx.doi.org/10.1038/ni.2366

Waldmann, T.A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.* 6:595–601. http://dx.doi.org/10.1038/nri1901

Wang, I.C., Y.J. Chen, D.E. Hughes, T. Ackerson, M.L. Major, V.V. Kalinichenko, R.H. Costa, P. Raychaudhuri, A.L. Tyner, and L.F. Lau. 2008. FoxM1 regulates transcription of JNK1 to promote the G1/S transition and tumor cell invasiveness. *J. Biol. Chem.* 283:20770–20778. http://dx.doi.org/10.1074/jbc.M709892200

Wang, Z., A. Ahmad, Y. Li, S. Banerjee, D. Kong, and F.H. Sarkar. 2010. Forkhead box M1 transcription factor: a novel target for cancer therapy. *Cancer Treat. Rev.* 36:151–156. http://dx.doi.org/10.1016/j.ctrv.2009.11.006

Williams, M.A., A.J. Tyznik, and M.J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature*. 441:890–893. http://dx.doi.org/10.1038/nature04790

Wirth, T.C., H.H. Xue, D. Rai, J.T. Sabel, T. Bair, J.T. Harty, and V.P. Badovinac. 2010. Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. *Immunity*. 33:128–140. http://dx.doi.org/10.1016/j.immuni.2010.06.014

Xiao, Z., K.A. Casey, S.C. Jameson, J.M. Curtsinger, and M.F. Mescher. 2009. Programming for CD8 T cell memory development requires IL-12 or type I IFN. *J. Immunol.* 182:2786–2794. http://dx.doi.org/10.4049/jimmunol.0803484

Xue, H.H., P.E. Kovanen, C.A. Pise-Masison, M. Berg, M.F. Radovich, J.N. Brady, and W.J. Leonard. 2002. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc. Natl. Acad. Sci. USA*. 99:13759–13764. http://dx.doi.org/10.1073/pnas.212214999

Xue, L., L. Chiang, B. He, Y.Y. Zhao, and A. Winoto. 2010. FoxM1, a forkhead transcription factor is a master cell cycle regulator for mouse mature T cells but not double positive thymocytes. *PLoS ONE*. 5:e9229. http://dx.doi.org/10.1371/journal.pone.0009229

Yang, C.Y., J.A. Best, J. Knell, E. Yang, A.D. Sheridan, A.K. Jesionek, H.S. Li, R.R. Rivera, K.C. Lind, L.M. D'Cruz, et al. 2011. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat. Immunol.* 12:1221–1229. http://dx.doi.org/10.1038/ni.2158

Zhou, B.P., Y. Liao, W. Xia, B. Spohn, M.H. Lee, and M.C. Hung. 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell Biol.* 3:245–252. http://dx.doi.org/10.1038/35060032