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LXR Signaling Couples Sterol Metabolism to Proliferation in the Acquired Immune Response

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SUMMARY

Cholesterol is essential for membrane synthesis; however, the mechanisms that link cellular lipid metabolism to proliferation are incompletely understood. We demonstrate here that cellular cholesterol levels in dividing T cells are maintained in part through reciprocal regulation of the LXR and SREBP transcriptional programs. T cell activation triggers induction of the oxysterol-metabolizing enzyme SULT2B1, consequent suppression of the LXR pathway for cholesterol transport, and promotion of the SREBP pathway for cholesterol synthesis. Ligation of LXR during T cell activation inhibits mitogen-driven expansion, whereas loss of LXRβ confers a proliferative advantage. Inactivation of the sterol transporter ABCG1 uncouples LXR signaling from proliferation, directly linking sterol homeostasis to the antiproliferative action of LXR. Mice lacking LXRβ exhibit lymphoid hyperplasia and enhanced responses to antigenic challenge, indicating that proper regulation of LXR-dependent sterol metabolism is important for immune responses. These results implicate LXR signaling in a metabolic checkpoint that modulates cell proliferation and immunity.

INTRODUCTION

The capacity of lymphocytes to undergo rapid expansion in response to antigenic challenge is essential for adaptive immunity. Mounting evidence suggests that metabolism plays a key role in the determination of lymphocyte responses. For example, activated T lymphocytes are functionally glycolytic despite sufficient oxygen tension (Fox et al., 2005). As a consequence of this metabolic program, T cell activation requires high glucose availability (Roos and Loos, 1973). In the absence of sufficient glucose, T cells fail to proliferate, and they undergo apoptosis (Alves et al., 2006; Rathmell et al., 2000; Vander Heiden et al., 2001). The influence of cellular lipid metabolism on immune responses, however, is poorly understood.

Cholesterol is an important component of cell membranes and has long been recognized to be necessary for cell growth and proliferation (Brown and Goldstein, 1974; Chen et al., 1975; Chen et al., 1974). Early studies defined a relationship between the pathway for de novo sterol synthesis and movement through the cell cycle in lymphocytes. Blocking of mevalonate production with certain cholesterol derivatives or HMG-CoA reductase inhibitors (statins) inhibits DNA synthesis and mitosis (Chakrabarti and Engleman, 1991; Chen et al., 1975). A complicating factor in these studies, however, is the requirement for the mevalonate pathway in production of nonsterol mevalonate derivatives such as geranylgeraniol and farnesol. Indeed, much of the immunomodulatory effects of statins have been attributed to the biology of these products (Kwak et al., 2000; Weitz-Schmidt et al., 2001). The question of whether intracellular cholesterol distribution or availability represents a dynamic signal for control of immune responses has not been adequately explored, and the molecular mechanisms that link lipid metabolism to cellular growth and the cell-cycle machinery remain unknown.

The liver X receptors (LXRα and LXRβ) are members of the nuclear receptor superfamily that regulate cholesterol homeostasis. The endogenous activators of these receptors are oxysterols and intermediates in the cholesterol biosynthetic pathway (Janowski et al., 1996). The two LXR isotypes share considerable sequence homology and respond to the same ligands, but their tissue distribution differs markedly. LXRα is highly expressed in liver, adipose tissue, and macrophages, and LXRβ is expressed ubiquitously (Repa and Mangelsdorf, 2000). Many LXR target genes are involved in cholesterol and fatty acid metabolism, such as ABCA1, ABCG1, SREBP-1c, and fatty acid synthase (Tontonoz and Mangelsdorf, 2003). Other targets, such as AIM/SPα, are involved in the regulation of apoptosis and innate immune responses (Joseph et al., 2004; Valledor et al., 2004).

LXRs also have the capacity to negatively regulate inflammatory gene expression via transrepression (Joseph et al., 2003). The LXR pathway has been shown to impact inflammatory responses in models of atherosclerosis, contact dermatitis, sepsis, and multiple sclerosis (Hindinger et al., 2006; Joseph et al., 2003; Joseph et al., 2002; Tangirala et al., 2002). Although the mechanisms underlying signal-specific transrepression remain incompletely understood, it is clear that activation of LXR inhibits inflammatory responses to LPS or cytokines in part via blockade of NF-κB signaling (Joseph et al., 2003; Ogawa et al., 2005). Recent studies have suggested that LXR-dependent repression of inflammatory target genes occurs via an N-CoR- and sumoylation-dependent process (Ghisletti et al., 2007). Interestingly, activation of TLR3 or TLR4 inhibits LXR cholesterol function via the transcription factor IRF-3, suggesting that LXR functions to regulate the crosstalk between inflammatory and metabolic pathways (Castrillo et al., 2003). Despite recent progress in understanding of the links between LXR and innate immunity, little is known regarding the impact of LXR signaling on T and B cell biology and acquired immunity.

We demonstrate here that transcriptional regulation of intracellular cholesterol homeostasis impacts cell proliferation and acquired immune responses. We show that T cell activation is accompanied by the downregulation of LXR target genes involved in cholesterol transport and the simultaneous induction of the SREBP-2 pathway for cholesterol synthesis. Ligand activation of LXR inhibits mitogen-driven T cell expansion by altering cellular sterol content through a pathway requiring the transporter ABCG1. Conversely, loss of LXR expression confers a proliferative advantage to lymphocytes, resulting in enhanced homeostatic and antigen-driven responses. LXR-dependent coupling of cholesterol metabolism and proliferation represents a previously unrecognized mechanism for the regulation of immune responses.

RESULTS

Age-Dependent Lymphoid Hyperplasia in Mice Lacking LXRβ Expression

To investigate the role of LXRs in lymphoid cells, we examined the immune system of young *Lxrα* and *Lxrβ* null mice (Peet et al., 1998) on a C57BL/6 background (greater than ten generations backcrossed). Flow cytometric analysis of spleen, lymph node (LN), and thymus from 6- to 8-week-old *Lxrα* and *Lxrβ* null mice revealed no significant differences in the frequency of $CD4^+$ and $CD8^+$ T and $CD19^+B220^+$ B lymphocyte populations when compared to LXR-sufficient controls (Figure S1A available online and data not shown). Thus, global loss of either LXR isotype does not grossly alter the development of T or B lymphocytes. However, analysis of hematopoetic tissues from older *Lxrβ* null mice (5–6 months) revealed moderate splenomegaly and an expansion in the total number of lymphocytes in spleen ($p = 0.01$) and LN ($p = 0.01$) (Figures 1A and 1B). No significant difference in the frequency of T and B cells was noted in the spleen, whereas LNs from *Lxrβ* null mice consistently showed a modest expansion in the B cell compartment (Figure 1C).

Since $Foxp3^+$ Regulatory T cells (Tregs) play an important role in maintaining lymphocyte homeostasis, we considered the possibility that the lymphoid hyperplasia might reflect a loss of Tregs. However, FACS analysis of *Lxrβ* null spleen and LN cells revealed no difference in the frequency of $CD4^+Foxp3^+$ T cells (Figure S1B), nor were differences noted in the activation markers CD69, CD25, and CD44 (Figure S1C; data not shown). Similarly, no differences were observed in the frequency of dendritic cells (DCs) from spleens of *Lxrβ* null mice or in MHC class II, CD86, and CD40 expression levels. These observations suggest that antigen-presenting cells are not activated and are not directly mediating the lymphoid hyperplasia (Figure S2).

Next, we examined the expression pattern of *Lxrα* and *Lxrβ* in murine immune cells. *Lxrα* was expressed at high levels in peritoneal-derived macrophages and bone-marrow-derived macrophages, whereas little or no mRNA was detected in resting purified B and T cells (Figure S3A). *Lxrβ* was expressed in macrophages, T cells, and B cells. We considered the possibility that activation of lymphocytes might alter *Lxrα* expression, but we failed to detect expression in splenic T cells from C57BL/6 mice activated with plate-bound anti-CD3ε (pbCD3) mAb in the presence or absence of 2 μM GW3965 (synthetic LXR agonist; Figure S3B). Human T cells were also found to express LXRβ but not LXRα protein (Figure S3C). As expected, expression of LXRα in THP-1 macrophages was induced by GW3965 as a result of autoregulation (Laffitte et al., 2001) (Figure S3C). Activation of LXR induced target gene expression in human T cells but did not lead to the upregulation of *LXRα* mRNA (Figure S3D; data not shown).

LXRβ Is an Intrinsic Regulator of Lymphocyte Proliferation

We hypothesized that LXR may regulate proliferation and/or acquisition of effector functions in lymphocytes. To test this possibility, we examined ^{3}H-thymidine incorporation into whole spleen cells from WT, *Lxrα* null, or *Lxrβ* null mice stimulated with a panel of mitogenic stimuli. Interestingly, anti-IgM-$(Fab')_2$-, concavalin A- and PMA/ionomycin-stimulated spleen cells from *Lxrβ* null mice incorporated significantly more thymidine than did those from WT controls (Figure 1D; data not shown). So that it could be determined whether this effect was due to an intrinsic difference in cell proliferation, purified CFSE-labeled T cells were stimulated with pbCD3 and analyzed by flow cytometry for proliferative response and viability. No difference was observed in cell viability or the dilution of CFSE-labeled T cells at 24 hr, indicating that *Lxrβ* null T cells do not have an inherent early survival advantage (Figure 1E). However, analysis at 72 hr and 96 hr revealed that the absolute number of T cells that had divided in response to TCR stimulus was markedly greater in *Lxrβ* null T cells than in *Lxrα* null and WT cells (Figure 1F). Thus, LXRβ expression intrinsically regulates lymphocyte expansion in response to either TCR, BCR, or pharmacologic activation.

Ligand Activation of LXR Inhibits Lymphocyte Expansion

In complimentary studies, we asked whether LXR activation altered the proliferative capacity of lymphocytes in vitro. Spleen cultures from C57BL/6 mice were stimulated with anti-IgM-$(Fab')_2$ or pbCD3 in the presence or absence of GW3965, LG68 (RXR agonist), or a physiologic LXR ligand, 22(R)-hydroxycholesterol. After 48–96 hr, cultures were pulsed with ^{3}H-thymidine

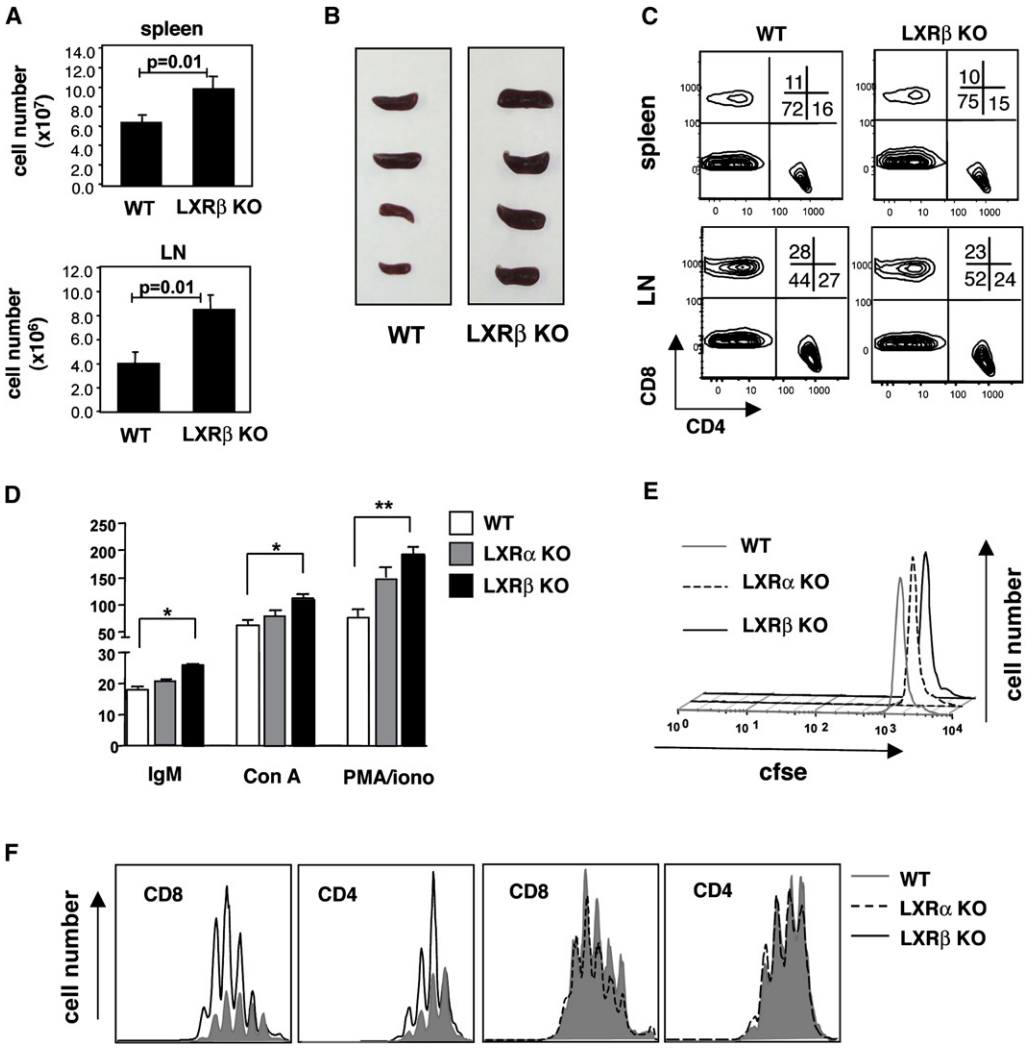

Figure 1. Splenomegaly and Lymphocyte Expansion in Mice Lacking LXRβ

(A) Total cellularity of spleen and LN from 6-month-old WT and LXRβ KO mice.

(B) Gross morphology of spleens.

(C) Frequency of CD4 and CD8 T cells from spleen and LN.

(D) Mitogen-driven proliferation of spleen cells from 6- to 8-week-old WT, LXRα KO, or LXRβ KO mice. Cells were stimulated with anti-IgM(Fab')$_2$ (10 μg/mL), Con A (10 μg/mL), or PMA (0.5 μM) and ionomycin (100 mM). ^{3}H-thymidine was added to cultures after 72 hr for the final 16 hr. *$p < 0.01$; **$p < 0.001$.

(E and F) TCR-driven proliferation of spleen cells from WT, LXRα KO, or LXRβ KO mice. Purified CFSE-labeled T cells were stimulated with pbCD3 (10 μg/mL). Cells were harvested at 24 hr (E) and 72 hr (F), stained with anti-CD4, CD8, and 7-AAD. Counting beads (5×10^4) were added to samples to serve as an internal control. Error bars represent ± SEM.

overnight so that proliferation could be determined. Activation of LXR by LXR and/or RXR agonists markedly reduced the proliferative capacity of cultured cells (Figure 2A). Similarly, proliferation of B and T cells was inhibited by 22(R)-hydroxycholesterol in a dose-dependent manner.

To determine whether the inhibitory effect of LXR agonists on lymphocyte proliferation was receptor dependent, cultures were stimulated with a panel of T and B cell mitogens in the presence or absence of receptor agonists. Activation of LXR alone or in combination with RXR markedly reduced ^{3}H-thymidine incorporation in WT spleen cell cultures stimulated with Con A, anti-IgM, or LPS (Figure 2B and Figure S4). A similar reduction in ^{3}H-thymidine incorporation was seen in spleen cells from *Lxrα* null mice. In contrast, LXR activation had little or no effect on ^{3}H-thymidine incorporation into mitogen-stimulated *Lxrβ* null splenocytes (Figure 2B and Figure S4). A modest decrease in ^{3}H-thymidine incorporation was observed in LG268-treated splenocytes stimulated with LPS and anti-IgM, indicative of an RXR-dependent, LXR-independent effect in B cells (Figure 2B and Figure S4). However, this decrease was not observed in Con A-treated spleen cells, suggesting that RXR activation alone does not perturb T cell expansion in this system (Figure 2B).

We considered the possibility that activation of LXR in whole spleen cultures might indirectly impact lymphocyte expansion

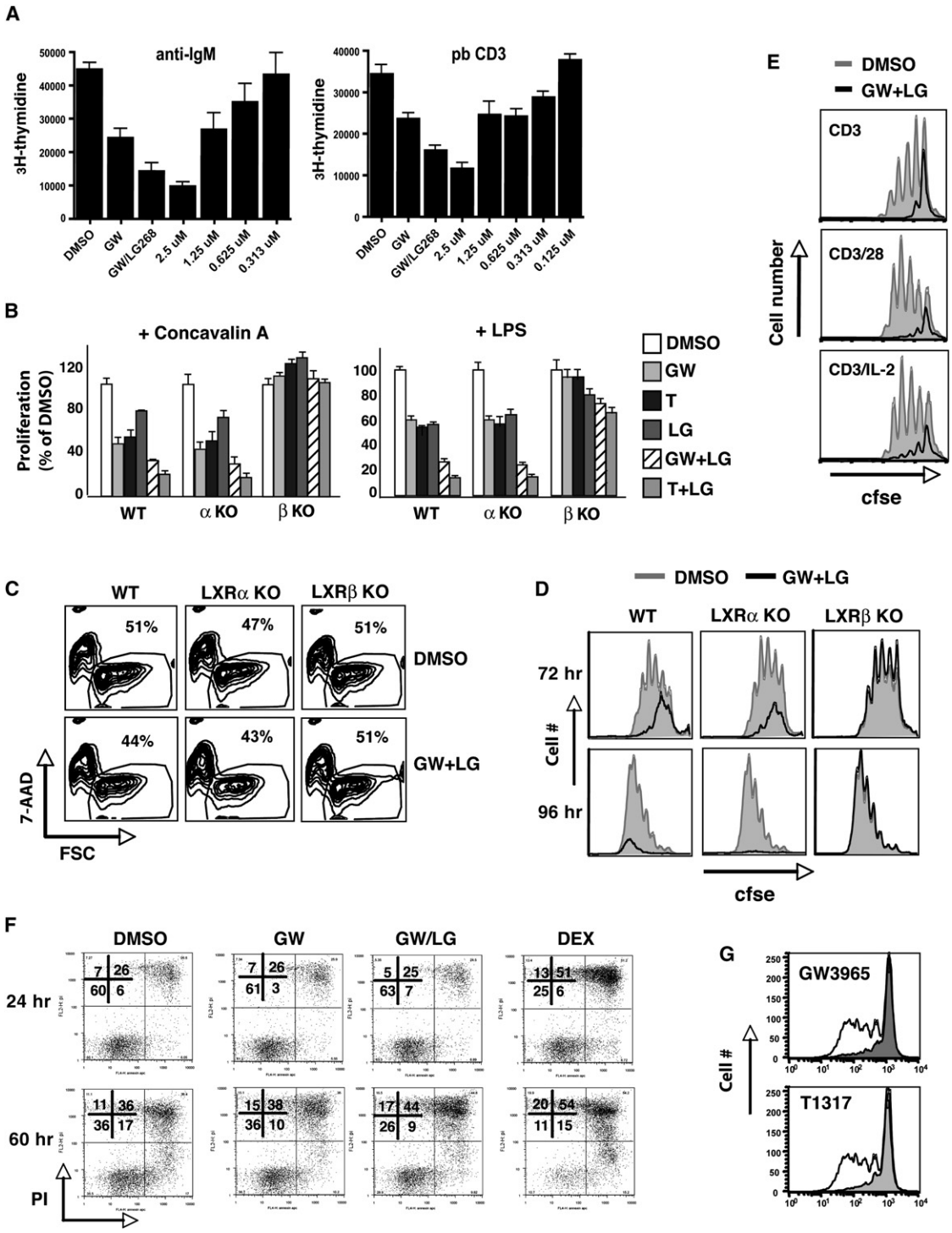

Figure 2. LXR Activation Inhibits Lymphocyte Proliferation

(A) Decreased mitogen-driven proliferation of WT spleen cells stimulated with anti-IgM(Fab')$_2$ (10 μg/mL) or pbCD3 (10 μg/mL) and LXR ligands GW3965 (2 μM), 22(R)-hydroxycholesterol (0.156 μM-2.5 μM), or RXR ligand LG268 (100 nM) as indicated. ^{3}H-thymidine was added to cultures after 72 hr for the final 16 hr.

(B) WT, LXRα KO, or LXRβ KO splenocytes were stimulated with ConA (10 μg/mL) or LPS (100 μg/mL) and treated with LXR ligands GW3965 (2 μM), T1317 (1 μM), and RXR ligand LG268 (100 nM) as indicated. ^{3}H-thymidine was added to cultures after 72 hr for the final 16 hr.

(C and D) CFSE dilution and viability of purified WT, LXRα KO, or LXRβ KO T cells stimulated with pbCD3 in the presence of GW3965 and LG268 was determined at 24–96 hr.

through actions on other cell types. So that this issue could be addressed, purified T cells were CFSE labeled and stimulated with pbCD3 in the presence of either receptor agonists or vehicle. Importantly, there was no significant difference in the viability of T cells cultured with LXR/RXR ligands at 24 hr, indicating that they are not inherently toxic to lymphocytes at these concentrations (Figure 2C). FACS analysis of cultures at 48–96 hr revealed that LXR activation inhibited proliferation of WT and *Lxrα* null T cells, but not *Lxrβ* null cells (Figure 2D). Thus, LXR activation has an intrinsic effect on lymphocyte proliferation that is mediated by LXRβ. We have also observed LXR-dependent inhibition of proliferation in developing bone-marrow-derived monocytes cultured with M-CSF in vitro (data not shown). Interestingly, we failed to observe an effect of LXR ligands on the proliferation of a number of transformed T and B cell lines (including Jurkat and Ramos; data not shown). This observation suggests that many transformed cells have lost their sensitivity to the LXR antiproliferative effect.

Efficient T cell activation requires two signals. The first is mediated via the TCR, whereas the second can be transmitted from a number of cell-surface receptors (Bromley et al., 2001). To determine whether costimulation could restore proliferation in the presence of ligand, we activated purified WT T cells with pbCD3 and added either soluble anti-CD28 or rIL-2. As expected, LXR activation markedly reduced proliferation in response to pbCD3 (Figure 2E); however, the addition of anti-CD28 or IL-2 to cultures did not rescue.

Next, we investigated whether the effects of LXR on lymphocyte expansion were also shared with other members of the nuclear receptor superfamily. In addition to LXR, both PPARγ and GR are known to inhibit inflammatory responses in immune cells (Ogawa et al., 2005). Treatment of T cells with dexamethasone caused cell death in 75%–90% of cells after 24 hr, whereas activation of LXR, RXR, or PPARγ had little effect (Figure 2F and Figure S5C). Interestingly, in contrast to LXR activation, PPARγ signaling conferred a modest survival advantage to proliferating T cells in vitro at later time points (data not shown). Thus, LXRβ activation regulates lymphocyte expansion in a manner distinct from glucocorticoid receptor and PPARγ.

For the determination of whether the transcriptional program initiated by LXR also regulates human lymphocytes, purified T cells were collected from peripheral blood of normal donors. Purified T cells were treated with vehicle or receptor ligands and stimulated with pbCD3 +/− soluble anti-CD28. In contrast to what is seen in murine lymphocytes, LXR/RXR activation did not induce apoptosis in cultures analyzed up to 72 hr (Figure S5D). Similar to murine lymphocytes, LXR activation also decreased the proliferative capacity of human $CD8^+$ or $CD4^+$ T cells stimulated with pbCD3 (Figure 2G and Figure S5A). In contrast to murine T cells, the addition of anti-CD28 was able to overcome the LXR-mediated inhibition of proliferation (Figure S5B), suggesting subtle differences in the biology of LXR in mouse and human lymphocytes.

LXR Activation Blocks Cell-Cycle Progression in Lymphocytes

One possible explanation for the antiproliferative effects of LXR is that the receptor is engaging an apoptotic pathway that decreases the total number of lymphocytes proliferating. Alternatively, it could be that LXR-dependent transcription is regulating cell-cycle progression. Analysis of forward scatter (FSC) and side scatter (SSC) characteristics of cultured T cells demonstrated that neither the activation nor ablation of LXR altered cellular enlargement in response to pbCD3 (Figure 3A; data not shown). However, examination of T cell blasts at 36–48 hr clearly established that LXR-activated cells did not divide efficiently (Figure 3B). Transgenic overexpression of the antiapoptotic factor Bcl-xL or addition of the caspase inhibitor ZVAD-fmk provided a modest survival advantage over their wild-type counterparts but did not restore proliferation in the presence of LXR ligand (Figure 3C; data not shown), consistent with a primary effect of LXR on proliferation rather than cell viability.

Next, we analyzed the DNA content of activated T cells in the presence or absence of LXR ligand. Interestingly, *Lxrβ* null T cells displayed an increased fraction of cells in S and G2/M phase and a decreased sub2N fraction at 36–48 hr compared to WT cells (Figure 3D and Figure S6). WT but not *Lxrβ* null cells treated with LXR agonist showed a markedly reduced fraction of cells moving through the cell cycle and an increase in the apoptotic sub2N fraction (Figure 3D and Figure S6). Analysis of DNA content in pbCD3-stimulated human T cell cultures also indicated a significant reduction in cells entering S phase at 24 hr with no difference in the sub2N fraction (data not shown). Thus, activation of LXRβ limits the capacity of T cells to move through the cell cycle, resulting in decreased proliferation.

We also analyzed the expression of cell-cycle proteins. qPCR analysis of GW3965-treated lymphocytes showed normal upregulation of cylin D2, D3, and c-Myc, suggesting movement into G1 (data not shown). Similarly, we observed normal upregulation of proliferation-associated antigen Ki-67 and CDK 4 (Figures 3E and 3F). However, ligand-treated cells maintained increased levels of the cell-cycle inhibitor p27kip, indicating a decreased ability to move through cell cycle. Accordingly, viable ligand-treated cells showed decreased PCNA expression at 48 hr (Figure 3E). We also observed decreased mRNA expression of cyclin E1 and E2 (data not shown), further supporting our assertion that enforced LXR activation decreases cell-cycle progression in activated lymphocytes.

LXR Signaling Does Not Inhibit Lymphocyte Activation

The NF-κB signaling pathway plays an important role in lymphocyte activation (Schulze-Luehrmann and Ghosh, 2006). Since LXR is known to block NF-κB-dependent gene expression, we

(E) CFSE dilution of purified WT T cells at 72 hr stimulated with pbCD3, soluble CD28, rIL-2, and/or LXR/RXR agonist as indicated.
(F) Annexin and PI staining of pbCD3 stimulated WT T cells cultured with GW3965 and LG268.
(G) CFSE dilution of human T cells stimulated with anti-CD3 crosslinked with pb goat anti-mouse and cultured with GW3965 or T1317. Counting beads (5×10^4) were added to samples (C, D, E, and G) to serve as an internal counting control and analyzed via flow cytometry.
Error bars represent ± SEM.

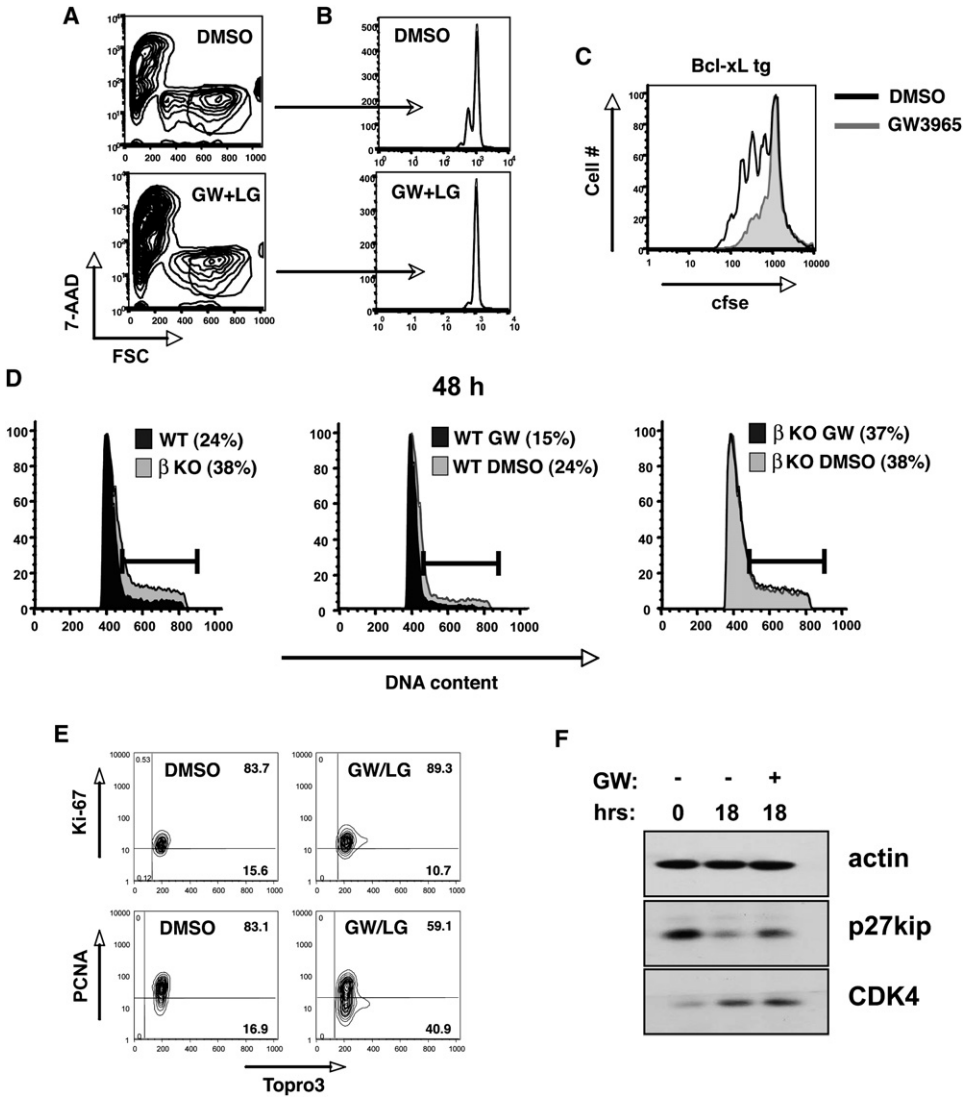

Figure 3. LXRβ Signaling Regulates Cell-Cycle Progression

(A–C) CFSE dilution of WT and Bcl-xL tg T cells stimulated with pbCD3 (10 μg/mL) for 36–96 hr in the presence of GW3965 (2 μM) and LG268 (100 nM). Cells were stained with 7-AAD and analyzed by flow cytometry.

(D) Cell-cycle analysis of WT and LXRβ KO T cells stimulated with pbCD3 and GW3965 and LG268 as indicated. Cells were stained for DNA content with propidium iodide at 48 hr and analyzed by flow cytometry.

(E and F) Cell-cycle proteins of WT T cells stimulated with pbCD3, GW3965, and LG268 as indicated. (E). Cells were permeabilized and stained for intracellular proliferation antigens Ki-67, PCNA and topro-3 for DNA content at 36 hr. Whole-cell lysates were collected and analyzed by western blot for p27kip and CDK4 expression at 18 hr (F).

considered the possibility that the anti-inflammatory action of LXR might underlie its effects on lymphocyte expansion. However, LXR activation did not significantly impact the upregulation of the activation markers CD69 and CD25 at 24 hr (Figure S7A; data not shown). LXR/RXR activation also did not perturb IL-2 production (Figure S7B). Furthermore, restimulation of resting T cells with IL-2 led to comparable induction of the IL-2-responsive genes lymphotoxin A and granzyme B (Figure S7C) (Beadling and Smith, 2002; Kovanen et al., 2005). Coupled with the observations that *Lxrβ* null T cells do not express higher levels of CD25, CD44, or CD69 ex vivo (Figure S1D and data not shown) and do not upregulate these markers to a greater extent in response to TCR crosslinking (Figure S7D), these data strongly suggest that the regulation of proliferation by LXR is not achieved through alterations of TCR or IL-2 signaling or inhibition of proximal lymphocyte activation.

We also addressed when LXR activation must occur in relation to TCR signals in order to block proliferation. To that end, CFSE-labeled T cells were activated with pbCD3 for 24 hr before LXR/RXR ligands were added to cultures. Interestingly, activation of LXR after 24 hr of TCR signaling perturbed expansion only in later rounds of division (Figure S8). If LXR ligands were washed out

2 hr after stimulation, then proliferation was restored (Figure S8), indicating that sustained LXR signaling is required to inhibit proliferation. These observations suggested that the effects of LXR were likely to be mediated by activation or repression of downstream target genes (see below).

Reciprocal Regulation of the SREBP-2 and LXR Transcriptional Programs during Lymphocyte Activation

To determine the mechanism by which LXR modulates proliferation, we profiled gene expression in splenic murine T lymphocytes before or after stimulation with pbCD3 in the presence or absence of LXR agonists. In particular, we examined genes involved in de novo cholesterol biosynthesis, reverse cholesterol transport, and cholesterol uptake. Consistent with previous reports showing that cholesterol synthesis is stimulated in activated T cells (Chen et al., 1975), our findings indicate that the entire pathway of SREBP-2 target genes was induced after activation (Figure 4A). Pathways for fatty acid and phospholipid synthesis were also induced, consistent with activation of SREBP-1 (data not shown). Unexpectedly, TCR activation also resulted in the concomitant and profound downregulation of LXR target genes, including two key genes involved in cholesterol transport, *Abca1* and *Abcg1* (Figure 4A). In contrast, culturing of activated lymphocytes with LXR led to a robust induction of these genes. The transcriptional effects of LXR were notably more limited in T cells as compared to other cell types, such as macrophages. A relatively small number of genes were altered by LXR activation, and the vast majority were linked to cholesterol metabolism. In fact, many LXR target genes expressed in other cell types (e.g., apoE, PLTP, apoD, ABCG5, ABCG8) are absent in T cells (data not shown). We also noted that culturing activated lymphocytes with LXR agonist modestly promoted SREBP-2 target gene expression (Figure 4A). This effect may be secondary to increased SREBP-1 expression driven by LXR or may reflect alterations in cholesterol content in the endoplasmic reticulum (ER) (see below).

Analysis of LXR and SREBP-1 target gene expression by real-time PCR confirmed that T cell activation is accompanied by the rapid and reciprocal regulation of these transcriptional pathways. For example, *Abca1* and *Abcg1* expression begins to decline, whereas *Ldlr* and *Hmcgr* expression begins to increase as early as 2 hr after activation (Figure 4B). The regulation of *Abca1* and *Abcg1* during T cell activation is LXR dependent, because deletion of LXRβ in T cells abolished both basal and ligand-inducible target gene expression (Figure 4C). To determine whether LXR target genes were also downregulated during lymphocyte activation in vivo, C57Bl/6 mice were injected intraperitoneally (i.p.) with anti-CD3ε antibody. Spleens were harvested 12 hr after injection, and mRNA was collected from purified T cells. In line with our in vitro observations, the LXR target genes *Abca1*, *Abcg1*, and *Srebp-1c* were markedly suppressed upon activation, whereas *Lxrβ* expression was maintained (Figure 4D).

Reciprocal regulation of the LXR and SREBP pathways by proliferative stimuli was also observed in primary murine B cells activated via the BCR and with PMA/ionomycin (Figure 4E; data not shown). A similar reciprocal relationship between LXR and SREBP target genes was observed in human lymphocytes activated with PMA/ionomycin (Figure S9). We also examined C57BL/6 mouse embryonic fibroblasts (MEFs) under basal conditions, serum starvation (G1 arrest), and refeeding (re-entry in to cell cycle). Real-time PCR confirmed that proliferative stimuli trigger suppression of LXR targets and induction of SREBP-2 targets in MEFs. Interestingly, serum deprivation for 24 hr upregulated LXR target gene expression in MEFs (Figure 4F). Release from cell-cycle arrest via refeeding for 6 hr with serum upregulated the SREBP-2 target genes *Ldlr* and *17β-Hsd* and downregulated expression of LXR target genes. These data mirror the gene expression patterns observed in quiescent and activated lymphocytes, suggesting that the reciprocal regulation of LXR and SREBP-2 activity is a general feature of cells moving through early stages of the cell cycle.

Proliferative Stimuli Induce SULT2B1 Expression and Reduce LXR Ligand Availability

The loss of LXR target gene expression despite preserved expression of the receptor strongly suggests that the abundance of endogenous LXR ligand is reduced during T cell activation. Consistent with this hypothesis, culturing primary T cells in the presence of the HMG-CoA reductase inhibitor simvastatin, previously shown to block production of LXR agonists, led to a reduction in *Abca1* and *Abcg1* expression (Figure 5A). Moreover, the addition of mevalonic acid restored their expression, indicating that oxysterol production from the mevalonate pathway is required for the maintenance of LXR activity in quiescent lymphocytes. Finally, target gene expression was also restored by the direct addition of the endogenous LXR agonist 22(R)-HC (data not shown).

To investigate the potential mechanism for regulation of LXR ligand availability during T cell activation, we returned to our transcriptional profiling analysis. Remarkably, expression of the oxysterol-metabolizing enzyme SULT2B1 was rapidly induced in response to T cell activation. SULT2B1 catalyzes the transfer of sulfate groups to oxysterols, inactivating them as LXR ligands and facilitating their export from the cell by ABCC1 and other membrane transporters (Chen et al., 2007; Javitt et al., 2001; Zelcer et al., 2003). Real-time PCR revealed that both SULT2B1 and ABCC1 were rapidly induced by proliferative stimuli (pbCD3 or PMA/ionomycin) in T cells (Figure 5B; data not shown). Importantly, a pronounced upregulation of SULT2B1 was also observed in T cells activated in vivo (Figure 5C). This observation strongly suggests that LXR target gene expression is suppressed during T cell activation through active metabolism of endogenous oxysterol LXR agonists. In support of this hypothesis, adenoviral expression of SULT2B1 in resting primary T cells from adenoviral receptor-expressing transgenic mice (Wan et al., 2000) recapitulated the effects of proliferative stimuli on both LXR and SREBP-2 target gene expression (Figure 5D).

LXR Inhibits Proliferation through ABCG1-Dependent Alteration of Cholesterol Homeostasis

The observation that T cell activation is associated with a reduction in endogenous LXR activators, coupled with the prominent regulation of LXR-dependent sterol transporters, suggested LXR may modulate T cell proliferation through control of cholesterol metabolism. We ruled out the possibility that limiting extracellular cholesterol availability was involved in the effects

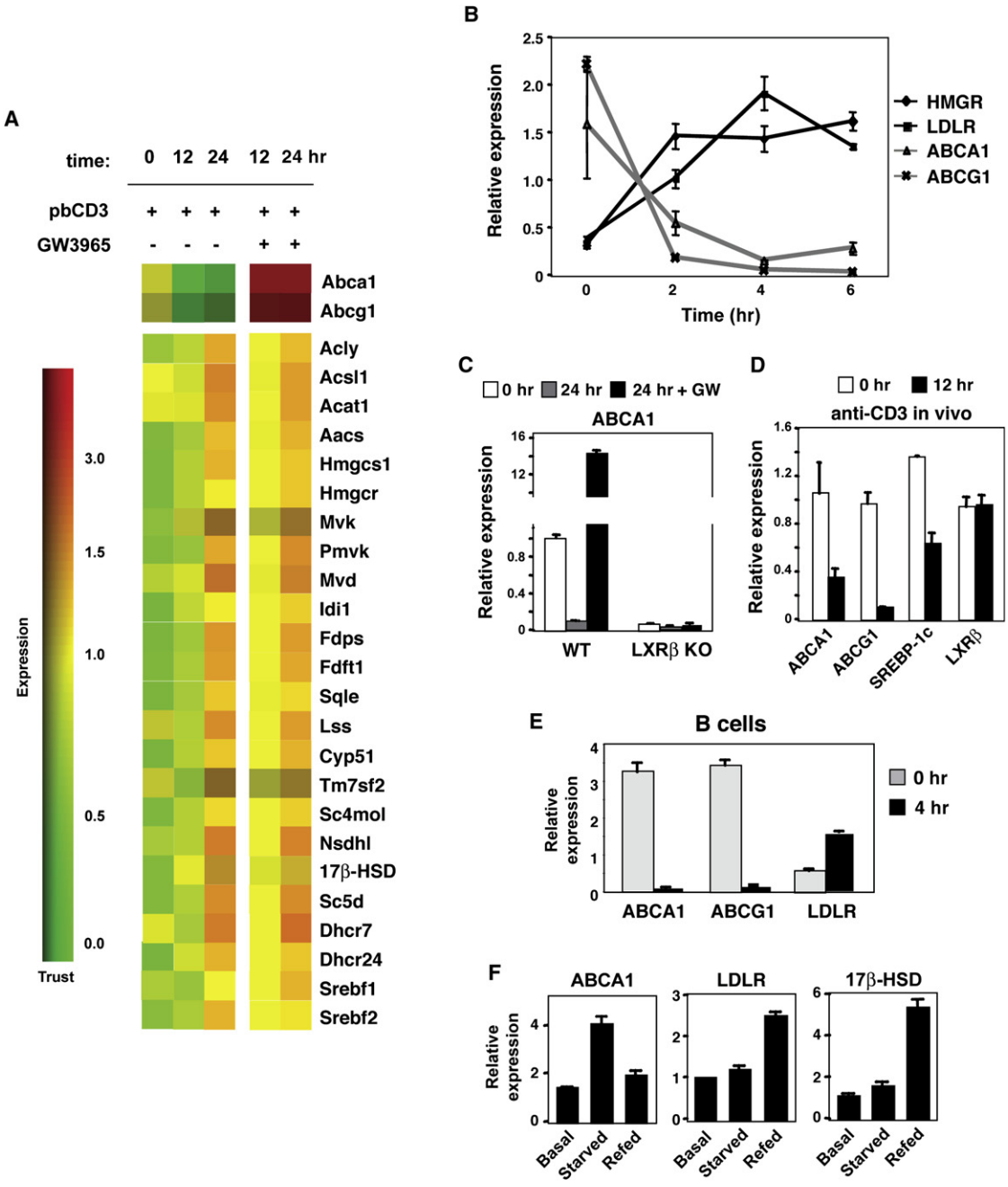

Figure 4. Reciprocal Regulation of the LXR and SREBP-2 Transcriptional Programs during Lymphocyte Activation

(A) DNA microarray analysis of SREBP-2 and LXR target genes of purified WT T cells stimulated with pbCD3 and GW3965 (2 μM).

(B) Real-time PCR analysis of mRNA from purified WT T cells stimulated with pbCD3 (10 μg/mL) for the indicated time.

(C) Real-time PCR analysis of mRNA from purified WT and LXRβ KO T cells stimulated for 24 hr with pbCD3 and GW3965 as indicated.

(D) Real-time PCR analysis of LXR and LXR target genes from mRNA of purified WT T cells of mice treated with 20 μg anti-CD3e i.p. for 12 hr.

(E) Real-time PCR analysis of mRNA from purified WT B cells stimulated for 4 hr with anti-IgM and GW3965 as indicated.

(F) Real-time PCR analysis of mRNA from proliferating (basal), serum-starved (starved), and refed WT mouse embryonic fibroblasts.

Error bars represent ± SEM.

of LXR agonist on T cell proliferation by supplementing cultures with excess LDL. Addition of as much as 100 μg protein/ml LDL had no effect on cell proliferation in the presence or absence of LXR agonist (Figure 6A; data not shown). We next addressed the possibility that LXR-dependent alteration of intracellular cholesterol metabolism was linked to proliferation. Assessment of global membrane cholesterol content by fillipin staining showed increased total cholesterol content in blasting T cells but no

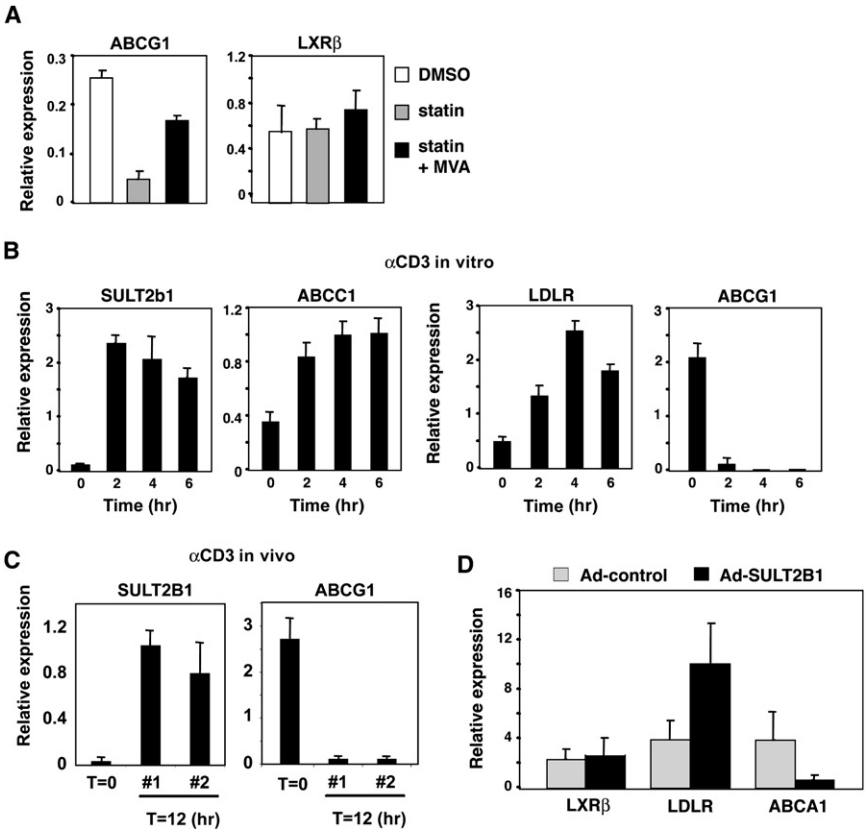

Figure 5. Regulation of LXR Signaling by SULT2b1 during T Cell Activation

(A) Real-time PCR analysis of LXRβ and ABCG1 from quiescent WT T cells cultured with simvastatin and 5 mM mevalonic acid.

(B) Real-time PCR analysis of SULT2b1, ABCC1, LDLR, and ABCG1 mRNA from purified WT T cells stimulated with anti-CD3 in vitro for the indicated times.

(C) Real-time PCR analysis of SULT2b1and ABCG1 mRNA from purified WT T cells stimulated for 12 hr with anti-CD3 in vivo.

(D) Real-time PCR analysis of LXRβ, LDLR, and ABCA1 mRNA from CAR-tg T cells transduced with SULT2b1-containing or control adenovirus. Error bars represent ± SEM.

gross difference between vehicle and LXR agonist treatment (Figure 6B). However, intracellular cholesterol trafficking is complex, and intracellular pools are not necessarily in equilibrium with the plasma membrane (Chang et al., 2006; Simons and Ikonen, 2000). Therefore, this crude analysis does not rule out more subtle or compartment-specific changes in sterol levels or changes in the abundance of rare sterol species [such as oxysterols; (Yancey et al., 2003)].

Previous work has established that ABCG1 and ABCA1 play central roles in both LXR-dependent sterol efflux and intracellular sterol trafficking. We therefore tested the requirement for these transporters in the antiproliferative effects of LXR agonists. Purified WT and *Abcg1* null lymphocytes were activated with PMA/ionomycin in the presence or absence of GW3965 and/or LG268. After 48–96 hr, CFSE proliferation profiles were assessed by flow cytometry. As expected, LXR agonist reduced the proliferative capacity of WT T cells (Figure 6C and Figure S11). Remarkably, however, the effects of LXR activation were greatly attenuated in cells lacking ABCG1. We also analyzed effects of LXR agonists on ABCA1 null and background-matched (B6/129) *ABCA1*$^{+/+}$ controls. Unfortunately, the ABCA1 null mutation is lethal in the C57BL/6 background, precluding a direct comparison of ABCG1 and ABCA1 on the same background. Nevertheless, loss of ABCA1 expression did not alter the response to LXR agonist (Figure 6D), suggesting that this transporter is not required for the LXR effect.

The above observations indicate that transport of sterols by ABCG1 is required for LXR agonists to inhibit cell proliferation. In strong support of this mechanism, the inhibitory effect of LXR activation could be completely overcome by providing lymphocytes with an excess of mevalonate (5 mM), the precursor for cholesterol and oxysterols (Figure 6E). Importantly, providing cells with levels of mevalonate sufficient to allow nonsteroidal modifications, such as protein prenylation, but insufficient to drive sterol synthesis (100 μM) had no effect on LXR-dependent inhibition. These data identify ABCG1 as a key component of a sterol trafficking pathway that must be downregulated during T cell activation. Enforced expression of ABCG1 during T cell activation by exogenous LXR agonists engages a metabolic checkpoint that blocks proliferation through alteration of intracellular sterol metabolism.

Endogenous LXR Signaling Regulates Acquired Immune Responses

To definitively determine whether the intrinsic loss of LXRβ signaling in T cells would translate into a proliferative advantage in vivo, we used a competitive adoptive transfer model of homeostatic proliferation. For these studies, 1 × 10^6 *Lxr*β null (Thy1.2$^+$) T cells and an equivalent number of congenic WT (Thy1.1$^+$) T cells were coadoptively transferred into the same B6.RAG null host and allowed to undergo homeostatic proliferation for 1 week. Strikingly, FACS analysis of spleen and blood from host animals revealed that the frequency of *Lxr*β null (Thy1.1$^-$, CD3$^+$) T cells was much higher ($p = 0.003$) than that of WT (Thy1.1$^+$, CD3$^+$) T cells isolated from the same host (Figure 7A).

In complimentary studies, we asked whether the activation of LXR would prevent the expansion of WT T cells in vivo. To that end, we pretreated purified WT Thy1.2$^+$ T cells and WT Thy1.1$^+$ T cells with GW3965 or vehicle, respectively, for 18 hr in vitro to allow for initiation of the LXR transcriptional program. Live cells were collected, and 1 × 10^6 vehicle- (Thy1.1$^+$) and GW3965- (Thy1.2$^+$) treated cells were injected into B6.RAG null mice. Peripheral blood and spleen were subsequently analyzed for Thy1.1$^+$ and Thy1.2$^+$CD3$^+$ T cells on days 5–7. Consistent

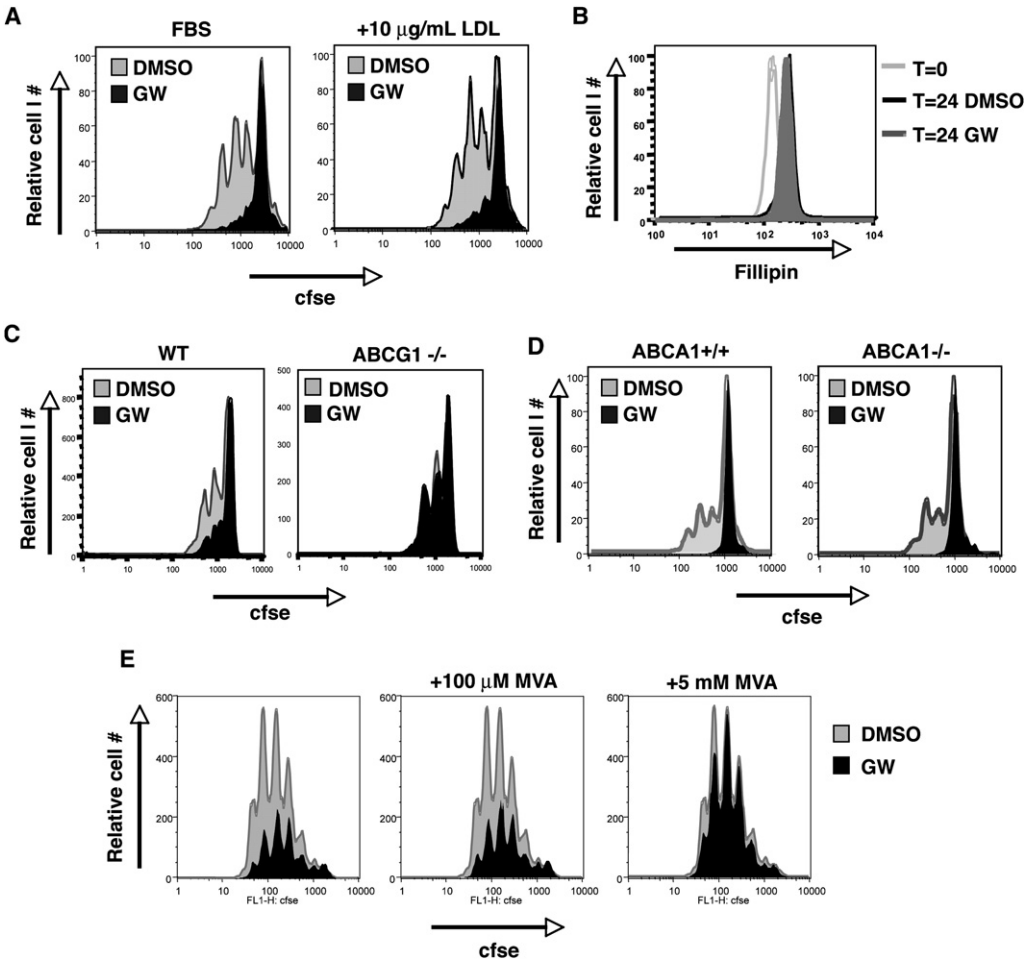

Figure 6. LXR Inhibits Proliferation through *Abcg1*-Dependent Alteration of Cholesterol Homeostasis

(A) CFSE dilution of WT T cells stimulated with pbCD3 and GW3965 in the presence of 10 μg/mL LDL for 60 hr.

(B) Cholesterol content of plasma membrane from WT T cells revealed by fillipin staining ex vivo or after 24 hr stimulation with pbCD3 and GW3965 as indicated.

(C and D) CFSE dilution of purified *abcg1*$^{-/-}$, *abca1*$^{-/-}$, and control T cells stimulated with PMA and ionomycin in the presence of LXR ligands for 60 hr.

(E) CFSE dilution of purified WT T cells stimulated with pbCD3 for 96 hr in the presence of GW3965 and mevalonic acid (MVA) as indicated.

with the above loss-of-function studies, pretreatment of T cells with LXR ligand markedly reduced the frequency of T cells undergoing homeostatic proliferation (Figure 7B).

The ability of LXR to modulate lymphocyte expansion strongly suggested that endogenous LXR signaling might have a functional impact on acquired immune responses. To determine whether loss of LXRβ would augment antigen driven proliferation in vivo, we immunized mice with 1 × 10^7 TAP-deficient mouse embryo cells (MECs) expressing the human adenovirus type 5 early region 1 (Ad5E1). Antigen-specific CD8$^+$ T cells were enumerated ex vivo 1 week after immunization by intracellular IFN-γ and TNF-α staining after a short-term in vitro restimulation with the E1B antigen $E1B_{192\text{-}200}$ (VNIRNCCYI) (Toes et al., 1998). Remarkably, FACS analysis indicated that the frequency of antigen-specific *Lxr*β null IFN-γ$^+$ ($p = 0.02$) or TNF-α$^+$ ($p = 0.01$) CD8+ T cells was 2- to 3-fold higher than that of their WT counterparts (Figures 7C and 7D). Thus, antigen-driven expansion of CD8$^+$ T cells is negatively regulated by LXRβ in WT mice. Taken together, these data establish LXR-dependent sterol metabolism as a novel signaling pathway regulating T cell function and immune responses.

DISCUSSION

An important characteristic of adaptive immunity is the capacity of antigen-specific lymphocytes to undergo rapid and extensive proliferation in response to antigenic challenge. Thus, understanding the signaling pathways that impact proliferation is critical to understanding how immune responses are generated and controlled. Previous work from a number of laboratories has outlined the importance of glycolytic metabolism in T cell responses (MacDonald and Cerottini, 1979; Rathmell et al., 2000; Vander Heiden et al., 2001). The influence of lipid metabolism on lymphocyte function, however, is poorly understood. We have shown here that intracellular sterol metabolism has a previously unrecognized regulatory role in the control of acquired immune

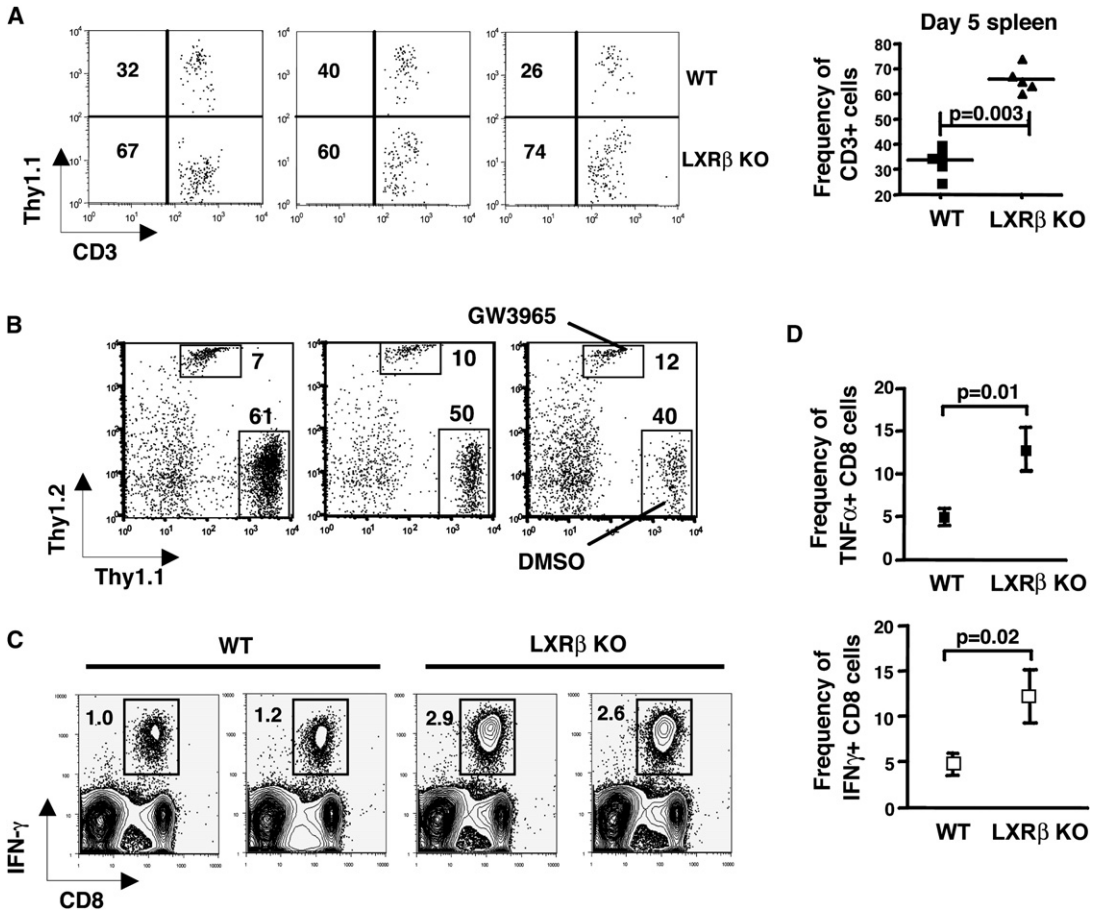

Figure 7. LXR Signaling Regulates T Cell Proliferation and Immunity

(A) Homeostatic proliferation of WT and LXRβ KO T cells. One million purified WT (Thy1.1$^+$) and LXRβ KO T cells (Thy1.2$^+$) were coadoptively transferred into *B6Rag*$^{-/-}$ hosts. After 5 days, spleens were harvested and cells stained with anti-CD3 and Thy1.1 to determine the frequency of recovered T cells. Each FACS plot represents an individual mouse, and the graph represents five mice from one experiment.

(B) Homeostatic proliferation of WT T cells stimulated with LXR/RXR ligands. WT (Thy1.2$^+$) T cells were pretreated with GW3965 in vitro for 18 hr. Control (Thy1.1$^+$) T cells were pretreated with DMSO in vitro for 18 hr. One million live cells per treatment group were coadoptively transferred into *B6Rag*$^{-/-}$ hosts. After 5 days, spleens were harvested and cells stained with anti-CD3, Thy1.1, and Thy1.2. Each FACS plot represents an individual mouse of five mice from one experiment.

(C and D) Increased antigen-specific immune response in LXRβ KO mice. WT and in LXRβ KO mice were immunized with 1 × 10^7 syngeneic MECs transfected with the human adenovirus type 5 early region 1 (Ad5e1). The frequency of antigen-specific CD8+ T cells in whole spleen was enumerated ex vivo 1 week after immunization by intracellular IFN-γ and TNF-α staining after a short-term in vitro restimulation with the $E1B_{192\text{-}200}$ (VNIRNCCYI) peptide (C). FACS data presented is two mice per genotype representative of four mice per group. The frequency of antigen-specific IFN-γ- and TNF-α-producing CD8 T cells within the CD8 T cell compartment is shown (D). Data is presented as the mean ± SEM of four mice per group. Each experiment was repeated twice.

Error bars represent ± SEM.

responses. Cholesterol is an essential component of membranes and therefore the requirement for adequate cholesterol in cell reproduction is obvious. However, our data reveal that the intracellular availability of sterols is dynamically regulated during T cell activation and that this is linked to transcriptional responses mediated by SREBP and LXR, as well as to cell-cycle control. Moreover, we show that the ability of LXR to alter cellular sterol metabolism through regulation of the ABCG1 sterol transporter impacts both lymphocyte proliferation and antigen-stimulated immune responses.

The initial clue to a potential role for LXR signaling in innate immune cells came from analysis of LXR null mice, which exhibit age-dependent splenomegaly and lymphadenopathy. This phenotype reflects expansion in both the T and B cell compartments and is linked to the previously unrecognized ability of LXRβ to regulate mitogen- and antigen-driven lymphocyte proliferation. Analysis of purified primary lymphoid cultures established that activation of LXRβ by physiologic or pharmacologic ligands diminishes the proliferative capacity of B and T cells. Conversely, genetic loss of LXRβ rescues cells from the inhibitory effect of LXR agonist and potentiates mitogen- and antigen-driven expansion. We also demonstrated that the ability of LXR to impact cell proliferation has a functional consequence for lymphoid homeostasis and antigen-driven immune responses in vivo. Lymphocytes lacking LXRβ expression show an exaggerated response in both homeostatic and vaccine-driven proliferation

models. Thus, endogenous LXR signaling is a physiologically important determinant of immune responses, and pharmacological LXR activation has the potential for immune modulation.

Given the ability of LXR to inhibit NF-κB signaling in macrophages, we initially suspected that LXR might interfere with TCR signaling and proximal pathways involved in lymphocyte activation. However, this appears not to be the case because markers of activation and IL-2 production were not affected by LXR ligands. We also excluded the possibility that LXR activation was causing apoptosis directly, as is known to occur with GR agonists. Rather, cell-cycle analysis indicated that LXR signaling was regulating the G1-to-S transition. Collectively, these observations suggested that LXR was controlling the expression of one or more genes whose action was inhibitory to cell-cycle progression. To identify such genes, we profiled gene expression during lymphocyte activation. We found that a battery of genes linked to cholesterol homeostasis is dynamically regulated during lymphocyte activation and that exogenous LXR ligand alters this metabolic program. In particular, the genes encoding the sterol transporters ABCA1 and ABCG1—both LXR targets—are rapidly downregulated upon T cell receptor crosslinking. The addition of LXR agonist strongly stimulates their expression. These findings led to the hypothesis that ABCG1 action alters sterol metabolism in lymphocytes in a manner that is inhibitory to proliferation. Definitive support for the requirement of ABCG1 in this model is provided by the demonstration that the ability of LXR to inhibit proliferation is markedly reduced in lymphocytes from *Abcg1* null mice. A definitive link to sterol metabolism is established by the observation that the inhibitory effect of LXR is completely blocked if lymphocytes are provided with an excess of mevalonate, the precursor for cholesterol and oxysterols.

Our results reveal the existence of an endogenous sterol signaling pathway that regulates lymphocyte proliferation through coordinate regulation of SREBP and LXR activity. The changes in SREBP and LXR target gene expression we observe during T cell activation are indicative of alterations in endogenous sterol regulators of both transcription factors. Brown and Goldstein have delineated an elegant mechanism for the regulation of cholesterol synthesis in which SREBP is held as an inactive precursor in the ER by the sterol-sensing protein SCAP (Goldstein et al., 2006). A logical conclusion of our data is that sterol content in the ER (cholesterol and/or oxysterols) must be decreasing rapidly upon lymphocyte activation. Furthermore, the reduction in LXR target gene expression during activation indicates that the nuclear availability of sterol LXR activators is also reduced. Indeed, basal expression of LXR target genes in lymphocytes is dependent on the production of endogenous LXR agonists by the mevalonate pathway.

Unexpectedly, the mechanism by which LXR activity is regulated during T cell activation does not involve alteration in ligand production; rather, it is due to induction of enzymatic ligand metabolism. The enzyme SULT2B1 transfers sulfate groups to oxysterols, inactivates them as LXR ligands, and facilitates their export from the cell. We have shown that expression of SULT2B1 is dramatically increased in T cells by proliferative stimuli. This induction would be predicted to deplete the cell of LXR ligand and suppress expression of LXR target genes (Chen et al., 2007; Fuda et al., 2007). In fact, expression of SULT2B1 in resting lymphocytes with an adenoviral vector recapitulates the effects of proliferative stimuli on both the LXR and SREBP gene expression programs. Thus, upregulation of SULT2B1 during cell proliferation provides an elegant mechanism to affect changes in cellular cholesterol metabolism required to support new membrane synthesis and cell division. Whether additional mechanisms also contribute to regulation of LXR activity during cell proliferation remains to be addressed.

The ability of ABCG1 expression to block proliferation implicates a sterol substrate of this transporter in a metabolic checkpoint that regulates cell-cycle progression. Early studies on cholesterol synthesis in lymphocytes found that manipulation of the mevalonate pathway by the addition of sterol metabolites, such as 25-hydroxycholesterol, resulted in a G1 arrest (Chakrabarti and Engleman, 1991). Similarly, HMG-CoA inhibitors block the proliferation of cells in multiple systems. A complicating factor in these studies is that suppression of the mevalonate pathway also perturbs synthesis of nonsterol mevalonate derivatives such as geranylgeraniol and farnesol. However, attempts to uncouple the cholesterol synthetic pathway from nonsteroidal protein modifications either with low-dose statins or inhibitors of downstream enzymes have revealed an absolute requirement for cholesterol in cell-cycle progression and mitosis (Martinez-Botas et al., 2001; Martinez-Botas et al., 1999). We have observed a similar arrest in the cell cycle; however, our gene profiling studies show that we are not blocking the SREBP-2 pathway. Rather, we are likely enforcing sterol efflux or redistribution, resulting in a localized depletion of sterols.

ABCG1 is known to play an important role in cholesterol and oxysterol efflux (Kennedy et al., 2005; Terasaka et al., 2007; Wang et al., 2004). Recent studies have also reported that ABCG1 is found in intracellular compartments, such as the ER and vesicles, and that ABCG1 expression stimulates SREBP-2 activity through the redistribution of sterols out of the ER (Tarr and Edwards, 2007). We hypothesize that adequate levels of one or more sterols in a particular cellular compartment, likely the ER, are read by the cell-cycle machinery as an indication of appropriate metabolic conditions for cell division. Downregulation of ABCG1 during activation may be necessary to maintain compartmentalization of these sterols. Forced induction of ABCG1 by LXR activation reduces the availability of this signaling sterol. In the absence of LXR, increased sterol levels act as a stimulus to proliferation. At present, we favor the hypothesis that cholesterol itself is the sterol being sensed by the cell-cycle machinery, because the ability of LXR agonists to block proliferation indicates that the signaling sterol is not an LXR agonist.

In summary, this work outlines a previously unrecognized role for LXRβ and sterol signaling in the regulation of lymphocyte function. Although our focus in this report has been on lymphocytes, the ability of the SULT2B1-LXR-ABCG1 axis to couple cellular cholesterol metabolism and proliferation is likely to be applicable to many cell types, particularly those undergoing rapid cell division. Finally, given that LXR responds to endogenous lipids whose availability may be altered in disease, our results raise the possibility that LXR signaling may impact acquired immune responses in human metabolic diseases such as dyslipidemia and atherosclerosis.

EXPERIMENTAL PROCEDURES

Mice and Cell Lines

C57BL/6 and C57BL/6 recombination activating gene 1-deficient mice were purchased from the Jackson Laboratory. *Lxrα* and *Lxrβ* null mice were a gift from D. Mangelsdorf (University of Texas Southwestern) and are greater than ten generations backcrossed to C57BL/6. BCL-xL transgenic mice were a gift from D. Green (St. Jude's). ABCG1 and ABCA1 null mice have been previously described (Kennedy et al., 2005; Timmins et al., 2005). Do11.10 CAR transgenic mice were purchased from the Taconic Laboratory. All mice were maintained under pathogen-free (SPF) conditions in the animal facilities of the University of California, Los Angeles (UCLA). TAP-deficient MECs expressing the human adenovirus type 5 early region 1 (Ad5E1) have been described (Schoenberger et al., 1998).

Media, Reagents, Antibodies, and Flow Cytometry

GW3965, T0901317, and GW7845 were provided by T. Willson and J. Collins (GlaxoSmithKline). Lymphocytes were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES (all from GIBCO), and 50 μM 2-ME (Sigma-Aldrich). 7-AAD, Propidium iodide, annexin V, anti-mouse CD25 (PC61), CD3 (2C11), CD4 (RM4-5), CD8 (53-6.7), CD28 (37.51), CD69 (HI.2F3), Foxp3 (FJK-16 s), and anti-human CD4 (RPA-T4), CD8 (RPA-T8), Ki-67 (B-56), and PCNA (PC-10) were purchased from BD Biosciences and eBioscience. Purified anti-human CD3 (OKT3) was purchased from Ortho Scientific. Murine rIL-2 was purchased from Peprotech. Flowbright counting beads (Invitrogen) were added to FACS tubes before analysis to normalize CFSE dilution profiles. Intracellular PCNA and Ki-67 staining was performed per the manufacturer's instruction (BD Biosciences). Cells were analyzed on FACSCalibur or LSR (Becton Dickinson) with FlowJo software (Treestar).

Cell Purification and Cell Counts

In some experiments, mouse or human T cells were enriched with negative selection. For mouse, T cell or B cell enrichment was performed with Pan T or B cell isolation kit (Miltenyi Biotec) and selection on MidiMACS columns (Miltenyi Biotec) per the manufacturer's instructions. For human T cells, enrichment was performed with Rosette Sep (Stem Cell Technologies) per the manufacturer's instructions. Purity was confirmed at > 95% by flow cytometry. Cells counts were performed on an Improved Neubauer Hemacytometer with trypan blue exclusion. All LN cell counts reported are from two inguinal and four axillary LNs. For adenoviral transductions, purified CAR transgenic T cells were incubated with adenovirus at a multiplicity of infection (MOI) of 10:1 for 1 hr in complete media. Cells were then washed twice and resuspended at 1×10^6 cells per mL in complete media.

Proliferation Studies

For ^{3}H-thymidine incorporation studies, single-cell suspensions of murine spleen were initially prepared with ACK lysis buffer. Whole spleen cells were plated in 96-well round bottom plates (2×10^5 cells/well) and stimulated with LPS (10 μg/mL), Concavalin A (10 μg/mL), anti-IgM $(Fab')_2$ (10 μg/mL), or PMA (0.5 μM) and ionomycin (100 mM). Cells were cultured for 24–96 hr at 37°C and 5% CO_2 and pulsed with ^{3}H-thymidine for the final 16 hr. For some studies, T cells or B cells were purified and enriched from spleen and LNs by negative selection as indicated above. Single-cell suspensions were labeled with CFSE (Molecular Probes) as described previously (Lyons and Parish, 1994). Cells were stimulated as above. Human T cells were CFSE labeled and stimulated with anti-CD3 (1 μg/mL) crosslinked with plate-bound goat anti-mouse (100 μg/mL) in 96-well flat-bottom plates (2×10^5 cells/well). Some cultures received 2 μg/well of soluble anti-CD28. Cells were stained for surface expression of CD8 and CD4 on the indicated days, incubated with 7-AAD, and 5×10^4 counting beads were added to tubes before analysis by flow cytometry.

Immunization and Homeostatic Proliferation Assays

For immunization studies, WT and *Lxrβ* null mice were immunized subcutaneously with 1×10^7 irradiated (3000 rad) TAP-deficient mouse embryonic fibroblasts expressing Ad5E1. Mice were sacrificed on day 7, and antigen-specific CD8+ T cells were enumerated from spleen as described (Schoenberger et al., 1998). Single-cell suspensions were incubated for 5 hr with $E1B_{192–200}$ peptide [(aa:VNIRNCCYI) A&A labs LLC)[at 2.5 μg/mL in the presence of Brefeldin A (BD Biosciences) ex vivo. Cells were stained for surface expression of CD8 and CD4, fixed, permeabilized with Cytofix/Cytoperm kit (BD Biosciences), and stained for intracellular IFN-γ and TNF-α according to the manufacturer's protocol. For homeostatic proliferation studies, 1×10^6 purified LXRβ null (Thy1.2$^+$) T cells and WT (Thy1.1$^+$) T cells were coadoptively transferred into the same B6.RAG null host. Peripheral blood and spleen was harvested and stained for CD3, and Thy1.1 expression was and analyzed by FACS.

RNA Isolation, DNA Microarray and Real-Time PCR Analysis

For DNA microarray, total RNA was isolated from cells with Trizol (Invitrogen) and further purified with RNAeasy columns (QIAGEN). Preparation and hybridization to Affymetrix 430 v2.0 was performed at the UCLA microarray core and data analyzed with GeneSpring GX 7.3 (Agilent Technologies). For real-time PCR, total RNA was isolated as above. One microgram of total RNA was reverse transcribed with random hexamers with the Taqman Reverse Transcription Reagents Kit (Applied Biosystems). Sybergreen (Diagenode) real-time quantitative PCR assays were performed with an Applied Biosystems 7900HT sequence detector. Results show averages of duplicate experiments normalized to 36B4.

Protein Isolation and Analysis

Total cell lysates were prepared in lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl [pH 7.4], 1% SDS) supplemented with protease inhibitors (Roche Molecular Biochemicals). Samples were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Membranes were probed with anti-hLXRα and anti-hLXRβ (Perseus Proteomics), anti-P27kip (Cell Signaling) and anti-CDK4 (Santa Cruz Antibodies) at 1:1000 overnight to detect expression. Goat anti-mouse and Donkey anti-rabbit (DAKO) were used at 1:3000 for 1 hr at room temperature and visualized with chemiluminescence (ECL, Amersham Pharmacia Biotech).

Statistical Methods

Real-time PCR data, in vitro proliferation assays, and cell counts are expressed as the mean ± standard deviation. The frequency of antigen-specific T cells and homeostatically proliferating T cells are presented as the mean ± standard error of the mean (SEM). All statistical analysis was done with the student's t test. A probability value of $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include eleven figures and can be found with this article online at http://www.cell.com/cgi/content/full/134/1/97/DC1/.

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