LXR Activation Reduces Proinflammatory Cytokine Expression in Human CD4-Positive Lymphocytes

Daniel Walcher, Andreas Kümmel, Bettina Kehrle, Helga Bach, Miriam Grüb, Renate Durst, Vinzenz Hombach, Nikolaus Marx

Background—CD4-positive lymphocytes, the major T-cell population in human atheroma, mainly secrete Th-1-type proinflammatory cytokines, like interferon (IFN)γ, tumor necrosis factor (TNF)α, and interleukin (IL)-2, thus promoting atherogenesis. Recent data suggest that the nuclear transcription factors liver X receptor-alpha and liver X receptor-beta (LXRα and LXRβ) limit plaque formation in animal models by modulating macrophage function. Still, the role of LXRs in CD4-positive lymphocytes is currently unexplored.

Methods and Results—Human CD4-positive lymphocytes express LXRα and LXRβ mRNA and protein. Activation of CD4-positive cells by anti-CD3 mAbs, anti-CD3/CD28 mAbs, as well as PMA/ionomycin significantly increased IFNγ, TNFα, and IL-2 in a concentration-dependent manner with a maximum at 1 μmol/L T0901317. Transient transfection assays revealed an inhibition of IFNγ promoter activity by T0901317 as the underlying molecular mechanism. Such anti-inflammatory actions were also evident in cell–cell interactions with medium conditioned by T0901317-treated CD4-positive cells attenuating human monocyte CD64 expression.

Conclusions—Human CD4-positive lymphocytes express both LXRα and LXRβ, and LXR activation can reduce Th-1 cytokine expression in these cells. These data provide new insight how LXR activators might modulate the inflammatory process in atherogenesis and as such influence lesion development. (Arterioscler Thromb Vasc Biol. 2006;26:1022-1028.)

Key Words: atherosclerosis ■ IFNγ ■ LXR ■ T-lymphocytes

CD4-positive lymphocytes play a critical role in early atherogenesis.1 During endothelial dysfunction, these cells are attracted by chemokines released from endothelial cells (ECs) and cells in the subendothelium and enter the vessel wall as naive Th0-cells. In the intima they encounter antigens like oxidized low-density lipoprotein (oxLDL) and subsequently differentiate to Th1-cells, releasing proinflammatory cytokines, like interferon (IFN)γ and tumor necrosis factor (TNF)α.2 These cytokines then orchestrate the inflammatory process during plaque development by activating ECs, monocyte/macrophages, and vascular smooth muscle cells (VSMCs).3 Moreover, interruption of the IFNγ signaling pathway reduces the extent of atherosclerotic lesions in apolipoprotein E (apoE)-deficient mice.4 Despite this large body of data implicating IFNγ in vascular disease, only a few studies addressed modulatory pathways to limit Th1-cytokine release from human CD4-positive lymphocytes, eg, by activation of nuclear transcription factors like peroxisome proliferators-activated receptors (PPARs).5 However, the role of the recently described liver X receptors alpha and beta (LXRα, LXRβ), both belonging to the same group of nuclear transcription factors, remains unexplored in CD4-positive lymphocytes.

LXRα and LXRβ are ligand-activated transcription factors, consisting of a ligand-binding and a DNA-binding domain. On activation, LXRs build heterodimers with another nuclear receptor, the retinoic X receptor (RXR), and bind to specific DR4-type LXR response elements (LXREs) in the promoter region of their target genes, thus regulating their expression.6 LXRs are activated by sterol metabolites like 22(R)- and 24(S)-hydroxycholesterol, as well as 24(S), 25-epoxycholesterol. In addition, synthetic LXR agonists include T0901317 as well as GW3965. The 2 LXR subtypes share substantial sequence homology and seem to respond to the same ligands. However, their tissue distribution is different. LXRα is mainly present in liver, intestine, adipose tissue, and macrophages, whereas LXRβ is ubiquitously expressed.7 Originally, LXRs have been described as regulators of gene expression in lipid and cholesterol metabolism, but recent data suggest that activation of these receptors may modulate atherogenesis. As such, LXR agonists reduce lesion development in mouse models of arteriosclerosis at least in part by influencing gene expression in cholesterol absorption and reverse cholesterol transport.8,9 Moreover, LXRs have been shown to exhibit anti-inflammatory effects by limiting the...
expression of pro-inflammatory mediators, like IL-6, COX-2, and matrix metalloproteinase (MMP)-9. Because CD4-positive lymphocyte-derived cytokine release is critical for the inflammatory response in lesion development, we examined the expression and functional role of LXRs in CD4-positive T-cells with respect to their effect on Th1-cytokine expression.

Methods

Cell Culture

Human CD4-positive T cells were isolated from freshly drawn blood of healthy volunteers by Ficoll-Histopaque (Sigma) gradient centrifugation to obtain mononuclear cells (PBMCs) and subsequent negative selection of CD4-positive T cells by magnetic bead separation (Miltenyi Biotech) as described by the manufacturer’s protocol. The purity of CD4-positive T cells was >97% as determined by flow cytometry. Human monocytes were isolated as previously described.

Reverse-Transcription Polymerase Chain Reaction

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Preparation of Nuclear and Cytosolic Extracts and Western Blot Analysis

For Western blotting, nuclear and cytosolic extracts of 10^7 cells were prepared as previously described. Standard Western blot analysis on total cell lysates was performed using mouse anti-LXRα and -β antibodies (mAbs, Perseus Proteomics).

Stimulation Assays, Enzyme-Linked Immunosorbent Assays, and Cell Viability Studies

Human CD4-positive T cells (1×10^6 cells/mL) were pretreated with the LXR activator T0901317 for 30 minutes (Alexis, dissolved in DMSO) and stimulated with anti-CD3 Abs for 48 hours (R&D Systems) or with a combined stimulation with anti-CD3 and anti-CD28 antibodies (Dynabeads) for 6 hours or with PMA (10 ng/mL)/ionomycin (0.5 μmol/L) for 6 hours, according to previously published time courses for these stimuli. Cells were then harvested and IFNγ, TNFα, IL-2, and IL-10 enzyme-linked immunosorbent assays (ELISA) (R&D Systems) were performed on cell-free supernatants as recommended by the manufacturer. In some experiments cells were stimulated with the LXR activator for 24 hours and the release of IL-10, a typical Th2-cytokine, was measured by ELISA (R&D Systems). Independent experiments were performed using cells from different donors. Cell viability was assessed by standard trypan blue exclusion.

KL67 Staining

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Northern Blot Analysis

For Northern blot experiments, cells were treated with anti-CD3 Abs for 24 hours or anti-CD3/CD28 antibodies for 4 hours or with PMA/ionomycin for 4 hours in the presence or absence of T0901317. Five micrograms of total RNA were used in standard Northern blot analysis using cDNA probes against IFNγ, TNFα, and IL-2, or against the house keeping gene GAPDH.

Transient Transfection Assays

To examine whether LXRs are functionally active in cells of the lymphocytic lineage, Jurkat cells were transiently transfected with a luciferase reporter construct containing 3 copies of a canonical LXRE and a pCMV-β-galactosidase construct using superfect according to the manufacturer’s protocol. For IFNγ promoter studies, we cloned 2 deletion promoter reporter constructs. For polymerase chain reaction (PCR), the following oligonucleotides were used to amplify the whole promoter of the human IFNγ: sense 5’-GGCGGATCCGCTCTGATGAAGGACTTCCTCAC-3’; anti-sense 5’-GGGTCGAGCCGTCCGAGAGAATTAAGCCA-3’.

The resulting PCR fragment was cloned into the BamHI and XhoI sites of the pGL3 luciferase reporter plasmid to achieve: [p(624)IFNγ-LUC]. The proximal fragment of the IFNγ-promoter lacking portions of the 5’-sequence, but containing the 2 critical AP-1–CREB-ATF binding sites [distal (−96 to −75bp) and proximal (−66 to −47 bp) element] for PMA/ionomycin-induced promoter activity, we got as a kindly gift from Dr J. Plutzky (Boston, Mass) (p108IFNγ-LUC). It was amplified by PCR with 2 BglII sites and cloned into the pGL3 luciferase reporter plasmid. Transfected cells were stimulated with PMA/ionomycin in the presence or absence of T0901317 (1 μmol/L) for 16 hours, before cells were harvested. Luciferase and β-galactosidase activity was measured using the Dual-Light assay (Tropix, Bedford, Mass).

Flow Cytometry

Human CD4-positive T cells were incubated with an equal volume of PBS containing saturating concentrations (10 mg/L) of fluorescein isothiocyanate (FITC)-conjugated anti-CD3 antibodies and PE-conjugated anti-CD4 antibodies for 30 minutes at room temperature. To examine the influence of T0901317 on the pro-inflammatory activity of T-cell supernatants toward other vascular cells, freshly isolated human monocytes were incubated with supernatants (50% original monocyte media and 50% conditioned media from T cells) derived from T cells after CD3 activation in the absence or presence of the LXR agonist (Figure 5A, upper panel). In some experiments, cells were first treated with conditioned media from anti-CD3-activated T cells (Figure 5B, upper panel) and then T0901317 was directly added to monocytes at similar concentrations. After 18 hours, cells were harvested for the investigation of monocyte CD64 expression on the cell surface. After washing, monocytes were stained with FITC-conjugated anti-CD64 antibodies. Finally, T cells or monocytes were washed 3 times and stored in 1% paraformaldehyde (Sigma) at 4°C until flow cytometric analysis was performed within 24 hours. To examine that T0901317 is active in human monocytes, we stimulated cells with IFNγ in the presence or absence of T0901317 and assessed TNFα protein content in cell-free supernatant.

Statistical Analysis

Results of the experimental studies are reported as mean±standard error of the mean (SEM). Differences were analyzed by 1-way-ANOVA followed by the appropriate post-hoc test. A P<0.05 was regarded as significant.

Results

Human CD4-Positive Lymphocytes Express LXRα and LXRβ mRNA and Protein

Isolated human CD4-positive lymphocytes express LXRα and LXRβ mRNA as determined by reverse-transcription (RT) PCR (Figure 1A). Western blot analysis revealed that both, LXRα and LXRβ protein are present in the nuclear fraction of isolated human CD4-positive T cells (Figure 1B), but not in the cytosol (data not shown). In addition, T-lymphocyte–like Jurkat cells also express LXRα and LXRβ mRNA and protein (Figure 1A and 1B).

To examine whether LXRs are functionally active in cells of the lymphocytic lineage, we transiently transfected Jurkat cells with a LUC reporter promoter construct containing 3 copies of a canonical LXRE. As shown in Figure 1C, the LXR activators T0901317 significantly induced reporter activity suggesting that LXRs are functionally active in these cells.
LXR Activation Inhibits IFNγ Expression in Human CD4-Positive T Cells

Unstimulated human CD4-positive T cells secreted only small amounts of IFNγ as determined by ELISA of cell-free supernatants, whereas incubation of cells with immobilized anti-CD3 Abs significantly increased IFNγ protein secretion from 2±3 to 466±222 pg/mL (P<0.05; n=13). Concomitant treatment with the LXR activator T0901317 inhibited this increase in a concentration-dependent manner with a maximal reduction to 40±18% at 1 μmol/L (P<0.05, compared with anti-CD3-activated cells, n=13). Similar data were obtained for TNFα with a maximal reduction of anti-CD3-induced cytokine release to 51±14% at 1 μmol/L (P<0.05, compared with anti-CD3-activated cells, n=18; Figure 2A). Stimulation of cells with the LXR activator T0901317 did not affect cell viability (by trypan blue exclusion) or cell surface CD3 expression as determined by flow cytometry (Table I, available online at http://atvb.ahajournals.org).

To examine whether LXR activation is capable of counteracting Th1-cytokine expression on treatment with a more potent stimulus, we performed similar experiments using a combined CD3/CD28 stimulation to induce cytokine release. As shown in Figure 2B, T0901317 decreased anti-CD3/CD28-induced IFNγ and TNFα release in a concentration-dependent manner with a maximal reduction at 1 μmol/L to 74±2% and 79±2%, respectively (P<0.05 compared with anti-CD3/CD28-activated cells, n=7) (Figure 2B). Because this stronger stimulation of cell with anti-CD3/CD28 antibodies (in contrast to the stimulation with anti-CD3 alone) also enhanced IL-2 protein secretion from 0 to 718±107 pg/mL, we next assessed the effect of LXR activation on this Th1-cytokine. Treatment of cells with T0901317 decreased IL-2 release in a concentration-dependent manner with a maximal reduction to 75±3% (P<0.05 compared with anti-CD3/CD28-activated cells, n=7) (Figure 2B).

To investigate whether LXR activator’s effect on CD4-positive lymphocyte-derived IFNγ expression depended on the stimulus used, we also used PMA/ionomycin to induce IFNγ release. PMA/ionomycin treatment of human CD4-positive T cells increased IFNγ protein content in the supernatant from 0 to 149±23 pg/mL. Treatment of cells with T0901317 reduced this IFNγ release to 46±10% at 1 μmol/L (P<0.05 compared with PMA/ionomycin-stimulated cells; n=6). Similar results were obtained for the release of TNFα with a reduction to 59±5% and IL-2 with a reduction to 55±4% at 1 μmol/L T0901317 (P<0.05 compared with PMA/ionomycin-stimulated cells; n=10) (Figure 2C). Solvent alone (DMSO) did not have an effect on Th1-cytokine expression (data not shown). In addition, to exclude that differences in cell proliferation account for the changes in cytokine expression observed, we stimulated cells with anti-CD3 or anti-CD3/CD28 antibodies, or with PMA/ionomycin in the presence or absence of T0901317 and assessed cell proliferation by nuclear Ki67 staining. Stimulation of cells with anti-CD3 or anti-CD3/CD28 antibodies markedly increased Ki67 expression, whereas PMA/ionomycin treatment had only a moderate effect on Ki67 positivity. Still, independent of the stimulus used, we did not find a significant effect of T0901317 on cell proliferation making it unlikely that differences in the number of Th1-cytokine secreting cells play a causal role in this context (please see Figure I, available online at http://atvb.ahajournals.org).

To examine the effect of LXR activation on the expression of counterbalancing Th2-cytokines, we stimulated CD4-positive lymphocytes with PMA/ionomycin and measured the release of IL-10, a typical Th2 cytokine. PMA/ionomycin induced IL-10 secretion from 0 to 241±43 pg/mL (P<0.01; n=4), but treatment of cells with T0901317 did not significantly alter the expression of this Th2-cytokine (214±45 pg/mL (P=not significant compared with PMA/ionomycin-stimulated cells; n=4). Moreover, T0901317 did not induce IL-10 expression, thus making it unlikely that LXR activation induces a Th2 response in these cells (data not shown).

LXR Activation Reduces Cytokine mRNA Expression in Human CD4-Positive Lymphocytes

To examine whether the decrease in pro-inflammatory cytokine expression by the LXR activator resulted from reduced cytokine mRNA expression, we treated CD4-positive T cells with T0901317 and performed Northern blot analysis. As shown in Figure 3, T091323 significantly reduced anti-CD3-induced, anti-CD3/CD28--induced, as well as PMA/ionomycin-induced Th1-cytokine mRNA, but did not affect mRNA levels of the constitutively expressed gene GAPDH.
LXR Activation Reduces IFNγ Promoter Activity

Next, we sought to investigate whether the effect of T0901317 on IFNγ mRNA expression is caused by a reduction in IFNγ promoter activity. To this end, we performed transient transfection assays in Jurkat cells using 2 different IFNγ promoter reporter constructs. PMA/ionomycin stimulation of cells transfected with a 624 bp IFNγ promoter construct [p(624)IFNγ-LUC], previously shown to exhibit highest activity on stimulation,14 led to a 12.8±1.9-fold increase in normalized promoter activity. Treatment with the LXR activator T0901317 significantly reduced this increase to 8.2±1.0-fold (P<0.05, compared with PMA/ionomycin-stimulated cells; n=3). Transfection studies with a 108 bp IFNγ promoter deletion construct, lacking portions of the 5′-sequence, but containing the 2 critical AP-1–CREB-ATF binding sites [distal (−96 to −75 bp) and proximal (−66 to −47 bp) element] for PMA/ionomycin-induced promoter activity,14 revealed a 4.1±0.6-fold increase in promoter activity on PMA/ionomycin stimulation. T0901317 treatment significantly reduced this increase to 2.7±0.3 (P<0.05 compared with PMA/ionomycin-stimulated cells; n=4), suggesting that the LXR activator may interact with these 2 sites (Figure 4). In the case of all constructs, T0901317 alone had no effect on promoter activity compared with control, consistent with the lack of a canonical LXRE in the IFNγ promoter.

T0901317 Reduces Pro-inflammatory Activity of CD4-Positive Lymphocytes on Human Monocytes

To examine potential functional effects of LXR-mediated reduced T-cell cytokine expression, we incubated supernatants from stimulated CD4-positive T cells with human monocytes and measured monocyte CD64 surface expression by flow cytometry. CD64 is the high-affinity receptor for IgG involved in phagocytosis and antigen capture, an IFNγ-regulated gene in human monocytes, and its expression on the cell surface indicates IFNγ activity on monocytes in vitro and in vivo.15 Incubation of freshly isolated human monocytes with supernatants from CD3-activated T cells significantly increased monocyte CD64 surface expression by 2.0-fold. Super-
natants taken from activated CD4-positive T cells after T0901317 treatment significantly reduced this increase (Figure 5A), consistent with reduced cytokine content in the media (data not shown). To exclude that these findings resulted from direct effects of residual LXR agonist in T cell supernatants, we stimulated human monocytes with conditioned media from CD4-positive cells to induce CD64 expression and then added T0901317 directly to the cells. As shown in Figure 5B, T0901317 did not have a direct significant effect on monocyte CD64 expression. To demonstrate that T0901317 was active in human monocytes, we stimulated cells with IFNγ in the presence or absence of T0901317 and measured monocytes TNFα secretion from 78±35pg/mL to 480±187pg/mL (P<0.05; n=6) and T0901317 significantly reduced this increase to 71±9% (P<0.05 compared with IFNγ-stimulated cells, n=6) (Figure 5B, inset), suggesting that the LXR activator is active in the monocytes used.

**Discussion**

The present study demonstrates the expression of LXRα and LXRβ in CD4-positive lymphocytes and shows that activation of LXR by the synthetic agonist T0901317 reduces Th1-cytokine expression in these cells. These data suggest that LXR, in addition to their modulatory action on macrophage function, may exhibit direct anti-inflammatory effects in CD4-positive lymphocytes, potentially contributing to the beneficial effects of LXR agonist on lesion development in animal models of arteriosclerosis.

Originally, LXR have been described as regulators of cholesterol metabolism and reverse cholesterol transport, but recent data suggest that these receptors may directly influence the expression of pro-inflammatory mediators in macrophages. Our study extends the knowledge on LXR by demonstrating their expression in CD4-positive lymphocytes, another cell type critically involved in the inflammatory processes during atherogenesis. We describe the expression of both LXRα and LXRβ in cells of the lymphocytic lineage and demonstrate that LXR is functionally active in these cells, because T0901317 is capable of activating a canonical LXRE in transfected cells. Future studies have to examine the presence of LXR in human atheroma.

The effect of T0901317 on Th-1 cytokine expression is most likely mediated by an activation of LXR. T0901317 is a
Figure 5. LXR activation reduces pro-inflammatory activity of T-cell supernatants on human monocytes in the absence of any direct effects on monocyte CD64 response. A, Freshly isolated human monocytes were incubated for 18 hours with conditioned media (SN) from CD4-positive T cells stimulated with anti-CD3 mAbs in the presence or absence of the LXR agonist (1 μmol/L T0901317) and mean fluorescence intensity of monocyte CD64 expression was measured by flow cytometry (upper panel). Results are expressed as percent of control (monocytes incubated with supernatants from activated T cells). Bars represent mean±SEM (n=5). *P<0.05. B, Human monocytes were incubated with conditioned media from CD3-activated T cells to induce CD64 expression and then directly stimulated with the LXR agonist T0901317 for 18 hours before CD64 expression was assessed by flow cytometry (upper panel). Results are expressed as percent of control (monocytes incubated with supernatants from activated T cells). Bars represent mean±SEM (n=7); no significant difference was seen except as compared with unstimulated cells. *P<0.05. Insert, T0901317 is active in human monocytes. Monocyte monocytes were stimulated with IFNγ in the presence or absence of T0901317 for 6 hours and monocyte TNFα release measured was measured by ELISA. Bars represent mean±SEM (n=6). *P<0.05 compared with IFNγ-stimulated cells.

well-characterized, synthetic LXR activator with no established activity on other receptors or transcription factors and the concentrations used in our study have previously been shown to regulate typical LXR target genes, like ABCA-1. Because no subtype-specific agonists for LXRα or LXRβ have been described so far, we, like others before in macrophages, cannot distinguish whether the effect on Th1 cytokines expression is mediated via LXRα, LXRβ, or both. Further studies are needed to establish subtype specific agonists, thus allowing further elucidation of the role of LXR subtypes in macrophage and T cell activation.

Our data suggest that the reduction in IFNγ expression is caused by an inhibition of IFNγ promoter activity. PMA/ionomycin-induced IFNγ expression is mediated via 2 AP-1–CREB-ATF binding sites in the proximal part of the IFNγ promoter. Transient transfection experiments using a promoter reporter construct containing these 2 binding sites, but lacking the 5′ part of the promoter revealed a significant reduction of reporter activity on LXR agonist treatment. Previous reports have shown that the inhibition of IFNγ expression by glucocorticoids is mediated by limiting the binding of AP-1–CREB-ATF to the promoter, suggesting that LXRs and GR, both members of the family of nuclear transcription factors, share similar pathways with respect to the regulation of IFNγ gene expression.

The effects of the LXR agonist on human T cells extend to inhibition of other pro-inflammatory Th1 cytokines, TNFα and IL-2, implicating LXRs as a potential nodal point for the regulation of T-cell–modulated inflammatory responses. In addition, the results obtained are unlikely to be derived from a shift of T cells toward a potential anti-inflammatory Th-2 response because T0901317 did not increase levels of IL-10, a classic TH2-cytokine, in CD4-positive T cells. Moreover, the LXR activator did not reduce PMA/ionomycin-induced IL-10 secretion. Still, we did not detect IL-4 and IL-5 in our system and thus cannot completely rule out an effect of T0901317 on these TH2 cytokines. Overall, these data suggest that LXR activation may shift the T-cell response toward a reduction of pro-inflammatory cell activation.

The reduction of IFNγ expression by LXR activators provides novel insight into LXRs anti-atherogenic effects. Data in animals have shown that LXR agonist treatment decreases lesion development in a mouse model of atherosclerosis. Because LXRs play an important role in cholesterol efflux from macrophages as well as in the reduction of lipid content in foam cells, such mechanisms have been proposed as the main mechanism at work. However, recent studies have shown that LXR agonists exhibit direct anti-inflammatory activity in macrophages, suggesting that a combined metabolic and anti-inflammatory effect of LXRs may contribute to such beneficial effects on atherogenesis. Our study now proposes that the anti-inflammatory action of LXRs not only is limited to macrophages but also extends to CD4-positive lymphocytes, major effectors of the inflammatory response in the vessel wall. T-cell–derived cytokines like IFNγ are critically involved in lesion development. As such, IFNγ has been shown to induce the expression of T-cell–specific chemokines from endothelial cells, thus facilitating the migration of additional T cells into the vessel wall, potentially creating a vicious circle of T cell recruitment and activation. Moreover, IFNγ has been implicated in plaque destabilization through its capacity to induce the expression of matrix-degrading MMPs. Therefore, a reduction of IFNγ release from activated T-cells, as described here, may at least in part contribute to the beneficial effects of LXR activator treatment on atherogenesis in mice, but this needs to be confirmed in additional in vivo experiments. Further studies are warranted to examine whether LXR activation may become a therapeu-
tical tool to also modulate the inflammatory processes in atherosclerosis in humans. To date in vivo application of LXR agonists is limited because of an increase in triglyceride during treatment, but the development of selective or partial agonists may help to overcome these problems and then potentially provide us with novel drugs to influence lesion development in patients at high-risk.

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**KI67-staining**

To examine the effect of LXR activation on CD4-positive cell proliferation, isolated cells were treated with anti-CD3 Abs for 48 hours or with a combined stimulation with anti-CD3 and anti-CD28 antibodies for 6 hours or with PMA (10 ng/mL) / ionomycin (0.5 µM) for 6 hours and nuclear staining for KI67 was performed as previously described.

**RT-PCR**

Total RNA from freshly prepared CD4-positive T cells was isolated for standard RT-PCR with amplification of LXRα, LXRβ and GAPDH cDNA employing the following primers:

LXRα (Sense: 5’-CTCATTGCTATCAGCATCTTCTGC-3’; antisense: 5’-AGTGCAAACACTTGCTCTGAGTGG-3’);
LXRβ (sense: 5’-GATGCTGGGCCACGAGCTTTGCCGTG-3’; antisense: 5’-GCCTCCTTGCACTTGCGCAGCCGGC-3’), and
GAPDH (sense: 5’-CCACCCATGGCAAATTCCATGGCA-3’; antisense: 5’-TCTAGACGGCAGGTCAGGTCCACC-3’).

Figure I: Effect of LXR activation on CD4-positive cell proliferation as assessed by nuclear KI67 expression. Isolated cells were treated with anti-CD3 Abs for 48 hours (A) or with a combined stimulation with anti-CD3 and anti-CD28 antibodies for 6 hours (B) or with PMA (10 ng/mL) / ionomycin (0.5 µM) for 6 hours (C) in the absence or presence of 1µM T0901317 and immunofluorescence staining for KI67 and DAPI was performed. A, B, and C show one representative experiment. D: Statistical analysis of KI67-positive cells of 4 independent experiments. Bars represent mean±SEM. *p<0.05 compared to anti-CD3-, anti-CD3/CD28- or PMA / ionomycin-stimulated cells.
Figure I

A. DAPI  KI67
    Co
    CD3
    CD3/28
    CD3/28 + T09

B. DAPI  KI67
    Co
    CD3/28
    CD3/28 + T09

C. DAPI  KI67
    Co
    PMA
    PMA + T09

D. KI67-positive cells (%)
   anti-CD3  -  +  +
   T09 (µM)  -  -  1

   KI67-positive cells (%)
   anti-CD3/CD28  -  +  +
   T09 (µM)  -  -  1

   KI67-positive cells (%)
   PMA / iono  -  +  +
   T09 (µM)  -  -  1

Figure I