

Liver X Receptors Control IgE Expression in B Cells¹

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B lymphocytes play a fundamental role in the development of IgE-dependent allergic immune reactions. Upon appropriate activation, IgE class switch recombination is initiated in B cells, followed by terminal differentiation to IgE-secreting plasmablasts. This process is controlled by different nuclear receptors, including receptors for vitamin D, retinoids, and peroxisome proliferator-activated receptor- γ ligands. In this study, we show constitutive expression of the nuclear liver X receptor (LXR) α and LXR β in peripheral human B cells. Activation of LXRs reduced secreted IgE ($-68\% \pm 11$) in CD40 and IL-4 activated B cells. The production of other isotypes, including IgG, IgM, IgA and B cell homeostatic parameters were not significantly altered by LXR activation. We identified inhibitory action of LXR activation on IgE production involving reduced phosphorylation of JNK and increased membrane CD23 expression ($38\% \pm 11$). The biological significance of our findings was validated by showing that systemic treatment of type I-sensitized BALB/c mice with LXR ligands reduced the serum concentrations of Ag-specific IgE in a dose-dependent manner (maximum, $-52\% \pm 14$). Thus, our data indicates that LXRs are involved in the control of IgE secretion by differentiating B cells. *The Journal of Immunology*, 2009, 182: 5276–5282.

Liver X receptors (LXRs)⁴ are members of the nuclear hormone receptor superfamily, which act as ligand-activated transcription factors (1). LXR α (also called NR1H3) is highly expressed in the liver and at lower levels in monocytes/macrophages, CD4 Th cells, and dendritic cells (2–4), whereas LXR β (NR1H2) is expressed ubiquitously (2, 5). LXRs form heterodimers with retinoid X receptors upon activation by oxidized cholesterol as well as intermediate products of the cholesterol biosynthetic pathway. The biological functions of LXRs include the control of gene expression not only linked to glucose and lipid homeostasis (6–9) but also show anti-inflammatory activity (1, 10, 11). LXRs modulate cytokines, including repression of IL-2, IL-6, or TNF- α in activated monocytes and T cells (3, 11). As these cytokines are also produced by B cells and involved in the regulation of IgE-synthesis, we investigated the biological impact of LXR activation in B cells.

IgE is the key effector molecule of type-I allergic immune reactions and its elevated serum concentrations are associated with atopic diseases as atopic eczema, hay fever, and allergic asthma (12). The initiation event in the pathogenesis of IgE-dependent

diseases is IgE class switch recombination in B cells, which is mediated by at least two different receptors (12, 13), namely CD40 and the IL-4 receptor. Stimulation of CD40, a TNF-receptor family member, results in activation of NF- κ B, JNK, and AP-1, whereas IL-4 receptor signaling mediates STAT-6 phosphorylation (12). Both signals synergistically induce two critical transcripts involved in IgE class switch recombination, namely the sterile ϵ -germline transcription (ϵ GLT) and activation-induced cytidine deaminase (AID) (12, 14). Direct activation in vitro by using monoclonal anti-CD40 Abs and rIL-4 is sufficient to induce IgE production by purified B cells (15). Additionally, indirect mechanisms modulate IgE production by activated B cells, e.g., by signaling of the low-affinity receptor for IgE (FC ϵ RII or CD23), intracellular adhesion molecule-1 (ICAM-1 or CD54), or IL-6 receptor (12, 15).

Previously, we have shown that nuclear hormone receptors, like retinoid-activated receptors and retinoid X receptors, the vitamin D receptor, and peroxisome proliferator-activated receptors control the allergic immune reaction through inhibition of IgE production (16–20). The mechanisms involved included direct inhibition of the ϵ GLT, as detected upon triggering the retinoid-activated receptors or vitamin D (16, 19). Additionally, retinoid-mediated inhibition of IgE production in B cells involved indirect mechanisms including inhibition of IL-6 production and induction of CD23 surface expression (17).

To date, no data on LXR expression and function in human B cells are available. Our data show that LXR α and LXR β are both constitutively expressed in human B cells and activated by T090137, a synthetic LXR ligand. LXRs mediate a strong inhibition of anti-CD40 and IL-4-mediated IgE secretion by human B cells. We show that indirect IgE-inhibitory mechanisms through reduced JNK-phosphorylation, but also through increased membrane CD23 expression, were relevant in LXR-mediated inhibition of IgE expression. In vivo data proves our findings as reduced allergen-specific IgE response was observed upon administration of LXR ligands to OVA-sensitized mice in a dose-dependent manner. Our data unravel a novel function of LXRs as modulators of B cell activation, suggesting a beneficial function of LXRs in anti-allergic therapy.

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⁴ Abbreviations used in this paper in this manuscript: LXR, liver X receptor; AID, activation-induced cytidine deaminase; ϵ GLT, ϵ -germline transcription; kgbw, kilogram body weight; sCD23, soluble CD23.

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Materials and Methods

Cell culture

After approval by the local ethics committee and informed consent was signed by the probands, B cells were isolated as described previously (19). Briefly, PBMC were isolated from buffy coats of healthy donors by density gradient separation (Ficoll hypaque, Biochrom) and B cells were purified using anti-CD19-coupled magnetic beads (Miltenyi Biotec) by magnetic cell sorting ($\geq 99.0\%$ CD19⁺ B cells, $<0.5\%$ CD3⁺ T cells, and $<0.5\%$ CD14⁺ monocytes, as assessed by flow cytometry). The purified human B cells (10^6 cells/ml) were cultured with and without the synthetic LXR ligand T0901317 (Alexis) at concentrations ranging from 10^{-8} M to 10^{-6} M in RPMI 1640 culture medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% heat inactivated FCS (all from Biochrom). Murine splenic B cells from female BALB/c mice (Federal Institute of Risk Assessment, Berlin, Germany) were obtained following magnetic depletion of other cells with CD43 and CD11b-specific magnetic beads (Miltenyi Biotec; purity $\geq 99.0\%$ B220⁺ B cells, $<0.3\%$ CD3⁺ T cells, $<0.4\%$ CD11b⁺ or CD11c⁺ cells). The murine B cells (7.5×10^5 /ml) were cultured under conditions described above, except that RPMI 1640 was replaced by IMDM culture medium (Biochrom). HL-60 and Jurkat cells were purchased from American Type Culture Collection and cultured according to the manufacturer's instructions. All cell cultures were conducted at 37°C and 5% CO₂ in humidified atmosphere.

RNA extraction and RT-PCR

LXR α/β mRNA expression was determined in freshly isolated purified human CD19⁺ B cells. After 24 h stimulation with the LXR ligand T0901317 (1 μ M), the mRNA expression of the LXR-inducible genes *abca1* and *abcg1* were assayed. For the analysis of α GLT and AID mRNA expression, B cells were stimulated with anti-CD40 (1 μ g/ml; R&D Systems) and IL-4 (5 ng/ml; Immunotools) for 3 days in the absence or presence of the LXR ligand. Total RNA, including a genomic DNase treatment, was extracted using RNeasy Mini Kit (Qiagen) and reverse transcribed by SuperScript synthesis system (Invitrogen) according to the manufacturer's protocol. For quantitative RT-PCR, Roche Lightcycler 1.5 system and FastStart DNA Master SYBR Green I was used. All oligonucleotides (TIB-Molbiol) are displayed in 5'-3' format: *lxra* tct gga gac atc tgc gag gta ca (sense), caa ggc aac ctc ggc atc atc (antisense), *lxrb* aac agc ggc tca aga act aat g (sense), ttg ctt agc gaa gtc cac gat (antisense), *aid* aga ggc gtg aca gtg cta ca (sense), atg tag cgg agg aag agc aa (antisense), *egl* as described in (16). Relative mRNA expression was normalized to the housekeeping gene *hprt* by amplification using atc aga ctg aag agc tat tgt aat gac ca (sense) and tgg ctt ata tcc acc act tgc tgc ccc tgt ggt gga cat agc aat g (antisense) or *pbgd* aga gtg att cgc gtg ggt acc (sense) and ccc tgt ggt gga cat agc aat g (antisense). Relative quantification was performed by $2^{-\Delta\Delta C_T}$ method. Conventional PCR was applied to determine the mRNA expression of the common LXR target genes *abca1* and *abcg1* using Rapidozym-PCR reagents (Rapidozym). The cDNAs were amplified for 30 cycles in a thermocycler (MWG Biotec) using the following oligonucleotides: *abca1* agc cag aaa gac acc agc at (sense), gga acc cag aga agg tt ca (antisense), *abcg1* ttc ttc gtc agc ttc gac ac (sense), gtg cg atc ttc ccg gtc ta (antisense).

Protein isolation and Western blotting

Nuclear extracts of B cells were prepared using NE-PER Extraction Reagent (Pierce) in the presence of protease inhibitor mixture (Protease Complete, Roche). Phosphorylation of JNK was analyzed in the nuclei of B cells preincubated with LXR ligand for 30 min before activation by anti-CD40 and IL-4 for 1 h. Proteins were separated by 12% SDS-gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The A γ s were detected by anti-phosphorylated JNK or anti-JNK Abs and matched secondary Abs conjugated with HRP (Santa Cruz Biotechnology) and visualized by chemiluminescence (ECL Plus, Amersham Biosciences). LXR proteins were detected by specific mAbs anti-LXR α or -LXR β (both Perseus Proteomics) and matched HRP-conjugated Abs (Santa Cruz Biotechnology) as described above.

ELISA

Human B cells (5×10^5 - 10^6 /ml) were cultured for 10 days and Igs were determined in the supernatants by ELISA as described in Ref. 19. Briefly, anti-human IgE clone HP6061 coated on 96-well Maxisorb plates (Nunc) and biotinylated HP6029 (SouthernBiotech) were used and captured IgE was detected by the enzymatic activity of streptavidin-alkaline phosphatase (Zymed Laboratories) and the colorimetric analysis of the cleaved *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) at 405 nm. Detection of IgA, IgG, and IgM were performed with matched Ab pairs (Jackson ImmunoResearch Laboratories) using alkaline phosphatase-conjugated detection

Abs and *p*-nitrophenyl phosphate substrate. Diluted human serum served as standard in which the respective Ig-levels were determined by the Charité Clinical Facility.

Human IL-10 expression was determined in cell-free supernatants of B cells activated by anti-CD40 and IL-4 in the absence or presence of LXR ligand T0901317 for 2–3 days by ELISA according to the manufacturer's instructions (IL-10 DuoSet, R&D Systems).

The serum concentrations of murine total and OVA-specific IgE and IgG subclasses were determined by ELISA as described previously (21). Briefly, 96-well Maxisorb plates were coated with anti-Ig mAbs or OVA (both 5 μ g/ml). Serial dilutions of serum samples and standards were diluted in 1% skimmed milk/PBS and incubated for 2 h. The amounts of subclass-specific Ig and OVA-specific IgG1 and IgG2a were detected by a biotinylated isotype-specific Ab (IgE, IgG1, IgG2a), respectively. The OVA-specific IgE serum levels were determined by anti-IgE (2 μ g/ml clone R35–72, BD Biosciences) coated 96-well Maxisorb plates after incubation of serially diluted sera and detection of bound OVA-IgE Abs following binding of biotinylated-OVA (1.25 μ g/ml). After incubation with streptavidin-HRP (R&D Systems), the colorimetric reaction of peroxidase substrate (tetramethylbenzidine; Sigma-Aldrich) was measured at 450/490 nm after addition of 1 M sulfuric acid. The concentration of Igs was calculated according the standard curve obtained from diluted, pooled serum of all OVA-sensitized mice or purified Igs respectively (all from BD Biosciences).

Ig-ELISPOT assay

The frequency of IgE secreting cells was determined by an ELISPOT assay. Peripheral human B cells were stimulated using anti-CD40 and IL-4 with or without the LXR ligand T0901317 (10^{-6} M) for 6 days. Anti-human IgE (HP6061) or anti-IgA (Jackson ImmunoResearch Laboratories) was coated on MultiScreen-High Protein Binding Immibition-P plates (Millipore). After blocking with 0.3% BSA/PBS, single-cell suspensions of stimulated B cells in serial dilutions were transferred for a 5 h secretion period at 37°C in humidified atmosphere. Secreted Igs were detected using biotinylated anti-IgE, -IgA, or -IgG (all BD Biosciences) and enzymatic staining by streptavidin-HRP of the membrane using 3-amino-9-ethylcarbazole-dimethylformamide (Sigma-Aldrich). Plates were read by an automatic ELISPOT reader (Cellular Technology) and Ig-secreting cells analyzed with Immunospot 4.0 software (Cellular Technology).

Proliferation (CFSE) and cytotoxicity (WST-1) assay

Proliferation of activated B cells was monitored on single-cell level by dilution of CFSE-fluorescence (Invitrogen) as described previously (22). Briefly, freshly isolated B cells were washed and resuspended in PBS at a final concentration of 10^7 cells/ml. CFSE (5 μ M) was added for 3 min at room temperature and the reaction was stopped by washing the cells with RPMI 1640 medium supplemented with 10% FCS. Human B cells were activated with anti-CD40 (1 μ g/ml), IL-4 (5 ng/ml), and IL-21 (50 ng/ml; Immunotools) in the presence or absence of the LXR ligand T0901317 (10^{-8} – 10^{-6} M) for 5 days. Murine B cell proliferation was induced by LPS (40 μ g/ml) and IL-4 (20 ng/ml) as described previously (22) and the impact of additional LXR-activation was assayed after 4 days. Cell proliferation was assessed by FACS analysis and dead cells were excluded using 1 μ M propidium iodide (Invitrogen).

Viability of B cells in the presence of the LXR ligand T0901317 was monitored by quantification of the mitochondrial activity using WST-1 (Roche). This cell-permeable tetrazolium salt is cleaved only in viable cells to a soluble formazan by a mitochondrial dehydrogenase, as described in Ref. 19. Briefly, 10^5 freshly isolated cells were cultured in 100 μ l for 4 days in flat-bottom 96-well plates and 10 μ l WST-1-reagent was added during the last 3 h of culture to each well. Cellular enzymatic activity was determined by measuring optical densities with a photometer at 450 nm.

Animal studies

Female 8-wk-old BALB/c mice (Federal Institute of Risk Assessment) were housed under specific pathogen-free conditions at the animal facility of the Charité Cardiac Research (Charité-Universitätsmedizin Berlin). All procedures of this study were conducted after approval by the local State Office of Health and Social Affairs. On days 1, 14, and 21, mice were sensitized i.p. with 10 μ g OVA adsorbed to 1.5 mg alum (Sigma-Aldrich) or vehicle PBS as control. Starting with the day before the first sensitization (day –1) until day 33, every second day the mice received i.p. either 4 or 20 mg LXR ligand per kilogram body weight (kgbw) or the vehicle (PBS). On day 35, mice were killed and serum was stored at –80°C until analysis.

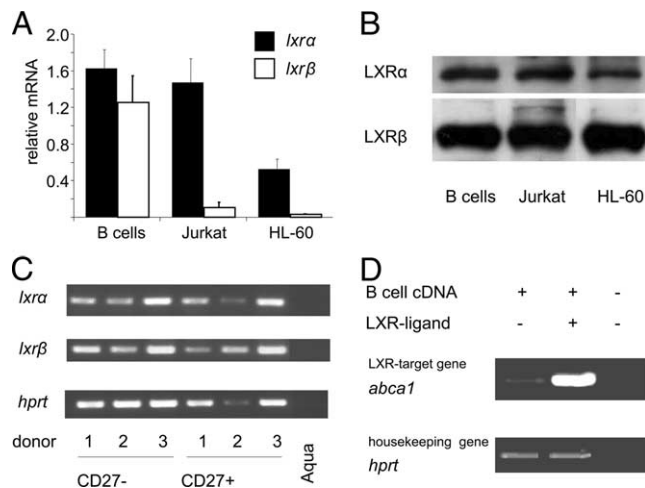


FIGURE 1. Expression of LXR α and LXR β in human B cells. **A**, Peripheral human CD19⁺ B cells, Jurkat (high control), and HL-60 cells (low control) were analyzed regarding their mRNA expression of *Lxr α* (black bars) and *Lxr β* (white bars) relative to the housekeeping gene *hprt* by quantitative RT-PCR. Data show the mean values and SEM of three individual experiments. **B**, The protein expression of LXR α and LXR β was analyzed by Western blot and one representative experiment of three is shown. **C**, LXR α / β mRNA in peripheral CD19⁺ naive B cells (CD27⁻) and memory B cells (CD27⁺) and data show a representative RT-PCR from three different donors. **D**, Expression of the mRNA of the LXR-inducible genes *abca1* in B cells activated by the pan-LXR-agonist T0901317 (10⁻⁶ M) after 18 h culture as detected by RT-PCR.

Statistical analysis

Data were compared by nonparametric analysis using Wilcoxon signed rank test for matched pairs and Mann-Whitney *U* test for independent groups (SPSS 12.0 software). Values of *p* < 0.05 were considered statistically significant.

Results

LXRs are constitutively expressed and functional in human B cells

We determined the molecular basis of LXR action by analysis of the expression of LXR α and LXR β mRNA and protein in peripheral B cells sorted according CD19 expression. Our data show that both LXRs were constitutively expressed in human B cells *ex vivo* (Fig. 1A). *Lxr α* mRNA expression in B cells is comparable with Jurkat cells (positive control) and higher than in HL-60 cells (low control; University of California, Santa Cruz Genome Browser; comparative expression profile GNF Expression Atlas 2 Data from U133A and GNF1H Chips) as detected by quantitative RT-PCR. The LXR α protein was clearly detectable by Western blot analysis of purified B cells (Fig. 1B). *Lxr β* mRNA expression was detected 11.6-fold higher in primary B cells than in Jurkat cells (positive control and Ref. 3) and 42.3-fold higher than in HL-60 cells (low control; University of California, Santa Cruz Genome browser; see above) (Fig. 1A). Accordingly, human B cells expressed LXR β protein (Fig. 1B). In both major peripheral human B cell subsets, naive and memory B cells sorted according to CD27 expression, *Lxr α* and *Lxr β* mRNA was constitutively expressed (Fig. 1C), and not altered by stimulation using anti-CD40 and IL-4 within the first 3 days (data not shown). To demonstrate biological functionality of the LXRs in B cells, we studied the induction of the common LXR-target genes *abca1* (also cholesterol efflux regulatory protein) and *abcg1* after 24 h of LXR-stimulation. Our data show strong induction of *abca1* mRNA expression in B cells in the presence of the LXR ligand T0901317 (Fig. 1D), suggesting that LXR-

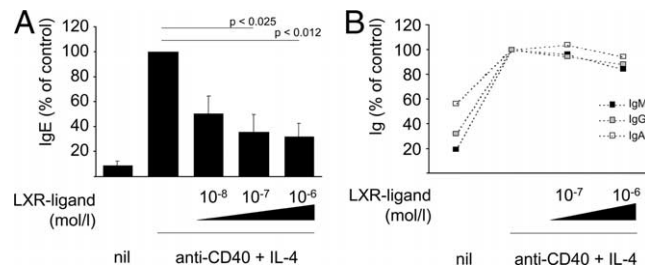


FIGURE 2. LXRs inhibit IgE expression in human B cells. Peripheral human B cells were activated by anti-CD40 (1 μ g/ml) and IL-4 (5 ng/ml) and the LXR-agonist T0901317 (10⁻⁸–10⁻⁶ M) and concentrations of IgE (**A**) and IgM (**B**, black squares), IgG (gray squares) and IgA (white squares) were determined after 10 days cell culture. Data show mean and SEM of six independent experiments.

specific signaling is activated by this treatment. Comparable data was obtained for *abcg1* (data not shown).

LXRs inhibit IgE synthesis of human B cells activated by anti-CD40 and IL-4

Next, we analyzed the impact of LXRs on IgE production in peripheral human B cells activated by anti-CD40 and IL-4 in the presence of the LXR ligand T0901317. Stimulation of B cells by anti-CD40 plus IL-4 induced IgE expression up to 18-fold (7.0 ng/ml $\pm$ 1.4; unstimulated 0.4 ng/ml $\pm$ 0.2) and was inhibited upon LXR activation in a dose-dependent manner starting at a concentration of 10⁻⁸ M with maximal inhibition of 73% at 10⁻⁶ M (2.6 ng/ml $\pm$ 1.2; Fig. 2A). The other Ig classes such as IgA, IgG, and IgM were less induced by anti-CD40 and IL-4 stimulation (2–4-fold regarding the isotype to 48.4 μ g/dl $\pm$ 12.3 IgA, 55.1 μ g/dl $\pm$ 9.6 IgG, 67.8 μ g/dl $\pm$ 21.2 IgM) than observed for IgE and not altered by LXRs at 10⁻⁶ M (Fig. 2B). Upon additional triggering of the anti-CD40 and IL-4-stimulated B cells with the mitogen IL-21 (23), the IgE induction was 10 times stronger (84.22 ng/ml $\pm$ 4.3). The secretion of IgA, IgG, and IgM by B cells was also 2–10-fold increased by IL-21 compared with anti-CD40 plus IL-4 (to 97.4 μ g/dl $\pm$ 5.1, 767.6 μ g/dl $\pm$ 141.6, and 129.5 μ g/dl $\pm$ 28.2, respectively; data not shown). In the presence of LXR ligands, the IgE expression was reduced in a dose-dependent manner with a maximal inhibition of 76.6% $\pm$ 3.1, whereas the other isotype classes IgA (+1.7%) and IgG (+5.3%) and IgM to a lesser extent (-36.2% $\pm$ 9.3) were not changed. Thus, although the amounts of secreted Igs of anti-CD40 and IL-4-stimulated B cells was different upon additional IL-21 stimulation, the magnitude of LXR-mediated modulation of the Ig-profile was comparable.

To dissect whether LXR-mediated signaling reduced the number of IgE-secreting cells or the amount of secreted Abs per cell, we determined the Ab secretion by ELISPOT assays. Initial studies of the kinetics show a peak Ab secretion after 6 days in anti-CD40 and IL-4-stimulated B cells (data not shown). Upon activation of B cells with anti-CD40 and IL-4, IgE secreting cells (Fig. 3A), but also IgA-secreting cells were detectable (data not shown). As shown in Fig. 3B, additional activation of LXRs clearly reduce the number of IgE-secreting cells (-64% $\pm$ 2.1). In contrast, the number of IgA-secreting cells was not significantly diminished by LXRs (-13% $\pm$ 10.0).

LXR activation does not impair B cell proliferation and viability

The impact of LXRs on the proliferation or viability of activated B cells was investigated by CFSE dilution of B cells on single-cell

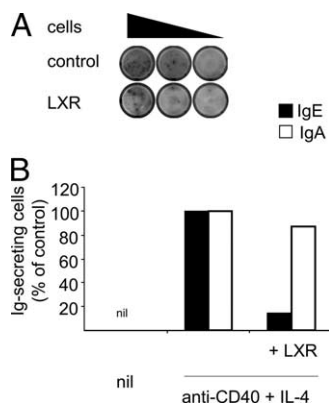


FIGURE 3. LXRs reduce the number of IgE secreting cells. *A*, CD19⁺ B cells were activated by anti-CD40 (1 μ g/ml) and IL-4 (5 ng/ml) with or without the LXR-agonist T0901317 (10^{-6} M) for 6 days and Ig-secreting cells were detected by ELISPOT from serial dilutions. *B*, Analysis of IgE and IgA secreting cells as described above. Data show one representative experiment of three.

level and mitochondrial enzymatic activity. CFSE-labeled human B cells were activated by anti-CD40, IL-4, and IL-21 in the presence or absence of LXR ligand T0901317 for 5 days. Cell counts and trypan blue staining showed comparable viable cell numbers of activated B cells independent from the presence of LXR ligand (data not shown). Flow cytometric analysis identified that B cell proliferation was strongly induced in the presence of anti-CD40, IL-4, and IL-21 in 46.1 to 77.0% of the cells, depending on the donor (Fig. 4*A*). Upon addition of the LXR ligand, the B cell proliferation was not altered significantly (-7.1% CFSE^{low} $\pm$ 2.7; mean fluorescence intensity $28.1\% \pm 1.7$; $p > 0.05$). Comparable data was obtained from anti-CD40 and IL-4-stimulated B cells in the absence or presence of the LXR ligand ($p > 0.05$), although

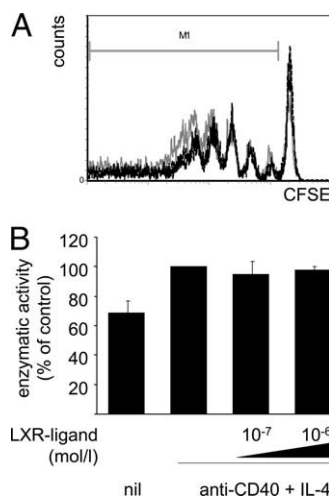


FIGURE 4. Proliferation and viability is not impaired by LXRs in human B cells. *A*, The proliferation of CFSE-labeled B cells stimulated by anti-CD40 (1 μ g/ml), IL-4 (5 ng/ml), and IL-21 (50 ng/ml) in the presence of additional LXR ligands (10^{-6} M, black line; 10^{-7} M, black dotted line) was determined after 5 days by flow cytometry. The histogram overlay is gated on live, activated B cells. Data show a representative of three similar experiments. M1 includes proliferated B cells. *B*, The mitochondrial NADH+H⁺ enzymatic activity of B cells correlating with cell viability was assayed using WST-1-assay after 3 days of activation by anti-CD40 (1 μ g/ml) and IL-4 (5 ng/ml). Data show mean values and SEM from three independent experiments.

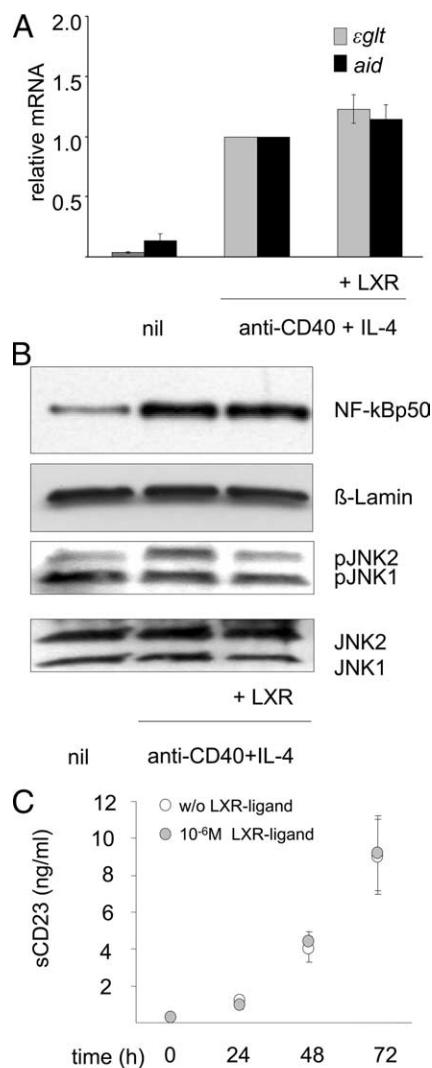


FIGURE 5. Mechanisms of activated LXRs mediating reduced IgE expression by human B cells. *A*, B cells were activated by anti-CD40 (1 μ g/ml) and IL-4 (5 ng/ml) and the LXR-agonist T0901317 (10^{-6} M) for 3 days and relative ϵ -switch transcript and *aid* mRNA expression was detected by quantitative RT-PCR. Data show mean values and SEM of four independent experiments relative to porphobilinogen desaminase house-keeping gene expression. *B*, Nuclear translocation of NF- κ Bp50 and phosphorylated JNK in activated B cells as described above and detected by Western blot. Data show one representative experiment of four. *C*, sCD23 expression in the supernatants of B cells activated for 1–3 days as indicated. Data show mean values and SEM of four independent experiments. w/o, Without.

the induction was less efficient and only 4–7% of the B cells proliferated 1–2 times.

To investigate whether LXR-activation hampers the B cell viability, mitochondrial enzymatic activity that was shown to correlate with cell viability was determined from activated B cells as described above after 3 days culture. Data show an increased mitochondrial activity of $\sim 30\%$ upon activation, which was not significantly altered by activation of LXRs (Fig. 4*B*).

These data indicate that anti-CD40 and IL-4-mediated B cell proliferation and viability was not impaired by activation of LXRs.

ϵ GLT and AID transcription are independent from LXR activation

Expression of the *εglt* and the enzyme AID are initial events and prerequisites for IgE class switch recombination (12). Therefore,

Table I. LXRs induce CD23 surface expression^a

	1	2	3	4
CD23				
Unstimulated	1.6/15.4	2.5/37.0	1.5/41.8	11.5/59.4
CD40 + IL-4	32.0/30.4	31.0/75.5	46.7/92.8	43.2/97.7
Add LXR	40.6/39.5	56.0/83.0	56.0/99.6	55.1/116.4
CD69				
Unstimulated	20.5/15.3	24.6/30.5	19.3/53.2	25.5/4.2
CD40 + IL-4	73.8/29.6	61.0/39.5	57.1/63.0	67.3/17.0
Add LXR	76.2/28.7	62.8/38.9	68.9/65.1	65.4/18.6

^a CD19⁺ cells were activated by anti-CD40 and IL-4 for 2 days and the role of LXRs (1 μ M) on CD23 and CD69 was analysed. Data show percentage of positive cells/mean fluorescence intensity of live CD19⁺ B cells from four independent experiments.

the expression of the *ε*glt and *aid* mRNA was examined in B cells activated by anti-CD40 and IL-4 in the presence of the LXR ligand T0901317 by quantitative RT-PCR. As shown in Fig. 5A, expression of *ε*glt and *aid* mRNA was induced by anti-CD40 plus IL-4 in B cells (30.2 ± 5.9 -fold and 14.8 ± 3.8 -fold, compared with unstimulated control, respectively). Upon additional LXR triggering, comparable results were obtained for both genes (*ε*glt: 39.3 ± 5.8 -fold; *aid* mRNA: 16.0 ± 4.0 -fold, compared with unstimulated control).

A key transcription factor initiating *ε*glt, but also *aid* transcription, is NF- κ Bp50. It is activated by CD40 signaling, translocates into the nucleus, and activates the promoter region of the *ε*-switch transcript and the *aid* gene. As different nuclear hormone receptors can reduce NF- κ B activation, we investigated the impact of LXR activation on NF- κ B translocation in activated B cells. Our data show that the expression of NF- κ Bp50 is increased in the nuclear extracts of B cells triggered by anti-CD40 and IL-4, though not significantly diminished by additional LXR activation (Fig. 5B).

As the activation of JNK has been reported to mediate IgE production independently from *ε*-switch transcript expression in B cells (24), we examined the role of LXR ligand T0901317 on the phosphorylation of JNK proteins in activated B cells. Initial studies on the kinetics of anti-CD40 plus IL-4-induced phosphorylation of JNK2 (p-JNK2) in human B cells showed a peak of phosphorylation after 10 min, whereas the p-JNK1 and the nonphosphorylated JNK protein remain unchanged (data not shown). Activation of B

cells by anti-CD40 and IL-4 increased pJNK2 nuclear expression (Fig. 4B). In B cells preincubated with the LXR ligand T0901317 for 30 min before stimulation, a marked reduction of JNK2 phosphorylation was detected, whereas pJNK1 remained unchanged.

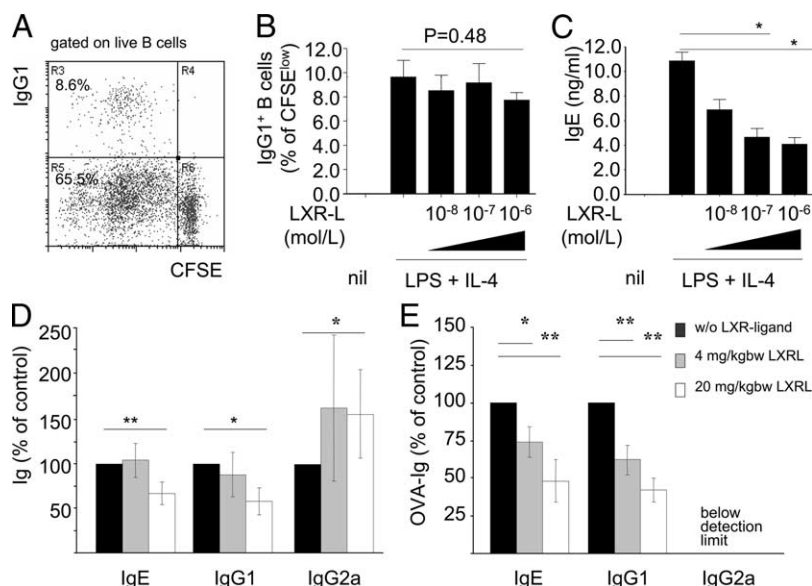
LXR action results in enhanced CD23 surface expression on B cells

The low-affinity receptor for IgE CD23 is induced in B cells by IL-4-mediated STAT6 phosphorylation (25). As membrane CD23 inhibits IgE production via a negative feedback loop (26–28), we investigated the role of LXR activation in CD23 expression by B cells. Our data show that membrane CD23 expression by B cells is strongly induced by anti-CD40 and IL-4 within the first 2 days of cell culture ($38.2\% \pm 3.4$ positive cells, unstimulated $4.3\% \pm 1.8$; Table I). Upon additional triggering of the LXRs, the frequency of membrane CD23⁺ B cells was increased up to 30% ($51.9\% \pm 2.8$). The surface expression of the very early activation Ag CD69 was not altered on activated B cells by LXRs (64.8 ± 2.8 vs 68.3 ± 2.1 ; Table I). By proteolytic shedding, the soluble form of CD23 (sCD23; IgE-binding factor) is cleaved and involved in promoting IgE synthesis (29). Our data show that paralleling the membrane CD23 expression by B cells, its soluble form is released in the supernatant upon activation by anti-CD40 and IL-4, which is not modulated by activated LXRs (Fig. 5C).

LXRs modulate the allergen-specific humoral immune response

To investigate whether the LXRs-mediated inhibition of IgE synthesis in human B cells may also be relevant in vivo, we determined the humoral immune response in OVA-sensitized mice undergoing systemic LXR-treatment and the impact of LXRs on murine B cell proliferation, isotype class switching, and Ig secretion in vitro. The data show that naive murine B cell proliferation is strongly induced by LPS and IL-4, as in $76.1\% \pm 2.3$. CFSE dilution was observed after 4 days (exemplary staining in Fig. 6A and data not shown). As isotype class switch recombination to IgG1 and IgE is induced by LPS and IL-4, but valid detection of IgE switched cells is technically limited, we determined the frequency of B cells expressing IgG1 and thus had completed DNA recombination ($9.6\% \pm 1.4$ of all proliferated cells, Fig. 6A). Upon additional LXR activation, neither the proliferation ($p > 0.05$; data not shown), nor the frequency of IgG1^{positive} B cells was significantly modulated (Fig. 6B). In contrast, the expression of

FIGURE 6. LXRs inhibit IgE production in vitro and in vivo. Murine naive splenic B cells were triggered by LPS (40 μ g/ml) and IL-4 (20 ng/ml). A and B, Proliferation of CFSE-labeled B cells and IgG1 expression was analyzed after 4 days of cell culture ($n = 4$). The dot blot was gated on live activated B cells. C, IgE secretion from murine B cells was determined from the supernatants after 8 days of cell culture as described above ($n = 4$). BALB/c mice were sensitized with OVA and concomitantly treated with the pan-LXR ligand T0901317 (LXRL; 4 and 20 mg/kg body weight; gray and white bars) or vehicle (black bars). Concentrations of total (D) and allergen-specific (E) Igs were determined from the sera by ELISA. Data show mean values and SEM of each group ($n = 12$). w/o, Without. *, $p < 0.05$; **, $p < 0.01$.



secreted IgE induced by LPS and IL-4 ($10.9 \text{ ng/ml} \pm 0.7$) was strongly reduced upon LXR triggering in a dose-dependent manner up to $-62.4\% \pm 3.6$ (Fig. 6C). These data show that the ex vivo induced IgE expression was directly inhibited by LXRs in murine B cells. As the murine B cell proliferation and activation-induced isotype class switch recombination were not altered by LXRs, these data were similar to those obtained from human B cells.

In vivo, OVA immunization of BALB/c mice induced prominent specific IgE and IgG1 responses and low IgG2a levels (Fig. 6). The serum-Ig concentrations of total IgE and IgG1 were decreased by LXR ligands in a dose-dependent manner (maximum, $-33\% \pm 13$ and $-42\% \pm 15$), while IgG2a was induced (maximum, $57\% \pm 50$, $p > 0.05$; Fig. 6A). Furthermore, treatment of sensitized mice with LXR ligands resulted in a decreased allergen-specific IgE response in the serum in a dose-dependent manner (4 or 20 mg/kgbw LXR ligand ($-32\% \pm 7$ or $-52\% \pm 12$, respectively); Fig. 6B). A similar action of LXRs was observed on OVA-specific IgG1 concentrations, upon treatment with 4 mg/kgbw LXR ligand ($-35\% \pm 5$) or with 20 mg/kgbw LXR ligand ($-42\% \pm 8$; Fig. 6B). OVA-specific IgG2a was not detectable in any sample.

Treatment of the mice with the LXR ligand was well tolerated. No increased mortality was observed and postmortem examinations did not show any visceral or vascular changes (data not shown).

Discussion

Here we show that B cells constitutively express the LXR- α and - β . Their activation limits anti-CD40 and IL-4-induced differentiation of B cells into IgE-secreting plasmablasts. Secretion of Ig classes such as IgA, IgG, and IgM, as well as B cell viability or proliferation, are not significantly affected by LXRs in this condition. IgE class switch recombination is not altered directly by LXR activation, while indirect IgE-inhibitory pathways are relevant. These include phosphorylation of the JNK in activated B cells and membrane CD23 expression. The biological relevance of our findings in vivo is highlighted by showing that LXR-agonist treatment in allergen-sensitized mice inhibits specific IgE and IgG1 humoral immune responses.

IgE is the key effector molecule of type-I-allergic immune reactions, which results from a Th2-dominated B cell activation leading to the differentiation of IgE-secreting cells (12). Exposition of sensitized individuals to the respective allergen generate new allergen-specific IgE-producing cells from memory B cells, e.g., as observed during pollen season (30). These newly formed plasmablasts can migrate to survival niches and become long-lived plasma cells, secreting IgE antibodies for many years (31). Because long-lived plasma cells are refractory to established treatments, strategies are needed to limit the generation of new IgE-producing cells.

Our data show that LXR-mediated inhibition of anti-CD40 and IL-4-induced IgE-production correlates to a decreased number of IgE-producing cells. The expression of ϵ -switch transcript expression and *aid* expression is not altered by LXRs, suggesting interference of LXRs with B cell differentiation and not IgE class switch recombination. This is in accordance with data obtained from a different nuclear receptor that was recently identified to limit the transition from activated B cells to Ab-secreting cells (32), although in the presence of IL-4 IgE class switching was additionally blocked (19).

Inhibition of the NF- κ B signaling pathway by LXRs through stabilization of I κ B was reported recently as the underlying mechanism in LXR-mediated inhibition of homocystein-driven IgG production in murine B cells (33). Our data provide evidence that CD40-mediated activation of NF- κ B is not impaired by LXRs, as

shown by unimpeded nuclear translocation of NF- κ Bp50. Additionally, the expression of two strictly NF- κ B-dependent genes (12), namely the ϵ -switch transcript and *aid* mRNA, are independent of LXR activation. These different results may be due to different in vitro conditions and/or species-dependent differences of LXR-mediated gene regulation (34).

AID plays an essential role in isotype class switch recombination and somatic hypermutation (14, 35). AID is induced in B cells by IL-4 and CD40-signaling through activated STAT6 and NF- κ Bp50, respectively (36). Our data show that activation of LXRs did not diminish *aid* mRNA expression, indicating that both required synergistic signaling pathways, namely NF- κ Bp50 and STAT6, are fully operational in the presence of LXRs. This hypothesis is also supported by our findings that nuclear translocation of activated NF- κ Bp50 was independent of LXR activation and the STAT6-dependent membrane CD23 expression was even enhanced by LXRs.

Modulation of JNK and membrane CD23 are possible candidates for inhibition of IgE production, independent of ϵ -switch transcript expression. Indeed, our data demonstrate that LXR-mediated inhibition of anti-CD40 and IL-4-driven IgE production is paralleled with reduced phosphorylation of JNK, whereas the ϵ -switch transcript expression is not affected by LXR activation. Similar results were previously reported upon addition of a JNK-specific inhibitor to anti-CD40 and IL-4 activated B cells (24), suggesting that LXR-mediated inhibition of JNK2-phosphorylation as a mechanism involved in LXR-mediated inhibition of IgE production.

The surface molecule CD23, also called low-affinity receptor for IgE, inhibits IgE expression in B cells via a negative feedback loop (26–28) by two independent mechanisms. Expression of CD23 on the surface of cells in the vicinity of activated B cells undergoing IgE isotype switching blocks their differentiation to high-producing IgE-secreting plasmablasts independently of ϵ -switch transcript expression (26). Additionally, CD23 signaling directly limits the anti-CD40 and IL-4 mediated IgE production, as shown in genetically targeted CD23-overexpressing B cells (26). These two mechanisms nicely correlate with our findings that upon LXR activation, the frequency of B cells expressing membrane CD23 was increased, but the number of cells secreting IgE was markedly decreased, independent of *εglt*. Our data indicate that differentiation of IgE-secreting plasmablasts may be blocked by LXRs through increased expression of membrane CD23. However, the shedding of sCD23, as detected in the supernatants of activated B cells, is not impaired by LXRs, indicating that transcriptional up-regulation of CD23 is relevant.

The biological significance of LXR activation on IgE production was also proven in vivo by administration of the synthetic pan-LXR agonist T0901317 to OVA type-I-sensitized BALB/c mice. Our data show that the total, but also allergen-specific IgE responses and to lesser extent IgG1 levels, were diminished upon LXR activation. In isolated murine B cells, as also observed in activated human B cells, activated LXRs strongly inhibited IgE expression, which is not associated with an impaired proliferation or isotype class switch recombination. Because both isotypes are induced by T cell help in the presence of IL-4 in mice and humans (12), LXRs might act exclusively directly in B cells on a parameter common to the plasmablast-secreting IgE and IgG1 but not IgG2a. This assumption is underlined by the observation that the IgG2a serum levels are not altered upon LXR treatment. Thereby, our data indicate that activated LXRs directly modulate activation of B cells in vivo.

To date, the therapeutic potential of LXRs in human is limited by some hurdles, such as transient hyperlipidaemia and intrahepatic lipogenesis in vivo (8, 37), which is mediated by SREBP1c (2). Upon development of specific LXR agonists mediating anti-allergic properties, species-dependent differences of the LXR-mediated genetic program must be considered (1, 34), e.g., CYP7a1, an important enzyme in bile acid synthesis, is induced by murine LXRs, but not by humans. Nevertheless, our data show that the inhibitory action on IgE production was conserved in human and mice. Still, the high similarity between the ligand-binding domains of LXR α and LXR β hampers selective receptor targeting (38), but LXR β -selective agonists are proposed to circumvent LXR α -mediated hepatic lipogenesis (1). The high expression of LXR β in human B cells and its control function on IgE production by activated B cells suggest that targeting LXR β in B cells may be beneficial in novel antiallergic therapy. A putative supportive function of LXRs in the treatment of allergic asthma was also observed recently in nonleukocytes. In human smooth muscle cells, LXRs inhibit the expression of multiple cytokines in response to proinflammatory mediators, including GM-CSF, and also reduces tissue remodelling through inhibited proliferation and migration (39).

In conclusion, our data show a novel function of LXRs in human B cells in the control of IgE synthesis. Thus, the development of cell- and receptor-selective LXR agonists modulating B cell activation more specifically may contribute to improve novel protocols in antiallergic therapy.

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Disclosures

The authors have no financial conflict of interest.

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