

# Liver X receptors regulate dendritic cell phenotype and function through blocked induction of the actin-bundling protein fascin

René Geyeregger,<sup>1</sup> Maximilian Zeyda,<sup>1</sup> Wolfgang Bauer,<sup>2,3</sup> Ernst Kriehuber,<sup>2,3</sup> Marcus D. Säemann,<sup>4</sup> Gerhard J. Zlabinger,<sup>5</sup> Dieter Maurer,<sup>2,3</sup> and Thomas M. Stulnig<sup>1</sup>

<sup>1</sup>Department of Internal Medicine III, Clinical Division of Endocrinology and Metabolism, Medical University of Vienna, Austria; <sup>2</sup>Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Austria; <sup>3</sup>Center of Molecular Medicine (CeMM) of the Austrian Academy of Sciences, Vienna, Austria; <sup>4</sup>Department of Internal Medicine III, Nephrology and Dialysis, Medical University of Vienna, Austria; <sup>5</sup>Institute of Immunology, Medical University of Vienna, Austria

**Liver X receptors (LXRs) are nuclear receptors regulating lipid and cholesterol metabolism. Recent data revealed a cross talk between LXR and Toll-like receptor signaling in macrophages, indicating a role in immunity. Here, we show that LXR $\alpha$  is expressed in human myeloid dendritic cells (DCs) and induced during differentiation of monocyte-derived DCs, whereas LXR $\beta$  is expressed constitutively at a very low level. LXR activation by 2 different LXR agonists strongly interfered with lipopolysaccharide (LPS)–**

**induced but not with CD40L-induced DC maturation by altering DC morphology and suppressing interleukin-12—but enhancing interleukin-10—secretion. LXR activation in DCs largely blocked their T-cell stimulatory ability despite essentially unaltered expression of various antigen-presenting and costimulatory molecules. Immunologic synapse formation was significantly inhibited by LXR activation along with a complete block in LPS—but not CD40L-induced expression of the actin-bundling protein fascin. Notably,**

**overexpression of fascin in LXR agonist-treated DCs restored immunologic synapse formation and restored their ability to activate T cells. In conclusion, our data reveal LXR as a potent modulator of DC maturation and function mediated in part by blocking the expression of fascin. Due to the central position of DCs in immunity, LXR $\alpha$  could be a potential novel target for immunomodulation. (Blood. 2007;109:4288–4295)**

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## Introduction

Liver X receptors (LXR $\alpha$  and LXR $\beta$ ) are nuclear receptors that are activated by oxidized cholesterol (oxysterols) or synthetic agonists such as T0901317 or GW-3965. LXRs dimerize with retinoid X receptors to bind to its response elements in the promoters of target genes.<sup>1–4</sup> LXR $\alpha$  is highly expressed in metabolic active organs such as liver, adipose tissue, and kidney, but also in immunocompetent cells such as macrophages, whereas LXR $\beta$  occurs ubiquitously.<sup>5</sup> LXRs have been shown to be implicated in cholesterol, fatty acid, and glucose metabolism.<sup>6</sup> Moreover, LXR cross talk with inflammatory signaling pathways in macrophages particularly Toll-like receptor (TLR) signaling has been shown.<sup>6–8</sup> LXR agonists inhibit the lipopolysaccharide (LPS)/TLR4-induced expression of inflammatory genes by interfering with nuclear factor- $\kappa$ B signaling in macrophages.<sup>8</sup>

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that are crucial as a linker between innate and adaptive immune responses and play a key role in inflammatory diseases such as atherosclerosis, rheumatoid arthritis, and allograft rejection.<sup>9–12</sup> After TLR activation, DCs transform from antigen-capturing immature DCs (iDCs) into mature DCs (mDCs), a process characterized by up-regulation of T-cell stimulatory cell surface molecules, secretion of inflammatory cytokines, and optimal T-cell stimulatory potency.<sup>13,14</sup> Activation of T cells by APCs in vivo depends on formation of an immunologic synapse (IS).<sup>15</sup> This specialized contact zone between T cells and the APC is dependent

on reorganization of cytoskeletal proteins in both participating cell types.<sup>16,17</sup> Active cytoskeletal changes result in the dynamic clustering of T-cell surface receptors and signaling molecules at the interface with the APCs.<sup>15,18</sup> During IS formation, DCs actively polarize filamentous actin (F-actin) toward the interface with resting T cells. Fascin is a DC-specific actin-bundling protein expressed only in mature but not in immature DCs<sup>19</sup> that is required for actin polarization in DCs during IS formation. Moreover, its expression is a prerequisite for full T-cell activation.<sup>16,19</sup> In addition to initiation of immune responses, DCs are implicated in the induction of central and peripheral tolerance under conditions of hampered DC maturation.<sup>9,20–23</sup> Hence, interfering with DC maturation could be a promising option for immunosuppressive strategies in organ transplantation and treatment of inflammatory diseases.<sup>24</sup>

The biologic role of LXRs in DCs is unknown so far. We show here that LXR $\alpha$  is highly expressed in human DCs and that treatment of DCs with LXR agonists affects DC phenotype and function particularly following LPS/TLR4-induced maturation. Thereby, LXR activation leads to impaired IS formation and markedly reduced capacity to promote T-cell activation in mDCs. Molecular studies revealed that the inhibitory effects of LXRs on DC-mediated T-cell activation are caused by a failure of LPS-stimulated DCs to express fascin. Thus, LXRs not only exert inhibitory effects on the innate immune system, but also affect adaptive immunity by interfering with DC maturation and function.

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These findings indicate that LXR $\alpha$  could be an interesting novel drug target for immunosuppressive agents.

## Materials and methods

### Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from acid citrate dextrose (ACD) buffy coats of healthy (70% male; no sex-specific differences were observed; data not shown) donors by density gradient centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). For isolation of myeloid DCs, PBMCs were depleted from monocytes by anti-CD14-conjugated magnetic MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood myeloid DCs (purity > 80%) were magnetically isolated from CD14<sup>+</sup> cells using the CD1c (BDCA-1) Dendritic Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Myeloid DCs ( $2 \times 10^5$  cells/mL) were directly analyzed or cultured for 2 days in culture medium consisting of RPMI 1640 (Invitrogen, Groningen, the Netherlands) including 50 U/mL penicillin and 50  $\mu$ g/mL streptomycin, 2 mM glutamine (Invitrogen), and 10% fetal bovine serum (Hyclone, Logan, UT).

For monocyte-derived DC differentiation, CD14<sup>+</sup> monocytes were isolated from PBMCs by magnetic cell sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec) achieving a purity of 95% to 99%. DCs were differentiated and matured as described<sup>25</sup> in RPMI including supplements as described for myeloid DCs. LXR agonists T0901317 (Calbiochem, San Diego, CA) and GW-3965 (generously provided by T. Willson and J. Collins; GlaxoSmithKline, Philadelphia, PA) were added at day 2 at 2  $\mu$ M and did not affect cell viability as assessed by propidium-iodide (Sigma, St Louis, MO) staining. Key experiments were repeated with addition of LXR agonists at day 5 yielding essentially the same results (not shown). For morphologic and immunofluorescence analysis, cells were embedded with mounting medium Vectashield (Vector Laboratories) and evaluated by light microscopy on an Aristoplan 307 microscope (Leitz, Wetzlar, Germany) using a Pl Fluotar Phaco 2 40 $\times$ /0.70 objective lens. Images were captured with a 300F camera (Leica Microsystems, Wetzlar, Germany) and analyzed by Leica IM500 software.

CD3<sup>+</sup> T cells (purity > 95%) were isolated by magnetic depletion of non-T cells, as described.<sup>26</sup> For highly purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells (purity > 98%), additional beads labeled with mAbs against CD19 (J4.119), CD56 (C218), CD34 (581), CD41 (P2), glycophorin A (11E4B7.6), and CD8 (B9.11) or CD4 [13B9.2] were included.<sup>27</sup> All antibodies were purchased from Immunotech (Marseille, France). Naive CD4<sup>+</sup>CD45RA<sup>+</sup> T-helper cells (purity > 85%) were isolated from PBMCs using the naive CD4<sup>+</sup> T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

### Gene expression analysis

Total RNA was isolated and reverse-transcribed as described,<sup>25</sup> with the modification of adding Glycoblue (Ambion, Austin, TX) for coprecipitation. Expression of LXR $\alpha$ , LXR $\beta$ , and RXR $\alpha$  mRNAs was quantitated by quantitative real-time reverse-transcription-polymerase chain reaction (QRT-PCR) using Taqman Assays-on-Demand (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and normalized to 18S RNA using the comparative dCt-method.<sup>28</sup>

### Cytokine production and cell surface marker expression

Cytokines secreted by DCs (interleukin-12p40 [IL-12p40], IL-12p70, and IL-10) and T cells (IL-2, IFN- $\gamma$ , IL-4, IL-5, IL-10, and IL-13) were analyzed by enzyme-linked immunosorbent assay (ELISA) as described<sup>29</sup> or by commercially available kits (all R&D Systems, Minneapolis, MN). Surface molecule expression was analyzed by FITC-labeled anti-CD40 (Immunotech) and anti-human leukocyte antigen-DR (HLA-DR), and PE-labeled anti-CD80, anti-CD86, anti-HLA-ABC, and anti-mannose receptor (MR; all BD Biosciences, San Jose, CA). For analyzing T-cell activation, T cells were stained with FITC-labeled

anti-T-cell receptor (TCR) or allophycocyanin-labeled anti-CD3, together with PE-labeled anti-CD25 or anti-CD69 (BD Biosciences), respectively. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

### Mixed leukocyte reaction (MLR)

DCs were irradiated (30 gray, <sup>137</sup>Cs source), washed, and subsequently added at increasing cell numbers to  $1 \times 10^5$  allogeneic CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup> T cells in 96-well culture plates, and proliferation was determined after pulsing with 1  $\mu$ Ci (0.037 MBq) [<sup>3</sup>H]-thymidine (ICN Pharmaceuticals, Irvine, CA) at day 4 of culture as described.<sup>26</sup> To assess cytokine production and activation markers of T cells in MLR, mature DCs ( $2 \times 10^5$ /well) were cocultured with  $1.5 \times 10^6$  CD3<sup>+</sup> T cells for 2 days in 12-well plates. For restimulation experiments, T cells from day 4 of the MLR were washed and rested for 24-hour culture medium including supplements as described above and restimulated with 10 ng/mL PMA (Sigma) and 1  $\mu$ M ionomycin (Sigma) for 96 hours. Subsequently, the cell-free supernatant was analyzed for cytokine production.

### Transfection of DCs

A pEGFP-C1-fascin-1 plasmid containing the full-length of human fascin-1 coding sequence (1.5 kb, GenBank U09873) was generously provided by Josephine C. Adams (Lerner Research Institute, Ohio) and used for transfection along with pEGFP-C1 empty vector plasmid (Clontech Laboratories, Palo Alto, CA). After 1-day maturation of DCs with LPS, the cells were harvested, washed with PBS, and resuspended in the specified electroporation buffer (Amaza, Cologne, Germany) to a final concentration of  $2 \times 10^6$  cells/mL. Plasmid DNA (15  $\mu$ g) was mixed with 0.1 mL cell suspension, transferred to a 2.0-mm electroporation cuvette, and transfected with an Amaza Nucleofector apparatus (Amaza). After electroporation, cells were immediately transferred to 2 mL culture medium and cultured for 24 hours in 6-well plates at 37°C. Optionally, transfected pEGFP-C1-positive and pEGFP-C1-fascin fusion protein-positive cells were subjected to fluorescence-activated cell sorting (FACS, FACSARIA; BD Biosciences) and were used for further experiments. The viability of DCs directly and 24 hours after transfection was 90% and 50%, respectively, whereas 48 hours after transfection the viability was essentially less than 20%, which was not sufficient for activating peripheral T cells in our study. The purity of sorted cells was generally more than 98%.

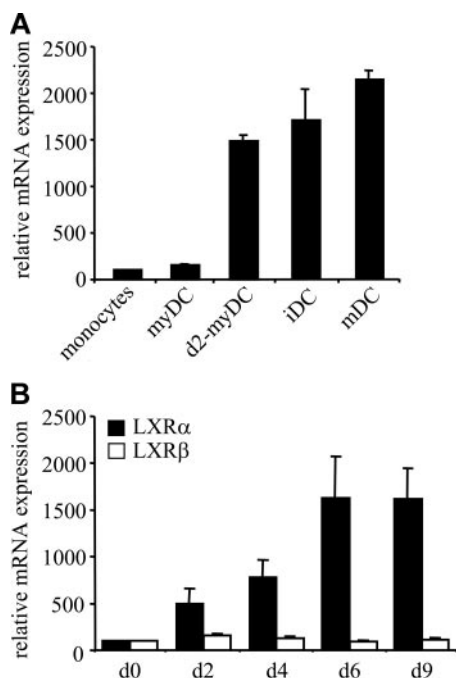
### Analysis of IS formation

For superantigen stimulation, transfected or CellTracker Orange (CMTMR; Molecular Probes, Eugene, OR)-labeled untransfected DCs were pulsed with 5  $\mu$ g/mL staphylococcal enterotoxin E (SEE; Toxin Technology, Sarasota, FL) in Hank balanced salt solution (HBSS) at 37°C for 30 minutes. After washing, DCs were incubated with Jurkat E6-1 T cells at a ratio of 1:1 at 37°C for 30 minutes. Of note, Jurkat T cells spontaneously form conjugates with DCs even in unstimulated cells (data not shown). Relocalization of CD3 and PKC $\theta$  toward the IS was analyzed as described.<sup>30</sup> The percentage of T cells forming conjugates with DCs was determined by counting at least 100 T-cell/DC conjugates per blinded sample by 2 individuals. No relocalization of CD3 was seen without SEE stimulation (data not shown).

### Intracellular fascin staining

For intracellular fascin staining, cells were permeabilized with methanol for 30 minutes at room temperature, and washed twice with PBS/1% BSA (Sigma). Cells were stained with anti-human fascin 1 mAb 55K-2 from Santa Cruz Biotechnology (Santa Cruz, CA) followed by staining with FITC-labeled goat anti-mouse F(ab')<sub>2</sub> Ab (DAKO, Glostrup, Denmark). All antibodies were incubated for 30 minutes at 4°C. After washing with PBS/1% BSA, fascin staining of cells was analyzed on a FACSCalibur.

For immunofluorescence images, cells ( $1 \times 10^6$ ) were mechanically detached from plates washed with PBS and allowed to settle on poly-L-lysine-coated slides (Marienfeld, Lauda-Koenigshofen, Germany) for 30 minutes on ice. For fascin staining, cells were permeabilized with



**Figure 1. LXR $\alpha$ , LXR $\beta$ , and RXR $\alpha$  expression in human DCs.** (A) mRNA expression of LXR $\alpha$  was analyzed in human peripheral blood CD14<sup>+</sup> monocytes (set to 100%), myeloid DCs (myDCs) directly after isolation and after 2 days in culture (2d-myDCs), as well as of monocyte-derived immature DCs (iDCs) and mature DCs (mDCs) by QRT-PCR. (B) LXR $\alpha$  and LXR $\beta$  induction during DC differentiation. LXR $\alpha$  and LXR $\beta$  mRNA expression were analyzed at different time points during DC differentiation from monocytes (d0 set to 100%). Data show means and SEM of 4 independent experiments.

methanol for 30 minutes at room temperature and treated with the anti-human fascin Ab in PBS/1% BSA followed by Alexa Fluor 488-labeled F(ab')<sub>2</sub> fragment of goat antimouse (Molecular Probes). All antibodies were incubated for 30 minutes at 4°C. After washing with PBS, slides were dried by a piece of paper and embedded with mounting medium (Vector Laboratories, Burlingame, CA).

## Statistics

Data are presented in means  $\pm$  SEM. Comparisons were performed by 2-tail unpaired Student *t* test, and a *P* value less than .05 was considered statistically significant.

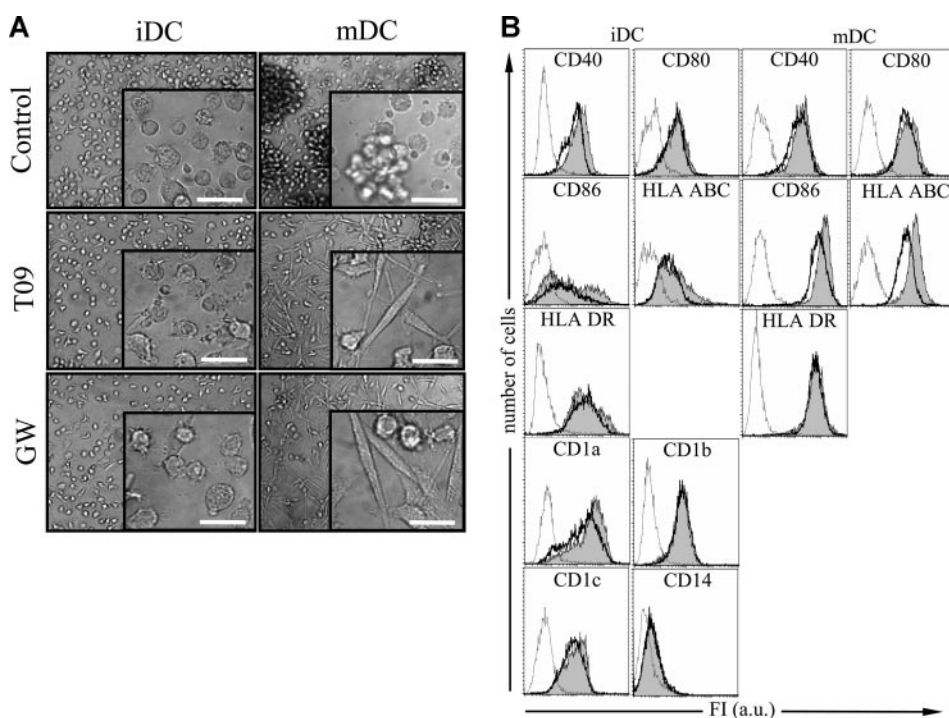
## Results

### Human DCs highly express LXR $\alpha$

To investigate whether human DCs express LXRs, we analyzed CD11c<sup>+</sup> myeloid DCs purified from peripheral blood. We found that freshly isolated myeloid DCs expressed significantly more LXR $\alpha$  mRNA compared with monocytes (+56%; *P* = .04). Notably, myeloid DCs strongly up-regulated LXR $\alpha$  expression (about 15-fold) after 2 days of culture (Figure 1A), when they resemble fully differentiated DCs as found in peripheral tissues.<sup>31</sup> Since monocyte-derived DCs are a useful model system for studying DCs in vitro, we examined LXR $\alpha$  expression in these cells. Immature DCs expressed about 16 times more LXR $\alpha$  mRNA than monocytes, and LXR $\alpha$  expression was not significantly up-regulated after 2 days of LPS-induced maturation (Figure 1A). mRNA concentrations of LXR $\alpha$  and LXR $\beta$  at day 0 (d0) were very similar to each other as estimated by nearly identical dCt values ( $9.2 \pm 0.4$  vs  $8.6 \pm 0.0$ ).<sup>3</sup> Taking into account the marked up-regulation of LXR $\alpha$  during DC differentiation, these data indicate that LXR $\alpha$  is by far the prominent LXR isoform in DCs. In addition, RXR $\alpha$ , the heterodimer partner of LXRs, was uniformly expressed in monocytes and different types of DCs (data not shown).

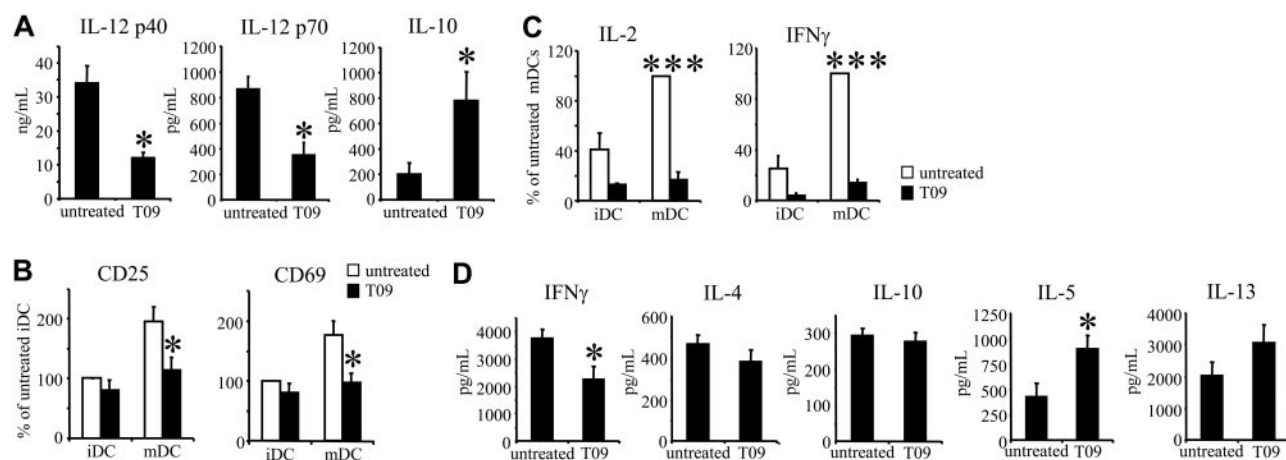
### LXR agonist treatment affects DC phenotype

The strong induction of LXR $\alpha$  in DCs points to a possible functional role of LXR in these cells. First, we examined the impact of LXR agonists (T0901317 and GW-3965) on the morphology of immature and mature monocyte-derived DCs in culture. iDCs show unaltered morphology when treated with LXR agonist (Figure 2A).



**Figure 2. Effect of LXR agonist treatment on DC morphology, differentiation, and maturation.** Immature DCs (iDCs) were differentiated for 7 days and left untreated (control) or treated from day 2 on with 2  $\mu$ M of the LXR agonists T0901317 (T09) or GW-3965 (GW). Mature DCs were obtained from iDCs by incubation with LPS for the last 2 days. (A) The maturation-induced clustering of DCs and morphologic changes were analyzed by a phase-contrast microscopy. Bar represents 30  $\mu$ m. Similar results were obtained in 4 different experiments. (B) Surface molecule expression as analyzed by immunofluorescence and flow cytometry. Histograms illustrate staining with isotype control mAb (open profile, fine line), and staining with mAb of the indicated specificity of untreated DCs (solid profiles) and of T0901317-treated (T09) DCs (open profiles, bold line). The logarithm of fluorescence intensities (FIs) is given in the abscissa spanning 4 orders of magnitude. One typical experiment of 10 is shown. Essentially identical results were obtained with GW-3965 (*n* = 4; data not shown).





**Figure 3. LXR agonist treatment of DCs leads to altered cytokine production and diminished ability to activate T cells.** (A) Immature monocyte-derived DCs (iDCs) were differentiated for 7 days and left untreated (control) or treated from day 2 on with 2  $\mu$ M of the LXR agonists T0901317 (T09). Mature DCs were obtained from iDCs by incubation with LPS for the last 2 days. After 2 days of LPS-induced DC maturation, cell-free supernatants were analyzed for IL-12p40, IL-12p70, and IL-10 secretion by ELISA in 7 different experiments. (B) Allogeneic T cells were cocultured with untreated or T0901317-treated (T09) iDCs and mDCs, respectively. CD25 and CD69 expression on CD3<sup>+</sup> T cells was analyzed by flow cytometry after 2 days of coculture in 3 independent experiments. (C) Allogeneic T cells were cocultured for 2 days with DCs that have been treated or not with T09 as in panel B, and cell-free supernatants were analyzed for IL-2 and IFN- $\gamma$  secretion ( $n = 3$ ). (D) T-cell restimulation. After 4 days of coculture, T cells were extensively washed, and restimulated with PMA/ionomycin. Cell-free supernatants of cocultures were analyzed for secretion of indicated cytokines. Significance versus untreated iDCs (\* $P \leq .05$ ) or mDCs (\*\* $P < .001$ ). Data show the means and SEM of 3 independent experiments.

After DC maturation by LPS, untreated mDCs become nonadherent to plastic and form large clusters (Figure 2A). However, LXR agonist-treated DCs remained adherent to the culture plate after LPS-induced maturation, formed only few cell clusters, and exhibited a widespread shape (Figure 2A).

Expression of a variety of cell surface molecules normally present on iDCs (CD1b, CD1c, CD14, CD80, CD86, HLA-ABC, and HLA-DR) was essentially unaltered by LXR agonist treatment ( $P > .05$ ), whereas expression of CD1a and CD40 was moderately but significantly decreased (Figure 2B). In DCs that have been stimulated by LPS to induce maturation, the expected up-regulation of distinct surface molecules CD40, CD80, and HLA-DR as well as their final expression in mDCs were essentially unaffected by both LXR agonists with the exception of CD86 and HLA-ABC expression, whose expression was reduced to a moderate but statistically significant extent (Figure 2B). Thus, despite the gross alterations in mDC shape, LXR activation had only a minor impact on surface molecule expression of immature and mature DCs.

#### LXR agonist treatment affects DC function

To investigate a potential functional role of LXRs in DCs, we first analyzed the impact of LXR agonists on DC cytokine production. Secretion of IL-12p40 and the functional form IL-12p70 was significantly decreased in LXR agonist-treated compared with untreated mDCs (Figure 3A). In contrast, production of IL-10 was increased by more than 5-fold in LXR agonist-treated compared with untreated mDCs.

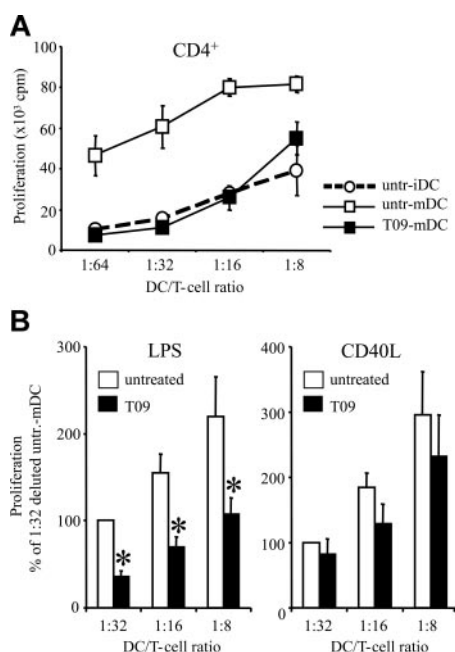
The most important function of DCs is their ability to activate T cells. When stimulated with LXR agonist-treated compared with untreated mDCs, allogeneic T cells showed markedly reduced CD25 and CD69 surface expression as well as significantly reduced production of the T-helper cell type 1 (Th1) cytokines IL-2 and IFN- $\gamma$  (Figure 3B-C; also see Figure S1, available on the *Blood* website by clicking on the Supplemental Figure link at the top of the online article). Since Th2 cytokine secretion was not detectable in primary allogeneic MLR (data not shown), we restimulated these cells with PMA/ionomycin.<sup>32</sup> The production of Th2 cytokine IL-5 was increased in restimulated cells, whereas IL-4, IL-10, and IL-13 were not significantly altered (Figure 3D). The production of IFN- $\gamma$

was also down-regulated in restimulated cells. These data suggest that LXR activation in DCs not only inhibits Th1 responses but also promotes polarization toward a Th2 response. The final hallmark of T-cell activation is induction of proliferation. Stimulation of CD4<sup>+</sup> T cells by DCs is considered to rely mainly on expression of major histocompatibility complex (MHC) class II molecules.<sup>13,33,34</sup> However, although MHC II (HLA-DR) expression was unaltered in LXR agonist-treated mDCs (Figure 2B), their ability to induce proliferation of allogeneic CD4<sup>+</sup>, CD8<sup>+</sup>, as well as naive CD4<sup>+</sup> CD45RA<sup>+</sup> T cells was drastically reduced to a level comparable with that of iDCs (Figure 4A; and data not shown). Notably, LXR agonists did not directly affect T-cell proliferation as assessed by stimulating T cells with CD3/28 antibodies in presence and absence of LXR agonist (data not shown and Walcher et al<sup>35</sup>).

The block in T-cell stimulatory capacity of LXR agonist-treated mDCs emphasized an interference of LXR with the process of DC maturation. Since DC maturation can be accomplished by different stimuli, we next tested whether the alterations found in LPS-induced mDCs were also apparent when DC maturation was induced by CD40Ligand (CD40L). However, the inhibitory effect of LXR agonist on DC-induced CD4<sup>+</sup> T-cell proliferation did not occur with CD40L-induced mDCs (Figure 4B). Taken together, LXR agonist treatment strongly impaired critical mDC functions including T-cell activation selectively after LPS- but not CD40L-induced maturation.

#### LXR agonist treatment strongly impairs fascin expression and IS formation

Several findings of this study (eg, alterations in cell morphology, DC clustering, and impairment of CD4<sup>+</sup> T-cell stimulation in MLR despite unaltered MHC II expression) pointed to a possible involvement of LXRs in regulating cytoskeletal functions in TLR4-matured mDCs. Since the actin-bundling protein fascin is strongly related to DC cytoskeletal functions such as dendrite formation and required for DC-mediated T-cell stimulation, we analyzed its expression in DCs following LXR agonist treatment.<sup>19</sup> LPS-induced expression of fascin as assessed by flow cytometry was essentially abolished by LXR agonists (Figure 5A). Along with unaltered induction of CD4<sup>+</sup> T-cell proliferation (Figure 3C), the



**Figure 4. LPS- but not CD40L-matured LXR agonist-treated DCs fail to stimulate T cells.** (A) Immature DCs (iDCs) were differentiated for 7 days and left untreated (control) or treated from day 2 on with 2  $\mu$ M of the LXR agonist T0901317 (T09). Mature DCs were obtained from iDCs by incubation with LPS for the last 2 days. At day 7, immature and LPS-matured DCs were washed extensively, irradiated, and cocultured with  $1 \times 10^5$  allogeneic purified CD4<sup>+</sup> T cells at the indicated ratios. After 4 days, proliferation was measured by [<sup>3</sup>H]-thymidine incorporation in 4 independent experiments. (B) Allogeneic MLRs were performed as described for panel A using LPS- and CD40L-matured DCs, respectively. \* $P \leq .05$  for difference between T09-treated versus untreated DCs. Data show the means and SEM of 4 independent experiments.

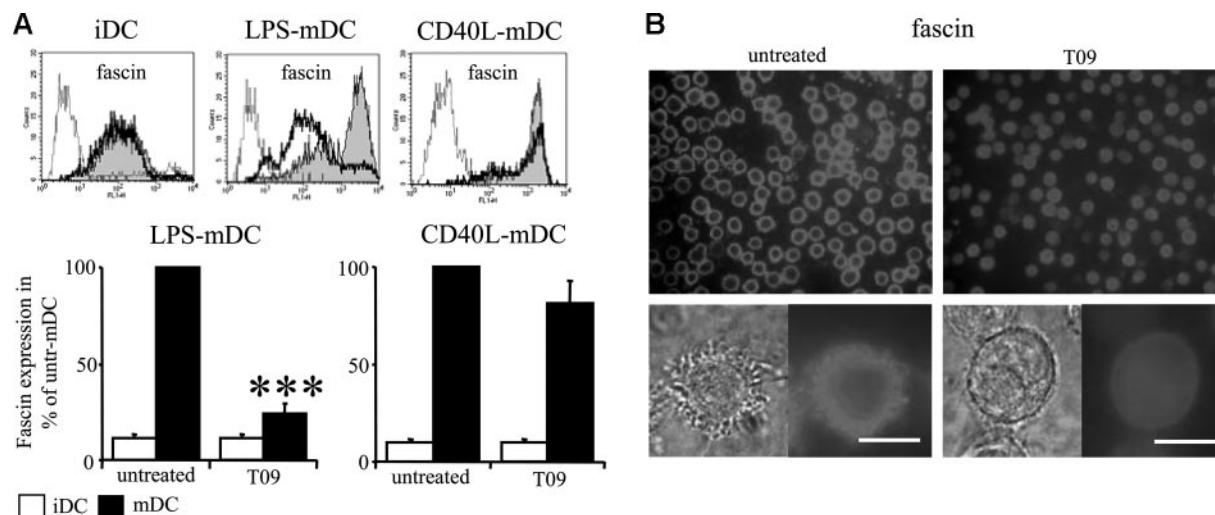
expression of fascin in LXR agonist-treated and CD40L-matured DCs remained essentially unaffected (Figure 5A). In addition, the LXR agonist-induced inhibition of fascin expression on LPS-matured DCs was further analyzed by immunofluorescence microscopy. Whereas untreated LPS-matured DCs showed numerous fascin-containing dendrites following mechanical detach-

ment from the plate for immunofluorescence analysis, LXR agonist-treated mDCs almost completely lacked fascin expression and dendrites (Figure 5B lower panel). These findings emphasize a critical role of blocked fascin expression in the LXR-mediated DC maturation defect.

Sustained TCR engagement by mDCs is important for full T-cell activation and depends on the formation of the IS. Actin cytoskeletal polarization in DCs that includes fascin was shown to be essential for IS formation.<sup>35</sup> As shown in Figure 6, IS formation (as assessed by the number of conjugates with relocalized CD3) was low with iDCs ( $18.5\% \pm 0.5\%$ ) and independent of LXR agonist treatment. IS formation was also not altered by LXR agonist treatment with mDCs that had been matured with CD40L ( $42.5\% \pm 1.5\%$  vs  $41.5\% \pm 8.2\%$  in LXR agonist-treated vs untreated DCs). In contrast, the number of conjugates with relocalized CD3 was markedly diminished in LXR agonist-treated compared with untreated mDCs ( $28.6\% \pm 4.4\%$  vs  $46.2\% \pm 4\%$ ) when DCs had been matured with LPS (Figure 6). Relocalization of PKC $\theta$  to the IS was not affected by LXR agonist treatment (Figure 6). Thus, the LXR-mediated blockage of fascin expression in LPS but not CD40L-matured DCs strikingly correlates with the blunted ability of mDCs to present the T-cell receptor/CD3 complex within the context of an IS and subsequent T-cell activation.

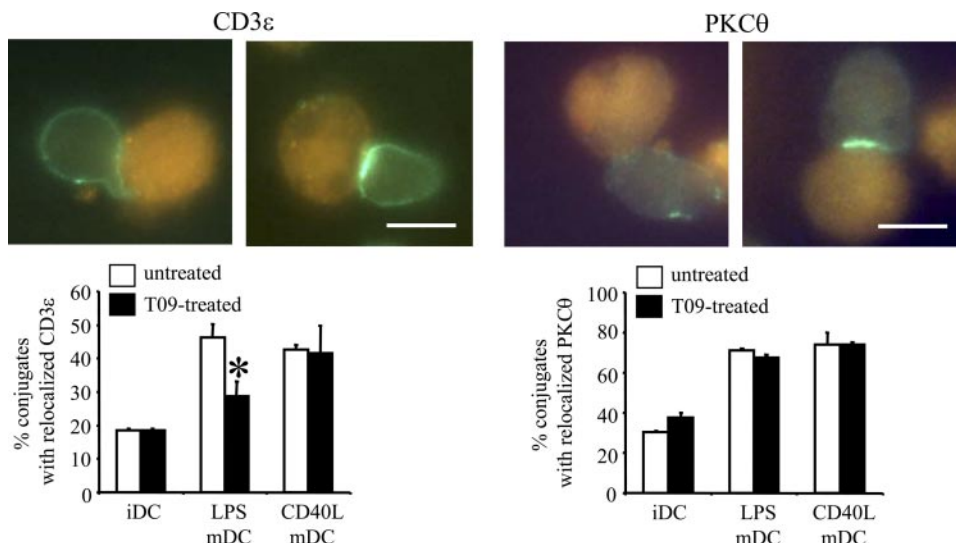
#### Overexpression of fascin in LXR agonist-treated mDCs restores their ability to form an IS and activate T cells

In order to test whether blunted fascin expression underlies LXR-mediated inhibition of IS formation and DC-induced T-cell activation, we transfected LXR agonist-treated and untreated iDCs and mDCs with either a plasmid encoding the GFP-linked full-length human fascin protein (fascin) or the empty GFP expression vector (vector). The transfection efficiency of iDCs with either vector or fascin plasmid was about 30% (data not shown). Transfection of mDCs resulted in 24% positive cells when transfected with the vector plasmid and about 14% when transfected with the fascin plasmid (data not shown). LXR agonist treatment reduced the transfection efficiency of mDCs transfected with the



**Figure 5. LPS- but not CD40L-matured LXR agonist-treated DCs fail to up-regulate fascin expression.** (A) Immature DCs (iDCs) were differentiated for 7 days and left untreated (control) or treated from day 2 on with 2  $\mu$ M of the LXR agonist T0901317 (T09). Mature DCs were obtained from iDCs by incubation with LPS or CD40L for the last 2 days. Histograms with open profiles (fine line) represent a staining pattern with an isotype control. T0901317-treated DCs (open profiles with bold line) and untreated DCs (solid profiles) show a staining pattern with the antifascin Ab. Typical histograms and diagrams showing mean of fluorescence intensities related to those obtained from untreated mDCs  $\pm$  SEM of at least 4 independent experiments are given. Significance versus untreated mDCs: \*\*\* $P \leq .001$ . (B) Typical light microscopy images and immunofluorescence staining of fascin of untreated and T09-treated LPS-matured DCs with different magnifications are shown. Bar represents 10  $\mu$ m. Similar results were obtained in 4 different experiments.

**Figure 6. LPS- but not CD40L-matured LXR agonist-treated DCs fail to induce IS formation.** Immature DCs (iDCs) were differentiated for 7 days and left untreated (control) or treated from day 2 on with 2  $\mu$ M of the LXR agonist T0901317 (T09). Mature DCs were obtained from iDCs by incubation with LPS or CD40L for the last 2 days. Mature DCs were pulsed with superantigen for 30 minutes and incubated with Jurkat T cells for an additional 30 minutes. CD3 $\epsilon$  and PKC $\theta$  relocalization was visualized by indirect immunofluorescence. Typical examples of conjugates, negative (left) or positive (right) for relocalization of CD3 $\epsilon$  and PKC $\theta$ , are shown. Bar represents 10  $\mu$ m. The diagram shows the percentage of conjugates counted positive for CD3 $\epsilon$  and PKC $\theta$  relocalization in means  $\pm$  SEM of 4 independent experiments. Significance versus untreated mDCs: \* $P \leq .05$ .



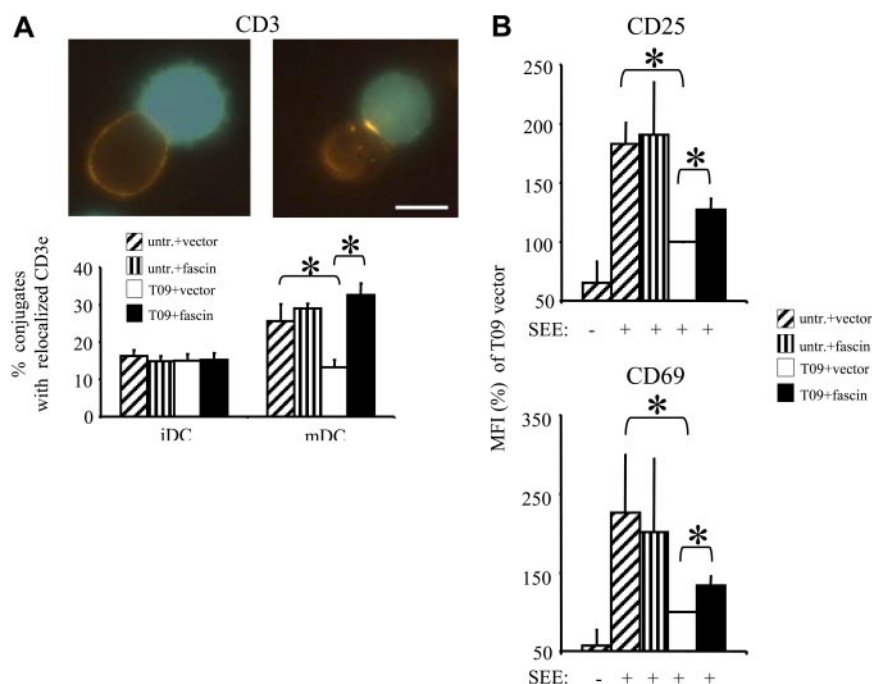
vector plasmid to 14% and fascin plasmid to 7% (data not shown). IS formation of DCs pulsed with superantigen SEE was assessed by CD3 relocalization to the T-cell/DC interface. Approximately 16% of conjugates between plasmid-expressing iDCs and T cells relocalized CD3 to the IS independently of LXR agonist treatment and the type of transfected plasmid (Figure 7A). As expected from untransfected cells (Figure 6), LXR agonist treatment inhibited CD3 relocalization of T cells to vector-transfected mDCs down to levels found in iDCs. However, CD3 relocalization to the IS was completely restored in LXR agonist-treated mDCs that were transfected with fascin (Figure 7A). These data show that fascin expression alone is sufficient to prevent the LXR-mediated block in IS formation of LPS/TLR4-stimulated DCs.

To further evaluate the biologic importance of decreased fascin expression for LXR-mediated inhibition of DC-induced T-cell activation, we analyzed activation marker expression of T cells stimulated with isolated vector- or fascin-transfected mDCs. Fascin transfection had no effect on induction of CD25 and CD69 on T

cells stimulated with untreated SEE-pulsed mDCs (Figure 7B). Induction of CD25 and CD69 expression was largely reduced by LXR agonist treatment in vector-transfected mDCs as expected from data with allogeneic T cells (Figure 7B; cf Figure 3A-B). Strikingly, when cells were transfected with fascin, the inhibitory effect of LXR agonist treatment on mDC-induced T-cell activation marker expression was significantly neutralized (Figure 7B). These data indicate that down-regulation of fascin expression constitutes one molecular mechanism by which LXR mediates its inhibitory action in mDCs to reduce their T-cell stimulatory ability.

## Discussion

Nuclear receptors regulate gene expression for a variety of cellular functions including metabolic processes. From the so-called orphan nuclear receptors that are mostly involved in metabolic regulation, only



**Figure 7. Overexpression of fascin in LXR agonist-treated DCs leads to enhanced IS formation and further T-cell activation.** Immature DCs were differentiated for 6 days and left untreated (untr) or treated from day 2 on with 2  $\mu$ M LXR agonist T0901317 (T09). Mature DCs were obtained from iDCs by incubation with LPS for the last day. iDCs and mDCs were then transfected with pEGFP-C1-vector (vector) or pEGFP-C1-fascin (fascin) plasmid. (A) At 24 hours after transfection, DCs were pulsed with superantigen for 30 minutes and incubated with Jurkat T cells for an additional 30 minutes. CD3 $\epsilon$  relocalization was visualized by indirect immunofluorescence. Typical examples of conjugates, negative (left) or positive (right) for relocalization of CD3 $\epsilon$ , are shown. Bar represents 10  $\mu$ m. The diagram shows the percentage of conjugates counted positive for CD3 $\epsilon$  relocalization in means  $\pm$  SEM of 4 independent experiments. Significance versus T09 + vector: \* $P \leq .05$ . (B) At 24 hours after transfection, GFP-positive DCs were FACS sorted to a purity of 98%. FACS-sorted pEGFP-C1-positive DCs were pulsed with superantigen for 30 minutes and incubated with Jurkat T cells at a DC/T-cell ratio of 1:8 and 1:16 for 24 hours. Expression of CD25 and CD69 was analyzed by 2-color flow cytometry. Diagram shows geometric mean fluorescence intensities (MFIs)  $\pm$  SEM related to those obtained from T09-treated vector-transfected DCs (T09 + vector, set to 100%) of 3 independent experiments. Significance versus T09-vector: \* $P \leq .05$ .



peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been described to control DC function so far.<sup>31,36,37</sup> Here we show that LXR $\alpha$ , the LXR paralogue known to be primarily involved in regulation of lipid metabolism, is highly expressed in peripheral myeloid as well as monocyte-derived DCs along with constitutive expression of LXR $\beta$  and the heterodimer partner RXR. The abundant expression of LXR $\alpha$  compared with LXR $\beta$  in DCs indicates that LXR $\alpha$  is the predominant LXR in DCs, and effects of unselective LXR $\alpha/\beta$  agonists as used here are mediated primarily via LXR $\alpha$ . Moreover, the strong induction of LXR $\alpha$  during DC differentiation suggests a physiological role of this nuclear receptor in DCs.

Data on the impact of LXRs in immunity have essentially been confined to the innate part of the immune system, particularly by controlling cholesterol efflux and inflammatory cytokine production in macrophages.<sup>8,38</sup> In contrast, few effects of LXR agonists have been reported on the adaptive immune system namely on T-cell cytokine production without directly affecting T-cell proliferation.<sup>35</sup> The data presented here point to a critical role of LXR $\alpha$  in DCs, which link innate and adaptive immunity. The functionally most significant finding in our study is that DC-induced T-cell proliferation was abolished when LXR agonist-treated DCs were used as stimulators. Since activation of primary T cells depends on DCs, these data suggest a profound block in primary T-cell responses upon LXR agonist treatment.

Our data on diminished T-cell stimulation despite essentially unaltered stimulatory molecule expression on the DC surface support the notion that surface expression of these molecules alone does not account for the exceptional ability of DCs to activate antigen-dependent immune responses.<sup>37,39</sup> However, functional actin cytoskeleton is required for full T-cell activation including IS formation and DC-driven T-cell activation independent of MHC II expression.<sup>19</sup> Thus, our data on blocked IS formation and unaltered MHC II expression in LXR agonist-treated DCs suggest that alterations in cytoskeletal function could underlie the observed defects in DC-mediated T-cell proliferation.

Induction of the actin-bundling protein fascin<sup>40,41</sup> following LPS-induced DC maturation was almost completely abolished by LXR agonist treatment along with impaired T-cell stimulatory capacity. Support for an involvement of fascin in LXR-mediated effects comes from our experiments with CD40L-matured DCs, in which LXR agonist treatment did not inhibit induction of fascin expression along with only minimal impairment of their T cell-stimulatory capacity. Based on these data, we hypothesized that lack of fascin expression is a crucial mechanism of the inhibitory effects of LXR in DCs. According to the function of cytoskeletal rearrangements in IS formation, overexpression of fascin in LXR agonist-treated DCs completely restored DC-induced CD3 relocalization to the IS, indicating that diminished fascin expression is a crucial mechanism by which LXR regulates IS formation. Moreover, these data illustrate the requirement of fascin in human DCs for IS formation. Unfortunately, the viability of human DCs rapidly declined within a few days following transfection thereby preventing induction of significant T-cell proliferation by transfected DCs and a direct test of restored DC fascin expression on this readout. Nonetheless, restoration of CD25 and CD69 expression on T cells stimulated with LXR agonist-treated DCs by overexpressing fascin strongly indicated the functional significance of impaired IS formation due to LXR-mediated abolishment of fascin expression in LPS-matured DCs. Since functional restoration by fascin overexpression was not complete, additional mechanisms of LXR action on DC ability to stimulate T cells cannot be excluded. Notably, neutralizing anti-IL-10 antibodies did not restore the T-cell stimulatory ability of LXR agonist-treated DCs in MLRs (data not shown). However, altered production of functional IL-12p70 during the MLR as well as polariza-

tion of T cells toward a Th2 response and secretion of cytokines other than those investigated here could contribute to LXR-mediated effects on DC stimulatory activity independently of abolished fascin expression.

The selective inhibitory effect of LXR activation on LPS- but not CD40L-induced DC maturation indicates that LXRs specifically interfere with LPS signaling. LXR-mediated inhibition of LPS/TLR4 signaling has been shown in macrophages by antagonizing NF- $\kappa$ B signaling thereby reducing proinflammatory cytokine expression.<sup>8</sup> TLR4 signals via myeloid differentiation primary response gene 88 (MyD88) and Toll-IL-1 receptor domain-containing adaptor-inducing interferon- $\beta$  (TRIF), which together activate nuclear factor- $\kappa$ B and mitogen-activated protein kinases (MAPKs), whereas CD40 activation implicates tumor necrosis factor (TNF) receptor-associated factor (TRAF) and Src family kinases,<sup>42,43</sup> giving rise to multiple possibilities for selective interaction by LXR. However, the precise mechanism how LXRs interfere with TLR4 signaling has not yet been resolved in any cell type.

DCs are critically involved in a variety of immune disorders such as rheumatoid arthritis, atherosclerosis, diabetes, and others.<sup>12,13,44-48</sup> Hence, alterations of DC phenotype and function such as those induced by LXR agonists could be an option for immunosuppression and treatment of these diseases. In addition, DCs are capable of inducing central and peripheral tolerance,<sup>9,20,22,23</sup> and impaired DC maturation leading to inadequately matured DCs has repeatedly been shown to be an inducer of tolerance.<sup>10,26,49-51</sup> Hence, LXR activation in DCs might be an attractive approach for tolerance induction that is highly desirable in various T-cell-mediated disorders such as autoimmune diseases and allograft rejection.<sup>9,20,22,23</sup>

In conclusion, the present study reveals a novel role of LXRs at the interface of innate and adaptive immune responses by critically interfering with DC maturation and function. Although the physiological role of LXRs and endogenous LXR agonists on DCs remains obscure, to date the marked impact of synthetic LXR agonists on DCs shown here bears novel possibilities for immunosuppressive strategies and tolerance induction. Since unselective LXR agonists exert dramatic side effects such as hypertriglyceridemia, highly selective LXR modulators targeting LXR $\alpha$  in distinct cell types are currently being developed.<sup>38</sup> Such selective LXR modulators specifically acting on DCs could become reasonable agents for novel immunosuppressive strategies for treatment of inflammatory and autoimmune diseases and prevention of allograft rejection.

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## Authorship

Contribution: All authors have contributed substantially to the present publication. R.G., M.Z., and T.M.S. principally designed the study and largely wrote the paper; W.B. and E.K. performed significant experimental procedures; and M.D.S., G.J.Z.,

and D.M. helped to analyze and interpret the data and helped to write the paper.

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Correspondence: Thomas M. Stulnig, Clin. Div. Endocrinology and Metabolism, Dept. Internal Med. III, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria; e-mail: thomas.stulnig@meduniwien.ac.at.

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