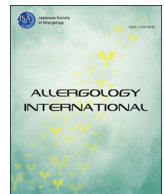




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Original article

Activation of LXRs using the synthetic agonist GW3965 represses the production of pro-inflammatory cytokines by murine mast cells

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LXR, liver X receptor;

OHC, hydroxycholesterol;

LPS, lipopolysaccharide;

DEX, dexamethasone; Ag, antigen

ABSTRACT

Background: The activation of liver X receptor (LXR) α or LXR β negatively regulates the expression of pro-inflammatory genes in mammalian cells. We recently reported that 25-hydroxycholesterol, a representative LXR-activating oxysterol, suppresses IL-6 production in mouse mast cells (MCs) following its engagement of the high-affinity IgE receptor (FcεRI). This finding suggests that murine MCs express functional LXRs; however, the mechanisms underlying the LXR-dependent repression of the MC-mediated production of pro-inflammatory cytokines, including IL-6, are poorly understood. Therefore, we employed the synthetic LXR ligand GW3965 to examine the functions of LXR α and LXR β in the production of pro-inflammatory cytokines by murine bone marrow-derived MCs (BMMCs).

Methods: We prepared BMMCs from wild-type (WT), LXR α ^{−/−}, and LXR α/β ^{−/−} mice. Each group of BMMCs was pretreated with GW3965 and then stimulated with IgE+antigen (Ag) or lipopolysaccharide (LPS). Cytokine production was then analyzed using specific ELISA kits.

Results: The activation of LXRs by GW3965 significantly attenuated the production of IL-1 α and IL-1 β , but not of IL-6, in the WT and LXR α ^{−/−} BMMCs stimulated with IgE+Ag. However, GW3965 treatment decreased the production of IL-1 α , IL-1 β , and IL-6 in WT and LXR α ^{−/−} BMMCs upon stimulation with LPS, while the GW3965-mediated suppression of cytokine production was nearly absent from the LXR α/β ^{−/−} BMMCs.

Conclusions: These findings demonstrate, for the first time, that the activation of LXRs by GW3965 attenuates the antigen- or LPS-induced production of pro-inflammatory cytokines, such as IL-1 α and IL-1 β , in murine MCs and that LXR β plays an important role in the LXR-mediated repression of cytokine production.

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Introduction

Liver X receptor (LXR) α and LXR β are representative nuclear receptors that were previously reported to negatively regulate the expression of pro-inflammatory genes in different types of mammalian cells, including macrophages,^{1–3} T cells,⁴ astrocytes,⁵ synovocytes,⁶ and airway smooth muscle cells.⁷ Furthermore,

topical or systemic administration of natural or synthetic LXR ligands resulted in the amelioration of glomerulonephritis,⁸ allergic dermatitis,^{9,10} chronic asthma,¹¹ and multiple sclerosis^{12,13} in experimental animal models. These findings suggest that the regulation of LXR activation in immune cells represents a promising therapeutic strategy for the above-mentioned immune disorders.

Mast cell (MC)-produced pro-inflammatory cytokines contribute to the pathogenesis of various chronic allergic and autoimmune disorders. Therefore, elucidating the biological mechanisms underlying LXR activation that reduce cytokine expression in MCs is valuable. Previously, we reported that 25-hydroxycholesterol (OHC), a natural LXR agonist, suppressed the production of the IL-6 protein in murine MCs activated via the high-

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affinity IgE receptor (FcεRI).¹⁴ However, oxysterols perform various functions in lipid metabolism. In addition to activating LXRs, oxysterols regulate the synthesis of cholesterol esters^{15–17} and the activation of HMG-CoA reductase.^{18,19} Indeed, treatment of MCs with 25-OHC considerably decreased the cell surface expression of FcεRI in an LXR-independent manner.¹⁴ Thus, it is unclear whether LXR activation directly suppresses FcεRI-dependent cytokine expression in MCs. To clarify the mechanisms by which LXRα and LXRβ are involved in the suppression of pro-inflammatory cytokine production, we employed GW3965, a widely used synthetic ligand that is specific for LXRα and LXRβ.^{16,9,20,21}

In this study, we found that treatment of murine bone marrow-derived MCs (BMMCs) with GW3965 decreased their IgE+antigen (Ag)- or LPS-stimulated cytokine expression. Furthermore, we investigated the functions of LXRα and LXRβ in the GW3965-mediated repression of cytokine expression.

Methods

Reagents

GW3965, dexamethasone (DEX), and LPS (*Escherichia coli* 0111: B4) were obtained from Sigma (St. Louis, MO, USA). Mouse anti-TNP IgE (IgE-3) and TNP-BSA (25 mol TNP per mol BSA) were purchased from BD Biosciences (San Diego, CA, USA) and BIOSEARCH Technologies (Petaluma, CA, USA), respectively. Recombinant murine IL-3 was obtained from PeproTech (Rocky Hill, NJ, USA), and PE-conjugated anti-mouse c-kit mAb (2B8), FITC/PerCP-conjugated anti-mouse FcεRIα mAb (MAR-1), and all ELISA kits were purchased from Affymetrix eBioscience (San Diego, CA, USA). Rabbit

anti-mouse LXRβ (N20) and β-actin (N21) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Animals

The LXRα^{-/-} and LXRβ^{-/-} mice were provided by Dr. Mangelsdorf²² and were backcrossed with C57BL/6J mice for at least eight generations; the LXRα/β^{-/-} mice were generated from the LXRα^{-/-} and LXRβ^{-/-} mice. The mice were bred in the animal facility of the Nihon University School of Medicine under specific pathogen-free conditions. Wild-type (WT) C57BL/6J mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan) and served as a wild-type control for the LXRα^{-/-} and LXRα/β^{-/-} mice. Following approval by the animal experimentation committee at Nihon University, all of the experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Nihon University.

Cell culture

The BMMC cultures were prepared from the femurs of mice as previously described,¹⁷ and after six to eight weeks in culture, the BMMCs were used for experiments.

GeneChip expression analysis

Murine genome-wide gene expression was assessed using the Mouse4 Genome 430 2.0 probe array (GeneChip, Affymetrix, Santa Clara, CA, USA), which contains an oligonucleotide probe set for approximately 39,000 full-length genes and ESTs, according to the

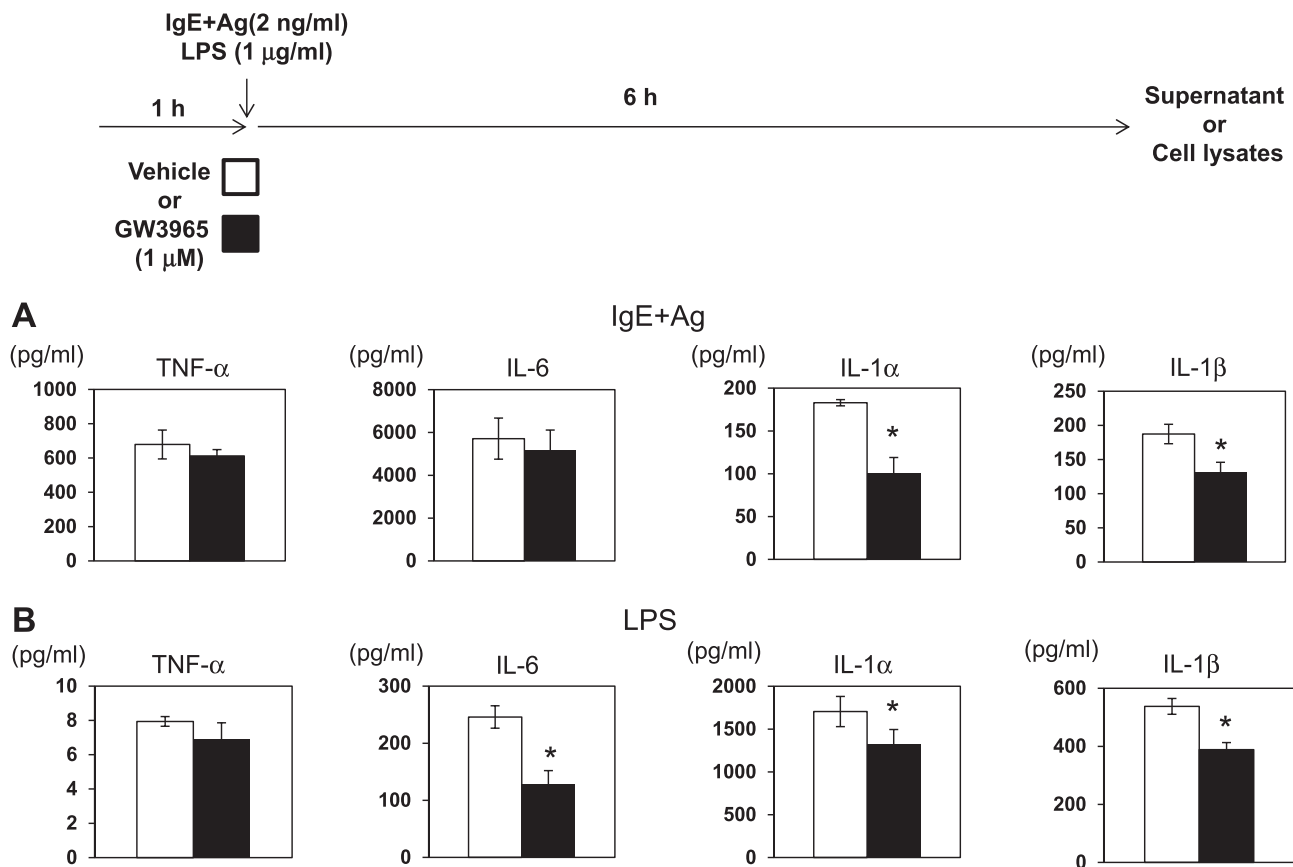


Fig. 1. The effects of GW3965 on the IgE+Ag or LPS-induced production of IL-1α, IL-1β, IL-6, and TNF-α by MCs. The IgE-sensitized WT BMMCs were treated with or without 1 μM GW3965 for 1 h and then stimulated with 2 ng/ml TNP-BSA (A) or 1 μg/ml LPS (B) for 6 h. The data are presented as the means ± SDs of quadruplicate experiments. **p* < 0.05 (Student's *t*-test). Similar results were obtained from three independent experiments.

manufacturer's instructions (Expression Analysis Technical Manual) and as described in previous reports.²³ The obtained data were analyzed using GeneSpring software, version GX 11.5 (Silicon Genetics, San Carlos, CA, USA). To normalize the staining intensity variations among the chips, the average differences for all of the genes on a given chip were divided by the median of all of the measurements on that chip. Based on these normalized values, the genes were classified as up-regulated (shown in red) or down-regulated (shown in green).

Measurement of cytokine production

BMMCs (1×10^7) were sensitized overnight with 0.5 $\mu\text{g/ml}$ anti-TNP IgE, and then 1×10^6 of the sensitized cells were washed twice with PBS and pretreated with 1 μM GW3965 or DMSO (vehicle) for 1 h. The pretreated cells were then stimulated with 2 ng/ml TNP-BSA or 1 $\mu\text{g/ml}$ LPS for 6 h. To detect spontaneous cytokine production, the cells were cultured in the absence of TNP-BSA or LPS for 6 h, and the levels of IL-6 and TNF- α in the supernatants and of IL-1 α and IL-1 β in the cell lysates were analyzed using specific ELISA kits. The data are presented as the net production levels of each cytokine.

Degranulation assay

The IgE-sensitized BMMCs (1×10^5) were washed twice with PBS and then stimulated with or without 2 ng/ml TNP-BSA for

30 min; A23187 (1 μM) was used as a non-immunological control. Degranulation was determined by measuring β -hexosaminidase release, as described previously,²⁴ and the results are presented as the percentage of released β -hexosaminidase, which was calculated as follows: (optical density of the supernatant from the cells)/(optical density of the total cell lysate) $\times$ 100.

Flow cytometric analyses

The BMMCs (1×10^5) were labeled with a PE-conjugated anti-mouse c-kit mAb and an FITC/PerCP-conjugated anti-mouse Fc ϵ RI α mAb at 4 $^\circ\text{C}$ for 30 min. After washing with PBS, the labeled cells were analyzed using a FACSCalibur (BD Biosciences).

Immunoblotting

The BMMCs (1×10^6) were lysed in lysis buffer (Tris-buffered saline containing 1% Nonidet P-40, 2 mM phenylmethanesulfonylfluoride, 10 $\mu\text{g/ml}$ aprotinin, 2 $\mu\text{g/ml}$ leupeptin and 2 $\mu\text{g/ml}$ pepstatin A) for 30 min on ice, centrifuged for 15 min at 15,000 g and resuspended in an equal volume of 2 $\times$ Laemmli buffer. Then, the samples were boiled, separated on a 12% SDS-PAGE gel, and transferred to an Immobilon-P membrane (Merck Millipore, Bedford, MA, USA). The membrane was incubated with a rabbit anti-mouse LXR β or β -actin polyclonal antibody followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary

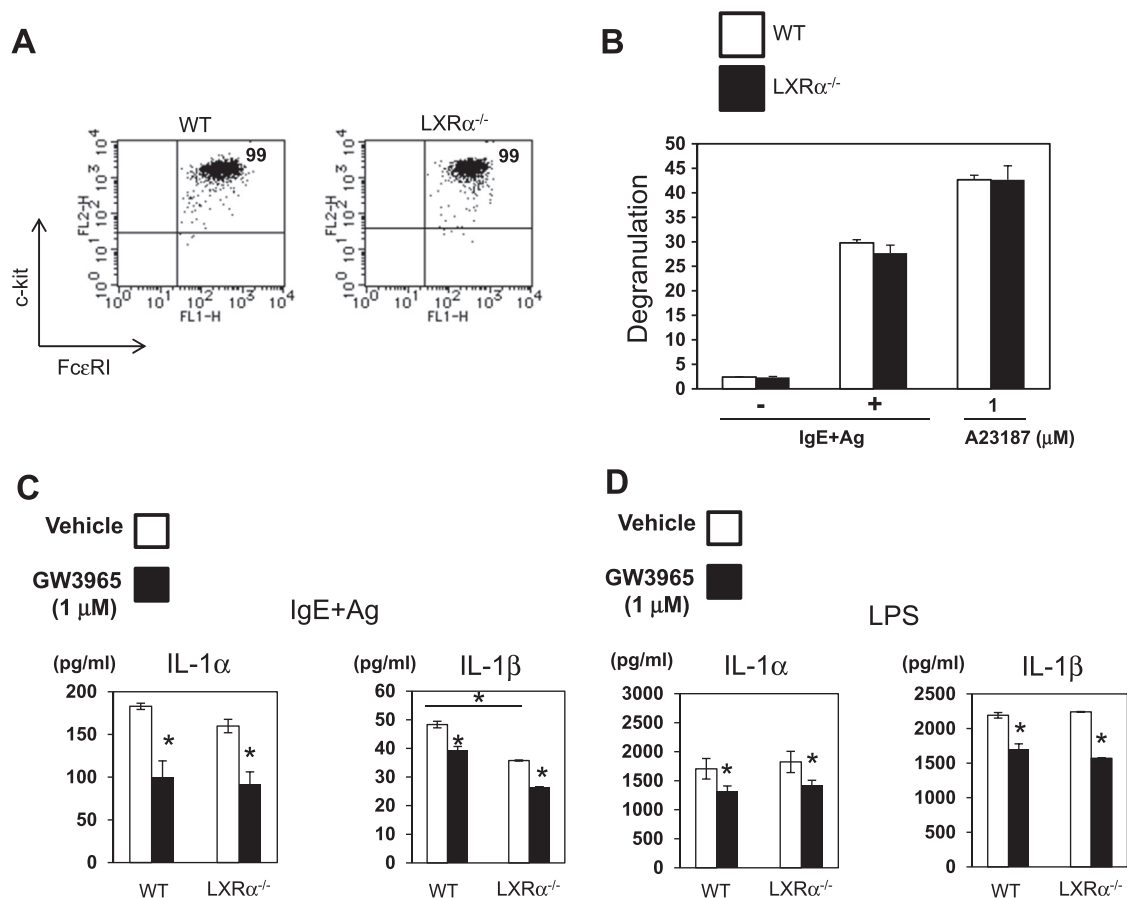


Fig. 2. The effects of GW3965 on the IgE+Ag or LPS-induced production of IL-1 α and IL-1 β by LXR $\alpha^{-/-}$ BMMCs. (A) Comparable expression of Fc ϵ RI and c-kit in WT and LXR $\alpha^{-/-}$ BMMCs. The results are representative of three independent experiments, which produced similar results. (B) The degranulation response was unaltered in LXR $\alpha^{-/-}$ BMMCs. IgE-sensitized WT and LXR $\alpha^{-/-}$ BMMCs were stimulated with 2 ng/ml TNP-BSA for 0.5 h. A23187 was used as a positive control. β -hexosaminidase release was analyzed as described in the Methods section. (C) The IgE-sensitized WT and LXR $\alpha^{-/-}$ BMMCs were treated with or without 1 μM GW3965 for 1 h and then stimulated with 2 ng/ml TNP-BSA (C) or 1 $\mu\text{g/ml}$ LPS (D) for 6 h. In (B)–(D), the data are presented as the means $\pm$ SDs of quadruplicate experiments. * $p < 0.05$ (Student's *t*-test). Similar results were obtained from three independent experiments.

antibody. The signals were detected via enhanced chemiluminescence (GE Healthcare Life Sciences, Tokyo, Japan).

Statistical analysis

The data are presented the means $\pm$ SD of at least three replicate samples. Statistical analysis was performed using Student's *t*-test, and probability values of less than 0.05 indicated significant differences.

Results

GW3965 attenuates the expression of mRNAs for IL-1 α , IL-1 β , IL-3, and IL-5 following Fc ϵ RI stimulation

First, we examined the effects of GW3965 on the expression of pro-inflammatory cytokine mRNAs in BMMCs via GeneChip expression analysis (Supplementary Fig. 1); DEX, a synthetic ligand of glucocorticoid receptors, was used as a positive control. Microarray analyses clearly indicated that DEX considerably suppressed the mRNA expression of 11 cytokines (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, IL-31, IL-33, and TNF- α) that was induced by IgE+Ag stimulation. In contrast, GW3965 failed to decrease the mRNA expression of IL-2, IL-4, IL-6, IL-13, IL-31, IL-33, or TNF- α but slightly or modestly reduced the mRNA expression of IL-1 α , IL-1 β , IL-3, and IL-5. These data suggest that GW3965 suppresses the expression of fewer cytokines than DEX.

GW3965 attenuates the protein expression of IL-1 α and IL-1 β following Fc ϵ RI stimulation

Of the cytokine genes induced by IgE+Ag stimulation, the expression of IL-1, IL-6, and TNF- α was induced in murine MCs upon stimulation with LPS.^{25–27} Thus, we examined the effects of GW3965 on the IgE+Ag- or LPS-induced protein expression of IL-1 α , IL-1 β , IL-6, and TNF- α . Consistent with our microarray data, GW3965 significantly attenuated the protein expression of IL-1 α and IL-1 β , but not of IL-6 or TNF- α , in MCs stimulated by IgE+Ag (Fig. 1A). In addition to IL-1 α and IL-1 β , IL-6 protein expression was suppressed in GW3965-treated MCs following stimulation with LPS (Fig. 1B).

LXR α is not required for the GW3965-mediated repression of cytokine production

To elucidate the biological function of LXR α in the GW3965-mediated repression of cytokine production, we prepared BMMCs from mice lacking LXR α . The LXR α ^{−/−} BMMCs displayed typical Fc ϵ RI and c-kit expression and degranulated in response to stimulation with IgE + Ag (Fig. 2A, B). Fig. 2C, D indicate that GW3965 reduced the production of IL-1 α and IL-1 β in LXR α ^{−/−} BMMCs in response to IgE + Ag or LPS stimulation. These results suggest that LXR α is not responsible for the GW3965-mediated repression of cytokine production in BMMCs.

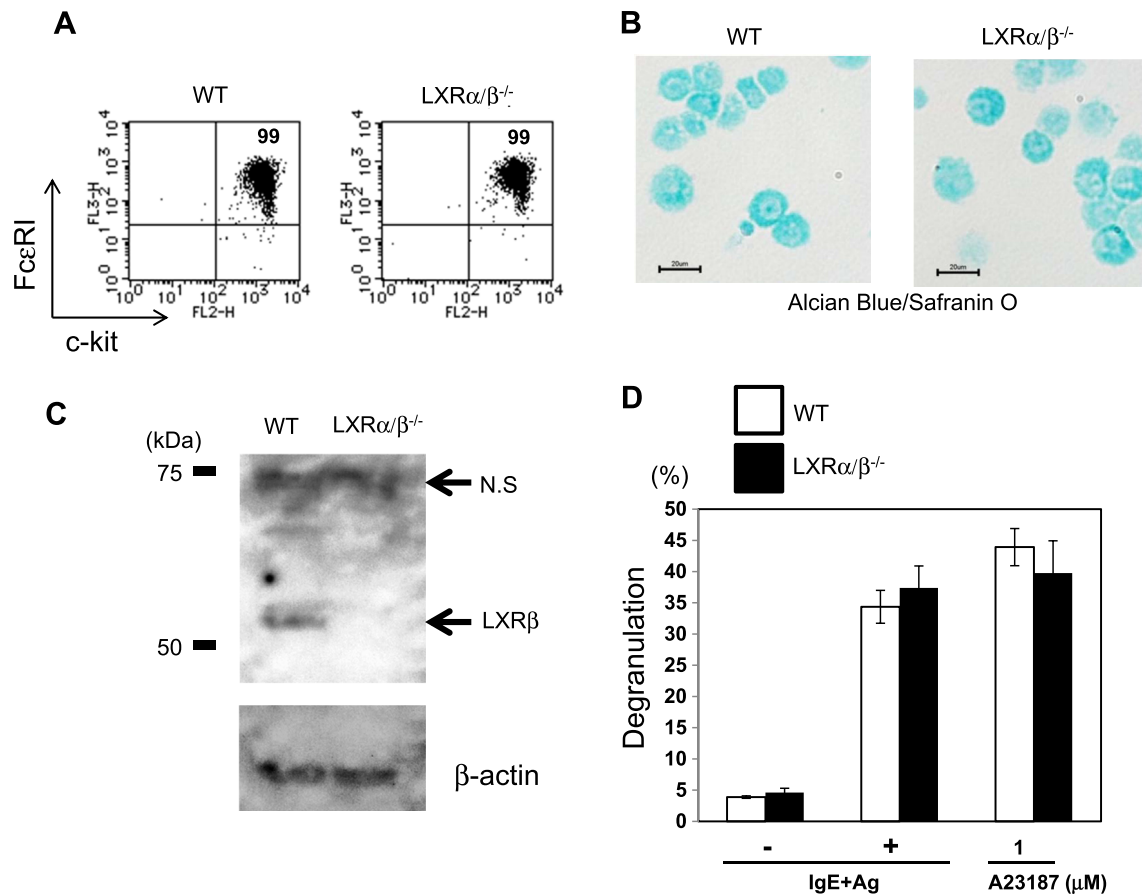


Fig. 3. LXR α/β ^{−/−} BMMCs exhibit a normal degranulation response upon Fc ϵ RI stimulation. (A) Comparable expression of Fc ϵ RI and c-kit in WT and LXR α/β ^{−/−} BMMCs. (B) Representative alcian blue/safranin O staining. (C) Western blot analysis of LXR β expression. In (A)–(C), the results are representative of three independent experiments, which produced similar results. (D) IgE-sensitized WT and LXR α/β ^{−/−} BMMCs were stimulated with or without 2 ng/ml TNP-BSA for 0.5 h; A23187 was used as a positive control. β -hexosaminidase release was analyzed, and the data are presented as the means $\pm$ SDs of triplicate experiments. Similar results were obtained from three independent experiments.

Neither LXR α nor LXR β is required for the degranulation response of murine MCs to stimulation by Fc ϵ RI

Similar to the LXR α ^{-/-} BMMCs, the LXR α / β ^{-/-} BMMCs expressed typical levels of Fc ϵ RI and c-kit (Fig. 3A). In addition, alcian blue/safranin O staining indicated that the maturity of the MCs was comparable between the WT and LXR α / β ^{-/-} BMMCs (Fig. 3B). We also confirmed that the LXR α / β ^{-/-} BMMCs indeed lacked LXR β expression (Fig. 3C). As shown in Fig. 3D, the Fc ϵ RI-induced degranulation response was not impaired in the LXR α / β ^{-/-} BMMCs. These results suggest that LXR α and LXR β are not essential for the degranulation response of MCs to stimulation by Fc ϵ RI.

LXR α / β ^{-/-} BMMCs are protected from the GW3965-mediated repression of cytokine production

Finally, we examined the effects of GW3965 on the expression of IL-1 α , IL-1 β , and IL-6 in LXR α / β ^{-/-} BMMCs stimulated with IgE+Ag or LPS. Following stimulation with IgE+Ag or LPS, GW3965 did not exert any suppressive effects on cytokine production by LXR α / β ^{-/-} BMMCs (Fig. 4A, B).

Discussion

In the present study, we have shown that the activation of LXRs by GW3965 resulted in the down-regulation of IL-1 and/or IL-6

expression in murine MCs stimulated with IgE+Ag or LPS. Because the inhibitory effects of GW3965 on cytokine expression were nearly absent from LXR α / β ^{-/-}, but not LXR α ^{-/-}, BMMCs (Fig. 4), we conclude that LXR β plays a crucial role in the GW3965-mediated repression of cytokine production. We and others independently reported that murine MCs predominantly express LXR β , but not LXR α .^{14,28} Therefore, we speculate that differences in the LXR β and LXR α expression levels may explain why LXR α is not required for the LXR-mediated repression of cytokine expression. In addition, the knockout of LXR α and LXR β did not significantly affect MC differentiation, Fc ϵ RI expression, or the degranulation response, as shown in Fig. 3. The inability of LXRs to regulate Fc ϵ RI expression and degranulation may be supported by our previous report, which demonstrated that Fc ϵ RI expression and Fc ϵ RI-induced degranulation are not altered in the presence of the synthetic LXR agonist T0901317.¹⁴ Collectively, these findings indicate that the activation of LXR α and LXR β does not interfere with the molecular mechanisms underlying Fc ϵ RI expression or the degranulation response of MCs.

In the absence of GW3965, we found that IgE+Ag-induced production of IL-1 β , as illustrated in Fig. 2C, and LPS-induced productions of IL-1 α and IL-6, as seen in Fig. 4B, were significantly decreased in the LXR α ^{-/-} and LXR α / β ^{-/-} MCs, respectively. We currently speculate that, in the absence of LXRs, the pro-longed culture of MCs interferes with cholesterol homeostasis and cytokine responses for the following reasons: (i) six to eight weeks were

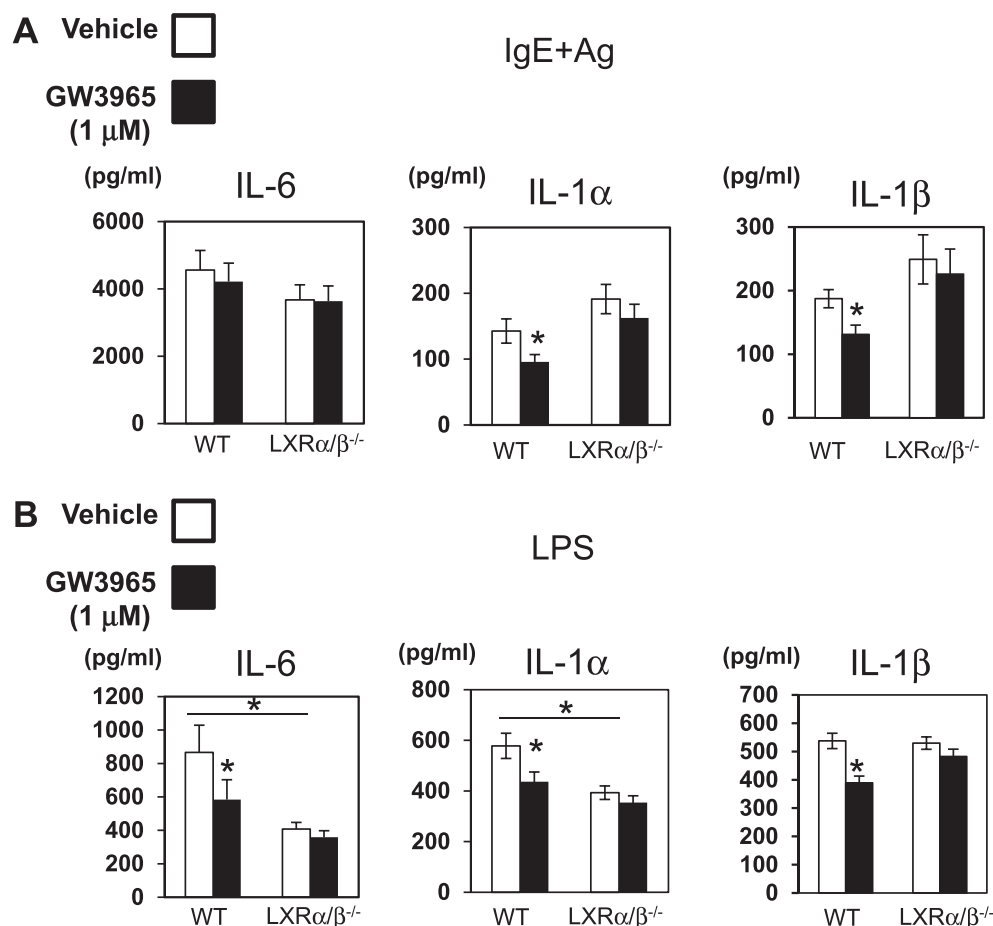


Fig. 4. The effects of GW3965 on the IgE+Ag or LPS-induced expression of IL-1 α , IL-1 β , and IL-6 by LXR α / β ^{-/-} BMMCs. IgE-sensitized WT and LXR α / β ^{-/-} BMMCs were treated with or without 1 μ M GW3965 for 1 h and then stimulated with 2 ng/ml TNP-BSA (A) or 1 μ g/ml LPS (B) for 6 h. In (A)–(B), the data are presented as the means $\pm$ SDs of quadruplicate experiments. * p < 0.05 (Student's t -test). Similar results were obtained from three independent experiments.

required for MC differentiation in this study; (ii) LXRs decrease intracellular cholesterol by increasing cholesterol efflux²⁹; and (iii) the shortage of intracellular cholesterol results in the constitutive up-regulation of cytokine production in MCs.³⁰

As we demonstrated, BMMCs produced substantial amounts of IL-6 protein upon stimulation by IgE+Ag or LPS; however, the activation of LXRs by GW3965 failed to suppress the IgE+Ag-induced production of IL-6 (Fig. 1). In this study, we did not fully elucidate the distinct effects of GW3965 on IL-6 production. In macrophages, the GW3965-dependent repression of pro-inflammatory gene expression in response to LPS is regulated by the LXR-mediated stabilization of the co-repressor complex.³¹ Ghisletti et al. reported that GW3965 stabilizes the binding of the co-repressor complex to the IL-1 β promoter in response to LPS but fails to stabilize the co-repressor complex when the cells are stimulated by Poly I:C.³² Future analysis of the binding of the co-repressor complex to the IL-6 promoter in MCs is required to explain the different sensitivities of the IL-6 gene to GW3965 when activated by distinct stimuli.

Previous studies employing mouse models suggested that the MC-mediated expression of IL-1 β is essential for the development of inflammatory arthritis^{33,34} and cryopyrin-associated periodic syndrome.³⁵ We expect that the administration of LXR agonists may have exerted suppressive effects on arthritic and skin inflammation in these experimental animals. Furthermore, epicutaneous application of LXR agonists ameliorated murine chronic atopic dermatitis by decreasing dermal Th₂ inflammation, for example, the infiltration of eosinophils, mast cells, and Th₂-positive lymphocytes.¹⁰ Regarding the cytokines associated with Th₂-mediated inflammation, the Fc ϵ RI-induced gene expression of IL-5, which is critical for the differentiation and proliferation of human and murine eosinophils,³⁶ was down-regulated in the presence of GW3965 treatment (Supplementary Fig. 1). Thus, it is likely that the LXR-mediated repression of IL-5 expression in MCs decreases the number of eosinophils in the dermis; however, the mechanisms by which MC-produced IL-5 promotes atopic skin inflammation in eosinophilia remain unclear. MC-produced IL-6 is required for LPS-induced lung inflammatory responses³⁷ and the TLR2-mediated inhibition of tumor growth,³⁸ and it protects the host from lethal sepsis by recruiting neutrophils.³⁹ Thus, MC-produced IL-6 exerts beneficial effects *in vivo*. However, treatment of MCs with GW3965 resulted in a reduction in LPS-induced IL-6 production. Therefore, we propose that the careful administration of LXR agonists to experimental animals *in vivo* should be performed in the future.

In conclusion, we addressed the biological roles of LXR α and LXR β in the LXR-dependent repression of pro-inflammatory cytokine expression in murine MCs, and our findings deepen our understanding of MCs and of the functions of LXRs.

Acknowledgments

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

SN, YO, NH, and KM performed the experiments and analyzed the data; SN and CR designed the research study and wrote the paper; KEU and MM provided essential materials and helpful suggestions; TT and CR supervised the research.

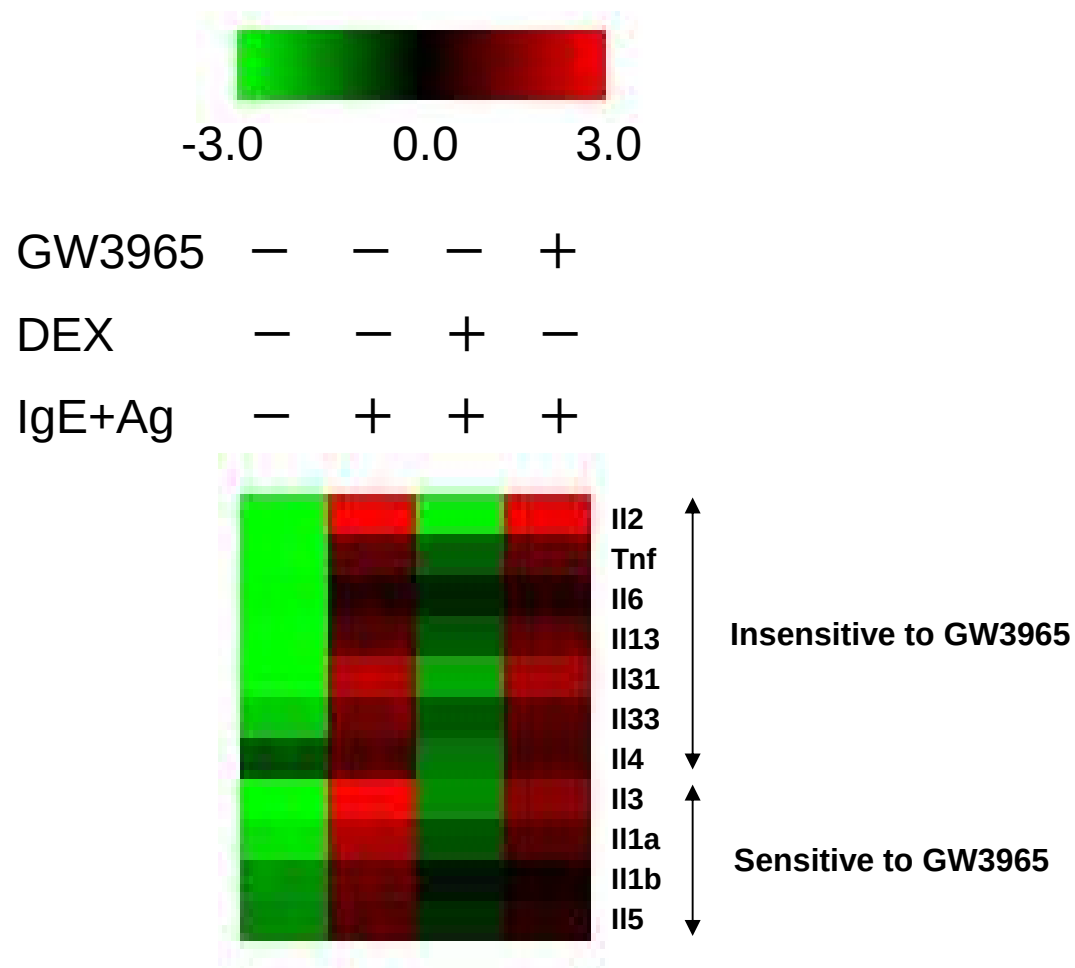
Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2015.03.001>.

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Supplementary Figure 1. The effects of GW3965 on FcεRI-dependent cytokine expression.

The IgE-sensitized WT BMMCs were treated with 1 μ M GW3965, 1 μ M DEX or DMSO (vehicle) for 1 h and then stimulated with 2 ng/ml TNP-BSA or medium alone for 1 h. Total RNA was prepared, and gene expression was analyzed via Affymetrix GeneChip analysis according to the manufacturer's instructions. (A) Heatmap displaying the expression of 11 cytokine genes in the four groups (DMSO+Medium, DMSO+TNP-BSA, GW3965+TNP-BSA, and DEX+TNP-BSA). A colorimetric scale corresponding to the expression levels is shown at the top of the figure.