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The liver X receptor promotes macrophage differentiation and suppresses osteoclast formation in mouse RAW264.7 promyelocytic leukemia cells exposed to bacterial lipopolysaccharide

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ABSTRACT

Lipopolysaccharide (LPS), the principal component of Gram-negative bacterial cell walls, is a stimulator of osteoclastogenesis and thus a key factor in inflammatory bone loss. We have recently reported that the important cholesterol and inflammatory regulator, liver X receptor (LXRα/β), can potently inhibit osteoclast formation from bone marrow-derived osteoclast precursors in a bacterial/LPS environment. In this manuscript, we further studied the effect of the LXR agonist GW3965 on osteoclast differentiation in RAW264.7 promyelocytic leukemia cells exposed to LPS. We found that not only did activation of the LXR potently inhibit the formation of TRAP-positive osteoclast-like cells, but promoted a population of TRAP-negative mononuclear cells with high phagocytic activity. We observed reduced expression of the osteoclast markers TRAP/*Acp5*, *Ctsk*, *Calcr* and *Oscar* after 3–4 days of GW3965 treatment, coinciding with an increase in the expression of the anti-osteoclastogenic factor *Irf8*. Expression of the macrophage/phagocytic marker *Cd68* was increased, however the "classical" macrophage markers *F4/80* and *Cd14* and the "alternatively" activated macrophage markers *Tgfβ* and *Il10* were not altered. Further, activation of LXR increased the expression of the macrophage survival gene *AIM/SPα*, a known LXR target gene, and osteoclast/macrophage-related markers (*Mitf, Pu.1, Usf1/2, Ostm1* and *Mfr*). Although Akt phosphorylation was reduced, GW3965 seemed to act independently of MAPKs (p38, ERK, JNK) and NFκB, and had no inhibitory effect on cytokine expression (*Tnfα, Il6,* or *Il1β*). Our results indicate that activation of the LXR not only inhibits the differentiation of osteoclast-like cells from RAW264.7 cells in a bacterial/LPS environment, but is also involved in the fate determination of myeloid progenitor cells into macrophages with high phagocytic capacity.

1. Introduction

Osteoclasts, dendritic cells and macrophages are derived from a common bone marrow progenitor cell [1]. It is the presence or absence of specific growth factors that can determine the lineage fate of the progenitor, for instance, macrophage colony stimulating factor (M-CSF) is important for stimulating the proliferation/differentiation of macrophages, whereas both M-CSF and receptor activator of NFκB ligand (RANKL) concomitantly drive these cells into osteoclasts. It is difficult to define the exact point at which a precursor cell becomes committed into a particular lineage, however in the case of osteoclasts it is believed to occur when they transition from a tartrate-resistant acid phosphatase (TRAP)-negative precursor into a TRAP-positive mononuclear pre-osteoclast. Following this, pre-osteoclasts acquire osteoclast specific markers, including calcitonin receptor, and relinquish macrophage-specific antigens and phagocytic activity [2]. However, the specific signaling pathways responsible for lineage commitment of the precursors have not been fully elucidated.

Large multinucleated osteoclasts are the primary bone resorbing cells and as such are crucial in the maintenance of bone quality. As excessive resorption by the osteoclasts is responsible for many common skeletal diseases, their differentiation is tightly regulated. Lipopolysaccharide (LPS), the main antigenic component of Gram-negative bacteria cell walls, is considered to be one of the major components that lead to bone loss in osteolytic diseases, in e.g. periodontitis, osteomyelitis, and arthritis [3,4]. LPS, through binding to toll-like receptor (TLR)-4, induces the production of pro-inflammatory cytokines such as TNFα, IL1β and IL6 that are able to stimulate osteoclast differentiation directly, or indirectly via increasing RANKL expression [5]. This increase in osteoclastogenesis ultimately results in the destructive erosion of bone.

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The liver X receptors (α/β) are nuclear transcription factors that are activated by naturally occurring ligands, specific derivatives of cholesterol, and by the synthetic agonist GW3965 [6]. There is increasing interest in the LXR as a drug intervention target, since once activated, LXR can inhibit inflammatory gene expression downstream of TLR4, IL1β and TNFα mediated signaling [7] and is able to prevent disease progression in numerous inflammatory models [8–11]. In our previous publications, we revealed another important function for the LXR in the specific regulation of osteoclast differentiation in both a physiological [12] and pathological environment [13]. In this study, we further explored the effect of GW3965 in LPS-induced differentiation of precursors using mouse RAW264.7 promyelocytic leukemia cells. We demonstrate that activation of the LXR not only inhibits LPS-induced osteoclast formation, but also promotes a population of macrophage-like cells with phagocytic characteristics.

2. Materials and methods

2.1. RAW264.7 cells

The mouse promyelocytic leukemia cell line RAW264.7 was purchased from ATCC. Cells were grown in α-MEM (Gibco) containing ribonucleosides and deoxyribonucleosides with 10% FBS (Invitrogen), 0.5% (10 mg/ml) gentamicin (Gibco) and 2 mM L-glutamine (Gibco). Then 10 μg/ml LPS (*Escherichia coli*-derived LPS, Sigma L4391) was added with or without 1 μM GW3965 (LXR agonist; Sigma) and cells were cultured for 6 days.

2.2. Phagocytic assay

RAW264.7 cells were seeded at a concentration of 20,000 cells in 96 well plates and cultured in α-MEM supplemented as described above. Phagocytosis was quantitatively measured on day 4 using the pHrodo Bio Particles Conjugates for Phagocytosis kit according to manufacturer's instructions (Invitrogen). Cells were then fixed in 4% paraformaldehyde for 20 min, permeabilized with PBS/0.1% Triton X-100 for 10 min then blocked with 4% BSA in 0.1% Tween-20 (PBS-T) for 30 min. Cells were incubated for 1 h with rabbit anti-TRAP 1:500 [14] followed by 1 h with Alexa flour 488 rabbit IgG 1:200 (Invitrogen). All antibodies were diluted in 2% BSA/PBS-T. The cells were visualized using a Leica, DM, IRB light microscope (Leica, Microsystems, Sweden). For analysis, photos of cells at high magnification with identical excitation and exposure settings were taken using OpenLab software (PerkinElmer, Sweden). Total number of TRAP positive cells (cells containing two or more nuclei) were counted in 3 wells/treatment.

2.3. RNA preparation and qPCR

RAW264.7 cells were seeded at a density of 5000 cells/cm^2 in 6 well plates and RNA extracted every 24 h for 6 days. For cytokine gene expression, cells were grown in α-MEM, supplemented as above, and pre-treated for 18 h with or without 1 μM GW3965. Then 10 μg/ml LPS was added and RNA extracted at time points indicated (1–24 h). Total RNA was isolated and qPCR performed as has been described previously [12]. Specific primers were designed using the Primer Express software (PE Biosystems, Foster City, CA, USA). *Lxrα*: forward 5′-ggagtgtcgacttcgcaaatg-3′, reverse 5′-cagcacacactcctccctca-3′; *Lxrβ*: forward 5′-gctctgcctacatcgtggtca-3′, reverse 5′-tgcgctcaggctcatcct-3′; interferon regulatory factor 8 (*Irf8*): forward 5′-gcagaaagccaagcagatgtg-3′, reverse 5′-gctctccttctctcagacaaaagc-3′; osteopetrosis associated transmembrane protein 1 (*Ostm1*): forward 5′-cggcagacagaatgcagatagt-3′, reverse 5′-cctgccacgtgctgttga-3′; macrophage fusion receptor/signal regulatory protein α *(Mfr/Sirpα)*: forward 5′-acatcttccacacggttgca-3′, reverse 5′-gtgtcctggatctgggttattt-3′; *Cd14*: forward 5′-agggtacagctgcaaggact-3′, reverse 5′-cttcagcccagtgaaagaca-3′; *Tnfα*: forward 5′-ggctgccccgactacgt-3′, reverse 5′-gactttctcctggtatgagatagcaa-3′; *Il6: forward* 5′-tgggactgatgctggtgaca-3′, reverse 5′-tgggagtggtatcctctgtgaa-3′: *Il1β*: forward 5′-ggacccatatgagctgaaagct-3′, reverse 5′-tgtcgttgcttggttctcctt-3′*; F4/80*: forward 5′-tgacaaccagacggcttgtg-3′, reverse 5′-gcaggcgaggaaaagatagtgt-3′; *Cd68:* forward 5′-cctccaccctcgcctagtc-3′, reverse 5′-ttgggtataggattcggatttga-3′. The following forward and reverse primer pairs were obtained from published studies: *Cfms, Oscar, Nfatc1, Calcr, Traf6, DC-stamp, Pu.1, Usf1, Usf2* [12], TRAP/*acp5* [14], cathepsin K (*Ctsk)* [14], *Aim/Spα* [15], microphthalmia-associated transcription factor (*Mitf)* [16], *Rank* [17], *Cfos* [18], *Tgfb, Il10* [19]. All genes were analyzed with the SYBR green detection method using the Applied Biosystems 7500 Real Time PCR machine as has been described previously [12]. All gene expression data were normalized against both β-actin and 18S and the control values expressed as 1 to indicate a precise fold change for each gene of interest.

2.4. TRAP enzyme activity

TRAP enzyme activity was measured in media collected from RAW264.7 cells cultured for 0–6 days as described previously [14].

2.5. Cytokine analysis

RAW264.7 cells were grown in α-MEM, supplemented as above, and pre-treated for 18 h with or without 1 μM GW3965. Then 10 μg/ml LPS was added and after 24 h cell culture media analyzed for TNFα, IL1β and IL6 (R&D systems) using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instruction.

2.6. Western blot

RAW264.7 cells were seeded at a density of 10,000 cells/cm^2 in 10 cm plates with α-MEM (as described above) and cultured for 2–3 days until 80% confluent. Cells were pre-treated for 18 h with or without 1 μM GW3965. Then 10 μg/ml LPS was added for 5, 15 and 30 min. Protein isolation and Western blots were performed as described previously [12]. Primary antibodies were incubated overnight at 4 °C; in 4% BSA, p38, p-p38, ERK, p-ERK, JNK, p-JNK, Akt, p-Akt and IκB-α (1:1000 Cell Signaling Technology). The levels of β-actin (1:30,000 for 30 min in 5% milk; Sigma) were analyzed as a control for constant loading for IκB-α. Densitometric values were quantified for each band with Image J 1.40. The control density value at each time point 0 (for both − and + GW3965) is presented as 1. Western blots were performed twice with consistent results.

2.7. Statistical analyses

Effects of the LXR agonist GW3965 between treated and untreated groups were analyzed either using a student's *T*-test or a one-way ANOVA with a posthoc Tukey test. All analyses were performed using the GraphPad Prism 5.0 software. Data were considered statistically significant when a *P* value less than 0.05 was obtained. All data are expressed as mean ± SEM.

3. Results

3.1. GW3965 stimulates the differentiation of macrophage-like cells in LPS exposed RAW264.7 cells

In our recent publication [13], we demonstrated that activation of LXR with the agonist GW3965 potently inhibits the formation of

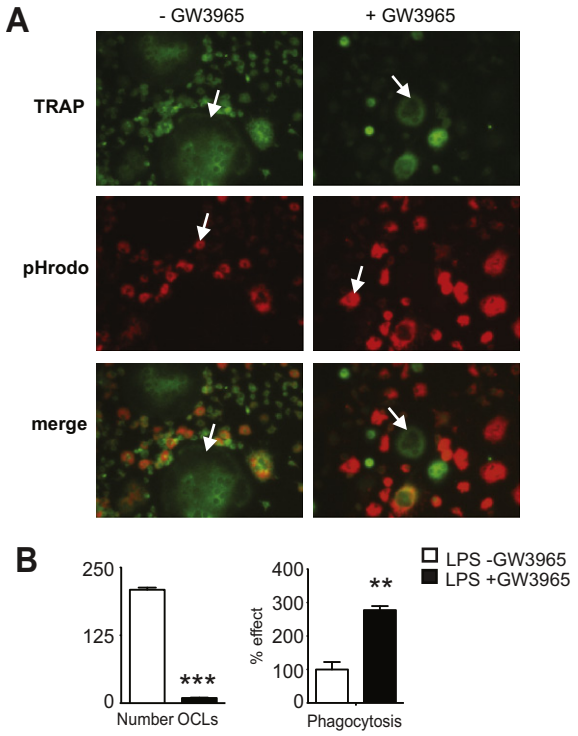

Fig. 1. RAW264.7 cells were cultured for 4 days in the presence of 10 μg/ml LPS with or without the LXR agonist GW3965 (1 μM). Cells were incubated with pHrodo Bio Particles Conjugates for 2.5 h at 37 °C. (A) Cells were fixed in 4% PFA and stained for TRAP (green stain) with pHrodo staining red. Arrows indicate multinucleated TRAP positive osteoclast-like cells (TRAP row), phagocytic cells (pHrodo row) and pHrodo negative osteoclast-like cells (merge row). (B) The number of $TRAP^+$ multinucleated osteoclast-like cells (OCLs) on day 4 with or without GW3965 were counted (from 3 wells/treatment). Phagocytosis was measured in a fluorescence plate reader and net uptake compared with cells cultured without GW3965 as a control set at 100%. Data expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ using a Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

large TRAP positive osteoclast-like cells from RAW264.7 cells in an LPS environment. In this study, we confirm this finding (Fig. 1A and B), and further demonstrate that the small osteoclast-like cells formed after GW3965 treatment retain typical osteoclast characteristics i.e. are TRAP positive and do not ingest particles (pHrodo) (Fig. 1A). However, we observed that activation of LXR with GW3965 stimulated a population of cells that displayed a macrophage-like phenotype, in that they were TRAP negative and able to phagocytose (Fig. 1A and B).

3.2. GW3965 inhibits osteoclast markers and induces certain macrophage genes

To further analyze the effect of GW3965 on macrophage and osteoclast differentiation, RAW264.7 cells were differentiated for 6 days in the presence of LPS and specific phenotypic markers analyzed. Activation of LXR had a significant effect on the expression of osteoclast-specific genes beginning on day 3 of LPS-induced differentiation, with an inhibition of TRAP/*Acp5, Ctsk, Calcr* and *Oscar* (Fig. 2A). Around the same time point, the expression of the anti-osteoclastogenic factor *Irf8* (Fig. 2B) and the macrophage/phagocytic marker *Cd68* increased (Fig. 2B). However, expression of the "classical" macrophage markers *Cd14* and *F4/80* and the "alternatively" activated macrophage markers *Tgfβ* and *Il10* were not changed (Fig. 2B and C). There was no change in the expression of *Lxrα* and *β* from day 0–6 of differentiation (Fig. 2D). The inhibition of osteoclast markers was also reflected in the TRAP activity assay (Fig. 2E). Finally, GW3965 also increased the expression of *Aim*, a macrophage survival gene and an LXR target gene, from day 2, indicating that the RAW264.7 cells were responding as expected to the GW3965 agonist (Fig. 2F).

3.3. LXR activation does not affect pro-inflammatory cytokine expression

As expected, LPS treatment of RAW264.7 cells dramatically increased the pro-inflammatory cytokines TNFα, IL6 and IL1β in the first 24 h after exposure (Fig. 3A), however the LXR agonist did not alter either the mRNA or protein expression of these cytokines (Fig. 3A and B).

3.4. Effect of GW3965 on the expression of differentiation genes

To further investigate the effect of GW3965 on differentiation, we analyzed the mRNA expression of osteoclast and macrophage specific signaling genes from day 0–6 of LPS exposure. Activating LXR did not affect the expression of *Cfms, Rank, Cfos* or *Nfatc1* in RAW264.7 cells exposed to LPS (Fig. 4A). However, the adaptor protein *Traf6*, *Mitf* with its collaborator *Pu.1*, the upstream regulatory factors *Usf1* and *2*, and finally *Ostm1,* which is coregulated by *Mitf,* were all increased at day 3–4 of differentiation (Fig. 4A). The macrophage fusion receptor (*Mfr*) was increased however there was no difference in *Dc-stamp*, also required for fusion (Fig. 4A).

3.5. Effect of GW3965 on MAPK and NFκB signaling pathways

We analyzed the downstream signaling pathways of TLR4/LPS and observed a reduction in phosphorylated Akt after GW3965 treatment (Fig. 4B), however, no reduction in phosphorylated ERK, p-38 or JNK or on degradation of the NFκB inhibitor Iκ-Bα.

4. Discussion

We have recently reported that activation of the LXR with the agonist GW3965 potently inhibited osteoclast formation from LPS-exposed bone marrow derived macrophages and RAW264.7 promyelocytic leukemia cells [13]. In the present study, we further investigated the role of GW3965 in LPS-induced differentiation of RAW264.7 cells, and clearly show that GW3965 not only inhibits LPS-induced osteoclast formation, but promotes a population of cells with predominantly macrophage-like characteristics.

RAW264.7 cells, a cell line initially derived from Balb/c mice infected with Abelson murine leukemia virus (MuLV), are widely used as a model for both osteoclast and macrophage differentiation. RAW264.7 cells respond to LPS stimulation through TLR4, inducing the secretion of inflammatory cytokines and the formation of large multinucleated osteoclast-like cells [13,20,21]. In this study, GW3965 significantly inhibited the formation of TRAP-positive osteoclast-like cells. This may be attributable to the significant increase in interferon regulatory factor-8 (*Irf8*), a key factor responsible for negativelyregulating osteoclast differentiation [22]. Instead, GW3965 promoted a population of cells that were negative for osteoclast markers (TRAP/*Acp5, Ctsk, Calcr* and *Oscar*) and had high phagocytic activity. One of the central roles of macrophages is to engulf and eliminate foreign pathogens [23], a phenotype lost when precursors are directed into the osteoclast lineage. Thus, the ability of these cells to phagocytose particles suggests these cells have macrophage-like characteristics, however macrophages are very heterogeneous and will alter their phenotype depending on in which tissue or environment they reside in [23,24].

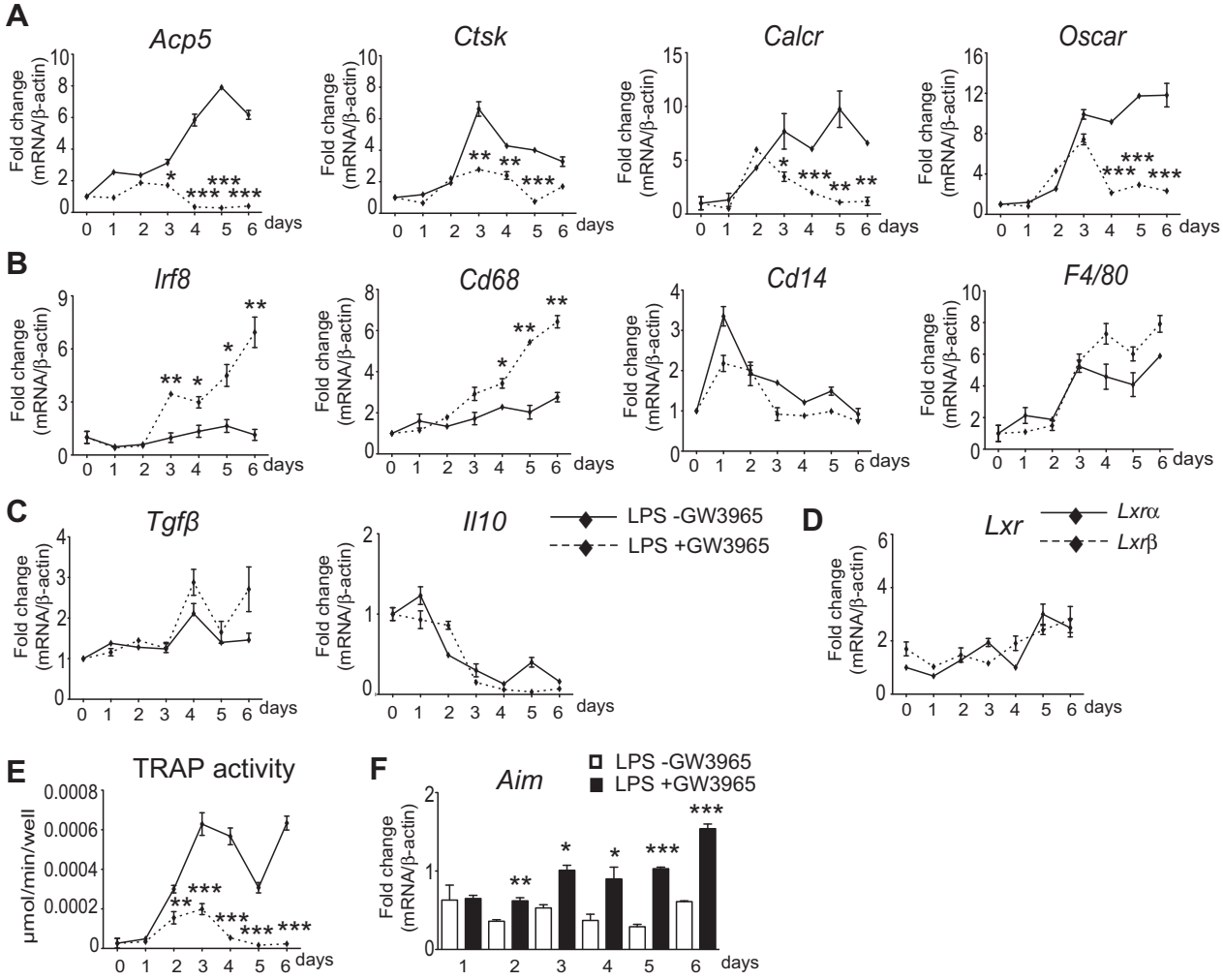

Fig. 2. RAW264.7 cells were cultured for 6 days in the presence of 10 μg/ml LPS with or without the LXR agonist GW3965 (1 μM). RNA was extracted every 24 h and qPCR ($n = 4$ wells/treatment/time) used to measure gene expression. Data were normalized against β-actin and the control values (day 0, no LPS treatment) expressed as 1. Data expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ using a one-way ANOVA with a posthoc Tukey test.

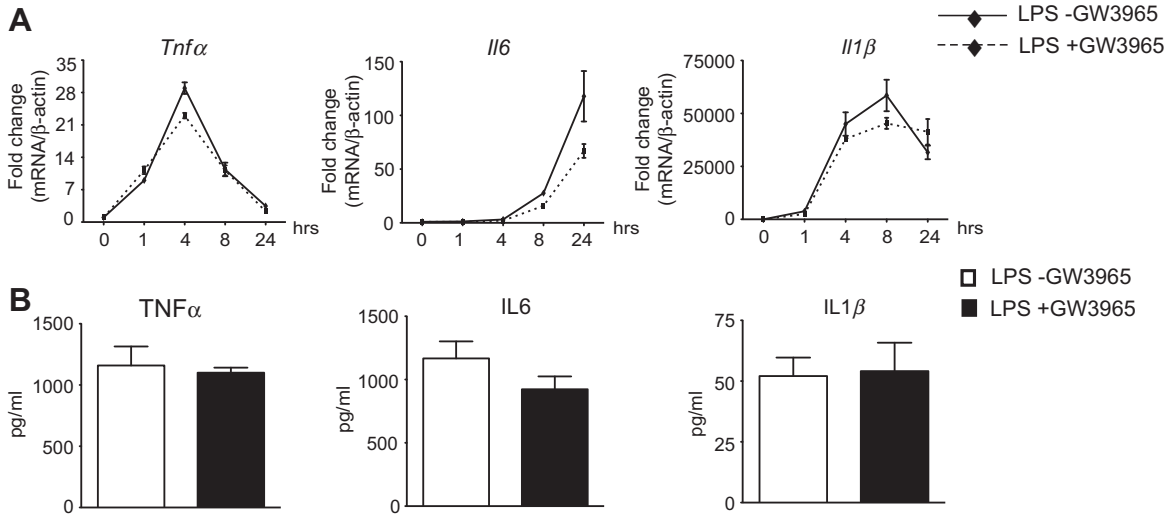

Fig. 3. RAW264.7 cells were cultured for 18 h with or without 1 μM GW3965 followed by 10 μg/ml LPS for 1, 4, 8 or 24 h. (A) RNA was extracted and qPCR ($n = 4$ wells/treatment/time) used to measure gene expression. Data were normalized against β-actin and the control values (day 0, no LPS treatment) expressed as 1. (B) The effect of GW3965 on cytokine levels was determined using ELISA on the cell media at 24 h. Data expressed as mean ± SEM.

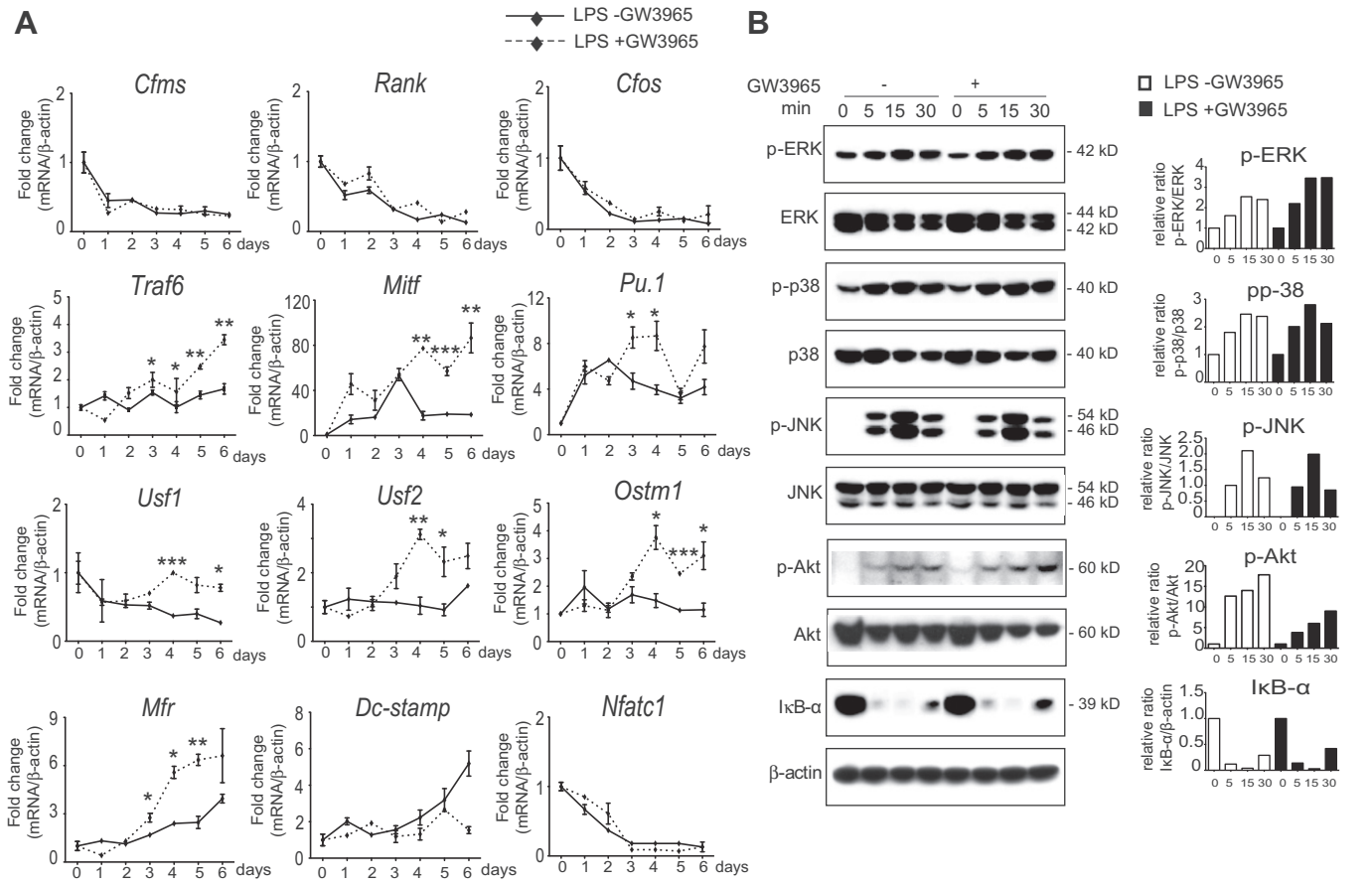

Fig. 4. (A) RAW264.7 cells were cultured for 6 days in the presence of 10 μg/ml LPS with or without the LXR agonist GW3965 (1 μM). RNA was extracted every 24 h and qPCR (n = 4 wells/treatment/time) used to measure gene expression. Data were normalized against β-actin and the control values (day 0, no LPS treatment) expressed as 1. Data expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ using a one-way ANOVA with a posthoc Tukey test. (B) RAW264.7 cells were cultured for 18 h with or without 1 μM GW3965 followed by 10 μg/ml LPS for 5, 15 or 30 min. Western blots were prepared from cell lysates with antibodies to MAPKs, Akt and IκB-α. Densitometric values of phosphorylated to non-phosphorylated forms, or with IκB-α to β-actin, were quantified using Image J. The control density value at time point 0 (for both − and + GW3965) is presented as 1.

Exposure of macrophages to LPS induces their activation and the secretion of proinflammatory cytokines via a TLR4 dependent pathway [25]. Consistent with this, exposing RAW264.7 cells to LPS for 24 h resulted in an increase in TNFα, IL6 and IL1β. GW3965 has been demonstrated to be a potent repressor of inflammation in LPS-exposed macrophages [26,27]. However, in RAW264.7 cells, we found no change in these cytokines following GW3965 treatment, suggesting this cell-line responds differently to LXR activation compared to primary macrophages. To investigate if these RAW264.7 cells had a macrophage-like phenotype following GW3965 treatment, we measured various macrophage markers with qPCR. LXR activated cells expressed high levels of *Cd68*, a macrophage and active phagocytosis marker [28], but not the macrophage markers *Cd14* and *F4/80*. In addition, they did not express high levels of *Tgfβ* or *Il10*, indicating they were probably not alternatively activated macrophages (M2). This suggested this population of cells displayed certain macrophage-like phenotypes, however not those related to pro-or anti-inflammatory actions, but rather to phagocytosis.

In order to characterize these cells further, we examined the downstream signaling pathways important in both osteoclast and macrophage differentiation. LPS activates its putative receptor TLR4 and signals via MyD88 (myeloid differentiation primary response gene) and either the adaptor molecule TRAF6 or phosphatidylinositol 3 kinase-Akt (PI3K-Akt), to activate NfκB and induce proinflammatory genes [25]. Following LXR agonist treatment, we observed increased expression of *Traf6,* suggesting activation of the Traf6 pathway. We also observed a reduction in Akt phosphorylation, similar to our previous study with RANKL-primed BMM exposed to LPS [13], suggesting that GW3965 could be involved in the induction of Akt following LPS infection. Also similar to what we reported previously [13], we saw no difference in the MAPKs (p-38 or JNK), although a slight increase in ERK, and no effect on degradation of the NFκB inhibitor IκB-α. As we observed no reduction in cytokine levels, and LXR is known to mediate its anti-inflammatory effects through transrepression of NFκB in a SUMOylation dependent mechanism, it may be acting differently in RAW264.7 cells.

Numerous other genes were increased following GW3965 treatment of LPS-stimulated RAW264.7 cells, most of which can be associated with osteoclastogenesis. For example, the ets family transcription factor PU.1, an important player in the differentiation of multipotent stem cells into the lymphoid and myeloid lineages and involved in promoting macrophage differentiation [29,30], was increased after LXR activation. However, PU.1 has also been found to increase as macrophages differentiate into osteoclasts, suggesting an important role for this factor in osteoclast lineage commitment [31]. Interestingly, PU.1's binding partner microphthalmia-associated transcription factor (MiTF), was also increased. MiTF is important in the regulation of osteoclast-specific genes including *Acp5* and *Ctsk* [32,33] as are the upstream stimulatory factors (Usf1 and 2) [34]. Further, osteoporosis-associated

transmembrane protein 1 (Ostm1), crucial in osteoclastogenesis [35], was also upregulated. This suggests that the LXR acts distally to the TRAF6/PU.1/MiTF regulatory pathways.

The results presented in this study clearly show that the cell population formed when LPS-stimulated RAW264.7 cells are treated with the LXR agonist have macrophage characteristics. These cells express reduced osteoclast-specific markers (*Acp5, Ctsk, Calcr* and *Oscar*), however display a macrophage phenotype with their ability to phagocytose and high expression of the macrophage/phagocytic marker *Cd68*. Interestingly, they also express increased common macrophage/osteoclast-related markers (*Mitf, Pu.1, Usf1/2* and *Ostm1*). In conclusion, the LXR ligand, in the presence of LPS as the differentiation factor, not only inhibits the differentiation of osteoclasts but promotes and augments the differentiation of macrophage-like cells. As this population of cells are highly phagocytic, they would be capable of ingesting foreign material such as apoptotic/necrotic cells and thus could play an important role in wound healing and the maintenance of healthy tissue.

Acknowledgments

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