LXR nuclear receptors prompt a pro-inflammatory gene and functional profile in human macrophages

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LXR nuclear receptors prompt a pro-inflammatory gene and functional profile in human macrophages

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4 Short title: LXR promotes a pro-inflammatory human macrophage signature

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17

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19

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23 **ABSTRACT**

24 Liver X Receptors (LXR) control cholesterol metabolism and exert anti-inflammatory actions in
25 activated macrophages. However, their contribution to human macrophage polarization in the
26 absence of pathogenic stimuli remains unclear. In fact, the LXR pathway has been reported to be
27 significantly enriched in pro-inflammatory synovial macrophages from rheumatoid arthritis patients
28 as well as in immunosuppressive tumors-associated macrophages from human metastatic colon
29 tumors. To determine the role of LXR on macrophage differentiation and polarization, we have
30 analyzed the contribution of LXR to the acquisition of the inflammatory and T-cell-activating
31 functions of human monocyte-derived macrophages. We now report that LXR activation prompts
32 the acquisition of a pro-inflammatory gene signature in human macrophages, whereas LXR
33 inactivation results in enrichment of an anti-inflammatory gene profile. Accordingly, activation and
34 inhibition of LXR oppositely alter the production of cytokines (e.g., TNF, IL1 β , CCL17, CCL19, IFN β 1)
35 and T cell stimulation activities associated to human macrophage polarization. Mechanistically, the
36 LXR-stimulated macrophage polarization shift relies on their ability to modulate the expression of
37 MAFB and MAF, which govern the acquisition of the macrophage anti-inflammatory profile. The
38 pathological significance of the LXR-mediated macrophage polarization shift was demonstrated by
39 the ability of LXR agonists to modulate macrophage polarization promoted by either tumor-derived
40 ascitic fluids or by synovial fluid from rheumatoid arthritis patients. As a whole, our results
41 demonstrate that LXR activation prompts the acquisition of a pro-inflammatory transcriptional and
42 functional specialization in human macrophages.

43

44 **KEYWORDS: Inflammation; Innate immunity; Macrophage; Macrophage Polarization**

45

46 INTRODUCTION

47 Macrophages defend the organism against endogenous danger signals and exogenous threats, and
 48 initiate and resolve inflammatory responses. To perform these assorted tasks, macrophages can
 49 display a huge spectrum of activation ("polarization") states, whose acquisition depends on their
 50 developmental origin, tissue location, and prevailing extracellular cues (1–3). During inflammation,
 51 macrophages exert pro-inflammatory and anti-inflammatory/resolving effector functions, whose
 52 fine-tuning and sequential occurrence are crucial for tissue injury removal and return to
 53 homeostasis, and whose unbalance is associated to chronic inflammatory diseases.

54
 55 M-CSF and GM-CSF have opposite instructing effects on macrophages during inflammatory
 56 responses (4, 5). M-CSF is indispensable for tissue-resident and monocyte-derived macrophage
 57 differentiation (6–9), and primes macrophages (M-MØ) for acquisition of an anti-inflammatory and
 58 immunosuppressive profile ($IL10^{high}$ TNF^{low} $IL23^{low}$ $IL6^{low}$) (5, 10–19). By contrast, GM-CSF is
 59 produced at sites of inflammation (6, 7), and primes macrophages (GM-MØ) for robust antigen-
 60 presenting, T cell-stimulatory and pro-inflammatory activity ($IL10^{low}$ TNF^{high} $IL23^{high}$ $IL6^{high}$). Thus, M-
 61 MØ resemble tissue-resident 'trophic' macrophages, whereas GM-MØ represent pro-inflammatory
 62 monocyte-derived macrophages. In line with their opposite effector functions, M-MØ and GM-MØ
 63 exhibit distinct transcriptional profiles (10, 13, 20–22) that resemble those of human resident and
 64 inflammatory macrophages *in vivo* (22–25), and also differ in their responsiveness to methotrexate
 65 (MTX) (26, 27).

66
 67 LXR α and LXR β (coded for by *NR1H3* and *NR1H2*, respectively) are ligand-activated transcription
 68 factors that regulate gene expression (positively and negatively) in a ligand-dependent manner, and
 69 that actively control macrophage differentiation and specialization (28, 29). LXR activity is crucially

involved in cellular cholesterol metabolism in most tissues. Upon binding of ligand (endogenous cholesterol derivatives, or synthetic agonists like T0901317 and GW3965), LXR positively control the expression of genes that collectively inhibit uptake and promote cholesterol efflux, thus contributing to prevent cellular lipid overload. LXR ligands also control inflammation in macrophages by antagonizing the induction of inflammation-related genes after activation (29, 30). These results have led to recognition of LXR as anti-atherogenic and anti-inflammatory factors (31, 32). However, LXR activation exacerbates inflammatory responses in human monocytes, dendritic cells and in a mouse arthritis model (33–36), and agonist-induced LXR activation elicits anti-tumor activity through immune-mediated mechanisms (37), specifically reducing the abundance of immunosuppressive Myeloid-Derived Suppressor Cells (MDSC) and enhancing anti-tumoral cytotoxic T lymphocytes (CTLs) activation (37). Of note, the LXR pathway is highly upregulated in pro-inflammatory macrophages from rheumatoid arthritis (RA) synovial fluid (RASf-MØ), where LXR potentiate TLR-driven cytokine release (38), but is also the most enriched pathway in large tumor-associated macrophages (large TAM) (39), which are immunosuppressive and correlate with a worse prognosis (40). This apparently discrepant findings illustrate the ambiguous contribution of LXR to inflammatory and immune responses, and raises the question of the role of LXR factors to human macrophage polarization (33, 41).

87

To address this issue, we have now determined the range of LXR target genes in anti-inflammatory M-MØ and pro-inflammatory GM-MØ (13, 22), and assessed the contribution of LXR to the acquisition of the transcriptional and functional signature of human macrophages through the use of specific LXR agonist (GW3965) and inverse agonist (GSK2033). We report that LXR controls human macrophage specialization, and that LXR activation primes monocytes towards the acquisition of the transcriptional and functional properties of pro-inflammatory macrophages.

94 **EXPERIMENTAL PROCEDURES**

95 **Generation of human monocyte-derived macrophages in vitro and treatments.** Human Peripheral
 96 Blood Mononuclear Cells (PBMCs) were isolated from buffy coats from anonymous healthy donors
 97 over a Lymphoprep (Nycomed Pharma) gradient according to standard procedures. Monocytes
 98 were purified from PBMC by magnetic cell sorting using anti-CD14 microbeads (Miltenyi Biotec).
 99 Monocytes (>95% CD14⁺ cells) were cultured at 0.5 x 10⁶ cells/ml in Roswell Park Memorial Institute
 100 (RPMI 1640, Gibco) medium supplemented with 10% fetal bovine serum (FBS, Biowest) for 7 days
 101 in the presence of 1000 U/ml GM-CSF or 10 ng/ml M-CSF (ImmunoTools) to generate GM-CSF-
 102 polarized macrophages (GM-MØ) or M-CSF-polarized macrophages (M-MØ), respectively (42).
 103 Cytokines were added every two days and cells were maintained at 37°C in a humidified atmosphere
 104 with 5% CO₂ and 21% O₂. Where indicated, macrophages were treated at different time points with
 105 one dose of LXR agonist GW3965 (1 µM, Tocris), LXR inverse agonist GSK2033 (1 µM, Tocris) or both,
 106 using dimethyl sulfoxide (DMSO) as vehicle. In the dual condition, the inverse agonist was added 1-
 107 hour prior to agonist treatment. When indicated, either Synovial Fluid from Rheumatoid Arthritis
 108 (RASf) patients or Ascitic Fluid from cancer patients (AF) was added to monocytes (0.2:1 in culture
 109 medium for RASf, 0.5:1 in culture medium for AF), and the cultures were maintained for 72 h.
 110 Synovial fluid samples were obtained by therapeutic arthrocentesis from the knee joints of patients
 111 with active Rheumatoid Arthritis (24). Ascitic fluids from metastatic tumors were kindly provided by
 112 Dr. M^a. Isabel Palomero (Oncology Department, Hospital General Universitario Gregorio Marañón).
 113 Samples were centrifuged (4000xg, 15 min) to remove cells and particulate material, sterile-filtered,
 114 aliquoted, and stored at -20°C until use. The patients provided informed consent and the Hospital
 115 General Universitario Gregorio Marañón ethics committee approved the study. For macrophage
 116 activation, cells were treated with 10 ng/ml E. coli 055:B5 lipopolysaccharide (Ultrapure LPS, Sigma-
 117 Aldrich). Human cytokine production was measured in supernatants collected from treated M-MØ

118 and GM-MØ using commercial ELISA [(TNF- α , CCL2 (BD Biosciences), IL-10, IL-6 (Biolegend), Activin
119 A, CCL19, CCL17, IL1 β , IFN β 1 (R&D Systems) and MMP12 (Bosterbio)] according to the procedures
120 supplied by the manufacturers.

121

122 **Quantitative real-time RT-PCR (qRT-PCR).** Total RNA was extracted using the total RNA and protein
123 isolation kit (Macherey-Nagel). RNA samples were reverse-transcribed with High-Capacity cDNA
124 Reverse Transcription reagents kit (Applied Biosystems) according to the manufacturer's protocol.
125 Real-time quantitative PCR was performed with LightCycler® 480 Probes Master (Roche Life
126 Sciences) and Taqman probes on a standard plate in a Light Cycler® 480 instrument (Roche
127 Diagnostics). Gene-specific oligonucleotides (Supplementary Table 1) were designed using the
128 Universal Probelibrary software (Roche Life Sciences). Results were normalized to the expression
129 level of the endogenous references genes *TBP*, *HPRT1* and *GAPDH* and quantified using the $\Delta\Delta CT$
130 (cycle threshold) method.

131

132 **Western blot.** M-MØ and GM-MØ cell lysates were subjected to SDS-PAGE (50 μ g unless indicated
133 otherwise) and transferred onto an Immobilon-P polyvinylidene difluoride membrane (PVDF;
134 Millipore). After blocking the unoccupied sites with 5% non-fat milk diluted in Tris-Buffered Saline
135 plus Tween 20 (TBS-T), protein detection was carried out with antibodies against LXR α (PPZ0412;
136 Biotechne), LXR β (PPK8917; Biotechne), MAFA (Sigma Aldrich) or c-MAF (sc-7866; Santa Cruz
137 Biotechnology). Protein loading was normalized using an antibody against GAPDH (sc-32233; Santa
138 Cruz Biotechnology) or vinculin (V9131; Sigma-Aldrich). Quimioluminescence was detected in a
139 Chemidoc Imaging system (BioRad) using SuperSignal™ West Femto (ThermoFisher Scientific).

140

141 **Small Interfering Ribonucleic Acid (siRNA) Transfection.** M-MØ or GM-MØ (1×10^6 cells) were
 142 transfected with human *NR1H3*-specific siRNA (siNR1H3, 50 nM) (s19568, Thermo-Fisher Scientific)
 143 and/or human *NR1H2*-specific siRNA (siNR1H2, 50nM) (s14684; Thermo-Fisher Scientific) using
 144 HiPerFect (Qiagen). Silencer™ Select Negative Control No. 1 siRNA (siCtrl, 50 nM) (#4390843;
 145 Thermo-Fisher Scientific) was used as negative control siRNA. Six hours after transfection, cells were
 146 allowed to recover from transfection in complete medium (18 h) and then lysed. Knock-down of
 147 NR1H3/LXR α or NR1H2/LXR β was confirmed by q-PCR and western blot. Monocytes (1×10^6 cells)
 148 were transfected with human MAFB-specific smart Pool (siMAFB, 25 nM) (M-009018; Dharmacon)
 149 using HiPerFect (Qiagen). siGenome non-targeting siRNA Pool#2 (siCtrl, 25 nM) (D-001206;
 150 Dharmacon) was used as negative control siRNA. Four hours after transfection, cells were treated
 151 with GSK2033 (1 μ M, Tocris) and allowed to recover from transfection in complete medium for 36
 152 h before lysis.

153

154 **Mixed Leukocyte Reaction (MLR).** Untreated or treated M-MØ and GM-MØ were detached using
 155 Trypsin-EDTA (Gibco) at 37°C, and re-plated in a 96-well U-bottom plates (10^4 cells/well) in RPMI
 156 with 5% human AB serum (Lonza) for 24 hours. Allogeneic T lymphocytes were isolated from PBMCs
 157 using CD3+ magnetic beads (Miltenyi Biotec), and co-cultured with macrophages (10:1 T
 158 lymphocyte:macrophage ratio) for 6 days in RPMI with 5% human AB serum. Then, 3 H-Thymidine (1
 159 μ Ci/well, Perkin Elmer) was added and, after 18 hours, radioactivity was transferred to a filter and
 160 thymidine counts measured in a scintillation counter (Perkin Elmer).

161

162 **RNA-sequencing and data analysis.** RNA was isolated from GM-MØ or M-MØ generated from
 163 monocytes exposed to a single dose of DMSO, GW3965, GSK2033 or both at the beginning of the
 164 differentiation process, and subjected to sequencing on a BGISEQ-500 platform

165 (<https://www.bgitechsolutions.com>). RNAseq data were deposited in the Gene Expression Omnibus
 166 (<http://www.ncbi.nlm.nih.gov/geo/>) under accession GSE156783 and GSE156696. On average,
 167 88.04 M reads per sample were generated and clean reads were mapped to the reference (UCSC
 168 Genome assembly hg38) using Bowtie2 (average mapping ratio to reference genome, 91.82%) (43).
 169 Gene expression levels were calculated by using the RSEM software package (44), and differential
 170 gene expression was assessed by using the R-package DESeq2 algorithms using the parameters Fold
 171 Change>2 and adjusted p value <0.05. Plots generated with the ggplot2 package, and heatmaps and
 172 clustering were done using the Genesis software (<http://genome.tugraz.at/genesisclient/>) (45).
 173 Differentially expressed genes were analysed for annotated gene sets enrichment using ENRICH
 174 (<http://amp.pharm.mssm.edu/Enrichr/>) (46, 47), and PANTHER (48), and enrichment terms
 175 considered significant with a Benjamini-Hochberg-adjusted p value <0.05. For gene set enrichment
 176 analysis (GSEA) (<http://software.broadinstitute.org/gsea/index.jsp>) (49), gene sets available at the
 177 website, as well as gene sets generated from publicly available transcriptional studies
 178 (<https://www.ncbi.nlm.nih.gov/gds>), were used. Detection of LXR-binding elements was done on
 179 the available CHIP-Seq data from GW3965-treated colorectal adenocarcinoma HT29 cells (GEO
 180 GSM2042848) (50) using the Cistrome Data Browser (<http://cistrome.org/db/#/>) and the DMINDA2
 181 (regulatory DNA motif identification and analysis) web server
 182 (<https://bmbl.bmi.osumc.edu/DMINDA2/index.php>) (51).

183

184 **Statistical analysis.** For comparison of means, and unless otherwise indicated, statistical significance
 185 of the generated data was evaluated using the paired Student t test in GraphPad Prism 8. In all cases,
 186 p<0.05 was considered as statistically significant.

187 RESULTS

188 **Differential expression of LXR α and LXR-regulated genes in GM-M ϕ and M-M ϕ .** We have
 189 previously defined two gene sets whose expression marks human monocyte-derived pro-
 190 inflammatory GM-M ϕ ("Pro-inflammatory gene set") and anti-inflammatory M-M ϕ ("Anti-
 191 inflammatory gene set") (Figure 1A) (13, 22), and also discriminates the gene profiles of pro-
 192 inflammatory and immunosuppressive macrophages *in vivo* (24). Accordingly, the GM-M ϕ
 193 transcriptome exhibits an over-representation of genes highly expressed by rheumatoid arthritis
 194 synovial fluid macrophages (RASf-M ϕ) (52), whereas the transcriptome of M-M ϕ shows a strong
 195 enrichment of genes preferentially expressed by tumor-associated macrophages (TAM) with
 196 significant prognostic relevance (39) (Figure 1B). Remarkably, however, the LXR pathway has been
 197 reported to be highly upregulated in both pro-inflammatory RASf-M ϕ (38) and immunosuppressive
 198 TAM from colorectal liver metastasis (39), thus raising the question of the role of LXR on human
 199 macrophage polarization. To clarify this apparent discrepancy, we sought to determine the role of
 200 LXR along the differentiation of both pro-inflammatory GM-M ϕ and anti-inflammatory M-M ϕ .

201
 202 Protein analysis on a large number of independent macrophage samples revealed a higher
 203 expression of LXR α (encoded by *NR1H3*) in GM-M ϕ , and a higher level of *NR1H2*-encoded LXR β in
 204 M-M ϕ (Figure 1C-D), a result that agrees with the relative expression of *NR1H3* and *NR1H2* mRNA
 205 in both macrophage subtypes (Figure 1E). Regarding LXR target genes [defined by positive
 206 responsiveness to desmosterol, (53)], their expression was not associated to a specific macrophage
 207 subtype (Figure 1F) and, in fact, GSEA revealed that the expression of LXR-target genes diverged
 208 between M-M ϕ and GM-M ϕ (data not shown). However, and unlike *LPL* (54) and *APOE* (55), the
 209 LXR target genes *ABCA1* (56), *ABCG4* (57), *MERTK* (58), *ARL4C* (59), *ARG2* (60) and *PLTP* (61) were

210 expressed at higher levels in anti-inflammatory M-MØ (Figure 1G), thus suggesting that the extent
211 of expression of classical LXR targets tends to be higher in M-MØ.

212

213 **LXR activation shifts the M-CSF-dependent differentiation of M-MØ towards the pro-
214 inflammatory side.** To address the extent of the LXR dependency of the transcriptome of M-MØ,

215 monocytes were exposed to a single dose of either the LXR agonist GW3965 (1 µM), the LXR inverse
216 agonist GSK2033 (1 µM) or both (GSK2033 1 hour prior to GW3965) at the beginning of the

217 differentiation process with M-CSF, and the transcriptome of the resulting macrophages was
218 determined (Figure 2A). As expected, GW3965 treatment enhanced the expression of LXR-

219 dependent genes (53), an effect that was abrogated or impaired by GSK2033 (Figure 2B). Exposure
220 to GW3965, and to a lower extent GSK2033, notably altered the transcriptome of M-MØ (Figure

221 2C). Indeed, expression of the M-MØ-specific and GM-MØ-specific gene sets were very significantly
222 altered in GW3965-M-MØ and GSK2033-M-MØ (Figure 2D), an effect also evident upon clustering

223 of the genes modulated by either ligand (Figure 2E, F). The most unambiguous effect was observed
224 in the case of GW3965-M-MØ, where the expression of the M-MØ-specific and GM-MØ-specific

225 gene sets was dramatically enhanced and impaired, respectively (Figure 2D), while expression of the
226 GM-MØ-specific gene set was reduced in GSK2033-M-MØ (Figure 2D). Of note, GW3965 treatment

227 impaired the expression of genes associated to the anti-inflammatory activities of M-MØ (*IL10*,
228 *FOLR2*, *CD209*) (22, 62, 63), and augmented the expression of paradigmatic genes of the GM-MØ-

229 specific gene sets (*CD226*, *EGLN3*) (Figure 2D, G). Therefore, LXR activation favors the acquisition of
230 a more pro-inflammatory transcriptional profile in anti-inflammatory M-MØ. Interestingly, the pro-

231 inflammatory effect of GW3965 varied along M-MØ differentiation, as its inhibitory effect on the
232 expression of the M-MØ-specific gene set (e.g., *IGF1*, *FOLR2*, *HTR2B*, *CD163L1*, *IL10*, *CCL2*) was more

233 profound at the beginning of the differentiation process (Figure 3A, B), and was weaker at later time

points (Figure 3A). By contrast, the inverse agonist GSK2033 had a negligible effect by itself, although it completely prevented the effect of the agonist (Figure 3A, B). Therefore, the pro-inflammatory consequences of LXR activation are maximal at the start of the M-MØ differentiation process and gradually weakens at later time points.

238

The functional significance of the LXR-dependent transcriptional changes in M-MØ was weighed by comparing the cytokine profiles and the allogeneic T lymphocyte-activating ability of M-MØ, GW3965-M-MØ and GSK2033-M-MØ. Compared to M-MØ, GW3965-M-MØ produced higher levels of IL-1 β and the immuno-stimulatory CCL19 chemokine, but lower levels of the anti-inflammatory cytokine IL-10 (Figure 3C). This trend towards a more pro-inflammatory cytokine profile was also observed upon LPS stimulation, as GW3965-M-MØ secreted higher levels of TNF, IFN β 1 and IL1 β (Figure 3D). Since the pro-inflammatory effect of GW3965 treatment was impaired or abolished in the presence of GSK2033 (Figure 3A, B), these results demonstrate that LXR activation prompts the acquisition of a more pro-inflammatory transcriptional and cytokine profile in anti-inflammatory M-MØ. Regarding immunogenic ability, GSK2033-M-MØ and GW3965-M-MØ triggered a weaker allogenic T cell proliferation than control M-MØ, an effect that was more significant and consistent in the case of GSK2033-M-MØ (Figure 3E). Therefore, modulation of LXR activity impacts the inflammatory and immunogenic activity of M-MØ, with LXR activation promoting the acquisition of a more pro-inflammatory phenotypic (transcriptional) and functional profile.

253

LXR inhibition modulates the GM-CSF-dependent macrophage differentiation towards the anti-inflammatory side. To assess the extent of the above findings, LXR activity was also pharmacologically modulated prior to the GM-CSF-driven differentiation of pro-inflammatory GM-MØ (Figure 4A). Like in M-MØ, exposure to GW3965 upregulated LXR-dependent genes (53),

258 whereas GSK2033 had the opposite effect (Figure 4B), and the transcriptome of GW3965-GM-MØ
 259 and GSK2033-GM-MØ were significantly distinct from that of control GM-MØ (Figure 4C). Indeed,
 260 GSK2033-GM-MØ displayed higher levels of the anti-inflammatory M-MØ-specific gene set and
 261 diminished expression of the pro-inflammatory GM-MØ-specific gene set (Figure 4D). The changes
 262 observed in GW3965-GM-MØ were less significant (Figure 4D), as also observed after clustering of
 263 the genes modulated by either LXR ligand, thus indicating that GM-MØ differentiation is more
 264 sensitive to the action of the inverse agonist GSK2033, that enhances the expression of genes of the
 265 anti-inflammatory M-MØ-specific gene set and limits the acquisition of the pro-inflammatory GM-
 266 MØ-specific gene set (Figure 4E-G). These conclusions were supported by gene ontology analysis
 267 using PROFILER and GSEA, that revealed a significant enrichment of the
 268 "HALLMARK_INFLAMMATORY_RESPONSE" and "HALLMARK_TNFA_SIGNALING_VIA_NFKB" gene
 269 sets in GW3965-GM-MØ, as well as a negative enrichment of the
 270 "HALLMARK_INFLAMMATORY_RESPONSE" gene set in GSK2033-GM-MØ (Supplementary Figure
 271 1A, B). Significantly, analysis of ChIP-Seq public datasets (GEO GSM2042848) identified functional
 272 LXR-binding elements (50) in the vicinity of pro-inflammatory genes upregulated by GW3965 in both
 273 GM-MØ and M-MØ (Supplementary Figure 2). Taken together, these results obtained on GM-MØ
 274 and M-MØ demonstrated that GW3965-activated LXR promotes the acquisition of a more pro-
 275 inflammatory macrophage profile, an effect limited by GSK2033 that, by contrast, enhances the anti-
 276 inflammatory specialization of monocyte-derived macrophages (that is, the acquisition of the anti-
 277 inflammatory M-MØ-specific gene set).

278

279 The strongest transcriptional effects of GSK2033 were observed at early stages of the differentiation
 280 of GM-MØ, a time at which the inverse agonist maximally impaired the expression of the GM-MØ-
 281 specific gene set and promoted the acquisition of genes of the M-MØ-specific gene set (Figure 5A,

282 B). In fact, neither ligand affected the expression of the GM-MØ-specific gene set if exposure was
 283 limited to 24-48 hours (Supplementary Figure 3A-C), and modulation of LXR activity for short time
 284 periods significant changed the expression of GM-MØ-specific genes when using lower
 285 concentrations of GM-CSF (Supplementary Figure 3D). These results, therefore, suggest that LXR
 286 influences the expression of genes whose expression reflects the pro-inflammatory or anti-
 287 inflammatory specialization of monocyte-derived human macrophages. Therefore, like in the case
 288 of M-MØ, the transcriptional consequences of LXR activity modulation are more profound at the
 289 start of the GM-MØ differentiation process.

290

291 **LXR activity shapes the inflammatory and immuno-stimulatory functions of GM-MØ.** To evaluate
 292 the influence of LXR activity on GM-MØ effector functions, we first determined the cytokine profile
 293 of GW3965-GM-MØ and GSK2033-GM-MØ. Compared to GM-MØ or GW3965-GM-MØ, GSK2033-
 294 GM-MØ produced significant lower levels of IL-1 β and the pro-inflammatory chemokine CCL17
 295 (Figure 5C), as well as lower levels of TNF, IL-10, CCL8, CCL19, IL-1 β and IFN β 1 in response to LPS
 296 (Figure 5D, E), in line with the anti-inflammatory effect of LXR inhibition. Conversely, GW3965-GM-
 297 MØ produced higher levels of TNF, IL-10, CCL19, IL-1 β and IFN β 1 after LPS stimulation, as well as an
 298 elevated TNF/IL10 ratio (Figure 5D, E). As a whole, this set of data indicates that the acquisition of
 299 the pro-inflammatory cytokine profile that characterizes GM-MØ is favored by LXR activation and
 300 impaired by LXR inhibition, what agrees with the respective transcriptional effects of GW3965 and
 301 GSK2033.

302

303 Regarding T cell activation, GW3965-GM-MØ showed higher allogeneic T lymphocyte-activating
 304 activity than untreated GM-MØ (Figure 5F,G), an increase that correlated with their higher
 305 expression of *CD80* (Figure 5H), which encodes a critical T cell co-stimulatory molecule. Therefore,

and like in the case of M-MØ, the level of activation of LXR determines the acquisition of T cell-stimulatory ability during GM-MØ differentiation. Indeed, the enhanced T cell stimulatory activity of GW3965-GM-MØ was prevented by the inverse agonist GSK2033 (compare GW3965-GM-MØ with GW/GSK GM-MØ, Figure 5F,G). Therefore, and in agreement with the transcriptional data, GW3965-dependent LXR activation favors the acquisition of GM-MØ-specific functions, an effect prevented by the GSK2033-mediated LXR inhibition.

Molecular mechanisms underlying the macrophage programming effect of LXR: Role of MAFB and

Activin A. While MAFB and MAF are master regulators for the differentiation of anti-inflammatory M-MØ (42, 64, 65), the GM-CSF-driven generation of GM-MØ is dependent on activin A (13, 22). Given the involvement of LXR factors on the acquisition of the transcriptional and functional profile of GM-MØ and M-MØ, we hypothesized that LXR might alter the relative levels of MAF/MAFB or activin A. The first hypothesis was supported by the significantly higher expression of MAFB-dependent genes in GSK2033-GM-MØ (Figure 6A). In fact, Western blot revealed that, compared to their corresponding controls, GSK2033-treated macrophages (GSK2033-GM-MØ and GSK2033-M-MØ) expressed higher levels of both MAFB and MAF (Figure 6B). Since GW3965-M-MØ exhibited reduced levels of MAF than M-MØ (Figure 6B), these results indicate that the influence of LXR on the generation of GM-MØ and M-MØ correlates with a distorted expression of MAFB and, to a lower extent, MAF. Conversely, although GW3965-M-MØ produced slightly higher levels of activin A than control M-MØ (Figure 6C), and GSK2033-GM-MØ exhibited lower activin A production (Figure 6C), the association of activin A production with the skewed generation of GM-MØ and M-MØ was not straightforward.

329 The implication of the enhanced expression of *MAFB* in the effect of GSK2033 was assessed by
 330 determining the expression of *MAFB* and *MAFB* targets in differentiating monocytes exposed to
 331 GSK2033 after siRNA-mediated silencing of *MAFB* (Figure 6D). In this experimental set-up, GSK2033
 332 increased the expression of *MAFB* and the *MAFB* target *LGIMN*, and this enhancing effect was
 333 inhibited when *MAFB* levels were knocked-down (Figure 6E). Conversely, *MAFB* levels did not
 334 significantly modified the ability of GSK2033 to blunt *ABCA1* expression (Figure 6E). Therefore, the
 335 effect of the LXR inverse agonist GSK2033 on monocyte-to-macrophage differentiation is mediated,
 336 at least partly, through the augmented expression of *MAFB*.

337

338 **Modulation of macrophage specialization by LXR: physio-pathological relevance.** Since the LXR
 339 pathway is highly upregulated in pro-inflammatory macrophages from rheumatoid arthritis (RA)
 340 synovial fluid (RASf-MØ) (38), and is also the most enriched pathway in immunosuppressive tumor-
 341 associated macrophages (39), we sought to analyze the apparently ambiguous contribution of LXR
 342 to macrophage polarization. To that end, we first evaluated whether LXR is involved in the
 343 macrophage polarizing ability of tumor-conditioned fluids, and specifically of ascitic fluids from
 344 tumors of various origins (Figure 7A). The M-CSF-induced expression of two functionally relevant
 345 markers of tumor-associated macrophages, IGF-1 (66, 67) and FOLR2 (63, 68), was prevented in the
 346 presence of the LXR agonist GW3965 (Figure 7B). Moreover, the upregulation of both genes in the
 347 presence of ascitic fluids was also impaired by the LXR agonist (Figure 7B), implying that the
 348 polarizing ability of tumor-conditioned media is dependent on LXR activity. Next, we evaluated the
 349 contribution of LXR to the polarizing ability of RA synovial fluid (Figure 7C). As shown in Figure 7D,
 350 all analyzed RA synovial fluids potentiated the expression of *NR1H3* and LXR targets (*ABCA1*), an
 351 effect completely abrogated by the LXR inverse agonist GSK2033. More importantly, RA synovial
 352 fluids also augmented, albeit variably, the expression of the GM-MØ-specific genes *CD1B* and

353 *INHBA*, whose enhancement was also prevented by GSK2033 (Figure 7D), indicating that LXR also
354 directly contributes to the pro-inflammatory polarization effect of RA synovial fluids. Altogether,
355 these results evidenced that LXR mediates the effect of two biological fluids (synovial and ascitic)
356 that promote the acquisition of opposite functional states in human macrophages, thus emphasizing
357 the critical role of LXR in human macrophage polarization.

358 DISCUSSION

359 LXR nuclear receptors have a prominent role in lipid and cholesterol homeostasis, but also regulate
 360 the expression of inflammatory mediators [reviewed in (69)] and are absolutely required for the
 361 generation of specialized splenic macrophages (70). To clarify the role of LXR in human innate cells,
 362 we have analyzed the role of LXR activation in macrophage differentiation and polarization,
 363 primarily through the use of pharmacological approaches. Our results indicate that LXR has a
 364 remarkable influence on the acquisition of the transcriptome that defines pro-inflammatory (GM-
 365 CSF-dependent) and anti-inflammatory (M-CSF-dependent) monocyte-derived macrophages, and
 366 that LXR over-activation promotes the acquisition of a pro-inflammatory transcriptional and
 367 functional profile, whereas abrogation of LXR activity favors the acquisition of anti-inflammatory
 368 capacities. Indeed, LXR modulates the cytokine profile of macrophages and modifies their T cell
 369 activation activity, a result that, to our knowledge, has not been previously reported.
 370 Mechanistically, modulation of LXR activity led to diminished levels of the factors that determine
 371 the pro- and anti-inflammatory polarization of human macrophages, namely activin A and
 372 MAF/MAFB, respectively. As a whole, our findings reveal a net pro-inflammatory effect of LXR during
 373 macrophage differentiation in the presence of either GM-CSF or M-CSF. The pathological relevance
 374 of our findings is illustrated by the LXR-dependency of the macrophage-polarizing action of both
 375 synovial fluid from Rheumatoid Arthritis patients (pro-inflammatory) and tumor-derived ascitic
 376 fluids (immunosuppressive).

377
 378 Numerous studies have evidenced that LXR suppress inflammation in the presence of pathogenic
 379 stimuli through direct and indirect mechanisms, including transactivation of anti-inflammatory
 380 genes and trans-repression of NF κ B- and AP1-dependent genes, and *in vivo* results have confirmed
 381 the net anti-inflammatory effects of LXR in the presence of macrophage-stimulating agents

382 [reviewed in (69)]. The pro-inflammatory action of LXR that we now report is compatible with those
 383 previous studies because we have assayed the effect of LXR activation on unstimulated monocytes
 384 that were subsequently exposed to either GM-CSF or M-CSF. Indeed, it is worth noting that the pro-
 385 inflammatory actions of LXR are not unprecedented and have been previously noted (30). Leaving
 386 aside the positive correlation between LXR activation and Rheumatoid Arthritis (34, 35, 38), long
 387 term pre-treatment of primary human macrophages to LXR ligands results in potentiated LPS
 388 responses (33), and LXR activation leads to increase dendritic cell maturation at the phenotypic,
 389 cytokine and functional levels (36). Further, LXR agonists have been recently reported to trigger
 390 trained immunity in human monocytes, whereby LXR activation primes macrophages for enhanced
 391 responses towards secondary stimuli (41) and impairs MDSC-mediated immunosuppression in
 392 cancer (37). While these findings are in line with the enhanced LPS-induced TNF production by
 393 GW3965-M-MØ (Figure 2), they can also be interpreted as LXR agonist having a macrophage re-
 394 polarizing action, because differentiation of M-MØ in the presence of GW3965 results in
 395 macrophages with an GM-MØ-like transcriptional and functional profile (enhanced expression of
 396 the "Pro-inflammatory gene set", diminished levels of MAF/MAFB, impaired basal IL-10 production).
 397 However, and regardless of the interpretation, all these results illustrate the ability of LXR for
 398 macrophage pro-inflammatory re-programming, and suggest that LXR activity modulates
 399 inflammatory responses in a cell- or activation-dependent manner, a suggestion that is reminiscent
 400 of the macrophage-specific effects of the LXR ligand desmosterol (53).

401

402 In the context of the net pro-inflammatory effect of LXR during human monocyte-to-macrophage
 403 differentiation, the macrophage re-polarizing action of LXR also shows a high degree of cell-
 404 specificity, as the sensitivity to LXR ligands greatly differ among monocytes, differentiating and fully
 405 differentiated M-MØ or GM-MØ. Globally, the effect of LXR activity modulators on the expression

406 of polarization-specific genes diminishes along monocyte-to-macrophage differentiation and is
407 inversely correlated with the acquisition of functional polarization. In fact, the sensitivity to LXR
408 modulation at later stages of differentiation is only observed when low doses of priming cytokines
409 are used. Therefore, the impact of LXR activity on macrophage polarization and functions is more
410 relevant at the monocyte stage and diminishes along macrophage differentiation. The more
411 prominent action of LXR at the beginning of the differentiation model is compatible with a
412 sequential and ordered involvement of different factors, including lineage-determining factors and
413 signal-dependent factors. In our opinion, the potent ligand-activation effect on LXR activity before
414 the growth factor trigger, unbalances the polarization towards a sustained pro-inflammatory profile.
415 It is possible that activated LXR at the monocyte level promotes its direct binding to regulatory
416 regions of pro-inflammatory and/or epigenetic changes that favor the recruitment of other pro-
417 inflammatory factors. Several mechanisms by activated LXRs might be altered that contribute to
418 these transcriptional changes.

419

420 One possible mechanism underlying the effect of LXR on macrophage pro-inflammatory polarization
421 can be interpreted by our pharmacological analysis. Inhibition of LXR was found to rise the
422 acquisition of an anti-inflammatory transcriptome and led to a significant increase in the expression
423 of MAF and MAFB, both of which determine the anti-inflammatory polarization of macrophages and
424 the production of IL-10 (42, 65, 71, 72). Whether LXR directly controls the expression of MAF/MAFB
425 in human macrophages would need further investigation. One possible clue came to our attention
426 through the analysis of available datasets from other cell lineages, which confirm the presence of
427 functional LXR-binding sites within GM-MØ-specific genes ("Pro-inflammatory gene set) that are
428 specifically upregulated in the presence of GW3965 (<http://cistrome.org/db/#/>; CistromeDB:
429 69799) (50). The link between LXR and MAFB has already been described in the case of the murine

430 osteoclast progenitors, where LXR agonists increased *Mafb* expression through Srebp-1c
431 transactivation of the *Mafb* promoter, and no evidence was found for direct LXR binding to the *Mafb*
432 promoter (73). Therefore, and regardless of the opposite effect of LXR on MAFB in human
433 macrophages and mouse osteoclast progenitors, which might reflect species- or cell-specific
434 differences (74), it appears that LXR is capable of pre-programming myeloid cells by altering the
435 expression of critical regulators of differentiation and polarization.

436

437 In summary, we have described a critical role of the LXR nuclear receptors during monocyte-to-
438 macrophage differentiation and in the acquisition of the transcriptional and functional profile of
439 human monocyte-derived macrophages. LXR activation skews macrophages to acquire a more pro-
440 inflammatory and immuno-stimulatory phenotype. Thus, and considering the enhanced activity of
441 LXR in animal models and human RA (34, 35, 38), modulation of LXR appears as a potential
442 therapeutic target, and LXR-dependent macrophage genes might be considered as useful
443 prognostic/therapeutic markers for human inflammatory diseases. In this regard, it is worth
444 mentioning that, when compared to TAM, RA synovial macrophages display the highest levels of
445 MMP12, INHBA and CCL17 (24), whose expression we have now shown to be dependent on LXR.
446 Therefore, it is tempting to speculate that enhancing the level of LXR activation might be beneficial
447 in other pathological settings where the presence of macrophages with deregulated anti-
448 inflammatory and immunosuppressive functions contribute to pathology (e.g., cancer).

449

450 **FIGURE LEGENDS**

451 **Figure 1. Differential expression of LXR and LXR-regulated genes in GM-MØ and M-MØ. A.**
 452 Schematic representation of the *in vitro* generation of pro-inflammatory (GM-MØ) and anti-
 453 inflammatory (M-MØ) macrophages from peripheral blood human monocytes. **B.** GSEA of the gene
 454 sets upregulated or downregulated in rheumatoid arthritis synovial macrophages (52) (GSE10500,
 455 left panels) or preferentially expressed by large and small TAM (39)(right panels) on the ranked
 456 comparison of the M-MØ and GM-MØ transcriptomes. Normalized Enrichment Score (NES) and
 457 False Discovery rate q value (FDRq) are indicated in each case. **C.** Protein levels of LXR α and LXR β in
 458 four independent samples (1-4) of GM-MØ and M-MØ, as determined by Western blot, and using
 459 Vinculin and GAPDH as protein loading controls. **D.** LXR α and LXR β expression in GM-MØ and M-
 460 MØ. Mean and SEM of 16 independent samples are shown. **E.** *NR1H3* and *NR1H2* mRNA expression
 461 in GM-MØ and M-MØ. Mean and SEM of 20 independent samples are shown. **F.** Heatmap of the
 462 expression of desmosterol-upregulated genes in GM-MØ and M-MØ. **G.** Relative mRNA expression
 463 of the indicated LXR target genes in GM-MØ and M-MØ. Mean $\pm$ SEM of six independent samples
 464 are shown. Statistical differences between groups were evaluated using paired t-test (*, $p<0.05$; **, $p<0.01$;
 465 ***, $p<0.001$; ****, $p<0.0001$).

466
 467 **Figure 2. Identification of LXR-regulated genes in anti-inflammatory M-MØ. A.** *In vitro* generation
 468 of control M-MØ, GW3965-M-MØ, GSK2033-M-MØ and GSK2033+GW3965-M-MØ before RNA
 469 isolation and RNA-sequencing. Control M-MØ were exposed to DMSO in parallel. **B.** GSEA of
 470 desmosterol-upregulated genes on the ranked comparison GW3965-M-MØ vs. M-MØ and
 471 GSK2033-M-MØ vs. M-MØ. **C.** Number of differentially expressed genes ($|\log_2FC|>1$, $adjp<0.05$,)
 472 relative to control M-MØ. **D.** Summary of GSEA of the "Pro-inflammatory gene set" and "Anti-
 473 inflammatory gene set" on the ranked comparisons of the indicated transcriptomes. The color of

the circles illustrates the type of enrichment of each comparison (positive, red; negative, blue). The area of each circle is proportional to the Normalized Enrichment Scores of each comparison, which is also indicated. Two representative GSEA profiles are shown at the top. **E.** Unsupervised clustering of differentially expressed genes ($|\log_2FC| > 1$) between control M-M ϕ and the transcriptomes of M-M ϕ generated in the presence of GW3965, GSK2033 or both. For each gene, mRNA expression level in the three donors are represented after normalizing gene expression and k-means clustering using Genesis (<http://genome.tugraz.at/genesisclient/>). **F.** Identification of "Pro-inflammatory gene set", "Anti-inflammatory gene set" and desmosterol-upregulated genes in each cluster. **G.** Comparison of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" with the genes significantly regulated after M-M ϕ differentiation in the presence of GW3965 (upper panel) or GSK2033 (lower panel).

Figure 3.- LXR activation in the generation of anti-inflammatory M-M ϕ : time-dependency and functional consequences. **A-B.** Relative mRNA expression of the indicated genes from the "Anti-inflammatory" (**A**) and "Pro-inflammatory" (**B**) gene sets in M-M ϕ generated under the indicated treatments. *ABCA1* expression was evaluated as a readout for LXR activation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). **C.** Production of the indicated cytokines in M-M ϕ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of six independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). **D.** LPS-stimulated (10 ng/ml, 18h) production of TNF, IFN β 1 and IL-1 β by M-M ϕ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of six independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). **E.** T cell-stimulatory capacity of M-M ϕ generated in the presence of GW3965 (1 μ M), or GSK2033 (1 μ M), as determined in Mixed Leukocyte Reactions (M-M ϕ vs. allogeneic CD3 $^+$ T lymphocyte), and using M-M ϕ generated in the presence of DMSO as a

control (CNT M-MØ). Mean $\pm$ SEM of six independent experiments are shown. The relative T cell-stimulatory capacity (% Proliferation rate) is shown in the right panel (*, $p < 0.05$; ****, $p < 0.0001$).

499

Figure 4. Identification of a novel set of LXR-dependent genes in proinflammatory GM-MØ. **A.** *In vitro* generation of control GM-MØ, GW3965-GM-MØ, GSK2033-GM-MØ and GSK2033+GW3965-GM-MØ before RNA isolation and RNA-sequencing. Control GM-MØ were exposed to DMSO in parallel. **B.** GSEA of desmosterol-upregulated genes in the ranked comparison GW3965-GM-MØ vs. GM-MØ and GSK2033-GM-MØ vs. GM-MØ. **C.** Number of differentially expressed genes ($|\log_2FC| > 1$, $\text{adj}p < 0.05$,) relative to control GM-MØ. **D.** Summary of GSEA of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" on the ranked comparisons of the indicated transcriptomes. The color of the circles illustrates the type of enrichment of each comparison (positive, red; negative, blue). The area of each circle is proportional to the Normalized Enrichment Scores of each comparison, which is also indicated. Two representative GSEA profiles are shown at the top. **E.** Unsupervised clustering of differentially expressed genes ($|\log_2FC| > 1$) between control GM-MØ and the transcriptomes of GM-MØ generated in the presence of GW3965, GSK2033 or both. For each gene, mRNA expression level in the three donors are represented after normalizing gene expression and k-means clustering using Genesis (<http://genome.tugraz.at/genesisclient/>). **F.** Indication of "Pro-inflammatory gene set", "Anti-inflammatory gene set" and desmosterol-upregulated genes in each cluster. **G.** Comparison of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" with the genes significantly regulated after GM-MØ differentiation in the presence of GW3965 (upper panel) or GSK2033 (lower panel).

518

Figure 5.- LXR activation in the generation of pro-inflammatory GM-MØ: time-dependency and functional consequences. **A-B.** Relative mRNA expression of the indicated genes from the "Pro-

inflammatory" (A) and "Anti-inflammatory" (B) gene sets in GM-MØ generated under the indicated treatments. *ABCA1* expression was evaluated as a readout for LXR activation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). C. Production of CCL17, IFN β 1 and IL-1 β in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of seven independent donors are shown (*, $p < 0.05$; ** $p < 0.01$). D. LPS-induced (10 ng/ml, 18h) production of TNF and IL-10 in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of seven independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). The TNF/IL-10 is shown in the right panel. E. LPS-induced (10 ng/ml, 18h) production of the indicated cytokines and chemokines in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of seven independent donors are shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). F-G. T cell-stimulatory capacity of GM-MØ generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as determined in Mixed Leukocyte Reactions (GM-MØ vs. allogeneic CD3+ T lymphocyte), and using GM-MØ generated in the presence of DMSO as a control (CNT GM-MØ). After a 6-day coculture, 3 H-thymidine incorporation was determined as a readout for T cell proliferation. Mean $\pm$ SEM of six independent experiments are shown. The relative T cell-stimulatory capacity (% Proliferation rate) is shown in G (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). H. *CD80* mRNA expression level in GM-MØ generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as measured by the Expectation Maximization RNAseq (RSEM) algorithm, and using GM-MØ generated in the presence of DMSO as a control (CNT GM-MØ). Adjusted p values of indicated comparisons are shown.

540

Figure 6. Molecular mechanisms underlying the macrophage polarizing effect of LXR. A. GSEA of genes downregulated by siRNA MAFB in the ranked comparison of the transcriptomes of GSK2033-GM-MØ and GM-MØ. B. Relative protein levels of MAFB or MAF in M-MØ (left) and GM-MØ (right) generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as determined by Western

545 blot and densitometry, and using GM-MØ generated in the presence of DMSO as a control (insert).
 546 For protein loading control purpose, Vinculin protein levels for determined in parallel. Mean $\pm$ SEM
 547 of the relative MAF and MAFB protein levels in M-MØ and GM-MØ from four independent donors
 548 are shown, and a representative Western blot experiment is shown in each case. **C.** Production of
 549 activin A in M-MØ and GM-MØ generated in the presence of the LXR agonist GW3965 (1 μ M), LXR
 550 inverse agonist GSK2033 (1 μ M) or both, as determined by ELISA, and using GM-MØ generated in
 551 the presence of DMSO as a control. Mean $\pm$ SEM of seven (GM-MØ) or eight (M-MØ) independent
 552 donors are shown (*, $p < 0.05$; **, $p < 0.01$). **D.** Schematic representation of the assay to determine
 553 the effect of the LXR inverse agonist GSK2033 on the expression of *MAFB* and the *MAFB* target gene
 554 *LGMN*. **E.** Relative mRNA expression of the indicated genes in differentiating GM-MØ generated in
 555 the presence of GSK2033 and after siRNA-mediated knock-down of *MAFB*. Mean $\pm$ SEM of seven
 556 independent experiments are shown (*, $p < 0.05$; ** $p < 0.01$).

557

558 **Figure 7. Physio-pathological relevance of the LXR-dependent expression of GM-MØ-specific**
 559 **genes.** **A.** Schematic representation for the generation of monocyte-derived macrophages in the
 560 presence of ascitic fluid from tumor patients (AF-MØ). **B.** Relative mRNA expression of the indicated
 561 genes in M-MØ and AF-MØ generated in the absence of presence of GW3965. The results obtained
 562 with each individual ascitic fluid are shown. **C.** Schematic representation for the generation of
 563 monocyte-derived macrophages in the presence of Rheumatoid Arthritis synovial fluid (RASf-MØ).
 564 **D.** Expression of the indicated genes in RASf-MØ generated in the presence or absence of GSK2033.
 565 Fold mRNA Expression indicates the expression of each gene relative to its expression in untreated
 566 monocytes kept in culture for 72 hours. The results obtained with each individual synovial fluid are
 567 shown.

568 **SUPPLEMENTARY FIGURE LEGENDS**

569 **Supplementary Figure 1. A.** Unsupervised clustering (<http://genome.tugraz.at/genesisclient/>) of
570 differentially expressed genes ($|\log_2FC| > 1$ and $\text{adj}p < 0.05$) between control GM-MØ and the
571 transcriptomes of GM-MØ generated in the presence of GW3965, GSK2033 or both. GSEA of the
572 indicated gene clusters on the ranked list of the M-MØ and GM-MØ transcriptomes, and Gene
573 ontology analysis of the gene clusters, as determined by statistical overrepresentation on the
574 PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System
575 (<http://www.pantherdb.org/about.jsp>). Fold Enrichment and p-value of the gene ontology terms
576 with a p value < 0.05 are shown. **B.** Normalized Enrichment Score (NES) of the transcriptomes of
577 GM-MØ generated in the indicated conditions (insert) after GSEA using the Hallmarks gene sets
578 from the Molecular Signatures Database v7.0 (<http://software.broadinstitute.org/gsea/index.jsp>)
579 (FDR, False Discovery Rate).

580

581 **Supplementary Figure 2.** Distribution of Flag-LXR α tag densities in the vicinity of the indicated genes
582 (highlighted in yellow) in colorectal adenocarcinoma HT29 cells treated with GW3965 (50). Data
583 were obtained from the Cistrome Data Browser (GEO GSM2042848). The LXR α binding regions of
584 interest (highlighted in orange) were scanned for LXRE motif using the HOMER motif matrix with
585 DMINDA (<https://bmbl.bmi.osumc.edu/DMINDA2/index.php>) (51), and the corresponding
586 statistical significance is shown.

587

588 **Supplementary Figure 3. A.** Expression of LXR α and LXR β in terminally differentiated GM-MØ
589 exposed to the LXR agonist GW3965 (1 μM) or the LXR inverse agonist GSK2033 (1 μM) for 24h or
590 48h, as determined by Western blot, and using GM-MØ treated with DMSO for 48h (-) as a control.
591 For protein loading control purpose, vinculin and GAPDH protein levels for determined in parallel.

592 **B.** Relative mRNA (NR1H2, NR1H3) and protein (LXR α , LXR β) levels in terminally differentiated GM-
593 M ϕ exposed to GW3965 (1 μ M) or GSK2033 (1 μ M) for 24h or 48h, as determined by qRT-PCR and
594 Western blot and densitometry, and using GM-M ϕ treated with DMSO for 48h (-) as a control. Mean
595 $\pm$ SEM of the relative levels of *NR1H2*, *NR1H3*, LXR α and LXR β in GM-M ϕ from 16 (mRNA) or 20
596 (protein) independent donors are shown (* $p < 0.05$, ** $p < 0.01$). **C.** Relative mRNA expression of the
597 indicated GM-M ϕ specific genes in terminally differentiated GM-M ϕ exposed to GW3965 (1 μ M) or
598 GSK2033 (1 μ M) for 24h or 48h, as determined by qRT-PCR. Shown is the expression level of each
599 gene relative to its expression in vehicle (DMSO)-treated GM-M ϕ . *SREBF1*, *MERTK* and *ABCA1* gene
600 expression levels were determined as readouts for LXR activation. Mean $\pm$ SEM of four independent
601 GM-M ϕ samples are shown (* $p < 0.05$, ** $p < 0.01$). **D.** Schematic representation of the experiment
602 shown in panel B. **E.** Relative expression of the indicated genes from the "Pro-Inflammatory gene
603 set" and "Anti-Inflammatory gene set" in GM-M ϕ generated under low concentrations of GM-CSF
604 (one dose of 1000U/ml, or three doses of 50 U/ml), and exposed to GW3965 (1 μ M), GSK2033 (1
605 μ M) or both, at day 5 of the differentiation process, and using GM-M ϕ generated in the presence
606 of DMSO as a control (insert). Mean $\pm$ SEM of three independent experiments are shown (* $p < 0.05$,
607 ** $p < 0.01$).
608

609 **DECLARATIONS**

610 **Ethics approval and consent to participate.** Biological samples were obtained from the Oncology
611 Department (Hospital General Universitario Gregorio Marañón) after patients had provided
612 informed consent. Approval was obtained from the ethics committee of Hospital General
613 Universitario Gregorio Marañón. The procedures used in this study adhere to the tenets of the
614 Declaration of Helsinki.

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625 **Authors' information** (optional)

626 **CONFLICTS OF INTEREST.** The authors declare no competing financial interests.

627 **AVAILABILITY OF DATA AND MATERIAL.** All transcriptional information described in the manuscript
628 has been deposited in Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/>)
629 under accession GSE156783 and GSE156696.

630 **AUTHORSHIP CONTRIBUTIONS.** AGA, MT-T, JVR, BA and EC-S performed research and analyzed
631 data; AGA, APK, MAV, AC and ALC designed the research and analyzed data; AGA, AC and ALC wrote
632 the paper.

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Figures

Figure 1

Differential expression of LXR and LXR-regulated genes in GM-MØ and M-MØ. A. Schematic representation of the in vitro generation of pro-inflammatory (GM-MØ) and anti-inflammatory (M-MØ) macrophages from peripheral blood human monocytes. B. GSEA of the gene sets upregulated or

downregulated in rheumatoid arthritis synovial macrophages (52) (GSE10500, left panels) or preferentially expressed by large and small TAM (39)(right panels) on the ranked comparison of the M-MØ and GM-MØ transcriptomes. Normalized Enrichment Score (NES) and False Discovery rate q value (FDRq) are indicated in each case. C. Protein levels of LXRα and LXRβ in four independent samples (1-4) of GM-MØ and M-MØ, as determined by Western blot, and using Vinculin and GAPDH as protein loading controls. D. LXRα and LXRβ expression in GM-MØ and M-MØ. Mean and SEM of 16 independent samples are shown. E. NR1H3 and NR1H2 mRNA expression in GM-MØ and M-MØ. Mean and SEM of 20 independent samples are shown. F. Heatmap of the expression of desmosterol-upregulated genes in GM-MØ and M-MØ. G. Relative mRNA expression of the indicated LXR target genes in GM-MØ and M-MØ. Mean ± SEM of six independent samples are shown. Statistical differences between groups were evaluated using paired t-test (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

Figure 2

Identification of LXR-regulated genes in anti-inflammatory M-MØ. A. In vitro generation of control M-MØ, GW3965-M-MØ, GSK2033-M-MØ and GSK2033+GW3965-M-MØ before RNA isolation and RNA-sequencing. Control M-MØ were exposed to DMSO in parallel. B. GSEA of desmosterol-upregulated genes on the ranked comparison GW3965-M-MØ vs. M-MØ and GSK2033-M-MØ vs. M-MØ. C. Number of differentially expressed genes (|log2FC|>1, adjp<0.05,) relative to control M-MØ. D. Summary of GSEA of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" on the ranked comparisons of the indicated transcriptomes. The color of the circles illustrates the type of enrichment of each comparison

(positive, red; negative, blue). The area of each circle is proportional to the Normalized Enrichment Scores of each comparison, which is also indicated. Two representative GSEA profiles are shown at the top. E. Unsupervised clustering of differentially expressed genes ($|\log_2FC| > 1$) between control M-MØ and the transcriptomes of M-MØ generated in the presence of GW3965, GSK2033 or both. For each gene, mRNA expression level in the three donors are represented after normalizing gene expression and k-means clustering using Genesis (<http://genome.tugraz.at/genesisclient/>). F. Identification of "Pro-inflammatory gene set", "Anti-inflammatory gene set" and desmosterol-upregulated genes in each cluster. G. Comparison of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" with the genes significantly regulated after M-MØ differentiation in the presence of GW3965 (upper panel) or GSK2033 (lower 483 panel).

Figure 3

Figure 3

LXR activation in the generation of anti-inflammatory M-MØ: time-dependency and functional consequences. A-B. Relative mRNA expression of the indicated genes from the "Anti-inflammatory" (A) and "Pro-inflammatory" (B) gene sets in M-MØ generated under the indicated treatments. ABCA1 expression was evaluated as a readout for LXR activation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). C. Production of the indicated cytokines in M-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of six independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). D. LPS-stimulated (10

ng/ml, 18h) production of TNF, IFN γ and IL-1 β by M-M ϕ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of six independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). E. T cell-stimulatory capacity of M-M ϕ generated in the presence of GW3965 (1 μ M), or GSK2033 (1 μ M), as determined in Mixed Leukocyte Reactions (M-M ϕ vs. allogeneic CD3+ T lymphocyte), and using M-M ϕ generated in the presence of DMSO as a control (CNT M-M ϕ). Mean $\pm$ SEM of six independent experiments are shown. The relative T cell- stimulatory capacity (% Proliferation rate) is shown in the right panel (*, $p < 0.05$; ****, $p < 0.0001$).

Figure 4

Figure 4

Identification of a novel set of LXR-dependent genes in proinflammatory GM-M ϕ . A. In vitro generation of control GM-M ϕ , GW3965-GM-M ϕ , GSK2033-GM-M ϕ and GSK2033+GW3965- GM-M ϕ before RNA isolation and RNA-sequencing. Control GM-M ϕ were exposed to DMSO in parallel. B. GSEA of desmosterol-upregulated genes in the ranked comparison GW3965-GM-M ϕ vs. GM-M ϕ and GSK2033-GM-M ϕ vs. GM-M ϕ . C. Number of differentially expressed genes ($|\log_2FC| > 1$, $\text{adj}p < 0.05$) relative to control GM-M ϕ . D. Summary of GSEA of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" on the ranked comparisons of the indicated transcriptomes. The color of the circles illustrates the type of enrichment of each comparison (positive, red; negative, blue). The area of each circle is

proportional to the Normalized Enrichment Scores of each comparison, which is also indicated. Two representative GSEA profiles are shown at the top. E. Unsupervised clustering of differentially expressed genes ($|\log_2FC| > 1$) between control GM-MØ and the transcriptomes of GM-MØ generated in the presence of GW3965, GSK2033 or both. For each gene, mRNA expression level in the three donors are represented after normalizing gene expression and k-means clustering using Genesis (<http://genome.tugraz.at/genesisclient/>). F. Indication of "Pro-inflammatory gene set", "Anti-inflammatory gene set" and desmosterol- upregulated genes in each cluster. G. Comparison of the "Pro-inflammatory gene set" and "Anti-inflammatory gene set" with the genes significantly regulated after GM-MØ differentiation in the presence of GW3965 (upper panel) or GSK2033 (lower panel).

Figure 5

Figure 5

LXR activation in the generation of pro-inflammatory GM-MØ: time-dependency and functional consequences. A-B. Relative mRNA expression of the indicated genes from the "Pro-inflammatory" (A) and "Anti-inflammatory" (B) gene sets in GM-MØ generated under the indicated treatments. ABCA1 expression was evaluated as a readout for LXR activation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). C. Production of CCL17, IFN γ 1 and IL-1 β in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of seven independent donors are shown (*, $p < 0.05$; ** $p < 0.01$). D. LPS-induced (10 ng/ml, 18h) production of TNF and IL-10 in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of seven independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). The TNF/IL-10 is shown in the right panel. E. LPS-induced (10 ng/ml, 18h) production of the indicated cytokines and chemokines in GM-MØ generated in the indicated conditions, as determined by ELISA. Mean $\pm$ SEM of

seven independent donors are shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). F-G. T cell-stimulatory capacity of GM-MØ generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as determined in Mixed Leukocyte Reactions (GM-MØ vs. allogeneic CD3+ T lymphocyte), and using GM-MØ generated in the presence of DMSO as a control (CNT GM-MØ). After a 6-day coculture, 3H-thymidine incorporation was determined as a readout for T cell proliferation. Mean $\pm$ SEM of six independent experiments are shown. The relative T cell-stimulatory capacity (% Proliferation rate) is shown in G (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). H. CD80 mRNA expression level in GM-MØ generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as measured by the Expectation Maximization RNAseq (RSEM) algorithm, and using GM-MØ generated in the presence of DMSO as a control (CNT GM-MØ). Adjusted p values of indicated comparisons are shown.

Figure 6

Figure 6

Molecular mechanisms underlying the macrophage polarizing effect of LXR. A. GSEA of genes downregulated by siRNA MAFB in the ranked comparison of the transcriptomes of GSK2033- GM-MØ and GM-MØ. B. Relative protein levels of MAFB or MAF in M-MØ (left) and GM-MØ (right) generated in the presence of GW3965 (1 μ M), GSK2033 (1 μ M) or both, as determined by Western blot and densitometry, and using GM-MØ generated in the presence of DMSO as a control (insert). For protein loading control

purpose, Vinculin protein levels for determined in parallel. Mean $\pm$ SEM of the relative MAF and MAFB protein levels in M-MØ and GM-MØ from four independent donors are shown, and a representative Western blot experiment is shown in each case. C. Production of activin A in M-MØ and GM-MØ generated in the presence of the LXR agonist GW3965 (1 μ M), LXR inverse agonist GSK2033 (1 μ M) or both, as determined by ELISA, and using GM-MØ generated in the presence of DMSO as a control. Mean $\pm$ SEM of seven (GM-MØ) or eight (M-MØ) independent donors are shown (*, $p < 0.05$; **, $p < 0.01$). D. Schematic representation of the assay to determine the effect of the LXR inverse agonist GSK2033 on the expression of MAFB and the MAFB target gene LGMN. E. Relative mRNA expression of the indicated genes in differentiating GM-MØ generated in the presence of GSK2033 and after siRNA-mediated knock-down of MAFB. Mean $\pm$ SEM of seven independent experiments are shown (*, $p < 0.05$; ** $p < 0.01$).

Figure 7

Figure 7

Physio-pathological relevance of the LXR-dependent expression of GM-MØ-specific genes. A. Schematic representation for the generation of monocyte-derived macrophages in the presence of ascitic fluid from tumor patients (AF-MØ). B. Relative mRNA expression of the indicated genes in M-MØ and AF-MØ generated in the absence of presence of GW3965. The results obtained with each individual ascitic fluid are shown. C. Schematic representation for the generation of monocyte-derived macrophages in the presence of Rheumatoid Arthritis synovial fluid (RASF-MØ). D. Expression of the indicated genes in RASF-

MØ generated in the presence or absence of GSK2033. Fold mRNA Expression indicates the expression of each gene relative to its expression in untreated monocytes kept in culture for 72 hours. The results obtained with each individual synovial fluid are shown.

Supplementary Files

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