THE LIVER X RECEPTOR PATHWAY IS HIGHLY UP-REGULATED IN RHEUMATOID ARTHRITIS SYNOVIAL MACROPHAGES AND POTENTIATES TLR DRIVEN CYTOKINE RELEASE.

Darren L Asquith[1†], Lucy E Ballantine[2†], Jagtar Singh Nijjar[1], Manhal Khuder Makdasy[1], Sabina Patel[3], Pamela B Wright[1], James H Reilly[1], Shauna Kerr[1], Mariola Kurowska-Stolarska[1], J Alastair Gracie[1], Iain B McInnes[1*]

Running title: LXR activation drives inflammation in RA

† These authors contributed equally to this work.

[1] College of Medical, Veterinary and Life Sciences, Institute of Infection, Immunity and Inflammation, Sir Graeme Davies Building, University of Glasgow, 120 University Place, Glasgow, UK G12 8TA.

[2] Work carried out at [1] but now works at Institute of Child Health, Alder Hey Children's Hospital, University of Liverpool, Liverpool, L12 8TA.

[3] Core Discovery Technology Group, GlaxoSmithKline, Gunnels Wood Road, Stevenage, SG1 2NY.

* Corresponding author:

Prof Iain B McInnes[1];

Tel: +44 (0)141 330 8412, Fax: +44 141 211 4878.

Email: Iain.McInnes@glasgow.ac.uk

Word Count: 2848

Keywords: Liver X Receptors (LXR), Toll-like receptors (TLR), Rheumatoid Arthritis (RA), Inflammation, Macrophages.

Abstract

Objectives Macrophages are central to the inflammatory processes driving rheumatoid arthritis (RA) synovitis. The molecular pathways that are induced in synovial macrophages and thereby promote RA disease pathology remain poorly understood.

Methods We used microarray to characterise the transcriptome of synovial fluid (SF) macrophages compared to matched peripheral blood monocytes from RA patients (n=8).

Results Using *in silico* pathway mapping, we found that pathways downstream of the cholesterol activated Liver X Receptors (LXRs), and those associated with toll-like receptor (TLR) signalling, were up-regulated in SF macrophages. Macrophage differentiation and TNFα promoted the expression of LXRα. Furthermore, in functional studies we demonstrated that activation of LXRs significantly augmented TLR driven cytokine and chemokine secretion.

Conclusion The LXR pathway is the most up-regulated pathway in RA synovial macrophages and activation of LXRs by ligands present within SF augments TLR driven cytokine secretion. Since the natural agonists of LXRs arise from cholesterol metabolism, this provides a novel mechanism that can promote RA synovitis.

Abstract word count: 160

Introduction

Rheumatoid arthritis (RA) is a debilitating autoimmune inflammatory condition of unknown etiology. RA affects approximately 1% of the population and is associated with increased morbidity, reduced life expectancy, a heightened degree of social burden and economic cost. The primary site of inflammation is the synovium, characterised by hyperplasia and vascularisation, inflammatory cell infiltration, hypoxia and destruction of adjacent cartilage and bone. This ultimately leads to irreversible destruction of the joint and impaired mobility. Although the use of biologic therapeutics has considerably improved clinical outcomes and prognosis, they remain effective in only a proportion of patients and rates of long-term remission achieved remain low. There is therefore an ever-greater need to understand the cellular and molecular processes by which pathology is mediated to develop future therapeutics.

Macrophages constitute the major leukocyte population within the synovial inflammatory infiltrate (≥40%).[1] Synovial macrophage numbers directly correlate with measures of disease activity and severity including; CRP, ESR, swollen joint count, synovial lining layer vascularity and thickness, the presence of citrillinated peptides and radiologic score.[2, 3] Furthermore, the number of synovial lining layer $CD68^{+}$ macrophages provide a useful biomarker of response upon successful clinical intervention in the context of clinical trials.[4-7] As macrophages are a major source of IL-6 and TNFα, the recent success of anti-cytokine therapeutics supports the notion that macrophage targeted therapies

may be beneficial for the treatment of RA (reviewed in,[8]) and highlight a central role for macrophages in the progression of human disease pathology.

There are multiple mechanisms by which synovial macrophages may contribute towards the inflammatory burden and joint destruction in RA.[9] Pro-inflammatory cytokines and immune complexes present in plasma induce early activation of $CD14^{+}$ monocytes, up-regulation of integrins and chemokine receptors (e.g. CCR1 and CCR2) and trans-endothelial migration of monocytes into the synovium. Monocytes accumulate in the synovium where high concentrations of M-CSF and GM-CSF enhance monocyte to macrophage differentiation and maturation.

The advent of transcriptional array techniques allows definitive characterisation of the pathways that are active within a given cell population. We therefore adopted a microarray-based approach to elucidate the molecular pathways that are activated in RA Synovial Fluid (SF) macrophages to identify novel pathways that could drive disease pathology. Using this approach we now report the unexpected prominence of the cholesterol activated Liver X Receptor (LXR) pathway that integrates with TLR pathways to enhance synovial cytokine secretion.

Methods

Reagents

GW3965 (Merck UK) was dissolved in DMSO.

Tissues and patient samples

Blood, SF and synovial membrane samples were obtained from patients with RA after obtaining their written informed consent. Patient numbers and characteristics are shown in Supplementary Table 1. This study complied with the World Medical Association Declaration of Helsinki and was approved by the Glasgow East Ethics Committee. All samples were processed immediately upon arrival by centrifugation on a histopaque density gradient to separate out the peripheral blood mononuclear cells followed by MACS CD14 positive selection.[10] The samples were lysed in Trizol and stored at -70^{o}C prior to RNA extraction. Cell purities were typically >95% (data not shown).

RNA purification and analysis

Single cell suspensions were lysed in buffer RLT or Trizol and the RNA extracted following the RNeasy micro kit (Qiagen). Gene expression was analysed using SYBR green QRT-PCR master mix on an ABI7900HT and SDS 2.3 software (Applied Biosystems). Gene expression was normalised to TATA binding protein (TBP) or GAPDH. Primer Sequences are shown in Supplementary Table 2.

Microarray analysis

The integrity of RNA was ensured by analysis of ribosomal 18S and 28S RNA intensity using an Agilent 2100 Bioanalyser (Agilent Technologies). Following

genomic DNA digestion cDNA was synthesised, fragmented and biotin labelled (FL-Ovation™ cDNA Biotin Module V2 kit -Nugen Technologies). An Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array was performed using a GeneChip® Scanner 3000. Background correction and normalisation for each probe set on the GeneChips was determined by the RMA algorithm using R and Bioconductor and quality assured using *arrayQualityMetrics.[11]* Further analysis and differential gene expression studies were carried out using *oneChannelGUI*. A pfp (predictor of false positive) value of 0.05 was chosen to determine differentially expressed genes. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA - Ingenuity Systems). The microarray data produced in this study is MIAME compliant (http://www.mged.org/Workgroups/MIAME/miame.html) and will be submitted to the ArrayExpress database (www.ebi.ac.uk/arrayexpress) once the manuscript is accepted for publication.

Cell culture

Cells were cultured at a density of 1.0 x10^5/ well in a 96 well plate (Corning) and matured to a macrophage phenotype as previously described.[10, 12] Cells were pre-incubated with GW3965 or vehicle at the indicated concentration for 48 hours prior to addition of TLR ligands; 100 ng/ml ultra pure lipoplysacchardide (LPS - Calbiochem), 10 µg/ml Lipoteichoic acid (LTA), 10 µg/ml Pam_3CSK4 or 1 µg/ml CL097 (Autogen) for 24 hours.

Cytokine analysis

The concentration of cytokines in cell culture supernatants was analysed by ELISA or luminex as previously described.[10]

Immunofluorescence

5 µM sections were deparaffinised, rehydrated and epitope retrieval was performed using citrate buffer followed by incubation with 20% horse serum. Primary antibodies were incubated overnight at 4^{o}C in 5% serum/ TBST at final concentration of 2.5 µg/ml LXRα (Abcam – clone PPZ0412), 2.6 µg/ml LXRβ (Santa Cruz – polyclonal), 2.5 µg/ml CD68 (Dako – clone PG-M1) or isotype controls (Dako). Biotinylated secondary antibodies were incubated for 30 min in 5% serum/TBST followed by incubation with Avidin-D fluorochrome conjugates (Vectorlabs; LXRα/β - Fluorescein or CD68 - Texas Red) for 45 min in PBS. Slides were mounted in vectashield containing DAPI (Vectorlabs) and visualised under a fluorescent microscope (Axiovert S100 and Openlab software).

Statistical analysis

Results are displayed as mean ± standard deviation. Statistical analysis was by paired Students t test, Wilcoxon paired t test or Two-Way Anova using the Graph Pad Prism 4 software. The significance of association between the differentially expressed genes and canonical pathways within IPA was determined using Fisher's exact test and corrected using Benjamini-Hochberg correction for multiple testing.

Results

Analysis of the synovial fluid derived macrophage transcriptome

To determine the gene expression profile that defines an RA SF macrophage, microarray analysis was performed on sorted SF macrophages and the transcriptome compared to that of matched peripheral blood monocytes obtained from eight subjects with RA. Only genes that were significantly differentially expressed with $p \leq 0.05$ between the blood and synovium in all eight donors were considered for further analysis. From a total of 54,675 detectable transcripts 8,303 were significantly differentially expressed between the blood and the synovium (Figure 1A & B). We examined the transcriptional relationships between SF macrophages and peripheral blood monocytes by principal component analysis. Importantly, the transcriptional profile for each of the sample populations clustered together but separately from each other indicating conservation of patterns of gene expression in each group but that SF macrophages have a transcriptionally distinct profile of gene expression from that of the peripheral blood monocytes (Figure 1C). It is likely therefore that the RA synovial micro-environment induces specific transcriptional changes upon entry of monocytes into the synovium that may drive disease pathology.

The LXR pathway is highly up-regulated in RA synovial fluid macrophages

We next examined the canonical biological pathways most associated with this transcriptional profile using IPA. The pathway most significantly induced in

SF macrophages was the LXR/RXR nuclear receptor activated pathway (Supplementary Table 3 and Figure 2A). By microarray LXRα expression was up-regulated by approximately 2.1 fold whilst the expression of LXRβ was not changed (Figure 2B and Supplementary Table 4). LXRs are nuclear receptor transcription factors that upon activation by oxidised cholesterol derivatives drive the expression of a large variety of transcriptional target genes. IPA analysis also revealed increased expression of known downstream target genes particularly, ATP Binding Cassette (ABC) A1 and ABCG1, Apolipoprotein (Apo) C1, Apo C2, Lipoprotein Lipase (LPL) and Phospholipid Transfer Protein (PLTP) (Figure 2A and Supplementary Table 4).

Confirmation of LXR activation and induction of downstream gene expression

To confirm the microarray results we used QRT-PCR to analyse changes in the level of gene expression. Consistent with the microarray results the expression of LXRα was significantly higher in SF macrophages compared to matched peripheral blood monocytes in all donors (Figure 3). In contrast, the expression of LXRβ was significantly downregulated. Furthermore, we confirmed that the expression of ABCA1, ABCG1 LPL, PLTP, ApoE, ApoC1 and ApoC2 were significantly increased (Figure 3) which suggests that the level of LXR activation is increased in monocytes upon entry into the RA synovial micro-environment.

LXR protein is present in synovial macrophages

We next sought to demonstrate the presence of LXRα and LXRβ protein particularly within synovial macrophages. Sections derived from RA synovial membrane biopsies were stained with antibodies against either LXRα or LXRβ in combination with CD68. We detected LXRα (40%) and LXRβ (36%) positive macrophages in RA synovial membranes (Figure 4A-C).

Macrophage differentiation up-regulates LXRα expression

Migration into the synovium induces the differentiation of monocytes into macrophages.[13, 14] We therefore examined the level of LXRα expression during the differentiation of healthy peripheral blood monocytes to a macrophage phenotype. In all four donors tested, LXRα was significantly increased between 10 and 150 fold over 6 days (Figure 5A). In contrast, LXRβ was significantly down-regulated by approximately two fold (Figure 5B) whereas the basal level of ABCA1 expression, as a reporter of LXR activation, was not significantly different between monocytes and macrophages, but could be increased by addition of the LXR agonist GW3965 (Figure 5C). Together these results suggest that whereas macrophage differentiation can alter the relative level of LXRα and LXRβ expression it is not sufficient alone to induce activation of the LXR pathway.

Studies have previously shown that administration of GW3965 to LPS-activated monocytes or macrophages potentiates the secretion of pro-inflammatory cytokines.[10, 15] To determine if the difference in the level of LXR expression between monocytes and macrophages impacts the level of subsequent cytokine secretion we treated syngeneic monocytes and

macrophages for 48 hours with GW3965 followed by stimulation with 100 ng/ml LPS. After 24 hours the concentration of TNFα in cell culture supernatants was measured by ELISA. In agreement with previous observations LXR agonism significantly increased the secretion of TNFα from LPS stimulated monocytes (Figure 5D and,[10]). In comparison to monocytes at the same concentration of GW3965, macrophages secreted significantly higher levels of TNFα in response to stimulation with LPS suggesting that the higher level of LXR expression in macrophages enhances inflammatory cytokine secretion.

TNFα augments the expression of LXRα in human macrophages

Differentiation of monocytes to macrophages enhances the level of LXR expression. However, TNFα is central to the pathology of RA and has previously been shown to increase the expression of LXRα in rabbit adipocytes.[16] To test whether TNFα effects LXR expression in human macrophage we treated M-CSF matured macrophages with 25 ng/ml TNFα for 4 hours. Addition of TNFα modestly, but significantly, increased LXRα expression two-fold whereas the expression of LXRβ and ABCA1 was unchanged (Figure 5 E-G). Treatment of macrophages with up to 100 ng/ml IL-6 did not change the expression of LXRα, LXRβ or ABCA1 (data not shown).

LXR activation potentiates TLR driven cytokine secretion in human macrophages

Our microarray analysis also showed that the expression of genes known to be involved in the inhibition of Retinoid X Receptor (RXR) function were highly

significantly up-regulated in SF macrophages (Supplementary Table 3). This pathway consisted mainly of genes that are downstream of Toll-like receptors (TLRs) and that are transcriptionally up-regulated upon TLR ligation. Furthermore, TLRs have been widely implicated as potential drivers of RA disease pathology. In particular TLR2, TLR4, TLR7 and TLR8 have all been shown to be expressed at higher levels in RA synovial macrophages and potential exogenous and endogenous TLR ligands have been identified in RA SF.[17-21] This was of particular interest as LXR agonism has previously been shown to potentiate macrophage cytokine secretion induced by TLR4 ligation with LPS (Figure 5D and,[10, 15]). However, the effect of LXR agonism upon cytokine secretion induced by ligation of TLR2, TLR7 and TLR8 is unknown. Human M-CSF matured macrophages were cultured in the presence of GW3965 for 48 hours prior to stimulation with TLR ligands; LPS (TLR4) and CLO97 (TLR7/8). TLR2 (TLR1/2 or TLR2/6) can be stimulated with either PAM_3CSK4 or LTA; however, these ligands exert differential effects in macrophages and therefore both of these TLR2 ligands were used in parallel.[22] In accordance with our previous findings, LXR agonism by addition of GW3965 significantly increased the secretion of TNFα from LPS stimulated human macrophages in a dose dependent manner (Figure 6A). Furthermore, LXR agonism augmented the secretion of TNFα from macrophages stimulated with ligands for TLR2 (Figure 6B and C) or TLR7/8 (Figure 6D). Luminex analysis of cell culture supernatants showed that this was not specific to TNFα as the secretion of other pro-inflammatory cytokines, IL-1β, IL-6, IL-12 and G-CSF, and inflammatory

chemokines, MIP-1α (CCL3) and MIP-1β (CCL4), that are typically secreted upon TLR ligation were also increased from macrophages stimulated with LPS (Figure 6E), LTA (Figure 6F) PAM_3CSK4 (Figure 6G) and CL097 (Figure 6H). There was a significant increase in IL-1RA but no significant difference in the concentration of IL-10 (Figure 6F, G and H and data not shown).

Discussion

By adopting a hypothesis free approach we have utilised microarray technology to elucidate the molecular pathways that are induced within RA SF macrophages. Importantly, we have shown that the transcriptome of SF macrophages differs considerably compared to peripheral blood monocytes. Furthermore, we have shown that the LXR pathway is highly induced and is a novel potential driver of RA disease pathology that is in part mediated by augmentation of TLR induced cytokine secretion.

Our studies show that the expression of LXRα is increased during macrophage differentiation *in vitro*, whilst the expression of LXRβ is down-regulated. This can be further augmented by the pro-inflammatory cytokine TNFα which is a hallmark of the inflamed synovium. LXRs are activated by a variety of oxidised cholesterol derivatives.[23] It is therefore interesting to find that a lipid-activated pathway is the most up-regulated pathway in SF macrophages as RA is associated with dyslipidemia i.e. high serum cholesterol and triglycerides and an atherosclerotic like phenotype.[24-26] Furthermore, several studies have demonstrated that the concentration of cholesterol and the cholesterol transport lipoproteins are elevated in SF.[27] Further studies are therefore required to characterise the synovial lipidome in detail. However, we speculate that the elevated levels of cholesterol in the synovium may lead to the subsequent induction of the LXR pathway, which may drive synovial inflammation. In agreement with this we have previously shown that dual activation of LXRα and LXRβ greatly enhances the onset and severity of disease in a murine model of

collagen-induced arthritis.[10, 28] Taken together these studies demonstrate a potential pro-inflammatory effect of LXR activation and for the first time show that LXR activated pathways contribute a major role in human pathology by driving inflammatory cytokine secretion, which may potentiate the progression of synovitis.

The mechanism(s) by which LXR activation drives RA disease pathology are unknown. However, the microarray analysis revealed that the pathways induced by TLR ligation were highly increased in the SF macrophages consistent with similar observations in the literature.[18, 29] The TLRs expressed on macrophages bind a variety of viral and bacterial derived products, e.g. bacterial lipoproteins (TLR1/2/6), lipopolysaccharide (TLR4) and single stranded RNA (TLR7/8). Whilst bacterial cell wall fragments, peptidoglycan and double stranded DNA have been identified within the synovium it is now well recognised that TLRs can be activated by endogenous self proteins such as heat-shock proteins and double stranded RNA released from necrotic synoviocytes.[18, 30] LXR activation is known to potentiate cytokine secretion from LPS activated human macrophages; this is in part achieved through increased expression of TLR4.[10, 15] Here we have extended these studies and shown that activation of the LXR pathway also leads to a dramatic increase in cytokine secretion driven by TLR 1/ 2, TLR2/6 and TLR 7/8. These results are of particular interest as the expression of TLR2 is up-reulgated upon differentiation of monocytes to macrophages, which can lead to the enhancement of Th17 cells.[31, 32] Furthermore, such studies suggest that TLR induced cytokine secretion in RA SF macrophages is mediated

mainly through TLR2. However, although we have confirmed that LXR activation increases the expression of TLR4, the expression of TLR1, TLR2, TLR6, TLR7 and TLR8 were not changed (data not shown). Therefore, the mechanism by which LXR activation promotes cytokine secretion induced by ligation of TLR1/2, TLR2/6 and TLR7/8 is unknown.

Overall, our results support the hypothesis that the dyslipidemia associated with arthritis may enhance the inflammatory aspect of disease and that this may be in part mediated through activation of the LXRs. Furthermore, our data help support clinical findings that reducing the atherosclerotic burden in RA may ameliorate the inflammatory aspect of disease and improve long-term prognosis in RA.

Acknowledgments and funding

Funding was provided by MASTER SWITCH European community grant, Medical Research Council (UK), the Nuffield Foundation Oliver Bird Rheumatism programme, Arthritis Research UK and the Iraqi Ministry of Higher Education and Scientific Research.

Competing interests

Sabina Patel was employed by GlaxoSmithKline.

Author contributions

Darren L Asquith, Lucy E Ballantine, Jagtar Singh Nijjar, Manhal Khuder Makdasy, Sabina Patel, Pamela B Wright, James H Reilly, Shauna Kerr, Mariola Kurowska-Stolarska and J Alastair Gracie, all contributed towards the provision of samples, analysis and interpretation of data, study design(s), the critical revision of the article and contributed towards the intellectual content.

Darren L Asquith, Lucy E Ballantine, Jagtar Singh Nijjar and Iain B McInnes also drafted the manuscript.

All authors approved the final version of the manuscript.

References

1. Sack U, Stiehl P, Geiler G. Distribution of macrophages in rheumatoid synovial membrane and its association with basic activity. *Rheumatol Int* 1994;**13**:181-186.
2. Baeten D, Kruithof E, De Rycke L, et al. Infiltration of the synovial membrane with macrophage subsets and polymorphonuclear cells reflects global disease activity in spondyloarthropathy. *Arthritis Res Ther* 2005;**7**:R359-369.
3. Mulherin D, Fitzgerald O, Bresnihan B. Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. *Arthritis Rheum* 1996;**39**:115-124.
4. Bresnihan B, Pontifex E, Thurlings RM, et al. Synovial tissue sublining CD68 expression is a biomarker of therapeutic response in rheumatoid arthritis clinical trials: consistency across centers. *J Rheumatol* 2009;**36**:1800-1802.
5. Haringman JJ, Gerlag DM, Zwinderman AH, et al. Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis* 2005;**64**:834-838.
6. Jahangier ZN, Jacobs JW, Kraan MC, et al. Pretreatment macrophage infiltration of the synovium predicts the clinical effect of both radiation synovectomy and intra-articular glucocorticoids. *Ann Rheum Dis* 2006;**65**:1286-1292.
7. Gerlag DM, Haringman JJ, Smeets TJ, et al. Effects of oral prednisolone on biomarkers in synovial tissue and clinical improvement in rheumatoid arthritis. *Arthritis Rheum* 2004;**50**:3783-3791.
8. Feldmann M, Williams RO, Paleolog E. What have we learnt from targeted anti-TNF therapy? *Ann Rheum Dis* 2010;**69 Suppl 1**:i97-99.
9. Kinne RW, Stuhlmuller B, Burmester GR. Cells of the synovium in rheumatoid arthritis. Macrophages. *Arthritis Res Ther* 2007;**9**:224.
10. Asquith DL, Miller AM, Hueber AJ, et al. Liver X receptor agonism promotes articular inflammation in murine collagen-induced arthritis. *Arthritis Rheum* 2009;**60**:2655-2665.
11. Kauffmann A, Gentleman R, Huber W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. *Bioinformatics* 2009;**25**:415-416.
12. Antoniv TT, Ivashkiv LB. Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages. *Arthritis Rheum* 2006;**54**:2711-2721.
13. Ridley MG, Kingsley G, Pitzalis C, et al. Monocyte activation in rheumatoid arthritis: evidence for in situ activation and differentiation in joints. *Br J Rheumatol* 1990;**29**:84-88.
14. Koch AE, Burrows JC, Skoutelis A, et al. Monoclonal antibodies detect monocyte/macrophage activation and differentiation antigens and identify functionally distinct subpopulations of human rheumatoid synovial tissue macrophages. *Am J Pathol* 1991;**138**:165-173.

15. Fontaine C, Rigamonti E, Nohara A, et al. Liver X receptor activation potentiates the lipopolysaccharide response in human macrophages. *Circ Res* 2007;**101**:40-49.
16. Zhao SP, Dong SZ. Effect of tumor necrosis factor alpha on cholesterol efflux in adipocytes. *Clin Chim Acta* 2008;**389**:67-71.
17. Enevold C, Radstake TR, Coenen MJ, et al. Multiplex screening of 22 single-nucleotide polymorphisms in 7 Toll-like receptors: an association study in rheumatoid arthritis. *J Rheumatol* 2010;**37**:905-910.
18. Ospelt C, Brentano F, Rengel Y, et al. Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. *Arthritis Rheum* 2008;**58**:3684-3692.
19. Huang Q, Ma Y, Adebayo A, et al. Increased macrophage activation mediated through toll-like receptors in rheumatoid arthritis. *Arthritis Rheum* 2007;**56**:2192-2201.
20. Radstake TR, Roelofs MF, Jenniskens YM, et al. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. *Arthritis Rheum* 2004;**50**:3856-3865.
21. Sacre SM, Lo A, Gregory B, et al. Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures. *J Immunol* 2008;**181**:8002-8009.
22. Long EM, Millen B, Kubes P, et al. Lipoteichoic acid induces unique inflammatory responses when compared to other toll-like receptor 2 ligands. *PLoS One* 2009;**4**:e5601.
23. Janowski BA, Willy PJ, Devi TR, et al. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 1996;**383**:728-731.
24. van Halm VP, Nielen MM, Nurmohamed MT, et al. Lipids and inflammation: serial measurements of the lipid profile of blood donors who later developed rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:184-188.
25. van Sijl AM, Peters MJ, Knol DK, et al. Carotid intima media thickness in rheumatoid arthritis as compared to control subjects: a meta-analysis. *Semin Arthritis Rheum* 2011;**40**:389-397.
26. Sattar N, McInnes IB. Vascular comorbidity in rheumatoid arthritis: potential mechanisms and solutions. *Curr Opin Rheumatol* 2005;**17**:286-292.
27. Ananth L, Prete PE, Kashyap ML. Apolipoproteins A-I and B and cholesterol in synovial fluid of patients with rheumatoid arthritis. *Metabolism* 1993;**42**:803-806.
28. Asquith DL, Miller AM, Reilly J, et al. Simultaneous activation of the liver X receptors (LXRalpha and LXRbeta) drives murine collagen-induced arthritis disease pathology. *Ann Rheum Dis* 2011;**70**:2225-2228.
29. Tamaki Y, Takakubo Y, Hirayama T, et al. Expression of Toll-like receptors and their signaling pathways in rheumatoid synovitis. *J Rheumatol* 2011;**38**:810-820.

30. van der Heijden IM, Wilbrink B, Tchetverikov I, et al. Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides. *Arthritis Rheum* 2000;**43**:593-598.
31. He Z, Shotorbani SS, Jiao Z, et al. HMGB1 promotes the differentiation of Th17 via up-regulating TLR2 and IL-23 of CD14+ monocytes from patients with rheumatoid arthritis. *Scand J Immunol* 2012;**76**:483-490.
32. Iwahashi M, Yamamura M, Aita T, et al. Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. *Arthritis Rheum* 2004;**50**:1457-1467.

Rheumatoid arthritis patient characteristics	
Number of patients	8
Mean age (years)	68
Female : Male	5 : 3
Mean disease duration (years)	12.2
Mean ESR (mm/hr)	86
Mean CRP (mg/L)	107
RF positive (%)	87.5
Disease modifying anti-rheumatic drug use (%)	62.5
Anti TNFα therapy (%)	1*

Supplementary Table 1**.** Clinical characteristics of the rheumatoid arthritis patients that contributed samples towards the microarray analysis; * Anti-TNF therapy ceased due to patient becoming resistant to therapy.

	Primer Sequence (5’ – 3’)
TBP	FWD: AGACCTTCCTGTTTACCCTTGCCA
	REV: TAGCTGTGGGTGACTGCTTGGATT
ABCA1	FWD: ACACCTGCAGTTCATCAGTGGAGT
	REV: ATAATGACCAGTGTGGCAGGGACA
LXRα	FWD: CAGGGCTGCAAGTGGAATTCATCA
	REV: GAGCAAGGCAAACTCGGCATCATT
LXRβ	FWD: ACTTCACCTACAGCAAGGACGACT
	REV: AAGATGTTGATGGCGATGAGCAGG
ApoC1	FWD: AGGACAGGACCTCCCAACCAA
	REV: GGCTGGGCCTTCCAAGACGAT
ApoC2	FWD: GGATTTGAGGTCCAGGGGACC
	REV: TGGGCGGCTGTCTTTGCTGAC
ApoE	FWD: GAACTGAGGGCGCTGATGGAC
	REV: CTCCGCCACCGGGGTCAGTT
LPL	FWD: TCATCAGTCGGTCCGCGCCT
	REV: AATCTCTTCTTTGGTCGGCGG
PLTP	FWD: GGATCCCGCTGGACGGATGAA
	REV: GCTGGTTGAGGAGGAAGCGCA

Supplementary Table 2. The sequence of the forward (FWD) and reverse (REV) primers used for analysis of human gene expression by SYBR green QRT-PCR.

Canonical pathways up-regulated in RA synovial macrophages	p value
LXR/RXR activation	3.96×10^{-7}
Hepatic fibrosis/ hepatic stellate cell activation	2.27×10^{-5}
Role of macrophages, fibroblasts and endothelial cells in RA	7.2×10^{-5}
LPS/IL-1 mediated inhibition of RXR function	1.07×10^{-4}
Coagulation system	1.98×10^{-4}

Supplementary Table 3. The top five significantly up-regulated canonical pathways in synovial fluid macrophages compared to syngeneic peripheral blood monocytes of patients with RA as assessed by use of Ingenuity Pathway Analysis. Fisher's exact test.

Gene	Gene expression Log2 fold change	pfp value	Reported functions
LXRα/β	2.12/1	0	Transcription factors that are activated by ligation of oxidised cholesterol derivatives.
ABCA1/G1	2.41	0	Lipid transporters that promote reverse cholesterol transport in macrophages.
ApoC1	6.38	0	Components of VLDL and HDL lipid particles, inhibits LPL and promotes LPS induced TNFα secretion from macrophages.
ApoC2	4.58	0	Part of VLDL particles and activates LPL to induced triglyceride hydrolysis.
ApoE	3.24	0	Transports cholesterol as part of LDL and VLDL particles from the periphery for degradation in the liver and excretion in bile.
LPL	6.09	0	A triglyceride hydrolase and acts as a receptor for HDL uptake.

PLTP	5.14	0	Transfer of phospholipids from VLDL particles to HDL during reverse cholesterol transport.

Supplementary Table 4. Log 2 fold change in the expression of genes that form the LXR/RXR pathway as identified by microarray analysis by comparison of the transcriptome of synovial fluid macrophages to peripheral blood monocytes. Liver X receptor (LXR), ATP binding cassette A1/ G1 (ABCA1/G1), Apolipoprotein (Apo), lipoprotein lipase (LPL) and phospholipid transfer protein (PLTP). n = 8 RA patients. Rank Product statistic.

Figure 1

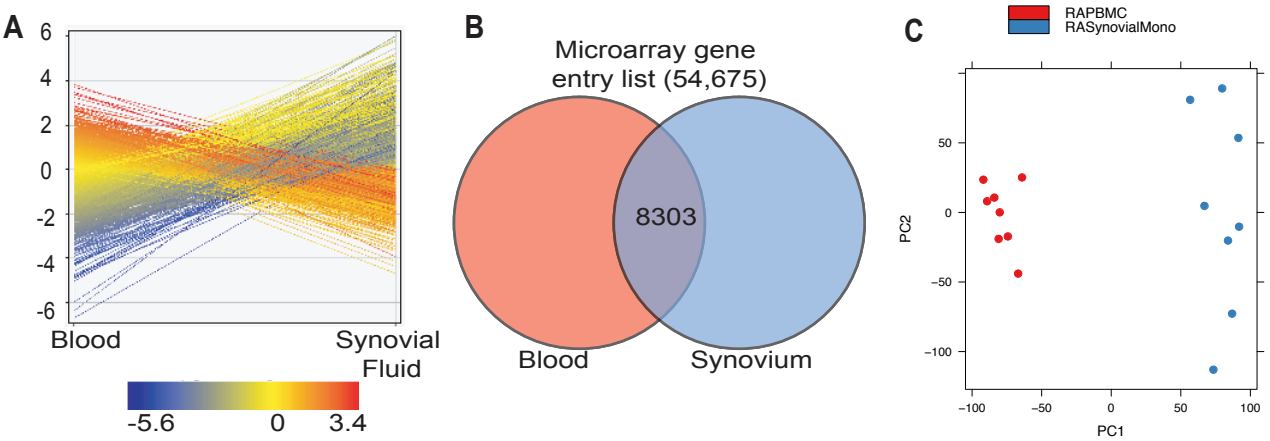
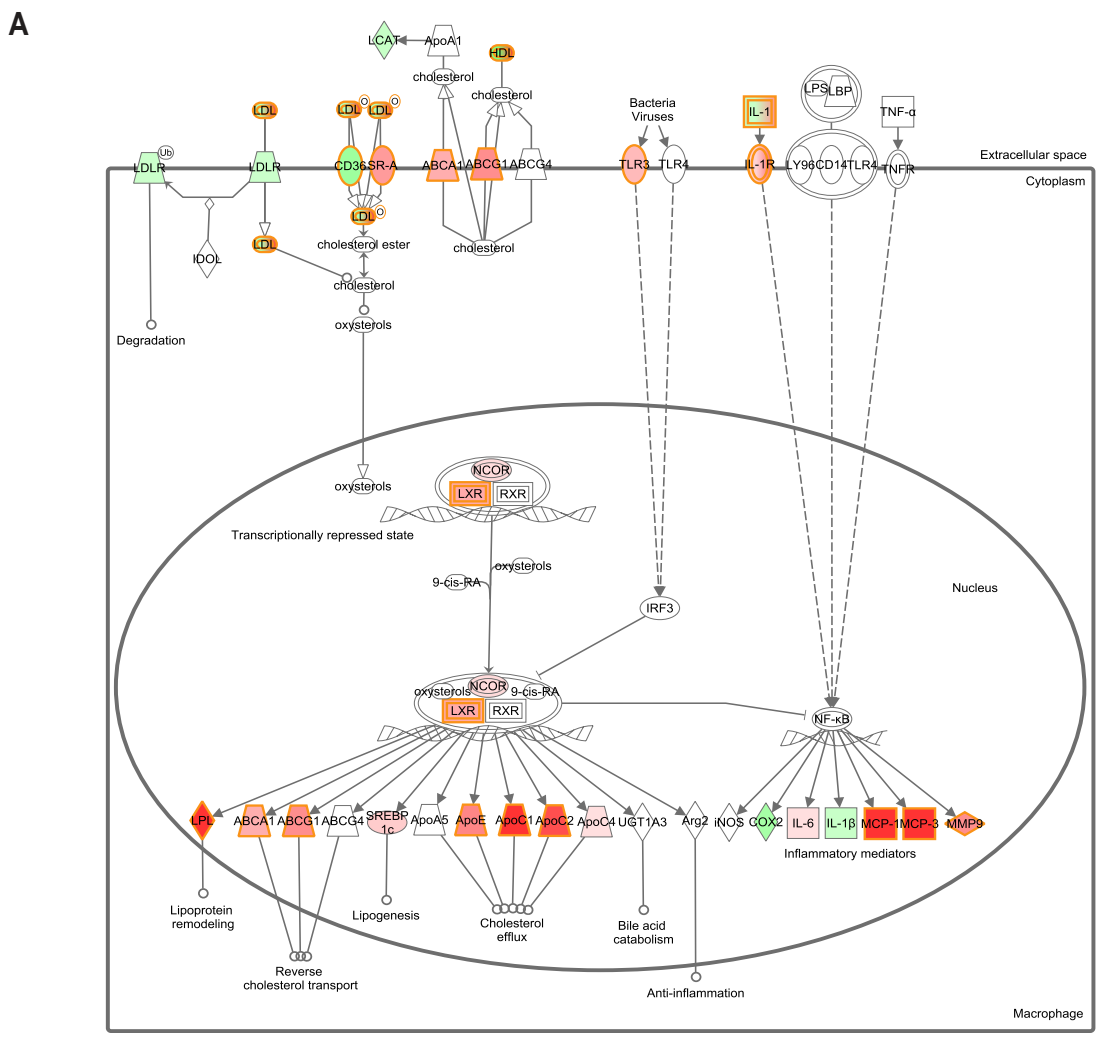


Figure 1. Microarray gene expression profiles of peripheral blood monocytes and synovial fluid resident macrophages. Microarray analysis was performed on RNA extracted from peripheral blood (PB) monocytes and synovial fluid (SF) resident macrophages of patients with rheumatoid arthritis (RA). (A) Graphical representation of the number and magnitude of transciptional changes between peripheral blood and synovial fluid macrophages. (B) Microarray analysis identified 8303 genes that were differentially expressed between the blood and synovial monocytes. (C) Principal Component Analysis revealed that synovial fluid monocytes (blue) have a uniquely distinct pattern of gene expression compared to syngenic peripheral blood monocytes (red). n = 8 independent donors.

Figure 2



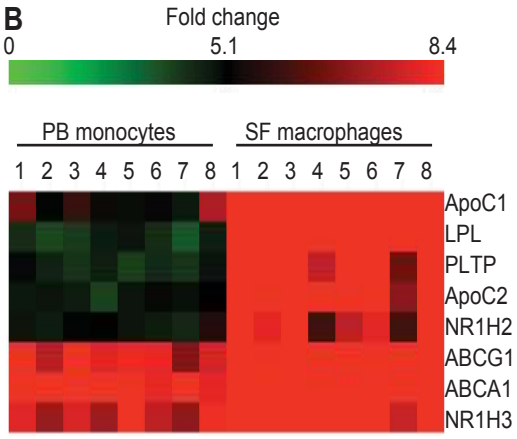


Figure 2. The Liver X Receptors pathway is the most significantly upregulated pathway in RA synovial macrophages. (A) A Schematic of the molecular pathways leading to the activation of the Liver X Receptors (LXRs) and the downstream LXR target genes. Cholesterol taken up into the cell by scavenger receptors (CD36) and the Low density lipopoprotein receptor (LDLR) is oxidised to form oxysterol ligands which can activate the LXRs. LXR activation induces the expression of down stream transcriptional target genes (ABCA1, ApoC1/C2/E) which in turn promote reverse cholesterol tranport. This pathway is a suggestive representation based on curated databases (Taken from Ingenuity Pathway Analysis). (B) Heatmap depicting the fold change of genes in the LXR pathway; Aplolipoprotein (Apo), Liopoprotein Lipase (LPL), Phospholipid Transfer Protein (PLTP), NR1H2 (LXRβ), ATP Binding Cassette (ABC), NR1H3 (LXRα). n=8 donors.

Figure 3

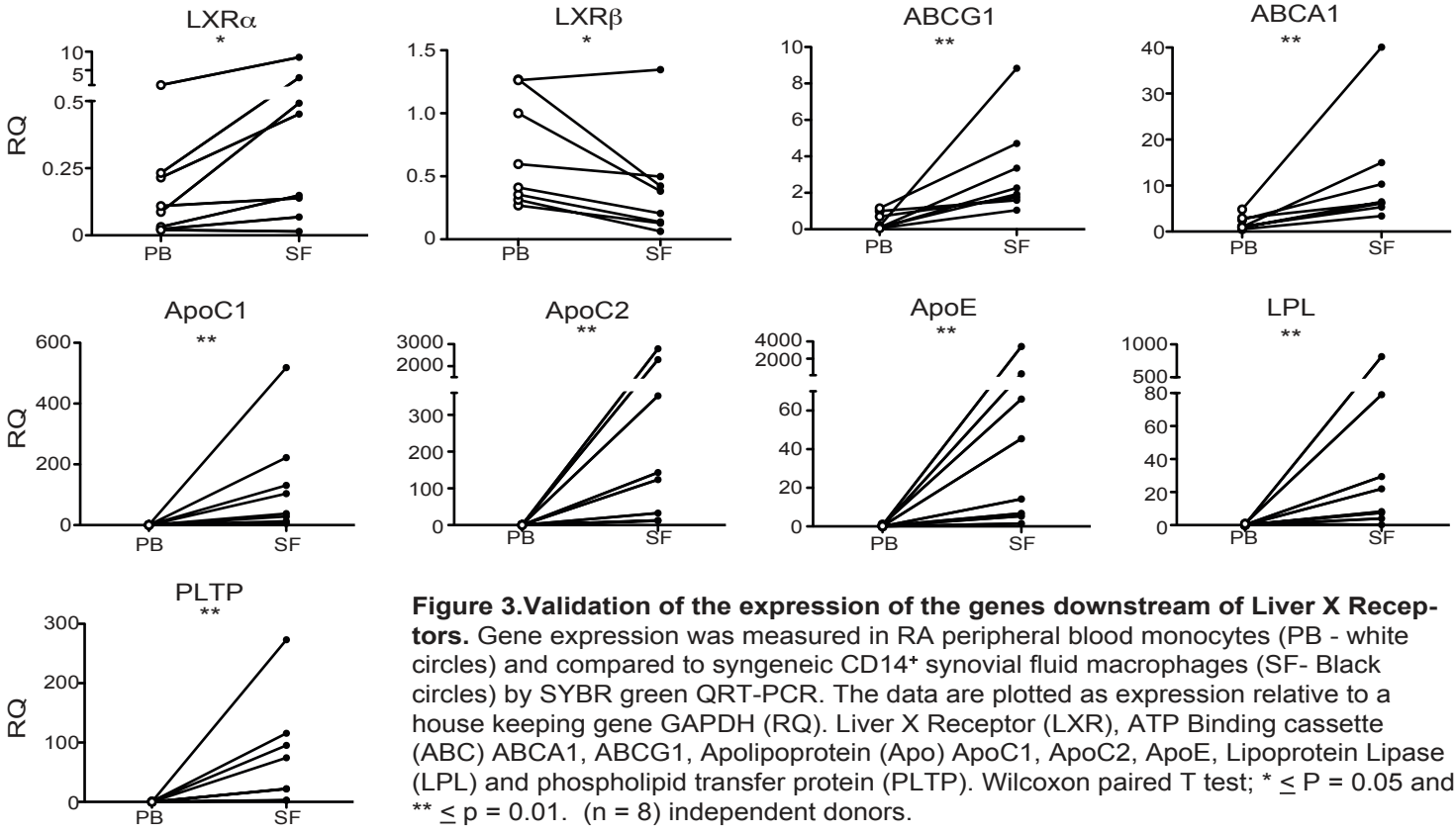
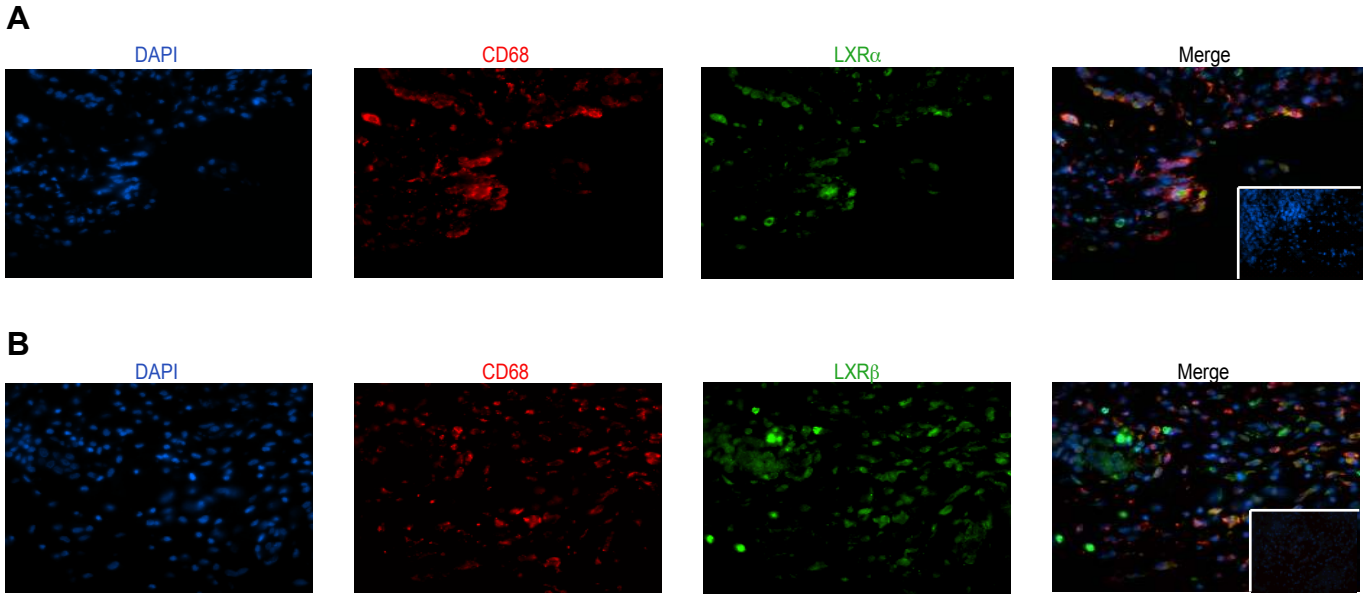


Figure 3.Validation of the expression of the genes downstream of Liver X Receptors. Gene expression was measured in RA peripheral blood monocytes (PB - white circles) and compared to syngeneic $CD14^+$ synovial fluid macrophages (SF- Black circles) by SYBR green QRT-PCR. The data are plotted as expression relative to a house keeping gene GAPDH (RQ). Liver X Receptor (LXR), ATP Binding cassette (ABC) ABCA1, ABCG1, Apolipoprotein (Apo) ApoC1, ApoC2, ApoE, Lipoprotein Lipase (LPL) and phospholipid transfer protein (PLTP). Wilcoxon paired T test; * ≤ P = 0.05 and ** ≤ p = 0.01. (n = 8) independent donors.

Figure 4



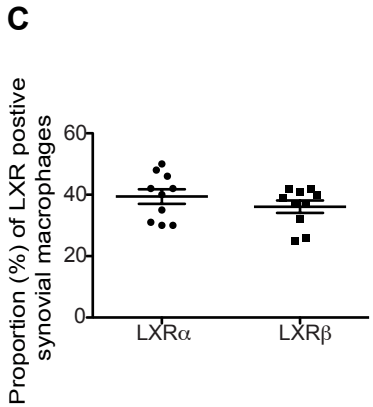


Figure 4. Liver X Receptors are expressed in synovial membrane macrophages. Representative 7 mm synovial membrane sections from patients with rheumatoid arthritis were stained for macrophages with anti-CD68, nuclear staining with DAPI and anti-LXRα (A) and anti-LXRβ (B). Images overlayed for detection of LXRa and LXRb in synovial macrophages shown in yellow. Isotype controls are shown in the bottom right hand corner. Magnification 40X. (C) The percentage of synovial macrophages that were positively stained for LXRα and LXRβ. One field of view/section/donor. Data from n = 10

Figure 5

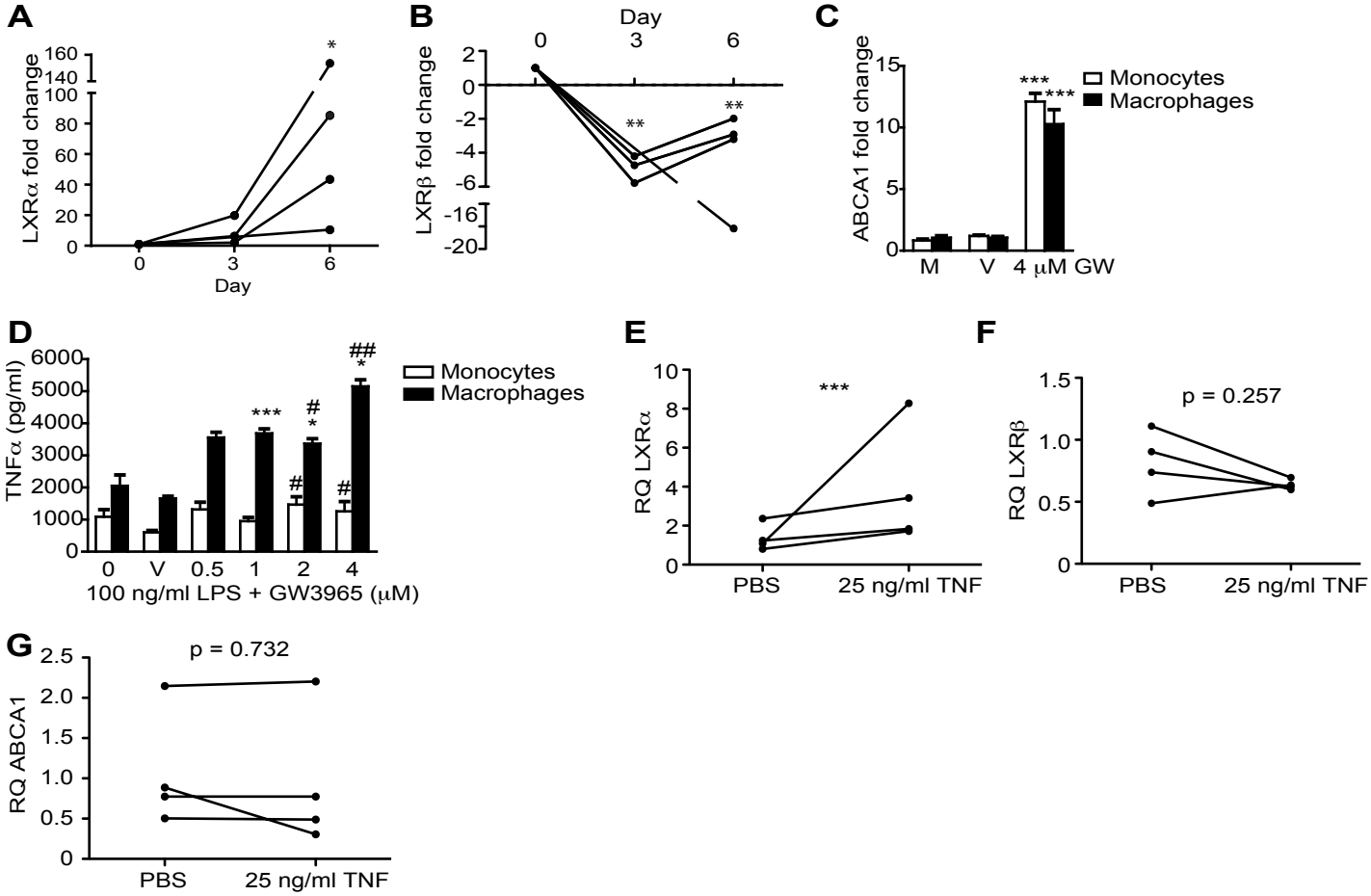


Figure 5. The synovial microenvironment promotes the expression and activation of the Liver X Receptors. CD14^{+} healthy human peripheral blood monocytes were treated with 50 ng/ml M-CSF for 6 days to induce macrophage differentiation. The expression of LXRα (A) and LXRβ (B) was measured by QRT-PCR. (C) M-CSF matured macrophages were treated with media alone (M), vehicle (V - DMSO) or 4 μM GW3965 (GW) - after 24 hours the expression of ABCA1 was measured by QRT-PCR. *** $p < 0.001$, Two-Way Anova. (D) M-CSF matured macrophages and syngeneic monocytes were cultured in the presence of GW3965 for 36 hours and then stimulated with100 ng/ml LPS. After 24 hours the concentration of TNFα in cell culture supernatants was measured by ELISA. Students paired T test * indicates T test between monocytes and macrophages at the same concentration of GW3965 whilst # indicates T test relative to vehicle control. (E - G) M-CSF matured macrophages were treated with TNFα or PBS. After 4 hours the cells were lysed for expression analysis of LXRα (E), LXRβ (F) and ABCA1 (G) relative to TATA-binding protein (TBP). Two-way Anova; *** $p = \leq 0.001$. n = 4 donors.

Figure 6

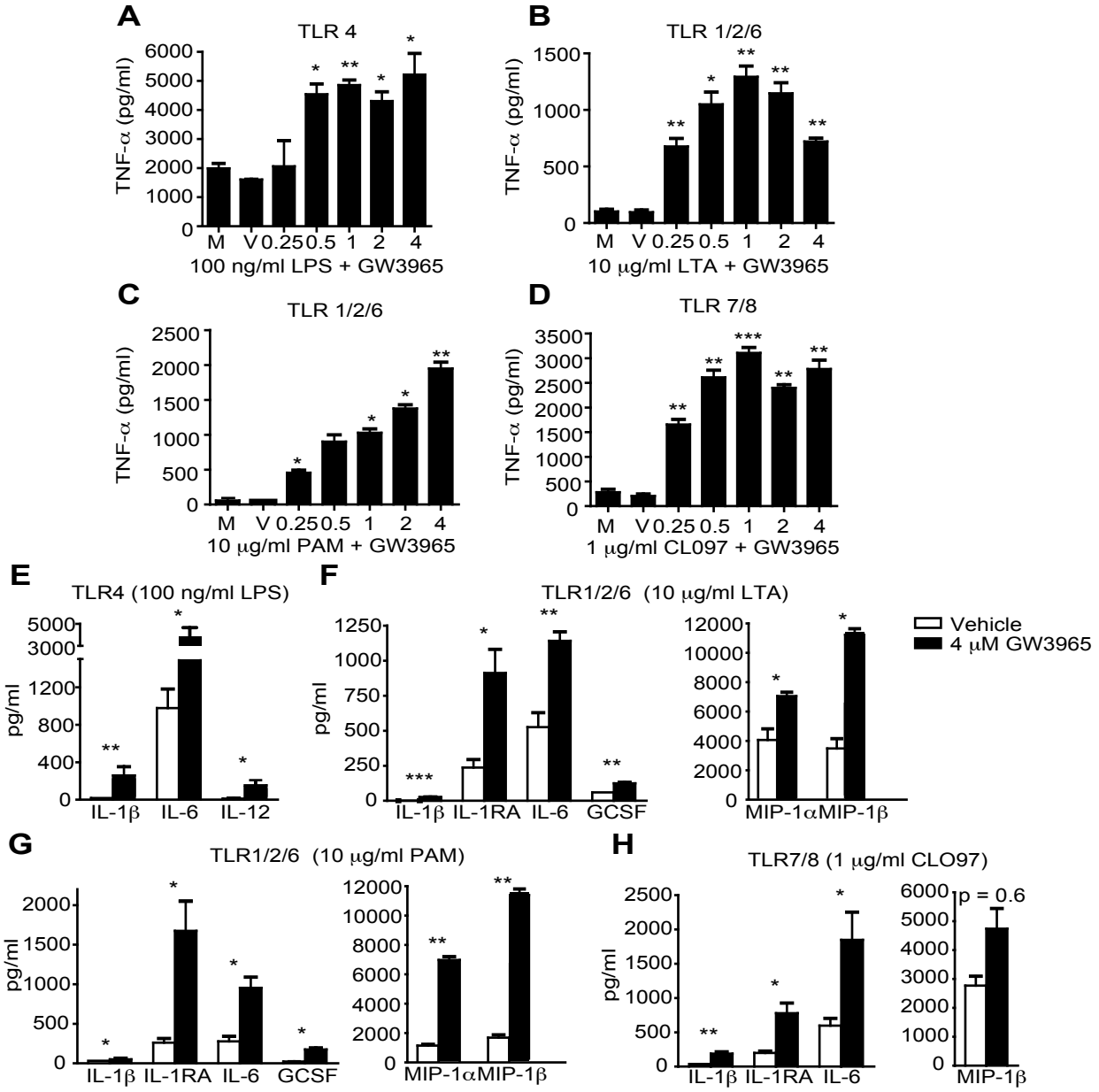


Figure 6. Liver X Receptor activation augments pro-inflammatory cytokine secretion in human TLR stimulated macrophages. Healthy human peripheral blood monocyte derived macrophages were treated with GW3965 at the indicated concentration (μM) for 24 hours. The cells were then stimulated with toll-like receptor ligands; TLR4 - 100 ng/ml LPS (A), TLR1/2/6 -10 μg/ml LTA (B) or 10 μg/ml PAM_3CSK4 (PAM) (C) or TLR 7/8 1 μg/ml CL097 (D) in the presence of GW3965 for a further 24 hours. (A - D) The concentration of TNFα in cell culture supernatants was measured by ELISA. (E - H) Luminex analysis of cell culture supernatants from macrophages stimulated with LPS (E), LTA (F), PAM_3CSK4 (G) or CL097 (H). Each condition was tested in triplicate and the results are representative of four independent experiments. Students paired T test; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.