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1 **Testosterone stimulates cholesterol clearance from human macrophages by activating LXR α**

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3

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23 **Testosterone stimulates cholesterol clearance from human macrophages by activating LXR α** 24 **Abstract**

25 **Aims:** Low testosterone in men is associated with increased cardiovascular events and mortality.
26 Testosterone has beneficial effects on several cardiovascular risk factors including cholesterol, endothelial
27 dysfunction and inflammation as key mediators of atherosclerosis. Although evidence suggests
28 testosterone is anti-atherogenic, its mechanism of action is unknown. The present study investigates
29 whether testosterone exerts anti-atherogenic effects by stimulating cholesterol clearance from
30 macrophages via activation of liver X receptor (LXR α), a nuclear master regulator of cellular cholesterol
31 homeostasis, lipid regulation, and inflammation. **Main Methods:** Using human monocyte THP-1 cells
32 differentiated into macrophages, the effect of testosterone (1-10nM) treatment (24-72 h) on the expression
33 of LXR α and LXR- targets apolipoprotein E (APOE), ATP-binding cassette transporter A1 (ABCA1),
34 sterol regulatory element-binding transcription factor 1 (SREBF1) and fatty acid synthase (FAS), was
35 investigated via qPCR and western blotting, with or without androgen receptor blockade with flutamide
36 or LXR antagonism with CPPSS-50. Cholesterol clearance was measured by monitoring fluorescent
37 dehydroergosterol (DHE) cellular clearance and ABCA1 cellular translocation was observed via
38 immunocytochemistry in testosterone treated macrophages. **Key Findings:** Testosterone increased
39 mRNA and protein expression of LXR α , APOE, ABCA1, SREBF1 and FAS. These effects were blocked
40 by flutamide and independently by LXR antagonism with CPPSS-50. Furthermore testosterone stimulated
41 cholesterol clearance from the macrophages and promoted the translocation of ABCA1 toward the cell
42 membrane. **Significance:** Testosterone acts via androgen receptor-dependent pathways to stimulate LXR α
43 and downstream targets to induce cholesterol clearance in human macrophages. This may, in part,
44 explain the anti-atherogenic effects of testosterone frequently seen clinically.

45

46 **Key Words:** Atherosclerosis, Macrophage, Liver X receptor, Androgen Receptor.

47 **Introduction**

48 An increase in cardiovascular mortality has been demonstrated in men with testosterone deficiency in
49 epidemiological studies [1] and also within a population of men with angiographically-proven coronary
50 heart disease [2] indicating low testosterone levels as an independent cardiovascular risk factor. The
51 administration of testosterone therapy (TTh) has been shown to improve risk factors for atherosclerosis,
52 the major underlying cause of cardiovascular disease (CVD), including reducing central adiposity and
53 insulin resistance and improving lipid profiles (in particular, lowering cholesterol), clotting and
54 inflammatory profiles and vascular function (reviewed in [3]). Despite this evidence the impact of
55 testosterone on the cardiovascular system remains controversial with concerns in regard to the safety of
56 TTh in men with cardiovascular disease [4-6]. These studies however have been highly criticised in their
57 methodological approach and interpretation of data. Systematic meta-analyses of TTh clinical trials have
58 not demonstrated an increased risk of adverse cardiovascular events or mortality [7,8]. The majority of
59 large observational studies with extended follow-up report TTh was associated with a significant
60 reduction in all-cause mortality, myocardial infarction, and stroke [9,10].

61 While there are no definitive randomised clinical trials of TTh that report direct effects on atherosclerosis,
62 some clinical and scientific studies have investigated the mechanistic effects of testosterone on
63 atherogenesis seen in testosterone deficient men. Carotid intimal media thickness (CIMT), considered a
64 surrogate marker of the degree of atherosclerosis, has been shown to be reduced by TTh in some
65 randomised clinical trials [11,12] yet was unchanged in others [13]. Conversely, coronary artery plaque
66 volume was increased as a result of TTh in hypogonadal men considered as a result of increased non-
67 calcified plaque volume and fibrous plaque volume compared to placebo [14]. Animal studies provide
68 consistent evidence that testosterone is atheroprotective. Aortic plaque formation is accelerated following
69 castration in models of atherosclerosis while replacing testosterone abrogates plaque development [15-
70 17]. These studies suggest that testosterone may act via both genomic androgen receptor (AR) -dependent
71 and non-genomic AR-independent mechanisms to protect against atherogenesis.

72 From their recruitment to the vascular sites of inflammation through to apoptosis and
73 destabilisation/rupture of the plaque, macrophages are considered the ‘driving force’ behind atherogenesis
74 with the formation of foam cells considered as the first sign of plaque development. Foam cell formation
75 is influenced by increased lipid uptake and the inability of macrophages to efflux sufficient amounts of
76 cholesterol to the reverse cholesterol transport (RCT) pathway. Indeed, macrophage RCT reflects the
77 removal of cholesterol from the arterial wall, and its impairment is associated with increased
78 atherosclerosis in several animal models [18-20]. Stimulation of cholesterol efflux from macrophages
79 therefore has the potential to be atheroprotective.

80 Testosterone has known immunomodulatory actions which may contribute to its observed
81 atheroprotective effects [21,22]. Although only relatively few studies have investigated the influence of
82 androgens on monocyte or macrophage function relevant to atherosclerosis, the majority of these indicate
83 anti-inflammatory actions [23-25] or improvements in cellular lipid processing [26,27]. Specifically, foam
84 cell induction by oxLDL is inhibited by dihydrotestosterone (DHT) in a cultured macrophage cell line
85 [27] and human monocyte-derived macrophages treated with androgens in vitro demonstrate inhibited
86 cholesterol accumulation [26]. In testosterone deficient mice whereby the primary cellular constituents in
87 the atherosclerotic plaque were identified as macrophages, TTh reduced overall plaque size compared to
88 placebo treatment suggesting a reduction in macrophage content in the arterial wall [16]. The molecular
89 events underpinning this androgen action are not yet known.

90 Liver X receptor (LXR), a member of the nuclear receptor superfamily, is a key regulator of cholesterol
91 metabolism within the cell and the LXR α isoform is expressed in macrophages[28]. Upon ligand-
92 activation LXR α regulates the expression of proteins involved in RCT, such as ATP-binding cassette
93 transporter A1 (ABCA1) which mediates cellular cholesterol efflux, and apolipoprotein E (APOE) which
94 binds cholesterol to form high density lipoprotein (HDL) subsequently preventing a build-up of
95 cholesterol within the cell [29,28]. LXR α is also known to regulate fatty acid synthesis, glucose
96 metabolism and suppresses inflammatory cytokines [30]. LXR agonists have been shown to significantly

97 reduce atherosclerotic plaque development in animal models [31]. One study has reported that in Swiss
98 white rabbits the effect of an LXR agonist has an equivalent effect to statin therapy in reducing plaque
99 size and, importantly, when both are administered together there is evidence of plaque regression [32].
100 The use of LXR agonists as a therapy has been confounded by the concomitant development of hepatic
101 steatosis in many of these studies. Testosterone has however been shown to inhibit enzymes of fatty acid
102 synthesis in the liver in animal studies [33]. In the present study we investigate whether testosterone
103 exerts anti-atherogenic effects through the stimulation of LXR α -mediated cholesterol clearance in
104 macrophages.

105

106 **Materials and Methods**

107 *Cell Culture*

108 Human acute monocytic leukemia, THP-1 (ECACC Cat# 88081201, RRID:CVCL_0006) and Mono Mac
109 6, MM6 (DSMZ Cat# ACC-124, RRID:CVCL_1426) cell lines utilised in this study were both originally
110 derived from male acute monocytic leukemia patients. Cell lines were maintained in RPMI-1640 medium
111 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% sodium pyruvate
112 and passaged every 3-4 days. Cells were used up to passage 20. Prior to experiments, THP-1 cells were
113 incubated in phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) in FBS-free medium for 72 hours to
114 induce differentiation to a M0 macrophage-like phenotype. All experiments were performed under FBS-
115 free conditions. Testosterone (Sigma-Aldrich) and flutamide (Sigma-Aldrich) were solubilised in 100%
116 ethanol and serially diluted in FBS-free media to final concentrations of 1-10 nM. The LXR agonist
117 T0901317 (Sigma-Aldrich) was dissolved in DMSO and diluted to a final concentration of 1 μ M. LXR
118 antagonist 5-Chloro-*N*-2'-*n*-pentylphenyl-1,3-dithiothalimide (5CPPSS-50; Wako) was dissolved in
119 methanol and diluted to a final concentration of 20 μ M. Control conditions used relevant vehicle solutions
120 of equivalent volumes to associated treatments.

121 *Cholesterol Clearance Assay*

122 5×10^4 THP-1 cells per well were seeded into 96-well plates. Following differentiation, cells were
123 incubated in 10 nM testosterone or vehicle for 24 hours. Dehydroergosterol (DHE; Sigma-Aldrich) was
124 added at a concentration of 320 μ M and cells were incubated for a further 18 hours to allow the
125 cholesterol analogue to be taken up by the cells. Cells were then washed in 1x PBS and fresh FBS-free
126 media was added. For direct observation of DHE clearance, cells were viewed using a Leica AF6000LX
127 inverted microscope. A single field of view was imaged every 15 minutes for 6 hours. A FLUOstar plate
128 reader, set to a wavelength of 390/460 nm, was used to measure changes in fluorescence at set time points

129 following careful media replacement. Fluorescence values were normalized against media only wells and
 130 are shown as percentage decrease in cellular fluorescence from baseline.

131 *Quantitative analysis of mRNA*

132 Following treatments, cells were harvested and total RNA extracted using an RNA mini kit (Ambion).
 133 RNA concentrations were measured spectrophotometrically. cDNA was synthesised from 250 ng of RNA
 134 using a superscript reverse transcription kit (Qiagen) and its quality was checked by performing end point
 135 PCR using GAPDH primers and agarose gel electrophoresis. Quantitative PCR was performed on a
 136 Stratagene system using SYBR Green (Applied Biosystems) according to the manufacturers'
 137 recommendations. Gene-specific primers were designed using PrimerBlast software and synthesised by
 138 Invitrogen (Table 1). Each sample was run in triplicate and mRNA levels were normalized to the
 139 housekeeping gene GAPDH and assessed relative to time-point and cell-type controls. Melting curve
 140 analysis was performed each time to check specificity of primers.

141 **Table 1. Primer sequences.**

Gene	NCBI Reference	Direction	Primer sequence	Product length
Androgen Receptor	NM_000044.2	F	AAG GCC TTG CCT GGC TTC CG	141
		R	AGG GGC GAA GTA GAG CAT CCT GG	
CD14	NM_000591.3	F	ACT GTC AGA GGC AGC CGA AGA GT	141
		R	CGC GCG CTC CAT GGT CGA TA	
GAPDH	NM_002046.3	F	GAA GGC TGG GGC TCA TTT GCA GG	150
		R	CAG TTG GTG GTG CAG GAG GCA T	
LXR α	NM_005693.2	F	CTC TGC AGA CCG GCC CAA CG	128
		R	GCA TCC GTG GGA ACA TCA GTC GG	
ApoE	NM_000041.2	F	GCT GGG AAC TGG CAC TGG GTC	124
		R	TGC TCC ATC AGC GCC CTC AGT T	
ABCA1	NM_005502.3	F	GGC CTT GGC CTT TGT CGG GG	122
		R	TGG TGC GGC CTT GTC GGT AT	
FAS	NM_004104.4	F	GCT GGA AGG CGG GGC TCT AC	124
		R	CGC AGC TGA GGG TCC ATC GT	
SREBF1c	NM_001005291.2	F	CGA CAT CGA AGG TGA AGT CGG CG	146
		R	GGC CAG GGA AGT CAC TGT CTT GGT T	

142 **Western Blot Analysis**

143 Following treatments, cells were harvested and lysed in RIPA buffer containing protease inhibitors.
 144 Protein concentration of cell lysates was determined by Bradford Assay and 50 µg protein was separated
 145 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 150 V, 90 min) and
 146 electrophoretically transferred to nitrocellulose membrane (100 V, 120 min). Membranes were blocked
 147 with bovine serum albumin (BSA) or milk in TBST, dependent upon the protein of interest (Table 2).
 148 Membranes were then incubated in primary antibodies against specific targets LXR α , APOE, ABCA1,
 149 SREBF1 and FAS (Table 2). GAPDH was used as a loading control. Subsequent incubation with
 150 horseradish peroxidase (HRP)-labelled goat polyclonal secondary antibody was carried out.
 151 Immunocomplexes were visualised using chemiluminescence development kit and images were captured
 152 and densitometry performed using GeneSnap and GeneTools software.

153 **Table 2. Antibody details.**

Protein	Size (kDa)	Blocking conditions	1° Antibody		2° Antibody
			Host	Dilution	Dilution
ABCA1	220	5% milk	Mouse	1 in 500 in 2.5% milk	1 in 1000 in 2.5% milk
APOE	36	0.5% BSA	Rabbit	1 in 250 in 2.5% BSA	1 in 1000 in 2.5% BSA
CALNEXIN	75	10% milk	Rabbit	1 in 20,000 in 10% milk	1 in 1000 in 10% milk
FAS	273	10% milk	Rabbit	1 in 500 in 10% milk	1 in 1000 in 10% milk
GAPDH	36	5% milk	Mouse	1 in 500 in 2.5% milk	1 in 1000 in 2.5% milk
LXR	50	0.5% milk	Mouse	1 in 250 in 2.5% BSA	1 in 1000 in TBST
SREBF1c	125	5% milk	Mouse	1 in 100 in 2.5% milk	1 in 1000 in 2.5% milk

154

155

156

157 ***Immunocytochemistry***

158 Cells were grown and differentiated on poly-L-lysine coated chamber slides followed by treatments for 6
159 hours. Cells were fixed using 4% paraformaldehyde, permeabilised with 0.25% Triton X-100, blocked
160 with 1% BSA, 0.3 M glycine and incubated in ABCA1 primary antibody (1/250) at 4°C overnight.
161 AlexaFluor 555 secondary antibody (1/300) was used to visualise ABCA1 staining. Nuclei were counter-
162 stained using DAPI and images were acquired using a Leica DM14000B inverted microscope. Total
163 ABCA1 area was assessed via fluorescence coverage in 5 randomised, blinded images from 3 repeated
164 experimental conditions (n=3) for treated and control cells using image j analysis software. Likewise,
165 mean pixel intensity was assessed via image j analysis. ABCA1 subcellular localisation was also
166 estimated in 5 randomised, blinded images from 3 repeated experimental conditions (n=3) for treated and
167 control cells and percentage of cells expressing perinuclear, cytoplasmic and cell membrane associated
168 ABCA1 staining calculated.

169 ***Statistical analysis***

170 Data is presented as mean \pm standard error (SEM) unless otherwise stated. Differences between groups
171 with normally distributed data were compared using two-tailed unpaired Student's t tests performed in
172 Excel. Non-normally distributed data was investigated with Mann-Whitney U tests for significance
173 between groups. Significance was accepted at $P \leq 0.05$.

174 **Results**175 ***Testosterone increases LXR α , APOE, ABCA1, SREBF1 and FAS gene and protein expression.***

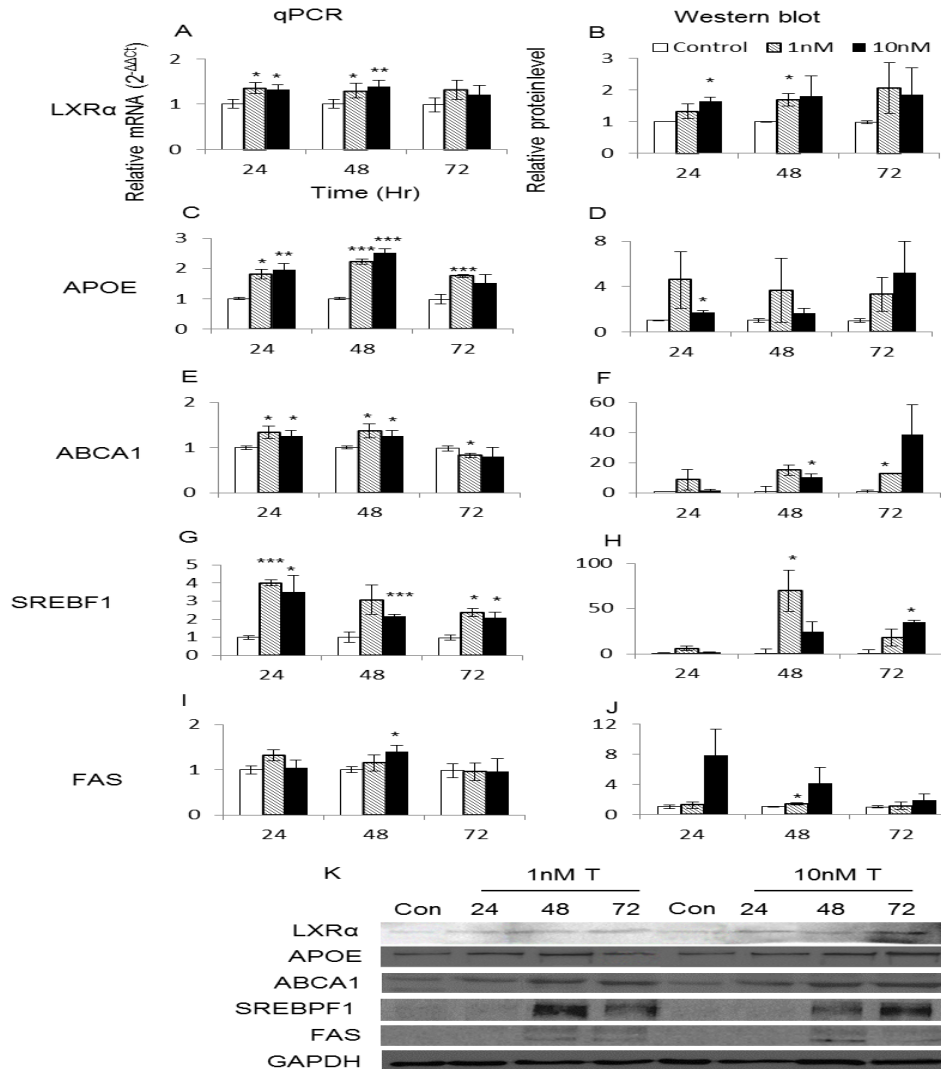
176 Exposing differentiated macrophages to both 1 nM and 10 nM testosterone resulted in a significant
177 increase in *LXR α* mRNA levels compared with control cells, after 24 and 48 hours ($n = 6$; Fig. 1 A).
178 Protein levels of *LXR α* were raised significantly after 48 hours in 1 nM testosterone ($n = 4$) and after 24
179 hours in 10 nM testosterone ($n = 3$; Fig. 1 B and K). Both concentrations of testosterone were found to
180 significantly increase *APOE* mRNA at all time points observed ($n = 3$; Fig. 1 C) and *APOE* protein levels
181 were increased significantly after 24 hours in 10 nM testosterone ($n = 3$; Fig. 1 D and K). Testosterone
182 significantly increased *ABCA1* mRNA at both 1 nM and 10 nM after 24 ($n = 8$) and 48 hours ($n = 9$; Fig.
183 1 E). This translated to a significant increase in *ABCA1* protein seen after 72 hours in 1 nM testosterone
184 and after 48 hours in 10 nM testosterone ($n = 3$; Fig. 1 F and K). 10 nM testosterone increased *SREBF1*
185 mRNA levels significantly at all of the time points observed whereas 1nM testosterone significantly
186 increased *SREBF1* mRNA levels after both 24 and 72 hours ($n = 3$; Fig. 1 G). *SREBF1* protein was
187 increased significantly after 48 hours in 1 nM testosterone ($n = 5$) and after 72 hours in the presence of 10
188 nM testosterone ($n = 4$; Fig. 1 H and K). Exposure to 10 nM testosterone caused *FAS* mRNA levels to
189 increase significantly after 48 hours ($n = 5$) whereas testosterone at 1 nM elicited a small but insignificant
190 increase in *FAS* mRNA ($n = 6$; Fig. 1 I). *FAS* protein levels were significantly increased after 48 hours in
191 1 nM testosterone ($n = 3$; Fig. 1 J and K). Thus testosterone increases the expression of *LXR α* and *LXR α* -
192 target genes in THP-1 monocyte-derived macrophages.

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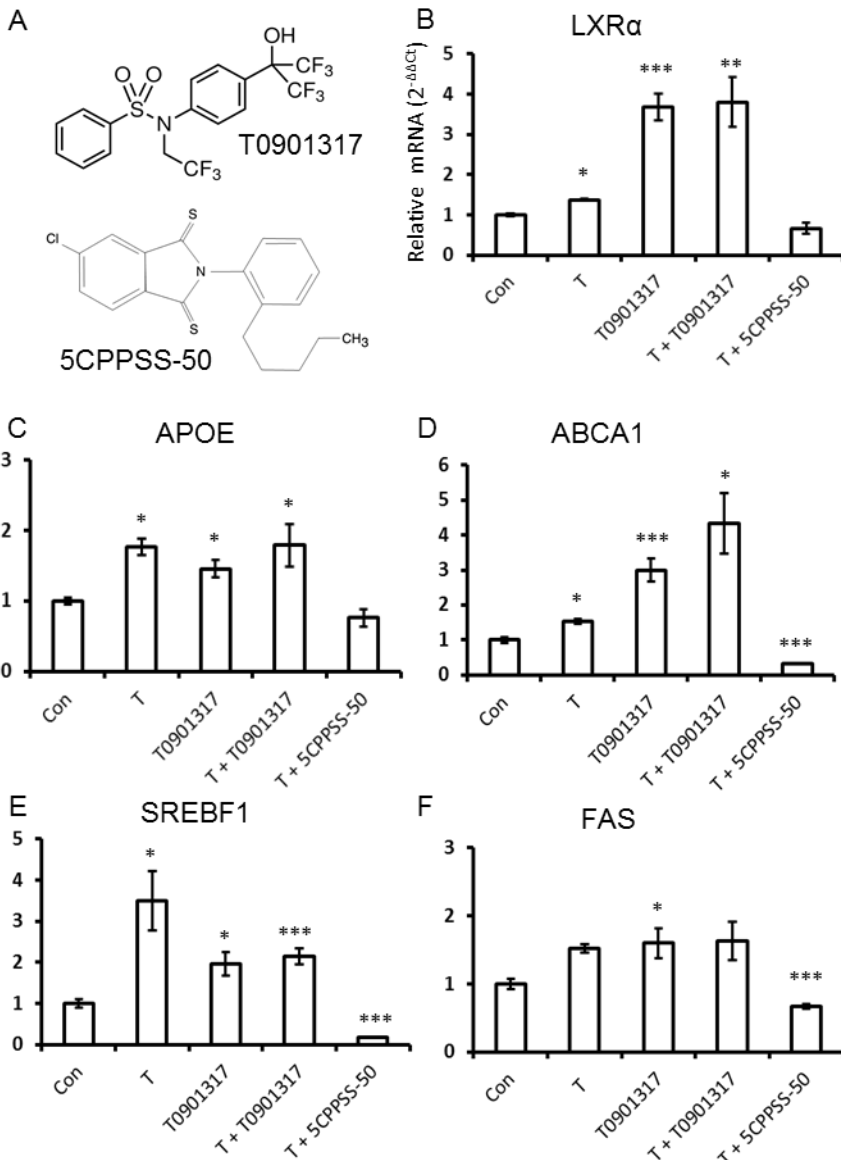


197

198 **Figure 1: Testosterone increases LXRα, APOE, ABCA1, SREBF1 and FAS gene and protein**
 199 **expression in THP-1 macrophages.** Analysis of gene and protein expression in differentiated THP-1
 200 macrophages by quantitative PCR and Western blot followed by densitometry, for LXRα (A and B),
 201 APOE (C and D), ABCA1 (E and F), SREBF1 (G and H) and FAS (I and J). THP-1 macrophages treated
 202 with testosterone (T) 1 nM (white columns) 10 nM (black columns) over time with data presented as
 203 mean ± SEM after normalisation to GAPDH as reference for both mRNA and protein analysis. qPCR
 204 values are presented as relative fold change from associated control treatments and western blot data as
 205 arbitrary densitometry units (A.U.). (K) Representative western blot images showing protein levels at 24,
 206 48 and 72 hours in response to testosterone 1 nM and 10 nM. n=3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

207 ***Testosterone increases APOE, ABCA1, SREBF1 and FAS gene expression via LXR α .***

208 THP-1 cells increased *LXR α* expression almost 4-fold in response to LXR agonist T0901317 ($n = 6$; Fig.
209 2 B). Exposure to T0901317 in combination with testosterone however did not elicit a further increase in
210 *LXR α* gene expression over that observed with T0901317 alone ($n = 6$; Fig. 2 B). Exposure to the LXR
211 antagonist 5CPPSS-50 in combination with testosterone completely abolished any increase in *LXR α* gene
212 expression observed with testosterone alone ($n = 6$; Fig. 2 B). The LXR agonist elicited an increase in the
213 expression of the *LXR α* target genes *APOE*, *ABCA1*, *SREBF1* and *FAS* ($n = 6$; Fig. 2 C-F). Testosterone
214 in combination with T0901317 did not further increase the expression of these target genes beyond mon-
215 treatment effects ($n = 6$; Fig. 2 C-F). Conversely, when LXR activity is blocked by the antagonist
216 5CPPSS-50, testosterone did not elicit an increase in the expression of *APOE*, *ABCA1*, *SREBF1* and *FAS*
217 ($n = 3$; Fig. 2 C-F).



218

219 **Figure 2: Testosterone increases APOE, ABCA1, SREBF1 and FAS gene expression in THP-1**220 **macrophages via LXR α .** (A) Molecular structure of the LXR agonist, T0901317 and LXR antagonist,221 5CPPSS-50. Relative mRNA levels of LXR α (B), APOE (C), ABCA1 (D), SREBF1 (E) and FAS (F) in

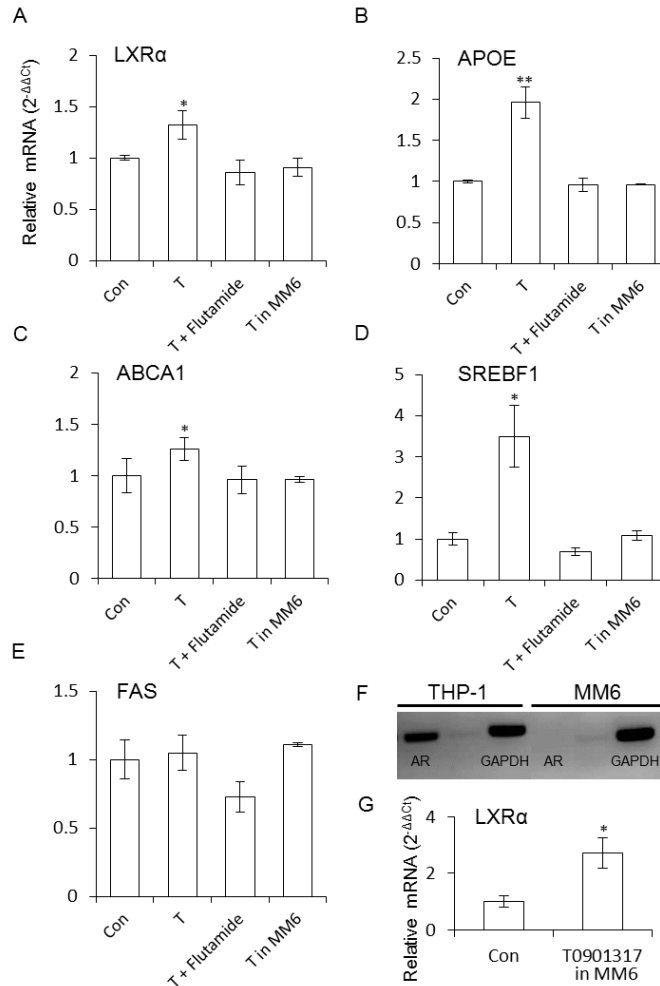
222 response to 24h treatment with testosterone 10nM (T), T0901317, T0901317 in combination with T, and

223 5CPPSS-50 in combination with T.

224

225 ***Testosterone acts through the AR to stimulate LXR α .***

226 When the activity of the AR is blocked with flutamide, testosterone did not increase the expression of
227 *LXR α* or any of its target genes ($n = 6$; Fig. 3 A-E). The cell line MM6 does not express the AR (Fig. 3 F)
228 and exposure of these cells to 10 nM testosterone for 24 hours did not increase the expression of *LXR α* or
229 any of its target genes ($n = 3$; Fig. 3 A-E). In order to demonstrate MM6 cells are able to respond to
230 experimental stimuli we applied the LXR agonist T0901317 for 24 hours and observed an increase in the
231 expression of *LXR α* ($n = 3$; Fig. 3 G).



232

233 **Figure 3: Testosterone acts through the androgen receptor to stimulate LXRα actions in THP-1**

234 **macrophages.** Relative mRNA levels of LXRα (A), APOE (B), ABCA1 (C), SREBF1 (D) and FAS (E)

235 in response to 24h treatment with testosterone 10 nM (T), T in combination with androgen receptor (AR)

236 blocker flutamide and the response to T in AR deficient MM6 cells. (F) End-point PCR shows AR

237 expression in THP-1 cells but not in MM6 cells, using GAPDH as a positive control. (G) Relative mRNA

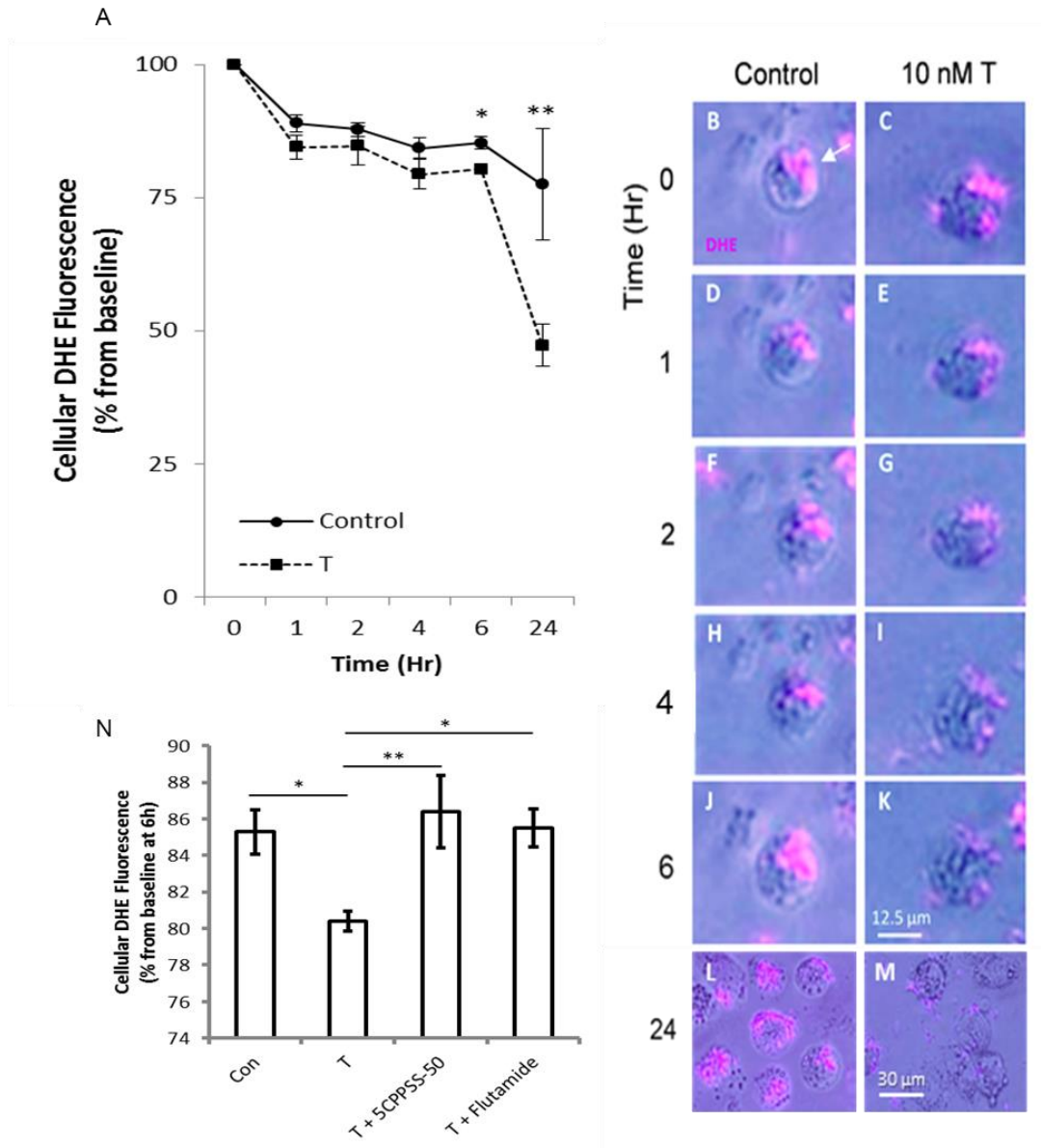
238 levels of LXRα in MM6 cells in response to LXR agonist, T0901317. Data presented as mean ± SEM

239 after normalisation to GAPDH as reference. n=3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

240

241 ***Testosterone stimulates cholesterol clearance from human macrophages.***

242 Within 6 hours of 10 nM testosterone treatment the rate of cholesterol clearance was significantly
243 increased with a reduction of 19.1% of the total DHE taken up by the cell ($n = 3$; black dashed line)
244 compared with a decrease of 14.7% of total DHE in control cells (black unbroken line; Fig. 4 A). Using
245 fluorescence microscopy, DHE (white arrow in B) was seen leaving the cell much faster in testosterone-
246 treated macrophages ($n = 3$; Fig. 4 C, E, G, I and K) than in controls (Fig. 4 B, D, F, H and J). After 24
247 hours testosterone-treated cells had cleared 53.2% of the DHE they originally contained ($n = 7$; black
248 dashed line; Fig. 4 A and M) compared with controls which had cleared only 23.1% (black unbroken line;
249 Fig. 4 A). Control cells at 24 hours can be seen to contain much higher levels of DHE when observed
250 directly using fluorescence microscopy ($n = 3$; Fig. 4 L Vs M). Thus testosterone increases the rate at
251 which THP-1 monocyte-derived macrophages clear cellular cholesterol. In cells treated with both
252 testosterone and the LXR antagonist 5CPPSS-50 or testosterone and flutamide for 6h, an increase in
253 cholesterol clearance was not observed compared to control cells ($n = 3$; Fig. 4 N).



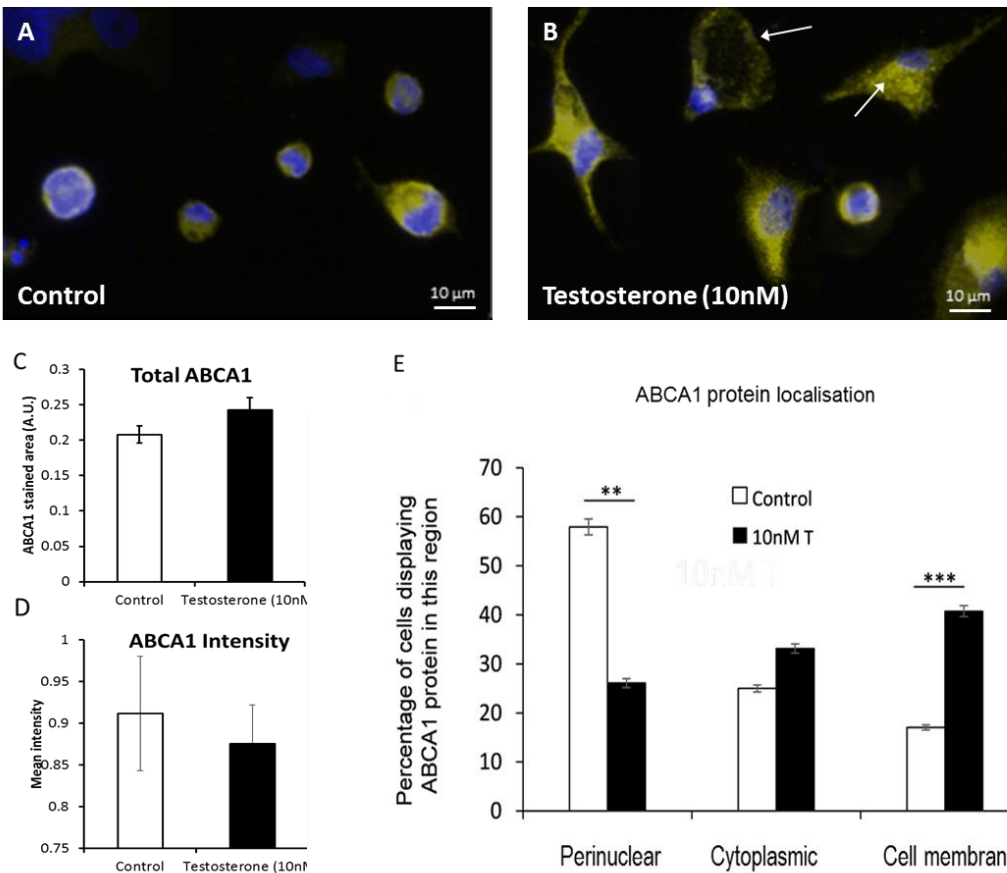
254

255 **Figure 4: Testosterone stimulates cholesterol clearance from THP-1 macrophages.** (A) Line graph
 256 shows the percentage cellular fluorescence over 24 hours in control cells (solid line) and cells treated with
 257 testosterone 10 nM (T) (dashed line). (B-M) Representative time-lapse fluorescence microscopy reveals
 258 dehydroergosterol (DHE: pink) clearance from a single cells over time (B-K, Scale bars, 12.5 μ m) and
 259 cell populations at 24 hours (L-M, Scale bar 30 μ m). (N) 6 hour percentage cellular fluorescence in
 260 control cells, treated with testosterone 10nM (T), T in combination with LXR antagonist 5CPPSS-50, or
 261 T in combination with flutamide. Data presented as mean \pm SD. n=3, * p <0.05, ** p <0.01 .

262 **Testosterone promotes the translocation of ABCA1 protein toward the cell membrane.**

263 Approximately 50% of control cells displayed ABCA1 protein in the perinuclear region (Fig. 5 D and
264 representative image in A), almost one quarter displayed ABCA1 protein throughout the cell in
265 cytoplasm, and a small percentage displayed ABCA1 protein at the cell membrane (Fig. 5 D). Following
266 exposure to 10 nM testosterone there was a significant drop in the number of cells displaying ABCA1
267 protein within the perinuclear region ($n = 8$; Fig. 5 D) and a significant increase in the number of cells
268 displaying ABCA1 at the cell membrane ($n = 8$; Fig. 5 D and representative image in C). A small increase
269 in the number of cells displaying ABCA1 protein throughout the cell in the cytoplasm was also seen in
270 cells treated with testosterone ($n = 8$; Fig. 5 D and representative image in B).

271



272

273 **Figure 5: Testosterone promotes the translocation of ABCA1 protein toward the cell membrane in**
 274 **THP-1 macrophages.** Representative fluorescence microscopy images reveal predominant localisation of
 275 ABCA1 protein (yellow) to the perinuclear region in a control cell (A). Cells treated with testosterone 10
 276 nM (T) demonstrate increased localisation of ABCA1 to the membrane and cytoplasm of the cell with the
 277 presence of cytoplasmic vesicles observed (B). White arrows indicate areas of high ABCA1 protein
 278 accumulation. Scale bar, 10 μ m. Semi-quantitative total ABCA1 expression as indicated by field of view
 279 fluorescence area (C) and mean fluorescence pixel intensity (D) compares control (white bars) and T
 280 treated (black bars) cells. (E) Percentage of cells displaying ABCA1 protein in the perinuclear region,
 281 cytoplasm and at the cell membrane is altered between control and T treated cells. Data presented as mean
 282 \pm SEM. n=3, * p <0.05, ** p <0.01, *** p <0.005.

283 Discussion

284 Low levels of testosterone are associated with increased incidence of CVD in men and TTh has been
285 shown in some studies to reduce several risk factors that contribute to the development of atherosclerosis
286 [21]. Here we provide evidence that testosterone may exert some of its anti-atherogenic properties
287 through the stimulation of LXR α -mediated cholesterol clearance in macrophages. Testosterone stimulates
288 expression of the *LXR α* gene in human THP-1 monocyte-derived macrophages, leading to an increase in
289 LXR α protein within the cell. LXR α functions as a master metabolic regulator controlling the
290 transcription of many genes encoding proteins involved in cholesterol and lipid metabolism. Indeed, the
291 LXR α -target genes ABCA1, APOE, SREBF1, and FAS, were also upregulated. Increased expression of
292 these targets was dependent upon nuclear receptor activity as LXR α antagonism abolished testosterone
293 effects. Furthermore, using the AR blocker flutamide, and a cell line which does not express the AR we
294 demonstrate that testosterone acts genomically via AR to increase LXR α and related target gene
295 expression. Functionally, testosterone promoted cholesterol clearance from cholesterol-loaded THP1
296 macrophages, an effect we suggest is via increased ABCA1 translocation to the cell membrane and
297 potentially as a result of AR-mediated LXR actions on ABCA1 and ApoE expression.

298 Within the early atherosclerotic plaque, a key feature of macrophages is their ability to internalise
299 modified low-density lipoprotein and promote removal of excess cholesterol from the developing
300 atheroma. As atherosclerosis progresses, there is a deregulation of cholesterol uptake and reverse
301 transport by macrophages leading to the development of lipid laden foam cells. Analogous to the present
302 study, it has been previously shown that primary human monocyte-derived macrophages treated with
303 testosterone demonstrated reduced cholesterol accumulation, a mechanism considered to be due to
304 increased cholesterol efflux and via upregulation of scavenger receptor-1B (a membrane receptor
305 involved in the binding of HDL) which consequently activates RCT [26]. No effect was observed on the
306 expression of ABCA1 although translocation to the membrane was not investigated. Similarly, the

307 induction of foam cell formation by oxLDL has been shown to be inhibited by DHT in cultured
308 macrophages [27]. Whether this was due to enhanced cholesterol processing and efflux was not clarified.

309 Many studies provide strong evidence that ABCA1-mediated cholesterol efflux from macrophages has
310 atheroprotective properties [34-36]. Furthermore in humans, mutations of ABCA1 lead to Tangier disease
311 which is characterised by severe HDL deficiency and severe generalised atherosclerosis [37]. Clinically,
312 testosterone therapy in hypogonadal men is often confounding in studies, either reducing, having no effect
313 or raising circulating HDL levels [38]. Lower HDL may putatively indicate pro-atherogenic actions, but
314 it is conversely considered that testosterone intensifies the RCT process thus reducing circulating HDL,
315 conditionally dependent upon subsequent hepatic uptake and catabolism of HDL cholesteryl esters, and
316 thereby exerting an overall anti-atherogenic rather than a pro-atherogenic effect [26]. Furthermore, HDL
317 efflux capacity can differ markedly among individuals with identical HDL-c concentrations an effect
318 considered via ABCA1 activity [39] and a large population study identified efflux capacity a better
319 predictor of existing coronary artery disease than HDL-c concentration [40]. The present study indicates
320 that testosterone increases ABCA1 expression in macrophages and suggests this may be via activation of
321 LXR as co-treatment with LXR antagonist, 5CPPSS-50, abrogated the effect. Similarly, previous studies
322 have shown that LXR activation time- and dose-dependently increases ABCA1 levels in macrophages
323 [41,29,42]. Due to the known inherent instability of ABCA1, these studies suggest that as well as
324 transcriptional regulation, LXRs may also promote stabilisation of the cholesterol transporter and
325 therefore the potential for prolonged cholesterol efflux. Indeed, we observed testosterone induced
326 upregulation of ABCA1 levels at 48 and 72h beyond gene expression changes suggesting that
327 testosterone or androgen receptor signalling may additionally promote ABCA1 stabilisation via LXR
328 activation, although the underlying mechanism for this remains unclear.

329 Evidence that LXR activation via alternate nuclear signalling pathways can lead to increased
330 macrophage-cholesterol efflux is apparent in studies utilising agonists of peroxisome proliferator-
331 activated receptor alpha (PPAR α) and PPAR γ which also upregulate LXR [43,44]. What was apparent in

332 the present study was that despite no increase in protein expression following 24h testosterone treatment
333 of THP1 macrophages, a translocation of ABCA1 from perinuclear regions occurred increasing ABCA1
334 density on the plasma membrane to potentially enhance intracellular cholesterol transport towards the
335 membrane ready for efflux. Indeed, testosterone promoted a greater than two-fold induction of cholesterol
336 clearance when compared to control cells within a 24 hour period.

337 In addition to ABCA1, the present study shows that testosterone increases macrophage APOE expression
338 in an LXR α -dependent manner as a potential mechanism to further promote RCT. Zanotti et al. [45]
339 demonstrate that macrophage, but not systemic, apoE is necessary for macrophage RCT in vivo.
340 Macrophages isolated from apoE $^{-/-}$ mice effluxed significantly less cholesterol than WT macrophages,
341 even in the absence of extracellular acceptors, suggesting that apoE selectively expressed in macrophages
342 may efficiently improve RCT in vivo by enhancing cholesterol availability for transport to liver and
343 faeces [45]. Indeed, reduced macrophage-derived cholesterol was observed in plasma, liver, and faeces
344 with concomitant increased atherosclerosis in healthy mice reconstituted with apoE-deficient
345 macrophages, independently of changes in the plasma lipoprotein profile [46]. Therefore, the increased
346 macrophage ABCA1 and ApoE expression observed in the present study may have resulted in enhanced
347 cholesterol efflux implicating a potentially beneficial effect of testosterone on atherosclerosis.

348 We have previously shown in vivo that testosterone increases *LXR α* and associated targets *APOE* and
349 ABCA1 expression in the liver of testicular feminized (Tfm) mice, which have a non-functional androgen
350 receptor and low circulating testosterone, following a high-cholesterol diet [47,33]. Marked hepatic lipid
351 accumulation was apparent in the Tfm mouse but not in the wild-type and testosterone treated Tfm mice.
352 The pathophysiology of hepatic steatosis is considered analogous to the processes that occur in
353 atherosclerosis and indeed early atherosclerosis was reduced in testosterone-treated Tfm mice suggesting
354 that testosterone influences lipid and cholesterol metabolism and/or transport at these sites. Contrary to
355 the present study however, this previous investigation suggests that the beneficial influence of

356 testosterone may act, at least in part, via AR-independent mechanisms indicating differences in either
357 testosterone or LXR α -regulation across different tissues and/or species [47].

358 Beyond effects on RCT, we show that testosterone partially increases targets of fatty acid metabolism
359 *SREBF1* and *FAS* in THP-1 macrophages via LXR activation. Cellular fatty acid and triacylglycerol
360 biosynthesis, upregulated by SREBF1 and FAS, triggers and is triggered by an inflammatory response.
361 Several studies show that enhanced fatty acid synthesis in macrophages is linked to inflammation,
362 although the dynamic relationship is not fully elucidated [48-50]. Srebf-1a isoform and LXR α are both
363 highly expressed in macrophages and known regulators of cytokine release. Specifically, Srebf-1 KO
364 macrophages exhibit reduced production of anti-inflammatory FAs resulting in late hyper-inflammatory
365 states and inhibited resolution of inflammatory responses [51,52]. While the present study did not
366 specifically investigate inflammation, it could be postulated that by altering macrophage lipid metabolism
367 testosterone may improve the inflammatory environment of the atherosclerotic plaque. Testosterone has
368 been previously shown to alter SREBF-1 expression in liver [53], as well as in subcutaneous adipose
369 tissue [47] and prostate cancer cell lines [54], although the involvement of LXR in these studies was not
370 assessed.

371 The influence of LXR in testosterone actions in the current study was demonstrated using a pan-LXR
372 antagonist which gives rise to the possibility that the increase observed in target gene expression may be
373 mediated by LXR β or a combination of both LXR subtypes. Evidence from previous studies however
374 suggests that LXR α plays a more dominant role in the regulation of cholesterol and fatty acid metabolism
375 than LXR β . For example, deletion of LXR α but not LXR β in APOE-knockout mice leads to an
376 accumulation of cholesterol as well as atherosclerosis and the expression of genes involved in cholesterol
377 and fatty acid metabolism, such as *SREBF1* and *FAS*, are additionally impaired in LXR α -knockout mice
378 but not in LXR β -knockout mice [55-57]. In the present study, co-treatment of THP-1 cells with
379 testosterone and pan-LXR agonist, T0901317, did not induce any further response in LXR-dependent
380 gene expression than treatments alone suggesting competing pathways of activation. Furthermore,

381 testosterone has no effect on gene expression in THP-1 monocyte-derived macrophages when the AR is
382 blocked or deficient demonstrating that testosterone requires a functional AR to stimulate LXR α and
383 LXR-associated target expression in these cells. The exact mechanisms of these nuclear receptor
384 interactions and cross-talk warrant further investigation.

385 While THP-1 macrophages are frequently used as a model for investigating human-derived macrophage
386 function and have been previously utilised to study LXR ligand responses [58,59], isolated primary
387 human male monocytes should be further investigated to translate current findings to the clinical situation
388 and highlight any potential role of testosterone and LXR in atherosclerosis. Furthermore, caution should
389 be applied when interpreting statistical significance as a representation of true biological meaning in small
390 data sets such as those presented in the current study. Additionally, the present study assessed cholesterol
391 clearance from DHE loaded macrophages as a potential indication of cholesterol efflux in the absence of
392 cholesterol acceptors as demonstrated previously [45]. However, further investigation is required to
393 confirm detailed mechanisms of testosterone action on cholesterol efflux pathways.

394 **Conclusion**

395 This study suggests that testosterone can stimulate cholesterol clearance from macrophages potentially
396 through membrane translocation of ABCA1 and via LXR α . Testosterone additionally influenced the
397 expression of cholesterol and lipid regulating proteins including ApoE via AR-dependent mechanisms,
398 which warrants further investigation to uncover the underlying mechanisms of action. This study
399 therefore highlights some important potential actions of testosterone which may explain how TTh reduces
400 vascular lipid accumulation in animal models and surrogate markers of atherosclerosis in some clinical
401 studies to improve cardiovascular risk in hypogonadal men.

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403

404 **Conflict of interest statement**

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