# Tanshinone IIA suppresses cholesterol accumulation in human macrophages: role of haem oxygenase-1

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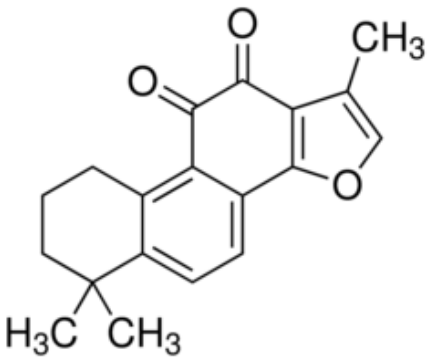
## Supplemental Methods

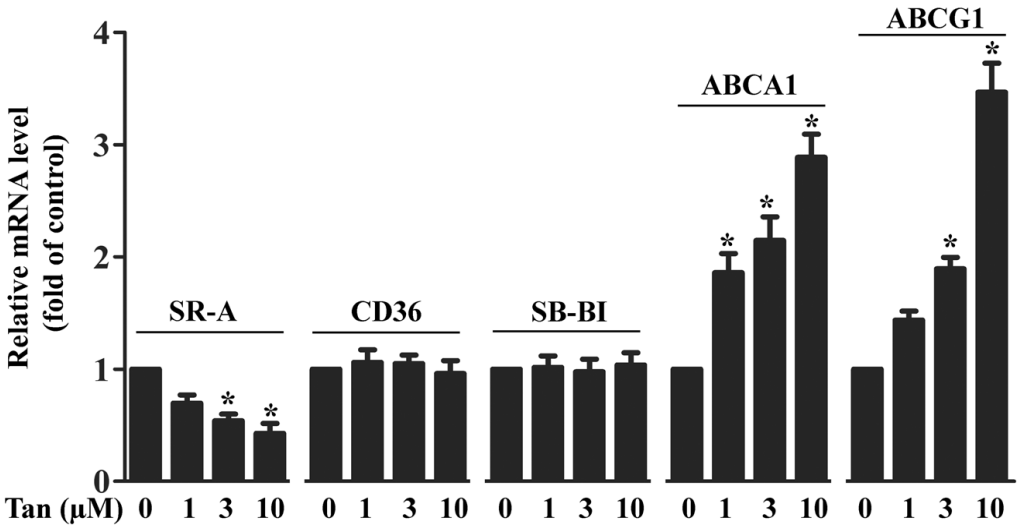
### *Assay for HO Enzyme Activity*

HO enzyme activity was measured by detecting the amount of bilirubin generated from cell lysates, as previously described (1). In brief, microsomes from harvested cells were added to a reaction mixture containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 IM hemin, and 100 mM potassium phosphate buffer, pH 7.4. Finally, 2 mg of rat liver cytosol was added as a source of biliverdin reductase. The reaction was allowed to proceed at 37 ℃ in dark for 1 h and terminated by the addition of 0.6 ml of chloroform. Extracted bilirubin was determined by calculating the difference in the absorbance at 464 and 530 nm. HO activity was expressed as picomoles of bilirubin formed per mg protein per hour.

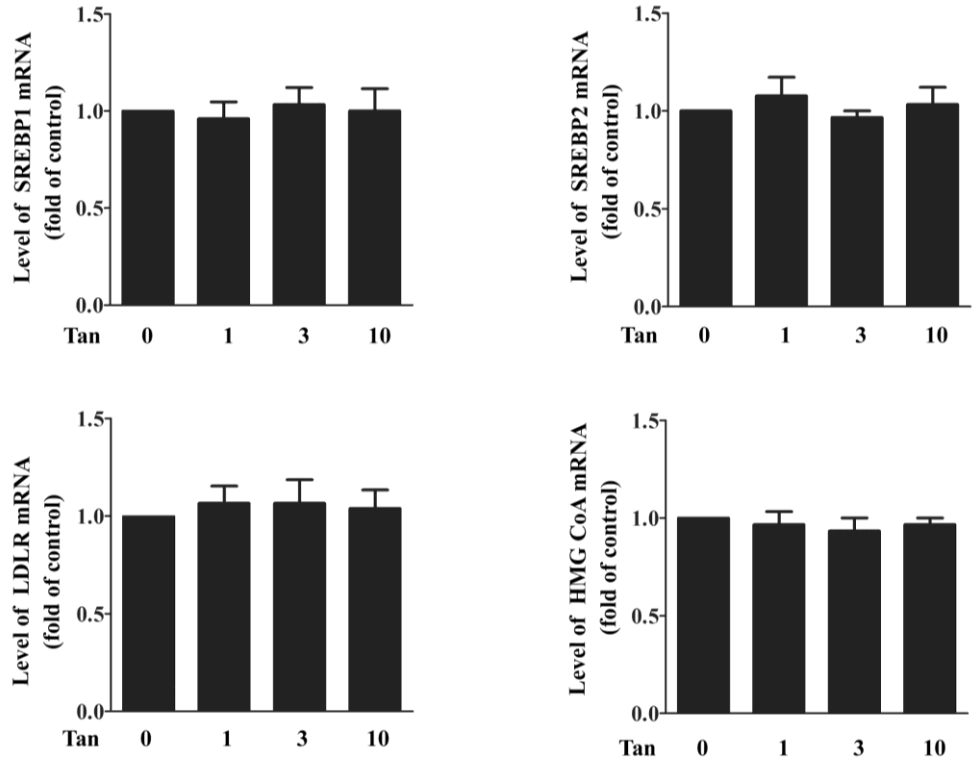
**Supplemental Figures and Tables**



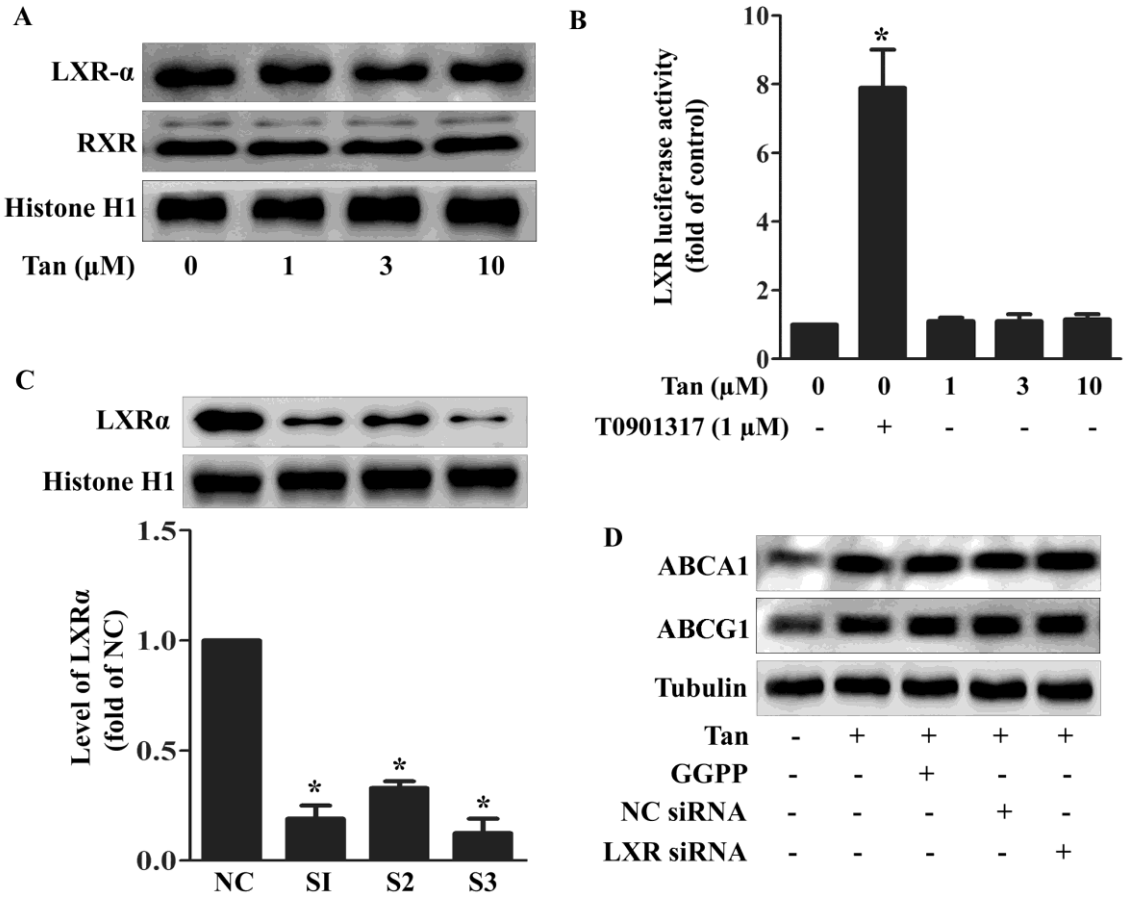

**Supplemental Figure I: The chemical structure of Tanshinone IIA.**




**Supplemental Figure II: Effects of Tan on mRNA expression of scavenger receptors and cholesterol transporters.** THP-1-derived macrophages were treated with indicated concentrations (1, 3, 10 μM) of Tan or vehicle (0.1% DMSO) for 24 h and subject to quantitative real-time PCR to determine the mRNA expression profile of SR-A, CD36, SR-BI, ABCA1, ABCG1, or GAPDH. **P*<0.05 vs. untreated group.

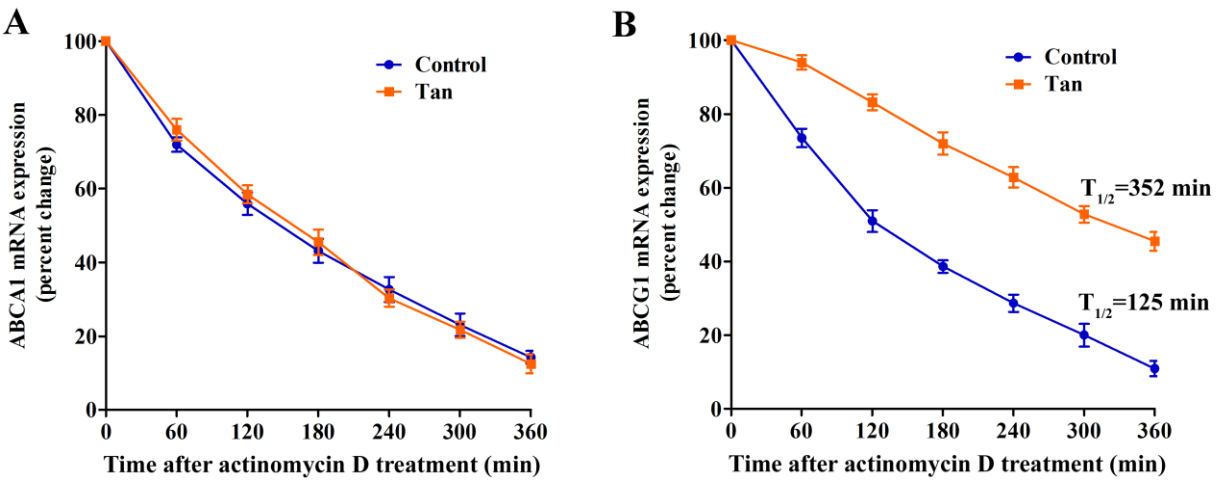



**Supplemental Figure III: Effects of Tan on lipid synthesis-related genes expression in THP-1 macrophages.** THP-1 macrophages were incubated with indicated concentrations of Tan (1, 3, 10 μM) for 24 h. Total RNA was extracted and subjected to the real-time quantitative PCR to determine gene expression levels of sterol regulatory element binding protein 1 (SREBP1), sterol regulatory element binding protein 2 (SREBP-2), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA) and low-density lipoprotein receptor (LDLR). The results are expressed as fold changes compared with the control from at least three independent assays.

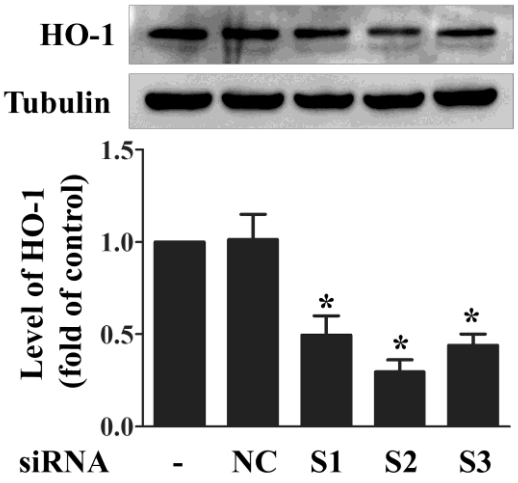



**Supplemental Figure IV: Tan did not alter LXR expression and transcriptional activity. A**: THP-1 macrophages were incubated with Tan (1, 3, 10 μM) for 12 h. Nuclear extracts were isolated and subjected to western blotting to determine the protein level of LXRα, RXR, and Histone H1. **B**: Macrophages were transfected with a luciferase reporter plasmid containing LXREs upstream of the thymidine kinase promoter (LXRE-tk-Luc) in the presence of β- galactosidase as a reference plasmid. 12 h after transfection, cells were treated with Tan (1, 3, 10 μM) or T0901317 (1 μM) for 24 h. Luciferase and β-galactosidase activities were then determined in the cell lysates. The values represent the means ± SEM which are expressed as luc/β-gal. **C**: Three independent siRNAs targeting LXRα (marked as S1, S2, and S3), as well as the negative control (NC), were transfected into THP-1 macrophages for 48 h. The protein

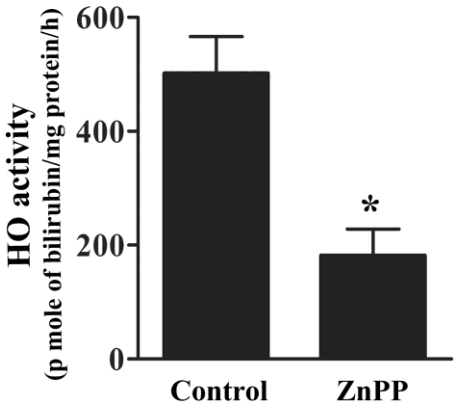
expression level of LXRα was measured by western blotting. α-tubulin was used as an internal control. *$P$<0.05 vs. control group. **D**: Macrophages were pretreated with LXRα inhibitor GGPP (20 μM) for 2 h, or transfected with LXRα specific siRNA (S3). After then, cell were incubated with Tan (10 μM) for 24 h and subjected to western blotting to determine protein expression of ABCA1 and ABCG1. α-tubulin was used as an internal control.



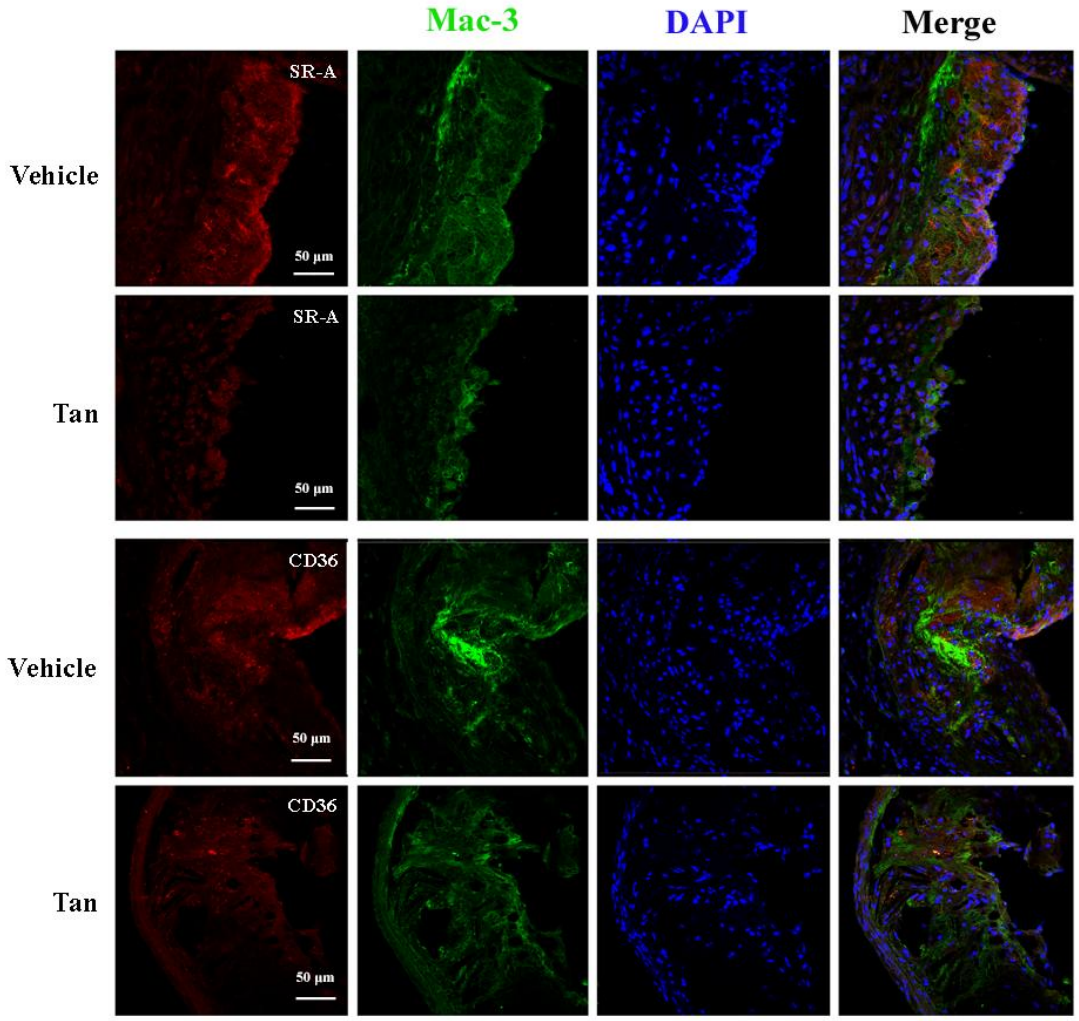

**Supplemental Figure V:** Effect of Tan on the regulation of ABCA1 and ABCG1 mRNA decay. Macrophages were incubated with Tan (10 μM) or vehicle (0.1% DMSO) for 24 h and actinomycin D (5 μg/ml) was added to the cells for different intervals. Total RNA was then isolated and subject to quantitative real-time PCR to determine the mRNA expression of ABCA1, ABCG1 and GAPDH. The normalized ABCA1 (**A**) and ABCG1 (**B**) mRNA signals were plotted as the percentage of the mRNA remaining. Decay curves were plotted versus time.



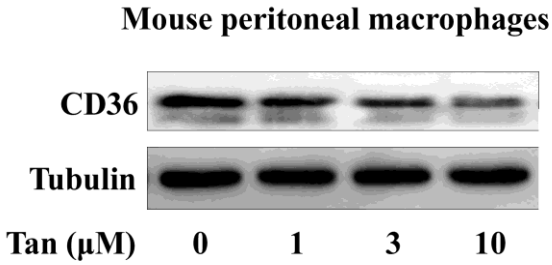

**Supplemental Figure VI: Silencing efficiency of different duplex siRNAs of HO-1.** Three independent siRNAs targeting HO-1(marked as S1, S2, and S3), as well as the negative control siRNA (NC), were transfected into THP-1 macrophages for 48 h. The protein expression level of HO-1 was measured by western blotting. α-tubulin was used as an internal control. **P*<0.05 vs. control group.




**Supplemental Figure VII: Effect of ZnPP on HO enzyme activity.** Three independent experiments were performed and data shown are means ± SEM obtained from triplicates of each experiment. **P*<0.05 vs. control group.




**Supplemental Figure VIII: Effect of Tan on the expression of SR-A and CD36 in murine atherosclerotic lesions.** SR-A and CD36 immunohistochemical staining (red) of aortic sinus from ApoE$^{-/-}$ mice treated with Tan or vehicle. Macrophages were stained with Mac3 (green) and nuclei were stained with DAPI (blue).




**Supplemental Figure IX: Effect of Tan on protein expression of CD36 in mouse peritoneal macrophages.** Mouse peritoneal macrophages were treated with indicated concentrations (1, 3, 10 μM) of Tan or vehicle (0.1% DMSO) for 24 h in the presence of oxLDL (50 μg/mL) and subject to western blotting to determine the protein level of CD36.

**Table I**

**Effect of Tan on body weight and serum lipid profile**

| | WT | ApoE$^{-/-}$ | ApoE$^{-/-}$+Tan |
|---|---|---|---|
| B.W. (g) | 26.9 ±2.6 | 32.8 ±2.3* | 33.3 ±1.9 |
| TC (mmol/L) | 2.8 ±0.6 | 25.8 ±4.2*** | 28.2 ±3.6 |
| TG (mmol/L) | 1.4 ±0.2 | 13.6 ±3.7*** | 12.3 ±2.6 |
| HDL (mmol/L) | 1.6 ±0.2 | 3.2 ±0.4** | 3.6 ±0.3 |
| LDL (mmol/L) | 0.3 ±0.3 | 11.3 ±2.5*** | 11.6 ±3.3 |

Data are represented as mean ± SEM. B.W., body weight; TC, total cholesterol; TG, triglycerides; LDL, low density lipoprotein-cholesterol; HDL, high density lipoprotein-cholesterol. *$P$<0.05, **$P$<0.01, and ***$P$<0.001 *vs* WT group ($n$=6-8 per group). No statistical significance was observed between the lipid profile and body weight of Tan versus ApoE$^{-/-}$ group (vehicle group).

No statistical significance was observed on the lipid profile and body weight in ApoE$^{-/-}$ mice treated with vehicle (ApoE$^{-/-}$) or Tan (ApoE$^{-/-}$+Tan).

**Supplemental References**

1. Yet, S.F., L.G. Melo, M.D. Layne, and M.A. Perrella. 2002. Heme oxygenase 1 in regulation of inflammation and oxidative damage. *Methods Enzymol.* **353**:163-176.