Supplemental Materials

Expanded granulocyte/monocyte compartment in myeloid-specific triple FoxO knockout increases oxidative stress and accelerates atherosclerosis in mice

Kyoichiro Tsuchiya, Marit Westerterp, Andrew J. Murphy, Vidya Subramanian,

Anthony W. Ferrante, Jr, Alan R. Tall, and Domenico Accili

ONLINE METHODS

Reagents. We purchased N-Acetyl-L-cysteine from Sigma. Sources of antibodies are: anti-p-InsR-β, phospho-FoxO1, FoxO3a, phospho-FoxO3a, FoxO4, Akt, p-Akt (Ser-473 and Thr-308), β-actin, and iNOS from Cell Signaling; anti-insulin-receptor, and α-tubulin from Santa Cruz Biotechnology; anti-nitrotyrosine, actin, and Irs1 from Millipore; anti-nitrosocysteine from Abcam.

Animal experiments. We measured body composition by NMR (Bruker Optics), blood glucose with a glucometer (One Touch Ultra, Lifescan), plasma insulin (Mercodia) by ELISA, triglyceride (Cayman chemical), cholesterol, and nonesterified fatty acids by colorimetric assays (Cholesterol E and NEFA C, Wako Pure Chemicals). We have described the procedure for intraperitoneal glucose (2g/kg^{-1}) and insulin (0.75U kg^{-1}) tolerance tests [1]. In some experiments WT and MYFKO mice are maintained with standard or high-fat (60% of calories from fat, Research Diets) diet for indicated periods.

Macrophage culture. We harvested peritoneal macrophages from mice by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycolate, and cultured them in DMEM supplemented with 10% fetal bovine serum and 20% L929 cell-conditioned medium. Bone marrow cells were collected from the femurs and tibias of mice, pooled, and differentiated in vitro by incubating for 7 days at 37°C in RPMI 1640 medium containing 10% FBS and 20% L929 cell-conditioned medium.

Blood analysis. Mouse peripheral blood was collected by tail vein puncture into heparin-coated tubes. Total RBC, WBC, and platelet counts were quantified using the FORCYTE Veterinary Analyzer (Oxford Science Inc.). The cells were subjected to RBC lysis and were stained using an antibody cocktail including CD45-APC-Cy7, CD115-APC, and Ly6C/G-PerCP-Cy5.5 (BD Pharmingen). Monocytes were identified as CD45^{+}CD115^{+} cells and further gated as Ly6C^{hi} or Ly6C^{lo} (Online Figure IA). For some experiments neutrophils and monocytes were identified as CD45^{+}CD11b^{+}Ly6G^{+} and CD45^{+}CD11b^{+}Ly6G^{-}, respectively. Multiparameter analyses were

performed using a LSR II flow cytometer (Becton Dickinson) with DiVa software. Data were analyzed using FlowJo software (Tree Star, Inc.).

Bone marrow analysis. Bone marrow (BM) analysis using flow cytometry was performed as described (Online Figure IB) [2-4]. We harvested BM from femurs and tibiae and lysed red cells, followed by resuspension in HBSS (0.2% BSA/0.5mM EDTA) and incubation with a cocktail of antibodies to lineage (lin) committed cells (B220, CD2, CD3e, CD4, CD8, CD11b, CD19, Gr-1, and TER-119; all FITC; eBioscience) and stem cell markers Sca1-PE-Cy7 and ckit-APC-Cy7. HSPCs were identified as $lin^{-}Sca1^{+}ckit^{+}$. Where further identification of hematopoietic progenitor cells was required, we used antibodies to CD16/CD32 (FcγRII/III) and CD34 to separate CMP ($lin^{-}Sca1^{-}ckit^{+}CD34^{int}FcγRII/III^{int}$), and GMP ($lin^{-}Sca1^{-}ckit^{+}CD34^{int}FcγRII/III^{hi}$).

Cell proliferation measurement. For cell cycle analysis in BM cells, we fixed and stained cells in 1μg/ml DAPI at 4°C for 30 minutes, followed by staining with lineage, stem and progenitor markers. We cultured thioglycollate-elicited peritoneal macrophages at $2.5x10^5$ cells/cm^2 and cultured them with DMEM supplemented with 10% fetal bovine serum and 20% L929 cell-conditioned medium. After 32h, we manually counted cell number and assessed their viability by MTT (Life Technologies). Staining of 5-bromo-2'-deoxyuridine (BrdU, Life Technologies) was performed using biotin-conjugated anti-BrdU antibody (Life Technologies) and streptavidin-Alexa Fluor 488 (Jackson Immunoresearch). We quantified percentage of BrdU-positive cells as the ratio of BrdU-positive to PI-positive cells.

Apoptosis detection. We loaded cultured peritoneal macrophages with free-cholesterol (58035, 10 mg/l and acetylated low-density lipoprotein, 100 mg/L) or 7-ketocholesterol (10μM) for 20h. We measured apoptosis by Alexa 488-labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen). For each condition, we randomly selected four separate fields, counted > 100 positive cells and plotted them as percentage of total cells.

Atherosclerotic Lesion Analysis. We pinned aortae on silicon dishes and performed Oil Red-O staining, using Image J software to quantify lipid-laden areas as a percentage of total area.

We isolated and fixed hearts in phosphate-buffered formalin then dehydrated and embedded them in paraffin.

Macrophage adhesion assay. Peritoneal macrophages were subjected to RBC lysis and were labeled with APC-anti-CD11b antibody (BD) for 30min. Then cells were washed and seeded ($5x10^5$ cells/cm^2) onto confluent MS1 cells stimulated with 20μg/ml LPS for 6 h. After 20 min, we washed non-adherent macrophages 3 times with PBS. We quantified adherent macrophages per field by fluorescent intensity and analyzed the data using ImageJ.

NO production. We loaded peritoneal macrophages with DAF-2 DA (0.5μM, invitrogen) for 30 min at 37 °C, washed 3 times with phenol red-free DMEM and incubated them in the dark. We used fluorescent microscopy to score cells, followed by ImageJ analysis. We assayed nitrate and nitrite levels in conditioned media of cell culture using Greiss assay kit (Enzo). Nitrate and nitrite levels are normalized by protein concentration of cell extracts.

Reactive oxygen species (ROS)/peroxynitrite, and superoxide production. We assessed intracellular ROS/peroxynitrite and superoxide using fluorescent dye 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA, Invitrogen) and dihydroethidium (DHE, Invitrogen), respectively. We treated serum- and growth supplement-starved cultured macrophages with or without LPS for 30 min, washed twice with Krebs-Ringer buffer and loaded them with CM-H2DCFDA (5 μM) or DHE (5 μM) for 20 min at 37°C. We visualized cells and quantified fluorescence using ImageJ software.

Hepatic lipid content. We measured liver lipid content by tissue saponification in ethanolic KOH after neutralization with MgCl2 [5], and hepatic triglyceride and cholesterol content by colorimetric assay (Wako Pure Chemicals).

mRNA analysis. We extracted RNA using TRIzol (Invitrogen), synthesized cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and performed quantitative RT-PCR using GoTaq SYBR Green qPCR Kit (Promega) in a Chromo4 Real-Time PCR Detection System (Bio-Rad). For analysis of GMP, we sorted cells using FACSAria (BD), collected them

into 0.35 ml of buffer-RLT and stored them at −80°C. RNA extraction and DNase treatment were performed with the RNeasy Micro kit (Qiagen Inc., California) according to manufacturers' instructions. Eluted RNA samples were reverse transcribed using SuperScript III and random hexamers (Invitrogen) according to protocol supplied by the manufacturer. Primer sequences are available on request.

Protein analysis. We lysed tissues or cells in buffer containing 2% SDS, 50mM Tris-HCl, and 5mM EDTA. For immunoprecipitation, we lysed liver or cells in RIPA buffer with protease/phosphatase inhibitors. We immunoprecipitated lysates with anti-InsR-β antibody, carried out immunoblotting and visualized the signal with ECL (GE Lifescience).

Statistical analysis. We show data as mean ± SE. We used the customary threshold $P < 0.05$ to declare statistically significant differences using unpaired t-test or ANOVA with Dunn's post hoc test.

Online Table I Serum chemistry panel of WTD-fed mice

	Ldlr$^{-/-}$ (n=11)	*Ldlr*$^{-/-}$: MYFKO (n=13)
ALP (U/l)	184 ± 28	218 ± 21
γ-GTP (U/l)	2.4 ± 0.9	4.1 ± 1.7
AST (U/l)	64 ± 18	78 ± 18
ALT (U/l)	59 ± 35	74 ± 26
Amylase (U/l)	1280 ± 118	1407 ± 105
BUN (mg/dl)	11 ± 0.9	10 ± 0.5
Albumin (g/dl)	2.4 ± 0.2	2.6 ± 0.2
UA (mg/dl)	2.5 ± 0.3	2.6 ± 0.5
TP (g/dl)	4.1 ± 0.1	4.2 ± 0.1
Globulin (g/dl)	2.1 ± 0.1	2.0 ± 0.2

Data were obtained after a 16-hr fast in 20-week-old mice fed WTD for 14 weeks. None of the differences is statistically significant.

ONLINE FIGURE LEGENDS

Online Figure I. Multiparameter flow cytometric analysis

(A) After selection of live blood cells and exclusion of doublets, leukocytes were gated as $CD45^+$ cells. Monocytes (Mo) were gated as $CD45^+CD115^+$, and neutrophils (Neu) as $CD45^+CD115^-$ $Ly6C^{hi}$. Monocytes consist of distinct $CD45^+CD115^+Ly6C^{lo}$ and $CD45^+CD115^+Ly6C^{hi}$ populations that correspond to $Ly6C^{hi}$ (hi) and $Ly6C^{lo}$ (lo) monocyte subsets, respectively. (B) After selection of live BM cells and exclusion of doublets, cells were gated for $Lineages^-$ (Lin^-). In Lin^- cells, HSPC were gated as $Sca\text{-}1^+c\text{-}Kit^+$ (gate 7). $Sca\text{-}1^-c\text{-}Kit^+$ progenitor (Pro) cells were further divided into GMP ($lin^-Sca1^-ckit^+CD34^{int}FcγRII/III^{hi}$) and CMP ($lin^-Sca1^-ckit^+CD34^{int}FcγRII/III^{int}$).

Online Figure II. FoxO expression in macrophages

FoxO mRNA and/or protein expression in (A and B) peritoneal and (C and D) bone marrow macrophages from WT and MYFKO mice (n=4). ** $p < 0.01$, *** $p < 0.001$ vs. WT.

Online Figure III. Peripheral blood analysis of single *Foxo1* knockouts and MYFKO mice

(A) WBC number, (B) percentage and (C) absolute number of $CD45^+CD11b^+Ly6G^-$ (neutrophils) and $CD45^+CD11b^+Ly6G^+$ (monocytes) cells in 6-weeks-old *LysM*-Cre: $FoxO1^{f/f}$ mice (n=4-5). (D) RBC, WBC and Plt number, and (E) percentage and (F) absolute number of neutrophils (Neu), lymphocytes (Ly), monocytes (Mo), eosinophils (Eo), and basophils (Ba) in 6-week-old WT and MYFKO mice with $Ldlr^{+/+}$ background (n=6-8). (G) Gene expression in peritoneal macrophages (n=4). (H) Representative pictures and (I) quantification of adherent CD11b-labelled peritoneal macrophages to MS1 cells (n=4). * $p < 0.05$, * $p < 0.05$, *** $p < 0.001$ vs. WT.

Online Figure IV. FoxO or cell cycle-related genes in HSPC, CMP, and GMP

Relative populations of cells in G1, S, and G2/M phase of (A) HSPC and (B) CMP (n=6). *Foxo* expression in (C) HSPC, (D) CMP, and (E) GMP sorted from BM of 6-week-old $Ldlr^{-/-}$ and $Ldlr^{-/-}$

: MYFKO mice (n=7-8). (F) Expression of cell cycle-related genes in GMP sorted from BM of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$: MYFKO mice (n=7-8). * $p < 0.05$ vs. *Ldlr*$^{-/-}$.

Online Figure V. Gene expression in aorta, caspase-3 immunostaining in aortic root , M1/M2 markers, and NF-κB signaling.

(A) Gene expression in whole aortae of mice after 14 weeks on WTD (n=4-6). (B) Representative pictures and (C) quantification of aortic root with active caspase-3 (n=6-7). (D) M1/M2 markers in peritoneal macrophages from WT and MYFKO mice (n=4). (E) LPS-induced p105 and p65 phosphorylation in peritoneal macrophages from WT and MYFKO mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *Ldlr*$^{-/-}$ or *WT*.

Online Figure VI. Insulin signaling in cultured macrophages

(A) Immunoblot of insulin signaling proteins in cultured peritoneal macrophages from WT and MYFKO mice. (B) Immunoblots of insulin signaling proteins in cultured peritoneal macrophages pretreated with insulin (10nM) or vehicle for 24 h prior to 3-hr incubation in serum-free medium followed by the addition of insulin for the indicated times.

Online Figure VII. Metabolic characterization of SD- or HFD-fed MYFKO mice

(A and B) Body weight (n=10-14) and (C and D) intraperitoneal glucose tolerance tests (n=6-10) after 18-hr fast in 12- or 16-week-old WT or MYFKO mice fed standard (SD) or high-fat diet (HFD), respectively. (E and F) Glucose and (G and H) insulin levels in 16- or 18-week-old mice fed SD or HFD fasted for 16 h (0h, Fasted), or fasted for 16 h and refed for 4 h (Refed) (n=6-8).

Online Figure VIII. Serum lipid and liver insulin signaling in WTD-fed *Ldlr*$^{-/-}$: MYFKO mice

Serum (A) TC, (B) TG, and (C) NEFA levels in 20-week-old mice fed WTD and fasted for 16 hr (n=6-9). Quantification of insulin-stimulated (D) p-InsRβ, (E) p-Akt (S473), and (F) p-FoxO1

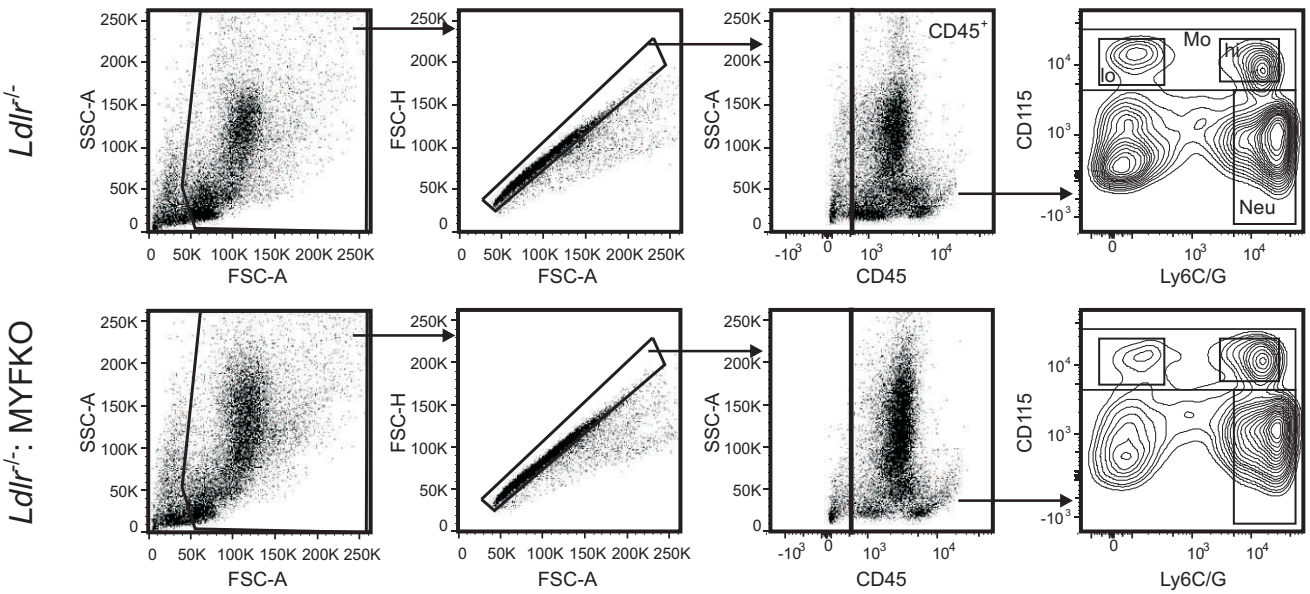
(S256) levels in liver of 14-week-old *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$: MYFKO mice fed WTD for 8 weeks. After a 16-hr fast, mice were injected with insulin or PBS and livers were collected 3 min later (n=3). * $p < 0.05$ vs. *Ldlr*$^{-/-}$.

REFERENCES

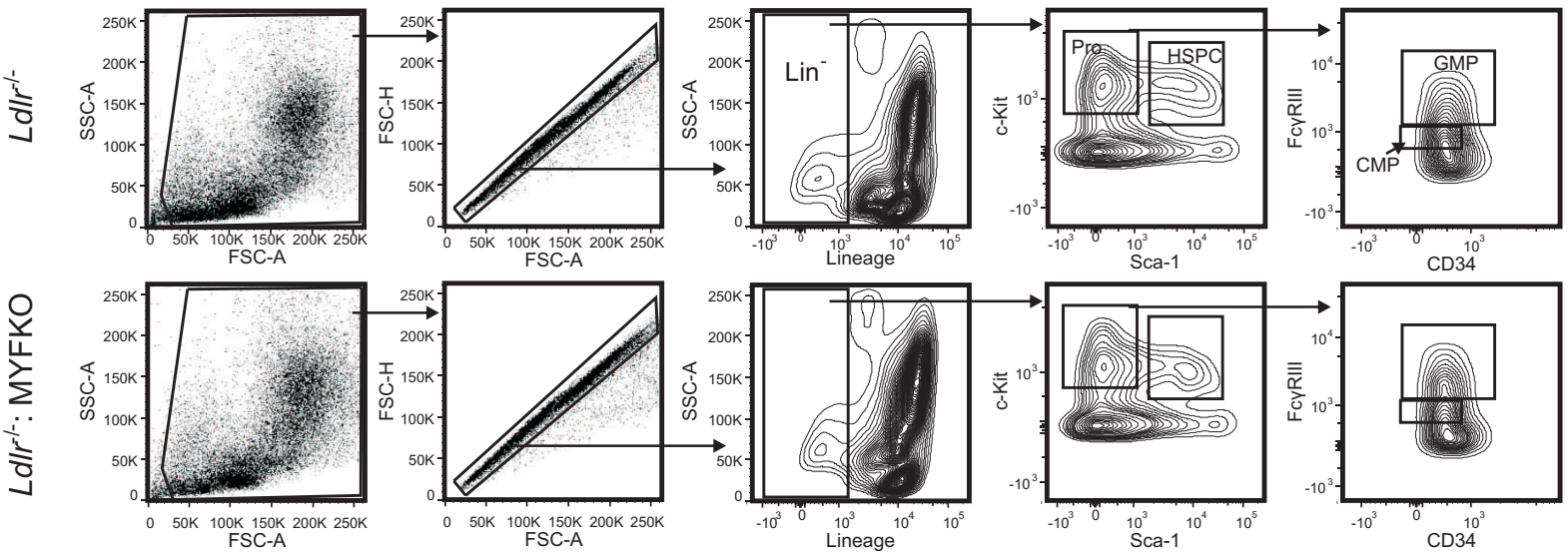
1. Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor foxo1 (fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest*. 2001;108:1359-1367
2. Kuo CL, Murphy AJ, Sayers S, Li R, Yvan-Charvet L, Davis JZ, Krishnamurthy J, Liu Y, Puig O, Sharpless NE, Tall AR, Welch CL. Cdkn2a is an atherosclerosis modifier locus that regulates monocyte/macrophage proliferation. *Arterioscler Thromb Vasc Biol*. 2011;31:2483-2492
3. Murphy AJ, Akhtari M, Tolani S, Pagler T, Bijl N, Kuo CL, Wang M, Sanson M, Abramowicz S, Welch C, Bochem AE, Kuivenhoven JA, Yvan-Charvet L, Tall AR. Apoe regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. *J Clin Invest*. 2011;121:4138-4149
4. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404:193-197
5. Norris AW, Chen L, Fisher SJ, Szanto I, Ristow M, Jozsi AC, Hirshman MF, Rosen ED, Goodyear LJ, Gonzalez FJ, Spiegelman BM, Kahn CR. Muscle-specific ppargamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *J Clin Invest*. 2003;112:608-618

Online Figure I

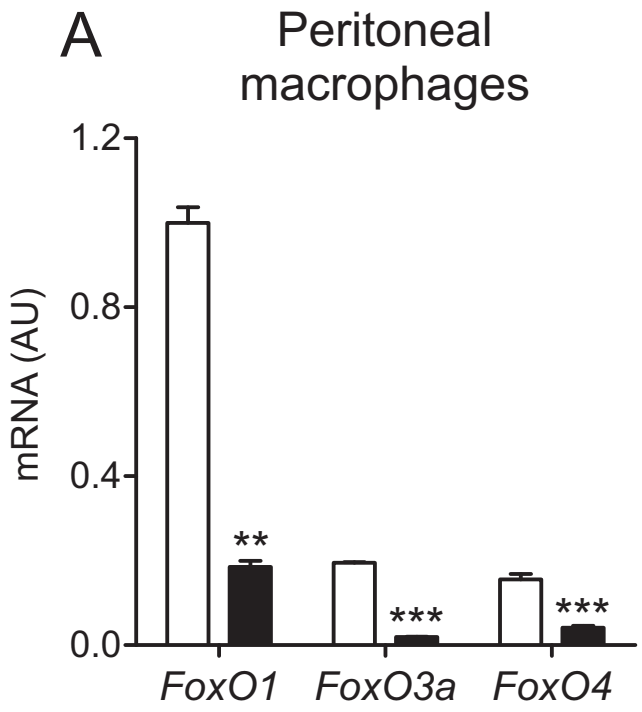
A

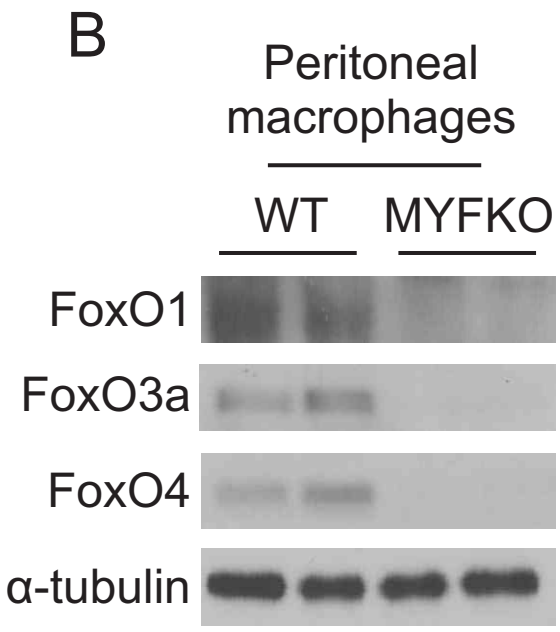


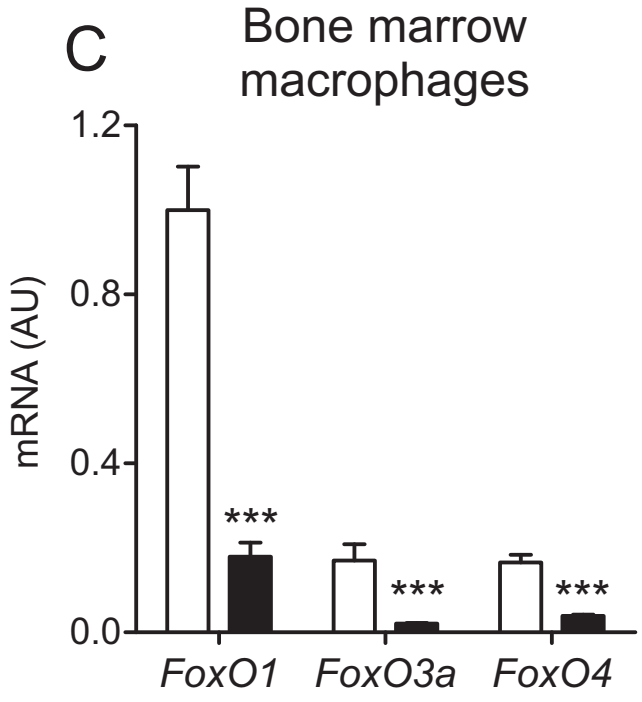
B

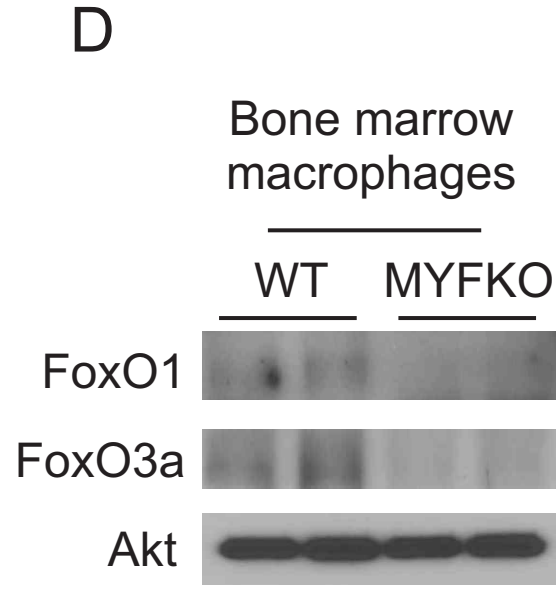


Online Figure II

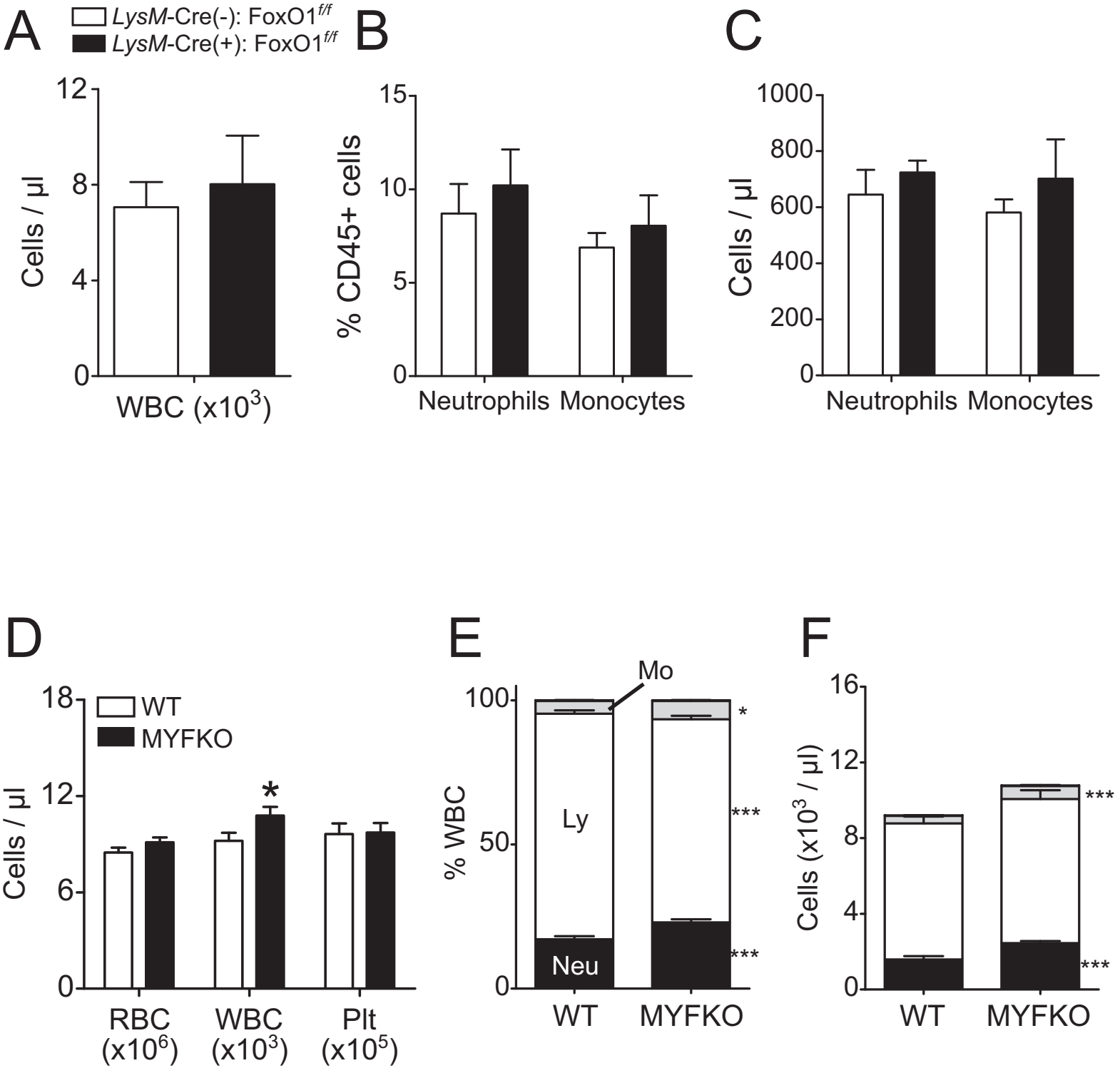




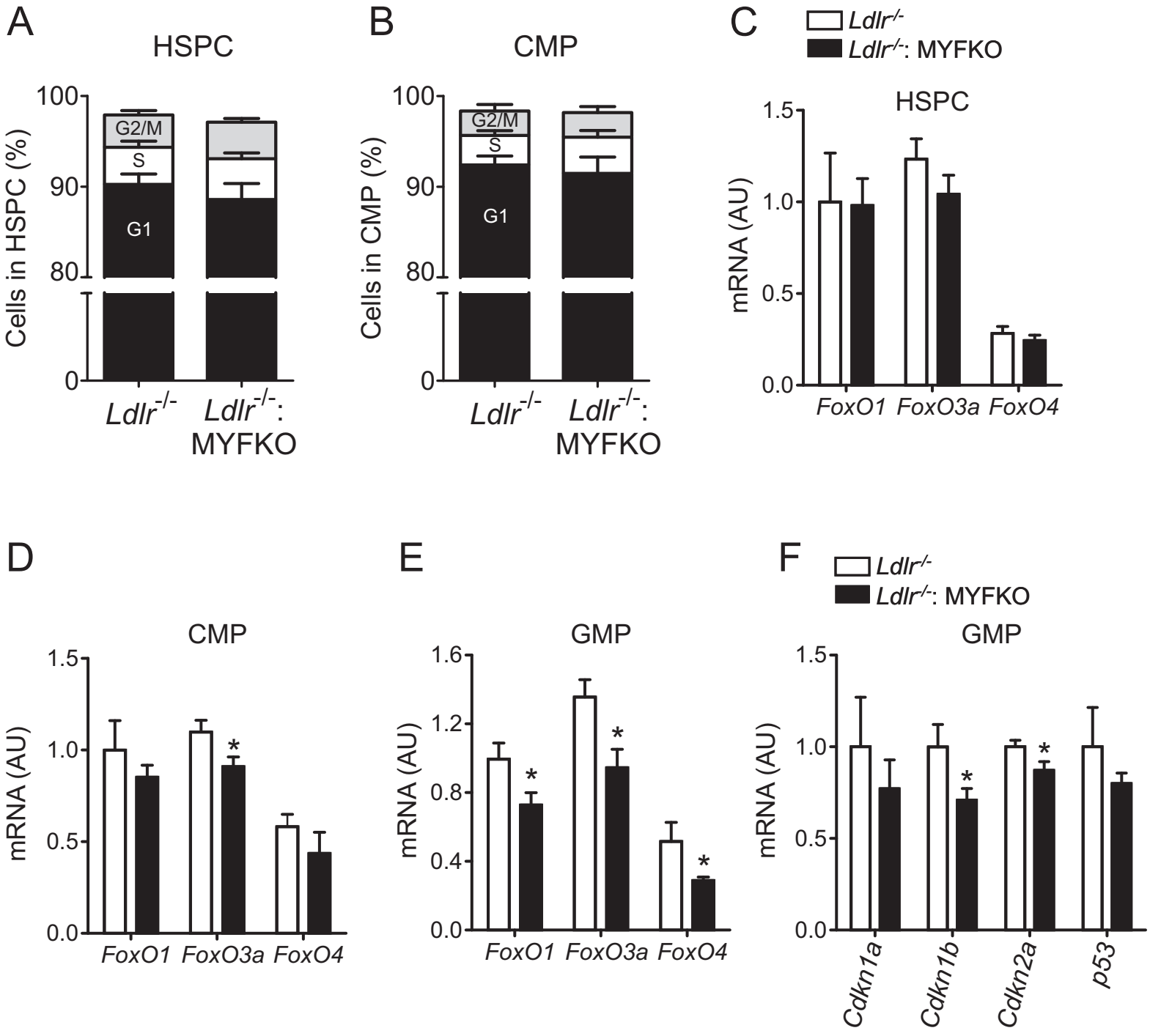




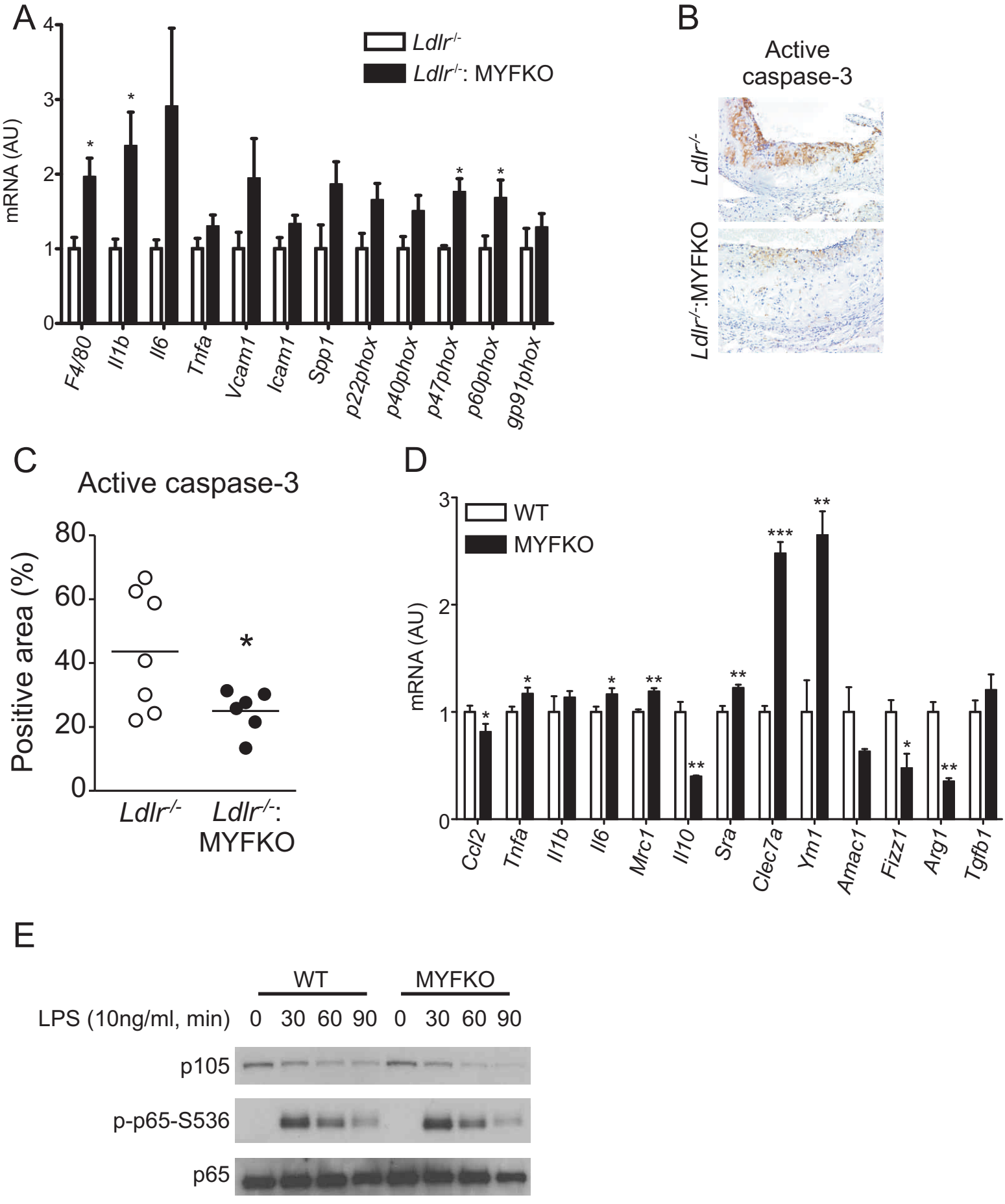
Online Figure III



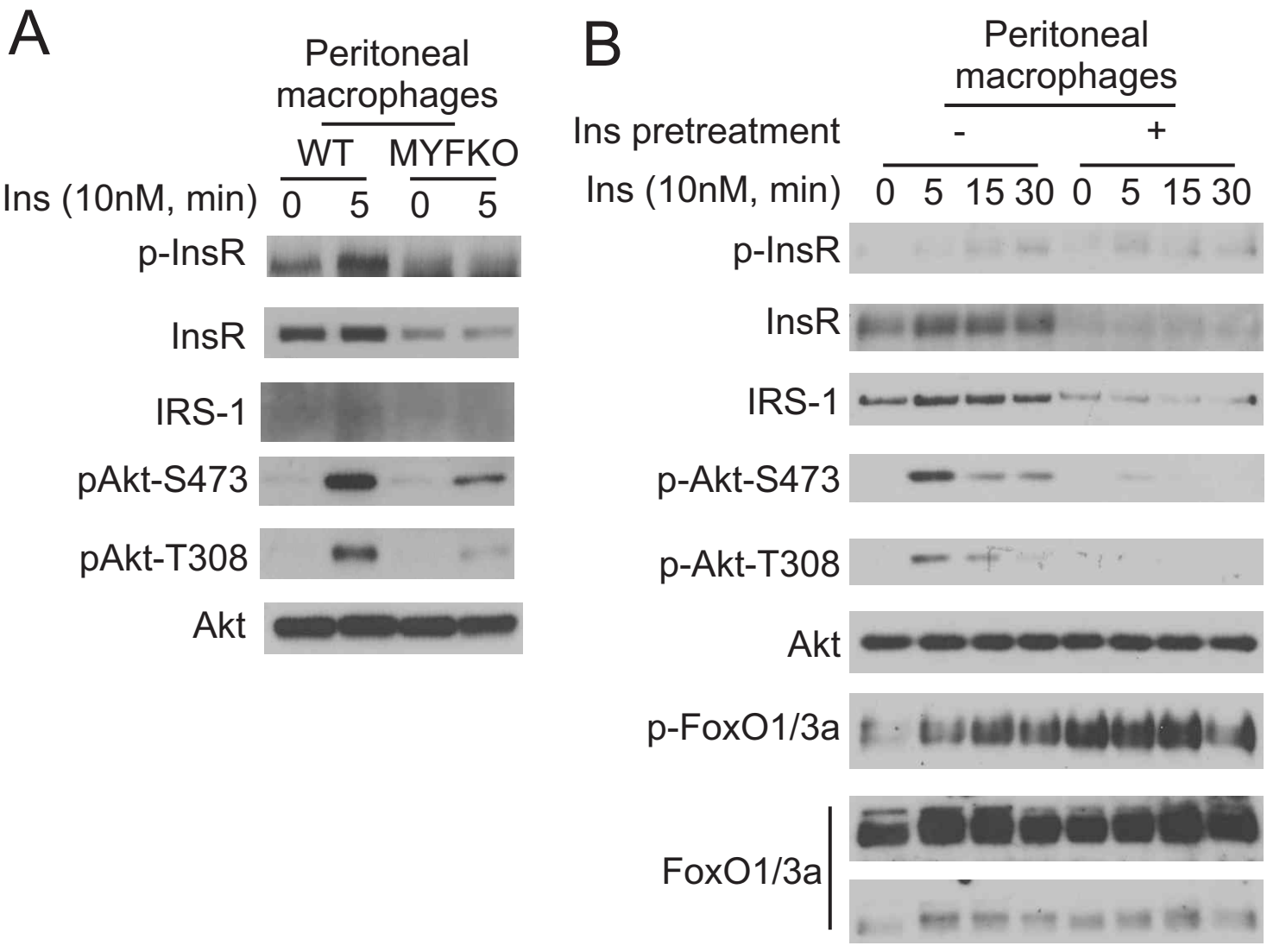
Online Figure IV



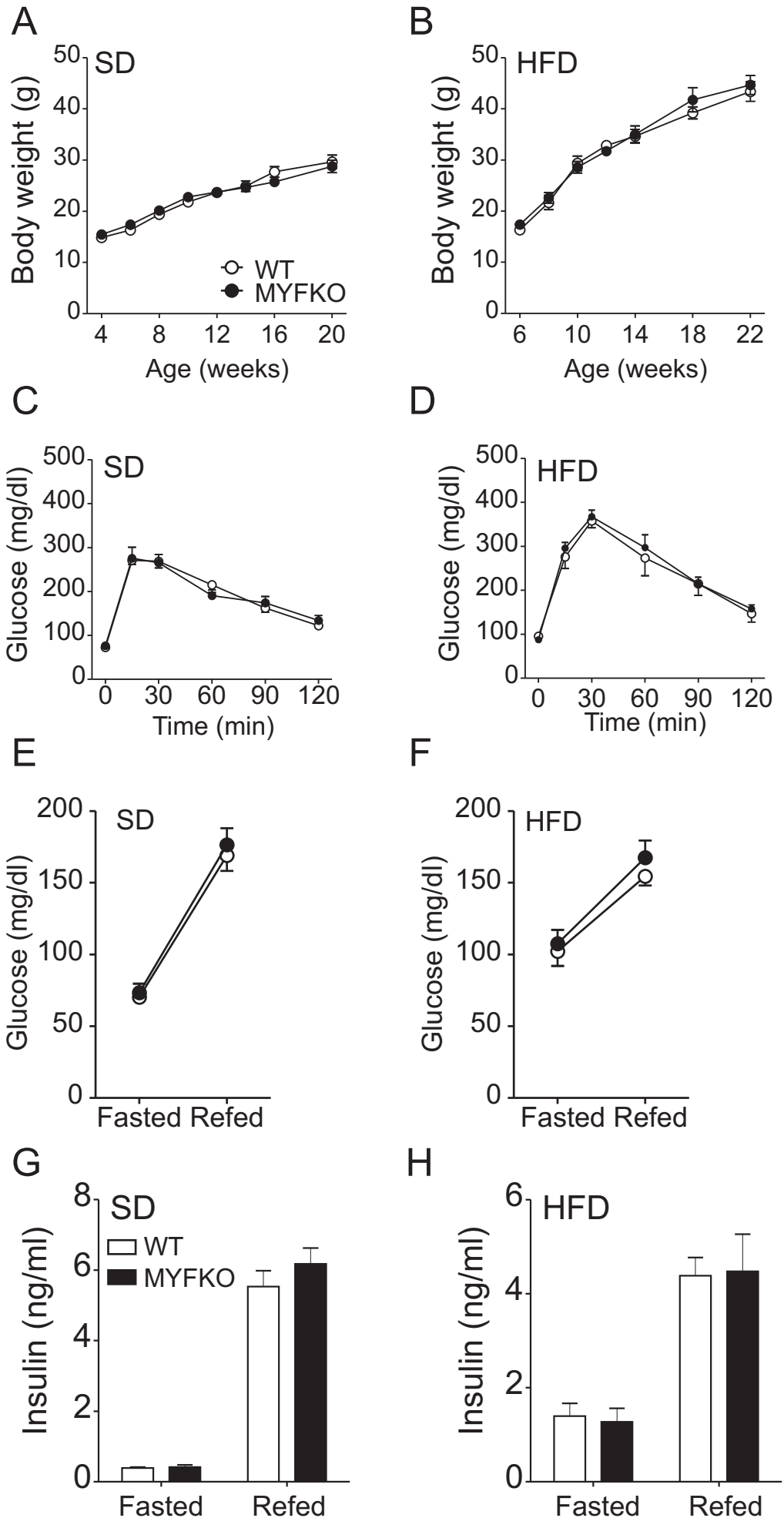
Online Figure V



Online Figure VI



Online Figure VII



Online Figure VIII