SUPPLEMENTAL MATERIALS
Chang et al.

METHODS AND MATERIALS

Animals and feeding protocols

β-Actin-driven tamoxifen-inducible-Cre (Mer/Cre/Mer) transgenic mice on a C57BL/6 background obtained from Jackson Laboratory were crossed with floxed LpL mice to obtain the β-actin-MerCreMer/LpLflox/flox offspring designated as TmLpL0 mice as described previously [1]. After confirming genotype by tail DNA, the TmLpL0 mice were given an intraperitoneal injection of 1 mg of 4-hydroxytamoxifen (Sigma) in peanut oil for 5 consecutive days. Plasma TG was determined 2 weeks after the last tamoxifen injection. LpL-knockout mice expressing LpL exclusively in muscle (MCKL0) were generated by breeding transgenic mice that carried the muscle-specific creatine kinase (MCK) promoter driving a human LpL minigene onto the LpL knockout background. All mice were on C57BL/6 background as previously described [2, 3]. For bone marrow (BM) LpL reconstitution, MCKL0 mice received BM transplants from wild type (Wt) C57BL/6 (Wt→MCKL0) or MCKL0 (MCKL0→MCKL0) mice following previously described protocols [2, 4]. In brief, recipient mice were given 100 mg/l neomycin (Sigma-Aldrich) 2 weeks before and after BM transplantation. Eight-week-old male mice were lethally irradiated with 13 Gy from cesium in two doses separated by 4 h. BM was collected from femurs and tibias of donor mice by flushing with sterile medium (RPMI 1640, 2% FBS, 5 U/ml heparin, 50 U/ml penicillin, 50 μg/ml streptomycin). Each recipient mouse was injected with ~ $3–5 \times 10^6$ BM cells. Four weeks after BM transplantation, peripheral blood was collected from the tail vein to verify donor BM reconstitution via PCR. Once reconstitution was confirmed, recipient mice were placed on a lard-rich high-fat diet (34.9% fat w/w, 0.28g cholesterol per kg of diet, Research Diets, Inc., D12492) for 8 weeks. Feeding of this diet in MCKL0 mice has been well-characterized and described in detail previously [2]. Both BM donor and recipient mice were male.

Mouse complete white blood cell counts with differentials

Mouse white blood cells (WBCs) were examined using the FORCYTE Veterinary Analyzer (Oxford Science Inc.) for total WBC and WBC subsets, including lymphocytes, neutrophils, monocytes basophiles and eosinophils. Blood was collected via tail vein from young adult mice at age of 8 to 16 weeks.

Blood leukocyte and hematopoietic stem cell subsets

MCKL0 and Wt mouse blood leukocytes were characterized for total monocytes and monocyte subsets by flow cytometry as previously described [5]. In brief, mouse blood was obtained via tail bleeding and collected into EDTA-containing tubes on ice. Red blood cells (RBCs) were lysed (lysis buffer, BD Pharmigen), and WBC were pelleted by centrifugation and resuspended in FACS buffer (HBSS containing 0.2% BSA w/v, 1 mM EDTA). Cells then were washed twice and stained with fluorochrome-conjugated antibodies against monocytes (APC Cy7-CD45^{hi}/ APC-CD115^{hi}, BD Pharmigen/eBioscience, respectively) and monocyte subsets (PerCP Cy5.5-Ly6C/G(Gr-1)^{hi} or Gr-1^{lo}, eBioscience). Neutrophils were identified as CD45^{hi}CD115^{lo}Ly6C/G (Gr-1)^{hi}.

Hematopoietic stem and progenitor cells (HSPC) were collected from BM by fluorescence-activated cell sorting (FACS) as previously described [5, 6]. BM harvested from femurs and tibias were subjected to a brief RBC lysis. BM was resuspended in FACS buffer and incubated in the dark on ice with a cocktail of antibodies specific for lineage-committed cells (lin, CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, and Ly-6G; all FITC; eBioscience) and stem cell markers Sca1 (APC, eBioscience) and ckit (APC Cy7, Biolegend). HSPC identified as lin$^-$Sca1$^+$ckit$^+$. CD16/CD32 (PE Cy7-

FcγRII/III, Biolegend) and CD34 (PE, Biolegned) were used to separate common myeloid progenitors (CMP, $lin^{-}Sca1^{-}ckit^{+}CD34^{int}Fc\gamma RII/III^{int}$) and granulocyte-macrophage progenitors (GMP, $lin^{-}Sca1^{-}ckit^{+}CD34^{int}Fc\gamma RII/III^{hi}$).

Cells were run on LSRII for flow analysis or FACSAria for FACS sorting, both running FACSDiva software (BD Bioscicence). The flow cytometry data were analyzed using FlowJo software (Tree Star Inc).

BM-derived monocyte differentiation

BM monocyte/macrophage progenitor cells (BMMP) were isolated and differentiated as previously described [7, 8]. Isolated BM cells were resuspended in PBS supplemented with 0.2% BSA and centrifuged (500 *g*, 10 min) to pellet BMMP. Isolated BMMP were placed on ice and counted using a hemocytometer. Cell survival rates were > 90%. 4 x 10^6 cells were resuspended in 10 ml BMMP differentiation media (Dulbecco's modified eagle medium containing 10% fetal bovine serum, 1% penicillin/streptomycin/L-glutamine, v/v, 10 ng/ml recombinant mouse M-CSF, w/v), plated onto non-tissue culture treated dishes and then incubated at 37ºC with 5% CO_2. Four days after seeding the cells, an additional 5 ml of fresh BMMP differentiation medium was added to each plate and incubated for another 3 days. To quantitate matured macrophages differentiated from BMMP, cells were lysed and centrifuged at 500 *g* for 5 min, and the pellet was resuspended in FACS buffer for flow cytometric analyses. Cells were incubated in the dark at 4°C for 30 min in FcBlock (BD Pharmingen), then for an additional 60 min with a cocktail of fluorochrome-conjugated primary antibodies including APC-CD115, APC Cy7-CD45, FITC-CD11b (BD Pharmagen) and PE-F4/80 (eBioscience). Mature macrophages were identified as $CD11b^{+}F4/80^{+}$. Cells were counted on a LSRII and analyzed using FlowJo software as described above.

Quantitative real-time RT-PCR for arterial inflammatory cells and BM HSPC

Total RNA was isolated from the proximal portion of the aorta with TRIzol reagent (Invitrogen). HSPC were isolated via fluorescent-activated cell sorting (FACS) directly into RLT lysis buffer (Qiagen). RNA from HSPC was extracted using a RNeasy Micro Kit (Qiagen). Single strand cDNA was synthesized from RNA using iScript reverse transcriptase according to the manufacturer's instructions (Bio-Rad). Quantitative real-time PCR was carried out on an iCycler (Bio-Rad) using the SYBR Green PCR kit (Applied Biosystems). Results were analyzed by comparing the threshold crossing (*Ct*) of each sample after normalization to control genes (ΔCt). Changes in the threshold crossing (ΔCt) were used to calculate relative levels of each mRNA using the formula $2^{-\Delta Ct}$.

Cell TG uptake and deposition

To study cell TG metabolism in BM-derived macrophages, a TG-rich emulsion (Intralipid) was radiolabeled with nondegradable 1α,2α (n)-[^{3}H] cholesteryl oleoyl ether to trace TG uptake and deposition as previously described [9, 10]. TG mass approximately equals TG deposition since basal cellular TG levels are low. We had demonstrated that cellular TG content was constant before all lipid emulsion experiments and therefore, it can be excluded from the equation: cellular TG utilization=(cell TG uptake + cell TG synthesis) – cell TG deposition. Cellular TG synthesis is negligible during the 4 h-incubation (i.e., less than 1% of TG uptake), as previously measured by our laboratory. Cellular utilization is thus defined as the ratio of TG uptake/TG mass [9-11].

M-CSF immunobinding assay

The immunoplates were coated with mouse recombinant M-CSF protein (mr-M-CSF-R, ~ 1μg/ml) for 18 h in coating buffer, followed by blocking and washing. Biotinylated M-CSF-R (0.5 to 1 μg /ml) was added to the coated plate for 2 h to examine M-CSF/ M-CSF-R interaction by HRP-conjugated streptavidin attached with the addition of TMB substrate. LpL or heparin was added to the 2 h incubation with M-CSF/M-CSF-R. Absorbance of TMB was analyzed using a plate reader [12].

Heparin-mediated cell-dissociation of LpL

To study the relation of LpL and aortic cell populations in response to heparin and dietary saturated fatty acids (FA), C57BL/6 mice purchased from the Jackson Laboratory were fed custom-made isocaloric diets manufactured by Harlan Teklad; a low-fat chow diet or high-fat, high-cholesterol diet enriched in either saturated fats (coconut oil) for 12 weeks as previously detailed [13, 14]. The high-fat diets contained 185 g fat and 2 g cholesterol per kg of diet and varied only in lipid and fatty acid composition. The low-fat chow diet contained 50 g fat and 0.2 g cholesterol per kg of diet.

At the end of feeding, mice were injected with heparin (100 IU/kg body weight) 10 min before sacrifice [15]. Because intravenous administration of heparin is known to increase LpL concentration in the circulation, plasma lipid profiles of these mice were examined to validate the heparin-mediated LpL release into blood. Fasting mouse plasma was collected and measured for levels of free FA (FFA) (NEFA C kit, Wako), TG, and total cholesterol (Chol) (Roche Diagnostics) according to the manufacturer's procedures as previously detailed [16] at the end of the 12-wk feeding period. Aortas were collected and assayed for LpL localization as previously described [13].

Immunofluorescent studies

The localization of arterial LpL with aortic cells was examined by immunofluorescence (IFC) similar to the process described previously [13, 17]. At the end of the feeding period, mice were euthanized and perfused extensively. Proximal aortas were dissected and fixed in 4% paraformaldehyde on ice for 2 h followed by the equilibration in 30% sucrose solution at 4°C overnight. The equilibrated samples were embedded in Tissue-Tek OCT (Sakura Finetek) and were first frozen on dry ice and then stored in a -80°C freezer. Embedded samples were sectioned with a cryomicrotome. Sections of proximal aorta were co-incubated with primary chicken anti-LpL antibody from Dr. Olivecrona's laboratory (Umeå University) /or goat anti-LpL (1:400~500, kindly provided by Dr. Andre Bensadoun, Cornell University), rat anti-EC (1:200, CD31, BD Pharmagen) and rabbit anti-macrophage (1:300~400, CD68, Santa Cruz) antibodies for 24 h at 4°C, followed by a 1-h incubation of the mixture of corresponding secondary antibodies, Alexa 647 anti-chicken /or goat IgG, Alexa 488 anti-rat IgG, and Alexa 546 anti-rabbit IgG. Immunofluorescence in the arterial wall was analyzed using a laser scanning confocal microscope (LSM-510 META or Nikon A1R MP). An argon laser (488 nm) and two helium-neon lasers (543 nm and 633 nm) were utilized for the excitation of Alexa 488, 546, and 647, respectively. Fluorescence of LpL and aortic cells was quantified as previously detailed [13, 17, 18]. To measure arterial immunostained macrophages in each optical section, regions of interest were defined based primarily on the location of EC staining, which highlights arterial structure. All macrophage markers within each region of interest were semiautomatically counted using computer software (ImageJ). Each optical section was adjusted for proper threshold and background subtractions for separating the correct signal from background. Particle sizes within 90 to 255 Gy were counted using a function (Analyze Particles) of the computer software (ImageJ). Each group consisted of 3 to 4 mice. For each artery sample, 10 to 20 sections were prepared and analyzed. Possible clusters of macrophage signals are also determined by the same function (Analyze Particles) because it provides options for segmentation when studying the circularity and size of particles.

Statistical analyses

Values were expressed as mean±SE. Statistical differences between group means were assessed by Student's *t*-tests to compare endpoints or ANOVA with Bonferroni's t-tests to evaluate potential interactions between groups. The variances are considered equal determined by F-tests or Brown-Forsythe tests. All statistical tests assumed a 95% level of confidence of a normal distribution (Shapiro-Wilk normality tests). Statistical significance was determined at the level of $p<0.05$. Statistical analyses were performed using Excel or GraphPad Prism 6.0.

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