Supplement Material.

Materials and Methods

Animal models

Male CD11c$^{-/-1}$ and C57BL/6 wild-type (WT) mice were used. Obesity was induced by high-fat diet (HFD; 21% w/w fat [41% of kcal from fat]; Dyets Inc., Bethlehem, PA), with mice fed normal diet (ND; 4.5% w/w fat [12% of kcal from fat], PicoLab Rodent Chow 5053) used as lean controls.[2] Some of the obese mice underwent weight reduction by switching from HFD to ND and being fed ND ad libitum for an additional 4 weeks. All animal studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, and all experimental procedures were in accordance with institutional guidelines.

Human studies

Human visceral adipose tissue (VAT; perigastric omentum) was collected from 21 morbidly obese patients (1 male, 20 females) at the time of bariatric surgery. Additional obese individuals (1 male, 8 females), who met the criteria of metabolic syndrome (MS)[3,4] (online Table I), and 9 gender- and age-matched lean healthy controls were recruited. Weight reduction in obese patients with MS was induced by a protein-sparing, very-low-calorie diet as previously reported.[5] Blood was taken at baseline (obese and healthy subjects) and after 4–6 weeks of weight loss (obese subjects) to examine monocyte CD11c expression and perform biochemical measurements. All biochemical measurements were performed at Quest Diagnostics Clinical Laboratory (Houston, TX). All human studies were approved by the Institutional Review Board of Baylor College of Medicine, and informed consent was obtained.

AT fractionation and flow cytometric (FACS) analysis

Collagenase digestion was performed to fractionate AT into adipocytes and stromal/vascular cells (S/Vs).[6,7] The following antibodies against mouse or human antigens were used to detect CD11c on mouse AT S/Vs, or on mouse or human blood monocytes, or to examine leukocytes in mouse AT: fluorescein isothiocyanate (FITC)–, phycoerythrin (PE), or PE-Cy5–labeled anti-mouse or anti-human CD11b or CD11c, FITC–anti-human CD14, FITC–anti-mouse T-cell receptor (TCR) β, PE–anti-mouse CD3, PE–anti-mouse Ly-6C (BD PharMingen, San Diego, CA), FITC–anti-mouse CD204, FITC–anti-mouse CD205, Alexa Fluor (AF) 488–anti-mouse macrophage galactose specific lectin 1 (MGL1) (ABD Serotec, Raleigh, NC), PE–anti-mouse F4/80, and PE-Cy5–anti-mouse I-A/I-E (MHC class II) (eBioscience, Inc., San Diego, CA). FACS analysis was performed with a FACScan using CellQuest software (Becton Dickinson, San Jose, CA) as described.[1,7]

Quantitation of mRNA and protein

mRNA of MCP-1, RANTES, macrophage inflammatory protein (MIP)-1β (CCL4), CD3, CD4, CD8, and F4/80 in mouse AT or mouse liver was examined by RNase protection assay (RPA).[2] mRNA of interferon-γ (IFN-γ), H2-Ab1 (MHC class II), arginase I, IL-10, iNOS, and adiponectin in mouse AT, and MCP-1, CD11b, and CD11c in human VAT was examined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using predesigned primers and probes (Applied Biosystems).

MCP-1 and RANTES protein in mouse AT homogenate and MCP-1 protein in mouse AT culture media and serum were measured using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).[2]

Injection of MCP-1 in lean mice

Male lean mice were injected daily with recombinant mouse MCP-1/JE (R&D Systems) through the tail vein at a dose of 2 ng/g/day; the same volume of saline was injected in control mice. On day 7, 6 hours after MCP-1 injection, blood was drawn for measurement of serum MCP-1, leukocyte counts, and CD11c expression on peripheral leukocytes.

Biochemical measurements and glucose tolerance test in mice

Blood was collected by orbital puncture from mice after fasting overnight. Fasting plasma levels of glucose and insulin were measured at the Mouse Metabolic Phenotyping Center, University of Cincinnati Medical Center. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) using the formula: fasting insulin (μIU/ml) x fasting glucose (mmol/l)/22.5. Glucose tolerance test was performed in mice after an overnight fast. Blood glucose concentrations were measured with a Glucometer Elite XL blood glucose meter (Bayer Corporation) before and 15, 30, 45, 60, 90, and 120 minutes after an intraperitoneal injection of dextrose dissolved in water (1 g/kg).

Statistical analysis

GraphPad Prism 4 was used to perform statistical analyses. Values are presented as mean±SEM. Student's t-test (for comparison between 2 groups) or one-way ANOVA (for comparisons of 3 or

more groups) followed by Bonferroni multiple comparisons test was used for statistical analysis; Spearman correlation coefficents were computed to examine correlations. Differences were considered significant at $P \leq 0.05$.

Figure legends:

Figure I. MCP-1 levels in AT and liver of obese and lean mice. mRNA levels were examined in mouse AT or liver by RPA, and protein levels were examined by ELISA in AT homogenate or in media conditioned from AT culture ex vivo for 8 hours with or without 10 ng/ml tumor necrosis factor–α (TNF-α). *A:* MCP-1 mRNA, protein levels, and secretion in mouse AT; n=12/group for mRNA, and n=4/group for protein and secretion. *B:* Representative RPA images of MCP-1 mRNA in mouse liver as compared with AT, and quantitation of MCP-1 mRNA level in mouse liver and AT; n=4/group. *C:* MCP-1 protein in mouse plasma. HF: obese mice; ND: lean mice; HF-WL: obese mice with normal diet–induced weight loss.

Figure II. MCP-1 administration and $CD11c^+$ monocytes. Serum MCP-1 level was elevated in lean mice after intravenous injection of MCP-1. At 6 hours after MCP-1 injection on day 7, serum MCP-1 was 182.8±10.2 pg/ml, significantly higher than that of control mice inoculated with saline (38.6±5.3 pg/ml, $P<0.01$, n=5). Total leukocyte counts were not significantly increased in blood of MCP-1–inoculated mice ($15.5\pm2.0 \times 10^6$/ml in MCP-1–inoculated mice vs. $13.1\pm1.5 \times 10^6$/ml in control mice, $P>0.05$, n=5/group), while the proportion of $CD11c^+$ monocytes in total leukocytes was significantly higher in the MCP-1–inoculated group (ND-MCP-1) than in the control group (ND-Con).

Figure III. αβT cells and γδT cells or CD4 and CD8α mRNA in AT of $CD11c^{-/-}$ and WT mice. *A:* αβT cells and γδT cells in AT S/V cells as examined by FACS analysis; n=8 each for $CD11c^{-/-}$ and WT mice on high-fat diet (HFD), and n=5 each for $CD11c^{-/-}$ and WT mice on

normal diet (ND). *B:* mRNA levels of CD4 and CD8α in AT of CD11c$^{-/-}$ and WT mice as examined by RPA; n=10/group. NS: not significant.

Figure IV. mRNA of arginase I, IL-10 and adiponectin in AT of CD11c$^{-/-}$ and WT mice. mRNA was examined by quantitative RT-PCR; n=12–16/group. Our data showed an increase in IL-10 mRNA in AT of obese WT compared to lean WT, which was consistent with Rocha's report,[8] but was in contrast with Lumeng's finding.[9]

Figure V. RANTES protein levels in AT homogenate of CD11c$^{-/-}$ and WT mice. RANTES protein was examined in mouse AT homogenate by ELISA. n=8/group.

Table I. Characteristics of obese subjects with MS and controls

	Obese subjects		Controls
	Baseline	Post–weight loss	Baseline
Gender	1 M, 8 F		1 M, 8 F
Age, years	45.1±2.2		42.2±2.8
Body weight, kg	121.7 ± 8.8	110.4 ± 8.0*	65.3 ± 2.4†
Weight loss, kg (% of initial weight)		11.3 ± 1.1 (9.2 ± 0.5%)	
BMI, kg/m^2	44.9 ± 3.5	40.9 ± 3.2*	23.3 ± 0.5†
Waist circumference, cm	121.4 ± 4.2		76.8 ± 1.9†
Fasting plasma glucose, mg/dl	94.5 ± 3.4	97.4 ± 3.6	89.1 ± 2.5
Fasting plasma insulin, μIU/ml	15.5 ± 1.7	11.6 ± 1.1‡	5.4 ± 1.0†
HOMA-IR	3.6 ± 0.4	2.8 ± 0.3	1.2 ± 0.3†
Total cholesterol, mg/dl	176.8 ± 7.1	169.2 ± 12.6	189.5 ± 14.6
LDL-cholesterol, mg/dl	109.3 ± 7.6	103.5 ± 8.8	107.7 ± 11.1
HDL-cholesterol, mg/dl	42.0 ± 2.7	44.3 ± 3.3	63.7 ± 5.0†
Triglyceride, mg/dl	142.3 ± 13.7	107.3 ± 11.9*	91.1 ± 12.1§

§ $P<0.05$, † $P<0.01$ for controls vs. obese subjects at baseline; ‡ $P<0.05$, * $P<0.01$ for post–weight loss vs. baseline in obese subjects.

References

1. Wu H, Gower RM, Wang H, Perrard X-YD, Ma RD, Bullard DC, Burns AR, Paul A, Smith CW, Simon SI, Ballantyne CM. Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation.* In press.
2. Wu H, Ghosh S, Perrard X-YD, Feng L, Garcia GE, Perrard JL, Sweeney JF, Peterson LE, Chan L, Smith CW, Ballantyne CM. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation.* 2007;115:1029-1038.
3. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA.* 2001;285:2486-2497.
4. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr., Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation.* 2005;112:2735-2752.
5. Vasudevan AR, Wu H, Xydakis AM, Jones PH, Smith EO, Sweeney JF, Corry DB, Ballantyne CM. Eotaxin and obesity. *J Clin Endocrinol Metab.* 2006;91:256-261.
6. Robker RL, Collins RG, Beaudet AL, Mersmann HJ, Smith CW. Leukocyte migration in adipose tissue of mice null for ICAM-1 and Mac-1 adhesion receptors. *Obes Res.* 2004;12:936-940.
7. Wu H, Rodgers JR, Perrard X-YD, Perrard JL, Prince JE, Abe Y, Davis BK, Dietsch G, Smith CW, Ballantyne CM. Deficiency of CD11b or CD11d results in reduced staphylococcal enterotoxin-induced T cell response and T cell phenotypic changes. *J Immunol.* 2004;173:297-306.
8. Rocha VZ, Folco EJ, Sukhova G, Shimizu K, Gotsman I, Vernon AH, Libby P. Interferon-γ, a Th1 cytokine, regulates fat inflammation: a role for adaptive immunity in obesity. *Circ Res.* 2008;103:467-476.
9. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* 2007;117:175-184.

Fig. I online

A

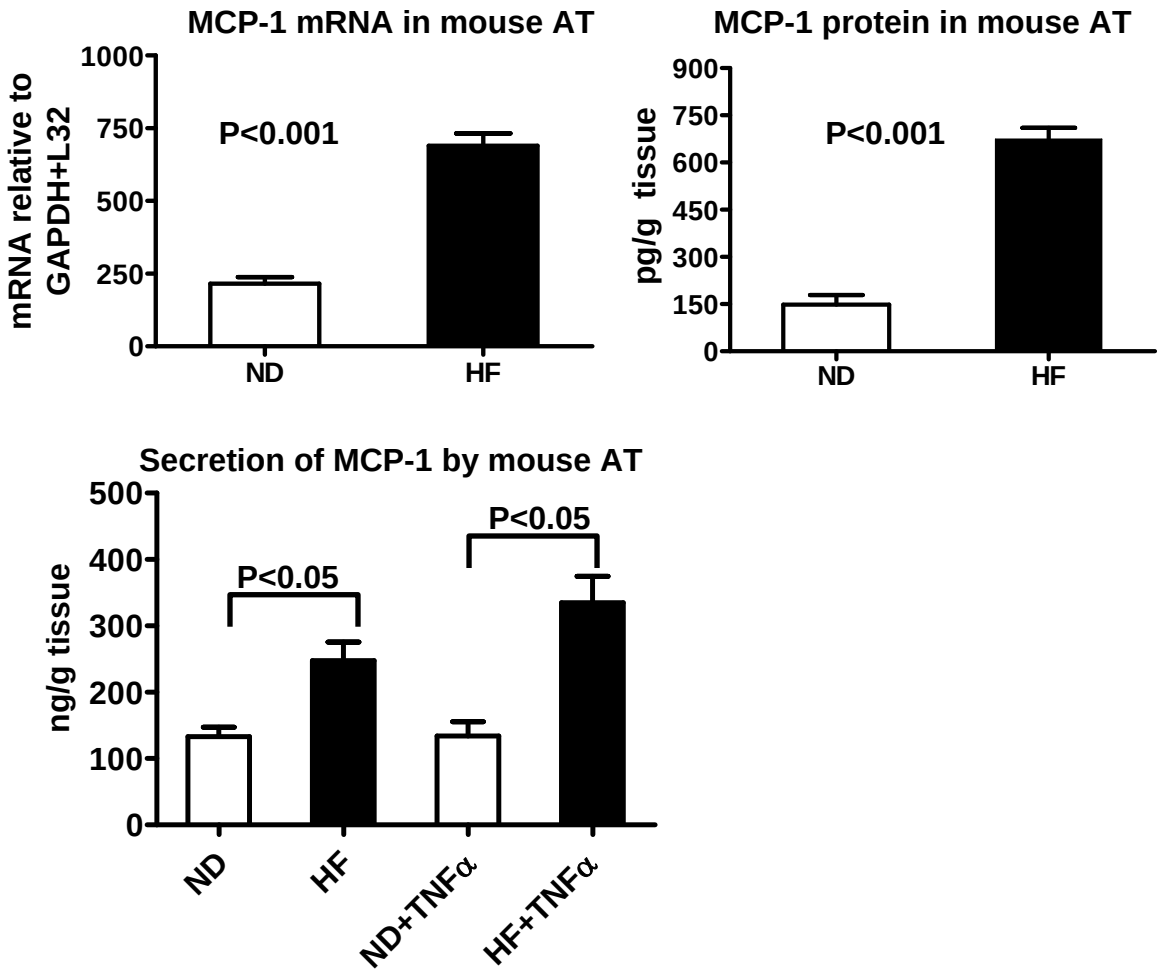
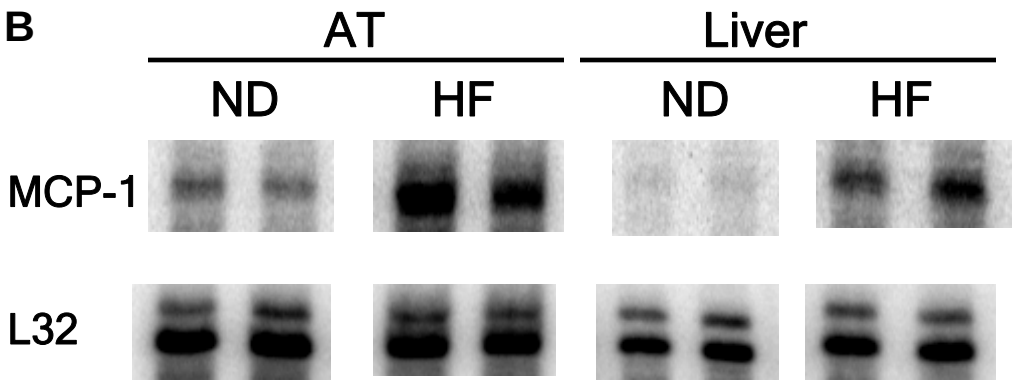


Fig. I online


B
AT
Liver
ND
HF
ND
HF
MCP-1
L32

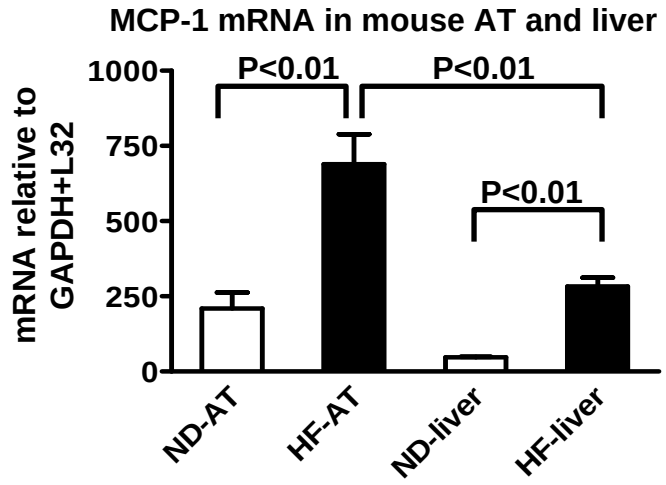

MCP-1 mRNA in mouse AT and liver
mRNA relative to GAPDH+L32
1000
750
500
250
0
P<0.01
P<0.01
P<0.01
ND-AT
HF-AT
ND-liver
HF-liver

Fig. I online

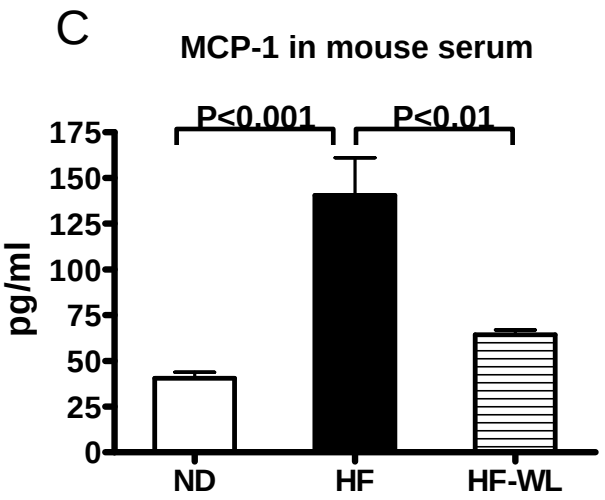
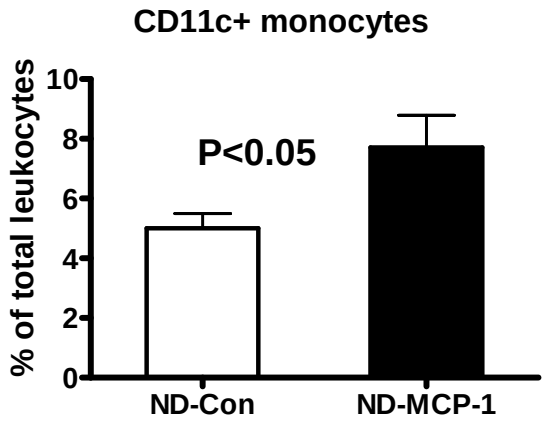

C
MCP-1 in mouse serum
P<0.001
P<0.01
pg/ml
175
150
125
100
75
50
25
0
ND
HF
HF-WL

Fig. II online



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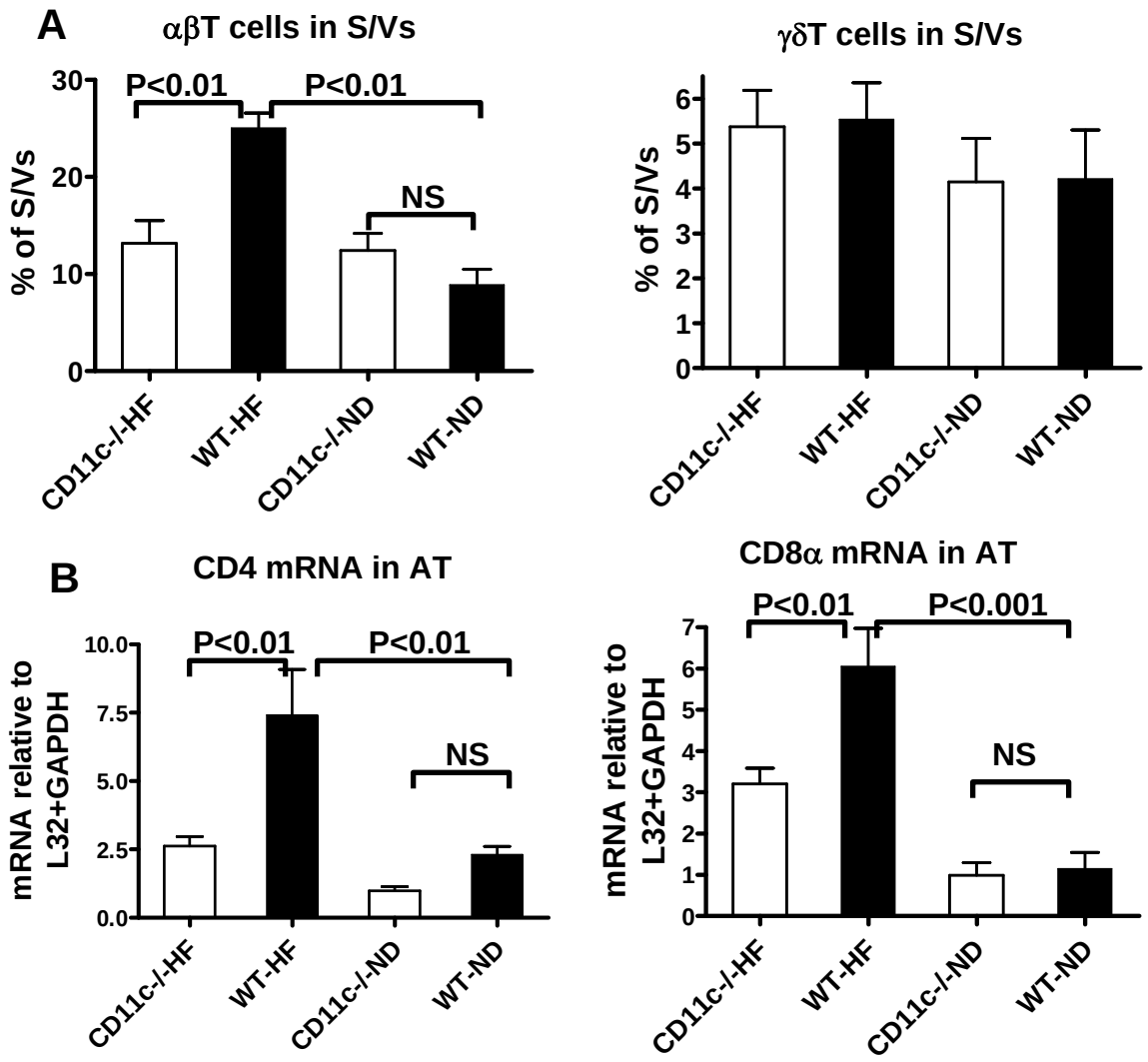

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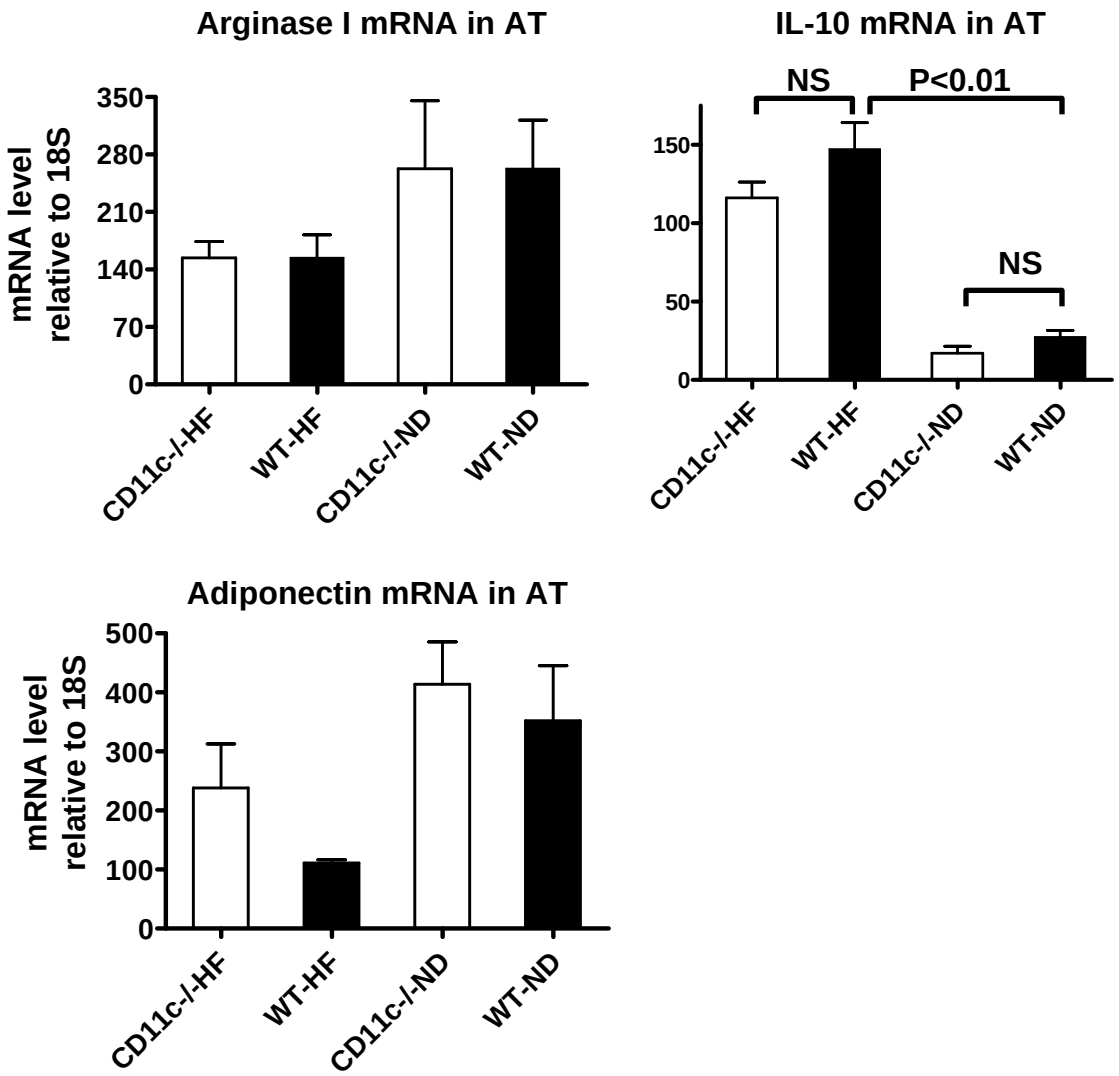

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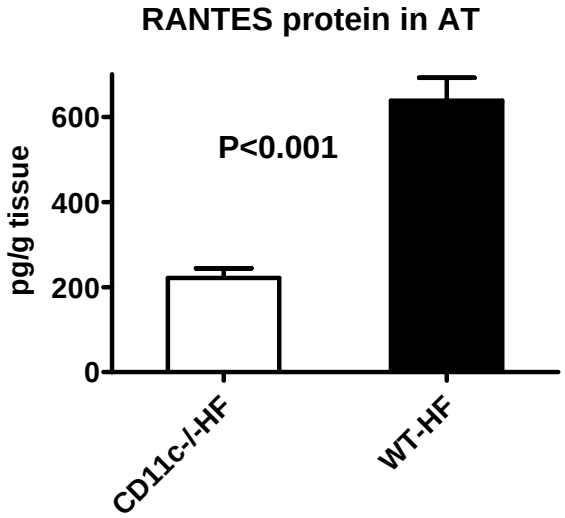
RANTES protein in AT
pg/g tissue
600
400
200
0
P<0.001
CD11c-/-HF
WT-HF