Epigenetic Regulation of Macrophage Polarization and inflammation by DNA methylation in Obesity

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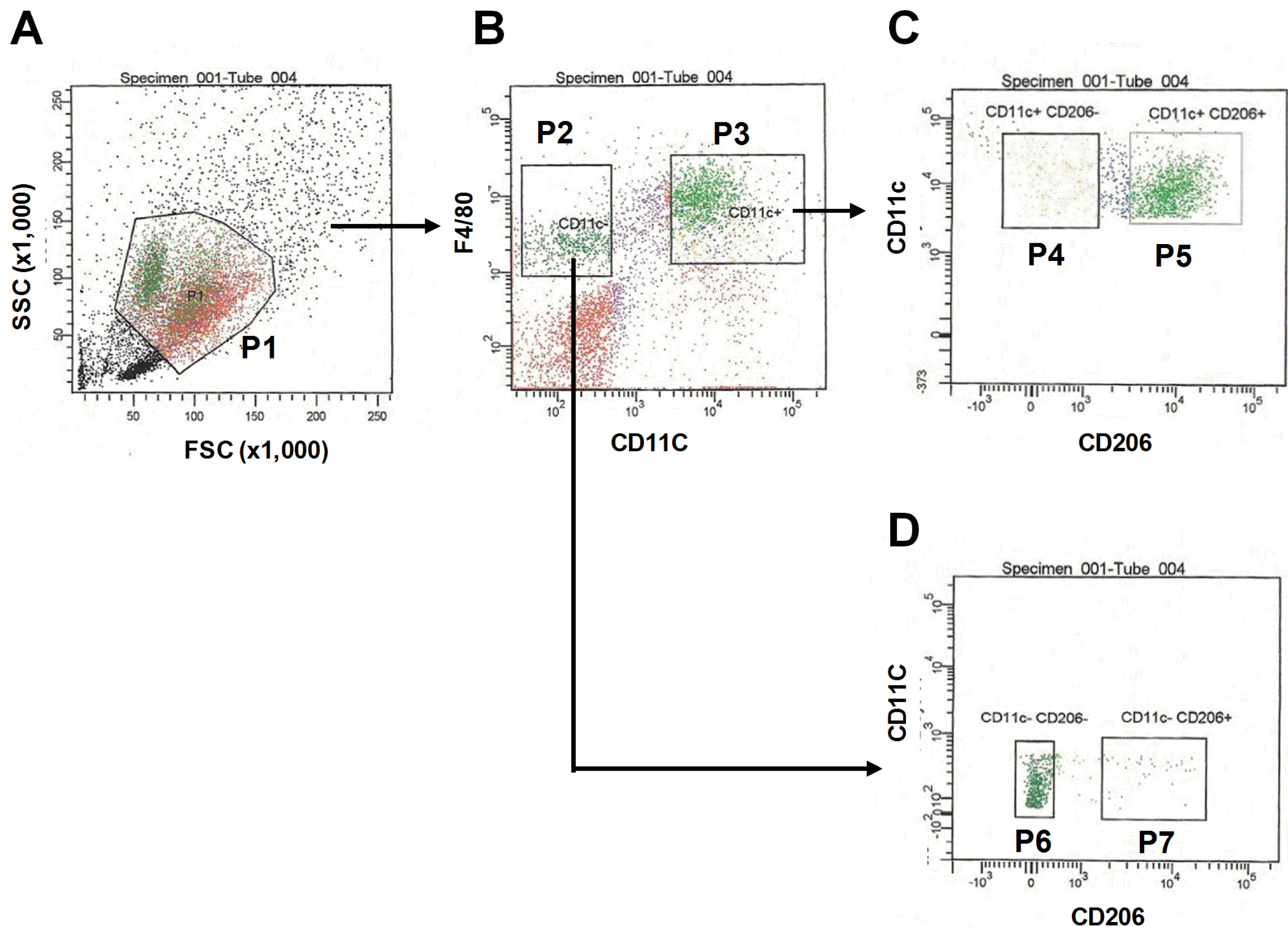
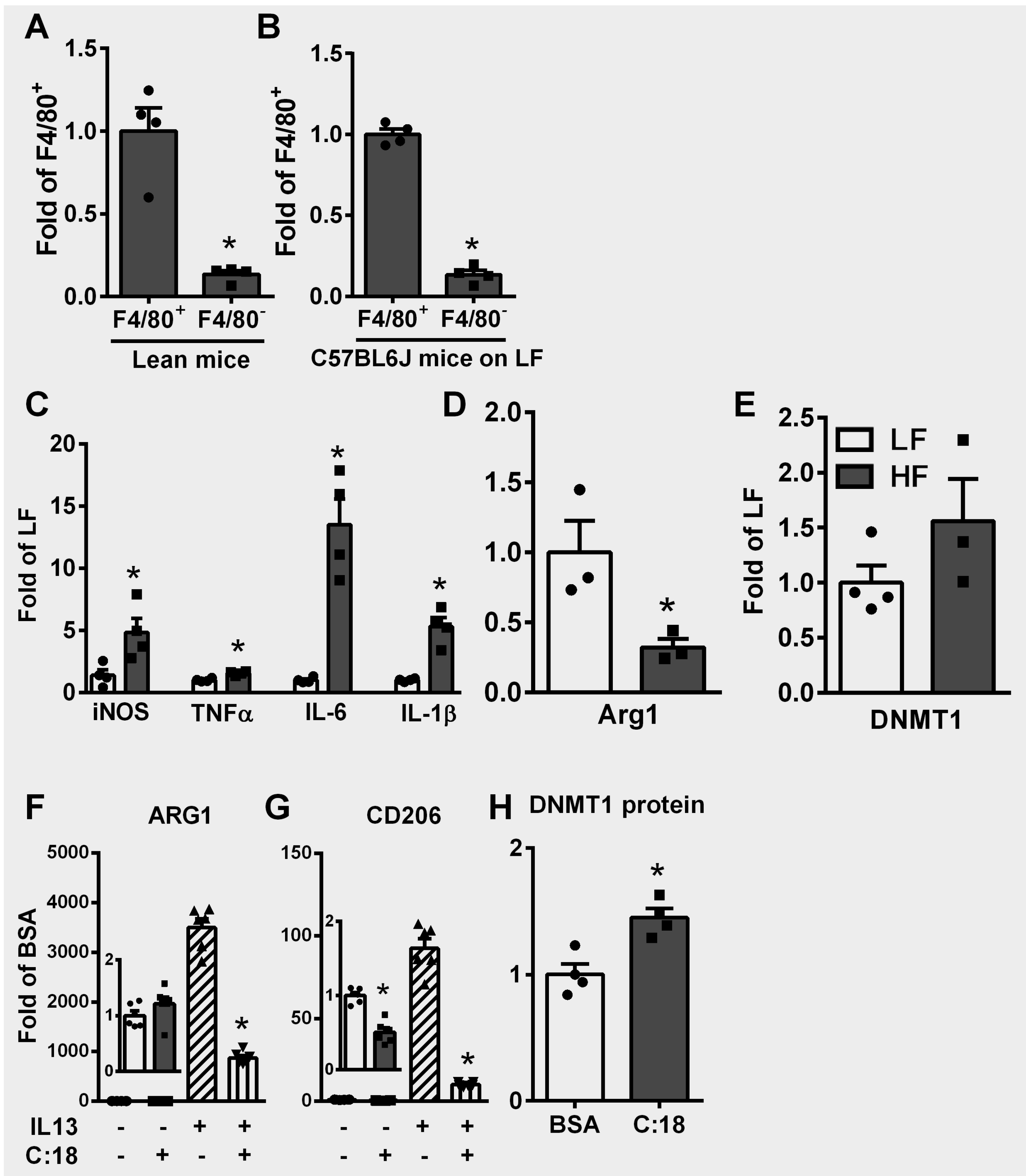
Supplemental Figures

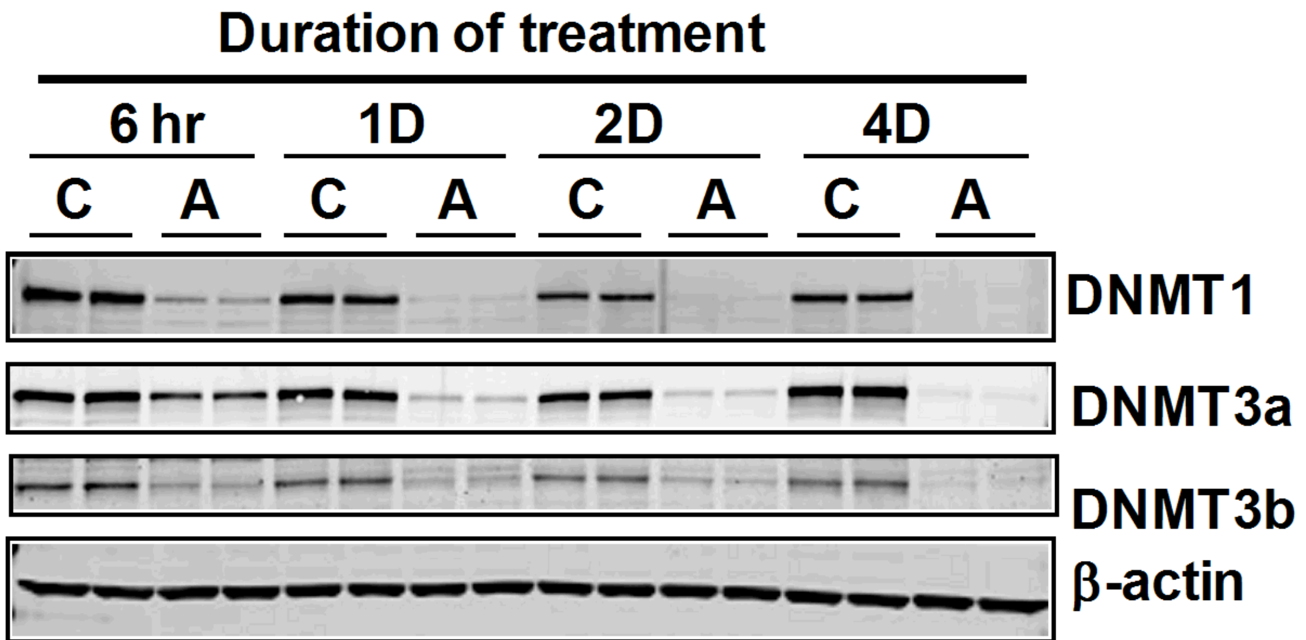


Supplement Figure 1. Isolation of M1 and M2 macrophages from adipose tissue. Stromal vascular cells from epidydimal adipose tissue were labeled with APC-F4/80, PE-Cy7-CD11c and PE-CD206, and M1/M2 macrophage subsets were isolated using BD FACSAria Cell Sorting machine as described in Methods. (A) Forward scatter (FSC) and side scatter (SSC) plot of adipose tissue stromal vascular cells. Cells in the P1 gate represented monocyte/macrophage population according to the FSC and SSC separation. (B) Cells in the P1 gate were separated into P2 ($F4/80^+CD11c^-$) or P3 ($F4/80^+CD11c^+$) populations. (C) P3 cells were then separated into P4 ($CD206^-$) and P5 ($CD206^+$) populations. (D) P2 cells were also separated into P6 ($CD206^-$) and P7 ($CD206^+$) populations. M1 macrophages in P4 gate ($F4/80^+CD11c^+CD206^-$), M2 macrophages in P7 gate ($F4/80^+CD11c^-CD206^+$), double positive cells in P5 gate ($F4/80^+CD11c^+CD206^+$), and double negative cells in P6 gate ($F4/80^+CD11c^-CD206^-$) were sorted and used for gene expression analysis.

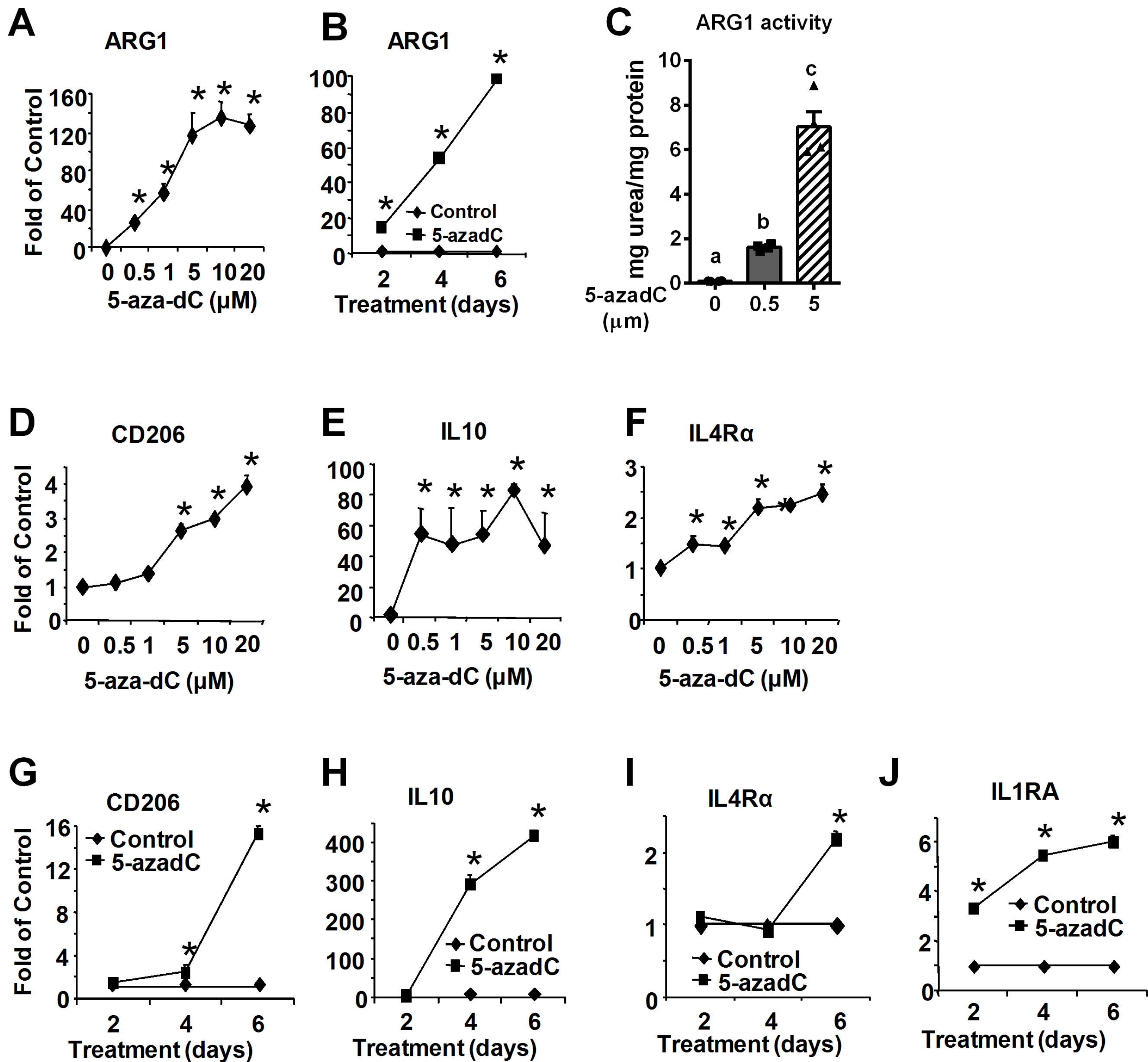


Supplemental Figure 2. The expression of M1 and M2 markers in macrophages under different physiological conditions.
(A-B) F4/80 expression in F4/80$^+$ ATMs and F4/80$^-$ cell populations from adipose tissue of lean mice (A) (control mice used in Figures 1I-K) and wild type C57BL/6J mice on a LF diet (B) (control mice used in Supplemental Figures 2C-E). SVF cells from mice epididymal adipose tissue were incubated with rat anti-mouse F4/80 antibody, followed by a pull down of F4/80-positive cells with sheep anti-rat

microbeads as described in the Methods. F4/80$^+$ cells enriched by the microbeads and F4/80$^-$ cells from the flow-through fraction were collected to measure F4/80 gene expression to confirm the purity of the isolated ATMs.
(C-E) M1 (C), M2 (D) marker and DNMT1 (E) expression in ATMs isolated from male C57BL/6J mice fed LF or HF diet for 16 weeks. Cells from 3-4 LF-fed mice are pooled; whereas cells from individual HF-fed mice were used for RNA isolation and gene expression measurements.
(F-G) ARG1 (F) and CD206 (G) expression in RAW264.7 macrophages stimulated with IL13 with or without stearate (C:18). RAW cells were pre-treated with 200μM stearate for 4 days and then treated with 10ng/ml IL13 for an additional 1 day.
(H) DNMT1 protein levels in RAW264.7 macrophages treated with 200μM stearate (C:18) for 4 days.
Data are expressed as mean +/- SEM. n=3-6. * $p<0.05$.



Supplemental Figure 3. 5-azadC reduces DNMT protein levels in RAW264.7 macrophages. RAW264.7 macrophages were treated with 0.5μM 5-azadC for indicated time and DNMT1, DNMT3a and DNMT3b protein levels were measured with immunoblotting.



Supplemental Figure 4. Inhibiting DNA methylation in macrophages promotes macrophage alternative polarization.

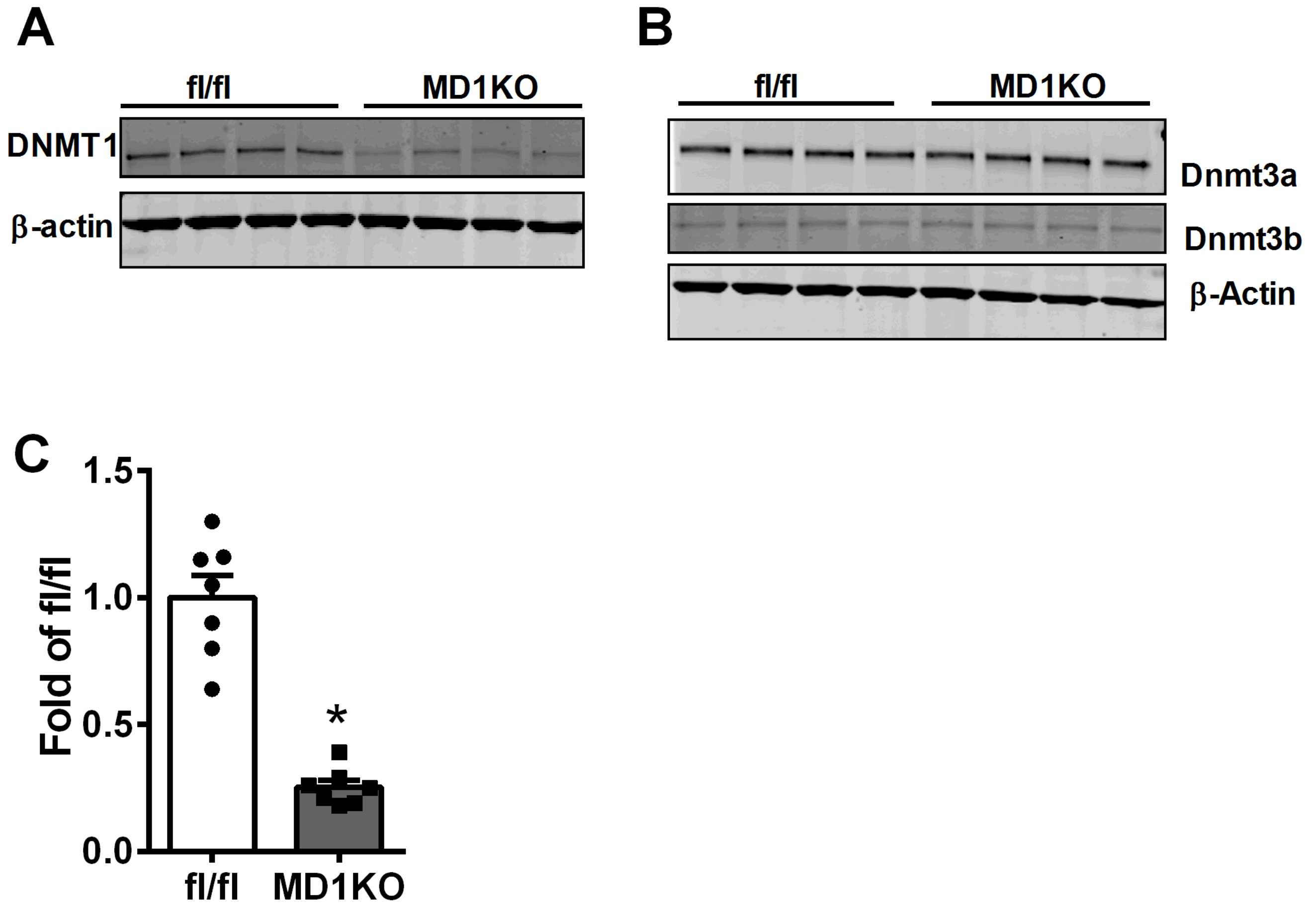
(A-B) ARG1 expression in RAW264.7 macrophages treated with 5-azadC at indicated doses for 2 days (A) or treated with 0.5μM 5-azadC for indicated time (B).

(C) ARG1 activity in RAW264.7 macrophages treated with 5-azadC at indicated doses for 4 days.

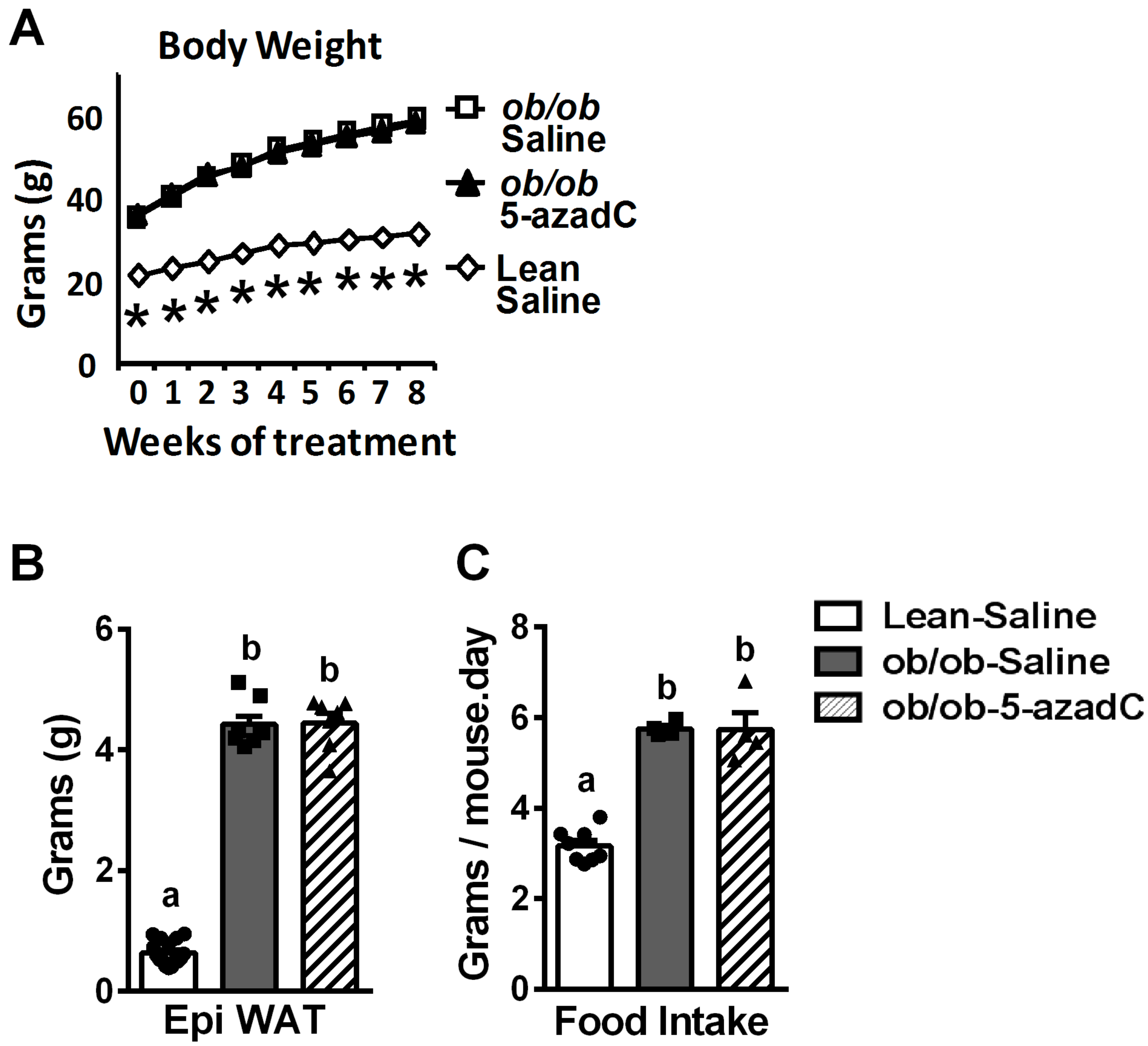
(D-F) CD206 (D), IL10 (E) and IL4Rα (F) expression in RAW264.7 macrophages treated with 5-azadC at indicated doses for 2 days.

(G-J) CD206 (G), IL10 (H), IL4Rα (I) and IL1RA (J) expression in RAW264.7 macrophages treated with 0.5μM 5-azadC for indicated time.

Data are expressed as mean +/- SEM. n=3-4. * $p<0.05$.



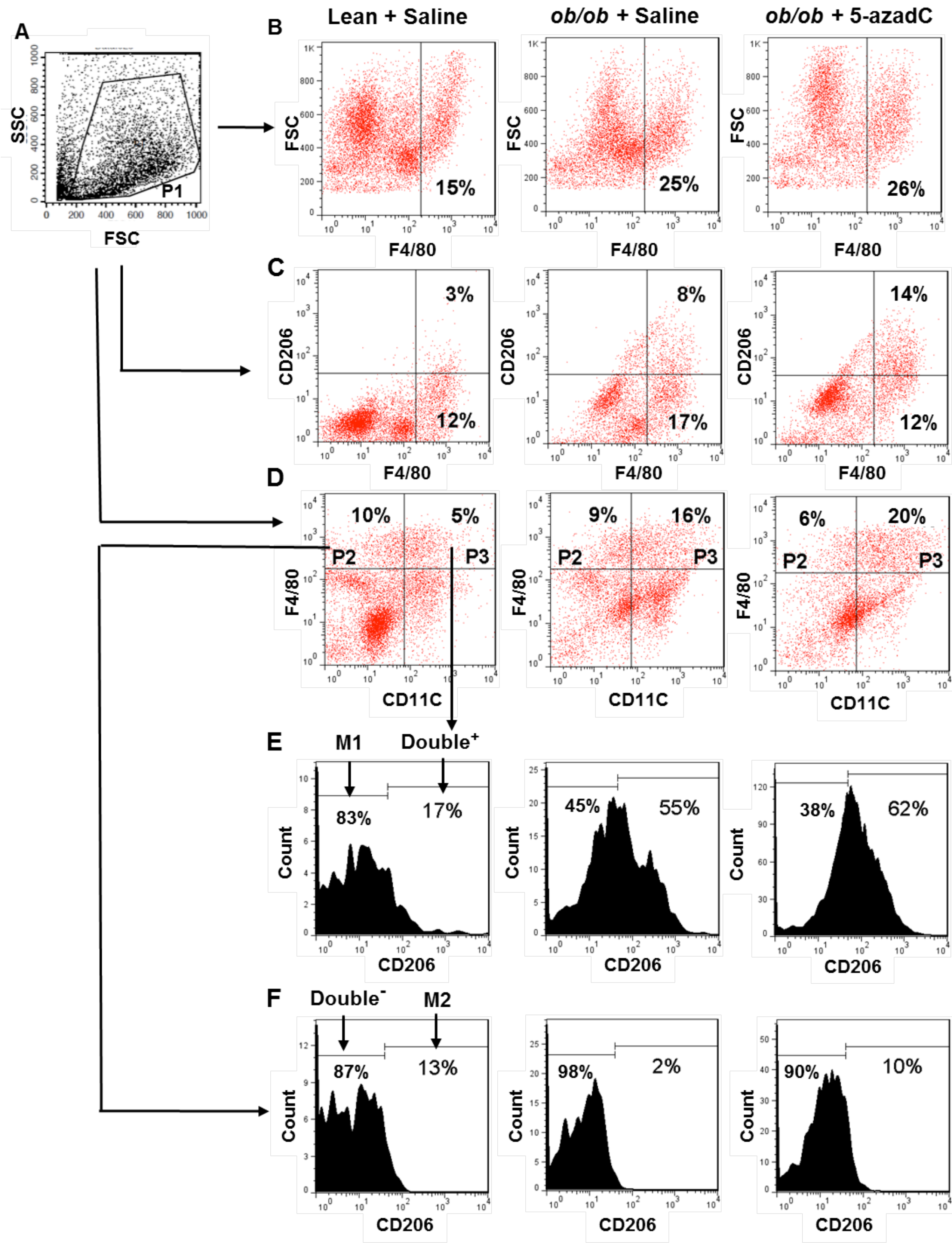
Supplemental Figure 5. DNMT protein levels in macrophages from MD1KO and fl/fl mice.
(A) DNMT1 protein levels in BMDMs isolated from MD1KO and fl/fl mice.
(B) DNMT3a and DNMT3b protein levels in BMDMs isolated from MD1KO and fl/fl mice.
Data are expressed as mean +/- SEM. Protein levels in (A) was normalized to β-actin. n=4-7. * $p<0.05$.



Supplemental Figure 6. Body weight, fat pad mass, food intake and ATM subset composition in lean and *ob/ob* mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

(A-C) Body weight (A), epididymal fat pad mass (B) and food intake (C) in lean and ob/ob mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

Data are expressed as mean +/- SEM. n=4-8. * $p<0.05$ vs. other groups. Groups labeled with different letters are statistically different from each other.



A
B
C
D
E
F
Lean + Saline
ob/ob + Saline
ob/ob + 5-azadC
SSC
FSC
P1
F4/80
CD206
CD11C
Count
15%
25%
26%
3%
12%
8%
17%
14%
12%
10%
5%
9%
16%
6%
20%
P2
P3
M1
Double+
83%
17%
45%
55%
38%
62%
Double-
M2
87%
13%
98%
2%
90%
10%

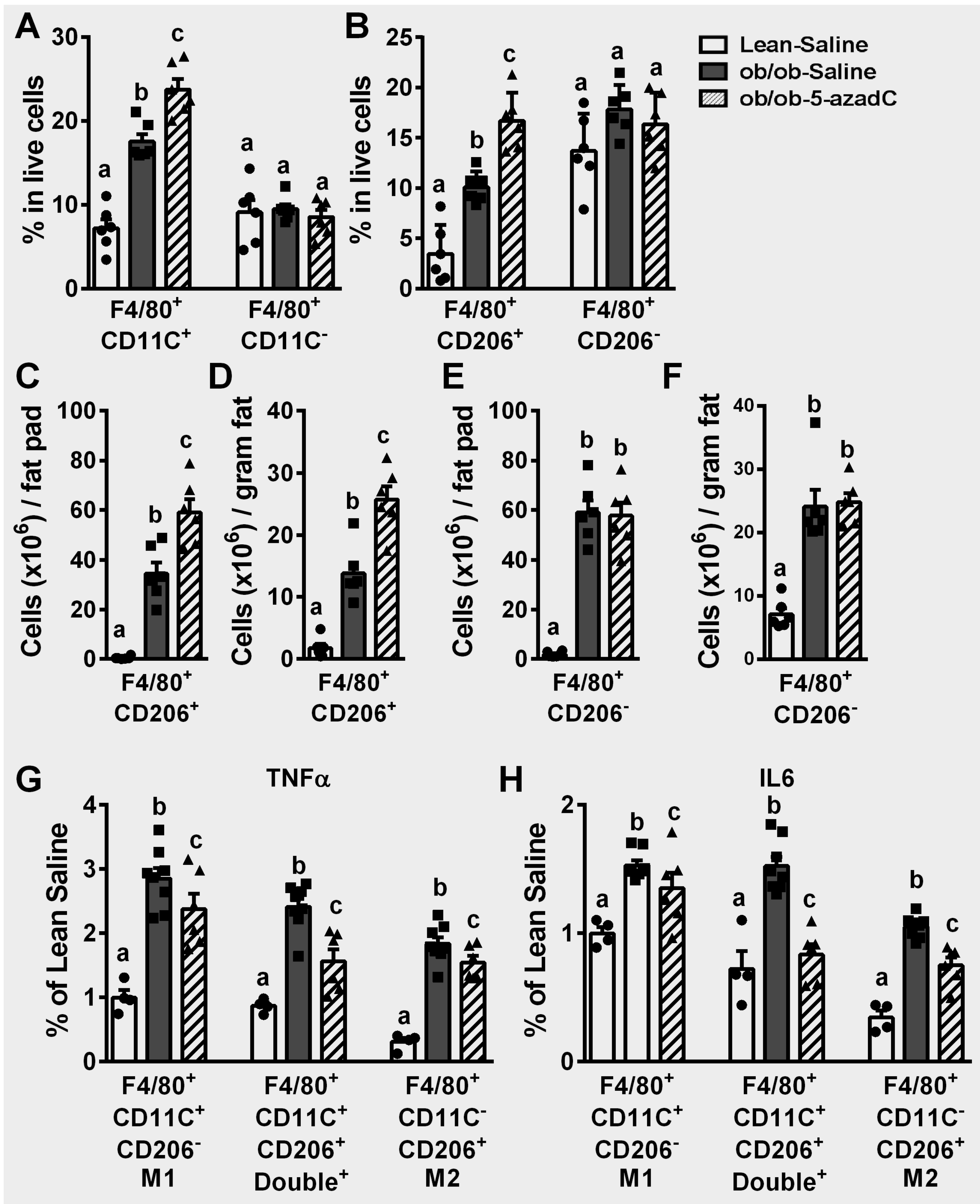
Supplemental Figure 7. Representative gating strategy of ATM subset composition in lean and ob/ob mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

SVF cells from epidydimal adipose tissue were labeled with APC-F4/80, PE-Cy7-CD11c and PE-CD206, and M1/M2 macrophage subsets were analyzed using BD FACS Calibur as described in Methods.

(A) FSC and SSC plot of adipose tissue SVF cells. Cells in the P1 gate represented live cell population according to the FSC and SSC separation.

(B-D) Cells in the P1 population were gated for F4/80 and FSC (B), F4/80 and CD206 (C) and F4/80 and CD11c (D).

(E-F) $F4/80^{+}CD11c^{+}$ cells in P3 gate and $F4/80^{+}CD11c^{-}$ cells in P2 gate were further gated for CD206 to study ATM subset composition of M1 ($F4/80^{+}CD11c^{+}CD206^{-}$) and $double^{+}$ ($F4/80^{+}CD11c^{+}CD206^{+}$) ATMs (E) and M2 ($F4/80^{+}CD11c^{-}CD206^{+}$) and $double^{-}$ ($F4/80^{+}CD11c^{-}CD206^{-}$) ATMs (F).



Supplemental Figure 8. ATM subset composition in lean and *ob/ob* mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

(A) Percentage of $F4/80^+CD11c^+$ and $F4/80^+CD11c^-$ ATMs in live SVF cells isolated from epididymal fat pads of lean and *ob/ob* mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

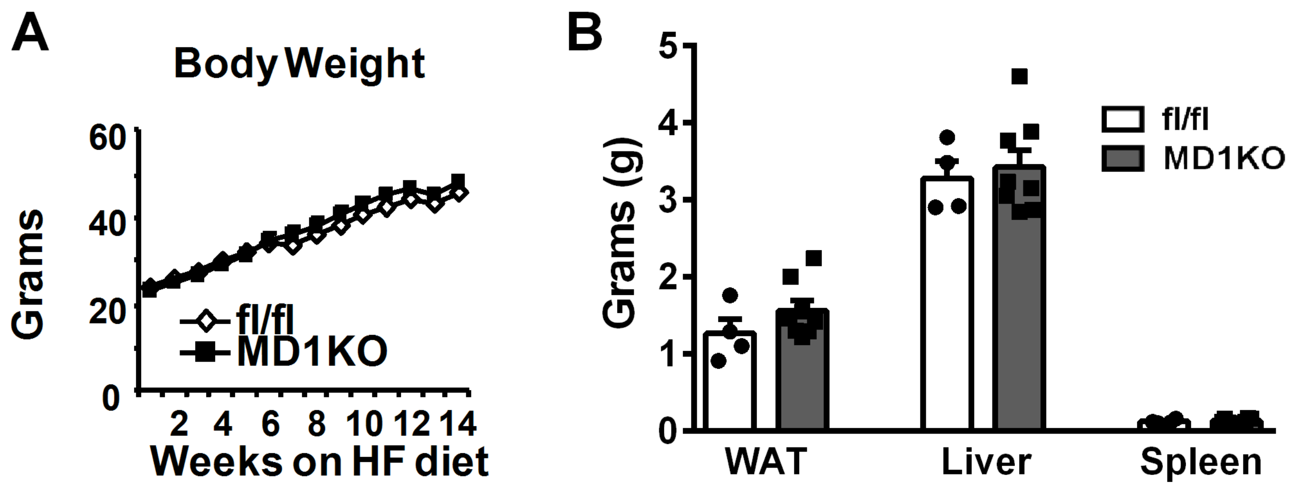
(B) Percentage of $F4/80^+CD206^+$ and $F4/80^+CD206^-$ ATMs in live SVF cells isolated from epididymal fat pads of lean and ob/ob mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

(C-D) $F4/80^+CD206^+$ ATM content in whole epididymal fat pad (C) or in per gram of fat (D) in lean and *ob/ob* mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks.

(E-F) $F4/80^+CD206^-$ ATM content in whole epididymal fat pad (E) or in per gram of fat (F) in lean and *ob/ob* mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks .

(G-H) TNFα (G) and IL6 (H) expression in M1, double+ and M2 ATMs isolated from lean and ob/ob mice treated with saline or 0.25mg/kg 5-azadC for 8 weeks. M1: $F4/80^+CD11c^+CD206^-$; M2: $F4/80^+CD11c^-CD206^+$; double+: $F4/80^+CD11c^+CD206^+$.

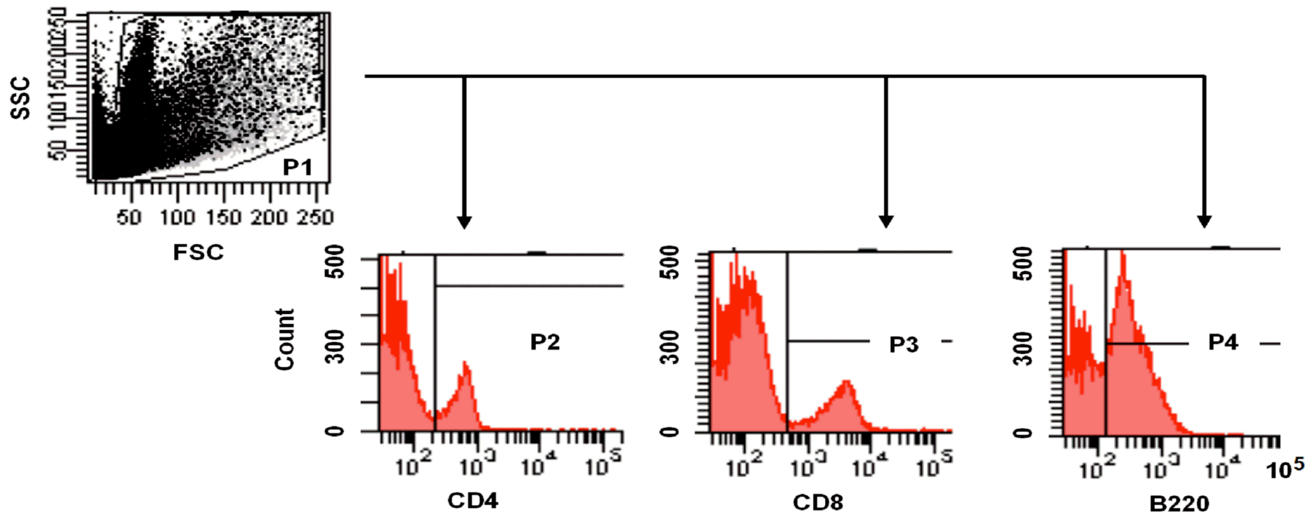
Data are expressed as mean +/- SEM. n=4-6. * $p<0.05$ vs. other groups. Groups labeled with different letters are statistically different from each other.



Supplemental Figure 9. Body and organ weight in fl/fl and MD1KO mice fed HF diet for 24 weeks. (A-B) Body weight (A) and organ weight (B) in fl/fl and MD1KO mice fed HF diet for 24 weeks.

Data are expressed as mean +/- SEM. n=4-8.

A



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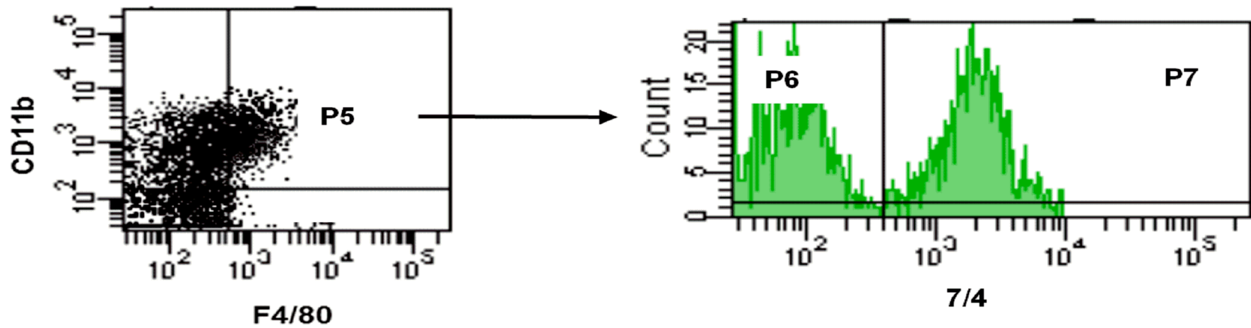

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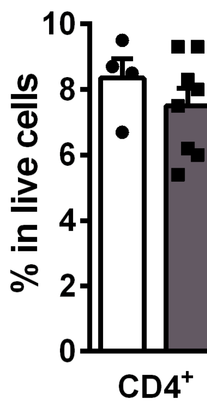

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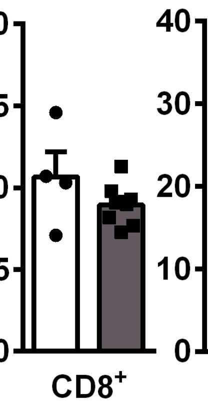
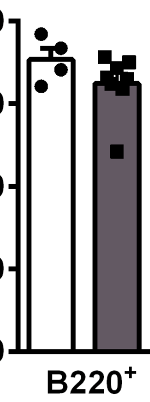


E



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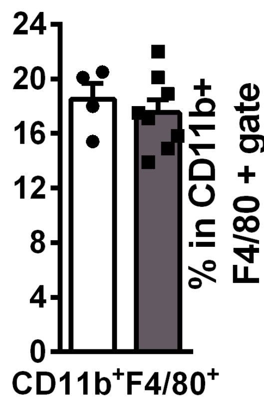

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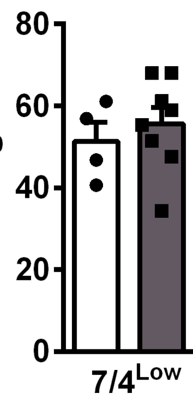

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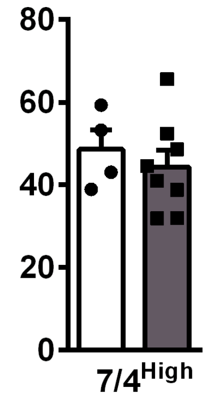
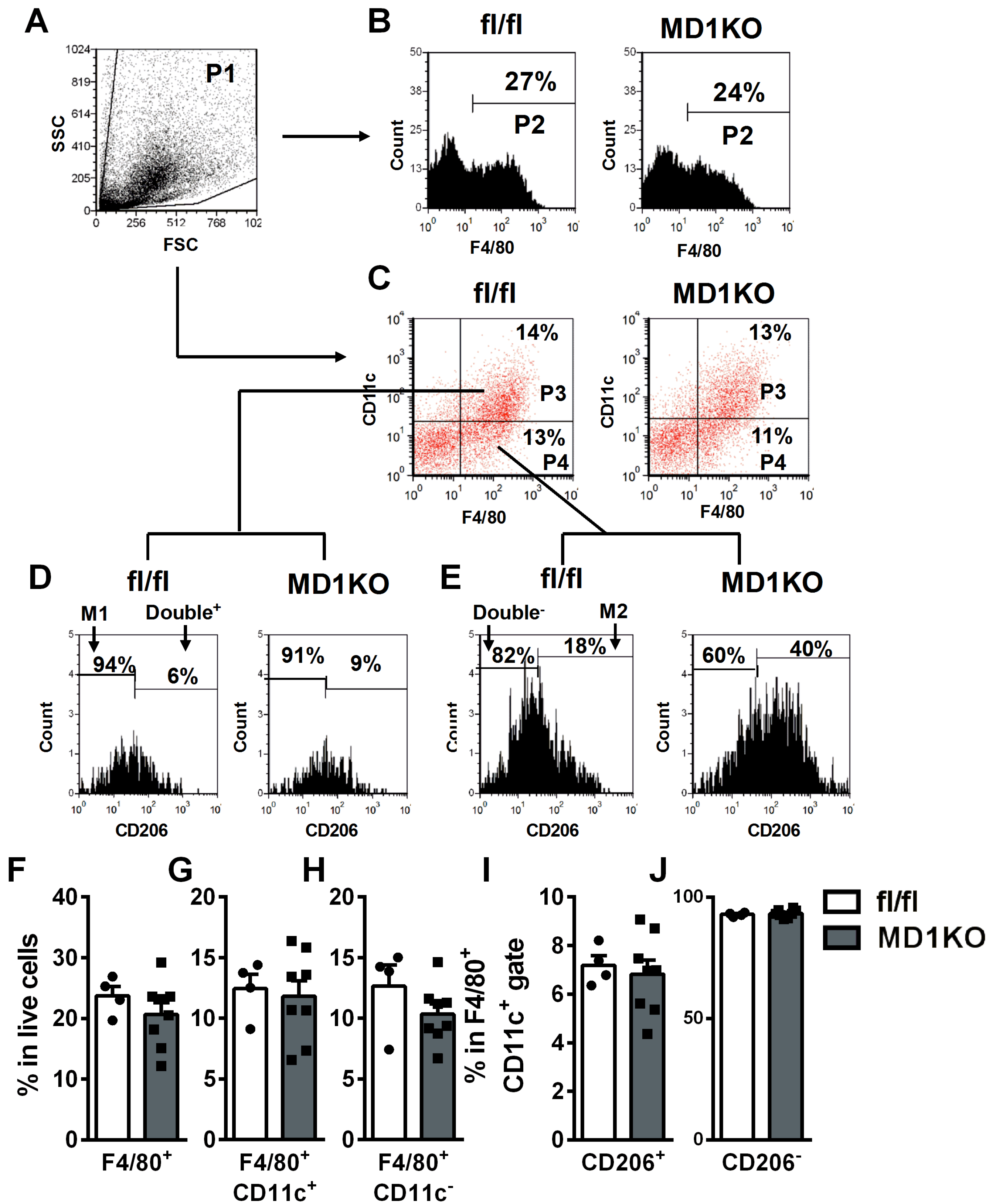


Supplemental Figure 10. Analysis of blood immune cell composition in fl/fl and MD1KO mice fed HF diet for 24 weeks.

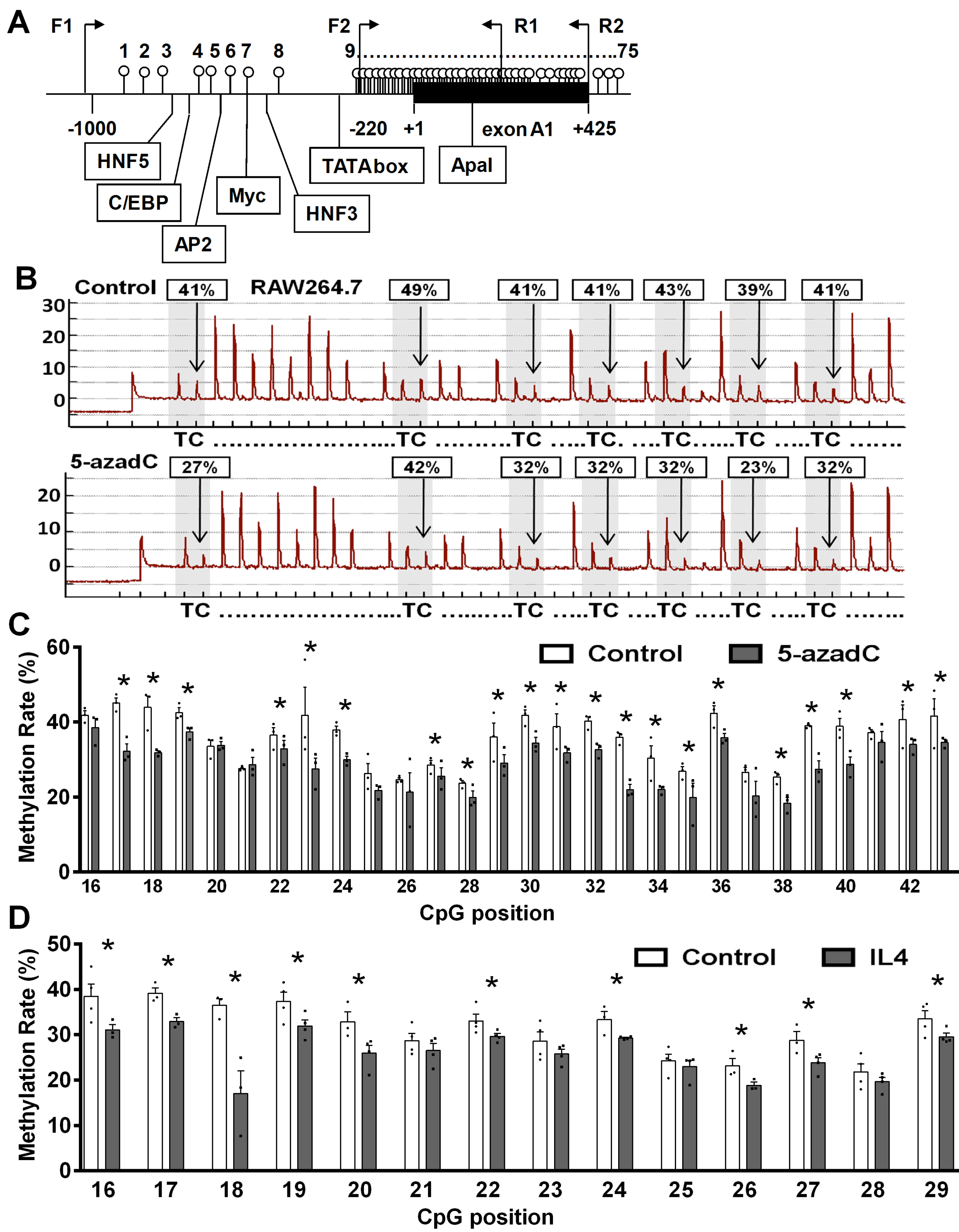
(A-B) Representative gating strategy for blood immune cell composition analysis using FACS. (A) FSC and SSC plot of white blood cells. Cells in the P1 gate represented live cell population according to the FSC and SSC separation. P1 cells were further gated for CD4, CD8 and B220. (B) White blood cells were gated for CD11b and the monocyte/macrophage-specific antigen F4/80 [1]. $CD11b^{+}F4/80^{+}$ monocyte fractions in P5 gate were then gated for 7/4 to analyze $7/4^{High}$ inflammatory and $7/4^{Low}$ resident monocyte subset [2].

(C-F) Blood composition of $CD4^+$ (C), $CD8^+$ (D) T lymphocytes, $B220^+$ B lymphocytes (E) and $CD11b^+F4/80^+$ monocytes (F) in fl/fl and MD1KO mice fed HF diet for 24 weeks.
(G-H) Percentage of $7/4^{Low}$ resident monocyte (G) and $7/4^{High}$ inflammatory monocyte (H) in $CD11b^+F4/80^+$ monocyte gate in fl/fl and MD1KO mice fed HF diet for 24 weeks.
Data are expressed as mean +/- SEM. n=4-8.



Supplemental Figure 11. Analysis of ATM subset composition in fl/fl and MD1KO mice fed HF diet for 24 weeks.

(A-E) Representative gating strategy and plots for ATM subset composition analysis using FACS. (A) FSC and SSC plot of adipose tissue SVF cells. Cells in the P1 gate represented live cell population according to the FSC and SSC separation. (B-C) Cells in the P1 population were gated for F4/80 (B) and F4/80 and CD11c (C). (D-E) $F4/80^{+}CD11c^{+}$ cells in P3 gate and $F4/80^{+}CD11c^{-}$ cells in P4 gate were further gated for CD206 to study ATM subset composition of M1 ($F4/80^{+}CD11c^{+}CD206^{-}$), $double^{+}$ ($F4/80^{+}CD11c^{+}CD206^{+}$) (D), $double^{-}$ ($F4/80^{+}CD11c^{-}CD206^{-}$) and M2 ($F4/80^{+}CD11c^{-}CD206^{+}$) ATMs (E).
(F-H) Percentage of $F4/80^{+}$ ATMs (F), $F4/80^{+}CD11C^{+}$ ATMs (G), $F4/80^{+}CD11c^{-}$ ATMs (H) in live SVF populations isolated from epididymal adipose tissue of fl/fl and MD1KO mice fed HF diet for 24 weeks.
(I-J) Percentage of $CD206^{+}$ (I) and $CD206^{-}$ (J) ATMs in $F4/80^{+}CD11c^{+}$ ATM populations in fl/fl and MD1KO mice fed HF diet for 24 weeks.
Data are expressed as mean +/- SEM. n=4-8.



A
F1
F2
R1
R2
1 2 3
4 5 6 7
8
9
75
-1000
-220
+1
exon A1
+425
HNF5
C/EBP
AP2
Myc
HNF3
TATAbox
ApaI
B
Control
RAW264.7
41%
49%
41%
41%
43%
39%
41%
TC
5-azadC
27%
42%
32%
32%
32%
23%
32%
C
Methylation Rate (%)
Control
5-azadC
CpG position
D
Methylation Rate (%)
Control
IL4
CpG position

Supplemental Figure 12. PPARγ1 promoter DNA methylation is decreased by 5-azadC and IL4 treatment in RAW264.7 macrophages.

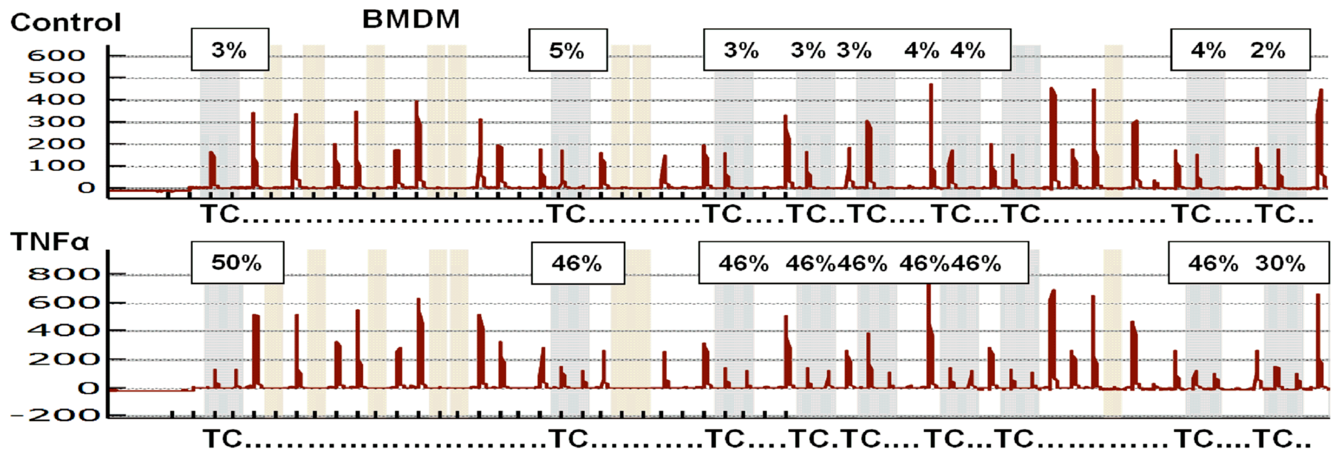
(A) Schematic illustration of PPARγ1 promoter and 5'-untranslated region [3]. The transcription start site is indicated as +1. TATA box, exonA1 are indicated. The CpG sites are indicated as upward vertical lines with open circles. The HNF5, C/EBP, Ap2, Myc and HNF3 motifs are indicated. Arrows indicate positions of forward and reverse primer used in cloning of the 1.5kb promoter (F1/R1 and F2/R2) and ChIP assays (F2/R1).

(B-C) Representative pyrosequencing analysis (B) and DNA methylation rate (C) of PPARγ1 promoter in RAW264.7 macrophages treated with 0.5μM 5-azadC for 4 days.

(D) DNA methylation rate of PPARγ1 promoter in RAW264.7 macrophages treated with 10ng/ml IL4 for 4 days.

Data are expressed as mean +/- SEM. n=3-4. * $p<0.05$.

A



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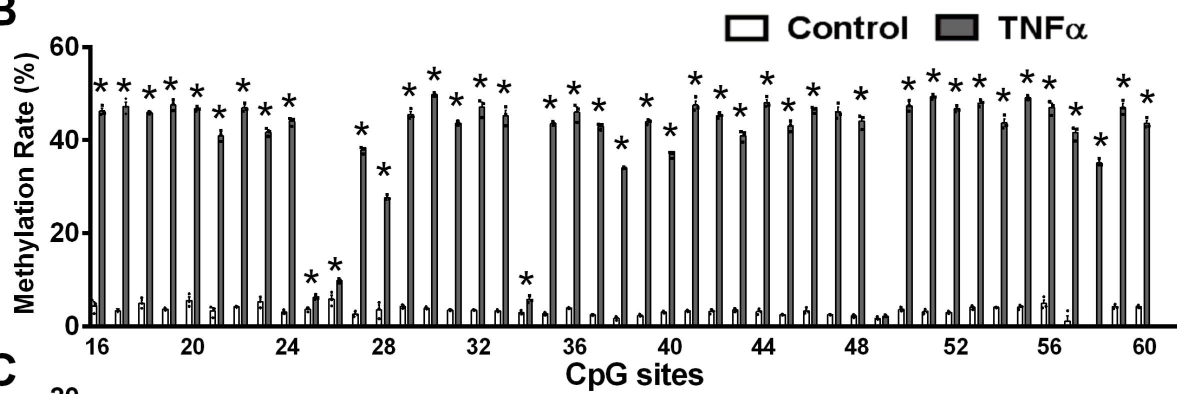

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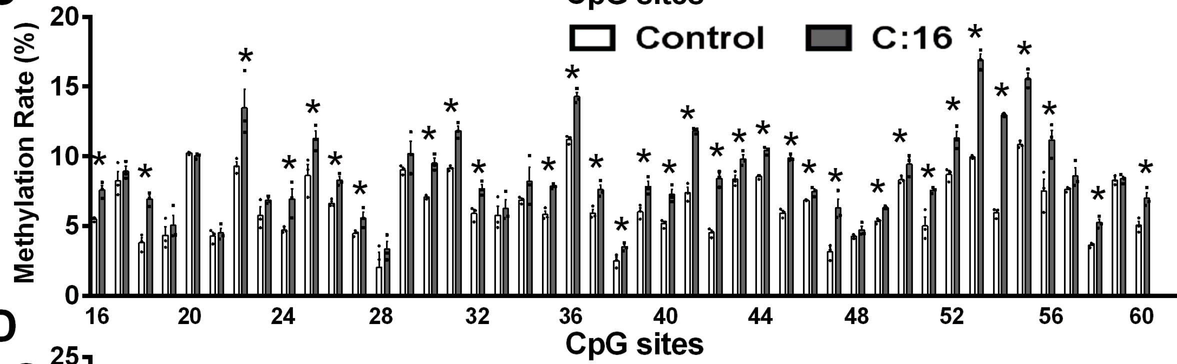

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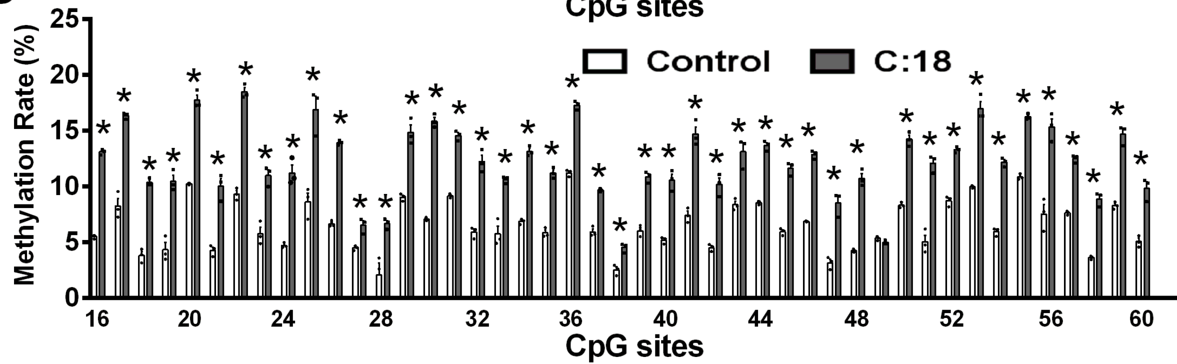
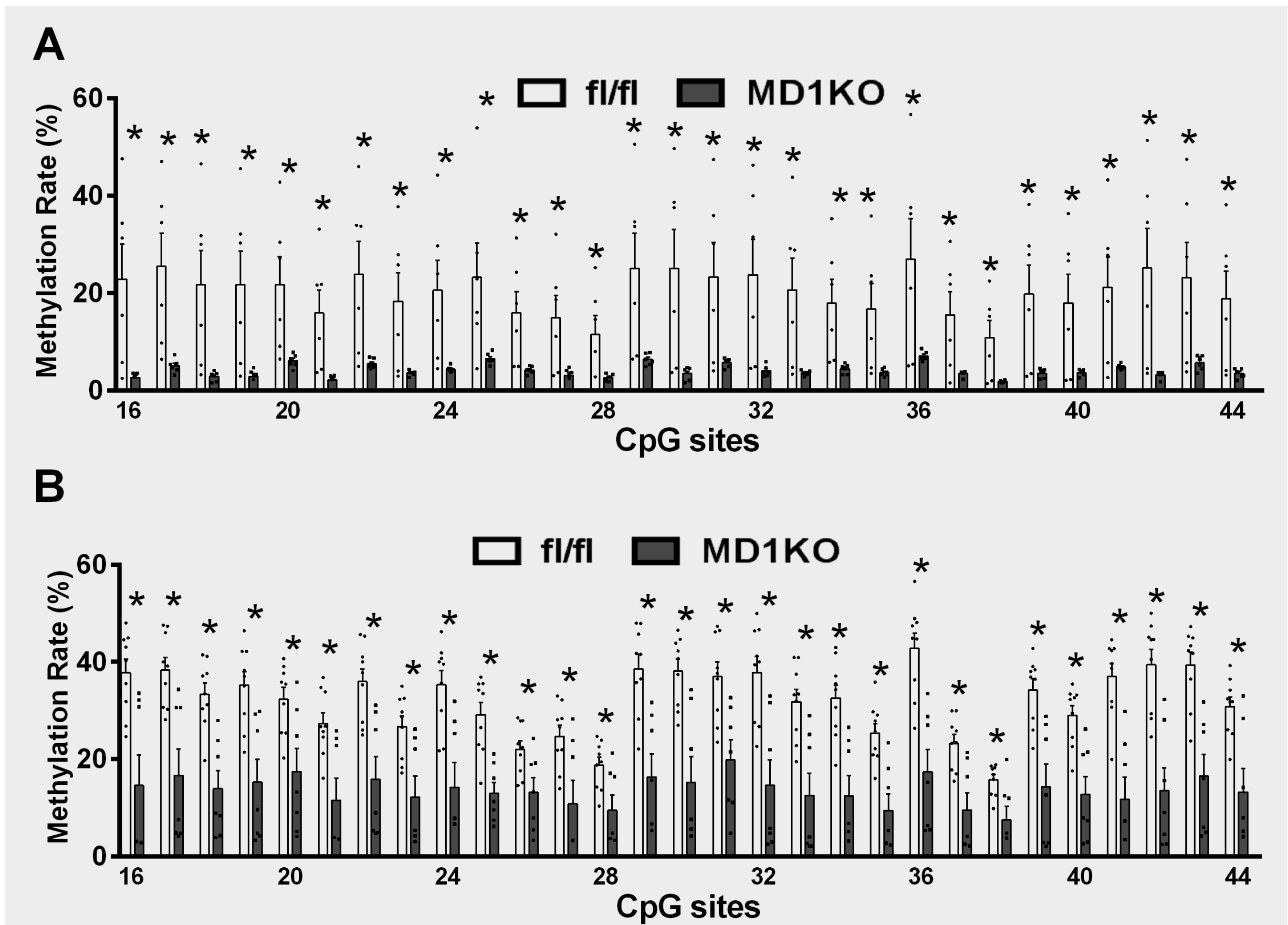


Supplemental Figure 13. PPARγ1 promoter DNA methylation is significantly increased by TNFα, stearate (C:18) and palmitate (C:16) in BMDMs.

(A-B) Representative pyrosequencing analysis (A) and methylation rate (B) of PPARγ1 promoter in BMDMs treated with 10ng/ml TNFα for 2 days.
(C-D) Methylation rate of PPARγ1 promoter in BMDMs treated with 200μM palmitate (C:16) for 2 days (C) or 200μM stearate (C:18) (D) for 4 days.
Data are expressed as mean +/- SEM. n=3. * $p<0.05$.



Supplemental Figure 14. PPARγ1 promoter DNA methylation is decreased in peritoneal macrophages and ATMs isolated from MD1KO compared to fl//fl mice.

(A) Methylation rate of PPARγ1 promoter in peritoneal macrophages isolated from fl/fl and MD1KO mice fed a regular chow diet for 10-12 weeks. n=6.

(B) Methylation rate of PPARγ1 promoter in ATMs isolated from fl/fl and MD1KO mice fed a HF diet for 24 weeks. n=7-9.

Data are expressed as mean +/- SEM. * $p<0.05$.

Supplemental References.

1. **Gordon S, Hamann J, Lin HH, Stacey M. F4/80 and the related adhesion-GPCRs. *European journal of immunology.* Sep 2011;41(9):2472-2476.**
2. **Rosas M, Thomas B, Stacey M, Gordon S, Taylor PR. The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *Journal of leukocyte biology.* Jul 2010;88(1):169-180.**
3. **Zhu Y, Qi C, Korenberg JR, et al. Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms. *Proc Natl Acad Sci U S A.* Aug 15 1995;92(17):7921-7925.**