Supporting Information

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SI Results

Recent reports have shown that T cells were also recruited into adipose tissue (1–4) and that accumulation of a $CD8^+$ T cell population appears to precede macrophage infiltration (3). However, because apoptosis inhibitor of macrophage (AIM) does not affect peripheral T cells (5), it is possible that AIM contributes to macrophage recruitment in a T cell-independent manner.

SI Materials and Methods

Antibodies and Reagents. Antibodies and reagents used for histological and biochemical experiments are as follows: F4/80 (RM2900; Calotag), IL-6 (AB1423; Chemicon), CD11b (M1/70; eBioscience), CD11c (N418; eBioscience), mannose receptor (MR) (clone MR5D3; BioLegend), CD16/32 (Fc blocker; clone 93; eBioscience), IkBα (rabbit polyclonal antibody; Cell Signaling Technology), JNK (G151-666 for total JNK; BD Bioscience; rabbit polyclonal antibody for phosphorylated JNK; Cell Signaling Technology), AKT (11E7 for total AKT; Cell Signaling Technology; 244F9 for phosphorylated AKT; Cell Signaling Technology), GSK3 (rabbit polyclonal antibodies for total GSK3 and phosphorylated GSK3β; Acris and Abcam, respectively), and AIM (SA-1 rabbit polyclonal; ref. 6). Neutralization of CD36 was performed using antimouse CD36 antibody (JC63.1 mouse IgA; Abcam) and control mouse IgA (Sigma). The TIRAP inhibitor was purchased from Imgenex.

Purification of rAIM. HEK293T cells were transfected with a pCAGGS-mAIM-HA plasmid and cultured in a serum-free culture medium (FreeStyle293 Expression Medium; Invitrogen) with 0.1% FBS for 3 d. rAIM was purified from the culture supernatant using anti-HA antibody column (Roche).

Isolation of Stromal-Vascular Cell Fraction (SVF) and FACS Analysis. SVF isolation was performed as previously described with some modifications. Briefly, mice were killed after an anesthesia and systemic heparinization. After a PBS infusion, epididymal fat pads were collected, minced into small pieces, washed in PBS containing heparin (1 U/ml) for 1 min to remove blood cells, and then centrifuged at 1,000 *g* for 5 min. Floating pieces of adipose tissues were collected and then incubate for 30 min in type 2 collagenase solution (2 mg/mL; type 2 collagenase purchased from Calbiochem dissolved in PBS). Thereafter, the digested tissue was centrifuged at 1,000 *g* for 5 min, and the resultant pellet containing enriched SVF was washed twice in a FACS wash buffer (PBS supplemented with 5% FBS), and filtered using a 0.45-μm mesh. The SVF cells, whose Fc receptors were blocked using antimouse CD16/32, were stained with APC-conjugated antimouse CD11b, eFluor 450-conjugated antimouse CD11c, and Alexa Fluor 488-conjugated antimouse MR, thereafter, analyzed using BD LSD II flow cytometer (Becton Dickinson). The numbers of M1 or M2 macrophages were calculated by multiplying the number of trypan blue negative living SVF cells by the proportion of each cell type.

In Vitro Adipogenesis and Preparation of Conditioned Medium. Differentiation of 3T3-L1 cells was carried out as described. Briefly, 2-d postconfluent 3T3-L1 preadipocytes were incubated in DMEM containing 1 μg/mL insulin, 1 μM DEX, and 0.5 mM IBMX, 10% FBS, for 2 d (from day 0 to day 2). The cells were then maintained in a maintenance medium (DMEM with 10% FBS and 1 μg/mL insulin) for the following 2 d (until day 4), and thereafter, insulin was removed from the culture medium. Culture medium was changed to a fresh one every 2 d. rAIM (25 μg/mL) or C75 (25 μM) was added to the day 6 adipocytes and cultured for the following 3 d without changing medium. The resulting culture supernatant was used as AIM-CM or C75-CM. As a control, supernatant from cells cultured from day 6 until day 9 (3 d) was used as none-CM. These CMs were directly used as stimulants for macrophage migration assay, IκBα degradation assay, or chemokine production assay.

Macrophage Chemotaxis Assay. The chemotaxis assay was performed using CytoSelect 96-well Cell Migration Assay kit (Cell Biolabs). Briefly, 1×10^6 cells per well were placed in the upper chamber with polycarbonate membrane at the bottom, and respective chemoattractant was added in the lower chamber. After incubation at 37 °C for 5 h, migrated cells were detached from the membrane, lysed, colored by CyQuant GR dye, and the relative fluorescence unit (RFU) was analyzed by a fluorometer at 480 nm/520 nm.

ELISA for Chemokine. For ELISA, commercially available ELISA kits were used as follows: MCP-1 (Thermo Scientific), MCP-2 (Immuno-Biological Laboratories), MCP-3 (eBioscience), CCL-5/RANTES (eBioscience), IL-6 (R&D Systems), TNFα (Shibayagi), and insulin (Mercodia). ELISA was performed according to the manufacturer's protocol.

Insulin Sensitivity Assay. Mice fasted for 5 h were challenged with 10 U/kg by i.p. injection. Fifteen minutes later, mice were killed, and epididymal fat, gastrocnemius, and liver tissues were used for immunoblotting. For GTT and ITT, mice fasted for 16 or 5 h were i.p. injected with 3 g/kg of glucose or 0.75 U/kg of insulin, respectively. Thereafter, at indicated time points, blood glucose levels were measured.

Insulin Secretion Assay. Isolation of islets from mice of each genotype was carried out as described previously with some modifications (7). After clamping the common bile duct at a point close to its opening into the duodenum, 2 mL Krebs-Ringer bicarbonate buffer containing collagenase (Sigma) was injected into the pancreatic duct. The swollen pancreas was removed and incubated at 37 °C for 30 min. The pancreas was dispersed by pipetting, and dispersed cells were washed twice with Krebs-Ringer bicarbonate buffer. Islets were manually collected through a stereoscopic microscope. To measure insulin secretion by isolated islets, islets were incubated with Krebs-Ringer bicarbonate buffer containing 2.8 mM glucose (basal glucose concentration) at 37 °C for 1 h. Preincubation was performed 15 islets per tube. After preincubation, islets were stimulated with Krebs-Ringer bicarbonate buffer containing 5.6 mM and 20 mM glucose, respectively, at 37 °C for 30 min. Insulin concentration was determined with insulin ELISA kit (Mercodia).

siRNA Transfection. A total of 150 nM of the ON-TARGET plus siRNA targeting the mouse sequence of TLR4 and a control Non-Targeting siRNA (both purchased from Thermo Scientific) were transfected into differentiated 3T3-L1 adipocytes (day 8) using X-tremeGENE siRNA Transfection Reagent (Roche). Twenty-four hours after transfection, cells were challenged with rAIM, and harvested after 72 h for analysis of *MCP-1* expression.

Quantitative PCR Assay. The quantitative evaluation of mRNA was performed by the $\Delta\Delta C_T$ method using a 7500 Fast Real-Time PCR system (Invitrogen). Values were normalized to those of GAPDH. Sequences of the oligonucleotides used are below:

Name	Sequence (5′–3′)
f-GAPDH	AACTTTGGCATTGTGGAAGG
r-GAPDH	GGATGCAGGGATGATGTTCT
f-F4/80	CCTGGACGAATCCTGTGAAG
r-F4/80	GGTGGGACCACAGAGAGTTG
f-CD11c	GAGCCAGAACTTCCCAACTG
r-CD11c	TCAGGAACACGATGTCTTGG
f-iNOS	CCAAGCCCTCACCTACTTCC
r-iNOS	CTCTGAGGGCTGACACAAGG
f-CD163	CCTGGATCATCTGTGACAACA
r-CD163	TCCACACGTCCAGAACAGTC
f-MR	CCACAGCATTGAGGAGTTTG
r-MR	ACAGCTCATCATTTGGCTCA
f-Arg-1	CTCCAAGCCAAAGTCCTTAGAG
r-Arg-1	AGGAGCTGTCATTAGGGACATC
f-MCP1	CATCCACGTGTTGGCTCA
r-MCP1	GATCATCTTGCTGGTGAATGAGT
f-MCP2	TTCTTTGCCTGCTGCTCATA
r-MCP2	AGCAGGTGACTGGAGCCTTA
f-MCP3	TTCTGTGCCTGCTGCTCATA
r-MCP3	TTGACATAGCAGCATGTGGAT
f-CCL5/RANTES	GTGCCCACGTCAAGGAGTAT
r-CCL5/RANTES	TCCTTCGAGTGACAAACACG
f-MIP1α	CAAGTCTTCTCAGCGCCATA
r-MIP1α	GGAATCTTCCGGCTGTAGG
f-IL1β	TGTAATGAAAGACGGCACACC
r-IL1β	TCTTCTTTGGGTATTGCTTGG
f-IL6	GATGGATGCTACCAAACTGGA
r-IL6	CCAGGTAGCTATGGTACTCCAGAA
f-TNFα	TCTTCTCATTCCTGCTTGTGG
r-TNFα	GGTCTGGGCCATAGAACTGA

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Fig. S1. Attenuated M1 polarization of adipose tissue macrophage in obese $AIM^{-/-}$ mice. (*A*) The SVF cells isolated from the epididymal fat tissue of lean and obese $AIM^{+/+}$ or $AIM^{-/-}$ mice, stained for F4/80 and CD11b (macrophage), CD11c (M1 marker), and MR. Thereafter, the number of M1 and M2 macrophages was determined by flow cytometry. A representative gating strategy for F4/80/CD11b/CD11c/MR is demonstrated (*Left*). Numbers of $F4/80^{+}CD11b^{+}$ total macrophages, $F4/80^{+}CD11b^{+}CD11c^{+}MR^{-}$ M1 macrophages, and $F4/80^{+}CD11b^{+}CD11c^{-}MR^{+}$ M2 macrophages present in an entire epididymal fat tissue are presented. (*B*) The ratio of M1 versus M2 macrophage number. $n = 6–8$ mice in each group. Averages ± SEM. (*C*) mRNA levels for *CD11c*, *iNos* (M1 markers), *CD163*, *MR*, and *Arginase* (M2 markers) were assessed by QPCR using RNA isolated from epididymal fat obtained from lean or obese $AIM^{+/+}$ and $AIM^{-/-}$ mice; $n = 6–8$ for each group. Values were normalized to those of GAPDH and presented as relative expression to those of lean $AIM^{+/+}$ mice. Error bar: SEM.

Fig. S2. Comparable apoptotic state in $AIM^{+/+}$ and $AIM^{-/-}$ adipose tissues. Epididymal fat tissues from $AIM^{+/+}$ and $AIM^{-/-}$ mice fed a HFD for 12 wk were challenged with a TUNEL staining. A comparison of $TUNEL^{+}$ cell number in $AIM^{+/+}$ and $AIM^{-/-}$ adipose tissues is also present. No significant difference in number of $TUNEL^{+}$ cell was observed. $n = 3–4$ for each. At least three different areas in three different sections per mouse were analyzed. Error bar: SEM.

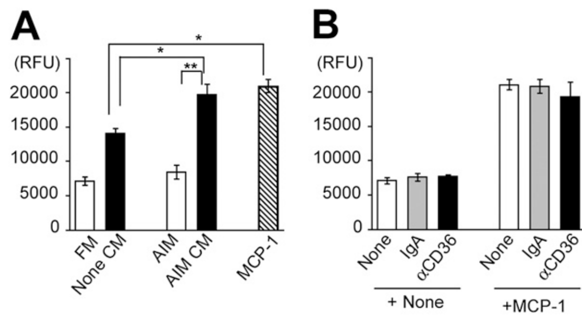

Fig. S3. AIM CM attracts both macrophage and monocyte. (*A*) Chemotaxis of J774.1 monocyte cells in response to specified stimulant. Attractants: rAIM (25 μg/mL), AIM CM, none CM, and FM (fresh DMEM culture medium containing 10% FBS). Averages from $n = 3 \pm$ SEM. MCP-1 (100 ng/mL) was used as a positive control. (*B*) To confirm that the anti-CD36 antibody itself has no direct effect on macrophage recruitment, chemotaxis of RAW 264.1 cells in response to MCP-1(100 ng/mL) was assessed in the presence of this antibody or control mouse IgA (10 μg/mL each). No influence of anti-CD36 antibody or mouse IgA to RAW 264.1 chemotaxis in response to MCP-1 was detected (*Right* three bars; +MCP-1). In addition, chemotaxis of RAW 264.1 cells in response to anti-CD36 antibody or mouse IgA was not observed (*Left* three bars; +none). Averages from $n = 3 \pm$ SEM.

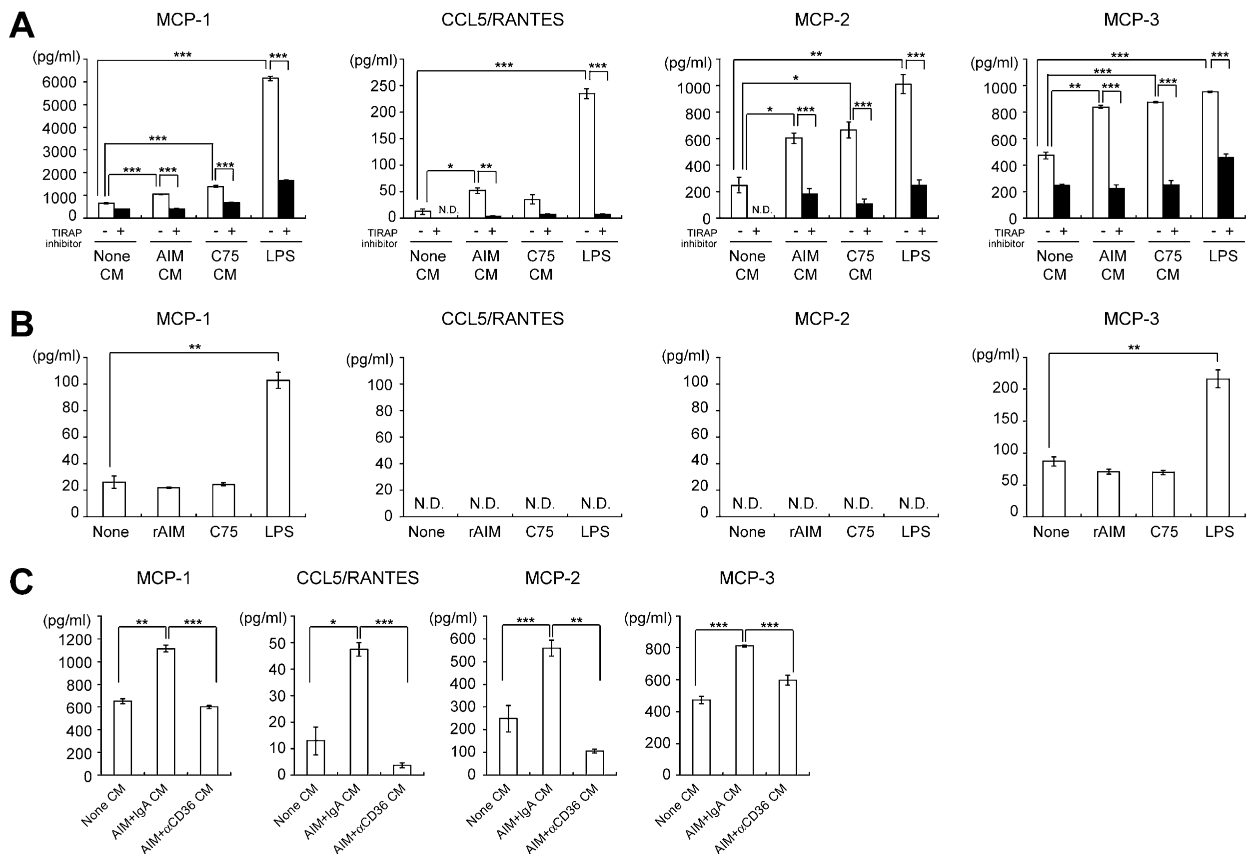

Fig. S4. ELISA analysis for chemokine production by adipocytes. (*A*) Protein levels for *MCP-1*, *CCL5/RANTES*, *MCP-2*, and *MCP-3* produced by 3T3-L1 adipocytes (day 6) in response to a treatment with AIM CM, C75 CM, or none CM in the absence (–) or presence (+) of a TIRAP inhibitor (25 μM) for 3 d. As a positive control, cells were treated with LPS (100 ng/mL) for 3 h in the absence or presence of a TIRAP inhibitor. $n = 3 \pm$ SEM. (*B*) Chemokine production by 3T3-L1 adipocytes in response to a treatment with rAIM (25 μg/mL) or C75 (25 μM) alone, LPS (100 ng/mL), or no stimulant (none) for 30 min. Note that such a short time (30 min) treatment by LPS was sufficient to induce a significant amount of MCP-1 or MCP-3 protein, but not of MCP-2 or CCL5/RANTES protein. Neither rAIM nor C75 alone induced any of the chemokines. $n = 3 \pm$ SEM. (*C*) Chemokine production by 3T3-L1 adipocytes in response to a treatment with AIM+αCD36 CM, or AIM+IgA CM, or none CM. $n = 3 \pm$ SEM. AIM CM/C75 CM: conditioned medium from 3T3-L1 adipocytes treated for 3 d with rAIM (25 μg/mL) or C75 (25 μM) respectively; AIM+αCD36 CM/AIM+IgA CM: conditioned medium from 3T3-L1 adipocytes treated for 3 d with rAIM (25 μg/mL) in the presence of anti-CD36 antibody or mouse IgA (10 μg/mL each), respectively; and none CM: control CM from 3T3-L1 adipocytes treated without rAIM or C75.

Fig. S5. Stimulation of TLR4 and chemokine production in 3T3-L1 adipocytes by saturated fatty acids. (*A*) Degradation of IkBα in 3T3-L1 adipoctytes in response to palmitic or stearic acid in the absence (–) or presence (+) of a TIRAP inhibitor. PA, palmitic acid; SA, stearic acid; and none, no treatment. Representative immunoblotting results from three independent experiments are presented. The density of the signal was quantified using National Institutes of Health Image J image analysis software and presented as values relative to those of prestimulation (*Bottom* two panels). $n = 3$. Error bar: SEM. *, versus the value at prestimulation (0 min). (*B*) QPCR analysis of mRNA levels for *MCP-1*, *CCL5/RANTES*, *MCP-2*, and *MCP-3* using RNA isolated from 3T3-L1 adipocytes treated with specified stimulant for 24 h in the absence (white bars) or presence (black bars) of a TIRAP inhibitor (100 μM). Values were normalized to those of GAPDH and presented as relative expression to those without stimulation (none). $n = 3$ for each. Error bar: SEM.

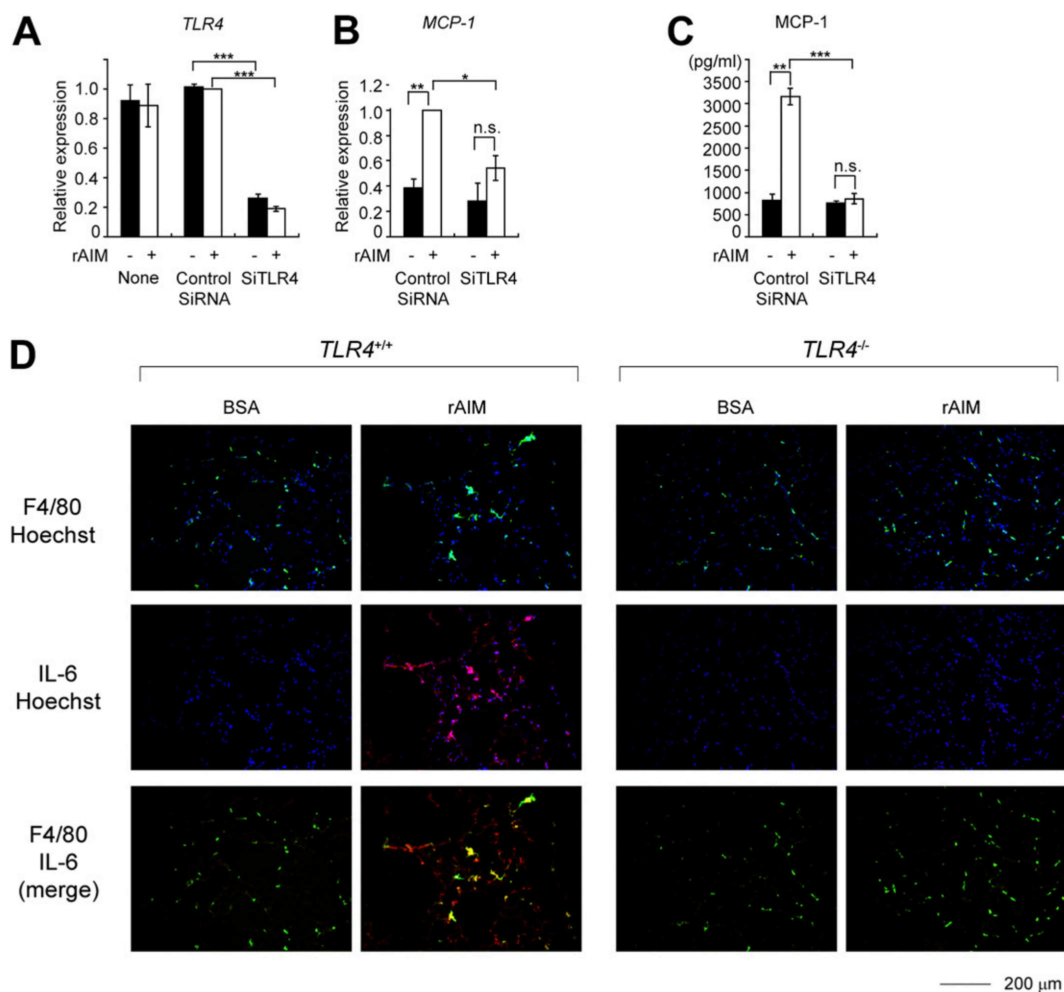

Fig. S6. Suppression of *TLR4* attenuated chemokine expression induced by AIM-dependent lipolysis. 3T3-L1 adipocytes (day 6) were tranfected with siRNA for *TLR4* or *GFP* (control) and thereafter treated with rAIM for 3 d. Cells were harvested and analyzed for the mRNA level for (*A*) *TLR4* and (*B*) *MCP-1*, by QPCR. Values were normalized to those of GAPDH and presented as relative expression to those without stimulation (none). (*C*) Resulting conditioned medium was assessed for MCP-1 protein by ELISA. None, no transfection of siRNA (*A*). $n = 3$ for each. Error bar: SEM. (*D*) $TLR4^{-/-}$and wild-type littermate mice ($TLR4^{-/-}$) were i.v. injected with rAIM or BSA three times every other day (400 μg in 200 μL PBS per injection). The day after the third injection (day 8 from the first injection), mice were killed, and infiltration of inflammatory macrophages in epididymal fat tissue was evaluated by staining specimens for F4/80 (green), IL-6 (red), and Hoechst (blue); $n = 5$ for each group. At least three different areas in three different sections per mouse were analyzed.

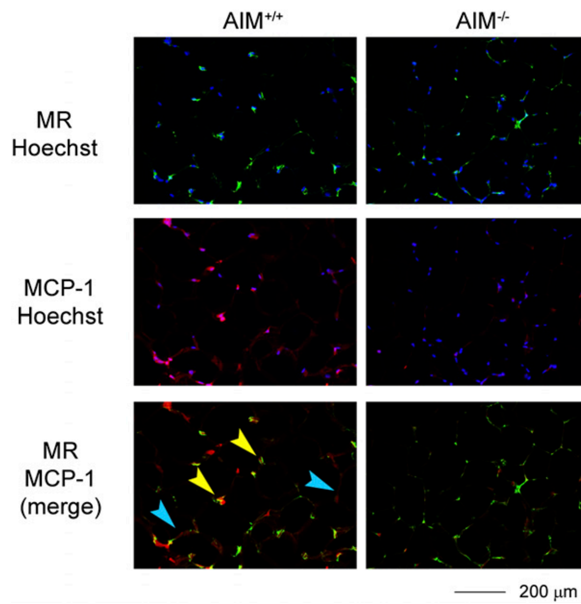

Fig. S7. Both adipocytes and resident M2 macrophages express MCP-1 in mice fed with a HFD. Epididymal fat tissue from wild-type $AIM^{+/+}$ or $AIM^{-/-}$ mice fed a HFD for 6 wk were stained for MR (green), MCP-1 (red), and Hoechst (blue). Both adipocytes (blue arrows) and MR^+ M2 macrophages (yellow arrows) expressed MCP-1. *n* = 5. At least three different areas in three different sections per mouse were analyzed.

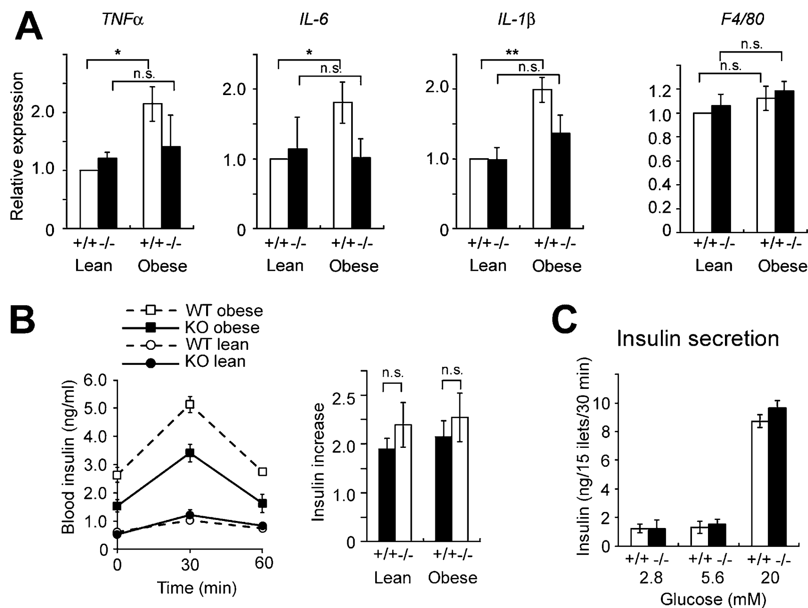

Fig. S8. Prevented inflammation in the liver and normal insulin secretion in obese $AIM^{-/-}$ mice. (*A*) QPCR analysis of mRNA levels for inflammatory cytokine genes and a macrophage marker F4/80 in the liver from $AIM^{+/+}$ or $AIM^{-/-}$ mice fed a HFD for 0 (lean) or 12 wk (obese). *n* = 6–8 for each group. Values were normalized to those of GAPDH and presented as relative expression to that in lean $AIM^{+/+}$ mice. Error bar: SEM. $AIM^{+/+}$ and $AIM^{-/-}$ mice fed a HFD for 0 (lean) or 12 wk (obese); *n* = 6–8 for each group. For ITT, two panels including absolute blood glucose levels (*Left*) and percentage of the initial (time 0) glucose level (*Right*) are presented. (*B*) Blood insulin levels during GTT performed on $AIM^{+/+}$ and $AIM^{-/-}$ mice fed a HFD for 0 (lean) or 12 wk (obese); *n* = 6–8 for each group (presented in Fig. 4*D*). Ratios of insulin increase in 30 min after the injection of glucose are also presented (*Right* graph). (*C*) Insulin secretion by pancreatic islets (15 islets per experiment) in response to different concentrations of glucose, isolated from $AIM^{+/+}$ or $AIM^{-/-}$ mice fed a HFD for 12 wk; *n* = 6–7 for each group. Error bar: SEM.

Fig. S9. A scheme for the putative role of AIM in establishment of adipose tissue inflammation and insulin resistance. The augmentation in blood AIM levels induces vigorous lipolysis in obese adipose tissues, increasing local extracellular fatty acid concentration to a sufficient level for stimulation of TLR4, which results in chemokine production by adipocytes, leading to macrophage recruitment. In *AIM*$^{-/-}$ mice, however, lipolysis cannot reach a sufficient level that influences macrophage infiltration. ATM, adipose tissue macrophage; FA, fatty acid.