Adipocyte-secreted IL-6 sensitizes macrophages to IL-4 signaling

Short running title: Adipocyte IL-6 signaling to macrophages

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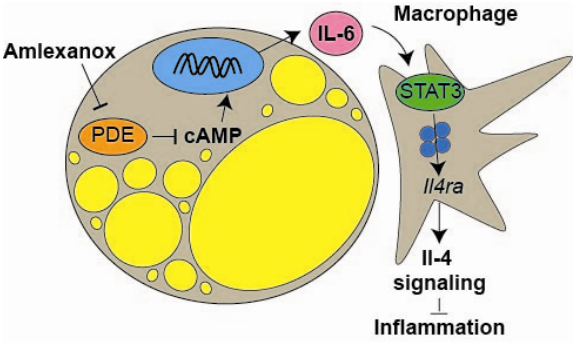
Abstract

Complex bidirectional crosstalk between adipocytes and adipose tissue immune cells plays an important role in regulating adipose function, inflammation, and insulin responsiveness. Adipocytes secrete the pleiotropic cytokine IL-6 in response to both inflammatory and catabolic stimuli. Previous studies suggest that IL-6 secretion from adipocytes in obesity promotes adipose tissue inflammation. Here we investigated catabolic stimulation of adipocyte IL-6 secretion and its impact on adipose tissue inflammation. In obesity, catecholamine resistance reduces cAMP-driven adipocyte IL-6 secretion in response to catabolic signals. By restoring adipocyte catecholamine sensitivity in obese adipocytes, amlexanox stimulates adipocyte-specific IL-6 secretion. Here we report that in this context, adipocyte secreted IL-6 activates local macrophage STAT3 to promote *Il4ra* expression, thereby sensitizing them to IL-4 signaling, and promoting a more anti-inflammatory phenotype. Supporting a paracrine adipocyte to macrophage mecnaism, these effects could be recapitulated using adipocyte conditioned media to pretreat bone marrow derived macrophages prior to polarization with IL-4. This cross talk may contribute to reduction in adipose tissue inflammation observed with amlexanox treatment in vivo. The effects of IL-6 signaling in the adipose tissue are complex and context specific. These results suggest that restoration of cAMP driven IL-6 secretion from obese adipocytes may reduce adipose tissue inflammation.

Graphic abstract



Chronic low-grade inflammation has been implicated in many of the common co-morbidities associated with obesity, including type 2 diabetes and non-alcoholic steatohepatitis. The non-canonical I-κB kinases IKKε and TBK1 are induced by chronic inflammation in adipocytes and perpetuate obesity by affecting catecholamine resistance via the activation of phosphodiesterase-3B (PDE-3B) [1-4]. Treatment of obese mice with amlexanox, a dual specific inhibitor of IKKε and TBK1, results in improved metabolic health via weight loss and resolution of adipose tissue inflammation [2]. By restoring cAMP signaling in adipocytes, amlexanox promotes the expression of IL-6 [5]. Adipocyte-secreted IL-6 activates hepatic STAT3 to suppress the expression of *G6pc* and gluconeogenesis [6]. However, the impact of amlexanox-induced adipocyte IL-6 secretion on adipose tissue inflammation has not been investigated.

In this study, we found that in vivo amlexanox treatment resulted in an IL-6 dependent Tyr705 phosphorylation of STAT3 in adipose tissue macrophages. The activation of STAT3 in macrophages resulted in upregulation of *Il4ra*, and sensitization to IL-4 and its anti-inflammatory effects. This effect could be recapitulated in vitro by treating bone marrow derived macrophages (BMDM) with conditioned media from adipocytes treated with amlexanox. These results suggest that adipocyte-secreted IL-6 may play a role in the anti-inflammatory effects of amlexanox treatment in vivo. This anti-inflammatory effect may be generalizable to adipocyte IL-6 secretion downstream of cAMP activation by physiological signals such as catecholamine signaling.

Research Design and Methods

Animals.

The following strains of mice from Jackson Labs were bred for littermate controlled experiments: C57BL/6J (RRID:IMSR_JAX:000664) *Stat3* floxed (RRID:IMSR_JAX:016923), LysM-cre (RRID:IMSR_JAX:004781), Adipoq-cre (RRID:IMSR_JAX:010803). Amlexanox was administered by daily oral gavage at a dose of 25 mg/kg to obese male mice. Mice were housed in a specific pathogen-free facility with a 12-h light, 12-h dark cycle, and given free access to food and water. All animal use was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California-San Diego and at the University of Michigan.

Stromal vascular cells (SVC) and mature adipocytes were isolated from adipose tissue by collagenase digestion followed by centrifuge at 500 *g* for 5 min. Serum IL-6 levels were quantified using Mouse IL-6 Quantikine ELISA from R&D (SM6000B) with 50 µl of serum or media.

Cells.

3T3-L1 media conditioning. Media from differentiated 3T3-L1s treated with 100 µM amlexanox or vehicle in RPMI 1640 was collected after 4 hours. For neutralization experiments, conditioned media were incubated with IL-6 NA 10 µg/mL (R&D Systems Cat# MAB406, RRID:AB_2233899) or normal goat IgG control antibody 10 µg/mL (R&D Systems Cat# AB-108-C, RRID:AB_354267) for 15 min.

BMDM. Dispersed bone marrow cells from 6-8 week old male mice were placed in culture media (RPMI 1640, 10% FBS, MCSF 20 ng/mL (Peprotech, no. 315-02), 20 mM HEPES, 2 mM L-Glutamine, and 1 mM Sodium Pyruvate). On day 6, cells were incubated with 50 ng/mL IL-6

(R&D, no. 406-ML-025) or conditioned medias prior to the addition of 10 ng/mL IL-4 (R&D, no. 404-ML-025/CF) on day 7 for either 30 minutes for western blot or 24 hours for qPCR.

Fluorescence activated cell sorting (FACS).

Adipose tissue macrophages were identified as $CD45^+$, $CD11c^+$, $Emr1^+$, $CD31^-$ were sorted into triazole for gene expression analysis. Each animal was sorted separately. BMDM identity confirmed by Emr1 and CD11b dual positivity. The following antibodies were purchased from Thermo Fisher Scientific: FC block (CD16/32) (14-0161-82, RRID:AB_467133), FITC conjugated anti-CD45(30-F11) (11-0451-82, RRID:AB_465050), Pacific Blue conjugated anti-CD45(30-F11) (MCD4528, RRID:AB_10373710), Brilliant Violet conjugated anti-CD45 (103147, RRID:AB_2564383), PE conjugated anti-CD64 (12-0641-82, RRID:AB_2735014), APC conjugated anti-CD11c (17-0114-82, RRID:AB_469346), Percp-Cy5 conjugated anti-CD3 (45-0031-82, RRID:AB_1107000), PE-Cy7 conjugated anti-CD31 (25-0311-82, RRID:AB_2716949), APC-Cy7 conjugated anti-LysG (25-9668-82, RRID:AB_2811793), eFluor-450 conjugated pTyr705 STAT3 (48-9033-42, RRID:AB_2574121). The following antibodies were purchased from BioLegend: APC-Cy7 conjugated anti-CD11c (117324, RRID:AB_830649), APC conjugated anti-Emr1 (123116, RRID:AB_893481), APC/Fire conjugated anti-CD11b (101262, RRID:AB_2572122), and PE/Cy7 conjugated anti-CD31 (102418, RRID:AB_830757). Alexa Fluor 647 conjugated anti-CLEC10A/CD301, from Novus Biologicals, (64874AF647, RRID: 2915978).

Western blot analysis.

Primary antibodies were purchased from Cell Signaling Technology: pTyr-705 STAT3 1:1000 (9145, RRID:AB_2491009), STAT3 1:4000 (9139, RRID:AB_331757) STAT6 (9362, RRID:AB_2271211), pTyr-641 STAT6 1:1000 (56554, RRID:AB_2799514) and β-Tubulin

1:1000 (2146, RRID:AB_2210545). Secondary antibodies were purchased from Thermo Fisher Scientific: Goat anti-mouse 1:10,000 (31430, RRID:AB_228307) and goat anti-rabbit 1:10,000 (31460, RRID:AB_228341).

Immunohistochemistry.

After blocking with 5% goat serum, the slides were incubated overnight with 1:200 anti-pTyr705 STAT3, and detected with DAB.

Data and Resource Availability.

All data generated or analyzed during this study are included in the published article (and its online supplementary files). No applicable resources were generated or analyzed during the current study.

Results

We have previously shown that amlexanox treatment of obese mice acutely promotes adipocyte IL-6 secretion via cAMP signaling [6]. Four hours after oral gavage with amlexanox, when serum IL-6 levels peak, Tyrosine 705 phosphorylation (pTyr705) of STAT3 is elevated in adipose tissue relative to vehicle control (Fig. 1A). Immunohistochemical analysis revealed many pTyr705 STAT3 positive nuclei in crownlike structures (Fig. 1B). Knockout (KO) of STAT3 in adipocytes reduced total adipose tissue levels of STAT3 but did not reduce relative STAT3 phosphorylation (Fig. 1C). To investigate STAT3 phosphorylation in immune cells, the stromal vascular cells (SVC) were collected and analyzed for pTyr705 STAT3 by fluorescence activated cell sorting (FACS). Amlexanox specifically increased STAT3 phosphorylation levels in both $Cd11c^{+}$ and $Cd11c^{-}$ adipose tissue macrophages (ATMs) (Fig. 1D-F). No effect was observed in neutrophils, dendritic cells, or T cells (Fig. 1G-I).

To determine whether the phosphorylation of STAT3 in macrophages is dependent on IL-6 we utilized whole body *Il6* KO animals. While littermate controls demonstrate the expected increase in serum IL-6 following amlexanox treatment, IL-6 levels were undetectable in the *Il6* KO animals (Fig. 2A). We have previously shown that hepatic STAT3 is activated by adipocyte-secreted IL-6 following amlexanox treatment [6]. As expected, amlexanox treatment induced hepatic STAT3 phosphorylation in the WT but not *Il6* KO animals (Fig. 2B and C). In the white adipose tissue of amlexanox treated WT controls, STAT3 phosphorylation was prominent in crown-like structures (Fig. 2D). However, in the *Il6* KO animals, no increase in STAT3 positive nuclei was observed with amlexanox treatment (Fig. 2D and E). Accordingly, the expression of the STAT3 target gene *Socs3* was elevated in the white adipose tissue of amlexanox-treated WT mice relative to vehicle control, while there was no effect of amlexanox treatment in the *Il6* KO

animals (Fig. 2F). The increase in macrophage STAT3 phosphorylation by amlexanox was dependent on IL-6, as it was not observed in the adipose tissue macrophages from *Il6* KO mice (Fig. 2G-K).

It has been previously demonstrated that IL-6 stimulation in macrophages activates STAT3-mediated expression of *Il4ra*, thereby sensitizing the cells to anti-inflammatory effects of IL-4 [7, 8]. Using bone marrow-derived macrophages (BMDM), we confirmed that IL-6 treatment increases the expression of *Il4ra* (Supplementary Fig. 1A), and the phosphorylation of STAT6 in response to IL-4 treatment (Supplementary Fig. 1B). IL-6 pretreatment increased the induction of *Arg1* by IL-4 (Supplementary Fig. 1C) and suppressed the expression of *Il1b* (Supplementary Fig. 1D).

To determine whether IL-6 secreted from amlexanox-treated adipocytes may function as a paracrine signal to adipose tissue macrophages, we treated BMDM with conditioned media from amlexanox-treated 3T3-L1 adipocytes (Fig. 3A). Amlexanox conditioned media, but not direct amlexanox treatment, increased the percentage of $Cd301^{+}$ cells in BMDM polarized with IL-4 (Fig. 3B). While conditioned media from adipocytes treated with vehicle control (VCM) did not significantly increase *Il4ra* expression in BMDM, conditioned media from adipocytes treated with amlexanox (ACM) increased *Il4ra* expression six-fold over non-conditioned control media (NCM) (Fig. 3C). Conditioned media from amlexanox-treated, but not vehicle-treated, adipocytes increased *Arg1* and decreased *Cd11c* expression in BMDM polarized with IL-4 (Fig. 3D and E). Importantly, conditioned media from adipocytes treated with amlexanox did not promote anti-inflammatory macrophage polarization in STAT3 KO BMDM (from LysM-Cre driven myeloid cell specific STAT3 KO (SMKO) mice), indicating that the effects of the

amlexanox-conditioned media on macrophage polarization were mediated through STAT3 (Fig. 3B).

To confirm that IL-6 in the adipocyte conditioned media mediates the effects of amlexanox, we treated the conditioned media with IL-6 neutralizing antibodies (IL-6NA), or IgG antibody control (Fig. 3F and Supplementary Fig. 2). Neutralization of IL-6 prevented the induction of STAT3 phosphorylation and blocked the induction of *Il4ra* in BMDM treated with amlexanox conditioned media (Fig. 3G and H). Accordingly, neutralization of IL-6 in the ACM blocked its ability to promote *Arg1* expression in IL-4 polarized macrophages (Fig. 3I). These results suggest that IL-6 secreted from adipocytes sensitizes macrophages to anti-inflammatory polarization via IL-4 signaling.

To determine if in vivo amlexanox treatment results in similar IL-4 sensitization in adipose tissue macrophages, we measured gene expression in mature adipocytes and adipose tissue macrophages isolated from mice 8 hours after a single dose of amlexanox or vehicle control. These experiments were performed in SMKO mice and floxed WT littermate controls. The reduction in *Adrb3* expression (due to feedback inhibition) by amlexanox treatment was significant in both the WT and SMKO mature adipocytes, with no significant differences between the genotypes (Fig. 4A). However, amlexanox treatment increased *Il4ra* expression in WT but not SMKO adipose tissue macrophages relative to vehicle control (Fig. 4B). Consistent with sensitization to IL-4 signaling in vivo, amlexanox treatment in WT mice also increased the expression of *Il10* in adipose tissue macrophages (Fig. 4C). Again, this effect was absent in the adipose tissue macrophages of SMKO mice treated with amlexanox (Fig. 4C). These data support a model in which adipocyte-secreted IL-6 resulting from oral amlexanox treatment plays a paracrine role activating local macrophage STAT3, which in turn upregulates the expression of

Il4ra, thereby increasing the sensitivity of the macrophages to anti-inflammatory IL-4 signaling (Fig. 4D).

To confirm the anti-inflammatory shift in adipose tissue macrophages in vivo, we, performed FACS analysis of macrophages in adipose tissue after amlexanox treatment. The frequency of pro-inflammatory $Cd11c^{+}$ adipose tissue macrophages decreased with amlexanox treatment in WT but not SMKO mice, again indicating dependence on macrophage STAT3 (Fig. 4E and F). Notably, we did not observe any change in the expression of macrophage recruiting factor *Mcp1* in the adipocytes from mice treated with amlexanox versus vehicle control (Fig. 4G). Overall, these data support a macrophage STAT3 mediated anti-inflammatory effect of adipocyte-secreted IL-6 during the acute response to amlexanox treatment.

Discussion

IL-6 has long been implicated in the pathogenesis of type 2 diabetes, as chronically elevated serum levels of IL-6 are associated with increased risk of developing type 2 diabetes in obese individuals [9-11]. Both adipocytes and cells in the stromal vascular fraction contribute to obesity-associated increases in adipose tissue IL-6 expression and secretion [12, 13]. Studies in obese adipocyte-specific *Il6* KO mice have suggested that adipocyte-secreted IL-6 promotes adipose tissue inflammation or has no net effect [13-15]. Importantly, obese adipocytes are characterized by increased inflammation and reduced cAMP signaling due to catecholamine resistance. In this context, other proinflammatory cytokines including TNFα are also secreted from adipocytes, which may shift the balance of adipocyte to macrophage crosstalk towards proinflammatory signaling.

Here, we report a pathway whereby amlexanox-induced IL-6 secretion from adipocytes reduces local macrophage inflammatory status. Importantly, we did not observe activation of this signaling pathway or anti-inflammatory effects in macrophages treated directly with amlexanox, although direct effects have been reported [16-18]. TBK1 has been shown to have anti-inflammatory effects, suggesting potential proinflammatory effects of direct TBK1 inhibition by amlexanox [19, 20]. Indeed, there are likely multiple pathways by which amlexanox affects adipose tissue inflammation of obese animals. Amlexanox-induced weight loss is a likely contributor to reduced adipose tissue inflammation with long-term amlexanox treatment [2, 5]. Interestingly, cAMP-driven adipocyte FGF21 secretion promotes adipose tissue beiging and contributes to amlexanox induced weight loss [5, 21]. Future work examining the interplay between the various adipokines secreted in different physiologic contexts will be important for understanding how adipocytes communicate both locally and systemically to impact metabolism

and inflammation in health and disease. The adipocyte to macrophage communication axis described here is just one of the signaling pathways regulating adipose tissue inflammation and metabolism downstream of catabolic stimulation of adipocytes.

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Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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Figure Legends

Fig. 1. STAT3 phosphorylation in adipose cells after amlexanox treatment. (A, C) Western blot analysis of STAT3 and Tyr705 phosphorylated STAT3 (pSTAT3) in epididymal and inguinal white adipose tissue (eWAT and iWAT respectively) isolated from male mice treated with amlexanox for 4 hours. (A) WT C57BL/6J mice. (B) Immunohistochemical analysis of pTyr705 STAT3, brown DAB staining. Slides also stained with H&E. Tissues harvested and immediately fixed 4 hours after amlexanox gavage, scale bar = 50 μm. (C) Adipocyte specific STAT3 KO mice and floxed littermate controls. (D-I) FACS analysis of the percent positivity of pTyr705 STAT3 in SVC populations. (D) All Cd45 positive immune cells. (E) Proinflammatory adipose tissue macrophages = $Cd45^{+}$, $Cd64^{+}$, $Cd11c^{High}$. (F) Anti-inflammatory adipose tissue macrophages = $Cd45^{+}$, $Cd64^{+}$, $Cd11c^{Low}$. (G) Neutrophils = $Cd45^{+}$, $Ly6G^{+}$. (H) Dendritic cells = $Cd45^{+}$, $Cd64^{-}$, $Cd11c^{+}$. (I) T-cells = $Cd45^{+}$, $Cd3^{+}$. * $p < 0.05$ by student's t-test vehicle versus amlexanox.

Fig. 2. STAT3 activation in adipose tissue immune cells is IL-6 dependent. Il6 KO mice and WT littermate controls treated with amlexanox or vehicle control for 4 hours. (A) Serum IL-6 levels. (B-E) Immunohistochemical analysis of pTyr705 STAT3, brown DAB staining. Slides also stained with Hematoxylin. Tissues harvested and immediately fixed 4 hours after amlexanox gavage. (B-C) Liver. (D-E) WAT. (B and D) Representative image from each genotype and treatment, scale bar = 100 μm. (C and E) Quantification of percent positive nuclei in sections from four animals per condition (three fields of view with approximately 200 nuclei each were averaged for each animal). (F) Relative expression of *Socs3* in WAT. (G-K) FACS analysis of the percent positivity of pTyr705 STAT3 in SVC populations. (G) Proinflammatory adipose

tissue macrophages = $Cd45^+$, $Cd64^+$, $Cd11c^{High}$. (H) Anti-inflammatory adipose tissue macrophages = $Cd45^+$, $Cd64^+$, $Cd11c^{Low}$. (I) Neutrophils = $Cd45^+$, $Ly6G^+$. (J) Dendritic cells = $Cd45^+$, $Cd64^-$, $Cd11c^+$. (K) T-cells = $Cd45^+$, $Cd3^+$. Statistical significance determined by post hoc analysis after significant two-way ANOVA. * $p < 0.05$ vehicle versus amlexanox within genotype. ˜ $p < 0.05$ WT versus KO within treatment group. # $p < 0.05$ vehicle versus amlexanox within genotype using fisher's exact test.

Fig. 3. Adipocyte-secreted IL-6 sensitizing macrophages to IL-4. (A) Schematic of adipocyte media conditioning and treatment of BMDMs. (B) Percent Cd301 positive staining of Emr1/Cd11b positive BMDM treated with amlexanox directly, or conditioned media from amlexanox treated adipocytes (ACM). (C-E) Gene expression analyzed by Q-PCR in BMDM pretreated with non-conditioned media (NCM), vehicle conditioned media (VCM) or amlexanox conditioned media (ACM) for 24 h before the addition of IL-4 for another 24 hrs. (C) Relative *Il4ra* expression. (D) Relative *Arg1* expression. (E) Relative *Cd11c* expression. (F) Schematic of adipocyte media conditioning with neutralizing antibodies and administration to BMDMs. (G-I) BMDM treated with vehicle or amlexanox conditioned media in which IL-6 was neutralized with IL-6NA or IgG control. (G) Western blot analysis of STAT3 Tyr705 phosphorylation. B-Tubulin serves as a loading control. (H) Relative *Il4ra* expression. (I) Relative *Arg1* expression. Statistical significance determined by post hoc analysis after significant two-way ANOVA. * $p < 0.05$ ACM versus VCM, unless otherwise indicated by line, ˜ $p < 0.05$ ACM versus VCM. # $p < 0.05$ IL-6NA versus IgG within media condition.

Fig. 4. In vivo sensitization of macrophages to IL-4 by amlexanox requires STAT3. (A) Q-PCR analysis of *Adrb3* expression in mature adipocytes from epididymal fat. (B-C) Q-PCR analysis of gene expression in epididymal adipose tissue macrophages ($Cd45^+$, $F4/80^+$, $Cd11b^+$,

$Cd3^-$) isolated by FACS from SVC. (B) Relative *Il4ra* expression in adipose tissue macrophages. (C) Relative *Il10* expression in adipose tissue macrophages. (D) Schematic of oral amlexanox treatment and activation of this adipocyte to macrophage communication axis. (E-F) FACS analysis of SVC isolated from the epididymal fat of mice treated with amlexanox or vehicle control for 52 hours. Macrophages defined as $Cd45^+$, $F480^+$, $Cd11b^+$, $Cd3^-$ cells. (E) $Cd11c^+$ macrophages as a percentage of Cd45+ cells. (F) $Cd11c^+$ macrophages as a percentage of total macrophages. (G) Q-PCR analysis of *Mcp1* expression in mature adipocytes from epididymal fat. Statistical significance determined by post hoc analysis after significant two-way ANOVA. * $p < 0.05$ vehicle versus amlexanox within genotype. ˜ $p < 0.05$ WT versus KO within treatment group.

Figure 1

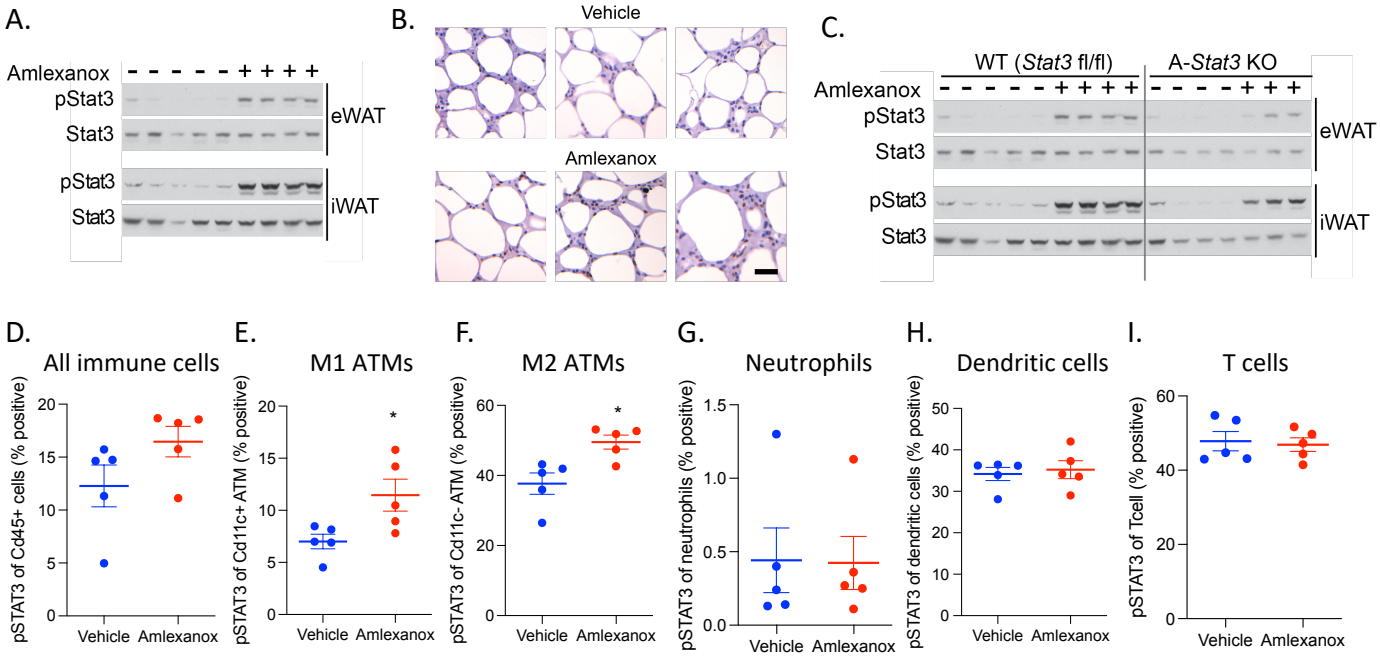
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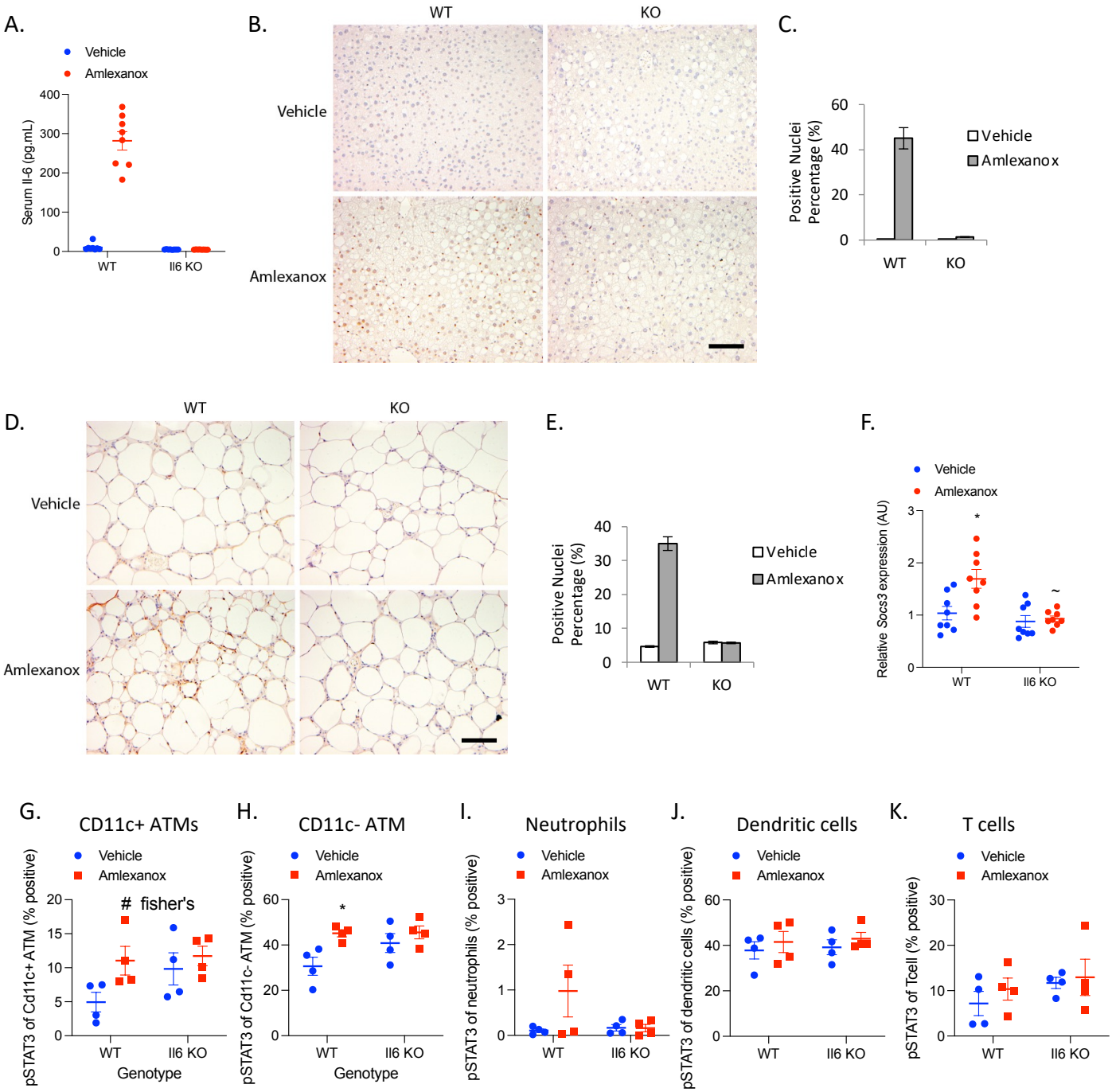
Figure 2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Vehicle
Amlexanox
*
pSTAT3 of Cd11c- ATM (% positive)
60
40
20
0
WT
Il6 KO
Genotype
I.
Neutrophils
Vehicle
Amlexanox
pSTAT3 of neutrophils (% positive)
3
2
1
0
WT
Il6 KO
J.
Dendritic cells
Vehicle
Amlexanox
pSTAT3 of dendritic cells (% positive)
60
40
20
0
WT
Il6 KO
K.
T cells
Vehicle
Amlexanox
pSTAT3 of Tcell (% positive)
30
20
10
0
WT
Il6 KO

Figure 3

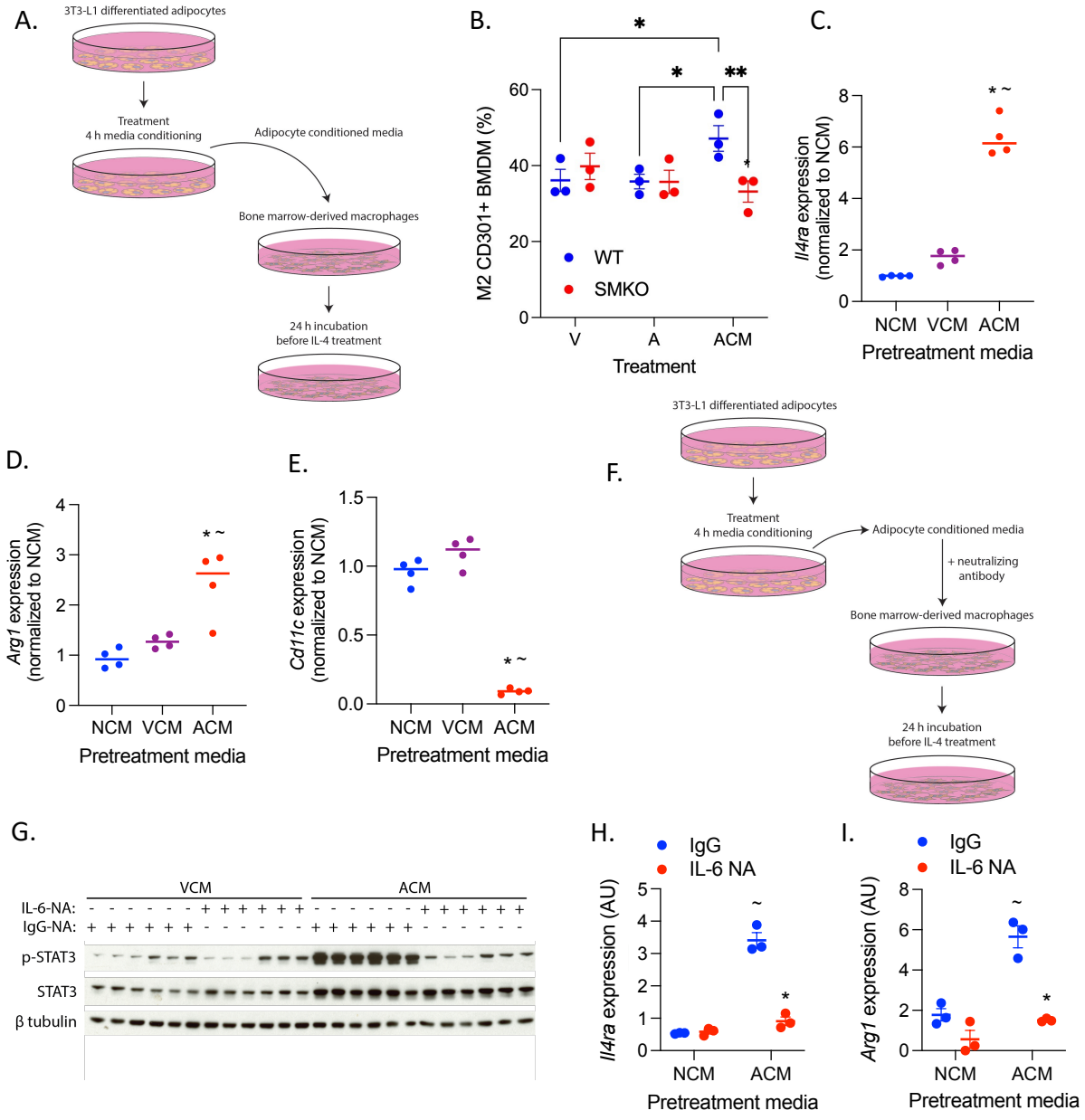
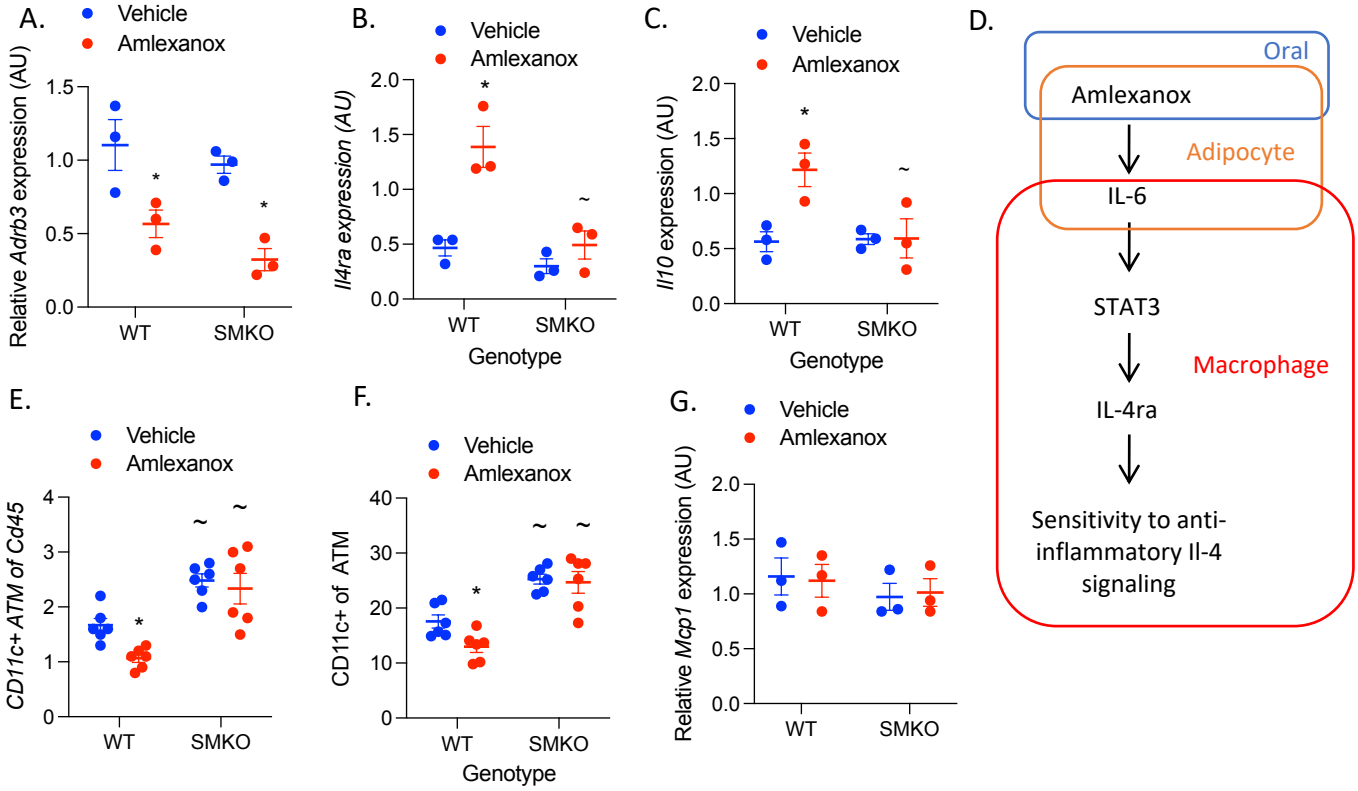

A.
3T3-L1 differentiated adipocytes
Treatment
4 h media conditioning
Adipocyte conditioned media
Bone marrow-derived macrophages
24 h incubation
before IL-4 treatment
B.
M2 CD301+ BMDM (%)
WT
SMKO
V
A
ACM
Treatment
C.
Il4ra expression
(normalized to NCM)
NCM VCM ACM
Pretreatment media
D.
Arg1 expression
(normalized to NCM)
NCM VCM ACM
Pretreatment media
E.
Cd11c expression
(normalized to NCM)
NCM VCM ACM
Pretreatment media
F.
3T3-L1 differentiated adipocytes
Treatment
4 h media conditioning
Adipocyte conditioned media
+ neutralizing
antibody
Bone marrow-derived macrophages
24 h incubation
before IL-4 treatment
G.
VCM
ACM
IL-6-NA:
IgG-NA:
p-STAT3
STAT3
β tubulin
H.
IgG
IL-6 NA
Il4ra expression (AU)
NCM ACM
Pretreatment media
I.
IgG
IL-6 NA
Arg1 expression (AU)
NCM ACM
Pretreatment media

Figure 4


A.
Vehicle
Amlexanox
Relative Adrb3 expression (AU)
1.5
1.0
0.5
0.0
WT
SMKO
B.
Vehicle
Amlexanox
Il4ra expression (AU)
2.0
1.5
1.0
0.5
0.0
WT
SMKO
Genotype
C.
Vehicle
Amlexanox
Il10 expression (AU)
2.0
1.5
1.0
0.5
0.0
WT
SMKO
Genotype
D.
Oral
Amlexanox
Adipocyte
IL-6
STAT3
Macrophage
IL-4ra
Sensitivity to anti-inflammatory Il-4 signaling
E.
Vehicle
Amlexanox
CD11c+ ATM of Cd45
4
3
2
1
0
WT
SMKO
F.
Vehicle
Amlexanox
CD11c+ of ATM
40
30
20
10
0
WT
SMKO
Genotype
G.
Vehicle
Amlexanox
Relative Mcp1 expression (AU)
2.0
1.5
1.0
0.5
0.0
WT
SMKO