ETC-1002 regulates immune response, leukocyte homing, and adipose tissue inflammation via LKB1-dependent activation of macrophage AMPK

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Abstract  ETC-1002 is an investigational drug currently in Phase 2 development for treatment of dyslipidemia and other cardiometabolic risk factors. In dyslipidemic subjects, ETC-1002 not only reduces plasma LDL cholesterol but also significantly attenuates levels of hsCRP, a clinical biomarker of inflammation. Anti-inflammatory properties of ETC-1002 were further investigated in primary human monocyte-derived macrophages and in vivo models of inflammation. In cells treated with ETC-1002, increased levels of AMP-activated protein kinase (AMPK) phosphorylation coincided with reduced activity of MAP kinases and decreased production of proinflammatory cytokines and chemokines. AMPK phosphorylation and inhibitory effects of ETC-1002 on soluble mediators of inflammation were significantly abrogated by siRNA-mediated silencing of macrophage liver kinase B1 (LKB1), indicating that ETC-1002 activates AMPK and exploits its anti-inflammatory effects via an LKB1-dependent mechanism. In vivo, ETC-1002 suppressed thioglycollate-induced homing of leukocytes into mouse peritoneal cavity. Similarly, in a mouse model of diet-induced obesity, ETC-1002 restored adipose AMPK activity, reduced JNK phosphorylation, and diminished expression of macrophage-specific marker 4F/80. These data were consistent with decreased epididymal fat-pad mass and interleukin (IL)-6 release by inflamed adipose tissue. Thus, ETC-1002 may provide further clinical benefits for patients with cardiometabolic risk factors by reducing systemic inflammation linked to insulin resistance and vascular complications of metabolic syndrome.—Filippov, S., S. L. Pinkosky, R. J. Lister, C. Pawloski, J. C. Hanselman, C. T. Cramer, R. A. K. Srivastava, T. R. Hurley, C. D. Bradshaw, M. A. Spahr, and R. S. Newton. ETC-1002 regulates immune response, leukocyte homing, and adipose tissue inflammation via LKB1-dependent activation of macrophage AMPK. J. Lipid Res. 2013. 54: 2095–2108.

Supplementary key words AMP-activated protein kinase • mitogen-activated protein kinases • liver kinase B1 • macrophages/monocytes • cytokines • adipose tissue • cardiometabolic risk factors • drug therapy • hypolipidemic drugs

With the rising prevalence of metabolic syndrome in the Western world (1), it is increasingly clear that chronic, low-grade inflammation serves as an important common denominator linking together visceral obesity, type 2 diabetes (T2D), and its vascular complications, including coronary artery disease (CAD) (2, 3). Although genetic and epidemiological data strongly indicate that therapeutic strategies designed to target inflammatory responses could be a viable approach to control various cardiometabolic risk factors (3–5, 6, 7), results from recent clinical trials have been rather disappointing. Antibodies designed to selectively block tumor necrosis factor (TNF)α (etanercept), interleukin (IL)-1β (gevokizumab), or IL-1β receptor (anakinra) failed to improve insulin sensitivity and showed only mild and inconsistent benefits in plasma glucose levels and β-cell function, despite their efficacy in other inflammatory conditions (8–10). Likewise, clinical benefits of reducing risk of MACE by nonlipid-lowering anti-inflammatory drugs targeting individual canonical mediators of inflammation have yet to be proven (11). Although somewhat surprising, these discrepancies between clinical efficacy and genetic and epidemiological data most likely reflect the complexity of immune responses associated with metabolic abnormalities in humans. Consequently, targeting more broad upstream signaling events, as opposed to a single inflammatory effector molecule, may offer greater clinical benefits to patients with cardiometabolic disorders, including T2D and CAD (9, 10).

ETC-1002 (8-hydroxy-2,2,14,14-tetramethylpentadecanoic acid), previously known as ESP55016, is a novel investigational drug being developed for the treatment of...
dyslipidemia and other cardiometabolic risk factors. Beneficial effects of ETC-1002 on lipid and glucose metabolism have been demonstrated in multiple animal models and have been linked to ETC-1002-mediated activation of AMP-activated protein kinase (AMPK) (12–15). AMPK represents an attractive therapeutic target, as it plays a fundamental role in maintaining cellular energy homeostasis via coordinating glucose and lipid metabolism as well as down-regulating proinflammatory signaling pathways (16–18). In clinical studies, ETC-1002 has not only demonstrated improved lipid profiles but also revealed significantly attenuated levels of hsCRP (19), an independent risk factor for CAD and a well-established clinical biomarker of inflammation (20, 21). Pharmacological activation of AMPK has been previously shown to limit inflammatory response in vitro and in vivo (22–26), and several signaling pathways, including Akt, GSK, SIRT, and NFκB, have been associated with anti-inflammatory consequences of AMPK activation (17, 27–29). While the exact mechanism of AMPK-dependent regulation of the immune response remains unclear, inhibition of mitogen-activated protein kinases (MAPK), such as JNK and p38, has been recently linked to AMPK-dependent anti-inflammatory signaling (30). Consistently, reduction in JNK phosphorylation has been verified with the AMPKβ1-specific activator A769662 (31).

Here we demonstrate that in primary human monocyte-derived macrophages (MDM), ETC-1002 mediates inhibition of a wide range of proinflammatory molecules that coincides with the modulation of AMPK and MAP kinase activities. AMPK phosphorylation and inhibitory effects of ETC-1002 on soluble mediators of inflammation were significantly abrogated by small inhibitory RNA (siRNA)-mediated silencing of macrophage liver kinase B1 (LKB1), indicating that ETC-1002 activates AMPK and exerts its anti-inflammatory effects via an LKB1-dependent mechanism. Furthermore, ETC-1002 treatment diminished leukocyte homing into inflammatory sites in vivo as well as reduced adipose tissue inflammation in a mouse model of diet-induced obesity (DIO). Thus, the anti-inflammatory properties of ETC-1002 may offer additional clinical benefits by controlling immune responses in patients with metabolic abnormalities.

MATERIALS AND METHODS

Reagents

RPMI 1640 media, DMEM media, nonessential amino acids, HEPES, phosphate buffered saline (PBS), and penicillin/streptomycin were obtained from Invitrogen® (Logon, UT). Fetal bovine serum (FBS) was obtained from Sigma Chemical Co. (St. Louis, MO). Proteome Profiler Human Cytokine Array Kit, Panel A, and Human Matrix Metalloproteinase Array 1 were purchased from R and D Systems (Minneapolis, MN) and RayBio (Norcross, GA), respectively. Antibodies to AMPKα (threonine 172), AMPKα (total), acetyl-CoA carboxylase (ACC) (Serine 79), ACC (total), Phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK (56G8), Phospho-p38 MAPK (Thr180/Tyr182), and p38 MAPK (D13E1) were obtained from Cell Signaling Technologies (Beverly, MA). Anti-F4/80 antibody (#ab74383) was purchased AbCam (Cambridge, MA). HPLC-grade reagents, solvents, Ultima Gold™ scintillation cocktail, and HISTOPAQUE®-1077 were obtained from Sigma-Aldrich (St. Louis, MO).

ETC-1002 formulation / ETC-1002-CoA synthesis

For in vitro assays, ETC-1002 was formulated using aseptic technique at 30 and 100 mM in sterile dimethylsulfoxide (DMSO) and stored in sterile microcentrifuge tubes at 4°C for up to four weeks (stability was assessed). Working solutions of ETC-1002 were prepared in serum-free RPMI 1640 containing 12 mM HEPES, 10,000 U/ml penicillin, and 100 μg/ml streptomycin. ETC-1002-CoA was synthesized using rat liver microsomes as described (32). For in vivo experiments, ETC-1002 dosing solutions were formulated by preparing a disodium salt aqueous solution using 2:1 molar ratio of NaOH to ETC-1002 in water. Carboxymethyl cellulose (CMC) and Tween-20 were added to make a final solution containing 0.5% CMC and 0.025% Tween with a final pH 7–8. Compound concentrations in dosing solutions were administered at a volume of 10 ml/kg body.

Hepatocyte isolation

All animal procedures were approved by an Institutional Animal Care and Use Committee at the Michigan Life Science and Innovation Center. Nutritionally staged male Sprague-Dawley (Crl:CD (SD)) rats were anesthetized with isoflurane, and livers were perfused for hepatocyte isolation as previously described (12). Hepatocytes were plated in high-glucose DMEM containing 20% FBS, 14 mM HEPES, 0.2% bovine albumin, 2 mM L-glutamine, 1x MEM nonessential amino acids, 100 μM insulin, 100 μg/ml dexamethasone, and 20 μg/ml gentamicin at a density of 1.5 × 10⁶ cells/cm² on collagen-coated 6-well plastic dishes. After an attachment period of 3–4 h, cells were washed once and cultured overnight in DMEM containing 10% FBS.

Macrophage preparation

Human blood was obtained from healthy volunteers under RCRC IRB (Independent Review Board) guidelines (Esperion IRB Protocol Number ESP001). For preparation of autologous serum, blood was allowed to clot and serum was separated with centrifugation at 2,500 rpm for 30 min. Peripheral blood mononuclear cells (PBMC) were isolated with density gradient using HISTOPAQUE®-1077 as previously described (33) and plated at 3 × 10⁶ cells per 12-well plate. Monocytes were adherence purified and differentiated for five days in RPMI 1640 supplemented with 40% autologous serum, 14 mM HEPES, 100 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine.

Protein arrays

At the end of differentiation period, macrophages were washed with PBS and switched to RPMI 1640 containing 5% autologous serum and supplemented with 14 mM HEPES, 100 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine. ETC-1002 at various concentrations (50 μM and 100 μM) was added to the media 1 h prior to stimulation with 100 ng/ml of lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO). Media conditioned by MDMs was collected 12 h following LPS stimulation and assayed with Proteome Profiler Human Cytokine Array Kit, Panel A, and Human Matrix Metalloproteinase Array, according to the manufacturer’s instructions. Data for cytokine and matrix metalloproteinase (MMP) arrays were captured and analyzed with a Kodak 4000MM Image Station. Net signal intensity for each analyte was expressed as percentage of the internal reference standard for each individual array membrane. Data are presented as mean ± SEM. Comparisons between groups
were performed by one-way ANOVA. A Bonferroni’s post hoc multiple comparison test was used to assess significant differences revealed by the ANOVA. Significance was accepted at $P \leq 0.05$.

**siRNA-mediated silencing of macrophage LKB1**

Macrophages were differentiated in 40% autologous serum for six days as described. At the end of the differentiation period, cells were washed with HBSS and switched to RPMI containing 5% autologous serum for 1 h prior siRNA treatment. LKB1 (STK11) (#43924290) and Negative control (Mock) (#4390843) siRNAs (Silencer Select PreDesigned siRNA, Invitrogen/Ambion, Logon, UT) were incubated for 15 min with polymer-based GenMute siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD) to form a siRNA transfection complex. LKB1 siRNA and mock siRNA transfection complexes were then added to MDMs at 50 nM siRNA final concentration for 5 h. At the end of 5 h incubation period, media containing siRNA transfection complexes was removed, cells were washed three times with HBSS, and then supplemented with RPMI containing 5% autologous serum. MDMs were allowed to recover for 24 h before stimulation with LPS and/or ETC-1002 treatment. Cell lysates were prepared as described in Western blot sections, and LKB1 expression and AMPK phosphorylation were assessed by Western blot. Conditioned media was collected, and levels of IL-6 and chemokine (C-C motif) ligand 2 (CCL2) / monocyte chemotactic protein-1 (MCP-1) were determined by ELISA (Human chemokine (C-C motif) ligand 2 (CCL2) / monocyte chemotactic protein-1 (MCP-1) - Two Pack Set, R & D Systems, Minneapolis, MN).

**Western blots**

Macrophage cell and adipose tissue lysates were prepared using approximately 150–400 μl 1x lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na 2 EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO 4, 1 μg/ml leupeptin, 1 mM PMSF, and 1x phosphatase inhibitor cocktail (Sigma). Cell and tissue lysates were sonicated with Branson Sonifier 150 (Danbury, CT) to disrupt cell membranes and release cellular/nuclear contents. Proteins in cell lysates were separated using SDS-PAGE (4–12%) Bis/Tris, MOPS running buffer (Invitrogen®, Logan, UT) and electrophoretically transferred to PVDF membranes. Nonspecific binding was blocked and membranes were probed with antibodies against β-actin, total and phosphorylated ACC, AMPKα, p38, JNK1/2, and F4/80 (Cell Signaling Technologies), and chemiluminescence was detected using ECL substrate (#RPQ9209, GE Healthcare, Buckinghamshire, UK) and captured with Kodak 4000MM Image Station. Density of specific immunoreactive bands was quantitated, and data are presented as ratio of phosphorylated to total proteins (mean ± SEM) or normalized to β-actin. Comparisons between groups were performed by one-way ANOVA and Bonferroni’s post hoc multiple comparison tests or unpaired two-tailed Student’s $t$-test. Significance was accepted at $P < 0.05$.

**HPLC-UV for ETC-1002-CoA determination**

A 15 μl aliquot from the extraction procedure described above was injected into the HPLC system utilizing an Alltima® C8 5μ, 250 × 4.6 mm ID HPLC column (Alltech Associates, Inc., Deerfield, IL) running 15–40% acetonitrile in 25 mM K 2 HPO 4 (pH 7.0) gradient before UV detection at 254 nm on a G1314A photodiode array detector (PDA) (Agilent Technologies, Inc., Santa Clara, CA). ETC-1002-CoA concentrations were determined by comparing the sample peak area to the peak area of an ETC-1002-CoA calibration standard.

**Thioglycollate-induced peritonitis**

All animal procedures were approved by an Institutional Animal Care and Use Committee at the Michigan Life Science and Innovation Center. C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with 1 ml of 4% sterile thioglycollate. Mice (n = 6 per each experimental group) received either vehicle alone or vehicle containing ETC-1002 at 30 mg/kg/day by oral gavage 24 h prior to thioglycollate challenge and for the duration of the study. Peritoneal cavity leukocytes were obtained from three lavage washes collected 24 h and 72 h after thioglycollate injection with 3 ml of cold PBS. Leukocytes were pelleted from fluid by centrifugation and resuspended in 5 ml of PBS. The number of cells migrated into peritoneal cavity 24 h (neutrophils) and 72 h (macrophages) after thioglycollate injection was determined with a hemocytometer. Data are expressed as mean ± SEM and comparisons between vehicle and ETC-1002-treated groups were performed using an unpaired two-tailed Student’s $t$-test. Significance was accepted at $P < 0.05$.

**Epididymal fat-pad tissue explants and ex-vivo inflammation studies**

All animal procedures were approved by an Institutional Animal Care and Use Committee at the Michigan Life Science and Innovation Center. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were singly housed in environmental isolators on ALPHA-dri paper bedding. Animals were provided free access to 60% high-fat diet (HFD) upon arrival beginning at 11 weeks of age. At 12 weeks of age, mice were randomly assigned to treatment groups (n = 10/group) and administered with vehicle alone or vehicle containing ETC-1002 at 30 mg/kg/day via oral gavage for nine weeks. At the time of necropsy, epididymal fat pads from the chow-fed animals, HFD-fed animals, and animals on HFD treated with ETC-1002 were removed, and weight of the fat pads from individual mice was determined. Adipose tissues were immersed into ice-cold, serum-free RPMI 1640 media and dissected into approximately 5 × 5 mm fragments under sterile conditions. Adipose tissue protein extracts were prepared, and expression of AMPK, JNK1/2, p38, and F4/80 in adipose tissue was determined by Western blot. Remaining fragments of epididymal fat pads were then placed into a 12-well tissue culture plate (four fragments per well) and incubated in serum-free RPMI 1640 at 37°C in humidified 0.5% CO 2 for 24 h. At the end of the incubation time, fat-pad fragments were removed from the plate and air-dried, and then the collective weight of tissues in each well was determined. IL-6 levels in media conditioned by tissue explants were measured with mouse IL-6 Quantikine ELISA Kit (R and D Systems), and the total amount of IL-6 released per well was normalized to the corresponding dry tissue weight. The fat-pad mass for individual animals and IL-6 levels released by tissue explants are expressed as mean ± SEM. Comparisons between groups were performed by one-way ANOVA. A Bonferroni’s post hoc multiple comparison test was used to assess significant differences revealed by the ANOVA. Significance was accepted at $P < 0.05$.

**RESULTS**

**Primary human MDMs do not form ETC-1002-CoA thioester in vitro**

Recent studies have identified AMPK and ATP citrate lyase (ACL) as distinct molecular targets for ETC-1002, mediating its beneficial effects on glucose and lipid metabolism (12). While ETC-1002-free acid activates liver AMPK, hepatocytes also readily convert the parent compound into
ETC-1002-CoA thioester, which has been shown to directly inhibit ACL (12). To determine whether macrophages metabolize ETC-1002 to the CoA thioester at levels comparable to primary hepatocytes, intracellular ETC-1002-CoA was measured in both cell types following incubation with ETC-1002 at concentrations of 30 µM or 100 µM. The model of primary human MDMs differentiated in autologous serum was selected since, unlike immortalized monocyte or macrophage cell lines, these cells more accurately reflect macrophage behavior at sites of chronic inflammation in human disease states (33).

Consistent with previously published data (12), ETC-1002-CoA was readily detectable in cell lysates from cultured hepatocytes (Fig. 1A). In contrast, analysis of MDM extracts failed to detect any measurable amounts of ETC-1002-CoA thioester at concentrations up to 100 µM, demonstrating that, unlike primary rat hepatocytes, macrophages do not convert ETC-1002-free acid into ETC-1002-CoA thioester at detectable levels. (Fig. 1A). Thus, in primary human MDMs, ETC-1002-free acid is likely to mediate pharmacological effects of ETC-1002.

ETC-1002 activates AMPK in primary human MDMs

In human hepatocellular carcinoma (HepG2) cells treated with ETC-1002, profound and concentration-dependent activation of AMPK has been previously attributed to ETC-1002-free acid as, similar to primary human MDMs, these cells do not metabolize parent molecules to ETC-1002-CoA thioester (12). To determine whether ETC-1002 activates AMPK in human macrophages, MDMs differentiated in autologous serum were treated with various concentrations of the compound, and cell lysates were probed with anti-phosphorylated AMPKα (T172) antibody. In vehicle-treated MDMs, basal levels of AMPK phosphorylation were readily detectable as demonstrated by the appearance of an immunoreactive band corresponding to the expected molecular mass of ~62 kDa (Fig. 1B). Concentration-dependent increases in phospho-AMPK-to-total-AMPK ratio in ETC-1002-treated cells indicate that ETC-1002 induces AMPK (T172) phosphorylation at levels comparable to those observed previously in HepG2 cells (12). ACC (serine 79) is a unique AMPK phosphorylation site and is commonly used as a marker of AMPK activity (34). As such, when cell lysates from MDMs treated with ETC-1002 were probed with anti-phosphorylated ACC (S79) antibodies, sustained and concentration-dependent increases in ACC (S79)-specific immunoreactivity were observed at the expected molecular mass of ~280 kDa. Consistently, the phospho-ACC to total-ACC ratio increased by 25% and 60% in cells treated with 50 µM and 100 µM ETC-1002, respectively (Fig. 1B). Taken together, these data confirm the AMPK-activating properties of ETC-1002 in primary human MDMs (Fig. 1B).

ETC-1002 inhibits release of proinflammatory cytokines and chemokines by stimulated human MDMs

Pharmacological activation of AMPK has been shown to reduce inflammatory responses and minimize organ damage in multiple animal models of acute and chronic inflammation (22, 23, 35). These effects of AMPK activation are linked, at least in part, to inhibition of Toll-like receptor (TLR)-mediated signaling and downregulation of proinflammatory molecules released by immune cells (23, 30, 31). To determine whether ETC-1002-mediated phosphorylation and activation of AMPK in primary human MDMs yields similar anti-inflammatory effects, macrophages were stimulated with 100 ng/ml of LPS in either the absence or presence of varying concentrations of ETC-1002.

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ETC-1002 activates AMPK and inhibits immune response

into MDM-conditioned media was then evaluated with cytokine arrays.

In resting MDMs, levels of proinflammatory cytokines and chemokines remained at the lower limits of detection. In contrast, stimulation with LPS triggered a strong and consistent inflammatory response as determined by a dramatic increase in the secretion of a variety of proinflammatory molecules (Figs. 2, 3, and 4). Acute-phase proteins, such as TNF-α, IL-1β, IL-6, and IL-8, are believed to be a hallmark of low-grade inflammation associated with metabolic abnormalities (2, 3). As shown in Fig. 2, treatment with ETC-1002 strongly suppressed the release of TNF-α, IL-1β, IL-6, and IL-8 by stimulated MDMs in concentration-dependent manner with inhibitory effects ranging from 15% to 77% (P < 0.05).

As leukocyte flux into inflammatory sites is controlled by chemotactic gradients (36, 37), we next investigated the effects of ETC-1002 on the expression of CC- and CXC-chemokine family members that are known to govern monocyte, lymphocyte, and neutrophil trafficking (36, 37). Similar to the stimulatory effects exerted by endotoxin on the upregulation of acute-phase proteins, LPS treatment increased the production of a wide array of chemotactic molecules (Fig. 3). Consistent with the inhibitory effects exerted by ETC-1002 on the expression of acute-phase cytokines, ETC-1002 suppressed the LPS-induced expression of CCL1, CCL2, CCL5, and CXCL10 chemokines to an even greater extent, with concentration-dependent inhibition ranging from 17% to 96% (P < 0.05) (Fig. 3).

Interestingly, and despite the broad anti-inflammatory effects exerted by ETC-1002, a subset of macrophage products associated with an activated state (e.g., MIP1α, IL1ra, TIMP-1) generally remained unchanged or were upregulated (TIMP-2, MMP-8, MMP-13; +41%, +56%, +98%, respectively; P < 0.05) in drug-treated cells (Fig. 4B).

Anti-inflammatory effects of ETC-1002 coincide with modulation of AMPK and MAPK signaling pathways

To investigate mechanism(s) underlying the anti-inflammatory properties of ETC-1002, activation of AMPK, ACC, and MAP kinases JNK and p38 were assessed in LPS-stimulated MDMs. Consistent with the established ability of proinflammatory stimuli to inhibit AMPK activity in macrophages (30, 31), LPS-challenged human MDM significantly reduced levels of phosphorylated AMPKα and ACC by 70% and 80%, respectively (Fig. 5). The LPS-induced reduction in AMPK phosphorylation coincided

Fig. 2. ETC-1002 attenuates expression of acute-phase cytokines by LPS-stimulated human MDMs. Macrophages differentiated in autologous serum for five days were stimulated with 100 ng/ml of LPS in either the absence or presence of different concentrations of ETC-1002. Levels of proinflammatory cytokines in the media conditioned by MDMs for 24 h were determined with cytokine arrays. Net signal intensity for each analyte was expressed as percentage of the internal reference standard for each individual array membrane (mean ± SEM). Comparisons between groups were performed by one-way ANOVA and Bonferroni’s post hoc multiple comparison tests, *P < 0.05.
LKB1 is required for ETC-1002-mediated AMPK activation (12). To determine whether the activation of macrophage AMPK by ETC-1002 is also LKB1-dependent, siRNA interference was utilized to reduce endogenous LKB1 protein levels and assess the effects of LKB1 knockdown on ETC-1002-mediated AMPK phosphorylation and inhibition of immune response.

Primary human MDMs were transfected with negative control “mock” or LKB1 siRNA for 5 h followed by a 24 h recovery period. Unstimulated or LPS-induced cells were then treated with vehicle or 100 µM ETC-1002, and AMPK phosphorylation as well as production of proinflammatory cytokines were assessed by Western blot and ELISA (Fig. 5). Macrophages transfected with LKB1 siRNA demonstrated a 63% reduction (\(P = 0.0002\)) in LKB1 protein with increased phosphorylation of MAP kinases p38 and JNK as shown by increased phosphorylated-to-total p38 and JNK protein ratio (Fig. 5). By contrast, when cells were stimulated with LPS in the presence of ETC-1002, AMPK and ACC phosphorylation was increased relative to LPS alone, whereas phosphorylated p38 and JNK levels were attenuated by 73% and 47%, respectively, when treated with 50 µM of ETC-1002 and by 90% and 71%, respectively, at 100 µM of ETC-1002 (Fig. 5).

**ETC-1002 activates macrophage AMPK and inhibits immune response via an LKB1-dependent mechanism**

Liver kinase B1 is an essential serine-threonine kinase that directly phosphorylates and activates AMPK (16, 18, 38, 39). Previously, we have demonstrated that in HepG2 cells, LKB1 is required for ETC-1002-mediated AMPK activation (12). To determine whether the activation of macrophage AMPK by ETC-1002 is also LKB1-dependent, siRNA interference was utilized to reduce endogenous LKB1 protein levels and assess the effects of LKB1 knockdown on ETC-1002-mediated AMPK phosphorylation and inhibition of immune response.

![Fig. 4. ETC-1002 regulates the expression of adhesion molecules and mediators of vascular inflammation by activated MDMs. Macrophages differentiated in autologous serum for five days were stimulated with 100 ng/ml of LPS in either the absence or presence of different concentrations of ETC-1002. Expression of the adhesion molecules and mediators of vascular inflammation in the media conditioned by MDMs for 12 h were determined with (A) cytokine and (B) matrix metalloproteinase arrays. Net signal intensity for each analyte was expressed as percentage of the internal reference standard for each individual array membrane (mean ± SEM). Comparisons between groups were performed by one-way ANOVA and Bonferroni’s post hoc multiple comparison tests, \(P < 0.05\).](image-url)
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ETC-1002 activates AMPK and exerts its anti-inflammatory effects via an LKB1-dependent mechanism. ETC-1002 attenuates homing of leukocytes into inflammatory site in vivo

Migration of circulating monocytes into adipose tissue followed by differentiation into proinflammatory macrophages is a hallmark of chronic, low-grade inflammation associated with excess of visceral adipose tissue (2, 40). As ETC-1002 inhibits the generation of proinflammatory chemokines in vitro (Fig. 3), the ability of ETC-1002 to affect chemotactic potential was assessed in response to thioglycollate-induced peritonitis. As shown in Fig. 7A.

compared with cells transfected with “mock” siRNA (Fig. 6A). As expected, unstimulated and LPS-induced “mock”-transfected MDMs showed increased AMPK (T172) phosphorylation in response to ETC-1002 treatment (Fig. 6B). In contrast, AMPK (T172) phosphorylation was largely abolished in cells transfected with LKB1 siRNA (Fig. 6B). Furthermore, consistent with anti-inflammatory effects of AMPK activation, ETC-1002 inhibited release of IL-6 and CCL2/MCP-1 by “mock”-transfected macrophages whereas cells transfected with LKB1 siRNA not only remained insensitive to ETC-1002 treatment but also revealed slightly stronger proinflammatory response (Fig. 6C). Thus, these data demonstrate that in primary human macrophages, ETC-1002 activates AMPK and exerts its anti-inflammatory effects via an LKB1-dependent mechanism.

ETC-1002 attenuates homing of leukocytes into inflammatory site in vivo

Migration of circulating monocytes into adipose tissue followed by differentiation into proinflammatory macrophages is a hallmark of chronic, low-grade inflammation associated with excess of visceral adipose tissue (2, 40). As ETC-1002 inhibits the generation of proinflammatory chemokines in vitro (Fig. 3), the ability of ETC-1002 to affect chemotactic potential was assessed in response to thioglycollate-induced peritonitis. As shown in Fig. 7A.
both 24 h and 72 h, indicating that ETC-1002 is likely to inhibit transmigration of both neutrophils and macrophages during the inception and propagation of inflammatory response (Fig. 7A).

During transmigration into inflammatory disease sites, monocytes and macrophages express a wide array of MMPs–proteolytic enzymes, which not only allow cells to negotiate extracellular matrix barriers but also may control chemotactic gradient by processing soluble mediators of inflammation and directly regulating inflammatory signaling pathways (42–44). To investigate whether inhibitory effect of ETC-1002 on leukocyte homing can, at least in part, be attributed to reduced expression of MMPs, media conditioned by human MDMs was assayed with MMP detection following thioglycollate challenge, leukocyte numbers in peritoneal lavage increased after 24 h and 72 h ($26.4 \times 10^6$/ml lavage fluid and $4.4 \times 10^6$/ml lavage fluid, respectively). By contrast, when mice were treated with 30 mg/kg of ETC-1002 prior to thioglycollate administration, leukocyte influx into the peritoneal cavity was significantly reduced by 48% at 24 h ($13.7 \times 10^6$/ml lavage fluid; $P < 0.008$) and by 61% at 72 h ($1.8 \times 10^6$/ml lavage fluid; $P < 0.002$) (Fig. 7A). Inflammatory response in mouse peritoneal cavity has defined sequential phases of the disease in which influx of neutrophils predominates at the early stages of inflammation (up to 24 h) followed by a macrophage-enriched infiltrates (48–72 h) (41). In ETC-1002-treated mice, the number of leukocytes in peritoneal lavage was reduced at following thioglycollate challenge, leukocyte numbers in peritoneal lavage increased after 24 h and 72 h ($26.4 \times 10^6$/ml lavage fluid and $4.4 \times 10^6$/ml lavage fluid, respectively). 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ETC-1002 activates AMPK and inhibits immune response

HFD-fed versus 0.89 ± 0.07 g, HFD-fed/ETC-1002; *P < 0.05). To evaluate the status of adipose tissue–associated inflammation, fragments of epididymal fat pads were maintained as ex vivo explants, and IL-6 levels were measured in media conditioned by tissue explants for 12 h. As shown in Fig. 8A, HFD-induced expansion of adipose tissue was accompanied by approximately 2-fold increase in IL-6 levels (16.6 ± 7.8 pg/mg chow-fed versus 35.7 ± 3.6 pg/mg HFD-fed; *P < 0.05). Importantly, and consistent with the in vitro anti-inflammatory effects of ETC-1002, IL-6 release was reduced by 63% in mice receiving ETC-1002 (IL-6 release: 35.7 ± 3.6 pg/mg HFD-fed versus 13.3 ± 2.2 pg/mg HFD-fed/ETC-1002; *P < 0.05).

Significantly, AMPK-activating properties of ETC-1002 were further demonstrated in vivo as AMPK phosphorylation attenuated by HFD was substantially restored in adipose tissues of ETC-1002-treated animals (Fig. 8B). Likewise, decreased phosphorylation of adipose JNK and p38 in tandem with reduced expression of macrophage-specific marker F4/80 strongly supports the role for AMPK/MAPK axis in mediating anti-inflammatory effects of ETC-1002 in vivo (Fig. 8B, Fig. 9).

DISCUSSION

In the present studies, we demonstrate that in stimulated primary human MDMs, ETC-1002 exerts broad anti-inflammatory effects consistent with LKB1-dependent anti-inflammatory effects of ETC-1002.

Recent studies have demonstrated that in a mouse model of DIO, a two-week treatment with ETC-1002 was sufficient to reduce body weight as well as to lower fasting plasma glucose and insulin levels (12). Since adipose tissue macrophages are believed to play a critical role in governing immune responses and insulin resistance in DIO (2, 40, 45), we next evaluated whether the beneficial effect of ETC-1002 on glycemic control can be linked to reduced levels of adipose tissue–associated inflammation. As such, male C57BL/6 mice were placed on HFD and orally dosed with either vehicle alone or ETC-1002 at 30 mg/kg/day for nine weeks. At the termination of the study, visceral adipose tissue from mice placed on HFD was macroscopically larger (Fig. 8A), with the average epididymal fat pad mass significantly increased relative to chow-fed animals (0.31 ± 0.04 g, chow-fed versus 1.3 ± 0.04 g, HFD-fed; *P < 0.05) (Fig. 7A). By contrast, ETC-1002-treated mice displayed a 32% reduction in fat-pad mass (fat-pad mass: 1.3 ± 0.04 g, HFD-fed versus 0.89 ± 0.07 g, HFD-fed/ETC-1002; *P < 0.05). To evaluate the status of adipose tissue–associated inflammation, fragments of epididymal fat pads were maintained as ex vivo explants, and IL-6 levels were measured in media conditioned by tissue explants for 12 h. As shown in Fig. 8A, HFD-induced expansion of adipose tissue was accompanied by approximately 2-fold increase in IL-6 levels (16.6 ± 7.8 pg/mg chow-fed versus 35.7 ± 3.6 pg/mg HFD-fed; *P < 0.05). Importantly, and consistent with the in vitro anti-inflammatory effects of ETC-1002, IL-6 release was reduced by 63% in mice receiving ETC-1002 (IL-6 release: 35.7 ± 3.6 pg/mg HFD-fed versus 13.3 ± 2.2 pg/mg HFD-fed/ETC-1002; *P < 0.05).

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**DISCUSSION**

In the present studies, we demonstrate that in stimulated primary human MDMs, ETC-1002 exerts broad anti-inflammatory effects consistent with LKB1-dependent anti-inflammatory effects of ETC-1002.
Polyphenols, has been shown to yield anti-inflammatory effects (22–25), resulting in diminished leukocyte accumulation in target organs, limited interstitial edema, and reduced levels of TNFα, IL-1β, IL-6, and IL-8 (22, 23). Similar to the anti-inflammatory effects demonstrated here with ETC-1002-treated human MDMs, pharmacological activation of AMPK in macrophage cell lines or bone activation of macrophage AMPK and the downregulation of MAPK signaling. Furthermore, in vivo, ETC-1002 attenuates leukocyte homing into inflammatory sites while inhibiting adipose tissue inflammation.

In multiple models of inflammation, AMPK activation by 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or synthetic, as well as plant-derived polyphenols, has been shown to yield anti-inflammatory effects (22–25), resulting in diminished leukocyte accumulation in target organs, limited interstitial edema, and reduced levels of TNFα, IL-1β, IL-6, and IL-8 (22, 23). Similar to the anti-inflammatory effects demonstrated here with ETC-1002-treated human MDMs, pharmacological activation of AMPK in macrophage cell lines or bone

**Fig. 8.** ETC-1002 reduces epididymal fat-pad mass and adipose tissue inflammation in a mouse model of diet-induced obesity. C57BL/6 mice were fed with chow (ChD) or 60% high-fat diet (HFD) for nine weeks. HFD groups (n = 10/group) were administered with vehicle alone or ETC-1002 at 30 mg/kg/day via oral gavage. (A) Macrograph of epididymal fat pads from the ChD- and HFD-fed animals administered with either vehicle (HFD vehicle) or 30 mg/kg of ETC-1002 (HFD ETC-1002). Left graph: Mass of epididymal fat pads obtained from the mice in different experimental groups. Right graph: IL-6 release into the media conditioned by adipose tissue explant ex vivo. Fragments of epididymal fat pads were maintained ex vivo in serum-free RPMI, and IL-6 levels were measured in a media conditioned by tissue explants for 12 h. (B) Phosphorylation of AMPK, p38, and JNK1/2 and expression levels of F4/80 in adipose tissue lysates were quantitated by densitometry of the respective immunoreactive bands. For AMPK, p38, and JNK1/2, data are presented as ratio of phosphorylated to total proteins (mean ± SEM). For F4/80 expression, data are normalized to β-actin (mean ± SEM). Comparisons between groups were performed by one-way ANOVA and Bonferroni’s post hoc multiple comparison test, *P < 0.05. Data are expressed as mean ± SEM.
marrow-derived macrophages largely reduces expression of TNFα, IL-1β, IL-6, and IL-8 (30, 31). Further, mechanistic links between AMPK activation and the inhibition of immune responses have been confirmed in recent genetic studies utilizing RNAi or dominant negative versus constitutively active forms of AMPKα (28, 29). Consistently, genetic LKB1 deletion supports the critical role for AMPK in regulating macrophage polarization/inflammatory status in vitro and in vivo (31). In the present study, siRNA interference targeting endogenous levels of LKB1 was utilized to confirm the mechanistic link between ETC-1002-mediated AMPK activation and its anti-inflammatory effects. LKB1 is a well-established, physiologically relevant kinase that phosphorylates and activates AMPK (16, 18, 38, 39). AMPK activation by agonists, such as AICAR, metformin, or phenformin, or in response to stress was absent in LKB1-deficient cells, identifying LKB1 as an obligatory AMPK-activating kinase (38, 46). Therefore, silencing of LKB1 in macrophages is likely to prevent activation of otherwise intact AMPKα protein. Indeed, 60% reduction of macrophage LKB1 expression was sufficient to diminish AMPK phosphorylation in ETC-1002-treated cells and to largely revoke ETC-1002-mediated inhibition of immune response, further confirming that ETC-1002 activates AMPK and exerts its anti-inflammatory effects via mechanisms dependent on the LKB1/AMPK axis. It is noteworthy that LKB1-deficient macrophages revealed slightly higher inflammatory response as shown by increased expression of IL-6 and CCL2/MCP-1. This may reflect inability of cell to adequately compensate for stress associated with LPS and/or compound treatment when the LKB1/AMPK axis is disrupted.

While numerous reports have demonstrated that AMPK can govern inflammatory response in immune cells, the signaling events leading to these effects remain unclear. Many signaling pathways, including Akt, GSK (29), SIRTs (28), NF-κB (17), and MAPK (30), have been proposed to mediate the anti-inflammatory effects that arise as a consequence of AMPK activation. However, the primary intracellular signaling cascades vary among published studies, depending on cell type, nature of proinflammatory stimuli, and experimental design. In our model using primary human MDMs, AMPK activation by ETC-1002 inhibits LPS-induced MAPK signaling as reflected in the reduced phosphorylation of JNK and p38. Similar inhibition of JNK and p38 phosphorylation has been previously demonstrated for AMPK activators in macrophage cell lines as well as in human monocytes where pharmacological activation of AMPK offsets immune responses in a MAPK-dependent fashion (26, 30).

Chronic low-grade inflammation has been regarded as a major pathogenic factor in obesity, T2D, and CAD (2, 3). Along with acute-response proteins, macrophage infiltration in adipose and vascular tissues orchestrates the expression of a complex network of chemokines and adhesion molecules that coordinate extravasation of leukocyte subsets into inflammatory sites (47, 48). Previous studies have established that AMPK activation reduces expression of CCL2/MCP-1 in macrophage-like cell lines as well as in adipose tissues of obese mice (30, 49). While ETC-1002 also attenuated macrophage CCL2/MCP-1 expression in our studies, it exerted a potent inhibitory effect on an array of chemotactic and adhesion molecules that play important roles in controlling vascular and adipose tissue inflammation, including ICAM-1, CXCL10 (IP-10), CCL5 (RANTES), and CCL1 (I-309) (2, 48). In addition to the inhibition of canonical mediators of inflammation, ETC-1002 significantly attenuated expression of CD40L and C5a. Interestingly, in clinical studies, plasma levels of CD40L and C5a are associated with an increased risk for the onset of acute cardiovascular events in humans (50, 51). Further, the ability of ETC-1002 to attenuate macrophage chemotactic potential in vitro was confirmed in a mouse model of thioglycollate-induced peritonitis in which administration of ETC-1002 largely reduced neutrophil and macrophage influx. Importantly, this inhibition of leukocyte homing likely to reflect reduced chemotactic gradient and be independent of proteolytic events, as MMP release by stimulated macrophages was either unaffected (MMP-1, MMP-10) or even upregulated (MMP-8, MMP-13) upon compound treatment. It is noteworthy that production of some macrophage gene products (IL-1α, MIP1α, TIMP-1) remained unchanged or was upregulated (TIMP-2, MMP-8, MMP-13) in drug-treated cells. While highlighting the complexity of the macrophage immune response, this may also indicate that ETC-1002-mediated inhibition of cytokine and
chemokine release is unlikely to result from off-target effects and/or cytotoxicity of the compound.

In DIO, circulating monocytes enter adipose tissue and differentiate into macrophages that display a proinflammatory phenotype and often reside in a form of crown-like structures juxtaposed to blood vessels and metabolically active as well as damaged adipocytes (2, 40, 52). Adipose tissue inflammation is believed to be a critical factor in promoting insulin resistance associated with visceral obesity (2, 3). Indeed, ablation of adipose tissue macrophages (ATM) or genetic disruption of the TLR4/JNK axis protects mice from developing obesity-related activation of the immune system and insulin resistance (45, 53). Likewise, selective therapeutic inactivation of JNK’s downstream effector molecules, TNFα or IL-1β, curbs hyperglycemia, lowers the proinsulin-to-insulin ratio, and improves insulin sensitivity in rodent models of obesity (54, 55). Similarly, ETC-1002 diminishes adipose JNK phosphorylation in mice on HFD and limits ATM content as shown by reduced expression of macrophage-specific marker 4F/80. Furthermore, adipose tissue mass was reduced in obese mice treated with ETC-1002 in tandem with decreased IL-6 levels in explant cultures of epididymal fat pads. Improved adipose tissue inflammation coincided with increased AMPK expression of macrophage-specific marker 4F/80. Furthermore, adipose tissue inflammation associated with metabolic abnormalities in humans is very conclusive (8–10). Similarly, although clinical evaluations of nonlipid-lowering anti-inflammatory drugs targeting individual canonical mediators of inflammation, such as CCR2, 5-LO, FLAP, Lp-PLA2, and sPLA2, are under way, clinical benefits resulting from these interventions remain to be established (11).

Since the pathophysiology of the immune response associated with metabolic abnormalities in humans is very complex, targeting more broad upstream signaling events, as opposed to single inflammatory effector molecule, may provide greater clinical benefit in patients with cardiometabolic disorders (9, 10). In this context, the anti-inflammatory benefits of AMPK activation have not been appreciated fully. Currently, the AMPK activator metformin is the most successful and widely prescribed drug to control hyperglycemia in subjects with T2D. In addition to well-documented effects of metformin on fasting plasma glucose and HbA1c (56), administration of metformin in T2D subjects is associated with significant lowering of plasma hsCRP levels (57). Interestingly, recent clinical studies suggest that these benefits may be directly linked to effects of metformin on monocyte and lymphocyte immune function (58, 59). Metformin administered to patients with impaired fasting glucose reduced plasma levels of hsCRP, soluble IGAM-1, as well as lymphocyte and monocyte release of IL-1, IL-2, IL-6, IL-8, INF-γ, TNF-α, and MCP-1 (58, 59). These recent clinical findings reveal a remarkable similarity to the anti-inflammatory “signature” of ETC-1002 (12, 13, 15). Given the clinical safety and efficacy profiles of ETC-1002, including consistent reduction in both LDL-C and hsCRP levels (19), these data reinforce its potential promise as a novel therapy for cardiovascular and metabolic diseases.

In summary, the present report describes anti-inflammatory properties of ETC-1002, a novel investigational drug being developed for the treatment of dyslipidemia and other cardiometabolic risk factors. We demonstrate that in stimulated primary human MDMs, ETC-1002 exerts strong anti-inflammatory effects consistent with LKB1-dependent activation of macrophage AMPK and downregulation of MAPK signaling. In vivo, ETC-1002 attenuates homing of leukocytes into the inflammatory site and inhibits adipose tissue inflammation in a mouse model of DIO. Thus, in addition to regulating imbalances of lipid and carbohydrate metabolism (12, 19), ETC-1002 may provide further clinical benefits for patients with cardiometabolic risk factors by reducing systemic inflammation associated with insulin resistance and vascular complications of metabolic syndrome (Fig. 9).

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