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(54) Title: METHODS FOR TREATING MACROPHAGE-MEDIATED DISEASES, AND METHODS OF IDENTIFYING AGENTS USEFUL THEREFORE

(57) Abstract: Provided herein are methods of treating macrophage-mediated inflammatory diseases and disorders. Also, disclosed are methods for screening for agents useful in such methods.

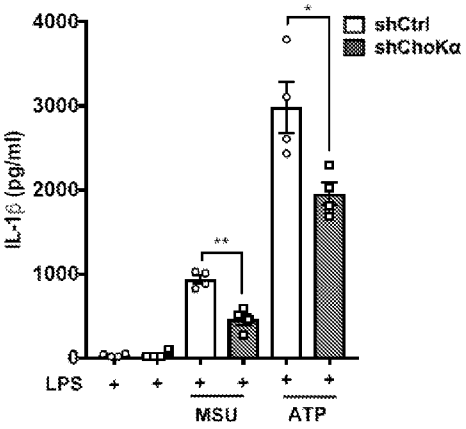

FIG. 3A

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METHODS FOR TREATING MACROPHAGE-MEDIATED DISEASES, AND METHODS OF IDENTIFYING AGENTS USEFUL THEREFORE

CROSS REFERENCE TO RELATED APPLICATION(S)

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) of US Serial No. 62/801,973, filed February 6, 2019, the entire content of which is incorporated herein by reference.

GRANT INFORMATION

[0002] This invention was made with government support under Grant Nos. AI043477 and ES010337 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 4, 2020, is named 20378-202455_SL.txt and is 49 kilobytes in size.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0004] The invention relates generally to inflammation and more specifically to methods and compositions for preventing CTL1 expression or choline phosphorylation to treat inflammation and degenerative diseases.

BACKGROUND INFORMATION

[0005] Macrophages respond to pathogens and tissue damage via pattern recognition receptors (PRR) that sense pathogen (PAMP) or damage (DAMP) associated molecular patterns (Guo et al., 2015; Martinon et al., 2009). Amongst PRR, Nod-like receptor pyrin domain containing 3 (NLRP3), a member of the Nod-like receptor (NLR) family that is induced upon macrophage activation, senses cytosolic oxidized mitochondrial DNA (ox-mtDNA) that is generated when activated macrophages are exposed to NLRP3-activating DAMPs, such as ATP or uric acid, and triggers IL-1β and IL-18 production and secretion (Zhong et al., 2018). While NLRP3 serves as its specific sensor subunit, the NLRP3 inflammasome also consists of the adaptor apoptosis-associated speck-like protein containing CARD (ASC), and the effector

enzyme pro-caspase-1, which undergoes autocleavage upon inflammasome activation, and is responsible for production of IL-1β and IL-18 (Elliott and Sutterwala, 2015; Martinon et al., 2009; Schroder and Tschopp, 2010).

[0006] Given the involvement of the NLRP3 inflammasome and IL-1β in many diseases, including type 2 diabetes, atherosclerosis, gout, rheumatoid arthritis, non-alcoholic steatohepatitis (NASH), lupus, and Alzheimer's disease (Busso and So, 2010; Heneka et al., 2013; Mridha et al., 2017; So and Martinon, 2017; Zhong et al., 2016b), it is not surprising that both initiation and termination of NLRP3 inflammasome activation are intricately regulated (Zhong et al., 2016a).

[0007] Choline is an essential human nutrient, serving as precursor for membrane phospholipids, acetylcholine, and functioning as a methyl group donor when metabolized to betaine and subsequently to S-adenosylmethionine (Aoyama et al., 2004; Glunde et al., 2011). Choline uptake is mediated by choline transporters, of which choline-transporter-like proteins (CTL) 1-5 are preferentially used to provide choline to Choline Kinase alpha (ChoKα), the first enzyme in phosphatidylcholine synthesis (Traiffort et al., 2005). Increased circulating choline and its enhanced uptake were observed in inflammatory diseases, including arthritis, cardiovascular diseases, and cancer (Al-Saffar et al., 2006; Glunde et al., 2011; Guma et al., 2015a; Hellberg et al., 2016; Seki et al., 2017). The use of choline as a tracer for enhanced cancer cell proliferation was established a few decades ago. More recently, the observation that activated cells, including fibroblasts and macrophages, take up choline at inflammatory sites suggested that choline may also have other biological functions. A need therefore exists for treatments that prevent or ameliorate macrophage-mediated inflammatory diseases and attenuate their progression.

SUMMARY OF THE INVENTION

[0008] The present invention is based on the observation that choline uptake via CTL1 and its phosphorylation by ChoKα and/or ChoKβ contribute to macrophage-mediated IL-1β-dependent inflammation. Thus, inhibition of choline uptake and/or phosphocholine synthesis reduces IL-1β production and ameliorates acute and chronic macrophage-mediated inflammation.

[0009] Accordingly, in one aspect, the invention provides a method of treating macrophage-mediated inflammatory and/or degenerative diseases or cancers in a subject. The method

includes administering to a subject in need thereof an effective amount of an inhibitor of choline-transporter-like protein 1 (CTL1) activity or expression or an inhibitor of choline phosphorylation. In various embodiments, the inhibitor of CTL1 activity or expression inhibits NLRP3 inflammasome activation, IL-1β production, or IL-18 production. In various embodiments, the subject is a mammal, such as a human. In various embodiments, the inhibitor of CTL1 activity or expression is a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment. In various embodiments, the inhibitor of choline phosphorylation is an inhibitor of ChoKα or ChoKβ. In various embodiments, the inhibitor of CTL1 activity or expression is an inhibitory nucleic acid that inhibits the expression of CTL1 or inhibits choline phosphorylation. In various embodiments, the inhibitory nucleic acid is selected from the group consisting of siRNA, shRNA, gRNA, oligonucleotides, antisense RNA or ribozymes that inhibit expression of CTL1 or inhibit choline phosphorylation. In various embodiments, the inhibitory nucleic acid is administered via a viral vector. In various embodiments, the macrophage-mediated inflammatory and/or degenerative disease is selected from the group consisting of cancer (especially lung cancer), lupus, gout, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, uveitis, Alzheimer's disease, Parkinson's disease, cryopyrin-associated periodic syndromes, nonalcoholic steatohepatitis (NASH), type 2 diabetes, atherosclerosis, macular degeneration, and geographical retinopathy.

[0010] In another aspect, the invention provides a method of inhibiting choline phosphorylation in a subject. The method includes administering to the subject an effective amount of an inhibitor of CTL1 activity or expression. In various embodiments, the inhibitor of CTL1 activity or expression is a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment. In various embodiments, the inhibitor of CTL1 activity or expression is an inhibitory nucleic acid that inhibits the expression of expression of CTL1 or inhibits IL-1β and/or IL-18 production. In various embodiments, the inhibitor of choline phosphorylation is an inhibitor of ChoKα or ChoKβ. In various embodiments, the inhibitor nucleic acid is selected from the group consisting of siRNA, shRNA, gRNA, oligonucleotides, antisense RNA or ribozymes that inhibit expression of CTL1 or inhibit IL-1β and/or IL-18 production. In various embodiments, the inhibitory nucleic acid is administered via a viral vector.

[0011] In another aspect, the invention provides a method of identifying an agent useful for treating macrophage-mediated inflammatory and/or degenerative diseases. The method includes contacting a sample of cells with at least one test agent, wherein a decrease in CTL1 expression

or choline phosphorylation in the presence of the test agent as compared to CTL1 expression or choline phosphorylation in the absence of the test agent identifies the agent as useful for treating macrophage-mediated inflammatory and/or degenerative diseases. In various embodiments, the test agent a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment. In various embodiments, the method may be performed in a high throughput format, such as contacting samples of cells of a plurality of samples with at least one test agent. In various embodiments, the plurality of samples may be obtained from a single subject or from different subjects.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-1J are pictorial and graphical diagrams showing that LPS stimulates macrophage choline uptake via NF-κB mediated CTL1 induction. Figure 1A shows the results from QPCR analysis of mRNAs encoding choline transporters and kinases in LPS stimulated BMDM. Mean ± SEM (n = 8). Figure 1B shows the results from immunoblot (IB) analysis of CTL1 at 4 hr after LPS stimulation. Figure 1C shows relative CTL1 protein expression in LPS stimulated BMDM shown as n-fold. Mean ± SEM (n = 4). Figure 1D shows intracellular choline in BMDM before and after LPS 4 hr treatment. Mean ± SEM (n = 4). Figure 1E shows the results of IB analysis of CTL1, IKKβ and tubulin in WT or IKKβ-deficient LPS-stimulated BMDM. **$p < 0.01$; ***$p < 0.005$. Phosphocholine (Figure 1F), glycero-3-phophocholine (Figure 1G), and phosphatidylcholine (PC) (Figure 1H) in were measured by ^{1}HMRS in RSM932A-pretreated BMDM stimulated for 4 hr. Mean ± SEM (n = 4). Figure 1I shows the results from QPCR analysis of *Slc44a1, Slc44a2, ChKa* and *ChKb* mRNAs in LPS-stimulated primary mouse microglia. Mean ± SEM (n = 5). Figure 1J shows the results from QPCR analysis of *Slc44a1* mRNA in BMS345541-pretreated BMDM and treated with LPS. Mean ± SD (n = 3). *$p < 0.05$; **$p<0.01$; ***$p < 0.005$; ****$p < 0.001$.

[0013] Figures 2A-2X are pictorial and graphical diagrams showing that choline deficiency or Slc44a1 knockdown reduce IL-1β production. Figure 2A shows the results from QPCR analysis of mRNAs encoding choline transporters and kinases in shSlc44a1 and shControl (shCtrl) immortalized BMDM (iBMDM). Mean ± SD (n = 3). The inserts demonstrate the efficacies of Slc44a1 silencing. Figure 2B shows IL-1β release by shSlc44a1 and shCtrl iBMDM primed with LPS for 4 hr and treated with ATP (40 min), nigericin (1 hr) or MSU (3 hr). Mean ± SD (n = 3). Figure 2C shows the results of IB analysis of caspase-1 in shSlc44a1 and shCtrl iBMDM that were LPS-primed and ATP-treated. Figure 2D show Caspase 1

activation shown as % of p20 release to culture medium by iBMDM treated as in Figure 2C. Mean ± SD (n = 3). Figure 2E shows IL-1β release by BMDM cultured either in control or choline-deficient medium and treated as in (Figure 2B). Mean ± SEM (n = 4). Figure 2F shows Caspase-1 in supernatants of LPS-primed and ATP-treated BMDM cultured either in control or choline-deficient medium. Figure 2G shows relative caspase 1 activation shown as % of p20 release to culture medium by BMDM treated as in Figure 2F. Mean ± SEM (n = 5). Figure 2H shows the results of IB analysis of caspase-1 and IL-1b in supernatants and lysates of LPS-primed and nigericin-treated BMDM cultured in either control or choline-deficient medium. *p < 0.05; **p < 0.01; ***p < 0.005. Figure 2I shows IL-18 production by shSlc44a1 and shCtrl iBMDM stimulated with LPS+ATP measured by ELISA. Mean ± SEM (n = 8). TNF (Figure 2J) and IL-6 (Figure 2K) secretion by shSlc44a1 and shCtrl iBMDM was measured by ELISA. Mean ± SD (n = 3) and Mean ± SEM (n = 5-6) respectively. Figure 2L shows IL-18 production by BMDM cultured in control or choline-free medium and stimulated with LPS+ATP. (n = 4). Figure 2M shows IL-1β release by BMDM cultured in control or choline-free medium and stimulated with the NLRP3 inflammasome activator nigericin and the AIM2 inflammasome activator poly(dA:dT). Mean ± SEM (n = 6). TNF (Figure 2N) and IL-6 (Figure 2O) secretion by BMDM cultured in control or choline-free medium was measured by ELISA. Mean ± SD (n = 3) and Mean ± SEM (n = 4) respectively. *Il1b* (Figure 2P), *Tnf* (Figure 2Q), and *Il10* (Figure 2R) mRNAs in LPS-stimulated shSlc44a1 and shCtrl iBMDM determined by QPCR. Mean ± SEM (n = 4). Figure 2S shows IL-1β release by WT and *Il10rb*$^{-/-}$ knockout BMDM cultured in control or choline-free medium. Mean ± SD (n = 3). Figure 2T shows the results from QPCR analysis of *Il10r*$^{-}$ and *Slc44a1* mRNAs in LPS-stimulated WT and *Il10rb*$^{-/-}$ BMDM. Mean ± SEM (n = 6). Figure 2U shows nitrite production by LPS-stimulated shSlc44a1 and shCtrl iBMDM was measured by Griess reaction. Mean ± SEM (n = 4). Figure 2V shows the results from QPCR analysis of *Nos2* mRNA in shSlc44a1 and shCtrl iBMDM. Mean ± SEM (n = 4). Calcium (Figure 2W) and potassium (Figure 2X) fluxes in BMDM cultured in control or choline-free medium, measured by Fura-2 AM and PBFI AM, respectively. Mean ± SD (n = 3). *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001.

[0014] Figures 3A-3J are pictorial and graphical diagrams showing that choline kinase knockdown and inhibition reduce IL-1β production. Figure 3A show IL-1β release by shChoKα and shCtrl iBMDM primed with LPS and stimulated with ATP or MSU. Mean ± SD (n = 3). Figure 3B shows IL-1β release by LPS-primed and ATP or MSU-stimulated BMDM pretreated overnight with 5 μM RSM932A. Mean ± SD. (n = 3). Figure 3C shows Caspase-1 in

supernatants of shChoKα and shCtrl iBMDM that were LPS-primed and ATP-treated. Figure 3D shows relative caspase 1 activation shown as % of p20 release to culture medium by iBMDM cultured as in Figure 3C. Mean ± SD (n = 3). Figure 3E shows the results of IB analysis of active caspase-1 in supernatants of LPS-primed and ATP-treated BMDM pretreated with RSM932A as indicated. Figure 3F shows relative caspase 1 activation in cells treated as above. Mean ± SEM (n = 4). Figure 3G shows the results of IB analysis of NLRP3 inflammasome subunits in shChoKα and shCtrl iBMDM or (Figure 3H) BMDM pretreated with RSM932A as indicated. *$p < 0.05$; **$p < 0.01$. Figure 3I shows that knockdown of single choline kinase isoform does not alter the expression of other isoforms or the NLRP3 inflammasome components. Figure 3I shows ChoKβ, ChoKα, pro-caspase 1, ASC, NLRP3, pro-IL-1β expression levels in iBMDM treated with or without LPS (100 ng/ml) for 4 hr. Figure 3J shows shCtrl and shChoKβ iBMDM were primed with 100 ng/ml LPS 4 hr and then treated with 400mM MSU for 3 hr and 3mM ATP for 1 hr. Culture supernatant was collected and IL-1β was measured by ELISA.

[0015] Figures 4A-4X are pictorial and graphical diagrams showing that choline deficiency alters mitochondrial lipids and depletes cellular ATP, resulting in AMPK activation and decreased IL-1β production. Figure 4A shows relative amounts of mitochondrial phospholipids in BMDM before and after LPS (24 hr) stimulation (n = 4). Figure 4B shows total mitochondrial phosphatidylcholine (mitoPC) and (Figure 4C) sphingomyelin (mitoSM), in BMDM cultured in control or choline-deficient medium and incubated with or without LPS for 24 hr. (n = 4). Figure 4D shows NAD^+/NADH ratio in BMDM cultured and treated as above. Mean ± SEM (n = 5). Figure 4E shows ATP synthase activity in mitochondria isolated from BMDM that were cultured and treated as above in the presence or absence of Oligomycin (10 μM). Mean ± SD (n = 2). Figure 4F shows relative cellular ATP presented as % of control in BMDM cultured and treated as above. Mean ± SEM (n = 6). Figure 4G shows the results of IB analysis of p-AMPK, ATPIF1 in BMDM cultured and treated as above. Figure 4H shows the results of IB analysis of p-AMPK after 24 hr LPS stimulation in the presence or absence of choline. (n = 3). Figure 4I shows IL-1β release by WT and *Ampkα1*$^{-/-}$ BMDM cultured in control or choline-deficient medium and stimulated with LPS+ATP. Mean ± SEM (n = 5). Figure 4J shows the results of IB analysis of ATPIF1 after 24 hr LPS stimulation in the presence or absence of choline. (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p<0.05$; ****$p < 0.001$. Figure 4K shows mitochondrial phospholipid changes analyzed by MS in BMDM cultured control or choline-deficient medium for 24 hr. (n = 4). Figure 4L shows total mitochondrial phosphatidylcholine (mitoPC) and (Figure 4M)

sphingomyelin (mitoSM), determined by mass spectrometry in BMDM cultured in control or choline-deficient medium and stimulated with LPS for 24 hr. Figures 4N and 4O show cellular ATP presented as % of control in shCtrl, shSlc44a1 (Figure 4N) and shChoKα (Figure 4O) stimulated with LPS for 24 hr. Mean ± SEM (n = 6). Figure 4P shows NAD^+/NADH ratio in LPS-stimulated shCtrl and shSlc44a1 iBMDM. Mean ± SEM (n = 9). Figures 4Q-4V show the results from IB analysis of indicated proteins and DRP1 amounts in mitochondria in shCtrl and shSlc44a1 (Figures 4Q and 4R), shCtrl and shChoKα (Figures 4S and 4T), and RSM932A-pretreated BMDM (Figures 4U and 4V), treated with or without LPS. Figure 4W shows IL-1β release by BMDM pretreated with the AMPK activator A769662, primed with LPS, and stimulated with ATP and nigericin for 40 min. Mean ± SEM (n = 4). Figure 4X shows succinate accumulation in LPS-stimulated BMDM cultured in control or choline-free medium. Mean ± SEM (n = 5). *p < 0.05; **p < 0.01; ***p<0.005; ****p < 0.001.

[0016] Figures 5A-5T are pictorial and graphical diagrams showing that impaired choline uptake and phosphorylation stimulate mitophagy and inhibit IL-1β production. Mitochondrial membrane potential (Ψm) was measured in (Figure 5A) shCtrl, shChoKα or (Figure 5B) RSM932A-pretreated iBMDM by TMRM staining. Mean ± SEM (n = 5 and 4, respectively). Figure 5C shows the results from immunofluorescence (IF) analysis of p62 recruitment to mitochondria stained by ATP5B antibody. Scale bar: 10 μm (images representative of three experiments). Figure 5D shows quantitation of mitochondrial p62 aggregates. Mean ± SEM. (n = 10 high-magnification fields per treatment in two independent experiments). Figure 5E shows the results from IF analysis of p62 and DRP1 recruitment to mitochondria in LPS-primed and ATP or nigericin-treated shChoKα and shCtrl iBMDM. Scale bar: 10 μm (images representative of three experiments). Figure 5F shows the results from IB analysis of p62, DRP1 and VDAC in mitochondria isolated from LPS-primed and ATP or nigericin-treated shChoKα and shCtrl iBMDM. Figure 5G shows quantitation of mitochondrial p62 aggregates in WT and *Ampkα1*$^{-/-}$ BMDM cultured in the presence or absence of choline and stimulated with LPS+ATP. Mean ± SEM. (n = 10-14 high-magnification fields per treatment in two independent experiments). Figure 5H shows the results from IF analysis of p62 recruitment to mitochondria. Scale bar: 20 μm (images representative of two experiments). Figure 5I shows the results from IF analysis of DRP1. Mitochondria were stained with ATP5B antibody. Scale bar: 20 μm (images representative of two experiments). *p < 0.05; **p<0.01; ***p < 0.005; ****p < 0.001. Figure 5J shows relative DRP1 in mitochondria isolated from shCtrl and shChoKα iBMDM that were LPS-primed and ATP-stimulated. (n = 3). Figure 5K shows the results from IB analysis of

indicated proteins in mitochondria isolated from RSM932A-pretreated BMDM that were LPS-primed and ATP-stimulated. Figure 5L shows relative DRP1 in mitochondria isolated from BMDM cultured and treated as in Figure 5K. (n = 3). Figure 5M shows quantitation of mitochondrial p62 aggregates. Mean ± SEM. (n = 13-20 high-magnification fields per treatment). Figure 5N shows the results from IF analysis of p62 recruitment to mitochondria that were stained with ATP5B antibody. Scale bar: 20 μm (images representative of three experiments). Figure 5O shows the results from IB analysis of p62, DRP1 and VDAC in mitochondria isolated from LPS-primed and ATP -treated BMDM cultured in control or choline-free medium. Figure 5P shows IL-1β release by shCtrl, shChoKα and shAtg7 iBMDM cultured in control or choline-free medium and treated with LPS+ATP. Mean ± SEM (n = 4). Figure 5Q shows mtROS accumulation in shCtrl and shChoKα iBMDM, and (Figure 5R) BMDM pretreated with RSM932A and treated with LPS+DOTAP or LPS+nigericin determined by MitoSox staining. Mean ± SEM (n = 4-5 and n = 3 respectively). Figures 5S and 5T show cytosolic mt-COX-1 and mt-D-Loop content determined by QPCR in (Figure 5S) shCtrl and shChoKα iBMDM and (Figure 5T) RSM932A-pretreated BMDM that were LPS-primed and stimulated with ATP or nigericin. Mean ± SD (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$.

[0017] Figures 6A-6G are pictorial and graphical diagrams showing that choline kinase inhibition reduces IL-1β production *in vivo*. Figure 6A shows that mice were pretreated with the choline kinase inhibitor MN58b (2.5 mg/kg) or vehicle daily for 3 days before intraperitoneal (i.p.) injection of 50 mg/kg LPS. % survival was determined by Kaplan Meyer analysis. (n = 6 animals per group). Figure 6B shows circulating IL-1β measured 3 hr after LPS injection in above mice. Mean ± SEM (n = 6 animals per group). Figures 6C-6E show that air pouches were created by s.c. injection of sterile air. Animals were treated with 2.5 mg/kg MN58b or vehicle 24 hr prior to injection of 3 mg/ml MSU crystals into the pouch. Tissue and intra-pouch wash were collected after 8 hr. Figure 6C shows the results from IF analysis of cells stained with macrophage marker F4/80 and CTL1 or ChoKα antibodies in skin collected from air pouch after MSU crystals injection. Scale bar 50 μm. Figure 6D shows pouch cell counts. Mean ± SEM. (n = 6 mice per group). Figure 6E shows IL-1β release into pouch cavity. Mean ± SEM (n = 6 animals per group). *$p < 0.05$; **$p < 0.01$. Circulating TNF (Figure 6F) and IL-6 (Figure 6G) 3 hr after LPS injection into mice treated as in Figure 6A. Mean ± SEM (n = 6 animals per group). n.s., not significant.

[0018] Figures 7A-7K are pictorial and graphical diagrams showing that choline kinase inhibition reduces MWS pathology. Figures 7A-7C show IL-1β release by BMDM from mice containing MWS (Figure 7A), FCAS (Figure 7B), and NOMID (Figure 7C) *Nlrp3* mutations. The cells were cultured in control or choline-deficient medium or in the presence of choline kinase inhibitor RSM932A, and stimulated with LPS or in case of FCAS activated at 32°C. Mean ± SEM. (n=10, MWS; n=4, FCAS and n=5, NOMID). *$p < 0.05$; ***$p<0.005$; ****$p<0.001$. Figures 7D-7K show MWS $Nlrp3^{A350VneoRCreT}$ mice were treated with the choline kinase inhibitor MN58b (2.5 mg/kg) or vehicle BID for 15 days. Citculating leukocytes (Figure 7D), granulocytes (Figure 7E), monocytes (Figure 7F), and lymphocytes (Figure 7G), were measured. Figure 7H shows spleen size as % spleen weight of body weight. Figure 7I shows images of spleens. Figure 7J shows liver size as % liver weight of body weight. Figure 7K shows the results from H&E staining of liver tissue from above mice. Mean ± SEM. (n = 3 vehicle- and n = 6 MN58b treatment). *$p < 0.05$; ***$p<0.005$.

DETAILED DESCRIPTION OF THE INVENTION

[0019] The present invention is based on the observation that choline uptake via CTL1 and its phosphorylation by ChoKα contribute to macrophage-mediated IL-1β-dependent inflammation. Thus, inhibition of choline uptake and/or phosphocholine synthesis reduces IL-1β production and ameliorates acute and chronic macrophage-mediated inflammation.

[0020] Before the present compositions and methods are described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0021] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0022] The term "comprising," which is used interchangeably with "including," "containing," or "characterized by," is inclusive or open-ended language and does not exclude

additional, unrecited elements or method steps. The phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. The present disclosure contemplates embodiments of the invention compositions and methods corresponding to the scope of each of these phrases. Thus, a composition or method comprising recited elements or steps contemplates particular embodiments in which the composition or method consists essentially of or consists of those elements or steps.

[0023] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0024] The term "subject" as used herein refers to any individual or patient to which the subject methods are performed. Generally the subject is human, although as will be appreciated by those in the art, the subject may be an animal. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of subject.

[0025] A subject "in need" of treatment with the invention's methods includes a subject that is "suffering from disease," i.e., a subject that is experiencing and/or exhibiting one or more symptoms of the disease, and a subject "at risk" of the disease. A subject "in need" of treatment includes animal models of the disease. A subject "at risk" of disease refers to a subject that is not currently exhibiting disease symptoms and is predisposed to expressing one or more symptoms of the disease. This predisposition may be genetic based on family history, genetic factors, environmental factors such as exposure to detrimental compounds present in the environment, etc.). It is not intended that the present invention be limited to any particular signs or symptoms. Thus, it is intended that the present invention encompass subjects that are experiencing any range of disease, from sub-clinical symptoms to full-blown disease, wherein the subject exhibits at least one of the indicia (e.g., signs and symptoms) associated with the disease.

[0026] As used herein, a "non-human mammal" may be any animal as long as it is other than human, and includes a transgenic animal and animals for which a production method of ES cells and/or iPS cells has been established. For example, rodents such as mouse, rat, hamster, guinea pig, rabbit, swine, bovine, goat, horse, sheep, dog, cat, or monkey are envisioned as non-human mammals.

[0027] The term "administering" to a subject means delivering a molecule, drug, or composition to a subject. "Administering" a composition to a subject in need of reducing a disease and/or of reducing one or more disease symptoms includes prophylactic administration of the composition (i.e., before the disease and/or one or more symptoms of the disease are detectable) and/or therapeutic administration of the composition (i.e., after the disease and/or one or more symptoms of the disease are detectable). When the methods described herein include administering a combination of a first composition and a second composition, the first and second compositions may be administered simultaneously at substantially the same time, and/or administered sequentially at different times in any order (first composition followed second composition, or second composition followed by first composition). For example, administering the second composition substantially simultaneously and sequentially in any order includes, for example, (a) administering the first and second compositions simultaneously at substantially the same time, followed by administering the first composition then the second composition at different times, (b) administering the first and second compositions simultaneously at substantially the same time, followed by administering the second composition then the first composition at different times, (c) administering the first composition then the second composition at different times, followed by administering the first and second compositions simultaneously at substantially the same time, and (d) administering the second composition then the first composition at different times, followed by administering the first and second compositions simultaneously at substantially the same time.

[0028] As used herein, an "effective amount" is an amount of a substance or molecule sufficient to effect beneficial or desired clinical results including alleviation or reduction in any one or more of the symptoms associated with macrophage-mediated inflammation such as, but not limited to, cancer, lupus, gout, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, uveitis, Alzheimer's disease, Parkinson's disease, cryopyrin-associated periodic syndromes, nonalcoholic steatohepatitis (NASH), type 2 diabetes, atherosclerosis, macular degeneration, and many more inflammatory and degenerative diseases. For purposes of this invention, an effective

amount of a compound or molecule of the invention is an amount sufficient to reduce the signs and symptoms associated with such disorders. In some embodiments, the "effective amount" may be administered before, during, and/or after any treatment regimens for the above-mentioned diseases.

[0029] The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when used in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, etc.), cell (*e.g.*, B cell, T cell, tumor cell), and/or phenomenon (*e.g.*, disease symptom), in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of molecule, cell and/or phenomenon in the first sample (or in the first subject) is lower than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis.

[0030] The terms "increase," "elevate," "raise," and grammatical equivalents (including "higher," "greater," etc.) when used in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, etc.), cell (*e.g.*, B cell, T cell, tumor cell), and/or phenomenon (*e.g.*, disease symptom), in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of the molecule, cell and/or phenomenon in the first sample (or in the first subject) is higher than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis.

[0031] As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, treatment of macrophage-mediated inflammation.

[0032] As used herein, the term "cancer" refers to a plurality of cancer cells that may or may not be metastatic, such as prostate cancer, liver cancer, bladder cancer, skin cancer (*e.g.*, cutaneous, melanoma, basal cell carcinoma, Kaposi's sarcoma, etc.), ovarian cancer, breast cancer, lung cancer, cervical cancer, pancreatic cancer, colon cancer, stomach cancer, esophagus cancer, mouth cancer, tongue cancer, gum cancer, muscle cancer, heart cancer, bronchial cancer, testis cancer, kidney cancer, endometrium cancer, and uterus cancer. Cancer may be a primary cancer, recurrent cancer, and/or metastatic cancer. The place where a cancer starts in the body is called the "primary cancer" or "primary site." If cancer cells spread to another part of the body

the new area of cancer is called a "secondary cancer" or a "metastasis." "Recurrent cancer" means the presence of cancer after treatment and after a period of time during which the cancer cannot be detected. The same cancer may be detected at the primary site or somewhere else in the body, *e.g.*, as a metastasis.

[0033] As used herein, the term "cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described (Pitot et al., Fundamentals of Oncology, 15-28 (1978)). This includes cells in early, intermediate and advanced stages of neoplastic progression including "pre-neoplastic" cells (*i.e.*, "hyperplastic" cells and dysplastic cells), and neoplastic cells in advanced stages of neoplastic progression of a dysplastic cell.

[0034] As used herein, a "metastatic" cancer cell refers to a cancer cell that is translocated from a primary cancer site (*i.e.*, a location where the cancer cell initially formed from a normal, hyperplastic or dysplastic cell) to a site other than the primary site, where the translocated cancer cell lodges and proliferates.

[0035] As used herein, the term "genetic modification" is used to refer to any manipulation of an organism's genetic material in a way that does not occur under natural conditions. Methods of performing such manipulations are known to those of ordinary skill in the art and include, but are not limited to, techniques that make use of vectors for transforming cells with a nucleic acid sequence of interest. Included in the definition are various forms of gene editing in which DNA is inserted, deleted or replaced in the genome of a living organism using engineered nucleases, or "molecular scissors." These nucleases create site-specific double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through nonhomologous end-joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations (i.e., edits). There are several families of engineered nucleases used in gene editing, for example, but not limited to, meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the CRISPR-Cas system.

[0036] As used herein, the term "test agent" or "candidate agent" refers to an agent that is to be screened in one or more of the assays described herein. The agent can be virtually any chemical compound. It can exist as a single isolated compound or can be a member of a chemical (*e.g.*, combinatorial) library. In one embodiment, the test agent is a small organic molecule. The term small organic molecule refers to any molecules of a size comparable to

those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (*e.g.*, proteins, nucleic acids, etc.). In certain embodiments, small organic molecules range in size up to about 5000 Da, up to 2000 Da, or up to about 1000 Da.

[0037] As used herein, the terms "sample" and "biological sample" refer to any sample suitable for the methods provided by the present invention. In one embodiment, the biological sample of the present invention is a tissue sample, *e.g.*, a biopsy specimen such as samples from needle biopsy (*i.e.*, biopsy sample). In other embodiments, the biological sample of the present invention is a sample of bodily fluid, *e.g.*, serum, plasma, sputum, lung aspirate, urine, and ejaculate.

[0038] The term "antibody" is meant to include intact molecules of polyclonal or monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as fragments thereof, such as Fab and F(ab')2, Fv and SCA fragments which are capable of binding an epitopic determinant. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art (Kohler, et al., Nature, 256:495, 1975). An Fab fragment consists of a monovalent antigen binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain. An Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner. An (Fab')2 fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A (Fab')2 fragment is a dimer of two Fab' fragments, held together by two disulfide bonds. An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains. A single chain antibody ("SCA") is a genetically engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

[0039] The terms "specifically binds" and "specific binding" when used in reference to the binding of an antibody to a target molecule (e.g., peptide) or to a target cell (e.g., immunosuppressive B cells), refer to an interaction of the antibody with one or more epitopes on the target molecule or target cell where the interaction is dependent upon the presence of a particular structure on the target molecule or target cell. For example, if an antibody is specific

for epitope "A" on the target cell, then the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody. In various embodiments, the level of binding of an antibody to a target molecule or target cell is determined using the "IC50," i.e., "half maximal inhibitory concentration" that refer to the concentration of a substance (e.g., inhibitor, antagonist, etc.) that produces a 50% inhibition of a given biological process, or a component of a process (e.g., an enzyme, antibody, cell, cell receptor, microorganism, etc.). It is commonly used as a measure of an antagonist substance's potency.

[0040] Reference herein to "normal cells" or "corresponding normal cells" means cells that are from the same organ and of the same type as any of the above-mentioned disease cell type. In one aspect, the corresponding normal cells comprise a sample of cells obtained from a healthy individual. Such corresponding normal cells can, but need not be, from an individual that is age-matched and/or of the same sex as the individual providing the above-mentioned disease cells being examined. In another aspect, the corresponding normal cells comprise a sample of cells obtained from an otherwise healthy portion of tissue of a subject having a macrophage-mediated inflammatory and/or degenerative disease.

[0041] The terms "choline transporter-like protein 1" and "CTL1" refer to a protein that in humans is encoded by the *SLC44A1* gene. The human CTL1 amino acid sequence is exemplified by SEQ ID NO: 1.

[0042] The terms "choline kinase" and "ChoK" (also known as choline phosphokinase) refer to an enzyme that catalyzes the first reaction in the chline pathway for phophatidylcholine (PC) biosynthesis. In mammalian cells, the enzyme exists as three isoforms: CKα-1,CKα-2 and CKβ. The human ChoKα and ChoKβ amino acid sequences are exemplified by SEQ ID NOs: 2 and 4, respectively.

[0043] The terms "interleukin-1 beta" and "IL-1β" (also known as leukocytic pyrogen, leukocytic endogenous mediator, mononuclear cell factor, and lymphocyte activating factor) refer to a cytokine that is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The human interleukin-1 beta amino acid sequence is exemplified by SEQ ID NO: 5.

[0044] The terms "interleukin-18" and "IL-18" (also known as interferon-gamma inducing factor) refer to a cytokine produced mainly by macrophages but also other cell types, stimulates various cell types and has pleiotropic functions. IL-18 is a proinflammatory cytokine that facilitates type 1 responses. Together with IL-12, it induces cell-mediated immunity following infection with microbial products like lipopolysaccharide (LPS). The human interleukin-18 amino acid sequence is exemplified by SEQ ID NO: 6.

[0045] The terms "interleukin 10" and "IL-10" (also known as CSIF; TGIF; GVHDS; IL10A) refer to a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. The human interleukin 10 amino acid sequence is exemplified by SEQ ID NO: 6.

[0046] The terms "interleukin-6" or "IL-6" refer to a cytokine that is a known mediator of fever and of acute phase responses. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production. The human interleukin 10 amino acid sequence is exemplified by SEQ ID NO: 7.

[0047] The terms "NLR Family Pyrin Domain Containing 3" and "NLRP3" refer to a specific protein coding gene. Diseases associated with NLRP3 include, but are not limited to, Muckle-Wells Syndrome and Cinca Syndrome. Among its related pathways are NOD-like receptor signaling pathway and Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signaling pathways. NACHT, LRR and PYD domains-containing protein 3 (NALP3), also known as cryopyrin, is a protein that in humans is encoded by the *NLRP3* gene located on the long arm of chromosome 1. NALP3 is expressed predominantly in macrophages and as a component of the inflammasome and detects products of damaged cells such as extracellular ATP and crystalline uric acid. Activated NALP3 in turn triggers an immune response. Mutations in the NLRP3 gene are associated with a number of organ specific autoimmune diseases. The human NALP3 amino acid sequence is exemplified by SEQ ID NO: 8.

[0048] The terms "inhibitor of nuclear factor kappa-B kinase subunit beta" and "IKKβ" refer to an enzyme that serves as a protein subunit of IκB kinase, which is a component of the cytokine-activated intracellular signaling pathway involved in triggering immune responses. Activated IKK-β phosphorylates a protein called the inhibitor of NF-κB, IκB (IκBα), which binds NF-κB to inhibit its function. Phosphorylated IκB is degraded via the ubiquitination pathway, freeing NF-κB, and allowing its entry into the nucleus of the cell where it activates various genes involved in inflammation and other immune responses. The human IKKβ amino acid sequence is exemplified by SEQ ID NO: 10.

[0049] Choline is a vitamin-like nutrient that is taken up via specific transporters and metabolized by choline kinase (ChoKα) which converts it to phosphocholine needed for *de novo* synthesis of phosphatidylcholine (PC), the main phospholipid of cellular membranes. Enhanced choline uptake was detected in cells within inflammatory sites, including tumors, inflamed joints, and atherosclerotic plaques (Hellberg et al., 2016; Matter et al., 2006; Roivainen et al., 2003; Schwarz et al., 2016). However, the biological impact of choline uptake and phosphorylation has only been studied in cancer, where choline feeds the phospholipid pool required for cell proliferation and migratory/invasive behavior (Al-Saffar et al., 2006; Glunde et al., 2011). Tracer studies demonstrated that under pathological inflammatory conditions choline is taken up by macrophages (Hellberg et al., 2016; Matter et al., 2006; Roivainen et al., 2003; Schwarz et al., 2016), but the effect of choline uptake on macrophage biology was heretofore unknown.

[0050] It has been found that Toll-like receptor (TLR) activation enhances choline uptake by macrophages (TLR4-mediated macrophage activation) and microglia resulting in upregulation of choline uptake due to NF-κB-dependent induction of the choline transporter CTL1. The newly taken up choline is rapidly converted to phosphatidylcholine via the Kennedy pathway (McMaster, 2018). Inhibition of CTL1 expression or ChoKα-mediated choline mobilization results in altered mitochondrial phospholipid composition and accumulation of defective mitochondria, that are rapidly eliminated through mitophagy. These results strongly suggest that choline uptake is essential for phospholipid remodeling and maintenance of mitochondrial function and integrity in metabolically challenged/stressed macrophages.

[0051] Macrophage activation involves extensive metabolic reprogramming, a glycolytic switch, elevated ROS production, and phospholipid remodeling that are needed for coping with the energetic cost of inflammatory cytokine production and bactericidal activity, and to ensure

proper membrane fluidity and plasticity (Chu, 1992; Grove et al., 1990; O'Neill et al., 2016; Tian et al., 2008; West et al., 2011). Ablation of macrophage choline cytidylyltransferase α (CCTα), which uses phosphocholine to generate CDP-choline, in the second step of the Kennedy pathway reduces phosphatidylcholine amounts with a subsequent decrease in accumulation of diacylgycerol (DAG), which interferes with TNF secretion (Tian et al., 2008). Unlike TNF and IL-6, bioactive IL-1β is not secreted via the classical secretory pathway. IL-1β and IL-18 production and release require macrophage priming, during which pro-IL-1β, pro-IL18 and the critical inflammasome sensor NLRP3 are made. Next, a variety of secondary stimuli, such as ATP, MSU crystals, microbial toxins, and various microcrystals and microfibers, all of which cause mitochondrial damage (Zhou et al., 2011), ROS production (West et al., 2011), the release of ox-mtDNA fragments (Zhong et al., 2018) trigger NRLP3 inflammasome assembly and activation. This results in conversion of pro-IL-1β and pro-IL18 to their mature forms, which are released from activated macrophages by a non-traditional protein secretion. Defective choline uptake or inhibition of phosphocholine synthesis interfere with IL-1β and IL-18 production by accelerating mitophagy and diminishing the cytosolic release of the ultimate NLRP3 inflammasome activator ox-mtDNA. Since sustained NLRP3 inflammasome activation and IL-1β production require some form of mitochondrial damage, ongoing mtROS production, and new mtDNA synthesis (Zhong et al., 2018), it appears that proper phosphatidylcholine synthesis is needed for maintaining mitochondrial membrane integrity after LPS priming, thereby preventing excessive damage that could result in defective ATP production and upregulation of AMPK-dependent mitophagy.

[0052] The effect of choline deficiency has been studied in liver. Mice fed a choline-deficient diet show alterations in hepatocyte mitochondrial membrane composition, and undergo depletion of phosphatidylcholine and phosphatidylethanolamine (Guo et al., 2005; Teodoro et al., 2008). These changes cause loss of mitochondrial membrane potential and reduced activity of the electron transport chain complex I and V (Guo et al., 2005; James et al., 1992). In choline-deficient macrophages, mitochondrial phosphatidylcholine and sphingomyelin are significantly reduced, mitochondrial membrane potential drops and ATP synthase, a part of complex V, activity declines. Of note, LPS also altered mitoPC and mitoSM and reduced ATP synthase activity, events that were strongly modified by choline deficiency. Choline taken up after LPS stimulation may maintain mitochondrial integrity and function, thereby preserving residual ATP synthase activity needed for IL-1β production, a suggestion that is consistent with previous observations (Mills et al., 2016). The reduction in ATP synthase activity caused by

choline deficiency is accompanied by accumulation of ATPIF1, a protein that not only inhibits the forward ATP synthase activity of complex V but also blocks the reverse reaction, in which ATP is hydrolyzed (Campanella et al., 2009). This protective response prevents excessive ATP expenditure during times of reduced mitochondrial ATP synthesis. At the same time, this response attenuates ROS production caused by ATP hydrolysis and reverse electron transport (Campanella et al., 2009). Although it is not currently known how choline deficiency and reduced mitoPC and mitoSM content affects mitochondrial function, without being bound by theory, it is plausible that changes in mitochondrial membrane composition interfere with complex V assembly or function without increasing mtROS production. It is also plausible that intracellular choline or its phosphorylated form, phosphocholine, are sensed by a mitochondrial protein, which induces protective responses that prevent mitochondrial failure during reduced phosphocholine availability.

[0053] Alternatively, insufficient cellular phosphatidylcholine interferes with ongoing membrane synthesis needed for maintenance of mitochondrial integrity. A regulatory role for membrane lipid composition in activated macrophages was also proposed by others. Phosphatidylinositol-4-phosphate in the trans-Golgi network (Chen and Chen, 2018) and cholesterol uptake and distribution in ER membranes (de la Roche et al., 2018) also contribute to NLRP3 inflammasome activation. Reduced mitochondrial ATP production results in decreased cellular ATP, elevated AMP/ATP ratio and activation of AMPK, which stimulates initiation of autophagy and accelerates mitophagic clearance of defective mitochondria. LPS stimulation rapidly represses AMPK activation, but the effect is transient and at later time points AMPK is activated in part via LPS-induced IL-10 to balance the energy and metabolic demands of activated macrophages (Ip et al., 2017; Nomura et al., 2015). Active AMPK downregulates inflammation, in part through inhibition of IL-1β production (Cordero et al., 2018; Guma et al., 2015b; Wang et al., 2016). In addition, it has recently been shown that mitophagy removes damaged mitochondria that release ox-mtDNA, needed for NLRP3 inflammasome activation (Zhong et al., 2018; Zhong et al., 2016c). AMPK stimulates mitophagy both through phosphorylation and activation of ULK1 (Egan et al., 2011), and by enhancing translocation of DRP1 to mitochondria (Toyama et al., 2016). Indeed, in the absence of AMPK, choline deprivation has no effect whatsoever on IL-1β production and secretion, due to DRP1 sequestration in the cytosol leading to defective mitophagy.

[0054] Prophylaxis with antibiotics is used to prevent septicemia; however, 40% to 50% of microorganisms at infection sites are antibiotic resistant (Li and Webster, 2018). Notably, prophylactic treatment with ChoKα inhibitor protects mice from lethal septic shock. ChoKα inhibition reduces IL-1β production and ameliorates MWS, a genetic disease caused by constitutive NLRP3 inflammasome activation, which so far can only be treated with IL-1 sequestering antibodies and decoy receptors. These results also indicate that ChoKα inhibitors may be useful for reducing IL-1β production in other diseases associated with excessive NLRP3 inflammasome activation, such as osteoarthritis, gout, diabetes, and the metabolic syndrome. Recently, it was found that in addition to decreasing the incidence of recurrent vascular events in atherosclerotic patients, treatment with an IL-1β blocking antibody led to reduced lung cancer incidence and mortality (Ridker et al., 2017a; Ridker et al., 2017b). Curiously, lung cancer is a type of cancer in which ChoKα is overexpressed, and is associated with high risk of recurrence (Huang et al., 2015; Ramirez de Molina et al., 2007). ChoKα inhibitors may exert a similar effect and an added benefit due to a more direct effect on cancer cell proliferation.

[0055] Accordingly, the present study links choline metabolism to the control of NLRP3 inflammasome dependent inflammation. The results implicate mitochondrial phospholipid remodeling as a key mechanism for preserving residual mitochondrial ATP synthase activity during the glycolytic switch that accompanies macrophage activation. The results provided herein indicate that CTL1 induction and elevated phosphocholine synthesis are important features of so-called macrophage priming, a process that enables macrophages to respond to a variety of NLRP3 activating challenges with IL-1β and IL-18 production and release.

[0056] Thus, in one aspect, the invention provides a method of treating macrophage-mediated inflammatory and/or degenerative diseases in a subject in need thereof. The method includes administering to the subject an effective amount of an inhibitor of phosphocholine synthesis. In various embodiments, the inhibitor of phosphocholine synthesis may be an inhibitor of CTL1 activity or expression, an inhibitor of choline phosphorylation, an inhibitor of ChoKα activity or expression, an inhibitor of ChoKβ activity or expression, or an inhibitor of IL-1β and/or IL-18 production. In various embodiments, the inhibitor may be a small molecule, peptide, antisense oligonucleotide, guide RNA, shRNA, antibody or antibody fragment.

[0057] In various embodiments, the inhibitor of CTL1 activity or expression, inhibitor of choline phosphorylation, inhibitor of ChoKα activity or expression, and/or inhibitor of ChoKβ activity or expression is an inhibitory nucleic acid that specifically inhibits expression of CTL1

and/or inhibits ChoKα activation and/or inhibits ChoKβ activation. As used herein, an "inhibitory nucleic acid" means an RNA, DNA, or a combination thereof that interferes or interrupts the translation of mRNA. Inhibitory nucleic acids can be single or double stranded. The nucleotides of the inhibitory nucleic acid can be chemically modified, natural or artificial. The terms "short-inhibitory RNA" and "siRNA" interchangeably refer to short double-stranded RNA oligonucleotides that mediate RNA interference (also referred to as "RNA-mediated interference" or "RNAi"). The terms "small hairpin RNA" and "shRNA" interchangeably refer to an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNAi. RNAi is a highly conserved gene silencing event functioning through targeted destruction of individual mRNA by a homologous double-stranded small interfering RNA (siRNA) (Fire, A. et al., Nature 391:806-811 (1998)). Mechanisms for RNAi are reviewed, for example, in Bayne and Allshire, Trends in Genetics (2005) 21:370-73; Morris, Cell Mol Life Sci (2005) 62:3057-66; Filipowicz, et al., Current Opinion in Structural Biology (2005) 15:331-41.

[0058] Methods for the design of siRNA or shRNA target sequences have been described in the art. Among the factors to be considered include: siRNA target sequences should be specific to the gene of interest and have about 20-50% GC content (Henshel et al., Nucl. Acids Res., 32: 113-20 (2004); G/C at the 5' end of the sense strand; A/U at the 5' end of the antisense strand; at least 5 A/U residues in the first 7 bases of the 5' terminal of the antisense strand; and no runs of more than 9 G/C residues (Ui-Tei et al., Nucl. Acids Res., 3: 936-48 (2004)). Additionally, primer design rules specific to the RNA polymerase will apply. For example, for RNA polymerase III, the polymerase that transcribes from the U6 promoter, the preferred target sequence is 5'-GN18-3'. Runs of 4 or more Ts (or As on the other strand) will serve as terminator sequences for RNA polymerase III and should be avoided. In addition, regions with a run of any single base should be avoided (Czauderna et al., Nucl. Acids Res., 31: 2705-16 (2003)). It has also been generally recommended that the mRNA target site be at least 50-200 bases downstream of the start codon (Sui et al., Proc. Natl. Acad. Sci. USA, 99: 5515-20 (2002); Elbashir et al., Methods, 26: 199-213 (2002); Duxbury and Whang, J. Surg. Res., 117: 339-44 (2004) to avoid regions in which regulatory proteins might bind. Additionally, a number of computer programs are available to aid in the design of suitable siRNA and shRNAs for use in suppressing expression of choline-transporter-like protein 1 (CTL1) or inhibiting choline phosphorylation.

[0059] Ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy target mRNAs, particularly through the use of hammerhead ribozymes. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. Preferably, the target mRNA has the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art.

[0060] Gene targeting ribozymes may contain a hybridizing region complementary to two regions, each of at least 5 and preferably each of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous nucleotides in length of a target mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA.

[0061] With regard to antisense, siRNA or ribozyme oligonucleotides, phosphorothioate oligonucleotides can be used. Modifications of the phosphodiester linkage as well as of the heterocycle or the sugar may provide an increase in efficiency. Phophorothioate is used to modify the phosphodiester linkage. An N3'-P5' phosphoramidate linkage has been described as stabilizing oligonucleotides to nucleases and increasing the binding to RNA. Peptide nucleic acid (PNA) linkage is a complete replacement of the ribose and phosphodiester backbone and is stable to nucleases, increases the binding affinity to RNA, and does not allow cleavage by RNAse H. Its basic structure is also amenable to modifications that may allow its optimization as an antisense component. With respect to modifications of the heterocycle, certain heterocycle modifications have proven to augment antisense effects without interfering with RNAse H activity. An example of such modification is C-5 thiazole modification. Finally, modification of the sugar may also be considered. 2'-O-propyl and 2'-methoxyethoxy ribose modifications stabilize oligonucleotides to nucleases in cell culture and *in vivo*.

[0062] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is an acronym for DNA loci that contain multiple, short, direct repetitions of base sequences. The prokaryotic CRISPR/Cas system has been adapted for use as gene editing (silencing, enhancing or changing specific genes) for use in eukaryotes (see, for example, Cong, Science, 15:339(6121):819-823 (2013) and Jinek, et al., Science, 337(6096):816-21 (2012)). By transfecting a cell with elements including a Cas gene and specifically designed CRISPRs, nucleic acid sequences can be cut and modified at any desired location. Methods of preparing compositions for use in genome editing using the CRISPR/Cas systems are described in detail in US Pub. No. 2016/0340661, US Pub. No. 20160340662, US Pub. No. 2016/0354487, US Pub. No. 2016/0355796, US Pub. No.

20160355797, and WO 2014/018423, which are specifically incorporated by reference herein in their entireties.

[0063] Thus, as used herein, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (*e.g.*, tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer", "guide RNA" or "gRNA" in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. One or more tracr mate sequences operably linked to a guide sequence (*e.g.*, direct repeat-spacer-direct repeat) can also be referred to as "pre-crRNA" (pre-CRISPR RNA) before processing or crRNA after processing by a nuclease.

[0064] There are many resources available for helping practitioners determine suitable target sites once a desired DNA target sequence is identified. For example, numerous public resources, including a bioinformatically generated list of about 190,000 potential sgRNAs, targeting more than 40% of human exons, are available to aid practitioners in selecting target sites and designing the associate sgRNA to affect a nick or double strand break at the site. See also, crispr.u-psud.fr, a tool designed to help scientists find CRISPR targeting sites in a wide range of species and generate the appropriate crRNA sequences.

[0065] Inhibitory nucleic acids, such as siRNA, shRNA, ribozymes, or antisense molecules, can be synthesized and introduced into cells using methods known in the art. Molecules can be synthesized chemically or enzymatically *in vitro* (Micura, Agnes Chem. Int. Ed. Emgl. 41 2265-9 (2002); Paddison et al., Proc. Natl. Acad. Sci. USA, 99:1443-8 2002) or endogenously expressed inside the cells in the form of shRNAs (Yu et al., Proc. Natl. Acad. Sci. USA, 99:6047-52 (2002); McManus et al., RNA 8, 842-50 (2002)). Plasmid-based expression systems using RNA polymerase III U6 or H1, or RNA polymerase II U1, small nuclear RNA promoters, have been used for endogenous expression of shRNAs (Brummelkamp et al., Science, 296: 550-3 (2002); Sui et al., Proc. Natl. Acad. Sci. USA, 99: 5515-20 (2002); Novarino et al., J. Neurosci., 24: 5322-30 (2004)). Synthetic siRNAs can be delivered by electroporation or by using lipophilic agents (McManus et al., RNA 8, 842-50 (2002); Kishida et al., J. Gene Med., 6: 105-10 (2004)). Alternatively, plasmid systems can be used to stably express small hairpin RNAs (shRNA) for the suppression of target genes (Dykxhoorn et al., Nat. Rev. Mol. Biol.,

4:457-67 (2003)). Various viral delivery systems have been developed to deliver shRNA-expressing cassettes into cells that are difficult to transfect (Brummelkamp et al., Cancer Cell, 2: 243-7 (2002); Rubinson et al., Nat. Genet., 33: 401-6 2003). Furthermore, siRNAs can also be delivered into live animals. (Hasuwa et al., FEBS Lett., 532, 227-30 (2002); Carmell et al., Nat. Struct. Biol., 10: 91-2 (2003); Kobayashi et al., J. Pharmacol. Exp. Ther., 308:688-93 (2004)).

[0066] Inhibitory oligonucleotides can be delivered to a cell by direct transfection or transfection and expression via an expression vector. Appropriate expression vectors include mammalian expression vectors and viral vectors, into which has been cloned an inhibitory oligonucleotide with the appropriate regulatory sequences including a promoter to result in expression of the antisense RNA in a host cell. Suitable promoters can be constitutive or development-specific promoters. Transfection delivery can be achieved by liposomal transfection reagents, known in the art (*e.g.*, Xtreme transfection reagent, Roche, Alameda, Calif.; Lipofectamine formulations, Invitrogen, Carlsbad, Calif.). Delivery mediated by cationic liposomes, by retroviral vectors and direct delivery are efficient. Another possible delivery mode is targeting using antibody to cell surface markers for the target cells.

[0067] In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a target cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. Accordingly, cleavage of DNA by the genome editing vector or composition can be used to delete nucleic acid material from a target DNA sequence by cleaving the target DNA sequence and allowing the cell to repair the sequence. As such, the compositions can be used to modify DNA in a site-specific, *i.e.*, "targeted" way, for example gene knock-out, gene knock-in, gene editing, gene tagging, etc., as used in, for example, gene therapy.

[0068] While the specifics can be varied in different engineered CRISPR systems, the overall methodology is similar. A practitioner interested in using CRISPR technology to target a DNA sequence can insert a short DNA fragment containing the target sequence into a guide RNA expression plasmid. The sgRNA expression plasmid contains the target sequence (about 20 nucleotides), a form of the tracrRNA sequence (the scaffold) as well as a suitable promoter and necessary elements for proper processing in eukaryotic cells. Such vectors are commercially available (see, for example, Addgene). Many of the systems rely on custom, complementary oligos that are annealed to form a double stranded DNA and then cloned into the sgRNA expression plasmid. Co-expression of the sgRNA and the appropriate Cas enzyme from the

same or separate plasmids in transfected cells results in a single or double strand break (depending of the activity of the Cas enzyme) at the desired target site.

[0069] As demonstrated herein, LPS increased CTL1 expression 6-fold, and boosted intracellular choline by 238% ($p<0.01$) and phosphocholine by 559% ($p<0.001$). Choline deficiency reduced MSU-induced IL-1β release by 60% ($p<0.001$), shCTL1 by 57% ($p<0.05$), and shChoKα by 89% ($p<0.05$) and ChoKα inhibition via RSM932A by 71% ($p<0.005$). Impaired choline uptake or ChoKα activity promoted AMPK activation and DRP1, LC3 and p62 recruitment to mitochondria. AMPK deletion blocked choline deficiency effect on IL-1β release. Of note, treatment with colchicine, previously defined to inhibit NLRP3 inflammasome activity and activate AMPK, also reduced CTL1 (90%) and ChoKα levels (85%). In vivo, after MSU crystal injection, cells collected from peritoneal cavity strongly expressed both CTL1 ($p<0.05$) and ChoKα ($p<0.01$). F4/80 positive myeloid cells recruited into the air pouch also expressed ChoKα and CTL1. Last, treatment with ChoKα inhibitor MN58b reduced MSU crystal-induced leukocyte recruitment by 47% ($p<0.05$) and IL-1β release by 66% ($p<0.01$).

[0070] Thus, inhibition of CTL1 expression or choline phosphorylation attenuated NLRP3 inflammasome activation and IL-1β and IL-18 production in stimulated macrophages. Mechanistically, reduced choline uptake altered mitochondrial lipid profile, attenuated mitochondrial ATP synthesis and activated the energy sensor AMP-activated protein kinase (AMPK). By potentiating mitochondrial recruitment of DRP1, AMPK stimulates mitophagy, which contributes to termination of NLRP3 inflammasome activation. Correspondingly, pharmacological ChoKα inhibition ameliorated several models of IL-1β-dependent inflammation *in vivo*, an effect that was also achieved by CTL1 targeting or by reduction in dietary choline.

[0071] Impaired choline uptake or phosphocholine production affects mitochondrial phosphatidylcholine and sphingomyelin, disrupts mitochondrial ATP synthesis, and triggers AMPK activation and mitophagy. By decreasing the amount of damaged mitochondria that produce oxidized- (ox)mtDNA, an NLRP3 activator, mitophagy attenuates IL-1β production. As demonstrated herein, inhibition of phosphocholine synthesis reduces IL-1β production and ameliorates acute and chronic macrophage-mediated inflammation.

[0072] In another aspect, the present invention provides a method of ameliorating macrophage-mediated inflammatory and/or degenerative diseases in a subject. As used herein,

the term "ameliorate" means that the clinical signs and/or the symptoms associated with macrophage-mediated inflammatory and/or degenerative diseases are lessened. The signs or symptoms to be monitored will be characteristic of a particular disease or disorder and will be well known to the skilled clinician, as will the methods for monitoring the signs and conditions thereof.

[0073] Administering may be done using methods known in the art (*e.g.*, Erickson et al., U.S. Pat. No. 6,632,979; Furuta et al., U.S. Pat. No. 6,905,839; Jackobsen et al., U.S. Pat. No. 6,238,878; Simon et al., U.S. Pat. No. 5,851,789). The compositions of the invention may therefore be administered prophylactically (*i.e.*, before the observation of disease symptoms) and/or therapeutically (*i.e.*, after the observation of disease symptoms). Administration also may be concomitant with (*i.e.*, at the same time as, or during) manifestation of one or more disease symptoms. In addition, the compositions of the invention may be administered before, concomitantly with, and/or after administration of another type of drug or therapeutic procedure (*e.g.*, surgery). Methods of administering the compositions of the invention include, but are not limited to, administration in parenteral, oral, intraperitoneal, intranasal, topical and sublingual forms. Parenteral routes of administration include, for example, subcutaneous, intravenous, intramuscular, intrastemal injection, and infusion routes.

[0074] In another aspect, the present invention provides a method of identifying an agent useful for treating a macrophage-mediated inflammatory and/or degenerative disease or disorder through the targeting of CTL1 expression or choline phosphorylation. The method includes contacting a sample of cells with at least one test agent, wherein a decrease in CTL1 expression or choline phosphorylation in the presence of the test agent as compared to CTL1 expression or choline phosphorylation in the absence of the test agent identifies the agent as useful for treating macrophage-mediated inflammatory and degenerative diseases. In one embodiment, a decrease in NLRP3 inflammasome activation, IL-1β production and/or IL-18 production in the presence of the test agent as compared to NLRP3 inflammasome activation, IL-1β production and/or IL-18 production in the absence of the test agent identifies the agent as useful for treating a macrophage-mediated inflammatory and degenerative disease. In various embodiments, the method may be performed in a high throughput format, such as contacting samples of cells of a plurality of samples with at least one test agent. In various embodiments, the plurality of samples may be obtained from a single subject or from different subjects.

[0075] An agent useful in a method of the invention can be any type of molecule, for example, a polynucleotide, a peptide, antisense oligonucleotide, antibody or antibody fragment,

a peptidomimetic, peptoids such as vinylogous peptoids, a small organic molecule, or the like, and can act in any of various ways to reduce or inhibit elevated NLPR3 inflammasome activation, CTL1 expression or choline phosphorylation. In various embodiments, the inhibitor of CTL1 expression or choline phosphorylation is an inhibitory nucleic acid that inhibits the expression of CTL1, NLRP3 inflammasome activation, IL-1β production and/or IL-18 production. For example, the inhibitory nucleic acid can be siRNA, shRNA, guide RNA (gRNA), oligonucleotides, antisense RNA or ribozymes that inhibit such activity or expression.

[0076] Further, the agent can be administered in any way typical of an agent used to treat the particular type of above-mentioned diseases or under conditions that facilitate contact of the agent with the target diseased cells and, if appropriate, entry into the cells. Entry of a polynucleotide agent into a cell, for example, can be facilitated by incorporating the polynucleotide into a viral vector that can infect the cells. Thus, the inhibitory nucleic acid can be delivered in, for example, a lentiviral vector, a herpesvirus vector or an adenoviral vector.

[0077] If a viral vector specific for the cell type is not available, the vector can be modified to express a receptor (or ligand) specific for a ligand (or receptor) expressed on the target cell, or can be encapsulated within a liposome, which also can be modified to include such a ligand (or receptor). A peptide agent can be introduced into a cell by various methods, including, for example, by engineering the peptide to contain a protein transduction domain such as the human immunodeficiency virus TAT protein transduction domain, which can facilitate translocation of the peptide into the cell.

[0078] Generally, an agent to be administered to a subject may be formulated in a composition (*e.g.*, a pharmaceutical composition) suitable for such administration. Such formulated agents are useful as medicaments for treating a subject suffering from any of the above-mentioned diseases, in part, by elevated or abnormally elevated CTL1 expression or choline phosphorylation.

[0079] Pharmaceutically acceptable carriers useful for formulating an agent for administration to a subject are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include,

for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the physico-chemical characteristics of the therapeutic agent and on the route of administration of the composition, which can be, for example, orally or parenterally such as intravenously, and by injection, intubation, or other such method known in the art. The pharmaceutical composition also can contain a second (or more) compound(s) such as a diagnostic reagent, nutritional substance, toxin, or therapeutic agent, for example, a cancer chemotherapeutic agent and/or vitamin(s).

[0080] In general, a suitable daily dose of a compound/inhibitor of the invention will be that amount of the compound/inhibitor that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient will range from about 0.0001 to about 100 mg per kilogram of body weight per day which can be administered in single or multiple doses.

[0081] When practiced as an *in vitro* assay, the methods can be adapted to a high throughput format, thus allowing the examination of a plurality (*i.e.*, 2, 3, 4, or more) of cell samples and/or test agents, which independently can be the same or different, in parallel. A high throughput format provides numerous advantages, including that test agents can be tested on several samples of cells from a single patient, thus allowing, for example, for the identification of a particularly effective concentration of an agent to be administered to the subject, or for the identification of a particularly effective agent to be administered to the subject. As such, a high throughput format allows for the examination of two, three, four, etc., different test agents, alone or in combination, on the macrophages of a subject such that the best (most effective) agent or combination of agents can be used for a therapeutic procedure. Further, a high throughput format allows, for example, control samples (positive controls and or negative controls) to be run in parallel with test samples, including, for example, samples of cells known to be effectively treated with an agent being tested.

[0082] A high throughput method of the invention can be practiced in any of a variety of ways. For example, different samples of cells obtained from different subjects can be examined, in parallel, with same or different amounts of one or a plurality of test agent(s); or two or more

samples of cells obtained from one subject can be examined with same or different amounts of one or a plurality of test agent. In addition, cell samples, which can be of the same or different subjects, can be examined using combinations of test agents and/or known effective agents. Variations of these exemplified formats also can be used to identify an agent or combination of agents useful for treating any of the above-mentioned diseases associated with elevated CTL1 expression or choline phosphorylation.

[0083] When performed in a high throughput (or ultra-high throughput) format, the method can be performed on a solid support (*e.g.*, a microtiter plate, a silicon wafer, or a glass slide), wherein samples to be contacted with a test agent are positioned such that each is delineated from each other (*e.g.*, in wells). Any number of samples (*e.g.*, 96, 1024, 10,000, 100,000, or more) can be examined in parallel using such a method, depending on the particular support used. Where samples are positioned in an array (*i.e.*, a defined pattern), each sample in the array can be defined by its position (*e.g.*, using an x-y axis), thus providing an "address" for each sample. An advantage of using an addressable array format is that the method can be automated, in whole or in part, such that cell samples, reagents, test agents, and the like, can be dispensed to (or removed from) specified positions at desired times, and samples (or aliquots) can be monitored, for example, for NLRP3 inflammasome activation and/or cell viability.

[0084] The invention also provides a method of determining whether any of the above-mentioned macrophage-mediated inflammatory and degenerative diseases or disorders is amenable to treatment with an inhibitor of CTL1 expression or choline phosphorylation, as disclosed herein. The method can be performed, for example, by measuring the amount of NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production in a cell sample of a subject to be treated, and determining that the measured NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production is elevated or abnormally elevated as compared to the level of NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production in corresponding normal cells, which can be a sample of normal (*i.e.*, not diseased) cells of the subject having any one of the above-mentioned inflammatory and/or degenerative diseases. Detection of elevated or abnormally elevated level of NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production in the cells as compared to the corresponding normal cells indicates that the subject can benefit from treatment with an inhibitor of CTL1 expression and/or

choline phosphorylation. A sample of cells used in the present method can be obtained using a biopsy procedure (*e.g.*, a needle biopsy), or can be a sample of cells obtained by a surgical procedure to remove and/or debulk the tumor.

[0085] In various embodiments, the method of identifying a disease or disorder amenable to treatment with an inhibitor of CTL1 expression and/or choline phosphorylation can further include contacting cells of the sample with at least one test agent known to inhibit NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production, and detecting a decrease in NLRP3 inflammasome activation, CTL1 expression, choline phosphorylation, IL-1β production and/or IL-18 production in the cells following said contact. Such a method provides a means to confirm that any of the above-mentioned diseases or disorders is amenable to treatment with an inhibitor of CTL1 expression and/or choline phosphorylation. Further, the method can include testing one or more different test agents, either alone or in combination, thus providing a means to identify one or more test agents useful for treating the particular symptoms of any of the above-mentioned diseases or disorders being examined. Accordingly, the present invention provides a method of identifying an agent useful for treating lupus, gout, osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, uveitis, Alzheimer's disease, Parkinson's disease, cryopyrin-associated periodic syndromes, type 2 diabetes, atherosclerosis, macular degeneration, lung cancer, and many more inflammatory and degenerative diseases in a subject.

[0086] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

[0087] *Macrophage culture and stimulation* – Femurs and tibias from C57BL/6 mice, *Ampkα1*$^{-/-}$ mice, and *Il10rb*$^{-/-}$ mice at 6-10 weeks of age were used to generate bone-marrow-derived macrophages (BMDM) as described (Hornung et al., 2008). Macrophages were cultured in DMEM supplemented with 10% FBS, 20% L929-cell conditioned medium, and 100 U/ml penicillin-streptomycin for 7-10 days. Bone marrow was isolated from MWS Nlrp3^{A350VneoRCreT}, FCAS Nlrp3^{L351PneoCreT}, and NOMID Nlrp3^{D301NneoCreT} conditional knock-in mice (Bonar et al., 2012; Brydges et al., 2009), and were allowed to differentiate over 7 days with addition of fresh mouse recombinant GMCSF every three days. (Z)-4-hydroxitamoxifen at 0.4 μg/ml was added to cells 24 hr prior to treatment to induce the mutant *Nlrp3* allele. Immortalized mouse BMDM were grown in DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. All cells were grown at 37°C with 5% CO_2. NLRP3 inflammasome activation was induced priming

for 4 hr with ultrapure LPS (100 ng/ml) followed by treatment with the NLRP3 activators ATP (4 mM) and nigericin (10 μM) for 45 min, unless otherwise indicated, and monosodium urate (MSU) crystals (400 μg/ml) and 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) liposomes (50 μg/ml) for 3 hr. In *Nlrp3* mutant macrophages IL-1b production was induced by LPS treatment for 16 hr at 37°C, whereas FCAS macrophages were incubated at 32°C without LPS addition. AIM2 inflammasome activation was induced by 4 hr LPS priming and transfection with lipofectamine 3000 and the AIM2 activator poly(dA:dT) (1 μg/ml) for 8 hr. For choline deprivation experiments, the cells were cultured in CMRL1066 medium with or without choline for 2-3 hr before priming with LPS and kept during the experiment.

[0088] *Microglia isolation* – Primary microglia was isolated from wild type mice as previously described (Saura et al., 2003). Briefly, confluent mixed glial cultures were prepared from cerebral cortices of 1-day-old male C57BL/6 mice by mechanical and chemical dissociation and culture mixed glial cell in DMEM-F12 until confluency was achieved after 10-12 days. Then, microglial cultures were prepared by mild trypsinization (0.05-0.12%) in the presence of 0.2-0.5mM EDTA and 0.5-0.8mM Ca^{2+} to detach an intact layer of astrocytes, leaving attached microglia. Microglia were cultured in DMEM-F12 supplemented with 10% FBS for 15-21 days, then cells were detached and seeded for experimentation.

EXAMPLE 2
In Vivo Animal Studies

[0089] *LPS-induced septic shock and air pouch model* – Eight- to twelve-week-old C57BL/6 mice were subjected to LPS-induced septic shock as described (Zhong et al., 2016c). Briefly, 50 mg/kg LPS was intraperitoneal (IP) injected, and when indicated, animals were pretreated with the ChoKα inhibitor MN58B 2.5 mg/kg or vehicle (sodium chloride) via IP injection daily, starting three days before LPS challenge. Mice were analyzed for survival and circulating cytokines (3hr after LPS challenge). None of the animals was excluded from the analysis.

[0090] *Synovium-like air pouch gout model* – Subcutaneous air pouches were generated by repeated injection of sterile air into 8-12-week old C57BL/6 mice to create an accessible space that develops a synovium-like membrane within 7 days as described (Wang et al., 2016). Afterward, mice were treated with MN58b IP (2.5 mg/kg) or vehicle, 24 hr before injecting 1ml MSU crystals (3 mg/ml) into the air pouch. Pouch tissue and fluids were harvested 8 hr later for analysis. None of the animals was excluded from the analysis.

[0091] *Muckle Wells Syndrome Mice* – Sixteen- to twenty-two-week-old male MWS $Nlrp3^{A350VneoRCreT}$ mutant mice were subjected to MN58b (2.5 mg/kg) or vehicle IP injection BID daily for 15 days. Tamoxifen (50 mg/kg) was administered daily via IP for 4 days after first MN58b (or vehicle) treatment, and once more on day 7 to maintain Cre expression. At the endpoint, blood, spleen and liver were collected for analysis. Complete blood counts were preformed using a ScilVet Animal Blood Counter (ABX Diagnostics). Livers were paraffin embedded and hematoxylin and eosine staining was performed to assess liver histology. None of the animals was excluded from the analysis and liver histology was blind examined.

EXAMPLE 3
Method Details

[0092] *shRNA Lentiviral Knockdown* – Knockdown of *Slc44a1, ChoKα*, and *Atg7* was done by lentiviral transduction of immortalized BMDM as described (Zhong et al., 2016c). Sequences of target shRNAs used in this study were obtained from the MISSION shRNA Library (Sigma). Briefly, HEK293T cells were plated at 60% confluence in 6-well plates, and were transfected with 20 μl lipofectamine 3000 and 10 μg shRNA, 1 μg VSV-G and 5 μg pLV-CMVΔ8.9 plasmids, following manufacturer's instructions. Supernatants were collected 36 hr after transfection, filtered through 0.45 μm and added to iBMDM. To increase infection efficiency 8 μg/ml of polybrene was added. Virus containing medium was washed away after 6 hr and the cells were cultured with fresh medium. A second round of infection was done at 48 hr after the first round. Infected iBMDM were expanded and selected with puromycin.

[0093] *Protein Immunoblotting and ELISA* – Mitochondria were isolated using Mitochondria Isolation kit. Whole cell lysates were prepared in NP40 buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail, and supernatants were analyzed by SDS-PAGE. Proteins were transferred into PVDF membranes, blocked in 5% BSA and 1X TBST for 1 hr, and incubated with indicated antibodies overnight. Secondary antibodies were added for 1 hr and detection was done using CLARITY™ Western ECL Substrate (Biorad). Paired antibodies (capture and detection) and standard recombinant mouse IL-1β (R&D Systems), and TNF and IL-6 (eBioscience) were used to determine cytokine concentrations according to manufacturer's instructions.

[0094] *RNA Isolation and Quantitative Real-Time PCT (QPCR)* – RNA was extracted using AllPrep DNA/RNA Mini kit, and cDNA was synthesized using SUPERSCRIPT™ VILO™ cDNA Synthesis Kit. mRNA expression was determined by QPCR in a CFX96 thermal cycler

(Biorad) as described (Zhong et al., 2016c). Data are presented in arbitrary units and were calculated by 2^(–delta delta CT) method. Primer sequences were obtained from the NIH qPrimerDepot (mouseprimerdepot.nci.nih.gov) and provided by Integrated DNA technologies, as set forth below.

[0095] *Immunofluorescence and Confocal Microscopy* – Treated BMDM and pouch tissue were fixed in 4% paraformaldehyde, permeabilized in 0.01% Triton X-100, and blocked in 1X PBS supplemented with 2% BSA and 5% normal horse serum. Primary antibodies were incubated in blocking buffer at 4°C overnight. Secondary Alexa antibodies from Life Technologies or Jackson Immuno Research Laboratories were added for 1 hr. Nuclei were counterstained with DAPI. Samples were imaged through a Leica SP5 confocal microscope. Quantitation of p62 aggregates was measured in high magnification fields (HMF) and plotted as p62 aggregates per cell.

[0096] *Choline and Phosphocholine Analysis by NMR* – BMDM were treated with LPS for 4 hr and then were collected and processed for NMR analysis as described (Guma et al., 2015a; Tiziani et al., 2009). Polar metabolite isolation was performed using a modified Bligh-Dyer procedure (Wu et al., 2008). Extracts were dried using a CentriVap refrigerated vacuum concentrator (Labconco, Kansas City, MO, USA). Dried extracts were reconstituted in 100 mM phosphate buffer (pH 7.0) prepared in 10% H_2O/90% D_2O (Sigma) that contained 0.5 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 (TMSP) as the internal standard. One-dimensional ^1H NMR spectra were acquired on a Bruker Avance III 500 MHz with 1.7 mm TCI MicroCryoProbe system (Bruker BioSpin Corp., Billerica, MA) equipped with an autosampler at 300 K and processed as previously described (Lodi et al., 2017; Lu et al., 2017; Ludwig and Günther, 2011). Metabolite assignment and quantification were performed using the Chenomx 8.2 NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada), the Birmingham Metabolite Library (Ludwig et al., 2012), and the Human Metabolome Database (Wishart et al., 2012).

[0097] *Total phosphatidylcholine and mitochondrial lipids analysis by mass spectrometry* – Total phosphatydilcholine (PC) analysis was measured in RSM932A-pretreated BMDM stimulated with LPS for 4 hr, while mitochondrial lipids were measured in mitochondria isolated from BMDM cultured in control or choline deficient medium and subjected to different treatments for 24 hr. Samples were processed for UHPLC-MS/MS analysis and analysis was performed on a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, Bremen, Germany) coupled to an Accela 1250 UHPLC system equipped with a quaternary

pump, vacuum degasser, and open autosampler with temperature controller (6°C; Fisher Scientific, San José, CA, USA). Chromatographic separation of metabolites was achieved by reverse phase (RP) analysis on a 150 mm × 2.1 mm Kinetex C18 (2.6 µm 100Å) column (Phenomenex Inc, Torrance, CA, USA) with the following conditions: solvent A, 60:40 water:acetonitrile with 10 mM ammonium formate and 0.1% formic acid; solvent B, 90:10 isopropanol:acetonitrile with 10 mM ammonium and 0.1% formic acid; separation gradient, initially 32% B, held for 2 minutes and then increased linearly from 32-99% B in 18 minutes, washing with 99% B for 5 minutes and column equilibration with 32% B for 10 minutes. The total run time was 35 minutes with a flow rate of 0.25 mL/min and an injection volume of 5 µL.

[0098] Ion detection was performed in full MS and MS/AIF modes with an electrospray (ESI) source simultaneously operating in fast negative/positive ion switching mode. The following acquisition settings were used for data collection in full MS mode: spray voltage, 4.0 kV; capillary temperature, 300°C; sheath gas, 51 (arbitrary units); auxiliary gas, 10 (arbitrary units); m/z range, 200-2000; data acquisition, centroid mode, microscans, 10; AGC target, 3e6; maximum injection time, 200 ms; mass resolution, 70,000 FWHM at m/z 200. The following parameters were modified as follows for full MS/AIF analysis: spray voltage, +3.5/-4.0 kV; capillary temperature, 250°C; sheath gas, 25 (arbitrary units); auxiliary gas, 15 (arbitrary units); higher-energy collisional dissociation (HCD), 10, 15, 20, 25, and 35 eV. The collision gas was nitrogen. Accuracy of analysis was ensured by calibrating the detector using commercial calibration solutions provided by the manufacturer. Mass tolerance was maintained at 5 ppm. The analytical platform was controlled by a computer operating the Xcalibur v. 2.2 SP1.48 software package (Thermo Scientific, San Jose, CA, USA). Raw files were processed using SIEVE 2.2.0 SP2 (Thermo Scientific, San Jose, CA, USA) and the MATLAB programming environment (MathWorks, Natick, MA, USA). MS/MS fragmentation patterns were used to differentiate lipid classes. Features that did not achieve a relative standard deviation (RSD) of less than 0.25 in the quality control (QC) were excluded from analysis. Integrated peak intensities were normalized by the total spectral area and summed by class. The total intensity for each class was scaled to the vehicle control average for analysis.

[0099] *Nitric oxide, calcium, and potassium flux measurements* – BMDM were treated with LPS for 24 hr. NO production was measured in conditioned media as the concentration of nitrite by Griess reaction using $NaNO_2$ as the standard as described (Terkeltaub et al., 2011). Briefly, 50 mL of conditioned medium, or sodium nitrite standards were incubated with 50 mL of equal

volumes of Griess reagents (Griess reagent A, 1% sulfanilamide in 5% phosphoric acid; and Griess reagent B, 0.1% Napthylenediamine in H_2O). Absorbance was measured at 490 nm. Calcium and potassium fluxes were detected in BMDM cultured in control or choline-free medium treated with LPS and ATP, using FURA-2, AM (Invitrogen), and PBFI, AM (Invitrogen) respectively and flux was calculated according to manufacturer's instructions.

[0100] *Mitochondrial Function* – Mitochondrial membrane potential (Ψm) was measured using TMRM (#T668, Life Technologies) according to manufacturer's instructions. Briefly, RSM932A-pretreated BMDM and shCtrl and shChoKα iBMDM were primed with LPS for 4 hr. Cells were stained with 200 nM TMRM for 30 min at 37°C, and then treated with CCCP (5 μM) for 5 min. After washing twice, fluorescence intensity was determined per manufacturer's instructions using a FilterMax F5 multimode plate reader (Molecular Devices), Mitochondrial reactive oxygen species (mtROS) was measured using MitoSOX (Invitrogen) as described (Zhong et al., 2016c). RSM932A-pretreated BMDM and shCtrl and shChoKα were primed with LPS for 4 hr and treated with nigericin and DOTAP for 30 min and 3 hr respectively. Cells were loaded with 4 μM MitoSOX for 20 min. After washing with PBS, fluorescence intensity was determined at 510/580 nm using a FilterMax F5 plate reader. NAD^+/NADH ratio was measured using NAD^+/NADH Cell-Based Assay kit according to manufacturer's instructions (Cayman Chemical Company). Briefly, BMDM were aliquoted into 96-well plates treated with LPS for 24 hr. Cells were lysed and centrifuged. Supernatants and standards were incubated in reaction solution for 1.5 hr. Absorbance was measured at 450 nm using FilterMax F5 plate reader. Complex V ATP synthase activity was measured in mitochondria isolated from BMDM treated with LPS for 24 hr using Complex V Activity Assay kit (Cayman Chemical Company) according to manufacturer's instructions. Absorbance was measured at 340 nm at 30-second intervals for 30 min using a FilterMax F5 plate reader. Cellular ATP was measured after 24 hr LPS stimulation using CellTiter-Glo Luminescent Assay (Promega) as described (Ip et al., 2017). Succinate was measured in BMDM treated with indicated concentrations of LPS for 24 hr in either control or choline-deficient medium using the Succinate Colorimetric Assay kit (Sigma) according to manufacturer's instructions. Briefly, cells were homogenized in ice-cold succinate assay buffer, and after centrifugation, supernatants and succinate standards were incubated with reaction solution for 30 min at 37°C. Absorbance was measured at 450 nm using FilterMax F5 plate reader.

[0101] *Cellular fractionation and measurement of cytosolic mtDNA* – BMDM were primed with LPS and stimulated with an NLRP3 inflammasome activator. Cellular fractionation was performed using Mitochondrial Isolation kit (ThermoScientific) according to manufacturer's instructions. Cytosolic mtDNA was analyzed as described (Nakahira et al., 2011). Briefly, DNA was isolated from 300 μl of the cytosolic fractions using All Prep DNA/RNA kit, and mitochondrial DNA encoding cytochrome *c* oxidase 1 and D-Loop were measured by QPCR with an equal volume of the DNA solution. Nuclear DNA encoding 18S ribosomal RNA and Tert was used for normalization. Primer sequences were obtained from the NIH qPrimerDepot (mouseprimerdepot.nci.nih.gov) and provided by Integrated DNA Technologies, as set forth below.

[0102] *Quantification and Statistical Analysis* – Data are shown as mean ± SD or mean ± SEM, as indicated. Statistical significance was determined using two-tailed student's t-test, and p values lower than 0.05 were considered statistically significant. Kaplan-Meier survival curves were analyzed by log rank test. All group numbers and detailed significant values are presented within the figure legends. Sample-sizes for mouse experiments were based on previous studies (Hoffman et al., 2010; Wang et al., 2016; Zhong et al., 2016c). GraphPad Prism was used for statistical analysis and graphing.

EXAMPLE 4
Results

[0103] *LPS induces macrophage CTL1/Slc44a1 expression and choline uptake* – Exposure of bone-marrow-derived macrophages (BMDM) to pathogen-associated molecular pattern (PAMP) lipopolysaccharide (LPS) increased *Slc44a1* mRNA (Figure 1A), which codes for the choline transporter CTL1 (Figures 1B and 1C). CTL1 induction correlated with enhanced choline uptake (Figure 1D). LPS also induced rapid choline mobilization via the Kennedy pathway, increasing cellular content of phosphocholine, glycerol-3-phosphocholine and phosphatidylcholine (PC), an effect that was blocked by the ChoKα inhibitor RSM932A (Figures 1F, 1G and 1H). In microglia, the myeloid cells of the central nervous system, LPS induced Slc44a1/CTL1 as well as *ChKa* mRNA (Figure 1I). Thus, enhanced choline uptake and phosphorylation is a general response to LPS stimulation of myeloid cells.

[0104] LPS rapidly activates NF-κB-dependent transcription to produce inflammatory mediators and cytokines (Ben-Neriah and Karin, 2011; Greten et al., 2007). NF-κB inhibition by

IKKβ ablation or the IKKβ inhibitor BMS345541 blocked *Slc44a1*/CTL1 induction (Figures 1E and 1J).

[0105] *Impaired choline uptake reduces NLRP3 inflammasome activation* – NF-κB activation also induces expression of pro-IL-1β and NLRP3, as well as numerous cytokines and chemokines (Vallabhapurapu and Karin, 2009). The impact of *Slc44a1* gene knockdown on LPS-induced responses was therefore examined. *Slc44a1* gene knockdown did not affect other choline transporters or *Chka* and *Chkb* mRNA expression (Figure 2A). Importantly, *Slc44a1* knockdown reduced IL-1β and IL-18 production in response to different NLRP3 inflammasome activators, including ATP, nigericin, and monosodium urate (MSU) microcrystals (Figures 2B and 2I). *Slc44a1* knockdown also led to decreased TNF production but had no effect on IL-6 release (Figures 2J and 2K). Reduced IL-1β production on *Slc44a1* knockdown correlated with diminished caspase-1 activation (Figures 2C and 2D). Culturing of BMDM in choline-deficient medium (so-called choline-free medium), also interfered with IL-1β (Figure 2E) and IL-18 production (Figure 2L), and caspase-1 activation (Figures 2F and 2G), but had no effect on AIM2 inflammasome-mediated IL-1β production (Figure 2M). Culture in choline-free medium, however had no effect on TNF and IL-6 production (Figures 2N and 2O). Choline deficiency did not cause intracellular accumulation of mature IL-1β, ruling out an effect on the secretory system involved in IL-1β release (Figure 2H).

[0106] *Slc44a1* ablation had no significant effect on synthesis of *Il1b* or *Tnf* mRNAs (Figures 2P and 2Q). The anti-inflammatory cytokine IL-10, which is induced upon LPS challenge, restrains IL-1β production by limiting glucose uptake and the glycolytic switch through autocrine induction of the mTORC1 inhibitor DDIT4 (Ip et al., 2017). *Slc44a1* ablation did not affect *Il10* mRNA expression (Figure 2R), and choline deficiency reduced IL-1β production in IL-10Rb-deficient BMDM (Figure 2S). IL-10Rb-deficient BMDM also exhibited normal upregulation of *Slc44a1* after LPS stimulation (Figure 2T), suggesting that the mechanisms by which IL-10 and choline deficiency control IL-1β production are different.

[0107] Nitric oxide (NO) limits IL-1β production independent of its antimicrobial function (Mishra et al., 2013). shSlc44a1 macrophages (iBMDM) showed similar nitrite secretion (Figure 2U) and *Nos2* mRNA expression (Figure 2V) to shCtrl macrophages, suggesting that NO is not involved in reduced IL-1β production after choline uptake impairment.

[0108] NRLP3 inflammasome activation and pro-IL-1β processing involve influx of calcium into the cytosol (Lee et al., 2012; Murakami et al., 2012; Zhong et al., 2013) and efflux of potassium (Munoz-Planillo et al., 2013). Incubation in choline-deficient medium did not affect calcium (Figure 2W) or potassium (Figure 2X) fluxes. Collectively, these results indicate that choline uptake regulates activation of the NLRP3 inflammasome and caspase-1. However, choline uptake via CTL1 does not affect the IL-10-dependent glycolytic switch or changes in intracellular calcium and potassium.

[0109] *Choline phosphorylation contributes to IL-1β production* – After LPS stimulation, choline is taken up and rapidly converted to phosphocholine by ChoKα (Figure 1F). ChoKα knockdown reduced IL-1β production (Figure 3A). Pretreatment with the ChoKα inhibitor RSM932A (Lacal and Campos, 2015) produced a similar effect (Figure 3B). Furthermore, ChoKα knockdown (Figures 3C and 3D) and RSM932A treatment (Figures 3E and 3F) inhibited caspase-1 activation. Inhibition or knockdown of ChoKα did not alter expression of NLRP3 inflammasome components, including caspase-1, NLRP3, ASC, or the amount of pro-IL-1β induced by LPS (Figures 3G and 3H).

[0110] *LPS alters mitochondrial phospholipid and sphingolipid profile* – LPS stimulation of BMDM altered mitochondrial lipid profile, decreasing PC and phosphatidylglycerol (PG) content, and increasing phosphatidylserine (PS) and sphingomyelin (SM) content (Figure 4A). LPS, however, did not alter mitochondrial phosphatidylethanolamine (PE) or phosphatidylinositol (PI) (Figure 4A). Choline deficiency also reduced mitochondrial PC (mitoPC) and increased mitochondrial SM (mitoSM), (Figure 4K), changes that were more pronounced after LPS stimulation (Figures 4B and 4C). Interruption of *de novo* synthesis of PC with the ChoKα inhibitor RSM932A showed similar results in mitoPC and mitoSM (Figures 4L and 4M).

[0111] *Choline deficiency activates AMPK to inhibit IL-1β production* – IL-1β production is tightly regulated by metabolic changes (Mills and O'Neill, 2016). LPS potentiates glycolysis and shuts down oxidative phosphorylation, which results in lower NAD^+/NADH ratio and intracellular ATP (Mills and O'Neill, 2016). Of note, phosphocholine preserves mitochondrial activity by facilitating removal of uncoupling free fatty acids and converting them to phospholipids (Rossi et al., 1962a; Rossi et al., 1962b). In addition, altered mitochondrial membrane integrity due to distorted lipid content affects mitochondrial function and biogenesis (Guo et al., 2005; James et al., 1992; Teodoro et al., 2008). LPS stimulation reduced the

NAD^+/NADH ratio, indicating diminished complex I forward activity, regardless of choline status (Figure 4D). Complex V activity and intracellular ATP were reduced after incubation in choline-deficient medium (Figures 4E and 4F). Knockdown of *Slc44a1* or ChoKα also reduced intracellular ATP after LPS stimulation (Figures 4N and 4O) without affecting the NAD^+/NADH ratio (Figure 4P), indicating a role of phosphocholine in preserving mitochondrial function. Low intracellular ATP results in AMPK activation which suppresses production of inflammatory cytokines, including IL-1β, through a poorly defined mechanism (O'Neill and Hardie, 2013; Steinberg and Kemp, 2009; Wang et al., 2016). Choline deficiency caused AMPK activation even after LPS stimulation (Figures 4G and 4H). *Slc44a1* or ChoKα knockdown and ChoKα pharmacological inhibition also promoted AMPK activation (Figures 4Q-4V). AMPK activation by the selective AMPK agonist A769662 significantly reduced IL-1β production (Figure 4W). Consistently, BMDM deficient in AMPKα1, the predominant catalytic subunit, showed enhanced IL-1β release, and choline deficiency in these cells no longer reduced IL-1β production (Figure 4I). Consistent with inhibition of ATP synthase activity, choline deficiency induced accumulation of ATP synthase inhibitory factor 1 (ATPIF1) (Figures 4G and 4J).

[0112] After LPS stimulation, and together with reduction of oxidative phosphorylation, the TCA cycle is also impacted. LPS induces accumulation of two TCA cycle intermediates, citrate and succinate (Tannahill et al., 2013), that potentiate production of NO and PGE2, and IL-1β respectively. However, culture in choline-free medium had no effect on LPS-induced succinate accumulation (Figure 4X).

[0113] *Impaired choline uptake and phosphorylation stimulate mitophagy* – AMPK activation stimulates autophagy/mitophagy via phosphorylation of ULK1 and mitochondrial fission factor (MFF), a mitochondrial outer-membrane receptor for DRP1, (Egan et al., 2011; Toyama et al., 2016). DRP1 binds to fission sites, which isolate mitochondria with reduced membrane potential, thereby enhancing recruitment of the mitophagy-promoting E3 ubiquitin ligase Parkin (Narendra et al., 2008). It has recently been shown that NF-κB-induced mitophagy facilitates removal of damaged mitochondria, which produce the NLRP3-activating ligand ox-mtDNA (Zhong et al., 2018; Zhong et al., 2016c). Knockdown of ChoKα or its pharmacological inhibition with RSM932A reduced mitochondrial membrane potential (Ψm), at least as effectively as the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Figures 5A and 5B). Loss of mitochondrial membrane potential enhanced mitochondrial recruitment of the autophagy adaptor p62/Sqstm1 (Figures 5C and 5D) and DRP1 (Figures 5E, 5F and 5J-5L),

indicating that lack of functional ChoKα activates mitophagy. Choline deficiency also potentiated p62 and DRP1 mitochondrial recruitment (Figures 5M-5O).

[0114] Importantly, AMPKa1 KO BMDM exhibited increased p62 recruitment to the mitochondria regardless of choline availability (Figures 5G and 5H), however DRP1 was mainly located in the cytosol, indicating impaired mitophagy (Figure 5I). Consistently, inhibition of mitophagy through *Atg7* knockdown enhanced IL-1β production and abrogated its downregulation in response to reduced choline uptake (Figure 5P). Consistent with mitophagic elimination of damaged mitochondria, ChoKα knockdown and inhibition attenuated production of mitochondrial reactive oxygen species (mtROS) in macrophages stimulated with various NLRP3 inflammasome activators (Figures 5Q and 5R). Furthermore, ChoKα knockdown or inhibition reduced release of fragmented mtDNA to the cytosol (Figure 5S). Inhibition of ChoKα with RSM932A also reduced the amount of cytosolic mtDNA (Figure 5T).

[0115] *ChoKα inhibition reduces IL-1β-dependent acute inflammation* – To validate these *in vitro* results under more physiologically relevant conditions, two different acute experimental models that are NLRP3-inflammasome-dependent were used. First, mice were subjected to LPS-induced septic shock by intraperitoneal (IP) injection of 50 mg/kg LPS. ChoKα was inhibited by IP injection of 2.5 mg/kg MN58b daily for 3 days prior to LPS challenge. Importantly, ChoKα inhibition prevented LPS-induced death (Figure 6A), and this was associated with reduced circulating IL-1β (Figure 6B). As found *in vitro*, ChoKα inhibition did not inhibit TNF or IL-6 production (Figures 6F and 6G). Synovium-like gout air-pouch model was also used. Once the air pouch was created, mice were pretreated with 2.5 mg/kg MN58b or vehicle 24 hr before injection with MSU crystals into the pouch to elicit NLRP3 inflammasome activation and acute gouty inflammation (Hoffman et al., 2010; Martinon et al., 2006; Wang et al., 2016). MSU crystal challenge caused recruitment of macrophages that expressed CTL1 and ChoKα into the pouch (Figure 6C). Pretreatment with the ChoKα inhibitor attenuated total leukocyte recruitment into the pouch and reduced production of IL-1β (Figures 6D and 6E). Together these results indicate that choline phosphorylation is important for mounting acute inflammatory responses and that ChoKα inhibition can attenuate inflammation *in vivo*.

[0116] *ChoKα inhibition reduces Muckel-Wells syndrome severity* – Cryopirin-associated periodic syndromes include three genetic diseases, Muckel-Wells syndrome (MWS), Familial cold autoinflammatory syndrome (FCAS) and Neonatal Onset multisystem inflammatory disease (NOMID), that arise from mutations in *NLRP3* gene that results in inflammasome

activation (Hoffman et al., 2001). IL-1β production in BMDM isolated from MWS Nlrp3^{A350VneoRCreT}, FCAS Nlrp3^{L351PneoCreT}, and NOMID Nlrp3^{D301NneoCreT} conditional knock-in mice, was decreased in the absence of choline or in the presence of ChoKα inhibitor (Figures 7A, 7B and 7C). Of note, treatment with ChoKα inhibitor MN58b attenuated MWS pathogenesis *in vivo*, as shown by reduction in total white blood cells count (Figure 7D), including granulocytes (Figure 7E) and monocytes (Figure 7F), without an effect on lymphocyte count (Figure 7G). In addition, treatment with MN58b reduced MWS splenomegaly (Figures 7H and 7I). MN58b also reduced liver size, most likely due to reduced immune cell infiltration (Figures 7J and 7K).

[0117] SEQUENCES

[0118] Human Choline Transporter-Like Protein 1 (CTL1) / Solute Carrier Family 44 Member 1 (SLC44A1), Isoform A, Accession No. NP_536856 (SEQ ID NO: 1):

MGCCSSASSAAQSSKREWKPLEDRSCTDIPWLLLFILFCIGMGFICGFSIATGAAARLVSGYDSYGNICGQKNTKLE
AIPNSGMDHTQRKYVFFLDPCNLDLINRKIKSVALCVAACPRQELKTLSDVQKFAEINGSALCSYNLKPSEYTTSPK
SSVLCPKLPVPASAPIPFFHRCAPVNISCYAKFAEALITFVSDNSVLHRLISGVMTSKEIILGLCLLSLVLSMILMV
IIRYISRVLVWILTILVILGSLGGTGVLWWLYAKQRRSPKETVTPEQLQIAEDNLRALLIYAISATVFTVILFLIML
VMRKRVALTIALFHVAGKVFIHLPLLVFQPFWTFFALVLFWVYWIMTLLFLGTTGSPVQNEQGFVEFKISGPLQYMW
WYHVVGLIWISEFILACQQMTVAGAVVTYYFTRDKRNLPFTPILASVNRLIRYHLGTVAKGSFIITLVKIPRMILMY
IHSQLKGKENACARCVLKSCICCLWCLEKCLNYLNQNAYTATAINSTNFCTSAKDAFVILVENALRVATINTVGDFM
LFLGKVLIVCSTGLAGIMLLNYQQDYTVWVLPLIIVCLFAFLVAHCFLSIYEMVVDVLFLCFAIDTKYNDGSPGREF
YMDKVLMEFVENSRKAMKEAGKGGVADSRELKPMASGASSA

[0119] Human Choline Kinase alpha (ChoKα), Isoform A, Accession No. NP_001268 (SEQ ID NO: 2):

MKTKFCTGGEAEPSPLGLLLSCGSGSAAPAPGVGQQRDAASDLESKQLGGQQPPLALPPPPPLPLPLPLPQPPPPQP
PADEQPEPRTRRRAYLWCKEFLPGAWRGLREDEFHISVIRGGLSNMLFQCSLPDTTATLGDEPRKVLLRLYGAILQM
RSCNKEGSEQAQKENEFQGAEAMVLESVMFAILAERSLGPKLYGIFPQGRLEQFIPSRRLDTEELSLPDISAEIAEK
MATFHGMKMPFNKEPKWLFGTMEKYLKEVLRIKFTEESRIKKLHKLLSYNLPLELENLRSLLESTPSPVVFCHNDCQ
EGNILLLEGRENSEKQKLMLIDFEYSSYNYRGFDIGNHFCEWMYDYSYEKYPFFRANIRKYPTKKQQLHFISSYLPA
FQNDFENLSTEEKSIIKEEMLLEVNRFALASHFLWGLWSIVQAKISSIEFGYMDYAQARFDAYFHQKRKLGV

[0120] Human Choline Kinase beta (ChoKβ), Accession No. NP_005189 (SEQ ID NO: 3):

MAAEATAVAGSGAVGGCLAKDGLQQSKCPDTTPKRRRASSLSRDAERRAYQWCREYLGGAWRRVQPEELRVYPVSGG
LSNLLFRCSLPDHLPSVGEEPREVLLRLYGAILQGVDSLVLESVMFAILAERSLGPQLYGVFPEGRLEQYIPSRPLK
TQELREPVLSAAIATKMAQFHGMEMPFTKEPHWLFGTMERYLKQIQDLPPTGLPEMNLLEMYSLKDEMGNLRKLLES
TPSPVVFCHNDIQEGNILLLSEPENADSLMLVDFEYSSYNYRGFDIGNHFCEWVYDYTHEEWPFYKARPTDYPTQEQ
QLHFIRHYLAEAKKGETLSQEEQRKLEEDLLVEVSRYALASHFFWGLWSILQASMSTIEFGYLDYAQSRFQFYFQQK
GQLTSVHSSS

[0121] Human Interleukin-1 beta (IL-1β), Accession No. NP_000567 (SEQ ID NO: 4):

MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPLDGGIQLRISDHHYSKGFRQAASVVVAMDKLRKM
LVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAYVHDAPVRSLNCTLRDSQQKSLVMSGPYELKALHLQGQDMEQ

QVVFSMSFVQGEESNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKNYPKKKMEKRFVFNKIEINNKLEFESA
QFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS

[0122] Human Interleukin-18 (IL-18), Isoform 1, Accession No. Q14116 (SEQ ID NO: 5):

MAAEPVEDNCINFVAMKFIDNTLYFIAEDDENLESDYFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDN
APRTIFIISMYKDSQPRGMAVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNKMQFESS
SYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNED

[0123] Human Interleukin 10 (IL-10), Accession No. Q6FGW4-1 (SEQ ID NO: 6):

MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGY
LGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGI
YKAMSEFDIFINYIEAYMTMKIRN

[0124] Human Interleukin 6 (IL-6), Isoform 1, Accession No. NP_000591 (SEQ ID NO: 7):

MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNM
CESSKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFESSEEQARAVQMSTKVLIQFL
QKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQDMTTHLILRSFKEFLQSSLRALRQM

[0125] NACHT, LRR and PYD domains-containing protein 3 (NALP3), Isoform A, Accession No. NP_004886 (SEQ ID NO: 8):

MKMASTRCKLARYLEDLEDVDLKKFKMHLEDYPPQKGCIPLPRGQTEKADHVDLATLMIDFNGEEKAWAMAVWIFAA
INRRDLYEKAKRDEPKWGSDNARVSNPTVICQEDSIEEEWMGLLEYLSRISICKMKKDYRKKYRKYVRSRFQCIEDR
NARLGESVSLNKRYTRLRLIKEHRSQQEREQELLAIGKTKTCESPVSPIKMELLFDPDDEHSEPVHTVVFQGAAGIG
KTILARKMMLDWASGTLYQDRFDYLFYIHCREVSLVTQRSLGDLIMSCCPDPNPPIHKIVRKPSRILFLMDGFDELQ
GAFDEHIGPLCTDWQKAERGDILLSSLIRKKLLPEASLLITTRPVALEKLQHLLDHPRHVEILGFSEAKRKEYFFKY
FSDEAQARAAFSLIQENEVLFTMCFIPLVCWIVCTGLKQQMESGKSLAQTSKTTTAVYVFFLSSLLQPRGGSQEHGL
CAHLWGLCSLAADGIWNQKILFEESDLRNHGLQKADVSAFLRMNLFQKEVDCEKFYSFIHMTFQEFFAAMYYLLEEE
KEGRTNVPGSRLKLPSRDVTVLLENYGKFEKGYLIFVVRFLFGLVNQERTSYLEKKLSCKISQQIRLELLKWIEVKA
KAKKLQIQPSQLELFYCLYEMQEEDFVQRAMDYFPKIEINLSTRMDHMVSSFCIENCHRVESLSLGFLHNMPKEEEE
EEKEGRHLDMVQCVLPSSSHAACSHGLVNSHLTSSFCRGLFSVLSTSQSLTELDLSDNSLGDPGMRVLCETLQHPGC
NIRRLWLGRCGLSHECCFDISLVLSSNQKLVELDLSDNALGDFGIRLLCVGLKHLLCNLKKLWLVSCCLTSACCQDL
ASVLSTSHSLTRLYVGENALGDSGVAILCEKAKNPQCNLQKLGLVNSGLTSVCCSALSSVLSTNQNLTHLYLRGNTL
GDKGIKLLCEGLLHPDCKLQVLELDNCNLTSHCCWDLSTLLTSSQSLRKLSLGNNDLGDLGVMMFCEVLKQQSCLLQ
NLGLSEMYFNYETKSALETLQEEKPELTVVFEPSW

[0126] Human Ubiquitin-like Modifier-Activating Enzyme ATG7, Isoform 1, Accession No. O95352-1 (SEQ ID NO: 9):

MAAATGDPGLSKLQFAPFSSALDVGFWHELTQKKLNEYRLDEAPKDIKGYYYNGDSAGLPARLTLEFSAFDMSAPTP
ARCCPAIGTLYNTNTLESFKTADKKLLLEQAANEIWESIKSGTALENPVLLNKFLLLTFADLKKYHFYYWFCYPALC
LPESLPLIQGPVGLDQRFSLKQIEALECAYDNLCQTEGVTALPYFLIKYDENMVLVSLLKHYSDFFQGQRTKITIGV
YDPCNLAQYPGWPLRNFLVLAAHRWSSSFQSVEVVCFRDRTMQGARDVAHSIIFEVKLPEMAFSPDCPKAVGWEKNQ
KGGMGPRMVNLSECMDPKRLAESSVDLNLKLMCWRLVPTLDLDKVVSVKCLLLGAGTLGCNVARTLMGWGVRHITFV
DNAKISYSNPVRQPLYEFEDCLGGGKPKALAAADRLQKIFPGVNARGFNMSIPMPGHPVNFSSVTLEQARRDVEQLE
QLIESHDVVFLLMDTRESRWLPAVIAASKRKLVINAALGFDTFVVMRHGLKKPKQQGAGDLCPNHPVASADLLGSSL
FANIPGYKLGCYFCNDVVAPGDSTRDRTLDQQCTVSRPGLAVIAGALAVELMVSVLQHPEGGYAIASSSDDRMNEPP
TSLGLVPHQIRGFLSRFDNVLPVSLAFDKCTACSSKVLDQYEREGFNFLAKVFNSSHSFLEDLTGLTLLHQETQAAE
IWDMSDDETI

[0127] Human inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), Isoform 1, Accession No. O14920-1 (SEQ ID NO: 10):

MSWSPSLTTQTCGAWEMKERLGTGGFGNVIRWHNQETGEQIAIKQCRQELSPRNRERWCLEIQIMRRLTHPNVVAAR
DVPEGMQNLAPNDLPLLAMEYCQGGDLRKYLNQFENCCGLREGAILTLLSDIASALRYLHENRIIHRDLKPENIVLQ
QGEQRLIHKIIDLGYAKELDQGSLCTSFVGTLQYLAPELLEQQKYTVTVDYWSFGTLAFECITGFRPFLPNWQPVQW
HSKVRQKSEVDIVVSEDLNGTVKFSSSLPYPNNLNSVLAERLEKWLQLMLMWHPRQRGTDPTYGPNGCFKALDDILN
LKLVHILNMVTGTIHTYPVTEDESLQSLKARIQQDTGIPEEDQELLQEAGLALIPDKPATQCISDGKLNEGHTLDMD
LVFLFDNSKITYETQISPRPQPESVSCILQEPKRNLAFFQLRKVWGQVWHSIQTLKEDCNRLQQGQRAAMMNLLRNN
SCLSKMKNSMASMSQQLKAKLDFFKTSIQIDLEKYSEQTEFGITSDKLLLAWREMEQAVELCGRENEVKLLVERMMA
LQTDIVDLQRSPMGRKQGGTLDDLEEQARELYRRLREKPRDQRTEGDSQEMVRLLLQAIQSFEKKVRVIYTQLSKTV
VCKQKALELLPKVEEVVSLMNEDEKTVVRLQEKRQKELWNLLKIACSKVRGPVSGSPDSMNASRLSQPGQLMSQPST
ASNSLPEPAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALDWSWLQTEEEEHSCLEQAS

[0128] An exemplary nucleic acid sequence of shSlc44a1 (SEQ ID NO: 11):

5′-CCGGGCATCAGTGAATCGCCTTATTCTCGAGAATAAGGCGATTCACTGATGCTTTTTG-3′

[0129] An exemplary nucleic acid sequence of shChka (SEQ ID NO: 12):

5′-CCGGGTTACTTGACTACATTCCAAACTCGAGTTTGGAATGTAGTCAAGTAACTTTTT-3′

[0130] An exemplary nucleic acid sequence of shAtg7 (SEQ ID NO: 13):

5′-CCGGCCAGCTCTGAACTCAATAATACTCGAGTATTATTGAGTTCAGAGCTGGTTTTTG-3′

[0131] Primers for mouse *Hprt1*:

Forward: CTGGTGAAAAGGACCTCTCG (SEQ ID NO: 14);
Reverse: TGAAGTACTCATTATAGTCAAGGGCA (SEQ ID NO: 15).

[0132] Primers for mouse *ChKa*:

Forward: GCTGCAGTATACTAGATCTCCAGTTGT (SEQ ID NO: 16);
Reverse: ATCAGCTTCCGCCTTTCA (SEQ ID NO: 17).

[0133] Primers for mouse *ChKb*:

Forward: GCAGAGGTTCAGAAGGGTGA (SEQ ID NO: 18);
Reverse: CCCCAGAAAAAGTGAGATGC (SEQ ID NO: 19).

[0134] Primers for mouse *Slc44a1*:

Forward: TTTGCCCAAGCTACCAG (SEQ ID NO: 20);
Reverse: GAGCACAGCGATGGAAGAA (SEQ ID NO: 21).

[0135] Primers for mouse *Slc44a2*:

Forward: CCTGGTGCTTGGCTATGG (SEQ ID NO: 22);
Reverse: CAAGGTCCAGGGAGA (SEQ ID NO: 23).

[0136] Primers for mouse *Slc44a3*:

Forward: GGTCATTTTGGGATTGCTGT (SEQ ID NO: 24);
Reverse: ACTGAGGTCGTTGGTGTAGTCA (SEQ ID NO: 25).

[0137] Primers for mouse *Slc44a4*:

Forward: ACTCTGTCCCCGTTTCCTTC (SEQ ID NO: 26);
Reverse: AAGTTGATGTTGGGGAGTGG (SEQ ID NO: 27).

[0138] Primers for mouse *Slc44a5*:

Forward: ATCCAAGTGGCCATCATCC (SEQ ID NO: 28);
Reverse: GATTAACGCACTGGGAAGGT (SEQ ID NO: 29).

[0139] Primers for mouse *Il1b*:

Forward: AGTTGACGGACCCCAAAAG (SEQ ID NO: 30);
Reverse: AGCTGGATGCTCTCATCAGG (SEQ ID NO: 31).

[0140] Primers for mouse *Il6*:

Forward: CCAGGTAGCTATGGTACTCCA (SEQ ID NO: 32);
Reverse: GCTACCAAACTGGCTATAATC (SEQ ID NO: 33).

[0141] Primers for mouse *Tnf*:

Forward: CCCTCACACTCAGATCATCTT (SEQ ID NO: 34);
Reverse: GCTACGACGTGGGCTACAG (SEQ ID NO: 35).

[0142] Primers for mouse *Il10*:

Forward: CAGAGCCACATGCTCCTAGA (SEQ ID NO: 36);
Reverse: TGTCCAGCTGGTCCTTTGTT (SEQ ID NO: 37).

[0143] Primers for mouse *Il10rb*:

Forward: TCTCTTCCACAGCACCTGAA (SEQ ID NO: 38);
Reverse: GAACACCTCGGCCTCCTC (SEQ ID NO: 39).

[0144] Primers for mouse *Nos2*:

Forward: CTTTGCCACGGACGAGAC (SEQ ID NO: 40);
Reverse: TCATTGTACTCTGAGGGCTGAC (SEQ ID NO: 41).

[0145] Primers for mouse *Cox1*:

Forward: GCCCCAGATATAGCATTCCC (SEQ ID NO: 42);

Reverse: GTTCATCCTGTTCCTGCTCC (SEQ ID NO: 43).

[0146] Primers for mouse *D-loop*:

Forward: AATCTACCATCCTCCGTGAAACC (SEQ ID NO: 44);
Reverse: TCAGTTTAGCTACCCCCAAGTTTAA (SEQ ID NO: 45).

[0147] Primers for mouse *18S*:

Forward: TAGAGGGACAAGTGGCGTTC (SEQ ID NO: 46);
Reverse: CGCTGAGCCAGTCAGTGT (SEQ ID NO: 47).

[0148] Primers for mouse *Tert*:

Forward: CTAGCTCATGTGTCAAGACCCTCTT (SEQ ID NO: 48);
Reverse: GCCAGCACGTTTCTCTCGTT (SEQ ID NO: 49).

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[0231] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of treating macrophage-mediated inflammatory and/or degenerative diseases in a subject in need thereof comprising administering to the subject an effective amount of an inhibitor of choline-transporter-like protein 1 (CTL1) activity or expression or an inhibitor of choline phosphorylation.

2. The method of claim 1, wherein the inhibitor of CTL1 activity or expression inhibits NLRP3 inflammasome activation, IL-1β production, or IL-18 production.

3. The method of claim 1, wherein the inhibitor of choline phosphorylation is an inhibitor of ChoKα or ChoKβ.

4. The method of claim 2, wherein the inhibitor of CTL1 activity or expression is a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment.

5. The method of claim 4, wherein the inhibitor of CTL1 activity or expression is an inhibitory nucleic acid that inhibits the expression of CTL1 or inhibits choline phosphorylation.

6. The method of claim 5, wherein the inhibitory nucleic acid is selected from the group consisting of siRNA, shRNA, gRNA, oligonucleotides, antisense RNA or ribozymes that inhibit choline phosphorylation.

7. The method of claim 6, wherein the inhibitory nucleic acid is administered via a viral vector.

8. The method of claim 1, wherein the macrophage-mediated inflammatory and/or degenerative disease is selected from the group consisting of cancer, lupus, gout, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, uveitis, Alzheimer's disease, Parkinson's disease, cryopyrin-associated periodic syndromes, nonalcoholic steatohepatitis (NASH), type 2 diabetes, atherosclerosis, and macular degeneration.

9. The method of claim 1, wherein the subject is a mammal.

10. The method of claim 9, wherein the mammal is a human.

11. A method of inhibiting choline phosphorylation in a subject comprising administering to the subject an effective amount of an inhibitor of CTL1 activity or expression.

12. The method of claim 11, wherein the inhibitor of CTL1 activity or expression is a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment.

13. The method of claim 11, wherein the inhibitor of choline phosphorylation is an inhibitor of ChoKα or ChoKβ.

14. The method of claim 11, wherein the inhibitor of CTL1 activity or expression is an inhibitory nucleic acid that inhibits expression of CTL1 or inhibits IL-1β and/or IL-18 production.

15. The method of claim 14, wherein the inhibitor nucleic acid is selected from the group consisting of siRNA, shRNA, gRNA, oligonucleotides, antisense RNA or ribozymes that inhibit expression of CTL1 or inhibit IL-1β and/or IL-18 production.

16. The method of claim 14, wherein the inhibitory nucleic acid is administered via a viral vector.

17. A method of identifying an agent useful for treating macrophage-mediated inflammatory and/or degenerative diseases, comprising contacting a sample of cells from a subject having a macrophage-mediated inflammatory and/or degenerative disease with at least one test agent, wherein a decrease in CTL1 expression or choline phosphorylation in the presence of the test agent as compared to CTL1 expression or choline phosphorylation in the absence of the test agent identifies the agent as useful for treating macrophage-mediated inflammatory and/or degenerative diseases.

18. The method of claim 17, wherein the test agent is a small molecule, peptide, antisense oligonucleotide, antibody or antibody fragment.

19. The method of claim 17, wherein a decrease in NLRP3 inflammasome activation, IL-1β production and/or IL-18 production in the presence of the test agent as compared to NLRP3 inflammasome activation, IL-1β production and/or IL-18 production in the absence of the test agent confirms that the agent is useful for treating macrophage-mediated inflammatory and/or degenerative diseases.

20. The method of claim 17, wherein the macrophage-mediated inflammatory and/or degenerative disease is selected from the group consisting of cancer, lupus, gout, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, uveitis, Alzheimer's disease, Parkinson's disease, cryopyrin-associated periodic syndromes, nonalcoholic steatohepatitis (NASH), type 2 diabetes, atherosclerosis, and macular degeneration.

21. The method of claim 17, which is performed in a high throughput format.

22. The method of claim 21, comprising contacting a plurality of samples of cells with at least one test agent.

23. The method of claim 22, wherein the plurality of samples is obtained from a single subject.

24. The method of claim 22, wherein the plurality of samples is obtained from different subjects.

25. The method of claim 22, comprising contacting a sample of cells with a plurality of test agents.

26. The method of claim 17, wherein the cells are mammalian.

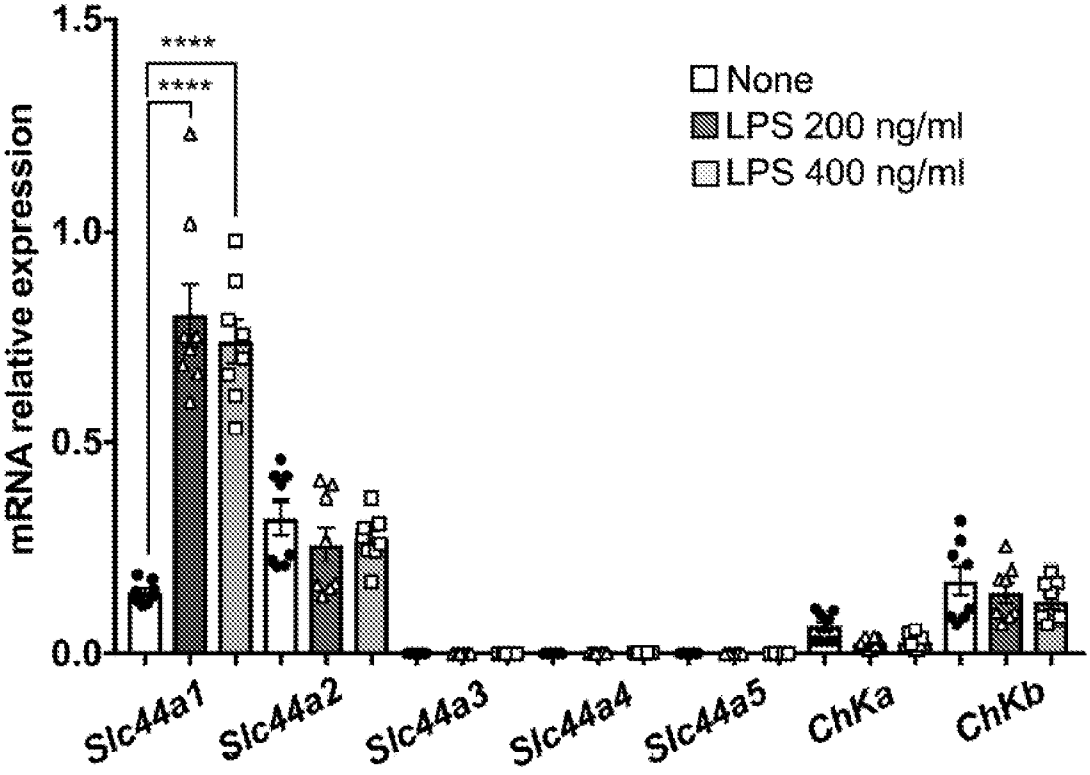

FIG. 1A

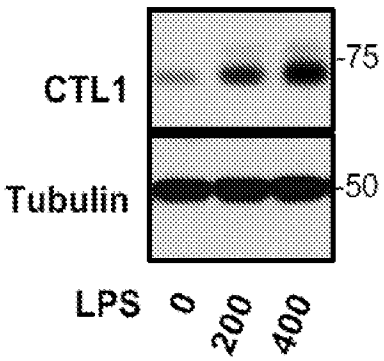

FIG. 1B

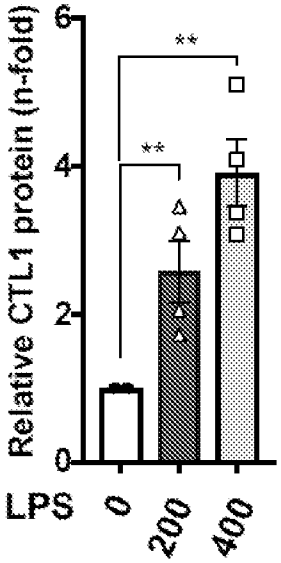

FIG. 1C

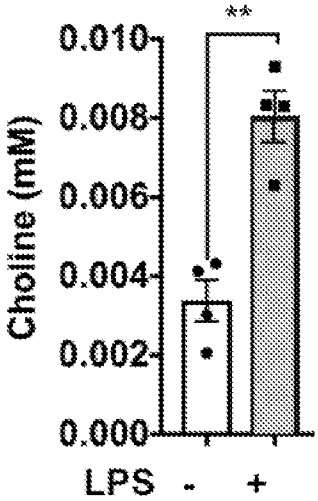

FIG. 1D

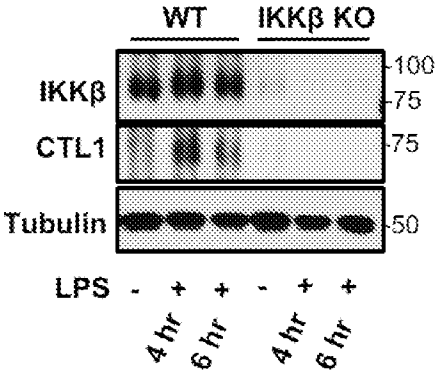

FIG. 1E

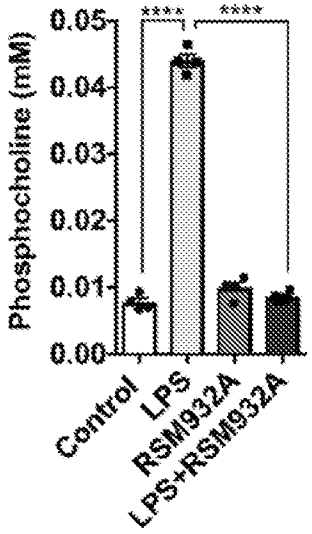

FIG. 1F

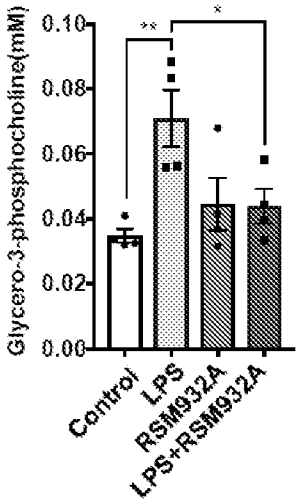

FIG. 1G

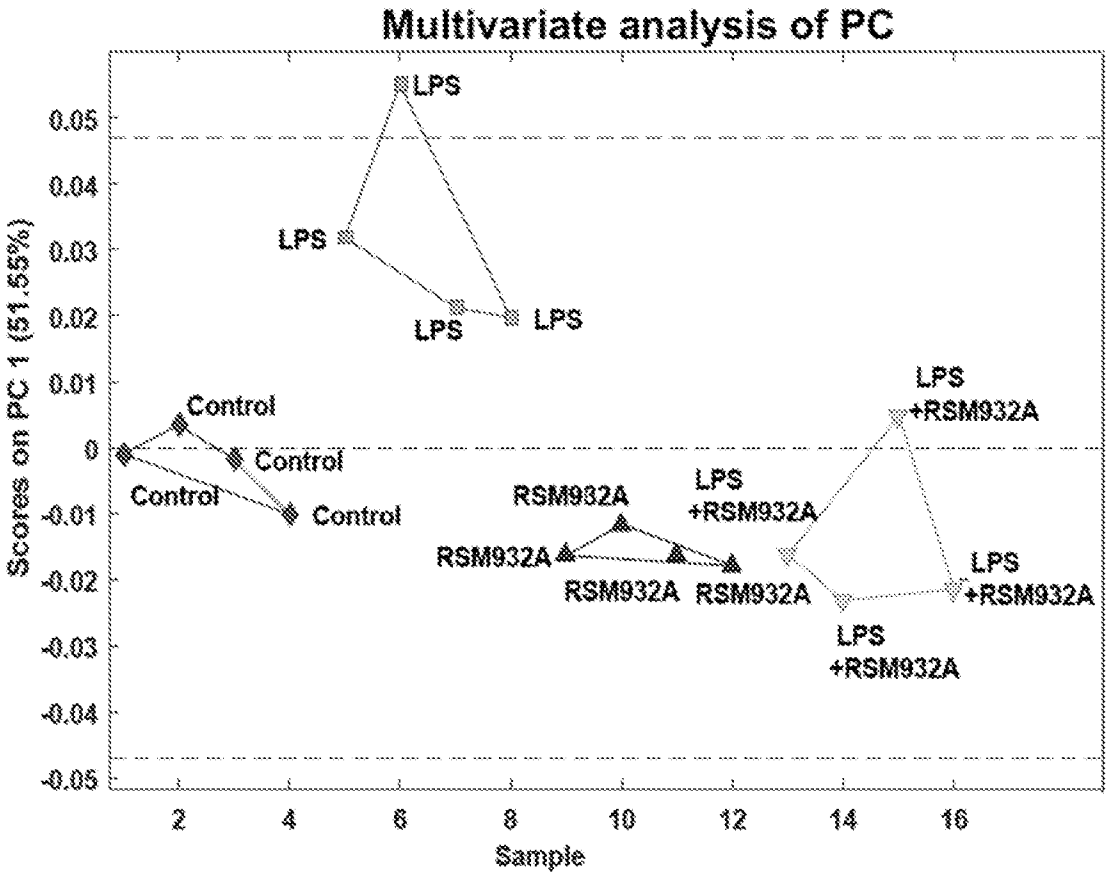

FIG. 1H

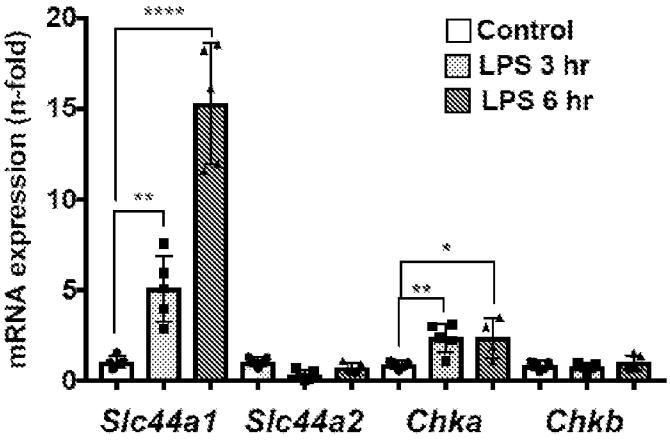

FIG. 1I

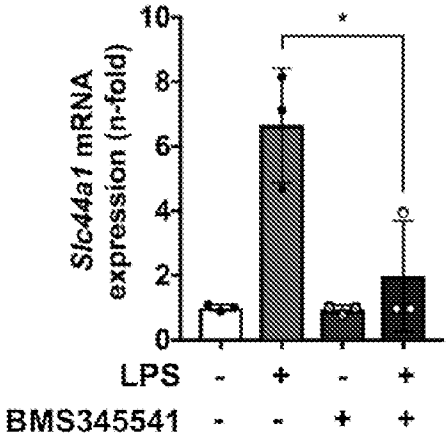

FIG. 1J

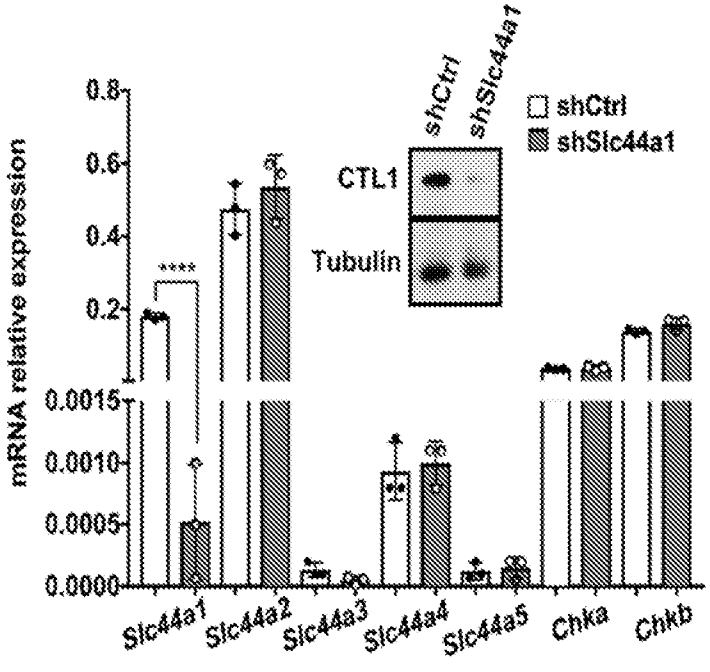

FIG. 2A

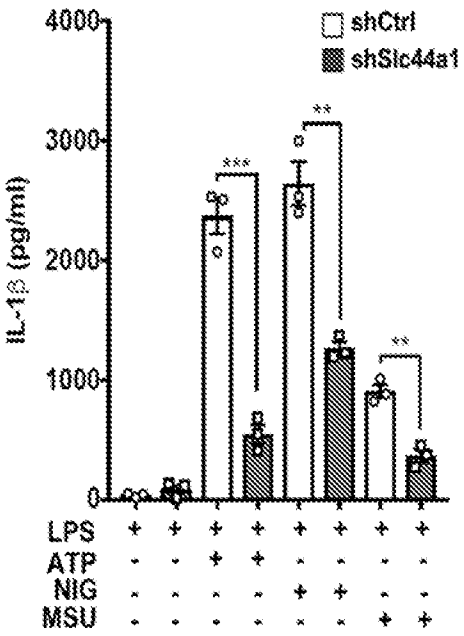

FIG. 2B

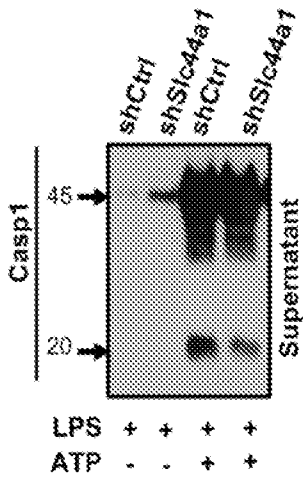

FIG. 2C

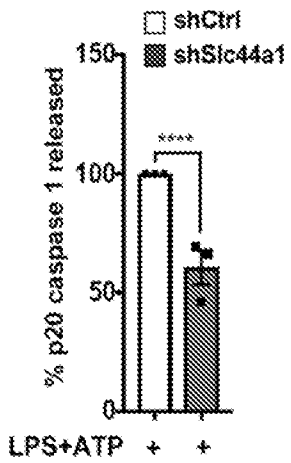

FIG. 2D

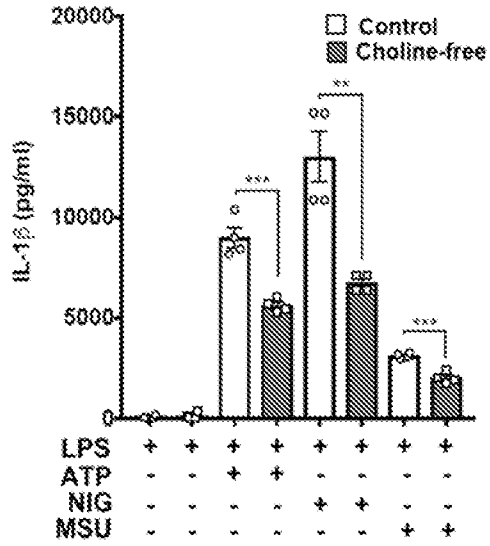

FIG. 2E

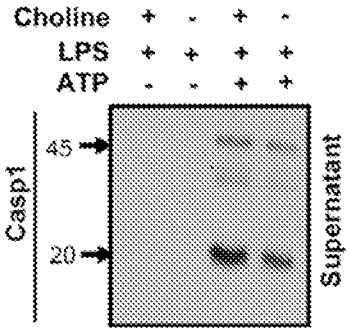

FIG. 2F

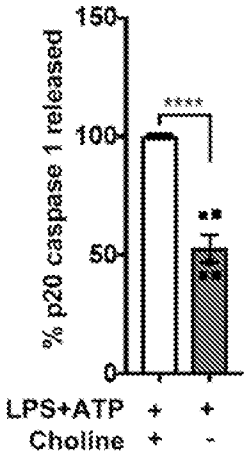

FIG. 2G

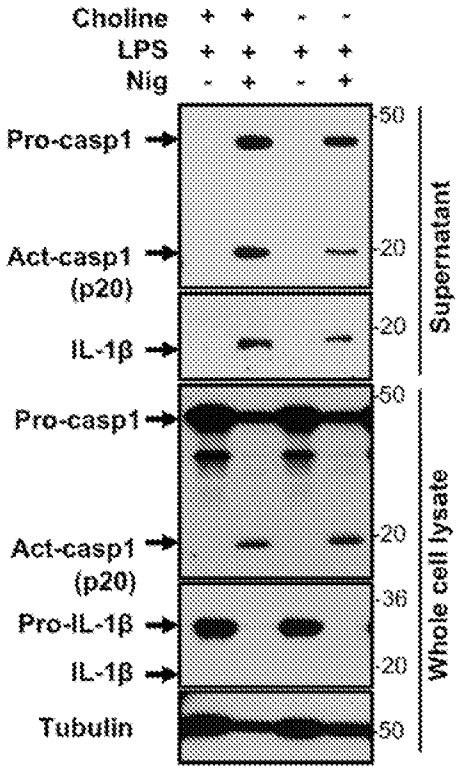

FIG. 2H

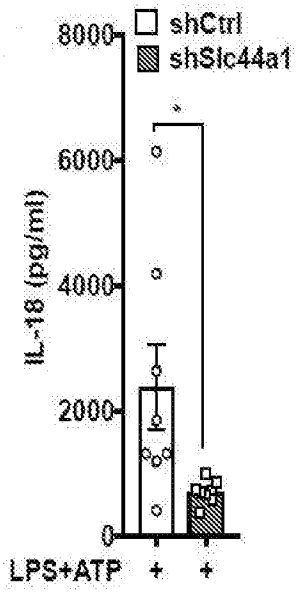

FIG. 2I

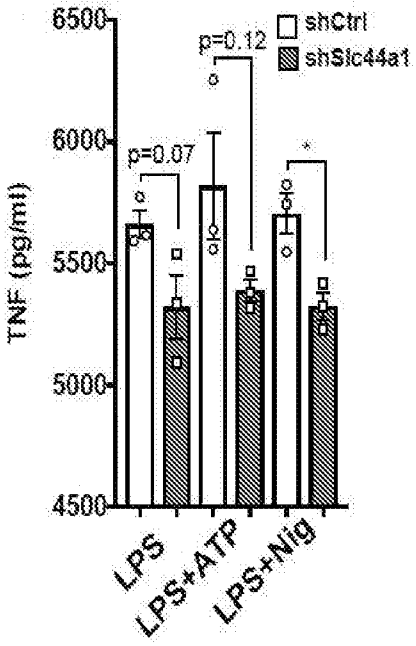

FIG. 2J

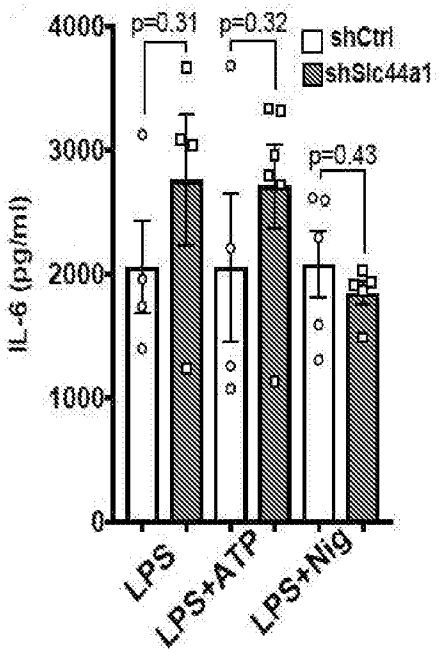

FIG. 2K

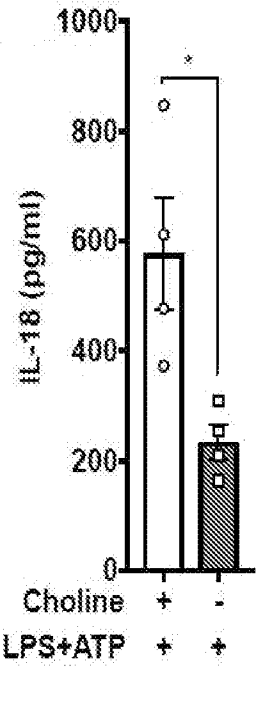

FIG. 2L

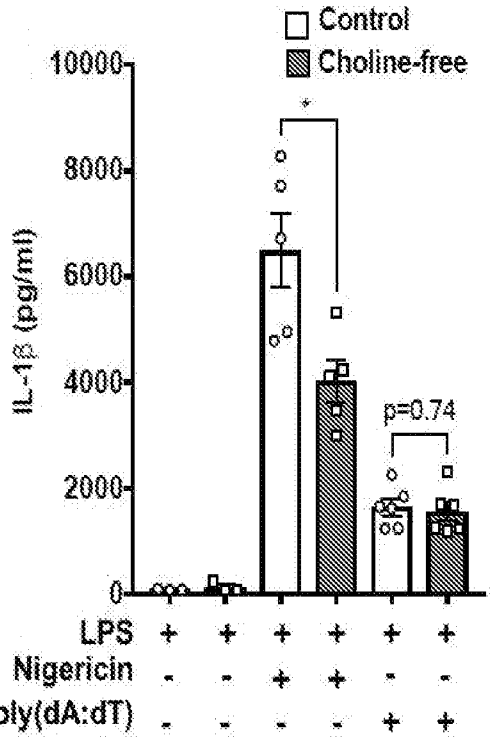

FIG. 2M

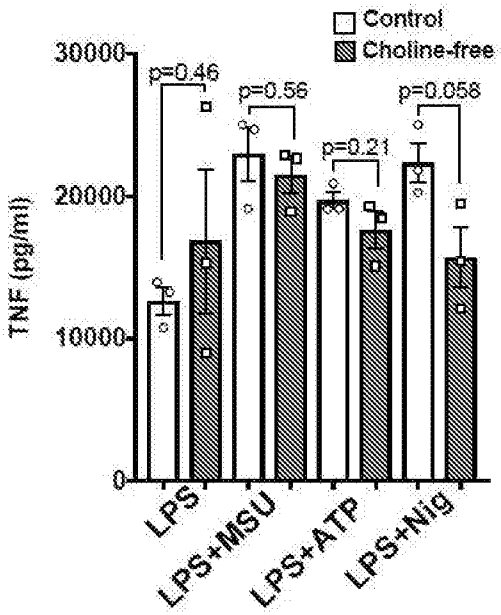

FIG. 2N

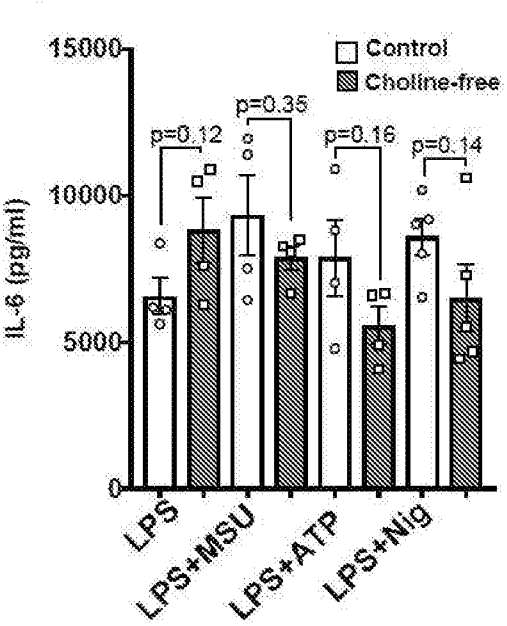

FIG. 2O

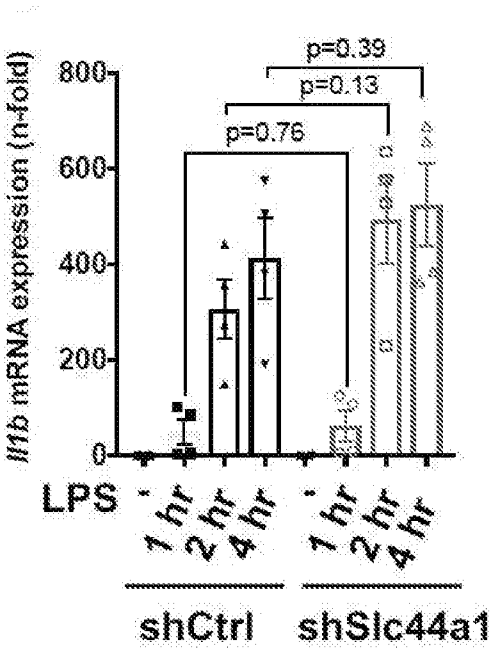

FIG. 2P

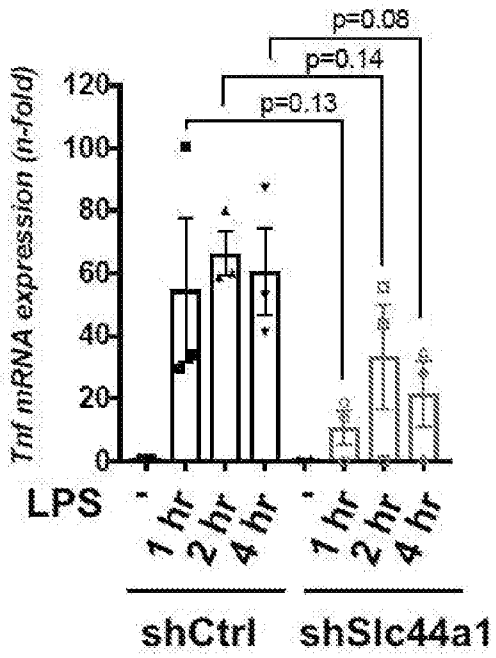

FIG. 2Q

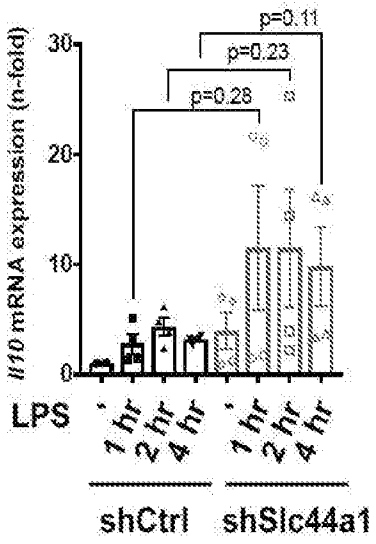

FIG. 2R

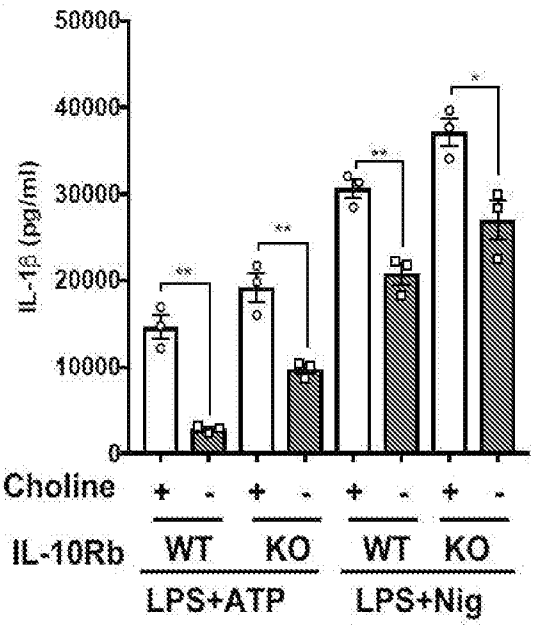

FIG. 2S

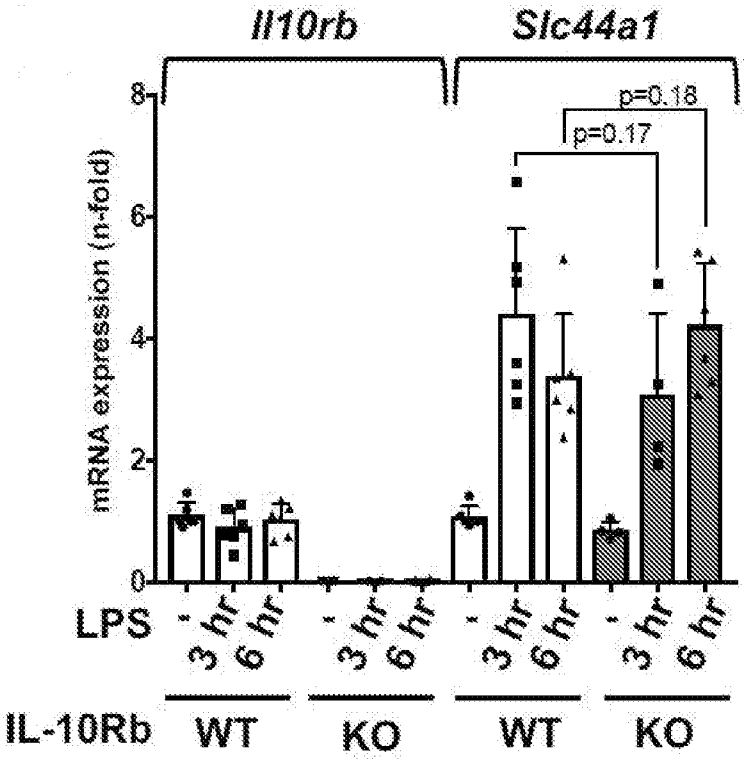

FIG. 2T

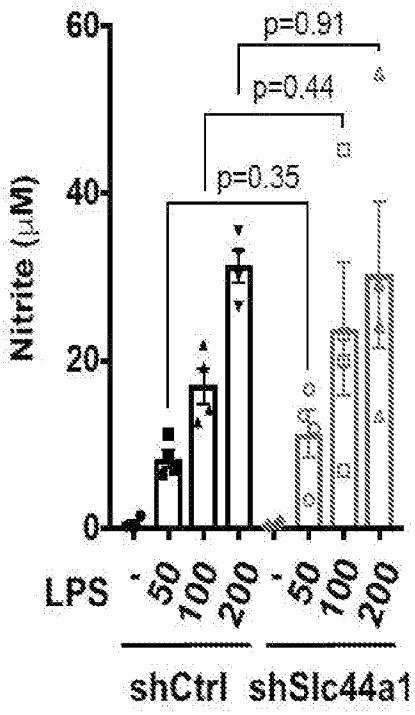

FIG. 2U

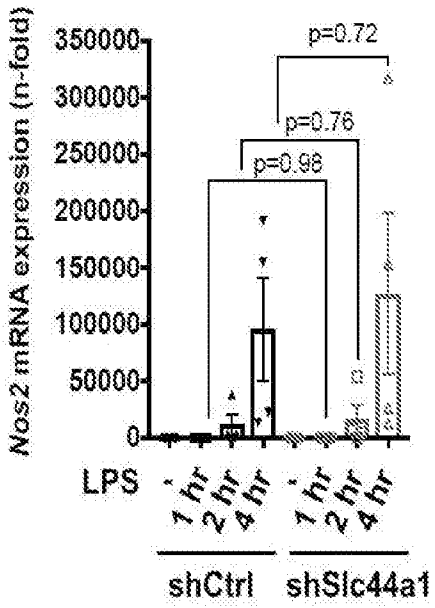

FIG. 2V

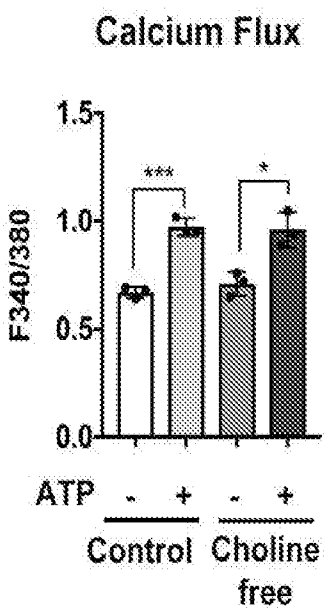

FIG. 2W

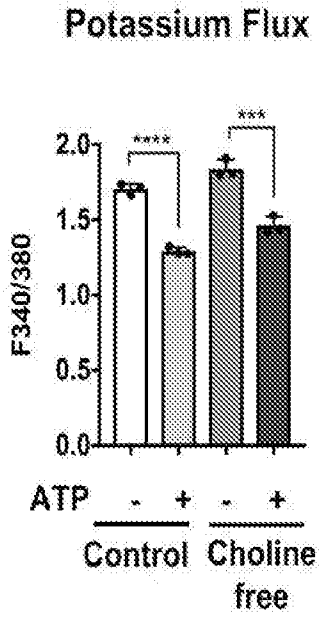

FIG. 2X

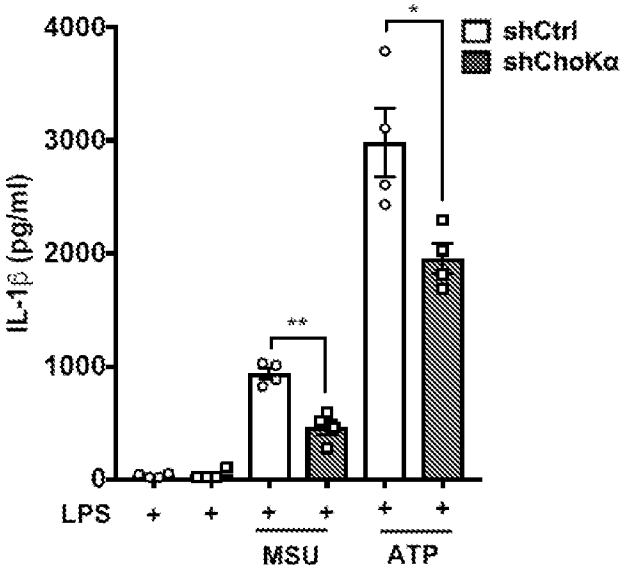

FIG. 3A

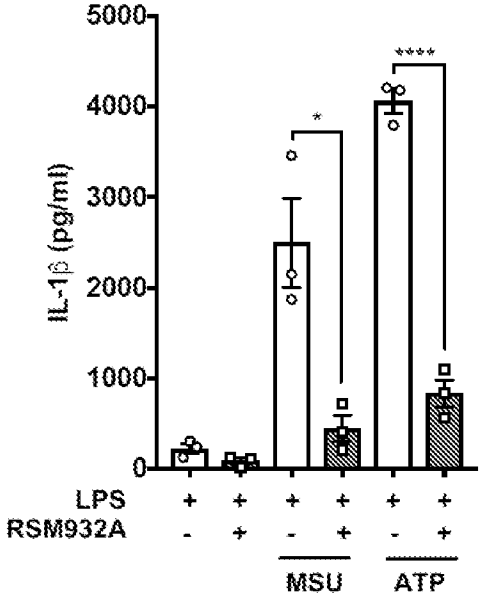

FIG. 3B

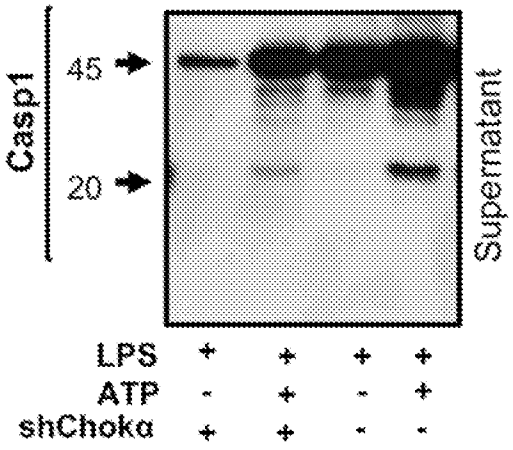

FIG. 3C

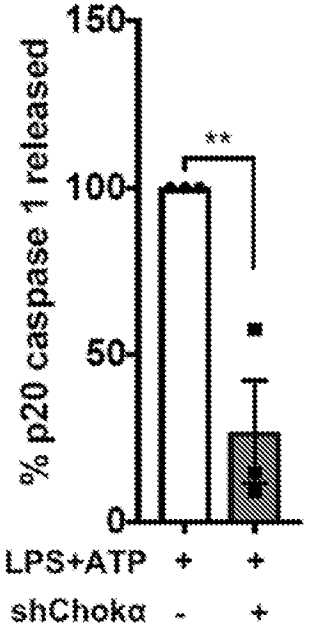

FIG. 3D

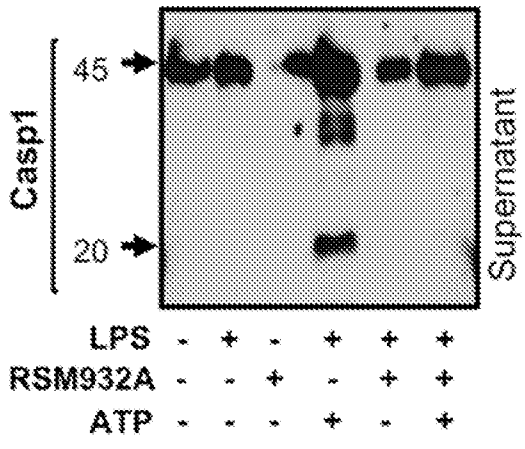

FIG. 3E

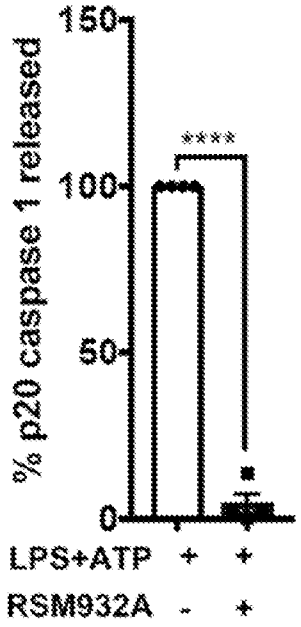

FIG. 3F

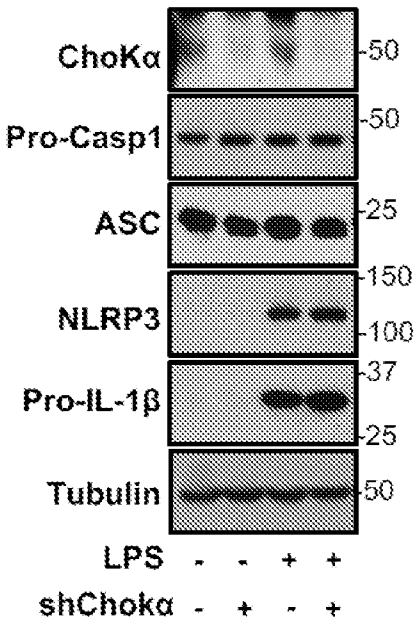

FIG. 3G

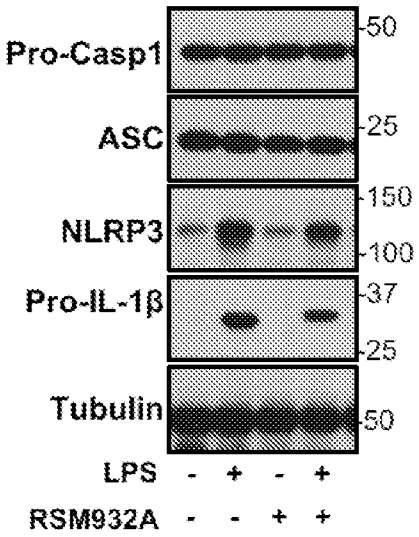

FIG. 3H

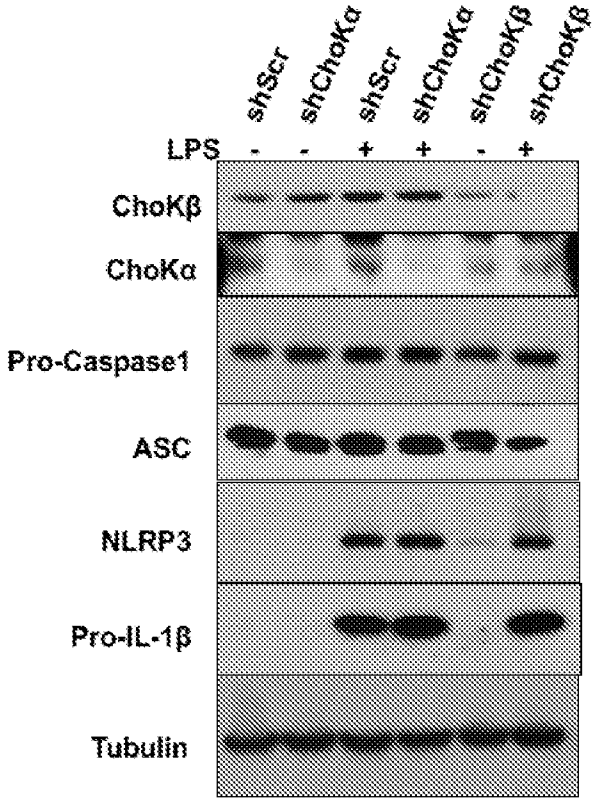

FIG. 3I

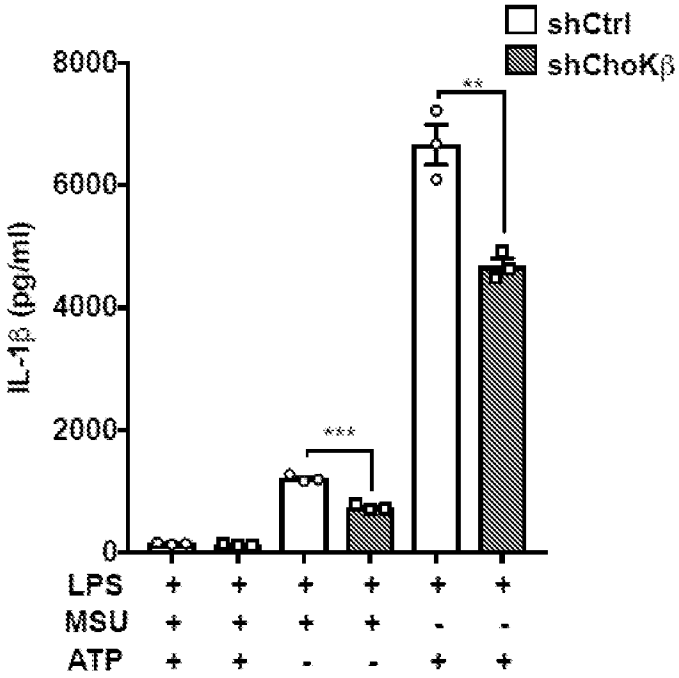

FIG. 3J

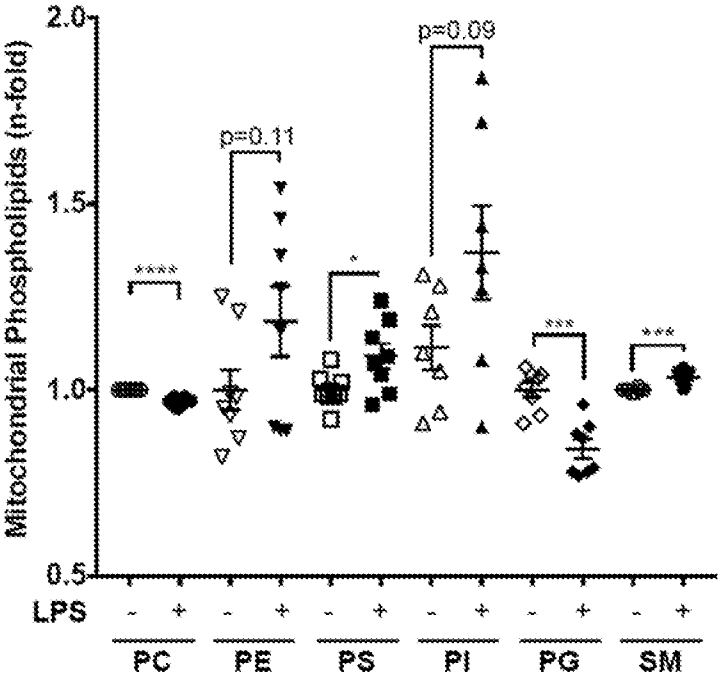

FIG. 4A

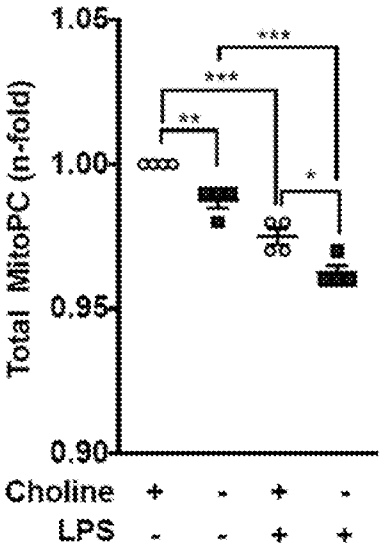

FIG. 4B

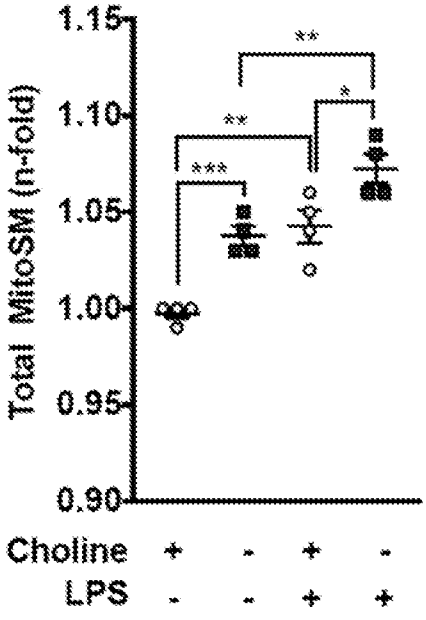

FIG. 4C

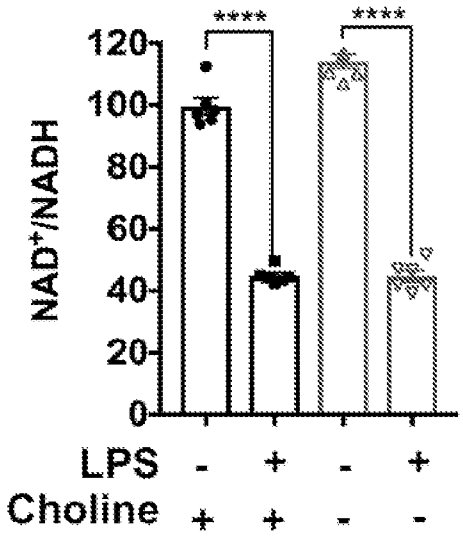

FIG. 4D

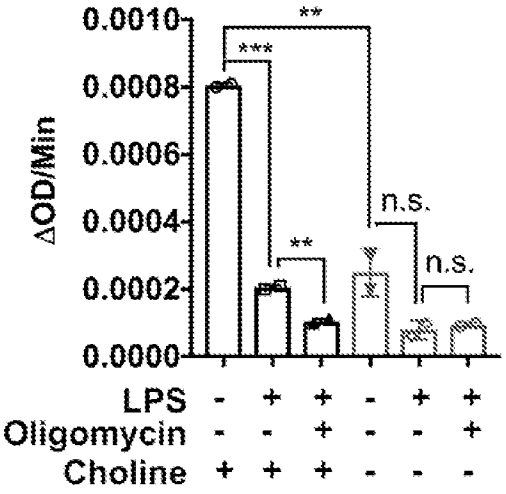

FIG. 4E

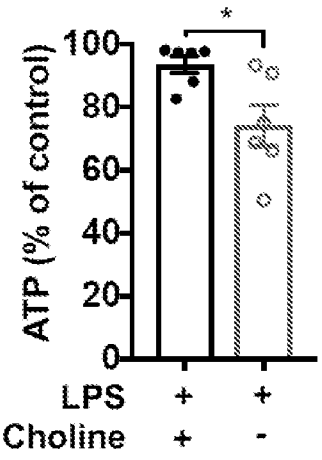

FIG. 4F

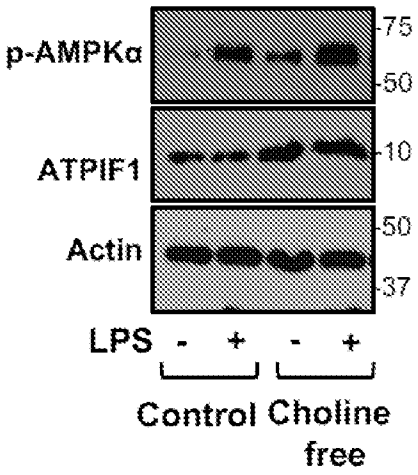

FIG. 4G

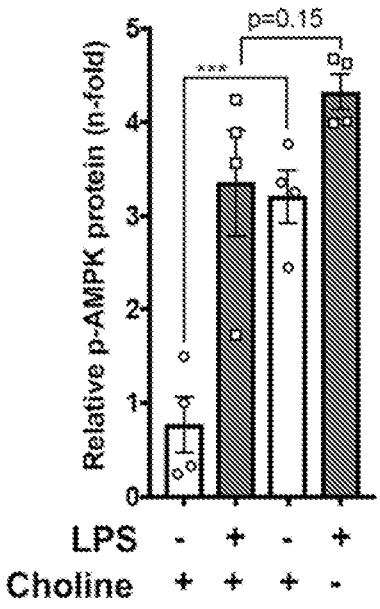

FIG. 4H

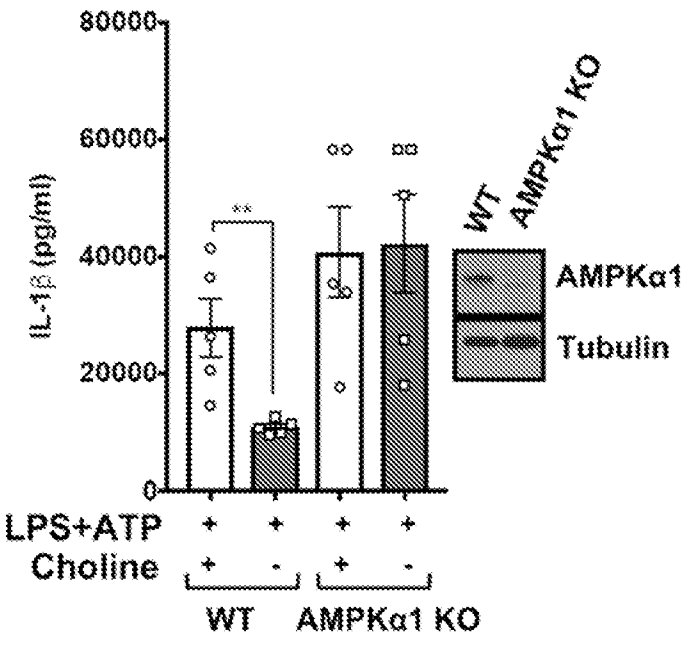

FIG. 4I

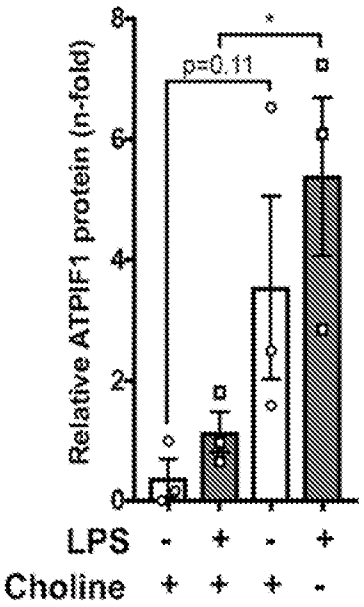

FIG. 4J

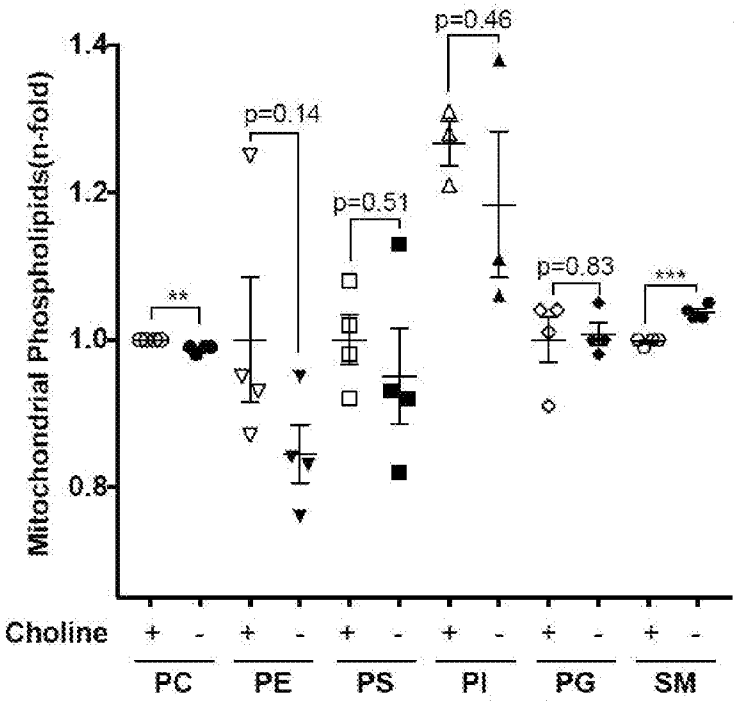

FIG. 4K

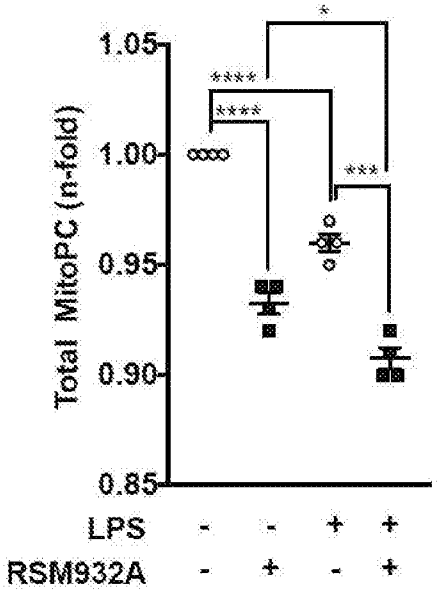

FIG. 4L

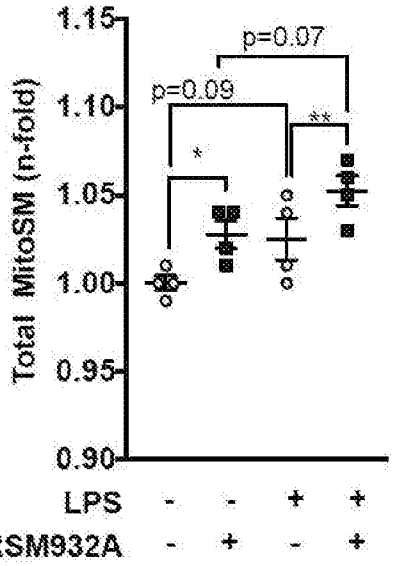

FIG. 4M

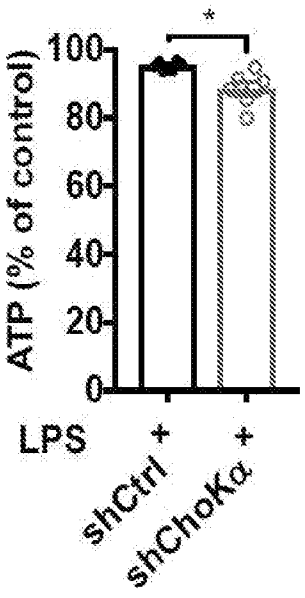

FIG. 4N

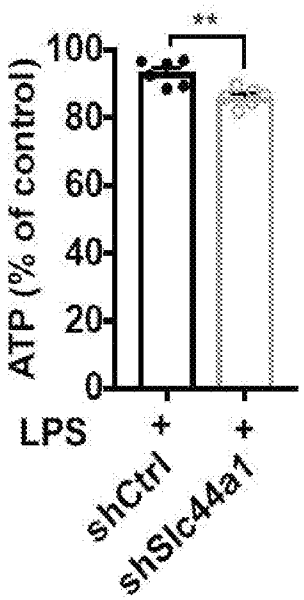

FIG. 4O

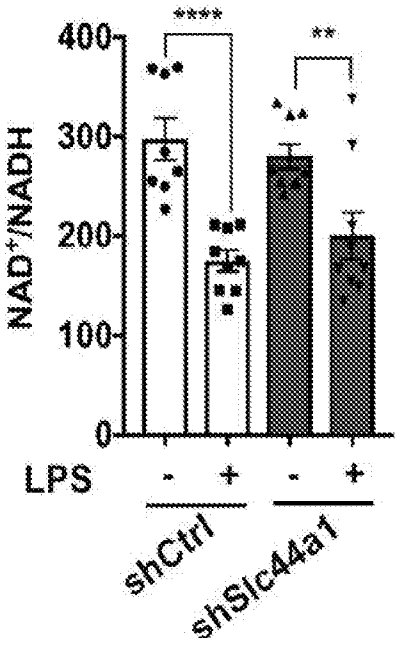

FIG. 4P

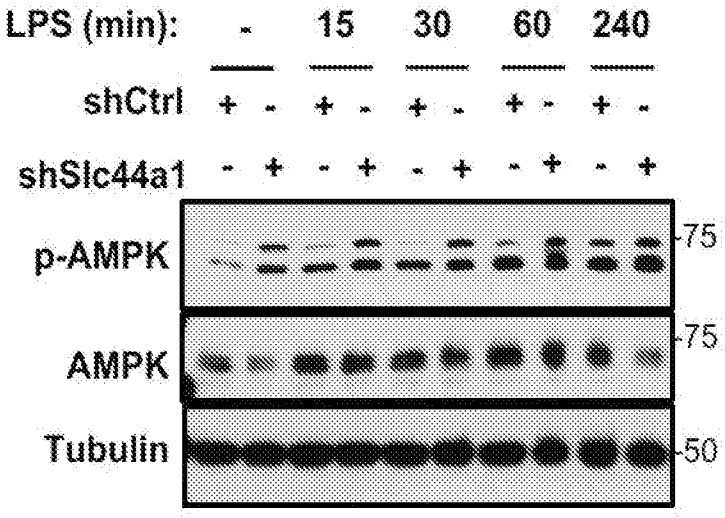

FIG. 4Q

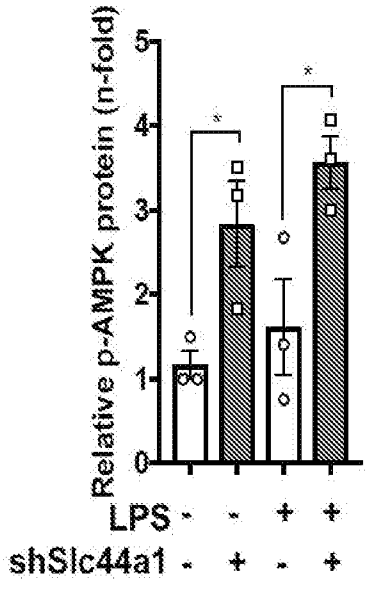

FIG. 4R

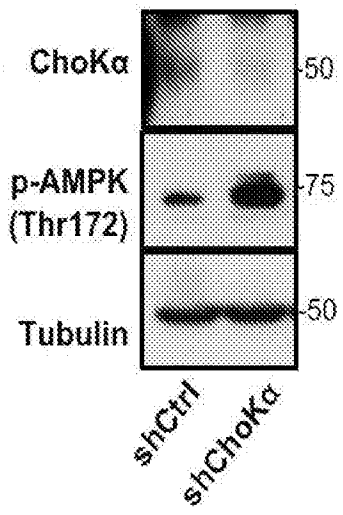

FIG. 4S

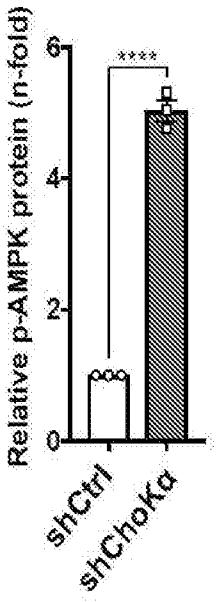

FIG. 4T

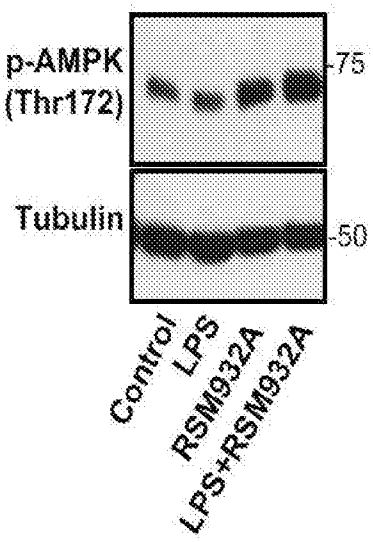

FIG. 4U

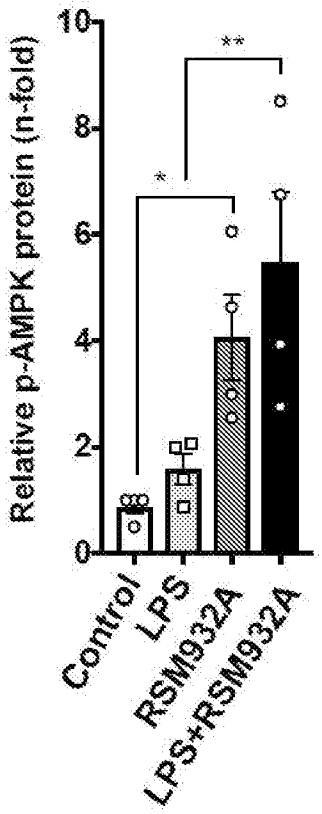

FIG. 4V

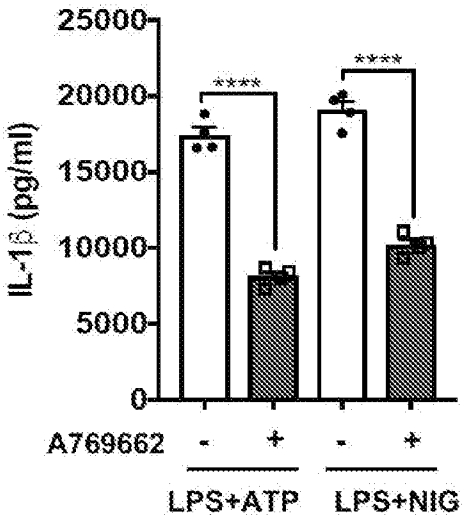

FIG. 4W

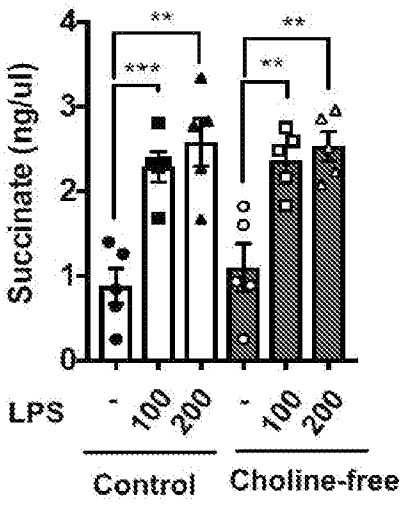

FIG. 4X

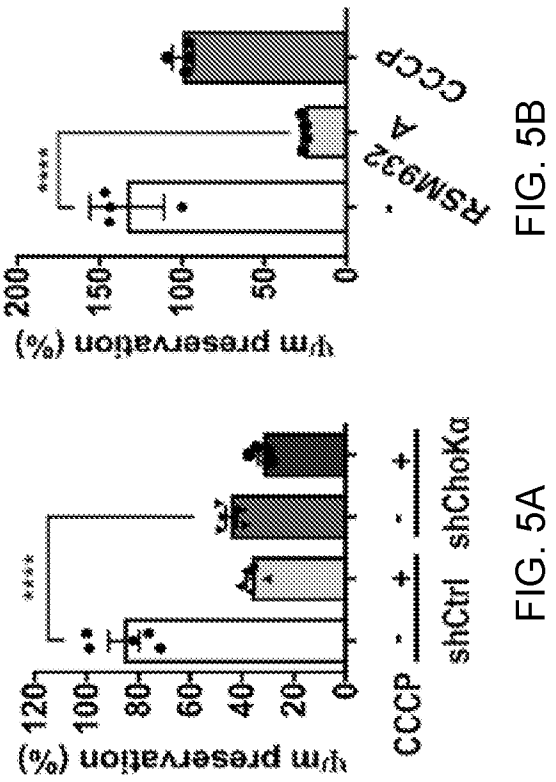

FIG. 5A

FIG. 5B

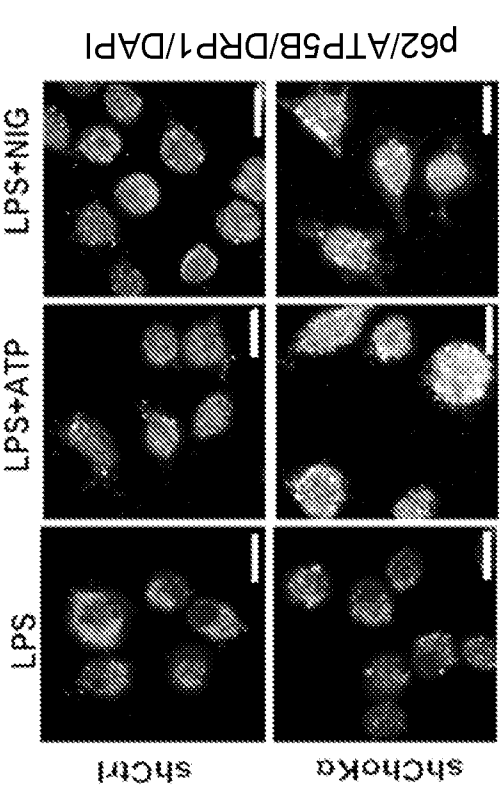

FIG. 5D

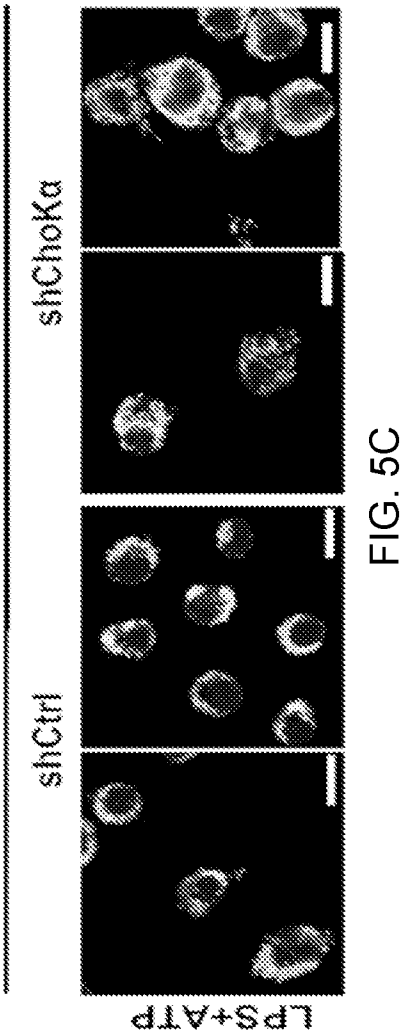

FIG. 5C

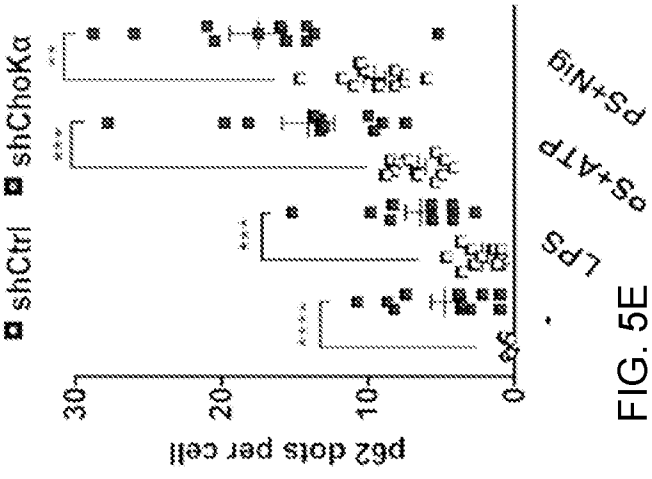

FIG. 5E

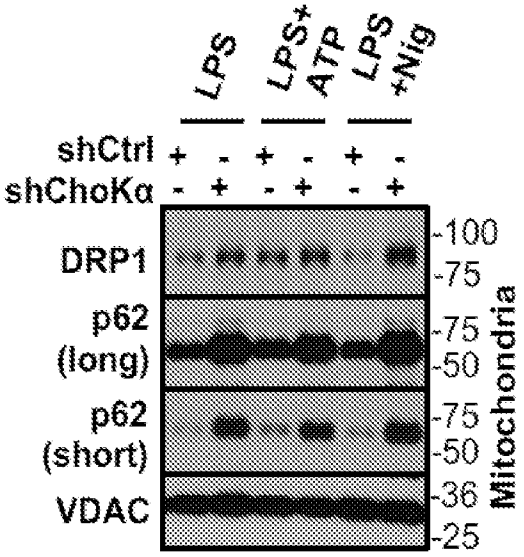

FIG. 5F

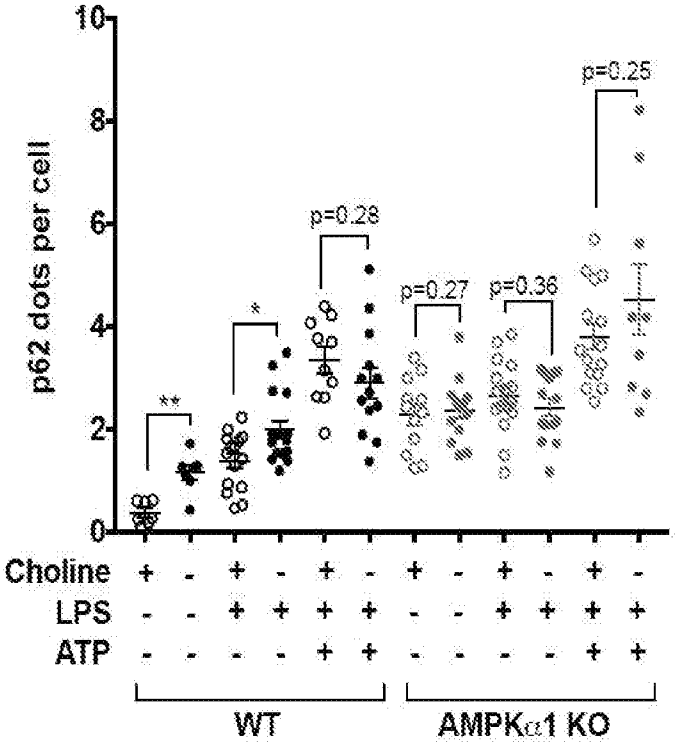

FIG. 5G

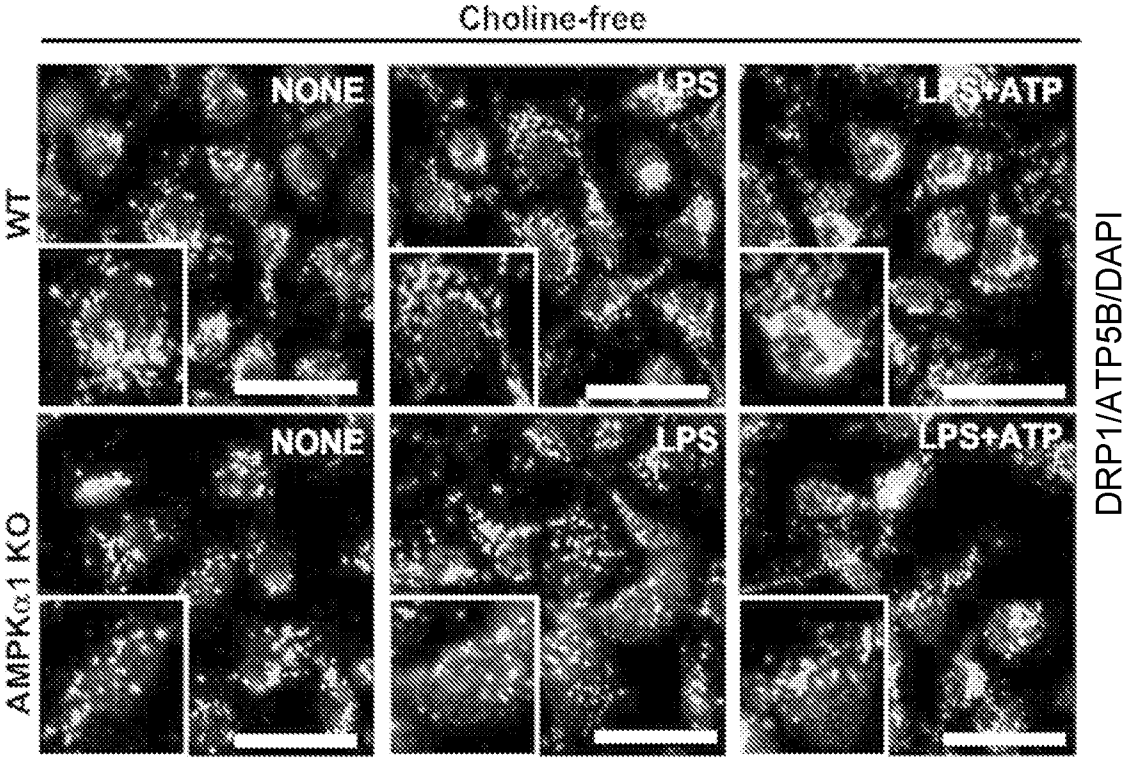

FIG. 5I

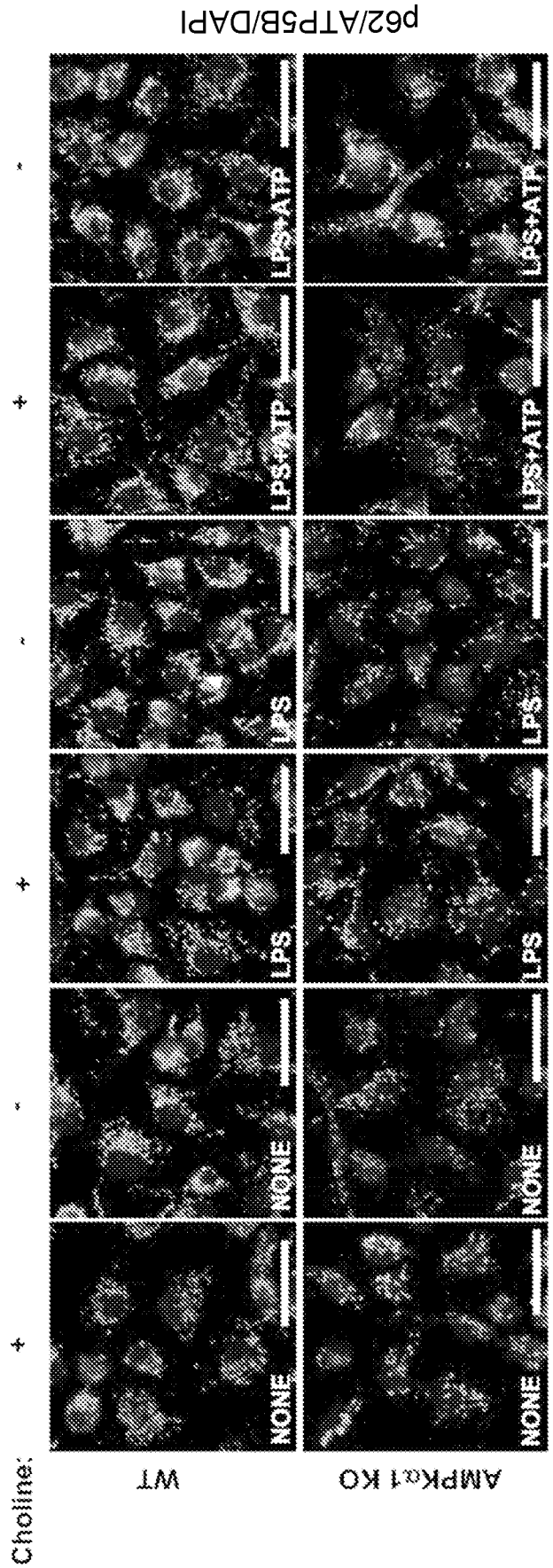

FIG. 5H

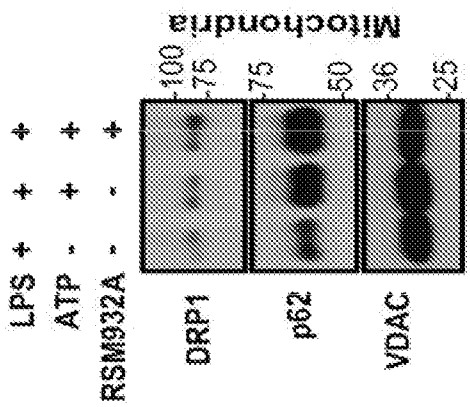

FIG. 5K

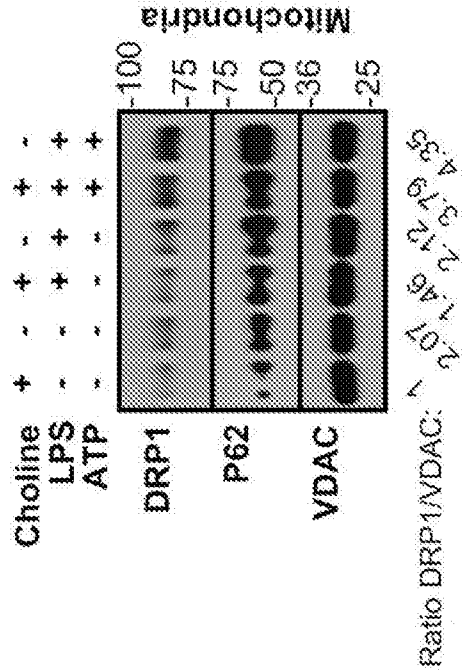

FIG. 5O

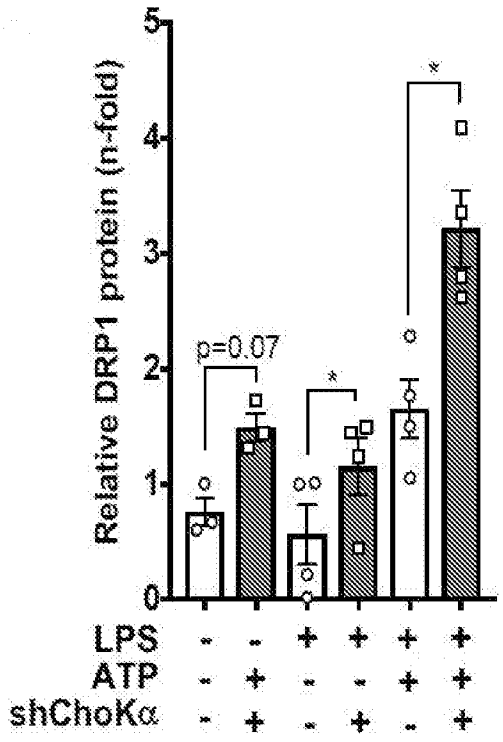

FIG. 5J

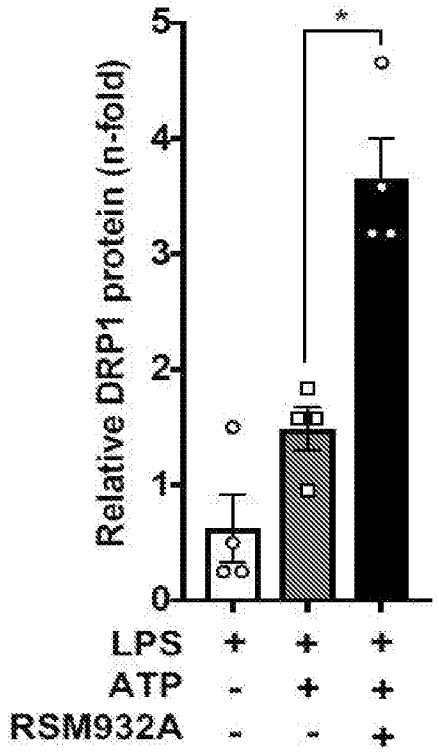

FIG. 5L

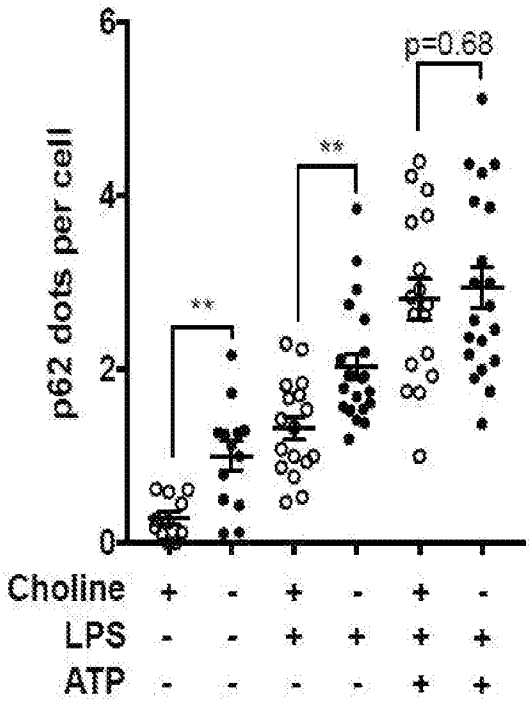

FIG. 5M

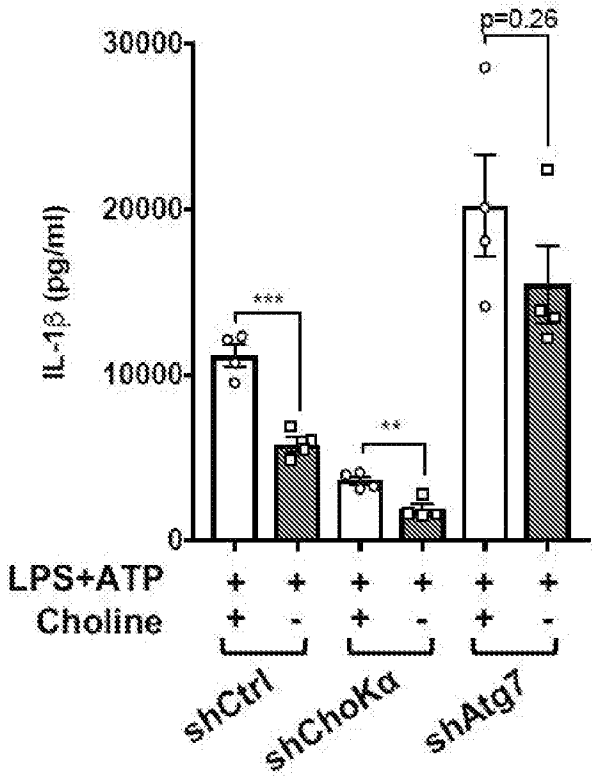

FIG. 5P

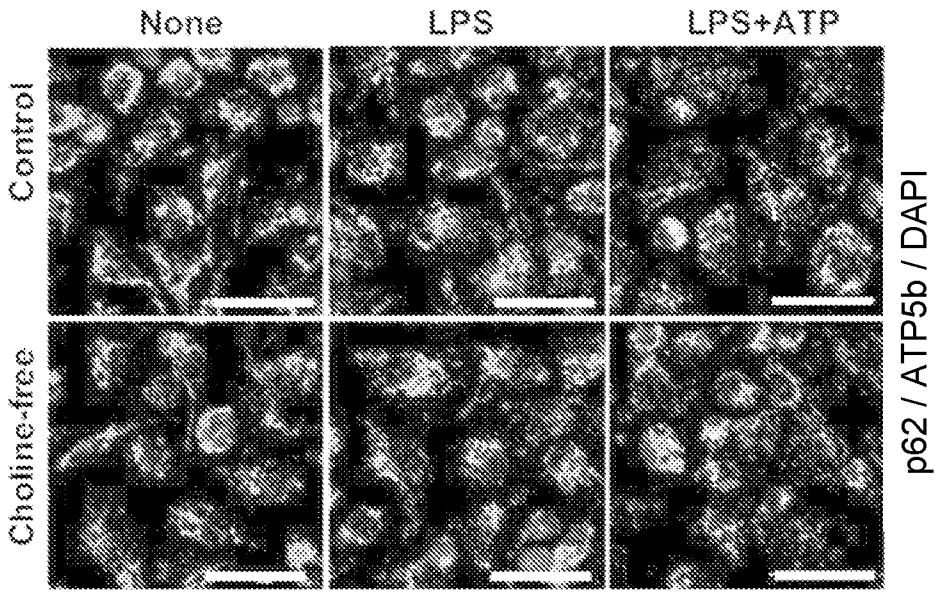

FIG. 5N

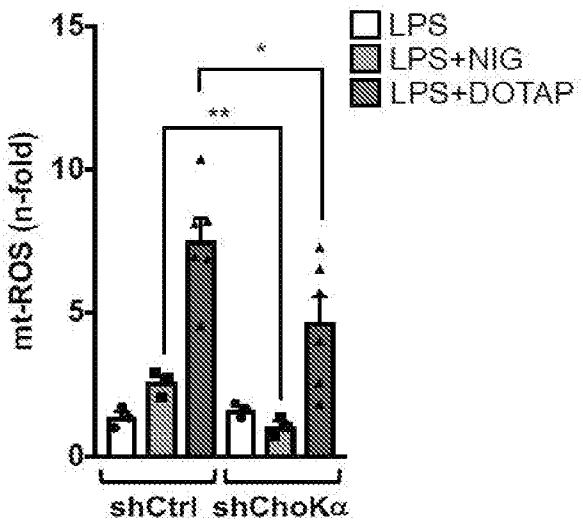

FIG. 5Q

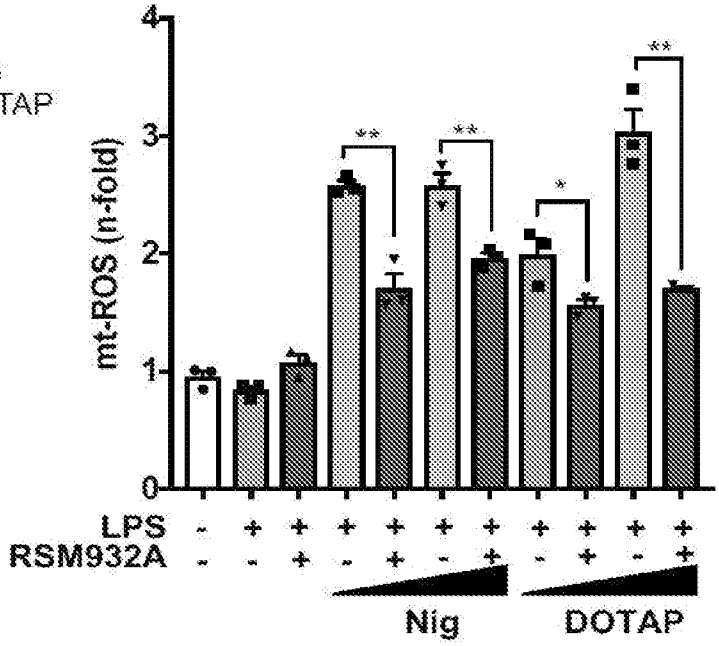

FIG. 5R

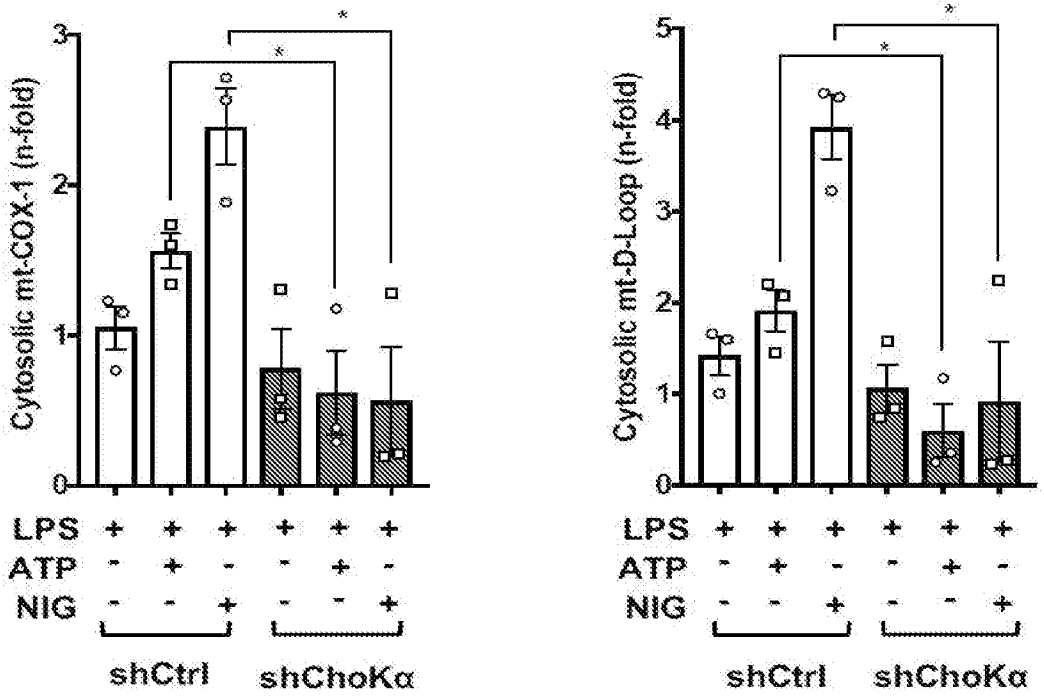
Cytosolic mt-COX-1 (n-fold)
Cytosolic mt-D-Loop (n-fold)
LPS
ATP
NIG
shCtrl
shChoKα

FIG. 5S

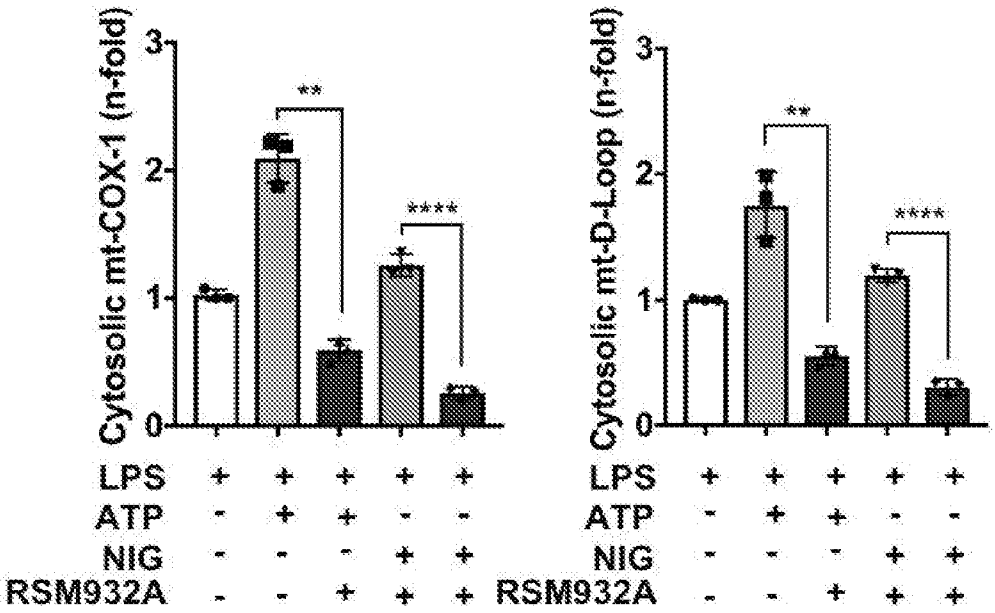
Cytosolic mt-COX-1 (n-fold)
Cytosolic mt-D-Loop (n-fold)
LPS
ATP
NIG
RSM932A

FIG. 5T

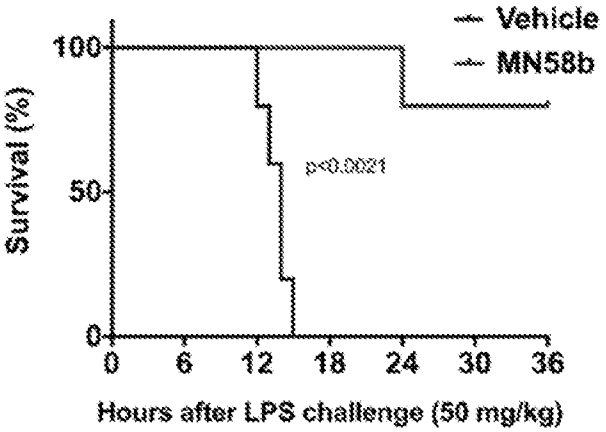

FIG. 6A

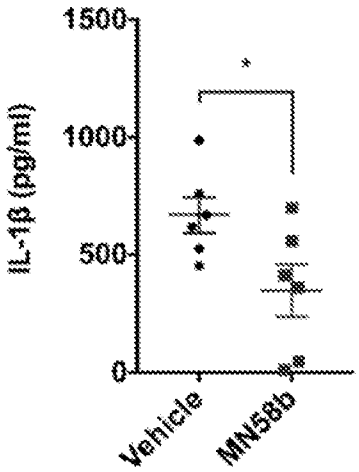

FIG. 6B

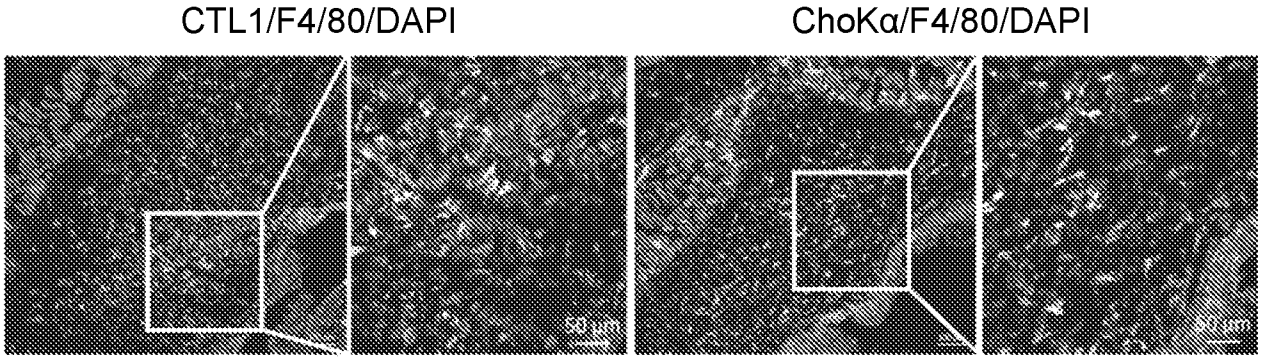

FIG. 6C

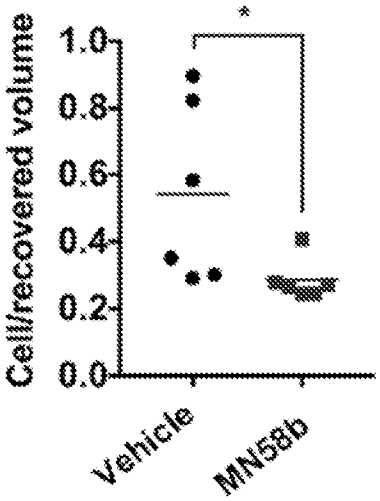

FIG. 6D

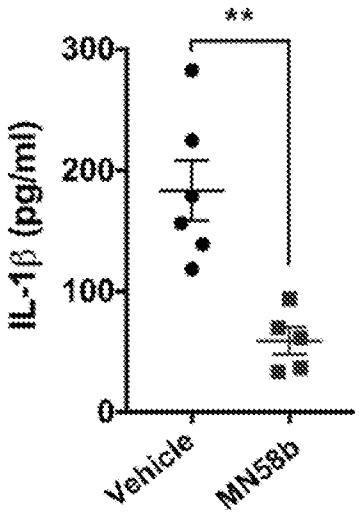

FIG. 6E

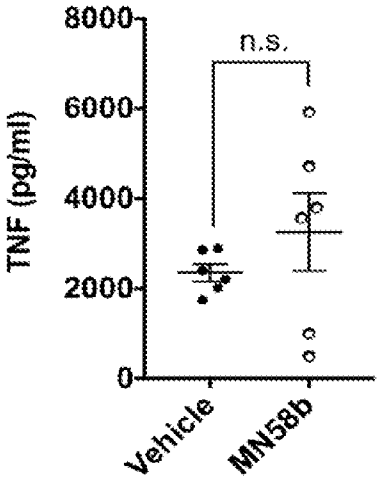

FIG. 6F

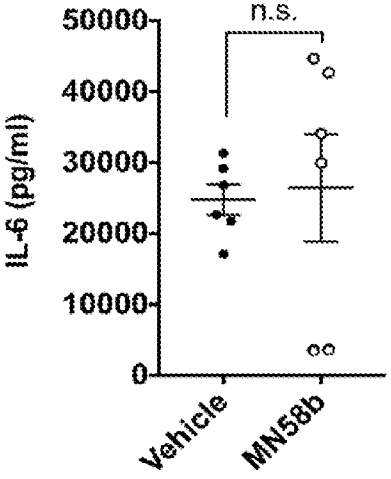

FIG. 6G

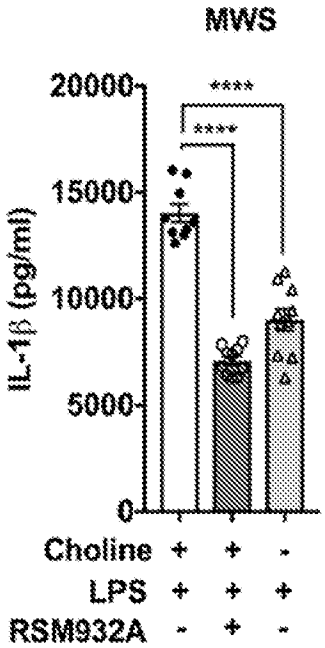

FIG. 7A

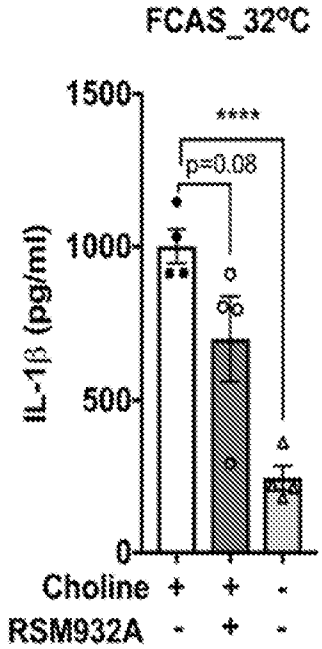

FIG. 7B

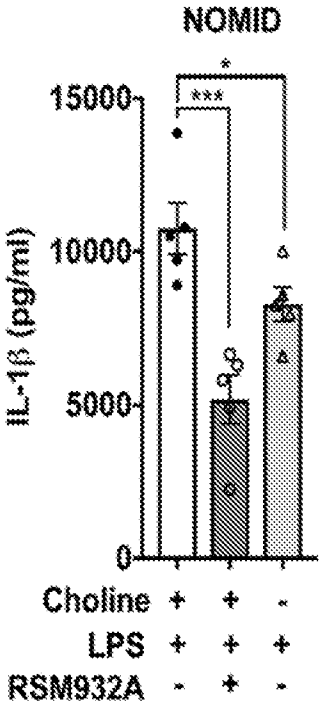

FIG. 7C

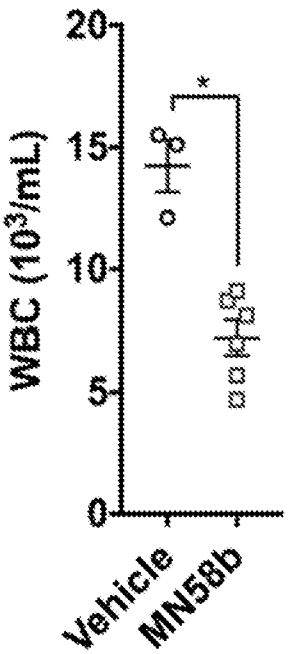

FIG. 7D

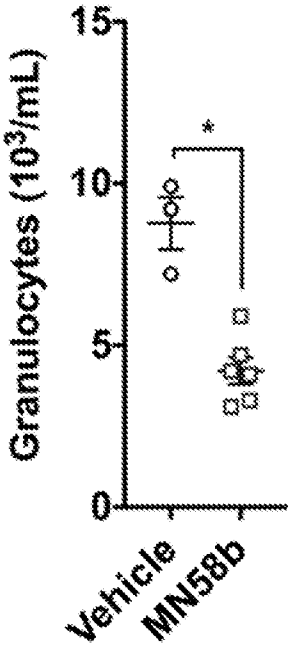

FIG. 7E

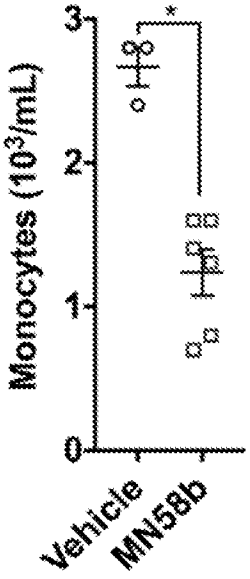

FIG. 7F

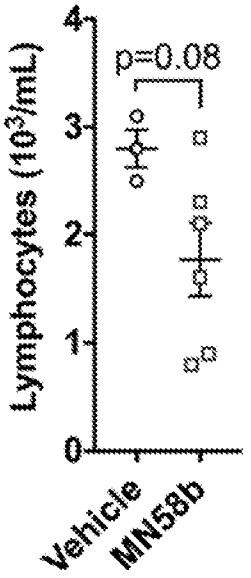

FIG. 7G

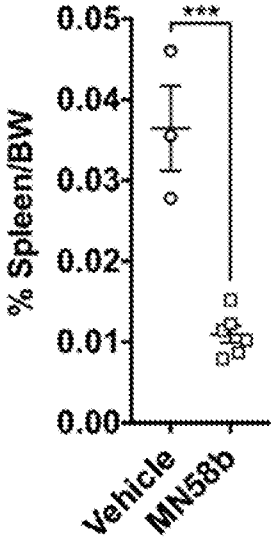

FIG. 7H

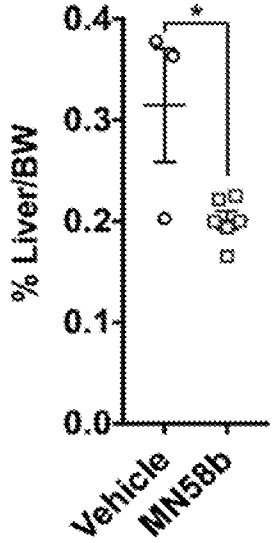

FIG. 7J

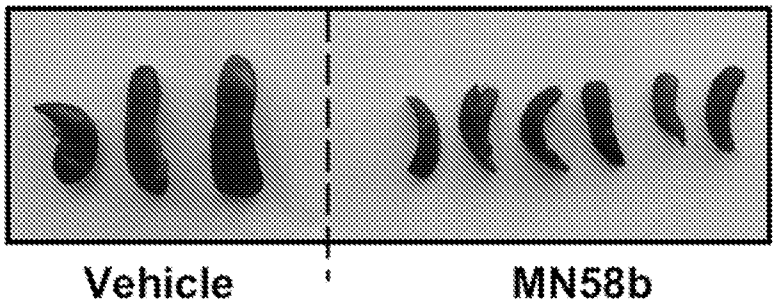

FIG. 7I

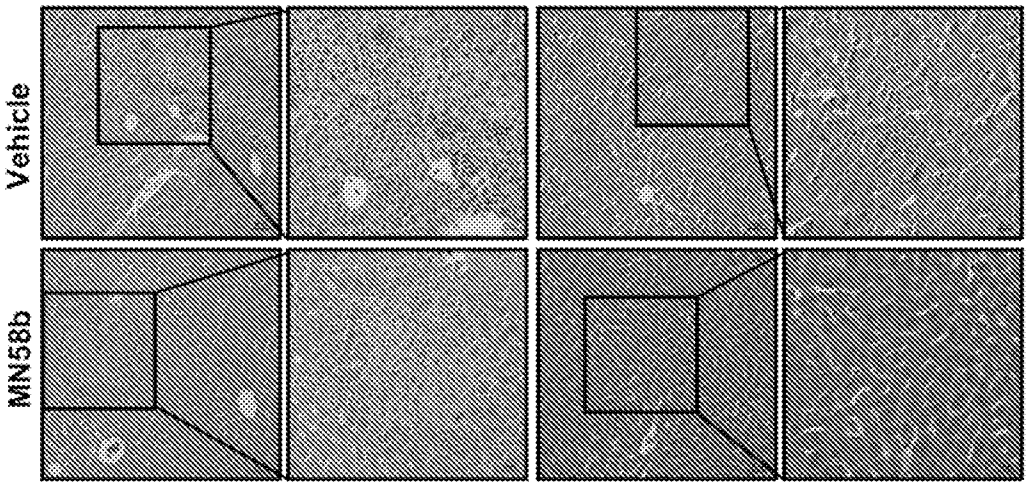

FIG. 7K

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/16637

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/16637

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+, claims 1-16, directed to a method of treating macrophage-mediated inflammatory and/or degenerative diseases, or inhibiting choline phosphorylation in a subject comprising administering an agent. The method will be searched to the extent that the agent encompasses an inhibitor of choline-transporter-like protein 1 (CTL1) activity or expression. It is believed that claims 1, 2, 4-12, 14-16 encompass this first named invention, and thus these claims will be searched without fee to the extent that the agent encompasses an inhibitor of CTL1 activity or expression. Additional agent(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected agent(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be the agent comprising an inhibitor of choline phosphorylation (claims 1, 3, 8-10, 13).
--continued on extra sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2, 4-12, 14-16, limited to an inhibitor of CTL1 activity or expression

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/16637

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/135, A61K 31/137, A61K 31/138, A61K 31/15 (2020.01)

CPC - A61K 35/66, A61K 38/00, A61K 45/00, A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	JP 2013/049646 A2 to UNIVERSITY OF TOKYO MEDICAL) 14 March 2013 (14.03.2013) abstract; para [0009]-[0022]	1, 8-12, 14-16 --- 2, 4-7
Y	FALCONER et al. Review: Synovial Cell Metabolism and Chronic Inflammation in Rheumatoid Arthritis. Arthritis & Rheumatology, July 2018, Vol 70, No 7, pg 984-99. Entire document, especially abstract; p. 988, Fig 2; p. 993, col 1	2, 4-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2020	23 JUN 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 20/16637

--continued from Box No. III Observations where unity of invention is lacking--

Group II, claims 17-26, directed to a method of identifying an agent useful for treating macrophage-mediated inflammatory and/or degenerative diseases.

The inventions listed as Group I+ and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features

Group I+ has the special technical feature of administering to a subject an effective amount of an inhibitor of CTL1 activity or expression or an inhibitor of choline phosphorylation, that is not required by Group II.

Group II has the special technical feature of contacting a sample of cells from a subject with at least one test agent, wherein a decrease in CTL1 expression or choline phosphorylation in the presence of the agent identifies the agent as therapeutically useful, that is not required by Group I+.

The inventions of Group I+ each include the special technical feature of a different type of agent, with a different effect, and is considered a distinct technical feature.

Common technical features

The inventions of Group I+ and Group II share the common technical feature of a method of treating macrophage-mediated inflammatory and/or degenerative diseases, and/or inhibiting choline phosphorylation in a subject in need thereof comprising administering to the subject an effective amount of an inhibitor of CTL1 activity or expression or an inhibitor of choline phosphorylation.

However, these shared technical features are previously made obvious by US 7,303,895 B1 to O'Regan et al., (hereinafter O'Regan), in view of the article by Brailoiu et al., entitled "Choline Is an Intracellular Messenger Linking Extracellular Stimuli to IP3-Evoked Ca2+ Signals through Sigma-1 Receptors", (Cell Reports, 08 January 2019, 26, 330-337), (hereinafter Brailoiu).

O'Regan teaches use of agents identified as an inhibitor of CTL1 in a composition with a pharmaceutically acceptable vehicle for use as a medicament intended for the treatment of diseases involving cells expressing the transcripts of a CTL gene, including neurodegenerative diseases (abstract "hCTL1 and hCTL2, involved in the metabolism and/or transport of choline in cells... the identification of CTL genes enables to develop new strategies for treating diseases of the nervous system, in particular neurodegenerative demyelenating diseases"; col 11, ln 11-27 "the use of the CTL genes and proteins to identify and to select compounds capable of modifying their activity is particularly targeted...allows the identification of a compound capable of inhibiting the activity of a polypeptide according to the invention..."; col 12, ln 50-65 "The subject of the invention is also a compound capable of being obtained from the method described above and a composition comprising said compound or a vector previously detailed and a pharmaceutically acceptable vehicle. Thus, one aspect of the invention relates to the use of said compound or vector for the manufacture of a medicament, in particular a medicament intended for the treatment of genetic diseases involving cells expressing the transcripts of a CTL gene such as...neurodegenerative, demyelinizing diseases, preferably Alzheimer's disease, Parkinson's disease and Huntington's disease. A close correlation in fact exists between Alzheimer's disease and the quantity of acetylcholine transferase...or the self-inhibitory action of endogenous acetylcholine in the brain of patients...demonstrating the involvement of the metabolism and the transport of choline in this disease"), yet does not specifically teach a treatment method comprising administering to a subject an effective amount of an inhibitor of CTL1 activity or expression.
Brailoiu teaches contacting a cell with an antisense inhibitor of CTL1 to inhibit CTL1 expression or activity in the cell (p. 334, col 2, para 2 "Potentiation of bradykinin-evoked Ca2+ signals by extracellular choline was substantially attenuated by loss of Sig-1R (shRNA) or CTL1 (small interfering RNA [siRNA]), but unaffected by scrambled shRNA or siRNA (Figures 4C-4E)"; p. e3, para 5 "Lipofectamine RNAiMax was used to transfect cells simultaneously with three different siRNAs against CTL1 (50 nM of each) to reduce CTL1 expression"). It would have been obvious to one of ordinary skill in the art to have administered a composition comprising any CTL1 inhibitors identified by the methods taught by O'Regan or those taught by Brailoiu to a subject with a neurodegenerative disease related to CTL1 activity for therapy, in order to reduce CTL1 activity that is involved in the pathology of the underlying neurodegeneration in said subject.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.