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(57) Abstract: The present invention is concerned with the use of agents that inhibit CD40L-induced inflammasome activity. Indeed, described herein are methods, compositions and pharmaceutical compositions useful in inhibiting CD40L-induced inflammasome activity without inhibiting CD40 biological activity, and in treating subjects suffering from disorders related to CD40L-induced inflammasome activity and IL-1 α expression.



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COMPOSITIONS AND METHODS FOR TREATING DISORDERS RELATED TO INFLAMMASOME ACTIVITY AND IL-1ALPHA EXPRESSION

FIELD OF THE INVENTION

5 The present invention is concerned with the use of agents that inhibit CD40L-induced inflammasome activity. Indeed, described herein are methods, compositions and pharmaceutical compositions useful in inhibiting CD40L-induced inflammasome activity without inhibiting CD40 biological activity, and in treating subjects suffering from disorders related to CD40L-induced inflammasome activity and IL-1 α expression.

INTRODUCTION

10 The inflammasome refers to a macromolecular complex formed by certain nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) following activation. The inflammasome consists typically of an NLR molecule, the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and a caspase
15 effector molecule (e.g., caspase-1). The best characterized inflammasomes are NLRP1 (also known as NALP1 or DEFCAP), NLRP3 (also known as NALP3, cryopyrin or CIAS1), and NLRC4 (also known as IPAF or CARD12) (see, Martinon F, et al., Annu Rev Immunol. 2009;27:229–265).

20 Inflammasomes are the central processing units (CPUs) responsible for decoding and integrating signals of foreignness, damage, danger, and distress released by pathogens, cells, and tissues. It was initially thought that the inflammasomes participated only in pathogen recognition and in the pathogenesis of a few, rare, hereditary inflammatory disorders. On the contrary, it is now clear that they have a central role in the pathogenesis of basically all types
25 of chronic inflammation, in metabolic diseases and cancer. A main function is to catalyze conversion of pro-IL-1 β and pro-IL-18 into their respective mature forms. However, the different inflammasome subtypes may also participate in additional responses (e.g., proliferation, regulation of glycolytic metabolism, and/or cell activation) albeit it is not clear whether these effects are still mediated through IL-1 β release or via modulation of other
30 caspase-1-dependent or -independent pathways.

Numerous medical disorders are related to inflammasome activity.

An improved understanding of the molecular mechanisms pertaining to such disorders related to inflammasome activity is needed.

In addition, improved techniques for treating such disorders related to inflammasome activity are needed.

The present invention addresses such needs.

5 SUMMARY OF THE INVENTION

Experiments were conducted during the course of developing embodiments for the present invention to investigate CD40L-induced IL-1 β and MCP-1 secretion in primary human retinal pigment epithelial (hRPE) cells. CD40L was shown to activate hRPE NALP1 and NALP3 inflammasomes and induced IL-1 β and IL-18 secretion through CD40L-
10 CD11b/ α 5 β 1 pathways. The induced IL-1 β secretion by CD40L was subject to negative regulation by PI3K. CD40L was shown to also trigger auto/paracrine-mediated MCP-1 secretion by hRPE demand IL-1 β and was shown to be sensitive to IL-1 β neutralizing antibodies. Such findings suggest previous unrecognized single (CD40L) ligand-induced, inflammasome-dependent mature IL-1 β secretion by any cell type.

15 Accordingly, the present invention is concerned with the use of agents that inhibit CD40L-induced inflammasome activity. Indeed, described herein are methods, compositions and pharmaceutical compositions useful in inhibiting CD40L-induced inflammasome activity without inhibiting CD40 biological activity, and in treating subjects suffering from disorders related to CD40L-induced inflammasome activity and IL-1 α expression.

20 In certain embodiments, the present invention provides methods for inhibiting inflammasome activity, assembly and/or activation in a subject. Such methods are not limited to a particular manner or technique. In some embodiments, such methods comprise administering to the subject a composition comprising one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity.

25 In some embodiments, such agents inhibit CD40L-induced inflammasome activity, assembly and/or activation without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome activity, assembly and/or activation through one or more of the following mechanisms:

without inhibiting interaction between CD40L and CD40;

30 through binding CD40L;

through inhibiting interaction between CD40L (CD154) and α 5 β 1 (CD49e/CD29);

through binding α 5 β 1 (CD49e/CD29);

through inhibiting interaction between CD40L (CD154) and α _M β ₂ (CD11b/CD18);

through binding $\alpha_M\beta_2$ (CD11b/CD18);
through inhibiting inflammasome related caspase-1 activation;
through inhibiting inflammasome related caspase-5 activation;
through reducing inflammasome related IL-18 expression, activation and secretion;
5 through reducing inflammasome related IL-1 β expression, activation and secretion;
through reducing or increasing inflammasome related intracellular MMP-9
expression;
through reducing inflammasome related PI3K signaling.

Such methods are not limited to specific types or kinds of agents inhibit CD40L-
10 induced inflammasome activity, assembly and/or activation. For example, in some
embodiments such agents are selected from the group consisting of an antibody (e.g., a
polyclonal antibody, a monoclonal antibody) or antigen binding fragment thereof, a peptide
(e.g., a mimetic peptide, a recombinant human or humanized peptide), an aptamer, a
peptibody, an adnectin, a nucleic acid, or a small molecule compound (e.g., a small molecule
15 compound having a molecular weight less than approximately 5000 Daltons).

In some embodiments, the one or more agents that inhibit CD40L-induced
inflammasome assembly, activation and/or activity are selected from the group including, but
not limited to, BD Pharmingen clone ICRF-44 anti-human CD11b mouse derived antibody,
Biolegend clone ICRF-44 PE anti-human CD11b antibody, Millipore clone JBS5 – anti- $\alpha 5\beta 1$
20 MAB1969, and a small peptide having the following amino acid sequence EQLKKSKTL
(SEQ ID NO: 1) (see, EP 2444101).

In some embodiments, the subject is a mammal. In some embodiments, the subject is
a human subject. In some embodiments, the subject is a human subject suffering from a
condition and/or disorder characterized by inflammasome activity.

25 Such embodiments are not limited to a particular type of condition and/or disorder
characterized by inflammasome activity. For example, in some embodiments, such a
condition and/or disorder is selected from the group consisting of the group including, but not
limited to, ocular diseases with an obvious inflammatory basis, including keratitis,
endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis,
30 vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis,
and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an
inflammatory basis including, age-related macular degeneration, diabetic retinopathy,
glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from the

group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

In certain embodiments, the present invention provides methods for preventing, attenuating, or treating a disorder related to inflammasome activity in a subject, comprising administering to the subject a composition comprising one or more of agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome activity, assembly and/or activation without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome activity, assembly and/or activation through one or more of the following mechanisms:

- without inhibiting interaction between CD40L and CD40;
- through binding CD40L;
- through inhibiting interaction between CD40L (CD154) and $\alpha 5\beta 1$ (CD49e/CD29);
- through binding $\alpha 5\beta 1$ (CD49e/CD29);
- through inhibiting interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18);
- through binding $\alpha_M\beta_2$ (CD11b/CD18);
- through inhibiting inflammasome related caspase-1 activation;
- through inhibiting inflammasome related caspase-5 activation;
- through reducing inflammasome related IL-18 expression, activation and secretion;
- through reducing inflammasome related IL-1 β expression, activation and secretion;
- through reducing or increasing inflammasome related intracellular MMP-9 expression;
- through reducing or increasing inflammasome related PI3K signaling.

Such methods are not limited to specific types or kinds of agents inhibit CD40L-induced inflammasome activity, assembly and/or activation. For example, in some embodiments such agents are selected from the group consisting of an antibody (e.g., a polyclonal antibody, a monoclonal antibody) or antigen binding fragment thereof, a peptide (e.g., a mimetic peptide, a recombinant human or humanized peptide), an aptamer, a

peptibody, an adnectin, a nucleic acid, or a small molecule compound (e.g., a small molecule compound having a molecular weight less than approximately 5000 Daltons).

In some embodiments, the one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity are selected from the group including, but not limited to, BD Pharmingen clone ICRF-44 anti-human CD11b mouse derived antibody, Biolegend clone ICRF-44 PE anti-human CD11b antibody, Millipore clone JBS5 – anti- α 5 β 1 MAB1969, and a small peptide having the following amino acid sequence EQLKKSKTL (SEQ ID NO: 1)(see, EP 2444101).

In some embodiments, the condition and/or disorder characterized by inflammasome activity is selected from the group consisting of the group including, but not limited to, ocular diseases with an obvious inflammatory basis, including keratitis, endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

In some embodiments, the condition and/or disorder characterized by inflammasome activity is characterized by elevated plasma levels of one or more of the following: CD40L, IL-1 β , IL-18, IL-1 α , IL-1Ra, c-reactive protein, STP2, MMP-9, and/or increased inflammasome activity.

In some embodiments, the composition is co-administered with and one or more additional agents. In some embodiments, the one or more additional agents are selected from the group consisting of a DAMP or PAMP inhibitor, agents binding to TLR1, TLR2, TLR3, TLR4, TLR7 and/or TLR9, agents modulating TLR receptors, agents that stabilize lysosomes, agents that inhibit gout crystal formation, anti-oxidant agents, agents that inhibit lipid peroxide formation, pannexin channel and potassium channel modulators including spironolactone or probenecid, calcium channel inhibitors, PI3K/mTor modulators, inhibitors

of interferons α , β , or γ , inhibitors of caspases 1, 4, or 5, and non-steroidal agents (e.g., ibuprofen, celecoxib, ketoprofen, naproxen, piroxicam, diclofenac).

In certain embodiments, the present invention provides methods for identifying a potential agent capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity, comprising a) assessing the binding ability of a test agent with $\alpha_M\beta_2$, CD40, and/or $\alpha 5\beta 1$; b) assessing the test agent's ability to inhibit intracellular inflammasome activation, reduce IL-1 β or IL-18 secretion, reduce intracellular IL-1 α expression, and/or reduce cellular MMP-9 expression while not inhibiting CD40 activity; c) identifying the test agent as a potential agent capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity if the test agent is capable of one or more of the following:

binding with $\alpha_M\beta_2$, CD40, and/or $\alpha 5\beta 1$ while not inhibiting CD40 activity;
inhibit intracellular inflammasome activation while not inhibiting CD40 activity,
reducing IL-1 β or IL-18 secretion, reducing intracellular IL-1 α expression while not inhibiting CD40 activity, and

reducing cellular MMP-9 expression while not inhibiting CD40 activity.

In certain embodiments, the pharmaceutical composition comprises one or more agents capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity and a pharmaceutically acceptable carrier.

In certain embodiments, the present invention provides kits comprising a pharmaceutical composition comprising one or more agents capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity, and one or more of a container, a pack, a dispenser, and instructions for administration.

In certain embodiments, the present invention provides methods for inhibiting or stimulating IL-1 α expression in a subject, comprising administering to the subject one or more agents that block or stimulate caspase-4 activation and/or one or more agents that block or stimulate IL-1 α expression by inhibiting or stimulating PI3K and/or inhibiting or stimulating IL-1 α expression by inhibiting or stimulating intracellular Ca ion signaling or Ca ion levels.

In some embodiments, the subject is a human subject. In some embodiments, the subject is a human subject suffering from a condition and/or disorder characterized by IL-1 α expression due to caspase-4 and/or PI3K activation and/or increasing intracellular Ca or blocking Ca signaling. In some embodiments, the condition and/or disorder characterized by IL-1 α expression by caspase-4 and/or PI3K activation and/or increasing intracellular Ca or

blocking Ca signaling is selected from the group consisting of the group including, but not limited to, ocular diseases with an obvious inflammatory basis, including keratitis, endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

In some embodiments, the one or more agents that block caspase-4 activation is LEVD-CHO. In some embodiments, the one or more agents that block IL-1 α expression by inhibiting PI3K includes, but is not limited to, LY294002, wortmannin, CAL-101, PI-103, or MK2206. In some embodiments, the one or more agents that blocking or stimulating caspase-4 activation and/or inhibit or stimulate IL-1 α expression by inhibiting or stimulating PI3K and/or inhibiting or stimulating IL-1 α expression by inhibiting or stimulating intracellular Ca ion signaling or Ca ion levels are selected from the group consisting of an antibody (e.g., a polyclonal antibody, a monoclonal antibody) or antigen binding fragment thereof, a peptide (e.g., a mimetic peptide, a recombinant human or humanized peptide), an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound (e.g., a small molecule compound having a molecular weight less than approximately 5000 Daltons).

In certain embodiments, the present invention provides methods for preventing, attenuating, or treating a disorder related to IL-1 α activity by inhibiting or stimulating caspase-4 and/or PI3K activation and/or increasing intracellular Ca or blocking Ca signaling in a subject, comprising administering to the subject a composition comprising one or more agents that inhibit or stimulate IL-1 α expression, and/or intracellular levels or signaling and/or extracellular signaling and/or extracellular secretion or release.

In some embodiments, the one or more agents is LEVD-CHO. In some embodiments, the one or more agents is one or more agents that block IL-1 α expression by inhibiting PI3K includes, but is not limited to, LY294002, wortmannin, CAL-101, PI-103, or MK2206. In

some embodiments, the one or more agents are selected from the group consisting of an antibody (e.g., a polyclonal antibody, a monoclonal antibody) or antigen binding fragment thereof, a peptide (e.g., a mimetic peptide, a recombinant human or humanized peptide), an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound (e.g., a small molecule compound having a molecular weight less than approximately 5000 Daltons).

In some embodiments, the subject is a human subject. In some embodiments, the disorder is selected from the group consisting of the group including, but not limited to, ocular diseases with an obvious inflammatory basis, including keratitis, endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Stimulation of CD40 mRNA synthesis (A) and protein production (B) in hRPE cells. The hRPE cells were treated either without (Ctrl) or with IFN- γ (IFN, 500 U/ml) for 1.5, 3, 6, 24, 48, 72 (A) or 24 hr (B). A, Steady-state CD40 mRNA expression as determined by RT-PCR. The fold changes in mRNA levels were calculated by comparison with untreated control after normalization against β -actin protein (A). Proteins from whole hRPE cell lysates were subjected to Western blotting analysis by anti-CD40 antibody.

FIG. 2: Immunofluorescence analysis of CD40, integrin $\alpha 5\beta 1$, and $\alpha M\beta 5$ (CD11b) expression in hRPE cells. The hRPE cells seeded in chamber slides were incubated with or without IFN- γ (1000 U/ml), for 24 hr. hRPE cells were untreated as control (Ctrl). In isotype control (Isotype Ctrl), normal rabbit IgG (A), normal mouse IgG (B) and normal mouse IgG1 (C) were used in IFN- γ treated cells. The cells were fixed as described in Materials and Methods. Expression of CD40 (A), $\alpha 5\beta 1$ (B) and CD11b (C) appears as green. The nuclei

were stained with biobenzimide (blue). Experiments were repeated twice. Images were taken at 400X magnification.

FIG. 3: Effects of CD40L on inflammasome activation. HRPE cells were cultured with or without 5 μ g/ml CD40L for 2 (B), 4 (B), 6 (A), 8 (B, E, G, F), 20 (B, C) or 24 hr (D).

5 To determine the steady-state mRNA levels of inflammasome components caspase-1, caspase-5, NALP1, and NALP3, total RNA was isolated and subjected to RT-PCR. The Fold changes were calculated by normalization against β -actin and comparison with untreated control mRNA levels in RT-PCR (A). Two hundred micrograms of proteins from the hRPE cell lysate were analyzed in each assay using a caspase-1 assay kit. Protein content in each
10 sample was determined by BCA assay. The data were from two independent experiments, shown as fold increases in caspase-1 activity. **P<0.01 (C). The caspase-1 and -5 protein cleavage (B, D) in hRPE cells was shown by Western blot analysis. To determine CD40L-induced cleaved-caspase-1 in extracellular media, two hRPE cell lines (627 and 6538) were treated with or without CD40L followed by Western blot analysis for whole cell lysate
15 (WCL) and the proteins secreted into the extracellular media (ECM). For Co-IP pull down, anti-NALP1 (E) or anti-caspase-1 (F, G) antibodies were used, followed by Western blotting using antibodies against NALP3, caspase-1, NALP1 or caspase-5.

FIG. 4: IL-1 β expression in hRPE cells. HRPE cells were cultured with or without LPS (1 μ g/ml), TNF α (20 ng/ml) and CD40L (5 μ g/ml) for 6 (A, RT-PCR) or 24 hr (B, C and
20 D). To determine the steady-state mRNA levels of IL-1 β and IL-18, total RNA was isolated and subjected to RT-PCR. The fold changes were calculated by normalization against β -actin and comparison with untreated control LPPE mRNA levels by RT-PCR (A). In B, ATP (5 mM) was also included in parallel with LPS or TNF α alone for 4 hr. (Ctrl, control; TNF, TNF α). In C, CD40L treatments were in the presence or absence of antibodies (Abs) against
25 CD40 (5 μ g/ml), CD11b (20 μ g/ml) or α 5 β 1 (10 μ l/m) as well as corresponding isotype controls (Ctrl). After incubation, the conditioned media were harvested and subjected to high sensitivity IL-1 β ELISA. *p<0.05; **p<0.01; ***p<0.001, as compared with untreated control (B) or CD40L-stimulated control (C). To determine CD40L-induced release of
30 mature IL-1 β secretion, two retinal pigmented epithelial lines (627 and 6538) were treated with or without CD40L, followed by Western blot analysis. (WCL, Whole cell lysate; ECM, proteins secreted into the extracellular media).

FIG. 5: Modulation of CD40L-induced IL-1 β secretion in hRPE cells. HRPE cells were cultured with or without CD40L (5 μ g/ml) for 24 hr in the presence or absence of IFN- γ

(IFN, 500U/ml), IL-4 (100 ng/ml), YVAD (2 μ M) or LY294002 (Ly, 50 μ M) for 24 hr. After incubation, the conditioned media were harvested and subjected to ELISA. IL-1 β secretion in unstimulated and IFN- γ stimulated hRPE cells were detected. * p <0.05; ** p <0.01; *** p <0.001, as compared with CD40L-stimulated control.

5 FIG. 6: Effect of CD40L on MCP-1 mRNA synthesis (A) and protein secretion (B, C, D and E) by hRPE cells. The hRPE resting cells were treated with or without 500 U/ml of IFN- γ (IFN) for 72 hr (E) or 20 pg/ml of IL-1 β for 24 hr in 10% serum media, then replaced by serum-free media with 0 or 5 μ g/ml of CD40L for 6 hr (A) or replaced by 10% serum media with 0 or 5 μ g/ml of CD40L for 24 (B, C, D and E), 48 and 72 hr (D) with or without
10 anti-CD40, - α 5 β 1, -CD11b or corresponding isotype control antibodies for additional 24 hr. In E, after IFN- γ -priming, the cells were incubated with or without LY294002 (LY). The data shown represent results from a typical experiment. To determine the steady-state mRNA levels of MCP-1, total RNA was isolated and subjected to RT-PCR. The fold changes were calculated by normalization against β actin and comparison with untreated control of mRNA
15 levels in RT-PCR (A). B to E, conditioned media were harvested and analyzed by ELISA. Data represent the mean $\pm$ SEM in triplicate determinations. Data shown are a representative of three experiments.

 FIG. 7: Effects of CD40L on cellular MMP-9 mRNA expression in hRPE. RT-PCR blot of hRPE cells exposed to CD40L (5 μ g/ml), LPS (1 μ g/ml), or IL-1 beta (2ng/ml) for 8 hr.
20 Extracted RNA was subjected to appropriate cycles of amplification and probed for MMP-9 and MMP-2 expression.

 FIG. 8: Immunofluorescence analysis of IL1 α expression in hRPE cells. The hRPE cells seeded in chamber slides were incubated with or without LPS (1000 ng/ml, F), IL-1 β (2 ng/ml, E) and tunicamycin (10 μ M, D) for 20 hr. hRPE cells were untreated as control (un,
25 A). In isotype control (Isotype Ctrl), normal rabbit IgG (C) was used in IL-1 β treated cells. In addition, no primary antibody (no 1st Ab) was used in IL-1 β treated cells. (B) The cells were fixed as described in Materials and Methods. Expression of IL-1 α appears as green. In G, image showed the distribution of IL1 α in the induced whole cells with higher magnified image. The nuclei were stained with Biobenzimide (blue) (A-F). Experiments were repeated
30 twice. Typical images were taken at 400X magnification (A-F).

 FIG. 9: IL-1 α and caspase-4 mRNA expression and caspase-4 activation in hRPE cells. The hRPE cells were cultured with or without LPS (1000 ng/ml), IL-1 β (2 ng/ml), tunicamycin (10 μ M) and IFN α , β , and γ (1000U/ml) for 6 hr (A-D), and IFN β (1000 U/ml)

for 0, 16, 24 hr (E). To determine the steady-state mRNA levels of IL-1 α and caspase-4, the total RNA was isolated and subjected to RT-PCR (A-D). The caspase-4 protein cleavage in hRPE cells was shown by Western blot analysis (E).

FIG. 10: IL1 α expression and the effect caused by caspase 4 in hRPE cells. HRPE cells were cultured with or without LPS (1 μ g/ml, A), Tunicamycin (10 μ M, B), and IL1 β (2 ng/ml, C) for 24 or 48 hr as well as adding or not adding caspase-4 (LEVD, 4 μ M) or 1 (YVAD, 4 μ M) inhibitors as well as neutralization antibody against IL-1 β and its isotype control antibody. After incubation, the whole cell lysates were subjected to IL-1 α ELISA. *p<0.05; **p<0.01; ***p<0.001, as compared between treated control and treated plus caspase-1 inhibitor or caspase-4 inhibitor as well as antibody -treated cells (A-C).

FIG. 11: Effects of IFNs and their priming on LPS-induced IL-1 α protein production in hRPE cells. The hRPE cells were primed or incubated with IFN α , β , and γ (1000 U/ml) for 16 hr, respectively. The primed cells were washed and incubated with LPS (1000 ng/ml) in the presence of absence of caspase-4 inhibitor (Z-LEVD-fmk) for 24 hr. After incubation, the whole cell lysates were harvested and subjected to ELISA. *p<0.05; **p<0.01; ***p<0.001, as compared with pre-treatment or adding caspase-4 inhibitor.

FIG. 12: Ca²⁺ signaling requirement in IL-1 α expression. The hRPE cells were cultured with LPS (1000 ng/ml) (A), tunicamycin (10 μ M) (Tu, C), IL-1 β (2 ng/ml) (D), or Pam3CSK4 (100 ng/ml) (Pam, E) for 24 hr. Inhibitors for Ca²⁺ signaling used were PD150606 (100 μ M) and BAPTA-AM (5 μ M, BAPTA). and caspase-4 inhibitor, Z-LEVD-fmk (4 μ M, LEVD) was used in B. After incubation, the whole cell lysates were harvested and subjected to ELISA. *p<0.05; **p<0.01; ***p<0.001, as compared with inducers alone (A, C-D) or with single inducer (B).

FIG. 13: IL-1 α expression is highly sensitive to PI3K blockade in hRPE cells. The hRPE cells were cultured with or without LPS (1000 ng/ml), Ionomycin (Io, 3 μ M), IL-1 β (2 ng/ml), tunicamycin (Tu, 10 μ M) and Pam3CSK4 (Pam, 100 ng/ml) for 24 hr in the presence or absence of Ly294002 (Ly, 75 μ M). After incubation, the whole cell lysates were harvested and subjected to ELISA. ***p<0.001, as compared with that without Ly294002.

FIG. 14: TLRs signaling for IL-1 α expression in hRPE cells. The hRPE cells were cultured with LPS (1000 ng/ml) (A, B), Pam3CSK4 (Pam, 100 ng/ml) or LPS plus Pam3CSK4 (B, C) for 24 hr in the presence of absence of TAK-242 (TAK, 75 μ M) (A), caspase-4 inhibitor (Z-LEVD-fmk, 4 μ M) (C), neutralized antibody against TLR2 or isotype

control antibody (C). After incubation, the whole cell lysates were harvested and subjected to ELISA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, as compared with inducers alone.

FIG. 15: Effects of CU-CPT22 on IL-1 α expression. The hRPE cells were cultured with CU-CPT22 (Cu, 10, 3, 1 μ M) (A, B, C) or in combination with LPS (1000 ng/ml) or Pam3CSK4 (Pam, 100 ng/ml) (B) for 24 hr in the presence of absence of TAK-242 (TAK, 100 μ M) (A), BAPTA-AM (BAPTA, 5 μ M) (A), caspase-4 inhibitor, Z-LEVD-fmk (4 μ M, LEVD) (A), Ly 294002 (Ly, 75 μ M) (A), and neutralized anti-TLR2 antibody (Ab T2) (C) as well as its isotype control antibody (Ab Ctrl) (C). After incubation, the whole cell lysates were harvested and subjected to ELISA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, as compared with inducers alone (A, C) or un-treatment and single inducer.

DETAILED DESCRIPTION OF THE INVENTION

Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in patients over 60 years old, affecting approximately 8 million individuals in the United States (see, Klein R, et al. 2011). Various inflammatory processes have been closely linked to the pathogenesis of AMD, including complement activation, pro-inflammatory cytokine release, and oxidative injury (see, Telander DG, et al. 2011; Kinnunen K, et al. 2012). Inflammasome complex assembly was recently shown to be elevated in the human retinal pigment epithelium (HRPE) of AMD eyes with geographic atrophy (see, Tarallo V, et al. 2012). The inflammatory mediator CD40L (CD154: TNFSF5) and its receptor, CD40, have been implicated in the pathogenesis of various ocular and neurodegenerative diseases, including ischemic retinopathy (see, Portillo et al. 2008), thyroid-associated ophthalmopathy (see, TAO; Hwang et al. 2009), and Alzheimer's disease (see, Togo et al, 2000), but it has never been evaluated as a possible inducer of inflammasome assembly in any cell type. Because IL-1 β is cleaved into its active form mainly by inflammasomes, experiments conducted during the course of developing embodiments were based upon a hypothesis that CD40L may be a novel, unrecognized inducer of inflammasome assembly.

Initially found on the surface of activated T-cells, CD40L expression has now been identified on a wide variety of cells, including platelets, B-cells, mast cells, macrophages, and natural killer endothelial, and epithelial cells (see, Schönbeck et al., 2001; Elgueta et al., 2009). CD40L is a 32-39 kDa, type II transmembrane glycoprotein that belongs to the TNF

superfamily. It can be cleaved into an 18 kDa, soluble, truncated form (sCD40L) with similar biological functions and is mainly released from activated platelets (see, Hassan et al. 2012).

Originally CD40L was thought to only bind to CD40, a co-stimulatory protein found on antigen presenting cells that is essential for their activation. However, three more CD40L
5 receptors (see, Hassan et al. 2012) have been found: α II β 3 (see, Andre et al. 2002), α 5 β 1 (see, Leveille et al. 2007), and CD11b (α M β 2 or MAC-1; Zirlik et al. 2007). Of these CD40L receptors, CD40, α 5 β 1, and CD11b have all been detected on hRPE cells (see, Willermain et al. 2000; Robbins et al. 1994; Elner et al. 2003, Elner VM et al. 1981). The CD40L/CD40
10 dyad is the best characterized interaction between CD40L and its receptors, being implicated in various immune and inflammatory diseases, (see, Elgueta et al. 2009; Hassan et al. 2012) including several ocular diseases (see, Bagenstose et al. 2012; Zhao et al. 2012; Umazume et al. 2010; Brignole et al. 2000). The other CD40 receptors in the eye, α 5 β 1 and CD11b, have been associated with cell migration, adhesion, proliferation and metastasis (see, Elner SG et al. 1996).

15 Inflammasomes contain Nod-like receptors (NLR) that recognize a wide spectrum of danger- and pathogen-associated molecular patterns (DAMPs and PAMPs), respectively (see, Kannegati et al. 2007). Of the 22 members of the NLR family, NALP1 and NALP3 represent two inflammasomes that are recognized to have important roles in innate immunity and inflammation. Assembly of these inflammasomes involves the activation of caspase-1 and
20 caspase-5, which we have recently reported in hRPE cells (see, Bian, et al. 2011). The NALP1 inflammasome consists of NALP1, caspase-1, caspase-5, and an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC or PYCARD). The NALP3 inflammasome lacks caspase-5 and has an additional caspase-1. These inflammasomes mediate the proteolytic cleavage of the pro-forms of IL-1 β and IL-18 to their
25 secreted active forms (see, Lamkanfi et al. 2012). Mature IL-1 β release through inflammasome activation has been recognized as a major player in inflammasome-mediated immune responses (see, Vyleta, et al. 2012).

Experiments conducted during the course of developing embodiments for the present invention determined that CD40L induced α 5 β 1- and α M β 2-dependent hRPE NALP1 and
30 NALP3 inflammasome assembly and caspase-1 and caspase-5 activation as well as gene expression and protein secretion of IL-1 β and IL-18. CD40L induced both CD-40-dependent monocyte chemoattractant protein (MCP)-1 secretion from hRPE cells primed with γ -INF and CD40 independent MCP-1 secretion from resting hRPE cells due to autocrine/paracrine

effects of CD40L induced IL-1 β and resulting in IL-1 β secretion. In addition, such experiments elucidated novel roles for CD40L and its receptors in differentially regulating inflammatory pathways through phosphatidylinositol 3-kinase (PI 3-kinases or PI3K).

Accordingly, the present invention is concerned with the use of agents that inhibit
5 CD40L-induced inflammasome activity. Indeed, described herein are methods, compositions and pharmaceutical compositions useful in inhibiting CD40L-induced inflammasome activity without inhibiting CD40 biological activity, and in treating subjects suffering from disorders related to CD40L-induced inflammasome activity and IL-1 α expression.

The present invention is not limited to a particular manner in which such agents
10 inhibit CD40L-induced inflammasome assembly, activation and/or activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome activity without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome activation without
15 inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome activity without inhibiting interaction between CD40L and CD40. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly without inhibiting interaction between CD40L and CD40. In some embodiments, such agents inhibit CD40L-induced inflammasome activation without inhibiting interaction between CD40L and
20 CD40. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity without inhibiting CD40 biological activity through binding CD40L.

In some embodiments, such agents inhibit CD40L-induced inflammasome activity through inhibiting (e.g., blocking, preventing) interaction between CD40L (CD154) and
25 α 5 β 1 (CD49e/CD29) without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly through inhibiting (e.g., blocking, preventing) interaction between CD40L (CD154) and α 5 β 1 (CD49e/CD29) without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly through inhibiting (e.g., blocking, preventing) interaction
30 between CD40L (CD154) and α 5 β 1 (CD49e/CD29) without inhibiting CD40 biological activation. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity without inhibiting CD40 biological activity through binding α 5 β 1 (CD49e/CD29).

In some embodiments, such agents inhibit CD40L-induced inflammasome activity through inhibiting (e.g., blocking, preventing) interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18) without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly through inhibiting (e.g., blocking, preventing) interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18) without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome activation through inhibiting (e.g., blocking, preventing) interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18) without inhibiting CD40 biological activity. In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity without inhibiting CD40 biological activity through binding $\alpha_M\beta_2$ (CD11b/CD18).

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through inhibiting inflammasome related caspase-1 activation without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through inhibiting inflammasome related caspase-5 activation without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through reducing (e.g., inhibiting, preventing, hindering) inflammasome related IL-18 expression, activation and secretion without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through reducing (e.g., inhibiting, preventing, hindering) inflammasome related IL-1 β expression, activation and secretion without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through reducing (e.g., inhibiting, preventing, hindering) inflammasome related intracellular IL- α expression without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through reducing (e.g., inhibiting, preventing, hindering) inflammasome related intracellular MMP-9 expression without inhibiting CD40 biological activity.

In some embodiments, such agents inhibit CD40L-induced inflammasome assembly, activation and/or activity through reducing (e.g., inhibiting, preventing, hindering) inflammasome related PI3K signalling without inhibiting CD40 biological activity.

The present invention is not limited to particular types or kinds of agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity. In some embodiments, such agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity include, but are not limited to, an antibody (e.g., a polyclonal antibody, a monoclonal antibody) or antigen binding fragment thereof, a peptide (e.g., a mimetic peptide, a recombinant human or humanized peptide), an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound (e.g., a small molecule compound having a molecular weight less than approximately 5000 Daltons).

In some embodiments, one or more of the following agents are provided for inhibiting CD40L-induced inflammasome assembly, activation and/or activity: BD Pharmingen clone ICRF-44 anti-human CD11b mouse derived antibody, Biolegend clone ICRF-44 PE anti-human CD11b antibody, Millipore clone JBS5 – anti- $\alpha 5\beta 1$ MAB1969, and a small peptide having the following amino acid sequence EQLKKSKTL (SEQ ID NO: 1)(see, EP 2444101).

In certain embodiments, such agents are useful in treating conditions and/or disorders characterized with inflammasome activity.

For example, the present invention provides methods for preventing, attenuating, and/or treating conditions and/or disorders characterized with inflammasome activity through administering to a subject (e.g., a human subject) a composition comprising one or more agents that inhibit CD40L-induced inflammasome activity, assembly and/or activation.

In some embodiments, such conditions and/or disorders characterized with inflammasome activity include ocular conditions and/or disorders. Examples of such ocular conditions and/or disorders include, but are not limited to, keratitis, endophthalmitis, blepharitis, conjunctivitis, ocular herpetic inflammation, uveitis, multiple sclerosis, vasculitis, arteritis, orbital inflammations; or non-obvious inflammatory ocular disease with an inflammatory basis including: age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and ocular edema.

In some embodiments, such conditions and/or disorders characterized with inflammasome activity include inflammatory conditions and/or disorders. Examples of such inflammatory conditions and/or disorders include, but are not limited to, rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis,

inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

5 In some embodiments, such conditions and/or disorders characterized with inflammasome activity include cryopyrin-associated periodic syndromes. Examples of such cryopyrin-associated periodic syndromes include, but are not limited to, familial cold-induced autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal onset multisystem inflammatory disorder (NOMID) otherwise known as chronic infantile
10 neurologic cutaneous and articular (CINCA) syndrome.

 In some embodiments, such conditions and/or disorders characterized with inflammasome activity include complex or acquired inflammasomopathies. Examples of such complex or acquired inflammasomopathies include, but are not limited to, gout, pseudogout, silicosis, asbestosis, and type II diabetes mellitus.

15 In some embodiments, such conditions and/or disorders characterized with inflammasome activity include NLRP3 extrinsic inflammasopathies. Examples of such NLRP3 extrinsic inflammasopathies include, but are not limited to, family Mediterranean fever (FMF), pyogenic arthritis with pyoderma gangrenosum and acne (PAPA) syndrome, hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), and Schnitzler's
20 syndrome.

 In some embodiments, such conditions and/or disorders characterized with inflammasome activity include NLRP1 associated disorders characterized with inflammasome activity. Examples of such NLRP1 associated disorders include, but are not limited to, Vitiligo-associated multiple autoimmune disease.

25 In some embodiments, such conditions and/or disorders characterized with inflammasome activity include NOD2 associated disorders characterized with inflammasome activity. Examples of such NOD2 associated disorders include, but are not limited to, Crohn's disease, and Blau syndrome.

 In some embodiments, such conditions and/or disorders characterized with
30 inflammasome activity include NLRP12 associated disorders. Examples of such NLRP12 associated disorders include, but are not limited to, Guadeloupe variant periodic fever syndrome.

In some embodiments, the condition and/or disorder characterized by inflammasome activity is characterized by elevated plasma levels of one or more of the following: CD40L, IL-1 β , IL-18, IL-1 α , IL-1Ra, c-reactive protein, STP2, MMP-9, and/or increased inflammasome activity.

5 In some embodiments, agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity and one or more additional agents are administered to a subject (e.g., a human subject suffering from a condition and/or disorder characterized by inflammasome activity). The present invention is not limited to a particular additional agent. Examples of additional agents include, but are not limited to, a DAMP or PAMP inhibitor, 10 agents binding to TLR1, TLR2, TLR3, TLR4, TLR7 and/or TLR9, agents modulating TLR receptors, agents that stabilize lysosomes, agents that inhibit gout crystal formation, anti-oxidant agents, agents that inhibit lipid peroxide formation, pannexin channel and potassium channel modulators including spironolactone or probenecid, calcium channel inhibitors, PI3K/mTor modulators, inhibitors of interferons α , β , or γ , inhibitors of caspases 1, 4, or 5, 15 and non-steroidal agents (e.g., ibuprofen, celecoxib, ketoprofen, naproxen, piroxicam, diclofenac).

 In some embodiments, such agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity and one or more additional agents are administered to a subject (e.g., a human subject suffering from a condition and/or disorder characterized by 20 inflammasome activity) under one or more of the following conditions: at different periodicities, at different durations, at different concentrations, by different administration routes, etc. In some embodiments, the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity is administered prior to the one or more additional agents, e.g., 0.5, 1, 2, 3, 4, 5, 10, 12, or 18 hours, 1, 2, 3, 4, 5, or 6 days, or 1, 2, 3, or 4 weeks prior to 25 the one or more additional agents. In some embodiments, the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity is administered after the one or more additional agents, e.g., 0.5, 1, 2, 3, 4, 5, 10, 12, or 18 hours, 1, 2, 3, 4, 5, or 6 days, or 1, 2, 3, or 4 weeks after the administration of the additional agent. In some embodiments, the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity and 30 the additional agent are administered concurrently but on different schedules, e.g., the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity is administered daily while the additional agent is administered once a week, once every two weeks, once every three weeks, or once every four weeks. In other embodiments, the agent

for inhibiting CD40L-induced inflammasome assembly, activation and/or activity is administered once a week while the additional agent is administered daily, once a week, once every two weeks, once every three weeks, or once every four weeks.

Compositions within the scope of this invention include all compositions wherein the agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity of the present invention are contained in an amount that is effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typically, the agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity may be administered to mammals, *e.g.* humans, orally at a dose of 0.0025 to 50 mg/kg, or an equivalent amount of the pharmaceutically acceptable salt thereof, per day of the body weight of the mammal being treated. In one embodiment, about 0.01 to about 25 mg/kg is orally administered to treat, ameliorate, or prevent such disorders. For intramuscular injection, the dose is generally about one-half of the oral dose. For example, a suitable intramuscular dose would be about 0.0025 to about 25 mg/kg, or from about 0.01 to about 5 mg/kg.

The unit oral dose may comprise from about 0.01 to about 1000 mg, for example, about 0.1 to about 100 mg of the agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity. The unit dose may be administered one or more times daily as one or more tablets or capsules each containing from about 0.1 to about 10 mg, conveniently about 0.25 to 50 mg of the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity or its solvates.

In a topical formulation, the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity may be present at a concentration of about 0.01 to 100 mg per gram of carrier. In one embodiment, the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity is present at a concentration of about 0.07-1.0 mg/ml, for example, about 0.1-0.5 mg/ml, and in one embodiment, about 0.4 mg/ml.

In addition to administering the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity as a raw chemical, such an agent may be administered as part of a pharmaceutical preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the agent into preparations which can be used pharmaceutically. The preparations, particularly those preparations which can be administered orally or topically and which can be used for one type of administration, such as tablets, dragees, slow release lozenges and capsules, mouth rinses

and mouth washes, gels, liquid suspensions, hair rinses, hair gels, shampoos and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by intravenous infusion, injection, topically or orally, contain from about 0.01 to 99 percent, in one embodiment from about 0.25 to 75 percent of active mimetic peptide(s), together with the excipient.

The pharmaceutical compositions of the invention may be administered to any patient that may experience the beneficial effects of the agent for inhibiting CD40L-induced inflammasome assembly, activation and/or activity. Foremost among such patients are mammals, *e.g.*, humans, although the invention is not intended to be so limited. Other patients include veterinary animals (cows, sheep, pigs, horses, dogs, cats and the like).

The agents for inhibiting CD40L-induced inflammasome assembly, activation and/or activity and pharmaceutical compositions thereof may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, buccal, intrathecal, intracranial, intranasal or topical routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The pharmaceutical preparations of the present invention are manufactured in a manner that is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active mimetic peptides with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as

magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethyl-cellulose phthalate, are used. Dye-stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active mimetic peptide doses.

Other pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active mimetic peptides in the form of granules that may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active mimetic peptides are in one embodiment dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations that can be used rectally include, for example, suppositories, which consist of a combination of one or more of the active mimetic peptides with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules that consist of a combination of the active mimetic peptides with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the agents in water-soluble form, for example, water-soluble salts and alkaline solutions. In addition, suspensions of the active mimetic peptides as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides or polyethylene glycol-400. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The topical compositions of this invention are formulated in one embodiment as oils, creams, lotions, ointments and the like by choice of appropriate carriers. Suitable carriers

include vegetable or mineral oils, white petrolatum (white soft paraffin), branched chain fats or oils, animal fats and high molecular weight alcohol (greater than C₁₂). The carriers may be those in which the active ingredient is soluble. Emulsifiers, stabilizers, humectants and antioxidants may also be included as well as agents imparting color or fragrance, if desired.

5 Additionally, transdermal penetration enhancers can be employed in these topical formulations. Examples of such enhancers can be found in U.S. Pat. Nos. 3,989,816 and 4,444,762.

Ointments may be formulated by mixing a solution of the active ingredient in a vegetable oil such as almond oil with warm soft paraffin and allowing the mixture to cool. A
10 typical example of such an ointment is one that includes about 30% almond oil and about 70% white soft paraffin by weight. Lotions may be conveniently prepared by dissolving the active ingredient, in a suitable high molecular weight alcohol such as propylene glycol or polyethylene glycol.

The present invention provides methods for identifying agents capable of inhibiting
15 CD40L-induced inflammasome assembly, activation and/or activity. For example, in some embodiments, such methods comprise determining the binding ability of a test agent with $\alpha_M\beta_2$, CD40, and/or $\alpha 5\beta 1$. Upon identification of a test agent that binds with $\alpha_M\beta_2$, CD40, and/or $\alpha 5\beta 1$, additional properties of the test agent can be ascertained including, but not limited to, assessing the test agent's ability to inhibit intracellular inflammasome activation,
20 reduce IL-1 β or IL-18 secretion, reduce intracellular IL-1 α expression, and/or reduce cellular MMP-9 expression while not inhibiting CD40 activity. Upon identification of such a test agent that meets any of the above criteria, additional testing, optimization, experimentation can be conducted including testing with mammalian subjects.

The present invention provides kits comprising one or more agents that inhibit
25 CD40L-induced inflammasome assembly, activation and/or activity, and at least one of the following: instructions for using such an agent, and one or more additional therapeutic agents (e.g., DAMP or PAMP inhibitor, agents binding to TLR1, TLR2, TLR3, TLR4, TLR7 and/or TLR9, agents modulating TLR receptors, agents that stabilize lysosomes, agents that inhibit gout crystal formation, anti-oxidant agents, agents that inhibit lipid peroxide formation,
30 pannexin channel and potassium channel modulators including spironolactone or probenecid, calcium channel inhibitors, PI3K/mTor modulators, inhibitors of interferons α , β , or γ , inhibitors of caspases 1, 4, or 5, and anti-inflammatory agents), and instructions for using such an agent in the treatment of one or more of the following disorders: keratitis,

endophthalmitis, blepharitis, conjunctivitis, ocular herpetic inflammation, uveitis, multiple sclerosis, vasculitis, arteritis, orbital inflammations; or non-obvious inflammatory ocular disease with an inflammatory basis including: age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, ocular edema, rheumatoid arthritis, lupus, infections including sepsis, vasculitis, arthritis, dermatitis, glomerulonephritis, hepatitis, inflammatory bowel disease, erythema, autoimmune disease such as Type 1 diabetes, Graves disease, periodontitis; or non-obvious inflammatory non-ocular or systemic disease with an inflammatory basis such as: Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

One of ordinary skill in the art will readily recognize that the foregoing represents merely a detailed description of certain preferred embodiments of the present invention. Various modifications and alterations of the compositions and methods described above can readily be achieved using expertise available in the art and are within the scope of the invention.

Having now fully described the invention, it will be understood by those of skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations, and other parameters without affecting the scope of the invention or any embodiment thereof. All patents, patent applications and publications cited herein are fully incorporated by reference herein in their entirety.

EXPERIMENTAL

Example I.

This example describes expression of receptors for CD40L in hRPE.

CD40 is the classical receptor for CD40L. Both positive and negative results have been seen in literature regarding whether hRPE cells express inducible CD40 (see, Willermain et al. 2000; Kanuga et al. 2002). Experiments conducted herein investigated CD40 mRNA synthesis and protein production by RT-PCR and Western blots, respectively. Because gamma-interferon (IFN- γ) is known to be an inducer of CD40, it was used to stimulate CD40 expression by hRPE cells. After treating hRPE cells with IFN- γ for 1.5, 3, 6, 24, 48 and 72 hr, total hRPE mRNA was isolated and subjected to RT-PCR analysis. In order to compare CD40 mRNA levels under different conditions, expression of the house keeping gene β -actin was used to monitor gel loading. IFN- γ , a priming agent known to be essential

for CD40 surface display and CD40L-dependent cytokine secretion by fibroblasts (see, Gelbmann et al. 2003) significantly increased CD40 mRNA expression by hRPE cells from very low levels in instrumental, residual hRPE cells (Fig. 1A). The time course showed that CD40 hRPE mRNA induction was detectable by 3 hr of exposure and that maximal CD40 mRNA expression was sustained for 24 to 48 hr. After 72 hr incubation, CD40 mRNA expression declined. Interference of FBS in the stimulation medium was also tested. The presence of FBS did not affect the CD40 mRNA expression (Fig. 1A).

To determine CD40 protein expression by IFN- γ the whole cell lysates from the hRPE cells were subjected to Western blotting analysis. As shown in Fig. 1B, IFN- γ increased CD40 protein production.

The enhanced hRPE CD40 protein expression by IFN- γ was further confirmed by immunofluorescence microscopy as shown in Fig. 2A. hRPE cells treated with IFN- γ (1000 U/ml) showed substantial CD40 expression that contrasted with absence or very weak expression when hRPE isotype control cells stained with CD40 primary antibody or with stimulated cells stain with lack of staining isotype when control was used as primary antibody or when primary antibody was omitted. Immunofluorescence analysis also was used to examine for expression of $\alpha 5\beta 1$ and CD11b, two other receptors for CD40L, by hRPE cells. Untreated, control hRPE cells, showed consistent expression of $\alpha 5\beta 1$ and CD11b. IFN- γ treatment only very mildly increased $\alpha 5\beta 1$ and CD11b hRPE expression. Staining was negative when in their isotype control antibody was used instead of primary antibody.

Example II.

This example demonstrates activation of inflammasomes by CD40L.

The emerging role of inflammasomes in ocular diseases has just been recently recognized, for example, activation of NALP3 is reported involved in hRPE oxidative stress response as well as hRPE, IL-1 α or lipofuscin stimulation (see, Kauppinen et al. 2012; Tseng et al. 2012; Anderson et al. 2013). Thus, experiments were conducted to determine if CD40L could also be involved in inflammasome activation and IL-1 β secretion in hRPE cells. Activation of NALP3 and NALP1 inflammasome complexes are involved in recruiting of pro-caspase-1 and converting it to mature caspase-1. Additionally, NALP1 recruits pro-caspase-5, which is then cleaved to mature caspase-5. The activated inflammasome complexes containing these mature caspases are capable of cleaving pro-IL-1 β and pro-IL-18

to corresponding active forms of IL-1 β and IL-18 that can then be secreted into the extracellular space (see, Lamkanfi & Dixit, 2012).

Experiments were conducted to determine if hRPE exposure to CD40L affects mRNA expression of the key components of the NALP3 and NALP1 inflammasome complexes, including NALP3, NALP1, caspase-1 and caspase-5. As shown in Fig. 3A, CD40L increased expression of caspase-1, caspase-5, NALP3 and NALP1 mRNA by 3.4, 1.9, 3.7 and 2.2 fold, respectively.

A caspase-1 assay kit was also used to monitor catalytic activity of caspase-1 after exposure to CD40L. HRPE cells were stimulated with CD40L (5 μ g/ml) for 20 hr. By comparing CD40L-treated sample with untreated control, the increase in caspase-1 activity was 78% (Fig. 3C).

Next, cleaved caspase-1 and caspase-5 by Western blotting were tested (Fig. 3B). HRPE cells were stimulated with CD40L (5 μ g/ml) for 2, 4, 8, or 20 hr. Comparisons of CD40L-treated samples with untreated control samples at the corresponding times showed that the levels of cleaved caspase-1 and caspase-5 were increased (Fig. 3B). The pro-caspase-1 was cleaved as early as 2 hr after the stimulation by CD40L and the cleaved product remained high up to 20 hr after stimulation (Fig. 3B, D). Similarly, active caspase-5 was absent in untreated cells, but the cleaved caspase-5 increased at 8 hr after CD40L treatment and was maintained at elevated levels up to 20 hr after stimulation (Fig. 3B). Fig. 3D shows that CD40L-induced cleaved-caspase-1 was detected in two hRPE cell lines (627 and 6538), both from whole hRPE cell lysate (WCL) and the extracellular media (ECM).

To confirm the formation of NALP3 and NALP1 inflammasome complexes in the CD40L-induced hRPE cells, Co-IP approach was performed. Immobilized rabbit anti-caspase-1 antibody was used to see if anti-caspase-1 antibody could pull down NALP3 protein from NALP3 inflammasome and caspase-5 proteins from the NALP1 inflammasome in CD40L-treated and untreated hRPE cell lysates. Similarly, immobilized rabbit anti-NALP1 antibody was used to examine if NALP1 protein is coprecipitation with caspase-1 and caspase-5 of the NALP1 inflammasome. Normal rabbit IgG was used as the co-precipitation control antibody. After washing, the samples were mixed with loading buffer and resolved by SDS-PAGE, and then the gels were transferred onto nitrocellulose for Western blot analysis with mouse anti-NALP3, rabbit anti-NALP1, mouse anti-caspase-5, or mouse anti-caspase-1 antibodies, respectively. The results showed that anti-NALP1 co-precipitated with caspase-1

and -5 (Fig. 3E), and anti-caspase-1 antibody co-precipitated with NALP-3 (Fig. 3F) and caspase-5 (Fig. 3G) to a much greater degree after hRPE cells were challenged with CD40L.

Example III.

5 This example demonstrates induction of hRPE IL-1 β by CD40L.

IL-1 β mRNA has been detected in ARPE19 and primary hRPE culture (see, Jaffe et al. 1992; Planck et al. 1993; Fukuoka et al. 2003) and enhanced secretion of hRPE IL-1 β and IL-18 proteins has been reported due to oxidative stress induced by 4-hydroxynoneal, a lipid peroxidative end product (see, Kauppinen et al. 2012). Experiments were conducted which
10 first evaluated IL-1 β and IL-18 mRNA expression post challenge with 5 μ g/ml of CD40L for 6 hr. RT-PCR results showed 4.9 and 1.9 fold increases by CD40L in the synthesis of IL-1 β and IL-18 mRNA, respectively (Fig. 4A).

Next, the secreted IL-1 β protein in the conditioned media was quantified by using a highly sensitive ELISA kit. Results showed that LPS and TNF α mildly increased IL-1 β
15 secretion (Fig. 4B). In clear contrast, CD40L induced IL-1 β secretion by as high as 8 fold and was found to increase IL-18 secretion by 3-fold (Fig. 4C).

ATP-driven activation of pannexin channels is thought to facilitate cytosolic access of LPS for activation of NALP3 by a two step mechanism (see, Kanneganti et al. 2007). In the experiment, however, ATP did not significantly enhance for LPS- or TNF α -induced IL-1 β
20 secretion. Like β -amyloid and monosodium urate, CD40L is likely to induce secretion at mature IL-1 β by receptor-redirected inflammasome activation.

To delineate possible involvement of the three known CD40L receptors in this process, neutralizing antibodies against CD40, CD11b, and α 5 β 1 were next used that were detected on hRPE cells. As shown in Fig. 4C, blocking CD40L receptors by either anti-CD11b or - α 5 β 1,
25 reduced the induced IL-1 β secretion by about 50%. In clear contrast, anti-CD40 did not inhibit the CD40L-induced IL-1 β secretion. Concurrent use of blocking antibodies to α 5 β 1 and CD11b did not further inhibit IL-1 β significant differences compared to using each alone. Furthermore, Western blotting was used to demonstrated mature IL-1 beta, 17 kD existed in the extracellular media of two hRPE cell lines stimulated by CD40L (Fig. 4D).

30

Example III.

This example demonstrates modulation of CD40L-induced IL-1 β expression.

To further delineate the involvement of the inflammasome in this process, the caspase-1 inhibitor, YVAD was used to test IL-1 β secretion by CD40L. As shown in Fig. 5, YVAD reduced the induced IL-1 β secretion.

CD40L-induced IL-1 β expression in monocytes is reported to be subject to PI3K negative control (see, Schmitz et al. 2008). Therefore, it was decided to investigate the role of PI3K pathway in CD40L-induced IL-1 β secretion. In agreement with the results from monocytes (see, Schmitz et al. 2008), blocking PI3K by Ly294002 significantly elevated CD40L induction of hRPE IL-1 β secretion by 1.3 fold (Fig. 5).

As CD40L exhibits pro-inflammatory effects, whether some immune regulatory cytokines, such as IFN- γ and IL-4, might block the CD40L-mediated pro-inflammatory hRPE responses was tested. Fig. 5 shows that in contrast to enhancing IFN- γ -induced MCP-1 production by CD40L, IFN- γ inhibited CD40L-induced hRPE IL-1 β secretion by 47%. Like IFN- γ , IL-4 also inhibited CD40L-induced hRPE IL-1 β secretion by 35%.

15 **Example IV.**

This example demonstrates CD40L induced hRPE MCP-1 secretion.

It has been known that CD40L induces secretion of MCP-1 in many cell types, such as human muscle cells (see, Sugiura et al. 2000), fibroblasts (see, Koumas et al. 2001; Gelbmann et al. 2003), microglia (see, D'Aversa et al. 2002), cervical carcinoma cell line (see, Altenburg et al. 1999), and retinoblastoma cell line (see, Usui et al. 2006). CD40L was also reported to induce IL-8 in IFN- γ -primed, but not in resting, hRPE cells (see, Willermain et al. 2000). Experiments were conducted which examined effects of CD40L on MCP-1 expression in resting and IFN- γ - or IL-1 β -primed hRPE cells. Primary hRPE cultures were either untreated or primed with IFN- γ (500 U/ml) for 72 hr or IL-1 β (0.02 ng/ml) for 24 hr, and then switched to fresh CD40L-containing media in the presence or absence of anti-IL-1 β , -CD40, - α 5 β 1, or -CD11b or isotypic (control) antibodies, or the PI3K inhibitor, Ly 294002, for additional 24, 48 or 72 hr.

As shown in Fig. 6, untreated hRPE cells exhibited a consistently basic level of MCP-1 mRNA, a result consistent with our previous studies (see, Bian et al 2003; Elner et al. 1997). In a typical experiment as shown in Fig. 6A, IFN- γ or CD40L alone each doubled hRPE MCP-1 mRNA synthesis. The level of secreted MCP-1 protein by CD40L was detectable by using highly sensitive ELISA (Fig. 6B). Because IL-1 β is a potent inducer for

hrPE MCP-1 secretion (see, Elner et al. 1991), modest levels of MCP-1 secretion induced by CD40L in resting cells might be through an auto- or paracrine mechanism. To test this assumption, experiments were conducted which included anti-IL-1 β . The data showed that neutralizing IL-1 β indeed reduced the CD40L-induced MCP-1 secretion by 70%. Although
5 CD40L-CD40 pathway has been shown involved in MCP-1 secretion in many cell types expressing cell surface CD40 (see, Li et al. 2002; D'Aversa et al. 2002; Urbich et al. 2001), the finding that hrPE derived IL-1 β may stimulate via an auto/paracrine route excludes the involvement of CD40L-CD40 pathway in CD40L negative [resting] RPE cells. Furthermore, the CD40L-induced MCP-1 secretion was insensitive to blocking anti-CD40 antibody even
10 when incubation time extended to 72 hr and extracellular MCP-1 levels reached about 15ng/ml, further confirming this conclusion (Fig. 6C, D).

In contrast to resting hrPE cells, CD40L synergistically enhanced MCP-1 production in IFN- γ -primed, but not IL-1 β -primed cells (Fig. 6C). The levels of MCP-1 protein induced by CD40L post IFN- γ priming was twice as high as that resulting from pre-stimulation by
15 IFN- γ and more than the sum of induction by IFN- γ priming and CD40L alone. This combined stimulation was sensitive to blocking anti-CD40 antibody and reduced by about 36%, whereas co-incubation with either anti-CD11b or - α 5 β 1 blocking antibodies had no effect. Furthermore, as the case with MCP-1 induction by many pro-inflammatory factors (see, Bian et al. 2001), the CD40L-induced MCP-1 production in IFN- γ primed hrPE cells
20 was also sensitive to PI3K inhibition with 70% reduction in MCP-1 secretion by 50 μ M Ly294002.

Example V.

This example demonstrates the effects of CD40L on hrPE MMP-9 expression.

25 HRPE cells incubated with LPS demonstrated robust increased MMP-9 gene expression compared to control (unstimulated) cells. HRPE cells incubated with human CD40L also exhibited markedly increased MMP-9 gene expression comparable to that obtained with LPS and as great as that obtained with IL-1 beta. Baseline hrPE MMP-2 expression was not significantly affected by exposure to CD40L. Fig. 7 shows the effects of
30 CD40L on cellular MMP-9 mRNA expression in hrPE.

Example VI.

This example describes the materials and methods for Examples I-V.

Materials. Recombinant human IL-1 β , TNF- α , IFN- γ , IL-4 and CD40L were purchased from R&D Systems (Minneapolis, MN). The caspase-1 inhibitor Z-YVAD-FMK and caspase-1 colorimetric assay kit were from Clontech and BioVision (Mountain View, CA), respectively. The rabbit polyclonal antibody against CD40 was from Santa Cruz
5 Biotechnology (Santa Cruz, CA). QIAshredder and RNeasy mini kit were purchased from Qiagen (Valencia, CA.). The monoclonal anti-MCP-1 and biotinylated anti-MCP-1 antibodies were purchased from R&D Systems. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and Fisher scientific (Pittsburgh, PA).

Cell isolation and culture. The hRPE cells were isolated within 24 hr of death from
10 the donor eyes as previously described in accordance with the Helsinki agreement (see, Elner VM, 1990; Elner SG, 1992). In brief, the sensory retina tissue was separated gently from the hRPE monolayer, and the hRPE cells were removed from Bruch's membrane with papain (5 U/ml). The hRPE cells were cultured in Dulbecco's modified Eagle's /Ham's F12 nutrient mixture medium (DMEM/Ham's F12), containing 15% fetal bovine serum, penicillin G (100
15 U/ml), streptomycin sulfate (100 μ g/ml), and amphotericin B (0.25 μ g/ml) in Falcon Primaria culture plates to inhibit fibroblast growth. The hRPE monolayers exhibited uniform immunohistochemical staining for cytokeratin 8/18, fibronectin, laminin, and type IV collagen in the chicken-wire distribution characteristic for these epithelial cells (see, Bian Z, 2004; Elner SG). Cells were sub-cultured, grown to reach near confluence, and exposed to
20 the same medium, but containing reduced serum (10 or 1%) for further experiments. For each experiment at least three donors were used, with typical results shown.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). The total cellular RNA was isolated from hRPE cells by QIAshredder and RNeasy mini kit according to manufacturer's protocol. The cDNA synthesis was set up according to the
25 protocol for a reverse transcription system. Briefly, 5 μ g of RNA was added to the reaction mixture with Superscript III reverse transcriptase (200 U/ μ l) and 1 μ l Oligod(T)₂₀ (0.5 μ g/ μ l) in a total volume of 20 μ l. PCR for each product was performed with 1, 0.1 ml of the cDNA solution and three different cycles (15, 25 and 35). PCR was accepted as semiquantitative, when individual amplicates were within the mid-linear portion of the response curve.
30 Specific cDNA was amplified by 35 (0.1 μ l cDNA), and 20 cycles (1 μ l cDNA) for MCP-1 and β -actin, respectively (see, Bian Z, 2003). The condition for caspase-5 PCR was as described by Lin et al and confirmed by examining three cycles (15, 25 and 35) first and then cycle 32 (1 μ l cDNA) was selected. For CD40, IL-1 β , IL-18, caspase-1, NALP1, and

NALP3, 32 cycles and 1 µl cDNA were used. The reaction was initiated by adding 0.15 µl of Taq DNA polymerase (5 u/ml) to a final volume of 20 µl. Each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of the ethidium bromide luminescence was measured by image sensor with a computer-controlled display. The synthetic oligonucleotide primers for human CD40, IL-1β, IL-18, caspase-1, caspase-5, NALP1, NALP3, MCP-1 and IL-8 genes were as shown in Table 1.

Table 1. Primer sequences used for RT-PCR

	Forward	Reward
CD40	5'- CAGAGTTCACTGAAACGGAATG CC-3' (SEQ ID NO: 2)	5'-TGCCTGCCTGTTGCACAACC-3' (SEQ ID NO: 3)
IL-1β	5'- CTTATTACAGTGGCAATGAGGAT G-3' (SEQ ID NO: 4)	5'- CTTTCAACACGCAGGACAGGTA CA-3' (SEQ ID NO: 5)
IL-18	5'- CCAGTAGAAGACAATTGCATCA AC-3' (SEQ ID NO: 6)	5'- TCTTATTATCATGTCCTGGGACA T-3' (SEQ ID NO: 7)
Caspase-1	5'- CACCACGGCAGGCCTGGATGAT GAT-3' (SEQ ID NO: 8)	5'- TCAAACATCTGGAAATTACCTTA ATAT-3' (SEQ ID NO: 9)
Caspase-5	5'- TAGACTCTTTGCGAAAGAATCGC GTGGCTCAT-3' (SEQ ID NO: 10)	5'- CACCTCTGCAGGCCTGGACAAT G ATGAC-3' (SEQ ID NO: 11)
NALP1	5'- CAGCTGCCTGACACATCTGGACG C-3' (SEQ ID NO: 12)	5'- GACTATGCGAGGTTCTTGGGTAT C-3' (SEQ ID NO: 13)
NALP3	5'-AACAGCCACCTCACTTCCAG-3' (SEQ ID NO: 14)	5'-GACGTAAGGCCAGAATTCAC- 3' (SEQ ID NO: 15)
MCP-1	5'- GCTCATAGCAGCCACCTTCATTC- 3' (SEQ ID NO: 16)	5'-GTCTTCGGAGTTTGGGTTTGC- 3' (SEQ ID NO: 17)

To ensure that equal amounts of templates were used in each amplification reaction, human β-actin sense (5'-GTGGGGCGCCCCAGGCACCA-3'(SEQ ID NO: 18)) and anti-sense (5'-GCTCGGCCGTGGTGGTGAAGC-3'(SEQ ID NO: 19)) primers were used in parallel.

ELISA. The levels of immunoreactive MCP-1 in the hRPE supernatants were determined by modification of a double ligand ELISA method as previously described (see, Bian et al. 2004). Briefly, diluted supernatants from hRPE were added and incubated for 1 hr.

The plates were then subject to sequential incubations with biotinylated rabbit anti-MCP-1 antibodies and streptavidin–peroxidase conjugate. Chromogen substrate (OPD) was added and the plates were incubated to desired extinction and the reaction was terminated with 3M H₂SO₄. Absorbance for each well at 490 nm was read. Standards included half-log dilution of corresponding chemokines at concentrations from 1 pg to 100ng/well. This ELISA method consistently detected chemokine concentrations greater than 10pg/ml in a linear fashion. Standards included 0.5 log dilutions of rMCP-1 (R&D Systems) from 5pg to 100ng/well. For IL-β, commercial human IL-1β high sensitivity ELISA kit (eBioscience) was used to detect IL-β from 0 to 10pg/ml in culture medium. For less range of MCP-1 Endogen Human MCP-1 ELISA kit (Pierce Biotechnology, Inc. Rockford, IL) was use.

Western blotting. Cellular extracts from hRPE cells for Western blots were processed according to the manufacturer's procedure (Sigma-Aldrich). 20–50µg of protein/sample was analyzed by SDS-PAGE. Protein was electro-transferred to nitrocellulose membrane, blocked with a solution of TBS containing 5% of non-fat milk and 0.1% Tween-20 (TBST) for 1 hr, and probed with primary antibodies overnight, followed by washing three times with TBST. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature, and washed three additional times with TBST. The membrane was then visualized using an enhanced chemiluminescent technique (ECL).

Co-immunoprecipitation (Co-IP). After removing the medium and washing hRPE cells with PBS, dithiobis[succinimidyl]propionate] (DSP) (Thermo Scientific, Rockford, IL) was used for intra-cellular crosslinking. Then, Pierce classic IP Kit (Thermo scientific) was used. Briefly, cell lysates were generated using ice-cold non-denaturing lysis buffer in the presence of Halt™ proteinase and phosphatase inhibitor cocktail (Thermo Scientific). The total protein of the lysates was measured by BCA protein assay kit (Sigma-Aldrich). A total of more than 100 µg proteins per sample were used for Co-IP experiments. All lysates were pre-cleared using the control agarose resin. Following pre-clear step, 2 µg affinity purified rabbit anti-human caspase-1 antibody (Bio Vision) and 4 µg affinity purified rabbit anti-human NALP1 antibody (Abcam) were combined with each pre-cleared cell lysates for overnight incubation. Next, the antibody/lysates samples were added to protein A/G plus agarose in the spin column with gentle end-over-end mixing for 1 hr. The resin was washed and the protein complexes bound to the antibody were eluted. The sub-sequent Western blot analyses were performed as described above. Proteins were separated by 4-15% gradient SDS-PAGE and transferred onto nitrocellulose membranes using a mini Trans Blot Cell (Bio-

Rad). Human NALP3 and NALP1 were detected by a mouse anti-human NALP3 (Abcam) and rabbit anti-NALP1 antibody (Abcam). Caspase-1 and casapase-5 were detected by mouse anti-human caspase-1 (Santa Cruz) and mouse anti-human caspase-5 (Thermo Scientific), respectively.

5 Immunofluorescence analysis of CD40 in hRPE cells. HRPE cells were plated in four-chamber glass slides (Lab-Tek, Polylabo, Strasbourg, France) in the indicated medium. After incubation for 48 h at 37 °C in a cell culture incubator, medium was aspirated and the adherent cells were fixed with 4% paraformaldehyde for 15 min. Then fixed cells were blocked in PBS solution containing 0.1% Triton X-100, 10% sheep serum and 5% BSA at
10 room temperature for 60 min. After three washes with 1% normal goat serum, then the cells were either incubated with or without primary antibody anti-CD40 (Santa Cruz Biotechnology, Inc), -CD11b (BioLegend), isotype control mouse IgG1 (BioLegend), - $\alpha 5\beta 1$ (Millipore), or non-immune rabbit IgG (Abcam) overnight. Next day, the cells were treated with secondary fluorescein isothiocyanate (FITC)-conjugated antibody, and diluted in PBS
15 solution containing 2% sheep serum and 1% BSA, at room temperature for 60 min in a humidified dark chamber, followed by two time washes with PBS solution. Finally, the cells were incubated with 1:10,000 Bis-benzimide for 2 min and washed with PBS again. The slides were mounted with prolong anti-fade kit mount (Molecular Probe, Inc) and sealed. The slides were examined under a fluorescence microscope equipped with an argon-krypton laser
20 with blue light for FITC excitation under 400X magnification.

Statistic analysis. Each experiment was confirmed by testing samples from three independent hRPE cell lines, thus results were representative of at least three independent experiments. For ELISA and functional assays, the results were representative of three independent experiments with similar results with each data point in triplicate. Various assay
25 conditions were compared using ANOVA and t-test by Statview software, and $p < 0.05$ was considered to be statistically significant. In ELISA and functional assays, values represent means $\pm$ SEM. In Western blots and RT-PCR data only results from one representative experiment were selected for the figures.

30 **Example VII.**

 Human RPE cells, located at the blood-retina burrier, are important immune-regulatory cells that play important key roles in innate and adaptive immunity involved in a variety of retinal pathologic processes. RPE cells and infiltrating leukocytes produce

inflammatory cytokines that are essential mediators of the innate immune response within the ocular microenvironment, either sterile or non-sterile inflammatory retinal diseases. The sterile inflammatory processes contribute to the pathogenesis of some retinal diseases. For example, in diabetic retinopathy, upregulation of IL-1 and caspase-1 activity occurs in retinal capillary cells (see, Kern TS, 2007; Mohrs, 2002). Various inflammatory processes have been closely linked to the pathogenesis of AMD, and emerging evidence suggests that inflammasome activation plays pivotal roles in the proinflammatory responses by RPE cells and involved in AMD pathogenic process (see, Klein R, et al. 2011; Doyle SL, 2012; Tseng WA, 2013).

More recently, Kayagaki N (2011) et al. found that in the murine system that cytosolic recognition of Gram-negative bacteria required the presence of functional caspase-11 in order to trigger NLRP3 inflammasome activation. To distinguish it from the previously known inflammasome pathway which is caspase-11-independent, this novel, caspase-11-dependent route of inflammasome engagement has been termed non-canonical inflammasome activation. It was also shown that the cytosolic delivery of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, was both necessary and sufficient to trigger non-canonical inflammasome activation (see, Kayagaki N 2013; Hagar JA, 2013). In humans, caspase-4 and caspase-5 have been thought to be the orthologs of rodent caspase-11. It has been previously demonstrated that caspase-4 is dually involved in hRPE pro-inflammatory and pro-apoptotic responses. Various pro-inflammatory stimuli and ER stress induce hRPE caspase-4 mRNA synthesis, protein production and activation (see, Bian 2009). Several reports have demonstrated that human caspase-4 mediates noncanonical inflammasome (see, Casson CN, 2015; Schmid-Burgk JL, 2015; Vigano E, 2015; Shi J, 2015).

Previous studies have shown RPE cells to be ideal targets for infectious agents (see, Moyer AL, 2008, p1358; Detrick B, 2001, p163; Nagineni CN, 2000, p407; Vann VR, 1991, p2462). Pathogen replication and elaboration of toxin by these agents can induce pro-IL-1 α production and may cause cell death. Multiple research groups have revealed that activators of caspase-1 can promote secretion of mature IL-1 α (see, Lukens JR, 2014]. In contrast, other studies suggest that calpain-like activities rather than inflammasomes are required for pro-IL-1 α processing (see, Lukens JR, 2014]. In non-hematopoietic cells either constitutively expressed or induced IL-1 α proteins mainly remain inside the cells, however upon membrane

disruption the intracellular IL-1 α is released into effected area, triggering inflammatory responses.

IL-1 α is one of pro-inflammatory, pleiotropic cytokines involved in inflammation and immunity. It belongs to a unique dual-function cytokine group and characterized by functioning both as membrane receptor agonist and transcription factor-like nuclear protein (see, Rider et al. 2013). IL-1 α is a central driver of immune responses generated in tissue damage, such as trauma, ischemia-reperfusion injury, hypoxia and cell pyroptosis, necrosis as well as apoptosis. It causes many inflammatory diseases (see, Cohen I, 2010). IL-1 α is released by cells and serves as an “alarmin” molecule to recruit other immune cells to the site of injury. IL-1 α can also lead to potent upregulation of inflammatory cytokines including proIL-1 β , IL-6, and TNF- α , followed by IL-1R-mediated NF- κ B signaling in nearby cells. IL-1 α has been proposed to be an apical initiator of autoinflammatory responses due to the fact that it does not require upregulation or processing to provoke inflammation once it is released (see, Lukens JR 2014). The released pro-IL-1 α and mature IL-1 α are both proinflammatory functional forms.

In as early as 1991, Jaffe GJ et al found that proinflammatory stimuli induce hRPE cells to accumulate, but not secrete IL-1 α . In the rat, IL-1 α was detected in the vitreous by vinpocetine treatment (see, Liu RT, 2014). However, so far no follow-up studies have been reported regarding IL-1 α expression, regulation and signaling pathways involved in hRPE cells. The following experiments demonstrated that multiple signaling pathways converge at PI3K to produce IL-1 α expression. Elevated intracellular Ca²⁺, activation of caspase-4 and caspase-1, secondary IL-1 β autocrine pathway are all involved in LPS-stimulated IL-1 α expression in hRPE cells.

Materials. Recombinant human IL-1 β was purchased from R&D System (Minneapolis, MN). IL-1 α antibody was from Abcam. Caspase-4 inhibitor Z-LEVD-fmk, toll-like receptor 4 (TLR4) inhibitor TAK-242, calpain calcium binding blocker PD150606, TLR2 antagonist CU-CPT22 were from EMD Minipore (Billerica, MA). Caspase-1 and -4 antibodies and caspase-1 inhibitor Z-YVAD-FMK were from BioVision (Mountain View, CA) and Clontech (Mountain View, CA), respectively. Recombinant human interferon gamma (IFN γ), human IL-1 α ELISA kit, and M-PER Mammalian Protein Extraction Reagent were obtained from Thermo Scientific (Rockford, IL). Recombinant INF α and β were obtained from PBL Aassay Science (Piscataway, NJ). QIAshredder and RNeasy mini kit were purchased from Qiagen (Valencia, CA.). TLR2 ligand Pam3CSK4 was purchased from Invivo

Gen (San Diego, CA). Ionomycin was from Cell Signaling (Danvers, MA). Tunicamycin, LPS, BAPTA-AM, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and Fisher scientific (Pittsburgh, PA).

Cell isolation and culture. The hRPE cells were isolated within 24 hr of death from the donor eyes as previously described in accordance with the Helsinki agreement (see, Elner VM, 1990; Elner SG, 1992). In brief, the sensory retina tissue was separated gently from the hRPE monolayer, and the hRPE cells were removed from Bruch's membrane with papain (5 U/ml). The hRPE cells were cultured in Dulbecco's modified Eagle's /Ham's F12 nutrient mixture medium (DMEM/Ham's F12), containing 15% fetal bovine serum, penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml), and amphotericin B (0.25 µg/ml) in Falcon Primaria culture plates to inhibit fibroblast growth. The hRPE monolayers exhibited uniform immunohistochemical staining for cytokeratin 8/18, fibronectin, laminin, and type IV collagen in the chicken-wire distribution characteristic for these epithelial cells (see, Bian 2004; Elner SG). Cells were sub-cultured, grown to reach near confluence, and exposed to the same medium, but containing reduced serum (10 or 1%) for further experiments. For each experiment at least two donors were used, with typical results shown.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). The total cellular RNA was isolated from hRPE cells by QIAshredder and RNeasy mini kit according to manufacturer's protocol. The cDNA synthesis was set up according to the protocol for a reverse transcription system. Briefly, 5 µg of RNA was added to the reaction mixture with Superscript III reverse transcriptase (200 U/µl) and 1 µl Oligod(T)₂₀ (0.5 µg/µl) in a total volume of 20 µl. PCR for each product was performed with 1, 0.1 ml of the cDNA solution and three different cycles (15, 25 and 35). PCR was accepted as semiquantitative, when individual amplicates were within the mid-linear portion of the response curve. Specific cDNA was amplified by 35 (1 µl cDNA), and 20 cycles (1 µl cDNA) for IL-1α and β-actin, respectively (see, Bian z, 2003). The condition for caspase-4 PCR was as described by Lin et al ³ and confirmed by examining three cycles (15, 25 and 35) first and then cycle 32 (1 µl cDNA) was selected. The reaction was initiated by adding 0.15 µl of Taq DNA polymerase (5 u/ml) to a final volume of 20 µl. Each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of the ethidium bromide luminescence was measured by image sensor with a computer-controlled display.

Table 2. Primer sequences used for RT-PCR

	Forward	Reward
IL-1 α	5'- CAGAAGACCTCCTGTCCTAT GAGG-3' (SEQ ID NO: 20)	5'-GTCAGGCATATTGGTGAGGCTGAC- 3' (SEQ ID NO: 21)
Caspase -4	5'- CAGACTCTATGCAAGAGAAG CAACGTATGGCAGGA-3' (SEQ ID NO: 22)	5'- CACCTCTGCAGGCCTGGACAATGATG AC-3' (SEQ ID NO: 23)

To ensure that an equal amount of templates was used in each amplification reaction,
 5 human β -actin sense (5'-GTGGGGCGCCCCAGGCACCA-3' (SEQ ID NO: 18)) and anti-
 sense (5'-GCTCGGCCGTGGTGGTGAAGC-3' (SEQ ID NO: 19)) primers were used in
 parallel.

ELISA. For IL- α , commercial human IL-1 α ELISA kit was used to detect IL- α from 0
 to 400 pg/ml in whole cell lysates. Briefly, hRPE cultures were washed by 4⁰C PBS then
 10 added by M-PER Mammalian Protein Extraction Reagent with Halt Protease Inhibitor
 Cocktail and PMSF. Next, whole cell lysates were harvested after centrifugation at 4⁰C,
 14000rpm for 15 minutes. The plates were added by sample and reconstituted standards. The
 following steps were sequential incubations with biotinylated antibody reagent and
 streptavidin–HRP solution. Chromogen substrate (TMB) was added and the plates were
 15 incubated to desired extinction and the reaction was terminated with 2M H₂SO₄. Absorbance
 for each well at 450 nm was read and corrected at 550nm.

Western blotting. Cellular extracts from hRPE cells for Western blots were processed
 according to the manufacturer's procedure (Sigma-Aldrich, St. Louis, MO). 20–50 μ g of
 protein/sample was analyzed by SDS-PAGE. Protein was electro-transferred to nitrocellulose
 20 membrane, blocked with a solution of TBS containing 5% of non-fat milk and 0.1% Tween-
 20 (TBST) for 1 hr, and probed with primary antibodies overnight, followed by washing three
 times with TBST. Next, the membranes were incubated with horseradish peroxidase-
 conjugated secondary antibody for 1 hr at room temperature, and washed three additional
 times with TBST. The membrane was then visualized using an enhanced chemiluminescent
 25 technique (ECL).

Immunofluorescence analysis of IL-1 α in hRPE cells. The hRPE cells were plated in four-chamber glass slides (Lab-Tek, Polylabo, Strasbourg, France) in the indicated medium. After incubation for 20 hr at 37 °C in a cell culture incubator, medium was aspirated and the adherent cells were fixed with 4% paraformaldehyde for 15 min. Then fixed cells were
5 blocked in PBS solution containing 0.1% Triton X-100, 10% sheep serum and 5% BSA at room temperature for 60 min. After three washes with 1% normal goat serum, then the cells were either incubated with or without primary antibody anti-IL-1 α (Santa Cruz Biotechnology, Inc), -CD11b (BioLegend), isotype control mouse IgG1 (BioLegend), - α 5 β 1 (Millipore) or non-immune rabbit IgG (Abcam) overnight. Next day, the cells were treated
10 with secondary fluorescein isothiocyanate (FITC)-conjugated antibody, and diluted in PBS solution containing 2% sheep serum and 1% BSA, at room temperature for 60 min in a humidified dark chamber, followed by two time washes with PBS solution. Finally, the cells were incubated with 1:10, 000 Bisbenzimidazole for 2 min and washed with PBS again. The slides were mounted with prolong antifade kit mount (Molecular Probe, Inc) and sealed. The
15 slides were examined under a fluorescence microscope equipped with an argon-krypton laser with blue light for FITC excitation under 40X magnification.

Statistic analysis. Each experiment was confirmed by testing samples from two or three independent hRPE cell lines, thus results were representative of two or three independent experiments. For ELISA and functional assays the results were representative of
20 three independent experiments with similar results with each data point in triplicate. Various assay conditions were compared using ANOVA and t-test by Statview software, and $p < 0.05$ was considered to be statistically significant. In ELISA and functional assays values represent means $\pm$ SEM. In Western blots and RT-PCR data only results from one representative experiment were selected to show in figures.

Proinflammatory agents and ER stress increased IL-1 α expression

IL-1 α expression was investigated in resting and stimulated hRPE cells by pro-inflammatory agents and ER stress inducer tunicamycin. A group of known pro-inflammatory agents including IL-1 β , LPS, and tunicamycin, were selected for immunofluorescence
30 analysis of intracellular IL-1 α protein in resting and stimulated hRPE cells. The hRPE cells were treated with IL-1 β (2 ng/ml), LPS (1000 ng/ml), and tunicamycin, (10 μ M) for 20 hr.

Immunofluorescence staining showed constitutively expressed basal levels of IL-1 α (Fig. 8A), in contrast to the negative immunostaining in no primary antibody or isotype

serum controls (Fig. 8B and C). Upon stimulation, IL-1 α proteins were markedly increased and existed in both cytosol and nucleus (Fig. 8D-G).

In parallel, after treating hRPE cells with LPS, tunicamycin, IL-1 β and IFN α , β , and γ for 6 hr, the total mRNA was isolated and subjected to RT-PCR analysis. In order to compare IL-1 α mRNA levels under different conditions, expression of the house keeping gene β -actin was used to monitor gel loading. As shown in Fig. 9 (A-D), LPS, tunicamycin and IL-1 β significantly increase hRPE IL-1 α mRNA synthesis (Fig. 9A-C). Compare with negative effect by type II IFN γ on IL-1 α expression, the type I IFN α and IFN β increased hRPE IL-1 α mRNA obviously (Fig. 9A, B, D). LPS and IFN β have synergistic effect on hRPE IL-1 α mRNA synthesis (Fig. 9A). Opposite with this result, IFN γ has no effect on hRPE IL-1 α mRNA synthesis by LPS (Fig. 9B). All IFNs all increased hRPE IL-1 α mRNA synthesis (Fig. 9D).

Next, the IL-1 α protein was tested in the hRPE whole cell lysates. The nearly confluent hRPE cells were challenged with or without LPS, tunicamycin and IL-1 β for 24 or 48 hrs, the condition medium and whole cell lysates were collected for IL-1 α ELISA. There was no detectable levels (>2 pg/ml) of IL-1 α protein found in the condition medium. The LPS (1000 ng/ml), tunicamycin (10 μ M) and IL-1 β (2 ng/ml) increased the IL-1 α protein production up to 17, 38, and 72 pg/ml, respectively. The induction by LPS and tunicamycin was transient, the induction peaked at 24 hr post stimulation, and dropped to about half of the peak value at 48 hr post stimulation (Fig. 10A, B).

The involvement of caspase-4 in IL-1 α expression by pro-inflammatory agents and ER stress inducer

Caspase-4 is intracellular LPS receptor with high specificity and affinity (see, Shi et al. 2015). Caspase-4 mediated noncanonical inflammasome activation is thought to play important roles in IL-1 α release (see, Casson et al. 2015; Vigano et al. 2015) and interaction with caspase-1 (see, Kajiwarra et al. 2014; Gross et al. 2012; Fettrlschoss et al. 2011). To investigate the roles of those caspases on IL-1 α expression, nearly confluent hRPE cells were stimulated by LPS (1000 ng/ml), tunicamycin (10 μ M) and IL-1 β (2ng/ml) in the presence or absence of caspase-4 inhibitor (Z- LEVD-fmk, 4 μ M) and caspase-1 inhibitor (YVAD-cmk, 4 μ M) for 24. The whole cell lysate ELISA showed that the LPS-, tunicamycin- and IL-1 β -inductions were 52, 36 and 27% reduced by Z- LEVD-fmk, and 47, 34 and 5 % by respectively (Fig. 10A, B, C).

The partial blockade of induced IL-1 α expression by caspase-1 inhibitor implicates that activation of caspase-1 is involved. One of major function of caspase-1 is to cleave pro-IL-1 β to produce secretion of mature IL-1 β . As IL-1 β is a potent inducer of IL-1 α (Fig. 10C), thus the caspase-1-dependent increase in IL-1 α could be due to the secondary effect though
 5 autocrine signaling by induced and secreted IL-1 β . To test this possibility IL-1 β neutralizing antibody was added before stimulating the cells with LPS which we demonstrated to cause mild induction of to mild IL-1b in hRPE cells. As shown in Fig. 10A, IL-1 β neutralization antibody blocked LPS-induced IL-1 α by 20%.

10 INFs priming augments LPS-induced IL-1 α expression

It has been shown that INF γ is able to ‘prime’ macrophage to evoke more rapid and heightened responses to LPS (see, Schroder et al. 2006). It is interesting to examine the roles of INFs for IL-1 α expression in hRPE cells. The cells were treated with INFs alone or in combination with LPS. When using alone, only type I INF α and INF β , but not type II INF γ ,
 15 induced IL-1 α mRNA synthesis (Fig. 9D). As a result, co-culture with INF α and INF β , but not INF γ , further enhanced LPS-induction (Fig. 9A and B). Meanwhile both type of INFs increased caspase-4 mRNA (Fig. 9D). Moreover, Western blots showed that INF β transiently induced pro-caspase-4 protein production and cleavage 16 hr post stimulation (Fig. 9E).

Next, intracellular IL-1 α protein production with IL-1 α ELISA kit was tested. Primary
 20 hRPE cultures were either un-treatment or primed with IFN α and β at 1000 U/ml for 16 hrs, then switched to fresh LPS-containing media for another 24 hrs. The whole cell lysates were subjected to ELISA. As shown in Fig. 11A, B), neither type I IFN α and IFN β priming, nor type II INF γ priming alone were able to stimulate IL-1 α protein production. The priming with type 1 IFN, IFN α and IFN β , all increase hRPE intracellular IL-1 α product under LPS
 25 induction by 1.5-, 1.9-fold of LPS treatment alone. It is interesting that, priming with IFN γ also resulted in 2.2-fold increase in that by LPS alone (Fig. 11B). In addition, the presence of caspase-4 blocker Z-LEVD-fmk eliminated 44 and 34% of IL-1 α production induced by pre-treatment of IFN β and INF γ , respectively.

30 Ca²⁺ signaling is required for intracellular IL-1 α expression

Ca²⁺ as an ubiquitous second messenger, is intricately involved in a wide spectrum of physiological functions, including signal transduction, secretion of proteins and gene expression. Increase in [Ca²⁺] is required for endocytosis of LPS receptor TLR4, a process

triggering the switchgear from pro-inflammatory to anti-inflammatory signaling pathways (see, Siegemund et al. 2012). To investigate the role of Ca^{2+} signaling in IL-1 α expression in hRPE cells, BAPTA-AM, a cytosolic Ca^{2+} chelator, ionomycin, a calcium ionophore, and PD150606, a blocker for calcium binding to calpain. Calpain is a calcium-dependent, non-lysosomal cysteine protease, involved in LPS signaling (see, Cui et al. 2013), TLR4 internalization (see, Siegemund et al. 2012) and pro-IL-1 α cleavage (see, Sultana et al. 2003), were employed. Primary hRPE cultures were either un-treated or treated by LPS, tunicamycin, IL-1 β and TLR1/2 ligand Pam3CSK4 in the presence or absence of inhibitors for 24 hr. Then whole cell lysates were subjected to ELISA assays. As shown in Fig. 12, BAPTA-AM reduced the LPS-, tunicamycin-, IL-1 β - and Pam3CSK4-induced IL-1 α expression induced by 68 (A), 96 (C) and 35 (D), 30% (E) respectively. Treating the cells with ionomycin induced higher levels of IL-1 α production as that of by LPS (Fig. 12B). The effect by PD 150606 exhibited only 13% inhibition.

Phosphatidylinositol-3-OH-kinase (PI3K) is essential for IL-1 α expression in hRPE cells.

PI3K has been known as one of key players for internalizing LPS receptor TLR4 and balancing pro- and anti-inflammatory TLR4 signaling (see, Siegemund et al. 2012). Therefore, experiments were conducted to determine if PI3K is involved in induced IL-1 α expression in hRPE cells. Ly294002 was used to confirm PI3K-mediated signaling. First, nearly confluent hRPE cells were pre-incubated with or without Ly294002 (75 μM) for 1 hr. Then, the cells were challenged with all the IL-1 α inducers used in this study, including LPS (1000 ng/ml), ionomycin (3 mM), IL-1 β (2 ng/ml), tunicamycin (10 μM) and Pam3CSK4 (100 ng/ml) for 24 hrs. The whole cell lysates were collected for IL-1 α ELISA. The results demonstrated that all the induced IL-1 α protein production by all tested inducers was almost completely abrogated (Fig. 13).

Involvement of TLR4 and TLR2 in IL-1 α expression

TLRs signaling play vital roles in the induction of the innate immune response. LPS and Pam3CSK4 are known ligands of TLR4 and TLR2, respectively. Expression of TLR4 and TLR2 has been reported in hRPE cells (see, Nazari et al. 2014). To further confirm the involvement of these two TLRs in IL-1 α expression, the TLR4 blocker TAK-22, neutralizing antibody to TLR2 and TLR2/1 dimerization blocker CU-CPT22 (Cheng et al. 2012), was

further used. First, the nearly confluent hRPE cells were pre-incubated with TAK-242 (75 μ M) for 0.5 hr. Then, hRPE cells were challenged with LPS (1000 ng/ml) for 24 hr. The whole cell lysates were collected for IL-1 α ELISA. The results showed that the TAK-242 blocked LPS-induced IL-1 α expression by about 55% (Fig. 14A). Pam3CSK4 alone (100
5 ng/ml) induced IL-1 α expression to the levels comparable to that by LPS. In addition Pam3CSK4 also enhanced LPS/TLR4-mediated IL-1 α expression, although the total induction was less than the sum of that by LPS and Pam3CSK4 alone (Fig. 14B). The Pam3CSK4-induced IL-1 α was reduced by caspase-4 inhibitor Z-LEVD-fmk and neutralizing antibody to TLR2 by 50 and 43 %, respectively (Fig. 14C).

10 Cu-CPT22 is a recently developed small molecule which can compete with the synthetic triacylated lipoprotein (Pam3CSK4) for binding to TLR1/2 with high potency and specificity. (see, Cheng K, 2012). Therefore, it was decided to use this compound to further confirm the role of TLR2 signaling for IL-1 α production in hRPE cells. However, it was unexpectedly found that Cu-CPT22 alone moderately induced IL-1 α expression and this
15 induction was totally eliminated by LY294002 (Fig. 15A). By contrast, BAPTA, TAK-242 and Z-LEVD-fmk did not result in statistically significant change in CU-CPT-22 induction. Moreover, CU-CPT22 in synergy with both LPS and Pam3CSk4 induced IL-1 α production by 4- and 3-fold, respectively. This synergistic effect fro Cu-CPT22 was in a dose-dependent manner at concentrations from 1 to 10 μ M. Neutralizing antibody to TLR2 inhibited CU-
20 CPT22 by 23% (Fig. 15C), which was less than that blocked for Pam3CSK4 induction (43%, Fig. 14C).

INCORPORATION BY REFERENCE

The entire disclosure of each of the patent documents and scientific articles referred to
25 herein is incorporated by reference for all purposes.

Each of the following references are herein incorporated by reference in their entireties:

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CLAIMS**What Is Claimed Is:**

- 5 1. A method for inhibiting inflammasome activity, assembly and/or activation in a subject, comprising administering to the subject a composition comprising one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity.
2. The method of Claim 1, wherein the one or more agents inhibit CD40L-induced
10 inflammasome activity, assembly and/or activation without inhibiting CD40 biological activity.
3. The method of Claim 1, wherein the one or more agents inhibit CD40L-induced inflammasome activity, assembly and/or activation through one or more of the following
15 mechanisms:
 - without inhibiting interaction between CD40L and CD40;
 - through binding CD40L;
 - through inhibiting interaction between CD40L (CD154) and $\alpha 5\beta 1$ (CD49e/CD29);
 - through binding $\alpha 5\beta 1$ (CD49e/CD29);
 - 20 through inhibiting interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18);
 - through binding $\alpha_M\beta_2$ (CD11b/CD18);
 - through inhibiting inflammasome related caspase-1 activation;
 - through inhibiting inflammasome related caspase-5 activation;
 - through reducing inflammasome related IL-18 expression, activation and secretion;
 - 25 through reducing inflammasome related IL-1 β expression, activation and secretion;
 - through reducing or increasing inflammasome related intracellular MMP-9 expression;
 - through reducing or increasing inflammasome related PI3K signaling.
- 30 4. The method of Claim 1, wherein the one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity are selected from the group consisting of an antibody or antigen binding fragment thereof, a peptide, an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound,

wherein the antibody is a polyclonal antibody or a monoclonal antibody,
wherein the peptide is a mimetic peptide, a recombinant human, or a humanized peptide.

5 5. The method of Claim 1, wherein the one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity are selected from BD Pharmingen clone ICRF-44 anti-human CD11b mouse derived antibody, Biolegend clone ICRF-44 PE anti-human CD11b antibody, Millipore clone JBS5 – anti- $\alpha 5\beta 1$ MAB1969, and a small peptide having the following amino acid sequence EQLKKSKTL (SEQ ID NO: 1).

10

6. The method of Claim 1, wherein the subject is a human subject.

7. The method of Claim 1, wherein the subject is a human subject suffering from a condition and/or disorder characterized by inflammasome activity.

15

8. The method of Claim 7, wherein the condition and/or disorder characterized by inflammasome activity is selected from

the group including, but not limited to, ocular diseases with an obvious inflammatory basis, including keratitis, endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from:

25 the group of systemic diseases with an, lupus, infections obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, 30 diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

9. A method for preventing, attenuating or treating a disorder related to inflammasome activity in a subject, comprising administering to the subject a composition comprising one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity.

5 10. The method of Claim 9, wherein the one or more agents inhibit CD40L-induced inflammasome activity, assembly and/or activation without inhibiting CD40 biological activity.

11. The method of Claim 9, wherein the one or more agents inhibit CD40L-induced
10 inflammasome activity, assembly and/or activation through one or more of the following mechanisms:

without inhibiting interaction between CD40L and CD40;

through binding CD40L;

through inhibiting interaction between CD40L (CD154) and $\alpha 5\beta 1$ (CD49e/CD29);

15 through binding $\alpha 5\beta 1$ (CD49e/CD29);

through inhibiting interaction between CD40L (CD154) and $\alpha_M\beta_2$ (CD11b/CD18);

through binding $\alpha_M\beta_2$ (CD11b/CD18);

through inhibiting inflammasome related caspase-1 activation;

through inhibiting inflammasome related caspase-5 activation;

20 through reducing inflammasome related IL-18 expression, activation and secretion;

through reducing inflammasome related IL-1 β expression, activation and secretion;

through reducing or increasing inflammasome related intracellular MMP-9
expression;

through reducing or increasing inflammasome related PI3K signaling.

25

12. The method of Claim 9, wherein the one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity are selected from the group consisting of an antibody or antigen binding fragment thereof, a peptide, an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound,

30 wherein the antibody is a polyclonal antibody or a monoclonal antibody,

wherein the peptide is a mimetic peptide, a recombinant human, or a humanized peptide.

13. The method of Claim 9, wherein the one or more agents that inhibit CD40L-induced inflammasome assembly, activation and/or activity are selected from BD Pharmingen clone ICRF-44 anti-human CD11b mouse derived antibody, Biolegend clone ICRF-44 PE anti-human CD11b antibody, Millipore clone JBS5 – anti- $\alpha 5\beta 1$ MAB1969, and a small peptide
5 having the following amino acid sequence EQLKKSKTL (SEQ ID NO: 1).

14. The method of Claim 9, wherein the condition and/or disorder characterized by inflammasome activity is selected from the group consisting of:

ocular diseases with an obvious inflammatory basis, including keratitis,
10 endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from:
15 the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease,
20 diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

15. The method of Claim 9, wherein the disorder characterized by inflammasome activity is characterized by elevated plasma levels of one or more of the following: CD40L, IL-1 β , IL-18, IL-1 α , IL-1Ra, c-reactive protein, STP2, MMP-9, and/or increased inflammasome
25 activity.

16. The method of Claim 9, wherein the composition is co-administered with one or more additional agents.

30 17. The method of Claim 16, wherein the one or more additional agents are selected from the group consisting of a DAMP or PAMP inhibitor, agents binding to TLR1, TLR2, TLR3, TLR4, TLR7 and/or TLR9, agents modulating TLR receptors, agents that stabilize lysosomes, agents that inhibit gout crystal formation, anti-oxidant agents, agents that inhibit

lipid peroxide formation, pannexin channel and potassium channel modulators including spironolactone or probenecid, calcium channel inhibitors, PI3K/mTor modulators, inhibitors of interferons α , β , or γ , inhibitors of caspases 1, 4, or 5, and anti-inflammatory agents selected from non-steroidal agents such as ibuprofen, celecoxib, ketoprofen, naproxen, piroxicam, and
5 diclofenac.

18. A method for identifying a potential agent capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity, comprising

a) assessing the binding ability of a test agent with $\alpha_M\beta_2$, CD40, and/or $\alpha_5\beta_1$;

10 b) assessing the test agent's ability to inhibit intracellular inflammasome activation, reduce IL-1 β or IL-18 secretion, reduce intracellular IL-1 α expression, and/or reduce or increase cellular MMP-9 expression while not inhibiting CD40 activity;

c) identifying the test agent as a potential agent capable of inhibiting CD40L-induced inflammasome assembly, activation and/or activity if the test agent is capable of one
15 or more of the following:

binding with $\alpha_M\beta_2$, CD40, and/or $\alpha_5\beta_1$ while not inhibiting CD40 activity;

inhibit intracellular inflammasome activation while not inhibiting CD40 activity,

reducing IL-1 β or IL-18 secretion, reducing intracellular IL-1 α expression while not inhibiting CD40 activity, and

20 reducing or increasing cellular MMP-9 expression while not inhibiting CD40 activity.

19. A pharmaceutical composition comprising a composition as recited in Claim 1 or 9.

20. A kit comprising (1) a pharmaceutical composition as recited in Claim 19, (2) a
25 container, pack, or dispenser, and (3) instructions for administration.

21. A method for inhibiting or stimulating IL-1 α expression in a subject, comprising administering to the subject one or more agents that block or stimulate caspase-4 activation and/or one or more agents that block or stimulate IL-1 α expression by inhibiting or
30 stimulating PI3K and/or inhibiting or stimulating IL-1 α expression by inhibiting or stimulating intracellular Ca ion signaling or Ca ion levels.

22. The method of Claim 21, wherein the one or more agents that block caspase-4 activation includes LEVD-CHO.

23. The method of Claim 21,

5 wherein the one or more agents that inhibit or stimulate IL-1 α expression includes inhibiting PI3K with LY294002, wortmannin, CAL-101, PI-103, or MK2206.

24. The method of Claim 21, wherein the one or more agents that inhibit or stimulate IL-1 α expression by blocking or stimulating caspase-4 activation and/or inhibit or stimulate IL-1 α expression by inhibiting or stimulating PI3K and/or inhibiting or stimulating IL-1 α expression by inhibiting or stimulating intracellular Ca ion signaling or Ca ion levels are selected from the group consisting of an antibody or antigen binding fragment thereof, a peptide, an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound, wherein the antibody is a polyclonal antibody or a monoclonal antibody,

10 wherein the peptide is a mimetic peptide, a recombinant human, or a humanized peptide.

25. The method of Claim 21, wherein the subject is a human subject.

20 26. The method of Claim 21, wherein the subject is a human subject suffering from a condition and/or disorder characterized by IL-1 α expression due to caspase-4 and/or PI3K activation and/or increasing intracellular Ca or blocking Ca signaling.

27. The method of Claim 25, wherein condition and/or disorder characterized by IL-1 α expression by caspase-4 and/or PI3K activation and/or increasing intracellular Ca or blocking Ca signaling is selected from the group consisting of:

the group of ocular diseases with an obvious inflammatory basis, including keratitis, endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from

the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious
5 inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease, diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

28. A method for preventing, attenuating, or treating a disorder related to IL-1 α activity by inhibiting or stimulating caspase-4 and/or PI3K activation and/or increasing intracellular
10 Ca or blocking Ca signaling in a subject, comprising administering to the subject a composition comprising one or more agents that inhibit or stimulate IL-1 α expression, and/or intracellular levels or signaling and/or extracellular signaling and/or extracellular secretion or release.

15 29. The method of Claim 21, wherein the one or more agents includes LEVD-CHO.

30. The method of Claim 21, wherein the one or more agents is one or more agents that block IL-1 α expression by inhibiting PI3K includes LY294002, wortmannin, CAL-101, PI-103, or MK2206.

20

31. The method of Claim 21, wherein the one or more agents includes wortmannin, CAL-101, PI-103, or MK2206.

25 32. The method of Claim 21, wherein the one or more agents includes ionomycin or calpain, PD150506, or BAPTA-AM.

33. The method of Claim 28, wherein the one or more agents are selected from the group consisting of an antibody or antigen binding fragment thereof, a peptide, an aptamer, a peptibody, an adnectin, a nucleic acid, or a small molecule compound,
30 wherein the antibody is a polyclonal antibody or a monoclonal antibody,
wherein the peptide a mimetic peptide, a recombinant human, or a humanized peptide.

34. The method of Claim 28, wherein the subject is a human subject.

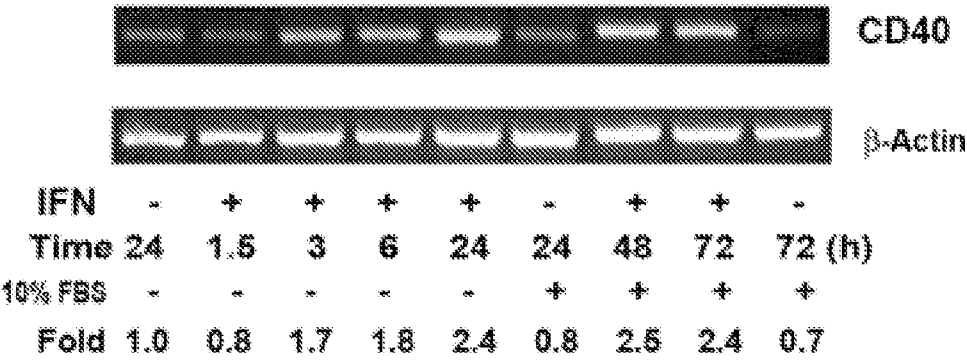
35. The method of Claim 28, wherein the condition and/or disorder is selected from the group consisting of:

the group of ocular diseases with an obvious inflammatory basis, including keratitis,
5 endophthalmitis, blepharitis, conjunctivitis, scleritis, herpetic inflammation, uveitis, vasculitis, arteritis, orbital inflammations, optic neuritis, sympathetic ophthalmia, retinitis, and other autoimmune diseases; or non-obvious inflammatory ocular diseases with an inflammatory basis including, age-related macular degeneration, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and corneal, uveal, or retinal edema; or from:

10 the group of systemic diseases with an obvious inflammatory basis, including various types of arthritis including rheumatoid arthritis including sepsis, vasculitis, dermatitis, glomerulonephritis, hepatitis, periodontitis, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, Graves disease and other autoimmune diseases; or non-obvious inflammatory systemic diseases with an inflammatory basis including, Alzheimer's disease,
15 diabetes, atherosclerosis, heart failure, obesity, and metabolic syndrome.

FIG. 1

A



B

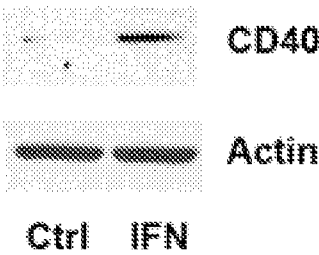


FIG. 2A

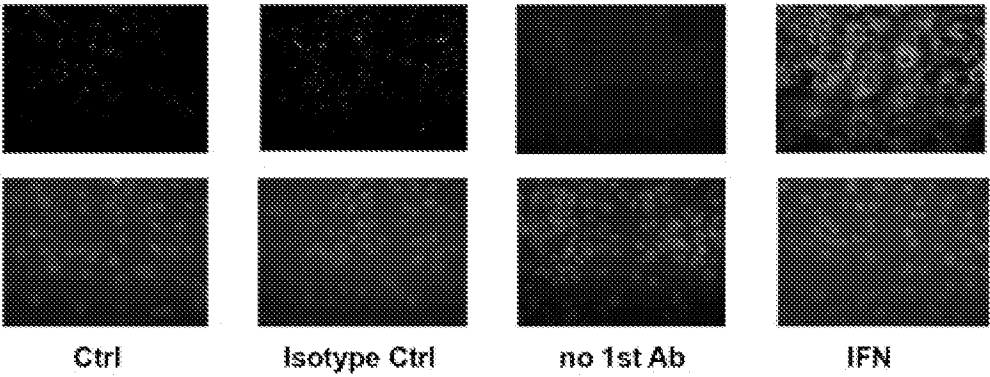
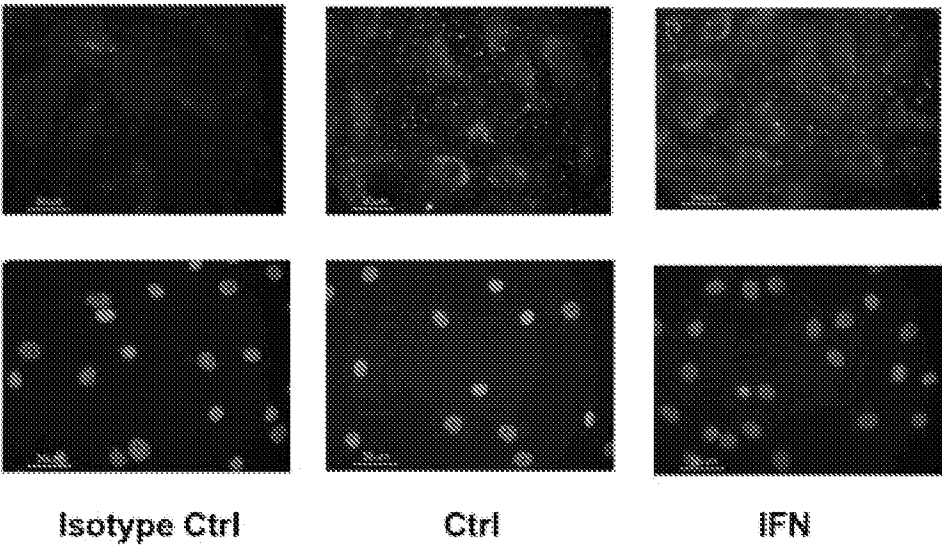


FIG. 2B



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FIG. 2C

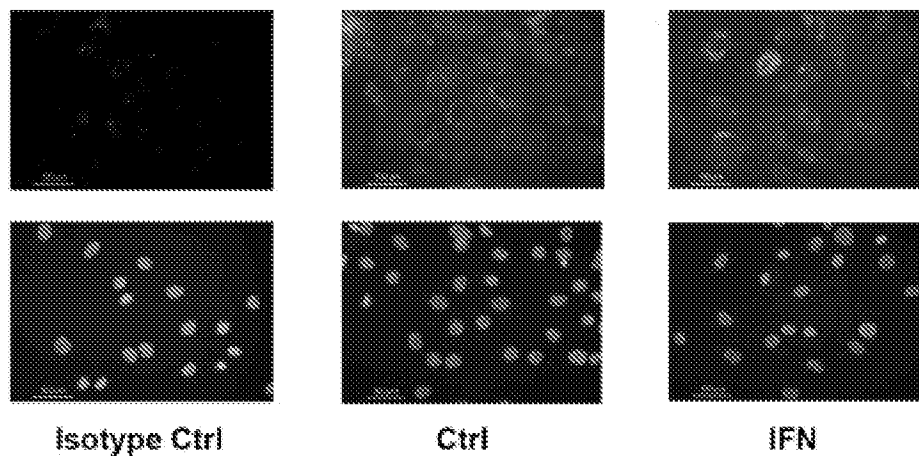


FIG. 3A

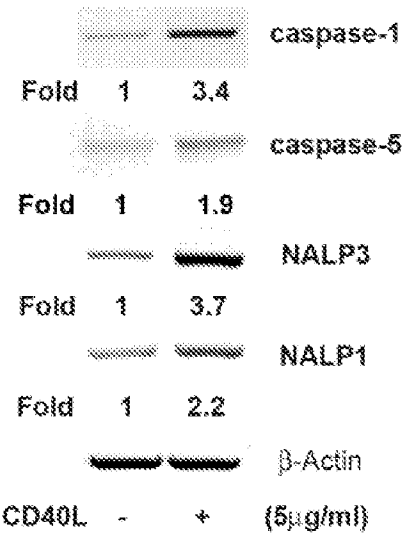


FIG. 3B

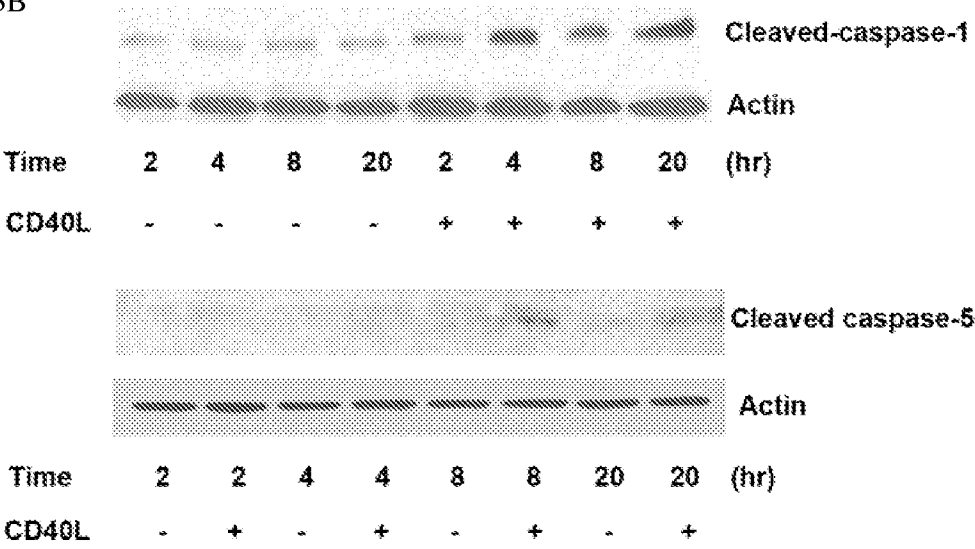


FIG. 3C

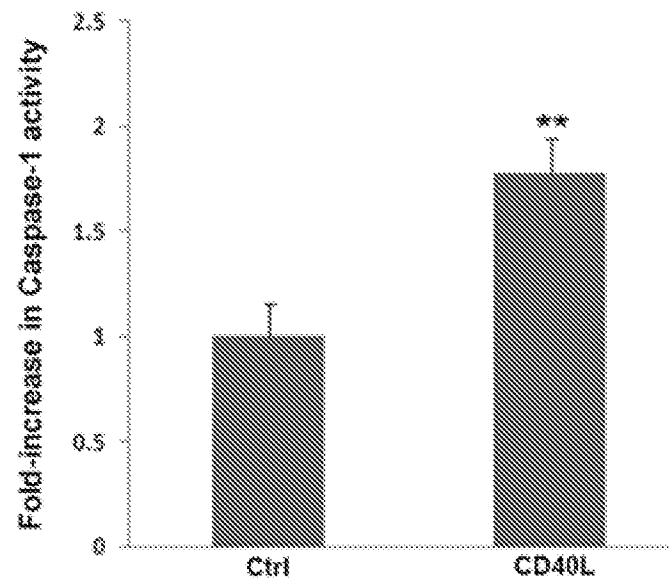


FIG. 3D

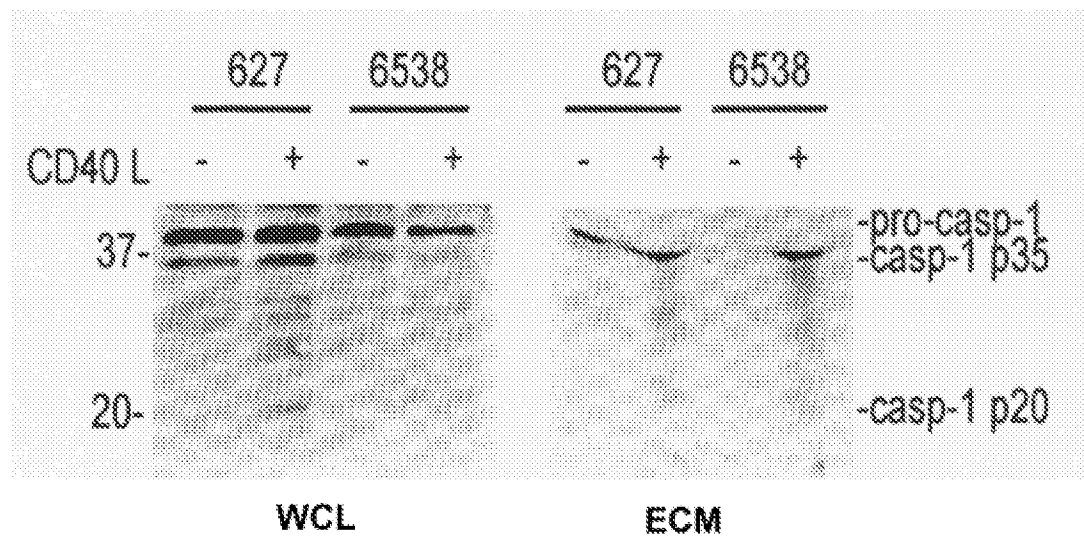


FIG. 3E

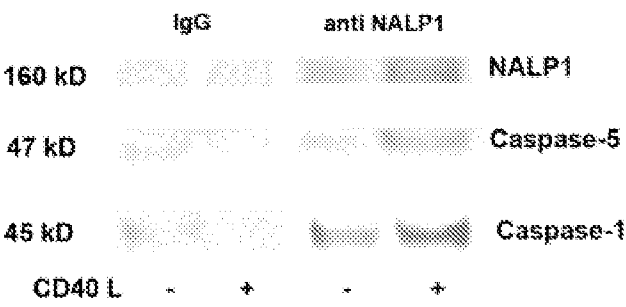


FIG. 3F

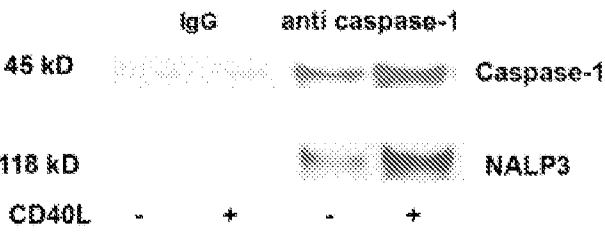
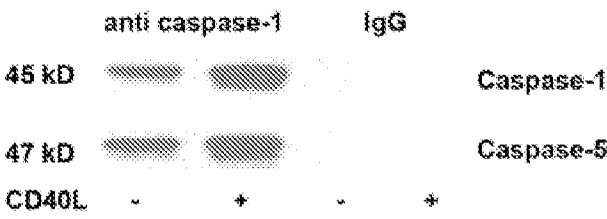


FIG. 3G



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FIG. 4A

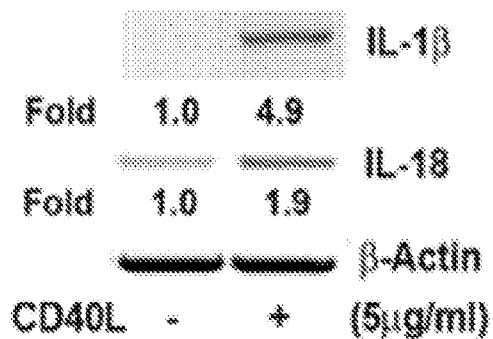


FIG. 4B

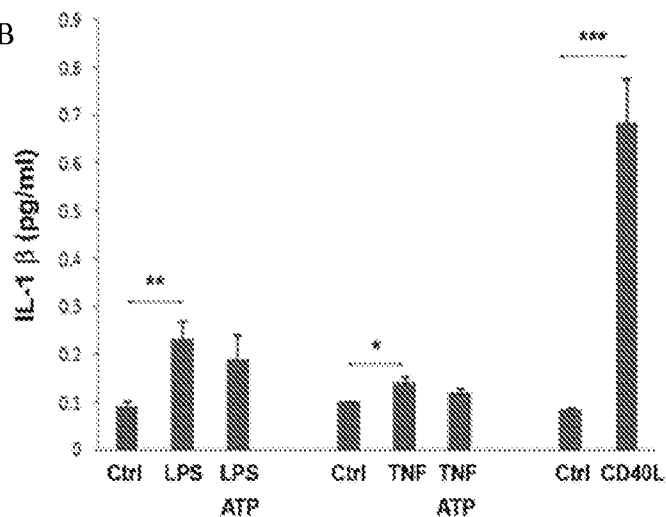


FIG. 4C

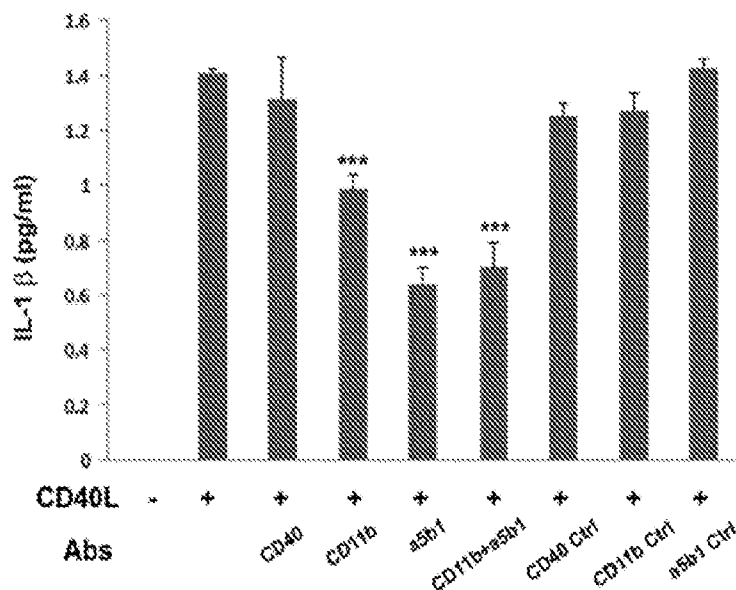
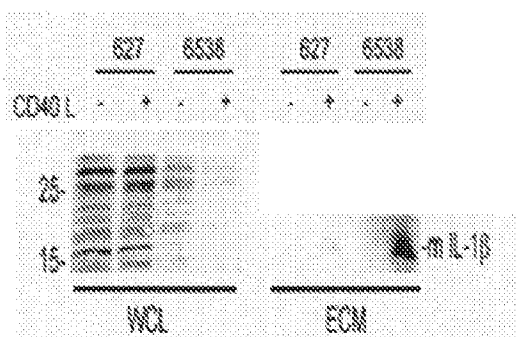
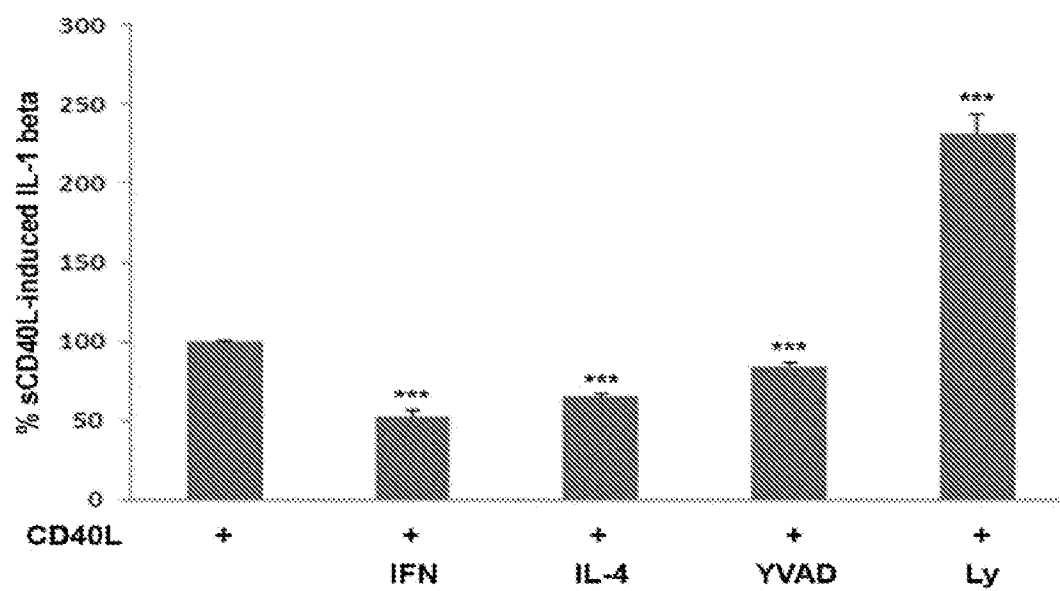


FIG. 4D



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FIG. 5



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FIG. 6A

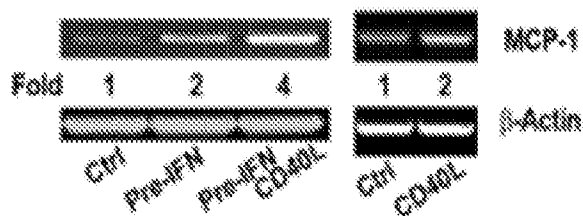


FIG. 6B

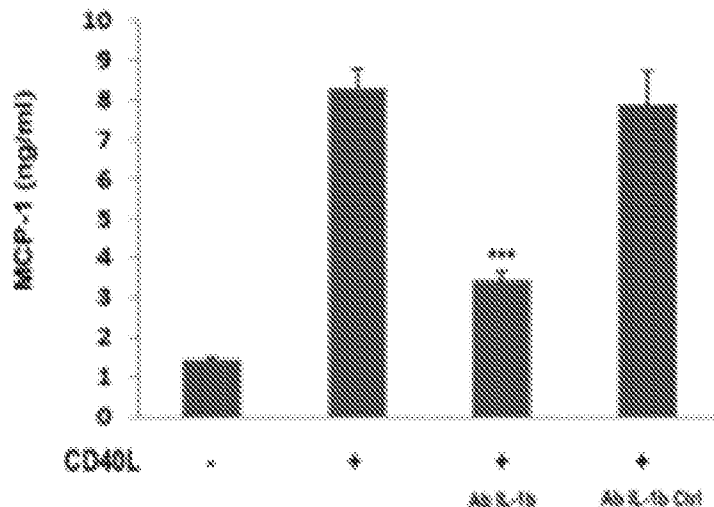


FIG. 6C

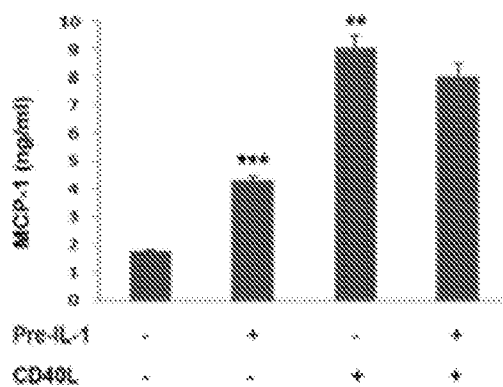


FIG. 6D

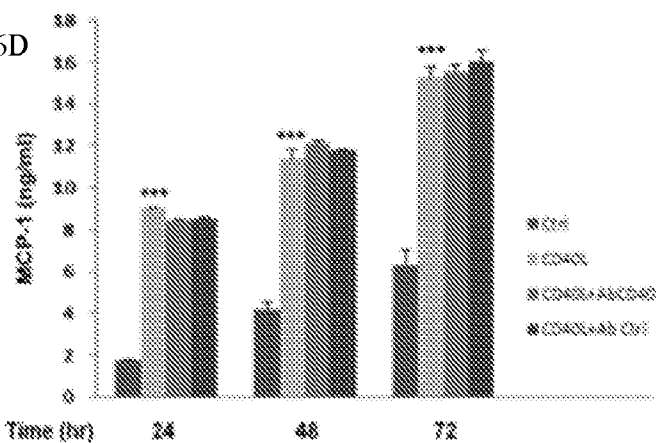


FIG. 6E

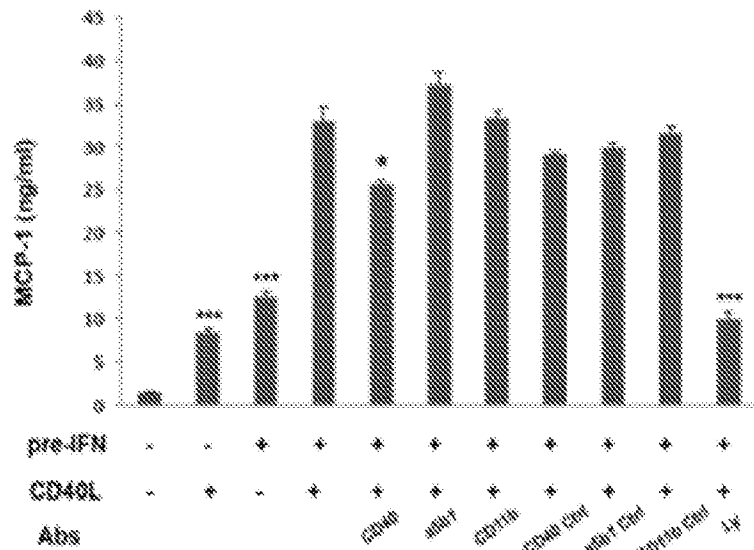


FIG. 7

MMP9 mRNA expression in hRPE cells

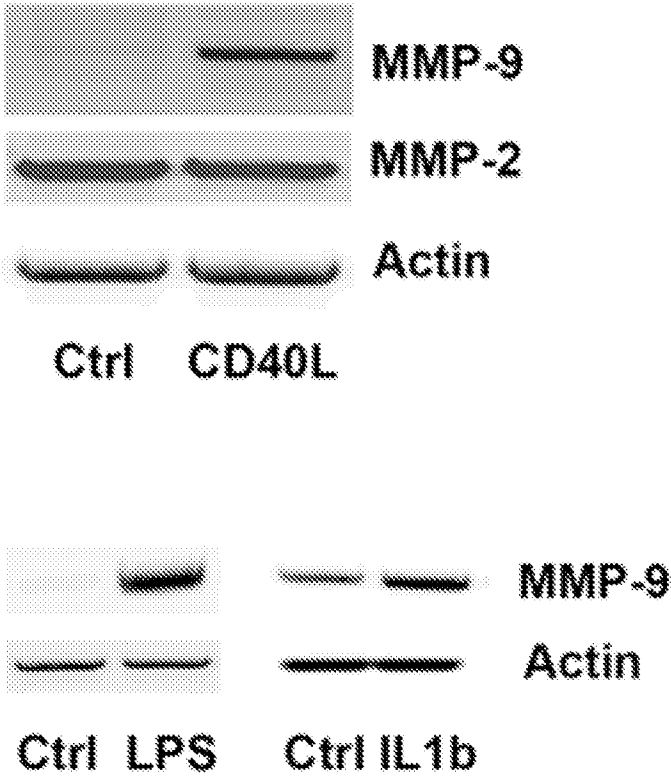
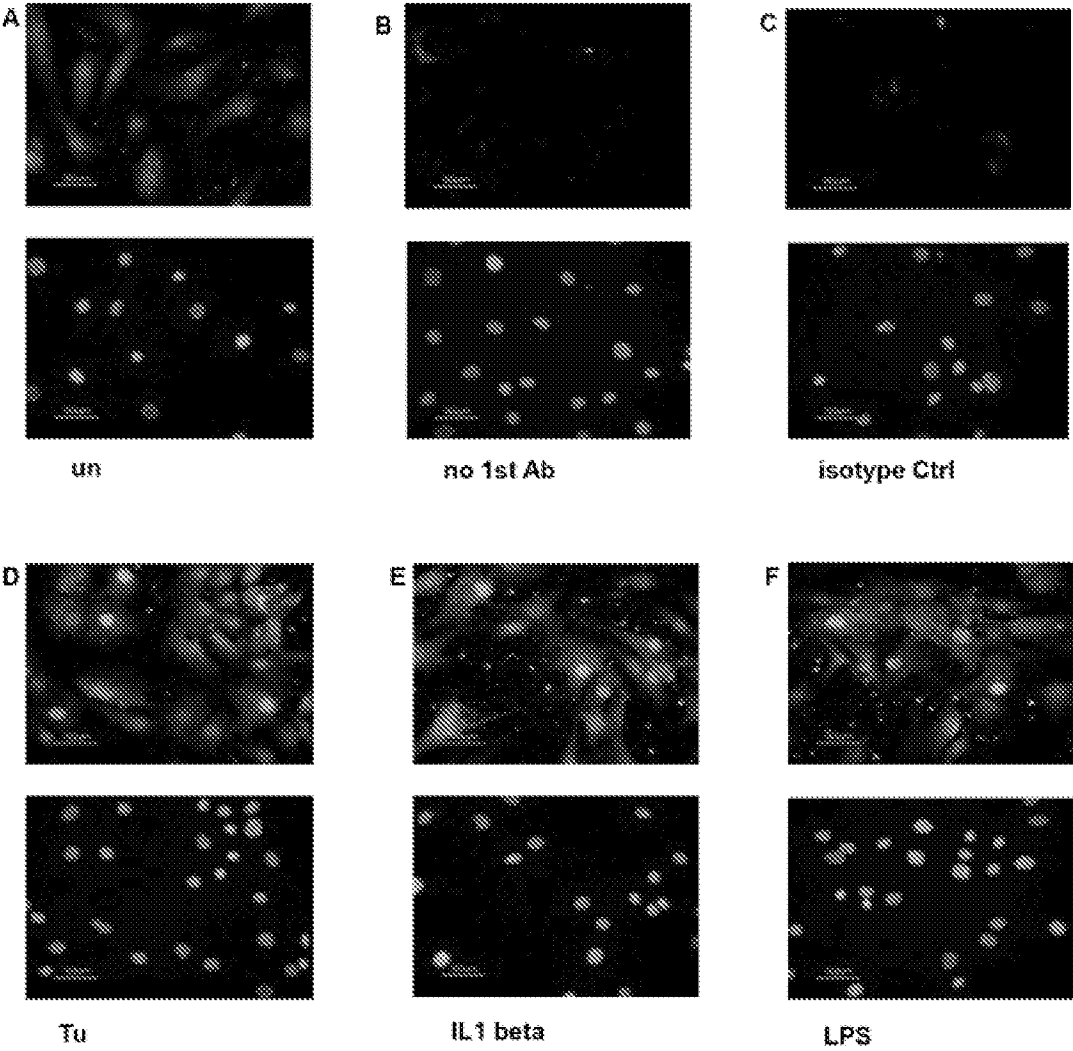


FIG. 8



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FIG. 8G

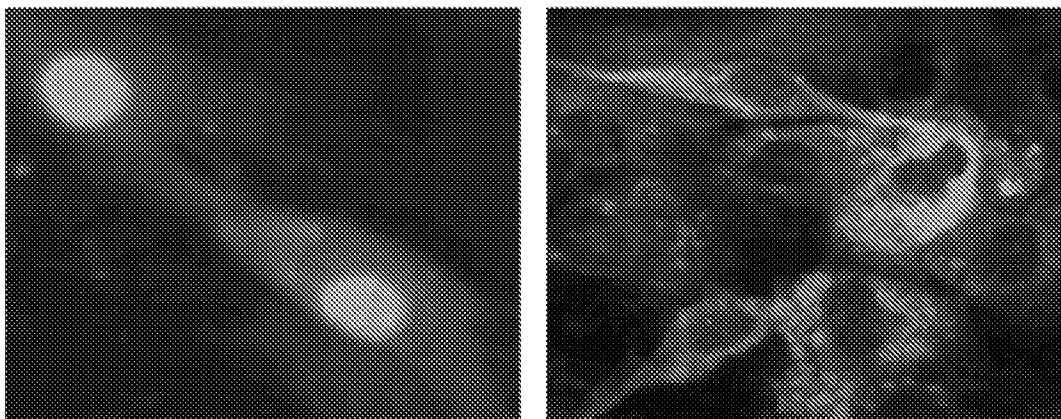


FIG. 9

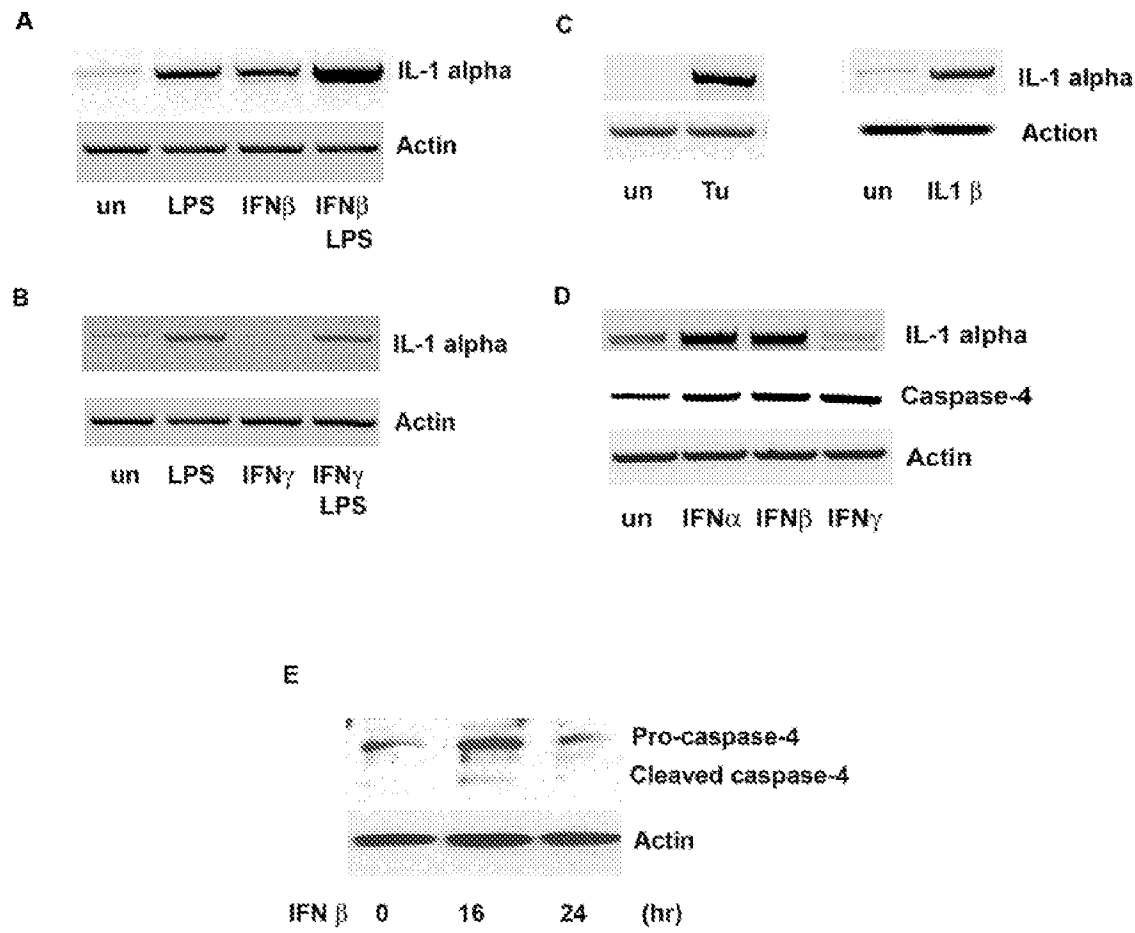


FIG. 10A

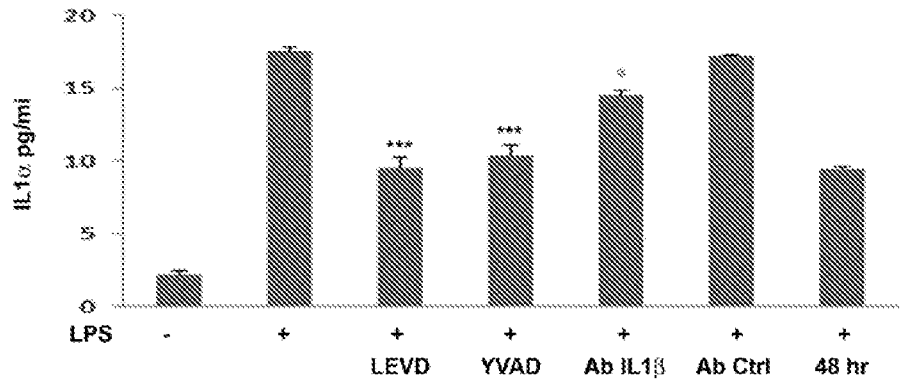


FIG. 10B

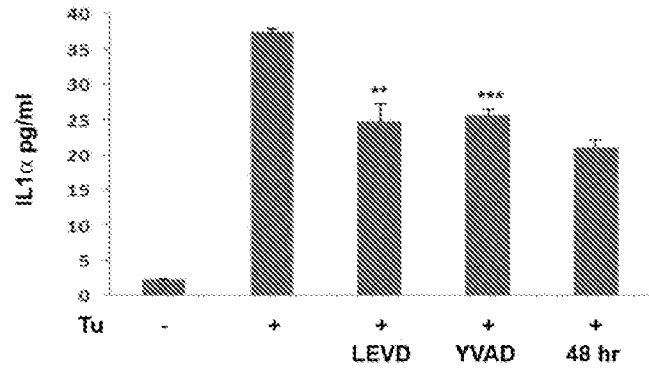


FIG. 10C

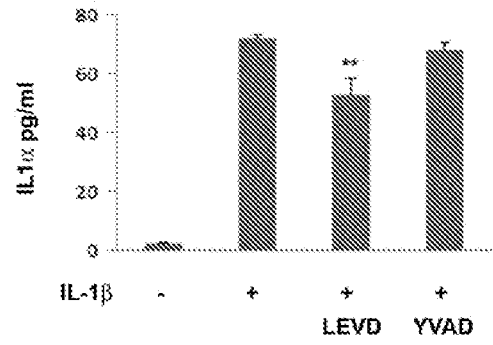
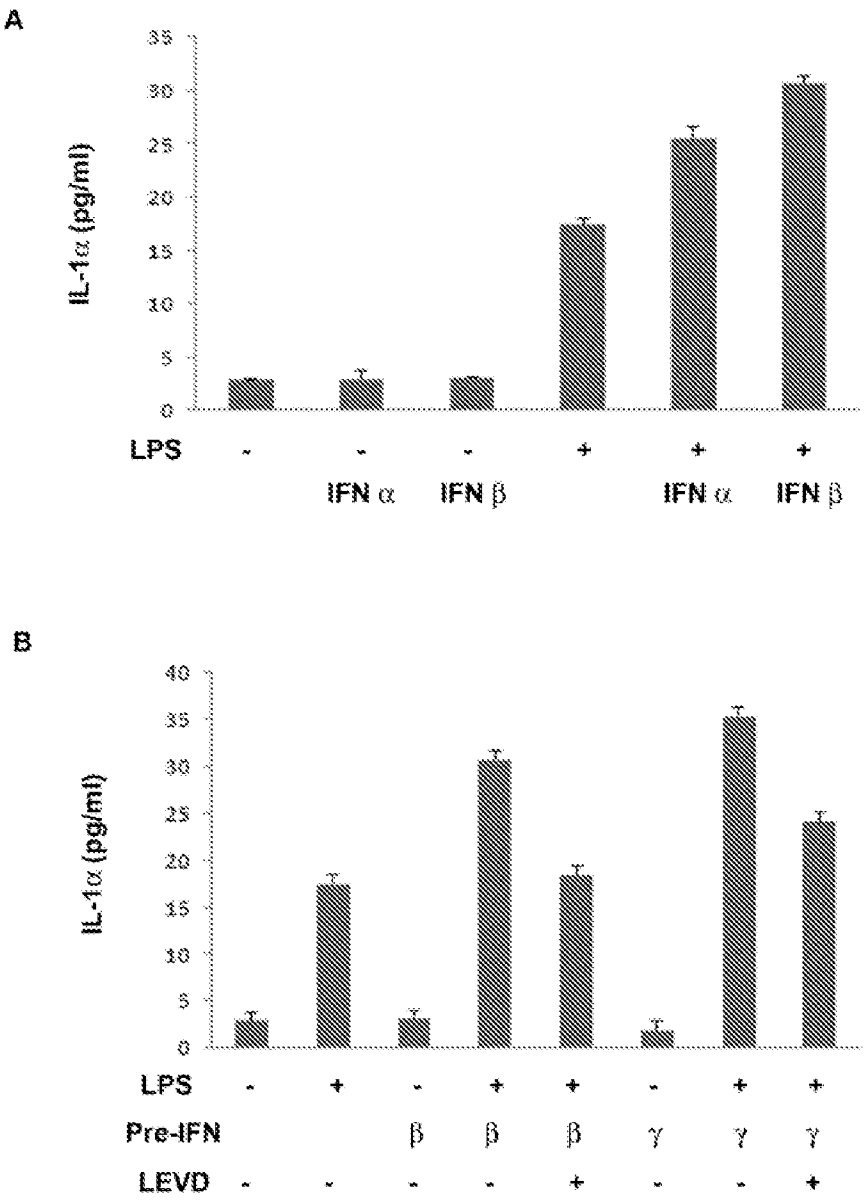


FIG. 10D

Laboratory Results - IL-1alpha expression in RPE cytosol			
60214	ELISA Results	pg/5x10 ⁶	Average
untreated cells	Inhibitor	2.44	2.22
		2.22	
		1.99	
CD40L 5 ug/ml		11.08	10.55***
		11.08	
		9.49	
CD40L 5 ug/ml	LEV D	10.40	9.34
		10.17	
		7.44	
CD40L 5 ug/ml	YVAD	8.58	7.82*
		7.44	
		7.44	
LPS 1000 ng/ml		17.89	17.66
		17.66	
		17.43	
LPS 1000 ng/ml	LEV D	9.26	8.5***
		8.12	
		8.12	
Tu 1 uM		22.43	21.89
		22.21	
		21.30	
Tu 1uM	LEV D	11.08	11.38***
		11.30	
		11.76	

FIG. 11



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FIG. 12A

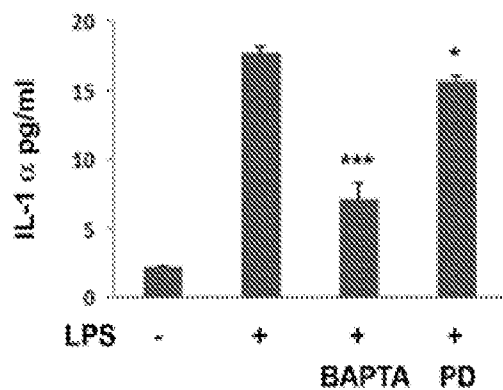


FIG. 12B

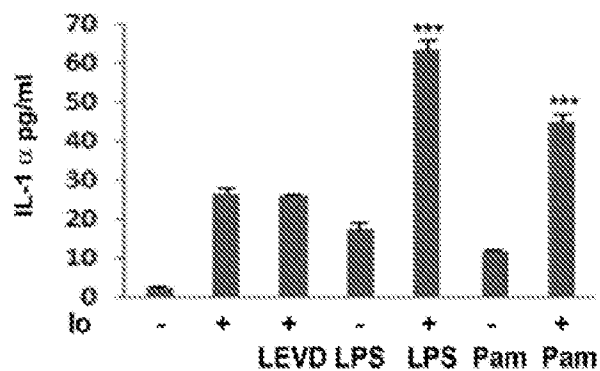


FIG. 12C

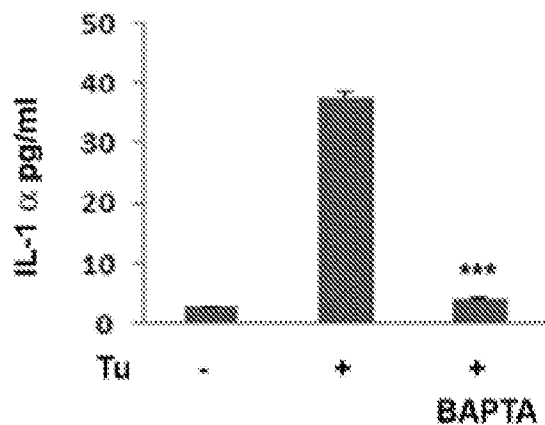


FIG. 12D

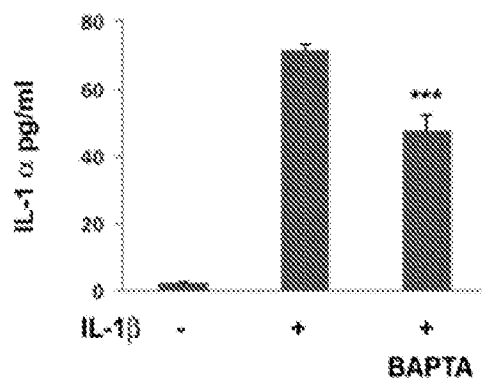


FIG. 12E

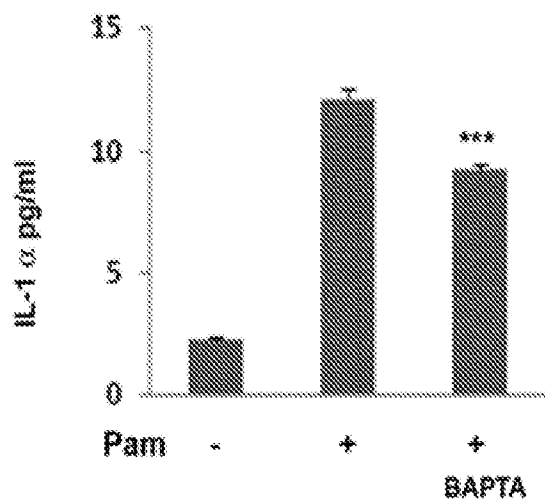


FIG. 13

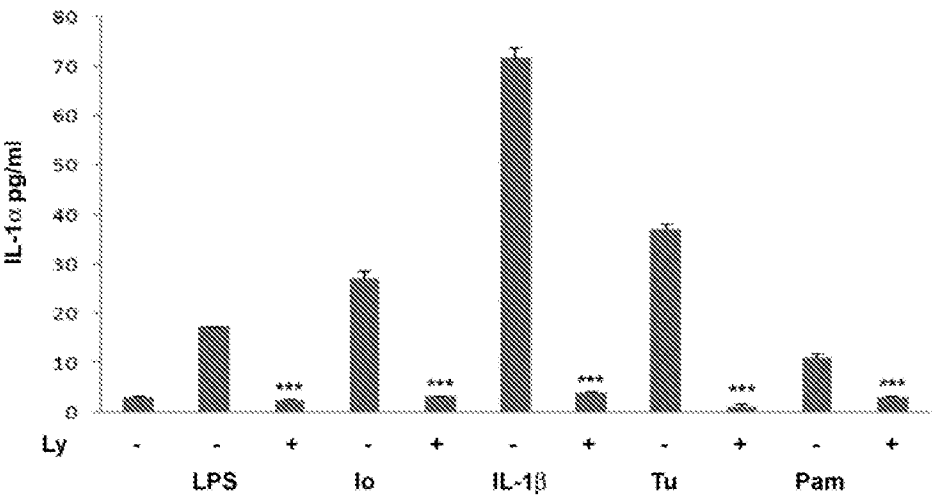


FIG. 14

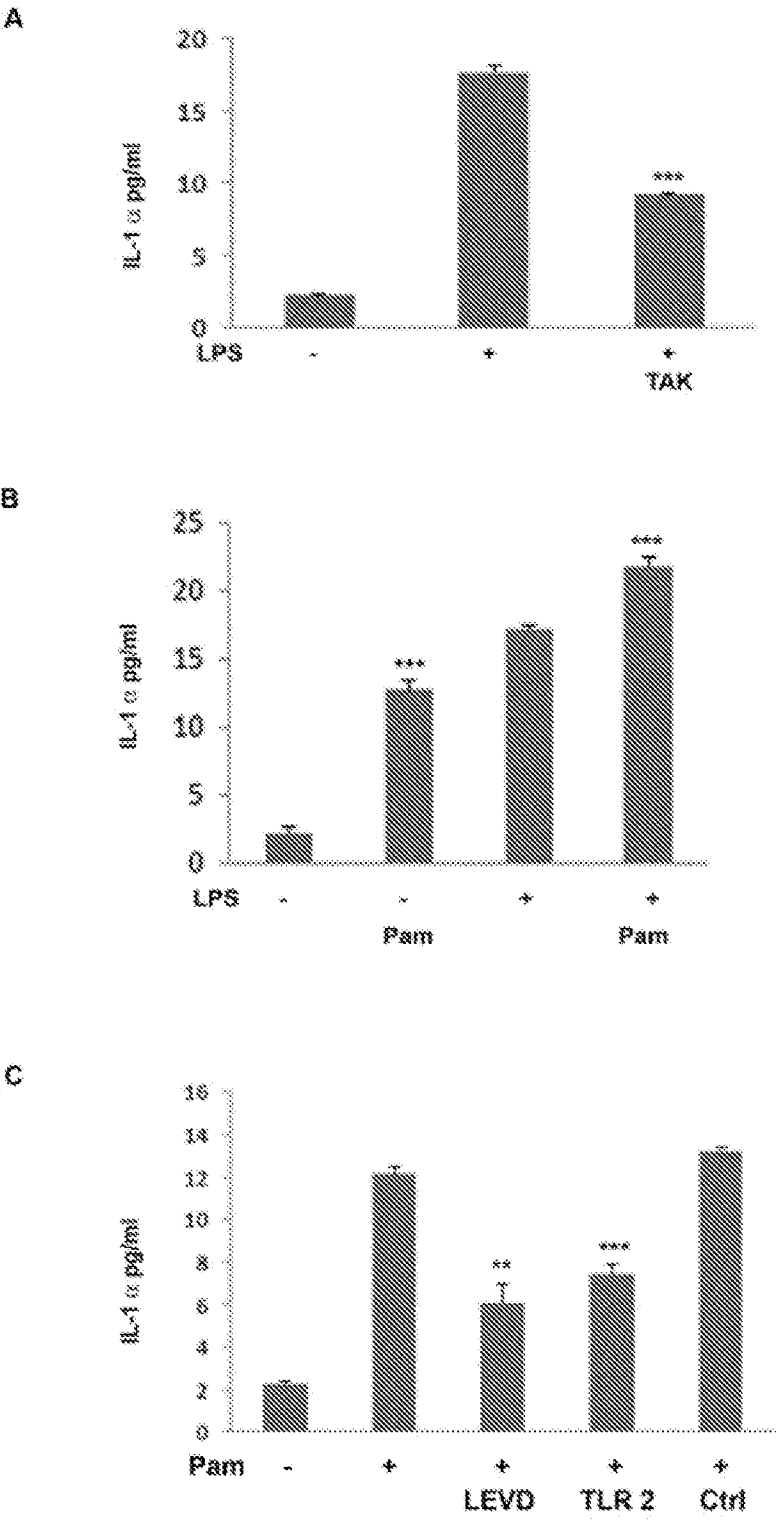


FIG. 15

