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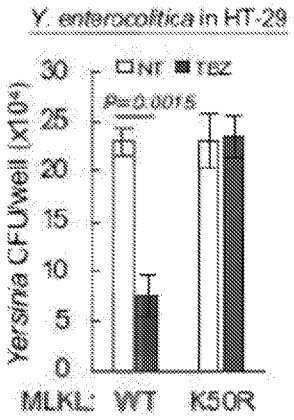
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(54) Title: CONTROLLING UBIQUITINATION OF MLKL FOR TREATMENT OF DISEASE

(57) Abstract: An agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) is disclosed for use in treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject. Use of the agent for additional diseases is also disclosed.

FIG. 10A



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CONTROLLING UBIQUITINATION OF MLKL FOR TREATMENT OF DISEASE

RELATED APPLICATIONS

This application claims the benefit of priority of Israeli Patent Application No. 279559 filed 17 December, 2020, the contents of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING STATEMENT

The ASCII file, entitled 90063SequenceListing.txt, created on 13 December, 2021, comprising 15,150 bytes, submitted concurrently with the filing of this application is incorporated herein by reference.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to method of treating diseases and, more particularly, but not exclusively, to infectious diseases, cancer and inflammatory bowel diseases (IBD) by controlling the level of ubiquitination of mixed lineage kinase-like molecule (MLKL).

Exploration of the mechanisms by which cytokines of the tumor necrosis factor (TNF) family induce cell death has led to the identification of a form of programmed cell death called "necroptosis", in which the mixed lineage kinase-like molecule (MLKL), upon its phosphorylation by the protein kinase RIPK3, triggers lethal rupture of the cellular membrane. This pathway was later shown also to be activated by a number of other inducers, including other cytokines and different pathogen components employing various proximally signaling proteins to activate RIPK3 (*1-7*).

Phosphorylation of MLKL by RIPK3 triggers in this pseudokinase a conformational change that results in its oligomerization, and also results in the exposure of clusters of charged residues at its N-terminus within a domain consisting of a four α-helical bundle (4HB). The exact mechanism of death mediation by MLKL is still not clear, except that the death seems to be inflicted by binding of the exposed charged residue clusters in MLKL to certain membrane lipids (*8*).

Prior its phosphorylation, MLKL resides mainly in the cytoplasm, but it can also be found in the nucleus, where it is activated by certain pathogens (*9-11*). Death mediation by MLKL appears to happen in the cell membrane (*12, 13*). It was previously reported that some of the activated MLKL molecules also associate with endosomal membranes, where they bind to ESCRT proteins and are then released from the cell within extracellular vehicles (EVs) whose generation they facilitate (*14*).

U.S. Patent Application No. 2016/0160189 provides methods and compositions for inducing necroptosis in target cells, including cancer cells. Specifically, according to U.S. 2016/0160189 necroptosis is induced using compositions including oligomers comprising RIPK3 proteins and RIPK1 proteins including, but not limited to, full length RIPK3 homodimers, truncated RIPK3 oligomers and/or full length RIPK3/RIPK1 heterodimers.

WO2018/033929 teaches agents which regulate MLKL for the treatment of inflammation in general and, more specifically of cancer.

Park et al., Cell Death and Disease (2020) 11:744 teaches that agents capable of down-regulation MLKL can be used to treat cancer.

SUMMARY OF THE INVENTION

According to an aspect of the present invention, there is provided a method of treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject, the method comprising administering to the subject a therapeutically effective amount of an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL), thereby treating the disease caused by the pathogen.

According to an aspect of the present invention, there is provided a method of treating a cancer sensitive to TRAIL receptor mediated cytotoxicity in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the cancer.

According to an aspect of the present invention, there is provided an article of manufacture comprising an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) and an antibacterial or antiviral agent.

According to an aspect of the present invention, there is provided an article of manufacture comprising an agent which downregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) and an agent that activates the TRAIL apoptotic pathway and/or TNF alpha.

According to an aspect of the present invention, there is provided an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) for use in treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject.

According to an aspect of the present invention, there is provided a method of treating inflammatory bowel disease (IBD) in a subject in need thereof comprising administering to the

subject a therapeutically effective amount of an agent that specifically up-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the IBD.

According to an aspect of the present invention, there is provided an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) for use in treating IBD.

According to an aspect of the present invention, there is provided method of treating a cancer in a subject in need thereof comprising co-administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL and TNF alpha, thereby treating the cancer.

According to an aspect of the present invention, there is provided a method of treating a disease associated with an upregulation of activity of EGF receptor in a subject comprising administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the disease.

According to embodiments of the present invention, the disease is selected from the group consisting of non-small-cell lung cancer, metastatic colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer and breast cancer.

According to embodiments of the present invention, the pathogen is a virus.

According to embodiments of the present invention, the virus is selected from the group consisting of Influenza A virus, Foot and mouth disease virus, Rhinovirus, Adenovirus, Ebolavirus, Kaposi Sarcoma Virus, Simian virus 40, Papillomavirus, Polio virus, Lymphocytic choriomeningitis virus (LCMV), Rhinovirus and Coronavirus.

According to embodiments of the present invention, the pathogen is a bacteria.

According to embodiments of the present invention, the bacteria is *Listeria monocytogenes.*

According to embodiments of the present invention, the agent enhances the activity and/or amount of a ubiquitin ligase that is capable of ubiquitinating lysine at position 50 of mixed lineage kinase domain-like protein (MLKL).

According to embodiments of the present invention, the agent is the ubiquitin ligase.

According to embodiments of the present invention, the agent is a polynucleotide encoding the ubiquitin ligase.

According to embodiments of the present invention, the ubiquitin ligase is E3 ubiquitin-protein ligase Itchy homolog (ITCH).

According to embodiments of the present invention, the agent enhances oligomerization of the MLKL.

According to embodiments of the present invention, the agent activates protein kinase RIPK3.

According to embodiments of the present invention, the agent is a multivalent agent that binds the MLKL.

According to embodiments of the present invention, the method further comprises administering to the subject an agent that directly targets the pathogen.

According to embodiments of the present invention, the agent is a peptide agent that is a substrate for ubiquitination by the E3 ubiquitin-protein ligase.

According to embodiments of the present invention, the peptide agent comprises at least 10 amino acids of MLKL.

According to embodiments of the present invention, the agent is a polynucleotide agent that is capable of down-regulation the amount of ITCH in cancer cells of the subject.

According to embodiments of the present invention, the method further comprises administering to the subject an agent that activates the TRAIL apoptotic pathway.

According to embodiments of the present invention, the agent that activates the TRAIL apoptotic pathway is selected from the group consisting of Mapatumumab, Contatumumab, TAS266, ONC201, Tigatuzumab, Dulanermin and Circularly permuted TRAIL (CPT).

According to embodiments of the present invention, the cancer is selected from the group consisting of myeloma, lymphoma, colorectal cancer, NSCLC, liver cancer, triple negative breast cancer, pancreatic cancer, cervical cancer, soft tissue sarcoma, ovarian cancer, glioblastoma.

According to embodiments of the present invention, the agent enhances the activity and/or amount of a ubiquitin ligase that is capable of ubiquitinating lysine at position 50 of mixed lineage kinase domain-like protein (MLKL).

According to embodiments of the present invention, the agent is the ubiquitin ligase.

According to embodiments of the present invention, the agent is a polynucleotide encoding the ubiquitin ligase.

According to embodiments of the present invention, the ubiquitin ligase is E3 ubiquitin-protein ligase Itchy homolog (ITCH).

According to embodiments of the present invention, the agent which downregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) is co-formulated with the agent that activates the TRAIL apoptotic pathway.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-G. Activated MLKL is ubiquitinated at specific lysine residues.

A. Time-dependent ubiquitination and phosphorylation of MLKL in human HT-29 cells in response to treatment with TBZ (TNF, 1000 units/ml; the bivalent IAP antagonist BV6, 1 μM; and the caspase inhibitor z-VAD-fmk, 20 μM).

B. MLKL ubiquitination in response to TBZ occurs as a consequence of its oligomerization. The extents of MLKL ubiquitination (top), aggregation (middle) and phosphorylation (bottom) were determined in MLKL knockout (KO) HT-29 cells expressing constitutively wild-type (WT) MLKL and its indicated mutants.

C. Accumulation of ubiquitinated MLKL in response to TBZ is not enhanced by treatment with MG132 (10 μM) or bafilomycin A1 (Baf A1, 0.1 μM).

D-F. Ubiquitination and oligome rization of the indicated mutants of (D, E) human MLKL expressed transiently in MLKL-KO HT-29 cells, and (F) mouse MLKL inducibly expressed in MLKL-KO MEFs, upon treatment with TBZ.

G. Ribbon diagram of the 4HB domain in mouse MLKL. The site of ubiquitination of MLKL (green) and the clusters of residues that appear to be crucially involved in the mediation of cell death (red and orange (*29*)) are indicated.

FIGs. 2A-L. Ubiquitinated MLKL associates with endosomal membranes.

A. Assessment of the occurrence of ubiquitinated and phosphorylated MLKL in the indicated subcellular fractions (15 μg protein applied per well). (PAM, plasma membrane associated membrane; PM, plasma membrane).

B-I. Immunocytochemical analysis of the effect of the K50R mutation in MLKL on the association of MLKL (B–E) and of both MLKL and ubiquitin (F–I) with early ($Rab5^+$) and late ($Rab7^+$) endosomes in MLKL-KO HT-29 cells that inducibly express MLKL, observed after 2.5 h of treatment with TBZ. B, C and F, G: Quantification of the data (~110 cells). (D, E) and (H, I): Examples of the immunofluorescence images. In D and E, green – MLKL, magenta – Rab5 or 7, white (and arrows) – MLKL+Rab5 or 7. In H and I, green – MLKL, red – Rab5 or7, blue – ubiquitin, magenta – Rab5 or 7 + ubiquitin, cyan – MLKL + ubiquitin, white (and arrows) – Rab5 or 7 + MLKL + ubiquitin. Scale bar, 10 μm.

J. Comparison of the amounts of MLKL and of ubiquitinated MLKL in 60 μg of proteins of the total cellular lysate and of EVs released from HT-29 cells in 3 h of treatment with TBZ.

K, L. Effect of the K50R mutation in MLKL on (H) the association of MLKL with ESCRT proteins and on (I) MLKL release in EVs from HT-29 cells treated for 2.5 h with TBZ.

FIGs. 3A-K MLKL ubiquitination is mediated by ITCH.

A. Comparison of the amounts of ITCH in 15 μg of proteins of the total cellular lysate and of EVs released from HT-29 cells in 3 h of treatment with TBZ.

B. ITCH binds to MLKL in 293T cells transiently overexpressing both proteins. Western analysis following the precipitation of MLKL via fused Strep tag.

C. In response to TBZ, ITCH binds inducibly to MLKL in HT-29 cells constitutively expressing wild-type MLKL but not its T357A/S358A or L162G/L165G mutant, and binds constitutively to the MLKL L58G/176G mutant.

D, E. Deletion analysis of ITCH for identification of the region in ITCH to which MLKL binds. (D) Western analysis of the binding of the various MLKL mutants to ITCH on transient expression in 293T cells. (E) Diagram showing the deletion mutants used and the location of the WW motifs in ITCH.

F, G. Immunocytochemical evidence for the colocalization of ITCH and activated MLKL in association with endosomes. (F) Quantification of the data (in 75 cells). (G) Immunofluorescence images. White (arrows), co-localization of both MLKL (green) and ITCH (blue) with either Rab5 or Rab 7 (red, upper and lower panels, respectively). Scale bar, 10 μm.

H. ITCH catalyzes *in vitro* the ubiquitination of MLKL but not of its K50R mutant.

I. Enhancement, by inducible expression of ITCH, of TBZ-induced ubiquitination of MLKL but not of its K50R mutant that are inducibly expressed in MLKL KO HT-29 cells.

J. Enhancement of TBZ-induced association of MLKL with ESCRT proteins by transient expression of ITCH, and inhibition of this association by its enzymatically inactive mutant.

K. Decrease, by ITCH knockdown, in TBZ-induced ubiquitination of MLKL.

FIGs. 4A-L. Ubiquitination of MLKL facilitates lysosomal destruction of *Lysteria.*

A. Amounts of viable *Listeria* in MLKL-KO MEFs inducibly expressing either wild-type mouse MLKL or its K50R, K51R mutant, 4 h after infection, and the effect of TBZ treatment for the last 1 h on the Listerial yields.

B, C. Comparison of the amounts of viable *Listeria*, 5 h (B) and 24 h (C) after infection, as well as of the effects of TBZ treatment for 2 h at the end of the infection period, in MLKL-KO HT-29 cells and in the KO cells inducibly expressing either wild-type MLKL or its K50R mutant.

D. Effects of various MLKL mutants expressed constitutively in MLKL-KO HT-29 cells and of treatment for 2 h with TBZ at the end of the infection period on the amounts of viable *Listeria* in the cells 5 h after infection.

E. Comparison of the effects of TBZ treatment for 2 h or 4 h after infection with *Listeria* on Listerial yield and on the extent of cell death, in wild-type and in MLKL-KO HT-29 cells.

F. Effect of ITCH knockdown on the amount of viable *Listeria* in HT-29 cells 5 h after infection, and on its modulation by TBZ treatment for 2 h at the end of the infection period.

G-J. Effects of chloroquine (CQ, 25 μM) and pepstatin A (PepA, 10 μg/ml) applied throughout the periods of *Listeria* infection of HT-29 cells as in B and C.

K-L. Immunocytochemical analysis of the location of *Listeria* in early and late endosomes, in lysosomes and in the cytosol, 5 h after infection, and their modulation by TBZ-treatment, in HT-29 cells inducibly expressing wild-type or K50R-mutated MLKL. (K) Ratios of the numbers of *Listeria* in the indicated compartments (analysis of 130 infected cells). (L) Typical immunofluorescence images. Blue–*Listeria*, green – MLKL, red – Rab7, magenta – Lamp1, (a lysosomal marker), yellow – MLKL + Rab7, white – MLKL + Rab7 + Lamp1, white arrows – *Listeria* in MLKL + Rab7 + Lamp1.

FIGs. 5A-F. Activated MLKL is ubiquitinated at specific lysine residues.

A-C.Assessment of the ubiquitination and/or phosphorylation of MLKL in (A) wild-type MEFs, in response to TBZ, (B) HT-29 cells in which FADD was knocked out by CRISPR/Cas9, in response to treatment with just TNF and Bv6 (TB), and (C) TBZ-treatment of HT-29 cells in which the endogenous MLKL was knocked out, and either the wild type or the K50R-mutated human MLKL was inducibly re-expressed under control of the GEV16/pF5x UAS system.

D. Conjugation of wild-type- and K63R-mutated HA-tagged ubiquitin to MLKL in HT-29 cells.

E. Comparison of the *in-vitro* effects of the indicated deubiquitinases on HA-tagged polyubiquitin chains that were conjugated to MLKL in HT-29 cells treated with TBZ for 3 h.

F. Effects of these deubiquitinases on synthetic K63-linked and K48-linked tetra-ubiquitin chains.

FIGs. 6A-F. Ubiquitinated MLKL associates with endosomal membranes.

A-D. Additional examples of the immunofluorescent images whose quantitative analysis is presented in Figure 2. A, B: Association of MLKL with early (Rab5$^+$, A) and late (Rab7$^+$, B) endosomes. C, D: Association of both MLKL and ubiquitin with early (Rab5$^+$, C) and late (Rab 7$^+$, D) endosomes. In A and B: green – MLKL, magenta – Rab5 or 7, white (and arrow) – MLKL + Rab5 or 7. In C and D: green – MLKL, red – Rab5 or 7, blue – ubiquitin, magenta – Rab5 or 7 + ubiquitin, cyan – MLKL + ubiquitin, white (and arrows) – Rab5 or 7 + MLKL + ubiquitin. Scale bar, 10 μm.

E, F. Comparative immunohistochemical analysis of the extent of association of MLKL and ubiquitin with (E) early endosomes and (F) the endoplasmic reticulum in HT-29 cells after 3 h of treatment with TBZ. Green – MLKL, red – early endosomes (in E) or endoplasmic reticulum (in F), blue – ubiquitin, cyan – MLKL + ubiquitin, magenta – early endosomes (in E) or endoplasmic reticulum (in F) + ubiquitin, yellow – MLKL + early endosomes (in E) or endoplasmic reticulum (in F), white (and arrows) – MLKL + early endosomes + ubiquitin.

FIGs. 7A-F. Functional consequences of MLKL ubiquitination

A, B. Immunoblot analysis of the kinetics of intracellular degradation of (A) EGF and EGFR and of (B) TNF, in TBZ-treated MLKL-KO HT-29 cells that inducibly express wild-type or K50R-mutated MLKL.

C, D. Increase in sensitivity to TBZ-induced cell death in (C) MEFs expressing the K50R, K51R-mutated mouse MLKL and in (D) HT-29 cells expressing the K50R human MLKL mutant. In both cells the endogenous MLKL was knocked out and then the wild-type and the mutant MLKL were expressed inducibly.

E, F. Immunocytochemical analysis of the extent of acidification of the lysosomes that contain *Listeria* in control and TBZ-treated HT-29 cells. (E) Quantification of the data (~100 infected cells). (F) Example of the immunostaining: green − Lamp1 (a lysosomal marker), red − staining of acidification by the LysoTracker reagent, blue – *Listeria*, yellow – Lamp1 + LysoTracker, magenta – *Listeria* + LysoTracker, cyan – *Listeria* + Lamp1, white− *Listeria* + Lysotracker + Lamp1. Scale bar, 10 μm.

FIG. 8 Diagrammatic presentation of the protocols of infection with *Listeria monocytogenes.*

FIG. 9A: Spectrum Annotation of Scan #21400 VLGLIKPLEMLQDQGKR (SEQ ID NO: 10)[Trypsin Digested:GG (Ubiquitination Site) on K]R.

FIG. 9B: Spectrum Annotation of Scan #3228 LHHSEAPELHGKIR (SEQ ID NO: 9) [Trypsin Digested:GG (Ubiquitination Site) on K]IR.

FIGs. 10A-D illustrate that MLKL ubiquitination facilitates destruction of Y. enterocolitica and E. coli bacteria.

FIGs. 10A-B. Yields of Y. enterocolitica bacteria 5 h after infection of HT-29 cells or their HT-29 K50R mutant, and 6 h after infection of L929 cells or their K50,51R mutant, and the modulation of these yields obtained by treatment of the HT-29 cells with TBZ and treatment of the L929 cells with TNF.

FIGs. 10C-D. Yields of E. coli 8 h after infection of L929 cells or their K50,51R mutant, and the modulation of these yields obtained by treatment of the L929 cells with TNF.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to method of treating diseases and, more particularly, but not exclusively, to infectious diseases, cancer and IBD, by controlling the level of ubiquitination of mixed lineage kinase-like molecule (MLKL).

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Phosphorylation of MLKL by the protein-kinase RIPK3 targets MLKL to the cell membrane, where it triggers necroptotic cell death.

The present inventors now report that conjugation of polyubiquitin chains (that are at least partly K63-linked) to distinct lysine residues in the phosphorylated MLKL molecule, is facilitated, at least partly, by the ubiquitin ligase ITCH that binds MLKL via its WW domains, targets MLKL to endosomes. This results in the release of phosphorylated MLKL in extracellular vesicles and decreased cell death. MLKL ubiquitination also enhances endosomal trafficking, which augments translocation of the bacterium *Listeria monocytogenes* to the lysosomes, leading to growth arrest of the bacteria (Figures 4A-J). The present inventors have identified the K50 site on MLKL as the major target for ubiquitination, as verified by mass spectrometry.

The present inventors propose taking advantage of this mechanism for the treatment of additional diseases which are associated with pathogenic agents. Thus, the present inventors contemplate agents that that up-regulate MLKL ubiquitination at the above mentioned site for the

treatment of diseases which are caused by (or associated with) pathogens that enter the cell via phagosomes, which then fuse with the endosomes.

The present inventors have shown that targeting MLKL to endosomes is dictated by ubiquitination of K50. Since this phenomenon endows cells with resistance to necroptotic death, the present inventors propose that agent that facilitate the ubiquitination of K50 will protect from the pathological consequences of necroptosis and be useful for treating inflammatory bowel disease (IBD) – see for example Sha li et al., World J Clin Cases 2018 November 26; 6(14): 745-752; Shindo et al., Shindo et al., iScience 15, 536–551, May 31, 2019 and Negroni et al., Biomolecules 2020, 10, 1431; doi:10.3390/biom10101431.

In addition, since downregulation of ubiquitination of MLKL at the K50 site leads to a significant decrease in the rate of degradation of cell-bound epidermal growth factor (EGF) and tumor necrosis factor (TNF), as well as of the EGF receptor (Figure 7A, B), the present inventors propose that agents that decrease ubiquitination can be applied to facilitate anti-cancer therapy with ligands of the TNF family such as TNF-related apoptosis-inducing ligand (TRAIL). The impediments in the degradation of anti-cancer ligands and receptors prompted by blockage of MLKL ubiquitination-mediated trafficking provoke cells vulnerable to TRAIL-mediated cell death.

Thus, according to a first aspect of the present invention, there is provided a method of treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject, the method comprising administering to the subject a therapeutically effective amount of an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL), thereby treating the disease caused by the pathogen.

According to another aspect of the present invention there is provided a method of treating inflammatory bowel disease (IBD) in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an agent that specifically up-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the IBD.

The term "MLKL" refers to the Mixed Lineage Kinase Domain-Like protein, a product of Gene ID: 197259. Exemplary MLKL amino acid sequences are set forth in GenBank accession nos. NP_001135969.1 and NP_689862.1.

In one embodiment, the amino acid sequence of MLKL is as set forth in SEQ ID NO: 6. According to a particular embodiment, the positioning of the ubiquitination (i.e. position 50) is according to the amino acid sequence as set forth in SEQ ID NO: 6.

Ubiquitination takes place by a cascade of enzyme activity (i.e. a plurality of enzymes which work together to bring about the same function – ubiquitination). For example, E1 activates the Ub;

then Ub is transferred to E2 ligase. E2 ligase together with E3 recognize a specific target and ligate the Ub to the target protein. In some cases Ub is transferred directly to E3 ligase.

The present invention contemplates upregulation of any of the components of the ubiquitination system such that ubiquitination at position 50 of MLKL is enhanced.

In one embodiment, the agent upregulates the activity and or amount of ubiquitinating enzyme that is responsible for adding a ubiquitin moiety at position 50 of MLKL.

In one embodiment, the ubiquitinating enzyme is a human ubiquitinating enzyme.

Ubiquitin-activating enzymes (E1s) have the EC number EC 6.2.1.45, ubiquitin-conjugating enzymes have the EC number EC 2.3.2.23 and ubiquitin ligases have the EC number 2.3.2.27.

According to a specific embodiment, the agent up-regulates the amount and/or activity of an E2 ligase.

Table 1, herein below provides nomenclature and most common synonyms used for E2 ubiquitin conjugating enzymes. The E2 nomenclature is in accordance with that used by the Human Genome Organization.

Table 1

Human Genome Organization Nomenclature	***Synonym***
UBE2V2	UEV2/MMS2
UBE2D1	UBC4/5/UBCH5A
UBE2D2	UBC4/5/UBCH5B
UBE2D4	HBUCE1
UBE2D3	UBC4/5
UBE2W	FLJ11011
UBE2B	UBC2/HHR6B/RAD6B/E217K
UBE2L6	RIGB/UBCH8
UBE2N	UBC13
UBE2L3	UBCH7
UBE2G1	UBC7/E217K
UBE2H	UBC8/E220K
UBE2M	UBC12
UBE2F	NCE2
UBE2E2	UBCH8
UBE2E3	UBCH9/UBCM2
UBE2S	E224K
UBE2U	MGC35130
UBE2R1	CDC34
UBE2R2	UBC3B/CDC34B
UBE2Z	HOYS7
UBE2J2	NCUBE2
Probable ubiquitin-conjugating enzyme E2 FLJ25076	LOC134111/FLJ25076

AKTIP	FTS/FT1
UBE2J1	NCUBE1
UBE2V1	UEV1/CROC1
UBE2Q2	DKFZ/UBCI
UBE2Q1	NICE5
	TSG101/VPS23/SG10
UEVLD	UEV3

In one embodiment, the agent upregulates the amount and/or activity of E3 ligase.

Exemplary E3 ligases contemplated by the present invention include which may be responsible for ubiquitination of MLKL, but are not limited to ITCH, Siah2, Smurf1, MDM2, BRCA1, PARKIN, UBE3A, TRIM5, NEDD4, UBR5, Huwe1, Arkadia, MuRF1, TRAF6, Trim32, UBR4, UBE3B and UBE3D.

In a specific embodiment, the E3 ligase is E3 ubiquitin-protein ligase Itchy homolog (ITCH).

Itch is an example of an E3-ubiquitin ligase that belongs to the Nedd4-like E3 family, and is characterized by a modular organization that includes: an N-terminal protein kinase C-related C2 domain; multiple WW domains; and a C-terminal HECT (homologous to the E6-associated protein carboxyl terminus) Ubiquitin (Ub)-protein ligase domain.

Human Itch is described by Perry W L, Hustad C M, Swing D A, O'Sullivan T N, Jenkins N A, Copeland N G. Nat Genet. 1998 18:143-6 and the sequences deposited under GenBank Accession numbers NM_031483 (nucleotide) (gi27477108) and NP_113671 (protein) (gi:27477109).

Other additional E3 ligases which may be responsible for ubiquitinating MLKL include AMFR, APC/Cdc20, APC/Cdh1, C6orf157, Cbl, CBLL1, CHFR, CHIP, DTL (Cdt2), E6-AP, HACE1, HECTD1, ECTD2, HECTD3, HECW1, HECW2, HERC2, HERC3, HERC4, HERC5, HUWE1, HYD, ITCH, LNX1, mahogunin, MARCH-I, MARCH-II, MARCH-III, MARCH-IV, MARCH-VI, MARCH-VII, MARCH-VIII, MARCH-X, MDM2, MEKK1, MIB1, MIB2, MycBP2, NEDD4, NEDD4L, Parkin, PELI1, Pirh2, PJA1, PJA2, RFFL, RFWD2, Rictor, RNF5, RNF8, RNF19, RNF190 and RNF20.

Agents which increase the amount of a ubiquitinating enzyme capable of ubiquitinating MLKL at position 50 (such as ITCH) include agents which are capable of increasing the transcription (for example a transcription factor known to interact with the 5'untranslated region of the ubiquitinating enzyme) of the ubiquitinating enzyme, the translation of the ubiquitinating enzyme or the stability of the ubiquitinating enzyme. Additionally, the agent which increases the

amount of the ubiquitinating enzyme, may be a polynucleotide which encodes the ubiquitinating enzyme, the protein itself or an active peptide thereof.

In one embodiment, the ubiquitinating enzyme (e.g. ITCH) (or the polynucleotide which encodes the ubiquitinating enzyme) which is administered to the subject is (or encodes a protein that is) at least 50 % homologous, more preferably at least 60 % homologous, more preferably at least 70 % homologous, more preferably at least 80 % homologous, and most preferably at least 90 % homologous to the polypeptide sequence as set forth in SEQ ID NO:7 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters) comprising ubiquitinating enzyme activity. The homolog may also refer to a deletion, insertion, or substitution variant, including an amino acid substitution, thereof and biologically active polypeptide fragments thereof.

Recombinant techniques are typically used to generate the ubiquitinating enzyme (e.g. ITCH) of the present invention. These techniques may be preferred due to the length of the protein and the large amounts required thereof. Such recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

To produce an expression vector for the expression of the ubiquitinating enzyme (e.g. ITCH) of the present invention, a polynucleotide encoding the ubiquitinating enzyme is ligated into a nucleic acid expression vector, which comprises the polynucleotide sequence under the transcriptional control of a cis-regulatory sequence (e.g., promoter sequence) suitable for directing constitutive, tissue specific or inducible transcription of the ubiquitinating enzyme in the host cells.

The phrase “an isolated polynucleotide” refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the ubiquitinating enzyme, as well as some intronic sequences interposing there between. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

As mentioned hereinabove, polynucleotide sequences of the present invention are inserted into expression vectors (i.e., a nucleic acid construct) to enable expression of the recombinant ubiquitinating enzyme. The expression vector of the present invention may include additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). Typical cloning vectors contain transcription and translation initiation sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals).

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the ubiquitinating enzymes of the present invention. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the ubiquitinating enzyme coding sequence; yeast transformed with recombinant yeast expression vectors containing the ubiquitinating enzyme coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the ubiquitinating enzyme coding sequence.

Other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the ubiquitinating enzyme), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed ubiquitinating enzyme.

Various methods can be used to introduce the expression vector of the present invention into the host cell system. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al.,

Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et al. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant peptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant ubiquitinating enzyme of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant ubiquitinating enzyme of may either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

Following a predetermined time in culture, recovery of the recombinant ubiquitinating enzyme is effected.

The phrase "recovering the recombinant ubiquitinating enzyme" used herein refers to collecting the whole fermentation medium containing the ubiquitinating enzyme and need not imply additional steps of separation or purification.

Thus, ubiquitinating enzymes can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

To facilitate recovery, the expressed coding sequence can be engineered to encode the ubiquitinating enzyme fused to a cleavable moiety. Such a fusion protein can be designed so that the ubiquitinating enzyme can be readily isolated by affinity chromatography; e.g., by

immobilization on a column specific for the cleavable moiety. Where a cleavage site is engineered between the ubiquitinating enzyme and the cleavable moiety, the ubiquitinating enzyme can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth *et al.*, Immunol. Lett. 19:65-70 (1988); and Gardella *et al.*, J. Biol. Chem. 265:15854-15859 (1990)].

The ubiquitinating enzyme of the present invention is preferably retrieved in "substantially pure" form.

As used herein, the phrase "substantially pure" refers to a purity that allows for the effective use of the ubiquitinating enzyme in the applications described herein.

In addition to being synthesizable in host cells, the ubiquitinating enzyme of the present invention can also be synthesized using *in vitro* expression systems. These methods are well known in the art and the components of the system are commercially available.

As mentioned, the ubiquitinating enzyme may be administered to the subject in need thereof as polynucleotides where they are expressed in vivo i.e. gene therapy.

The phrase "gene therapy" as used herein refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition or phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. For review see, in general, the text "Gene Therapy" (Advanced in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) ex vivo and (2) *in vivo* gene therapy. In ex vivo gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material in situ. The cells may be autologous or non-autologous to the subject. Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism in situ, that is within the recipient. These genetically altered cells have been shown to express the transfected genetic material in situ.

To confer specificity, preferably the nucleic acid constructs used to express the ubiquitinating enzyme comprises cell-specific promoter sequence elements.

Recombinant viral vectors are useful for *in vivo* expression of the ubiquitinating enzyme since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

It will be appreciated that as well as (or instead of) upregulating expression/activity of a ubiquitnating enzyme capable of ubiquitinating MLKL at position 50, the present inventors also contemplate downregulating a deubiquitinating enzyme that is capable of deubiquitinating ubiquitinated MLKL at position 50.

The term "deubiquitinating" enzyme refers to an enzyme that cleaves ubiquitin from proteins.

According to a specific embodiment, the deubiquitinating enzyme is a cysteine protease or a metalloprotease.

Exemplary deubiquitinating enzymes which may be capable of deubiquitinating ubiquitinated MLKL at position 50 include USP7, USP47, USP2, USP7, USP15, USP9X, USP28, USP30.

Downregulation of the deubiquitinating enzyme may be effected at the protein or nucleotide level as is further described herein below.

Since the present inventors have shown that mere oligomerization of MLKL suffices to dictate its ubiquitination, additional agents that are contemplated which can serve as therapeutics for diseases caused by pathogenic agents (e.g. infectious diseases and IBD) are those that enhance oligomerization of MLKL. For example agents that activate protein kinase RIPK3 are contemplated. RIPK3 phosphorylates MLKL and by this triggers its oligomerization. Examples

of such agents include, but are not limited to TRAIL, ligands of TLR (Toll-like receptors) such as BCG and Interferon alpha.

Other agents capable of oligomerizing MLKL include multivalent agents that bind MLKL (e.g. a chimeric oligomer of the antigen binding portion of anti-MLKL antibody, fused to cell-penetrating moiety – see for example Alewine et al., The Oncologist 2015;20:176–185).

As mentioned, the agents disclosed herein above (i.e. those which upregulates ubiquitination of lysine at position 50 of MLKL) may be used to treat a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject. Once the pathogen enters the cell, the pathogen is typically driven into the lysosome.

Advantageously, the agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL), is capable of performing its activity in live cells (i.e. increases the uptake of the pathogen into the lysosome), hence, preventing the release of live pathogen from infected cells in which MLKL is activated.

In one embodiment, the pathogen which is responsible for causing the disease is a bacteria.

In some embodiments, the bacterial pathogen is *Listeria monocytogenes*.

In other embodiments, the bacterial pathogen is of the genus Yersinia (e.g. *Yersinia enterocolitica* or *Yersinia pestis*. It will be appreciated that since Yersinia bacteria has been associated with various diseases such as Crohn's disease and reactive arthritis, the present inventors contemplate treating these diseases which upregulate ubiquitination of lysine at position 50 of MLKL.

In some embodiments, the pathogen is *E-coli*.

Additional disease causing bacteria include, but are not limited to *Mycobacterium Tuberculosis, Mycobacterium Leprae, Legionella pneumophila, Shigella flexneri* and *Francisella tularensis*, which are also contemplated by the present invention.

According to a particular embodiment, the bacteria is not *salmonella typhimurium*.

According to another embodiment, the pathogen is a parasite, including, but not limited to single-celled protozoans such as the agents of malaria, sleeping sickness, and amoebic dysentery.

According to another embodiment, the pathogen which causes the disease is a virus.

Examples of viruses that utilize the endosomal pathway include Influenza A virus, Foot and mouth disease virus, Rhinovirus, Adenovirus, Ebolavirus, Kaposi Sarcoma Virus, Simian virus 40, Papillomavirus, Polio virus, Lymphocytic choriomeningitis virus (LCMV), Rhinovirus and Coronavirus.

In some embodiments, the pathogen is an intracellular pathogen.

Additional viruses that cause disease are summarized in Table 2, herein below.

Table 2

Family	Baltimore group	Important species	envelopment
Adenoviridae	Group I (dsDNA)	Adenovirus	non-enveloped
Herpesviridae	Group I (dsDNA)	Herpes simplex, type 1, Herpes simplex, type 2, Varicella-zoster virus, Epstein–Barr virus, Human cytomegalovirus, Human herpesvirus, type 8	enveloped
Papillomaviridae	Group I (dsDNA)	Human papillomavirus	non-enveloped
Polyomaviridae	Group I (dsDNA)	BK virus, JC virus	non-enveloped
Poxviridae	Group I (dsDNA)	Smallpox	enveloped
Hepadnaviridae	Group VII (dsDNA-RT)	Hepatitis B virus	enveloped
Parvoviridae	Group II (ssDNA)	Parvovirus B19	non-enveloped
Astroviridae	Group IV (positive-sense ssRNA)	Human astrovirus	non-enveloped
Caliciviridae	Group IV (positive-sense ssRNA)	Norwalk virus	non-enveloped
Picornaviridae	Group IV (positive-sense ssRNA)	coxsackievirus, hepatitis A virus, poliovirus, rhinovirus	non-enveloped
Coronaviridae	Group IV (positive-sense ssRNA)	Severe acute respiratory syndrome virus	enveloped
Flaviviridae	Group IV (positive-sense ssRNA)	Hepatitis C virus, yellow fever virus, dengue virus, West Nile virus, TBE virus	enveloped
Togaviridae	Group IV (positive-sense ssRNA)	Rubella virus	enveloped
Hepeviridae	Group IV (positive-sense ssRNA)	Hepatitis E virus	non-enveloped
Retroviridae	Group VI (ssRNA-RT)	Human immunodeficiency virus (HIV)	enveloped

Family	Baltimore group	Important species	envelopment
Orthomyxoviridae	Group V (negative-sense ssRNA)	Influenza virus	enveloped
Arenaviridae	Group V (negative-sense ssRNA)	Lassa virus	enveloped
Bunyaviridae	Group V (negative-sense ssRNA)	Crimean-Congo hemorrhagic fever virus, Hantaan virus	enveloped
Filoviridae	Group V (negative-sense ssRNA)	Ebola virus, Marburg virus	enveloped
Paramyxoviridae	Group V (negative-sense ssRNA)	Measles virus, Mumps virus, Parainfluenza virus, Respiratory syncytial virus,	enveloped
Rhabdoviridae	Group V (negative-sense ssRNA)	Rabies virus	enveloped
Unassigned	Group V (negative-sense ssRNA)	Hepatitis D	enveloped
Reoviridae	Group III (dsRNA)	Rotavirus, Orbivirus, Coltivirus, Banna virus	non-enveloped

Examples of coronaviruses include: human coronavirus 229E, human coronavirus OC43, SARS-CoV, HCoV NL63, HKU1, MERS-CoV and SARS-CoV-2.

According to a particular embodiment, the coronavirus is SARS-CoV-2.

Exemplary viral diseases which may be treated according to embodiments of the present invention are summarized in Table 3, herein below.

Table 3

Diseases
• gastroenteritis • keratoconjunctivitis • pharyngitis • croup • pharyngoconjunctival fever • pneumonia • cystitis
• Hand, foot and mouth disease

Diseases
• pleurodynia • aseptic meningitis • pericarditis • myocarditis
• infectious mononucleosis • Burkitt's lymphoma • Hodgkin's lymphoma • nasopharyngeal carcinoma
• acute hepatitis
• chronic hepatitis • hepatic cirrhosis • hepatocellular carcinoma
• herpes labialis, cold sores - can recur by latency • gingivostomatitis in children • tonsillitis & pharyngitis in adults • keratoconjunctivitis
• Aseptic meningitis
• infectious mononucleosis • Cytomegalic inclusion disease •
• Kaposi sarcoma • multicentric Castleman disease • primary effusion lymphoma
• AIDS
• influenza • (Reye syndrome)
• measles • postinfectious encephalomyelitis
• mumps
• hyperplastic epithelial lesions (common, flat, plantar and anogenital warts, laryngeal papillomas, epidermodysplasia verruciformis

Diseases
• Malignancies for some species (cervical carcinoma, squamous cell carcinomas)
• croup • pneumonia • bronchiolitis • common cold[
• poliomyelitis
• rabies (fatal encephalitis)
• congenital rubella • German measles
• chickenpox • herpes zoster • Congenital varicella syndrome

According to a specific embodiment, the viral disease is COVID-19.

The present inventor contemplates administering agents that directly target the pathogen in combination with the agents described herein above.

Agents that directly target (e.g. bind specifically) bacteria include antibiotics and anti-bacterial peptides.

As used herein, the term "antibiotic agent" refers to a group of chemical substances, isolated from natural sources or derived from antibiotic agents isolated from natural sources, having a capacity to inhibit growth of, or to destroy bacteria, and other microorganisms, used chiefly in treatment of infectious diseases. Examples of antibiotic agents include, but are not limited to; Amikacin; Amoxicillin; Ampicillin; Azithromycin; Azlocillin; Aztreonam; Aztreonam; Carbenicillin; Cefaclor; Cefepime; Cefetamet; Cefinetazole; Cefixime; Cefonicid; Cefoperazone; Cefotaxime; Cefotetan; Cefoxitin; Cefpodoxime; Cefprozil; Cefsulodin; Ceftazidime; Ceftizoxime; Ceftriaxone; Cefuroxime; Cephalexin; Cephalothin; Cethromycin; Chloramphenicol; Cinoxacin; Ciprofloxacin; Clarithromycin; Clindamycin; Cloxacillin; Co-amoxiclavuanate; Dalbavancin; Daptomycin; Dicloxacillin; Doxycycline; Enoxacin; Erythromycin estolate; Erythromycin ethyl succinate; Erythromycin glucoheptonate; Erythromycin lactobionate; Erythromycin stearate; Erythromycin; Fidaxomicin; Fleroxacin; Gentamicin; Imipenem; Kanamycin; Lomefloxacin; Loracarbef; Methicillin; Metronidazole;

Mezlocillin; Minocycline; Mupirocin; Nafcillin; Nalidixic acid; Netilmicin; Nitrofurantoin; Norfloxacin; Ofloxacin; Oxacillin; Penicillin G; Piperacillin; Retapamulin; Rifaxamin, Rifampin; Roxithromycin; Streptomycin; Sulfamethoxazole; Teicoplanin; Tetracycline; Ticarcillin; Tigecycline; Tobramycin; Trimethoprim; Vancomycin; combinations of Piperacillin and Tazobactam; and their various salts, acids, bases, and other derivatives. Anti-bacterial antibiotic agents include, but are not limited to, aminoglycosides, carbacephems, carbapenems, cephalosporins, cephamycins, fluoroquinolones, glycopeptides, lincosamides, macrolides, monobactams, penicillins, quinolones, sulfonamides, and tetracyclines.

Antibacterial agents also include antibacterial peptides. Examples include but are not limited to abaecin; andropin; apidaecins; bombinin; brevinins; buforin II; CAP18; cecropins; ceratotoxin; defensins; dermaseptin; dermcidin; drosomycin; esculentins; indolicidin; LL37; magainin; maximum H5; melittin; moricin; prophenin; protegrin; and or tachyplesins.

The antiviral drug may be selected from the group consisting of remdesivir, an interferon, ribavirin, adefovir, tenofovir, acyclovir, brivudin, cidofovir, fomivirsen, foscarnet, ganciclovir, penciclovir, amantadine, rimantadine and zanamivir.

The agents which upregulate ubiquitination of MLKL may be formulated together with the antibacterial or antiviral agent (e.g. in a single pharmaceutical composition) or may be provided as separate compositions and packaged together in a single article of manufacture.

The agents may, if desired, be presented together in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing each of the active ingredients. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

As mentioned, the agents described herein above may also be used to treat inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease.

MLKL, when ubiquitinated in lysine 50, antagonizes TRAIL-induced cell death by

enhancing endosomal-trafficking. Conversely, agents that arrest MLKL ubiquitination will, by preventing the MLKL-induced endosomal-trafficking and lysosomal-destruction of anti-cancer ligands like TRAIL, and their receptors, facilitate the anti-cancer effects of these ligands in cancers such as colon cancer, gastric cancer, and pancreatic adenocarcinoma. Moreover, the extent of ubiquitination of MLKL in these cancers may serve as a potential biomarker for the extent of susceptibility to cell death induction by ligands such as TRAIL, when these ligands are applied for tumor therapy.

Thus, according to another aspect of the present invention there is provided a method of treating a cancer sensitive to TRAIL receptor mediated cytotoxicity in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the cancer.

Down-regulating of ubiquitination may be understood as being complete elimination of ubiquitination at position 50 of MLKL or at least a 50 % decrease in the number of molecules undergoing ubiquitination, a 60 % decrease in the number of molecules undergoing ubiquitination ubiquitination, a 70 % decrease in the number of molecules undergoing ubiquitination ubiquitination or higher.

Examples of cancer sensitive to TRAIL receptor mediated cytotoxicity include but are not limited to myeloma, lymphoma, colorectal cancer, NSCLC, liver cancer, triple negative breast cancer, pancreatic cancer, cervical cancer, soft tissue sarcoma, ovarian cancer, glioblastoma. Additional information regarding such cancers can be found in Yuan et al., Cancer Metastasis Rev. 2018 December ; 37(4): 733–748, the contents of which are incorporated herein by reference.

As mentioned, the present inventors have shown that downregulation of ubiquitination of MLKL at the K50 site leads to a significant decrease in the rate of degradation of receptors and ligands thereof. For example, the present inventors have shown that agents that downregulate ubiquitination of MLKL at the K50 site reduce the amount of cell-bound epidermal growth factor (EGF) and tumor necrosis factor (TNF), as well as of the EGF receptor (Figure 7A, B).

Thus, the present inventors propose use of agents that downregulate ubiquitination of MLKL at the K50 site to enhance the therapeutic activity of TNF alpha treatment (e.g. to enhance the effect of TNF alpha in the treatment of cancer using the limb perfusion approach). Thus, according to still another aspect of the present invention there is provided a method of treating cancer comprising co-administering a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL and TNF alpha to the subject,

thereby treating the cancer.

Typical cancers that are treated with TNF alpha include bulky melanoma metastases and soft tissue sarcomas. Additional chemotherapeutic agents may be combined with this treatment, according to the particular cancer type and status. For example, the chemotherapeutic agent melphalan may be combined with the TNF alpha and the agent that downregulates ubiquitination of MLKL at the K50.

Since the present inventors have shown that agents that downregulate ubiquitination of MLKL at the K50 site reduce the amount of cell-bound epidermal growth factor (EGF), the present inventors propose treating diseases associated with an upregulation of activity of EGF receptor by administering agents that specifically down-regulate ubiquitination of lysine at position 50 of MLKL.

Particular examples of diseases which are associated with an up-regulation of activity of EGF receptors include non-small-cell lung cancer, metastatic colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer and breast cancer.

Agents that are capable of specifically down-regulating ubiquitination of lysine at position 50 of MLKL include those that decrease the activity and/or amount of ubiquitin enzymes capable of same. The agent may be a polynucleotide agent which hybridizes with the ubiquitinating enzyme, a neutralizing antibody or a small molecule antagonist.

Down-regulation at the nucleic acid level

Down-regulation at the nucleic acid level is typically effected using a nucleic acid agent, having a nucleic acid backbone, DNA, RNA, mimetics thereof or a combination of same. The nucleic acid agent may be encoded from a DNA molecule or provided to the cell *per se*.

Thus, downregulation of a ubiquitinating enzyme can be achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of specifically inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g, the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA

silencing agents include non-coding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs.

In one embodiment, the RNA silencing agent is capable of inducing RNA interference.

In another embodiment, the RNA silencing agent is capable of mediating translational repression.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., ubiquitin kinase) and does not cross inhibit or silence other targets or a splice variant which exhibits 99% or less global homology to the target gene, e.g., less than 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81% global homology to the target gene; as determined by PCR, Western blot, Immunohistochemistry and/or flow cytometry.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs).

Following is a detailed description on RNA silencing agents that can be used according to specific embodiments of the present invention.

DsRNA, siRNA and shRNA - The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 base pairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is suggested to result from providing Dicer with a substrate (27mer) instead of a product

(21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned, the RNA silencing agent of some embodiments of the invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop.

It will be appreciated that, and as mentioned hereinabove, the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

miRNA and miRNA mimics - According to another embodiment the RNA silencing agent may be a miRNA.

The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single-stranded RNA molecules of about 19-28 nucleotides in length, which regulate gene expression. miRNAs are found in a wide range of organisms (viruses.fwdarw.humans) and have been shown to play a role in development, homeostasis, and disease etiology.

The term "microRNA mimic" or “miRNA mimic” refers to synthetic non-coding RNAs that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics imitate the function of endogenous miRNAs and can be designed as mature, double stranded molecules or mimic precursors (e.g., or pre-miRNAs). miRNA mimics can be comprised of modified or unmodified RNA, DNA, RNA-DNA hybrids, or alternative nucleic acid chemistries (e.g., LNAs or 2'-O,4'-C-ethylene-bridged nucleic acids (ENA)). For mature, double stranded miRNA mimics, the length of the duplex region can vary between 13-33, 18-24 or 21-23

nucleotides. The miRNA may also comprise a total of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides. The sequence of the miRNA may be the first 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may also be the last 13-33 nucleotides of the pre-miRNA.

Preparation of miRNAs mimics can be effected by any method known in the art such as chemical synthesis or recombinant methods.

It will be appreciated from the description provided herein above that contacting cells with a miRNA may be effected by transfecting the cells with e.g. the mature double stranded miRNA, the pre-miRNA or the pri-miRNA.

The pre-miRNA sequence may comprise from 45-90, 60-80 or 60-70 nucleotides.

The pri-miRNA sequence may comprise from 45-30,000, 50-25,000, 100-20,000, 1,000-1,500 or 80-100 nucleotides.

Antisense – Antisense is a single stranded RNA designed to prevent or inhibit expression of a gene by specifically hybridizing to its mRNA. Downregulation of a ubiquitinating enzyme can be effected using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the ubiquitinating enzyme.

Nucleic acid agents can also operate at the DNA level as summarized infra.

Downregulation of agents such as ubiquitinating enzymes can also be achieved by inactivating the gene (e.g., MLKL, RIPK3 or RIPK1) via introducing targeted mutations involving loss-of function alterations (e.g. point mutations, deletions and insertions) in the gene structure.

As used herein, the phrase "loss-of-function alterations" refers to any mutation in the DNA sequence of a gene (e.g., ubiquitinating enzyme) which results in downregulation of the expression level and/or activity of the expressed product, i.e., the mRNA transcript and/or the translated protein. Non-limiting examples of such loss-of-function alterations include a missense mutation, *i.e.*, a mutation which changes an amino acid residue in the protein with another amino acid residue and thereby abolishes the enzymatic activity of the protein; a nonsense mutation, *i.e.*, a mutation which introduces a stop codon in a protein, e.g., an early stop codon which results in a shorter protein devoid of the enzymatic activity; a frame-shift mutation, *i.e.*, a mutation, usually, deletion or insertion of nucleic acid(s) which changes the reading frame of the protein, and may result in an early termination by introducing a stop codon into a reading frame (e.g., a truncated protein, devoid of the enzymatic activity), or in a longer amino acid sequence (e.g., a readthrough protein) which affects the secondary or tertiary structure of the protein and results in a non-functional protein, devoid of the enzymatic activity of the non-mutated polypeptide; a readthrough mutation

due to a frame-shift mutation or a modified stop codon mutation (*i.e.*, when the stop codon is mutated into an amino acid codon), with an abolished enzymatic activity; a promoter mutation, *i.e.*, a mutation in a promoter sequence, usually 5' to the transcription start site of a gene, which results in down-regulation of a specific gene product; a regulatory mutation, *i.e.*, a mutation in a region upstream or downstream, or within a gene, which affects the expression of the gene product; a deletion mutation, *i.e.*, a mutation which deletes coding nucleic acids in a gene sequence and which may result in a frame-shift mutation or an in-frame mutation (within the coding sequence, deletion of one or more amino acid codons); an insertion mutation, *i.e.*, a mutation which inserts coding or non-coding nucleic acids into a gene sequence, and which may result in a frame-shift mutation or an in-frame insertion of one or more amino acid codons; an inversion, *i.e.*, a mutation which results in an inverted coding or non-coding sequence; a splice mutation *i.e.*, a mutation which results in abnormal splicing or poor splicing; and a duplication mutation, *i.e.*, a mutation which results in a duplicated coding or non-coding sequence, which can be in-frame or can cause a frame-shift.

Methods of introducing nucleic acid alterations to a gene of interest are well known in the art [see for example Menke D. Genesis (2013) 51: - 618; Capecchi, Science (1989) 244:1288-1292; Santiago et al. Proc Natl Acad Sci USA (2008) 105:5809-5814; International Patent Application Nos. WO 2014085593, WO 2009071334 and WO 2011146121; US Patent Nos. 8771945, 8586526, 6774279 and UP Patent Application Publication Nos. 20030232410, 20050026157, US20060014264; the contents of which are incorporated by reference in their entireties] and include targeted homologous recombination, site specific recombinases, PB transposases and genome editing by engineered nucleases. Agents for introducing nucleic acid alterations to a gene of interest can be designed publically available sources or obtained commercially from Transposagen, Addgene and Sangamo Biosciences.

Following is a description of various exemplary methods used to introduce nucleic acid alterations to a gene of interest and agents for implementing same that can be used according to specific embodiments of the present invention.

Genome Editing using engineered endonucleases - this approach refers to a reverse genetics method using artificially engineered nucleases to cut and create specific double-stranded breaks at a desired location(s) in the genome, which are then repaired by cellular endogenous processes such as, homology directed repair (HDR) and non-homologous end-joining (NFfEJ). NFfEJ directly joins the DNA ends in a double-stranded break, while HDR utilizes a homologous sequence as a template for regenerating the missing DNA sequence at the break point. In order to

introduce specific nucleotide modifications to the genomic DNA, a DNA repair template containing the desired sequence must be present during HDR. Genome editing cannot be performed using traditional restriction endonucleases since most restriction enzymes recognize a few base pairs on the DNA as their target and the probability is very high that the recognized base pair combination will be found in many locations across the genome resulting in multiple cuts not limited to a desired location. To overcome this challenge and create site-specific single- or double-stranded breaks, several distinct classes of nucleases have been discovered and bioengineered to date. These include the meganucleases, Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENs) and CRISPR/Cas system.

Meganucleases – Meganucleases are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG family are characterized by having either one or two copies of the conserved LAGLIDADG motif. The four families of meganucleases are widely separated from one another with respect to conserved structural elements and, consequently, DNA recognition sequence specificity and catalytic activity. Meganucleases are found commonly in microbial species and have the unique property of having very long recognition sequences (>14bp) thus making them naturally very specific for cutting at a desired location. This can be exploited to make site-specific double-stranded breaks in genome editing. One of skill in the art can use these naturally occurring meganucleases, however the number of such naturally occurring meganucleases is limited. To overcome this challenge, mutagenesis and high throughput screening methods have been used to create meganuclease variants that recognize unique sequences. For example, various meganucleases have been fused to create hybrid enzymes that recognize a new sequence. Alternatively, DNA interacting amino acids of the meganuclease can be altered to design sequence specific meganucleases (see e.g., US Patent 8,021,867). Meganucleases can be designed using the methods described in e.g., Certo, MT et al. Nature Methods (2012) 9:073-975; U.S. Patent Nos. 8,304,222; 8,021,867; 8, 119,381; 8, 124,369; 8, 129,134; 8,133,697; 8,143,015; 8,143,016; 8, 148,098; or 8, 163,514, the contents of each are incorporated herein by reference in their entirety. Alternatively, meganucleases with site specific cutting characteristics can be obtained using commercially available technologies e.g., Precision Biosciences' Directed Nuclease Editor™ genome editing technology.

ZFNs and TALENs – Two distinct classes of engineered nucleases, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have both proven to be

effective at producing targeted double-stranded breaks (Christian *et al.*, 2010; Kim *et al.*, 1996; Li *et al.*, 2011; Mahfouz *et al.*, 2011; Miller *et al.*, 2010).

CRISPR-Cas system - Many bacteria and archaea contain endogenous RNA-based adaptive immune systems that can degrade nucleic acids of invading phages and plasmids. These systems consist of clustered regularly interspaced short palindromic repeat (CRISPR) genes that produce RNA components and CRISPR associated (Cas) genes that encode protein components. The CRISPR RNAs (crRNAs) contain short stretches of homology to specific viruses and plasmids and act as guides to direct Cas nucleases to degrade the complementary nucleic acids of the corresponding pathogen. Studies of the type II CRISPR/Cas system of *Streptococcus pyogenes* have shown that three components form an RNA/protein complex and together are sufficient for sequence-specific nuclease activity: the Cas9 nuclease, a crRNA containing 20 base pairs of homology to the target sequence, and a trans-activating crRNA (tracrRNA) (Jinek et al. *Science (*2012) 337*:* 816–821.). It was further demonstrated that a synthetic chimeric guide RNA (gRNA) composed of a fusion between crRNA and tracrRNA could direct Cas9 to cleave DNA targets that are complementary to the crRNA in vitro. It was also demonstrated that transient expression of Cas9 in conjunction with synthetic gRNAs can be used to produce targeted double-stranded brakes in a variety of different species (Cho *et al.*, 2013; Cong *et al.*, 2013; DiCarlo *et al.*, 2013; Hwang *et al.*, 2013a,b; Jinek *et al.*, 2013; Mali *et al.*, 2013).

The CRIPSR/Cas system for genome editing contains two distinct components: a gRNA and an endonuclease e.g. Cas9.

The gRNA is typically a 20 nucleotide sequence encoding a combination of the target homologous sequence (crRNA) and the endogenous bacterial RNA that links the crRNA to the Cas9 nuclease (tracrRNA) in a single chimeric transcript. The gRNA/Cas9 complex is recruited to the target sequence by the base-pairing between the gRNA sequence and the complement genomic DNA. For successful binding of Cas9, the genomic target sequence must also contain the correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The binding of the gRNA/Cas9 complex localizes the Cas9 to the genomic target sequence so that the Cas9 can cut both strands of the DNA causing a double-strand break. Just as with ZFNs and TALENs, the double-stranded brakes produced by CRISPR/Cas can undergo homologous recombination or NHEJ.

The Cas9 nuclease has two functional domains: RuvC and HNH, each cutting a different DNA strand. When both of these domains are active, the Cas9 causes double strand breaks in the genomic DNA.

A significant advantage of CRISPR/Cas is that the high efficiency of this system coupled with the ability to easily create synthetic gRNAs enables multiple genes to be targeted simultaneously. In addition, the majority of cells carrying the mutation present biallelic mutations in the targeted genes.

However, apparent flexibility in the base-pairing interactions between the gRNA sequence and the genomic DNA target sequence allows imperfect matches to the target sequence to be cut by Cas9.

Modified versions of the Cas9 enzyme containing a single inactive catalytic domain, either RuvC- or HNH-, are called 'nickases'. With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or 'nick'. A single-strand break, or nick, is normally quickly repaired through the HDR pathway, using the intact complementary DNA strand as the template. However, two proximal, opposite strand nicks introduced by a Cas9 nickase are treated as a double-strand break, in what is often referred to as a 'double nick' CRISPR system. A double-nick can be repaired by either NHEJ or HDR depending on the desired effect on the gene target. Thus, if specificity and reduced off-target effects are crucial, using the Cas9 nickase to create a double-nick by designing two gRNAs with target sequences in close proximity and on opposite strands of the genomic DNA would decrease off-target effect as either gRNA alone will result in nicks that will not change the genomic DNA.

Modified versions of the Cas9 enzyme containing two inactive catalytic domains (dead Cas9, or dCas9) have no nuclease activity while still able to bind to DNA based on gRNA specificity. The dCas9 can be utilized as a platform for DNA transcriptional regulators to activate or repress gene expression by fusing the inactive enzyme to known regulatory domains. For example, the binding of dCas9 alone to a target sequence in genomic DNA can interfere with gene transcription.

There are a number of publically available tools available to help choose and/or design target sequences as well as lists of bioinformatically determined unique gRNAs for different genes in different species such as the Feng Zhang lab's Target Finder, the Michael Boutros lab's Target Finder (E-CRISP), the RGEN Tools: Cas-OFFinder, the CasFinder: Flexible algorithm for identifying specific Cas9 targets in genomes and the CRISPR Optimal Target Finder.

In order to use the CRISPR system, both gRNA and Cas9 should be expressed in a target cell. The insertion vector can contain both cassettes on a single plasmid or the cassettes are expressed from two separate plasmids. CRISPR plasmids are commercially available such as the px330 plasmid from Addgene.

Genome editing using recombinant adeno-associated virus (rAAV) platform - this genome-editing platform is based on rAAV vectors which enable insertion, deletion or substitution of DNA sequences in the genomes of live mammalian cells. The rAAV genome is a single-stranded deoxyribonucleic acid (ssDNA) molecule, either positive- or negative-sensed, which is about 4.7 kb long. These single-stranded DNA viral vectors have high transduction rates and have a unique property of stimulating endogenous homologous recombination in the absence of double-strand DNA breaks in the genome. One of skill in the art can design a rAAV vector to target a desired genomic locus and perform both gross and/or subtle endogenous gene alterations in a cell. rAAV genome editing has the advantage in that it targets a single allele and does not result in any off-target genomic alterations. rAAV genome editing technology is commercially available, for example, the rAAV GENESIS™ system from Horizon™ (Cambridge, UK).

Methods for qualifying efficacy and detecting sequence alteration are well known in the art and include, but not limited to, DNA sequencing, electrophoresis, an enzyme-based mismatch detection assay and a hybridization assay such as PCR, RT-PCR, RNase protection, in-situ hybridization, primer extension, Southern blot, Northern Blot and dot blot analysis.

Sequence alterations in a specific gene can also be determined at the protein level using e.g. chromatography, electrophoretic methods, immunodetection assays such as ELISA and western blot analysis and immunohistochemistry.

Ribozymes

Another agent capable of downregulating ubiquitinating enzyme is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding the ubiquitinating enzyme. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme

Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

DNAzymes

Another agent capable of downregulating ubiquitinating enzyme is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of ubiquitinating enzyme. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Down-regulation at the polypeptide level

One example, of an agent capable of downregulating a ubiquitinating enzyme is an antibody or antibody fragment capable of specifically binding ubiquitinating enzyme. Preferably, the antibody specifically binds at least one epitope of the ubiquitinating enzyme. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

As the ubiquitinating enzyme is typically localized intracellularly, an antibody or antibody fragment capable of specifically binding MLKL, RIPK3 or RIPK1 is typically an intracellular antibody.

It will be appreciated that targeting of a particular compartment within the cell can be achieved using intracellular antibodies (also known as “intrabodies”). These are essentially single chain antibodies to which intracellular localization signals have been added (e.g., ER, mitochondrial, nuclear, cytoplasmic). This technology has been successfully applied in the art (for review, see Richardson and Marasco, 1995, TIBTECH vol. 13: 306-310). Intrabodies have been shown to virtually eliminate the expression of otherwise abundant cell surface receptors and to inhibit a protein function within a cell (See, for example, Richardson et al., 1995, Proc. Natl. Acad.

Sci. USA 92: 3137-3141; Deshane et al., 1994, Gene Ther. 1: 332-337; Marasco et al., 1998 Human Gene Ther 9: 1627-42; Shaheen et al., 1996 J. Virol. 70: 3392-400; Werge, T. M. et al., 1990, FEBS Letters 274:193-198; Carlson, J.R. 1993 Proc. Natl. Acad. Sci. USA 90:7427-7428; Biocca, S. et al., 1994, Bio/Technology 12: 396-399; Chen, S-Y. et al., 1994, Human Gene Therapy 5:595-601; Duan, L et al., 1994, Proc. Natl. Acad. Sci. USA 91:5075-5079; Chen, S-Y. et al., 1994, Proc. Natl. Acad. Sci. USA 91:5932-5936; Beerli, R.R. et al., 1994, J. Biol. Chem. 269:23931-23936; Mhashilkar, A.M. et al., 1995, EMBO J. 14:1542-1551; PCT Publication No. WO 94/02610 by Marasco et al.; and PCT Publication No. WO 95/03832 by Duan et al.).

To prepare an intracellular antibody expression vector, the cDNA encoding the antibody light and heavy chains specific for the target protein of interest are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for the marker. Hybridomas secreting anti-marker monoclonal antibodies, or recombinant monoclonal antibodies, can be prepared using methods known in the art. Once a monoclonal antibody specific for the marker protein is identified (e.g., either a hybridoma-derived monoclonal antibody or a recombinant antibody from a combinatorial library), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from the display package (e.g., phage) isolated during the library screening process and the nucleotide sequences of antibody light and heavy chain genes are determined. For example, many such sequences are disclosed in Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database. Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods.

For cytoplasmic expression of the light and heavy chains, the nucleotide sequences encoding the hydrophobic leaders of the light and heavy chains are removed. An intracellular antibody expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes full-length antibody light and heavy chains such that a full-length antibody is expressed intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed intracellularly. In another embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible

peptide linker [e.g., $(Gly_4Ser)_3$ and expressed as a single chain molecule. To inhibit marker activity in a cell, the expression vector encoding the intracellular antibody is introduced into the cell by standard transfection methods, as discussed hereinbefore.

Once antibodies are obtained, they may be tested for activity, for example via ELISA.

Another agent which can be used along with some embodiments of the invention to downregulate the ubiquitinating enzyme is an aptamer. As used herein, the term "aptamer" refers to double stranded or single stranded RNA molecule that binds to specific molecular target, such as a protein. Various methods are known in the art which can be used to design protein specific aptamers. The skilled artisan can employ SELEX (Systematic Evolution of Ligands by Exponential Enrichment) for efficient selection as described in Stoltenburg R, Reinemann C, and Strehlitz B (Biomolecular engineering (2007) 24(4):381-403).

Another way of downregulating ubiquitination at position 50 of MLKL is by upregulating a deubiquitinating enzyme capable of deubiquitinating ubiquitinated MLKL.

Methods of upregulating expression of proteins (including deubiquitinating enzymes) are provided herein above (with respect to ubiquitinating enzymes).

Still another way of downregulating ubiquitination at position 50 of MLKL is by administration of a peptide agent that is a substrate for ubiquitination by the E3 ubiquitin-protein ligase. Thus, for example peptide agents derived from MLKL which comprise the ubiquitination site at position 50 are contemplated. The peptide agents typically comprise at least 5, 6, 7, 8, 9, 10, 15, 20 or more amino acids of MLKL. In one embodiment, the peptide comprises between 5-50, 5-40, 5-30, 5-20 amino acids of MLKL. The peptide may be attached to a cell penetrating moiety.

As used herein the phrase "penetrating agent" refers to an agent which enhances translocation of an attached polypeptide across a cell membrane.

According to one embodiment, the penetrating agent is a peptide and is attached to the C or N terminus of the peptide (either directly or non-directly) via a peptide bond.

Typically, cell penetrating peptides have an amino acid composition containing either a high relative abundance of positively charged amino acids such as lysine or arginine, or have sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids.

According to a particular embodiment, the peptides of the present invention are attached to the cell penetrating peptides via a linking moiety.

The agents which down-regulate ubiquitination of lysine at position 50 of MLKL may be provided to the subject for the treatment of the cancers specified herein with agents that activates the TRAIL apoptotic pathway.

Examples of agents which activate the TRAIL apoptotic pathway include Mapatumumab, Contatumumab, TAS266, ONC201, Tigatuzumab, Dulanermin and Circularly permuted TRAIL (CPT).

Additional agents known to activate the TRAIL apoptotic pathway are provided in Yuan et al., Cancer Metastasis Rev. 2018 December ; 37(4): 733–748, the contents of which are incorporated herein by reference.

The agents which activate the TRAIL apoptotic pathway may be co-formulated with the agents which down-regulate ubiquitination of lysine at position 50, or may be provided as separate compositions to the subject.

Thus, each agent included in the combination can be formulated separately for use in combination. The drugs are said to be used "in combination" when, in a recipient of both drugs, the effect of one drug enhances or at least influences the effect of the other drug.

The two agents in the combination cooperate to provide an effect on target cells that is greater than the effect of either drug alone. This benefit manifests as a statistically significant improvement in a given parameter of target cell fitness or vitality. In embodiments, the improvement resulting from treatment with the drug combination can manifest as an effect that is at least additive and desirably synergistic, relative to results obtained when only a single agent is used.

In use, each drug in the combination can be formulated as it would be for monotherapy, in terms of dosage size and form and regimen. In this regard, the synergy resulting from their combined use may permit the use of somewhat reduced dosage sizes or frequencies, as would be revealed in an appropriately controlled clinical trial.

According to one embodiment, the agent which down-regulates ubiquitination of lysine at position 50 of MLKL and the agent which activates the TRAIL apoptotic pathway are administered concomitantly.

According to another embodiment, the agent which activates the TRAIL apoptotic pathway and the agent which down-regulates ubiquitination of lysine at position 50 of MLK are administered sequentially, wherein the first agent is used, for example, 30 minutes, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, a week, a month or more after the second agent. Such a determination is well within the capacity of one of skill in the art. In another embodiment, the agent

which activates the TRAIL apoptotic pathway and the agent which down-regulates ubiquitination of lysine at position 50 of MLK are administered sequentially, wherein the second agent is used, for example, 30 minutes, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, a week, a month or more after the first agent.

The agents of the present invention may be provided per se or as part of a pharmaceutical composition.

which also includes a physiologically acceptable carrier. The purpose of a pharmaceutical composition is to facilitate administration of the active ingredient to an organism.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the downregulating agents, ligands or population of exosomes accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in “Remington’s Pharmaceutical Sciences,” Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport

peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

The term "tissue" refers to part of an organism consisting of cells designed to perform a function or functions. Examples include, but are not limited to, brain tissue, retina, skin tissue, hepatic tissue, pancreatic tissue, bone, cartilage, connective tissue, blood tissue, muscle tissue, cardiac tissue brain tissue, vascular tissue, renal tissue, pulmonary tissue, gonadal tissue, hematopoietic tissue.

Pharmaceutical compositions of some embodiments of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which 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 may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver

a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (e.g. downregulating agents, ligands or population of exosomes) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., a disease associated with activation of a necroptosis activation pathway, necroptosis or inflammation) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be

formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Below is a list of some exemplary animal models that can be used to determine the efficacy of agents that down-regulate ubiquitination of lysine at position 50 of MLKL.

Tumor formation in transgenic mice overexpressing an oncogene – A transgenic mouse model for cancer (e.g., breast cancer) such as the erb model (Shah N., et al., 1999, Cancer Lett. 146: 15-2; Weistein EJ., et al., 2000, Mol. Med. 6: 4-16) or MTV/myc model (Stewart TA et al., 1984, Cell, 38: 627-637), the c-myc model (Leder A., et al., 1986, Cell, 45:485-495), v-Ha-ras or c-neu model (Elson A and Leder P, 1995, J. Biol. Chem. 270: 26116-22) can be used to test the ability of agents that down-regulate ubiquitination of lysine at position 50 of MLKL to suppress tumor growth in vivo.

Tumor formation in mice administered with cancerous cell lines – For the formation of solid tumors, athymic mice can be injected with human or animal (e.g., mouse) cancerous cells such as those derived from breast cancer, ovarian cancer, prosate cancer or thyroid cancer, and following the formation of cancerous tumors the mice can be subjected to intra-tumor and/or systemic administration of an agent capable of down-regulating ubiquitination of lysine at position 50 of MLKL.

The following cell lines (provided with their ATCC Accession numbers) can be used for each type of cancer model:

For breast cancer:

Human breast cancer cell lines – MDA-MB-453 (ATCC No. HTB-131), MDA-MB-231 (ATCC No. HTB-26), BT474 (ATCC No. HTB-20), MCF-7 (ATCC No. HTB-22) and MDA-MB-468.

For ovarian cancer:

Human ovarian cancer cell lines – SKOV3 (ATCC No. HTB-77), OVCAR-3 HTB-161), OVCAR-4, OVCAR-5, OVCAR-8 and IGROV1;

For prostate cancer:

Human prostate cancer cell lines – DU-145 (ATCC No. HTB-81), PC-3 (ATCC No.CRL-1435);

For thyroid cancer:

Human derived thyroid cancer cell lines – FTC-133, K1, K2, NPA87, K5, WRO82-1, ARO89-1, DRO81-1;

For lung cancer:

Mouse lung carcinoma LL/2 (LLC1) cells (Lewis lung carcinoma) – These cells are derived from a mouse bearing a tumor resulting from an implantation of primary Lewis lung carcinoma. The cells are tumorigenic in C57BL mice, express H-2b antigen and are widely used as a model for metastasis and for studying the mechanisms of cancer chemotherapeutic agents (Bertram JS, et al., 1980, Cancer Lett. 11: 63-73; Sharma S, et al. 1999, J. Immunol. 163: 5020-5028).

For melanoma:

Mouse B16-F10 cells (Melanoma) – The cells are derived from mouse (C57BL/6J) bearing melanoma (Briles EB, et al., 1978, J. Natl. Cancer Inst. 60: 1217-1222).

Tumor formation in animal models by administration of cancerous cells - Athymic *nu/nu* mice (e.g., female mice) can be purchased from the Jackson Laboratory (Bar Harbor, ME). Tumors can be formed by subcutaneous (s.c.) injection of cancerous cells (e.g., 2 x 10^6 cells in 100 µl of PBS per mouse). Tumors are then allowed to grow *in vivo* for several days (e.g., 6-14 days) until they reach a detectable size of about 0.5 cm in diameter. It will be appreciated that injection of cancerous cells to an animal model can be at the organ from which the cell line is derived (e.g., mammary tissue for breast cancer, ovary for ovarian cancer) or can be injected at an irrelevant tissue such as the rear leg of the mouse.

Modes of administration of MLKL ubiquitination inhibitors to tumor - To test the effect of MLKL ubiquitination inhibitors on inhibition of tumor growth, the inhibitor is administered to the animal model bearing the tumor either locally at the site of tumor or systemically, by intravenous injection of infusion, via, e.g., the tail vein. The time of administration may vary from immediately following injection of the cancerous cell line (e.g., by systemic administration) or at predetermined time periods following the appearance of the solid tumor (e.g., to the site of tumor

formation, every 3 days for 3-10 times as described in Ugen KE et al., Cancer Gene Ther. 2006 Jun 9; [Epub ahead of print]).

Evaluation of solid tumor inhibition **-** Tumor sizes are measured two to three times a week. Tumor volumes are calculated using the length and width of the tumor (in millimeters). The effect of MLKL ubiquitination inhibitor treatment can be evaluated by comparing the tumor volume using statistical analyses such as Student's *t* test. In addition, histological analyses can be performed using markers typical for each type of cancer.

Altogether, once the tumors are formed, the agent capable of down-regulating MLKL ubiquitination at position 40 is administered to the individual in need thereof, e.g., the animal model bearing the tumor, either locally or systemically, and the effect of the agent on tumor growth is detected using methods known in the art.

Models for testing agents that up-regulate ubiquitination of lysine at position 50 of MLKL for treatment of IBD include dextran sulfate sodium-induced colitis, Oxazolone-induced colitis, acetic acid-induced colitis and salmonella-induced colitis.Transgenic and gene knockout animal models of ulcerative colitis include IL-7 Tg mice and TCRα KO mice.

Dosage amount and interval may be adjusted individually to provide the active ingredient at a sufficient amount to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

As used herein the term "about" refers to ± 10 %

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps

and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

The subject of the present invention typically refers to a mammalian subject – e.g. a human subject.

The present inventors further propose that uncovering the position on which MLKL is ubiquitilated, paves the way for a novel screening assay which can be used to identify agents that are capable of treating diseases associated with a necroptosis pathway.

Such diseases include, but are not limited to a renal disease, a pulmonary disease, a hepatic disease, cancer, infectious disease, neurodegenerative disease, inflammatory bowel disease, ischemic reperfusion, psoriasis and a cardiovascular disease. Further diseases are described in Choi et al., doi.org/10.1172/jci.insight.128834, the contents of which are incorporated herein by reference.

Thus, according to another aspect of the present invention, there is provided a method of identifying an agent capable of treating a disease associated with activation of a necroptosis pathway comprising:

(a) contacting the agent with MLKL or a polynucleotide encoding same;

(b) analyzing ubiquitination of lysine at position 50 of said MLKL, wherein a change in the amount of ubiquitination at said position is indicative of an agent that is capable of treating the disease associated with a activation of the necroptosis pathway. The method may be carried out in a cell mixture or a cell-free mixture, comprising MLKL and the necessary components for ubiquitination. Ubiquitination may be observed by any means suitable for detection of protein ubiquitination - such as mass spectroscopy and additional methods described in the Examples section herein below. An exemplary method for detecting ubiquitination is disclosed in US Patent Application No. 2019-0185902, the contents of which are incorporated herein by reference.

Depending on whether the agent causes an up or down regulation in the amount of ubiquitination the agent can be proposed as a candidate for treating a particular disease. Preferably, the agent causes at least a 10 %, 20 %, 30 %, 40 %, 50 % or greater increase/decrease in the amount of ubiquitination of MLKL compared to a control assay which is carried out in the absence of the agent.

Additional experiments may be carried out to corroborate that the agent is a suitable agent for treating the disease - both *in vitro* and *in vivo*, for example using animal models for the disease.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); “Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

MATERIALS AND METHODS

Cell culture: Cells of the human HT-29 colorectal adenocarcinoma line were grown in McCoy's 5A medium. Normal mouse embryonic fibroblasts (MEFs) and embryonic fibroblasts of MLKL-knockout mice were immortalized by expression of the SV40 large T antigen. MEFs and human embryonic kidney (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium. The cell-growth media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Gene knockout and knockdown in vitro in human HT-29 cells: MLKL was knocked out *in vitro* using the MLKL CRISPR/Cas9 KO plasmid (Santa Cruz, sc-401248) that was transfected with Lipofectamine 3000. FADD was knocked out by lentiviral infection with two gRNA sequences (5'-GTTCCTATGCCTCGGGCGCGT (SEQ ID NO: 1) and 5'-ACGCGCCCGAGGCATAGGAAC (SEQ ID NO: 2)) in the pX459 plasmid (Addgene, 62988). For knockdown of ITCH, SMARTpool ON-TARGETplus human ITCH siRNA(L-007196-00-0050, Dharmacon) was transiently tranfected into HT-29 cells, and the cells were further incubated for 3 days.

Constitutive and inducible expression of proteins: Unless otherwise indicated, transfected MLKL and its various mutants were fused C-terminally to a double strep tag (**WSHPQFEK**-GGGSGGGSGGGS-**WSHPQFEK** - SEQ ID NO: 3). The human MLKL L357A/S358A, L58G/I76G and L162G/165G mutants (and, as a control, the wild-type protein) were expressed constitutively in MLKL knocked-down cells as previously described (Yoon, 2016 #4038).

The GEV16/pF5x UAS system (*30*) was used to inducibly express, in MLKL knockout cells, the various human and mouse MLKL mutants in which lysine residues were replaced with arginine (and, as controls, the wild-type proteins) and human MLKL fused C-terminally to green fluorescent protein (GFP). In the experiment presented in Figure 3J, this system was also used for the expression of ITCH and the ITCH C830A mutant that were fused C-terminally to a myc tag (EQKLISEEDL (SEQ ID NO: 4)). Unless otherwise indicated, induction by treatment with 4HT (1 μM) was carried out for 6 h. In the experiment presented in Figure 3D, the various ITCH-deletion mutants were fused C-terminally to the FLAG-tag (DYKDDDDK - SEQ ID NO: 5). HA-ubiquitin and its K63R mutant, as well as Rab5 and Rab7 fused N-terminally to mCherry, were expressed constitutively by lentiviral infection. Transfections for transient expression of genes were done by Lipofectamine 3000 for HT-29 cells and by JetPEI for HEK293T cells.

Reagents: Bafilomycin A1 (11038-500) and pepstatin A (9000469-10) were from Cayman Chemical, and MG132 (M7449), chloroquine (C6628), and 4-hydroxytamoxifen (H6278) were from Sigma-Aldrich. Human EGF from ProSpec was biotinylated using an EZ-Link Sulfo-NHS-biotin kit from Thermo Fisher Scientific (21326). TNF was from YbdY Biotech, and the bivalent IAP (inhibitor of apoptosis protein) antagonist BV6 and the caspase inhibitor z-VAD-fmk were both from WuXi App Tec. Strep-Tactin Sepharose 50% suspension (2-1201-010) and Strep-Tactin elution buffer (2-1042-025) were from IBA Lifesciences. Agarose-coupled TUBE1 (UM401) was from LifeSensors. Brij O10 (polyoxyethylene (10) oleyl ether, P6136) was from Sigma-Aldrich. The Lipofectamine 3000 transfection reagent (L3000015) was from ThermoFisher, and the JetPEI transfection reagent (101-10N) was from Polyplus-transfection. LysoTracker™ Red DND-99 (L7528) was from ThermoFisher. The cDNAs encoding wild-type HA-tagged ubiquitin (17608) were from Addgene.

Antibodies: The following antibodies were applied for Western blot analysis: anti-mouse MLKL (Sab1302339), Anti-HA (H6908), anti β-actin (A5441), anti-Flag M2 (13026) and anti-MVB12B (HPA043683) from Sigma; anti-human MLKL (ab184718), anti-human phospho-MLKL (ab187091), and anti-Rab7 (ab 137029) from Abcam; anti-TSG101 (612696), anti-ITCH (611199), anti-EEA1 (610456) and anti-phosphotyrosine (61000) from BD Biosciences; anti-LDH (sc-27230), anti-STIM-1 (sc-166840) and anti-TOM20 (sc-17764) from Santa Cruz Biotechnology; anti-ubiquitin (VU-1) from LifeSensors; anti-myc tag (05-724) from Millipore; anti-EGFR (6F1) from Enzo Life Sciences; anti-HSP70 (EXOAB-Hsp70A-1) from System Biosciences; anti-Strep tag (34850) from Qiagen; anti-Na+/K+ ATPase (ANP-001) from Alomone Labs, and anti-VPS28 (NBP1-85976) from Novus Biologicals. Horseradish peroxidase-conjugated antibodies from Jackson ImmunoResearch were used for western blot analyses. Normal rabbit IgG control (3900) was from Cell Signaling.

For immunofluorescence analysis Alexa Fluor 647-conjugated anti-mouse IgG (715-605-150) was used and Cy3-conjugated anti-rabbit IgG (111-165-144) from Jackson ImmunoResearch, anti-Lamp1-Alexa Fluor 647 (NBP2-25183AF647, Novus Biologicals) and Alexa Fluor 405-conjugated anti-mouse IgG (ab175660) from Abcam. For staining of infected *Listeria* anti-Listeria antibody (ab35132) from Abcam was used. Ubiquitin was stained by anti-ubiquitin antibody (FK2, BML-PW8810-0500) from Enzo Life Sciences. For visualization of early and late endosomes, either Rab5 or Rab7 fused with mCherry was expressed constitutively. In the experiments presented in Figure 6E and F, CellLight Early Endosomes-GFP (C10586) and CellLight ER-GFP (C10590) (Thermo Fisher) was constitutively expressed in the cells, and these cells were used to

assess co-localization of MLKL with early endosomes and the endoplasmic reticulum, respectively, according to the manufacturer's protocol.

Immunoprecipitation and Western analyses: Proteins were immunoprecipitated as described (*14*). To estimate the extent of oligomerization of MLKL, cells were extracted with 1% NP-40 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40) including protease and phosphatase inhibitors (1 mM PMSF, 40 mM beta-glycerophosphate, 50 mM NaF and 1 mM sodium vanadate), and this was followed by solubilization in Laemmli SDS–PAGE buffer devoid of reducing agent, as described (*12*). In Western analyses where some nonspecific bands could be seen, specific bands are indicated by arrows. To assess interactions of proteins with MLKL, HT-29 cells constitutively expressing MLKL that was fused C-terminally to the double Strep-tag (*31*) were used. Interactions of ESCRT proteins with MLKL were assessed following extraction of the cells in a buffer containing 1% Brij, as described (*14*). Immunoblotting was done as described (*9*). For identification of the binding site for MLKL in ITCH (Fig.ure 3D), MLKL was eluted by biotin after its precipitation by Strep-Tactin magnetic beads.

Cytological and immunocytological analyses: For immunocytological analyses, cells were fixed with 4% paraformaldehyde for 15 min at room temperature in phosphate buffered saline (PBS), permeabilized with ice-cold methanol for 10 min at −20°C, and blocked with MAXblock Blocking Medium (Active Motif, 15252) overnight at 4°C. The cells were then incubated with the indicated antibodies for 1 h at room temperature, and then with fluorescent dye-conjugated secondary antibodies for 2 h at 4°C in MAXbind Staining Medium (Active Motif, 15253).

Images were acquired with an Olympus IX 81 confocal microscope (Olympus Imaging) using the UPLSAPO 60 x /1.35 NA oil objective, and were processed using the FluoView FV1000 (Olympus Imaging) and ImageJ (NIH) software. Colocalization was determined manually using unsaturated single Z-stack images, by measuring the overlap of signals between channels with constant threshold values. Data are presented as means (%) ± standard deviations (S.D.) in approximately equal numbers of images acquired in at least 3 independent experiments; *P* values were calculated using a two-tailed Student's t-test.

Collection of extracellular vesicles: Extracellular vesicles generated by TBZ-treated HT-29 cells were collected 3 h after TBZ application and were isolated as previously described (*14*).

Assessment of EGF uptake and of uptake and phosphorylation of its receptor: Western blot analyses of the uptake of biotinylated EGF and of the uptake and phosphorylation of the EGF receptor were as previously described (*14*).

Quantification of cell death: Cell death was quantified based on the concentration of lactic dehydrogenase (LDH) in the cell-culture media, using the Cytotoxicity Detection Kit 4744934001 (Sigma-Aldrich).

Assessment of MLKL ubiquitination: Samples of 6 million HT-29 cells and 2 million MEFs, seeded 1 day prior to the experiment, were treated with 1 μM 4HT for 12 h to induce expression of wild-type or mutant MLKL. Ubiquitinated MLKL was isolated from cell extracts using TUBE1-agarose (LifeSensors) according to the manufacturer's protocol, except that the extraction buffer also contained protease and phosphatase inhibitors (1 mM PMSF, 40 mM beta-glycerophosphate, 50 mM NaF, and 1 mM sodium vanadate).

For assessment of the incorporation of HA-tagged ubiquitin chains into MLKL, wild-type and mutant HA-tagged ubiquitins were first expressed constitutively by lentiviral infection in cells inducibly expressing MLKL fused to Strep-tag. Following further growth of the cells for 12 h and, when indicated, stimulation by TBZ, the cells were lysed by boiling in a buffer containing 1% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM dithiothreitol (DTT). This was followed by 10-fold dilution of the lysates in a buffer containing 1% NP-40, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA and the protease and phosphatase inhibitors specified above. MLKL was affinity-purified using Strep-Tactin beads, and conjugation of the tagged ubiquitin chains to it was assessed by western blotting using anti-HA antibody.

Comparison of the effects of various deubiquitinases on the ubiquitin chains conjugated to MLKL: For the *in-vitro* MLKL ubiquitination assay, wild-type or K50R MLKL, affinity-purified by Strep-Tactin beads following denaturation in SDS as described above, were incubated for 1 h at 37°C with recombinant E1 (UBE1, E-305; 50 nM), E2 (UBE2L3, E2-640; 200 nM) and E3 (ITCH, E3-260; 1 μM) enzymes, ubiquitin (U-100H, 80 μM), Mg-ATP (B-20, 10 mM) and 10× E3 ligase reaction buffer (B-71).

To assay the deubiquitination *in vitro*, MLKL, affinity-purified by Strep-Tactin beads or—as controls—synthetic K63-linked and K48-linked tetra-ubiquitin chains (UM-K630 and UM-K480, R&D Systems) were incubated with the indicated recombinant deubiquitinases (USP2(E-506), 200 nM; Otubain1(E-522B), 1 μM; AMSH(E549), 1 μM; CYLD(E-556), 20 nM), for 1 h at 37°C in a reaction buffer containing 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 25 mM KCl and 1 mM DTT. All enzymes and recombinant proteins were from R&D Systems.

Isolation of subcellular fractions: Subconfluent cultures of HT-29 cells in eight 150-mm plates were incubated with TBZ for 3 h. After the cells were rinsed twice with PBS they were collected by scraping, and homogenized in a buffer containing 225 mM mannitol, 75 mM sucrose,

0.5 mM EGTA and 30 mM Tris-HCl pH7.4, using a loose-fitting glass/Teflon Potter-Elvehjem homogenizer and a 22-gauge syringe. Fractionation was performed by differential centrifugation, as previously described (*32*).

Listeria monocytogenes infection and quantification: HT-29 cells and MEFs were seeded 2 days before infection and incubated, prior to infection, for 10 h in BIOGRO-2 serum-free medium (Biological Industries) without antibiotics. *Listeria monocytogenes* (strain 10403S, kindly provided by Dr. Steffen Jung), was applied in serum-free medium to the HT-29 cells at a multiplicity of infection (MOI) of 20, and to the MEFs at a MOI of 5, and this was followed by centrifugation at 300 x g for 10 min and incubation at 37°C for 1 h to allow invasion of the bacteria. The cells were then rinsed with PBS, incubated with 100 μg/ml gentamycin for 10 min and then with fresh growth medium containing 5 μg/ml of gentamicin. Subsequent treatments of the cells were as described in Figure 8.

To quantify the intracellularly grown bacteria, the infected cells were rinsed twice with PBS and then lysed in 0.2% Triton X-100 in PBS (1 ml per 2.5-cm well). After serial dilutions the lysates were spread on Brain Hear Infusion (BHI) agar plates (BD diagnostic, 211065). Values presented in the figures are means ± S.D. of colony forming units (CFU); *P* values were calculated using a two-tailed Student's t-test.

RESULTS

Besides phosphorylating MLKL, treatment of cells by agents that activate the protein kinase RIPK3 also triggers a time-dependent increase in MLKL ubiquitination (Figures 1A and 5A–C). Mutational ablation of the phosphorylation (*4*) or of the oligomerization of MLKL(*12*) also prevents its ubiquitination. In contrast, a mutation yielding spontaneous oligomerization of MLKL (L58G/I76G) results in spontaneous ubiquitination, suggesting that this ubiquitination is dictated by a conformational change that occurs in MLKL upon its oligomerization (Figure 1B).

Treatment of cells with a proteasomal inhibitor did not result in increase in ubiquitinated MLKL. Nor was such an increase observed in cells treated with bafilomycin A1, which arrests lysosomal degradation (Fig. 1C). Both comparing the effectiveness of incorporation of wildtype and of K63R mutant ubiquitin into the MLKL-conjugated ubiquitin chains (Figure 5D) and assessing the effects of various deubiquitinases on ubiquitinated MLKL (Figure 5E) indicated that the ubiquitin moieties in these chains are interlinked via their K63 residues.

In a mutational study in which different lysine residues in MLKL were replaced with arginine, It was found that mutation of a single residue in human MLKL (K50) and of two adjacent ones in mouse MLKL (K50 and K51) in the loop that connects helices a2 and a3 in the MLKL

4HB domain fully abolished the ubiquitination (Figures 1D–G and 5C). These findings suggested that ubiquitination affects only specific residues in MLKL and does not serve to dictate its degradation.

In comparing the amounts of ubiquitinated MLKL in various subcellular fractions, this protein was found to be specifically associated with the microsomal membranes (Figure 2A).

Consistently, immunostaining of human colon cancer HT-29 cells following activation of MLKL in them revealed colocalization of MLKL with Rab5 and with Rab7—markers of early and late endosomes, respectively—as well as co-association of ubiquitin and MLKL with these endosomal markers. In contrast, cells in which lysine 50 in MLKL was replaced with arginine (K50R) did not display such associations (Figures 2B-I and 6A-D), nor was association of ubiquitinated MLKL with the endoplasmic reticulum or with the mitochondria observed (Figure 6E, F).

It was previously found that the association of MLKL with endosomal membranes occurs pursuant to its binding to certain components of the ESCRT system and that it eventually results in exclusion of these MLKL molecules in EVs (*14*). As shown in Figure 2J, the MLKL molecules found in EVs are highly ubiquitinated, to a much greater extent than those found intracellularly. Arrest of MLKL ubiquitination by the K50R mutation greatly decreased the association of MLKL with ESCRT proteins as well as its exclusion within EVs (Figure 2K, L). It thus seems that the association of MLKL with endosomes and its eventual exclusion in EVs are dictated by its ubiquitination.

On examining the list of MLKL-binding proteins that were identified in the exosomes, it was found that these proteins also contain the ubiquitin ligase ITCH (*16, 17*) (Fig. 3A). Consistently, it was found that transiently overexpressed ITCH and MLKL effectively bind to each other (Figure 3B) and that oligomerization of MLKL prompts its association with ITCH (Figure 3C). Characteristically of HECT-type E3 ligases, ITCH was found to bind MLKL via its WW motifs (Figure 3D, E). Immunostaining confirmed that following phosphorylation of MLKL, in response to combined treatment with TNF, BV6 and zVAD (TBZ), it associates with ITCH in both early and late endosomes (Figure 3F, G). In an *in-vitro* test, ITCH catalyzed the ubiquitination of MLKL but not of its K50R mutant (Figure 3H). Overexpression of ITCH consistently enhanced the ubiquitination of MLKL but not of K50R MLKL (Figure 3I). It also enhanced the association of MLKL with ESCRT proteins, whereas overexpression of an enzymatically dead ITCH mutant compromised MLKL association with those proteins (Figure 3J). These findings indicate that ITCH contributes to the ubiquitination of MLKL.

The association of MLKL with endosomes has been found to have two functional consequences—facilitation of endosomal trafficking of certain cell-surface receptors, and decrease in cell-death induction, the latter apparently as a result of the release of phosphorylated MLKL in EVs (*14*). Prevention of this association by mutation of those lysine residues in MLKL that are ubiquitinated was found here to consistently result in a significant decrease in the rate of degradation of cell-bound epidermal growth factor (EGF) and tumor necrosis factor (TNF), as well as of the EGF receptor, in cells in which MLKL phosphorylation was induced (Figure 7A, B), and it increased the effectiveness of necroptosis induction (Figure 7C, D).

MLKL was recently shown to suppress the growth of *Listeria monocytogenes* (*18*). *Listeria* enters cells by co-opting receptor-uptake mechanisms. The extent to which the *Listeria* cells are then destroyed in the lysosomes, or keep replicating in the cytoplasm or in membrane encased niches, is subject to modulation by antagonistic mechanisms elaborated by this pathogen and its host cells (e.g. (*19-22*)). It was therefore decided to examine whether the ubiquitination-dependent association of MLKL with endosomes contributes to its effect on these bacteria.

Both in mouse embryonic fibroblasts (MEFs) (Figure 4A) and in human HT-29 cells (Figure 4B, C), MLKL was found to have an inhibitory effect on *Listeria* replication, and that this was enhanced by treatment with TBZ. As with the ubiquitination of MLKL, this anti-Listerial effect seemed to be exerted as a result of MLKL oligomerization (Figure 4D). It was observed significantly earlier than any sign of cell death (Figure 4E) and, unlike the cytotoxic effect, was abolished by mutation of the ubiquitination sites in MLKL (Figure 4A−C). It was also eliminated by knockdown of ITCH (Figure 4F). Knockout of MLKL resulted in enhancement of Listerial growth to higher levels than those observed in cells expressing the non-ubiquitinatable MLKL mutant. In cells infected for 24 hours, the relative extent of the inhibitory effect of nonubiquitinated MLKL on Listerial growth, compared to that of ubiquitinated MLKL, was greater than in cells infected for only 5 hours (compare Figure 4C to 4B). Treating the HT-29 cells with chloroquine or with a cathepsin D inhibitor fully blocked the inhibitory effect of TBZ on Listerial growth in MLKL-expressing cells, while having no effect on the extent of inhibition of Listerial growth that resulted from mere expression of MLKL in the cells (Fig. 4G-J).

These findings suggested that MLKL affects Listerial growth both constitutively and in a way that is enhanced upon phosphorylation and ubiquitination of MLKL. They further suggest that the anti-Listerial effect induced upon phosphorylation and ubiquitination of MLKL depends on lysosome functioning, whereas the constitutive anti-Listerial effect of MLKL does not. In exploring the nature of the effect of MLKL on lysosomal function, it was found that stimulation of MLKL

phosphorylation has no effect on the relative amounts of acidified lysosomes (Figures 7E, F). However, immunostaining of the *Listeria*-infected cells with endosomal markers revealed that stimulation of MLKL ubiquitination dramatically enhances the translocation of *Listeria* from the early to the late endosomes and from there to the lysosomes, and that this enhancement does not occur in cells expressing the K50R MLKL mutant (Figure 4K, L).

Altogether, these findings suggested that the arrest of *Listeria* replication by MLKL occurs through at least two distinct functions of this protein: enhancement of lysosomal destruction of the bacteria via the effect of MLKL on the endosomes, and a slower inhibitory effect, independent of lysosomal function, that might correspond to a direct toxic effect of MLKL on the cytoplasmically located bacteria (*18*). This sequence of events is consistent with the fact that *Listeria* enter the cells within phagosomes that either fuse with the endosomes or break open into the cytosol (*22*).

EXAMPLE 2

Corroboration of the K50 site of MLKL as the substrate for ubiquitination

MLKL, tagged C-terminally with the Strep-tag, was affinity purified using Strep-Tactin beads from 4×10^8 human HT-29 cells that had been treated for 3 hours with TBZ- TNF (1000 units/ml) plus the bivalent IAP antagonist BV6 (1 μM) and the caspase inhibitor z-VAD-fmk (20 μM) or, as control, from the same number of untreated HT-29 cells. The purified proteins were digested by trypsin. Following trypsinization Ubiquitinated peptides were isolated using the PTMScan Ubiquitin Remnant Motif (K-e-GG) Kit (Cell Signaling). The isolated peptides were then analyzed by liquid chromatography tandem mass spectrometry (MS/MS).

RESULTS

The results of the mass spectrometry analysis are provided in Table 4, herein below.

Table 4

Modified sequence	Base sequence	Intensity – No treatment	Intensity After TBZ
FK[Trypsin Digested:GG (Ubiquitination Site) and AALEEANGEIEK (SEQ ID NO: 11)	FKAALEEANGEIEK (SEQ ID NO: 8)	0	0
LHHSEAPELHGK – SEQ ID NO: 12 [Trypsin Digested:GG (Ubiquitination Site) and IR	LHHSEAPELHGKIR (SEQ ID NO: 9)	90786.9	298573.6

VLGLIKPLEMLQDQGK[Trypsin Digested:GG (Ubiquitination Site) [Common Variable:Oxidation on M] (SEQ ID NO: 13) and R	VLGLIKPLEMLQDQGKR (SEQ ID NO: 10)	0	375021.4
VLGLIKPLEMLQDQGK[Trypsin Digested:GG (Ubiquitination Site) (SEQ ID NO: 14) and R	VLGLIKPLEMLQDQGKR (SEQ ID NO: 10)	0	378655.1

The peptides having the sequence as set forth in SEQ ID NO: 10 (both the oxidized (row 3) and non-oxidized form (row 4) was found to be ubiquitinated in lysine 50. Both show high ubiquitination after TBZ treatment and no ubiquitination prior to the treatment. Oxidation probably occurred during the manipulation of the samples for the mass spec analysis). The peptide listed having the sequence as set forth in SEQ ID NO: 8 was on the border of detection. SEQ ID NO: 9 was ubiquitinated in lysine 331. Unlike lysine 50, lysine 331 is not evolutionary conserved and its ubiquitination was only partially dependent on TBZ treatment.

Confirmation of the identity of the ubiquitinated peptides, by spectral annotation is also seen in Figures 9A-B.

EXAMPLE 3

MLKL ubiquitination also facilitates destruction of some other intracellular bacteria

Similarly to *L. monocytogenes,* the Gram-negative bacterium *Yersinia enterocolitica* enters cells by co-opting receptor-uptake mechanisms, and its replication within the infected cells is subject to modulation by antagonistic effects exerted both by the bacteria and by cellular defense mechanisms. On assessing the yields of these bacteria in infected HT-29 and L929 cells it was found that, as with *Listeria,* in cells that express WT MLKL the yield of *Y. enterocolitica* was effectively decreased by their treatment with TBZ and TNF, respectively, whereas in HT-29 cells expressing the K50R MLKL mutant and in L929 cells expressing the K50,51R MLKL mutant, these treatments had no effect on their bacterial yields (Figures 10A, B).

The impact of MLKL activation on the infection of these cells by *Escherichia coli*, a bacterium that mainly grows extracellularly but can also adhere to mammalian cells and be phagocytized by them, was also examined. It was found that the yields of *E. coli* that could be recovered from HT-29 and L929 cells were decreased by TBZ and TNF treatments, similarly to the effects exerted by those respective treatments on the yields of *L. monocytogenes* and *Y. enterocolitica*, and that these effects were also compromised by mutation of lysine 50 in human MLKL or of lysines 50 and 51 in mouse MLKL (Figures 10C, D).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting. In addition, any priority document(s) of this application is/are hereby incorporated herein by reference in its/their entirety.

WHAT IS CLAIMED IS:

1. A method of treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject, the method comprising administering to the subject a therapeutically effective amount of an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL), thereby treating the disease caused by the pathogen.

2. An agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) for use in treating a disease caused by a pathogen which utilizes the endosomal pathway to enter a cell of a subject.

3. The method of claim 1 or agent of claim 2, wherein said pathogen is a virus.

4. The method or agent of claim 3, wherein said virus is selected from the group consisting of Influenza A virus, Foot and mouth disease virus, Rhinovirus, Adenovirus, Ebolavirus, Kaposi Sarcoma Virus, Simian virus 40, Papillomavirus, Polio virus, Lymphocytic choriomeningitis virus (LCMV), Rhinovirus and Coronavirus.

5. The method of claim 1 or agent of claim 2, wherein said pathogen is a bacteria.

6. The method or agent of claim 5, wherein said bacteria is *Listeria monocytogenes.*

7. A method of treating inflammatory bowel disease (IBD) in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an agent that specifically up-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the IBD.

8. An agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) for use in treating IBD.

9. The method or agent of any one of claims 1-7, wherein said agent enhances the activity and/or amount of a ubiquitin ligase that is capable of ubiquitinating lysine at position 50 of mixed lineage kinase domain-like protein (MLKL).

10. The method or agent of claim 9, wherein said agent is said ubiquitin ligase.

11. The method or agent of claim 9, wherein said agent is a polynucleotide encoding said ubiquitin ligase.

12. The method or agent of claims 10 or 11, wherein said ubiquitin ligase is E3 ubiquitin-protein ligase Itchy homolog (ITCH).

13. The method or agent of any one of claims 1-6, wherein said agent enhances oligomerization of said MLKL.

14. The method or agent of claim 13, wherein said agent activates protein kinase RIPK3.

15. The method or agent of claim 13, wherein said agent is a multivalent agent that binds said MLKL.

16. The method or agent of any one of claims 1-6, further comprising administering to the subject an agent that directly targets the pathogen.

17. A method of treating a cancer sensitive to TRAIL receptor mediated cytotoxicity in a subject in need thereof comprising administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the cancer.

18. A method of treating a cancer in a subject in need thereof comprising co-administering to the subject a therapeutically effective amount of an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL and TNF alpha, thereby treating the cancer.

19. A method of treating a disease associated with an upregulation of activity of EGF receptor in a subject comprising administering to the subject a therapeutically effective amount of

an agent that specifically down-regulates ubiquitination of lysine at position 50 of MLKL, thereby treating the disease.

20. The method of claim 19, wherein the disease is selected from the group consisting of non-small-cell lung cancer, metastatic colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer and breast cancer.

21. The method of any one of claims 17, 18 or 19, wherein said agent is a peptide agent that is a substrate for ubiquitination by said E3 ubiquitin-protein ligase.

22. The method of any one of claims 17, 18 or 19, wherein the peptide agent comprises at least 10 amino acids of MLKL.

23. The method of any one of claims 17, 18 or 19, wherein said agent is a polynucleotide agent that is capable of down-regulation the amount of ITCH in cancer cells of the subject.

24. The method of any one of claims 17-22, further comprising administering to the subject an agent that activates the TRAIL apoptotic pathway.

25. The method of claim 24, wherein said agent that activates the TRAIL apoptotic pathway is selected from the group consisting of Mapatumumab, Contatumumab, TAS266, ONC201, Tigatuzumab, Dulanermin and Circularly permuted TRAIL (CPT).

26. The method of claims 17 or 18, wherein said cancer is selected from the group consisting of myeloma, lymphoma, colorectal cancer, NSCLC, liver cancer, triple negative breast cancer, pancreatic cancer, cervical cancer, soft tissue sarcoma, ovarian cancer, glioblastoma.

27. An article of manufacture comprising an agent which upregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) and an antibacterial or antiviral agent.

28. The article of manufacture of claim 27, wherein said agent enhances the activity and/or amount of a ubiquitin ligase that is capable of ubiquitinating lysine at position 50 of mixed lineage kinase domain-like protein (MLKL).

29. The article of manufacture of claim 28, wherein said agent is said ubiquitin ligase.

30. The article of manufacture of claim 28, wherein said agent is a polynucleotide encoding said ubiquitin ligase.

31. The article of manufacture of claims 29 or 30, wherein said ubiquitin ligase is E3 ubiquitin-protein ligase Itchy homolog (ITCH).

32. An article of manufacture comprising:

(i) an agent which downregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL); and

(ii) an agent that activates the TRAIL apoptotic pathway and/or TNF alpha.

33. The article of manufacture of claim 32, wherein said agent which downregulates ubiquitination of lysine at position 50 of mixed lineage kinase domain-like protein (MLKL) is co-formulated with said agent that activates the TRAIL apoptotic pathway.

34. A method of identifying an agent capable of treating a disease associated with activation of a necroptosis pathway comprising:

(a) contacting the agent with MLKL or a polynucleotide encoding same;

(b) analyzing ubiquitination of lysine at position 50 of said MLKL, wherein a change in the amount of ubiquitination at said position is indicative of an agent that is capable of treating the disease associated with a activation of the necroptosis pathway.

35. The method of claim 34, wherein said disease is a renal disease, a pulmonary disease, a hepatic disease, cancer, infectious disease, neurodegenerative disease, inflammatory bowel disease, ischemic reperfusion, psoriasis and a cardiovascular disease.

36. The method of claim 34, wherein when said change is an increase, said disease is a cancer promoted by constitutive signaling of the EGF receptor.

37. The method of claim 35, wherein said cancer is selected from the group consisting of colorectal cancer, non-small cell lung cancer and head and neck cancer.

38. The method of claim 34, wherein when said change is a decrease, said disease is a cancer sensitive to the TRAIL receptor cytotoxic effect.

FIG. 1A

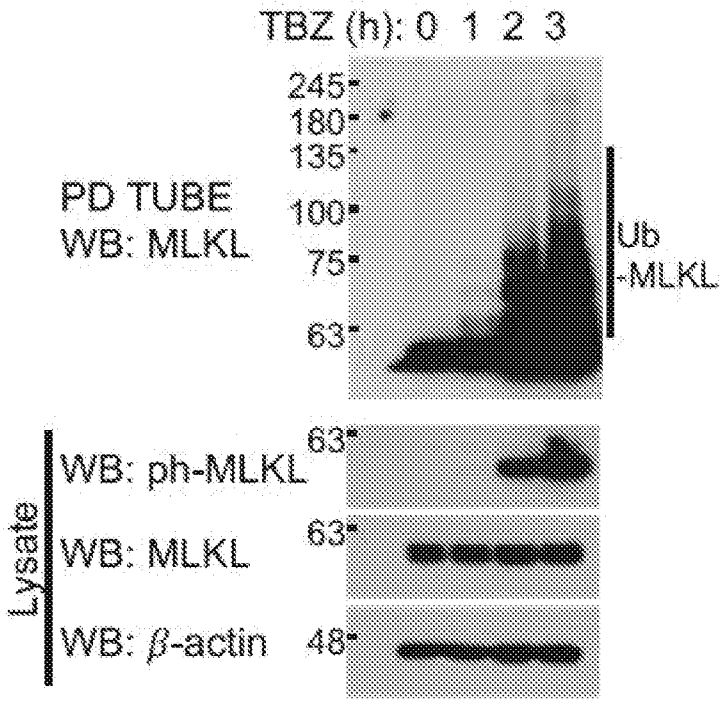

TBZ (h): 0 1 2 3
245
180
135
100
75
63
*
PD TUBE
WB: MLKL
Ub
-MLKL
Lysate
WB: ph-MLKL
63
WB: MLKL
63
WB: β-actin
48

FIG. 1B

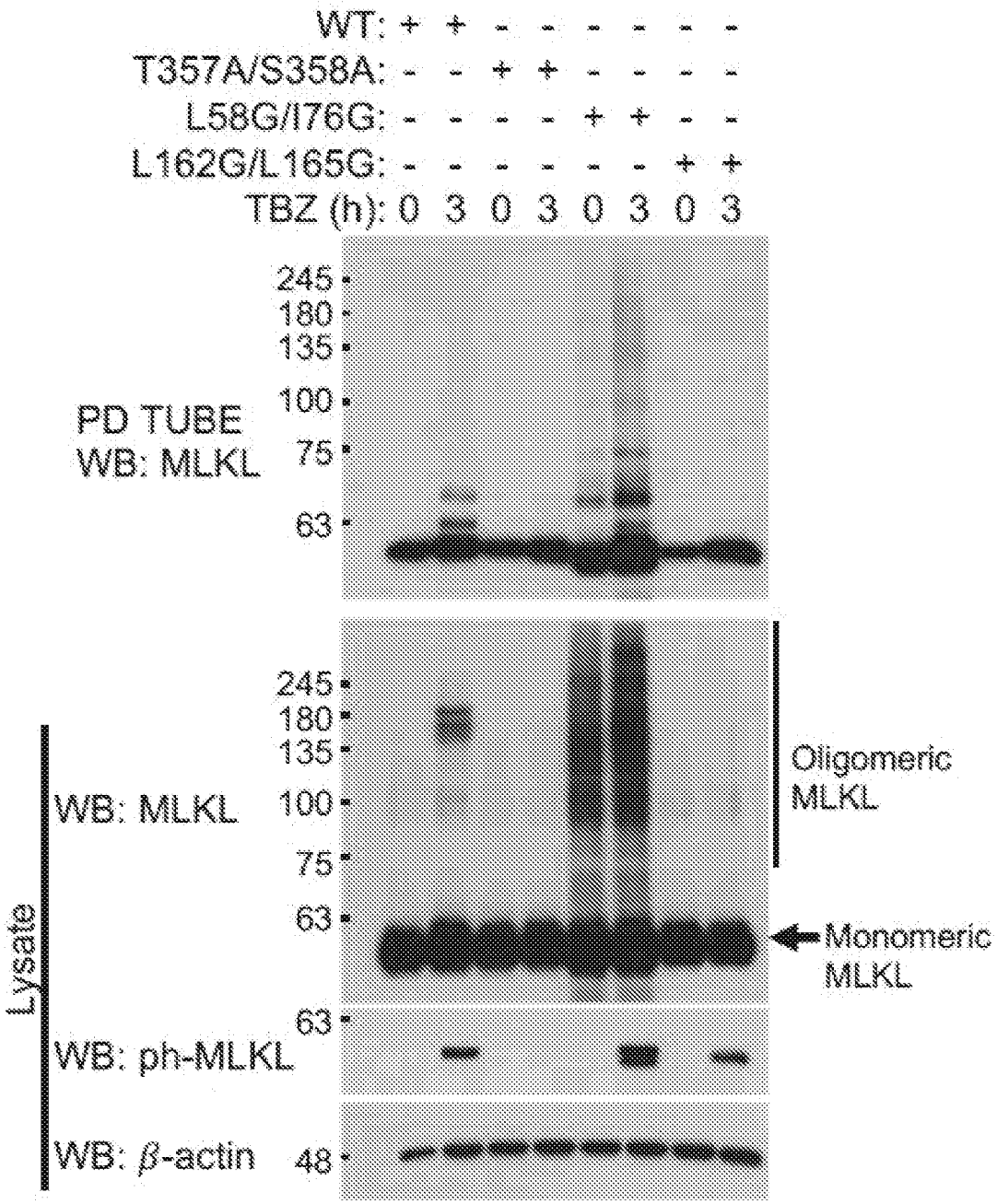
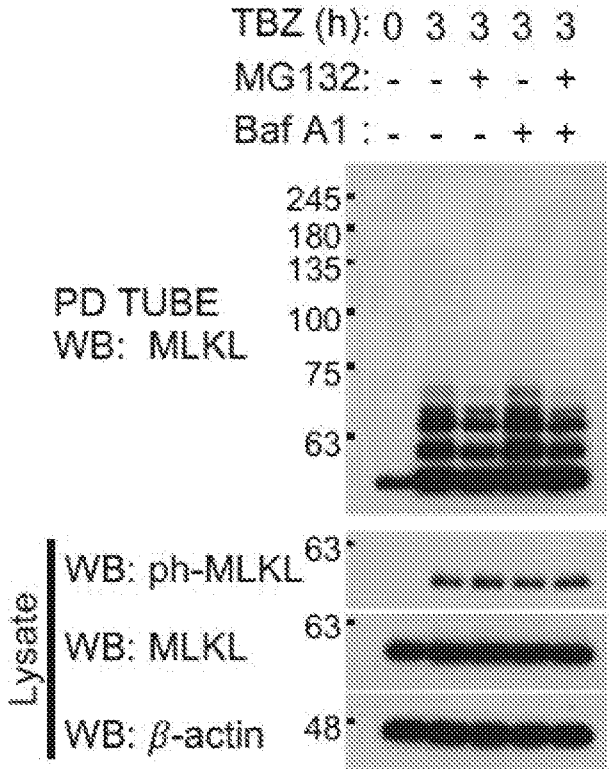

WT: + + - - - - - -
T357A/S358A: - - + + - - - -
L58G/I76G: - - - - + + - -
L162G/L165G: - - - - - - + +
TBZ (h): 0 3 0 3 0 3 0 3
245
180
135
100
75
63
PD TUBE
WB: MLKL
245
180
135
100
75
63
63
48
Lysate
WB: MLKL
WB: ph-MLKL
WB: β-actin
Oligomeric MLKL
Monomeric MLKL

FIG. 1C


TBZ (h): 0 3 3 3 3
MG132: - - + - +
Baf A1 : - - - + +
PD TUBE
WB: MLKL
245
180
135
100
75
63
Lysate
WB: ph-MLKL
63
WB: MLKL
63
WB: β-actin
48

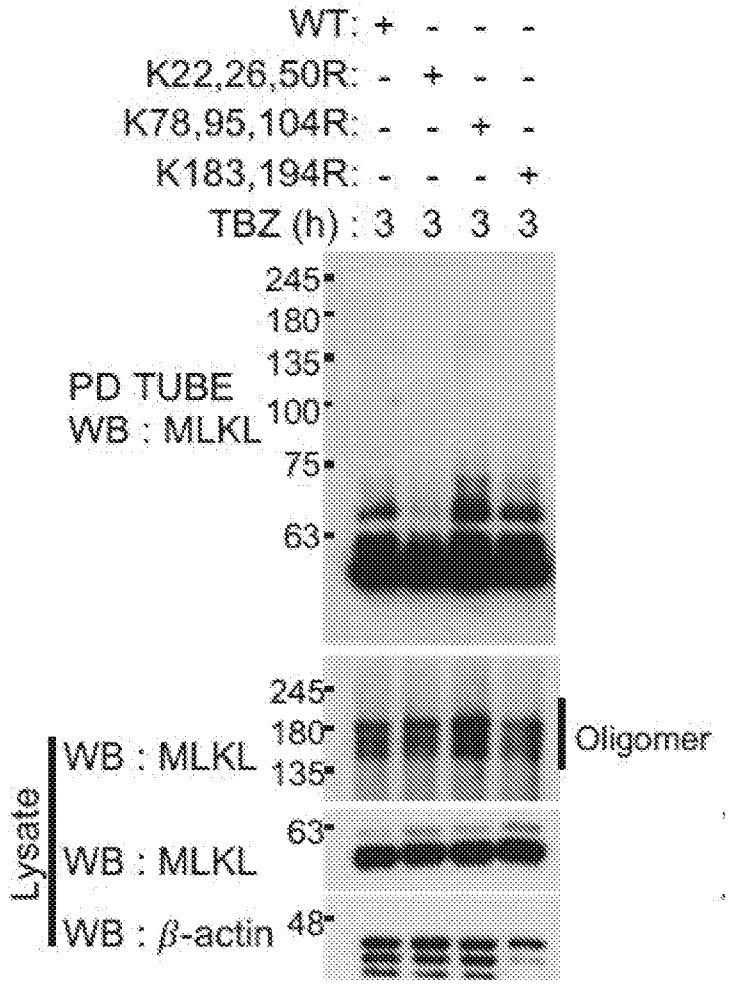

WT: + - - -
K22,26,50R: - + - -
K78,95,104R: - - + -
K183,194R: - - - +
TBZ (h) : 3 3 3 3
PD TUBE
WB : MLKL
245
180
135
100
75
63
Lysate
WB : MLKL
WB : MLKL
WB : β-actin
245
180
135
63
48
Oligomer

FIG. 1D

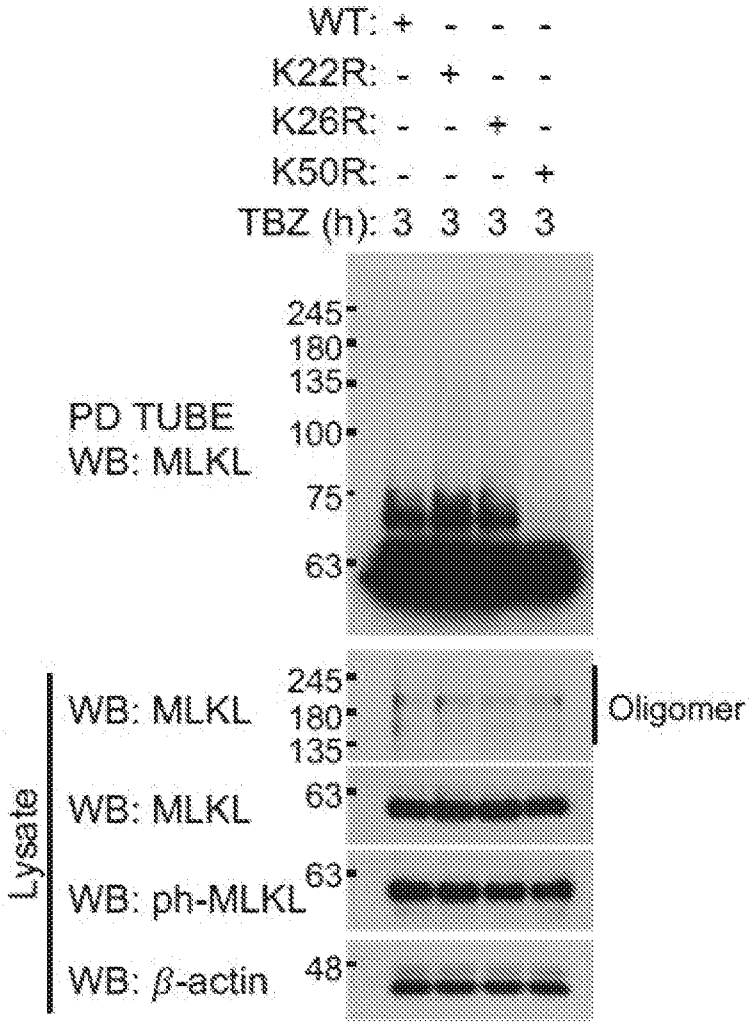

WT: + - - -
K22R: - + - -
K26R: - - + -
K50R: - - - +
TBZ (h): 3 3 3 3
PD TUBE
WB: MLKL
245
180
135
100
75
63
Lysate
WB: MLKL
WB: MLKL
WB: ph-MLKL
WB: β-actin
245
180
135
63
63
48
Oligomer

FIG. 1E

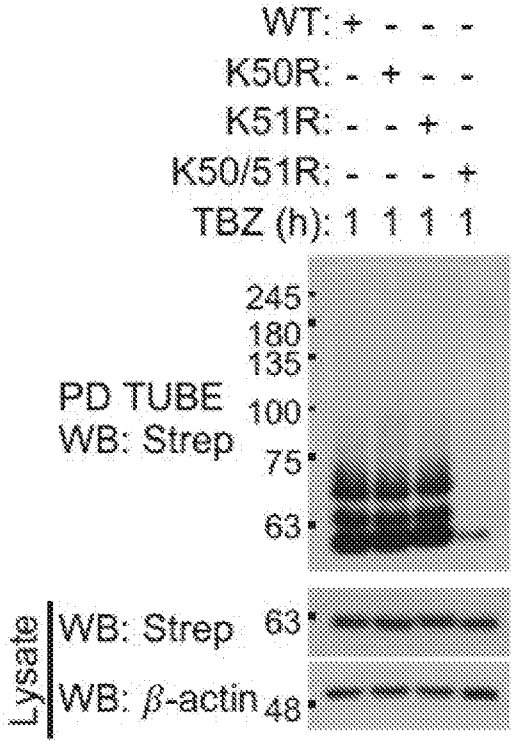

WT: + - - -
K50R: - + - -
K51R: - - + -
K50/51R: - - - +
TBZ (h): 1 1 1 1
245
180
135
100
75
63
PD TUBE
WB: Strep
Lysate
WB: Strep
63
WB: β-actin
48

FIG. 1F

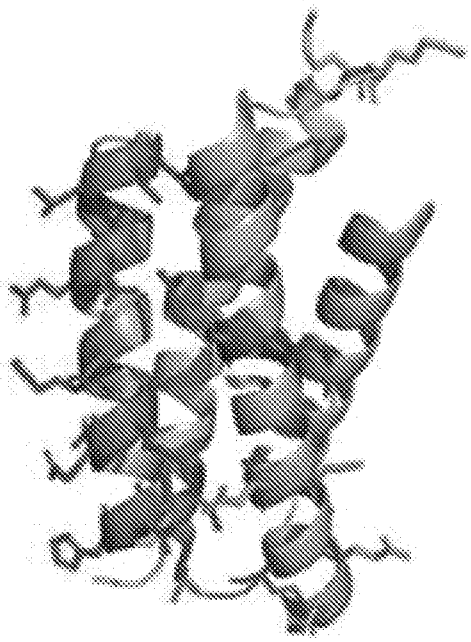


FIG. 1G

FIG. 2A

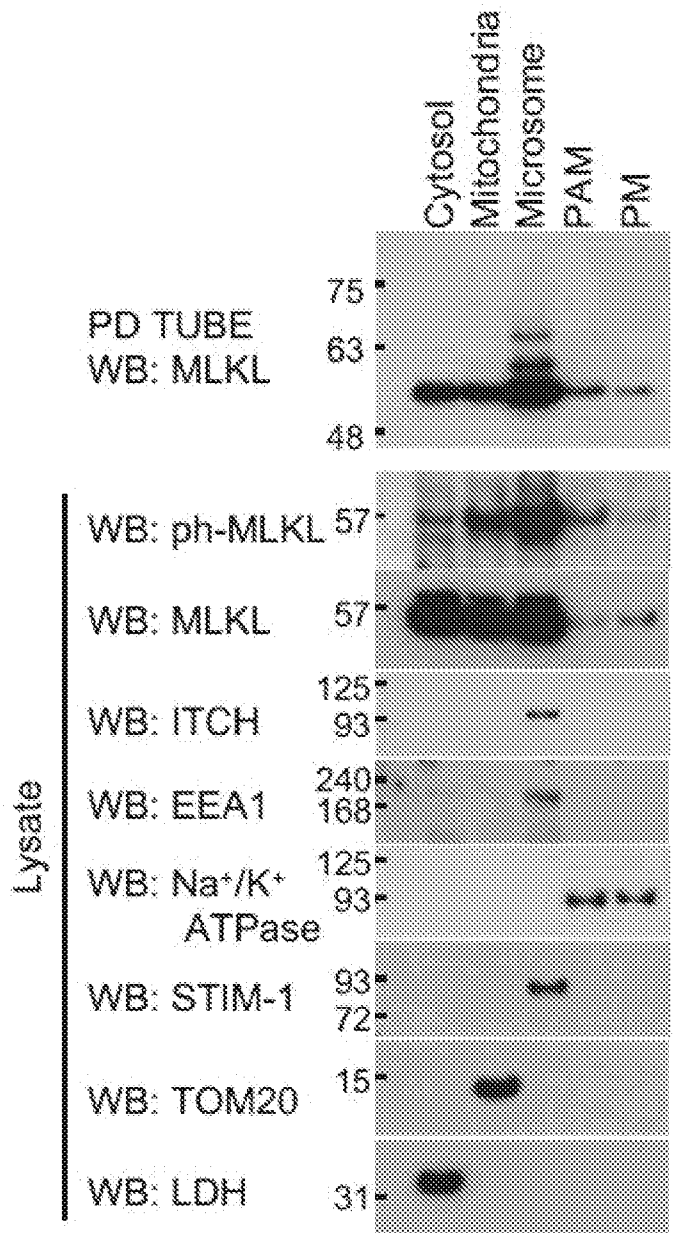

Cytosol
Mitochondria
Microsome
PAM
PM
PD TUBE
WB: MLKL
75
63
48
Lysate
WB: ph-MLKL
57
WB: MLKL
57
WB: ITCH
125
93
WB: EEA1
240
168
WB: Na+/K+ ATPase
125
93
WB: STIM-1
93
72
WB: TOM20
15
WB: LDH
31

FIG. 2B

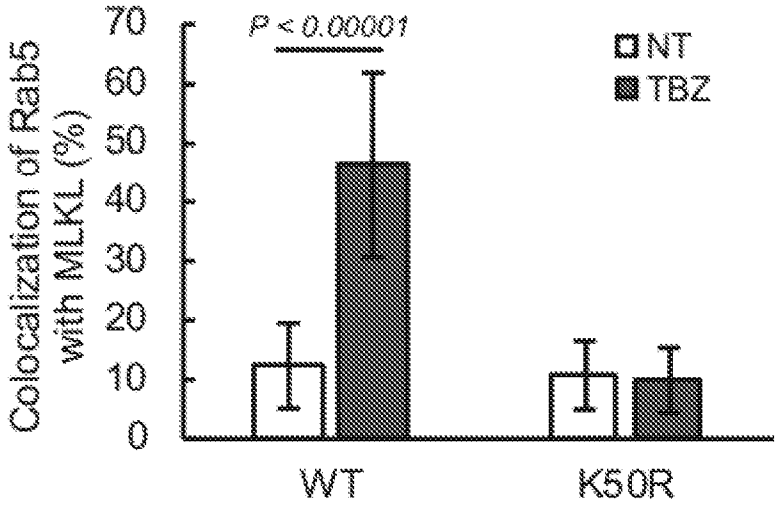

Colocalization of Rab5 with MLKL (%)
70
60
50
40
30
20
10
0
P < 0.00001
NT
TBZ
WT
K50R

FIG. 2C

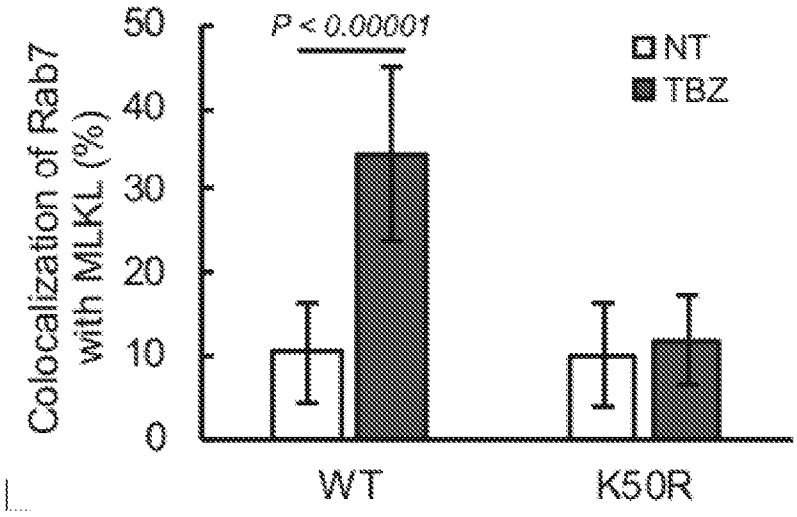
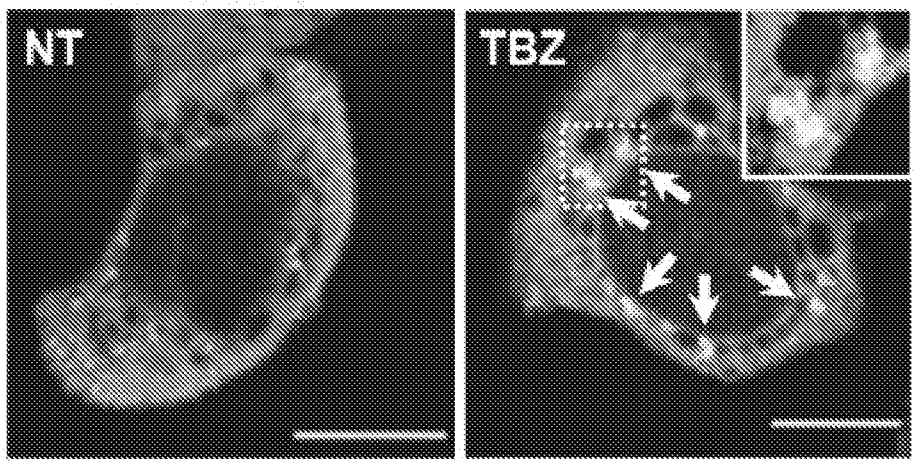

Colocalization of Rab7 with MLKL (%)
50
40
30
20
10
0
P < 0.00001
NT
TBZ
WT
K50R

FIG. 2D



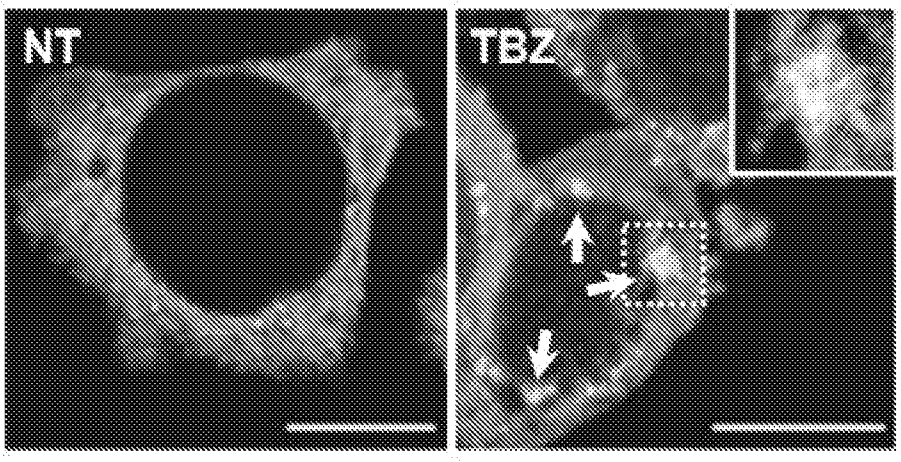
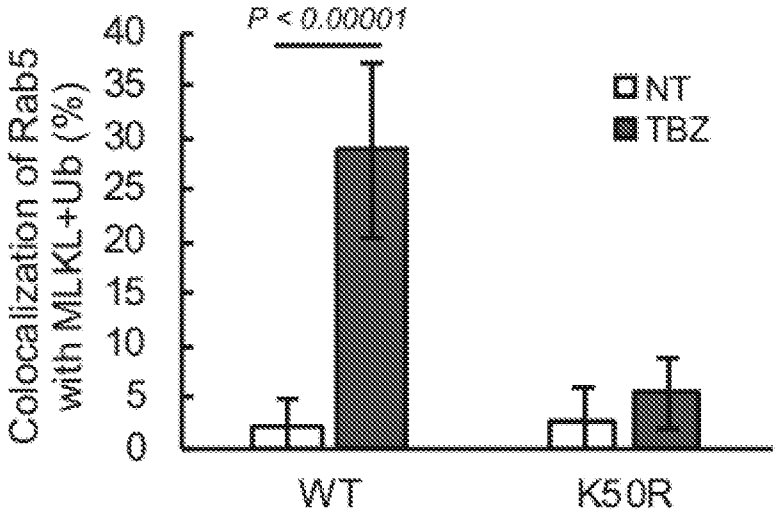


FIG. 2E

FIG. 2F



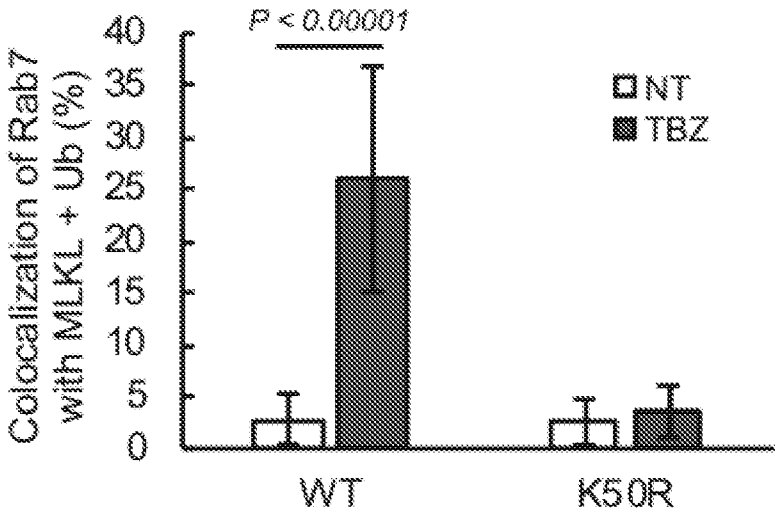
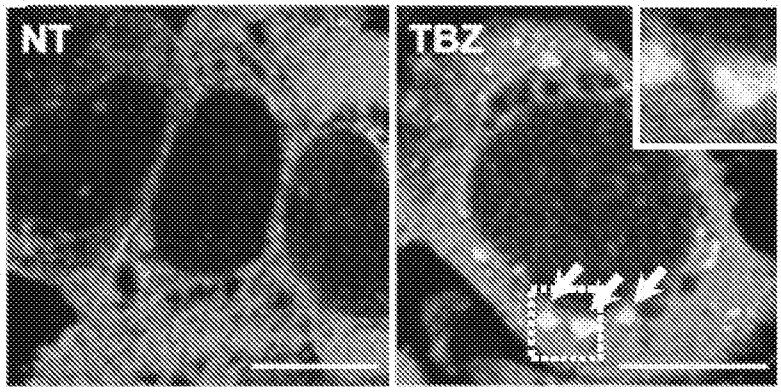


FIG. 2G

FIG. 2H



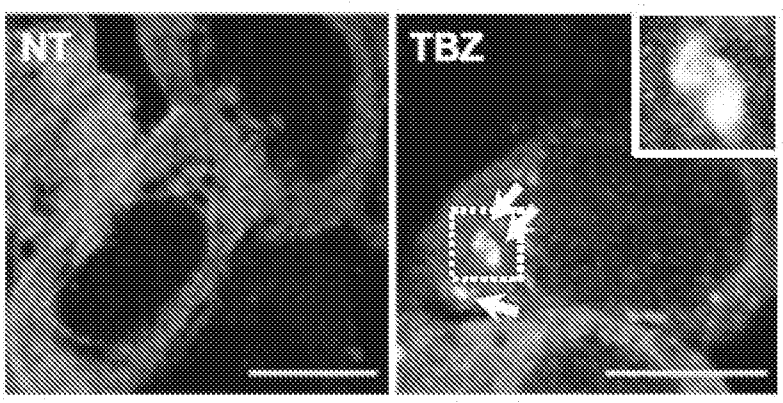
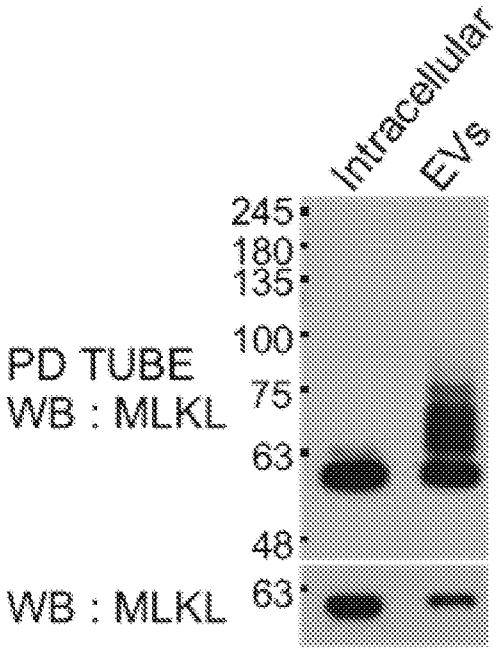


FIG. 2I

FIG. 2J


Intracellular
EVs
245
180
135
100
75
63
48
PD TUBE
WB : MLKL
WB : MLKL
63

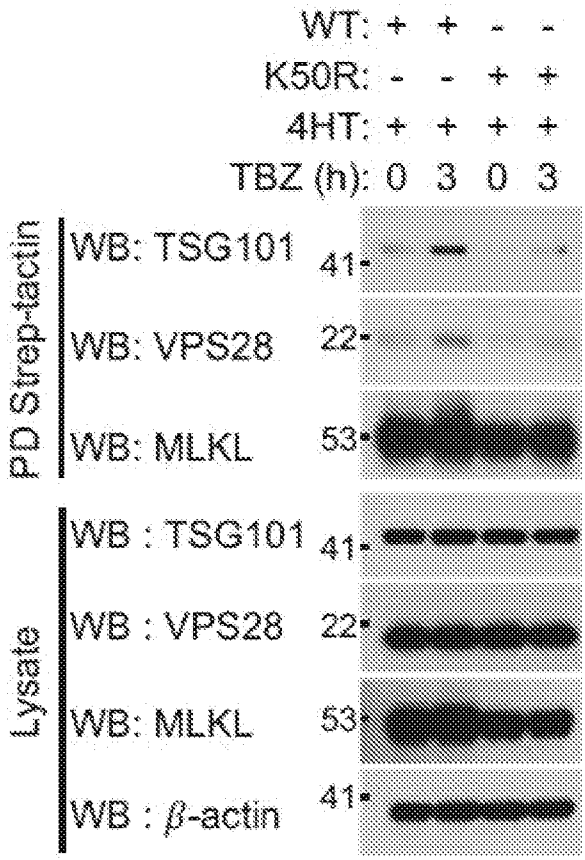

WT: + + - -
K50R: - - + +
4HT: + + + +
TBZ (h): 0 3 0 3
PD Strep-tactin
WB: TSG101
41
WB: VPS28
22
WB: MLKL
53
Lysate
WB : TSG101
41
WB : VPS28
22
WB: MLKL
53
WB : β-actin
41

FIG. 2K

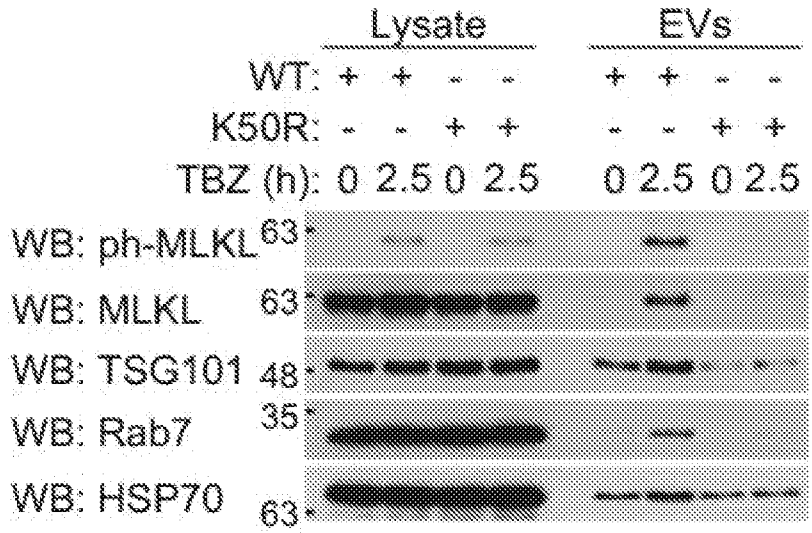


FIG. 2L

FIG. 3A

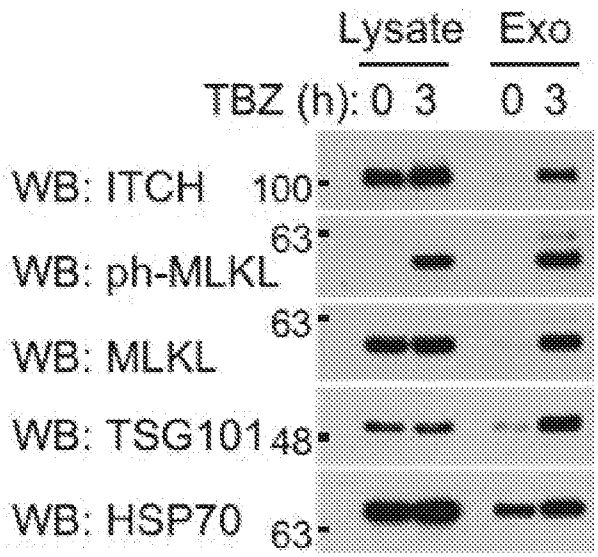


FIG. 3B

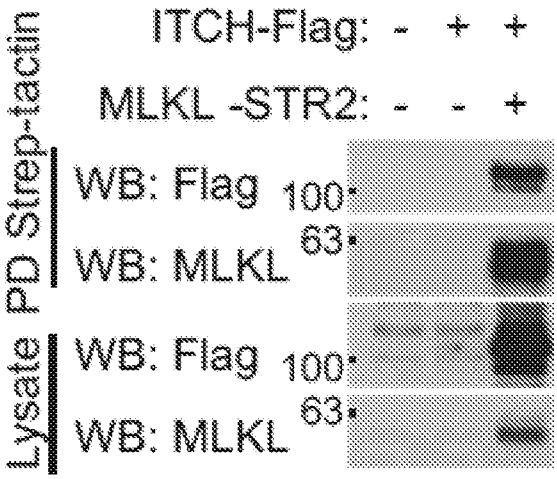


FIG. 3C

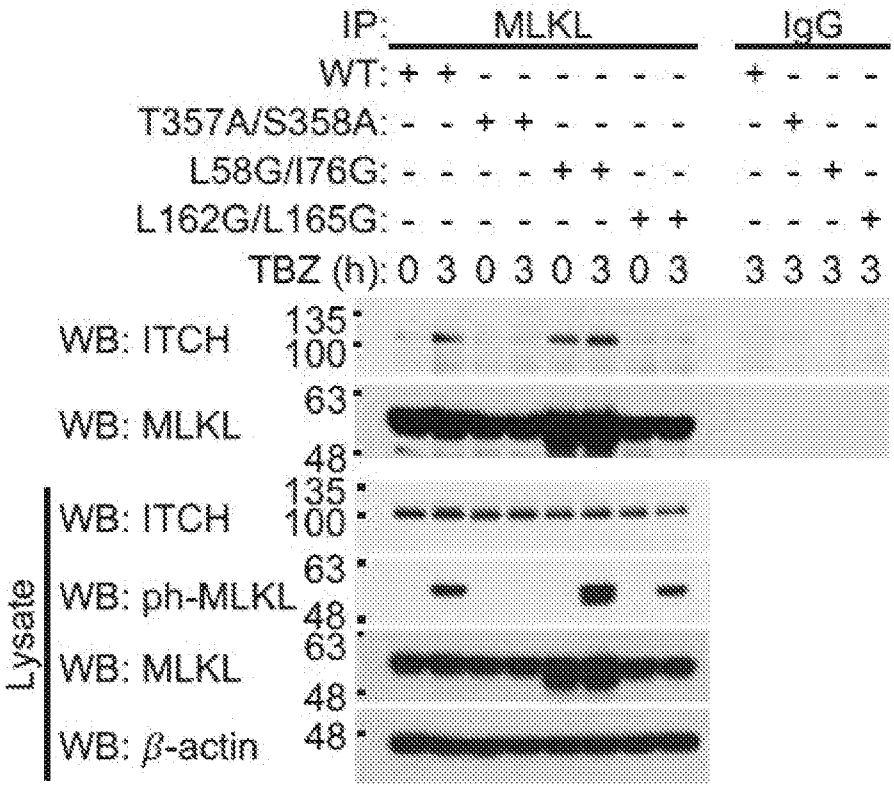

IP: MLKL IgG
WT: + + - - - - - - + - - -
T357A/S358A: - - + + - - - - - + - -
L58G/I76G: - - - - + + - - - - + -
L162G/L165G: - - - - - - + + - - - +
TBZ (h): 0 3 0 3 0 3 0 3 3 3 3 3
WB: ITCH
135
100
WB: MLKL
63
48
Lysate
WB: ITCH
WB: ph-MLKL
WB: MLKL
WB: β-actin
48

FIG. 3D

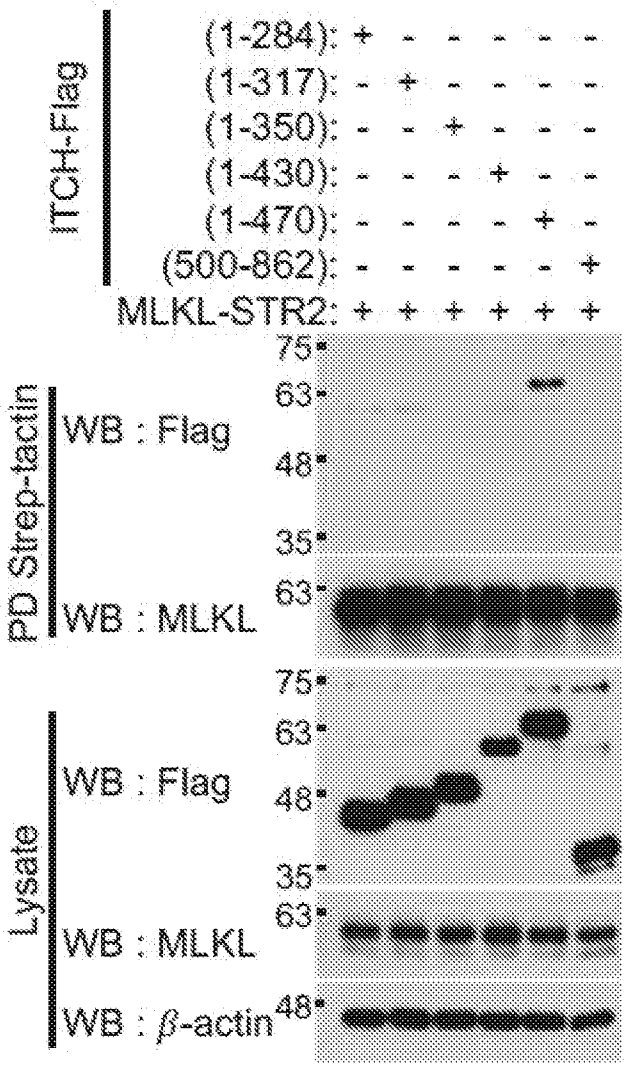

ITCH-Flag
(1-284): + - - - - -
(1-317): - + - - - -
(1-350): - - + - - -
(1-430): - - - + - -
(1-470): - - - - + -
(500-862): - - - - - +
MLKL-STR2: + + + + + +
PD Strep-tactin
WB : Flag
WB : MLKL
Lysate
WB : Flag
WB : MLKL
WB : β-actin
75
63
48
35
63
75
63
48
35
63
48

FIG. 3E

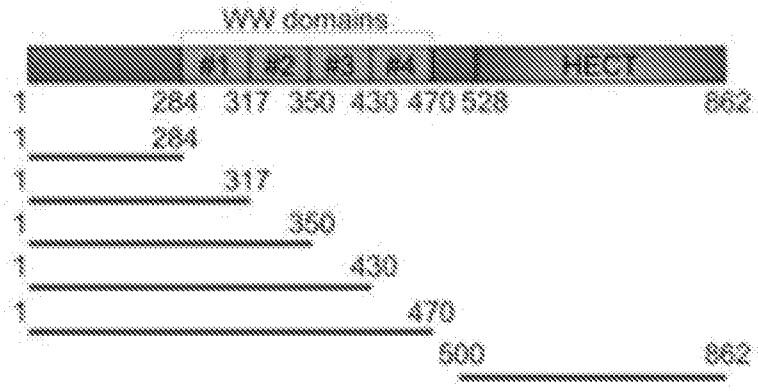

WW domains
#1
#2
#3
#4
HECT
1
284
317
350
430
470
528
862
1
284
1
317
1
350
1
430
1
470
500
862

FIG. 3F

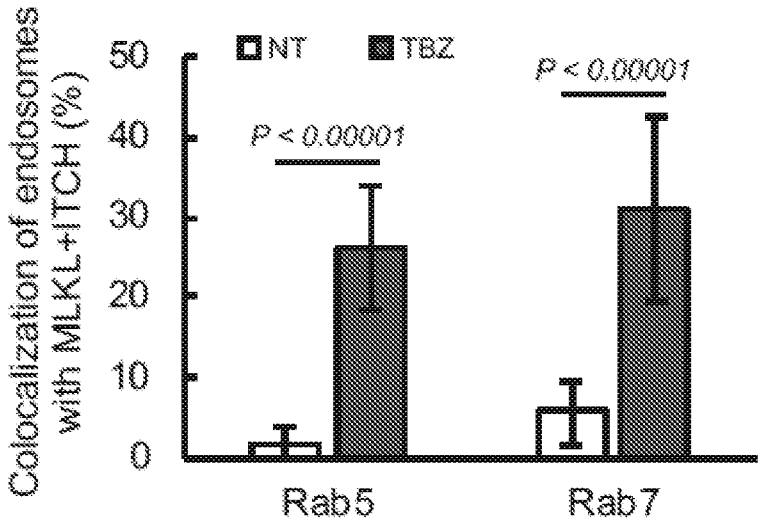
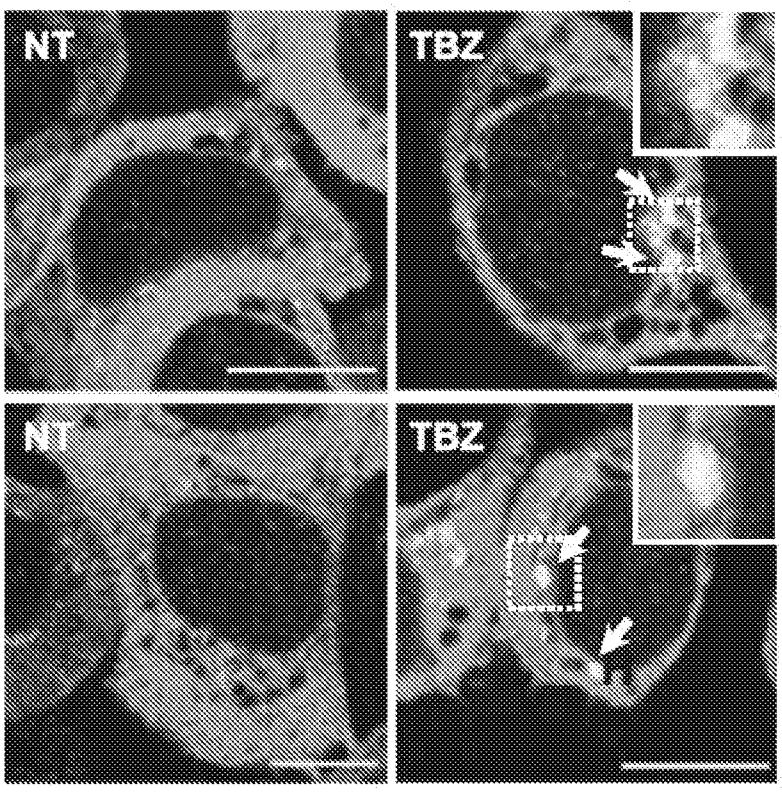

Colocalization of endosomes with MLKL+ITCH (%)
50
40
30
20
10
0
NT
TBZ
P < 0.00001
P < 0.00001
Rab5
Rab7

FIG. 3G



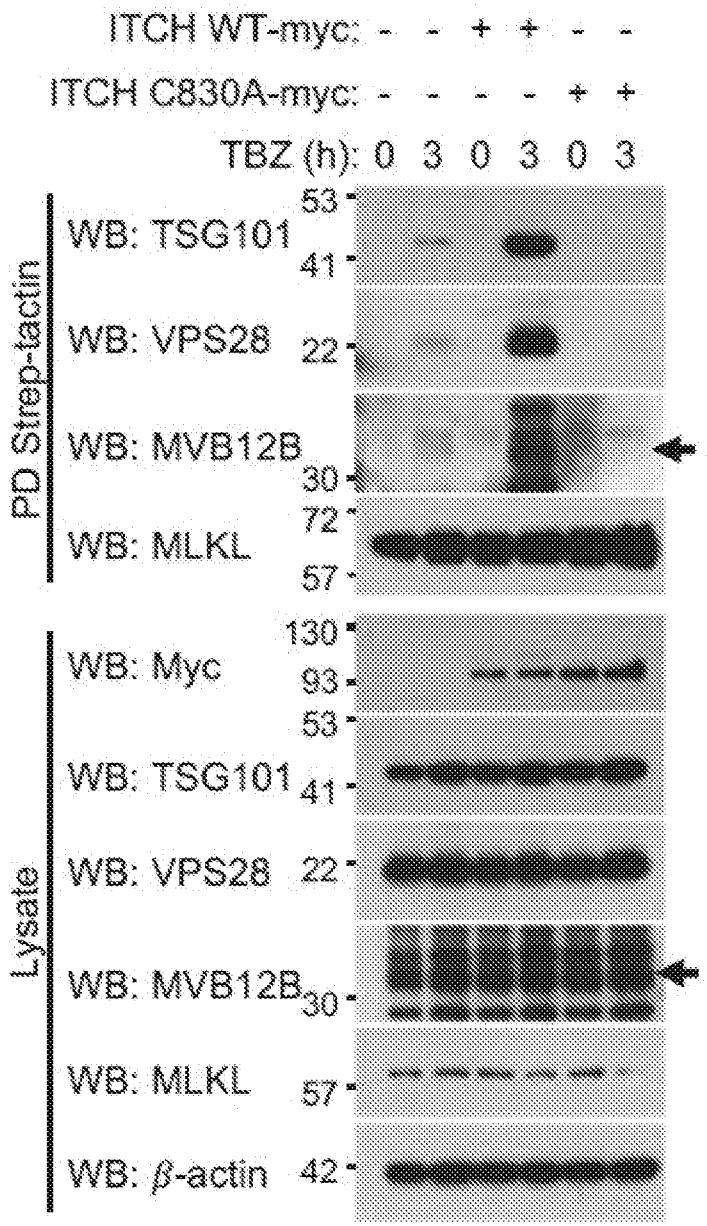

ITCH WT-myc: - - + + - -
ITCH C830A-myc: - - - - + +
TBZ (h): 0 3 0 3 0 3
PD Strep-tactin
WB: TSG101
WB: VPS28
WB: MVB12B
WB: MLKL
Lysate
WB: Myc
WB: TSG101
WB: VPS28
WB: MVB12B
WB: MLKL
WB: β-actin
53
41
22
30
72
57
130
93
53
41
22
30
57
42

FIG. 3J

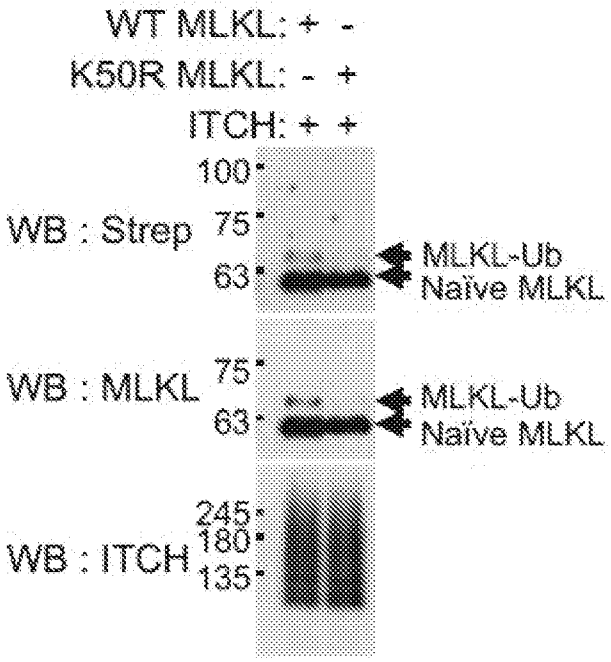

WT MLKL: + -
K50R MLKL: - +
ITCH: + +
WB : Strep
100
75
63
MLKL-Ub
Naïve MLKL
WB : MLKL
75
63
MLKL-Ub
Naïve MLKL
WB : ITCH
245
180
135

FIG. 3H

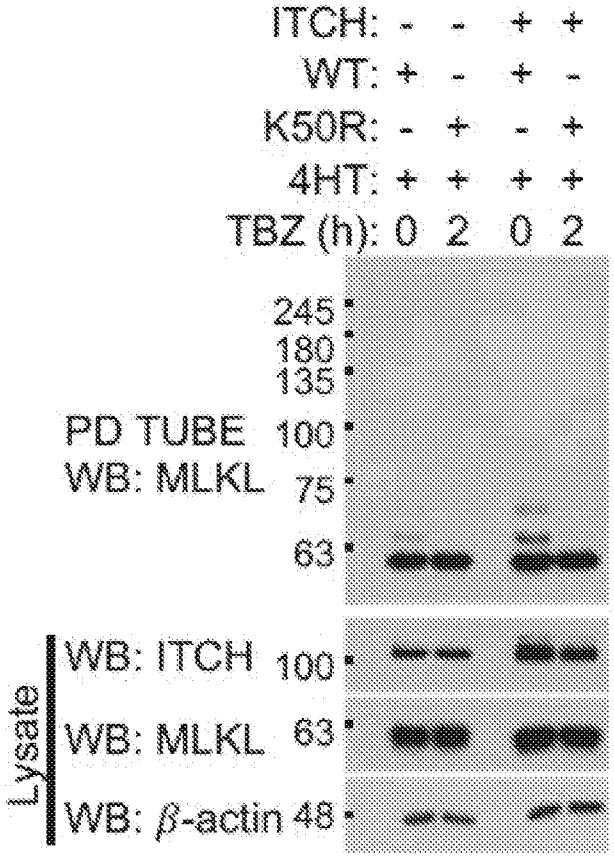

ITCH: - - + +
WT: + - + -
K50R: - + - +
4HT: + + + +
TBZ (h): 0 2 0 2
PD TUBE
WB: MLKL
245
180
135
100
75
63
Lysate
WB: ITCH
100
WB: MLKL
63
WB: β-actin
48

FIG. 3I

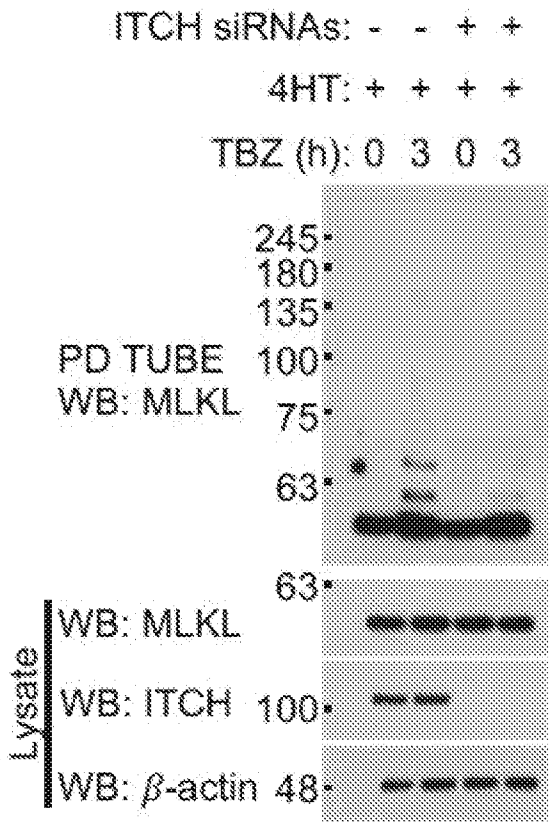

ITCH siRNAs: - - + +
4HT: + + + +
TBZ (h): 0 3 0 3
245
180
135
PD TUBE
WB: MLKL
100
75
63
63
Lysate
WB: MLKL
WB: ITCH
100
WB: β-actin
48

FIG. 3K

FIG. 4A

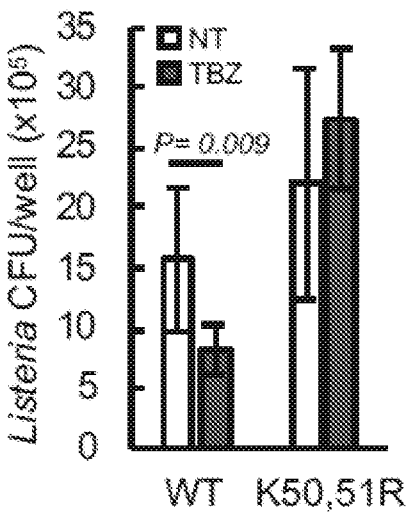


FIG. 4B

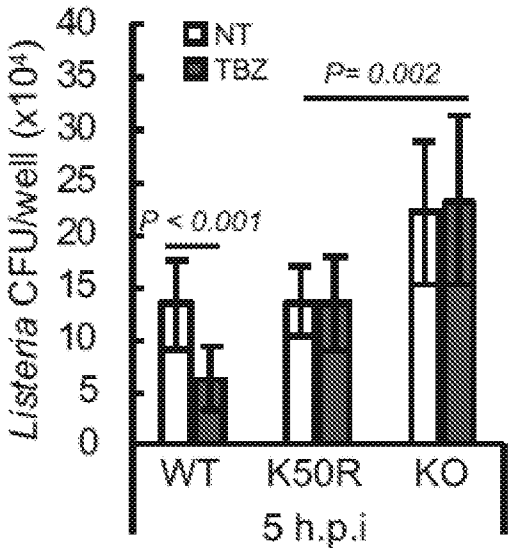


FIG. 4C

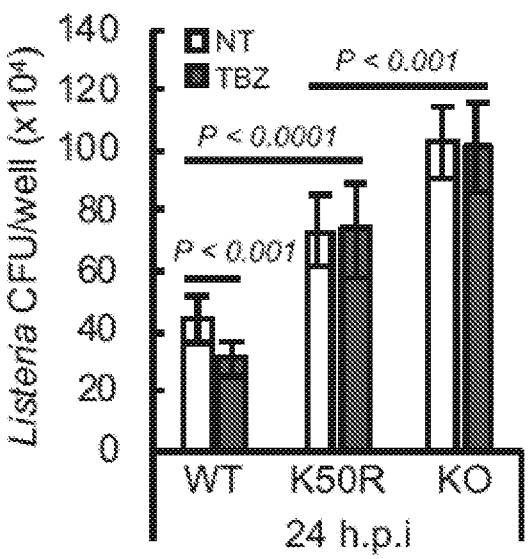


FIG. 4D

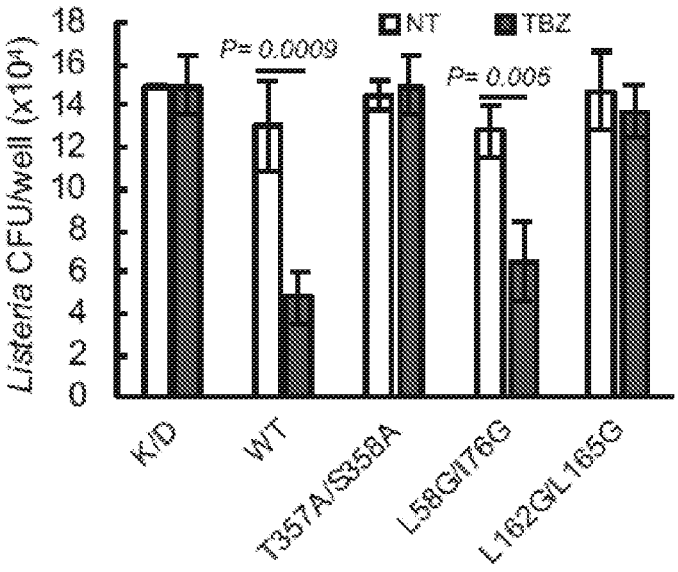


FIG. 4E

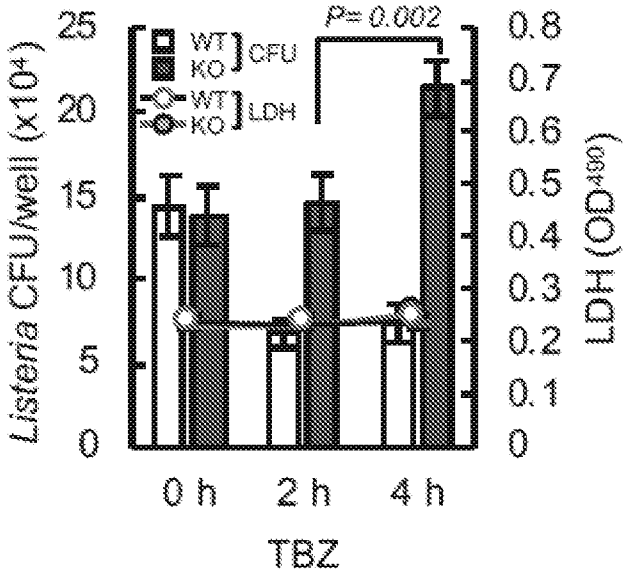


FIG. 4F

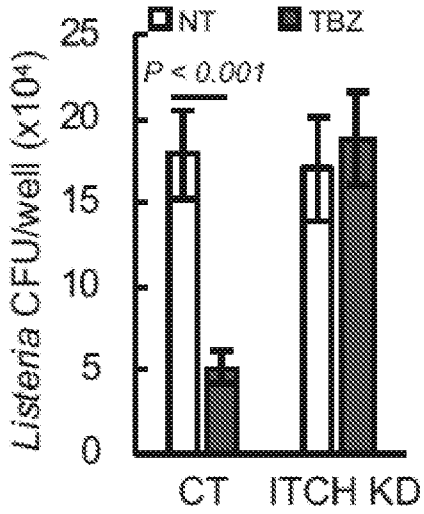
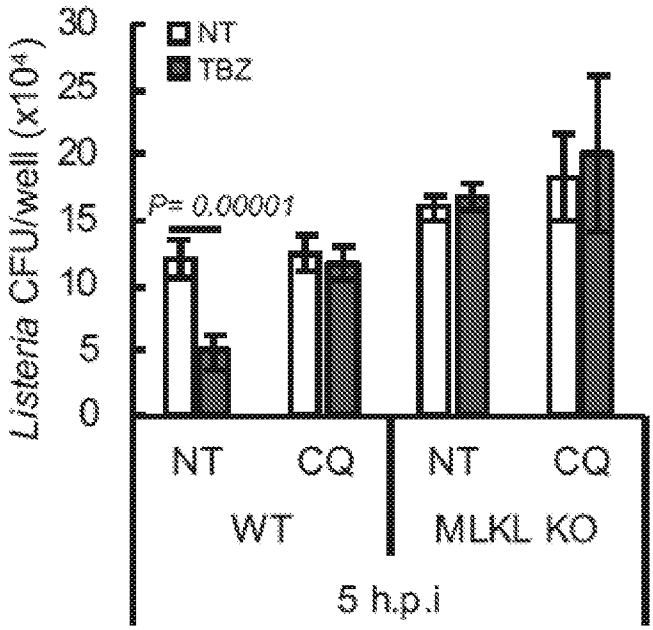


FIG. 4G



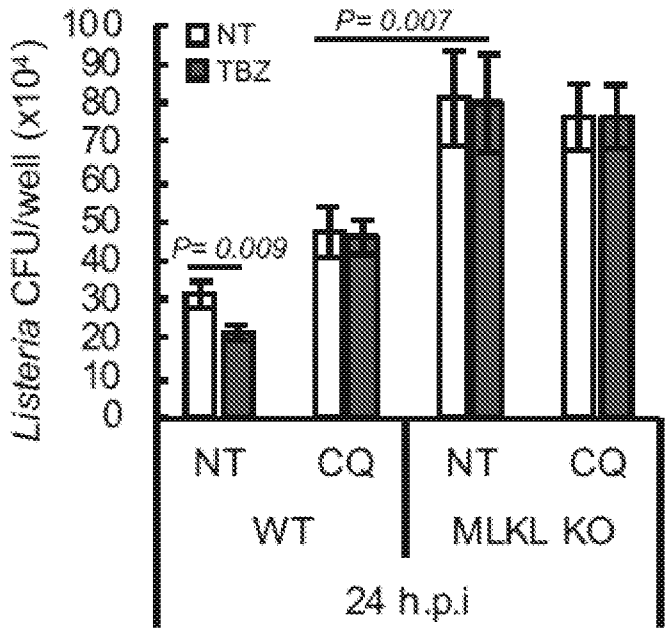


FIG. 4H

FIG. 4I

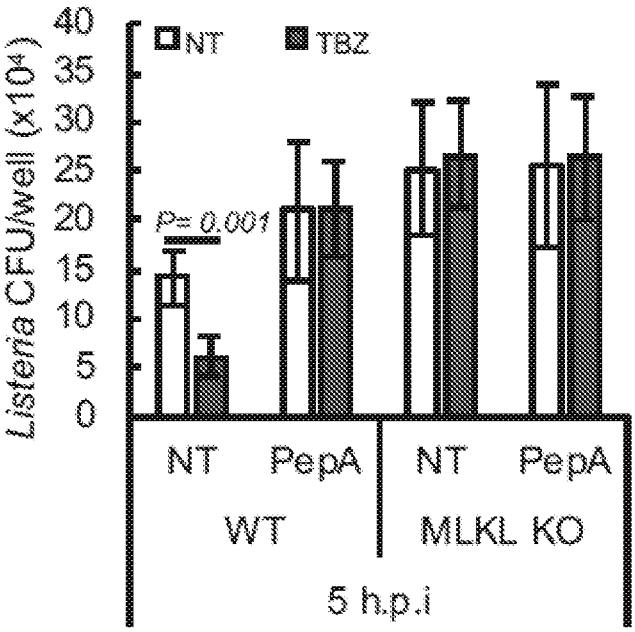
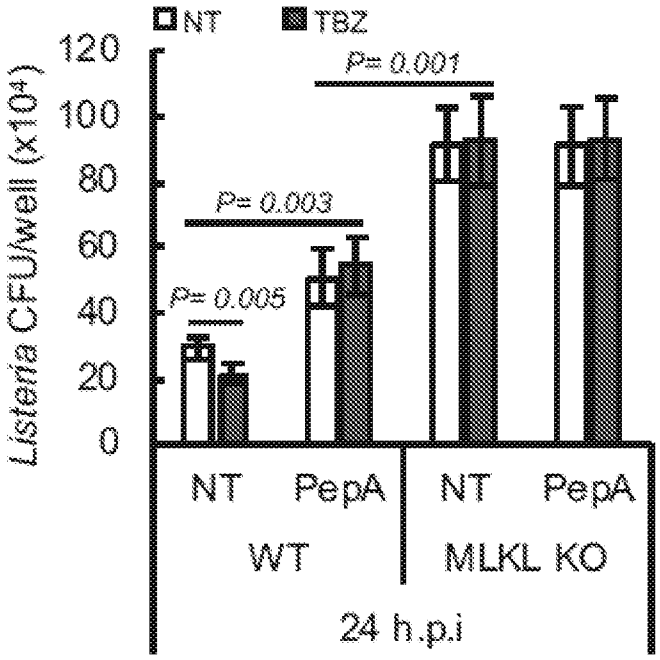


FIG. 4J



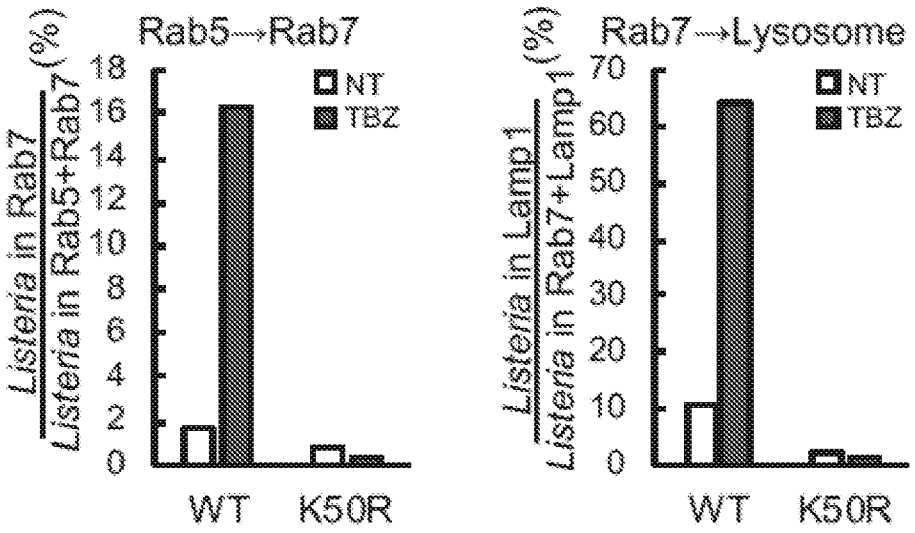

Rab5→Rab7
Listeria in Rab7 / Listeria in Rab5+Rab7 (%)
0
2
4
6
8
10
12
14
16
18
NT
TBZ
WT
K50R
Rab7→Lysosome
Listeria in Lamp1 / Listeria in Rab7+Lamp1 (%)
0
10
20
30
40
50
60
70
NT
TBZ
WT
K50R

FIG. 4K

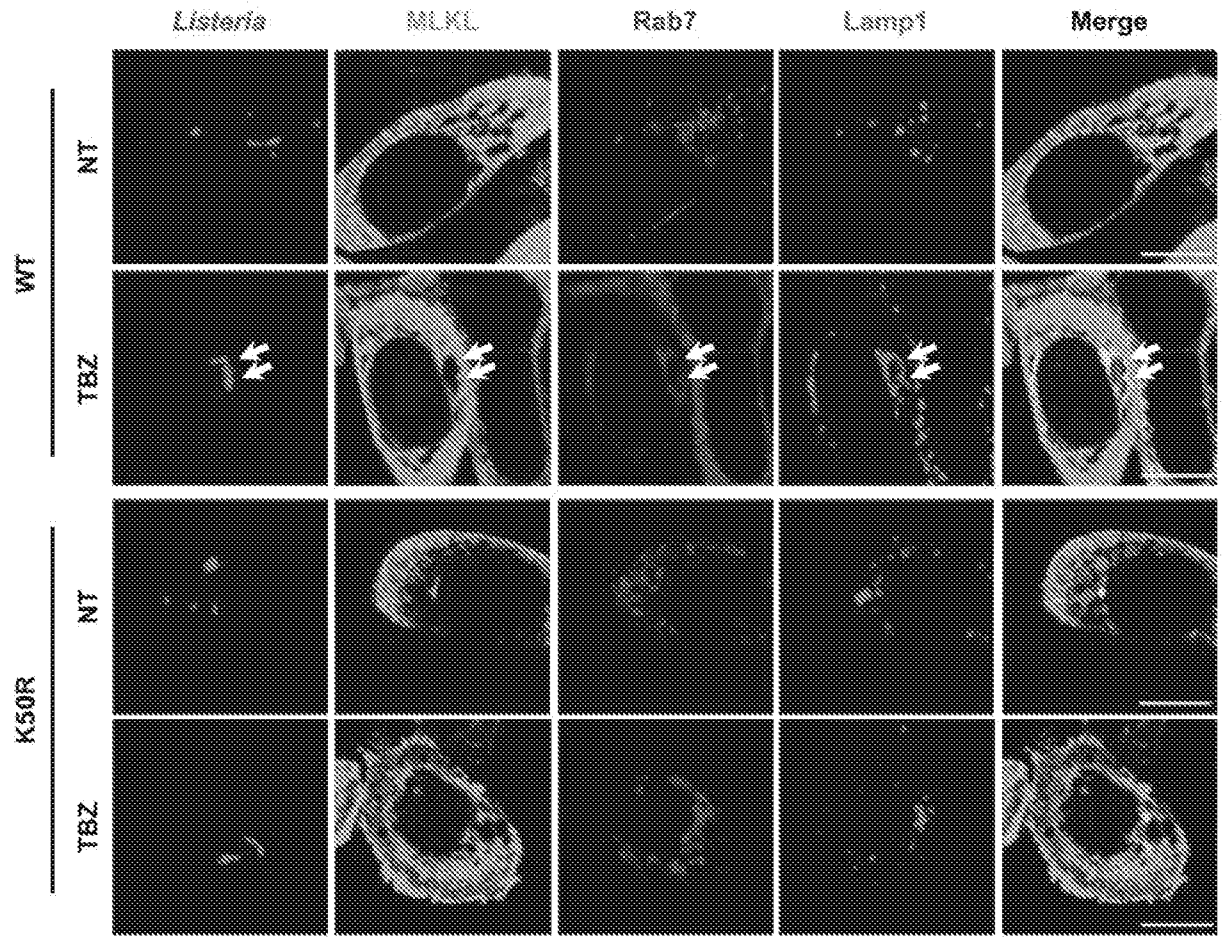

Listeria
MLKL
Rab7
Lamp1
Merge
WT
NT
TBZ
K50R
NT
TBZ

FIG. 4L

FIG. 5A

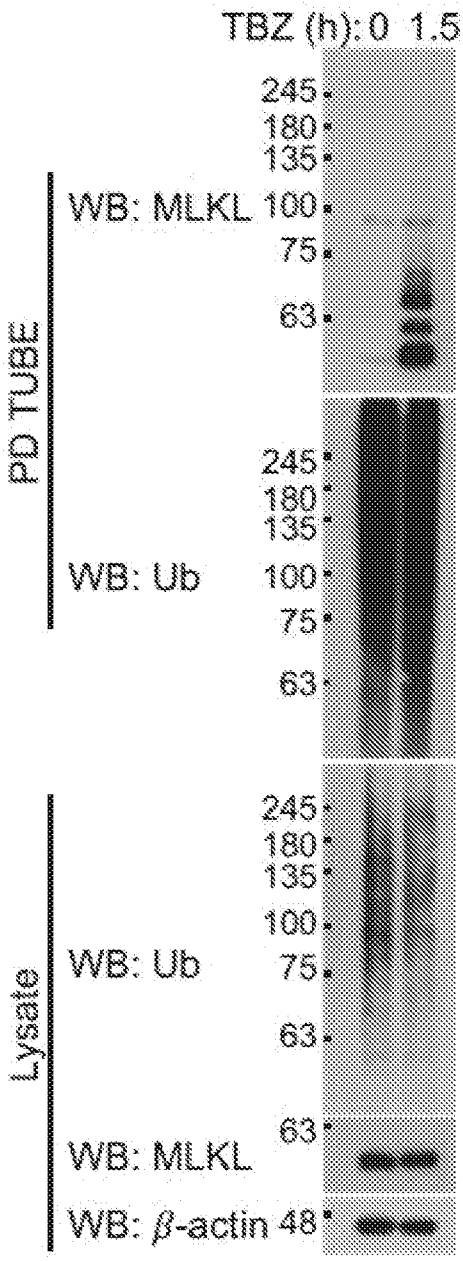

TBZ (h): 0 1.5
PD TUBE
WB: MLKL
WB: Ub
Lysate
WB: Ub
WB: MLKL
WB: β-actin
245
180
135
100
75
63
48

FIG. 5B

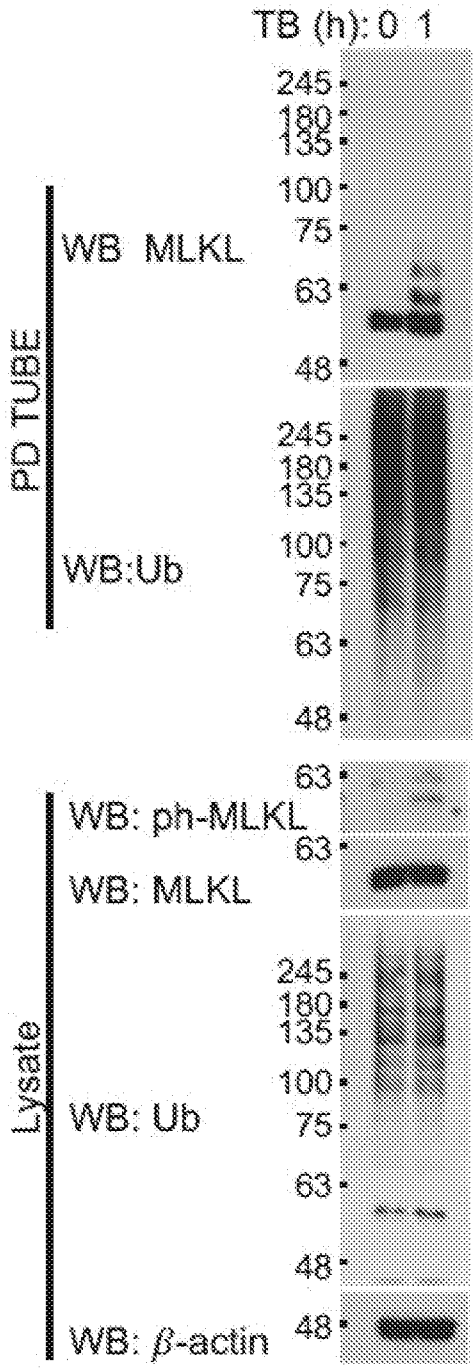

TB (h): 0 1
PD TUBE
WB MLKL
WB:Ub
Lysate
WB: ph-MLKL
WB: MLKL
WB: Ub
WB: β-actin
245
180
135
100
75
63
48

FIG. 5C

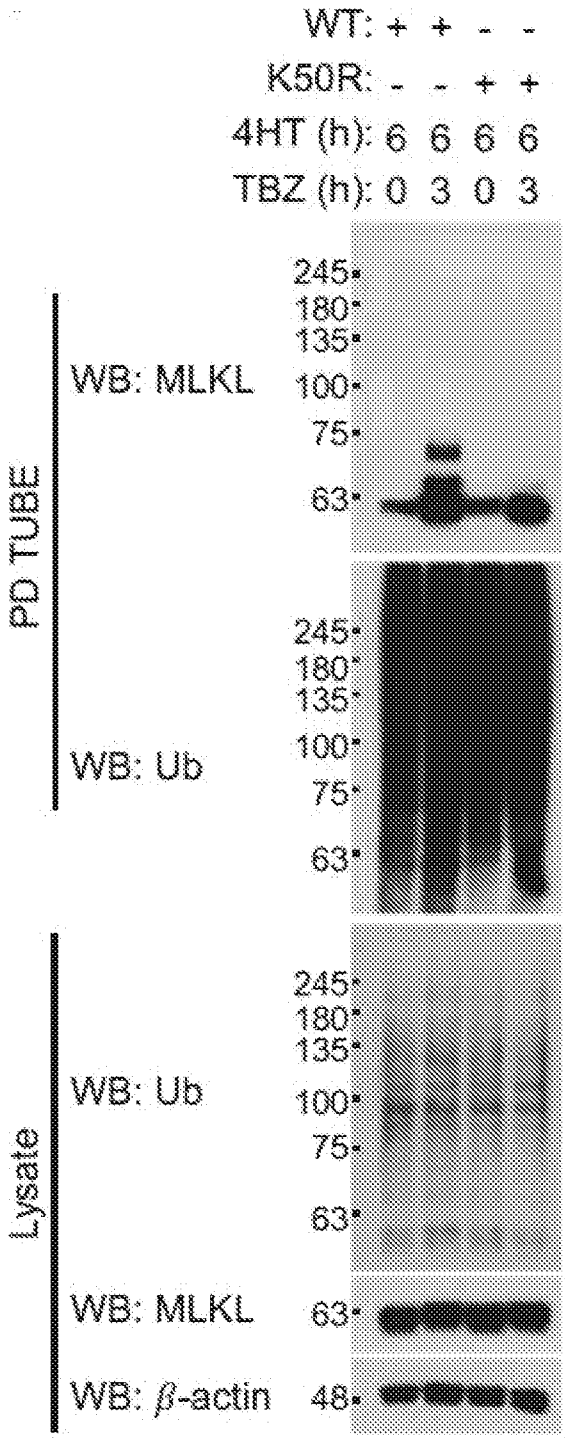

WT: + + - -
K50R: - - + +
4HT (h): 6 6 6 6
TBZ (h): 0 3 0 3
PD TUBE
WB: MLKL
WB: Ub
Lysate
WB: Ub
WB: MLKL
WB: β-actin
245
180
135
100
75
63
48

FIG. 5D

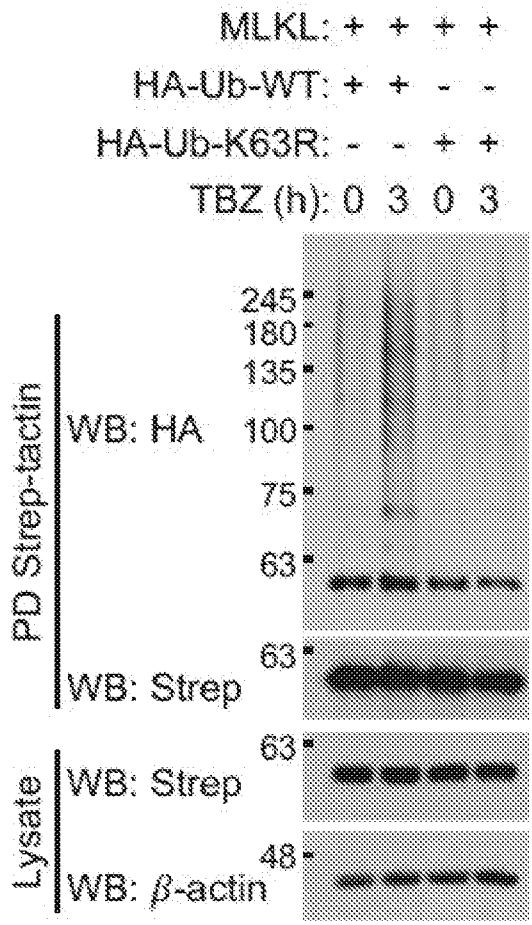

MLKL: + + + +
HA-Ub-WT: + + - -
HA-Ub-K63R: - - + +
TBZ (h): 0 3 0 3
PD Strep-tactin
WB: HA
WB: Strep
Lysate
WB: Strep
WB: β-actin
245
180
135
100
75
63
48

FIG. 5E

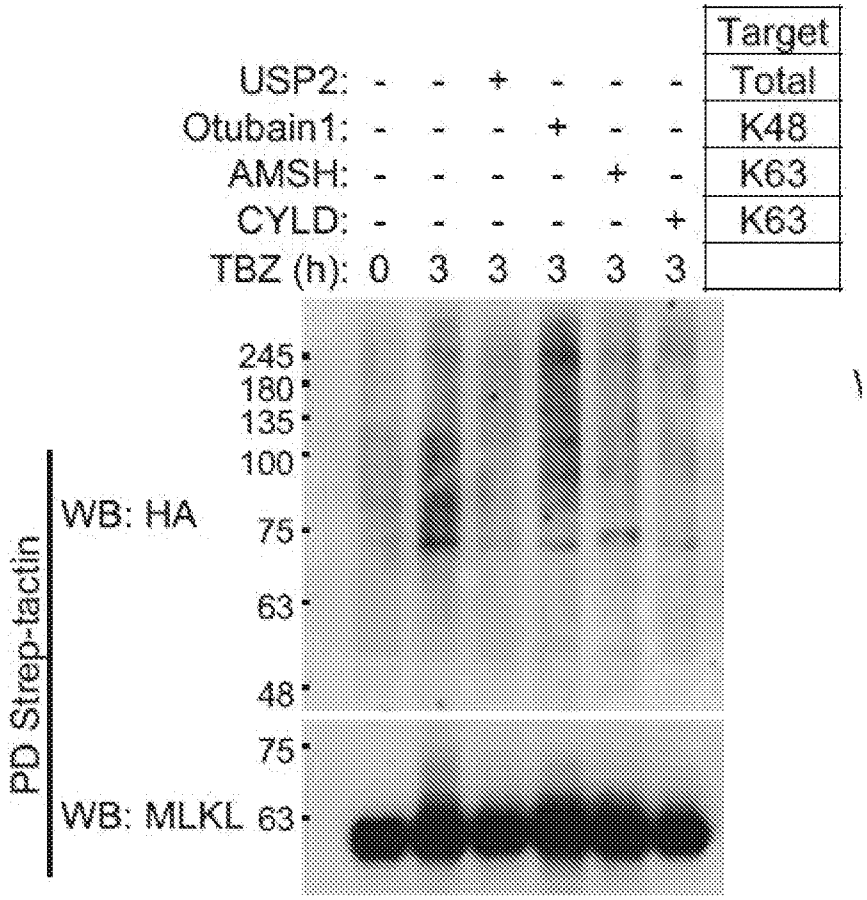

Target
USP2: - - + - - -
Total
Otubain1: - - - + - -
K48
AMSH: - - - - + -
K63
CYLD: - - - - - +
K63
TBZ (h): 0 3 3 3 3 3
245
180
135
100
75
63
48
WB: HA
75
63
WB: MLKL
PD Strep-tactin

FIG. 5F

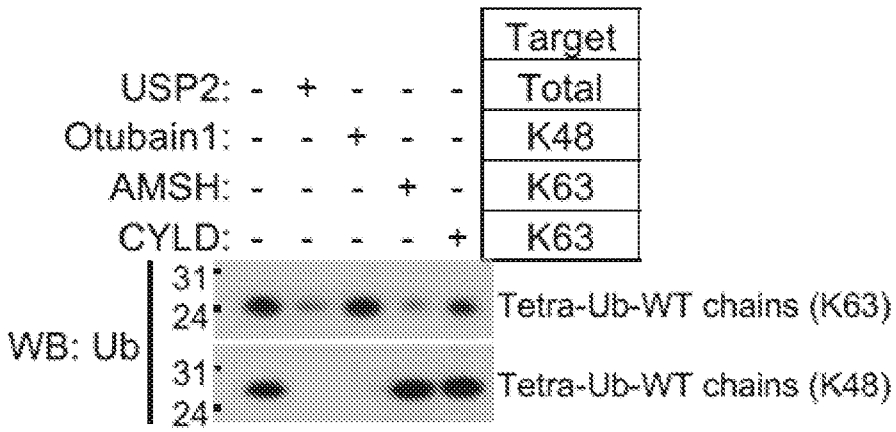

Target
USP2: - + - - -
Total
Otubain1: - - + - -
K48
AMSH: - - - + -
K63
CYLD: - - - - +
K63
31
24
Tetra-Ub-WT chains (K63)
31
24
Tetra-Ub-WT chains (K48)
WB: Ub

FIG. 6A

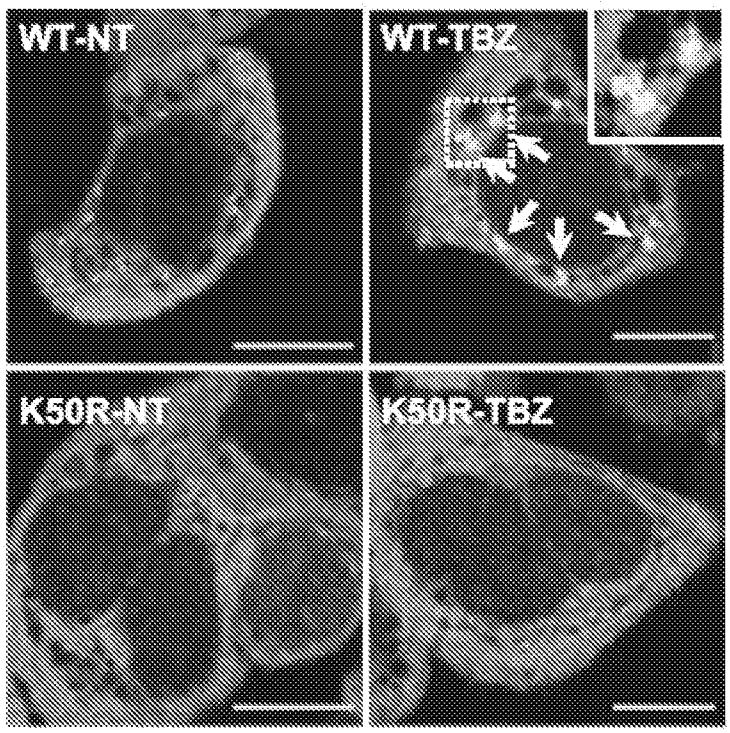


FIG. 6B

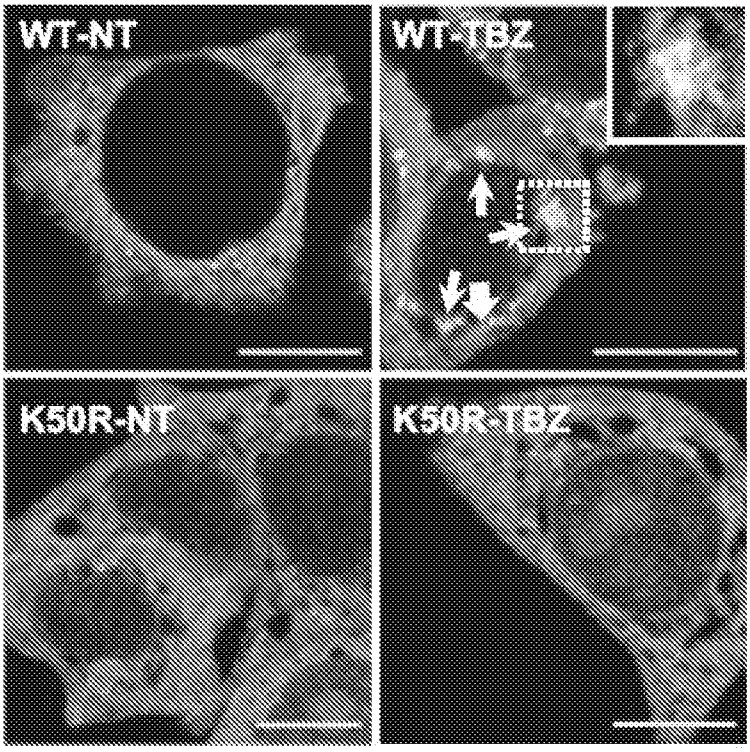
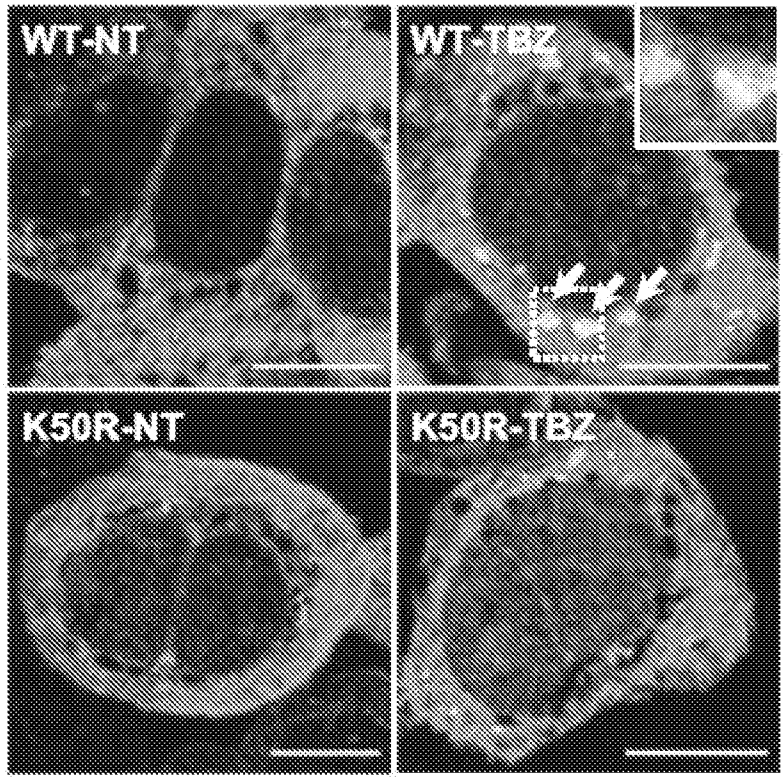


FIG. 6C



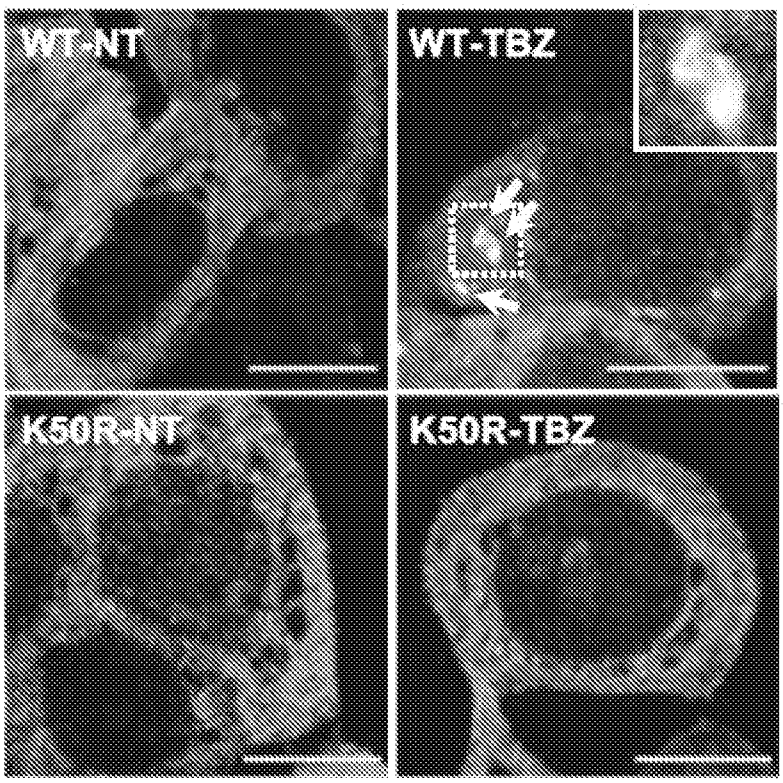
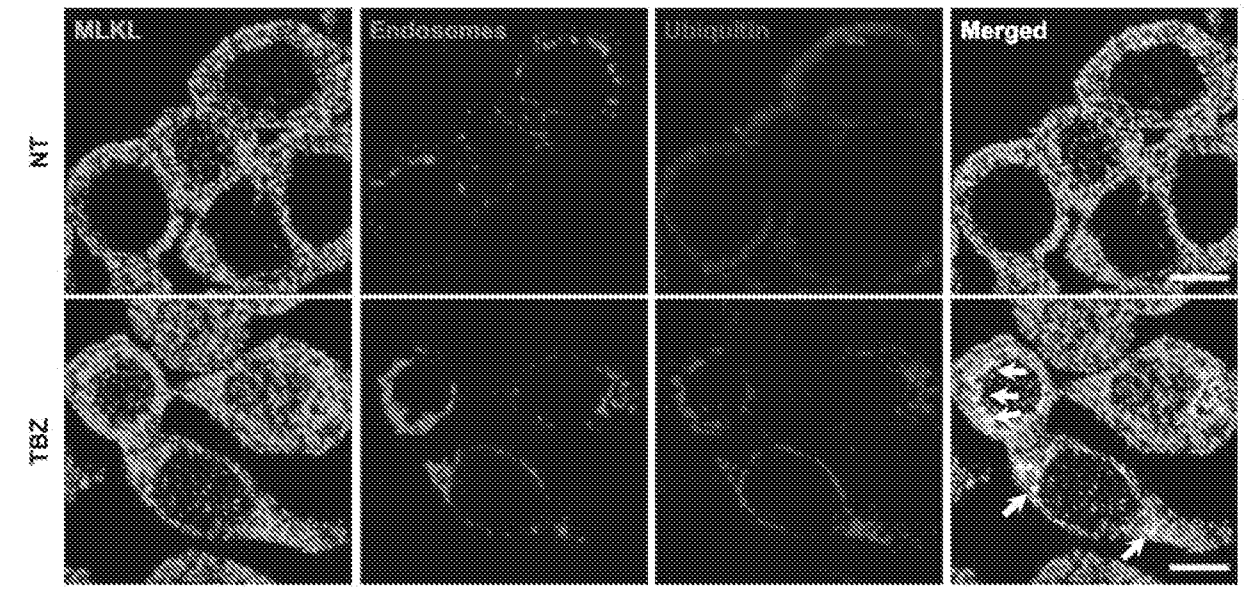


FIG. 6D

FIG. 6E



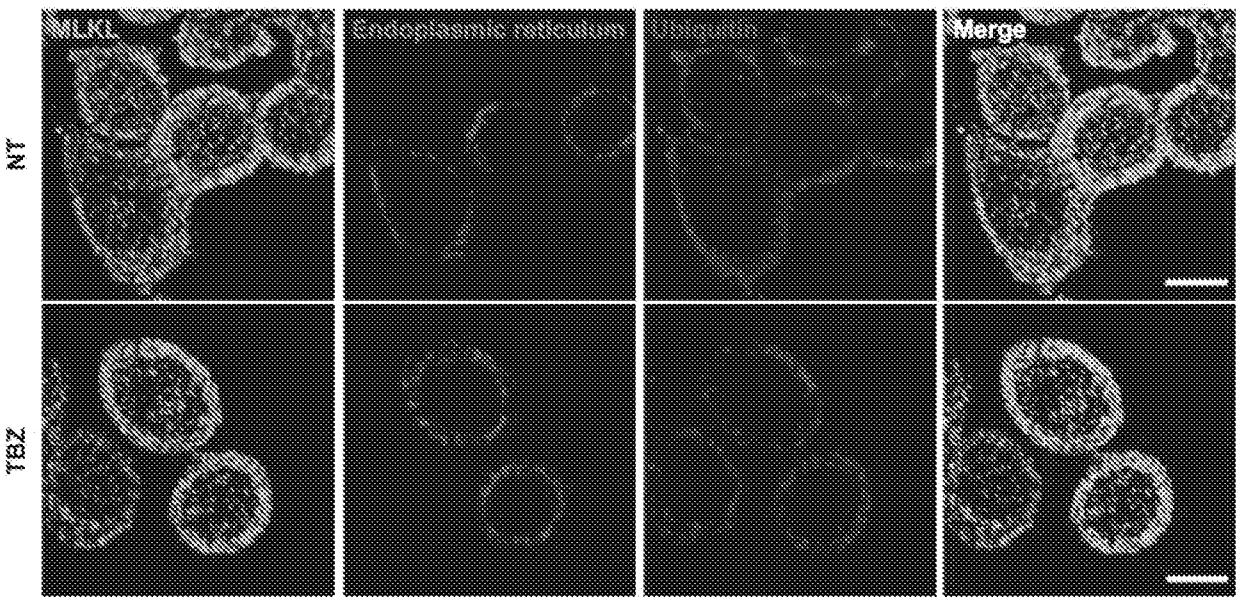


FIG. 6F

FIG. 7A

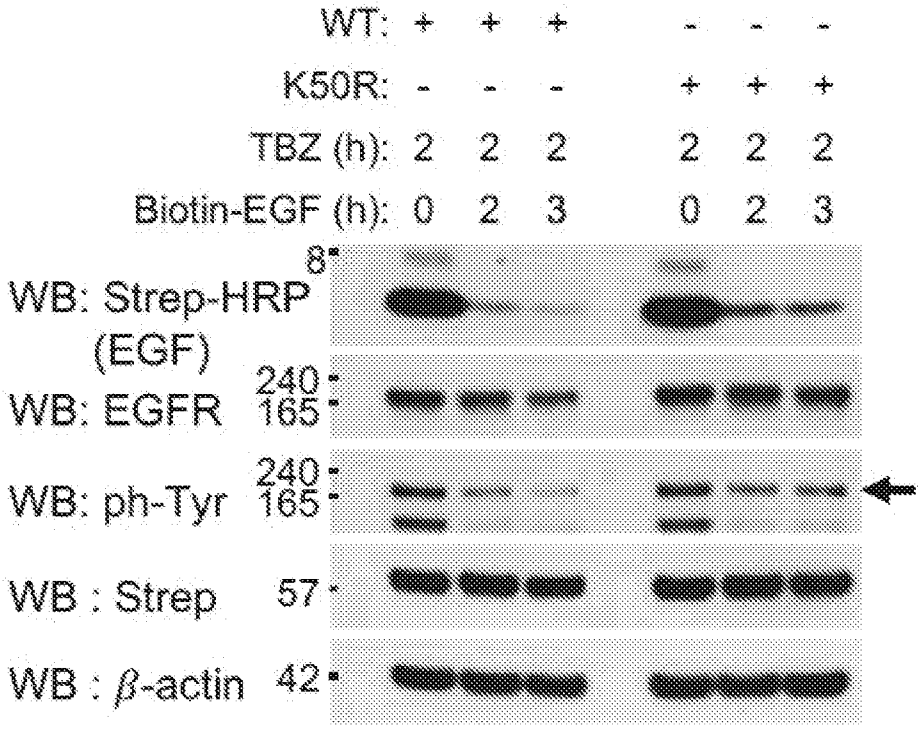

WT: + + + - - -
K50R: - - - + + +
TBZ (h): 2 2 2 2 2 2
Biotin-EGF (h): 0 2 3 0 2 3
WB: Strep-HRP (EGF)
WB: EGFR
WB: ph-Tyr
WB : Strep
WB : β-actin
8
240
165
240
165
57
42

FIG. 7B

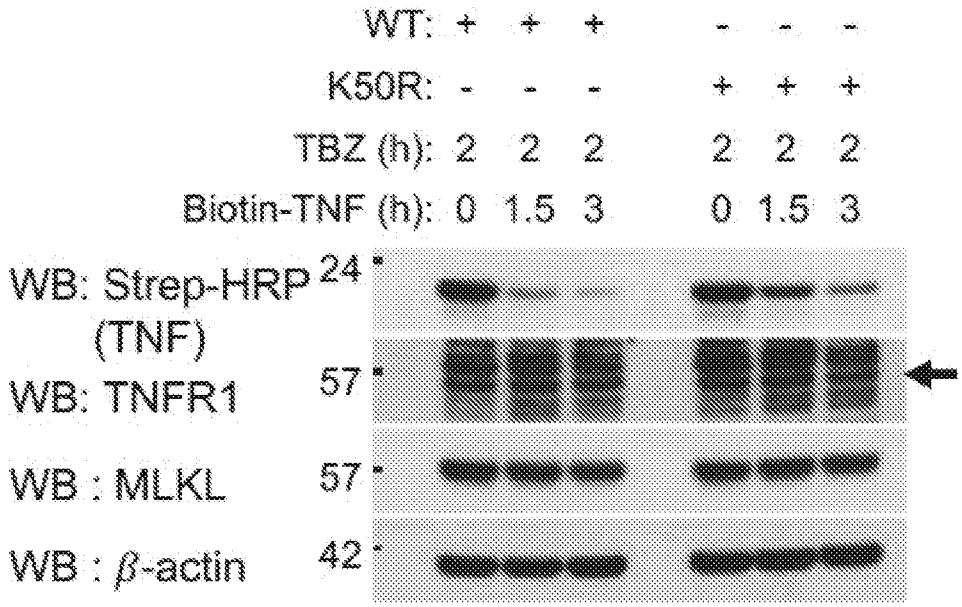

WT: + + + - - -
K50R: - - - + + +
TBZ (h): 2 2 2 2 2 2
Biotin-TNF (h): 0 1.5 3 0 1.5 3
WB: Strep-HRP (TNF)
WB: TNFR1
WB : MLKL
WB : β-actin
24
57
57
42

FIG. 7C

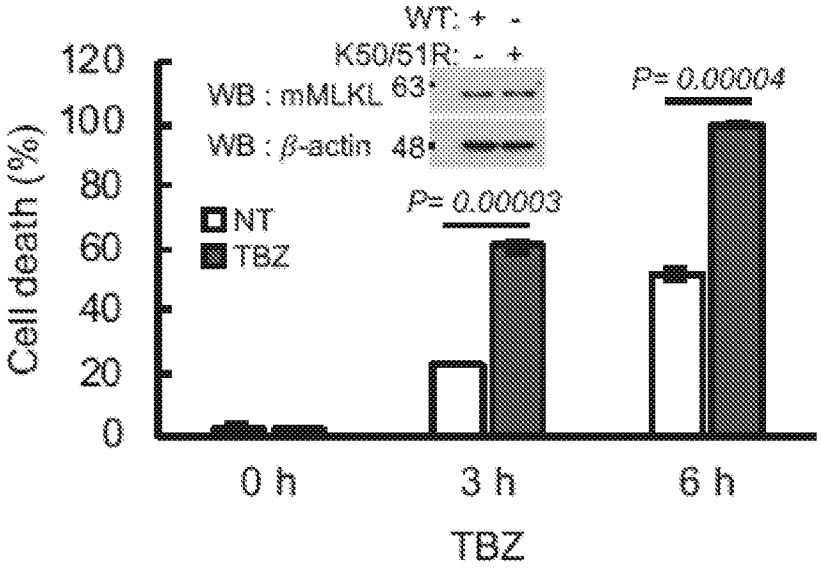
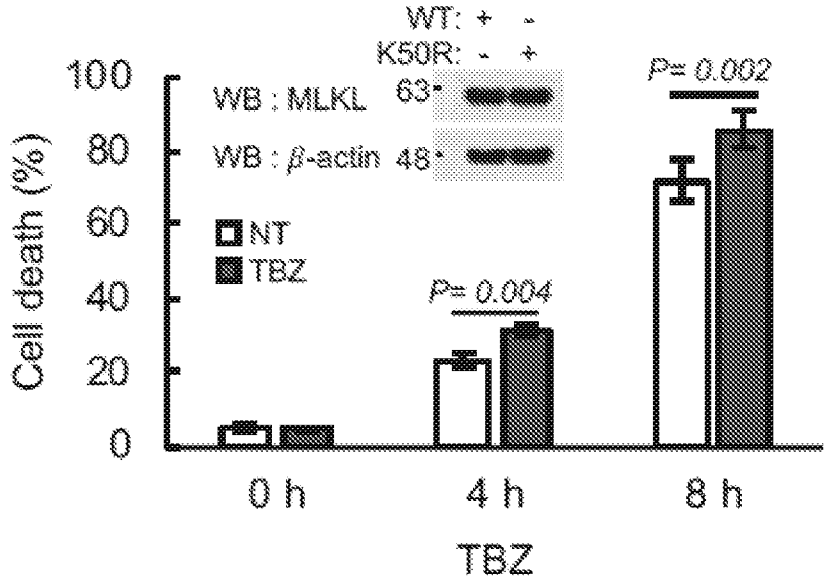

WT: + -
K50/51R: - +
WB : mMLKL 63
WB : β-actin 48
P= 0.00004
P= 0.00003
NT
TBZ
Cell death (%)
120
100
80
60
40
20
0
0 h
3 h
6 h
TBZ

FIG. 7D


WT: + -
K50R: - +
WB : MLKL 63
WB : β-actin 48
P= 0.002
P= 0.004
NT
TBZ
Cell death (%)
100
80
60
40
20
0
0 h
4 h
8 h
TBZ

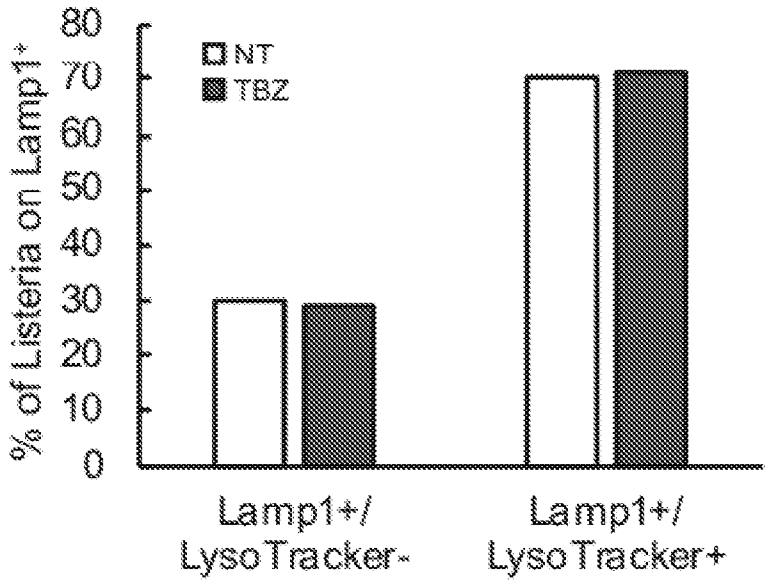

% of Listeria on Lamp1+
80
70
60
50
40
30
20
10
0
NT
TBZ
Lamp1+/
LysoTracker-
Lamp1+/
LysoTracker+

FIG. 7E

FIG. 7F

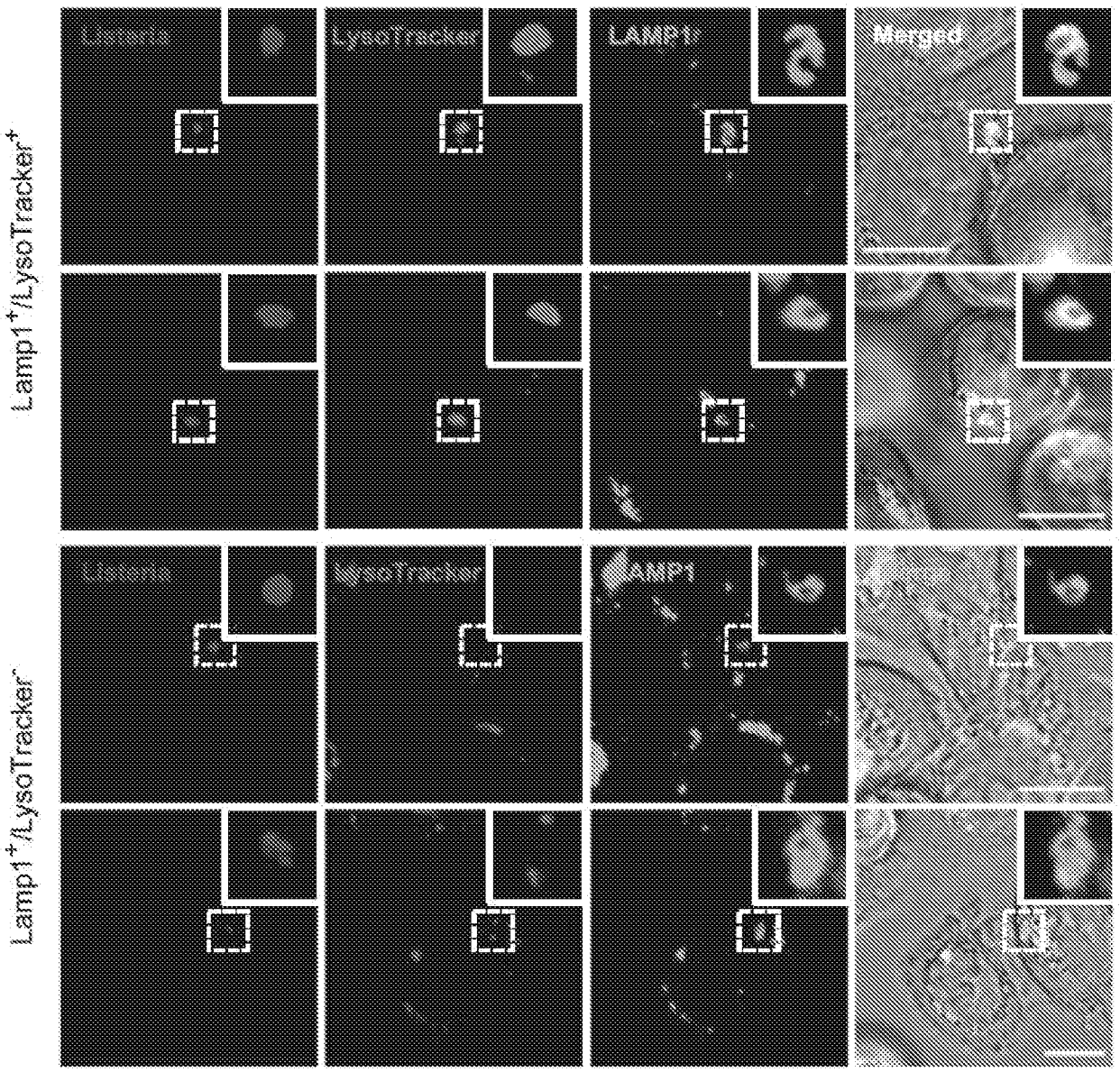

Lamp1+/LysoTracker+
Lamp1+/LysoTracker-
LysoTracker
LAMP1
Merged

FIG. 8

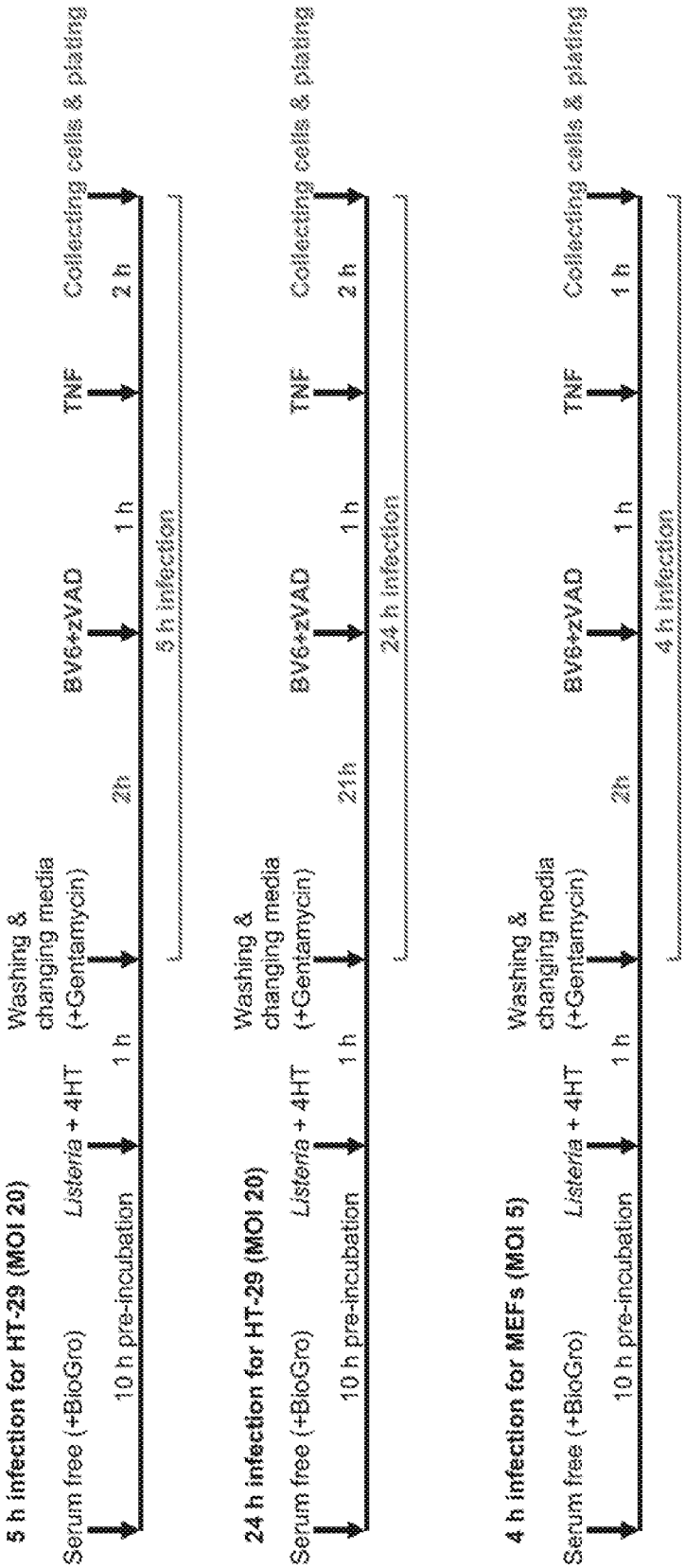
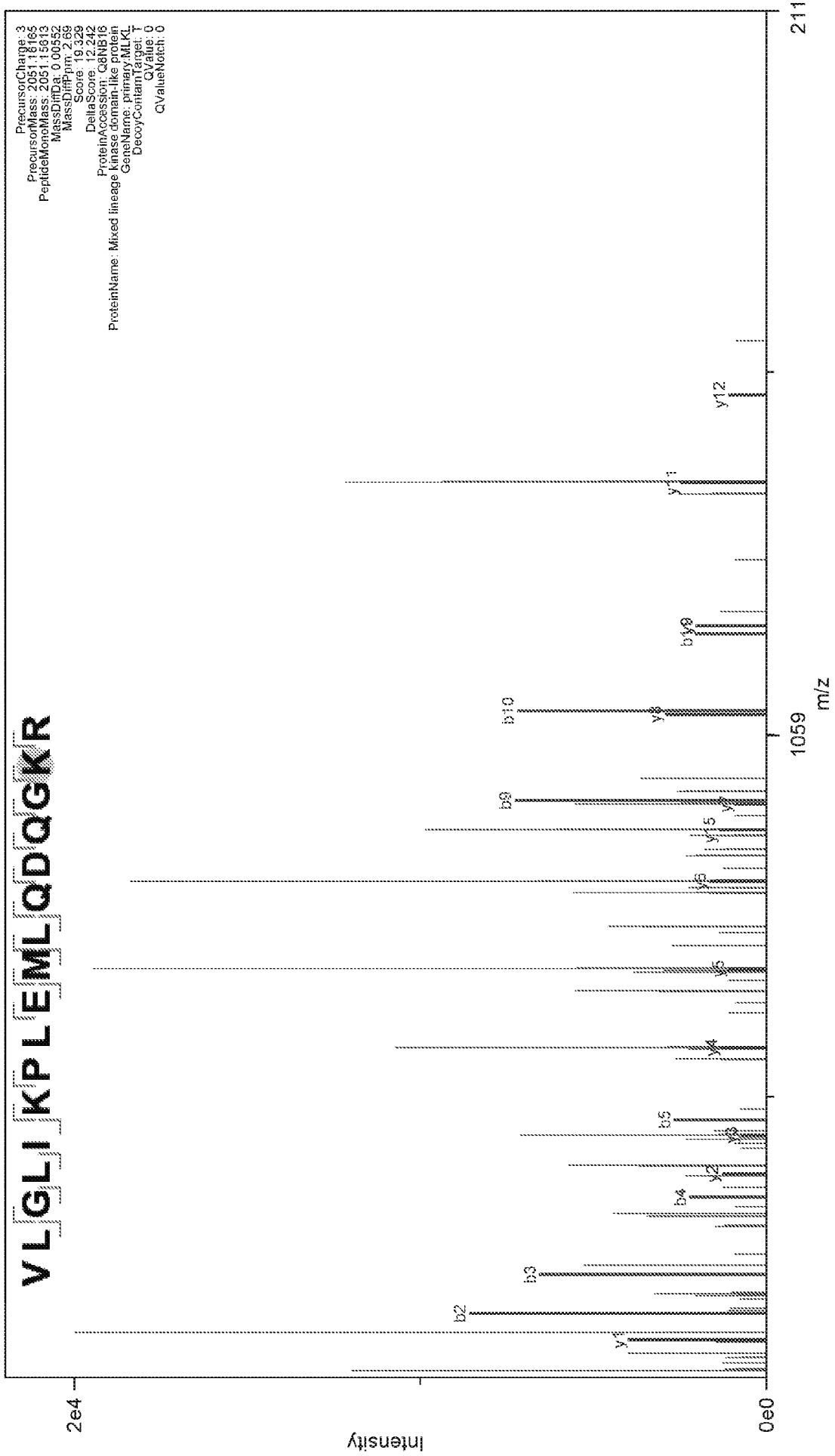

5 h infection for HT-29 (MOI 20)
Serum free (+BioGro)
10 h pre-incubation
Listeria + 4HT
1 h
Washing & changing media (+Gentamycin)
2h
BV6+zVAD
1 h
TNF
2 h
Collecting cells & plating
5 h infection
24 h infection for HT-29 (MOI 20)
Serum free (+BioGro)
10 h pre-incubation
Listeria + 4HT
1 h
Washing & changing media (+Gentamycin)
21h
BV6+zVAD
1 h
TNF
2 h
Collecting cells & plating
24 h infection
4 h infection for MEFs (MOI 5)
Serum free (+BioGro)
10 h pre-incubation
Listeria + 4HT
1 h
Washing & changing media (+Gentamycin)
2h
BV6+zVAD
1 h
TNF
1 h
Collecting cells & plating
4 h infection

FIG. 9A


PrecursorCharge: 3
PrecursorMass: 2051.18165
PeptideMonoMass: 2051.15613
MassDiffDa: 0.00552
MassDiffPpm: 2.69
Score: 19.329
DeltaScore: 12.242
ProteinAccession: Q8NB16
ProteinName: Mixed lineage kinase domain-like protein
GeneName: primary:MLKL
DecoyContainTarget: T
QValue: 0
QValueNotch: 0
V L G L I K P L E M L Q D Q G K R
Intensity
2e4
0e0
1059
m/z
2111
b2
b3
b4
b5
b9
b10
y1
y2
y4
y5
y6
y8
y11
y12

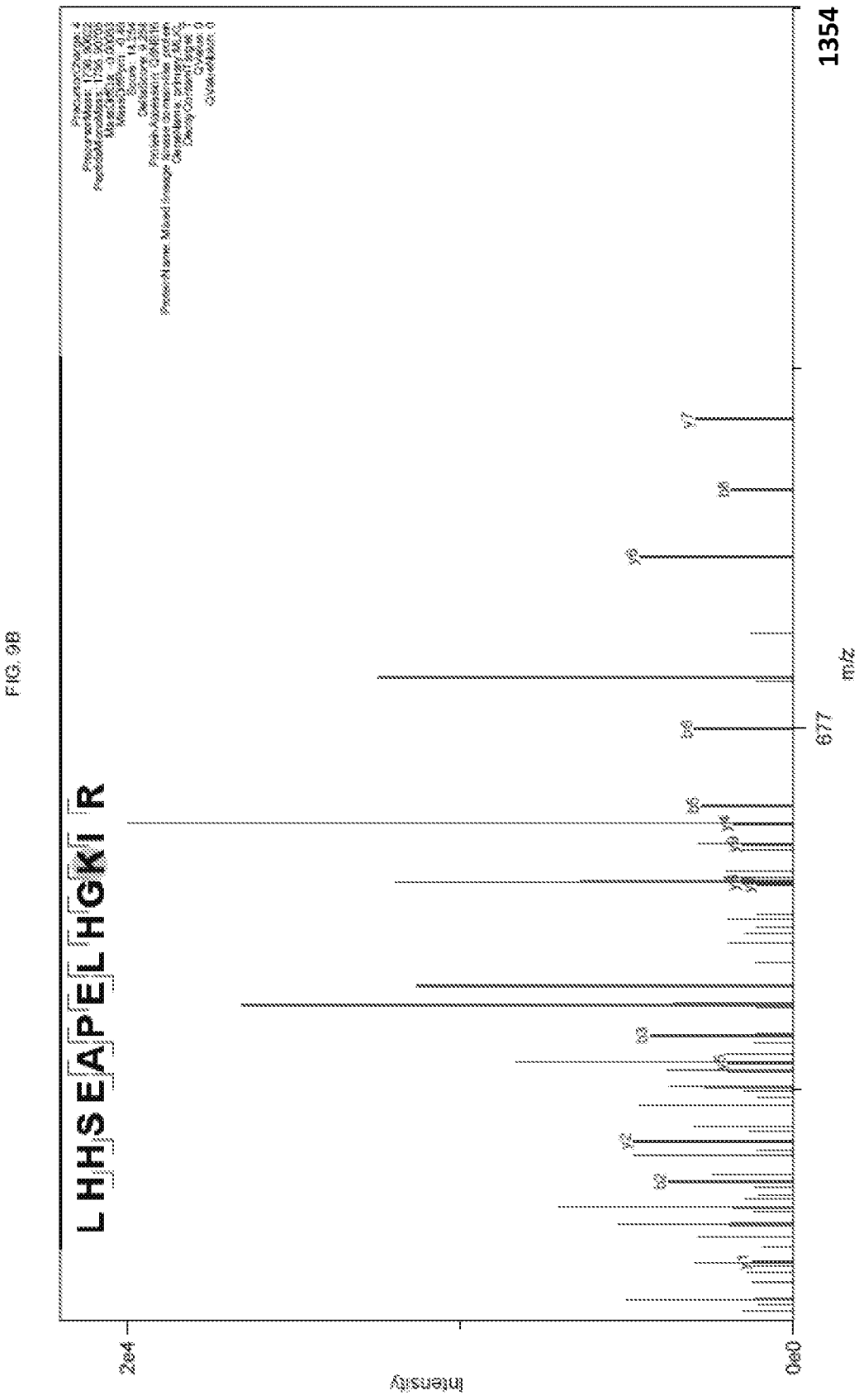


FIG. 9B

FIG. 10A

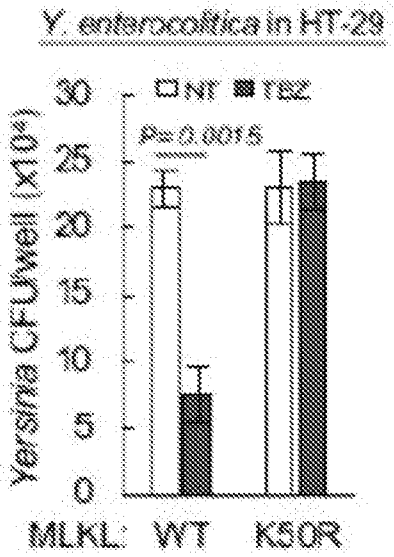


FIG. 10B

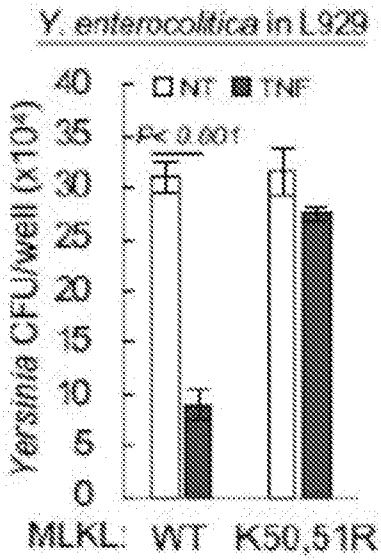


FIG. 10C

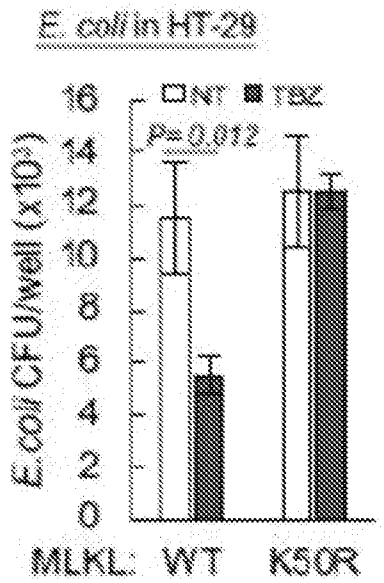
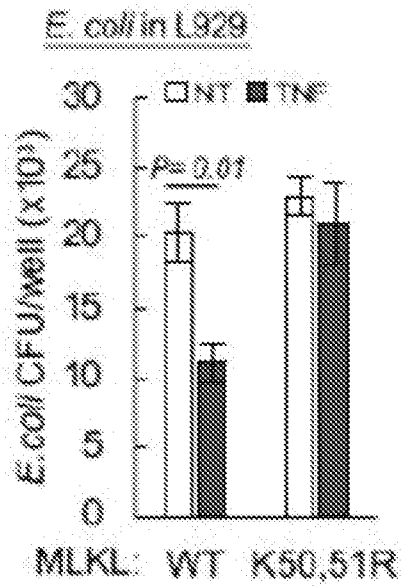


FIG. 10D



INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2021/051498

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/53 A61P31/00 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DONDELINGER YVES ET AL: "Poly-ubiquitination in TNFR1-mediated necroptosis", CMLS CELLULAR AND MOLECULAR LIFE SCIENCES, BIRKHAUSER VERLAG, HEIDELBERG, DE, vol. 73, no. 11, 11 April 2016 (2016-04-11), pages 2165-2176, XP035738829, ISSN: 1420-682X, DOI: 10.1007/S00018-016-2191-4 [retrieved on 2016-04-11] fig 3; ----- -/--	1-38

[x] Further documents are listed in the continuation of Box C.
[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2022	21/04/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilch, Bartosz

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2021/051498

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

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

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2021/051498

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WU CHAOYI ET AL: "Interconnections among major forms of regulated cell death", APOPTOSIS, LONDON, GB, vol. 25, no. 9-10, 5 September 2020 (2020-09-05), pages 616-624, XP037258659, ISSN: 1360-8185, DOI: 10.1007/S10495-020-01632-2 [retrieved on 2020-09-05] page 618, right-hand column; -----	1-38

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