Engineered Cells for Controlled Pyroptotic Signaling and Therapeutic Applications

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October 15, 2025

Technical Field

The present invention relates to engineered cells comprising modified mitochondrial anchored protein ligase (MAPL) expression systems, methods for controlling pyroptotic cell death pathways, and therapeutic applications thereof. More specifically, the invention encompasses novel cellular platforms that exploit the MAPL-mediated mitochondrial-lysosomal axis to regulate inflammatory signaling and programmed cell death for treating cancer, inflammatory disorders, and neurodegenerative diseases.

Background Art

Pyroptosis represents a specialized form of inflammatory programmed cell death characterized by gasdermin-mediated plasma membrane pore formation and rapid cellular rupture accompanied by release of pro-inflammatory cytokines. This process plays critical roles in innate immunity, pathogen defense, and various disease states including cancer, neurodegeneration, and autoinflammatory conditions. Recent advances in understanding pyroptotic mechanisms have revealed complex interactions between mitochondrial dysfunction, lysosomal membrane permeabilization, and cytosolic detection of mitochondrial DNA (mtDNA) by the cGAS-STING pathway.

MAPL is an outer mitochondrial membrane-localized E3 ligase that catalyzes both SUMOylation and ubiquitination of diverse protein substrates. Previous studies have demonstrated that MAPL participates in mitochondrial dynamics, mitophagy regulation, and apoptotic cell death through SUMOylation of the fission GTPase DRP1. Genetic ablation of MAPL in mice confers protection against various forms of cellular stress, including neonatal anesthesia-induced neuronal death, septic cardiomyopathy, and hepatocellular transformation, indicating that MAPL functions as a critical determinant of cell survival and death decisions.

Recent mechanistic investigations have uncovered that MAPL overexpression induces a BAX/BAK-independent form of cell death distinct from classical intrinsic apoptosis. Genome-wide CRISPR knockout screening has identified essential components of the pyroptotic machinery, including NLRP3 inflammasome constituents, gasdermin family members (GSDMD and GSDME), and the cGAS-STING DNA sensing pathway, as requisite mediators of MAPL-induced cell death. Furthermore, multiple genes associated with Parkinson disease pathogenesis, including VPS35, LRRK2, GBA, and VPS13C, have emerged as regulators of this MAPL-dependent pyroptotic pathway, suggesting convergence between mitochondrial quality control mechanisms and inflammatory cell death programs.

The molecular pathway involves MAPL-dependent formation of mitochondrial-derived vesicles (MDVs) containing mtDNA cargo that traffic to a specialized subset of lysosomes via MIRO1/2 and VPS35-dependent mechanisms. Subsequent lysosomal membrane permeabilization, mediated by GSDME pore formation and requiring LRRK2 function, releases mtDNA into the cytosol where it activates cGAS-STING signaling. This activation amplifies NLRP3 inflammasome assembly and caspase-1-mediated GSDMD cleavage, culminating in plasma membrane rupture and pyroptotic cell death. The pathway operates independently of canonical mitochondrial outer membrane permeabilization through BAX/BAK pores and does not require voltage-dependent anion channel (VDAC) activity.

Despite these mechanistic insights, existing cellular platforms for therapeutic exploitation of pyroptotic pathways suffer from significant limitations. Uncontrolled pyroptosis can precipitate excessive inflammation and tissue damage, while insufficient pyroptotic activity permits immune evasion by cancer cells and persistence of infected cells. Current approaches to modulate pyroptosis lack spatial and temporal precision, cell-type specificity, and fail to integrate multiple regulatory checkpoints that govern the decision between inflammatory cell death and cellular survival.

Therefore, substantial unmet need exists for engineered cellular systems that provide precise, tunable control over MAPL-dependent pyroptotic signaling pathways. Such systems would enable therapeutic applications requiring controlled inflammatory responses, targeted elimination of diseased cells, or enhancement of anti-tumor immunity while minimizing off-target toxicity and chronic inflammation.

Summary of Invention

The present invention addresses multiple technical challenges inherent in therapeutic modulation of pyroptotic cell death pathways. First, existing methods for inducing pyroptosis lack cell-type specificity and temporal control, resulting in unintended inflammation and tissue damage. Second, constitutive activation of pyroptotic machinery is incompatible with stable cellular platforms required for therapeutic delivery. Third, the complex multi-step nature of MAPL-dependent pyroptosis, involving mitochondrial vesicle formation, lysosomal trafficking, membrane permeabilization, and inflammasome activation, presents challenges for rational pathway modulation. Fourth, no existing cellular platforms integrate the newly discovered mitochondrial-lysosomal axis with controllable pyroptotic output suitable for therapeutic applications.

The invention solves these problems by providing engineered cells comprising inducible MAPL expression systems coupled with modular regulatory elements that control distinct steps of the pyroptotic pathway, thereby enabling precise spatiotemporal control over inflammatory cell death while maintaining cellular viability under baseline conditions.

The present invention provides engineered cells comprising a modified MAPL expression cassette under control of an inducible promoter system. The engineered cells further comprise at least one regulatory module selected from: (i) a mitochondrial-derived vesicle (MDV) trafficking module comprising modified expression levels of MIRO1, MIRO2, or VPS35 polypeptides; (ii) a lysosomal targeting module comprising modified expression levels of RAB GTPases, HOPS complex components, or CORVET complex components; (iii) a gasdermin regulation module comprising modified expression levels of GSDMD, GSDME, or GSDMC polypeptides or fragments thereof; (iv) an inflammasome modulation module comprising modified expression levels of NLRP3, ASC, or caspase-1 polypeptides; and (v) a cGAS-STING pathway module comprising modified expression levels of cGAS, STING, or downstream effectors thereof.

In preferred embodiments, the inducible promoter system is selected from tetracycline-responsive elements, hypoxia-responsive elements, radiation-inducible promoters, small molecule-responsive promoters, or tissue-specific promoters. The MAPL expression cassette encodes wild-type human MAPL (MUL1) protein or a functional variant thereof retaining at least 85% sequence identity and SUMO E3 ligase activity. The engineered cells optionally comprise a MAPL variant with enhanced SUMOylation activity, altered substrate specificity, or modified subcellular localization signals.

The engineered cells of the invention maintain viability under non-inducing conditions while retaining capacity for rapid pyroptotic cell death upon induction. The cells produce mitochondrial-derived vesicles containing mitochondrial DNA cargo in response to MAPL induction, wherein said vesicles traffic to lysosomes via MIRO1/2-dependent and VPS35-dependent mechanisms. The lysosomes undergo membrane permeabilization mediated by GSDME pore formation, resulting in cytosolic release of mitochondrial DNA. The released mitochondrial DNA activates the cGAS-STING pathway and NLRP3 inflammasome, leading to caspase-1 activation, GSDMD cleavage, plasma membrane pore formation, and pyroptotic cell death accompanied by release of inflammatory cytokines including IL-1β, IL-6, and IL-18.

In certain embodiments, the engineered cells comprise modifications that enhance specific steps of the pyroptotic pathway. For example, cells may overexpress MIRO1 and MIRO2 to increase mitochondrial-derived vesicle formation, thereby amplifying the pyroptotic response. Alternatively, cells may comprise partial knockdown of LRRK2 to delay lysosomal membrane permeabilization, providing an extended window for therapeutic delivery before cell death. In other embodiments, cells comprise conditional expression of GSDME fragments that are activated by specific proteases present in disease microenvironments, conferring disease-site-specific pyroptotic activation.

The invention further provides engineered cells comprising biosensor modules that report on pyroptotic pathway activation. Such biosensors include fluorescent reporters responsive to cGAS-STING activation, NLRP3 inflammasome assembly, gasdermin pore formation, or mitochondrial DNA release. These biosensor-equipped cells enable real-time monitoring of pyroptotic progression and facilitate optimization of therapeutic regimens.

In advanced embodiments, the engineered cells comprise multiple orthogonal inducible systems controlling distinct nodes of the pyroptotic pathway. For instance, a first inducible system controls MAPL expression while a second independent inducible system controls GSDME expression or activation. This dual-control architecture provides enhanced safety through requirement of two independent activation signals before commitment to pyroptotic cell death.

The engineered cells of the invention are derived from various cellular sources including primary human cells, immortalized cell lines, induced pluripotent stem cells, or differentiated progeny thereof. Preferred cell types include macrophages, dendritic cells, natural killer cells, T cells, mesenchymal stem cells, or cancer cell lines. The choice of parental cell type is determined by the intended therapeutic application and desired biodistribution, persistence, and immunological properties.

The engineered cells of the present invention provide multiple advantageous effects over existing cellular therapeutics and pyroptosis-inducing agents. First, the inducible MAPL expression system enables precise temporal control over pyroptotic activation, allowing therapeutic cells to be delivered, distribute to target tissues, and undergo verification of localization before pyroptotic induction. Second, the modular regulatory elements permit independent optimization of distinct pathway steps, facilitating fine-tuning of inflammatory output intensity and kinetics. Third, integration of the mitochondrial-lysosomal axis provides a novel mechanistic route to pyroptosis that bypasses certain resistance mechanisms such as BCL-2 family overexpression. Fourth, the engineered cells maintain normal viability and function under non-inducing conditions, enabling complex cellular behaviors such as trafficking, antigen

presentation, or local cytokine production prior to terminal pyroptotic activation. Fifth, the inflammatory cytokines and damage-associated molecular patterns released upon pyroptosis create a pro-immunogenic microenvironment that enhances anti-tumor immunity or pathogen clearance. Sixth, the discovery that Parkinson disease-associated proteins regulate this pathway enables development of cell-based models for mechanistic investigation and drug screening. Seventh, the cells provide a platform for investigating the physiological roles of the MAPL-mitochondrial-lysosomal axis in contexts ranging from innate immunity to neurodegeneration.

Detailed Description of the Invention

The present invention arises from the comprehensive understanding that mitochondrial anchored protein ligase (MAPL), encoded by the MUL1 gene located on human chromosome 1p36.11, functions as a critical regulator of a specialized inflammatory cell death pathway designated pyroptosis. This pathway operates through a previously uncharacterized mitochondrial-lysosomal axis involving formation of mitochondrial-derived vesicles containing mitochondrial DNA, trafficking of these vesicles to lysosomes via MIRO-dependent and VPS35-dependent mechanisms, gasdermin E-mediated permeabilization of lysosomal membranes, release of mitochondrial DNA into the cytosol, activation of cyclic GMP-AMP synthase and stimulator of interferon genes (cGAS-STING) pathway, amplification of NLRP3 inflammasome signaling, caspase-1-mediated cleavage of gasdermin D, and terminal plasma membrane rupture characteristic of pyroptotic cell death. The discovery that this pathway operates independently of classical BAX/BAK-dependent mitochondrial outer membrane permeabilization and functions as an essential component of innate immune responses in primary macrophages provides the foundation for the present invention.

The engineered cells of the invention comprise precisely designed genetic modifications that confer controllable activation of this MAPL-dependent pyroptotic pathway while maintaining cellular viability and functionality under baseline non-inducing conditions. The fundamental innovation resides in the integration of inducible expression systems with the newly elucidated mitochondrial-lysosomal-pyroptotic axis, enabling therapeutic applications previously unattainable with existing cellular platforms. The invention provides both the engineered cellular compositions and the methods for their production and therapeutic use.

MAPL is a 352 amino acid transmembrane protein localized to the outer mitochondrial membrane through an N-terminal transmembrane domain spanning approximately residues 10-30. The protein comprises several functional domains essential for its E3 ligase activity and substrate recognition. The RING finger domain, located at residues 104-142 according to InterPro domain annotation (IPR001841), provides the catalytic activity for both ubiquitination and SUMOylation reactions. This RING domain coordinates two zinc ions through a characteristic cross-brace arrangement of cysteine and histidine residues conforming to the consensus sequence C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-C-X2-C-X(4-48)-C-X2-C where X represents any amino acid.

The RING domain specifically interacts with E2 conjugating enzymes including UBC9 for SUMOylation reactions and various E2 enzymes of the UBE2 family for ubiquitination reactions. Crystal structure studies of related RING domain E3 ligases, such as c-Cbl (PDB ID: 1FBV) and TRAF6 (PDB ID: 3HCS), provide structural models for understanding MAPL's catalytic mechanism. The RING domain positions the E2-conjugating enzyme and substrate in proximity to facilitate transfer of ubiquitin or SUMO from the E2 active site cysteine to substrate lysine residues.

MAPL contains additional regulatory regions including a predicted coiled-coil domain at residues 200-250 that likely mediates protein-protein interactions with substrates and regulatory partners. The C-terminal region, residues 250-352, contains multiple predicted phosphorylation sites (Ser275, Ser298, Thr312 based on PhosphoSitePlus database accession P021147) suggesting regulation by kinase signaling pathways. The subcellular localization of MAPL is determined by the N-terminal transmembrane anchor, which inserts into the outer mitochondrial membrane with the bulk of the protein including the RING domain oriented toward the cytosol, enabling access to cytosolic and outer membrane-associated substrates.

MAPL's SUMOylation activity employs the small ubiquitin-like modifier SUMO1 (96 amino acids, 11.6 kilodaltons) which is conjugated to lysine residues within substrate proteins. The SUMOylation consensus sequence is typically ψKXE where ψ represents a large hydrophobic residue (often isoleucine, leucine, or valine), K is the acceptor lysine, X is any amino acid, and E is glutamic acid. Known MAPL SUMOylation substrates include DRP1 (dynamin-related protein 1, encoded by DNM1L gene) at lysine 597, resulting in stabilization of DRP1 oligomers at mitochondrial constriction sites and promotion of mitochondrial fission. Other identified substrates include AKT1 (protein kinase B), where SUMOylation by MAPL reduces AKT1 activity and attenuates pro-survival signaling.

The ubiquitination activity of MAPL conjugates ubiquitin (76 amino acids, 8.6 kilodaltons) to substrates, either as monoubiquitination or polyubiquitin chains with various linkage topologies. MAPL has been shown to catalyze K48-linked polyubiquitination, which typically targets substrates for proteasomal degradation, and K63-linked polyubiquitination, which often serves regulatory signaling functions. Identified ubiquitination substrates include MFN2 (mitofusin 2, a mitochondrial fusion GTPase) where MAPL-mediated ubiquitination promotes MFN2 degradation and mitochondrial fragmentation.

The gasdermin family comprises six members in humans: GSDMA, GSDMB, GSDMC, GSDMD, GSDME (also designated DFNA5), and PJVK (pejvakin). Each gasdermin protein shares a conserved structural architecture consisting of an N-terminal pore-forming domain (approximately 240-280 amino acids) and a C-terminal autoinhibitory domain (approximately 160-200 amino acids) connected by a flexible linker region. In the full-length inactive state, the C-terminal domain binds intramolecularly to the N-terminal domain, preventing membrane interaction and pore formation.

Gasdermin D (GSDMD), encoded by the GSDMD gene on chromosome 8q24.3, comprises 484 amino acids with a molecular weight of 52.7 kilodaltons. The N-terminal pore-forming domain spans residues 1-275, while the C-terminal autoinhibitory domain comprises residues 310-484. The interdomain linker (residues 276-309) contains the primary caspase-1 cleavage site at aspartic acid 275 (Asp275), conforming to the caspase-1 recognition sequence FLTD. Upon cleavage by activated caspase-1, the N-terminal fragment (GSDMD-N, approximately 31 kilodaltons) dissociates from the C-terminal domain and undergoes oligomerization. Structural studies using cryo-electron microscopy (cryo-EM) have revealed that GSDMD-N assembles into large ring-shaped oligomers comprising 26-28 monomers with an outer diameter of approximately 18 nanometers and an inner pore diameter of approximately 10-15 nanometers (Nature 2018, volume 557, pages 62-67). These oligomeric rings insert into lipid membranes through hydrophobic interactions, with preference for membranes containing cardiolipin and phosphatidylinositol phosphates.

Gasdermin E (GSDME), encoded by the GSDME/DFNA5 gene on chromosome 7p15.3, comprises 496 amino acids with a molecular weight of 54.3 kilodaltons. GSDME is cleaved by caspase-3 at aspartic acid 270 (Asp270) within the sequence DMPD, generating an N-terminal pore-forming fragment (GSDME-N, approximately 30 kilodaltons). GSDME-N shares structural similarity with GSDMD-N and similarly forms oligomeric pores in membranes. The critical distinction is that GSDME activation is downstream of caspase-3 rather than caspase-1, connecting apoptotic caspase activation to pyroptotic membrane permeabilization. Expression of GSDME is tissue-restricted, with high expression in brain, small intestine, and kidney, and low or absent expression in many transformed cell lines. Restoration of GSDME expression in cancer cells through demethylation of the GSDME promoter converts apoptotic stimuli into pyroptotic cell death.

The membrane selectivity of gasdermin pores is determined by lipid composition. GSDMD-N and GSDME-N exhibit preferential binding to membranes enriched in phosphatidylinositol phosphates, particularly phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), and in cardiolipin. The inner leaflet of the plasma membrane is enriched in these lipids, explaining preferential pore formation at the plasma membrane. However, lysosomes also contain elevated levels of PI(4,5)P2 and bis(monoacylglycero)phosphate (a cardiolipin isomer), rendering lysosomal membranes susceptible to gasdermin pore formation. The present invention exploits this membrane selectivity wherein GSDME pores preferentially form in lysosomal membranes containing mitochondrial-derived vesicles, creating the initial breach that releases mitochondrial DNA into the cytosol.

The NLRP3 inflammasome is a multiprotein complex central to innate immune responses and pyroptotic cell death. The core components are NLRP3 (NOD-like receptor family pyrin domain-containing 3, also designated NALP3 or cryopyrin), ASC (apoptosis-associated speck-like protein containing a CARD, encoded by PYCARD gene), and pro-caspase-1.

NLRP3, encoded by the NLRP3 gene on chromosome 1q44, comprises 1036 amino acids with a molecular weight of 118 kilodaltons. The protein contains multiple functional domains arranged from N-terminus to C-terminus: a pyrin domain (PYD, residues 1-93) mediating homotypic interactions with ASC, a central nucleotide-binding domain (NBD or NACHT domain, residues 213-534) providing oligomerization activity and ATPase function, and C-terminal leucine-rich repeats (LRRs, residues 731-1036) functioning in autoinhibition and potentially in sensing activating stimuli. In the inactive state, NLRP3 exists as monomers or dimers maintained in an autoinhibited conformation through intramolecular interactions between the LRR domain and the NACHT domain.

Upon activation by diverse stimuli including ATP, pore-forming toxins, crystalline materials, and mitochondrial dysfunction, NLRP3 undergoes conformational changes releasing autoinhibition and exposing the NACHT domain. The NACHT domain then mediates ATP-dependent oligomerization, forming a wheel-like structure with 10-12 NLRP3 subunits (Nature Immunology 2016, volume 17, pages 250-258). The pyrin domains project outward from this oligomeric platform, creating multiple binding sites for recruitment of ASC.

ASC, encoded by the PYCARD gene on chromosome 16p11.2, comprises 195 amino acids with a molecular weight of 21.5 kilodaltons. ASC contains an N-terminal pyrin domain (residues 1-91) and a C-terminal CARD domain (caspase recruitment domain, residues 101-195) connected by a short linker. The pyrin domain of ASC binds to the pyrin domain of NLRP3 through homotypic PYD-PYD interactions. Upon recruitment to oligomerized NLRP3, ASC undergoes nucleation-dependent polymerization forming extended filamentous structures. Structural studies using cryo-EM have revealed that ASC filaments comprise helical assemblies where adjacent ASC molecules interact through PYD-PYD interfaces (Cell 2014, volume 156, pages 1193-1206). These ASC filaments grow to micrometer lengths, creating a dense meshwork of CARD domains projecting from the filament surface.

The CARD domains of polymerized ASC recruit pro-caspase-1 through CARD-CARD interactions. Pro-caspase-1, encoded by the CASP1 gene on chromosome 11q22.3, comprises 404 amino acids with a molecular weight of 45 kilodaltons. The protein contains an N-terminal CARD domain (residues 1-95) and a C-terminal caspase domain comprising a large subunit (p20, residues 120-297) and a small subunit (p10, residues 317-404). In the monomeric state, pro-caspase-1 possesses negligible enzymatic activity. However, upon recruitment to ASC filaments, pro-caspase-1 molecules are brought into close proximity, inducing dimerization and autocatalytic cleavage at Asp297 and Asp316. This cleavage separates the large and small subunits, which reassemble into the active caspase-1 heterotetramer comprising two p20 subunits and two p10 subunits arranged as a (p20-p10)2 complex.

Activated caspase-1 is a cysteine protease with specificity for cleavage after aspartic acid residues, particularly recognizing the tetrapeptide sequence WEHD or YVAD. Principal substrates include pro-IL-1β (cleavage at Asp116 to generate mature 17 kilodalton IL-1β), pro-IL-18 (cleavage at Asp36 to generate mature 18 kilodalton IL-18), and gasdermin D (cleavage at Asp275 to generate the pore-forming GSDMD-N fragment). The enzymatic activity of caspase-1 propagates the inflammatory signal through cytokine maturation and executes pyroptotic cell death through gasdermin activation.

The cGAS-STING pathway is a cytosolic DNA sensing system that detects aberrant DNA in the cytoplasm and initiates type I interferon responses. This pathway plays essential roles in antiviral immunity, responses to self-DNA released from damaged organelles, and anti-tumor immunity.

Cyclic GMP-AMP synthase (cGAS), encoded by the MB21D1 gene on chromosome 6p21.2, comprises 522 amino acids with a molecular weight of 58.7 kilodaltons. The protein contains an unstructured N-terminal region (residues 1-160) and a C-terminal catalytic nucleotidyltransferase domain (residues 161-522) belonging to the CD-NTase family. Crystal structure studies have revealed that cGAS binds double-stranded DNA through an extended positively charged surface on the nucleotidyltransferase domain, with a binding site accommodating DNA lengths from approximately 45 base pairs to several kilobases (Science 2013, volume 341, pages 1390-1394). DNA binding induces conformational changes that activate the catalytic site.

Upon activation, cGAS catalyzes synthesis of 2'3'-cyclic GMP-AMP (2'3'-cGAMP) from ATP and GTP substrates. The reaction proceeds through formation of a cGAS-DNA-ATP-GTP quaternary complex, with cGAS catalyzing formation of a phosphodiester bond between the 2'-hydroxyl of GMP and the 5'-triphosphate of AMP, followed by formation of a second phosphodiester bond between the 3'-hydroxyl of AMP and the 5'-triphosphate (after pyrophosphate release) to close the ring, generating the unique 2'-5' and 3'-5' mixed-linkage cyclic dinucleotide. This 2'3'-cGAMP product is distinct from bacterial cyclic dinucleotides which contain exclusively 3'-5' linkages.

Stimulator of interferon genes (STING), encoded by the TMEM173 gene on chromosome 5q31.2, comprises 379 amino acids with a molecular weight of 42 kilodaltons. STING is an endoplasmic reticulum-resident transmembrane protein containing four transmembrane domains (residues 1-20, 24-44, 48-68, and 332-352) with N-terminus and C-terminus both oriented toward the cytosol, and a large cytosolic ligand-binding domain (residues 138-340). The ligand-binding domain forms a V-shaped butterfly-like homodimer, with the 2'3'-cGAMP binding pocket located at the dimer interface. Binding of 2'3'-cGAMP induces conformational changes that trigger STING trafficking from endoplasmic reticulum to Golgi apparatus and activate downstream signaling.

Upon 2'3'-cGAMP binding, STING recruits and activates TANK-binding kinase 1 (TBK1, encoded by TBK1 gene on chromosome 12q14.2, 729 amino acids, 84 kilodaltons). TBK1 is a serine/threonine kinase of the IKK family that phosphorylates STING at a C-terminal serine cluster (Ser365, Ser366 in human STING) creating a binding site for interferon regulatory factor 3 (IRF3). TBK1 phosphorylates IRF3 at Ser385, Ser386, Ser396, Ser398, and Ser402, inducing IRF3 dimerization, nuclear translocation, and transcriptional activation of type I interferon genes (IFNA, IFNB1). STING also activates the NF-κB pathway through recruitment of IKKα/IKKβ complex, leading to phosphorylation and degradation of IκBα and nuclear translocation of NF-κB heterodimers (p65/p50) to activate pro-inflammatory gene expression.

In the context of MAPL-induced pyroptosis, mitochondrial DNA released into the cytosol is recognized by cGAS, leading to 2'3'-cGAMP synthesis and STING activation. The resulting type I interferon and NF-κB signaling provides amplification of NLRP3 inflammasome activity through transcriptional upregulation of NLRP3, pro-IL-1β, and other inflammasome components. Additionally, recent evidence indicates that STING possesses direct non-transcriptional functions including proton channel activity that acidifies the cytosol and enhances NLRP3 activation, and trafficking to lysosomes where it may directly participate in lysosomal membrane permeabilization.

Mitochondrial-derived vesicles are cargo-selective vesicles that bud from mitochondria independently of wholesale mitophagy. MDV formation involves selective packaging of mitochondrial content (proteins, lipids, metabolites, or DNA) into vesicles that range from 70 to 150 nanometers in diameter, consistent with incorporation of both outer and inner mitochondrial membranes. The machinery governing MDV formation includes MIRO GTPases, motor proteins, and cargo recognition factors.

MIRO1 (mitochondrial Rho GTPase 1), encoded by the RHOT1 gene on chromosome 17q11.2, comprises 618 amino acids with a molecular weight of 68 kilodaltons. MIRO2 (mitochondrial Rho GTPase 2), encoded by the RHOT2 gene on chromosome 16p13.3, comprises 617 amino acids with a molecular weight of 68 kilodaltons. Both MIRO proteins share similar domain architectures: an N-terminal GTPase domain (residues 1-180), two EF-hand calcium-binding domains (residues 200-270 and 290-360), a second GTPase domain (residues 380-560), and a C-terminal transmembrane domain (residues 580-618) that anchors MIRO to the outer mitochondrial membrane.

MIRO proteins function as adaptors linking mitochondria to motor proteins and the cytoskeletal network. The N-terminal GTPase domain interacts with kinesin motor proteins (particularly KIF5 family members) mediating anterograde transport along microtubules toward the plus-end. The C-terminal GTPase domain interacts with dynein-dynactin complex mediating retrograde transport toward the minus-end. The EF-hand domains provide calcium-dependent regulation, where elevated calcium binding causes conformational changes that reduce motor protein interactions and arrest mitochondrial movement.

In the context of MDV formation, MIRO1 and MIRO2 mediate the formation of thin tubular protrusions from mitochondria that extend along microtubules. Live-cell imaging studies have documented that these MIRO-dependent tubules can extend several micrometers from the main mitochondrial network before undergoing scission to release vesicular carriers. The mechanism likely involves MIRO-mediated recruitment of motor proteins that exert pulling forces on mitochondrial membranes, combined with local membrane curvature generation and eventual dynamin-related protein 1 (DRP1)-mediated scission at the base of the tubule.

VPS35 (vacuolar protein sorting 35), encoded by the VPS35 gene on chromosome 16q11.2, comprises 796 amino acids with a molecular weight of 91.7 kilodaltons. VPS35 is a core component of the retromer complex, which functions in endosomal cargo sorting and retrograde trafficking from endosomes to the trans-Golgi network. The retromer comprises a cargo recognition subcomplex formed by VPS35, VPS26 (isoforms VPS26A and VPS26B), and VPS29, along with membrane deformation components of the sorting nexin family (SNX1, SNX2, SNX5, SNX6).

Crystal structure studies of the VPS35-VPS29-VPS26 trimer reveal that VPS35 forms an extended α-solenoid architecture creating a curved scaffold that interacts with cargo proteins through recognition of specific sorting motifs (Nature Structural & Molecular Biology 2011, volume 18, pages 608-614). VPS35 binds cargo proteins including cation-independent mannose-6-phosphate receptor, sortilin, and Wntless through recognition of cytoplasmic tail sequences containing aromatic residues and specific spacing motifs.

The role of VPS35 in mitochondrial-derived vesicle trafficking was initially identified through studies showing VPS35 interaction with MAPL and requirement for trafficking of peroxisome-targeted MDVs (Molecular Cell 2016, volume 61, pages 507-519). In the context of the present invention, VPS35 is required for efficient delivery of mtDNA-containing MDVs to lysosomes. The mechanism likely involves VPS35-mediated recognition of specific cargo proteins within MDVs, sorting of MDVs within the endolysosomal system, and routing to lysosomes rather than alternative destinations. The D620N mutation in VPS35 associated with familial Parkinson disease may alter these trafficking properties, potentially contributing to disease pathogenesis through aberrant MDV routing and altered inflammatory signaling.

Lysosomes are membrane-bound organelles containing hydrolytic enzymes maintained at acidic pH (4.5-5.0) through the activity of vacuolar-type H-ATPase (V-ATPase) proton pumps. Lysosomal trafficking and identity are regulated by RAB GTPases, tethering complexes, and SNARE proteins mediating membrane fusion events.

RAB7, encoded by the RAB7A gene on chromosome 3q21.3, comprises 207 amino acids with a molecular weight of 23.5 kilodaltons. RAB7 is the master regulator of late endosome and lysosome identity and function. RAB7 cycles between GDP-bound inactive and GTP-bound active states, with GTP-loading catalyzed by the Mon1-Ccz1 guanine nucleotide exchange factor complex and GTP hydrolysis stimulated by TBC1D2 and TBC1D5 GTPase-activating proteins. Active RAB7-GTP recruits multiple effector proteins including RILP (RAB-interacting lysosomal protein) which mediates coupling to dynein motors for centripetal lysosome movement, PLEKHM1 which recruits the autophagy machinery, and the HOPS tethering complex which mediates lysosome-lysosome and autophagosome-lysosome fusion.

The HOPS complex (homotypic fusion and protein sorting complex) is a hexameric protein complex comprising VPS11, VPS16, VPS18, VPS33A, VPS39, and VPS41 subunits. HOPS functions as a tethering factor capturing membranes at distances of 10-20 nanometers and as a SNARE chaperone facilitating assembly of SNARE complexes that execute membrane fusion. VPS33A within HOPS binds syntaxin 17 (STX17) on autophagosomes or syntaxin 7/8 on late endosomes, while VPS39 and VPS41 contain RAB7-binding domains that provide specificity for late endosomes and lysosomes.

The CORVET complex (class C core vacuole/endosome tethering complex) is related to HOPS but contains VPS3 and VPS8 subunits replacing VPS39 and VPS41. CORVET preferentially binds RAB5 rather than RAB7 and functions at early endosomes. The transition from CORVET to HOPS during endosome maturation accompanies the RAB5-to-RAB7 conversion and progression from early endosomes to late endosomes and lysosomes.

In the context of MAPL-induced pyroptosis, the identification of multiple HOPS and CORVET subunits (VPS33B, VPS41) and RAB GTPases (RAB5B, RAB8B, RAB1A, RAB3D, RAB42, RAB30, RAB7B) in the genome-wide screen indicates that proper endolysosomal trafficking and organelle identity are essential for the pathway. This suggests that mitochondrial-derived vesicles traffic through the endosomal system encountering RAB5-positive early endosomes and RAB7-positive late endosomes before final delivery to LAMP1-positive lysosomes. Disruption of any step in this trafficking cascade by loss of specific RAB proteins or tethering complexes prevents efficient MDV delivery to lysosomes and protects against pyroptotic cell death.

Leucine-rich repeat kinase 2 (LRRK2), encoded by the LRRK2 gene on chromosome 12q12, comprises 2527 amino acids with a molecular weight of 286 kilodaltons, making it one of the largest kinases in the human genome. LRRK2 contains multiple functional domains arranged in a specific architecture: ARM (armadillo) repeats (residues 1-510), ANK (ankyrin) repeats (residues 589-953), LRR (leucine-rich repeats, residues 1010-1285), a ROC (Ras of complex proteins) GTPase domain (residues 1335-1503), a COR (C-terminal of ROC) domain (residues 1518-1870), a kinase domain (residues 1875-2132), and a WD40 domain (residues 2231-2527).

The kinase domain belongs to the ROCO family and exhibits serine/threonine kinase activity with specificity for phosphorylation of substrates at threonine residues within the consensus motif Φ-X-T where Φ represents a hydrophobic residue. Identified LRRK2 substrates include a subset of RAB GTPases, particularly RAB8A, RAB10, RAB12, RAB35, and RAB43, which are phosphorylated at conserved threonine residues (Thr72 in RAB8A, Thr73 in RAB10) within the switch-II region of the RAB GTPase domain (eLife 2016, volume 5, article e12813). This phosphorylation alters RAB protein interactions with guanine nucleotide exchange factors and effector proteins, modulating membrane trafficking pathways.

The GTPase domain provides regulatory control over kinase activity through a molecular mechanism where GTP binding to the ROC domain promotes kinase activation while GTP hydrolysis reduces activity. The COR domain is hypothesized to function as a dimerization interface enabling LRRK2 homodimerization or heterodimerization with LRRK1. The N-terminal repeats (ARM, ANK, LRR) mediate protein-protein interactions with scaffolding proteins, membranes, and regulatory partners.

LRRK2 localizes to multiple subcellular compartments including cytosol, mitochondria, endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes. Lysosomal localization is enhanced under conditions of lysosomal stress or damage induced by lysosomotropic agents such as L-leucyl-L-leucine methyl ester (LLOME), chloroquine, or monosodium urate crystals. Upon lysosomal damage, LRRK2 is recruited to damaged lysosomes through interactions with RAB7-positive lysosomal membranes. At these sites, LRRK2 phosphorylates a subset of RAB proteins, recruiting effectors involved in lysosomal membrane repair through ESCRT-dependent mechanisms.

Pathogenic mutations in LRRK2 are the most common genetic cause of familial Parkinson disease and are also found in sporadic cases. The most prevalent mutation is G2019S, located within the kinase domain activation loop, which increases kinase activity approximately 2-3 fold relative to wild-type. Other pathogenic mutations include R1441C/G/H in the ROC GTPase domain, which reduce GTPase activity and consequently increase kinase activity, and Y1699C in the COR domain. These gain-of-function mutations result in excessive phosphorylation of RAB substrates and dysregulation of membrane trafficking, contributing to neurodegeneration through mechanisms that remain incompletely understood but may involve altered lysosomal function, autophagy defects, and inflammatory dysregulation.

In the context of MAPL-induced pyroptosis, LRRK2 is required for efficient lysosomal membrane permeabilization and release of mitochondrial DNA from damaged lysosomes into the cytosol. Loss of LRRK2 does not prevent delivery of mtDNA-containing mitochondrial-derived vesicles to lysosomes but blocks subsequent lysosomal membrane breach. This positions LRRK2 as a regulator of lysosomal membrane integrity or repair, where LRRK2 may normally function to repair minor membrane damage but becomes overwhelmed or dysregulated under conditions of extensive gasdermin-mediated permeabilization. Alternatively, LRRK2 activity may actively promote membrane permeabilization under inflammatory conditions through phosphorylation of RAB proteins that normally stabilize lysosomal membranes or through recruitment of permeabilization factors.

The tetracycline-inducible gene expression system, also designated the Tet-On system, provides reversible control of transgene expression through administration of tetracycline or its derivative doxycycline. The system comprises two components: a tetracycline-controlled transactivator protein and a tetracycline-responsive promoter element controlling the gene of interest.

The reverse tetracycline-controlled transactivator (rtTA) is a fusion protein comprising the tetracycline repressor DNA-binding domain from Escherichia coli transposon Tn10 fused to the activation domain of VP16 from herpes simplex virus. Several generations of rtTA have been developed with improved properties. The original rtTA (designated rtTA-1) exhibited moderate expression levels and required high doxycycline concentrations (1000 nanograms per milliliter). The improved rtTA2 superscript S -M2 variant incorporates four amino acid substitutions (F86Y, E19V, L106W, P110S) that increase doxycycline sensitivity approximately 10-fold, enabling effective induction at 10-100 nanograms per milliliter doxycycline. The most recent rtTA3 variant (also designated rtTA-V16) incorporates additional mutations providing faster induction kinetics, lower background expression, and improved expression levels.

For construction of engineered cells of the present invention, the rtTA3 coding sequence (GenBank accession KF650445) is cloned into an expression vector under control of a constitutive promoter. Suitable constitutive promoters include the human elongation factor 1 alpha (EF1α) promoter, the phosphoglycerate kinase (PGK) promoter, the ubiquitin C (UBC) promoter, or the cytomegalovirus immediate early (CMV) promoter. The EF1α promoter is particularly preferred due to strong and consistent expression across diverse cell types without silencing in primary cells or upon differentiation. A specific implementation employs the EF1α promoter sequence from nucleotides -1176 to +67 relative to the transcription start site, derived from the human EEF1A1 gene (GenBank accession J04617), which provides robust expression while maintaining relatively compact size suitable for viral vector packaging.

The rtTA3 expression cassette comprises the following elements arranged 5' to 3': EF1α promoter, rtTA3 coding sequence (636 base pairs encoding 212 amino acids), and a polyadenylation signal (simian virus 40 late poly(A) signal or bovine growth hormone poly(A) signal). The cassette is inserted into a lentiviral transfer vector backbone such as pLenti (Addgene plasmid 17447) or pCDH (System Biosciences catalog CD510B-1). The specific insertion site is chosen to avoid interference with lentiviral long terminal repeats (LTRs) and packaging signal sequences. For example, insertion between the HIV-1 Rev response element (RRE) and the central polypurine tract (cPPT) maintains efficient viral packaging and transduction.

The tetracycline-responsive promoter element (TRE) comprises multiple copies of the tetracycline operator (tetO) sequence positioned upstream of a minimal promoter. The tetO sequence is a 19 base pair palindromic sequence (5'-TCCCTATCAGTGATAGAGA-3') derived from the E. coli tetracycline resistance operon. In the absence of doxycycline, rtTA exhibits minimal DNA binding. Upon doxycycline binding, rtTA undergoes conformational changes that enable high-affinity binding to tetO sequences. When multiple tetO sequences are arrayed in tandem, cooperative binding of multiple rtTA molecules creates a potent activation complex that recruits the transcriptional machinery through the VP16 activation domain.

Optimized TRE constructs employ seven tandem repeats of the tetO sequence (TRE7 or P subscript tet-7) positioned 5' to a minimal promoter such as the minimal CMV promoter (comprising nucleotides -53 to +75 relative to the CMV immediate early transcription start site) or the minimal human β-globin promoter. The TRE7 sequence provides strong inducible expression with low background in the absence of doxycycline. For maximal induction strength while maintaining low background, alternative designs incorporate bidirectional TRE promoters where tetO repeats are flanked by minimal promoters on both sides, enabling expression of two genes (for example, MAPL and a selectable marker or fluorescent reporter) from a single bidirectional TRE.

The MAPL coding sequence for expression in human cells employs the human MUL1 reference sequence (GenBank accession NM underscore 007293.4) encoding 352 amino acids. The coding sequence is synthesized with optimized codon usage for human cells while maintaining the amino acid sequence identical to the reference. Codon optimization improves expression by replacing rare codons with frequently used synonymous codons, removing cis-acting regulatory elements that reduce expression (upstream open reading frames, AU-rich elements, cryptic splice sites), and optimizing GC content to approximately 50-60% which is optimal for mammalian expression.

A specific optimized MAPL coding sequence incorporates the following modifications relative to the reference sequence: codon optimization increasing codon adaptation index from 0.78 to 0.92, removal of a cryptic splice donor site at nucleotide position 462 (achieved by synonymous substitution of CAG to CAA at codon 154), and removal of ATTTA pentamers which function as AU-rich destabilizing elements in 3' untranslated regions but can reduce expression when present in coding regions. The optimized sequence maintains 83% nucleotide identity to the reference sequence while encoding identical amino acid sequence.

For detection and validation, an epitope tag sequence is appended to the C-terminus of MAPL. Suitable tags include the FLAG tag (DYKDDDDK, 8 amino acids), HA tag (YPYDVPDYA, 9 amino acids), or Myc tag (EQKLISEEDL, 10 amino acids). The FLAG tag is particularly preferred due to high affinity monoclonal antibodies (such as M2 antibody, Sigma-Aldrich catalog F1804) enabling efficient immunoprecipitation and detection. The tag is connected to the MAPL C-terminus through a flexible linker sequence such as GGGGS or GAPGSAAA to minimize interference with MAPL folding or function.

The complete inducible MAPL expression cassette comprises the following elements arranged 5' to 3': TRE7 promoter (358 base pairs comprising seven tetO repeats and minimal CMV promoter), 5' untranslated region (UTR) including Kozak consensus sequence for optimal translation initiation (GCCACCATGG where ATG is the start codon), MAPL coding sequence with codon optimization (1056 base pairs), FLAG tag coding sequence (24 base pairs), and poly(A) signal (234 base pairs for SV40 late poly(A) or 220 base pairs for bovine growth hormone poly(A)). This cassette totaling approximately 1.7 kilobases is inserted into a lentiviral transfer vector for cell transduction.

While tetracycline-inducible systems provide versatile and widely used control, alternative inducible systems offer advantages for specific applications including small molecule inducers with better pharmacokinetics, systems with lower basal expression, or orthogonal systems enabling independent control of multiple genes.

The ecdysone-inducible system employs the heterodimeric receptor comprising the ecdysone receptor (EcR) from Drosophila melanogaster and retinoid X receptor (RXR) from mammals. In the presence of the ecdysone analog ponasterone A or synthetic muristerone A, the EcR-RXR heterodimer binds to ecdysone response elements (EcRE) and activates transcription. The VgEcR variant incorporating amino acid substitutions from Drosophila virilis EcR exhibits reduced background and improved induction. The system requires constitutive expression of both EcR and RXR components (or an EcR-RXR fusion protein) along with an EcRE-controlled response promoter driving MAPL expression. The ecdysone system exhibits minimal background, though inducer availability is more limited than doxycycline.

The rapamycin-inducible system (or synthetic rapalog-inducible system) employs chemically induced dimerization. Two fusion proteins are expressed: one comprising the FKBP12 rapamycin-binding domain fused to a transcription activation domain (such as p65 activation domain), and another comprising the FRB rapamycin-binding domain of mTOR fused to a DNA-binding domain (such as zinc finger domains engineered to recognize specific DNA sequences). Upon addition of rapamycin or synthetic rapalog AP21967, FKBP12-FRB heterodimerization brings the activation domain to the DNA-binding domain positioned at the response element, initiating transcription of MAPL. This system exhibits very low background but requires more complex vector design incorporating both fusion proteins.

The light-inducible system employs optogenetic components such as the CRY2-CIB1 heterodimerization system from Arabidopsis thaliana or the GAVPO system combining GAL4-VP16 fusion with light-dependent nuclear localization. In the CRY2-CIB1 system, blue light (450-490 nanometers) induces heterodimerization, which can be coupled to transcriptional activation through fusion of CRY2 to a transcription activation domain and anchoring CIB1 to a promoter-bound DNA-binding domain. Light-inducible systems provide exceptional spatiotemporal control including subcellular spatial resolution and sub-second temporal resolution, though requiring specialized illumination equipment.

For tissue-specific or disease-site-specific induction without exogenous inducer molecules, hypoxia-inducible systems employ hypoxia-response elements (HREs) from genes such as VEGF or EPO. HREs are bound by hypoxia-inducible factor 1 (HIF-1α/HIF-1β heterodimer) specifically under hypoxic conditions (typically oxygen tension below 2-5%). Solid tumors characteristically exhibit regions of hypoxia due to abnormal vasculature, enabling tumor-selective MAPL expression. A specific implementation employs five tandem copies of the HRE from the human VEGF gene (sequence 5'-TACGTGCT-3', the core HIF-1 binding site) positioned upstream of a minimal promoter driving MAPL expression. This cassette is silent under normoxic conditions (21% oxygen, typical of culture and most normal tissues) but activates under hypoxia typical of tumor microenvironments.

Construction of expression vectors employs standard molecular biology techniques including restriction endonuclease digestion, DNA ligation, polymerase chain reaction amplification, and Gibson assembly or related seamless cloning methods.

For lentiviral expression, third-generation self-inactivating (SIN) lentiviral vectors derived from HIV-1 are employed. These vectors comprise the following components in the transfer plasmid: 5' long terminal repeat (LTR) with deletion in the U3 region rendering the LTR transcriptionally inactive after integration (self-inactivating design), packaging signal (Psi), Rev response element (RRE), central polypurine tract (cPPT), transgene expression cassette, Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) enhancing transgene expression, and 3' LTR. The specific vector backbone pCDH-EF1-MCS-T2A-Puro (System Biosciences catalog CD532A-1) provides a suitable starting point, where the EF1α promoter drives a multiple cloning site (MCS) permitting insertion of the rtTA cassette, a T2A ribosomal skip sequence enabling co-expression of puromycin resistance for selection, and convenient restriction sites for insertion.

The rtTA expression cassette is amplified by PCR from a template plasmid (such as pLVX-Tet-On Advanced, Takara Bio catalog 632162) using forward primer incorporating an NheI restriction site and reverse primer incorporating a NotI restriction site. PCR employs high-fidelity DNA polymerase such as Q5 (New England Biolabs catalog M0491) or Phusion (Thermo Fisher Scientific catalog F530) with 30 cycles of amplification (98 degrees Celsius denaturation for 10 seconds, 60 degrees Celsius annealing for 20 seconds, 72 degrees Celsius extension for 30 seconds). The PCR product is gel-purified using a gel extraction kit (Qiagen QIAquick catalog 28704), digested with NheI and NotI restriction enzymes (New England Biolabs catalog R3131 and R3189), and ligated into the vector backbone similarly digested and dephosphorylated using Antarctic phosphatase (New England Biolabs catalog M0289). Ligation employs T4 DNA ligase (New England Biolabs catalog M0202) with overnight incubation at 16 degrees Celsius using a 3:1 insert:vector molar ratio.

The ligation product is transformed into chemically competent Escherichia coli bacteria (Stellar competent cells, Takara Bio catalog 636763 or DH5α, Thermo Fisher Scientific catalog 18258012) by heat shock (42 degrees Celsius for 45 seconds) and plated on LB agar plates containing ampicillin (100 micrograms per milliliter) or kanamycin (50 micrograms per milliliter) depending on the antibiotic resistance marker in the vector backbone. Individual colonies are picked after overnight incubation at 37 degrees Celsius, cultured in LB medium with antibiotic selection, and plasmid DNA is isolated using miniprep kits (Qiagen QIAprep Spin catalog 27104). Candidate clones are screened by restriction digest analysis to identify clones with correctly sized insert, followed by Sanger sequencing across the entire insert and junctions to confirm sequence fidelity.

For construction of the TRE-MAPL expression vector, a similar approach employs restriction digest and ligation, or alternatively Gibson assembly for seamless cloning. Gibson assembly is particularly advantageous when combining multiple fragments or avoiding restriction sites within the MAPL coding sequence. For Gibson assembly, linear vector backbone and insert fragments with 15-40 base pair overlapping ends are mixed with Gibson Assembly Master Mix (New England Biolabs catalog E2611) containing T5 exonuclease (creates 3' overhangs by removing nucleotides from 5' ends), Phusion DNA polymerase (fills in gaps), and Taq DNA ligase (seals nicks), and incubated at 50 degrees Celsius for 1 hour. The TRE promoter, MAPL-FLAG coding sequence, and poly(A) signal are assembled in a single reaction into a lentiviral backbone, followed by transformation and clone verification as described above.

A specific complete construct designated pLenti-EF1α-rtTA3-P2A-Puro comprises: HIV-1 5' LTR (634 base pairs), packaging signal (360 base pairs), RRE (589 base pairs), cPPT (116 base pairs), EF1α promoter (1200 base pairs), rtTA3 coding sequence (636 base pairs), P2A self-cleaving peptide sequence (66 base pairs, amino acid sequence ATNFSLLKQAGDVEENPGP derived from porcine teschovirus-1), puromycin N-acetyltransferase coding sequence (600 base pairs), SV40 poly(A) (234 base pairs), WPRE (592 base pairs), and 3' LTR (636 base pairs), totaling 5663 base pairs. A second construct designated pLenti-TRE-MAPL-FLAG-IRES-GFP comprises: HIV-1 5' LTR, packaging signal, RRE, cPPT, TRE7 promoter (358 base pairs), MAPL-FLAG coding sequence (1080 base pairs), encephalomyocarditis virus internal ribosome entry site (IRES, 596 base pairs), enhanced green fluorescent protein coding sequence (720 base pairs), bovine growth hormone poly(A) (220 base pairs), WPRE, and 3' LTR, totaling 5547 base pairs.

Lentiviral particles are produced by transient transfection of HEK293T cells (human embryonic kidney cells expressing SV40 large T antigen, ATCC catalog CRL-3216) with the transfer plasmid encoding the transgene, packaging plasmids encoding viral structural proteins (Gag, Pol) and enzymes (reverse transcriptase, integrase), and envelope plasmid encoding a viral envelope glycoprotein.

For third-generation lentiviral packaging, four plasmids are employed: (1) the transfer plasmid (pLenti-EF1α-rtTA3-P2A-Puro or pLenti-TRE-MAPL-FLAG-IRES-GFP described above), (2) pMDLg/pRRE encoding HIV-1 Gag and Pol proteins (Addgene plasmid 12251, 8670 base pairs), (3) pRSV-Rev encoding HIV-1 Rev protein (Addgene plasmid 12253, 4072 base pairs), and (4) pMD2.G encoding vesicular stomatitis virus glycoprotein (VSV-G) for pseudotyping (Addgene plasmid 12259, 5824 base pairs). This system separates packaging functions across multiple plasmids with no overlapping sequences, minimizing risk of generating replication-competent lentivirus.

HEK293T cells are seeded in 15-centimeter tissue culture dishes at 8 times 10 to the power 6 cells per dish in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific catalog 11965092) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific catalog 16000044, heat-inactivated at 56 degrees Celsius for 30 minutes), 2 millimolar L-glutamine (Thermo Fisher Scientific catalog 25030081), and 1% penicillin-streptomycin (Thermo Fisher Scientific catalog 15140122). Cells are cultured overnight at 37 degrees Celsius with 5% CO subscript 2 in a humidified incubator to reach 70-80% confluency at time of transfection.

Transfection employs calcium phosphate precipitation or polyethylenimine (PEI). For PEI-based transfection, 20 micrograms transfer plasmid, 15 micrograms pMDLg/pRRE, 7.5 micrograms pRSV-Rev, and 7.5 micrograms pMD2.G are mixed in 2 milliliters serum-free DMEM. Linear PEI (25 kilodalton, Polysciences catalog 23966-1) at 1 milligram per milliliter stock concentration is added at 3:1 PEI:DNA mass ratio (150 microliters of 1 milligram per milliliter PEI for 50 micrograms total DNA), mixed gently, and incubated at room temperature for 15 minutes to allow DNA-PEI complex formation. The DNA-PEI mixture is added dropwise to cells while gently swirling the dish, and cells are incubated overnight. The following morning, medium is replaced with fresh complete medium to remove residual PEI and cellular debris.

Viral supernatant is harvested at 48 hours and 72 hours post-transfection. At each timepoint, culture supernatant (approximately 25 milliliters per dish) is collected, centrifuged at 500 times g for 10 minutes at 4 degrees Celsius to pellet cellular debris, and the clarified supernatant is filtered through 0.45 micrometer pore size cellulose acetate filters (Corning catalog 431220) to remove remaining cells and large aggregates. The filtered viral supernatant is used immediately for cell transduction or concentrated for long-term storage.

For concentration, filtered viral supernatant is centrifuged at 25,000 times g for 2 hours at 4 degrees Celsius in ultracentrifuge tubes (Beckman Coulter catalog 355618) using a fixed-angle or swinging-bucket rotor. The supernatant is carefully aspirated and the viral pellet is resuspended in 1/100th original volume of serum-free DMEM or Hanks' Balanced Salt Solution (HBSS) by gentle pipetting and overnight incubation at 4 degrees Celsius on a rocker platform to

ensure complete resuspension. The concentrated virus is aliquoted in 50-100 microliter volumes in cryovials and stored at negative 80 degrees Celsius. Avoid freeze-thaw cycles which reduce viral titer by approximately 50% per cycle.

Lentiviral titer is quantified by transduction of a target cell line (HEK293T or HeLa cells) with serial dilutions of viral supernatant, followed by quantification of transduced cells by flow cytometry for fluorescent markers or by quantitative PCR for integrated proviral DNA.

For flow cytometry-based titering of vectors encoding fluorescent proteins (such as pLenti-TRE-MAPL-FLAG-IRES-GFP), HEK293T cells are seeded in 12-well plates at 1 times 10 to the power 5 cells per well and cultured overnight. Serial 10-fold dilutions of viral supernatant (undiluted, 1:10, 1:100, 1:1000) are prepared in complete medium and 1 milliliter of each dilution is added to wells in duplicate or triplicate. Polybrene (hexadimethrine bromide, Sigma-Aldrich catalog H9268) is added at final concentration of 8 micrograms per milliliter to enhance transduction efficiency by neutralizing electrostatic repulsion between viral particles and cell membranes. Cells are incubated with virus for 24 hours, then medium is replaced with fresh complete medium and cells are cultured for an additional 48-72 hours to allow transgene expression.

Cells are harvested by trypsinization (0.25% trypsin-EDTA, Thermo Fisher Scientific catalog 25200056 for 5 minutes at 37 degrees Celsius), resuspended in FACS buffer (phosphate-buffered saline containing 2% fetal bovine serum and 2 millimolar EDTA), and analyzed by flow cytometry (BD FACSCanto II or similar instrument). GFP-positive cells are quantified using appropriate gating to exclude autofluorescence background (established using non-transduced control cells). The percentage of GFP-positive cells and total cell number are used to calculate transducing units (TU): TU per milliliter equals (number of GFP-positive cells) times (dilution factor) divided by (volume of virus added in milliliters). Titer is calculated from dilutions giving 5-30% transduction efficiency to ensure single-copy integration and linear dose-response.

For vectors lacking fluorescent markers, quantitative PCR (qPCR) quantifies integrated proviral DNA. Cells are transduced as described above, genomic DNA is isolated using DNeasy Blood & Tissue Kit (Qiagen catalog 69504), and qPCR is performed using primers and probe specific for the WPRE sequence or other vector-specific sequence not present in host genome. Copy number is calculated by comparison to standard curve generated from plasmid DNA standards, and transducing units are calculated considering DNA amount analyzed and cell number.

Typical lentiviral titers achieved with VSV-G pseudotyped third-generation vectors are 1 times 10 to the power 6 to 1 times 10 to the power 7 TU per milliliter for unconcentrated supernatant and 1 times 10 to the power 8 to 5 times 10 to the power 8 TU per milliliter for 100-fold concentrated preparations. Titers are influenced by plasmid quality, transfection efficiency, cell density, and harvest timing.

The first step in generating engineered cells with inducible MAPL expression is establishing cell lines stably expressing the rtTA transactivator. This is accomplished by transduction with lentiviral vector encoding rtTA, selection of transduced cells, and isolation of clonal cell lines with appropriate characteristics.

U2OS cells (human osteosarcoma cell line, ATCC HTB-96) are employed as an exemplary cell line due to robust growth characteristics, amenability to genetic modification, and relevance for studying cell death pathways. Cells are maintained in McCoy's 5A medium (Thermo Fisher Scientific catalog 16600082) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, cultured at 37 degrees Celsius with 5% CO subscript 2. For lentiviral transduction, cells are seeded in 6-well plates at 2 times 10 to the power 5 cells per well and cultured overnight to reach 30-40% confluency at time of transduction.

Lentiviral vector pLenti-EF1α-rtTA3-P2A-Puro is added to cells at multiplicity of infection (MOI) of 5, corresponding to 5 transducing units per cell. For a well containing 3 times 10 to the power 5 cells (after overnight culture), 1.5 times 10 to the power 6 TU is added. For virus stock at 1 times 10 to the power 7 TU per milliliter, this requires 150 microliters of viral supernatant, which is diluted in 2 milliliters complete medium containing 8 micrograms per milliliter polybrene. The medium is replaced 24 hours post-transduction to remove virus and polybrene. MOI of 5 achieves greater than 90% transduction efficiency while maintaining predominantly single-copy integration, as multiple-copy integration increases with higher MOI and may cause aberrant expression due to gene dosage effects or integration site interference.

Selection of transduced cells begins 48 hours post-transduction by adding puromycin (Thermo Fisher Scientific catalog A1113803) at 2 micrograms per milliliter. Puromycin concentration is optimized for each cell type by kill curve analysis, where non-transduced cells are exposed to puromycin concentrations from 0.5 to 10 micrograms per milliliter and concentration causing complete cell death within 7 days is selected. For U2OS cells, 2 micrograms per milliliter puromycin achieves complete kill of non-transduced cells by day 5. Medium containing puromycin is replaced every 2-3 days during selection. After 10-14 days of selection, surviving colonies comprise cells stably expressing rtTA and puromycin resistance, which are expanded as a polyclonal population or subjected to clonal isolation.

For clonal isolation, puromycin-selected cells are trypsinized, counted, and plated at limiting dilution in 96-well plates to achieve approximately 0.5-1 cell per well. This ensures that wells containing colonies are derived from single cells. Cells are cultured for 2-3 weeks to allow colony expansion, with medium changes every 3-4 days. Wells containing single colonies are identified by microscopic examination and expanded progressively to 24-well, 6-well, and ultimately T75 flasks. Approximately 48 clonal lines are established for characterization.

Characterization of rtTA-expressing clones includes assessment of basal expression (expression in absence of doxycycline), induced expression (expression in presence of doxycycline), induction fold-change, and viability/growth characteristics. For a screening assay, clones are seeded in 24-well plates at 5 times 10 to the power 4 cells per well and cultured overnight. The following day, half of wells receive doxycycline (Sigma-Aldrich catalog D9891) at 1 microgram per milliliter (final concentration, corresponding to 2.2 micromolar) while control wells receive vehicle (water). After 24 hours, cells are lysed and rtTA protein expression is assessed by immunoblotting using anti-rtTA antibody (Takara Bio catalog 632310, mouse monoclonal) or by proxy through expression of a TRE-controlled reporter if the cells also express such a construct.

Alternatively, rapid clone screening employs transient transfection of a TRE-luciferase reporter plasmid. Clones are transfected with pTRE-Tight-Luc (Takara Bio catalog 631059) using Lipofectamine 2000 reagent, cultured for 24 hours in presence or absence of doxycycline, and luciferase activity is measured using Bright-Glo Luciferase Assay System (Promega catalog E2610). Clones exhibiting low background (luminescence ratio in absence vs. presence of doxycycline less than 0.1) and high induction (greater than 100-fold increase with doxycycline) are selected for further characterization.

Based on screening results, 3-5 top-performing clones are subjected to detailed characterization including dose-response analysis (measuring reporter expression across doxycycline concentrations from 1 nanogram per milliliter to 10 micrograms per milliliter), time-course analysis (measuring reporter expression at 6, 12, 24, 48 hours post-doxycycline addition), and reversibility analysis (measuring reporter decay after doxycycline removal). An ideal clone exhibits less than 5% background expression without doxycycline, greater than 200-fold induction at 100 nanograms per milliliter doxycycline, rapid induction kinetics (50% maximal expression by 12 hours), and rapid reversibility (50% signal decay within 24 hours after doxycycline removal).

rtTA-expressing cells characterized as described above are transduced with the second lentiviral vector pLenti-TRE-MAPL-FLAG-IRES-GFP encoding inducible MAPL expression. This creates cells wherein doxycycline addition induces MAPL expression through the rtTA-TRE system.

Transduction employs the same methodology as described for rtTA introduction, with cells seeded at 2 times 10 to the power 5 per well in 6-well plates, transduced with pLenti-TRE-MAPL-FLAG-IRES-GFP at MOI of 3-5, and cultured for 24 hours before medium change. Because this vector encodes GFP, transduction efficiency is directly assessed by flow cytometry 72 hours post-transduction, with or without doxycycline induction. In the absence of doxycycline, cells exhibit minimal GFP fluorescence (background less than 5% GFP-positive). Upon 24-hour doxycycline treatment (1 microgram per milliliter), cells exhibit strong GFP expression with typically greater than 80% of cells GFP-positive in successfully transduced populations.

For applications requiring homogeneous expression, fluorescence-activated cell sorting (FACS) is employed to enrich cells with desired expression characteristics. Cells are transduced at low MOI (0.3-1.0) to favor single-copy integration, cultured for 72 hours without doxycycline to allow transgene integration and equilibration, then sorted on a FACSAria or similar cell sorter (BD Biosciences). The GFP-negative population (corresponding to cells with low basal expression) is collected as a bulk sorted population, which is then expanded and subjected to clonal isolation by limiting dilution as described previously.

Individual clones derived from sorted cells are expanded and characterized for MAPL expression and function. Clones are seeded in 6-well plates at 2 times 10 to the power 5 cells per well and treated with doxycycline (1 microgram per milliliter) or vehicle for 48 hours. Cells are harvested for protein lysate preparation and immunoblot analysis. Cell pellets are washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in RIPA lysis buffer (50 millimolar Tris pH 7.4, 150 millimolar NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche cOmplete Mini EDTA-free catalog 11836170001) and phosphatase inhibitor cocktail (Roche PhosSTOP catalog 4906845001), and incubated on ice for 30 minutes with periodic vortexing. Lysates are clarified by centrifugation at 16,000 times g for 15 minutes at 4 degrees Celsius and protein concentration is determined by BCA assay (Thermo Fisher Scientific catalog 23225).

Equal amounts of protein (typically 30 micrograms) are mixed with 4 times Laemmli sample buffer (Bio-Rad catalog 1610747) containing 10% β-mercaptoethanol, heated at 95 degrees Celsius for 5 minutes to denature proteins, and separated by SDS-PAGE using 4-20% gradient polyacrylamide gels (Bio-Rad Mini-PROTEAN TGX catalog 4561096). Proteins are transferred to 0.2 micrometer pore size nitrocellulose membranes (Bio-Rad catalog 1620112) using wet transfer (100 volts for 1 hour at 4 degrees Celsius in transfer buffer comprising 25 millimolar Tris, 192 millimolar glycine, 20% methanol). Membranes are blocked for 1 hour at room temperature in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% non-fat dry milk (Bio-Rad catalog 1706404).

Primary antibodies are diluted in TBST with 5% bovine serum albumin (BSA, Sigma-Aldrich catalog A7906) and membranes are incubated overnight at 4 degrees Celsius with gentle rocking. Anti-FLAG M2 antibody (Sigma-Aldrich catalog F1804, mouse monoclonal) at 1:2000 dilution detects FLAG-tagged MAPL. Anti-β-actin antibody (Cell Signaling Technology catalog 4970, rabbit monoclonal) at 1:2000 dilution serves as loading control. Following primary antibody incubation, membranes are washed 3 times for 10 minutes each in TBST, incubated with HRP-conjugated secondary antibodies (anti-mouse IgG-HRP, Cell Signaling Technology catalog 7076 at 1:5000 for FLAG; anti-rabbit IgG-HRP, Cell Signaling Technology catalog 7074 at 1:5000 for β-actin) for 1 hour at room temperature, washed 3 times for 10 minutes in TBST, and developed using enhanced chemiluminescence substrate (Bio-Rad Clarity Western ECL catalog 1705061). Chemiluminescence is detected using ChemiDoc imaging system (Bio-Rad) or X-ray film.

Expected results show no detectable or minimal MAPL-FLAG expression in vehicle-treated samples (uninduced), and robust MAPL-FLAG expression appearing as a band at approximately 42 kilodaltons (352 amino acid MAPL plus FLAG tag gives predicted molecular weight of 40.3 kilodaltons, running slightly higher due to post-translational modifications or gel mobility) in doxycycline-treated samples (induced). β-actin should appear as a 42 kilodalton band with equal intensity across all samples. Clones exhibiting tight regulation (uninduced/induced ratio less than 0.05) and strong induced expression (greater than 50-fold over background) are selected.

Functional validation confirms that induced MAPL expression leads to pyroptotic cell death. Clones are seeded in 96-well plates at 1 times 10 to the power 4 cells per well, cultured overnight, then treated with doxycycline (0, 0.01, 0.1, 1, 10 micrograms per milliliter) in triplicate wells per condition. Cell viability is assessed at 24, 48, 72, and 96 hours post-induction using CellTiter-Glo 2.0 Luminescent Cell Viability Assay (Promega catalog G9241) which measures ATP content as a proxy for viable cell number. Reagent is added according to manufacturer instructions (100 microliters reagent per 100 microliters culture medium in each well), plates are incubated for 10 minutes at room temperature on orbital shaker to induce cell lysis, and luminescence is measured using SpectraMax i3x plate reader (Molecular Devices) or similar luminometer.

Expected results show dose-dependent and time-dependent cell death, with higher doxycycline concentrations and longer treatment durations producing lower viability (lower luminescence signal). At 1 microgram per milliliter doxycycline for 72 hours, viable cell mass typically decreases to 20-40% of vehicle-treated controls. The EC subscript 50 (doxycycline concentration producing 50% maximal cell death) is typically in the range of 50-200 nanograms per milliliter at 72 hours.

Pyroptotic characteristics are confirmed through additional assays. SYTOX Green nucleic acid stain (Thermo Fisher Scientific catalog S7020) is a cell-impermeant dye that only enters cells with permeabilized plasma membranes, providing a direct measure of membrane rupture characteristic of pyroptosis. Cells are seeded in 96-well black-walled clear-bottom plates (Corning catalog 3904) at 1 times 10 to the power 4 cells per well, cultured overnight, and treated with doxycycline (1 microgram per milliliter) or vehicle. SYTOX Green is added at final concentration of 50 nanomolar in the culture medium and fluorescence is monitored over time using a plate reader with excitation 488 nanometers and emission 525 nanometers, with measurements every 2 hours. Increased fluorescence indicates SYTOX Green entry through membrane pores and binding to intracellular DNA. Expected results show progressive fluorescence increase in doxycycline-treated cells beginning around 24-36 hours post-treatment, while vehicle-treated cells maintain low baseline fluorescence. The time to 50% maximal signal typically occurs at 40-60 hours post-induction.

Inflammatory cytokine release, another hallmark of pyroptosis, is assessed by enzyme-linked immunosorbent assay (ELISA) for IL-6 and IL-1β. Cells are seeded in 6-well plates, treated with doxycycline or vehicle, and culture supernatants are collected at 24, 48, and 72 hours. Supernatants are clarified by centrifugation at 500 times g for 5 minutes to remove detached cells and debris, and stored at negative 80 degrees Celsius until analysis. Human IL-6 DuoSet ELISA (R&D Systems catalog DY206) and Human IL-1β/IL-1F2 DuoSet ELISA (R&D Systems catalog DY201) are performed according to manufacturer protocols. Briefly, 96-well plates are coated overnight with capture antibody, blocked, incubated with samples and standards, washed, incubated with detection antibody, washed, incubated with streptavidin-HRP conjugate, washed, developed with TMB substrate, stopped with sulfuric acid, and read at 450 nanometers with 540 nanometers wavelength correction. Expected results show progressive increase in IL-6 secretion from 24 to 72 hours in doxycycline-treated cells, with concentrations typically reaching 500-5000 picograms per milliliter by 72 hours, while vehicle-treated cells show less than 50 picograms per milliliter. IL-1β shows similar kinetics but lower absolute levels (100-500 picograms per milliliter at 72 hours for induced cells).

Based on comprehensive characterization, a final master cell line is selected exhibiting: (1) tight basal regulation (no detectable MAPL expression without doxycycline), (2) robust inducible expression (greater than 100-fold increase with 1 microgram per milliliter doxycycline), (3) dose-dependent and time-dependent pyroptotic cell death, (4) membrane permeabilization confirmed by SYTOX Green uptake, and (5) inflammatory cytokine secretion. This master cell line is expanded, characterized extensively, cryopreserved in multiple vials as a master cell bank, and used for subsequent experiments and therapeutic development.

Industrial Applicability

The engineered cells and methods of the present invention find broad industrial applicability in biopharmaceutical development, cellular therapy manufacturing, and therapeutic treatment of human diseases. The invention provides novel cellular platforms for controlling pyroptotic signaling pathways with precision unattainable using existing approaches. The cells enable development of next-generation cellular immunotherapies combining cell-mediated effector functions with controlled inflammatory cell death, providing therapeutic strategies for cancers resistant to existing treatments. The invention further provides research tools for investigating pyroptotic mechanisms, screening therapeutic compounds targeting pyroptosis, and developing cellular models of diseases involving dysregulated inflammatory signaling. Manufacturing processes for the engineered cells employ established cell culture, genetic engineering, and quality control technologies, enabling industrial-scale production under good manufacturing practice conditions suitable for clinical development and commercialization. The therapeutic methods address significant unmet medical needs in oncology, infectious disease, and other conditions, offering potential for improved patient outcomes and commercial value through regulatory approval and market introduction.

Theoretical Basis of the Present Invention

Mathematical Formulation of MAPL-Dependent Pyroptotic Pathway

The theoretical foundation of the present invention can be expressed through a series of mathematical equations describing the kinetics and dynamics of MAPL-induced pyroptotic cell death. These equations provide quantitative framework for understanding pathway progression, optimizing inducible expression parameters, and predicting therapeutic outcomes.

Equation 1: MAPL Expression Kinetics

$$\frac{d[MAPL]}{dt} = k_{syn} \cdot \frac{[Dox]^n}{K_d^n + [Dox]^n} - k_{deg}[MAPL]$$

This equation describes the temporal dynamics of MAPL protein concentration in engineered cells following doxycycline administration. The variable [MAPL] represents the intracellular concentration of MAPL protein in nanomolar units at time t measured in hours. The parameter k subscript syn denotes the maximum synthesis rate of MAPL protein in nanomolar per hour, determined by the strength of the TRE promoter and translational efficiency. The variable [Dox] represents the intracellular doxycycline concentration in nanomolar units, which equilibrates rapidly with extracellular concentration. The parameter K subscript d is the dissociation constant for doxycycline binding to rtTA in nanomolar units, typically ranging from 10 to 100 nanomolar for rtTA3 variants. The exponent n is the Hill coefficient representing cooperativity of rtTA binding to TRE elements, typically between 2 and 4 reflecting multiple rtTA molecules binding to the seven tetO repeats. The parameter k subscript deg represents the degradation rate constant for MAPL protein in per hour units, determined by ubiquitin-proteasome-mediated turnover and cellular autophagy, typically 0.1 to 0.3 per hour corresponding to protein half-life of 2 to 7 hours.

Equation 2: Mitochondrial-Derived Vesicle Formation Rate

$$\frac{d[MDV]}{dt} = k_{MDV} \cdot [MAPL] \cdot [MIRO] \cdot [VPS35] \cdot f(\Delta\Psi_m) - k_{fus}[MDV][Lyso]$$

This equation quantifies the rate of mitochondrial-derived vesicle formation and subsequent fusion with lysosomes. The variable [MDV] represents the number of mtDNA-containing mitochondrial-derived vesicles per cell at time t. The parameter k subscript MDV is the rate constant for MDV biogenesis in per hour units, representing the efficiency of MAPL-mediated initiation of vesicle formation. The variable [MIRO] represents the concentration of MIRO1 and MIRO2 proteins in nanomolar units, which mediate the tubulation and transport processes required for MDV formation. The variable [VPS35] represents VPS35 protein concentration in nanomolar units, essential for cargo sorting and vesicle trafficking. The function f(ΔΨ subscript m) represents the dependence on mitochondrial membrane potential ΔΨ subscript m measured in millivolts, where f(ΔΨ subscript m) equals 1 for normal mitochondrial potential (approximately negative 180 millivolts) and decreases sigmoidally as potential dissipates, reflecting reduced MDV formation from depolarized mitochondria. The parameter k subscript fus represents the fusion rate constant between MDVs and lysosomes in per nanomolar per hour units. The variable [Lyso] represents the number of lysosomes per cell, typically 200 to 500 in mammalian cells.

Equation 3: Lysosomal mtDNA Accumulation

$$\frac{d[mtDNA_{lyso}]}{dt} = k_{fus}[MDV][Lyso] - k_{release}[mtDNA_{lyso}] \cdot [GSDME_N]$$

This equation describes the accumulation of mitochondrial DNA within lysosomal compartments and its subsequent release into cytosol. The variable [mtDNA subscript lyso] represents the concentration of mitochondrial DNA within lysosomes in arbitrary units per cell. The term k subscript fus [MDV] [Lyso] represents the delivery rate of mtDNA to lysosomes via MDV fusion as defined in Equation 2. The parameter k subscript release is the rate constant for mtDNA release from damaged lysosomes in per nanomolar per hour units, dependent on lysosomal membrane permeabilization. The variable [GSDME subscript N] represents the concentration of cleaved N-terminal gasdermin E

fragments in nanomolar units, which form pores in lysosomal membranes enabling mtDNA escape.

Equation 4: Gasdermin E Activation

$$\frac{d[GSDME_N]}{dt} = k_{casp3}[Casp3_{act}][GSDME_{FL}] - k_{oligo}[GSDME_N]^m$$

This equation models the proteolytic activation of gasdermin E by caspase-3 and subsequent oligomerization into membrane pores. The variable [GSDME subscript N] represents concentration of GSDME N-terminal pore-forming fragment in nanomolar units. The parameter k subscript casp3 is the catalytic rate constant for caspase-3-mediated cleavage of GSDME in per nanomolar per hour units, typically 0.5 to 2 per nanomolar per hour. The variable [Casp3 subscript act] represents concentration of activated caspase-3 in nanomolar units, generated through both intrinsic apoptotic signaling and inflammasome-mediated caspase-1 activation. The variable [GSDME subscript FL] represents concentration of full-length uncleaved GSDME in nanomolar units, which varies significantly across cell types due to epigenetic silencing in many transformed cells. The second term represents consumption of monomeric GSDME subscript N through oligomerization into membrane-inserted pores, where k subscript oligo is the oligomerization rate constant and m is the order of oligomerization reaction, typically 26 to 28 reflecting the number of monomers per pore structure.

Equation 5: Cytosolic mtDNA and cGAS-STING Activation

$$\frac{d[mtDNA_{cyto}]}{dt} = k_{release}[mtDNA_{lyso}][GSDME_N] - k_{deg,DNA}[mtDNA_{cyto}]$$

$$[cGAMP] = k_{cGAS} \cdot \frac{[cGAS][mtDNA_{cyto}]}{K_{m,DNA} + [mtDNA_{cyto}]}$$

These coupled equations describe cytosolic mtDNA accumulation and cGAS-STING pathway activation. The variable [mtDNA subscript cyto] represents concentration of mitochondrial DNA in the cytosolic compartment in arbitrary units. The term k subscript release [mtDNA subscript lyso][GSDME subscript N] represents release from permeabilized lysosomes as derived from Equation 3. The parameter k subscript deg,DNA represents the degradation rate constant for cytosolic DNA by cytosolic nucleases including TREX1 and DNase II, typically 0.5 to 2 per hour. The variable [cGAMP] represents concentration of 2'3'-cyclic GMP-AMP, the second messenger product of cGAS enzymatic activity, in nanomolar units. The parameter k subscript cGAS is the catalytic rate constant for cGAS-mediated synthesis of cGAMP in nanomolar per hour units. The variable [cGAS] represents cGAS protein concentration in nanomolar units, constitutively expressed in most cell types. The parameter K subscript m,DNA is the Michaelis constant representing the concentration of cytosolic DNA at which cGAS achieves half-maximal catalytic rate, typically 5 to 20 nanomolar.

Equation 6: NLRP3 Inflammasome Assembly

$$\frac{d[NLRP3_{oligo}]}{dt} = k_{nuc}[NLRP3]^p \cdot g([cGAMP], [ROS], [K^+]) - k_{dis}[NLRP3_{oligo}]$$

This equation captures the nucleation-dependent assembly of NLRP3 inflammasome complexes. The variable [NLRP3 subscript oligo] represents concentration of oligomerized NLRP3 inflammasome platforms in nanomolar units. The parameter k subscript nuc is the nucleation rate constant in per nanomolar to the power p-1 per hour units. The variable [NLRP3] represents monomeric NLRP3 protein concentration in nanomolar units, upregulated by NF-κB transcriptional activity downstream of STING activation. The exponent p represents the critical nucleus size for NLRP3 oligomerization, typically 10 to 12 monomers. The function g([cGAMP], [ROS], [K to the power +]) represents the combined activating effects of multiple NLRP3 stimuli including cGAMP concentration [cGAMP] in nanomolar, reactive oxygen species concentration [ROS] in arbitrary units, and cytosolic potassium concentration [K to the power +] in millimolar, where g approaches 1 under strong activation and approaches 0 under basal conditions. The parameter k subscript dis represents the disassembly rate constant for NLRP3 oligomers in per hour units, relatively slow once formed.

Equation 7: Caspase-1 Activation and GSDMD Cleavage

$$\frac{d[Casp1_{act}]}{dt} = k_{auto}[NLRP3_{oligo}][ProCasp1]^2 - k_{inhib}[Casp1_{act}]$$

$$\frac{d[GSDMD_N]}{dt} = k_{casp1}[Casp1_{act}][GSDMD_{FL}] - k_{pore}[GSDMD_N]^q$$

These equations describe the autocatalytic activation of caspase-1 and subsequent cleavage of gasdermin D. The variable [Casp1 subscript act] represents activated caspase-1 concentration in nanomolar units. The parameter k subscript auto is the rate constant for proximity-induced autocatalytic activation of pro-caspase-1 recruited to NLRP3 oligomers, in per nanomolar squared per hour units reflecting the bimolecular nature of the reaction. The variable [ProCasp1] represents pro-caspase-1 concentration in nanomolar units. The parameter k subscript inhib represents inactivation rate of caspase-1 by endogenous inhibitors in per hour units. The variable [GSDMD subscript N] represents cleaved GSDMD N-terminal fragment concentration in nanomolar units. The parameter k subscript casp1 is the catalytic rate constant for caspase-1-mediated GSDMD cleavage in per nanomolar per hour units, typically 1 to 5 per nanomolar per hour. The variable [GSDMD subscript FL] represents full-length GSDMD concentration in nanomolar units. The parameter k subscript pore represents the rate constant for GSDMD subscript N oligomerization and insertion into plasma membrane, and q is the oligomerization order, typically 26 to 28 monomers per pore.

Equation 8: Cell Death Probability

$$P_{death}(t) = 1 - \exp\left(-\int_0^t \lambda(\tau)d\tau\right)$$

$$\lambda(t) = \lambda_0 + \lambda_{GSDMD} \cdot [GSDMD_N](t) + \lambda_{GSDME} \cdot [GSDME_N](t)$$

These equations provide a probabilistic framework for cell death timing based on gasdermin pore accumulation. The variable P subscript death (t) represents the cumulative probability that a cell has undergone pyroptotic death by time t. The function λ(t) represents the instantaneous hazard rate or death rate at time t in per hour units. The parameter λ subscript 0 represents the baseline death rate from non-pyroptotic causes, typically 0.001 to 0.01 per hour. The parameter λ subscript GSDMD represents the contribution to death rate per nanomolar of GSDMD subscript N pores in plasma membrane, in per nanomolar per hour units. The parameter λ subscript GSDME represents the contribution to death rate per nanomolar of GSDME subscript N, in per nanomolar per hour units. The exponential form reflects that cell death is a stochastic process where the probability of survival decreases exponentially with accumulated membrane damage.

Equation 9: Inflammatory Cytokine Release

$$\frac{d[IL1\beta_{ext}]}{dt} = k_{mat}[Casp1_{act}][ProIL1\beta] + k_{leak}[IL1\beta_{int}] \cdot P_{pore}(t)$$

$$P_{pore}(t) = 1 - \exp(-\alpha \cdot [GSDMD_N](t))$$

These equations model the maturation and release of interleukin-1β as a representative inflammatory cytokine. The variable [IL1β subscript ext] represents extracellular concentration of mature IL-1β in picograms per milliliter. The parameter k subscript mat is the rate constant for caspase-1-mediated cleavage of pro-IL-1β to mature form in per nanomolar per hour units. The variable [ProIL1β] represents pro-IL-1β concentration in nanomolar units, upregulated by NF-κB signaling. The parameter k subscript leak represents the rate constant for passive release of intracellular IL-1β through gasdermin pores in per hour units. The variable [IL1β subscript int] represents intracellular concentration of mature IL-1β in nanomolar units. The variable P subscript pore (t) represents the probability that a cell has formed plasma membrane pores at time t. The parameter α is a scaling constant in per nanomolar units relating GSDMD subscript N concentration to pore formation probability.

Equation 10: LRRK2-Dependent Lysosomal Membrane Stability

$$k_{release} = k_{release,0} \cdot \left(1 + \frac{[LRRK2]}{K_{LRRK2}}\right)^{-1} \cdot h([GSDME_N])$$

This equation describes how LRRK2 kinase activity modulates the rate of mtDNA release from lysosomes by regulating membrane repair or permeabilization resistance. The parameter k subscript release,0 represents the baseline lysosomal release rate in the absence of LRRK2 regulatory effects. The variable [LRRK2] represents active LRRK2 kinase concentration in nanomolar units. The parameter K subscript LRRK2 is the concentration of LRRK2 at which lysosomal membrane stabilization is half-maximal, in nanomolar units. The inverse relationship indicates that higher LRRK2 activity reduces mtDNA release by enhancing membrane repair, while LRRK2 inhibition or knockout increases release. The function h([GSDME subscript N]) represents the opposing effect of GSDME pores overwhelming membrane repair capacity, where h increases sigmoidally with GSDME subscript N concentration.

Equation 11: Therapeutic Efficacy in Tumor Microenvironment

$$\frac{dT}{dt} = r_g T\left(1 - \frac{T}{K}\right) - k_{kill} \cdot I(t) \cdot T$$

$$\frac{dI}{dt} = s_0 + s_{pyro} \cdot N_{dying}(t) - d_I \cdot I$$

These equations model tumor growth dynamics and immune-mediated killing following administration of engineered pyroptotic cells. The variable T represents tumor cell number. The parameter r subscript g represents tumor intrinsic growth rate in per day units. The parameter K represents tumor carrying capacity determined by nutrient availability and space constraints. The parameter k subscript kill represents the rate constant for immune cell-mediated tumor cell killing in per immune cell per day units. The variable I(t) represents the concentration or number of activated immune effector cells (cytotoxic T cells, NK cells, activated macrophages) in the tumor microenvironment. The parameter s subscript 0 represents baseline immune cell infiltration rate in immune cells per day. The parameter s subscript pyro represents the recruitment rate of immune cells per dying pyroptotic cell, quantifying the inflammatory amplification effect. The variable N subscript dying (t) represents the number of engineered cells undergoing pyroptosis at time t, calculated from Equation 8 applied to the

administered cell population. The parameter d subscript I represents the death or emigration rate of immune effector cells in per day units.

Equation 12: Optimal Doxycycline Dosing Schedule

$$D_{opt}(t) = D_{max} \cdot \frac{\exp(-t/\tau_{accum})}{1 + \exp\left(\frac{t - t_{induce}}{\tau_{switch}}\right)}$$

This equation provides a time-dependent doxycycline dosing function optimized for therapeutic applications. The variable D subscript opt (t) represents the optimal extracellular doxycycline concentration at time t post-cell administration in micrograms per milliliter. The parameter D subscript max represents maximum safe doxycycline dose in micrograms per milliliter, typically 5 to 10 for human applications. The parameter τ subscript accum represents the time constant for engineered cell accumulation at target tissue in hours or days, typically 24 to 72 hours for intravenous administration and 6 to 24 hours for local injection. The parameter t subscript induce represents the optimal timing to initiate MAPL induction in hours post-cell administration, determined from biodistribution studies. The parameter τ subscript switch represents the transition time constant in hours, controlling how rapidly doxycycline is increased from baseline to maximum, typically 12 to 48 hours to allow gradual MAPL accumulation rather than acute toxicity.

These mathematical formulations provide quantitative framework for understanding MAPL-dependent pyroptotic pathway dynamics, enabling rational design of engineered cellular systems, prediction of therapeutic outcomes, and optimization of treatment parameters including cell dose, timing of induction, and combination with other therapeutic modalities.

Prior Art Reference

Nguyen, M., Collier, J.J., Ignatenko, O. *et al.* MAPL regulates gasdermin-mediated release of mtDNA from lysosomes to drive pyroptotic cell death. *Nat Cell Biol* (2025). https://doi.org/10.1038/s41556-025-01774-y