Chapter 17

Modulation of Host Programmed Cell Death Pathways by the Intracellular Protozoan Parasite, *Toxoplasma gondii* — Implications for Maintenance of Chronic Infection and Potential Therapeutic Applications

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http://dx.doi.org/10.5772/61529

Abstract

Programmed cell death (PCD) pathways are genetically programmed mechanisms that can trigger the cell to die or commit "cell suicide". There are three major forms of programmed cell death that are now recognized: apoptosis (type I), autophagy (type II) and necrotic cell death or necroptosis (type III). While these cell death processes were once thought to occupy discrete cell states, evidence suggests that apoptosis, autophagy and necrosis are often regulated by similar pathways and share initiator and effector molecules and some subcellular compartments indicating that crosstalk exists between these three main forms of cell death pathways, resulting in a balanced interplay by which the cell decides its fate. PCD pathways have important roles in many cellular processes such as development and oncogenic transformation, but PCD pathways also play important roles in host defense and elimination of pathogens. *Toxoplasma gondii* is a microbial pathogen for which programmed cell death pathways are a key part of the host defense. *T. gondii* is an obligate intracellular protozoan parasite that infects approximately one-third of the world's population. In most immunocompetant individuals, the chronic infection is asymptomatic due to an effective immune response that eliminates active parasite replication. The parasite has evolved immune evasion strategies that enable it to survive and persist long enough in the host however to establish a chronic infection in which the cyst stage persists within neurons in the brain and skeletal muscle in the periphery. *T. gondii* has evolved multiple mechanisms to resist killing by apoptotic, autophagic and necrotic cell death pathways, and the parasite's manipulation of host PCD pathways plays a crucial role in host–parasite interactions and maintenance of the chronic infection. While most individuals chronically infected with *T. gondii* are asymptomatic, severe disease can occur in immunocompromised individuals where the infection reactivates from the brain causing severe necrotizing encephalitis, and increasing evidence indicates chronic cerebral toxoplasmosis in some individuals may lead to neuropsychiatric disorders such as schizophrenia and suicidal behavior. This review will focus on the role of PCD pathways in host defense of *T. gondii* and the parasite manipulation of these PCD pathways. A bet-

ter understanding of the molecular components underlying the PCD pathways and the parasite manipulation of these pathways may yield new therapeutic targets for treatment of clinical sequelae of cerebral toxoplasmosis.

Keywords: Apoptosis, autophagy, necroptosis, necrosis, toxoplasmosis

1. Introduction

Overview of Programmed Cell Death (PCD) pathways and applications to *Toxoplasma gondii* infection

Programmed cell death (PCD) pathways are genetically programmed mechanisms that can trigger the cell to die or commit "cell suicide". There are three major forms of programmed cell death which are now recognized: apoptosis (type I), autophagic cell death (type II) and necrotic cell death or necroptosis (type III) [1–4]. Apoptosis, the best-characterized and dominant form of PCD, is a controlled physiological process of cellular self-destruction that removes dead cells without damage to the host. Apoptosis is critically important during development and morphogenesis and eliminates damaged cells such as cancerous cells or infected cells that may interfere with normal function. Necrosis, conversely, is a cell death process that is observed in response to severe stresses such that occurs after physical injury or prolonged ischemia and has been long thought to be an unregulated process. However, a programmed form of necrotic death, called necroptosis, is now recognized that can occur during physical traumas, in neurodegeneration, in cell death due to ischemia or infection and that, in contrast to unordered necrosis, has dedicated molecular pathways controlling necrotic cell death. Autophagy is predominantly a strategy for survival and not death, serving as a housekeeping mechanism of normal turnover of long-lived proteins and whole organelles and crucial to maintenance of healthy cells. However, autophagy can promote cell death during normal development and excessive autophagy, stimulated in times of stress such as nutrient deprivation and some diseases, can lead to what is now recognized as autophagic cell death [5–7]. While these cell death processes were once thought to occupy discrete cell states, evidence suggests apoptosis, autophagy and necroptosis are often regulated by similar pathways, share initiator and effector molecules and some subcellular compartments indicating a complex crosstalk exists between these three main forms of cell death pathways, resulting in a balanced interplay by which the cell decides its fate [8].

PCD pathways are also an important part of host defense against intracellular pathogens. The intracellular protozoal pathogen, *Toxoplasma gondii,* is a common infection that causes a chronic infection of central nervous system that persists for the lifetime of the individual. PCD pathways are a key part of the host defense against *T. gondii*, but the parasite has evolved multiple mechanisms to resist killing by host cell death pathways. Parasite manipulations of host cell death pathways are essential components leading to successful establishment of infection and for maintenance of the chronic infection in the host. In this review, the molecular components and signaling pathways of each of the three main types of PCD pathways will be

http://dx.doi.org/10.5772/61529

discussed with the role of these PCDs in the pathogenesis of the intracellular pathogen, *T. gondii*, and potential therapeutic targets for toxoplasmosis and other diseases, the focus of this review.

2. Apoptosis, necroptosis and autophagic cell death pathways: Morphological features and molecular components

Apoptosis, necroptosis and autophagic cell death pathways operate via distinct genetically programmed molecular mechanisms containing dedicated molecular components and leading to characteristic morphological features (Figure 1). Apoptosis operates via a caspase-dependent mechanism while necroptosis and autophagic cell death are caspase-independent processes, with autophagic cell death being cathepsin-dependent and necroptosis mediated via the receptor interacting protein kinase 3 (RIP3) and its substrate, the mixed lineage kinase-like domain protein, MLKL [8]. Apoptosis results in mitochondrial membrane permeabilization, chromatin condensation and DNA fragmentation, resulting in cells become smaller (pknotic) and membrane blebbing into apoptotic bodies. Phagocytes, such as macrophages, subsequently engulf apoptotic cells and hence apoptosis is considered a noninflammatory form of programmed cell death. Necrotic cell death, in contrast to apoptosis, is marked by swelling of ER and mitochondrial organelles, increase in cell size, rapid rupture of the plasma membrane resulting in lysis of the cell and release of danger-associated molecular patterns (DAMPs) which stimulate inflammation. While necrosis has traditionally been considered to be "pathological cell death", necroptosis is now recognized to occur via a regulated mechanism that is inhibited by caspases [9, 10]. Autophagy is the process by which cells recycle cellular constituents and involves engulfment of cytoplasmic material and intracellular organelles within double-membrane vesicles called autophagosomes that fuse with lysosomes to make autophagolysosomes that degrade the cellular cargo via cathepsins located in the autolysosomes. The molecular components and signaling pathways of each cell death processes are reviewed below, followed by the current understanding of the crosstalk that exists between these PCD pathways.

2.1. Apoptosis

The term apoptosis was first used in 1972 by Kerr, Wyllie and Currie [11] to describe a form of cell death morphologically distinct from necrosis. Apoptosis has since been well studied and is now understood to be a regulated energy-dependent process mediated via cysteine-dependent aspartate-directed enzymes called caspases [3]. Apoptosis is a homeostatic process that balances cell numbers and plays a crucial role in several physiological processes including embryogenesis to shape morphological structures such as fingers, in the establishment of functional synaptic connections in the nervous system, in development of the immune response to remove self-reactive lymphocytes and at the termination of the immune response to remove antigen-specific lymphocytes. Apoptosis is also used to rid the body of cells in various pathological conditions such as removal of cancerous cells, infected cells or cells damaged by noxious agents [12].

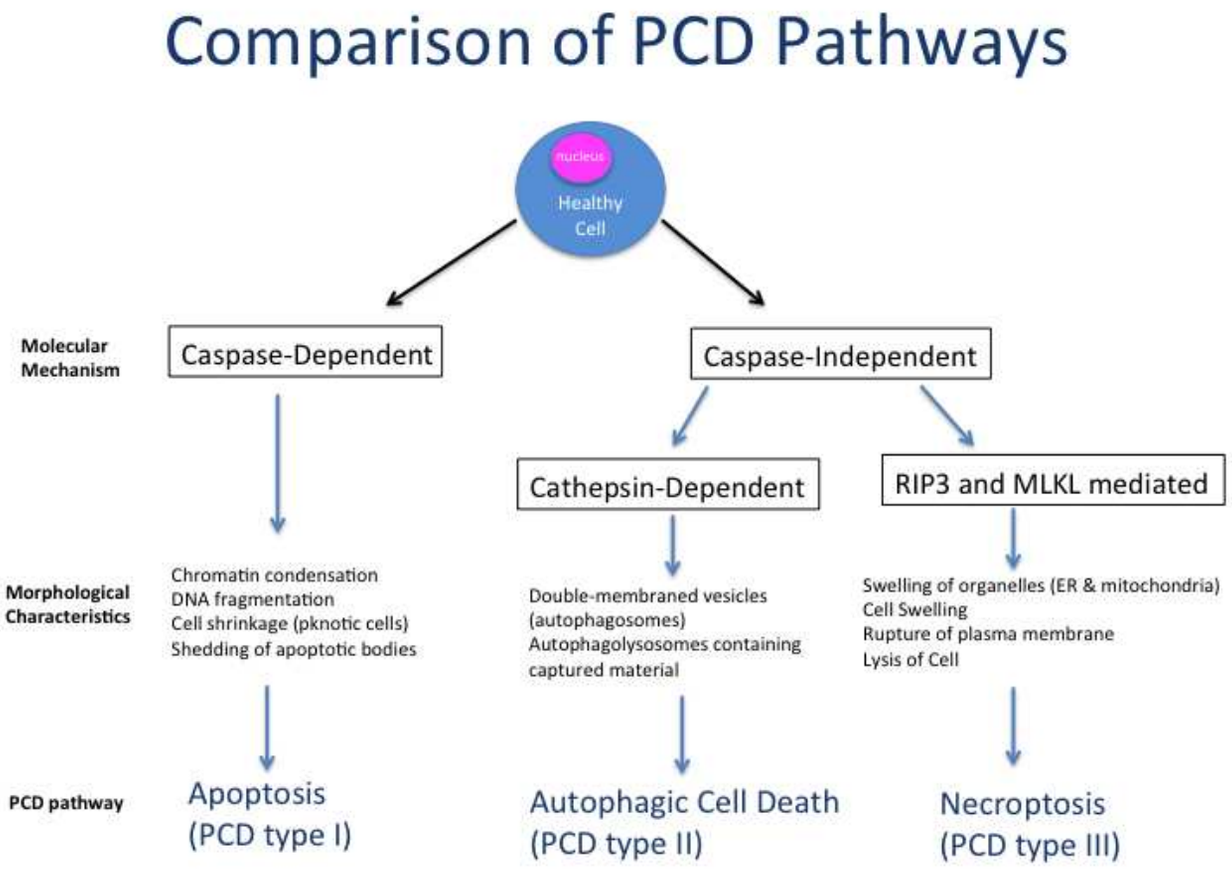

Figure 1. Comparison of three types of Programmed Cell (PCD) Pathways. The molecular mechanisms and morphological characteristics of Apoptosis, Autophagic Cell Death and Necroptosis cell death pathways, so-called PCD types I, II and III, respectively, are indicated. RIP3 = receptor-interacting protein kinase 3; MLKL = mixed lineage kinase-like domain protein

2.1.1. Signaling pathways and molecular components of apoptosis

Apoptosis is mediated via caspases which are present in the cytoplasm as pro-enzymes and when activated initiate a proteolytic cascade that results in apoptosis of the cell. There are three activation pathways that can initiate apoptosis: the extrinsic (death receptor) pathway, the intrinsic (mitochondrial) pathway and the granzyme pathway (Figure 2). There are about 14 known caspases which are divided into initiator and effector caspases. Initiator caspases, caspases 8, 9 and 10, are triggered by either the extrinsic, intrinsic or granzyme pathway, respectively. All three pathways converge on caspase 3, which activates effector caspases 6 and 7 that lead to apoptosis. A major target of effector caspases is poly-ADP-ribose polymerase (PARP) which is involved in DNA repair, cell survival, proliferation and differentiation.

The extrinsic pathway is initiated by binding of cell membrane receptors of Fas (CD95) or tumor necrosis factor (TNF) family receptor (TNFR), which after binding its relevant ligand of FasL or TNFα, respectively, trimerizes and attracts the docking proteins Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD), respectively. FADD

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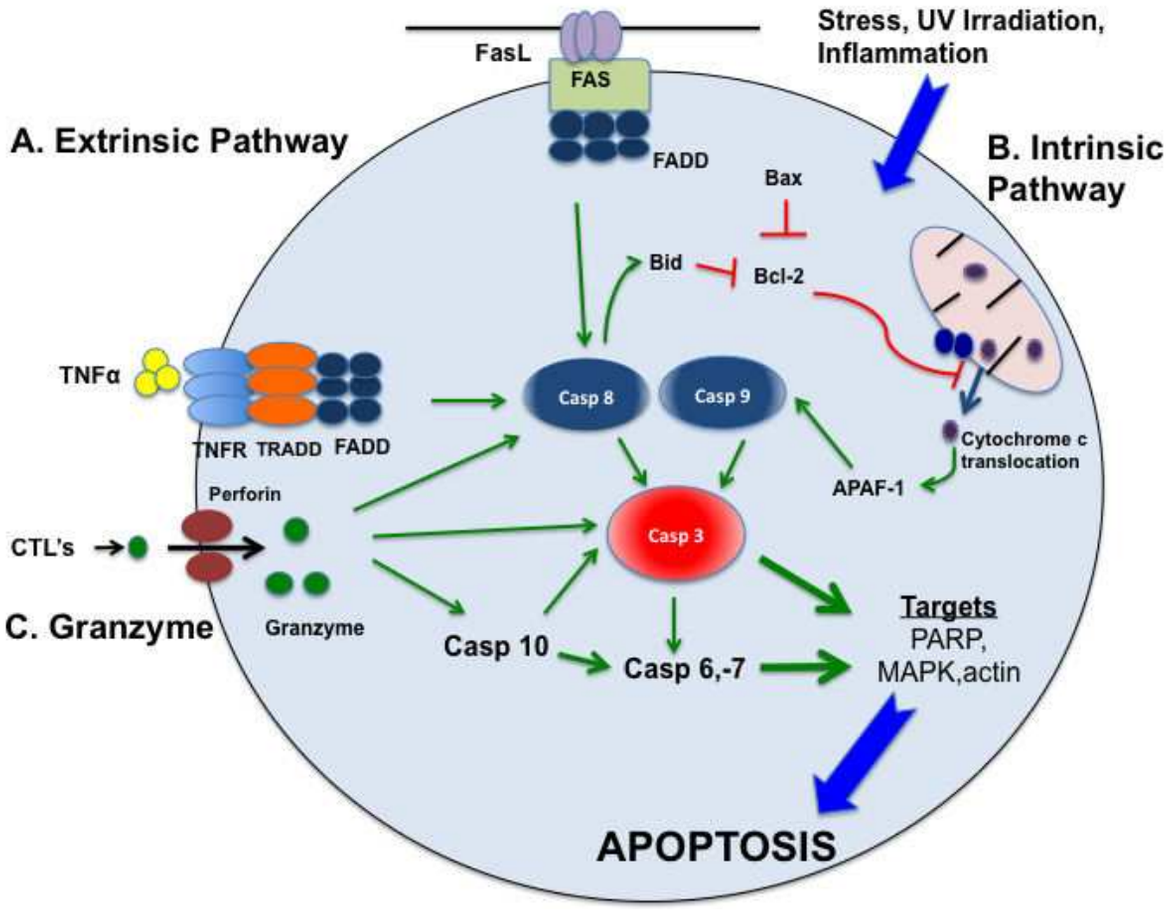

Figure 2. Signaling Pathways of Apoptosis. Apoptosis is induced through either the extrinsic pathway (A), intrinsic pathway (B) or the granzyme pathway (C). Activation of initiator caspases 8 and 9 (Casp 8,-Casp9), leads to apoptotic cell death through activation of Caspase 3 (Casp3) and subsequent activation of effector caspases 6 and 7 (Casp 6-Casp7). The extrinsic pathway (A) is initiated via extracellular molecules, FasL and TNFα, which bind to TNFR family members, Fas and TNFR respectively which activate Casp8 (green lines). The intrinsic pathway (B) is initiated by stress, UV irradiation and inflammation which act on the mitochondria through the pro-apoptotic Bcl-2 family members, such as Bax, resulting in the blockage of the anti-apoptotic activity of Bcl-2 (red lines). As a result, Cytochrome c is released into the cytoplasm and activates Casp9 through APAF-1 (green lines). Casp8 may also trigger the intrinsic pathway through activation of Bid, which inhibits the anti-apoptotic activity of Bcl-2. The granzyme pathway (C) is activated via cytotoxic T cells (CTLs) that introduce granzyme molecules into the target cells via secretion of perforin, which through a multimerization process forms a pore in the cell membrane allowing granzyme into the target cell. Granzymes cleave multiple caspases, including Caspase 10.

contains two death effector domains that can bind and cleave caspase 8. TRADD lacks death effector domains but binds FADD that then cleaves caspase 8. Activated caspase 8 then activates caspase 3. The intrinsic pathway is activated via changes in the mitochondria membrane potential which can be triggered via stress, toxic reagents, UV irradiation and inflammation. Changes in mitochondria membrane result in translocation of cytochrome c from the inner mitochondrial membrane into the cytoplasm. Cytochrome c binds to apoptosis protease activating factor 1 (APAF-1) which in the presence of ATP cleaves caspase 9. The intrinsic pathway is regulated by Bcl-2 which localizes to the outer mitochondria membrane. Bcl-2 members (Bcl-2 and Bcl-x) are anti-apoptotic while others such as Bax and Bad are pro-

apoptotic. The granzyme activation pathway is stimulated by cytotoxic T lymphocytes (CTLs) or NK cell secretion of perforin, which creates pores in the target cell membrane allowing granzymes into the cytoplasm activating caspase 10 which can then activate caspase 3.

2.1.2. Apoptosis in physiological and pathological conditions and potential therapeutic application

Apoptosis is a tightly regulated process and is rarely observed in healthy animals because phagocytes rapidly remove apoptotic cells. Abnormalities in cell death regulation can be a significant component of diseases such as cancer, AIDS and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. Some of these conditions are characterized by insufficient apoptosis and others excessive apoptosis. Excessive apoptosis, for example, is a feature of neurodegenerative diseases while resistance to apoptosis characterizes many cancers. Understanding the mechanisms of apoptosis at the molecular level may provide therapeutic interventions in these diverse disease processes.

2.1.2.1. Autophagic cell death

Autophagy, which means literally to "eat oneself", is a physiological response to stress such as starvation and is considered the major cellular mechanism for generating the needed metabolic sources of energy and metabolites during times of nutrient deprivation. Autophagy can also be used to remove damaged or unwanted organelles, such as mitochondria and long-lived proteins. Autophagy is a homeostatic mechanism that is predominantly a cytoprotective process [7, 13]. Other substrates are also now recognized as potential cargo for autophagy including lipids, nucleic acids, reticulocytes and intracellular pathogens and viruses. The concept of autophagic cell death has been a matter of debate, but it is now recognized as a type of programmed cell death mechanism that can lead to both apoptotic and necrotic cell death in certain circumstances such as extreme stress conditions [14, 15]. Autophagic cell death or type II programmed cell death is now used to refer to cell death process distinct from apoptosis that is caspase-independent and occurs with accumulation of double-membrane organelles called autophagosomes.

2.1.3. Signaling pathways and molecular components of autophagy

Autophagy delivers cytosolic materials to the interior of the lysosomes for degradation via a process involving the "*de novo*" formation of cytoplasmic double-membrane vacuoles called autophagosomes which then fuse with lysosomes forming an autolysosome that degrades the cellular cargo (Figure 3). Autophagy is regulated by a genetic program with a number of autophagy-related genes (Atg) whose gene products (ATG proteins) regulate distinct steps in autophagy. The autophagic pathway proceeds through the following defined steps: (i) initiation phase involves the formation of an isolation membrane or phagophore, (ii) elongation of the phagophore, (iii) maturation of an autophagosome with accumulation of cytosolic cargo, (iv) fusion of the mature autophagosome with the lysosome and (v) degradation of the contents via lysosomal proteases (i.e., cathepsins) in the autolysosome. The initiation phase involves formation of an initiation complex comprised of the class III PI3 kinase, VSP34, which

http://dx.doi.org/10.5772/61529

converts phosphatidylinositol to phosphatylinositol-3-phosphate (PI3P). The activity of VSP34, which binds to beclin 1, requires the activity of VSP15, the regulator beclin 1 and ATG14L (Figure 3a). Elongation of the autophagosome membrane requires action of two ubiquitin-like conjugation systems, the Atg5–Atg12 conjugation system and the microtubule-associated protein-1 light chain (LC3). The phosphatidylethanolamine-conjugated form of LC3, LC3-PE (also called LC-II) is generated by ATG4-dependent proteolytic cleavage of LC3 and the action of the E1 ligase, ATG7, the E2 ligase, ATG3 and the E3 ligase complex, ATG12/ATG5/ATG16L. LC3-PE stably associates with the autophagosome and is commonly used as a marker for autophagosomes.

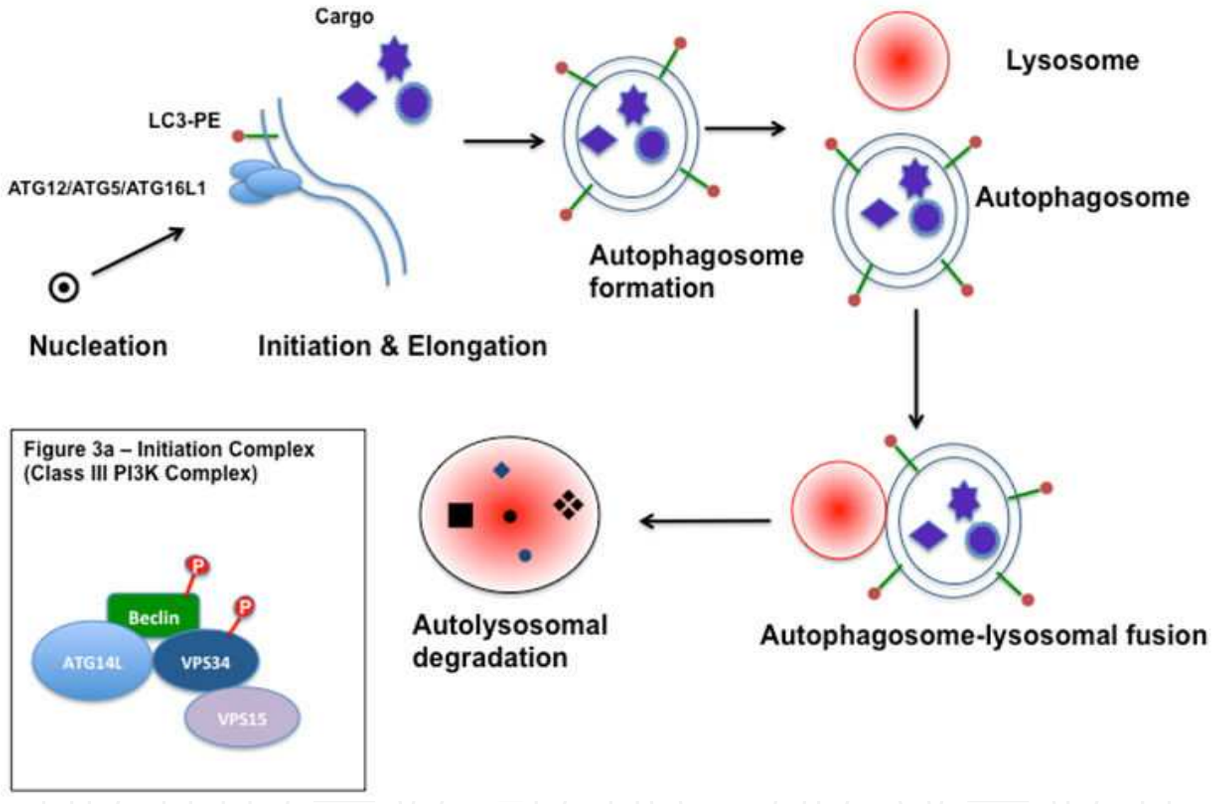

Figure 3. Autophagy Pathway. Autophagy is a membrane-dependent pathway that involves a series of defined steps beginning with a nucleation step which then leads to: 1). Initiation of the isolation membrane or phagophore, 2). Formation of the autophagosome around cellular cargo, 3). Fusion of autophagosome with lysosomes, and 4). Degradation of cargo by lysosomal proteases in autolysosomes. Formation of the initiation complex (also called Class III PI3K Complex) as shown in Figure 3a, consists of Beclin-1, ATG14L, VPS34 and VPS15. Activation of the initiation complex requires disruption of binding of Bcl-2 to Beclin-1. The Class III PI3K complex generates PI3P at the site of nucleation of the isolation membrane, which leads to binding of PI3P-binding proteins and the subsequent recruitment of proteins involved in the 'elongation reaction' of the isolation membrane. These proteins contribute to membrane expansion resulting in the formation of a double-membrane structure, the autophagosome that surrounds cellular cargo. The phosphatidylethanolamine-conjugated form of the LC3 (LC3-PE) is generated by the ATG4 dependent proteolytic cleavage of LC3 and the action of E1 ligase, E2 ligase and the E3 ligase ATG12/ATG5/ATG16L. LC3-PE stably associates with the autophagosome and is a marker of autophagosomes and therefore LC3 is commonly used as a marker of autophagy pathway.

While the autophagy pathway is primarily a homeostatic process promoting cell survival, increased autophagosomal formation can occur coincidentally with cell dying indicating autophagy may be involved in regulated cell death pathways, most notably apoptosis [7, 13]. For example, in apoptosis-comprised cells, cells die via a caspase-independent mechanism characterized by autophagosome accumulation, implicating a role for autophagy in the cell death process. Autophagy and apoptosis may also be regulated coordinately as anti-apoptotic proteins that downregulate apoptosis can also downregulate autophagy. For example, members of the Bcl-2 family can bind to beclin-1, thus inhibiting the formation of initiation complex that is necessary to stimulate autophagy. Other studies indicate once apoptosis is activated, apoptosis effector molecules may suppress autophagy as beclin-1 is cleaved and inactivated by caspases. Finally, studies also indicate autophagy proteins may play a dual role in regulation of apoptosis and autophagy. For example, Atg5 may affect the extrinsic apoptotic pathway through interaction with FADD proteins, while Atg12 is an effector of intrinsic apoptotic pathway with both Atg5 and Atg12 acting as pro-apoptotic regulators.

Autophagy pathway has also been implicated in necrosis and necroptosis cell death. Evidence indicates autophagy may be able to act as an inhibitor of necrosis/necroptosis by preserving cellular functions, removing toxic products and maintaining cellular energy. For example, knockdown studies of components of autophagy pathways, such as beclin-1, have found cytotoxicity is exacerbated suggesting that autophagy has a cytoprotective role. Autophagy may be able to act as a buffer to metabolic stress, providing a mechanism to generate ATP to maintain metabolic viability and thus prevent necrotic cell death. Conversely, studies using a specific inhibitor of necroptosis, necrostatin-1, found both necroptosis and autophagy were inhibited, indicating autophagy may be induced by necrosis. Molecular components underlying the relationship between autophagy and necroptosis/necrotic cell death at this point are poorly understood.

2.1.4. Physiological and pathological roles of autophagic cell death and therapeutic implications

Thus, while autophagy is now recognized to play a role in apoptotic and necrotic cell death programs, this process is complex and incompletely understood. However, increasing evidence indicates autophagic cell death may be necessary for cell death in certain circumstances. It has been suggested autophagic cell death represents a failed adaptive mechanism that may have prevented death under milder conditions. Understanding of the relationships of autophagic cell death with other programmed cell death modalities will require further study. The study of autophagy in disease is an emerging area of research. Autophagy pathway and/or autophagic cell death pathways which result in failure to remove damaged organelles and/or damaged cells are now thought to contribute to various diseases such as cancer, neurodegenerative diseases such as Parkinson's disease, aging and inflammation. Elucidation of the influence of autophagy with other cell death programs will be essential to the development of therapeutics targeting autophagy for treatment of these various diseases. In the context of infectious disease, autophagy also is now recognized to play an important role in intracel-

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lular pathogen defense, and it has been suggested that intracellular pathogen load could be a factor that disrupts the balance between cell survival and cell death [16].

2.2. Necroptosis

Pathologists first used the term "necrosis" in the early 19th century to describe tissue destruction. Necrosis is characterized by organelle swelling and cell lysis, releasing the cellular content of cells and resulting in inflammation. The first indication that necrosis could occur in a regulated manner arose from observation that tumor necrosis factor (TNFα) could trigger apoptosis and necrotic forms of cell death. Caspase inhibition strongly exacerbates necrotic cell death, indicating caspase activity negatively regulates necrosis. This accumulating evidence led to the concept of "programmed necrosis" and the coining of the term necroptosis in 2005 to designate regulated necrotic cell death [4, 9]. Receptor-interacting protein kinase 3 (RIP3) and its substrate, the mixed lineage kinase-like domain protein (MLKL), are now recognized as the key activation steps and the molecular hallmarks of regulated necrotic cell death or necroptosis.

2.2.1. Signaling and molecular components of necroptosis

Programmed necrotic cell death can be activated when cells are stimulated by ligation of death receptors including CD95 (also known as FAS), TNF receptors (TNFRs) and TNF-related apoptosis-inducing ligand (TRAIL). Ligation of cell death receptors leads to the formation of complex 1, comprised of TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2), receptor-interacting kinase 1(RIP1) and cellular inhibitors of apoptosis (CIAPs) (Figure 4). Upon the release of second mitochondria-derived activator of caspases (Smac) from the mitochondria, RIP1 is de-ubiquinated and dissociates from the death receptor and induces the formation of FADD-RIP1-caspase 8 pro-apoptosis complex II. Conversion of complex 1 to complex II indicates a change in cell fate from pro-survival to cell death. When caspase 8 is inhibited, RIP1 and RIP3 form the necrotic death domain complex, the necrosome, and their kinase activities become activated. RIP3 recruits it substrate monomeric MLKL from the cytoplasm and phosphorylates it. The phosphorylation destabilizes MLKL and drives its oligomerization which enables it to bind to phosphatidylinositol phosphate lipids (PIPs) and cariolipin (CL). Different PIPs and CLs orchestrate the translocation of MLKL to different membrane compartments including the mitochondria, endoplasmic reticulum, the Golgi, lysosomes and plasma membranes. In addition to activation via death receptors, it is also now clear that necroptosis can also be initiated by pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) and cytokines via activation of RIP3 through distinct upstream mechanisms.

2.2.2. Physiological and pathological roles of necroptosis and potential therapeutic applications

Programmed necrosis has been found to be important in host antiviral responses and a variety of tissue-damage related diseases such as acute pancreatitis, ischemic reperfusion injury, retinal detachment, atherosclerosis, neuronal loss in Gaucher's disease, amongst others. It has

been a paradigm that apoptotic cell death is anti-inflammatory with dead cells cleared by phagocytes while necrotic cell death leads to inflammation and tissue damage. Necrotic cells, however, can be internalized by macrophages although via macropinocytosis and thus in a manner distinct from phagocytosis of apoptotic cells, but they are nonetheless efficiently cleared by professional and nonprofessional phagocytes and hence rarely found in tissues. Large numbers of dying cells such as may occur in excessive tissue injury, autoimmune diseases such as systemic lupus erythematosus or infectious disease may lead to the defective clearance of necrotic cells and contribute to the persistent inflammation and tissue damage. However, smaller number of cells dying via necrosis or necroptosis may not necessarily lead to massive tissue damage and hence controlled necrotic cell death may not necessarily lead to massive tissue damage commonly associated with necrotic cell death.

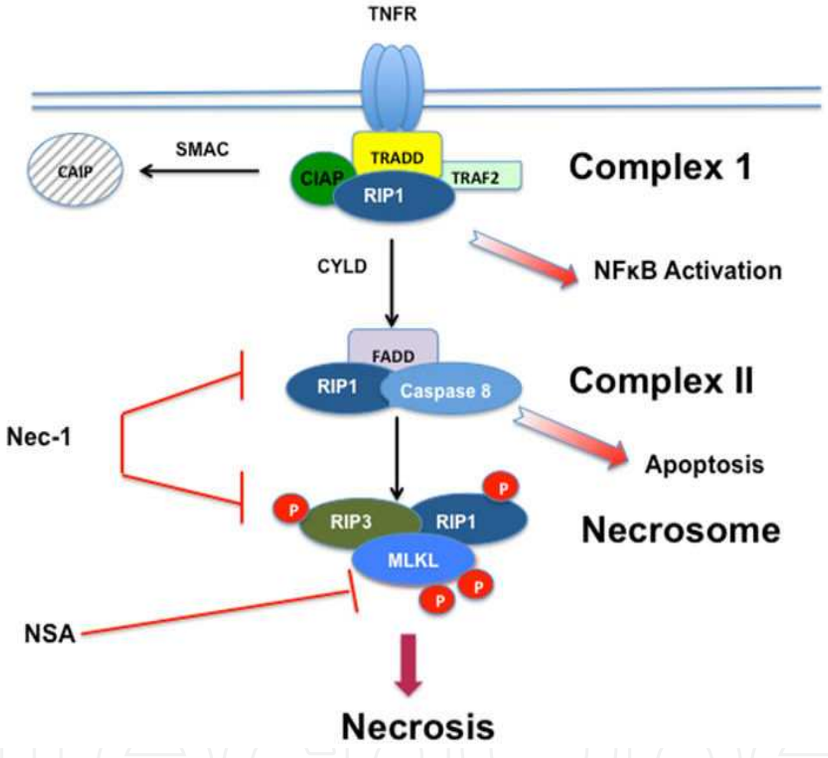

Figure 4. Necroptosis Signaling Pathway. Ligation of tumor-necrosis factor 1 (TNFR1) leads to the formation of Complex 1 that is comprised of TNFR-1, TFNR1-associated death domain (TRADD), TNFR-associated factor 2 (TRAF-2), Receptor-interacting kinase 1 (RIP1) and cellular inhibitors of apoptosis (CIAPs). RIP1 is initially modified by ubiquitin chains within Complex I after recruitment to the membrane and serves as the signaling platform for NF-κB activation. Upon release of the second mitochondria-derived activation of caspases (Smac) from the mitochondria, CIAPs are auto-degraded. RIP1 is subsequently de-ubiquinated by CYLD and dissociates from the death receptor, and associates with FADD-RIP1-Caspase8 forming Complex II, which is pro-apoptotic. Within Complex II, Caspase 8 activates Caspase 3/7 to induce apoptosis and blocks necrosis by cleaving and inactivating RIP1 and RIP3. When apoptosis is inhibited, as for example by Z-VAD, RIP1 and RIP3 form the necrotic death complex, the necrosome, and their kinase activities become activated. RIP3 recruits the mixed lineage kinase domain-like protein, MLKL, and phosphorylates MLKL, causing its activation. Activated MLKL then associates with membranes such as the plasma membrane, mitochondrial members and others, causing lysis of these membranes and death of the cell. Inhibitors of necroptosis include, Necrostatin 1 (Nec1) which inhibits RIP1 dependent necrosis by inhibiting the kinase activity of RIP1 to prevent necrosome formation and Necrosulfonamide (NSA), which inhibits MLKL-mediated necrosis.

Knockout of either RIP3 or MLKL genes in mice has been used to assess the physiological and pathological roles of programmed necrosis. These types of studies have confirmed that necroptosis is important in host antiviral responses and in some tissue-damage related diseases. Several inhibitors have been identified that can inhibit necroptosis. Necrostatins, for example, are small molecules that inhibit RIP1 kinase activity and thus inhibit necroptosis (Figure 4). RIP1 also contributes to other processes such as apoptosis and NK-kB activation, however, indicating other processes may also be affected by Nec-1. Targeting RIP3 or MLKL offer more specific inhibitors for study or treatment of necroptosis. A chemical inhibitor of MLKL called necrosulfonamide (NSA), for example, blocks necrosis by preventing oligomerization of MLKL and thus is a specific inhibitor of necroptosis (Figure 4). Use of these specific inhibitors of necroptosis will be essential to elucidate the role of necroptosis in cell death. As the role of programmed necrotic death is now known to occur in several human diseases, development of specific inhibitors of neroptosis could also have therapeutic uses.

2.3. Crosstalk between apoptosis, necroptosis and autophagy PCD pathways

As molecular components and regulation of each pathway has become better understood, it has become apparent that significant crosstalk exists between the three major pathways of programmed cell death, the focus of which has been the topic of several recent reviews [3, 8, 10, 13]. Extensive crosstalk exists between apoptosis and autophagy as discussed above in the section on autophagic cell death. The stimuli for apoptosis and autophagy are often the same and evidence indicates instances where they can cooperate, antagonize or assist each other. Caspases, Beclin-1 and/or the TOR kinase pathway have all been implicated in participating in the complex crosstalk between apoptosis and autophagy. Crosstalk between apoptosis and necrosis also appears to exist as receptors that stimulate apoptosis can also stimulate necroptosis. Energy is thought to play a central role in determining the interplay between these two pathways where instances of high ATP levels would enable a cell to undergo apoptosis while low ATP levels would favor necrosis. Other factors such as p53, Bcl-2 proteins and PARP1 are also suggested to play a role. Evidence also suggests a complex interplay between autophagy and necroptosis. Autophagy and necrosis can be activated in parallel or sequentially and can have either opposite or the same effects. Autophagy, for example, can protect against some types of necroptotic cell death with autophagy serving as the last resort before cell death via necrosis. The molecular basis for interlinked processes in autophagy and necroptosis and the impact of autophagy on necroptosis and other cell death pathways [13], however, remain unclear. While the molecular details are not fully understood, it is clear that a complex crosstalk and molecular interplay between apoptosis, autophagy and necroptosis occur and determine the ultimate fate of the cell to survive or die in a given situation or under a given stress signal. A better understanding of the mechanisms of apoptosis, autophagic cell death and necroptosis at the molecular level and the crosstalk between these cell death pathways would provide a deeper insight into different disease processes and may provide novel therapeutic strategies.

3. *Toxoplasma gondii:* Manipulation of programmed cell death pathways and impact on host–parasite interactions and maintenance of chronic infection

PCD can be important host cell defense mechanisms to eliminate intracellular pathogens. *Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes a chronic infection with approximately one-third of the world's population chronically infected, making *T. gondii* one of the most prevalent human parasitic infections worldwide [17–19]. In humans, infection with *T. gondii* is characterized by rapid parasite replication and dissemination throughout the body with the parasite capable of infecting all types of nucleated cells. The acute infection is followed by a chronic infection that lasts for the lifetime of the individual with the parasite harbored within neurons in the brain and muscle tissue in intracellular cysts. In most immunocompetant individuals, the infection is asymptomatic due to an effective immune response that eliminates active parasite replication. In immunocompromised individuals such as AIDS patients that are chronically infected, the parasite reactivates from cysts in the brain leading to severe and potentially fatal encephalitis. The parasite can also be transmitted transplacentally and can cause severe neurological complications to the fetus including mental retardation, hydrocephaly and chorioretinitis which can reactivate through the first two decades of life potentially leading to blindness. Finally, increasing evidence indicates that in some immunocompetant individuals, chronic infection with *T. gondii* may lead to development of serious psychiatric disorders such as schizophrenia or suicidal tendencies [20–22].

While the host mounts an effective immune response against *T. gondii*, the parasite has evolved immune evasion strategies that enable it to survive and persist long enough in the host to establish the cyst stage of the infection that can persist for the lifetime of the host. PCD pathways are a key part of the host defense against *T. gondii*, but the parasite has evolved multiple mechanisms to manipulate these pathways. For example, the parasite induces apoptosis in selected immune cell lineages, thus suppressing the host immune response and helping to establish infection but the parasite also has multiple mechanisms to resist killing by apoptotic and, to lesser degree, autophagic and necrotic cell death pathways in different host cell types. Thus, parasite manipulations of host cell death pathways appear to be an essential component to the successful establishment of infection and maintenance of the chronic infection. A brief summary of the mechanisms by which the parasite manipulates these cell death pathways is reviewed below followed by a discussion of the potential therapeutic interventions.

3.1. Apoptosis

T. gondii has the ability to promote apoptosis of immune effector cells early after infection in selected cell types. In murine models of toxoplasmosis, apoptosis of CD4+, CD8+ T cells, B cells, NK cells and granulocytes is observed in the spleen early after infection [23]. Apoptosis

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in Peyer's patch T cells in perorally infected mice has also been observed [24]. Acute infection of *Toxoplasma* in both mice and humans induces a state of transient immunosuppression as determined by decreased antibody and T lymphocyte responses. Triggering of apoptosis of T cells and other immune effector cells by *T. gondii* may be one factor by which the parasite restricts the immune response, thus allowing the establishment of infection. Induction of apoptosis of immune effector cells may be somewhat parasite strain-dependent as, in murine models, high levels of apoptosis in T cells was induced when mice were infected with virulent RH strain while this phenomenon was not seen in mice infected with avirulent strains.

Inhibition of apoptosis by infection with *T. gondii* has been found to occur in a wide range of cell types including macrophages and a wide variety of non-immune effector cells such as fibroblasts, endothelial cells, muscle cells and astrocytes [23, 25, 26]. Infected host cells are resistant to induction of apoptosis to a wide range of stimuli including CTL-mediated cytotoxicity, irradiation, growth factor withdrawal, TNFα and several toxic reagents. Blockage of apoptosis in host cells establishes an anti-apoptotic condition of the host cell and favors parasite persistence. Neighboring uninfected cells are also rendered resistant to apoptosis. Inhibition of host cell apoptosis of non-immune effector cells likely ensures the host cell stays alive long enough to facilitate intracellular replication of the parasite in the tissues while suppression of apoptosis of uninfected neighboring cell may help create a microenvironment in different tissues in which the parasite can persist and replicate.

While blockage of apoptosis is known to occur in a wide variety of host cells, modulation of apoptosis in cells of the central nervous system (CNS) is less well studied and results are ambiguous. *T. gondii* can infect neurons, astrocytes and microglia supporting growth of the rapidly replicating tachyzoite form while bradyzoite stage and cysts develop only in neurons and astrocytes. In murine astrocytes, infection blocks apoptosis beginning at 6–24 h after infection, allowing time for the parasite to replicate, egress and initiate infection of a new host cell [26]. Thus, inhibition of apoptosis in astrocyte host cells may allow an increase in parasite numbers in the brain and help establish the chronic infection in the brain. Conversely, several studies indicate the parasite induced apoptosis in neurons. For example, tachyzoites induced apoptosis in mouse brain cells in adult mice including in neurons [27, 28]. Likewise, in a murine model of congenital toxoplasmosis, infection resulted in a decrease in neuron number and markers of apoptosis were found indicating infection induced apoptosis in CNS tissues [29]. In murine neural stem cells, infection was also found to induce apoptosis although only apoptosis of neighboring neurons were assessed in this study [30]. A study in murine brain indicated infected neurons were resistant to apoptosis and apoptosis was only induced in the uninfected neurons [31]. Microglia cells which are activated in the brains of mice infected with *T. gondii* with toxoplasmic encephalitis produced nitric oxide (NO), which induces neuronal apoptosis indicating activated microglia may be part of the mechanism leading to neuronal loss of uninfected neurons [32]. Collectively, the above results are suggestive of a mechanism in which *Toxoplasma* infection of neural tissues induces apoptosis of neurons, thus leading to loss of neurons which could lead to neurological abnormalities. However, it is unclear if apoptosis of infected neurons was induced or, alternatively, if infected neurons are resistant to apoptosis. Suppression of apoptosis of infected neuronal cells may facilitate development

of the cysts in neurons and allow persistence of the parasite in the brain. A better understanding of parasite modulation of apoptosis in infected neurons is thus of importance. A better understanding of the modulation of apoptosis in infected neurons could lead to the development of antiparasitic drugs and other therapeutic interventions to control and manage the chronic phase of infection.

3.1.1. Parasite mechanisms modulating apoptosis

The parasite replicates within a membrane-bound compartment called the parasitophorous vacuole (PV), and hence it is not in direct contact with the host cell cytoplasm and regulators or effectors of apoptosis. However, parasite secretory molecules, which are either released into the host cell at invasion, secreted across the PV membrane (PVM) or act at the PVM cytosolic surface, have been identified which can modulate host cell apoptosis [25, 33]. These mechanisms are briefly summarized below.

3.1.2. Parasite mechanisms that stimulate apoptosis

Several mechanisms have been identified by which the parasite can stimulate apoptosis. In parasite-infected murine macrophages, supernatants can induce apoptosis of neighboring uninfected macrophages via nitric oxide [34]. Induction of apoptosis has also been found to occur via nitric oxide secretion from activated microglia. Nitric oxide secretion from activated microglia can lead to neuronal cell death and may be an important mechanism by which infection leads to neuronal loss in the brain. The parasite secretory molecule, GRA1, has been identified as capable of inducing apoptosis of infected monocytes and uninfected bystander cells [35]. Secreted GRA1 from sites of ongoing *T. gondii* replication could also induce apoptosis of monocytes recruited to the site of parasite replication. Given monocytes are essential to control parasite replication at the site of primary infection, inhibition of apoptosis in monocytes could serve to downregulate host responses early in infection, helping to establish the infection in the host.

3.1.3. Parasite mechanisms that inhibit apoptosis

Multiple mechanisms have been identified by which the parasite can inhibit apoptosis [25]. Activation of NF-κB is a mechanism that inhibits apoptosis in fibroblasts and some other host cells [36–38]. Early after infection, the parasite induces rapid translocation of host cell transcription factor, NF-kB, into the nucleus, which activates cell survival pathways and induction of an anti-apoptotic state of the host cell. Nuclear translocation of NF-κB and subsequent gene expression requires activity of the host IκB kinase (IKK). The activation of NF-κB was associated with localization of phosphorylated IκBα subunits to the PVM that was mediated by a parasite-derived IκB kinase (TgIKK). TgIKK is produced as the parasite replicates, and thus allows for a continued phosphorylation of IκB and sustained inhibition of apoptosis. Thus, *T. gondii* modulation of NF-κB gene expression and induction of anti-apoptotic state of the host cell involves both the host cell IKK and TgIKK activity at different phases of infection. NF-kB

http://dx.doi.org/10.5772/61529

activation has not been found to occur in infected macrophages. Rather in macrophages, *T. gondii*, inhibits apoptosis by G_i-protein-mediated signaling, activating PI 3-kinase leading to phosphorylation of protein kinase B (PKB/AKt) as an inhibitor of apoptosis [39]. A micro-RNA-mediated mechanism has also recently been identified in macrophages that inhibited apoptosis via a reduction in Bim, a pro-apoptotic effector of the Bcl-2 family [40]. Other mechanisms have been identified in various cell types that can inhibit caspase cascade including blockage of caspases 8 and 9 and 3, increased expression of anti-apoptotic molecules of the Bcl-2 family, decreased activity of poly (ADP)-ribose polymerase and inhibition of granzymes [33, 41].

Only a few parasite proteins that modulate apoptosis have been identified. The parasite serine–threonine phosphatase, called TgPP2C, was recently identified which downregulates apoptosis in host cells [42]. TgPP2C is secreted into host cells from the PV and translocates to the host cell nucleus and has been shown to regulate growth and survival of the parasite. Using a yeast two-hybrid system, TgPP2C was found to interact with host cell protein, SSRP1 (structure-specific recognition protein 1), which binds to DNA and regulates DNA repair genes. It is speculated that SSRP1 might also be involved in expression of other genes involved in cell survival and apoptosis. Identification of other parasite molecules by which the parasite manipulates host cell apoptosis would facilitate a deeper understanding of host–parasite relationship and may lead to development of new therapeutic targets and antiparasitic drugs.

3.1.4. Parasite modulation of apoptosis: significance to pathogenesis

In conclusion, *T. gondii* has the ability to both promote and inhibit apoptosis of its own host cell and of uninfected neighboring cells by multiple mechanisms. The ability to modulate apoptosis varies by host cell type, parasite load and parasite virulence. Manipulation of apoptosis has been proposed to be crucial to promoting a stable parasite–host interaction and allowing establishment of persistent infection [23]. The multitude of anti-apoptosis mechanism that the parasite *T. gondii* employs is likely reflective of its status as an obligate intracellular parasite and the essentiality of maintenance of host cell viability to sustain parasite replication and survival. However, intracellular replication eventually leads to lysis of the host cell and typically occurs between 48 and 72 h post-infection, a temporal scale similar to apoptosis. Given the similar temporal scales of lytic death of the host cell and cell death by apoptosis, it can be argued inhibition of apoptosis of the host cell is not necessary to allow for parasite replication. However, as the parasite needs to obtain purine, cholesterol, tryptophan and other components from the host cell, the parasite inhibition of host cell apoptosis may also serve to maintain the host cell in a pro-survival state to enhance availability of host cell nutrients. Inhibition of apoptosis may also be critical for the chronic phase of infection allowing differentiation of bradyzoites and development of the cyst stage in neurons. A better understanding of manipulation of apoptosis by bradyzoites in host cells and, specifically in neurons, is of great importance as the CNS is the compartment in which the parasite predominantly persists in the chronic infection and the cause of serious clinical sequelae in the CNS of immunocompromised individuals causing reactivated toxoplasmosis, in immature immune system of the fetus and newborns causing congenital toxoplasmosis, and in immunocompetant individuals possibly contributing to the development of serious neuropsychiatric disorders.

3.2. Autophagy

Autophagy has been found to play a role in host defense against many intracellular pathogens as the autophagy pathway can degrade intracellular pathogens via autophagolysosomes including *T. gondii* [16]. *T. gondii* replicates within a membrane compartment in the host cell called the parasitophorous vacuole (PV) that is a non-fusogenic compartment and does not fuse with cell lysosomes. However, the PV can be delivered to the lysosomes via autophagy-mediated delivery in IFN-γ-activated macrophages resulting in killing of the parasite [43]. This autophagic-mediated parasite killing is mediated by the immunity-related GTPases (IRGs) which are stimulated by IFN-γ. Engagement of CD40 also has been found to induce killing of *T. gondii* via autophagy in many nonhematopoetic cells including endothelial cells lines, human and mouse retinal pigment epithelial cells as well as in hemopoietic cells such as macrophages [44, 45].

Interestingly, in non-immune-stimulated cells, evidence indicates the parasite can use autophagy to enhance its own survival. In the first 24 h after infection, host cell autophagy is upregulated and the parasite uses autophagy to acquire host cell nutrients, while at 24–36 h after infection when significant parasite replication has occurred, host cell autophagy is suppressed [46, 47]. This biphasic response may be due to the crosstalk that exists between apoptosis and autophagy. That is, inhibition of apoptosis occurs in infected cells during the first 24 h after infection and as a result of crosstalk, it has been suggested that autophagy is stimulated and then, vice versa, during the later stages of the intracellular infection cycle (> 24 h post-infection), as apoptosis inhibition wanes, suppression of autophagy increases. Thus, regulation of host cell autophagy by the parasite may be part of a cell survival mechanism in *T. gondii* infected host cells related to inhibition of apoptosis. Interestingly, parasite-mediated activation of AKt signaling has recently been found to prevent autophagy degradation of the parasite [48]. As AKt activation has also been linked to inhibition of apoptosis in *T. gondii* infected cells, this event suggests AKt signaling may be a pathway by which the parasite can regulate both host cell autophagy and apoptosis pathways [39, 48]. Dual manipulation of host cell apoptosis and autophagy pathways indicates a fine level of control of host PCD pathways by the parasite to promote parasite persistence in the host. Further work on the interplay between apoptosis and autophagy and the parasite modulation of these two processes is needed to further elucidate these mechanisms.

3.3. Necrotic cell death

A few *in vitro* studies have reported *T. gondii* inducing necrotic cell death of host cells [49–52]. In IFN-γ stimulated fibroblasts and astrocytes, infection with avirulent parasite strains of the parasite, rather than stimulating autophagic-mediated mechanism of parasite killing, induces disruption of the PV membrane via IRG proteins resulting in parasite death and subsequently triggering of necrotic host cell death [50, 53]. This type of necrotic death of the host cells involved killing of intracellular parasites and thus would promote host survival via elimination of the parasite. In these experiments, the parasite was found to egress within hours after infection, thus limiting replication in host cells which also would promote clearance of the parasite by immune cells. Experiments have similarly found early egress of the parasites in

http://dx.doi.org/10.5772/61529

IFN-γ-stimulated astrocytes, indicating this mechanism may also function in the brain to limit parasite numbers and aid in elimination of the parasite [54].

Conversely, *Toxoplasma*-specific primed T cells were found to be able to induce rapid egress of the parasite, causing necrotic cell death of host cells via ligation of the death receptor or perforin, but as egress was rapid the intracellular parasites escaped killing [55]. These egressed parasites were capable of infecting neighboring cells, thus suggesting that during *Toxoplasma* infection of T cells, death receptor-and perforin-mediated parasite egress may contribute to parasite dissemination of the parasite. Furthermore, as necrotic cell death induced by death receptor or perforin-dependent parasite egress contributes to inflammatory processes, it could also lead to further spread of the parasite due to infection of invading leukocytes. This process may also contribute to the establishment of infection in the brain as infected leukocytes can invade brain. Finally, death receptor-induced or perforin-mediated egress could be involved during the reactivation of *T. gondii* in chronically infected individuals, leading to reinfection of egressed parasites in the brain. Thus, parasite evasion of this cell death pathway may have significant consequences on the clinical sequelae of the chronic infection in the brain. Experiments in fibroblasts found necrotic host cell death was not dependent on the necroptosis mediator RIPK3 or caspases [52]. Further studies are needed to ascertain if necroptosis occurs in other cell types that have been associated with necrotic-type cell death in *T. gondii* infected cells.

4. Parasite manipulation of PCD pathways: Conclusions and potential therapeutic implications

A better understanding of the mechanisms by which the parasite modulates apoptosis, autophagic and necrotic host cell death pathways will enhance our understanding of the host/parasite relationship in toxoplasmosis and may yield new therapeutic targets to treat pathological consequences of cerebral toxoplasmosis. For example, while the infection is asymptomatic in immunocompetant individuals, recent evidence indicates chronic infection may lead to psychiatric and other neurological disorders in some individuals. Some of these effects may be due to modifications of processes leading to cell death or resulting in dysregulated cell death, resulting in reduction in CNS cell numbers and specifically of neurons. Conversely, inhibition of apoptosis of astrocytes in the brain could be crucial for establishment of the parasite in this compartment, while inhibition of apoptosis of infected neurons may be essential for development of bradyzoite stage and thus crucial to long-term persistence of the parasite in the brain. The evidence indicates the parasite can manipulate both host cell apoptosis and autophagy pathways, indicating a sophisticated level of parasite control of host PCD pathways and elucidation of the molecular details of these mechanisms could lead to new therapeutic targets with which to control the infection. A better understanding of manipulation of apoptosis and other cell death pathways in the brain cells such as microglia, astrocytes and neurons is of particular importance. The identification of molecular components of PCD pathways crucial to parasite–host interactions in chronic toxoplasmosis could lead to development of new antiparasitic drugs and/or yield new potential therapeutic targets to treat

pathological consequences of chronic cerebral toxoplasmosis in immunocompetant individuals and reactivated toxoplasmosis in immunosuppressed individuals. A similar harnessing of apoptotic, autophagic and programmed necrosis cell death pathways has been proposed to treat cancers, neurodegenerative disorders and other diseases, where PCD pathways have been identified as involved in pathogenesis [56]. The dissection of the molecular mechanisms by which *T. gondii* manipulates apoptosis and other cell death pathways thus could also serve as a benefit to probe pathways in normal cells and in diseases in which apoptosis and other cell death pathways play a central role.

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