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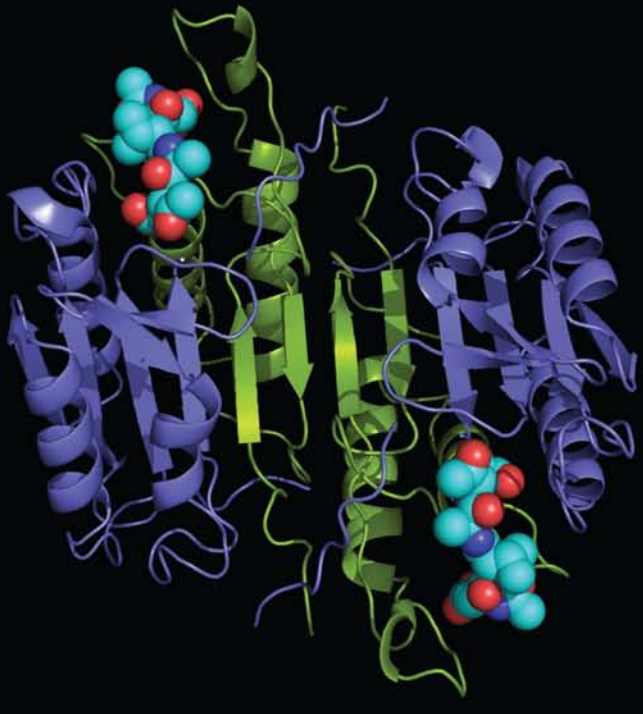
Design of Caspase Inhibitors as Potential Clinical Agents



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Preface

In 1992 the first report was published describing the identification of caspase-1 or Interleukin-1β Converting Enzyme (ICE), the founding member of a new class of cysteine based proteases. Since then, there has been an enormous effort to discover small molecule inhibitors of this therapeutically important class of enzymes. During this period, a large volume of literature has emerged describing the identification of a diverse range of inhibitors, ranging from substrate-like peptidic to non-peptidic heterocycles. Despite this significant effort, only four different inhibitors have initiated clinical trials: Pralnacasan (VX740) for rheumatoid arthritis (subsequently withdrawn during phase II trials); VX765, a second generation caspase-1 inhibitor (entered Phase II trials for psoriasis, but no current status is available); Emricasan (PF-03491390/IDN-6556), an anti-fibrotic agent for the treatment of chronic liver disease (apparently discontinued after completing Phase II trials); and LB-84451, for undisclosed indications, but possibly focusing on its anti-fibrotic activity (currently in Phase II trials). This emphasizes the difficulty in progressing a caspase small molecule inhibitor from discovery into the clinic, and, in this book, we intend to outline these efforts and highlight the complex issues that have been encountered. We also will present the current status of clinical trials and the future potential for caspase inhibitors as therapeutic agents.

The first three chapters outline what is currently known about the inflammatory and apoptotic caspase pathways. Logue and Martin (Chapter 1) present a comprehensive overview of the key caspase proteolytic pathways, both apoptotic and inflammatory, and the importance of these pathways in normal cellular activities. Chapters by Kersse et. al. (Chapter 2) and Matsui (Chapter 3) provide an in-depth coverage of the potential therapeutic value of an inflammatory or apoptotic (respectively) caspase inhibitor. In each of these chapters, the authors present an overview of the relevant pathways, along with *in vitro* and *in vivo* evidence supporting the use of a caspase inhibitor for each indication. In Chapter 4, Nalley reviews the catalytic properties of caspases, how to use these properties in designing inhibitors, and the potential difficulties involved therein. In particular, one of the key outstanding questions in caspase small molecule discovery is whether a specific or a pan-caspase inhibitor is preferred. As different indications will likely require a caspase inhibitor with a unique specificity profile, Nalley illustrates that by understanding the architecture of each caspase active site and its known substrate specificities, it should be possible to design inhibitors with different profiles.

We then turn to applying this knowledge to the design of caspase inhibitors and, in subsequent chapters, review the progress already made toward discovering small molecule inhibitors. Ivachtchenko et al (Chapter 5) outline progress towards discovering non-peptide inhibitors and give a very comprehensive overview spanning a large number of different chemical classes. This is followed by a chapter by Wos and Demuth (Chapter 6) that summarizes the current status of the discovery of

inflammatory caspase inhibitors and by Ullman (Chapter 7) that gives a perspective of the discovery of apoptotic caspase inhibitors. Chapter 8 presents an example of the preclinical approach undertaken to identify an apoptotic caspase inhibitor. In this case, Holgen presents a case-study of the discovery and characterization of Emricasan, a compound that completed Phase II clinical trials, but is now reported as discontinued from further development.

As described in the previous chapters, caspase inhibitors have been predominantly identified either by functional screens, structure-based design, or by computational modeling. In most cases, a combination of all three approaches has been used. However, as new technologies have emerged so have the approaches taken to inhibitor discovery, and some of these novel approaches are presented in Chapter 9 (Scheer and Romanowski). The advantage of using novel approaches has been validated by the discovery of an allosteric regulatory site that lies at the dimeric interface between the caspase large and small subunits. The presence of an allosteric site presents an opportunity to inhibit catalytic activity that avoids the limitations associated with designing a molecule that binds to the active site.

In the final chapter (Chapter 10), Eda gives an overview of the current status of ongoing clinical trials with caspase inhibitors. A survey of the literature indicates that potent caspase inhibitors can be discovered; however, advancing these compounds into the clinic has been challenging. Some of the key issues that are discussed revolve around questions such as what is the desired selectivity profile, whether reversible or irreversible inhibition is more relevant to the indication, and what impact the mode of inhibition has upon the toxicity profile. The answers will likely depend upon the indication being pursued and whether the caspase being targeted is apoptotic or inflammatory.

Despite the difficulties involved in caspase inhibitor discovery, considerable progress has been made and early clinical studies have shown promise. However, of the four compounds that have entered clinical trials, two have been discontinued and the fate of the remaining two compounds remains unclear. Nevertheless, the potential therapeutic benefits are tremendous, and there appears to be a renewed enthusiasm for caspase small molecule drug discovery. If one of the current compounds shows clinical benefit and makes it to market as a “first-in-class” drug, we have no doubt that this will fuel an enhanced discovery effort for additional compounds that could be “best-in class.”

Editors

Dr. Steve Linton has been involved with San Diego biotech for over 15 years, not only focusing on modulators of apoptosis while at Idun, but also working in several therapeutic areas, including inflammation and oncology, as well as metabolic and CNS disorders. Dr. Linton graduated with a BS degree in chemistry from Texas Christian University and received his PhD in the area of natural product synthesis from Rice University under the direction of Dr. Tohru Fukuyama. After beginning his medicinal chemistry career at Gensia Pharmaceuticals in 1992, Dr. Linton joined Idun Pharmaceuticals in 1995. He contributed to the development of Idun's caspase inhibitor drug discovery platform as well as investigated other modulators of apoptosis and is widely published in this area. Dr. Linton was promoted to medicinal chemistry section head and was part of the core team that presented Idun technology to potential investors. Idun was acquired by Pfizer in 2005, and its flagship caspase inhibitor, Emricasan, is currently in late-stage clinical development. Dr. Linton has also participated in other start-ups, such as Synstar, Inc., a custom synthesis contract research organization (Hangzhou, San Diego), as well as Novasite Pharmaceuticals, where he served as director of chemistry. He is currently director of project management at Halozyme Therapeutics.

Dr. Tom O'Brien graduated from Trinity College, Dublin, Ireland, with a BA (Mod) degree in genetics. Dr. O'Brien completed his PhD degree at Cornell University, Ithaca, New York, in the laboratory of Dr. John Lis, and subsequently moved to the University of California at Berkeley where he pursued postdoctoral research studies in the laboratory of Dr. Robert Tjian, where his research focused on dissecting the biochemical and molecular regulation of eukaryotic transcription. In 1999 Dr. O'Brien moved to the newly formed company Sunesis Pharmaceuticals, where his work focused on the discovery of novel small-molecule caspase inhibitors. As the biology project leader for their caspase small-molecule programs, Dr. O'Brien was one of the key people that helped optimize and validate their fragment-based approaches to small-molecule drug discovery. During his time at Sunesis, Dr. O'Brien was also the lead biologist for a number of additional programs, one of which recently entered clinical trials. In 2006 Dr. O'Brien joined Genentech, Inc., in their newly formed Department of Cell Regulation.

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1 Mammalian Caspase Activation Pathways in Apoptosis and Inflammation

Susan E. Logue and Seamus J. Martin

CONTENTS

1.1 INTRODUCTION

Members of the caspase family of cysteine proteases play key roles in signal transduction cascades in apoptosis (programmed cell death) and inflammation. Caspases are normally expressed as inactive precursor enzymes (zymogens), a subset of which become activated during apoptosis and coordinate the demolition of the cell from within. To date, three major apoptosis-associated pathways to caspase activation have been elucidated. Certain caspases, such as caspases-1, -4, and -5, also play key roles in signaling pathways associated with immune responses to microbial pathogens.

In this situation, caspase activation results in the maturation of pro-inflammatory cytokines, such as IL-1β and IL-18. Here we discuss the current understanding of how caspases are activated during apoptosis and inflammation and the roles these proteases play in either context.

1.2 MAMMALIAN CASPASES

Early studies directed toward identifying genes involved in the regulation of programmed cell death (PCD) were conducted in the nematode worm *Caenorhabditis elegans* and led to the identification of the cell death defective gene-3 (*ced-3*).[1] Worms defective for *ced-3* failed to eliminate many of the 131 cells that normally undergo PCD during worm development and implicated this gene as a major regulator of PCD in this organism. Ensuing searches for human homologues of *ced-3* resulted in the publication of a landmark paper by Horvitz and colleagues in 1993 describing interleukin-1β converting enzyme (ICE) as a human homologue of CED-3.[1] ICE, or caspase-1, as it is now commonly known, became the founding member of the family of aspartic acid–specific proteases, called caspases. To date, twelve members of the human caspase family have been identified (caspases-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -12, -14). Based upon the functional data available, these caspases fall into two distinct groups; apoptotic caspases (caspases-2, -3, -6, -7, -8, -9, -10) and inflammatory caspases (caspases-1, -4, -5, -12) with the role of caspase-14 somewhat poorly defined at present (Figure 1.1). Irrespective of their function, all members of this protease family are thought to cleave their substrates following an aspartate (Asp) residue.[2] Caspases recognize the Asp residues they cleave within a tetrapeptide motif, P4-P3-P2-P1, with substrate cleavage occurring at the peptidyl bond distal to the P1 residue. Depending upon the caspase in question, residues P2 to P4 can vary; however, position P1 has a near absolute requirement for Asp.[2]

Caspases are highly active proteases that are initially expressed as largely inactive precursors (pro-caspases) that require further proteolytic processing to achieve their active forms. Pro-caspases are comprised of three distinct domains: an N-terminal pro-domain, a large subunit containing the active site cysteine within a conserved QACXG motif, and a small C-terminal subunit (Figure 1.1). An Asp cleavage site frequently demarcates the N-terminal pro-domain from the large subunit. Similarly, a linker domain, containing one or two Asp cleavage sites, divides the large and small subunits.[2] Receipt of an activation signal initiates proteolytic processing of pro-caspases via a two-step process. Initial proteolytic cleavage at the Asp residues within the linker domain separates the large and small subunits. The caspase pro-domain is frequently, but not always, removed by proteolytic cleavage at the Asp residue located between this domain and the large subunit.[3] This series of proteolytic events results in the formation of active heterotetramers, comprised of two large subunits, two small subunits, and two active sites.[4–6] The substrate specificity of active caspases for Asp residues, combined with their own requirement for cleavage at specific Asp resides, suggested that caspase activation occurred either by autoproteolytic means or via cleavage by other caspases.

Apoptotic caspases can be further subdivided on the basis of their domain structures (Figure 1.1). Initiator caspases (caspases-2, -8, -9, -10) possess long pro-domains

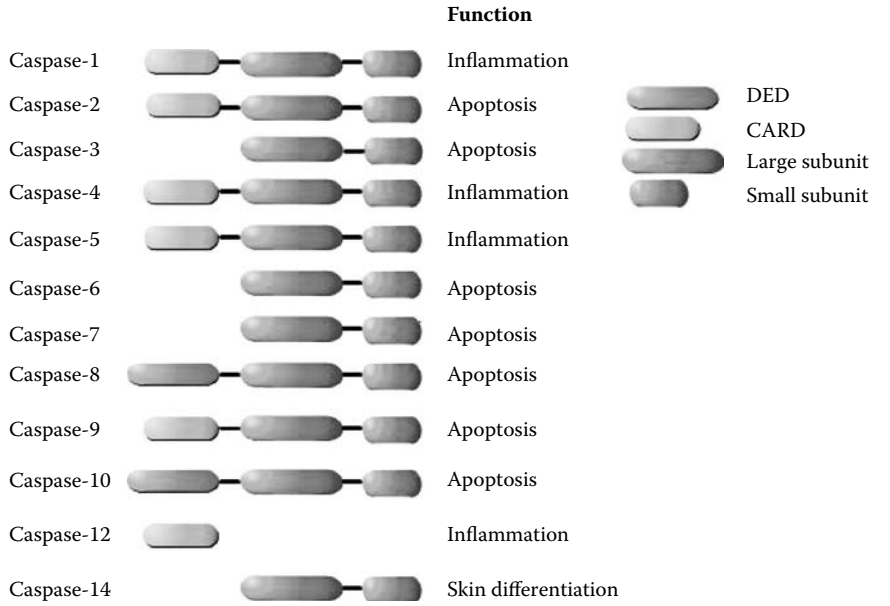

FIGURE 1.1 (See color insert.) Domain structures of the human caspase family.

with protein-protein interaction motifs, such as caspase recruitment domains (CARDs) or death effector domains (DEDs).[2] These motifs enable initiator caspase clustering upon scaffold molecules following receipt of activation signals. The clustering of multiple initiator caspases into close proximity induces dimerization followed by autoprocessing, a mechanism referred to as the induced-proximity model.[7] Conversely, effector caspases (caspases-3, -6, -7, -14) have short pro-domains, lacking protein interaction motifs, and are dependent upon upstream initiator caspases for processing.[2,8] Therefore, caspase activation occurs in a hierarchical manner with initiator caspase activation both preceding and facilitating downstream effector caspase activation.

Apoptotic cell death is characterized by a specific morphology, which includes blebbing of the plasma membrane, nuclear condensation, and fragmentation.[9] This characteristic morphology is a consequence of effector caspase-mediated cleavage of numerous cellular substrates, the precise details of which remain obscure. To date, over 400 effector caspase substrates have been identified.[10] However, the cleavage of only a small subset of these substrates has been definitively linked to specific features of apoptosis. The serine/threonine kinase rho-associated kinase I (ROCK I), structural proteins vimentin, Gas2, and plectin, and the nuclear protein ICAD have all been linked to the morphological changes associated with apoptosis. For example, caspase-3-mediated cleavage of the inhibitor of caspase-activated DNase (ICAD), breaks the inhibitory association of ICAD with caspase-activated DNase (CAD), allowing CAD to initiate DNA fragmentation.[11] Targeting of cytoskeletal proteins vimentin,[12,13] Gas2,[14] and plectin[15] by caspases contributes to changes in cell shape, while proteolysis of ROCK I has been associated with nuclear fragmentation and plasma membrane blebbing.[16,17]

1.3 CASPASE ACTIVATION PATHWAYS

Caspase activation pathways have been the focus of intense research over the past 10 years. Presently, the most studied and accepted pathways leading to caspase activation are the mitochondrial pathway, the death receptor pathway, and the granzyme B–initiated pathway. Other, less well-defined caspase activation pathways, such as the inflammasome and endoplasmic reticulum stress-induced caspase activation pathways, have also been described.

1.3.1 The Intrinsic Pathway to Caspase Activation

Early studies examining cell death initiated by cell damage, such as cytotoxic drugs or in ionizing radiation, found that overexpression of Bcl-2, a protein localized to mitochondria, blocked cell death.[18–20] These observations suggested the mitochondria, in addition to acting as the "powerhouse" of the cell, may be involved in cell death signaling.

Bcl-2 is the founding member of a large family of proteins important in the regulation of cellular life and death decisions.[21] Over the past years our knowledge of this protein family has expanded dramatically, and we now know that the Bcl-2 family is comprised of twenty-two members, some of which promote apoptosis, while others suppress this form of cell death. Although functionally distinct, each member of this family possesses at least one Bcl-2 homology (BH) domain. Pro-survival members of this family, Bcl-2, Bcl-xL, Bcl-w, Bcl-b, Mcl-1, and A1, contain three or four BH domains, while pro-apoptotic members contain between one and three BH domains. The pro-apoptotic members of the Bcl-2 family can be divided into two distinct groups, those that contain Bcl-2 homology (BH) domains 1–3 (Bax, Bak, and Bok) and those containing only a single BH-3 domain, referred to as BH3-only proteins (Noxa, PUMA, Bad, Bim, Bid, Bmf, HRK, Bik, and BLK).[22]

Intense research over the past 10 years, investigating the mechanisms by which Bcl-2 proteins regulate cell death decisions, has revealed a complex network of interactions between family members in which the ratio of pro- to anti-apoptotic Bcl-2 family members controls the release of cytochrome *c* from mitochondria. Pro-apoptotic members Bax and Bak have essential functions in regulating cytochrome *c* release. Normally, Bax is localized to the cytoplasm where it is maintained in an inactive conformation, possibly via interactions with pro-survival proteins Bcl-2, Bcl-xL, and Mcl-1.[23,24] Similarily, Bak, an integral membrane protein localized to the outer mitochondrial membrane, is restrained through binding to anti-apoptotic Bcl-2 proteins. Following receipt of pro-apoptotic signals, levels of active BH3-only proteins increase by a mixture of transcriptional upregulation (PUMA, Noxa) and posttranslation modification (Bim, Bad, Bid), depending upon the initiating stimulus.[25] Activation of the BH3-only cohort of proteins shifts the balance in favor of apoptosis by relieving the inhibition placed upon Bak and Bax. As a consequence, Bax and Bak undergo conformational changes that permit oligomerization of these proteins within the mitochondrial outer membrane and release of intermembrane space proteins, the most important of which is cytochrome *c*.

BH3-mediated repression of pro-survival Bcl-2 family members was thought, until recently, to be a relatively nonselective process. The use of peptides mimicking

the α-helical BH3 domain permitted studies examining interactions between BH3-only proteins and other members of this family and found that the BH3-only subfamily can be divided into direct activators and de-repressors. Direct activators, such as Bid and Bim, have the ability to directly target and activate Bax and Bak.[26] Other BH3-only proteins, such as Bad, Bik, and PUMA, while not directly activating Bax or Bak, do so indirectly by neutralizing pro-survival Bcl-2 proteins. Furthermore, there is significant selectivity among the interaction of de-repessors with pro-survival Bcl-2 proteins. For instance, Bad has been demonstrated to interact with Bcl-2 and Bcl-xL, but not Mcl-1, while Noxa interacts with Mcl-1 but not with Bcl-2 or Bcl-xL.[27]

Ultimately, the balance of pro- and anti-apoptotic Bcl-2 family proteins controls permeabilization of the outer mitochondrial membrane and release of intermembrane space proteins. Cytochrome *c* resides in the mitochondrial intermembrane space and is released in response to diverse stress signals. *In vitro* systems, artificially reconstituting the intrinsic pathway of caspase activation, identified three apoptotic protease activating factors (Apafs) required for caspase activation. Further analysis identified these as Apaf-1, a homologue of *Caenorhabditis elegans* CED-4, caspase-9, and cytochrome *c*.[28–30]

1.3.1.1 Apoptosome Formation

Apaf-1 is comprised of an N-terminal CARD motif, a nucleotide binding and oligomerization domain, and thirteen WD40 repeats. Upon release from the mitochondrial intermembrane space, cytochrome *c* binds to the WD40 repeats on Apaf-1 initiating a conformational change and unmasking the CARD motif. Pro-caspase-9, like Apaf-1, contains a CARD motif within its pro-domain permitting association with Apaf-1.[31,32] ATP binding to the Apaf-1/pro-caspase-9/cytochrome *c* complex triggers further conformational changes culminating in the formation of a heptameric wheel-shaped complex called the apoptosome. The CARD domains of Apaf-1 and pro-caspase-9 are located at the center of the complex, while the WD40 domains form the "spokes" of the wheel.[33] Pro-caspase-9 undergoes autoprocessing within the apoptosome from which it triggers downstream caspase processing (Figure 1.2).

Elegant analysis of cytochrome *c*-initiated caspase activation cascades established that all intrinsic caspase activation is dependent on caspase-9.[34] Following activation within the apoptosome caspase-9 targets and simultaneously processes pro-caspases-3 and -7.[34,35] Caspase-3, in turn, cleaves pro-caspases-2 and -6, followed by caspase-6-mediated processing of caspases-8 and -10.[34] Pro-caspase-9 is also cleaved by caspase-3 generating a positive feedback loop between the initiator and effector caspase[34] (Figure 1.2). Knockout mouse studies have confirmed the importance of caspase-9 and Apaf-1 in the intrinsic pathway to caspase activation. Cells derived from *CASP-9* null animals demonstrated resistance to internal stress agents, such as cytotoxic drugs and radiation.[36,37] A similar resistance to apoptotic stimuli was also evident in *APAF-1* knockout animals, reinforcing the importance of apoptosome formation to intrinsic caspase activation.[38]

Apart from cytochrome *c*, other mitochondrial matrix proteins, including Smac/DIABLO, OMI/Htra2, and endonuclease G, are released from the mitochondrial intermembrane space as a result of outer membrane permeabilization during apoptosis.[39] Endonuclease G translocates to the nucleus and may contribute to oligonucleosomal

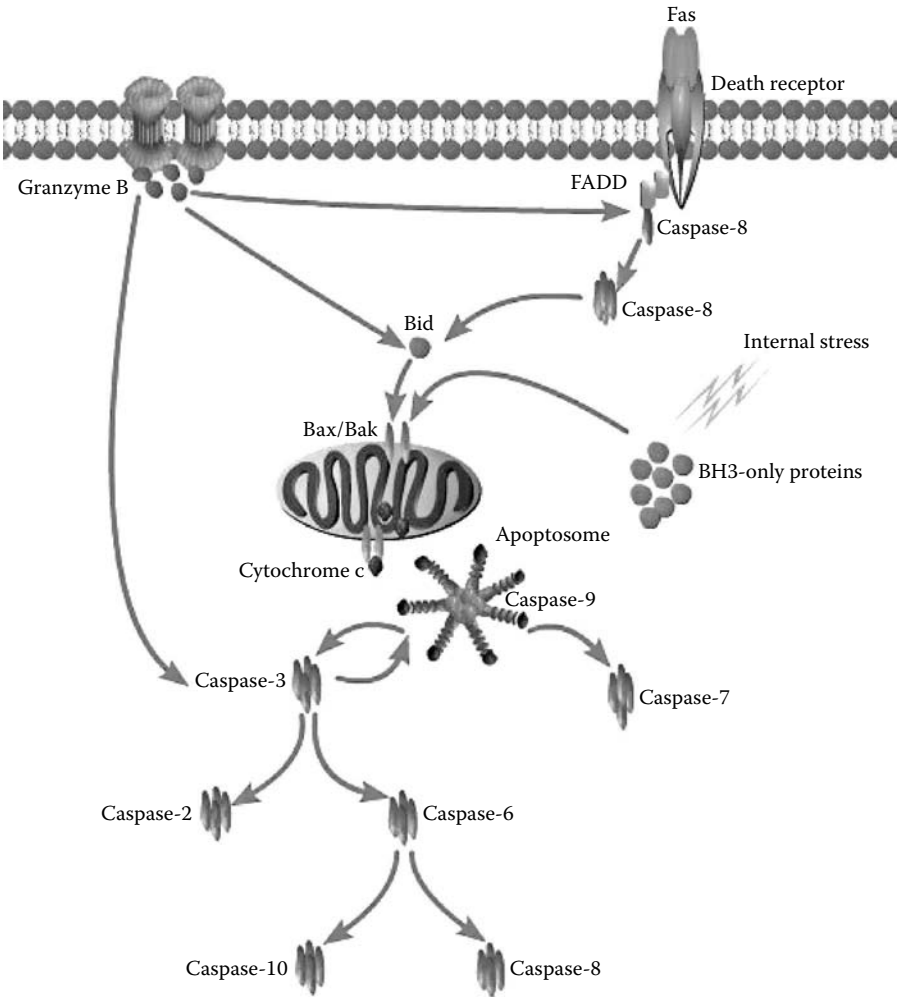

FIGURE 1.2 (See color insert.) The major routes to apoptosis-associated caspase activation. See main text for further details.

DNA fragmentation.[40] Both Omi/Htra2 and Smac/Diablo interact with inhibitor of apoptosis proteins (IAPs). Such interactions are thought to dissociate IAPs from effector caspases, effectively freeing these caspases for activation by upstream initiator caspases such as caspase-9.[41–43]

1.3.2 Extrinsic or Death Receptor–Initiated Caspase Activation Pathways

Engagement of death receptors, present on the cell surface, can also induce apoptotic cell death. For example, activated cytotoxic T cells recognize virally infected cells, leading to caspase activation and cell death via death receptors present on the target cell surface, such as tumor necrosis factor alpha (TNFα) or Fas/CD95.[44]

Such cytotoxic T cells can also kill via the granule-dependent granzyme B pathway, as described later. Structurally, death receptors are comprised of an extracellular cysteine-rich domain (ligand binding region) and an intracellular region containing a death domain (DD). Ligand binding to death receptors initiates receptor trimerization and recruitment of adapter proteins, such as FADD and TRADD, forming the death-inducing signaling complex (DISC).[45] Similar to the apoptosome, the DISC functions as a caspase activation platform and recruits, via death effector domain (DED) interactions, multiple pro-caspase-8 or pro-caspase-10 molecules, leading to their activation.[46] Active caspase-8 transmits and propagates the apoptotic signal by cleaving and activating pro-caspase-3, which in turn cleaves pro-caspases-6 and -2 (Figure 1.2).

Two distinct pathways of Fas-mediated apoptosis have been identified, leading to the classification of cells as either type-I or -II.[47] Type-I cells generate sufficient quantities of active caspase-8 at the DISC leading to pro-caspase-3 activation and cellular demolition. In contrast, type-II cells fail to produce sufficient active caspase-8 at the DISC (for reasons that remain obscure) and require cross talk with the mitochondrial pathway to trigger downstream caspase cascades. In type-II cells, DISC-activated caspase-8 cleaves the BH3-only protein, Bid, generating truncated Bid (tBid). The amino terminus of tBid is subsequently myristolated, targeting it to mitochondria where it induces cytochrome *c* release via BAX or BAK.[48–51] Death in type-II cells is thus routed through the apoptosome pathway.

Both type-I and type-II Fas-induced apoptosis can be blocked by the cellular-FADD-like inhibitory protein (c-FLIP).[52] Like caspase-8, c-FLIP contains DEDs, allowing it to compete with the latter for recruitment to the DISC. However, c-FLIP lacks protease activity and its incorporation within the DISC inhibits progression of the signaling cascade.[53]

1.4 GRANZYME B-MEDIATED CASPASE ACTIVATION

Virally infected and transformed cells are removed from the body through natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). Initial secretion of TNFα and IFN_9 by NK cells limits viral replication and spread. CTLs form the second wave of attack by seeking out and specifically targeting virally infected cells. Engagement of virally infected cells by a CTL results in the initiation of apoptosis by delivery of cytotoxic granules.[54] Perforin, a pore-forming protein, present in cytotoxic granules generates pores in the target cell that facilitate delivery of cytotoxic granzymes into the target (Figure 1.2). The precise mechanism by which perforin mediates delivery of granule components has not been resolved. However, the importance of perforin to this process is clearly illustrated by perforin-deficient mice, which display impaired clearance of viral pathogens.[55]

Granzyme B, a serine protease, is a key component of cytotoxic granules. Artificial systems have demonstrated that addition of perforin and granzyme B alone are sufficient for the induction of apoptosis.[56] Granzyme B, similar to the caspases, cleaves its substrates after aspartate residues,[57,58] suggesting that this protease has the ability to directly activate members of the caspase family. Indeed, caspase-3 was the first substrate identified for granzyme B.[59,60] Experiments utilizing Jurkat

cell-free extracts demonstrated that addition of granzyme B resulted in processing of pro-caspase-3 and multiple caspase substrates.[59] The cohort and activation cascade of caspases activated in the granzyme B–mediated pathway has recently been elucidated.[61] Caspases-3, -7, -8, and -10 are directly processed by granzyme B, whereas caspases-2, -6, and -9 are processed in a second, caspase-3-dependent wave of processing.[61] Because granzyme B directly cleaves and activates caspases, addition of caspase-specific inhibitors might be expected to inhibit granzyme B–mediated cell death. However, although preincubation of Jurkat cells with caspase-3-like specific inhibitor DEVD-fmk or the broad range caspase inhibitor zVAD-fmk reduced DNA fragmentation, in response to granzyme B and perforin, no long-term protection was evident, indicating granzyme B has other noncaspase targets.[62] This is not entirely surprising when we consider that many viruses encode caspase inhibitors, such as Crm A or p35, as a means to aid their survival and replication in host cells. Therefore, by incorporating a caspase-independent route to cell death, granzyme B has the ability to overcome these viral defenses.

Subsequent studies demonstrated that overexpression of Bcl-2 clonogenically protected cells against granzyme B and perforin-mediated apoptosis.[62–64] Protection by Bcl-2 implied that granzyme B may also act upstream of mitochondria. Indeed, the BH3-only Bcl-2 family member Bid was subsequently identified as a target of granzyme B.[65] Cleavage of Bid by granzyme B generates a truncated fragment that translocates to mitochondria, initiating release of intermembrane space proteins such as cytochrome *c* (Figure 1.2). Both granzyme B and caspase-8 target and cleave Bid, although at distinct sites. Overexpression studies with a mutant, granzyme B–resistant Bid (D75E) demonstrated inhibition of granzyme B and perforin-induced apoptotic features, while overexpression of D59E Bid (mutated at the caspase-8 site) failed to abolish the apoptotic phenotype.[66] The latter result illustrates that granzyme B directly cleaves Bid rather than doing so indirectly by caspase-8-mediated cleavage.

Several studies now indicate that Bid, rather than caspases, is the preferential substrate for human granzyme B, with Bid cleavage being evident within minutes of granzyme B entry to the target cell. Upon entry into the target, granzyme B rapidly induces mitochondrial permeabilization, via Bid, and release of intermembrane space proteins leading to apoptosome assembly and caspase activation (Figure 1.2). Concurrently, granzyme B can also directly target and cleave caspases, thereby amplifying the level of caspase activation.

1.5 EMERGING CASPASE ACTIVATION PATHWAYS

The endoplasmic reticulum and inflammatory-mediated pathways of caspase activation are relatively poorly understood, compared to the death receptor (extrinsic) and mitochondrial-mediated (intrinsic) pathways. Our current knowledge of each pathway and the mechanisms utilized to achieve caspase activation is outlined below.

1.5.1 Endoplasmic Reticulum Stress-Induced Caspase Activation

The endoplasmic reticulum (ER) is responsible for the synthesis, folding, and maturation of proteins within the cell. Stresses negatively regulating energy availability or

intracellular calcium levels, such as ischemia, can have a detrimental affect on protein folding, leading to the accumulation of unfolded proteins, a condition referred to as ER stress. Cells have evolved response mechanisms aimed at reducing levels of unfolded proteins and restoring cellular homeostasis. However, in certain situations, these survival mechanisms are insufficient and cell death ensues.[67] Unlike the death receptor or mitochondrial-mediated pathways the cohort of caspases and their activation mechanisms have not been firmly established in ER stress-induced apoptosis and are still a matter of debate. Processing of pro-caspases-12, -3, -6, -7, -8, and -9 in response to ER stress has been reported.[68–70] However, as yet, the order in which these caspases are activated is unknown, as is the apical caspase in this context.

Early work proposed caspase-12 as the initiator caspase in ER stress-induced apoptosis.[68] Mouse embryonic fibroblasts from caspase-12-deficient animals displayed partial resistance to the ER stress-inducing agents, brefeldin A, and tunicamycin.[68] However, recent studies using mouse caspase-12 knockout cells, from a different source, have cast doubt upon this claim. Saleh and colleagues reported that caspase-12, rather than being implicated in apoptosis, functions in pro-inflammatory responses as a negative regulator of IL-1β processing.[71] To date, no substrates for mouse caspase-12, aside from caspase-12 itself, have been reported. Indeed, the importance of caspase-12 for ER stress-induced apoptosis has been further undermined by the discovery that the majority of humans lack full-length caspase-12. A frameshift mutation, producing a premature stop codon, is present in most humans, resulting in the production of a short CARD-only protein.[72] Certain individuals of African descent lack this mutation and are able to produce full-length and presumably active caspase-12.[73] Studies examining the outcome of caspase-12 expression in this subset of the population determined that expression of full-length caspase-12 correlated with increased susceptibility to severe sepsis.[73] Collectively, these data argue that caspase-12 processing does not trigger ER stress-induced caspase activation but is involved as a negative regulator of inflammatory responses.

Additional data indicate that ER stress-induced apoptosis is dependent upon mitochondrial-mediated processes to promote caspase activation. Mitochondrial translocation of Bax and cytochrome *c* release has been observed in cells treated with tunicamycin or thapsigargin. Moreover, studies using cells devoid of a functional mitochondrial pathway (*BAX/BAK* null or *APAF-1* null mouse embryonic fibroblasts), or overexpressing anti-apoptotic Bcl-2, fail to activate caspases in response to ER stress signals.[74,75] These observations indicate that ER stress-induced caspase activation is dependent upon the intrinsic or mitochondrial pathway. As yet, the signaling pathways employed by the ER to trigger cytochrome *c* release have not been delineated but most likely occur by regulation of Bcl-2 family members.

1.5.2 Inflammatory-Mediated Caspase Activation

As previously described, caspases can be subdivided based upon their sequence identity and chromosome location. Based upon these criteria, caspases-1, -4, -5, -11, and -12 form the inflammatory branch of the caspase family. Caspase-1 is the most intensively studied member of this group and is the protease responsible for the cleavage and maturation of IL-1β, permitting its secretion from monocytes and macrophages

in response to pathogens and other pro-inflammatory stimuli.[1] Caspase-1 activity is also required for the maturation of IL-18, a cytokine involved in IFNγ secretion, and more recently this caspase has also been implicated in the cleavage of IL-33, a cytokine involved in T helper cell type 2 (Th2) polarization.[76–78] Although early studies identified caspase-1 as the first homologue of *Caenorhabditis elegans* CED-3, suggesting it played a role in cell death, the generation of *CASP-1* null mice failed to support this observation and suggested an inflammatory rather than a cell death role for this protease.[79–81]

Other members of the inflammatory caspase subfamily include caspases-4, -5, -11, and -12. *CASP-11* null mice, similar to *CASP-1* null mice, do not process IL-1β and, as a direct consequence of this, are resistant to lipopolysaccharide (LPS)-induced endotoxic shock.[82] Unlike caspase-1, caspase-11 expression is inducible by inflammatory activators, such as LPS, via NFkB and STAT-1 signaling.[83] No direct homologue of murine caspase-11 has been identified in humans. However, human caspases-4 and -5 share a high degree of homology with murine caspase-11 and are thought to have arisen from a gene duplication of mouse caspase-11.[84] Like caspase-11, caspase-5 is inducible by LPS and has been implicated in IL-1β processing.[84] At present, little data concerning caspase-4 are available. It has been suggested to function as the human equivalent of mouse caspase-12[85] and has been implicated in ER stress-induced apoptosis, but as yet no convincing data are available to support this hypothesis.

Although caspase-1 was the founding member of the caspase family, relatively little is known about its activation mechanisms and substrates compared to other members of the caspase family. Structurally, owing to the presence of a long pro-domain containing a CARD motif, caspase-1 is a member of the initiator caspase family alongside caspases-8 and -9. Therefore, it is likely that caspase-1 requries a scaffold protein or complex to facilitate its activation. Unlike caspases-8 and -9, the activation platform for caspase-1 (dubbed the inflammasome) has not been fully resolved. Indeed, it is only in the past few years that a model for caspase-1 activation has been proposed.

1.5.2.1 Inflammatory Caspase Activation Pathways

The Nod-like receptor (NLR) family of cytoplasmic adaptor proteins has been implicated in the processing of inflammatory caspases. Members of this family are composed of an N-terminal pyrin, CARD or BIR (baculovirus inhibitor of apoptosis repeat) domain, a central NAIP, CIITA, HET-E TP-1 (NACHT) domain, and C-terminal leucine-rich repeats (LRRs). The N-terminal domain is believed to be important for protein-protein interactions, while the C-terminal LRRs are essential for pathogen detection. The central NACHT region, which is related to the NB-ARC domain of Apaf-1, is thought to induce oligomerization following LRR stimulation. Similar to Apaf-1, oligomerization of some members of the NLR family has been reported following LRR activation.[86] Furthermore, constitutive activation following removal of the LRRs has been reported, analogous to the constitutive activation observed by removal of the WD40 repeats from Apaf-1.[86] Based upon phylogenetic analyses, NLRs can be divided into four subfamilies, class II transactivator (CIITA),

Nods, ICE protease activating factor (IPAF), and NACHT leucine-rich repeat and pyrin domain-containing proteins (NALPs). Presently, only the NALP and IPAF subfamilies have been implicated in inflammatory caspase activation. The putative activation platforms mediating inflammatory caspase activation are described below. However, it should be emphasized that these activation platforms have been postulated based upon overexpression studies and as yet have not been purified as native complexes.

1.5.2.1.1 The NALP Subfamily

The NACHT-, LLR-, and PYD-containing protein (NALP) subfamily is the largest subfamily within the NLR family, encompassing fourteen members.[87] With the exception of NALP1, all members of the NALP family are composed of an N-terminal pyrin domain and C-terminal LRRs. The C terminus of NALP1 differs from that of all other members because it contains both FIIND and CARD interaction motifs in addition to the LRRs.[87] The NALP family has been implicated in the processing and activation of inflammatory caspases via formation of activation platforms referred to as inflammasomes (Figure 1.3). Although each member of the NALP family could potentially form inflammasomes, to date, the NALP3 and NALP1 inflammasomes are the best characterized.

1.5.2.1.2 NALP3 Inflammasomes

NALP3 is the most well studied of all the proposed inflammasome platforms and has been implicated in caspase-1 activation stimulated by ATP, monosodium sodium urate, and the bacteria *Listeria monocytogenes*.[88–90] NALP3 lacks a CARD motif and therefore is unable to directly bind and recruit caspases. To enable caspase recruitment, an adapter molecule, apoptosis-associated Speck-like protein containing a CARD (ASC), is recruited to NALP3.[91] ASC is a bipartite protein comprised of both a pyrin motif facilitating binding to NALP1 and a CARD region, enabling recruitment of caspase-1 to the complex. A second caspase-1 molecule may be recruited by the inclusion of a second adapter molecule, CARDINAL, within the complex (Figure 1.3). Structurally, CARDINAL resembles the FIIND and CARD interaction motifs, which are present on the C terminus of NALP1 but missing in all other NALP family members.[92–94] Recruitment of CARDINAL to the NALP3 inflammasome can recruit a second caspase-1 molecule to the activation platform via CARD-CARD interactions.[94,95] Assembly of the NALP3 inflammasome is thought to promote activation of caspase-1. The importance of NALP3 and the adapter molecule, ASC, in caspase-1 activation is clearly illustrated in cells lacking either molecule. Cells deficient in NALP3 or ASC display impaired activation of caspase-1 and release of cytokines in response to LPS.[96] Consequently, *ASC* null mice are resistant to endotoxic shock induced by LPS injection.[91] Conversely, individuals suffering from Muckle-Wells syndrome, an autoinflammatory disorder, express a mutated and constitutively active NALP3 resulting in unrestrained caspase-1 activation and cytokine release.[97]

1.5.2.1.3 NALP1 Inflammasomes

By virtue of its C-terminal CARD motif, NALP1 can directly recruit caspases (Figure 1.3). Studies have suggested that caspase-5 is recruited to the CARD region

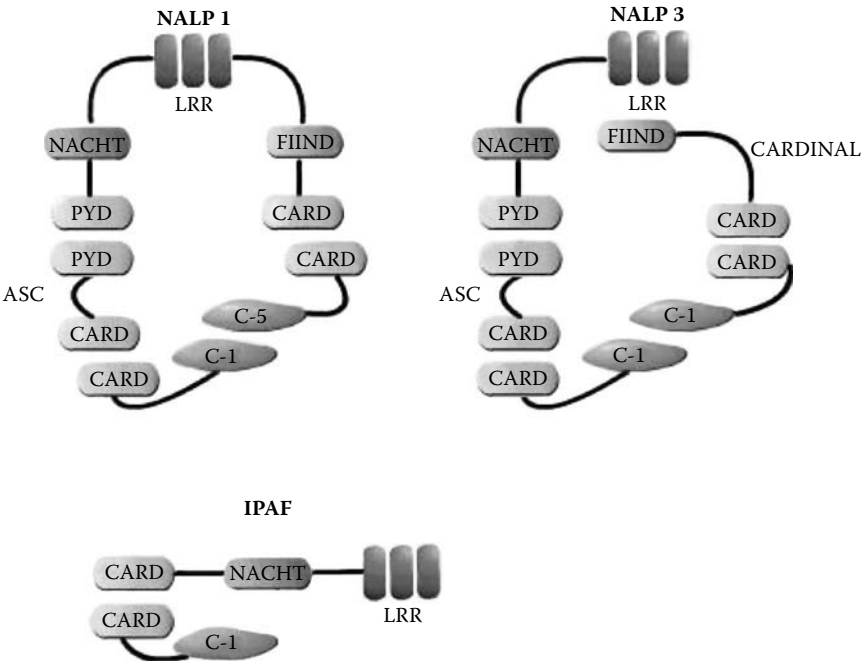

FIGURE 1.3 (See color insert.) Proposed composition of the NALP1, NALP3, and IPAF inflammasomes. See main text for further details.

of NALP1, while binding of caspase-1 to the complex is dependent upon the adapter molecule ASC[95] (Figure 1.3). NALP1 inflammasome formation therefore results in the activation of both caspases-1 and -5. The ligands capable of specifically triggering assembly of the NALP1 inflammasome have not been extensively determined. However, anthrax toxin has been reported to trigger assembly of NALP1 inflammasomes and inflammatory caspase activation.[98]

1.5.2.2 IPAF Subfamily

Members of the IPAF subfamily are composed of an N-terminal CARD, a central NACHT domain, and C-terminal LRRs. Caspase-1 activation, via assembly of the IPAF inflammasome, occurs by direct recruitment to IPAF.[86] Unlike the NALP1 or NALP3 models of caspase-1 activation, IPAF does not require adapter molecules such as ASC or CARDINAL, but rather, directly recruits caspase-1 via the N-terminal CARD domain. The C-terminal LRR in IPAF has autoactivation properties, as loss of the LRRs results in constitutive activation of IPAF.[86] Currently, flagellin is the only reported activator of IPAF-dependent caspase-1 processing.[99,100] Neuronal apoptosis inhibitor protein (NIAP), due to its high-sequence homology with the NACHT and LRR regions of IPAF, has been classified as an IPAF subfamily member.[87] Unlike IPAF, NIAP possesses a baculovirus inhibitor of apoptosis repeats (BIR), a motif associated with caspase inhibitors such as XIAP, at the N terminus rather than a

CARD domain. It has been suggested that NIAP and IPAF may interact within the same caspase-1 activation platform,[101] but the precise mechanism of interaction or the functional consequence of this interaction have not been determined.

1.6 CONCLUSIONS

Apoptotic cell death is required for the removal of damaged, infected, or aged cells from the body. Unlike other forms of cell death, such as necrosis, apoptosis is a highly ordered and regulated process characterized by the activation of caspases-2, -3, -6, -7, -8, -9, and -10. The caspase family consists of initiator and effector caspases. Initiator caspases are activated by scaffold molecules such as the DISC, the apoptosome, or the recently described inflammasome. Upon activation, initiator caspases proteolytically process the effector caspases, resulting in their activation. Effector caspases process hundreds of cellular substrates, some of which contribute to the classical morphology associated with apoptosis. Over the past 10 years, a huge amount of work has deciphered the major caspase activation pathways and has resulted in the defining of the apoptosome, death receptor, and granzyme B–mediated pathways to caspase activation. Deciphering less well-established pathways, such as inflammatory caspase activation mechanisms, is the challenge that currently preoccupies workers in this fast-moving field.

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2 Role of Caspases in Inflammation-Driven Diseases

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2.1 INTRODUCTION

Caspases constitute an evolutionarily conserved family of cysteinyl-dependent proteases that fulfill essential roles in apoptosis, inflammation, cell survival, proliferation, and differentiation, and act by aspartate-specific cleavage of a wide number of cellular substrates.[1,2] Ten murine (caspase-1, -2, -3, -6, -7, -8, -9, -11, -12, -14) and eleven human (caspase-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -14) catalytically active caspases have been reported so far.[3] They are synthesized as inactive proenzymes that

include an N-terminal prodomain of variable length, followed by two domains with conserved sequences: a large subunit (~20 kDa, p20) and a small C-terminal subunit (~10 kDa, p10). Caspases can be subdivided according to the length of the prodomain into two main categories. Large-prodomain caspases contain an N-terminal homotypic protein-protein interaction motif belonging to the death domain (DD) superfamily, in particular the caspase recruitment and activation domain (CARD; caspase-1, -2, -4, -5, -9, -11, and -12) and the death effector domain (DED, caspase-8 and -10). These domains are used to recruit inactive monomers to multiprotein complexes, each of which is specific for a particular caspase. In these complexes the proenzyme undergoes conformational changes required for its activation.[1,4] The short-prodomain caspases (caspase-3, -6, -7, and -14) contain a prodomain of only a few amino acids and exist as preformed dimers. They acquire enzymatic activity upon proteolytic maturation by large-prodomain caspases or other proteases. Caspases can also be classified according to their functions, which correlate with their phylogeny: the apoptotic initiators, the apoptotic executioners, and the inflammatory caspases. The latter group encompasses caspase-1, -4, -5, -11, and -12; caspase-4 and -5 are most likely the human paralogs of murine caspase-11.[3]

Caspase-1, the prototypic member of the inflammatory caspases, is responsible for the maturation of pro-interleukin (IL)-1β, pro-IL-18, and pro-IL-33, three related cytokines playing critical roles during inflammation.[5–8] Indeed, the requirement for caspase-1 in maturation of pro-IL-1β and pro-IL-18 was confirmed in caspase-1-deficient mice.[9,10] The enzymatic activation of caspase-1, a member of the CARD-containing large-prodomain caspases, occurs in large, multimeric protein platforms (~700 kDa) commonly referred to as inflammasomes.[11] In general, the assembly of these complexes involves the orchestrated recruitment of three functionally distinct building blocks: a sensor, an adaptor, and an effector.[12,13] Inflammasome formation is initiated when a sensor-platform protein belonging to the NOD-like receptor (NLR) family detects the presence of an instigating factor through its leucine-rich repeats (LRRs), an evolutionarily conserved ligand-sensing domain. Distinct NLR-family members sense distinct pathogen-associated molecular patterns (PAMPs) and danger signals known as damage-associated molecular patterns (DAMPs). NALP1b detects *Bacillus anthracis* toxin; NALP3 senses gout-associated uric acid crystals, bacterial RNA, and pathogens, such as *Staphylococcus aureus* and *Listeria monocytogenes*; Ipaf specifically detects the presence of flagellin of intracellular bacteria, such as *Salmonella typhimurium* and *Legionella pneumophilia*.[14–21] Direct or indirect binding of a ligand to the LRR induces conformational changes that expose the NACHT domain and thereby trigger oligomerization of the NLR proteins and consequent inflammasome assembly.[22] NLR proteins also contain at their N-termini a homotypic interaction motif belonging to the DD superfamily, either a CARD or a PYRIN. These motifs mediate the recruitment of procaspase-1 in one of two ways: (1) CARD-containing NLR members directly recruit caspase-1 through a CARD-CARD interaction, or (2) PYRIN-containing NLR members recruit caspase-1 via the adaptor protein ASC/PYCARD, which contains both a CARD and a PYRIN domain (Figure 2.1).[23,24] In addition, NALP3 inflammasomes encompass a second adaptor, CARDINAL, which recruits procaspase-1 through its CARD domain.[12] In this way, multiple procaspase-1 molecules are brought close