REVIEW ARTICLE

Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members*

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ABSTRACT. Interleukin-33 (IL-33) is the latest member of the IL-1 family that has become very attractive since the discovery of its major target cells, the innate lymphoid cells type 2 (ILC2), involved in the initiation of the type 2 immune response (secretion of IL-5 and IL-13) during parasitic infection and allergic diseases such as asthma. IL-33 is a chromatin-associated protein as it possesses in its N-terminus, a chromatin-binding domain, and is constitutively expressed in the nuclei of endothelial cells and in epithelial cells of tissues exposed to the environment. It is however, essential for IL-33 to be extracellularly released to bind to its receptor ST2 through the C-terminus portion of the protein in order to induce the inflammatory and type 2 responses. Like other IL-1 family members, IL-33 does not possess any signal peptide and may be released through unconventional secretory mechanisms or following cell damage and necrosis. It was initially believed that IL-33, like IL-1β and IL-18, requires processing by caspase-1 to be released, and for biological activity. On the contrary, full length IL-33 is biologically active, and processing by caspases results rather in IL-33 inactivation. Moreover, it has been recently shown that the bioactivity of IL-33 can be increased by inflammatory proteases secreted in the microenvironment, similarly to IL-1α, IL-1β and IL-18. This review will summarize recent progress on how IL-33 is released and processed compared with the other IL-1 family members, and how the immune cells recruited to the site of injury can regulate the disease-associated function of IL-33.

Key words: IL-33, IL-1 family, inflammatory proteases, caspase-1, neutrophil serine proteases

Interleukin (IL)-1 family is composed of 11 members including IL-1α, IL-1β, IL-18 and IL-33. They play a central role in the activation of inflammation and in the first steps of innate immunity against pathogens or tissue damage [1]. They share the same three-dimensional structure characterised by 12 strands organised in a β trefoil [2, 3], and activate the same family of receptors. They also induce the same signalling cascade in their target cells, involving MAPK or NF-κB pathways, and the final transcription of inflammatory genes. And yet, they each have their own unique function probably because they are expressed by different cell types, either constitutively or after being induced, and because they stimulate different target cells through their own receptors [1]. IL-33 is the latest member of the family, discovered initially as a nuclear factor present in endothelial cells [4], and first named NF-HEV (nuclear factor from high endothelial venules). Two years later, IL-33 has been rediscovered as the extracellular ligand for the orphan IL-1 receptor family member ST2, and named IL-33 [5]. The N-terminal region ($IL\text{-}33_{1\text{-}65}$) is necessary and sufficient for IL-33 to be expressed in the nucleus and is associated with chromatin [6, 7]. A role for nuclear IL-33 has been suggested in chromatin compaction and transcription regulation [6, 7]. However, no target genes have yet been identified that are regulated by intracellular IL-33. Moreover, the IL-33-KO mice phenotype looks like ST2 KO, supporting a predominant, extracellular role through ST2. IL-33 might be stored in the nucleus in order to be protected from cleavage and inappropriate release, but is ready to be secreted upon necrosis or damage. Once in the extracellular space in their full-length form IL-33 (and IL-1α), can activate their receptors, and have therefore been proposed to be endogenous danger signals or alarmins [8, 9], such as HMGB1 [10], in charge of alerting the immune system where there is tissue injury. Binding of IL-33 to ST2 occurs through the C-terminal part (the IL-1-like domain), and is decisive in initiating a type 2 innate immune response during infectious, inflammatory or allergic diseases [11, 12]. The role of the different IL-1 family members has already been well described [13]. Recent findings have been obtained that contribute to the understanding of IL-33 function, including the discovery of major target cells of IL-33, the innate lymphoid cells type 2 (ILC2) found in mouse [14-16] and human [17, 18]. These non-T and non-B lymphoid cells, localized in mucosal tissues (lung, intestine), adipose tissue and lymphoid organs (spleen, lymph node), express high levels of ST2, and

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secrete significant quantities of type 2 cytokines, IL-5 and IL-13, and pro-inflammatory IL-6 [19] when stimulated with IL-33. Activation of these cells is essential in the initiation of a type 2 response against worm infection [16], or during an allergic response such as asthma [19, 20]. In addition to ILC2, IL-33 can potently activate not only mast cells, but also other immune cells, such as granulocytes, macrophages, NK andTh2 cells [21]. Analyses of IL-33-knockout mice revealed that IL-33 plays key roles in innate immunity, in particular in the initiation and amplification of a type 2 response, independently of antigen recognition [22, 23].

A striking characteristic of the IL-1 family members is the lack of a signal peptide that addresses the proteins to the endoplasmic reticulum and Golgi pathway. Accordingly, they are released through unconventional secretory mechanisms. This peculiarity will be discussed in the first paragraph. It has also been shown that the IL-1 domain, in the C-terminal part of the protein, is necessary to activate the IL-1 family receptor. However, some differences between the members of the IL-1 family exist in the requirement or not of processing of the protein to confer activity to the cytokine. If IL-1β and IL-18 need processing, IL-1α and IL-33 are active in their full-length form. The mechanisms involved in getting rid of the N-terminal portion also vary between these cytokines, in particular the role played by the inflammasome, and the caspase-1 that cleaves and activates IL-1β and IL-18, but inactivates IL-33, while having no enzymatic effect on IL-1α. Proteases released by different inflammatory cells are also involved in regulating cytokines from the IL-1 family. This important issue has also been recently addressed for IL-33, giving new insight into the biological function of IL-33. In this review, we will focus on particular mechanisms of secretion and activation of IL-1 family members, emphasizing recent advances that have contributed to our understanding of IL-33.

EXPRESSION AND RELEASE OF IL-33: A NEW DANGER SIGNAL

Unlike IL-1β and IL-18, IL-33 and IL-1α are not mainly expressed by hematopoietic cells. Human, full-length IL-33 protein is found in endothelial cells all along the vascular tree, in epithelial cells in tissue exposed to the environment (lung and respiratory tract, stomach and digestive tract, vagina, skin,. . .), in fibroblastic cells in lymphoid organs (spleen , lymph node), and in the brain [24]. IL-33 expression is similar in mouse tissues (pneumocytes in the lung, epithelial cells in the vagina, fibroblastic reticular cells and activated fibroblasts in lymphoid organs, in the brain), with the exception of the endothelium as the protein is, unexpectedly, not constitutively expressed in endothelial cells [25]. In contrast to IL-1β and IL-18 that reside in the cytosolic compartment and are expressed upon pathogenic stimulation, IL-33 and IL-1α are, surprisingly for cytokines, constitutively expressed and stored in the nucleus of the cells. So, they are already translated when cells undergo necrosis and can be immediately released into the extracellular space after cell damage [8, 9] (*figure 1*). Accordingly, the full length IL-33 is found in the supernatant of cells following physical injury when the plasma membrane is broken and when cell integrity is lost. This has been shown in human endothelial cells [8], LPS-activated macrophages [26], and inflammatory cytokine-activated fibroblastic synoviocytes [27] treated with detergent or that have been physically damaged. The release of IL-33 during necrosis has also been demonstrated during parasitic infection [28]. *In vitro*, co-culture of epithelial cells with a gastrointestinal nematode leads to necrosis and to the release of full length IL-33 in the extracellular medium. The study also shows that nematode extracts are not sufficient to induce necrosis and IL-33 release, and that parasite motility is necessary. This suggests that intestinal damage and tunnels formed by nematodes could induce the release of full-length IL-33. Extracellular IL-33 has been detected in human blood and synovial fluids [27], and in mouse peritoneal and bronchoalveolar lavage fluid [29], in pathological conditions where cells have been damaged (during rheumatoid arthritis or infection with influenza virus respectively).

IL-1β and IL-18 do not possess a classical signal sequence that would allow them to be secreted via the endoplasmic reticulum and Golgi pathway. As they also need to be released in order to bind to their receptor, they are secreted by unconventional mechanisms. These mechanisms involve cytokine compartmentalization in vesicles. The vesicles might be secretory lysosomes [30], exosomes [31] or exocytosis vesicles [32]. This transit is regulated by purinergic receptor activation by extracellular ATP, essential for IL-1β release in vesicles [31]. ATP is found extracellularly following cell damage, but can also be secreted by activated cells (neurons, T cells, neutrophils). Purinergic receptor activation induces calcium entry into the cytoplasm, a key point in the phospholipase activation needed for vesicle formation and for the formation of the inflammasome and caspase-1 activation. Caspase-1 is involved, on one hand, in IL-1β and IL-18 maturation (this will be discussed further), on the other hand, and independently of this processing role, caspase-1 is involved in the secretion of proteins without signal peptides such as IL-1β [33], IL-1α [34-36], and HMGB1 [37]. Caspase-1 might play a role in secretion, by cleavage of a substrate important in trafficking [33] or by forming complexes with the secreted protein [34]. More recently, autophagy has been demonstrated to be an amplifier of IL-1β and IL-18 secretion, again in an inflammasome-dependent manner [38]. The possibility of an active release of IL-33, without losing cell integrity, is currently being investigated. Indeed, in recent studies, IL-33 has been proposed to be released without the necessity of cell damage and necrosis. Using extracts of *Alternaria*, a common fungus causing allergic respiratory diseases such as asthma, on human bronchial epithelial cells, IL-33 is translocated into the cytoplasm and released as a full-length form into the supernatant without affecting cell viability [39]. IL-33 secretion depends, in this case, on the intracellular calcium concentration regulated by ATP and purinergic receptor stimulation. This ATP-dependent release of IL-33 is supported by detection of IL-33 in the supernatant of corneal epithelial cells [40], astrocytes and glial cells [41] when stimulated with TLR ligands associated with ATP.

The unconventional secretion mechanisms used by IL-1 family members avoid the endoplasmic reticulum and Golgi pathway, and are probably allows them to be more rapidly secreted. Indeed, the secretion of IL-1 family

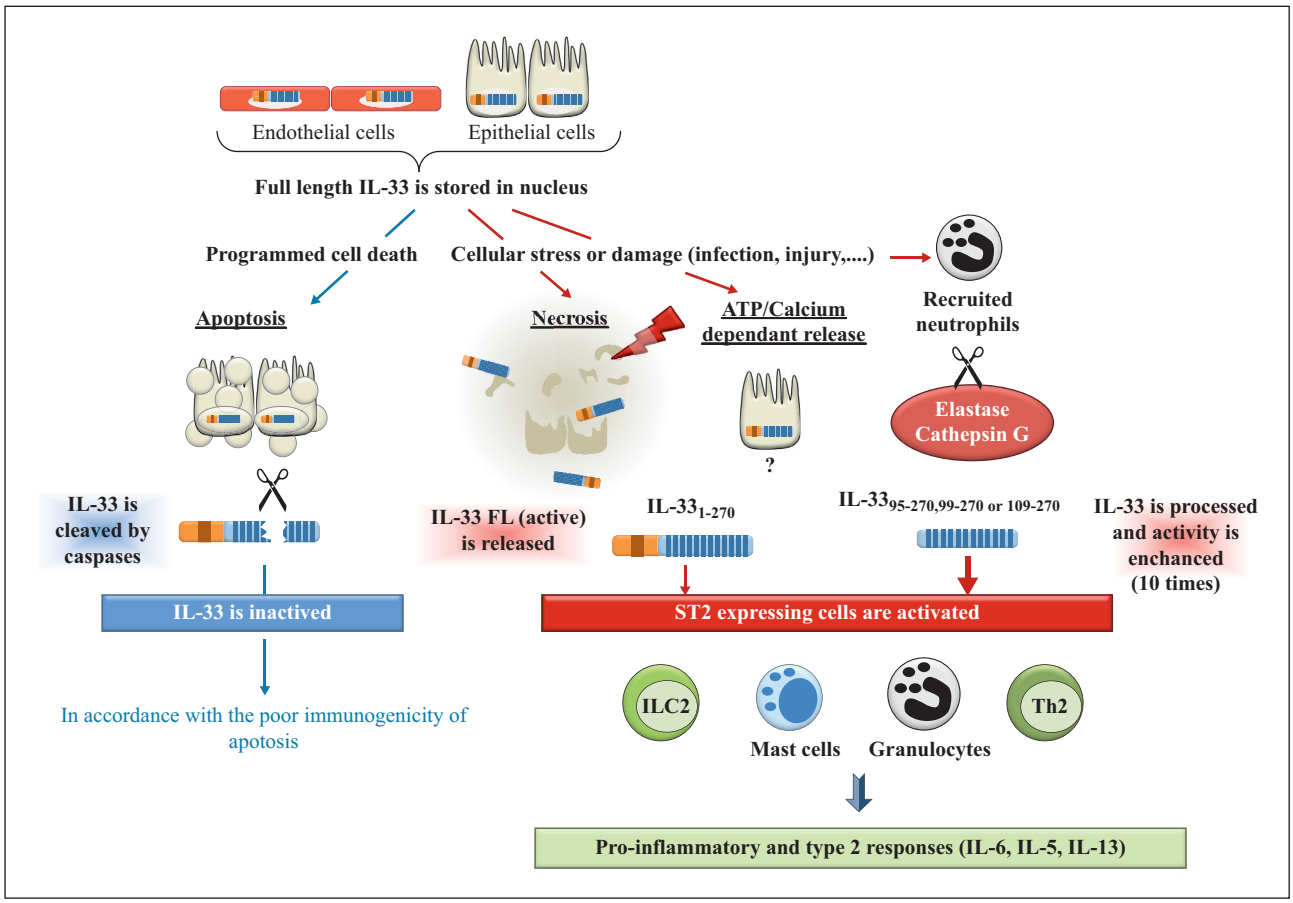

Figure 1

Schematic representation of IL-33 secretion and processing mechanisms.
IL-33, as a danger signal, is constitutively expressed and stored in the nucleus of endothelial cells and mucosal epithelial cells. During a programmed cell death, IL-33 is cleaved and inactivated by caspases, whereas it is released as an active full-length (FL) protein during necrosis. Neutrophils, recruited to the site of injury, secrete serine proteases, cathepsin G and elastase that are able to cleave and amplify IL-33 bioactivity. IL-33 functions, characterized by the initiation of a type 2 and/or inflammatory responses (secretion of IL-5, IL-13 or IL-6), are thus exacerbated.

cytokines is linked with cellular stress (infection, starvation, mechanical or chemical stress, injury,. . .), where quick activation of the immune system is important for a rapid response to the stress. Among IL-1 family members, IL-33 and IL-1α are already expressed and stored in the nucleus of the cell, and are immediately released upon cell damage when membrane integrity is lost, in order to act as an alarmin.

PROCESSING OF IL-33 AND REGULATION OF ITS ACTIVITY

IL-1 family members are synthesized as full length propeptides containing an N-terminal domain upstream of the IL-1 cytokine domain. Whereas IL-1β and IL-18 need to be cleaved to get rid of the amino terminal part and acquire their biological activity, IL-1α [42] and IL-33 [8, 43] are active in their full-length form.

It is well known that IL-1β and IL-18, synthesized as 31 and 24 kDa preforms respectively, can be cleaved in the cytoplasm of monocytes and macrophages by caspase-1 to release the active 17 kDa IL-1β and 18 kDa IL-18 [44, 45]. The generation of active IL-1β and IL-18 depends thus on two stimuli: TLR stimulation to induce preform cytokine synthesis and inflammasome activation to activate caspase-1. The second leading to caspase-1 activation can be a danger signal from a pathogenic (LPS, flagellin, anthrax, RNA, DNA,. . .) or endogenic (ATP, uric acid,. . .) origin [46]. It was thus proposed that similarly, the 32 kDa full-length IL-$33_{1\text{-}270}$ could be cleaved and activated by caspase-1 generating IL-$33_{112\text{-}270}$ [5]. However, the supposed cleavage site, after serine 111, is not a consensus cleavage site for caspase-1, which preferentially cleaves protein after an aspartic acid. In fact, recent findings have demonstrated that caspase-1, and apoptotic caspases 3 and 7, do not activate IL-33 [8, 47, 48]. On the contrary, they cleave the protein inside the IL-1 domain, after aspartic acid D178, resulting in cytokine inactivation during apoptosis. Therefore, the IL-$33_{112\text{-}270}$ form, which was supposed to be generated by caspase-1 and which is actually the commercially available mature form of IL-33, does not exist biologically. Murine IL-$33_{1\text{-}266}$ is similarly cleaved after D175, inside the IL-1 domain and is inactivated by caspase-3 [49]. Caspases cleave IL-33 within the C-terminal domain, in a non-structured region between β4 and β5 that is absent in the tertiary structure of other IL-1 family members [2]. This could explain the discrepancy between the role of caspases in IL-1 family member processing. The biological significance of IL-33 inactivation by caspases during apoptosis could be to prevent IL-33 release during programmed cell death, which does not require an inflammatory response. Indeed, unlike IL-1β and IL-18, IL-33 is active as a full-length protein and is constitutively expressed in epithelial and endothelial cells. If uncontrolled, IL-33 could be released inappropriately,

leading to an undesirable, inflammatory response. This is in accordance with the role of IL-33 as a danger signal: released after necrosis, but inactivated during apoptosis [8, 50] (*figure 1*).

Until recently however, it was not known if the full-length IL-33$_{1\text{-}270}$ form was the only active form, or if proteases other than caspases would be able to cleave the protein, liberating the cytokine domain. It has now been demonstrated that IL-33 is a substrate for serine proteases released by inflammatory cells recruited to the site of injury. Indeed, proteases from neutrophils (elastase, cathepsin G and proteinase 3) [43, 51] cleave the full-length IL-33, liberating active forms: IL-33$_{95\text{-}270}$, IL-33$_{99\text{-}270}$ and IL-33$_{109\text{-}270}$ between 18 and 21 kDa in human and 20 kDa mIL-33$_{102\text{-}266}$ in mouse [43] (*figure 2*). These mature IL-33 forms possess a 10 times greater potency than the full-length protein to activate ST2. They are active *in vivo* and can be detected in the bronchoalveolar lavage fluid in a mouse model of acute lung injury where neutrophils are recruited. This suggests that IL-33 activity could be amplified by the inflammatory environment thanks to the cleavage of the full-length form by protease from innate immune cells recruited to the injured tissue. This is an important advance in the understanding of IL-33 biology and pathologies such as asthma, rheumatoid arthritis or intestinal inflammation, where IL-33 is involved. IL-33 has been shown to initiate a type 2 inflammatory response in these diseases, and neutrophils, which play a critical role in these diseases [52-54] could lead to the generation of superactive forms of IL-33 which could exacerbate the immune response (*figure 1*).

Extracellular regulation by immune cells proteases has also been described for other members of the IL-1 family cytokines (*table 1*). In inflammatory models where cell damage is induced, caspase-1 is not the only protease able to activate IL-1β [55] or IL-18 [56], as their activity is still observed in caspase-1-KO mice. Furthermore, in human, various cleaved forms of IL-1β have been observed in synovial fluid from arthritic patients and in bronchoalveolar lavage fluid from patients suffering from sarcoidosis [57]. Indeed, neutrophil and mast cell proteases have been involved in the generation of active IL-1β and IL-18. *In vitro*, IL-1β is cleaved and activated by cathepsin G, elastase [57], proteinase 3 [58, 59], and chymase [60]. This

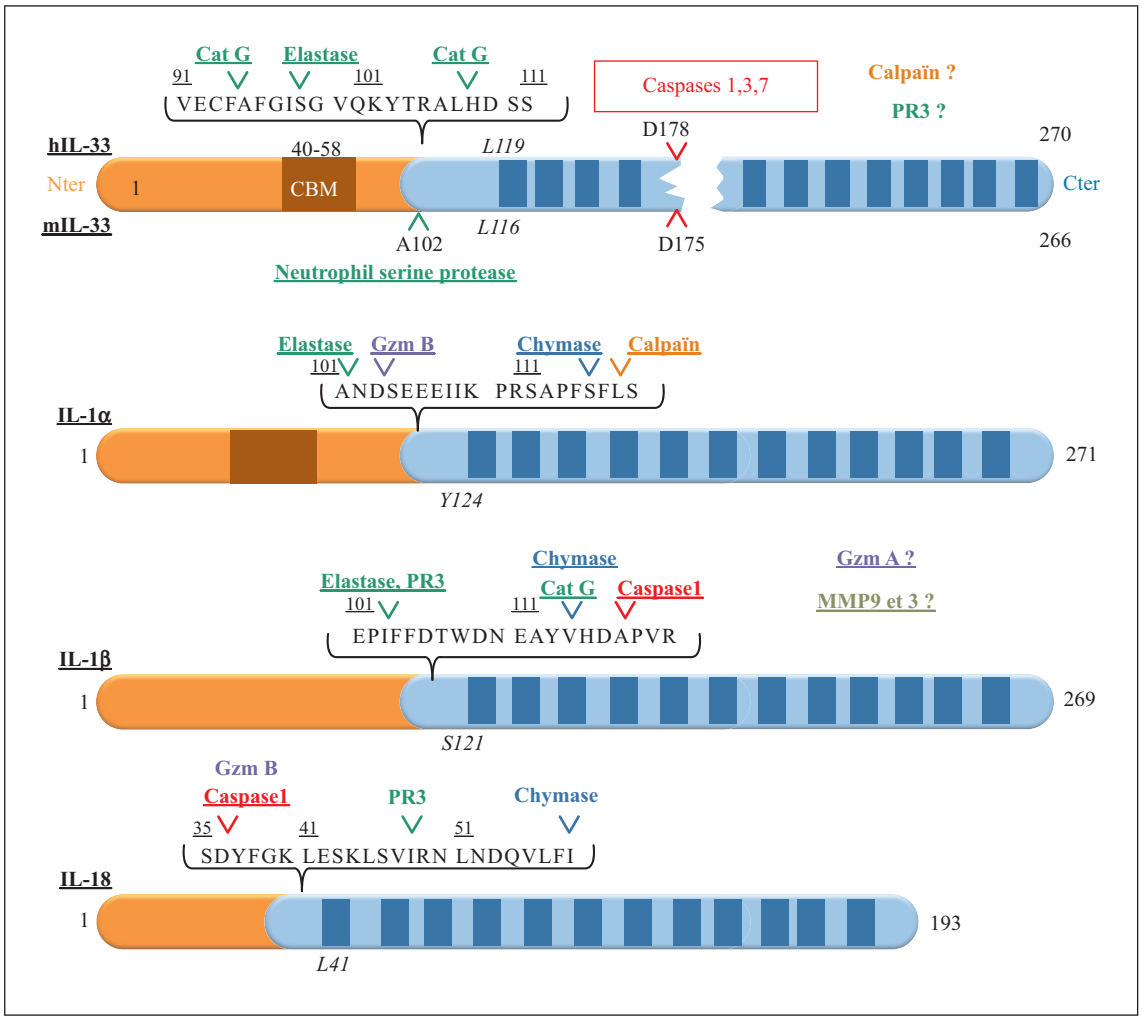

Figure 2

Processing sites of IL-1 family members: IL-33, IL-1α, IL-β and IL-18.
Intracellular proteases (caspases or calpain) or extracellular proteases from neutrophils (in green), mast cells (in blue) or NK cells or T cells (purple) cleave IL-1 family members. The amino acid recognition sites, when identified, are indicated with an arrow. The IL-1 domain is represented in blue, and the first of twelve β strands starts from the amino acid in italics. Underlined proteases activate the cytokine, liberating the IL-1 domain, whereas boxed proteases inactivate them. The consequence of the other protease cleavage has not been demonstrated. For IL-33, the chromatin binding motif (CBM) is indicated in the N-terminal part and human or mouse protein are indicated above or below the illustration, respectively.

Table 1
Effect of protease processing on the biological activity of IL-1 family members IL-33, IL-1α, IL-β and IL-18. The amino acid after which cleavage occurs is indicated.

		Intracellular cleavage			**Extracellular cleavage**						
	Protease	**Caspase-1**	**Apoptotic Caspases (3 and 7)**	**Calpain**	**Neutrophil proteases**			**Mast cell proteases**	**Granzymes**		**MMPs**
					Elastase	**Cathespin G**	**Proteinase G**	**Chymase**	**Granz A**	**MMP 3,9**	**MMP 3,9**
IL-33	Effect	Inactivation	Inactivation	Cleavage	Activation		Activation-Inactivation				
	Cleavage site	D175	D175	?	198	F94 and L108	?				
	References	8. 48	47	71.72	43	43	43.51				
IL-1a	Effect	No cleavage		Activation	Activation			Activation		Activation	
	Cleavage site			F118	A101			F116		D103	
	References	67		42, 69, 70	42			42		42	
IL-1ß	Effect	Activation		No cleavage	Activation	Activation	Activation	Activation	Activation	No cleavage	Activation-Inactivation
	Cleavage site	D116			Y113	I103	I103	Y113	R120		?
	References	44		70	57	57	58, 59	60	62	62	63
IL-18	Effect	Activation					Cleavage	Cleavage		Cleavage	
	Cleavage site	D35					V47	F57		D36	
	References	45					64	65		66	

seems to be also relevant *in vivo,* in a mouse model of arthritis, since the cleaved form of IL-1β, still observed in the absence of caspase-1, disappears when elastase or chymase inhibitor is used or when neutrophils and mastocytes are deficient in DPPI, the serine protease-activating enzyme [58, 61]. Authors suggest that IL-1β activation depends, in the first stages of the disease, on neutrophil proteases (particularly proteinase 3), and later, on caspase-1. *In vitro*, other proteases have been shown to cleave IL-1β: granzyme A from cytotoxic cells [62] and metalloproteases MMP-9, MMP-3 and MMP-2 [63]. Similarly, IL-18 can be cleaved by proteinase-3 from neutrophils [64], chymase from mast cells [65] and granzyme B from cytotoxic cells [66]. Cleavage sites are indicated in *figure 2*. IL-1α processing and activation mechanisms resemble that of IL-33. IL-1α is not a caspase-1 substrate [67] and is active as a full-length protein [68]. However, it can be cleaved by various proteases, increasing its bioactivity. Calpain, an intracellular, calcium-dependent protease was the first to be described [69, 70], but elastase, chymase and granzyme B are also able to activate IL-1α extracellularly [42]. Calpain also seems to cleave IL-33, but precise identification of the forms generated, and their activity have not been characterized [71, 72].

CONCLUSION AND REMAINING QUESTIONS

IL-33 can have a protective role in parasitic infection or a deleterious effect in allergic inflammation, initiating a type 2 response. To manipulate the IL-33/ST2 pathway in the clinic, it is thus important to consider the mechanisms of release and the regulation of the cytokine. IL-33 is part of the IL-1 family and is structurally similar to IL-18 [73]. However, IL-33 is more closely related to IL-1α in terms of expression, secretion, processing and activation mechanisms. With regards to expression and secretion, IL-33 and IL-1α have been described as "danger signals" that is to say they are constitutively expressed by cells in contact with the environment and released in their full-length forms after necrosis when cells undergo injury or infection [8, 9]. During apoptotic cell death, IL-33 is, by contrast, inactivated by caspases [8, 47]. It has been suggested that for some "danger signals", such as IL-1α or HMGB1, active mechanisms of release might exist. It remains however, to be clarified whether IL-33 could be secreted by means other than necrosis. Indeed, some studies have described extracellular IL-33 that did not alter cell viability [39, 74], however, the exact mechanisms of release have not been studied precisely. Vesicular traffic, dependent on calcium entry, described for the other members of IL-1 family, might be a way for IL-33 to exit the cell [74]. If cellular stress is usually associated with this unconvenionnal release, stimuli that would be able to induce active release of IL-33 need to be elucidated.

With respect to activation, IL-33 and IL-1α, in contrast to IL-1β and IL-18, are both active as a full-length protein and cannot be activated by caspase-1. The microenvironment can, however, modulate the activity of all the IL-1 family members. Inflammatory proteases from immune cells recruited to or present at the inflammatory site (neutrophils, mast cells or lymphocytes) have been demonstrated to amplify IL-1α [42] and IL-33 [43], as well as IL-1β [57-60] and IL-18 [64-66] functions. Neutrophils have

been shown to be regulators of IL-33 [43, 51], and active forms of IL-33 have recently been identified for the first time: IL-$33_{95\text{-}270}$, IL-$33_{99\text{-}270}$ and IL-$33_{109\text{-}270}$. However, it remains to be considered whether other cells (mast cells, lymphocytes. . .) or other proteases (MMPs. . .) are able to modify IL-33 activity. The reason why neutrophil proteases increase IL-33 bioactivity is linked to the release of the IL-1-like domain, freed from its N-terminal domain. This is a general mechanism, observed among all of the IL-1 family. We can formulate various hypotheses to explain why full-length IL-33 is less active than the newly identified C-terminal forms. The full-length protein could have a reduced affinity to its receptor, firstly due to the steric hindrance of the N-terminal part, secondly, the reduced affinity could be due to electronic changes, given that IL-33/ST2 interaction is polar [2, 48] and that protein global charge is modified by the cleavage. Indeed, full-length IL-33 is more basic than the super active forms. Only the most studied members of the IL-1 family have been described here, but to compare quickly with other members, it seems that IL-36, similarly to IL-1α and IL-33, is active as a full-length protein. Its activity can also be increased when the N-terminal part of the protein is removed [75]. This cleavage probably does not involve caspase-1, but the proteases responsible for this activation have not been identified. IL-37, for its part, can be activated by caspase-1, similarly to IL-1β and IL-18.

In summary, IL-1 family member processing and secretion mechanisms are unusual and complex compared with other cytokines. It is, however, essential to define them fully and to understand their particular role in the initiation of immune responses following cellular stress during various inflammatory, infectious or autoimmune diseases.

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