Chapter 8
Regulation and Function of the Inhibitory C-Type Lectin Clec12A/MICL

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Abstract The C-type lectin domain family 12 member A (Clec12A/MICL) is an inhibitory receptor encoded in the Dectin-1 cluster. It is widely expressed in myeloid cells and was identified as a specific marker for cancer stem cells in acute myeloid leukemia. Clec12A possesses an immunoreceptor tyrosine-based inhibition motif (ITIM), which can counteract activating signals from immunoreceptor tyrosine-based activation motifs (ITAMs). The receptor can sense necrotic cell death and limit ITAM-coupled receptor-induced inflammation in response to cell death or tissue damage. One Clec12A agonist released from dead cells was identified as uric acid in its crystallized form. Clec12A limits ITAM-dependent respiratory burst and IL-8 release from neutrophils in response to uric acid crystal binding but does not interfere with crystal-induced inflammasome activation. This review discusses recent insights into the regulation and biological functions of Clec12A.

Keywords Clec12A • MICL • CLL1 • CLL-1 • DCAL2 • DCAL-2 • KLRL1 • MSU • Sodium urate • Uric acid • Crystals • ITAM • ITIM • Sterile inflammation

8.1 Structure and Expression Pattern of Clec12A

The C-type lectin domain family 12 member A (Clec12A) is alternatively called myeloid inhibitory C-type lectin-like receptor (MICL) (Marshall et al. 2004), C-type lectin-like molecule 1 (CLL-1) (Bakker et al. 2004), dendritic cell-associated lectin 2 (DCAL-2) (Chen et al. 2006), or KLRL1 (Han et al. 2004). The Clec12A gene is located in the Dectin-1 cluster of C-type lectin receptors (CLRs). Similar to other members encoded in this cluster, it is a type II transmembrane protein with an extracellular C-type lectin domain (CTLD), which lacks the amino acid motif of classical CTLDs required for Ca^{2+} complexation and carbohydrate binding. Clec12A and the closely related orphan CLR Clec12B (Hoffmann

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et al. 2007) are the only inhibitory receptors encoded in the Dectin-1 cluster. Their intracellular domains contain an immunoreceptor tyrosine-based inhibition motif (ITIM). Receptors harboring ITIMs generally antagonize activating immune receptors harboring immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth 1989). While ITAM-coupled receptors signal via tyrosine kinases like Syk, ITIMs upon phosphorylation recruit the lipid phosphatase SHIP or protein tyrosine phosphatases SHP-1 or SHP-2 (Lanier 2003). Clec12A and B were shown to associate both with SHP-1 and SHP-2 (Han et al. 2004; Marshall et al. 2004; Pyz et al. 2008; Hoffmann et al. 2007). Several isoforms of Clec12A exist (Gerhard et al. 2004; Marshall et al. 2004). Notably, one isoform has an additional tyrosine-based signaling motif that might transduce ITIM-independent signals. Another isoform lacks the transmembrane domain, which potentially gives rise to an intracellular protein, as Clec12A is a type II transmembrane protein. A functional relevance for these isoforms is, however, unknown to date.

Clec12A is mainly expressed on myeloid cells including monocytes, granulocytes (neutrophils, eosinophils, basophils), both myeloid and plasmacytoid dendritic cells, macrophages, and nearly absent in lymphocytes and NK cells (Marshall et al. 2004, 2006; Bakker et al. 2004). Intriguingly, while the expression level of Clec12A does not change during the differentiation of human monocytes into macrophages, the amount of Clec12A glycosylation was shown to increase during macrophage differentiation (Marshall et al. 2006). However, similar effects have not been reported for murine Clec12A. Whether the differential glycosylation of Clec12a has functional significance, e.g., by changing the affinity to its ligands or to other receptors on the cell surface, remains to be determined.

Human Clec12A was transcriptionally downregulated after TLR stimulation in vitro, and human granulocytes and monocytes recruited to the site of acute inflammation had reduced expression of Clec12A (Marshall et al. 2006). Similarly, mouse Clec12A was downregulated on myeloid cells after TLR stimulation (Pyz et al. 2008) and on cells recruited to the peritoneum during peritonitis induced by uric acid crystals or thioglycolate broth (Heng et al. 2008). These findings indicate a role for Clec12A in limiting immune responses in the absence of microbes or danger. However, Clec12A-deficient mice do not develop spontaneous autoimmune or autoinflammatory syndromes indicating that Clec12A is largely dispensable for immune homeostasis (Neumann et al. 2014).

8.2 Clec12A as a Dead Cell Receptor That Recognizes Uric Acid Crystals

Most of the activating CLRs encoded in the Dectin-1 and the Dectin-2 clusters are pattern recognition receptors (PRRs) that either recognize pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or both (Iborra and Sancho 2014). ITIM-containing immunoreceptors often recognize

endogenous ligands to prevent autoimmunity, e.g., inhibitory NK cell receptors recognize MHC class I (MHC I) molecules to prevent NK cell activation by healthy host cells. Upon virus-induced downregulation of MHC I, ITAM-coupled activating NK cell receptors are no longer blocked by MHC I-specific ITIM receptors and the host cell is attacked (Long 2008). Given the homology of Clec12A to inhibitory NK cell killer cell lectin-like receptors, it was initially speculated that Clec12A could be an inhibitory NK cell receptor (Han et al. 2004). Yet, NK cells do not seem to express Clec12A under homeostatic conditions. Recombinant mouse Clec12A (Clec12A-Fc fusion protein) still bound to cells from diverse primary mouse tissues indicating that Clec12A can sense endogenous ligands. Furthermore, reporter cells expressing mouse Clec12A fused to the intracellular signaling domain of CD3ζ responded to stimulation with mouse tissues (Pyz et al. 2008) further supporting the notion that Clec12A binds to endogenous agonists. By searching for cell types and conditions that expose the ligand of Clec12A, we observed that in all tested tissues, murine or human Clec12A selectively bound to dead cells that had lost the integrity of the plasma membrane. In addition, reporter cells expressing either human or mouse Clec12A fused to the intracellular domain of CD3ζ did not respond to viable cells. However, when certain cell types were killed by freeze-thaw cycles, they were able to activate Clec12A reporter cells (Neumann et al. 2014). These findings indicate that Clec12A is a sensor of cell death suggesting that Clec12A could potentially inhibit cell death-induced immune cell activation by ITAM-coupled PRRs like Mincle (Clec4E), which recognizes the intracellular protein SAP130 (Yamasaki et al. 2008), and DNGR-1 (Clec9A), which recognizes filamentous (F) actin (Zhang et al. 2012; Ahrens et al. 2012; Sancho et al. 2009). Indeed, we found enhanced sterile inflammatory responses in Clec12A-deficient mice in a model of low-dose X-ray irradiation, in which inflammation is sensitive to an antibody that blocks Mincle (Clec4E) and MCL (Clec4D) (Yamasaki et al. 2008; Miyake et al. 2013) indicating that Clec12A can indeed inhibit Mincle-induced inflammation in vivo. Whether Clec12A also regulates DNGR-1-dependent immune responses is unclear at this point.

Since Clec12A reporter cell activation by dead cells was sensitive to pretreatment of the killed cells with proteases, we anticipated that Clec12A recognizes proteinaceous ligands. However, we have so far been unable to isolate a proteinaceous ligand from dead cells using recombinant Clec12A, while we could easily purify actin with recombinant DNGR-1 and SAP130 with recombinant Mincle (K. Neumann, unpublished). Therefore, we additionally searched for other danger-associated molecular patterns released from dead cells, including uric acid (Shi et al. 2003). Uric acid is the end product of purine catabolism. The degradation of nucleic acids after cell death leads to a local increase in uric acid concentration that favors its crystallization (Kono et al. 2010).

Allopurinol is a drug, which is used to lower uric acid levels in gout patients by inhibiting the conversion of xanthine to uric acid by xanthine dehydrogenase (XDH). Interestingly, we found that allopurinol inhibited Clec12A-reporter cell activation by dead cells. Moreover, both human and mouse recombinant Clec12A specifically bound to uric acid crystals in vitro. In addition, reporter cells expressing

human or mouse Clec12A were specifically activated by uric acid crystals but not by the Dectin-1 ligands zymosan or curdlan or by other crystalline structures like silica, calcium pyrophosphate (CPPD), or polystyrol beads (Neumann et al. 2014). Together, these findings indicate that Clec12A is a uric acid crystal-specific receptor.

Dead cell-mediated activation of Clec12A reporter cells or binding of Clec12A to dead cells was not completely blocked by allopurinol or uricase treatment, respectively (Neumann et al. 2014). Therefore, we speculate that Clec12A may sense additional agonists exposed or released by dead cells. Clec12A may bind to parts of the protein complex containing the Mincle ligand SAP130. Given the high expression of both Clec12A and DNGR-1 on $CD8^+$ dendritic cells in the mouse (Heng et al. 2008; Kasahara and Clark 2012; Lahoud et al. 2009), it is also conceivable that certain forms of cell death generate Clec12A binding sites associated with the DNGR-1 ligand F-actin. Alternatively, ions released from the cytoplasm of dead cells may crystallize with calcium ions in the extracellular space, creating other crystalline structures that may be recognized by Clec12A. These possibilities need to be further explored.

While we identified Clec12A as the first mammalian crystal-recognition receptor, it is interesting to note that C-type lectin-like domains had been identified as crystal-binding domains before. They constitute one of three classes of antifreeze proteins found in cold-water fish that prevent ice crystal formation, thereby lowering the serum freezing temperature (Gronwald et al. 1998; Zelensky and Gready 2005). Thus, the C-type lectin domain is in principle well suited to bind to crystals. The future will show if there are other mammalian C-type lectin domains or CLRs that recognize other crystals like bone mineral or cholesterol.

8.3 Clec12A Regulates Inflammatory Responses

The inflammatory properties of uric acid crystals (in the form of monosodium urate, MSU) have been intensively investigated since they were discovered as the cause of gout (Shi et al. 2010; Mccarty and Hollander 1961). It is now widely accepted that uric acid has inflammatory properties only in its crystalline state and that crystallization is facilitated in vivo by crystal-specific antibodies (Kanevets et al. 2009). The crystals activate the complement cascade leading to the release of inflammatory breakdown products (e.g., C5a) (Hasselbacher 1979; Russell et al. 1982), and complement-deficient animals show reduced inflammation in response to uric acid crystals (Tramontini et al. 2004). In myeloid cells, the crystals activate intracellular NLRP3 inflammasomes, which leads to maturation of IL-1β and a form of inflammatory cell death called pyroptosis. NLRP3-deficient mice have a severely diminished inflammatory response to crystals (Martinon et al. 2006). It has further been shown that in human whole blood, C5a release by complement activation is required to prime the NLRP3 inflammasome (An et al. 2014). Whether specific recognition of the uric acid crystals by myeloid cell surface receptors is required for

NLRP3 inflammasome activation is unknown. It is becoming clear though that most crystals or crystal-like structures activate the NLRP3 inflammasome, indicating that probably there is not one specific crystal-recognition receptor linking to the NLRP3 inflammasome. Still, interaction of crystalline structures with the cell membrane is essential for inflammasome activation (Hari et al. 2014).

Such interactions may occur via recognition of crystal-attached proteins like complement or antibodies that are bound by complement receptors or Fc receptors, respectively, but their contribution has not been investigated. The activation of human neutrophils by pure unopsonized crystals depends on CD11b and/or CD16, as antibodies targeting these receptors block crystal-induced neutrophil activation (Barabe et al. 1998; Ryckman et al. 2004). Whether one of these receptors directly binds to the uric acid crystals is currently unknown. Another receptor implicated in the recognition of uric acid crystals is CD14, a co-receptor for TLR4. CD14 was shown to bind to uric acid crystals and facilitate activation of macrophages by uric acid crystals in the absence of serum, but CD14 is considered to neither have sufficient affinity nor specificity in the presence of other proteins (Scott et al. 2006).

Last, it was shown that uric acid crystals directly interact with lipids in cellular plasma membranes. The binding to cholesterol leads to membrane reorganization (lipid sorting) that induces activation of Syk, presumably by cross-linking ITAM-coupled receptors independent of their specificity (Ng et al. 2008). Alum crystals were also shown to activate Syk in a similar manner, which seems to be required for its adjuvanticity (Flach et al. 2011). Together, there seem to be various direct and indirect modes of recognition for uric acid crystals by innate immune cells. All of them depend on ITAM-coupled receptors and the downstream tyrosine kinase Syk. Therefore, independent of how myeloid cells recognize uric acid crystals in a given situation, their activation by the ITAM-coupled kinase Syk should be sensitive to ITIM receptor-mediated inhibition by Clec12A. In line with this notion, neutrophils deficient of Clec12A showed enhanced Syk-dependent reactive oxygen species (ROS) production in response to uric acid crystals, which was accompanied by enhanced phosphorylation of the NADPH oxidase (Neumann et al. 2014). Similarly, antibody-mediated downregulation of Clec12A on human neutrophils or siRNA-mediated downregulation of Clec12A on a human neutrophil cell line led to enhanced IL-8 production in response to uric acid crystals (Gagne et al. 2013). Most importantly, Clec12A has nonredundant functions in regulating the inflammatory response to uric acid crystals in vivo, as Clec12A-deficient mice showed increased neutrophil influx in response to intraperitoneal injection of uric acid crystal (Neumann et al. 2014). Although crystal-induced activation of the NLRP3 inflammasome and IL-1β secretion is dependent on Syk (Gross et al. 2009; Hara et al. 2013; Shio et al. 2009), blocking of Clec12A on neutrophils did not enhance IL-1β secretion in response to uric acid crystals (Gagne et al. 2013). This suggests that Syk activity may not be the limiting factor for inflammasome activation. The role of Clec12A in regulation of neutrophil activation in response to uric acid crystals is summarized in Fig. 8.1.

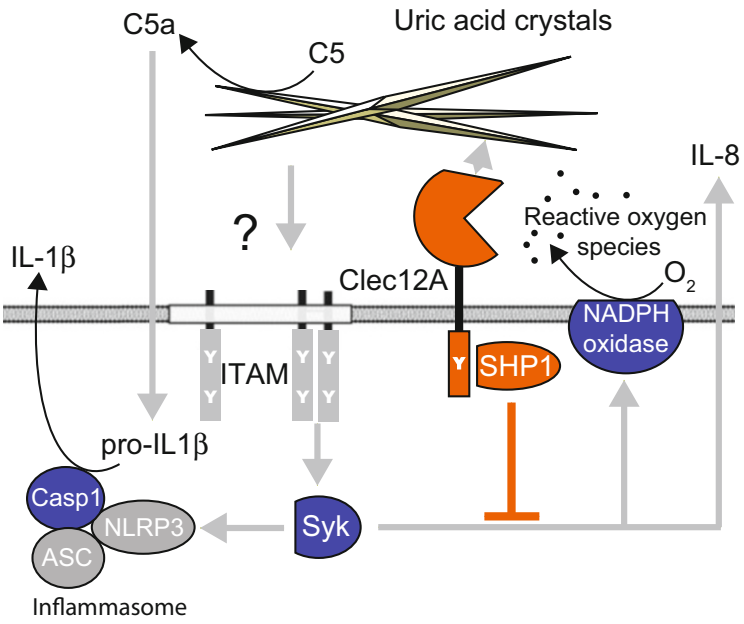

Fig. 8.1 Clec12A limits uric acid crystal-induced respiratory burst (ROS production) and IL-8 but not IL-1β secretion. Uric acid crystals induce activation of the complement system, which leads to cleavage of C5 to C5a and C5b. C5a induces priming of the inflammasome by upregulating transcription of pro-IL-1β. Direct or indirect interaction of the crystals with the plasma membrane induces ITAM-dependent activation of Syk, which is required for NLRP3 inflammasome activation and IL-1β secretion. Syk also activates a signaling cascade leading to NADPH oxidase activation that produces reactive oxygen species and to production of the chemotactic cytokine IL-8. Upon cross-linking to the activated ITAMs, Clec12A is phosphorylated and recruits via its ITIM protein phosphatase SHP-1 or SHP-2, which counteract Syk-induced NADPH oxidase activation and IL-8 production

8.4 Function of Clec12A on Dendritic Cells

While Clec12A-mediated suppression of neutrophil inflammatory responses is genetically established, the role in other cell types is unclear. Clec12A is strongly expressed on various dendritic cell subsets (Lahoud et al. 2009; Kasahara and Clark 2012). As dendritic cells are the major antigen-presenting cells that initiate and shape T cell responses, Clec12A may also regulate adaptive immunity. In this context, it was shown that antibody-mediated targeting of antigen to Clec12A for antigen delivery to dendritic cells enhances humoral immune responses (Lahoud et al. 2009). In the original study, Lahoud et al. used a monoclonal IgG antibody against Clec12A, which probably does not extensively crosslink Clec12A. Since this Clec12A antibody did not lead to activation of dendritic cells, co-injection of adjuvant (LPS or CpG) was required to induce strong humoral immune responses to Clec12A-targeted antigen (Lahoud et al. 2009). In a similar approach, in which the

activating receptor DNGR-1 was targeted for antigen delivery, an adaptive humoral immune response was achieved even in the absence of adjuvant (Lahoud et al. 2011). Thus, targeting of an antigen to Clec12A does not activate dendritic cells but seems to be sufficient for antigen internalization, processing, and presentation. When uric acid crystals were co-injected with an antigen in vivo, these crystals significantly enhanced the generation $CD8^+$ T cell responses (Shi et al. 2003), which requires antigen cross-presentation. Since ROS production favors antigen cross-presentation (Hari et al. 2015; Savina et al. 2006) and since Clec12A can in principle regulate ROS production, this process might also be sensitive to Clec12A inhibition.

It should be noted that cross-linking of Clec12A on human dendritic cells with a monoclonal IgM antibody specific for Clec12A induced activation of the mitogen-activated protein kinase (MAPK) pathway and CCR7 upregulation. This signal synergized with the CD40 signaling pathway to enhance cytokine expression (Chen et al. 2006). Whether this is physiologically relevant or a property of this specific cross-linking antibody remains to be determined.

Together, these findings along with the expression of Clec12A on dendritic cells indicate that Clec12A might modulate certain adaptive immune responses during sterile injury or crystal recognition, but additional experiments are required to address these questions.

8.5 Clec12A Expression on Acute Myeloid Leukemia Cells

Clec12A was independently identified in a screen for novel antibody targets for acute myeloid leukemia (AML) (Bakker et al. 2004). Novel therapies are required for this malignancy as tumor cell progenitors, or leukemic stem cells confer resistance to chemotherapy (Dick 2005). Therefore, antibodies targeting surface markers of leukemic stem cells could be useful to eradicate these cells. A preclinical study using the surface marker CD44 already showed a promising outcome (Jin et al. 2006). Interestingly, Clec12A is expressed in the malignant $CD34^+CD38^-$ stem cell compartment in the majority of $CD34^+$ AML patients, while it is absent on normal $CD34^+CD38^-$ resting bone marrow cells (van Rhenen et al. 2007). Indeed, it was recently shown that Clec12A could serve as a diagnostic marker to quantify minimal residual disease (Roug et al. 2014; Larsen et al. 2012). Research on the possible use of Clec12A as a therapeutic target for immunotherapies against AML is currently conducted both in academia and industry (Zhao et al. 2010; Hangalapura et al. 2014; Noordhuis et al. 2010; Lu et al. 2014). Whether Clec12A is simply a marker for AML cells or whether this receptor has also a functional role in AML biology is currently unclear. Yet, it is conceivable that an inhibitory signal delivered by Clec12A within the right microenvironment could keep leukemic stem cells in a quiescent state.

8.6 Conclusion

Work over the last few years identified Clec12A as an important inhibitory pattern recognition receptor that regulates inflammatory responses to cell death. Such reactions are important for the maintenance of homeostasis and tissue regeneration, but inflammation can also cause further tissue damage that can even lead to organ failure (Arslan et al. 2010; Imaeda et al. 2009; Jiang et al. 2005). Because cell damage is often induced during infection, CLRs that sense dead cells may also play critical roles in immune response against pathogens that have few or successfully hide their PAMPs. The activating dead cell receptor DNGR-1 has already been shown to be essential for protective immune responses against certain viruses (Iborra et al. 2012; Zelenay et al. 2012). The circumstances in which Clec12A is essential to either prevent excessive inflammation that is harmful to the host or regulate protective immune responses against pathogens are currently unknown. The further analysis of Clec12A-deficient mice will identify these circumstances and broaden our understanding of the immunological consequences of cell death recognition. During production of this article a recent study has shown that Clec12A indeed regulates immune responses against pathogens, as Clec12A-deficient mice were shown to be more susceptible to *Salmonella* infection (Begun et al. 2015).

Conflict of Interest The authors declare no conflict of interest.

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