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Sensing tissue damage by myeloid C-type lectin receptors

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ABBREVIATIONS

ATP: Adenosine TriPhosphate

CAR-T: Chimeric Antigen Receptor T-Cell

CLEC: C-type LECtin

CLR: C-type Lectin Receptor

DC: Dendritic Cell

IFN: Interferon

IRF: Interferon Response Factor

ITAM: Immunoreceptor Tyrosine-based Activating Motif

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motif

LDL: Low Density Lipoprotein

LPS: Lipopolysaccharide

PRR: Pattern Recognition Receptor

ROS: Reactive Oxygen Species

SHP-1/ -2: Src-Homology 2 domain containing Phosphatase -1 / -2

SYK: Spleen tYrosine Kynase

TLR: Toll-Like Receptor

ABSTRACT

After both sterile or infectious insults, damage is inflicted to tissues leading to accidental or programmed cell death. In addition, events of programmed cell death also take place under homeostatic conditions, such as in embryo development or in the turnover of hematopoietic cells. Mammalian tissues are seeded with myeloid immune cells, which harbor a plethora of receptors that allow the detection of cell death, modulating immune responses. The myeloid C-type Lectin Receptors (CLRs) are one of the most prominent family of receptors involved in tailoring immunity after sensing dead cells. In this chapter, we will cover a diversity of signals arising from different forms of cell death and how they are recognized by myeloid CLRs. We will also explore how myeloid cells develop their sentinel function, exploring how some of these CLRs identify cell death and the type of responses triggered thereof. In particular, we will focus on DNGR-1 (*CLEC9A*), MINCLE (*CLEC4E*), CLL-1 (*CLEC12A*), LOX-1 (*OLR1*), CD301 (*CLEC10A*) and DEC-205 (*LY75*) as paradigmatic death-sensing CLRs expressed by myeloid cells. The molecular processes triggered after cell death recognition by myeloid CLRs contribute to the regulation of immune responses in pathologies associated to tissue damage, such as infection, autoimmunity and cancer. A better understanding of these processes may help to improve the current approaches for therapeutic intervention.

KEYWORDS: C-type lectin receptors, cell death, macrophages, dendritic cells, myeloid.

1. *Introduction*

Quoting the famous movie "Forrest Gump" in its 25th anniversary, "*Momma always said dyin' was a part of life*". Independently of the meaning of this quote in our daily life, this is an important assertion when we take a closer look at our bodies. Based on a cycle of cell death and regeneration, our cells are in a constant turnover under physiological conditions (Pellettieri and Alvarado 2007). This process is even more patent in pathological situations where massive tissue damage can be caused by both sterile or infectious insults. Immune cells populating all tissues constitute a sentinel network that surveys and reacts to cell death (Kroemer 2017).

Pattern recognition receptors (PRRs) allow the sensing of conserved structures released or exposed during cell death (Shekarian et al. 2017). Importantly, cell death can be triggered through diverse programs, which deliver distinct signals. Sensing these signals by myeloid cells originates different immune responses depending on the PRR involved in the recognition. Among the PRRs, C-type lectin receptors (CLRs) exert a prominent function in death recognition (Sancho and Reis e Sousa 2013). Herein, we will review the type of signals generated during cell death and how these signals are produced depending on particular cell death programs. All this information will be integrated from an immune perspective, analyzing how myeloid cells recognize these signals through a panel of CLRs and their subsequent inflammatory outcomes.

2. *Molecular signatures associated to microorganisms and self-damage*

Immune cells express different combinations of pattern recognition receptors (PRRs) that allow them to sense Pathogen-Associated Molecular Patterns (PAMPs), conserved molecular entities present in microorganisms (Janeway 1989). However, this does not fully explain the initiation of immunity in the apparent absence of infections. The danger theory defends that the immune system acts not only discriminating foreign molecules from the host's own, but also by identifying evolutionarily-conserved endogenous molecular patterns associated with threats to homeostasis, that are exposed, released or produced in response to stress (Matzinger 2002). These Damage-Associated Molecular Patterns (DAMPs) are self-molecules that can be detected by immune cells, inducing either immunity or promoting wound healing. The interplay between DAMPs and PAMPs and their PRRs may instruct immune responses.

Cell death comprises a variety of modalities whereby cells cease to carry out their functions in an irreversible manner. Cell death generates DAMPs, and it is an excellent example of a situation where immune cells have to discriminate homeostatic death from menacing situations. The nature of released DAMPs upon cell death embraces a wide range of molecules that can be pre-formed or newly synthesized (Yatim et al. 2017). For instance, constitutive intracellular components released upon loss of membrane integrity, such as nucleic acids or cytoskeleton proteins. On the other hand, tissue injury can induce the specific modification of endogenous molecules, such as the glycation of ligands recognized by the Receptor for Advanced Glycation End-products (RAGE) or the production of cytokines, such as IL1α or IFN-α, which can act as immunomodulators (Roh and Sohn 2018).

There is evidence that the nature of DAMPs is well conserved along evolution. For instance, the hydrophobic portions (*hyppos*) of different molecules are embedded

within biological membranes or hydrophobic cores in macromolecules, and their exposure can be linked to cell stress or death (Seong and Matzinger 2004). Another example of ancient DAMPs may be nucleic acids, which are usually restricted to the nuclear or cytoplasmic space, and their exposure can be indicative of cell death or viral infection. Mammalian cells are armed with an ample repertoire of receptors that detect nucleic acids, both at the intracellular and extracellular level (Roers et al. 2016). The detection of nucleic acids is also a common feature of invertebrate animals, yeasts and bacteria. Additionally, other properties of the microenvironment can condition the binding of defined DAMPs to those receptors (Rajamäki et al. 2013; Santoni et al. 2015). For instance, bacterial infections and tumors can acidify the cellular microenvironment, impacting sensing of DAMPs and signaling.

3. *Types of cell death*

Cell death occurs both under steady-state conditions (*e.g.* embryo development) and upon infection or sterile tissue damage. Cell death can be broadly categorized either as an accidental phenomenon (Accidental cell death – ACD) or as a regulated cellular process (Regulated cell death – RCD). This is actually the current classification system updated by the Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al. 2015), which provides guidelines to define all aspects of cell death. According to the classic paradigm, ACD occurs only under pathological conditions and, alternatively, RCD mostly happens during homeostatic situations (Green et al. 2009). However, this paradigm has been challenged by the discovery of different forms of RCD. We next describe the

triggers, morphological features, underlying molecular mechanisms and relationship with immune responses of the main forms of cell death.

3.1 Necrosis

Defined as uncontrolled cell death following severe injury (or ACD), necrosis is accompanied with plasma membrane rupture. This results in the release of the intracellular content out to surrounding tissues, which may generate further damage (D'Arcy 2019). Under this view, the trigger and molecular mechanisms underlying necrosis are unspecific, and involve any form of chemical or physical insult that overwhelms control mechanisms and trigger this indiscriminate cell death. However, cells can die displaying necrotic features under a number of RCD processes such as necroptosis, pyroptosis or NETosis among others, which are driven by defined molecular mechanisms (Tang et al. 2019).

Necrosis was classically considered an immunogenic process compared with RCD, due to the random exposition of cellular components/antigens that otherwise should be sheltered under the plasma membrane (Kroemer et al. 2009). However, this notion has been challenged by several observations where certain types of RCD can be immunogenic, while ACD is still considered a pro-inflammatory event associated with uncontrolled tissue damage.

3.2 Apoptosis

Apoptosis represents the first described RCD program (Kerr et al. 1972). It is characterized by cell rounding, nuclear condensation, DNA fragmentation, membrane blebbing and the eventual formation of membrane-surrounded apoptotic bodies (Nagata 2018). Apoptosis can be triggered extrinsically by the engagement of membrane receptors such as death receptors (*e.g.* FAS, also known as CD95) or intrinsically by mitochondrial outer membrane permeabilization due to exposition to different toxic stimuli (irradiation, toxins, hypoxia) (Reed and Pellecchia 2005). Additionally, granzymes can trigger apoptosis in cells targeted by cytotoxic T and NK cells (Elmore 2007).

Apoptosis is a finely regulated process based on the sequential activation of the Caspase family of cysteine proteases (McIlwain et al. 2013). These enzymes exist in steady-state as inactive zymogens. Stimuli that trigger apoptosis activate initiator caspases. Caspase-8 and -9 are prototypical initiator caspases of the extrinsic and intrinsic apoptosis, respectively. The proteolytic action of these caspases activates downstream executor caspases such as Caspase-3. Executor caspases cleave a wide range of proteins, giving rise to destruction of subcellular structures, thus compromising the cellular integrity (Galluzzi et al. 2016).

The immune consequences of apoptosis have been classically considered as silent or anti-inflammatory, such as the induction of T cell tolerance to antigens associated to apoptotic cells (Steinman et al. 2000). In fact, the removal of apoptotic cells by phagocytosis, namely, efferocytosis, is considered an anti-inflammatory process (Morioka et al. 2019). However, certain types of caspase-mediated cell death induced upon anthracycline-based anti-tumor chemotherapies are immunogenic (Casares et al. 2005; Obeid et al. 2007). In addition, apoptotic cells that are not efficiently cleared

undergo a process termed secondary necrosis defined as dissolution of the cell following apoptosis (Green et al. 2009). It is difficult to distinguish between secondary and primary necrosis, as both imply loss of plasma membrane integrity and are typically immunogenic due to the release of intracellular pro-inflammatory cell components (Bell et al. 2006). However, secondary necrotic cells have gone through various modifications during the process of apoptosis and consequently, molecules released during secondary necrosis may drive distinct immunologic responses (Sachet et al. 2017).

Therefore, assigning a silent or tolerogenic role to apoptosis may not be accurate. A systematic characterization of the molecular mechanisms, the clearance of apoptotic bodies and their location may help to characterize the immunogenicity of specific apoptotic processes. Importantly, apoptosis and its dysregulation underlies both physiological and pathological processes, including cell homeostasis, tissue remodeling, organ transplantation and cancer (Linkermann et al. 2014; Singh et al. 2019). In addition, a number of pathologies have been linked to defective clearance of apoptotic cells (Morioka et al. 2019).

3.3 Pyroptosis

Pyroptosis represents an alternative Caspase-dependent RCD driven by the activation of the inflammasome, a cytosolic multiprotein complex responsible for the release of IL-1α/β and IL-18 (Fink et al. 2008; Tang et al. 2019). Pyroptosis is characterized by the formation of cell membrane pores, leading to membrane rupture and release of cytosolic contents to the extracellular environment (Wang et al. 2019). Pyroptosis can be triggered by many different PAMPs (*e.g.* bacterial peptidoglycans,

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viral dsRNA) and DAMPs (ATP, elevated intracellular ROS) through the canonical Caspase-1 pathway (Liu and Lieberman 2017) or after direct recognition of LPS by Caspase-4/5 in humans or Caspase-11 in mice, in a non-canonical pathway (Shi et al. 2014). In addition, pyroptosis can be activated by alternative caspases such as Caspase-8 during *Yersinia* infection (Orning et al. 2018) or Caspase-3 in response to chemotherapy in certain cancer cells (Wang et al. 2017).

The final step for the development of pyroptosis involves the cleavage of Gasdermins, a family of proteins that comprises functionally diverse enzymes with pore-forming potential. In particular, Gasdermin-D (GSDMD) is cleaved in most models of pyroptosis (Shi et al. 2015). GSDMD is a proteolytic substrate of Caspases-1/4/5/8/11 and its cleavage releases the N-terminal effector domain from the inhibitory domain. The N-terminal domain of GSDMD oligomerizes in the cell membrane and forms cytotoxic pores, through which caspase-substrates of small size, such as IL-1β/-18 and the alarmin IL-1α are spread to the extracellular milieu. The accumulation of eventually leads to membrane rupture and, releasing the entire cellular content (Feng et al. 2018). Additionally, chemotherapy drugs induce pyroptosis by a similar mechanism through Caspase-3 cleavage of Gasdermin-E (Wang et al. 2017).

Due to the involvement of pro-inflammatory cytokines IL-1α/-1ß/-18 and the eventual release of the intracellular content, pyroptosis is a highly inflammatory form of cell death (Tang et al. 2019). The main function of pyroptosis is the defense and rapid clearance of bacterial and viral infections (Doitsh et al. 2014; Robinson et al. 2019). However, persistent inflammasome activation can produce excessive and chronic

inflammation, eventually contributing to metabolic disorders, autoinflammatory diseases and cancer (Davis et al. 2011).

3.4 Necroptosis

Necroptosis is a form of necrotic RCD sharing morphological features to necrosis, with the rupture of the plasma membrane as the main characteristic (Pasparakis and Vandenabeele 2015). It can be triggered by multiple inflammatory stimuli, both sterile and infectious (Tang et al. 2019). Of note, molecules that induce apoptosis, such as TRAIL or Fas Ligand (FasL), can also ignite necroptosis when apoptosis is chemically inhibited (Vercammen et al. 1998).

Mechanistically, necroptosis relies on the phosphorylation of mixed lineage kinase domain-like pseudokinase (MLKL) by the receptor-interacting serine/threonine kinase 3 (RIPK3) conforming the critical RIPK3-MLKL axis for necroptosis induction (Sun et al. 2012). Phosphorylated MLKL can bind phosphatidylinositol phosphates present in the inner face of the plasma membrane (Dondelinger et al. 2014). Once translocated to the membrane, MLKL inserts into it and multimerizes with the final consequence of membrane permeabilization (Galluzzi et al. 2017).

Current findings support that necroptosis is an immunogenic cell death important for the activation of innate and adaptive immunity. For instance, RIPK3-deficient mice are highly sensitive to viral infections (Cho et al. 2009; Huang et al. 2015) and the presence of RIPK3 in tumor cells is required for eliciting cytotoxic antitumor responses (Yatim et al. 2015). In fact, necroptosis in tumor cells promotes anti-tumor immunogenicity. Thus, *Ripk3* expression in colorectal carcinoma patients is reduced

compared with adjacent healthy tissue and high *Ripk3* expression constitutes an independent good-prognostic factor (Feng et al. 2015). In line with these findings, the oncogenes BRAF and AXL drive the loss of *Ripk3* expression (Najafov et al. 2018). Consequently, tumor cells dampen necroptosis to become poorly immunogenic and hence escape immunosurveillance; therefore promoting necroptosis in cancer cells has a great potential as adjuvant for antitumor therapy (Cho 2018). Necroptosis has not only been involved in infections and malignancies, but it is also implicated in a variety of pathologies such as myocardial infarction and stroke, atherosclerosis or inflammatory bowel disease (Linkermann and Green 2014).

3.5 NETosis

Netosis is an RCD process driven by Neutrophil Extracellular Traps (NETs) extrusion. Despite its name, coined after its first characterization (Brinkmann et al. 2004), NETs are not only produced by neutrophils but by other granulocytes as well (Araźna et al. 2015). NETs are web-like DNA structures formed after chromatin decondensation and release to the extracellular environment following plasma membrane rupture; in addition, several proteins adhere to these DNA-based nets, including histones and components of cytoplasmic granules (Delgado-Rizo et al. 2017). Interestingly, NETosis has also been described without involving cell death, a process termed vital-Netosis (Timp et al. 2015).

The molecular mechanisms implicated in the induction of NETosis are quite diverse (Tang et al. 2019), with notorious differences between cell death-based NETosis, vital-NETosis or even NET formation through release of mitochondrial DNA (Yousefi et

al. 2019). In fact, the pyroptosis driver GSDMD (Sollberger et al. 2018) and pyroptosis-related IL-1β (Mitroulis et al. 2011) have been involved in triggering NETosis, indicating the complexity of this process. However, production of Reactive Oxygen Species (ROS) is of capital relevance for triggering most forms of the NETotic processes (Araźna et al. 2015).

NETosis is triggered in response to infection but also in sterile conditions, such as autoimmune disorders, ischemia-reperfusion injury and cancer (Branzk and Papayannopoulos 2013). In fact, the diversity of the molecular mechanisms involved in NETosis initiation is associated with the plethora of stimuli that ignite this process.

A main function of NETosis is to content the spread of infections by trapping pathogenic microorganisms (Mesa and Vasquez 2013), thus contributing to control the infection-mediated inflammation. However, in the development of this process, DAMPs are also released, which can be sensed by surrounding innate immune cells, triggering further inflammation. Due to this feed-back loop, NETosis becomes a highly inflammatory process (Tang et al. 2019), which has clinical implications in autoimmune diseases (Lee et al. 2017b).

3.6 Relevance of oxidative stress in cell death

In previous sections, we have explored in detail forms of PCD with well-described pathophysiological roles such as apoptosis, pyroptosis, necroptosis and NETosis (Jorgensen et al. 2017). Nevertheless, there are some other forms of cell death whose importance needs to be studied in further detail (Tang et al. 2019). However, a common feature for all the cell death programs is that, to some extent, the oxidative stress

contributes to their triggering. This oxidative stress results from the imbalance between ROS production and the antioxidant capacity of the cell (Ghosh et al. 2018). One of the main consequences of an excessive ROS presence in a cell is the peroxidation of lipids, main components of membranes which are indispensable for maintaining the structure and the functionality of the cell (Gaschler and Stockwell 2017). Lipid peroxidation drives different forms of cell death, such as ferroptosis (Dixon et al. 2012), pyroptosis (Kang et al. 2018), necroptosis (Canli et al. 2016) or NETosis (Palladino et al. 2018), among others.

As a consequence of all these death processes, cell rupture occurs, what would drive the activation of the immune system. However, ROS activity can regulate immune activation by altering the structure of otherwise pro-inflammatory components. This is the case for the High Mobility Group B1 (HMGB1) protein. HMGB1 is a nuclear component that acts as a prototypic DAMP that can be recognized by TLR4, triggering inflammation (Andersson and Tracey 2011). However, once oxidized by mitochondrial ROS during apoptosis, HMGB1 induces immunological tolerance (Kazama et al. 2008). Therefore, each specific cell death-associated situation needs to be deeply characterized in order to know its immunological impact.

4. *C-type lectin receptors in myeloid cells*

The myeloid lineage comprises a group of cells that derive from common myeloid progenitors in the bone marrow. Common myeloid progenitors give rise to diverse cell types, including mast cells, basophils, neutrophils, eosinophils, monocytes, macrophages and DCs, which belong to the innate immune system, and erythrocytes and megakaryocytes, which can contribute to the immune response, but have a

different primary function (Weiskopf et al. 2016). Immune myeloid cells comprise the main core (or classical core) of the innate immune system, characterized by their readiness to respond to insults and for not being antigen-specific. Among myeloid cells, DCs and macrophages continuously surveil tissues, having a dual role in the modulation of both innate and adaptive immunity. They are equipped with a diverse arsenal of PRRs composed by Toll-Like Receptors (TLRs), Nucleotide-binding domain, Leucine-Rich repeat-containing (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), AIM2-like receptors (ALRs) and RIG-I-like receptors (RLRs) (Brubaker et al. 2015). These receptors allow to specifically detect signals from their microenvironment, acting as central hubs that integrate information about environmental cues and possible threats. Thus, myeloid cells orchestrate immune function, collaborating in tissue homeostasis or healing, or instructing both innate and adaptive immunity.

The family of CLRs are of prominent relevance in sensing cell death by myeloid cells (Sancho and Reis e Sousa 2013). CLRs are characterized for bearing C-type lectin-like domains (CTLD), which in many cases includes a carbohydrate recognition domain (CDR). Their domain architecture is the base for grouping CLRs in up to 17 groups (Zelensky and Gready 2005). The CDR domain allows CLRs to recognize a wide range of glycans exposed on self and non-self-ligands (Iborra and Sancho 2014). In addition, we proposed a classification of myeloid CLRs based on their intracellular signaling motifs, in an attempt to classify this complex receptor family based on functional criteria (Sancho and Reis e Sousa 2012). Thus, myeloid CLRs can be broadly classified as Immunoreceptor Tyrosine-based Activating Motif (ITAM)-coupled CLRs, hemi-ITAM-(hemITAM)-bearing CLRs, Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)-containing CLRs, and a group of CLRs lacking typical signaling motifs (del Fresno et al. 2018). In here, we will

explore some selected myeloid CLRs that sense cell death, modulating inflammatory responses. The selection of these CLRs has not been done based on structural criteria, but on their paradigmatic roles in the detection of tissue damage by myeloid cells.

4.1. DNGR-1 (CLEC9A)

Dendritic cell Natural killer lectin Group Receptor-1 (DNGR-1, CLEC9A) is a CLR encoded in chromosomes 12 in humans and 6 in mice (Sancho et al. 2008; Huysamen et al. 2008). The expression pattern of DNGR-1 is very restricted to the dendritic cell lineage and, more specifically, to type I conventional DCs and common DC progenitors (Sancho et al. 2008; Schraml et al. 2013). Additionally, DNGR-1 is also expressed by mouse but not human, plasmacytoid dendritic cells (Sancho et al. 2008; Zilionis et al. 2019).

Structurally, DNGR-1 is a transmembrane protein that belongs to the group V of CLRs. These CLRs show an NK cell receptor-like architecture, having no CRD motifs and whose binding capacity to their ligands is calcium-independent (Sancho and Reis e Sousa 2012). In its intracellular region, DNGR-1 features a hemITAM signaling module, that can signal through the Spleen tyrosine kinase (SYK) (Sancho et al. 2009; Zelenay et al. 2012). DNGR-1 acts as a homodimer through a cysteine in its neck region. Under conditions of low pH or ionic strength, the neck region of DNGR-1 undergoes changes in its tertiary structure, driving resistance to disassembly of the homodimer (Hanč et al. 2016).

The only known ligand for DNGR-1 is filamentous actin (F-actin), a major component of the cellular cytoskeleton that can be accessible to immune cells upon loss of plasma membrane integrity, as occurs in different forms of cell death (Ahrens et al. 2012; Zhang et al. 2012). The docking site for DNGR-1 homodimers in F-actin involved

two contiguous monomers from one protofilament and the adjoining one from the other (Hanč et al. 2015). Moreover, myosin II helps stabilizing the interaction between F-actin and DNGR-1 (Schulz et al. 2018).

Upon recognition of F-actin, SYK is recruited to the hemITAM of DNGR-1. DNGR-1/SYK signaling promotes cross-presentation of dead cell-associated antigens in MHC-I, contributing to priming of CD8^{+} T cells. However, and contrary to Dectin-1, the recruitment of SYK to DNGR-1 hemITAM does not result in NF-κB activation or increased production of inflammatory cytokines (Zelenay et al. 2012). Also, DNGR-1 does not act as a scavenger receptor promoting the uptake of dead cells (Sancho et al. 2009). Instead, DNGR-1 signaling promotes the diversion of phagocytic cargo into non-acidic, non-lysosomal compartments, which prevents antigen degradation and promotes cross-presentation (Zelenay et al. 2012; Iborra et al. 2012). This phenomenon participates in shaping the repertoire of primary antiviral immune responses, without affecting the global effector response (Zelenay et al. 2012; Iborra et al. 2012). However, cDC1s and crosspriming of anti-viral CD8^{+} T cells through DNGR-1 contributes to the generation of tissue-resident memory CD8^{+} T cells specific for the viral threat (Iborra et al. 2016b).

Moreover, DNGR-1 can additionally limit inflammation in situations of tissue damage, a phenomenon termed disease tolerance (Soares et al. 2017). Recently, cDC1s have been involved in the recruitment of neutrophils to inflammation foci (Janela et al. 2019). In different models of tissue-specific injury, such as acute pancreatitis induced by caerulein and kidney aggression during systemic candidiasis, sensing of tissue damage through DNGR-1 in cDC1s restricts immunopathology (Del Fresno et al. 2018). Mechanistically, engagement of F-actin by DNGR-1 activates the phosphatase SHP-1,

which restrains NFκB activation by heterologous receptors and subsequent expression of proinflammatory mediators, such as TNF and CXCL2/MIP-2α that contribute to immunopathology (Del Fresno et al. 2018).

To date, whether SYK and SHP-1 signaling occur simultaneously or alternatively remains obscure. Intriguingly, the inhibitory ITAM configuration by which SHP-1 binds to the hypophosphorylated ITAM requires the transient binding and activation of Syk to the ITAM (Mkaddem et al. 2014; Iborra et al. 2016a). The conditions that determine the overall outcome of F-actin recognition in DNGR-1 signaling remain unknown. Here, the ionic composition of the microenvironment, which may fine-tune the conformation of DNGR-1 homodimers (Hanč et al. 2016), may be an important cofactor, providing versatility to DNGR-1 signaling. In this context, F-Actin constitutes the DAMP recognized by DNGR-1, while the environmental ionic strength would represent a modulating factor.

Considering the lack of activating signaling after DNGR-1 engagement and its regulatory role in heterologous pathways, the net inflammatory outcome after death recognition by DNGR-1 may be regulatory. However, surrounding signals to the DNGR-1-mediated dead cell sensing, such as adjuvants in the context of infection or vaccination, may switch these responses towards a pro-inflammatory result, considering the role of this receptor in antigen cross-priming.

4.2. MINCLE (*CLEC4E*)

Macrophage INducible C-type LEctin (MINCLE, CLEC4E) is a CLR encoded in chromosome 12 in humans and in chromosome 6 in mice. Mincle is expressed by

neutrophils (Lee et al. 2012), macrophages (Yamasaki et al. 2008), DCs (Martínez-López et al. 2019) and monocytes (http://www.immgen.com; (Heng et al. 2008)).

From the structural point of view, Mincle is a transmembrane protein that belongs to the group II of CLRs, which comprises CLRs with a single CRD relying in calcium for their ligand binding (Sancho and Reis e Sousa 2012). Mincle forms heterodimers with Macrophage C-type Lectin (MCL, CLEC4D), with which it shares a very similar structure (Furukawa et al. 2013; Lobato-Pascual et al. 2013). Mincle coordinates two Ca^{2+} ions that are responsible for the recognition of sugar moieties in its ligands (Furukawa et al. 2013). Neighboring its sugar binding sites, Mincle features a surface hydrophobic groove that binds long fatty acids (Feinberg et al. 2013; Decout et al. 2017). At the intracellular region, Mincle presents a short domain that does not contain any tyrosine-based regulatory motif. Instead, Mincle bears an arginine residue in its transmembrane region that mediates association with immunoreceptor tyrosine-based activation motif (ITAM)-containing FcRγ chain (Yamasaki et al. 2008; Ishikawa et al. 2009, 2013).

Mincle was identified as the long-sought receptor for chord factor, a proinflammatory molecule from *Mycobacterium* spp (Ishikawa et al. 2009; Schoenen et al. 2010). Since then, multiple microbial components have been shown to engage and activate Mincle, driving activation of myeloid cells (Lu et al. 2018). For instance, it promotes protective immune responses against bacterial sepsis after cecal ligation (Lee et al. 2017a) and also drives the activation of macrophages upon sensing *Malassezia* spp (Yamasaki et al. 2009), an infectious skin fungus whose surface glycolipids were defined as the specific Mincle ligand (Ishikawa et al. 2013). Furthermore, recognition of gut microbiota through Mincle contributes to reinforce the immune response in the gut

barrier (Martínez-López et al. 2019). In all these cases, engagement of Mincle induces the phosphorylation of SYK and downstream signaling through CARD9 and NFκB, leading to increased production of proinflammatory mediators, such as CXCL2/MIP-2α and TNF, promoting the recruitment of neutrophils to inflammation foci (Werninghaus et al. 2009).

However, Mincle also binds the endogenous Spliceosome-Associated Protein 130 (SAP130) (Yamasaki et al. 2008). This protein is part of the U2 Small Nuclear RibonucleoProtein (snRNP) complex, implicated in removal introns, and with a nuclear localization under steady-state conditions (Kiss 2004). The recognition of SAP130 by Mincle has been characterized in different settings. Upon SAP130 ligation, Mincle induces SYK-dependent production of the proinflammatory mediators TNF and CXCL2/MIP-2α. In irradiated thymi, Mincle promotes the production of CXCL2/MIP-2α in macrophages, driving inflammation (Yamasaki et al. 2008). In more physiologically relevant scenarios, the proinflammatory signals triggered by Mincle can be observed in mouse models of alcoholic liver injury. During alcoholic liver disease, microbiota-derived LPS can be detected by liver macrophages (Parlesak et al. 2000). Sensing of LPS induces the expression of Mincle in macrophages in an Interleukin-1 receptor-associated kinase M (IRAK-M)-dependent manner (Zhou et al. 2016). Mincle can sense endogenous SAP130 in alcoholic livers, amplifying SYK- and inflammasome-mediated IL1β production (Zhou et al. 2016; Kim et al. 2018). Thus, Mincle aggravates alcoholic liver disease, whereas Mincle-deficient mice display reduced neutrophilia, steatosis and fibrosis in their livers (Zhou et al. 2016; Kim et al. 2018).

In a tumor context, pancreatic ductal adenocarcinomas display stable expression of the necroptotic components RIP1 and RIP3. The genetic deletion of RIP3 or the chemical inhibition of RIP1 protected against tumor development, what was associated to an immunogenic infiltrate. Mechanistically, pancreatic adenocarcinoma cells exploited necroptosis induction to promote their growth by promoting the recruitment of tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs) in a CXCL1/KC-dependent manner, limiting the efficacy of chemotherapeutic agents. But additionally, necroptosis of pancreatic ductal adenocarcinoma cells also promotes the release of SAP130, with a parallel Mincle upregulation in tumor-infiltrating myeloid cells. Interestingly, Mincle-deficient mice phenocopied RIP3-decifient mice and were also protected against oncogenesis (Seifert et al. 2016). Furthermore, administration of trehalose dimycolate, a synthetic analog of the *Mycobacterium tuberculosis* ligand for Mincle, accelerates the growth of both RIP3-competent and -deficient tumors, suggesting that both SAP130 and the bacterial ligands for Mincle induce similar signaling and immune responses (Seifert et al. 2016).

Recently, two new endogenous ligands have been identified for Mincle, β-glucosylceramide (Nagata et al. 2017) and cholesterol sulfate (Kostarnoy et al. 2017). β-glucosylceramide is a ubiquitous metabolite released upon cell death. β-glucosylceramide induces the activation of DCs, promoting the production of proinflammatory mediators, such as TNF and CXCL2/MIP-2α. Thus, in a mouse model, thymic irradiation or mice deficient for the enzyme in charge of β-glucosylceramide degradation in the hematopoietic compartment show an increased recruitment of neutrophils to irradiated thymi, resulting in increased destruction of the organ. Of note, β-glucosylceramide can also induce the expression of costimulatory molecules in DCs *in*

vitro, and boosts priming of adaptive immune responses (Nagata et al. 2017). Similarly, Mincle can sense cholesterol sulfate, an abundant molecule in barrier epithelia. Sensing of cholesterol sulfate by bone marrow DCs promotes their maturation and the production of proinflammatory mediators such as IL1α, IL1β, KC, CCL3/MIP1α and CCL4/MIP1β. This is important in models of contact dermatitis, where Mincle plays a detrimental role, aggravating inflammation and increasing tissue abnormalities (Kostarnoy et al. 2017).

The discovery of β-glucosylceramide and cholesterol sulfate as ligands for Mincle with proinflammatory properties similar to SAP130 makes it difficult to discriminate their individual contribution to the inflammatory response induced by Mincle during tissue injury, as SAP130 is fundamental for the cell machinery and the others are widely produced metabolites. Overall, it could be concluded that cell death recognition by Mincle generates pro-inflammatory responses. Whether these responses eventually regulate inflammation by a third-party component, as described in the pancreatic adenocarcinoma context, illustrate the complexity of the outcome after cell death recognition. In this regard, sensing of *Leishmania* through Mincle can induce SHP-1 activation through an inhibitory ITAM configuration, restricting DC activation (Iborra et al. 2016a). Whether any endogenous ligand is capable of inducing Mincle signaling through an inhibitory ITAM configuration remains unknown. In any case, Mincle is a great example of a receptor that identifies *hyppos* as danger cues and modulates immunity accordingly.

4.3. CLL-1 (*CLEC12A*)

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CLEC12A is a receptor also known as MICL, CD371, KLRL1 or DCAL-2, but based on the usage frequency of these names, we proposed the consensus alias CLL-1 together with its gene name, *CLEC12A* (del Fresno et al. 2019). It is codified in chromosome 12 in humans and chromosome 6 in mice. CLEC12A is expressed by human monocytes, macrophages, polymorphonuclear cells (Marshall et al. 2004) and DCs (Han et al. 2004; Gurka et al. 2015), while in the mouse, it is expressed by both CD8$^+$ and CD8$^-$ spleen DCs, pDCs, B cells, thymic DN, DP and CD8$^+$ T cells, most of the granulocytes and myeloid cells, along with bone marrow NK cells (Kasahara and Clark 2012) (http://www.immgen.com; (Heng et al. 2008)). CLEC12A surface expression is downregulated in myeloid cells upon activation, both in humans (Marshall et al. 2006) and mice (Pyz et al. 2008), although receptor levels were increased upon experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS) (Sagar et al. 2017), suggesting a complex implication of CLEC12A in the control of myeloid activation during inflammation.

Structurally, CLEC12A is a transmembrane protein that belongs to the group V of CLRs (Sancho and Reis e Sousa 2012). It bears an ITIM motif in its intracellular domain that couples to the phosphatases SHP-1 and SHP-2, responsible for the regulatory role of CLEC12A upon inflammation (Marshall et al. 2004).

Early studies indicated that CLEC12A recognizes a non-microbial endogenous ligand (Pyz et al. 2008). Although it is now known that the receptor also senses *Plasmodium*-derived hemozoin (Raulf et al. 2019), CLEC12A is a well-established receptor for dead cells that recognizes monosodium urate (MSU) crystals and delivers regulatory signals. Consequently, *Clec12a*-deficient mice show exacerbated

inflammatory responses when challenged with MSU, necrotic cells or sublethal thymic irradiation (Neumann et al. 2014). In this line, CLEC12A-lacking mice are more sensitive to collagen antibody-induced arthritis (CAIA), which was reproduced by administering CLEC12A-blocking antibodies (Redelinghuys et al. 2016). Indeed, genetic variants of CLEC12A associate with rheumatoid arthritis in humans (Michou et al. 2012). These data support the regulatory role of CLEC12A in inflammation.

However, there are a number of reports showing a pro-inflammatory role for CLEC12A. Indeed, both antibody-based targeting and genetic deletion of CLEC12A, protect mice from EAE development, where CLEC12A facilitates the binding and transmigration of DCs across the blood-brain barrier (Sagar et al. 2017). Considering that CLEC12A recognizes MSU from dying cells (Neumann et al. 2014) and that serum uric acid reverses EAE progression (Scott et al. 2002), the maintenance of high uric acid levels upon CLEC12A-deficient conditions was argued as an explanation for the beneficial effect of its loss (Sagar et al. 2017). In any case, the distinct underlying mechanisms triggering different autoimmunity models (Lyons et al. 1999) may explain these apparently controversial results. Thus, during EAE, CLEC12A could show a "hijacking" effect over uric acid plus its contribution to DC infiltration, while for CAIA, CLEC12A may act in myeloid cells as an intrinsic regulator of the inflammatory response after sensing tissue damage. In addition, MSU recognition by CLEC12A amplifies cytosolic RNA-mediated IFN-I production, and therefore, *Clec12a*-deficient mice are sensitive to lymphocytic choriomeningitis virus (LCMV) infection. In this case, the molecular mechanism relies on Src kinase activation downstream CLEC12A, what promotes TBK1-IRF3 signaling and IFN-I production (Li et al. 2019).

Interestingly, CLEC12A expression is one of the most differentially upregulated surface markers in leukemic stem cells (Daga et al. 2019). Its combined expression with some other markers has been proposed as an efficient tool for detecting minimal residual disease in acute myeloid leukemia (AML) and, high CLEC12A levels along with CD123 are a strong prognostic marker for leukemia relapse (Roug et al. 2014). In fact, CLEC12A has been proposed as a therapeutic target in human AML (Williams et al. 2019), with successful antibody-drug conjugate (Jiang et al. 2018), bispecific antibody (van Loo et al. 2019) or CAR-T cell (Laborda et al. 2017) strategies. The relevance of cell death recognition in this context is a matter of debate. However, it is tempting to speculate that CLEC12A expression by tumor cells may help them to boost an anti-inflammatory environment after the recognition of surrounding dead cells, facilitating thus the scape from immunosurveillance. Overall, these data indicate that the sensing of tissue damage by CLEC12A can give rise to both pro-inflammatory and regulatory responses.

4.4. LOX-1 (*OLR1*)

The CLR LOX-1 is encoded by the *OLR1* gene and it is also known as LOXIN (del Fresno et al. 2019). It is codified in chromosome 12 in humans and chromosome 6 in mice. LOX-1 was first described as an endothelial receptor (Sawamura et al. 1997), but it is also expressed by human macrophages (Yoshida et al. 1998), monocytes (Draude et al. 1999) and DCs (Nickel et al. 2009), with a similar expression pattern in the mouse, where it is also expressed by bone marrow neutrophils (http://www.immgen.org; (Heng et al. 2008)). LOX-1 expression is low under steady-state conditions, but diverse inflammatory stimuli induce the surface expression of this CLR (Kattoor et al. 2019).

From the structural point of view, similarly to CLEC12A, LOX-1 is a transmembrane protein that belong to the group V of CLRs (Sancho and Reis e Sousa 2012). It does not bear any distinguishable ITAM or ITIM motif in its intracellular domain, what it is usually associated to CLRs with endocytic capacity that mediate antigen capture for further processing and presentation to T cells (Geijtenbeek and Gringhuis 2009). Notably, the lack of a defined intracellular module facilitates the involvement of this type of CLRs in flexible signaling pathways triggered by themselves or heterologous receptors (del Fresno et al. 2018).

The first described ligand of LOX-1 are oxidized-low density lipoproteins (LDLs) (oxLDL) (Sawamura et al. 1997). The involvement of this receptor in atherosclerosis was uncovered in atherosclerosis-prone mice ($LDLR^{-/-}$), which showed reduced pathology when crossed with $Olr1$-deficient mice, indicating that LOX-1 is pro-atherogenic (Mehta et al. 2007). OxLDL internalization after recognition by LOX-1 is a key event in the development of coronary artery disease, with LOX-1 genetic variants associated to increased risk (Tian et al. 2019). In fact, a serum-soluble cleaved form of LOX-1 is used as a diagnostic and prognostic tool in this pathology (Tian et al. 2019).

In addition to oxLDL, LOX-1 recognizes other endogenous ligands such as modified lipoproteins, activated platelets, heat shock proteins (HSP) (Huysamen and Brown 2009) and apoptotic cells (Oka et al. 1998). The recognition of apoptotic cells may occur by two alternative mechanisms. On the one side, LOX-1 directly recognizes phosphatidylserine on the membrane of cells undergoing apoptosis (Murphy et al. 2006). On the other side, the recognition and internalization of apoptotic cells may occur through LOX-1 binding to HSPs. HSPs are cytoplasmic compounds identified as stress-

responsive proteins, which are also translocated to the cell surface during different processes of cell death (Goh et al. 2011). In myeloid cells, LOX-1 binds different members of this family such as HSP-60 (Liu et al. 2019) or HSP-70 (Parlato et al. 2010). In this sense, HSPs facilitate the uptake of dying cells (Zhu et al. 2016). The functional consequences of HSP-60 recognition by LOX-1 is the activation of macrophages resulting in cytokine production (Liu et al. 2019) and the induction of antigen presentation in DCs after binding HSP-70 (Delneste et al. 2002), being the HSP-70-LOX1 interaction an underlying mechanism for the uptake of apoptotic cells (Parlato et al. 2010).

LOX-1 expression is upregulated in circulating and tumor-infiltrating neutrophils, while its expression is negligible in healthy volunteers. Interestingly, LOX-1+ neutrophils show transcriptional and suppressive characteristics of myeloid-derived suppressor cells (Condamine et al. 2016). Indeed, the expression of LOX-1 on polymorphonuclear cells defines highly immunosuppressive cells that correlates with recurrence of glioblastoma and disease progression (Chai et al. 2019). LOX-1-mediated delivery of immunosuppressive signals in a cell death-rich environment such as a tumor could be a strategy to escape from immunosurveillance. In any case, these data suggest that LOX-1 could be a marker for human MDSCs. The regulatory role for LOX-1 observed in these settings and whether it is neutrophil-specific deserves further investigation.

LOX-1-deficient mice show improved survival in a Cecal Ligation and Puncture (CLP) sepsis model. This phenotype is accompanied by a dampened systemic production of pro-inflammatory cytokines, while neutrophil infiltration into the peritoneum was boosted, favoring bacterial clearance (Wu et al. 2011). In this model LOX-1 plays and intriguing role, promoting inflammatory responses but suppressing neutrophil

mobilization, although indirect effects cannot be ruled out. LOX-1 has also been described as a surface receptor for both Gram-positive and Gram-negative bacteria (Shimaoka et al. 2001), where a bacterial HSP-60 homolog could be the specific LOX-1 ligand (Zhu et al. 2013). Therefore, it is difficult to interpret in this context whether the observed effects are dependent of bacteria or cell death recognition.

In summary, the immune balance after cell death sensing by LOX-1 could be described as pro-inflammatory due to its repercussion in myeloid activation and antigen presentation. However, although the implication of cell death recognition is not fully characterized in LOX-1$^+$ intratumor MDSCs or after infection, the triggering of LOX-1-mediated immunosuppressive signals cannot be discarded. An interesting hypothesis would be that different LOX-1 ligands trigger diverse inflammatory responses, either regulatory or pro-inflammatory.

4.5. CD301 (*CLEC10A*)

CLEC10A can be also named as CD301, CD301a, MGL-1, HML or CLECSF14 but based on their usage frequency, we proposed the consensus alias CD301 together with its gene name, *CLEC10A* (del Fresno et al. 2019). It is encoded in chromosome 17 in humans. In mice there are two homologous genes, *Clec10a*/CD301 and *Mgl2*/CD301b (Tsuiji 2003), both encoded in chromosome 11. CLEC10A is expressed by human monocyte-derived DCs (Higashi et al. 2002), CD1c$^+$ DCs (Heger et al. 2018) and macrophages, specifically in alternative activated macrophages (Raes et al. 2005), with a similar expression pattern in mouse DCs and macrophages (http://www.immgen.org; (Heng et al. 2008)). CLEC10A surface expression is downregulated upon DC maturation

while anti-inflammatory agents such as dexamethasone enhances CLEC10A expression both in macrophages and DCs (van Vliet et al. 2006). Structurally, CLEC10A is a transmembrane protein that belong to the group II of CLRs (Sancho and Reis e Sousa 2012). Similar to LOX-1, it does not bear any distinguishable ITAM or ITIM motif in its intracellular domain.

CLEC10A recognizes N-Acetylgalactosamine (GalNAc) residues present in glycoproteins and glycosphingolipids, which can be found in the surface of helminth parasites (van Vliet et al. 2005), tumors (Mortezai et al. 2013) and apoptotic cells (Malagolini et al. 2009). CLEC10A mediates the internalization of these cells and diverts the cargo to the phagolysosomal route (van Vliet et al. 2007).

Clec10a-deficient mice were generated to explore the role of this CLR in tissue homeostasis. The only noticeable defect of *Clec10a*-deficient mice is a mild increase in blood erythrocytes, which is caused by an impaired scavenging of aged erythrocytes by macrophages (Onami et al. 2002). However, the implication of CLEC10A in the sensing and removal of apoptotic cells is evident upon X-ray irradiation of pregnant mice, where massive apoptosis is induced in the developing embryos. Here, the number of pups was reduced in the absence of *Clec10a*, with a defective clearance of apoptotic cells in tissues suffering a vast apoptosis generation, such as the neural tube (Yuita et al. 2005).

In the same line, the lack of *Clec10a* originated evident phenotypes under pathological conditions running in the presence of massive cell death. This is the case for multiple sclerosis (MS). *Clec10a* expression is upregulated in MS lesions at the brain, particularly in P2Y12R$^+$ M2-polarized microglia (Ilarregui et al. 2019). In accordance with an immunosuppressive function for CD301 in this model, *Clec10a$^{-/-}$* mice display a

worsened outcome during EAE development at the resolution phase of the pathology (Ilarregui et al. 2019). Despite *ex vivo* experiments showed that CLEC10A induces the apoptosis of T cells, whether a defect in apoptotic cell removal accounts for the observed phenotype cannot be ruled out. Similarly, *Clec10a*-deficient mice are more susceptible to pulmonary infections by *Klebsiella pneumoniae* due to a severe lung immunopathology, despite a comparable bacterial burden (Jondle et al. 2016). In this case, also, the phenotype could rely in a defective clearance of dead cells due to the lack of CLEC10A.

Overall, CLEC10A acts as a regulatory CLR after sensing apoptotic events. This is in line with the anti-inflammatory signals generated by efferocytosis, i.e. the engulfment and clearance of apoptotic cells (Morioka et al. 2019). In fact, CLEC10A also regulates inflammation by recognizing stimuli different from apoptotic cells, such as commensal bacteria during colitis (Saba et al. 2009) or CD45 on the surface of effector T cells (van Vliet et al. 2006).

4.6. DEC-205 (*LY75*)

DEC-205 receptor is also known as Gp200-MR6 or CD205, but after analyzing the usage frequency of these names, we proposed the consensus alias DEC-205 together with its gene name *LY75* (del Fresno et al. 2019). It is encoded in the second chromosome in both human and mouse. While essentially any human hematopoietic cell express DEC-205 (Kato et al. 2006), in the mouse, it is expressed at low levels by B and T cells and granulocytes (Sancho and Reis e Sousa 2012), with a high expression in DCs (Jiang et al. 1995), in particular CD8^{+} DCs (Heath et al. 2004)

(http://www.immgen.com; (Heng et al. 2008)). From the structural point of view, DEC-205 belongs to the group VI of transmembrane CLRs, which includes CLRs bearing multiple CRD whose ligand binding capacity is calcium-dependent. The intracellular domain of DEC-205 is characterized by the absence of any identifiable ITAM or ITIM motif (Sancho and Reis e Sousa 2012).

DEC-205 can bind bacterial ligands (Zhang et al. 2008), oxLDL (Nickel et al. 2009) and apoptotic cells (Shrimpton et al. 2009). Using antibodies that bind DEC-205, early studies showed that this receptor internalizes antigens to the endo/lysosome in thymic epithelial cells and DCs (Jiang et al. 1995). Afterwards, it was demonstrated that this DEC-205-mediated endocytic capacity participated in the clearance of apoptotic thymocytes by thymic epithelial cells (Small and Kraal 2003). Eventually, DEC-205 was defined as a death-sensing receptor that recognizes cells undergoing apoptosis and secondary necrosis (Shrimpton et al. 2009). This binding is pH-dependent, requiring an acidic environment (Cao et al. 2015). This is evolutionarily consistent with the function of DEC-205 as a death receptor, because extracellular acidification usually associates with inflammation, constituting a danger signal (Rajamäki et al. 2013). Eventually, keratins have been described as the actual ligand for DEC-205 (Cao et al. 2016).

Most of the latest studies on DEC-205 are focused on its use as a target in antigen delivery strategies due to its expression pattern and endocytic capacity leading to antigen presentation (Iberg and Hawiger 2019). In this sense, the immune outcome can be modulated to promote immunity against viral infections (Padilla-Quirarte et al. 2019) or cancer (Johnson et al. 2008), or tolerance for diabetes treatment (Petzold et al. 2012).

There is not a clear picture of the inflammatory responses triggered after natural recognition of dead cells by DEC-205. As indicated for CLEC10A, the clearance of apoptotic cells may be silent for the immune system, but further studies are required to clarify this point. In addition, lessons learnt from targeting studies suggest that, depending on the surrounding context, DEC-205 may be quite plastic in the signaling delivered after its engagement, which constitutes a core property of CLRs (del Fresno et al. 2018).

5. *Conclusions*

During virtually any stress conditions, tissue damage and cell death take place, and the recognition of associated molecular cues by myeloid CLRs in these circumstances is key for tailoring immune responses. However, programmed cell death is also a relevant process that occurs under homeostatic conditions for the maintenance of tissue fitness. Here, we have dissected different forms of cell death and how these events condition the inflammatory responses triggered after their sensing.

Myeloid CLRs are main players in tissue damage sensing, displaying great functional plasticity, both in terms of ligand recognition and the inflammatory outcomes of this sensing. As CLRs are prompted to crosstalk, their specific ligand, the surrounding environment and other receptors simultaneously activated, are important factors to consider. All these variables can affect the functional consequences of the engagement of a particular CLR.

As described in this chapter, the recognition of cell death by myeloid CLRs can generate either regulatory or inflammatory reactions, impacting both innate and

adaptive immune responses. Therefore, understanding how myeloid CLRs (and other receptors) recognize certain cell death programs is fundamental to harness these responses for therapeutic purposes, as CLRs are surface molecules whose function can be easily targeted.

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