Clec2d Joins the Cell Death Sensor Ranks

Carlos del Fresno[1] and David Sancho[1]

[1] Immunobiology lab. Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC). Madrid 28029, Spain.

Contact details:

David Sancho: dsancho@cnic.es; @SanchoLab

SUMMARY

Sensing tissue damage is an ancient function of immune cells, that regulates inflammation, tissue repair and immunity. In this issue of *Immunity*, Ken Rock and colleagues uncover the role of Clec2d as a cell death receptor sensing released histones during necrosis, which contributes to inflammation and immunopathology.

PREVIEW

While physiological clearance of cells undergoing apoptosis is mostly immunologically silent, inflammation triggered after sensing of necrosis or certain forms of immunogenic apoptosis constitutes a warning during tissue damage events. This is related to Polly Matzinger's danger theory (refined in (Matzinger, 2002)), proposing that immune cells may become activated after sensing molecular traits released or exposed following tissue damage, defined as Danger-Associated Molecular Patterns (DAMPs), which would be otherwise sheltered intracellularly. In this line, macrophages and dendritic cells (DCs) are early damage sensors, as they are present in virtually any tissue, and are equipped with DAMP-sensing receptors. Activation of macrophages and DCs ignites the recruitment of other immune cells and the production of cytokines that contribute to inflammation and immunity, which can help to contain the insult and initiate tissue repair processes. However, production of proinflammatory cytokines, reactive oxygen species and some other immune effector mechanisms can cause undesired immunopathological damage to the host tissue. Therefore, it is fundamental to understand the mechanisms involved in the fine-tuning of the balance between the protective and detrimental effect of immune responses following sensing of tissue damage by myeloid cells. Ken Rock and

colleagues (Lai et al., 2019) seeked for new cell death receptors expressed in macrophages. They focused their attention in C-type lectin receptors (CLRs) as a versatile group with a C-type lectin domain that binds many endogenous ligands, and acting as DAMP sensors such as DNGR-1/*Clec9a* and MINCLE/*Clec4e*.

The authors generated T cell lines expressing the hemagglutinin (HA)-tagged ectodomain of the investigated CLRs fused to the CD3ζ intracellular domain and coupled to an NFAT/luciferase-based reporter {Sancho, 2009}. When these chimeric receptors are triggered either by binding their ligands or crosslinking with specific antibodies against the CLR or the HA epitope, luciferase is expressed, thus acting as a reporter for specific CLR binding. To test necrosis-derived ligands with these reporter cell lines bearing different CLR-CD3ζ chimeras, the authors treated mouse and human cell lines and primary mouse splenocytes either with UV-irradiation, freeze and thaw cycles or heat-shock, followed by 8 hours of incubation with the reporter cell line. Both supernatant from necrotic cultures and paraformaldehyde-fixed necrotic cells activated Clec2d-expressing reporter cells (but not Clec1b- or Clec4a2-expressing), while live cells did not. Using an alternative approach, ligands were FACS-stained on the surface of necrotic but not on live cells by Clec2d ectodomain fused to mouse IgG Fc. These data indicated that Clec2d was able to recognize ligand/s exposed and released by necrotic cells and evolutionarily conserved in mouse and human.

The authors next deciphered the biochemical nature of this ligand, showing that it was sensitive to proteases, but rather resistant to SDS-denaturation, suggestive of a primary polypeptide sequence. Taking advantage of this, the authors tested SDS-PAGE fractions in the cell reporter assay followed by mass spectrometry of those fractions that triggered the reporter assay, containing the Clec2d ligand/s. This clever approach led to the identification of histones as the Clec2d ligand. The activation of the Clec2d-expressing reporter cell line by thymus-purified and recombinant histones, and complementary pull-down experiments using histone-coated beads with soluble Clec2d confirmed that all five histones (H1, H2A/B, H3, H4) are ligands for Clec2d. This is a very relevant finding, as histones were already known DAMPs (Silk et al., 2017), but whether there was a dedicated receptor for them was not clear. A detailed mapping uncovered that, rather than a particular aminoacid sequence, a poly-basic motif found in all the histones was essential for binding to Clec2d. The alteration of this basic nature (either by mutation or acetylation) impaired the Clec2d binding capacity of histones.

Clec2d was early shown to bind sulfated glycosaminoglycans in a calcium-independent manner (Gange et al., 2004). Interestingly, histones can bind sulfated polysaccharides through their basic N-terminus region (Watson et al., 1999), the same that Lai *et al*. show now as critical for binding Clec2d (Lai et al., 2019). Taking all together, it is tempting to speculate that the binding of Clec2d to histones *in vivo* occurs via sulfated glycans bound to histones. In this sense, it would be interesting to know whether Clec2d activation by purified histones is sensitive to heparinase treatment.

To investigate the potential physiological relevance of Clec2d sensing of histones, the authors used a well-established model of hepatic injury by mouse exposure to an overdose of

acetaminophen/paracetamol (APAP), where histones are released systemically as a consequence of liver damage (Xu et al., 2011). Of note, serum from APAP-treated mice induced the activation of the Clec2d-expressing reporter cell, correlating with high serum levels of the hepatic enzyme alanine aminotransferase (ALT) as a readout of liver injury. To demonstrate that histones are the DAMPs recognized by Clec2d in this model, purified histones from the serum of APAP-treated mice activated the Clec2d-expressing reporter cell line, and this activation was abolished when histones were immunodepleted from the serum. The authors next addressed what are the pathophysiological consequences of sensing histones by Clec2d in this tissue damage model. *Clec2d*-deficient mice were protected during APAP-induced liver injury, showing reduced serum ALT levels and better survival. To connect this phenotype with histone recognition, intravenous administration of purified histones induced the production of serum proinflammatory cytokines in WT mice, which was reduced in *Clec2d*-deficient settings. All these evidences support the notion that Clec2d-sensing of histones released upon tissue injury in this model mediates cytokine-driven inflammation, leading to further late liver damage, although a direct demonstration is missing.

Exposure of Clec2d expressing macrophages to purified histones did not result in inflammatory cytokine production, suggesting that Clec2d is not a signaling receptor *per se*. The authors reasoned that histones are released as complexes with DNA that could be sensed intracellularly by endosomal TLR9. As they showed that Clec2d is expressed in macrophages at the surface but also in endo-phagosomes, they hypothesized that Clec2d could be trafficking histone-DNA complexes to endosomes to initiate TLR9-mediated inflammatory responses. After checking that the TLR9 ligand CpG binds to histones but does not activate Clec2d by itself, they showed that CpG and histone co-stimulation boosts the inflammatory response in WT macrophages. This synergistic response was dampened in *Clec2d*-deficient cells while *Tlr9*-deficient macrophages do not respond at all. The Clec2d-dependent synergistic effect on the inflammatory capacity of DNA was confirmed when using whole nucleosomes (complexes of histones and DNA) and again, this response was DNA-dependent, as DNAse I treatment killed cytokine production in response to nucleosomes. Finally, co-existence of DNA and Clec2d ligands was also shown in Neutrophil Extracellular Traps (NETs) after neutrophil activation. This is reminiscent of the role of High Mobility Group B (HMGB) proteins in binding immunogenic nucleic acids facilitating sensing by endosomal TLRs (Yanai et al., 2009). Since HMGB1 has polybasic regions, this opens the additional possibility that Clec2d may bind HMGB1 in an SDS-sensitive manner, and contribute to some of its effects.

The take-home message from this work is that Clec2d recognizes histones released during tissue damage and those histones are coupled to DNA; Clec2d carries the histone-DNA complexes to endosomal compartments where DNA can be sensed by TLR9, leading to inflammatory cytokine production (Figure 1). This damage-sensing pathway contributes to further inflammation and collateral tissue damage.

The precise Clec2d-dependent factors accounting for this secondary tissue injury are unexplored in this work. Direct inflammatory responses triggered in resident macrophages could

contribute to that tissue damage. Neutrophils and monocytes are recruited to the liver upon APAP treatment (Krenkel et al., 2014), with potential to mediate immunopathology. However, the relationship between Clec2d and Natural Killer (NK) cells deserves some attention. NKRP1d, an inhibitory NK cell-associated receptor was identified as a Clec2d ligand and therefore, NKRP1d dampens NK-mediated cytotoxicity when bound to Clec2d (Iizuka et al., 2003). Considering the discussed role of NK cells in the APAP-induced liver damage model (Krenkel et al., 2014), it would be interesting to elucidate the role of Clec2d in NK-mediated cytotoxicity, especially to translate this knowledge to other fields such as cancer immunotherapy.

In the original characterization of *Clec2d*-deficient mice generated by the lab of Dr. Gillespie, they described that 10 to 16 weeks-old mice show an osteopenic phenotype (Kartsogiannis et al., 2008). This is primarily due to the osteoblast-derived inhibitor role of Clec2d over osteoclast formation (Zhou et al., 2002), accounting for the OsteoClast Inhibitory Lectin (OCIL) nomenclature for Clec2d. In that seminal work, they reported “no apparent immune function defect” of *Clec2d*-deficient mice, as the loss of the receptor did not impact on the cellularity of immune populations nor the expression of activation markers in the steady-state (Kartsogiannis et al., 2008). Interestingly, mice used by Lai *et al*. were from the same source, which were further backcrossed to C57BL/6J for this work. For the APAP-induced liver injury experiments, 10 to 14 weeks old mice were used, which might be beginning to show bone alterations. It could be speculated that this fact could impact the immune phenotype upon experimental tissue damage conditions. Therefore, performing these studies in young mice would merit further studies.

In summary, this work places Clec2d as a new death cell receptor sensing histones as DAMPs. This bears important clinical implications as it would allow the targeting of just a single receptor instead of diverse histones in order to reduce immunopathology in certain tissue damage-causing pathologies. In addition, sensing histones from solid tumors with massive necrosis through Clec2d could be exploited to favor a pro-inflammatory anti-tumor response. In fact, tumor cells can show reduced global levels of histone modification, which may favor Clec2d sensing. This work thus opens a fascinating avenue of research in order to therapeutically exploit this new tissue damage sensing pathway.

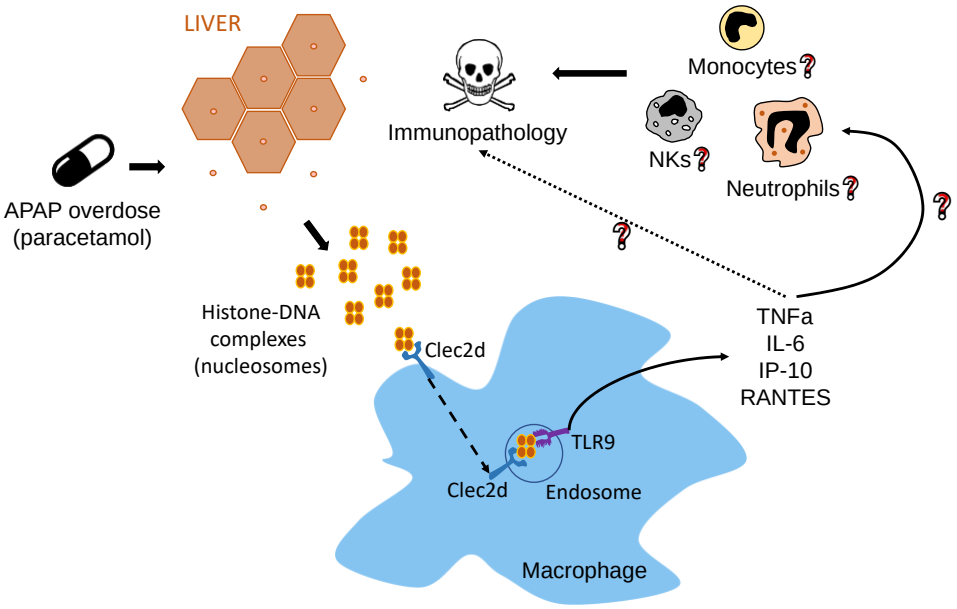

FIGURE LEGEND

Figure 1. Histones recognition by Clec2d contributes to immunopathology upon tissue damage conditions. Overdose administration of APAP (acetaminophen / paracetamol) triggers necrosis of hepatocytes, with a subsequent release of the intracellular content, such as nucleosomes containing histone-DNA complexes. Clec2d, expressed on the surface of macrophages, recognizes poly-basic motifs on histones, trafficking histone-DNA complexes into endosomal compartments. Endosomal TLR9 can then gain access to immunostimulatory DNA, triggering the expression of proinflammatory cytokines such as TNFα, IL-6, IP-10 and RANTES. This inflammatory response further contributes to liver injury by unaddressed mechanisms that may include direct collateral damage by the inflammatory cytokines or further recruitment of immune cells that would contribute to immunopathology.

REFERENCES

Gange, C.T., Quinn, J.M.W., Zhou, H., Kartsogiannis, V., Gillespie, M.T., and Ng, K.W. (2004). Characterization of sugar binding by osteoclast inhibitory lectin. J. Biol. Chem. *279*, 29043–29049.

Iizuka, K., Naidenko, O. V, Plougastel, B.F.M., Fremont, D.H., and Yokoyama, W.M. (2003). Genetically linked C-typed lectin-related ligands for the NKRP1 family of natural killer cell receptors. Nat. Immunol. *4*, 801–807.

Kartsogiannis, V., Sims, N.A., Quinn, J.M.W., Ly, C., Cipetić, M., Poulton, I.J., Walker, E.C., Saleh, H., McGregor, N.E., Wallace, M.E., et al. (2008). Osteoclast inhibitory lectin, an immune cell product that is required for normal bone physiology in vivo. J. Biol. Chem. *283*, 30850–30860.

Krenkel, O., Mossanen, J.C., and Tacke, F. (2014). Immune mechanisms in acetaminophen-induced acute liver failure. Hepatobiliary Surg. Nutr. *3*, 331–33143.

Lai, J.-J., Cruz, F.M., and Rock, K.L. (2019). Immune sensing of cell death through c-type lectin-receptor-2d recognition of poly- basic histone sequences causes inflammation and tissue injury. Immunity.

Matzinger, P. (2002). The danger model: A renewed sense of self. Science (80-.). *296*, 301–305.

Silk, E., Zhao, H., Weng, H., and Ma, D. (2017). The role of extracellular histone in organ injury. Cell Death Dis. *8*, e2812.

Watson, K., Gooderham, N.J., Davies, D.S., and Edwards, R.J. (1999). Nucleosomes bind to cell surface proteoglycans. J. Biol. Chem. *274*, 21707–21713.

Yanai, H., Ban, T., Wang, Z., Choi, M.K., Kawamura, T., Negishi, H., Nakasato, M., Lu, Y., Hangai, S., Koshiba, R., et al. (2009). HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature *462*, 99–103.

Zhou, H., Kartsogiannis, V., Quinn, J.M.W., Ly, C., Gange, C., Elliott, J., Ng, K.W., and Gillespie, M.T. (2002). Osteoclast inhibitory lectin, a family of new osteoclast inhibitors. J. Biol. Chem. *277*, 48808–48815.

ACKOWLEDGMENTS

CdF is supported by AECC Foundation (INVES192DELF). Work in the DS laboratory is funded by the CNIC; by the European Research Council (ERC-2016-Consolidator Grant 725091); by the European Commission (635122-PROCROP H2020); by Ministerio de Ciencia, Innovación e Universidades (MICINN), Agencia Estatal de Investigación and Fondo Europeo de Desarrollo Regional (FEDER) (SAF2016-79040-R) ; by Comunidad de Madrid (B2017/BMD-3733 Immunothercan-CM); by FIS-Instituto de Salud Carlos III, MICINN and FEDER (RD16/0015/0018-REEM); by Acteria Foundation; by Atresmedia (Constantes y Vitales prize) and by Fundació La Marató de TV3 (201723). The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the MICINN and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (SEV-2015-0505).