Charcot–Leyden crystals and other protein crystals driving type 2 immunity and allergy

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Protein crystals derived from innate immune cells have been synonymous with a Type-2 immune response in both mouse and man for over 150 years. Eosinophilic Galectin-10 (Charcot–Leyden) crystals in humans, and Ym1/Ym2 crystals in mice are frequently found in the context of parasitic infections, but also in diseases such as asthma and chronic rhinosinusitis. Despite their notable presence, these crystals are often overlooked as trivial markers of Type-2 inflammation. Here, we discuss the source, context, and role of protein crystallization. We focus on similarities observed between Galectin-10 and Ym1/2 crystals in driving immune responses; the subsequent benefit to the host during worm infection, and conversely the detrimental exacerbation of inflammation and mucus production during asthma.

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Introduction

Most proteins can only function properly in the soluble state and the occurrence of spontaneous protein aggregation or crystallization *in vivo* remains an unsolved scientific enigma. Although protein aggregation is generally seen as detrimental, the *in vivo* formation of protein crystals can offer selective advantages for the organism. The specific properties of the closely packed crystalline state allow space-efficient storage or sequestration of proteins for later usage, protection from proteolytic degradation and prevention of toxicity [1]. Conversely, through their physicochemical properties and triggering of specific pathways, crystalline protein deposits can also damage cells and tissues, compromise cell viability, and cause deleterious structural and functional tissue damage [1], a pathogenic phenotype often seen in type 2 inflammation associated with parasitic infections and asthma. How crystal deposits can be tolerated under physiological conditions in particular tissues, yet contribute to disease progression in others is poorly understood. In most cases, the impact of a phase transition to the crystalline state on the protein bioactivity is unknown and it is not clear whether crystals are disease-drivers, harmful side-effects, or harmless bystanders reporting high local protein concentration [2]. Here, we will discuss two proteins which form highly abundant crystals in humans and mice during pathological type 2 immunity such as asthma and chronic rhinosinusitis with nasal polyps (CRSwNP). We will highlight the impact of the crystal structure on protein immunogenicity as well as the potential pathophysiological effect of a phase transition to the crystalline state.

Charcot–Leyden crystals (CLCs)

Many human diseases driven by type 2 immune responses such as asthma and CRSwNP are characterized by accumulation of Charcot–Leyden Crystals (CLC) in the tissues. CLCs were first described in the late 1800s by Charcot [3] and Leyden [4], who identified needle-shaped bipyramidal crystals in a leukemic patient and asthmatic sputum, respectively. Since their identification over 150 years ago, many descriptions of CLCs, ranging in sizes from <10 to >70 μm, have been published in various organs ranging from the sputum to the lymph nodes and spleen [5]. Since their first description, a unifying feature of CLC deposition has been the colocalization with excessive tissue eosinophil infiltration, a finding eventually also leading to the purification and biochemical characterization of CLCs from purified eosinophils and eosinophilic leukemic cells [6,7]. CLCs are comprised solely of galectin-10, a highly abundant protein in the cytoplasm of eosinophils, which is involved in granule biogenesis [8]. Galectin-10 represents 7–10% of the cytoplasmic content of these cells [7], making it the

fifth most abundant protein in peripheral blood eosino-phils [9]. Galectin-10 mRNA and protein expression, as well as the presence of extracellular CLCs have thus become markers of tissue eosinophilia and/or activation across a number of diseases. Importantly, in respiratory diseases such as asthma, CRSwNP and allergic bronch-opulmonary *aspergilliosis* (ABPA), which can present with multiple endotypes, galectin-10 protein and CLCs are more frequently associated with a type 2 signature, and potentially a sign of intense eosinophil activation [10–14]. Even in COVID-19, where eosinophil activation seems to be linked to disease severity, high levels of Gal-10 were measured in critically ill compared to patients with mild disease, but so far CLCs have not been reported [15].

While galectin-10 has proven to be a useful biomarker of eosinophilic and type 2 inflammation, the extracellular crystallization of this protein is far more enigmatic and our understanding of the role of CLCs in inflammation is woefully lacking. Since galectin-10 expression is limited to humans and a small number of non-human primates, with no equivalents found by protein sequence compari-son in rodents, mechanistic research on CLCs in disease pathogenesis has been lagging behind [16]. CLCs form when eosinophils undergo intense activation, often releasing their nuclear DNA in a process called eosinophil extracellular trap (EET) formation [17●●,18]. Also hypoxia and alterations in extracellular pH seem to favor CLC formation, at least in *in vitro* experiments [17●●,19]. The formation of CLC crystals is by no means an immunolog-ically inert process. The administration of recombinant CLCs to mice revealed several important contributions of these crystals to inflammation and type 2 immunity [17●●]. Firstly, the administration of crystalline, but not soluble galectin-10 to the airways of mice proved to be a potent immune stimulus, inducing a swift local production of inflammatory cytokines (IL-1β, IL-6, TNFα) as well as the recruitment of neutrophils, Ly6C+ monocytes and dendritic cells. These results are paralleled in human studies, where CLCs induced IL-1β induction from human macrophages, in a process involving crystal phago-cytosis and triggering the NLRP3 inflammasome [20]. The recruitment of neutrophils and accompanying cyto-kine production was also seen following stimulation of nasal polyp tissue from CRS patients with CLCs, but not soluble protein [21●]. Furthermore, it has been demon-strated that, once recruited, GM-CSF-primed neutrophils undergo NETosis following stimulation with CLCs [21●]. NETosis, in which neutrophils release their internal contents, including long DNA fibers to form webs which trap and combat pathogens [18], itself can act as an immune stimulus and propagate inflammation. Both human macrophages and murine lung dendritic cells (DCs) are capable of phagocytosing CLCs, the latter of which traffic to the lymph node to initiate T cell responses [17●●,20]. The administration of CLCs together with innocuous antigen, that is, ovalbumin, was able to boost the antigen-specific T cell response in mice which was associated with increased antigen-specific antibody responses [17●●]. High levels of galectin-10 coming from a subset of CD16+ human eosinophils may also have a role in suppression of proliferating T cells [22].

Apart from recruiting and activating immune cells, CLCs can induce mucus production by epithelial cells. Over-production of mucus is an important clinical manifesta-tion of many CLC-associated pathologies including asthma, CRS and ABPA; in which the crystals are often found impacted in the characteristically sticky and eosin-ophil-rich 'allergic mucin' of these diseases. As well as their ability to induce *Muc5ac* and mucus production in mouse lungs [17●●], CLCs may additionally interact with the mucus via a carbohydrate recognition domain (CRD) to provide a physical scaffold, similar to barbed wire, which can make the mucus more tenacious and difficult to expectorate. The further induction of eosinophil and neutrophil extracellular traps has also been shown to contribute to the increased elasticity and viscosity of allergic mucin, and extracellular DNA can be found in the sputum of a subset of severe asthmatics [23,24●]. Ligands capable of interacting with the CRD of galec-tin-10 remain elusive, and so far only ribose and mannose have been demonstrated as binding partners [17●●,25], while many glycan arrays using galectin-10 have proved disappointing [8]. However, many of these studies used galectin-10 as a dimeric protein. We speculate that the organization of galectin-10 protein molecules in a crystal-line lattice may afford multiple binding sites with carbo-hydrate ligands, and thereby increase the avidity of what may be a low-affinity interaction, potentially not identi-fied using soluble protein alone.

Inflammatory crystalline proteins in mice
In mice, 'type 2-high' responses also trigger the formation of protein crystals. These murine crystals have been documented in mouse tissues since 1905 and are fre-quently observed in aged mice, genetically altered mice particularly those generated on a C57BL/6 or Sv/129 background [26], but also in the context of asthma and parasitic and fungal infections [27,28], or mice subjected to inhalation toxicity studies for particulates and tobacco [29]. The lung appears to be the main site of crystal accumulation, associated with increased mucus produc-tion [26,30,31] with manifestations generally known as eosinophilic crystalline pneumonia, or alternatively referred to as acidophilic macrophage pneumonia or crystalline pneumonitis [31–33]. It was not until the 2000s that these murine crystals could be purified from the bronchoalveolar lavage fluid of viable motheaten mice [26] and hyaline gastric lesions of aged 129S4/SvJae and B6,129 CYP1A2 null mice [34]. These pseudo-CLCs are made up of the two closely related proteins, Ym1 (*Chil3*) and Ym2 (*Chil4*); members of the family of chitinase-like proteins (CLPs), thus called due to their chitin binding

domain, yet absence of chitinolytic activity. The main cellular sources of Ym1 and Ym2 are macrophages and neutrophils, but expression can also be induced in dendritic cells, monocytes, mast cells and airway epithelial cells [35,36[•]]. In the lung, Ym1 is the most prominently expressed CLP under steady state conditions and can be detected from embryonic day 18.5 on [37]. Decreased lung *Chil3* transcript levels in late embryonic and adolescent lung are a genetic determinant of pulmonary function associated with lower basal lung capacity [38]. Ym2, in contrast, is barely detectable in the lung, but high in the stomach [39–41]. A dramatic induction of Ym2 is seen during Th2-high pathologies, in which Ym2 replaces Ym1 as the major lung CLP. In this context, Ym proteins are best known as IL-4/IL-13-inducible and highly expressed markers of alternatively activated macrophages (AAMs) that are involved in wound healing, tissue repair and initiation of fibrosis [28,42–44], type 2-dependent processes that contribute to normal lung development but also pathologic airway remodeling [45,46]. Whether this is mediated through the binding of Ym1 to components of the extracellular matrix such as heparin and heparan sulfate is still debated [47,48]. In addition, neutrophil-derived Ym1 was suggested to be taken up by wound-healing macrophages [44] and impaired wound healing in diabetic mice was associated with reduced Ym1 levels [49]. Ym1-deficient macrophages showed an enhanced AAM phenotype, suggesting that Ym1 might act as a brake to control or limit the induction of AAM polarization [50[••]].

There is a high similarity between Ym1 and Ym2, and currently there is a lack of specific tools capable of distinguishing these two proteins. Therefore, the distinct roles of Ym1 versus Ym2 is difficult to address, and we refer to these as Ym1/2 where they cannot be reliably discriminated in the literature. During parasite infection, Ym1/2 protein has been suggested to be directly chemotactic for eosinophils [51]. However, no or only weak eosinophil chemotactic activity could be detected either in *in vitro* or *in vivo* assays with purified Ym1/2 [28,47] and Ym1/2 expression was actually found to be dispensable for eosinophil recruitment during allergic peritonitis or *Trypanosoma brucei brucei* infection [42,43]. More recently, an anti-parasite effector mechanism of Ym1/2 was highlighted, not by recruiting eosinophils, but by inducing neutrophil accumulation through the IL-1 dependent expansion of IL-17A-producing γδ T cells. The Ym-dependent regulation of anti-helminth type 2 immune responses was found to be bi-phasic, showing opposing effects in early versus late phases of infection. While innate IL-4Rα-independent Ym1/2 promoted the development of an early reparative type 2 response, adaptive IL-4Rα-dependent Ym1/2 was important for limiting the magnitude of type 2 response, by reducing type 2 cytokine production from both ILCs and CD4^{+} T cells in the lung at later stages of infection [52[•]]. Adaptive Ym1/2 also

triggered lung repair via enhanced RELMα expression by epithelial cells, which regulated the collagen cross-linking enzyme lysyl hydroxylase 2, a pathway partly responsible for the pro-repair effects of Ym1/2 [52[•]].

Apart from parasitic infection, Ym1 and Ym2 are among the most strongly induced proteins in the airways after allergen exposure [53–56]. Ym1/2, expressed by DCs in response to IL-13, primed DCs to stimulate Th2 cytokine production by CD4^{+} T cells and promoted the development of allergic airway inflammation by inhibiting the production of 12-hydroxyeicosatetraenoic acid by 12/15 (*S*)-lipoxygenase. Blocking Ym1/2 has been shown to attenuate mediastinal lymph node production of IL-5 and IL-13 [57] and to significantly reduce ovalbumin-induced allergic airway inflammation [50[••],58,59].

Outlook and conclusion

Type 2 immunity is thought to have evolved as a parasite defense mechanism and as an innate tissue repair process to mitigate the damage caused by the infection. These processes, however, are also associated with more 'modern' diseases such as asthma and allergies as a consequence of an exaggerated immune response to innocuous environmental antigens (Figure 1). Although found in different animal species and produced by distinct cell types, both galectin-10 and Ym1/2 crystals are prominent components of these prototypic type 2-driven pathologies. The convergent evolution of CLCs and Ym1/2 crystals in type 2 immunity strongly suggests a selective advantage of these protein crystals in the enhancement of type 2-driven immune responses. The phase transition from a soluble state to a crystalline lattice in type 2-polarized environments might provide structural support and initiate remodeling processes and repair after tissue damage. Alternatively, these proteins exert their function in the soluble state, and phase transition to the crystal form is a rapid, potentially reversible way to contain their bioactivity in an anatomically restricted manner, as seen within and around plugged airways. While such enhancement proves beneficial during normal organ development or after parasite infections, mechanisms that transform these tissue-regenerative type 2 responses into progressive fibrotic disorders, as in the case of asthma, remain unclear. Studies into the biology of CLCs and pseudo-CLCs *in vivo* have been hampered since mice do not express the gene encoding for galectin-10 and no Ym1/Ym2 (*Chil3/Chil4*) knock out strains have been described to date. The potential cell-surface receptors for galectin-10 and Ym1/Ym2 that mediate their pro-type 2 effects are still unknown, as are the natural ligands associating with the crystal structure. In addition, due to the high amino acid sequence identity of Ym1/2 proteins and the lack of specific tools to discriminate between the two homologues, it has so far proved problematic to define their unique functional characteristics and individual contribution to steady state and disease conditions.

Figure 1
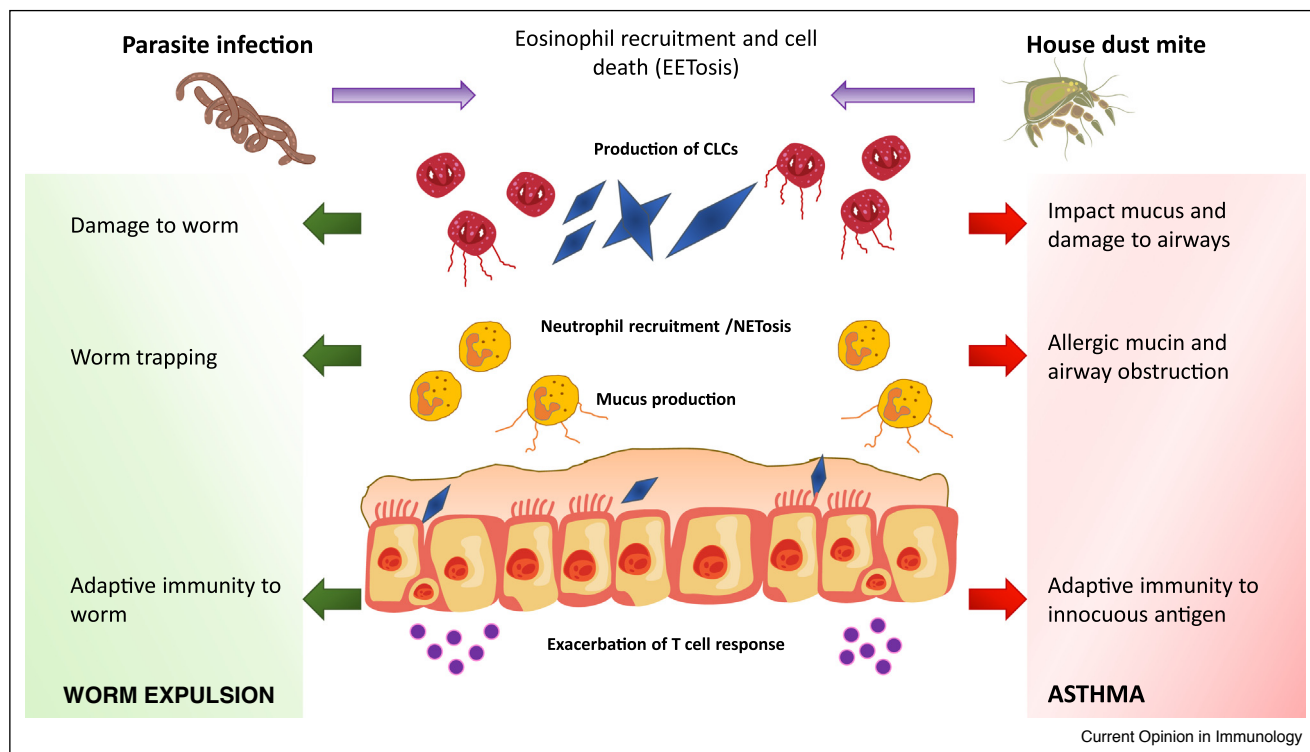
Overview of the role of CLCs in type 2 immune responses in humans. Many of these responses are postulated for the murine protein crystals Ym1/2 in type 2 immunity.

The temporal, context and cell-specific expression profiles of Ym1/Ym2 proteins, however, strongly suggest that these proteins might exhibit essential non-redundant roles in periods of maximal tissue remodeling.

Eosinophils are well established to be one of the most important effector immune cells in parasitic infections and eosinophil-derived CLCs have been identified in the presence of parasitic infections [60]. CLCs were present in 20% of over 10 000 stool samples of patients with *Entamoeba histolytica* [61], particularly in the vicinity of mucus of an amoebic ulcer. Although the role of CLCs has not been directly studied in these contexts, there are many functions of CLCs which could be easily conceived as beneficial to combat worm infection. Firstly, the production of CLCs is a result of EETosis, in which eosinophils undergo a programmed form of cell death to exude significant chromatin fibers as well as granular proteins with anti-helminth properties [18]. The webs formed by eosinophil cell death could assist worm trapping and the needle-shaped protein crystals may themselves induce significant damage to the parasite. Additional NETs can be induced by neutrophils, recruited after CLC production, that further trap the parasite and contribute to tenacious mucus. It is apparent that CLCs have a more potent effect than soluble protein in terms of immune cell

activation. However, in its soluble form, galectin-10 plays important roles in eosinophil development [8] and it is conceivable that extracellular soluble protein may have as yet undescribed roles. One report demonstrated the presentation of galectin-10 on EETs to suppress Th2 cells [22] and galectin-10 bound to secreted vesicles may be taken up by other cells to initiate cell activation.

Understanding the requirements for CLC and pseudo-CLC formation and their function during Th2-high pathologies might help identify pathways associated with excessive crystal-driven type 2 inflammation. Therefore, much more work is needed to characterize the concentration and crystallization state of these proteins, together with their role in diseases such as asthma and CRSwNP. Recently, a series of antibodies were developed that could rapidly dissolve existing CLCs by binding to a key amino acid residue in the crystal-packing interface. These antibodies not only helped to dissolve *in vitro* generated CLCs, but also CLCs obtained from the mucus of CRSwNP patients [17••]. It is an exciting prospect that further clinical development of these antibodies could address the real contribution of CLC protein crystals in chronic airway diseases characterized by intense eosinophil activation. Like for most biologicals, the challenge will be to define the correct patient that could benefit from this type of therapy, and

finding out what this biological might offer over other type 2 and eosinophil-targeting therapies.

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Conflict of interest statement

Bart Lambrecht has received consultancy fees from AstraZeneca, Sanofi, GSK, Novartis, Oncoarendi and Argenx. These do not affect content of this article.

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