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Chitins and Chitinase Activity in Airway Diseases

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Abstract

Chitin, one of the most abundant biopolymers on earth, is bound and degraded by chitinases, specialized enzymes that are similarly widespread in nature. Chitin catabolism impacts global carbon and nitrogen cycles through a host of diverse biological processes, but recent work has focused attention on systems of chitin recognition and degradation conserved in mammals, connecting an ancient pathway of polysaccharide processing to human diseases influenced by persistent immune triggering. Here, we review current advances in our understanding of how chitin-chitinase interactions impact mucosal immune feedback mechanisms essential to maintaining homeostasis and organ heath.

Introduction

Understanding the interactions between natural environmental constituents and mucosal surfaces is a critical element in deciphering human health and disease. Among these constituents, polysaccharides represent abundant structural elements of plant and animal biomass that are degraded in a wide range of processes that not only provide nutrients but also dictate microbial, epithelial, and immune cell composition at barrier tissues (1,2). The polysaccharide chitin has long been studied as an energy source, bacterial substrate, and structural component of terrestrial and aquatic invertebrates, as well as a molecular pattern that triggers immune responses and morphogenesis in several sophisticated recognition systems in plants and animals. As a structural component of parasites, fungi, insects, and crustaceans, chitin represents an insoluble scaffold on which a multitude of antigenic glycoproteins are arrayed, and thus its degradation plays a crucial role in determining subsequent immune responses. Although chitin-binding proteins and chitinase enzymes are highly conserved throughout evolution, their roles in mammalian physiology have been

elusive. New appreciation of naturally-occurring chitin structures along with novel mouse models, however, has revealed conserved function for these family members in mediating feedback between mucosal immune responses and environmental drivers of disease.

An evolutionary perspective

Chitin and cellulose are the most abundant polysaccharides on earth, representing stable structural elements in most invertebrates and plants, respectively. Like cellulose, chitin is a simple linear homopolysaccharide, consisting of β(1–4)-linked N-acetyl-D-glucosamine (GlcNAc), generated by chitin synthases of chitin-producing organisms and likely extruded from the plasma membrane in a manner similar to cellulose (3,4). De-acetylation of chitin generates chitosan, comprised of N-glucosamine polymers that allow less interchain hydrogen bonding and thus are more soluble in weak acid than chitin. In nature, extended chitin polysaccharide chains form microcrystalline fibers that arrange into highly hydrogen-bonded antiparallel (α-chitin), parallel (β-chitin), or mixed (γ-chitin) arrays. The most common form is α-chitin, which is a major structural element in fungal cell walls and invertebrate exoskeletons; notably, some animals employ multiple forms of chitin for seemingly different biological functions, e.g., the squid (*Loligo*), synthesizes α-chitin in its beak, β-chitin in its pen (gladius), and γ-chitin in stomach lining (5). The extensive interchain hydrogen bonding endows chitin with unique stability that places it among the toughest and most resilient natural materials on earth, remaining recalcitrant under the most extreme temperature and pressure conditions (6), and surviving intact within fossilized insects, egg capsules, and even a 505-million-year-old fossil marine sponge from the Burgess Shale (7,8,9). As a structural component, chitin is often mineralized, or in complex with proteins, phenols, lipids, or other carbohydrates such as β-glucans, that crosslink to form highly ordered macromolecular scaffolds. As such, the mechanical properties, stability, and organization of chitin-containing composite structures such as insect cuticle, squid beak, nacre, etc., have inspired their use as natural and synthetic templates for a wide range of bioengineered materials (10,11).

The formidable resilience and abundance of chitin does not translate into large-scale accumulation of chitin in the environment, however, due to the similarly widespread distribution of specialized chitin-binding proteins that include chitinases, enzymes that digest polymeric chitin. Chitinolytic enzymes can be broadly grouped by mode of cleavage: endochitinases (EC 3.2.1.14), which hydrolyze internal chain linkages, and exochitinases (EC 3.2.1.52), which catalyze β(1–4)-hexosaminidase activity that cleaves GlcNAc residues from the non-reducing end of a chitin chain (Figure 1). Further classification into several glycosyl hydrolase (GH) families is based on sequence and structural homology, with GH18, GH20, and GH84 sharing a similar $(\beta/\alpha)_8$ triosephosphate (TIM)-barrel catalytic domain; many chitinases additionally include highly specific chitin-binding domains (CBDs) that enhance anchoring to insoluble substrates. GH18 domain architectures and structural features are highly conserved and show remarkable phylogenetic diversity, including all mammalian chitinases and enzymatically inactive chi-lectins, as well as chitinases from viruses, bacteria, fungi, plants, and animals (12,13).

Strategies for disassembling and utilizing natural chitin substrates, like the substrates themselves, are necessarily complex, often coordinated in chitinolytic organisms by several chitin-binding proteins and active chitinases comprising a multimodular molecular machine, akin to cellulosomes that deconstruct plant cell wall polysaccharides (1,14). In marine environments, *Vibrios* initiate chitin sensing, chemotaxis, attachment, cleavage, uptake and catabolism to mediate the turnover of enormous amounts of crustacean chitin (15). A multi-protein chitinolytic system is also employed by *Serratia marcescens*, in which a lytic polysaccharide mono-oxidase (LPMO) chitin-binding protein introduces intra-chain breaks which allows an endo-acting non-processive chitinase and two processive chitinases moving in opposing directions on the chitin polymer to liberate smaller oligosaccharides, which can be then be converted to GlcNAc by an additional chitobiase (16). A polysaccharide utilization locus specific for chitin conversion, like the starch utilization system (Sus) in *Bacteroides thetaiotaomicron*, has been described for the soil saprophyte *Flavobacterium johnsoniae*, which couples efficient chitin hydrolysis with scavenging, importing, and metabolizing chitin oligomers via multiple GH18 chitinases, two SusC/D-like outer membrane proteins, an inner membrane transporter, β(1–4)-hexosaminidase, and a glucosamine-6-phosphate deaminase (17). Other bacteria induce multiple enzymatic activities in concert with chitinases to achieve maximal growth in a competitive environment, such as *Clostridium phytofermentans*, which expresses high levels of cellulases and chitinases that it uses to degrade and consume both plant cellulose as well as the chitin from accompanying fungal species, respectively (18).

Plants themselves also enlist sophisticated strategies for recognizing and responding to environmental chitin signals across a wide range of symbiotic and defensive functions. In ripening grape skin, for example, increased chitinase and glucanase activities may ward off attack by fungi and insects (19), and form prominent components of pathogenesis-related (PR) proteins that are induced in response to wounding, stress, or pathogen exposure. Rice plants and *Arabidopsis* express LysM receptor-like kinases that recognize chitin and peptidoglycan (20), although certain fungal plant pathogens deploy similar LysM-domain proteins in a competitive manner to conceal chitin as a counter-strategy (21). Chitin-based defense signals show a high degree of specificity and may have evolved in parallel with comparable lipo-chitooligosaccharide recognition systems in plant roots that are critical to establishing symbiotic relationships with mycorrhizal fungi and nitrogen-fixing bacteria (20). Intriguingly similar systems for chitooligosaccharide recognition form the basis of the symbiotic colonization by the luminescent bacteria *Vibrio fisheri* within the light-emitting organ of the Hawaiian bobtail squid (22).

Conservation in mammals

Although mammals do not synthesize chitin, they retain a battery of chitin-binding proteins and active chitinases that show evolutionarily conserved functions on chitin substrates. Phylogenomic evidence indicates a significant expansion of homologous GH18 family chitinases and chi-lectins coincident with a duplication event early in the vertebrate linage, after the divergence of jawless fish, that generated ancestral forms of genes encoding the two active endochitinases in mice and humans, chitotriosidase (Chit1) and acidic mammalian chitinase (AMCase; 13). Interestingly, the proximity between chitinase loci and

histocompatibility complex paralogon genes on human chromosome 1 indicates that the expansion of the chitinase family corresponded with the acquisition of adaptive immunity (23). Evolutionary pressure is evident in syntenic chitinase genomic regions in mammals and other vertebrates, where genes encoding active chitinases occur in multiple copies, often flanked by inactive chi-lectins and pseudogenes as a result of gene duplication and loss. In mouse, for example, the chi-lectin Brp39 (*Chil1*) is positioned near to chitotriosidase (*Chit1*), while AMCase (*Chia1*) is flanked by the chi-lectins oviductin (*Ovgp1*), Ym1 (*Chil3*), Ym2 (*Chil4*), Bclp (*Chil5*), BYm (*Chil6*), and a Chia1 pseudogene. Similarly, human YKL-40 (CHI3L1) is positioned next to CHIT1, and AMCase (CHIA) is flanked by oviductin (OVGP1), YKL-39 (CHI3L2) and several CHIA pseudogenes.

Mammalian chitinases and chi-lectins contain N-terminal signal peptides consistent with their characterization as secreted proteins, and both Chit1 (24,25) and AMCase (26,27) have been crystallized, revealing the conserved chitin-binding cleft and DxxDxDxE active site motif common to GH18 family members. Chi-lectins, such as YKL-39, retain this fold and chitooligosaccharide binding specificity, but contain substitutions in critical active-site residues required for catalysis that can be reverted to restore chitinase activity (28). In Chit1 and AMCase, the catalytic domain is linked via a serine-threonine-rich hinge domain to a highly specific C-terminal chitin-binding domain, another widely conserved feature of GH18 family members (12). Recombinant Chit1 and AMCase both exhibit processive endochitinase activity on chitooligosaccharide and crystalline chitin substrates, generating mostly chitobiose and GlcNAc as reaction products (29,30,31,32,33,34,35,36)

Implications for health and disease

Consistent with their conserved GH18 family structure and function, mammalian chitinases bind and degrade chitin, providing homeostatic defense and clearance mechanisms that are further boosted by immune activation. Although elevated chi-lectins, chitinases and chitinase activity have been documented extensively in the context of a wide array of inflammatory diseases, often providing useful biomarkers indicating disease severity (37), how several of these factors mediate interactions with natural chitin substrates in physiologic settings remains unclear. Chit1, primarily expressed by macrophages, was originally described in the context of lysosomal storage dysfunction in Gaucher's disease, accumulating in lipid-laden macrophages and elevated in several diseases involving macrophage accumulation and activation, consistent with original observations that human chitinases may participate in anti-parasite and anti-fungal defense (38,39). Indeed, recombinant Chit1 inhibits fungal growth in vitro and shows protective effects in mouse models of lethal fungal challenge (30). In humans, a common 24-bp duplication in CHIT1 renders the enzyme inactive, however, and phenotypic consequences of this loss are unremarkable, even in populations exposed to chitin-rich diets and parasites (40). Whether Chit1 is regulated in specific subsets of macrophages and / or other cell types during development, however, may yield clues to understanding how these cells contribute to inflammatory or infectious diseases.

The other active mammalian endochitinase, AMCase, is expressed in mucosal epithelium, prominently lung and stomach, where it is secreted constitutively as a stable active enzyme. Originally named for its chitinase activity at acid pH, AMCase is expressed by secretory

cells, such as gastric chief cells, and retains activity in the presence of stomach proteases, presumably functioning to digest chitin from dietary sources (29,41,42). Interestingly, the presence of dietary chitin influences chitinase gene expression in the stomach and may have exerted evolutionary pressure on CHIA gene selection among insectivorous primates (43,44). In the lung, AMCase is expressed by secretory epithelial cells lining proximal and distal airways, club cells and type 2 alveolar cells, respectively, and is secreted into the airway lumen where it comprises the major endochitinase in airway fluid (45,46). Notably, chitinase enzyme activities can be influenced by factors such as temperature, pH, and ionic strength, and considerable confusion in the literature exists due to the ability of both Chit1 and AMCase to enzymatically cleave chitotrioside substrates (e.g., 4-methylumbelliferyl-β-d-*NNN*-triacetylchitotrioside) commonly used in chitotriosidase activity assays. Also complicating the validation of specific chitinases or chitinase activities in complex biological materials are potential contributions from blood cells, serum components, or contaminating fungal, plant or bacterial chitinases. AMCase, however, appears to non-redundantly mediate both chitobiosidase and chitotriosidase activity in mouse bronchalveolar lavage (BAL) fluid, while human BAL fluid shows much lower relative chitobiosidase and chitotriosidase activity, likely owing to inefficient enzymatic activity among common human AMCase isoforms, which prior studies have associated with increased asthma risk (36,45,46,47,48).

Although expressed constitutively, AMCase becomes further induced by STAT6-activating signals, such as IL-13, in the context of type 2 immune challenge or allergic lung disease (32,49), providing a specific example of how type 2 immunity mediates feedback mechanisms to maintain homeostasis. AMCase digests insoluble chitin particles, which elicit a multifaceted immune response at airway mucosal barrier tissues that shares features with other types of epithelial stress and injury resulting from mechanical or chemical damage. At the nexus of such pathways lies activation of group 2 innate lymphoid cells (ILC2s), sentinel tissue-resident cells which can produce IL-5 and IL-13 in response to acute signals emanating from surrounding epithelial and stromal cells. Within hours after local chitin deposition, ILC2s increase IL-5 and IL-13 production in response to upstream activators such as IL-33, TSLP, IL-25, and leukotrienes, thereby mediating the local infiltration of eosinophils into lung tissue and alternative activation of macrophages (Figure 2; 32,50,51,52,53,54,55). Similar activating signals underpin the terminal differentiation of tissue effector T-helper type 2 (Th2) cells during adaptive immune responses to a wide array of tissue perturbations including chitinous fungal, helminth, and insect material, comprising a common checkpoint for type 2 lymphoid cell activation in the lung (56). Chitin particles additionally generate a separate suite of cytokines that concomitantly controls activation of lung resident γδ T cells that produce IL-17 and mediate neutrophil accumulation. In this setting, genetic ablation of ILC2s leads to enhanced γδ T cell activation and prolonged neutrophil recruitment to tissues, suggesting that cross-talk between resident innate and innate-like lymphoid cells can influence chitin-mediated tissue recruitment and retention of specific types of infiltrating myeloid effector cells (52).

AMCase activity determines the extent and kinetics of chitin degradation in the airways and subsequent magnitude and duration of the accompanying immune responses. This is particularly relevant to the turnover and dwell time of complex environmentally-derived

chitin substrates, such as fungal cell walls and insect excreta, immunostimulatory compounds comprised of highly ordered chitin lattices and crosslinked antigenic glycoconjugates (57,58). Mice expressing a lung-specific AMCase transgene exhibit attenuated inflammatory responses to such extracts, whereas mice that lack AMCase or have reduced AMCase activity show increased inflammation and delayed resolution after challenge with chitin or chitin-containing fungal or dust mite extracts (32,45,46,51,52,53). Additionally, illustrating the evolutionarily conserved function for chitinases in mammalian physiology, AMCase-deficient mice spontaneously accumulate environmentally-derived chitin in the airways over time and develop age-related lung fibrosis, which can be ameliorated by genetic or therapeutic restoration of chitinase activity (46). Evidence that chitin particles accumulate abnormally in the airways of human patients with interstitial lung disease (ILD), such as idiopathic pulmonary fibrosis (IPF), also points to a role for this process in the context of human fibrotic lung disease and presents unexplored therapeutic opportunities for such conditions that are increasingly recognized to involve both epithelial cell dysfunction and environmental drivers (46,59).

Conclusions / Future Work

The appreciation of conserved functions for chitinases throughout evolutionary history has led to recent advances in the understanding of these proteins in mammalian physiology, broadening the view of how type 2 immunity has evolved to participate in the homeostatic maintenance of tissues. In particular, the induction of AMCase by type 2 immune cytokines IL-4 and IL-13 through STAT6 activation by many chitin-containing stimuli connects a type 2 immune effector molecule with its biochemical substrate, providing a mechanism for specific recognition and feedback regulation of epithelial function by the mammalian immune system; such circuits have been associated with small intestinal homeostasis as well (60,61,62), suggesting a more general role for type 2 immunity in mucosal health. STAT6 targets in addition to AMCase, however, appear to mediate lung health and homeostasis after perturbation and damage induced by chitin and related challenges, as mice deficient in IL-4/IL-13-producing cells or signaling components show abnormal repair processes or worsened disease after injury (46,63,64,65). Notably, these targets include chi-lectins such as Ym1 and Ym2, whose participation in mammalian chitin recognition and degradation remain unclear. The ubiquitous presence of chitin substrates in the environment and their steady-state accumulation in AMCase-deficient animals also prompts new consideration of how systemic effects of this process might impinge upon other mucosal tissues, including the gastrointestinal tract (42,46,66). In this regard, how the microbiota is selected in the presence of natural chitin substrates or how chitinase-expressing pathogens, such as *Listeria monocytogenes*, *Legionella pneumophila*, and *Pseudomonas aeruginosa*, among others, may utilize chitin or chitinases in the context of host mucosal tissues remains to be elucidated. Finally, technical advances have enabled unprecedented direct observations of chitinase movement on crystalline chitin substrates, allowing calculation of single-molecule kinetic parameters for *Serratia* chitinase A on insoluble chitin microfibrils (67,68). X-ray-free electron laser (XFEL) radiation has also recently been utilized to resolve the structure of naturally-occurring eosinophil MBP-1 nanocrystals *in situ* (69). Similar microscopic and

biochemical characterizations of chitinases in complex with natural chitinous substrates will be critical to further understanding how the chitin degradative system operates in mammals.

Acknowledgements

Abbreviations

AMCase	acidic mammalian chitinase
CBD	chitin-binding domain
Chit1	chitotriosidase
GH	glycosyl hydrolase
GlcNAc	$\beta(1\text{–}4)$-N-acetyl-D-glucosamine
IL	interleukin
ILC	innate lymphoid cell
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis
LPMO	lytic polysaccharide mono-oxidase
LysM	lysin motif
PR	pathogenesis related
STAT6	signal transducer and activator of transcription 6
Th2	T-helper type 2 cell
TSLP	thymic stromal lymphopoietin

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69. Soragni A , Yousefi S , Stoeckle C , Soriaga AB , Sawaya MR , Kozlowski E , et al. Toxicity of Eosinophil MBP Is Repressed by Intracellular Crystallization and Promoted by Extracellular Aggreg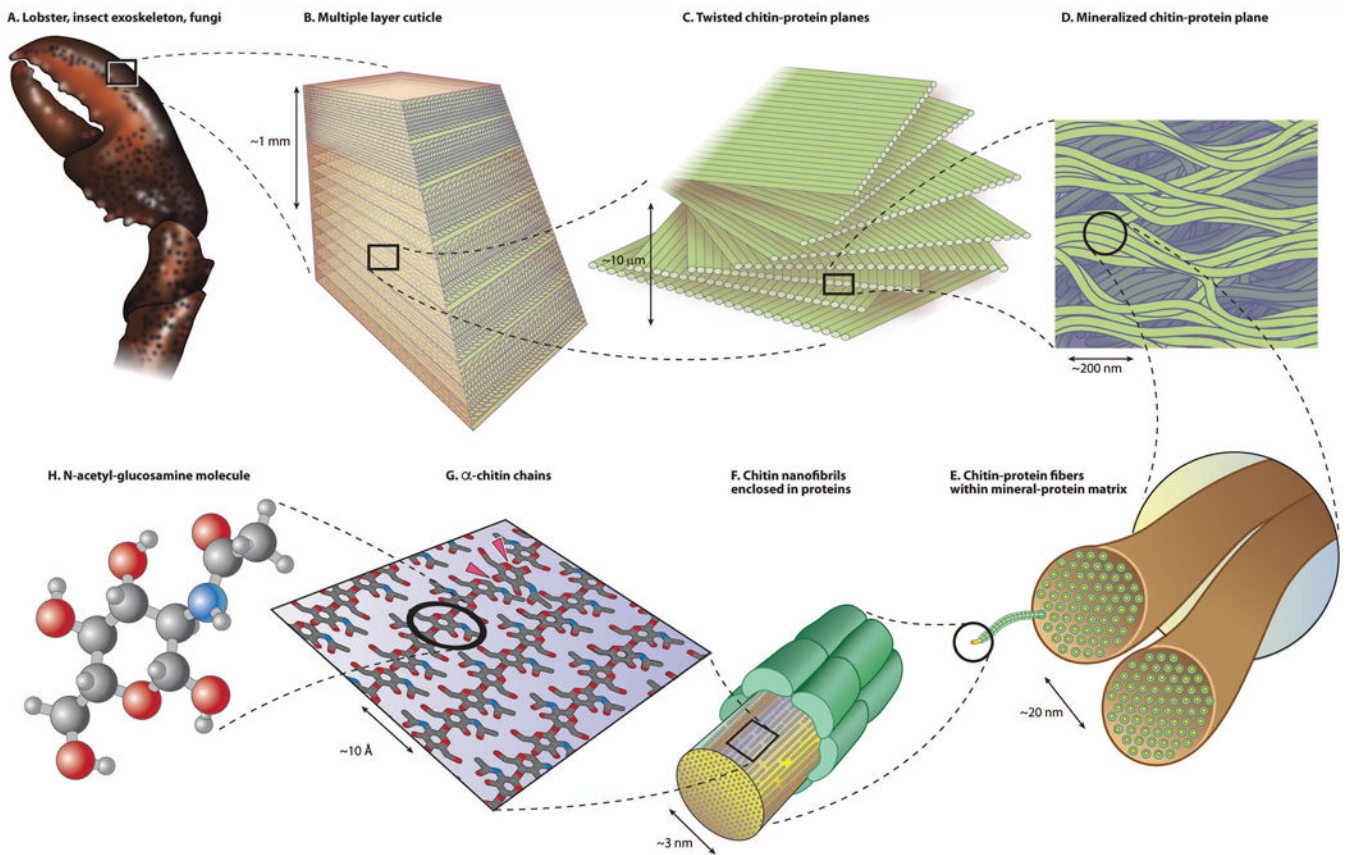ation. Mol Cell [Internet]. 2015;57:1011–21. Available from: http://www.sciencedirect.com/science/article/pii/S1097276515000490

Figure 1. Organization of natural chitin structures.

Chitin (exemplified by crustacean exoskeleton; A) is layered in repeating planar arrays comprised of mineralized chitin-protein fibers (B-E). Within these fibers, extensive hydrogen bonding organizes linear polymeric N-acetyl-glucosamine chains into nanofibrils (F-H), which can be internally cleaved by endochitinases, or at free non-reducing ends by exochitinases (G; arrows). Illustration based on Nikolov et al., *Adv. Mater.* 22:519–26; 2010.
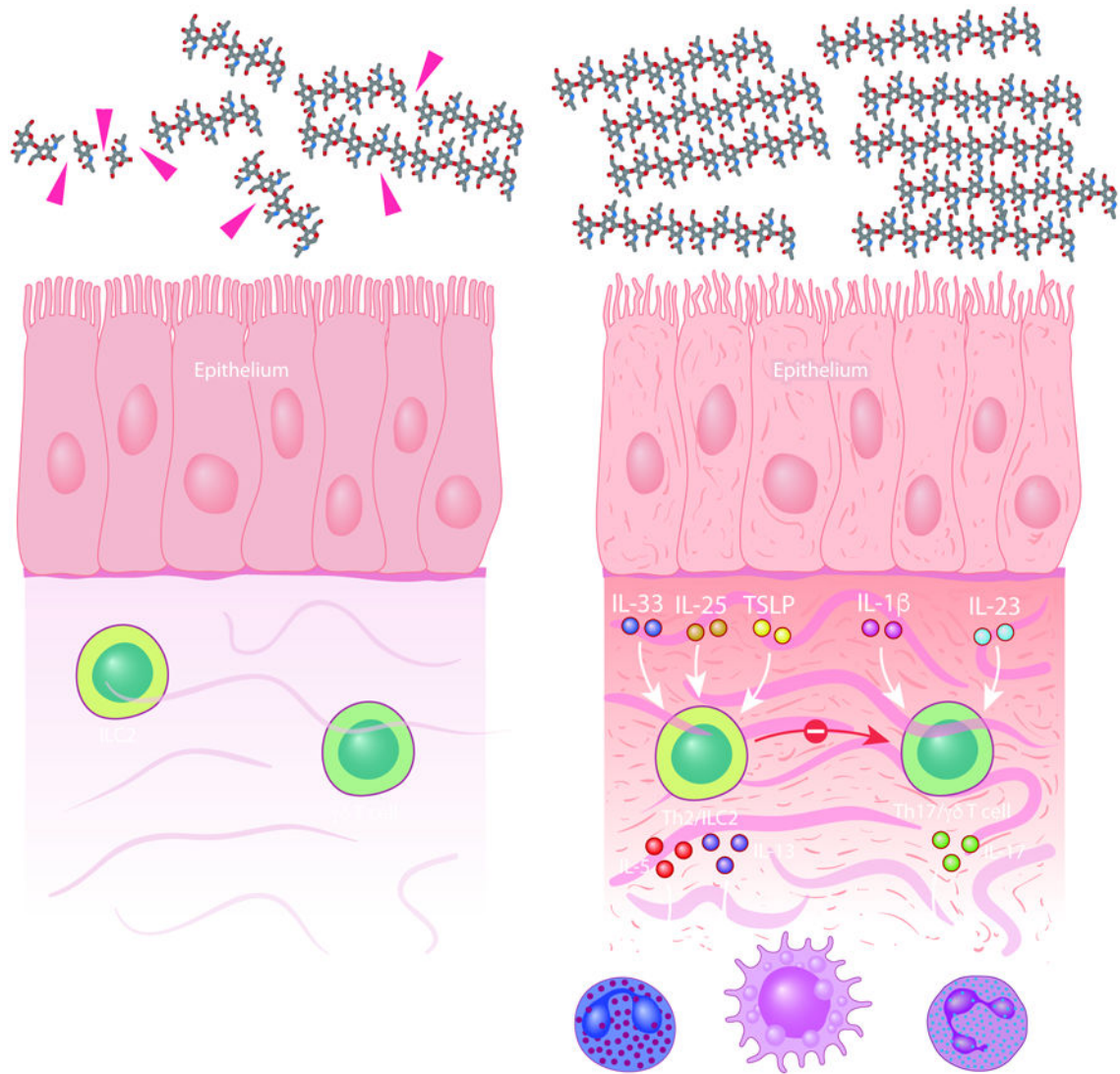
Figure 2. Airway chitinase activity maintains lung health and homeostasis.
In normal healthy airways (left panel), acidic mammalian chitinase (AMCase; arrows) degrades environmentally-derived chitin polymers. In the absence of AMCase (right panel), chitin polymers accumulate in the airways, leading to epithelial stress, chronic activation of resident lymphoid cells, inflammatory cell and cytokine production and age-related fibrosis.