

Chitin, Chitinases and Chitinase-like Proteins in Allergic Inflammation and Tissue Remodeling

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Chitin, the second most abundant polysaccharide in nature after cellulose, consist exoskeleton of lower organisms such as fungi, crustaceans and insects except mammals. Recently, several studies evaluated immunologic effects of chitin *in vivo* and *in vitro* and revealed new aspects of chitin regulation of innate and adaptive immune responses. It has been shown that exogenous chitin activates macrophages and other innate immune cells and also modulates adaptive type 2 allergic inflammation. These studies further demonstrate that chitin stimulate macrophages by interacting with different cell surface receptors such as macrophage mannose receptor, toll-like receptor 2 (TLR-2), C-type lectin receptor Dectin-1, and leukotriene B4 receptor (BLT1). On the other hand, a number of chitinase or chitinase-like proteins (C/CLP) are ubiquitously expressed in the airways and intestinal tracts from insects to mammals. In general, these chitinase family proteins confer protective functions to the host against exogenous chitin-containing pathogens. However, substantial body of recent studies also set light on new roles of C/CLP in the development and progression of allergic inflammation and tissue remodeling. In this review, recent findings on the role of chitin and C/CLP in allergic inflammation and tissue remodeling will be highlighted and controversial and unsolved issues in this field of studies will be discussed.

Key Words: Chitin, chitinases, chitinase-like proteins, immunity, remodeling

INTRODUCTION

Chitin, β -(1-4)-poly-N-acetyl D-glucosamine, is widely distributed in nature and is the second most abundant polysaccharide in nature after cellulose. It is found in the cell walls of bacteria

and fungi, mushrooms, exoskeleton of crustaceans (crabs, shrimp, etc.) and insects, the microfilarial sheath of parasitic nematodes, and the lining of the digestive tracts of many insect.¹⁻⁸ In these locations, chitin is used by the chitin-containing organisms to protect it from the harsh conditions in its environment and host anti-parasite/pathogen immune responses. The mammalian counterpart of chitin has not been described. Despite its ubiquity, chitin does not accumulate in the environment mainly because chitinolytic bacteria or saprophytes efficiently recycle most of chitin.⁹ As some chitin derivatives are known to be non-toxic, non-allergenic, biodegradable, and biocompatible, a number of prostheses such as artificial skin, contact lenses, surgical stitches have been produced from chitin derivatives and are widely used in medical practice.¹⁰ Thus, it is very common for humans to be exposed to chitin/chitin derivatives in daily life.

Although mammals cannot synthesize or metabolize chitin, a number of chitinolytic enzymes [true chitinases, e.g., chitotriosidases, acidic mammalian chitinase (AMCase)] or chitin-binding proteins [chitinase-like proteins (CLPs), e.g., Ym-1, Ym-2, breast regression protein 39 (BRP-39, chondrocyte protein-39)] were discovered in mammals.^{5,11} Many of the chitinase family proteins are constitutively expressed in macrophages and epithelial cells of the lung and digestive tracts, consisting of the body's first line of defense against exogenous agents including chitin-containing pathogens.^{12,13} Interestingly, some of the chitinases or CLPs (C/CLPs) have been reported to be expressed in an inducible manner in certain inflammatory and allergic conditions.¹⁴⁻¹⁶ Increased expression of AMCase in the lung was reported in the develop-

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ment of Th2 inflammation in allergic animal models and the human asthmatic airway.¹⁴ Genetic studies in human populations have shown linked polymorphisms in AMCase to asthma susceptibility in children, suggesting that inherent defects in this chitinase could underlie airway inflammation and allergic responses.¹⁷ Recently, a similar role has been proposed with a chitinase-like protein YKL-40. The serum and tissue expression of YKL-40 was significantly correlated with the severity of asthma, and genetic association of serum YKL levels and asthmatic airways has been identified.^{18,19} These studies also raise the possibility that the chitin, as a major binding partner of C/CLPs in mammals, might play an important role as a modulator (initiator or enhancer) of allergic immune responses, in that C/CLPs play a critical role. However, interactions between chitin and C/CLPs have not been adequately studied.

CHITIN REGULATES INNATE IMMUNE RESPONSES

Two decades ago, a number of studies demonstrated that chitin and chitin derivatives stimulated macrophages to produce cytokines that confer non-specific host resistance to bacterial and viral infections and anti-tumor activity.¹⁹⁻²⁴

Since then, a number of studies describing more specific immunologic activities of chitin have been reported. Shibata et al. re-evaluated the immunological effects of chitin *in vivo* and *in vitro* using phagocytosable small-sized chitin particles that demonstrated significant priming effects of chitin particles in alveolar macrophages and NK cells in mice.²⁵ They showed intravenous administration of fractionated chitin particles (1 to 10 μm) into the lung activates alveolar macrophages to express cytokines such as IL-12, tumor necrosis factor (TNF)- α , and IL-18, leading to INF- γ production mainly by NK cells.²⁵ Subsequent studies by the same group of investigators demonstrated that the cytokine production was through a mannose-receptor mediated phagocytosis process.²⁶ The macrophage plasma membrane mannose receptors serve to mediate the internalization of the chitin particles that, eventually, are degraded by the lysozyme and N-acetyl- β -glucosaminidase in the

macrophages of human and experimental animals.²⁷ Those studies were the first demonstration of the direct interactions between chitin and cell surface receptors and raised the possibility that chitin uses specific signaling pathways in immune regulation.

Recently, intriguing direct *in vivo* immune modulatory function of chitin has been described.²⁸ The investigators administered chitin beads directly into the lungs of mice expressing a green fluorescent protein (GFP)-enhanced transcript of IL-4 (4get mice) via intranasal application. Within several hours after chitin exposure, IL-4 GFP positive cells, in particular eosinophils (GFP+, siglec F+) and basophils (GFP+, IgE+, cKit-), were recruited to the lungs of these mice. The chitin-induced eosinophil recruitment was shown to be dependent on leukotriene B₄, because eosinophilic recruitment was significantly decreased in the leukotriene B₄ receptor null mice (BLT1). They further demonstrated that chitin alternatively activates alveolar macrophages and macrophage response was critical event in recruitment of eosinophils because depletion of macrophages by clodronate liposome treatment prevented recruitment of eosinophils. These studies raise the possibility that chitin can be directly involved in the generation of allergic responses and provide another clues to explain high asthma frequency in the workers predicted to have high environmental exposure of chitin.^{29,30}

CHITIN MODULATES ADAPTIVE TYPE 2 IMMUNE RESPONSES

As described above, there are multiple evidences indicating that chitin is a potent innate immune stimulator of macrophages and other innate immune cells. This raises the possibility that chitin could affect allergen-induced adaptive type II responses as well. Generally, type I cytokines are produced by innate immune cells and it has been shown that type I cytokines down-regulate type 2 allergic immune responses.³¹ In addition, the administration of IFN- γ or IL-12 significantly inhibited Th2 driven inflammatory responses in allergic animal models.^{32,33} Thus, it is reasonable to speculate that chitin could negatively modulate allergen-induced type 2 inflammatory responses if

chitin does stimulate type I cytokines production. Several studies strongly support this contention. Shibata et al. has elegantly demonstrated that orally given chitin significantly down regulates allergen-induced IgE production and lung inflammation in a ragweed-immunized allergic animal model.³¹ The allergen-stimulated Th2 cytokines, such as IL-4, IL-5, and IL-10 production was significantly inhibited by the presence of chitin in spleen cell culture. They further demonstrated that IFN- γ produced by NK cells and ragweed-specific Th1 cells was responsible for the inhibition of allergen-induced Th2 cytokine production.³¹ In a separate study, the same group of investigators have shown that chitin is a strong Th1 adjuvant that up-regulates heat-killed *Mycobacterium bovis* Calmette-Guerin bacillus (HK-BCG)-induced Th1 immunity, but down regulates mycobacterial protein (MPB-39)-induced Th2 immunity.³⁴ The Th1 adjuvant effect of chitin microparticles (CMP) in inducing viral specific immunity has also been reported.³⁵ Later studies by Strong et al. further demonstrated that direct intranasal application of chitin microparticles into the lung also significantly down-regulated allergic response to *Dermatophagoids pteronyssinus* (Der p) and *Aspergillus fumigatus* in a murine model of allergy.³⁶ The chitin treatment substantially reduced these allergen-induced serum IgE levels, peripheral eosinophilia, airway hyper-responsiveness, and lung inflammation. They noted the elevation of Th1 cytokines IL-12, IFN- γ and TNF- α and reduction in IL-4 production in the chitin-treated mice compared to sham controls. Similarly, intranasal application of water soluble chitosan also significantly attenuated *Dermatophagoids farinae* (Der f)-induced lung inflammation and mucus production.³⁶ Subsequent studies by Ozdemir et al. further demonstrated that application of microgram quantities of chitin microparticles had a beneficial effect in preventing and treating histopathologic changes in the airways of asthmatic mice.³⁷ All these studies strongly support the contention that chitin negatively regulate the development of adaptive type 2 allergic responses. As a regulatory mechanism, down regulation of allergen-induced arginase I and thymic stromal lymphopoietin (TSLP) expression in the bronchial epithelium was suggested.³⁸ The significant role of

TSLP and arginase I in Th2 polarization and tissue remodeling process has been previously described, respectively.^{39,40} From the clinical point of view, the regulatory function of chitin on Th2 adaptive immune response is therapeutically important because it can be applied to control a variety of type 2 allergic diseases.

Although many studies strongly suggest that chitin or chitin derivatives enhance Type I immunity while suppressing Type II inflammatory responses, a number of issues remain to be clarified to generalize these effects. The animal models that were used to assay chitin effects in these studies may represent only a specific type of allergic response. This needs to be tested in other type 1 as well as type 2 allergic animal models such as animal models using Ovalbumin (OVA) as a immunizing allergen.^{41,42} As discussed in the above section, *in vivo* chitin also stimulated innate immune cells, such as eosinophils and basophils, the cells that are closely associated with allergic responses. Type 2 cytokines produced from these innate immune cells can further enhance allergen-induced inflammatory and tissue responses. These studies suggest that *in vivo* regulation of chitin on adaptive Th2 immune response may not be simple or uni-directional. In addition, a Th2 adjuvant effect of chitin in a Th2 allergic animal model has been observed (unpublished observations). When viewed in combination, these studies demonstrate that chitin or chitin derivatives have complex *in vivo* regulatory mechanisms on adaptive immune responses and this necessitates clarification for future clinical applications of chitin in allergic diseases.

C/CLPS AND ALLERGIC INFLAMMATION

The chitinases Der p 15 and Der p 18 originated from house dust mites have been identified as major dog allergens⁴³ and also suggested to be allergenic to humans.⁴⁴ The potential role of endogenous mammalian C/CLPs in allergic immune response only recently been investigated (for review see.^{5,45} One of the prototypic chitinase AMCase was first noted to be induced during Th2 inflammation through an IL-13-dependent mechanism.¹⁴ It was also shown to play an important role in the

pathogenesis of Th2 inflammations and IL-13 effector pathway activation.¹⁴ In support of this contention, subsequent studies also demonstrated that potential role of AMCase in human asthma,¹⁷ ocular allergy,⁴⁶ and allergic airway inflammation.⁴⁷ However, in contrary to the mice system, recent studies demonstrated that AMCase expressed in human lung was mostly inactive and chitotriosidase was mainly responsible for the chitinase activity in the lung.⁴⁸ Thus, whether the chitotriosidase in humans has the same immunologic activity in allergic responses as AMCase in mouse is an interesting question that need to be addressed in further studies. Recently more exciting stories were developed for role of CLPs in allergic diseases. In the mouse allergic models, Ym-1 and Ym-2 was recognized as a allergy-associated protein.^{44,47,49} In humans, our laboratory demonstrated that levels of YKL-40/BRP-39 in sera and lung tissues were significantly associated with asthma development and even severity of the disease.¹⁸ Subsequent extensive population studies also demonstrated that YKL-40 polymorphisms are functionally and genetically associated with asthma patients,¹⁹ suggesting an important role of YKL-40/BRP-39 in the pathogenesis of asthma or Th2 allergic diseases. Interestingly, a significant role of YKL-40/BRP-39 in the generation of intestinal bowel disease (IBD) as a active pathogenic mediator in acute colitis has been suggested.^{13,15} When viewed in combination, these studies provide noble insights on the biological role of C/CLPs in a number of allergic and inflammatory diseases including asthma, asthma, and rhinosinusitis, and IBD. However, specific functional role of C/CLPs in allergic diseases remains to be defined.

C/CLPS IN TISSUE REMODELING

Recently a number of studies suggest that important role of C/CLPs in disease pathogenesis that characterized by pathologic tissue remodeling. Bargagli et al.⁵⁰ reported that significantly higher activity of chitotriosidase, in serum and BAL of patients with sarcoidosis, especially in those with progressing disease and lung involvement, than in controls. They also showed that the increase of the chitotriosidase activity was specific in sarcoidosis

patients because the activity was in normal range in the patients with tuberculosis or with idiopathic pulmonary fibrosis and pulmonary fibrosis associated with systemic sclerosis.^{50,51} In addition, potential role of chitotriosidase in the evolution of nonalcoholic fatty liver disease such as non-alcoholic steatohepatitis (NASH) has been suggested.^{52,53} In those studies, Kupffer cells in NASH patients overproduce chitotriosidase contributed to the progression from uncomplicated steatosis to steatohepatitis with progressive fibrosis.⁵³ Several studies also suggested that CLPs such as YKL-40 or mouse Ym-1 or Ym-2 could be involved in tissue remodeling process. Serum YKL-40 was significantly related to the degree of liver fibrosis and staining of YKL-40 antigen was higher in areas with fibrosis, particularly areas with active fibrogenesis.^{54,55} The serum levels of YKL-40 was correlated with the stage of hepatic fibrosis in patients infected with *S. japonicum*, further support potential role of YKL-40 in the pathogenesis of fibrogenesis.⁵⁶ In the animal models that accompanying tissue remodeling process also demonstrated significant changes in the C/CLPs expression at the site of inflammation or remodeling. Th2-inducing pathogens *Schistosoma mansoni* and *Nippostrongylus brasiliensis* cause granulomatous inflammation and liver fibrosis in the infested mice. In that model, AMCase and Ym-1 expression were significantly increased along with type 2 cytokines such as IL-13 and IL-4.⁵⁷ In the mice with pulmonary fibrosis induced by crystalline silica exposure,⁵⁸ or herpesvirus⁵⁹ also demonstrated that close associations between expression of C/CLPs and degree of tissue remodeling. However, it is still not clear whether C/CLPs actively participate in the tissue remodeling process or indirectly modulate the process through regulation of other cytokines and/or growth factors. The elevated expression of C/CLPs could only represent specific type of macrophage activation, such as alternative activation of macrophages, that produce a number of potent growth factors capable of inducing tissue remodeling. In this case, C/CLPs only involves in the activation process of macrophages and other cells. Further mechanistic studies using specific gene targeted animal models or transgenic models will be required to define more specific function of C/CLPs in tissue remodeling.

CONTROVERSIAL AND UNSOLVED ISSUES

Macrophage activation by chitin

The macrophages response to exogenous chitin is not consistent when similar studies are compared. The earlier studies indicated that direct stimulation of macrophages with chitin or chitin derivatives significantly enhanced the expression of type I pro-inflammatory cytokines such as TNF- α and IL-12 and IFN- γ .^{21,60} Later, Shibata et al. further supported the contention that chitin stimulates macrophages to express type I cytokines such as TNF- α , IL-18 and IL-12 that resulted in the expression of IFN- γ by NK cells.^{25,26} IFN- γ is well known cytokine that classically stimulates macrophages.⁶¹ However, studies from Reese et al. demonstrated that chitin alternatively activated macrophages (AAM) that were critical for chitin-induced inflammatory responses.²⁸ It has been shown that chitin or chitin derivatives stimulate wound healing via AAM.¹⁰ Other studies indicated that chitin directly stimulates macrophages to express TNF- α and IL-17 via TLR-2 and MyD-88-dependent but TLR-4-independent pathways.⁶² These studies suggest a number of pathways, and cytokines are possibly involved in chitin-induced macrophages activation and cytokine production. Also, the discrepancies in chitin-induced macrophage activation between similar studies were possibly originated from the difference in the experimental system itself. The possibility of other contaminants, such as lipids or β -glucan, was excluded by demonstrating that chitin-induced responses were abolished by the treatment of chitin with chitinase before challenging animals.²⁸ LPS contamination has been also excluded because the chitin-induced response was known to be TLR-4 independent.^{28,62} However, the chitin preparation (size difference), doses, duration, and route of chitin administration could differently affect inflammatory and tissue responses.^{9,28} When viewed in combination, it is more reasonable for now to speculate that chitin has the capacity to stimulate macrophages in multiple directions depending on experimental conditions or pathways involved. Chitin can interact with diverse cell surface receptors, such as macrophage mannose receptor, TLR-2 receptors,^{25,62} and a C-type lectin receptor

dectin-1 (unpublished observation). It would be very interesting to define specific chitin-receptor interactions for macrophage activation. The alternative activation of macrophages by chitin could be a secondary event followed by the initial influx of eosinophils or other inflammatory cells expressing type II cytokines. In this scenario, chitin initially activates macrophages to express pro-inflammatory mediators, such as LTB₄, that recruit eosinophils and other innate immune cells expressing type II cytokines. Then, the macrophages can be alternatively activated by the cytokines expressed by those inflammatory cells. To understand the nature of chitin-induced immune regulation, further characterization of underlying mechanisms leading to distinct macrophage activation will be required in future studies.

Chitin effects on other innate immune cells

Epithelial cells and other antigen presenting cells (APCs) in the airways or the gut are often exposed to a variety of exogenous allergens and potentially chitin-containing pathogens by inhalation or ingestion. Epithelial cells endogenously express chitinases and chitin binding proteins or their expression is significantly induced by allergenic stimulation.^{14,44,49} The specific role of epithelial cells in chitin-induced immune response has not been carefully evaluated, and the effects of chitin on innate immune cells other than macrophages and NK cells remains largely unknown. As epithelial and dendritic cells are key players in allergen (or pathogen) recognition and processing, it is important to define the response of these cells to chitin to understand the immune regulatory function of chitin *in vivo*. Because dendritic cells express a number of toll-like receptors and C-type lectin receptors endogenously and in a inducible manner like macrophages,^{63,64} it is reasonable to speculate that exogenous chitin can directly interact with dendritic cells, and modulate functional phenotypes that lead to specific polarized T cell responses. It has been shown that when airway epithelial cells are exposed to allergen or injury, several alarmins, such as TSLP, along with pro- and anti-inflammatory cytokines are produced from epithelial cells, that could affect dendritic cell phenotypes and functions.³⁹ Interestingly,

chitosan has been shown to down-regulate TSLP expression from allergen-stimulated epithelial cells,³⁸ suggesting potentially important roles of epithelial cells and dendritic cells in the immunological activity of chitin. Cytokines, chemokines and other mediators produced from chitin stimulated epithelial cells or dendritic cells will be interesting targets for future studies.

CHITIN INTERACTION WITH ENDOGENOUS CHITINASES OR CLPS

Although several *in vivo* and *in vitro* studies have provided substantial evidence that chitin uses certain receptors expressed on the surface of macrophages, the exact interacting molecule(s) has not yet been defined. As discussed in the previous section, although chitin is not produced in mammals, many chitinases or CLPs are endogenously expressed in mammals. Chitinases and CLPs belong to family 18 glycosyl hydrolase members which contain a catalytic domain and a chitin binding domain.^{5,65} The physical binding properties between chitin and a CLP have been characterized in previous studies.⁶⁵⁻⁶⁷ However, the exact role of these chitinase or chitinase-like proteins in the regulation of chitin-induced inflammatory and immune regulatory function remains to be determined. Previous studies demonstrated that the expression of a number of chitinases and CLPs were induced in allergic animal models and humans with allergic inflammation. However, it is undetermined whether the chitinase or CLPs expression is induced or not by *in vivo* chitin stimulation. Recently, Reese et al. reported that after *in vivo* administration of chitin into the lungs of chitinase overexpressing transgenic mice, chitin-induced eosinophil and basophil recruitment was abolished.²⁸ These studies suggest a protective role of chitinases against chitin or chitin-containing pathogens such as parasites. However, previous studies from our laboratory and others demonstrated significant induction of chitinases and CLPs in an animal model of allergy using non-chitin containing allergen such as OVA.^{14,49} Chitinases and CLPs are also impressively induced in the lung of IL-13 overexpressing transgenic mice, and specific inhibition of chitinase activity in these animal

models using chemical (allosamidine) or neutralization antibody against specific chitinases, significantly reduced OVA- or IL-13-induced inflammation.¹⁴ These studies suggest that endogenous chitinases or CLPs could be involved in general host response, irrespective of chitin. When viewed in combination, chitin or allergen modulated the expression of chitinases or chitin-binding proteins, and these chitinases may have a direct protective role against chitin by degrading the chitin itself, but also may function to amplify inflammatory responses against chitin or other allergens. The question as to what kinds of chitinases or chitinase-like molecules are induced in response to exogenous chitin, and how this endogenous chitinase family of proteins interacts with chitin in immune responses, is still waiting to be answered.

CONCLUSION

A number of studies indicate that chitin and chitin derivatives have diverse biological activities. Not only the abundance of chitin in the environment or the growing use of chitin or chitin derivatives in various biomedical fields but also the potent immunologic activity of chitin has prompted us to consider chitin as a potential pro- or anti-allergenic molecule. Recent studies demonstrated that chitin primarily stimulate innate immune cells to generate type 1 or type 2 inflammatory responses depending on the chitin size or composition and/or ways of chitin administration (Fig. 1). *In vivo* chitin also directly or indirectly modulates allergen-induced adaptive inflammation through a number of ways (Fig. 1). However, exact *in vivo* immune regulatory effects of chitin has been controversial and the underlying mechanisms of chitin regulation of allergic response has not been clearly defined. Also a number of recent studies strongly suggest that C/CLPs are involved in the development or progression of allergic diseases and tissue remodeling. Further characterization on the interactions between chitin and C/CLPs, the specific role of endogenous C/CLPs in allergic immune responses and tissue remodeling, and underlying signaling pathways will be required to understand the exact biological role of chitin, C/CLPs in the pathogenesis of allergic and

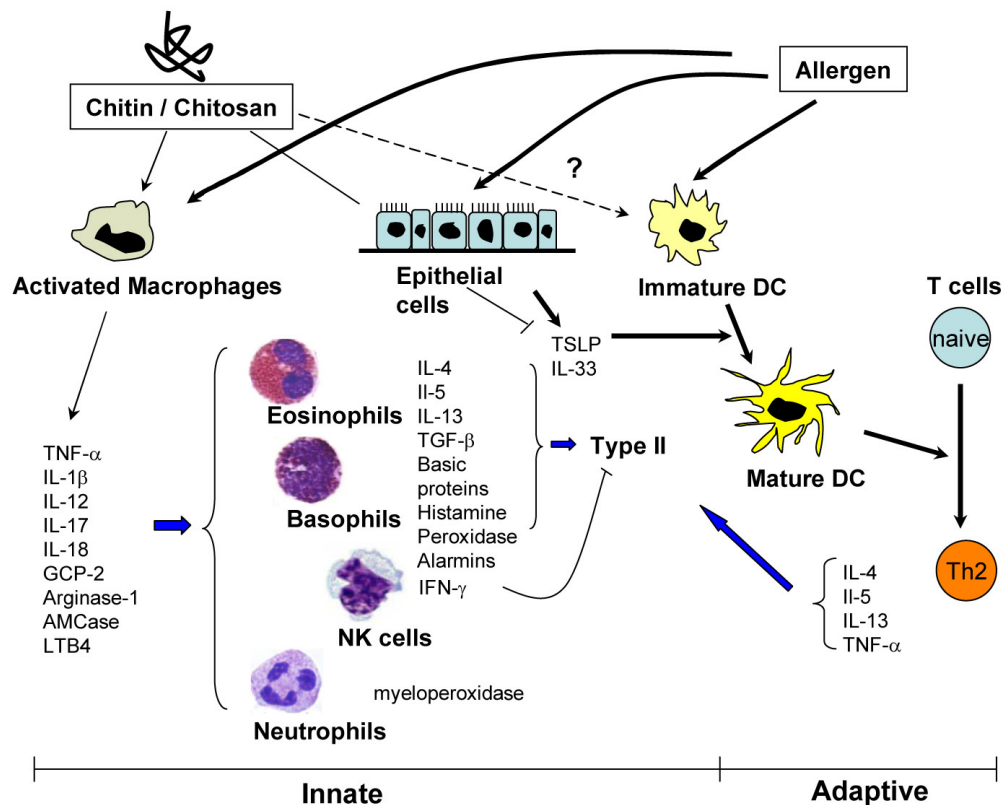


Fig. 1. Chitin stimulation of innate immune cells and regulation of adaptive allergic immune responses. Chitin or chitin derivatives activate macrophages to express a number of pro-inflammatory cytokines, chemokine (e.g., GCP-2) and other mediators (LTB4) via macrophage mannose receptor, TLR-2, and C-type lectin receptor. Then the eosinophils, basophils and neutrophils that are recruited by these inflammatory mediators present type II allergic responses by secretion of Th2 cytokines (IL-4, IL-5, IL-13) and other allergic mediators (histamines, peroxidases). Thus, chitin-induced eosinophils and basophils further enhance allergen-induced adaptive Th2 inflammatory responses. On the other hand, many studies also support that chitin activates macrophages, NK cells and neutrophils to produce type I cytokines (TNF- α , IL-12, IL-1 β and IFN- γ), and suppress allergen-induced adaptive type II immune responses. Chitosan down-regulate allergen-induced TSLP expression from epithelial cells, and inhibits Th2 polarization by TSLP-dendritic cell interaction. The direct effect of chitin and chitin derivatives on dendritic cells remains to be determined.

inflammatory diseases.

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