Review

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Recent Progress of Chitosan Nanoparticles for the Development of Superior Delivery of Vaccines

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Chitosan seems to be an innovative biological material potentially utilized as a nanoparticle carrier for drug delivery, which could be low toxic, biocompatible, and easy to prepare. Chitosan nanoparticles have been employed in gene delivery. As a type of multifunctional adjuvant, chitosan nanoparticles could activate the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway to induce cell protection and/or proliferation via the modulation of autophagy within dendritic cells. In general, adjuvants may improve the innate and/or adaptive immune responses to a vaccine antigen by facilitating the antigen presentation of antigen presenting cells such as dendritic cells. The choice of a suitable adjuvant has become vital for improved safety and/or expanded application of vaccines. Fortunately, chitosan nanoparticles could be designed to target the dendritic cells to be enhanced by its adjuvant effect and for stimulating robust immune responses. Therefore, chitosan nanoparticles may be a good immune stimulant with encouraging properties for the development of superior vaccine delivery. Indeed, vaccines could play a key role in human health. In this review, we summarize the concept and/or recent progress in the field of chitosan nanoparticles, providing a valuable resource for investigating the molecular mechanisms of chitosan for the development of a greater vaccine.

Keywords: vaccine; chitin; chitosan; nanoparticle; dendritic cell; PI3K; mTOR; autophagy

Introduction

Of all polymers used to deliver nano-transporters, naturally occurring chitosan may be one of the most promising materials in the field of nanomedicine, which may be employed to create chitosan nanoparticles. Chitosan nanoparticles could be cost-effectively and easily made [1], which might be an interesting transport particle due to their positive charge with protonated amino acids to interact with the negatively charged mucin within various epithelial cells [2]. In general, chitosan holds amine groups that could gain a positive charge when protonated at acidic pH phases. In addition, the preference may be documented for many reasons including low toxicity and/or biocompatibility [3] (Fig. 1). For example, chitosan production has been detected to elicit superior protection when compared to the other nano-emulsion [2]. Investigating these valuable properties, chitosan nanoparticles have been used to develop ultimate antiviral, antibacterial, and/or clean supply vehicles of vaccines. Again, natural polymers like chitosan might be preferred to design nanoparticles because of their biocompatibility and long lifetime, which could deliver smart prevention as a vaccine-related material for infectious diseases [4]. Up to now, however, there is not sufficient clinical data that states about the chitosan nanoparticles [4]. Several features may render chitosan and/or its derivatives an ideal paradigm for the procedure as vaccine and/or antigen carriers [5]. Chitin is a natural polymer that can be straightforwardly extracted from molds' mycelium or lobsters' shells [6]. Deacetylating the amino terminus of the chitin could yield chitosan, an amino polysaccharide that is indeed well-matched and low-toxic for bio-usage. Since chitosan is soluble in acidic pH and insoluble in alkaline pH, the physiological alkaline or neutral pH of sub-epithelial space might make chitosan insoluble or unstable. Interestingly, trimethyl chitosan could accelerate the maturation of dendritic cells [5] (Fig. 2). This characteristic could induce a wide response to antibodies being soluble even at physiological pH levels [7,8]. Furthermore, the trimethyl chitosan could simply attach to mucin on the surface of mucosal cells, which might hold the encapsulated vaccine or antigen with the trimethyl chitosan on mucosal cells. Generally, this property is familiar as a paste property of trimethyl chitosan [9]. Therefore, trimethyl chitosan has been commonly employed in the intranasal delivery of several antigens such as hepatitis B virus surface antigen and/or tetanus toxin antigen [10,11]. Nasal vaccines could manage to overcome the limitation associated with the antigen countersignature, which may be a notable entrance point for several infectious vaccine agents. In addition, adjuvant and antigen transport systems are greatly important in order to induce an appropriate mucosal immune response against valued antigens [12].

Most DNA complexes may candidly bind to cationic delivery molecules. Hence, cationic polymers such as chi-

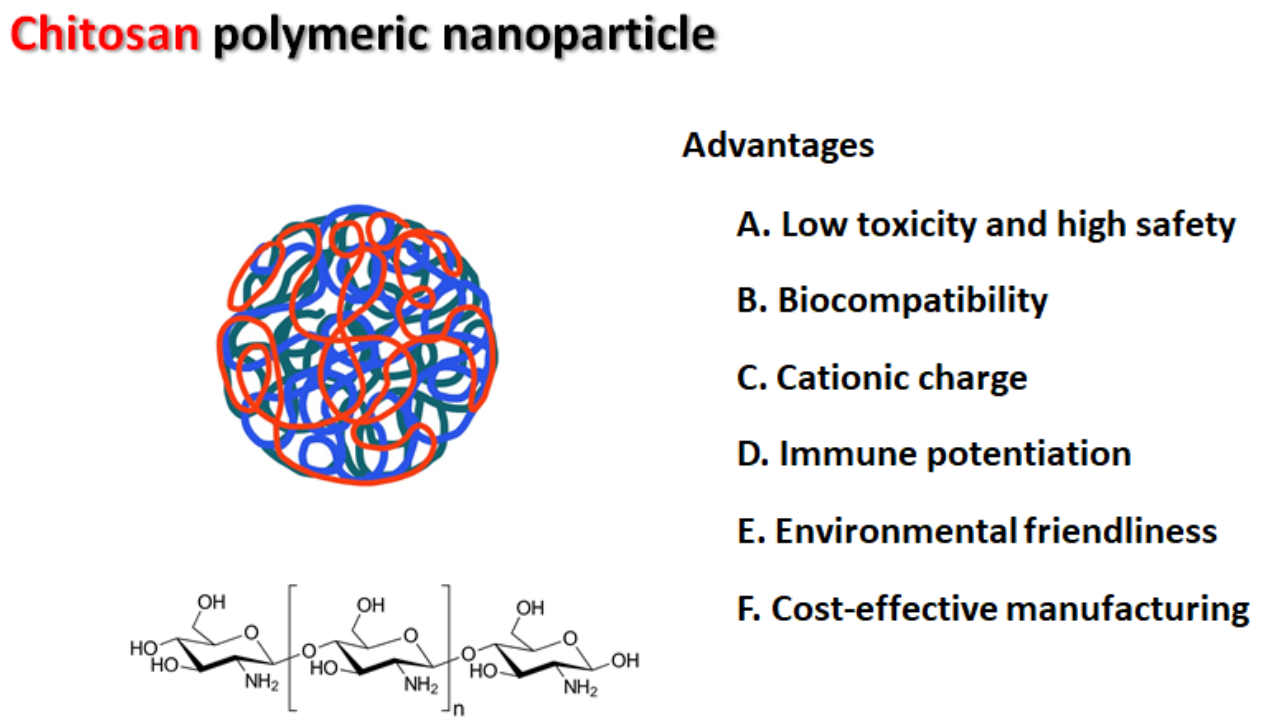

Fig. 1. Schematic image of chitosan nanoparticle. Several advantages of the chitosan nanoparticle are shown on the right side. The chemical structure of chitosan is also shown at the bottom. This illustration has been created with Microsoft PowerPoint 2013.

tosan have been broadly investigated as candidates for gene delivery. In addition, chitosan nanoparticles could be also a carrier for drug delivery with an immunogen packaging in addition to the strong adjuvant for antigen vaccination [13]. Indeed, successful vaccination depends on the association with potent adjuvants that could enhance the immunogenicity of antigens and/or stimulate the immune system [14]. However, the precise mechanism of chitosan as a potentiation of immunological responses has remained uncertain. Subsequently, multi-cationic metal chelator polyethyleneimine (PEI) has been used to connect with the chitosan to increase the total positive charge of nanoparticles, which may be qualified to promote DNA binding [15]. The biocompatible nano vector with the great capability of DNA binding by PEI-chitosan could become a promising carrier for various gene expressions to enhance innate and/or adaptive immunity [16].

Immuno-stimulatory Effects of Chitosan as Adjuvants

Chitosan is a natural polymer along with the popular dietary fiber, which could be derived from the cuticles of shellfish such as lobster, shrimp, crab, and/or the cell wall of mushrooms. Chitin is also a natural substance existing in the exoskeletons of insects and/or in fungal cell walls [17]. As mentioned above, chitosan is a deacetylated product of chitin, which has been extensively utilized in various medical applications [18,19]. Remarkably, chitosan could be easily degraded into chitooligosaccharides by conventional heating and/or microwave irradiation, which possesses several biological activities including antibacterial, antiviral and/or antifungal as well as robust immune-regulatory activities [20]. In particular, the chitooligosaccharides degraded by microwave irradiation might exhibit the best activity, which may exhibit an extremely wide range of biological activities and/or a noteworthy potential to be applied in various industries [19,21]. Chitosan and chitooligosaccharides could promote innate and/or adaptive immunity by strong immune-stimulatory activity in immunosuppressed mice [22]. Several chitooligosaccharide-derived products have been further investigated for conceivable immune-stimulatory applications in pharmaceutical and/or homeopathic levels [23].

These valuable properties including high charge density, low toxicity and/or biocompatibility are of great interest in medical research. In addition, the muco-affinity of chitosan may create vast potential for various biomedical applications [8,24]. Chitosan could work both as a matrix for distribution and as an adjuvant of antigen or vaccine [25]. In this regard, it might be helpful that chitosan nanoparticles could be reproducibly arranged by ionic gelation as an antigen [26]. Chitosan nanoparticles are positively charged, spherical in shape, showing good stability in dispersion form into hydrogels for several months [26]. Based on the observed cellular immune responses, this plat-

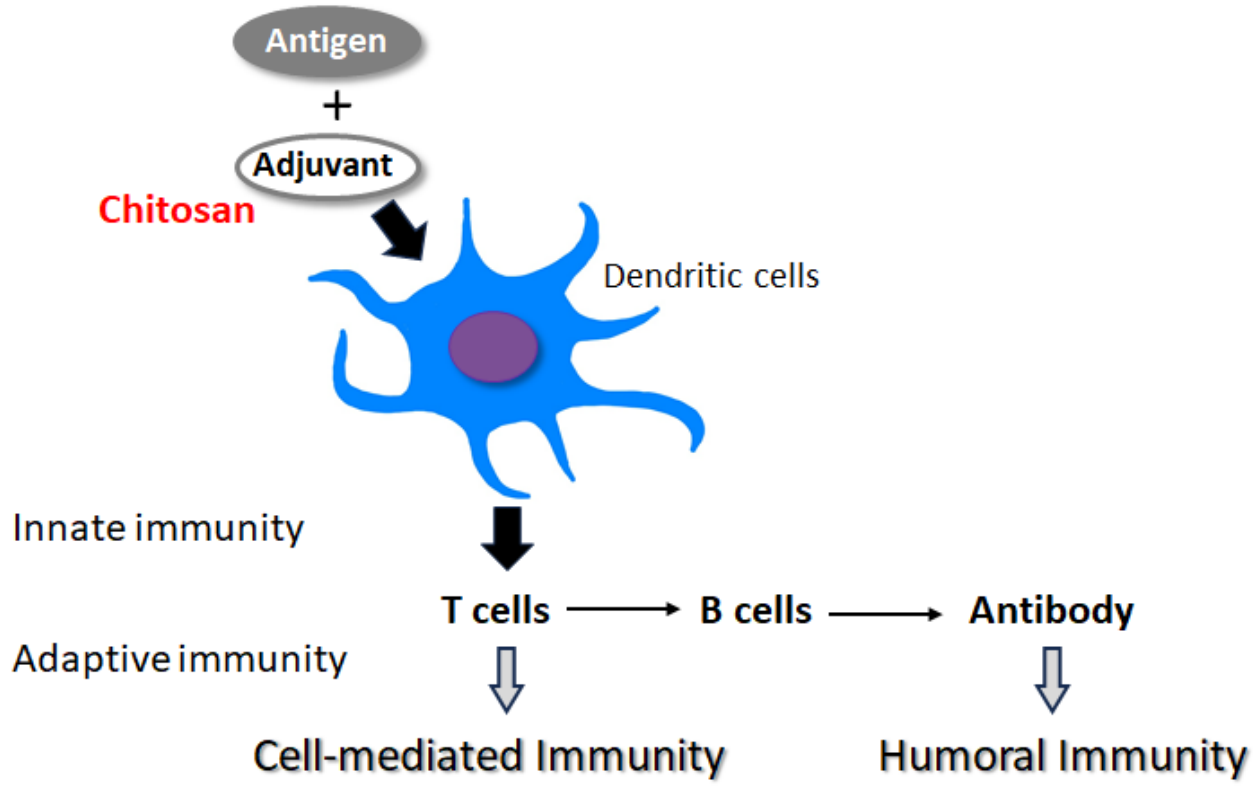

Fig. 2. Simplified schematic representation of innate and adaptive immunity. Dendritic cells may organize a distinctive system of immune cells for the induction of several immune responses. In general, with the help of some adjuvants such as chitosan in innate immunity, dendritic cells could transfer information from the external antigen to the effector immune cells in adaptive immunity. This illustration has been created with Microsoft PowerPoint 2013.

form of antigen delivery might be also a good cooperative for application in other indications [27]. For example, chitosan may enable the antigen administration via less-invasive mucosal routes such as sublingual or pulmonic application route [27]. In combination with the mucosal adjuvant technology, chitosan nanocarriers might be a favorable platform for the development of innovative mucosal therapeutic vaccines. A point that warrants future investigation might be exploiting the clinical efficacy of chitosan nanoparticles for the relevant treatment [26,27].

Some chitosan with an average molecular weight of less than 10,000 Da might be simply absorbed through the intestine and might be mainly excreted from the kidney [28], which are useful biological properties in various fields including cosmetics, nutrition, pharmaceuticals, biotechnology and/or medicine [29,30]. The chitosan with a molecular weight of about 5000 Da could be the most active polymer supporting junction barriers through the activation of adenosine 3′, 5′-cyclic monophosphate (AMP)-activated protein kinase (AMPK) [31], which might be mediated by calcium sensor mediated calcium release from the endoplasmic reticulum of epithelial cells in the intestine [31]. Therefore, chitosan might be valuable in the treatment of secretory diarrheas. In addition, it has been described that chitosan could also inhibit the expression of programmed cell death ligand 1 (PD-L1) via the AMPK activation in several kinds of cancer [32]. Therefore, chitosan could be employed as an advantageous way to improve the efficacy of existing chemotherapies by effective PD-L1 downregulation. Interestingly, metformin could modify the function of chitosan, which might prevent the overexpression of PD-L1 in various cancer cells [33]. Treatment with chitosan and/or chitooligosaccharides may be beneficial for the prevention of several cancers.

PI3K/AKT Signaling Pathway May be Involved in the Immune-stimulatory Effect of Chitosan

It has been revealed that chitosan could bind to the phosphoinositide 3-kinase (PI3K) p85 subunit to prevent the signaling of the PI3K/AKT pathway, possibly by suppressing the activity of MAPK/ERK and/or MEK1/2, suggesting insights into the development of new targets to enhance the pharmacological effect of chitosan against cancers [34]. In addition, chitosan might be a potential immune activator and/or a good vaccine adjuvant via the activation of PI3K/AKT signaling, indicating that chitosan may also activate cancer cells via the PI3K/AKT signal-

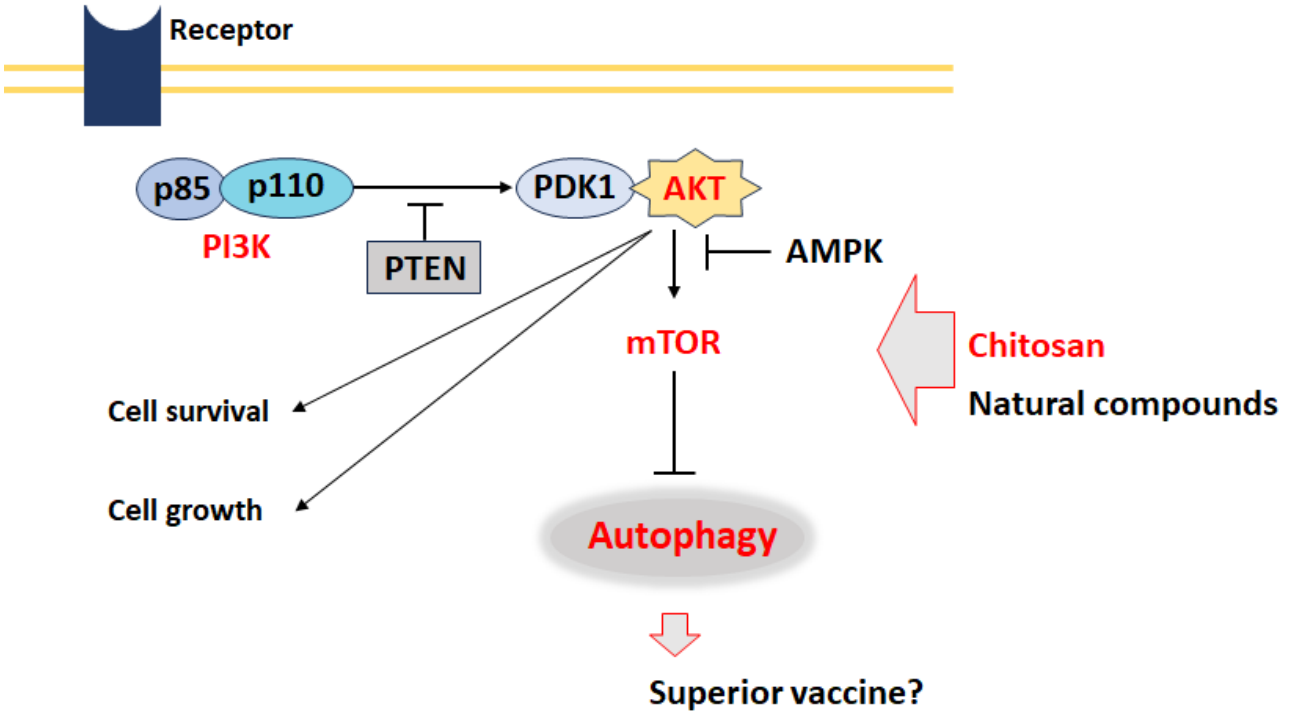

Fig. 3. A hypothetical schematic image and overview of the PI3K/AKT signaling pathway for autophagy. The PI3K/AKT/AMPK/mTOR signaling pathway might be intricately related to the regulation of autophagy. The arrowhead indicates stimulation, whereas the hammerhead shows inhibition. Chitosan and/or several natural compounds might influence autophagy via the modulation of the PI3K/AKT/AMPK/mTOR signaling pathway. Note that several important activities such as inflammatory-related reactions have been omitted for clarity. Abbreviation: AMPK, adenosine 3′, 5′-cyclic monophosphate (AMP)-activated protein kinase; mTOR, mammalian/mechanistic target of rapamycin; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10. This illustration has been created with Microsoft PowerPoint 2013.

ing pathways and might be a possible immune potentiator [35]. Chitosan and/or chitooligosaccharides could also induce the activation of AMPK, which might negatively regulate the cAMP-induced chloride secretion, which could reverse the afatinib-induced AKT inhibition via the AMPK-independent mechanism [36]. Therefore, chitosan may act as a promising natural polymer-derived compound for the development of treatment for afatinib-associated diarrheas (Fig. 3).

Protein tyrosine kinases (PTKs) could activate the PI3K/AKT signaling by a specific ligand such as epidermal growth factor or nerve growth factor binding to the corresponding receptor. Activation of PI3K by PTKs may lead to the production of several inositol phospholipids including phosphatidylinositol 3,4-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3), which could activate the phosphoinositide-dependent kinase 1 (PDK1) by enrolling it. Activated PDK1 might phosphorylate and then trigger AKT activation [37]. Janus kinase 1 (JAK1) and the signal transducer could also stimulate the PI3K/AKT signaling pathway after the stimulation of receptors by interleukin 2 (IL-2) and/or interferon gamma (IFNγ) [38]. Activated AKT could directly and/or indirectly modulate the function of the mammalian/mechanistic target of rapamycin (mTOR) that could form mTOR complex 1 (mTORC1), which might contribute to the integration of signals controlling cell survival, proliferation, and/or cell metabolism [39]. One of the negative regulators of mTORC1 may be the activity of AMPK and/or the level of AMP/ATP. Therefore, the PI3K/AKT/mTOR signaling pathway might play a critical role in many physiological and/or pathological conditions, particularly in cell growth, differentiation, cell death/apoptosis, and/or survival. Various upstream regulators of the PI3K/AKT/mTOR signaling pathway have been documented [40]. Remarkably, the PI3K/AKT/mTOR signaling may be involved in the regulation of several pathways to inflammation or apoptosis via the modification of autophagy [41,42]. For example, a study has revealed that pre-treatment with alginate sodium sulfate of propylene glycol could diminish hepatocellular apoptosis and/or autophagy by regulating the PI3K/AKT/mTOR signaling pathway [43]. Interestingly, methylprednisolone could also improve hepatocyte apoptosis and/or autophagy via the PI3K/AKT/mTOR signaling

pathway [44]. In line with these results, itaconate could activate the PI3K/AKT/mTOR signaling pathway to inhibit autophagy, which might also decrease the number of autolysosomes preventing autophagic cell death [45]. Activation of the mTOR signaling may decrease the amount of endogenous antigens of major histocompatibility complex class II (MHC class II) molecules by blocking autophagy in dendritic cells [46]. On the contrary, inhibition of the mTOR signaling with some inhibitors such as rapamycin could enhance the secretion of proinflammatory cytokines [47], which may be related to the activation of NF-κB signaling via the activation of PI3K/AKT signaling pathway [48]. Therefore, the PI3K/AKT signaling in dendritic cells might play imperative roles in activating cellular immunity. In addition, the maturation of dendritic cells might be also achieved through the modification of the PI3K/AKT signaling, which may play an important role in initiating cellular immunity of T cell responses [49].

Dendritic Cells and Chitosan

Dendritic cells are fundamental architects of the specific immune response to antigens, which could promote the development and/or proliferation of effector T cells to protect the host from various pathogens (Fig. 2). Interestingly, microenvironments such as low oxygen, nutrition, and/or starvation levels may determine the control of dendritic cells [50]. In this context, the activation and/or inhibition of dendritic cells by controlling the availability of oxygen and/or nutrient levels might be crucial [41,51]. Some reports have shown that dendritic cells may be influenced by hypoxia in their cell survival, differentiation, and/or activation [50–52]. Prolonged exposure to hypoxia may result in apoptosis of dendritic cells via the inhibition of the PI3K/AKT signaling pathway [53]. Therefore, the PI3K/AKT pathway may also play a significant role in the survival of dendritic cells [54], which may be closely associated with the induction of hypoxia-inducible factor (HIF) that is the major regulator of various responses in hypoxia [55]. In the presence of adequate oxygen, an oxygen-regulated α subunit of HIF1 (HIF-1α) is prolyl-hydroxylated then leading to ubiquitination and rapid destruction in proteasomes [56]. In the condition of hypoxia, the HIF-1α could promote the expression of target genes including B-cell lymphoma 2 (*BCL-2*) family-related proteins, which may be involved in the survival/apoptosis of dendritic cells [57].

Chitosan has been utilized for conveying nucleic acid-based therapeutics including short interference RNA (siRNA) owing to its nature of cationic charge and/or low toxicity [58,59], which might be also a striking drug delivery cargo including for vaccine delivery compared to the other vehicles. In addition, chitosan-based nanoparticles could be modified for the delivery of dendritic cells, which have been employed to initiate dendritic cells for the whole-cell lysates from cancer cells, in which tumor growth has been significantly delayed in mice attributable in part to cytotoxic T lymphocytes response [60]. Autophagy is the main pathway to eliminate abnormal accumulated proteins. Therefore, autophagy in dendritic cells might play a crucial role in antigen presentation and/or potent T cell induction during the antigen presentation. Importantly, the PI3K/AKT/mTOR signaling pathway has been involved in the process of autophagy, which could also activate the function of dendritic cells [61]. Interestingly, inhibiting the PI3K/AKT/mTOR signaling could occasionally induce autophagy [62]. In addition, an enhanced level of autophagy in dendritic cells has been found by the significant promotion of AMPK [63]. In general, autophagy could be induced by two different ways of upstream signaling based on either the inactivation of mTOR or the activation of AMPK. In both meanings, chitosan could activate autophagy in dendritic cells. Activated autophagy could make dendritic cells migrate to secondary lymphoid tissues to interact with naïve T cells to initiate effector T cell responses [64,65].

Downregulation of autophagy by the activation of mTOR could also inhibit antigen presentation [66]. In contrast, rapamycin-mediated stimulation of autophagy in dendritic cells could considerably restore the $CD4^+$ T cell responses [66]. Starvation is an efficient way to induce autophagy, which may subsequently result in boosted antigen presentation and/or robust T-cell responses [67]. By augmenting autophagy-mediated antigen presentation, it has been shown that a DNA vaccine could directly acquire greater efficacy [68]. The PI3K/AKT/mTOR signaling might be involved in the occurrence/development of several inflammations with dysfunctional autophagy [41,69]. Interestingly, the majority of oncoviruses may evade the immune detection of hast and could activate some signaling cascades including the PI3K/AKT/mTOR pathway associated with modified autophagy and/or angiogenesis [69,70]. Consistently, pre-treatment with propylene glycol could decrease hepatocellular apoptosis and autophagy by regulating the PI3K/AKT/mTOR pathway [43,71]. Treatment with methylprednisolone could improve apoptosis and/or autophagy via the modification of the AKT/mTOR pathway in hepatocytes [44,72]. In line with these results, activated mTOR could consequently impede autophagy and autolysosomes for preventing autophagic cell death [45,73]. Consequently, activation of mTOR could limit the T cell activation via the reduction of endogenous antigen presentation [74]. In contrast, inhibition of mTOR with rapamycin may enhance the production of proinflammatory cytokines, which may activate the PI3K/AKT pathway with aberrant proliferation of some cells [47,75]. Interestingly, chronic mTOR activation could impair keratinocyte differentiation and contribute to the phenotypical changes realized in the psoriatic epidermis [75]. It has been shown that a chitosan-modified nanoparticle could enhance the uptake efficiency of gut epithelial cells via the modulation of the PI3K/AKT

pathway, which could promote regulatory T cells inhibiting dendritic cell maturation for keeping the immune homeostasis [76].

Adjuvants Enhance Vaccine Efficacy through Autophagy

The immune system is a vast network with some crosstalk of cells from innate and/or adaptive immunity. Autophagy might be an important mechanism including neutrophils, monocytes, macrophages, and dendritic cells. A number of studies have emphasized the potential of targeting autophagy for the control of several infections [77–83]. This information has also attracted substantial interest in developing some modulators of autophagy as a novel approach for superior vaccination [77]. Dendritic cells might be one of the most powerful l antigen-presenting cells, which could link to the association between innate and adaptive immune systems. It has been shown that autophagy is involved in various functions of dendritic cells in physiological and/or pathological situations with T-cell activation [78,79]. Autophagy and immunity may share the property of being protective for foreign organisms. Interestingly, autophagy has been shown to be involved in antigen processing and presentation in dendritic cells, which may be frequently achieved from lysosomal degradation [80]. Autophagy may be a process destined to degrade intracellular components and/or antigens by guiding them to lysosomes.

Several vaccines may be derived from attenuated forms of pathogens. Removing dangerous virulence genes may increase the key safety of the vaccine, however, it might occasionally decrease the immunogenicity of the vaccine. In particular, the lost genes may be associated with autophagy and/or related functions. For boosting the host immune responses, autophagy should be enhanced with adjuvants to increase the phagocytosis as well as the antigen presentation by dendritic cells as much as possible [81]. For example, an additional application of rapamycin could enhance the MHC class II presentation by dendritic cells via the activation of autophagy, which could enhance the efficacy of the vaccine [82]. Consequently, prompting innate pathways in combination with the activation of autophagy could enhance the immune response and then improve the vaccine efficiency [83]. Interestingly, curcumin may be recognized for prompting autophagy, which would have been tried for cancer immunotherapy. Therefore, a nanoparticle version of curcumin could optimize autophagy and might finally enhance the vaccine efficacy [84]. Furthermore, the antioxidant glutathione could also improve the vaccination by increasing the autophagy [85]. However, the possible effect of those adjuvants may be pathogen-specific.

Autophagy might play imperative roles in both innate and adaptive immunity, which may be an inflexibly regulated mechanism, which may represent the most basic host defense system within eukaryotes [86]. Autophagy stimulates antigen processing not only for MHC class II but also for the MHC class I pathway [87]. In the conventional model, MHC class I molecules are limited to surveying the cytosol for endogenous antigens from viruses to tumors. Further studies are necessary to more obviously outline the relationship between the several autophagy pathways and MHC class I and/or class II antigen presentation throughout the immune responses to intracellular pathogens [87]. Inhibition of autophagy could remove the cross-presentation nearly totally, whereas the induction of autophagy may deeply enhance the cross-presentation. Interestingly, autophagosomes could work as effective antigen carriers for enhancing the cross-presentation. In addition, autophagosomes might disrupt the antigen storage in dendritic cells [88]. Dendritic cells could deposit the antigen for a long time within endolysosomal compartments and thereby keep up MHC class I antigen cross-presentation to $CD8^+$ T cells [88]. The effect of the increased antigen cross-presentation may be independent of altered enzymatic activity in the proteasome. Again, autophagy could be involved in supporting the antigen cross-presentation by dendritic cells.

Future Perspectives of Chitosan

Pharmacological induction of autophagy could increase the pathogen clearance in phagocytes as well as in dendritic cells. Therefore, combined adjuvants with an autophagy modulator have been proven to boost host immune responses by increasing immune cell functions including cytokine/chemokine production. Interestingly, another option to enhance autophagy is known to be attained through the activation of vitamin D receptors. The active form of vitamin D, 1,25-dihydroxyvitamin D3 could powerfully induce autophagy in human monocytes and may initiate the transcription of autophagy-related genes such as Beclin1 and/or *ATG5* [89]. TLR2/1/CD14 stimulation might also activate the antibacterial autophagy through the vitamin D receptor in human primary monocytes [89]. Remarkably, a combination of retinoic acid (RA) and vitamin D3 could enhance the levels of reactive oxygen species (ROS) and autophagy [90]. Vitamin D is frequently used as the modulator of bone formation in regenerative medication. Interestingly, it has been shown that chitosan could be employed for the continuous delivery of efficient vitamin D [91]. All-trans retinoic acid (ATRA) incorporated nanoparticles of methoxy polyethylene glycol grafted chitosan may have cytotoxicity against cancer cells, which is further effective in inhibiting the invasion of tumor cells rather than free of all-trans retinoic acid [92]. Apoptosis of tumor cells seems to be progressed by treated with chitosan. Furthermore, vitamin D may be associated with the stimulation of innate immunity. In some cases, certain small molecules including vitamin D could be a novel therapeutic modality with a promising potential for the better performance of immune checkpoint blockade cancer therapies [93].

Targeting the regulation of autophagy in dendritic cells might be an attractive approach to augment the efficacy of vaccination and/or to improve immunotherapeutic strategies against infectious diseases or cancers. Given the potent anti-inflammatory effects associated with autophagy, employing the functions in vaccination might support regulating inflammations in some infectious diseases. Vaccines that are based on genetic fusion of antigens to important components of autophagy might improve adaptive immune responses by enhancing antigen presentation and/or processing. However, the favorable effector mechanism applied by autophagy might differ among cell types and/or the type of pathogens. In addition, the impact of autophagy might comprise serious cross-talk within the immune system. Therefore, therapeutic tactics should be fixed individually for a specific condition. For example, an innovative way to enhance the immune response to an RNA/DNA vaccine bagged with chitosan has been revealed through the induction of autophagy [94]. Additionally, a number of natural compounds capable of modulating autophagy have been reported [95,96] (Fig. 3). In addition, several studies have investigated the advantages of combining these natural compounds with chemotherapeutic drugs. In particular, more and more phytochemicals are now applied to the treatment of metabolic diseases [97,98]. Therefore, it might be necessary to have a more comprehensive understanding of the potential mechanisms of various diseases. Nowadays, the aluminum hydroxide gel (alum) adjuvant has been successfully used as the principal adjuvant in clinical vaccines. However, the adjuvant with alum may induce long-duration of the inflammatory reaction with severe local tissue irritation. Therefore, there has been a vital requirement for the development of alternative adjuvants. It has been shown that chitosan nanoparticles are comparable to the alum-based adjuvant in efficacy but greater in inducing cell-mediated immune response with a balanced Th1/Th2 immune pathway [99,100]. This information would also contribute to developing further tactics for superior vaccines with adjusted efficiency and safety against several diseases. Anyway, future studies should focus on investigating the role of autophagy in the specific interplay between vaccine and host dendritic cells in an organized manner.

Conclusion

Chitosan might possess immune-stimulatory effects as a potential good adjuvant, which might be related to the alteration of autophagy via the modification of PI3K/AKT/mTOR signaling in dendritic cells. Therefore, chitosan nanoparticles could be one of the most promising materials for the development of superior vaccine delivery.

Author Contributions

Conceptualization, MN, NS, YI, SY, and SM; original draft preparation and editing, MN, NS, YI, SY, and SM; visualization, MN, NS, YI and SM; supervision, SM. Each author (MN, NS, YI, SY, and SM) has participated sufficiently in this work of drafting the article or revising the article for the important rational content. Then, all authors gave final approval of the version to be submitted. Finally, all authors have read and agreed to the published version of the manuscript. All authors have also agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

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Conflict of Interest

The authors declare no conflict of interest.

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