REVIEW ARTICLE

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CpG Oligodeoxynucleotides as TLR9 Agonists

Therapeutic Application in Allergy and Asthma

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Abstract

Unmethylated cytosine-phosphate-guanine (CpG) dinucleotides in microbial DNA sequences activate Toll-like receptor (TLR) 9, and previous studies have shown that oligodeoxynucleotides (ODNs) containing CpG in specific base sequence motifs (CpG ODNs) can reiterate the majority of the immunomodulatory effects produced by bacterial DNA. Many of the manifestations in allergic diseases are primarily due to T helper (T_h)-2 cell-type responses. CpG ODNs can induce T_h1 and T-regulatory (T_{reg}) cell-type cytokines that can suppress the T_h2 response. The therapeutic application of TLR9 has been explored extensively in recent years, and many studies are being conducted to assess the safety and efficacy of TLR9 agonists in various diseases, including atopic and infectious diseases, and cancer. Studies in murine models have shown that the development of atopic airway disease can be prevented by treatment with CpG ODNs. Various clinical trials are currently ongoing to determine the efficacy of CpG ODNs as a therapeutic tool for atopic diseases. In this review, we discuss the therapeutic application of CpG ODNs in allergy and asthma. CpG ODNs may be used alone or as an adjuvant to immunotherapy to treat these disorders.

The immune system protects against invasion by pathogenic micro-organisms by discriminating between self and non-self antigens and eliminating the latter. The mammalian immune system can be divided into two branches: 'innate immunity' and 'adaptive immunity'. Toll receptors were first described in *Drosophila* as being involved in innate immunity. Soon after the discovery of *Drosophila* Toll, a structurally related family of proteins was identified in mammals, known as Toll-like receptors (TLRs).[1] These TLRs play a major role in the detection of invading micro-organisms by recognizing specific, conserved microbial component patterns and promoting activation of both innate and adaptive immunity.[1,2] These conserved patterns involve six-base self-complementary 'palindromes' in microbial DNA, which appear to be responsible for the stimulatory effect on the immune system.[3,4] All palindromes capable of immune stimulation were shown to contain at least one cytosine-phosphate-guanine (CpG) dinucleotide.[3] The palindrome ACCGGT and other palindromes that consisted

entirely of A and T did not cause stimulation. AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC, and TCGCGA were active palindromes.[3,4] Secondary structures formed by the palindromes that included CG were thought to be the underlying mechanism responsible for stimulation because CG base pairs conferred greater stacking stability.[3]

CpG DNA leads to adaptive immune responses by producing strong T helper (T_h)-1-type innate immune responses.[5] Activation of T_h1 cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ, by CpG DNA is effective against various infectious diseases.[5-8] In allergy and asthma, T_h2-type immune response plays a major role. In this context, CpG DNA not only induces the T_h1 cytokines but also has the capacity to reverse and prevent T_h2 immune response.[5,9-12]

1. Toll-Like Receptors (TLRs)

The TLR family represents the mammalian homolog of *Drosophila* Toll, and has at least 13 members.[1,13,14] The structure of TLR family members comprises a leucine-rich repeat (LRR) domain in the extracellular domain and Toll/IL-1 receptor (TIR) domain in the intracellular domain. In humans, the TLR family can be divided into subfamilies on the basis of comparison of amino acid sequences. The TLR2 subfamily is composed of TLR1, 2, 6, and 10.[15] The TLR9 subfamily is composed of TLR7, 8, and 9.[16]

TLRs are distributed in a variety of human cell populations.[17] Messenger RNAs (mRNAs) for most of the TLRs, except TLR3, are expressed in monocyte/macrophage.[18] Subsets of dendritic cells differ in their distribution of TLRs.[19,20] There are two different subsets of dendritic cells in human blood: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs).[21-23] pDCs exclusively express TLR7 and 9, whereas mDCs express TLR1, 2, 4, 5, and 8. TLR9 is also expressed on B lymphocytes.[24] Some studies have suggested that mDCs also express TLR7.[19,20,25,26]

TLR signal-transducing pathways in mammals are both similar and dissimilar from those in *Drosophila*. Toll pathways and immune deficiency pathways are responsible for antifungal and anti-Gram-negative bacterial responses in *Drosophila*. Pathways originating from the common TIR domain of TLRs mediate the host defense against micro-organisms in mammals. The signal pathway of the TLR family and IL-1 receptor (IL-1R) family is exceedingly homologous and interacts with an adaptor protein, myeloid differentiation primary response gene 88 (MyD88), which is vital for the production of TLR family-induced inflammatory cytokines. MyD88 has a TIR domain in the C-terminal portion and a 'death domain' in its N-terminal portion. The serine/threonine kinase IL-1R-associated kinase (IRAK), which contains a death domain, is recruited by MyD88 upon stimulation. Activation of IRAK by phosphorylation, followed by its association with tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 ultimately leads to the activation of mitogen-activated protein kinase (MAPK)8 (also known as c-JUN N-terminal Kinase [JNK]) and nuclear factor (NF)-κB.[1] NF-κB induces transcription of many pro-inflammatory cytokine genes, including those encoding TNFα and IL-6. Activation of TLR9 in pDCs by CpG DNA induces production of large amounts of TNFα and IFNβ. This process is MyD88 dependent and is mediated by IFN regulatory factor 7 (IRF7). IRF7 forms a signaling complex with MyD88, IRAK, and TRAF6, and becomes activated. Translocation of IRF7 to the nucleus induces TNFα and IFNβ production[13] (figure 1).

2. TLR9 and CpG DNA

In humans, nucleic acid features from natural and synthetic sources have been shown to be recognized by TLR3, 7, and 9.[5] Direct sequence-specific binding of unmethylated CpG dinucleotides in microbial DNA activates TLR9.[27,28] These unmethylated CpGs are primarily responsible for the immunostimulatory property of bacterial DNA. Vertebrate DNA lacks this property,[29] as most of the CpGs in their DNA are methylated at position 5 of cytosine.[3] Although most of the TLRs are located in the cell membrane, TLR9 is found intracellularly in the endosome.[5,30] For immunostimulation, CpG DNA must be internalized intracellularly and be co-localized with TLR9 in endosomes[5,31] (figure 1). Acidification of CpG DNA occurs, and acidified CpG DNA is taken up by B lymphocytes and monocytes by adsorptive endocytosis in an intracellular endosomal compartment. Reactive oxygen species are generated by the endosomal acidification of DNA, which leads to the activation of NF-κB and consequent expression of cytokines.[32,33]

Although recognition of CpG oligodeoxynucleotide (ODN) by TLR9 is well ascertained, structural details of receptor-ligand interaction are not yet known.[34] TLR9 has 25 LRR motifs in its extracellular domain, 5 of these motifs bear inserting sequences that do not conform to the LRR consensus motif.[34] A study by Peter et al.[34] showed that deletion of individual LRR motifs results in loss of functional integrity of the extracellular domain of murine TLR9. When deleting only inserting sequences, it was observed that LRR2, 5, and

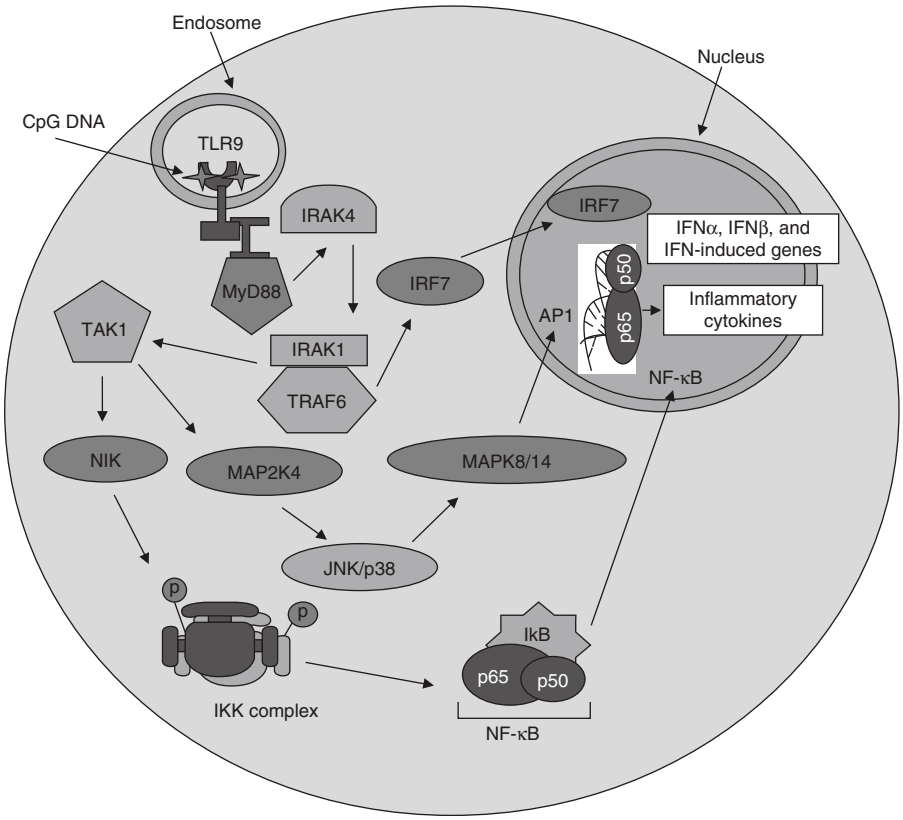

Fig. 1. Toll-like receptor (TLR) 9 signaling pathway: cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODNs) are internalized into endosomes and interact with endosomally located TLR9. An intracytoplasmic activation signal transduction occurs following interaction of CpG ODN and TLR9. Recruitment of myeloid differentiation primary response gene 88 (MyD88) is initiated, and the MyD88-TLR complex promotes association with interleukin (IL)-1 receptor-associated kinase (IRAK) 4. Activation of IRAK4, in turn, induces hyperphosphorylation of IRAK1, which induces interaction of tumor necrosis factor receptor-associated factor (TRAF) 6 with the complex. TRAF6 activates transforming growth factor-β-activated kinase (TAK) 1 and this leads to mitogen-activated protein kinase (MAPK) kinase 4 (MAP2K4)-mediated activation of both MAPK8 and MAPK14. and inhibitor of nuclear factor (NF)-κB kinase (IKK) complexes, resulting in the upregulation of transcription factors, including NF-κB and activator protein-1 (AP1), which in turn activate production of pro-inflammatory cytokine genes. In plasmacytoid dendritic cells (pDCs), TRAF activates interferon regulatory factor (IRF)-7 and induces interferon (IFN)-α and IFNβ production. **JNK** = c-JUN N-terminal kinase; **NIK** = NF-κB-inducing kinase.

8 contribute to CpG-mediated receptor activation.[34] Deletion of these LRRs inhibit only CpG-DNA binding; receptor dimerization is not affected.[34] These investigators also identified a positively charged region in the N-terminus that is essential for CpG DNA-mediated TLR9 activation on the basis of a homology modeling approach.[34] TLR9 on B cells, dendritic cells, and other cell types are engaged by CpG ODN, which results in the induction of T_h1-type and T-regulatory (T_{reg})-type immune responses.[35] In comparison to other TLR ligands, expression of TLR9 is restricted to certain dendritic cells and B cells that provide relative specificity in the response.[36]

The underlying mechanism for recognition of CpG DNA is not fully known. A study by Hurtado and Peh[37] found that LL-37, a cationic antimicrobial peptide derived from keratinocytes and neutrophils, promotes quick and efficient sensing of CpG ODN by human pDCs and B cells.[37] With LL-37, CpG ODN was recognized within minutes, and without LL-37, 20- to 30-fold more CpG was required to produce the same effect.[37] The LL-37-promoted CpG detection was observed in both ODN with modified phosphorothioate and ODN with natural phosphodiester backbones.[37] It is noteworthy that not only bacterial DNA but also CpG-motif-containing deoxynucleotides can demonstrate immunostimulatory activities in human antigen-presenting cells (APC) and lymphocytes. TLR9 specifically recognizes the CpG motifs in synthetic DNAs containing deoxy-CpG (dCpG) dinucleotides in specific sequence contexts.[38]

Expression of the TLRs is regulated by several cytokines. TLR9 expression can be downregulated in macrophages by colony-stimulating factor (CSF) 1, which is induced after infection and can downregulate TLR9 expression in macrophages. Thus, CpG DNA-induced inflammatory cytokine response can be suppressed by CSF1.[1] Based on the activation

of either pDCs or B lymphocytes by oligonucleotide containing a CpG motif (CpG ODN), three different classes have been identified.[2] Class A CpG ODNs cause maximum natural killer (NK) cell stimulation and IFNα secretion by pDCs, while class B CpG ODNs cause cytokine production by strong B lymphocyte and NK cell stimulation. The class C CpG ODNs combine the effect of both class A and class B.[39]

3. Immunobiology of Allergy and Asthma

CD4+ T cells play a key role in the pathogenesis and immunobiology of atopic conditions and asthma.[29,40] Based on their cytokine production, CD4+ T cells can be divided into T_h1 and T_h2 cell types. The effect of these cells is counter regulatory on each other. T_{reg} cells are a specialized subpopulation of T cells that secrete IL-10 and cause down-modulation of allergic inflammation.

Strong T_h2 immune deviation against otherwise benign specific antigen has been implicated in the pathogenesis of allergic diseases[40,41] (figure 2). Mast cells release IL-4 on exposure to allergens in sensitized individuals. When APCs present antigen in the presence of IL-4, T_h2 cells are activated. Production of T_h2-type cytokines from these cells causes activation of B lymphocytes, eosinophils, and basophils. Production of chemokines and cytokines, such as IL-4, IL-5, and IL-13, from T_h2 cells leads to various manifestations of asthma, including eosinophilia, airway hyper-responsiveness (AHR), and B-cell isotype switching to IgE production.[24,29,42] Products of mast cells, eosinophils, and basophils induce hyperplasia of myofibroblasts, goblet cell hyperplasia/metaplasia, denudation of the epithelial layer, extracellular matrix/collagen production, and angiogenesis (figure 2).

T_h17 cells are a recently identified lineage of pro-inflammatory CD4+ T effector cells that is different from T_h1 and T_h2 cells.[43-45] They secrete different cytokines, including IL-17A, IL-17F, and IL-22.[43] Recently, a role of T_h17 cells and T_h17 cytokines in the pathogenesis of allergic asthma has been suggested.[44] For example, IL-17 is able to trigger inflammation

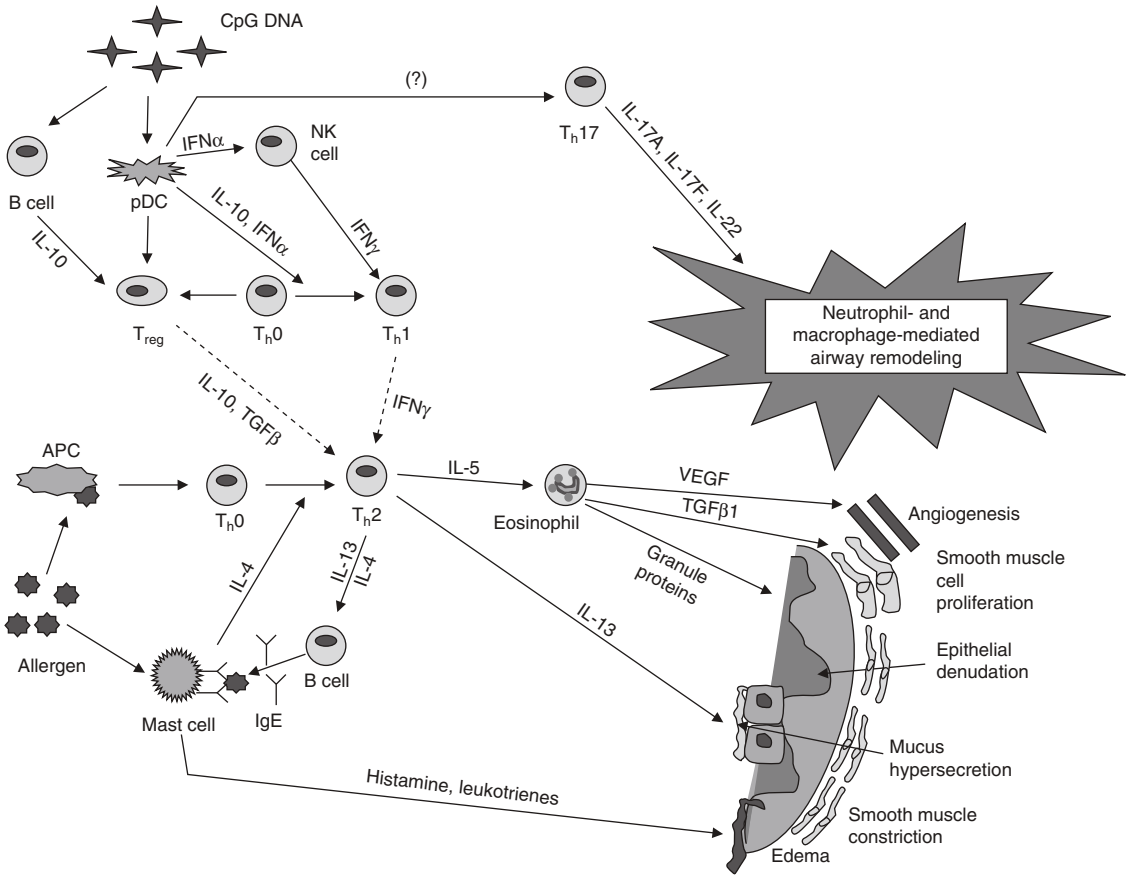

Fig. 2. Modulation of immune response in atopic asthma by cytosine-phosphate-guanine (CpG) DNA: plasmacytoid dendritic cell (pDC) and B-cell-mediated T helper (T_h)-1 and T-regulatory (T_{reg}) responses are induced by CpG DNA. Induction of T_h1 and T_{reg} responses lead to secretion of interferon (IFN)-γ from T_h1 cells, and interleukin (IL)-10 and transforming growth factor (TGF)-β from T_{reg} cells, which suppress the T_h2 activation. **APC** = antigen-presenting cell; **NK** = natural killer; **VEGF** = vascular endothelial growth factor.

in the airways by inducing neutrophils and stimulating innate immunity[46] (figure 2). IL-22 is a member of the IL-10 family and is involved in chronic inflammation. Both IL-17 and IL-22 regulate granulocyte CSF and production of CXC chemokines in the lung.[47] CpG ODNs activate pDCs by endosomal TLR9 and control both innate and adaptive immunity by producing type 1 IFN.[48] The role of pDCs in the differentiation of T_h17 is not quite known as yet. In a recent study, Ishifune et al.[49] used CpG-activated bone marrow-derived pDCs and concluded that pDCs have a strong ability to induce T_h17 cells.[49] Further studies are warranted to determine the mechanism underlying pDC-induced T_h17 differentiation; however, CpG-activated pDCs might cause some toxic effects (figure 2).

4. CpG DNA in Allergy and Asthma

Short-acting β2 adrenergic agonists (albuterol [salbutamol], terbutaline), long-acting β2 adrenergic agonists (salmeterol, formoterol) in combination with corticosteroids,[50] and more recently leukotriene antagonists (montelukast, zafirlukast), are currently the most commonly used agents in the treatment of asthma.[12] Although corticosteroids do not help in modifying or curing the disease, they are often successful in helping with the manifestations of inflammation. Use of immunotherapy in allergic diseases is an uncommon mode of treatment and its efficacy in asthma has been questioned. However, there are many controlled studies that support the use of immunotherapy in many cases of asthma.[40,51,52] Immunotherapy is believed to act against specific allergens through the alteration of T_h2 to T_h1 immune response. Activation of APCs by DNA motifs with a central unmethylated CpG dinucleotide in specific base sequences leads to secretion of a wide array of chemokines and cytokines that promote activation of naive CD4+ cells and augment their differentiation into T_h1 cells[29,40] (figure 2); one or more CpG motif-containing ODNs can be used to reproduce these responses.[53] Bacterial DNA motifs containing unmethylated CpG dinucleotides can cause swift and coordinated release of IL-6, IL-12, and IFNγ.[54]

The role of CpG DNA in the prevention of antigen-induced asthma in mice has been reported.[32,40] In acute allergic inflammation, production of IL-4 and IL-5 can be inhibited by CpG DNA.[29,55] Mice that are deficient in IL-4 and IL-5 have restricted infiltration of eosinophils in their airways and less AHR after allergen sensitization and challenge.[56,57] The role of IL-13 in the development of allergen-induced AHR does not depend upon eosinophilic airway inflammation. IL-13-induced AHR may be due to the direct effect of the IL on airway epithelial cells and its promotion of goblet cell hyperplasia.[29] Established AHR can be reversed by suppression of IL-13,[29,58] and it has been shown in a murine model that CpG DNA can inhibit IL-13 and thus prevent goblet cell hyperplasia.[29,59] Acute inflammation in asthma leads to chronic airway response. Chronic airway response leads to airway remodeling that includes goblet cell hyperplasia/metaplasia and subepithelial matrix/collagen deposition. Current treatment modalities for asthma, such as β2-agonists and corticosteroids, do not appear to prevent these structural changes and thus do not effect airways remodeling. Bronchoalveolar lavage after treatment with CpG ODNs exhibits increased levels of transforming growth factor (TGF)-β, which suggests that T_{reg} cells might be playing some role in these protective effects (figure 2). This might explain why CpG ODN-mediated protection is independent of T_h1 cytokines.[12]

The CpG motifs supplied by bacterial DNA induce regulator responses in CD4+ T cells. As hypothesized earlier, T_h1 response is not the only mechanism responsible for CpG DNA-induced manifestations. Kline et al.[24] reported that treatment of mice with CpG ODN suppressed airway eosinophilia and airway hyperactivity and resulted in decreased levels of IL-4 in the bronchoalveolar lavage fluid, with a modest rise in T_h1-type cytokines such as IL-12 and IFNγ. Kline and colleagues[55] also found the inhibition of T_h2 response in IFNγ/IL-12 knockout mice following treatment with CpG ODN, which suggests that switching from a T_h2-type to a T_h1-type response to antigen is not sufficient to explain the protective effects of CpG ODN. In another study using an *in vitro* allergen rechallenge model of atopic responses, Kitagaki et al.[40] reported that on stimulation of ovalbumin (OVA)-sensitized splenocytes with CpG ODN, release of IL-5 was inhibited in a concentration-dependent manner. Although T_h1-type cytokines were released from the CpG ODN stimulated splenocytes, maximal suppression of IL-5 did not occur at maximal concentration of Th-1 cytokines.[40] Interestingly, T_h2-type response was inhibited in IFNγ/IL-12 knockout mice. In addition, IL-10 was shown to be released from OVA-sensitized splenocytes in a concentration-dependent manner, with an inverse correlation between its concentration and IL-5 suppression.[40] This suggests that IL-10 plays an important role in the protective effects of CpG DNA.

CpG DNA also regulates the activity of indoleamine 2,3-dioxygenase (IDO), which is a rate-limiting enzyme in the catalysis of tryptophan to kynurenunes.[24] Kynurenunes are immunomodulatory molecules responsible for the regulation of T-cell function. Human pDCs can drive naive CD4+CD25– T cells to differentiate into CD4+CD25+FOXP3+ regulatory cells.[60] A study by Chen et al.[60] showed that human pDCs

express high levels of IDO, and activation of TLR9 by CpG ODNs results in increased expression of IDO and generation of inducible T_{reg} cells from CD4+CD25– T cells. T_h1 response from eosinophils is suppressed after IDO expression,[61] and CpG DNA-induced IDO expression in dendritic cells results in enhanced regulatory cell numbers and function.[62]

5. CpG Oligodeoxynucleotides in Murine Models of Allergy and Asthma

Many studies in the recent past have shown significant results with the use of CpG ODN in murine models of allergy and asthma. In one study, administration of CpG ODN along with antigen prevented the induction of T_h2 cytokines, airway eosinophilia, and IgE production.[55] In another study in a murine model, CpG ODN treatment was not only beneficial in preventing acute inflammation, but it also appeared to decrease airway remodeling.[12]

CpG ODN treatment may help not only in the prevention of the development of asthma, but may also be useful in reversing the hallmarks of established asthma. The co-administration of OVA allergen with CpG ODN can reduce already established airway disease in mice.[63] Eosinophilic airway inflammation and bronchial hyper-reactivity were decreased in mice treated with the combination of OVA and CpG ODN compared with a control group that was given experimental antigen alone.[63]

Treatment with CpG ODN has also shown promising results in other atopic diseases. Intraperitoneal administration of CpG ODN with OVA in mice during sensitization resulted in decreased symptoms of allergic rhinosinusitis compared with the control group.[64] A study by Jain et al.[65] showed that CpG-based immunotherapy is both antigen specific and antigen nonspecific to a lesser extent. In this study, mice were sensitized to two different antigens (OVA and schistosome eggs), challenged with OVA through inhalation, and treated with CpG ODN immunotherapy based on OVA antigen. Challenge of these animals with schistoma antigen showed moderately reduced eosinophilia and significantly reduced AHR.[65] Previously Kitagaki et al.[66] reported protection and reversal of airway inflammation by CpG ODN treatment through either the parenteral or the mucosal route. The authors demonstrated that oral administration of OVA and CpG combination can reverse and prevent the airway inflammation induced by OVA antigen.[66] A home-made allergen, *Chenopodium album* (Ch.a) extract, has been used in combination with CpG ODN in another study.[67] Intraperitoneal administration of ODN and Ch.a combination in Ch.a pollen allergen extract-sensitized Balb/c mice showed increased levels of IL-10 and IFNγ. Interestingly, the ratio of IgG2a/IgG1 was also increased in comparison with mice treated with antigen alone or with buffer (phosphate-buffered saline [PBS]) alone. Thus, the T_h2-mediated immune response was inhibited by CpG ODN and Ch.a combination via the regulatory effects of IL-10.[67] In another study, the same authors concluded that intranasal administration of the CpG ODN and Ch.a combination in the same model can induce increased levels of IFNγ and IL-10.[68] A study of co-administration of CpG ODN/Ch.a by intranasal or subcutaneous routes showed that the induction of IL-10 and IFNγ was less for the subcutaneous route than the intranasal route. Thus, CpG/Ch.a immunotherapy stimulates regulatory responses more effectively by the intranasal route in Ch.a-induced asthma.[69]

In another study in mice, Martínez Gómez et al.[70] used a CpG ODN immunotherapy based on phospholipase A2 (PLA2), the major allergen of bee venom. Results showed that titers of IgG2a were higher in the animals immunized with the CpG/PLA2 combination than with PLA2 alone, and higher titers of IgG2a were protective against allergen challenge in sensitized mice.[70] Suzuki et al.[71] conducted a study using the combination of CpG DNA and T-cell epitope peptide in a murine model of asthma and concluded that CpG DNA and T-cell peptide conjugate-based immunotherapy is useful in the treatment and prevention of asthma.

6. CpG-Mediated Response in Human Atopic Diseases

Human trials of CpG DNA in allergy and asthma have shown promising results. An *in vitro* study in peripheral blood mononuclear cells (PBMCs) from patients with ragweed allergy showed that there is induction of T_h1-type response by ragweed pollen allergen/CpG ODN conjugates (Amb a 1/immunostimulatory DNA conjugate [AIC]), while only T_h2-type response was induced when ragweed pollen allergen was used alone in these patients.[72] A randomized, double-blind, placebo-controlled pilot study by Creticos et al.[73] showed that treatment with AIC in ragweed allergic patients was effective and well tolerated. Twenty-five adult patients who had a history of seasonal allergic rhinitis and were allergic to ragweed extract were enrolled in the study and were randomly divided into two groups. Both AIC and placebo groups received a total of six injections at weekly intervals before the onset of the first ragweed season. Doses for the six AIC injections were 0.06, 0.3, 1.2, 3.0, 6.0, and 12.0 μg. These doses were adjusted accordingly

in the event of any reaction or missed dose. Compared with the placebo group, the AIC-treated group showed significant improvement in peak-season rhinitis scores on the visual analog scale, peak-season daily nasal symptom diary scores, and mid-season overall quality-of-life scores.[73] There was a transient increase in Amb a 1-specific IgG, but seasonal Amb a 1-specific IgE and IL-4-positive basophils were decreased in AIC-treated patients. There was no significant change in IgE antibody titer, but seasonal IgE response was decreased.[73] AIC-treated patients also showed improved peak-season rhinitis visual analog scores and peak-season daily nasal symptom diary scores over placebo in the following ragweed seasons. No systemic reaction or clinical abnormality related to the vaccine was seen. These findings suggest that AIC vaccine treatment offers long-term treatment in patients with ragweed allergic rhinitis. However, this study had a small sample size and did not provide sufficient data on long-term safety and the effectiveness of the therapy.[73]

In another randomized, double-blind, placebo-controlled study, 40 subjects with mild atopic asthma were enrolled.[74] Subjects received 36 mg of placebo or immunostimulatory DNA sequences (ISS 1018) weekly by inhalational route for 4 weeks. Quantitative measurement of gene expression was done by polymerase chain reaction in sputum cells and PBMCs. Twenty-four hours after the second and fourth doses, allergen inhalational challenge was performed to measure early and late fall in forced expiratory volume in 1 second. ISS 1018 was well tolerated and did not show any exacerbation in the symptoms of asthma.[74] Cells from patients who received ISS 1018 showed a significant increase in the levels of IFNγ, and IFN-induced genes such as chemokine CXCL10, IFN-stimulated gene 54, and monocyte chemotactic protein (MCP)-1 and MCP-2. However, the physiologic parameters of the patients, such as pulmonary function, AHR, and airway eosinophilia, did not show any favorable changes.[74] This study inferred that in asthmatics, induction of IFNγ and IFNγ-inducible genes is not adequate to inhibit the allergen-induced responses. This could be due to inadequate dose, duration, or route of administration of treatment, and may not have inhibited all the immune cells responsible for allergic response.[74]

In a phase I/IIa clinical trial of subcutaneous allergen-specific immunotherapy, 21 subjects with a history of mild to moderate allergic rhinoconjunctivitis with asthma due to house dust mite (HDM) were enrolled.[75] Subjects first received only the allergen in two sessions, once weekly, and received three subcutaneous injections. Six subcutaneous injections of immunotherapy with QbG10 (A-type CpG ODN packaged into virus-like particles) were given at 1- to 2-week intervals. A-type ODNs have G-rich 3′ and 5′ ends with phosphorothioate-modified ends and a phosphodiester palindromic center portion.[76] Subjects were assessed clinically by physicians before inclusion, as well as 12, 34, and 48 hours after treatment. No intolerance to the antigen was identified, and within 10 weeks, all the subjects were free of symptoms of allergic rhinitis and allergic asthma.[75] Allergen-specific IgG was increased and also there was a transient increase in IgE titers after injection of HDM allergen and QbG10 in these subjects. Patients were symptom free for 38 weeks post-treatment.[75] Although subcutaneous immunotherapy of HDM-allergic patients with QbG10 was well tolerated and almost all the allergic symptoms were gone after 10 weeks of treatment, this therapy is not highly popular among allergy patients because it is a time-consuming and laborious procedure. Nonetheless, the use of QbG10 adjuvant immunotherapy could reduce the number of injections and make the procedure less time consuming.[75]

An *in vitro* study determined the effects of stimulation of sino-nasal tissue from patients with asthma and controls via local application of CpG ODN, *Staphylococcus aureus* enterotoxin B (SEB), and CpG in combination with SEB.[77] The expression levels of IL-5, IL-12, IFNγ, and TLR9 were determined. CpG ODN alone or in combination with SEB significantly reduced the expression of the pro-inflammatory cytokine IL-5 in the sino-nasal tissue of non-asthmatic compared with asthmatic rhinosinusitis patients. There was no significant difference in the levels of IL-12, IFNγ, and TLR9 expression.[77] Since local application of CpG ODN decreases the expression of pro-inflammatory cytokine, it could be used as immunotherapeutic agent.[77]

Tulic et al.[78] conducted a study in ragweed-sensitive allergic rhinitis patients in which they determined the effect of short-course immunotherapy with AIC on cytokine mRNA expression and eosinophilia in the nasal mucosa. Fifty-seven subjects with a history of seasonal symptoms of rhinoconjunctivitis and with ragweed allergy participated in this study. Just before the 2001 ragweed season, six increasing doses of subcutaneous injection with increasing concentrations of AIC (0.06, 0.3, 1.2, 3.0, 6.0, and 12.0 μg) or placebo were given weekly. A subset of patients agreed to undergo biopsy, and specimens were collected before allergen challenge, preseason, and postseason 24 hours after allergen challenge. Cytokines and inflammatory cell responses were assessed.[78] Results showed that numbers of allergen-induced major basic protein, IL-4-, IL-5-, or IFNγ-positive cells were not different between the placebo and AIC groups in the first weeks.[78] However, increased IFNγ mRNA-positive cells and significantly reduced increases in eosinophils and IL-4 mRNA-positive cells were seen on rebiopsy after the

end of ragweed season in the AIC group as compared with the placebo group. There were no differences in symptom scores or medication use between the placebo and AIC group in the first ragweed season, but in the second ragweed season, symptoms were significantly decreased in the AIC group. None of the subjects showed any intolerance to AIC. It was concluded that short-course immunotherapy with AIC modified the nasal mucosa response to allergen challenge by decreasing the eosinophilia and T_h2 cytokine production and increasing the T_h1 cells 4–5 months after completion of immunotherapy and seasonal exposure to ragweed pollen.[78] Evidence of clinical efficacy was observed in these patients in the second ragweed season without any additional immunization with AIC.[78]

Thus, the present data from human studies suggest that CpG ODN-based immunotherapy could be a novel therapeutic tool for atopic disease. CpG ODNs have proven to be potent immunomodulators in mice, but their stimulatory effect is not as striking in large animals and humans. Studies are being conducted to explore the possibility of enhancing its stimulatory effect by its co-administration with other TLR9 agonists.[79]

7. Possible Side Effects of TLR-Based Therapies

TLRs are implicated in a variety of human diseases, including asthma, malignancy, autoimmune disorders, and atherosclerosis.[30] They play dual roles as physiologic and pathologic mediators; hence, excessive activation of these molecules is potentially hazardous.[30]

A study by Boukhvalova et al.[80] treated influenza and respiratory syncytial virus infection in cotton rats (*Sigmodon hispidus*) with the immunostimulant poly ICLC, a synthetic double-stranded RNA and TLR3 agonist. Results showed that at antiviral doses of poly ICLC, lung inflammation was increased.[80] A study by Knuefermann et al.[81] concluded that TLR9 agonist causes lung inflammation. In this study, CpG ODN was administered intraperitoneally in TLR-deficient (TLR-D) and wild-type (WT) mice, and after 6 hours, inflammatory markers were observed.[81] Results showed that in WT mice there was strong activation of pulmonary NF-κB and increased levels of pulmonary TNFα and IL-1β. Serum levels of inflammatory cytokines were also increased in these mice, while in contrast these responses were abolished in TLR9-D mice.[81] In another study, Knuefermann et al.[82] found that synthetic bacterial DNA-mediated TLR activation caused loss of cardiomyocyte contractility and increased myocardial cytokine production. WT and TLR9-D mice were challenged with CpG ODN, and markers of inflammation were determined. Increased activity of NF-κB and increased levels of IL-1β, TNFα, and inducible nitric oxide synthase were observed in WT mice.[82] Sarcomere shortening was observed in isolated ventricular cardiomyocytes in TLR9-D mice after CpG ODN exposure.[82] In a study by Thaxton et al.,[83] it was found that TLR9 activation can cause fetal resorption and preterm birth in IL-10(–/–) mice. Administration of low doses (25 μg/mouse) of CpG in IL-10(–/–) mice by intraperitoneal route on day 6 and day 14 of gestation caused fetal resorption and preterm birth, respectively. WT mice failed to show any such effects on comparable doses, but interestingly, pups born to WT mice with high doses (400 μg/mouse) showed craniofacial/limb defects.[83]

The expression of TLRs on a wide variety of tumors suggests their role in the pathogenesis of cancer.[84] Activation of TLRs in tumor cells induces tumor cell proliferation, synthesis of immunosuppressive molecules, and resistance to apoptosis.[84] Thus, TLR signaling pathway activation may be subverted by the neoplastic process for progression of advanced cancer.[84] A study by Chiron et al. [85] concluded that phosphorothioate-modified TLR9 ligands could protect cancer cells against apoptosis induced by TRAIL (TNF-related apoptosis-inducing ligand). Results of this study showed that CpG ODN protected TLR9+ (NCL-H929, NAN6, KMM1) and TLR9– multiple myeloma cells from TRAIL-induced apoptosis.[85] Thus, a broad diversity of pathways mediated by TLRs need to be considered and should be studied further to prevent deleterious activation.

8. Future Developments

Modulation of the immune response by manipulation of activity of TLRs for therapeutic purpose has been pursued intensely since the discovery of human TLRs. These activities have been focused in various areas, including allergy, asthma, infectious diseases, malignancies, and vaccine adjuvants. Although initial trials showed promising results in malignancies and infectious diseases, results of long-term trials have been disappointing.[86]

In mouse models, CpG ODNs have shown antitumor activity as monotherapy and also in combination with various other therapies, including chemotherapies, vaccines, and other immunotherapies.[87] TLR9 agonists have also been proved beneficial in primate and rodent models of allergy and asthma, with some promising results in early human trials.[87] Although TLR9 agonists have enormous clinical application, their safety and efficacy needs to be determined.

Data from preclinical and early clinical studies support the hypothesis that TLR9 agonists enhance both the cellular and humoral response to different antigens and can be used as vaccine adjuvants.[87] The role of TLR agonists as vaccine adjuvants has less safety concerns than other applications because only small doses are given.[86] This application seems to hold a lot of potential and is being pursued actively.

TLRs have the unique property of linking innate and adaptive immunity, which makes them novel targets for vaccine development.[88] TLR cross talk by stimulation with multiple TLR agonists could be synergistic or inhibitory to innate immune response. A study by Booth et al.[89] investigated the effect of co-stimulation of sheep B cells and PBMCs with TLR3, 4, 7/8, and 9 agonists. Interestingly, results showed that co-stimulation of PBMC with CpG plus ORNs (TLR7/8 agonists) or CpG plus imiquimod (TLR7/8 agonists) significantly reduced CpG-induced IFNα production, B-cell proliferation, and IgM response.[89] This suggests that crosstalk could possibly be a downregulatory mechanism.[89] In the near future, we will learn more about TLRs and TLR agonists, which may teach us new ways to therapeutically use this knowledge for various diseases.

9. Conclusion

Asthma affects people of all ethnic groups and ages, and has been estimated to affect approximately 300 million people worldwide.[90] The prevalence of asthma and allergy has increased considerably in developed countries over the past few decades[90,91] and the same trend has been shown by developing countries more recently.[92] Therapies based on immunomodulation have gained considerable attention in the treatment of such diseases in recent times. As suggested by the hygiene hypothesis, the recent rise in atopic disorders may be due to less exposure to the microbes and their products in modern life. Bacterial DNA is one such product and has immunostimulatory properties. Many studies have been done in animals showing an immunomodulatory effect of CpG DNA in allergy and asthma, but limited studies are available to advance our understanding of the effect of CpG ODNs in clinical asthma. Human trials for CpG DNA are currently ongoing and our knowledge on the immunologic and pharmacologic effects of CpG DNAs has significantly increased in recent years. Early and preclinical studies suggest that CpG ODNs have the potential to be a novel approach in the treatment and prevention of atopic diseases. The CpG ODNs may be used in asthma as a therapeutic or disease-modifying agent. Obviously, additional studies are required to confirm the potential use of CpG DNA in the treatment of allergy and asthma.

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