

New Biological Approaches in Asthma: DNA-Based Therapy

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Abstract: Asthma is characterized by airway inflammation, bronchial hyper-responsiveness, and reversible airway obstruction. Medications for asthma include corticosteroids, β_2 -adrenergic receptor agonists, chromones, methylxanthines, and leukotriene modifiers. Despite these advances in therapy, many symptoms are not well controlled. Since asthma is a chronic airway inflammation with a bias towards a type 2 T helper (Th2) cell response, some new approaches are targeted towards the Th2 inflammation pathway. These include anti-IgE therapy, anti-Th2 cytokine therapy, and therapies aiming at increasing Th1 cytokines. This article will focus on DNA-based therapy, a novel therapeutic strategy for asthma. Immunostimulatory gene therapy using CpG oligodeoxynucleotides with central deoxycytidyl-deoxyguanosine (CpG) dinucleotide, in which the cytosine nucleobase is unmethylated, can stimulate the innate immunity and augment Th1 response. With DNA gene therapy, genes can be introduced to target Th1 cytokines or other mediators in the airway in order to counteract Th2 inflammation in asthma. Also, antisense oligonucleotides can target mRNA species of interest in asthma. Through these therapies, we can expect long-lasting effects, better control of symptoms, and reducing medication in the future.

Keywords: Asthma, Th1/Th2, cytokine, CpG, gene therapy, antisense oligonucleotides.

INTRODUCTION

Asthma is an increasing chronic disease worldwide. The "hygiene hypothesis" is thought to be a major reason for the dramatic increase in asthma prevalence in developed countries over the past 40 years [1, 2]. The developing immune system of humans has a type 2 T helper cell (Th2) bias in utero and in early life. Exposure to pathogens (during infections and daily life) provides Th1 stimuli, which results in a gradual decrease of the Th2 bias. However, the decreased frequency of Th1-like infections during childhood due to improvements in hygiene, increased vaccination, use of antibiotics, and smaller family sizes may result in a lack of Th1 stimulation, thereby skewing the immune response towards a Th2 response and increasing the likelihood of developing asthma [2].

Asthma is a heterogeneous disease characterized by reversible airflow obstruction, bronchial hyper-reactivity, and airway inflammation. The inflammatory and immune response associated with asthma includes prominent infiltration of the airway with eosinophils [3] and Th2 lymphocytes [4]. After IgE cross-linkage of the high-affinity IgE receptor (Fc ϵ RI), mast cells are activated and degranulated, which leads to the release of mediators and the activation of eosinophils [5]. Eosinophils release pro-inflammatory mediators such as preformed major basic protein, newly synthesized leukotrienes, platelet activating factor, tumor necrosis factor (TNF), interleukin (IL)-5, and other cytokines [3], leading to bronchoconstriction, mucus secretion, and denudation of the airway epithelium [6]. Recruitment of Th2 lymphocytes results in the late phase of the inflammatory response [7]. The ongoing inflammatory response may lead to airway remodeling characterized by bronchial wall thickening, smooth muscle hypertrophy, and thickening of the basement membrane [8], which results in a progressive decline in lung function [9].

Anti-inflammatory therapy can reduce airway inflammation and prevent later airway remodeling. The gold standard treatment and the most effective anti-inflammatory therapy for chronic asthma consists of inhaled glucocorticoids. Other medications such as methylxanthines, cromones, long-acting β_2 -agonists, and leukotriene modifiers in combination with

glucocorticoids can achieve a good control of asthma. Despite all these medications, some cases of severe asthma may not be well controlled. These medications generally do not alter the immune response to allergens in sensitized patients. New therapies such as anti-IgE monoclonal antibodies (Omalizumab) could reduce acute exacerbations in asthma patients receiving inhaled glucocorticoids [10]. Cytokine therapy such as anti-IL-4, anti-IL-5, or interferon (IFN)- γ therapy has shown promising effects in murine models of asthma but has failed in clinical trials due to low efficacy or to side effects [11-13]. This article will focus on a novel type of therapy for asthma, DNA-based therapy, which aims at enhancing the Th1 response, decreasing the Th2 response, or down-regulating certain RNA transcripts to modulate the immunopathogenesis of asthma. This paper will review DNA-based therapies using immunostimulatory oligonucleotides, DNA gene therapy, antisense oligonucleotides, and RNA interference and will discuss results from studies using animal models of asthma and from clinical trials. Overall, these studies show that DNA-based therapies for asthma may hold a promising future.

IMMUNOSTIMULATORY OLIGONUCLEOTIDES

Introduction

The first immunostimulatory therapy for nonbacterial diseases was reported by Dr. Coley in the 1890s. To treat cancer patients, he used live streptococci and later "Coley's toxin" (consisting of heat-killed Gram-positive and gram-negative bacteria), and achieved more than 40% sustained clinical remissions [14]. Almost 100 years later, Yamamoto *et al.* and Messina *et al.* found that bacterial DNA itself could enhance natural killer (NK) cell activity as well as B cell activation [15, 16]. Krieg *et al.* found that some sequence motifs containing a central deoxycytidyl-deoxyguanosine (CpG) dinucleotide in which the cytosine nucleobase is unmethylated, were responsible for the observed immunostimulatory effects on B cell activation [17]. Bacterial DNA has a higher frequency of CpG dinucleotide (1 in every 16 dinucleotides) than vertebrate DNA (1 in every 125 dinucleotides). Furthermore, 70% to 90% of the cytosine bases at position 5 in CpG dinucleotides are methylated in mammals, while less than 5% are methylated in bacterial DNA [18, 19]. Therefore, vertebrates have developed some immune defense against bacteria through the recognition of unmethylated CpG dinucleotides.

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Immunostimulatory Mechanisms of CpG Oligodeoxynucleotides

The recognition of unmethylated CpG dinucleotides within specific sequence contexts in bacterial DNA and synthetic oligodeoxynucleotides (ODNs) occurs through the Toll-like receptor 9 (TLR9) [20]. TLR9-mediated signaling occurs through the recruitment of an adaptor molecule, MyD88, followed by the engagement of IL-1 receptor-associated kinases (IRAKs) and the adaptor molecule TNF receptor-activated factor 6 (TRAF6) [21-24], and then by the downstream activation of mitogen-activated protein kinases (MAPK) and nuclear factor κ B (NF- κ B) [20, 25, 26]. CpG ODNs can activate several types of cells such as B cells, NK cells, and antigen-presenting cells (APCs), including monocytes, macrophages, and dendritic cells [17, 27, 28]. This results in the secretion of cytokines such as IFN- α , IFN- β , IL-12, IL-6, TNF- α , IFN- γ , and chemokines [14, 28-31], and the upregulation of costimulatory cell surface markers or the major histocompatibility complex (MHC) [32, 33]. Thus, CpG ODNs are extensively studied as an adjuvant of immunization and as a treatment for cancer or allergic diseases.

Types of CpG ODNs

Although CpG ODNs are recognized by pathogen-associated molecular patterns, the immunostimulatory effects are sequence-specific. Immune cells respond efficiently to 6-mer nucleotides with the general sequence: purine-purine-CG-pyrimidine-pyrimidine [17]. GACGTT activates mouse immune cells efficiently [17] while the optimal sequence for human immune cells is GTCGTT or TTCGTT [34]. Differences between species in response to various CpG ODNs appear to be caused by genetically determined TLR9 sequence-binding selectivity [35].

There are two types of CpG ODNs that activate human immune cells with different effect [36]. The "D" or "A" type contains phosphorothioate G-rich sequences at the end, a phosphodiester palindromic sequence with a CpG dinucleotide in the middle, and a poly G tail 4~6 bases in length at the 3' end [36]. The "D" or "A" type CpG ODNs preferentially stimulate NK cells to produce IFN- γ [37], stimulate plasmacytoid dendritic cells to produce IFN- α/β [38], and have strong *in vivo* antitumor activity in mouse models [38-40]. The other type is the "K" or "B" type, which has an entirely phosphorothioate backbone with multiple CpG dinucleotides [36]. This type of CpG ODNs can activate B cells and dendritic cells, and can increase IL-10,

IL-6, and IgM production [34, 41-43]. Most of the studies in asthma models have been carried out with the "K" type CpG ODNs.

Chemical Modifications of CpG ODNs

Several modifications within synthetic CpG ODNs can be performed to improve their immunostimulatory effect and their stability (Fig. 1). These include nucleobase (purines and pyrimidines) modifications, internucleotide linkage modifications, and sugar modifications.

Deletion of either C or G nucleotide in the CpG dinucleotide and replacement of cytosine by 5-methyl cytosine results in the loss of immunostimulatory effect, showing that sequence specificity is very important for immune recognition [17, 44]. Studies of the effects of each functional group of CpG ODNs using deletion or modification showed that 2-keto, 3-imino, and 4-amino groups of cytosine and the 1-imino, 2-amino, and 6-keto groups of guanine are important for the observed immunostimulatory activity [45]. The examples of modifications of the nucleobases in CpG ODNs which result in decrease or loss of immunostimulatory activity are shown in (Fig. 2) [45].

The natural phosphodiester backbone (R=O in Fig. 1) present in bacterial DNA is highly susceptible to nuclease degradation. The phosphorothioate-modified backbone (R=S in Fig. 1) provides resistance to cellular nuclease and better cellular uptake [36, 46] and provides enhanced immunostimulatory effects [17]. The immunostimulatory activity is abolished if the natural 3'-5'-internucleotide linkage is replaced by the unnatural 2'-5'-linkage (Fig. 3) [47]. Also it is reported that the synthetic stereo-enriched [Sp] and [Sp-Rp-Sp] chimeric and stereo-random phosphorothioate CpG ODNs evoked higher cell proliferation response than [Rp] phosphorothioate CpG ODNs (Fig. 3) [48]. Unlike the negatively charged phosphodiester and phosphorothioate ODNs, replacement of non-ionic methylphosphonate linkage (Fig. 3) within 3 nucleotides in the 5'-flanking sequence to the CpG motif decreases the cell proliferation and cytokine secretion in mouse spleen cell culture [49].

CpG ODNs with a 2'-O-methyl modification (Fig. 4) at CpG impedes immunostimulatory activity [50], suggesting that the receptors have a strict preference for deoxyribonucleosides in the CpG dinucleotide. Modifications with 2'-O-methyl or 3'-O-

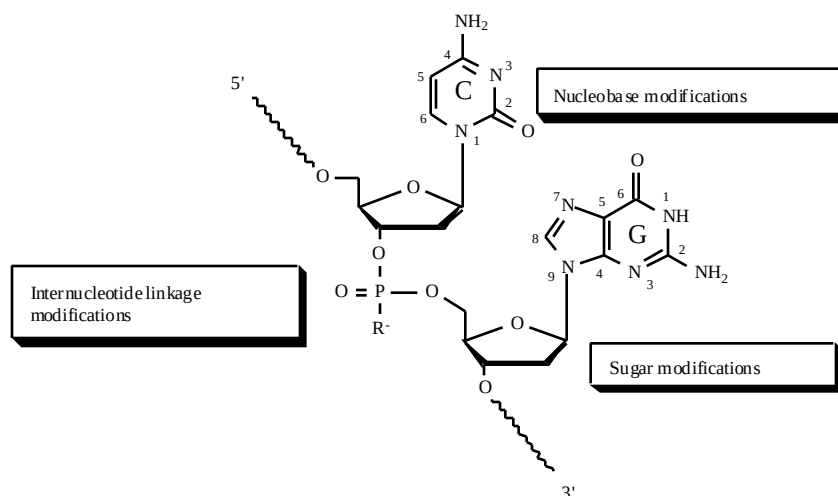


Fig. (1). Chemical structure of CpG dinucleotides and modification sites.

Natural CpG DNA in bacteria consists of phosphodiester backbone (R=O), while the synthetic phosphorothioate backbone (R=S) is more stable and resistant to nuclease. The three important sites for modification are shown.

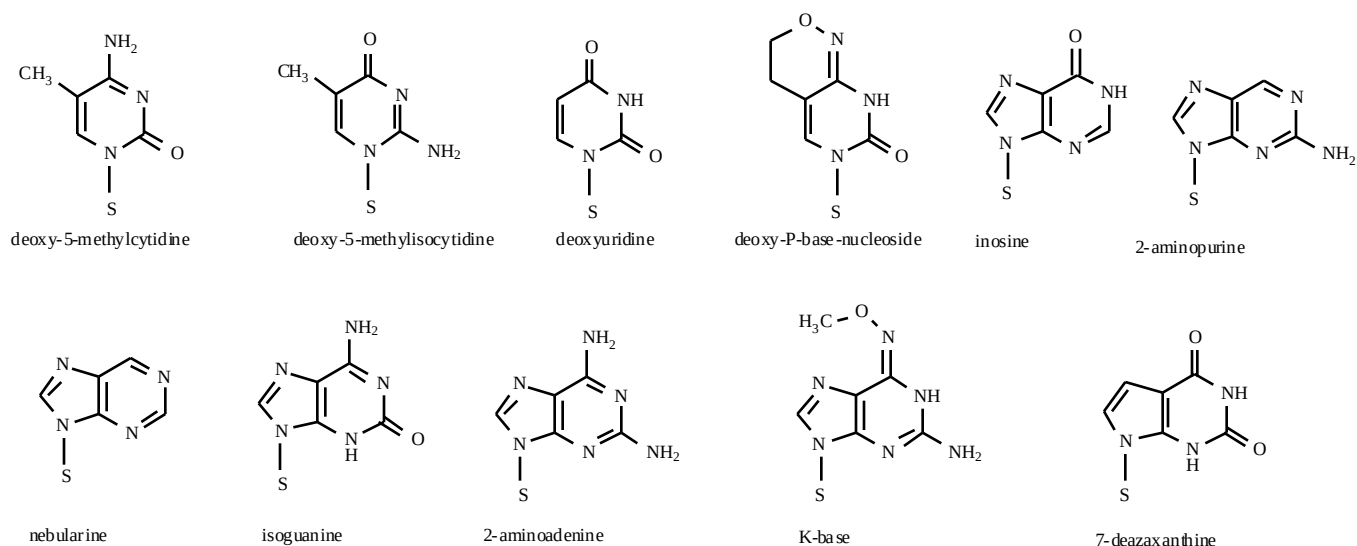


Fig. (2). Examples of nucleobase modifications in CpG ODNs.

These nucleobase modifications result in decrease or loss of immunostimulatory activity. S represents a 2'-deoxyribose.

methyl (Fig. 4) deoxynucleotides immediately 5'- or 3'- to the CpG motif significantly suppress immunostimulatory activity [51]. However, an increase in immunostimulatory activity is observed if the modifications are made in the flanking region at least 2 nucleotides away from the CpG motif, as well as bulkier 2'-O-methoxyethyl modification [51]. Abasic modifications (Fig. 4) in the 3'-flanking sequence to the CpG motif does not

change the immunostimulatory activity, while similar modifications in the 5'-flanking sequence increase the immunostimulatory activity [44].

Recent studies show that conjugation of CpG ODNs through their 5' ends (5'-5'-linked DNA) significantly reduces their immunostimulatory activity [52]. Thus, ligands such as

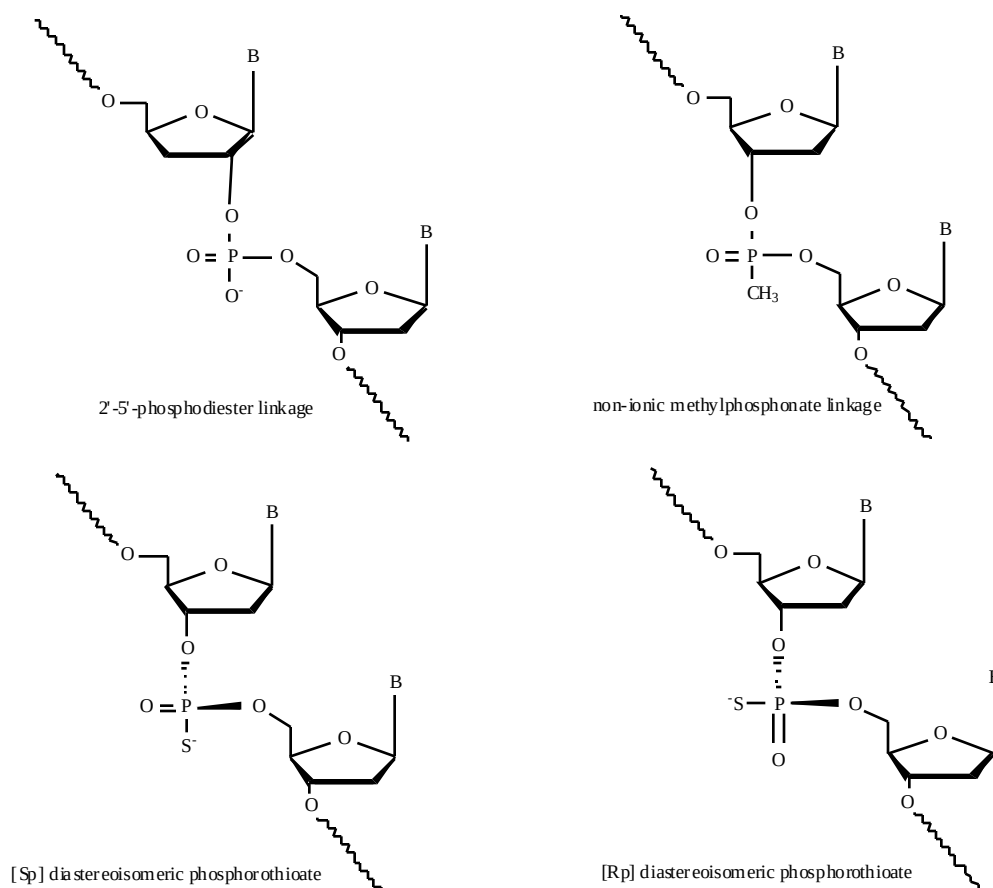


Fig. (3). Examples of internucleotide linkage modifications in CpG ODNs.

B represents a heterocyclic base.

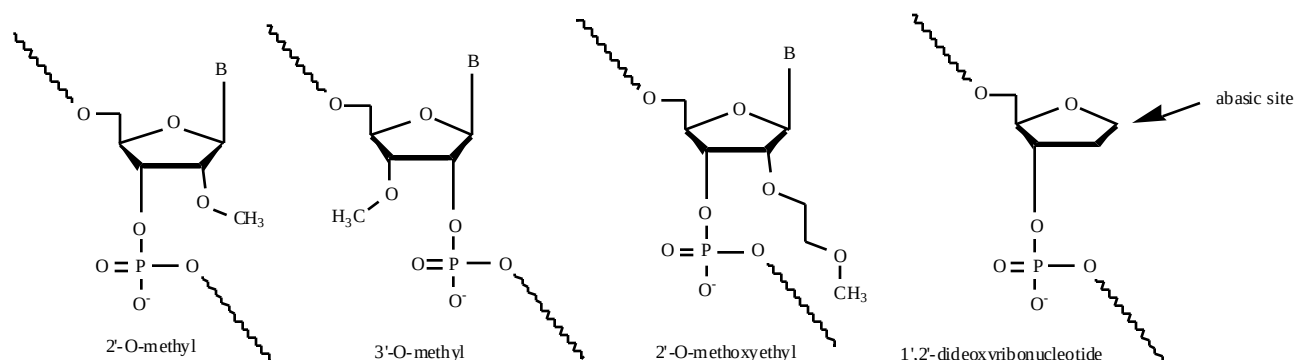


Fig. (4). Examples of sugar modifications in CpG ODNs.

B represents a heterocyclic base.

antigens, antibodies, or allergens should not be attached at the 5' end of CpG ODNs, because they could interfere with the recognition of CpG ODNs and therefore prevent immunostimulatory activation. The addition of poly-guanosine runs to the 3' end of CpG ODNs enhance the immunostimulatory activity *in vivo* and *in vitro* [53]. Taken together, these findings have provided important clues for the creation of the synthetic oligonucleotides with greater immunostimulatory effect.

Toxicity

Since CpG ODNs can activate B cells and APCs, they could potentially trigger autoimmune processes. Studies in animal models are inconclusive; some showing an aggravation of autoimmune disease [54] while some not [55, 56]. Further study is needed to elucidate this problem.

The Therapeutic Potential of CpG ODNs for Asthma

CpG ODNs trigger the production of IFN- γ , which inhibits the proliferation of Th2 cells and induces a Th1 environment. Thus, CpG ODNs are extremely effective at inducing a Th1-like immune response and preventing Th2 immune stimulatory effects which cause allergic asthma [57]. Kline *et al.* showed that systemic CpG ODNs could prevent the development of IgE production, airway eosinophilia, and airway hyper-reactivity (AHR) in mice sensitized to schistosome eggs [58]. IL-4 was decreased and IFN- γ and IL-12 were increased in bronchoalveolar lavage (BAL). Others confirmed that CpG ODN treatment can decrease airway eosinophilia, blood and bone marrow eosinophilia, AHR, IgE, and Th2 cytokine expression (IL-4, IL-5, and IL-13), and increase the expression of Th1 cytokines (IFN- γ and IL-12) and anti-inflammatory cytokines (transforming growth factor [TGF]- β 1) [58-62]. A p38 MAPK-dependent pathway initiated by CpG ODNs was shown to produce IL-12 which acts against allergic lung inflammation [63]. The cytokine production in splenocytes from antigen- and alum-immunized mice showed that not only IFN- γ but also IL-10 production correlated with the inhibition of IL-5 production [64]. Surprisingly, the decrease in AHR induced by CpG ODNs does not require the presence of IL-12 or IFN- γ , although their absence leads to an altered dose-response curve [65]. CpG ODNs decrease the expression of the costimulatory molecule B7.2 and slightly increase the expression of B7.1 [62], which have been shown in many models to preferentially influence Th1 and Th2 responses [66]. Furthermore, CpG ODN treatment of asthmatic mice leads to a decrease in the expression of transcription factor GATA-binding protein 3 (GATA-3), an important factor for Th2 development [67, 68]. CpG ODN treatment before allergen challenge can also prevent antigen presentation to Th2 cells but not to Th1 cells [69]. Taken together, these results show that

CpG ODNs can suppress Th2 cell development and Th2 cytokine production, increase Th1 and anti-inflammatory cytokine production, thus decreasing pulmonary inflammation and AHR in asthmatic animal models.

Considering the possible side effects associated with systemic administration, Broide *et al.* have shown that CpG ODNs administration via mucosal delivery (intranasal and intratracheal) is as effective as systemic delivery (intraperitoneal) at inhibiting airway eosinophilia and at switching the cytokine response from Th2 to Th1 in a mouse model of asthma [70]. Others also confirmed that CpG ODNs are effective when given through the transmucosal route [61, 70, 71]. Recently, Hessel *et al.* reported the effect of intranasal delivery of CpG ODNs before allergen challenge in Th2-primed mice [69]. They found that CpG ODNs could prevent the induction of Th2 cytokines and could inhibit the IgE-dependent release of Th2 cytokines.

In a murine model of chronic asthma induced by repeated allergen exposures, CpG ODNs could inhibit subepithelial collagen deposition, with protection lasting up to 6 months [72, 73]. CpG ODNs could inhibit goblet cell hyperplasia, peribronchial smooth muscle layer thickness, and peribronchial angiogenesis in a chronic asthma model [73-75]. Inhaled CpG ODNs could also reduce AHR and attenuate airway remodeling with thinner reticular basement membranes in rhesus monkeys repeatedly exposed to house dust mite (*Der farninae*) [76]. Thus, CpG ODNs may be a novel form of therapy that attenuates allergen-induced airway remodeling such as subepithelial collagen deposition, goblet cell metaplasia, airway smooth muscle hyperplasia, and peribronchial angiogenesis.

Comparing CpG ODNs treatment to standard daily asthma treatment, Broide *et al.* found that a single dose of CpG ODNs was at least as effective as seven daily injections of dexamethasone at inhibiting airway eosinophilia and protecting sensitized mice from inflammation with nebulized allergen challenge [60]. Combination of CpG ODNs and corticosteroid was not superior to either agent alone [59]. Moreover, the effects of CpG ODNs therapy appear to be transient, suggesting that optimal CpG ODNs therapy may require repeated administrations [77].

Clinical Trials Using CpG ODNs for Asthma Treatment

Several clinical trials have been conducted using CpG ODNs for asthma and allergy, as well as cancer and infections. Interim results from phase I/II clinical studies involving ~300 individuals suggest that CpG ODNs are well tolerated and activate the immune system, which seems promising for the treatment of allergic airway inflammation [78, 79]. Gauvreau *et al.* evaluated the therapeutic effect of 1018 ISS (a "K" type CpG

ODNs) in mild persistent asthma patients. They found that weekly inhalation of 1018 ISS for 1 month using a nebulizer significantly increased the expression of IFN- γ and IFN-inducible genes, and that 1018 ISS was safe and pharmacologically active in asthma patients. However, 1018 ISS did not inhibit the fall in FEV1 or other characteristic responses to inhaled allergen challenge [80]. Thus, further experimentation and refinement for future clinical trials in asthma are required.

DNA GENE THERAPY

Introduction

DNA gene therapy was first used for immunization against a specific allergen [81], and many studies have focused on allergens such as pollen or mite in different sensitization models [82, 83]. We will first introduce the different DNA delivery systems to the lung and then focus on genes encoding cytokines, signaling pathway molecules, and other mediators which have immunomodulatory effects on asthma pathogenesis.

Pulmonary Gene Delivery

The delivery of exogenous genes to the lung can be divided into viral and non-viral gene delivery systems. Viral gene transfer vectors such as adenovirus (Ad), adeno-associated virus (AAV), and lentivirus vectors are considered the most efficient gene delivery vehicles. Recombinant Ad (rAd) vectors with deleted E1 and E4 genes (to avoid vector replication) have a moderate transfection efficiency [84]. The viral genes may provoke an immediate inflammatory response and a cytotoxic T lymphocyte (CTL) response that limits the persistence of expression [85]. Therefore, gene expression is transient and repeated administration is necessary [86]. One of the most

serious limitation for the use of rAd as a gene therapy vector is that 55% of adult humans have pre-existing Ad-specific antibodies [87], and that the inflammatory response is higher than with other types of vectors.

AAV is a small single-stranded DNA parvovirus that requires co-infection with an adenovirus or a herpesvirus for propagation [84]. AAV vectors express the gene of interest in a stable manner. AAV vectors lack viral coding sequences and do not activate CTLs [88]. But pre-existing antibodies to AAV limit the usefulness of this vector [89]. Lentiviruses are positive-strand RNA viruses capable of infecting nondividing cells [90]. Gene expression from lentiviral vectors is also stable, but the safety issues associated with the integration of lentiviral vectors into the genomic DNA need to be carefully addressed [91].

Nonviral gene delivery systems are non-infectious, less immunogenic, and have low toxicity [92], but one of their major drawbacks is the low transfection efficiency. Liposomes, cationic polymers, receptor-mediated gene delivery, endosome escape systems, nuclear targeting systems, and episomal vectors are all nonviral gene delivery systems [92]. Cationic liposomes bind to DNA via electrostatic interaction and spontaneously form into discrete lipid/DNA particles. These particles are resistant to nuclease degradation and result in efficient transfection both *in vitro* and *in vivo* [92]. The transfection efficiency is dependent on the lipid-DNA particle size and the lipid:DNA ratio. Gene expression from liposomes is transient [92].

Cationic polymers such as poly-L-lysine, polyethyleneimine (PEI), and polyamidoamine dendrimers have also been used in gene transfer studies [92]. The transfection efficiency of cationic polymers is related to particle size and surface charge (zeta potential) [92]. Receptor-binding ligands linked to short DNA-binding peptides such as oligolysine can target DNA

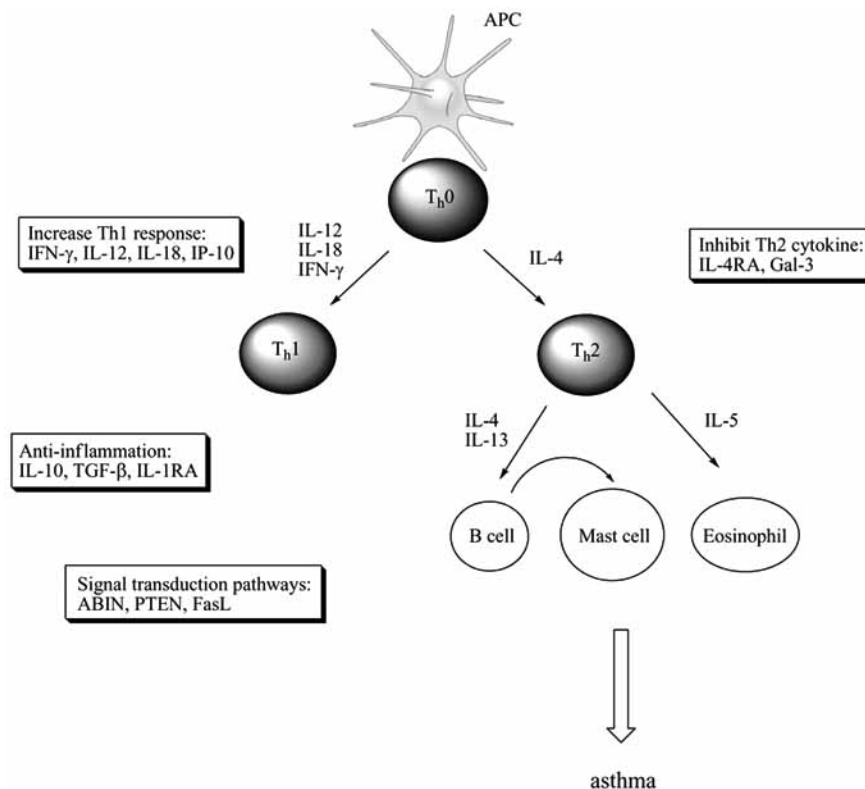


Fig. (5). Targets for DNA gene therapy.

Asthma is a chronic airway inflammation with a dominant Th2 response. Strategies for DNA gene therapy include increasing the Th1 response and anti-inflammatory cytokines, inhibiting Th2 cytokines, and targeting signal transduction pathways such as NF- κ B, PTEN, and FasL.

complexes to specific cell surface receptors [93]. In systems with endosome escape and nuclear targeting for example, synthetic peptides that mimic the membrane-fusing domain of influenza virus haemagglutinin are used to mediate endosomal escape of peptide-DNA complexes [94]. Episomal vectors have the capacity to replicate and to be maintained extrachromosomally in human cells [92]. They contain a viral origin of replication, a bacterial plasmid origin of replication, and a viral early gene product that binds to the viral origin and promotes extrachromosomal replication and episomal maintenance [95]. One of the main advantages of episomal

vectors is that they have the capacity to carry large human genomic inserts (100-300 kb) and that gene expression is relatively stable [92].

Therapeutic Potential of DNA Gene Therapy for Asthma

DNA gene therapy for asthma includes strategies aiming at increasing the Th1 response and anti-inflammatory cytokines, inhibiting Th2 cytokines, and targeting signal transduction molecules or bronchodilator mediators (Fig. 5). The results from

Table 1. Summary of Effects of Airway Administered DNA Gene Therapy in Animal Models of Asthma

Gene	Function	Animal model	Route of administration / vector	Effects	Reference
IFN- γ	Th1 cytokine	OVA-sensitized mice	Intratracheal / liposome	$\downarrow$ AHR $\downarrow$ eosinophil, IL-5 in BAL	[96]
IFN- γ	Th1 cytokine	Conalbumin-sensitized mice	Intratracheal / liposome	$\downarrow$ eosinophil, IL-4, IL-5 in BAL	[97]
IFN- γ	Th1 cytokine	OVA-sensitized mice	Intranasal / adenovirus	$\downarrow$ AHR $\downarrow$ airway eosinophilia $\downarrow$ IL-4, IL-5 in bronchial LN $\uparrow$ IFN- γ , IL-12 in bronchial LN	[98]
IL-12	Th1 cytokine	Dermatophagoides pteronyssinus (Der p) 1-sensitized mice	Intratracheal / liposome	$\downarrow$ AHR $\downarrow$ airway eosinophilia $\uparrow$ IFN- γ , IL-12 in BAL $\downarrow$ IL-5, eosinophil in BAL	[100]
IL-12	Th1 cytokine	OVA-sensitized mice	Intranasal / vaccinia virus	$\downarrow$ AHR $\downarrow$ eosinophil in BAL $\downarrow$ IL-4, IL-5 in culture lung cells	[101]
IL-18	Th1 cytokine	OVA-sensitized mice	Intranasal / adenovirus	$\downarrow$ AHR $\downarrow$ mucus production $\downarrow$ airway eosinophilia $\downarrow$ IL-4 in bronchial LN $\uparrow$ IFN- γ in bronchial LN	[103]
IP-10	Th1 chemokine	OVA-sensitized mice with airway GM-CSF expression	Intranasal / adenovirus	$\downarrow$ eosinophil in BAL $\downarrow$ IL-4, IL-13 in BAL $\uparrow$ IFN- γ , MCP-1, RANTES in BAL	[106]
IL-10	Anti-inflammatory cytokine	OVA-sensitized mice	Intratracheal / adenovirus	$\downarrow$ AHR $\downarrow$ eosinophil and neutrophil in BAL $\downarrow$ airway eosinophilia	[108]
IL-10	Anti-inflammatory cytokine	OVA-sensitized mice with airway GM-CSF expression	Intranasal / adenovirus	$\downarrow$ AHR $\downarrow$ eosinophil and neutrophil in BAL $\downarrow$ airway eosinophilia	[109]
TGF- β	Anti-inflammatory cytokine	OVA-sensitized mice	Intratracheal / liposome	$\downarrow$ eosinophil and neutrophil in BAL $\downarrow$ eotaxin in BAL $\downarrow$ airway eosinophilia	[110]
IL-1RA	IL-1 antagonism	OVA-sensitized mice	Intranasal / adenovirus	$\downarrow$ AHR $\downarrow$ eosinophil and neutrophil in BAL $\downarrow$ airway eosinophilia $\downarrow$ IL-5, eotaxin in BAL $\uparrow$ IFN- γ in BAL	[112]
IL-4RA	IL-4 antagonism	OVA-sensitized mice	Intratracheal / AAV	$\downarrow$ AHR $\downarrow$ IL-5, IL-13 in BAL $\downarrow$ airway eosinophilia	[113]
Galectin-3	IgE binding protein	OVA-sensitized mice	Intratracheal / plasmid	$\uparrow$ F75, FVC $\downarrow$ pulmonary pressure $\downarrow$ eosinophil and T cell in BAL	[116]
Galectin-3	IgE binding protein	OVA-sensitized mice with RAE	Intranasal / plasmid	$\downarrow$ AHR and airway eosinophilia $\downarrow$ IL-5, IL-10 mRNA in lung tissue $\downarrow$ mucus secretion, subepithelial fibrosis	[117]
ABIN-1	NF- κ B inhibitory protein	OVA-sensitized mice	Intratracheal / adenovirus	$\downarrow$ airway eosinophilia $\downarrow$ serum IgE $\downarrow$ eotaxin, IL-4, IL-1 β in BAL	[119]
PTEN	Phosphatase to block PI3K/Akt pathway	OVA-sensitized mice	Intratracheal / adenovirus	$\downarrow$ AHR $\downarrow$ IL-4, IL-5, ECP in BAL $\downarrow$ airway eosinophilia	[121]
FasL	Ligand in apoptotic pathway	OVA-sensitized mice	Intratracheal / adenovirus	$\downarrow$ AHR $\downarrow$ IL-5, IL-13, eotaxin, TNF- α in BAL $\uparrow$ eosinophil apoptosis	[122]
ANP	Bronchodilator peptide	OVA-sensitized mice	Intranasal / lipofectamine	$\downarrow$ AHR	[129]

studies using local DNA gene delivery in animal models of asthma are summarized in (Table 1).

Th1 cytokine genes such as IFN- γ , IL-12, and IL-18 have been used in murine models of asthma to downregulate Th2 cytokines and attenuate AHR. Systemic or intratracheal delivery of the IFN- γ gene significantly inhibits AHR and airway eosinophilia [96, 97]. The effects of IFN- γ gene delivery are dependent on IL-12 and on signal transducers and activators of transcription 4 (STAT-4) via intranasal administration [98]. Systemic IL-12 gene delivery inhibits AHR, eotaxin in BAL, and airway eosinophilia [99]. A single-chain IL-12 DNA plasmid delivered intratracheally was capable of inhibiting AHR, airway eosinophilia, and Th2 cytokine production [100]. A similar effect was shown using intranasal transfer of a vaccinia virus vector carrying the IL-12 gene; in this case, AHR attenuation was sustained for up to 4 weeks [101]. Ad-mediated IL-12 gene transfer can counteract the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced airway inflammation [102]. Intranasal IL-18 gene delivery using a rAd vector attenuates AHR, airway eosinophilia, mucus production, and IL-4 production, and increases IFN- γ production [103]. These effects are dependent on IL-12/IFN- γ . IFN- γ inducible protein 10 (IP-10) is a chemokine that attracts T cells, monocytes [104], and NK cells and facilitates the selective recruitment of Th1 cells that preferentially express the receptor CXCR3 [105]. Intranasal administration of a rAd harboring the IP-10 gene results in the inhibition of eosinophilia and IL-4 in BAL, while IL-5 and eotaxin are unchanged. The effect is dependent on IFN- γ [106].

Anti-inflammation is another approach. IL-10 and TGF- β , which are pleiotropic cytokines with significant anti-inflammatory and immunosuppressive properties, can downregulate Th2 responses [107]. Intratracheal delivery of an Ad expressing the IL-10 gene, an anti-inflammatory cytokine, could inhibit AHR, decrease eosinophils and neutrophils in BAL, and decrease pulmonary inflammation [108]. Similar results were noted after intranasal transfer of the IL-10 gene in ovalbumin (OVA)-sensitized mice with GM-CSF expression in the airway [109]. Intratracheal administration of the TGF- β gene decreases eosinophils and neutrophils in BAL as well as eotaxin, and improves airway eosinophilia [110]. IL-1 receptor antagonist (IL-1RA), an endogenous receptor antagonist, inhibits the proinflammatory functions of IL-1 by occupying the same receptor without inducing signal transduction [111]. Intranasal delivery of an Ad carrying the human IL-1RA in OVA-sensitized mice significantly ameliorates AHR, reduces airway eosinophilia and neutrophilia, decreases IL-5 and eotaxin in BAL, and increases IFN- γ increased in BAL [112].

Other studies have focused on Th2 cytokine antagonists. Zavorotinskaya *et al.* reported that intratracheal delivery of AAV carrying the IL-4 receptor antagonist (IL-4RA) gene reduced AHR and airway eosinophilia as well as IL-5 and IL-13 production [113]. Nishikubo *et al.* developed a double mutant IL-4 gene (murine IL-4 mutant Q116D/Y119D), which prevented serum IgE production and eosinophil infiltration in BAL after intraperitoneal injection [114]. Galectin-3 (Gal-3) is an IgE-binding protein that down-regulates IL-5 gene expression [115]. Intratracheal delivery of the Gal-3 gene inhibits eosinophils and T cells in BAL and IL-5 mRNA in lung tissue [116], which improves pulmonary function parameters such as pulmonary pressure and expiratory flow. The same group used intranasal delivery of the Gal-3 gene in a chronic asthma model with repeated allergen exposure, and showed an attenuation of AHR as well as improvements in airway eosinophilia, subepithelial fibrosis, and mucus secretion [117].

Another gene therapy strategy consists of targeting signaling pathways. NF- κ B is a well-known inflammatory

signaling pathway mediator and a pleiotropic transcription factor important in Th2 differentiation [118]. Intratracheal delivery of the ABIN-1 gene, an inhibitor of NF- κ B, using an Ad vector reduces airway eosinophilia, serum IgE production, and decreases the expression of eotaxin, IL-4, and IL-1 β in BAL [119]. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) blocks the action of the phosphoinositide 3-kinase (PI3K)/Akt pathway which is crucial for the regulation of cell survival [120]. Kwak *et al.* showed that intratracheal administration of an Ad carrying the PTEN gene reduced AHR and airway inflammation [121]. Fas ligand (FasL) is another target that mediates the apoptotic pathway in lymphocytes. Chuang *et al.* delivered FasL intratracheally using an adenoviral vector and showed an attenuation of AHR and airway eosinophilia (due to an increase in eosinophil apoptosis), as well as a decrease in IL-5, IL-13, and eotaxin in BAL [122].

Other mediators may be targeted for asthma treatment. The novel human hematopoietic growth factor fms-like tyrosine kinase 3 ligand (Flt3-L) has been reported to prevent the development of and reverse asthma-like conditions in murine models of asthma [123, 124]. Intramuscular delivery of a Flt3-L plasmid attenuates AHR for at least 24 days and increases IFN- γ in BAL, as well as IL-10 and anti-OVA IgG_{2a} in serum [125]. In contrast, IL-4, serum IgE, IL-5, and eosinophilia in BAL are significantly decreased. Atrial natriuretic peptide (ANP), which has vasodilator, natriuretic, and diuretic properties [126], reduces airway resistance in healthy human subjects and asthma patients [127, 128]. Kumar *et al.* delivered a plasmid encoding ANP intranasally in a murine model of asthma and showed an attenuation of AHR [129].

ANTISENSE OLIGONUCLEOTIDES AND RNA INTERFERENCE

Introduction

Several strategies for sequence-specific post-transcriptional inhibition of gene expression have been growing, such as antisense oligonucleotides (ASOs), ribozymes, DNA enzymes, and RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) [130]. Because multiple copies of a protein (approximately 5000 copies) are produced by each mRNA molecule, targeting mRNA rather than the protein itself is potentially more efficient. There are many possible targets for the treatment of asthma, including cell surface receptors, adhesion molecules and ligands, ion channels, cytokine and related factors, intracellular signal transduction molecules, and transcription factors [130]. The new generation of respirable ASOs (RASOs), external guide sequence (EGS) ribozymes, and RNAi-based therapies are promising drugs for allergy [130].

ASOs

ASOs are single-stranded synthetic nucleic acid polymers (usually 18~25 nucleotides), which hybridize with the target mRNA in a sequence-dependent complementary manner via hydrogen bonds [130]. ASO mechanism of action involves forming a duplex with the mRNA, inhibiting splicing, inhibiting translation, and disrupting ribosome assembly. ASO-mRNA duplex formation activates RNase H, which degrades mRNA and blocks protein translation [131].

Chemistry Backbone of ASOs

First generation ASOs are phosphorothioate oligodeoxynucleotides (PS-ONs) (where one of the

noncovalently bonded oxygen atoms is replaced by a sulfur atom), which are more resistant to nuclease than standard phosphodiester oligonucleotides [130]. Second generation ASONs contain nucleotides with alkyl modifications at the 2' position of the ribose. This includes the 2'-O-methoxyethyl oligonucleotides (2'MOE-ONs) which have an increased target binding affinity and an increased resistance to degradation [130]. Third generation ASONs include peptide nucleic acids, N3'-P5' phosphoroamidates, 2'-deoxy-2'-fluoro- β -D-arabino nucleic acids, locked nucleic acids, morpholino oligonucleotides, cyclohexene nucleic acids, and tricyclo-DNA oligonucleotides [132].

Toxicity

PS-ONs are metabolized through nucleases and not through cytochrome p450, and therefore do not have drug interaction toxicity [133]. However, PS-ONs may have some unexpected effects, such as the inhibition of nonintended targets containing homologous sequences, or CpG motif stimulation of B cells. These side effects could be minimized by proper sequence design and screening [133]. The non-sequence related side effects, such as complement activation, hypotension, and prolongation of clotting time, which results from nonspecific interactions of the negatively charged backbone, could be minimized by selecting appropriate backbone chemistries and by using local routes of delivery [133].

Therapeutic Potential of ASONs for Asthma

The targets for ASONs include receptors (adenosine A1 receptor, the α chain of IL-5 receptor, and the common β chain of IL-3/IL-5/GM-CSF receptor), cytokines (IL-4, IL-5, and stem cell factor [SCF]), signal transduction molecules (Syk, Lyn, and NF- κ B), transcription factors (STAT-6 and GATA-3), etc. Results from studies using transmucosal delivery of ASONs are summarized in (Table 2). Results from studies using RASONS delivered by aerosol will be discussed later.

IL-3, IL-5, and GM-CSF receptors share a common β chain, and are all involved in eosinophil, mast cell, and macrophage

activation and survival [134]. Intratracheal delivery of ASONs for the common β chain of the IL-3/IL-5/GM-CSF receptor attenuates AHR and BAL eosinophilia in a dose-dependent manner [135].

IL-4 and IL-5 are important Th2 cytokines that aggravate airway inflammation. Intranasal IL-4 ASONs complexed with PEI suppress IL-4 expression in BAL, and decrease serum IgE level and airway inflammation [136]. Systemic IL-5 ASONs (2'MOE-ONs) reduce eosinophils in BAL and AHR [137]. SCF (also c-kit ligand) is an important activating and chemotactic factor for mast cells and eosinophils [138]. Intranasal SCF ASONs reduce intracellular SCF expression, IL-4 production, and eosinophilic infiltration in BAL [139].

NF- κ B is an important inflammatory signal transduction molecule. Intravenous delivery of ASONs to the p65 subunit of NF- κ B (PS-ONs) inhibits airway inflammation, AHR, as well as IL-4, IL-5, and TNF- α production in BAL [140]. GATA-3 is a transcription factor important in Th2 cell development [67]. Intranasal delivery of GATA-3 ASONs (PS-ONs lacking CpG dinucleotides) significantly inhibits Th2 cytokine production, airway eosinophilia, and AHR [141].

Nakanishi *et al.* identified a gene called *gob-5* which is highly expressed in hyperreactive airways [142]. Intratracheal administration of adenovirus expressing an antisense to *gob-5* reduced mucous overproduction and responsiveness to allergens [142].

RASONS

RASONS are effective at doses smaller than other routes of administration. Since they are delivered as aerosols directly to the lungs, the potential for non-antisense systemic side effects and toxicity can be minimized [143]. The lung is uniquely lined with surfactant, which is primarily composed of dipalmitoylphosphatidylcholine, a zwitterionic lipid with cationic properties in the lung, therefore enhancing the uptake of oligonucleotides into cells [144].

The first developed and extensively studied RASON is EPI-2010. It is a 21-mer RASON (PS-ONs, 5'-GATGGAGGGCGGC

Table 2. Summary of Effects of Airway Administered ASONs in Animal Models of Asthma

Target	Target function	Animal model	Route of administration	Size/type	Effects	reference
Adenosine A1 receptor	GPCR for smooth muscle contraction	House dust mite - sensitized rabbit Ascaris-sensitized primate Human with asthma	aerosol	21-mer / PS-ON	$\downarrow$ AHR only modest effect in human with mild asthma	[133, 145, 147-149]
Common β chain of IL-3/IL-5/GM-CSF receptor	Receptors for cytokine/chemokine	OVA-sensitized BN rat	Intratracheal	18-mer / PS-ON	$\downarrow$ AHR $\downarrow$ BAL eosinophilia	[135]
IL-4	Th2 cytokine	OVA-sensitized mice	Intranasal	22-mer / PS-ON	$\downarrow$ airway inflammation $\downarrow$ serum IgE $\downarrow$ IL-4 in BAL	[136]
c-kit (SCF)	Growth factor	OVA-sensitized mice	Intranasal	18-mer / PS-ON	$\downarrow$ Airway eosinophilia $\downarrow$ IL-4 in BAL	[139]
GATA-3	Transcription factor	OVA-sensitized mice	Intranasal	18-mer / PS-ON	$\downarrow$ AHR $\downarrow$ airway eosinophilia $\downarrow$ IL-4 in BAL	[141]
<i>gob-5</i>	Ca ²⁺ -dependent chloride channel	Mouse	Intratracheal	Adenoviral vector	$\downarrow$ AHR $\downarrow$ Mucus overproduction	[142]
P38 α MAPK	Kinase	OVA-sensitized mice	Aerosol	20-mer / 2'MOE-ON	$\downarrow$ AHR $\downarrow$ airway eosinophilia $\downarrow$ IL-4, IL-5, IL-13 in BAL	[152]
Syk tyrosine kinase	Kinase	Nippostrongylus larvae / OVA-sensitized BN rats	Aerosol	60-mer / PS-ON	$\downarrow$ airway eosinophilia and neutrophilia $\downarrow$ ICAM-1, β 2 and α 4 integrins expression in BAL leukocyte	[153]

ATGGCGGG-3') targeting the initiation codon of the adenosine A1 receptor mRNA (-9~+11). EPI-2010 sequence is conserved in rabbit, monkey, and man [145]. The absorption, distribution, metabolism, excretion, and safety properties of EPI-2010 were evaluated by Ali *et al.* [146]. Using a nebulizer, they delivered aerosolized [³⁵S]-labeled EPI-2010 to normal rabbits. They found that, in contrast to studies involving parenteral delivery of ASON where the highest concentrations of drug are found in the liver and kidney, the majority of EPI-2010-derived radioactivity was found in the target lung tissue. The combined EPI-2010-derived [³⁵S] radioactivity found in the heart, liver, and kidney was only 6.9% of the administered dose. The half-life of EPI-2010 in the lung was approximately 30 hours. EPI-2010 and its metabolites were mainly excreted through the urine with some minor fecal excretion. There were no significant cardiovascular side effects noted. Autoradiograms of RASONS indicated that penetration occurred even to the deeper tissues of the lung in RASONS-treated animals. Additionally, post-labeling studies indicated that intact EPI-2010 could be detected only in the lung.

The adenosine A1 receptor (a G-protein-coupled-receptor) is upregulated both in animal models of asthma and in asthmatic patients [147]. EPI-2010 reduces the number of adenosine A1 receptors in bronchial smooth muscles and attenuates AHR in rabbits and primates [147-149]. In phase I clinical trials conducted in the UK, EPI-2010 was effective at single inhaled doses, thus reducing the need for a bronchodilator to control asthma symptoms. Furthermore, the duration of effect was 6.8 days (range 4~11 days) with a once-per-week treatment. EPI-2010 was safe and well tolerated, without any coagulation or cardiovascular side effects [145]. However, the phase II clinical trials performed in patients with moderate persistent asthma who were receiving inhaled corticosteroids showed insufficient

effects [133]. Therefore, the development of EPI-2010 was discontinued.

The p38 MAPK pathway is important for the activation of IL-5 and eotaxin receptors in eosinophils, which result in the survival, activation, degranulation, and chemotaxis of eosinophils [150, 151]. RASONS targeting p38 α MAPK reduce IL-4, IL-5, and IL-13 expression, eosinophils in BAL, and decrease AHR [152]. Syk protein kinase mediates the inflammatory response after cross-linking of the IgE/IgG receptor (Fc ϵ R/Fc γ R) in many cell types, and Syk RASONS reduce antigen-induced pulmonary inflammation and NO and TNF- α expression in alveolar macrophages [153, 154]. Furthermore, Syk RASONS inhibit airway eosinophilia and neutrophilia, intercellular adhesion molecule-1 (ICAM-1), and β 2 and α 4 integrin expression in BAL leukocytes [153].

Other RASONS targeting GATA-3, the IL-4 receptor α chain, the very late activation antigen-4 (VLA-4) α subunit, and TNF- α are under study [130].

RNA Interference (RNAi) Using Small Interfering RNAs (siRNAs)

Long double-stranded RNAs (dsRNAs) are processed into 21~23 nucleotide siRNAs by the dicer enzyme (an RNase III-like dsRNA-specific endonuclease). Then, siRNA molecules are incorporated into a nuclease complex, the RNA-induced silencing complex (RISC), which includes helicase, RecA, and exo- and endonucleases, as well as other proteins. RISC guides the RNA degradation machinery to the target mRNA complementary to the siRNA [130]. siRNAs are more potent than ASONs: siRNAs more effectively knock down their targets at lower concentrations than ASONs [130]. However, siRNAs may

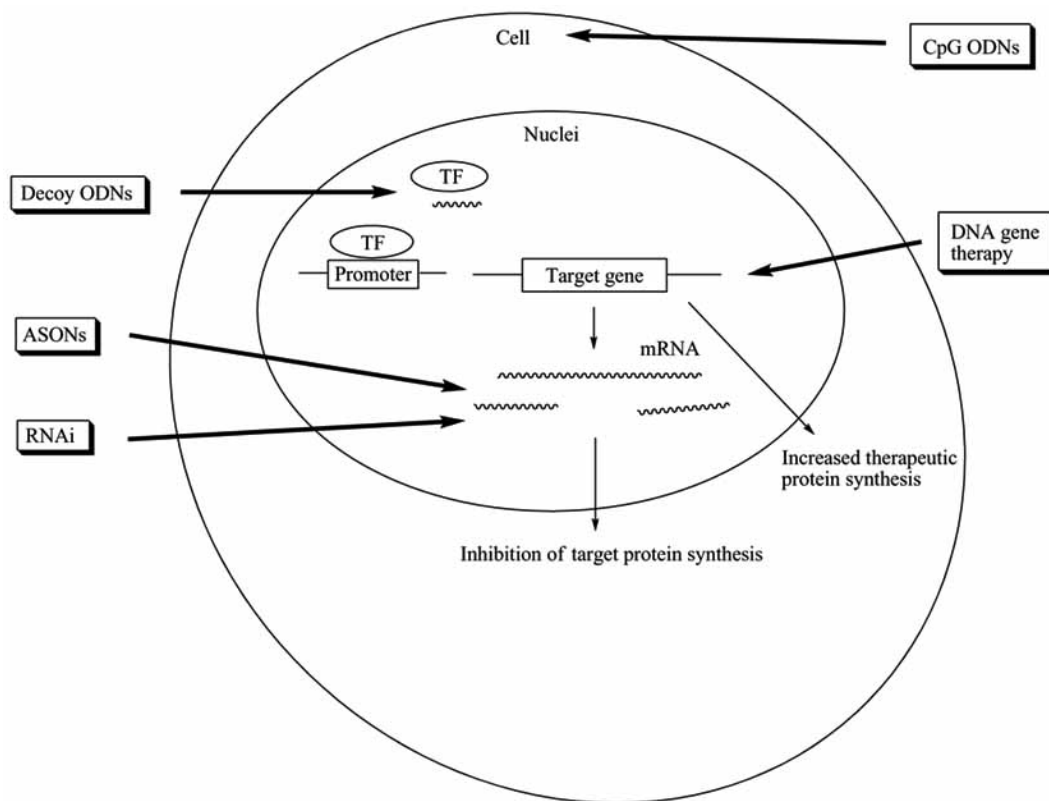


Fig. (6). Summary of DNA-based therapy.

While CpG ODNs act at the cell level to enhance Th1 response, DNA gene therapy acts at the gene level, decoy ODNs act at the transcription level, and ASONs and RNAi act at the mRNA level.

have the potential to elicit immune responses via interactions with TLR3, and trigger interferon responses such as long dsRNA and its analogs [155]. Thus, one should be cautious of the potential immune response to siRNAs to minimize unwanted non-specific effects.

Our studies on GATA-3 RNAi showed that intratracheal administration of a lentivirus carrying GATA-3 siRNA attenuates AHR, airway eosinophilia, as well as IL-5, IL-13, and eotaxin expression in BAL (unpublished data).

Transcription Factor Decoy Oligonucleotides

Double-stranded DNA with high affinity for a target transcription factor can be introduced into target cells as a "decoy" cis element [156]. The decoy ODNs attenuate the authentic cis-trans interaction, leading to the removal of trans-factors from the endogenous cis-element with subsequent modulation of gene expression [157]. However, the majority of decoy ODNs is sequestered and degraded in lysosomes and never reaches the nucleus. Thus, ribbon ODNs (which consist of two identical molecules enzymatically ligated) have been developed to increase the nuclease resistance of ODNs [158].

STAT-1 is constitutively expressed in epithelial cells of asthmatic patients [159]. Quarcoo *et al.* showed that intranasal decoy ODNs specific for STAT-1 (PS-ONs) inhibit AHR, eosinophils, IL-5 levels in BAL, and vascular cell adhesion molecule 1 (VCAM-1) expression in vascular endothelial cells [160]. NF- κ B is a pleiotropic transcription factor important in Th2 differentiation [118]. Intratracheal administration of NF- κ B decoy ODNs inhibits AHR, airway inflammation, local production of mucus, as well as IL-5, IL-13, and eotaxin production [161].

CONCLUSION

Because the current anti-inflammation drugs for asthma treatment are not satisfactory, the development of novel immunomodulatory drugs is warranted. DNA-based therapy (Fig. 6) can modulate the immune system at the genetic level (DNA gene therapy), the epigenetic level (ASONS, RNAi, or decoy ODNs), or augment the Th1 response (CpG ODNs). Systemic delivery of DNA or oligonucleotides may result in severe side effects. Thus, local delivery of DNA or oligonucleotides intranasally or via a nebulizing system may minimize unwanted systemic side effects and provide a therapeutic effect. Although clinical trials failed to show a significant effect of EPI-2010 on asthmatic patients, there are still many ongoing clinical trials. Advances in this field are promising and may soon lead to the development of new treatments for asthma.

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