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Review Article

Immunomodulatory and Therapeutic Potential of CPG and Probiotic DNA – A Review

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ABSTRACT

The in depth studies on the immune response shows its application in diseases other than infections. There are hardly any chemical based drugs which are given as immunomodulators. Most of the disease treatment drugs available in the market may have side effects and are costly. Hence, alternatively the immunotherapy can be employed to manage immune related diseases by the use of natural agents like probiotics. Moreover, the literature shows that prokaryotic DNA can activate immune response and has potential therapeutic applications. This immune efficacy of DNA is due to the presence of unmethylated DNA i.e. CpG motifs (regions of DNA where Cytosine and Guanine are separated by only one phosphate) in prokaryotes. Hence, the various applications of prokaryotic as well as probiotic DNA are being explored in the treatment of immune related disorders. The status of Probiotic DNA and synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) from various microorganisms are reviewed here, with reference to some key examples where these have succeeded in prevention and treatment of diseases.

Keywords: Probiotics, DNA, CpG-ODN, Immunomodulatory Therapy, Cytokines

1. INTRODUCTION

The immune system is a versatile defence system and has evolved to protect animals, humans and other living beings from invading pathogens and infectious diseases. The increase in knowledge about the immune response mechanisms unveils the relationship of immune response and related disorders. This made clear that besides immunological disorders, many other diseases can be modified by modulating Immune response. The rise in infectious diseases as well as non infectious immune related disorders demand the need for the development of efficient immunomodulators. Various medications including chemical drugs as well as natural therapies have been tested for curing such diseases. The chemical based drugs employed to cure these diseases may have the side effects and are costly. Moreover, consumer awareness about the harmful effects of chemical drugs raised a need to search for natural/alternative therapies for the treatment of diseases. Immunotherapy is one of the alternative ways of modification of diseases. Probiotics, the beneficial bacteria have been proved to have immunostimulating efficacy. These bacteria have GRAS (Generally Regarded As Safe) status, which made them most consumer acceptable alternative therapeutic agent. The advances in techniques in molecular biology, immunology and microbiology lead towards finding that not only the probiotic

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but their DNA alone also is capable of immune activation. Preliminary experiments revealed that probiotic DNA is a better immune enhancer than its whole cell. This immunomodulating potential is due to the presence of CpG motifs in the DNA of probiotic microorganism, being a prokaryote.

2. PROBIOTICS

Probiotics are defined as viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects. Probiotics are live bacteria which are intended to colonize the large intestine and confer physiological health benefits to the host, when administered in adequate amounts and they promote or support a beneficial balance of microbes to live in the gastrointestinal tract[1]. The growing awareness of relationship between diet and health and the side effects of allopathic medication has led to an increase in the demand for the food products that support health beyond providing nutrition and act as an alternative source of medicine. Hence, the use of probiotics appear as a new tool for the treatment of diseases[2]. In addition, the bioefficacy of the probiotics can be enhanced by the use of prebiotics. Prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health. Thus, the synergistic effect of prebiotics and probiotics as synbiotics is useful in improving the health of the gut. In addition, prebiotics also have following health effects[3]:

- Improvement of blood glucose responses.
- Relieve acute and chronic constipation.
- Inhibition of secondary bile acid formation.
- Treatment of salmonellosis.
- Enhancement of mineral absorption.

2.1 Specific roles and benefits of probiotics in the body

- Prevention of genital and urinary tract infections4,5
- Nutrient synthesis and bioavailability6
- Prevention of infection by Pathogenic bacteria7
- Alleviation of the symptoms of Rheumatoid Arthritis8,9,10
- Establishment of a healthy microbiota in preterm infants11
- Increased cellular immunity (e.g. increased natural killer cell activity)12
- Alleviation of Lactose maldigestion13
- Anti- allergic potential14,15
- Immune tolerance16
- Lowered levels of cold and influenza-like symptom in children17
- Lowered incidence of diarrhoea18
- Immunostimulatory capability19
- Anti-carcinogenic activity20
- Lowering of blood cholesterol levels21,22
- Hypoglycemic activity23
- Reduction of pro-inflammatory cytokine expression24

Due to the above mentioned properties, probiotic bacteria lead the researchers to place them as one of the food supplements and fortifying agents. Food sources of probiotic bacteria also include fermented dairy products like yoghurt, milk with live culture and also sold as kefir, koumiss and yakult, etc.

2.2 Mechanism of action of Probiotics

Besides showing their antimicrobial activity directly by releasing bacteriocins, the mechanism by which probiotics exert their bioactivity in gut include their effect on immune system of host as has been given in fig.1 by Shida and Nanno[25]

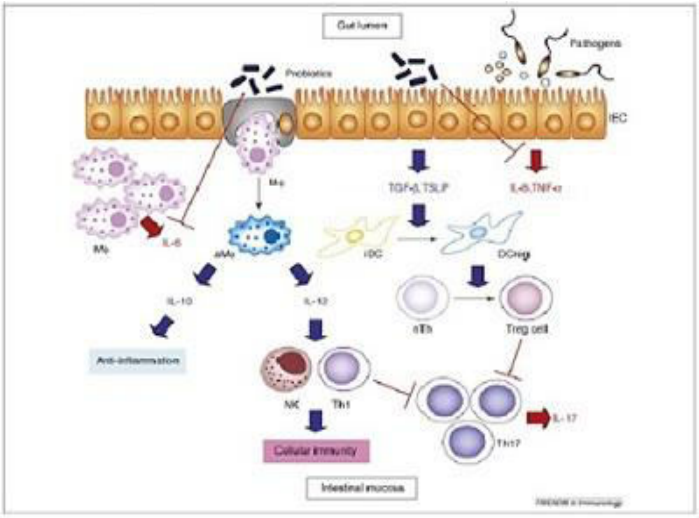

Fig.1: Mechanism of Probiotics in Gut

On exposure to pathogen microorganisms, intestinal epithelial cells (IEC) produce pro-inflammatory mediators such as IL8 and TNFα. The production of these cytokines is inhibited by probiotics and anti-inflammatory mediators such as TGF-β are produced, which can stimulate the differentiation of immature dendritic cells (DCs), followed by the induction of regulatory T cells (Treg). These cells exercise an anti-inflammatory function

by controlling Th1, Th2 and probably Th17 cells. The macrophages (M) produce large amounts of IL6 in the inflamed mucosa. The probiotics may determine the reduction of this production and the increase of the production of IL10. The intestine usually produces small quantities of IL12, but probiotics have the potential to increase this production.

2.3 Cellular components of Probiotic Bacteria and their effect on host immunity

It has been reported that cellular components of Lactic acid Bacteria (LAB) as well as live bacteria[26] and inactivated or heat killed bacteria[27,28] could exert immunoregulating effects. Other components of bacteria such as peptidoglycan & teichoic acid[29]; cell surface protein[30,31]; exopolysaccarides[32], etc provide immunoregulatory effects.

Bacterial DNA is also being explored as an immune enhancer[33]. The vertebrate immune system recognizes 'CpG motifs' of probiotic DNA as foreign and trigger protective immune responses which are strongly Th1-based. A study conducted by Krieg et al.[34] and Weeratna et al.[35], proved that bacterial DNA acts as a strong vaccine adjuvant, for inducing humoral immunity. These cellular components are known as pathogen associated molecular patterns (PAMPs) and they exert their effect by interacting with Pattern Recognition Reeptors (PRRs) of immunocompetent cells[36,37] because, PAMPs are considered as the ligands of PRRs.

Probiotic DNA has the following benefits over whole cell bacteria-

- Ameleoration of bioactivity by the use of DNA is more than whole cell probiotic bacteria[38].
- Whole cell probiotics are not useful in certain immunocompromised persons and the persons who are already extremly ill[39].

2.4 Health effects of Probiotic DNA

Over the past few years, there has been a dramatic increase in understanding the molecular and cellular effects of CpG DNA and its effects *in vivo* in animal models. Studies till date suggest that CpG DNA can be used in the treatment of a variety of diseases including infection, allergy and cancer.

The immunodulatory effects of probiotic DNA has been observed by Lammers et al.[40]. In this study, peripheral blood mononuclear cells (PBMC) from healthy donors were incubated with pure DNA of eight probiotic strains and with total bacterial DNA from human faeces collected before and after probiotic ingestion. It was concluded that *Bifidobacterium* genomic DNA induced secretion of the anti-inflammatory interleukin-10 by PBMC. Iliev et al.[33] found that DNA from *Lactobacillus rhamnosus* GG (LGG) has a strong stimulatory effect on murine B cell proliferation. It was investigated that the whole genomic DNA isolated from LGG applied onto the murine splenic B cell cultures had mitogenic activity and that the chromosomal DNA from the strain at a concentration 50 µg/ml raised strong proliferation response in murine B cells.

Rachmilewitz et al.[41] demonstrated that the immunostimulatory activity of the genomic DNA together with other factors of probiotic activity is involved in the mechanism of protection of gut by probiotic bacteria. Probiotic bacteria DNA can also suppress systemic inflammatory responses to pathogenic bacterial DNA[42,43]. Moreover, many CpG motifs have been identified in probiotic bacteria[44] .Takahashi et al.[45] proved the anti-allergic activity of probiotic DNA. It was investigated that ODN (oligodeoxynucleotides) BL07 of *Bifidobacterium longum* BB536 strain, significantly inhibited secretion of IgE in B cells in the presence of IL-4 and anti- CD40.

The experiments were conducted by Medina et al.[46] to evaluate the ability of different strains of *Bifidobacterium longum* along with their DNA to induce cytokine production by peripheral blood mononuclear cells (PBMCs). It was proved that live cells of all *B. longum* strains stimulated regulatory cytokine interleukin (IL)-10 and proinflammatory cytokine tumour necrosis factor (TNF-α) production. Genomic DNA of some strains stimulated the production of the Th1 and pro-inflammatory cytokines, interferon (IFN)-γ and TNF-α, but not that of IL-10. Ghadimi et al.[14] experimentally proved that the Gram-positive (probiotic) *Lactobacilli* and B*ifidobacteria* tested, as well as their genomic DNA, dose-dependently modulated the TH1/TH2 response to allergens. DNA seemed to contribute to about 50% of the immunomodulatory effects exerted by live bacteria.

Zhong et al.[47] investigated the effect of living probiotics, probiotic DNA and the CpG-ODNs on both immune response and intestinal barrier function in ovalbumin-sensitized rat and the underlying mechanisms. It was concluded that the probiotic genomic DNA and CpG-ODN was comparable with living probiotics in preventing food allergic response by immune modulation and intestinal barrier function enhancement, and the activation of TLR9/NF-κB signal pathway might be involved in this process.

In our own lab, Randhawa et al.[38] conducted a study to compare the *in vivo* efficacy of 3 probiotic strains having maximum (*Lactobacillus delbrueckii* 405), moderate (*Lactobacillus brevis* 403), minimum *(Bifidobacterium bifidium* BD 234) immune activity and their isolated DNA (DNA LB 405, DNA 403, DNA Bif 234) on the basis of evoking the immune response in Swiss albino mice. The results demonstrated that a substantial augmentation in immune efficacy was observed in the animals receiving genomic DNA over the group receiving viable bacteria. Hence, it was concluded in their thesis that to get the immune effects, it is not necessary to give whole bacterial cell in the host. Instead, bacterial DNA of immunoactive probiotic can be used as a safe immunobiotherapeutic agent (anti-diabetic, anti-cholesteremic, immunorestorer) even in immunocompromised host. The immunostimulating activity of DNA depends on the presence of CpG motifs.

Various researchers explored the significance and application of CpG-ODNs in modulating immune system as well as preventing and curing various immune related diseases.

3. CpG DNA

Current knowledge indicates that prokaryotic DNA can activate immune response and has potential therapeutic application. The initial discovery of this phenomenon was reported by Tokunaga et al.[48], who found out that in vivo DNA purified from *Mycobacterium* sp. (but not DNA from vertebrates) activated Natural Killer (NK) cells fostered the release of IFN-γ by these cells and caused tumor regression.

Krieg et al.[34] confirmed that bacterial DNA was immunostimulatory in a way that mammalian DNA was not, and identified a sequence pattern of bases, or motif, responsible for this property. Indeed, methylation of cytosine residues in the bacterial DNA or in the corresponding oligodeoxynucleotides destroyed their immunostimulatory activities[34]. CpG motifs are present in significantly greater frequency in bacterial DNA than vertebrate DNA. Specifically, bacterial DNA is thought to activate inflammatory cells because of its high content unmethylated CpG dinucleotides[49].

3.1 Mechanism of action of CpG DNA

Bacterial DNA and CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. CpG–ODNs interact with CpG binding protein at the cell surface. After endocytosis and recognition by Toll like receptor 9 (TLR-9), (one of the members of the Toll family of innate immune receptors, that detect molecular patterns related to intracellular pathogens), TLR9 + CpG then interact with myeloid differentiation primary response gene 88 (MYD88) and the inhibitor of nuclear factor κB- (NF-κB) kinase (IKK) complexes and the complex is translocated to the cell nucleus. The location of TLR-9 within the endosome provides both rapid and specific detection of pathogenic DNA and protection against activation by non-pathogenic "self" DNA[50]. Once in the cell nucleus, the CpG-ODNs activate NF-κB and initiate the induction of cytokines and chemokines (Fig. 2) by Fonseca and Kline[51].

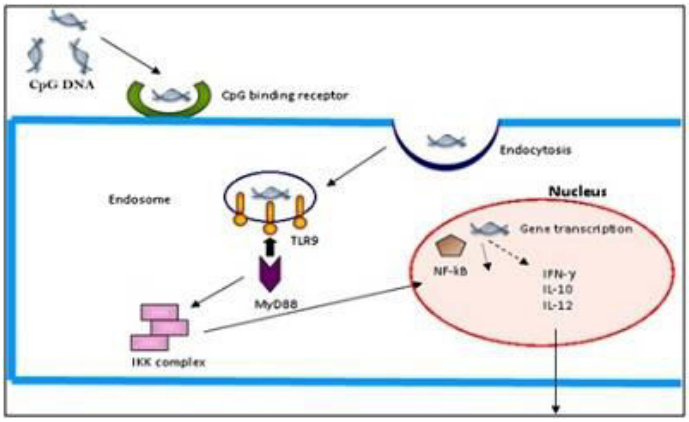

Fig. 2: Mode of action of CpG DNA

CpG-DNA also induces the monocytes and macrophages to produce inflammatory cytokines such as Il-6, Il-12, IFN-α, IFN-β, TNF-α, IL-1β, IL-18, which mediate ADCC (Antibody Dependent Cell Cytotoxicity), express inducible nitric oxide synthase and promotes lytic activity of NK cells and the secretion of IFN-γ[52,53]. As shown in fig. 3, by Agrawal and Kandimalla[54], CpG DNA

exhibit several immunological effects that has led to their use as therapeutic agents and adjuvants for various diseases.

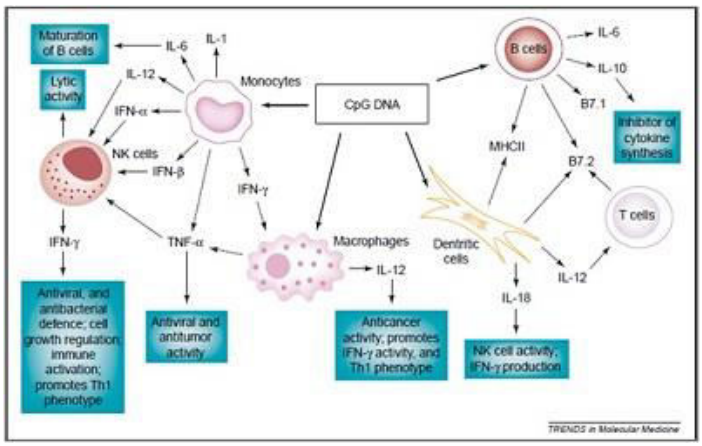

Fig. 3: Effects of CpG DNA on host immune cells

3.2 Immunotherapeutic applications of CpG DNA

The immune efficacy of CpG-ODNs resulted in the testing of their therapeutic potential. The potential applications CpG-ODNs (as mentioned in fig. 4) are as follows:

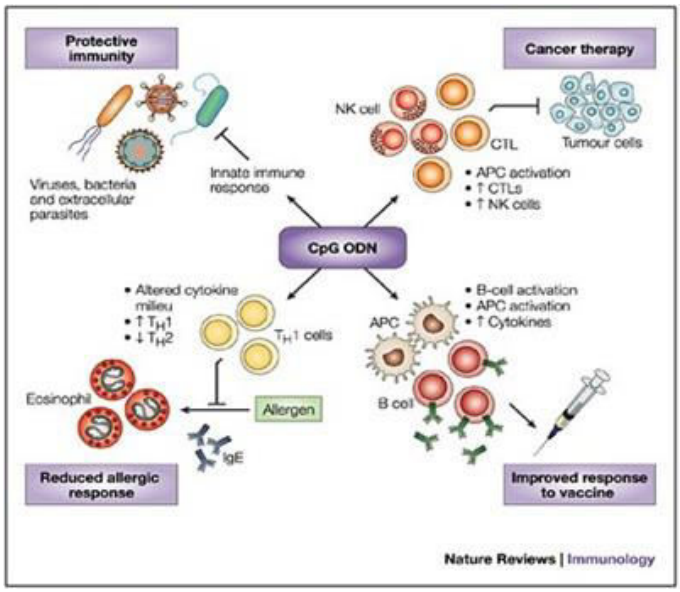

Fig. 4: Potential therapeutic uses of CpG oligodeoxynucleotides[55]

3.2.1 Protection against infectious diseases

Krieg et al.[56] experimentally proved that when mice were injected with bacterial DNA or CpG-ODNs, a rapid production of IL-12 and IFN-was noticed. The serum levels of IL-12 remain increased for at least 8 days after a single injection of CpG-ODNs, but IFN-γ levels returned to baseline within 24 h. It was concluded that this Th1-like cytokine response to CpG motifs induces a state of resistance to infection by *Listeria monocytogenes* in susceptible specific pathogen-free BALB/c mice. Gomis et al.[57] observed anti-infectious effect of DNA containing CpG motifs, when chickens were shown to be protected by infection from *Escherichia coli* due to the provision of intramuscular or subcutaneous injection doses to chickens prior to *E.coli* challenge. The study conducted by Jørgensen et al.[58] suggested that CpG DNA induces antiviral protection in Atlantic salmon fish.

Huang et al.[59] demonstrated that bacterial DNA plays an important role in the macrophage response to a heat killed pathogen *Brucella abortus*. Talati et al.[60] also reported the role of bacterial DNA/TLR9 pathway in implicating early host defense against *Streptococcus pneumonia*. Zhu et al.[61] proved protective effect of CpG DNA against mastitis induced by *Escherichia coli* infection in rat model. Easton et al.[62] conducted a study and proved that intranasal delivery of CpG oligodeoxynucleotides provided significant protection to susceptible BALB/c mice against the Gram-negative bacterium *Burkholderia pseudomallei*, which is the causative agent of melioidosis. Judy et al.[63] investigated that the prophylactic application of CpG oligonucleotides augments the early host response and confers protection in acute melioidosis.

3.2.2 Development of Vaccine/Vaccine adjuvant

McCluskie and Krieg[64] reviewed that CpG-ODNs greatly enhance the speed and strength of the immune responses if used as vaccine adjuvants. Krieg et al.[34] proposed the mechanisms contributing to the strong adjuvant activity of the CpG motifs for inducing humoral immunity may include:

i) Synergy between TLR9 and the B cell receptor (BCR) preferentially stimulating antigen specific B-cells.

ii) Inhibition of B-cell apoptosis improving B-cell survival[65].

iii) Enhanced IgG class switch DNA recombination which may enhance the maturation of the immune response[66,67].

Kojima et al.[68] observed adjuvant effects of multi CpG motifs on an HIV DNA vaccine. The results suggested that the immunogenicity of DNA vaccines can be augmented by the addition of CpG motifs. Cooper et al.[69] evaluated CpG DNA in human clinical trials as adjuvants for Hepatitis B surface antigen either in combination with alum or alone. Sugai et al.[70] proved that a CpG-ODN acts as an efficient adjuvant counterbalancing

the Th1/Th2 immune response in Diptheria-Tetanus-Pertusis (DPT) vaccine.

Maurer et al.[71] examined that CpG-ODN boost cytokine and stimulatory molecule expression on murine bone marrow-derived dendritic (mBMDC). It was concluded that CpG-DNA acts as potent adjuvant for vaccination therapies. Zhao et al.[72] investigated the protective response against *Treponema pallidium* (Tp) infection of a DNA vaccine enhanced by an adjuvant CpG-ODN. It was proved that CpG-ODN stimulated the secretion of IL-2 and IFN-γ and protective effect was observed due to decrease in the incidence of Tp-infection of skin lesions ulceration lesions. Wang et al.[73] evaluated CpG-ODN for its immunostimulatory activity in mice which were vaccinated with recombinant outer membrane protein P56 (rP56Δ) of *Orientia tsutsugamushi*. It was concluded by this study that CpG-ODN adjuvant could help in enhancing the rP56Δ immune responses, elicit memory immune response (IgM antibody) quickly and shorten its vaccination schedule.

3.2.3 **Antiallergic potential**

Horner et al.[74] conducted *in vitro* study with PBMCs from both nonatopic and atopic donors to characterize the antiallergic potential of phosphorothioate ISS-ODN in human subjects. This study demonstrated that phosphorothioate ISS-ODN elicits an innate immune response by PBMCs, which inhibits IL-4–dependent IgE synthesis. In addition, ISS-ODN also stimulated the production of IgM, IgG, and IgA.

Kitagaki et al.[75] quoted that the oral administration of CpG-DNA can protect against eosinophilic airway inflammation in murine model of asthma in association with reduction of antigen specific IgE in a dose dependent manner. CpG-ODNs may be useful as a component of oral immunotherapy to promote tolerance in established asthma. Suzuki et al.[76] demonstrated, for the first time, that immunotherapy with CpG DNA conjugated with a T-cell peptide is useful in preventing and treating allergic conditions. Xu et al.[77] proved the ability of immunomodulatory CpG–ODN, which are potent inducers of Th1 cytokines to prevent allergic symptoms in mice immunized and sensitized with allergen. Ashino et al.[78] investigated that co-administration of CpG-ODNs with an antigen prevented airway eosinophilia and IgE production as well as bronchial hyperreactivity in a murine model of asthma.

Miyazaki et al.[79] investigated the suppressive mechanism of CpG treatment in allergic conjunctivitis induced experimental mice. It was concluded that administration of the CpG-ODN induced significant splenomegaly and adoptive transfer of the splenocytes isolated from CpG-treated mice conferred resistance to inflammation in allergen-induced recipient mice. Farrokhi et al.[80] performed a study to evaluate the potential effects of Co-administration of *Chenopodium album* allergens and CpG-ODN (CpG/Ch.a) in the immune deviation from Th2 to Th1 responses in case of Allergic Rhinitis (AR) patients. Several cytokines such as IFN-γ, IL-4, IL-13, IL-10 and Ch.a specific IgE were measured in culture supernatants of PBMCs of patients. This study suggested that co-administration of CpG/Ch.a is effective in vitro in suppression of Th2 and stimulation of Th1 cytokine production. Kaburaki et al.[81] suggested that Japanese cedar pollen allergen (Cry j 1) conjugated with CpG-ODNs immunostimulation can induce Cry j1 specific Th1 responses, thereby inhibiting IgE response to the pollen allergen. Hence, CpG-ODN may be an effective novel method of inducing protection against atopic disorders.

3.2.4 **Antitumor immunization**

Weeratna et al.[35] reported that CpG-ODNS can be used successfully in tumor immunotherapy in animal models as well as in human clinical trials. Preliminary studies in several animal models of cancer suggest that CpG DNA have the ability to induce tumor regression by activating innate immunity, enhancing antibody dependent cellular cytotoxicity and elicit a specific, protective immune response[82]. Cornet et al.[83] proved that CpG DNA activate dendritic cells *in vivo* and induce a functional and protective immunity. Study of the CD8 response obtained after antigenic challenge suggested that a functional memory response is induced upon vaccination with CpG-ODN. Thus, MHC class I – restricted epitope in combination with CpG DNA is a promising and rather simple cancer formulation.

Vaccination with CpG-conjugated tumor cells induces the expansion of tumor-specific cytotoxic T lymphocytes (CTL) that reduce the growth of established tumors and prevents their metastatic spread[84]. They established that this conjugation of

CpG to the surface of tumor cells improves the uptake of tumor cell vaccines by Antigen Presenting Cells (APCs) and further triggeres the functional maturation of the APCs.

3.3 Risks of using CpG-ODN *in vivo*

Though many benfits of CpG-DNA have been described but the application of these CpG-DNA motifs do involve some risks in vivo. Few toxic effects of CpG DNA were indicated such as it may trigger autoimmune diseases like Systemic lupus erythematosus[85]. Authors cautioned that excessive activation of immune system can cause systemic inflammatory response syndrome. A toxicity study of probiotic DNA was done on Swiss Albino Mice by Randhawa et al.[38] and it was concluded that toxicity is dose dependent. It is safe to administer 50-75µg/ml Probiotic DNA *in vivo*, but 100µg/ml and above dose lead to loss of hair and more aggressive behavior.

4. Future Recommendations

The efficacy of DNA can also be enhanced by loading it onto nanoparticles before introducing it in the body. So, nanoparticle assisted delivery may be a promising approach to alleviate the problem of instability and degradation of DNA and the studies over the nanoparticle approach for DNA delivery are going on.

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