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A Study on the immune effects of synergistic oligodeoxynucleotide from probiotics

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Thesis Title

A Study on Immune Effects of Synergistic Oligodeoxynucleotide from Probiotics

Abstract

Bacterial genomes span a significant portion of diversity, reflecting their adaptation strategies; these strategies include nucleotide usage biases that affect chromosome configuration also known as genomic DNA. The genomic DNA has recently been shown to elicit a highly evolved immune defense. This response can be selectively triggered for a wide range of therapeutic applications, including use as a vaccine adjuvant to immunotherapies for allergy, cancer, and infectious diseases. Here, I explore an immuno-synergistic oligodeoxynucleotide (iSN-ODN, named iSN34), derived from *Lactobacillus rhamnosus* GG (LGG) genomic sequences, that exhibits a synergistic effect on immune response to CpG-induced immune activation. Bacterial DNA is a potent stimulator of the host immune response that mediated by unmethylated CpG motifs which leads to abrogation of the immunostimulatory activity. Synthetic oligodeoxynucleotides (ODNs) which contains CpG motifs that are like those found in bacterial DNA stimulate a similar response and these immunomodulatory ODNs have various potential therapeutic effects to combat. Bacterial DNA is a PAMP that is mediated by members of the Toll-like family of receptors(TLRs). Here, I extend that observation by demonstrating the synergistic induction (in mouse splenocytes) of IL-6 by the combination of iSN34 with cell wall components of bacteria and fungi. Likewise, TLRs have been implicated in the recognition of the fungal pathogens.

Several components located in the cell wall or cell surface to fungi have been identified as potential ligands. The sequence of iSN34 was designed based on the genomic sequences of LGG. Pathogen-free mice were purchased from Japan SLC and maintained under temperature- and light-controlled conditions. I tested the effects of iSN34 exposure *in vitro* and *in vivo* by assessing effects on mRNA expression, protein levels, and cell type in murine splenocytes. I demonstrate that iSN34 has a significant stimulatory effect when administered in combination with CpG ODN, yielding enhanced interleukin (IL)-6 expression and production. I also observed that splenocytes pretreated with iSN34 and then co-stimulated with agonists for TLR1/2 (Pam_3CSK_4), TLR4 (LPS), or TLR2/6 (Zymosan) exhibited enhanced accumulation of IL-6. IL-6 is a pleiotropic cytokine that has been shown to prevent epithelial apoptosis during prolonged inflammation.

My results are the first report of a bacterial-DNA-derived ODN that exhibits immune synergistic activity. The potent over-expression of IL-6 in response to treatment with the combination of CpG ODN and iSN34 suggests a new approach to immune therapy. Additionally, suggested that the combination of iSN34 with TLR1/2, TLR4, or TLR2/6 agonists may permit the induction of a potent immune response. This finding may lead to novel clinical strategies for the prevention or treatment of dysfunctions of the innate and adaptive immune systems.

Abbreviation

2-ME	2-mercaptoethanol
CD19	Cluster of differentiation 19
CD86/CD69	Cluster of differentiation 86/ Cluster of differentiation 69
CpG ODNs	Cytosine-phosphate-guanine oligodeoxynucleotides
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
i.p. injections	Intraperitoneal injections
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IL-	Interleukin
IFN-γ	Interferon-γ
iSN34	Immune-synergistic ODN 34
iSN-ODN	Immune-synergistic Oligodeoxynucleotide
JCA	Juvenile chronic arthritis
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
NK cells	Natural killer cells
Pam_3CSK_4	N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-(Lys)4
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PRRs	Pattern recognition receptors
PS ODN	Phosphorothioated oligodeoxynucleotide
qPCR	Real-time quantitative PCR
RA	Rheumatoid arthritis
SD	Standard deviation
soJIA	Systematic-onset juvenile idiopathic arthritis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

Contents

Chapter 1

General Introduction

Innate and Adaptive Immunity

The Immune system is the body's primary defense system against invasion by microbes. The human body has evolved natural barriers to prevent entry by microbes. The immune system is organized into innate and adaptive components. The innate immune response is the first line defense for the body and it uses nonspecific cells, such as phagocytes, and molecules, such as complement components, to attempt to eliminate invading organisms. This response is coordinated by a variety of specialized cells including monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, natural killer (NK) cells, and NK T cells. These cells are activated germline-encoded Pattern Recognition Receptors (PRRs). PRRs bind to molecular signatures, often-essential structural or genetic components, which are conserved among pathogens. This allows the innate immune system to respond rapidly to a broad range of infectious agents that have breached the skin and mucous membranes. Toll like receptors (TLRs) are one of the pattern recognition molecule used by innate immune system. On the other hand, the adaptive immune system usually triggered by the innate system and it is slow to mobilize but provides a highly antigen specific response. This is accomplished through the somatic recombination of T and B cell receptors and provides a mechanism for long-lived specific immunity.

The innate immune system relies on preexisting molecules and cells that nonspecifically attack invaders; this system protects us well against a wide range of infections. In general, the innate system protects against infection by removing the infectious agent. However, it is unable to respond specifically to microbes or other foreign material (antigen). In adaptive system which depends on gene rearrangement to generate many preexisting receptors (repertoire), expressed on

lymphocytes, that can identify essentially any antigen. Thus, a balance between activation and suppression must be struck to ensure an appropriate and effective immune response. Understanding how the innate immune system is activated and how this response may be controlled will help in designing safe and effective therapeutic interventions.

Probiotics

It is widely believed that fermented products were probably found, or better to say, discovered spontaneously. The legend tells that yogurt is most likely resulted from a fermentation process within the animal skin bags used for transportation of water and milk in regions with low humidity and high temperatures (Middle Asia and Middle East). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." At the beginning of 1900s Louis Pasteur identified the microorganisms responsible for the process of fermentation, whereas E. Metchnikoff associated the enhanced longevity of Bulgarian rural people to the regular consumption of fermented dairy products such as yogurt. Metchnikoff tried first to find out the possible effect of these microbes on human health. In 2013 (Hill et al., 2014) an expert consensus document had been published on the scope and appropriate use of the term probiotic: live microorganisms which when administered in adequate amounts confer a health benefit on the host.

Lactobacillus rhamnosus GG (LGG) is one of the most widely used probiotic strains with various health benefits. LGG was identified as a potential strain for its resistance to acid and bile, faster growth characteristics and adhesion capacity to the intestinal epithelial layer (Doron et al., 2005). Specific lactic acid bacterial (LAB) strains, such as *Lactobacillus* strains, have been considered as probiotics because of their health benefits (Marike and Sarah, 2014; Balish and Warner, 2002; Rath

2003; Sartor 2005). These strains have a long history of consumption in traditional fermented foods as natural inhabitants of healthy human gastrointestinal tracts. Probiotic bacteria are required to express high resistance to acid and bile, adhere to intestinal surfaces, and colonize in the gastrointestinal tract. Research findings have shown that several *Lactobacillus* bacterial strains possess the resistance to acid and bile, inhibitory activity toward the growth of pathogenic bacteria, and positive effects on the host health (Matsumoto et al., 2005; Christensen et al., 2002; Cross et al., 2004; Matsuguchi et al., 2003). The key foundation for improving the functional properties and the biotherapeutic action of probiotic foods and pharmaceutical products is the selection of suitable probiotic candidates. Studies have discovered that some specific strains of lactobacilli can induce the production of pro-inflammatory cytokines (interleukins IL-1 and IL-6) and anti-inflammatory cytokines (interleukins IL-12 and IL-10) in animal/human body. We observed an immune synergistic ODN named iSN34 which is derived from *Lactobacillus rhamnosus* GG.

CpG Oligodeoxynucleotide and Immunosynergistic Oligodeoxynucleotide

Oligodeoxynucleotides (ODNs) containing with unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides (CpG ODN) mimic the immunostimulatory activity of bacterial DNA. The strong stimulatory effect of bacterial DNA is largely due to the presence of unmethylated CG-containing motifs, which are the foundation for the design and testing of synthetic CpG-containing oligodeoxynucleotides (CpG ODN) as immune adjuvants and stand-alone therapeutics (Kuramono et al., 1992; Krieg et al., 1995; Krieg 1998). It has been reported that bacterial DNA represents a PAMP that is recognized by the vertebrate immune system leading to a coordinated on immune responses including innate and acquired immunity (Krieg et al., 1995) as well as unmethylated CG dinucleotide are present at high frequency in prokaryotic DNA but rare in eukaryotic DNA (Razin

et al, .1981; Cardon et al., 1994). Another approach has been explored that bacterial DNA is able to associate or alone with other bacterial products, can trigger the release of IL-6, IL-12. IFN-γ, and IgM (Klinman et al., 1996**).** Cellular recognition of CpG motifs is made possible by TLR9, a member of an evolutionarily conserved family of proteins that are responsible for mediating innate immune reactions, especially against bacterial infections, through the recognition of pathogen-associated molecular patterns (Akira et al., 2001; Takeda et al., 2001).

Immunosynergic effects of ODN is somehow new knowledge of ODN research. In earlier studies, researcher was done research about the immunomodulatory effect (Wang et al., 2015), immunosuppressive (Shirota et al., 2005; Sato et al., 2008) and immunostimulatory (Wooldridge et al., 1997) effect of ODN. In 2017, Nigar et. al. explores a novel ODN (named iSN34) which is synthesized by *Lactobacillus rhamnosus* GG ATCC53108 and have synergistic effect on immune response on CpG-induced immune activation. The sequence of iSN34 is TTCCTAAGCTTGAGGCCT which contains 18 bases. Initially, we were screening for inhibitory/suppressive ODN (iODNs) with a TTAGGG motif. In 2013, we successfully designed a strong iODN (named 'iSG3') that has a six-base loop head structure and a 3′-oligo $(dG)_{3–5}$ tail sequence and showed that iSG3 possesses potent immunosuppressive activity (Ito et al., 2013).

iSG3: CCTCATTAGGGTGAGGG

In the initial stages of the present study, we sought to further characterize iSG3 and highly homologous sequences in the LGG genome. Although we generated 50 candidates of ODNs (iSN01-50). When we assessed these molecules by the same assay as that used to identify iSG3, diametrically opposed results were obtained. I demonstrate that treatment of iSN34 with CpG-ODN leads to increased IL-6 expression and that this increased expression enhances various clinical symptoms and abnormalities and therapeutic target in candidate inflammatory diseases.

iSN34 has synergistic effects not only associated in TLR9 but also TLR1/2, TLR4 and TLR6 in immune activation. This finding may open novel perspectives for clinical strategies to prevent or treat innate and adaptive immune systems.

Immune function of CpG ODN

Bacterial DNA contains unmethylated "CpG motifs" that strongly activate the mammalian immune system (Yamamoto et al., 1992; Kreig et al., 1995; Klinman et al., 1996; Sato et al., 1996 and Yamamoto et al., 1995). Synthetic oligodeoxynucleotides containing such CpG motifs stimulate B cells (Kreig et al. 1995; Yi et al. 1996)], natural killer (NK) cells (Ballas et al., 1996; Verthelyi et al., 2001), and professional antigen-presenting cells (APCs) (Sparwasser et al., 1997; Stacey et al.,1996; Sparwasser et al., 1998; Behboudi et al., 2000) to proliferate and/or secrete a variety of cytokines, chemokines, and immunoglobulins (Ig). Certain CpG motifs (CpG-A) are especially potent at activating NK cells and inducing IFN-α production by PDCs, while other motifs (CpG-B) are especially potent B cell activators. Preclinical and early clinical trials indicate synthetic oligodeoxynucleotides containing CpG ODN have potent immunostimulatory effects and activate host defense mechanisms leading to innate and acquired immune responses. Until now CpG-driven immune activation can exacerbate inflammatory tissue damage, promote the development of autoimmune disease, and increase sensitivity to toxic shock (Krieg et al., 1995; Zeuner et al., 2003; Sparwasser et al., 1997; Cowdery et al., 1996; Heikenwalder et al., 2004; Deng et al., 1999).

CpG DNA has been explored as a therapeutic agent for cancer, asthma, allergy, and infectious diseases and as an adjuvant in immunotherapy, but it generally requires phosphorothioate or other chemical modification. Such modification may have disadvantages associated with toxicity, such

as a transient anticoagulant effect, activation of complement cascade, and inhibition of basic fibroblast growth factor binding to surface receptors, because of non-specific protein binding (Henry et al., 1997). Thus, CpG DNA can induce T helper type 1 cytokine production; this promotes a cytotoxic T-lymphocyte response with enhanced immunoglobulin production, which has been used in the treatment of a broad spectrum of diseases, including cancer, viral and bacterial infections, allergic diseases and inflammatory disorders (Krieg 2002; Sato et al., 1996; Dalpke et al.,2002; Kandimalla et al., 2002).

Toll-like receptors as Pattern-recognition receptor

Toll-like receptors (TLRs) are an important family of pattern-recognition receptors with at least 11 members (Kumagai et al., 2008; Takeda et al., 2003) that are key molecules for microbial elimination, such as the recruitment of phagocytes to infected tissues and subsequent microbial killing. TLR family members are sub-divided to cell membrane-associated and endosome-associated receptors. While the surface receptors that target molecular pathogenic patterns, examined activation events using agonists employed primarily by bacterial or fungal agents as well as Pam_3CSK_4 is a synthetic triacylated lipoprotein signaling through the heterodimers TLR1/2 (Patel et al., 2005). Lipoprotein from the Gram-positive bacteria -induced signaling pathways is mediated, in part, through TLR2 and other receptors (Kaisho et al., 2001), lipopolysacchride (LPS) the Gram-negative bacteria cell wall component induced signaling is mediated by TLR4 (Akashi et al., 2000), flagellin (TLR-5 agonist), and Zymosan, an extract from the cell wall the yeast *Saccharomyces cerevisiae*, is recognized by another heterodimers TLR-2/6 and has properties of a potent immune stimulant with the capability of activating adaptive immune response that mainly recognize microbial components including lipids, lipoproteins and flagella. Whereas TLR3 and TLR7/8 recognizes double and single-stranded RNA, TLR9 recognizes bacterial DNA or single-

stranded synthetic oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs (Alexopoulou et al., 2001; Heil et al., 2004; Takeshita et al., 2001; Kawai et al., 2010) are expressed in intra-cellular vesicles of the endoplasmic reticulum, endosomes and lysosomes. All TLRs share a common architecture consisting of extracellular leucine-rich repeats and a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain (Matsushima et al., 2007). These receptors 4 signal as dimers, differentially recruiting the adapter proteins: Myeloid differentiation primary response gene 88 (MyD88) and MyD88 adapter-like (Mal also known as TIRAP) and/or TIR-domain-containing adapter inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM). Adapters initiate signal cascades culminating in the activation of nuclear factor kappa b (NF-κB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors 1, 3, 5 and 7 (IRF-1, 3, -5 and -7) (Fitzgerald et al., 2003). Together, these transcription factors not only drive expression of interferons, cytokines and chemokines, but also influence cellular proliferation, maturation and survival. However, we explored the synergistic activity of iSN34 combined with nucleic acid ligand-responsive TLRs (TLR-1/2,4,6) in mouse splenocytes which include a variety of cell populations such as T and B lymphocytes, dendritic cells, macrophages, NK-cells, and NK T cells with different immune functions. Thus, TLR family is an important group of receptors through which innate immunity recognizes invasive microorganisms. Recent gene targeting studied have revealed that TLRs sense organisms ranging from bacteria to fungi, Protozoa and viruses. Here we show it in table 1.

IL-6 induction

More than 30 years have elapsed from the discovery of IL-6. During these years of mostly non-profit driven basic research has elucidated many facets of IL-6 biology, including the receptor complex composition, the signal transduction pathways and the role of IL-6 in inflammation and cancer. IL-6-type cytokines form a subfamily of the helix bundle cytokines. All IL-6-type cytokines comprise four long α helices termed A, B, C and D, which are arranged in a way that leads to an up-up-down-down topology. All nine family members of the interleukin (IL)-6 cytokine family (IL-6, IL-11, CNTF, LIF, OSM, CLC, CT-1, NNT-1, and IL-27) share the membrane protein glycoprotein 130 (gp130) as one signal transducing beta-receptor subunit. Some IL-6-type cytokines need additional membrane-bound signal transducing beta-receptors and/or non-signaling alpha-receptor proteins to induce signal transduction (Garbers et al., 2012). In case of IL-6, the cytokine initially binds to the membrane-bound or soluble non-signal-transducing IL-6R. Only in complex with IL-6R, IL-6 can recruit two molecules of the signal-transducing receptor glycoprotein 130 kDa (gp130) (Garbers and Scheller, 2013).

IL-6 type cytokines are mainly involved in inflammation by controlling differentiation, proliferation, migration, and apoptosis of target cells. IL-6 has additional roles in a variety of other processes such as metabolism, embryonic development and memory consolidation. A dysfunction of the complex regulatory cytokine network might lead to acute and chronic inflammation, autoimmune diseases or neoplastic disorders. Being IL-6 is a multifunctional cytokine, originally identified as a T-cell derived factor that induces activated B-cells to differentiate into antibody-producing cells with biological activities including regulation of immune response, inflammation and hematopoiesis (Kishimoto 2005). Because IL-6 is multifunctional, *in vivo* overproduction of it causes various clinical symptoms and abnormalities in laboratory test results, which may well

explain the manifestations observed in patients with various inflammatory diseases, including rheumatoid arthritis(RA) and systematic-onset juvenile idiopathic arthritis soJIA (Kishimoto 2005; Mima et al., 2009). During infections and tissue injuries, IL-6 is promptly produced by monocytes and macrophages and contributes to removal of infectious agents and restoration of damaged tissues through activation in immune, hematological and acute-phase responses (Tanaka et al., 2016).

Aims of the present study

The aims of the study were to be screening a novel oligodeoxynucleotide and its immune function in innate and adaptive immune system. We observe a significant stimulatory effect of iSN34 when administered in combination with CpG ODN, yielding enhanced interleukin (IL)-6 expression and production. IL-6 is a pleotropic cytokine that has been shown to prevent epithelial apoptosis during prolonged inflammation. I also extend our observation by demonstrating the synergistic induction (in mouse splenocytes) of IL-6 by the combination of iSN34 with cell wall components of bacteria and fungi. Further I observed that splenocytes pretreated with iSN34 and then co-stimulated with agonists for TLR1/2 (Pam_3CSK_4), TLR4 (LPS), or TLR2/6 (Zymosan) exhibited enhanced accumulation of IL-6.

The specific findings were as follows

1. Synergistic oligodeoxynucleotide strongly promotes CpG-induced interleukin-6 production.
2. Immune synergistic oligodeoxynucleotide from *Lactobacillus rhamnosus* GG enhances the immune response upon co-stimulation by bacterial and fungal cell wall components.

Table 1. TLRs and its ligands

Bacterial components	Species	TLR usage	Location of TLR	Signaling adaptor	Effector cytokine induced
LPS	Gram-negative bacteria	TLR4	Plasma membrane (cell surface)	TIRAP, MyD88, TRAM and TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Diacyl lipopeptides	Mycoplasma	TLR2/TLR6	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Triacyl lipopeptides	Bacteria	TLR2/TLR1	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Peptidoglycans	Gram-positive bacteria	TLR2(?)	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Lipoteichoic acid	Gram-positive bacteria	TLR2/TLR6	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Phenol-soluble modulin	Staphylococcus aureus	TLR2	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Glycolipids	Treponema maltophilum	TLR2	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Atypical LPS	Non-entero bacteria	TLR2(?)	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Flagellin	Flagellated bacteria	TLR5	Plasma membrane (cell surface)	MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
CpG DNA	Bacteria	TLR9	Endosome	MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Not determined	Uropathogenic bacteria	TLR11	Plasma membrane (cell surface)	MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Fungal components					
Zymosan	Saccharomyces cerevisiae	TLR2/TLR6	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Mannan	Saccharomyces cerevisiae	TLR4	Plasma membrane (cell surface)	TIRAP, MyD88, TRAM and TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
	Candida albicans		Plasma membrane (cell surface)	TIRAP, MyD88, TRAM and TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Phospholipomannon	Candida albicans	TLR2	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
Glucuronoxylomannon	Cryptococcus neoformans	TLR4	Plasma membrane (cell surface)	TIRAP, MyD88, TRAM and TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Viruses					
DNA	Viruses	TLR9	Endosome	MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.), type
dsRNA	Viruses	TLR3	Endosome	TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
ssRNA	RNA Viruses	TLR7/TLR8	Endosome	MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Envelope proteins	RSV,MMTV	TLR4	Plasma membrane (cell surface)	TIRAP, MyD88, TRAM and TRIF	Inflammatory cytokines (TNF-α, IL-6 etc.), type
Hemagglutinin protein	Measles viruses	TLR2	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)
ND	HCMV,HSV1	TLR2	Plasma membrane (cell surface)	TIRAP, MyD88	Inflammatory cytokines (TNF-α, IL-6 etc.)

This table is modified by Uematsu and Akira, 2008

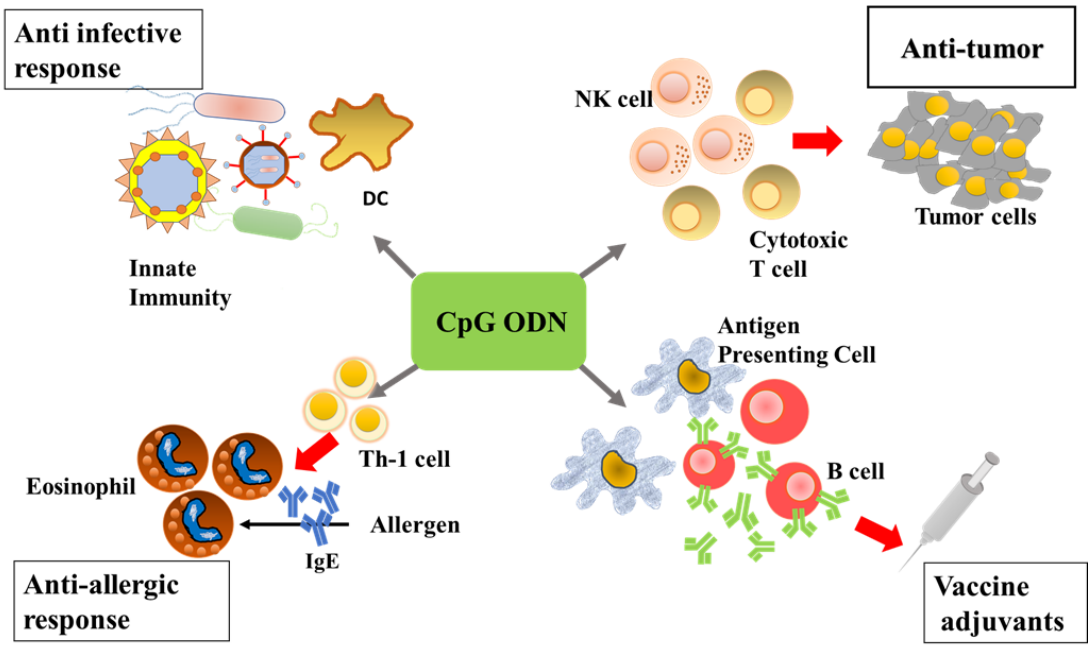

Figure 1 | Potential therapeutic uses of CpG oligodeoxynucleotides (ODNs). Four major applications for CpG ODNs have been developed. The innate immune response elicited by CpG motifs has developed to protect the host from infectious pathogens. Therefore, CpG ODNs might be used as stand-alone agents to reduce susceptibility to infection. When combined with allergen, these ODNs stimulate an antigen-specific T helper 1 (TH1)-cell response that inhibits the development of TH2-cell-mediated allergic asthma. CpG ODNs also improve the function of professional antigen-presenting cells (APCs) and create a cytokine/chemokine milieu that is conducive to the development of an adaptive immune response to co-administered vaccines. Finally, the immune cascade that is elicited by CpG ODNs results in the activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) that facilitates (alone or in combination with other therapies) the treatment of cancer. This figure is modified from Nature Reviews Immunology. (Klinman, 2004)

Chapter 2

Synergistic oligodeoxynucleotide strongly promotes CpG-induced interleukin-6 production

2.1. Introduction

Nucleic acids have been shown to be predominantly potent molecular triggers of the innate immune response, not only providing a quick response against pathogens but also playing a role in shaping the adaptive immune response. These nucleic acids include CpG oligodeoxynucleotides (CpG ODNs) that are responsible for the immune stimulatory effect of bacterial DNA (Krieg 2002). Several studies have confirmed that bacterial DNA, as well as synthetic CpG ODNs (ODNs containing unmethylated CG dinucleotides and a phosphorothioate or chimeric backbone that renders these molecules nuclease resistant), have potent immunostimulatory effects (Weiner 2009). Toll-like receptors(TLRs) are extensively expressed on innate immune cells recognizing a variety of pathogen-associated molecules including bacterial DNA(TLR9) within the endosomal compartment. TLRs activation involves specific DNA sequences containing an unmethylated CG dinucleotide (CpG motif). TLR9 is predominantly expressed on B-cells, macrophages, and dendritic cells in mouse, and B cells and plasmacytoid cells (pDC) in human (Krieg 2002). It became progressively clear that triggering CpG-TLR9 signaling pathway leads to upregulate of proinflammatory genes such as interleukin (IL)-6, Tumor necrosis factor (TNF)-α and type-1 interferons (IFN-α and IFN-β), via activation of MyD88 adapter proteins leading to recruitment of kinases and downstream activation of IRF and NF-$_k$B signaling (Klinman 2004). In 1998 Krieg et al. demonstrated in one figure of activation of CpG that triggers all subsets of B cell and can drive more than 95% of them into the cell cycle. In the present study, I investigated the immune response using ODN candidates from the *Lactobacillus rhamnosus* GG (LGG) genome. Notably, the genomes of diverse bacteria share DNA motifs that are rarely found in higher vertebrates (Bird 1987; Gilkeson et al., 1989; Messina et al., 1991). LGG is one of the most widely used probiotic

strains and has been associated with various health benefits. LGG has been observed to exhibit increased acid and bile resistance, enhanced growth characteristics, and improved adhesion to the host intestinal epithelial layer (Doron et al., 2005). LGG's potent health benefits are presumed to reflect the production of a range of molecules that contribute to host interaction, including bacterial pili and fimbriae, lipoteichoic acid molecules, major secreted proteins, galactose-rich exopolysaccharides, and specific DNA motifs (Marijke and Sarah, 2014). These motifs include non-methylated CG dinucleotides that trigger cells expressing Toll-like receptor 9 (TLR9); this trigger results in the activation of natural killer (NK cells), B cells, monocytes, macrophages, and dendritic cells (Weiner 2009; Klinman et al., 2005; Cowdery et al., 1996; Sparwasser et al., 1998; Stacey et al., 1996) , yielding an innate immune response characterized by the production of T-helper type(Th)-1 cells and proinflammatory cytokines (Weeratna et al., 200;Sun et al., 1998; Stacey et al., 1996) . Th1 favor T-cell mediated immunity and defense against intracellular pathogens or antibody mediated immunity and defense against extracellular pathogens.

It is figured that some intestinal bacteria exert beneficial effects, whereas others demonstrate deleterious effects. LGG chromosomal DNA has been shown to be a potent inducer of splenic B cell proliferation, CD86/CD69 expression, and cytokine production in mouse (Ilive et al., 2005) and an efficient suppressor of allergic activity (Ilive et al., 2008). In addition, various bacterial strains (including *Lactobacillus, Lactococcus, Bifidobacterium, Enterobacter cloacae, Bacteroides fragilis*, *Enterococcus faecalis*, and *Escherichia coli*) have been shown to induce pro inflammatory mediators that produced predominantly by activated macrophages such as TNF-α, IL -6, IL-12p70, and IL-23; these mediators may in turn be responsible for the induction and maintenance of chronic inflammatory responses (Stallmach et al., 2004; Hart et al., 2005; Kamada et al., 2005; Fuss et al., 2006). Cytokines are redundant in their activity, meaning similar functions

can be stimulated by different cytokines. They are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically or antagonistically. Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Cytokines may be produced in and by peripheral nerve tissue during physiological and pathological processes by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells (Xie et al., 2006).

One component shared among these inducing organisms is the nature of bacterial DNA known as synthetic oligodeoxynucleotides as responsible for immune activation. Bacterial DNA constitutes a PAMP that is recognized by the vertebrate immune system, leading in turn to coordinated immune responses comprising both innate and acquired immunity (Krieg et al., 1995). Unmethylated CpG dinucleotides, which are present at high frequency in prokaryotic DNA but are rare in eukaryotic DNA (Razin et al., 1981; Cardon et al., 1994), are considered one such PAMP. Further research has suggested that bacterial DNA, alone or in combination with other bacterial products, triggers the release of IL-6, IL-12, interferon (IFN) -γ, and immunoglobulin (Ig) M (Klinman et al., 1996). In this context, the ability of bacterial DNA to induce IL-6 is of special interest. In fact, overproduction of IL-6 *in vivo* has been shown to cause various clinical symptoms and abnormalities such as spiking fever, skin rash, arthritis, pericarditis, hepatosplenomegaly, and growth retardation. In laboratory models, which may explain the manifestations observed in patients with various inflammatory diseases, including rheumatoid arthritis (RA) and systematic-onset juvenile idiopathic arthritis (soJIA) (Kishimoto 2005; Mima and Nishimoto, 2009). Now, it is shown that stimulation of mouse splenocytes with the combination of CpG-ODN and an immuno-synergistic ODN (iSN-ODN, which designated iSN34 derived from LGG genomic sequences yields significant up-regulation of IL-6 expression. I further determine that this

combination treatment leads to increased IL-6 expression *in vivo*, and thus increased expression enhances clinical symptoms and abnormalities. Indeed, CpG-motif-containing ODNs are widely studied as promising adjuvants for vaccines against a range of diseases, including infections, cancer (including kidney, skin, breast, uterine and immune malignancies (Jahrsdorfer et al., 2003; Carpentier et al., 2003), and allergies. Thus, these results are consistent with those previous studies; our observation of selective enhancement of IL-6 expression suggests that synergistic ODNs might be useful as vaccine adjuvants.

2.2. Materials and methods

2.2.1. Animals

Pathogen-free female C57BL/6 mice (4 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and maintained under temperature- and light-controlled conditions. Mice were provided with *ad libitum* access to a standard diet of Labo MR Breeder (Nihon Nosan Co., Kanagawa, Japan) and sterile water. Mice were 6 to 7 weeks of age at the start of the study.

2.2.2. ODNs

Endotoxin-free desalted phosphorothioated (PS) ODNs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Each of the PS-ODNs was reconstituted in endotoxin-free water and passed through a 0.22-μm pore microfilter (Nihon Millipore K.K., Tokyo, Japan) prior to use. Mouse splenocytes were treated with equimolar amounts of CpG ODN 1555 (Klaschik et al., 2007), control ODN 1612 (Sato et al., 2010), CpG ODN 1585 (Ballas et al., 2001), CpG ODN 2395(Marshall et al., 2005), or MsST (defined below) (Shimosato et al., 2009) (Table 2).

2.2.3. Cells and cell culture

Splenocytes were prepared using standard methods (Ito et al., 2013; Wang et al., 2015). and were then cultured in triplicate or quadruplicate wells of a 24-well plate (Nalge Nunc International K.K., Tokyo, Japan) at 2×10^6 cells/well in volumes of 1 mL/well of RPMI 1640 medium (Sigma-Aldrich St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, and 0.0035% 2-mercaptoethanol (2-ME).

2.2.4. qPCR analysis

For analysis of gene expression, total RNA was isolated from ODN-stimulated mouse splenocytes and treated with DNase I (Macherey-Nagel GmbH & Co., Duren, Germany) for 15 min at room temperature; the nuclease was then heat-inactivated by incubation of the mixtures at 70˚C for 15 min (Katoh et al., 2004; Shimosato et al., 2010; Shigemori et al., 2012; Ballas et al., 2001). Thereafter, cDNA was prepared by reverse transcription from 1 μg of total RNA per sample using the Prime Script® RT reagent kit (TaKaRa Bio, Inc., Tokyo, Japan). Equal volumes of cDNA were used for quantification of various cytokine cDNAs via real-time quantitative PCR (qPCR) using a Thermal Cycler Dice® Real Time System (TaKaRa Bio, Inc., Tokyo, Japan). The qPCR analyses were performed with SYBR Premix Ex Taq (TaKaRa Bio) using gene-specific primers, as described previously (32). Primers for the β-actin- and IL-6-encoding genes were purchased from TaKaRa Bio. As a control, poly $(A)^+$ RNA samples were used as templates to check for the presence of contaminating genomic DNA. Each pair of gene-specific primers included one primer designed to span an exon-intron junction, and the other designed to span the actual exon-intron boundary. The sensitivity of the reaction and amplification of contaminating products, such as self-

annealed primers, were evaluated by amplifying serial dilutions of cDNA. For cross-sample comparison of results obtained following various treatments, levels of cytokine-encoding mRNA were first normalized to those of β-actin-encoding mRNA. Data are shown as the mean + standard deviation (SD) of one experiment representative of three independent experiments that yielded similar results.

2.2.5. Wash-out assay

Splenocytes were prepared using standard methods (Shimosato et al., 2010). Cells were cultured in a 24-well plate (Nalge Nunc International K.K.) at 1×10^7 cells/well in volumes of 1 mL/well of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, and 0.0035% 2-ME. Cells were incubated in the presence of 3 μM iSN34 for 24 h at 37°C in a 5% CO_2 environment. Cells were then washed with fresh medium to remove the iSN34. Cells were resuspended in fresh medium supplemented with 3 μM CpG-B (ODN 1555) for 6 h and cytokine expression was detected by qPCR as described above. Data are shown as the mean + SD of one experiment representative of three independent experiments that yielded similar results.

2.2.6. Cytokine quantification

IL-6 levels in cell culture supernatants after 48 h of various treatments were quantified using a commercially available ELISA kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions.

2.2.7. Intracellular staining

Splenocytes (2×10^6 cells/well) were pre-incubated for 3 h in medium supplemented with either 0.625 μM iSN34 or an equivalent volume of water. Cells then were washed with medium to remove the ODNs, resuspended in medium containing 3.0 μM CpG-B (ODN 1555), and incubated for 12 h. After stimulation, cells were cultured for 4 h at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 μg/mL of brefeldin A, 2 μg/mL of ionomycin, and 20 ng/mL of phorbol 12-myristate 13-acetate. For intracellular staining, cells were fixed in 4% PFA for 15 minutes at room temperature, washed, and permeabilized by incubation for 15 minutes on ice; cells were then further incubated with phycoerythrin-labeled anti-mouse IL-6 antibody (Biolegend, San Diego, CA). Splenocytes were first stained with anti-mouse IL-6 antibody for 60 minutes on ice. Cells were washed and the percentages of $CD19^+$ $IL\text{-}6^+$ cells were determined using FACS Calibur (BD Biosciences). Data were acquired and analyzed using Flow Jo software. All analyses were carried out at least in triplicate; representative results are presented.

2.2.8. *In vivo* study

In vivo experiments employed 4-week-old C57BL/6 female mice obtained and maintained as described above. After a preliminary acclimatization period of 2 weeks, mice (6 weeks of age) were sensitized by a total of 3 intraperitoneal (i.p.) injections (administered once every other week) of 200 μL of phosphate-buffered saline (PBS) + PBS, PBS (100 μL) + CpG-B (100 μg), iSN34 (20 μg) + PBS, or iSN34 (20 μg) + CpG-B (100 μg). For this study, MsST was used as the CpG-B. MsST is a strong immunostimulatory CpG ODN that is derived from the *lacZ* gene of *Streptococcus thermophilus* ATCC19258 and has an ability similar to that of the murine prototype

CpG ODN (ODN 1555) to induce inflammatory cytokine production and cell proliferation [31]. Mice were subjected to euthanasia and necropsy at 1 week after the final i.p injection (i.e., at 11 weeks of age). At sacrifice, we measured body weight, spleen weight, and spleen length, and collected blood for further analysis.

2.2.9. Statistical analysis

All statistical analyses were performed using a statistical software package (Prism 7, GraphPad, Inc., La Jolla, CA, USA). Two-tailed One-way ANOVA with a post-hoc Tukey-Kramer test was used to determine the significance of the differences in all experiments except for the body weight trends. Body weight gain changes analyzed by Two-way ANOVA with post-hoc Bonferroni to compare treatment and time effects. Differences were considered significant at $p< 0.05$. Values for the *in vivo* experiment (spleen weight and spleen length) are presented using box and whisker plots. Other *in vitro* values are presented as means + SDs of three independent experiments (n=9).

Table 1. ODN sequences

Name	5'-sequence-3'	Reference
Ctr (1612)	G*C*T*A*G*A*G*C*T*T*A*G*G*C*T	Klaschik et al.,2007
CpG-A (1585)	G*GGGTCAACGTTGAG*G*G*G*G*G	Ballas et al.,2001
CpG-B (1555)	G*C*T*A*G*A*C*G*T*T*A*G*C*G*T	Sato et al.,2010
CpG-B (MsST)	C*A*G*G*A*C*G*T*T*G*T*A*T*C*A*C*T*A*A	Shimosato et al.,2009
CpG-C (2395)	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G	Marshall et al., 2005
iSN34	T*T*C*C*T*A*A*G*C*T*T*G*A*G*G*C*C*T	Nigar et al., 2017

*Phosphorothioate bond

2.3. Results

2.3.1. Immune synergistic activity of LGG ODN and expression of IL-6 mRNA by murine immune cells

In this study, I analyzed the synergistic effects of ODNs that had been designed based on LGG DNA sequences. Candidates that were positive for synergistic effects in a preliminary screen were subjected to confirmation of synergistic activity by assessing the stimulation of *IL-6* mRNA expression in splenocytes (Fig. 3). To determine the effect of iSN34 on the expression and secretion of cytokines, I assessed *IL-6* transcription in mouse splenocytes exposed to the combination of iSN34 + CpG-B. To determine the optimal concentration of iSN34 for this assay, I examined *IL-6* mRNA accumulation in the presence of iSN34 at concentrations ranging from 0.01 to 10 μM. As shown in Fig. 2a, iSN34 (in the presence of CpG-B) exhibited dose-dependent stimulation of *IL-6* expression at iSN34 concentrations of 0.01 to 2.5 μM and the highest concentration of iSN34 determined at 0.63 μM. Next, I compared the activity of iSN34 (0.63 μM) when combined with CpG-A (ODN 1585), CpG-B (ODN 1555), CpG-C (ODN 2395), or negative control (ODN 1612) ODN. As shown in Fig. 2b, iSN34 exhibited synergy in the induction of *IL-6* mRNA expression when combined with A-type, B-type, or C-type CpG ODNs, while the combination of iSN34 and C-type CpG ODN did not yield significant induction of *IL-6* mRNA expression. These results suggested that synergy was strongest for the combination of iSN34 and CpG-B (ODN 1555). To further explore the activity of iSN34, we also examined the molecule's effect on murine splenocytes in a wash-out assay. The culture was exposed to 3 μM iSN34 + CpG-B or control (water) for 24 h to monitor the immune synergistic activity after iSN34 removal. Interestingly, as shown in Fig. 2c, immune synergistic activity was maintained in splenocytes after

iSN34 was removed from the culture, such that *IL-6* mRNA levels were effectively unchanged after wash-out of the ODNs.

2.3.2. Induction of IL-6 production by exposure to low-concentration iSN34

I next investigated the secretion of IL-6 by murine splenocytes grown for 48 h in the presence of different concentrations of iSN34 (0.01 to 10 μM) + CpG-B; in this experiment, IL-6 levels in the spent medium were detected by ELISA. The results showed that the secretion of IL-6 was elevated in a dose-dependent fashion following iSN34 exposure, with peak IL-6 secretion detected in the presence of 0.63 μM iSN34 (Fig. 4). This result indicated that a low concentration of iSN34 can induce IL-6 production.

2.3.3. Enhancement of the IL-6^+ subpopulation in murine splenocytes

Next, I sought to investigate the effect of iSN34 exposure on a specific subpopulation of the splenocytes. Specifically, I used flow cytometry to classify the identity of IL-6^+ mouse splenocyte cells following stimulation of the CpG-induced immune reaction by the synergistic ODN. I observed that the population of $CD19^+$ IL-6^+ cells was increased following induction with iSN34 + CpG-B (Fig. 5a). As shown in Fig. 5a, the percentage of $CD19^+$ cells were significantly elevated in the cells treated with iSN34 + CpG-B. Additionally, this stimulation rendered the $CD19^+$ IL-6^+ subpopulation a significantly larger fraction of the total cell population (Fig. 5b). Thus, the synergistic effects of iSN34 + CpG-B reflected increased IL-6 expression in $CD19^+$ B-lymphocytes.

2.3.4. Effect of i.p. injection of iSN34 + CpG

I next extended our analysis of the synergistic effects of iSN34 + CpG by testing this treatment *in vivo* (Fig. 6a). All four groups of mice (n=4/group; injected i.p. with PBS + PBS, PBS + CpG-B, iSN34 + PBS, or iSN34 + CpG-B) exhibited typical weight gain during the study, and there was no significant difference among the 4 groups in terms of terminal body weight (Fig. 7). I did, however, note hypertrophy (splenomegaly) of the spleen in mice treated with iSN34 + CpG-B compared to the spleens of the other groups (Fig. 8); these effects were significant whether assessed as spleen weight (Fig. 6b) or length (Fig. 6c). My findings suggest that the combined use of iSN34 and CpG-B may find application in modifying the innate immune response.

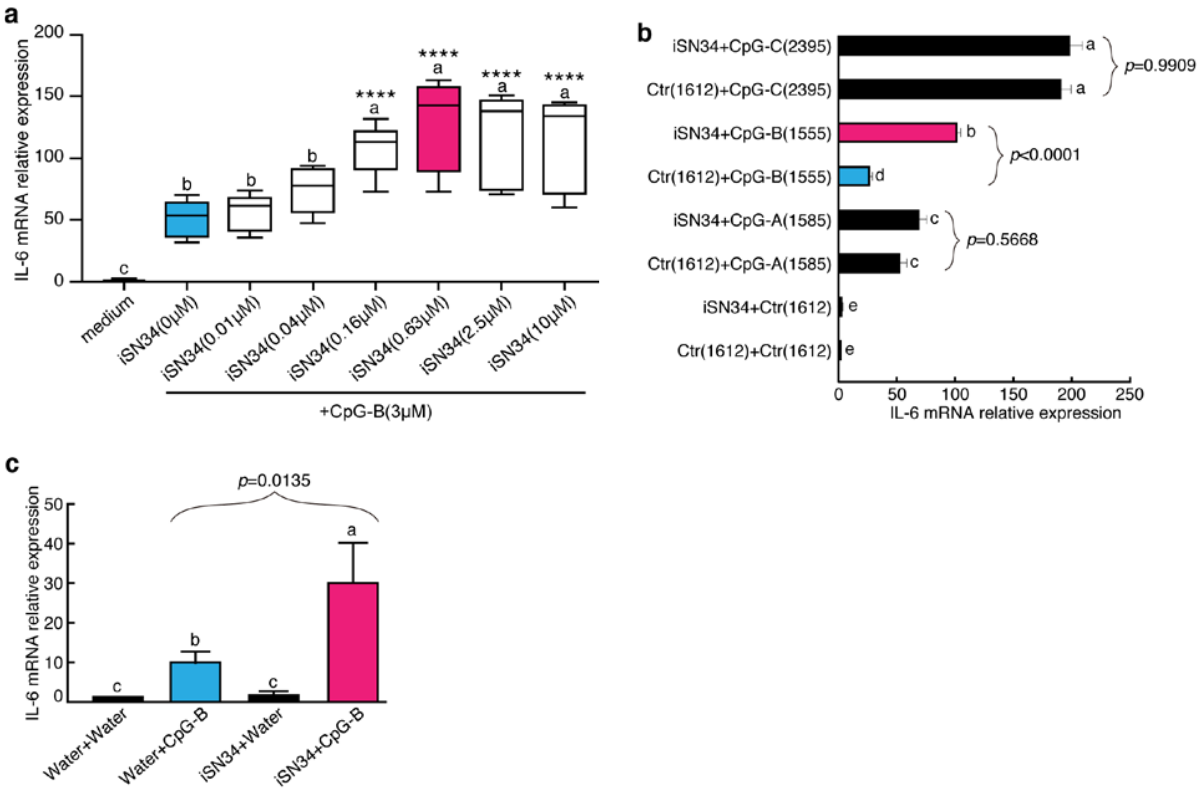

Figure 2. iSN34 was used to determine the optimal concentration of ODNs. a Mouse splenocytes were pre-incubated in medium for 3 h prior to exposure to iSN34 (at 0.01, 0.04, 0.16, 0.63, 2.5, or 10 μM) + CpG-B (ODN1555; at equimolar levels) or to ODN 1612 (control) for 6 h. Accumulation of *IL-6* mRNA was determined by qPCR. Results are shown as the ratio of *IL-6* mRNA levels for stimulated (iSN + CpG-B) versus ODN 1612-treated cells. b The synergistic effects of iSN34 (0.63 μM) were assessed in combination with CpG-A (ODN 1585), CpG-B (ODN 1555), CpG-C (ODN 2395), and Ctr 1612 (Control ODN). c Mouse splenocytes (1×10^7 cells/mL) were pre-incubated in medium for 3 h prior to exposure to 3 μM ODN 1612 or iSN34 for 24 h. Cells then were washed with medium (to remove the ODNs) and resuspended in medium with 3 μM CpG-B (ODN 1555) for 6 h. Results are shown as *IL-6* mRNA expression (normalized to β-actin-encoding mRNA; see qPCR method) in stimulated cells in the wash-out assay. All assays were carried out at least three independent times in triplicate. Similar results were obtained from at least three different mice. Values are presented as mean + SD of three independent experiments, each performed in triplicate (n=9). Values with different letters (*i.e.,* a, b, c, d, and e) were significantly different. ****p<0.0001 *vs.* iSN34 (0 μM). This figure is taken from BMC Immunology.

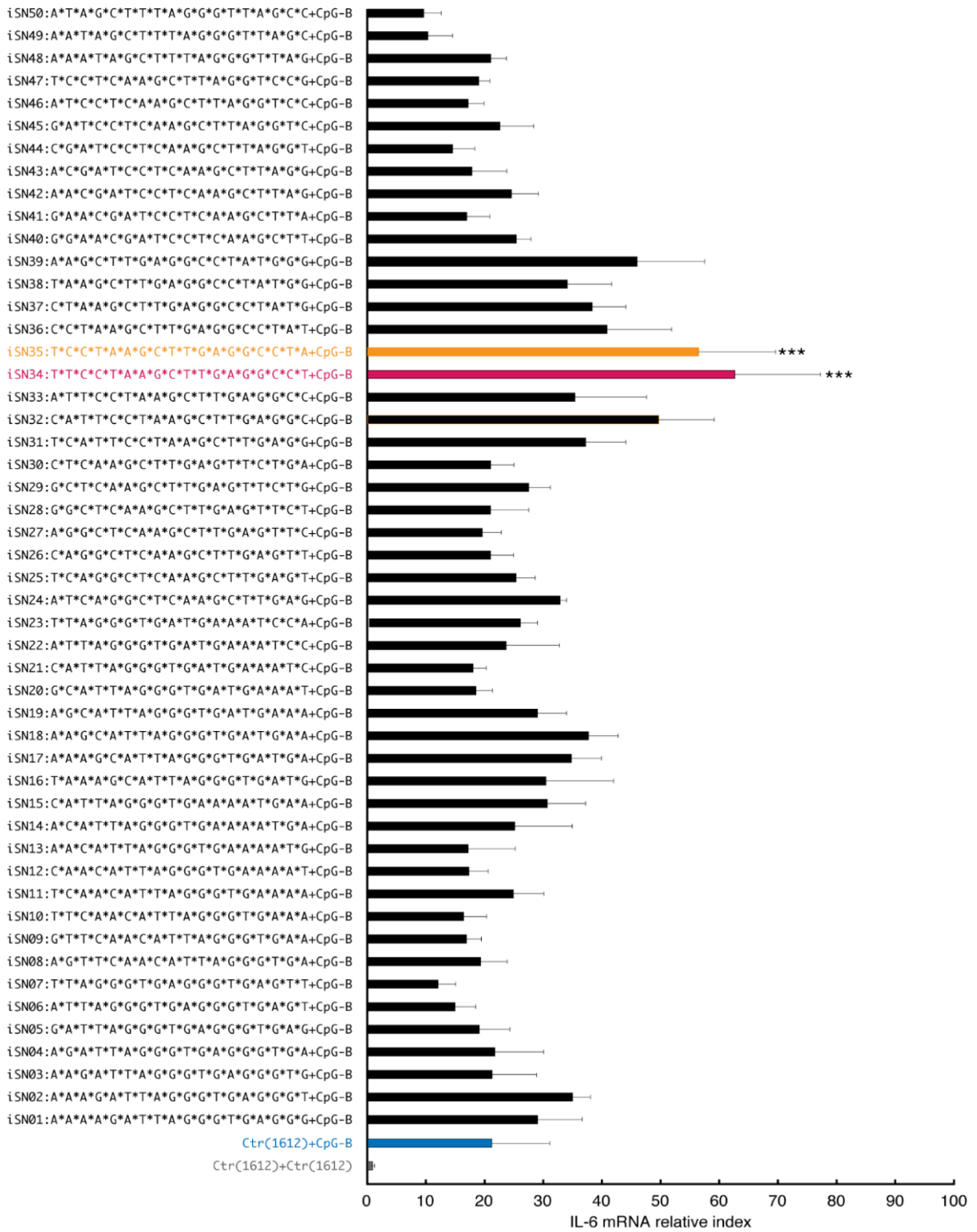

Figure 3. Analysis of *IL-6* mRNA expression in mouse splenocytes, as assessed by qPCR. Mouse splenocytes (2×10^6 cells/mL) were pre-incubated in medium for 3 h prior to exposure to 3 µM iSN candidates (No. 1-50), to CpG-B (ODN 1555), or to ODN 1612 (control) for 6 h. The results are presented as the mean + SD of at least three independent experiments, each performed in triplicate. **** $p<0.0001$ *vs.* [Ctr (1612) + CpG-B] (blue). Red: [iSN34 + CpG-B], Orange: [iSN35 + CpG-B]. This figure is taken from BMC Immunology.

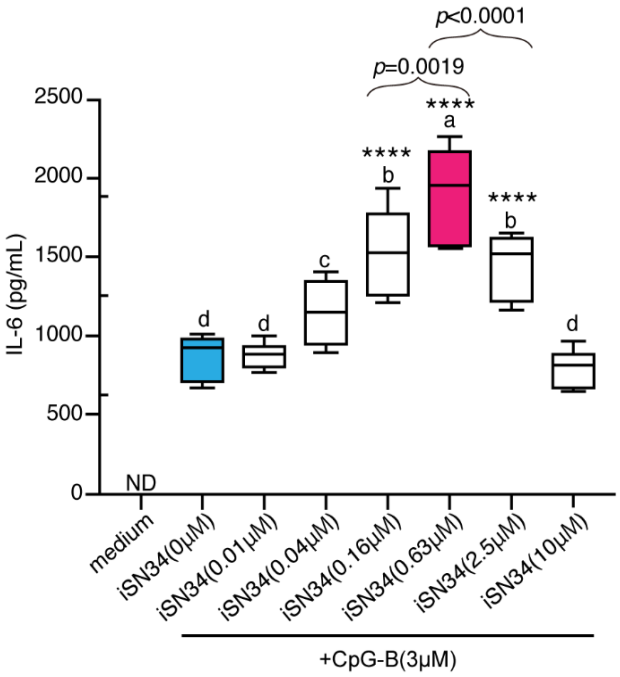

Figure 4. iSN34 with CpG-B enhances IL-6 production. Supernatants from stimulated cells were collected and IL-6 protein levels were measured by ELISA. Mouse splenocytes were harvested 48 h later and intracellular IL-6 protein levels were determined by ELISA. All assays were carried out at least three independent times in triplicate. Similar results were obtained from at least three different mice. Values are presented as mean + SD of three independent experiments, each performed in triplicate (n=9). Values with different letters (*i.e.*, a, b, c, d, e, and f) were significantly different. ****p<0.0001 *vs.* iSN34(0 μM). ND; not detectable. This figure is taken from BMC Immunology.

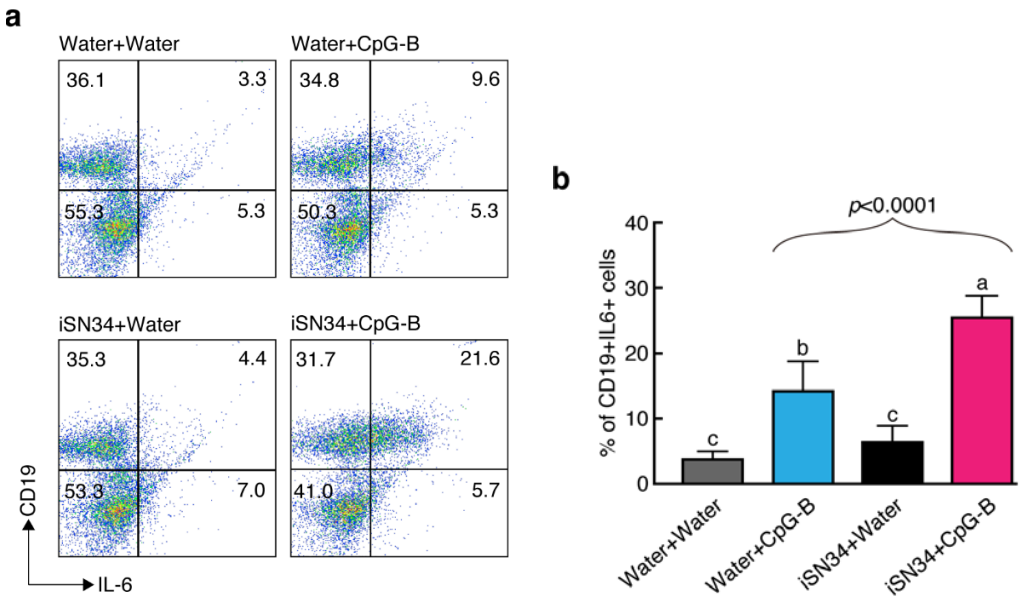

Figure 5. Representative flow cytometry plots. Dot plot of forward-angle versus right-angle light-scattering properties; the oval marks indicate the electronic windows used for analysis of fluorescence data for lymphocyte precursors; the rectangular boxes indicate the electronic windows used for analysis of fluorescence data for monocyte precursors with percentages. a A quadrant has been set to delineate the CD19 and IL-6 cells. Murine splenocytes were stimulated with water + water, water + CpG-B, iSN34 + water, or iSN34 + CpG-B for 12 h, and then sorted into $CD19^+$ $IL\text{-}6^+$ cells. b Mean percentage of $IL\text{-}6^+$ $CD19^+$ cells in the total population was determined in each group. Similar results were obtained from at least three different mice. Values are presented as mean + SD of three independent experiments, each performed in triplicate (n=9). Values with different letters (*i.e.*, a, b, c, and d) were significantly different (p<0.01). This figure is taken from BMC Immunology.

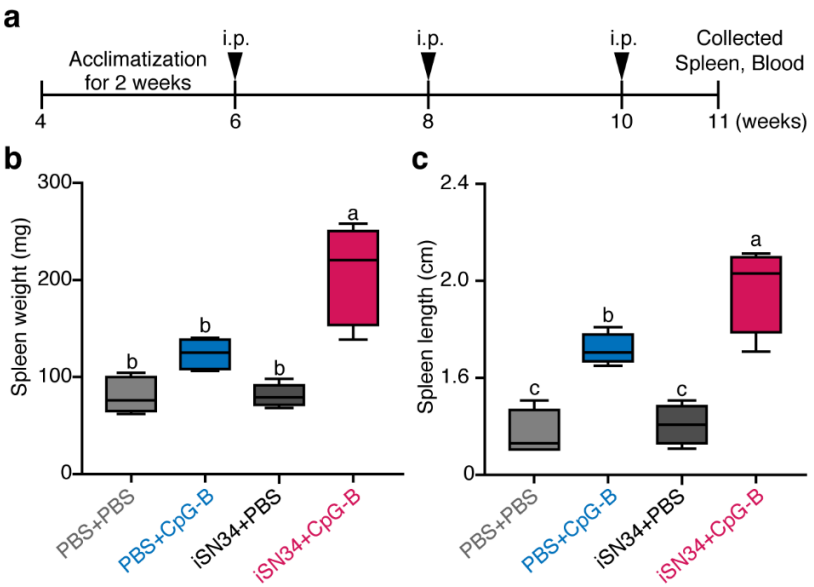

Figure 6. Cellular changes in spleens of mice following the *in vivo* trial. a Schematic design of the *in vivo* experiment performed in female C57BL/6 mice. Six-week-old mice were sensitized by a total of three i.p. injections (administered once every other week) with 200 µL of PBS + PBS, PBS (100 µL) + CpG-B (100 µg), iSN34 (20 µg) + PBS, or iSN34 (20 µg) + CpG-B (100 µg). Mice were euthanized at 11 weeks of age. b Spleen weight (mg). c Spleen length (cm). Results in panels b and c are presented using box and whisker plots. The central bar in each box indicates the median, with boxes extending to the 25th and 75th percentile values; whiskers extend to the minimum and maximum values. Values with different letters (*i.e.*, a, b, c, and d) were significantly different ($p<0.01$). This figure is taken from BMC Immunology.

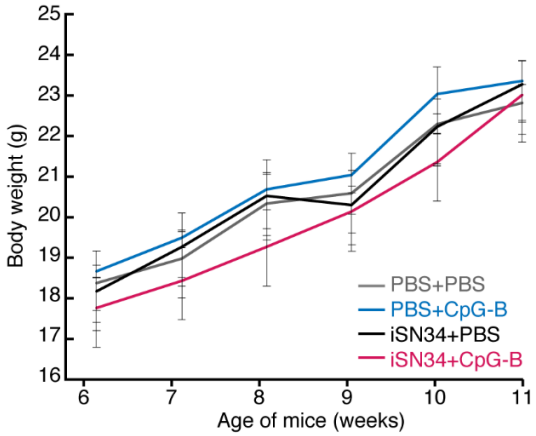

Figure 7. Body weight trends in female C57BL/6 mice (from 6-11 weeks of age) during treatment with iSN34 + CpG-B or control regimens. Body weights were measured once weekly. Data presented are the average weight per group. Data are presented as mean ± SE. This figure is taken from BMC Immunology.

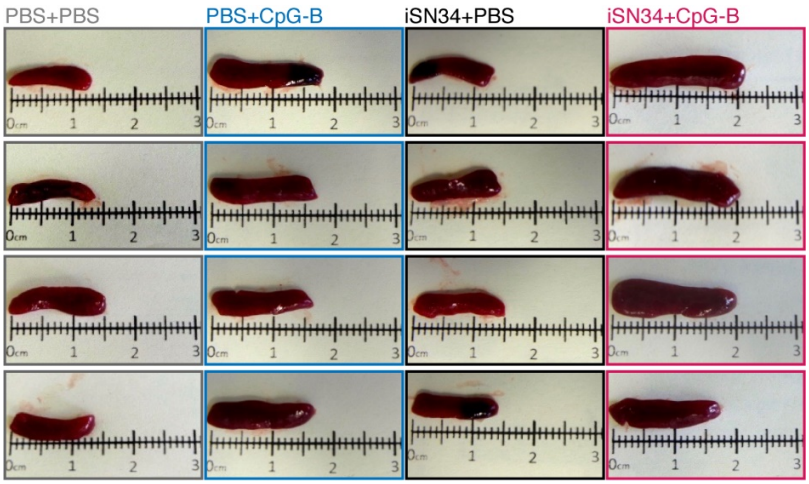

Figure 8. Changes in spleen size in mice after administration of iSN34 + CpG-B or control regimens. The picture shows representative spleens from one animal of each of the four groups: PBS + PBS, PBS + CpG-B, iSN34 + PBS, and iSN34 + CpG-B. This figure is taken from BMC Immunology.

2.4. Discussion

This study exposed that iSN34 shows immune synergistic effects after combined with CpG-ODN. The activity of iSN34 was related with multiple types of CpG-ODNs. This hypothesize showed the synergistic activity of iSN34 + CpG at the level of *IL-6* mRNA accumulation, IL-6 protein secretion, and IL-6^{+} cell production following *in vitro* exposure of cultured splenocytes. Above mentioned tested ODNs, only iSN34 exhibited these effects, which were observed only when combined with CpG ODN. In previous work has shown that CpG ODN is a potent stimulator of dendritic cells and macrophages which causes the induction of Th-1-associated cytokines such as IL-6 and IL-12 (Wanger et al., 2004). In the current work, iSN34 was able to combine with CpG to stimulate the production of IL-6 which suggest that iSN34 would be a highly effective adjuvant for Th-1-mediated vaccines that defense against intracellular pathogens or antibody-mediated immunity and defense against extracellular pathogens.

Therefore, iSN34 might be useful for the prevention or treatment of diseases associated with inflammatory disorders including RA, inflammatory bowel disease (IBD), multiple sclerosis, systematic-onset juvenile chronic arthritis (JCA), osteoporosis, and psoriasis (Kopt et al., 1994; Yamamoto et al., 2000; Atreya et al., 1995; Ulevitch et al., 1995). However, different research showed that IL-6 acts as a potent stimulator of B-cell proliferation (Collins et al., 2015), plasma cell survival (Tosato et al., 1988), and antibody production (Minges et al., 2002). Thus, these outcomes indicated that the combination of iSN34 and CpG strongly induces the expression of IL-6. It is anticipated that IL-6 overproduction effects that might be at odds with the prevention or treatment of some diseases. Further work will be needed to address the combination of these distinct effects.

Next observed that iSN34 exhibited greater synergy when combined with CpG-B (Fig. 2b) in compare to CpG-A and CpG-C. These results specified that CpG-B is the appropriate synergistic inducer for use with iSN34. Where CpG ODN is classified based on their action and different response. Type A CpG have maintains of terminal G residues and a palindromic central CpG motifs resulting nanoparticle ODN structures passage within pDC into transferrin receptor-positive endosomes and give potent induction of IFN-α. On the other hand, the short single-stranded type B ODN traffic in pDCs to a lysosome-associated membrane protein-1-positive late endosomal compartment and lead to pDC maturation and inflammatory cytokine production but minimal IFN-α production (Honda et al., 2005; Guidcci et al., 2006; Honda et al., 2005). In contrast, Type C ODN combine features of both A and B ODN and nearly stimulate all responses. In my studies also exposed that co-administration of iSN34 and CpG ODN had synergistic effects in a wash-out assay. Particularly when demonstrated that splenocytes pretreated with iSN34 maintain the ability to stimulate the immune response even after the iSN34 is washed out, suggesting that the immune synergistic effect of iSN34 may be mediated via stimulation of cell signaling. Assessment of the immune synergistic effect of iSN34 and CpG-B by measuring IL-6 protein secretion (via ELISA) confirmed that 0.63 μM iSN34 yielded peak synergistic efficacy under our experimental conditions.

Further investigated CD19 B-lymphocytes cells that is one of the most reliable biomarkers for B cells. CD19 can receive positive stimuli from a variety of cytokines, including IFN, IL-4 and IL-6; such stimulation leads to proliferation, differentiation, cytokine production, and other effector functions (Troys et al., 2004). In present studies, B cells exposed to the combination of CpG and iSN34 exhibited significant up-regulation of CD19 expression compared to controls. To clarify whether specific subpopulations of cells were selectively activated by iSN34, further characterized

IL-6-producing splenocytes using flow cytometry. This flow cytometric analysis revealed that the proportion of IL-6-secreting $CD19^+$ B-cells in the spleen increased over two-fold in mice treated with iSN34 + CpG-B, compared to the levels seen in the control group (Fig. 5c). These findings could be relevant for the study of inflammation.

It also found that co-administration of iSN34 and CpG-B *in vivo* in healthy mice was adequate to cause expansion of this cell type, yielding increased spleen weight and length. Notably, this treatment did not result in significant changes in terminal body weight of iSN34 and CpG group compared to control groups and another iSN34+control and CpG+ control group, suggesting that iSN34 was not associated with gross toxicity (as might be implied by weight loss or attenuation of weight gain). I did, however, detect hypertrophy of the spleen in mice sensitized by i.p. injection with iSN34 + CpG-B. In previous work, it was demonstrated that CpG-B is involved in activating the innate immune response and induces splenomegaly (Yamamoto et al., 2016; Ballas et al., 2001). Indeed, more than 100 years splenomegaly has been considered an important complication of acute and chronic disease in inflammation research (Pozo et al., 2009; Delano et al., 2009; Osler et al., 1908).

Notably, iSN34 does not provide significant synergistic induction of inflammatory cytokines (TNF-α, IFN-γ) other than IL-6 (data not shown). Given this observation, it has been assessed that combined therapies incorporating iSN34 may significantly complement and further enhance the positive effects of mucosal immunity. In summary, our study demonstrated that iSN34 acts synergistically in combination with CpG ODN in the induction of IL-6, the multifunctional cytokine that was originally identified as a T-cell-derived factor and has subsequently been shown to induce the differentiation of activated B-cells into antibody-producing cells with biological

activities and involve the regulation of immune response, inflammation, and hematopoiesis (Kishimoto 2005). This synergy may represent a new modality for the treatment of systematic inflammatory disorders.

2.5. Conclusions

The new insights provided by this study suggest a novel concept of synergistic ODN activity distinct from that described in previous ODN studies. However, the implications of this synergy for clinical symptoms or disease risk are unknown. These facts suggest that this immune synergy represents a wide-open research field with good scientific prospects and could yield improvements in the prevention and treatment of immune disorders. However, further preclinical and clinical investigations will be needed to understand how specific targeting of the IL-6 pathway can be applied in disease treatment.

Chapter 3

Immune synergistic oligodeoxynucleotide from *Lactobacillus rhamnosus* GG enhances the immune response upon co-stimulation by bacterial and fungal cell wall components

3.1. Introduction

Bacterial DNA is a potent stimulator of the host immune response. The host TLR9 recognizes bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODNs) that harbor unmethylated CpG motifs which characterize of bacterial DNA (Alexopoulou et al., 2001; Heil et al., 2004; Takeshita et al., 2001; Kawai et al., 2010); and such recognition leads to abrogation of immunostimulatory activity (Hemmi et al., 2000). In retrospect, we described an immune-synergistic oligodeoxynucleotide (iSN34), derived from LGG genomic sequences that exhibited a synergistic effect on the immune response to CpG-induced immune activation (Nigar et al., 2017). Among the nucleic acids, CpG-ODNs are responsible for the immune stimulatory effect of bacterial DNA (Krieg et al., 2002). In 1995 Dr. Arthur Krieg identified the unmethylated, dinucleotide CpG motifs present in bacterial DNA that responsible for immune activation and these single-stranded CpG oligodeoxynucleotides act as synthetic immunoadjuvants. These synthetic immunoadjuvants, known as nucleic acid-based Toll-like receptor (TLR) ligands are promising immunotherapeutic agents that play a critical role in the innate immune response by facilitating the recognition of pathological antigens. TLR ligands involves in innate immune system which is an evolutionarily conserved host defense mechanism against pathogens initiating immune responses based on the activation of pattern recognition receptors (PRRs) that are expressed by cells of the immune system. PRRs are evolutionarily conserved germline-encoded

receptors that recognize pathogen-associated molecular patterns (PAMPs) from a range of microorganisms; various microbial motifs serve as distinct ligands (Kawai et al., 2009; Mogensen et al., 2009). Upon recognition of molecular motifs specific for microbial molecules, TLRs mediate the secretion of pro-inflammatory cytokines (Bekerdjian-ding et al., 2009) that work together to orchestrate the early host response to infection. The secretion of these cytokines is a prerequisite for the subsequent activation and shaping of adaptive immunity. It is important to note that PAMP of innate immune recognition of pathogens differ markedly from recognition mechanisms associated with the adaptive immune system. Upon recognition of foreign antigen, particularly in the presence of PAMPs, DCs help to initiate an adaptive immune response by B cells which express antigen-specific T cell receptors. Toll-like receptors (TLRs) are an important family of pattern-recognition receptors with at least 11 members (Kumagai et al., 2008; Takeda et al. 2003) that are key molecules for microbial elimination, such as the recruitment of phagocytes to infected tissues and subsequent microbial killing. TLR family members are sub-divided to cell membrane-associated and endosome-associated receptors. Endosomal TLRs are specialized to sense nucleic acids. While TLR3 and TLR7/8 recognizes double and single-stranded RNA, TLR9 recognizes bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs (CpG ODNs hereafter) (Alexopoulou et al., 2001; Heil et al., 2004; Takeshita et al., 2001; Kawai et al., 2010). Multiple TLR triggering could synergistically activate immune response upon multiple agonists engagement (Tan et al., 2014). In the present report, it demonstrates that iSN34 exhibits an immune-synergistic effect on cytokine secretion upon co-stimulation of other TLR families.

Cell membrane-associated TLRs are displayed on the cell surface. This class of TLRs recognize a variety of molecular pathogenic patterns, including microbial (bacterial or fungal) components

such as lipids, lipoproteins, and flagellar proteins. Examples of natural agonists include Gram-positive bacterial lipoprotein, which induces signaling via TLR2 and other receptors (Kaisho et al., 2001); lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component that induces signaling via TLR4 (Akashi et al., 2000); flagellin, a bacterial protein that acts through TLR5; and Zymosan, an extract from the cell wall of the yeast *Saccharomyces cerevisiae* that is recognized by the heterodimeric TLR2/6 receptor. Surface receptors can also recognize artificial ligands; one such agonist is Pam_3CSK_4, a synthetic triacylated lipoprotein that has been shown to activate signaling through the heterodimeric TLR1/2 receptor (Patel et al., 2005). Notably, the agonists of surface TLRs (e.g., Zymosan) can serve as potent immuno-stimulants capable of activating adaptive immune responses (Ganter et al., 2003).

There is now overwhelming evidence for the effects of TLR stimulation on B cells. This work typically has employed LPS (to stimulate TLR4) or CpG-ODN (to stimulate TLR9), since these receptors are the most abundant TLRs on the surface of naive B cells (Buchta et al., 2014; Bekerdjian-Ding et al., 2009). However, B cells also express other TLRs, albeit at lower levels, that may have different effects on B cell differentiation. Indeed, combinations of two of more TLR ligands have been reported to act synergistically to enhance the activation of a variety of innate immune cells *in vitro* (Matthews et al., 2012; Napolitani et al., 2005) and to boost responses to vaccination (Wells et al., 2008). There is limited information on the effect of stimulating B cells with multiple TLR ligands *in vitro* and the use of TLR agonist combinations to enhance antibody responses *in vivo*. TLR3 and TLR1/2 are expressed at low levels on naive B cells, yet B cells respond to stimulation to their respective ligands, poly I:C and Pam_3CSK_4 *in vitro* (Barr et al., 2007; Gururanjan et al., 2007). Stimulation of the TLR1/2, TLR4, and TLR2/6 signaling pathways

results in activation of a series of signaling proteins leading to expression of genes encoding pro-inflammatory cytokines and chemokines (Akira et al., 2001).

In the present work, I investigated the immune-synergistic activity of an ODN (named iSN34) when combined with stimulation of the cell membrane-associated TLRs (TLR1/2, 4, 6) displayed on mouse splenocytes. I demonstrate here that IL-6 is over-expressed in response to the treatment of mouse splenocytes with the combination of iSN34 and bacterial or fungal cell wall components. These novel results propose a new modality for the treatment of systematic inflammation and immuno-therapy and the demonstration expands the immune-synergistic potential of iSN34 to include effects on extracellular TLR-mediated activity. Thus, iSN34 could facilitate the release of other signal mediators (e.g. those released when an organism is infected, or sustains trauma and tissue injury, thereby producing an acute phase inflammatory response). These outcomes indicate that it may be possible to develop drugs that treat inflammatory diseases by targeting upstream processes in innate immune cells.

3.2. Materials and methods

3.2.1. Ethics statement

All experimental procedures were carried out in accordance with the regulations for Animal Experimentation of Shinshu University, and the animal protocol approved by the Committee for Animal Experiments of Shinshu University. Consistent with national regulations and guidelines, and in keeping with Law No. 105 and Notification No. 6, all experimental procedures were reviewed by the Committee for Animal Experiments and approved by the president of Shinshu University as protocol No. 230070.

3.2.2. Mice

Pathogen-free female C57BL/6 mice (4 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and maintained under temperature- and light-controlled conditions. Mice were provided with *ad libitum* access to a standard diet of Labo MR Breeder (Nihon Nosan Co., Kanagawa, Japan) and sterile water. Mice were 6 to 7 weeks of age at the start of the study.

3.2.3. Reagents

Endotoxin-free desalted phosphorothioated (PS) ODNs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA), or by Gene Design, Inc. (Osaka, Japan). Each PS-ODN was reconstituted in endotoxin-free water and passed through a 0.22 μm pore microfilter (Nihon Millipore K.K., Tokyo, Japan) prior to use. Mouse splenocytes were treated with iSN34, an ODN with the sequence 5′-TTCCTAAGCTTGAGGCCT-3′ that is derived from *Lactobacillus rhamnosus* GG (Nigar et al., 2017). Phosphate-buffered saline (PBS; pH 7.4) was used as a control.

3.2.4. TLR agonists

In this study, I tested four innate immune modulators: N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-(Lys)4 (abbreviated as Pam_3CSK_4), Gram-negative lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, Gram-negative flagellin from *Salmonella typhimurium*, and Zymosan from the cell wall of the yeast *Saccharomyces cerevisiae*; all modulators were purchased from InvivoGen (CA, USA).

3.2.5. Cells and cell culture

Mouse splenocytes were prepared using standard methods (Ito et al., 2013; Wang at al., 2015) and were then cultured in triplicate or quadruplicate wells of a 24-well plate (Nalge Nunc International K.K., Tokyo, Japan) at 2×10^6 cells/well in volumes of 1 mL/well of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, and 0.0035% 2-mercaptoethanol (2-ME). Mouse splenocytes were pre-incubated in medium for 3 h prior to exposure to the low-concentration iSN34 (0.63 μM) or to PBS (control), alone and in combination with Pam_3CSK_4 (1 μg/ml), LPS (20 ng/ml), or Zymosan (1 μg/ml) for 6 h (for use in qPCR analysis) or for 24 hours (for use in ELISA and flow cytometric analysis).

3.2.6. qPCR analysis

Real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analyses were performed with SYBR Premix Ex Taq (TaKaRa Bio) using specific primers, as previously described (Yamamoto et al., 2017). Primers for the β-actin- and IL-6-encoding genes were purchased from TaKaRa Bio. As a control, poly $(A)^+$ RNA samples were used as templates to check for the presence of contaminating genomic DNA. The sensitivity of the reaction and amplification of contaminating products, such as self-annealed primers, were evaluated by amplifying serial dilutions of cDNA. For cross-sample comparison of results obtained following various treatments, levels of cytokine-encoding mRNA were first normalized to those of β-actin-encoding mRNA. Data are shown as the mean + standard deviation (SD) of one experiment (consisting of 3-4 technical replicates) representative of three independent experiments that yielded similar results.

3.2.7. Cytokine quantification

I measured the concentration of the cytokine IL-6 in cell culture supernatants after 24 hours of various treatments. IL-6 levels were quantified using a commercially available ELISA kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions.

3.2.8. Intracellular staining

Splenocytes (2×10^6 cells/well) were pre-incubated for 3h in medium supplemented with either 0.625 μM iSN34 or an equivalent volume of PBS. Cells then were resuspended in medium containing 1 μg/ml of Pam_3CSK_4, 20 ng/ml of LPS, or 1 μg/ml of Zymosan; and incubated for 24 h at 37°C; following this 24h stimulation, the cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 μg/mL of brefeldin A, 2 μg/mL of ionomycin, and 20 ng/mL of phorbol 12-myristate 13-acetate; and cultured for 4 h at 37°C. For intracellular staining, cells were fixed in 4% Paraformaldehyde(PFA) for 15 minutes at room temperature, washed, and permeabilized by incubation for 15 minutes on ice; cells were then further incubated with phycoerythrin-labeled anti-mouse-IL-6 antibody (Biolegend). Splenocytes were first stained with anti-mouse-IL-6 antibody for 60 minutes on ice. Cells were washed and the percentages of $CD19^+$ $IL\text{-}6^+$ cells were determined using a FACS Calibur (BD Biosciences). Data were acquired and analyzed using Flow Jo software (Ver. 10). All analyses were carried out at least in triplicate; representative results are presented.

3.2.9. Statistical analysis

All statistical analyses were performed using a statistical software package (Prism 7, GraphPad, Inc., La Jolla, CA, USA). Two-tailed One-way ANOVA with a post-hoc Tukey-Kramer test was used to determine the significance of the differences in all experiments. Differences were considered significant at $p < 0.05$. Other *in vitro* values are presented as means + SDs of three independent experiments (n=3).

3.3. Results

3.3.1. iSN34 co-stimulates TLR agonist-induced proinflammatory cytokine expression in splenocytes

Splenocytes (2×10^6 cells/well) were cultured with iSN34 and stimulated with various TLR agonists (Pam_3CSK_4, LPS, or Zymosan). These cells were harvested at 6 hours after stimulation with TLR agonists, and the *IL-6* mRNA levels in these cells measured by real-time RT-qPCR. I observed that stimulation with iSN34 in combination with Pam_3CSK_4 (TLR1/2 agonist), LPS (TLR4 agonist), or Zymosan (TLR2/6 agonist) strongly enhanced the expression of *IL-6* mRNA in compared to the expression obtained upon stimulation with PBS alone or in combination with the respective individual reagents (PBS + PBS, iSN34+ PBS, PBS+ Pam_3CSK_4, PBS+ LPS, and PBS + Zymosan) (Fig. 9A, B, and C). In accordance with the lack of TLR5 expression on these splenocytes, [Was this lack of TLR5 shown in the present work (how?) or should this point cite a previous paper?]1 μg/ml flagellin (a TLR5 agonist) did not significantly alter *IL-6* mRNA expression in iSN34-pretreated splenocytes (n=3; data not shown). These results suggested that synergy was generated not only for the combination of iSN34 and Pam_3CSK_4 (a synthetic bacterial-like molecule) but also for that of iSN34 with LPS (bacterial cell wall component) and iSN34 with Zymosan (fungal component).

3.3.2. IL-6 production is synergistically enhanced by iSN34 in combination with TLR1/2, TLR4, or TLR2/6 ligands

After assessing effects on *IL-6* mRNA expression in mouse splenocytes, I next investigated potential effects at the IL-6 protein level. I measured the secretion of IL-6 by mouse splenocytes grown for 24 hours in the presence of iSN34 + Pam_3CSK_4 (1 μg/ml), iSN34 + LPS (20 ng/ml), or

iSN34 +Zymosan (1 μg/ml). In this experiment, IL-6 levels in the culture supernatants were detected by ELISA. Each of the tested combinations (iSN34 and Pam_3CSK_4, iSN34 and LPS, and iSN34 and Zymosan) yielded significantly enhanced production of IL-6 compared to the levels observed in cultures grown in the absence of agonists or with single agonists (Fig. 10).

3.3.3. Stimulation with iSN34 in combination with TLR1/TLR2, TLR4, or TLR2/6 agonists enhances the generation of IL-6^+ cells from $CD19^+$ B cells

Next, I sought to investigate the effect of iSN34 exposure on a defined subpopulation of the splenocytes. Specifically, I used flow cytometry to classify the identity of IL-6^+ mouse splenocyte cells following stimulation of the TLR-mediated immune reactions with and without the synergistic ODN. Dot plots of FACS results for cells exposed to PBS + PBS, iSN34 + PBS, PBS + Pam_3CSK_4, iSN34 + Pam_3CSK_4, PBS+ LPS, iSN34 + LPS, PBS + Zymosan, or iSN34 + Zymosan are presented in Fig. 11A. I observed that the population of $CD19^+$ IL-6^+ cells increased following exposure to the combination of iSN34 and Pam_3CSK_4 (Fig. 11B). As shown in Fig. 11B, the percentage of $CD19^+$ cells were significantly elevated in the cells treated with iSN34 and Pam_3CSK_4 compared to the $CD19^+$ population in the cells treated with PBS + Pam_3CSK_4, iSN34 + PBS, or PBS + PBS. I hypothesized that the combination of iSN34 and Pam_3CSK_4 activates IL-6 production by B cells. Interestingly, I obtained similar results with iSN34 + LPS (a TLR4 bacterial ligand) and iSN34 + Zymosan (a TLR2/6 fungal ligand). Thus, our FACS analysis confirmed the above observations, demonstrating that iSN34 synergistically stimulates the activation of immune cells by both bacterial components and fungal components.

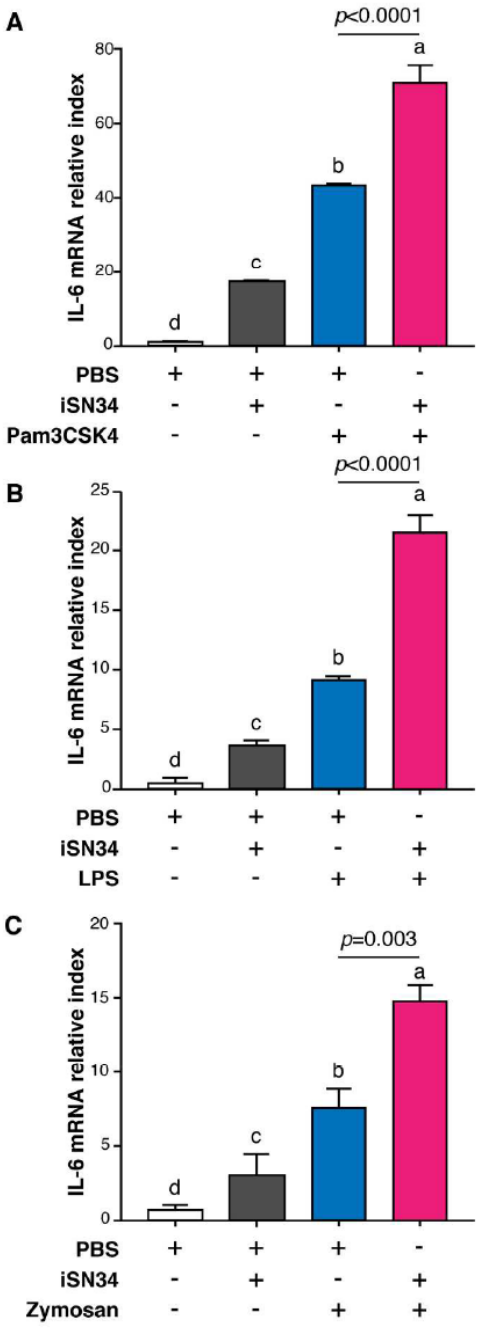

Figure 9. Analysis of *IL-6* mRNA expression in mouse splenocytes. Mouse splenocytes were pre-incubated in medium for 3 h prior to exposure to iSN34 (0.63 μM) or to PBS (control), alone and in combination with Pam_3CSK_4 (1 μg/ml), LPS (20 ng/ml) or Zymosan (1 μg/ml), for 6 h. The accumulation of *IL-6* mRNA was determined by qPCR. Results are shown as the values of *IL-6* mRNA transcript levels relative to expression with PBS alone. Values are presented as mean + SD of three independent experiments (i.e., using the splenocytes from at least three different mice), each performed in triplicate (n=9). Values with different letters (*i.e.,* a, b, c, and d) were significantly different. This figure is taken from Animal Science Journal.

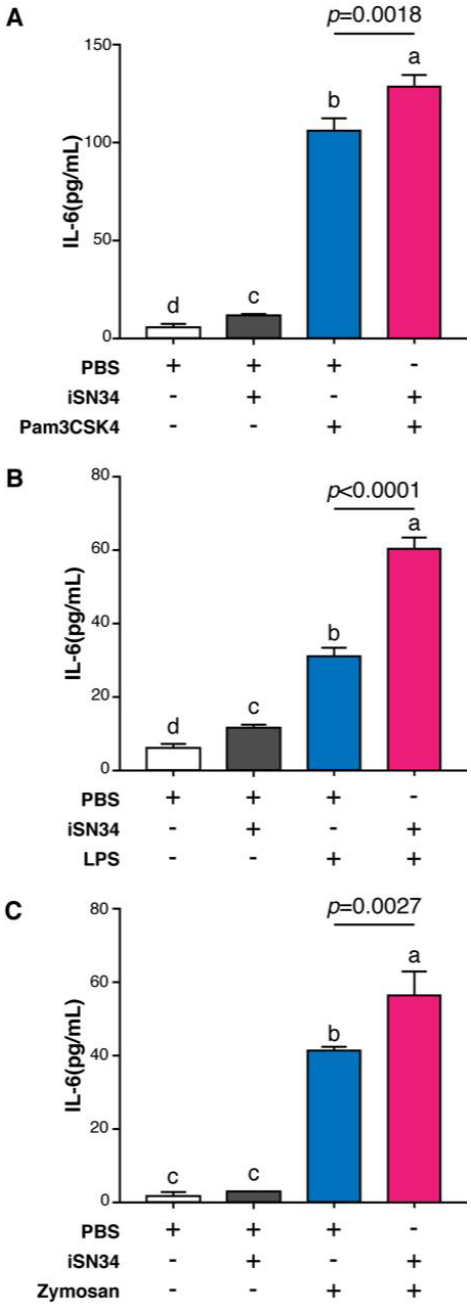

Figure 10. The combination of iSN34 with TLR ligands (Pam_3CSK_4, LPS, or Zymosan) enhances IL-6 production. Supernatants from stimulated cells were collected and IL-6 protein levels were measured by ELISA. Murine splenocytes were stimulated with PBS + PBS, iSN34 + PBS, PBS + Pam_3CSK_4, iSN34 + Pam_3CSK_4, PBS + LPS, iSN3 + LPS, PBS + Zymosan, or iSN34 + Zymosan for 24 h. (A) The synergistic effect of iSN34 was assessed in combination with Pam_3CSK_4. (B) The synergistic effect of iSN34 was assessed in combination with LPS. (C) The synergistic effect of iSN34 was assessed in combination with Zymosan. All assays were carried out at least three independent times, each time in triplicate. Similar results were obtained with the splenocytes from at least three different mice. Values are presented as mean + SD of three independent experiments, each performed in triplicate (n=3). Values with different letters (*i.e.*, a, b, c, and d) were significantly different. This figure is taken from Animal Science Journal.

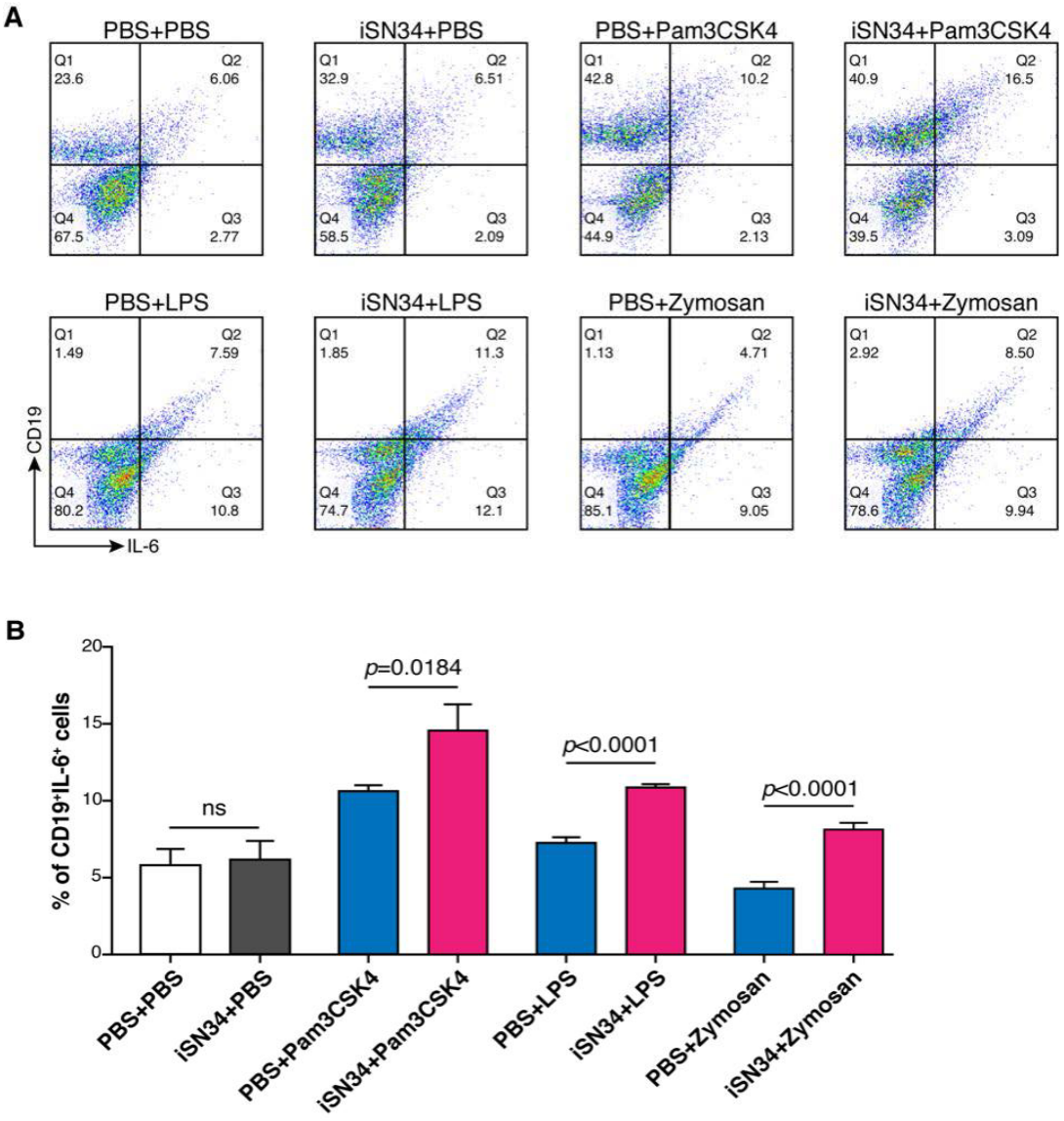

Figure 11. (A) Representative flow cytometry plots. Dot plot of forward-angle versus right-angle light-scattering properties; the oval marks indicate the electronic windows used for analysis of fluorescence data for lymphocyte precursors; the rectangular boxes indicate the electronic windows used for analysis of fluorescence data for monocyte precursors with percentages. A quadrant has been set to delineate the CD19 and IL-6 cells. (A, B) Murine splenocytes were stimulated with PBS + PBS, iSN34 + PBS, PBS + Pam_3CSK_4, iSN34 + Pam_3CSK_4, PBS + LPS, iSN34 + LPS, PBS + Zymosan, and iSN34 + Zymosan for 24 h, and then sorted to determine the number of $CD19^+$ $IL\text{-}6^+$ cells as percentages of the total population in each group. (B) Data (mean percentages of $CD19^+$ $IL\text{-}6^+$ cells in total population) for the groups. Similar results were obtained with the splenocytes from at least three different mice. Values are presented as mean + SD of three independent experiments, each performed in triplicate (n=9). Significant differences between the means of different groups were determined by a post-hoc Tukey-Kramer test. ns, not significant. This figure is taken from Animal Science Journal.

3.4. Discussion

These studies deliver the primary evidence, to my knowledge, that co-stimulation with iSN34 and TLR ligands such as TLR1/TLR2, TLR4, and TLR2/6 induces the production of an inflammatory cytokine. These up regulatory effects were observed with the TLR2 ligand Pam_3CSK_4, the TLR4 ligand LPS, and the TLR2/6 ligand Zymosan; each of the 3 exhibited synergistic induction of IL-6 expression at both the mRNA and protein level in mouse splenocytes. Further investigation confirmed that purified $CD19^+$ B cells are directly activated by Pam_3CSK_4, LPS, and Zymosan (ligands recognized by TLR1/2, TLR4, and TLR2/6, respectively) to produce IL-6. CD19 is specifically expressed in normal and neoplastic lymphoid cells as a type I transmembrane protein, with a single transmembrane domain and a biomarker for normal and neoplastic B cells as well as follicular dendritic cells. CD19 is critically involved in establishing intrinsic B cell signaling thresholds through modulating both B cell receptor (BCR)-dependent and independent signaling (Fujimoto et al., 1998; Poe et al., 2012). It takes part of antigen-independent development as well as the immunoglobulin-induced activation of B cells and other surface molecules to allow both direct and indirect recruitment and binding of various down-stream protein kinases (Zhou et al., 1992; Van Zelm et al., 2006). Therefore, the present work demonstrated B cell activation *in vitro* by measuring changes in surface receptor expression (after 24 hours), cytokine production (after 6 hours), and protein (after 24 hours).

TLR agonists have been proposed broadly as candidate vaccine adjuvants because of the ability to enhance initiation of immune responses to antigens by activating DCs. Though, TLRs are also expressed on B cells and may contribute to effective B cell activation, promoting differentiation into antigen-specific, antibody-producing plasma cells *in vivo* (Genevieve et al., 2017). In recent research established that each agonist enhanced B cell activation through increased expression of

surface receptors and cytokine secretion. When B cells were stimulated with the combination of iSN34 + Pam_3CSK_4, iSN34 + LPS, or iSN34 + Zymosan, synergistic enhancement of cell activation (compared to that with each agonist alone) was observed.

Zymosan stimulates the signaling cascade by binding to TLR2 (Akira et al., 2006), a PRR that is expressed in many cell types, though primarily in innate immune cells such as dendritic cells (DCs) and macrophages (Mills et al., 2011). The activation of TLR2 leads to secretion of inflammatory cytokines, including TNF-a, IL-6, IL-10, IL-12, and IL-23 (Volman et al., 2005; Veldheon et al., 2006). Recent demonstration of the significant induction of IL-6 secretion by stimulated splenocytes is dependable with the previous literature. IL-6 displays pleiotropic effects, including effects on the maturation and activation of macrophages, osteoclasts, chondrocytes and endothelial cells. Other research laboratory has shown the crucial role of IL-6 in the pathogenesis of many chronic inflammatory and autoimmune diseases, including rheumatoid arthritis (Yue et al., 2010; Ishihara et al., 2002), multiple sclerosis (Vladic et al., 2002), diabetes (Wedrychowicz et al., 2004), and asthma (Wong et al., 2001). Additionally, IL-6 appears to play a dominant role in chronic inflammation and tumor growth (Gasperini et al., 1999). Above mentioned results show that Zymosan enhanced production of IL-6 treated with iSN34 on splenocytes, in agreement with Gasperini et al. (1999). Moreover, TLR agonists are commonly used as vaccine adjuvants to boost antibody production with corporation the contribution of direct stimulation of B cell TLRs to the development of an *in vivo* antibody response is not well understood (Bekerdjian-ding et al., 2009). This study clearly demonstrated that these agonists resulted in a characteristic activation profile of B cells, suggesting non-redundancy in signaling pathways. Notably, CD19 has been the target of the clinical development of therapeutic monoclonal antibodies targeting lymphoma, leukemia, and autoimmune disorders (Wang et al., 2012). Since these receptors are strongly expressed on murine

B cells (Buchta et al., 2014), nearly all studies of intrinsic TLR activation in B cells have focused on TLR4 and TLR9. Based on our investigation, the combination of iSN34 and Pam_3CSK_4 activated murine B cells, inducing IL-6 production by purified $CD19^+$ cells. Parallel activation of murine $CD19^+$ B cells from splenocytes was detected with iSN34 + LPS and iSN34 + Zymosan, consistent with the presence on these cells of TLR4 and TLR2/6. Taken together, these TLR agonists induced the potentiality of TLRs as regulators of B cell activation, especially in the context of $CD19^+$ cells. CD19 is known to be specifically expressed in normal and neoplastic B cells as well as in follicular DCs (Tedder et al., 2009).CD19 has been shown to play a role in antigen-independent development as well as in immunoglobulin-induced activation of B cells; the surface density of CD19 is highly regulated throughout B cell development and maturation, until the expression of this marker is lost during terminal plasma cell differentiation (Haas et al., 2005; Bradbury et al., 1992). The present study largely confirmed the existing data, but also extended these results to the protein level, demonstrating the presence of TLR1/2, TLR4, and TLR2/6 by flow cytometric analysis. Furthermore, the protein level of TLR2 was quite high, in compare to the expression level of corresponding mRNA (TLR4 and TLR6) (Fig. 9). In summary, the whole results explored the novel biological effects of iSN34 on the modulation and activation of TLR ligands during the immune response. The novel features of iSN34 may be of interest for laboratories developing novel therapeutics for inflammatory diseases that are driven by TLR-mediated immune activation.

3.5. Conclusion

In conclusion, the findings demonstrated that the combination of low-concentration iSN34 with TLR1/2, TLR4, or TLR2/6 agonists yielded a synergistic stimulation of B cells. The stimulated

cells exhibited unique activation profiles, such that the combined ligands contributed to enhanced induction of B cell activation. These results confirm the importance of TLRs in human defense against invading pathogens and this article will highlight some of the research emerging at the landmarks of bacterial and fungal membrane and innate immunity. Therefore, iSN34 displays great potential as a promising and unthreatened agent for incorporation into immune response therapies.

Chapter 4
General discussion

Bacterial DNA containing unmethylated CpG motifs exhibit potential as immune adjuvants, antigens, anticancer and immunoprotective agents. Cellular recognition of CpG motifs is made possible by TLR9, a type I transmembrane protein with an extracellular domain composed of leucine-rich repeat motifs and a cytoplasmic TIR signaling domain (Gay et al., 2006; Watters et al., 2007) , is localized in the endoplasmic reticulum (ER) of antigen presenting cells and B cells (Leifer et al. 2004); Iwasaki et al. 2004) that are responsible for mediating innate immune reactions, especially against bacterial infections, through the recognition of Pathogen-associated molecular pattern (PAMP) (7,8). The interaction between CpG ODNs and TLR9 occurs within the acidified endosome/lysosome (Hacker et al., 1998), which leads to induction of cytokines, including interleukin-6 (IL-6) and interferon-a (IFN-a) (Klinman et al., 1996; Kawai et al., 2004). Synthetic CpG ODNs, used in clinical applications, can be divided into at least 4 classes based on the potential of cytokine induction, depending on their sequence properties. Of these, class A and class B CpG ODNs are well characterized. Class A CpG ODNs mainly activate TLR9 in plasmacytoid dendritic cells (Krug et al., 2001), leading to IFN-a induction. On the other hand, class B CpG ODNs stimulate IL-6 induction from B cells, and do not induce IFN-a (Hartmann et al., 2000). The potential for induction of IFN-a in class A CpG ODNs is thought to be attributed to a self-assembled higher order structure formed by their palindromic and poly-guanine sequences (Kerkmann et al., 2005; Klein et al., 2010) because class B CpG ODNs do not form such structures. Cells of the innate and adaptive immune systems utilize a variety of Pathogen recognition receptors (PRRs) to recognize bacterial DNA. Endosomal nucleic acid receptors such as TLR7, TLR8, and

TLR9 are crucial for host defense, yet can have deleterious functions in immune-mediated diseases. TLR9 responds to ssDNA containing unmethylated CpG motifs (Kreig et al., 1995). In humans TLR9 expression is limited to plasmacytoid dendritic cells and B cells while it is more widely expressed in mice. As our understanding of TLR9 has expanded, attaching TLR-driven cellular responses to modulate inflammation and immunity has become reality. Short synthetic ODN containing CpG motifs have remarkable immunostimulatory properties such as driving cytokine and IFN production, enhancing APC functional, and lymphocyte maturation (Hemmi et al., 2000). These CpG ODNs have tantalizing immunotherapeutic potential. By means of their stimulatory properties they can be used to enhance vaccine responses, as anti-cancer agents, and to reduce the transmission of HIV (Krieg 2006; Murad et al., 2009; Becker et al., 2005). Remarkably, these CpG ODN have been reported to suppress pathological immunity as evidenced by their beneficial effects on allergic diseases such as asthma, and autoimmune diseases such as diabetes (Kline et al., 2008; Kline et al., 2007; Fallarino et al., 2009). Still, CpG treatment can reduce certain inflammatory disease and exacerbate others is still poorly understood (Krieg 2012). It has been suggested that TLR9-dependent immunostimulatory properties of CpG ODNs depend on their ability to promote immune cell maturation and effector functions, including cytokine secretion and enhanced T cell stimulatory functions of dendritic cells (DCs) (2-4). However, this study exhibits a novel oligodeoxynucleotide (iSN34) which have the synergistic effect with association of CpG as well as bacterial and fungal cell wall components.

Using several different theories, we noticed that while total guanine or adenine content affected inhibitory potency the specific sequence did not appear to be of great importance. For example, class B stimulatory ODNs 1826 and 10104 inhibited AIM2 with a similar potency to A151. This off-target mechanism of action may contribute to many of the anti-inflammatory effects of

suppressive and stimulatory ODN alike. Evidence that PS backbone ssDNA constructs, even those containing stimulatory CpG motifs, can mediate inhibition of cytosolic receptor signaling has not been previously reported and is an important concept. Particularly considering the several clinical trials in all phases of development using CpG ODN constructs (Krieg 2012). With widespread use as vaccine adjuvants it is possible CpG ODN-mediated suppression of cytosolic sensing pathways may enhance susceptibility to certain pathogenic infections. This was evinced in a study by Trieu *et al*. which demonstrated that treatment with the suppressive 2114 construct increased bacterial loads of the intracellular pathogen113 *Salmonella typhimurium* by suppressing NF-κB-dependent cytokine responses in a TLR9-independent manner (Trieu et al., 2009).

This study speculated the synergistic activity of association of iSN34 and CpG ODN at the level of IL-6 mRNA accumulation, IL-6 protein secretion and IL-6^{+} cell production following in vitro exposure of cultured splenocytes. Interestingly the ODN iSN34 was effective to stimulate the production of IL-6 when combined with CpG and may be distinctly active adjuvants for Th-1 vaccines. Moreover, IL-6 is known as a potent stimulator of B-cell proliferation, plasma cell survival and antibody production. Several studied showed the usefulness of IL-6 for the prevention or treatment of diseases associated with inflammatory disorders, including RA, inflammatory bowel disease (IBD), multiple sclerosis, systematic-onset juvenile chronic arthritis (JCA), osteoporosis, and psoriasis [BMC41-44]. I further exhibited the proportion of IL-6 screening CD19+ B-cells in the splenocytes. Additionally, this stimulation concentrated the $CD19^{+}$ IL-6^{+} subpopulation a significantly larger fraction of the total cell population that reflects synergistic effects of iSN34 +CpG-B by expressing IL-6 in $CD19^{+}$ B-lymphocytes. IL-6 involved mainly in inflammation by controlling differentiation, proliferation, migration, and apoptosis of target cells as well as other processes such as metabolism, embryonic development, memory consolidation. A

dysfunction of the complex regulatory cytokine network might lead to acute and chronic inflammation, autoimmune diseases or neoplastic disorders. Taken together, IL-6 can promptly produce by monocytes and macrophages and contributes to removal of infectious agent's through activation of immune, hematological and acute-phase responses during contagions and tissue injuries (Tanaka et al., 2016). These research results support this notion.

Therefore, in another study continue to investigate the synergistic effects of association with bacterial and fungal components. Bacteria and fungi are also able to exploit the TLR system to envade host immune responses. Pam_3CSK_4 is a synthetic derivative of triacylated lipoproteins that preserves most of the immune stimulatory activity of full-length lipoproteins (Jin et al.2008). In this study, the Pam_3CSK_4 TLR2 agonist associated with iSN34 significantly increased the production of IL-6. Ongoing studies successfully showed the effects of iSN34 and TLR cell membrane associated ligands such as TLR2 ligand Pam_3CSK_4, the TLR4 ligand LPS, and the TLR2/6 ligand Zymosan; each of the three-exhibited synergistic induction of IL-6 expression both the mRNA and protein level in mouse splenocytes. Zymosan activates the signaling cascade by binding to TLR2 and Dectin-1 (Akira et al., 2006). PRRs such as TLRs and Dectin-1 are expressed in many cell types, mainly innate immune cells such as DCs and macrophages (Mills 2011). Zymosan-dependent responses by DCs and macrophages lead to secretion of inflammatory cytokines, including TNF-α, IL-6, IL-10, IL-12 and IL-23 (Volmer et al. 2005; Vedhoenbet al. 2006). Several studies have shown IL-6 appears to play a dominant role in chronic inflammation and tumor growth (Gasperini et al., 1999). I demonstrated that purified $CD19^+$ B cells are directly activated by Pam_3CSK_4, LPS, and Zymosan (ligands recognized by TLR1/2, TLR4, and TLR2/6, respectively) to produce IL-6. Proinflammatory cytokines (e.g. IFN-γ or IL-6) play an important role and are induced by microbial agents, or parts thereof. These are recognized predominantly by

Toll-like receptors (TLRs) and have immunostimulatory properties. Activation of an immune cell via TLRs can induce and modify cytokine production after activating downstream kinases and transcription factors (Saitoh et al., 2009; Jeong et al., 2011). Proinflammatory cytokines are essential mediators of the immunopathology associated with microbial sepsis. The fungal cell wall component zymosan and bacterial DNA are well-studied experimental tools for investigating these processes, simulating the presence of fungal or bacterial infection. Despite emerging evidence of this study demonstrated B cell activation *in vitro* by measuring changes in surface receptor expression, cytokine expression, and protein production. Genevieve and colleagues describes the expression of TLRs on B cells and able to contribute effective B cell activation, promoting differentiation into antigen-specific, antibody-producing plasma cells *in vivo.* In the present work, each agonist enhanced B cell activation through increased expression of surface receptors and cytokine secretion. When B cells were stimulated with the combination of iSN34 + Pam_3CSK_4, iSN34 + LPS, or iSN34 + Zymosan, synergistic enhancement of cell activation (compared to that with each agonist alone) was observed. TLR agonists are commonly used as vaccine adjuvants to boost antibody production. In my investigation, the combination of iSN34 and Pam_3CSK_4 activated murine B cells, inducing IL-6 production by purified $CD19^+$ cells. Similar activation of murine $CD19^+$ B cells from splenocytes was observed iSN34 associated with LPS and Zymosan, consistent with the presence on these cells of TLR4 and TLR2/6. It was also demonstrated that purified $CD19^+$ B cells are directly activated by Pam_3CSK_4 mediated via TLR1/TLR2 to produce IL-6.

These studies provide the first evidence that iSN34 with LPS and Pam_3CSK_4 induced inflammatory cytokines and found that TLR4 ligand LPS and TLR2 ligand Pam_3CSK_4 induced IL-6 production in mouse splenocytes and IL-6 in splenocytes. Collectively, the effects induced

by these TLR agonists highlight the potential of TLRs as regulators of B cell activation, especially in the context of $CD19^+$ cells. CD19 has been shown to play a role in antigen-independent development as well as in immunoglobulin-induced activation of B cells; the surface density of CD19 is highly regulated throughout B cell development and maturation, until the expression of this marker is lost during terminal plasma cell differentiation (Haas et al. 2005; Bradbury et al. 1992). It is noteworthy that we found the protein level of iSN34 and Pam_3CSK_4 was rather high, in contrast to expression level of the corresponding mRNA of iSN34 with LPS and iSN34 with Zymosan respectively. Understanding the mechanism of TLR activation in immune cells is essential to develop novel strategies to amplify immune responses to antigens in vaccine preparations (Pulendran 2004).

Finally, evidence even suggests that Finally, evidence even suggests that in combination of our findings iSN34 have synergistically stimulated with endosomal compartments (CpG ODN is known to the ligand of TLR9) as well as cell surfaces (Pam_3CSK_4, LPS and Zymosan known to the ligands of TLR1/2, TLR4 and TLR2/6). Further study is obviously necessary to clarify the screening of mechanism and advanced function in immune system.

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