C-DI-GMP BASED IMMUNE STIMULATORY FORMULATIONS AS VACCINE ADJUVANTS

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ABSTRACT

CYCLIC-Di-GMP BASED IMMUNE STIMULATORY FORMULATIONS AS VACCINE ADJUVANTS

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3′,5′-Cyclic diguanylic acid (c-di-GMP) is a bacteria-derived small cyclic di-nucleotide that functions as the universal bacterial secondary messenger. To date, studies concerning its immuno-stimulatory and immuno-modulatory effects show that cytosolic sensing of c-di-GMP by the innate immune sensor receptor STING, induces a robust type-I Interferon production in antigen presenting cells and leads to their maturation as evidenced by increased co-stimulatory molecule expression. However, the chemical structure and the anionic nature of c-di-GMP limit its efficient entry through cellular membranes, requiring its transfection to the cytosol. In this study, we explored the possibility of using three different strategies that would improve the intracellular delivery and/or boost the immunostimulatory activity of c-di-GMP. Specifically, we show that cationic peptide complexation of c-di-GMP or its encapsulation in bacteria-derived membrane vesicles (MVs) boost antigen-specific immune responses and induce a potent protective anti-tumor response in mice, relative to the free ligand. Moreover, we demonstrate that c-di-GMP shows synergistic immunostimulatory activity when used in combination with the TLR9 ligand, CpG motif containing oligodeoxynucleotide (CpG ODN). The results of this study suggest that the immune stimulatory activity of c-di-GMP can be enhanced by the use of these strategies and could contribute to the development of new vaccine adjuvants/immunotherapeutic agents for use in the clinic.

Keywords: c-di-GMP, cationic peptides, bacterial membrane vesicles, CpG ODN,vaccine adjuvant

ÖZ

AŞI ADJUVANTI OLARAK C-Dİ-GMP KÖKENLİ
İMMÜN UYARICI FORMÜLASYONLAR

Yıldız, Soner

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3',5'-siklik diguanilik asit (c-di-GMP) bakterilerce üretilen ve evrensel ikincil mesajcı olarak görev yapan küçük bir siklik di-nükleotitdir. İmmün modülator ve immune uyarıcı etkileri hakkında yapılmış olan çalışmalar, memeli hücrelerinde yer alan STING algacı üzerinden tanınan siklik diguanilatın ko-uyaran molekül ifadesini arttırarak antijen sunum hücrelerinin olgunlaşmasına ve bu hücrelerden tip-1 interferon salımına yol açtığını göstermiştir. Ancak siklik diguanilatın kimyasal yapısı ve anyonik doğası, molekülün hücre membranlarından serbest geçişini kısıtlamakta ve sitozole transfekte edilmesine ihtiyaç doğurmaktadır. Biz bu çalışmada c-di-GMP'nin hücre içine transferini arttıracak ve/veya immune uyarıcı özelliklerini iyileştirecek üç farklı stratejinin uygulanabilirliklerini inceledik. Özellikle c-di-GMP'nin katyonik peptitlerle komplekslenerek veya bakteri-kökenli membran kesecikleri içerisine enkapsüle edilerek serbest liganta göreceli olarak antijene-özgü immün yanıtı iyileştirdiğini ve etkin bir anti-tümör koruyucu yanıta sebep olduğunu gösterdik. Bununla birlikte siklik diguanilatın CpG motifi içeren oligodeoksinükleotidlerle birlikte kullanımının sinerjistik immune uyarıcı etki gösterdiğini bulduk. Bu tez çalışmasından elde edilen sonuçlar, yukarıda belirtilen stratejilerin kullanımıyla c-di-GMP'nin immune uyarıcı aktivitesinin iyileştirilebileceğini ve klinikte kullanıma yönelik yeni aşı adjuvantları/ immünoterapötik ajan geliştirilmesine katkıda bulunabileceğini düşündürmektedir.

Anahtar Kelimeler: Siklik di-guanilat, katyonik peptitler, bakteri kökenli membran kesecikleri, CpG ODN, Aşı adjuvanı

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

2’-Fluo-AHC-c-di-GMP	2'- O- (6- [Fluoresceinyl] aminohexyl carbamoyl)
APC	Antigen presenting cell
AGE	Agarose Gel Electrophoresis
BCIP	5-Bromo-4-chloro-3’-indolyphosphate p-toluidine salt
BcR	B cell receptor
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
c-di-GMP	Cyclic di-guanosine monophosphate
cGMP	Cyclic guanosine monophosphate
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
CXCL	CXC-chemokine ligand
DAMP	Danger/damage associated molecular pattern
DC	Dendritic cell
DDX41	DEAD box protein 41
DGC	Diguanylate Cyclase
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
ELISpot	Enzyme Linked-Immunosorbent Spot
ER	Endoplasmic reticulum
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GC	Germinal Center
GTP	Guanosine Triphosphate
hPBMC	Human peripheral blood mononuclear cell
IFN	Interferon
IFNβ	Interferon Beta
Ig	Immunoglobulin
IKK	Inhibitor kappa B kinase
IRF	Interferon-regulatory factor
IL	Interleukin
IP 10	Interferon gamma-induced protein 10
LGP-2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
Mϕ	Macrophages
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma Differentiation-Associated protein 5
MHC	Major histocompatibility complex

MV	Membrane Vesicles
MyD88	Myeloid differentiation factor-88
NF-κB	Nuclear factor- kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PDE	Phosphodiesterase
PNPP	Para-nitrophenyl pyro phosphate
poly I:C	Polyriboinosinic polyribocytidylic acid
RPMI	Roswell Park Memorial Institute
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene-I
RLR	Retinoic acid-inducible gene-I like receptor
RNA	Ribonucleic acid
R848	Resiquimod
SA-AP	Streptavidin-alkaline phospahatase
ssRNA	Single-stranded RNA
STING	Stimulator of Interferon genes
TAE	Tris-base, Acetic Acid, EDTA
TBK-1	TANK-binding kinase 1
TcR	T cell receptor
T_H1	T helper type 1
T_H2	T helper type 2
T_H17	T helper type 17
T_{REG}	Regulatory T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR domain containing adaptor inducing IFN-β

CHAPTER 1

INTRODUCTION

1.1 Immune System

1.1.1 General concepts on Immune System

Biological organisms, including simple unicellular microorganisms and complex multi-cellular systems, evolved distinctive types of self defense mechanisms (i.e. immunity) to other organisms that may potentially harm them. For example, bacteria possess a rudimentary immune system, using enzymes to protect against bacteriophage infections[1], whereas anti-microbial peptides provide protection against pathogen entry in fruit flies[2]. In higher taxa of vertebrates, such defense oriented mechanisms are collectively known as the immune system and the branch of science that studies the structure and function of the immune system is known as immunobiology.

To achieve the task of protection from pathogens, the immune system has to fulfill four essential tasks. First, presence of a pathogen has to be recognized so that an immune response could be established against that invader. This process is known as **immunological recognition**, and is ministered by specialized cells of the innate immune system. Discrimination of self from non-self or altered-self holds the basis for recognition of an outsider in the body[3]. Following successful recognition, the infection must be eliminated promptly to minimize damage. This is achieved through **immune effector functions** whereby an appropriate response to the pathogen is initiated[4]. However, if left unchecked, such effector functionalities are essentially self-damaging to the host and are therefore kept under control via **immune regulatory** mechanisms[5]. Last task that the immune system has to comprise is rapid protection against recurrent or persistent infections through a recall mechanism known as **immunological memory**[6].

Immune system consists of different compartments. Physical and chemical barriers surrounding the host comprise the first line of defense against infectious agents. For example, tight cell junctions in the epidermis create a physical barrier and restrict pathogen entry[7], whereas anti-microbial defensin peptides secreted from epithelial cells combat microorganisms[8]. These barriers protect the individual by providing an isolated outer stratum to prevent casual entry of infectious agent[9]. When the first line of defense is breached by the invader molecules or organisms, tissue resident innate immune cells, such as dendritic cells

(DCs) and macrophages are activated[10]. Innate immune system evolved to recognize general patterns of molecules expressed by pathogens and has the capacity to clear out infection in a non-specific manner. It is also responsible to invoke an adaptive response to infectious agents via specialized function of Antigen Presenting Cells (APCs), including DCs, macrophages and monocytes[11]. Where the innate response to a pathogen remains inadequate and fails to clear an infection, the adaptive immune system consisting of T and B lymphocytes generate a highly specific response to the pathogen. In this context, certain pathogen derived-molecules such as toxins or membrane proteins are considered as antigens where an antigen is defined as any substance capable of triggering an adaptive immune response. In contrast to innate responses, adaptive responses develop in a delayed fashion, and display high specificity to the antigen in question[12]. To sum up, together with the primary barriers to invasion by infectious agents, innate and adaptive arms of the immune system comprise the whole of the immune system and provide protection against pathogens.

1.1.2 Lymphoid tissues in the body

All blood cells differentiate from common hematopoietic progenitor stem cells residing in one of the primary lymphoid organs, the bone marrow[13]. During early developmental stage, hematopoietic stem cells separate into two major cell lineages: the common myeloid progenitor cells give rise to cells of the myeloid lineage including megakaryocytes, erythrocytes, granulocytes, and macrophages; whereas common lymphoid progenitor cells give rise to B cells, T cells, and Natural Killer (NK) cells[14,15]. However, dentritic cells, considered as the most important antigen presenting cells, have various types and may originate from both progenitors[16]. Most cell lineages explained above complete their maturation either in the bone marrow or in tissues they home to. However, immature T lymphocytes migrate to the thymus, the second primary lymphoid organ in the body, where they develop into functional T cells[17]. Hence, formation of immune cells, i.e. white blood cells, depends on these two important primary lymphoid tissues.

Following their maturation, white blood cells distribute throughout the body and some, such as macrophages, become tissue resident, and others such as T and B lymphocytes circulate between blood and the secondary lymphoid tissues including spleen and lymph nodes[18]. Antigen presenting cells including DCs, monocytes, macrophages, may migrate from tissues to secondary lymphoid organs through the lymphatic vessels, a complex network that constantly drains tissue fluid and collectively integrates lymphoid organs and blood circulation[19]. Secondary lymphoid organs such as spleen display a distinctive architecture of segregated T cell and B cell zones, where each lymphocyte population is in close contact with the APCs[20].

1.1.3 Innate Immune System and Pattern Recognition Receptors

Initial encounter with an infectious agent usually takes place in tissues where resident macrophages and DCs are the first cells that initiate a response by secreting cytokines and chemokines[21]. Such secreted factors contribute to the inflammatory response, characterized by vasodilation of nearest veins and increased entry of monocytes and neutrophils into infected tissue, leading to redness, pain, and swelling[22]. The effects of some of the important cytokines and chemokines secreted during inflammation are listed in **Table 1.1**[23]. DCs and macrophages are equipped with specialized "danger sensing" receptors known as Pattern Recognition Receptors (PRRs) that are located in several cellular compartments, including the membrane surface, endosomes and the cytosol[24]. PRRs are able to recognize conserved molecular patterns expressed by pathogens, collectively known as Pathogen Associated Molecular Patterns (PAMPs). PRR family has several sub-families, including the Toll-like receptor (TLR) family, retinoic acid inducible gene-I (RIG)-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, C-type lectins, cytosolic DNA sensors and Mannose Binding Receptors[25]. These receptors have invariant binding sites indicating that they are only able to bind to certain PAMPs, such as gram negative bacterial cell wall component Lipo-polysaccharides (LPS) is recognized by plasma-membrane resident TLR4, whereas unmethylated cytosine-phosphate-guanine motifs (CpG motifs) seen in bacterial genome are sensed in the endosomes through TLR9. Some of the important PRRs and the ligands they recognize are listed in **Table 1.2**[26]. Many of the PRRs are signaling receptors, where following ligand binding, a cascade of signal transduction events is initiated, culminating in expression of several immune-system related genes. Downstream signaling mediated by certain PRRs is summarized in **Figure 1.1**[27]. Of this vast family of "danger-sensing" receptors, some are dedicated to sensing nucleic acids of bacterial or viral origin. This special group of nucleic acid sensing PRRs will be detailed in later sections.

1.1.4 Adaptive Immune System

Another distinctive arm of the immune system is known as the adaptive immunity which is entirely dependent on T and B cell mediated responses. Each T cell and B cell expresses specialized antigen-recognition receptors, also known as the T cell receptor (TcR) and the B cell receptor (BcR), respectively, that uniquely bind to a single antigen among trillions of possibilities observed in nature. The repertoire of these antigen-recognition receptors is predetermined during the maturation process of these cells, basically through a process called as somatic recombination[28]. After antigenic stimulation, the specific T or B lymphocyte that recognized the antigen expands clonally, i.e. go through several cycles of cell division to increase their numbers. By doing so, lymphocytes that are capable of binding to antigens are increased in number. The process of antigen recognition in B cells is direct (i.e. BcR binds to a specific region of antigen directly). Following antigen recognition, B

Table 1.1 List of important cytokines and chemokines and their immune regulatory effects

Cytokines	**Produced by**	**Function**	
IL-1β	Mϕ, keratinocytes	Fever, induction of acute-phase protein secretion, T cell and Mϕ activation	
TNFα	DC, NK and T cells	Local inflammation and endothelial activation	
IL6	Mϕ, DC	Fever, T and B cell growth and differentiation	
IL-12	Mϕ, DC	Activation of NK cells, induction of CD4 T-cells to differentiate into T_H1	
IL-15	non-T cells	CD8 memory T cell survival, stimulation of NK and T cell growth	
IL-18	Activated Mϕ	Induction of IFNγ secretion via NK and T cells, favors T_H1 immunity	
IFNα	DC	Anti-viral immunity, induction of MHC I expression	
IFNγ	T cells, NK cells	Suppression of T_H2 immunity, Mϕ activation, increased expression of antigen processing components	
Chemokines	**Produced by**	**Attracted cells**	**Major effect**
CXCL8 (IL-8)	Monocytes, Mϕ, DC	Neutrophils, naïve T cells	Mobilization, activation and degranulation of neutrophils
CCL3 (MIP-1α)	Monocytes, T and Mast cells, fibroblasts	Monocyte, NK, T cells basophils, DC	Promotes T_H1, antiviral defense, competes with HIV-1
CCL4 (MIP-1β)	Monocytes, Mϕ, Neutrophils, endothelium	Monocyte, NK, T, DC	Competes with HIV-1
CXCL10 (IP10)	T, fibroblast, endothelial, monocytes, keratinocyte	Resting T cells, NK, monocytes	Promotes T_H1, antiangiogenic, immunostimulant

Table 1.2 Important Pattern Recognition Receptors and their ligands

PRR	Cellular expression		Ligands	
	Immune cells	Non-immune cells	PAMP	DAMP
MDA5	Ubiquitous	Ubiquitous	Long dsRNA [polyI:C], EMCV	
RIG-I	Ubiquitous	Ubiquitous	5′triphosphate dsRNA, short Poly[I:C], RNA viruses (NDV, VSV, SeV, flu ΔNS1, HCV, JEV)	
TLR1/2	Monocyte, mDC, B cell, NK, neutrophil, basophil	Ubiquitous	LAM, PGN, GPI (*Toxoplasma gondii*), LTA, triacyl lipopeptide, zymosan	
TLR2/6	Monocyte, mastocytes, mDC	Epithelial cell, neural cell, MSC, endothelial cell	LTA, diacyl lipopeptide	
TLR3	mDC	Epithelial cell, neural cell, MSC	RNA virus (WNV, RSV, MCMV), synthetic dsRNA (Poly[I:C], poly [A:U])	
TLR4	Monocyts, macrophages, mDC, mastocytes, basophil	Renal cell, hepatic cell, keratinocytes, MSC, endothelial cell	LPS, viral proteins (HIV, VSV, RSV, retroviruses)	HSP, fibronectin, hyaluronic acid, fibrinogen
TLR5	mDC, monocyte, NK, T cell	Gastric epithelial cell, keratinocytes, MSC	Flagellin	
TLR7	pDC, B cell, esosinophil		RNA virus (influenza), synthetic ssRNA, imidazoquinoline (R848/CL097/imiquimod)	
TLR8	mDC, T and B cell, monocyte		Synthetic ssRNA (AU-rich) imidazoquinoline (R848/CL075)	
TLR9	pDC, B cell, basophil, eosinophil	Epithelial cell, keratinocytes, MSC, endothelial cell	Unmethylated CpG dsDNA, dsDNA virus (HSV, MCMV)	DNA/LL-37
TLR10	pDC, neutrophil, B cell, basophil		Unknown	

mDC myeloid DC, *NK* natural killer cell, *pDC* plasmacytoid DC, *MSC* mesenchymal stem cell, *LTA* lipotechoic acid, *LAM* lipoarabinomannan, *PGN* peptidoglycan

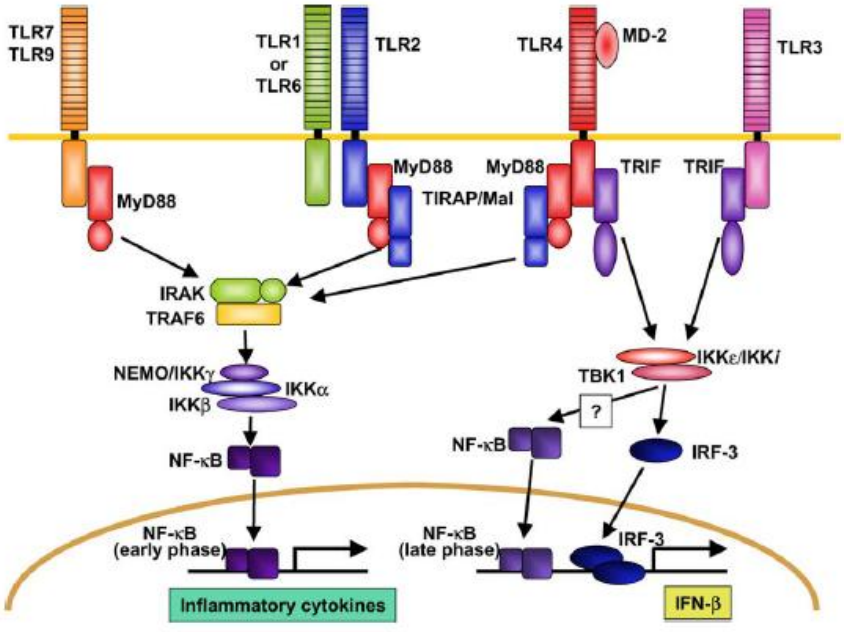

Figure 1.1 Downstream signalling of Toll-like Receptors

cells differentiate into antibody secreting plasma cells. In contrast, T cells are incapable of direct antigen recognition. TcRs can only bind to peptides derived from antigens presented in association with major histocompatibility complex (MHC) molecules. Of these, MHC Class I is expressed on all nucleated cells and functions in presentation of cytosolic antigens to $CD8^+$ T cells. MHC Class II expression is restricted to APCs and functions in presentation of endocytosed antigens to $CD4^+$ T cells. Following antigen recognition through MHC Class I, $CD8^+$ cytotoxic T cells become activated and kill infected target cells through the release of specialized enzymes (Perforin and Granzyme B). Antigen-experienced $CD4^+$ helper T cells display a variety of unique abilities, as summarized in **Figure 1.2**[29].

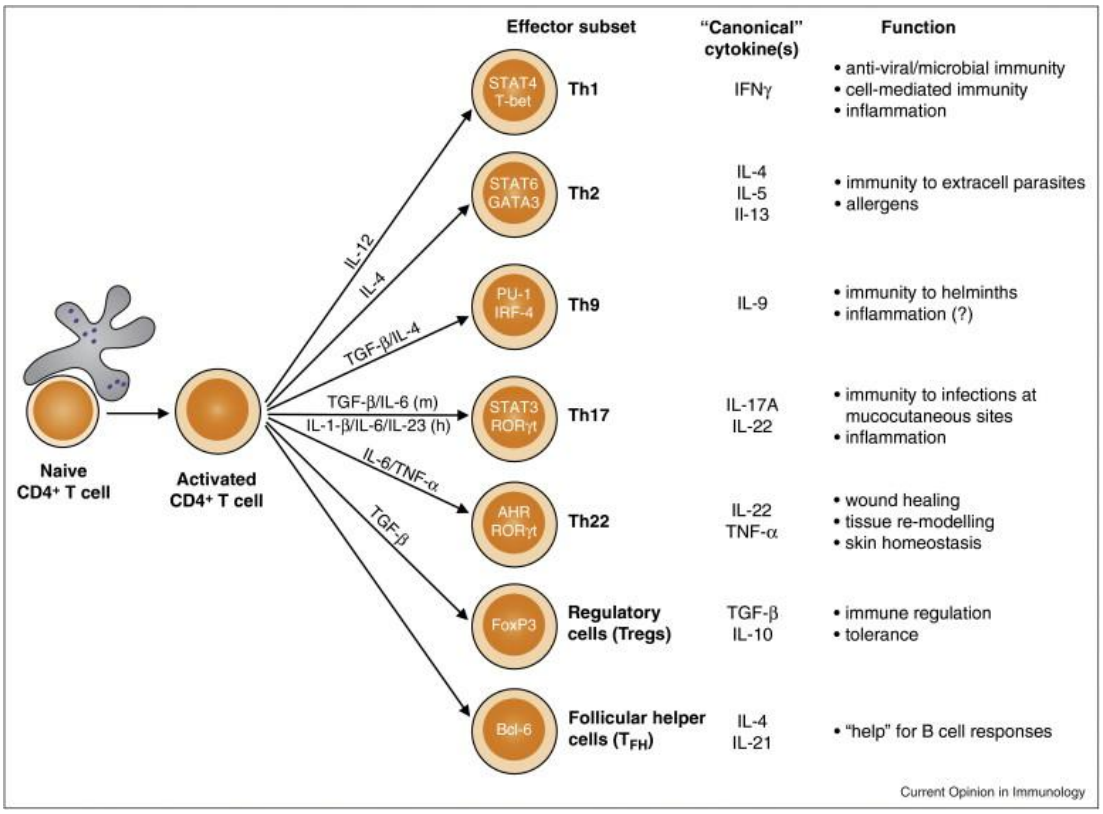

Figure 1.2 Helper T cell differentiation and the biological functions of helper T cell subsets

Although the adaptive immune responses entirely depend on T and B lymphocytes, the nature of the response is heavily influenced by innate immunity. For example, certain intracellular pathogen components are sensed by DCs through the PRRs they express, leading to the release of cytokines. Depending on the combination of cytokines present in the environment, $CD4^+$ T cells that are primed by these DCs may differentiate into various T

helper types, including T_H1, T_H2, T_H17 or induced T_{REG} cells, that provide help to combat intracellular pathogens, parasitic infections, extracellular bacteria or suppress over-exuberant immune responses, respectively. For example, IL12 secretion from APCs during peptide presentation to T cells causes T_H1 type helper T cell formation, whereas IL4 presence induces T_H2 development. These helper T cell types provide help to antigen-experienced B cells so that depending on the type of help provided, the B cells switch the class of antibody they secrete. During this process, B cells change the type of antibody they secrete from IgM to different types of Immunoglobulin molecules, such as IgG or IgE (**Table 1.3**)[30,31,32].

In summary, the type of adaptive immunity that is most effective against a specific pathogen is shaped by innate immunity.

Table 1.3 Classes and major functions of Immunoglobulin molecules

Name	Types	Description
IgA	2	Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens. Also found in saliva, tears, and breast milk.
IgD	1	Functions mainly as an antigen receptor on B cells that have not been exposed to antigens. It has been shown to activate basophils and mast cells to produce antimicrobial factors.
IgE	1	Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy. Also protects against parasitic worms.
IgG	4	In its four forms, provides the majority of antibody-based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to the fetus.
IgM	1	Expressed on the surface of B cells (monomer) and in a secreted form (pentamer) with very high avidity. Eliminates pathogens in the early stages of B cell mediated (humoral) immunity before there is sufficient IgG.

1.2 Bacterial secondary messenger cylic di-guanylic acid, c-di-GMP

1.2.1 Biosynthesis of c-di-GMP and its effects in bacteria

3', 5'- Cyclic diguanylic acid (c-di-GMP) is a naturally occurring small cyclic dinucleotide found in bacteria. It consists of two identical guanosine monophosphate molecules, bonded by phosphodiester bonds through the phosphate groups, thus forming a cyclic structure (**Figure 1.3**)[33]. There are several examples of small nucleotide-based second messenger signaling systems (ex. cAMP signalling, cGMP coupled protein signaling)

studied in both eukaryotes and bacteria[34,35]. As one of them, c-di-GMP was initially identified as an allosteric regulator of cellulose synthase in *Gluconacetobacter xylinus*[36]. It is now accepted as a universal bacterial secondary messenger that is involved in a variety of intracellular signaling mechanisms in many bacterial species.

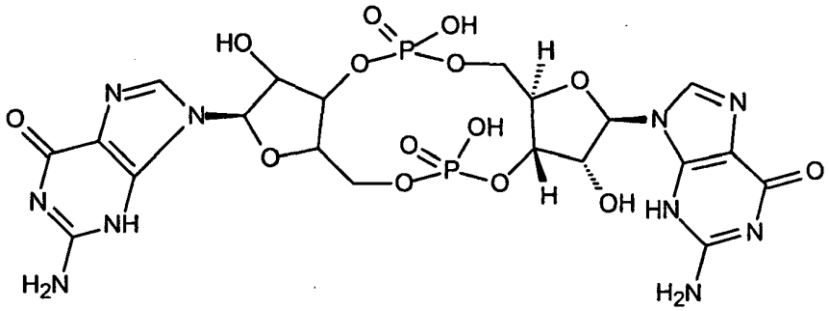

Figure 1.3 Chemical structure of c-di-GMP

In the last decade, there have been numerous studies concerning c-di-GMP biosynthesis, degradation and signaling. Two important protein families have been identified that regulate the levels of the second messenger in bacteria: diguanylatecyclases (DGC), and phosphodiesterases (PDE). The former synthesizes c-di-GMP from two guanosine triphosphate (GTP) molecules whereas the latter cleaves the phosphodiester bonds to generate an intermediate, which is subsequently converted into two guanosine monophosphate molecules (**Figure 1.4**)[37]. Depending on the environmental conditions surrounding the bacteria, sensory domains located on the plasma membrane[38] transduce signals, culminating in elevated expression of DGC. Opposing activities of DGCs and PDEs control cellular levels of c-di-GMP.

Depending on the environmental cues, c-di-GMP levels affect a variety of cellular functions, including biofilm formation, alterations in the cell surface, host colonization and regulation of bacterial flagellar motility[33]. For example, *Gluconacetobacter xylinus*, the bacterial species in which c-di-GMP was first identified, is able to sense oxygen availability in the environment through membrane receptors and increase cellulose anabolism through c-di-GMP signaling, providing an inducible shift in cellulose synthase activity depending on anaerobic or aerobic conditions[39]. Another example of c-di-GMP regulated metabolism is seen in *Escherichia coli*, *Pseudomonas aeruginosa*and *Salmonellatyphimurium*, in which increase in cellular concentrations of c-di-GMP causes biofilm formation and expression of virulence factors at the same time[40]. It is thought that the secondary signals provided by c-di-GMP act as "lifestyle switch regulators", regulating environment depended responses, cell adhesion and cell to cell communication[34].

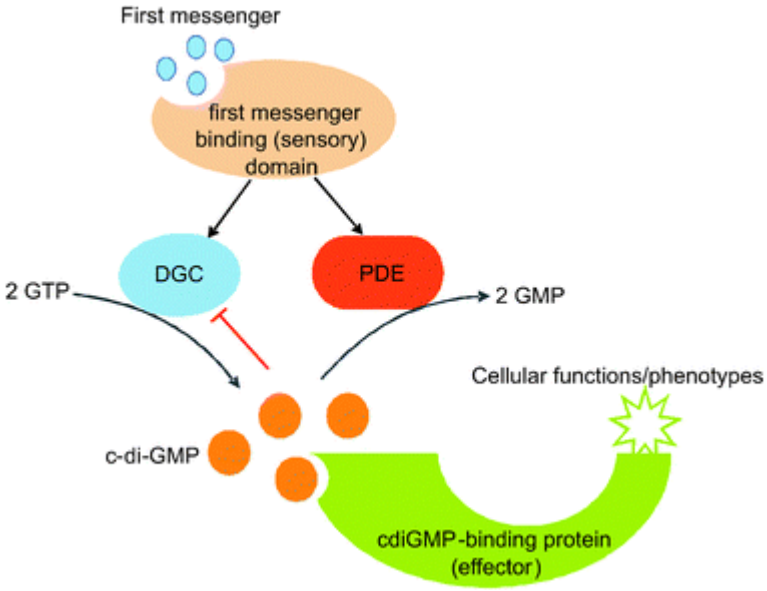

Figure 1.4 Downstream signalling of c-di-GMP in bacteria

The universal abundance of c-di-GMP in bacteria also brings up the question whether it is recognized by the immune system of higher eukaryotic organisms. David K.R. Karaolis *et al.* was the first to show that c-di-GMP was able to activate human immature dendritic cells to secrete several key cytokines and chemokines. Other studies confirmed the immune-stimulatory activity of c-di-GMP and the molecule is now accepted as a PAMP. Mechanism of c-di-GMP mediated signaling and its immune-modulatory effects are detailed in the next section.

1.2.2 Nucleic acid sensors and c-di-GMP signaling

As previously explained, innate immune system has distinctive responsive mechanisms that are able to recognize unique molecular signatures conserved among microbial organisms, which are not normally present in the host (PAMPs). As a newly identified PAMP member, c-di-GMP possesses distinct immune-stimulatory properties which raise the question of recognition mechanism of the compound.

One of the central strategies in recognition of an infection by the innate immune system is the detection of pathogen-derived nucleic acids. Pathogen-derived nucleic acids are discriminated from self-nucleic acids based on their sequence, structure, modifications and their intracellular localization. However, mislocalized self DNA or RNA can be recognized

as a "danger-associated molecular pattern" (DAMP), indicating cellular damage and may result in inappropriate responses as seen in certain autoimmune diseases, ex. systemic lupus erythematosus[41]. Several nucleic acid sensors have been identified, such as the endosomal TLR3, TLR7 and TLR9), cytosolic RIG-I, MDA5 or LGP2, either targeting ssRNA, dsRNA of viruses or unmethylated CpG motifs observed in bacteria (**Figure 1.5**)[42]. Yet, none of these receptors were shown to function in the detection of bacteria derived cyclic di-nucleotides, indicating that a novel receptor was involved in recognition of the molecule. Considering the evolutionary history of biological organisms, it is only logical to predict the presence of a "sensor" dedicated for the detection of cyclic di-GMP, the universal secondary messenger in bacteria. However, the question of how c-di-GMP was recognized by the host immune system remained undefined until the discovery of a novel cytosolic receptor, called Stimulator of Interferon Genes (STING, also known as TMEM173, MPYS, MITA and ERIS).

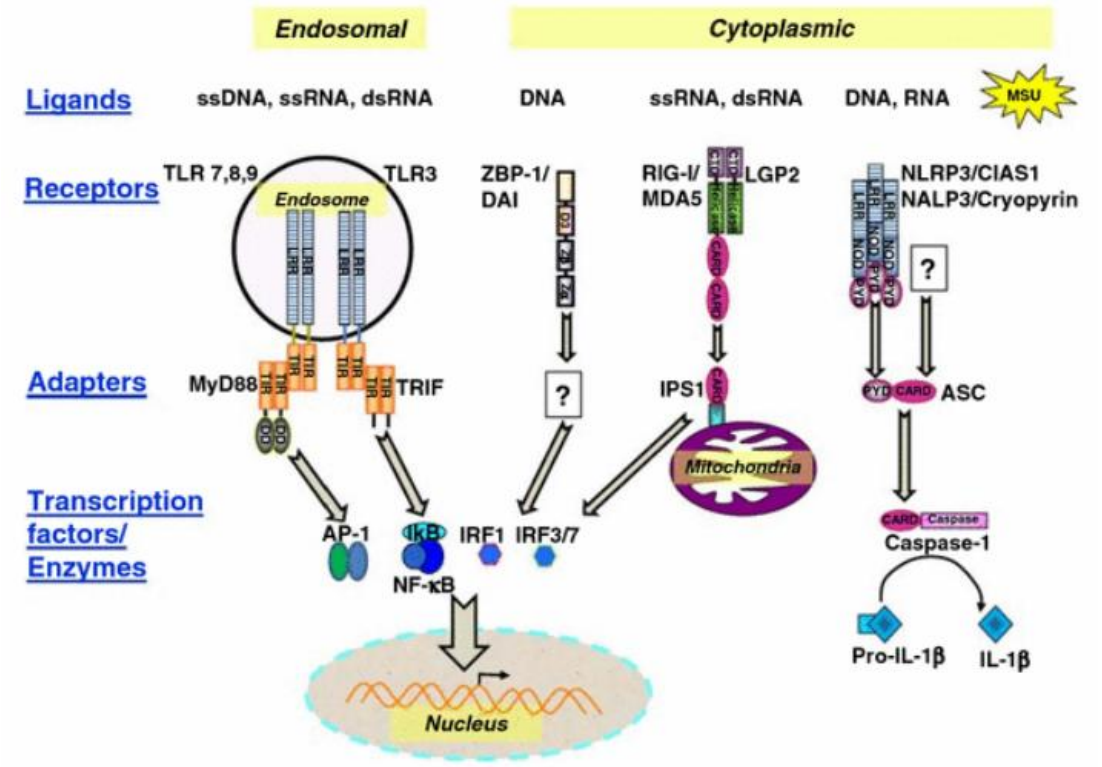

Figure 1.5 Major nucleic acids sensors of the innate immune system

Several groups independently identified STING which was previously described as an adaptor protein functioning in intracellular signaling pathways, as a key receptor in innate immune response to nucleic acids[43,44]. Following sensing of nucleic acids (dsDNA, bacteria

derived cyclic di-nucleotides including c-di-AMP and c-di-GMP) in the cytosol, STING induces increased expression of type-I Interferon production (specifically IFNβ) and co-related genes (**Figure 1.6**)[45]. The receptor resides on the membrane of the endoplasmic reticulum through its four transmembrane domains and its carboxy terminal domain extends to the cytosol[46]. STING has a major role in detecting the presence of intracellular bacteria, viruses and eukaryotic pathogens and functions in clearing the infection via cytokine induction provided by downstream signaling of the receptor[47].

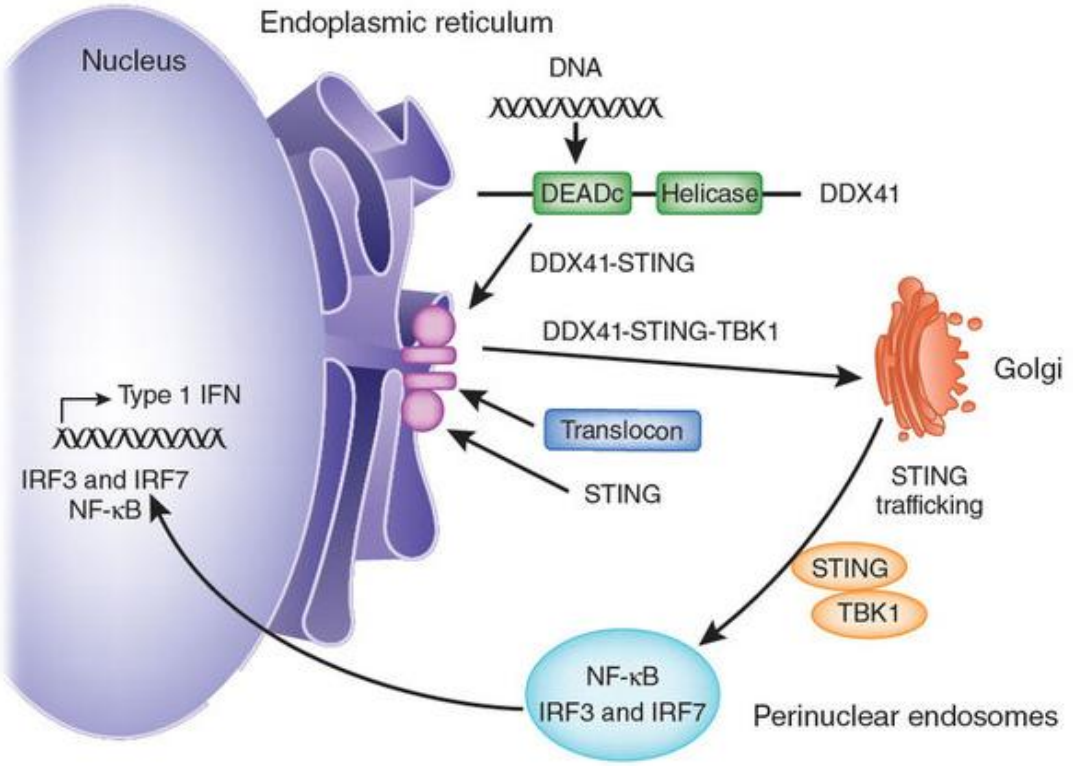

Figure 1.6 STING dependent signalling

Although STING has important signaling functions in recognition of cytosolic nucleic acids, additional adaptor proteins, such as DDX41, are required for appropriate responses to dsDNA[48]. However, the receptor is a direct immune sensor of c-di-GMP, since structural analysis of the protein revealed binding of the cyclic di-nucleotide to STING in the absence of other adaptors[49]. Moreover, expression of the receptor in cells those are normally unresponsive to c-di-GMP results in type-I Interferon production following stimulation with c-di-GMP, indicating the presence of a direct detection mechanism. Analysis of the crystal structure of the receptor revealed that STING exists as a homo-dimer that creates an open inner surface for ligand binding, enabling direct interaction of c-di-GMP with the receptor[50].

It has been suggested that in response to ligand engagement, homo-dimeric STING relocates from the endoplasmic reticulum to the Golgi apparatus and then to the cytoplasmic punctate structures where it recruits and activates the kinase TBK1[51]. Active TBK1 phosphorylates IRF3, the key transcription factor required for IFNβ production[52]. STING-mediated signaling was also associated with initiation of NFκB and MAPK pathways, leading to pro-inflammatory cytokine secretion and maturation in dentritic cell[46]. c-di-GMP through STING mediated immune activation holds promise in development of novel immunotherapeutic agents such as vaccine adjuvant applications. In this context, the amino acid sequence similarity between human and mouse STING proteins (81% similar, 68% identical)[53] creates an opportunity for testing of cyclic di-nucleotide based immunotherapeutic agents in mouse models that would be relevant to future clinical studies in humans.

1.2.3 Immune-stimulatory properties of c-di-GMP

The original finding that c-di-GMP induced maturation of human DCs *in vitro*, attracted attention to the immune stimulatory properties of this molecule. Numerous *in vivo* studies were conducted to test its immunotherapeutic potential[54]. In one such study, pre-treatment of mice with c-di-GMP resulted in a striking protective effect against subsequent systemic or mucosal bacterial challenge with *S. aureus*, confirming the stand-alone immune-protective potential of c-di-GMP *in vivo*[55]. The fundamental mechanisms that underlie the c-di-GMP mediated immune stimulation and vaccine adjuvanicity have been recently explored[56]. Karaolis *et al.* showed that c-di-GMP upregulated co-stimulatory molecule expression *in vitro* in human and mouse myeloid DCs but had no effect on human plasmacytoid DCs. Myeloid DCs stimulated with c-di-GMP responded by secreting pro-inflammatory cytokines (TNFα, IL-12), IFNγ, IP-10 and IL-8, which may explain the promising *in vivo* immune activator properties of the molecule[56]. c-di-GMP was also shown to trigger IL-6 and IFNβ secretion in various cell types including, mouse peritoneal exudate cells (PECs), RAW (Mouse Monocyte/Macrophage Cell line) and DCs. Similar cytokine secretion profiles were also observed in hPBMC and mouse splenocytes[45]. Of the aforementioned cytokines, IFNβ holds a unique importance since type-I Interferon inducers are known to have potent vaccine adjuvant activities[57]. Furthermore, IFNβ is known to prevent progression of certain aggressive tumors[58] and shows therapeutic effects in treatment of certain autoimmune diseases, such as multiple sclerosis[59]. Vaccines adjuvant studies conducted with c-di-GMP demonstrated favorable antigen-specific IgG responses comparable to those seen with potent vaccine adjuvants (such as LPS and CpG ODN), characterized by numerous Germinal Center (GCs) formation, a hallmark of memory B cell development. Several researches presented that antibody profiles were more skewed towards IgG2a than IgG1, indicating a T_H1 dominated response, consistent with the secreted cytokines associated with c-di-GMP stimulation[60]. Intranasal or sublingual administration of H5N1 virosomes adjuvanted with c-di-GMP effectively induced local and systemic H5N1-specific humoral and cellular immune responses in mice, suggesting applicability as a novel mucosal vaccine adjuvant[61].

1.3 Aim of the study

In the last decade, numerous studies demonstrated the potent immune-stimulatory activity of c-di-GMP, including its use as a successful vaccine adjuvant in mice. Recognition of c-di-GMP through the cytosolic pattern recognition receptor STING, leads to dendritic cell maturation, pro-inflammatory cytokine production and induction of type-I Interferons in mouse and humans. Having such important characteristics, c-di-GMP is considered as a candidate adjuvant for vaccine studies as well as an anti-tumor therapeutic agent. However, the chemical structure and anionic nature of the molecule restricts its efficient entry into the cytosol, requiring development of novel delivery strategies that would improve its immune-stimulatory activity. The aim of this thesis was to develop different strategies that would increase the immunogenicity of c-di-GMP. To this end, we first aimed to test the immune activator potential of a c-di-GMP/cationic peptide based formulation. A second approach involved the preparation of host-guest complexes of the drug and the macrocyclic compound cucurbituril. The third approach explored the possibility of enhancing the immunostimulatory activity of the drug following its encapsulation in bacteria-derived membrane vesicles. Lastly, we tested a possible synergistic immune activator potential of dual PRR ligand usage by targeting receptors residing in different subcellular compartments: c-di-GMP signaling through cytosolic STING and CpG ODN signaling through endosomal TLR9. The effectiveness of the above mentioned strategies were evaluated in in vitro and in vivo experiments.

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Cell culture media, Buffers and other standard solutions

RPMI 1640 cell culture media, Na-pyruvate, HEPES, L-Glutamine, Penicillin/ Streptomycin, Non-essential amino acid solution and DNase RNase free double distilled water (ddH_2O) were purchased from Hyclone, USA. Fatal Bovine Serum (FBS) and Phosphate Buffer Saline (PBS) were obtained from Lonza, Switzerland. TAE buffer10X and Tween 20 were from Fisher Scientific, USA. Other buffers, cell culture media and solutions used in this study (e.g., 6X Loading Dye, PBS, T-cell buffer, Blocking Buffer, ELISA Wash buffer, FACS Buffer) are detailed in **Appendix A**.

2.1.2. Reagents

Trypan Blue Dye and Ficoll-Hypaque density gradient medium were obtained from Lonza, Switzerland. Lipofectamine 2000 was purchased from Invitrogen. 50 bp ladder, 1 kb DNA ladder, and ssRNA ladder were obtained from NEB, UK. For cell fixation and permeabilization protocols, Fixation and Permeabilization Medium A and Medium B (Invitrogen, USA) were used.

2.1.3. Cyclic nucleotides, Peptides, and Chemical carriers

Cyclic diguanosine monophosphate (c-di-GMP), 2'- O- (6- [Fluoresceinyl] aminohexyl carbamoyl)- cyclic diguanosine monophosphate (2'-Fluo-AHC-c-di-GMP), and Guanosine-2', 3'- cyclic monophosphate (cGMP), were at least 97 % pure (HPLC, UV, 252 nm) and were purchased from Biolog, Germany. Nonamer arginine peptide (Arg(9)), polylysine peptide (Lys(9)), Ovalbumin (OVA) complete protein and the $CD8^+$ T-cell epitope SIINFKL peptide were obtained from Anaspec, USA. Cucurbituril was a kind gift from Associate Professor Dönüs Tuncel (Bilkent University, Chemistry Department). Polyethyleneimine (PEI) was a kind gift from Associate Professor İhsan Gürsel.

2.1.4. Toll like Receptor (TLR) Ligands and Oligodeoxynucleotides (ODNs)

TLR ligands and ODNs used in stimulation experiments are listed in the **Table 2.1**. All TLR ligands and ODNs were endotoxin and protein free. Human commensal bacteria-derived membrane vesicles (MVs) were a kind gift from Mrs. Esin Alpdundar.

Table 2.1 TLR ligands and ODNs used in this study

Lipopolysaccharide (LPS) -isolated from *E.coli*-	Sigma, USA
polyinosidicacid:cytidilic acid (polyI:C)	Amersham, UK
K3 CpG ODN *Seq* -ATCGACTCTCGAGCGTTCTC-	Alpha DNA, Canada

2.1.5. Antibodies and related Reagents

For cytokine ELISA studies, monoclonal antibodies (coating antibody), monoclonal biotinylated antibodies (detection antibody) and streptavidin-ALP conjugates were obtained from Biolegend, USA or Mabtech, USA. For antigen-specific antibody measurements by ELISA, alkaline phosphatase conjugated goat anti-mouse total IgG, IgG1 and IgG2c were used (Southern Biotech, USA). *p*-nitrophenyl phosphate di-sodium salt (PNPP) substrate was from Thermo Scientific, USA. Immulon 1B and Immulon 2HB plates were from Thermo Scientific, USA. BCIP substrate used in ELISPOT protocol was prepared in the laboratory (**Appendix A**). All flourescent-dye conjugated antibodies were from Biolegend, USA.

2.2. METHODS

2.2.1. Preparation of complexes and delivery agents

Complexes used in experiments were prepared by mixing cyclic diguanosine monophosphate (c-di-GMP) with cationic peptides Arg(9), Lys(9), cationic polymer polyethyleneimine (PEI) or the macrocyclic molecule cucurbituril at different molar ratios (1:1, 1:2, 1:4, and 1:8). The samples were then incubated for 30 minutes at room temperature, in order to allow for the complexation to proceed. For experiments involving bacteria-derived membrane vesicles (MVs), c-di-GMP was either directly mixed with MVs, or the same amount was encapsulated within MVs using the dehydration-rehydration method as described previously[62]. For this, c-di-GMP (15 μg, 100 μl) and MVs (10 μg, 100 μl) were mixed and then lyophilized. The resultant powder was then re-hydrated using a minimal volume of ddH2O (50 μl) to generate MVs encapsulating c-di-GMP. All complexes and delivery agents were diluted with an appropriate volume of PBS prior to further use.

2.2.2. Characterization of Complexes

2.2.2.1. Visualization of complex formation by Agarose Gel Electrophoresis (AGE)

To investigate whether c-di-GMP was complexed with cationic peptides, cucurbituril or polyethyleneimine (PEI), 2'- O- (6- [Fluoresceinyl] amino hexylcarbamoyl)- cyclic diguanosine monophosphate (2'-Fluo-AHC-c-di-GMP) was mixed with the complexation agents at the indicated molecular ratios given for the unlabeled c-di-GMP. Uncomplexed free form of 2'-Fluo-AHC-c-di-GMP was used as the reference sample. 10 µl of each complex or an equivalent concentration of fluoresceinated free c-di-GMP was mixed with 2 µl of 6X loading dye (**Appendix A**). Samples were then loaded on a 2% agarose gel and run using 1X TAE buffer (**Appendix A**) at 70 V for 30 minutes. The bands were visualized under a UV Transilluminator (VilberLourmat, France). The distance traveled by free 2'-Fluo-AHC-c-di-GMP was accepted as the reference point and samples were evaluated for complexation based on the band shifts caused relative to the reference.

2.2.3. Cell Culture

2.2.3.1. Cell lines and Culture Conditions

2.2.3.1.1. EG7

E.G7-OVA, derived from C57BL/6 (H-2b) mouse lymphoma, is a mouse thymoma cell line. Initial isolates of cells were transfected with pAc-neo-OVA plasmid which results in stable expression of the complete chicken Ovalbumin (OVA) protein. For storage, cells were kept in culture media (complete RPMI 1640, 10% FBS) supplemented with 10% dimethyl sulfoxide (DMSO), in liquid nitrogen. 4-5 days prior to the experiment, cells were thawed in water bath, at 37ºC for 3-4 minutes and suspended in complete RPMI, 10% FBS after primary media was washed away. Cells were grown overnight in T-75 tissue culture flasks containing 1 mg/ml neomycin, washed and then expanded for 4-5 days. For their maintenance, cells were passaged every 2 days by replacing 9/10 of culture with fresh media and grown in a humidified 37°C, 5% CO_2 incubator.

2.2.3.1.2. Culture of primary cells

Single cell suspensions, prepared from mice spleens (Splenocytes Preparation, **Section 2.2.3.2**), were cultured in complete RPMI 1640 medium supplemented with 5% regular FBS in a humidified 37°C, 5% CO2 incubator.

2.2.3.2. Preparation of single cell suspensions from mice spleen

C57BL/6 and BALB/c mice were housed at the Animal House Facility in Molecular Biology and Genetics Department, Bilkent University. All mice used for *in vitro* stimulation studies were adult female or male BALB/c (8-12 weeks old). Animals were sacrificed by cervical dislocation. Animal handling and experimental procedures were in accordance with

ethical considerations, and approved by the animal ethical committee of Bilkent University (Bil-AEC). Following removal of spleens, the organs were placed into 35 mm petri dishes containing 3 ml of media (complete RPMI 1640 containing 2% FBS). To prepare single-cell suspensions, the spleens were mashed using the rubber end of a sterile syringe plunger, collected using a sterile plastic Pasteur pipette and transferred into 15 ml conical falcon tubes while taking care to avoid transferring the fibrous connective tissues. The tubes were then filled with pre-warmed RPMI 1640 (2% FBS) and centrifuged at 1700 rpm for 10 minutes. Supernatants were aspirated and cells were resuspended in 1 ml culture media. The washing step was repeated two more times and the cell pellets were resuspended in 1 ml of RPMI 1640 (5% FBS). Following cell counting (**Section 2.2.3.3**), the cell concentration was adjusted according to the requirement of each experiment.

2.2.3.3. Cell counting

hPBMC, mouse splenocytes or cell lines were resuspended in an appropriate volume of culture medium (usually 1 ml). 10 µl of this cell solution was diluted 10X, mixed with Trypan Blue solution (0.4 %) at 1:1 ratio and then applied to a hematocytometer by capillary action. Cells were counted in 16 small squares, separated as 4 big squares, under a compound microscope. Total cell number was calculated according to the formula given below.

$$\boldsymbol{Number of cells counted \times 2500 \times Dilution factor = Total\ number\ of\ cells\ in\ 1\ ml}$$

Cells were then diluted in RPMI 1640 (5% FBS) to the desired concentration. Working cell concentrations were $4x10^6$ cells/ml for ELISA, and $2x10^6$-$4x10^6$ cells/ml for flow cytometric analyses, unless otherwise stated.

2.2.4. Determination of Immunostimulatory Activities of Complexes

2.2.4.1. *In vitro* Stimulation of Cells with Complexes

Free c-di-GMP (1,66-15 µM), its complexes (diluted in FBS free complete RPMI 1640) and their controls were prepared as described before, at least 30 minutes before stimulation. Depending on the experiment, cell lines, splenocytes or hPBMCs were adjusted to required concentration. For ELISA and flow cytometric analyses, final volumes were adjusted to 200 µl (130 µl cells + 70 µl stimulants) in 96-well flat-bottom plates. Following incubation for 24 hours, plates were spun at 1700 rpm, 5 minutes, and supernatants (~185 µl) were collected and stored at -20ºC until further use for cytokine determination by ELISA. Remaining cells were re-suspended in 100 µl PBS and stained for cell-surface markers as described in the following sections. For cellular internalization experiments and intracellular cytokine staining protocols, cells ($2.5x10^6$/ml, 400 µl) and labeled or unlabeled stimulants (15 µM, 100 µl) were mixed in 15 ml falcon tubes and incubated for 1-7 h, respectively.

Lipofectamine 2000 (Invitrogen, USA) was used according to the manufacturer's protocol. Stimulants were used at the indicated concentrations shown in **Table 2.2**.

Table 2.2 Concentration of stimulants used in experiments

c-di-GMP	1,66-15 µM
c-GMP	1,66-15 µM
Lipofectamine 2000	0,7 µl per sample
Arg(9)	3,32-30 µM
Cucurbituril	3,32-30 µM
CpG	1-3 µM

2.2.4.2. Determination of Cytokine Production by Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants of *in vitro* stimulation assays were obtained as described in **Section 2.2.4.1**. Immulon 2 HB plates were coated with 50 µl of monoclonal antibody in PBS and incubated overnight at 4ºC. Next, contents were expelled by inversion of plates and 200 µl of Blocking buffer (**Appendix A**) was added to wells. Plates were left at room temperature (RT) for 2 hours and then washed with ELISA wash buffer (**Appendix A**). Washing step involved immersion of plates in wash buffer to fill the wells completely, incubation for 5 minutes and inversion of plates to empty the well contents. This process was repeated 5 times, followed by a final wash with ddH_2O 4 times. Plates were tap-dried, culture supernatants were added into wells (50 µl) and incubated for 2 hours at RT. Serially diluted (2-fold) recombinant cytokine standards were similarly prepared. Plates were washed as previously described and 50 µl of biotinylated detection antibody diluted in T-cell Buffer (**Appendix A**) was added to the wells. Coating and detection antibodies diluted 1:200 in PBS prior to use, as manufacturer's protocol suggested, Biolegend, USA. Plates were left overnight at 4ºC and then washed as before. Fresh streptavidin-alkaline phosphatase conjugate (Strep-ALP) solution was prepared at least 1-2 hours prior to use, (1/1000 dilution in PBS buffer) and then 50 µl of this solution was introduced to wells followed by incubation for 1 hour at RT. Plates were washed and developed using 50 µl of PNPP substrate addition. Color development was recorded at 405 nm using an ELISA plate reader, Multiskan FC Microplate Photometer (Thermo Scientific, USA) at 30 minute intervals.

2.2.4.3. Fluorescence-Activated Cell Sorting (FACS) Analysis

2.2.4.3.1. Cell Fixation

Cells (100 µl in PBS; see **Section 2.2.4.1**) were transferred to U-bottom plates and centrifuged at 1700 rpm, 10 minutes. Pellets were fixed using 100 µl of 4% paraformaldehyde (Fixation Medium A - Invitrogen, USA) for 15 minutes at RT and washed

as before using 200 µl of FACS Buffer (**Appendix A**). Fixed cells were kept for a maximum of 1 week at 4°C in FACS buffer until further use.

2.2.4.3.2. Cell surface and intracellular staining

Fixed or live cells were centrifuged and supernatants were discarded. Pellets were resuspended in 100 µl FACS buffer (**Appendix A**) containing 1 µg/ml of fluorescently labeled antibody against the indicated cell surface markers, and incubated for 30 minutes in dark. Following washing in 200 µl FACS Buffer 2-3 times, cells were re-suspended in 200 µl FACS buffer and analyzed using an Accuri C6 Flow Cytometer. Live cells were stained and washed at 4ºC. Where an intracellular component was investigated, staining was performed in 100 µl of permeabilization buffer (Permeabilization Medium B, Invitrogen, USA) containing fluorochrome conjugated antibody (1 µg/ml). Fluorochrome conjugated antibodies used throughout the experiments are listed in the **Table 2.3**.

Table 2.3 Fluorescently labeled antibodies used in this study

Name	Fluorescent Conjugate
Anti-mouse I-A/I-E	PE
Anti-mouse CD86	Alexa Fluor 647
Anti-mouse B220	PE/Cy5

2.2.4.3.3. Analysis of cellular internalization of c-di-GMP and its complexes

Complexes of c-di-GMP/Arg(9) (at molar ratio of 1:2) were prepared as described in **Section 2.2.1**, using either free c-di-GMP or 2'-Fluo-AHC-c-di-GMP. 100 µl of labeled c-di-GMP or its complexes (15 µM) were added to 400 µl of cells ($5x10^6$ cells/ml) and incubated at 37ºC for 2 hours. After incubation, cells were washed; fixed and analyzed using an Accuri C6 Flow Cytometer and Mean Fluorescence Intensities were evaluated.

2.2.5. Vaccination Studies

2.2.5.1. Maintenance of animals

Male or female C57BL/6 (6-8 week old) mice were used for vaccination studies. Animals were kept in Animal House Facility located at Department of Molecular Biology and Genetics, Bilkent University. Mice were maintained in rooms under controlled ambient conditions (22ºC ± 2) regulated with 12 hours light and 12 hours dark cycles. Unlimited access of food and water was supplied to animals in the course of study. All experimental procedures were in accordance with ethical considerations and were approved by the Animal Ethical Committee of Bilkent University (Bil-AEC).

2.2.5.2. Immunization of mice with model antigen OVA adjuvanted with c-di-GMP formulations

6-8 week old C57BL/6 mice were immunized intraperitoneally on days 0 and 14 with 100 µl of PBS, OVA or OVA mixed with different adjuvant formulations including c-di-GMP, its complexes and appropriate controls. On days 15 and 33, blood samples were collected into glass tubes from tail veins by introducing a 1 mm cut using a scalpel blade. Bleeding was stopped by applying pressure. Following clot formation, sera were prepared (**Section 2.2.5.3**) and stored at -20ºC until use. Antigen and adjuvant doses used in immunization experiments are detailed in **Table 2.4**.

Table 2.4 Dose of individual components used in vaccination studies

Components	**Dose**
OVA	7.5 µg/mouse
c-di-GMP	15.0 µg/mouse
MVs	10.0 µg/mouse
K3	10.0 µg/mouse

2.2.5.3. Preparation of Sera

Blood samples collected from immunized mice were tightly capped and incubated in a water bath (37ºC) for 2 h. Following full clot formation, sera were transferred to fresh eppendorfs, and then centrifuged at 8000 rpm for 10 minutes at 25ºC. Sera, separated from red blood cells were collected, transferred to 96-well flat bottom plates and stored at -20ºC until use.

2.2.5.4. IgG ELISA

To determine antigen specific total IgG, IgG1 and IgG2c levels in blood of immunized mice, collected sera were subjected to IgG ELISA protocol. For this purpose, Immulon 1B plates were coated with 50 µl of OVA protein (7.5 µg/ml), diluted in PBS. Plates were gently tapped for uniform distribution of volumes over the surface of wells, and were incubated overnight at 4ºC. Blocking and washing steps were as described (**Section 2.2.4.2**). 50 µl of 16X diluted sera (in PBS) were then introduced to the first wells of plates and 4-fold serially diluted (total of 8 dilutions for each serum sample). Plates were incubated at 4ºC overnight, washed, followed by 50 µl of goat anti-mouse total IgG/ALP, goat anti-mouse IgG1/ALP or goat anti-mouse IgG2c/ALP addition (diluted 1000X in T-cell buffer). After 3 hours of incubation, plates were washed and PNPP substrate was added to wells. Color development was followed at 405 nm using an ELISA reader (Thermo Scientific, USA), as described before.

2.2.5.5. Tumor Challenge using OVA Expressing E.G 7 Cell Line

34 days after the booster injection (day 51), OVA immunized mice were challenged with E.G7-OVA tumorigenic cell line. As described previously, E.G7 cells synthesize OVA protein constitutively and thus OVA is considered as a tumor antigen in this model. E.G7 cells were expanded as described in **Section 2.2.3.1.2**. $4x10^6$ tumor cells were injected subcutaneously into the dorsal flank of each mouse. After the tumors reached a palpable size (~50 mm^3, one week after tumor initiation), tumor volumes were measured using a caliper every other day and recorded as Length x Width x Height.

2.2.5.6. IFN-gamma ELISPOT

To detect antigen specific CD8+ T-cell responses, we performed IFN-gamma ELISPOT using immunized mice splenocytes stimulated in vitro with the H-2Kb-restricted OVA class I epitope SIINFEKL. 1 day prior to experiment, Immulon 2HB plates were coated with 50 µl of anti-mouse IFNγ antibody (5 µg/ml in PBS). Plates were incubated for 4-5 h at room temperature, blocked with 200 µl of Blocking Buffer and incubated overnight at 4ºC. On the day of the experiment, plates were washed as described before (**Section 2.2.4.2**) and were kept in a sterile environment until use. Mice were sacrificed (day 67) and single cell splenocytes were prepared according to the protocol described in **Section 2.2.3.2**. Splenocyte concentrations were adjusted to $20x10^6$ cells/ml. Template plates for serially diluted splenocytes were prepared as follows: First wells of the template plate was filled with 200 µl of $20x10^6$ splenocytes/ml from individual mice in duplicate and serially diluted ¼-fold by transferring 50 µl from the 1st wells to 150 µl cell culture medium containing 2nd wells. Following pipette-aided mixing, the same process is repeated for the 3rd and 4th wells to create 4 different cell concentrations. Meanwhile, 150 µl of cell culture medium, with or without the H-2Kb-restricted OVA class I epitope SIINFEKL (20 µg/ml), were added into the coated plates and 50 µl of the above described template plate associated cell suspensions were added to the first 4 rows of the coated plates so that from top to bottom, each row contained $1x10^6$, $2.5x10^5$, $6.25x10^4$ and $1.56x10^4$ cells/well, respectively. Plates were then incubated at 37ºC, 5% CO^2 for 14-18 hours, taking care not to move any of the plates during the entire incubation period. Next day the plates were washed as in **Section 2.2.4.2**, with a minor difference in the Wash Buffer (PBS is replaced by ddH2O). The modified wash buffer ensures complete removal of cells from the assay plates. For detection antibody, 50 µl of biotinylated goat anti mouse IFNγ antibody (1 µg/ml diluted in T-cell buffer, **Appendix A**) was added to wells, and plates were incubated for 2 h, at room temperature. Following washing, 50 µl of Streptavidin-Alkaline phosphatase solution (diluted 1:1000 in T-cell Buffer, **Appendix A**) were added to wells, incubated for 1 h and washed for the final time. To develop the spots, BCIP-low melting agarose substrate was used (70 µl/well). To prepare the BCIP-Agarose substrate, 4 ml of BCIP solution (heated to 45ºC) was mixed with microwave-dissolved low melting agarose (0.03 g in 1 ml of ddH2O). This substrate solution was kept at 45ºC to prevent hardening of agarose before addition to wells. 1 h after substrate addition and agarose solidification, plates were sealed with a plate sealer and spots were counted using a dissecting microscope on the next day.

2.2.5.7. Statistical Analysis

Data obtained from different experimental groups were statistically analyzed using IBM SPSS v17.0 software. Student T-test (one tailed unpaired comprasion) was conducted between untreated and treated groups. A P value of < 0.05 was considered as significant.

CHAPTER 3

RESULTS & DISCUSSION

3.1. c-di-GMP forms efficient complexes with nonamer arginine peptide

The physicochemical properties of native c-di-GMP prevent its free passage across cellular membranes[63]. Since the target receptor for c-di-GMP, stimulator of interferon genes (STING), resides on the endoplasmic reticulum membrane, it is vital for its ligand (c-di-GMP) to achieve efficient entry into the cytosol in order to trigger an observable response[45]. To overcome this challenge by enhancing the cellular uptake of c-di-GMP, we evaluated the feasibility of a simple complexation strategy that would not interfere with target (STING) recognition. To this end, we chose certain cationic peptides/polymers, Arg(9), Lys(9), and polyethyleneimine (PEI), as complexation agents since these molecules are well known membrane transduction agents. For example, Arg(9) is a positively charged small peptide, with known cell penetrating properties. It is taken into cells via the endosomal route and has no observable cytotoxicity to eukaryotic cells[64,65]; on the other hand Lys(9) and PEI are also positively charged and can act as transfection reagents for their associated cargo [66,67]. To evaluate whether these molecules could form complexes with c-di-GMP, fluorescently labeled form of the cyclic di-nucleotide (2'-Fluo-AHC-c-di-GMP) was mixed with different molar ratios (1:1, 1:2) of Arg(9), Lys(9) or pEI and complexation was followed on an agarose gel as described in **Section 2.2.2.1**. Assesment of complex formation was done based on band shifts of the samples relative to the reference sample (2'-Fluo-AHC-c-di-GMP alone). Of the three cationic molecules tested, only c-di-GMP/Arg(9) showed increased retardation on the gel, suggesting complexation between these two molecules (**Figure 3.1**). Complexation efficiency increased when a higher concentration of Arg(9) was used. In contrast, Lys(9) or PEI did not cause a significant band shift. These results suggest that c-di-GMP/Arg(9) complexation is not a simple electrochemical attraction process and might involve specific interaction of the cyclic dinucleotide with the positively charged guanidino group of Arginine[68]. Of interest, Arg is the only amino acid with this functional group, and the absence of the guanidino group in Lys(9) may explain lack of complex formation even if the net charges of both peptides are identical (= +9).

Following elimination of Lys(9) and PEI as ineffectual complexation agents, the utility of an alternative macrocylic drug delivery vehicle was also tested in complexation trials. Cucurbit(n)urils are small barrel-shaped structures composed of 5, 6, 7, 8 or 10 glycoluril subunits that can form host-guest complexes with various organic and inorganic small molecule drugs[69]. To test whether cucurbit(6)uril could facilitate transfer of c-di-GMP into

cytosol, fluorescently labeled c-di-GMP was mixed with cucurbituril at 1:1, 1:2 and 1:4 molar ratios. Parallel to this study, additional molar ratios of c-di-GMP/Arg(9) complexes (1:0.5, 1:1, 1:2 and 1:4) were also tested to further analyze the effect of peptide amount on

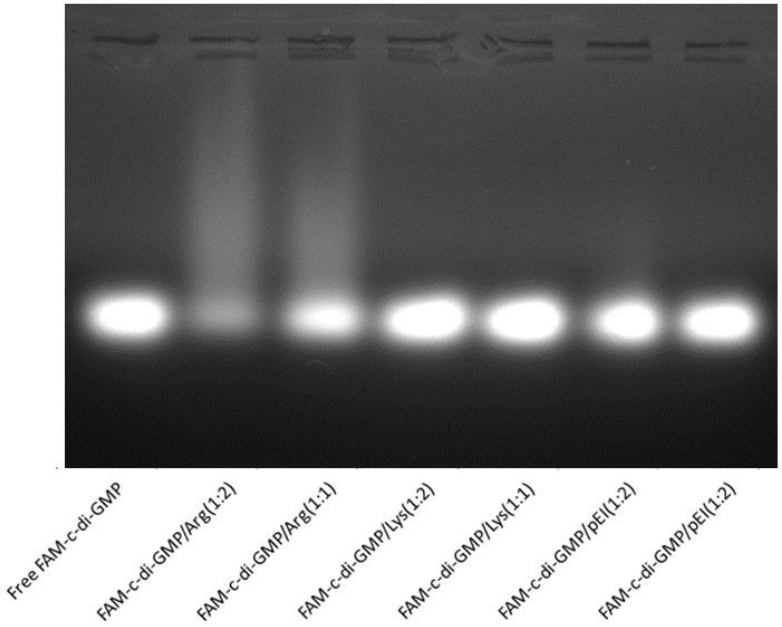

Figure 3.1 Agarose gel electrophoresis of c-di-GMP/cationic peptide or polymer complexes Fluorescently labeled 2'-Fluo-AHC-c-di-GMP (0.67 μg each based on c-di-GMP amount) and its complexes were loaded onto a 2% agarose gel) and run at 70 V for 45 minutes. The bands were visualized under a UV Transilluminator. The gel image is a representative of three independent experiments, each giving similar results.

complexation efficiency. **Figure 3.2** shows that c-di-GMP/Arg(9) complexation efficiency increased when molar ratios of 1:2 and 1:4 were used. At these molar ratios, c-di-GMP-associated bands were more delayed relative to the reference sample. In contrast, c-di-GMP/cucurbituril demonstrated no such band shifts.

c-di-GMP is a quite distinct molecule, capable of forming bimolecular, tetramolecular and octamolecular structures in the presence of monovalent cations[70], such as Na^+ and K^+. To assess whether c-di-GMP/Arg(9)complexation may be further enhanced in the presence of such ions, c-di-GMP was mixed with Arginine at molar ratio of 1:1, 1:2 and 1:4 either in presence or absence of KCl. Furthermore, to investigate the role of Arginine residues in complexation Arg(9) homopolymer was replaced with another cationic cell-penetrating peptide, Tat(45-57). Tat peptide is an HIV Tat protein-derived 11-mer peptide with a net charge of +8 and has the following sequence; YGRKKRRQRRR[71]. Being rich in arginine

residues, it was included in the study to see whether it would show the same effect seen with nonamer Arginine. The gel image of one representative study is shown in **Figure 3.3**. Compared to Arg(9), Tat(11) formed inefficient complexes even when used at the highest ratio of 1:4. On the other hand, presence of cations in the solution had no significant effect on efficiency of complexation with Arg(9) as comparable amounts of band shifts took place with or without K^+ ions.

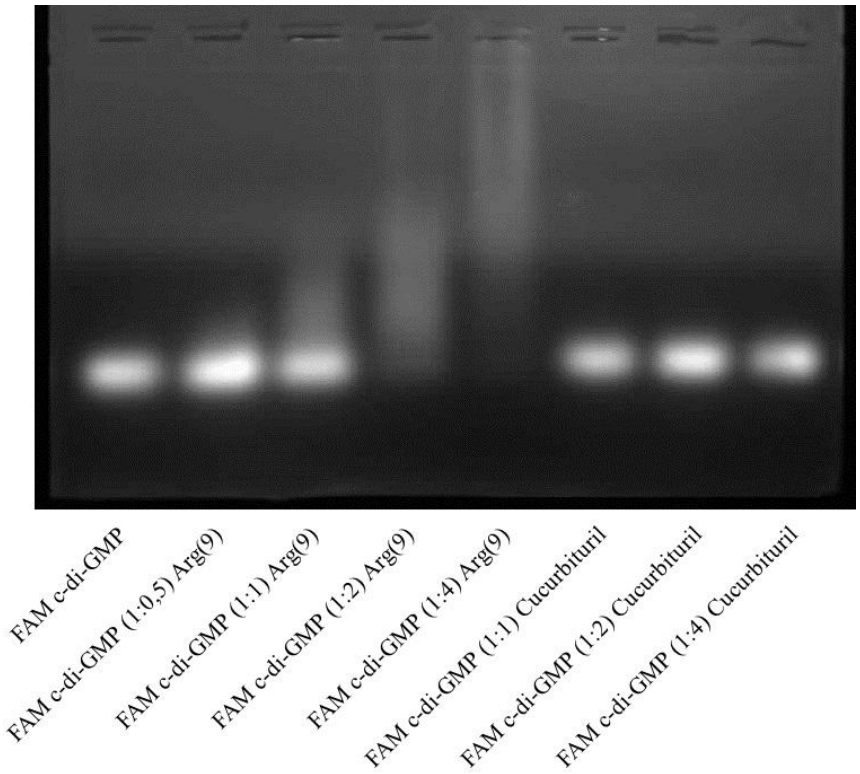

Figure 3.2 Agarose gel electrophoresis of c-di-GMP/Arg or c-di-GMP/cucurbituril complexes. Fluorescently labeled 2'-Fluo-AHC-c-di-GMP (0.67 μg) was mixed with increasing molar ratios of Arg(9) (1:0.5, 1:1, 1:2 and 1:4) or cucurbituril (1:1, 1:2 and 1:4). Samples were loaded onto a 2% agarose gel and run at 70 V for 30 minutes. The bands were visualized under a UV transilluminator. The gel image is a representative of three independent experiments, each giving similar results.

In summary, based on the results presented in **Figures 3.1-3**, Lys(9), PEI, and Tat peptide were excluded from later studies. However, since cucurbituril is a relatively small molecule, agarose gel electrophoresis may not be an ideal method to observe a miniscule band-shift to support the presence of a host-guest interaction with c-di-GMP. Therefore, although positive data for c-di-GMP/cucurbituril interaction was absent, this approach was further tested in *in*

vitro stimulation assays. For Arg(9) incorporating complexes, since molar ratios of 1:2 and 1:4 showed equivalent complexation efficiencies, later studies included only the 1:2 formulation. The finding that Arg(9) uniquely forms complexes with c-di-GMP is an interesting outcome and is in support of published results showing the importance of Arginine 231/232 in STING-mediated response to the cyclic nucleotide[72]. Absence of covalent bonding between c-di-GMP and Arg(9) is of benefit since the complexes are readily dissociable in cellular compartments and the ligand is free to interact with its receptor. Another advantage of using Arg(9) for c-di-GMP delivery is its distinct property to rupture

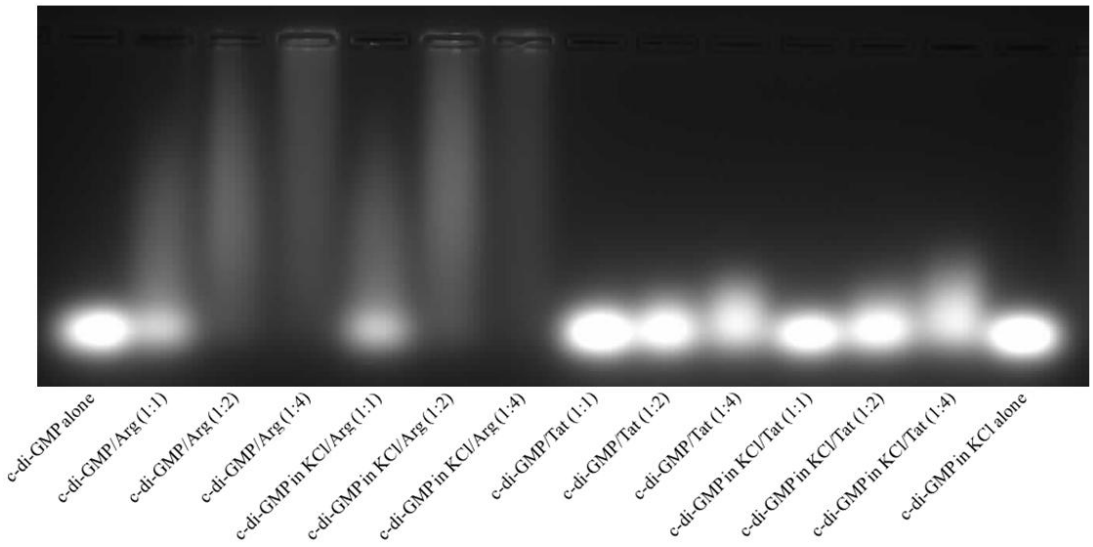

Figure 3.3 Agarose gel electrophoresis of c-di-GMP/Arg(9) or c-di-GMP/Tat complexes prepared in the absence or presence of KCl. Fluorescently labeled 2'-Fluo-AHC-c-di-GMP (0.67 μg) was mixed with increasing molar ratios of Arg(9) or Tat peptide (1:1, 1:2 and 1:4) in the absence or presence of KCl (15 mM). Samples were loaded onto a 2% agarose gel and run at 70 V for 30 minutes. The bands were visualized under a UV transilluminator. The gel image is a representative of three independent experiments, each giving similar results.

of the endosomal barrier[73], enabling transfer of its associated cargo into the cytosol. Thus, use of this peptide may not only enhance uptake of c-di-GMP by cells but could also contribute to its delivery to the cytosol. Moreover, the simplicity of this strategy, i.e complexation following mixing of two components, could be harnessed in clinical development of this agent as a potential vaccine adjuvant.

3.2. c-di-GMP/Arginine complexes are internalized more efficiently in mouse splenocytes.

Having identified c-di-GMP/Arg(9) at 1:2 nonaarginine to c-di-GMP ratio as the optimum molar quotient to achieve complexation, we next analyzed whether these complexes would be internalized more efficiently than the free c-di-GMP. For this purpose, complexes prepared in the presence of fluorescently labeled cyclic nucleotide were incubated with mouse splenocytes for 2 hours and internalization was assessed based on Mean Fluorescence Intensities associated with the cells. Results show that, FAM labeled c-di-GMP/Arg(9) complexes were internalized at least 4-fold more when compared to FAM labeled c-di-GMP alone (**Figure 3.4-A**). To evaluate whether a specific population of cells internalized the complexes at different rates, samples were stained for the B cell marker B220/CD45R and extent of FAM labeled c-di-GMP signal was quantitated in $B220^+$ (**P3 gate, Figure 3.4-B**) and $B220^-$ cells (**P4 gate, Figure 3.4-B**). Results revealed that (**Figure 3.4-B**) extent of cellular internalization of free FAM-c-di-GMP (**red histograms**) or its Arg(9) complexes (**blue histograms**), did not vary among different cell types (**upper panel for B220+ cells, lower panel for B220- cells**). For the P3 or the P4 gated cells, FAM-c-di-GMP associated fluorescence signal was 5,5-6 fold of the free cyclic nucleotide. In conclusion, these results show that complexation enhances the cellular uptake of c-di-GMP for all cell types.

3.3. Assessment of in vitro immunostimulatory activity of c-di-GMP and its complexes in mouse splenocytes

Mouse spleen contains several subsets of myeloid and lymphoid cell lineages. Among these, dendtritic cells are pivotal in presenting antigens to T lymphocytes, forming the link between innate and adaptive immunity[74]. In such antigen presenting cells (APCs), surface MHC Class II molecules are required to present antigen-derived peptides to specific helper T cells. Moreover, expression of co-stimulatory molecules (such as CD80, CD86, and CD40) on APCs is required for full activation of T cells[75]. In the absence of an insult, APCs have a basal level of MHC Class II and co-stimulatory molecule expression. Following exposure to components of a pathogen, there is a distinct upregulation in expression levels of MHC Class II and co-stimulatory molecules. Since c-di-GMP is perceived as a danger signal, as other PAMPs such as LPS or dsRNA, it would be expected to induce upregulation of aforementioned cell surface molecules in antigen presenting cells[76]. Consequently, these two cell surface proteins (CD86 and MHC Class II) were chosen as specific markers to evaluate the immunostimulatory activity of c-di-GMP.

To evaluate maturation of APCs, splenocytes were first stimulated with various concentrations of free c-di-GMP (5 μM, 15 μM, 45 μM, and 135 μM) for 24 hours. TLR4 ligand lipopolysaccharaide (LPS) was used as a positive control, whereas unstimulated cells served as negative controls. Following incubation, cells were fixed and stained for MHC Class II and CD86. Flow cytometric analysis of stained cells shows that the increase in

percent of MHC Class II/CD86 double positive cells correlated with an increase in c-di-GMP concentration up to a dose of 15 µM (**Figure 3.5-A**). Higher doses of c-di-GMP did not contribute to an increase in the double positive cell numbers, indicating a saturation point for the immunostimulatory effect of c-di-GMP. For further evaluation of ligand concentration and stimulation capacity, splenocytes were incubated with additional concentration of c-di-GMP (1.66 µM, 5 µM, 15 µM, and 45 µM, **Figure 3.5-B**). Based on these results, a dose interval of 1.66-15 µM was chosen to be the suitable range for future studies.

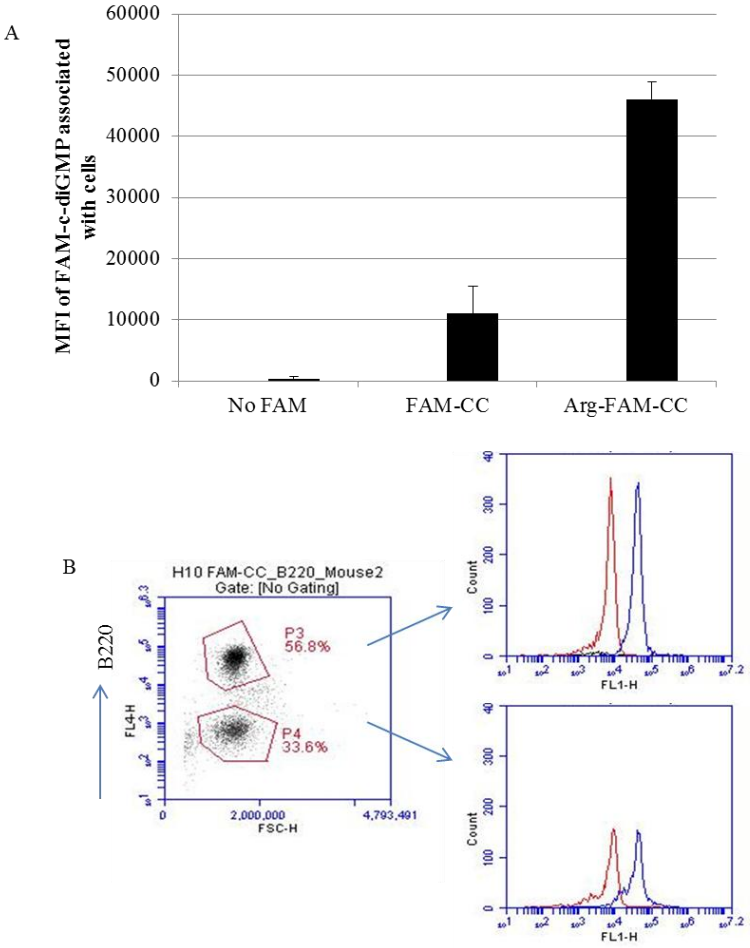

Figure 3.4 Complexes of c-di-GMP/Arg(9) are internalized more efficiently than free cyclic dinucleotide. Free 2'-Fluo-AHC-c-di-GMP (15 µm) or an equivalent amount of its complex with Arg(9) (at 1:2 molar ratio) was incubated with splenocytes ($5x10^6$ cells/ml) at 37ºC for 2 hours. **(A)** Mean fluorescence intensity in whole splenocytes (MFI) ± S.D (average of four individual measurements). **(B)** MFI associated with $B220^+$ (P3gate and upper right histograms) and $B220^-$(P4 gate and lower right histograms) is shown for free 2'-Fluo-AHC-c-di-GMP (red histograms) and its complex with Arg(9) (blue histograms).

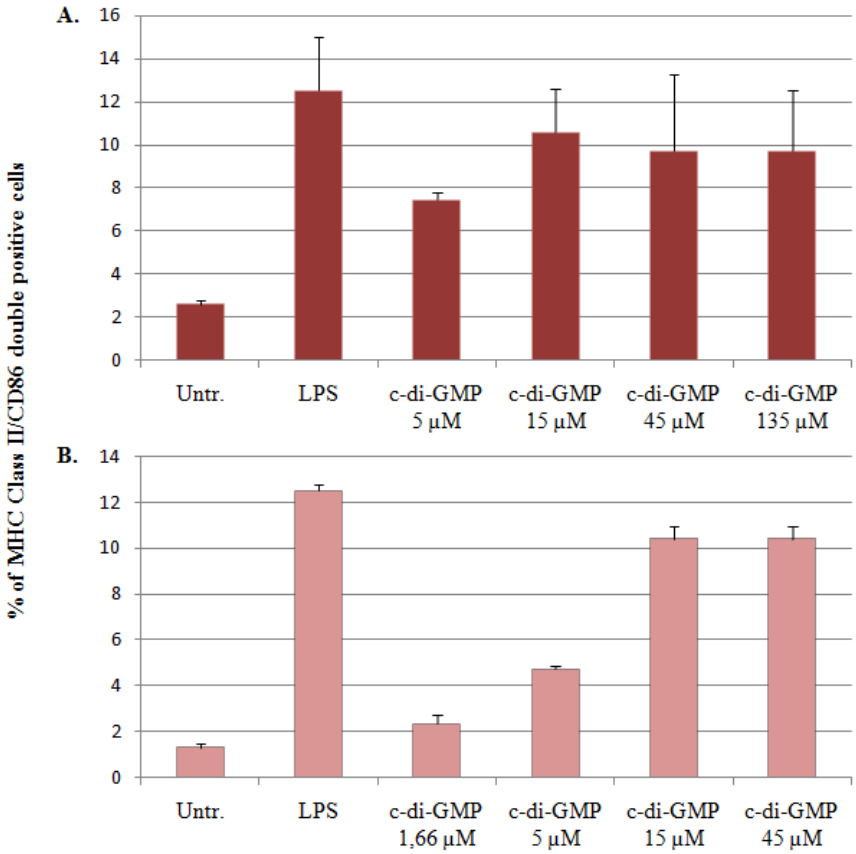

Figure 3.5 Determination of stimulation capacity of c-di-GMP at different doses in mouse splenocytes. Spleen cells (2.5x 10^6/ml) were stimulated with 5 µM, 15 µM, 45 µM, and 135 µM **(A)** or 1.66 µM, 5 µM, 15 µM, and 45 µM **(B)** c-di-GMP. 24 h later, % of MHC Class II/CD86 expressing cells were determined by flow cytometry

Next, in order to assess whether Arg(9) complexation or cucurbituril association had a beneficiary effect on the immunostimulatory activity of c-di-GMP, mouse splenocytes were stimulated in vitro with various formulations and appropriate positive controls. For Arg(9) incorporating complexes, molar ratio of 1:2 was chosen since those complexes yielded the optimum complexation (see **Figure 3.2**). For cucurbituril host-guest association, the ratio of drug to host was also adjusted to be 1:2. All assays were performed using 3 different c-di-GMP concentrations (1.66 µM, 5 µM, 15 µM). cGMP, a structurally similar analog of c-di-GMP without the immunostimulatory activity was used at the maximum dose (15 µM) as the negative control. Following 24 h of incubation, stimulated cells were stained with fluorescently labeled anti-mouse I-A/I-E (MHC Class II) and anti-mouse CD86 as explained before. Results presented in **Figure 3.6-A** show that c-di-GMP/Arg(9) complexes significantly increased the percentage of MHC Class II/CD86 double positive cells relative to free c-di-GMP containing groups. Of note, 5 µM of c-di-GMP/Arg(9) showed equivalent

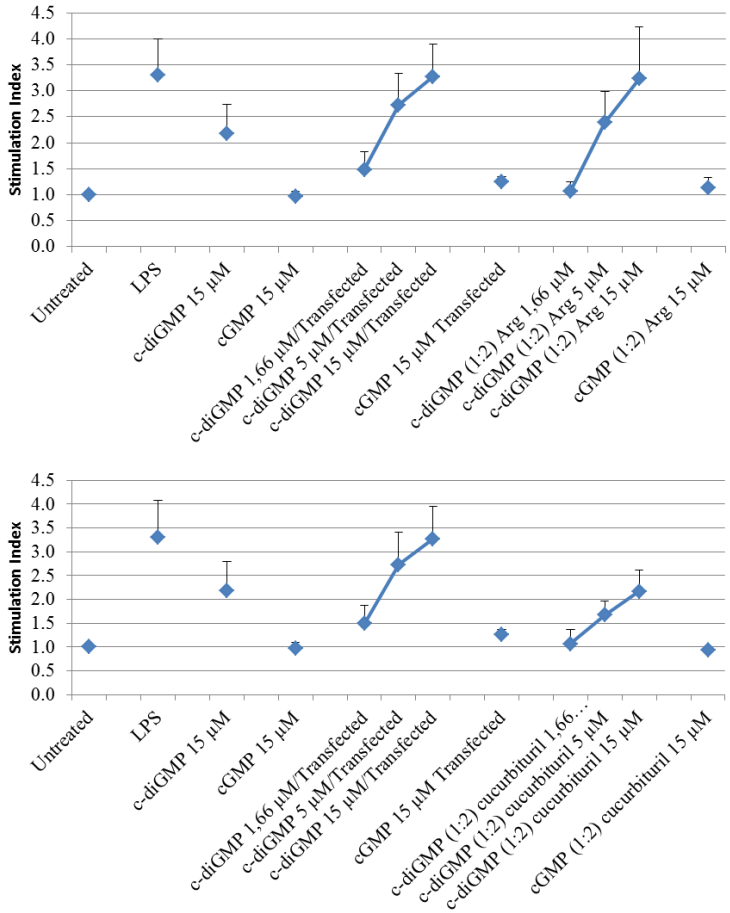

Figure 3.6 Stimulation indexes of complex formulations in mice splenocytes. Complexes and transfected or free controls were cultured with splenocytes ($2.5x10^6$ cells/ml) for 24 hours. Stimulation indexes were calculated according to fold increase in MHC Class II^+ $CD86^+$ double positive cells. c-di-GMP/Arg(9) complexes **(A)** and c-di-GMP/cucurbituril mixes **(B)** were compared to the stimulatory properties of c-di-GMP alone or its transfected controls. Values are average of six individual mice. Significance among groups were tested using stundent's t-test, P value < 0.05*

stimulatory activity observed with a 3X dose of the free drug (15 µM) (**Figure 3.6-A**). The immunostimulatory activity of c-di-GMP/(Arg9) was comparable to Lipofectamine 2000 transfected groups, suggesting that the compound was efficiently transferred to the cytosol. The negative controls (cGMP, lipofectamine2000 transfected cGMP and cGMP(1:2)Arg(9)) did not change the expression levels of the cell-surface markers, indicating that the response was specific to the cyclic di-nucleotide and not to the complexation agents. Cucurbituril containing complexes also increased the number of double positive cells in a dose dependent manner (**Figure 3.6-B**). However, there was no significant improvement when compared to the free drug. Therefore, further studies with cucurbituril complexation were abandoned.

In summary, these results show that complexation with Arg(9) peptide increases the cytosolic availability and hence the immunostimulatory activity of c-di-GMP and may replace the need to transfect the drug through the use of cytotoxic lipid-based transfection reagents.

3.4. In vitro cytokine responses of mouse splenocytes stimulated with c-di-GMP and its formulations

Studies concerning the immunogenicity of PRR ligands indicate that besides the ability to stimulate antigen presenting cell maturation, profile of cytokines induced is also important in eliciting appropriate inflammation at the infection site, and to achieve differentiation of antigen-stimulated lymphocytes in the lymph node[77,78]. The nature of the cytokines secreted from antigen presenting cells is considered as the third signal that shapes the differentiation of helper T cells. For example, IL-12 drives T_H1 type helper cell development[79,80]. Having such importance in shaping of adaptive immunity, we next focused on determining the cytokine profile induced by c-di-GMP and its complexes.

Published results indicate that c-di-GMP stimulates the secretion of pro-inflammatory cytokines such as IL-12, TNFα and in particular the type I Interferon, IFNβ[56,45]. To assess the activity of Arg(9) complexed c-di-GMP, secretion of pro-inflammatory cytokines (TNFα, IL-12 and also IL-6) from stimulated splenocytes were assessed by cytokine ELISA (**Figures 3.7-A, B and C**, respectively). The lipofectamine 2000 transfected c-di-GMP and its complex with Arg(9) induced significantly higher levels of TNFα than the free drug at the highest dose tested ($P<0.05$, **Figure 3.7-A**). c-di-GMP/Arg(9) and the free drug triggered similar levels of IL-6 whereas transfection significantly elevated IL-6 secretion (**Figure 3.7-B**). In contrast, for IL-12, only the Arg(9) complexed group triggered significantly higher levels of IL-12 (**Figure 3.7-C**). Among the three cytokines tested, IL-6 is unique as it is classified as both a pro-inflammatory and an anti-inflammatory cytokine[81]. It can activate the immunosuppressive cytokines such as IL-1 receptor antagonist and IL-10[82]. In this context, lack of significant IL-6 secretion in c-di-GMP/Arg(9) stimulated samples coupled with favorable levels of IL-12 and TNFα suggests that this formulation may drive development of T_H1-type of immunity; and hence could be of value as a vaccine adjuvant to provide specific protection against intracellular pathogens.

3.5. c-di-GMP synergizes with the synthetic TLR 9 agonist CpG oligo deoxy nucleotide (ODN) to induce co-stimulatory marker upregulation and proinflammatory cytokine secretion

Emerging evidence suggests that to induce an effective immune response, use of multiple pattern-recognition receptor ligands can result in synergistic effects that modulate the innate and the adaptive immunity[83]. Rationale behind such studies is to mimic a natural infection by

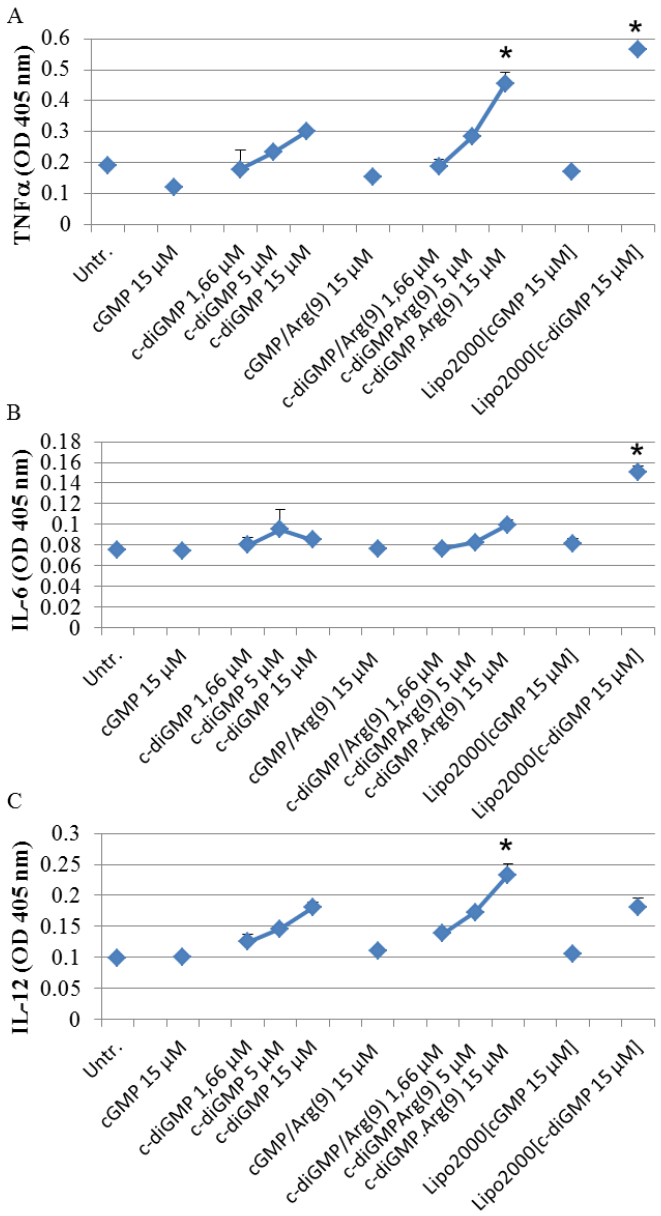

Figure 3.7 Pro-inflammatory cytokine secretions in supernatants of splenocytes cultured with the indicated groups. TNFα **(A)**, IL6 **(B)**, and IL12 **(C)** were quantitated from culture supernatants using ELISA. Results are the average of three individual mice. Significance among groups were tested using stundent's t-test, P value < 0.05*

a pathogen, which usually involves more than one Pathogen Associated Molecular Pattern (PAMP). In theory, multiple ligands targeting a variety of PRRs are expected to increase the immunogenicity of the vaccine formulation in question. There are studies confirming the synergistic activity of certain PRR agonist pairs[84,85]. Yet, depending on the PRR ligand

combination used, antagonistic effects have also been reported[86]. Based on the aforementioned information, we intended to explore possible synergism between c-di-GMP and CpG-ODN mediated immune activation. The rationale was to combine two different PRR ligands that utilized receptors residing in different subcellular compartments: c-di-GMP signaling through cytosolic receptor, STING, and CpG ODN signaling through endosomal receptor, TLR9. Of interest, there have been no published studies exploring the adjuvant activity of such a combination.

To evaluate the imminustimulatory activities of c-di-GMP and CpG-ODN in combination, increasing concentration of c-di-GMP was mixed with a fixed amount of CpG-ODN (Optimum CpG-ODN dose of 1 μM) and percentage of MHC Class II/CD86 expressing cells were compared to those stimulated with single ligands. Flow cytometric analyses of cells stimulated with 3 different doses of c-di-GMP alone showed a dose-dependent increase in the number of double positive cells (**Figure 3.8, middle panels**) and were consistent with our previous results. Similar to c-di-GMP alone (15 μM), optimum dose of CpG-ODN alone (1 μM) induced ~11 % of MHC Class II/CD86 double positives as opposed to ~1.5 and ~1.9 % observed with unstimulated or cGMP treated cells (negative controls), respectively. When used alone at their optimum doses, immunostimulatory activities of free c-di-GMP and CpG-ODN were comparable to LPS control. Interestingly c-di-GMP synergized with CpG-ODN to induce significantly elevated levels of double positive cells (**Figure 3.8, lower panels**). The combination of 15 μM c-di-GMP and 1 μM CpG ODN improved marker upregulation ~22-fold and ~3-fold when compared to unstimulated or CpG ODN stimulated samples, respectively.

Similarly, c-di-GMP synergized with CpG ODN and induced significantly higher levels of IL-12, IL-6 and TNFα from mouse splenocytes with respect to the maximum doses of the single ligands ($P<0.05$, **Figure 3.9**, 15 μM c-di-GMP+1 μM CpG ODN combination). Particularly, IL6 and TNFα secretion was drastically augmented. These results suggest that these two PRR ligands may be used in combination as an effective vaccine adjuvant.

3.6. In vivo vaccine adjuvant activity of c-di-GMP, its formulations and combined c-di-GMP+CpG

Thus far, several successful approaches were developed to increase the immunogenicity of c-di-GMP *in vitro*, including cationic peptide complexation and synergistic behavior with CpG ODN. Moreover, encapsulation in commensal bacteria-derived membrane vesicles (MVs of *Pediococcus pentosaceus*) was evaluated as a good delivery system in consideration of successful encapsulation of c-di-GMP, as previously shown. However, whether these strategies improved the vaccine adjuvant potential of the drug was yet to be determined, since there are several cases which show no efficacy *in vivo* although having promising *in vitro* stimulatory properties[87].

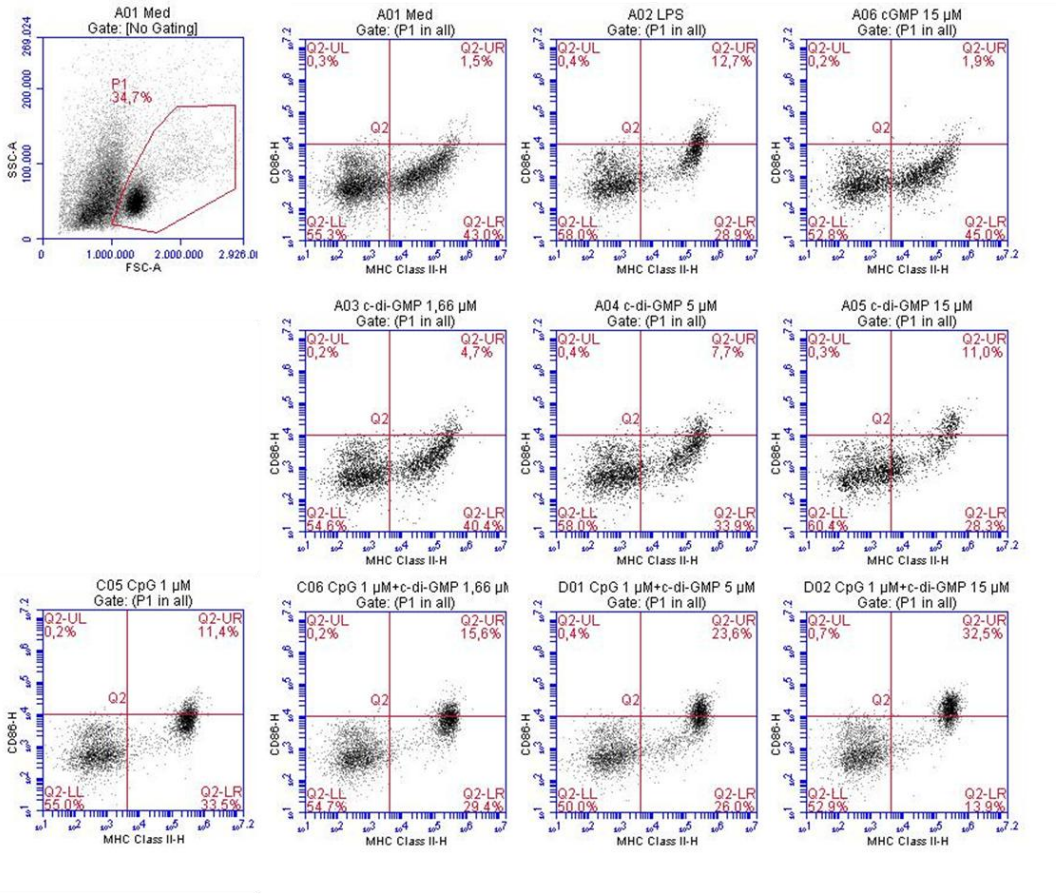

Figure 3.8 Dot plot graphs of mice splenocytes cultured with the indicated formulations for 24 h. Antigen presenting cells were gated based on their FSC SSC characteristics (P1). MHC Class II (x-axis) and CD86 (y-axis) expression was determined using fluorescently labeled specific antibodies. Cells were analyzed using Flow Cytometer. Graphs are representative of three independent experiments, with similar results.

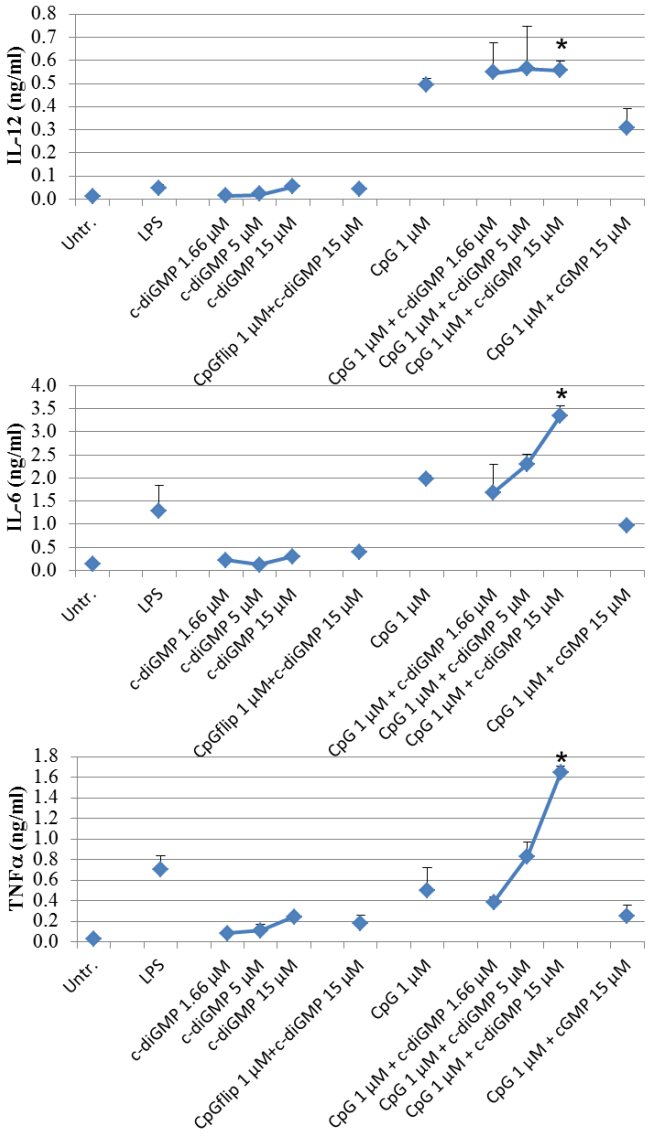

Figure 3.9 Pro-inflammatory cytokine secretions in supernatants of splenocytes cultured with the indicated groups. TNFα **(A)**, IL6 **(B)**, and IL12 **(C)** were quantitated were quantitated from culture supernatants using ELISA. Results are the average of three individual mice. Significance among groups was tested using stundent's t-test, P value < 0.05*

To test these formulations *in vivo*, C57BL/6 mice were immunized (five animals per group) intraperitoneally with PBS, Ovalbumin (OVA), or OVA adjuvanted with c-di-GMP and its formulations on days 0 and. 14 days after both injections, and sera were collected from animals as described in **Section 2.2.5.3**, to quantitate OVA specific antibody levels. OVA protein is one of the most widely used model antigens in immunization studies and hence was chosen for testing the potency of our vaccine adjuvant formulations[88]. For the antibody subclasses to be measured, IgG1 and IgG2c (IgG2a isotype in C57BL/6 mice) were

chosen since class switching to these subtypes depends on the type of T cell help provided to B lymphocytes (T_H2 versus T_H1, respectively)[89,90]. **Figure 3.10** presents the average OVA-specific IgG1 and IgG2c responses of groups of 5 mice immunized with the formulations indicated in the legends. The serial dilutions of individual mice sera aid to show that the response (i.e. the observed color development) correlates with the amount of antibody present and is not just an artifact. Moreover, it enables comparison of OD values between groups at a specific serum dilution. Based on the general results presented below, the response of individual mice from each group was compared using a serum dilution of 1/32768 as indicated on the figures.

In terms of IgG1 responses, c-di-GMP/Arg(9) triggered significantly higher levels OVA-specific antibody production with respect to OVA+c-di-GMP immunized group ($P<0.05$, **Figure 3.11-A**). This may be explained by the higher internalization patterns that were observed *in vitro*. Moreover, complexation may have improved half-life of c-di-GMP in blood circulation, as the cationic peptide may have provided protection from phosphodiesterases that naturally cleave the phosphodiester bonds present in the structure of the cyclic dinucleotide. Of interest, c-di-GMP alone significantly increased OVA-specific IgG1 levels ($P<0.05$), whereas CpG ODN had no effect on this subclass of Immunoglobulin G. This is to be expected since CpG ODN is known to be a strong T_H1 skewing adjuvant[91]. Combination of c-di-GMP+CpG did not change the specific IgG1 titers when compared to c-di-GMP adjuvanted group. The alternative strategy to use commensal bacteria-derived MVs as a carrier to deliver c-di-GMP was tested in this vaccination model using 3 different formulations; i) c-di-GMP was administered as a mixture with MVs and OVA (OVA+c-di-GMP+MV), ii) it was encapsulated in MVs and then mixed with OVA (OVA+[c-di-GMP+MV]) or iii) both the drug and the antigen was co-encapsulated in MVs [OVA+c-di-GMP+MV].The rationale lies behind was to see any immunomodulatory behaviour of MVs on their own (i), to assess whether encapsulation in MVs could improve the immunostimulatory activity of c-di-GMP (ii), and to investigate whether co-delivery of the antigen and the adjuvant simultaneously would improve immunogenicity (iii). Results show that compared to c-di-GMP adjuvanted group, c-di-GMP+MV did not alter levels of OVA specific IgG1. In contrast, encapsulation in MVs significantly increased specific IgG1 titers ($P<0.05$, OVA+[c-di-GMP+MV]). Co-encapsulation displayed no added benefit. A separate study from our laboratory demonstrated that commensal bacteria-derived membrane vesicles exert immune-modulatory effects possibly because the combination of PRR ligands present in the structure of these MVs negatively regulate production of pro-inflammatory cytokines and enhance secretion of the immune-modulatory cytokines, IL-10. However, following c-di-GMP encapsulation, MVs would be enriched in their "danger signal" content, and the drug would be protected from phosphodiesterase attack, resulting in augmented adjuvanticity. In summary, encapsulation of c-di-GMP in MVs proved to be a successful approach to increase the immune stimulatory potential of the drug.

Evaluation of IgG2c responses indicated that when used on their own, both CpG ODN and c-di-GMP stimulated T_H1 development and thus enhanced the OVA-specific titers ($P<0.05$ for both groups, **Figure 3.11-B**). In contrast to IgG1 responses, c-di-GMP/Arg(9) did not improve OVA-specific titers when compared to the cyclic nucleotide alone. Co-administration of c-di-GMP+CpG-ODN strongly elevated the IgG2c antibody titers, in

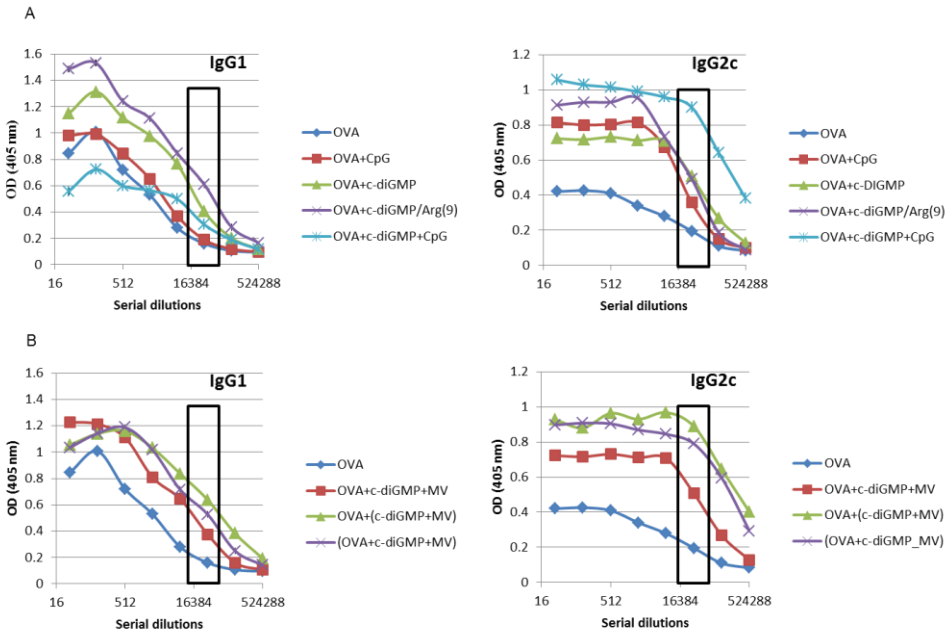

Figure 3.10 Average OD versus serial dilution graphs of sera collected from immunized mice (average of 5 mice/group). Ova specific IgG1 and anti-IgG2c levels of c-di-GMP/Arg(9), c-di-GMP+CpG or the single use ligand groups **(A)** and MV capsulated samples **(B)** were assessed using ELISA. Results are average of five individual mice.

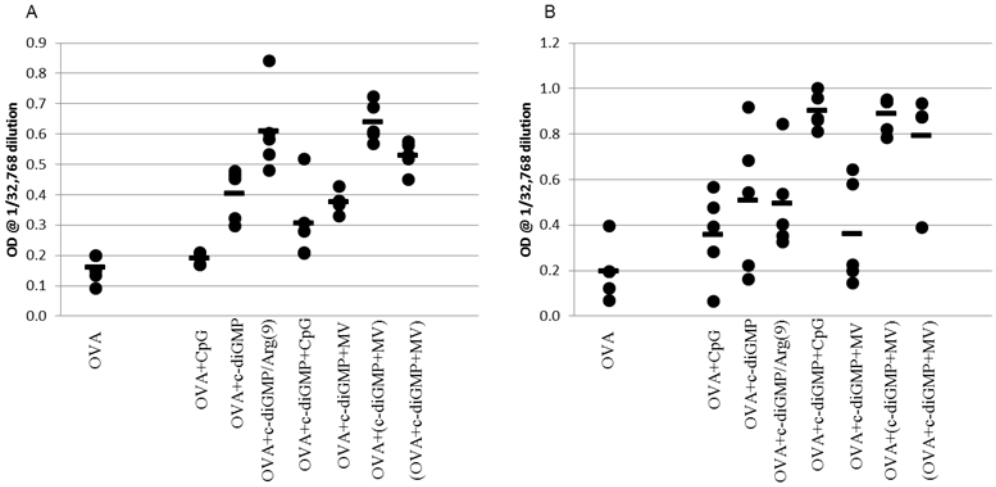

Figure 3.11 Antibody titers of individual mice immunized with the indicated groups were measured at 1/32,768 dilution. Ova specific IgG1 **(A)** and anti-IgG2c levels **(B)** of individual mice.

which analysis of IgG2c/IgG1 ratio was highest, indicating a very strong T_H1-biased response. Of the MV containing groups, OVA+[c-di-GMP+MV] was the most effective in inducing OVA-specific IgG2c.

In summary, all formulations tested improved the overall antibody responses. Strongest Th1 polrizations were observed with c-di-GMP+CpG-ODN combination and c-di-GMP encapsulated in MVs.

3.7. Effect of c-di-GMP containing formulations in a tumor protection model

As summarized above, all c-di-GMP formulations tested, except the c-di-GMP+MV mixture and Arg(9) complexed groups, triggered a T_H1 dominated response. Arg(9) complexation elevated IgG1 levels but did not alter the IgG2c levels relative to the free drug, indicating a mixed T_H1/T_H2 type of response. Having evidence in support of a T_H1 polarized responses with formulations, we next focused on assessing the development of cell-mediated immunity in these mice. Specifically, mice previously vaccinated with OVA antigen plus c-di-GMP and/or its formulations (see the previous section) were challenged subcutaneously with OVA expressing tumorogenic cell line, EG.7 at day 51. After the tumors reached a palpable size in the control group, tumor volumes were measured every second day using a caliper and expressed as length x width x height (mm^3). Tumor volumes recorded 12 days after tumor injections and images of tumors from representative mice are presented in **Figures 3.12**.

Results of the tumor challenge study show that in contrast to sham-immunized (PBS), OVA+CpG-ODN or OVA+c-di-GMP immunized mice, all c-di-GMP containing formulations significantly reduced the tumor burden. Interestingly, the OVA+c-di-GMP/Arg(9) and c-di-GMP encapsulated in MVs showed complete protection from subcutaneous EG.7 tumor challenge. (**Figure 3.12**). OVA+c-di-GMP/CpG adjuvanted groups also showed significantly reduced tumor volumes.

These results suggest that the above-mentioned formulations that were found to be effective in the tumor protection model stimulated strong tumor antigen-specific $CD8^+$ T-cell responses. However, other immune system cells may have also contributed to anti-tumor activity (i.e Natural Killer Cells, NK cells). Thus, to further evaluate the antigen-specific cytotoxic T cell responses induced following immunization, mice were sacrificed and splenocytes were stimulated *ex vivo* with the MHC Class I epitope of OVA (SIINFEKL peptide). Production of antigen specific IFNγ was assessed using ELISPOT assay as described in **Section 2.2.5.6**. In assay design, only source of IFNγ secretion was the OVA-specific CD8+ T-cells following exposure to the re-call antigen. Samples without the SIINFEKL peptide stimulation served as the negative control, to determine the background levels of this cytokine.

Consistent with the ability of these formulations to induce anti-tumor immunity, ex-vivo SIINFEKL re-stimulation of spleen cells harvested from immunized mice resulted in 8 to 10 fold increase in IFN-γ production compared to no-peptide negative controls (**Figure 3.13**),

suggesting increased tumor-specific CD8 T cell responses. Highest increase in the cytokine response was observed with c-di-GMP/Arg(9) and MV encapsulated groups.

These findings show that c-di-GMP/Arg(9) complexes, c-di-GMP+CpG-ODN combination and c-di-GMP encapsulated in MVs are effective vaccine adjuvants and can generate a protective anti-tumor response

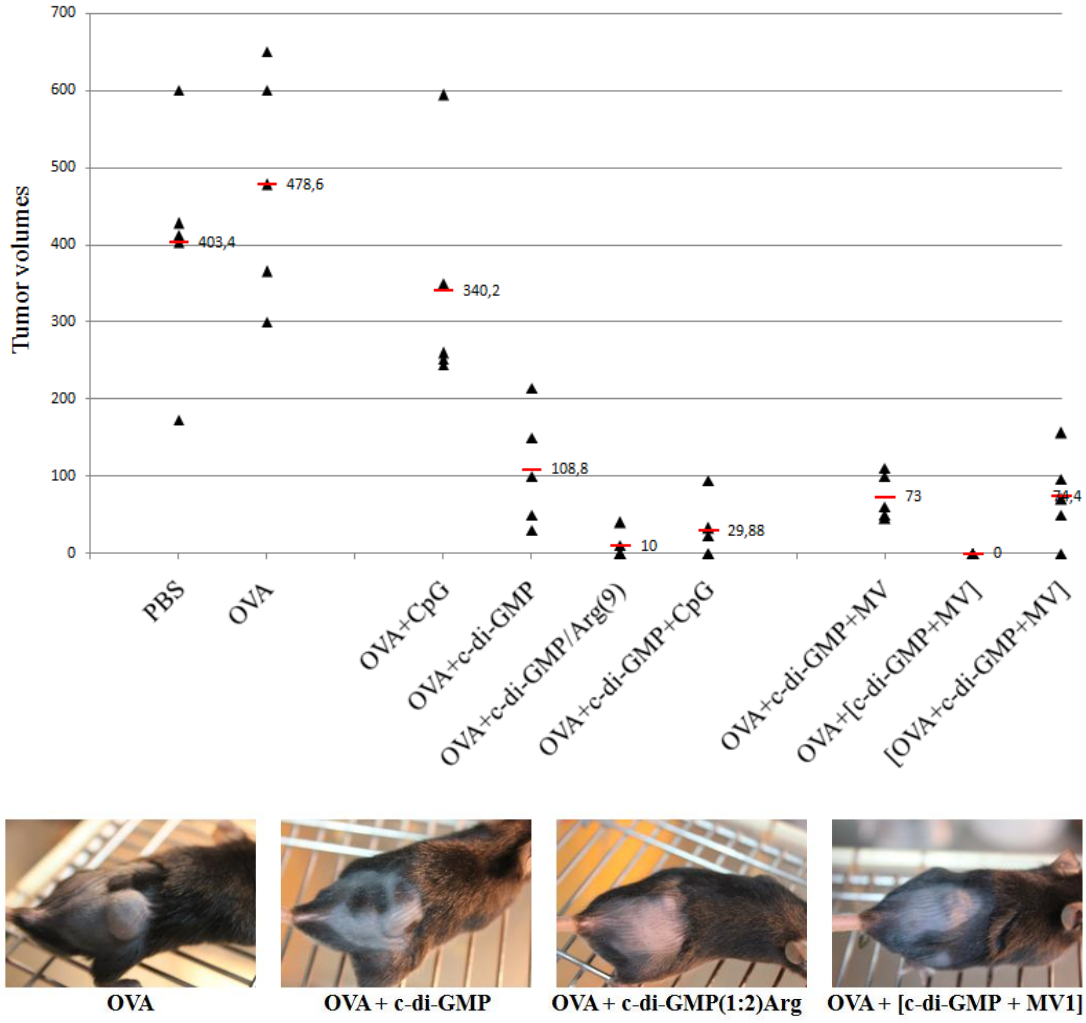

Figure 3.12 Tumor volumes in immunized mice 12 days after challenge with EG.7 tumor cells. Tumor volumes were measured in length x width x height using a caliper (A). Mean of each groups are given as lines where dots represent an individual mouse. Images of representative groups (B)

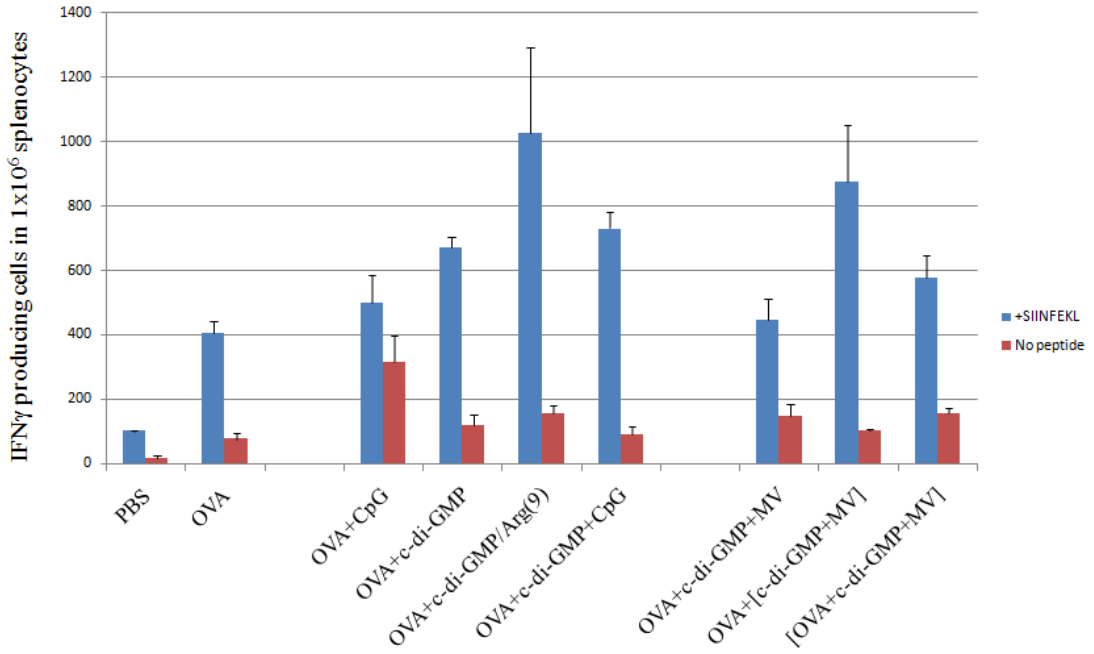

Figure 3.13 IFNγ ELISPOT results of spleenocytes stimulated *ex-vivo* with SIINFEKL peptide. Bars are representative of 3 individual mice±SD.

CHAPTER 4

CONCLUSION

As a recently identified pathogen associated molecular pattern (PAMP), c-di-GMP was demonstrated to be a successful vaccine adjuvant, inducing maturation of antigen presenting cells, and triggering secretion of Type I interferons. However, c-di-GMP is not cell permeable and requires use of high doses to see an effect or transfection to the cytosol. In this study, we aimed to develop alternative delivery strategies that would enhance the cellular transfer and/or the immune stimulatory activity of c-di-GMP.

For this purpose, we investigated various cationic compounds (nonaarginine, nonalysine, polyEthyleneImine, Tat peptide) that may possibly form complexes with c-di-GMP. We showed that c-di-GMP forms stable complexes with Arg(9) peptide in the presence or absence of K^+, and not with any other cationic peptides, indicating a specific interaction between the c-di-GMP and Arg(9) other than a simple electrostatic one. On the other hand, cucurbituril, another delivery vehicle forming guest-host complexes with the drug, failed to show a significant benefit.

In vitro uptake studies revealed that in mice splenocytes c-di-GMP/Arg(9) complexes were internalized 4-fold more efficiently with respect to its free counterpart. In parallel with this internalization profile, c-di-GMP/Arg(9) complexes were shown to increase the immune-stimulatory properties of c-di-GMP comparable to transfected controls, as evidence by higher MHC Class II/CD86 upregulation and elevated pro-inflammatory cytokine production from mouse splenocytes. These results show that of the tested molecules, Arg(9) peptide was the only one that could form stable complexes and stimulate higher levels of pro-inflammatory cytokine production.

Attempts to improve the immunogenicity of the cyclic nucleotide also included its use together with the TLR9 ligand CpG ODN. When both ligands were administered simultaneously, there was a drastic increase in MHC Class II/CD86 expression on antigen presenting cells, indicating synergistic immune-stimulatory activity. Dual stimulation improved the levels of IL6 and TNFalpha production, whereas IL12 secretion remained unchanged when compared to CpG ODN. This data suggests the presence of a strong synergism between c-di-GMP and CpG ODN signaling pathways.

Arg(9) complexes of the drug or its combined administration with CpG ODN were tested in in vivo vaccination studies using C57BL/6 mice and OVA as the model antigen. Furthermore, similar to drug encapsulation in liposomes, c-di-GMP was encapsulated in membrane vesicles secreted from bacteria and their adjuvanicity was tested in the abovementioned system. Immunization studies revealed that all formulations tested

generated higher OVA-specific IgG antibodies. Based on OVA-specific IgG2c/IgG1 ratios, it was concluded that c-di-GMP+CpG ODN and MV encapsulated c-di-GMP generated a Th1 skewed immune response whereas Arg(9)peptide complexes induced a mixed Th1/Th2 response. Of interest, mice vaccinated with the above adjuvanted formulations showed complete protection from subcutaneous tumor challenge, suggesting improved CD8+ T cell responses. Further analysis of cytotoxic T cells responses via ex-vivo re-stimulation of immunized mice spleen cells with the SIINFEKL peptide revealed that IFNgamma production was significantly higher in groups injected with the formulations developed in this thesis. These results suggest that Arg(9) complexation, encapsulation in bacteria-derived vesicles or co-stimulation together with CpG ODN enhance the adjuvanicity of c-di-GMP.

Vaccine adjuvants are essential for enhancing and directing the adaptative immune response to antigens. Yet, most vaccines are still adjuvanted with the Th2-skewing alum formulations. Development of more effective adjuvants would improve the vaccine efficacy and is gaining importance as a new field of research. In this context, c-di-GMP has a significant importance as a newly identified and potentially effective compound. We showed that membrane transport and immune-stimulatory properties of this cyclic dinucleotide can be increased by either Arg(9) peptide complexation, synergistic use with CpG ODN, and encapsulation in membrane vesicles. These formulations enhanced both the humoral and the cellular immunity, suggesting that they may be of value when used as adjuvants for viral vaccines or as anti-tumor therapeutic agents. The results of this thesis could be important in understanding the immunostimulatory behaviour of cyclic di GMP and could encourage its use in future studies.

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APPENDIX A

BUFFERS, SOLUTIONS AND CULTURE MEDIA

Blocking Buffer (ELISA)

500 ml 1x PBS
25 grams BSA (5%)
250 µl Tween20 (0,025%)

BCIP-low melting agarose substrate solution

4 ml BCIP
1 ml water
0,03 g low melting agarose

Loading Dye (Agarose gel)

0,009 grams Bromophenol blue
0,009 grams Xylen cyanol
2,8 ml ddH2O
1,2 ml 0,5M EDTA
11 ml glycerol

PBS (Phosphate Buffered Saline) [10x]

80 grams NaCl
2 grams KCl
8,01 grams Na2HPO4 . 2H2O
2 grams KH2PO4
1 lt with ddH2O

PBS-BSA-Na azide (FACs Buffer)

500 ml 1x PBS
5g BSA (1%)
125mg (0,25%)

T-cell Buffer (ELISA)

500 ml 1x PBS
25 ml FBS (5%)
250 µl Tween20 (0,025%)

Wash Buffer (ELISA)

500 ml 10x PBS
2,5 ml Tween20
4,5 lt ddH2O

RPMI-1640 (Hyclone)

2 % : 10 ml FBS (FBS = inactivated at 55°C)
5 % : 25 ml FBS
10 % : 50 ml FBS

5 ml Penicillin/Streptomycin (50 µg/ml final conc.)
5 ml HEPES (Biological Industries), (10 mM final conc.)
5 ml Na Pyruvate, (0,11 mg/ml final conc.)
5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x conc. stock)
5 ml L-Glutamine, (2 mM final conc.)