DEVELOPMENT OF NOVEL DNA VACCINE AND IMMUNOTHERAPEUTIC APPROACHES

Maroof Hasan
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Maroof Hasan MD

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To my parents
Many potential DNA vaccination and immunotherapeutic strategies have been explored in both animal models and human settings in recent years. However, the failure of efficient and safe method development suggests that it is time to rethink, since more innovative approaches are needed for better control of infection and immunotherapy.

DNA-based vaccination is a relatively new technology. Such a vaccine consists of a circular piece of DNA (plasmid), which contains an immunity-inducing gene. Once inside a cell, the DNA vaccine vector produces the protein antigen, which is seen as foreign and leads to the generation of an immune response. However, DNA vaccination has so far met with limited success likely due, at least in part, to the lack of strong elicited immune responses in humans. There is therefore a need for the development of appropriate adjuvant, which can be used together with DNA vaccines.

Bioplex technology is a novel non-viral approach, which makes it possible to overcome some of the barriers for efficient delivery of antigen encoding plasmid DNA into the cell. In order to incorporate different functional entities into plasmid DNA for specific purposes, highly stable anchors of either Locked nucleic acid (LNA) or bisPNA (Peptide nucleic acid) can be used to attach several and different functional entities.

In the first paper, we investigated the possibility of stabilizing sequence-specific binding of PNA-anchored functional peptides to plasmid DNA by hybridizing PNA and LNA oligomers as "openers" to partially overlapping sites on the opposite DNA strand. This procedure allows hybridization at reduced PNA-to-plasmid ratios, allowing greater than 80% hybridization even at ratios as low as 2:1. Using significantly lower amounts of PNA-peptides combined with shorter incubation times reduces unspecific binding and facilitates purification.

In the second study, we developed a novel branching technique to generate multiple anchor binding sites per plasmid. The LNA oligonucleotides were synthesized with an extension of bases that is not used for plasmid hybridization, but designated for hybridization to secondary oligonucleotides. This universal anchor system is versatile and can give an exponential growth of number of functional entities presented per plasmid. This new branching approach is very effective for attaching nucleic acid-based toll-like receptor (TLR) ligands for DNA vaccination and immunotherapy.
In third study, we wanted to determine the potential role of Bruton's tyrosine kinase (Btk) in the TLR signaling pathway in mouse splenic B cells. Inhibition of attenuators represents a generally applicable and alternative strategy for enhancing the potency of various forms of prophylactic and therapeutic vaccines. Our data provide evidence in support of the theory that Btk negatively regulates both TLR9 activation and expression in mouse splenic B cells. Thus, down regulation of Btk with plasmid expressing short hairpin RNA (shRNA) and attaching TLR9 ligands in the same construct might be a powerful tool for enhancing immune responses.

In the fourth study, we investigated the effect of simultaneous administration of TLR3 ligand (poly I:C) and single stranded DNA oligonucleotides (ssDNA ODN) on human monocyte-derived dendritic cells (moDCs). Using poly I:C as a DC maturing agent, we show that the maturation and cytokine production can be significantly inhibited by ssDNA ODN. The addition of ssDNA together with poly I:C blocks phosphorylation of IRF3, a downstream component of this pathway. Taken together our findings indicate that ssDNA inhibits some yet to be identified pathway(s) leading to maturation of DCs that might be promising candidates for the development of novel immunotherapeutic approaches.

In conclusion, the present work describes the development of new technologies for vaccination and immunotherapy. The ideal non-viral technological platform might include an immune cell-targeting molecule, shRNA construct against negative regulators of immune signaling and addition of TLR agonists in the same setting. Through this work we have developed a strategy, which could serve as an embryo for future development.
LIST OF PUBLICATIONS


*Authors contributed equally


*Authors contributed equally
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LIST OF ABBREVIATIONS

APC  Antigen presenting cell
Ag   Antigen
BS   Binding site
BCG  Bacille Calmette-Guérin
Btk  Bruton’s tyrosine kinase
Btk KO Btk knockout
BCR  B-cell receptor
cDNA Complementary DNA
CD   Cluster of differentiation
CTL  Cytotoxic T lymphocyte
DC   Dendritic cell
DNA  Deoxyribonucleic acid
dsDNA Double-stranded DNA
ER   Endoplasmic reticulum
FE   Functional entity
HSP  Heat-shock protein
IFN-α Interferon-alpha
IFN-β Interferon-beta
IFN-γ Interferon-gamma
IRAK IL-1 receptor associated kinase
IRF  Interferon Regulatory Factor
IL   Interleukin
Ig   Immunoglobulin
IM   Intramuscular
LNA  Locked nucleic acid
LPS  Lipopolysaccharide
LTA  Lipoteichoic acid
MHC  Major histocompatibility complex
MZ   Marginal zone
MAPK Mitogen activated protein kinase
MAL  MyD88 adaptor-like
moDC Monocyte derived DC
MyD88 Myeloid differentiation primary response protein 88
NLS  Nuclear localization signal
NF-κB Nuclear factor kappa B
NK cell Natural killer cell
ODN  Oligonucleotide
PAMP Pathogen-associated molecular pattern
PRR  Pattern-recognition receptor
pDNA Plasmid DNA
PNA Peptide nucleic acid
PCR  Polymerase chain reaction
PBM C Peripheral blood mononuclear cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>SUN</td>
<td>Self-assembling UNiversal anchors</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing IFN-β</td>
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<tr>
<td>TRAF</td>
<td>Tumour-necrosis-factor receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Xid</td>
<td>X-linked immunodeficiency</td>
</tr>
<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
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1. INTRODUCTION

1.1 Vaccine

A vaccine is a preparation given to an individual to create a state of protection against a disease. Historically, vaccines have been designed using empirical approaches. Most successful vaccines consist of live-attenuated pathogens. For example, the smallpox vaccine, which has been administered to over 1 billion people to eradicate the disease worldwide ¹, consists of a live-attenuated vaccinia virus. Furthermore, the yellow fever vaccine 17D has been administered to nearly half a billion people worldwide ²,³. Bacille Calmette-Guérin (BCG) is an attenuated TB vaccine, used to immunize infants in most developing countries ⁴. Despite the great successes of such vaccines, we do not have a detailed mechanistic understanding of how these vaccines work. Clearly, insights into how they function may provide vital clues in guiding the rational design of future vaccines against pandemics and emerging infections.

1.2 DNA vaccine

DNA-based vaccination is a relatively new technology. The science behind DNA vaccination had its beginnings over a half-century ago with early tumorigenesis studies. Both Stasney et al. ⁵ and Ito ⁶ in separate studies showed that injections of nucleic acid containing extracts of papillomatous tissue from wild cottontail rabbits can induce tumour in domestic rabbits. In the 1980s, studies into the in vivo expression of injected DNA, both linear and plasmid, expanded ⁷. In 1990, there was the important discovery that naked plasmid DNA when injected into mouse muscle generated an in vivo expression of the plasmid encoded reporter gene ⁸. This suggested that plasmid DNA could be used to express an immunity-inducing gene. Once inside a cell, the DNA vaccine vector produces the antigen, which is seen as foreign and leads to the generation of an immune response. Some other studies also demonstrated the in vivo activity in a variety of animal models, including studies showing that Hepatitis B Virus DNA could induce hepatitis in chimpanzees ⁹ and that injection of insulin and growth hormone genes could cause the production of these hormones in rats ¹⁰. Following these initial reports the field of DNA vaccines took hold with numerous studies investigating various DNA constructs in a wide range of animal disease models ¹¹-¹⁴. Subsequently, DNA vaccines
were moved to clinical examination where they have been studied for their ability to
drive relevant immune responses against a host of human diseases. After IM injection of
a plasmid, myocytes are the predominant cells transfected; however, other cells located
within the muscle including dendritic cells (DCs) and to a high degree monocytes are
also transfected. Although the exact mechanism of immune induction has yet to be
elucidated, the transfection of these cell types including DCs allows immune induction
through MHC I and/or MHC II pathways. This method of immunization is advantageous
in terms of simplicity, adaptability, and cost of vaccine production. However, the entry of
DNA vaccines and expression of antigen are subject to physical and biochemical barriers
imposed by the host.

In small animals such as mice, the host-imposed impediments have not prevented DNA
vaccines from inducing long lasting, protective humoral, and cellular immune responses.
In contrast, these barriers appear to be more difficult to overcome in large animals and
humans. Several of these strategies, such as altering codon bias of the encoded gene,
changing the cellular localization of the expressed antigen, and optimizing delivery and
formulation, have led to improvements in DNA vaccine efficacy in animals. In July
2005, the first DNA vaccines obtained licensure; one was for the prevention of disease
cased by infectious hematopoietic necrosis virus (IHNV) in farm raised Atlantic salmon
and the other to prevent disease caused by West Nile virus in horses. In March 2007,
a therapeutic DNA vaccine to treat melanoma in dogs received approval from US
Department of Agriculture. Thus, total DNA vaccine successes to date include two
preventive vaccines for infectious diseases and a therapeutic vaccine for cancer, but still
there is not a single licensed DNA vaccine for humans.

1.3 DNA vaccine delivery systems and its barriers

The uptake of DNA plasmids by cells upon injection is very inefficient: only a small
proportion of the injected material is internalized by cells and results in successful
transfection (a process of introducing nucleic acids into cells by non-viral methods).
Transfection is a complex process involving several steps: adsorption of the transfection
complex to the cellular surface, membrane translocation or endocytosis, escape from the
endosome/lysosome, nuclear translocation with or without chromosomal integration and
finally the effect of the nucleic acid by expression of a reporter/therapeutic gene. Transfection of plasmids into eukaryotic cells in culture often results in high levels of
protein production, mostly when used with products that facilitate the entry of the plasmid into the cells. However, when these eukaryotic cells are part of a larger system, such as the host, the plasmid appears to encounter difficulties in transfecting the cells. This emphasizes the need for improved delivery systems. The ideal gene delivery system must protect the nucleic acid from degradation, have the ability to deliver it to target cells, and should induce efficient gene expression in the presence of body fluids, such as serum and interstitial fluids, concomitant with being nontoxic and stable during storage and treatment.\textsuperscript{22,23}

The barriers are physical and biochemical, intracellular or extracellular.\textsuperscript{24} Serum proteins and enzymes, such as endonucleases, can degrade the plasmid, thereby reducing the amount that is available in the host to express the antigen. The number of hurdles for the plasmid increases upon entry into a host cell.\textsuperscript{25,26} One possible mechanism of plasmid uptake is endocytosis, after which the plasmid travels through the endosome-lysosome compartment, where it can be degraded or by using some techniques like adding some specific functional entity that helps to destabilize endosomal membrane, it is possible to escape from degradation.\textsuperscript{29}

Two basic strategies that have been used for increasing DNA-vaccine potency include physical delivery technology by electroporation (EP) in situ to increase antigen production by facilitating plasmid-DNA delivery directly into cells and formulation with microparticles to target antigen-presenting cells.\textsuperscript{30,31,32} Both the approaches are effective in animal models, but have yet to be evaluated fully in human clinical trials. Other plasmid DNA delivery methods showed some promises include-intramuscular injection with hydrodynamic pressure, biojector needle free delivery method, gene gun immunization and ‘tattooing’, the latter two as a means to administer DNA to skin cells.\textsuperscript{33,34,35,36,37,38}
14 Nanotechnology and DNA

Deoxyribonucleic acid (DNA) was first isolated in 1869 by the Swiss scientist Friedrich Miescher from the nuclei of human white blood cells. He described the substance as white, slightly acidic chemical of unknown function. By the late 1940s, scientists knew what DNA contained—phosphate, sugar, and four nitrogen-containing chemical "bases": adenine (A), thymine (T), guanine (G), and cytosine (C). But no one had figured out what the DNA molecule looked like until the structure of DNA duplex was delineated by Watson and Crick in 1953. By the late 1950s, their work had been widely accepted by the scientific community.

DNA has been recognized not only for carrying of genetic code but also for serving as an attractive building block in bottom-up research. It has preferentially been used in the self-assembly of functional nanomaterials since they are more readily available by synthetic chemical means and are more convenient to handle than proteins. DNA has been used to create simple structures as junctions and DNA lattices as well as more complex origami type structures and nanoelectric circuits or DNA box with a controllable lid. Thus, DNA can be used as a structural material rather than as a carrier of biological information. DNA nanotechnology is a subfield of nanotechnology where the unique molecular recognition properties of DNA and other nucleic acids are used to
create novel, controllable structures out of DNA. It almost exclusively uses ssDNA oligonucleotides and hybridizes them with other ssDNA oligonucleotides, generating duplexes with sticky ends. Duplexes are then connected through hybridization of the sticky ends to eventually form supramolecules with rigid three-dimensional structure of the complex. Sometimes branched DNA structures are also used to create DNA complexes with useful biological properties. We used a Self-assembling Universal (SUN) anchoring technique as a possible strategy for using plasmid, dsDNA, as a backbone in the self-assembly of supramolecules and lattices. Single-stranded oligonucleotides can also be used in the form of aptamers, which adopt a three-dimensional configuration allowing them to bind to target molecules.

1.5 Bioplex technology

Originally, novel Bioplex (Biological complex) technology was developed to overcome some of the barriers for efficient delivery of plasmid DNA into the cell. However, incorporating different functional entities into plasmid DNA for specific purposes is not straightforward. Highly stable anchors of either Locked nucleic acid (LNA) or bisPNA (Peptide nucleic acid) can be used to attach several and different functional entities. By choosing certain biological functions the complex can be targeted to specific organs/cells and also increase the cellular and nuclear uptake of the complex.

![Figure 1.2](image)

**Figure 1.2**: Schematic presentation of Bioplex concept.
By using this non-viral delivery system it is possible to circumvent some of the problems occurring with viral vectors such as endogenous virus recombination, oncogenic effects caused by integrating viruses and immune responses. Furthermore, non-viral vectors have advantages in terms of simplicity of use, ease of large-scale production. Plasmid-based vectors normally give short-term expression since they are lost over time. The transient expression is beneficial for vaccine antigen expression, which should be limited in time. For some potential purposes including DNA vaccination and immunotherapy development we have modified our plasmid based non-viral vector (Bioplex) in such a way that we can attach different functional entities for particular purposes. We call this novel branching technology SUN (Self-assembling UNiversal anchors). LNA oligonucleotides are hybridized to the displaced strand of the plasmid, caused by the strand invading bis PNA. The LNA oligonucleotides have an extension of bases that are not used for plasmid hybridization but designated for hybridization to secondary oligonucleotides.

Figure 1.3: Schematic presentation of modified Bioplex platform and how the secondary and tertiary oligonucleotide can be attached to it.

The complex is generated through self-assembling hybridization of single-stranded oligonucleotides. The self-assembling universal anchor system is versatile and can give an exponential growth of number of functional entities presented per plasmid. This new branching approach is very effective for attaching nucleic acid-based toll-like receptor
(TLR) ligands (like CpG oligonucleotides) for DNA vaccination for enhancing immune responses.

1.6 PNA and LNA

In this present study we have used two DNA analogs with superior binding affinity. These “genetic anchors” have significantly different properties; Peptide Nucleic Acid (PNA) $^{59,60}$ having a flexible and neutral N-(2-aminoethyl)glycine backbone and Locked Nucleic Acid (LNA) $^{61-64}$, having a rigid C3'-endo furanose conformation by a 2'-O, 4'-C lock and a negative phosphate backbone. In the first study we used LNA or linear PNA as openers to facilitate hybridization of bisPNA. During the development of SUN technology we used bisPNA as opener to facilitate hybridization of SUN LNA. Moreover, this technology may have applications that go beyond the addition of biologically active molecules. We used LNA anchors due to the following reasons:

- Sequence specificity $^{65-68}$
- Mismatch discrimination capacity $^{69,70}$
- High stability. It has been shown that improved serum stability and reduced off targeting effects in vitro by incorporation of LNA in siRNA molecules $^{71,72}$
- Low toxicity $^{73}$
- Can bind and form triplexes with dsDNA $^{74,75}$
- Adding functionality to plasmid DNA is possible $^{76}$
- Can be synthesized with modifiers and labels $^{76}$
- Water soluble $^{77}$
- Gel separation possible $^{77}$

1.7 Immunotherapy

Immunotherapy (also known as biologic therapy or biotherapy) refers to an array of treatment strategies based upon the concept of modulating the immune system to achieve a prophylactic and/or therapeutic goal.

This can be done in a couple of ways:

i. Stimulating own immune system to work harder or smarter.

ii. Providing immune system components, such as man-made immune system proteins.

Immunotherapy is most often used along with, or after, another type of treatment to add to its effects. It is sometimes used to treat cancer, like BCG $^{78,79}$ immunotherapy for early stage (non-invasive) bladder cancer. Since the late 1980s evidence has become available
that instillation of BCG into the bladder is an effective form of immunotherapy in this disease 79. It utilizes instillation of attenuated live bacteria into the bladder, and is effective in preventing recurrence in up to two thirds of cases. BCG also used for immunotherapy of colorectal cancer 80 and there are a number of cancer vaccines in development that use BCG as an adjuvant to provide an initial stimulation of the patient’s immune system. Topical immunotherapy utilizes an immune enhancement cream (imiquimod) which is an interferon producer causing the patients own killer T cells to destroy warts 81, actinic keratoses, basal cell cancer, vaginal intraepithelial neoplasia 82, squamous cell cancer 78,83, cutaneous lymphoma 84, and superficial malignant melanoma 85. Lung cancer also has been demonstrated to potentially respond to immunotherapy 86.

Thus immunomodulation could be a potential approach for treating different diseases. By titrating the type and number of immune adjuvants, targeting specific immune cells, sites and methods of delivery techniques and some other modifications, it is possible to get the desired response either Th1, Th2 or mixed Th1 and Th2. Bioplex technology is very appropriate for achieving this purpose.

1.8 Immune system and toll-like receptor (TLR)

All living organisms are exposed constantly to microorganisms that are present in the environment and need to cope with the invasion of these organisms into the body. The vertebrate immune responses can be divided into innate and adaptive immunity, with innate immunity being the first line of defense against pathogens 87,88. The innate system identifies pathogen-associated molecular patterns (PAMPs) on the pathogens by host pattern-recognition receptors (PRRs). By contrast, adaptive immune responses are slower processes mediated by T and B cells, both of which express highly diverse antigen receptors that are generated through DNA rearrangement and are thereby able to specifically respond to a wide range of antigens. The adaptive immune response provides the vertebrate immune system with the ability to recognize and remember specific pathogens and to mount stronger attacks each time the pathogen is encountered. It is adaptive immunity because the body’s immune system prepares itself for future challenges.
PRR discovery came from an entirely different line of research. Christiane Nüsslein-Volhard of the Max Planck Institute in Tübingen analyzed mutations in fruit flies. In 1985, she saw a weird-looking fly larva in which the ventral portion of the body was underdeveloped. Her spontaneous comment was "Das war ja toll!" meaning "That was weird!" and she coined the name Toll for the mutated gene. The protein product of the Toll gene was found to cause ventralization, and normal functional activity of Toll is necessary for dorsoventral polarity in the fly. The discovery of Toll was one in a series of discoveries of genes controlling early embryogenesis, which led to a Nobel prize for Nüsslein-Volhard in 1995. A decade after Nüsslein-Volhard’s discovery of Toll, Jules Hoffmann’s laboratory in Strasbourg reported that Toll not only controls dorsoventral polarity but also has a role in the immune defense in Drosophila. With Hoffmann’s discovery, Toll was, for the first time, associated with host defense.

Since then, Toll-like receptors are the most studied members of PRR family. TLRs are classical transmembrane proteins whose ligand-binding domains—composed of leucine-rich repeats (LRRs)—pointing towards the extracellular milieu or to the topologically equivalent lumen of membrane-enclosed intracellular compartments. This structural conformation help TLRs to recognize either extracellular or membrane-bound foreign organisms. In either case, the TLR signal transduction domains, known as Toll/IL-1 receptor (TIR) domains, are directed towards the cytoplasm and transduce their signals through interaction with cytoplasmic adaptor proteins, which also contain TIR domains. Ligand-induced receptor and adaptor dimerization results in recruitment and activation of additional signalling proteins and eventual triggering of host-defence responses. Lately, other families of PRRs were described: the RIG-I like receptors (RLRs) and NOD-like receptors (NLRs). Unlike TLRs, these families consist of soluble proteins that survey the cytoplasm for signs that broadcast the presence of intracellular invaders.

The innate immune system needs to identify the pathogen classes because, defending against different pathogens requires radically different types of immune responses, and these must be initiated by innate immune cells. Recent studies have shown that the innate immune system has a greater degree of specificity and it is highly developed in its ability to discriminate between self and foreign pathogens. Although emerging result points to an important role for NLRs, and RLRs in modulating adaptive immunity, much of our
understanding comes from analyses of TLR function and their roles in modulating the magnitude and quality of T cell immunity against pathogens and vaccines.

Accumulating evidence indicates that activation of the innate immune system is a prerequisite for the induction of acquired immunity, particularly for the induction of a T helper 1 (Th1)-cell response. TLRs recognize microbial products and initiate a complex immune response designed to eliminate invading pathogens 95.

If the invader is an extracellular parasite, then the immune system must mount an appropriate immune response involving the early activation of eosinophils and the subsequent production of interleukin-4 & IgE, which is termed a T-helper 2 (Th2) response. In contrast, clearing the body of an intracellular viral infection requires a different type of immune response, termed T helper 1 (Th1) response, in which NK cells are activated as quickly as possible to control the infection until specific interferon (IFN-\(\gamma\)) secreting T cells and cytotoxic T lymphocytes (CTLs) are produced. It has been shown that it is possible to skew the immune response to vaccination either to a Th1 or Th2 or a mixed Th1/Th2 outcome, simply by titrating the concentrations of immunomodulatory oligonucleotides 96.

Th1 vaccines should be particularly useful for the prevention and treatment of allergic diseases and asthma, since interferon-\(\gamma\) released by Th1 cells and NK cells can downregulate IgE synthesis, as well as inhibit Th2 cells that control the late phase component of the allergic response. It is conceivable that the administration of an immunostimulatory oligonucleotide sequence with a weak tumour antigen could stimulate a delayed hypersensitivity response sufficient to eliminate malignant cells. By increasing endogenous IFN-\(\gamma\) synthesis, therapeutic Th1 vaccines could promote recovery from chronic viral or parasitic infections 96.

1.9 TLR signaling

Cells of the immune system have evolutionarily conserved germ line-encoded receptors, toll-like receptors, that recognize distinct microbial component and elicit different, but sometimes overlapping, immune responses 87,94,97,98. TLRs constitute a family of at least 11 receptors that recognize a broad range of microbial stimuli, including bacterial cell wall components, carbohydrates and viral or bacterial nucleic acids 91,99-103.
Subfamilies of TLRs are expressed in distinct cellular compartments, for example, TLR1, 2, 4, 5, 6 and 10 and 11 are present on the surface of cells, while TLR3, 7, 8 and 9 are located in endosomes and within the endoplasmic reticulum\textsuperscript{104-107}. The expression of TLRs is also different among species\textsuperscript{108-110}, which could be one explanation for the different findings like differences regarding TLR3 and TLR9 ligand combinations between mice, chicken, fish and human\textsuperscript{111-114}. Once activated by the appropriate ligands, TLRs transduce signals through distinct pathways involving adaptor proteins containing Toll/IL-1R (TIR) domains\textsuperscript{115,116}. The signaling pathways initiated by most TLRs are regulated by the adaptor protein MyD88 (myeloid differentiation factor 88), with some variations by the additional recruitment of MAL (MyD88 adaptor-like), TRAM (TRIF-related adaptor molecule), and TRIF (TIR-domain-containing adaptor inducing interferon-\(\beta\))\textsuperscript{117}. TLR3 is unique, since it does not use MyD88 but instead recruits only TRIF, which mediates the activation of NF-\(\kappa\)B and IRF3\textsuperscript{118}. TLR4, the receptor for LPS, is the only TLR that can use either of the two adaptors (MyD88 or TRIF)\textsuperscript{118}. TLR signaling promotes the antigen presenting cell maturation by activating mitogen-activated
protein kinase and nuclear factor-κB (NF-κB), which then mediate the expression of cytokines, resulting in the induction of innate and adaptive immunity 91,119.

Simplified presentation of TLR signaling pathways:

![TLR signaling pathways diagram](image)

Intracellular nucleic-acid sensors and signaling pathways:

![Intracellular nucleic-acid sensors and signaling pathways diagram](image)

**Figure 1.5:** Schematic presentation of intracellular nucleic-acid sensors and signaling pathways. Reproduced from Gilliet M, Cao W and Liu Y-J 120.
1.10 Bruton's tyrosine kinase (Btk) and its role in TLR signaling

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that is essential for B cell development. Mutations in the human BTK gene lead to X-linked agammaglobulinemia (XLA), a primary immunodeficiency characterized by the absence of B-lymphocytes with subsequent susceptibility to pyogenic bacterial infections and enteroviral disease. Recent studies have described the involvement of Btk in some form of TLR signaling. In particular, it has been shown that Btk interacts with the conserved cytosolic domain termed the Toll/interleukin-1 receptor (TIR) of TLRs 4, 6, 8, and 9. Btk was also found to specifically associate with MyD88, Mal, and IRAK1. However, the exact relationship between Btk and the different TLRs remains largely unexplored and inconclusive.

We identified Btk as a potential negative regulator in TLR9 signaling pathway in mouse splenic B cells. It has been shown that combining immunostimulation and inhibitory cytokine gene-silencing in one single small interfering RNA (siRNA) molecule enhanced immune responses by human monocyte-derived DCs. Since inhibition of attenuators represents a generally applicable and alternative strategy for enhancing the potency of various forms of prophylactic and therapeutic vaccines, thus, down regulation of Btk with plasmid expressing short hairpin RNA (shRNA) and attaching TLR9 ligands in the same construct might be a powerful tool for enhancing immune responses.

1.11 Dendritic cell (DC)

Dendritic cells were first described by Paul Langerhans (Langerhans cells) in the late nineteenth century. It wasn't until 1973, however, that the term "dendritic cells" was coined by Ralph M. Steinman and Zanvil A. Cohn. During certain developmental stages they grow as branched projections, the dendrites, that give the cell its name. However, these do not have any special relation with neurons, which also possess similar appendages. DCs are potent professional antigen-presenting cells (APCs) and have a central role in directing the adaptive immune response to pathogens. The ability of DCs to stimulate naïve T cells has long been thought to be crucial in initiating an effective immune response. Recent studies corroborate a concept of different DC subsets generating quantitatively and qualitatively distinct types of adaptive immunity. DCs use toll-like receptors (TLRs) to recognize conserved microbial structures. TLR
signaling promotes DC maturation by activating mitogen-activated protein kinase and nuclear factor-κB (NF-κB), which then mediate the expression of various cytokines, resulting in the induction of innate and adaptive immunity ⁹¹,¹¹⁹. Hence, exploiting the full immunostimulatory potential of DCs is likely the key to achieving an effective immune response to prevent or control infections and development of novel immunotherapeutic approaches.

1.12 Monocyte derived dendritic cell (moDC)

Immature DCs, similar to those found in peripheral tissues, can be generated by culturing human monocytes with GM-CSF and IL-4 and have been used to identify the activation signals that induce DC maturation ¹³⁹,¹⁴⁰. These cells have a high level of endocytic activity but low T cell stimulatory capacity. However, once activated they reduce their phagocytic capacity and gain a more migratory phenotype and mature into immunostimulatory DCs. The maturation process results in up regulation of adhesion and costimulatory molecules, down regulation of the endocytic activity and provides an optimal window for loading exogenous antigens on MHC class II molecules and MHC class I molecules by cross-presentation ¹⁴¹,¹⁴². It is believed that the maturation of DC plays a central role in determining the outcome of immune responses. Maturation can be induced by several stimuli, such as LPS (TLR4 ligand), Flagellin (TLR5 ligand), inflammatory cytokines (e.g. TNF-α), ligation of cell-surface receptor (e.g. CD40 by CD40 ligand) and double-stranded RNA or the double-stranded RNA mimic poly I:C ¹⁴³-¹⁴⁷. Upon activation, DCs mature into potent immunostimulatory cells that can drive T cell clonal expansion and, through production of immunomodulatory cytokines, promote the development of T helper 1 (Th1), Th2, Th17 or T regulatory (Tregs) effectors ¹⁴⁸-¹⁵⁰. Detailed knowledge of TLR expression on DCs and subsequent functional consequences of TLR ligation will be useful for the development of novel therapeutic approaches.
Figure 1.6 Schematic design how modified Bioplex DNA vaccine-construct might work.
2. AIMS OF THE THESIS

The aim of the study was to develop novel DNA vaccination and immunotherapeutic approaches using Bioplex technology. Though some progress has been made in this field over the last decade DNA vaccination in humans remains suboptimal compared to other immunization regimens. There is therefore a need for the development of a new generation of vaccines that are more efficient and safe to use. We aimed to attach toll-like receptor (TLR) ligands either alone or in combination directly to antigen-encoding plasmid DNA. This combinatorial approach could potentially activate different TLRs like Yellow fever vaccine YF-17D that activates multiple TLRs (one of the most efficient vaccine so far, activating TLR2, 7, 8 & 9). By manipulating different TLRs we aimed to fine-tune immune responses in desired direction.

The specific aims of this thesis were:

- To improve sequence-specific binding of nucleic acid analogue with functional entities to plasmid DNA for efficient gene delivery.
- To develop a novel branching technique for different gene and immunotherapeutic purposes.
- To find out potential regulators involving in toll-like receptor signaling pathway.
- Develop different means to modulate the functions human monocyte-derived dendritic cells.
3. METHODS

3.1 PNA and LNA
Unlabeled PNA and PNA-peptide chimeras were synthesized at Eurogentec Ltd., UK. The fluorophore-labeled PNA-peptide chimeras were synthesized and labeled at Light-Up Technologies AB, Sweden. The fluorophore SYBR103 (Molecular Probes Inc., OR), a derivative of the asymmetric cyanine dye thiazole orange, was added to the N-terminus of the PNA via a ten-carbon ethylene linker, while the hydrophilic spacer NC6O4H12 (AEEA) was used as linker (L) between PNA and peptides. All synthesis reactions were performed from the C-terminal end. Each N-terminal AEEA linker provides a positive charge at the pH used, as do the free amine groups of lysine and arginine residues. The LNA “openers”, other LNAs and LNA anchors were synthesized at Proligo SAS, France.

3.2. Plasmids and hybridization conditions
The plasmids used in these studies are all based on the pEGFPLuc plasmid (Clontech, BD Biosciences, CA) and modified as described previously \(^{151}\). In the mixed-base “linear” PNA experiments, the plasmids contained six binding sites for the labeled PNA and five sites for “opener” PNAs 528 and 664. The “opener” molecules were added at a PNA/binding site ratio of 5:1, and were incubated at 37 °C overnight in 10 mM phosphate buffer, pH 6.8. The fluorophore-labeled PNA was added at a PNA/binding site ratio of 5:1, with the final concentration adjusted to 1 μM, and incubated at 37 °C for at least 5 h. To remove excess unbound probe, a 45-base oligonucleotide containing the probe-binding site was added at four times excess. Alternatively, the hybridization mixture was purified on a Jet Quick PCR purification micro spin column (Genomed Inc., MO), and the DNA concentration was then determined by measuring OD\(_{260}\).

3.3 Quantification of hybridization efficiency
Quantification of bound PNA using the fluorophore probe and analysis for the presence of unspecific binding were done by mobility shift in 0.8% agarose gels. Quantification of bisPNA hybridization was done by mobility shift in polyacrylamide gel electrophoreses (PAGE), after digestion with the enzymes EcoRI and NotI (for the B site), or with EcoRI and SpeI (for the G site). This gives a fragment of between 137 and 234 base pairs (depending on the plasmid), containing the specific PNA binding sites. In addition, two larger fragments of 2048 and 4490 base pairs without any binding sites are obtained. The digested plasmids were subjected to PAGE (8%) in 1× TBE buffer, stained with SYBR-
Gold (Molecular Probes) and analyzed in the Fluoro-S gel documentation equipment (BioRad Laboratories, CA) with a CCD camera, and further evaluated using the Quantity One software. By analyzing the signals from unshifted DNA in the 137–234 base pair fragment with PNA-binding sites and the 2-kb fragment without binding sites, and calculating the ratio between these two bands, the amount of unhybridized plasmid was determined. In this way, the differences in loading of the samples were also normalized.

### 3.4 Construction of SUN plasmid and oligonucleotide anchors

The B4.7 and Clone5 plasmids (in paper 2) are based on the pEGFPLuc plasmid (Clontech, BD Biosciences, CA) and modified as described previously 151. The pPCR plasmid with the construct U6 (7ab)3 containing the LNA and PNA anchor binding domains was ordered from Geneart Gmbh, (Regensburg, Germany), and was slightly modified by adding the U6-promoter. The binding sites in the control pPCR plasmid was deleted by digestion with the flanking restriction enzymes Nhel and Apal and blunted with Klenow (NEB, New England Biolabs Ltd., MA, USA), prior to self-ligation. PNA oligonucleotides were synthesized at Eurogentec SA (Seraing, Belgium), LNA oligonucleotides at Proligo SAS, France and DNA oligonucleotides at DNA technology A/S (Aarhus, Denmark).

### 3.5 Hybridization in SUN plasmid

Plasmid B4.7 was incubated with LNA oligonucleotide αLNA9a [α is used to denote the fact that these oligonucleotides represent the first layer of hybridized material], αLNA11a, αLNA13a, αLNAmix13a or αSUN-LNAa, alone or simultaneously with PNA866. Both LNA and PNA were added in 10-fold excess in relation to the number of anchor binding domains presented by the plasmid, and incubated at 37°C overnight in 20 mM NaPO4 and 1 mM EDTA buffer pH 6.8, at a final concentration of 0.1 mg/ml. Plasmid B4.7 has 4 anchor binding domains for LNA and PNA, respectively. Clone5, lacking anchor-binding domains, was used as negative control. Unhybridized oligonucleotides and hybridized plasmids were separated through agarose gel electrophoresis and the fluorescent signal from respective LNA oligonucleotide was detected and quantified for the band corresponding to super-coiled plasmid.
3.6 Quantification of secondary and tertiary hybridization

\( \gamma \text{LNAG2} \) (200 pmol) and an equimolar mixture of \( \beta \text{Ray-4Sa} \) and \( \beta \text{Ray-4Sb} \) (100 pmol each), was 5’-end labeled with 50 \( \mu \)Ci 32P \( \gamma \)-ATP (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using 10 units of T4 polynucleotide kinase (Promega UK Ltd., Southampton, UK) according to the manufacturers’ protocol. The reaction was stopped after 10 min at 37° C with 2 \( \mu \)l 0.5 M EDTA. U6(ab)3 plasmid (50 nM) was SOP hybridized with either radioactively labeled \( \beta \text{Ray} \) oligonucleotide or \( \beta \text{Ray} \) hybridized with radioactively labeled \( \gamma \text{LNAG2} \) or \( \gamma \text{6-fam} \) labeled DNA oligonucleotide, depending on if the secondary or tertiary hybridization was to be quantified. Both secondary and tertiary oligonucleotides were added in a 1:1 ratio of anchors to binding sites, and incubated in presence, or absence, of salt at room temperature overnight. Samples were analyzed through PAGE electrophoresis and the radioactivity was detected using a Fuji phosphor imager screen and scanned by Molecular Imager FX pro plus with the QuantityOne software (Bio-Rad Laboratories Inc.).

3.7 ODNs and TLR ligands

Synthetic, endotoxin-free, completely phosphorothioate-modified oligonucleotides (S-ODN) were supplied by DNA Technology A/S (Risskov, Denmark) and used at a final concentration of 5 \( \mu \)g/ml unless otherwise stated. The sequences of S-ODN used were 5’-GTCGTTTTGTCGTT TTGTCGTTGTTGGTGGTGGTG -3’ (CpG-DNA ODN) and 5’-GAAGTTTTGAGGTTTTGAAGTT GTTGGTGGTGGTG -3’ (Non-CpG-DNA ODN). \textit{Escherichia coli} LPS (O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO). Poly I:C was purchased from Amersham Biosciences and used at a concentration of 25 \( \mu \)g/ml unless otherwise stated. Poly I, Poly A, Poly U, Poly G, Poly A:U, Poly G:U and chloroquine were from Sigma-Aldrich (St. Louis, MO). In some cases, Poly I, Poly A, Poly U, and Poly C were obtained from Midland Certified Reagent Company (Midland, Texas, USA).

3.8 Limulus assay

The endotoxin content of different reagents was measured with Limulus assay (QCL-1000, Cambrex Bio Science Walkersville, MD, USA) according to the manufacturer’s instruction. Only batches of Poly I:C, Poly I, CpG, Non CpG, other ODNs with no detectable levels of endotoxin and in one case of Poly I (after purification), less than 0.8 EU/ml were used in this study.
3.9 Mouse strains and preparation of splenic mature B cells

Xid/CBA mice (6-8 week old) were obtained from Charles River Laboratories (Sweden). Btk−/−/CBA mice were created by back-crossing Btk−/−/SW129 as described. Btk−/− (C57BL/6 background) were provided by Johan Forssell Department of Cell and Molecular Biology, Division of Tumour Biology, Umeå University (Umeå, Sweden). Wild type (WT) CBA and C57BL/6 mice were used as controls. Mouse splenic B cells were enriched using a high gradient magnetic separation column (Miltenyi Biotec). The spleen cells, which had been stained with anti-B220-Ab coupled micromagnetic particles (Miltenyi Biotec), were retained on the separation column under a high gradient magnetic field; these cells were subsequently eluted from the column. The purity of the cells was examined by flow cytometric analysis following staining with PE conjugated rat anti-mouse anti-CD19 antibodies. The purity of the cells was >95%.

3.10 Cytokine detection

In mouse experiments, 3×10^6 purified B cells/well (in 1 ml of medium) were stimulated for 24 h with 5 µg/ml CpG-DNA, 5 µg/ml non-CpG-DNA or 1 µg/ml of LPS. For all assays, cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES and 50 µM 2-ME. Amounts of cytokines in the supernatants were assessed according to the manufacturer’s recommendations using commercially available ELISA kits (IL-6, TNF-α) and sets (IL-12p40, IL-10) from BD Biosciences (San Diego, CA).

All supernatants from human DC cultures were taken at indicated time points and frozen in -20°C until use. Cytokine levels were determined in a Luminex reader using a multiplex assay (Millipore Corporation, Billerica, MA) according the manufacturer’s protocol. In some experiments cytokines were assessed according to the manufacturer’s recommendations using commercially available ELISA sets TNF-α from from BD Biosciences (San Diego, CA) and IL-6 from R & D Systems (Minneapolis, MN).

3.11 FACS analysis

Intracellular expression of TLR9 was detected by flow cytometry using FITC-anti-mouse TLR9 (clone M 9.D6 from eBioscience, San Diego, CA, USA), gated on B cells using R-phycoerythrin (R-PE) anti-CD19 (BD Biosciences, CA, USA). Cells were fixed and
permeabilized according to the manufacturer’s procedure using Fix and Perm reagents (CALTAG laboratories, Vienna, Austria). Rat IgG2a-FITC and Rat IgG2b-PE were used as isotype controls (BD Biosciences).

Identification of T1, T2 and mature B cell subpopulations were based on previous report 153. Briefly, 1x10^6 cells were stained with anti-B220 PECy5 (BD Pharmingen), anti-CD21 (eBioscience) and anti-CD24 PE (BD Pharmingen). Cells were incubated for 15 minutes with these antibodies and washed with PBS containing 5% FBS, anti-rat IgG APC (CALTAG Laboratories) was used as a secondary antibody for anti-CD21. Finally, cells were fixed with PBS containing 1% formalin. Cell acquisition was performed in a FACS DIVa (BD, Biosciences). For all samples 100,000 events were computed and analyzed for T1, T2 and M B cell subpopulations in WinMDI 2.8 software.

3.12 Quantitative RT-PCR and Microarray analysis

Total RNA from splenic B cells was extracted with RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (100 ng) was reverse-transcribed into cDNA with AMV reverse transcriptase using random hexamer primers (Roche Applied Science, IN). 18S rRNA was used as endogenous control. Primers and probes for mouse TLR9, IL-23 and IL-27 were purchased as pre-developed TaqMan assays (Assays-on-DemandsTM, Applied Biosystems). Quantitative RT-PCR was performed as previously described 152. The Affymetrix small sample protocol consisting of two cycles of amplification was used to obtain a larger amount of cRNA. 100 ng cRNA was hybridized to MOE430 2.0 GeneChips®; one array per sample were run and around 39,000 transcripts were analysed. The cRNA synthesis and hybridizations were performed in the BEA core facility at Department of Biosciences and Nutrition (www.ki.se/sea), Karolinska Institutet, Novum, Huddinge, Sweden.

3.13 In vitro differentiation of DC

CD14+ monocytes were enriched from buffy coats from healthy blood donors by negative selection using RosetteSep Human Monocyte Enrichment (1 ml/10 ml blood; Stem Cell Technologies, Vancouver, BC, Canada). Monocytes were then separated using lymphoprep (Nycomed, Oslo, Norway) density gradient. The cells were adhered for 2 hours in medium (RPMI 1640 supplemented with 1% HEPES [N-2-
hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 1% Sodiumpyruvate, 2 mM L-glutamine, 1% Streptomycin and penicillin, 10% endotoxin-free fetal bovine serum (FBS; GIBCO Life Technologies, Paisley, UK), washed with PBS and cultured for six days in complete medium containing recombinant human cytokines interleukin 4 (IL-4; 6.5 ng/ml; R&D Systems, Minneapolis, MN) and granulocyte macrophage-colony-stimulating factor (GM-CSF; 250 ng/ml; Peprotech, London, UK), to obtain immature dendritic cells. After three days, half of the media was replaced with fresh, cytokine-containing media and the cells were transferred to new flasks.

3.14 Phenotypic characterization of DC

At day six immature dendritic cells were washed and resuspended in PBS. They were incubated for 30 min in 4°C with the following anti-human mAbs: CD1a (clone NA1/34; DAKO, Glostrup, Denmark), CD3 (clone SK7; BD Biosciences, San Diego, CA), CD14 (clone TÜK 4; DAKO), and CD19 (clone HD37; DAKO) and characterized by flow cytometric analysis in the FACSCalibur flow cytometer. The mean purity of the cells was 92.2 ± 5.0 % CD1a−CD14+.

3.15 Stimulation of immature DCs

On day six, immature dendritic cells were counted and 5x10^5 cells plated in 24-well plates in 1 ml medium (RPMI supplemented with 10% FBS, HEPES, streptomycin and penicillin) containing recombinant human IL-4 and GM-CSF. Lipopolysaccharide (LPS; 100 ng/ml, Sigma-Aldrich) was added in one well as a positive control for maturation of DCs and just medium in another as a negative control. Poly I:C, poly I and CpG was added in mentioned concentrations. In some experiments, 20 µM chloroquine was added to the DCs 2 hours before addition of stimulus. 200 µl supernatant from all wells was collected and frozen at 24 hour for ELISA and multiplex. At 48 hours all samples were collected and DC maturation was characterized by flow cytometry. The cells were incubated for 30 min in 4°C with the mAbs CD1a (clone NA1/34; DAKO), CD80 (clone L307.4), HLA-DR (clone L243) and CD86 (clone 2331/FUN-1; all from BD Biosciences). The dead cells in the culture were determined by staining with 7-AAD (BD Biosciences). For each sample, 10^5 cells were collected.
4. RESULTS

4.1 Paper I
Increased stability and specificity through combined hybridization of peptide nucleic acid (PNA) and locked nucleic acid (LNA) to supercoiled plasmids for PNA-anchored "Bioplex" formation.

Summary: Low cellular uptake and poor nuclear transfer hamper the use of non-viral vectors in gene therapy. Addition of functional entities to plasmids using the Bioplex technology has the potential to improve the efficiency of transfer considerably. We have investigated the possibility of stabilizing sequence-specific binding of peptide nucleic acid (PNA) anchored functional peptides to plasmid DNA by hybridizing PNA and locked nucleic acid (LNA) oligomers as "openers" to partially overlapping sites on the opposite DNA strand. The PNA "opener" stabilized the binding of "linear" PNA anchors to mixed-base supercoiled DNA in saline. For higher stability under physiological conditions, bisPNA anchors were used. To reduce nonspecific interactions when hybridizing highly cationic constructs and to accommodate the need for increased amounts of bisPNA when the molecules are uncharged, or negatively charged, we used both PNA and LNA oligomers as "openers" to increase binding kinetics. To our knowledge, this is the first time that LNA has been used together with PNA to facilitate strand invasion. This procedure allows hybridization at reduced PNA-to-plasmid ratios, allowing greater than 80% hybridization even at ratios as low as 2:1. Using significantly lower amounts of PNA-peptides combined with shorter incubation times reduces unspecific binding and facilitates purification.

4.2 Paper II
Self-assembling supramolecular complexes by single-stranded extension from plasmid DNA.

Summary: Self-assembling supramolecular complexes are of great interest for bottom-up research like nanotechnology. DNA is an inexpensive building block with sequence-specific self-assembling capabilities through Watson-Crick and/or Hoogsteen base pairing and could be used for applications in surface chemistry, material science, nanomechanics, nanoelectronics, nanorobotics, and of course in biology. The starting point is usually single-stranded DNA, which is rather easily accessible for base pairing and duplex formation. When long stretches of double-stranded DNA are desirable,
serving either as genetic codes or electrical wires, bacterial expansion of plasmids is an inexpensive approach with scale-up properties. Here, we present a method for using double-stranded DNA of any sequence for generating simple structures, such as junctions and DNA lattices. It is known that supercoiled plasmids are strand-invaded by certain DNA analogs. Here we add to the complexity by using "Self-assembling UNiversal (SUN) anchors" formed by DNA analog oligonucleotides, synthesized with an extension, a "sticky-end" that can be used for further base pairing with single-stranded DNA. We show here how the same set of SUN anchors can be utilized for gene therapy, plasmid purification, junction for lattices, and plasmid dimerization through Watson-Crick base pairing. Using atomic force microscopy, it has been possible to characterize and quantify individual components of such supra-molecular complexes.

4.3 Paper III
Defective Toll-like receptor 9-mediated cytokine production in B cells from Bruton's tyrosine kinase-deficient mice.

Summary: Bruton's tyrosine kinase (Btk), a member of the Tec family of tyrosine kinases, plays an important role in the differentiation and activation of B cells. Mutations affecting Btk cause immunodeficiency in both humans and mice. In this study we set out to investigate the potential role of Btk in Toll-like receptor 9 (TLR9) activation and the production of pro-inflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF)-alpha and IL-12p40. Our data show that Btk-deficient B cells respond more efficiently to CpG-DNA stimulation, producing significantly higher levels of pro-inflammatory cytokines but lower levels of the inhibitory cytokine IL-10. The quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis presented in this work shows that mRNA production of one of the important new members of the IL-12 family, IL-27, was significantly increased in Btk-deficient B cells after CpG-DNA stimulation. In this study, we demonstrate significant differences in CpG responsiveness between transitional 1 (T1) and T2 B cells for survival and maturation. Furthermore, TLR9 expression, measured both as protein and as mRNA, was increased in Btk-defective cells, especially after TLR9 stimulation. Collectively, these data provide evidence in support of the theory that Btk regulates both TLR9 activation and expression in mouse splenic B cells.
4.4 paper IV

Down-modulation of poly I:C-induced human monocyte-derived dendritic cell maturation and cytokine production by single-stranded DNA oligonucleotides.

Summary: Dendritic cells (DCs) are potent antigen-presenting cells (APCs) and exploiting the full immuno-modulatory potential of DCs is likely the key for fine-tuning of immune responses. DCs use toll-like receptors (TLRs) to recognize conserved microbial structures. TLR signaling promotes DC maturation, which then mediates the expression of various cytokines, chemokines and co-stimulatory molecules. Very few studies have been devoted to address the impact of TLR ligand combinations on DC maturation and functions especially in the human system. Using synthetic TLR3 ligand, poly I:C as a DC maturing agent, we show that monocyte-derived DC (moDC) maturation and cytokine production can be significantly inhibited by single-stranded DNA oligonucleotides (ssDNA ODN). Combined treatments of moDCs with ssDNA and poly I:C resulted in inhibition of IL-12p40, IL-12p70, TNF-α, IFN-α, IL-6, MIP1-α, MIP1-β and MCP-1 production. Simultaneous stimulation also led to inhibition of poly I:C-induced expression of CD80, CD86 and HLA-DR. Furthermore, TLR3 stimulation activated IRF-3 in moDCs and subsequently led to production of the antiviral response chemokine IP-10. The addition of ssDNA together with poly I:C blocked phosphorylation of IRF3, a downstream component of this pathway. Thus a better knowledge of DC function and the cross-talk between different immune receptors or adaptors could be relevant for the development of new immunotherapeutic approaches.
5. CONCLUSION AND PERSPECTIVES

The present work describes the development of new technologies for vaccination and immunotherapy. The ideal non-viral technological platform might include an immune cell- targeting molecule (which could be achieved through our newly developed SUN anchors), shRNA construct to down-regulate negative regulators of immune signalling (like against Btk) and addition of TLR agonists in the same setting. Through this work we have developed a strategy, which could serve as an embryo for future development.
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