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Preparation and Evaluation of Single-Stranded DNA Aptamer-Based Immunological Adjuvant in Broiler Chickens

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Preparation and Evaluation of Single-Stranded DNA Aptamer-Based Immunological Adjuvant
in Broiler Chickens

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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ABSTRACT

Mineral oils and metal salts are commonly used as adjuvants to enhance acquired immunity. Recently, monoclonal antibodies (MAbs) and recombinant peptides agonist CD40 receptor have shown remarkable promise for induction of rapid and robust immune responses. Limitations of this approach MAb production costs and multiple administrations due to anti-MAb immune responses. Here we demonstrate the development of a unique and sophisticated DNA aptamer-based alternative for CD40-directed delivery of universal antigens as an alternative in chickens, and potentially other vertebrate species. This receptor, expressed by antigen-presenting cells, acts as a costimulatory molecule for activated T helper lymphocytes. After initially selecting for high affinity aptamers of independent sequence, we utilized a polymerization process where multiple aptamers were simultaneously and sequentially arranged through rolling circle amplification products (RCA-p), potentially capable of binding multiple recognition sites and causing receptor clustering. Selected sequences were demonstrated to effectively activate chicken macrophage HD11 cell line through CD40 receptors, demonstrating proof of concept *in vitro*. Additionally, using limited proteolysis liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS), we deduced the actual amino acid sequences targeted by these aptamer products, which are essential for activation. In chapter III, we produced a biotinylated version of an effective CD40-activating RCA-p, and streptavidin-conjugated this RCA-p to a biotinylated peptide of the highly conserved type-A influenza peptide M2e. This conjugate was administered subcutaneously at either (25µg/bird) or (50µg/bird) at age 7 and 21 days. Anti-M2e IgG responses were determined at 14, 21, 28 and 35 days of age by ELISA. The RCA-p-M2e complex, at 50µg/bird, was able to induce robust seroconversion as early as 7 days post-immunization, with high antibody titer consistent through the end of the experiment. The lower dose, however, showed delayed responses

and required the second administration. Taken together, these results constitute proof of concept for RCA-p-directed antigen delivery for peptide antigens and a potential tool for rapid generation of new vaccines against animal diseases.

In chapter IV we discuss PCR artifacts that develop during traditional DNA aptamers selection and proposed a repetitive thermal correction cycles [(65°C/10min, 20°C/1min)x6] of the PCR. This may improve future selection of aptamers for similar purposes.

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DEDICATION

This piece of work is dedicated to..

The souls of

My deceased father Sabr Akar Al-Ogaili

My deceased mother and

My martyr Master Advisor and Mentor, Dr. Ali Abdul-Hussein Shalash.

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LIST OF ABBREVIATIONS

Ags – antigens

APCs – antigen presenting cells

Apt – aptamer

BCR – B-cell receptor

CD40 – cluster of differentiation 40

CD40L – CD40 ligand

chCD40ED – chicken CD40 extra-domain

CTL – cytotoxic T lymphocyte

DAMPs – danger-associated molecular patterns

DCs – dendritic cells

dLN – draining lymph node

ELISA – enzyme-linked immunosorbent assay

HMGB1 – high mobility group box 1

IFN- γ – interferon gamma

IL – interleukin

IgG – immunoglobulin G

LC-ESI-MS – limited proteolysis electrospray ionization liquid chromatography mass
spectrometry

M2e – matrix protein 2

MAbs – monoclonal antibodies

MALDI-TOF-MS – matrix-assisted laser desorption ionization time of flight mass spectrometry

MHC-II – major histocompatibility complex class II

NLRs – NOD-like receptors

NO – nitric oxide

PAMPs – pathogen-associated molecular patterns

RAGE – receptor of advanced glycation end products

PRRs – pattern recognition receptors

RCA – rolling circle amplification

RLRs – RIG-like receptors

SELEX – systemic evolution of ligands by exponential enrichment

SEQ – sequence

SCS – spacer complementary sequence

TCR– T cell receptor

T_H – T helper

TLR – Toll-like receptor

VLPs – virus-like particles

LIST OF PUBLISHED PAPERS

Chapter IV

Al-Ogaili, A. S., B. M. Hargis, and Y. M. Kwon. Efficient correction of heteroduplex DNA formed during PCR amplification of DNA aptamer libraries. (Submitted for publication at BioTech.).

INTRODUCTION

High density rearing of commercial poultry has led to concentrate the effect of many infectious agents with resulting economic losses (Nair, 2005; Morris *et al.*, 2007; CIWF, UK, 2007). Many strategies are now in use to confront the hazard of these infectious agents such as the use of sanitation, biosecurity, antibacterials, antiprotozoals, anthelmintics and vaccinations (Bermudez, 2008). The emergence of evolutionary resistance of these pathogens to such drugs has restricted their use in modern poultry (Peek and Landman, 2003; Dibner and Richard, 2005; Hume, 2011). Seeking alternatives for traditional antibacterials and antiprotozoals encourages relying on other strategies such as vaccination to control the diseases.

Since the first description of vaccines, the definition of vaccinology has expanded to include vaccine manufacturing technologies and other (non-biological) considerations such as ethical and economical evaluations (Berzofsky *et al.*, 2004; Hardt *et al.*, 2016; Barrett, 2016). From the perspective of immunoprotection, vaccines must achieve minimum levels of efficacy, potency, purity and safety. Veterinary vaccines, however, consider economic gain as well. In more broad application for vaccines, vaccines can be applied to non-infectious diseases or cancers in humans. In contrast, in veterinary vaccinology the principle applications are for the protection against animal disease, there are still very significant contributions to human health and economy (Meeusen *et al.*, 2007; Roth, 2011).

The aim of vaccination is to introduce protective antigens of virulent pathogens to the host immune system safely. Individually, animals may be protected by increasing the infectious dose 50 (ID₅₀) for the pathogen. Epidemiologically, a phenomenon known widely as herd immunity can occur when most susceptible animals are protected by vaccination. This decreases the rate of

transmission within populations by decreasing susceptibility to infection, and/or decreasing the quantity and duration of shedding of the pathogen by infected animals. As reviewed by several authors (Fenton and Pedersent, 2005; Fine *et al.*, 2011; Kim *et al.*, 2011; Balakrishnan and Rekha, 2018) herd immunity, defined as “*The level of resistance to highly transmittable pathogen within population*”, is also referred to as “*The minimum proportion of the population that show complete protection against particular pathogen threat*”. In completely susceptible population, the number of likelihood secondary infections, which result from the initial index case, is known as basic reproductive ratio (R_0 , *r naught*), which is also known as *case reproductive rate*. Three potential values for R_0 : 1) $R_0 > 1$, the index case produces more than one new secondary infection. 2) $R_0 = 1$, the index case produces one new secondary infection. 3) $R_0 < 1$ the index case produces less than one new secondary infection. R_0 is usually higher for aerosol-transmitted pathogens as opposed to other routes of transmission such as fecal-oral or trans-epithelial. Based upon the principle of R_0 , it is critical to estimate the number of protected individuals within the herd to secure the population against epidemics. For most pathogens, successful vaccine should induce $\geq 80\%$ herd immunity (Fenton and Pedersent, 2005; Fine *et al.*, 2011; Kim *et al.*, 2011; Balakrishnan and Rekha, 2018).

With most vaccines, delivering an effective immunogen requires the contribution of an effective and safe adjuvant or adjuvant combination to link the innate and the adaptive immune responses, thereby guiding and exaggerating the immune response, and/or to reduce the antigenic mass within vaccine. Thus, effective adjuvants increase the potency and improve the efficacy of vaccines (Coffman *et al.*, 2010; Bonam *et al.*, 2017; Garçon *et al.*, 2011).

Acquired immune responses begins with stimulation of innate immunity. Antigen-presenting cells (APCs), especially dendritic cells (DCs), link the innate and acquired immune responses. These cells are specialized in foreign antigens uptake, processing and presentation (Schuijs *et al.*, 2019).

The recognition of foreign material by APCs is through highly conserved pattern recognition receptors (PRRs), which attract cognate highly conserved pathogen-associated molecular patterns (PAMPs) on viral, bacterial and fungal pathogens such as bacterial LPS, peptidoglycan, unmethylated CpG DNA and viral single-stranded RNA and DNA (Thompson *et al.*, 2011; Juul-Madsen *et al.*, 2012). There are several PRRs such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs). These receptors are soluble, membranous or cytosolic. The PRRs-PAMP interaction pathway represents signal-0 in the course of immune response. However, many sterile inflammatory processes appear to contradict these known processes, which likely indicates alternative mechanisms for induction of inflammation. In many cases, the immune system ignores the “*non-self*” antigens unless they encounter PRRs on DCs and they are accompanied by *damage* to tissue. Matzinger (1994) stated that immune response elicited against damage-associated molecular patterns (DAMPs) is greater than that those generated against PAMPs alone. Inactivated pathogens, or their highly purified products, are not ideal for DC activation alone; therefore, traditional adjuvants are essential for efficacy of inactivated vaccines. Some examples of these adjuvants are aluminum salts (aluminum hydroxide and aluminum phosphate), oil emulsions, and purified PAMPs or similar products (Schijns *et al.*, 2008, Kool *et al.*, 2012; Wilson *et al.*, 2012; Bonam *et al.*, 2017).

Well-designed vaccines with specifically effective adjuvants are favorable in modern vaccines to compensate the lack of virulent action of the weakened or inactivated, or low antigenic mass as in subunit or recombinant vaccines. In this way, adjuvants exchange false exaggeration of low-virulent pathogens to robust immune response (McKee *et al.*, 2007; Garçon *et al.*, 2011; Milligan, 2015; O’Hagan and Fox, 2015).

In the first generations of vaccines, aluminum salts, oil emulsions such as water-in-oil or oil-in-water emulsions, and synthetic biological compounds had been utilized as vaccines' adjuvants interchangeably. Such adjuvants rely on their chemical composition or biological activity to trigger local immune 'recognition' response in non-specific manner (Garçon *et al.*, 2011; Di Pasquale *et al.*, 2015). With the better understanding of immune response and vaccine-host relationships, the era of more sophisticated vaccines has emerged. These new vaccines needed new generation of integrated adjuvants. This has led to development of innovated molecular adjuvants such as monoclonal antibodies (MAbs), cytokines and recombinant peptides. These next-generation adjuvants have relatively recently begun to replace first generation adjuvants in order to offer more flexible and more efficient vaccines (Hatzifoti and Heath, 2007; Chen *et al.*, 2010; Knudsen *et al.*, 2015). For example, with recombinant vaccines, immune enhancer peptide(s) are co-expressed with the vaccine's immunogen. In other subunit vaccines, the adjuvant may be linked chemically to the immunogen compartment to form one multi-molecule unit (Layton *et al.*, 2009; Chen *et al.*, 2012; Noh *et al.*, 2015). As stated earlier, the first generation adjuvant targeting is induction of innate immune response, primarily through activation of PRRs. Unlike those traditional adjuvants, next-generation adjuvants are designed to target immune elements that lead to induction of a specific immune response. Examples include adjuvants that provide agonistic activity of specific costimulatory molecules on the surface of the APCs or interleukins. Thus, next-generation adjuvants trigger and modulate specific type and/or pathway of the immune response due to their high specificity (O'Hagan, 2015; De Veer and Meeusen, 2011).

There are three primary objectives for this thesis. The first part discusses the use of single-stranded DNA aptamers, which have been specifically selected against the extra-domain of the chicken CD40 receptor, as a novel immunological agonist for this receptor. The process of the aptamers

selection was as described by Tuerk and Gold (1990), and Ellington and Szostak (1990) simultaneously to produce highly specific enriched aptamers. Physiologically, the CD40 and the CD40 ligand (CD40L) are presented as trimers. The oligomerization of the receptor-ligand requires the participation of the receptor and the ligand motifs, with cross-linking of the CD40 subunits as a requisite for activation. To simulate this naturally occurring oligomerization, and to exaggerate the agonistic effect of our aptamers on the chicken CD40 receptor, the aptamers were amplified by rolling circle amplification (RCA) technique. Technically, the RCA technique yields single stranded DNA, presenting tandem repeats of the template. Accordingly, RCA is ideal potential option for spanning and crosslinking the trimer receptor and in increasing the possibility of receptor stimulation.

The rationale behind targeting chicken CD40 receptor as that this costimulatory molecule acts synergistically with other costimulatory molecules and cytokines to modulate the acquired immune response. In fact, this molecule is required to amplify the action of APCs and activated T helper lymphocytes. Previously, Chen and coworkers (2010, 2012) have developed an anti-chicken CD40 MAb that effectively stimulated a chicken macrophage cell line (HD11) and as an adjuvant *in vivo*. Therefore, we sought to mimic the action of this novel MAb by replacing it with DNA aptamers. Similarly, our multivalent-polyvalent RCA products-produced aptamers, containing repeating monomeric aptamers, caused detectable stimulation of the chicken HD11 macrophage cell line.

The second objective of this dissertation is focused on the evaluation of our sophisticated immunological adjuvant *in vivo*. We tested the ability of our aptamers-based RCA products to increase the efficacy and the potency of an immune response against a small, low immunogenic avian influenza virus (AIV) extracellular domain of the matrix protein 2 (M2e) peptide. The

extracellular domain, which is a 24-amino acid peptide, forms ion channels to deliver the viral RNA into the host's cell. This peptide is a highly conserved peptide among all AIVs. However, this peptide induces very low immune response *per se* (Tarigan *et al.*, 2015; Cho *et al.*, 2015; Vuong *et al.*, 2018). We prepared an immune complex composed of streptavidin as an immunologically inert central molecule. The streptavidin links two biotinylated RCA molecules with two biotinylated synthetic M2e peptides. In fact, we replicated our previous work with this particular peptide with MAb as the adjuvant. We administered this immune complex [(2)RCA-SA-(2)M2e] in two doses (25µg/bird) and (50µg/bird) subcutaneously to broiler chickens at two time intervals. The results were encouraging and demonstrated that our aptamers could enhance the production of anti-M2e IgG in chicken sera.

The third objective of this thesis deals with developing a method, which able to correct the common artifacts that commonly develop in PCR products during DNA aptamer selection processes. Commonly, this method depends on thermal treatment of the PCR byproducts to reduce probable heteroduplex formation. The results showed significant reduction in these byproducts and improved linear enrichment yield of the treated pools of aptamers.

CHAPTER I

REVIEW OF LITERATURE

1.1.INTRODUCTION

In commercial poultry industry, there are many vaccines and vaccination programs are in use. Successful vaccine requires utilization of the proper route of administration, the proper timing, the proper antigenic mass and, with many vaccines, the proper adjuvant (Schijns, 2000). Generally, all inactivated, subunit and inactivated recombinant vaccines strictly require the input of adjuvants. As discussed above, the main objective of vaccination is to introduce the foreign antigen safely to the immune system aiming to decrease the pathogenicity of wild-type challenge by increasing the infectious dose 50 (ID₅₀). In poultry, the purpose of vaccination is to increase the herd immunity or to hyperimmunize breeders in order to protect progeny by maternal antibodies (Collett, 2013). Inactivated vaccines, however, usually only provide protection in advance of challenge with pathogens causing clinical disease and rarely prevent infection *per se*. Consequently, this applies selection pressure on wild-type pathogens toward either more virulent variants of the original pathogen, or toward antigenic change (escape mutants). To keep in pace with this phenomenon, vaccine developmental is a continuous process (Gandon *et al.*, 2001; Davison, 2008, Gimeno, 2008). Accordingly, development of adjuvants moved hand-in-hand with that of vaccines. Adjuvants have relatively recently moved from first-generation inorganic salts-based, and mineral oil-based adjuvants to highly sophisticated molecular adjuvants (Di Pasquale *et al.*, 2015). Better understanding for several branches of disciplines such as immunology, microbiology and molecular biology has enabled designing better adjuvants. The demand for developing new

generations of adjuvants is highly linked to the mechanisms by which the virulent pathogens evade the host immune system (Furman and Davis, 2015).

1.2.THE DISCOVERY OF VACCINES

Chronologically, the power of vaccines in controlling infectious agents was recognized by medieval Far East, Central Asian, and Ottoman societies (Andre, 2003; Leung, 2011; Boyston, 2012). The generally acknowledged birth of modern vaccinology, the science of vaccines, was first described in Europe in 1796 by Edward Jenner who utilized cowpox virus from infected humans to inoculate naïve humans, which reduced the incidence and severity of smallpox in inoculated people (Smith, 2011). Later, Louis Pasteur developed the first live attenuated bacterial vaccine against chicken cholera in 1879 (Pasteur, 1880; Smith, 2012). Contributions from other scientists such as Paul Ehrlich and Elie Metchnikoff were considerably valuable breakthroughs. These significant contributions from vaccinology were the fundamental baseline that suited better understanding for immunology basics such as the host's primary and secondary immune responses (Grieg, 1902; Gordon, 2008; Valent *et al.*, 2016).

Since their discovery, vaccines have become a very important economic industry. Nowadays, the vaccine chosen depends on several epidemiological or biological factors. Of emerging diseases, the time line required for initial developing a vaccine and final approval usually takes 15 years on average with tremendous economic investment (Beasley, 2015). Fundamentally, there are two major types of vaccines, live vaccines and inactivated vaccines. However, the spectrum of this category extends to include many vaccine subtypes. Despite a prodigious number of recent

publications regarding poultry vaccination, most are struggling experimentally and have not yet become commercially viable.

1.3.TYPES OF VACCINES

1.3.1. Live vaccines

Commercial live poultry vaccines are available for almost all avian pathogens. Technically, the development of this type of vaccines vary widely. This type may include traditional live vaccines, recombinant live vaccines, and vectored vaccines (Schat, 2015). This type is suitable for mass application methods with various routes, which largely meets low labor requirements for use in commercial poultry (Sharma. 1999).

Live vaccines usually require minimal antigenic mass for protection since the immunogen is capable of multiplication within the host. This type has great advantages economically as it enhances rapid immune responses and provides for protection of mucosal surfaces. In many cases where very high levels of immunity are desired, live vaccines precede booster vaccinations with inactivated and/or subunit vaccines to prime the immune response. However, the immune response to these vaccines in poultry often relatively short-lived, and often requires multiple booster vaccinations for breeders or layers prior to onset of sexual maturity. Another disadvantage of live vaccination is that these vaccines are not easy to handle, they demand specific storage conditions and exhibit relatively short shelf life, and require specific procedures prior use such as mixing with skim milk or other chlorine neutralizing agents (Box, 1984; Sharma, 1999; Gimeno, 2008; Collett, 2013). Mucosal delivery of live Newcastle disease (ND) vaccine in combination with chitosan as the adjuvant showed elevated level of immune response (Rauw *et al.*, 2010).

1.3.1.1. Live attenuated vaccines

This type of vaccine, such as infectious bronchitis (IB) vaccine and infectious bursal disease (IBD) vaccine is commonly produced by the artificial attenuation of naturally virulent pathogens (Müller *et al.*, 2012; Lim *et al.*, 2012). The process of attenuation includes the passing of the pathogen in abnormal host (tissue culture, embryonating chicken eggs, or media), or inducing mutation(s) by knocking out certain gene(s) where the pathogen loses some pathogenicity but retains infectivity. (Yamaguchi *et al.*, 1996; Steel *et al.*, 2009).

Some bacterial vaccine strains are genetically stable such as *Salmonella Typhimurium* χ 3985, however, live attenuated vaccines are not always safe because of the possibility of reverting to virulence when used in the natural host, as there is evolutionary pressure for selection of increased reproductive fitness of the pathogen. One method to develop live attenuated bacterial vaccines is transposon mutagenesis, which is sometimes a limitation of live bacterial vaccines due to fair randomness and large number of mutations obtained (Sampson *et al.*, 2003). On the other hand, the possibility of recombination of attenuated viral vaccines has to be considered with live viral vaccine selection. Nevertheless, there is potentially tremendous reward as compared to relatively modest risk in that protection from common wild-type pathogens is possible (Hassan and Curtiss, 1996; Drake, 1999; Watanabe *et al.*, 2008; Barrett, 2015; Eschke *et al.*, 2018). Live attenuated IBD vaccine strains are resident in most poultry farms and they may interfere with the future vaccination and maternal immunity leading to vaccine insufficiency (Müller *et al.*, 2012).

A new emerging type of live attenuated vaccines prepared by insertion certain antigen(s) related to virulence into vector genome such as live avirulent viral or bacterial vectors. Well-known viral vector systems that have been used in preparation of recombinant poultry vaccines are fowlpox

virus, Marek's disease, herpesvirus of turkey (HVT), ND virus and baculovirus. Many of these viral vectors expressing H5 avian influenza virus (AIV) antigen or ND virus antigen have been shown to be successful vaccine candidates. Many bacterial vector systems have been described in poultry including *Salmonella* spp. (Curtiss and Kelly, 1987; Boursnell *et al.*, 1990; Beard *et al.*, 1991; Capua and Alexander, 2008). Yeast, such as *Pichia pastoris*, is also a good vector candidate for expression the protozoan antigens such as *Eimeria* spp (Zhang *et al.*, 2014).

1.3.1.2.Live avirulent vaccines

Many variants of certain viral or bacterial pathogens that lack significant pathogenicity but retain infectivity have been utilized as live vaccines. Hitchner B1 and LaSota are good examples of lentogenic and mesogenic strains of the ND virus, respectively (intracerebral pathogenicity index –ICPI of the Hitchner B1 strain is 0.2, whereas the ICPI of the LaSota is 0.4). Avirulent stains naturally cause subclinical infections and may go unnoticed. The development of modern molecular biology has greatly increased investigations in this regard, as clinical signs are not useful or reliable. Choosing low virulence strains as vaccine candidates must meet certain criteria such as immunogenicity and stability under field conditions. (Westbury *et al.*, 1984; Alexander and Senne, 2008; Kapczynski *et al.*, 2013). Genetically engineered bacteria can serve this purpose as well. There are many salmonellae and *E.coli* in use as vaccines or vaccine vectors (see below).

1.3.2. Inactivated vaccines

Inactivated vaccines are composed of whole killed viral or bacterial soma or any non-replicating antigen vaccine such as purified peptides and recombinant subunit vaccines (Schat, 2015). Due to inactivation or high selection and purity of the epitopes, these vaccines lack productivity and pathogenicity, which in turn affect their immunogenicity. Therefore, these types of vaccine often

required adjuvant, high antigenic mass, and more than one dose to maintain sufficient duration of protective immune response (Meeusen *et al.*, 2007; Marangon and Busani, 2006). With the inactivated vaccines, the type of the adjuvant is highly directing and interfering the most features of the elicited immune response. Moreover, the most robust and uniform immune responses to inactivated vaccines usually follow priming by live vaccines. Priming-boosting strategy is most commonly used for relatively long-lived poultry such as breeders or layers. The immune response for inactivated vaccines is different from that induced by live vaccines. In inactivated vaccines, cellular immune response is relatively poor whereas, humoral immune response is the dominant and largely influenced by the presence and type of the adjuvant (Muir *et al.*, 2000; Schijns *et al.*, 2014). Live vaccines usually administered via mucosal route and capable of inducing secretory antibody (SIgA), which can reduce infectivity in addition to protection from disease, whereas inactivated vaccines are often administered via injection (Jayawardane and Spradbrow, 1995).

Advantages of inactivated vaccines include increased safety, low cost of development for most vaccines, and relatively long shelf life. Killed vaccines are prepared by the inactivation of the pathogen by exposure to heat, gamma radiation or chemicals such as formaldehyde and β -propiolactone (Astill *et al.*, 2018).

1.3.3. Toxoid vaccines

This type of vaccine relies on using highly purified inactivated toxins of some bacteria. Such bacteria are known to induce the disease by releasing toxins (exotoxins mainly). This type of vaccines, such as diphtheria toxoid and tetanus toxoid vaccines, are quite common in human medicine and veterinary medicine other than avian medicine. However, in commercial poultry, few publications describing toxoid vaccines against necrotic enteritis and turkey cellulitis caused

by *Clostridium perfringens* (*C. perfringens*) and *Clostridium septicum* (*C. septicum*), respectively. *C. perfringens*-causing necrotic enteritis is with not clearly understood pathogenicity. However, recent publications have described the application of toxoid anti-*C. perfringens* vaccines to chickens with variable success (Keybum *et al.*, 2013; da Costa *et al.*, 2013).

C. septicum, on the other hand, causes cellulitis and dermatitis in turkey during the production period. The alpha toxin of *C. septicum* is responsible for the clinical disease. In one approach, our laboratory developed a formalin-inactivated and adjuvanted toxoid vaccine against this disease with success (unpublished data). Formalin-induced detoxification occurs through chemical modification of the toxin. However, the exact chemical mode of modification is not clearly demonstrated (Thaysen-Andersen *et al.*, 2007). Other chemical or heat treatments are used to detoxify the bacterial toxins (Galazka, 1993; Jones *et al.*, 2008). Recently, recombinant, genetically engineered inactivated toxins have been tested in mammals as vaccine candidates (Gentshev *et al.*, 2001). This kind of vaccines requires adjuvant and the immune response against this kind of vaccines is similar to that of the inactivated vaccines in many facets except, the antibody profile against toxoid vaccines is with narrow range due to fewer epitopes (Baxter, 2007).

1.3.4. Recombinant vector vaccines and subunit vaccines

The strategy of preparing recombinant vaccines depends on the presence of low virulent or avirulent live carrier, which gained the capacity to carry and deliver heterologous antigen(s) as a vaccine. Genetically engineered attenuated salmonellae and *E. coli* strains commonly used as delivery systems for many vaccines antigens. Insertion of specific antigen sequences into vector plasmids can produce co-expression of this particular antigen with the outer membrane proteins of the vector such as *OmpA* or *LamB*, thus improving the immune response against the selected

antigen(s). Immune enhancing effectors may be designed to co-express with the vaccine antigen, such as co-presentation as fusion protein. Examples include *E. coli* heat-labile toxin subunit B, avian CD154 or ligands for PRRs such as IL-4. (O’Callaghan *et al.*, 1990; Pistor and Hobom, 1990; Cardenas and Clements, 1993; Wolfenden *et al.*, 2010; Sawant *et al.*, 2011). Genetically engineered yeast vectors (e.g. *Pichia pastoris*) are able to carry other pathogenic eukaryote genes to overcome post-translational modification and protein folding issues in eukaryotic proteins (Higgins, 1995). As discussed above, several avian viruses have been utilized as vaccine vectors. Those are all large DNA viruses with relatively large genomes (see above).

Genetically re-assorted live attenuated AI vaccines have been described (Nogales and Martínez-Sobrido, 2017). To produce this kind of vaccine, embryonating eggs (or susceptible cell line) are co-infected simultaneously with two viral strains, e.g. wild-type HPAI virus and reference attenuated virus that has high replication rate in the embryonating chicken egg or cell line. Re-assorted strains can then be selected using serological tests. Recombination between the attenuated and the wild type viruses will result in some combinations, which include the attenuated HA cleavage site with the wild-type HPAI antigens.

Reverse genetics vaccines are another type of recombinant (live) viral vaccines. DNA viruses and positive-sense RNA viruses were the first targets for reverse genetics vaccines development (Nogales and Martínez-Sobrido, 2017). However, several systems have been described for development reverse genetics vaccine against influenza virus (Horimoto and Kawaoka, 2006; Uchida *et al.*, 2014). In one example, the HA and NA gene segments of the wild-type virus (e.g., H5N1) are inserted in the backbone of the laboratory attenuated virus (A/Puerto Rico/8/34 (PR8)). In plasmid-based technique, each of the viral cassettes were inserted into individual vector plasmids, i.e. 8 plasmids represent RNA polymerase I for 8 segments cDNA, and 4 plasmids

expressing polymerase II for viral proteins ([RdRp complex: PB2, PB1 and PA] and NP for viral RNP assembly). The cassettes include the viral RNA polymerase I transcription cassettes with polymerase promoter and terminator. Multiple basic amino acids of HA from LPAI virus replace the motif of the HPAI virus HA counterpart. All the viral HA, NA, and other viral proteins complementary DNA (cDNA) are all combined in the cassettes between the promoter and the terminator (Neumann *et al.*, 2005; Uchida *et al.*, 2014). Recently, less plasmids based systems have been described. Adjuvants reliable for this type of vaccines could be aluminum salts, or squalene-based oil emulsions or other next-generation adjuvants (Nogales and Martínez-Sobrido, 2017).

1.3.5. Conjugate vaccines

Some antigens of pathogens express low immunogenicity, especially in newborns, such as bacterial polysaccharides and bacterial capsular polysaccharides. Because these antigens are non-proteinaceous and less antigenic, these molecules directly activate B cells with no T cells recruitment (T cell-independent B cell activation). The sequelae of this pathway is absence of memory cells, nevertheless, antibody class switching truly occurs (von Bülow *et al.*, 2001; Shulgakovskaya *et al.*, 2004). Other pathogens have certain strategies of evading the host immune system by concealing their surface antigens (antigenic disguise) or inhibiting certain immunological pathways (Finlay and McFadden, 2006).

As the name refers, conjugate vaccines act to link host immune responses to well identified immunogen in conjugation with low immunogenic antigens of a disguise invader. With this type of vaccines, low immunogenic *haptens* covalently conjugated to carrier protein render them

inducting well-defined immune response (Avery and Goebel, 1929; Finn, 2004; Colombo *et al.*, 2018).

1.3.6. DNA vaccines

This type of vaccine makes benefit of vector plasmids harboring pathogen encoded gene(s) together with promotor/terminator sequences. This plasmid promotes the expression of the pathogen gene(s) directly onto the recipient avian cells (Meunier *et al.*, 2016). In fact, this type of vaccine is a developed recombinant vaccine; it utilizes the recipient somatic cells as the expression system for the vaccine antigen rather than other unicellular or viral vectors. In this way, recipient somatic cells will present vaccine antigens on their MHC class I molecules resulting in the cytotoxic T cell activation pathway. Many poultry DNA vaccines have been developed against avian pathogens such as anti-AI vaccine (Robinson *et al.*, 1993; Suarez and Schultz-Cherry, 2000).

The limitation of this approach, however, is selection of the plasmid administration tool and the target tissue for vaccination. Some publications describe using viable attenuated viruses or bacteria as the plasmid injection tools. These organisms protect the plasmid from the degradation by the recipient nucleases and promote the uptake of the plasmid by APCs. With these delivering systems, humoral immune response will develop (Sizemore *et al.*, 1995; Liu, 2003).

1.3.7. Virus-like particles and virosome vaccines

These types of vaccines consist of the non-genetic antigenic components of enveloped viruses, which are specially prepared. Virus-like particles (VLPs) and virosomes are alike with few differences. They both lacking the genetic materials but retaining the outer viral coat assembly and native antigens (Moser *et al.*, 2013; Lua *et al.*, 2014). VLPs are produced by recombinant

technology from naked and enveloped viruses by inserting certain viral protein genes in recombinant vector plasmid of higher eukaryotic cells of plants, insects, yeast, or embryonating chicken eggs. After the vector replication, the resulted VLPs are self-assembled and could be harvested using several techniques such as ultracentrifugation. Eventually, the VLPs consist of all virus antigens and lack the viral genome (Lua *et al.*, 2014).

Virosomes, on the other hand, are produced *in vivo* from enveloped viruses such as AIV by three steps: 1) detergent solubilization of the viral coat, 2) removing the viral nucleocapsid by ultracentrifugation, and 3) elution of the detergent (Stegmann *et al.*, 1987; Huckriede *et al.*, 2005). Influenza virus VLPs and virosomes have been shown to have the ability to bind to the host cell membrane due to the presence of biologically active hemagglutinin (HA). The immunological significance of the HA is that this viral protein is a major target of anti-influenza immune response (Huckriede *et al.*, 2005). Recently, recombinant HA protein of influenza virus has been expressed in a bacterial vector, which was then conjugated with bacteriophage-derived VLPs, showed protection against the challenge with the wild-type virus (Jegerlehner *et al.*, 2013).

1.4.THE DISCOVERY OF VACCINE ADJUVANTS AND THEIR TYPES

Chronologically, the discovery of the vaccine adjuvants and their development is closely related to the development of inactivated vaccines (Di Pasquale *et al.*, 2015). There are several definitions for vaccine adjuvants or immunological adjuvants in literature. Generally, vaccine adjuvants are substances that promote potency, efficacy and persistence of adaptive immune response to an antigen. In essence, suitable adjuvants have low or non-immunogenicity *de novo*. Therefore, the development of adjuvant is closely related to the better understanding of immunology and biology.

With the basic level of understanding for these sciences, simple chemical adjuvants were common. When the discipline of immunology and microbiology expanded, more adjuvants that are more sophisticated then emerged. The first attempt to develop an adjuvant was reported by Gaston Ramon, a French veterinarian, in 1924 when he injected inactivated diphtheria or tetanus toxoid in combination with starch, breadcrumbs or tapioca in horses. He recorded high anti-diphtheria and anti-tetanus antibodies in horses that developed abscesses at the injection site (Cited by Vogel and Hem, 2004). Further investigation by other scientists demonstrated that aluminum salts and mineral oil compounds had the similar effects. In 1932, aluminum salts were approved for use in human vaccines. However, this adjuvant was only successful with killed vaccines and those vaccine that targeting humoral immune response (as reviewed by Spickler and Roth, 2003). For viral pathogens or intercellular pathogens in general, the cell-mediated branch of the acquired immune response is required. Thus, different adjuvants were required. Unlike old killed vaccines which may harbor traces of elements of the pathogen that can serve as a casual adjuvant, modern highly purified vaccines are less successful in induction an immune response *de novo* (O'Hagan, 1997; Schijns, 2000). It is difficult task to create one adjuvant category because of the huge diversity of these molecules (biologically and chemically) and rudimentary understanding for required modes of action. However, with recently improved understanding of molecular immunology, the mechanisms of actions of adjuvants became clearer. Adjuvants can now be classified according to their chemical composition or their immunological action. As described by Schijns (2000), Schijns and co-workers (2008), and Schijns (2017), there are two immunological groups of adjuvants described as signal-1 enhancers/potentiators and signal-2 enhancers/potentiators (please see below).

Signal-1 potentiators' category includes TCR-peptide-MHC class II molecular interactions. However, this interaction is not always sufficient to ignite full immune response process because signal-0 (danger signal) is also required (signals 0, 1, 2 and 3 models are reviewed in detail below). Traditional adjuvants such as aluminum salts and oil emulsions that prolong the vaccine antigen release from the inoculation site are all under this category (Schijns, 2017). Unless adjuvants are included, inactivated vaccines may be ignored by the acquired immune system despite the presence of sufficient antigenic mass. It is now well understood that innate immune responses provides signal-0 via the interactions of PRRs-PAMPs (Spickler and Roth, 2003). As stated earlier, DAMPs could provide robust innate immune responses in sterile inflammation. These molecules, such as extracellular high mobility group box 1 (HMGB1), and extracellular heat shock proteins (HSPs), show the ability to trigger innate immune response via receptor for advanced glycation end products (RAGE) and TLR receptors, respectively (Matzinger, 1994; Tang *et al.*, 2012). It is now well understood that the following adjuvants category depends on adjuvant input within the time course of innate and acquired immune response to adjuvanted vaccines. The exception are VLPs and virosomes vaccines. These vaccines may elicit protective immune response in absence of adjuvants (viral genomic RNA or DNA, or artificial adjuvant). This might be attributed to the highly concentrated and highly ordered epitopes that show tandem repeat order. In addition, these vaccines directly activate APCs (Schijns, 2017).

Signal-2 potentiators, on the other hand, act to stimulate the APCs (DCs in particular) with concurrence of costimulatory molecules upregulation. These molecules act as adjuvants via agonistic engagement with specific type of PRRs on the APCs (Schijns, 2017).

1.4.1. Signal-1 enhancers/potentiators

Adjuvants under this category act directly by influencing the introduction of the vaccine antigen to innate immune cells, more specifically, APCs. This category includes the adjuvants that extend the availability time of the vaccine antigen, and create danger signal to attract the interested inflammatory and innate immune cells. For example, this kind of adjuvants may include aluminum salts, oil emulsions, TLR agonists, interferon (IFN- γ), and interleukins (IL-1 β) (Gupta *et al.*, 2014a; Gupta *et al.*, 2014b; Ramakrishnan *et al.*, 2015). The introductory effect of these adjuvants has to be accompanied by danger signal through irritation and local damage. As discussed above, natural immune response starts with signal-0, which represented by the DAMPs/PAMPs interaction with PRRs on the DCs. Next, DCs become mature with changed cytokine profile, which lead to homing of the mature DCs to marginal draining lymph nodes (dLN). Naïve T cells, on the other hand, do not have the receptors or cytokines profile to leave the secondary lymphoid organs or draining lymphatic vessels in mammals. Within the secondary lymphoid organs, naïve T cells only encounter processed and presented foreign antigen by mature DCs homing to dLN in mammals. DCs require input from injured tissue and other inflammatory cells in the form of chemokines and cytokines to direct lymphocytic chemotaxis.

As discussed above, PAMPs-PRRs and/or coupling with DAMPs are crucial events to trigger innate immune responses (Coffman *et al.*, 2010; Matzinger, 1994). The nature of the pathogenic antigen determines the course of the subsequent acquired immune response. When foreign antigens are introduced parenterally, they must cause stimulation of DCs to act as a vaccine. For example, with aluminum salts-based adjuvants, the traditional thinking has been that these adjuvants simply adsorb and slowly release the antigen locally. However, they also offer a degree of irritation, stimulating innate immune responses, thereby stimulating immature DCs via their PRRs. This

phenomenon provides multiple mechanisms for interaction of vaccine antigens with DCs. In this way, adjuvants such as aluminum salts may promote a prolonged immune response signal-0 (Marrack *et al.*, 2009). Evidence suggests that aluminum salts result in local necrosis and uric acid production. This pathway leads to stimulation of DCs via NOD-like receptor (NLRs) and NLRP3 inflammasome activation (Mutwiri *et al.*, 2011). However, several more recent observations suggest that the actual mechanisms involved may be more complicated. For example, in mammals, the cascade of DC innate activation has been reported to be dependent upon inflammasome formation rather than TLRs interaction. NLRP3 inflammasome signaling could instruct the production of pro-inflammatory cytokines such as IL-1 β and IL-18 by macrophages (Eisenbarth *et al.*, 2008; Vance *et al.*, 2009; Wilson *et al.*, 2014). As described above, this pathway is dependent upon priming with PAMPs-PRRs. Not all aluminum salts-based vaccines carry microbial PAMPs (Savelkoul *et al.*, 2015).

Recent studies have shown that aluminum salts have the capacity for complement fixation, mammalian eosinophil and neutrophil recruitment and granulomatous lesion formation but not direct DC maturation (Shamri *et al.*, 2011; Ghimire, 2015). Thus, the ability of aluminum salts to mediate PRRs is questionable. Moreover, the mode of action of aluminum salts-based, oil-based emulsions, Freund's complete and Freund's incomplete adjuvants is not well understood. Instead, aluminum salts require synergistic effect of LPS to stimulate the action of caspase-1 in DCs with subsequent release of IL-1 β and IL-18. Conversely, MyD88-knockout mice, which lack IL-1 β and IL-18, were able to respond normally to aluminum salts-based adjuvanted vaccines (Franchi and Núñez, 2008; McKee *et al.*, 2009; Mosaheb *et al.*, 2017). Studies on water-in-oil emulsions suggested that the vaccine antigen enclosed in the aqueous phase of the adjuvant, whereas oil-in-water adjuvants trap the antigen in the oil phase. Therefore, hydrophilic antigens work better with

water-in-oil adjuvants and hydrophobic antigens work better with oil-in-water adjuvants. Oil emulsions and Freund-based adjuvants suggested to work by depot-slow release of the antigen hypothesis (Stills, 2005; Awate *et al.*, 2013). Furthermore, there is solid evidence that the DCs function to carry a vaccine antigen with labeled adjuvant to marginal LNs in mammals (Herbert, 1968; Dupuis *et al.*, 1998; Jansen *et al.*, 2007).

1.4.2. Signal-2 enhancers/potentiators

As stated above, PRRs are the receptors responsible for recognizing the cognate PAMPs (see above). These receptors are the keystone in innate immune recognition pattern (O'Hagan *et al.*, 2017). In fact, PRRs are highly conserved among vertebrates and are able to recognize their counterparts of highly conserved PAMPs of bacteria, viruses and fungi. The class of PRRs involved in initiation of the innate immune response determines the PPR-dependent acquired immune response. For example, Toll-like receptors (TLRs) guide acquired immune responses differentially from those guided by NLRs with initiation of innate immunity. Conversely, Matzinger (1994) hypothesized that the immune system is more activated by danger signals rather than *non-self* signals. Accordingly, danger signal recognition via DAMPs, leads to direct or indirect upregulation of APC costimulatory molecules (signal-2).

Unlike signal-1 enhancer adjuvants, signal-2 enhancer adjuvants act to deliver the vaccine antigen directly to immune cells instead of storage behavior. Adjuvants under this category act by altering the cytokines profile and/or expression of costimulatory molecules (Sprikler and Roth, 2003). When humoral immune response is required for protecting against certain types of pathogens, cytokine profiles play the critical role in determining the antibody abundance and isotype. Different antibody isotypes harbor different abilities in neutralization and opsonization of the

pathogen and complement fixation. Cytokines such as interleukins (IL-1 β , IL-2 and IL-18), type I interferons (α and β), MGF and G-CSF may be processed chemically or by co-expression with the pathogen gene to act as potential adjuvants (Hilton *et al.*, 2002; Rahman and Eo, 2012). For example, type I interferons induce upregulation of CD40, CD80, CD86, MHC class I and class II molecules. When recombinant influenza vaccine carrying interferon was administered, significant increased levels of protection were reported (Proietti *et al.*, 2002; Hervas-Stubbs *et al.*, 2011).

Our lab group have established that using extracellular HMGB1 as an adjuvant was quite successful (Kuttappan *et al.*, 2013). Extracellular HMGB1 is a ligand for RAGE, which mediates DC maturation (Grover *et al.*, 2013). Finally, costimulatory molecule receptor-ligand pathways have been recently investigated, and shown to be effective. For example, anti-CD40 MAb showed sustainable adjuvant effects in this category in mice (Hatzifoti *et al.*, 2008). More recently, an anti-CD40 MAb has been demonstrated to be similarly effective in chickens when linked to a variety of antigens (Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016; Martin *et al.*, 2017; Vuong *et al.*, 2018).

1.5.CD40 RECEPTOR

Communication among cells occurs physically and chemically via various receptors and ligands. Cell interaction and cell signaling are essential tasks to induct, sustain and terminate an immune response. Several molecules have been characterized as costimulatory molecules within the adaptive immune response. Cluster of differentiation 40 (CD40) is one of these molecules that participates significantly in the course of developing a humoral immune response. This molecule was firstly reported as a surface antigen protein expressed on human bladder carcinoma cells

(Koho *et al.*, 1984). Further studies have shown that this molecule is naturally expressed as a B-lymphocyte cell surface receptor that regulates humoral immune response (Paulie *et al.*, 1985), as well as on other APCs. (Stout and Suttles, 1996; van Kooten and Banchereau, 1997; Ma and Clark, 2009; Suttles and Stout, 2009). This receptor, which belongs to tumor necrosis factor receptor (TNF-R) superfamily, is a 48kDa glycoprotein with extracellular domain, leader sequence, transmembrane domain and intracellular domain. The extracellular domain is a trimer whereas; the cytoplasmic domains are several TNFR-associated factors (TRAFs) (Braesch-Andersen *et al.*, 1989; Bajorath and Aruffo, 1997; McWhirter *et al.*, 1999).

The ligand for the CD40 receptor (CD154) is a 34-39kDa protein with trimeric extracellular domain that belongs to TNF superfamily. The ligand is transiently expressed by activated CD4⁺ T_H lymphocytes. However, recent studies have shown that CD40L (CD154) is also expressed on DCs and activated B cells. In DCs, the regulation of adaptive immune response via this receptor is well identified (Ma and Clark, 2009). As described above, the expression of CD40 and CD40L requires preceding signal-0 and signal-1. Naïve B cells become activated through the engagement of B cell receptors with foreign peptide antigen at the periphery, thereby acting as the APC for resting T cells at the T zone within dLN.

Engagement of TCR on naïve T cell with presented peptide that is in association with the MHC class II molecule on the B cell leads to activation and differentiation of the naïve T cells into T_H cells. Both activated cells begin to express costimulatory molecules such as CD40, CD80, CD86 and MHC class II molecules on B cells as well as transient expression of CD40L on activated T cells. Therefore, activated T cells also participate in activation of B cells via CD40-CD40L pathway. The subsequent downstream cell signaling within activated B cells is differentiation into

plasma cells, which produce abundant antibodies with high affinity and specificity to the target antigen, as well as leading to memory cell development (Parker, 1993; Ratcliffe, 2008).

1.6. CD40 RECEPTOR AGONISTS AND ANTAGONISTS

Most publications on CD40 agonists and antagonists are dealing with mammalian anti-cancer therapy research and autoimmunity, respectively (Vonderheide *et al.*, 2013; Karnell *et al.*, 2018). Recent studies showed that the use of different agonistic MAbs to CD40 might lead to induction of adaptive immune responses or mediate cytotoxicity against specific CD40-expressing lymphomas. These approaches are promising tools in cancer immunotherapy (Tai *et al.*, 2005; Vonderheide and Glennie, 2013). Activation of tumor-associated macrophages via CD40 revert the suppressive action of tumor cells, and render them active antitumor phagocytes instead of tumor-promoting cells. CD40 agonists stimulate other APCs by triggering tumor-specific T cell immune response (Beatty *et al.*, 2011; Palma and Lewis, 2013; Vonderheide *et al.*, 2013). Anti-CD40 MAbs, soluble CD40L or viral-vector expressing CD40L have been shown to mimic humoral or cellular immune responses to a variety of T cell-dependent or independent immune responses (Hanyu *et al.*, 2008; Thompson *et al.*, 2015).

Anti-CD40 MAbs seem to be promising vaccine adjuvants that act alone or in combination with other molecules in several species (Thompson *et al.*, 2015; Martin *et al.*, 2017). In one approach, our group has recently shown that targeting chicken CD40 receptors for activation with anti-chicken CD40 MAb was extremely successful for induction of robust and long lasting immune responses (Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016). Anti-CD40 MAbs have also been demonstrated to induce either agonistic or antagonistic effects dependent upon site(s) of

CD40 binding (Yu *et al.*, 2018). Clearly, anti-CD40 agonistics are promising immunological adjuvants (Martin *et al.*, 2017).

1.7.DNA APTAMERS AS RECEPTOR AGONISTS

Aptamers, artificially prepared oligonucleotide molecules or peptides, have drawn attention as alternatives to MAbs in many biological systems with multiple advantages (Reverdatto *et al.*, 2015). Each RNA or single-strand DNA (ssDNA) molecule expresses a unique three-dimensional structure according to its sequence. The almost infinite number of folding patterns created by unique nucleotide sequences creates multiple possibilities, with low frequency, which are able to uniquely bind to specific complex molecules such as receptors. By creating random ssDNA sequences and sequentially enriching those expressing binding to a target molecule, individual aptamers can be selected with high affinity for those targets. This phenomenon enables aptamers to have affinities and specificities to target molecules that may exceed those of MAbs to the same target molecules. In practice, aptamers are identified via an *in vitro* repetitive selection process from a combinatorial oligonucleotides library, which is abbreviated as SELEX, standing for Systematic Evolution of Ligands by EXponential enrichment. The characterization of the enriched aptamers is with mathematical prediction via bioinformatics. Conversely, MAbs require animals for production. Other characteristics of aptamers include thermostability, chemical resistance and reproducibility. For example, they can be shared as a known sequence rather than a biological product, providing an additional advantage over MAbs (Tuerk and Gold, 1990; Ellington and Szostak, 1990; Irvine *et al.*, 1991; Hermann and Petel, 2000; Baker, 2015; Bradbury, 2015; Jeddi and Saiz, 2017). There are many proposed and proven uses of selected aptamers including

therapeutic applications, purification of molecules, studying biological pathways, and developing biomarkers (Centi *et al.*, 2007; Dollins *et al.*, 2008; Kunii *et al.*, 2011; Zhang *et al.*, 2012; Wang and Li, 2013).

Few publications describe aptamers as potent immunological adjuvants. Pastor and coworkers (2013) showed that anti-CD28 aptamers could act as a potential adjuvant. They described different aptamers that acted to stimulate or block the receptor. Another study has shown that anti-OX40 aptamers could enhance the action of an antitumor vaccine (Weinberg, 2002).

There is currently a dearth of published information discussing the economic value of adjuvants. Generally, optimal price of vaccine/vaccination unit to the price of the protected individual/production is critical in poultry. Herein, cheaper vaccines with cheaper application processes are favorable for commercial poultry. Monoclonal antibody use as a commercial vaccine adjuvant would likely be cost-prohibitive for commercial poultry. As anti-CD40 MAb have been demonstrated to be highly effective in poultry (Chou *et al.*, 2016; Vuong *et al.*, 2018), the potential for cost-effective mimicry of this response through selected anti-CD40 aptamers is potentially appealing.

Even within well-accepted commercial adjuvants, costs of specific adjuvants vary widely, as do their efficacy and duration of elicited response. For example, aluminum salts are markedly less expensive than emulsion oils, but the immune response is generally higher with oil-based adjuvanted vaccines (Astudillo and Auge de Mello, 1980 as cited by James and Rushton, 2002; Rushton, 2009). Thus, it is possible that effective aptamer-directed antigen delivery methods could potentially be appealing from both a cost and efficacy perspective, although this has not been evaluated.

1.8.CONCLUSIONS

The increasing demand for antibiotic-free animal products in concurrence with emergence of antibiotic resistant bacteria, and the emergence of highly devastating viral pandemics have contributed to strong efforts toward vaccination-mediated prophylaxis for commercial poultry (Hoelzer *et al.*, 2018; de Vries *et al.*, 2018). Developing new vaccines and updating old vaccines is a promising field within animal agriculture and for poultry production specifically. Improved understanding of poultry pathogens and avian immune responses will likely enable development of increasingly sophisticated and more effective vaccines. Subunit vaccines are currently the most intensely investigated vaccines for poultry. However, such vaccines are less immunogenic and need the participation of adjuvant to achieve effective immune response (Garçon *et al.*, 2011; Miligan, 2015).

Adjuvants are currently required for most candidate subunit vaccines, due to the relatively low immunogenicity of these antigens, and adjuvants are required for durable responses for vaccination of pullets intended to protect breeders for approximately one year of production. Currently used traditional commercial vaccine adjuvants typically enhance the immune response by facilitating the engulfment of the vaccine antigen. This enhancement is by promoting signal-1 or signal-2 during the adaptive immune response. Signal-1 enhancer adjuvants promote the engulfment of the foreign antigen via PRRs and may promote other innate immune response elements such as complement fixation (Coffman *et al.*, 2010; Schijns, 2017). Signal-2 enhancer adjuvants target costimulatory receptors such as CD40 as one of the central molecules in the adaptive immune response activation. This costimulatory molecule orchestrates the T-dependent humoral immune response by providing co-stimulation to both T cells and APCs. Targeting this molecule enhances the action of APC in multi-dimensional ways. On DCs, this molecule acts a costimulatory receptor,

which when engaged with CD40L on activated T cell; both cells will pass through downstream activation resulting in differentiation of activated T cell into T_H cell subsets such as T_H1, T_H2, T_H9, T_H17 or T_{reg} (Ma and Clark, 2009). The T_H subset differentiation depends on the nature of the antigen that affects the cytokine profile of the DC, which in turn determines the T_H subtype. For B cell mediated antibody production, isotype switching and affinity maturation are all events, which follow CD40-CD40L interaction through the T-dependent B cell activation pathway. Involvement of B cell presentation of certain antigens for naïve T cells is an essential event for effective humoral immune responses (Parker, 1993; Ratcliffe, 2008).

Several publications have showed that agonists for the CD40 could trigger an immune response resembling the natural immune response against wild-type pathogens. These CD40 agonists could act successfully as adjuvants such as soluble CD40L and anti-CD40 MAbs. The MAb-adjuvanted vaccines showed robust immune response with haptens of low immunogenicity. One of the limitations of using MAbs as an adjuvant for commercial poultry vaccines is the cost and handling requirements of this kind of adjuvanted vaccine. Additionally, the MAbs are immunogenic themselves and may interfere with subsequent booster-initiated immune responses (Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016; Martin *et al.*, 2017; Vuong *et al.*, 2018).

This dissertation describes investigations related to selection and design of anti-CD40 aptamers as a potential alternative to MAb-directed CD40 activation and presentation of antigenic cargo. The potential advantages of this approach could include improved stability, low cost of production, and robust induction of immune responses as compared to traditional adjuvants for chickens.

In broader range, our anti-CD40 aptamers could lead to new insight of receptor activation. For example, the activation of the CD40 receptor by ligation with CD40 agonists on tumor-associated

macrophages – M2 cells reverts the inhibitory effect of these cells on cytotoxic T cell and enhance phagocytic activity of the M1 TAMs (Hoves *et al.*, 2018).

1.9. REFERENCES

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CHAPTER II

Activation of Chicken HD11 Macrophage Cell Line via CD40 Receptor by Single-Stranded DNA Aptamers-Based Rolling Circle Amplification Products

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ABSTRACT

Cluster of differentiation 40 (CD40) is a costimulatory receptor molecule on antigen presenting cells (APCs) that mediates specific immune responses when interacts with its ligand (CD40L/CD154) on activated T helper cells. Previously, anti-chicken CD40 monoclonal antibody has been proven to induce similar effect on the APCs. In this study, we aimed to mimic the naturally or artificially occurring APCs activation by developing DNA aptamer agonist to the CD40 receptor. First, we developed DNA aptamers to chicken CD40 extra domain as a bait. Next, the selected aptamers served as templates for rolling circle amplification (RCA) procedure. RCA products would enhance aptamers effect by spanning larger space with stronger binding affinity to CD40. Eight DNA aptamer candidates were used to generate four RCA products (namely, RCAI-RCAIV). The signal transduction ability of the RCA was evaluated on chicken HD11 macrophage cell line. The results showed that all RCA products (RCA I in particular) were able to activate the macrophage HD11 cell line. Additionally, limited proteolysis liquid chromatography electrospray ionization mass spectrometry experiment showed inhibition in trypsin digestion of CD40 by Aptamers and determined the amino acid sequences where the candidate aptamers attach the CD40 receptor. Our work could bring new insight into the use of aptamers, in the form of RCA, for stimulation/blocking of many receptor-ligand interactions.

Keywords: Chicken CD40 receptor agonist, DNA aptamers, Rolling circle amplification, Mass spectrometry

2.1.INTRODUCTION

Cluster of differentiation 40 (CD40) was firstly reported as a surface antigen protein expressed on human bladder carcinoma cells (Koho *et al.*, 1984). Further studies have shown that this antigen is naturally expressed as a B-lymphocyte cell surface receptor that regulates humoral immune response. Now, CD40 is a costimulatory receptor that expressed on variety of immune and non-immune cells including antigen-presenting cells (APCs) and regulates adaptive immune response. The ligand for the CD40 receptor (CD40L/CD154) transiently expressed by activated CD4⁺ T helper (T_H) lymphocytes (Amitage *et al.*, 1992; Banchereau *et al.*, 1994; Foy *et al.*, 1996; Stout and Suttles, 1996; van Kooten and Banchereau, 1997; Bajorath and Aruffo, 1997; Ma and Clark, 2009; Suttles and Stout, 2009; Kawabe *et al.*, 2011).

Upon maturation, DCs express CD40 as costimulatory molecules. In a classical adaptive immunity, four signals (0-3) are required for optimal DC/T_H activation and differentiation (see Chapter I, Review of Literature). Immature peripheral DCs recognize highly conserved PAMPs and DAMPs via different receptors such as the PRRs and RAGE receptor (signal-0) (Matzinger, 1994; Sousa, 2004; Hou *et al.*, 2008; Kagan, 2012). Once the DC engulfs foreign antigenic peptide, it starts a process of maturation with processing and presenting of this peptide on its own major histocompatibility complex class II (MHC class II) molecule. Here, maturing DCs exhibit changes in cytokines production profile with concurrence homing to T zone at the draining lymph nodes. At this point, the DC is with less phagocytic capacity and more presenting activity. At the T zone of the lymph node, naïve T cell receptor (TCR) engages processed peptide on cognate MHC class II molecule on the DC (signal-1). This engagement activates T cell and render it expressing CD40L transiently with other costimulatory molecules and cytokines. The crosslinking of these costimulatory molecules on both cells represents signal-2. This will lead to downstream cell

signaling in both DCs and T cells. Cytokines from the DC such interleukin (IL)-1 family and IL-2 serve as signal-3 for the T cells differentiation. Similarly, activated T cell produces cytokines that affect the same cell or neighboring cells such as IL-2 and IL-4. The type of the T cell cytokines depends on the type of the foreign peptide that was initially presented by the DC. The antigen-specific signal-1 is necessarily inefficient to stimulate naïve T cell full differentiation into effector T_H cells (subsets). T cell needs all the signals to complete proliferation and subsets differentiation such as T_H1 or T_H2 (Curtsinger *et al.*, 1999; Erf, 2004; Corthay, 2006; Thaïss *et al.*, 2011; Abbas *et al.*, 2012). When B cells involved in antigen presentation, CD40-CD40L engagement is crucial event in T-cell-dependent B-cell activation and proliferation. CD40 participates in T-lymphocyte-dependent antibody class switching, antibody affinity maturation, development of memory B cells and formation of the germinal centers (Noelle *et al.*, 1992; Alvarez *et al.*, 2008; Stavnezer, *et al.*, 2008; Elgueta, *et al.*, 2009; Wu *et al.*, 2010).

Recent studies showed that the use of different agonists to CD40 leads to induction of adaptive immune response useful in cancer immunotherapy (Vonderheide and Glennie, 2013). CD40 agonists revert the suppressive effect of tumor cells on macrophages, rendering them active antitumor phagocytes instead of tumor-promoting cells. CD40 agonists stimulate other APCs by triggering tumor-specific T cell immune response (Beatty *et al.*, 2011; Palma and Lewis, 2013; Vonderheide *et al.*, 2013). Anti-CD40 monoclonal antibodies (MAbs), soluble CD40L or viral-vector expressing CD40L have shown to mimic humoral or cellular immune responses to a variety of T cell-dependent or independent immune responses (Dullforce *et al.*, 1998; Hanyu *et al.*, 2008; Thompson *et al.*, 2015).

In one approach, our group has recently shown that targeting chicken CD40 receptor for activation with anti-chicken CD40 MAb was extremely successful with robust immune responses against

haptens (Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016; Vuong *et al.*, 2018). Anti-CD40 MAbs showed several biological capacities such as agonistic or antagonistic effects. Anti-CD40 agonistics are promising immunological adjuvants (Martin *et al.*, 2017; Vuong *et al.*, 2018). The limitation of using MAbs as vaccine adjuvants is the presence of the MAbs themselves due to their high immunogenicity that might interfere with the vaccine action, difficult and costly production (Chames *et al.*, 2009).

Therefore, the use of alternative, biologically accepted and low-immunogenic molecules, would be more beneficial for practical *in vivo* applications. Accordingly, we sought to utilize aptamers to substitute anti-chicken CD40 MAb. The folding pattern of single stranded oligonucleotides creates a molecular structure in which the ligand becomes incorporated. This phenomenon enables aptamers to have affinities and specificities to target molecules that may exceed those of MAbs to the same molecule. Many advantages of aptamers over MAbs; aptamers selection is via *in vitro* process. In addition, aptamers are thermostable, chemically resistant, reproducible and low immunogenic (Irvine *et al.*, 1991; Hermann and Petel, 2000; Dollins *et al.*, 2008; Baker, 2015; Bradbury, 2015; Jeddi and Saiz, 2017).

In this study, we sought to stimulate chicken macrophage HD11 cell line by developing agonistic anti-chicken CD40 DNA aptamers. First, we developed anti-chicken CD40 extra domain DNA aptamer by systemic evolution of ligands by exponential enrichment (SELEX) procedure (Tuerk and Gold, 1990; Ellington and Szostak, 1990). Eight aptamer candidates reproduced in form of four rolling circle amplification (RCA) products (namely, RCAI –IV) (Ali *et al.*, 2014; Lv *et al.*, 2015; Mohsen and Kool, 2016). RCA products exhibit thousands of tandemly repeated sequences of the selected aptamers to mimic the naturally occurring receptor-ligand oligomerization more

closely. We tested the RCA products on the HD11 cells line to record their stimulation via nitric oxide liberation by Griess assay (Crippen *et al.*, 2003).

Moreover, we specified the amino acid sequences crucial for receptor ligand interaction and receptor stimulation by limited proteolysis electrospray ionization liquid chromatography mass spectrometry (LC-ESI-MS) experiment. The pairing LC-ESI-MS with limited proteolysis studies to map the surface of proteins with unknown three-dimensional structures is becoming an invaluable technique (Sides *et al.*, 2012). Using similar concept, we determined those sites that hindered to the enzyme of proteolysis and they can be detected by reduction in signal corresponding to the tryptic peptide associated with the site.

2.2.MATERIALS AND METHODS

2.2.1. Target protein, generation of recombinant extracellular domain of the chicken CD40.

Our target protein was the chicken CD40 receptor; however, the recombinant protein used for the aptamers selection was chicken CD40 extracellular domain protein (chCD40_{ED}). The protein was a kind gift from Dr. Luc R. Berghman at the University of Texas A&M. The recombinant chCD40_{ED} protein was expressed in hybridomas, human embryonic kidney (HEK)-293 Freestyle cells in protein-free medium (Antagene Inc., Santa Clara, CA, USA) with concentration of 1mg/ml. Briefly, total RNA was isolated from chicken spleen and cDNA was synthesized accordingly. The sequence encoding the chCD40ED, which was already determined, was amplified with nested PCR and the gene encoding the protein was cloned into expression vector pcDNA5 (Invitrogen) (Chen *et al.*, 2010).

According to the mass-spectrometry analysis, the protein was with MW around 25712 Da (Figure 2.5). The protein's stock was diluted (w/v) with sterile 1X phosphate buffer saline (1XPBS) to concentration of 15µg/L and aliquoted into 1ml in sterile tubes and stored at -80°C.

2.2.2. Single stranded DNA library and primers

A randomized ssDNA library, forward and reverse primers were all purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa, USA). The oligonucleotide library design included flanking the variable region (40 nucleotides) with constant 20 nucleotides forward primer sequence at the upstream and constant 20 nucleotides reverse primer-binding site at the downstream. The randomized region of the library, which was with 40 nucleotides, was with theoretical diversity of 1×10^{24} random sequences (Table 2.1). The random region of the library designation based on equal incorporation of A, C, G and T bases at each position. The reverse primer was ordered as phosphorylated at the upstream to facilitate the action of the λ -exonuclease enzyme in the later step during the enrichment cycles. The ssDNA library and the primers were dissolved in sterile 1XTE buffer (Cat# T9285, Sigma-Aldrich), to 100µM according to the manufacturer and stored at -20°C.

2.2.3. *In vitro* aptamers selection procedure

The procedure of aptamers selection was carried out using SELEX procedure as described elsewhere (Tuerk and Gold, 1990; Ellington and Szostak, 1990; Irvine *et al.*, 1991). At the first round, 35µl of the ssDNA library (1µg/µl) equivalent to $\sim 6 \times 10^{14}$ units was diluted with 115µl binding buffer (50mM Tris HCl, 25mM NaCl, 5mM MgCl₂, 10mM DTT (dithiothreitol), pH 7.5) (Wang *et al.*, 2013). A denaturation process carried out by heating to the DNA library-in buffer to 95°C for 10min. After, the mixture cooled down immediately to 4°C for 5min, and incubated at

room temperature (RT) for 10min. To exclude non-specific binders and filter-binders, the ssDNA library was passed three times through a 0.45 μ M nitrocellulose acetate membrane (HAWP, Millipore, MA, USA) using filter holder (“pop-top”, Millipore, MA, USA). The library and the target protein (50 μ l, 15 μ g/L) incubated together with rotation for 1hr at RT in the binding buffer. After the time passed, the library-protein mixture passed through the filter, and the filter washed 3x with the binding buffer. The filter then immersed in 200 μ l of pre-heated (60°C) elution buffer (0.4 M sodium acetate, 5mM EDTA, 7M urea; pH 5.5) for 5min. The eluted material was diluted with 200 μ l ddH₂O and the DNA was precipitated with ethanol precipitation method (Wang *et al.*, 2013; Sytabekova *et al.*, 2017). Each following SELEX enrichment round used only 75% of the volume of the target protein in the previous round while the protein concentration remained constant.

2.2.4. PCR and dsDNA digestion into ssDNA

After each enrichment cycle, the resulted dsDNA was digested into ssDNA with λ exonuclease enzyme (see below). The ssDNA was amplified with 25 PCR amplification cycles. The PCR was conducted using G2 Hot Start Green Master Mix (Cat# M7422, Promega). The 25 μ l reaction mix was 1 μ l (10 μ M) from the of the DNA library (enriched aptamers in the later cycles) as the template, 2 μ l of each forward and phosphorylated reverse primers (4 μ M), and 12.5 μ l of 2X reaction buffer (pH 8.5). The reaction buffer composed of 2.5U of the polymerase enzyme, 400 μ M of each dNTPs and 4mM MgCl₂. Nuclease-free water was added to adjust the final reaction volume to 25 μ l. The thermal cycles composed of 95°C for 5min as the denaturing step, followed by 25 thermal cycles composed of 95°C for 30sec, 56°C for 15sec and 72°C for 15sec. The final extension was at 72°C for 5min. The PCR products were monitored on 1% agarose gel stained with SYBR Safe dye (ThermoFisher Scientific, USA). The reverse primer was order as phosphorylated to facilitate this

task (Table 2.1). The PCR-resulted double-stranded DNA (dsDNA) was subjected to the action of λ exonuclease enzyme (Cat# M0262S, NEB, USA) to retain the ssDNA for the next round. The digestion reaction conditions were as recommended by the manufacturer.

2.2.5. Cloning and sequencing

2.2.5.1. Sanger sequencing

As a process of monitoring, after six rounds of selection, the PCR products were cloned and sequenced using Sanger sequencing method (Sanger *et al.*, 1977). The cloning process utilized the pGEM-T and pGEM-T easy vector systems (Cat# A1360, Promega). As instructed by the manufacturer, the PCR product was inserted into the kit vector in ligation buffer. The insert-containing plasmid DNA was transformed into electro-competent TOP10 *E. coli* (Invitrogen, Carlsbad, CA, USA) by pulsed high voltage electroporation (1750V for 5 milliseconds) (ECM™ 830 electroporation generator, Fisher Scientific). After the transformation, 500 μ L of SOC media (Invitrogen, Carlsbad, CA) was added immediately and the bacteria was incubated for 2hrs at 37°C with shaking. The SOC medium-containing bacteria was streaked on Luria-Bertani (LB) medium supplemented with ampicillin (100 μ l/plate) (Cat # R110846, Thermo Fisher Scientific), IPTG (Calbiochem) (Cat # 420291, Sigma-Aldrich), and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) (Cat # B1690, Thermo Fisher Scientific). After overnight incubation, the culture was screened and 15 white colony candidates were harvested. The identification of positively cloned colonies depends on the color differentiation. Bacteria do not harbor the pUC18 plasmid will be killed by the ampicillin. Whereas, bacteria do not carry the insert will appear in blue color colonies because of the X-Gal, and the bacteria that harbor the insert will appear in white colonies. One extra blue bacterial colony was included as a positive control for the assay. Those chosen colonies were lysed and the DNA was purified using QIAprep Miniprep kit (Qiagen, Hilden, Germany) and

sent for Sanger sequencing (Eurofins MWG Operon, Louisville, KY, USA). Results were analyzed with ClustalW2 2.1 software (University College Dublin) (Singh and Bhatia, 2016)

2.2.5.2. Illumina sequencing

The procedure of high throughput Illumina sequencing was as described elsewhere (Schütze *et al.*, 2011; Scoville *et al.*, 2017). The results were analyzed using “Galaxy Bioinformatics” (open source), and FastQC Version: 0.11.5 (Babraham Bioinformatics, Babraham Institute, UK) (Thiel and Giangrande, 2016; Thiel, 2016; Randrianjatovo-Gbalou *et al.*, 2018).

Briefly, enriched aptamer samples from rounds 1, 2, 6, 8 and 10 were prepared for Illumina sequencing platforms. Each selected round's ssDNA was barcode-tagged and used as a template for PCR amplification using Illumina sequencing primers (Table 2.1). The ssDNA were diluted with nuclease free water to have concentration of 10uM, then, the primers, which were already included with unique barcodes representing each SELEX cycle, have been utilized to amplify the templates. Twenty-five PCR cycles have been applied using KOD hot start DNA polymerase and buffer (Cat # 71086-3, Sigma-Aldrich, CA, USA). Then, PCR products were mixed together in the same volume within each category. The mixed DNA samples were PAGE-purified using 4-20 % polyacrylamide gel (Life Technologies, NY, USA). Then, these DNA samples were mixed together so that approximately the same quantity of DNA from each of six samples would be included in the final mixed sample, based on the measurement with Qubit 2.0 Fluorometer. Finally, 20µl of the final mixed sample (50.4 ng/ul) was sent to Genomics Core Facility at the University of California at Riverside, USA for Illumina sequencing using MiSeq with 151 cycles in single-end mode.

The total number of the reads by the Illumina sequencing were about ~16 million reads; however, about 5% of the total reads were neglected due to their improper length or quality. The rest of the reads were tracked according to their barcodes. After analyzing the results, the highly-yielded enriched aptamers were tested for the free energy change ($\Delta G[\text{kcal.mole}^{-1}]$) of the three dimensional structure using the OligoAnalyzer 3.1 Tool at Integrated DNA Technologies (available online by Integrated DNA Technologies (IDT) website), and the most reliable ones have been chosen to be tested by the next step.

2.2.6. Dot-blot hybridization assay

Dot-blot assay was applied to detect the affinity of the candidate aptamers to the target protein. The assay was as described elsewhere (Vivekananda and Kiel, 2006; Wang and Li, 2013). According to the Sanger sequencing and the Illumina sequencing results, the candidate aptamers were ordered as biotinylated at the upstream (IDT, Coralville, Iowa, USA). Immune-Blot PVDF membrane (0.2 μm , Cat # 162-0174, Bio Rad laboratories, Ca.) cut into 8 strips of 6 cm^2 (1 cm^2 /treatment). Ten microliters (15 $\mu\text{g}/\text{mL}$) of the target protein was dropped onto the center of five of the test squares. After drying, the stripes blocked with KPL blocking buffer for 30min and washed for three times with 1X KPL washing buffer (Cat # 50-63-00, KPL, Gaithersburg, MD). Concentrations of (12, 25 and 50) μM of the aptamers (10 μl each) were dropped onto the center of the test squares on the membranes. The membranes sat to air dry and washed (3X) with 1X KPL washing buffer. Streptavidin-conjugated alkaline phosphatase (Cat # 475-300, KPL, Gaithersburg, MD) (diluted 1:500) poured on the membrane for 30min and another course of washing followed. Finally, the color developed by adding 5-bromo-4-chloro-3-indoxyl-phosphate and nitro blue tetrazolium (BCIP/NBT) substrate (Cat # 50-81-07, KPL, Gaithersburg, MD, USA). The reaction stopped by pouring tap water thoroughly.

2.2.7. Rolling circle amplification

According to the dot-blot assay results, the enriched aptamers were with good affinity to the target protein, yet, their ability to sustain the clustering and oligomerization of the CD40 receptor in chicken HD11 macrophage cell line was questionable. To achieve the highest possibility for this process, RCA technique was suggested. Multivalent RCA products-containing polyvalent aptamers were acquired via aptamers-based template design (Ali *et al.*, 2014). All the eight candidate aptamers were grouped into four groups with two aptamers each to design four complementary templates for RCA technique (Tables 2.1 and 2.3, Figure 2.1). The upstream end of all RCA templates was phosphorylated to sustain enzymatic ligation. The primer designation as 20 nucleotides length complementary sequence to the upstream and the downstream extremities of the template together to assist the ligation (Table 2.1). Finally, a spacer complementary sequence (SCS) annealed thermally to ensure rigid regions among the repetitive aptamers sequences. The procedure of the RCA started with annealing 15-20pmol of the primer (equivalent up to 132ng/ μ l) with primer binding site on the template (1-2pmol equivalent to 129ng/ μ l) making the template as a circle with the aid of the primer, despite the template was still being nicked right in the middle of the primer. The primer-template mixture was heated first to 95°C for 10min, to 56°C for 5min and then cooled down to RT in 50 μ L TE buffer. To ligate the nicked template, the ligation of the template with T4 DNA ligase enzyme (Cat# M0202S, NEB, USA) followed the annealing procedure. The ligation reaction conditions were as recommended by the manufacturer. For the polymerization, ϕ 29 DNA polymerase enzyme (Cat# M0269L, NEB, USA) was used. All the reaction conditions were as recommended by the manufacturer, except that the reaction was hold for 16hr at 30°C and then heat-inactivated at 65°C for 10min. After each enzymatic treatment, the DNA was purified by ethanol precipitation method. For SCS adding, the RCA products quality

and quantity were evaluated using microplate reader (BioTeck). Two molecular weights of the SCS were mixed with one molecular weight of the RCA products. Again, the RCA products-SCS mixture was held on heating for 10min at 95°C and for 50°C for 5min and then ice-cooled immediately to apply the annealing status.

2.2.8. Measuring activation of chicken HD11 macrophage cell line

Chicken HD11 macrophage cell line (Crippen *et al.*, 2003) was propagated on 96-well plate at 37°C in a humidified atmosphere having 5% CO₂ in Dulbecco's modification of eagle's medium (DMEM) (Mediatech, Manassas, VA) enriched with 8% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 5% chicken serum (Sigma, St. Louis, MO). All treatments were applied when the confluence of the batch was 80% (Hussein and Qureshi, 1997; Crippen *et al.*, 2003). After the treatment, the plate was incubated at the same conditions for extra four hours. When the time passed, the contents of every well was collected, centrifuged and the supernatant was subjected to Griess assay to detect the nitric oxide liberation in the medium. All cell line work was done in Dr. Berghman's laboratory, University of Texas A&M.

2.2.9. MALDI-TOF-MS, LC-ESI-MS/MS, and LC-ESI-MS

For these experiments, we used SEQ1 and SEQ2 as the candidate aptamers individually or in combination because they showed best stimulation pattern on tissue culture experiment in form of RCA I. First, both protein and the individual aptamers were subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) using Bruker Daltonics, MALDI relex III time of flight instrument to confirm the expected molecular weight for both protein and the aptamers. The second experiment tested the identity of the target protein. The protein (1µg) was subjected to full digestion with trypsin (20ng) (Promega) in 25mM

$\text{NH}_4\text{HCO}_3 \sim 7.5$ pH buffer at 37°C for 1hr. Following, liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), using Agilent Bruker Daltonics Amazon-SL, used to confirm the protein identity as the CD40 receptor from *Gallus gallus* as using standard method described in elsewhere (Kannan *et al.*, 2007).

Last experiment was designed to understand aptamers-protein binding process properly. We conducted a limited proteolysis liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) experiment. The LC-ESI-MS experiment has been done using Bruker ESQUIR-LC quadrupole ion trap (QIT) without MS/MS and intensities of the extracted ion chromatograms corresponding each tryptic peptide, which were used to determine the relative inhibition for the enzyme activity toward the chCD40_{ED} protein due to aptamers binding. LC-ESI-MS was operated using standard method protocols described elsewhere (Kannan *et al.*, 2007; Kannan *et al.*, 2009). One microgram of the chCD40_{ED} protein was subjected to the digestion with 20ng of trypsin (Promega) containing 25mM $\text{NH}_4\text{HCO}_3 \sim 7.5$ pH buffer at 37°C for 1h with and without the candidate aptamers (individually or in combination). In addition, negative control ssDNA aptamers random sequence was included. After the incubation period passed, the enzyme action was chemically inactivated by adding 5% FA in 60% acetonitrile (ACN) and the resulted digest was subjected to LC-ESI-MS.

2.2.10. Statistical analyses

Data from nitric oxide liberation are presented as mean \pm standard error. Treatments with RCA products were in triplicates. Multi-Way Analysis of Variance (ANOVA) followed by Tukey's honest significant difference (HSD) post hoc test were used to compare means of nitric oxide liberation among treatments to detect significance differences between and among several

treatments with RCA products on tissue culture with JMP Pro software (version 13, SAS Institute Inc., NC, free version by University of Arkansas). The p -values are reported as significant at $p < 0.001$.

2.3.RESULTS AND DISCUSSION

2.3.1. *In vitro* selection, identification and characterization of the aptamers

The naturally occurring CD40/CD40L interaction is a central event in orchestrating both branches of any given acquired immune response. In a recent study, our group established a novel anti-chCD40 MAb. This well-characterized MAb showed the ability to induce significant stimulation in chicken HD11 macrophage cell line. When this MAb administered with different routes in combination with a desired peptide, the CD40-targeted immunogen induces robust immune response and several desired effects such as immunoglobulin class switching occur (Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016). However, like all MAbs, the anti-chCD40 MAb production would make it impractical to commercialize this as vaccine adjuvant due to high cost and intense labor. To avoid these undesirable side effect and cost, we investigated the ability of *in vitro*-selected DNA aptamers to mimic the same effect as in MAb as agonist to the CD40 receptor on the same cell line.

The SELEX procedure that applied for selection aptamers against chCD40_{ED} started with random ssDNA library and continued as repetitive 10 enrichment cycles of SELEX as described by Tuerk and Gold (1990). Following each enrichment cycle, PCR procedure was carried out using the resulted aptamers as the template to amplify the enriched aptamers for the next round. PCR is useful tool to increase the yield and the purity of the enriched aptamers. To resume the SELEX

process, the amplified dsDNA was then being digested with λ -exonuclease. To avoid losing aptamers with high affinity to the target protein, the molar ratio of the protein to ssDNA library was shifted toward protein. The protein amount was reduced by 25% in every next round with the same concentration to apply selection pressure toward aptamers specificity (Berens *et al.*, 2001).

To evaluate the selection process, ssDNA products from round six was clonally sequenced with Sanger sequencing method (Sanger *et al.*, 1977). The data obtained were analyzed using ClustalW2 2.1 software (University College Dublin) (Singh and Bhatia, 2016). The result showed that there were two identical aptamers sequences and a third sequence with considerable consensus among the others. One aptamer sequence was repeated two times over the 15 samples and the other was with 60% consensus sequence with these two sequences (Figure 2.1). These three aptamers sequences were evaluated by IDT OligoAnalyzer 3.1 tool software (available online by Integrated DNA Technologies (IDT) website, IDT, USA) to judge their fitness. The two different sequences were ordered as biotinylated DNA sequence at their 5' prime end (IDT, USA) to apply dot-blot assay. The highly enriched aptamers may not be always suitable for the receptor stimulation. As stated by Yu *et al.* (2018), only those MAbs who are able to bind the receptor at certain sites will promote agonistic activities despite of high binding affinity (Figure 2.9D).

In the next step, the ssDNA aptamers from rounds 1, 2, 6, 8 and 10 were sequenced using high throughput Illumina sequencing method. According to the barcodes we included for each round, there were around 4×10^6 reads/barcode from each round. Significantly, about 95% of the raw reads were retained as high quality aptamers sequences when filtered with the bioinformatics tools. Analyzing the results with Galaxy bioinformatics and FastQC showed increasing in the selected aptamers enrichment over rounds (Thiel and Giangrande, 2016; Thiel, 2016; Randrianjatovo-Gbalou, 2018). The analyzed data showed that there were highly enriched six sequences (SEQ1-

SEQ6) over the aptamers pool (Table 2.2, Figure 2.2). When blotting these abundant six sequences against each selection round, the highest represent sequences proportion were (SEQ6 and SEQ5) consistently. To achieve calculations, round 1 considered to be the base line or round 0 as all the possible sequences are represented theoretically in the pool. When measuring the proportion of the enriched sequences/round and relative frequency, the six sequences were variable in enrichment. SEQ3 and SEQ5 were with the highest relative frequency observance, both steadily enriched over the SELEX cycles (Table 2.2).

Depending on the results from Sanger sequencing and Illumina sequencing, eight candidate aptamers sequences assessed (two sequences from Sangers and six sequences from Illumina, Table 2.3). Dot-blot assay evaluated the ability of the eight candidate aptamers to attach the target protein *in vitro*. Candidate aptamers were ordered as biotinylated at the upstream to evaluate their ability to interact with the protein on Immune-Blot PVDF membrane. The results showed that all aptamers candidate sequences were with significant binding affinity to the target protein at concentration of 25 μ M or more (Figure 2.3). All the eight aptamers were able to detect the target protein with this assay and the detection limit was (15:10-50) μ M protein : aptamers. It is convenient to refer that the candidate aptamers were with the primers and primer binding sites sequences when applied this assay due to the possibility that these sites might share in protein interaction as well.

2.3.2. Rolling circle amplification

As stated earlier, due to the smaller size of our aptamers candidates in comparison to the target receptor (the MW of each individual candidate aptamer is estimated to be around 24kDs whereas, the MW of the chicken CD40 receptor extra domain trimer is around 75kDa – Figure 2.7), RCA technique was suggested (Figure 2.4). RCA products would increase the chance of the CD40

receptor activation on the chicken macrophage cell line by spanning wider area on the receptor and by increasing the overall affinity of the ligand(s). To achieve this task, the complimentary of the eight candidate aptamers sequences were dual designed as templates for RCA procedure. RCA technique gives long tandem repetitive sequences (several hundreds) of the original template. Herein, RCA products make benefit of this feature and to increase the ability of the aptamers-CD40 receptor clustering. The RCA products were polyvalent and multivalent products. Over the run of the RCA procedure, the RCA products were with high molecular weight (more than 10K nucleotides) (Figure 2.5). The ssDNA aptamers sequences within each RCA product were separated by short SCS representing dsDNA to apply rigid and inert stretches within the ssDNA of the RCA products long tail. This RCA products-SCS complex hybridization was to exclude any possibility of non-specific cell line activation by RCA products. Experimentally, each RCA products is with tandem repeats of the two included aptamers candidates and, each neighboring aptamers are separated by spacer-SCS; in other words, the RCA products is a repeated sequence of ssDNA (aptamers) and dsDNA of spacer-SCS, reciprocally. Here, the spacer-SCS stretches provide stability over the structure and to act as hinge region as in antibody. Additionally, we noticed that using any random RCA products with no spacer-SCS added could stimulate the nitric oxide liberation from the chicken macrophage cell line (data not shown). Furthermore, adding the SCS has prevented the clumping and precipitation of the RCA products.

The RCA procedure passes through several enzymatic treatments in exchange with DNA precipitation and purification procedures. Therein, the confirmation we had on the RCA products configuration and RCA products-SCS annealing was the digestion with restriction enzyme (AgeI). The SCS was included with restriction sequence. The digestion pattern of the RCA products with

this enzyme showed that we actually had the correct product and correct RCA products-SCS hybridization, thus, there was no need for the sequencing at this step (Figure 2.6, Table–2.1).

2.3.3. Nitric oxide liberation and Griess assay.

The RCA products-SCS complex were used to treat the chicken HD11 macrophage cell line (Crippen *et al.*, 2003). The four aptamers-dependent templates were amplified into four RCA products namely RCA I to RCA IV.

The results of Griess assay showed that there were detectable elevation in nitric oxide liberation in the media after the incubation with the RCA products for all sequences. However, there was significant variability in the efficacy and potency among RCA products. RCA I was with the highest response. The steep of the slope response was dose-dependent and significantly appeared in RCA I. Of course, the results were compared with negative control RCA products (NC-RCA); which are RCA products that had been designed by having two DNA sequences that have not been enriched according to the Illumina sequencing results to develop RCA template as in positively selected aptamers. The results were also compared with anti-chicken CD40 MAb that is known to stimulate the chicken HD11 cells as the positive control (Chou *et al.*, 2016).

The activity and viability of the chicken HD11 cell was also measured by adding lipopolysaccharide (LPS) as the general positive controls for this assay. All the RCA products induced a detectable liberation of the nitric oxide in the media with marked peak elevation with RCA I (Figure 2.9A). The stimulation of chicken macrophage HD11 cells with RCA I was with the highest nitric oxide liberation values at all concentrations and comparable with that of the anti-chicken CD40 MAb (Figure 2.9B) (Hussein and Qureshi, 1997; Brightbill and Modlin, 2000; Crippen *et al.*, 2003; Sun *et al.*, 2003).

The results also confirmed that the highly expressed aptamers were not the ideal aptamers for receptor stimulation. Indeed, there are specific site(s) where ligand could only promote clustering and oligomerization of receptor (Yu *et al.*, 2018). There are several anti- CD40 MAb, yet only those who interact with cysteine-rich region apically can promote receptor oligomerization.

2.3.4. MALDI-TOF-MS, LC-ESI-MS/MS, and LC-ESI-MS

The rationale behind using MALDI-TOF MS experiment was to verify the identity of the chCD40_{ED} protein (Bonk and Humeny, 2001). The results of this experiment showed that the MW of this protein was 25712 Dalton (Figure 2.7). Another LC-ESI-MS/MS experiment was used to discover the binding sites of CD40 partially protected by DNA (Figure 2.10) (Sundqvist *et al.*, 2007). For this experiment, we used SEQ1 and SEQ2 as these sequences represented by RCA I and gave best stimulation on tissue culture. Intensities of the tryptic peptides involving the detection the site(s) where these two aptamers attach to the target protein were low in comparison to the control chCD40_{ED} protein without the aptamers. These complemented results showed that there where physical linking between the target protein and the aptamers at least at three different peptide segments, and, this configuration was strong enough to protect the protein from the action of the trypsin enzyme after the period of digestion. Each peak indicted the protected sequence within the protein by the action of the aptamers separately or reciprocally. Similar to previous cell line experiment where the NC-RCA was able to stimulate mild nitric oxide liberation (Figure 2.9), the negative control aptamers showed detectable protection activity against the action of trypsin enzyme too. Still, the two positive control aptamers are more consistent with their protection level. Moreover, to determine the sites where the aptamers attract the target protein, the data from the LC-ESI-MS were subjected to virtual protein tertiary structure configuration analysis (PyMOL, free software at: www.pymol.org). According to this software, the two aptamers are attaching the target protein at the amine terminate (Figure 2.11B)

and the addition of the two aptamers together acted synergistically. However, the actual sites where each aptamers links the protein did not investigated at this point. In the process of building the tertiary structure of our target protein, significant amino acid sequences participated in attraction the aptamers sequences. The fact that this region is *flabby*, herein, highly resonant and cannot be detected easily by many 3D protein-building software. It is suitable to demonstrate that in the actual CD40-CD40L interaction, the ligand is spanning the inner of the receptor pulling it inward. This receptor-ligand clustering causes the oligomerization of the receptor (Figure 2.11D) (Reyes-Moreno *et al.*, 2004; Elgueta *et al.*, 2009), whereas, in our receptor-aptamers clustering model, the aptamers link the receptor apically (tandem repeats of the RCA products). The CD40 receptor is a trimer, herein, clustering at the proximal extremities of the receptor trimer is what may cause the same effect as in receptor-ligand clustering and oligomerization (Figure 2.11C).

2.4.CONCLUSIONS

Recently, developing aptamers for variety of molecules has become well-established technology. Aptamers and aptamers-based molecules are to replace the traditional MAbs in molecular biology. Indeed, expansion in this field is yet to overcome the limitations of the use of the aptamers due to poorly understood molecular properties of these molecules. We proved that the DNA aptamers-based RCA products are ideally capable of conducting the activation of chicken macrophage HD11 cell line via the CD40 receptor. In this study, we report the first description of using ssDNA aptamers-dependent RCA products as an agonist for the chicken CD40 receptor. The *polyduplex* synthetic aptamers in the RCA products mimics the naturally occurring receptor/ligand interaction and subsequent receptor oligomerization. We also utilized the use of the DNA aptamers to halt the

action of the trypsin enzyme on the target protein by utilizing LC-ESI-MS experiment. Herein, we determined the actual site where our aptamers attach the protein. We confirmed the cell line results by showing three amino acid stretches at the amine site of the protein where the aptamers (or the RCA products) attach. Despite using monomer instead of the receptor trimer as our target protein for aptamers selection, yet, our protein was expressed in eukaryotic cell line system, which should support the post-translation modification. In naturally occurring CD40-CD40L interaction stoichiometry, upon initial physical contact of the ligand with its cognate receptor, the receptor undergoes clustering at the extracellular domain, thus, signal transduction occurs through the cytoplasmic domain (Grassme *et al.*, 2002). The receptor's extracellular crosslinking is what leads to receptor oligomerization and this observation was recorded with the natural and artificial agonists of the CD40; i.e., CD40L and anti-CD40 MAbs (Bjorck *et al.*, 1994). The ligand has to configure with the receptor in a specific manner to induce the response (Figure 2.11). Interestingly, soluble CD40L was not able to induce the expected receptor signaling nor many anti-chicken CD40 MAbs. The conclusion was that there is a certain pattern and site for the ligand to act as an agonist (Baccam and Bishop, 1999; An *et al.*, 2011). Our aptamers did not show any agonistic activity when administered to the macrophage cell line as an individual nor combined aptamers. The macrophage stimulation only occurred with RCA products. Similarly, we have proven that not all the aptamers we have selected were able to oligomerize the receptor significantly; only those who were able to link specific receptor sites were the successful. However, we did not investigate nor report any antagonistic activity of the studied RCA products (Cerchia *et al.*, 2012; Soldevilla *et al.*, 2016).

Our goal is to create synthetic molecule could be preferably beneficial when loaded with appropriate antigen to act as specifically designed and sophisticated adjuvant. The flexibility we

provide with the use of aptamers-based RCA products makes it easier to introduce the aptamers into the biological systems without any molecular concerns. Indeed, the RCA products are naturally protected against any nuclease enzyme activity at the downstream due to the attached circular template (data not shown) and the intentionally biotinylated at upstream end to upload appropriate antigen.

2.5.TABLES

Table 2.1. DNA Library, primers, RCA templates and spacer complementary sequences designs. The random library, which is with theoretical 1×10^{24} unique sequences, flanked with forward primer sequence at the upstream and with the reverse primer-binding site at the downstream. Phosphorylated reverse primer ordered to facilitate exonuclease activity. Illumina primers were included with barcodes at the upstream to split the sequences after NGS. RCA templates phosphorylated at upstream to apply enzymatic ligation.

Description	DNA Sequence
Library	CCG AAT TCG AAG GAC AAG AG (N) ₄₀ [†] TCT TT TAT GCT ACG TCC CGC
Forward primer (FP)	CCG AAT TCG AAG GAC AAG AG
Reverse primer (RP)	Phosph/GCG GGA CGT AGC ATA AAA GA
Illumina FP	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNN GG(BC) [‡] TT- (Library FP)
Illumina RP	(Library RP)-TAG AGC ATA CGG CAG AAG ACG AAC
RCA-I template	<u>GCA TCT GAA C</u> GCG GGA CGT AGC ATA AAA GAC GAA TTC ACT GCT CCA TTT ACA CCC CAT TAC CAA TTC CAC CTC TTG TCC TTC GAA TTC GG <u>GAT CCA CCG GTA GCA</u> GCG GGA CGT AGC ATA AAA GAC CTA GCC CTG CTC TTC TGA TCC CTA TTC CAT GTA GCC CTA CTC TTG TCC TTC GAA TTC GG <u>GGA ACG TCT T*</u>
RCA-II template	<u>GCA TCT GAA C</u> GCG GGA CGT AGC ATA AAA GAG CGG GAC GTA GCA TAA AAG AAA ACC CAA ACT GCA CTT ACT CTT GTC CTT CGA ATT CGG <u>GAT CCA CCG GTA GCA</u> GCG GGA CGT AGC ATA AAA GAG AAC AAT CGG AAC TGG TCA TCC TAG GAC GTC GTG ACT TAC CTC TTG TCC TTC GAA TTC GG <u>GGA ACG TCT T</u>
RCA-III template	<u>GCA TCT GAA C</u> GCG GGA CGT AGC ATA AAA GAG AGA TGA CCA ACC ATA CTG ACT TGA CAG CAT CCC TCA TCT CTC TTG TCC TTC GAA TTC GG <u>GAT CCA CCG GTA GCA</u> GCG GGA CGT AGC ATA AAA GAA TGG GGG TAG TTG CAA AGG ATA GGG GTA AAA GAA ATG TTG CTC TTG TCC TTC GAA TTC GG <u>GGA ACG TCT T</u>
RCA-IV template	<u>GCA TCT GAA C</u> GCG GGA CGT AGC ATA AAA GAT GAG TCG TGC TAC CGT TCT GTC CTA ATA CTC GTT TGT CTG CTC TTG TCC TTC GAA TTC GG <u>GAT CCA CCG GTA GCA</u> GCG GGA CGT AGC ATA AAA GAG GAC TCG ACA CGC CGG TCT GTC CTG GGA TTC GTT TTT TCT CTC TTG TCC TTC GAA TTC GG <u>GGA ACG TCT T</u>
RCA-NC template	<u>GCA TCT GAA C</u> ATG GGC AGT TCA AAC TAC GAC ATA GCA GAC CAA GGT ATA G <u>GAT CCA CCG GTA GCA</u> TGA TGC AGG TCC TGT AAT TGG ACT GAT CAC ATA CCT AGA T <u>GGA ACG TCT T</u>
RCA primer	GTT CAG ATG CAA GAC GTT CC
Spacer-complement	GAT CCA CCG GTA GCA

[†] Represents random sequence

[‡] Represents barcode sequence

*Single underline represents the primer-binding site and the double underline represents the spacer complementary sequence

Table 2.2. Aptamers proportion and relative frequency at each enrichment SELEX round. Sequences of each rounds marked with certain barcode to ensure accurate splitting with the proper software. The proportion of six highly enriched aptamer sequence/round and their relative frequency upon each round have been estimated.

Round	Barcode	No. of Sequences/round	Sequence	% of enriched seq./round	Relative frequency
1	TAGATCGC	4.438 X10 ⁶	SEQ1	0.00006	0.028
			SEQ2	0.00011	0.0467
			SEQ3	0.00155	0.6449
			SEQ4	0.00048	0.1962
			SEQ5	0.00018	0.075
			SEQ6	0.00002	0.0095
2	CTCTCTAT	3.9 X10 ⁶	SEQ1	0.00231	0.0945
			SEQ2	0.00198	0.0809
			SEQ3	0.01408	0.5766
			SEQ4	0.00408	0.167
			SEQ5	0.00139	0.0568
			SEQ6	0.00059	0.0243
6	TATCCTCT	4.368 X10 ⁶	SEQ1	0.00104	0.0442
			SEQ2	0.00133	0.0569
			SEQ3	0.00319	0.1364
			SEQ4	0.00591	0.2531
			SEQ5	0.00053	0.0226
			SEQ6	0.01136	0.4867
8	AGAGTAGA	4.038 X10 ⁶	SEQ1	0.02725	0.053
			SEQ2	0.07678	0.1489
			SEQ3	0.21052	0.4083
			SEQ4	0.05697	0.1104
			SEQ5	0.04211	0.0817
			SEQ6	0.10194	0.1977
10	ACTGCATA	2.739 X10 ⁶	SEQ1	0.16631	0.0529
			SEQ2	0.31121	0.0652
			SEQ3	3.20593	0.6712
			SEQ4	0.66189	0.1385
			SEQ5	0.17813	0.0374
			SEQ6	0.16631	0.0349

Relative frequency calculations: Summation of the count of each enriched sequence per round (SEQ1+ SEQ2+...+ SEQ6) = SEQ_{total}, and divided each enriched sequence count by the SEQ_{total} = (SEQ1/ SEQ_{total}, SEQ2/ SEQ_{total},..., SEQ6/ SEQ_{total})

Table 2.3. Candidate aptamers. Eight highly enriched aptamers sequences from Sanger sequencing and Illumina sequencing were used to design four RCA templates. Each template, which composed of primer binding site, spacer and two aptamers sequences, enzymatically circularized with the aid of the primer.

Description	Aptamers												RCA combination
SEQ1 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGG	TGG	AAT	TGG	TAA	TGG	RCA I
	GGT	GTA	AAT	GGA	GCA	GTG	AAT	TCG	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ2 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGT	AGG	GCT	ACA	TGG	AAT	RCA I
	AGG	GAT	CAG	AAG	AGC	AGG	GCT	AGG	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ3 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGT	AAG	TGC	AGT	TTG	GGT	RCA II
	TTT	CTT	TTA	TGC	TAC	GTC	CCG	CTC	TTT	TAT	GCT	ACG	
	TCC	CGC											
SEQ4 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGG	TAA	GTC	ACG	ACG	TCC	RCA II
	TAG	GAT	GAC	CAG	TTC	CGA	TTG	TTC	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ5 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGA	GAT	GAG	GGA	TGC	TGT	RCA III
	CAA	GTC	AGT	ATG	GTT	GGT	CAT	CTC	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ6 (Illumina)	CCG	AAT	TCG	AAG	GAC	AAG	AGC	AAC	ATT	TCT	TTT	ACC	RCA III
	CCT	ATC	CTT	TGC	AAC	TAC	CCC	CAT	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ7 (Sanger)	CCG	AAT	TCG	AAG	GAC	AAG	AGC	AGA	CAA	ACG	AGT	ATT	RCA IV
	AGG	ACA	GAA	CGG	TAG	CAC	GAC	TCA	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										
SEQ8 (Sanger)	CCG	AAT	TCG	AAG	GAC	AAG	AGA	GAA	AAA	ACG	AAT	CCC	RCA IV
	AGG	ACA	GAC	CGG	CGT	GTC	GAG	TCC	TCT	TTT	ATG	CTA	
	CGT	CCC	GC										

*Each two aptamers sequences within the template separated by spacer complementary sequence

2.6.FIGURES

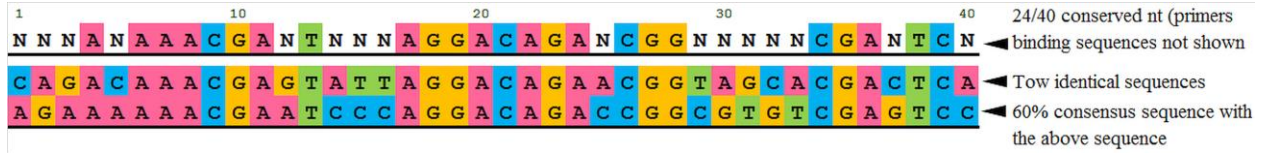


Figure 2.1. Sanger sequencing results. Sequences of the aptamers identified after six rounds of SELEX selection against chCD40ED protein. The aligned sequences represent the variable regions only with no primer binding sites.

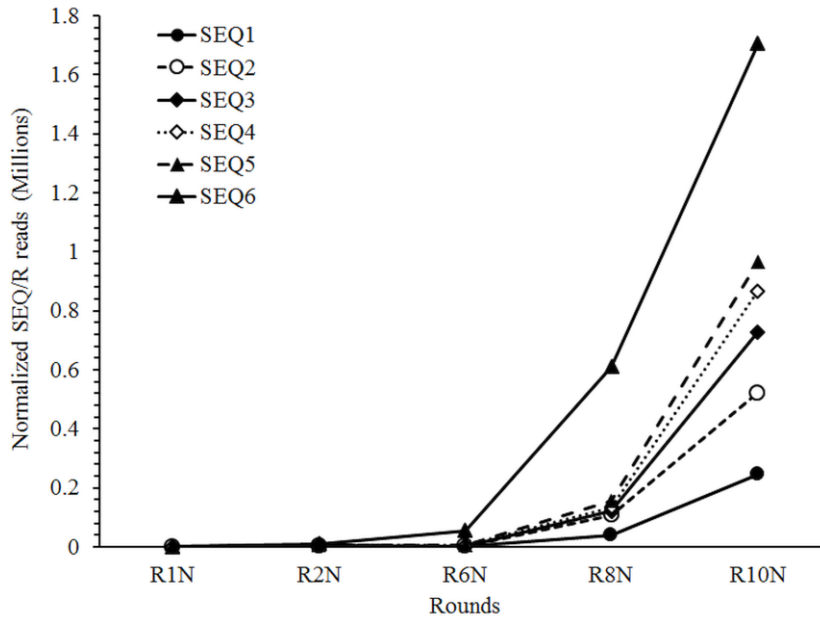


Figure 2.2. Illumina sequencing results. Normalized exponential increase of six highly enriched aptamer sequences among DNA pool over rounds of the enrichment cycles of SELEX. Normalization calculation $[(\text{frequency of aptamer sequence} \times 100 / \text{total number of reads}) / (\% \text{ the aptamer sequence in R1})]$. R1 as the reference round (R0) since all sequences should be represented hypothetically.

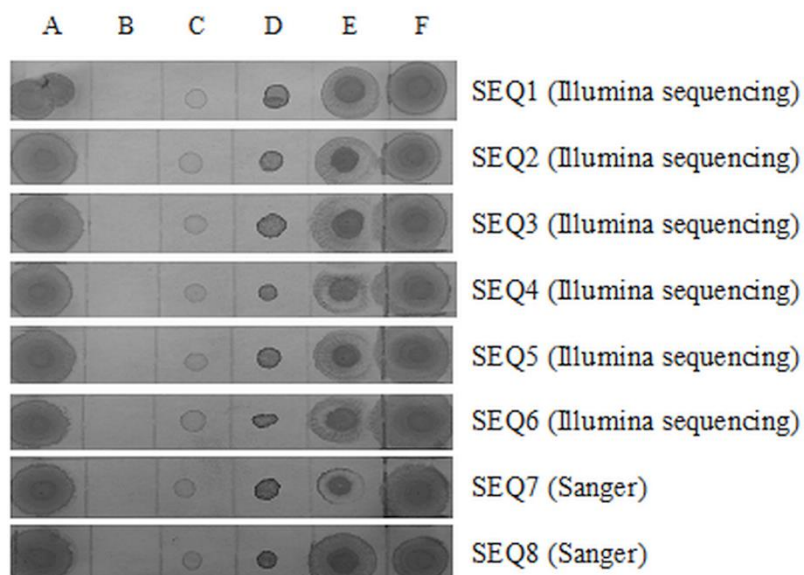


Figure 2.3. Dot-blot assay results. Eight Immuno-Blot PVDF membrane stripes, each divided into 6cm² activated with concentrated methanol and washed. 10µl of the chCD40_{ED} protein (15µg/mL) dropped onto the center of (**A, B, D-F**), air dried and blocked with commercial blocking buffer. 20µl of each of eight biotinylated aptamers sequences (Table 3.3) dropped on the center of each stripe within (**D-F**) at concentrations 12, 25 and 50µM, respectively. 20µl biotinylated anti-CD40 MAb positive control (10µg/mL) (**A**) and 1XIPBST (**C**) were the positive and the negative controls, respectively. The stripes set for 30min, washed (3x) and streptavidin-alkaline phosphatase was added followed by BCIP/NBT substrate.

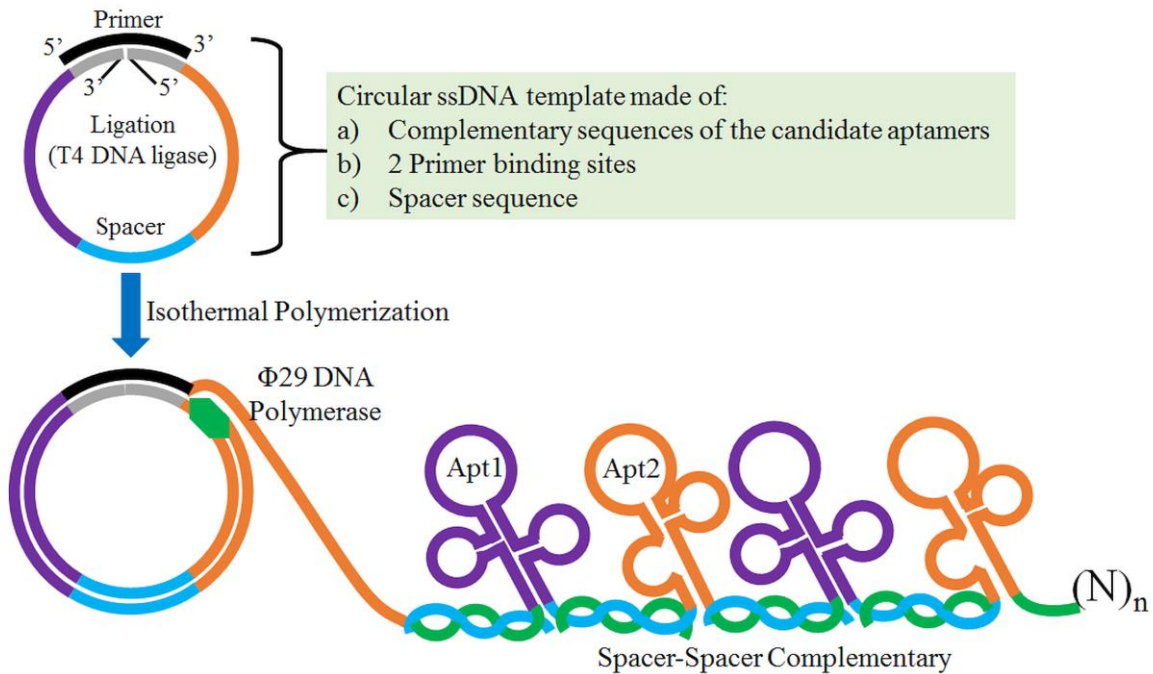


Figure 2.4. Schematic illustration represents the process of ssDNA aptamers-based RCA. The candidate aptamers were dual configured in one template. Other constituents, such as primer binding site and spacer were included too. The products of the RCA are then annealed with spacer complementary sequence to sustain dsDNA between each two-neighbor aptamers, which in turn, sustains rigidity at this particular site. The two adjacent aptamers sequences would be flabby and the spacer-spacer complementary will serve as the hinge region.

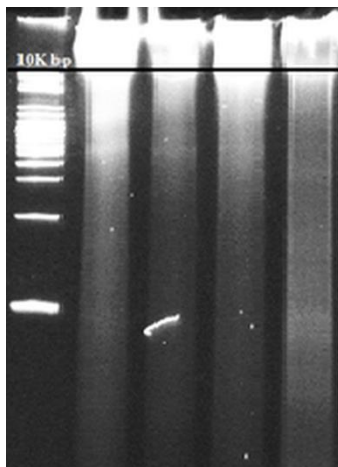


Figure 2.5: RCA products on denaturing 15%TBE urea gel. According to Illumina and Sanger sequencing, eight aptamers candidates were chosen to prepare four RCA templates (RCA I-IV, lanes 2-5, respectively). First, the templates were annealed with single spanning primer. Then, the templates were ligated with T4 DNA ligase, and last, the primer-template was polymerized with ϕ 29 DNA polymerase. The resulted RCA products were hybridized with spacer complementary to produce alternating repeat regions of ssDNA and dsDNA. This is to sustain the rigidity and to reduce aptamer-aptamer interaction configuration.

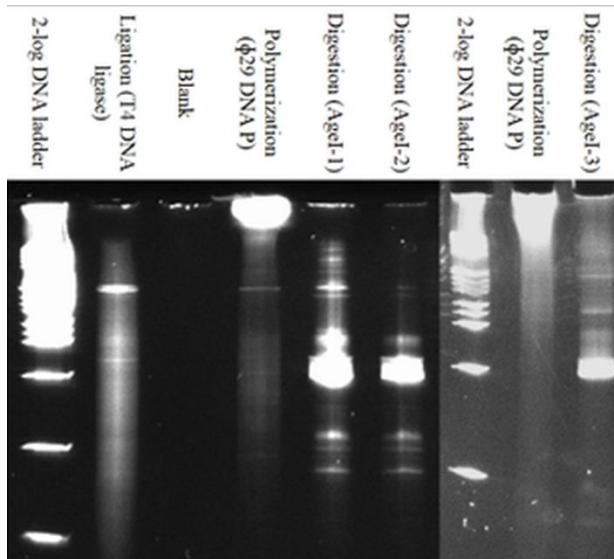


Figure 2.6. RCA products assessment after the digestion with AgeI restriction enzyme (loaded on 15% TBE urea denaturing gel). RCA products were prepared by the ligation of the linear ssDNA template with T4 DNA ligase (Cat# M0202S, NEB) with the presence of primer. After incubation with ϕ 29 DNA polymerase (Cat# M0269L, NEB), the resulting RCA products were digested with AgeI restriction enzyme according to manufacturer instructions (Cat# R0552S, NEB). The templates were designed to produce products containing the spacer complementary to serve as a restriction site for this enzyme. To prove successful incorporation of this spacer-AgeI region, the AgeI enzyme was incubated with RCA products in three types of buffers (NEBuffer 1.1, NEBuffer 2.1 and NEBuffer 3.1 represented by AgeI-1, AgeI-2 and AgeI-3, respectively) to show cleavage of the RCA product

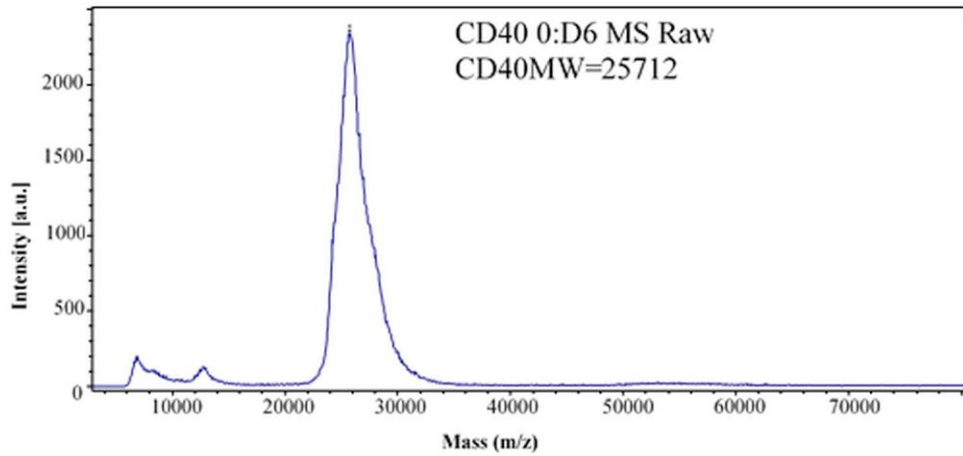


Figure 2.7. Identification of the MW of the CD40 receptor protein by mass spectrometry. SDS-PAGE gel loaded protein (1mg/mL) was sliced-cut and tested with MALDI-TOF-MS of the protein produced by trypsin digestion. The peak molecular weight of the protein is 25712 Dalton.

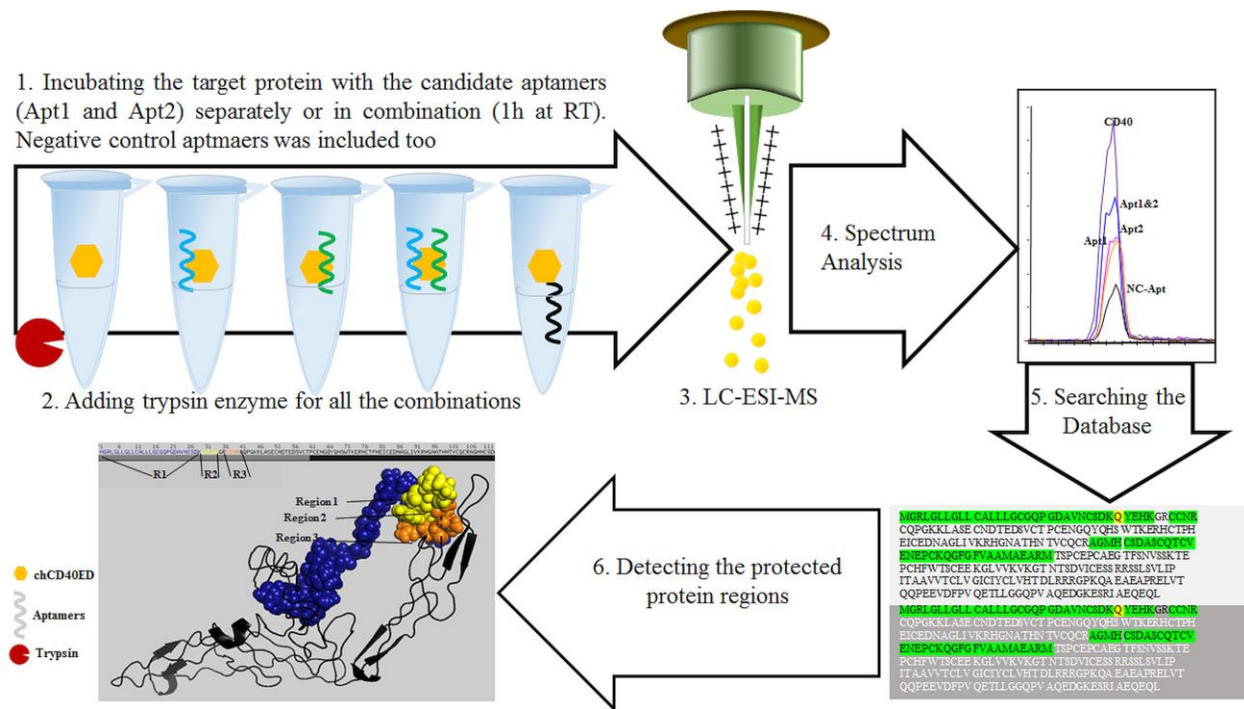


Figure 2.8. Schematic illustration represents the experimental design of LC-ESI-MS. LC-ESI-MS was used to discover the binding sites of CD40 that were partially protected by DNA and to test the ability of the candidate aptamers to attach and protect the target protein from the action of trypsin enzyme. The enzyme was added after the incubation of the protein with the aptamers-1 (blue), aptamers-2 (green) separately or in combination. Of course, NC aptamers (black) was included in the process of testing the digestion. Mass-spectrometry assay and spectrum analysis were used to detect the undigested amino acid sequences.

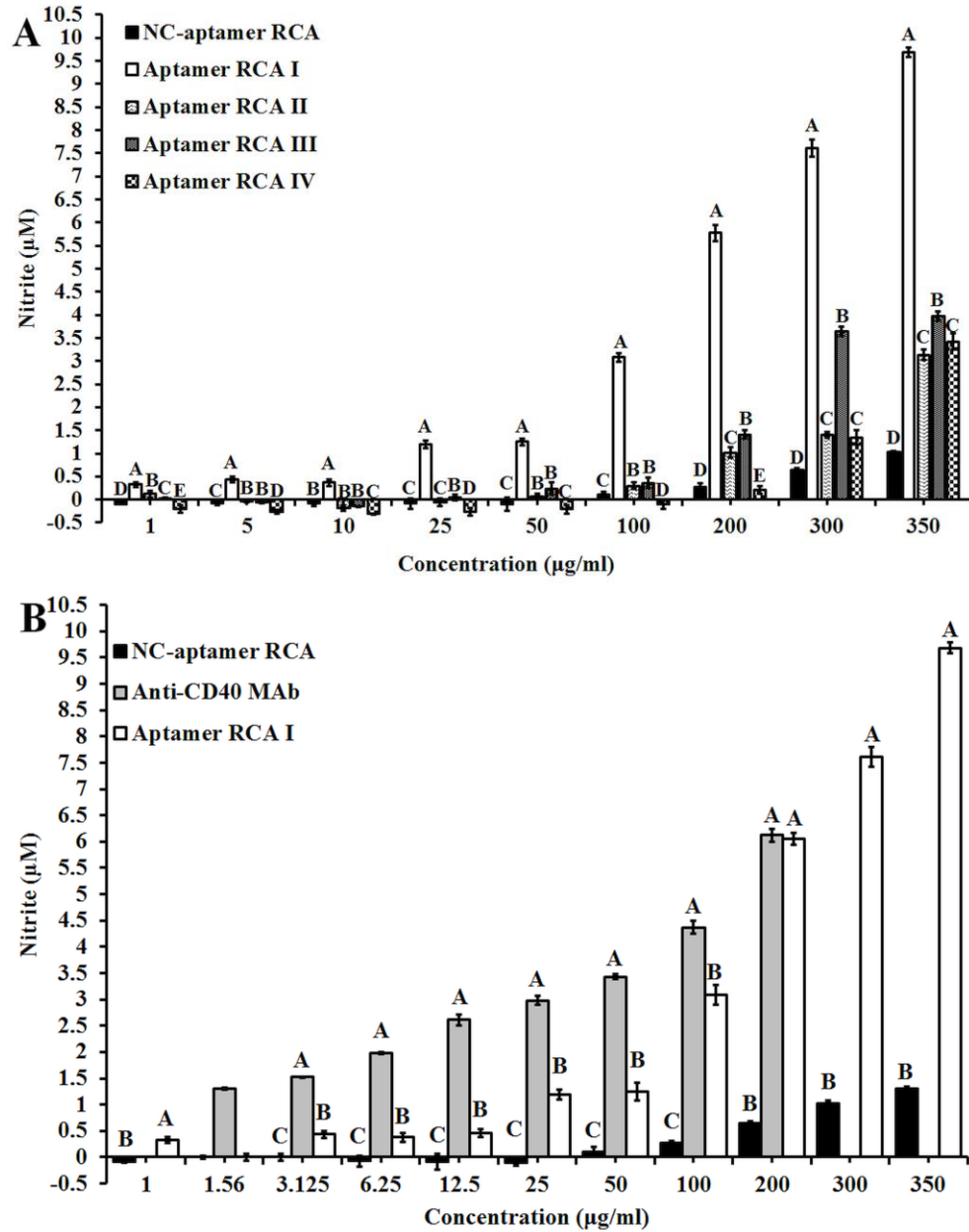


Figure 2.9. Nitric oxide liberation from chicken HD11 macrophage cell line over increased concentrations of RCA products (A: RCA I to RCA IV in comparison with NC-RCA, B: RCA I in comparison with NC-RCA and anti-chCD40_{ED} MAb). The RCA products were incubated overnight with the HD11 macrophage cell line in concentrations 1, 5, 10, 25, 50, 100, 200, 300 and 350 (µg/ml) in triplicates. The nitric oxide liberated was measured using Griess assay and showed slop increasing in regard to dose applied. The result was compared to anti-chicken CD40 MAb which is known to have the effect on this cell line (Chen *et al.*, 2010) and negative control RCA (two aptamer candidates that were not enriched in the Illumina sequencing results). Multiple way ANOVA and Tukey’s honest significant difference (HSD) post hoc test were applied to detect significant differences within treatments. Different uppercase letters represent significant difference at $p < 0.0001$.

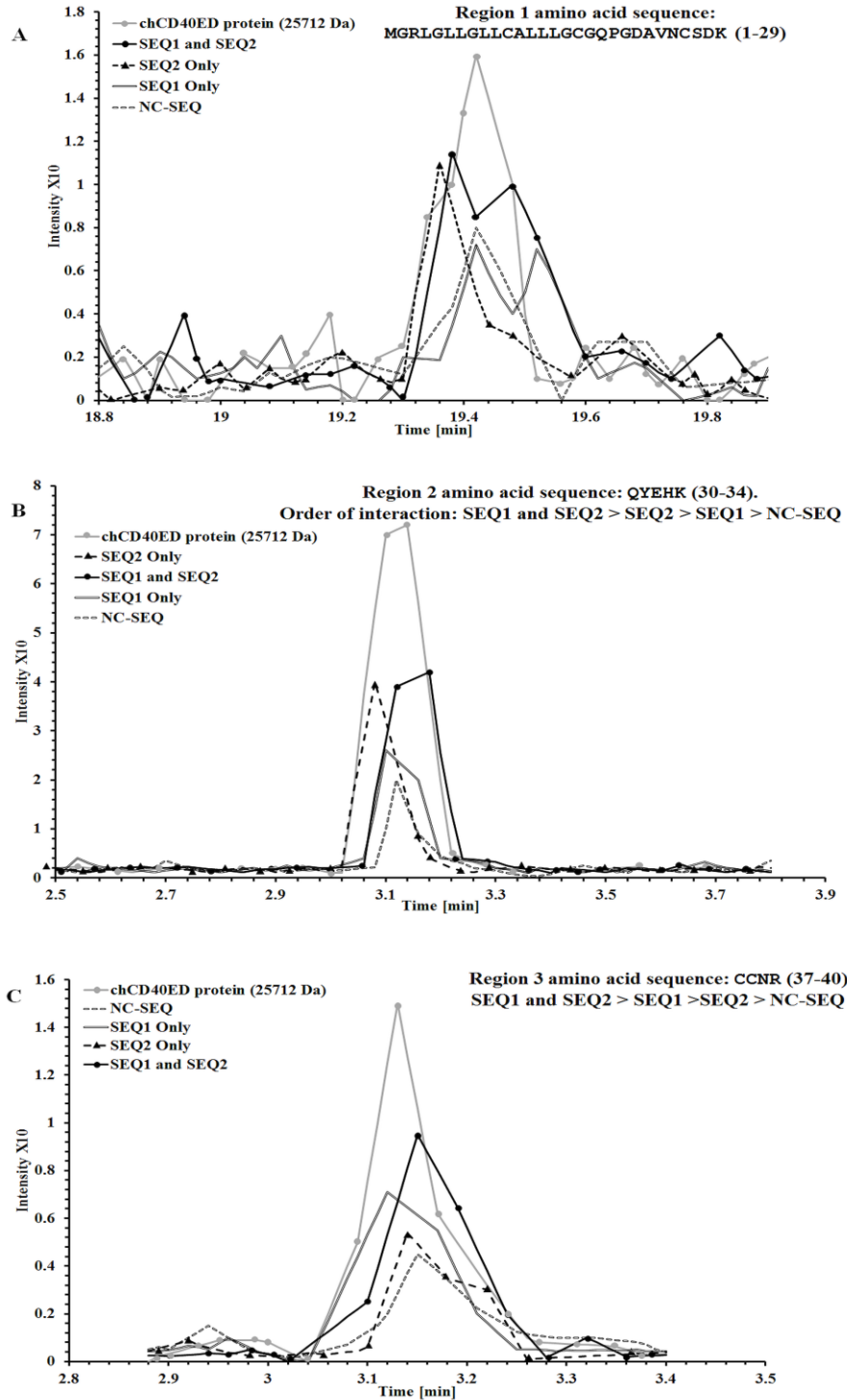


Figure 2.10. LC-ESI-MS results. After incubation of recombinant chCD40_{ED} protein (25712 D, gray line, gray circle) with the aptamers [SEQ1 (double line), SEQ2 (dished line, black triangle), SEQ1 and SEQ2 (black line, black circle), and NC-SEQ (dashed gray line)], the protein was subjected to trypsin digestion overnight. Three distinct regions on the protein sequence were protected from digestion (A, B and C). The peptide sequences were most effectively protected by the aptamers in combination.

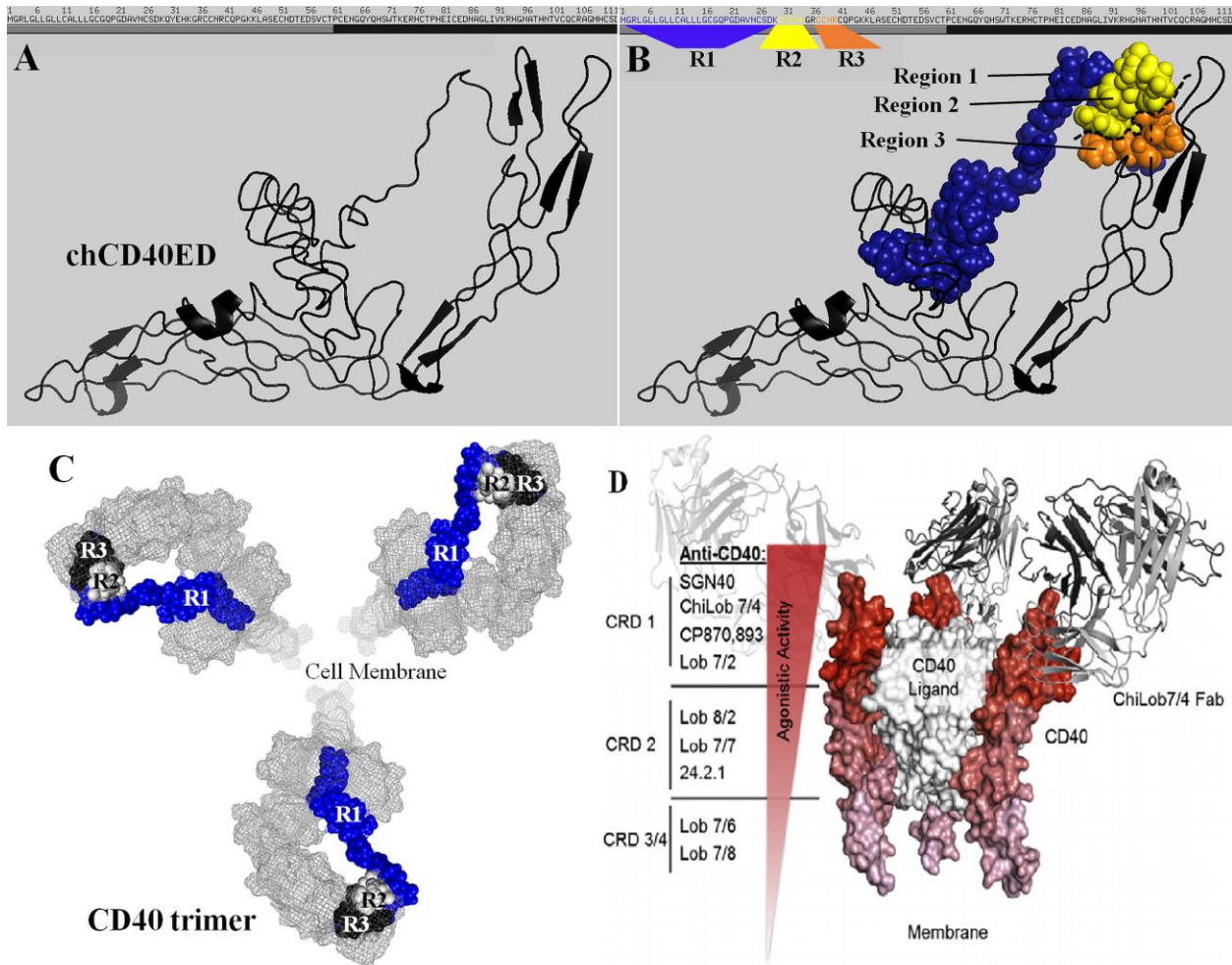


Figure 2.11. Virtual tertiary structure of chCD40_{ED} showing the potential aptamers linking sites. **A)** Cartoon modeling shows the tertiary structure of the protein. **B)** Space-filling model representing the virtual aptamers-protein interaction sites. Three regions (blue, yellow and orange) determined by MALD-TOF-MS experiment. The blue region represents long *flabby* peptide sequence where CD40L attaches. **C)** Top view of virtual trimer 3D structure of the chCD40_{ED} (Mesh model, gray) with the potential aptamer linking sites (space filling model, blue, white and black). These sites located in the inner of the receptor resembling the actual ligand binding sites (see below). **D)** CD40-CD40L interaction (human) where the CD40 and the CD40L are incorporated. In addition, anti- CD40-MAb Fc interaction regions. With the receptor-MAb model, agonistic activity of the MAb increases proximally over the receptor, whereas, the antagonistic activity increases distally.

Sources: **A, B & C**, PyMOL 2.2 software, W. L. DeLano, Schrodinger, Inc., **D**, cited: Yu, X. *et al.*, 2018. Cancer Cell. 33, 664-675.

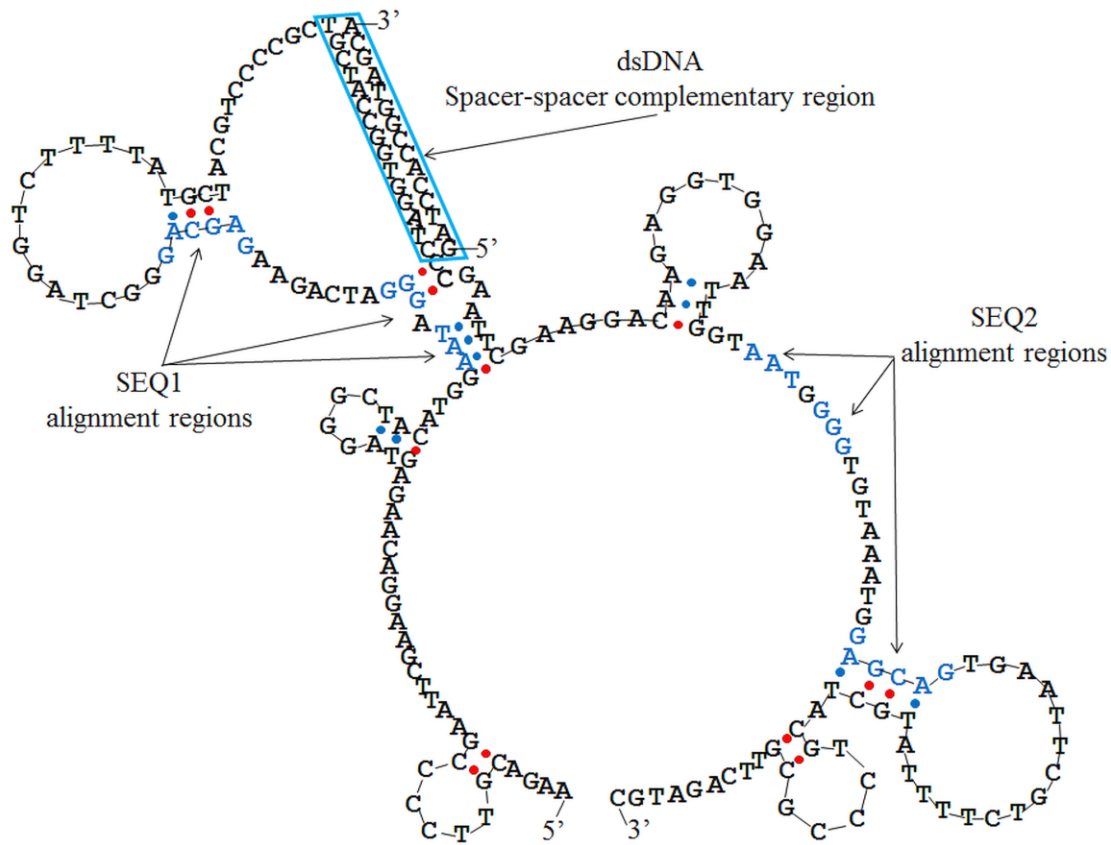


Figure 2.12. Alignment of one RCA I products unit. Hypothetically, this figure represents one unit of the RCA products showing the aligned three regions that are highly conserved between the two sequences (SEQ1 and SEQ2) (blue font). These regions might be essential in the aptamers-protein interaction. The hairpin prediction done manually due to the presence of the spacer-spacer complementary region (blue trapezium), which is a dsDNA and we could not find software can predict the hairpin of the mixed ssDNA and dsDNA molecule.

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CHAPTER III

Evaluation of a Novel Adjuvant Composed of DNA Aptamers-Based Rolling Circle Amplification Products Targeting Chicken CD40 Receptor and Conjugated to a Peptide Antigen in Broiler Chickens

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ABSTRACT

Cluster of differentiation 40 (CD40) receptor on antigen presenting cells, including B cell, engagement with CD40 ligand (CD40L/CD154) on activated T helper (T_H) cell provides the ignition signal required for B cell proliferation, differentiation and antibody production. Subsequently, there would be upregulation of many required molecules during the path of acquired immune response. Previously, we developed DNA aptamers that were specifically selected against chicken CD40 receptor extracellular domain. We showed that these aptamers –in form of rolling circle amplification products (RCA-p) – were capable of induction stimulation in chicken macrophage HD11 cell line. Here, we sought to test the potential ability of these aptamers to enhance an acquired immune response to low immunogenic peptide hapten *in vivo* by actin as immunological adjuvant. We tested the ability of our aptamers to increase the efficacy of the immune response to this low-immunogenic peptide hapten by increasing IgG response in broiler chickens after the immunization with RCA-peptide immune complex. For this purpose, we prepared an immune complex composed of two biotinylated aptamers-based RCA products molecules conjugated with streptavidin, which in turn, conjugated with two biotinylated peptide molecules. We administered this immune complex to broiler chickens at two different doses (25µg/bird) or (50µg/bird) at age 7 and 21 days via subcutaneous route at the nape of the neck. The later immune response quantification of the anti-peptide IgG titer in the serum samples by ELISA showed that the RCA products-peptide immune complex at dose of 50µg/bird was able to induce robust immune response as early as 7 days post-immunization and the antibody titer remained high until the end of the experiment. On the other hand, immunization with the lower dose showed gradual increase in the antibody titer and needed a booster dose. The use of aptamers

as sophisticated immunological adjuvants gave promising results, and would lower the cost of vaccines and would change the strategy of vaccination programs.

Key words: Broiler, DNA aptamers adjuvant, rolling circle amplification products, CD40.

3.1.INTRODUCTION

Cluster of differentiation 40 (CD40) is a surface receptor glycoprotein that expressed on antigen presenting cells (APCs) and other non-immune cells. The ligand for this receptor is the CD40 ligand (CD40L/CD154) which expressed by activated T helper (T_H) lymphocyte transiently. This receptor is a member of tumor necrosis factor receptor superfamily, whereas the ligand is a member in tumor necrosis factor superfamily (Stout and Suttles, 1996; van Kooten and Banchereau, 1997; Bosson *et al.*, 2006; Ma and Clark, 2009; Suttles and Stout, 2009). In essence, naïve T (T_H/T_H0) lymphocyte requires three constative signals for activation and differentiation. Signal-1 gained by T cell receptor (TCR) engagement with cognate major histocompatibility complex (MHC) class II/peptide on APCs, signal-2 provided by costimulatory molecules engagement on both cell types, and signal-3 comes from cytokines released by both activated cell types. When the third signal received, activated T_H cell starts to differentiate into subsets of effector T_H cell such as T_H1 and T_H2 . The type of the T cell subset depends on the type of the foreign peptide, which in turn, guides the cytokine profile of both cell types. (Caux *et al.*, 1994; Pinchuk *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Ma and Clark, 2009; Okoye and Wilson, 2011; Gerlach *et al.*, 2012; Tubo and Jenkins, 2014). APCs, on the other hand, require signal-0 down from chemokines for maturation and homing (Thaiss *et al.*, 2011). The resulted immune response upon the engagement of the CD40 receptor with its ligand is different according to the type of the APC and the type of the foreign peptide. A well-studied pathway has demonstrated that T_H cell causes DCs to promote $CD8^+$ cytotoxic T lymphocyte (CTL) activation via CD40-CD40L pathway (Vonderheide and Glennie, 2013). The three-cell type activation model requires that both T_H and CTL cell receptors must engage cognate MHC class II or I co-expressed foreign antigens on the same DC. This triple cell engagement leads to DC activation, T_H and CTL cells activation/differentiation. Schoenberger

et al. (1998), documented that priming DCs with agonistic anti-CD40 monoclonal antibody (MAb) could provide help for CTL in CD4-depleted mice.

Macrophages produce IL-12 and receive activation from T_H1 via CD40L and interferon-gamma (IFN- γ) in classical macrophage activation pathway. IFN- γ , which is a potent activator for macrophage, greatly enhances the activity of macrophages against the intracellular pathogens. Both CD40L and IFN- γ share synergetic effect on macrophages to facilitate their production of MHC class II molecule and several enzymes such as phagocyte oxidase and inducible nitric oxide synthase, which stimulate the production of reactive oxygen species and nitric oxide in the phagolysosomes, respectively. CD40L and IFN- γ are also enhance the production of lysosomal enzymes in the macrophages presenting peptide/MHC class II molecule to the T_H cells. Similarly, in innate immune response, macrophages receive the same signal of activation by natural killer cells (Ma *et al.*, 2003; Scott *et al.*, 2004; Abbas *et al.*, 2015). It has been shown that CD40 ligation in macrophages promoted a promising anti-cancer activity within these cells. Macrophages play role in stimulation of angiogenesis at the site of cancerous tissue, hence, promote cancer metastasis. In addition, macrophages suppress anti-cancer innate and adaptive immune response by suppressing NK and T cells (Noy and Pollard, 2014). Ligation of CD40 on these macrophages reverted their tumor-enhancing activity; and render them active antitumor participant cells. However, other studies showed that CD40 is expressed by the cancer cells themselves. Anti-CD40 agonists such as MAb adjuvants induced antitumor activity by several mechanisms (Rakhmilevich *et al.*, 2012). Controversially, CD40 ligation promotes anti-apoptosis in B cells while soluble CD40L and agonistic anti-CD40 MAb may promote apoptosis of certain CD40 expressing tumors without any participation of CD40 expressing immune cell (Eliopoulos *et al.*, 2000, Vonderheide and Glennie, 2013).

Upon TCR-peptide/MHC class II engagement, immunological synapse (IS) forms. On the T lymphocyte side, TCR is the central molecule within this synapse, however, other costimulatory molecules participate considerably such as CD3 and CD28 and CD40L (Abbas *et al.*, 2012). When B lymphocyte involved in formation of IS with T_H, rapidly accumulated CD40L is lost shortly (Yellin *et al.*, 1994). The molecular mechanism of diminishing CD40L after the T_H activation explained by several publications. Detection of soluble form of the CD40L in the media after T cell activation suggested the usefulness of this form for further activation of other uninvolved immune components (Graf *et al.*, 1995). In a paradox, Hollenbaugh *et al.* (1992) were able to induct costimulation, but not full activation, for B cells by the soluble form of the CD40L. Other publications suggested that after the CD40-CD40L engagement, the CD40L detached from the T_H cells and endocytosed by the antigen-presenting B cell (Gardell and Parker, 2017). This phenomenon, which is antigen-dependent, is to sap activated T_H cells render them recruiting bystander B cells. The outcome of CD40-dependant B cell activation with natural CD40L, soluble CD40L or agonistic anti-CD40 MAb resulted in B cell proliferation with increased MHC class II molecule expression and differentiation into plasma cells and memory cells. Plasma cells undergo profound production of antibody with high affinity maturation, and antibody isotype class switching. (Kawabe *et al.*, 1994; Han *et al.*, 1995; Kawabe *et al.*, 2011; Gardell and Parker, 2017).

Recent studies showed that the use of different agonists to CD40 receptor would lead to induction of acquired immune response. Agonists, such as anti-mouse CD40 monoclonal antibodies (MAbs) or soluble CD40L, were able to mimic the course of natural acquired immune response (Richman and Vonderheide, 2014a; Beatty *et al.*, 2017). In one approach, our group had shown that the use of the chicken CD40 receptor, as the target for activation with anti-chicken CD40 MAb, was extremely successful with robust immune responses. (Chen *et al.*, 2010; Chen *et al.*, 2012).

Recently, we showed that DNA aptamers that had been specifically selected against chicken CD40 receptor extracellular domain (chCD40ED) were capable of stimulation of chicken macrophage HD11 cell line (data unpublished). Thus, our DNA aptamers are one additional agonist to the CD40 receptor. We aimed to substitute the anti-chicken CD40 MAb with DNA aptamers. Aptamers have many advantages over the monoclonal antibodies such as thermostability, chemical resistance, reproducibility, and low cost (Jayasena, 1999; Song *et al.*, 2012).

Anti-CD40 agonists could act as immunological adjuvants. We sought to test the ability of our anti-chCD40_{ED} DNA aptamers (in form of rolling circle amplification products – RCA products) to act as immunological adjuvant in chicken. Herein, we might induce guided immune response toward peptide hapten in chickens with better potency and efficacy. For this purpose, the RCA products become chemically loaded with short low immunogenic peptide hapten with the aid of streptavidin. The peptide was a synthetic sequence of the external domain of matrix protein 2 (M2e). This peptide is a short peptide that is unlikely to induce immune response by its own (Feng *et al.*, 2006; Vuong *et al.*, 2018). We administered the RCA products-M2e peptide immune complex to broiler chickens. Briefly, we conjugated two molecules of aptamers-based RCA products with streptavidin, which in turn, conjugated with two molecules of the peptide as the hapten. We administered this immune complex subcutaneously in two different doses according to the RCA products concentration (50µg/bird or 25µg/bird) to broiler chickens in priming and boosting doses at 7 and 21 days. In either RCA products dose, the peptide concentration was 0.2µg/bird.

The results showed that this immune complex could induce significant level of anti-peptide IgG antibody titer in the chicken sera as early as seven days post-immunization when 50µg/bird was administered. This antibody titer remained high significantly to the fourth week post-

immunization. However, birds that had been immunized with the lower dose needed the boosting dose to sustain detectable level of the antibody titer at the end of the experiment. Our work would bring provide new foundations for peptide vaccine adjuvanted with aptamers-based adjuvants.

3.2.MATERIALS AND METHODS

3.2.1. Chickens, random mixing and management

The conditions for animal use in this study were met the requirements of and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas (permit # 18127, see appendix). Total 120 broiler one-day-old chicks were divided into six groups (n=20) by different color tags in comingle pen. The chicks were Cobb-Vantress Off Sex – Male obtained on the day-of-hatch from the Fayetteville hatchery, Fayetteville, AR. The chicks were mechanically block randomized by moving 1/3 of each of the chicks from three boxes to a single new chick box, thus equally re-mixing each of the boxes. This was done in case boxes were derived from different hatchers and/or potentially different breeder sources (this variability has historically been the most critical for randomization effects). The management practice took the consideration of the broiler management guide of Cobb (cobb-ventress.com).

3.2.2. Aptamers, rolling circle amplification products and M2e peptide

3.2.2.1.Aptamers.

Previously, we developed DNA aptamers targeting chicken CD40 extracellular domain (chCD40_{ED}) protein in process of systemic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold, 1991; Ellington, and Szostak, 1990) (unpublished data). Briefly, ten

rounds of enrichment via SELEX procedure needed to develop these DNA aptamers against the target protein. We run several assays to prove the affinity, the specificity and the biological activity of our aptamers toward this target protein *in vitro*. We successfully stimulated nitric oxide liberation from chicken macrophages HD11 cell line with our aptamers in form of RCA products. In addition, we specified the sites where our aptamers attached the target protein by running an LC-ESI-MS experiment (see Chapter II).

3.2.2.2.Rolling circle amplification products.

The anti-chCD40_{ED} aptamers we developed were prepared in form of biotinylated RCA products. The biotinylation process achieved by hybridizing upstream-biotinylated primer with the template. Biotinylation of the RCA is to facilitate the conjugation with streptavidin in the later step. The process of aptamer-dependent RCA products was as described elsewhere (Blanco *et al.*, 1989; Johne *et al.*, 2009). Briefly, our RCA technique relied on preparing RCA template depending on our DNA aptamers sequences. The template composed of two aptamer sequences separated by a spacer sequence. We circularized the template by annealing primer (20 nucleotides) that complementary to the upstream and the downstream of the ssDNA template and ligated of the nicked sequence between the primer and the template with T4 DNA ligase enzyme (Cat# M0202S, NEB, USA). The RCA template ordered as phosphorylated at the upstream (IDT, Coralville, Iowa, USA) to sustain the enzymatic ligation.

The procedure of the RCA started with annealing 15-20pmol of the primer (equivalent up to 132ng/μl) with primer binding site on the template (1-2pmol equivalent up to 129ng/μl). The primer-template mixture was heated first to 95°C for 10min, to 56°C for 5min and then cooled

down to RT in 50 μ L 1xTE buffer. To ligate the nicked template, the ligation of the template with T4 DNA ligase enzyme as recommended by the manufacturer (Ali *et al.*, 2014).

The polymerization of the template was with ϕ 29 DNA polymerase enzyme (Cat# M0269L, NEB, USA). All the reaction conditions were as recommended by the manufacturer except that the reaction was hold for 16hr at 30°C and then heat-inactivated at 65°C for 10min. After each enzymatic treatment, the DNA was purified by ethanol precipitation. For SCS adding, two molecular weights of the SCS were mixed with one molecular weight of the RCA products. Again, the RCA products-SCS mixture was hold on heating for 10min at 95°C and for 50°C for 5min and then ice-cooled immediately to apply the annealing status. The RCA products represented tandem repeats of the original aptamers, hence; increase the chance for CD40 receptor activation *in vivo*. Adding SCS piece to ensure rigid sequences composed of double stranded DNA in exchange with our aptamer sequences to serve several purposes such as conserving the original DNA aptamers sequence by avoiding any form of dimerization and increasing the spanning area of our aptamers.

3.2.2.3. Peptide immunogen.

A commercial synthetic amino-terminus biotin labeled M2e peptide was a kind gift from Dr. Luc R. Berghman at the University of Texas A&M. The peptide was a biotinylated sequence (GenScript Inc. Piscataway, IL). This peptide, which was with the sequence “NAWSKEYARGFAKTGK”, representing the ectodomain of the matrix protein 2 of the influenza virus (Vuong *et al.*, 2018). Conceptually, the biotinylation is to promote the conjugation of this peptide with streptavidin to form unit in the immune complex (see below).

3.2.3. Immunizing complex preparation, doses, timing and routes for administration

Immunization complexes were prepared as described by Chen and co-workers (2012). Briefly, streptavidin (Thermo Fisher Scientific Inc., Rockford, IL) was used as the central molecule that conjugate other molecules in the immune complex (Figure 3.1). As mentioned above, the M2e peptide was ordered as biotinylated (GenScript Inc. Piscataway, IL), whereas the biotinylation of the RCA products was by ordering a biotinylated primer at the upstream to apply the RCA procedure. The biotinylated M2e peptide (0.2µg) was mixed with the biotinylated RCA products (50µg) in 1 to 1 ratio on molecular basis in 1xPBS with rotation at room temperature (RT) for 3hrs. The peptide-RCA products mixture then added to streptavidin in 2 to 1 ratio and stirred at 4°C for 3hrs. Biotinylated anti-chCD40ED MAb was used as a control for our immune complex. This MAb was another kind gift from Dr. Luc R. Berghman at the University of Texas A&M.

The same procedure was followed when preparing the anti-chCD40_{ED} MAb-based immune complex, the M2e peptide alone, or the RCA products alone. Similarly, the peptide or the RCA products alone ratio to the streptavidin was 2 to 1.

The immunizing doses and timing were as described in Table 3.1. The total volume of the calculated doses for either immunizing formula was 0.4mL/bird. The formulation of the immunizing complexes was in an emulsified PBS containing [5% (v/v) squalene, 0.4% (v/v) Tween 80 (Sigma-Aldrich, St. Louis, MO) in PBS].

3.2.4. Blood sampling

Blood sampling was at weekly intervals in a sterile procedure. The blood were collected from the jugular vein at week 1 and from the brachial wing vein at the next four weeks. In the first week,

approximately 1 mL of blood was collected using 25G needles, 1 inch long in 5 ml syringes. In the next four collection procedures, 2-3 mL were collected using the same needle-syringe conformance. The blood samples were transferred to sterile and labeled tubes with lids. After blood complete clotting, blood samples were subjected to centrifugation for 5 min at 500xg and the sera were stored at -20°C (Lumeij, 1987; Kelly *et al.*, 2013).

3.2.5. Quantification of specific IgG by ELISA

Antigen-capture ELISA was used to quantify the level of the anti-peptide IgG titer in the chicken sera. All serum samples were loaded in duplicates in a 96-well plate throughout the assay. The ELISA procedure started by incubation of the biotinylated M2e peptide (MW 2267.2Da) with 5µg/mL goat anti-biotin antibody (MW 150kDa) (ThermoFisher Scientific Inc., Rockford, IL), in 1 to 1 molecular ratio in 10mL 0.05M carbonate-bicarbonate buffer (pH 9.6) for 2hrs at RT with rotation. 100µL of this peptide-antibody complex then coated a 96-well microtiter plate (MaxSorb, Thermo Fisher Scientific Inc., Rochester, NY) overnight at 4°C. Next, the un-adsorbed supernatants were removed and the plate was washed (3x) with 200µL 1xPBS-T for 15sec/each and blocked with 200µL commercial blocking buffer (Thermo Fisher Inc.) for 1h at RT. After a course of washing with 1xPBS-T for three times, the plate was incubated with the investigated sera [(100µL), diluted 1:500 in PBS to blocking buffer (1:1), 1% normal goat serum (v/v) and 1% normal rabbit serum (v/v)] overnight at 4°C. After a another course of washing, the plate was loaded with 100µL of horseradish peroxidase-conjugated, affinity purified isotype-specific rabbit anti-chicken IgY antibody (Thermo Fisher Scientific Inc., Rochester, NY).

The dilution of the secondary antibody was 1:30,000 in bovine serum albumin (BSA) in blocking buffer and 1% normal goat serum (v/v). The plate was set for 1h at RT and a course of 5x washing

followed. For developing the color reaction, we used OptEIA™ TMB substrate (BD, Lakes, NJ). The color developing process was following the manufacturer instructions and the reaction terminated by adding 1N sulfuric acid (50µL/well) after 5min for all the trials.

Absorbance was measured by using BioTeK microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450nm using Gen5 version data analysis software (BioTek Inc) (Layton *et al.*, 2009; Kuttappan *et al.*, 2015).

3.2.6. Statistical analyses

The relative average levels of the anti-peptide antibody in the sera was normalized by calculating the value of each sample (n=20) to the mean value of the non-immunized group as the negative control (S-N). The average mean of the readings of the non-immunized birds was the baseline to calculate the S-N values of all groups. Results were analyzed using one-way analysis of variance (ANOVA) with least significant differences (L.S.D.) of the means of the S-N values and Student's *t*-test were used to determine the significant differences between the treatments. All data were analyzed using JMP software (SAS institute Inc., Cary, NC, software by the University of Arkansas). Statistical differences of the RCA-peptide immunized group were at level $p < 0.0001$ (Chou *et al.*, 2016).

3.3.RESULTS AND DISCUSSION

3.3.1. Evaluation of the anti-Me2 antibody titers

The pioneer work of activating the CD40 receptor by artificial agonists had encourage many laboratories to further investigating the outcome of this activation (Li and Ravetch, 2011; Richman and Vonderheide, 2014b). In essence, natural or artificial activation of this receptor could lead to many desirable effects over the course of the acquired immune response. When B cell involved in CD40-CD40L crosslinking, high antibody titer, antibody isotype-switching and affinity maturation are obvious (Amitage and Alderson, 1995; Merluzzi *et al.*, 2004). In this experimental design, we tested the ability of DNA aptamers-based RCA products to act as an adjuvant directed toward the CD40 receptor and indirectly conjugated with influenza M2e peptide. The same RCA products has previously shown the ability to agonist the CD40 receptor in chicken macrophage HD11 cell line (data unpublished). We compared our immune complex [(2)RCA products-SA-(2)peptide] (Figure 3.1) in two different doses with several negative control groups [SA-(2)RCA products alone, SA-(2)peptide alone, or non-immunized group) and with our previous immune complex design [(2)MAb-SA-peptide(2)] (Table 3.1). As stated by our group previously, chicken immunized with agonistic anti-CD40 MAb conjugated with low immunogenic peptide could induce high antibody titer directed against this particular peptide (Chen *et al.*, 2012). Currently, our results showed that the administration of 50µg/bird of RCA products-peptide immune complex could induce significant level of anti-peptide IgG titer for long term at level $p=0.0001$ (Figure 3.2B). The high antibody titer was detected as early as 7 days post immunization and remained high till the end of the experiment especially for birds with the high dose. These results are ideally match with our group novel work when the MAb was used as the adjuvant with the same dose and route (Figures 3.2A and 3.2B).

The same immune complex at lower dose (25µg/bird) failed to induct significant level of anti-peptide IgG in the sera at 7 or 14 days post first immunization. However, this particular immunization needed boosting at day 21 to induct significant level of the antibody titer at 28 days post immunization (Figures 3.2A and 3.2B, Figure 3.3). Our results showed no need to use booster dose to gain satisfactory level of anti-peptide IgG titer at dose 50µg/bird (Table 3.2). Boosting could apply additional undesired effects especially in broiler chickens such as immune stress and cost. All birds showed retarded immune response at day 14 post immunization (boost timing) (Figure 3.3A). We recorded some management issues during the day before the onset of blood sampling. This might stressed out the birds at that particular time. However, it seems that the birds have compensated the immune response against the immunogen in later weeks.

3.4.CONCLUSIONS AND SIGNIFICANCE

The M2e protein is highly expressed on the surface of the naturally infected cells with influenza virus. However, the M2e is poorly immunogenic by its own (Black *et al.*, 1993; Feng *et al.*, 2006). It is obvious that MAb adjuvants attached to soluble peptide hapten targeting the CD40 receptor yields better-enhanced long lasting immune response than traditional adjuvants (Barr *et al.*, 2003; Martin *et al.*, 2017; Vuong *et al.*, 2018). Despite the use of relatively low concentration of the hapten (M2e peptide), our RCA products-peptide immune complex succeeded to induct high level of anti-M2e IgG antibody titer in the chicken sera at dose (50µg/bird). With this dose, the elevation of the antibody titer started as early as seven days post-immunization and remained high to the end of the experiment. The action of this immune complex proves that this complex is with high potency and efficacy (Deng *et al.*, 2015). These results are comparable to our lab group previous

work with MAb as the adjuvant with the same peptide (Chou *et al.*, 2016; Vuong *et al.*, 2018). However, lower dose of the same complex (25µg/bird) yielded only minor level of the antibody titer and needed a booster dose to sustain relatively acceptable level of the same antibody titer at the end of the experiment.

Our immune adjuvant could guarantee the work as a universal adjuvant with any short soluble antigenic peptide. The immune response course with this DNA aptamers-based RCA products immunogenic adjuvant would pass through APCs-T_H cells interaction pathway.

Finally, aptamers are replacing the MAbs in many in vitro assays such as ELISA especially in vaccines assessment (Yoon *et al.*, 2017; Trausch *et al.*, 2017). We are first reporting targeting the CD40 receptor with DNA aptamers as a vaccine adjuvant. This would offer a cheaper molecule and easier to prepare molecule with high stability. This would improve the effectiveness of many vaccines not in the husbandry field but also in vaccinology in general.

3.5.TABLES

Table 3.1. RCA Template, primer and spacer complementary sequence designs. The RCA template composed of partial primer-binding sequences at the upstream and the downstream. The primer utilized to circularize the template with the aid of T4 DNA ligase enzyme. The template composed of the complementary sequences of two aptamers separated by spacer to ensure individual effect of each aptamer and to avoid dimerization.

Description	Sequence (5'→ 3')
RCA products template	<u>GCA TCT GAA C</u> GCG GGA CGT AGC ATA AAA GAC GAA TTC ACT GCT CCA TTT ACA CCC CAT TAC CAA TTC CAC CTC TTG TCC TTC GAA TTC GG <u>GAT CCA CCG GTA GCA</u> GCG GGA CGT AGC ATA AAA GAC CTA GCC CTG CTC TTC TGA TCC CTA TTC CAT GTA GCC CTA CTC TTG TCC TTC GAA TTC GG <u>GGA ACG TCT T</u>
RCA primer	GTT CAG ATG CAA GAC GTT CC
Spacer	GAT CCA CCG GTA GCA

Single underline represents the primer-binding site and the double underline represents the spacer complementary sequence

Table 3.2. Description of the experimental design. Six groups (n=20) were raised in a comingle pen and immunized with two different doses of RCA-SA-peptide immune complex (i.e. low: 25µg/bird and high: 50µg/bird) for two times. This immune complex composition was compared with anti-chicken CD40 monoclonal antibody-based immune complex that has been given at (50µg/bird) for two times as well, and with three negative control groups.

Group	Description	Dose/bird	Prime/route	Boost/route
Non-immunized	NA	NA	NA	NA
NC	SA-peptide(2)	0.2µg	D7/SC	D21/SC
NC	SA-RCA(2)	50µg	D7/SC	D21/SC
MAB-based adjuvant	(2)MAB-SA-peptide(2)	50µg-0.2µg	D7/SC	D21/SC
RCA-based adjuvant	(2)RCA-SA-peptide(2)	25µg-0.2µg	D7/SC	D21/SC
RCA-based adjuvant	(2)RCA-SA-peptide(2)	50µg-0.2µg	D7/SC	D21/SC

†Immune complexes was prepared in a volume of 0.4mL/dose/bird emulsified PBS (containing 5% [v/v] squalene and 0.4% [v/v] Tween 80, pH=7.4)

*Doses of SA-(2)peptide and (2)MAB 2C5-SA-(2)peptide were calculated according to Chen *et al.*, 2012. Values represent RCA and M2e concentrations, respectively.

Table 3.3. Normalized S-N values of the ELISA results of the anti-M2e IgG antibody in chicken sera. The (S) is the average sample reading and (N) is the average mean of the readings of the non-immunized group. Chickens were divided randomly into 6 groups in a comingle pen (n=20) and immunized at two timing (days 7 and 21). Investigated immune complex (RCA-M2e) was with two different doses (i.e. low: 25µg/bird and high: 50µg/bird). Blood collected at weekly intervals to apply ELISA assay. The sera diluted at 1:500 and the assay run in duplicates. Significant differences between groups were calculated by comparing means \pm SE of the S/N values by ANOVA and student *t*-test (JMP software, SAS institute Inc., Cary, NC, software by the University of Arkansas). Different letters represent significant differences at level $p=0.0001$

Group	D7PI (Mean\pmSE)	D14PI (Mean\pmSE)(Boost)	D21PI (Mean\pmSE)	D28PI (Mean\pmSE)
Non-immunized	1 \pm 0.0341 ^d	1 \pm 0.0155 ^d	1 \pm 0.0178 ^d	1 \pm 0.0176 ^d
M2e only	1.1368 \pm 0.0315 ^{bc}	1.0935 \pm 0.0128 ^{bc}	1.0281 \pm 0.0122 ^d	1.0724 \pm 0.022 ^{cd}
RCA only	1.0901 \pm 0.0326 ^{cd}	1.0597 \pm 0.0301 ^{cd}	1.0956 \pm 0.0167 ^c	1.1131 \pm 0.0141 ^c
MAb-peptide (50µg/bird)	1.2190 \pm 0.0693 ^a	1.557 \pm 0.0267 ^{ab}	1.2206 \pm 0.0280 ^b	1.4735 \pm 0.0269 ^a
RCA-peptide (25µg/bird)	1.0487 \pm 0.0573 ^{cd}	1.0561 \pm 0.0159 ^{cd}	1.2170 \pm 0.0223 ^b	1.2220 \pm 0.0215 ^b
RCA-peptide (50µg/bird)	1.2990 \pm 0.0347 ^a	1.1742 \pm 0.0271 ^a	1.3017 \pm 0.0257 ^a	1.4343 \pm 0.0462 ^a

3.6.FIGURES

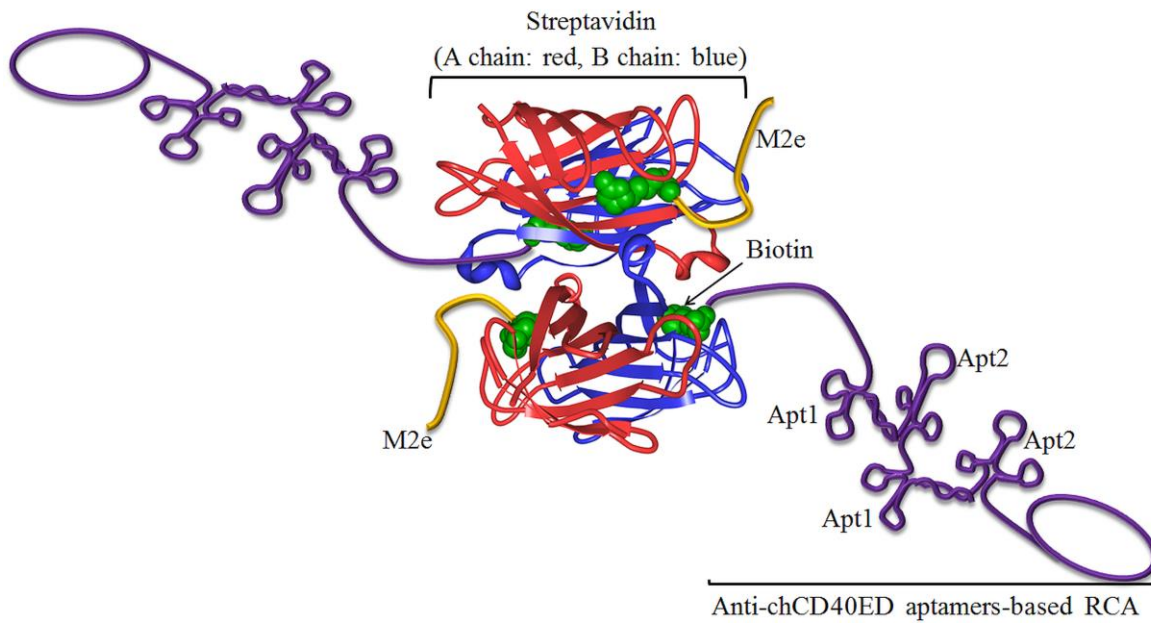


Figure 3.1: schematic figure represents the design of the immune complex. Streptavidin (SA) is the central molecule that holds the biotinylated components of the immune complex tightly. RCA molecule was biotinylated to conjugate with the SA. Similarly, the biotinylated M2e peptide was mixed with the RCA in 1 to 1 ratio on the molecular basis. RCA-M2e mix then added to SA in 2 to 1 ratio on molecular basis as well. The design followed what described by Chen *et al.* 2010. Streptavidin-biotin model: Trong *et al.*, 2011, M2e model: Cho *et al.*, 2015.

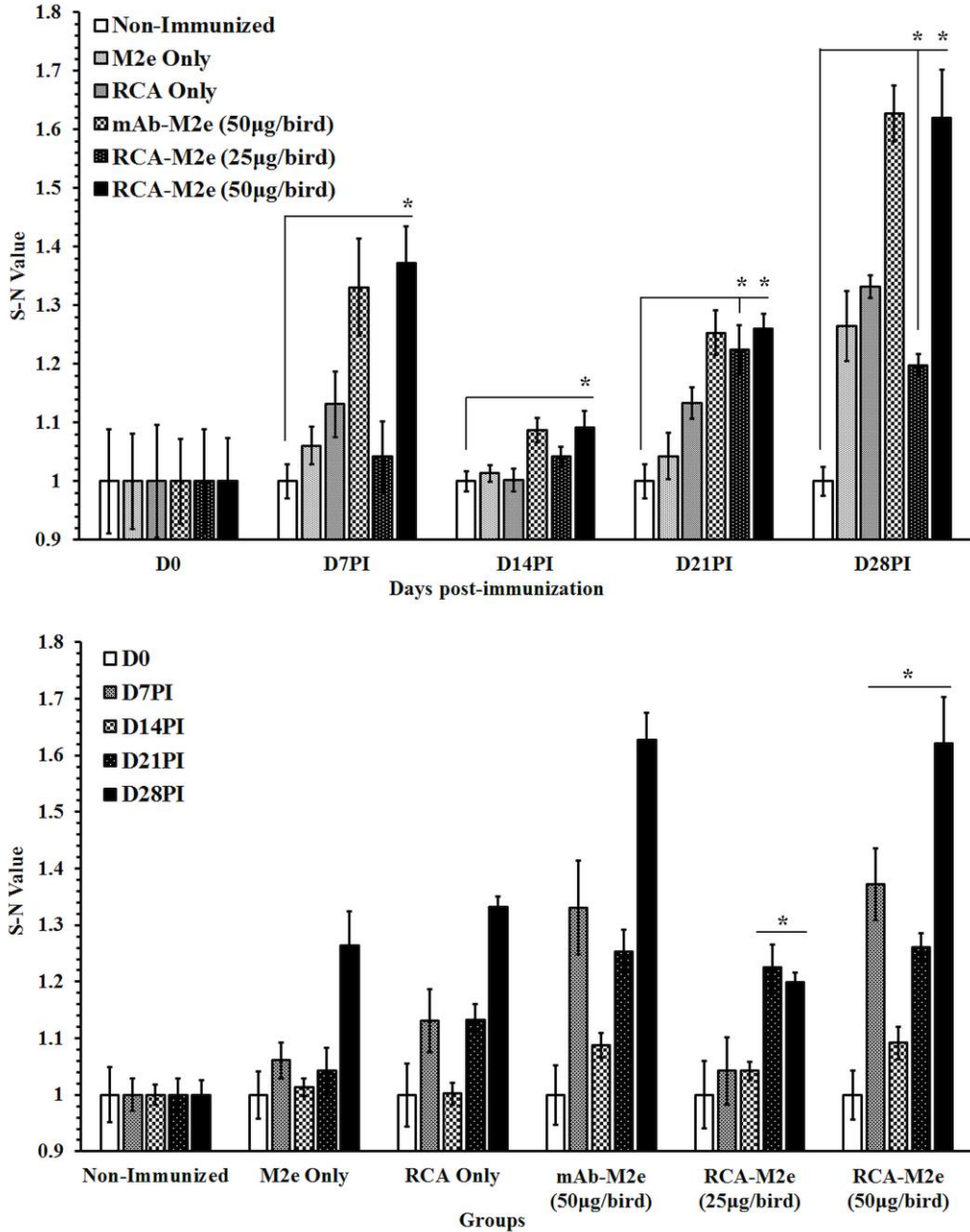


Figure 3.2. Normalized ELISA results representing S-N values of the anti-M2e IgG in chicken sera at weekly intervals after immunization. Birds were immunized at day 7 and day 21 via s/c route with RCA-peptide complex in two different doses (50µg/bird) or (25µg/bird) (n=20). The normalization calculations $S-N = \frac{\text{average of (sample read - mean reads of the non-immunized group)}}{\text{mean of each group}}$ calculated accordingly. The significant differences of the antibody titers between the group that received 50ug/bird of the RCA-peptide immune complex and the non-immunized group are according to ANOVA and student *t*-test. Asterisks represent statistical differences ($p=0.0001$) from the non-immunized control group. **A)** Results according to the timing after immunization; **B)** Results according to groups.

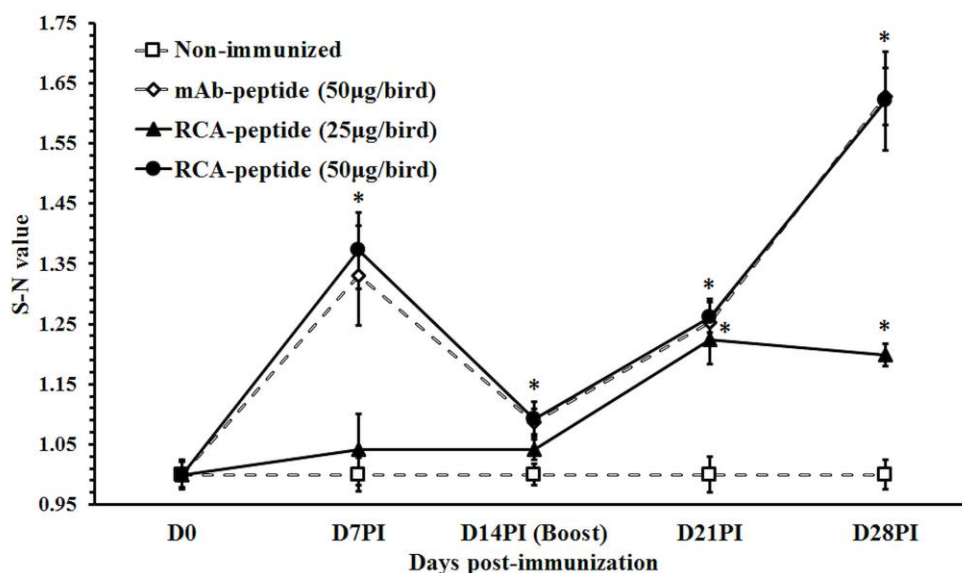


Figure 3.3. Normalized ELISA results representing S-N values of anti-peptide IgG in chicken sera at weekly intervals. Results shown at D0-D28 post-immunization with RCA-peptide (50µg/bird) or RCA-peptide (25µg/bird). Birds were immunized at day 7 and day 21 subcutaneously (n=20/group) and blood sampling performed at weekly intervals. Birds immunized with the higher dose showed significant differences to the non-immunized group as early as D7PI, whereas, birds immunized with the lower dose showed significant differences only at D21PI. Results show mean \pm SE and the differences are according to ANOVA and student *t*-test. Asterisks represent statistical differences (* $p=0.0001$) from the non-immunized group.

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CHAPTER IV

Efficient Correction of Heteroduplex DNA Formed during PCR Amplification of DNA Aptamer Libraries

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ABSTARCT

DNA aptamers are now emerging as a promising target-recognizing biomolecule of choice in various biomedical and biotechnological applications with multiple advantages. Traditional aptamer selection process is through systemic evolution of ligands by exponential enrichment (SELEX) procedure, which includes multiple PCR steps to amplify the enriched aptamers. During amplification, unwanted DNA byproducts of considerable amount are often formed. These spurious byproducts as characterized by DNA fragments, which appear in variable lengths on gel electrophoresis, are considered as the result of formation of heteroduplexes within the randomized region of the DNA aptamer library. These PCR byproducts negatively alter the diversity and composition of the DNA library. Here we describe a simple method that corrects these PCR byproducts efficiently. When repetitive cycles of heating-cooling ($60^{\circ}\text{C}\rightarrow 20^{\circ}\text{C}$) were applied to the enriched aptamers, our results showed that this procedure has efficiently removed the formation of the byproducts, suggesting that this method can be used widely in removing heteroduplexes or other type of spurious byproducts formed during amplification of DNA aptamer library.

Keywords: DNA aptamer, PCR byproducts, DNA heteroduplexes, SELEX.

4.1.INTRODUCTION

The technique of systemic evolution of ligands by exponential enrichment (SELEX) was initially developed independently and simultaneously by two laboratories in 1990 (Tuerk and Gold, 1990; Ellington and Szostak, 1990). This technique has been used extensively to develop nucleic acid aptamers with high specificity and affinity to various targets for variable applications. In fact, aptamers have been replacing monoclonal antibodies in many biological and biochemical applications (Jayasena, 1999; Dollins *et al.*, 2008; Scoville *et al.*, 2017).

The procedure of SELEX requires randomized oligonucleotide library that is flanked by two constant regions at the upstream and the downstream, which serve as primer binding sites for subsequent PCR amplification. Oligonucleotide library generation depends on equal opportunity of the four-nucleotide bases incorporated at any position within the variable region. Depending on the length of the random region, the predicted diversity of the oligonucleotide library is 4^n where (4) represents the four nucleotides, i.e., A, C, G and T and n is the length of the random variable region. For example, a library with 40n at the random region form 10^{24} different sequence hypothetically (Vorobyeva *et al.*, 2018). At the ends of each SELEX enrichment cycle, the PCR is used to amplify the enriched aptamers exponentially. In the later step, the double-stranded DNA products of the PCR are typically converted into single stranded DNA (ssDNA) by the digestion with an exonuclease enzyme (e.g. lambda exonuclease – λ - exonuclease) to resume the original library format for the new enrichment cycle (Avci-Adali *et al.*, 2010). However, due to highly diverse sequence of the library, there is always a possibility to develop amplify spurious byproducts during the PCR steps. Many sequences in the random library might undergo partial hybridization between different sequences to form PCR byproducts with variable lengths (Smyth *et al.*, 2010; Tolle *et al.*, 2014; Thompson *et al.*, 2002). Another source of the PCR byproducts can

arise when one or both primers fully or partially hybridize to other regions than the constant primer-annealing sites. In essence, the correct PCR products are usually mixed with one or two types of byproducts; i.e. smearing bands and/or ladder-like byproducts when analyzed by agarose gel electrophoresis. In either way, the PCR byproducts might affect negatively the diversity of the enriched aptamers. There are studies suggesting practical methods to resolve this issue through PCR re-conditioning (Smyth *et al.*, 2010; Tolle *et al.*, 2014; Thompson *et al.*, 2002; Qiu *et al.*, 2001).

Here, we describe a method that could efficiently correct the size and the shape of the PCR byproducts that occur during SELEX DNA aptamer selection. We hypothesized that the application of appropriate thermal cycles might partially denature these heteroduplexes, facilitating homoduplexes formation. PCR byproducts are considered to be formed due to the formation of DNA heteroduplexes when mixing more than one template sequence *en masse* (Ruano and Kidd, 1998; Kalle *et al.*, 2014). Our thermal cycle involves raising the temperature of the DNA byproducts to 65°C for 10 min and cool them down to 20°C for 1 min, which was repeated for six times.

With our method, however, the fully hybridized homoduplexes would not be affected due to their very low free energy change (ΔG) value (Petruska and Goodman, 1995). Our results showed marked reduction in the ladder-like and/or smearing band PCR byproducts. Analysis of the resulting library by Illumina sequencing demonstrated that there was a marked reduction in the proportion of the shorter and longer reads in the enriched library by these corrective cycles in comparison to the negative control library. Here, we describe a simple and an effective method that corrects the undesirable PCR byproducts to improve overall quality of the aptamers library.

4.2.MATERIALS AND METHODS

4.2.1. DNA library and primers.

A randomized single-stranded DNA (ssDNA) library, forward and reverse primers were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). The library was composed of two parts; a variable region of 40 nucleotides, which was flanked by two constant regions that consisted of 20 nucleotides each at the upstream and the downstream. The fixed regions consisted of the sequence for annealing of the forward primer at the upstream and reverse primer binding site sequence at the downstream. The library sequence was 5'-CCG AAT TCG AAG GAC AAG AG (N)₄₀ TCT TT TAT GCT ACG TCC CGC-3', whereas the forward and the reverse primers sequences were: 5'-CCG AAT TCG AAG GAC AAG AG-3' and 5'-GCG GGA CGT AGC ATA AAA GA-3, respectively. Our library was with 10²⁴ sequence hypothetically. The reverse primer was phosphorylated at the upstream for digestion with λ -exonuclease enzyme in the later step during the enrichment cycles. The library and the primers were diluted in 1X Tris-EDTA buffer (TE buffer, ThermoFisher Scientific, USA) to sustain 100 μ M concentration as recommended by the manufacturer and were stored in -20°C.

4.2.2. Target protein

The target protein used for the aptamers selection was a recombinant protein of chicken CD40 extracellular domain protein (chCD40_{ED}). The protein was a kind gift from Dr. Berghman at the University of Texas A&M. Briefly according to Chen and co-workers (2010), total RNA was isolated from chicken spleen and cDNA was synthesized accordingly. The sequence encoding the chCD40_{ED}, which was already determined, was amplified with nested PCR and the gene encoding the protein was cloned into expression vector pcDNA5 (Invitrogen). The chCD40_{ED} recombinant

protein was expressed in hybridomas, human embryonic kidney (HEK)-293 Freestyle cells in protein-free medium (Antagene Inc., Santa Clara, CA, USA) with concentration of 1mg/mL. The protein's stock was diluted with sterile 1X phosphate buffer saline (1XPBS, w/v) to concentration of 15µg/L and aliquoted into 1ml in sterile tubes and stored at -80°C.

4.2.3. *In vitro* SELEX procedure

Ten enrichment cycles were applied to develop the specific aptamers to the target protein. The procedure for aptamer selection was performed as described elsewhere (Tuerk and Gold, 1990; Ellington and Szostak, 1990). Briefly, a 35µL from the ssDNA library (1µg/µl) was mixed with 115µL binding buffer (50mM Tris HCl, 25mM NaCl, 5mM MgCl₂, 10mM DTT (dithiothreitol), pH 7.5) and was denatured by heating at 95°C for 10min, cooled to 4°C for 5 min and incubated at room temperature (RT) for 10min. The mixture filtered three times through nitrocellulose filter membrane (0.45µm) (Ref# HAWP01300, Merck Millipore Ltd, Ireland) hold by filter holder ("pop-top", Millipore, MA, USA) to get rid of non-specific bounders. 50µL (15µg/L) of the target protein was incubated with library-in-binding buffer with rotation for 1hr at RT. The library-protein mixture passed through another nitrocellulose filter membrane to trap the enriched aptamers. The filter then immersed in 200µl of pre-heated (60°C) elution buffer (0.4 M sodium acetate, 5mM EDTA, 7M urea; pH 5.5) for 5min. The eluted material was diluted with 200µl ddH₂O and the DNA was precipitated with ethanol precipitation method (Wang and Li, 2013).

4.2.4. Amplification and digestion of the DNA

After each enrichment cycle, the enriched ssDNA aptamers were amplified with 25 cycles of PCR amplification. The PCR was conducted using G2 Hot Start Green Master Mix (Cat# M7422, Promega). The reaction mix was 1µl (10µM) from the of the DNA library (enriched aptamers in

the later cycles) as the template, 1 μ l of each forward and phosphorylated reverse primers (4 μ M), 12.5 μ l of 2X reaction buffer (pH 8.5). The reaction buffer composed of 2.5U of the polymerase enzyme, 400 μ M of each dNTPs and 4mM MgCl₂. Nuclease-free water was added to adjust the final reaction volume to 25 μ l. The thermal cycles composed of 95°C for 5min as the denaturing step, followed by 25 thermal cycles composed of 95°C for 30sec, 56°C for 15sec and 72°C for 15sec. The final extension was at 72°C for 5min. The PCR products were monitored on 1% agarose gel stained with SYBR Safe dye (ThermoFisher Scientific, USA). The PCR-resulted double-stranded DNA (dsDNA) was subjected to the action of λ exonuclease enzyme (Cat# M0262S, NEB, USA) to retain the ssDNA for the next round. The reverse primer was order as phosphorylated to facilitate this task (Table 4.1). The digestion reaction conditions were as described by the manufacturer.

4.2.5. Correcting thermal cycles

After each amplification of the enriched aptamers, the PCR products were ethanol precipitated, purified and re-suspended initially in 100 μ L 1X TE buffer (v/v). In general, the final concentration of the DNA in the 1X TE buffer (w/v) was \sim 150 ng/ μ l. We aliquoted the diluted DNA into 50 μ l in PCR tubes (Cat# N80105580, ThermoFisher). Then, the purified DNA were subjected to six correcting thermal cycles using thermocycler (PTC-200, Peltier Thermal Cycler). The protocol involved raising the temperature to 65°C for 10 min, cool down to 20°C for 1min and this cycle was repeated for six times. After the sixth cycle, the DNA was monitored on 1% agarose gel (see Amplification and digestion of the double-stranded DNA).

4.2.6. Illumina sequencing

The procedure for Illumina sequencing was as previously described (Scoville *et al.*, 2017). The bioinformatics analysis was performed using the free online software “Galaxy Bioinformatics” (open access software) (Thiel and Giangrande, 2016; Kinghorn *et al.*, 2018). Briefly, samples from rounds 1, 6, 8 and 10 were prepared for Illumina sequencing platforms. The enriched DNA aptamers from each selected rounds were barcode-tagged and were used as a template for PCR amplification using primers with Illumina adapter sequences. The DNA aptamers were diluted in nuclease-free water to the concentration of 10 μ M. The Illumina adapter primers, which also include unique index sequences for different samples, were utilized to amplify the templates. Twenty-five PCR cycles were applied using Q5 hot start high-fidelity DNA polymerase and buffer (Cat # M0493S, NEB). The resulting PCR products were combined at ultimately the same amount and concentration (50.4 ng/ μ l). The mixed DNA samples were PAGE-purified using 4-20 % polyacrylamide gel (Life Technologies, NY, USA), and was sent for Genomics Core Facility at the University of California at Riverside, USA for Illumina sequencing using MiSeq with 151 cycles in single-end mode.

The results from the Illumina sequencing were analyzed using multiple commands in the Galaxy tool. The total number of the reads by the Illumina sequencing were about 16 millions. These reads were tracked according to their index sequences. The quality of the reads, the lengths of the reads and the conserved sequences were investigated as described previously (Thiel and Giangrande, 2016; Thiel, 2016). The total number of the reads by the Illumina sequencing were about ~16 million reads, however, about 5% of the total reads was neglected due to their improper length or quality. The rest of the reads were tracked according to their barcodes. After analyzing the results, the highly-yielded aptamers were tested for the free energy change (ΔG [kcal.mole⁻¹]) of the three

dimensional structure using the OligoAnalyzer 3.1 Tool available online by Integrated DNA Technologies (IDT) website, and the most reliable ones have been chosen to be tested by the next step.

4.3.RESULTS AND DISCUSSION

Standard SELEX procedure has a number of limitations that intervene the overall goal of this procedure of selecting specific aptamers with high affinity and specificity to the target (Lakhin *et al.*, 2013). The combination of randomized ssDNA library and SELEX procedure applies selection pressure resembles the natural selection of function-to-structure relation in RNA (Meyers *et al.*, 2004; Thiel *et al.*, 2011). Conventional PCR procedure is an additional player that causes bias when a random library is used as the template. The resulting issues such as incorrect hybridization events between primer-template, template-template, and mutation are common (Katilius *et al.*, 2007; Spill *et al.*, 2016). Therefore, when using random library as the template for PCR, the artifacts should increase logically. Eventually, the evolution of selection pressure favor the highly amplifying aptamer sequences over the highly specific aptamer in later SELEX cycles (Shao *et al.*, 2011; Takahashi *et al.*, 2016; Acinas, *et al.*, 2005; Yufa *et al.*, 2015; Witt *et al.*, 2017).

Many researchers require dealing with the formation of the PCR byproducts in order to reduce the development of non-specific aptamers (Blind *et al.*, 2015; Eaton *et al.*, 2015). In the process of aptamers selection, there are two common artifacts in the PCR products; these are ladder-like PCR products and smearing band (Figure 4.1). These PCR byproducts can appear as early as in the second selection round during aptamer selection and increase linearly (Figure 4.2).

PCR byproducts formed because of several undesirable events during the aptamers amplification by PCR. The random design of the variable region of the library reveals multiple potential sites other than the primer binding regions on the library where primers can anneal (Tolle *et al.*, 2014; Yufa *et al.*, 2015). Additionally, the partial annealing of two different DNA strands could form the heteroduplexes, i.e. two DNA strands act synergistically with the primer(s) to produce the amplicon (Musheev and Krylov, 2006; Shao *et al.*, 2011).

In order to investigate the template-template hybridization, we amplified the library with one primer only (forward or reverse) (Figure 4.3). With extension using one of the primers only, ladder-like byproduct or smear band were efficiently removed from the library. However, the length of the PCR products was not typical for the library length. This could be attributed to the fact that there are specific sequences act to hybridize to each other to act as a primer. Therefore, using one primer was enough to amplify these duplexes. With the presence of one primer, the complementary sequence act as the second primer and vice versa. Therein, the size of the PCR byproducts depends on the site of template-template hybridization more than the site of primer-template hybridization. We concluded that this byproduct could be <2X the library size at least at the first PCR cycle. When the cycles continue, the byproducts size increase gradually (Acinas *et al.*, 2005; Yufa *et al.*, 2015; Witt *et al.*, 2017).

To decrease the potential of heteroduplexes formation, we sought to apply a course of thermal treatment for the PCR products. The upper limit of the temperature was meant to be below the denaturing temperature of the fully-hybridized templates and above the annealing temperature of the primer-template. We tested several different temperatures (data not shown) and then we chose to use 65°C as the partial denaturing temperature. The rest of the thermal course is to give the chance for the homoduplexes to re-anneal at 20°C (Figure 4.4). Therefore, there is no denaturing

for the homoduplexes due to their very low ΔG value; instead, the heteroduplexes would be applied under the pressure to find their correct complementary sequences. After testing different number of thermal cycles, we found that six cycles of the thermal correction protocol gives the best results. Both the ladder-like and smearing band byproducts are sensitive to the correction with this method and only traces of either byproducts could be detected aftermath (Figure 4.4).

For the Illumina sequencing results, there are several stretches over the aptamers sequences where they were partially matching with the reverse primer sequence (Table 4.1D). This agrees with our prediction for the origin of the PCR byproducts and supports Tolle and co-workers (2014) results. In addition, there were extra-piece in the downstream where it, in most cases, expressing partial or full forward primer sequence, reverse primer sequence or both as an overlapping sequence (Table 4.1A, B, C and E).

The format of typical Illumina sequence results is shown in (Table 4.1 A). After ten round of aptamers selection, the common PCR byproducts are listed in Table 4.1 (B-E). Some aptamers showed incorrect sequence of the reverse primer (RP). This could be attributed to the polymerase enzyme proofreading capacity (McInerney *et al.*, 2014). Other aptamer sequences showed longer sequence than usual with the extra piece carrying partial or complete RP sequence or forward primer (FP) binding site sequence. This result matches with the conclusion of previous publication (Tolle *et al.*, 2014). In some instances, the long extra piece showing overlapped FP binding site and RP sequences.

There were short sequences that do not carry any primer sequence. Both short and long sequences were increasing linearly over rounds. However, PCR products that were treated with the correction protocol showed lower tendency to express these artifacts. In such PCR products, the short reads

proportion was with steep increase in the proportion in comparison to the total reads. This could be explained as the short reads are the results of the amplification of homoduplexes and heteroduplexes. Whereas, the long reads is the results of the heteroduplexes only (Table 4.2). Application the correction thermal cycle for the PCR products of the 10th round succeeded to shift this proportion toward the normal sequence size (Table 4.3). In our results, the smearing bands PCR byproducts were more sensitive to the thermal correction protocol than the ladder-like ones (Figure 4.4). This could be explained that the smearing bands are due to the formation of three-dimensional structure hairpin whereas, the ladder-like byproduct are due to formation of heteroduplexes. Unfortunately, we were facing the ladder-like byproducts in our experiment mostly.

4.4.CONCLUSIONS

The use of PCR is the only known reliable method to exponentially amplify the enriched aptamers in the SELEX method. The PCR, however, could decrease the efficiency of the SELEX method itself due to the presence of DNA artifacts or bias over the enriched aptamers (Tolle *et al.*, 2014; Thiel *et al.*, 2011). In fact, the presence of the artifacts in the aptamers might interfere with ligand-target interaction yield, which would result in low quality aptamers such as aptamers with no specificity to the target or aptamers with low affinity to the target. There are several methods are in use to decrease these artifacts in PCR products. PCR reconditioning is a common procedure to reduce the effects of these artifacts (Thiel, 2016).

In the current article, we described a simple, yet, effective protocol to reduce the well-known common PCR byproducts confronting the process of SELEX. Our method treat both ladder-like

and smearing band PCR byproducts at the same efficiency. Visually, the agarose gel images showed that the method was capable of effective correction of the ladder-like and the smearing band that had been detected previously on the PCR byproducts. Similarly, the Illumina sequencing results showed that there was marked decrease in proportion of the short and long sequences after the application of this protocol. The formation of these short or long PCR byproducts might be due to the presence of matched sequences within the random library sequence with that of forward primer binding site or reverse primer sequences. Additionally, the formation of heteroduplexes between two random sequences within the library, could act as a primer to form multiple-length PCR products. Finally, the ladder-like byproducts were more refractory to correction than the smearing bands byproducts.

4.5. TABLES

Table 4.1. The common artifacts in PCR products after the 10 rounds of aptamers selection by SELEX procedure. A) The common library design with barcode included. **B)** Reverse primer (RP) shows incorrect sequence. Many aptamer sequences showed longer sequence than usual with the extra piece carrying partial RP sequence. **C)** The extra piece of a long aptamers showing forward primer binding site (FPBS) sequence. **D)** The long extra piece showing overlapped FPBS and RP sequences. **E)** Normal aptamer length with partial RP sequence.

A			
Barcode	FPBS	Variable region	RP
CTTCGGGTATCCTCTT	TCCGAATTCGAAGGACAAGAG	N ₄₀	TCTTTTATGCTACGTCCCGC

B			
FPBS	Aptamer	RP*	Extra piece with RP [†] sequence
TCCGAATTCGAA GGACAAGAG	N ₄₀	TCTTTTTTTTCTTCCTCCCC	(N) ₃₀₋₄₀ TCTCTTTTTCCTTCTTCTT

*RP sequence with incorrect sequence
[†]Extra piece with partial RP sequence (bold font)

C			
FPBS	Aptamer	RP	Extra piece with FB* sequence
TCCGAATTCGA AGGACAAGAG	N ₄₀	TCTTTTATGCTACGTCCCGC	NNNNTCCGAATTCGAAGGACAAGA G (N) ₂₀₋₄₀

*The extra piece is with RP sequence (bold font)

D			
FPBS	Aptamer	RP	Extra piece with overlapped FB-RB* sequence
TCCGAATTCGA AGGACAAGAG	N ₄₀	TCTTTTATGCTACGTCC CGC	(N) ₃₀₋₄₀ TCTTTTATGCTACGTCCCGC

* The extra piece showing overlapped partial FPBS binding site (large font) and RP sequence (small and bold font)

E		
FPBS	Aptamer with partial RP sequence	RP
TCCGAATTCGAAGGACAAGAG	(N) ₂₀₋₂₅ TCTTTNNTGNTACNTCCCG	TCTTTTATGCTACGTCCCGC

Table 4.2. The proportion of the short (<80nt%) and long reads (>80nt%) in comparison to normal length sequences (80nt%). The total reads showed that there were increase in the short and long read percentage in the later rounds

Round	Total reads	<80nt%	80nt%	>80nt%
1	4, 441, 562	0.66	95.68	3.66
6	4, 379, 389	1	97	2
8	4, 087, 274	4.33	90.01	5.66
10	3, 777, 547	23.86	67.89	9.33

Table 4.3. The proportion of short (<80nt%) and long reads (>80nt%) in comparison to normal length sequences (80nt%) with and without the course of the thermal correction. Round 1 used as the reference round for all lengths of sequences.

Round	Total reads	<80nt%	80nt%	>80nt%
1	4, 441, 562	0.66	95.68	3.66
10 (not corrected)	3, 777, 547	23.86	67.89	8.25
10 (corrected)	4, 549, 398	0.88	95.56	3.56

4.6.FIGURES

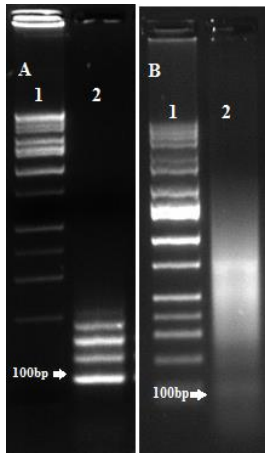


Figure 4.1. Patterns of PCR byproducts that occur during SELEX procedure. PCR byproducts appear in two different patterns on 1% agarose gel. (A) ladder-like pattern of PCR byproducts. (B) Smear band pattern PCR byproducts. According to the DNA library design, the correct size of the amplicon should be 80bp. These PCR products are from rounds 10 and 8 of the SELEX procedure, respectively.

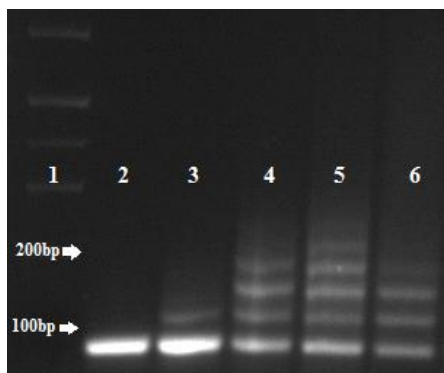


Figure 4.2. Evolution of the PCR byproducts over the SELEX rounds. Lanes 2 represent the DNA library, lanes 3, 4, 5 and 6 represent PCR products from rounds 1, 2, 3, and 4 respectively. PCR products were loaded on 1% agarose gel.

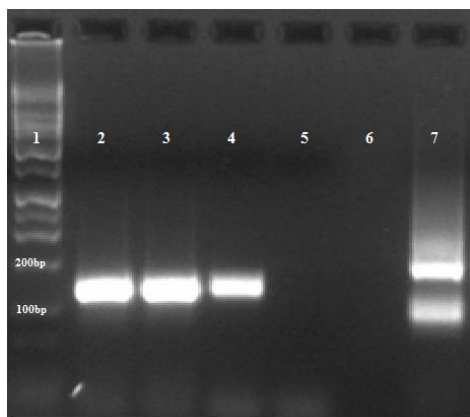


Figure 4.3. Amplification of aptamers pool with single primer. Aptamers from round 6 were used as a template for PCR. The process of amplification was carried out using forward primer alone (lane 2, 45 thermal cycles), reverse primer alone (lane 3, 45 thermal cycles), forward primer alone (lane 4, 30 thermal cycles), or forward and reverse primers together (lane 7, 45 thermal cycles). Lanes 5 and 6 represent negative controls with no template or no primer, respectively.

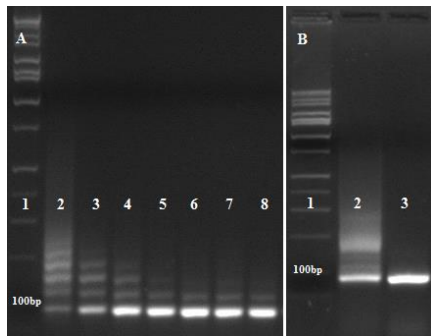


Figure 4.4. Application of thermal cycles to correct PCR byproducts. (A): PCR byproduct from the 10th enrichment cycle of SELEX procedure. The PCR byproducts appear as a ladder-like smear pattern (lane 2). The correction protocol included application of 65°C for 10min, followed by 20°C for 1min for 1, 2, 3, 4, 5 and 6 cycles (lanes 3-8, respectively). (B): correction of smearing band PCR byproducts by applying 6 cycles of the thermal protocol (lane 2 before and lane 3 after the correction).

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CHAPTER V

5.1. CONCLUSIONS AND SIGNIFICANCE

Restrictions and strict scrutiny on antimicrobial use with absence of effective antiviral remedies in commercial poultry expose the vaccines as the main tool to confront the infectious agents (Kennedy and Read, 2017; Kennedy and Read, 2018). Traditional vaccines act to stimulate less effective immune responses, mainly, due to low specificity or/and low immune enhancing capacities of their accompanying adjuvants. Assembling an efficient vaccine with potent adjuvants is demanding due to emerging of low-immunogenic and more sophisticated vaccines such as subunit vaccines (Nobel, 2013).

The studies presented in this dissertation aimed to determine the ability of DNA aptamers-based RCA products to act as an immunological adjuvant in broiler chickens. In Chapter II, I describes the development of agonistic DNA aptamers to chicken CD40 receptor. We hypothesized that ligation of CD40 receptor on macrophages with DNA aptamers would promote activation of these cells. First, we prepared the candidate aptamers in form of RCA products to combine the specificity of our aptamers with the higher affinity and large spanning are offer by the RCA products. These aptamers were capable to induce stimulation of chicken macrophage HD11 cell line characterized by dose-dependent nitric oxide liberation. In addition, I have shown the actual sites where these aptamers interact with the target protein, herein, specified the active sites on the receptor.

Several attempts have achieved to develop CD40 agonistic adjuvants, mainly, by developing monoclonal antibodies in mammalian models (Carlring *et al.*, 2004; Smyth and Kershaw, 2011; Thompson *et al.*, 2015). Recent publications from our lab showed that anti-CD40 monoclonal antibody-based adjuvant could guide robust immune response when loaded with hapten peptide

(Chen *et al.*, 2010; Chen *et al.*, 2012; Chou *et al.*, 2016). Traditionally, adaptive immune response ignition requires the interaction of TCR on the resting lymphocytes with peptide/MHC class II molecule on APCs as the first signal. Once the first signal sparked, activation of both the T lymphocyte and the APC with concurrence upregulation of costimulatory molecules and cytokines accompanies. Follows, the second signal comes from costimulatory molecules, such as CD40, CD80, on the APCs with cognate ligands on activated T lymphocytes. Costimulatory molecules-ligands interaction optimizes the T lymphocytes and APCs activation. The third signal comes from the cytokines that have releases by the two types of the activated immunological cells.

When B cells present the foreign antigen to the T cell, activated B cells trigger the differentiation of a subset of T_H cells called follicular helper T cells (T_{FH}) in the lymphoid follicles. The T_{FH}, in turn, mediate B cell differentiation and proliferation. CD40-CD40L interaction plays crucial role in this two-way activation pathway and formation of germinal centers. Commonly, in the dark zone within the germinal centers of lymphoid follicles, B cells proliferate, differentiate and expand within the light zone of the germinal centers. This is accompanied by emerging of long-living memory B cell and antibody-producing plasma cells with antibody class switching and antibody affinity maturation (Crotty, 2012; Mesin *et al.*, 2016; Vaccari and Franchini, 2018). However, CD40 receptor interaction pathways within these immune cells is only discussed briefly in mammalian species, we only predict the same effects in avian species.

The study discussed in Chapter III presents the use of the DNA aptamers specifically selected against the chicken CD40 receptor *in vitro* to act as adjuvant when engaged with low immunogenic peptide hapten. Encouraged by the results from the first experiment (Chapter II), we proceeded to develop an immune complex composed of the enriched aptamers (in form of RCA products) in conjugation with peptide hapten. These aptamers should be replacing the agonistic anti-CD40

MAB that our lab group developed recently. To determine the ability of our DNA aptamers to work as an adjuvant, we created an immune complex composed of RCA-streptavidin-peptide. We sought to use M2e peptide in our immune complex. M2e is a conserved peptide among influenza viruses and it is known to induce no or low immune response by its own.

Our immune complex showed ability to stimulate the production of anti-M2e IgG in chicken sera as early as 7 days post immunization. This antibody titer remained high until the age of 35 days 28 days post immunization. However, the lower dose of the same immune complex needed a boost to sustain a detectable anti-M2e IgG titer. In essence, the immune complex could have been created easier if we selected DNA aptamers against biotin to replace the streptavidin. However, the streptavidin is high molecular weight protein that creates immune response by its own and may interfere with final immune response (Meyer *et al.*, 2000; Yumura *et al.*, 2012).

Chapter IV presents an effective method for correction the PCR byproducts that confront the use of SELEX procedure as the main tool to develop DNA aptamers. When multi-sequence template used to develop PCR, as in the use of DNA library to select aptamers, heteroduplexes formed considerably. The formation of these heteroduplexes is due to partial matching and hybridization among different sequencing within the library pool or due to incorrect primer-template hybridization site. For either cause, the high variability of the DNA library promotes these undesirable hybridization. Anyway, the PCR byproducts may take ladder-like shape or smearing bands. These byproducts affect the yield and quality of the enriched aptamers. In this chapter, I described a simple and effective thermal correction course that was applied to reduce the heteroduplexes formation in the PCR products. The thermal correction course composed of six cycles of elevation the temperature of the purified PCR products to 65°C for 10min and then cool

them down to 20°C for 1min, with no need for enzymatic treatment. The results confirmed high quality DNA yield of this method in all samples.

Taken together, our results suggest that our DNA aptamers are qualified candidate for immunological adjuvant in chickens. These aptamers could be considered signal-2 enhancers because they facilitate an acquired immune response at the level of costimulatory molecules. Using low immunogenic DNA aptamers as adjuvants could mount better immune response, increase the safety of vaccines and offer better mate for subunit vaccines. In addition, using DNA aptamers-based adjuvant would simultaneously lower the cost of many modern vaccines.

The significance of this dissertation, as we proved, that ligation of CD40 receptor on APCs promotes induction of acquired immune response in chickens. At least, we showed this in macrophages and B-lymphocytes.

As mentioned earlier, tumor-associated macrophages (TAMs) promote tumor cells proliferation and metastasis by enhancing angiogenesis within the tumor mass and by inhibiting the T-cell dependent anti-tumor immunity. The ligation of CD40 receptor on TAMs would revert their tumor-enhancing activity and suppressive effect on immune response in mammals. Several chemokines and cytokines are responsible for attraction of monocytes to tumor microenvironment and polarizing these monocytes into TAMs such as macrophages colony-stimulating factor and IL-4 and IL-10. However, the mechanisms by which TAMs enhance tumor metastasis are poorly understood (Wang and Joyce. 2010; Liu and Cao, 2015; Hoves *et al.*, 2018).

In chicken, we know that macrophages play crucial role in the pathogenesis of lymphoid leucosis and Marek's disease infections. In lymphoid leucosis, medullary macrophages appear to enhance the virus replication and transmission to the lymphoid cells in the bursa of Fabricius. In addition,

macrophages suppress immune response by suppressing the normal spleen cells response in Marek's disease (Lee *et al.*, 1978; Gilka and Spencer, 1987; Barrow *et al.*, 2003).

Apart from using our aptamers as immunological adjuvant, these aptamers would provide spectacular input for dealing with oncoviruses infections in chicken such as lymphoid leukosis and Marek's disease. These aptamers could polarize macrophages, herein; revert their suppressive effect during the pathogenesis of these diseases (Wang *et al.*, 2018; Chakraborty *et al.*, 2019).

The knowledge we have that the ligation of the CD40 receptor on the tumor-associated macrophages reverts their tumor enhancing activity and render them active antitumor cells. Therefore, the aptamer-RCA coupling might have an impact on mammalian cancer research as well.

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APPENDIX



Office of Research Compliance

To: Billy Hargis
Fr: Craig Coon
Date: June 5th, 2018
Subject: IACUC Approval
Expiration Date: May 31st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 18127: *Evaluation of novel adjuvant composed of ssDNA aptamers-based RCA products targeting chicken CD40 receptor and conjugated with M2e-based peptide in broiler chicken.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the modification form) prior to initiating the changes. If the study period is expected to extend beyond May 31st, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Adil Al-Ogaili, Christine Vuong, and Callie McCreery. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciated your cooperation in complying with the University and Federal guidelines involving animal subjects.

CNC/tmp