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A novel atheroprotective role of MF59-like adjuvant when co-administered with CETP vaccine in rabbit model of atherosclerosis

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ABSTRACT

Objective(s): In this study, for the first time, MF59 adjuvant was used to develop a cholesteryl ester transfer protein (CETP) vaccine. The efficacy of the vaccine was compared with the efficacy of CETP vaccine formulated with Alum/CpG, the formulation that its immunogenicity has been already demonstrated in rabbit and mice.

Materials and Methods: Tetanus toxoid- CETP peptide (TT-CETP) was mixed with Alum/CpG or MF59-like and administered subcutaneously for total five times in rabbit model of atherosclerosis. Anti-TT-CETP specific antibody, CETP activity in sera and mRNA level of cytokine IL-4 and IFN-γ in peripheral mononuclear cells were determined. Therapeutic response was also examined by tracking serum lipoprotein levels and pathologic observation of atherosclerotic lesions at aortic site.

Results: More anti-TT-CETP antibody was found in Alum/CpG vaccinated rabbits compared to buffer (P<0.001). Antibody induced by MF59-like formulation was not significantly higher than buffer. CETP activity and lipoprotein levels were not significantly different between vaccinated and control rabbits. The mRNA level of IL-4 was significantly lower than buffer while, IFN-γ gene expression was significantly higher in both vaccinated groups. Atherosclerosis thickness grade of aorta was dramatically lower than buffer (P<0.01) in both vaccinated groups.

Conclusion: It is concluded that MF59-adjuvanted CETP vaccine showed anti-atherosclerosis properties, but the protective effect could not be directly attributed to the immune response induced by anti TT-CETP antibody and CETP inhibition. Further studies are needed to explain the anti-atherosclerosis properties of MF59 in the presence of TT-CETP peptide.

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Introduction

The inverse relationship between the concentration of high density lipoprotein-cholesterol (HDL-C) and the risk of cardiovascular disease is well demonstrated (1). Inhibition of cholesterol ester transfer protein (CETP), based on some epidemiologic evidences and animal studies, provides a promising strategy for increasing HDL-C (2-4). CETP transfers cholesteryl ester (CE) from HDL into low density lipoprotein (LDL) and very low density lipoprotein (VLDL). The function of CETP in plasma leads to lower levels of HDL-C and increase the levels of LDL-C and VLDL-C (5). Several classes of CETP inhibitors have been described before (6) and some of them (Anacetrapib and Evacetrapib) are in advanced clinical trials (7-9). CETP vaccine works based on inducing antibodies which bound and neutralize CETP activity in plasma (10). Clinical trial of tetanus toxoid-CETP (TT-CETP) vaccine, termed Ti-CETP, was stopped in phase *II* due to the lack of immunogenicity (11). TT-CETP is a synthetic peptide containing C-terminal 16 amino acid of human CETP linked to 14 amino acid sequence of TT. CETP

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peptide acts as a B cell epitope for generating antibody response and TT peptide acts as T helper cell epitope. TT sequence has been introduced in vaccine to overcome immune tolerance against self-antigen of CETP (10). Strategies aims to increase the immunogenicity of CETP vaccine are under investigating now.

MF59™ is a biodegradable and biocompatible adjuvant that was approved for human use in 1997. The MF59™ is an oil-in-water emulsion containing the naturally occurring squalene oil (12). Clinical trials on immunogenicity and safety of various MF59-adjuvanted vaccines have shown that MF59 induces a strong antibody response against co-administered antigen (13). These properties make the MF59 probably an appropriate adjuvant for inducing antibody against CETP vaccine.

In the present study, for the first time, we examined the effectiveness of CETP vaccine formulated with MF59-like, which has the same formulation as MF59™, in preventing the atherosclerosis lesion formation in rabbit model of atherosclerosis. Moreover, based on the previous study reporting Alum/CpG7909 as a potent adjuvant used for increasing the immunogenicity of TT-CETP vaccine (14), we compared therapeutic effects of MF59-adjuvanted CETP vaccine with the vaccine formulated with Alum/CpG.

Materials and Methods

Materials

New Zealand white rabbits were obtained from Pastour Institute of Tehran (Tehran, Iran). TT-CETP Peptide was ordered from Peptrone (Peptrone, South Korea) which was demonstrated by the company to be >95% pure with high-performance liquid chromatography (HPLC) analysis. The amino acid sequence of peptide was as follows:
CQYIKANSKFIGITE-FGFPEHLLVDFLQSLS-amide.
CpG7909 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') was ordered from Microsynth (Microsynth, Switzerland). All other materials were provided from other commercial sources with highest purity available.

Preparation of MF59-like nanoparticles

MF59 is an emulsion consisting of 4.7% v/v squalene, 0.5% v/v Tween 80 and 0.5% v/v Span 85 in citrate buffer (10 mM, pH 6.5). MF59-like was prepared by homogenization at 20,000 psi with a high pressure homogenizer (Emolsiflex-C3, Avestin, Canada) as described elsewhere (15). The emulsion was sterilized by passage through a polysulfone filter (0.22 µm pore size; Millipore, USA) and stored at 4 °C. The mean particle size, polydispersity index (PdI) and surface charge of the emulsion droplets were determined with a Dynamic Light Scattering instrument (Nano-ZS; Malvern, UK). MF59-like was mixed with an equal volume of antigen solution prior to injection (16).

Animals

Animals were kept individually in animal house, under 12/12 hr light/dark cycle at a temperature controlled (20-24 °C) with free access to food (standard laboratory diet, Javaneh Khorasan Co, Mashhad, Iran) and water. All animal experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts.

Vaccination and atherosclerosis model

Male rabbits were divided into three groups (n= 6-8) with the same weight and serum lipoprotein level. Animals were vaccinated subcutaneously (SC) in a volume of 100 µl, by 50 µg TT-CETP peptide mixed with MF59-like or Alum/CpG. To prepare formulations, TT-CETP peptide (1 mg/ml) was dissolved in sterile 10 mM histidine/10% sucrose buffered (pH 6.5), with or without CpG ODN7909 (200 µg/ml), and mixed (1:1 v/v) with MF59-like or alumminum hydroxid 2% (15). negative control group was only inoculated with histidine buffer. Rabbits were boosted SC 4 times at 3 weeks intervals with the same formulations. From week 11, animals were placed on a diet supplemented with 1% cholesterol and maintained on high cholesterol diet for 8 weeks. Blood samples were collected from fasted rabbits at weeks 0, 8, 11, 19 and centrifuged (1500 g, 15 min, 4 °C) to separate serum. Sera were kept frozen at -20 °C until being used.

Anti-TT-CETP antibody analysis

Anti-TT-CETP was determined by ELISA as described before (14). Endpoint antibody titers were determined based on the highest dilution of serum sample that gives twice times the mean absorbance obtained from control (17). Dot blot assay were also used for detection of anti-TT-CETP in studied group as the same manner as ELISA with some differences (18). Antigen spots on nitrocellulose were detected using G:BOX chemiluminescence imager system (Syngene,UK) with chemiluminescence substrate (Thermo Scientific, USA).

CETP activity assay

CETP activity in sera of rabbits (RFU (relative fluorescence unit /µl serum/hr) was measured with commercially available fluorimetric kit (Abcam, USA) according to manufacturer's instructions.

Lipoprotein analysis

Total cholesterol (total-C), LDL-C, HDL-C and VLDL-C were measured using commercial biochemical test kits with the enzymatic method (Biosystems, Spain). Atherogenic index was calculated with equations i.e. LDL/HDL. Non HDL-C was calculated from lipid profile (Total-C minus HDL-C).

Table 1. Primers used for RT-PCR amplification of rabbit cytokines

Cytokine	Sense primer 5'–3'	Anti-sense primer 5'–3'	Amplicon size (bp)
IL-4	GTCACTCTGCTCTGCCTCCT	GCAGAGGTTCCTGTCGAGTCC	302
IFN-γ	TTCCCAAGGATAGCAGTGGT	TGAAGCCAGAAGTCCTCAAAA	160
GAPDH	GAATCCACTGGCGTCTTCAC	CGTTGCTGACAATCTTGAGAGA	160

Quantitation of cytokine transcripts of IL-4 and IFN- γ by real-time RT- PCR

Cytokine IL-4 and IFN-γ mRNA levels were quantitated in blood samples collected at week 11.Total RNAs were extracted from rabbit peripheral mononuclear cells using RNAlater-conserved blood and a RiboPure™ Blood Kit (Invitrogen, USA). RNAs were quantified using a Nanodrop-2000 spectrophotometer (Thermo Scientific, USA) and the purity was assessed by determining the A_{260}/A_{280}. Real-time RT-PCR was performed using a one-step Quantitect SYBER Green RT-PCR Kit (Qiagen, USA).

The primer sequences of rabbit cytokines and housekeeping gene were listed in Table 1. Quantitative RT-PCR was performed using the Step one Thermal Cycler (Applied Biosystems, USA) for 40 cycles. Reverse transcription was performed at 50 ºC for 30 min followed by PCR initial activation step at 95 ºC for 15 min. Quantitative PCR was performed at 94 ºC for 15 sec, primer-specific annealing temperature for 30 sec, and 72 ˚C for 30 sec. A melting curve analysis was performed after the amplification phase to eliminate the possibility of nonspecific amplification or primer-dimer formation. Fold changes in cytokine gene expression, relative to the control, were analyzed by the comparative ΔΔCT method (19).

Atherosclerotic lesion analysis

At week19, rabbits were euthanized by overdose of anesthesia according to the guidelines (20). Aortic arch was harvested and fixed in 10% formalin. The aorta was embedded in the optimum cut temperature medium and sectioned. Sections were stained with hematoxylin and eosin (H&E). The extent of atherosclerotic lesions was analyzed by histological determination of the intima to medial thickness assessed by histopathologist in a blinded manner using a microscope (Olympus, Japan) equipped with a 10 Achroplan objective. The atherosclerotic thickness grade was assessed based on scale 1-4; *Grade 1)* Plaque less than half as thick as the media with some form of endothelial dysfunction; increases in permeability to plasma constituents including lipids; evidence of adherence of blood components (macrophages and platelets) to endothelium; macrophages and isolated foam cells inside the endothelium. *Grade 2)* Plaque at least half as thick as media with accumulation of intracellular lipid, macrophages, and smooth muscle cells. *Grade 3)* Plaque as thick as the media with an abundance of macrophages, smooth muscle cells, and connective tissue (indicating proliferation and synthesis of extracellular matrix components by smooth muscle cells, leading to the accumulation of collagen and proteoglycans). *Grade 4)* Plaque thicker than the media with a large extracellular intimal lipid core and inflammatory cell infiltration, including macrophages, foam cells, and calcification in the lipid core (21). Calcification at aortic cite identified by expert pathologist using histochemistry and light microscopy (22, 23).

Statistical analysis

Statistical analyses were performed using SPSS software, version 16. Differences between groups of anti-TT-CETP antibody, serum CETP activity and lipoprotein levels were analyzed by a one-way analysis of variance (ANOVA) with a Tukey-Kramer post-hoc test. Atherosclerosis thickness grades were analyzed using Kruskal-Wallis test. A value of $P<0.05$ was considered significant.

Results

Physicochemical characterization of MF59-like

Particle size, polydispersity index (PdI) and zeta potential of vaccine formulations are shown in Table 2.

Anti-TT-CETP antibody

Anti-TT-CETP (week 0, 8, 11) was determined in sera using ELISA method. Figure 1, A depicts anti TT-CETP specific antibody induced in immunized rabbits. Alum/CpG vaccine generated significantly more anti- TT-CETP specific antibody rather than the control ($P<0.001$). Antibody induced by Alum/CpG was also significantly higher than MF59-like ($P<0.05$). Induced antibody was not significantly different between animal received MF59-like and buffer (Figure 1A). Figure1B shows dot blot assay of studied group.

CETP activity

CETP activity was measured in serum (week 0, 8, 11). As showed in Figure 2, no significant difference was found in enzyme activity of MF59-like vaccinated and control rabbits. Although Alum/CpG induced high antibody in vaccinated rabbits but, the activity of CETP was not different when compared to the buffer.

Table 2. Physicochemical properties of vaccine formulations. Values represent mean±SD (n=5)

	Z-average Size (nm)	PdI[a]	Zeta Potential (mv)
MF59-like	185.6±8.48	0.127±0.003	-23.43±2
Alum/CpG	1219.6±366	0.316±0.29	15.8±2.17

a: Poly dispersity Index

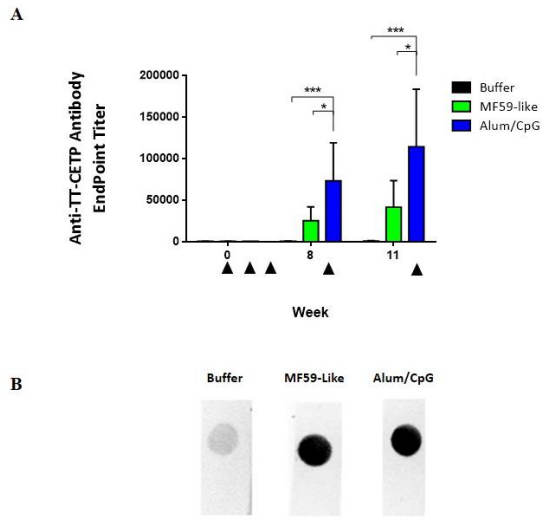

Figure 1. A) Anti-TT-CETP antibody. Antibody against TT-CETP in vaccinated and control rabbits (n=6-8) were analyzed by ELISA. Values represent mean±SD. Arrows indicate the time vaccines were administered. * P<0.05, *** P<0.001. B) Dot blot analysis. Anti-TT-CETP antibody in studied groups was analyzed with dot blot assay at week 11

Lipoprotein analysis

The level of total-C, LDL-C and HDL-C and VLDL were determined in the sera of fasted rabbit at week 0, 8, 11 and 19. When rabbits were placed on high cholesterol diet, all lipoprotein levels increased in vaccinated and control rabbits, but no significant difference in lipoprotein profile were found between MF59-like and Alum/CpG vaccinated rabbits with buffer. There was also no significant difference between animals vaccinated with Alum/CpG and MF59-like (Figure3).

Cytokine transcripts quantitation

Cytokine IL-4 and IFN-γ gene expression in peripheral mononuclear cells were measured by RT-PCR at week 11. IL-4 mRNA level was significantly lower

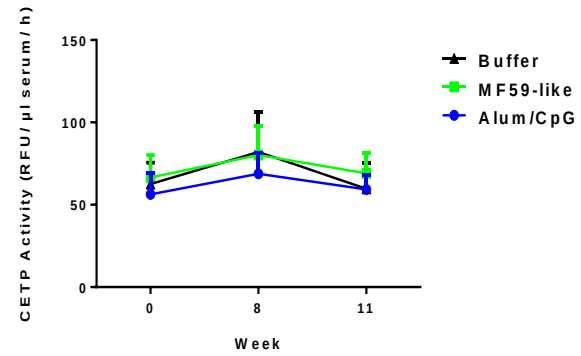

Figure 2. CETP activity. CETP activity in serum sample of vaccinated and control rabbits (n=6-8) were determined with a commercial kit. Values represent mean+SD

than negative control (P<0.0001) in both vaccinated groups. While the level of IFN-γ gene expression was significantly higher than negative control (P<0.01 and P<0.001 for MF59-like and Alum/CpG, respectively). Rabbits vaccinated with Alum/CpG and MF59-like CETP vaccine expressed 1.35 and 1.2 fold more IFN- γ than buffer, respectively. The level of IFN-γ in Alum/CpG vaccinated rabbits was higher than MF59-like (P<0.05). IFN- γ / IL-4 ratio was also higher in both vaccinated group rather than buffer (P<0.0001). These results suggested that immune response conferred by TT-CETP vaccine formulated with MF59-like and Alum/CpG can be associated with increase in IFN-γ secreting by Th1 cells. Figure 4 shows fold change in IFN-γ and IL-4 mRNA level in the studied groups.

Aorta lesions analysis

Atherosclerotic lesions were reduced in vaccinated rabbits compared to the control (P<0.01). There was no significant difference between animals vaccinated with Alum/CpG and MF59-like. Figure 5, 6 show atherosclerosis thickness grade and aortic section images in vaccinated and control rabbits, respectively.

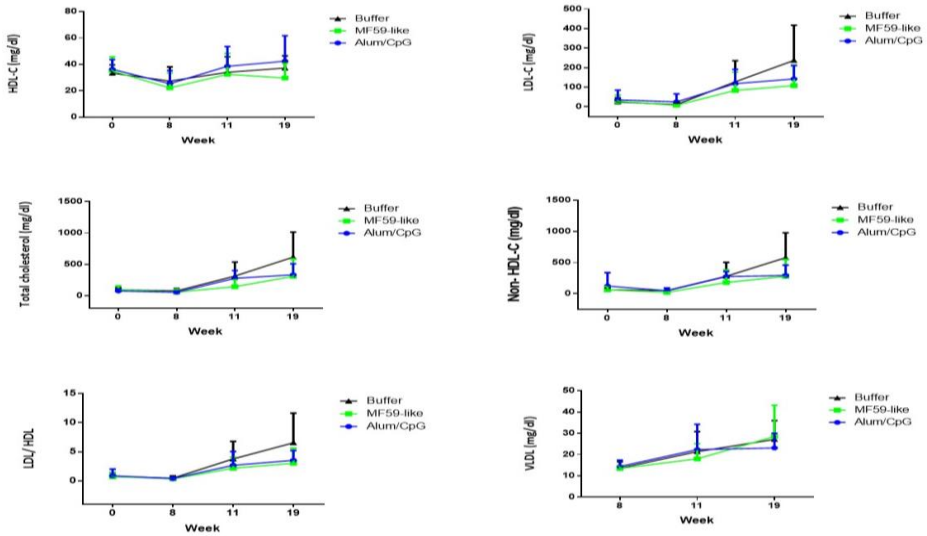

Figure 3. Serum lipoprotein levels in vaccinated and control rabbits. Lipoprotein levels (n=6-8) were measured with commercial biochemical test kits. Values represent as average concentration (mg/dl) with SD

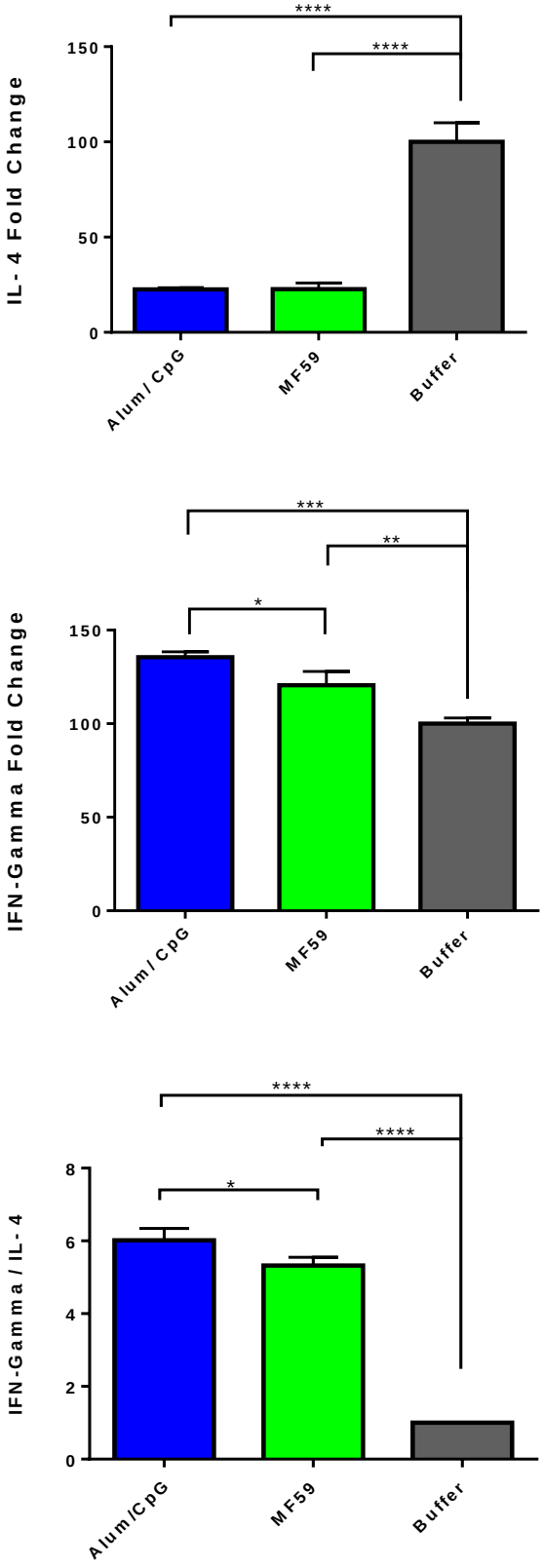

Figure 4. Cytokine IL-4 and IFN-γ mRNA expression levels. The levels of IL-4 and IFN-γ mRNA in peripheral blood mononuclear cells of vaccinated and control rabbits were determined by real time RT-PCR at week 11. All values represent means±SD (n=4)

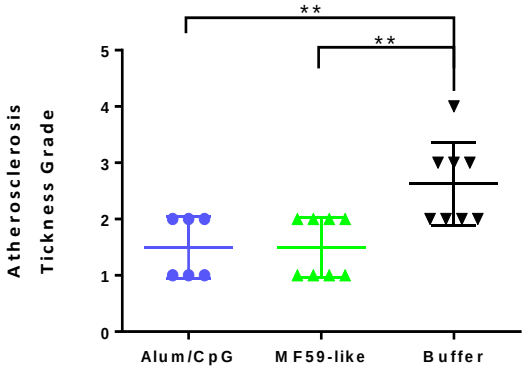

Figure 5. Analysis of aortic lesions. At the end of study, aortic arch (n=6-8) was removed, sectioned and stained with H&E Atherosclerosis thickness grade in each rabbit were classified into scale 1-4. Graph represents mean±SD, ** $P< 0.01$

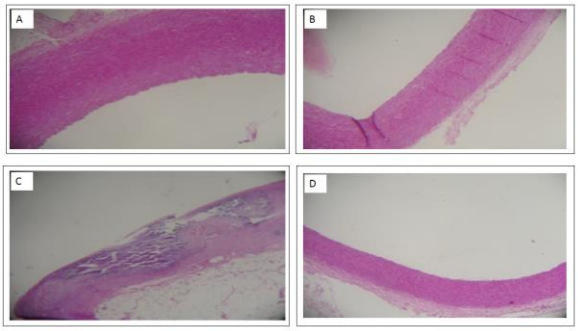

Figure 6. Aortic arch cross-section images in studied groups. After 8 weeks of feeding rabbits by high cholesterol diet, atherosclerotic lesions at aortic arch were classified into 1-4 grades. A) Aortic section from a rabbit vaccinated with CETP vaccine formulated with Alum/CpG. B) Aortic section from a rabbit vaccinated with CETP vaccine formulated with MF59-like. C) Aortic section from negative control (buffer) rabbit with a calcified plaque. D) Aortic section from regions with no evidence of atherosclerosis

Discussion

Several CETP vaccine formulations containing different kind of antigens, carrier molecules and adjuvants have been evaluated in animal models (24-30). *Liaw et al* Used linear array of 6 repeats of a CETP epitope linked to rabbit IgG Fc domain, as a carrier (28). Other investigators used other vaccine formulations containing heat shock protein 65 (Hsp-65) of *Mycobacterium tuberculosis* fused with the linear polypeptide epitope of CETP (24, 25) and asparagianse linked to TT and CETP epitope with alum adjuvant (26, 27). They showed that these vaccines efficiently elicited antibodies against CETP and reduced susceptibility to atherosclerosis. Intranasal immunization with chitosan/CETP nano-particles (30) and intramuscular immunization with DNA vaccine containing CETP epitope and CpG ODN (29) also inhibited atherosclerosis in a rabbit model

of atherosclerosis. The clinical trial of TT-CETP vaccine, named Ti-CETP, showed that the vaccine needs to make more immunogenic in order to be more effective (31). In the present study, we used MF59 to enhance the immune responses against CETP vaccine. The results of our study indicated that the vaccine has anti-atherosclerosis properties at the site of the aortic lesions. However, the atheroprotective properties could not be attributed directly to the CETP inhibition. This study is the first report of using MF59 as an adjuvant with CETP vaccine.

MF59™ is an adjuvant used in influenza vaccine (FLUAD™) since 1997 (32). Epidemiologic studies have concluded that viral illnesses such as influenza can lead or exacerbate cardiovascular disease (33) and the effectiveness of influenza vaccine in preventing cardiovascular events has been reported in some studies (34-37). It also has been reported that Influenza vaccine used MF59 as an adjuvant, shown more protective results rather than non MF59-adjuvanted vaccine. (38-40). The exact underlying mechanism explaining the relationship between MF59-influenza vaccine and the protection against cardiovascular disease is not clear yet. In all of these studies, the protecting effects of the MF59 containing influenza vaccine has been attributed to the enhanced immune response induced by adding adjuvant and the consequent reduction of cardiovascular morbidity and mortality caused by the influenza virus infection. Our results demonstrated that MF59 in the presence of TT-CETP also showed anti-atherosclerosis effects in rabbit. However, the hypothetical mechanism, which was based on the CETP inhibition, was not met in our study. Based on our knowledge, the effect of MF59-CETP vaccine on the prevention of atherosclerosis lesions has not been reported before. The following evidences may be related to anti-atherosclerosis properties found with MF59-CETP.

Moreover, since atherosclerosis is recognized as an inflammatory disease (41), the adjuvants induce regulatory T-cell (T_{reg}), may show some atheroprotective effects in this disorder. T_{reg} inducing properties of some adjuvants including alum, polylactic-co-glycolic-acid (PLGA) and incomplete Freund's adjuvant (IFA) have been reported recently (42-45). We suggest that MF59, which is chemically similar to IFA, may also induce T_{reg} immune response.

We also evaluated therapeutic effects of Alum/CpG-adjuvanted TT-CETP vaccine, which its immunogenicity in rabbits and mice has been already demonstrated in a previous study (14). Adding Toll-like receptor 9 (TLR9) agonist CpG7909 to alum resulted in more stronger anti-CETP antibody response than the CETP/Alum vaccine (14). Consistent with this study, our results showed that Alum/CpG-CETP vaccine elicited strong antibody response which was significantly higher than antibody induced by MF59-CETP vaccine. Alum/CpG-CETP vaccine also displayed atheroprotective effects while it has been shown that this effect could not be associated with CETP inhibition.

It has been shown that aluminum hydroxide adjuvant (alum), has anti-atherogenic properties (46). In consistent with our finding, a study has reported that alum dramatically decreased atherosclerotic lesion size in Apo E knockout mice (46). The mechanisms by which alum activates immune suppression are not fully characterized. However, activation of natural immune suppressive T_{regs} and down regulation of CD4 cells activation markers may be associated (47). A mRNA levels of two cytokines IL-4 and IFN-γ were quantified to correlate cytokine profile of the rabbits with the protection results. Our results showed that vaccinated animals developed detectable levels of cytokines, with significant predominance of IFN-γ over IL-4 (Figure 3). IFN- γ is involved in activating cellular immune response (19). These findings indicate that both Alum/CpG and MF59 could induce cellular immune response in the presence of TT-CETP. For the formulation containing CpG, the immune mechanism could be related to the stimulation of TLR9. It was reported that mixed Th1/Th2 responses can be promoted by MF59 (48) while, in the present study we found strong Th1-polarization properties by MF59-like when co-administered with TT-CETP (Figure 3).

Conclusion

Both CETP vaccine formulated with MF59 and Alum/CpG dramatically reduced atherosclerosis lesions in vaccine treated rabbits. However, surprisingly, we found that this clinical benefit cannot be attributed to the CETP inhibition. Based on our knowledge, it is the first report of the beneficial effects of MF59-CETP vaccine in preventing atherosclerosis lesions in rabbits. Our results suggest that MF59 and Alum/CpG may have a potential for using in vaccines being developed for atherosclerosis. The objective of this study was not to elucidate the mechanism of atheroprotective effects of these two formulations, except the potential for CETP inhibition. So, more studies are needed to clarify the novel role of MF59 in preventing cardiovascular disease.

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