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Immunogenicity of DNA- and recombinant protein-based Alzheimer Disease epitope vaccines

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Alzheimer disease (AD) process involves the accumulation of amyloid plaques and tau tangles in the brain, nevertheless the attempts at targeting the main culprits, neurotoxic β-amyloid (Aβ) peptides, have thus far proven unsuccessful for improving cognitive function. Important lessons about anti-Aβ immunotherapeutic strategies were learned from the first active vaccination clinical trials. AD progression could be safely prevented or delayed if the vaccine (1) induces high titers of antibodies specific to toxic forms of Aβ; (2) does not activate the harmful autoreactive T cells that may induce inflammation; (3) is initiated before or at least at the early stages of the accumulation of toxic forms of Aβ. Data from the recent passive vaccination trials with bapineuzumab and solanezumab also indicated that anti-Aβ immunotherapy might be effective in reduction of the AD pathology and even improvement of cognitive and/or functional performance in patients when administered early in the course of the disease. For the prevention of AD the active immunization strategy may be more desirable than passive immunotherapy protocol and it can offer the potential for sustainable clinical and commercial advantages. Here we discuss the active vaccine approaches, which are still in preclinical development and vaccines that are already in clinical trials.

Keywords: Alzheimer disease, DNA vaccine, protein vaccine, liposome-based vaccination, adjuvants, electroporation, immune responses

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Introduction

According to World Health Organization, there were an estimated 35.6 million people with dementia worldwide, and this number is projected to nearly double every 20 y. AD is the most common cause of dementia and may contribute to 60–70% of all dementia cases.[1] It is characterized clinically by an insidious onset and progressive cognitive decline that impacts memory, language, judgment, and orientation to time and space, eventually resulting in death, usually within 10 y of diagnosis. The neuropathological features of the disease include extracellular plaques composed primarily of Aβ, and intracellular neurofibrillary tangles composed mainly of a cytoskeletal protein, tau.[2-5] These pathological changes result in a profound loss of neuronal synapses over the course of the disease, thereby contributing to a progressive reduction in the functional capacity of the patients. Aβ abnormalities precede and accelerate tau pathology, therefore, the first immunotherapy strategy was aimed at eliminating Aβ peptide from the brain of AD patients. However, it is well known that the clinical trials with the first-in-human anti-Aβ vaccine (e.g., AN1792 which is a fibrillar Aβ formulated in QS21 adjuvant with or without polysorbate B) has been halted due to induction of meningoencephalitis in the small subset of vaccinated AD patients.[6] To eliminate the harmful effect of autoreactive Th cells and have

Table 1. Ongoing clinical trials for Alzheimer disease

Company	Vaccine	Composition	Adjuvant	Trial phase	References
Novartis	CAD106	Aβ1–6 on Qβ VLP	VLP; VLP/Alum; VLP/MF59	Phase III	32
AFFiRiS AG	AD02/AD03	Aβ1–6 Mimatope + carrier	Alum	Phase II	33
Pfizer/J&J	ACC-001	Aβ1–6 + carrier CRM197	QS-21	Phase II	10, 23, 24
Merck	V950	N-terminal region from $Aβ_{1–42}$	ISCOMATRIX; ISCOMATRIX+Alum	Phase II	10, 23, 24
United Biomedical	UB-311	Aβ1–14 + T cell epitope from MVF, HBVsa, PT, TT.	CpG+Alum	Phase II	10, 23, 24
AC Immune	ACI-24	Aβ1–15 on a liposome membrane	Liposome and MPLA inside the liposome	Phase I/II	10, 26, 27

therapeutically relevant concentrations of anti-Aβ antibodies several groups and companies decided to use passive vaccination strategy.[7-10] Recently announced results of phase III clinical trials that evaluated frequent administration of high concentrations of 2 humanized monoclonal antibodies (bapineuzumab and solanezumab) into patients with mild to moderate AD indicated that the cognitive and functional primary endpoints were not met.[11-13] However, reduction of the amyloid burden and a significant decrease of CSF p-tau were detected in some AD patients in Pfizer/Janssen AIP studies.[12,13] Administration of high dose of solanezumab (400 mg per wk) was well tolerated by vaccinated subjects,[14] but unfortunately it was not effective in 2 separate studies conducted by Eli-Lilly. At the same time analysis of pooled data from the mild AD, but not in the moderate AD subgroup of these 2 studies showed a small, statistically significant advantage over placebo on a cognitive measure in the patients given solanezumab,[11] suggesting that immunotherapy should be initiated at the earliest stages of AD or even in asymptomatic people at AD risk to minimize synaptic and neuronal loss. Based on these results 3 passive vaccinations studies have been initiated: (1) 770-patient phase II/III gantenerumab (Roche) study in prodromal and mild AD[15]; (2) 210-patient prevention study in individuals with inherited autosomal-dominant mutations (the Dominantly Inherited Alzheimer Network [DIAN] study)[16]; and ADCS/A4 trials in normal individuals with positive "Aβ/PetScan" test.[17] Our team believes that frequent and long-term administration of high dose of expensive humanized Aβ-specific antibodies in patients at very early stages of sporadic AD and, a fortiori, in asymptomatic pre-AD patients is not feasible for conventional treatment. Instead, active immunization is a more practical approach if vaccine is safe, fairly immunogenic in elderly people, and will not activate potentially harmful autoreactive Th cells in vaccinated subjects.

Immunogenicity of Protein-Based AD Epitope Vaccines Formulated in Various Adjuvants

Almost 8 y ago we proposed a vaccine strategy[18] and based on that strategy generated various peptide/recombinant protein epitope vaccines composed of a small immunodominant self-B cell epitope of $Aβ_{42}$ and one or two universal foreign Th epitopes. These epitope vaccines induced high levels of therapeutically potent anti-$Aβ_{42}$ antibodies without activation of potentially harmful autoreactive Th cells.[18-21] Immunizations of Tg2576 mouse model of AD with peptide and recombinant protein based epitope vaccines did not activate autoreactive Th cells as well. Importantly, vaccinations induced production of therapeutically relevant titers of anti-Aβ antibodies (≥50 μg/mL), which in turn inhibited accumulation of $Aβ_{42}$ pathology in the brains of older mice, reduced glial activation and prevented the development of behavioral deficits in aged animals without increasing the incidence of microhemorrhages.[21,22] Five other protein vaccines based on the same strategy are already being tested in various phase I–III clinical trials[23-25] and one vaccine, ACI-24 has been added to this list recently,[26,27] (**Table 1**).

Although, all protein-based AD vaccines share some common characteristics they are vary from one another and each presents distinct challenges which must be addressed on a case-by-case basis. However, the major challenge for all type of protein/peptide-based AD vaccines based on self-Aβ and tau antigens is how to induce a sufficiently high and long lasting immune response to immunizations. In order to achieve this objective it is necessary to formulate protein antigens in an adjuvant, the compound that can boost the immune response against a vaccine antigen. There are several adjuvants that can enhance immune responses to protein antigens without causing significant harmful side effects: (1) Mineral salts/gels (e.g., alum); (2) Oil-in-water emulsions (e.g., MF59, AS03, Montanides/ISA); (3) Water-in-oil emulsions (Mas-1/MER5); (4) Saponin-based (e.g., QS21); (5) Delta-Inulin-based (e.g., Advax™); (6) Microbial derivatives (e.g., TLR agonists such as MPLA, imiquimod, CpG, LT, etc); (7) Endogenous human immunomodulators (cytokines); (8) Virosomal/particle (e.g., VLP); (9) Cationic liposomes (10) Combinations of these adjuvant systems (e.g., Iscomatrix [structural complex of saponin with phospholipids/cholesterol] or AS04 [combination of alum and MPLA]).[28] However, from all these adjuvants only Alum is generally used

to enhance immune responses to many human vaccines,[29] while, 2 other adjuvants recently have been licensed in Europe for use as the components for few viral vaccines (MF59 for flu vaccine in elderly people and AS04 for HBV and HPV vaccines[30]). Although, Alum is a relatively weak adjuvant it has been used either alone (for AD01/92) or in combinations with VLP (CAD106), ISCOMATRIX (V950), and CpG (UB-311) to enhance immune responses to AD vaccines (**Table 1**) and.[31] In addition, AC Immune is using liposomes in combination with MPLA in ACI-24 vaccine for enhancing the antibody responses to $A\beta_{1-15}$ B cell epitope, and Novartis is using VLP particles (CAD106) formulated in MF59 adjuvant. Unfortunately, there are only few published results on the immunological efficacy of 2 vaccines, CAD106[32] and AD01/02.[33]

More specifically, CAD106, which is composed of $A\beta_{1-6}$ B cell epitope coupled to the coat protein of bacteriophage Qβ on the surface of virus-like particles, was shown to be safe in subjects with mild-to-moderate AD. Antibody responses directed to Aβ were detected in 62% of low dose and 82% of high dose subjects; however, quantification of the antibody titers was done relative to serum from rhesus macaques immunized with CAD106, making it difficult to interpret the actual magnitude of the humoral responses and possible therapeutic value of these concentrations of antibodies.[32] The one of advantages of this vaccine has been the assumption that the repetitive and ordered exposure of $A\beta_{1-6}$ peptides on the surface of viral particles should lead to the induction of high titers of anti-Aβ antibodies without adjuvant. However, apparently, the antibody response was still low, since the adjuvant (Alum or MF59) was incorporated into the vaccine formulation.[34]

According to AFFiRiS[33] they have completed clinical phase I studies with AD01/02 composed of 6 aa peptide mimicking N-terminus of $A\beta_{42}$ formulated with Alum adjuvant and demonstrated safety for both vaccine candidates in 48 mild-moderate AD patients. The company stated that: (1) AD02 formulation demonstrated stabilization of cognitive parameters over the 18 mo observation period in 9 from 12 patients; (2) Immunological data supported a potential correlation between post-vaccination antibody levels and cognitive function.[35] Although, phase I trials by AFFiRiS were completed and they decided to move to phase II trials with 420 patients with early AD, unfortunately, the data analyses are not published yet. Of note, AFFiRiS are also testing an AD03 vaccine targeting N-terminal-truncated and pyroglutamated Aβ in phase I trials.[33]

United Biomedical uses UB-311 vaccine based on N-terminal $A\beta_{1-14}$ and formulated with CpG/Alum adjuvants. Phase I open-label study to evaluate the safety, tolerability, and immunogenicity of this vaccine was initiated 4 y ago in Taiwan and completed in 2010, but the results of the study are not yet disclosed.[36]

V-950 is a multivalent Aβ vaccine conjugated with Alum/Iscomatrix adjuvant that triggers production of anti-Aβ antibodies in serum and CSF of animal models that are targeting various N-terminal truncated fragments of Aβ.[24,37] At present there is no information about exact B cell epitope(s) of amyloid and possible carrier molecule that can provide Th cell immune responses to these B cells.

ACC-001 vaccine, a short N-terminal $A\beta_{1-7}$ fragment attached to a carrier protein, CRM197 (non-toxic variant of diphtheria toxin) and formulated in the QS-21 adjuvant is currently tested in 6 different phase II studies.[23,24]

Thus, at least 6 peptide/protein based AD vaccines are being tested in clinical trials in patients with mild-moderate AD and at least one more vaccine, Lu AF20513 vaccine recently tested in our laboratory[38] is moving to phase I trial in Europe in 2014.[39] If the safety and at least immunogenic efficacy of these vaccines will be proved in the above mentioned trials, we believe that the most immunogenic AD vaccine(s) should be used as a preventive measure in subjects with very early AD pathology (prodromal AD[40]) or even in asymptomatic subjects at AD risk.

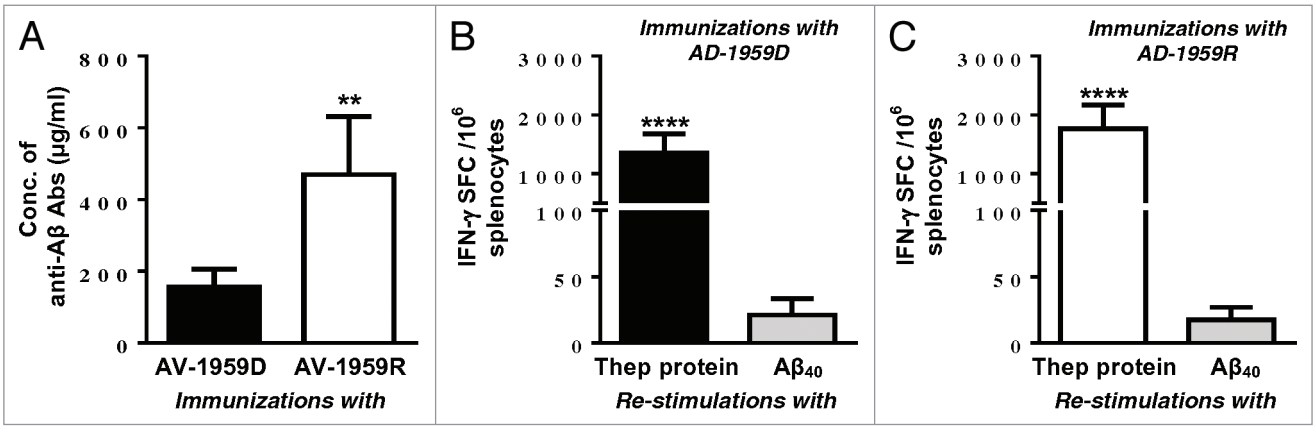

Figure 1. Humoral and cellular immune responses generated in mice by DNA-based epitope vaccine using TDS-IM EP system and protein-based AD epitope vaccine formulated with Quil-A adjuvant. (**A and B**) Cellular responses are specific to Thep protein, but not $A\beta_{40}$ peptide. Splenocytes were re-stimulated with 10 µg/mL protein or $A\beta_{40}$ peptide. (**C**) Concentrations of anti-Aβ antibodies were detected after 3rd immunizations in sera from individual mice. Bars indicate average ± SD (n = 5 per group, $^{**}P \le 0.01$, $^{****}P \le 0.0001$).

Table 2. Liposomal dose formulations composition and characterization

Composition[a], mg					Dose characteristics			
Liposomal Formulation	SUV (lipid)	DNA (AV-1959D)	Protein (AV-1982R)	Sucrose	Size nm, PDI	Zeta mV	DNA dose µg	Protein dose µg
A (DNA and Protein)	6.250	0.025	0.050	18.750	149 ± 2, 0.46 ± 0.05	18 ± 4	9.7 ± 1.0	18.6 ± 0.8
B (DNA)	6.250	0.025	Nil	18.750	134 ± 9, 0.35 ± 0.09	18 ± 5	10.0 ± 0.3	NA
C (Protein)	6.250	Nil	0.050	18.750	112 ± 3, 0.25 ± 0.01	17 ± 8	NA	22.3 ± 2.5

[a]2.5 dose vial fill, ave ± stdev with n > 3.

DNA Based AD Vaccines and Electroporation System for Enhancing of Immune Responses

Unlike to protein based AD vaccines there is no reports on DNA vaccine that are in clinical trials.

However, DNA vaccines exhibit several significant advantages when compared with a recombinant protein or peptide-based vaccines including less complicated technologies of production, high stability, the capability to modify genes encoding desired antigen/s, the ability to make changes in the cellular localization of an antigen by means of adding or removing signal sequences or transmembrane domains, and the ability to target the desired type of immune response. However, the application of DNA immunization methods used in mice did not provide encouraging results in humans or large animals.[41] More specifically, although DNA vaccines can be immunogenic without any adjuvant, the efficacy of in vivo transfection of DNA vaccines in humans and large animal species is low and delivery devices such as gene gun or electroporation (EP) are required to make these vaccines immunogenic in humans.[42,43] The gene gun delivers gold particles coated with plasmid into the epidermal and dermal layers of the skin.[44] It is believed that gene gun directly delivers DNA into the cell and even the nucleus and that is why the immune responses can be induced with significantly lower doses of naked DNA than in other delivery systems.[41] Based on this we tested $A\beta_{42}$-based DNA vaccine strategy and demonstrated the immunogenicity of this vaccine in wild type mice.[45] Dr Rozenberg's group also showed that gene-gun–administration of $A\beta_{42}$ dimer gene can effectively elicit humoral immune responses not only in wild type, but also in APP/Tg mice.[46] While this and other groups continue to test DNA vaccines-based on full-length $A\beta_{42}$[47-50] in preclinical models of AD we decided to move to another direction. More specifically, to avoid potentially harmful autoreactive Th cell responses generated by full-length $A\beta_{42}$ (AN1792), we designed a DNA epitope vaccine composed of $3A\beta_{11}$ and a non-self, universal Th cell epitope, PADRE.[20,51,52] Other groups supported this strategy for DNA vaccines against AD using short peptides spanning $A\beta_{42}$ and various viral[53,54] and non viral carriers.[55]

More recently, we hypothesized that to make this vaccine more immunogenic in humans with highly polymorphic MHC genes additional universal Th epitopes may be needed. Accordingly we developed a novel MultiTEP platform based DNA epitope vaccines, AV-1955D and AV-1959D and tested the efficacy of these vaccines in mice, rabbits, and rhesus macaques.[38,56,57] In these studies we decided to enhance immune responses to DNA vaccinations with electroporation device from Ichor Medical Systems acceptable for humans instead of using gen gun system from Bio Rad that can be used only for animals. It was shown that EP destabilizes the cell membrane for a short time period to allow DNA to enter the cells more efficiently.[58] In fact, EP could increase gene expression in vivo by 100- to 1000-fold compared with needle injection of naked plasmid DNA[59,60] inducing a strong immune response to DNA vaccines. Importantly, EP-mediated delivery of DNA vaccines is now being tested for safety and immunogenicity in several phase I clinical trials (http://www.clinicaltrials.gov). Although, EP delivery of DNA vaccines, AV-1955D and AV-1959D activated both humoral and cellular immune responses in all tested species the most interesting data have been developed in monkeys.[38,56,57] More specifically, data showed that both vaccines activated a broad and individualized repertoire of Th cells specific to peptides from different pathogens incorporated into the MultiTEP platform design and induced high titers of potentially protective anti-Aβ antibodies. We further hypothesized that MultiTep platform based vaccine may (1) provide broad coverage of human population with highly polymorphic MHC class molecules and (2) activate in vaccinated subjects pre-existing memory T cells, formed after conventional vaccinations and infections received during the lifespan. Finally, recruitment of memory T cells may overcome nonresponsiveness of elderly people to new vaccines due to immunosenescence.

Recently, we decided to compare the immunogenic efficacy of DNA-based vaccine, AV-1959D to homologous protein-based vaccine, AV-1959R in wild type mice. Delivered by EP device AV-1959D vaccine induces cellular immune responses comparable with that generated after immunizations of mice with AV-1959R formulated in a strong adjuvant, Quil-A (analog of QS21 for animals) (**Fig. 1 A and B**). As shown in **Figure 1C**, both vaccines also induced strong humoral immune responses after 3 immunizations, however AV-1959R generated significantly higher levels of anti-Aβ antibodies than DNA vaccine, AV-1959D. We believe that this superior antibody response might be associated with Quil-A, which is a strong, Th1-type adjuvant. In fact, our recent

data generated with the same, AV-1959D vaccine delivered by AgilePulse in vivo EP system form Cellectis (SA/BTX-Harvard Apparatus) supported this hypothesis, since vaccinated mice of the same haplotype induces significantly higher humoral immune responses (data not shown, paper in preparation). Based on these data we concluded that both DNA- and protein-based AD epitope vaccines described above can be immunogenic in humans if appropriate adjuvant or delivery system will be used in clinical trials.

DNA- and Protein-Based AD Epitope Vaccines and Liposomes for Enhancing of Immune Responses

Cationic mannosylated liposomes are very promising adjuvants and delivery systems for DNA, proteins and other biological molecules and drugs. They are discrete particulate structures based on lipid bilayers with characteristics that depend on the exact components and the protocol of manufacturing. Entrapment of protein/peptide antigen or DNA encoding the antigen into the liposomes can protect them from interaction with plasma, alter pharmacokinetic characteristics and the distribution compared with free compounds. These changes may lead to more effective uptake of antigen by APC and longer half-life of antigen therefore increasing immune responses. Previously it was shown that mice injected with liposome containing plasmid encoding HBsAg induced much greater (up to 100-fold) antibody responses against the

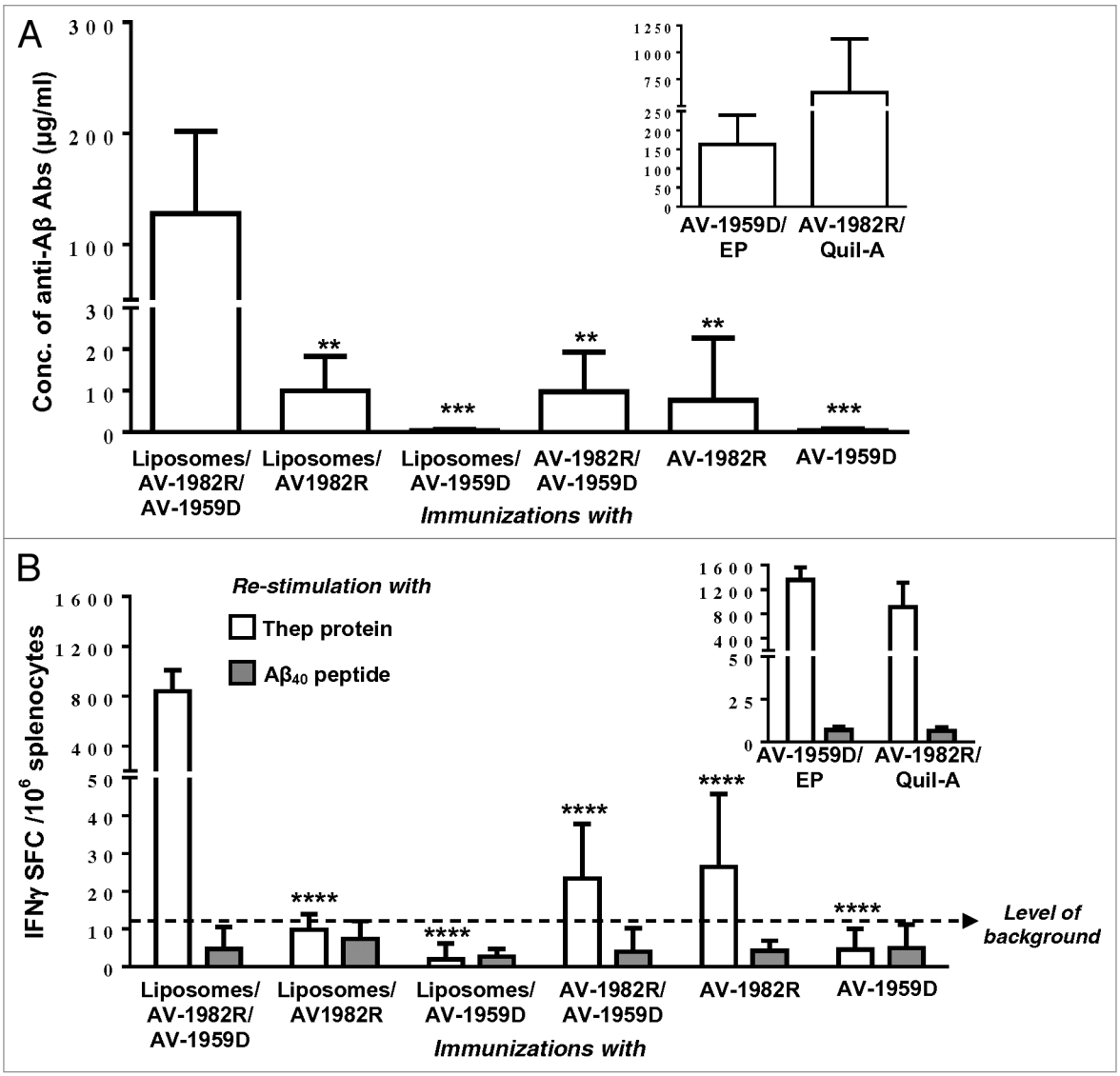

Figure 2. Humoral and cellular immune responses generated by different formulations of protein- and DNA-based AD epitope vaccines, AV-1982R and AV-1959D, respectively. (**A**) Concentrations of anti-Aβ antibodies were detected after 3rd immunizations in sera from individual mice. (**B**) Cellular responses are specific to Thep protein, but not $A\beta_{40}$ peptide. Splenocytes were re-stimulated with 10 μg/mL Thep protein or $A\beta_{40}$ peptide. Bars indicate average ± SD. Statistical differences in all groups were calculated relative to Liposomes/AV-1982R/AV-1959D immunized group using two-tailed *t* test (n = 6 per group, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$).

encoded antigen than animals immunized with the naked DNA.[61] In addition, we studied the effects of various combinations of DNA and protein vaccines entrapped in the liposomes on the antibody response using hemagglutinin and hepatitis-B surface antigen. We observed a strong synergistic effect on the immune response when protein antigen and DNA encoding the same antigen were entrapped in the same liposomal compartment. This synergistic response was not seen when the protein and DNA materials were contained in separate liposomal vehicles and administered as a combination immunisation dose suspension.[62] Here we decided to test this strategy for enhancing of immune responses against self-antigens such as Aβ peptide.

More specifically, wild-type mice were immunized with liposomes containing DNA vaccine, protein vaccine, and DNA/protein together. Briefly, Liposomes were prepared from >95% purity lipid from Lipoid GmbH as described previously.[62] The liposomes were characterized in terms of particle size / zeta potential, and DNA/protein dose content (**Table 2**). As DNA vaccine we chose AV-1959R, while, to avoid the interference with endotoxins, in this study we used our GMP grade protein vaccine composed of 3 copies of $A\beta_{1-11}$ fused to promiscuous synthetic Th epitope PADRE and universal epitope from tetanus toxin, P30 (designated as AV-1982R). As control groups, mice were immunized with naked AV-1959D, AV-1982R, and mix of both. In addition, as a positive control, mice were immunized with AV-1982R formulated in Quil-A adjuvant and AV-1959D delivered by EP device. As shown in **Figure 2A**, AV-1959D and AV-1982R entrapped into the liposomes together induced significantly higher anti-Aβ antibody response compared with either liposomes/AV1959D or liposomes/AV1982R. We observed also significantly higher immune response in mice immunized with liposome containing DNA and protein vaccines regardless are they used alone or in combination. Animals immunized with AV-1959D/EP generated comparable level of humoral immune responses to vaccinations with liposome containing DNA and protein based vaccines, in contrary to immunizations with AV-1982R/Quil-A (**Fig. 2A**, inscribed Figure).

Cellular immune responses specific to Th epitopes or to $A\beta_{40}$ peptide were analyzed in splenocytes of immunized mice re-stimulated in vitro with recombinant protein composed of Th epitopes as well as with $A\beta_{40}$ peptide. Again, in splenocytes of mice immunized with AV1959D and AV-1982R entraped into the liposomes together we detected significantly higher number of splenocytes producing IFNγ than in splenocytes of mice from other groups. Importantly, no Aβ-specific response was seen in all mice (**Fig. 2B**). Positive control groups of mice generated similar level of cellular responses after immunization with AV-1959D/EP or AV-1982R/Quil-A (**Fig. 2B**, inscribed Figure). This new vaccination approach has been termed "co-delivery" and may derive from the simultaneous presentation of antigen via MHC class-I (DNA) and MHC class-II (protein) pathways to CD8+ and CD4+ cells at the same antigen presenting cell -a mode of presentation that would commonly occur with live viral pathogens. Additionally the liposome composition employed a surface presented mannose moiety, ManDOG lipid, to specifically target liposomal uptake in APCs (dendriric cells) via the mannose receptor.[63] Although it is recognized an extensive formulation control immunological response study would be required to prove these mechanisms in this specific vaccine scenario.

Conclusions and Perspectives

These studies highlight the importance to consider the advantages and disadvantages of a particular approach to the development of vaccines in order to avoid undesirable side effects while achieving the desired result. It is obvious that protein based vaccine may be a better choice in case of availability of safe and effective adjuvant. Although, DNA based vaccines are less immunogenic but they are considered to be safe and if good EP device is available, they can be quite effective. Finally, liposomes containing DNA and Protein based vaccines together are interesting approach for development of AD vaccine, however are premature for translation into the clinic yet.

Disclosure of Potential Conflicts of Interest

A.B. is an employee and shareholder of Xenetic Biosciences.

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