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Adjuvants Derived from *Neisseria meningitidis* Serogroup B Induce a Cross Reactive Response against *Neisseria gonorrhoeae* in Mice

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Abstract

Introduction: *Neisseria* is a large genus of bacteria that colonize mucosal surfaces. Of the 11 species that colonize humans, only two are pathogens, *N. meningitidis* and *N. gonorrhoeae*. Both are obligate human pathogens; the last is causal agent of gonorrhea disease. Although curable with timely antibiotic treatment, an annual incidence of more than 62 million cases is estimated by the World Health Organization and there are no successful vaccines available. In contrast, several prophylactic vaccine options for *Neisseria meningitidis* meningitis do exist. Of note, there is trace of cross parenteral response induced between *N. meningitidis* and *N. gonorrhoeae*, and Proteoliposome (PL, also named outer membrane vesicles, OMV) vaccine has shown high impact on this response. **Objective:** To determine effect of VAMENGOC-BC™ and its derivates (AFPL1 and AFCo1) at mucosal and systemic level and possible cross response against *Neisseria gonhorroeae* in Balb/c and C57Bl/6 mice. **Methods:** We evaluated cross response against *N. gonorrhoeae* in mouse serum IgG and saliva IgA after mucosal immunization with VA-MENGOC-BC or its derivatives (AF, Adjuvant Finlay PL1 or AFCo (cochleate) 1). **Results:** Immunizations with AFPL1 or AFCo1 induce anti *N. gonorrhoeae* at saliva IgA and serum IgG responses similar to VA-MENGOC-BC™ vaccine. **Conclusions:** Such data confirms a new possible window of prime-boost vaccination strategy against gonorrhea and extends our knowledge regarding the effect of PL vaccines on cross responses.

Keywords

N. meningitidis, *N. gonorrhoeae*, Vaccine, Cross Reactivity

1. Introduction

Neisseria is a large genus of bacteria that colonize mucosal surface. Of the 11 species that colonize humans, only two are pathogens, *N. meningitidis* and *N. gonorrhoeae*. Both are obligate human pathogens; the last is the causal agent of gonorrhea disease. Although curable with timely antibiotic treatments, high numbers of resistant strains had an annual incidence of more than 62 million cases estimated by the World Health Organization and there are no successful vaccines available [1]. This is also a neglected disease that can be associated with human immunodeficient virus (HIV)-co-infection. However, there are reports of some potential vaccine candidates against *N. gonorrhoeae* [2] [3] and cross IgG response between *N. meningitidis* and *N. gonorrhoeae* has been reported [4]. This is mainly consequence of the similar structural proteins important for immunogenicity. In addition, proteoliposome (PL, also named outer membrane vesicles, OMV) vaccines against *N. meningitidis* have been demonstrated to reduce the incidence of human Gonorrhea infection as first reported using VA-MENGOC-BC™ vaccination in Cuba [5] followed by MeNZB vaccination in New Zealand [6].

While *N. gonorrhoeae* infects and colonizes at mucosal surfaces where IgA is the main protecting antibody, both VA-MENGOC-BC™ and MeNZB vaccines are parenteral vaccines mainly generating systemic specific IgG protective responses. However, evaluation of mucosal immunization has been limited due to its lower immunogenicity and higher dose requirement as compared to parenteral routes and the absence of mucosal adjuvants [7].

We have developed a PL-derived cochleate (AFCo1), which contains similar proteins and pathogen-associated molecular patterns as PL. It induces specific systemic IgG and mucosal IgA in mice and is thus a promissory mucosal adjuvant. AFCo1 applied by nasal route induces mucosal (regional and distal) and systemic immune responses (specific saliva IgA, serum IgG, and IgG2a subclass), and IFN-γ polarizing to a Th1 pattern [8] [9] [10]. Successful results were obtained using nasal immunization of AFCo1 containing recombinant glycoprotein 2 from Herpes simplex virus type 2 [11]. It also demonstrated adjuvant effect with *N. meningitidis* and *Salmonella typhi* polysaccharides inducing memory B cells, Th1 cytokines and specific IgA production with similar anti polysaccharide C response to the bivalent VA-MENGOC-BC™ vaccine without the requirement of covalent conjugation [12].

As noted above, one of the shortcomings of parenteral immunization is its relatively poor ability to induce genital-tract-specific IgA [13] [14]. IgA is considered important in protecting the genital tract from infection, as its presence is

correlated with a protective role against *Chlamydia* and human immunodeficiency virus (HIV) [15] [16]. In mice, intranasal immunization has been promising in terms of eliciting genital-tract antigen-specific IgA and IgG [17] [18], primates [19], and humans [20] [21]. Therefore, we conducted this study of gonococcal cross protection by meningococcal vaccination to obtain more information on this subject.

2. Material and Methods

Animals. Female C57Bl6 or BALB/c mice, six to eight weeks olds, were used for all the experiments (Taconic M&B, Denmark or Cenpalab, Cuba). C57Bl6 were housed in microisolators under specific-pathogen-free conditions at animal facility, Sahlgrenska Academy, Göteborg University, Sweden or BALB/c in conventional animal facilities at Finlay Institute, Cuba. All the experiments were performed with the approval from Ethical Committees for Laboratory Animals in Sweden or Finlay Institute, respectively. The studies were performed at least twice.

Preparation of the membrane from *N. gonorrhoeae*. The *N. gonorrhoea* ATCC 19424 strain was cultured in chocolate agar plates at 5% CO_2, and 35˚C - 36˚C. Then, they were transferred to 3 mL liquid media for 1 h and then transferred to 500 mL Erlenmeyer under shaker agitation overnight at 35˚C - 36˚C. They were centrifuged at 3000 rpm, 10 minutes at 4˚C and the pellet extracted in 5 mL of lithium chloride buffer (200 mM LiCL, 100 mM $C_2H_3O_2Li$, pH 6.0) over glass spheres for 2 h at 50˚C under shaker 100 rpm. Then, they were centrifuged at 13,000 rpm for 20 min and the supernatant at 40,000 rpm for another 2 h. The pellet containing the outer membrane proteins was resuspended in distilled water with 0.02% sodium azide and maintained at 4˚C until used.

Preparation of proteoliposome (PL) and AFCo1. PLs from outer membrane vesicles detergent-extracted from *N. meningitidis* serogroup B were prepared and supplied as an ethanol precipitate by the Finlay institute's vaccine production unit, Havana, Cuba. PL-derived AFCo1 was prepared as previously described 8, 11.

Animal immunization and sample collection. In the pathogen free mice: two groups of seven female C57Bl/6 mice each were inoculated (i.n) with AFCo1 or AFPL1m respectively, using 3 doses of 100 μg with 7 days-intervals in 12.5 μL per nostril per dose per mouse. One group was inoculated (i.m) with AFPL1 absorbed onto $Al(OH)_3$ using 2 doses of 25 μg with 7 days-intervals in 100 μL. Another group was used as control. In the conventional mice: three groups of seven female BALB/c mice each were inoculated i.m with AFCo1, AFPL1 or VA-MENGOC-BC™ using 2 doses of 12 μg with 14 days-intervals in 100 μL per mouse. Another two similar age and sex groups were immunized i.n with AFCo1 or AFPL1 using 3 doses of 100 μg with 7 days-intervals in 12.5 μL per nostril per dose per mouse. Other three groups were immunized with VA-MENGOC-BC™ 2 doses spaced 14 days by i.m routes. The second and third ones received and

additional boost of AFPL1 or AFPL1 by i.n route, respectively. To compare the production of IgA by different strain of mice BALB/c and C57Bl/6 were immunized by nasal route with AFCo1 or AFCo1 as adjuvant of N. gonorrhea antigens in 3 doses spaced 7 days. Vaginal or saliva anti PL or Ng IgA responses in both strains of mice were measured, respectively. In all groups, blood samples were obtained from the tail vein at 21 days after the last dose. Vaginal samples were collected at 21 days after the last immunization rinsing the vaginal cavities 3 times with 50 µL phosphate buffer saline (PBS). Saliva samples were collected at 7 days after the last immunization by Pilocarpine 0.5% intraperitoneal stimulation. These were immediately inactivated at 56˚C for 15 min and the supernatants after centrifugation (9000 g) were stored as well as sera at −20˚C until used.

Antibody response by ELISA. Maxisorp 96-well plates (Nunc) were coated with 100 µL of PL form *N. meningitidis* (20 µg/mL) or membrane extracted proteins from *N. gonorrhoeae* (5 µg/mL) in 0.05 M carbonate buffer, pH 9.6 for 4 h at room temperature, followed by overnight incubation at 4˚C. The plates were blocked with 2% bovine serum albumin (BSA) in PBS for 30 min at 37˚C. Serial dilutions starting in 1:10 of sera or 1:50 of vaginal washes obtained were incubated for 1 h at 37˚C in % BSA-PBS buffer. After washing with 0.05% Tween-20, plates were incubated 1 h at 37˚C with goat anti-mouse immunoglobulin (Ig) G, anti-mouse IgG1 (Southern Biotechnology Associates, Inc., Birmingham, AL), anti-mouse IgG2c (Research Diagnostics Inc., NJ), anti-mouse IgG2b, or anti-mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, AL) conjugated to horseradish peroxidase in 1% BSA-PBS buffer. Plates were then washed with 0.05% Tween-20 and developed using 100 µL of 1 mg/mL o-phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate buffer (pH 4.5) in the presence of 0.04% H_2O_2. The reaction was stopped with H_2SO_4. The absorbance was read at 492 nm. Positive IgG and IgA titers in sera and vaginal washes were defined as the reciprocal of the sample dilution giving an optical density (OD) of 0.4 and 0.3 above the background value, respectively and were expressed by calculating the geometric mean of titers per group.

Statistical analysis. Statistical analyses were done by ANOVA multiple comparison test followed by Tukey and by Student's t-test and 2 way ANOVA followed by Bonferroni post-test using Graph Pad Prism 5 software (CA, USA).

3. Results

Parenteral immunizations with VAMENGOC-BC™ or its derivatives (AFPL1 and AFCo1) induced systemic specific IgG response without a mucosal mucosal specific IgA response, but intranasal AFPL1 and AFCo1 did induce salivary specific IgA in BALB/c mice

We confirmed that i.m applications of VA-MENGOC-BC™ vaccine, AFPL1 or AFCo1 induce specific systemic IgG response in BALB/c mice. VA-MENGOC-BC™ and AFCo1 induced similar specific IgG response to each other, but they were higher than that induced by AFPL1 (Figure 1A). Neither VA-MENGOC-BC™ nor AFPL1 and AFCo1 applied by i.m route in naïve mice induced saliva specific

IgA (Figure 1B). Nevertheless, i.n applications of AFPL1 or AFCo1 induced a systemic specific IgG and mucosal specific IgA in BALB/c mice. Both immune responses were higher in AFCo1 than AFPL1 immunized mice (Figure 1C and Figure 1D). Overall, the mucosal routes induced specific IgA but parenteral administration did not.

Parenteral VA-MENGOC-BC™ did not induce salivary IgA but a mucosal boost did

In order to further evaluate the prime-boost strategies using two routes of immunization to explore the possible influence of mucosal challenge after a previous i.m immunization with VA-MENGOC-BC™, we immunized mice with vaccine i.m doses and then, boosted them with a dose of AFPL1 or AFCo1. Three doses of AFPL1 or AFCo1 by i.n route induce significant anti PL IgA in saliva. Two i.m doses of VA-MENGOC-BC™ did not induce mucosal IgA response but an i.n booster with AFPL1 or AFCo1 i.n induced a salivary specific IgA similar than to 3 i.n doses. All cases AFCo1 response was higher than AFPL1 (Figure 2). Overall, these results demonstrated the requirement of mucosal immunization for the induction of mucosal specific IgA response and how one mucosal boost is enough when previous parenteral encounters has been occurred.

Intranasal immunization with AFPL1 or AFCo1 as well as the intramuscular VA-MENGOC-BC™ induced anti-*Neisseria gonorrhoeae* IgG cross reactive responses in C57Bl/6 mice

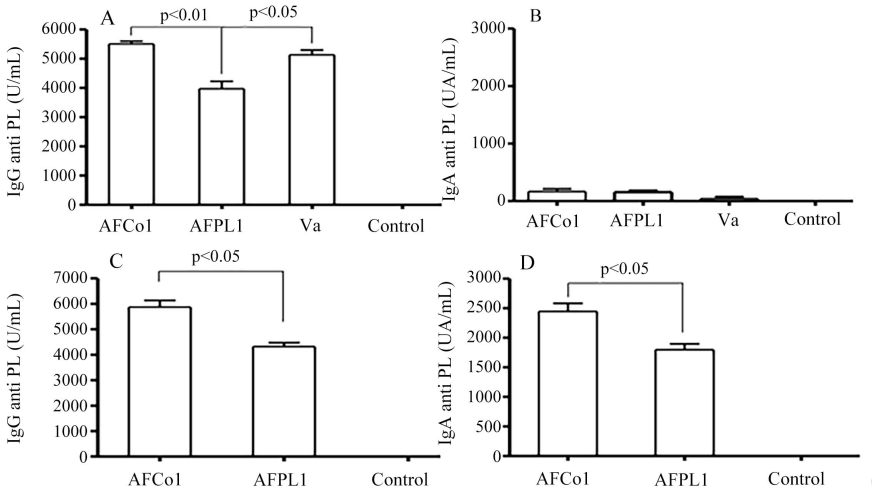

Figure 1. Parenteral immunization with VAMENGOC-BC™ (Va) and its derivates (AFPL1 and AFCo1) induce systemic specific IgG response but not mucosal specific IgA response, but nasal AFPL1 and AFCo1 did in Balb/c mice. A, shows anti proteoliposome (PL, main antigen of this vaccine) serum IgG and mucosal IgA levels induce by different formulations applied in 2 doses by intramuscular route. B, shows anti PL IgA levels induce by different formulations applied in 2 doses by intramuscular route. C and D, AFPL1 and AFCo1 by 3 intranasal route induce serum anti PL IgG and saliva anti PL IgA, respectively. Statistical analyses were done by ANOVA multiple comparison test followed by Tukey using Graph Pad Prism 5 software.

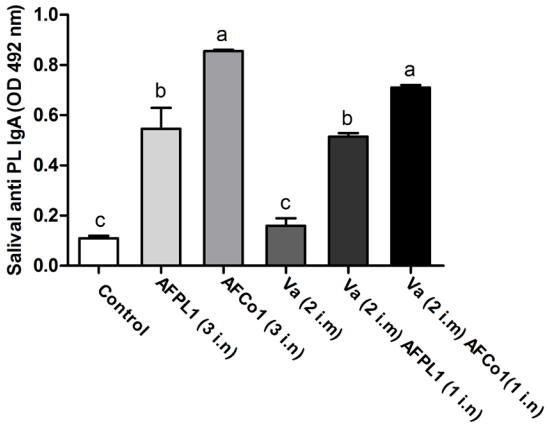

Figure 2. Parenteral VA-MENGOC-BC™ did not induce salival IgA but a mucosal boost did. Three intranasal (i.n) doses or 2 intramuscular (i.m) doses of VA-MENGOC-BC™ (Va) followed by 1 i.n dose induce saliva anti Proteoliposome (PL) IgA in mice, which is boosted by AFPL1 and AFCo1 in Balb/c mice. Different letters denote significant differences ($p < 0.05$) according to 1 way ANOVA followed by Tukey analysis using Graph Pad Prism 5.

To explore the possible cross reactive response aginst *N. gonorrhoeae* antigens induce by different formulations C57Bl/6 mice were immunized with 3 i.n doses of AFCo1 or AFPL1 or 2 i.m doses of VA-MENGOC-BC™. Three i.n doses of AFPL1 or AFCo1 induce anti PL and anti *N. gonorrhoeae* response at serum IgG responses similar to 2 i.m doses of the vaccine. Nevertheles, in all cases the anti PL response was higher than cross reactive response (**Figure 3A**).

Evaluation of IgG1 and IgG2c subclasses as a Th2 or Th1 polarized response, respectivelly, was also conducted. Serum anti PL IgG1 was higher than anti *N. gonorrhoeae*, in concordance with their specific serum IgG. The anti *N. gonorrhoeae* IgG induced by AFPL1 was less than 0.4 OD value. This negative value did not mean a negative response but a response lower than the cut off value of the assay (**Figure 3B**). Anti PL IgG2c responses were similar among all formulations but significantly higher than anti *N. gonorrhoeae* antigens which were below the cut off (**Figure 3C**).

Anti PL and *N. gonorrhoeae* vaginal IgG responses were both induced. Note that these results are presented in OD because there were below the stringent cut off but still significant induced (**Figure 3D**) as also represented above for anti *N. gonorrhoeae* IgG2c (**Figure 3C**). Neither anti PL nor anti *N. gonorrhoeae* antigens vaginal IgA responses were detected in C57Bl/6 mice. Overall, these results pointed out the induction of cross reactive anti *N. gonorrhoeae* antigens IgG responses induced by VA-MENGOC-BC™ and its derivatives AFPL1 and AFCo1

BALB/c induces more specific IgA than C57Bl/6 mice at vaginal and saliva levels

In order to further evaluate the poor of IgA specific response in C57Bl/6 mice the anti PL or anti *N.* Ng IgA responses induce by AFCo1 alone or AFCo1 + *N. gonorrhoeae* antigens i.n in BALB/c mice were conducted. In BALB/c mice

salival and vaginal specific IgA was induced using AFCo1 or Ng adjuvanted with AFCo1 applied by nasal route (Figure 4A and Figure 4B). These results pointed out the induction of cross reactive anti *N. gonorrhoeae* antigens IgA responses in mucosa induced by AFCo1 + *N. gonorrhoeae* antigens.

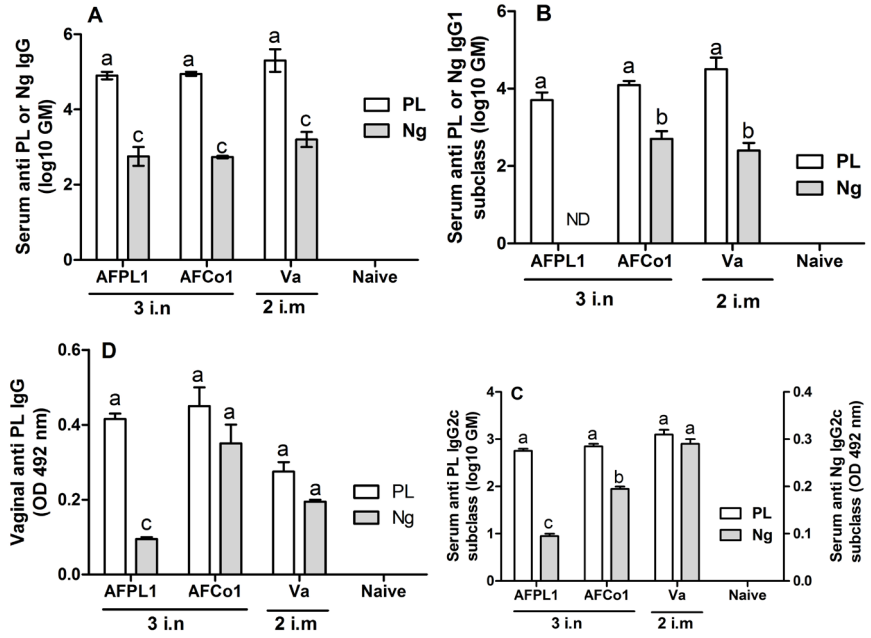

Figure 3. Intranasal (i.n) immunization with AFPL1 or AFCo1 as well as intramuscular (i.m) VA-MENGOC-BC™ induce anti *Neisseria gonorrhoeae* IgG cross responses in C57Bl/6 mice. The i.n immunization with AFPL1 or AFCo1 was given in 3 doses spaced 7 days. The i.m immunization with VA-MENGOC-BC™ onto Aluminium hydroxide (Alum) was given in 2 doses space 14 days. A and B, serum specific IgG and IgG1 responses against *N. meningitidis* serogroup B (PL) and *N. gonorrhoeae* (Ng) antigens, respectively; C serum specific IgG2c subclass against PL and Ng antigens; D, vaginal anti PL IgG responses, respectively. GM, geometric mean. Different letters denote significant differences ($p < 0.05$) according to 2 way ANOVA followed by Bonferroni post-test analysis using Graph Pad Prism 5 software.

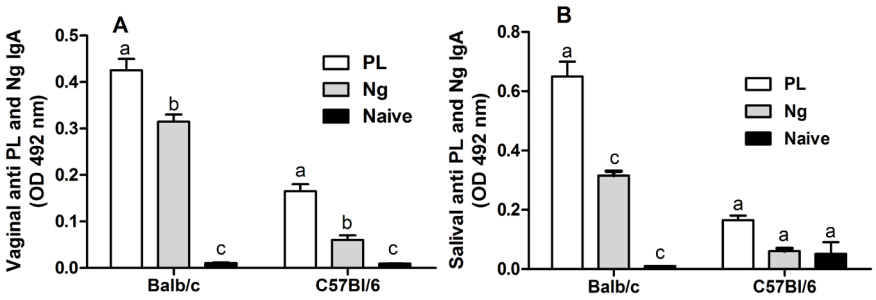

Figure 4. The Balb/c induces more specific IgA than C57Bl/6 mice at vaginal and saliva levels. Mice were immunized by i.n route with Proteoliposome (PL) transformed into AFCo1 or *Neisseria gonorrhoea* antigens (Ng) in 3 doses spaced 7 days. A and B, vaginal or salival anti PL IgA responses in both strains of mice, respectively. Different letters denote significant differences ($p < 0.05$) according to 2 way ANOVA followed by Bonferroni post-test analysis using Graph Pad Prism 5 software.

4. Discussion

Parenteral immunization with VAMENGOC-BC™ and its derivatives (AFPL1 and AFCo1) which conserve core protective antigens induced systemic specific IgG response, but not mucosal specific IgA responses. In contrast, intranasal AFPL1 and AFCo1 induce mucosal specific IgA in BALB/c mice. This is in concordance with the well-known dictum that mucosal administration is required to induce mucosal response. This route induces similar IgG responses to the parenteral route but also induced potent mucosal IgA responses [22] [23]. This is in contrast to VAMENGOC-BC™ applied by parental route which requires a mucosal boost (AFPL1 or AFCo1) to induce a specific IgA response. AFCo1, with a multilayer microparticle containing calcium is more stable and more potent than the AFPL1 nanoparticle administered by the mucosal route as was previously described [7] [24].

VA-MENGOC-BC™ and its derivatives, AFPL1 or AFCo1, by i.n or i.m immunizations induce anti *Neisseria gonorrhoeae* IgG cross reactive responses in C57Bl/6 mice. Specific IgG1 was higher than IgG2c against both antigens, PL and *N. gonorrhoeae*; but the former antigens induced higher specific IgG1 levels than the later one. IgG2c is also induced against both antigens but more so for PL than *Ng* antigens. In addition, mucosal specific IgA responses against both antigens were poorly detected in C57Bl/6 mice.

Cross reactive responses of genus *Neisseria* has been investigated, mainly between pathogenic species *N. meningitidis* and *N. gonorrhoeae* [4] [25]. These studies followed demonstration that VA-MENGOC-BC™ human vaccination decreased the gonococcal incidence in Cuba as reported by us as early as 2009 [5], later referred by others authors in 2018 [26] and 2019 [27] and extended to other similar vaccines in 2017 [6]. Certainly, PL (OMV) vaccines have a high impact in this type of cross reactive response.

The complex constitution of the PL [28] [29] [30] as protective antigenic core of VA-MENGOC-BC™ with its adjuvant properties [8] [24] [31] are certainly involved in the induction of cross protection. These proteins have a structural homology with *N. gonorrhoeae* and have been described as capable of producing a cross-response [26]. Another possibility is based on the structural homology that exists between commensal species of the genus; such is the case of *N. lactamica* since it is closely related to *N. meningitidis* and *N. gonorrhoeae* as it colonizes the upper respiratory tract [32]. In addition, genetic analyses of both pathogenic and non-pathogenic *Neisseria* show they are closely related, with evolutionary studies suggesting frequent exchange of genetic material including virulence genes [33]. Of note is that a decline of gonococcal incidence was also observed in New Zealand using MeNZB which also contains PL (OMV) [6].

The induction of more IgG1 (Th2 polarizing) than IgG2c (Th1 polarizing) against Ng formulations seems to be more related to the antigens *per se* than the adjuvant because this adjuvant present in VAMENGOC-BC™, AFPL1, or AFCo1 induces mainly a Th1 response in mice and human [7] [24] [25] [26]. Neverthe-

less, the induction of a Th2 pattern is important for IgA mucosal response but the initial selections of C57Bl/6 for the experiment seem to be wrong. The use of BALB/c mice induces this specific IgA response against PL and *N. gonorrhoeae*.

5. Conclusion

In conclusion, we have shown that VAMENGOC-BC™ and its derivatives (AFPL1 and AFCo1) induce systemic specific IgG response without a mucosal one, but intranasal immunization with AFPL1 and AFCo1 induces both systemic and mucosal responses in BALB/c mice. The induction of cross reactive IgG response with Ng antigens was demonstrated in C57Bl/6 mice and extended to specific IgA in BALB/c mice.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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