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Taylor & Francis Group

COMMENTARY

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Experimental studies using OMV in a new platform of SARS-CoV-2 vaccines

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ABSTRACT
Although COVID-19 vaccines have recently been approved for emergency use, search for new vaccines are still urgent, since the access of the countries, especially the poorest, to the vaccines, has shown to be slower than the necessary to rapidly control the pandemic. We proposed a novel platform for vaccine using recombinant receptor binding domain (rRBD) from Sars-Cov-2 spike protein and *Neisseria meningitidis* outer membrane vesicles (OMVs). The antigen preparation produced a humoral and cellular immune response. Taken together our findings suggest a good immunostimulatory patter in response to immunization with rRBD plus *N. meningitidis* OMV.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a recently identified coronavirus that causes coronavirus disease 2019 (COVID-19)[1] characterized by its severe 'flu'-like symptoms that can progress to acute respiratory distress (ARDS), pneumonia, renal failure, and death.[2] COVID-19 was declared by WHO a public health emergency of international concern on 30 January 2020. Due to the great pathogenicity and rapid spread of SARS-Cov-2, COVID-19 became the deadliest, fastest-moving pandemic since 1918, leading to hundreds of thousands of deaths and huge economic impact.[3] Although the first vaccines have recently been approved for emergency use, the access of the countries, especially the poorest, to the vaccines, has shown to be slower than the necessary to rapidly reach the herd immunity and slowdown the pandemic. Thus, researches for new vaccines and adjuvants are urgent.

The spike (S) protein is a structural glycoprotein which plays a key role in the receptor recognition and cell membrane fusion process.[4] This protein is composed of two subunits, S1 and S2. The S1 subunit possess the receptor-binding domain (RBD) that binds to the host cell receptor angiotensin-converting enzyme 2 (ACE2) mediating viral cell entry.[5] Due to this characteristic, RBD is a critical target for neutralizing antibodies.[4]

The Outer membrane vesicles (OMVs) are nano-sized spherical blebs shed by Gram-negative bacteria that reflects outer membrane composition. It is composed of bacteria surface-exposed antigens in native conformation and orientation, and immunostimulatory molecules, such as lipopolysaccharide (LPS), lipoproteins or peptidoglycans. These vesicles mimic the outside of bacteria, resembling a pathogen but, as they are non-living, lack the ability to cause associated disease and have been proposed as vaccines since their discovery.[6] Vaccines against *Neisseria meningitidis* serogroup B (MenB) based on OMVs have proved to be very useful against epidemic strains in Cuba,[7] Norway[8] and New Zealand.[9] On the other hand, the 4CMenB (Bexsero) vaccine that also contains outer membrane vesicles (NZ OMV) and additionally contains three surface-exposed recombinant proteins (fHbp, NadA, and NHBA), shows important cross-reactivity with other MenB strains and it is estimated to provide 66–91% coverage against MenB strains worldwide.[10] Besides being approved for use against MenB around the world, the OMV-based vaccines have also been tested against other diseases, such as *N. gonorrhea, Mycobacterium tuberculosis, Klebsiella pneumoniae, Salmonella* and has proved to be more immunogenic than classical vaccines.[6]

Here, we propose a preliminary study of a Covid-19 vaccine with rRBD as antigen and *N. meningitidis* OMV and aluminum hydroxide as adjuvants, with two intramuscular and two intranasal doses.

The critical role of S protein to Sars-Cov-2 cell invasion makes this protein an obvious vaccine candidate.[11] Thus, the S protein, including RBD, is actually, the main target to anti-COVID-19 vaccines development,[12] and its effectivity as immunogen has proved to be satisfactory even in phase III studies, with different effectiveness, depending on the vaccine platform used.[13–15] Besides the choice of the antigenic target, the choice for a good adjuvant is crucial to vaccine development. OMVs from Gram-negative bacteria have shown to have adjuvant properties, since due to its membrane composition, it retains several pathogen-associated molecular pattern (PAMPs), recognized by pattern recognition receptors (PPRs) in antigen presenting cells (APC), what is crucial for a strong adaptive immune response.[16] Its application to deliver heterologous vaccine antigens has been demonstrated to bacterial, viral, parasitic, and even cancer antigens.[17–20]

Aluminum hydroxide was chosen as a compound of our vaccine design, since it has been used as an adjuvant for the licensed vaccines containing OMV, such as Bexsero®[21] and its use is allowed in humans. The intramuscular route of administration is the choice for inactivated vaccines that contain aluminum hydroxide in the preparation[22] Boost with intranasal antigen[23] free of aluminum hydroxide aimed to verify whether the animals would intensify or modulate the response after this administration.
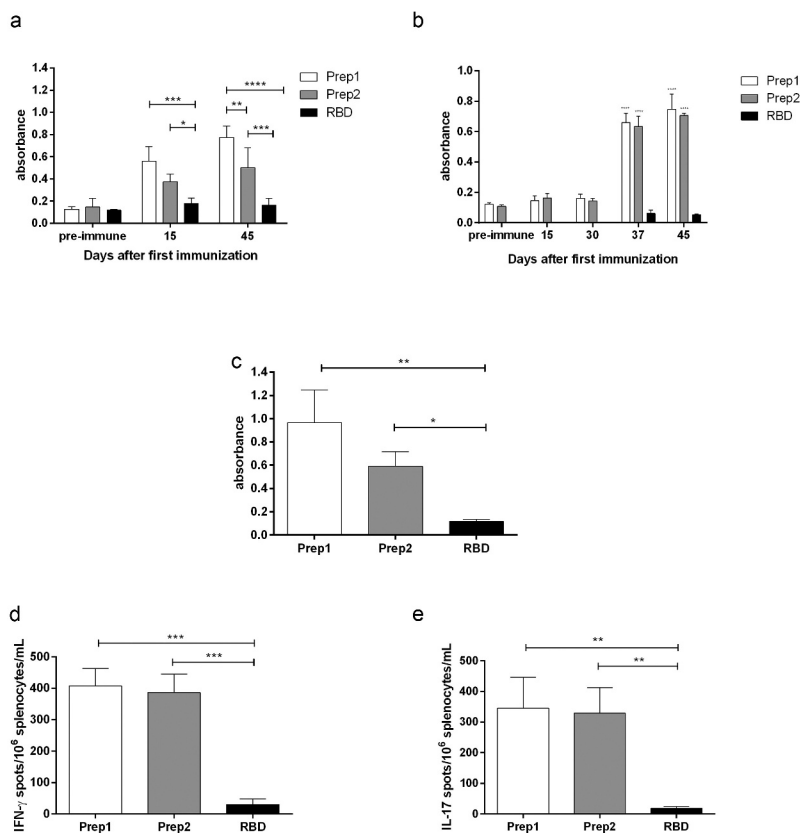
Figure 1. Immune response after immunization with rRBD from Sars-Cov-2 with aluminum hydroxide plus OMV from *Neisseria meningitidis* strains B:8:P1.6 (prep1) or C:2a.P1.5 (prep2). The IgG production in sera collected 15 and 45 days after the first immunization dose was measured by ELISA and can be observed in (a). The IgA production was accessed 15, 30, 37 and 45 days after the first immunization dose (b). Additionally, 45 days after the first immunization dose, the animals were euthanized and the splenocytes were cultured under rRBD stimuli. The IgG production in culture supernatant was evaluated by ELISA (c) and the number of cells/10[6] splenocytes producing IFN-γ (d) or IL-17 (e) was evaluated through ELISpot.

In our study, although immunization with rRBD alone induced a small production of sera with IgG directed toward SARS-CoV-2, we observed an increase in IgG production when rRBD was mixed to OMV plus aluminum hydroxide, in both, prep1 and prep2, 15 and 45 days after the first immunization dose (Figure 1(a)). As a versatile delivery system, OMVs can be used in a simple mixture with the antigen,[24] chemically conjugated to them[25] or can be genetically engineered to express the antigen proteins.[26] Although there are some evidence that chemical conjugation improves the OMV adjuvant property,[25] the simple mixture of the OMV with antigens, as we have done in this work, is capable of inducing serum production of IgG heterologous to OMV, that is, specific to the vaccine antigen.[17,18,24,27] We have recently demonstrated that this vaccine schedule induces anti-RBD IgG with intermediary avidity,[28] what is not completely surprising, since, even natural infection has shown to fail in inducing high avidity antibody directed toward SARS-CoV-2 antigens, probably due to incomplete avidity maturation.[29] Since the interaction between SARS-CoV-2 and human ACE2 possesses strong affinity, the induction of high avidity anti- SARS-CoV-2 antibodies are desirable and can be necessary to truly block SARS-CoV-2 adherence and infection[30] and can be a challenge. We also observed IgA production, but only 37 days after the first immunization dose, i.e., only after the first boost with intranasal antigen for both prep1 and prep2 immunization (Figure 1(b)), what is interesting since IgA seems to dominate the early neutralizing antibody response to SARS-CoV-2[31] We also verified the anti-rRBD IgG production in

the supernatant of the splenocytes stimulated in vitro with rRBD. In this experiment, IgG production was higher in the groups immunized with prep1 and prep2, compared to the animals immunized with rRBD alone. Although the total amount of antibody in animals immunized with prep1 was slightly higher than in animals immunized with prep2, no statistical significance was observed (Figure 1(c)).

We found an increase in IFN-γ producing cells in splenocytes culture from animals immunized with prep1 and prep2. The animals immunized with preparations containing OMV produced around 13 times more IFN-γ producing cells in splenocytes culture, than animals immunized with rRBD alone (Figure 1(d)). This finding is in accordance to others, that also find higher number of IFN-γ-producing cells[32] or higher levels of IFN-γ in the sera[33] or culture supernatant[34] of the animals receiving OMV in their vaccine preparations. We also found an increase in IL-17 producing cells in splenocytes derived from animals immunized with prep1 and prep2 after stimuli with rRBD (Figure 1(e)). Kim et al.,[34] although used OMV as a homologous vaccine, also found an increase in IL-17 production by culture splenocytes. These authors suggest the protection against bacteria-induced lethality occurs via Th1 and Th17 cell responses, since they also found increase in IFN-γ production. Il-17 production was elicited after immunization with SARS-CoV-2 pre-fusion stabilized (S-2P) spike protein adjuvanted with CAF®01 but not with aluminum hydroxide, nor a squalene-based oil-in-water emulsion system.[35] Although IL-17 has been

strongly associated with immunopathology, it also has an important role in host defense.[36] This cytokine plays a key role in defense from both extracellular bacteria and viruses that infect airway mucous membranes, regulates homeostasis, and contributes to the repair of epithelial cells, damaged previously by an extracellular inflammatory stimulus.[37] The exact role of IL-17 against Sars-CoV-2 is not completely elucidated. Its production seems to be enhanced in Sars-CoV-2 infected people with heterogeneous commitment degrees, but its association with disease severity is not clear.[38,39]

Although our data are preliminary, and more studies are necessary specially to improve avidity of anti-SARS-CoV-2 antibodies, taken together our findings suggest a good immunostimulatory pattern in response to immunization with rRBD plus *N. meningitidis* OMV, showing a promising platform to anti-COVID-19 vaccine development. Additional studies with animal models as OMV without adjuvants or others are required to assess the potential safety and effectiveness of this vaccine approach in humans.

Methodology

Outer membrane vesicles (OMV) from *N. meningitidis* strains, serogroup B: (B:8:P1.6) and serogroup C (C:2a.P1.5) were obtained as previously described.[40] These two strains have been used for our group to produce OMVs with protein compounds slightly different (data not shown), so they deserved to be tested in separated vaccine preparations. Moreover, they belonged to two different serogroups, B and C. Five Swiss female mice per group were immunized with 3 µg/animal of recombinant receptor-binding domain (rRBD) from SARS-Cov-2 protein S complexed to aluminum hydroxide at 0.1 mM plus 10 µg/ml of OMV from *N. meningitidis* strains B:8:P1.6 (prep1) or C:2a.P1.5 (prep2) or rRBD alone, as control. The OMVs were detoxified as previously described.[41] Animals were immunized with two doses of antigenic preparations intramuscullarlly (IM), 15 days apart and two doses intranasally (IN), 7 days apart.[28] The intranasal inoculation was performed with RBD plus OMV, without aluminum hydroxide. Blood samples were obtained by retro-orbital plexus puncture in anesthetized animals, 15 and 45 days after the first immunization dose. Forty-five days after immunization the animals were anesthetized with Dopalen and Anasedan (CEVA, Brazil), sacrificed by cervical dislocation and the spleens were collected for the ELISpot assay. The supernatant from splenocyte culture was collected to IgG measurement. All procedures involving animals were performed following the guidelines of the Brazilian Code for the Use of Laboratory Animals and approved by the ethics committee of CEUA-IAL/Pasteur (protocol number 03/2012 extended to 2022).

Enzyme-Linked Immunosorbent assay (ELISA) from mice sera or from splenocyte supernatant was performed as previously described,[40] except for the adsorption step, in which polystyrene plates (Maxisorp Nunc – Thermo Fisher Scientific, Rochester, NY, USA) were adsorbed with 100 µL/well of a solution containing 1 µg/mL of rRBD diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6, and incubated overnight at 4°C, in a humid chamber. The secondary antibodies (dilution) used were anti-mouse IgG (γ) (1:5.000) or IgA(α)(1:2000) (Kirkegaard & Per Laboratories, KPL, Gaithersburg, MD, EUA).

The Enzyme-Linked Immunosorbent Spot (ELISpot) assay was also performed as described by Trzewikoswki de Lima et al.,[40] with minor modifications. The splenocytes derived from immunized animals were added to the wells at 1×10^6 cells/mL and the cells were stimulated with 2 µg/mL of rRBD, 10 µg/mL concanavalin A (ConA) (Sigma Chemical Company, St. Louis, MO, USA) or added without stimulus.

The significance of the results was evaluated by ANOVA with Tukey post test. *P* values were considered significant when p ≤ 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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