DIPARTIMENTO SCIENZE DELLA VITA

DOTTORATO DI RICERCA IN SCIENZE DELLA VITA

CICLO XXXV

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Immunogenicity evaluation of next-generation vaccine platforms for prevention of Influenza and COVID-19

SETTORE SCIENTIFICO-DISCIPLINARE: MED/42

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A.A. 2022-2023

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1. ABSTRACT

Background: Over the past hundred years, humans have experienced a long list of microbial threats to health. Among them, pandemics represent one of the major burdens for public health, economy and society. Influenza and most recently Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), being two pandemic-risk viruses both transmitted through the respiratory tracts, share many common features, and the constant emergence of new viral variants brings difficulties to permanently control the disease they cause [1]. Development of new, safer, cheaper, high-throughput vaccine platforms (such as DNA- or vector-based vaccines) targeting most conserved and immunodominant viral epitopes, might be the turning point to ensure control of infectious diseases and prevention of pandemics [2].

Aim: The aim of this thesis is to report immunogenicity findings of new vaccine platforms, which may contribute to their development as future vaccination strategies against pandemic-causing infectious diseases, such as Influenza and COVID-19.

Methods: In Project 1, serum samples from BALB/c mice immunized intramuscularly with two different DNA-based Influenza vaccine constructs expressing the Influenza Neuraminidase (NA) protein have been tested in a pseudotype-based Enzyme-Linked Lectin Assay (pELLA) assay, in order to evaluate the presence of post-vaccination NA-inhibiting antibodies.
In Project 2, we evaluated the potential of a *Leishmania tarentolae* (Lt)-purified SARS-CoV-2 recombinant RBD-SD1 antigen ("Lt-RBD") and of a Lt-based vaccine platform expressing SARS-CoV-2 S protein ("Lt-spike") combined with the Lt-RBD (LeCoVax-2) in inducing antigen-specific T cell mediated responses when administered to BALB/c mice via the mucosal (rectal, R) or systemic (subcutaneous, SC) route of immunization. Both Lt-RBD and LeCoVax-2 were used either adjuvanted or not. T cell responses (release of IFN-γ, IL-4, TNF-α) raised after vaccine administration were assessed by Enzyme-Linked ImmunoSpot (ELISpot) assay upon isolation of splenocytes.

Results and Conclusions: Results from both Projects highlight an elicitation of immune responses after BALB/c mice immunization with either DNA-based or Lt-based vaccines.

In Project 1, a positive titer of NA-inhibiting antibodies was especially detected in serum samples belonging to the mice group immunized with a high dose (10 μg) of the two different dbDNA™ vaccines, "Construct 1" and "Construct 2", but an overall positive result was also detected in the group treated with a low dose (1μg) of "Construct 2". These results support the use of the innovative dbDNA™ as DNA-based vaccine platform for Influenza NA and the suitability of the pELLA assay for the immunogenicity assessment of this kind of NA-expressing vaccines.

In Project 2, promising data have been obtained upon evaluation of antigen-specific cytokines-producing T-cell capacity of both the Lt-based platform LeCoVax-2 and the Lt-purified antigen RBD-SD1. When administered via the R route, the purified adjuvanted RBD-SD1 did not induce any detectable T-cell-mediated immune responses, in comparison with the relatively high production of Th1/Th2 cytokines observed after immunization with LeCoVax-2 (especially when adjuvanted). LeCoVax-2, however, was also effective when administered enterally without adjuvant. These results show immunogenicity of this innovative Lt-based platform also by mucosal immunization and pose the basis for further investigations.

In conclusion, our results support further development of the two novel vaccine platforms evaluated. These studies are worthy to be conducted as they might give us a clue not only about the most immunogenic but also affordable and scalable vaccination strategy to use in order to promptly react to the next pandemic [4].

2. INTRODUCTION

2.1 Vaccines for prevention of infectious diseases

Vaccines represent one of the major medical breakthroughs which saved millions of lives in our recent history (e.g., smallpox has been completely eradicated [36]). They rely on the ability of our immune system to respond to, and remember, encounters with specific pathogen antigens. A vaccine is indeed nothing more than a biological product capable of safely inducing an immune response that confers protection against infection and/or disease on subsequent exposure to specific pathogens [6]. For this reason, vaccines are regarded as the primary strategy in the prevention and control of infectious diseases such as Influenza and the recently emerged COVID-19, particularly in high-risk groups**.**

Currently licensed seasonal flu vaccines can be mainly of two types: live attenuated and inactivated influenza vaccines. Both contain glycoproteins from Influenza viruses capable of triggering an immune response in the host, and they can be either trivalent or quadrivalent in their composition [40].

So far, multiple vaccines against COVID-19 have either completed clinical trials or are currently in clinical evaluation. The most common vaccine types include traditional vaccines like inactivated and recombinant protein vaccines, as well as some novel vaccines like RNA-based, DNA-based and non-replicating viral vector vaccines.

Like influenza vaccines, COVID-19 vaccines rely on inducing immune responses against the main immunogenic antigen(s) of the virus [41].

2.2 Vaccine Immunity

Vaccines mediate protection by inducing effector mechanisms (cells or molecules) capable of controlling pathogen infection. B cells have a predominant role in the efficacy of current vaccines, by production of antibodies (“humoral immunity”) that bind specifically to foreign antigens and neutralize pathogens. The early protective efficacy is indeed conferred by antigen-specific antibodies. Among them, systemic IgG and mucosal IgA responses have a

crucial role in viral disease protection, neutralizing viral particles and mediating effector cells control of infection [37, 38].

However, the importance of T cells response ("cellular immunity") should not be underestimated. Cellular immunity is essential for the development of high-affinity antibodies and immune memory to reach an effective long-term protection, thus representing the principal target for future vaccination strategies [8]. The value of T cell immunity is exemplified by recent evidence, which suggest that a key role in recovery from COVID-19 disease is played not only by neutralizing antibodies, but also by memory T cells. As example, Zhang et al. [47] found that, while antibody levels tended to decline, memory T cells remained comparatively stable over time, suggesting that T cell responses might provide more durable protection than antibody-mediated immunity.

T cells are mainly divided into cytotoxic CD8+ (Tc) and helper CD4+ (Th) T lymphocytes. Tc are effector cells capable to limit the spread of infectious agents by recognizing and killing infected cells or secreting specific antiviral cytokines. On the other hand, Th cells contribute to protection through cytokine production and provide support to the generation and maintenance of T and B cell responses. There are two main subclasses of Th cells, Th1 and Th2, depending on their main cytokine production (e.g., IFN-γ and TNF-α for Th1, IL-4 for Th2) [8] (**Figure 1**).

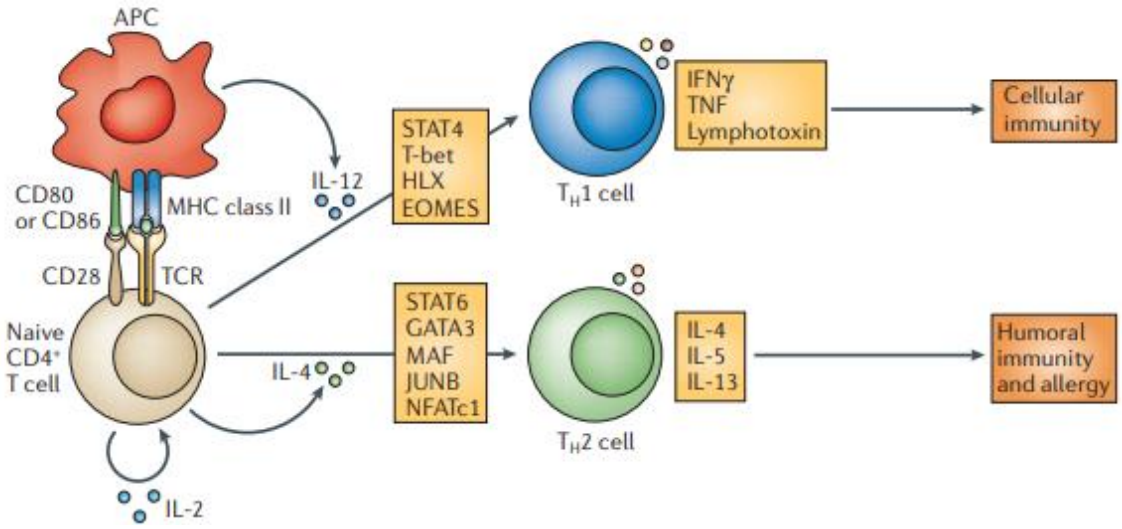


Figure 1. T helper cell populations. The Th1 subset of CD4+ T cells produces pro-inflammatory cytokines, such as IFN-γ and TNF, and induces cell-mediated immune responses. The Th2 subset generally secrets cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate, and is associated with humoral immunity and allergy. [42].

The nature of the vaccine antigen directly affects the type of effector cells involved into the host immune response. It is therefore of primary importance to identify the best candidate vaccine antigen able to elicit a balanced response from all counterparts.

In fact, prevention of infection is achieved only through vaccine-induced antibodies, but disease attenuation and protection against complications is supported by T cells in the so-called T-dependent immune response [8].

2.3 Next-generation vaccines

Vaccination represents the best way to control the spread of infectious diseases among the population [7]. However, current vaccine platforms show some limitations, as for example the high costs of production and the low safety and immunogenicity profile.

Furthermore, many diseases are not yet fully preventable through vaccination, and specific high-risk populations cannot be efficiently and safely targeted by current vaccine programs (e.g., children, elderly, pregnant women, immunodeficient and chronically ill patients) [4].

Currently available prophylactic vaccines are indeed mainly based upon conventional and well-established platforms, such as live-attenuated, inactivated or subunit vaccines. However, many of them required multiple doses to reach an effective long-lasting immunity, or even annual reformulations (as in the case of Flu vaccines). Some formulations (e.g., live-attenuated vaccines) could also be at risk of side effects for the host [36].

For all these reasons, researchers around the world daily focus their attention on the discover of more effective, safer, inexpensive and with long-lasting immune response vaccines.

An ideal vaccine should be quick and easy to produce in large quantities, safe and capable to induce a proper immune response against specific antigens effectively presented to the host immune system. Indeed, effective containment of an infectious disease depends on the immediate availability and application of a prophylactic vaccine.

The unexpected global emergency caused by COVID-19 pandemic has underlined the urgent need of discovering new innovative platforms allowing the rapid development of more cost-

effective vaccines targeting infectious diseases [20]. Recently, new approaches offering advantages over limitations of traditional vaccines have been developed.

2.4 DNA-based vaccines

Manufacture of vaccines using synthetic methods of production made possible the development of much safer vaccines, as well as the prevention and/or attenuation of infectious diseases previously untargeted through early vaccination methods.

The best example is represented by nucleic acid-based vaccines, which combine the advantages of *in situ* expression of antigen with the safety of subunit vaccines. It was demonstrated in the 1990s that when antigen-encoding nucleic acid is introduced into the body it is capable to induce an immune response against the encoded antigen [10].

DNA-based vaccines also offer the potential to be relatively simple and inexpensive to manufacture, allowing large-scale production [4].

The field of DNA vaccinology is developing very rapidly. Together with the improvement of vaccine safety and stability (at different temperatures and pH), DNA vaccines have the great ability to elicit both B- and T-cell immune responses [9].

However, although they are now widely licensed and commercially available for veterinary use, and despite they show promising results in pre-clinical models, the progression of DNA vaccines into clinic does not seem to take fold, likely due to a poor delivery into human cells combined with an insufficient stimulation of the human immune system [4].

Additionally, most DNA vaccines are plasmids and must be grown into bacterial cultures (generally of *E. Coli*), necessitating unnecessary and/or undesirable elements (e.g., antibiotic resistance genes), thus going to affect vaccine safety profile. Furthermore, using bacteria as site of production can likely result into events of recombination which can in turn lead to the loss of the antigenic determinant expressed by the plasmid [10].

Synthesizing the vaccine entirely *in vitro* without a bacterial step could overcome these limitations and ensure a batch-to-batch uniformity. This has recently become possible by using enzymes derived from bacteria and bacteriophages in a proprietary method from

Touchlight Genetics company. Indeed, through controlled batch reactions, this company was able to synthetically develop novel, covalently closed, double-stranded, linear DNA constructs, named as "Doggybones" (dbDNA™) for their proposed shape (**Figure 2**). These constructs solely encode an antigen expression cassette, composed by antigen, promoter, polyA tail and telomeric ends [10].

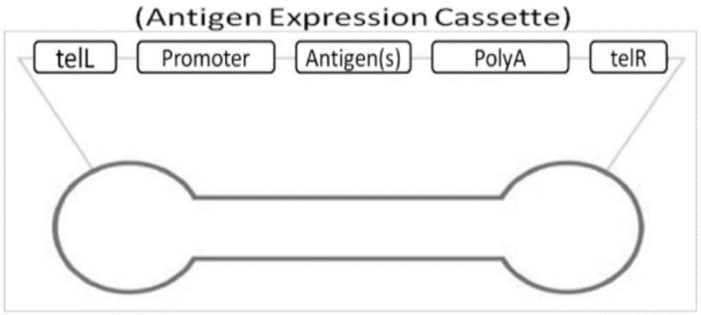


Figure 2. Schematic representation of a dbDNA™ construct (Doggybone). Doggybones are generated after *in vitro* amplification of a linear double-stranded covalently closed DNA using Rolling Circle Amplification (RCA) strand displacement DNA polymerase in combination with TelN protelomerase. Rolling circle replication takes place from a starting plasmid resulting in concatamers which are then resolved through the actions of TelN on the *telRL* sites included in the sequence. Addition of restriction enzymes and exonuclease removes any contamination from the plasmid backbone sequences to leave the cassette Doggybone only. The end product is a linear dsDNA construct flanked by ssDNA hairpins (Doggybone) [10].

The developers have demonstrated that Doggybones are comparable to conventional plasmids in terms of expression and immunogenicity, even if administered at a lower dosage [10], thus representing a valid, minimalistic, more stable, affordable and cost-effective alternative. Furthermore, being an effective DNA vaccine easily producible on a large scale by enzymatic processes, Doggybones would ensure rapid vaccine design and manufacture [10, 12].

2.5 *Leishmania tarentolae* as an antigen delivery platform

Leishmania tarentolae (*L. tarentolae*, or Lt) is a eukaryotic protozoan sand fly-transmitted parasite having reptiles (mainly geckos) as vertebrate host. It has recently been discovered as an advantageous and promising biotechnological expression tool. One of its major applications is as an easy-to-handle vehicle for human recombinant DNA/protein expression [19] (**Figure 3**). It was indeed demonstrated that most strains of the *Leishmania* species are

non-pathogenic to humans and other mammals (e.g., the Jena Bioscience commercially available Lt strain P10, mostly based upon the Lt TARII/UC strain [19]). They are classified as biosafety level class I organisms, therefore representing a perfect recombinant protein antigens production machinery for both prevention and detection of emerging viral epidemics [20].

This alternative microbial eukaryotic expression system has indeed many advantages compared to other heterologous ones, such as prokaryotes (*E. Coli*), yeasts, insect cells or even mammalian cell lines (HEK293). It is relatively quick, easy and cost-effective to culture, hence applicable to a large-scale industry production even in countries that lack high-technology cell factories. It allows the production of robust recombinant protein yields, following an extraordinary homogeneous human-like glycosylation pattern (missing in prokaryotic systems). As a vehicle for antigen delivery, it has also the capacity to overcome major drawbacks of plasmid DNA vaccine candidates, generally inefficient in intracellular delivery, therefore resulting in low gene expression levels followed by a limited elicitation of the immune responses [19, 20]. Protozoa of the genus *Leishmania* have the capability of effectively targeting immune phagocytic cells, such as macrophages and dendritic cells (DCs), delivering an expressed and/or surface-exposed protein antigen to these cells; indeed, after inoculation of the engineered Lt into subcutaneous tissues of mammals, they are internalized and therefore able to activate DCs, which in turn maturate and initiate a polarized (Th1-like, especially if adjuvanted with immune-modulating molecules) adaptive immune response against the specific antigen of interest [21].

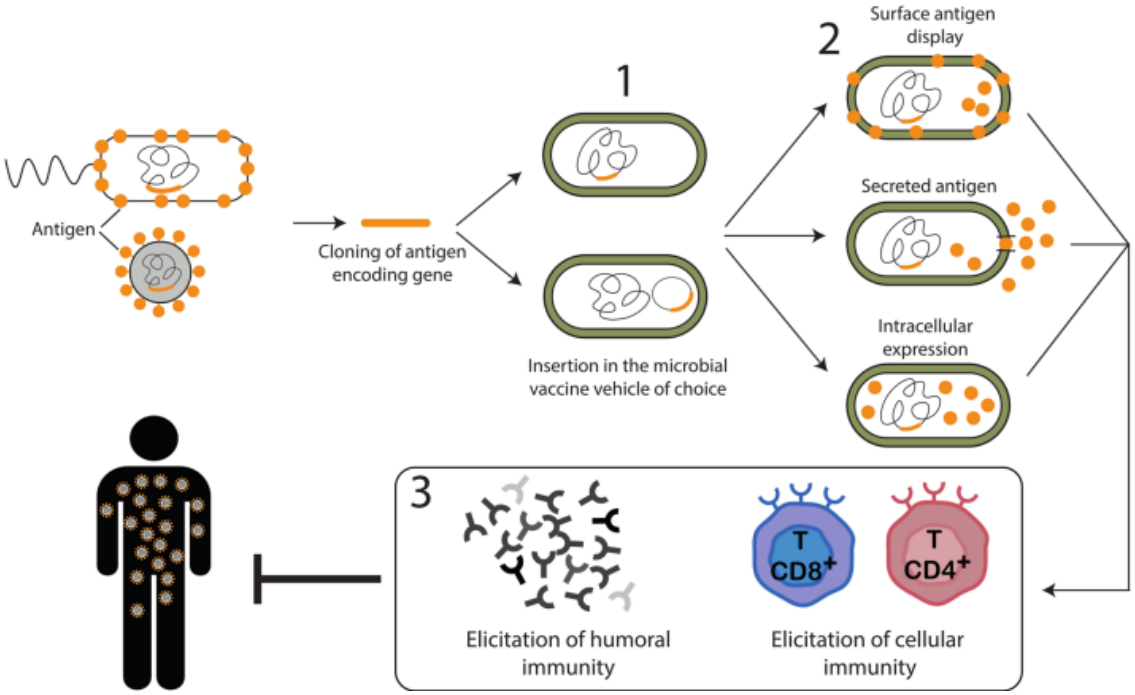


Figure 3. Schematic representation of a microbial vector in novel vaccine strategies. A gene encoding for a pathogen antigen is cloned and expressed into a microbial vaccine vehicle (such as *L. tarantolae*). Different types of expression could be exploited, such as surface antigen display, secretion of the antigen, or intracellular expression of the antigen. The selected microbial vehicle is then used for the immunization of the host with the aim of eliciting cellular and humoral immunity against the expressed antigen of interest [43].

Thanks to all these features, *L. tarantolae* could be one of the best candidates as antigen expression system to face outbreaks of new infectious pathogens (such as SARS-CoV-2), giving us the possibility to rapidly reproduce and deliver emerging viral antigens of interest mimicking as best as possible what is produced by a human virus during its natural infection cycle [20].

2.6 RNA viruses with pandemic potential

Among viruses, those with RNA genome have been recognised as the leading cause of pandemics in our recent history. We can currently identify Influenza and SARS-CoV-2 as the two major representatives of this category. Both transmitting through the respiratory tracts and causing mild, severe or even asymptomatic respiratory syndromes, these two pandemic-risk viruses can spread globally in a short time [1].

Influenza and SARS-CoV-2 viruses undergo through antigenic changes over time (known as "antigenic drift"), which allows the emergence of new viral strains able to escape any pre-existing protective immunity acquired by population with previous infections and/or vaccinations. This is the reason why, for example, we need to regularly update Influenza vaccines every year against predicted circulating strains [13], and this is most likely what we should expect (and in part already do) with the recently emerged SARS-CoV-2 virus.

The main characteristics and structure of Influenza and SARS-CoV-2 viruses are reported in **Figure 4**.

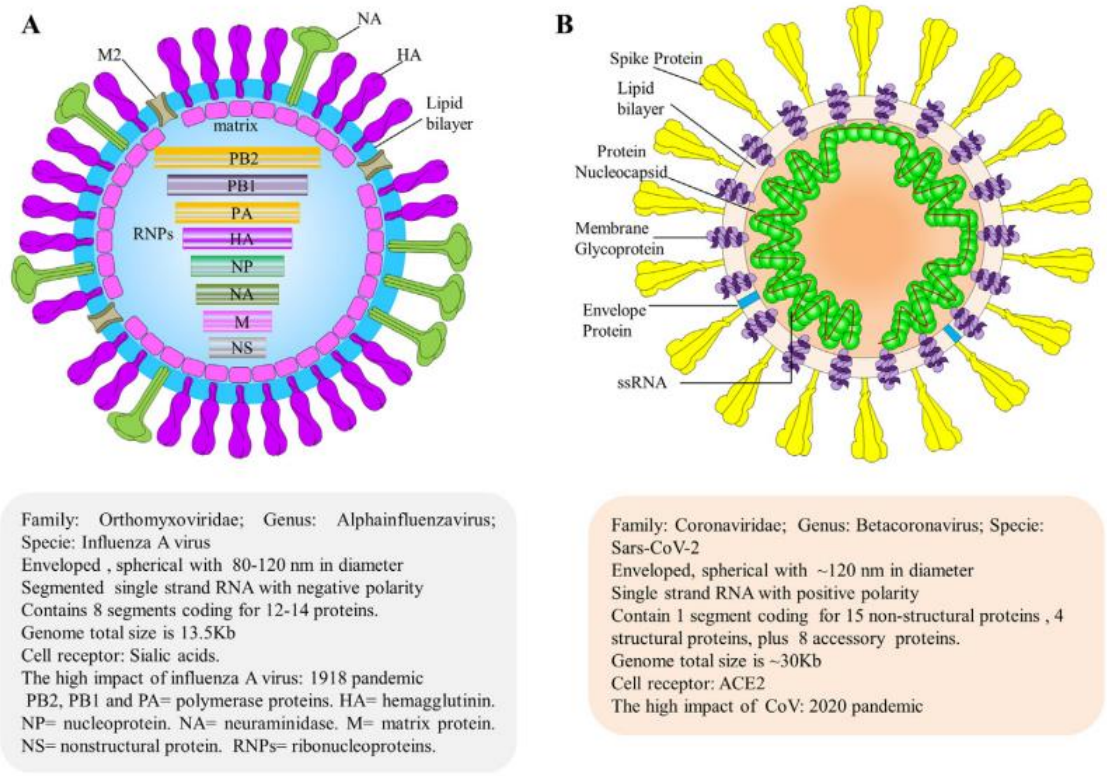


Figure 4. Human Influenza A (A) and SARS-CoV-2 (B) virions, with main structural and genome properties [23].

Influenza A virus (IAV) is the most common cause of worldwide human annual epidemics and occasional pandemics [27], causing 3-5 million cases and a mean of 500'000 deaths every year [13, 26]. Hemagglutinin (HA) and Neuraminidase (NA) are its major surface glycoproteins, which can combine to give rise to many different IAV subtypes. At the same time, these two proteins cooperate and have opposite functions, both necessary for virus infection. In fact, both interact with human sialic acids (SAs) attached to host cell surfaces

glycolipids and glycoproteins: HA binds them allowing viral entry by endocytosis, while NA cleaves and removes them in order to release virus progeny from infected cells [26].

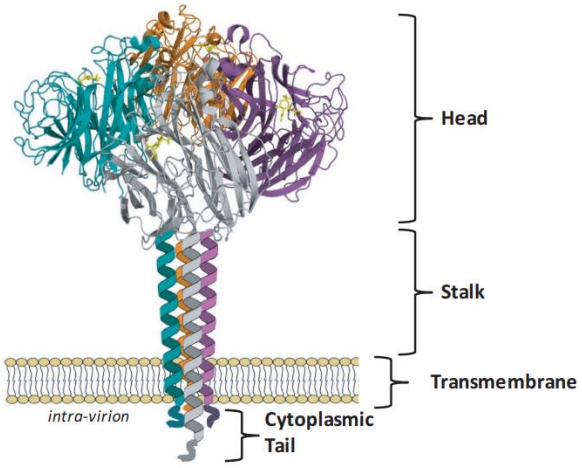


Figure 5. Influenza virus exposed tetrameric NA protein. NA is a tetramer of four identical monomers, each consisting of four distinct structural domains: the catalytic head, the stalk, the transmembrane region and the cytoplasmic tail [24].

NA (**Figure 5**) is the second most abundant surface glycoprotein on influenza viruses (generally expressed in a 1:4 ratio respect to HA), and as a target for current influenza vaccines contributes towards protection [15, 22].

With its enzymatic sialidase activity, NA is fundamental for the virus not only to mediate viral budding and so the release and spread of newly formed viral particles from infected cells. It also prevents aggregation of viral progeny and improve human mucus penetration (freeing virus from sialylated host mucins) [15, 16].

Like the immunodominant HA protein, also the NA is always subjected to antigenic changes over time, but with a lower rate. Therefore, for its consistency and all the reasons mentioned above, NA is being investigated as an alternative or adjunct antigen within currently available HA-targeting vaccines, giving more possibilities for the development of an “universal” or “cross-subtype” Influenza vaccine. Since antibodies elicited against NA, for its intrinsic viral activity, do not prevent viral entry, they cannot be classified as “neutralizing” antibodies. However, NA-inhibiting antibodies are important to prevent disease and reduce the severity of symptoms, decreasing viral load and cell-to-cell transmission [15, 16].

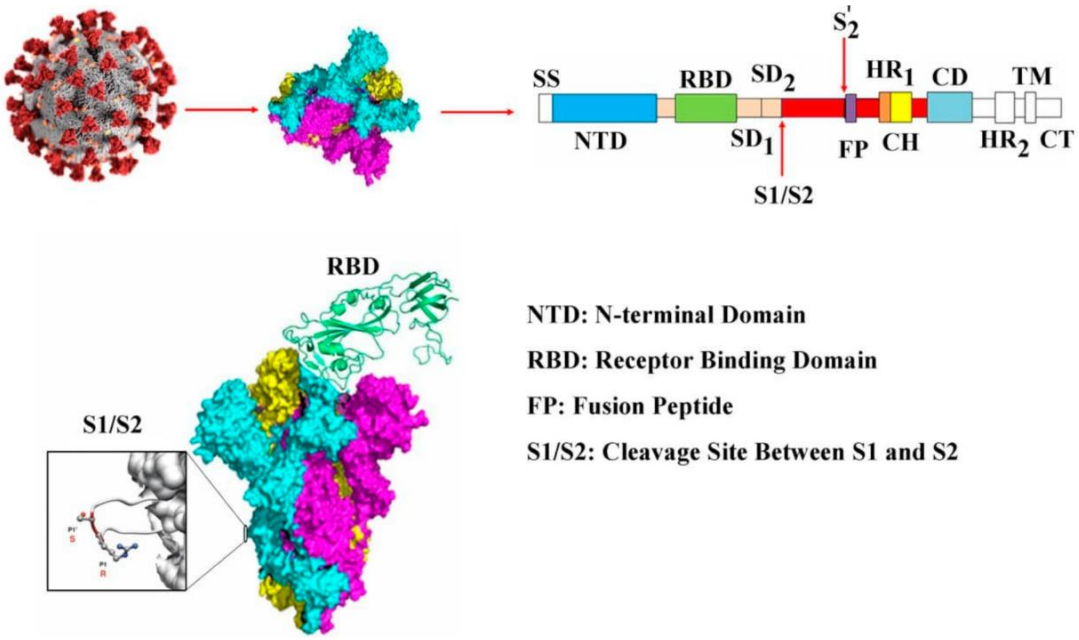


Figure 6. Schematic representation of SARS-CoV-2 spike protein. Different domains are shown by different colors. SS, single sequence; NTD, N-terminal domain; RBD, receptor-binding domain; SD1, subdomain 1; SD2, subdomain 2; S1/S2, S1/S2 protease cleavage site; S2', S2' protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail [25].

The novel SARS-CoV-2 virus is the causative agent of COVID-19 pandemic started in 2020 [28], which has caused more than 600 millions of confirmed global cases and more than 6 millions of deaths up to now [30]. SARS-CoV-2 viral entry into host cells is mediated by the Angiotensin-Converting Enzyme 2 (ACE2) receptor present on different human cell types, which is bound by the Receptor Binding Domain (RBD) of the viral surface Spike (S) glycoprotein. Upon this binding, S protein undergoes proteolytic activation and different conformational changes, allowing membrane fusion and subsequent viral genome entry [28].The S protein (**Figure 6**), which gives to the virus its unique "crown-like" appearance (hence the term Coronavirus), is composed by two functional subunits: S1, including the Receptor Binding Domain (RBD), which is responsible for binding to the ACE2 receptor; and S2, which includes the fusion peptide, responsible for viral entry into target cells [25, 28, 31]. Due to its central role in virus infection and propagation, the S protein represents the main immunodominant protein of SARS-CoV-2 viruses, and it is targeted by neutralizing antibody responses arised through natural infection and/or vaccination [29]. However, given that residues present on S-RBD portion mutate quite frequently, SARS-CoV-2 is able to escape pre-existing immunity and to give rise to new viral variants periodically [28].

Among all S domains, subdomain 1 (SD1), present on S1 subunit and probably involded in spike homotrimers association and stability, has been recognized as one of the most conserved neutralizing epitopes among SARS-CoV-2 viruses, even if it is hard to target since generally occluded and only accessible during transient conformational changes [29]. Indeed, being SD1-targeting antibodies only a small percentage of circulating neutralizing antibodies compared to those against the trimeric S-RBD, they represent a minor cause of immune selective pressure for virus evolution, hence they could be effective against all variants [29].

2.7 New correlates of protection

Currently available vaccines mainly elicit preventive humoral immune responses, which are identified as the universal correlate of protection for common infectious diseases. Antibody responses triggered by vaccination or natural infection are mostly directed towards viral immunodominant surface antigens included in the vaccine or present in the circulating variant. The result is the development of a strain-specific immunity [27] which is however easily escapable by the costant emergence of new viral variants. Targeting more conserved viral epitopes with future vaccination strategies could be a promising strategy to achieve a broader heterosubtypic immunity, with a superior and long-lasting efficacy in the population. To reach this goal, the collaboration and the homeostatic balance between humoral and cellular responses become of primary importance.

The assessment of humoral responses through classical serological methods (e.g., the standardized haemagglutinin inhibition assay used to evaluate and license new Influenza vaccines) is well estabilished globally. On the contrary, evaluation of cell-mediated immune responses is still confined to expolarotory research purposes, due to a general misalignment among laboratories around the world and a certain variability related to methods' procedures (including reagents and equipment used) [27].

This brings into focus the need for more harmonized and standardized cell-mediated immunity (CMI) assays, such as flow-cytometry based and ELISpot assays, in order to facilitate site-to-site comparisons in the field of clinical trials for "universal vaccines" development [27].

The ELISpot assay (**Figure 7**), first described in 1983 [32], has recently obtained a lot of popularity among vaccines-related clinical trials. In fact, it is widely recognised as an extremely sensitive assay, capable to give an accurate and quantitative measure of vaccine immunogenicity in terms of specific T and B cell responses [27, 32]. For example, upon appropriate *in vitro* stimulation of isolated human peripheral blood mononuclear cells (PBMCs) or murine splenocytes with a pre-determined concentration of a specific antigen, it is possible to enumerate how many specific single T cells have secreted one or more particular cytokines in response to that specific stimulation [27]. In this way, we are able to mimick *in vitro* the behaviour that T cells would have *in vivo* during a second encounter with a pathogen, against which a recipient (animal or human) has been immunized through previous infection and/or vaccination. Therefore, the ELISPOT assay represents one of the best means to evaluate immunogenicity of specific vaccine antigens in terms of cell-mediated responses.

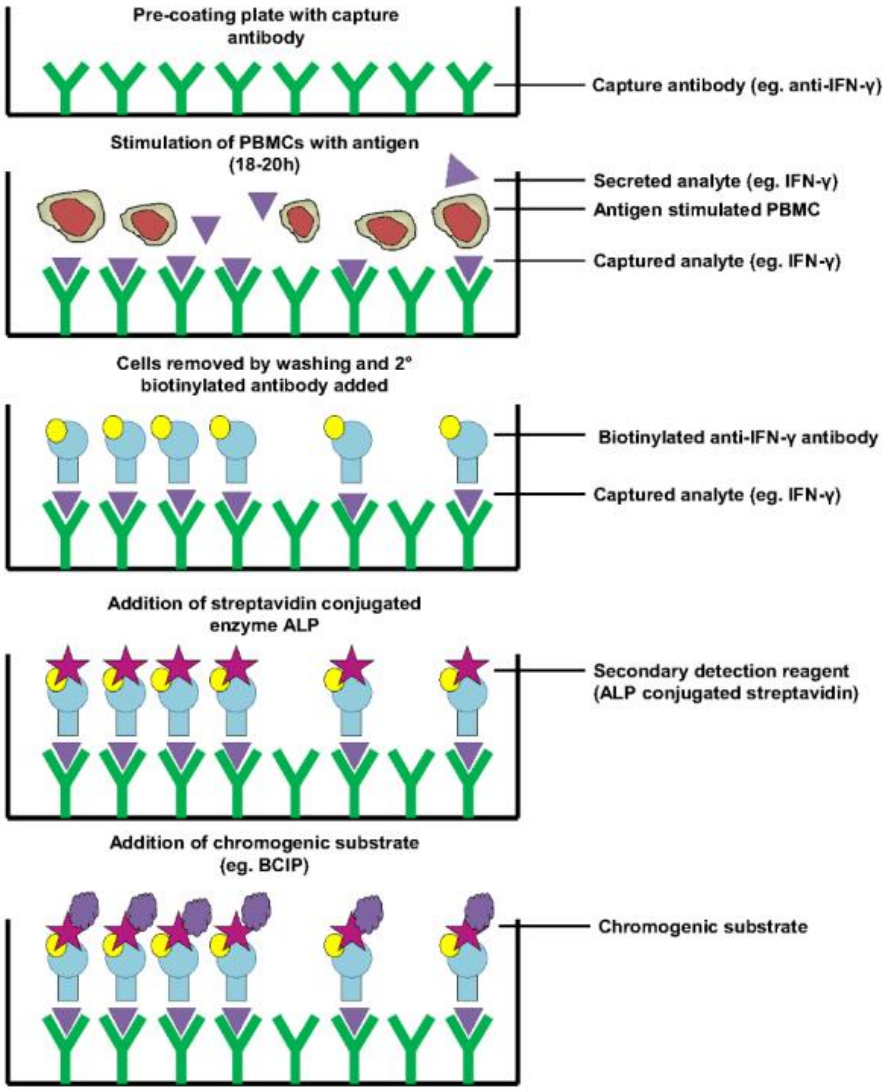


Figure 7. Schematic illustration of the ELISpot assay. The ELISpot assay involves the isolation of cells such as peripheral blood mononuclear cells (PBMCs) or splenocytes, and their addition to a capture antibody-coated plate (e.g., anti-IFN-γ). Cells are then stimulated with a specific antigen. In the presence of the stimulus, antigen-specific T-cells present in the cell population will secrete cytokines (e.g., IFN-γ) which can be captured by the antibody used to coat the plate. Following stimulation period (usually 18–20 h), the cells are removed by washing and the bound cytokine typically detected using a secondary detection reagent conjugated to an enzymatic label (e.g., alkaline phosphatase—ALP). The enzyme catalyzes the colorimetric spot formation when in the presence of a chromogenic substrate (e.g., 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt—BCIP). Other enzymes and chromogenic substrates can be used for development of ELISpots, such as horseradish peroxidase followed by addition of the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) [27].

3. AIM OF THE THESIS

The aim of the present thesis is to report immunogenicity findings of two novel vaccine platforms for the prevention and control of Influenza and COVID-19 infections.

Project 1 is focused on Influenza prevention through DNA-based vaccine constructs (dbDNA^{TM}) expressing NA, which elicit NA-inhibiting antibodies that can be evaluated through a pseudotype-based ELLA assay. Being NA a more conserved antigen as compared to HA (which is commonly used as Influenza vaccine antigen), vaccine formulations that include NA have the potential to confer a more cross-reactive response, that may possibly cover mismatched Flu strains of the same subtype. Using dbDNA^{TM} as vectors to express NA may provide a rapid, easy to reproduce and broadly protective Influenza vaccination strategy.

Project 2 reports the outcomes of one of the first attempts of Italy to develop its own COVID-19 vaccine. In 2021, thanks to the collaboration between the University of Milano and VisMederi, an innovative *Leishmania tarentolae* (Lt)-based SARS-CoV-2 RBD-SD1 production system and a Lt-based vaccine platform expressing SARS-CoV-2 S protein were developed. In our study, a Lt-purified RBD-SD1 (or "Lt-RBD") and a Lt-based vector expressing SARS-CoV-2 whole spike protein ("Lt-spike") combined with RBD-SD1 (LeCoVax-2) were investigated as vaccine strategies (adjuvanted or not) either administered subcutaneously or via the enteral way to BALB/c mice. Antigen-specific T cell responses were evaluated by ELISpot to assess the effectiveness of the different vaccines in the elicitation of cellular immune responses. Our aim was to demonstrate the suitability of the Lt, a protozoan parasite, as a potential antigen vehicle for mucosal immunization. This latter may have advantages over systemic immunization not only at a practical level but also in terms of induction of T-cell-specific responses.

The promising results obtained in Project 1 and Project 2 may contribute to the development of the above-mentioned antigen-expressing platforms (NA-expressing dbDNA^{TM} constructs and LeCoVax-2) as future vaccine candidates. Their use may potentially provide the effective, scalable, affordable, easy to administer and universally protective vaccination strategy needed to protect people worldwide and promptly react to the next pandemic.

4. PROJECT 1

4.1 Aim of the project

The aim of this project was to evaluate the immunogenicity potential of two dbDNA™ vaccine constructs against Influenza virus infection.

4.2 Materials and Methods

BALB/c mice were immunized with two different doses of two different dbDNA™ vaccine constructs expressing Neuraminidase 1 (N1) protein from H1N1 A/California/07/2009 Influenza virus. Intramuscular immunization in 50 µl PBS was followed by electroporation (EP) of the injection site, which has been demonstrated to improve immunogenicity increasing cell transfection and thus leading to improved antigen expression and adjuvanticity [12]. At Day 28 after priming, mice were challenged with A/California/07/2009 virus (2000 PFU). Bleed was performed at day 32 and serum samples obtained were tested on a Pseudotype-based Enzyme-Linked Lectin Assay (pELLA) in order to evaluate the presence of NA-inhibiting antibodies.

4.2.1 Pseudotypes-based Enzyme Linked Lectin Assay (pELLA)

ELLA is a semi-quantitative assay capable of detecting the presence of antibodies directed against the Neuraminidase (NA) protein of the Influenza virus. The assay was developed in order to assess NA-inhibiting (NI) antibody titers in serum samples following Influenza virus natural infection and/or vaccination with NA-containing vaccines. First described in 1990 [17] and optimized in 2014, ELLA is a subtype-specific and reproducible assay for routine evaluation of human antibody responses to NA [18]. Antigen source can vary between mismatched viruses, purified NA, treated whole virus and pseudovirus particles, also known as pseudotypes. Pseudotyped viral particles (PVs) are safe chimeric “viruses” in which the outer (lipid envelope exposed) surface glycoproteins of a virus of interest (e.g., Influenza virus) are combined with the replication-defective viral core of another virus (e.g., HIV in the case of lentiviral pseudotypes). PVs allow for accurate, rapid, sequence-directed antiviral screening in a low containment biosecurity level, offering a safe and efficient alternative to reassortant mismatched or Triton-X-treated wild-type viruses for serological assays made to study emerging RNA viruses with pandemic potential [13, 14]. In this project, Influenza

lentiviral pseudotypes bearing on their surfaces a combination of an avian haemagglutinin (H11) and a human neauraminidase (N1) have been developed and used as the antigenic source for a pseudotypes-based ELLA (pELLA). This combination enables the detection of specific antibody responses against the human circulating NA subtype [15], avoiding interference of the immune responses generally elicited by current Influenza vaccines against the immunodominant HA protein. At the same time, it was shown that co-expression of HA with NA improves the release of newly formed pseudotyped lentiviruses [15].

The ELLA assay is based on the enzymatic activity of the NA which allows it to cleave sialic acids (SAs) bound to the terminal regions of oligosaccharides present on cellular and viral glycoproteins expressed by infected cells [16]. To perform the pELLA assay, 96-wells plates have to be coated with fetuin, a highly glycosylated serum protein which represents our source of SA-terminal galactose linkages. Adding a NA protein source to each well, fetuin terminal SAs will be cleaved exposing the penultimate galactose sugar. Peanut agglutinin (PNA), which is a lectin from *Arachis hypogaea* with a specificity for the galactose, conjugated with a horseradish peroxidase (HRPO), will bind to galactose residues. The final addition of a chromogenic peroxidase liquid substrate such as the 3,3′,5,5′-Tetramethylbenzidine (TMB), followed by that of a 0.5M Hydrochloric Acid solution (HCl) to stop the reaction, allows colorimetric quantification of the extent of desialylation, and so a measure of the NA enzymatic activity. The Optical Density (OD) measured through a luminometer at 450 nm is indeed proportional to this activity [22].

A schematic illustration of the pELLA assay is shown in **Figure 8**.

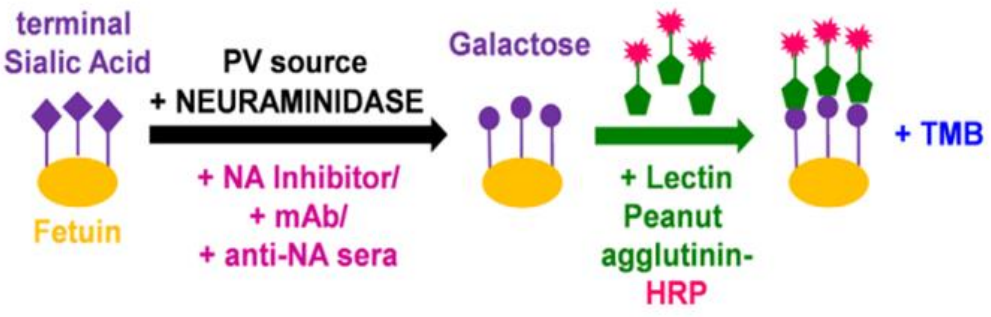


Figure 8. Schematic illustration of a pseudotypes-based Enzyme-Linked Lectin Assay (pELLA). In the pELLA, the substrate fetuin is coated on each well of a 96-well plate. Once added, the Neuraminidase (NA) Pseudovirus (PV) cleaves the terminal sialic acid (SA) residues of the substrate fetuin, exposing galactose residues. The terminal galactose residues exposed by NA cleavage are specifically recognized by the substrate lectin peanut agglutinin conjugated to horseradish peroxidase (HRP), and addition of a peroxidase substrate such as TMB results in a detectable color change that can be measured at OD_{450}. In the presence of NA-inhibing antibodies, NA cannot exert its sialidase action, and this can be indirectly measured via the pELLA [16].

To assess the capability of sera from naturally infected or appropriately vaccinated subjects to inhibit this NA activity through the presence of NA-inhibiting (NI) antibody titers, serial dilutions of the sera are incubated on fetuin-coated plates with a fixed quantity of NA source at 37°C overnight.

Prior to the execution of a pELLA, the antigenic source to use in the assay must be titrated. The appropriate dilution of pseudovirus (PV) to use is the one which gives us the 90% of the maximum OD_{450} signal obtained, and it should be at least 10-fold higher than that of the background (example of a PV titration in **Figure 9**).

Virus Titration	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Background
Column/Row	1	2	3	4	5	6	7	8	9	10	11	12
Row A	3,5	3,5	3,5	3,141	2,372	1,68	0,915	0,57	0,752	0,383	0,367	0,585
Row B	3,49	3,5	3,5	3,227	2,553	1,723	1,034	0,544	0,635	0,275	0,375	0,166
Row C	3,428	3,5	3,332	3,176	2,491	1,687	0,986	0,776	0,459	0,659	0,295	0,213
Row D	3,5	3,371	3,5	3,044	2,171	1,582	0,878	0,545	0,536	0,34	0,306	0,234

Figure 9. Example of a PV titration in ELLA. Four replicates (A-D rows) of the same PV source have been 2-fold diluted from column 1 to column 11. Column 12 was reserved to the background. The mean of the replicates OD_{450} values obtained for each PV dilution and for the background has been considered for calculation, and 1:6 has been chosen as the appropriate dilution of PV to use in the pELLA assays of this project.

Once obtained the information on the appropriate fixed amount of PV to use, it can be used both as viral input and as positive virus control (VC) (column "VC" in **Figure 10**) during serum samples testing. The 50% of the mean of all OD_{450} values obtained in this column represents the cut-off to discriminate the presence of NI in each serum. The reciprocal of the highest serum dilution which results in at least 50% inhibition of the maximum signal is regarded as the NI titer ("positive" if ≥ 10), reported as the 50% end-point titer.

VC	10	20	40	80	160	320	640	1280	2560	5120	Background	Titre	Sample ID
3,454	3,493	3,345	3,332	3,275	3,212	3,155	3,282	3,211	3,135	3,17	0,253	5	1
3,16	3,207	3,271	3,276	3,292	3,316	3,343	3,353	3,34	3,391	3,361	0,229	5	
3,194	0,98	1,087	1,346	1,679	1,89	2,302	2,745	3,107	3,153	3,308	0,295	40	2
3,421	0,958	1,136	1,382	1,664	2,066	2,342	2,78	3,094	3,296	3,307	0,214	80	
3,408	0,628	0,715	0,961	1,164	1,688	1,925	2,497	3,011	3,122	3,286	0,184	80	3
3,372	0,657	0,723	1,052	1,156	1,489	2,036	2,469	2,887	3,038	3,16	0,367	160	
3,44	2,406	2,695	3,017	3,018	3,114	3,222	3,156	3,153	3,248	3,242	0,376	5	4
3,386	2,454	2,693	3,01	3,118	3,253	3,101	3,211	3,184	3,212	3,212	0,5	5	

Figure 10. Example of a pELLA plate. Four different samples have been tested in duplicate and 2-fold diluted (starting from a 1:10 dilution) from column 2 to column 11, followed by the addition of the previously determined appropriate amount of PV. Column 1 (VC) has been reserved to the positive control (PV only), while column 12 to the background (wells without antigen and NI sources). On the right, sample IDs information has been reported, as well as NI titer results obtained for each replicate of each sample (one-fold difference between the two replicates titers is accepted).

4.2.1.1 Antigen titration

Coating buffer 1X is prepared diluting 10 ml of coating buffer (10X) with 90 ml of deionized water. The coating buffer is then used to prepare both stock (25 mg/ml, stored in aliquots at -20°C) and working (25 µg/ml, prepared immediately before use) fetuin solutions. Nunc® flat-bottom 96-wells plate with MaxiSorp™ surface (with high-protein binding capacity) are then coated with 100 µl of fetuin working solution, covered with plate sealers and stored at 2-8°C until use (at least 24-48 hours and for a maximum of 2 months). On test Day 1, plates are washed 3 times with 300 µl of wash buffer (0.01M PBS, pH 7.4, 0.05% Tween 20 solution in 1 l of deionized water) and placed under a biosafety cabinet, where 50 µl of sample diluent (1% BSA, 0.5% Tween 20 solution in 100 ml of Dulbecco's 1X PBS with 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$) are added to all wells in rows A-D, columns 2-11. 100 µl of pure PV are added to column 1 (rows A-D) and 2-fold serial dilutions are performed by transferring 50 µl from one well to the next one (in rows A-D, columns 1-11). Then, 50 µl of sample diluent are added to each well of rows A-D, columns 1-11. Only 100 µl of sample diluent are added to column 12 (rows A-D) as background. The plates are then covered with a plate sealer and incubated overnight (16-18 hours) at 37°C in a humidified incubator. On Day 2, plates are washed 6 times with 300 µl of wash buffer. Then, 100 µl of PNA-HRPO solution, prepared at 1 mg/ml in conjugate diluent (1% BSA in 100 ml of Dulbecco's 1X PBS with 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$), stored in aliquots at -20°C and further diluted 1:1000 in conjugate diluent immediately before use, are added to each well. Plates are then incubated for 2 hours at room temperature (RT) in the dark and washed again 3 times with 300 µl. After that, 100 µl of TMB are added to each well, followed by a short incubation of 10-15 minutes at RT in the dark and by the adding of 100 µl/well of 0.5 M HCl to stop the reaction and read the plates.

4.2.1.2 Serum titration

Serum samples were heat-inactivated 30-60 minutes at 56°C for complement inactivation, in order to avoid related unspecific binding signals in immunological assays.

The general assay protocol for serum titration is the same described above for antigen titration. Under the biosafety cabinet, 50 µl of sample diluent are added to whole columns 1 (PV control) and 3-11 of washed fetuin-coated plates. 90 µl of sample diluent are instead added to all wells of column 2. Afterwards, 10 µl of each serum sample are added in duplicate to the wells of column 2 (e.g., 10 µl of the first sample are added in wells 2A and 2B). Serial

2-fold dilutions of each serum replicate is performed by transferring 50 µl from each well of column 2 to the next until column 11. In the meantime, PV solution at the previously chosen dilution is prepared in sample diluent and 50 µl are added to all wells except for column 12, in which only 100 µl of sample diluent are added as background.

4.2.2 Pseudotypes production

Different plasmids are needed for production of Influenza pseudotypes (**Figure 11**). For H11N1 lentiviral pseudotypes production, the following plasmids quantities have been used: 0.5 µg of human NA1 protein expressing plasmid, 0.5 µg of avian HA11 plasmid, 0.5 µg of p8.91 (Gag-Pol genes from HIV packaging structure) and 0.75 µg of pCSFLW as reporter plasmid (firefly derived luciferase). The total amount of plasmid DNA was of 2.25 µg. This amount was used to transfect each well of the 6-wells plates with HEK293T cells at the right confluence. Polyethylenimine (PEI) has been selected as transfection reagent, in a ratio of 3 µl of PEI per each µg of DNA, so in our case the total amount of PEI considered per each well was 6.75 µl.

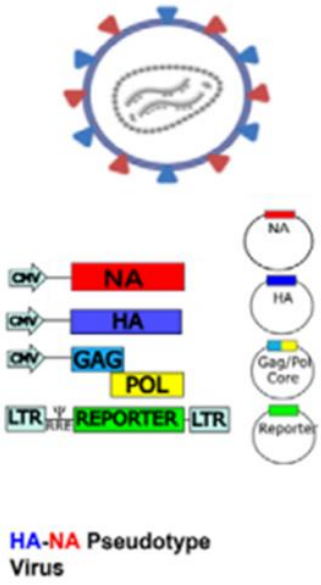


Figure 11. Plasmids needed to build Influenza HA-NA PV and its final conformation. Quadruple plasmid transfection system (including HA: Hemagglutinin; NA: Neuraminidase; Gag/Pol Core: Gag-Pol genes from HIV packaging structure; and a reporter plasmid) can be used to produce pseudotypes expressing HA and NA on the PV surface [modified from 16].

4.2.2.1 Plasmids transformation, amplification and purification

Plasmids transformation into *E. Coli* bacteria cells has been performed as follows. A tube of NEB 10-beta Competent *E. Coli* cells was thawed on ice for 10 minutes. An amount of 1 – 5 µl containing 1 pg – 100 ng of plasmid DNA was added to 50 µl of cells, and the transformation tube was then carefully mixed 4 – 5 times in order to mix cells and DNA. After that, the mixture was placed 30 minutes on ice and heat-shocked in a water bath at 42°C for exactly 30 seconds to allow plasmid entry; then again 5 minutes back on ice. After that, 950 µl of NEB 10-beta/Stable Outgrowth Medium at RT was added to the mixture which is put to grow into a shaking incubator at 37°C for 1 hour. This outgrowth step allows bacteria to express antibiotic resistance proteins. Finally, 100 µl of the 10-fold dilution of the transformation tube mixture was spread onto a 10 cm LB agar plate containing the appropriate selective antibiotic (in our case the Ampicillin). Incubation of plates overnight at 37°C allowed only bacteria which have incorporated the plasmid of interest, with the appropriate antibiotic resistance gene, to grow and divide in single colonies [11].

On the following day, the single colonies were isolated and put into an appropriate amount of LB broth for another overnight outgrowth step at 37°C into a shaking incubator, in order to amplify population of plasmid-expressing bacteria.

After last overnight incubation, transfection-grade plasmid purification was performed. The purification was made following QIAGEN® Plasmid *Plus* Maxi Kit high-yield protocol. First, bacterial culture was harvested and centrifuged at 6000 g for 15 minutes at 4°C. Pelleted bacteria were then resuspended into 8 ml of Buffer P1 (containing RNase A solution). In order to lyse cells, 8 ml of Buffer P2 were added and gently mixed by inverting until the lysate appeared viscous. After an incubation of 3 minutes at room temperature (15 – 25°C), the lysis was stopped adding 8 ml of Buffer S3 and mixing immediately by gently inverting 4 – 6 times. The lysate was then centrifuged at 4000 g for 10 minutes; the pellet was discarded, and the supernatant transferred into the QIAfilter cartridge, where it was filtered by the plunger into a new tube. After that, 5 ml of Buffer BB (able to bind plasmid DNA) were added and mixed by inverting 4 – 6 times. The lysate was then transferred to a QIAGEN Plasmid *Plus* spin column with a tube extender attached on the QIAvac 24 Plus. Applying approximately –300 mbar vacuums the liquid was drawn through the column, in order to remove the extender and wash DNA with 0.7 ml of Buffer ETR reapplying vacuum. Buffer ETR is crucial to remove endotoxins (also known as lipopolysaccharides or LPS, components

of Gram-negative bacteria cell membranes) released during the lysis step of plasmid purification, which could significantly reduce transfection efficiency and influence results outcome, interpretation, reproducibility and comparison [44]. The column was then washed again upon vacuum with 0.7 ml of Buffer PE (at 96 – 100% of ethanol). To completely remove residual wash buffer, the column was then placed into a 2 ml collection tube and centrifuged at 10000 g for 1 minute. After that, the column was placed into a clean 2 ml tube and 0.4 ml of Buffer EB were then added to the centre of the column. After 1 minute of incubation at room temperature, plasmid DNA was eluted through 1 minute centrifugation.

4.2.2.2 Quantification

Quantification of obtained plasmid DNA was performed through Nanodrop spectrophotometer, able to measure DNA concentration in a 2-µl drop on a pedestal. The DNA concentration in ng/µl was reported after readout of absorbance at A260 nm.

4.2.2.3 Transfection

Once obtained all plasmid DNA of interest, it was possible to proceed with H11N1 pseudotypes production through transfection on a suitable producer cell line, in our case the Human Embryonic Kidney 293 cells transformed with the SV40 large T antigen (HEK293T). This cell line is highly susceptible to transfection and make good retroviral packaging cells [13]. The following paragraph describes the protocol we used to obtain our Influenza PV: a rapid, reliable, cost-effective and safe production of lentiviral pseudotypes characterized by a lentiviral core containing a reporter and a surface with the avian Influenza haemagglutinin HA11 and the human neuraminidase NA1 glycoproteins. This was made using the widely available, highly efficient, less cytotoxic and low-cost PEI transfection reagent [3,14].

First, HEK293T cells were sub-cultured into 6-wells plates 24 hours prior transfection. Seeding 4 x 10^5 cells/well, it is possible to reach the desired confluence of 60-90% to perform transfection the day after seeding. A representation of cells after transfection is shown in **Figure 12**.

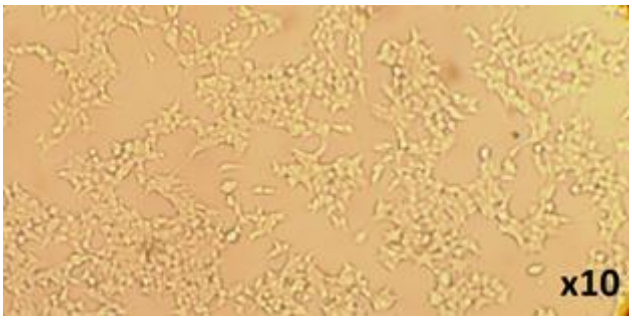


Figure 12. Visual representation of the right HEK293T cell confluence needed to transfect [14].

We used DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin and 1% L-Glutamine as Complete cell culture medium, while Opti-MEM™ was used as Transfection medium. Prior to start transfection, both mediums were pre-warmed in a water bath at 37°C. Then (under a class II biosafety cabinet to avoid any contamination) two different sterile tubes were prepared, respectively for Transfection medium - Plasmids (Tube 1) and Transfection medium – PEI (Tube 2) mixes. 200 µl of Transfection medium/well were added into both mixes (e.g., for a whole plate, add 200 µl x 6 wells, so add 1.2 ml of Transfection medium to the mixes). After that, appropriate amounts (see paragraph *4.2.2* and multiply for the total number of wells) of plasmids were added into Tube 1, as well as of PEI into Tube 2. Both tubes were gently flicked, incubated for 5 minutes at RT and then mixed by adding the content of Tube 2 into Tube 1. After gentle resuspension and incubation of the mixture for 15 minutes at RT, the tube was gently inverted every 3 to 4 minutes. During incubation, the spent culture medium of the pre-seeded HEK293T 6-wells plates was replaced with 2 ml of the fresh one (it is very important to carefully remove the old medium and slowly add the new one to one side of each well, to avoid detaching cells). After incubation, we added 200 µl of the transfection mixture dropwise throughout the total surface area of each well (do also a gently swirl of the plates to ensure dispersal). Then, after 48 hours of incubation at 37°C - 5% CO_2, cells supernatant containing the viral pseudotypes was harvested and filtered (using sterile syringes and Millex-HA cellulose acetate 0.45 µm filters), prior to be aliquoted and stored at -80°C until use.

4.2.2.4 Statistical Analyses

To evaluate the NI antibody response of immunized mice, serum samples were tested in pELLA assay in duplicate and the resulting 50% endpoint titers were determined. The 50% endpoint titer was defined as the reciprocal of the highest serum dilution that resulted in at least 50% inhibition of the maximum signal represented by the viral control (VC) minus the blank [(VC-BLANK)/2)]. Geometric mean of the 50% endpoint titers obtained from two replicates of the same sample was defined as the GMT for that specific serum. To allow statistical comparison of results, to titers measured as < 10 (1:10 was the first dilution of sera), was assigned a titer of 5.

Descriptive statistical analyses were reported in terms of Geometric Mean Titer value of each immunized group, Geometric Standard Deviation and 95% Confidence Interval of the Geometric Mean. Differences between groups *vs* control were evaluated by one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis test) followed by post-hoc analysis via Dunn's test. All the statistical tests were conducted with a significance level $\alpha = 0.05$. Statistical analyses were performed with Graphpad Prism Software version: 9.0.2®.

4.3 Preliminary Results

In order to evaluate NA-inhibiting antibody response of mice towards the two different N1-expressing dbDNA™ vaccines constructs, serum samples from *(i)* mice non-immunized, *(ii)* immunized with dbDNA™ Construct 1 or *(iii)* immunized with dbDNA™ Construct 2 were collected post-viral challenge and tested in pELLA assay.

As reported in **Figure 13a**, five mice were assigned to each of the five groups. The first two groups have been immunized, respectively, with a low (1 µg) and a high (10 µg) dose of Construct 1. To Group 3 and 4 were administered, respectively, a low and a high dose of Construct 2. The last group is the control group, treated with PBS only. Then, all groups received the viral challenge at day 28.

Serum samples obtained from bleed at day 32 have been tested in duplicate in pELLA assay, with a 1:6 dilution of the PV, chosen as the optimal one after ELLA titration results analysis.

Positive titers (>10) were found in all groups except for the control one (**Figure 13a-b**, "Control PBS"), in which all the samples yielded a negative titer (reported as "5", i.e., half of the reciprocal of the first detectable titer (10) (**Figure 13a**)), as expected. Most of the mice

belonging to the first group (i.e., receiving priming with a 1 μg of Construct 1), however, showed predominantly negative responses, suggesting that a low dose of Construct 1 might not be able to induce an immune memory necessary for the development of an effective immune response after priming. Increasing 10-fold the dose of Construct 1 ("Construct 1 High Dose", 10 μg) resulted in a raise of NI titers, with more than half of mice showing titers ≥40 (**Figure 13a**). Despite a correlate of protection for NI titers has not been established yet, Memoli et al. [35] found that a NI titer of ≥40 may be more predictive of protection than HI titers, making these results (which derive from a single immunization only) very promising. Administration of Construct 2 at low or high dose yielded NI GMT which are comparable to those obtained after priming with Construct 1 at the highest dosage (**Figure 14**). The Kruskall-Wallis non-parametric test followed by post-hoc analysis via Dunn's method showed statistically significant differences ($p = 0.0426$) between Group 4 ("Construct 2 High Dose") and the control group (**Figure 14**), suggesting that the N1-expressing dbDNA™ Construct 2 at the highest dosage might be the most immunogenic condition among those evaluated. Although these results are preliminary and further investigations are being conducted, they show that priming with dbDNA™ vaccine constructs could be a good strategy to induce acceptable NI antibody responses, even after priming only; and suggest that the two dbDNA™ vaccines should be considered as a booster dose as well.

a)

Treatment	Group	Sample n°	NI Titre - Replicate 1	NI Titre - Replicate 2
Construct 1 Low dose	1	1	5	5
		2	5	5
		3	5	5
		4	20	20
		5	5	5
Construct 1 High dose	2	6	80	40
		7	40	40
		8	5	5
		9	5	5
		10	40	80
Construct 2 Low dose	3	11	80	160
		12	5	5
		13	10	20
		14	5	5
		15	40	40
Construct 2 High dose	4	16	5	10
		17	80	40
		18	40	40
		19	20	10
		20	20	20
Control (PBS only)	5	21	5	5
		22	5	5
		23	5	5
		24	5	5
		25	5	5

b)

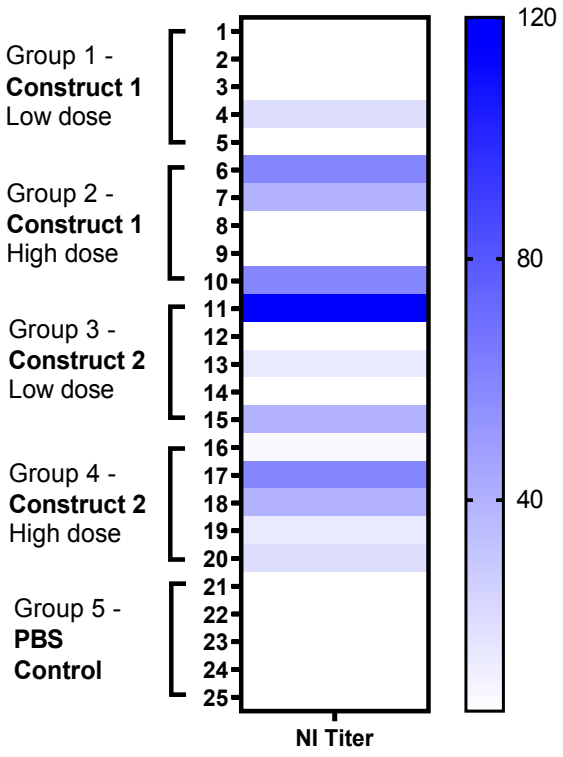
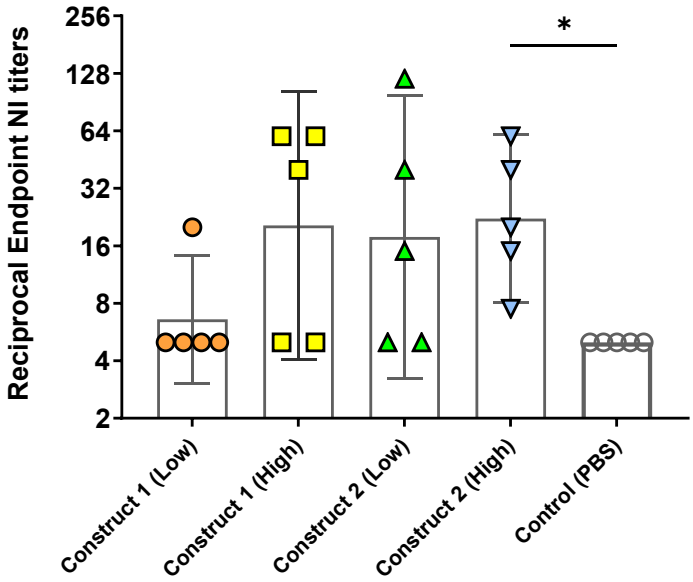


Figure 13. NA-inhibiting (NI) antibodies titer results. Five groups of mice (sample size per group: n=5) were immunized either with two different N1-expressing dbDNA™ vaccines constructs or PBS in order to evaluate NI antibody responses. The first two groups of mice (Group 1 and Group 2) were immunized, respectively, with a low (1 µg) and a high (10 µg) dose of a N1-expressing dbDNA™ vaccines construct (Construct 1). To Group 3 and Group 4 were administered, respectively, the same low and high dose but of a different N1-expressing dbDNA™ vaccine construct (Construct 2). To Group 5 (Control group) was administered PBS only. All groups received the viral challenge at day 28. Serum samples were collected 4 days post-viral challenge with the A/California/07/2009 virus (2000 PFU) and were tested in duplicate in pELLA assay to determine NI antibody titers. Figure a) shows the duplicate NI titers (Replicate 1 and Replicate 2) obtained for each mouse of each group. In Figure b), a heat map of the Geometric Mean Titers ("Geo. Mean") (GMT) obtained for each sample is shown.



	Construct 1 (Low)	Construct 1 (High)	Construct 2 (Low)	Construct 2 (High)	Control (PBS)
Geometric mean	6.598	20.48	17.83	22.21	5.000
Geometric SD factor	1.859	3.661	3.951	2.266	1.000
Lower 95% CI of geo. mean	3.055	4.088	3.237	8.041	5.000
Upper 95% CI of geo. mean	14.25	102.6	98.17	61.33	5.000

Figure 14. Graphic representation of all NI titers obtained by pELLA. pELLA testing was performed on serum samples collected 32 days post-priming of mice immunized with either N1-expressing dbDNA™ vaccines Construct 1 (low dose – 1μg – or high dose – 10 μg) or Construct 2 (low dose – 1μg – or high dose – 10 μg) or PBS, and challenged with A/California/07/2009 virus (2000 PFU) 28 days after immunization. Each symbol represents the geometric mean of the two NI endpoint titers (replicates) obtained per each sample. Data are expressed as Geometric Mean NI titer (horizontal bars) with 95% Confidence Intervals (CI). Geometric Mean NI Titer value of each group ("Geometric Mean"), the Standard Deviation (SD) (geometric SD factor) as well as 95% CI of the Geometric Mean are shown at the bottom of the graph. Comparison of the mean rank of each group with the control group was performed by Kruskall-Wallis non-parametric test and for the post-hoc analysis the Dunn's multiple comparison test was used. Statistical significance was defined as $P<0.05$. * $P = 0.0426$.

5. PROJECT 2

5.1 Aim of the project

The aim of Project 2 was to assess and compare the immunogenicity potential of both a Lt-purified SARS-CoV-2 RBD-SD1 antigen ("Lt-RBD") and a Lt-based SARS-CoV-2 vaccine platform expressing the whole S protein ("Lt-spike") combined with the Lt-RBD (LeCoVax-2) (**Figure 15**), upon either subcutaneous or mucosal administration into BALB/c mice. Within this project, I was mainly engaged on the experimental design and analyses performed to assess cell-mediated immune responses, which were evaluated via ELISpot testing of isolated splenocytes. This contribution was included into the following publication: "*Efficacy of mucosal vaccination using a protozoan parasite as a vehicle for antigen delivery: IgG and neutralizing response after rectal administration of LeCoVax-2, a candidate vaccine against COVID-19"* [34]. In this work we showed how we can safely manipulate *L. tarentolae* in order to express protein antigens from Wild Type SARS-CoV-2 strain and use this platform as a new mucosal vaccine vehicle to effectively prevent and/or reduce virus infection.

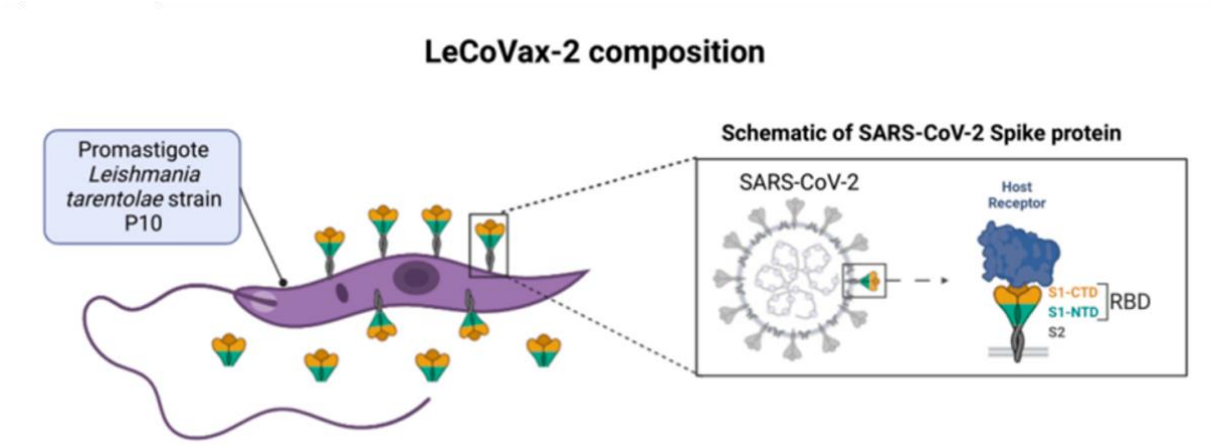


Figure 15. LeCoVax-2 vaccine composition. The LeCoVax-2 vaccine formulation is defined as the combination of the protozoan parasite *Leishmania tarentolae* engineered to express the SARS-CoV-2 virus Spike protein on its surface, and the purified recombinant protein RBD-SD1 overexpressed in *L. tarentolae* [34].

5.2 Materials and Methods

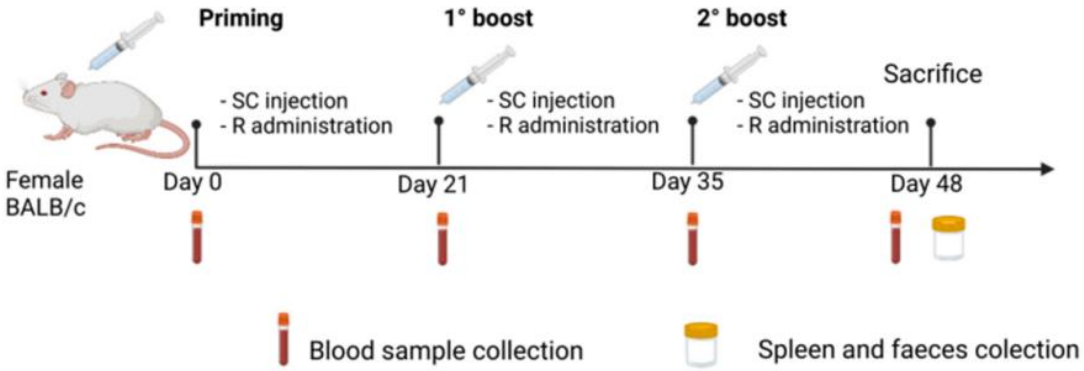


Figure 14. Schematic overview of the immunization experiment. Female BALB/c mice were assigned to 10 different groups (sample size per group: n=10) and immunized at day 0 (priming), day 21 (1st boost) and Day 35 (2nd boost) with different *L. tarentolae* (Lt) – based vaccine formulations or PBS either via the rectal (R) or subcutaneous (SC) route. Serum samples were collected on Day 0 (prior to immunization) and at each following immunization as well as upon sacrifice, for characterization of the antibody response. Spleens were collected at sacrifice (Day 48 post-priming) and used to isolate splenocytes for peptide stimulation and subsequent ELISpot analysis [34].

BALB/c mice were divided into ten groups (n=10 mice per group) and immunized by subcutaneous (SC) injection or rectal (R) administration with (*i*) RBD-SD1 ("Lt-RBD", a purified recombinant polypeptide including both the RBD and the SD1 portion of the SARS-COV-2 Spike glycoprotein, obtained by engineering the Lt P10 strain for secretory expression) or (*ii*) the Lt-based platform LeCoVax-2, which is the combination of Lt-RBD and Lt-spike (this latter being a clone of the *L. tarentolae* P10 strain designed to express on its surface the whole Spike protein of the ancestral SARS-CoV-2 virus). Both vaccines were administered either adjuvanted or not (adjuvants evaluated: AddaVax; Alum (Adju-Phos 2%); and Resiquimod (R848); all from Invivogen). One of the five groups assigned to each route of immunization was reserved to the control (PBS only) (**Table 1**).

Each experimental group received three prime-boost immunizations on day 0, 21 and 35. On day 48 the mice were sacrificed, spleens were collected (**Figure 16**) and used to isolate splenocytes for antigen-specific *in vitro* stimulation and subsequent ELISpot analysis.

Only samples IgG-positive at day 48 post-administration were evaluated in ELISpot (serum IgG determination results obtained through in-house Enzyme-Linked Immunosorbent Assay (ELISA) are *not shown* in the present thesis).

Experimental group	Formulation	Dose/ volume
Subcutaneous injection		
PBS-SC	PBS solution (control)	100 µl
LeCoVax-2 +Alum-SC	2×10^7 cells Lt-spike + 10 µg RBD + Alum	100 µl
LeCoVax-2 +AddaVax-SC	2×10^7 cells Lt-spike + 10 µg RBD + AddaVax	100 µl
RBD+Alum-SC	20 µg RBD + Alum	100 µl
RBD+AddaVax-SC	20 µg RBD + AddaVax	100 µl
Rectal administration		
PBS-R	PBS solution (control)	50 µl
LeCoVax-2-R	1×10^8 cells Lt-spike + 20 µg RBD	50 µl
LeCoVax-2 +R84810-R	1×10^8 cells Lt-spike + 20 µg RBD + 10 µg R848	50 µl
LeCoVax-2 +R84825-R	1×10^8 cells Lt-spike + 20 µg RBD + 25 µg R848	50 µl
RBD+R84825-R	20 µg RBD + 25 µg R848	50 µl

Lt: *Leishmania tarentolae*; RBD: Receptor Binding Domain; SC: subcutaneous; R: rectal; Alum: aluminium phosphate gel; AddaVax: squalene-based oil-in-water (w/o) nano-emulsion; R848: Resiquimod.

Table 1. Summary of the experimental groups per each route of immunization, with the assigned vaccine formulation and its specifics. Five groups of mice were subcutaneously immunized (three prime-boost immunization) on day 0, 21 and 35 with different treatments: i) Lt-spike and Lt-RBD antigen (RBD-SD1, referred to as "RBD" in the picture), mixed with AddaVax adjuvant (AddaVax; InvivoGen) at a 1:1 ratio (v:v); ii) Lt-spike and Lt-RBD, mixed with aluminium phosphate gel adjuvant (Alum) (Adju-Phos 2%; InvivoGen) at a 1:1 ratio (v:v); iii) Lt-RBD and AddaVax at a 1:1 ratio (v:v); iv) Lt-RBD antigen mixed with Alum at a 1:1 ratio (v:v); v) PBS as placebo. Five groups were rectally immunized on day 0, 21 and 35 with different formulation as follows: i) Lt-spike and Lt-RBD antigen mixed with 10 µg Resiquimod (R848, Invivogen); ii) Lt-spike with Lt-RBD and 25 µg R848; iii) Lt-spike and Lt-RBD antigen without the addition of any adjuvant; iv) Lt-RBD antigen mixed with 25 µg R848; v) PBS as placebo [34].

5.2.1 Splenocytes isolation

Splenocytes were isolated from each fresh murine spleen by gently pressing and smashing it through a 70 µm cell strainer using a syringe plunger. Cells were washed with Complete Medium (RPMI-1640 with L-Glutamine and 25 mM HEPES supplemented with 10% FBS and 1% Penicillin/Streptomycin 100X) and centrifuged at 250g for 10 minutes at RT. After centrifugation the supernatant was discarded and Red Blood Cells (RBCs) were lysed resuspending the pellet with 2 ml of RBC Lysis solution (RBC Lysis buffer 10X diluted 1:10

in distilled sterile water). The suspension was incubated 2 minutes in ice and the lysing reaction was stopped by adding 30ml of Complete Medium. Cell suspension was then centrifuged 250g for 10 min. Once discarded the supernatant, the pellet was resuspended in 10ml of Complete Medium to perform the counting of cells (see paragraph **5.2.2 "ELISpot assay"** below).

Once assessed each cell viability, we centrifuged each sample at 250g for 10 minutes at RT. After that, the pellet was resuspended in 1ml of FBS supplemented with 7.5% Dimethyl Sulfoxide (DMSO; Sigma-Aldrich), put in a cryovial placed in an appropriate Cell Freezing Container, left 24 hours at -80°C, and then stored in a liquid nitrogen tank until use.

5.2.2 ELISpot assay

The T cell responses of immunized mice were analyzed using a Mouse IFN-γ/IL-4 Double-Color ELISPOT kit (CTL ImmunoSpot) and a pre-coated Mouse TNF-α Single-Color ELISPOT kit (CTL ImmunoSpot), following the manufacturer's protocols.

On Day 0, we performed activation (with a 70% ethanol solution prepared in distilled sterile water) and coating (with the *Murine Mouse IFN-γ/IL-4 Capture Antibodies Solution*) of the high-protein binding PVDF membrane-bottom filter 96-well plates of the IFN-γ/IL-4 kit, prior to left them incubate overnight at 4°C. Plates of the TNF-α kit were ready to use.

On Day 1, we started preparing stimuli solutions (for negative, positive and antigen-specific *in vitro* stimulation) at 2X their final concentration in plate, where at 100 µl/well of each stimulus we added 100 µl/well of cells. Stimuli solutions were prepared in Complete CTL Test Medium (CTL Test Medium supplemented with 1% of L-Glutamine), considering for the final volume that each condition is seeded in triplicate per each sample. In the specific, negative control was represented by Complete CTL Test Medium only. The positive control by the Invitrogen™ eBioscience™ Cell Stimulation Cocktail (500X), a cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin (generally used to activate many cell types and induce cytokine production in *in vitro* cell cultures for subsequent detection in immunoassays), diluted 1:250 in Complete CTL Test Medium to have a final dilution of 1:500 (1X concentration) in plate. For the antigen-specific stimulation, we used the PepMix™ SARS-CoV-2 (Spike Glycoprotein) from JPT (a provider of peptide pools efficient for T cell

in vitro stimulation in T cell assays), prepared at 2 µg/ml in Complete CTL Test Medium in order to have it at 1 µg/ml final concentration in plate.

After overnight incubation of plates at 4°C, plates of the IFN-γ/IL-4 kit were decanted by the *Capture Solution* and washed once with 150 µl/well of PBS. Plates of the TNF-α kit were ready to use. After that, we proceeded plating 100 µl of each 2X stimuli solution in the corresponding wells (in triplicate per each sample condition). Plates were then placed into a 37°C humified incubator, 5-9% CO_2, until cell seeding (at least 15 minutes).

In the meantime, frozen mouse splenocytes samples were thawed by transferring the cryovials from the liquid nitrogen tank to a 37°C water bath until thawed (approximately 1 minute, taking care to leave the cap out of water to avoid any contamination of samples). Thawed samples were then transferred under a biosafety cabinet, where the content of each cryovial was added to a corresponding labeled 15 ml sterile conical tube pre-filled with 8 ml of pre-warmed FBS at 37°C (already sterile-filtered and de-complemented through a 30 minutes incubation in a 56°C water bath). 1 ml of pre-warmed FBS was then used to rinse each cryovial, in order to collect also residual cells, reaching a total volume of 10 ml for each cell suspension. Each sample was then incubated for 10 minutes at room temperature for cell resting, prior to be centrifuged at 250 g for 10 minutes. Supernatants were then discarded, and each cell pellet was resuspended in 10 ml of pre-warmed Complete CTL Test Medium, prior to be washed again by centrifuge at 250 g for 10 minutes.

Once discarded the supernatant, each pellet was then resuspended in 10 ml of Complete CTL Test Medium in order to perform cell viability assessment and counting using the CTL-LDC™ Live/Dead Cell Counting Kit. Briefly, 50 µl of each cell suspension was added to a different 1.5 ml eppendorf tube pre-filled with 50 µl of the CTL-LDC™ Live/Dead Cell Counting Dye (light-sensitive reagent) and well mixed. 10 µl from each tube was put onto a chamber of the 2-chamber hemocytometer slides provided with the kit. Hemocytometer were then read at the CTL Immunospot® S6 ULTIMATE Analyzer instrument.

Once assessed each cell viability, cell concentration was adjusted in order to have final concentration of 3 x 10^6 cells/ml per each sample. 100 µl/well, containing 3 x 10^5 cells, are then seeded in triplicate upon the 100 µl/well of each pre-filled sample condition (negative, positive and peptide stimulation). Plates with cells and stimuli in a 1:1 ratio in each well

(reaching a total volume of 200 µl/well) were then placed into a 37°C humified incubator, 5-9% CO_2, for 24 hours.

On Day 3, after 24 hours of incubation without perturbations, plates were washed (two times with PBS and two times with 0.05% Tween-20 – PBS solution, 200 µl/well each time), seeded with 80 µl/well of *Anti-murine IFN-γ* (*FITC*-conjugated primary antibody) / *IL-4* (*Biotin*, i.e. a biotinylated primary antibody)) *Detection Antibodies Solution* or, depending on the kit, *Anti-murine TNF-α (Biotin) Detection Antibody Solution* (filtered through a 0.1 µm low protein binding filter), and incubated for two hours at room temperature. Plates were then washed again three times (0.05% Tween-20 – PBS solution, 200 µl/well), prior to add 80 µl/well of the *Tertiary Solution* containing either *FITC-HRP* (anti-FITC HRP conjugated antibody) and *Strep-AP* (Streptavidin – Alkaline Phosphatase conjugate) both diluted 1:1000 in the appropriate kit diluent for the IFN-γ/IL-4 kit, or just the Strep-AP for the TNF-α kit. Plates were then incubated at room temperature, respectively, 1 hour for the IFN-γ/IL-4 kit and 30 minutes for the TNF-α kit. Plates were then washed again (two times with 0.05% Tween-20 – PBS solution and two times with distilled water, 200 µl/well), prior to add 80 µl/well of the *Blue Developer Solution* (constituted by the appropriate chromogenic *Substrate Solutions*) in order to develop IL-4 spots in the IFN-γ/IL-4 kit and TNF-α spots in the TNF-α kit. Plates were incubated 15 minutes at room temperature. Then, just for the IFN-γ/IL-4 kit, 80 µl/well of the *Red Developer Solution* were added in order to develop IFN-γ spots after having stopped the reaction by gently rinsing membrane with tap water, decanting and repeating this step three times, prior to wash once more with 200 µl/well of distilled water. Plates of the IFN-γ/IL-4 kit were then incubated at room temperature for other 5-10 minutes. It was important to keep Developer Solutions protected from direct light, and to do not exceed the ten minutes between preparation and usage. Last, front (four times) and back (by removing the protective underdrain) of both kit plates were gently washed with tap water, prior to be left 24 hours to air-dry face down on paper towels on bench top.

Plates are then scanned (**Figure 17**) and counted at the CTL Immunospot® instrument for each well Spot Forming Units (SFU) determination and Quality Control (QC) analysis through the Immunospot® Double-Color (DC, for the IFN-γ/IL-4 kit) or Single-Color (SC, for the TNF-α kit) ELISPOT Enzymatic Software Suite.

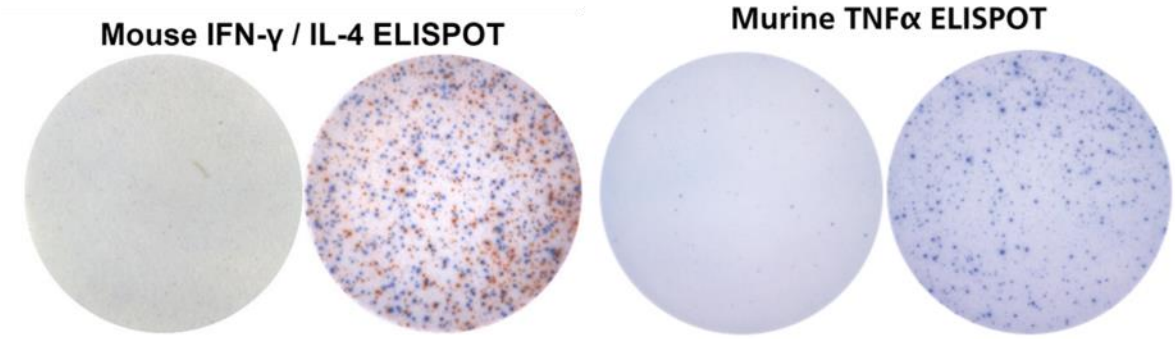


Figure 17. Representative images of unstimulated and stimulated wells of the Murine IFN-γ/IL-4 and TNF-α ELISPOT kits [51].

5.2.3 Statistical analyses

Statistical analyses were performed for data collected in the immunization experiment. The Mann-Whitney test was used to compare number of IFN-γ, IL-4 and TNF-α secreting cells among different mice treatment groups. Due to the reduced number of samples (< 5) available for some groups (only samples IgG positive at day 48 post-administration were evaluated in ELISpot), a biologically relevant pairwise statistical comparison was performed only when the sample size of each of two different treatment groups was considerable (≥ 5).

5.3 Results

SARS-CoV-2-specific T cell responses were measured through ELISpot analysis, using splenocytes from mice immunized with three vaccine doses. IFN-γ-, IL-4- and TNF-α-secreting cells elicited upon *in vitro* stimulation were quantified and expressed as SFU/10^6 cells. Only samples IgG positive at day 48 post-administration of the first vaccine dose were evaluated in ELISpot (serum IgG determination results obtained through in-house Enzyme-Linked Immunosorbent Assay (ELISA) are *not shown* in the present thesis).

Our goal was to evaluate whether, after an antigen-specific stimulation, cells were able to recognize antigen upon which they were primed/boosted, showing a cell-mediated immune response in terms of cytokines secretion. Obtained results were used to evaluate the best vaccine composition, as well as the most effective route of immunization.

All vaccinated mice showed antigen-specific T cell responses. Statistical analyses of results were performed only for groups with the number of IgG-positive animals ≥ 5 for both groups being compared in pairwise tests.

In detail, as reported in **Figure 18**, splenocytes from mice vaccinated by SC injection with Lt-RBD plus adjuvants (AddaVax or Alum) yielded sensibly higher IL-4 secretion (compared to the control value) upon *in vitro* re-stimulation with the Spike glycoprotein peptide pool, with significantly higher value in the RBD+AddaVax-SC group compared to the LeCoVax-2+AddaVax-SC (Mann-Whitney test, $p = 0.036$). No significant differences in terms of both IFN-γ and TNF-α T-cell responses to the stimulation were obtained by analyzing same treatment groups. Only the group subcutaneously immunized with RBD+Alum-SC showed a statistically higher frequency of TNF-α-secreting cells compared to the group treated with Lt-RBD+AddaVax-SC (Mann-Whitney test, $p = 0.036$).

On the other side, analysis of mice groups immunized via the rectal way with LeCoVax-2 plus adjuvants showed detectable IFN-γ and TNF-α responses, compared to the negative control (PBS only) and other treatment groups. In this case, no increased IL-4 secretion was detected in vaccinated mice compared to PBS treated ones (**Figure 18**).

Overall, the results highlight the ability of these Lt-based vaccines to elicit different Th1/Th2 immunogenicity profiles, with a more Th2-oriented (IL-4) immune response related to the adjuvanted Lt-RBD administered subcutaneously compared to the Th1-like (IFN-γ and TNF-α) response induced by the adjuvanted LeCoVax-2 administered via the rectal route. In addition, LeCoVax-2 vaccination through the mucosal (R) route showed a certain immunogenicity also in absence of adjuvants.

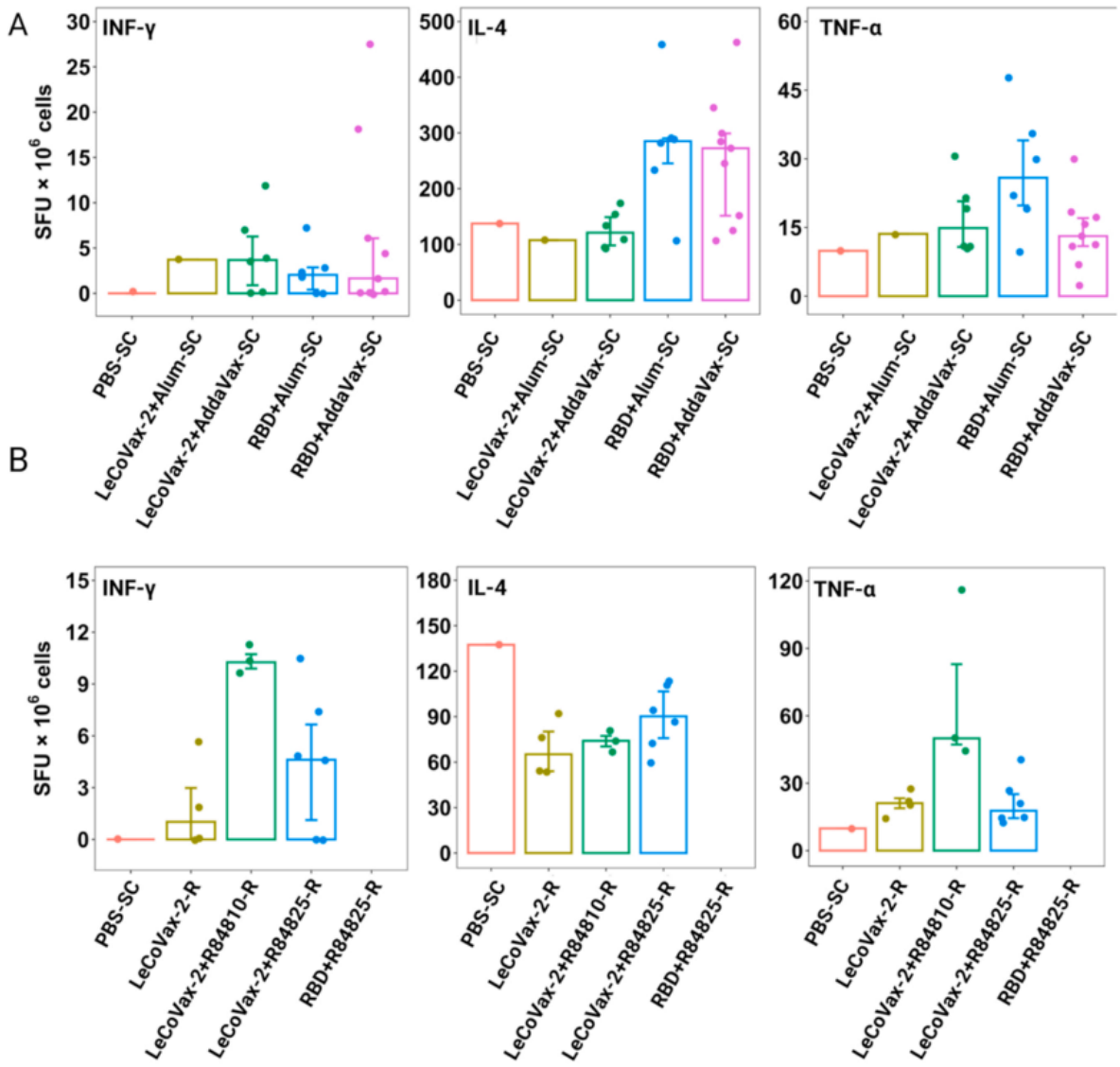


Figure 18. Evaluation of antigen-specific cytokine-producing T cells capacity. The number of IFN-γ, IL-4 and TNF-α secreting cells per million splenocytes (Spot Forming Units, SFU) was determined by ELISpot analysis at day 48 post-administration of LeCoVax-2 through the SC route (A) and R route (B). Only samples that were IgG positive at day 48 post-administration were evaluated. Bars represent median values, error bars 25th and 75th percentiles. For rectal administration, we lacked PBS treated individuals (see Methods): for comparison, we thus reported the values obtained from the single individual which received a SC injection. [34].

6. DISCUSSION

The immunological response induced by current Influenza vaccines is generally evaluated through classical and well standardized serological assays, such as hemagglutination inhibition (HI), single-radial haemolysis (SRH) and microneutralization (MN) assays. However, these assays only permit to assess HA-directed antibody responses, able to prevent Influenza disease [33], but not NA-directed responses, able to reduce disease severity and duration. Innovative methods capable of measuring antibodies triggered in response to stimulation with this secondary immunogenic antigen have been developed. Among them, the ELLA assay is the most common, practical, and widely used serological method currently used for the evaluation of NA-inhibiting responses [15, 16].

In Project 1, serum samples obtained from BALB/c mice intramuscularly immunized with two doses of two NA-expressing dbDNA™ vaccine constructs were tested in ELLA. Results obtained demonstrate an overall immunogenicity of both constructs, with positive titer results particularly observed upon immunization with Construct 2, especially at the highest dose evaluated (10 μg). Construct 2 (proprietary composition) encodes a nuclear localization signal, therefore it has more likelihood to effectively express the carried antigen, and hence to induce a protective immunogenic response against the pathogen of interest.
dbDNA™ constructs, with their easy and rapid-to-reproduce technology as well as high expression profile, represent indeed an excellent vaccine platform to address emergency situations like pandemics. Expression of NA (which is more conserved than the commonly used HA and hence able to elicit a more cross-reactive response) on such constructs may have the potential for a broadly protective and scalable vaccination strategy against (pandemic) Influenza.

Innovative and rapidly available vaccine platforms are particularly sought after since they could be promptly deployed to prevent pandemic-causing infectious diseases, such as the recently emerged COVID-19. In addition to the formulation or composition of the vaccine itself, it is also very important to consider the best route of immunization, ideally capable of triggering both humoral and cell-mediated immune responses. Although the subcutaneous (SC) and intramuscular (IM) injections remain the most common vaccination routes, mucosal administration (as example, via oral, nasal or enteral route) would deserve to be studied further. The importance of the mucosal immunity has been underscored by the continued

transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by infected vaccinated individuals [48].

Mucosal vaccination has demonstrated to confer protection against many infectious diseases [34] and provides several potential advantages over SC or IM routes, including protection at the site of pathogen entry, clearance of microorganisms on mucosal surfaces, induction of tissue-resident effector and long-lived memory T cells at mucosal surfaces, or the ability to produce immune crosstalk that provides protection at distal mucosal surfaces [49].

Worthy of note the analysis of cell-mediated immune responses which is catching on in the field of vaccine immunogenicity evaluations, with T-cell assays such as ELISPOT or intracellular cytokine staining by flow cytometry analysis being the most used methods to establish phenotype and magnitude of T cell responses as new correlates of protection. Indeed, T $CD4^+$ responses, which have a key role in B-cell help and cytokine production, might sometimes be better correlates of protection than antibody titers [52].

To evaluate the potential of mucosal immunization in inducing SARS-CoV-2-specific T cell responses, we evaluated a novel *L. tarentolae* (Lt)-based vaccine platform expressing SARS-CoV-2 S protein ("Lit-spike") combined with Lt-purified recombinant RBD-SD1 ("Lt-RBD") (LeCoVax-2) upon both rectal (R) and subcutaneous (SC) immunization of BALB/c mice (Project 2). In this study, animals were assigned to 10 different groups (consisting of 10 mice each) and subjected to three prime-boost immunizations with different Lt– based vaccine formulations (adjuvanted or not) or PBS either via the R or SC route. Serum samples were collected on Day 0 (prior to immunization) and at each following immunization (day 21, 1st booster dose; day 35, 2nd booster dose) as well as at sacrifice, for characterization of the antibody response via ELISA and neutralization assay (results *not shown*). Spleens were collected at sacrifice (Day 48 post-priming) and used to isolate splenocytes for peptide stimulation and subsequent ELISpot analysis.

RBD-specific T cell responses of splenocytes from mice showing IgG-positive titers at Day 48 post-priming have been evaluated in ELISpot in terms of cytokines production (IFN-γ, IL-4, TNF-α) and quantified as Spot Forming Units (SFU).

Comparing subcutaneous and rectal administration of Lt-RBD alone and Lt-spike + Lt-RBD (LeCoVax-2), we could highlight a different type of elicited T cells responses. Indeed,

adjuvanted Lt-RBD subcutaneous immunization relies on the elicitation of a Th2 – IL-4 mediated immune response. On the contrary, the rectal administration of the adjuvanted LeCoVax-2 vaccine formulation seems to induce a more Th1-banced immune response, as suggested by the release of the other two inflammatory and main anti-viral cytokines (IFN-γ and TNF-α).

In terms of cytokines secretion, the adjuvanted LeCoVax-2 showed to be an efficient delivery vehicle for SARS-CoV-2 antigens, especially when administered enterally rather than subcutaneously. The formulation containing LeCoVax-2 plus 10 μg of R848 and injected via the R route is particularly interesting in that it stimulates the highest production of IFN-γ and TNF-α observed in the study, although no statistical differences were observed among groups and there is still lack of a clear correlate of T-cell mediated protection. The fact that a response to RBD was observed, in rectal administration, only when Lt-spike cells were present (i.e., in the LeCoVax-2 preparation) provides strong evidence for the role played by *Leishmania* cells in the generation of the immune response. This, coupled with the observed seroconversion in animals immunized with LeCoVax-2 through the R route [34] poses the basis for considering LeCoVax-2 as a promising vaccine candidate by enteral immunization.

Notably, antigen administration through the R route can stimulate B-cell clones that home to the respiratory apparatus, in addition to the intestine. This would thus guarantee specific mucosal responses, at both the gut and respiratory levels [34].

Indeed, the enteral route does not come without limitations. Defecation or leakage of the rectal antigen within a short time post-immunization may account for variable effectiveness of the administered formulation. The method itself of administration may hamper delivery into the small intestine, hence the rectal antigen may reach the colon but not the ileum, where most Peyer's patches are located. Preparation of LeCoVax-2 as oral tablets coated for protection against gastric acids and formulated for a controlled release in the distal part of the small intestine may allow a higher retention time of the antigen in the gut, compared to the time ensured by rectal administration. It may also ensure the potential translocation of the antigen to lymphoid cells in the ileum and through the M cells and the Peyer's patches [34].

More in general, both oral and rectal drug administration are expected to imply rather variable interindividual adsorption of the active compound [34], however they might be particularly

indicated in some context (e.g., resource-limited settings) and in specific populations (e.g., toddlers and infants) as they may overcome some drawbacks associated to the SC administration (e.g., requirement of trained medical personal, use of needles).

Further studies are worthy to be conducted to assess LeCoVax-2 as an oral formulation, which may have several potential practical advantages compared to classic SC injection - such as easier vaccine production procedures (in terms of sterility and purity of the components), ease of administration and the possibility of partially overcoming vaccination.

In summary, Project 2 presents a first example of a candidate COVID-19 vaccine tested in rectal administration. Results from Project 2 show that (i) *L. tarentolae* is an efficient and safe vaccine platform, which can be used for production and delivery of viral antigens, naturally adjuvanted for enteral immunization [34]; and (ii) further corroborate the ability of the mucosal immunization in triggering cell-mediated responses, potentially at a higher level than that induced in a systemic immunization regimen. The relevance of T-cell mediated immune response in protection against COVID-19 is underscored by the fact that most of the Spike-specific CD4+ T cell response is conserved against SARS-CoV-2 variants of concern (VOC), whereas a decline in antibody responses against VOC has been observed. This suggests that T-cell immunity may provide an efficacious line of defense that can protect from the development of severe COVID-19, and advocate for deeper evaluation of this branch of immunity when assessing COVID-19 vaccine formulations [50].

Importantly, both *L. tarantolae* and dbDNA^{TM} could likely be rapidly available, safe and effective vehicles for antigens other than those assessed. The versatility and scalability of these vaccine platforms, as well as their ability to induce humoral and/or cell-mediated immune response, might make them an excellent solution to be exploited in a pandemic scenario.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

These projects represent a step towards the design of superior, long-lasting and broadly protective vaccines [16], which could become an ally of current vaccine platforms against emerging and pandemic viruses. The promising results obtained in Project 1 and Project 2 suggest that the two different vaccine platforms could be also evaluated as a booster dose to current vaccines in order to improve the breadth of their elicited immune responses.

At the same time, it would be of great importance to progress in the process of harmonization and standardization of novel assays used for the evaluation of humoral and cell-mediated immune responses elicited through vaccination and/or natural infection, with the aim of identify a clear correlate of protection for each of the investigated disease and have a better overview of vaccines immunogenicity [27].

The hope is that thanks to these innovative technologies more diseases will be addressed, and more people reached by novel preventative and therapeutic vaccines [4].

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9. PUBLICATIONS

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10. ACKNOWLEDGEMENTS

At the end of this journey, I would like to thank people who made it possible.

Professor Emanuele Montomoli, for the constant inspiration, support and trust he had in me throughout all these years, giving me the chance to be part of the VisMederi family.

Dr. John S. Tregoning, for giving me the great opportunity to join his lab at Imperial College London, guiding me during the time abroad.

Dr. Alessandro Manenti, for being the reference point of these working years, always ready to advise and help when needed.

Dr. Giulia Piccini, for her kindness and willingness to follow me during the drafting process of the present work.

Dr. Pietro Piu, for his precious statistical advice.

My colleagues and friends, for all the moments spent together at work and outside, which gave me the energy necessary to start everyday with good intentions.

My family and my partner, for always standing by me and believing in me, encouraging me to achieve my goals and helping me to realize all my dreams.