A respirable HPV-L2 dry-powder vaccine with GLA as amphiphilic lubricant and immune-adjuvant

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Abstract:	Vaccines not requiring cold-chain storage/distribution and suitable for needle-free delivery are urgently needed. Pulmonary administration is one of the most promising non-parenteral routes for vaccine delivery. Through a multi-component excipient and spray-drying approach, we engineered highly respirable dry-powder vaccine particles containing a three-fold repeated peptide epitope derived from human papillomavirus (HPV16) minor capsid protein L2 displayed on Pyrococcus furious thioredoxin as antigen. A key feature of our engineering approach was the use of the amphiphilic endotoxin derivative glucopyranosyl lipid A (GLA) as both a coating agent enhancing particle de-aggregation and respirability as well as a built-in immune-adjuvant. Following an extensive characterization of the in vitro aerodynamic performance, lung deposition was verified in vivo by intratracheal administration in mice of a vaccine powder containing a fluorescently labeled derivative of the antigen. This was followed by a short-term immunization study that highlighted the ability of the GLA-adjuvanted vaccine powder to induce an anti-L2 systemic immune response comparable to that of the subcutaneously (s.c.) administered liquid-form vaccine. Despite the very short-term immunization conditions employed for a preliminary vaccination experiment, the intratracheally administered dry-powder, but not the s.c. injected liquid-state, vaccine induced consistent HPV neutralizing responses. Overall, the present data provide proof-of-concept validation of a new formulation design to produce of a dry-powder vaccine that may be easily transferred to other antigens.

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DIPARTIMENTO DI SCIENZE
DEGLI ALIMENTI E DEL FARMACO

Parma, September 14th, 2021

Prof. Dr. Christine Allen
Editor-in-Chief
Journal of Controlled Release

Dear Professor Allen,

enclosed, please find the revised version of our manuscript " **A respirable dry-powder vaccine containing GLA as an amphiphilic lubricant and immune-adjuvant: proof-of-concept testing with an inhalational human papillomavirus L2-based immunogen**" to be considered for publication in *Journal of Controlled Release.*

We carefully considered all the points raised by the Reviewers and modified the manuscript accordingly.
As also reported in the attached point-by-point reply to the Reviewers' comments, we are grateful to the Reviewers for the time they dedicated to our work and for the useful suggestions they provided. We have addressed all the points raised by the Reviewers and substantially improved the overall quality (and readability) of the manuscript.

As to the criticism of Reviewer n. 3 which, by the way, is in stark contrast with the overall positive judgments expressed by the other Reviewers, we are convinced that the key factor for successful vaccine production has been clearly stated at the end of the 'Introduction' section of our manuscript.
We also believe that our paper, which has a clearly spelled out rationale, reports a full characterization of an innovative formulation and a preliminary, yet quite extensive and convincing set of *in vivo* data on the distribution and immunogenicity of the PfTrx-HPV-L2-GLA powder vaccine.

I look forward to your kind reply.

Sincerely yours,

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Answer to Reviewers' comments

First of all, we would like to thank the Reviewers for the time they have dedicated to our work and for the useful suggestions. We have addressed the points raised by the Reviewers to improve the quality of the manuscript.

Below our answers (in red) to the Reviewers comments.

Reviewer #1: In this manuscript, Bettini and co-authors developed a method to achieve a respirable dry-powder vaccine not requiring cold-chain storage/distribution. First, authors validated the excipient (mannitol, lactose and trehalose) to prevent the protein aggregation and achieve a high production yields. Next, authors evaluated the immunogenicity of the intratracheally delivered dry-powder vaccine compared with s.c injected protein vaccine or reconstituted dry-powder vaccine. Intratracheally delivered dry-powder vaccine showed comparable immune response compared to controls. However, before the acceptance of the manuscript, there are several points that need to be addressed. Below are specific comments.

1. In the legend of figure 1, 0.83%, 1.33%, 2.00% represents different amounts of the antigen which is not coincide with the table 1.
Table 1 refers to a first set of antigen-lacking powders produced with different amounts of sodium stearate as a surfactant and a technological surrogate of the (highly expensive) amphiphilic immune-adjuvant monophosphoryl lipid A. Then we moved to the production of powders with 1% sodium stearate and different contents of antigen (Figure 1). We realize that the presentation of the different powder sets was not clear enough. Therefore, we added a Table in the Supplementary, (new Table S1), summarising the compositions of the various powder prepared and investigated. We also explained in the text the coding of the second set of powders.

2. In table 1, the production yield is quite low, so is there a purification step to remove the not-dry-powder proteins? If not, it is hard to tell if the immunogenicity is owing to the dry-powder protein. Please include description to explain this.
The ratio between the protein antigen and the bulking agent is very low and strongly favors the latter component (antigen= 2% w/w, mannitol nearly 98% w/w as reported in the new Table S1), so it is hard to imagine that even a small fraction of the protein was not incorporated into the powder. Also, the marked difference between the liquid and the dry-powder form of the vaccine we observed in immune responses measured by ELISA or PBNA could not be explained if a large fraction of the antigen were free, i.e., not entrapped into powder particles.

3. In figure 1, the size of the dry-powder vaccine is more than 1um, which is quite large. What is the size of the original protein? Please include description to explain the size difference.
The size difference between the produced powders and the antigen molecules is at least two orders of magnitude. Powders have a size in the range of a few microns, while the protein antigen, which in SEC analysis elutes as a dimer, has an estimated hydrodynamic diameter (determined by DLS) of approximately 300 nm. We believe there is no need for any further explanation.

4. In figure 3, size of 2.00% w/w PfTrx-HPV-L2 is smaller than 1.33% w/w of PfTrx-HPV-L2. Please include description to explain the size difference between two.
Figure 3 reports images of powder samples selected to highlight the morphological features of the two powders rather than the size distribution. The difference between the median particle size of

the two powders was not statistically significant ($D_{v,50}$ of 2.39 ± 0.24 μm for Powder #5 and 2.65 ± 0.18 μm for Powder #6).

5. A bar graph or actual value description would be helpful to supply in Figure 4A and Figure 5A to summarize the fluorescence.
A reference false-color scale, from which the actual values can be extrapolated, was already included in Figure 4A.
A similar false-color scale has been added to the revised Fig 5 A.
Actual fluorescence values were also added to the revised Figures 4A and 5A.

6. In the legend of figure 6, there is no explanation on #1 group. Figure 6c and d are missing #1 group.
Thanks for pointing out this insufficient disclosure. Figure 6 legend has been modified to fix this information gap.

Reviewer #2: This manuscript investigates respirable vaccine dry-powder containing GLA as multifunctional additive. It has good rationale and shows meaningful scientific data. But it needs to check some point for the publication of JCR.
We are grateful for the positive evaluation of our work.

Main concern:
First of all, the main concern of this manuscript is that most are just explanations of the results and lack the discussion in result part. Especially, there is no direct data on lubricant function of GLA. This must be provided.
To highlight the lubricant functionality of GLA, we have added a paragraph reporting the flow data of a GLA-containing powder *vs* the one containing sodium stearate. A corresponding methodological paragraph has been added to the 'Methods' section as well.

Minor concern:
1. page 2: Abbreviation of PSD should be "particle size distribution". IVIS should be "In vivo Imaging System"
The typos have been corrected.

2. line 154: in order to avoid confusion, the Formulations should be explained using a Table to mention the components in detail.
Thanks for pointing out this insufficient disclosure. Indeed, he presentation of the different powder sets was not clear enough. Therefore, according to the Reviewer suggestion, we added a Table to the Supplementary material (presented as Table S1 in the revised ms.), summarising the various powders prepared and investigated. We also better explained in the text the coding of the second set of powders.

3. line 156: Balking agent -> bulking agent???
The typo has been corrected.

4. line 165: Outlet temperature should be described as a dependent value.
We definitively agree with the point raised by the Reviewer. The outlet temperature is a dependent parameter that changes with several factors including equipment geometry, formulation composition, feed rate, drying air humidity and ambient temperature. That is why we usually do not

report this parameter because we believe it is of little, if any, usefulness for the purposes of reproducibility of the spray-drying process.

3. line 205: it needs to define about fine particle fraction with cut-off diameter of stages. FPF% refers to the percentage deposited on a stage that is typically less than 5 m. How did the author calculate it? The ACI result should be displayed for cut-off diameter of stages and the FPF% calculation equation should be attached. The FPF% cut-off diameter must be clearly stated. It also needs to explain why a glass microfiber filter was used without using a coating method.
We have added the cut-offs ~~s~~ diameters of the stages as they have been calculated according to the USP, plus a sentence to better explain how the FPD was calculated.

4. line 315: It would be better if it mentioned with exact number how much vaccine powder emitted from the insufflator in test group.
We added this information in the Methods.

5. line 393: It is difficult to mention mannitol as the best bulking agent "the superior production yields it afforded and the superior aerodynamic performance of the protein-containing powder" just based on Yield (%), EF (%) and RF (%). it was just prepared by one condition without consideration of particle properties.
We added a sentence to clarify that the above statement should not be taken in absolute terms but rather referred to the specific conditions that have been tested.

6. line 400: in Table1, what is the reason of the low yield of Powder #2 compared to other formulations? What is the size distribution?
That was a mistake. We apologize and thank the Reviewer for having pointed it out. The actual value is 31.53. We corrected the value in Table 1. It is still the lowest one, but not so different from the other values and falls within the typical range of yields for powders produced with this type of equipment.

7. line 435: Figure 2 (A) What is the migration artifact? It is appeared that the molecular weight in changed by spray drying process. No y axis title for Fig 2b.
What we meant by 'migration artifact' is the so-called 'smile effect', that is a difference in apparent electrophoretic mobility between samples loaded into a right-most lane (in this case DP) and an inner lane (in this case UT). Indeed, as shown by the results of a similar electrophoretic analysis presented in panel 3C, there is no appreciable difference in the apparent molecular weights of the untreated and spray-dried *Pf*Trx-HPV-L2 antigen. However, to avoid any possible misunderstanding, we removed from Figure 2 legend the sentence referring to a migration artefact and this slight difference in electrophoretic mobility.
A y-axis descriptor has been added to the revised Figure 2B.

8. line 459-461: this sentence to explain that it prepared same molecular concentration 1% fractional amount of sodium stearate. For this that it should be prepared 5.75% GLA not 0.17%?? Please confirm them.
We added a sentence to better explain this point. We assumed that a lower amount of a larger molecule is needed to cover the same surface, thus the GLA concentration was scaled down according to the ratio 306.5/1763.5, which turns out to be approximately 0.17.

9. line 486: The SEM images of prepared particle showed broad particle size and the magnified SEM images (X20K) showed the most of particle are under 1 micron. It needs to explain with span value or Dv10 or Dv90 values. No y-axis title for Fig 3d.
The requested values have been added to the revised manuscript.
A y-axis descriptor has been added to the revised Figure 3D.

10. line 492: please insert "mean ± SD"
As specified at the end of the Methods section (line 371 of the original manuscript), unless otherwise indicated, all values are expressed as the mean ± standard deviation. So, we think it is not really essential to repeat this specification throughout the text.

11. line 492: What is the reason to choose Powder #6 final formulation? Please explain.
We added a sentence to explain that powder #6 was selected as it was the one containing the highest amount of antigen.

12. line 501: please insert the Standard deviation
Done.

13. line 505-509: please mention concrete numbers, and it should be noted if there is a statistical difference. For this discussion, specific experimental data for moisture reduction should confirmed such as Karl Fischer or surface charge depletion.
We deleted the sentence dealing with water loss or charge dissipation as potential explanations of the observed effect because the differences between EF and FPM, before and after storage, turned out not to be statistically significant**.**

14. line 532: Fig4A/4B lacks the legibility of the figure. Fig4C: What is the value? (Average?) If the value is average, the intensity legend is not matched with values and images.
Figure 4A presents the results of total fluorescence signal quantification within the thorax region that is enclosed by the white cube shown in the background. Figure 4B shows the results of Micro-CT imaging. False-color scales and the actual fluorescence intensity values measured in those areas are now reported in the revised Figure 4.
Figure 4C (whole mouse image) is just a representative epifluorescence picture obtained with the IVIS instrument and as such it is not quantifiable in a precise manner.
Lung, liver, kidneys and spleen fluorescence data were obtained by *ex-vivo* imaging and the amount/intensity of fluorescence in each district was quantified and expressed as a total fluorescence signal normalized per second and surface area (cm^2).

15. line 591: The Group # No. and Formulation # No can be confused. Please confirm that.
We added the word Group before # to resolve this ambiguity and avoid any possible confusion.

16. line 596: There is no explanation for group #4
Group #4 was actually described at the very beginning of this paragraph (line 592), but for the sake of clarity and in response to the Reviewer's point, in the revised manuscript it is now described sequentially, together with the other groups, at the end of the paragraph.

17. line 600: Fig 6B is not shown the standard deviation.
Since the results in Figure 6B are presented as single-animal data-points, there is actually no reason for providing a standard deviation value.

In Fig6D, Whether HPV16 neutralization titers were evaluated using the same sample as the GST-L2 ELISA. Why the number of samples is different?

As specified in the revised legend to Figure 6D, only a subset of the sera analyzed by GST-L2-ELISA (Figure 6B) were actually assayed for virus neutralization capacity using the L2-PBNA. Leaving out the sera from the negative control group (#1), which were found to be completely devoid of HPV16-L2 reactive antibodies, for the determination of neutralizing antibody titers with the L2-PBNA, we focused on a subset of five immune-sera/group (groups #2, #3 and #4) derived from the animals in each group that displayed the highest (top five) anti-HPV-L2 total antibody titers by GST-L2 ELISA (Figure 6B).

Reviewer #3: The authors developed an antigen powder formulation for the respirable vaccine. As a component to stabilize it, the an adjuvant GLA was used. The authors reported on the physicochemical property, tissue distribution and its antigen-producing activity.

Overall, it is not clear what is a key factor for the successful antigen production. The authors developed a 4 set of formulation (Table 1). However, the tissue distribution, intra-lung distribution was systemically analyzed. I think the manuscript is too preliminary to be accepted in JCR. The other comments are listed below:

We disagree with the Reviewer's criticism, which contrasts with the overall positive judgments expressed by the other Reviewers. In our humble opinion, the key factor for successful vaccine production has been clearly stated at the end of the Introduction (lines 109-117 in the original manuscript).

In fact, we are convinced that our paper, which has a clearly explained rationale, reports a full characterisation of an innovative formulation and a preliminary, yet quite extensive and convincing set of *in vivo* data on the distribution and immunogenicity of the PfTrx-HPV-L2-GLA powder vaccine.

1. It is not clear how the authors optimized their system. Especially, what is a key parameter for the optimization.

As explained in the manuscript (lines 426-428), the optimization was based on process yield and powder respirability.

2. In table 1, the authors developed 4 set of formulation. In this case, the content of sodium stearate was varied. In this formulation the amount of GLA is same?

These formulations did not contain GLA. As stated in the manuscript, sodium stearate was used in preliminary set-up experiments as a surfactant and a technological surrogate of the amphiphilic immune-adjuvant glucopyranosyl lipid A. However, we realize that the presentation of the different powder sets was not clear enough, and we thank the Reviewer for underlining the point. Therefore, we added a Table in the Supplementary, summarising the various powder prepared and investigated. We have also explained in the text the coding of the second set of powders.

3. The authors reported that the antigen is distributed to the liver. I worry that the activated immune-system attack the antigen presenting cells in liver. The toxicity, especially in repeated injection must be analyzed.

As mentioned in section 3.3 (lines 547-563) a fairly strong in-vivo fluorescence signal was indeed observed in the liver. Importantly, however, subsequent ex-vivo analyses, most notably SDS-PAGE fractionation coupled to Alexa-based near-infrared antigen visualization, clearly revealed the

absence of any detectable, protein antigen-associated fluorescence signal in the case of liver extracts. In our opinion (see lines 562-564), this indicates that Alexa labelled peptide fragments, rather than the intact *Pf*Trx-HPV-L2 antigen, were actually transferred to the liver, probably on the way toward further degradation and, ultimately, elimination. Based on this result, we do not see any risk of 'activated immune-system attack of antigen presenting cells in the liver', nor long-term toxicity of our respiratory tract-delivered dry-powder vaccine.

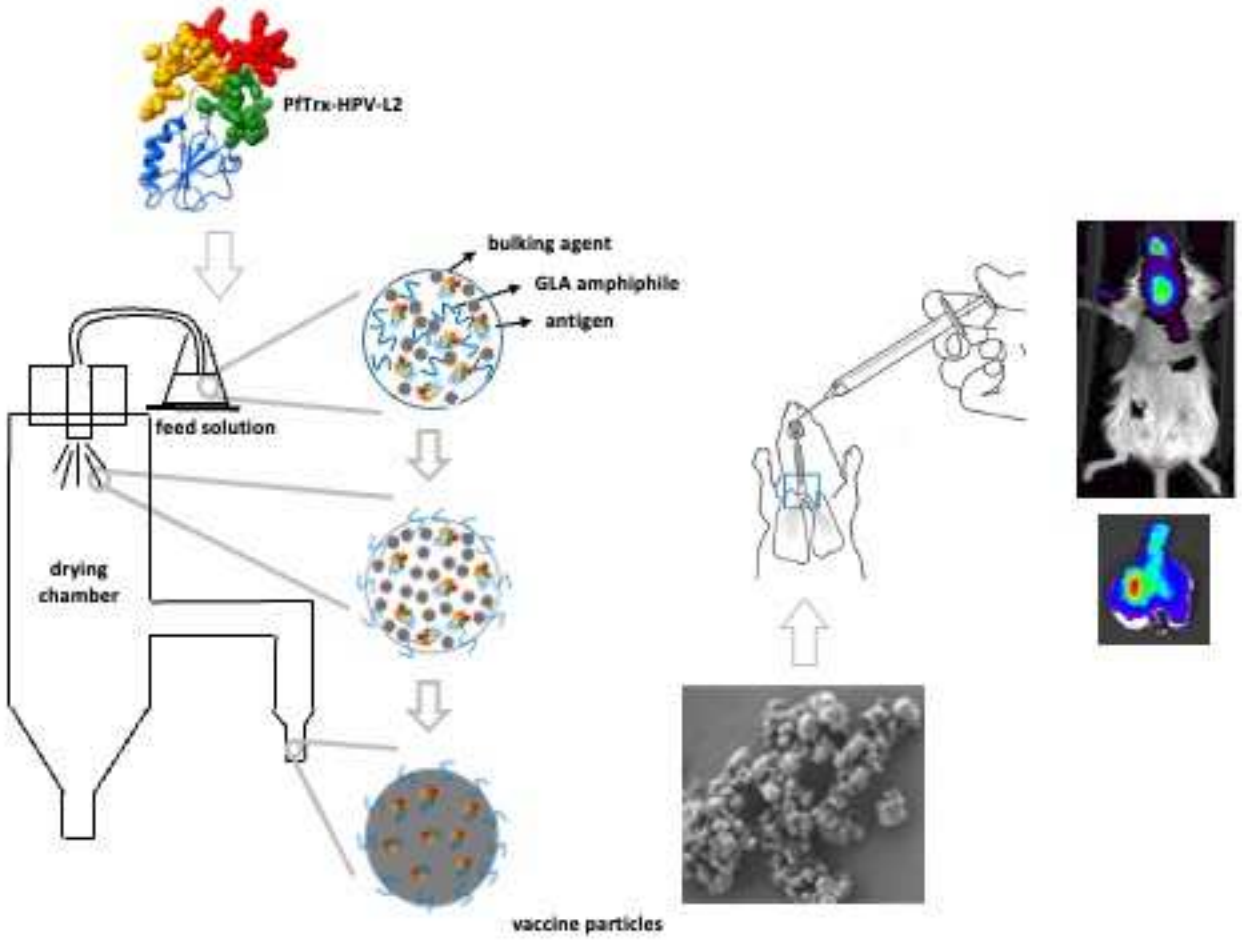
4. In Fig 6, the antibody production in #4 seems to be highest. What is a mechanism?
Since total anti-HPV16-L2 antibody titers measured in the three groups (#2, 3, 4) by ELISA (panel 6B) are quite similar, we suppose that the Reviewer is actually referring to the data presented in panel 6D. These are the results of pseudovirion-based neutralization assays (PBNA), which specifically measure neutralizing (not total) anti-L2 antibodies, i.e., antibodies that are capable to prevent virus entry into engineered target cells. As pointed out by the Reviewer, the production of these particular (virus-neutralizing) antibodies seems to be highest in group #4, which is composed of mice that were immunized with the intratracheally delivered dry-powder *Pf*Trx-HPV-L2 vaccine. The actual reasons for this apparent superiority are presently unknown. However, given the short immunization schedule utilized for our preliminary immunogenicity assessment, as briefly mentioned at the end of section 3.4 (lines 643-646), we speculate that this might reflect a faster affinity-maturation of anti-HPV-L2 antibodies and a more effective production of virus-neutralizing antibodies.

5. In relation to the comment 4, the antigen production appeared in all-or-none pattern. Why?
This comment, that, we suppose, again refers to the L2-PBNA data in Figure 6D, is not entirely correct. In fact, although the best responses (especially with regard to the number of responding animals) were measured in group #4 (intratracheally delivered dry-powder PfTrx-HPV-L2 vaccine), a good response was also observed for group #3 (PfTrx-HPV-L2-GLA powder, dissolved in PBS prior to subcutaneous injection). It would be tempting to speculate that the antigen+GLA formulation *per se* (i.e., even after solubilization and subcutaneous injection), in addition to intratracheal delivery of the dry-powder vaccine, somehow also positively influences neutralizing antibody production, but the data we have collected so far are not sufficient to support this hypothesis. To further clarify the conceptual difference between the data reported in Figure 6B and 6D, specific y-axis descriptors have been added to both figures.

Reviewer #4: Vaccines not requiring cold-chain storage/distribution and suitable for needle-free delivery are
urgently needed. Pulmonary administration is one of the most promising non-parenteral routes for vaccine delivery.
The aim of this study to develope and test a dry-powder inhalatory (DPI) formulation of a
HPV vaccine based on a previously described L2 antigen , in which three copies of the HPV16
L2 major cross-neutralization epitope comprised between amino acids 20-38 are grafted
intramolecularly to Pyrococcus furiosus thioredoxin (hereafter designated as PfTrx-HPV-L2)
The PfTrx-HPV-L2 DPI vaccine was produced by spray-drying through a particle engineering
approach based on the molecular deposition of an amphiphilic immune-adjuvant (glucopyranosyl
lipid A; GLA) on the surface of the spray-dried particles.It is interesting and well designed study

We are grateful to the Reviewer for her/his nice comments and extremely honoured for her/his great consideration of our work.

Graphical Abstract



A respirable HPV-L2 dry-powder vaccine with GLA as amphiphilic lubricant and immune-adjuvant

~~A respirable dry-powder vaccine containing GLA as an amphiphilic lubricant and immune-adjuvant: proof-of-concept testing with an inhalational human papillomavirus L2-based immunogen~~

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Abbreviations

GLA= Glucopyranosyl Lipid A
MPLA= Monophosphoryl Lipid A
HPV16= Human Papillomavirus
S.C.= Subcutaneous
I.T.= Intratracheal
DPI= Dry Powder Inhaler
EM= Emitted Mass
EF= Emitted Fraction
RM= Respirable Mass
RF= Respirable Fraction
MMAD= Mass Median Aerodynamic Diameter
GSD= Geometrical Standard Deviation
FSI= Fast Screening Impactor
ACI= Andersen Cascade Impactor
PSD= Particle Size Distribution
SEM= Scanning Electron Microscopy
DVS= Dynamic Vapor Sorption
TGA= Thermogavimetric Ananlysis
ELISA= Enzyme-Linked Immunosorbent Assay
SDS-PAGE= Sodium Dodecyl Sulphate Polyacrylamide Gels
Micro-CT= Micro-Computed Tomography
IVIS= *In vivo* Imaging System

Abstract

Vaccines not requiring cold-chain storage/distribution and suitable for needle-free delivery are urgently needed. Pulmonary administration is one of the most promising non-parenteral routes for vaccine delivery. Through a multi-component excipient and spray-drying approach, we engineered highly respirable dry-powder vaccine particles containing a three-fold repeated peptide epitope derived from human papillomavirus (HPV16) minor capsid protein L2 displayed on *Pyrococcus furious* thioredoxin as antigen. A key feature of our engineering approach was the use of the amphiphilic endotoxin derivative glucopyranosyl lipid A (GLA) as both a coating agent enhancing particle de-aggregation and respirability as well as a built-in immune-adjuvant. Following an extensive characterization of the *in vitro* aerodynamic performance, lung deposition was verified *in vivo* by intratracheal administration in mice of a vaccine powder containing a fluorescently labeled derivative of the antigen. This was followed by a short-term immunization study that highlighted the ability of the GLA-adjuvanted vaccine powder to induce an anti-L2 systemic immune response comparable to that of the subcutaneously (s.c.) administered liquid-form vaccine. Despite the very short-term immunization conditions employed for a preliminary vaccination experiment, the intratracheally administered dry-powder, but not the s.c. injected liquid-state, vaccine induced consistent HPV neutralizing responses. Overall, the present data provide proof-of-concept validation of a new formulation design ~~for the production of~~to produce a dry-powder vaccine that may be easily transferred to other antigens.

Key words: pulmonary vaccination, dry powder for inhalation, spray drying, human papillomavirus, glucopyranosyl lipid A.

1. Introduction

Vaccination is an established cost-effective strategy for the prevention of infectious (and some non-infectious) diseases [1,2] and its value as a life-saving tool is all the more appreciated during the present SARS-CoV2 pandemics [3]. Regardless of their attenuated/inactivated whole virus or recombinant nature, most present-day vaccines require a strict cold-chain maintenance ~~in order to~~to remain active. Indeed, cold-chain maintenance accounts for approximately 50% of vaccine cost on average and a similar fraction of the total vaccine production is thought to be wasted or damaged due to cold-chain breakage [4,5]. This is both an economical and a healthcare problem, which especially (but not exclusively) impacts on low-and-middle-income countries (LMICs), that is the very places where vaccination is most needed. An additional drawback associated to most current vaccines is that they are commonly administered via intramuscular (i.m.) or subcutaneous (s.c.) injection. This mode of delivery requires trained medical personnel, may face religious and/or ethnical barriers, and comes with the inherent risk, especially in LMICs, of needle-stick related injuries and potential infections. Even in high-income countries, adverse reactions to parenteral, needle-requiring vaccine administration (so-called 'lipothymic reaction' or 'needle-phobia') [6] as well as the burden of this mode of administration on the healthcare system have been documented. Novel vaccine formulations, compatible with viral and protein-based subunit antigens, suitable for non-parenteral administration are thus actively sought as a possible solution to the ~~above described~~above-described limitations of current vaccines. Solid-dosage vaccines (i.e., lyophilized or powder form vaccines) are reportedly more stable than their corresponding liquid forms, both from the biochemical (i.e., antigen structural integrity) and microbiological point of view, due to the absence of water [7]. This makes them amenable to non-cold-chain distribution and a more extensive stockpiling. However, due to the general sensitivity of proteins to dehydration, water removal is a risky step for the maintenance of antigen integrity. These potentially adverse effects can be supressed, or at least minimized, by the addition of sugars (e.g. trehalose), polyols (e.g. mannitol), polysaccharides (e.g. dextran) or amino acids (e.g. L-leucine) as stabilizing agents and antigen-protective excipients [8-14].

Among the various routes of non-parenteral vaccine administration, pulmonary vaccination is gaining momentum due to ~~a number of~~several favorable features. These include the large surface area, extended vascularization and abundance of dendritic cells and macrophages for antigen capture of the respiratory tract, without exposure to a harsh environment such as the one found in the gastro-intestinal tract. This, coupled with direct antigen delivery to a mucosal site, may result in a more effective mucosal immunity without any interference with systemic immunity, thus allowing for a broad application of this mode of immunization. An additional advantage of pulmonary vaccination is its needle-free administration -a mode of delivery that in humans is facilitated by the availability of well-developed dry powder inhalers [14].

The first attempts at pulmonary vaccination date back to the late 60's. These were based on the inhalatory administration of either very low amounts of living attenuated *Mycobacterium bovis* [15] or a liquid-form of aerosolized influenza vaccine [16], both of which led to successful immunization and protection. Despite these promising initial results, follow-up studies on pulmonary vaccination were discontinued, likely due to the lack of easy-to-use administration devices. They were resumed

more than 35 years later and extended to non-airborne infections such as hepatitis B (HBV), diphtheria and measles (see #7 for a review). HBV and influenza have been the predominant test-bench of pulmonary vaccination, and different types of variously adjuvanted immunogens, relying on inactivated whole-virus but also on recombinant antigens, formulated by spray-freeze-drying and delivered in either a liquid or a powder form have been investigated in different preclinical settings for their lung deposition and immunogenicity [7,17,18].

In addition to the specific types of antigen and immune-adjuvant, the physical state of the vaccine as well as its formulation and manufacturing, have also been shown to strongly influence the immune performance of inhalatory vaccines [10,17,19-22]. Of note, powder vaccine formulations have generally been found to be comparable, or superior, to liquid antigen formulations with regard to immunogenicity, but with the added benefits of long-term stability and ease of delivery [7,10,17,18,20-22]. Particle size and shape, and particularly an aerodynamic diameter smaller than 5 µm, are key features for achieving a suitable lung deposition [17-19]. Among the different drying processes used ~~for the production of~~to produce respirable particles, spray-drying stands up as the most easily scalable procedure. Also, the aerodynamic performance of spray-dried powders has been shown to be significantly improved by a particle engineering approach involving the addition of small amounts of surfactant or amphiphilic molecules with lubricant functionality [23,24].

Similar to most licensed vaccines, current human papillomavirus (HPV) vaccines, which rely on different combinations of major capsid protein L1 virus-like particles (VLP) [25], also have to be continuously refrigerated during storage/distribution [26]. Following-up to the encouraging results obtained with liquid-state, monotypic (HPV16) VLPs delivered through the airways via nasal nebulization or bronchial aerosolization [27], two different dry-powder HPV vaccine formulations were developed and tested for thermal stability and immunogenicity upon i.m. injection [28-30]. These powder vaccine formulations were produced by either a multicomponent excipient, spray-dry system [28,29], or with the use of glass forming polymers and trehalose as spray-drying excipients followed by atomic layer deposition to coat the resulting particles with defined layers of the alum adjuvant [30]. Except for one study dealing with the thermal stability of a spray-dried formulation of the nonavalent, Gardasil® 9 HPV vaccine [28], the other studies were focused on simplified, intrinsically more thermostable HPV antigens, such as L1 capsomeres [30] and a peptide epitope from minor capsid protein L2 incorporated into MS2 bacteriophage VLPs [29]. A substantial increase in thermal stability along with an immunogenicity comparable to, or even higher than, that of liquid vaccine controls was observed for both spray-dried and alum-coated vaccine formulations. However, the resulting vaccine powders were always dissolved prior to immunization, which was only performed by intramuscular injection, thus obviously precluding any information on dry-powder vaccine respirability, lung deposition and immunogenicity upon pulmonary delivery.

To begin to fill these gaps, we developed and tested a dry-powder inhalatory (DPI) formulation of a HPV vaccine based on a previously described L2 antigen [31,32], in which three copies of the HPV16 L2 major cross-neutralization epitope comprised between amino acids 20-38 are grafted intramolecularly to *Pyrococcus furiosus* thioredoxin (hereafter designated as *Pf*Trx-HPV-L2). The thermal stability and immunogenicity of this particular antigen [31,32] as well as the preclinical efficacy and cross-neutralization capacity of other antigens based on a similar L2 epitope [33] have been documented previously.

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The *Pf*Trx-HPV-L2 DPI vaccine was produced by spray-drying through a particle engineering approach based on the molecular deposition of an amphiphilic immune-adjuvant (glucopyranosyl lipid A; GLA) on the surface of the spray-dried particles. The rationale behind this approach was to improve both particle respirability as well as surface exposure of the adjuvant and its recognition by lung immune cells, especially antigen presenting cells such as dendritic cells and macrophages [18]. The dry powder vaccine thus produced was extensively characterized with respect to aerodynamic properties, including the assessment of its *in vivo* lung deposition capacity. The systemic immunogenicity of the intratracheally delivered *Pf*Trx-HPV-L2 DPI vaccine was also preliminarily evaluated in a short-term immunization study in mice.

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2. Materials and Methods

2.1 Materials

Mannitol, Pearlitol® SD 200 batch no. E556G, was a kind gift by Roquette Freres. Sodium stearate was purchased from Magnesia GmbH. Glycopyranoside Lipid A (GLA, PHAD), synthetic version of Monophosphoryl Lipid A (MPLA), was supplied by Avanti Polar Lipids. All solvents were at analytical grade and ultrapure water (0.055 µS/cm, TOC 1 ppb) was produced by reverse osmosis (Purelab Pulse + Flex ultrapure water, Elga-Veolia).

2.2 Methods

***2.2.1 Pf*Trx-HPV-L2 and GST-L2 constructs**. Codon-usage optimized *Pyrococcus furiosus* (*Pf*) Trx (*Pf*Trx) coding sequences were chemically synthesized (Eurofins MWG Operon) and inserted into the *NdeI* site of a His-tag-lacking pET26 plasmid. Three tandemly repeated copies of the HPV16 L2 aa 20-38 peptide grafted to the display site of *Pf*Trx was generated by inserting the corresponding sequences into the Trx *CpoI* sites of pre-assembled pET-*Pf*Trx plasmids as described previously [31,32,34]. The resulting constructs were sequence-verified and transformed into *Escherichia coli* BL21 codon plus (DE3) cells for recombinant protein expression.
Glutathione S-transferase (GST)-L2 fusion to be used as capture antigen for ELISA was produced by cloning the L2 (aa 1-120) coding sequence into the *SmaI* site of the GST expression vector pGEX-4T-2.

2.2.2 Expression and purification of the *Pf*Trx-HPV-L2 antigen. Recombinant *Pf*Trx-HPV-L2 was produced by overnight induction at 30°C in auto-inducer medium (10 g/L yeast extract, 25 mM NH_4SO_4, 50mM KH_2PO_4, 50 mM Na_2HPO_4, 2 mM glucose, 6 mM α-lactose, 1.2 mM $MgSO_4$). Following cell lysis, the untagged *Pf*Trx-HPV-L2 protein was purified by cation exchange chromatography and detoxified by a three-fold repeated Triton X-114 (1% v/v) treatment [35] . Endotoxin (LPS) removal, to levels lower than 2 EU/mL, was verified for each antigen batch by the LAL QLC-1000 test (Lonza). The GST-L2 protein was affinity-purified on glutathione-sepharose columns (GE Healthcare) according to the manufacturer's instructions. The composition and purity of individual protein preparations were assessed by electrophoretic analysis on 11-15% SDS-polyacrylamide gels (SDS-PAGE). Protein concentration was determined by measurement of absorbance at 280 nm using calculated extinction coefficients [36] and with the use of a QubitH 2.0 Fluorometer (Life Technologies).

2.2.3 Spray drying. Dried engineered powders for inhalation containing the antigen were produced by spray drying with a mini Spray Dryer Büchi B-290 (Büchi Laboratoriums-Technik) in aspiration and open mode. The feed solution was prepared by dissolving the bualking agent in ultrapure water whereas the amphiphilic compound was dissolved in ethanol (95% v/v). The protein solution in 25 mM potassium phosphate buffer (pH 7.4) was, then, added to the water portion and ethanol solution was added drop wise to the aqueous one under continuous magnetic stirring at 200 rpm in

~~order to~~to reach a final solution with water to ethanol ratio 70:30 v/v. The total concentration of solutes was always kept at 0.6% w/v.

The concentration of the bulking agent in the solution was adjusted for each formulation to complement 0.6% w/v of the total solute taking into consideration the concentration of the protein and the amphiphilic compound. Formulations of the main powder tested are summarized in Table S1 in Supplementary. The drying parameters were set as follow: inlet temperature, 125 °C; drying air flow rate, 601 L/h; aspiration, 35 m^3/h; solution feed rate, 3.5 mL/min and nozzle diameter, 0.7 mm.

2.2.4 Particle size distribution by laser diffraction. The particle size distribution (PSD) of spray dried powders was measured by laser light scattering (Spraytec®, Malvern Instruments Ltd). The diffractometer was equipped with a 300 mm focal lens, which measures particle size in the range 0.1-900 μm. Samples were prepared by suspending 10 mg of powders in 10 mL of cyclohexane containing 0.1% w/v of Span 85 (Honeywell Fluka™). To improve homogeneity, the dispersion was put in an ultrasonic bath (8510, Branson Ultrasonics Corporation,) for 5 minutes before PSD measurements that were carried out in triplicate for each sample with an obscuration threshold of at least 10%. Data were expressed as volume diameter of 10^{th} ($D_{v,10}$), 50^{th} ($D_{v,50}$) and 90^{th} ($D_{v,90}$) percentile of the particle population and as Span value [($D_{v,90}$-$D_{v,10}$)/$D_{v,50}$].

2.2.5 Scanning electron microscopy and powder flow. A Scanning Electron Microscope (FESEM-FIB Zeiss Auriga Compact, Carl Zeiss) was used to investigate particle morphology, shape and surface characteristics of the spray dried powders. Powders were deposited on adhesive black carbon tabs pre-mounted on aluminum stubs; powder in excess was removed with a gentle nitrogen flow, and samples imaged without any metallization process. The microscope was operated after 30 min of depressurization (1.87 10^{-4} Pa) with an accelerating voltage of 1.0 kV and a working distance of 4.9 mm. Images were taken at different magnification.

Dynamic angle of repose was determined as an indicator of the powder flow properties. Measurements were carried out according to Ph. Eur. 10^{th} ed. employing a Friability tester (model TA3R, Erweka GmbH) where the drum had been removed and replaced with a transparent glass vial (volume 10 mL) filled with about 50 mg of powder. The vial was, then, attached to the tester arm and rotated for 60 seconds at 20 rpm. A video was recorded with an iPhone 6 (Apple), and single frames were extracted and analyzed with the software Image J64 (NIH) to measure the angle between the horizontal lane and the powder avalanche line during the so-called rolling stage. Six independent measurements were performed each tested powder.

2.2.6 *In vitro* aerodynamic performance assessment. The aerodynamic performance of the powders was firstly investigated using a Fast Screening Impactor, FSI, (Copley scientific Ltd,). 10 mg of powder were loaded manually in a hypromellose Quali-V-I capsule size 3 (Qualicaps) and one single capsule for each test was aerosolized using a high resistance RS01® device (RPC, Plastiape~~, Osnago, Italy~~). The entire system was connected to a vacuum pump (Mod. 1000, Erweka GmbH,) which created the air flow to aerosolize and distribute the powder in the FSI. The flow rate used

during each test was adjusted, according to current USP monograph, with a Critical Flow Controller TPK (Copley Scientific Ltd) in order to produce a pressure drop of 4 kPa over the inhaler. Thus, flow rate was set at 60 L/min before each experiment using a Flow Meter DFM 2000 (Copley Scientific Ltd). The pump was activated for 4 seconds for each test so that to withdraw a volume of 4 L of air from the inhaler. The aerosolization performance was tested in triplicate. The following parameters were calculated: the emitted mass (EM) was the amount of powder that was emitted from the device; the emitted fraction (EF) was calculated as the ratio between the EM and the amount of powder loaded in the capsule. The respirable mass (RM) was the mass of powder with aerodynamic diameter lower than 5 µm, ~~i.e.~~i.e., the amount deposited on the filter (type A/E, diameter 76 mm, Pall Corporation) of the Fine Fraction Collector (FFC); the respirable mass (RF) was calculated as the ratio between RM and the amount of powder loaded in the capsule. EM and RM were calculated from the weight of the device loaded with the filled capsule and the filter before and after the aerosolization of the powder.

Andersen Cascade Impactor (ACI, Apparatus 3, USP 41, Copley scientific Ltd) was employed to determine the aerodynamic distribution of the powder produced for the *in vivo* study. The measurements were carried out according to USP-NF <601>; thus, at the flow rate of 60 L/min (see below), the cut-offs diameters of the stages -1, -0, 1, 2, 3, 4, 5, 6 were the following: 8.60, 6.50, 4.40, 3.20, 1.90, 1.20, 0.55 and 0.26 µm. A glass microfiber filter of diameter 82.6 mm (Whatman plc~~, Little Chalfont, UK~~) was placed right below stage six ~~in order~~ to collect particles with a diameter lower than that ~~of the stage 6 cut-off~~0.26 µm. 20 mg of each powder were loaded in Quali-V-I size 3 capsules and the RS01® device was again employed for the aerosolization. Before running the experiment, 2 mL of a solution of Tween® 1% w/v in ethanol was applied on the particle collection surface of each stage; after complete solvent evaporation a thin layer of surfactant was obtained on the stage surfaces that ensured efficient particle capture avoiding particle bouncing. An air flow rate of was set at 60 L/min, (Flow Meter DFM 2000) was generated by a vacuum pump (SCP5, Copley scientific Ltd) activated for 4 seconds, using the TPK to adjust the flow. After the powder aerosolization, the powder deposited in the device was cumulated with that remained in the capsule and collected in with ultrapure water in a 50 mL volumetric flask~~s~~; similar collection was done for the powder deposited in the induction port. The powders deposited onto stages -1, -0 and 1 were solubilized individually with 25 mL of ultrapure water, while for all the other stages 10 mL were used. The filter was removed from the system and put in a crystallizer with 10 mL of ultrapure water and put 5 minutes in an ultrasonic bath. The solutions obtained from the filter as well as that from device plus capsule were filtered through a 0.45 µm cellulose acetate syringe filters (Labservice Analytica), before injection in HPLC for the quantification of mannitol that was taken as tracer of the particles. Starting at the final collection site (filter), the cumulative mass versus cut-off diameter of the respective stage was derived. The mass of mannitol with an aerodynamic diameter less than 5 µm (Fine Particle Dose or Respirable Mass, RM) was calculated by interpolation. ~~Different~~ Several different aerodynamic parameters were also calculated: Emitted Mass, EM, the mannitol mass collected from the induction port to the filter; Emitted Fraction, EF, the ratio % between the EM and amount of powder loaded in the capsule; ~~Respirable Mass, RM, the mass of the aerosolized mannitol with an aerodynamic diameter < 5 µm;~~ Respirable Fraction, RF, percentage of the RM with respect to the EM. Moreover, the Mass Median Aerodynamic Diameter (MMAD) defined as the diameter

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which separates the powder in two populations with equal weight was determined by plotting the cumulative percentage of mass less than the cut-off diameter for each stage on a probability scale versus the relevant aerodynamic diameter of the stage on a logarithmic scale. MMAD is the slope of the line obtained by linear regression of the experimental points. Finally, the Geometrical Standard Deviation (GSD), a parameter indicating how wide the particle size distribution, was calculated as the square root of the ratio between the size of the 84.13% of the particle population in the log-normal distribution and the size of the 15.87% of the particle population in the log-normal distribution.

2.2.7 Mannitol HPLC analysis. A Shimadzu VP (Shimadzu Corporation) high performance liquid chromatographic (HPLC) system coupled with refractive index detector (RID-10A, Shimadzu) set at 40 °C was used for mannitol quantification, following the analytical method reported in the relevant monography by United States Pharmacopeia (USP 41). Ultrapure water was employed as mobile phase at a flow rate of 1 mL/min and injection volume was set at 100 µL on a Waters 717 plus autosampler (Waters Corporation). As a stationary phase an Aminex® HPX-87H Ion Exclusion, 300 mm x 7.8 mm (Bio-Rad, Hercules) column was used. The column was equilibrated at 80°C for 2 hours with the mobile phase pumped at 0.2 mL/min then, maintained at that temperature during the chromatographic runs. A Shimadzu Class VP software was used for data acquisition and analysis. Each sample was injected 6 times and the 3 closer values were used to calculate mean and standard deviation. Linearity of the responses was assessed between 0.01 mg/mL and 1 mg/mL (R^2= 0.999). The limit of detection and the limit of quantification were 2.6 µg/mL and 8 µg/mL, respectively.

2.2.8 Fluorescent labelling of *Pf*Trx-HPV-L2. The *Pf*Trx-HPV-L2 protein was dissolved in 0.1 M sodium bicarbonate buffer, mixed with the reactive dye solution (Alexa Fluor 750; Life Technologies) and incubated for 1 hr at RT with continuous stirring according to the manufacturer's instructions. Separation of the labelled protein from the unreacted, free dye was performed by size fractionation (Econo-Pac 10DG Desalting columns, Bio-Rad). Labelling stoichiometry was determined by comparing the absorbance at 280 nm of equally concentrated solutions of the ~~unlabelled~~unlabeled and the Alexa-labelled protein. The Alexa-conjugated *Pf*Trx-HPV-L2 antigen incorporated into the DPI at 2 % w/w of the resulting product, was administered intratracheally to female BALB/c mice, in either a PBS pre-dissolved liquid form or in powder form.

2.2.9 Animal experimentation. Female inbred (7–8 weeks-old) BALB/c mice were purchased from Envigo Laboratories (Italy). Prior to use, animals were acclimatized for at least 5 days to the local vivarium conditions (room temperature: 20–24°C; relative humidity: 40–70%; 12-h light–dark cycle), with free access to standard rodent chow and softened tap water. All animal experiments were approved by the intramural animal welfare committee for animal experimentation (authorization n. 31/2015-PR to Chiesi Farmaceutici for imaging experiments; authorization n. 985/2019-PR to University of Parma for immunization experiments) to comply with the European Directive 2010/63 UE, Italian D. Lgs 26/2014 and the revised "Guide for the Care and Use of Laboratory Animals" (Natl. Res. Council, US Committee, 8th Ed, 2011).

For intratracheal administration, animals were lightly anesthetized with either 2.5% isoflurane delivered in a transparent plastic box or ketamine/xylazine (80 and 10 mg/kg, respectively, administered intraperitoneally) and positioned on the intubation platform, hanging by their incisors placed on the wire. Using a small laryngoscope to visualize the trachea, the Alexa-conjugated *Pf*Trx-HPV-L2 vaccine was intratracheally administered by either a syringe-operated micro-cannula (liquid-form vaccine) or with the use of a modified Penn-Century, Dry Powder Insufflator™ (Model DP-4M) device (dry-powder vaccine) [37].

2.2.10 *In vivo* and *ex-vivo* imaging. Three different experimental approaches were utilized for *in vivo* imaging. For fluorescence molecular tomography (FMT 2500; PerkinElmer), the anesthetized animals were carefully positioned in the imaging cassette, which was then placed into the FMT imaging chamber. A near infrared (NIR) laser diode transilluminated the thorax region (*i.e.*, passed light through the body of the animal to be collected on the opposite side), and the resulting signal was detected with a thermoelectrically cooled charge-coupled device camera placed on the opposite side of the imaged animal. Appropriate optical filters allowed the collection of both fluorescence and excitation data sets, and the multiple source-detector fluorescence projections were normalized to the paired collection of laser excitation data. Cumulative fluorescence data were reconstructed using the FMT 2500 system with TrueQuant software version 2.2 (Perkin Elmer) in order to obtain a three-dimensional fluorescence signal quantification within the lungs.
The same animals were analyzed by Micro-Computed Tomography (Micro-CT) imaging, performed 15 min after administration with the use of a Quantum GX Micro CT apparatus (Perkin Elmer), as well as by IVIS Lumina II imaging (Perkin Elmer). Micro-CT images were acquired with an intrinsic retrospective two-phase respiratory gating technique using the following parameters: 90 KV, 88 µA over a total angle of 360° for a total scan time of 4 min.
After *in vivo* imaging, mice were euthanized by anesthetic overdosing, followed by bleeding from the abdominal aorta and organ explantation. *Ex-vivo* imaging was then performed on the lungs, trachea liver, ~~splеen~~spleen, and kidneys by IVIS Lumina II.
Portions of the explanted lungs, trachea and liver (5 mg of frozen tissue each) were suspended in 300 µL of RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS, pH 8.0], disrupted by sonication (Misonix Sonicator 3000; 20 min, 20% power) and, after centrifugation for 20 min at 10000 x g, the resulting supernatants were fractionated by SDS-PAGE, followed by detection of the Alexa-labelled *Pf*Trx-HPV-L2 protein by near-infrared fluorescence at 800 nm, using a ChemiDoc MP Imaging System (Bio-Rad).

2.2.11 Animal immunization. Mice were subdivided into four (three control and one test) groups, which were injected subcutaneously (s.c.) with four doses (1 priming and 2 boosts) of a solubilized control powder (#1), liquid antigen (#2), solubilized vaccine powder (#3) or the same powder administered intratracheally (#4) at weekly intervals. Group #1 was comprised of five animals, which were s.c. injected with 1 mg of GLA-containing but antigen-lacking powder dissolved in 50 µl of PBS right before administration. Group #2, comprised of 7 animals, was s.c. injected with the detoxified and filter-sterilized liquid *Pf*Trx-HPV-L2 antigen (20 µg) adjuvanted with 50 µg of aluminum hydroxide (Brenntag) and 10 µg of GLA. Group #3, also comprised of seven animals, was s.c. injected

with 1 mg of GLA-containing, *Pf*Trx-HPV-L2 vaccine powder, solubilized into 50 μL of PBS right before administration Test group #4 was comprised of ten mice, to which the GLA-containing, *Pf*Trx-HPV-L2-vaccine powder (about 2 mg) was administered intratracheally with the use of a modified Dry Powder Insufflator™ device as described above. The average amount of vaccine powder emitted from the insufflator was pre-determined by weighting the device before and after insufflation. As revealed by these measurements, approximately 50% of the loaded powder was emitted from the device and available for delivery; in detail, the average amount of vaccine powder emitted from the insufflator was 2.43±0.07 mg, corresponding to 48.7±1.37 μg of antigen). One week after the last immunization (total duration of the immunization protocol: 21 days) blood samples were collected by cardiac puncture, and the resulting sera, obtained by blood centrifugation (20 min at 10000 x g) after 3 hr at room temperature, were used to evaluate the immune responses in ELISA and neutralization assay.

2.2.12 ELISA. GST–Trx capture ELISA for anti-L2 antibody detection was carried out in 96-well microtiter plates pre-coated with glutathione-casein and subsequently blocked with casein buffer as described previously [38]. Individual immune sera were analyzed in duplicate by progressive (two-fold) serial dilutions starting from a 1:50 dilution, using a pool of pre-immune sera as a control. Following serum addition and 1 hr incubation at 37°C, plates were washed three times and incubated with a HRP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich) previously diluted 1:5000 in PBS containing 0.3% v/v Tween-20 (PBS-T). Plates were incubated for 1 h at 37°C, washed three times, and developed by adding the KPL ABTS Peroxidase Substrate [2,29-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)] staining solution (SeraCare; 100 μL/well). Absorbance at 405 nm was measured after 30 minutes at 30°C with a microplate reader (iMark™, Bio-Rad).
To evaluate protein antigen integrity, 96-well microtiter plates were coated with *Pf*Trx-HPV-L2 (5 μg/mL in PBS, 100 μL/well; o/n incubation at 4°C) pre-exposed to different concentrations of ethanol (0, 1, 5, 10, 15, 20, 30, 50, 70%) and/or to spray-drying . After three washes with PBS-T, plates were blocked with 3% skim milk in PBS for 1 hr at 37°C. Serial dilutions of a monoclonal anti-L2 antibody [(K4L2(20-38), [39]] or an anti-*Pf*Trx mAb were then added, followed by three washes as above and incubation for 1 hr at 37°C with a HRP-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich) diluted 1:5000 in PBS-T. After three additional washes, plates were developed by adding the KPL ABTS Peroxidase Substrate staining solution (100 μl/well) and read at 405 nm with a microplate reader after a 30 minutes incubation at 30°C (iMark™, Bio-Rad).

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2.2.13 Immunoglobulin isotyping. Immunoglobulin subclasses in sera from mice immunized with the different antigen formulations and modes of delivery were determined by ELISA using a Pierce mouse antibody isotyping kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A pool composed by equal volumes (100 μl each) of sera from the four best responding animals was employed as a test sample (50 μl fixed volume) that was added to ELISA strip-wells pre-coated with isotype-specific (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) anti-mouse heavy-chain capture antibodies and assayed in duplicate. Following incubation (1 hr at RT under gentle shaking conditions) with horseradish peroxidase (HRP)-conjugated, goat anti-mouse IgG+IgA+IgM detection

antibodies and washing, the TMB substrate was added, incubated for 5-15 minutes, and color development was read with a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad) at 405 nm.

2.2.14 Neutralization assay. L2 pseudovirion-based neutralization assays (L2-PBNA) were performed as described previously [40,41]. Briefly, following MCF10A cells plating, propagation/lysis, extracellular matrix (ECM) deposition and washing, a pseudovirion solution, prepared in conditioned medium from CHO furin cells and supplemented with 5 µg/ml heparin (Sigma H-4784) in a total volume of 120 µL/well, was added to the ECM-coated wells. The virus-furin-heparin mixture was incubated overnight at 37°C. The medium was then ~~removed~~removed, and the wells were washed twice with PBS. The final wash was then replaced with the immune sera (100 µL/well of the same sera utilized for immunoglobulin isotype profiling) serially diluted in pgsa-745 growth medium. The plate was incubated at 37°C for 6 h to allow for antibody binding to the target L2 epitope, followed by addition of pgsa-745 reporter cells (50 µL) at a concentration of $8x10^3$/well. For all assays, sera were serially diluted in 3-fold dilution steps starting from an initial 1:50 dilution. The secreted Gaussia luciferase was determined with the coelenterazine substrate and Gaussia glow juice using a microplate luminometer (Victor3, PerkinElmer). Data were analyzed and 50% inhibitory concentrations (IC50) were determined using the GraphPad Prism software program.

2.2.15 Statistical analysis. Unless otherwise indicated, all values are expressed as the mean ± standard deviation. Statistical significance of differences was evaluated using two-tailed unpaired t-test with significance level set at a p-value ≤ 0.05. Statistical analysis was performed with Microsoft Excel version 16.18 (Microsoft Corporation) and the Prism 7 software version 7.0d (GraphPad Software).

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3. Results and Discussion

3.1 Pre-formulation of respirable particles loaded with the *Pf*Trx-HPV-L2 antigen

Building upon previous work by Parlati et al. [23], we initially focused on the basic set-up of a DPI vaccine prototype using sodium stearate as a surfactant and a technological surrogate of the amphiphilic immune-adjuvant monophosphoryl lipid A (MPLA).

Spray-drying, which entails a temporary (in the order of milliseconds) exposure to fairly high temperatures (typically from 100°C to 140 °C) and a rapid interface conversion, may be a rather harsh process, especially when applied to protein-based bioactive components that may undergo irreversible denaturation as well as oxidation. Based on the above considerations, in addition to the use of a thermostable protein antigen, we adopted a particle engineering approach, paying special attention to two key steps, namely, i) the use of a water-ethanol solution in order to dissolve the amphiphilic component of the final formulation, which, on one hand, would allow to reduce the inlet drying temperature but, on the other, may be detrimental to protein stability; and ii) the total amount of solute in the feed solution in order to obtain a suitable amount of powder at the end of the process. In fact, should the solute only be comprised of the antigen, extremely high protein concentrations would be required, with protein aggregation and subsequent precipitation. We thus resorted to the supplementation of the feed solution with a bulking agent. To this end, we conducted a pre-formulation study with mannitol, lactose and trehalose as potential excipients. As a result of this study, mannitol was selected as the best bulking agent in the tested experimental conditions due the superior production yields it afforded and the superior aerodynamic performance of the protein-containing powder (see Supplementary Table ~~S1~~S2).

A further preliminary study indicated 70% v/v ethanol as the highest organic solvent content of the protein-containing (*Pf*Trx-HPV-L2: 1 mg/mL) hydro-alcoholic solution compatible with protein stability (see Supplementary Figure ~~S1~~S2). Thus, a 70:30 (v/v), water:ethanol solution was selected for the subsequent production of *Pf*Trx-HPV-L2 DPIs.

Table 1. Manufacturing parameters and aerodynamic properties of antigen-lacking powders containing different amounts of sodium stearate[1]

Powder #	Sodium Stearate (% w/w)	pH of Feed Solution	Yield (%)	$D_{v,50}$	EF (%)	RF (%)
1	-	5.89	54.63	6.08 (0.74)	87.51 (2.01)	43.52 (4.01)
2	0.33	5.88	31.53	18.58 (2.44)	75.16 (5.17)	8.02 (3.04)
3	1.00	7.94	43.72	3.40 (0.73)	89.89 (1.32)	48.10 (9.39)
4	2.00	8.14	59.46	26.35 (3.09)	94.46 (2.69)	13.59 (7.21)

[1]Sodium stearate content (as percentage by weight of the solutes contained in the feed solution), production yield, pH of the feed solutions, median volume diameter ($D_{v,50}$), Emitted Fraction (EF, as percentage of the loaded amount) and Respirable Fraction (RF, as percentage of the loaded amount) of powders prepared with different amounts of sodium stearate. Mean values and standard deviations in parenthesis (n=3).

In parallel, the concentration of sodium stearate in the feed solution (from 0.33 to 2.00% w/w of the solute) was investigated with respect to the particle size distribution (Table 1) and *in vitro* (fast screening) deposition of the resulting powders. The respirability *vs* sodium stearate concentration profile could be fitted to a quadratic equation with a maximum around 1% w/w sodium stearate, thus confirming previous data obtained in a similar experimental set-up [23].

Based on these pre-optimized experimental conditions, we then investigated the effect of an increased antigen content (i.e., a *Pf*Trx-HPV-L2 starting concentration higher than 1 mg/ml and a correspondingly higher fractional protein content) on the physico-chemical properties of the resulting powders prepared with 1% sodium stearate. Three antigen concentrations, expressed as solute weights in the feed solution of 0.83, 1.33 and 2.00% w/w, were tested (Powders #3a, #3b and #3c). The particle size distribution and the aerodynamic behavior of the resulting powders are reported in Figure 1.

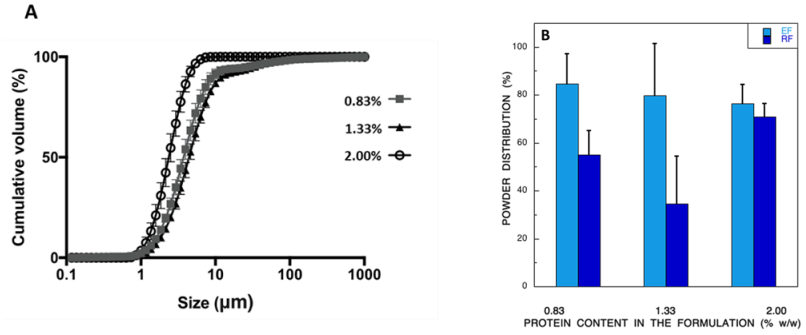

Figure 1. (A) Particle cumulative undersize distribution evaluated by laser light diffraction of spray-dried powders 3a, #3b and #3c containing different amounts of the antigen: 0.83% w/w (*squares*), 1.33% w/w (*triangles*), 2.00% w/w (*empty circles*) (mean values and standard deviation bars are indicated; n=3). **(B)** Emitted fraction (EF) and Respirable Fraction (RF) aerodynamic properties of three powders containing different amounts of the *Pf*Trx-HPV-L2 antigen as indicated. Mean and standard deviation values (n=3) are indicated.

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At a 2.00% w/w antigen concentration (obtained by adding 1.5 mL of an 8 mg/mL protein solution to 100 mL of feed solution), a monomodal powder (span 1.28 ± 0.04, Figure 1A) composed of highly respirable microparticles (RF=70.9% ± 5.6) (Figure 1B) was produced, with a spray-drying yield of approximately 65%. SDS-PAGE analysis (Figure 2A) confirmed the presence of an apparently intact, dry-powder associated antigen, whose functional integrity (i.e., the ability to be recognized by anti-HPV-L2 and anti-*Pf*Trx mAbs) was verified by ELISA (Figure 2B).

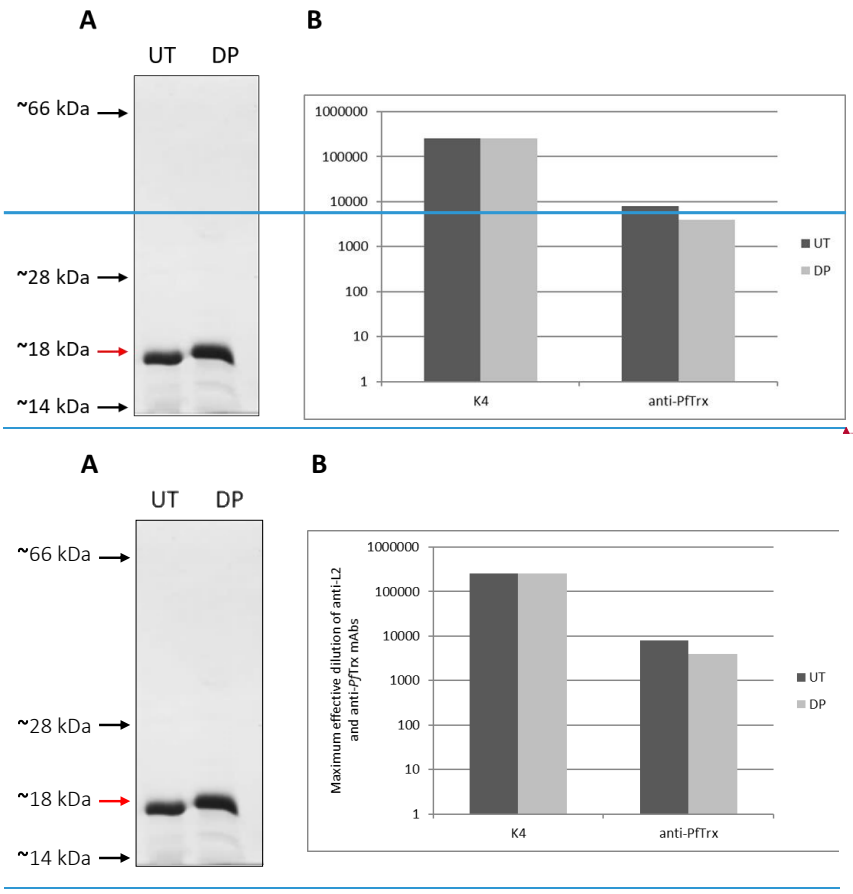

Figure 2. (A) SDS-PAGE analysis of the untreated (UT) liquid-form and the dry-powder formulated (DP) *Pf*Trx-HPV-L2 antigen. The expected migration position of the *Pf*Trx-HPV-L2 protein is indicated by a *red arrow*~~, the slight difference in electrophoretic mobility between the UT and the DP samples is due to a migration artifact~~; the migration positions of molecular mass markers are indicated by *black arrows*. **(B)** Comparative ELISA analysis of the immunoreactivity of the untreated (UT, *dark-grey*) and the dry-powder formulated (DP, *light-grey*) *Pf*Trx-HPV-L2 antigen. mAbs directed against the L2 epitope (K4) or the *Pyrococcus* thioredoxin scaffold (anti-*Pf*Trx) were used as primary antibodies. The results are the average of two technical replicates, which differed by no more than 5% ~~from~~ of the mean.

3.2 Conversion of *Pf*Trx-HPV-L2 into a prototype respiratory vaccine using GLA as a particle lubricant and a built-in immune-adjuvant

The presence of an immune-adjuvant is an essential prerequisite for subunit vaccine immunogenicity [1,2], including vaccines such as Gardasil and Cervarix that are made up by

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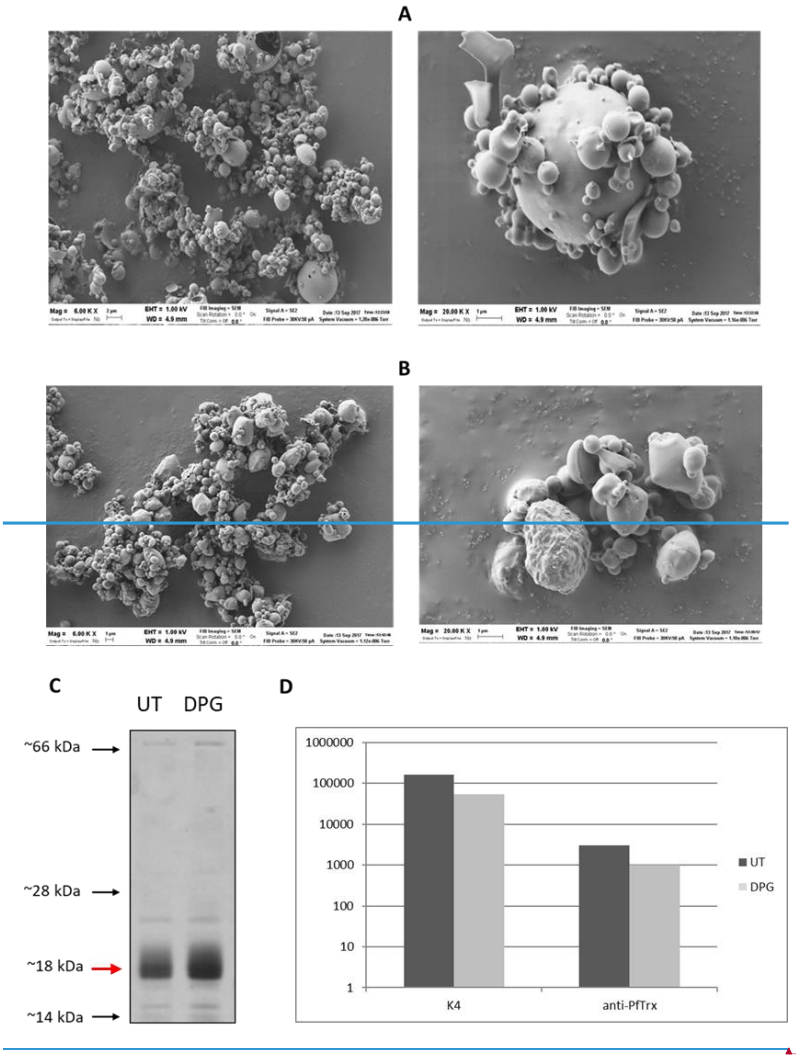
intrinsically highly immunogenic HPV-L1 pseudovirion components [25,26,28]. GLA is a synthetic derivative of MPLA, a low-toxicity lipopolysaccharide that is present as a biological adjuvant in various licensed vaccines, including Cervarix. GLA is an amphiphilic molecule that acts as an agonist of toll-like receptor 4 (TLR4) [42]. GLA itself [43] and its parent compound MPLA [20,27,44] have proved to be effective immune adjuvants when included in formulations for nasal and pulmonary administration. The amphiphilic nature of GLA prompted us to test its possible use as a sodium stearate replacement, capable of acting as both a coating agent enhancing particle flow, de-aggregation and ultimately respirability, as well as a built-in immune-adjuvant.

We thus set out to incorporate GLA as a sodium stearate substitute in our *Pf*Trx-HPV-L2 microparticle formulation. As a first approximation, we estimated the amount of GLA that would be required to obtain a particle surface coating ~~similar to~~like that achieved with sodium stearate, starting from the assumption that a lower amount of a larger molecule is needed to cover the same surface. Based on the molecular weight ratio between sodium stearate (306.5 g/mol) and GLA (1763.5 g/mol) ~~and sodium stearate (306.5 g/mol)~~, a 1% (w/w) fractional amount of sodium stearate corresponds to approximately 0.17% w/w GLA. With respect to the GLA concentration in the feed solution, this amount corresponds to 1.02 µg/ml, which is one order of magnitude lower than the reported CMC for MPLA [45]. Thus, also considering that the feed solution contained 30% v/v ethanol, we assumed that GLA was completely dissolved without micelle formation, which represents a key feature for the surfactant migration at the air/liquid interface of the droplets during the spray-drying process [23,46,47]. The adjuvant concentration we employed is significantly higher than that reported in the literature [20], thus allowing to assume that a relevant portion of GLA distributed to the droplet air liquid interface during the drying process and, ultimately, on the microparticle surface.

A new powder, containing GLA as both an amphiphilic technological excipient with particle lubricant functionality and an immune adjuvant, was thus produced, starting from 4 and 12 mg/mL solutions of detoxified and filter-sterilized *Pf*Trx-HPV-L2 antigen dissolved in phosphate buffer. In this way, an antigen concentration in the final powder of 1.33% w/w (Powder #5) or 2 % w/w (Powder #6) was obtained.



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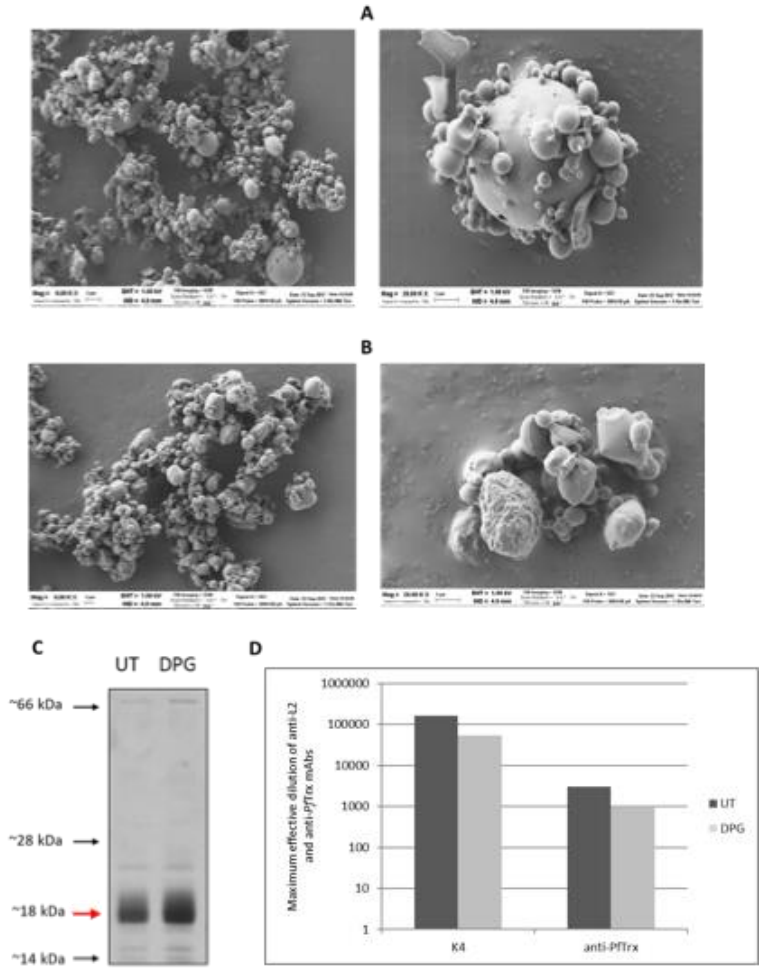

Figure 3. SEM images of the GLA-containing powder #5 (1.33% w/w of *Pf*Trx-HPV-L2) **(A)** and powder #6 containing 2.00% w/w *Pf*Trx-HPV-L2 **(B)**. Magnification 6.000x (*left-hand image*) and 20.000x (*right-hand image*). **(C)** SDS-PAGE analysis of the untreated liquid-form (UT) and the dry-powder-GLA formulated (DPG) *Pf*Trx-HPV-L2 antigen. The expected migration position of the *Pf*Trx-HPV-L2 protein is indicated by a *red arrow*; the migration positions of molecular mass markers are indicated by *black arrows*. **(D)** ELISA analysis of the untreated (UT, *dark-grey*) and the dry-powder-GLA formulated (DPG, *light-grey*) *Pf*Trx-HPV-L2 antigen. mAbs directed against the L2 epitope (K4) or the *Pyrococcus* thioredoxin scaffold (anti-*Pf*Trx) were used as primary antibodies for detection. The results are the average of two technical replicates, which differed by no more than 5% ~~from~~ of the mean.

The resulting particles (production yields of 79 and 65%, respectively), which were mainly spherical in the case of 1.33% *Pf*Trx-HPV-L2 and more irregularly shaped for the higher (2.00% w/w) antigen

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concentration (Figures 3A and 3B), featured a narrow mono-modal size distribution: ~~with a~~for Powder #5 $D_{v,10}$= 1.27±0.12 µm, $D_{v,50}$ of 2.39 ± 0.24 µm, $D_{v,90}$ =4.30±0.32 µm, Span value= 1.27; for Powder #~~5 6 and~~ $D_{v,10}$= 1.43±0.09 µm, $D_{v,50}$ =2.65 ± 0.18 µm, $D_{v,90}$ =4.78±0.55 µm~~,~~, Span value= 1.26~~for Powder #6~~. The solution obtained upon particle solubilization in ultrapure water displayed a slightly basic pH (7.5 and 8.0 for Powder #5 and #6, respectively).

The lubricant functionality of GLA was compared to that of sodium stearate by evaluating the powder flow properties through the determination of the dynamic angle of repose according to Ph. Eur. 10th ed. The measurement was carried out on powders containing the same amount of antigen (1.33%). The GLA-containing powder had an angle of repose of 18.74±1.48 ° indicative of excellent flow properties, as opposite to the sodium stearate-containing powder which showed poor flowability (angle of repose = 46.6 ± 0.60°).

Aerosolization performance analysis, initially conducted with the RS01® device and a Fast Screening Impactor (FSI), yielded an Emitted Fraction (EF) of 86.0% ± 0.8 (Powder #5) and 81.3% ± 11.2 (Powder #6) and a Fine Particle Fraction (FPF) of 61.2% ± 8.6 (Powder #5) and 60.0% ± 2.0 (Powder #6).

To gain more detailed information on the aerodynamic particle size distribution of powder #6 (the one with the highest antigen content), aerodynamic performance was also assessed with an Andersen Cascade Impactor (ACI) through quantitative detection of mannitol in the impactor stages by a HPLC/RID method. Since the protein is embedded within GLA-coated, mannitol-containing particles, it was assumed that mannitol could be used as a reliable indicator of antigen emission from the device and its distribution within the different stages of the impactor. Mannitol emitted from RS01® was 12.5± 1.5 mg (EF = 64.2%±7.85), with a Fine Particle Mass (FPM) of approximately 9±0.7 mg (RF = 72.3%± 5.59) and a Median Mass Aerodynamic Diameter (MMAD) of 2.5± 0.1 µm.

A subset of powder #6-filled capsules was stored at room temperature under light-shielded conditions and re-tested 5 months after production. The mannitol EF was found to be approximately 10% higher compared to time zero; RF also increased (83.2%±12.1), although for both parameters the difference was not statistically significant with respect to the values recorded at time zero (p>0.5); ~~whereas the~~ MMAD remained essentially the same. ~~Surprisingly, a better aerodynamic performance was observed after storage, likely due to either loss of residual humidity or surface charge dissipation and a consequent improvement of powder deaggregation upon aerosolization.~~

The presence of residual free water was excluded by Thermo-Gravimetric Analysis, which indicated a negligible water loss (<0.1%) upon heating (not shown), while Isothermal Dynamic Vapor Sorption measurements carried out at both 25°C and 40°C revealed the very low and completely reversible tendency of the powder to absorb humidity (see Supplementary Figure S2). This can be ascribed both to the partial hydrophobic coating of the particle surface with GLA and to the fact that the spray-drying process yielded the β-form of mannitol (melting peak at 166°C revealed by DSC analysis; not shown), which represents the thermodynamically stable and non-hygroscopic crystal form of the bulking agent (i.e., the most abundant component of the powder). As revealed by semi-quantitative SDS-PAGE analysis (Figure 3C), the *Pf*Trx-HPV-L2 content of the GLA powder was in line with the amount expected ~~on the basis of~~based on the input concentration and fractional content of the protein. Importantly, the immunoreactivity of the solubilized spray-dried antigen (i.e., the

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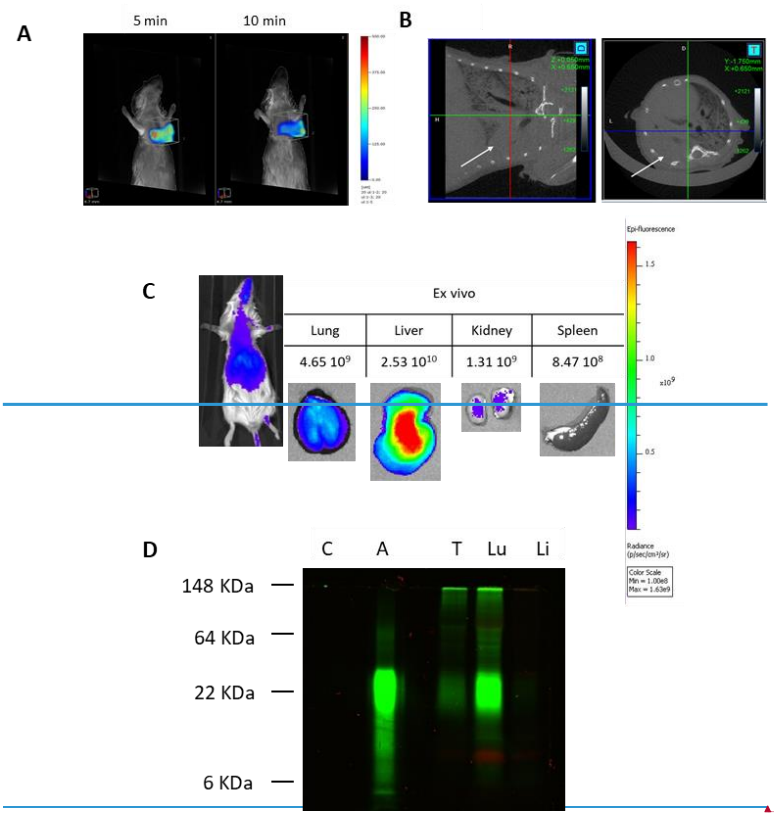
antigen retrieved from particles dissolved in aqueous buffer) revealed by ELISA was the same as that of the native antigen (Figure 3D).

3.3 Lung deposition of the *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine administered intratracheally

To gain insight on the lung deposition capacity of the *Pf*Trx-HPV-L2-[GLA] vaccine (Powder #6) administered intratracheally, we set-up conditions for direct *in vivo* imaging of the antigen. To this end, we used the NIR–dye Alexa Fluor 750 to label the *Pf*Trx-HPV-L2 protein. The labeled protein was then incorporated into GLA-coated particles using the previously developed formulation procedure (spray-dry yield of 57.8% w/w). Prior to intratracheal delivery, the powder was subjected to an FSI aerodynamic evaluation, which yielded Emitted Fraction and Respirable Fraction values (87.2% $\pm$ 6.1 and 67.0% $\pm$ 4.8, respectively) superimposable to those obtained with the unlabeled protein-containing powder.



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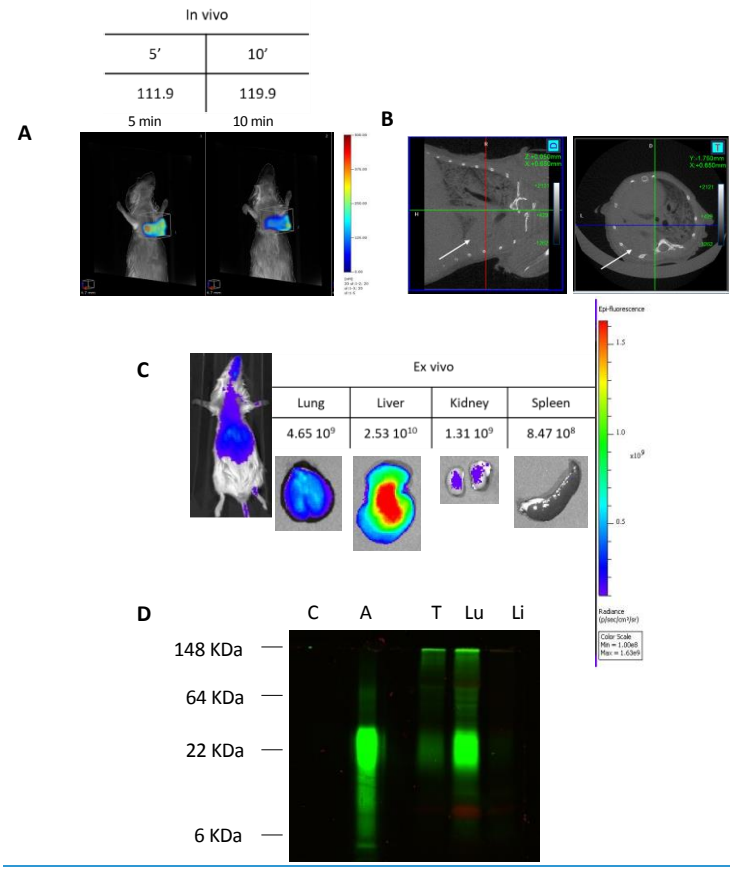

Figure 4. Tissue distribution of the Alexa Fluor750-labeled *Pf*Trx-HPV-L2 antigen delivered intratracheally in liquid form. Intratracheally administered, pre-dissolved Alexa-labeled vaccine powder visualized by FMT, 5 and 10 min after intratracheal delivery **(A)** and by Micro-CT **(B)** imaging (transversal and coronal views are shown in the left- and right-side images, respectively, where the arrows indicate the accumulation sites of the delivered liquid vaccine). *In vivo* **(C**, *left-side panel*) and *ex-vivo* multi-organ **(C**, *right-side panel***)** IVIS imaging of the Alexa-labeled *Pf*Trx-HPV-L2 antigen 15 min after intratracheal administration. **(D)** SDS-PAGE fractionation and fluorescence-based visualization of trachea (T), lung (Lu) and liver (Li) tissue homogenates derived from a mouse, to which the pre-dissolved, Alexa-labeled dry-powder vaccine was administered intratracheally~~-~~; equivalent amounts (5 ng) of the unlabeled (C) and the Alexa-labeled (A)~~,~~ *Pf*Trx-HPV-L2 protein served as controls for this experiment. The results are from a representative experiment that was performed in parallel on two different animals.

In a preliminary experiment aimed at setting-up mouse pulmonary administration conditions, the labeled dry-powder vaccine was dissolved in aqueous buffer prior to intratracheal delivery. As shown in Figure 4A, the Alexa-labeled *Pf*Trx-HPV-L2 protein could be tracked in the lungs by FMT,

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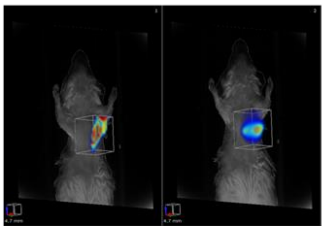
with nearly the same fluorescence intensity at 5 and 10 min after delivery and a slightly preferential (but likely random) accumulation in the left lobe. This kind of distribution was confirmed by Micro-CT imaging, which allowed~~s~~ to visualize the fine anatomical details of the lung delivery site (Figure 4B). As revealed by IVIS imaging, after 15 min the fluorescence signal became more diffuse and apparently spread to the liver and other sub-thoracic organs (Figure 4C). Indeed, an intense fluorescence was detected not only in the lungs but also in the liver and other organs explanted after mouse sacrifice. To distinguish between protein-associated fluorescence and a spurious signal arising from protein degradation and release into the circulation of the Alexa dye (either free or associated to *Pf*Trx-HPV-L2 peptide fragments), trachea, lung and liver tissue homogenates from the same animal were examined by SDS-PAGE. As shown in Figure 4D, a strong and a slightly weaker but well detectable fluorescence signal was found to be associated -in lung and trachea extracts, respectively- with a polypeptide displaying the same electrophoretic mobility as the authentic Alexa-labeled *Pf*Trx-HPV-L2 protein, whereas no fluorescence was detected in the case of the liver homogenate. This indicates that liver fluorescence was indeed associated to small-sized degradation products of the labeled *Pf*Trx-HPV-L2 protein, that ran out of the gel upon electrophoretic fractionation.

Next, a similar experiment was conducted with the *Pf*Trx-HPV-L2-[GLA] powder (2 mg containing 12 μg of Alexa-labelled antigen), delivered intratracheally with the use of a modified dry powder insufflator device. As shown by the data presented in Figure 5, also under these conditions, which closely mimic the actual set-up of mouse pulmonary vaccination, a fairly even antigen distribution within the respiratory tract, with a marked accumulation in the lungs, was revealed by *in-vivo* and *ex-vivo* analyses. Specifically, *in vivo* imaging by FMT revealed fluorescence signals of similar intensity in the lungs at both 5 and 10 min after administration (Figure 5A). A more targeted localization to the chest region compared to the solubilized vaccine was revealed by IVIS imaging (*cf*. Figure 5C with Figure 4C). As shown in Figure 5B, these results were confirmed by the more anatomically detailed visualization afforded by Micro-CT analysis.

Altogether, these data fully support the favorable aerodynamic properties and suitability for pulmonary administration of the *Pf*Trx-HPV-L2-[GLA] powder initially pointed out by the results of *in vitro* aerosolization analyses.

A



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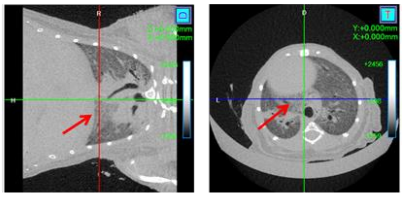
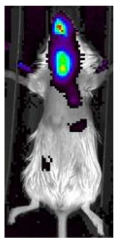
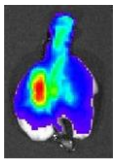
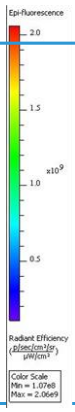


C

In vivo		Ex vivo
Trachea	Lung	5.86 10^9
1.03 10^9	3.53 10^8	







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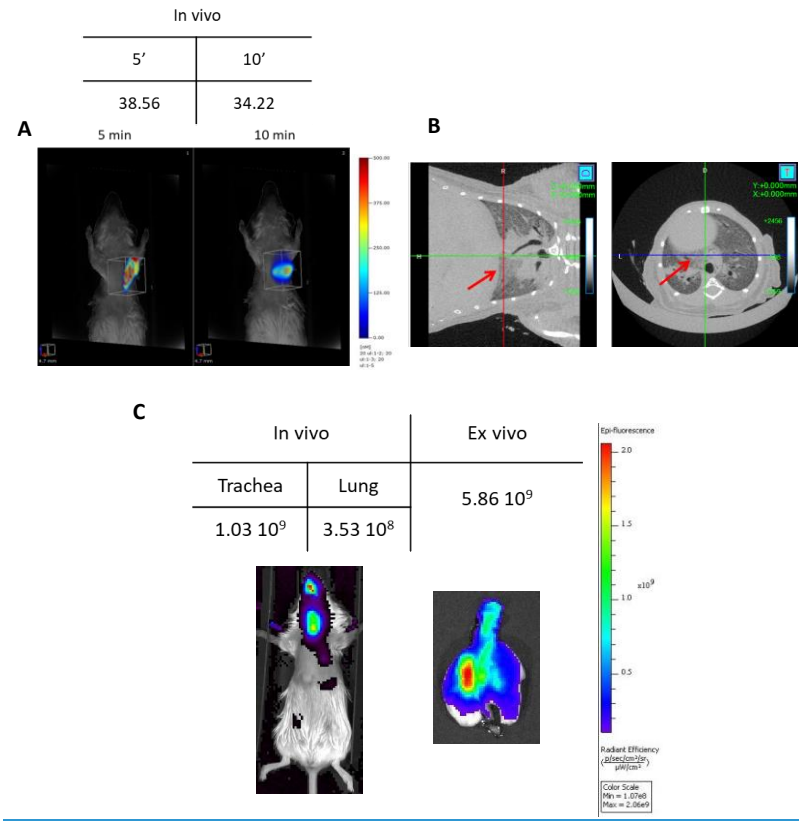

Figure 5. Lung deposition of the intratracheally administered dry-powder vaccine. (A) *In vivo* total body FMT imaging of the Alexa Fluor750-labelled *Pf*Trx-HPV-L2 antigen distribution at two different time-points (5 and 10 min) after administration of the vaccine powder. (B) Micro-CT imaging of antigen deposition into the lungs; transversal and coronal views are shown in the left- and right-side images, respectively, where the arrows indicate the vaccine powder deposition sites. (C) *In vivo* (*left panel*) and *ex-vivo* (*right panel*), IVIS analysis of Alexa Fluor 750-labelled *Pf*Trx-HPV-L2 accumulation in the respiratory tract, 15 min after intratracheal delivery of the dry-powder vaccine.

3.4 Preliminary evaluation of the immunogenicity of the pulmonary delivered *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine

A comparative, pulmonary *vs*. subcutaneous (s.c.) vaccination experiment was then set-up. ~~In addition to the test sample (#4), i.e., the *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine administered intratracheally,~~ This included the following treatment and control groups: group #1) s.c. injection of a solubilized, GLA-containing dry-powder lacking the antigen (negative control); group #2) s.c.

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injection of the liquid-form, alum+GLA-adjuvanted *Pf*Trx-HPV-L2 antigen; group #3) s.c. injection of the *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine solubilized immediately before administration; group #4 the *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine administered intratracheally.

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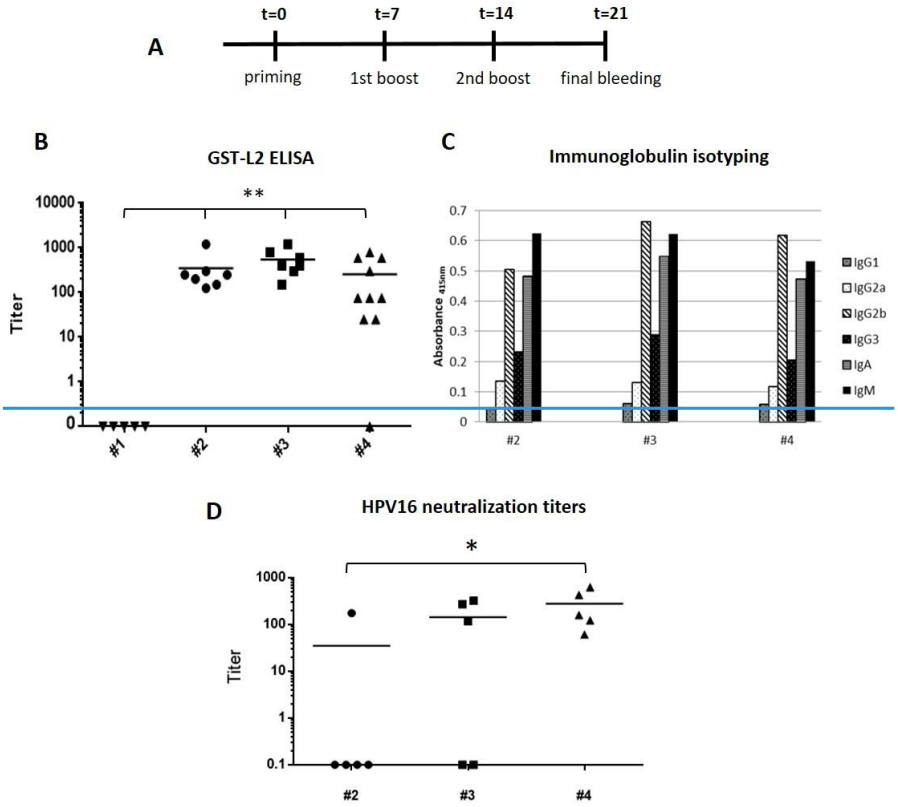

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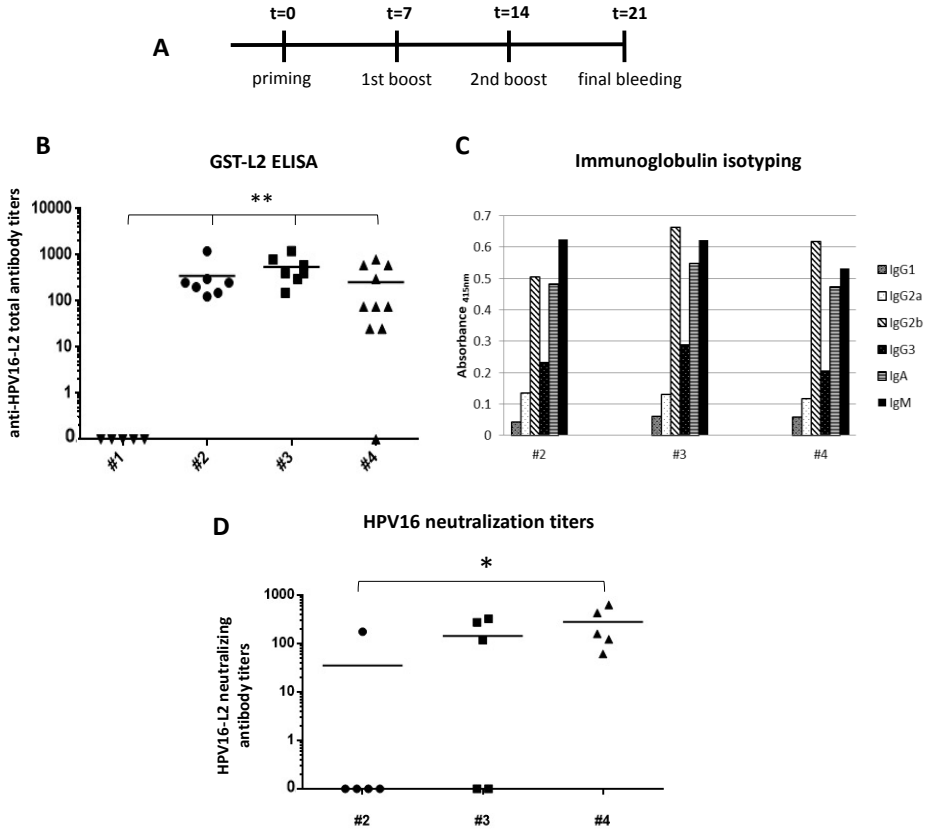

Figure 6. Immunogenicity profiling. **(A)** Timeline of the short-term immunization experiment performed with the GLA-adjuvanted, *Pf*Trx-HPV-L2 DPI delivered intratracheally. **(B)** GST-L2 ELISA performed on sera from differently treated groups of mice, collected one week after the last immunization. The following treatments were applied to individual groups: #1, negative control, subcutaneous immunization with solubilized, GLA only containing powder with no antigen; #2, subcutaneously injected, liquid form, GLA+alum adjuvanted *Pf*Trx-HPV-L2 antigen; #3 subcutaneously injected *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine solubilized in PBS immediately before administration; #4, *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine administered intratracheally. Data represent anti-HPV-L2 total antibody titers measured in individual mice; the means of the titers for each group are indicated by horizontal lines (**$p \leq 0.01$). **(C)** Immunoglobulin isotyping of pooled sera from the mice groups (#2, #3 and #4) shown in panel B, ~~with the exception of~~except for the completely unresponsive, negative control group #1. **(D)** HPV16 neutralization titers determined by the L2-PBNA on a subset of five immune-sera/group (#2, #3 and #4) derived from the animals in each group that displayed the highest (top five) anti-HPV-L2 total antibody titers by GST-L2 ELISA (panel B). Data represent neutralizing antibody titers measured in individual mice; mean values of the titers for each group are indicated by horizontal lines (*$p \leq 0.05$).

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~~In order to~~To minimize the risk (and potential confounding effects) of bacterial infections associated to intratracheal delivery, we decided to use a short-schedule immunization protocol. This only involved two boost immunizations at a one-week interval, followed by final bleeding and immune sera collection after one more week (Figure 6A). As shown by the ELISA data reported in Figure 6B, very similar titers of total anti-L2 antibodies were detected in all groups, except for the antigen-lacking, negative control. The fact that absolute titers (average value=1:370) were significantly lower than those detected previously with the same antigen delivered subcutaneously [48] is likely explained by the short-term immunization protocol adopted in the present exploratory study (21 days with only two boost injections and final bleeding all one-week a part), compared to 72 days with three biweekly boost injections and a 1-month time-interval before sera collection [31,32,34]. We also determined the immunoglobulin isotype-profile of the antibodies elicited by the different immunogens and modes of delivery. As shown in Figure 6C, the immunoglobulin isotype distributions associated to the dry-powder and the liquid vaccines were quite similar. In both cases, the Th1-associated IgG2b and IgG3 isotypes were considerably (10-fold on average) more represented than the Th2-associated IgG1 isotype. This indicates a marked Th1 polarization of the immune response that was previously shown to be causally associated to the use of the GLA immune-adjuvant [42,43]. Also highly represented were the IgM and IgA immunoglobulins, with a slight prevalence of the latter isotype in the case of the dry-powder vaccine.

Despite the short immunization schedule employed for this exploratory experiment, we also determined the ability of the induced anti-L2 antibodies to neutralize HPV16 pseudovirions. To this end, we used the highly sensitive L2-based pseudovirion neutralization assay [41] to determine the presence of HPV16 neutralizing antibodies. As shown in Figure 6D, the highest and most consistent titer (5 positives out of 5 tested serum samples) was detected in sera from mice immunized intratracheally with the dry-powder formulation (#4). Interestingly, a slightly lower and less consistent (3 positives out of 5 tested sera) but well detectable neutralizing response was also observed in mice subcutaneously immunized with the solubilized dry-powder vaccine (#3). Instead, a poor neutralizing response, with only one out of five tested sera capable of HPV16 pseudovirion neutralization, was observed for sera from mice immunized subcutaneously with the liquid vaccine formulation (#2). The latter result, which contrasts with the neutralization capacity previously documented for the liquid-form, Montanide ISA720-, Alum/MPLA- or AddaVAx-adjuvanted *Pf*Trx-HPV-L2 antigen administered subcutaneously using the standard 72 days immunization protocol [31,32,34,48], likely reflects the short-term immunization schedule utilized for this experiment and perhaps the lack of an adequate length of time for antibody affinity-maturation. Under these sub-optimal but highly stringent immunization conditions, the dry-powder formulated *Pf*Trx-HPV-L2-[GLA] vaccine administered intratracheally, thus appears to be superior to the liquid-form vaccine, at least with respect to the short-term induction of HPV16 neutralizing antibodies.

4. Conclusions

We have provided proof-of-concept validation of a novel particle engineering approach that allows to produce a highly respirable powder vaccine by exploiting GLA as a double-purpose component capable of acting as both a technological lubricant improving the aerodynamic properties of the powder and an immune adjuvant. Both features stem from the amphiphilic nature of the GLA molecule that partitions on the particle surface during spray-drying manufacturing.

We also exploited the thermostability of the *Pf*Trx-HPV-L2 antigen, which proved to be resistant to a fairly high ethanol concentration, for immediate drying of a mannitol-supplemented, antigen-containing solution. The resulting dry-powder vaccine, which was always handled under non-refrigerated conditions, did not change its physico-chemical or aerodynamic properties, and retained a fully intact antigen upon storage for five months at room temperature. Indirect evidence, such as powder vaccine immunogenicity and the marked Th1 polarization of the induced immune response, indicates that the relatively harsh conditions associated to spray-drying are also well tolerated by the GLA adjuvant.

An open question regards the applicability of the present approach to other, less sturdy antigens. However, preliminary results (not presented in this work) indicate that a good quality and immunogenic dry powder vaccine could also be obtained with a more complex and less thermostable antigen, in which *Pf*Trx-HPV-L2 is genetically fused to an oligomerization domain that converts the primary antigen into a heptameric nanoparticle form [49-50].

Pulmonary delivered powder vaccines have often been found to induce a superior immune response compared to their parenterally injected counterparts [7,21,22]. It is thus not so surprising that even in our preliminary, and largely sub-optimal immunization experiments, the intratracheally administered DPI vaccine outperformed the subcutaneously injected vaccine in the short-term induction of HPV16 neutralizing antibodies. The reasons for this apparent superiority remain to be clarified. They might be related to either the particulate nature of a dry-powder vaccine, which has previously been shown to enhance immunogenicity through a more efficient targeting of antigen presenting cells [7,21], the closer physical association between the antigen and the GLA adjuvant inherent to the dry powder formulation, and/or to the large surface area and extensive vascularization of the lungs [18]. Interestingly, even the DPI vaccine solubilized right before subcutaneous injection, induced higher and more consistent neutralizing antibody titers than its liquid-form counterpart. Whether this is due to residual micro-particle aggregates that resisted complete dissolution or to a tighter association between the GLA adjuvant and the antigen is presently not known. Also unknown, and worth of future, more detailed investigations is the ability of the *Pf*Trx-HPV-L2 vaccine (and its polytopic derivatives [49-50]) to induce a mucosal response in the lungs, but also in the genital apparatus. This, together with a more extensive characterization of the HPV neutralizing immune responses elicited by a longer-term immunization protocol, will also shed light on the cross-neutralization potential of the pulmonary delivered *Pf*Trx-HPV-L2 vaccine. Altogether, the data presented in this work represent a step forward toward the development of needle-free and easy to handle, next-generation vaccines.

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Conflicts of interest

The authors declare no conflict of interest. A European patent application based on the results of this study has been filed (WO2019EP78277).

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Table 1. Manufacturing parameters and aerodynamic properties of antigen-lacking powders containing different amounts of sodium stearate[1]

Powder #	Sodium Stearate (% w/w)	pH of Feed Solution	Yield (%)	$D_{v,50}$	EF (%)	RF (%)
1	-	5.89	54.63	6.08 (0.74)	87.51 (2.01)	43.52 (4.01)
2	0.33	5.88	31.53	18.58 (2.44)	75.16 (5.17)	8.02 (3.04)
3	1.00	7.94	43.72	3.40 (0.73)	89.89 (1.32)	48.10 (9.39)
4	2.00	8.14	59.46	26.35 (3.09)	94.46 (2.69)	13.59 (7.21)

[1]Sodium stearate content (as percentage by weight of the solutes contained in the feed solution), production yield, pH of the feed solutions, median volume diameter ($D_{v,50}$), Emitted Fraction (EF, as percentage of the loaded amount) and Respirable Fraction (RF, as percentage of the loaded amount) of powders prepared with different amounts of sodium stearate. Mean values and standard deviations in parenthesis (n=3).

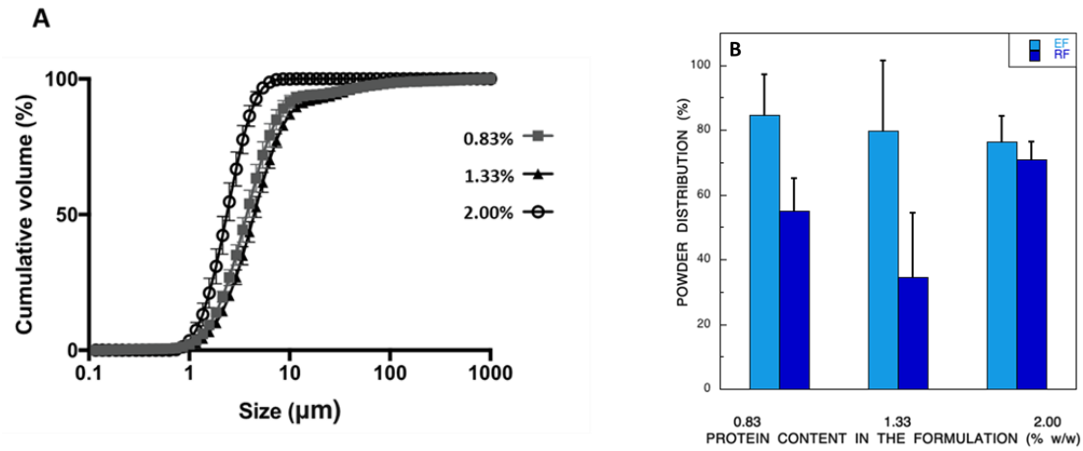

Figure 1. (A) Particle cumulative undersize distribution evaluated by laser light diffraction of spray-dried powders 3a, #3b and #3c containing different amounts of the antigen: 0.83% w/w (*squares*), 1.33% w/w (*triangles*), 2.00% w/w (*empty circles*) (mean values and standard deviation bars are indicated; n=3). **(B)** Emitted fraction (EF) and Respirable Fraction (RF) aerodynamic properties of three powders containing different amounts of the *Pf*Trx-HPV-L2 antigen as indicated. Mean and standard deviation values (n=3) are indicated.

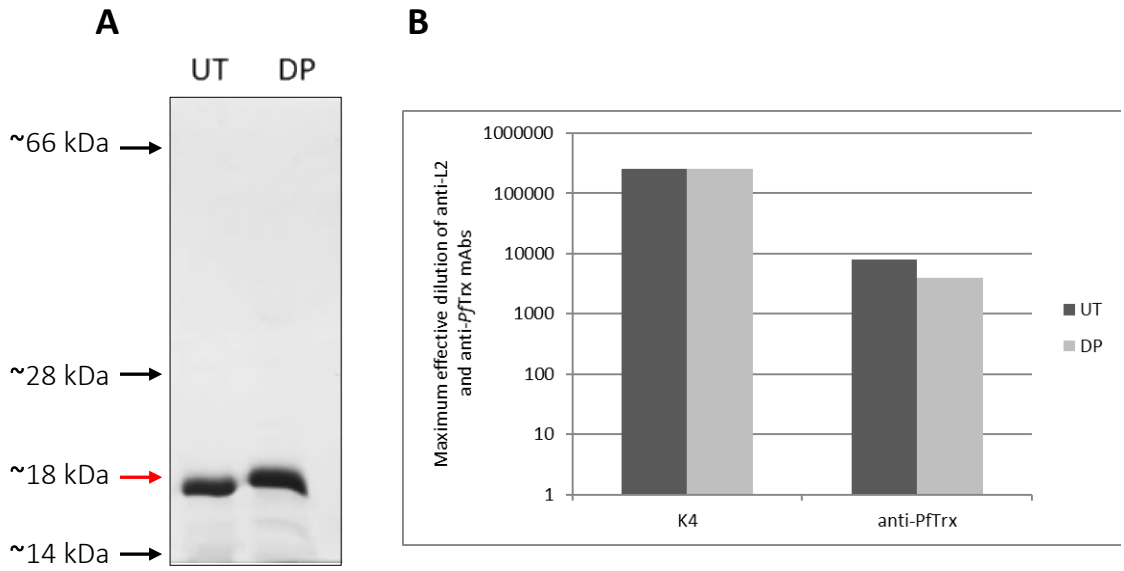

Figure 2. (A) SDS-PAGE analysis of the untreated (UT) liquid-form and the dry-powder formulated (DP) *Pf*Trx-HPV-L2 antigen. The expected migration position of the *Pf*Trx-HPV-L2 protein is indicated by a *red arrow*; the migration positions of molecular mass markers are indicated by *black arrows*. **(B)** Comparative ELISA analysis of the immunoreactivity of the untreated (UT, *dark-grey*) and the dry-powder formulated (DP, *light-grey*) *Pf*Trx-HPV-L2 antigen. mAbs directed against the L2 epitope (K4) or the *Pyrococcus* thioredoxin scaffold (anti-*Pf*Trx) were used as primary antibodies. The results are the average of two technical replicates, which differed by no more than 5% of the mean.

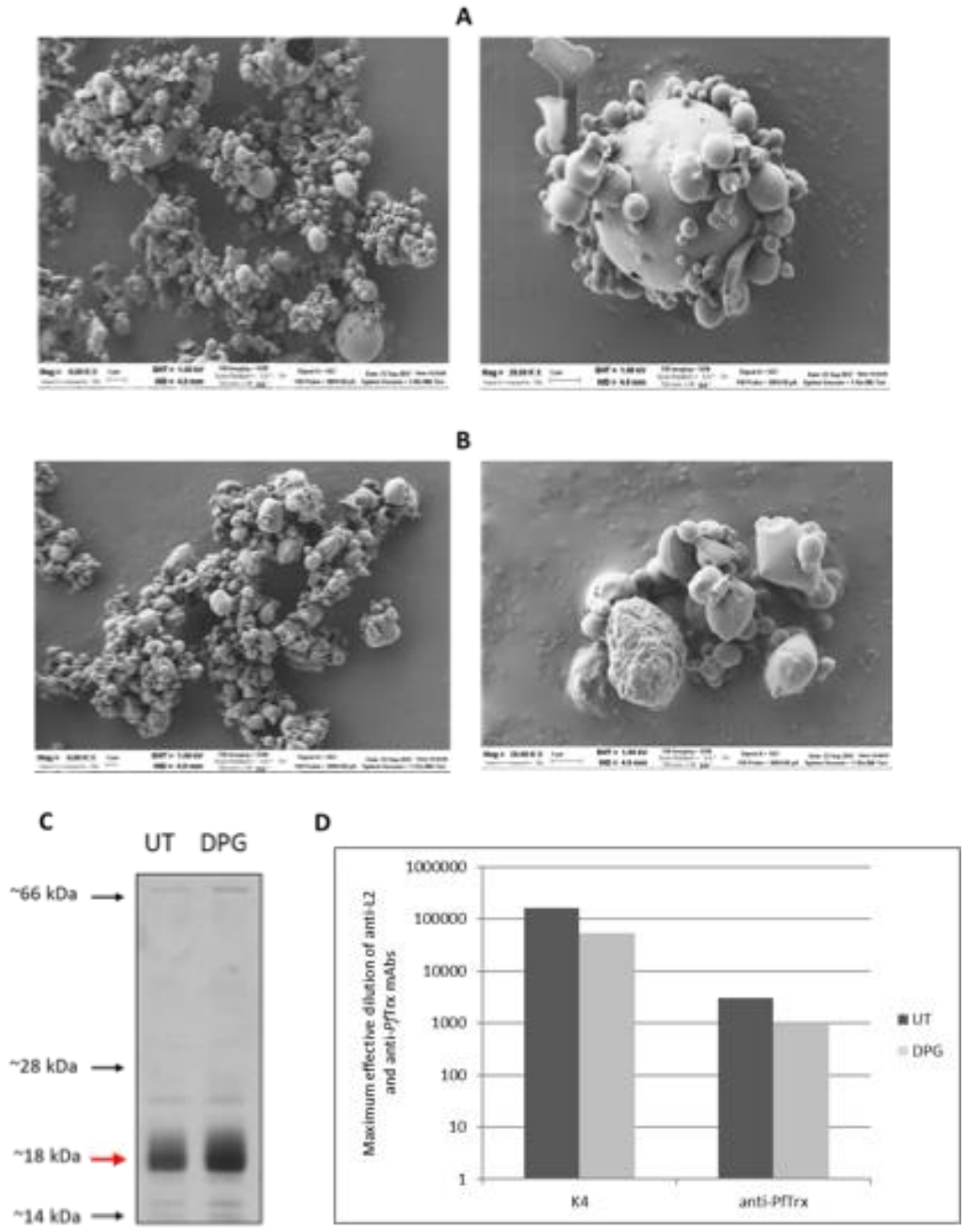

Figure 3. SEM images of the GLA-containing powder #5 (1.33% w/w of *Pf*Trx-HPV-L2) **(A)** and powder #6 containing 2.00% w/w *Pf*Trx-HPV-L2 **(B)**. Magnification 6.000x (*left-hand image*) and 20.000x (*right-hand image*). **(C)** SDS-PAGE analysis of the untreated liquid-form (UT) and the dry-powder-GLA formulated (DPG) *Pf*Trx-HPV-L2 antigen. The expected migration position of the *Pf*Trx-HPV-L2 protein is indicated by a *red arrow*; the migration positions of molecular mass markers are indicated by *black arrows*. **(D)** ELISA analysis of the untreated (UT, *dark-grey*) and the dry-powder-GLA formulated (DPG, *light-grey*) *Pf*Trx-HPV-L2 antigen. mAbs directed against the L2 epitope (K4) or the *Pyrococcus* thioredoxin scaffold (anti-*Pf*Trx) were used as primary antibodies for detection. The results are the average of two technical replicates, which differed by no more than 5% of the mean.

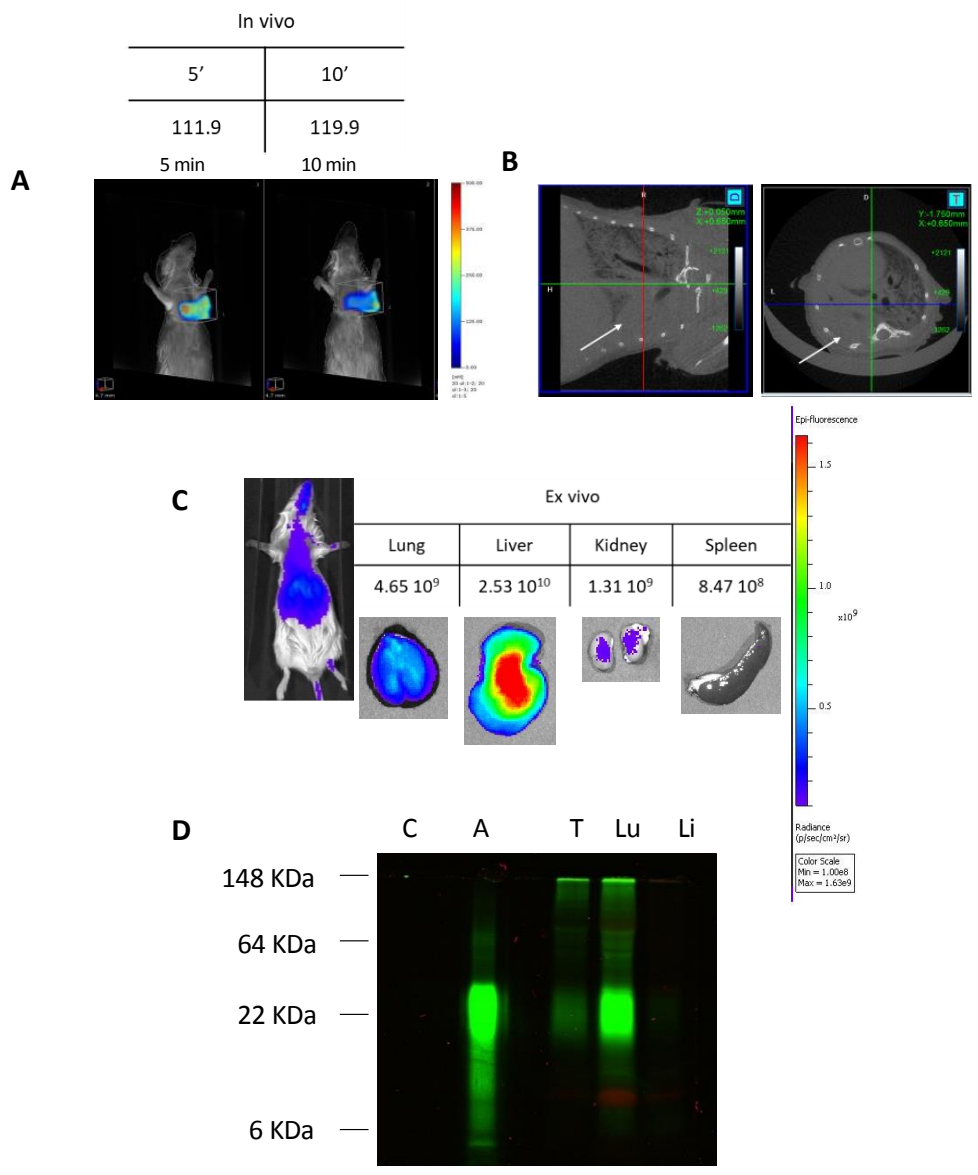

Figure 4. Tissue distribution of the Alexa Fluor750-labeled *Pf*Trx-HPV-L2 antigen delivered intratracheally in liquid form. Intratracheally administered, pre-dissolved Alexa-labeled vaccine powder visualized by FMT, 5 and 10 min after intratracheal delivery **(A)** and by Micro-CT **(B)** imaging (transversal and coronal views are shown in the left- and right-side images, respectively, where the arrows indicate the accumulation sites of the delivered liquid vaccine). *In vivo* **(C**, *left-side panel*) and *ex-vivo* multi-organ **(C**, *right-side panel***)** IVIS imaging of the Alexa-labeled *Pf*Trx-HPV-L2 antigen 15 min after intratracheal administration. **(D)** SDS-PAGE fractionation and fluorescence-based visualization of trachea (T), lung (Lu) and liver (Li) tissue homogenates derived from a mouse, to which the pre-dissolved, Alexa-labeled dry-powder vaccine was administered intratracheally; equivalent amounts (5 ng) of the unlabeled (C) and the Alexa-labeled (A) *Pf*Trx-HPV-L2 protein served as controls for this experiment. The results are from a representative experiment that was performed in parallel on two different animals.

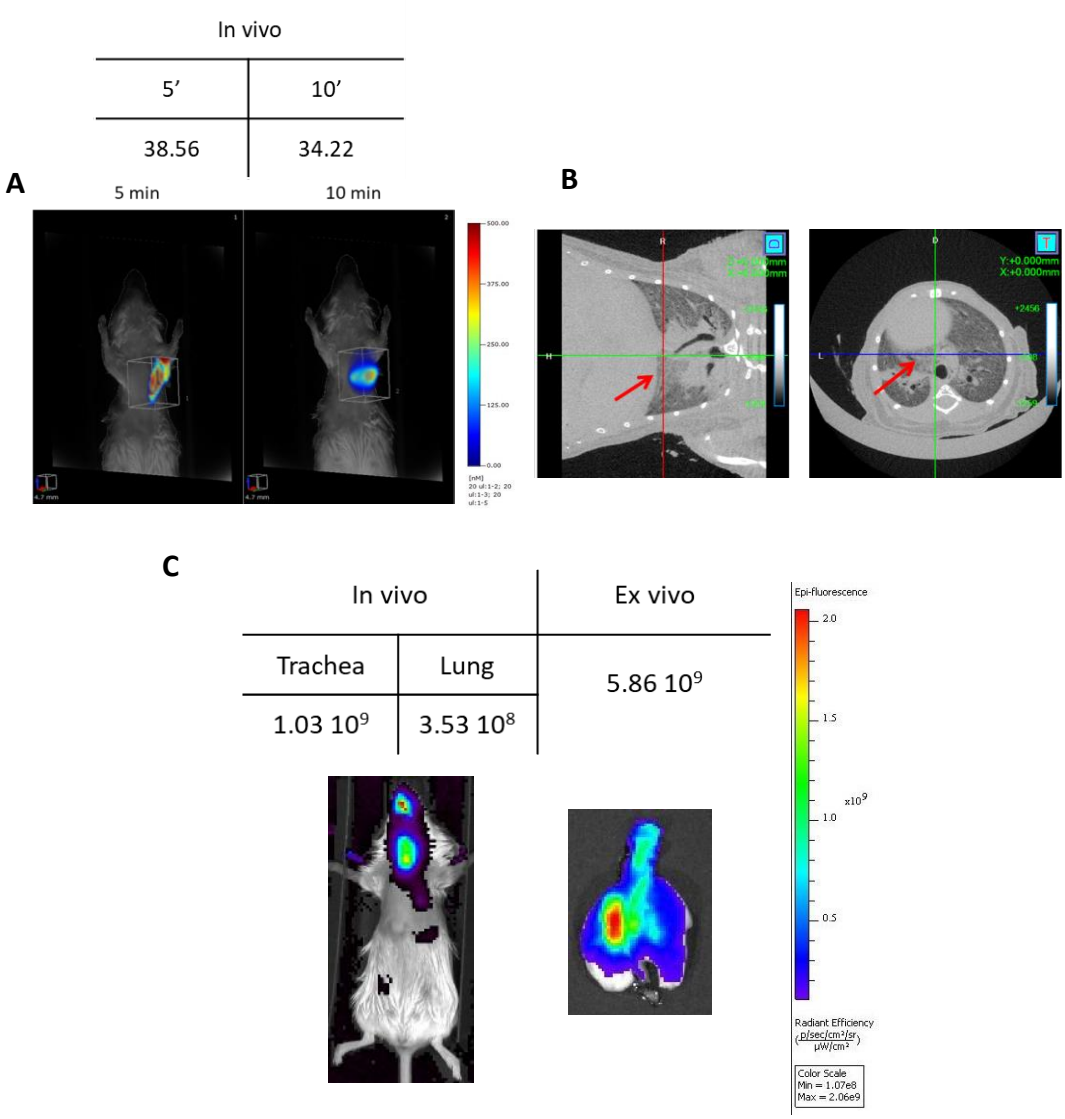

Figure 5. Lung deposition of the intratracheally administered dry-powder vaccine. (A) *In vivo* total body FMT imaging of the Alexa Fluor750-labelled *Pf*Trx-HPV-L2 antigen distribution at two different time-points (5 and 10 min) after administration of the vaccine powder. (B) Micro-CT imaging of antigen deposition into the lungs; transversal and coronal views are shown in the left- and right-side images, respectively, where the arrows indicate the vaccine powder deposition sites. (C) *In vivo* (*left panel*) and *ex-vivo* (*right panel*), IVIS analysis of Alexa Fluor 750-labelled *Pf*Trx-HPV-L2 accumulation in the respiratory tract, 15 min after intratracheal delivery of the dry-powder vaccine.

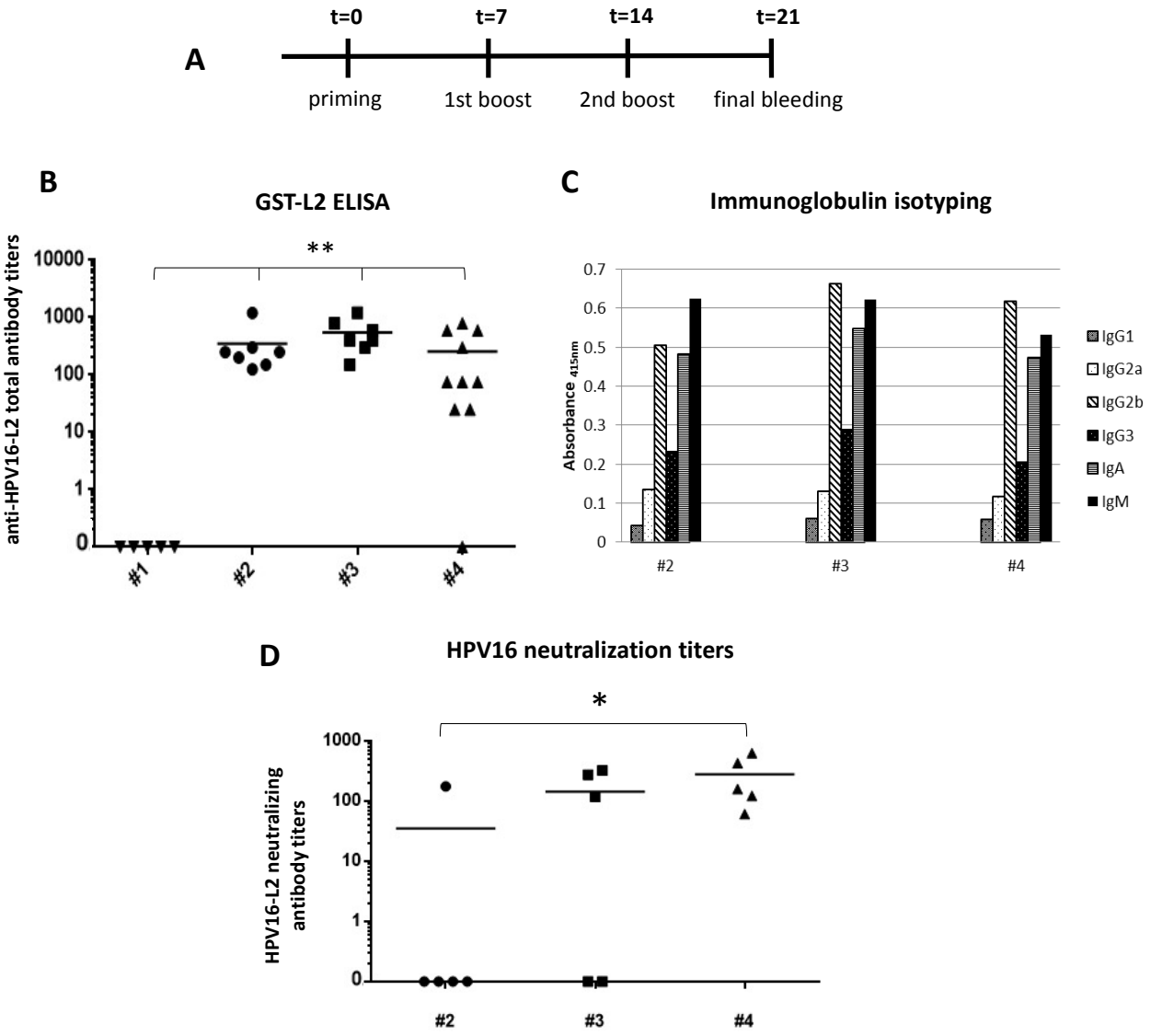

Figure 6. Immunogenicity profiling. **(A)** Timeline of the short-term immunization experiment performed with the GLA-adjuvanted, *Pf*Trx-HPV-L2 DPI delivered intratracheally. **(B)** GST-L2 ELISA performed on sera from differently treated groups of mice, collected one week after the last immunization. The following treatments were applied to individual groups: #1, negative control, subcutaneous immunization with solubilized, GLA only containing powder with no antigen; #2, subcutaneously injected, liquid form, GLA+alum adjuvanted *Pf*Trx-HPV-L2 antigen; #3 subcutaneously injected *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine solubilized in PBS immediately before administration; #4, *Pf*Trx-HPV-L2-[GLA] dry-powder vaccine administered intratracheally. Data represent anti-HPV-L2 total antibody titers measured in individual mice; the means of the titers for each group are indicated by horizontal lines (**$p \leq 0.01$). **(C)** Immunoglobulin isotyping of pooled sera from the mice groups (#2, #3 and #4) shown in panel B, except for the completely unresponsive, negative control group #1. **(D)** HPV16 neutralization titers determined by the L2-PBNA on a subset of five immune-sera/group (#2, #3 and #4) derived from the animals in each group that displayed the highest (top five) anti-HPV-L2 total antibody titers by GST-L2 ELISA (panel B). Data represent neutralizing antibody titers measured in individual mice; mean values of the titers for each group are indicated by horizontal lines (*$p \leq 0.05$).

Credit author statment

Irene Rossi, and **Gloria Spagnoli**, Investigation, Writing original draft; **Fabio Stellari** and **Quigxin Chen**: investigation; **Francesca Buttini** and **Fabio Sonvico**: Validation, Methodology; **Davide Cavazzini** and **Angelo Bolchi**: Resources, Data curation; **Martin Müller**: Formal analysis; **Simone Ottonello** and **Ruggero Bettini**: Conceptualization, Writing Reviewing and Editing, Funding aquisition