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A Bacterium from the Human Microbiota as a Vaccine Vector. Efficient Priming of the murine immune system by vaginal delivery of recombinant *Streptococcus gordonii*

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Abstract

The ability to prime the immune system against an antigen, and to rapidly recall this response upon antigen reencounter is a fundamental characteristic of the adaptive immune response. The association of an antigen recognized by the immune system in a certain tissue, with the same antigen encountered at a later timepoint in a different tissue, is of primary importance to obtain a systemic and effective immune response and is the foundation behind the utilization of vaccines. The study of vaccine delivery platforms that may activate the immune system in such a manner is therefore of primary importance in the effort to design novel vaccine formulation and prime-boost strategies.

The aim of the present work was to study the *in vivo* priming effect induced by a recombinant *Streptococcus gordonii* vaccine vector expressing a heterologous antigen and delivered to the vaginal tract, a unique mucosal tissue.

To study the priming effect induced by the recombinant *Streptococcus gordonii*, we employed a prime-boost strategy and compared the cellular an humoral immune response towards the soluble antigen between recombinant- and WT-immunized mice. Using this model, we show that vaginal immunization with the recombinant *Streptococcus gordonii* elicited systemic production of antigen-specific antibodies, shifted the IgG subclasses profile, led to an increase in plasma cells in the lymph nodes, a higher number of antigen-specific antibody-secreting cells in the spleen and modulated the cytokine expression profile of splenocytes. The longevity of the priming effect induced by the recombinant vector was also analyzed by comparing three and six months boosting schedules. We found that the priming is boostable with a similar efficacy for at least six months (Chapter 3). These data demonstrate that vaginal priming with the recombinant *S. gordonii* vector results in a systemic activation of both cellular and humoral immune compartments, and that this priming effect is long-lived without significant immune waning.

In this study, we also assessed the transcriptomic response of splenocytes from *S. gordonii*-immunized mice towards the antigen. We observed differences in immune pathways between recombinant- and WT-immunized mice, and also between the three- and six-months boosted groups

(Chapter 4). In addition, we observed a gene signature correlated with antigen-specific IgG titers. These findings suggest that the immune system's encounter with the antigen on the surface of the recombinant *S. gordonii* in the vaginal tract results in a differential immune activation in in response to the antigen.

This work contributes to the knowledge on the capability of recombinant live vaccine vectors delivered mucosally to prime and modulate the immune response, and has important implications in the design of innovative vaccination strategies.

Chapter 1 - Introduction

1.1 Mucosal tissues and the immune system

Mucosal surfaces are boundaries between the host and the external environment (1), and can be divided into two major types: Type I mucosal tissues are covered by a simple columnar epithelium and include the upper female genital, respiratory, and gastrointestinal tracts. Type II has a much more robust architecture, based on multilayers of stratified squamous epithelium containing keratinocytes and includes the lower female genital, the esophageal, corneal and oral tracts (2). These different mucosal tissues harbor differences in immune mechanisms. Type I tissues contain organized mucosal-associated lymphoid tissues (MALT) and express polymeric Ig receptors (pIg) which bind primarily secretory Immunoglobulin A (sIgA). Type II tissue do not contain MALT, and activated antigen-presenting cells within these tissue must migrate to a nearby lymph nodes in order to present the antigen to lymphocytes. Additionally, instead of pIgR, type II tissues express FcRn, which makes IgG the dominant protective immunoglobulin subtype in these tissues (3).

Owing to their nature as immunological interfaces, much attention has been given to mucosal immunizations in the context of vaccinology. Vaccination through mucosal routes can stimulate immune responses both locally and systemically (4). Mucosal vaccine systems have been developed for application in various tissues, including nasal, oral, ocular, rectal, and vaginal routes (5). However, the most widely explored vaccine routes in both preclinical and clinical research are by far the nasal and oral, owing to their simplicity of application and years of experience in pharmaceutical drugs delivery (6). Compared to other mucosal tissues, the female genital tract is unique in that it contains both type I and type II mucosal tissues (3). Thus, the vaginal mucosa offers a promising and underexplored route of immunization due to its diverse immune activation mechanisms, and its ability to induce both local and systemic immune responses. Indeed, several studies have shown the beneficial effects of vaccines administration via the vaginal tissue in both humoral and cellular immune responses (7–11). However, despite its advantages, the vaginal tissue's unique features, along with the anatomical and immune cells localization during the periodic menstrual cycle,

necessitate adequate antigen delivery vehicles to facilitate the induction of an immune response. In fact, without proper adjuvants and temporal administration, vaginal immunizations often fail to induce an immune response owing to the dynamics of antigen processing at mucosal tissues (12–16).

1.2 The microbiome, bacterial engineering and *Streptococcus gordonii*

The host microbiome, the endogenous flora consisting of various microorganisms and thousands of species of bacteria, has adapted to occupying different niches and exerts crucial functions in metabolism, immunomodulation, and tissue homeostasis (17). The majority of the human microbiome organisms are found in the gastrointestinal tract (18), however colonizing bacteria are also found in great numbers in the skin, oral, respiratory and urogenital tracts (19). Initially, it seemed improbable that such vast numbers of foreign microorganisms residing within the host would not drive an excessive host-wide inflammatory response, leading to tissue damages, and thus it was suggested that these microorganisms evade immune recognition (19). However, studies have shown that the immune system does indeed recognize the commensal microflora within the host, and this recognition is crucial for maintenance of homeostasis and immunity (20–23). In the context of the vaginal tissue, studies have shown that disturbances in the composition of the vaginal microbiome, specifically loss of the commensal *Lactobaccili* species, induces epithelium rupture and increases susceptibility to sexually transmitted diseases (24).

Due to their unique interaction with the host immune system and advancements in genetic manipulation tools, opportunities arise to engineer commensal bacteria, and other non-pathogenic bacteria, as vaccine vehicles for mucosal delivery (25,26). In the past three decades, we and others have demonstrated various health-related applications of recombinant bacteria platforms, primarily of Lactic Acid Bacteria strains (27–34). One notable advantage of these platforms is the continuous production of the vaccine antigen *in situ*. In the context of mucosal vaccines, surface anchoring of the heterologous antigen allows higher exposure that can facilitate interaction with epithelial and immune system components.

Streptococcus gordonii is an α-hemolytic Gram-positive bacterium and a member of the human oral microbiome. It was first isolated in 1958 and has been used for decades for genetic studies and manipulations, owing to its natural competence for genetic transformations (35). We have previously presented a host-vector system (36) in *S. gordonii* that facilitates stable heterologous antigen expression due to chromosomal integration of the construct. Chromosomal integration is based on homology between DNA regions flanking the heterologous antigen sequence in the donor plasmid and regions on the bacterial chromosome. Recombination is verified by loss of Kanamycin resistance, acquisition of Erythromycin resistance, and expression of the heterologous protein. Recombinant *S. gordonii* expressing TTFC antigen was shown to protect mice from a lethal toxin challenge (37), and to induce both humoral and cellular antigen-specific immune response when administered orally, nasally and intragastrically (33,34,38–42). When administered intravaginally to mice (43,44) and non-human primates (45), recombinant *S. gordonii* successfully colonized the vaginal tissue, induced local and systemic antigen-specific antibodies, and resolved candidiasis infection. Additionally, In a phase I clinical trial with 150 volunteers, *S. gordonii* administered both nasally and orally successfully colonized various regions, was well tolerated, and rapidly eradicated by an azithromycin treatment as a safety mechanism (46).

1.3 *Chlamydia trachomatis* and MOMP

Chlamydia trachomatis (C.t) is Gram-negative human pathogen, an obligate intracellular bacterium with a biphasic development cycle, in which elementary bodies (EBs) enter the cell and transform into reticulate bodies (RBs), which in turn replicate in a specific membrane-enclosed compartment. Following replication, RBs transform back into EBs that can infect surrounding cells (47). C.t is the leading global cause of Sexually Transmitted Infection (STI), and it is estimated that globally, 131 million new cases of Chlamydial-derived genital infections occur annually (48,49). C.t STI is associated with serovars D-K (50), while other serovars are associated with ocular trachoma (A-C) and lymphogranuloma venereum (L_1-L_3). When diagnosed at an early stage, primarily by nucleic acid amplification tests (NAATs) of vaginal swabs or urine samples, *Chlamydia* infection can

be treated effectively using antibiotics such as doxycycline or azithromycin. However, the majority of chlamydial infections are asymptomatic, and during this undetected period, can cause adverse sequelae. The most severe sequelae of this infection is the ascension of the infection to the upper genital tract, where it may cause pelvic inflammatory disease, infection of the uterus, ovaries, pelvic peritoneum and fallopian tubes. This may lead to long-term health issues, including ectopic pregnancy, infertility and chronic pain in the pelvic (51,52). In addition, urogenital tract infection by *Chlamydia trachomatis* was associated with increased risk of acquiring HIV infection (53,54).

At present, high income countries rely on screening programs to detect and treat *Chlamydia* infections (55–57). However, these programs require considerable funding and even so, are difficult to conduct in large quantities. Thus, an effective and economically viable vaccine against *Chlamydia trachomatis* is of urgent need in order to control the rate of infection and subsequent pathologies. However, to date no such vaccine has been clinically approved (50).

Major Outer Membrane Protein (MOMP) is a highly immunogenic chlamydial protein in both humans and mice and is considered a promising vaccine target (50,58). It is an adhesin that participates in Chlamydial cell entry (59), and studies have shown that blocking its exposed variable domains hampered binding to the host cell (60). CTH522 is a multivalent Chlamydial antigen containing portion of MOMP from serovar D, and the extended variable domains of MOMP from serovars D, E, F and G (61). In pre-clinical models, CTH522 was shown to have a protective effect against C.t challenges (61,62). Recently, it was demonstrated to be safe and immunogenic in a phase I clinical trial when administered with (63).

1.4 Heterologous prime boost

Heterologous prime-boost strategy involves the a vaccination regiment in which the antigen is administered in differential routes or formulations between one dose and the other. Such strategies are the focus of current vaccine research in order to improve the immune response induced by the vectors. Following the SARS-COV-2 pandemic, heterologous prime-boost schemes were used extensively in several countries, as means to reduce adverse effects and, enhance immune system's

recognition of new variants and to mitigate waning immunity (64). Indeed, several studies demonstrated that heterologous prime-boost is favorable when compared to homologous prime-boost in certain formulations (65–69).

1.5 Transcriptomic signature of vaccination

The host's response to a vaccine involves several levels of responses, such as changes in gene expression, protein production, metabolism and cellular composition. These modifications, interwoven into a complex network of interactions is what enables the ability to mount an effective immune response towards the antigen. The field of systems vaccinology has emerged in an attempt to generate a clearer picture of these complicated biological networks and to identify transcriptomic signatures associated with the favorable responses, i.e. vaccine-induced immunity (70,71). Recently, systems vaccinology studies have identified a favorable transcriptional signature induced in myeloid cells following vaccination with Pfizer's BNT162b2 SARS-COV-2 vaccine (72).

While in humans the utilization of systems vaccinology studies to study transcriptomic response is largely limited to PBMCs from blood samples, mouse models allow the analysis of transcriptomic response in lymphoid tissues such as the spleen. This in turn allows to associate transcriptomic signature with immunological parameters such as serum antibody levels and cytokines production (72–74).

In this work, a recombinant *S. gordonii* expressing the CTH522 antigen, named FR368, was used as a live vaccine vector, and delivered vaginally as part of a heterologous prime-boost strategy.

Chapter 2 – Aims of the study

The aim of the present work was to examine the immunogenicity of an engineered *S. gordonii* strain expressing on its surface the multivalent Chlamydial MOMP antigen CTH522, referred to as FR368, and administered to mice intravaginally. We characterized the immune response induced before, and after heterologous subcutaneous boost with a soluble CTH522 protein by comparing it to the response in mice vaginally immunized with the WT strain of *S. gordonii*. Importantly, we characterized the longevity of the priming effect by our recombinant *S. gordonii* vector by employing two different boosting schedules, at either three or six months from the vaginal priming. Understanding the capacity of a mucosally-administered bacterial vaccine vector to stimulate a systemic and long-lasting immune priming effect towards an antigen, even when encountered in a different tissue or formulation, is of importance to the efforts to develop novel vaccination strategies.

Using ELISA, we studied and characterized the production of systemic antigen-specific IgG in response to the vaginal colonization by the recombinant *S. gordonii* vector, and following the heterologous boost with the purified protein at the two boosting schedules. The numbers of antibody-secreting cells (ASCs) was studied using B-cell ELISPOT on splenocytes from mice immunized with either recombinant or WT *S. gordonii*. Additionally, Using flow cytometry, we studied the induction and persistence of plasma cells, as well as cytokines production by antigen-specific T cells.

The differences in the transcriptomic response of splenocytes from WT- or recombinant *S. gordonii*-immunized mice to the CTH522 antigen was studied using RNA sequencing and Systems Vaccinology approach.

Chapter 3 – Vaginal immunization by the human microbiota bacterium *Streptococcus gordonii* expressing a *Chlamydia trachomatis* antigen efficiently primes the immune system in mice

In this chapter we describe the priming effect induced in mice following mucosal immunization with a recombinant strain of *Streptococcus gordonii* expressing an heterologous antigen. We used this model as a tool to study the extent in which the bacterial vaccine vector delivered mucosally could prime the immune system towards the antigen, when the antigen is reencountered in a different formulation and administration route.

Recombinant *Streptococcus gordonii* expressing on its surface the *Chlamydia trachomatis* multivalent MOMP antigen, CTH522, named FR368, was used to immunized mice intravaginally. Three or six months after the vaginal priming, purified CTH522 protein administered subcutaneously was used as a booster.

Our data show that vaginal immunization with the recombinant *Streptococcus gordonii* successfully primes the immune systems towards the heterologous boost.

Vaginal immunization by the human microbiota bacterium *Streptococcus gordonii* expressing a *Chlamydia trachomatis* antigen efficiently primes the immune system in mice

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Abstract

Mucosal surfaces are active immunological interfaces, and as such are promising avenues for vaccines delivery. However, their unique characteristics and the nature of immune system activation in these tissues require the utilization of appropriate adjuvants or delivery systems. Recombinant *Streptococcus gordonii* expressing a heterologous antigen can colonize mucosal tissues and activate the immune system when delivered mucosally in mice. In the present study, we examined whether intravaginal administration of a recombinant *S. gordonii* vector, named FR368, genetically engineered to express the *Chlamydia trachomatis* multivalent MOMP antigen, CTH522, could prime the immune system in a heterologous prime-boost scheme. First, we show that the vaginal colonization itself triggered a systemic antigen-specific IgG response. Heterologous subcutaneous boosting with unadjuvanted CTH522 antigen at either three or six months after vaginal immunization resulted in a marked increase in serum CTH522-specific IgG levels in FR368-immunized mice, compared to WT-immunized ones. Concomitantly, a significant increase in the levels of CTH522-specific antibody secreting cells, alongside a rise in the percentage of plasma cells in the spleen and lymph nodes, respectively, were observed in the FR368-immunized mice. Vaginal colonization with FR368 also induced a shift in cytokines expression profile in antigen-specific T cells upon restimulation, dominated by an IL-17 increased expression, suggesting a mucosal association of antigen first encounter context. Our results highlight the potential of immunization with recombinant *S. gordonii* as a potent mucosal delivery system able to stimulate a systemic immune response in a heterologous prime-boost scheme, which is currently the focus of many vaccines' development.

Introduction

Mucosal surfaces serve as barriers between the host and the external environment, in addition to their many physiological roles (1). Owing to their nature as immunological interfaces, much attention has been given to mucosal immunizations in the context of vaccinology. Vaccination through mucosal routes can stimulate immune responses both locally and systemically (2). Mucosal vaccine systems have been developed for application via nasal, oral, ocular, rectal, and vaginal routes (3). However, the most widely explored vaccine routes in both preclinical and clinical research are by far the nasal and oral, owing to their simplicity of delivery and years of experience in pharmaceutical drugs delivery (4). The vaginal mucosa offers a promising and underexplored route of immunization due to its ability to induce both local and systemic immune responses. Indeed, several studies have shown the beneficial effects of vaccines administration via the vaginal tissue in both humoral and cellular immune responses (5–9). However, the vaginal tissue's unique features necessitate adequate antigen delivery vehicles to facilitate the induction of an immune response. In fact, without proper adjuvants, vaginal immunizations often fail to induce an immune response owing to the dynamics of antigen processing at mucosal tissues (10–13).

The host microbiome, the endogenous flora consisting of various microorganisms and thousands of species of bacteria, has adapted to occupying mucosal niches and exerts crucial functions in metabolism, immunomodulation, and tissue homeostasis (14). For example, the oral microbiome presents capabilities to modulate the immune landscape (15), particularly in the context of Th17 response, which is considered important for the development of mucosal immunity (16). Due to their unique interaction with the host, and advancements in genetic manipulation tools, these bacteria present opportunities to be engineered as vaccine vehicles for mucosal delivery (17). In the past three decades, we and others have demonstrated various health-related applications of recombinant bacteria platforms, primarily of Lactic Acid Bacteria strains (17–21). One notable advantage of these platforms is the continuous production of the vaccine antigen *in situ*. In the context of mucosal

vaccines, surface anchoring of the heterologous antigen allows higher exposure that can facilitate interaction with epithelial and immune system components.

Streptococcus gordonii is a Gram-positive bacterium and a member of the human oral microbiome. We have previously presented a host-vector system (22) in *S. gordonii* that facilitates stable heterologous antigen expression due to chromosomal integration of the construct. Recombinant *S. gordonii* expressing TTFC antigen was shown to protect mice from a lethal challenge (23), and to induce both humoral and cellular antigen-specific immune response when administered orally or nasally (24–29). When administered intravaginally to mice (30,31) and non-human primates (32), recombinant *S. gordonii* successfully colonized the vaginal tissue, induced local and systemic antigen-specific antibodies, and resolved candidiasis infection. In a phase I clinical trial with 150 volunteers, *S. gordonii* administered both nasally and orally successfully colonized various regions, was well tolerated, and rapidly eradicated by an azithromycin treatment as a safety mechanism (33). *Chlamydia trachomatis* (C.t) is a human pathogen and the leading global cause of sexually transmitted bacterial infection (34). To date, no vaccine against C.t has been clinically approved (35). Major Outer Membrane Protein (MOMP) is a highly immunogenic chlamydial protein in both humans and mice and is considered a promising vaccine target (36). CTH522 is a multivalent chlamydial antigen containing regions of MOMP from C.t serovars D, E, F and G (37). In pre-clinical models, CTH522 was shown to have a protective effect against C.t challenges (37,38) and to be safe and immunogenic in a phase I clinical trial (39).

In the present work, we examined the immunogenicity of an engineered *S. gordonii* strain expressing on its surface the CTH522 antigen, referred to as FR368, and administered to mice intravaginally. Immunogenicity profile was characterized before, and after heterologous subcutaneous boost with a soluble CTH522 protein, in terms of production of CTH522-specific antibodies, elicitation of antibody secreting cells, induction and persistence of plasma cells, and cytokines production by antigen-specific T cells. We also studied the duration of the priming effect by comparing two different bosting intervals. Our analysis showed that vaginal colonization with

FR368 successfully primed the immune system inducing systemic antigen-specific immune response that could be recalled by boosting both three- and six-months later.

Materials and Methods

Bacterial strains and growth conditions.

FR368 expressing the CTH522 antigen from *Chlamydia trachomatis*, and the recipient control strain GP1295 (WT) were used for immunization experiments. Bacteria were grown at 37°C with 5% CO_2 in Brain-Heart infusion (BHI; #CM1135, OXOID Thermo Fisher Diagnostics S.p.A.), except for the cytofluorimetric analysis experiments and immunizations, for which they were cultured in TSB without dextrose (#286220, BD Difco) until late exponential phase. For solid medium, BHI was supplemented with 1.5% Agar and 3% sheep blood (#83297, Liofilchem). Antibiotics were added at the following concentrations: For liquid cultures, Erythromycin (#6376, Sigma) at 0.5µg/ml and Kanamycin (#J60668, Alfa Aesar) at 100µg/ml, for solid cultures Erythromycin at 3µg/ml and Kanamycin at 500µg/ml.

Construction of recombinant *S. gordonii* strain FR368.

S. gordonii strain surface displaying the M6-CTH522-3xHA fusion protein was constructed by using the host-vector system previously described (22). The *emm6*-based gene fusion includes the DNA sequence coding for the CTH522 protein. Construction of the above-mentioned *emm6*-based gene fusion vector was carried out as follows: The amino acids sequence of CTH522 (40) was codon-optimized for expression in *S. gordonii,* synthesized with *BglII* upstream and *EcoRI* downstream, and cloned into cloning vector pJExpress412 (ATUM Bio, USA). CTH522 (*ompA(cth522*)) fragment was digested using *BglII* and *EcoRI and* cloned into pSMB55 donor DNA plasmid, yielding pSMB55-*ompA(cth522*). The resulting plasmid was linearized and used to transform competent *S. gordonii* GP1295 cells. Erythromycin-resistant transformants were selected, and one clone was named FR368. Procedures for cloning of the gene fusions in *Escherichia coli*, transformation of *S. gordonii*, and genetic analysis of streptococcal transformants were performed as previously described (41,42).

Analysis of recombinant bacteria.

The expression of the M6-CTH522-3xHA fusion protein on the surface of FR368 was verified by flow cytometry analysis. Bacteria were grown in TSB without dextrose to early stationary phase,

washed in phosphate-buffered saline (PBS; pH 7.4), resuspended in PBS containing 2% bovine serum albumin (BSA; #A4503, Sigma) and incubated at 37 °C for 30 min while shaking. Cells were incubated with either anti-MOMP rabbit serum (1:100; obtained from Staten Serum Institute, Denmark), anti-M6 rabbit serum (1:40; prepared by Cesare Berneri, Istituto Zooprofilattico Sperimentale of Lombardia and Emilia, Brescia, Italy) or Rat anti-HA mAb (1:100, #11867423001, Clone 3F10, Merck) for 1 h at 4 °C. After two washes with PBS, PE-conjugated anti-rabbit (1:40, #SC-3753, Santa Cruz Biotechnology) or PE-conjugated anti-rat IgG (1:100, #405406, Biolegend) was added to bacteria for 20 min at 37 °C. Cells were washed twice in PBS and finally resuspended in 0.3 ml of PBS and acquired by flow cytometry (LSRFortessa x-20, Becton Dickinson, San Diego, CA). Data analysis was performed using FlowJo v10 (TreeStar, Ashland, OR, USA).

Dot-Blotting

Purified CTH522 protein, recombinant strain FR368, and the WT strain GP1295 were blotted through circular templates vacuum-manifold onto a nitrocellulose membrane in twofold serial dilutions, starting from 12.5ng and 1×10^8 CFU, respectively. Membrane containing blotted cells and protein was dried at 80◦C for 15 min. The membrane was blocked with Tris-Buffer-Saline (150mM NaCl, 20 mM Tris-Cl; pH 7.5) supplemented with 0.05% (v/v) Tween 20 (#P2287, Sigma-Aldrich) and 5% milk (#A0830, AppliChem) (TBS-T 0.05% with 5% milk) for 2 hours at room temperature, followed by an overnight incubation with rabbit anti-MOMP (diluted 1:10,000 in TBS-T 0.05% with 5% milk). Membrane was washed three times with TBS-T 0.05% and incubated with and alkaline phosphatase (AP)-conjugated anti-rabbit IgG secondary antibody (1:1,500, #4050-04, Southern Biotech) in TBS-T 0.05% with 5% milk. The antigen-antibody reaction was developed by using BCIP (5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt #11383221001) and NBT (nitro-blue tetrazolium chloride, # 11383213001, both from Sigma-Aldrich) diluted in working solution (0.05M MgCl2, 0.1M Tris pH9.5, 0.1M NaCl). The mass of CTH522 protein on the surface of recombinant bacteria, per CFU, was calculated by densitometric analysis using the ImageJ 1.53e Intensity Density measurement tool.

Western-Blotting

To prepare cell-free protein extract, 1×10^8 CFU for either FR368 or WT strains were washed once in 1mL H_2O and centrifuged for 5 min at 10,000 x g at 4 °C. Supernatant was discarded, bacterial pellet was resuspended in 100µL H_2O and transferred to an Eppendorf tube containing 0.04g glass beads (#G1145, Sigma-Aldrich). Samples were placed in TissueLyser (QIAGEN) for 2 min at 30Hz for 2 cycles. Samples were centrifuged for 5 min at 10,000 x g at 4 °C and supernatant containing proteins was transferred into a new tube. Samples were processed and run using NuPage Bis-Tris mini gel electrophoresis (life technologies) per manufacturer's instructions. Novex Sharp protein standard (Life Technologies, #LC5800) was used to determine protein size. Proteins were transferred to nitrocellulose membrane and blocked with TBS-T 0.05% with 5% milk for 2 hours at room temperature, followed by overnight incubation with rabbit anti-MOMP (1:10,000) diluted in TBS-T 0.05% with 5% milk. Membrane was washed with TBS-T 0.05% and then incubated for 1 hour with (AP)-conjugated anti-rabbit IgG secondary antibody (1:1,500 Southern Biotech) in TBS-T 0.05% with 5% milk. The antigen-antibody reaction was developed by using BCIP/NBT (as above).

Genome Sequencing

Bacterial genomic DNA (gDNA) was purified using the Raffinose protocol for DNA purification, as described elsewhere (43). In short, bacterial pellet was resuspended in Raffinose buffer (50mM Tris, 5mM EDTA pH 8, 20% v/v Raffinose (Sigma-Aldrich) and 200µL of 100mg/mL lysozyme (final concentration 2.6mg/mL) (#L6876 Sigma-Aldrich). Samples were incubated for 1 hour at 37°C, followed by centrifugation and resuspension in 0.1mg/mL Proteinase K (#P2308, Sigma-Aldrich) and incubated for 30 min at 37°C. 15 min into the incubation, 400µL SDS 10% was added, followed by 1mL of 5M NaCl. Solution was washed three times using the SEVAG (24:1 Chloroform: Isoamyl alcohol) method, each time collecting the light phase into a new tube. Finally, DNA was precipitated using ice-cold isopropanol. DNA was quantified using Qubit dsDNA quantification kit per manufacturer's instructions. DNA was sequenced using Oxford Nanopore sequencing kit SQK-LSK109 and Expansion Barcoding kit (EXP-NB103), manufactured by Oxford

Nanopore Technologies (ONT) following manufacturer's instructions on a flow cell using GridION instrument.

Mice

Seven-weeks old female BALB/C mice, purchased from Charles River (Lecco, Italy) were housed under specific pathogen-free conditions in the animal facility of the Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Department of Medical Biotechnologies at University of Siena, and treated according to national guidelines (Decreto Legislativo 26/2014). Experiments were planned and conducted utilizing the three R's principles (Reduce, Replace and Refine), which included environmental enrichment and nesting, veterinary oversight, numbers reflecting statistical significance, and the use of anesthesia followed by cervical dislocation for the sacrifice. All animal studies were approved by the Ethics Committee "Comitato Etico Locale dell'Azienda Ospedaliera Universitaria Senese" and the Italian Ministry of Health (number 1004/2015-PR on September 22, 2015).

Hormone treatment and estrous cycle stage determination

On day -5 prior to the beginning of the experiment, the estrous cycle of experimental animals was synchronized by a subcutaneous administration of 0.1 mg of β-estradiol 17-valerate (#E1631, Sigma-Aldrich) resuspended in ethanol and diluted in olive oil. Estrus cycle synchronization was verified by assessing vaginal smears on slides using LEICA DM1000 microscope. The stage of the estrous cycle was determined by examining the proportions and morphology of leukocytes, cornified and epithelial cells present in the smear.

Immunizations

Mice were immunized three times on weeks 0, 1, and 2 by the intravaginal route (IVAG) with 10^9 CFU in a volume of 20µL PBS of either Wild-Type (GP1295) or recombinant (FR368) *S. gordonii* bacterial vector expressing the vaccine antigen CTH522. Mice were subcutaneously boosted with either CTH522 antigen (5µg/mouse) administered in a volume of 100µl/mouse in NaCl 0.9%

(Fresenius Kabi, Italy) or Saline (NaCl 0.9%) two times, on weeks 13 and 17 (3-months boosting), or 27 and 31 (6-months boosting). Mice were sacrificed at day 10 post 2nd boost (week 18.5 or 32.5).

Sample collection and cell preparation

Blood samples were taken from individual mice by the temporal plexus periodically, and by cardiac puncture at day 10 post second boost upon sacrifice. Samples were incubated for 30 min at room temperature and then centrifuged at 1,200g for 10 min. Sera were collected and stored at -20°C until analysis. Vaginal samples for assessment of bacterial colonization were taken from individual mice weekly as follows: the vagina was flushed with 20µL sterile PBS, and then sampled with rayon tips (#160C, Copan). Tip was placed in a tube containing PBS 1% BSA and then vortexed for 1 min. Rayon tip was discarded and the tube was centrifuged at 1,200g for 10 min at 4°C. Supernatant was transferred to a new tube, protease inhibitor was added (1:100 #P8340, Sigma-Aldrich) and samples were stored in -20°C until assayed for antibodies by ELISA. Pellet was resuspended in sterile BHI-10% glycerol and stored in -80°C until plated on selective BHI blood-agar for colonization assessment. Draining lymph nodes (inguinal and iliac) and spleens were mashed onto 70 µm nylon screens (Sefar Italia, Italy) and washed two times in RPMI medium (# BE12-167F, Lonza, Belgium) supplemented with 100 U/ml penicillin/streptomycin (#P0781, Sigma-Aldrich) and 10% fetal bovine serum (#10082, Gibco, USA). Samples were treated with red blood cells lysis buffer according to manufacturer instruction (#00-4300-54, eBioscience, USA).

Multiparametric flow cytometric analysis.

For cellular population characterization, cell samples from spleens and draining lymph nodes were incubated for 30 min at 4°C in Fc-blocking solution (cRPMI with CD16/CD32 mAb 1:100 [#14-0161-85, clone 93; eBioscience]). Cells were stained with AF700-conjugated anti-CD45R/B220 (1:80 #557957 clone RA3-6B2; BD Biosciences), FITC-conjugated anti-GL-7 (1:50 #562080 clone GL-7; BD Biosciences), PE-Cy7-conjugated anti-CD95 (1:50 #557653 clone Jo2; BD Biosciences), BV510/BV605-conjugated anti-IgD (1:50 #563110 clone 11-26c.2a; BD Biosciences), PE-conjugated anti-CD138 (1:50 #553714 clone 281-2; BD Biosciences), BUV395-conjugated anti-

CD3e (1:100 #563565 clone 145-2C11; BD Biosciences), BB700-conjugated anti-CD4 (1:100 #566407 clones RM4-5, BD Biosciences), BV421-conjugated anti-TACI (1:50 #742840 clone 8F10; BD Biosciences) mixed in BD Brilliant Stain Buffer (BD Biosciences) for 30 min. at 4°C.

For intracellular cytokine staining, cells were stimulated for 6 h in the presence of CTH522 antigen (5 µg/ml) and co-stimulatory antibodies CD28 and CD49d (#16-0281-85 clone 37.51 and #16-0492-85 clone R1-2, respectively, both at 2 µg/ml, Invitrogen). Brefeldin A (5 µg/ml, #B7651, Sigma-Aldrich) and Monensin solution (1x, # 00-4505-51, eBioscience) were added to all samples for the last 4 h of incubation. Cells were washed twice in PBS and labeled with Live/Dead Fixable Viability Stain 780 per manufacturer's instructions (#565388, BD Biosciences, USA). Fixation and permeabilization were performed using BD Cytofix/Cytoperm kit according to the manufacturer instruction (#554714, BD Biosciences) before Fc-blocking and stained with BV421-conjugated anti-IL-17A (1:50 #5633354 clone TC11-18H10; BD Biosciences), BV786-conjugated anti-IFN$_Y$ (1:50 #563773 clone XMG1.2; BD Biosciences), BV650-conjugated anti-TNF (1:50 #563943 clone MP6-XT22; BD Biosciences), PE-Cy7-conjugated anti-IL-4 (1:50 #560699 clone 11B11; BD Biosciences), PE-Cy7-conjugated anti-IL-13 (1:50 #25-7133-82 clone eBio13A; eBioscience), PE-CF594-conjugated anti-IL-2 (1:50 #562483 clone JES6-5H4; BD Biosciences), APC-R700-conjugated anti-CD44 (1:400 #565480 clone IM7; BD Biosciences), BV510-conjugated anti-CD8a (1:50 #563068 clone 53-6.7; BD Biosciences), BB700-conjugated anti-CD4 (1:100 #566407 clones RM4-5, BD Biosciences) and BUV395-conjugated anti-CD3e (1:100 #563565 clone 145-2C11; BD Biosciences). All antibodies were titrated for optimal dilution. Samples were acquired on BD LSRFortessa X20 flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v10 (TreeStar, USA).

B-cell ELISPOT

Antibody secreting cells (ASCs) within splenocytes were evaluated by mouse IgG Single-Color ELISPOT assay (#MIGG-SCE-2M/2, CTL Europe GmbH) 10 days post 2nd boost. Multiscreen filter 96-well plates were coated overnight at 4 °C with either CTH522 (5µg/ml) for the detection of

antigen-specific IgG or with anti-IgG capture antibody for the detection of total IgG. Plates were washed and blocked for 1 hour with serum-free CTL-Test B culture medium (CTL, Europe GmbH) supplemented with 1% L-glutamine (#G8540, Sigma-Aldrich, USA) at room temperature. $1x10^6$ cells/well were added in a volume of 100µl of CTL-Test B medium for the analysis of CTH522-specific IgG ASCs. Plates were incubated for 18 hours at 37°C with 5% CO_2, washed with PBS-0.05% Tween 20, and anti-murine IgG Detection Solution was added for 2 hours at room temperature. Plates were washed, incubated with 80 µl/well of Tertiary Solution Strep-AP (diluted 1:1,000) at room temperature for 30 min, washed again and reacted with Blue Developer Solution for 10 minutes. The number of spots was determined by plate scanning and analysis was performed with an Immunospot S6 ULTIMATE Analyzer (CTL, Europe GmbH).

ELISA

Serum CTH522-specific IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) at different time points. Flat bottomed Maxisorp microtitre plates (Nunc, Denmark) were coated with CTH522 (1 µg/ml) overnight at 4°C in a volume of 100µl/well. Plates were washed and blocked with 200µl/well of PBS containing 1% BSA (Sigma-Aldrich) for 2 hours at 37°C. Serum samples were added and titrated in three to five-fold dilutions in 100 µl/well diluent buffer. After incubation for 2 hours at 37°C samples were washed and incubated with the AP-conjugated goat anti-mouse IgGs (IgG1 #1070-04, IgG2a #1080-04, IgG2b #1090-04, Anti-IgG3 #1100-04 diluted 1:1,500 for total IgG, 1:1,000 for specific subclasses, Southern Biotech, USA) in diluent buffer for 2 hours at 37°C in 100 µl/well and developed by adding 200µl/well of 1mg/ml AP substrate (#P5994, Sigma-Aldrich). The optical density was recorded using Multiskan FC Microplate Photometer (Thermo Scientific). Antibody levels were expressed as ng/mL following subtraction of blank signal. Vaginal wash samples were analyzed similarly to serum samples, with the following modifications: Following coating for 3 hours at 37°C, vaginal wash samples were incubated overnight at 4°C and diluted 1:100 for total IgG or IgA and 1:2 for antigen-specific antibodies. Concentrations of antibodies in samples were calculated using a standard curve of IgG (as above) or IgA (#1040-04). Positive controls were

included in all assays as follows: anti-IgG coating (1:1000, #1010-01), IgG standards (#0107-01), anti-MOMP rabbit serum (SSI, Denmark) and anti-rabbit IgG-AP (#4050-04).

Statistical analysis

All samples were tested individually. The levels of serum antibodies were expressed as Mean ± SEM, and statistical analysis of antibody concentration was performed. Kruskal-Wallis or Mann-Whitney tests were used to assess the statistical difference between the immune response induced between the differently primed groups. A P-value ≤ 0.05 was considered significant. Statistical analysis was performed using Graph Pad Prism version 9 (GraphPad Software, USA).

Results

Expression of the Chlamydial MOMP antigen by recombinant vector *S. gordonii*

The CTH522 MOMP antigen was expressed on the surface of *S. gordonii* as a fusion with the M6 protein, using the host-vector system for heterologous gene expression (22). A representative clone, FR368, was verified by DNA sequencing, analyzed for CTH522 MOMP antigen expression, and used as a vaccine in mouse immunization experiments.

Based on our previous sequencing of the WT strain's chromosome (22), we confirmed the chromosomal integration of the heterologous antigen in FR368 using Nanopore sequencing (**Figure 1**). The WT has the homologous recombination sequences flanking the Kanamycin resistance (KmR) gene *aph(3')-IIIa* at the recombination site, located downstream of bp number 955,470 in the original parental *S. gordonii* strain's genome (V288, ATCC 35105, GenBank accession CP000725) (44). In FR368, the KmR gene was replaced with the sequence encoding the M6-CTH522 antigen together with the completion of the Erythromycin resistance (EmR) gene, *ermC*, downstream of the construct. Aligning the sequence of the integrated construct in FR368 genome with the sequence of the donor plasmid pSMB55-*ompA(cth522)* revealed the preservation of sequence integrity and frame (data not shown).

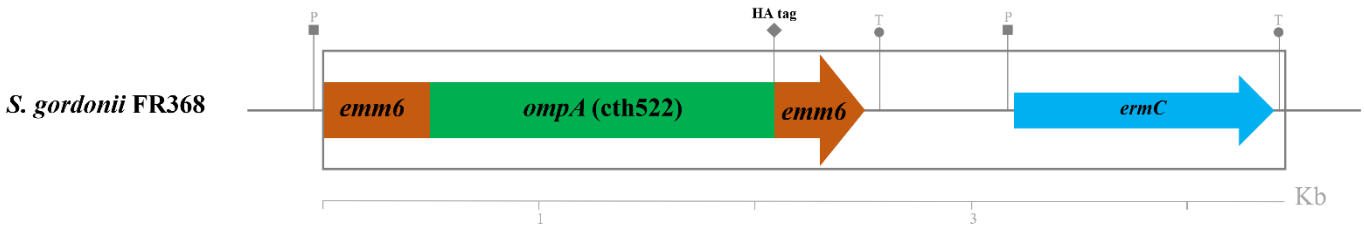

Figure 1 | Structure of the heterologous construct integrated in the chromosome of *S. gordonii* FR368. FR368 is a recombinant *S. gordonii* strain expressing the *C. trachomatis* recombinant MOMP (*ompA*) multivalent antigen named CTH522, comprised of 226 amino acids (aa) of rMOMP of Chlamydia trachomatis serovar D, 68aa of MOMP extended variable domain 4 (exVD4) of serovar D, 68aa of exVD4 of serovar E, 69aa of exVD4 of serovar F and 69aa of the exVD4 of serovar G.. The amino acid sequence of CTH522 previously published (40) was used to generate and synthesize an *S. gordonii*-codon optimized DNA sequence encoding the CTH522 protein. The CTH522-encoding sequence (*ompA cth522*) was cloned in frame with *S. pyogenes emm6* sequence in a plasmid carrying Erythromycin resistance gene, *ermC*. The resulting plasmid was used to transform competent *S. gordonii* using the host-vector system previously described (22). The 4,465-bp genetic construct designed for the expression of M6-CTH522 fusion protein on the surface of the *S. gordonii*, integrated

downstream of nucleotide 955,470 of V288 chromosome (GenBank accession no. CP000725) as confirmed by Nanopore DNA sequencing. The construct contains a fusion gene encoding the: i) 42 aa of the leader peptide of the *S. pyogenes* M6 protein, ii) first 122 aa of the N-terminal of M6, iii) 500 aa of CTH522 antigen, iv) 31 aa 3xHA tag, v) last 140 aa of the C-terminal of M6, and the *ermC* Erythromycin resistance gene. Genes and their direction of transcription are represented as arrows. The fragments of the fusion gene are labeled with the name of the encoded genes. Promoter (P) and transcription termination (T) sequences are indicated. Scale is in kb.

An anti-MOMP rabbit serum was used to analyze heterologous antigen expression in *S. gordonii* (**Figure 2**). Flow cytometric analysis of whole cells show expression of the CTH522 MOMP antigen on the bacterial surface (**Figure 2A**). Staining with anti-M6 and anti-HA antibodies demonstrated their co-expression on the bacterial surface (data not shown). Western Blot analysis on whole cells extracts revealed two bands with a molecular weight (M.w) of approximately 105 and 116 kDa (**Figure 2B**). The band at 105 kDa represents the M6-CTH522 fusion protein, while the band at 116 kDa is postulated to result from an uncleaved signal peptide of the fusion protein, estimated at 11 kDa, as the analysis was carried out on whole cell lysates. While the fusion protein was predicted to have a M.w of 90.91 kDa based on its amino acids sequence, this 15% variance from the predicted M.w is consistent with previous findings when assessing the M.w of M6-fusion proteins using western blotting (30,45). Soluble CTH522 recombinant protein displayed a band at 54 kDa, as previously demonstrated (40). Lastly, the mass of CTH522 expressed on the bacteria surface was calculated using Dot Blot assay (**Figure S1**). By using a standard curve of purified protein vs signal intensity, we found that CTH522 was expressed at approximately 370ng per 10^9 CFU, the CFU dosage previously found to be the optimal immunization dose using recombinant *S. gordonii* (30). Collectively, these data demonstrate that the DNA sequence was successfully incorporated into the chromosome of FR368 at the designated position while preserving the nucleotide and amino acids sequences, and that FR368 successfully translates the M6-CTH522 heterologous antigen and expresses it on the bacteria's surface.

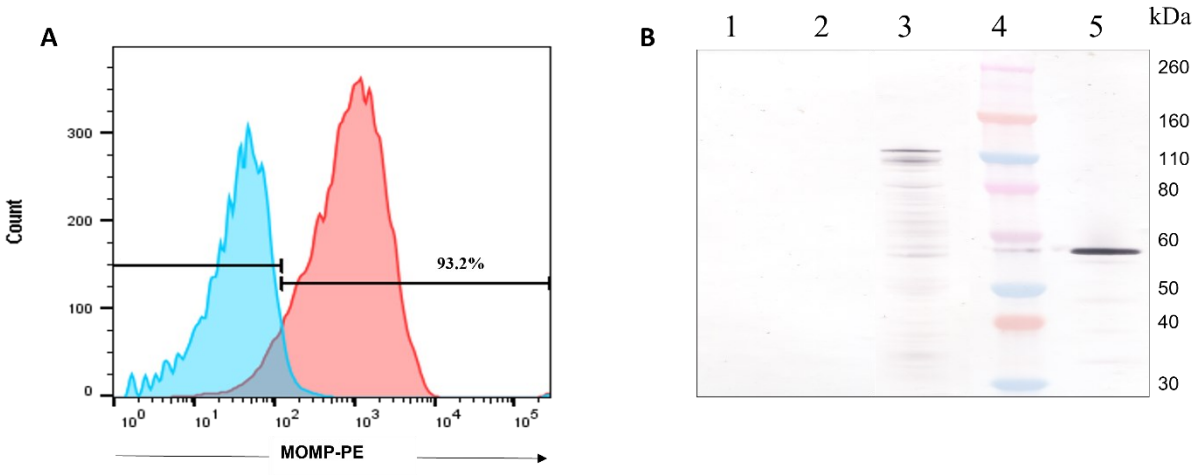

Figure 2 | Expression of CTH522 antigen on FR368 recombinant *S. gordonii*. A) Flow cytometric analysis of *S. gordonii* FR368 expressing CTH522. Recombinant FR368 and WT strain GP1295 were grown in Tryptic Soy Broth without dextrose until late exponential phase. $2x10^7$ cells of either recombinant (red histogram) or WT control (blue histogram) were reacted with anti-MOMP rabbit serum and with a secondary anti-rabbit PE-conjugated antibody and analyzed using BD LSRFortessa X20 flow cytometer. 93.2% of the FR368 cells were positive for MOMP expression. Representative histogram of n=3 experiments of biological replicates. **B)** Western blot analysis of protein preparations. Recombinant and WT strains were grown in Brain Heart Infusion until late exponential phase. Bacterial samples were washed once in H_2O, centrifuged at 10,000 x g and the pellet was resuspended in 100µL H_2O. Pellet was then transferred to an Eppendorf tube containing 0.04g glass beads and placed in a tissue lyser for 2 min at 30Hz for 2 cycles. Samples were centrifuged and supernatant containing proteins was subjected to reducing agent (DTT). 1) Blank, 2) WT Strain ($1x10^8$ CFU), 3) FR368 ($1x10^8$ CFU), 4) kDa molecular weight standard, 5) CTH522 protein (12.5ng). Two bands were observed in FR368, at 105 kDA and 116 kDA. The band representing M6-CTH522 fusion protein is seen at 105 kDa, while the band at 116 kDa is postulated to represent a fusion protein with an uncleaved signal peptide, estimated at 11 kDa. M6-CTH522 fusion protein was predicted to have a M.w of 90.91 kDa based on its amino acids sequence. This 15% variation between expected and observed M.w for M6-fused proteins is consistent with previous observations assessing the fusion protein's size using western blotting (30,45). Purified CTH522 was measured at 54 kDa, as expected.

Vaginal colonization by FR368 primes the immune system towards the CTH522 antigen and promotes antigen-specific systemic IgG production.

The immunogenicity of the FR368 vaccine vector was assessed *in vivo* in mice. Following estrous cycle synchronization using 0.1 mg of β-estradiol 17-valerate, female BALB/c mice were immunized intravaginally (IVAG) with 10^9 CFU of either WT or FR368 strains on weeks 0, 1 and 2. Bacterial colonization of the vaginal tract was assessed using plating of vaginal swabs on selective agar plates. WT and FR368 *S. gordonii* efficiently colonized the mouse vaginal tract, with 100% of

the mice colonized one week after the first immunization, and approximately 50% of mice colonized at week four (**Figure 3A**), as expected from previous works (46,47). No colonization was detected in any of the groups by week 12, and no significant difference in average colonization duration was observed between FR368 and WT strains (4.16 ± 0.6 vs 3.47 ± 0.37 weeks, respectively, p=0.59. **Figure 3B**).

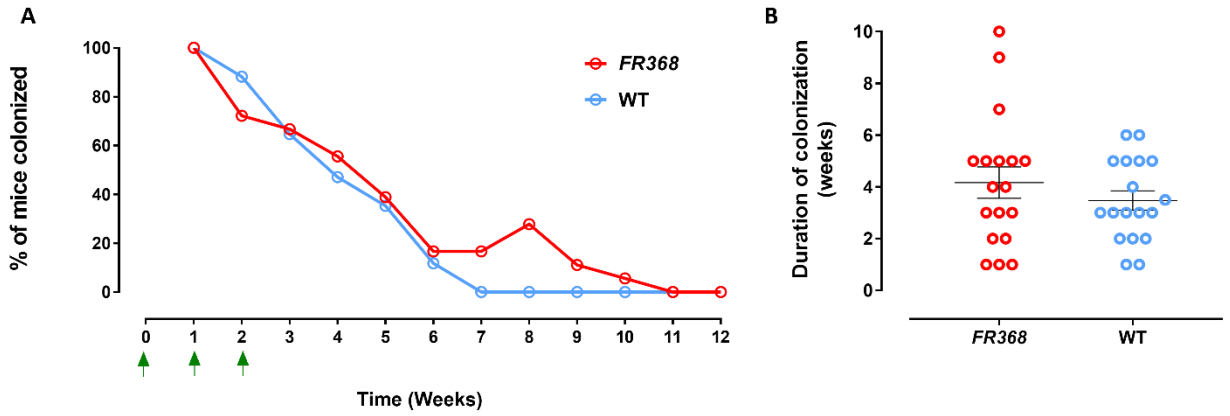

Figure 3 | **Vaginal colonization by *S. gordonii.*** Recombinant *S. gordonii* FR368 and WT strains were grown in TSB without dextrose until late exponential phase, washed and resuspended in 1/1000 of the original volume and stored in -80°C until use. Mice were intravaginally immunized with 10^9 CFU of either recombinant *S. gordonii* (FR368) or WT, delivered in 20µL PBS on weeks 0, 1 and 2 (green arrows). Vaginal swabs were collected weekly by washing the vagina with 20µL sterile PBS, and then sampling using Rayon tips. The Rayon tip was placed in an Eppendorf tube containing PBS 1% BSA and vortexed for 1 min. Vaginal pellet was resuspended in 100uL BHI 10% glycerol and stored in -80°C until plating. Pellet was plated on selective BHI 3% sheep blood agar plates to assess colonization by FR368 (Erythromycin selection, 3µg/mL) and WT (Kanamycin selection, 500µg/mL). **A)** Colonization of the murine vaginal tract with *S. gordonii*. Individual dots represent percentage of mice with a positive vaginal sample for either FR368 (red) or WT (light blue) at a given week. After 4 weeks, approximately 50% of mice immunized with either strain was still colonized. No colonization was detected in either strain by week 11. **B)** Average duration of positive colonization per strain in weeks. Individual circles represent the duration of positive sampling in weeks for individual mice. Horizontal lines represent groups' means, vertical lines represent SEM. No significant difference was measured in colonization duration between FR368 and WT strains (4.16 ± 0.6 vs 3.47 ± 0.37 weeks, respectively, p=0.59). Statistical significance was determined using Mann-Whitney test. n=18 per group.

ELISA assays were conducted on serum and vaginal samples collected periodically to assess whether intravaginal immunization with FR368 induced production of anti-CTH522 antibodies. Measurements of CTH522-specific serum IgG demonstrated that vaginal colonization with FR368 induced a significant systemic immune response already two weeks (W2) after the first immunization

(**Figure 4**). Firstly, FR368-immunized mice's values at W2 presented a significant increase from the group's baseline levels at W0 (67.58 ± 11.03 vs 3.86 ± 2.86 ng/mL, p=0.0006. **Figure 4**). Furthermore, this increase was significantly elevated compared to WT-immunized mice values at W2 (1.97 ± 0.63 ng/mL respectively, p<0.0001. **Figure 4**). By W6, serum CTH522-specific IgG levels of the FR368-immunized group returned to levels comparable to those of the WT-immunized groups and remained non significantly different for the following six weeks (**Figure 4**). We also examined the presence of CTH522-specific IgG and IgA in vaginal washes, but no differences were detected between the groups at the examined timepoints (data not shown).

We then assessed the extent of immune priming facilitated by the intravaginal immunization with the recombinant *S. gordonii* by heterologously boosting mice with two doses of 5µg of purified CTH522 administered subcutaneously (S.C.) and analyzing serum CTH522-specific antibodies response using ELISA. To that end, on week 12 (W12), the two groups were further divided into four groups of n=6 each as follows: FR368-immunized mice were divided to either three-months boosting (W13 and W17) or six-months boosting (W27 and W31, "*Late*") of subcutaneously administered 5µg CTH522 protein. WT-immunized mice were split into either subcutaneously administered 5µg CTH522 protein, or saline, both administered on W13 and W17 (**Figure 4**). From this point onward, the WT-immunized group that received the subcutaneously administrated CTH522 boosting is referred to as WT-immunized, while the group that received saline is referred to as antigen-naïve.

One week post the first S.C boost (W14), FR368-immunized mice undergoing the three-months boosting schedule showed a rapid increase in CTH522-specific serum IgG, significantly higher compared to WT-immunized mice (49.13 ± 20.2 and 3.27 ± 2.27 ng/mL, respectively, p=0.008. **Figure 4**), and to its own pre-boost levels at W12 (9.87 ± 2.70 ng/mL, p=0.009). The magnitude of the response of the three-months boosted FR368-immunized mice at W14 was slightly lower than, yet comparable to the group's own W2 levels (p=0.25). FR368-immunized mice undergoing the six-

months boosting schedule presented a marginally significant increase in their CTH522-specific IgG levels one week after the first boost (W28) compared to their pre-boost levels at W27 (16.1 ± 4.85 vs 7.01 ± 3.65 ng/mL, p=0.061. **Figure 4**). At W14, WT-immunized mice did not produce a serum IgG response different to the antigen-naïve group (2.62 ± 1.03 ng/mL, p>0.9. **Figure 4**). This remained the case in the following three weeks until the second dose of boosting.

Four weeks after the first boost (W17 or W31), a second boost of equal dosage was delivered subcutaneously, and ten days later (W18.5 or W32.5) serum CTH522-specific IgG were reanalyzed using ELISA.

Three-months-boosted FR368-immunized mice showed a robust CTH522-specific systemic IgG response, significantly elevated compared to the WT-immunized group (2,425 ± 1,591 and 50.78 ± 26.04 ng/mL, respectively, p=0.017. **Figure 4**), and to its own W2 values (p=0.026). CTH522-specific IgG levels of the WT-immunized mice at W18.5 were not significantly different compared to the values of FR368-immunized mice at W2 (p=0.37). The six-months boosted FR368-immunized mice also demonstrated a significant increase in CTH522-specific serum IgG ten days following the second boost (W32.5), compared to its own levels before the second boost (W31) (690 ± 530.4 vs 7.41 ± 6.44 ng/mL, respectively, p=0.015). No statistically significant difference was found between the three- and six-months boosting FR368-immunized groups at ten days following the second boost (W18.5 vs W32.5, p=0.13).

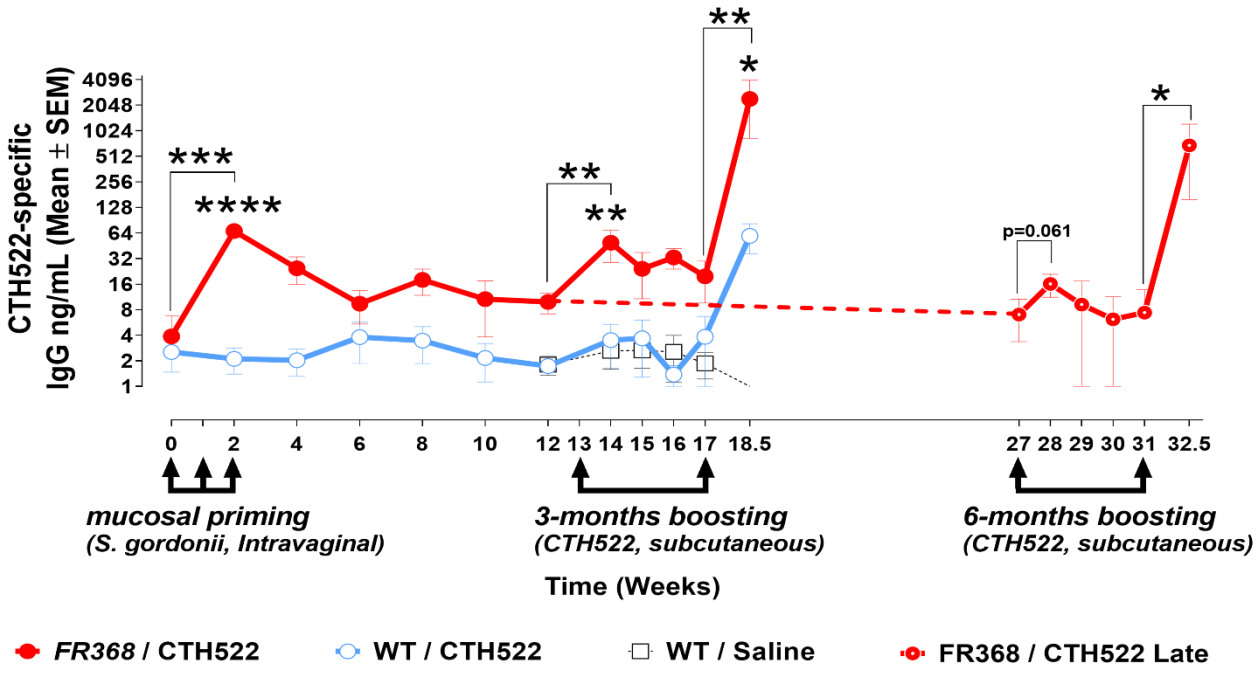

Figure 4 | CTH522-specific serum IgG response measured by ELISA. Serum anti-CTH522 IgG levels were determined at the indicated timepoints using ELISA. Serum CTH522-specific IgG antibodies concentration in ng/mL. Female Balb/c mice were intravaginally immunized with 10^9 CFU of either FR368 recombinant (n=12) or WT strain (GP1295, n=12) on weeks 0, 1 and 2 (three arrows, intravaginally). On week 12, groups were further divided into four groups of n=6 each as follows: FR368-immunized mice were divided to either 3-months boosting (weeks 13 and 17, red) or 6-months boosting (weeks 27 and 31, dotted red, "*Late*") of subcutaneously administered 5µg CTH522 protein each (arrows). WT-immunized mice were split to either subcutaneously administered 5µg CTH522 protein (light blue) or saline (dotted black line), both administered on weeks 13 and 17. Serum samples from individual mice were serially diluted and added to CTH522-coated ELISA plates and antigen-specific IgG levels were measured based on an IgG standard curve. At week 2 (W2), mean IgG values were measured at 67.58 ± 11.03 and 1.97 ± 0.63 ng/mL for the FR368- and WT-immunized groups, respectively, $p<0.0001$, n=12 per group. W2 values presented a significant increase from FR368-immunized mice's baseline levels at W0 (3.86 ± 2.86 ng/mL, p=0.0006, n=12). Seven days after the first boost (W14), FR368-immunized mice undergoing the three-months boosting schedule and WT-immunized groups had mean CTH522-specific IgG levels of 49.13 ± 20.2 and 3.27 ± 2.27 ng/mL respectively, p=0.008. The FR368-immunized mice's CTH522-specific IgG concentration at W14 were also significantly elevated compared to the group's pre-boost values at W12 (9.87 ± 2.70 ng/mL, p=0.008). At day 10 after the second boost (W18.5) mean IgG concentrations for FR368- and WT-immunized groups were measured at 2,425 ± 1,591 and 59.25 ± 22.88 ng/mL, respectively, p=0.017. FR368-immunized mice's values at this time point were also significantly higher than the group's values at W2, W14 and W17 (p-values: p=0.026, p=0.0087, p=0.002, respectively). At day ten after the second boost (W18.5) WT-immunized mice showed a significant increase compared to their W14 and W17 values (p=0.008 and p=0.015, respectively, not marked). CTH522-specific IgG levels of the WT-immunized mice at W18.5 were not significantly different compared to the values of FR368-immunized mice at W2 (p=0.37). On week 28 (W28), 7 days after their first boost, FR368-immunized mice undergoing the six-months boosting schedule ("*Late*") displayed a marginally significant increase in CTH522-specific IgG levels compared to their pre-boost values at week 27 (16.1 ± 4.85 and 7.01 ± 3.65 ng/mL, p=0.061). At 10 days following the second boost (W32.5), six-months boosted group showed significantly elevated levels of CTH522-specific IgG compared to W31 (pre second boost) (690 ± 530.4 vs 7.41 ± 6.44 ng/mL, p=0.015). No

significant differences were found between three- and six-months boosting strategies on either seven days post first boost or 10 days post the second boost (d7 PB1: 49.13 ± 20.2 and 16.1 ± 4.85 ng/mL, respectively, p=0.17; d10 PB2: 2,425 ± 1,591 vs 690 ± 530.4 ng/mL, respectively, p=0.13). Individual points represent group's mean ± SEM. Statistical significance was determined using Mann-Whitney and Kruskal-Wallis tests. Statistical significance is shown as follows: *, $p < 0.05$. **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

Next, we analyzed the anti-CTH522 IgG subclasses profile ten days after the second boosting, at W18.5 or W32.5, using ELISA. FR368-immunized mice undergoing the three-months boosting schedule had a mean IgG2a/IgG1 ratio of 0.83 ± 0.48, with four out of six mice expressing higher levels of IgG1 compared to IgG2a, trending towards a Th2 polarization. WT-immunized mice presented an opposite phenotype, with a ratio of 1.19 ± 0.11, with five out of six mice presenting higher levels of IgG2a compared to IgG1, towards a Th1 polarization. FR368-immunized mice undergoing the six-months boosting schedule had a more balanced Th1/Th2 polarization in terms of IgG subclasses produced, with an IgG2a/IgG1 ratio of 0.96 ± 0.36, with 50% of the mice presenting higher levels of IgG2a, and the other 50% showed higher levels of IgG1 (data not shown).

Vaginal colonization with FR368 increases plasma cells in the lymph nodes and CTH522-specific Antibody Secreting Cells in the spleen following heterologous boost.

The presence of plasma cells in the draining lymph nodes (dLN) and of CTH522-specific antibody-secreting cells in the spleens was assessed ten days after the second boost (W18.5 or W32.5) using flow cytometric analysis and B-cell ELISPOT.

Flow cytometric analysis revealed that FR368-immunized mice, in both three- and six-months boosting schedules, had a significantly higher percentage of plasma cells ($TACI^+$ $CD138^+$) in the lymph nodes draining the vaginal tissue and the subcutaneous injection site, compared to WT-immunized mice (0.6% ± 0.2 and 0.76% ± 0.33 vs 0.2% ± 0.02, p=0.03 and p=0.015, respectively, **Figure 5A,B**). In addition, a significant increase in splenic plasma B cells ($TACI^+$ $CD138^+$) was detected in the six-months FR368-immunzied group compared to three-months FR368-immunized

group (6.12% ± 0.21 vs 2.65% ± 0.38, respectively, p=0.002. data not shown). We did not detect any differences in Germinal Centre B cells' (GL-7^{+} CD95^{+}) frequencies or numbers in either the dLN or the spleen between the WT- and FR368-immunized groups (data not shown).

B-cell ELISPOT assay using CTH522-coated plates revealed that FR368-immunized mice, both in the three- and six-months boosting schedules, had a significantly higher number of CTH522-specific IgG-secreting B cells compared to WT-immunized mice (4 ± 0.85 and 3.67 ± 0.76 vs 1.5 ± 0.5 ASCs per 10^6 splenocytes, p=0.02 and p=0.039, respectively, **Figure 5C**). No differences were found between the two FR368-immunized groups (p>0.9) and between the WT-immunized and antigen-naïve groups (p>0.5). In addition, no differences were detected in the number of total non-antigen-specific IgG ASCs (data not shown).

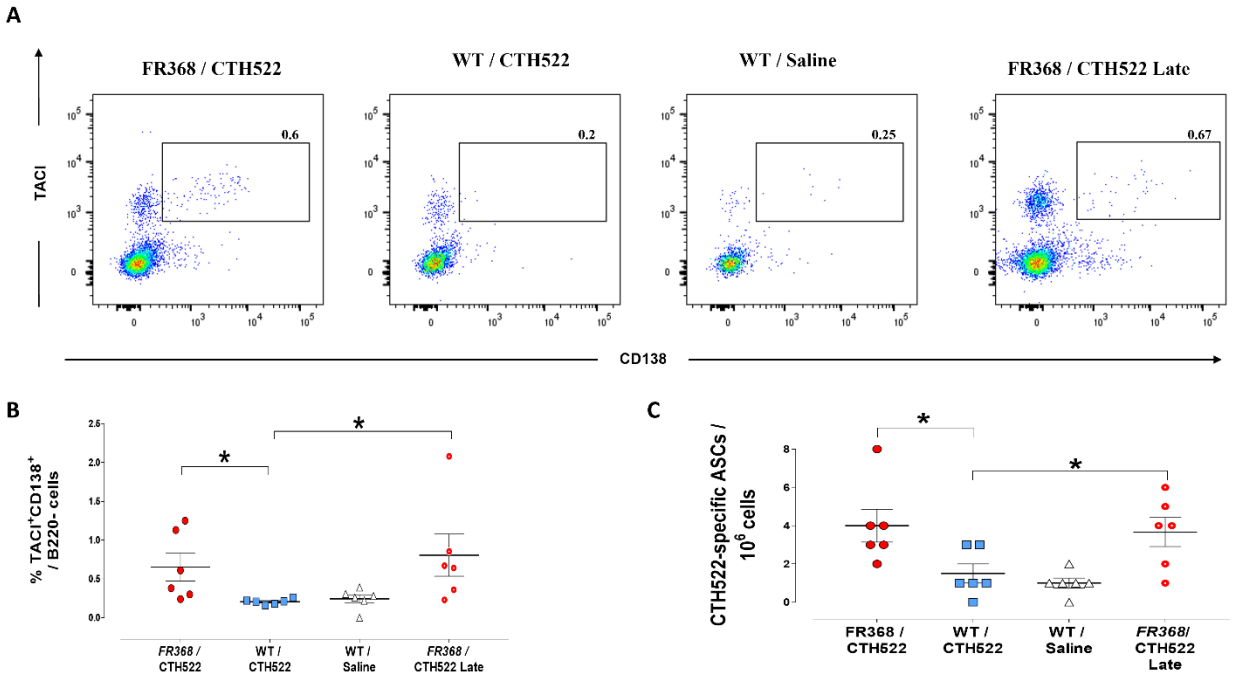

Figure 5 | Presence of plasma cells and antibody secreting cells in draining lymph nodes and spleen. The presence of plasma cells and antibody secreting cells was analyzed using flow cytometry and B cells ELISPOT 10 days after the second boost (W18.5 or W32.5). Female Balb/c mice were intravaginally immunized with 10^9 CFU of either FR368 recombinant (n=12) or WT strain (GP1295, n=12) on weeks 0, 1 and 2. On week 12, groups were further divided into four groups of n=6 each as follows: FR368-immunized mice were divided to either three- (weeks 13 and 17, red) or six-months boosting (weeks 27 and 31, red and white, "Late") of subcutaneously administered 5µg CTH522 protein. WT-immunized mice were split to either subcutaneously administered 5µg CTH522 protein (light blue) or saline (white), both administered on weeks 13 and 17.

A) Representative dot plot showing plasma cells ($TACI^+CD138^+$) from the iliac draining lymph nodes, gated on $B220^-IgD^-CD3^-$ live cells. **B)** Frequencies of positively stained $TACI^+CD138^+$ plasma cells were measured as percentage of $B220^-$ cells in the iliac draining lymph nodes using flow cytometry. FR368-immunzied in the three-months boosting scheme (red circles), WT-immunized with CTH522 boosting- (light blue squares), WT-immunized with saline (white triangles) and FR368-immunzied in the six-months boosting scheme (red circles with a white dot). FR368-immunized mice undergoing both three- and six-months boosting schedules had a significantly higher percentage of plasma cells compared to WT-primed mice (0.6% ± 0.2 and 0.76% ± 0.33 vs 0.2% ± 0.02, p=0.03 and p=0.015, respectively). No significant difference was found between the three- and six-months boosting schemes. Individual dots represent individual mice, results are reported as mean ± SEM. **C)** Number of CTH522-specific IgG-secreting cells per 10^6 splenocytes. 10^6 Splenocytes from either FR368- and WT-immunized mice were plated on CTH522-coated ELISPOT plates (5µg/mL) and incubated for 18 hours at 37 °C with 5% CO2. Following incubation and antibodies detection, ASCs were quantified by counting spots on the surface of the CTH522-coated membrane using Immunospot S6 ULTIMATE Analyzer. FR368-immunized mice in both three- and six-months boosting scheme had significantly more ASCs compared to WT-immunized mice (4 ± 0.85 and 3.67 ± 0.76 vs 1.5 ± 0.5 ASCs per $1x10^6$ splenocytes, p=0.02 and p=0.039, respectively). WT-immunized mice with saline boost (antigen-naïve) had 1 ± 0.25 ASCs per $1x10^6$ splenocytes. No difference was found between FR368-immunized in the three-months and the six-months boosting schedules (p=0.9). Individual dots represent individual mice, results are reported as mean ± SEM of n=6 mice per group. Statistical significance was determined using Mann-Whitney test. Statistical significance is shown as follows: *, $p < 0.05$.

FR368 vaginal priming induces a differential cytokine-expression profile in *in vitro* stimulated splenocytes.

We used flow cytometry to analyze the cytokines expressed by splenocytes *in vitro*. Splenocytes from the four different groups were harvested ten days after the second boost (W18.5 and W32.5) and restimulated *in vitro* with the CTH522 antigen for six hours.

Flow cytometric analysis of intracellular cytokine staining revealed that splenocytes from FR368-immunized mice in both the three- and six-months boosting schedules presented significantly higher levels of IL-17^+ $CD4^+CD44^{high}$ cells compared to WT-immunized mice (0.39% ± 0.05 and 0.68% ± 0.1, p=0.01 and p=0.002 vs WT-immunized mice, respectively. **Figure 6A**) Within the FR368-immunized groups, the six-months group's IL-17^+ $CD4^+CD44^{high}$ cells levels were significantly higher than the three-months boosting group's (p=0.015). Additionally, the FR368-immunized mice undergoing the three-months boosting schedule presented significantly higher levels of IL-17^+ $CD8^+$ cells compared to both the WT-immunized group and the FR368-immunized six-months group

(p=0.008 and 0.004, respectively. data not shown). By contrast, $CD4^{+}CD44^{high}$ T cells from WT-immunized mice showed significantly higher levels of Interferon-γ (IFN_{γ},), IL-2^{+} and TNF-α^{+} compared to both three- and six-months FR368-immunized groups (**Figure 6B-D**). Specifically, WT-immunized group had 0.75% ± 0.09 of $CD4^{+}CD44^{high}$ T cells positive for IFN_{γ} (p=0.004 and p=0.073 vs three- and six-months FR368-immunized mice, respectively. **Figure 6B**), 2.29% ± 0.20 for IL-2 (p=0.006 and p=0.002, **Figure 6C**), and 2.33% ± 0.49 positive for TNF-α (p=0.009 and p=0.004, respectively. **Figure 6D**). Interestingly, FR368-immunized mice in the six-month boosting schedule had significantly lower IL-2^{+} $CD4^{+}CD44^{high}$ T cells compared to their three-months counterparts (p=0.036). No differences were observed in the levels of expression of IL-4^{+} and IL-13^{+} (data not shown).

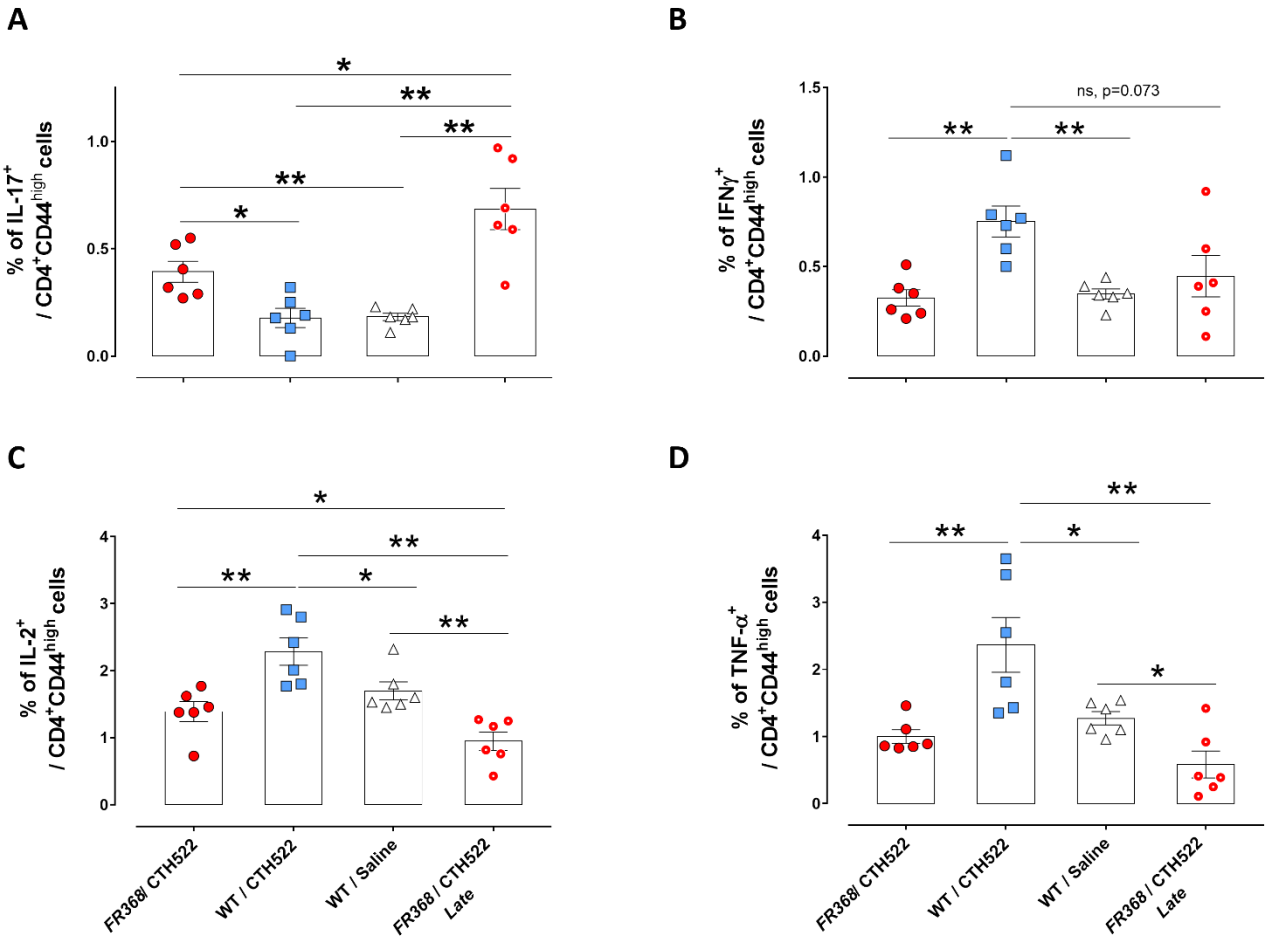

Figure 6 | Intracellular cytokine production after *in vitro* stimulation. Ten days after the second boost (W18.5 and W32.5), mice were sacrificed and $2x10^{6}$ Splenocytes were cultured for 6 hours in the presence CTH522 antigen (5 µg/ml) and co-stimulatory antibodies CD28 and CD49d (both at

2 µg/ml). Brefeldin A (5 µg/ml, Sigma-Aldrich) and Monensin solution (1x, eBioscience) were added to all samples for the last 4 h of incubation. Frequencies of cytokines-producing CTH522-specific T cells ($CD4^+CD44^{high}$) were measured by intracellular staining using flow cytometry (LSRFortessa x-20). Percentages of either IL-17^+, IFN_γ^+, IL-2^+, or TNF-α^+ out of $CD4^+CD44^{high}$ T cells are reported. **A**) $CD4^+CD44^{high}$ T cells from FR368-immunized mice on both three- and six-months boosting schedules showed a significant increase in IL-17 expression compared to WT-immunized mice (0.39% ± 0.05 and 0.68% ± 0.1 vs 0.18% ± 0.05, p=0.01 and p=0.002, respectively). Between the FR368-immunzied groups, the six-months boosted group showed an increase compared to the three-months group (p=0.015). No differences were found between the two WT-immunized and the antigen-naïve groups (p=0.9). **B**) $CD4^+CD44^{high}$ T cells from WT-immunized mice boosted with CTH522 showed a significant increase in In IFN_γ^+ cells compared to FR368-immunized mice in the three-months boosting schedule (0.75% ± 0.09 vs 0.32% ± 0.04, respectively, p=0.004), and marginally significant increase compared to the six-months boosting schedule (0.45% ± 0.12, p=0.073). **C**) WT-immunized mice showed a significant increase in the expression of IL-2 compared to both three- and six-months boosted FR368-immunized groups (2.29% ± 0.20 vs 1.39% ± 0.15 and 0.95% ± 0.14, p=0.006 and p=0.002, respectively). A significant reduction in IL-2^+ cells was found between the three- and six-months boosting groups (p=0.036). **D**) WT-immunized mice boosted with CTH522 showed a significant increase in TNF-α^+ $CD4^+CD44^{high}$ T cells compared to both three- and six- months boosted FR368-immunized mice (2.33% ± 0.49 vs 1% ± 0.1 and 0.59% ± 0.2, p=0.009 and p=0.004, respectively). A significant decrease in TNF-α^+ T cells was also found between the FR368-immunized mice undergoing the six-months boosting schedule and the WT-immunized mice receiving saline as a boost (antigen-naïve) (p=0.03). Columns represent group's Mean ± SEM, individual dots represent individual mice. n=6 per group. Statistical significance was determined using Mann-Whitney test. Statistical significance is shown as follows: *, $p < 0.05$. ** $p < 0.01$.

Discussion

In this work, we examined whether mucosal delivery of a vagina-colonizing *S. gordonii* expressing a heterologous antigen could efficiently prime the immune system towards a heterologous boost in a murine model. To that end, a recombinant *S. gordonii* expressing the multivalent MOMP antigen of *C. trachomatis*, CTH522, was delivered mucosally as a priming vector in a heterologous prime-boost immunization regiment. We show that mucosal immunization with the bacterial vector successfully primes the immune system and modulates the humoral and cellular immune responses towards a heterologous subcutaneous protein boost administered either three- or six-months after the priming.

Firstly, we used Nanopore sequencing the confirm the successful integration of the heterologous construct encoding the CTH522 antigen into FR368's chromosome, while preserving the base-pair sequence and the reading frame. To our knowledge, this is the first time that a recombinant *S. gordonii* strain expressing a heterologous antigen has been sequenced using NGS technology in order to confirm the fidelity of the antigen sequence.

The utilization of recombinant bacteria as a vaccine delivery platform to mucosal surfaces, and particularly of Gram-positive bacteria, has been studied for decades (17). However, colonizing bacteria have been speculated to be poor vaccine vectors owing to the induction of tolerance following the prolonged exposure (48). However, Laver et al. have demonstrated an immune response induced by a colonizing recombinant commensal bacterium expressing a heterologous antigen and administered nasally in a recent phase I clinical trial (NCT03630250) (49). In addition, while several studies have found CTH522 to be a promising *Chlamydia* vaccine candidate (38,39), it is weakly immunogenic when delivered unadjuvanted (50). In the context of mucosal delivery, a recent study by Nguyen et al. showed that multiple immunizations with CAF01-adjuvanted CTH522 delivered at the upper genital tract, failed to induce a systemic humoral immune response (51). Here we show that our recombinant *S. gordonii* strain FR368 delivered intravaginally successfully elicited the production of serum CTH522-specific IgG as early as W2 following the first vaginal immunization.

This response, elicited by an aggregated dosage of approximately 0.75µg CTH522, was superior in absolute values to the response generated by the WT-immunized mice in response to the two boosts of 5µg unadjuvanted CTH522. However, as our delivery system relies on administration of live, colonizing bacteria, capable of *in vivo* proliferation and *in-situ* antigen production, the exact effective mass of antigen delivered is extremely difficult to quantify.

The intravaginal administration of FR368 also facilitated a robust immune response following subcutaneous boosting with the unadjuvanted CTH522 protein. FR368-immunized mice produced significantly more CTH522-specific serum IgG compared to the WT-immunized mice, and in similar efficacy when boosted at both three- and six-months. These findings are in line with recently published findings by us, demonstrating that a longer time interval between priming and boosting did not significantly affect serum IgG titers (52). The WT-immunized low IgG response following the protein boosts is also in line with previous findings (50). The priming effect observed in the FR368-immunized mice may result due to complexing of the CTH522 antigen with the bacterial peptidoglycans, which were suggested to serve as immunostimulatory molecules and natural adjuvants (53,54). Indeed, it has been shown that components of *S. gordonii* cell wall can interact with various PRRs (55), as well as activate TLRs expressed on the murine vaginal epithelium (56). Thus, our data supports the idea that vaginal colonization by recombinant *S. gordonii* can elicit a long-lasting priming effect of the immune system towards the heterologous antigen and induces a memory response which is triggered upon antigen reencounter, even after six months.

The IgG subclasses profile is suggested to be influenced by the type of T-helper response elicited (57). When analyzing the profile of CTH522-specific IgG subclasses at W18.5 and W32.5, we observed a trend towards a Th2 response in FR368-immunized mice in the three-months boosting schedule, a Th1 response in WT-immunized mice, and a balanced Th1/Th2 response in the FR368-immunized mice in the six-months boosting schedule. The IgG2 dominance in WT-immunized mice is in line with previous finding of IgG subclasses composition elicited by unadjuvanted CTH522, however with a different mouse strain (C57bl/6) (50). Interestingly, in our own previous works with

recombinant *S. gordonii* vectors expressing different antigens and administered mucosally, the serum IgG response was typically predominated by the IgG2a subclass compared to IgG1 (27,58), or had comparable levels (23,30). Thus, the divergence in IgG subclasses profile may derive from the heterologous prime-boost strategy employed in the current study, in which the antigen was first encountered in a mucosal setting associated with the *S. gordonii* as a natural adjuvant. This work involves a heterologous prime-boost regiment in which both the formulation and route of administration are modified. Therefore, pinpointing whether the shift in IgG subclasses profile is owing to the formulation (*S. gordonii*-bound), route (vaginal immunization) or age of mice (three vs six months boosting) requires further investigations which are beyond the scope of this study.

Production of antigen-specific antibodies is the focus of an overwhelming majority of vaccine formulations (59). This response involves several types of B-cells (60). Indeed, we have demonstrated that the increase in serum IgG production following boosting was accompanied by a higher percentage of $TACI^{+}CD138^{+}$ plasma cells in the iliac lymph nodes draining both the vaginal tissue and the S.C. injection site, and higher numbers of CTH522-specific ASCs in the spleen compared to the WT-immunized group. Our data suggest that vaginal immunization with FR368 facilitates rapid proliferation of plasma cells in the dLN upon antigen reencounter (60), and induces a differentiation and retainment of CTH522-specific ASCs in the spleen. The lack of significant differences between the two boosting schedules with regards to B cells populations further supports the longevity of the priming effect induced by the vaginal colonization.

In our study, *in vitro* stimulated splenocytes from mice intravaginally immunized with the FR368 *S. gordonii* vector showed a clear increase in IL-17 expression by $CD4^{+}CD44^{+}$ T cells. Th17 T cell response is highly associated with mucosal immunity in infections and vaccines (61). In the context of the female genital tract, the role of IL-17 is not clear, and its expression was associated with both resolution (62) and dampening (63) of inflammatory response. Its increased expression does suggest, however, that the mucosal interaction of the immune system with the CTH522 antigen on the surface of the *S. gordonii* had impacted the cytokines profile produced by T-helper cells upon

restimulation. On the other hand, the vaginal priming with FR368 resulted in reduced expression of IFN_{γ}, IL-2 and TNF-α compared to the WT-immunized mice. The higher expression of IFN_{γ} and IL-2 in WT-immunized mice is indeed in line with their Th1-trending IgG2a subclasses profile observed (60,64). The reduction in inflammatory cytokines expression in FR368-immunized mice compared to the WT-immunized mice may suggest that the responses observed (increased in plasma cells, ASCs, and serum CTH522-specific IgG) result from antigen presentation carried out mainly by affinity-matured memory B cells (60), developed following the vaginal immunization with FR368. Further studies are necessary to determine the *in vivo* dynamics of *S. gordonii*-derived antigen processing and presentation in the vaginal tract.

In conclusion, in this work we show that intravaginal immunization with a recombinant *S. gordonii* successfully primed the immune response in a heterologous prime-boost immunization scheme. Our data suggests that long-term boosting interval is as effective as the short-term. FR368 has demonstrated a potential as a mucosal priming candidate formulation, and merit further studies in additional immunization schemes and protection models.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors Contributions

GP, EP and BP conceived and designed the experiments. BP constructed, cultured, and prepared bacteria for *in vitro* and *in vivo* experiments, with the help of EP, FI, and FS. BP, EP and FF performed *in vivo,* and *ex vivo* experiments. GP, EP and BP discussed and interpreted the findings. BP wrote the manuscript. GP and EP edited the manuscript. GP provided financial support. All contributing authors have read and approved the submitted version.

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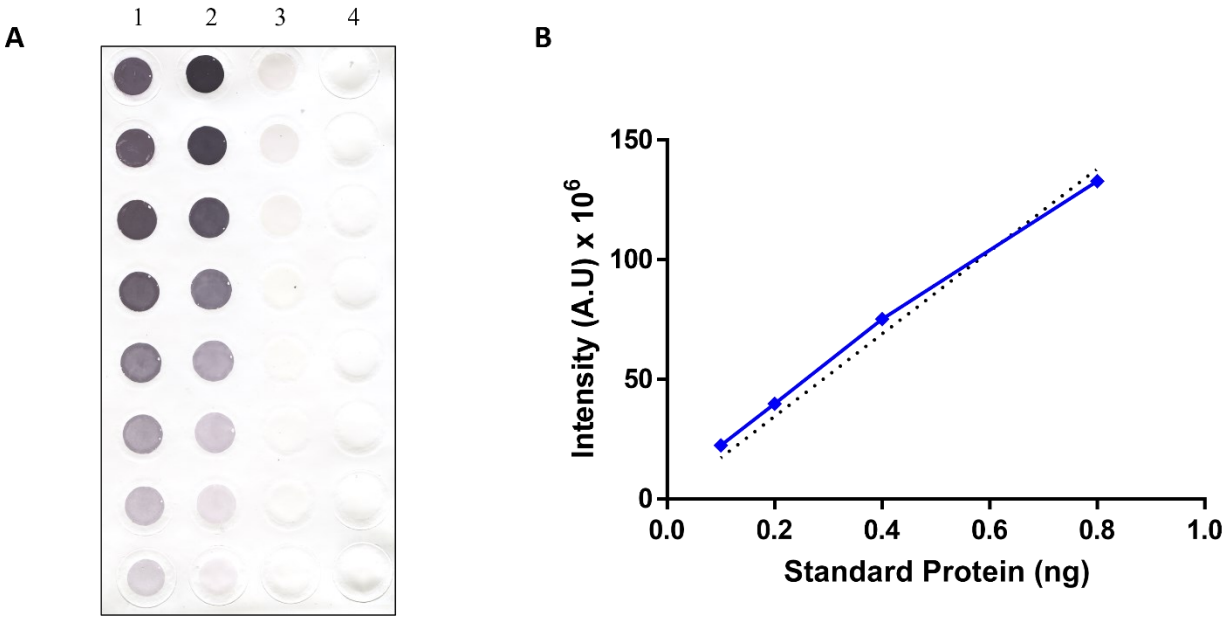
Supplementary Material

Supplementary Figure 1: CTH522 mass quantification on bacteria's surface using dot blot.



Supplementary Figure 1 | CTH522 mass quantification on bacteria's surface using dot blot. Recombinant FR368 and WT *S. gordonii* strains were grown in Brain Heart Infusion until late exponential phase. The reported CFU or mass were diluted in 100µL PBS and blotted using a vacuum-manifold onto a nitrocellulose membrane in two-fold serial dilutions, starting from 1×10^8 CFU of bacteria or 12.5ng of the CTH522 protein. Membranes were dried, blocked using TBS-T 0.05% with 5% milk, and reacted with anti-MOMP rabbit serum ON at 4°C. Membranes were then washed and reacted with anti-Rabbit Alkaline phosphatase conjugated antibody. **A**) Dot blot analysis of 1) FR368, 2) CTH522 protein, 3) WT strain and 4) Blank. **B**) Signal intensity quantification Signal intensity quantified by scanning the membrane in high resolution and measurement of the intensity using ImageJ software. Measurements were used to generate a standard curve of intensity in A.U. vs. nanograms of CTH522. Signal quantification revealed that $1x10^9$ CFU express ~370ng of M6-CTH522 antigen. Representative membrane of n=3 experiments.

Chapter 4 – Transcriptional Analysis After Mucosal Priming by a Recombinant Vaccine Vector *Streptococcus gordonii* Expressing the MOMP Chlamydial antigen Reveals Enrichment of Specific Immune Pathways and identifies a Signature Correlated with Antibody Titers

In this chapter we analyze the transcriptomic response of *in vitro* stimulated splenocytes from immunized mice to the antigen. This method allows us to study how vaginal priming with a live bacterial vector affects gene expression profile when the antigen is reencountered *in vitro*.

The transcriptomic response of *in vitro* stimulated splenocytes from mice immunized with either WT or FR368 towards the CTH522 antigen was analyzed in order to identify the differentially expressed genes, the immune pathways activated and the correlation between gene expression and antibodies production.

Our data show that vaginal immunization with the recombinant *Streptococcus gordonii* activates different immune pathways and generates a gene signature associated with antibody titers.

Transcriptional Analysis After Mucosal Priming by a Recombinant Vaccine Vector *Streptococcus gordonii* Expressing the MOMP Chlamydial antigen Reveals Enrichment of Specific Immune Pathways and identifies a Signature Correlated with Antibody Titers

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Abstract

Mucosal surfaces are particularly vulnerable to infection, and vaccines targeting these areas would be of great importance. However, understanding the responses and protective mechanisms induced by mucosal vaccines have been challenging, particularly with regards to the genital tract, impacting the development of this type of vaccines. The transcriptomic analyses and Systems Biology approach emerges as potential tools to better study these immunological mechanisms and find new correlates of immunogenicity.

Streptococcus gordonii is a Gram-positive bacterium, member of the human oral microbiome and has been studied as a vaccine vector for different antigens and and sites of immunization. In this work we studied the transcriptomic response to the vaginal colonization with the Wild-Type or a recombinant *S. gordonii* expressing the CTH522 protein, a multivalent antigen composed of regions of the major outer membrane protein (MOMP) of *Chlamydia trachomatis*.

Combining the intravaginal immunizations and a subcutaneous boost with the CTH522 protein, we had access to the systemic responses through the transcriptomic analysis of the splenocytes from mice primed with either the WT or recombinant strain. The priming with the recombinant bacteria modulated different biological processes, including the activity of IL-1 and IL-2 signaling networks, the activation of transcription factors and T cell modules and the expression of genes like Ccl3. Moreover, a signature of genes correlated with the antibody response was identified, implicating the Interferon type I pathway, the cell cycle activity and genes like Ccl3 and Il18bp.

Introduction

Streptococcus gordonii is a Gram-positive bacterium and a member of the human oral microbiome, that can be genetically manipulated to express heterologous antigens based on chromosomal integration of a donor construct (1), and used as a vaccine delivery vector. We have previously shown that *S. gordonii* vectors expressing various antigens, and delivered in mucosal tissues, could protect from lethal toxin challenge (2) and activate different immune compartments such as antibody production and T-cell proliferation (3–9). In vaginal delivery to both mice and non-human primates, recombinant *S. gordonii* vectors successfully colonized the vaginal tract, induced antibody production both locally and systemically and resolved vaginal yeast infection by *Candida albicans* (10–12). *S. gordonii* vector was also found to be safe in a phase I clinical trial when administered nasally (13).

The ability of mucosal vaccination or infection to stimulate a systemic immune response is greatly dependent on the type of mucosal tissue (14). The vaginal mucosa is unique in its characteristics as it contains both type I and type II mucosal tissues, with their respective immunological features (15). Numerous studies have demonstrated that vaginal immunization can induce systemic cellular and humoral responses (16–20). However, despite these features it remains an underexplored route of immunization compared to other mucosal tissues such as the nasal and oral, due to their presence in both sexes, the simplicity of administration and decades of experience with pharmaceuticals delivery (21). Additionally, the vaginal tract is considered a complex site for antigen processing and requires adequate adjuvants (22–25).

Given the complexity of this route of immunization, new approaches may be useful for better understanding the response generated after vaccination. Systems Vaccinology presents itself as a possibility to assess the complexity behind the immune mechanisms that lead to protection (26). Different vaccines had their responses characterized through this approach, including Influenza (27,28), Yellow Fever (29) and Ebola (30,31).

In the context of mucosal vaccines, Systems Vaccinology has been pointed as promising to support the search for correlates of protection and provide important insights into the underlying mechanisms driving protective immunity (26). Vaccines against Tuberculosis (32), Influenza (33) and enteric diseases such as cholera and enterotoxigenic *Escherichia coli* (34), have recently had important mechanisms elucidated thanks to this approach. Moreover, important advances in understanding the mechanisms behind protection against simian immunodeficiency virus (SIV) infection were also achieved. This systems approach allowed the identification of early blood transcriptional signatures that correlate with antigen-specific antibody responses in vaginal secretions (35), and has contributed to the identification of synergic factors between host restriction factors and vaccine-induced immune responses (36).

Widely used in Systems Biology studies, transcriptomics offers the possibility of following and comparing the gene expression in various conditions, allowing the study of the biological processes after vaccination. In the present work, we examined the *in vitro* transcriptomic signature observed in splenocytes harvested from mice intravaginally immunized with either WT or recombinant *S. gordonii* strain expressing the *Chlamydia trachomatis* (C.t) multivalent MOMP antigen, CTH522 (37–39), and then boosted with the purified CTH522 protein.

Here, we show that vaginal priming with a recombinant *S. gordonii* expressing on its surface the CTH522 molecule modulates the transcriptomic response of splenocytes stimulated *in vitro* with the CTH522 protein. Additionally, we identified a gene signature correlated with anti-CTH522 IgG levels. Lastly, we demonstrate that the boosting schedule, at three or six months after the priming, influences the immune modules activated upon antigen reencounter. Our analysis showed that vaginal colonization with recombinant *S. gordonii* induced persistent and noticeable changes in the transcriptomic response of *in vitro* stimulated splenocytes to the antigen.

Materials and Methods

Mice

Seven-weeks old female BALB/C mice from Charles River (Lecco, Italy) were housed under specific pathogen-free conditions in the animal facility of the Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Department of Medical Biotechnologies at University of Siena, and treated according to national guidelines (Decreto Legislativo 26/2014). Experiments were planned and conducted utilizing the three R's principles (Reduce, Replace and Refine), which included environmental enrichment and nesting, veterinary oversight, numbers reflecting statistical significance, and the use of anesthesia followed by cervical dislocation for the sacrifice. All animal studies were approved by the Ethics Committee "Comitato Etico Locale dell'Azienda Ospedaliera Universitaria Senese" and the Italian Ministry of Health (number 1004/2015-PR on September 22, 2015).

Experimental Design

Mice were intravaginally (IVAG) primed three times on weeks 0, 1 and 2 with either Wild-Type (GP1295) or recombinant (FR368) *S. gordonii*. Three or six-months after the priming, mice were subcutaneously boosted with 5µg of purified unadjuvanted CTH522 protein (**Figure 1**). The transcriptomic response was characterized 10 days after boosting in *in vitro* stimulated splenocytes, while the induction of CTH522- specific IgG serum response was evaluated at the same time point on serum samples.

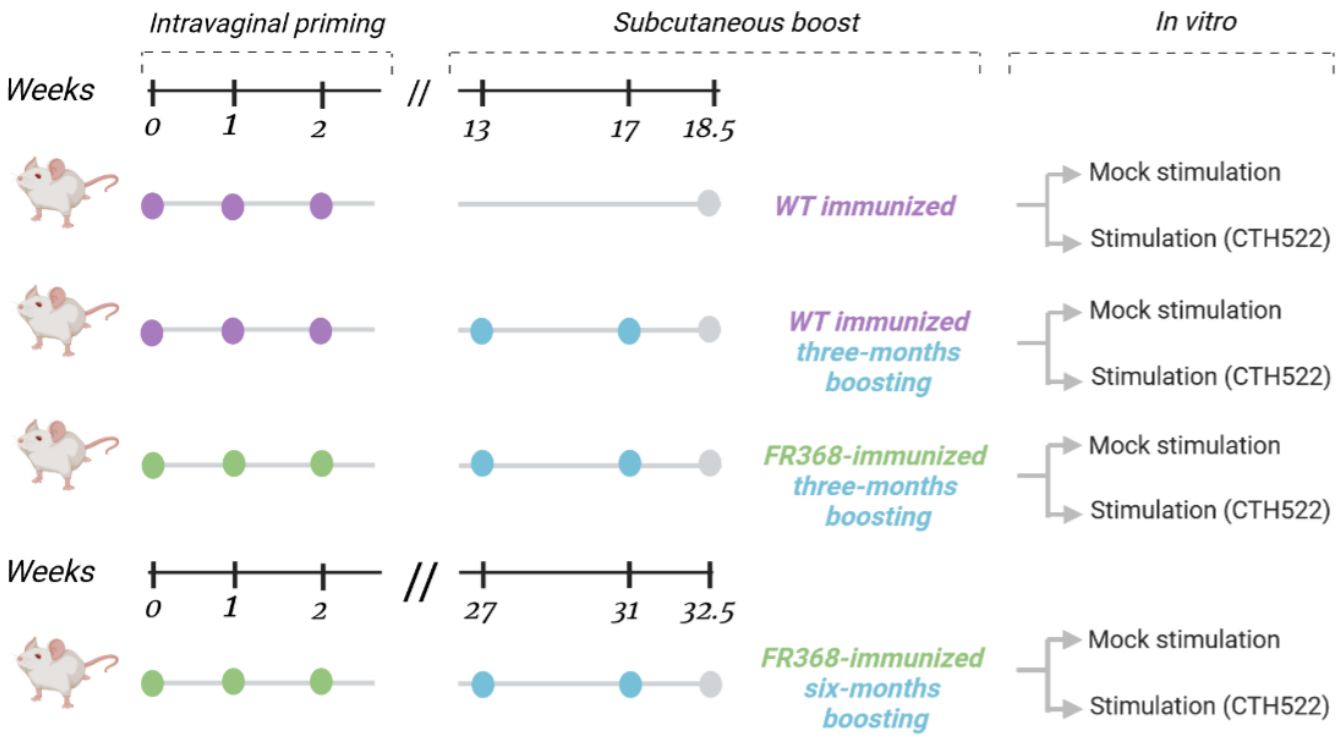

Figure 1 | Experimental Design. Groups of 6 mice were intravaginally primed with Wild-Type (WT) or recombinant (FR368) *S. gordonii.* Three primings were performed on weeks 0, 1 and 2. For the boosted groups, subcutaneous administrations of the CTH522 protein were performed on weeks 13 and 17 for the three-months boosting schedule and on weeks 27 and 31 for the six-months boosting schedule. Spleens and blood were collected 10 days after the final boost and splenocytes were seeded in the presence (Stimulation) or absence (Mock-stimulation) of the purified CTH522. Blood samples were used for the assessment of IgG response by ELISA.

Immunizations

Following estrous cycle synchronization with subcutaneously delivered 0.1 mg of β-estradiol 17-valerate (#E1631, Sigma-Aldrich) resuspended in ethanol and diluted in olive oil, mice were immunized three times on weeks 0, 1, and 2 by the intravaginal route (IVAG) with 10^9 CFU in a volume of 20μL PBS of either Wild-Type (GP1295) or recombinant (FR368) *S. gordonii* bacterial vector expressing the vaccine antigen CTH522 (Philosof et al. 2022, unpublished). On weeks 13 and 17 (three-months boosting) or 27 and 31 (six-months boosting), mice were subcutaneously boosted with either CTH522 protein (5μg/mouse) administered in a volume of 100μl/mouse in NaCl 0.9% (Fresenius Kabi, Italy) or Saline (NaCl 0.9%). Mice were sacrificed ten days after the second boost (week 18.5 or 32.5).

Sample collection and cell preparation

Blood samples were taken from individual mice by cardiac puncture at day ten post second boost upon sacrifice. Samples were incubated for 30 min at room temperature and then centrifuged at 1,200 x *g* for 10 min. Sera were collected and stored at -20°C until analysis by ELISA. Spleens were mashed onto 70 µm nylon screens (Sefar Italia, Italy) and washed two times in RPMI medium (#BE12-167F, Lonza, Belgium) supplemented with 100 U/ml penicillin/streptomycin (#P0781, Sigma-Aldrich) and 10% fetal bovine serum (#10082, Gibco, USA). Samples were treated with red blood cells lysis buffer according to manufacturer instruction (#00-4300-54, eBioscience, USA) and quantified.

ELISA

Serum CTH522-specific IgG levels were determined by enzyme-linked immunosorbent assay (ELISA). Flat bottomed Maxisorp microtitre plates (Nunc, Denmark) were coated with CTH522 (1 µg/ml) overnight at 4°C in a volume of 100µl/well. Plates were washed and blocked with 200µl/well of PBS containing 1% BSA (Sigma-Aldrich) for 2 hours at 37°C. Serum samples were added and titrated in three to five-fold dilutions in 100 µl/well diluent buffer. After incubation for 2 hours at 37°C samples were washed and incubated with the Alkaline-Phosphatase-conjugated goat anti-mouse IgGs (IgG1 #1070-04, IgG2a #1080-04, IgG2b #1090-04, Anti-IgG3 #1100-04 diluted 1:1,500 for total IgG in 100 µl/well and developed by adding 200µl/well of 1 mg/ml AP substrate (#P5994, Sigma-Aldrich). The optical density was recorded using Multiskan FC Microplate Photometer (Thermo Scientific). Positive controls were included in all assays as follows: anti-IgG coating (1:1000, #1010-01), IgG standards (#0107-01), anti-MOMP rabbit serum (SSI, Denmark) and anti-rabbit IgG-AP (#4050-04).

Splenocytes *in vitro* stimulation

10^6 splenocytes from each mouse were suspended in 100 µl of cRPMI and plated in a 96-well plate in 5 replicates per mouse per condition. For stimulation, 100 µl of 10 µg/mL CTH522 were added to each well, at a final concentration of 5 µg/mL. In mock samples 100 µl of cRPMI were

added. Cells were incubated for 6 hours at 37°C with 5% CO_2. Replicates were pooled down together in a same-sample same-condition manner, centrifuged for 10 min at 500g 4°C and supernatant was discarded. Cell pellets were resuspended in 50 µl lysis buffer RA1 (#740955 NucleoSpin kit, Machery-Nagel) and flash frozen in liquid nitrogen. Samples were stored in -80°C until library preparation. RNA was extracted from frozen samples (#740955 NucleoSpin kit, Machery-Nagel), quantified using Qubit RNA quantification kit per manufacturer's instructions and Quality controlled using Agilent Bioanalyzer RNA 6000 nano kit (#5067-1511, Agilent) per manufacturer's instructions.

Illumina sequencing

Libraries were prepared using Stranded mRNA prep kit (Illumina, USA) according to manufacturer's instructions, using 60 ng of total RNA input per sample with dual indexing. Pooled libraries were sequenced on a single run of an Illumina NovaSeq 6000 instrument with 100 bp single end reads. Base calling was performed using Illumina's basespace FASTQ Generation pipeline. Basecalled reads were transferred on a local server and trimmed with Trimmomatic to remove low quality bases (Q treshold=20) at the beginning of the read and within the read using a sliding window size of 5 nucleotides with a required quality of 4. Trimmed reads under 36 nucleotides in length were discarded. Trimmed reads were aligned to the mouse reference transcriptome using STAR and reads were counted using HTSeq.

Data Analysis

All the transcriptomics data analyses were carried out in R software, version 4.1.2 (2021-11-01), running under Windows 10 x64. Scripts can be found on Github at the following link: https://github.com/IsaMoscardini/VacPath/tree/main/Src.

Low expressed genes were filtered out and counts were normalized by log2CPM (40) to assess data variability by Principal Component Analysis (PCA), performed using the mixOmics package (41). Differential Expression Analysis was performed using the DESeq2 package (42). Genes with an adjusted p value less than 0.05 were considered Differentially Expressed (DEG). The *Wild-Type*

immunized no boosting Mock-stimulated group was used as a baseline for the overall comparison between groups and the *Wild-type three-months boosted stimulated* group was used as baseline for the comparison with the *FR368-primed three- and six-months boosting stimulated* schedules.

Mouse Ensembl genes were converted into human gene Symbol (HGNC) by biomaRt (43,44) and enrichment analysis was carried out using the Blood Transcription Modules (BTM) database (45) and the CERNO test from the tmod package (46), using genes ranked by the adjusted p-value.

Correlation analysis was performed in log2CPM normalized data, after filtering low expressed genes. Gene expression values were correlated with the log2 of IgG titers using Spearman correlation. The Uniprot database (47), the web tool EnrichR (48,49), and the MSigDB gene set database (50,51) were used to access biological information about these genes.

Results

Data variability

Principal Component Analysis (PCA) was performed to assess intra- and inter-group variability (**Figure 2**). The baseline group (WT saline mock-stimulated) presented a small intra-group variability, clustering together (in grey). The other immunization schedules presented a higher variability and samples had higher dispersion. The *in vitro* stimulation led samples to spread towards the lower part of the graph, driven by genes negatively correlated to the second component, including Irf8, Socs3, Stat3, Socs1 and Il21 (data not shown). No outliers were detected.

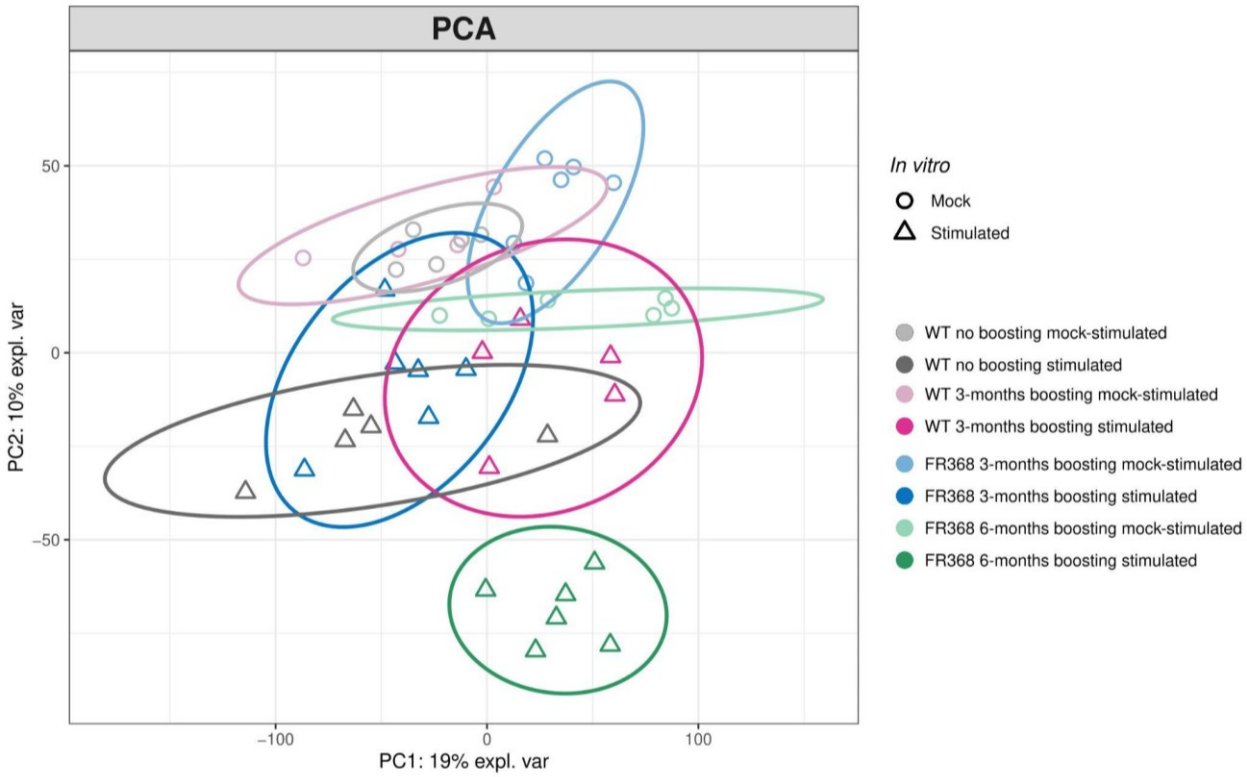

Figure 2 | Principal Component Analysis. To evaluate gene expression data distribution and the presence of possible outliers, PCA analysis was performed by mixOmics package, using the normalized expression values. 19% of the total variance is explained by the first component and 10% by the second component, which seems important for the distinction between stimulated and mock-stimulated samples.

***In vitro* stimulation with the purified CTH522 generates a strong signal and provides insight into the host's response to different immunization schemes.**

To understand the transcriptomic changes driven by each *in vivo* immunization scheme and by the *in vitro* stimulation process, we performed a Differential Expression (DE) analysis with the DESeq2 package, using the *Wild-Type no boosting mock-stimulated* group as baseline. The total number of differentially expressed genes (DEGs) for each group is provided in **Table 1**.

Immunization Schedule	Intravaginal priming	Boost (time after priming)	In vitro Stimulation	Up-regulated genes	Down-regulated genes
WT no boosting stimulated	Wild-Type	No	Yes	379	308
WT 3-months boosting mock-stimulated	Wild-Type	Yes (3 months)	No	0	0
WT 3-months boosting stimulated	Wild-Type	Yes (3 months)	Yes	345	185
FR368 3-months boosting mock-stimulated	Recombinant FR368	Yes (3 months)	No	131	82
FR368 3-months boosting stimulated	Recombinant FR368	Yes (3 months)	Yes	243	64
FR368 6-months boosting mock-stimulated	Recombinant FR368	Yes (6 months)	No	782	352
FR368 6-months boosting stimulated	Recombinant FR368	Yes (6 months)	Yes	1387	1284

Table 1. The number of Differentially Expressed Genes (DEGs) for each immunization schedule. Differential Expression Analysis was performed using the DESeq2 package and genes with an adjusted p-value of less than 0.05 were considered differentially expressed. The WT no boosting mock-stimulated group was used as baseline.

The *in vitro* stimulation process leads to a transcriptomic perturbation that is slightly higher in samples that never encountered the antigen before (WT no boosting stimulated) compared to the WT and FR368 three-months boosting schedules. The exception is the FR368 six-months boosting schedule, which presents a higher number of differentially expressed genes compared to the other groups. These DEGs are mainly related to metabolism, especially glucose metabolism, and in a smaller number to the interferon pathway (data not shown).

Following the DE analysis, Gene Set analysis was performed using a Functional Class Scoring method and the Blood Transcription Modules (BTM) database. A strong signal was generated in *in vitro* stimulated samples, regardless of the immunization scheme, characterized by the up-regulation of DNA and viral sensing, innate antiviral pathways, and Interferon and chemokines responses (**Figure 3**). Although these modules were shared, differences between groups were observed. For example, the activation of the *proinflammatory cytokines and chemokines* module was stronger in the

FR368-immunized mice subjected to the three-months boosting schedule, owing to a higher fold-change of genes like TNF, CCL3 and CCRL2. On the other hand, the *signaling in T cells (I)* module was stronger in the FR368-immunized mice in the six-months boosting schedule, driven by genes differentially expressed only in this condition, such as IFNG, EGR1, JUNB, PRF1 and TNFRSF9.

In addition to the observed common signature, the stimulation process allowed the identification of specific modules enriched only in the FR368-immunized groups. Both three- and six-months boosting schedules activated modules linked to T cells, cell cycle, putative targets of PAX3 and the AP-1 transcription factor network. Specific DEGs for these two immunization schedules include CD83, ATF3, LEF1 and CCL3. Moreover, modules related to dendritic cells and monocytes were specifically activated in the three-months boosting schedule, by unique DEGs such as TNFRSF1B, GRINA, SLC7A11 and IL36G. On the other hand, the *cytokines − receptors cluster* and different T cell modules were unique to the six-months boosting schedule, enriched by DEGs specific for this immunization schedule, like CSF2, GZMA, GATA3, IL12RB1 and NLRC3.

The mock-stimulated groups did not bring relevant biological information, highlighting the importance of the *in vitro* stimulation process for assessing responses through DE and Gene Set Analyses.

Figure 3 | Gene Set Enrichment Analysis. The significance of the activation of each Blood Transcription Module for each group was assessed through a multivariate enrichment analysis. A strong antiviral response is detected in the *in vitro* stimulated groups, while specific signatures distinguish different immunization schedules, such as the FR368-primed groups.

Key transcriptional differences in response to *in vitro* stimulation in splenocytes from recombinant or WT-immunized mice

To further explore the transcriptional differences driven by the priming with the recombinant *S. gordonii*, a distinct DE analysis was carried out comparing *in vitro* stimulated samples from the FR368-immunized three-months and six-months boosting schedules to the WT-immunized group. In total, 46 differentially expressed genes were found in common between the two FR368-immunized groups, 16 downregulated, 13 upregulated and 17 genes presenting different directions in each group.

Genes that belong to the IL-1 and IL-2 signaling network, or that are activated by these cytokines, were up-regulated in both schedules, including Il1b, Mapkapk2, Nampt, Peli1, Pfkfb3, Psmb1, Pten and Stk17b. Moreover, the Neutrophil cytosol factor 1 (Ncf1) is described as coexpressed with Il1b and it was also found to be up-regulated.

In fact, after *in vitro* stimulation, all groups showed an increase in the Il1b gene when compared to *WT no boosting mock-stimulated* samples, as seen in the first DE analysis. This increase is not only much more pronounced in FR368-primed animals, but also followed by the increase in other genes related to these pathways,

Correlation between gene expression values and serum IgG titers highlights genes and biological pathways possibly linked to increased antibody production.

To investigate genes whose expression could be linked to the antibody response, normalized gene expression values were correlated with the IgG titers. Since the *in vitro* stimulation led to a strong effect in the gene expression observed in the Gene Set analysis, the correlation with the IgG titers was performed with the gene expression profile of the 17 mock-stimulated samples that had *in vivo* contact with the antigen (WT three-months boosting, FR368 three-months boosting and FR368 six-months boosting). These samples, obtained at the same time point as the measurements of antibody titers, represent a more biologically relevant sample than the stimulated ones for the correlation with serum IgG titers.

The expression of 553 genes was found significantly correlated (p-value < 0.05) with the log2 of the IgG titers in the serum. Looking at the biological function, these genes enriched for pathways related to Myc targets V1 (cell proliferation pathway), interferon alpha response, mTORC1 signaling and IL2/STAT5 signaling, activated mainly by genes negatively correlated with IgG titers (**Figure 4A**). Many of the significantly correlated genes are linked to the interferon pathway, especially type I, as represented in the network of Figure 4B. Genes such as Azi2, Trim25, Pum2 and Eftud2 encode proteins that activate this pathway and were negatively correlated with the total IgG titers, as well as Jak1, a kinase that plays a major role in the interferon signal transduction. On the other hand, genes like Yipf2, which inhibits the cGAS-STING signaling, were positively correlated with the IgG titers, suggesting that indeed the interferon signaling pathway may be less expressed in samples with higher titers.

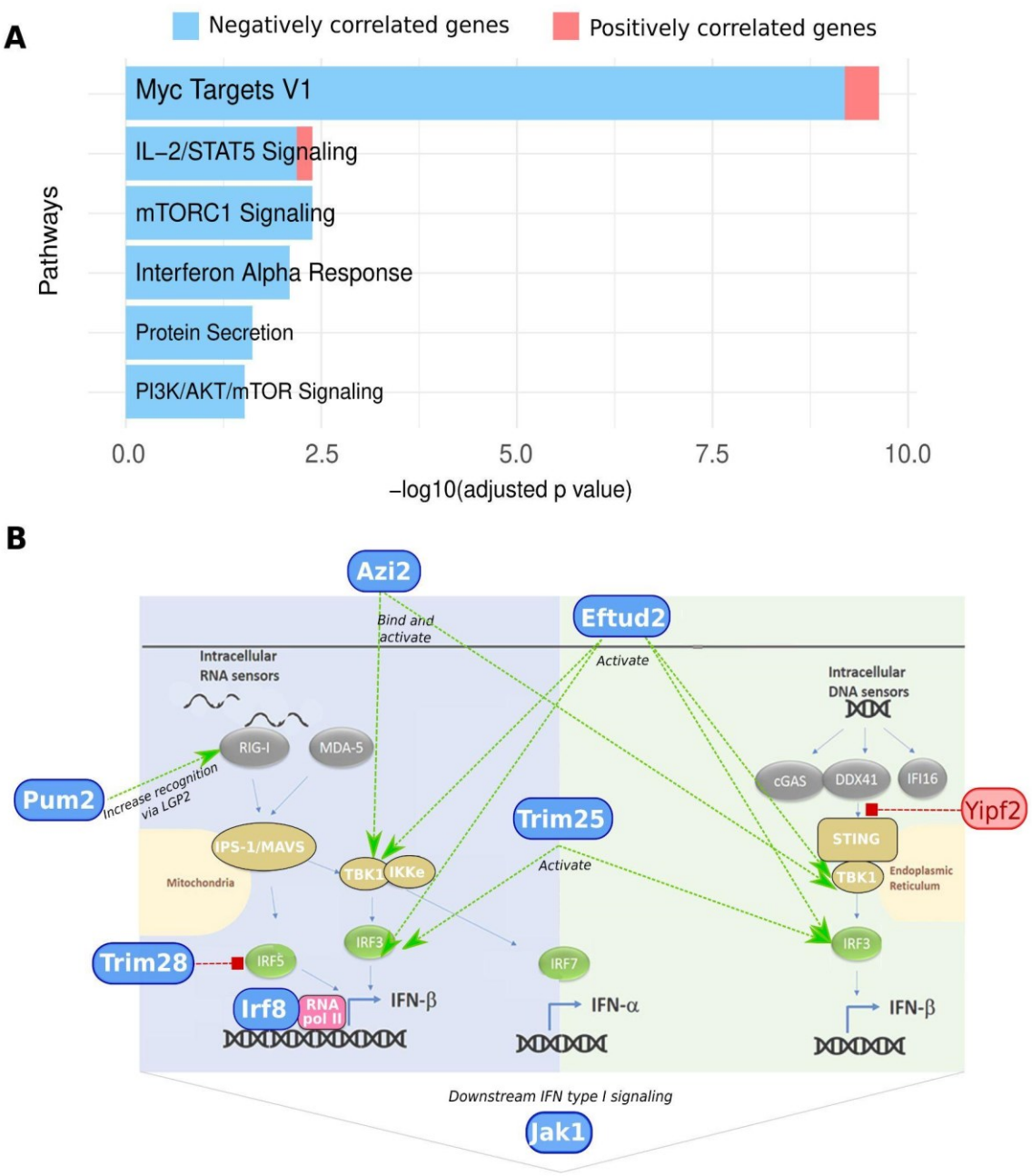

Figure 4 | Correlation of gene expression and serum IgG titers. A. Gene set analysis of the 553 genes significantly correlated with serum IgG levels. Performed with enrichR web tool using the MSigDB Hallmark 2020 database. **B.** Interferon type I activation network. The 7 genes found negatively correlated with the IgG titers are represented in blue while the gene positively correlated with the IgG titers is represented in red. Green arrows indicate activation of the protein/pathway, while the red arrow represents the inhibition of the protein/pathway. The figure was adapted from Jefferies CA, 2019 (52). The link between the genes and the Interferon pathways was found in previously published works (52–60).

Figure 5A displays the barplot indicating the log2 of the IgG titers for each sample, colored by immunization groups and a heatmap with z-score of normalized expression values for the 50 genes most significantly correlated with IgG titers. Among the top negatively correlated features, there are genes involved in the interferon type I network, such as Azi2, Phb2 and Eftud2, but also Anp32b, that might participate in the regulation of adaptive immune responses. On the other hand, among the top positive correlated genes, there are Ccl3 (an important chemoattractant), Il18bp (the encoder of the natural antagonist of IL-18) and Pi4kb (involved in Golgi-to-plasma membrane trafficking).

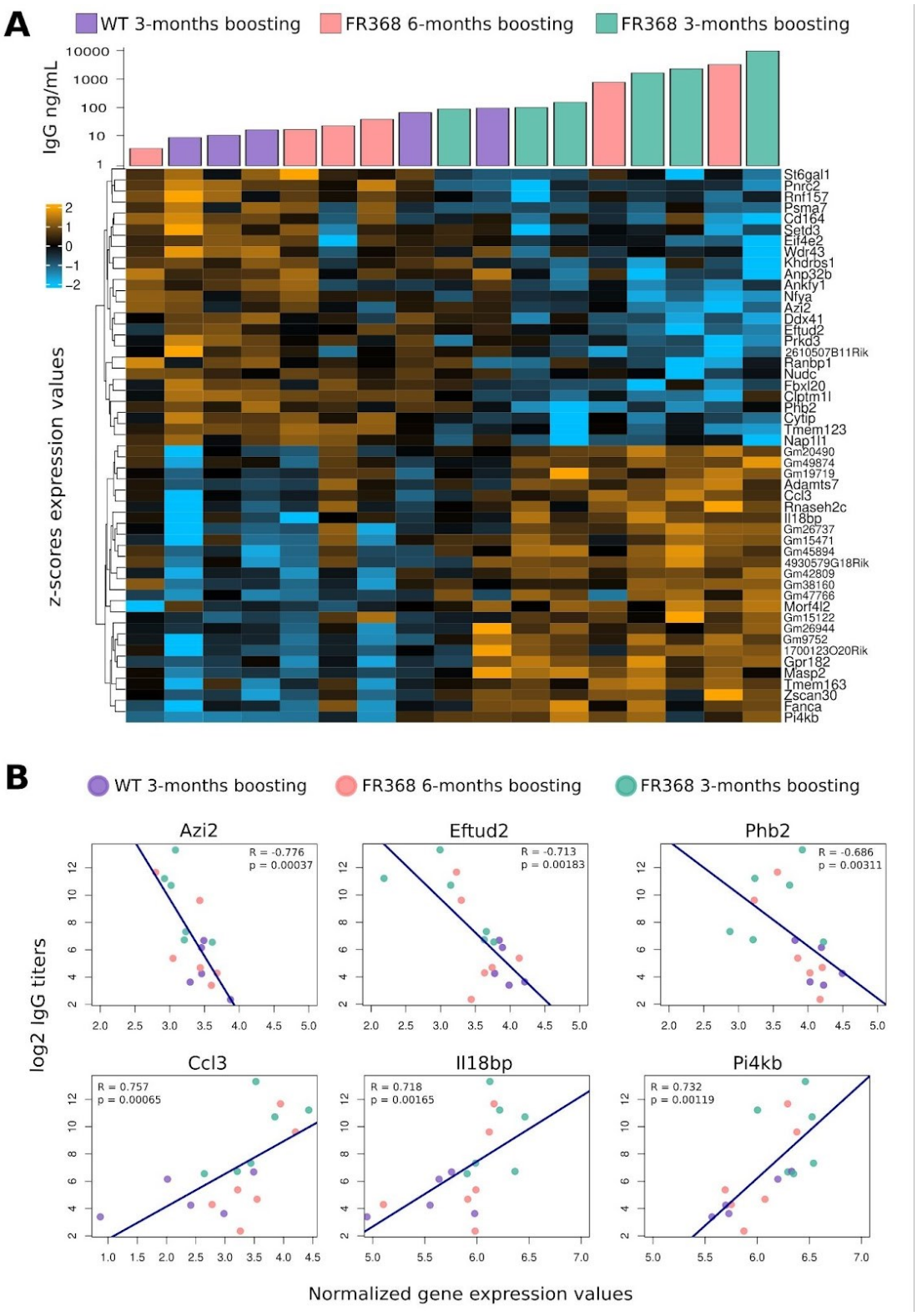

Figure 5 | Top 50 genes correlated with serum IgG titers. A. Barplot displaying the log2 of IgG titers in the serum, bars are colored by immunization schedule, followed by the heatmap representing the z-score of the normalized expression values of the 50 genes with the lowest p-values for the correlation with IgG. **B.** Dot plots for 6 of the top correlated genes, displaying the normalized expression value on the x-axis and the log2 of the IgG titers on the y-axis. Dots are colored by immunization schedule, as the bars in panel A.

Discussion

In this work, we leverage transcriptomic analysis to study the host's response to different immunization schedules. Mice were intravaginally primed with either Wild-Type or a recombinant *S. gordonii* expressing the CTH522 protein, a multivalent chlamydial antigen containing regions of MOMP from *C. trachomatis* serovars D, E, F and G found to be safe and immunogenic in a phase I clinical trial (39), and then received two subcutaneous boosts of the unadjuvanted CTH522 protein. Splenocytes were collected 10 days after the final boost and seeded in the presence or absence of the CTH522 protein. The *in vitro* stimulation process is important to characterize the response generated by a local infection (61). In the present study, we took advantage of this method to assess the systemic response generated by the intravaginal priming and the subsequent subcutaneous boosts.

The stimulation process allowed us to retrieve information on the differences between intravaginally priming mice with the wild-type (WT) or with the recombinant bacteria expressing the *C. trachomatis* antigen (FR368). In the enrichment analysis, compared to the baseline group, the T cell signaling pathway was significantly activated only in FR368-primed samples, as well as the AP-1 transcription factor network module. The AP-1 transcription factor network is involved in different bacterial and viral infections (62,63), including in *Chlamydia trachomatis* (64) and *Chlamydia pneumoniae* (65) infections, in the latter case being a key factor of the host's response, regulating inflammatory mediators like IL6, IL8, and IFN.

Differences between the FR368-primed and the WT-primed groups after stimulation have also emphasized the role of the IL-1 and IL-2 signaling pathways in the response generated by different priming schedules. IL-1 is commonly mentioned for its roles in innate immunity and inflammation, however, this cytokine plays essential roles in the bridging of adaptive responses (66). IL-1 has been shown to improve the differentiation of naive T cells through the regulation of DC activation (67–69) and to enhance the persistence and response of memory cells when administered together with the antigen (70). In addition, IL-1 is known for its role in favoring the differentiation of CD4+ T cells during priming, acting as a driver of Th17 responses - a key mediator of mucosal immunity (71).

Therefore, the increase in this pathway after stimulation of FR368-primed samples could indicate an important mechanism of this immunization schedule.

The Interferon type I signaling network was negatively correlated with the IgG titers in the mock-stimulated samples. However, whether the antigen encounter *in vivo* upon subcutaneous boosting leads to an activation of the interferon pathways, as seen in the *in vitro* stimulated samples, requires further investigations. Since our samples were collected 10 days after the final boost, the detected transcriptomic responses are probably related to downstream processes occurring as a consequence of the boosting and may not be tightly linked to the response to the CTH522 protein itself.

The Ccl3 gene was differentially expressed after stimulation only in FR368-primed samples, both in three- and six-months boosting schedules. Interestingly, this gene was also positively correlated with the serum IgG. Ccl3, also known as macrophage inflammatory protein 1 alpha (MIP-1α), is an important chemoattractant secreted by various cells, including fibroblasts, epithelial cells, lymphocytes, resident and recruited monocytes, and macrophages (72,73). Besides acting as an attractant for immune cells like monocytes (74,75) and natural killer cells (76), CCL3 has a well described role in the migration of dendritic cells (77–79) and lymphocytes (80).

In a study examining the immunogenicity of an adenovirus-based vaccine vector, co-expression of CCL3 with the retroviral antigens increased vaccine protection from infection by enhancing neutralizing antibody titers and virus-specific CD4+ T cell responses (81). Indeed, previous studies suggest that CCL3 can enhance humoral and cellular responses in both mucosal and systemic immunity. A study comparing the nasal administration of Chicken egg albumin (OVA) in the presence or absence of CCL3 found enhanced systemic antibody responses marked by higher levels of IgM and all the IgG subtypes. The CD4+ T cells in Peyer patches, cervical lymph nodes and spleens of mice immunized in the presence of CCL3 exhibited marked increases in OVA-specific proliferative responses. Moreover, CCL3 promoted mucosal and systemic CD8+ CTL responses (82).

It has been suggested that CCL3 is an important cytokine for sustaining and amplifying a previously primed T-cell response (83). Our data suggest that intravaginal immunization with the recombinant bacteria expressing the CTH522 antigen induces Ccl3 expression upon antigen reencounter through *in vitro* stimulation.

Taken together, our data suggest that the *in vivo* site and context of antigen encounter modulate the transcriptomic signature of *in vitro* stimulated splenocytes. We demonstrated a differential activation of inflammatory pathways genes, which was associated with higher systemic antibody response. Moreover, we have shown that Ccl3 is a marker of the recall responses in mice primed with the recombinant *S. gordonii,* and it is associated with the IgG titers after immunization, being a possible biomarker of vaccine response.

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Chapter 5 - Conclusions

The study of *in vivo* immune priming is of significant value for the development of new vaccination strategies and to the efforts to optimize existing prime-boost regiments. In the present work we characterized the ability of a recombinant *Streptococcus gordonii* vaccine vector, named FR368 and delivered intravaginally, to prime a systemic immune response.

To study the priming effect induced by our vector, we applied a heterologous prime-boost design in which mice were vaginally primed by either the WT or FR368, and then boosted subcutaneously with the purified protein expressed on the surface of the FR368. This model allows to identify the extent of immune priming induced by the encounter of the antigen on the surface of the *S. gordonii* vector, delivered mucosally, compared to the immunogenicity of the soluble antigen alone. Additionally, applying two different boosting schedules enabled us to characterize the durability of the priming effect. Our data show that this prime-boost model is a useful tool to dissect the extent of systemic immune priming by a mucosally administered bacterial vaccine vector.

The vaginal immunization with the FR368 alone triggered the production of antigen-specific serum IgG. Following boosting with the purified protein, the production of serum antigen-specific IgG has rapidly and significantly increased in FR368-immunized mice. In line with that, higher numbers of antigen-specific antibody-secreting B cells (ASCs) were observed the spleens of FR368-immunized mice, together with an increase in the presence of plasma cells ($TACI^{+}$ $CD138^{+}$) in the draining lymph nodes (Chapter 3). These findings are important as the systemic production of antibodies is the principal measurement of most vaccine formulations (75).

Our observation that no significant differences were observed in terms of IgG production, ASCs and plasma cells between the three- and the six-months boosting schedules of FR368-immunized mice demonstrates that the priming effect is long lasting and potent (Chapter 3). These findings are of importance since the longevity of the priming induced by a vaccination, or the waning of the immune response gained, are the focus of current vaccine studies (76–79), and efforts to identify long-lasting heterologous prime-boost strategies are underway.

We have also identified that vaginal priming with FR368 induced a shift in the cytokines expression profile of antigen-specific $CD4^+$ T when stimulated *in vitro* with the purified protein. This shift was dominated by an increase in IL-17 and a decrease in pro-inflammatory cytokines IFN_γ, IL-2 and TNF-α, and these observations were similar in both boosting schedules. The profile of cytokines secreted by activated $CD4^+$ T-cells is central to the understanding of the type of immune response activated in reaction to the antigen. Our data suggest that the first encounter of the immune system with the antigen on the surface of the *S. gordonii* vector induced a differential activation profile, in which mucosal association is evident and inflammation is reduced, while maintaining high levels of antibodies production (Chapter 3). These findings are important to the efforts to develop vaccination strategies that result in an enhanced immune response while avoiding excessive inflammation.

We hypothesized that the differences in IgG levels and cytokines profile was due to activation of different immune pathways in response to the antigen. To that end, we analyzed the *in vitro* gene expression profile in splenocytes from WT- or FR368-immunized mice in response to the CTH522 antigen (Chapter 4). We observed a higher activation of *pro-inflammatory cytokines and chemokines* module in the FR368-immunized mice undergoing the three-months boosting schedule, while the *signaling in T cells (I)* module was stronger in the FR368-immunized mice undergoing the six-months boosting schedule. In addition, FR368-immunized mice exclusively exhibited increased expression of *Ccl3*, an important chemoattractant. These findings may assist in the search for correlates of efficacy in vaccine-induced immune response (Chapter 3).

We also observed a gene signature correlated with antibody titers. Within this signature, interferon-related genes were negatively correlated with higher antibody levels in the serum (Chapter 4). These findings suggest a novel view on the correlation between interferon signaling and antibodies production, which are traditionally positively correlated. In the context of vaccine development this may assist in developing vaccines that elicit high antibodies production without inducing systemic inflammation.

In conclusion, this work expands the knowledge of the priming effect induced by mucosal immunization with a recombinant *S. gordonii*, and has important implications for the future design of new prime-boost vaccination strategies.

Chapter 6 - References

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Appendices
A
Curriculum vitae di Bar Philosof

EDUCATION

PhD in Medical Biotechnologies | University of Siena, Department of Medical Biotechnology — 2019 – present, Italy

- Marie Sklodowska-Curie PhD candidate
- Awarded the prestigious European Commission's Marie Sklodowska-Curie Doctoral Scholarship (10 students among two hundred applicants) to carry out research as part of the VacPath consortium to develop novel vaccine delivery vectors.

M.Sc. in Neuroscience | Tel Aviv University, Sagol School of Neuroscience — 2016 – 2018, Israel

- Full Scholarship
- GPA in M.Sc.: 93.86

The Adi Lautman Interdisciplinary Program For Outstanding Students | Tel Aviv University — 2014 – 2018, Israel

- GPA in the Programme: 92
- Awarded a competitive full Scholarship (15 students among hundreds of applicants annually) to attend this unique four-year program that offers a highly interdisciplinary curriculum from the first year of undergraduate studies through to graduation with an MSc or MA degree.

International Baccalaureate Diploma | United World College of the Adriatic (UWC) — 2006 – 2008, Italy

Won a full scholarship to UWC, an educational movement of 18 schools in 4 continents, which brings together excelling students aged 16-19 from 155 countries to live and study together for two years, thereby building inter-cultural education, bridges, and leadership capacity towards meaningful social impact.

SCIENTIFIC RESEARCH EXPERIENCE

Immune system priming by an engineered *S. gordonii* vector expressing heterologous antigen — 2019 - Present

PhD.; Advisor: Prof. Gianni Pozzi | University of Siena

- Investigating the ability of an engineered recombinant bacteria delivered mucosally to induce immune priming against a vaccine antigen in an immunocompetent *in-vivo* models.
- Characterization of the immune response using Flow-Cytometry, ELISA, and transcriptomic analyses.

The Role of Astrocytes in Glioma Progression — 2016 - 2018

M.Sc.; Advisor: Dr. Lior Mayo | Tel Aviv University

- Investigated of the role of astrocytes in the progression of Glioblastoma Multiforme (GBM) in mice using immunocompetent *in-vivo* models.
- Characterization of the tumor's microenvironment using Bioluminescence, Flow-Cytometry, and transcriptomic analysis.

2016

The role of RAI14 in promoting Glioblastoma proliferation

Undergraduate research assistant; Advisor: Prof. Marcello Ehrlich | Tel Aviv University

- Investigated the influence of RAI14 on U-87 human tumor cell line using proliferation assays.
- Western-blot analysis of the influence of RAI14 on the activation of the JNK pathway.

Reducing cancer metastases in two murine models using TLR-4 and TLR-9 immunostimulators and attenuation of stress-induced immunosuppression 2016

Undergraduate research assistant; Advisor: Prof. Shamgar Ben-Eliyahu | Tel Aviv University

- Investigated the influence of a prolonged stress exposure on the efficacy of immune stimulation.

PUBLICATIONS

- Perelroizen, R*., Philosof, B*., et al (in press). Astrocyte immunometabolic regulation of the tumour microenvironment drives glioblastoma pathogenicity. **Brain** (2022) doi: 10.1093/brain/awac222

- Philosof, B., et al. (2022). Vaginal immunization by the human microbiota bacterium *Streptococcus gordonii* expressing a *Chlamydia trachomatis* antigen efficiently primes the immune system in mice. **In preparation**.

RESEARCH SKILLS & TECHNIQUES

❖ *In-vivo* mouse models of tumor development and bacterial infection	❖ Mammalian and bacterial cultures
❖ Murine tissues cells isolation: CNS, lymph nodes, spleen, blood, vaginal tract	❖ Bacterial isolation from tissues
❖ Flow cytometry analysis and FACS: designing panels, compensation and FlowJo analysis	❖ Bacterial genome sequencing using Oxford Nanopore
❖ ELISA and Bioplex	❖ Molecular Cloning, Viral vectors construction, Gel electrophoresis, Bacterial transformation, and Plasmids editing
❖ Isolation of RNA and transcriptomic analysis by qPCR and Illumina mRNA sequencing	❖ Lentiviral and retroviral production and *in-vivo* and *in-vitro* transfections
❖ Transgenic mice lines establishment, colony maintenance and genotyping	❖ Immunoblotting

PROFESSIONAL EXPERIENCE

Executive Assistant to the CEO | Clariter Recycling and Refining Center SA 2012-2014 Israel

- Supported the executive management of Clariter, a start-up company that has developed chemical patents to convert plastic waste into crude-oil derived products.
- Led and wrote the company's "Social and Community Impact" plan for its Industrial Scale Plant in South Africa.

Board Member | UWC Israel National Committee 2009 – 2014 Israel

- Elected to serve as a member of the UWC Israel National Committee Board for six consecutive years by the UWC Israel Alumni Association. Served as Head of Student Recruitment, Head of Admissions and Head of Student Preparation.
- Established a think-tank comprising experienced UWC alumni, psychologists, and educational advisors, forming the new admissions process.

MILITARY SERVICE

Sergeant, Non-commissioned Officer | Strategic Planning Division, IDF J5 — 2009-2011, Israel

- Full military service in the IDF's principal military cooperation unit.
- Awarded an Excellency Award by the Head of the IDF J5 directorate (2-star general) for Outstanding Performance.

SKILLS AND INTERESTS

- LANGUAGES: **Hebrew** - Mother tongue | **English** - Native speaker | **Italian** - Fluent
- INTERESTS: Science Fiction | Travelling | Football

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