Developing an experimental vaccine against Cryptosporidiosis using an attenuated *Salmonella typhimurium* delivery vector

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Abstract

Human cryptosporidiosis caused by *Cryptosporidium hominis* and *C. parvum* is an important disease causing severe diarrheal symptoms in infants and adults around the world. To date, no curative therapeutics or prophylactic vaccines against cryptosporidiosis exist. A recombinant Gp45 vaccine, developed in our laboratory, has shown to be partially protective against *C.parvum* in mice. However, to develop a more effective vaccine, we propose to use an attenuated *Salmonella typhimurium* model as an oral vaccine delivery vector for the Gp45 recombinant protein. We hypothesize that mucosal immunity, i.e. IgA levels, will be greater following the use of an oral YS1646 vaccine in addition to the intramuscular Gp45 vaccine in comparison with the intramuscular Gp45 vaccine alone.

The overall goal of this M.Sc. was to produce various vaccine candidates against *C.parvum*, assess the expression of Gp45 of each candidate and evaluate the immunogenicity of our vaccine candidates in mice. Three constructs were constructed to express Gp45 in *S. typhimurium* and were transformed in an attenuated strain of *S.typhimurium*. We were successful in creating various plasmid constructs for expression of recombinant Gp45 in *S.typhimurium*. Unfortunately, we did not succeed in demonstrating expression of the recombinant Gp45 in any of our expression constructs. Despite failure to express Gp45 from *Salmonella in vitro*, two of our vaccine candidates were used in a preliminary study in mice. Following an IM/oral vaccination regimen and a 6-week study period, one of our vaccine candidates showed a statistically significant response compared to a negative control and no vaccine candidate produced greater serum IgG or intestinal IgA compared to a single dose of intramuscular Gp45. However, our YS1646 vaccine elicited a strong *S.typhimurium* specific serum IgG and intestinal IgA response suggesting the vaccine vector, and

not the protein of interest, was the target of the murine immune system. Further optimization of our constructs is needed to obtain detectable levels of protein expression *in vitro* and enhance humoral immunity by our oral YS1646 vaccine *in vivo*. This investigation provides a first insight regarding the use of a vaccine vector against cryptosporidiosis.

Résumé

La cryptosporidiose humaine causée par *Cryptosporidium hominis* et *Cryptosporidium parvum* est une maladie importante causant une diarrhée importante chez les enfants ainsi que chez les adultes. À ce jour, il n'existe aucun traitement curatif ou prophylactique contre la cryptosporidiose. Par conséquent, étant donné la forte morbidité reliée à la crypstosporidiose, nous avons cherché à développer un vaccin prophylactique. Un vaccin recombinant Gp45 adjuvé, développé dans notre laboratoire, s'est révélé partiellement protecteur contre *C.parvum* chez la souris. Cependant, pour développer un vaccin plus efficace, nous proposons d'utiliser un modèle atténué de *Salmonella typhimurium* comme vecteur d'administration de vaccin oral pour la protéine recombinante Gp45. Nous émettons l'hypothèse que l'immunité de la muqueuse, c'est-à-dire les taux d'IgA intestinaux, sera supérieure suite à l'utilisation d'un vaccin oral YS1646 en plus du vaccin intramusculaire Gp45.

L'objectif global de cette Maitrise était de produire divers candidats de vaccins contre *C.parvum*, évaluer l'expression de Gp45 de chaque candidat et évaluer l'immunogénicité de nos vaccins candidats chez la souris. Trois plasmides ont été clonés pour exprimer Gp45 dans *S. typhimurium* et ont été transformés dans une souche atténuée de *S. typhimurium*. Nous avons réussi à créer diverses constructions plasmidiques pour l'expression de Gp45 recombinant dans *S.typhimurium*. Malheureusement, nous n'avons pas pu démontrer l'expression de la protéine Gp45 recombinante dans aucun de nos plasmides. Malgré l'impossibilité d'exprimer Gp45 à partir de *S.typhimurium* in vitro, deux de nos candidats de vaccins ont été utilisés dans une étude préliminaire chez la souris. Après un régime de vaccination IM / orale et une période d'étude de 6 semaines, un candidat-vaccin a montré une réponse statistiquement significative comparée à un

contrôle négatif et aucun vaccin n'a produit une IgG sérique ou une IgA intestinale supérieure à une dose unique de Gp45 intramusculaire. Cependant, notre candidate de vaccin YS1646 a induit une forte réponse IgG sérique spécifique de S.typhimurium et une réponse IgA intestinale suggérant que le vecteur, et non la protéine d'intérêt, était la cible du système immunitaire murin. Une optimisation supplémentaire de nos constructions est nécessaire pour obtenir des niveaux détectables d'expression des protéines in vitro et améliorer l'immunité humorale par notre vaccin oral YS1646 in vivo. Ce projet de recherche fournit un premier aperçu concernant l'utilisation d'un vecteur de vaccin contre la cryptosporidiose.

Acknowledgements

Foremost, I would like to thank my supervisor and mentor, Dr. Momar Ndao, for his support and his guidance throughout my Master's. Thank you for pushing me to get involved in all these amazing teaching and training opportunities; it was an amazing networking experience. I will also never forget the hours spent in your office discussing experiments, calling 15 different people for collaborations and drinking Bissap. I would also like to thank Dr. Brian Ward for his great support and guidance in this project. Your incredible wisdom and perspective have greatly helped me in both my academic career and personal life.

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Thank you everyone, et à bientôt!

Contribution of Candidate

The author designed and performed the experiments presented in this thesis under the supervision of Dr. Momar Ndao. The author also performed data analysis and wrote this thesis with editorial contributions from Dr. Momar Ndao. Sections from the literature review of this thesis were adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis" (Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).) This review paper was co-authored by the author and Dr. Karine Sonzogni-Desautels. Dr. Sonzogni-Desautels approved of the review's adaptation in this thesis.

Literature review

Human cryptosporidiosis

Brief history

Cryptosporidiosis is a water-borne disease caused by the protozoan parasite *Cryptosporidium spp*. *Cryptosporidium spp.* are unicellular apicomplexan parasites measuring 2-6μm in diameter [1]. The two main species responsible for disease in humans are *C.parvum* and *C.hominis* [1]. *Cryptoporidium spp.* have been infecting humans for thousands of years, as recent analysis of dried faecal samples from 3,000 years ago and beyond showed that Andean native Americans[2] and ancient Peruvians[3] and Mexicans[4] were infected with *Cryptosporidium*. However, it is only in 1907 that this parasite was discovered and first characterized by Ernest Edward Tyzzer[5]. After Tyzzer's discovery of *Cryptosporidium*, little attention was given to it; it is only in the 1980s, when HIV patients were found to have severe complications when co-infected with *Cryptosporidium spp.*, that the importance of this parasite was determined. The magnitude of the 1989 Swindon & Oxfordshire, UK and 1993 Milwaukee, USA outbreaks brought great public and scientific attention to this parasite and research on *Cryptosporidium spp.* was enhanced[5].

Immunocompetent

Immunocompetent individuals commonly get infected with *Cryptosporidium spp.*[6]. Symptoms in immunocompetent individuals depend widely on age and previous exposure to the parasite, but many infections are asymptomatic and most are self-limited.[6]. Immunocompetent individuals usually recover spontaneously from a transient gastroenteritis characterized by watery diarrhea and abdominal cramps[1,7,8]. Clinical signs in immunocompetent individuals include gastroenteritis-like symptoms affecting the upper gastro-enteric tract, watery diarrhea, abdominal cramps and loss of appetites[9]. In a lower proportion of

infected people, vomiting and fever may present[9]. Symptoms usually clear after 12.7 days, but may last for up to a month[10].

Immunocompromised

Immunocompromised patients, such as HIV/AIDS patients, people under immunosuppressive treatments (cancer patients undergoing chemotherapy or patients with solid-organ transplants), patients with inheritable immunodeficiency syndromes (children with Severe Combined Immunodeficiency Syndrome (SCID)) and infants can develop chronic, severe and even life-threatening clinical signs[1]. AIDS patients were particularly at risk of lethal cryptosporidiosis before the availability of effective antiretroviral therapy [11,12]. Patients with immunodeficiency that are infected with *Cryptosporidium spp.* typically present with much more severe symptoms. Symptoms present in the entire gastro-enteric tract, including the gall bladder and pancreatic duct, and this can lead to complications such as pancreatitis and biliary cirrhosis[9]. Symptoms such as sinusitis have also been observed in HIV patients co-infected with *Cryptosporidium spp.*[13]. Recent advances in antiretroviral therapy (ART) have markedly reduced the risk of cryptosporidiosis in HIV-infected individuals[14,15]. As a result, patients co-infected with HIV and *Cryptosporidium* spp. have much lower mortality rates compared to 15 years ago[16-18].

Children are also a high-risk group for cryptosporidiosis as they can suffer from long-lasting symptoms after *Cryptosporidium spp.* infection. Studies have shown that cryptosporidiosis led to permanent impaired physical fitness and cognitive function in young children, particularly in those who were malnourished[19,20].

Life cycle

Although *Cryptosporidium* was discovered in 1907 and its life cycle was fairly well described early on, the currently accepted life cycle model was established years later in 1986 with the help of electron microscopy. [2]. The life cycle begins with the ingestion of thick-walled oocysts by the host (Figure 1). The oocysts will migrate in the digestive tract to the small intestine where it excysts. Four sporozoites are released in the lumen and infect intestinal epithelial cells. In the apical end of the cells, the sporozoites mature to become trophozoites and form type 1 meront (type 1 schizont), followed by maturation to merozoites[2,5] (Figure 1). Merozoites that become trophozoites form the asexual cycle while those that become type 2 meronts continue to the sexual cycle of the parasite (Figure 1). Type 2 meronts (type 2 schizonts) then become merozoites and can differentiate in either a microgamont or a macrogamont (Figure 1). The macrogamont matures to a zygote while the microgamont necessitates an intermediate microgamete stage. Two types of oocysts are sporulated from the zygote: thin-walled oocysts, which remain in the host and contribute to auto-infection, and thick-walled oocysts, which are released in the faeces and to the environment (Figure 1). Oocysts that are shed from infected hosts can then be ingested by and infect other animals or humans.

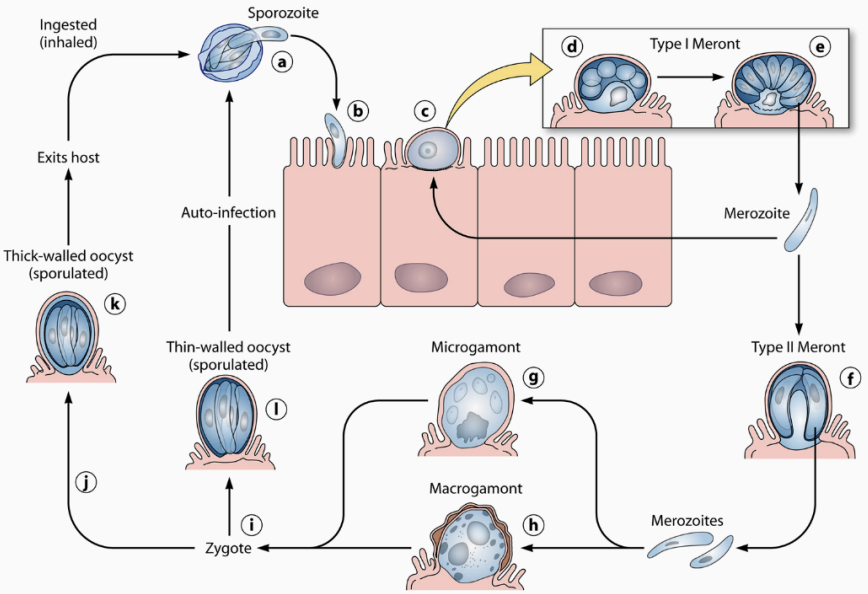


FIG 1 Schematic representation of the *Cryptosporidium parvum* life cycle. After excysting from oocysts in the lumen of the intestine (a), sporozoites (b) penetrate host cells and develop into trophozoites (c) within parasitophorous vacuoles confined to the microvillous region of the mucosal epithelium. Trophozoites undergo asexual division (merogony) (d and e) to form merozoites. After being released from type I meronts, the invasive merozoites enter adjacent host cells to form additional type I meronts or to form type II meronts (f). Type II meronts do not recycle but enter host cells to form the sexual stages, microgamonts (g) and macrogamonts (h). Most of the zygotes (i) formed after the fertilization of the microgamont by the microgametes (released from the microgamont) develop into environmentally resistant, thick-walled oocysts (j) that undergo sporogony to form sporulated oocysts (k) containing four sporozoites. Sporulated oocysts released in feces are the environmentally resistant life cycle forms that transmit the infection from one host to another. A smaller percentage of zygotes (approximately 20%) do not form a thick, two-layered oocyst wall; they have only a unit membrane surrounding the four sporozoites. These thin-walled oocysts (l) represent autoinfective life cycle forms that can maintain the parasite in the host without repeated oral exposure to the thick-walled oocysts present in the environment. (Modified from reference 33 with permission.)

Figure 1: Life cycle of *Cryptosporidium spp*[3]. Source: Bouzid, M., Hunter, P., Chalmers, R. & Tyler, K. Cryptosporidium Pathogenicity and Virulence. *Clin Microbiol Rev* **26,** 115–134 (2013). Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3553671/. Accessed on May 12th, 2018.

Epidemiology

Adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis"

Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).

Among the causes of mortality worldwide, diarrheal-associated deaths are in the top 10 causes of mortality in humans and the fourth leading cause in children under 5 years of age (around 499,000 deaths every year)[4]. Human cryptosporidiosis caused by *Cryptosporidium hominis* and *C. parvum* is the second most common cause (following only rotavirus) of moderate-to-severe diarrhea in 0–11 month-old infants and the third most common in 12–23 month-old toddlers in sub-Saharan Africa and south Asia[21]. For example, in rural Bangladesh, 77% of children less than 2 years old were infected with *Cryptosporidium* spp.[22]. This infection was associated with failure to thrive and impaired cognitive functions in young children in developing countries[22-24]. More worryingly, around 202,000 deaths are attributable to cryptosporidiosis among children younger than 24 months old in sub-Saharan Africa, India, Pakistan, Bangladesh, Nepal and Afghanistan[25]. Among these deaths, around 59,000 are in excess in comparison if these children were *Cryptosporidium* spp.-negative[25]. *C. hominis* was isolated in 77.8% of cryptosporidiosis cases in children in sub-Saharan Africa and south Asia, with *C. parvum* present in 9.9%[25]. *C. parvum*-positive cases can arise from human-to-human transmission[25], but *C. parvum* is a zoonotic protozoan parasite and can also be transmitted from animal hosts to humans[7]. For this reason, veterinary students can sometimes get infected through contact with *C. parvum*-infected calves[26-28]. Also, *C. parvum* oocysts obtained from livestock can contaminate water[29], and *Cryptosporidium* spp. constitute a significant public health concern in developed and developing countries due to its ubiquitous nature[30]. *C. hominis* or *C. parvum* contamination of water can lead to foodborne outbreaks[7] following consumption of fruits and vegetables irrigated or washed with *Cryptosporidium* spp.-contaminated water[31]. Many waterborne outbreaks have been reported in developed countries following contamination with *C. hominis* or *C. parvum* oocysts of drinking (untreated surface water, water-treatment limitations, water-testing limitations, etc.) or recreational water (swimming pools, etc.)[32]. As mentioned above, one of the most important waterborne outbreaks happened in Milwaukee, USA in 1993, in which 403,000 people showed symptoms of watery diarrhea following *Cryptosporidium* spp. infection[8,32] and many immunocompromised people died of the infection[33]. While the source of the infection is still debated[34], filtration system of Milwaukee's water treatment plants was deficient and did not remove all oocysts from the treated water[8]. This outbreak cost

USD 64.6 million in lost productivity and USD 31.7 million in medical costs[35]. More recent outbreaks of cryptosporidiosis, although not as sizeable compared to Milwaukee, have been reported in the past years. Swimming pools and water parks with appropriate operating standards were found to be contaminated with *Cryptosporidium spp.* and outbreak size varied from 12 to 358 reported cases[36]. Another recent outbreak reported in April of 2018 in the La Crosse County in Wisconsin is a testimony of the timelessness of this parasite[37].

From a veterinary point of view, *Cryptosporidium* spp. infect a variety of mammals, including calves, dogs, cats, rabbits and birds [[32,38]]. Bovine cryptosporidiosis caused by *C. parvum* is a major problem in the dairy industry because infection is extremely prevalent[39-41] in newborn dairy calves and can cause life-threatening disease[42,43]. Because *C. parvum* is a zoonotic parasite and *Cryptosporidium* spp. complete the life cycle within a single individual[1], bovine cryptosporidiosis is also a public health concern. The risk is compounded by the fact that morbidity associated with infection in both humans and animals occurs at a very low parasite inoculum: 30 *C. parvum* oocysts are sufficient to cause a symptomatic human infection[44].

Innate Immunity

Adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis"

Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).

The critical role of the innate immune response to *C. parvum* infection has been covered elsewhere[45,46]. Briefly, innate immunity is required for controlling the intensity of *Cryptosporidium* spp. infection[47]. After ingestion of *Cryptosporidium* spp. oocysts, sporozoites are released in the intestinal lumen and migrate to IECs; while IECs are the first physical barrier against infection, they also are the main target

for *Cryptosporidium spp.* sporozoites[1,47]. Chemokines are first released by *C. parvum*-infected IECs to promote chemotaxis at the site of infection; chemokines induce migration of dendritic cells in the ileum and the draining lymph nodes[48] (Figure 2). Inflammatory monocytes will also migrate to the subepithelial space in response to *C. parvum* infection and secrete TNFα and IL-1β[49]. These cytokines will increase permeability, therefore weakening the integrity of the intestinal epithelial barrier[49]. Also, nitric oxide NO is important in *C. parvum* infection clearance and reduces oocyst shedding in chronically infected nude mice[50]. NO is produced independently of IFNγ in *Cryptosporidium* spp. infections[51]. Inhibition of inducible nitric oxide synthase (iNOS) led to increased parasitism and oocyst shedding in *C. parvum*-infected piglets[52]. During *C. parvum* infection, the protective effect of iNOS depends on arginine availability in mice[50]. In fact, Leitch and He showed that supplementation with L-arginine decreases oocyst shedding in athymic nude mice[50]. IFNγ mediated production of chemokines by IECs recruits dendritic cells which clear *C. parvum* infection[46]. IECs also release antimicrobial peptides to destroy free parasites or can enter apoptosis if *C. parvum* infection already occurred[47] (Figure 2). Infection of IECs by *Cryptosporidium* spp. activates the MyD88 and NF-kB signalling cascade through Toll-like receptors (TLRs); in particular, TLR2 and TLR4 induce the production of human β-defensin 2 to help clear parasites[53,54].

Mucosal immunity is important for clearance of *Cryptosporidium* spp., as reviewed elsewhere[55]. The activation of antigen-presenting cells such as macrophages and dendritic cells is also important in *Cryptosporidium* spp. infections[56-60]. Dendritic cells can clear *Cryptosporidium* spp. from the site of infection via activation of adaptive immune response [60,61]. But, dendritic cells, neutrophils and IFNγ are also important in *Cryptosporidium* spp. infection because they play a crucial role in pathogen recognition and clearance of the parasite through direct contact[59,60,62,63] (Figure 2). The crucial role of mucosal natural killer (NK) cells (non-T, non-B lymphocytes[64]) in *Cryptosporidium* spp. infection is an active field of research[65-67]. The important contributions of NK cells and IFNγ in innate immune responses against *C. parvum* infection have previously been reviewed[68]. Briefly, NK cells are an important source of IFNγ in cryptosporidiosis and they are key players in controlling the infection in mice[65,69]. In the acute phase of

infection, *C. parvum* sporozoites induce production of IL-12 by macrophages and dendritic cells[70]. IL-12 acts synergistically with IL-18 and TNFα to activate NK cells[1,71,72] (Figure 2). Thus, the production of IFNγ by NK cells and macrophages in response to *Cryptosporidium* spp. is promoted by IL-12 and IL-18[73,74]. Secreted IFNγ can inhibit *C. parvum* invasion and intracellular development by acting directly on enterocytes and preventing parasite invasion[59,63] (Figure 2). Moreover, NK cells can efficiently kill *Cryptosporidium* spp.-infected human IECs[67] by inducing programmed cell death via the action of released cytotoxic granules[71] (Figure 2).

Apart from its role in inducing IFNγ production by NK cells, IL-18 also has a NK cell-independent role (Figure 2). $Rag2^{-/-}gammac^{-/-}$ adult mice (deficient for NK, T and B cells) can clear *C. parvum* infection due to NK cell-independent IFNγ production[66]. In this case, IFNγ is probably produced by IL-18- and IL-12-activated macrophages[66]. However, NK, T and B cell-deficient $Rag2^{-/-}gammac^{-/-}$ adult or neonate mice have more severe *C. parvum* infections than T and B cell-deficient $Rag2^{-/-}$ adult mice[65]. Consequently, even if both NK cell-dependent and -independent IFNγ have a protective role in innate immunity against *C. parvum* infection, presence of NK cells significantly helps to contain infection[65].

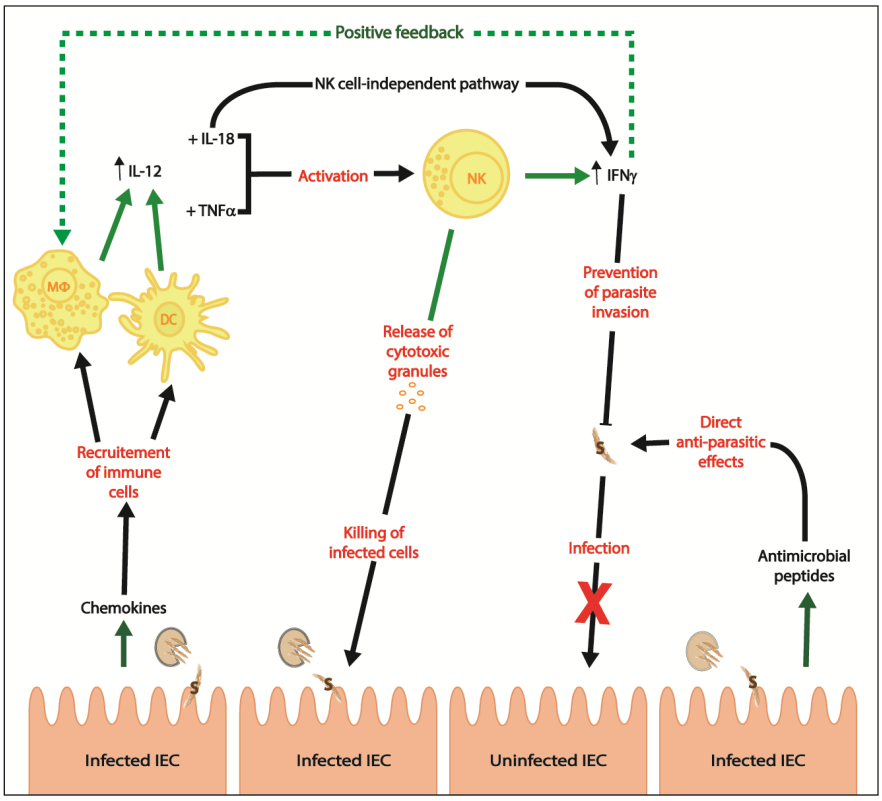


Figure 2. Innate immune responses during *Cryptosporidium* spp. infection. Green solid lines show release of molecules, green dotted line illustrates positive feedback, solid black lines present direction of effects and red wording define effects. NK = NK cells, MΦ = macrophages, DC = dendritic cells, S = sporozoites and IEC = intestinal epithelial cells.

The susceptibility of interferon gamma receptor knockout (IFNγR-KO) mice to *Cryptosporidium* spp. infection is an excellent example of the essential role of IFNγ for the control of cryptosporidiosis [[75-77]]. SCID-IFNγKO mice have heavier infections than SCID mice[78] and IL-12KO mice are highly susceptible to *Cryptosporidium* spp. infection [[79,80]]. In addition, treatment of newborn SCID mice with anti-IL-12 neutralizing antibodies exacerbates cryptosporidiosis[73].

Adaptive Immunity

Adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis"

Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).

Cell Mediated Immune Responses

The innate response is important for initial control of *Cryptosporidium* spp. infection, but adaptive immune responses are required for resolution of this disease[1,47]. The gut-associated lymphoid tissue (GALT) of the intestine is the main line of defence against pathogenic and commensal organisms of the gastrointestinal tract[81]. The intestinal environment contains a very diverse pool of antigens from food and microorganisms[82]. The mucosal immune system is therefore an important barrier to protect against pathogenic organisms and to confer tolerance against food antigens and the gut microbiota[83]. The gut immune responses therefore encompass high numbers of pro-inflammatory cells to prevent infection and regulatory T cells that regulate homeostasis[84]. *Cryptosporidium* spp. infection is more severe (potentially fatal) and longer lasting in immunocompromised individuals with defective adaptive immune response [85,86]. The crucial role of T-cell responses in *Cryptosporidium* spp. infection is obvious when studying HIV-infected patients[87] and patients with an immunodeficiency affecting T-cells[85].

As reviewed[69], CD4+ T cells are key actors in mounting adequate immune responses against cryptosporidiosis. Indeed, during the acute phase of infection involving innate immunity, CD4+ T cells are essential to clear *Cryptosporidium* spp.[1]. *Cryptosporidium* spp. infection is particularly frequent in AIDS patients with CD4+ T cell counts of <100 cells/μL[88]. CD4+ T cell counts <50 cells/μL are correlated with worse disease outcomes in immunocompromised patients[59,85,89-91]. T_H17 cells constitute the first subset of

CD4+ T helper cells to differentiate upon exposure of antigen-presenting cells to pathogens and are therefore important during the early stages of an infection[71]. T_H17 cells differentiate from naive CD4+ T cells in presence of IL-6 and TGFβ (produced by dendritic cells), but in the absence of IL-12 and IL-4[71] (Figure 3). IL-23 stimulates T_H17 cells to produce IL-17, but not IFNγ or IL-4[71]. Because IL-17 is involved in cytokine and chemokine secretion, which will have a chemo tactic effect on neutrophils at the site of infection, IL-17 supports innate immunity against pathogens[71] (Figure 3). Among other T_H17 cytokines, IL-17, IL-6, TNFα, TGFβ and IL-23 are found in increased levels in the gut-associated lymphoid tissue and spleen of immunosuppressed BALB/c mice infected with *C. parvum*[92] (Figure 3).

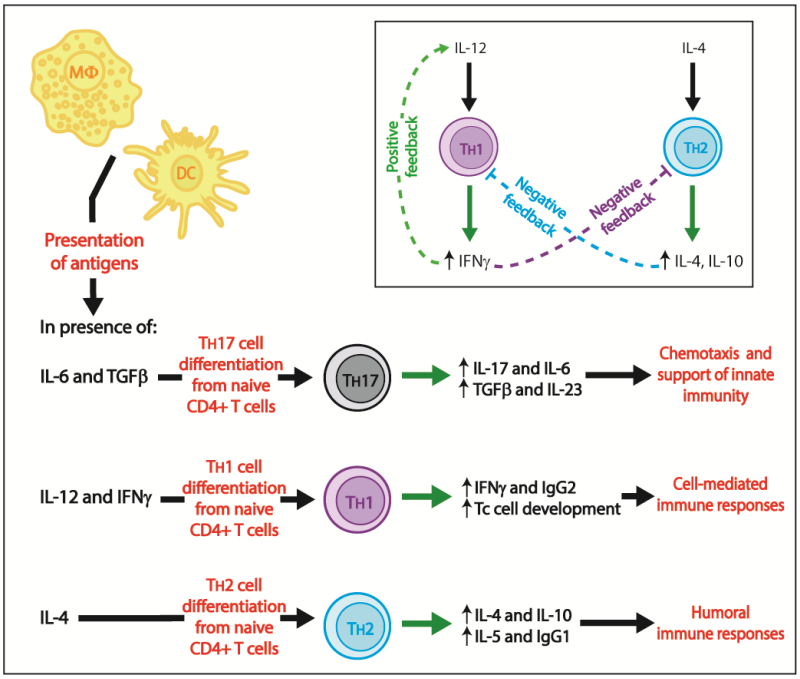


Figure 3. Adaptive immune responses during *Cryptosporidium* spp. infection. Green solid lines show release of molecules, green dotted line illustrates positive feedback, light blue and purple dotted lines represent negative feedback, solid black lines present direction of effects and red wording define effects. MΦ = macrophages, DC = dendritic cells, T_H1 = T_H1 T cells, T_H2 = T_H2 T cells, T_H17 = T_H17 T cells.

Promotion of cell-mediated immune responses and killing of infected cells resulted, in part, from macrophages and dendritic cells secretion of IL-12 and activated NK cells secretion of IFNγ[93] (Figure 4).

In fact, IL-12 and IFNγ induce differentiation of naive CD4+ T cells to T_H1 cells which will, among other effects, secrete IFNγ, produce IgG2 and promote differentiation of cytotoxic T cells from CD8+ precursors[93] (Figure 4). IFNγ has a positive feedback on IL-12 secretion by activating macrophages, while having a negative feedback on the T_H2 differentiation of naive CD4+ T cells (Figure 4); consequently, IFNγ strongly promotes a T_H1 environment[93]. In contrast, Il-4 induces differentiation of CD4+ T_H2 cells which, among other effects, induce production of IgG1, activate eosinophils and secrete IL-5, IL-4 and IL-10 (Figure 3); IL-4 and IL-10 have a negative feedback on T_H1 cells[93] (Figure 3). There is therefore a balance between T_H1 and T_H2 immune responses; cytokines secreted in a T_H1 environment inhibit T_H2 differentiation and vice versa. During *Cryptosporidium* spp. infection, CD4+ intraepithelial lymphocytes (IELs) produce IFNγ which is essential for innate immunity and adaptive T_H1 immune responses and has a direct inhibitory effect on *Cryptosporidium* spp. development in host enterocytes[46,69].

The role of cytokines in *Cryptosporidium* spp. infection has been reviewed elsewhere[78]. Because of their importance in the immune response to *C. parvum* infection, they will briefly be reviewed here as well. As mentioned, IFNγ has a vital role in controlling early phase infection as a major component of the innate immune response. However, this pro-inflammatory cytokine also has an important role in adaptive immunity[78]. IL-12 and IFNγ promote development of naive CD4+ T cells into T_H1 cell [78,93] (Figure 3) which contribute to the killing of intracellular microorganisms, such as *Cryptosporidium spp.*, by stimulating phagocytosis, neutrophil degranulation, and release of reactive oxygen species[78,94-96]. In addition, IL-4 has a protective role in *Cryptosporidium* spp. infection via IL-4-induced differentiation of naive CD4+ T cells into T_H2 cells[78] (Figure 2). In C57BL/6 adult mice, IFNγ-producing CD4+ T cells were essential in the initial phases of *C. parvum* infection to control the severity of infection, while IL-4-producing CD4+ T cells were important to accelerate resolution of infection[97]. Therefore, even if cytokines associated with T_H1 immune responses (e.g., IFNγ and IL-12) are essential to clear *C. parvum* infection, some cytokines associated with T_H2 immune responses (e.g., IL-4) have an important supporting role[98]. Wild-type, but not IFNγKO, mice treated with IL-4 neutralizing antibodies were less susceptible to *C. parvum* infection than

untreated mice; IL-4 can therefore have an IFNγ-dependent protective role[98]. Thus, typical T_H2 cytokines (i.e., IL-4) can potentially protect against cryptosporidiosis via T_H1 immune responses[78] (Figure 4), as already reported for *Leishmania major* infection[99].

CD8+ T-cells are also important for clearance of the parasite. CD8+ T-cells also produce IFNγ in response to infection (Figure 4) and potentially lyse *Cryptosporidium* spp.-infected IECs through the secretion of anti-parasitic cytotoxic granules[100]. However, CD8+ T-cells are not major actors in adaptive immune responses against *Cryptosporidium* spp. infection. *C. parvum*-infected SCID mouse recipients of splenocytes from immunocompetent mice cleared infection unless treated with anti-CD4+ or anti-INFγ monoclonal antibodies, while anti-CD8+ monoclonal antibodies had no effect on the outcome[101]. SCID mice injected with IELs from immune BALB/c donors shed fewer oocysts and recovered more rapidly from *C. muris* infection; protection was abrogated by depletion of CD4+ T cells, but not CD8+ T cells, from IELs[102]. In addition, BALB/c mice infected with *C. muris* and treated with anti-CD4 monoclonal antibodies had increased duration of patent infection and oocyst shedding, while mice treated with anti-CD8 monoclonal antibodies had only a moderate increase in oocyst shedding[103].

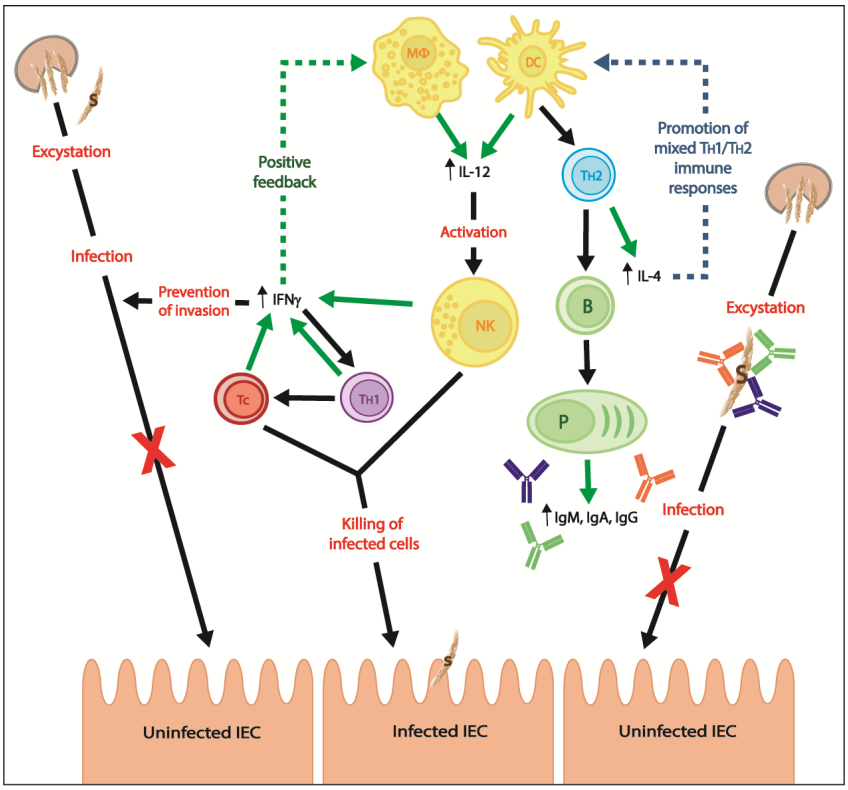


Figure 4. Protective immune responses during *Cryptosporidium* spp. infection and targets for vaccination. Green solid lines show release of molecules, green dotted line illustrates positive feedback, dark blue dotted line represents induction of mixed immune responses, solid black lines present direction of effects and red wording define effects. MΦ = macrophages, DC = dendritic cells, NK = NK cells, T_H1 = T_H1 T cells, T_H2 = T_H2 T cells, Tc = cytotoxic T cells, B = B cells, P = plasmocytes, S = sporozoites and IEC = intestinal epithelial cells.

Humoral Immune responses

Although the important role of cell-mediated immune responses is well described in *Cryptosporidium* spp. infection, the importance of humoral immune responses is not fully understood[55]. As part of mucosal immune responses, B-cells represent a major subset of GALT immunity[104] and gut resident B-cells undergo V(D)J recombination to produce secretory IgA[105,106]. Also, systemic *Cryptosporidium* spp.-specific antibodies, notably serum IgM, IgA and IgG, are generated following infection[107-109]. Generally, these antibodies are insufficient to prevent and control *Cryptosporidium* spp. infection[107] and are not essential for

recovery and clearance of the parasite[110]. However, antibodies may play a supportive role in protection, as hyperimmune bovine colostrum (HBC) has undeniable prophylactic and therapeutic effects[111-113]. In fact, many studies report that administration of hyperimmune colostrum/antibodies protects newborn animals against *Cryptosporidium* spp. infection[1]. The ability of antibodies to prevent cryptosporidiosis has not been thoroughly characterized in human medicine and lessons learned from veterinary medicine will be reviewed here.

Treatment of Immunocompromised Cryptosporidium spp.-Infected Patients With Hyperimmune Bovine Colostrum

It is important to note that the importance of *Cryptosporidium* spp.-specific antibodies for protection against cryptosporidiosis might not be equal between humans and animal models. For example, high levels of faecal *C. parvum*-specific IgA and IgM antibodies following infection correlate with reduced oocyst shedding in *C. parvum*-infected athymic C57BL/6 nude mice[114]. IgA antibodies are present in *Cryptosporidium* spp.-infected AIDS patients, but this response is insufficient to protect against cryptosporidiosis[107,115]. In other words, anti-*C. parvum* antibodies alone cannot clear infection in immunocompromised *Cryptosporidium* spp.-infected patients without the support of CD4+ T cells[115]. Therefore, conclusions drawn from immunocompromised mouse models may not always be applicable for immunocompromised humans. In fact, contradictory results are reported in the literature. On one side, some studies suggest a partial protective role of antibodies from HBC against cryptosporidiosis in immunocompromised patients[111,116,117] and HBC in concentrate powder form was an effective therapeutic approach in *C. parvum*-infected HIV patients as it significantly decreased stool weight and frequency[118]. On the other side, two studies showed that only some patients had reduced oocyst shedding after treatment[119] and that HBC had no protective effect compared to a placebo to decrease stool volume or oocyst shedding[120].

Diagnostics

Microscopy

Microscopy remains a gold standard in the diagnosis of many protozoan infections[121]. In the case of *Cryptosporidium spp.* acid fast staining of oocysts faecal samples followed by direct examination was the first technique to be used as a routine diagnostic test[121]. Many different staining techniques have been developed and microscopy is still used today to diagnose cryptosporidiosis[122]. However, the main limitation of this technique is sensitivity, given the small size of the oocyst and the variability of excreted oocysts in patients. Faecal smear staining and observation following sucrose concentration of oocysts allowed for a 100-fold increase in sensitivity (10^6 oocyst/g of faeces limit to ~10^4 oocyst/g of faeces detection limit)[121,123]. To achieve higher sensitivity, fluorescently labelled antibodies against oocyst wall proteins can be used for detection of the parasite in faecal smear [124,125]. FITC conjugated antibodies are now commercially available and used for routine faecal and environmental screening of cryptosporidiosis[125,126].

Immunoassays

Indirect enzyme-linked immunosorbent assays (ELISA) for detection of *C.parvum* antigens in faecal samples could allow for faster and more efficient diagnosis of protozoan parasites. ELISA diagnostic tests could help provide further insight where microscopic results are not conclusive. Rapid enzyme immunoassays (EIA) diagnostic tests have been developed and are commercially available. However, their sensitivity and specificity were found to be very low compared to a commercially available direct fluorescent antibody (DFA) kit[127]. This suggests that, while EIA kits would be a fast and accurate diagnostics tool in high prevalence areas, it is not the method of choice for diagnosis in populations with low prevalence of cryptosporidiosis because of the increased false positives[127,128]. In a low prevalence context, there would be fewer true positives which could result in the proportion of false positives to be higher than the number of true positives.

PCR-Based

While coproscopic methods and immunoassays are valuable techniques for diagnosing cryptosporidiosis, PCR-based techniques are at least as sensitive[123,125] and allow for accurate diagnosis and genotyping[125]. The most common gene targets for PCR-based *Cryptosporidium* diagnosis are the 18s ss rRNA gene[129], the *Cryptosporidium* oocyst wall protein (COWP)[130], a 70kDa heat shock protein (hsp70)[131] and a 60kDa glycoprotein (GP60)[132]. Nested PCR combined with restriction fragment length polymorphism (RFLP) is a novel promising technique[133]. It is simple and inexpensive, is very sensitive for the detection of *Cryptosporidium* oocysts (1 oocyst in water or stool sample) and allow for specie genotyping for more in-depth analysis[134].

Vaccines against *Cryptosporidium spp.* Infection

Adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis"

Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).

DNA Vaccines and Subunit Vaccines

Many types of vaccines exist, such as DNA vaccines, subunit vaccines, live-attenuated vaccines and vector vaccine [135,136]. Many promising vaccine approaches for cryptosporidiosis have been reviewed elsewhere[1,55,110]; briefly, some DNA and subunit vaccine candidates will be reviewed here. DNA vaccines encoding some surface proteins of *C. parvum* (such as Cp12 and Cp21[137] or cp15 and p23[138] or CP15/60[139]) lead to protective immune responses via production of high IgG level [137,138], elevated T_H1 cytokines[138] and/or increase in the numbers of CD4+ and CD8+ T cells[137]. Protection from DNA vaccines resulted in up to 77.5% reduction in oocyst shedding after challenge[137,138].

Subunit vaccines have been commonly used in vaccine development against cryptosporidiosis and several immunodominant proteins have been identified as potential vaccine candidates[140]. As mentioned previously, pregnant cows were vaccinated with *C. parvum* sporozoite p23 surface protein and resulting HBC was protective for *C. parvum*-challenged calves[141]. Also, in another study, anti-P23 HBC-treated calves showed no clinical sign of cryptosporidiosis and reduced and delayed oocyst shedding[142]. HBC from pregnant cows immunized with CP15/60 recombinant protein successfully transferred antibodies to calves via colostrum intake; however, challenge of treated calves was not presented[143]. In mice, divalent recombinant Cp15-23 led to significant antibody and T_H1 cytokine production and elevated numbers of CD4+, but did confer only partial protection against *C. parvum* challenge[144].

Live-Attenuated Vaccine

Live-attenuated vaccines have historically been shown to be best at eliciting long lasting memory immune responses, whereas subunit vaccines elicit a more modest memory response, often requiring subsequent booster doses to achieve long-lasting immunity[136]. Attenuated vaccines were first developed for viral and bacterial pathogens because of the inherent complexity of parasitic organisms; however, some vaccine development is ongoing for a few pathogenic parasites[145].

Live-attenuated vaccines elicit strong T_H1 biased immune responses and offer protective cell-mediated immunity[136]. Several live-attenuated vaccines have been developed against protozoan parasites causing enteric disease, i.e., *Eimeria*[146,147]. Early studies showed that chickens receiving irradiated *E. maxima* oocysts were protected against coccidiosis-induced weight loss[148]. Also, live-attenuated *Toxoplasma gondii* induced protective immunity against toxoplasmosis in sheep for at least 6 months[149,150]. In addition, live vaccines against another parasite, *Leishmania* spp., have recently been studied[151]. A non-pathogenic species, *L. tarentolae*, elicits strong protective T_H1 immune responses in mice against *L. donovani*[152]. Similar responses were observed in mice vaccinated with attenuated *L. donovani*[153]. Although this approach is promising, a live-attenuated vaccine may not be ideal for cryptosporidiosis due to its host requirement for replication[1]. This is further exacerbated by the lack of a continuous *in vitro* culture system allowing oocyst production for *Cryptosporidium spp*[154]. Nonetheless, γ-irradiation has been used on *Cryptosporidium spp.* oocysts or sporozoites to reduce their viability and infectivity[155]. Irradiated *C. parvum* oocysts were shown to elicit protective immune responses in calves challenged at 3 weeks post-vaccination[156].

Vaccine Vectors

Vaccine vectors came into play in the early 1990s[157], but the first vaccine vector to be licensed is a chimeric yellow fever attenuated strain in 2010[158]. A vaccine against *Cryptosporidium* spp. should stimulate mucosal immune responses by promoting uptake of antigens by microfold cells (M cells), specialized epithelial cells adjacent to enterocytes that facilitate the passage of antigens to Peyer's patches[71]. Intestinal antigen delivery to the M cells could be achieved using a vaccine delivery system such as attenuated bacterial or viral vectors[159,160]. To our knowledge, no viral vectors have been used in candidate vaccines for *Cryptosporidium* spp., but several live bacterial vectors have been studied[161-163].

Bacterial vaccine vectors are very promising for vaccine antigen delivery as they can elicit protective immune responses against bacterial, viral and protozoan pathogens in both mice and humans[164]. For instance, delivery of influenza hemagglutinin and neuraminidase using an attenuated *S. typhimurium* vector induced strong protective cellular and humoral immunity against Influenza A virus[165]. Also, delivery of *Trichinella spiralis* DNA using an attenuated *S. typhimurium* elicited protective mixed T_H1/T_H2 immune responses in mice[166]. Moreover, *Plasmodium falciparum* tCSP genes fused to secretion signals were delivered through *S. typhimurium* and boosted with a DNA vaccine and elicited strong cellular T_H1 immune responses[167]. Overall, the many advantages of this vaccine approach (ease of administration and low production cost) engender excellent candidates for vaccine development[168]. Fusing the protein of interest to a secretion signal and a chaperone binding domain of *S. enterica* allows secretion of the antigen of interest through the type III secretion system-dependent for delivery to antigen-presenting cells[169].

A number of attenuated *S. typhimurium* vectors expressing *Cryptosporidium* spp. antigens have been generated[161-163]. Promising humoral and cellular immune responses were obtained from a prime-boost technique with *Salmonella enterica* serovar Typhi CVD-908-*htrA* and cytolysin A (ClyA) fused to either *C. hominis* apyrase (CApy), profilin or Cp15[161]. In mice, these vaccines elicited strong humoral immune

responses with high production of IgG1 and IgG2b and interesting cellular immune responses via production of different levels of several cytokines (IFNγ, IL-2, IL-6, and IL-12)[161]. Attenuated *Salmonella enterica* serovar Typhimurium vaccine strain SL3261 expressing *C. parvum* Cp23 or Cp40 fused to fragment C of tetanus toxin elicited humoral immune responses when delivered as an oral boost after subcutaneous immunization with cp23 or cp40 DNA[162]. An attenuated *Salmonella enterica* serovar Typhi CVD 908-*htrA* expressing Cp15 delivered intranasally in mice showed high production of IL-6, IFNγ and Cp15-specific IgG[163]. However, vaccination did not result in protection against *C. parvum* infection in mice[163]. Another vector system used for *Cryptosporidium* spp. antigen delivery is *T. gondii*[170]. Immunization of mice with *T. gondii* expressing *C. parvum* P23 antigen resulted in high levels of serum IgG, predominantly IgG1, which is characteristic of a T_H2 immune response [[170,171]]. In another study, *Lactobacillus casei* Zhang (a probiotic bacterium[172]) was used to deliver *C. parvum* P23 to mice and generated increased levels of IFNγ, IL-6, serum IgG and faecal IgA[173].

Overall, vaccine vectors show promising immunological results and appear to be an interesting option for vaccine development against cryptosporidiosis. Although one challenge study showed no protection after vaccination[163], more studies using various vectors and immunogens are needed to assess the true potential of this method. It will be very interesting to determine if they show better protection against *Cryptosporidium* spp. infection than their non-vector strategies. The high carrying capacity of vectors is also an advantage, as they can deliver multiple antigens and even adjuvants to the target site[174]. Vaccine vectors can also be used either alone or in combination with DNA or antigen-based vaccine candidates as a 'prime-pull' method[161,167].

Prime-Pull Vaccine Approach

The prime-pull vaccine approach primes the immune system with an antigen to elicit strong systemic T cells immune responses and then 'pulls' T cell immune responses at the site of infection using local delivery of immunogens and/or pro-inflammatory molecules to elicit local protective and long-lasting memory responses[175]. In other words, the 'prime' immunization using intramuscular delivery of antigen(s) elicits systemic T cell immune responses while the 'pull' immunization allows for the formation of a strong pool of tissue-resident T cells[175]. As mentioned above, the prime-pull approach has been used in combination with vaccine vectors against *Cryptosporidium* spp. infection in various delivery schedules and methods[161-163]. In some studies, *C. parvum* DNA was used to 'prime' mice and the *C. parvum* antigen-expressing *Salmonella* spp. vector was given as a boost[162]. In other studies, the *Salmonella* spp. vector vaccine was given as a 'prime' and then boosted with recombinant protein given intraperitoneally[161,163]. Overall, the prime-pull method elicits much stronger immune responses than the vector or the antigen alone[161-163].

Salmonella

Salmonella enterica are gram negative bacilli. There are currently two species, six subspecies and over 2,500 serovars of these flagellated facultative anaerobic bacteria[176,177]. *Salmonella enterica* is the leading foodborne pathogen in the US, causing over 19,000 hospitalizations and over 370 deaths annually[178]. *Salmonella spp.* enter hosts through the oral route and migrate to the gut, where they colonize the intestinal epithelia[179]. In humans, *S,enterica* serovars utilize type 3 secretion systems (T3SS) to inject effector proteins which cause pedestal formation and internalization of the bacterium in a *Salmonella* containing vacuole (SCV)[180]. In this niche, effector protein expression causes bacterial replication and death of the infected cell by pyroptosis[181,182]. This will in term cause damage to the intestinal epithelia and may result in diarrhea and dehydration of the host[183].

Human *Salmonellosis* is a dangerous and serious threat caused by a well-adapted bacterium which utilizes needle-like apparatus to specifically inject proteins in gut epithelial cells[184]. Pathogenicity aside, this bacterium is an ideal vector to deliver antigen in the intestinal lumen and epithelia. Many researchers are working on attenuating and adapting *S. enterica* for various uses[185,186]. *S. typhimurium* YS1646 is an S.*enterica* serovar which was attenuated to prevent pathogenesis in hosts and used as a cancer therapy candidate because of its promising specific targeting of tumor cells[187].

Salmonella typhimurium YS1646 (VNP20009)

The original hyper invasive YS72 strain is *purI*$^{-}$, requiring external adenine and vitamin B1 for optimal growth and *xyl*$^{-}$[188]. YS72 was then used to create the YS1646 strain. The *waaN* gene (previously *msbB*), which is involved lipid A acetylation in *Salmonella spp.* [189], was deleted from YS72, reducing pathogenicity of the strain[190]. Because of this mutation, the YS1646 strain is incapable of inducing TNFα and the risk of septic shock is therefore reduced[191].

The YS1646 strain was originally used as an experimental antitumor agent. It was shown to target and infect various types of solid tumours in C57BL/6 mice[190]. It was further demonstrated that YS1646 could inhibit tumour growth of subcutaneously implanted tumours at a dose of $1x10^5$ cfu/mice intravenously (IV) and growth of lung metastases at a dose of $2x10^6$ cfu/mice IV in C57BL/6, athymic nude mice and SCID mice[192].

Treatments Against Cryptosporidiosis

Anti-parasitic treatment for cryptosporidiosis has been widely studied, yet very limited. Nitazoxanide is the only FDA-approved drug for the treatment of cryptosporidiosis in individuals of one year of age and older. This drug has been shown to be 93% effective in parasite clearance in immunocompetent individuals[193]. This drug, however, was found to be ineffective in patients suffering from HIV-related immunosuppression[194] and in organ transplant recipients[195]. Azithromycin and paromomycin have also been shown to have some effect against cryptosporidiosis, but remain inefficacious in immunocompromised individual [196,197]. Rifamycin, in combination with nitazoxanide, was shown to significantly decrease burden of disease in immunocompetent patients[198]. Rifaximin, a non-absorbed derivative of rifamycin, was even shown to be effective in treating diarrheal symptoms in HIV patients co-infected with *Cryptosporidium* spp.[199].

In recent years, drug development has been focused on developing compounds targeting key enzymes of *Cryptosporidium* spp.[197]. Some of those compounds inhibiting the calcium-dependent protein kinase 1 of *Cryptosporidium* spp. have been shown to be effective at reducing *Cryptosporidium* spp. burden in immunocompromised mice[200]. K11777, a compound targeting a clan CA cysteine protease of *Cryptosporidium* spp., was successful in rescuing mice from a lethal infection with *C.parvum*[201].

Despite novel experimental approaches to developing treatments against cryptosopriosis, some are not totally efficacious and very few are available on the market. This calls for the development of novel vaccines to prevent this disease as well as novel effective treatments to

Conclusions and Future Directions

Adapted from "lessons learned from protective immune responses to optimize vaccine against cryptosporidiosis"

Lemieux, M. W., et al. (2017). "Lessons Learned from Protective Immune Responses to Optimize Vaccines against Cryptosporidiosis." Pathogens **7**(1).

As *Cryptosporidium* spp.-infected immunocompetent individuals only present with transient diarrhea while immunocompromised patients and infants in developing countries can have very severe and life-threatening cryptosporidiosis, the competency of the host immune system to raise adequate immune responses is the key factor to clear *Cryptosporidium* spp. parasites. The pathogenesis of cryptosporidiosis is incompletely understood because this protozoan parasite induces complex host immune responses. Innate immunity can contain *C. parvum* infection via the action of IL-18- and IL-12-activated macrophages and NK cells which induce NK cells-dependent and NK cells-independent IFNγ production (Figures 2 and 4). Adaptive immunity will clear *C. parvum* infection via the action of CD4+ T_H1 cell-mediated immune responses which induce IFNγ production and killing of infected IEC; T_H2 immune responses and humoral immunity have a non-negligible supportive role (Figures 3 and 4).

To sum up, protective immune responses against *Cryptosporidium* spp. infection requires strong mucosal T_H1 cell-mediated immune responses with the support of a T_H2-dependant *Cryptosporidium* spp.-specific humoral immunity (Figure 4). A vaccine that induces such immune responses, if safe for use in children and immunocompromised individuals, should be the best candidate to prevent cryptosporidiosis. Furthermore, because *Cryptosporidium spp.* infects the intestinal epithelia, a vaccine against cryptosporidiosis would ideally elicit strong mucosal immune responses[55]. Vaccine vectors using the 'prime-pull' approach represent a new era in vaccine development and we believe that these new techniques have the potential to elicit more targeted immune responses and localized protection against

Cryptosporidium spp. infection[162]. Results from ongoing studies will determine potential of this new vaccine approach against *Cryptosporidium* spp. infection.

We hypothesize that an oral *S.typhimurium* YS1646 vaccine vector expressing our recombinant Gp45 in addition to the intramuscular Gp45 will elicit a greater mucosal immune response against *C.parvum* compared to an intramuscular Gp45 vaccine alone.

Rationale and research objectives

Cryptosporidiosis poses a serious threat for developed and developing countries. Treatment of cryptosporidiosis in immunocompetent hosts relies on Nitazoxanide, while this treatment is not efficacious in immunocompromised individuals. Furthermore, the lack of better therapeutic drugs and prophylaxis, coupled with the important burden of this infectious disease warrants the development of a vaccine against cryptosporidiosis. This thesis describes novel research to adapt an attenuated *Salmonella* strain to express and deliver gp45, a *Cryptosporidium spp.* antigenic protein, and determine the immunogenicity of this novel vaccine approach. Gp45 is a valuable vaccine candidate as it is expressed on the sporozoite stage of the parasite and plays an important role in adhesion and locomotion to the host's intestinal epithelium. Attenuated *Salmonella* have been demonstrated to be useful in targeted protein delivery. Their biological content, such as lipopolysaccharide enhances the immune response, therefore acting as an adjuvant.

Gp45, a surface glycoprotein of *Cryptosporidium spp.* has been demonstrated to be a good and effective vaccine candidate. It elicits a strong antibody response which was shown to confer protection to *C.parvum* infection (Karine Sonzogni-Desautels, Investigating Cryptosporidium parvum surface proteins as candidates for a vaccine against bovine cryptosporidiosis, 2017). The first objective of this thesis was to develop constructs with an insert of a codon optimized gp45 sequence. The second objective was to

transform those constructs into *Salmonella* YS1646 and analyze the protein expression in axenic culture. Finally, we wanted to assess the immunogenicity of our vaccine in a prime-boost immunization approach in C57BL/6 mice. This final objective was to compare the previously studied IM gp45 vaccine with a prime-boost vaccine using the available gp45 candidate followed by YS1646-gp45 administration.

Methods

Construct preparation

Our laboratory designed and developed constructs for antigen expression in *S. typhimurium*. Constructs were engineered with the SspH1 or SspH2 secretion signal of *S.typhimurium* both preceded with their corresponding promoters. The SspH1 promoter and secretion signal guide proteins into either *Salmonella* pathogenicity island 1(SPI1) or SPI2 type III secretion system, which translocate proteins across the plasma membrane or the vacuolar membrane respectively[202]. SspH2 and its promoter, however, are only translocated through the vacuolar membrane via SPI2[203]. The nucleotide positions and sequences for *S. typhimurium* promoters and secretion signals can be found on GenBank under the accession number NZ_CP007804. Because this strain has not been characterized yet, we used the gene sequences of *S.typhimurium* LT2 strain (Genbank CP001363.1) for which the sequences of SspH1, and SspH2 were characterized, and we aligned them with the full genome of *S.typhimurium* YS1646 to determine the correct sequence. SspH1 secretion signal was found under Genbank CP001363.1 locus STM14_1483 and SspH2 secretion signal was found under Genbank CP001363.1 locus STM14_2769. Primers were designed to flank each promoter-secretion signal sequence with the Xho1 and Not1 at the 5' and 3' end respectively (**SspH1_fwd**: 5'- CGC CTC GAG CGC TAT ATC ACC AAA AC-3'; **SspH1_rev**: 5'- CTC TGC GGC CGC GGT AAG ACC TGA CGC TC-3'; **SspH2_fwd**: 5'- CGC CTC GAG GTT TGT GCG TCG TAT -3'; **SspH2_rev**: 5'- CTC TGC GGC CGC ATT CAG GCA GGC ACG CA -3'). The promoters and secretory signals were then inserted in the pQE-30 backbone (Qiagen; Hilden, Germany).

Codon Optimization of GP45

Cryptosporidium parvum GP45 (Genbank accession number AF114166) cDNA was optimized using the Java Codon Adaptation Tool (JCat)[204]. A 6x His tag was added at the 3'-end of the gene followed by a stop codon. Furthermore, the optimized sequence was flanked with Not1 and Asc1 at the 5'and 3'-end respectively. The optimized sequence cDNA was synthesized by ThermoFisher's GeneArt services in pMK-RQ-Bs.

Construction of plasmids expressing the GP45 *C.parvum* antigen.

Digestion of optimized Gp45

The optimized GP45 sequence from pMK-RQ-Bs was inserted into the pSSPH1 and pSSPH2 constructs using Not1 (ThermoFisher Scientific; Waltham, USA) and Asc1 (ThermoFisher Scientific; Waltham, USA) enzymes. 14µL of pMK-RQ-Bs-GP45 was digested in a 1.7mL Eppendorf tube with 0.5µL of Not1 and Asc1, 2µL of Tango buffer (included with the enzymes) and 3µL of sterile demineralized distilled water. The digestion tube was incubated at room temperature overnight. The next day, 10uL of the digestion was mixed with 5uL of 6x loading dye (ThermoFisher Scientific; Waltham, USA) and loaded on a 0.8% agarose gel (Wisent bioproducts; St-Burno, Canada). A 1kB ladder (New England Biolabs; Ipswitch, USA) was also loaded on the gel. TAE buffer (ThermoFisher Scientific; Waltham, USA) was added to fill the tank and the gel was run for 65 minutes at 90 volts.

Gel Extraction of Digested Optimized Gp45

The gel was visualized on a UV table and the digested fragments of 633bp were excised using a scalpel blade. DNA from the gel fragments was then isolated using a Gel Extraction kit (BioBasic; Amherst, USA).Briefly, the excised fragment was put in a 1.5mL microfuge tube and the DNA was purified and eluted as per manufacturer's protocols.

Ligation of the GP45 DNA Fragment Into the YS1646 Constructs

In a 1.5mL microfuge tube, add 2μL of 10x T4 DNA ligase buffer,5μL of purified digested vector, 5μL of purified digested insert, 7μL of nuclease-free water and 1μL of T4 DNA ligase. We are using the pSspH1 and pSspH2 vectors with the codon optimized Gp45 insert. The tubes were gently mixed and incubated at room temperature overnight. The next morning, the ligase was heat inactivated by incubating the tubes at 56°C for 10 minutes. The tubes were chilled on ice and the ligated DNA was transformed into Subcloning Efficiency™ DH5α™ Competent Cells (ThermoFisher Scientific; Waltham, USA).

Transformation of Ligated Plasmids Into DH5α Cells

50μL of DH5α cells were thawed on ice for 10 minutes and mixed gently. 10uL of the ligation mixture was then added to the cells and mixed gently without vortexing before being placed on ice for 30 minutes. The tubes were placed at 37°C for 1.5 minutes to heat shock the cells then placed back on ice for 5 minutes. We then added 100μL of S.O.C. medium (New England Biolabs; Ipswitch, USA) at room temperature and put the tubes in a shaking incubator at 37°C and 250rpm for one hour. 100μL of cells were then spread on pre-warmed agar plates containing ampicillin

(50μg/mL) and incubated at 37°C overnight. The following day, we selected three colonies and inoculated 5mL of liquid LB with ampicillin (50μg/mL) in 15mL polypropylene round bottom tubes using P200 tips. Liquid cultures were grown overnight at 37°C and shaking at 250rpm.

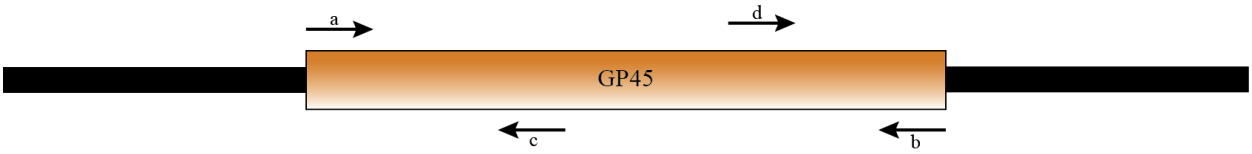
Miniprep DNA Extraction of Our Constructs

The following day, 2mL of each culture was used to perform a mini-prep plasmid DNA extraction using the BioBasic miniprep kit (BioBasic; Amherst, USA). 2mL of the overnight cultures were used to purify plasmid DNA as per manufacturer's protocol. Purified plasmids were stored at -20°C.

Sequencing of Constructs

To ensure proper insertion of the Gp45 gene in the plasmids, primers were designed to sequence the gene and the flanking plasmid sequences. The following primers were used: Primer a (gp45 opt fwd seq), 5-CCG GCG GCC GCG ATG TGG TTC-3', primer b (gp45 opt rev seq), 5'-CCG GGC GCG CCC TTA TTA GTG-3', primer c (gp45 opt outbegin seq), 5'-ATC GGC GCG TAA ACG ATG GTG-3' and primer d (gp45 opt outend seq), 5'-CAT CGT TCC GTC TGT TTT CGC-3'.

Primer binding sites and orientation are illustrated in the following figure.



Purified construct DNA and primers were sent to Genome Quebec for Sanger Sequencing. The new constructs were designated SspH1-Gp45 and pSspH2-Gp45. The empty pQE-30 backbone was also designated pQE-Null.

Intramuscular vaccine preparation: Purification of 6xHis-tagged Gp45 recombinant protein

Recombinant Gp45 antigen was purified from pET-28a(+) constructs developed by Karine Sonzogni-Desautels, a former PhD student in our laboratory.

Protein Expression

500mL of LB kanamycin (50µg/mL) were inoculated with the pET-28a(+)-Gp45 clone and culture was grown overnight at 37°C and shaking at 250rpm. The next day, 200mL from the pre-culture was added to 2L of LB kanamycin (50µg/mL). Cultures were incubated at 37°C and 250rpm for one hour (until optical density reached 0.8-1) and 10mL of IPTG 0.1M (final concentration: 1mM) was added. Cultures were incubated for another three hours at 37°C and 250rpm. Culture was transferred to 1L centrifugation tubes and centrifuged at 4000rpm for 15 minutes at 4°C. Supernatant was discarded in bleach and both pellets were transferred into one pre-weighed 50mL Falcon™ tube (StemCell technologies; Vancouver, Canada) and centrifuged at

4000rpm for another 15 minutes at 4°C to remove as much supernatant as possible. The pellet was weighed.

Protein Purification

The bacterial pellet was resuspended in 4mL of B-PER™ (ThermoFisher Scientific; Waltham, USA) per gram of pellet. 10µL of EDTA-free protease inhibitor (Millipore-Sigma; Burlington, USA) per 100µL of B-PER™, 1µL of DNAseI for 1mL of B-PER™ and 10µL of lysozyme (Millipore-Sigma; Burlington, USA) were added to the mixture. The suspension was pipetted up and down until homogeneous whilst avoiding formation of foam. The mixture was incubated in a rotary incubator one hour at 37°C. The mixture was then transferred to 50mL Corning™ tubes and centrifuged at 12,500g for 20 minutes at 4°C to separate soluble proteins from the insoluble proteins. The lysate was transferred to a 50mL Falcon® tube and filtered using a 0.22µm Steriflip® filter unit (Millipore-Sigma; Burlington, USA).

Purification of His-tagged Protein with NI-NTA using gravity flow

Purification of our recombinant Gp45 antigen was performed using a Ni-NTA purification column per manufacturer's protocol. 1mL of HisPur Ni-NTA resin (ThermoFisher Scientific; Waltham, USA) per 10mL of bacterial lysate was washed with PBS. The washed resin was added to the clear lysate and rotated at 4°C for two hours. The tube was centrifuged at 3000rpm for 5 minutes and supernatant was removed. 20mL of wash buffer was added to the resin and the mixture was loaded to an Econo-pac® chromatography column. The wash buffer was drained by gravity flow and another 20mL of wash buffer was added to the resin in the column. The protein was

eluted with 0.5mL five times, with each elution being collected in different tubes. Fractions were analyzed by SDS-PAGE and pooled together to perform dialysis against PBS using a Slide-a-lyzer 3500 MCWO dialysis cassette (ThermoFisher Scientific; Waltham, USA). The protein was then concentrated using an AmiconUltra-15 3000 MCWO centrifugal filter device (Millipore-Sigma; Burlington, USA).

Transformation of YS1646

The constructs pSspH1-Gp45, pSspH2-Gp45 and pQE-Null were transformed into *S. typhimurium* YS1646 by electroporation following a protocol developed by Dr. Li Xing in our laboratory. Electro competent YS1646 cells were thawed on ice and 40µL of cells were transferred to a 1.5mL microfuge tube. 20µL of plasmid DNA from a midiprep was added to the cells and the mixture was incubated on ice for 1 minute. The mixture was transferred to an electroporation cuvette (Fisher #FB102) and loaded in the Gene Pulser Xcell (BioRad; Hercules, USA). The cells were pulsed at 3.0kV, 200Ω and 25µF. After pulsing, 1mL of cold LB broth was immediately added to the cuvette and the mixture was transferred to a 15mL round bottom polypropylene tube. The tube was incubated at 37°C shaking at 250rpm for 1 hour. The tubes were then centrifuged and bacterial pellets were resuspended in 200µL of LB. 100µL of the mixture was plated on LB agar plates (50µg/mL) and incubated at 37°C overnight. The next day, colonies were picked and grown into 10mL LB broth with 50µg/mL ampicillin. The cultures were spun down and suspended in 5mL of 15% glycerol.

Western Blot Analysis

Recombinant strains of *S. typhimurium* were grown overnight in LB broth containing Ampicillin (50μg/mL) at 37°C shaking at 250rpm in aerobic conditions. 200μL from bacterial lysates were prepared by isolating bacterial cells through centrifugation at 10,000rpm for 10 minutes at 4°C. Supernatant was separated from the pellet and 100μL western blot sample buffer (3mL 20% SDS, 3.75mL 1M Tris buffer at pH6.8, 9mg bromophenol blue, 2.4mL β-mercaptoethanol, 4.5mL glycerol; bring volume to 15mL with ddH_2O) was added to the cell pellet. 150μL of supernatant was mixed with 50μL sample buffer. Samples were loaded and run on a 15% SDS-PAGE gel. Protein content of the gel was then transferred to a nitrocellulose membrane using the BioRad Semi-dry transfer kit (BioRad; Hercules, Canada). The membrane was incubated in blocking buffer (PBST with 5% skim milk) for 30 minutes and washed three times in PBS (pH 7.4; 0.01 M phosphate buffer, 0.14 M NaCl) containing 0.05% Tween 20 (Sigma-Aldrich). It was then incubated with a solution of 1:2000 monoclonal anti-polyhistidine mouse antibody in PBST with 5% BSA (Sigma-Aldrich; Oakville, Canada) and 0.05% Sodium Azide (Sigma-Aldrich; Oakville, Canada) overnight at 4°C with gentle agitation. The membrane was washed four times with PBST and was incubated with 1:10,000 anti-mouse IgG horseradish peroxidase goat antibody (Abcam; Toronto, Canada) in blocking buffer for one hour at room temperature with gentle agitation. The membrane was washed four times with PBST and incubated with Clarity™ chemiluminescent substrate (BioRad; Hercules, USA) for 5 minutes. The membrane was finally developed using an auto radiography cassette (Sigma-Aldrich; Oakville, Canada), film (Denville Scientific; Holliston, USA and Kodak X-OMAT 2000 system (Kodak; Rochester, USA). To blot using anti-Gp45 antibodies, the same protocol was used, to the exception of primary and secondary

antibody type and concentration. The primary antibody used was a rabbit polyclonal anti-Gp45 antibodies developed by Dr. Sonzogni-Desautels in our laboratory, which was diluted 1:10,000 in PBST with 5% BSA (Sigma-Aldrich; Oakville, Canada) and 0.05% Sodium Azide (Sigma-Aldrich; Oakville, Canada). Secondary antibodies were anti-rabbit IgG horseradish peroxidase goat antibody (Abcam; Toronto, Canada) diluted 1:20,000.

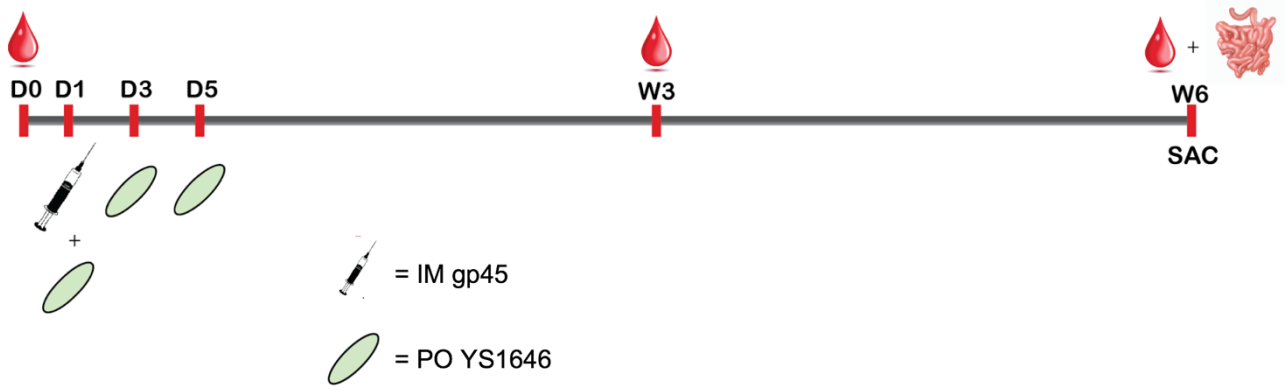
YS1646 Vaccine Preparation

100mL of LB ampicillin (50μg/mL) were inoculated with the vaccine glycerol stocks for pSspH1-Gp45, pSspH2-Gp45 and pQE-Null. Aerobic cultures were grown overnight at 37°C and 250rpm. The next morning, the cultures were centrifuged for 10 minutes at 3000rpm at 4°C. The supernatant was discarded and pellets were washed in 10mL LB with 15% glycerol before being centrifuged again (same conditions) and being resuspended in 10mL LB with 15% glycerol. 1mL aliquots were prepared, flash frozen using a dry-ice and ethanol mixture and stored at -80°C. One aliquot was thawed for colony forming unit (cfu) counting. 1:10 serial dilutions were performed and the 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} dilutions were plated on ampicillin plates (50μg/mL) in duplicates and incubated at 37°C overnight. The next day, colonies were counted to obtain the total cfu of the vaccine stocks. The volume and dilution factor for each vaccine dose (200μL) to contain 10^{9}cfu was calculated. On the day of vaccination, vaccine aliquots were thawed and the calculated volume was centrifuged at 3000rpm for 10 minutes at 4°C. Pellets were resuspended in the same volume of cold PBS (pH 7.4; 0.01 M phosphate buffer, 0.14 M NaCl) and kept on ice until immunization.

Immunization Protocol

Six to eight-week-old male and female C57BL/6 mice (Jackson Laboratories) were bread in our animal facilities at the MUHC research institute. Five groups of mice with 5 mice/group were used for this study. Two groups of mice were immunized intramuscularly with 40µg of recombinant Gp45 from clone pET-28a(+)-Gp45 followed by three doses of 10^9cfu of either YS1646-pSspH1-Gp45 (Group C) or YS1646-pSspH2-Gp45 (Group D) vaccine given by oral gavage every other day. One group was administered PBS intramuscularly and orally using the same immunization schedule (Group A). One group was immunized intramuscularly with a single dose of 40µg of recombinant Gp45 from clone pET-28a(+)-Gp45(Group B). The last group of mice was immunized with three doses of YS1646-pQE-Null (Group E) once every other day. IM injections were performed in the left hind leg of the mice using 0.5mL syringes. Oral gavage was performed using disposable animal feeding needles (Fisherbrand; Ottawa, Canada) and animals were given 200µL of either PBS or corresponding YS1646 vaccine diluted in PBS to reach a concentration of 10^9cfu in 200µL. All animal procedures were performed in accordance with Institutional Animal Care and Use Guidelines and were approved by the Animal Care and Use Committee at McGill University.

Study Timeline:



Humoral response: Enzyme-Linked Immunosorbent assay

Serum IgG ELISA

Throughout the immunization timeline, blood was collected by saphenous bleed from each mouse at week 3 and by cardiac puncture at week 6 in BD microtainer® tubes. Sera were obtained by centrifugation. The humoral immune responses were analyzed by enzyme-linked immunosorbent assay (ELISA). Serial dilutions of IgG standard antibodies were performed and coated on 96-well plates. The remaining wells of the 96-well plates were coated with the recombinant Gp45 from clone pET-28a(+)-Gp45 (50ng/well). Plates were incubated overnight at 4°C. The next day, plates were washed three times with PBST. Plates were then incubated for 60 minutes with 150µL/well of blocking buffer (PBS, 5% bovine serum albumin (Sigma-Aldrich; Oakville, Canada), 0.1% Tween 20 (Sigma-Aldrich; Oakville, Canada)) at 37°C. 1 in 50 dilutions of the sera in blocking buffer were added in duplicates to wells (50µL/well) and incubate for 1 hour at 37°C. Plates were washed four times with PBS/0.05% Tween 20. Anti-Mouse IgG (Fc specific)–Peroxidase antibody produced in goat (Sigma) were diluted 1:50,000, added in all wells (75µL/well) and plates were incubated for 30 minutes at 37°C. The plates were washed and the 3,3',5,5'-Tetramethylbenzidine (Millipore, Billerica, MA) substrate was added (100µL/well). Plates were incubated at Room Temperature (RT) for 15 minutes and 50µL/well of 0.5M sulfuric acid (Sigma-Aldrich) was added to stop the reaction. The plates were read at 450nm. IgG results are expressed as concentrations (ng/mL)

Intestinal IgA ELISA

At day of sacrifice, 10cm of the small intestine was collected and homogenized in Protease Inhibitor (PI) Cocktail (Sigma-Aldrich; Oakville, Canada) at a 1:5 dilution (weight/volume). The tissue was homogenized (Homogenzier 150; Fisher Scientific, Ottawa, ON), centrifuged at 2500xg at 4ºC for 30 minutes and the supernatant was collected. Supernatants were stored at -80ºC until analyzed by ELISA.

Serial dilutions of IgA standard antibodies were performed and coated on 96-well plates. The remaining wells of the 96-well plates were coated with the recombinant Gp45 from clone pET-28a(+)-Gp45 (50ng/well). Plates were incubated overnight at 4°C. The next day, plates were washed three times with PBST. Plates were then incubated for 60 minutes with 150µL/well of blocking buffer (PBS, 5% bovine serum albumin (Sigma-Aldrich; Oakville, Canada), 0.1% Tween 20 (Sigma-Aldrich; Oakville, Canada)) at 37°C. 1 in 2 dilutions of the intestinal homogenate in blocking buffer were added in duplicates to wells (50µL/well) and incubate for 1 hour at 37°C. Plates were washed four times with PBS/0.05% Tween 20. Anti-Mouse IgA (Fc specific)–Peroxidase antibody produced in goat (Sigma-Aldrich; Oakville, Canada) were diluted 1:10,000, added in all wells (75µL/well) and plates were incubated for one hour at 37°C. The plates were washed and the 3,3',5,5'-Tetramethylbenzidine (Millipore, Billerica, MA) substrate was added (100µL/well). Plates were incubated at Room Temperature (RT) for 15 minutes and 50µL/well of 0.5M sulfuric acid (Sigma-Aldrich; Oakville, Canada) was added to stop the reaction. The plates were read at 450nm. IgA results are expressed as concentrations per gram of intestine (ng/mL/g).

Statistical Analysis

Weight data and IgG ELISA data was analyzed using the software GraphPad Prism 6 (LaJolla, CA). Each time point of each group was compared using a two-way ANOVA with Tukey's multiple comparison test. Our first factor was the different vaccines and the second factor was the time variable. P-values less than 0.05 were considered significant.

IgA ELISA data was analyzed using the software, GraphPad Prism 6 (LaJolla, CA). Each time point of each group was compared using a one-way ANOVA with Tukey's multiple comparison test. P-values less than 0.05 were considered significant.

Results

pET-28a(+) recombinant Gp45 constructs protein expression

Four constructs were previously developed by Karine Sonzogni-Desautels for expression and purification of recombinant Gp45, P2, P23 and Gp900. We wanted to transform these constructs in our vaccine vector *S. typhimurium* YS1646 to evaluate protein expression. Plasmids were isolated from BL21 *E.coli* cells and transformed into YS1646 by electroporation. Protein expression in YS1646 as well as BL21 was then evaluated after IPTG induction. In BL21 cells, expression of the four *C. parvum* recombinant proteins in the bacterial lysates was obtained after IPTG induction via Western Blot analysis (Figure 5A). Proteins detected in the bacterial lysates matched the size of their corresponding recombinant protein in the control wells for the exception of Gp45 (Figure 5A). The recombinant protein appears to be around 60kDa, which was already observed by Dr. Sonzogni-Desautels and is caused by the protein aggregating and forming polymers. In YS1646, no protein was detected in either the supernatants or the bacterial lysates for other cultures (Figure 5B). Non-specific banding at 45kDa in the bacterial lysates of all four cultures and in the negative control group was observed (Figure 5B). Non-specific banding was not found in the supernatant wells. pET-28a(+)-based plasmids were successful in expressing the four antigens in *E.coli* BL21, but not in *S.typhimurium* YS1646 (Figure 5).

Cloning and expression of *C.parvum* Gp45 in pQE30-based constructs.

Two constructs were built through the insertion of a recombinant codon optimized for *C.parvum* Gp45 in the pSspH1 and pSspH2 constructs (Figure 6). The sequences of those constructs were verified by Sanger sequencing (McGill University Genome Quebec innovation centre) and the accuracy of the cloning was demonstrated by sequence alignment (Figure 7). Sequences demonstrated that the promoters, the secretion signals and the optimized recombinant Gp45 gene, form a single open reading frame (Figure 7). Sequences also aligned with a 6X histidine tag at the C-terminal of the constructed gene (Figure 3). Five missense mutations were observed in the SspH2 region of pSspH2-Gp45 at nucleotides 1018, 1194, 1291 and 1310 of sequence 1 (Figure 7A). It is difficult to determine whether missense mutations may have an effect on protein function and binding. Research groups have succeeded to predict such effects by using databases and protein model [205,206]. However, we did not possess the necessary tools to determine the effects of our mutations in the function and binding of the SspH2 secretion signal. Further analysis and modelling is required to fully assess the consequences of those mutations on the folding and function of our chimeric protein.

The constructs were transformed by electroporation into the *S.typhimurium* YS1646 strain. Expression of the target antigen linked to either secretion signal was evaluated by immunoblotting against a histidine tag. The chimeric proteins (SspH1-Gp45 and SspH2-Gp45) were not detected in bacterial lysates or supernatants of axenic cultures (Figure 8A). To determine if the His-tag was problematic, we performed immunoblotting using polyclonal anti-Gp45 primary antibodies. Although the recombinant protein was detected, the chimeric proteins SspH1-Gp45 and SspH2-

Gp45 were not detected in either bacterial lysates or supernatants of axenic cultures (Figure 8B). It is to be noted that we did observe non-specific binding of the anti-Gp45 antibodies to proteins in both supernatants and bacterial lysates of *S.typhimurium* YS1646 (Figure 8B). To verify that protein abundance was not an issue for detection of our chimeric proteins, we lysed the YS1646 vaccine candidates transformed with pSspH1-Gp45 or pSspH2-Gp45 and purified the proteins via column chromatography. After purifying one litre of either vaccine candidate and a negative control using column chromatography, chimeric proteins were not detected by immunoblotting with α-his tag antibodies (Figure 9).

Cloning and Expression of *C.parvum* Gp45 in pWSK129-based Constructs

According to studies assessing various protein-coding genes in *E.coli*, many proteins may be toxic to bacterial cells when overexpressed[207]. Therefore, to control for potential toxicity, we used a low copy number plasmid to evaluate protein expression in our vaccine vector. One construct was built through the insertion of a recombinant codon optimized for *C.parvum* Gp45 in the pWSK129 construct[208] (Figure 10). The sequences of this construct were submitted for Sanger sequencing. However, we were unsuccessful in receiving successful sequences yet and we are in contact with Genome Quebec to optimize sequencing conditions. Nonetheless, expression of the target antigen was evaluated by immunoblotting using α-his tag antibodies. Our recombinant protein was not effectively expressed by the pWSK129 plasmid YS1646 (Figure 11. Altogether, our data suggests that our chimeric proteins cannot be expressed in *S.typhimurium* YS1646 cells. Given that the pET-28a(+)-Gp45 showed successful expression in BL21 but not in *S.typhimurium*,

it is possible that *S.typhimurium* does not have the necessary machinery to express or fold Gp45 properly.

Protective Potential of YS1646 Transformed With pQE30-based Constructs *in Vivo*

Our vaccine candidates did not alter mice weights as they were stable among groups throughout the 6-week study period. (Figure 12). Sera collected throughout the study was analyzed by ELISA to determine Gp45-specific IgG levels. No detectable IgG levels were observed in two of the control animal groups (saline and YS1646 containing pQE-Null). We also observed a significant increase of IgG in the group having received a single dose of Gp45. In the experimental animals having received Gp45 and YS1646 containing pSspH2-Gp45, Gp45-specific IgG was significantly increased at six-week post-vaccination compared to week 0 (Figure 13A). However, there was no significant difference in Gp45-specific IgG in the animals having received Gp45 and YS1646 containing pSspH2-Gp45 compared to animals having received a single dose of recombinant Gp45 (Figure 13A).

In animals having received Gp45 and YS1646 containing pSspH1-Gp45, no significant increase in antigen specific IgG was detected (Figure 13A). The titres of intestinal IgA were also analyzed. None of the mice were found to have significant levels of antigen specific IgA titres compared to the saline control mice (Figure 13B).

S.typhimurium specific antibody titres were also assessed to determine whether the vaccine elicited an unspecific response to the vaccine vector. Heat inactivated *S.typhimurium* YS1646 was coated on a 96-well plate and serum IgG was analyzed. Mice vaccinated with the YS1646 vector had

elevated IgG titres against the vector itself (Figure 14A). In addition, the YS1646-pQE-Null negative control had also increased IgG titres suggesting that mice responded to the vaccine vector independently of the Gp45 expressing plasmids. All groups immunized with the YS1646 vector developed a vector-specific response while a limited response was observed among the saline-treated group and the recombinant protein group (Figure 14A). The immune response was limited in those groups because plates were coated with heat inactivated *S.typhimurium*. Although all groups immunized with a *S.typhimurium* vector showed increased *S.typhimurium* specific IgG titres, only the IM Gp45+PO SspH1-Gp45 group showed a statistically significant increase in IgG titres specific for *S.typhimurium* (Figure 14A).

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Intestinal IgA titres was also analyzed. Mice that received any form of the YS1646 construct showed a significant increase in intestinal IgA titres compared to the PBS group (Figure 14B). As expected, mice immunized with Gp45 alone did not develop a humoral response to the *S.typhimurium* vector (Figure 14). This data shows that our vaccine candidates elicited a strong immune response to the vaccine vector itself.

Figures

Figure 5: Immunoblotting of Gp45, P23, Cp2 and Gp900 in BL21 and YS1646

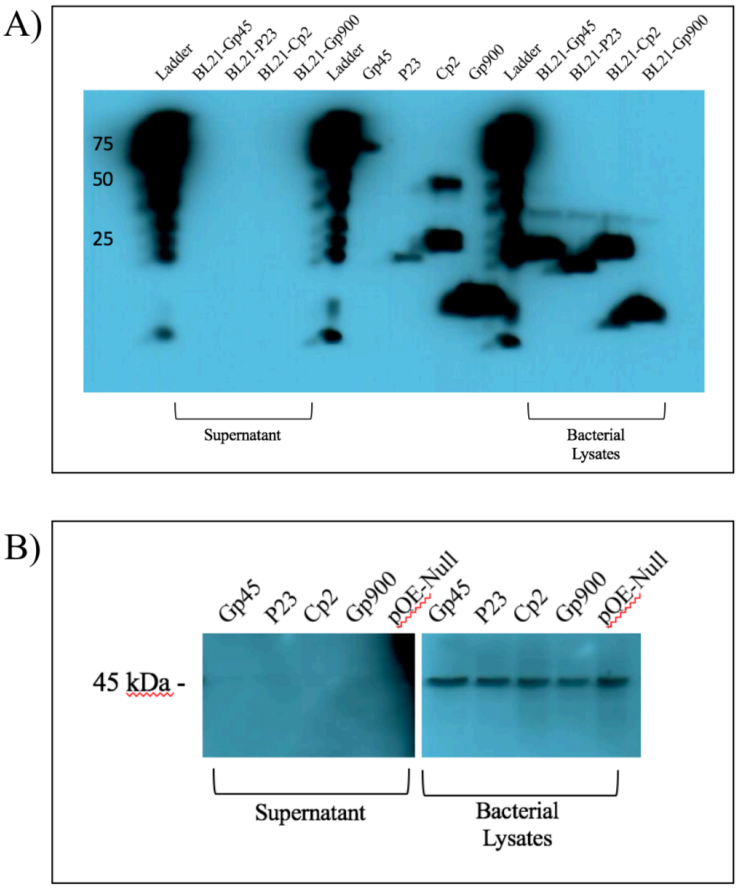

Figure 5: Immunoblotting of Gp45, P23, Cp2 and Gp900 in BL21 and YS1646

The plasmids pET-28-Gp45, pET-28-P23, pET-28-Cp2 and pET-28-Gp900 were transformed into E.coli BL21 (A) and S.typhimurium YS1646 (B). Cultures were induced with IPTG and axenic culture supernatant and bacterial cell lysates were analysed for the expression of recombinant Gp45, P23, Cp2 and Gp900. Recombinant proteins were loaded in (A): labeled Gp45, P23, Cp2 and Gp900 (A). Proteins are detected using a-His tag antibodies.

Figure 6: digestion of pQE-30 based plasmids and recombinant Gp45 gene

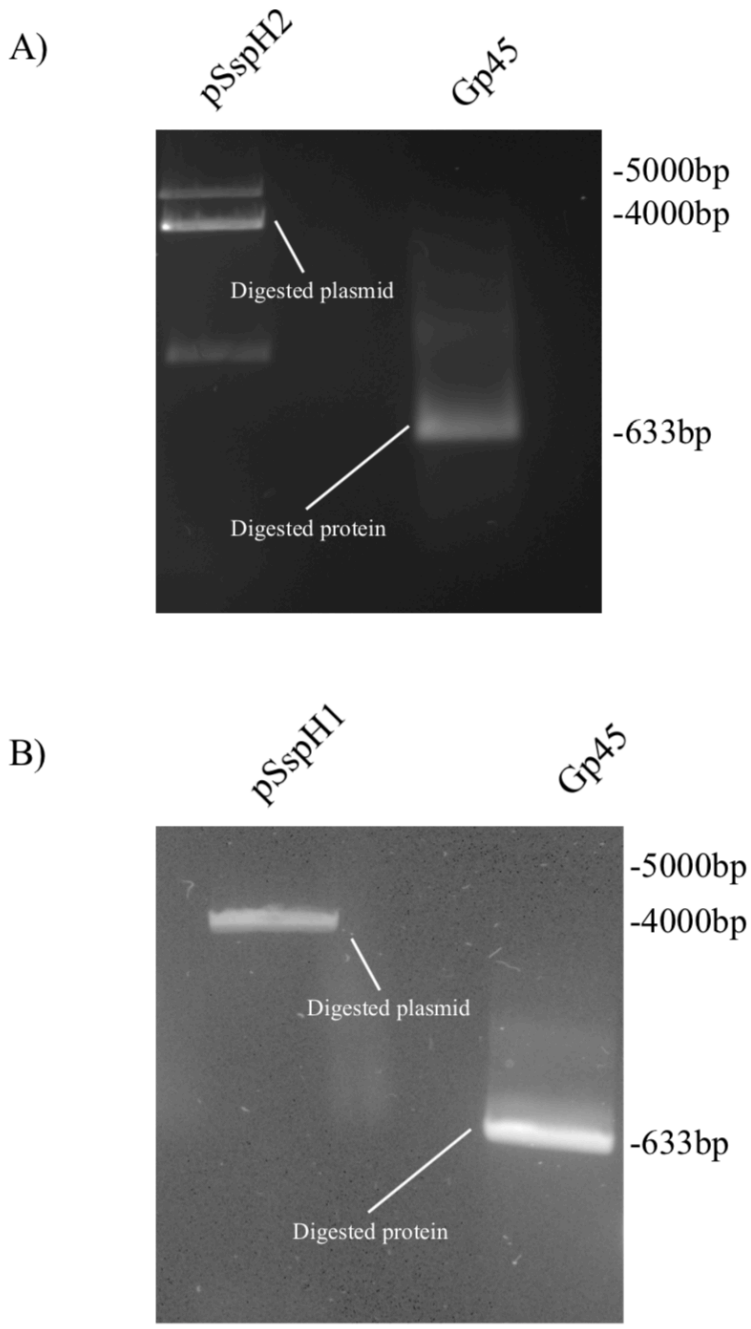

Figure 6: digestion of pQE-30 based plasmids and recombinant Gp45 gene

pQE-30-SspH2 (A) and pQE-30-SspH1 (B) were digested using Not1 and Asc1 restriction enzymes and run on a 0.8% DNA gel. Codon optimized recombinant Gp45 was digested with Not1 and Asc1.

Figure 7: Sequence alignment of pSspH2-Gp45 and pSspH1-Gp45

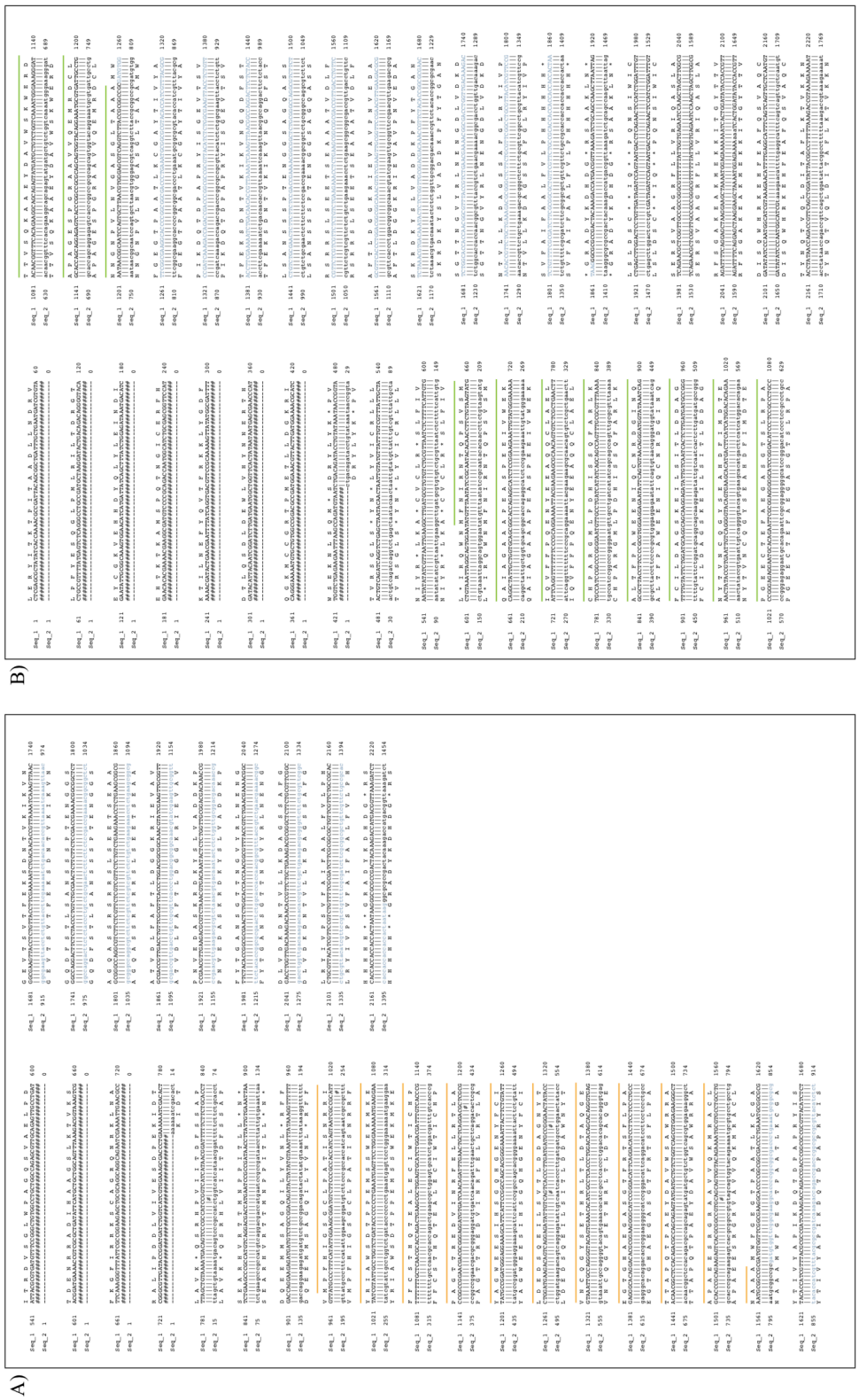

Figure 7: Sequence alignment of pSspH2-Gp45 and pSspH1-Gp45
Sequences in blue are the recombinant Gp45 gene. The sequence labelled by an orange line corresponds to the SspH2 promoter and secretory signal. The sequence labelled by a green line corresponds to the SspH1 promoter and secretory signal.
A) Our constructed pSspH2-Gp45 (seq2) was aligned with the expected theoretical sequence of the plasmid (seq1). Five missense mutations were observed in the SspH2 region of our obtained sequence of pSspH2-Gp45 at nucleotides 1018, 1194, 1291 and 1310.
B) Our constructed pSspH1-Gp45 (seq2) was aligned with the expected theoretical sequence of the plasmid (seq1). We observed no point mutations in the obtained sequence when compared to the theoretical sequence.

Figure 8: Recombinant Gp45 expression

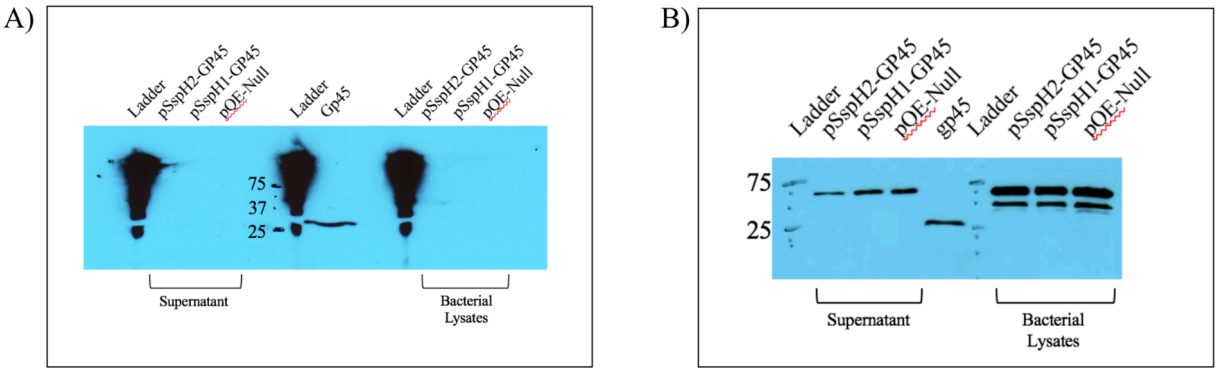

Figure 8: Recombinant Gp45 expression
The plasmids pSspH2-Gp45, pSspH1-Gp45 and pQE-Null were transformed into S.typhimurium YS1646. Axenic culture supernatant and bacterial cell lysates were analysed for the expression of fusion SspH2-Gp45 and SspH1-Gp45. Proteins are detected using a-His tag antibodies (A) or a-Gp45 antibodies (B).

Figure 9: Chromatography purification of pSspH2-Gp45, pSspH1-Gp45 and pQE-Null

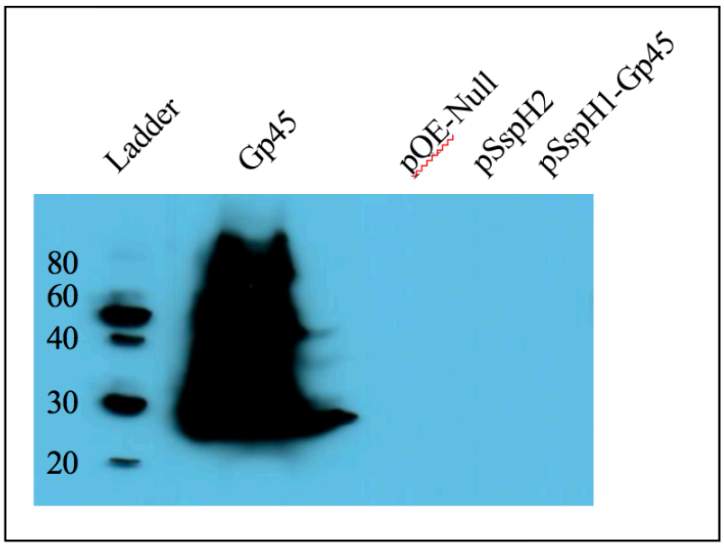

Figure 9: Chromatography purification of pSspH2-Gp45, pSspH1-Gp45 and pQE-Null
The plasmids pSspH2-Gp45, pSspH1-Gp45 and pQE-Null were transformed into S.typhimurium YS1646. Cells were lysed and the chimeric proteins were purified through column chromatography. Protein elutions were analysed for expression of fusion SspH2-Gp45 and SspH1-Gp45. Proteins are detected using a-His tag antibodies.

Figure 10: Digestion of pWSK129 based plasmids and recombinant Gp45 gene

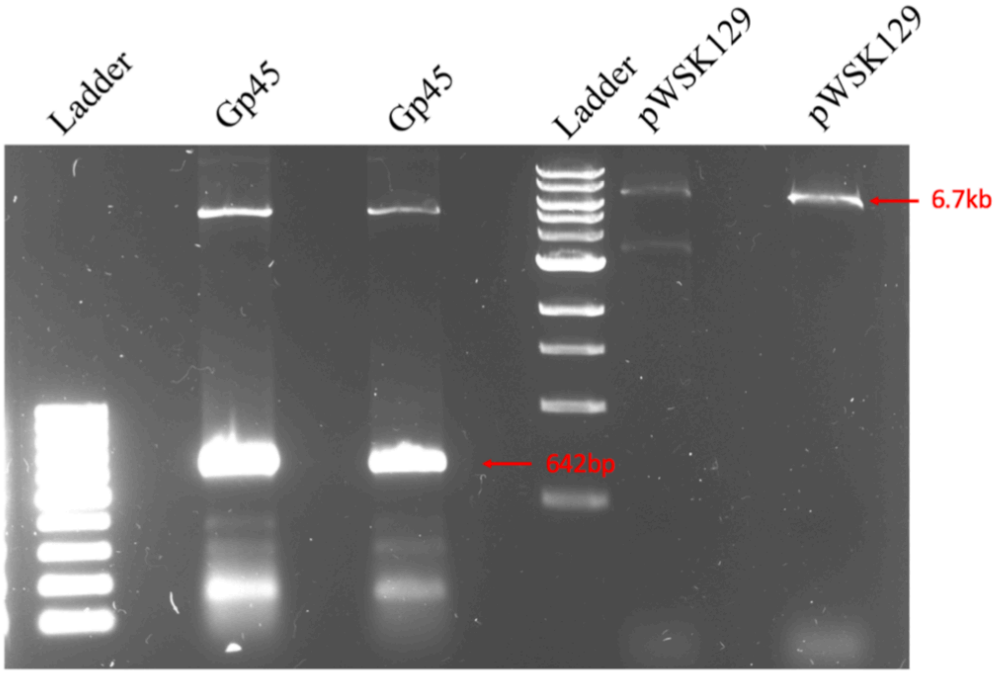

Figure 10: Digestion of pWSK129 based plasmids and recombinant Gp45 gene

Recombinant Gp45 and pWSK129 were digested using Not1 and BamH1 restriction enzymes and run on a 0.8% DNA gel. Both Gp45 and pWSK129 were performed in duplicates.

Figure 11: Immunoblotting of the pWSK129-Gp45 construct in YS1646

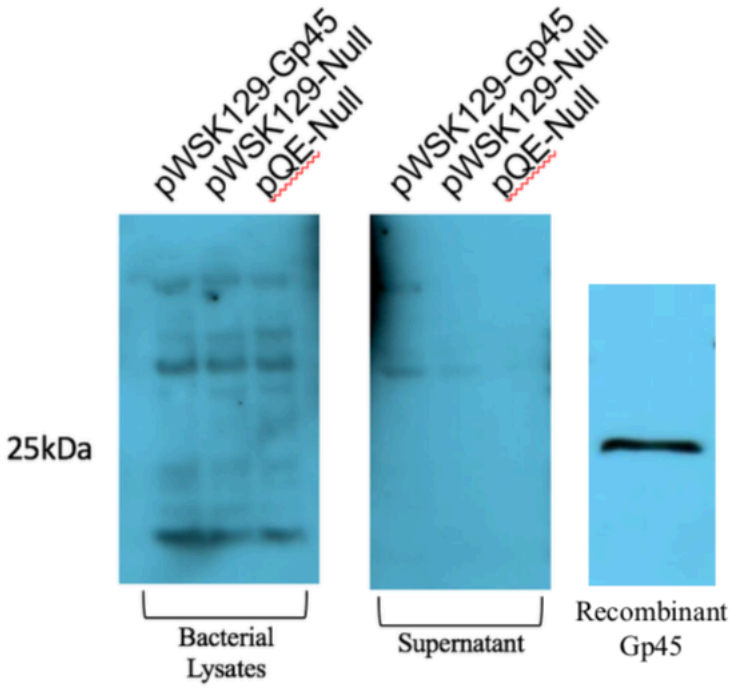

Figure 11: Immunoblotting of the pWSK129-Gp45 construct in YS1646

The plasmids pWSK129-Gp45, pWSK129 (empty vector) and pQE-30 were transformed into S.typhimurium YS1646. Axenic culture supernatant and bacterial cell lysates were analysed for the expression of recombinant Gp45. Proteins are detected using a-His tag antibodies.

Figure 12: weight changes in mice afterYS1646 immunization

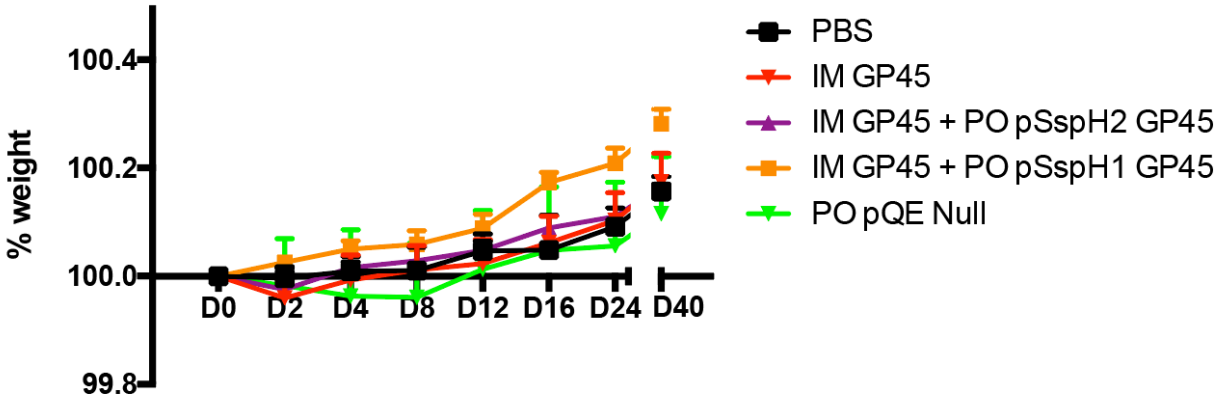

Figure 12: weight changes in mice afterYS1646 immunization
The weight of each mouse was recorded for six weeks after immunization. Mice were immunized at days 1, 3 and 5 and sacrificed on day 40.Weights are displayed as the percent of original weight on day 0 ± standard error. n=5

Figure 13: Humoral IgG response against Gp45

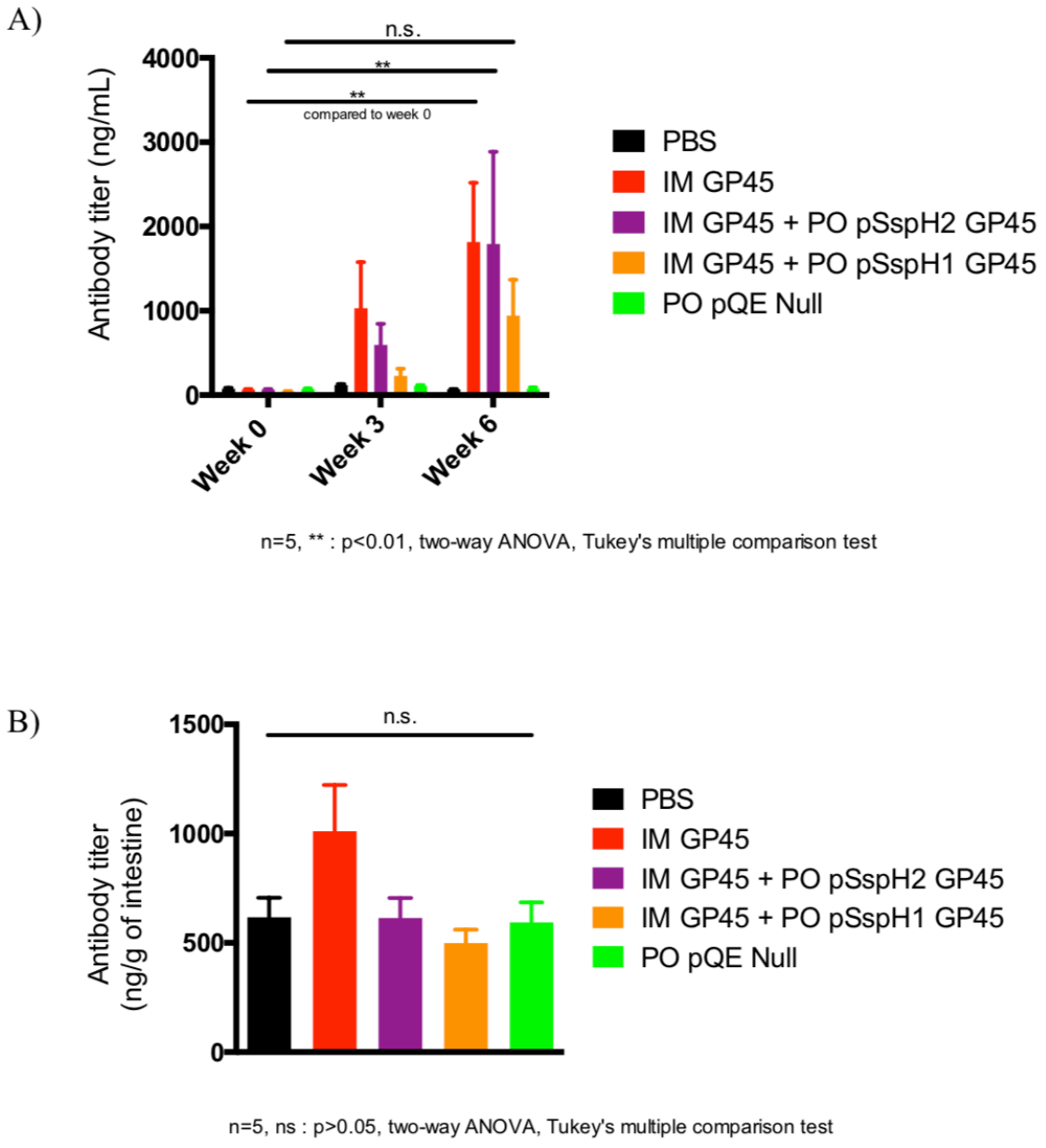

Figure 13: Humoral IgG response against Gp45

Gp45 specific total serum IgG (A) and intestinal IgA (B) in immunized mice: saline control animals received three doses of saline, YS1646 controls received three doses of YS1646-pQE-Null, Gp45 controls received a single dose of 40µg Gp45 IM, and the experimental animals received a single dose of 40µg Gp45 in combination with three doses of 10^9cfu YS1646 transformed with pSspH2-Gp45 or pSspH1-Gp45. Serum IgG results are presented for weeks 0, 3 and 6 and expressed as antibody titers (ng/mL) ± standard error (A) and intestinal IgA results are presented for week 6 and expressed as antibody titers per gram of intestine (ng/g) ± standard error (B).

Figure 14: Humoral response against *S.typhimurium* YS1646

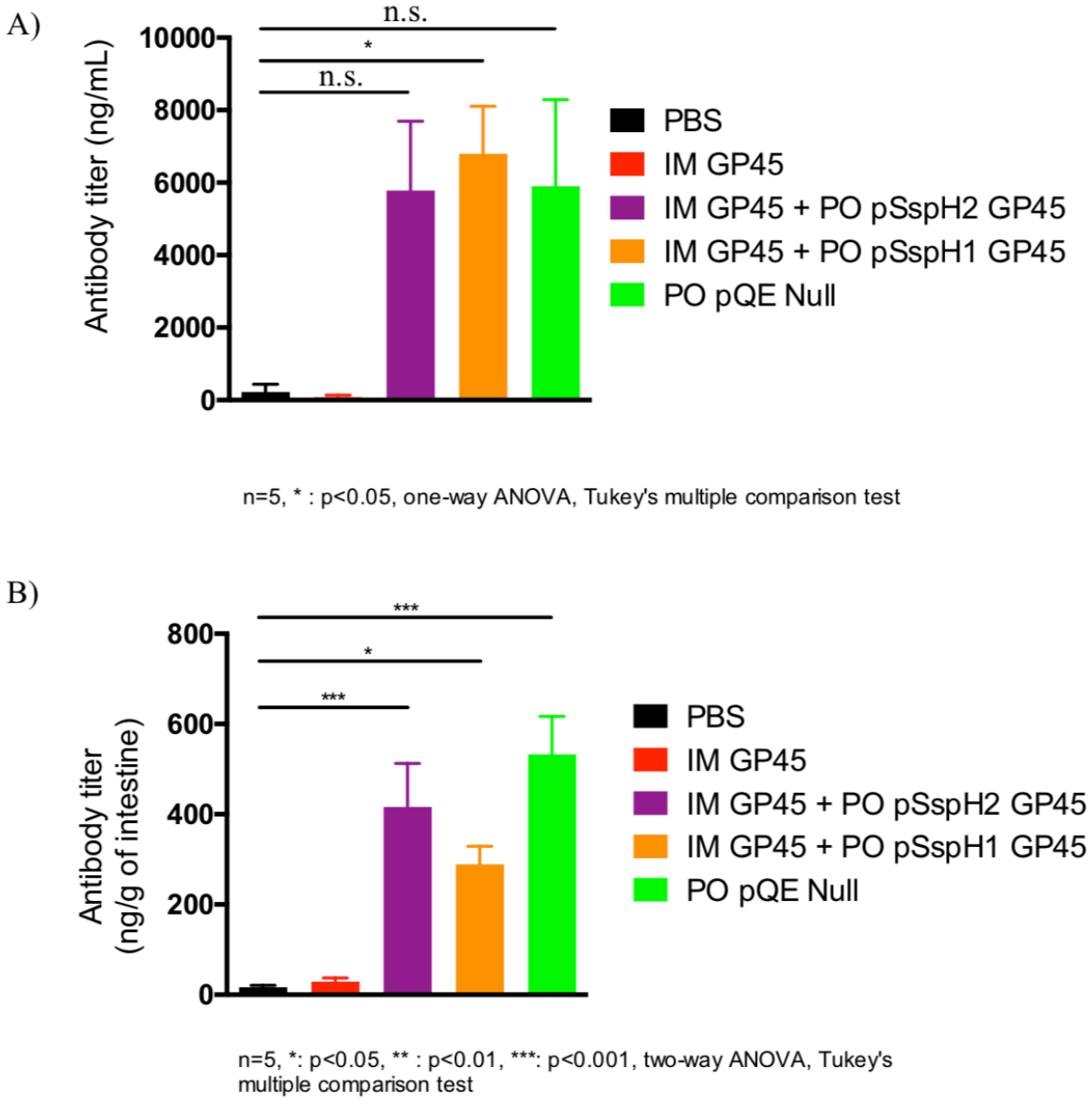

Figure 14: Humoral response against *S.typhimurium* YS1646

YS1646 specific total serum IgG (A) and intestinal IgA (B) in immunized mice: saline control animals received three doses of saline, YS1646 controls received three doses of YS1646-pQE-Null, Gp45 controls received a single dose of 40µg Gp45 IM, and the experimental animals received a single dose of 40µg Gp45 in combination with three doses of 10^9cfu YS1646 transformed with pSspH2-Gp45 or pSspH1-Gp45. Serum IgG results are presented for week 6 and expressed as antibody titers (ng/mL) ± standard error (A) and intestinal IgA results are presented for week 6 and expressed as antibody titers per gram of intestine (ng/g) ± standard error (B).

Discussion

The lack of effective treatment against cryptosporidiosis and the gravity of this disease emphasizes the need for an effective vaccine which would be the key in reducing the worldwide rate of diarrhea and co-morbidities associated with this disease[23,209]. No human vaccine is currently available to prevent cryptosporidiosis, but we believe that using novel approaches and technology, developing one is attainable. Previous research in our laboratory has demonstrated the protective potential of four *C.parvum* antigens, notably the recombinant Gp45. In combination with various adjuvants, intramuscular recombinant Gp45 was proven to be immunogenic and protective against *C.parvum* in mice (Karine Sonzogni-Desautels, in preparation). We wanted to use a novel vaccine vector approach to improve on this vaccine and develop a vaccine candidate that is easy and inexpensive to produce, while being orally administrable for ease of delivery in developing countries.

The attenuated *S.typhimurium* strain YS1646 passed phase 1 clinical trial and was therefore shown to be safe when administered IV in cancer patients[210]. This strain was also proven to be safe when orally delivered to mice up to 10^9cfu[211]. Similar attenuated *Salmonella* strains have previously been used to deliver *Cryptosporidium spp.* antigens to mice. A group demonstrated the immunological potential of *S.tpyhimurium* SL3261 expressing Cp23 or Cp40[162]. Other successes support the attainability of our project[161,163].

We initially used four inducible plasmids designed by a PhD student in our lab in the YS1646 bacteria to assess for protein expression in *S.typhimurium*. The pET-28a(+)-based constructs were originally developed to express and purify four *C.parvum* recombinant antigens: Gp45, Cp2, P23 and Gp900 (Karine Sonzogni-Desautels, in preparation). The constructs were transformed in *E.coli* BL21, a strain used for protein expression, and *S.typhimurium* YS1646. Bacteria were grown in axenic culture and induced with IPTG. In *E.coli* BL21, protein expression was detected in the bacterial lysates of all four constructs, but not in the supernatant (Figure 5A). This suggests that proteins were expressed intracellularly and not secreted. This is to be expected since the plasmids did not contain secretion signals. In *S.typhimurium* YS1646 however, we did not detect protein expression from any of the four constructs in either bacterial lysates or supernatants (Figure 5B). It is important to note that other groups have successfully induced protein expression with IPTG in various *S.typhimurium* strains[212,213].

The absence of protein detection may be caused by the absence of protein expression by the four constructs in YS1646. *S.typhiurium* was reported to be biased towards certain codons (Brandis, 2016). This would result in the bacteria not being able to synthesize the proteins because of a lack of complementary tRNA. We therefore proceeded to optimize the codon sequence of our recombinant Gp45 and make our own custom constructs specifically designed for *S.typhimurium* expression. We were successful in the construction of two plasmids for the expression of recombinant Gp45 in *S.typhimurium*. Sanger sequencing confirmed the experimental sequence of our recombinant Gp45 to be identical with our predictions (Figure 7); however, we did observe missense mutations in the SspH2 gene leading to non-conservative amino-acid changes (Figure 7A). The first substitution mutation at amino acid 1018 (in reference to sequence 1) falls in the

promoter region of SspH2 and therefore could have an impact in expression of the SspH2-Gp45 fusion protein expression if the mutation alters any aspect of protein binding[214]. The other mutations in SspH2 may have an effect on the proteins structural stability or folding[9]. We decided nonetheless to move forward with both constructs.

We performed western blotting on axenic cultures of three constructs in YS1646: pSspH1-Gp45, pSspH2-Gp45 and pQE-Null (an empty vector for control). Using α-His tag antibodies, chimeric Gp45 expression could not be detected for any of our constructs in either the bacterial lysates or the supernatants (Figure 8A). To confirm the absence of protein expression from our constructs in YS1646 and to rule out the possibility of the His tag not being properly detected because of improper folding or truncation, we performed immunoblotting using polyclonal antibodies specific for recombinant Gp45 (Karine Sonzogni-Desautels, in preparation). We observed non-specific binding in both bacterial lysates and supernatants, but we could not detect the expression of our chimeric protein from our constructs (Figure 8B).

To assess whether protein abundance was an issue, we performed column chromatography purification on 1L cultures for each of our constructs. Using α-His tag antibodies, chimeric Gp45 expression was not detected from any of our constructs (Figure 9).

The absence of protein detection from our constructs may be due to incorrect protein folding or inability of *S.typhimurium* to express our recombinant antigens[215]. As an attempt to solve this issue, we transformed our codon optimized recombinant Gp45 in pWSK129, a low copy number plasmid which was originally used for this purpose[208] (Figure 10). We hoped that reducing

plasmid number and protein expression would be sufficient to prevent bacterial cell death. We then successfully transformed our construct in *S.typhimurium* YS1646 and performed western blotting on axenic cultures. It is to be noted that we experienced much less difficulty with the cloning of this construct: we obtained more colonies and had higher transformation efficiency. We did not detect protein expression in either culture supernatants or bacterial lysis for our pWSK129-based constructs (Figure 11). As this did not solve our issue, it is possible that the protein cannot be expressed by the *S.typhimurium* YS1646 vector because of issues with expression or improper folding.

A member of our lab had demonstrated that a YS1646 vaccine against *Clostridium difficile* with no detectable protein expression was successful in inducing a specific and protective immune response (Kaitlin Winter, in preparation). We therefore wished to determine the immunological potential of our vaccine strains. We orally immunized mice with 10^9cfu of YS1646 transformed with SspH1-Gp45 and SspH2-Gp45 vaccines. Mice did not seem to be affected by this vaccine as mice from all group steadily gained weight throughout the experiment (Figure 12). Mice were euthanized at week 6 and serum samples from week 0, 3 and 6 were analyzed by ELISA to determine the IgG and IgA response. At week 6, we observed a significant increase in IgG titres in mice vaccinated with a single dose of Gp45 and mice vaccinated with Gp45 followed by YS1646 containing SspH2-Gp45 compared to week 0 (Figure 13A). However, mice having received Gp45 followed by YS1646 containing SspH1-Gp45 did not show a significant increase in IgG titres at week 6 compared to week 0 (Figure 13A). The IM Gp45 + PO YS1646-SspH1-Gp45 group's IgG titres were half of the other two experimental groups. A reduction in IgG titres may be explained by the isotype switching caused by the adjuvant properties of our live attenuated *S.typhimurium*

vaccine. There was no significance difference in IgA titres between groups at week 6 (figure 13B). These results indicate that our vaccine candidate transformed with SspH2-Gp45 was as effective as a single dose of recombinant Gp45 at inducing an IgG-type humoral immune response. None of our vaccine candidates were shown to be effective at inducing a mucosal IgA immune response.

We investigated whether mice developed an immune response against the vaccine vector. Our findings show that mice immunized with our YS1646 vector developed a humoral and mucosal response to *S.typhimurium* YS1646. We observed elevated titres of serum IgG and intestinal IgA specific to *S.typhimurium* YS1646 (Figure 14B). These results indicate that our vaccine candidates transformed with SspH2-Gp45 or SspH1-Gp45 elicited a response against the YS1646 vector. This may represent an issue for the use of similar vaccine vectors in the future.

This suggests that our YS1646 vaccine candidates are not a beneficial addition to the current recombinant Gp45 vaccine developed in our laboratory. Furthermore, the use of the SspH1-Gp45 construct was detrimental of the humoral response elicited by the recombinant Gp45 and all *S.typhimurium* YS1646 vaccine vectors elicited a specific immune response which could potentially prevent effective future immunization with this same vector. A potential solution to this, however, may be to use different strains to prevent pre-existing immunity to affect effective vaccine delivery in subsequent vaccinations[216].

In summary, we were able to construct and evaluate several expression vectors for the delivery of recombinant Gp45 through *S.typhimurium* YS1646 as vaccine candidates. We failed to demonstrate expression of our chimeric or recombinant protein in the YS1646 vector in various

conditions using different purification and detection methods. Our vaccine candidates did show immunological potential in mice as our mice did develop an immune response against the vaccine vector. However, our vaccines did not show an improvement of the immunological potential compared to the recombinant Gp45 protein alone. Nonetheless, other experimental vaccine vectors against cryptosporidiosis are available and were did not appear to have similar issues. We therefore remain confident in the capacity of vaccine vectors in effectively preventing cryptosporidiosis and alleviating burden in those affected by it.

Future Directions

Several steps are needed to move this project forward and optimized an effective vaccine candidate for future research. Groups have developed software to determine the toxicity of certain peptides[217,218]. Even if we never tested for possible Gp45 toxicity in *S.typhimurium,* using such software might help us determine the best peptide region from Gp45 to use for our YS1646 vector. This could improve the chances of developing an efficient vaccine candidate. Another issue we can encounter when expressing and secreting proteins in *S.typhimurium* is improper protein folding, resulting in failure of protein secretion from the type 3 secretion system. Several tools are available to determine protein structure, but for the purpose of our work, it would be less expensive to create several versions of our recombinant protein and perform secretion assays.

Additionally, we may wish to develop other vaccine vectors. We performed our immunization study in immunocompetent mice, although our primary targets for this cryptosporidiosis vaccine are children and immunocompromised individuals. There is fear that a

live attenuated vaccine may be dangerous and potentially life threatening for those groups. To assess the safety and efficacy of our vaccine in an immunocompromised model, we could deliver our vaccine to mice lacking the IFNγR, which is an immunocompromised mice model we have access to.

In the event that the vector is lethal to those mice, we may wish to use a more attenuated vector for vaccine delivery. Another group at McGill University developed several attenuated strains of Entero-pathogenic *E.coli* (EPEC)[219]. EPEC contains a T3SS that closely resembles the one from *S.typhimurium* and we therefore believe that an attenuated strain of EPEC could serve as an effective vaccine candidate for future studies. More importantly, the various levels of attenuation would give us more control to make our vaccine candidate safe yet effective in immunocompromised mice.

Furthermore, once a secreting construct is developed, we would need to investigate different vaccine administration schedules to determine the most immunological one. Multimodal and prime-boost vaccine approaches have been proven to be very effective[220]. Therefore, it would be interesting to have a similar approach with the YS1646 vaccine candidates and evaluate the effect of different immunization schedule on the immune response in mice.

This work demonstrates the first steps of our team in developing a vaccine vector against cryptosporidiosis. There is a growing need for prevention against cryptosporidiosis in both developing and developed countries, as it is an important threat and cause of child mortality and stunting. Parasitic diseases are extremely complex and there is currently no marketed vaccine for

cryptosporidiosis. New technological advancements and tools are therefore crucial in developing prophylactic vaccines against parasites. We believe this study brings us a step further in understanding the complexity of such task and brings us closer to the development of an effective vaccine against cryptosporidiosis.

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