Generation and evaluation of SMT-expressing *Lactococcus lactis* live vaccines against visceral leishmaniasis

Raya Mahbuba

Department of Microbiology and Immunology
McGill University, Montreal

June 2011

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract

Lactococcus lactis is a Gram-positive lactic acid bacterium commonly used in the dairy industry. It has been deemed safe for human consumption by the United States Food and Drug Administration and holds Generally Recognised As Safe (GRAS) status. *L. lactis* is non-pathogenic, non-colonizing and non-invasive. The utility of this bacterium as a cell "factory" able to synthesis heterologous proteins is now well established. Previous work in our lab has also shown that *L. lactis* has the ability to stimulate the innate immune system. All these properties make *L. lactis* a suitable vector for biological delivery of antigens to stimulate an immune response.

Visceral leishmaniasis is a disseminated protozoan infection, caused by the species of the *Leishmania donovani* complex, which affects the liver, spleen and bone marrow. There are 500,000 new cases of visceral leishmaniasis every year and 90% of clinical cases are fatal if left untreated. In addition to the disadvantages of high cost and side effects of chemotherapy, there is also emerging resistance of parasites to anti-leishmanial drugs. In light of these facts, the development of a vaccine against visceral leishmaniasis is a priority.

We have engineered *L. lactis* (strains NZ9000 and PH3960) to heterologously express the protective *Leishmania* antigen, sterol 24-c-methyltransferase (SMT), in the cytoplasm. In separate studies, these strains of *L. lactis* were assessed for their protective capability as live vaccines against *L. donovani* in BALB/c mice, both individually and adjuvanted with IL-12-secreting *L. lactis*. Immune responses to subcutaneous immunizations were monitored by measuring serum antibody titers against SMT and cytokine production from splenocytes upon stimulation by soluble *Leishmania* antigen at the end point of infection. Mice immunised with SMT-expressing clones of *L. lactis* NZ9000 had high titers of both serum IgG1 and IgG2a as well as splenocyte cytokine response profiles which demonstrated a mixed Th1/Th2 response. The co-administration of SMT- and murine IL-12-producing *L. lactis* clones was able to reduce parasite load in the liver significantly in the case of

L. lactis NZ9000. Overall, we demonstrated that *L. lactis* is a suitable vehicle for the delivery of vaccine antigens and generation of protective immune responses against visceral leishmaniasis.

Résumé

Lactococcus lactis est une bactérie lactique Gram-positive couramment utilisée dans l'industrie laitière. Elle a été jugée sans danger pour la consommation humaine par la “United States Food and Drug Administration” et elle détient le statut “GRAS” (Generally Recognised As Safe). *L. lactis* est non pathogène, non-colonisatrice et non-invasive. L'utilité de cette bactérie comme une cellule «usine» capable de synthétiser des protéines hétérologues est maintenant bien établie. Des travaux antérieurs dans notre laboratoire ont également montré que *L. lactis* a la capacité de stimuler le système immunitaire inné. Toutes ces propriétés font de *L. lactis* un vecteur approprié pour l’administration biologique d’antigènes afin de stimuler une réponse immunitaire.

La leishmaniose viscérale est une infection protozoaire diffuse, causée par les espèces du complexe *Leishmania donovani*, qui affecte le foie, la rate et la moelle osseuse. Chaque année, 500,000 nouveaux cas de leishmaniose viscérale sont déclarés et 90% des cas cliniques sont mortels s'ils ne sont pas traités. En plus des inconvénients de coût élevé et les effets secondaires de la chimiothérapie, un autre problème majeur est l’émergence de parasites résistants aux médicaments anti-*Leishmania*. C’est pourquoi le développement d'un vaccin contre la leishmaniose viscérale est une priorité.

Nous avons modifié *L. lactis* (souches NZ9000 et PH3960) afin qu’elle exprime l'antigène protecteur de *Leishmania*, stérol 24-C-méthyltransférase (SMT), de façon hétérologue dans le cytoplasme. Dans des études distinctes, ces souches de *L. lactis* ont été évaluées pour leur capacité de protection comme vaccins vivants contre *L. donovani* chez la souris BALB/c, individuellement, mais aussi en présence de *L. lactis* sécrétant de l’IL-12 en tant qu’adjuvant. Les réponses immunitaires à ces vaccinations sous-cutanées ont été suivies en mesurant les titres d'anticorps sériques dirigés contre SMT et la production de cytokines provenant de splénocytes stimulés par les antigènes solubles de *Leishmania* au point final de l'infection. Les souris immunisées avec les clones de *L. lactis* NZ9000 exprimant SMT montrent des taux sériques élevés d’IgG1 et

d’IgG2a, ainsi qu’un profil de cytokines splénique démontrant une réponse mixte Th1/Th2. La co-administration de clones de *L. lactis* exprimant SMT et de l’IL-12 murin a permis de réduire la charge parasitaire dans le foie de manière significative dans le cas de *L. lactis* NZ9000. Dans l'ensemble, nous avons démontré que *L. lactis* est un vecteur approprié pour l’administration d’antigènes vaccins et la génération de réponses immunitaires protectrices contre la leishmaniose viscérale.

Acknowledgements

First and foremost, I thank my supervisor, Dr. Benoit Cousineau for giving me the opportunity to work in his lab and further my education. Thank you Benoit for your guidance and wisdom over the past two years. You've made me a better scientist and for this I am extremely grateful. I will remember the lessons always.

I also thank Dr. Martin Olivier, both for his contribution to our research project and for taking a chance on me three summers ago and letting me work in his lab. I would not be doing a Master's now if I wasn't a summer student first.

Team Vaccine! Karen: I've said this before and I'll say it again, I would be running around like a headless chicken if it weren't for you. Thank you for all that you've done for me- in the lab, outside the lab, even after you left the lab.
Felix: Thank you for putting up with my never-ending questions and helping take blood even though it melts your brain.

Team Intron! Cecilia: Thank you for your advice and hugs in the lab and as well as the training outside the lab. Lauren: My second year would not have been nearly as fun if you weren't there, nor would my knowledge of memes have been as complete. Caroline: Thank you for always being calm and patient even when I didn't follow "the system" as diligently as I should have.

MOL lab! It has been a wonderful three years knowing you. Thank you for all the fantastic lunch conversations and making things more fun and sillier. I could not have asked for better neighbours. Ben: Thank you for always being across the hall with a warm hug and an attentive ear.

A big thank you to my friends and family for putting up with me even though I disappeared for months at a time and staying in touch.

Ma: Thank you for *everything*!

Finally I would like to acknowledge the Canadian Institutes for Health Research (CIHR), McGill University and the Centre for Host-Parasite Interactions (CHPI) for their financial of this research.

1. Literature review

1.1 *Lactococcus lactis*

Lactococcus lactis is part of a taxonomically diverse group known as lactic acid bacteria (LAB). LAB are Gram-positive, non-sporulating bacteria characterised by their ability to produce lactic acid upon carbohydrate fermentation. They occupy niches such as milk and plant surfaces [1]. Certain species of LAB are also part of the endogenous mucosal microbiota of vertebrates e.g. *Lactobacillus plantarum* and *Streptococcus gordonii*. LAB have been used throughout history in the production of fermented dairy products (cheese and yoghurt) and other fermented food such as pickled vegetables. Since LAB have a long history of safe consumption by humans, dietary LAB have been given Generally Recognised as Safe (GRAS) status by the United States Food and Drug Administration (US FDA) as well as Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) [2]. In addition to their use in the food industry, LAB are used as unicellular "factories" for heterologous proteins.

Various genetic tools have been developed for use with *L. lactis* and the complete genome sequence of several strains is available (*L. lactis* subsp. *cremoris* MG1363 [3,4], *L. lactis* subsp. *cremoris* NZ9000 [4], *L. lactis* subsp. *cremoris* SK11 [5], *L. lactis* subsp. *lactis* IL1403 [6], *L. lactis* subsp. *lactis* CV56 [7] and *L. lactis* subsp. *lactis* KF147 [8]); thus making *L. lactis* a model LAB. Based on certain metabolic phenotypes, there are two subspecies and one biovar of *L. lactis*– subsp. *cremoris*, subsp. *lactis* and subsp. *lactis* biovar *diacetylactis* [9]. *L. lactis* may also be divided genotypically into two identically named subspecies– subsp. *cremoris* and subsp. *lactis* – although, genotype and phenotype classifications do not always agree. The *lactis* subspecies (genotype and phenotype) is more commonly found in nature. Based on chromosomal diversity, it has been hypothesised that the dairy starter strains of *L. lactis* (phenotypically subsp. *cremoris* and subsp. *lactis* biovar *diacetylactis*) evolved from *L. lactis* found on plants [9]. These dairy starter strains lack several

metabolic gene functions, thought to be an adaptation to being cultured in nutrient-rich milk, and consequently, their chromosomes are also smaller than wild-type strains.

The most widely used LAB for research purposes are the *L. lactis* strains MG1363 and NZ9000. MG1363 has a *lactis* phenotype and a *cremoris* genotype. It is a plasmid-free and prophage-cured derivative of the NCDO712 dairy strain [10]. NZ9000 is derived from MG1363 and its *nisRK* genes are integrated into the *pepN* locus (discussed in section 1.1.2). The NZ3900 strain is also derived from MG1363. It is a *lacF* deletion mutant and, like NZ9000, its *nisRK* genes are located in the *pepN* locus [11]. A further mutation in the gene encoding alanine racemase in NZ3900 created the PH3960 strain [12] (discussed in section 1.1.2).

1.1.1 Inflammatory properties of *L. lactis*

Caution must be taken when extrapolating observed immune responses between *L. lactis* strains. In one study, 46 *L. lactis* strains were incubated with macrophages to monitor the resulting interleukin (IL)-6, IL-12 and tumour necrosis factor (TNF) secretion [13]. These strains, representing the two subspecies and one biovar *L. lactis*, were isolated from milk, plants and their products. The induction of immune responses were strain-specific only and unrelated to subspecies, biovar or origin of isolate.

Norton and colleagues examined mucosal and serum antibodies in BALB/c mice before and after inoculation with *L. lactis* MG1363 [14]. They found that uninoculated young mice already possessed serum and mucosal antibodies against *L. lactis* protein extracts. These antibodies cross-reacted with protein extracts from other LAB, namely *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Streptococcus thermophilus*. Serum IgG levels declined as mice aged indicating that these may be maternal antibodies. However, serum anti-lactococcal IgM increased with age. The authors suggest that these might be a result of exposure to environmental LAB.

Yam and colleagues evaluated chemokine mRNA and cytokine expression from leukocytes recruited into murine air pouches containing *L. lactis* NZ9000

[15]. 95% of recruited cells were neutrophils, followed by eosinophils, monocytes, macrophages (Mϕ) and lymphocytes. These cells were found to contain CCL1, CCL2, CCL3, CCL4, CXCL2 and CXCL10 mRNA at significantly higher levels than the negative control. These chemokines are involved in recruiting various leukocytes [16]. However, an *in vitro* co-incubation assay of bone marrow-derived Mϕ and *L. lactis* showed lower levels of chemokine mRNA. *In vitro* data also showed that *L. lactis* is able to induce expression of IL-1β, IL-12p40 and IL-10 cytokine mRNA as well as maturation of dendritic cells (DC).

More recently, *L. lactis* subsp. *cremoris* MG1363 was found to possess an unusual pellicle, a thin compact outer layer composed of hexasaccharide phosphate repeating units [17]. The authors suggest that the pellicle may prevent interaction between the host's innate immune receptors and the bacterial cell wall, thus preventing phagocytosis. Indeed, wild-type bacteria were more resistant to phagocytosis by murine macrophages compared to pellicle-deficient mutants in an *in vitro* phagocytosis assay. Thus the pellicle may be similar in function to cell wall capsules of Gram-positive pathogenic bacteria which also provide resistance to phagocytosis.

1.1.2 *Lactococcus lactis* as a delivery vector

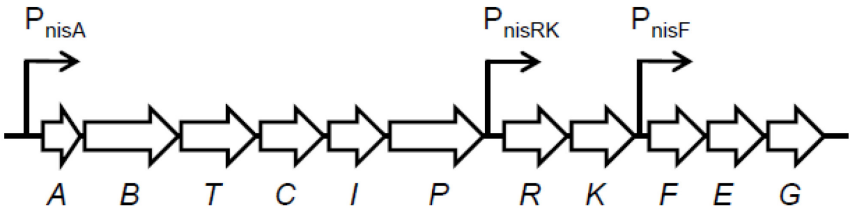
Over the past two decades, a vast body of research has helped establish *L. lactis* as an excellent delivery vector for biological molecules [18]. A large number of proteins have been produced from *L. lactis* using various plasmids and gene expression systems. A widely used expression system is the Nisin-Controlled Gene Expression (NICE) system which has been used in medical, pharmaceutical and food technology fields [19]. The NICE system is easy to use, can be tightly controlled and allows for the production of large amounts of protein. Other inducible and constitutive promoters have been developed but the NICE system is one of the most widely used.

Nisin is a 34-amino acid pore-forming antibacterial peptide (or bacteriocin) produced by *L. lactis*. Nisin, which is effective against other Gram-

positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *Enterococci*, *Staphylococci* and *Streptococci*, is commonly used as a preservative in the food industry. Like *L. lactis*, nisin holds GRAS and QPS status from the US FDA and the EFSA, respectively. [20]. The nisin operon consists of three promoters and eleven genes (Fig. 1A). P_{nisA} and P_{nisF} are nisin-inducible and P_{nisRK} is constitutive. P_{nisA} controls transcription of nisin precursor (NisA), proteins that modify, translocate and process nisin (NisB, NisT, NisC and NisP) and a protein involved in immunity against nisin (NisI). P_{nisRK} controls transcription of the regulator-sensor proteins (NisR and NisK). PnisF controls transcription of more proteins involved in immunity against nisin (NisF, NisE and NisG). The strains typically used in the NICE system have inactivated *nis* operons such that only the nisin-inducible promoter, P_{nisA}, and the regulator-sensor two-component system, *nisRK*, are functional. Exogenous nisin binds the histidine-protein kinase, NisK, causing it to phosphorylate itself and the response regulator, NisR (Fig. 1B). Activated NisR is then able to induce transcription from P_{nisA}. The minimum inhibitory concentration (MIC) of nisin is 14 ng/ml [10]. Since protein production by nisin induction is carried out at concentrations below the MIC (0.01 to 10 ng/ml), mutant strains lacking genes for nisin immunity are able to tolerate induction conditions and remain viable. There is a linear dose-response to protein production induced by nisin and levels of induced protein can be as high as 50%-60% of total cell protein [10].

The *L. lactis* expression system can also be used to direct recombinant protein to one of three locations– the cytoplasm, the cell wall or outside the cell by secretion [21]. Cytoplasmic expression is achieved with the absence of signal peptides or anchoring domains in the final protein. Different N-terminal signal peptides have been used in the *L. lactis* system to allow protein secretion. Of these, the most efficient and widely used is the signal peptide of Usp45, the major secreted protein of *L. lactis*. At the cell wall, cleavage of the signal peptide releases recombinant protein to the outside environment. Addition of a nine-residue peptide containing negatively charged residues to the Usp45 signal peptide further enhances protein expression and secretion [22]. Addition of an

(A)



(B)

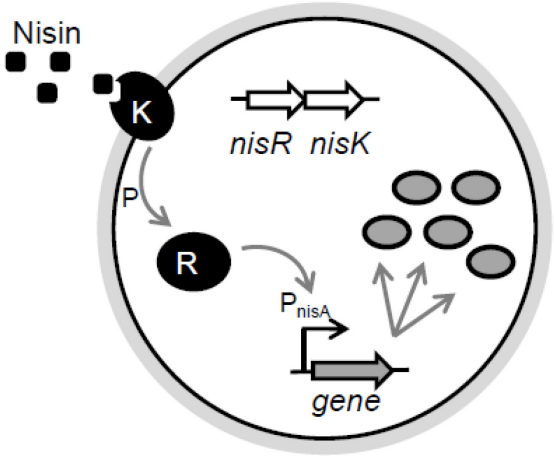


Figure 1. Overview of the Nisin-Controlled Gene Expression (NICE) system. **(A)** Schematic diagram of the nisin gene cluster. P_{nisRK} is a constitutive promoter and controls production of NisR and NisK, the two-component system which regulates the nisin gene cluster. P_{nisA} and P_{nisF} can be induced by nisin. *A* encodes the nisin A structural gene; *B*, *T*, *C* and *P* are involved in post-translational modifications and translocation of nisin; *I*, *F*, *E* and *G* are involved in immunity against nisin. **(B)** Expression of heterologous gene products. NisK and NisR are constitutively expressed. Nisin is recognised by NisK which phosphorylates NisR. NisR induces transcription of the desired gene product which is under control of the P_{nisA} promoter. Adapted from [20].

N-terminal signal peptide as well as a C-terminal cell wall anchoring tag allows for attachment of recombinant protein to the bacterial cell wall. After the signal peptide is cleaved, recombinant protein stays attached to peptidoglycan by an amide bond via the anchoring tag. The most prevalent cell wall anchor motif is that of the M6 protein from *Streptococcus pyogenes* [21].

Production of heterologous protein by *L. lactis* can be increased by using mutant strains deficient in either HtrA, an extracellular protease, or HtrA and ClpP, an intracellular protease These *L. lactis* mutants have higher production of heterologous proteins since degradation of recombinant protein by proteases is decreased [23].

The cell wall of an *L. lactis* strain has been modified to enhance protein release. *L. lactis* strain PH3960 is deficient in alanine racemase (Alr), an enzyme which converts L-alanine to D-alanine [12]. D-alanine, a critical component of the cell wall, is required for cross-linking of peptidoglycan chains. These alr^- mutants have weakened cell walls and are more immunogenic via intragastric and intravaginal immunisation [12,24]. In the absence of D-alanine, PH3960 cells become more permeable but cell viability is unaffected when the bacteria are orally administered. This could be due to presence of D-alanine derived from gut commensal bacteria [25].

Another modification to increase protein production from *L. lactis* is codon optimisation [26]. Organisms vary in their GC content. The probability of *L. lactis* successfully expressing a heterologous protein is higher if the GC content of the heterologous gene is similar to that of its own genes [20]. Codon optimisation replaces rarely used codons in a heterologous gene with those that are most commonly used in *L. lactis*. Although codon usage was an impediment to protein production in the past, with the advent of custom gene synthesis technology, this is no longer the case.

1.1.2.1 Delivery of antigens by *L. lactis* and efficacy of *L. lactis*-based vaccines

As a GRAS organism, *L. lactis* is an attractive alternative to attenuated pathogenic bacterium as vaccine vehicles. Attenuated organisms may revert to

their pathogenic forms and are especially unsafe for use in children and immuno-compromised individuals. Whereas, with *L. lactis*, there is no safety concerns since these bacteria are unable to replicate and persist in the human body. Lack of colonisation, however, may reduce the immunogenicity of the live vaccine when used orally [27]. Bacterial vaccines do not require extensive downstream processing, thus making them relatively inexpensive to produce. There is also the added advantage that Gram-positive bacteria are stable when lyophilised [28] and do not require cold chains for storage.

There is much published work documenting the use of *L. lactis* as live vaccines. Most of this research has focused on combatting bacterial or viral infections (Tables 1A and B). However, some groups have used *L. lactis* as live bacterial vaccines against the parasites *Plasmodium falciparum*, *Plasmodium yoelli* and *Giardia lamblia* (Table 1C). Ramasamy and colleagues expressed Merozoite surface antigen (MSA) 2, a *Plasmodium falciparum* antigen, on the cell wall of *L. lactis* [29]. Nasal and oral immunisation of rabbits with this clone elicited production of serum anti-MSA2 IgG. The highest titers of antibodies were achieved by oral immunisation. Oral immunisation also caused production of intestinal anti-MSA2 IgA. They also found that young adult mice (four to six weeks old) develop higher levels of serum antibodies upon immunisation than neonate (one week old) or old mice (24 to 30 weeks old) [30]. In later experiments, they were also able to show MSA2-specific interferon (IFN)-γ production from splenocytes of immunised C57BL mice [31].

Zhang *et al*. tested merozoite surface protein (MSP) 1 of *Plasmodium yoelli* expressed in *L. lactis* as an oral vaccine in BALB/c and C57BL/6 mice [32]. The protection observed was mouse strain-specific; all BALB/c mice were able to resolve infection themselves by 18 days, however immunised mice displayed more than ten-fold less parasitemia than control mice. Immunised C57BL/6 mice were able to resolve infection by 25 days whereas unimmunised C57BL/6 mice displayed uncontrolled parasitemia and had all died by this time-point. Interestingly, the authors observed that C57BL/6 mice immunised with 1×10^8 antigen-expressing bacteria displayed lower parasitemia than mice immunised

Table 1. Vaccine antigens expressed by *L. lactis*

(A) Bacterial antigens expressed in *L. lactis*

Origin	**Antigen and references**
Bacillus subtilis	Neutral protease [33]
Brucella abortus	Ribosomal protein (L7/L12) [34,35], heat-shock protein (GroEL) [36]
Clostridium tetani	Tetanus toxin fragment C (TTFC) [37,38,39]
Enterohaemorrhagic *Escherichia coli*	Type III secreted protein (EspA) [40]
Enterotoxic *Escherichia coli*	F4 fimbrial adhesin (FaeG) [41,42]
Erysipelothrix rhusiopathiae	Surface protective antigen (SpaA) [43]
Group B *Streptococcus*	Pilin island 1 [44]
Helicobacter pylori	Urease B (UreB) [45,46,47], Type IV secretion system cap (Cag7) [48]
Listeria monocytogenes	Invasion-associated protein (P60), Listeriolysin O (LLO) [49,50]
Proteus mirabilis	Fimbrial protein (MrpA) [51]
Streptococcus pneumoniae	Pneumococcal surface protein A (PspA) [52], Pneumococcal protective protein (PppA) [53,54,55]
Streptococcus pyogenes	C-repeat region of M protein [56]
Yersinia pseudotuberculosis	Low calcium response (Lcr) V [57]

(B) Viral antigens expressed in *L. lactis*

Origin	Antigen and references
Bovine rotavirus	Non-structural protein (NSP) 4 [58]
Dengue virus serotype 2	Envelope domain III (EDIII) [59]
Human immunodeficiency virus (HIV)-1	V2-V4 loop of envelope protein (gp120) [60]
Human papillomavirus (HPV)16-induced tumours	Oncogene protein (E7) [61,62]
Porcine circovirus type 2 (PCV2)	Capsid (Cap) protein [63]
Porcine transmissible gastroenteritis virus (TGEV)	Spike (S) protein [64]
Rotavirus	Viral protein (VP) 4 [65], VP7 [66], VP8 [24,67]
Severe acute respiratory syndrome (SARS)-associated coronavirus	Nucleocapsid (N) protein [68]

(C) Parasitic antigens expressed in *L. lactis*

Origin	Antigen and references
Giardia lamblia	Cyst wall protein (CWP) 2 [27,69]
Plasmodium falciparum	Merozoite surface antigen (MSA) 2 [29,30,31], Glutamate-rich protein (GLURP)-Merozoite surface protein (MSP) 3 [70]
Plasmodium yoelli	Merozoite surface protein (MSP) 1 [32]

with 5×10^9 bacteria. The authors suggest this may, in part, be a result of immune tolerance generated by antigen "overdose".

Finally, Lee and Faubert expressed cyst wall protein (CWP) 2 from *Giardia lamblia* on the cell wall of *L. lactis* for oral immunisation in BALB/c mice [69]. They observed intestinal anti-CWP2 IgA after immunisation and 63% reduction in cyst shedding in these mice. In a later study, they showed a balanced T helper (h) 1/Th2 response in the mesenteric lymph nodes and Peyer's patches of immunised mice, involvement of $CD4^+$ T cells and $CD19^+$ B cells and 71% reduction in cyst shedding [27].

The report of a live lactococcal vaccine against *Leishmania donovani* is discussed in section 1.2.3.1.1.

1.1.2.2 Delivery of cytokines by *L. lactis*

L. lactis has also been used to deliver various cytokines which serve therapeutic and prophylactic purposes [71] (Table 2). *L. lactis* secreting recombinant IL-12 has been used as an adjuvant to stimulate a Th1 response in vaccine studies utilising mouse models of various diseases. IL-12 is composed of two subunits, p35 and p40, which heterodimerise via disulphide bonds. The p40 subunit can also homodimerise and has regulatory functions unrelated to the upregulation of IFN-γ. The p40 homodimer is a receptor antagonist for the IL-12 and IL-23 receptors and has certain diseases-specific roles e.g. it promotes susceptibility to cutaneous leishmaniasis [72] but has a protective function during *Mycobacterium* infections [73,74]. Therefore, to prevent p40 homodimerisation and ensure production of only IL-12, recombinant IL-12 from *L. lactis* is expressed as single peptide chains– the p35 and p40 subunits being joined by a flexible linker [75]. The N-terminus of single-chain IL-12 (scIL-12) has the usp45 signal peptide, thus allowing secretion of the molecule. ScIL-12 is biologically active and can induce *ex vivo* IFN-γ production from mouse splenocytes [75]. In mice, co-administration of *L. lactis* secreting scIL-12 and *L. lactis* expressing E7 protein of human papillomavirus type 16 (HPV-16) was able to enhance specific immunity to this antigen, specifically the cellular immune response.

Table 2. Non-antigenic proteins expressed from *L. lactis* for therapeutic and prophylactic purposes

Protein	**References**
Cu/Zn superoxide dismutase	[76]
Epidermal growth factor (EGF)	[77,78]
Human Trefoil factor 1	[79]
Interferon (IFN)-γ	[80,81]
IFN-1β	[82]
Interleukin (IL)-10	[83,84]
IL-12	[61,75,85,86]
IL-2	[87]
IL-6	[87]
IFN-γ-inducible protein (IP)-10	[88,89]
Lymphotactin	[90]

L. lactis secreting IL-10 has also been met with success. Treatment with this clone reduced chemically-induced colitis in mice by 50% and prevented gut pathology in IL-10-deficient mice [91]. A biologically-containable clone of this *L. lactis* clone was also used in a phase I clinical trial (discussed in the next section).

1.1.2.3 A clinical trial with recombinant *L. lactis* secreting IL-10

A phase I safety and tolerability clinical trial examining efficacy of *L. lactis* expressing IL-10 as a therapy for Crohn's disease was completed in 2006 [92]. Individuals with Crohn's disease suffer from chronic, unregulated gut inflammation. In healthy humans, IL-10 plays a crucial role in regulating the immune response towards normal gut flora. The exact causes for Crohn's disease have not been ascertained but immunomodulatory cytokines, such as IL-10, may be able to reduce the pathogenic inflammation [93]. Patients were administered ten capsules containing 10^{10} CFU of lyophilised, genetically modified, IL-10-expressing *L. lactis* twice daily for seven days in conjunction with a bile acid sequestrant and a proton pump inhibitor to increase viability of the bacteria. One of the ten trial patients had to be withdrawn from the study due to adverse side effects (persistent vomiting which led to non-compliance with the indicated treatment dosage). Eight patients displayed clinical benefits and four relapsed when *L. lactis* therapy was stopped. In 2009, the authors reported that a Phase IIA dose escalation trial was in progress [94]. These clinical trials are encouraging as they show the potential of recombinant *L. lactis* in a clinical setting. Finally, the success of these clinical trials also indicates feasibility of recombinant *L. lactis* as vaccines.

1.1.3 Safety concerns regarding usage of *L. lactis* for medical purposes

L. lactis has a long history of being consumed safely by humans. However, a few incidences of human infections have been reported, mostly involving immunocompromised patients. Usually, *L. lactis* infections occur after ingestion of unpasteurised dairy products by individuals with other co-morbidities

[95]. For example, liver abscesses were found in a 62-year-old man who had consumed unpasteurised home-made yoghurt [96]. Further investigation revealed that his sigmoid colon had been pierced by a 43 mm piece of bone which disrupted the mucosa and allowed passage of bacteria into portal circulation. Other infections caused by *L. lactis* include bacterial endocarditis, peritonitis, necrotizing pneumonitis, septic arthritis septicaemia, cerebellar abscess, deep neck infection, osteomyelitis, canaliculitis and meningitis [95]. However, *L. lactis* infections are rare and antibiotic treatment is effective.

Another concern is the release of genetically modified *L. lactis* into the environment. This has been overcome by engineering auxotrophic mutants that are unable to survive outside a host. Steidler and colleagues created a clone of *L. lactis* which secretes human IL-10 [92]. The IL-10 expression cassette was cloned such that it replaced the thymidylate synthase gene (*thyA*) in the bacterial chromosome. The resulting mutant is unable to survive in the environment due to lack of thymine or thymidine. Should this clone regain *thyA*, it would occur via replacement of the IL-10 expression cassette, thus no longer making this clone a genetically modified organism.

In summary, *L. lactis* has been developed into a successful delivery vehicle and a vast amount of research on *L. lactis*–based vaccines is on-going. A *L. lactis*-based vaccine would be safe, easy to administer and inexpensive to manufacture. We, therefore, aim to develop a *L. lactis*-based vaccine against leishmaniasis.

1.2 Leishmaniasis

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*. The disease is endemic in the tropics, subtropics and Mediterranean Basin where it is spread through the bite of female phlebotomine sandflies, although transmission via shared needles has been documented [97,98]. *Leishmania* is dimorphic, existing as flagellated metacyclic promastigotes in the alimentary tract of the sandfly vector and transforming to amastigotes upon phagocytosis by Mϕ and DC of the mammalian host. Inside the phagolysosome, amastigotes are able to multiply and lyse the host cell in order to re-infect other phagocytic cells as well as non-phagocytic cells; for example, in the case of visceralizing *Leishmania*, infected phagocytic cells circulating in the blood carry amastigotes to sites such the bone marrow, lymph nodes, spleen and liver which consequently become heavily parasitized.

Depending on the parasite species, there are four forms of the disease– cutaneous leishmaniasis, mucocutaneous leishmaniasis, visceral leishmaniasis (VL; also known as kala-azar) and post-kala-azar dermal leishmaniasis (PKDL). Additionally, the same form of leishmaniasis can be caused by different parasites in the "Old World" and "New World". Thus cutaneous leishmaniasis, wherein ulcerating localized lesions form on the skin, is caused by *L. tropica*, *L. aethiopica* and *L. major* species complexes in the Old World and *L. mexicana*, *L. guyanensis* and *L. braziliensis* species complexes in the New World. Infections with *L. aethiopica*, *L. mexicana* and *L. braziliensis* may also manifest as greatly disseminated skin lesions. In the New World, cutaneous leishmaniasis, caused by *L. braziliensis* and *L. panamensis*, may lead to chronic and progressive lesions in the oral and nasal mucosa. PKDL and VL (which is a focus of the work presented) are discussed in the next section.

1.2.1 Visceral leishmaniasis

VL is endemic in more than 60 countries, however 90% of the disease occurs in the Indian subcontinent (India, Nepal, Bangladesh) where it is caused by *Leishmania donovani*, and in East Africa (Sudan, Ethiopia) and Brazil where it is

caused by *Leishmania infantum* (sometimes referred to as *L. chagasi*). All infections do not cause clinical disease; ratios of incident asymptomatic infection to incident clinical cases vary, for example, between 1:2.6 and 11:1 in Sudan, between 8:1 to 18: 1 in Brazil and 5.6:1 in Ethiopia [99]. Individuals who have been diagnosed with VL will die if left untreated. There are 500,000 new cases of VL every year and 50,000 deaths resulting from it, although these numbers are probably underestimations as VL cases often go unreported [100].

Over the course of infection, these visceralizing parasites will disseminate to the bone marrow, as well as the liver and spleen which become grossly enlarged. Other symptoms include fever, fatigue, weakness, loss of appetite and weight loss [99]. Patients may also become immunosuppressed and acquire secondary infections such as pneumonia, diarrhoea and tuberculosis. More recently, co-infection with HIV has also made treatment difficult [101]. In addition, individuals who have had VL may have recurrent infection in the form of PKDL, a nodular rash which appears on the face but can spread to other areas of the skin over time.

There are, at present, no commercially available human vaccines against VL. The disease is conventionally treated with pentavalent antimonials, which are highly toxic with life-threatening side effects such as cardiac arrhythmia and acute pancreatitis [99]. In the light of parasite resistance to these drugs, amphotericin B and miltefosine are also being used [102]; however, these drugs are not devoid of their own sets of adverse side effects, e.g. nephrotoxicity [103] and gastrointestinal toxicity [104], respectively. Until recently, these drugs were also very expensive but are now subsidized as part of a World Health Organisation initiative to eliminate VL in the Indian subcontinent. As an anti-leishmanial drug, paromomycin has a better safety profile and is also comparatively inexpensive [99]. Another disadvantage is that all of the anti-leishmanial drugs mentioned above (except miltefosine) must be given by injection or infused intravenously. These drugs have long regimens and therefore require extended hospitalisation of VL patients.

1.2.2 Animal models used for VL research

Immune responses to *Leishmania* have been widely studied *in vitro* and *in vivo* with animal models and human patients. Four main animal models are in use– mouse, hamster, dog and monkey [105]. The dog and monkey models are used for secondary testing and pre-clinical trial studies, respectively. Rodent models, mainly BALB/c mice and Syrian golden hamsters, are used in most laboratory settings for primary testing. It should be noted, however, that none of these models truly reproduce the disease which is seen in humans.

While natural infections occur via sandfly bites, laboratory models of infection are most commonly induced by intravenous or intracardiac injection of a large number of amastigotes or metacyclic promastigotes (10^7 or higher; subcutaneous, intraperitoneal and intradermal modes of infection are sometimes also used) [106]. A relatively newer but less used intradermal model of infection which uses a lower infective dose (10^4 parasites or higher) also exists [107]. Hamsters are highly susceptible to infection and thus die as a result, whereas infection in BALB/c mice is self-healing and chronic.

Infected hamsters display various symptoms of active and progressive human VL such as weight loss, hepatosplenomegaly and increasing parasite burden [105]. However, their susceptibility to *Leishmania* is much greater than seen in humans, making hamsters a good model to study the mechanisms of immunosuppression and pathogenesis. Fewer reagents exist that are compatible with biological samples from hamsters and as a consequence there is less immunological data. Hamsters are also used for passage and maintenance of virulent parasites and field isolates.

Mice do not display the symptoms seen in hamsters, thus representing the response seen in infected but asymptomatic individuals. This makes mice useful for the study of protective responses [105]. Working with mice also has the added advantage of greater availability of reagents and immunological data. When mice are infected with *Leishmania*, the parasites reach the liver and multiply rapidly inside Kupffer cells (KC), the liver's resident macrophages. The liver immune response is able to clear the parasites within six to eight weeks, depending on the

strain of mouse and parasite [108]. Parasites in the spleen and bone marrow grow more slowly and persist in these tissues.

1.2.2.1 Immune responses in the liver during VL

Both $CD4^+$ and $CD8^+$ T cells become activated during infection with the generation of Th1 and Th2 responses. Granulomas start to form after an initial period of silent parasite replication [109]. This is a complex process involving production of chemokines (e.g. CCL2, CCL3 and CXCL10) leading to recruitment of monocytes, neutrophils, $CD4^+$ and $CD8^+$ T cells [110,111,112]. Secretion of pro-inflammatory cytokines such as TNF [113,114], IL-2 [115], IL-12 [116,117], IFN-γ [118], lymphotoxin (LT) [113] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [119] from natural killer (NK) and natural killer T (NKT) cells is also observed . These cytokines cause production of chemokines and parasiticidal molecules from KC. The role of $CD8^+$ T cells is more important during subsequent infections and these cell types are mostly found in granulomas that form during re-infection [120]. Mice deficient in TNF and reactive nitrogen intermediates (RNI) are able to form granulomas but cannot control parasite growth [113,121,122]. Reactive oxygen intermediates (ROI) also play important roles in the control of parasite growth during early infection [122]. B cells are able to regulate granuloma formation; mice lacking B cells form granulomas and clear parasites faster and have a lower spleen parasite burden [112]. The Th2 cytokine, IL-4, is important for the reduction of liver parasites and priming of $CD8^+$ T cells [123]. IL-4 is also essential for maintenance of IFN-γ production and controlling infection in the spleen [124].

Immunoregulatory cytokines such as IL-10, IL-27 and transforming growth factor (TGF)-β from $CD4^+$ T cells have been shown to suppress immune responses during infection. IL-10, which is co-expressed with pro-inflammatory cytokines such as IFN-γ and TNF, suppresses Mϕ activation, DC migration and responses to IL-12 and IFN-γ [125]. High levels of IL-10 signal disease progression in humans and mice. IL-27 is important for protection from immuno-pathogenesis caused by the pro-inflammatory responses to *Leishmania* [126].

TGF-β greatly suppresses immune responses during *L. chagasi* infection but has minor effects during *L. donovani* infection [127,128]. Proper formation of granulomas is essential for resistance to VL; presence of granulomas seems to correlate with spontaneous control and maintenance of infection in a subclinical state. Granulomas are either absent or poorly formed in the highly susceptible hamster model, immunodeficient mice and human patients with advanced disease [129].

1.2.2.2 Immune responses in the spleen during VL

In the spleen, parasite growth is difficult to control even though early immune responses are the same as those in the liver. The spleen enlarges to up to 15% of the mouse body weight in six to eight weeks [108]. There is an absence of granulomas and disruption of lymphoid tissue microarchitecture. Th1 inflammatory cytokines, such as IL-12 and IFN-γ, and inhibitory cytokines, such as TGF-β and IL-10, are co-expressed in the spleen. Th2 cytokines, such as IL-4 are absent or found at low levels [130].

Normally, Mϕ in the marginal zone (MZ) and red pulp take up antigen from blood flowing through the marginal sinus. DC are also thought to pick up antigen by phagocytosis of infected Mϕ or their remnants in the MZ. These antigen-presenting cells (APC) which express CCR7 on their surface then migrate to the periarteriolar lymphoid sheath (PALS) in response to chemokine (CCL19 and CCL21) secretion from stromal cells [131]. In the PALS, DC present antigen to T cells with IL-12 co-stimulation so that antigen-specific T cell responses may be generated. IL-12 co-stimulation causes naive $CD4^+$ T cells to become Th1 cells which secrete IFN-γ thus activating macrophages to eliminate parasites. However, in the later stages of infection in the spleen, heavily parasitised Mϕ migrate to the PALS. Here they produce TNF which reduces chemokine production from stromal cells and, as a consequence, migration of DC. T cells are unresponsive to *Leishmania* antigens and undergo apoptosis [132].

Although TNF secretion is strictly regulated in the liver, high amounts of TNF are secreted in the spleen and cause damage to the organ. TNF secretion also

leads to secretion of large amounts of IL-10 [133], causing immunological dysfunction and suppression of TNF which allows parasites to survive in the spleen. Also, IL-10 further reduces migration of DC into the PALS by down-regulating their surface CCR7. Overall, there is disruption of splenic architecture, loss of germinal centers and marginal macrophages and destruction of follicular DC. Destruction and alteration of splenic tissue leads to immunodeficiency and increased susceptibility to secondary infections [109].

1.2.2.3 Role of host genetics in resistance to VL

Immunity to VL is partly mediated by host genetics [134]. *Slc11a1* (solute carrier family 11, member 1) encodes a proton efflux pump which translocates protons from Mϕ phagolysosomes to the cytosol. Mutation in this gene results in increased parasitemia in the liver and spleen during *L. donovani* infection [135]. *Slc11a1* also controls susceptibility to some intracellular bacteria e.g. *Salmonella typhimurium* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG).

Lyst encodes a lysosomal trafficking protein. Homozygous *Lyst* mutant mice are unable to clear *L. donovani* parasites from the spleen [136]. Mutant mice have a defect in NK cell activity. Oppositely, overexpression of *Lyst* causes murine macrophages and fibroblasts to have smaller parasitophorous vacuoles which inhibit growth of *L. amazonensis*, which causes cutaneous leishmaniasis, and results in control of infection [137].

H2 is the major histocompatibility complex (MHC) class II gene which is involved in antigen presentation and the adaptive immune response. Resistance to *L. donovani* or *L. infantum*, as demonstrated by low liver parasitemia, is observed in mice with the haplotypes $H2^{k/b}$, $H2^{b}$, $H2^{r}$, $H2^{a}$, $H2^{k}$ and $H2^{s}$ whereas susceptibility occurs in mice with the haplotypes $H2^{d}$, $H2^{q}$ and $H2^{f}$ [134].

1.2.3 Requirements for a successful VL vaccine

The relatively simple life cycle of the *Leishmania* parasite and the fact that disease resolution leads to life-long immunity makes leishmaniasis one of the few parasitic diseases against which generation of a successful vaccine is an attainable

goal. However, as a result of immune responses observed during VL and lack of a clear-cut Th1/Th2 paradigm, the immune responses that effective VL vaccines are expected to generate are complex. Although protection correlates with cell-mediated immunity (CMI) and a Th1 response, simultaneous Th2 and humoral responses are also beneficial and may be necessary. Indeed, a growing body of vaccine studies suggests that a mixed Th1/Th2 response coupled with a humoral response is protective against VL [138].

Years of research have also brought forward various approaches to vaccine formulations i.e. the antigen, the adjuvant and the delivery system. In first generation vaccines, antigens were provided in the form of live, attenuated or killed parasites. Second generation vaccines consist of antigens that are purified from *Leishmania* or expressed as recombinant protein from bacteria or viruses containing *Leishmania* genes. Newer, third generation vaccines being studied are DNA vaccines in which DNA encoding antigen is injected and translated by the host's machinery. An alternative approach has been to immunise with sandfly salivary proteins [139,140,141,142], which augment the infectivity of the parasites [143]. Finally, synthetic peptides based on *Leishmania* epitopes found to stimulate T cell are also being researched for their utility as vaccines [144].

1.2.3.1 Use of adjuvants in VL vaccines

Adjuvants and delivery systems used in VL vaccines are selected by their ability to generate Th1 and CMI responses (Table 3). Adjuvantation with such microorganisms or their pathogen-associated molecular patterns (PAMP) is especially important for purified antigens which do not possess any of the immunostimulatory molecules from their originating species to stimulate pattern recognition receptors (PRR) or APC. Use of adjuvants also reduces the number of antigens and/or immunisations required to elicit a strong memory response. Immunostimulatory adjuvants are simply Toll-like receptor (TLR) stimulants such as attenuated or heat-killed whole bacteria or viruses, or components derived from these organisms; whereas vaccine delivery systems combine antigen and adjuvant to further concentrate the effects of a vaccine for uptake by APC, thus lowering

Table 3. Adjuvants and delivery systems used in experimental VL vaccines

(A) Immunostimulatory adjuvants

Type of adjuvant	**Advantages**
BCG and other microorganisms (e.g. *Propionibacterium acnes*)	T helper (h) 1-stimulating adjuvant
Saponin	Functions mainly through induction of Th1 cytokines
Monophosphoryl lipid A (MPL)	T cell adjuvant; acts through Toll-like receptor (TLR) 4 to activate innate and acquired responses
CpG	TLR9 ligand; induces innate and Th1-biased adaptive immune responses
Recombinant IL-12	Modifies and redirects immune response towards Th1

Adapted from [145]

(B) Delivery systems and/or immunostimulatory adjuvants

Type of adjuvant	**Advantages**
Water-in-oil emulsions (e.g. Freund's adjuvant, Montanide 720)	Enhance level of response or focus response through a desired pathway e.g. Th1
DNA vaccines	Drives expression of antigen in eukaryotic cells; elicits humoral, $CD4^+$ and $CD8^+$ T cell immune responses
Heterologous prime-boost	Priming with DNA and boosting with recombinant viral vectors or recombinant proteins to enhance cellular immunity
Dendritic cells	Antigen carriers and professional antigen-presenting cells (APC) for induction of cellular immunity
Liposomes	Immunostimulatory adjuvant presenting antigen to APC
Microorganisms (e.g. BCG, *L. lactis*)	Expression, carrier and adjuvant system for protein

Adapted from [145]

effects on non-immune cells. This further reduces number of immunisations and quantity of adjuvant required and is especially advantageous in cases where adjuvants may cause some toxicity.

1.2.3.1.1 Bacterial adjuvants

BCG, attenuated *M. bovis*, is a Th1-stimulating adjuvant allowed for human use. It has been shown to be effective as an adjuvant for *Leishmania* vaccines in several animal models (mice, dogs and monkeys). Live BCG expressing LCR1, a *L. chagasi* antigen shown to stimulate a cellular immune response [146], was used as a vaccine delivery system in BALB/c mice challenged with *L. chagasi* [147]. Mice immunised subcutaneously but not intraperitoneally with the live bacterial vaccine were protected, as demonstrated by their low liver parasitemia. When stimulated with live *L. chagasi* promastigotes, splenocytes of protected mice secreted higher IFN-γ and lower IL-10 compared to control mice. Interestingly, intraperitoneally BCG-immunised hamsters displayed either increased susceptibility or fared no better than control animals after *L. donovani* challenge [148].

Another microorganism, *Propionibacterium acnes* (commonly found in the skin flora), has also been evaluated for its ability as an adjuvant. Mice were protected from *L. donovani* challenge after three intraperitoneal injections of purified A2, an amastigote-specific protein and putative virulence factor [149], adjuvanted with heat-killed *P. acnes* [150]. High serum titers of IgG1 and IgG2a were seen after immunisation. Stimulation of splenocytes with recombinant A2 caused proliferation and secretion of IFN-γ and IL-4 [150]. Mice were also protected from *L. chagasi* challenge when immunised three times with freeze-thawed *Leishmania* antigen adjuvanted with *P. acnes* [151]. As before, mixed Th1/Th2 responses were seen from the splenocytes of immunised mice.

Live *L. lactis* has also been evaluated for its adjuvanticity in a VL vaccine [152]. Three clones of *L. lactis*, expressing A2 cytoplasmically, attached to the cell wall or secreting A2 outside the cell, were evaluated for their ability to protect BALB/c mice against *L. donovani* infection. Subcutaneous immunisation with the

cell wall-anchored construct generated A2-specific IgG antibody and resulted in lower liver parasitemia against low dose parasite infection but not a high dose infection. Although an antibody response was not generated against cytoplasmically expressed A2, immunisation with this construct resulted in lower parasitemia against both low and high dose challenge. Splenocytes from mice immunised with either *L. lactis* expressing cytoplasmic or cell wall-anchored A2 and subsequently challenged with *L. donovani* secreted IFN-γ, IL-10 and IL-2 when stimulated with soluble *Leishmania* antigen (SLA).

Monophosphoryl lipid A (MPL) is a non-toxic derivative of the lipopolysaccharide of *Salmonella enterica* serovar Minnesota that has been approved for human use [153]. It is a potent Th1 immunostimulant that acts through TLR4. MPL as a stable emulsion (MPL-SE) has been most commonly used with the polyprotein vaccine LEISH-F1 (also known as Leish-111f and MML); a combination found to protect mice against cutaneous and visceral leishmaniasis [154,155]. The results of another study using sterol 24-c-methyltransferase in combination with MPL-SE are detailed in section 1.3. In two clinical studies with dogs, Leish-F1 adjuvanted with MPL-SE failed to protect from canine VL, however a third group, using a different immunisation protocol, was able to demonstrate protection [156,157,158].

1.2.3.1.2 Plant-derived adjuvants

Saponins are a class of glycosides found in plants. Immunisation with FML (fucose-mannose ligand surface glycoprotein purified from *L. donovani* promastigotes) using saponin as an adjuvant in mice, hamsters and dogs showed production of IgG1 and IgG2a antibodies and IFN-γ production in response to FML, in addition to significant protection from parasite challenge [159,160,161]. Phase III trials in dogs have shown vaccine efficacies of up to 80% [162]. This formulation has been licensed as Leishmune® for use in Brazil to vaccinate dogs, a major reservoir for *Leishmania* in the region. Although widespread use of the vaccine has not occurred, its use has led to declining VL rates in dogs and humans [163]. This vaccine is currently being developed further into a third generation

vaccine using specific T cell synthetic epitopes found in FML antigen. Since saponin is haemolytic, its use in humans has been limited [164].

1.2.3.1.3 Cytokines

Recombinant IL-12 has also been used as an experimental adjuvant. As discussed earlier, during *Leishmania* infection, IL-12 is a critical cytokine required to induce secretion of IFN-γ and other Th1 responses. Addition of IL-12 in vaccines as an adjuvant has shown mixed results in murine and canine models of VL. Some groups were able to show increased vaccine efficacy [165,166] whereas others observed that although IL-12 adjuvantation increased humoral and intradermal reaction to *Leishmania* antigen, no reduction in parasitemia occurred [167]. With yet another antigen, IL-12, supplied in the vaccine as DNA or protein, abrogated the protection conferred by antigen alone [168]. In dogs, IL-12 adjuvanted vaccines were either non-protective [169] or showed no improvements over the non-adjuvanted counterpart [170]. In the case of the non-protective vaccine, it was suggested that the dose and timing of IL-12 administration may need to be fine-tuned in order to see effects. Indeed, it has been shown with *L. major* that persistence of IL-12 is a contributing factor to the outcome of vaccines [171].

1.2.3.2 Clinical trials to evaluate potential VL vaccines

Very few clinical trials for VL vaccines have been carried out. The earliest report of a vaccine study in humans involved inoculating 119 volunteers from a VL-endemic area in Kenya with live *L. donovani* parasites which were isolated from a ground-squirrel and found to be dermatropic [172]. Inoculation with this strain resulted in the formation of a nodular lesion or "leishmanioma" on the skin at the site of immunisation. Within six to eight weeks of immunisation, 95 volunteers showed positive immune reactions to *Leishmania* antigen (leishmanin skin test (LST)). 70 volunteers were followed for up to two years and showed no incidence of VL. Of these 70 volunteers, 53 were again tested for immune response to *Leishmania* antigen; only six showed a negative response. Finally, six

volunteers were infected with a human strain of *L. donovani* soon after their initial inoculation. None of these six volunteers developed leishmaniomata or VL for up to one and half years after challenge. However, there was no control group in this trial and the sample size was not large enough for the results to be statistically relevant. Such a study would also be deemed unethical by today's standards.

Less encouraging results were obtained from a clinical trial using killed *L. major* adjuvanted with BCG [173]. 1155 volunteers were immunised with two doses of autoclaved *L. major* (ALM) adjuvanted with BCG while 1151 volunteers received BCG alone. The rationale behind this approach was the observation that individuals with prior exposure to *L. major* were at lower risk of contracting VL. 30% of immunised individuals showed a positive reaction to LST after immunisation. After two years of follow-up, the authors did not find any significant differences in the incidence of VL between immunised and control volunteers (11.5% and 12.3%, respectively); however, only 7.2% of the immunised volunteers who showed a positive LST developed VL. The overall vaccine efficacy was 6%. The ALM+BCG vaccine was improved by using alum as a second adjuvant [174,175]. Intradermal injections were shown to be safe and immunogenic with positive LST which were sustained even at two years post-immunisation. In addition, it was reported that four placebo subjects in the extended phase II study had developed VL during the course of the trial. However, one disadvantage of alum usage is that it may cause an inflammatory myopathy known as macrophagic myofasciitis [176].

More recently, LEISH-F1, in combination with MPL-SE, has been evaluated for safety and immunogenicity in humans in four clinical trials in Colombia, Peru, Brazil and India [177,178,179,180]. A phase I, dose-escalating trial to assess the safety and immunogenicity of LEISH-F1+MPL-SE vaccine against VL in 36 Indian volunteers was carried out. Subjects were followed for 24 weeks after subcutaneous vaccination. No severe adverse events were reported; most volunteers experienced malaise, myalgia, fever and headache. The vaccine was found to be safe in both naive individuals and those who had had prior exposure to *L. donovani*. The peripheral blood of immunised volunteers was

tested for presence of IFN-γ, TNF, IL-2, IL-4, IL-5 and IL-10. All tested cytokines except IL-10 were elevated. In addition, only *L. donovani*-naive individuals had elevated IL-4. The authors concede that larger group sizes are required in order for their findings regarding cytokine levels to be significant.

1.3 Introduction to conducted research

Yam and colleagues have shown the potential of *L. lactis* as a vaccine adjuvant in a mouse model [15]. They went on further to show that the A2 antigen of *Leishmania* can be expressed by *L. lactis* and directed to different cellular locations [152]. These clones were able to elicit A2-specific humoral and Th1/Th2 responses in mice. Furthermore, immunised mice had lower liver parasitemia as a result of immunisation.

The work presented here expands on the previous research by using another protective *Leishmania* antigen, sterol 24-c-methyltransferase (SMT). SMT is an enzyme which is highly conserved among *Leishmania* species. It synthesises ergosterol, a major membrane sterol in trypanosomatid parasites. The enzyme is found in both promastigotes and amastigotes.

SMT was identified as an *L. infantum* antigen by screening an *L. infantum* expression library with pooled sera from *L. infantum*-infected hamsters or Sudanese VL patients with the active disease [181]. Cured Indian VL patients showed IFN-γ production when their peripheral blood was incubated with purified recombinant SMT. Brazilian VL patients also displayed anti-SMT IgG. When mice were immunised with SMT adjuvanted with MPL-se three times at three week intervals, they had high levels of anti-SMT IgG1 and IgG2a. Splenocytes of these mice produced IFN-γ and IL-10 in response to stimulation with SMT [182]. It was also shown that immunised mice possessed multi-functional (secreting IL-2, IFN-γ and TNF) CD4+ and CD8+ T cells. Finally, these mice had reduced parasitemia in their liver and spleen at four weeks after challenge with *L. infantum*.

Here we show that immunisation with *L. lactis* expressing SMT cytoplasmically generated an immune response from BALB/c mice. We also

show that adjuvantation of SMT-expressing *L. lactis* with IL-12-secreting *L. lactis* lowers liver parasitemia.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for cloning and *E. coli* strain BL21 (DE3) was used for production of N-terminal decahistidine-tagged SMT (His-SMT). These strains were grown in LB broth (Wisent; St. Bruno, QC, Canada) supplemented with appropriate antibiotics at 37°C with shaking.

L. lactis subsp. *cremoris* strains NZ9000 and PH3960 were used for immunisations. These strains were grown at 30°C in M17 medium (Oxoid; Basingstoke, Hampshire, England) supplemented with 0.5% glucose (GM17) and appropriate antibiotics without shaking. In addition, GM17 medium was supplemented with 200 μg/ml D-alanine (Sigma; St. Louis, MO) when culturing *L. lactis* subsp. *cremoris* strain PH3960.

Antibiotic concentrations used are as follows: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; spectinomycin, 300 μg/ml.

2.2 Cloning and expression of recombinant SMT in *E. coli* and *L. lactis*

The codon-optimised SMT gene in pUC57 was obtained from GenScript (Piscataway, NJ). The nisin-inducible promoter, P_{nisA}, was PCR amplified from pDL278 with the following set of primers: 5'-CCG GAA TTC AGT CGA CCT AGT CTT ATA ACT ATA CTG ACA ATA G-3' and 5'-TGC GGA ATT CAT GCA TTT TGA GTG CCT CCT TAT AAT TTA T-3'. The PCR product was digested with EcoRI and NsiI to introduce it upstream of the SMT gene in pUC57. This construct was digested with SalI to introduce it into pLE1, an *E. coli-L. lactis* shuttle plasmid. Competent *L. lactis* was transformed with this pLE1 plasmid, containing codon-optimised SMT under the control of P_{nisA} promoter, by electroporation. Expression of cytoplasmic SMT was induced by nisin and assessed by Western blot of *L. lactis* whole cell extracts.

For the expression of His-SMT, codon-optimised SMT was blunt cloned into pET-16b using NsiI and BamHI restriction sites directly downstream of and in-frame with the N-terminal decahistidine sequence. Competent *E. coli* strain

BL21 (DE3) was transformed with this construct by heat-shock. Saturated overnight cultures of this transformed strain were diluted at a ratio of 1:100 into fresh media containing 100 μg/ml ampicillin and grown to OD_{600} of 0.4-0.5. His-SMT expression was then induced for 3 hours by adding 1 mM IPTG. Purification of His-SMT was carried out according to the manufacturer's protocol (Novagene; Darmstadt, Germany). Protein expression was assessed by Western blot.

2.3 Preparation of *L. lactis* for immunisations

Saturated overnight cultures of the various *L. lactis* strains were diluted into fresh media (containing chloramphenicol) at a ratio of 1:20 and grown to OD_{600} of 0.4-0.5. Nisin (10 ng/ml) was then added to induce production of SMT or IL-12 for 3 hours. In the case of *L. lactis* strain PH3960, after 1 hour of nisin induction, cells were centrifuged at 3000×g and resuspended in the same volume of media lacking D-alanine for a further 2 hours of nisin induction.

After 3 hours of induction, cells were washed twice in PBS and finally resuspended in PBS containing 25% glycerol. 500 μl and 1 ml aliquots of this suspension were frozen at -80°C until further use. The correct dilution of the aliquots for immunisation were determined and confirmed by carrying out serial dilutions and counting colony-forming units (CFU). Protein expression was assessed by Western blot.

On the day of immunisation, aliquots were thawed on ice, centrifuged at 5.6 krpm at 4°C in a microcentrifuge and resuspended in the appropriate volume of endotoxin-free PBS (Gibco; Burlington, ON) to yield ~1×10^{10} CFU/ml. For immunisation with two clones of *L. lactis*, diluted bacterial suspensions were mixed in equal volumes to yield a final concentration of ~1×10^{10} CFU/ml.

2.4 Preparation of *L. lactis* total cell lysates

1 ml of an induced *L. lactis* culture was centrifuged at 13 krpm in a micro-centrifuge, resuspended in TES (10 mM Tris-HCl, 1 mM EDTA, 25% sucrose, pH 8.0) containing lysozyme (1 mg/ml), mutanolysin (50 U/ml) and RNase A (0.1 mg/ml) and incubated at 37°C for 1 hour to digest the cell wall. Protoplasts were

then lysed by adding TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 4% SDS and incubating in a boiling water bath for 5 minutes.

2.5 Western blot analysis

L. lactis cell lysates and purified protein from *E. coli* were run on 12% SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore; Billerica, MA). Membranes were blotted with mouse polyclonal anti-SMT IgG (kindly provided by Dr. Steven G. Reed) at a dilution of 1:10,000, followed by goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Sigma) at a dilution of 1:20,000. ScIL-12 was detected by using anti-mouse IL-12 neutralizing antibody (R&D Systems; Minneapolis, MN, USA) at a dilution of 1:10,000 followed by anti-goat antibody conjugated to HRP (Sigma) at a dilution of 1:10,000. Bands were revealed using Immobilon Western Chemiluminescence HRP Substrate (Millipore) and visualised on radiographic film.

Antibodies were diluted in PBS containing 1% Tween-20 (Fisher; Ottawa, ON) and 5% non-fat dried milk. Membrane washes were carried out using PBS containing 1% Tween-20.

2.6 Parasite culture and SLA preparation

L. donovani strain 1S2D promastigotes were grown in SDM medium containing 10% FBS (Gibco) at 25°C. Soluble *Leishmania* antigen (SLA) was prepared by resuspending late-stationary phase promastigotes in PBS at a concentration of ~10^8 parasites/ml and subjecting the parasite suspension to five freeze-thaw cycles. The suspension was centrifuged at 16,000 rcf for 15 minutes at 4°C in a microcentrifuge. The supernatant was collected and its protein concentration was quantitated by the Bradford method.

2.7 Immunisation of mice and *L. donovani* challenge

At week 0, 5-10 BALB/c mice (six-weeks-old; Charles River Laboratories; Montreal, QC) per group were immunised subcutaneously with

200 µl bacterial suspension containing ~2×10^9 CFU of *L. lactis* in endotoxin-free PBS (Table 4). Mice in the PBS group received 200 µl endotoxin-free PBS. Immunisation was repeated twice more at two week intervals (weeks 2 and 4). Two weeks after the third immunisation (week 6), mice were infected with late-stationary phase *L. donovani* promastigotes by intravenously injecting the tail vein with 100 µl endotoxin-free PBS containing ~4×10^7 parasites. Mice were sacrificed 4 weeks after infection (week 10).

Throughout the study, blood from the lateral saphenous vein was collected from each mouse. Collected blood was kept at 4°C for 1 hour before centrifugation at 16,000 rcf for 20 minutes at 4°C in a microcentrifuge. The serum was stored at -80°C until required.

2.8 SMT-specific antibody detection

Individual humoral responses of mice were measured by enzyme-linked immunosorbent assay (ELISA). High affinity protein binding 96-well plates (Costar; Corning, NY) were coated with purified His-SMT (2.5 µg/ml) overnight at 4°C. Plates were blocked with PBS containing 2.5% FBS for 1 hour at room temperature. Two-fold serial dilutions of serum samples (starting at 1:50 in PBS containing 2.5% FBS) were applied to the corresponding wells and incubated for 2 hours. Plates were then incubated for 1 hour with goat anti-mouse IgG antibody conjugated to HRP (Sigma) (for assessment of total anti-SMT IgG titers) or with goat anti-mouse IgG1 antibody (Sigma) or goat anti-mouse IgG2a antibody (Sigma) followed by a further 1 hour incubation with rabbit anti-goat IgG antibody conjugated to HRP (Sigma) (for assessment of anti-SMT IgG1 or IgG2a). Finally, plates were incubated for 20 minutes with activated ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) ELISA substrate (Sigma) and the absorbance was read at 405 nm using an ELISA microplate reader. For total IgG, the end-point was the last dilution that resulted in a positive readout compared to naive sera. For IgG1/IgG2a, the OD_{405} reading of sera diluted to 1/50 is shown.

Table 4. Immunisation protocol

(A) Schedule of immunisation, infection and sample collection

Week	**Protocol**
0	Immunise with PBS or 2×10^9 *L. lactis* (#1)
2	Immunise with PBS or 2×10^9 *L. lactis* (#2)
4	Immunise with PBS or 2×10^9 *L. lactis* (#3)
6	Infect with 4×10^7 *L. donovani*
10	Assess parasitemia and immune responses
Serum anti-SMT IgG was assessed at weeks 1, 3, 5, 8 and 10 for *L. lactis* NZ9000 vaccine trial	
Serum anti-SMT IgG1 and IgG2a was assessed at weeks 4 and 10 for *L. lactis* NZ9000 vaccine trial	
Serum anti-SMT IgG1 and IgG2a was assessed at weeks 4 and 10 for *L. lactis* PH3960 vaccine trial	
10 mice per group were used *L. lactis* NZ9000 vaccine trial. 5 mice per group were used for *L. lactis* PH3960 vaccine trial.	

(B) Group designations and corresponding *L. lactis* clones administered

Group	Bacteria (CFU and type) administered
Vector	2×10^9 *L. lactis* containing empty pLE1
IL-12 half	1×10^9 *L. lactis* containing empty pLE1 + 1×10^9 *L. lactis* secreting IL-12
SMT	2×10^9 *L. lactis* expressing SMT
SMT half	1×10^9 *L. lactis* containing empty pLE1 + 1×10^9 *L. lactis* expressing SMT
SMT+IL-12	1×10^9 *L. lactis* expressing SMT + 1×10^9 *L. lactis* secreting IL-12
Mice in "PBS" group received 200 µl endotoxin-free PBS. Mice in other groups received indicated numbers of either NZ9000 or PH3960 strain in a total of 200 µl.	

2.9 Splenocyte isolation and cytokine assays

Upon sacrifice, spleens were removed from each mouse. Single cell suspensions were prepared in PBS. Cells were centrifuged at 1400 rpm at 4°C for 5 minutes and resuspended in ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2-EDTA) to lyse red blood cells. Cells were washed twice more in PBS and finally resuspended in RPMI (Wisent) containing 10% FBS, 0.3 mg/ml L-glutamine, 1 mM penicillin-streptomycin and 0.5 mM β-mercaptoethanol.

For the re-stimulation assay, 2×10^6 cells per well were plated in 24-well plates with 50 μg/ml SLA, anti-CD3 (0.4 μg/ml) and anti CD28 (5 μg/ml) as a positive control or RPMI as a negative control and incubated for 72 hours. Culture supernatants were collected for assessment of cytokine levels by sandwich ELISA (ELISA Ready-SET-Go!, eBiosciences; San Diego, CA) according to manufacturer's protocol.

2.10 Assessment of parasite load after challenge

Upon sacrifice four weeks post-infection, the liver was removed from each mouse. Liver impression smears were obtained and stained using Diff-Quick solutions (Dade Behring; Newark, DE). Parasitemia was determined by microscopic examination of stained smears and calculating Leishman-Donovan units (number of amastigotes per 1000 host cell nuclei × liver weight (mg)). Only parasites that were whole and showed no abnormal morphology were counted.

2.11 Statistical analysis

Data are displayed as the mean ± standard error of the mean. Differences among groups were assessed by unpaired t-test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1.1 SMT expression from bacterial strains

We obtained a codon-optimised, synthesised SMT gene (from GenScript) to achieve greater expression of heterologous SMT from *L. lactis*. In order to codon-optimise a gene, one starts from the amino acid sequence of a given gene and selects the most commonly used codon for each amino acid, while also taking into consideration the effect of the codon-optimised nucleotide sequence in the overall mRNA structure. The codon-optimised SMT gene was placed directly downstream of the P_{nisA} promoter in the pLE1 plasmid to allow nisin-inducible expression. Cytoplasmic SMT from whole cell extracts of *L. lactis* (NZ9000 and PH3960) was recognised by mouse anti-SMT IgG as a protein weighing ~40 kDa (Fig. 2A). This is the expected length of the protein [182].

The codon-optimised SMT sequence was also placed under the control of an IPTG-inducible promoter in the pET-16b plasmid. This sequence also possessed an N-terminal decahistidine sequence. Purification resulted in a single band slightly higher than 40 kDa which was seen by Coomassie stain and was also recognised by anti-SMT IgG by Western blot (Fig. 2B).

Single-chain IL-12 (scIL-12) secreted from *L. lactis* is also nisin-inducible and is secreted via the usp45 secretion signal (Hugentobler *et al.*, manuscript in preparation; scIL-12 was codon-optimised and synthesised by GenScript). Expression of IL-12 from *L. lactis* (NZ9000 and PH3960) prepared for immunisations was checked by Western blot and the single chain protein appears as a band at around 70 kDa (Fig. 2C).

3.1.2 Antibody responses against SMT from *L. lactis* NZ9000

Mice were injected subcutaneously with PBS or *L. lactis* NZ9000 every two weeks for a total of three immunisations. In order to confirm that all mice were immunised with ~2×10^9 bacteria, dilutions of the bacterial suspensions used for immunisation were plated on each immunisation day.

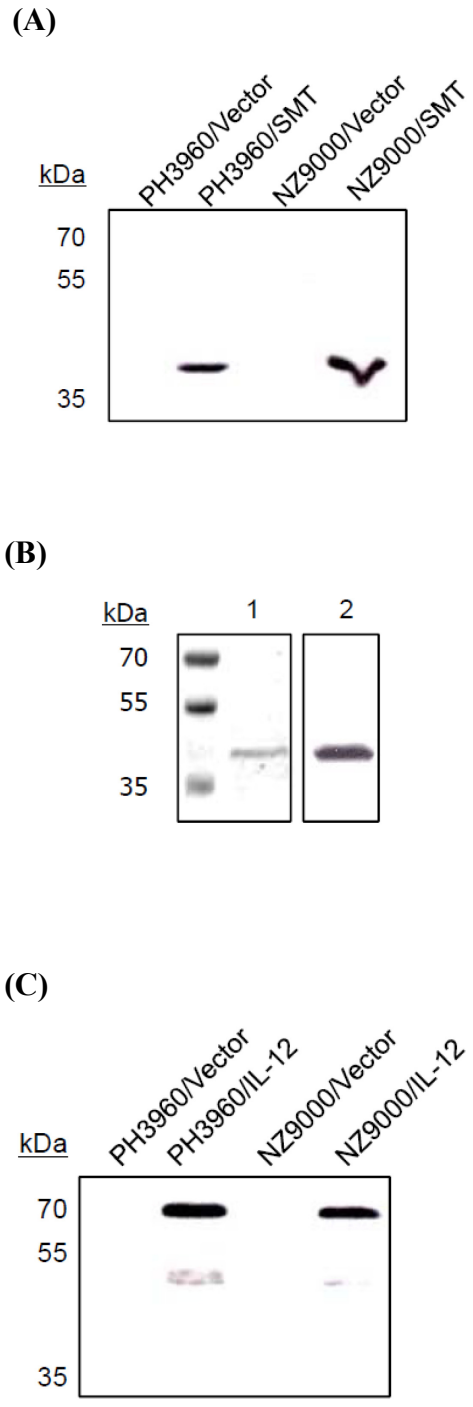


Figure 2. Protein expression from *L. lactis*. **(A)** Whole *L. lactis* cell extracts analysed by Western blot with anti-SMT antibody. **(B)** Purified His-SMT from *E. coli* on a Coomassie gel (lane 1) or analysed by Western blot with anti-SMT antibody (lane 2). **(C)** Whole *L. lactis* cell extracts analysed by Western blot with anti-IL-12 antibody.

Anti-SMT IgG levels in serum samples taken a week after each immunisation were measured by ELISA (Fig. 3A). Mice injected with PBS, *L. lactis* NZ9000/Vector or *L. lactis* NZ9000/IL-12 displayed background levels of anti-SMT IgG. Mice immunised with 1×10^9 or 2×10^9 *L. lactis* NZ9000/SMT only had gradually increasing levels of anti-SMT IgG which were significantly higher a week after the third immunisation. Co-administration of *L. lactis* NZ9000/IL-12 with SMT-expressing bacteria resulted in lower anti-SMT IgG levels than were seen in mice given the same number of SMT-expressing bacteria but not adjuvanted with IL-12.

The Th1/Th2 balance of an immune response can be inferred upon by measuring the IgG1 and IgG2a levels in sera. High levels of IgG2a indicate a predominantly Th1 immune whereas high levels of IgG1a indicate a Th2 response [183]. We assessed the anti-SMT IgG1 and IgG2a levels in sera collected a week after the last immunisation (Fig. 3B). Again, mice injected with PBS, *L. lactis* NZ9000/Vector or *L. lactis* NZ9000/IL-12 had basal levels of both antibody isotypes. Mice given 1×10^9 *L. lactis* NZ9000/SMT (supplemented with *L. lactis* NZ9000/Vector) also demonstrated basal levels of anti-SMT IgG2a and had higher levels of anti-SMT IgG1, although not significantly. These mice had an IgG2a:IgG1ratio of 0.61 (a higher ratio indicates a stronger Th1 response). Mice that were co-administered *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/IL-12 had similar IgG1 and IgG2a levels and an IgG2a:IgG1 ratio of 0.60). Mice given 2×10^9 *L. lactis* NZ9000/SMT had significantly higher IgG1 and IgG2a levels compared to the controls and an IgG2a:IgG1 ratio of 0.54. Thus, a predominantly Th2 response was observed in SMT-immunised mice.

3.1.3 Protection from *L. donovani* infection by *L. lactis* NZ9000/SMT

Two weeks after the final immunisation, mice were challenged intravenously with liver metacyclic promastigotes. Upon infection, parasites injected intravenously reach the liver, as well as some other organs. Here, they multiply rapidly inside Kupffer cells, the resident macrophages of the liver, causing the liver to enlarge. The parasite load in the liver can be measured by

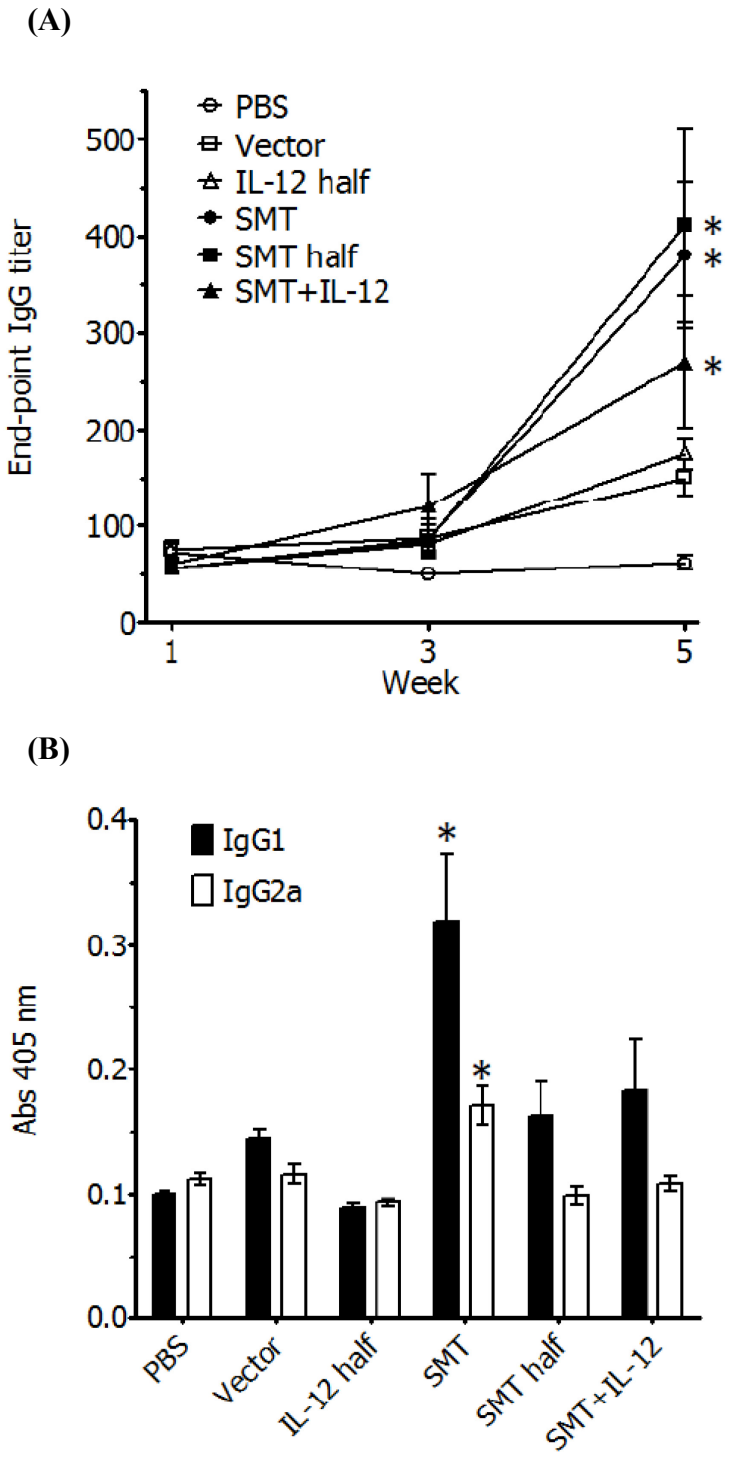


Figure 3. Antibody responses of mice immunised with *L. lactis*/NZ9000 clones. (A) Anti-SMT IgG levels in mouse sera collected one week after each immunisation. **(B)** Levels of anti-SMT IgG1 and IgG2a in mouse sera collected one week after the last immunisation. "*" denotes $p < 0.05$ to PBS and Vector.

calculating Leishman-Donovan Units (LDU). This measurement takes into account the weight of the liver and ratio of *L. donovani* amastigotes to host cells (as seen in stained liver impressions). Liver parasitemia was measured four weeks from the start of infection (Fig. 4). Mice co-immunised with *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/IL-12 had significantly lower parasitemia. While not of the same magnitude, lower parasitemia was also observed in mice immunised with *L. lactis* NZ9000/IL-12 (complemented with *L. lactis* NZ9000/Vector).

3.1.4 Immune responses during *L. donovani* challenge

Serum samples taken from mice at two and four weeks post-infection were used to measure anti-SMT IgG, IgG1 and IgG2a levels. All mice immunised with *L. lactis* NZ9000/SMT, had higher anti-SMT IgG levels at week eight than at week five (Fig. 5A). In the case of mice immunised with 2×10^9 *L. lactis* NZ9000/SMT, anti-SMT IgG continued to increase at week ten, the end-point of the study; whereas anti-SMT IgG levels had decreased in mice that had received 1×10^9 *L. lactis* NZ9000/SMT.

Anti-SMT IgG1 and IgG2a levels were higher in all *L. lactis* NZ9000/SMT-immunised mice (Fig. 5B). Anti-SMT IgG1 was significantly higher in mice that were immunised 2×10^9 *L. lactis* NZ9000/SMT. These mice also had significantly higher IgG2a however the IgG2a:IgG1 ratio at week ten had decreased to 0.43. Mice that were co-administered SMT- and IL-12-expressing *L. lactis* also had significantly higher IgG1 and an IgG2a:IgG1 ratio of 0.57. Mice that received only 1×10^9 *L. lactis* NZ9000/SMT supplemented with *L. lactis* NZ9000/Vector had an IgG2a:IgG1 ratio of 0.66. Overall, a predominant Th2 response was indicated.

Four weeks post-infection, splenocytes from infected mice were re-stimulated to assess the cytokines secreted in response to SLA. Secretion of IFN-γ, IL-4 and IL-10 was seen from all splenocyte samples (Fig. 6). Mice administered *L. lactis* NZ9000 had higher secretion of all three cytokines compared to mice given PBS only, although not significantly. Interestingly, some

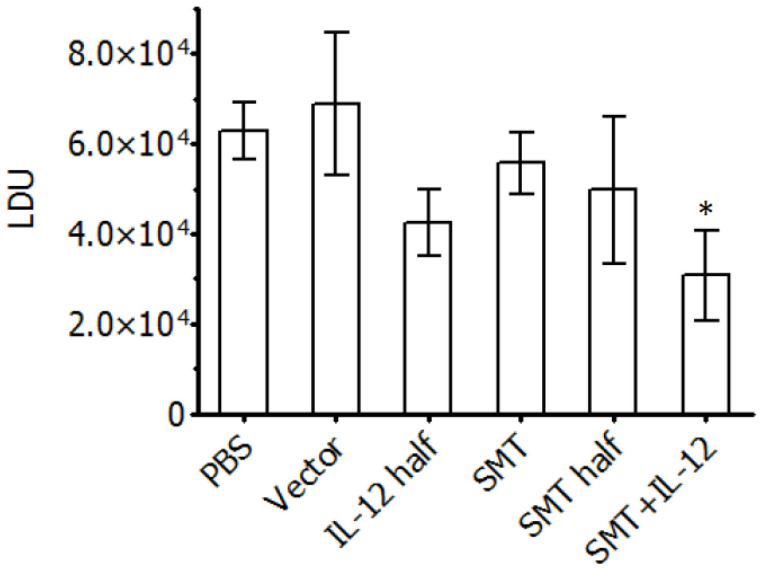


Figure 4. Protection from VL in mice immunised with *L. lactis* NZ9000 clones and infected with *L. donovani*. Leishman-Donovan Units (LDU) were determined from liver impression smears of mice, immunised with *L. lactis* NZ9000 clones, fours week after *L. donovani* infection. "∗" denotes $p < 0.05$ to PBS and Vector.

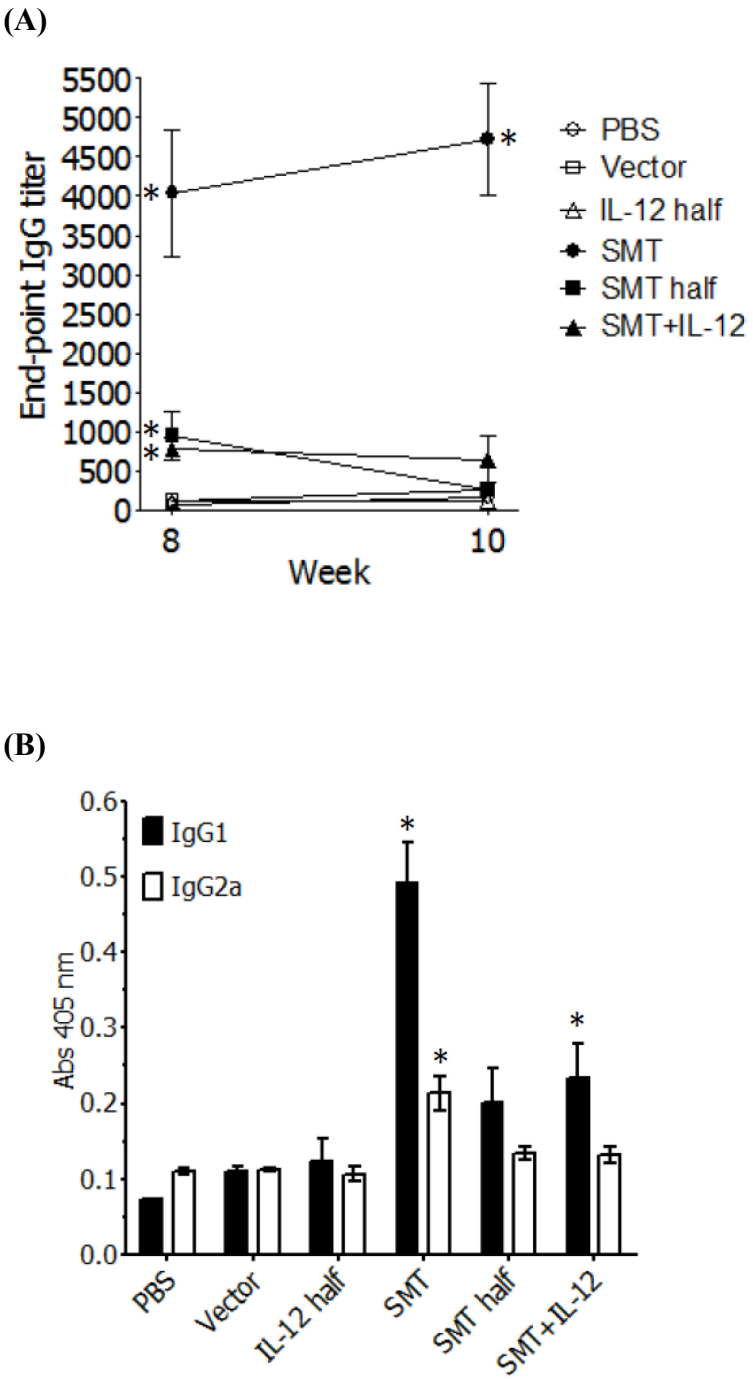


Figure 5. Antibody responses of mice immunised with *L. lactis* NZ9000 clones and infected with *L. donovani*. (A) Anti-SMT IgG levels in mouse sera at two and four weeks after infection. **(B)** Levels of anti-SMT IgG1 and IgG2a in mouse sera collected four weeks after infection. "*" denotes $p < 0.05$ to PBS and Vector.

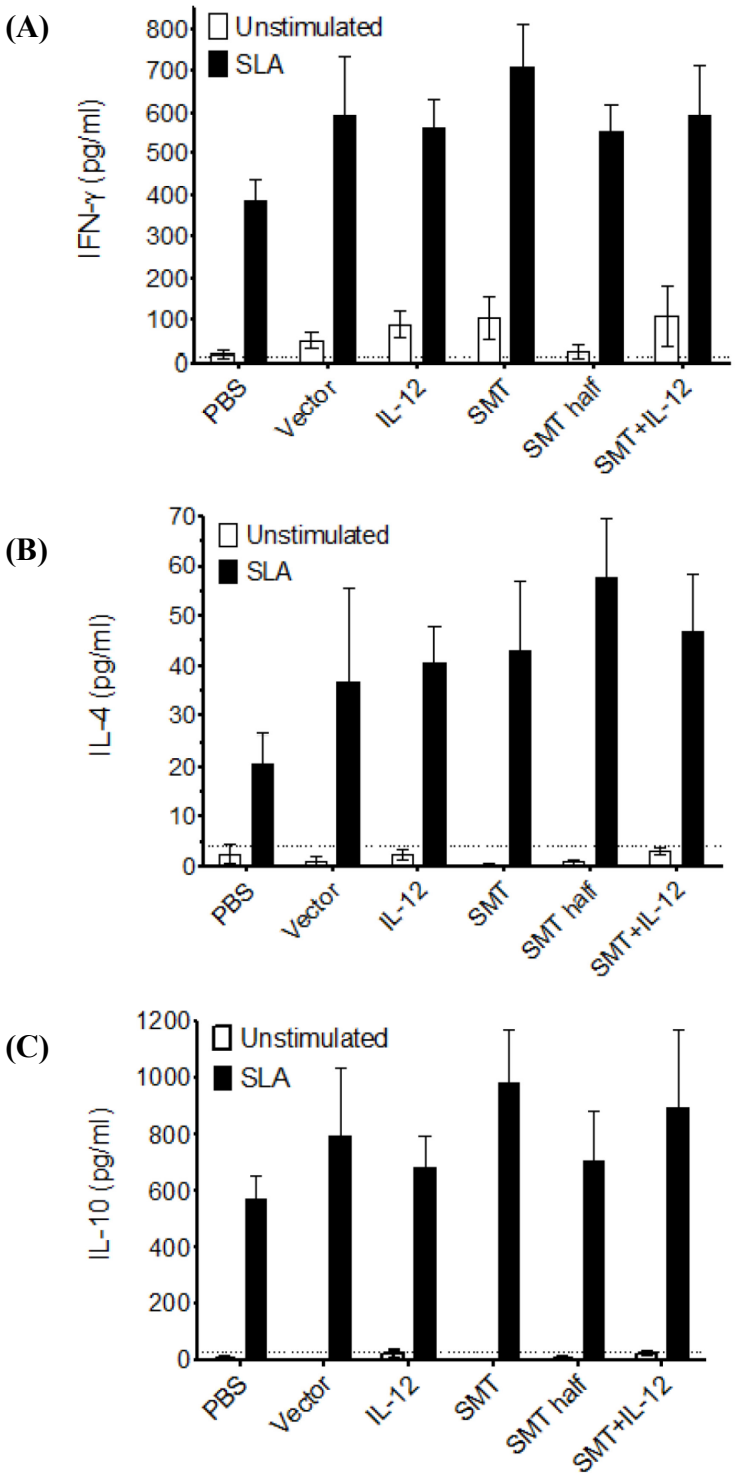


Figure 6. *Ex vivo* responses to SLA of splenocytes from mice immunised with *L. lactis* NZ9000 clones and infected with *L. donovani*. Supernatants from splenocytes re-stimulated with SLA for 72 hours were analyzed for **(A)** IFN-γ, **(B)** IL-4 and **(C)** IL-10 by ELISA. Dotted line represents assay cut-off.

groups of mice given *L. lactis* also secreted IFN-γ, but not IL-4 or IL-10, in the absence of stimulation.

3.2 Rationale for use of *L. lactis* PH3960 strain

Based on the protection seen in mice immunised with *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/IL-12, we aimed to improve the immune response generated and tease out the protection conferred by *L. lactis* NZ9000/IL-12 alone. To that end, we used *L. lactis* PH3960, an alanine racemase-deficient strain which has been shown to enhance immune responses to intracellular recombinant antigens [12,24]. Deficiency in the alanine racemase affects the proper cross-linking of peptidoglycan chains in the bacterial cell wall, therefore, making the cell walls weaker. Otherwise, the immunisation and infection protocol remained unchanged.

3.2.1 Antibody responses against SMT from *L. lactis* PH3960

Serum samples were taken from immunised, uninfected mice two weeks after the last immunisation to measure IgG1 and IgG2a levels (Fig. 7). Mice administered PBS, *L. lactis* PH3960/Vector or *L. lactis* PH3960/IL-12 had basal levels of IgG1 and IgG2a against SMT. A predominantly Th2 response was indicated by the significantly higher levels of IgG1 and IgG2a in all mice immunised with *L. lactis* PH3960/SMT. As expected, the highest IgG1 and IgG2a levels were seen in mice immunised with 2×10^9 *L. lactis* PH3960/SMT. Compared to immunisation with NZ9000, mice immunised with PH3960 had much more anti-SMT IgG1 than the control mice. The IgG2a:IgG1 ratios for the "SMT", "SMT half" and "SMT+IL-12" groups were 0.32, 0.35 and 0.52. This indicated that a stronger Th2 response was elicited by PH3960 immunisation than by NZ9000 immunisation but also demonstrated the role of secreted IL-12 from *L. lactis* in causing a stronger Th1 response than was seen in mice not receiving IL-12 adjuvantation.

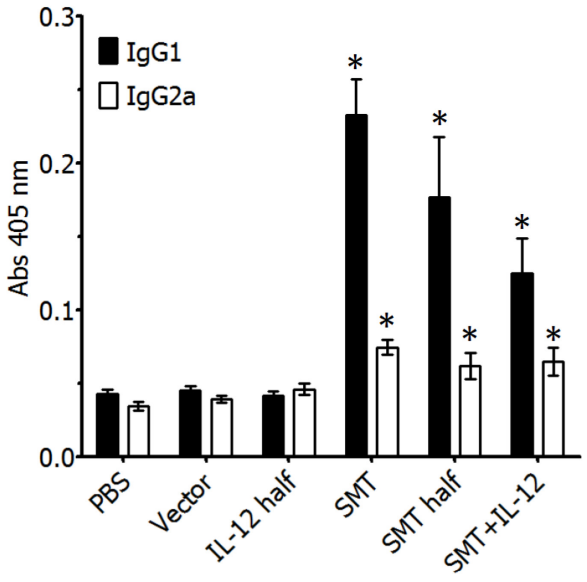


Figure 7. Antibody responses in mice immunised with *L. lactis* PH3960 clones. Anti-SMT IgG1 and IgG2a levels in mouse sera collected two weeks after the last immunisation. "*" denotes $p < 0.05$ to PBS and Vector.

3.2.2 Protection from *L. donovani* infection and immune responses during infection after immunisation with *L. lactis* PH3960

Mice were infected with metacyclic *L. donovani* promastigotes two weeks after the last immunisation. Four weeks post-infection, liver parasitemia was assessed (Fig. 8). There were no differences in parasitemia between groups. Groups of mice given *L. lactis* tended to have a greater variation in their LDU compared to mice given only PBS. It should also be noted that although mice were infected with the same number of parasites in both studies, parasitemia in the second study (immunisation with PH3960) was about one-third of that seen in the first. This may indicate reduced virulence in our stocked parasites.

IgG1 and IgG2a levels in mice sera were analyzed at week ten (Fig. 9). Compared to control groups, all *L. lactis* PH3960/SMT-immunised mice had significantly higher anti-SMT titers of both isotypes. At week ten, mice given *L. lactis* PH3960/SMT (with or without *L. lactis* PH3960/Vector) had higher ratios of IgG2a to IgG1 compared to that at week six ("SMT", 0.47; "SMT half", 0.47 and "SMT+IL-12", 0.58). Thus, although the immune response remained predominantly a Th2 response, the strength of the Th1 arm had increased.

Four weeks after infection, splenocytes of mice were re-stimulated with SLA assessed for cytokine production (Fig. 10). All mice given *L. lactis* PH3960 had higher IFN-γ expression compared to mice given only PBS. Although, mice that were co-administered *L. lactis* PH3960/SMT and *L. lactis* PH3960/IL-12 had the highest level of IFN-γ compared to mice given PBS or *L. lactis* PH3960/Vector, this group of mice also had significantly higher IL-10 secretion. This may explain why no reduction in parasitemia was seen in this group. However, all groups secreted IL-10 in the absence of stimulation. IL-4 secretion from SLA re-stimulation could not be detected from any of the mice. IL-4 is a Th2 cytokine; therefore, the cytokine expression profile contrasted with the Th2-skewed immune response suggested by the IgG isotype profile. However, it may be possible that other Th2 cytokines were expressed. The Th1 immune response was suppressed by the large amounts of the suppressive cytokine, IL-10; thus, leading to the lack of differences in parasitemia. Lack of IL-4 across all groups

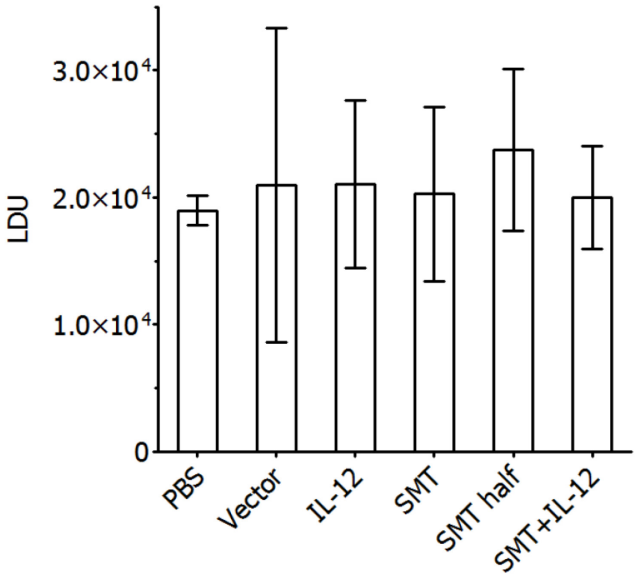


Figure 8. Live parasitemia in mice immunised with *L. lactis* PH3960 clones. Leishman-Donovan Units (LDU) were determined from liver impression smears of mice, immunised with *L. lactis* PH3960 clones, fours week after *L. donovani* infection.

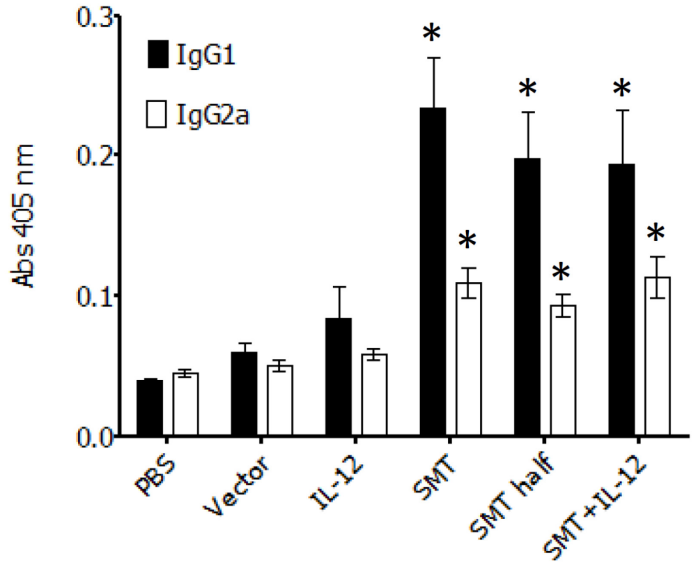
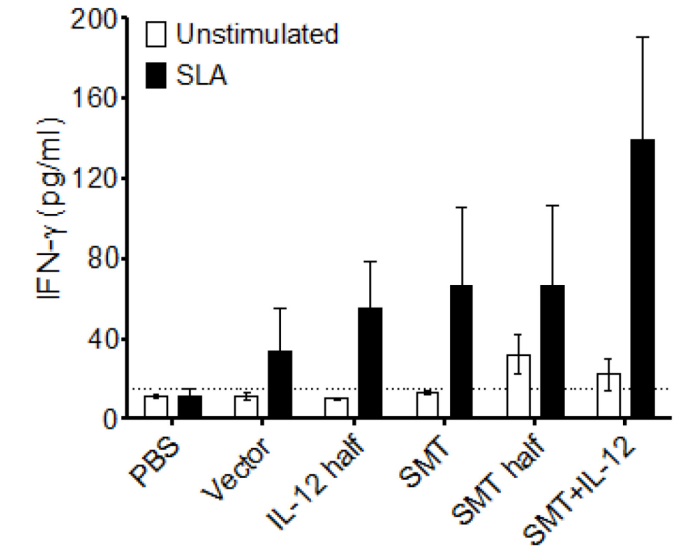


Figure 9. Antibody responses in mice immunised with *L. lactis* PH3960 clones and infected with *L. donovani*. Levels of anti-SMT IgG1 and IgG2a in mouse sera collected four weeks after infection. "*" denotes $p < 0.05$ to PBS and Vector.

(A)



(B)

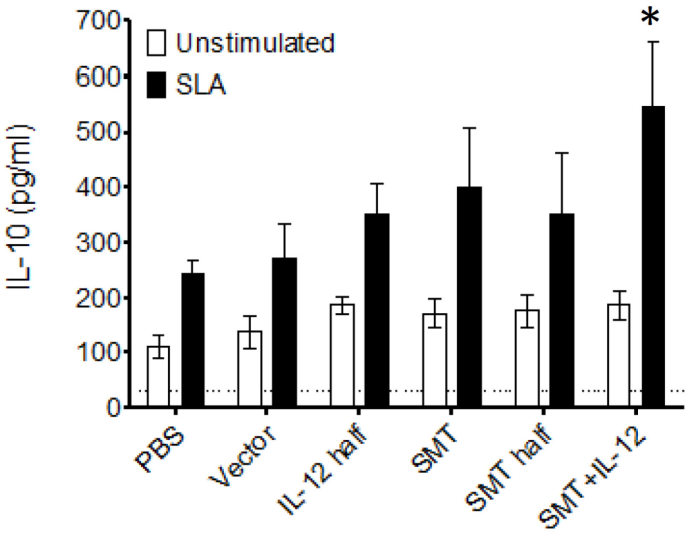


Figure 10. *Ex vivo* responses to SLA of splenocytes from mice immunised with *L. lactis* PH3960 clones and infected with *L. donovani*. Supernatants from splenocytes re-stimulated with SLA for 72 hours were analyzed for **(A)** IFN-γ and **(B)** IL-10 by ELISA. "*" denotes $p < 0.05$ to PBS and Vector. Dotted line represents assay cut-off.

could also explain why no differences in parasitemia were seen, as low IL-4 levels are indicative of progressive VL.

4. Discussion and conclusions

The purpose of the work presented was to develop a live *L. lactis*-based vaccine against visceral leishmaniasis (VL) and further understand the type of responses generated by *L. lactis* immunisation. We used a BALB/c mouse model of *L. donovani* infection and two strains of *L. lactis*, NZ9000 and PH3960, expressing the *Leishmania* antigen SMT cytoplasmically, either unadjuvanted or adjuvanted with the corresponding *L. lactis* strain secreting single-chain (sc) IL-12.

Work in our lab has shown that immunizing BALB/c mice with A2-expressing *L. lactis* is able to reduce *L. donovani* parasitemia in the liver when immunisation doses of 2×10^9, but not 2×10^7 or 2×10^5, bacteria are administered subcutaneously (R. Mahbuba *et al.*, unpublished data). This indicates that subcutaneous injection with higher doses of bacteria could lead to even better protection. However, this is not feasible as injecting mice with more than 2×10^9 *L. lactis* causes them to develop ulcers on the skin. It may be possible to increase the amount of protein contained by the bacteria by altering the duration of induction or using other promoters, which may be constitutive or inducible like P_{nisA}.

Our approach was to use codon-optimisation which has been shown to increase production of recombinant proteins [26]. In codon-optimisation, a desired gene is synthesised using the most commonly used codons for a given amino acid of the desired protein, while also taking into consideration the effect of the new codons on the overall structure of the resulting mRNA. Thus, codon-optimisation of the SMT gene resulted in the replacement of those codons in the original *Leishmania* gene which are rarely used in *L. lactis* by those which are more commonly used in the bacteria.

Immunisation with *L. lactis* expressing an antigen generally induces a balanced Th1/Th2 response. Therefore, it was not surprising when immunisation with 2×10^9 *L. lactis* expressing SMT resulted in mostly anti-SMT IgG1 production. This predominantly Th2 response was not decreased greatly when

using *L. lactis* secreting IL-12 as an adjuvant to better stimulate a Th1 response. Nevertheless, lower parasitemia was observed when *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/IL-12 were co-administered. A greater Th2 immune response resulted when mice were immunised with *L. lactis* PH3960/SMT which may explain why a protective response was not seen in these mice, even when adjuvanted with IL-12-secreting *L. lactis* PH3960. Previous work by Goto and colleagues has shown that immunisation of BALB/c mice with purified SMT results in a predominantly Th2 response [184]. However, adjuvantation of purified SMT with MPL-SE enhanced the Th1 response such that, based on IgG1 and IgG2a levels, the overall response was a balanced Th1/Th2 response. Therefore, it appears that SMT inherently stimulates a more Th2-skewed immune response. *Leishmania* antigens that intrinsically stimulate a greater Th1 response may be better suited from *L. lactis*-based vaccines against leishmaniasis. Alternatively, we could use a delivery vector that drives a stronger Th1 response to express SMT, e.g. *Streptococcus gordonii* [27].

Although we saw similar antibody responses from immunisation with *L. lactis* NZ9000/SMT and *L. lactis* PH3960/SMT (PH3960 induced production of slightly more anti-SMT IgG1 compared to IgG2a), the cytokine expression from splenocytes and the protection from infection were different. When mice were administered *L. lactis* NZ9000 clones and their splenocytes were stimulated with soluble *Leishmania* antigen (SLA) at four weeks after infection, the overall IFN-γ, IL-4 and IL-10 expression was higher in mice that received bacteria than those that received PBS, without much variation within the mice that received bacteria (one exception was high expression of IL-4 from mice that received *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/Vector in combination). When the same was done with splenocytes of mice given the PH3960 strain, we again saw higher cytokine expression from these mice compared to that from mice given only PBS. However, distinctly higher IFN-γ and IL-10 expression was seen from mice that received both *L. lactis* PH3960/SMT in combination with *L. lactis* PH3960/IL-12. In contrast to the *L. lactis* NZ9000 vaccine trial, splenocytes of mice immunised with the PH3960 strain expressed much lower amounts of IFN-γ

compared to the amount of IL-10 expressed. Another contrast between the two *L. lactis* strains was that splenocytes of mice immunised with NZ9000 secreted IFN-γ in the absence of stimulation whereas splenocytes of mice immunised with PH3960 secreted IL-10 in the absence of stimulation. In addition, none of these groups expressed IL-4. In the case of PH3960, the high amounts of IL-10 relative to IFN-γ, as well as the lack of IL-4 production, seem to indicate progressive VL. As mentioned before, immune responses to *L. lactis* are strain specific and our research highlights some differences between the NZ9000 and PH3960 strains. Specifically, our findings show that the PH3960 strain induces a stronger Th2 response and may also cause production of immunosuppressive cytokines such as IL-10. These strain-specific inflammatory properties of *L. lactis* should be taken into consideration when developing live lactococcal vaccines in order to generate the required immune response which confers immunity against a certain disease.

Goto and colleagues showed that when the splenocytes of SMT-immunised C57BL/6 mice were re-stimulated with the antigen, very low amounts of IFN-γ and high amounts of IL-10 were secreted [182]. However, when SMT immunisation was adjuvanted with MPL-SE, IFN-γ secretion increased greatly and IL-10 expression only doubled. Adjuvantation with MPL-SE also greatly enhanced the ability of SMT to stimulate secretion of TNF and IL-2 from splenocytes. Therefore, in the absence of effective adjuvantation, immune responses to SMT are very low and almost negligible. Similar results were seen with immunised BALB/c mice [184]. Thus it seems that SMT requires a very strong Th1-stimulating adjuvant such as MPL-SE in order to protect against *Leishmania* infection.

We observed that mice that were co-immunised with *L. lactis* NZ9000/SMT and *L. lactis* NZ9000/IL-12 had significantly lower parasitemia compared to control mice. Therefore, *L. lactis* secreting IL-12 is a promising adjuvant for live lactococcal vaccines against *Leishmania*. We used murine scIL-12 to stimulate a Th1 response towards SMT. Our scIL-12 is similar to that used by Bermudez-Humaran and colleagues [61]. It is biologically active and induces secretion of IFN-γ from murine splenocytes (Hugentobler *et al*.,

manuscript in preparation). Surprisingly, *L. lactis* NZ9000/IL-12 was also able to lower parasitemia in the absence of SMT, although not to the same degree or to statistical significance. Further studies will be required to investigate this phenomenon. Perhaps, *L. lactis* secreting IL-12 was still present subcutaneously at the time of *L. donovani* infection, thus providing stimulus to elicit a Th1 response. We will need to examine how long after immunisation *L. lactis* persists subcutaneously and ensure that infections are carried out when these bacteria are no longer present. We may also need to increase the amount of IL-12 given to the mice, either by increasing production of IL-12 by *L. lactis* or increasing the ratio of IL-12-expressing bacteria to antigen-expressing bacteria. Alternatively, we could modify the immunisation schedule. Variable results have been observed with regard to IL-12 adjuvantation in *Leishmania* vaccines and the immunisation schedule is a critical factor (discussed in section 1.2.3.1.3). We also chose the end-point of the mouse study at four weeks following infection as this is the time-point where peak parasitemia is observed [185]. It may be worthwhile to monitor parasitemia at various time-points to ascertain how parasitemia in immunised mice varies from that in unimmunised mice.

In our future work, various approaches can be taken in order to stimulate a greater Th1 response when using live *L. lactis* as a vaccine delivery system. DNA vaccination has been used in animal studies of *Leishmania* vaccines [145] and *L. lactis* has also been developed for delivery of DNA vaccines [18]. DNA vaccination is able to elicit humoral, as well as, $CD4^+$ and $CD8^+$ T cell responses. Since *L. lactis* is non-invasive, it can be engineered to express internalin A (InlA) of *Listeria monocytogenes* or fibronectin-binding protein A (FnBPA) of *Staphylococcus aureus* so that *L. lactis* can be internalised [186]. This approach enhances the expression of the desired protein from host cells. DNA vaccination can also be incorporated into a heterologous prime-boost vaccination strategy where the immune system is primed with a DNA vaccine and subsequently boosted with the same protein antigen. Heterologous prime-boost has been shown to enhance cellular immunity against infectious diseases [187]. Thus, we could

use *L. lactis* to first deliver the antigen sequence as the priming DNA vaccine and then the protein as a boost.

Other vaccination routes could also be explored. We used a subcutaneous, parenteral mode of immunisation. Mucosal vaccination by oral or intranasal routes is non-invasive and induces both local and systemic immune responses [1]. *L. lactis* is widely used for mucosal delivery of antigens in animal models of various diseases; in addition, oral delivery of *L. lactis* expressing IL-10 in humans was shown to be safe. Most tested *Leishmania* vaccines are given parenterally; but recently, mucosal routes have been evaluated and show promise. Increased IFN-γ production and protection from cutaneous leishmaniasis is observed upon intranasal administration of Leish-F1 [154] and oral administration of *L. amazonensis* antigens [188]. Mice are protected against VL when intranasally vaccinated with LACK (*Leishmania* analogue of the receptors for activated C kinase) DNA [189]. Immunised mice had higher IFN-γ and IL-4 production compared to control mice. Interestingly, intranasal administration of LACK protein did not offer protection to cutaneous leishmaniasis whereas LACK DNA was protective [190].

We used IL-12 as an adjuvant; similarly, we could also use *L. lactis* expressing IFN-γ either subcutaneously or mucosally [80]. Alternatively, cytokine or chemokine genes could be incorporated into DNA vaccines or heterologous prime-boost strategies [191].

In conclusion, we generated two strains of *L. lactis* expressing the *Leishmania* SMT antigen and showed that when coupled with IL-12 as an adjuvant, *L. lactis* NZ9000 expressing SMT was able to reduce parasitemia in BALB/c mice as well as elicit a mixed Th1/Th2 response. Further work will involve exploring other adjuvants, different routes of immunisation and alternative immunisation regimens. Overall, our work demonstrates that *L. lactis* is a suitable vehicle for the expression *Leishmania* antigens and delivery of VL vaccines.

5. References

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