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Hookworm Na-ASP-2 – putative functions, allergenicity and implications for vaccine development

PhD Thesis

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I have stated clearly and fully in this thesis the extent of any collaboration with others and that to the best of my knowledge and belief, the thesis contains no material previously published by any other person except where due acknowledgment has been made.

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Abstract

Necator americanus is one of several hookworms that infect humans. The other notable species are *Ancylostoma duodenale* and *Ancylostoma ceylanicum*. Hookworms infect approximately 540-730 million people worldwide with more than a billion at risk however there are currently no vaccines to limit the global burden of disease. Activation associated secreted protein 2 (ASP-2) is one of the most highly up-regulated genes upon the transition of hookworm larvae from the free-living to the infective stage. Vaccine trials in numerous animal models of hookworm disease showed that ASP-2 was efficacious and worthy of development as a human hookworm vaccine. *Na*-ASP-2 was safe and immunogenic in a phase 1a human clinical trial in hookworm-naive volunteers in the U.S. However in a phase 1b trial in a hookworm-endemic area of Brazil, a subset of the trial patients exhibited immediate systemic hypersensitivity reactions, due to circulating IgE antibodies against the vaccine as a result of prior hookworm exposure, hence the vaccine was shelved. These studies highlighted the fact that there was insufficient knowledge about the biology of ASP-2 and its interactions with the human host.

My hypothesis was twofold: 1) De-allergenisation (reduce IgE reactivity) of *Na*-ASP-2 to get a better vaccine; 2) ASP-2 has specific molecular and/or cellular binding partners within the human host to exert its effector functions.

In this thesis, I mapped the IgG and IgE epitopes of *Na*-ASP-2 to both recombinant fragments of the protein as well as overlapping synthetic 13mer peptides. I then used this information to design and express site-directed mutants where single amino acids in major IgE epitopes were substituted to Alanine in an attempt to de-allergenise the

protein. Four distinct IgE mutants were expressed in recombinant form and screened for reduced IgE reactivity using sera from phase 1b trial patients. Single point mutations resulted in reduced IgE binding, albeit non-significant and future efforts should focus on a single protein that incorporates all four mutations.

The crystal structure of *Na*-ASP-2 from *N. americanus* reveals a putative equatorial binding groove containing a conserved tandem Histidine motif located in the centre of the groove. This putative binding groove was thought to provide a yet to be determined catalytic activity, prompting speculation that it interacts with proteins from its human host. In this thesis I employed two distinct approaches to elucidate putative host-derived binding partners for ASP-2. Firstly, by panning a random 12-mer peptide phage library, 16 peptides were identified after three successive rounds of panning. Most of the identified peptides were enriched for Glutamine and Histidine residues, and 3 peptides possessed a HXXQH motif, and a fourth peptide contained the highly similar HXXXH motif. BLASTp searches of public databases with an emphasis on the human proteome identified many distinct proteins containing varying degrees of similarity to different regions within the peptides; however, no peptide had a perfect match across its entire sequence length (12 residues) to known proteins, implying that binding of *Na*-ASP-2 to its native ligand is dependent on shorter peptides, possibly those containing an HXXQH motif. One such protein was the SK3 small conductance calcium-activated potassium channel, a transmembrane protein that contained a HNHQH motif within its extracellular domain. Binding of the synthetic SK3 peptide to recombinant *Na*-ASP-2 was confirmed by differential scanning fluorimetry. Potential binding modes of the peptide to *Na*-ASP-2 were studied by molecular dynamics simulations which clearly identify a preferred topology of the *Na*-ASP-2:SK3 peptide complex.

To further explore potential binding partners of *Na*-ASP-2, the recombinant hookworm protein was used to probe a human proteome microarray, whereupon it bound selectively to the B cell Igα receptor CD79a. This is the first description of a host-pathogen protein:protein interaction identified using proteome microarrays. Using flow cytometry I confirmed the association that *Na*-ASP-2 bound to human B lymphocytes *ex vivo.* To investigate the biological effects of ASP-2 on human cells, B cell RNA was extracted and submitted to Next Generation RNA Sequencing, and revealed downregulated transcription of approximately 1000 B cell mRNAs while only approximately 100 mRNAs were upregulated, compared with control-treated cells. The expression of a range of molecules was affected by *Na*-ASP-2, including factors involved in leukocyte transendothelial migration pathways and the B cell signaling receptor pathway. Of note was the downregulated transcription of lyn and pi3k, molecules that are known to interact with CD79A and control B cell receptor signaling processes. Together, these results highlight a previously unknown interaction between a hookworm-secreted protein and B cells, which has implications for helminth-driven immunomodulation and vaccine development. Further, the novel use of human protein microarrays to identify host–pathogen interactions, coupled with *ex vivo* binding studies and subsequent analyses of global gene expression in human host cells, demonstrates a new pipeline by which to explore the molecular basis of infectious diseases.

Publications relevant to this thesis

1. **Tribolet L**[1], Cantacessi C[1,2], Pickering D A[1], Navarro S[1], Doolan D L[3], Trieu A[3], Fei H[4], Chao Y[4], Hofmann A[5], Gasser R B[6], Giacomin P R[1*] and Loukas A[1*] (2015). Probing of a human proteome microarray with a recombinant pathogen protein reveals a novel mechanism by which hookworms suppress B cell receptor signalling. Journal of Infectious Diseases; Feb; 211(3): 416-25.

2. **Mason L****; **Tribolet L****; Simon A; von Gnielinksi N; Taylor P; Willis C; Jones M K, Sternberg P W; Gasser R B; Loukas A; Hofmann A. (2014). Probing the equatorial groove of the hookworm SCP/TAPS protein and vaccine candidate antigen, *Na*-ASP-2. The International Journal of Biochemistry & Cell Biology. May; 50: 146-55 (**Co-first author)

3. Pearson MS, **Tribolet L**, Cantacessi C, Periago MV, Valero MA, Jariwala AR, Hotez P, Diemert D, Loukas A, Bethony J (2012) Molecular mechanisms of hookworm disease: stealth, virulence, and vaccines. J Allergy Clin Immunol. Jul;130(1):**13**-21.

4. Diemert DJ, Pinto AG, Freire J, Jariwala A, Santiago H, Hamilton RG, Periago MV, Loukas A, **Tribolet L**, Mulvenna J, Correa-Oliveira R, Hotez PJ, Bethony JM. (2012) Generalized urticaria induced by the Na-ASP-2 hookworm vaccine: implications for the development of vaccines against helminths. J Allergy Clin Immunol. Jul;130(1):**169**-76.

Publications not directly relating to, but relevant to this thesis

5. Osman A, Wang CK, Winter A, Loukas A, **Tribolet L**, Gasser RB, Hofmann A (2012). Hookworm SCP/TAPS protein structure--A key to understanding host-parasite interactions and developing new interventions. Biotechnol Adv. May-Jun;30(3):**652**-7.

6. Pearson MS, Pickering DA, **Tribolet L**, Cooper L, Mulvenna J, Oliveira LM, Bethony JM, Hotez PJ, Loukas A (2010). Neutralizing antibodies to the hookworm

hemoglobinase Na-APR-1: implications for a multivalent vaccine against hookworm infection and schistosomiasis. J Infect Dis. May 15;201(10):1561-9.

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Chapter 1:

Introduction and Literature Review

1.1 Hookworm Infections and their Global Health Burden

Soil transmitted helminths (STH) infect between 1.5 and 2 billion people worldwide. Of these, hookworms are among the most abundant and infect approximately 740 million people in the warm, tropical and subtropical climates of Sub-Saharan Africa, East Asia and the Pacific Islands, India, Southern Asia, Latin America and China [1] (**Figure 1.1**). Hookworm infections are most common in impoverished or developing tropical countries and thus people infected with hookworms and other STHs already suffer poor diet and nutrition, thereby compounding the problems associated with infection such as iron deficiency anaemia and the lethargy due to continued intestinal blood loss [2, 3].

The annual number of deaths attributable to hookworm varies widely, from 12,000 to 135,000 depending on the source of information [4-6]. However the true burden of hookworm disease is more insidious than solely deaths per annum - the Disability Adjusted Life Years (DALYs) statistic tells a more complete story. One DALY refers to one year of life lost due to the combined effects of premature mortality and time lived in states of less than full health. The global DALY estimates attributed to hookworm have been as much as 22.1 million [4]. This statistic indicates that hookworm is second only to malaria in terms of DALYs attributed to parasitic infections. A major factor in this calculation is the impact on childhood development

and economic productivity [7]. Hookworm disease is more prevalent in males than females [8], possibly due to the immunosuppressive effects of male hormones such as testosterone [9] and its effect on preventing the development of partial immunity. Another reason for the higher burden in males could be as a result of increased activity and contact with infected soil due to occupational risk.

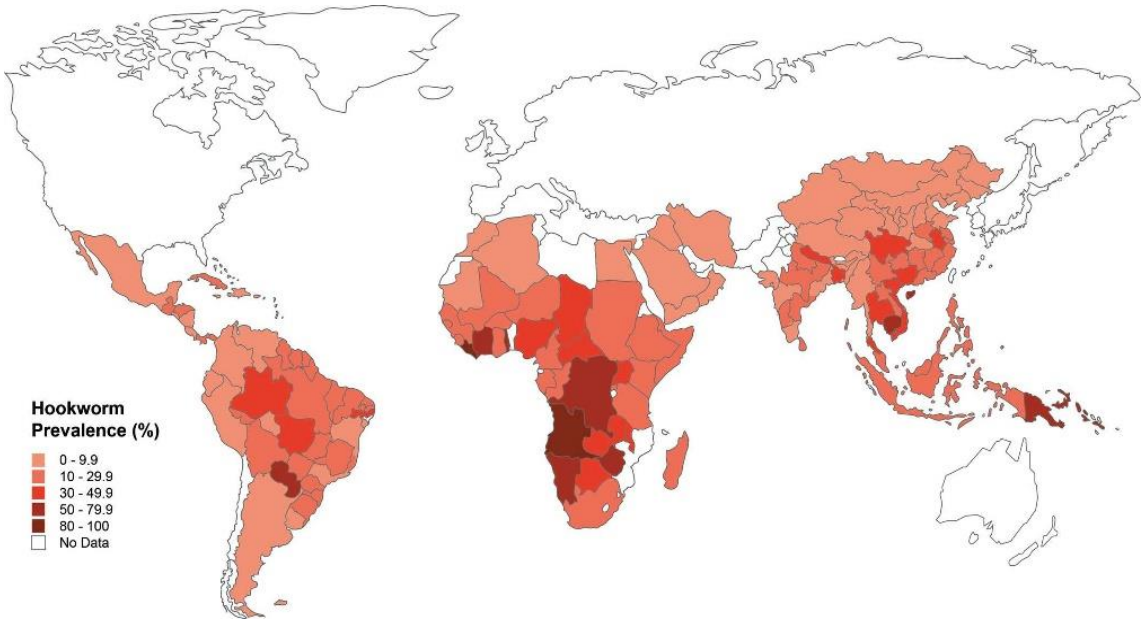

Figure 1.1 Global Distribution of Human Hookworm Infection, 2003 [10]

Illustration: Margaret Shear, Public Library of Science, adapted from [11], copied from [10].

1.2 Hookworm infection history

Hookworms have co-evolved with humans and have been infecting them for as long as records indicate. Intestinal worm infections are mentioned in the Papyrus Ebers, the earliest complete medical text dating from 1550 B.C. [4, 12]. The Vedas from 1500 B.C, an Indian religious text also mentions “abdominal diseases that depend on worms”

[13]. Although hookworm is considered a 3rd world or developing nation affliction, it was present in significant numbers in the southern United States until the early 20th century due to lack of sanitation and poor hygiene standards. The Rockefeller Sanitary Commission began in 1909 and used a combination of chemotherapeutics, improved access to clean water, increased sanitary facilities and education in hygiene standards to significantly reduce infection rates [14]. After its eradication in the U.S., the same group successfully controlled hookworm infection in northern Australia [15].

In 1929 Fulleborn discovered that in the Corrientes region near the border of Argentina and Paraguay almost 100% of the population were infected with hookworm [16]. High prevalence of hookworm infections was not only restricted to developing countries - 77% of the population in an aboriginal community in the northern regions of Western Australia in the mid-1990s were infected with hookworm, resulting in high levels of anaemia and low levels of serum iron and ferritin [17, 18]. Biannual treatment with albendazole over a 6 year period showed hookworm prevalence was reduced to 6% [19], thus demonstrating that although laborious and expensive, sustained chemotherapy, improved education and increased living standards, result in significant health improvements and a reduction in hookworm prevalence.

The emergence of drug failure in treating hookworm infections poses a threat to the sole reliance on anthelmintic drugs to control the infection. For example, mebendazole (the drug of choice in WHO treatment programs) was reported to have diminished efficacy in reducing hookworm burdens in Mali [20]. Moreover, resistance to pyrantel, another commonly used anthelmintic against hookworm infection, has been observed in the Kimberley region of Australia [21]. Clearly, the emergence of reduced anthelmintic

efficacy is a problem for human hookworm infection much like the reduced efficacy against strongylid nematodes of ruminants [22-24] and is already a major issue for the control of livestock helminth parasites [25-27]. Therefore, not only is there a necessity for new chemotherapeutic drugs, but a prophylactic vaccine is needed to avoid the rapid reinfection that follows curative therapy with anthelmintic drugs.

1.3 Hookworm species

Hookworms are members of the phylum Nematoda, order Strongylida. They display common features of nematodes, including a cylindrical appearance and dioecious adult stages. Adult worms are 1-2 cm in length. There are two major species of hookworm that infect humans, *Necator americanus* and *Ancylostoma duodenale.* Three other species, *Ancylostoma braziliense*, *Ancylostoma ceylanicum* and *Ancylostoma caninum* are primarily parasites of dogs but the larvae can penetrate human skin, and *A. ceylanicum* can develop to patency in humans [28, 29]. Indeed, recent reports show that *A. ceylanicum* is the major hookworm species parasitizing humans in some areas of Cambodia, even in the presence of *N. americanus* [30]. *A. braziliense* causes cutaneous *larva migrans* or "creeping eruption" where the larvae of zoonotic hookworms arrest in the dermis and cannot penetrate the sub-dermal layers of human skin. The majority of human infections are caused by *N. americanus* and *A. duodenale*. *N. americanus* is more widespread and accounts for the greatest burden of disease however *A. duodenale* causes the most severe symptoms, primarily blood loss and reduced serum iron levels; this is mainly due to its increased size and voracity of appetite for blood [31], possibly accounting for its increased fecundity [4]. Unlike *N. americanus*, *A. duodenale* can infect humans by both oral and percutaneous transmission [31]. However, *N.*

americanus is more successful at percutaneous entry than *A. duodenale,* possibly due to the more developed burrowing spine in the 3rd stage larva [32].

1.4 Hookworm life cycle

Hookworms require adequate moisture, sandy soil and warm temperatures in order for the eggs to hatch and develop in the soil into the infective third stage larvae (L3). Eggs hatch within 48 hours and become the non-infective rhabditiform larvae (L1). The L1 then moult and enter the 2nd larval phase (L2), before migrating to the surface of the soil or grass where they become the filariform larvae or L3, the infective stage for human hosts. During this stage they become developmentally arrested [33], do not feed and are adapted to surviving in the environment for weeks until they come into contact with human skin [34]. The L3 then migrate to the top of a blade of grass (if in a grassy environment) to increase the chance of contact with a human host [35].

Upon infecting a human host, *Necator* L3 become activated by sensing elements of the human host tissue and serum [35], and actively burrow through the skin. Once the larvae reach the circulatory system they migrate via the afferent vascular system to the heart then to the pulmonary capillaries where they break through the blood vessels into the alveolar spaces and migrate up the trachea, and are finally coughed up and swallowed into the digestive tract [35]. Upon making their way through the stomach to the upper small intestine they moult again and develop to the blood feeding adult stage hookworms, latch onto the mucosa of the duodenum with their teeth (*Ancylostoma*) or cutting plates (*Necator*) [33] and consume a blood meal in order to obtain nutrition and sexually reproduce (**Figure 1.2**).

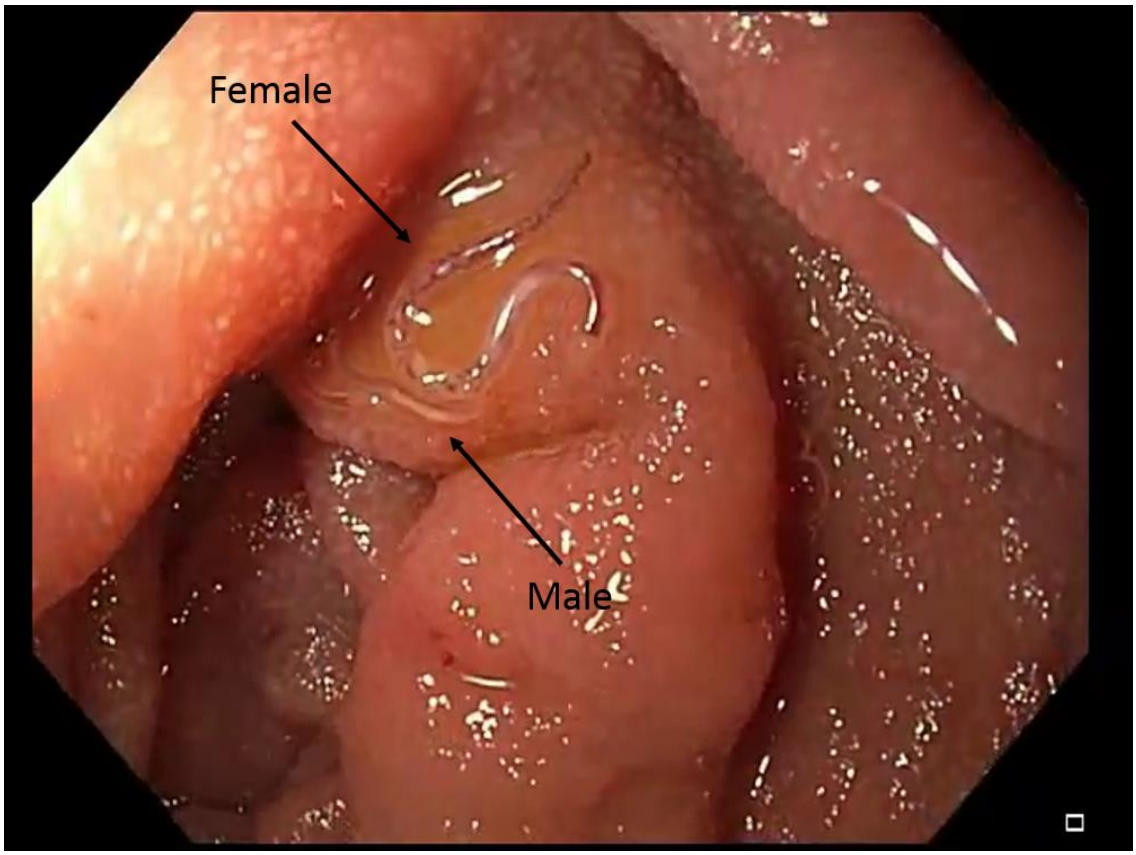

Figure 1.2 Adult female (top) and male (bottom) *N. americanus* mating in the duodenum of an experimental infected human volunteer. Photo captured from an endoscopy video taken from Croese et al., 2015 [36].

Adult hookworms in the duodenum secrete enzymes into the mucosa and rupture capillaries. The worms avidly feed on the extravasated blood and digest their blood meal using a cascade of proteolytic enzymes, breaking down haemoglobin and serum proteins into smaller more easily digestible forms [37-39]. The dioecious hookworms find a mate, copulate, and the females begin to lay thousands of eggs per day. The eggs pass in the faeces of the host, and the eggs per gram of faeces (epg) can be calculated and used as a marker for worm burden (Kato-Katz method) [40, 41]. It takes between 5-

9 weeks from L3 penetrating the skin of the human host to the sexually mature adult stage worms arriving in the gut [42] (**Figure 1.3**). Once they have migrated their way through the gut to the host's intestines, they can survive there for more than 15 years [43].

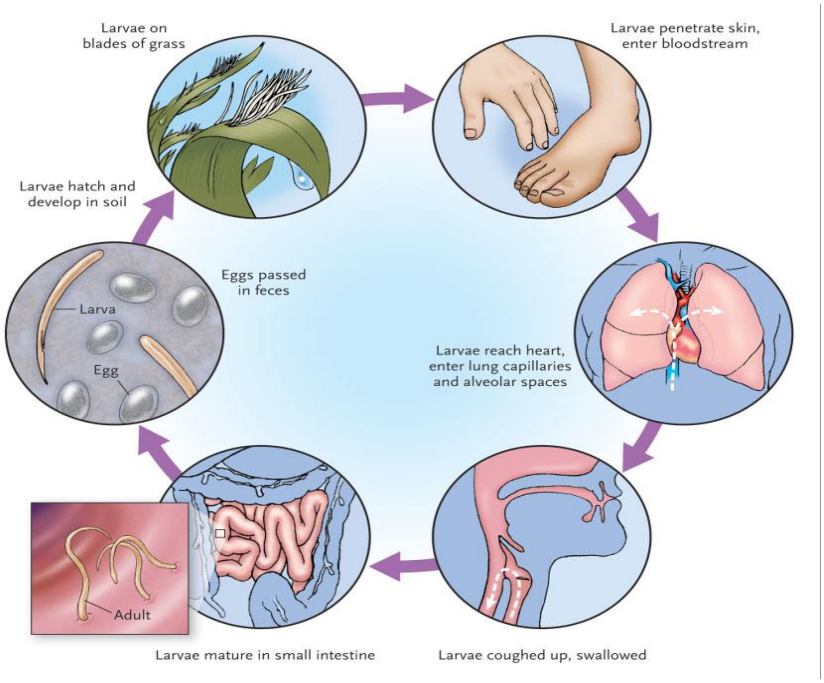

Figure 1.3 The Life Cycles of *Necator americanus* and *Ancylostoma duodenale*. [42]

1.5 Pathology

Iron deficiency anaemia is the most common pathologic sequela of a heavy hookworm burden in both adults and children [16, 44, 45]. Following primary infection, dermatitis, oedema, stomach discomfort and mild nausea are often encountered [46]. Chronic hookworm infections can lead to lethargy, reduced physical fitness, reduced productivity, protein deficiency, stunting of growth, learning difficulties, mental retardation or delayed mental development [31], all the result of blood loss induced by feeding worms. A hookworm infection of 40-160 worms can be associated with

anaemic haemoglobin levels (<11g per decilitre) [47]. In severe cases, extreme hookworm burden combined with lack of sufficient nutrition can result in death [48].
In well-nourished individuals an infection of 20 worms is well tolerated [36] and in fact results in an increase in Hb. A study in Zanzibar using multivariate analyses, showed that intensity of hookworm infection determined via epg, correlated with lowered hemoglobin and serum ferritin [49]. However there are several variables at play that influence the level of anemia/malnutrition and iron deficiency in the hookworm infected individual. The end result being dependent upon levels of infection, overall health, adequate nourishment, immune compromise and comorbidities along with the presence or absence of regular anthelmintic therapy.

Hookworm infection often reaches peak intensity in the elderly [50]; this could be caused by repeated exposure, the longevity of hookworms that are able to live for years inside their human hosts [32], and a reduction in immune competence due to ageing and hookworm burden [51]. This lack of naturally acquired immunity despite (in some cases) decades of hookworm exposure, indicates that a vaccine is not only desirable but essential in the pursuit of a solution to hookworm disease and its eradication on a global scale.

1.6 Current methods for treating helminth infection

Provision of adequate sanitary facilities along with an increase in hygiene standards are absolute requirements to minimize hookworm disease prevalence, combined with education and awareness of risks of transmission. Unfortunately, the challenge of dragging hundreds of millions of people out of poverty is overwhelming, so hookworm disease and other neglected tropical diseases will continue to be a problem for the

foreseeable future until vaccines that prevent reinfection and provide long lasting protection are developed. At present we must rely on anthelmintic treatment exclusively, yet this does nothing to prevent the rapid reinfection that characterises hookworm infection.

1.7 Anthelmintic Chemotherapy

The major families of anthelmintic drugs used to treat hookworm infection are the benzimidazoles, the nicotinic acetylcholine acting agents (cholinergic) and avermectins [52]. Benzimidazole compounds such as mebendazole and albendazole are drugs that bind to nematode β-tubulin and inhibit parasite microtubule polymerisation, thereby causing the death of adult worms. Trials with mebendazole have resulted in varying degrees of success at eliminating hookworm infection, with reported efficacies ranging from 11% [53] to 98% [54], however repeated mebendazole treatments in the same area results in diminished treatment efficacy [55]. Albendazole seems to be more effective at eliminating hookworm infection [4], but all treatments have some degree of variability and therefore reliability. A 2012 study in Lao PDR showed that albendazole was more efficient than mebendazole however a single dose treatment was largely inefficient, providing cure rates of 33% or less and moderate egg reduction [56]. In recent years there has been an increasing body of literature on reduced efficacy of benzimidazoles for human helminths [20, 57, 58].

Levamisole and pyrantel (Combantrin) are cyclic amidines [59] and are examples of cholinergic anthelmintics that are agonists of the nicotinic acetylcholine receptors (AChRs), and cause spastic paralysis of the body cells of the hookworm [52, 60]. Levamisole and pyrantel are effective against roundworms (*Ascaris*) and hookworms

but are not as effective against whipworms [52, 61]. Resistance has been noted with cyclic amidine anthelmintics as shown by Reynoldson et al [21].

Avermectins such as ivermectin are macrocyclic lactones that are produced by *Streptomyces avertimilis* [62]. They are one of the most recently developed of the commercially available anthelmintics and act on glutamate activated chloride channels in nerve or muscle cells of nematodes, causing hyperpolarisation by increasing the permeability of chloride ions through the membrane, which results in paralysis and death of the parasite [63]. Hookworm resistance to ivermectin has not yet been reported, probably due to its recent introduction into treatment programs. However, given its recent widespread use as a broad spectrum antibiotic, hookworm resistance to ivermectin may well develop in the near future, as seen in 2009 and 2011 with the nodule worm of humans, *Onchocercus volvulus* [64].

Monepantel (MOP), a new anthelmintic drug from a group of amino-acetonitrile derivatives has been shown to be effective in sheep and goats [65]. Two other relatively recent anthelmintic are dequantel which is used in conjuction with abamectin and results in acetylcholine receptor inhibition in somatic muscle [66] and also been proven effective against gastrointestinal nematodes in sheep [67]. Thus far these newer anthelmintics have yet to be investigated in human trials.

A combination of 2 or more anthelmintics is more effective than one alone due to differing modes of action; e.g. levamisole/pyrantel and mebendazole given together are better than either one alone [4]. Additionally, there are some promising new drugs on the horizon that are currently being tested in clinical trials, such as tribendimidine

which has a higher efficacy than albendazole in single dose form [68, 69]. Cycling drug use may also reduce chances of emergent drug resistant strains of hookworm; for example using albendazole then pyrantel then tribendimidine with each successive treatment [70].

The major obstacles to successful implementation of uniform mass drug administration (MDA) to control soil transmitted helminths in developing areas is the multitude of factors involved such as funding, manpower, compliance and commensurate improvements in infrastructure to encourage adequate sanitation and hygiene so as to minimise chances of continued transmission [11, 71]. Regardless of efficacy in single dose treatments, none of the drugs available at present effectively prevent hookworm reinfection post-treatment. Reinfection commonly occurs within 4-6 months in highly endemic areas, and 6 months post-treatment, faecal epg have returned to pre-treatment levels [72]. Additionally, if large scale de-worming is undertaken via anthelmintic administration, it must encompass the entire population of a given area, for any heavily infected people that remain untreated will provide a reservoir of contamination for further infections [73].

1.8 Hookworm Vaccines

Despite the lack of evidence of naturally acquired immunity against hookworm infections, no vaccine needs to achieve sterilizing immunity in order for it to be effective in lowering disease burden across populations over time.

The general consensus in combating hookworm infection seems to be a combination of vaccines (when available) and chemotherapy, often referred to as integrated control, or vaccine-linked chemotherapy: as shown previously by dynamic modelling systems

[74]. Populations are first de-wormed with an anthelmintic drug and are then vaccinated once their worm burdens have been eliminated. Over a period of time the overall disease burden is expected to lower to an acceptable level, and with combined improvements in hygiene and sanitation, eradication of hookworm disease in developing countries becomes entirely possible. It has been postulated that a vaccine for hookworm need only be 50% effective to produce a significant reduction in morbidity and infection rates in a given population over time using a stochastic model [75].

Other challenges in both developing and delivering a hookworm vaccine include the global scale of infected populations and the fact most are in rural and remote communities. Additionally considering hookworms are considered a Neglected Tropical Disease (NTD) and primarily in the developing or 3rd world, they are low on the list of priorities for large pharmaceutical companies as they may not provide the guarantee of profit desired by most commercial partners [1].

1.8.1 Live attenuated larval vaccine

There have been several strategies implemented in the pursuit of an effective hookworm vaccine. From live attenuated larvae, deceased larvae, recombinant excretory/secretory proteins and more recently peptide based subunit vaccines attached to immune targeting molecules. Despite many attempts there is currently no commercially available hookworm vaccine. In 1964 Miller created a live, radiation-attenuated *A. caninum* L3 vaccine which provided up to 97% protection in dogs [76]. Miller used X-rays of 40 kilorads (40 kr) to irradiate *A. caninum* larvae, which when used to inoculate dogs, afforded 88-97% protection against larval challenge [76]. However, the vaccine had drawbacks, including the ready availability of sufficient

numbers of hookworm larvae for industrial sized scale-up and the cost of processing the larvae into a vaccine after the necessary irradiation to ensure attenuation and safety.

The *A. caninum* attenuated L3 vaccine was available commercially for a short while in the United States in the 1970's [77] but was discontinued due to a combination of poor sales and complaints from veterinarians about the presence of low numbers of eggs in the faeces after vaccination. In comparison, influenza virus vaccine use is both commonplace and widespread in the developed world, despite proven vaccine effectiveness (VE) of only 44% [78]. To have a long-term impact on reducing transmission, a vaccine need not be sterilising to prevent widespread propagation of hookworm disease. It merely needs to be effective at reducing symptoms and transmission to sufficiently low levels as to provide benefit to the most susceptible groups (such as children and the elderly) and to eradicate the disease in a cost effective manner over a reasonable time frame. Moreover hookworms do not produce asexually within the host, further supporting the development of partially efficacious vaccines.

1.8.2 Use of rodent models

Most of the early work in the development of hookworm subunit vaccines was conducted in mouse models due to their relative ease of accessibility and low cost. The mouse model of hookworm, however, is far from ideal due to the limited developmental capacity of the parasite in mice. As a result, researchers have focussed on establishing animal models of hookworm infection/disease, both naturally occurring and experimental, where adult worms mature and produce patent infections, thereby facilitating access to adult worms and allowing the effect of a vaccine on fecundity of female worms to be readily assessed. The dog-*A. caninum* hookworm relationship has

been utilized as a naturally occurring host-parasite model for the development of human hookworm vaccines [79-81], but is very expensive and comes with ethical considerations. Both *N. americanus* and *A. ceylanicum* can complete their development in hamsters [82, 83], although the yield of adult *N. americanus* obtained from hamsters is sub-optimal. *N. americanus* infection has been established in the common marmoset, *Callithrix jaccus,* which exhibits many characteristics of the immunology of hookworm infections in humans [84], but is difficult to establish in terms of logistics, ethical considerations and cost.

1.8.3 Promising recombinant protein vaccines

There are several potential vaccine candidates in early stage trials that show promise in animal models. Hotez *et al* 2010 summarised the current vaccine candidates in terms of efficacy at reducing worm burdens and epg, protection against blood loss, and the predicted ease of scale-up for industrial production [3]. The most promising thus far from the adult stage parasite is *Necator americanus*-Aspartic Protease-1_{mut} (*Na*-APR-1_{mut}), a catalytically inactivated form of the major haemoglobinase used by hookworms to digest their blood meal [37, 85, 86]. *Na*-APR-1 initiates the digestion of haemoglobin, cutting it into smaller more digestible pieces that can be further digested by a suite of other hookworm proteolytic enzymes, granting the worm its primary source of nutrition [38, 39]. Dogs vaccinated with APR-1 had reduced anaemia, worm burden and faecal egg counts [37, 86].

The importance of L3 secreted antigens was illustrated by the larval attenuation vaccine developed by Miller [77], as well as the findings of Hawdon and Hotez [35] who reasoned that L3 excretory/secretory proteins were eliciting the protective immune

response seen in the attenuated larval vaccine. Therefore, some of the major L3 secreted proteins are being developed as one component of a human hookworm vaccine by the Human Hookworm Vaccine Initiative (HHVI) [1]. Indeed, one of the lead L3 antigens for a human hookworm vaccine is *Necator americanus*-Activation associated Secreted Protein-2 (*Na*-ASP-2), a member of the ASP family of proteins that will be discussed at length in the section on hookworm ASPs (**Section 1.12**).

Antigen selection for a hookworm vaccine has been guided in part by efforts to develop a vaccine for the related nematode parasite of ruminants, *Haemonchus contortus. H. contortus* produces a multi-glycoprotein complex in the gut wall of the adult blood-feeding stage, H-gal-GP, a mixture of glycosylated proteases, protease inhibitors, lectins and other proteins thought to be involved in nutrient acquisition and digestion. Vaccination with the H-gal-GP complex elicits high levels of protection against *H. contortus* challenge infections in sheep [87]. The cattle nematode vaccine against *C. oncophora* is based on an ASP produced by the adult stage worm and is proving effective in reducing fecundity [88]. Therefore given the efficacy of attenuated parasites (irradiated hookworm L3) and antigen complexes (*H. contortus* H-gal-GP) as anti-nematode vaccines, investigating both larval and adult hookworm antigens is a logical approach in the pursuit of developing a prophylactic subunit vaccine [89]. A bivalent vaccine containing a larval protein such as *Na*-ASP-2 and an adult protein such as *Na*-GST-1 [90, 91] or *Na*-APR-1 would be an optimal approach, thereby developing protective antibodies against the two major life cycle stages of the parasite that reside inside the human host. This is the major approach to developing a human hookworm vaccine currently being undertaken by the HHVI.

1.8.4 Challenges in developing a hookworm vaccine

Recent studies have shown that certain hookworm antigens that are immunogenic and well recognized by infected individuals can also pose a problem via antigen-specific circulating IgE antibodies [92] in adults and the elderly. This can result in allergic reactions upon vaccinations which then disqualifies these antigens as vaccine candidates. However this is less likely to be a problem in young infants due to their lack of repeat exposure and reduced opportunity to develop antigen-specific IgE antibodies against hookworm. Thus in endemic areas, a hookworm vaccine could be given along with current measles, mumps and rubella vaccines.

Additionally targeting antigens not or poorly seen in natural immunity such as in the case of "hidden antigens" may limit the uptake or effectiveness in protective antibodies. Although when examples of such vaccines have been administered in animal models via multiple boosts there is evidence of protection [37].

1.9 Hookworm ES proteins

As outlined in section 1.8, there is much interest in the biology of hookworm ES proteins as vaccine candidates. However, there is also interest in the properties of parasite ES proteins based on their purported immunomodulatory capacities [93], which could be used as novel therapeutics. Hookworm ES products are the molecules secreted by hookworms and represent the primary molecular interface between the parasite and host. ES products consist of a complex mix of proteins, carbohydrates and lipids secreted from the surface or oral openings of the parasite [94]. ES products contain molecules that are essential for establishment and maintenance of a parasitic existence and include proteins that are essential for invasion, migration, feeding and immune evasion. The secretomes of hookworms have been characterised at the protein [94] and

genome/transcriptome [95, 96] levels, shedding light on the complex molecular interactions between the parasites and their host tissues.

ES products include proteases that allow the larvae entry into the human host through the epidermis and dermal layers [97] and into the circulatory system. Hookworms also secrete a variety of factors to suppress the immune system, allowing the parasite's survival whilst existing inside the circulatory system that is abundant with immune cells, antibodies, complement and other factors that are deleterious to pathogen survival [98]. Hookworms secrete different molecules at different developmental stages to overcome the various hurdles presented to it on its transit through tissues, i.e. entering the skin, lungs and gastrointestinal tract.

1.10 ES antigen discovery

Antigens on the surface of adult *Necator* worms were being investigated as early as 1987 by Behnke and Pritchard [99]. Using immunoblots they noted different migration profiles of the secreted proteins released by different developmental stages of *N. americanus* [100]. Some of the first proteins to be biochemically characterised from hookworms were acetylcholinesterases [101, 102], hyaluronidases [103] and a suite of gelatinolytic proteases including metalloproteases [82, 83]. Meanwhile, efforts were underway to improve hookworm L3 larval culturing techniques *in vitro* [104] to facilitate their maintenance in a laboratory environment. With the development of cDNA phage library technology, cDNAs encoding many more hookworm ES products were identified, with a particular focus on proteases and ASPs [105].

Early efforts in high throughput gene discovery resulted in an expanding dataset of hookworm genes encoding secreted proteins [106], culminating in the description of 20,000 *N. americanus* cDNA contigs [95] and approximately 90% predicted coverage of the *A. caninum* transcriptome [107]. Further, the tissue-specific transcriptomes of adult hookworms have been determined using laser microdissection microscopy of specific organs of adult *N. americanus* and *A. caninum*, to make tissue specific cDNA libraries for EST sequencing [108]. Most recently, the draft genome of *N. americanus* was sequenced, revealing more than 19,000 genes [96].

DNA microarrays have recently been used to characterise the differentially transcribed mRNAs of *A. caninum* L3 upon activation with host serum [109]. The two largest families of up regulated mRNA transcripts in activated hookworm larvae encoded members of the Pathogenesis Related Proteins (PRP) family (of which ASPs are members) and proteases. Interestingly, two of the three leading vaccine antigens for development of a human hookworm vaccine are an ASP (*Na*-ASP-2) and a protease (*Na*-APR-1) [1]. Proteomic strategies have been used to characterise the secreted proteome of adult *A. caninum* using off-gel electrophoresis and tandem mass spectrometry [94]. Twenty-eight percent of the identified proteins were members of the ASP family. A total of 34 of these ASP proteins were identified, providing further evidence and implication that this particular protein family plays an integral role in host-parasite interactions and appears to have undergone a massive expansion in the hookworms in particular [94, 110].

Massive parallel sequencing has been used to identify 19,997 contiguous sequences (contigs) and of these 6771 had known orthologues in *C. elegans* [95]. Use of the recent

development of online databases of nematodes allows comparative analysis of the experimentally discovered contigs with known contigs, allowing estimation of families of functional proteins and associations with known molecular pathways. Further prioritization identified 18 putative drug targets with no human homologues [95].

Advances in genomics along with the commercial availability of genomic analyses have seen an increasing number of whole genomes being published in recent years. The *N.americanus* genome revealed that there were 137 predicted SCP/TAPS proteins [96].

1.11 ASP Proteins – SCP/TAPS

ASPs are a member of the Pathogenesis Related Protein super family (PRP) and the sterol carrier protein/Tpx-1/Ag-5/PR-1/Sc-7 (or SCP/TAPS) family [94]. The first PR-1 protein was discovered in plants and was associated with pathogen infection [111]. There has since ensued a steady stream of discovery of SCP/TAPS proteins from tomato plants to soil transmitted helminths, however the designated nomenclature has remained inconsistent and confusing and includes acronyms such as PRPs [112], venom allergen-like proteins (VALs) and ASPs. Sequence analysis and phylogenetic comparison of the SCP/TAPS within and between eukaryotic species may lead to a more definitive classification system [113]. Other members of the PR-1 family are from such disparate sources as fungi and maize [112], to the human brain tumour proteins - P25Ti and GliPR [114].

Advances in next generation sequencing and bioinformatics applications have resulted in a wealth of sequence information on PRPs or SCP/TAPs. This approach has been

used to analyse the SCP/TAPs proteins in parasitic trematodes including multiple species of liver and blood flukes [115], utilising algorithms that detected the presence of specific cysteine residues conserved in SCP/TAPS proteins along with secondary structure predictions and homology modelling revealed a total of 151 proteins that had significant homology to known SCP/TAPS proteins.

PRPs are present in plants and are normally expressed in response to pathogenic infections and diseases. An early example was discovered in 1982 by Camacho and Sanger and dubbed p14 [97]. P14 is expressed in tomato plant leaves in response to viral and fungal infection and is thought to be a general pathophysiological response of the tomato plant to infection [116]. Some other examples of PRPs in plants include a bean protein called PR-4 [117], PR-1c in tobacco [118], PRb-1 in barley [119], CR-16 in carrots [120], CaPR-10 from hot peppers and PdPR5-1 in prunes [121]. Almost all are expressed in response to pathogenic infection and are produced as a self-defence mechanism by the plant [122]. PRPs are found in nematodes other than hookworms, including *O. volvulus* and the plant parasitic nematodes such as *Heterodera schachtii*, although the exact function of these proteins and their modes of action are still to be ascertained [123]. As previously mentioned the nematode vaccine against *Cooperia oncophora* is based on an ASP produced by the adult worm and reduces parasite egg output. It is believed that all nematodes possess SCP/TAPs/PRPs although in hookworms for unknown reasons, they are expanded in number [96].

Considering the ASP family of molecules is also present in such a diverse range of organisms from fungi to plants, to parasitic nematodes and mammals suggests a certain conservation of function [124] and/or a necessity of some of these proteins to fulfil

basic, essential roles for the organism. It begs the question: If plants produce SCP/TAPS or ASPs as a defence mechanism in response to infection/invasion, is this similar to the role they play in parasitic nematodes such as hookworm, when they are attacked by the host's immune system?

1.12 Hookworm ASPs

ASPs are highly up-regulated in hookworm L3 upon the transition from a free-living to a parasitic state [109]. The fact that hookworm ASPs are so highly expressed upon contact with serum would indicate they may have a function involved with host invasion and/or immune modulation or evasion [94, 109, 125]. ASPs have been topical over the last 10 years due to their efficacy as hookworm vaccine antigens in animal models [79], their adjuvant properties [79, 126, 127] and their potential anti-inflammatory properties [128].

ASPs were first described from *A. caninum* L3. *Ac*-ASP-1 was first identified as the major protein produced by the L3 upon entry into the canine host [129], and a major component of the L3 ES products. Recombinant *Ac*-ASP-1 adjuvanted with alum has been used to vaccinate mice (which are not fully permissive hosts for hookworms) and showed a reduction of 79% of L3 migrating to the lungs [130]. Similarly, ASP-1 orthologues from three different types of hookworms have been shown to protect mice against larval challenge [131].

Using structure based alignment of known ASPs and ASP transcriptomes [109], they can be classified into 3 structural families. Generally across all ASPs, dioether bonds 1-3 occur in the central core of the protein and dioether bonds 4 and 5 if present, occur in

the c-terminal moiety. Both type 1 and 3 ASPs possess 5 dioether bonds (apart from a couple of specific exceptions), whereas type 2 ASPs are lacking the dioether bond 2 from the core [132]. The major difference between groups 1 and 3 is group 1 is characterized by 2 conserved Histidine residues (His-69 and His-129) while group 3 is missing these conserved Histidine residues [132].

Neutrophil inhibitory factor (NIF) found in ES from *A. caninum* was the only ASP-like protein that has a known function prior to commencement of my PhD project. It binds to β-2 integrin found on the surface of leukocytes and suppresses neutrophil recruitment [133]. It was trialled as a vaccine in animals and although it showed significant reductions in fecundity via reduced faecal egg counts, it was not significantly protective in reducing worm burden [128]. *Ac*-ASP-3, *Ac*-ASP-4, *Ac*-ASP-5, *Ac*-ASP-6 [125] and *Ac*-ASP-7 [109] have also been characterised to some degree yet none of their functions are known, although the crystal structure of *Ac*-ASP-7 has been solved [132].

1.13 *Na*-ASP-2 as a vaccine

In 1999 Hawdon cloned and characterized ASP-2 from *Ancylostoma caninum* L3 [134]. Serum antibodies isolated from dogs that were vaccinated with recombinant *Ac*-ASP-2 were used to immunoprecipitate *Ac*-ASP-2 protein from L3 extracts, indicating recognition of native ASP-2 from L3 in vaccinated dogs [79]. Recombinant *Na*-ASP-2 was produced in insect cells as a secreted protein, and the ability of anti-recombinant ASP-2 antibodies to pull-down the native parasite-derived protein implied that the recombinant disulphide bond-rich protein had faithfully replicated the fold of the wild type protein secreted by L3. Vaccinated dogs had high titre IgG1 and IgG2 responses

and significantly reduced adult worm burdens and egg counts after challenge infection with *A. caninum* L3 [135].

Recombinant *Na*-ASP-2 was expressed in the yeast, *Pichia pastoris* and underwent pre-clinical evaluation as part of the HHVI development program [135]. The crystal structures of *Necator Na*-ASP-1 and *Na*-ASP-2 have been solved, allowing further insights into the ASP family of proteins [136, 137] (**Figure 1.4**). Critically, screening of sera from patients living in endemic areas of hookworm infestation in Hainan China and Minas Gerais, Brazil- revealed that a significant protective association was seen between the presence of IgE antibodies to *Na*-ASP-2 and a reduced likelihood of heavy hookworm infection in both humans and laboratory animals [79]. Following this discovery, animal studies ensued with *Na*-ASP-2 as a vaccine candidate for human hookworm disease.

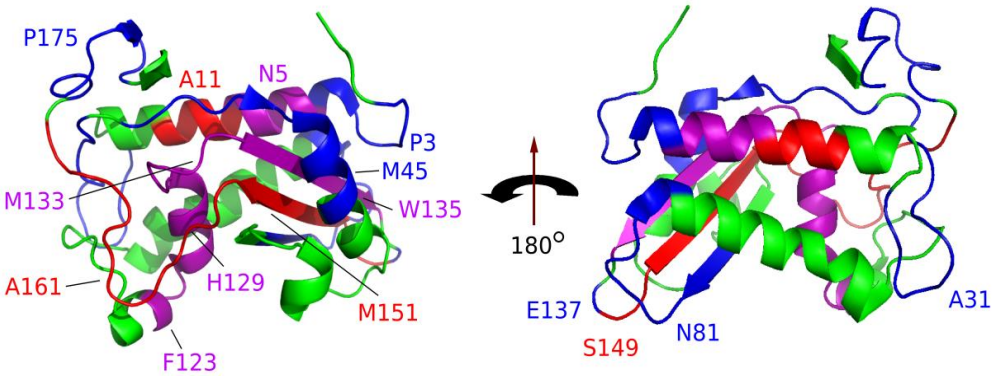

Figure 1.4 Crystal structure of *Na*-ASP-2 [137].

Animal trials of the recombinant *Na*-ASP-2 hookworm vaccine showed that the adjuvanted protein was immunogenic well tolerated, and stimulated high antibody titres in laboratory animals [82]. Additionally, upon challenge with hookworm L3 the vaccinated dogs had significantly lowered faecal egg counts and intestinal worm

burdens, providing further evidence of the suitability of *Na*-ASP-2 as a vaccine candidate. A phase 1a human clinical trial was then undertaken in the United States. The trial was placebo controlled and blinded, with naïve volunteers, namely those with no prior hookworm exposure. This phase 1a trial yielded high geometric mean titres, was well tolerated and induced a lasting immune response in the trial subjects [138].

Following the safe completion of the *Na*-ASP-2 phase 1a trial in the USA, a phase 1b trial commenced in Brazil in subjects resident in a hookworm endemic area but whom were treated with albendazole prior to vaccination. Three of the 9 subjects that received the vaccine suffered immediate hypersensitivity reactions in the form of full body urticaria [92]. The trial was immediately terminated and the subjects with allergic reactions were successfully treated with antihistamine. The subjects who developed urticarial responses were subsequently shown to have high circulating anti-ASP-2 IgE titres prior to vaccination. Despite its promise as a vaccine target in animal models and human trials in hookworm-naïve individuals, *Na*-ASP-2 was shelved as a vaccine candidate until further notice. Furthermore, surveys post-trial were conducted in hookworm endemic areas in Brazil and a large proportion of the community demonstrated significant levels of IgE against *Na*-ASP-2 [92]. One notable effect of this trial is the major changes to the way helminth vaccine antigens will be selected and trials will be designed in the future, where potential vaccine candidates will be screened against banks of sera for IgE reactivity in order to ensure that only safe vaccines are developed.

1.14 "De-allergenising" *Na*-ASP2

Efforts are underway to "revive" *Na*-ASP-2 as a viable hookworm vaccine, with two major approaches being considered. The first strategy involves re-engineering of the recombinant protein to include the human immunoglobulin Fc gamma antibody fragment so as to target the protein to a specific receptor in order to bypass allergy-inducing pathways and abrogate histamine release in vaccinated subjects. An Fcγ-ASP-2 chimera protected hamsters against hookworm challenge to the same extent as wild type recombinant ASP-2 [139]. Moreover, the chimeric protein efficiently reduced the release of histamine in human basophils sensitized with anti-*Na*-ASP-2 IgE obtained from individuals living in a hookworm-endemic area. In dogs infected with canine hookworm, the chimera resulted in significantly reduced immediate-type skin reactivity when injected intra-dermally compared with wild type recombinant *Na*-ASP-2. There is no literature evidence thus far that long term use of Fcγ-chimeras promoting or inducing autoantibodies, however this could theoretically present a problem.

The second strategy to "de-allergenise" *Na*-ASP-2 is to re-engineer the protein via targeted mutagenesis of IgE-eliciting epitopes identified in the *Na*-ASP-2 molecule from the phase 1b clinical trial [92], and this approach underpins a major component of my PhD thesis.

1.15 Immune responses to helminths and allergens;

1.15.1 Type 2 cytokine responses

CD4+ helper T cells possess the ability to differentiate into a number of specialized subsets in order to facilitate distinctly different roles for the immune system depending on the stimulus and response required [140, 141]. Th2 cells are of particular interest in helminth infections [142, 143], producing cytokines such as IL-4, IL-5 and IL-13 that stimulate humoral responses such as IgG1, IgG4 and IgE and effector granulocyte

responses that cooperate to damage parasites [144]. However, these same Type 2 immune responses are pathogenic during allergic inflammation, where the body reacts inappropriately to harmless environmental stimuli and host tissues can be attacked.

1.15.2 IgE and Allergy

There are 5 isotypes of Immunoglobulin (Ig) in humans - IgA, IgD, IgE IgG and IgM. IgG antibodies also have 4 further subclasses IgG1, 2, 3 and 4 [145]; each of these classes and subtypes play a specific role in the immune response. IgA is mainly found in the body's mucous membranes, IgD is predominantly membrane-bound on the surface of B cells; IgM, IgG and IgE are commonly produced by B lymphocytes in response to invading pathogens. Specific cytokine presence or absence in the immune milieu can also dictate or drive Ig-specific immune responses via T-cell dependent immune responses involving both mast cells and B lymphocytes; for example IFNγ can promote class switching toward IgG2a that is important for immunity to bacteria and viruses while IL-4 promotes IgG1, IgG4 and IgE responses that are involved in responses to allergens and helminths.

IgE is generally the lowest antibody by concentration in circulation and has long been associated with allergic responses [146]. IgE binds to its specific antigen and cross links with mast cell and basophil Fcepsilon(ε)Receptor1 in a crucial element of the allergic pathway which leads to histamine release and classic allergic symptoms. As noted above, IgE is commonly elevated in patients with allergies or parasitic infections [147]. When antigen specific IgE binds to FcεRI-sensitized mast cells or basophils, the cells degranulate, releasing a variety of inflammatory mediators such as histamine, serotonin, heparin, mast cell proteases, tumor necrosis factor (TNF), platelet activating factor and Type 2 cytokines. These cytokines, such as IL-4, IL-5 and IL-13 increase

IgE production and drive Th2 responses, such as eosinophilia, mucus production and smooth muscle hyper-reactivity [148]. These mediators are sometimes referred to as a "cytokine storm", and are the main drivers in the appearance and pathology of allergic symptoms.

1.17 Immunology of hookworm infections

Hookworms elicit a Type 2 immune response in infected individuals, characterised by eosinophilia, high IgE titres and IL-4, IL-5, IL-9 and IL-13 production [149]. IL-5 is associated with resistance to g.i. nematode infection [150]. Human hookworm infections elicit IgG1, IgG4, IgM, IgD IgA and IgE antibodies that bind to hookworm antigens [151]. The order of the antibodies arising is usually IgM>IgG>IgE, the latter arising slowly over repeated exposures, as shown from experimental infections [152, 153]. Interestingly, a protective association has been observed between increasing levels of IgE against *Na*-ASP-2 and lowered risk of hookworm infection [79]. This indicates that IgE production against worm antigens can be beneficial for natural immunity. Despite this anecdotal evidence, vaccination with the same antigen in animal models produced high IgG1 antibody titres and resulted in significant protection, and highlighted the distinct anti-ASP-2 antibody phenotypes that ensue from natural hookworm infection versus recombinant protein vaccination.

Very little research has been conducted regarding the molecular mechanisms by which hookworms elicit Type 2 immune responses. *Na*-ASP-2 administered to rodents in recombinant form elicits a type 2 cytokine response typical of helminth infections, along with *Na*-ASP-2 specific antibodies and neutrophil recruitment [154, 155]. Despite these observations, no direct interactions between *Na*-ASP-2 and a defined cell

type or molecule had been demonstrated prior to the commencement of my PhD project.

1.18 Introduction to this study

There is a dire need to develop an effective vaccine against human hookworm infection. Based on evidence from naturally resistant humans from endemic areas, and vaccine studies in dogs and previously hookworm naïve humans, *Na*-ASP-2 is one of the best candidates for such a vaccine. However, its progression to phase 2 clinical trials has been hampered by obvious safety concerns related to risk of urticaria in individuals from endemic areas with prior hookworm exposure. As a consequence, the overall aim of my thesis will be to increase our understanding of the immunobiology and allergenicity of *Na*-ASP-2.

The hypotheses underpinning this thesis are that:

1. The *Na*-ASP-2 protein contains specific residues that determine its IgE reactivity
2. Mutation of these residues will result in a less allergenic vaccine
3. *Na*-ASP-2 interacts with its human host via specific cell surface proteins, most likely elements of the host immune system

The first specific aim was to explore the possibility of de-allergenising *Na*-ASP-2 by identifying the predominant IgE epitopes recognised by hookworm infected individuals and mutating these residues in the recombinant protein to render the vaccine less allergenic. Other allergens from plants and food have been re-engineered to remove IgE epitopes, often reducing the allergenicity of a protein by more than 95%. The next specific aim was exploring the biological role and potential binding partner of at least one member of each structural group of ASPs (including *Na*-ASP-2 as a representative group 1 member). Several methods will be utilized in order to achieve this objective. Due to time constraints, only the structural group 1 ASPs (to which *Na*-ASP-2 belongs) was investigated.

It is expected that this study will provide a detailed analysis of the biology of Na-ASP-2, potentially enabling its revival as a vaccine candidate and also discovering potential host-parasite interactions that shed light on hookworm mechanisms of immune modulation, and their potential exploitation as immunotherapeutics for treating inflammatory diseases.

Chapter 2:

De-allergenising the human hookworm vaccine candidate, *Na*-ASP-2, via targeted mutagenesis.

2.1 Introduction

Based on the significant protection induced against hookworm challenge infection in laboratory animals following vaccination [79, 82], *Na*-ASP-2 was selected to progress into human clinical trials as part of the development of a recombinant vaccine for human hookworm disease [3]. Despite being well tolerated and immunogenic in a phase Ia clinical trial conducted in helminth-naïve individuals living in the United States [138], future development of the *Na*-ASP-2 vaccine was suspended as it induced an immediate hypersensitivity response including urticaria, mild pain and swelling when administered to individuals in a phase Ib trial in a helminth-endemic area of Brazil [92]. The allergenicity of the vaccine was subsequently linked to pre-existing IgE to native, parasite-derived *Na*-ASP-2 induced by prior hookworm infection [92].

These findings provided the imperative to "de-allergenise" *Na*-ASP-2 before any further development of this vaccine was considered. Numerous reports in the literature have described the de-allergenisation of allergy-inducing molecules such as the shrimp allergen Tropomyosin Pen a 1 [156], the White Birch pollen allergen Bet v 1 [157], the Ves v 5 wasp venom allergen [158], the Der f 2 house dust mite allergen [159, 160] and the Timothy grass pollen allergen Phl p 5 [161] through the ablation of IgE-inducing epitopes by targeted mutagenesis. This approach has also been successfully used to

reduce binding of human and mouse IgE antibodies to the high affinity receptor, FcεRI [162].

Allergic symptoms are initiated by the binding of allergens to IgE antibodies which stimulates the cross linking of IgE, leading to aggregation of the IgE receptor on mast cells and basophils which triggers the release of histamines and other chemical mediators of allergy [163]. Targeted mutagenesis of allergens that reduce IgE binding and subsequent allergic manifestation while maintaining some degree of immune reactivity has been well documented. For example, hypoallergenic derivatives of the Hymenoptera wasp venom allergen Ves v 5 [158], the common house dust mite allergens Der f 2 [164] and Lep d 2 [165], the major peanut allergen Ara h 6 [166] and the Timothy grass pollen allergen Phl p 5 [161], have all been produced by selective disruption of disulfide bonds or deletion of proline residues that destabilise secondary structure and abrogate IgE epitopes. A report of allergen mutagenesis most pertinent to this study documents the ablation of a dominant IgE epitope from Pen ch 18, a major allergen of the fungus *Penicillium chrysogenum* whereby charged amino acids crucial to IgE epitope formation were substituted with alanine residues [167].

Firstly, this chapter describes the identification of IgE-inducing epitopes of *Na*-ASP-2, a necessary first step in the de-allergenisation of this molecule. Overlapping recombinant *Na*-ASP-2 fragments (spanning the length of the protein) were expressed in *E. coli* and probed by Western blotting with human sera from *Na*-ASP-2 phase Ib clinical trial volunteers [92] to roughly identify the regions of the molecule that contained IgE- and IgG-inducing epitopes. It was hoped that the IgG and IgE dominant areas of the protein would be mutually exclusive or in clustered locations on the

molecule, in which case the overlapping peptide approach could be focused on a more specific area. Unfortunately this was not the case and it was necessary to have the entire ASP-2 protein epitope mapped. Elucidation of minimal epitopes was then achieved by the synthesis of overlapping 13-mer peptides across the entire *Na*-ASP-2 protein and probing these peptides with phase Ib trial patient sera using an ELISA-based approach. In an attempt to de-allergenise the protein, charged and/or polar amino acids contained within two of the major identified IgE-inducing epitopes of *Na*-ASP-2 were substituted for non-polar alanine residues by site directed mutagenesis in order to disrupt epitope formation. Four single substitution mutants of *Na*-ASP-2 were produced and tested (along with the wild-type protein) for their ability to bind anti-*Na*-ASP-2 IgE and IgG from phase Ib trial patient sera [92].

2.2 Materials and methods

2.2.1. Cloning of recombinant Na-ASP-2 fragments

The crystal structure of *Na*-ASP-2 [137] was used to design short fragments with optimal stability for recombinant protein expression. Oligonucleotide primers incorporating *Nco*I and *Xho*I restriction sites were designed (**Table 2.1**) to amplify five overlapping cDNAs spanning the entire length of mature *Na*-ASP-2 (minus the signal peptide) from a *N. americanus* cDNA library available in our laboratory using *pfu* turbo DNA polymerase (Stratagene).

Fragment	Position	Primer
1	Gly-1 – Met-48	Fw: catgccatgggaggttgtcctgacaatggaatg
		Rev: cgctcgagcatcgtcttcatcttagcagc
2	Gln-30 – Leu-78	Fw: catgccatgggacaggccaaggatggagctggtggaaatgcc
		Rev: cgctcgagcaatcctttcctttggttaggc
3	Gln-64 – Lys-110	Fw: catgccatgggacaatgtgtattcaagcactcg
		Rev: cgctcgagtttttctgcaagttcgccgaa
4	Asp-87 – Gly-128	Fw: catgccatgggagatagcggtatggacaaagca
		Rev: cgctcgagcccgactcctctgctgaacaa
5	Gly-120 – Gly173	Fw: catgccatgggaggaggcttgttcagcagagga
		Rev: cgctcgagtcctttctcgtagatatcctt

Table 2.1 Primers used to generate overlapping *Na*-ASP-2 fragments. Amino acid numbering starts at the beginning of the mature protein after cleavage of the predicted signal peptide. Underline denotes restriction sites, XhoI or NcoI.

Amplicons were then ligated into the *Nco*I and *Xho*I sites of the pET32a expression vector (Novagen) such that they were in frame with the vector's N-terminal thioredoxin and C-terminal 6xHis tags, transformed into chemically competent *E. coli* TOP10 cells (Invitrogen), plated onto Luria Bertani agar plates supplemented with 100 μg/mL ampicillin (LB_{amp}) and incubated overnight at 37°C. Recombinant colonies were confirmed by colony PCR, plasmids were extracted by mini-prep and transformed into

chemically competent *E. coli* BL21(DE3) and *E. coli* rosetta-gami cells (Invitrogen), plated onto LB_{amp} plates and incubated overnight at 37°C.

2.2.2. Expression of recombinant Na-ASP-2 fragments

Fragments were pilot-expressed by inoculating 10 mL of LB_{amp} broth with a single recombinant *E. coli* BL21(DE3) colony and grown overnight at 37°C with shaking (225 rpm). Glycerol stocks were made from each overnight culture and stored at -80°C. Overnight cultures were diluted 1:100 in LB_{amp} broth (10 mL) and grown to log phase (OD_{600} ~ 0.5) for 3 hours at 37°C with shaking (225 rpm). To find the optimal temperature for protein induction, each culture was divided in half, induced by adding 1 mM isopropyl-b-d-1-thiogalactopyranoside (IPTG) and incubated for a further 18 h with shaking (225 rpm) at either 25°C or 37°C.

2.2.3. Detection of Na-ASP-2 fragment protein expression

One milliliter of each culture was harvested, resuspended in 200 µl of resuspension buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) and lysed by 3 cycles of freeze/thawing. The insoluble fraction was pelleted by centrifugation for 5 mins at 4°C and 16,000 *g* and samples of the soluble fraction were subjected to 12% SDS-PAGE and visualised by Coomassie Blue staining.

2.2.4. Scale-up expression and purification of Na-ASP-2 fragments

Expression cultures were grown from glycerol stocks as described in section 2.2.2 except that overnight cultures were diluted in 200 mL LB_{amp} and induced at 25°C. Bacteria were pelleted by centrifugation at 5000 *g* for 20 min at 4°C and resuspended in

3 mL of resuspension buffer. *E. coli* were then disrupted by 3 passes through a pre-chilled French pressure cell (KIN020; Sim Aminco) at 16,000–18,000 psi. Homogenates were sonicated at 40% duty cycle for 30 s at 4°C, centrifuged at 39,800 *g* at 4°C for 30 min and the resulting supernatants filtered through a 0.45 μm syringe filter (Pall). Each protein solution was passed through a pre-packed 5.0 mL Hi-Trap immobilized metal ion affinity chromatography–fast flow column (GE Healthcare) that was charged with 100 mM nickel sulfate and equilibrated with 10 column volumes of resuspension buffer at a flow rate of 1.0 mL/min. Bound proteins were purified by washing with resuspension buffers containing an increasing concentration of imidazole (40–150 mM) and by elution in 5 column volumes of elution buffer (500 mM imidazole). Various fractions taken throughout the purification process were analysed by 12% SDS-PAGE and eluate fractions that contained protein were pooled. Protein concentrations were adjusted to 2.0 mg/mL by use of Amicon Ultra-15 centrifugal concentration devices (Millipore) and concentrated samples were visualized by 12% SDS-PAGE with Coomassie Blue staining.

2.2.5. Western blotting of Na-ASP-2 fragments

Each *Na*-ASP-2 fragment was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane for 1 hour at 200 mA. Membranes were then blocked with Western blocking buffer (5% skim milk powder/PBS/0.1% Tween). Fragments were confirmed by probing with anti-6xHis-HRP (Invitrogen) diluted 1:5000 in blocking buffer. To determine which fragments were the targets of IgG and/or IgE, membranes were probed with pooled sera from *Na*-ASP-2 phase 1b trial patients [92], followed by either mouse anti-human IgG-HRP (Jackson) diluted 1:2000 in blocking buffer or mouse anti-human IgE-HRP (Jackson) diluted 1:2000 in blocking buffer. All antibody

incubations were performed for 1 hour at RT with washing (3 x 5 mins with PBS/0.1% Tween) between each probing step. Bound antibody was detected with the chemiluminescent detection substrate ECL Prime (VWR) on a gel imaging system (Biorad).

2.2.6. IgG and IgE epitope mapping using overlapping Na-ASP-2 peptides

ELISAs were used to identify epitopes recognized by anti *Na*–ASP-2 IgG and IgE antibodies in sera from participants who developed urticaria after vaccination with *Na*-ASP-2 [92]. IgG epitopes were assessed using purified IgG via protein G sepharose, whereas IgE epitope mapping was performed with sera that had been depleted of IgG via the use of protein G sepharose (GE Healthcare), according to the manufacturer's instructions.

Ninety-six–well flat-bottom, polystyrene microtiter plates containing streptavidin were coated with biotinylated sequential linear 13-mer *Na*-ASP-2 peptides incorporating an SGSG spacer with an overlap of 2 amino acids (Appendix 2.1) (Mimotopes, Victoria, Australia). Coated plates were incubated with ELISA blocking buffer (2% BSA/PBS) for 1 hour at 37°C then probed with primary antibodies (IgE - 1:20 in ELISA blocking buffer or IgG – 1:200 in ELISA blocking buffer) and incubated at 4°C overnight. Plates were then probed for 2 hours at RT with either goat anti-human IgG-HRP diluted 1:2000 in ELISA blocking buffer (Chemicon International) or murine anti-human IgE-HRP diluted 1:2000 in ELISA blocking buffer (Invitrogen, San Diego, Calif). Plates were washed 5 times with PBS/0.1% Tween-20 between incubations. Bound secondary antibody was detected at 405 nm using a microplate spectrophotometer (Benchmark

Plus; Bio-Rad Laboratories) A positive result was determined as 4 x standard deviations above background wells containing no sera.

2.2.7. Site-directed mutagenesis of Na-asp-2

Four *Na-asp-2* mutant cDNAs, each identical to wild-type *Na-asp-2* except for a single alanine codon in place of the nucleotide triplet encoding either Glu-137, Lys-140, Ser-149 or Asp-150, were generated using overlapping PCR. For each mutant, a pPICZαA plasmid encoding wild-type full-length *Na*-ASP-2 protein was used as template and a set of two overlapping "first-round" PCR products were produced with (A) a forward primer containing the mutation and a reverse vector primer (AOX 3') and (B) a complementary reverse primer containing the mutation and a forward vector primer (AOX 5') (**Table 2.2**). Cycling conditions were: initial denaturation at 95°C for 5 mins followed by 35 cycles of denaturation (95°C for 1 min), annealing (55°C for 30 secs) and extension (72°C for 1 min) with a final extension step (72°C for 10 mins). These sets of PCR products were purified by gel extraction and 1 μL of each product in a set was used as template in a "second round" PCR which was performed as for the first round products except that, for the first ten cycles, the annealing temperature was lowered to 50°C and no primers were added to facilitate annealing and extension of complementary first round product ends to form a product containing full-length, mutant *Na-asp-2*. To specifically amplify (now mutant) *Na-asp-2*, flanking oligonucleotides (with the forward and reverse primers incorporating *Eco*RI and *Xba*I restrictions sites, respectively, to facilitate cloning into pPICZαA) were added for the rest of the PCR. A proof-reading DNA polymerase (*pfu* turbo – Stratagene) was used for all reactions.

Na-asp-2 Mutant	Forward Primer	Reverse Primer
Na-asp-2$_{E137A}$	(gaa-gcg) atggtatggcaagcgaccgttaagctt	(ttc-cgc) aagcttaacggtcgcttgccataccat
Na-asp-2$_{K140A}$	(aag-gcg) caagaaaccgttgcgcttggatgctat	(ctt-cgc) atagcatccaagcgcaacggtttcttg
Na-asp-2$_{S149A}$	(tca-gcg) gtggaagcgtgctcaaatatgtgttat	(tga-cgc) ataacacatatttgagcacgcttccac
Na-asp-2$_{N150A}$	(aat-gcg) gaagcgtgctcagcgatgtgttatgtg	(att-cgc) cacataacacatcgctgagcacgcttc

Table 2.2 Primers used to generate *Na-asp-2* mutants*.

* Nucleotide changes from wild-type are in parentheses and their position in the primer is underlined. Each mutant primer was used in combination with the appropriate forward (gcggaattcggttgtcctgacaatgga) or reverse (gcgtctagaccagcactgcagagtcccttctcctt) flanking primer to generate first round PCR products.

2.2.8. Cloning and expression screening of Na-asp-2 mutants

Na-asp-2 mutant amplicons were ligated into the *Eco*RI and *Xba*I sites of the pPICZαA expression vector (Invitrogen) such that they were in frame with the vector's alpha-

factor secretion signal (to facilitate secretion of the expressed protein into culture media) and C-terminal 6xhis tag, transformed into chemically competent *E. coli* TOP10 cells (Invitrogen), plated onto LB agar plates supplemented with 25 μg/mL zeocin (LB_{zeo}) and incubated overnight at 37°C. Recombinant colonies were confirmed by colony PCR and plasmids were extracted by mini-prep and verified by sequencing. Plasmids (40 μg) were linearized by an overnight, 37°C digestion with SacI, purified by phenol:chloroform extraction and ethanol precipitation and resuspended in 20 μl of H_2O. Linearized vector was transformed into electrocompetent *Pichia pastoris* X-33 cells by electroporation (2 ms in 2 mm cuvettes using a BioRad Gene Pulser Xcell - 2 kV, 25 μF capacitance, 200 Ohms resistance, square wave electroporation), plated onto YPD agar plates supplemented with 2000 μg/mL zeocin, covered in foil and incubated overnight for 3 days at 28°C.

Integration of the expression cassette into the yeast chromosome was confirmed by colony PCR. Ten positive colonies were each seeded into 2 mL of BMGY in 50 mL tubes, incubated at 30°C overnight with shaking at 250 rpm and the resultant cells centrifuged (2,500 *g* for 5 mins at 10°C), resuspended in 3 mL of BMMY to induce expression and similarly incubated for 96 hours. Cultures were supplemented with 0.5% methanol every 24 h to maintain promoter activity and 20 μL samples of culture media were taken every day and analysed for protein expression by Western blotting with anti-His-HRP (**Section 2.2.5**).

2.2.9. Large-scale expression and purification of Na-ASP-2 mutants

A 10 μL sample of the highest expressing culture of each *Na*-ASP-2 mutant was used to inoculate 25 mL of BMGY in a 250 mL conical flask, which was cultured overnight

with shaking (250 rpm) at 30°C. The 25 mL culture was then used to inoculate 250 mL of BMGY in a 2.5 L conical flask, which was cultured overnight with shaking (250 rpm) at 30°C. To induce protein expression, the cells were pelleted at 2,500 *g* for 30 mins at 10°C, and then resuspended in 2 L of BMMY and split between four 2.5 L conical flasks, which were incubated with shaking (250 rpm) at 30°C for 96 hours. Methanol was added to a final concentration of 0.5% (2.5 mL/flask) every 24 hours to maintain promoter activity. Cells were pelleted at 2,500 *g* for 30 mins at 10°C. The supernatant was decanted, filtered through a 0.45 μm filter and concentrated under pressure to approximately 100 mL through a 3 kDa filter with a Filtron Ultrapump II pump concentrator (Pall). The concentrate was then transferred to an Amicon Ultra-15 centrifugal concentration device (Millipore) and further concentrated to 5 mL by repeatedly centrifuging (3,000 *g* for 30 mins at 10°C). This concentrate was then buffer exchanged into binding buffer (50mM Na_2HPO_4/NaH_2PO_4, pH 8, 300mM NaCl, 10mM imidazole) by repeatedly filling the unit to capacity and concentrating to 5 mL for a total of 4 times. Proteins were then purified by IMAC (**Section 2.2.4**) and fractions analysed by 12% SDS-PAGE and Western blotting with anti-6xHIS-HRP (**Section 2.2.5**). Positive fractions were then pooled, the concentration adjusted to 1.0 mg/mL using an Amicon Ultra-15 centrifugal concentration device and verified using gel densitometry in comparison with BSA standards.

2.2.10. IgE and IgG reactivity of Na-ASP-2 mutants

The IgE and IgG reactivity of phase I clinical trial patients to the *Na*-ASP-2 mutants was determined by ELISA using standard techniques. Plates were washed 10 times in PBST between incubations. Each mutant and wild-type *Na*-ASP-2 protein was coated on microtiter plates at 5.0 μg/mL and then blocked with BPBST (5% BSA/PBS/0.1%

Tween-20). Patient sera (n=9) or a pool of hookworm-naïve sera (each 1:50 in BPBST) was added to each antigen in quadruplicate for 1 hour at 37°C. Either goat anti-human IgG-biotin (Pierce) or mouse anti-human IgE-biotin (Human Reagent Laboratory) secondary antibodies (1:5000 in BPBST) were added for 1 hour at 37°C. A streptavidin-HRP tertiary antibody (OptEIA – BD Biosciences) (1:250 in BPBST) was then added for 1 hour at 37°C. Peroxidase activity was detected by incubation with tetramethyl benzidine chromogenic substrate (Life Technologies) for 20 mins and measured at 655 nm.

2.3 Results

2.3.1. Na-asp-2 fragment design

The position of each *Na-asp-2* fragment within the 3D crystal structure of *Na*-ASP-2 [137] is shown in **Figure 2.1**. Fragment 1 is predicted to form a largely solvent-exposed sheet-helix. Fragment 2 contains a putative sheet-sheet-helix where the helix and one strand are partly buried within the protein, and includes the putative catalytic Ser. Fragment 3 is predicted to form a largely solvent-exposed sheet-helix and includes the binding cavity Glu and Ser. Fragment 4 contains a putative sheet-sheet, includes a higher percentage of solvent exposed residues and spans the predicted ligand-binding domain. Fragment 5 is composed of a short predicted helix-sheet-sheet and includes the catalytic His.

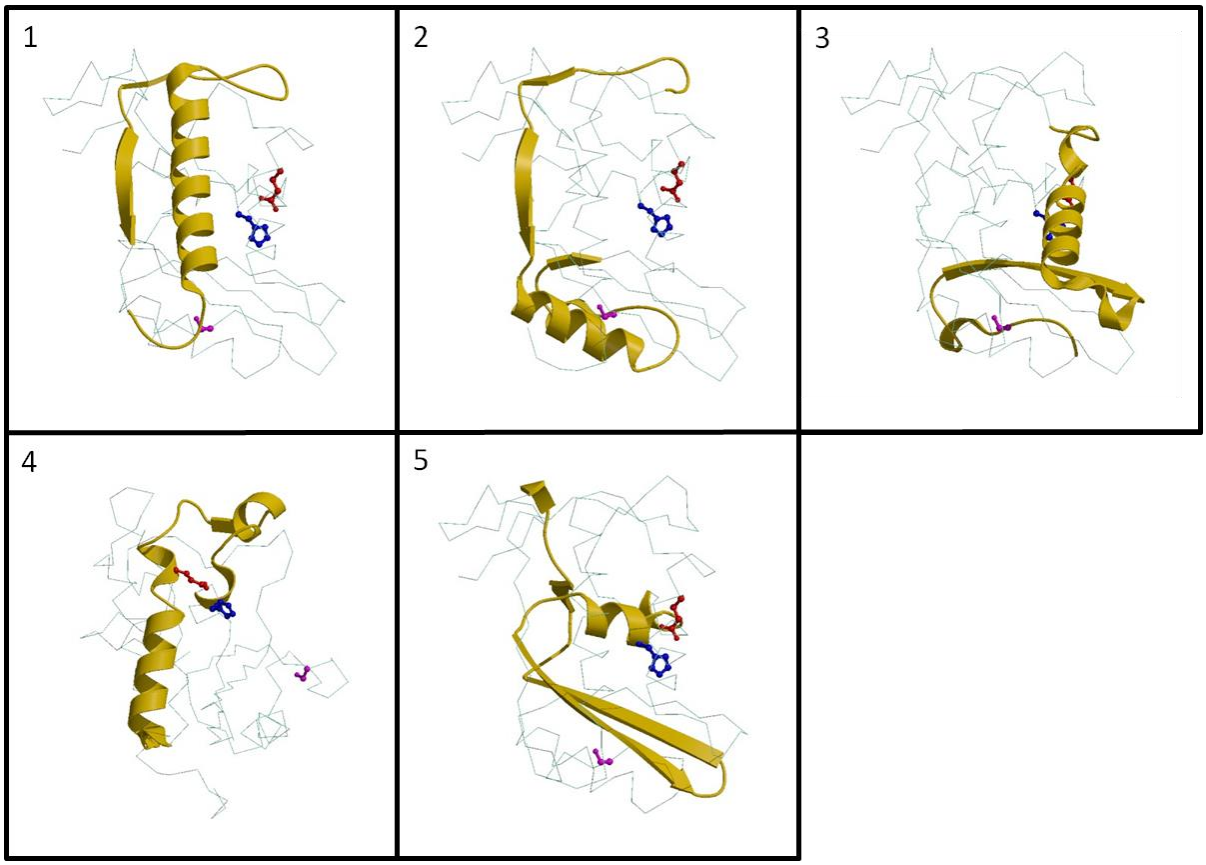

Figure 2.1 *Na*-ASP-2 overlapping fragments. Each fragment (1-5) is shown in gold on the carbon trace backbone of *Na*-ASP-2. Side chains of the putative binding cavity residues Ser, His and Glu [137] are shown in magenta, blue and red respectively. Images generated with MODELLER software.

2.3.2. Na-ASP-2 fragment expression

Expression of all *Na*-ASP-2 fragments was tested in a number of different expression strains and conditions (data not shown). Yields were highest when expressed in *E. coli* BL21 (DE3) cells at 25°C (**Figure 2.2**). Proteins corresponding to each fragment were successfully purified using IMAC and their identities and size confirmed by SDS-PAGE (**Figure 2.3**) and Western blotting with anti-6xHis-HRP (**Figure 2.4**).

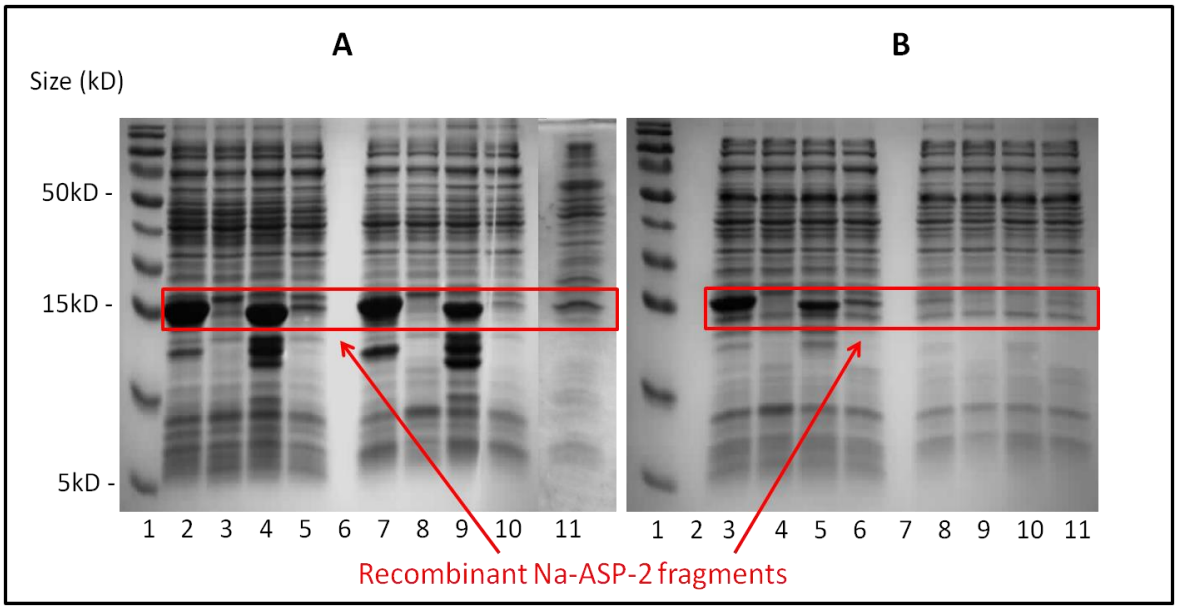

Figure 2.2 SDS-PAGE gels stained with Coomassie brilliant blue showing expression of *Na*-ASP-2 fragments 1-5. Fig. 2A BL21 DE3 expression - Lane 1: Pageruler prestained protein Ladder, Lanes 2-5: Fragments 1,3,4,5 expressed at 25°C, Lane 6: Blank, Lanes 7-10: 1,3,4,5 expressed at 37°C Lane 11: Fragment 2 expressed in BL21 DE3 at 25°C. Fig. 2B Rosetta-Gami Expression - Lane 1: Pageruler protein Ladder, Lane 2: Blank, Lanes 3-6: Fragments 1,3,4,5 expressed at 25°C, Lane 7: Blank, Lanes 8-11: Fragments 1,3,4,5 expressed at 37°C.

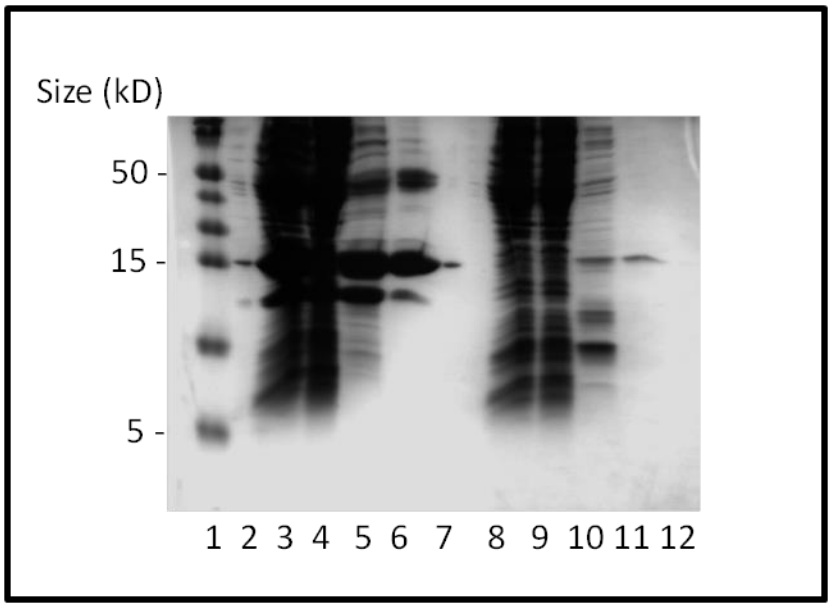

Figure 2.3 SDS-PAGE showing representative purification of *Na*-ASP-2 fragments 1 and 3. Lane 1: Pageruler prestained protein ladder, Lane 2: Blank, Lane 3: Fragment 1 Starting material, Lane 4: Unbound fraction, Lane 5: Fragment 1 60 mM Imidazole, Lane 6: Fragment 1 200 mM Imidazole fraction, Lanes 7-8: Blank, Lane 9: Fragment 3 Starting material, Lane 10: Fragment 3 Unbound fraction, Lane 11: Fragment 3 60 mM Imidazole, Lane 12: Fragment 3 200 mM Imidazole. Due to high concentrations of protein there is overflow in several lanes*.

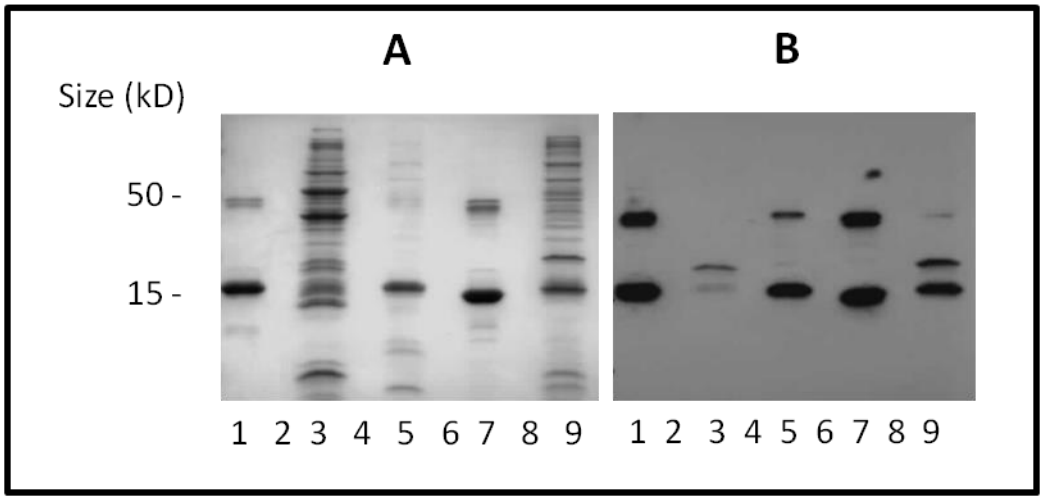

Figure 2.4 SDS PAGE gel (A) and identically loaded Western blot probed with anti-6xHis HRP (B) to detect recombinant fragments. Lane 1: Fragment 1, Lane 2: Blank, Lane 3: Fragment 2, Lane 4: Blank, Lane 5: Fragment 3, Lane 6: Blank, Lane 7: Fragment 5, Lane 8: Blank, Lane 9: Fragment 6.

2.3.3. Crude IgG and IgE epitope mapping of Na-ASP-2

IgG and IgE epitopes of *Na*-ASP-2 were mapped to recombinant fragments by Western blotting with pooled sera from patients enrolled in the phase Ib *Na*-ASP-2 clinical trial in Brazil [92]. All fragments were bound by antibodies from pooled patient IgG, and at least two fragments were positive for IgE (**Figure 2.5**).

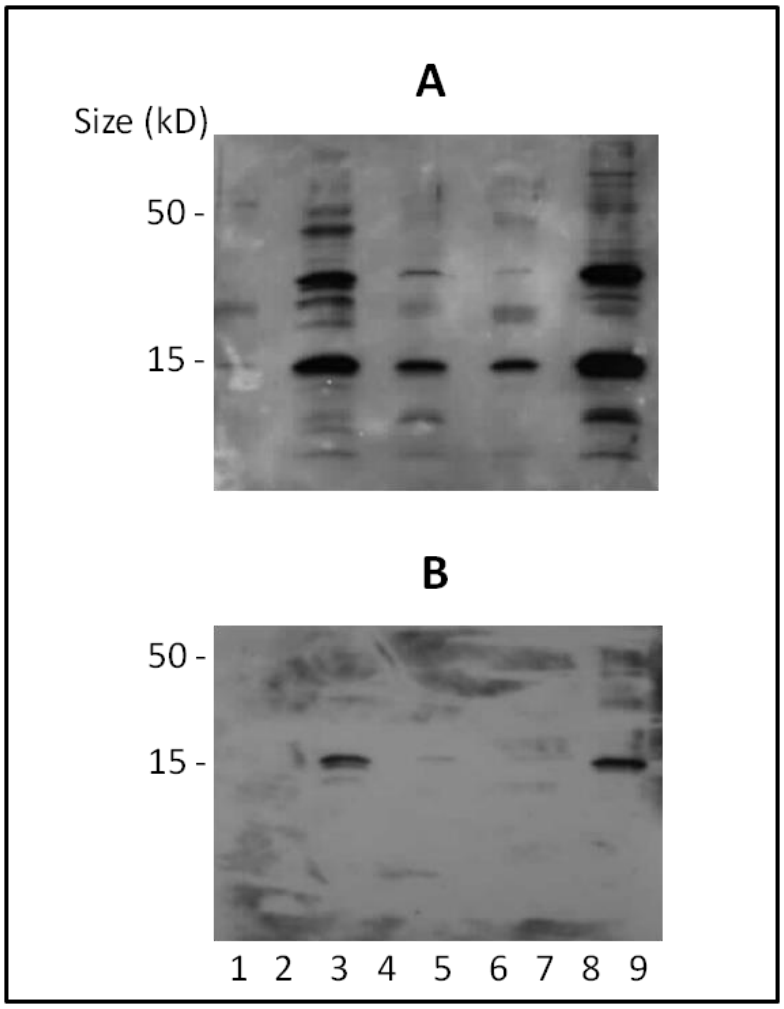

Figure 2.5 Western blots of recombinant *Na*-ASP-2 fragments probed with (A) anti-IgG and (B) anti-IgE purified from pooled trial patient sera. Lanes 1: Fragment 1, Lane 2: Blank, Lane 3: Fragment 2, Lane 4: Blank, Lane 5: Fragment 3, Lane 6: Blank, Lane 7: Fragment 4, Lane 8: Blank, Lane 9: Fragment 5.

2.3.4. Fine IgG and IgE epitope mapping of Na-ASP-2

To further delineate epitope binding, a Pepset of plated *Na*-ASP-2 peptides was probed with IgG and IgE from *Na*-ASP-2 phase Ib trial patient sera. IgG- and IgE-reactive peptides are shown in Table 2.3. The most reactive IgE epitopes (both recognized by 8/9 patients) were W135 (WQETVKLGCYVEA) and S149 (SNMCYVVCQYGPA).

The most reactive IgG epitope (recognised by 8/9 patients was also W135. A 3D crystal structure of *Na*-ASP-2 displaying those epitopes recognized by IgG and/or IgE patient antibodies is also presented (**Figure 2.6**).

Patient ID		IgE peptides	OD at 405nm		IgG peptides	OD at 405nm2
AO1					F123	0.297
					M133	0.283
					W135	1.3
					E137	0.337
AO2		W135	0.305		M45	0.226
		S149	0.398		F123	0.48
					H129	0.344
					W135	0.94
AO3		F123	0.22		P3	0.187
		W135	0.221		F123	0.368
		S149	0.492		W135	1.02
		M151	0.247			
AO4		F123	0.218		M45	0.196
		W135	0.419		F123	0.285
		S149	0.435		W135	0.757
					P175	0.177
AO5		W135	0.162		W135	0.569
		S149	0.329			
		A161	0.141			
AO6		E97	0.26		A31	0.205
		W135	0.197		M45	0.16
		S149	0.246		N81	0.44
					F123	0.173
AO7		A11	0.177		F123	0.78
		W135	0.312		W135	0.854
		S149	0.398			
		A161	0.272			
AO8		W135	0.434		F123	0.259
		S149	0.502		W135	0.496
					E137	0.195
AO9		W135	0.257		N5	0.351
		S149	0.432		W135	0.415

Table 2.3 Epitopes recognized by circulating anti–*Na*-ASP-2 IgG and IgE in sera collected from the 9 participants of the phase Ib trial of the *Na*-ASP-2 vaccine conducted in Brazil

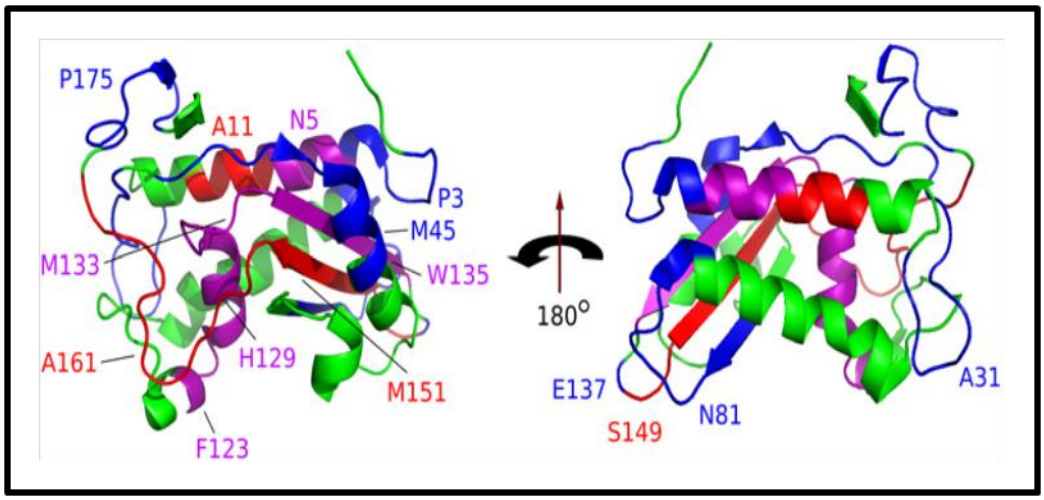

Figure 2.6 A 3D representation of *Na*-ASP-2 highlighting the epitopes that were recognized by IgG and/or IgE antibodies from *Na*-ASP-2 phase Ib trial patient sera. Epitopes in blue are those recognized by IgG, those in red are recognized by IgE, and those in purple are recognized by both IgG and IgE [137].

2.3.5. Selection of mutagenic sites

The 13mer peptides containing the most highly reactive IgE epitopes were W135 and S149 (**Section 2.3.4**) Based on the crystal structure of *Na*-ASP-2 [137], the residues selected for mutation (alanine substitution) were the four solvent-exposed, polar and/or charged amino acids within these epitopes (Glu-137, Lys-140, Ser-149 or Asp-150) (**Figure 2.7**).

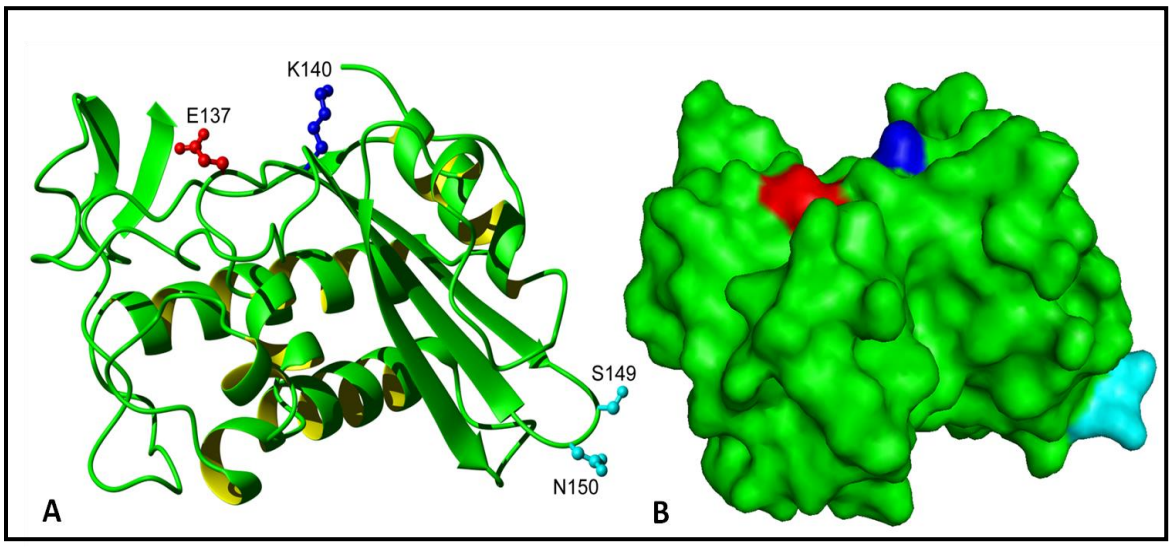

Figure 2.7 *Na*-ASP-2 crystal structure with mutated amino acids highlighted. Solvent-exposed residues targeted for mutation and their location in a ribbon (A) and space-filling (B) model of *Na*-ASP-2. Glu-137 (acidic, polar) is shown in red; Lys-140 (basic, polar) is shown in blue and Ser-149 and Asn-150 (neutral, polar) are shown in cyan.

2.3.6. Site-directed mutagenesis

For each reaction, appropriate combinations of mutant and vector primers were used to generate first round PCR products which were then used as templates in second round reactions with ORF flanking primers to produce mutant *Na-asp-2* amplicons (**Figure 2.8**). All first round products were of the expected size and second round amplicons were the size of full-length *Na-asp-2* with the flanking segments of the vector included.

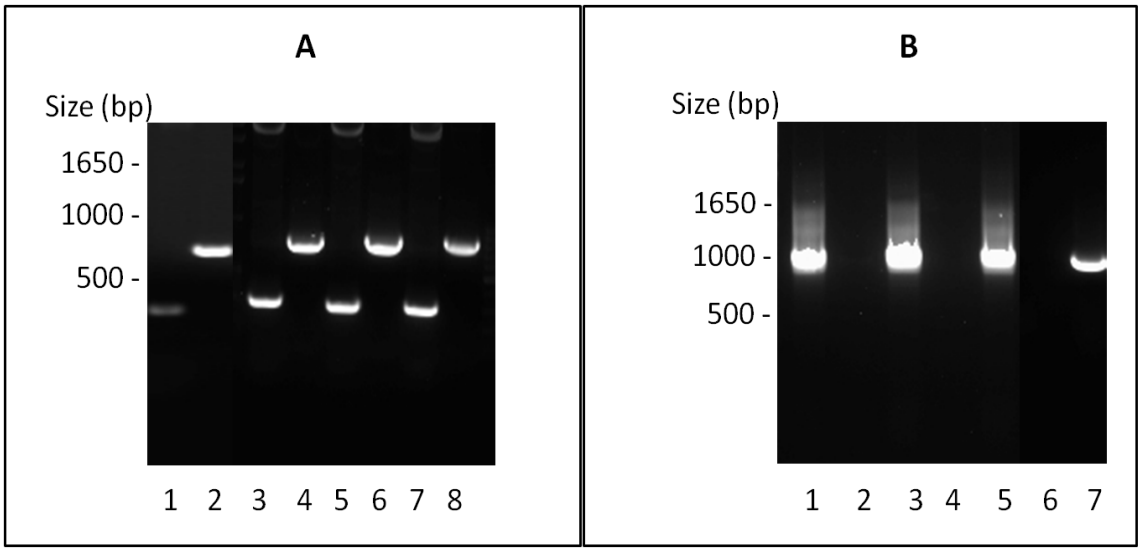

Figure 2.8 Generation of mutant *Na-asp-2* amplicons. (A) Odd-numbered lanes contain first round products produced with forward mutant and reverse vector primers and even-numbered lanes contain first round products produced with reverse mutant and forward vector primers. Lane 1 and 2 - *Na-asp-2*$_{E137A}$, lane 3 and 4 - *Na-asp-2*$_{K140A}$, lane 5 and 6 - *Na-asp-2*$_{S149A}$, lane 7 and 8 - *Na-asp-2*$_{N150A}$. (B) Second round PCR products generated with ORF flanking primers. Lane 1 - *Na-asp-2*$_{E137A}$, lane 2 - *Na-asp-2*$_{K140A}$, lane 3 - *Na-asp-2*$_{S149A}$, lane 4 - *Na-asp-2*$_{N150A}$.

2.3.7. Na-ASP-2 mutant expression in Pichia pastoris

The mutants were expressed in *Pichia pastoris* and purified via IMAC using an AKTA FPLC. A representative purification of one of the mutants (*Na*-ASP-2$_{E149A}$) is shown in **Figure 2.9** with the purification profile showing a major elution peak (marked with an asterisk) over fractions A5 – A7. SDS-PAGE and anti-6xHis-HRP Western blot analysis of eluted *Na*-ASP-2$_{S149A}$ fractions (**Figure 2.10**) revealed bands of the expected size.

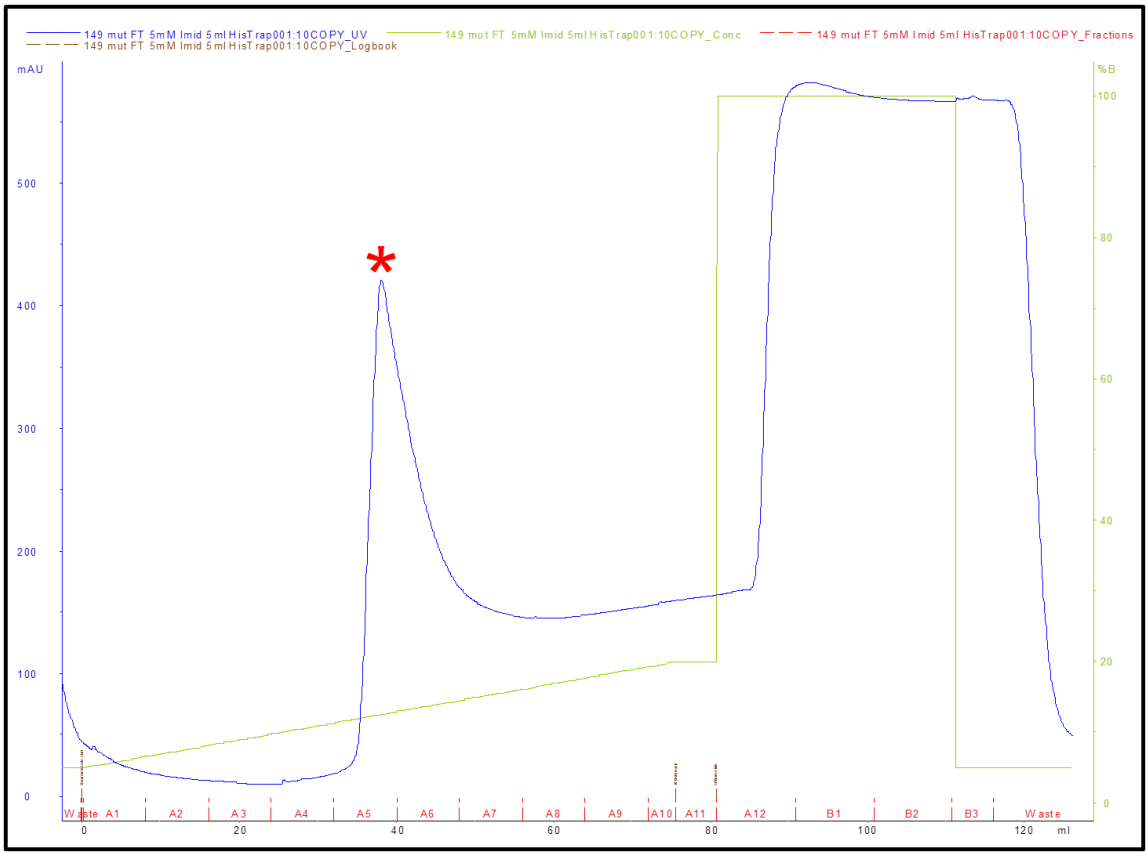

Figure 2.9 Chromatogram of *Na*-ASP-2_{S149A} purified on an AKTA FPLC purification workstation. The blue line shows the UV trace of the elution profile (elution peak marked with a red asterisk); the green line denotes the imidazole gradient; the X-axis shows the eluate fractions.

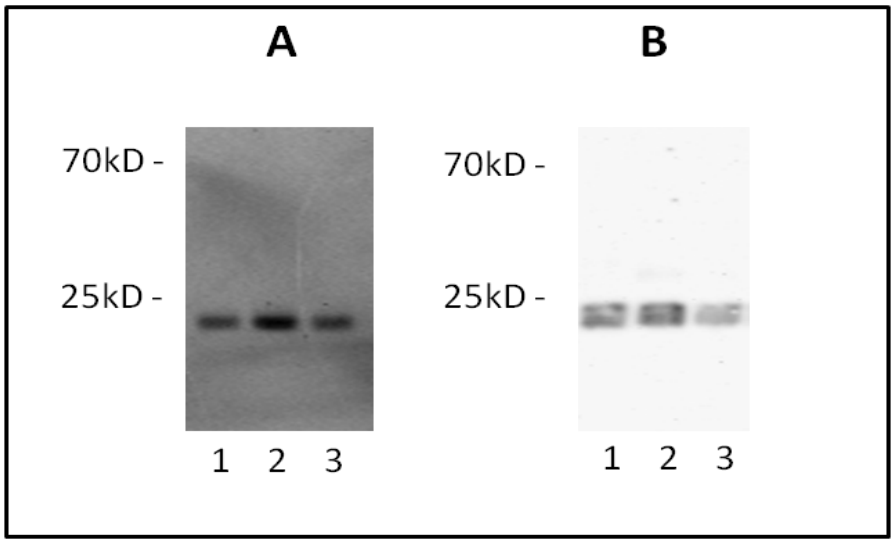

Figure 2.10 SDS PAGE (A) and anti-6xHis-HRP Western blot analysis (B) of eluted *Na*-ASP-2_{E149A} fractions. Lane 1- fraction A5, lane 2 – fraction A6, lane 3 – fraction A7.

2.3.8. IgE and IgG reactivity of Na-ASP-2 mutants

Na-ASP-2_{wt} and all 4 *Na*-ASP-2 mutants were probed with antibodies from the *Na*-ASP-2 phase Ib trial patients to investigate the changes in IgG and IgE reactivity caused by site-directed mutagenesis of IgE epitopes. *Na*-ASP-2_{E137A} showed the largest reduction in IgE reactivity for all patients (**Figure 2.11**). However, subsequent SDS-PAGE analysis of all proteins revealed *Na*-ASP-2_{E137A} to be substantially degraded after short term storage at 4°C, accounting for the reduced antibody binding observed by ELISA. Of the remaining mutants, *Na*-ASP-2_{K140A} exhibited the least IgE reactivity with reductions of 39.6% of OD values with regards to IgE antibody binding from patient 2, 38.1% for patient 7 and 9.7% for patient 9. The *Na*-ASP-2_{K149A} mutant proved the best in terms of expression and had OD reductions of 26.0%, 10.3% and 7.4% for patients 2, 7 and 9 respectively. The *Na*-ASP-2_{K150A} mutant provided 40.3% reduction in binding for patient 2, however it also produced a 4.5% increase in IgE

binding for patient 7 which is somewhat detrimental and a 20.9% reduction for patient 9All *Na*-ASP-2 mutants displayed the same level of IgG reactivity as *Na*-ASP-2_{wt}.

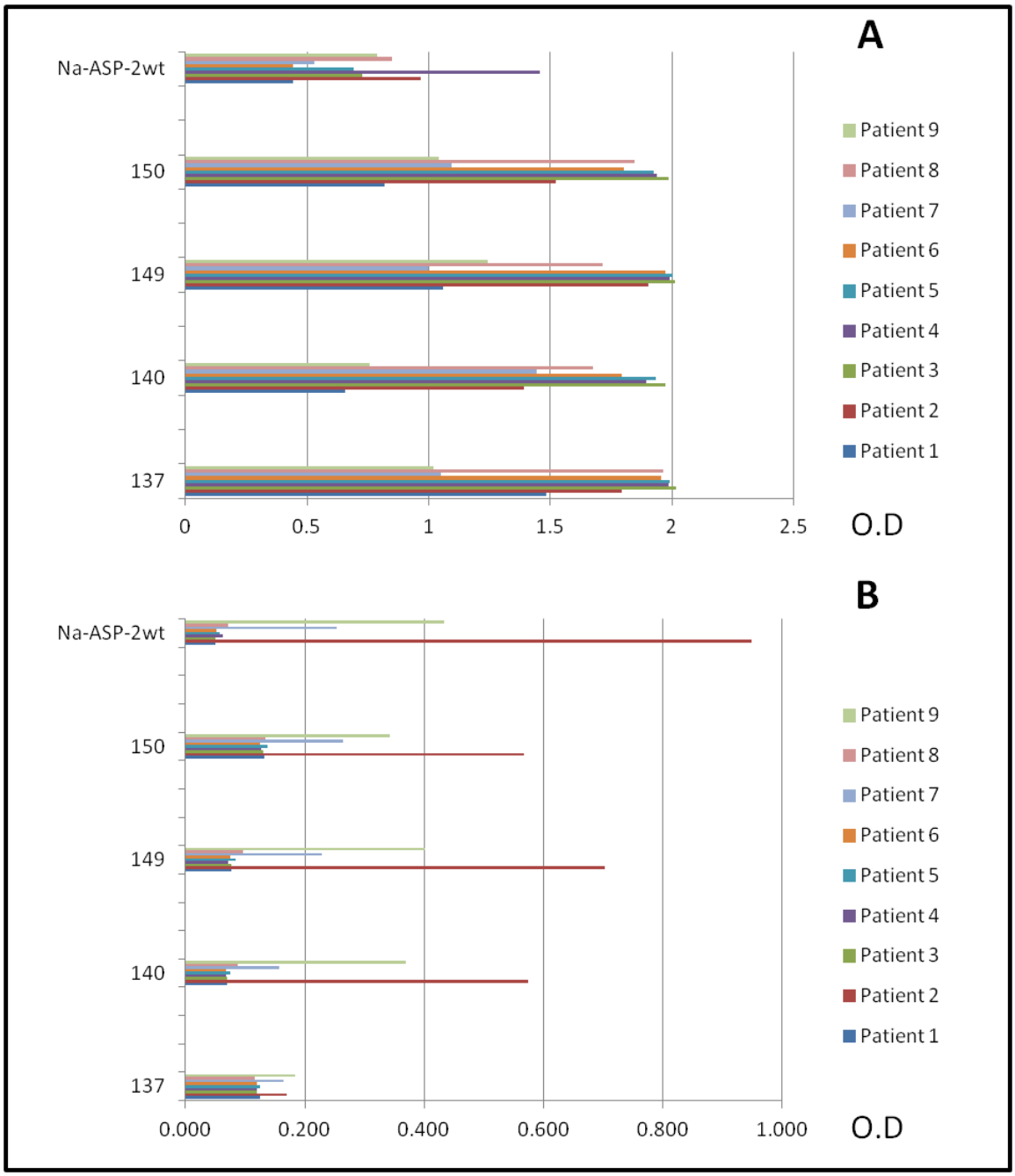

Figure 2.11 IgG (A) and IgE (B) levels from *Na*-ASP-2 phase 1b clinical trial subjects against purified recombinant *Na*-ASP-2wt and site-directed mutants of *Na*-ASP-2.

2.4 Discussion

The phase 1b clinical trial of the human hookworm vaccine *Na*-ASP-2 resulted in several subjects exhibiting generalized urticarial reactions upon vaccination which was a direct result of circulating IgE specific to *Na*-ASP-2, likely from prior hookworm exposure [92]. Th2 responses are typically seen in the presence of helminth infection and IgE antibodies are subsequently common [51], despite allergic reactions being negatively associated with helminth infections [168]. Nonetheless, a vaccine that induces a generalized hypersensitivity response is clearly unacceptable for further clinical trials in humans.

Accordingly, the aim of this chapter was to identify the major IgG and IgE epitopes of the hookworm vaccine antigen *Na*-ASP-2 with a view to mutating the major IgE epitopes in an attempt to re-engineer a less allergenic but equally protective form of *Na*-ASP-2. To facilitate this, antibodies from *Na*-ASP-2 phase Ib trial patients, including those that developed an urticarial response upon vaccination, were used to probe 5 overlapping recombinant fragments or a Pepset of 13mer overlapping peptides that spanned the length of *Na*-ASP-2. Ideally it would have been ideal to test all 4 mutants in the wheel and flare skin sensitivity test along with histamine release assays. However it was deemed beyond the scope of this study.

All fragments were recognized by the pooled patient IgG samples, but only 2 fragments were recognized by IgE – fragment 2 and fragment 5. This was consistent with peptide data, as the 2 peptides most strongly recognized (W135 and S149 - 8/9 patients) are contained within fragment 5. No peptides contained within fragment 2 were IgE-

reactive which could be due to a conformational epitope(s) that was not reproduced with linear peptides in the Pepset.

The 3 subjects from the *Na*-ASP-2 phase Ib clinical trial who exhibited allergic symptoms (urticaria) as a result of the vaccination not only had the highest serum IgE titres but all 3 responded to the same 2 major IgE epitopes - W135 and S149. It was reasoned, therefore, that disruption of these epitopes would form part of the strategy to de-allergenise *Na*-ASP-2, despite the fact that the same epitopes were also targets of IgG; in 8 of the 9 subjects, at least one of the two epitopes was positive for IgG or IgE via ELISA. Ablating epitopes that elicit both IgG as well as IgE responses may well affect the molecule's efficacy as a vaccine due to potentially lowered IgG binding as a result of epitope disruption, as IgG is traditionally considered the ideal antibody isotype to target for vaccination due to its relative abundance and antigenic specificity [169, 170].

Another epitope of interest was F123, as it was recognized by IgE from two subjects and IgG from four subjects. However, in the interests of time, the two epitopes displaying the highest IgE prevalence and reactivity among patients were prioritized. Additionally, IgE reactivity of F123 was low in comparison to IgG reactivity, further supporting the lower prioritization of this epitope for disruption. Excluding peptides W135, S149 and F123, there were 9 different ELISA positive IgG epitopes and 4 IgE epitopes.

The mapping strategies described herein have provided insight into the both the IgG and potentially harmful IgE linear epitopes of *Na*-ASP-2. The immunodominant CD4+

T cell epitopes of some allergens are conformational [171] so, while it is possible that conformational *Na*-ASP-2 antibody epitopes are yet to be discovered, the approaches documented in this chapter have provided an informed starting point in the process of *Na*-ASP-2 de-allergenisation.

Once the major IgE–inducing epitopes of *Na*-ASP-2 had been determined, we sought to ablate these epitopes through mutagenesis of residues within these regions, while still retaining its IgG immunogenicity. To this end, four *Na*-ASP-2 mutants were made, each differing from *Na*-ASP-2_{wt} by a single alanine substitution of a charged and/or polar residue within the epitope. Alanine substitutions were chosen to cause minimal disruption to protein conformation. Preservation of *Na*-ASP-2_{wt} molecular structure was desired, so that IgG epitopes (and, therefore, B cell immunoreactivity) was retained [79]. It is worthy of note that the majority of literature on de-allergenisation in this manner is concerned only with the retention of T cell reactivity (which is important for allergen specific immunotherapy in the treatment of allergic diseases) and there is only a handful of reports dealing with IgE epitope disruption and the preservation of B cell reactivity. All four mutations were predicted to be solvent exposed (**Figure 2.7**) and therefore more easily accessible to and recognised by the immune system in comparison to other "hidden" epitopes located in less solvent exposed areas, maximising the likelihood that disruption of these regions would result in reduced IgE reactivity.

The wild-type and four mutant forms of *Na*-ASP-2 were assessed for their reactivity with IgE and IgG from the trial patient sera with particular attention being paid to the patients exhibiting the highest IgE titres (subjects 2, 7 and 9), as these 3 patients were

the same that presented with urticarial allergic reactions upon vaccination. While the remaining 6 patients still had detectable *Na*-ASP-2-specific IgE responses, none of them developed urticaria after a single vaccination with adjuvanted *Na*-ASP-2. To induce an allergic response, the IgE antibody level needs to be high enough to induce cross linking of antigen-antibody complexes on mast cells, leading to the release of cytokines and chemokines which initiate the inflammation process of urticaria. All mutants displayed lowered IgE reactivity compared to *Na*-ASP-2_{wt}, with *Na*-ASP-2_{E137A} showing the greatest reduction. This was initially encouraging; however, when the mutants were subjected to further SDS-PAGE analysis it was noted that *Na*-ASP-2_{E137A} had undergone substantial degradation over time, thus invalidating the ELISA results for this mutant protein. Of the remaining mutants (none of which had undergone degradation in storage), *Na*-ASP-2_{K140A} had the greatest reduction in IgE antibody reactivity. *Na*-ASP-2_{S149A} showed lowered reactivity for all 3 allergic patients (2, 7 and 9) and the *Na*-ASP-2_{E150A} mutant showed a substantial reduction in IgE binding for patient 2, however less of a reduction for patient 9 and an increase in IgE binding for patient 7.

In terms of IgG binding, all 4 mutants displayed comparable or increased IgG binding in comparison to *Na*-ASP-2_{wt}. This was somewhat unexpected given the IgE epitopes that were disrupted were also shared IgG epitopes. There is a possibility that the disruption of IgE binding resulted in less steric hindrance of the epitope and surrounding residues, thereby facilitating easier accessibility of IgG to the region. The synthesised overlapping peptides were 13mers yet the average IgG epitope ranges from 5-20 amino acids; minimal epitopes were not determined in this study so it is possible that disruption of IgE binding resulted in stronger binding of IgG to those same IgE

epitopes, as well as increasing the number of surrounding epitopes to which IgG could bind. For example, disruption of the W135 IgE epitope might have improved accessibility to E137, an IgG epitope that was only recognised by two patients, A01 and A08 (**Table 2.3**).

Given that there was no decrease in IgG immunogenicity for any one mutant, future work could involve the production of a *Na*-ASP-2 variant containing all 4 mutations, and/or other mutations identified by the epitope mapping described herein, which might exhibit an even greater reduction in IgE reactivity due to the ablation of multiple IgE epitopes. Such a mutant would then need to be tested in an animal model of hookworm infection, to ensure vaccine efficacy before progressing towards clinical development but, if successful, might facilitate the resurrection of an efficacious vaccine candidate and a leap forward in the development of a safe vaccine against hookworm disease.

Introduction to Chapter 3

Finding a binding partner and exploring the possibility of a biological function of *Na*-ASP-2 was one of the primary aims of my thesis. Several methods were implemented in this part of the investigation in order to ascertain the possibility of a putative binding partner and following that, assigning a biological function to the *Na*-ASP-2 hookworm secreted protein.

The first approach used was the panning of a random peptide phage display library, the process of probing a surface coated with the protein of interest, with a recombinant phage expressing a random peptide on the surface of a viral capsid. With each round of replication in host bacteria, the viral particles amplify logarithmically and there are billions of possible peptide permutations. This makes phage display panning a popular method for probing a protein of interest for binding partners or binding epitopes to peptides, proteins or antibodies of interest.

Chapter 3:

Phage display determination of binding partners of *Na*-ASP-2

3.1 Introduction

Excretory/secretory proteins or ES proteins are the primary way in which the hookworm interacts with its surrounds while inside the human host. ES proteins facilitate a variety of essential functions for the hookworm necessary for its survival and ASPs are the largest component of hookworm ES, comprising >30% of the total secreted proteins [94]. *Na*-ASP-2 is an activation-associated, ES protein produced by the infective stage of *N. americanus* [172]. The crystal structure of *Na*-ASP-2 indicates a putative binding groove, suggesting a ligand-binding site [137]. The identification of a *Na*-ASP-2 binding partner within the human host may aid in deducing the biological role of the protein, and shed light on its mechanism of vaccine-mediated protection. The only ASP with a well characterized binding partner and physiological role is Neutrophil Inhibitory Factor (NIF), a group 3 ASP that binds to the β2 integrin domain of CD11b and inhibits neutrophil recruitment, an essential element in the inflammatory

response process [133, 173]. Vaccination trials with NIF in hamster models resulted in significantly reduced fecundity in the hookworm *Ancylostoma ceylanicum* [128] but did not result in significantly reduced adult worm burdens. There remain dozens of hookworm ASPs with as yet unidentified biological roles [174].

Bacteriophage library panning or phage display panning involves probing a protein of interest with a library of recombinant phage that has engineered random peptides of specific size, expressed and displayed on the surface of the viral capsid coat via a short peptide linker [175]. Phage display panning has been successfully utilized in identifying ligands involved in interactions for a variety of proteins such as the Hepatitis C envelope protein E2 [176], human CD40 [177], rat aquaporin-2 expressed [178] and human carbonic anhydrase IX [179]. Phage display peptide library panning has been used to identify peptide binding ligands for a variety of purposes [176, 180, 181].

In this chapter a recombinant random peptide phage display library (m13ke) was used to pan and identify random 12-mer peptides bound to the hookworm protein *Na*-ASP-2. Using the sequence data of the peptides to identify a consensus sequence, it was possible to identify candidate proteins to which ASP-2 might bind *in vivo*.

3.2 Materials and Methods

3.2.1. Phage Display

A phage display-12 peptide library kit with lambda bacteriophage M13KE (New England Biolabs (NEB)) was used to identify 12-mer peptides that bound to

recombinant pichia derived *Na*-ASP-2. The phage were engineered to express a random peptide as a fusion with the pIII coat protein of the M13 bacteriophage, resulting in a peptide displayed on the surface of a virion. The random peptide is followed by a spacer (Gly-Gly-Gly-Ser) then the wild type sequence, of which there are 2.7 X 10^9 possible sequences. Phage virions were then used to infect *E. coli* ER2738 F' lacZ M15. The vector carries the lacZα gene and the F-factor of ER2738 contains a mini transposon which confers tetracycline resistance. Selection was carried out on LB agar plates containing Xgal and IPTG and supplemented with tetracycline as described by the manufacturer; positive, non-lytic plaques appeared blue on the plated agar. Three rounds of panning were carried out in triplicate at 4°C in 24 well plates coated with *Na*-ASP-2 at concentrations of 50 µg/mL (first pan), then 25 and 10 µg/mL for the second and third pans respectively. Phage libraries were plated out at varying dilutions (10^{-2} to 10^{-6}) in order to obtain approximately 50-100 plaques per plate. Twelve plaques were selected from each plate after the third round of panning, and single stranded phage DNA was purified according to the manufacturer's instructions and submitted for automated dideoxy sequencing (Macrogen, China). The spacer of Gly-Gly-Gly has the reverse nucleotide sequence of CCTCCACCT, allowing rapid identification of sequencing data.

3.2.2. Database searches

Peptides were submitted to Blastp searches [182] against the human refseq genome at NCBI. Peptide features were calculated using the peptide property calculator (https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi). Peptide comparisons were conducted using CLUSTALW (http://www.genome.jp/tools/clustalw/).

3.2.3. Molecular dynamics (MD) simulations and differential scanning fluorimetry

MD simulations were conducted using the program Gromacs [183] installed on in-house servers (Intel XEON X3220 Quad Core; AMD Phenom X4 9850 Quad Core CPUs). The Gromacs 43a1 force-field and the spc water model were used. A peptide comprising residues 244-255 of the human SK3 channel was generated in an extended conformation, and manually placed above the equatorial groove of *Na*-ASP-2 with O [184], in two opposite directions. To ensure a charge-neutral cell, chloride counter ions were added by replacing solvent molecules at sites of high electrostatic potential. A position-restrained dynamics simulation of 20 per second was performed to equilibrate the solvated protein-ligand complex and to gradually heat the simulation cell to 300 K. The production MD simulation was carried out for a simulation time of 50 ns, with periodic boundary conditions being applied in all three dimensions, and employing the Particle Mesh Weald (PME) method to treat the long-range electrostatic interactions. Trajectories were analysed using Gromacs tools.

3.3 Results

3.3.1. Phage Panning

Phage display panning with a 12-peptide library phage was used to ascertain a binding peptide to the human hookworm protein *Na*-ASP-2. Collaborators at Griffith University (L. Mason and A. Hofmann) used this data to conduct molecular binding simulations using kinetic modeling and illustrated metal binding capacity of the tandem histadine motif, the results of which have recently been published [185] and clearly identified a preferred topology of the *Na*-ASP-2:SK3 peptide complex.

To ensure the appropriate concentration of phage was used in each step of the panning process, a series of titrations were performed to ensure accurate quantification of phage, commonly dilutions of 10^{-2} to 10^{-8} of purified phage. Shown below (**Figure 3.1**) are examples of actual MK13E bacteriophage titrations between *Na*-ASP-2 pans; the IPTG/Xgal blue/white selection via the LacZ operon allows for rapid identification of phage-plaques, where unlike the usual convention, blue, non-lytic plaques are positive for the phage.

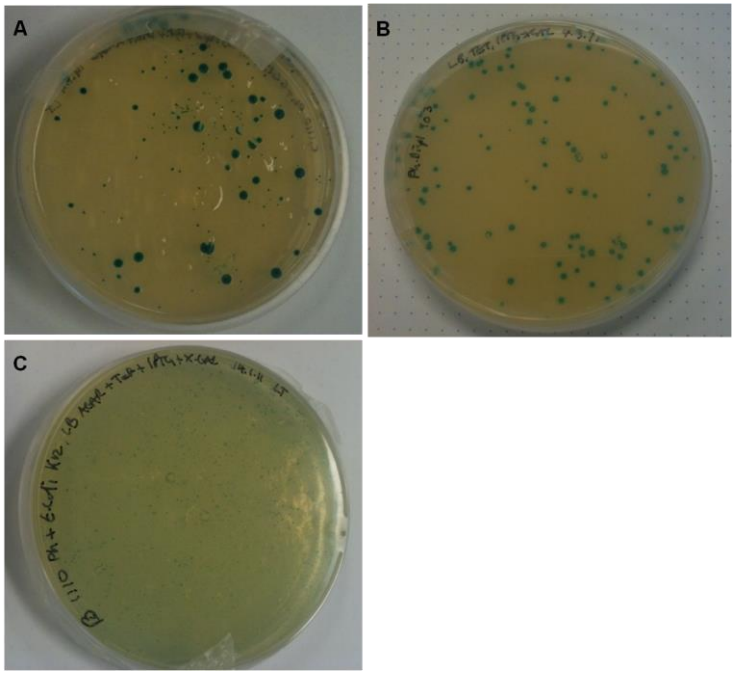

Figure 3.1 M13KE phage titrations in *Ecolab* ER2738, plated in decreasing dilutions. Dilutions are required in order to calculate the correct dilution for successive pans. Tetracycline and IPTG/X-Gal were used for selection and identification due to the *Ecolab* vector containing the gene for tetracycline resistance and the Lac-Z gene. A – 1:10,000 dilution, B – 1:1000 dilution, C – 1:100 dilution. 10^{-2} to 10^{-7} was the standard range of dilutions used in order to obtain meaningful titers of 20-100 plaques.

3.3.2. Alignment of Na-ASP-2 binding peptides with the putative SK3 binding protein domain

From five separate panning experiments, a total of 16 peptides were identified after three successive rounds of panning. One peptide (KLIGHNQQHAIL) was identified in two independent screens of the library, while two additional peptides (LHQPKDW/RHSRQH) were identified with just a single amino acid difference (**Table 3.1**). All of the peptides except for one had a net positive charge, with 11 having pI values greater than 9.0. Most of the peptides were enriched for Glutamine and Histidine residues. Three peptides possessed a HXXQH and a fourth peptide contained a HXXXH motif. Blast searches of public databases with an emphasis on the human proteome identified many distinct proteins containing varying degrees of similarity to different regions within the peptides, however no peptide had a 100% match across its entire length (12 residues) to known proteins, implying that binding of *Na*-ASP-2 to its native ligand is dependent on shorter peptides, possibly those containing an HXXQH motif. Some of the dissimilar sequences may have bound as a result of conformational similarity to the biological target/binding partner, however to ascertain this would require further studies. One such protein was the small conductance calcium-activated potassium channel 3 (SK3), a trans-membrane protein that contained a HNHQH motif within its extracellular domain of LHHPNATHNHQHAGT.

Peptide sequence	pI	Net charge	Gln/His content/motifs	Attribute
I V L P L S A P T A L K	9.69	+1	-	Basic
T P K N W P L V S S L R	11.66	+2	-	Basic
K L I G H N Q Q H A I L*	9.69	+3	HXQQH	Basic
Q S Q P R S L Q P P P A	10.55	+1	Q(3)	Basic
H T A N W H L T T H S A	7.81	+3	HXXXH	Basic
L H Q P K D W H S R Q H#	9.69	+4	HXXQH	Basic
L H Q P K D R H S R Q H#	11.48	+5	HXXQH	Basic
A P W H Q D T P K P P V	7.55	+1	XHQX	Basic
A F N V H K P G E P S Y	7.54	+1	H(1)	Basic
Q P R Q L H N G S P Y N	9.35	+2	QXXQXH	Basic
H Y K T E H A I R T S P	9.31	+3	H(1)	Basic
S S A A R L T A H P Y T	9.35	+2	H(1)	Basic
I N A A Y P N R V S S P	9.34	+1	-	Basic
V Y S A Q P Q Q L R P T	9.34	+1	QXQQ	Basic
Q L A S L K N F S L P A	9.69	+1	Q(1)	Basic
Q S V K E Q T D R P S A	6.51	0	Q(2)	Neutral

Table 3.1 Summary of 12-mer peptides from the phage panning that bound to immobilized *Na*-ASP-2. *peptide identified twice in two independent screens. #two peptides differ by a single residue (Trp-7 for Arg-7).

3.3.3. In silico modeling reveals probable binding site of Na-ASP-2 to SK3 peptide

As shown below in Table 3.2, there is a portion of the SK3 domain that shares amino acid sequence homology with the common Phage Display sequence shown above in Table 3.1 - HXQQH - however in the SK3 domain this motif is HNHQH. SK3 is a calcium activated potassium channel found on many human cells, including the central nervous system, muscle, liver, pituitary, prostate, kidney, pancreas and vascular endothelium hyper polarization [186] along with the intestine, and plays an important role in regulating muscle tone [187]. The SK3 ion channels play a major role in human physiology, particularly in smooth muscle relaxation/vasodilation and these channels are down regulated in the non-junctional membranes of skeletal muscle [188].

Sequence	**CLUSTAL 2.1 multiple sequence alignment**
1	--IVLPLSAPTALK---
2	--TPKNWPLVSSLR---
3	----KLIG-HNQQHAIL
4	--QSQPRSLQ--PPPA-
5	--HTANWHLTTHSA---
6	--LHQPKDWHSRQH---
7	--LHQPKDRHSRQH---
8	----APWHQDTPKPPV-
9	---AFNVHKPGEPSY--
10	----QPRQLHNGSPYN-

11	--HYKTEHAIRTSP---
12	---SSAARLTAHPYT--
13	--INAAYPNRVSSP---
14	VYSAQPQQLR---PT--
15	--QLASLKNFSLPA---
16	----QSVKEQTDRPSA-
SK3(239-253)	**-LHHPNATHNHQHAGT**

Table 3.2 Clustal Sequence Alignment

3.3.4. In silico binding model via differential scanning fluorimetry

In order to investigate the feasibility of the consensus sequence resulting from the phage display panning of *Na*-ASP-2, colleagues from Griffith University constructed an *in-silico* model of the binding interaction between the SK3 peptide and *Na*-ASP-2 protein (**Figure 3.2**). The peptide is predicted to sit in the "binding groove" of the protein and is the lowest binding energy conformation, therefore representing the most probable binding position of the peptide and the protein.

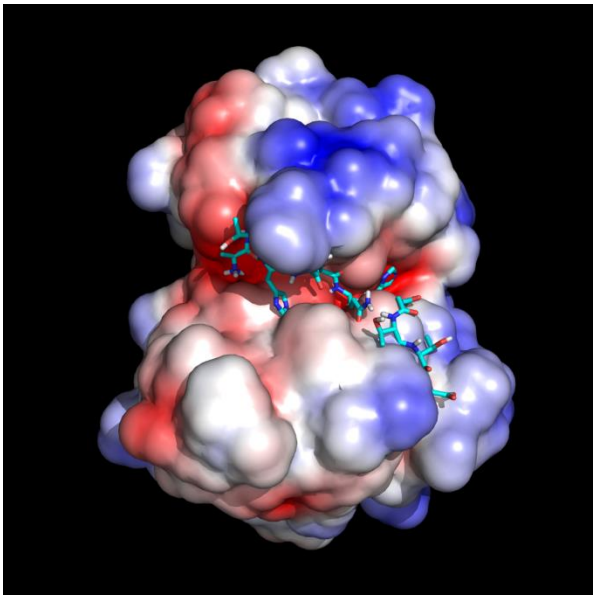

Figure 3.2 3-dimensional crystal structure of the *Na*-ASP-2 molecule binding the consensus peptide in its putative binding groove. The bound peptide is derived from the extracellular region of the SK3 protein (HNHQH). Electrostatic potentials are shown: red indicating - acidic amino acids, blue – basic, white - neutral. Figure prepared with Pymol [189].

Binding of the synthetically made peptide from the SK3 molecule, to *Na*-ASP-2 was confirmed via differential scanning fluorimetry see Table 3.3 [185], proving consistent with the theoretical binding deduced from the peptide phage library results.

Ligand	**Interactions observed in crystal structure[a]**	**Differential scanning fluorimetry $\Delta T_{1/2}$ (K)[b]**
Mn^{2+}	not bound	-0.23 (0.0; 3)
Co^{2+}	His 88-NE2 (2.3) His 148-NE2(2.6) H2O 60-O (2.3) H2O 91-O (2.5) H2O 172-O (2.8) H2O 194-O (2.4)	+0.03 (0.15; 3)
Ni^{2+}	His 88-NE2 (2.3) His 148-NE2(2.5) H2O 59-O (3.3) H2O 74-O (3.5)	-0.42 (0.0; 3)
Cu^{2+}	His 88-NE2 (2.3) His 148-NE2(2.5) H2O 32-O (2.6)	+6.0 (0.06; 3)
Zn^{2+}	His 88-NE2 (2.2) His 148-NE2(2.3) H2O 24-O (2.5) H2O 74-O (2.7) H2O 77-O (2.5)	+1.1 (0.13; 3)
SK3 peptide	not bound	+1.1 (0.15; 6)
SK3 peptide, Mn^{2+}	not bound	+1.0 (0.13; 3)
SK3 peptide, Co^{2+}	not bound	+1.0 (0.13; 3)
SK3 peptide,	not bound	+1.3 (0.05; 3)

Ni^{2+}		
SK3 peptide, Cu^{2+}	not bound	+1.4 (0.12; 3)
SK3 peptide, Zn^{2+}	not bound	+1.2 (0.16; 3)

Table 3.3 Differential scanning fluorimetry of SK3 peptide binding to *Na*-ASP-2. [a] - Distances are given in Å. [b] - $\Delta T_{1/2}$ is defined as the difference between $T_{1/2}$ (protein:ligand) and $T_{1/2}$ (protein:H2O). Values given are the average of N repeats, with the standard deviation and N given in brackets. The effect of water addition to protein caused a temperature shift of $\Delta T_{1/2} = T_{1/2}$ (protein:H2O) - $T_{1/2}$ (protein) = 0.13 K (0.0; 3) [185].

3.4 Discussion

Phage display panning was used to find a consensus sequence of bound library peptide to *Na*-ASP-2 protein, using a recombinant 12-mer peptide library in the lambda phage. The results of several rounds of panning and 20 different sequence results, led to 16 disparate sequences and a common consensus sequence of HXQQH. Although some of the peptides sequenced lacked this motif, they may have possessed conformational similarity. Using this information and the BLAST sequence homology tool, the first hit was shown to be a peptide within the SK3 potassium ion channel protein on the surface of human cells, which had a similar sequence of HNHQH. *In silico* modeling provided insight into the likelihood of such a potential interaction [185].

SKCa channels, also known as small conductance calcium-activated potassium channels, are activated by low concentrations of intracellular calcium and consist of homomeric or heteromeric assemblies of α–subunits named SK1, SK2 and SK3. They are involved in integrating changes in intracellular calcium with membrane potentials

[190]. SKCa channels are important in multiple physiological roles, such as cellular homeostasis, as evidenced by the wide variety of literature articles on the subject.

Ion channels such as the SK3 calcium activated potassium channel are renowned for their role in action potential activation and suppression, however a role has been described in mediating reactive oxygen species (ROS) [191]. If *Na*-ASP-2 does indeed bind to and interfere with the function of the SK channel, it may putatively suppress the expression of ROS that could otherwise be harmful to the hookworm larvae during their journey from the skin to the lungs. Considering the potent anti-inflammatory properties of hookworm ES products [192] of which *Na*-ASP-2 is a member, it is reasonable to assume that *Na*-ASP-2 is one of the many ES proteins that collectively facilitate the larvae's safe passage through the human host's circulatory system.

Although *in silico* permutations indicate a favorable binding energy state and therefore a likelihood of protein-protein interactions between ASP-2 and SK3, there remains no physiological evidence to indicate that this binding is occurring *in vivo*. There appears to be no obvious beneficial effects for the hookworm larvae from blocking SK3 channels, except potentially increased smooth muscle relaxation and reduction of ROS, which may ease its journey through the circulatory system, en route to the lungs. Further investigation is required before a clear physiological role for ASP-2/SK3 interactions can be confirmed.

At least one ASP has previously been implicated in immunomodulation of the host by blood-feeding hookworms [113]. The abundance of these ES proteins during the intra-mammalian stages of the hookworm is contrasted by a paucity of known host receptors.

The only known interaction underpinned by experimental evidence is that of the Group 3 ASP, neutrophil inhibitory factor (NIF) with a human integrin [173]. Several other members of the PR-1 family of proteins are known toxins, i.e. effectors of host ion channels [193]. Group 2 ASPs, including *Na*-ASP-2, possess a conserved equatorial binding groove with a prominent tandem Histidine motif [132]. Herein, we provided a molecular and functional characterisation of this structural feature, and also identified the human SK3 channel as a potential target of *Na*-ASP-2. Considering the emerging importance of the SK3 channel as a therapeutic target for disorders involving neuron hyper-excitability, these current findings highlight the potential of hookworm ASPs as therapeutic tools for a variety of disorders, including addiction, pain, and autoimmune diseases.

Introduction to Chapter 4

In order to address the validity of the interaction between the SK3 peptide and *Na*-ASP-2, I decided to re-address potential binding partners of *Na*-ASP-2 using protein ligands instead of peptides. Potential protein:protein interactions would then be confirmed (if possible) using human tissues to further address potential host-parasite functional interactions. To do this, I decided to probe a human proteome array with biotinylated recombinant *Na*-ASP-2. The protein array technology allows rapid scanning of tens of thousands of human proteins in a single experiment. Positive interactions were then further analyzed using *ex vivo* co-culture of human cells with ASP-2 followed by global gene expression analyses on host cells using next generation sequencing.

Chapter 4:

Probing of a human proteome microarray with a recombinant pathogen protein reveals a novel mechanism by which hookworms suppress B cell receptor signaling

4.1 Introduction

Although a large amount of research has investigated ASPs as vaccines, little is known about their biological roles. *Na*-ASP-2 is highly up-regulated in *Necator americanus* infective L3 stage larvae upon exposure to serum [194], however once the larvae reach the lung, gene expression diminishes. This suggests that the ASP-2 protein may be

involved in immune regulation whilst the parasite is within the circulatory system. *Na*-ASP-2 administered to rodents in recombinant form elicits a type 2 cytokine response typical of helminth infections, along with *Na*-ASP-2-specific antibodies and neutrophil recruitment [154, 155]. Despite these observations, no direct interactions between *Na*-ASP-2 and a defined cell type or molecule has been demonstrated. Based on X-ray crystallographic studies, the ASP family has 3 distinct structural subtypes [132]. *Na*-ASP-2 as a member of the group 2 ASPs contains a putative binding groove flanked by Histidine and Glutamic acid residues [137], suggesting the presence of an unknown ligand or binding partner. Neutrophil Inhibitory Factor (NIF), another hookworm derived ASP-like protein, binds directly to host CD11b, thereby inhibiting neutrophil function [133]. Therefore it remains likely that *Na*-ASP-2 similarly binds to a cell surface receptor from its human host to exert its effector function(s).

In the previous chapter, it was shown that a peptide identified via phage display panning (HNQQH) was homologous to a peptide in the extra cellular fragment of the human SK3, cell surface protein (HNHQH). *In silico* structural modeling of binding kinetics showed a favourable low energy binding state of the SK3 indicating a favorable conformation of protein-protein interactions if the SK3 peptide was docked in the putative binding groove of *Na*-ASP-2 [185]. However with no *in vivo* or *ex vivo* evidence to confirm these findings, we wanted to confirm the binding using proteins rather than peptides and array technology provided the perfect way to do so. As a result of which further investigation focused on the potential of other binding partners with a greater focus on biological relevance and cellular interactions.

This chapter aimed to confirm and extend the previous investigations regarding potential human host binding partners of *Na*-ASP-2. The recent development of a human V5 proteome array, which contains over 9,000 human proteins printed on a single slide, enabling the first ever probing of these arrays with a pathogen-derived protein to identify a novel host-pathogen molecular interaction, and showed that *Na*-ASP-2 binds to human CD79a. We confirmed the identified protein-protein interactions using blood cells obtained from human volunteers and undertook a broad analysis of changes in host gene expression via RNA sequencing and bioinformatic analysis.

4.2 Materials and Methods

4.2.1. Ethics

Healthy Caucasian volunteers (aged 30-45) from Cairns, Australia, a region that is not endemic for human hookworm infection, were used as a source of peripheral blood under informed consent using a protocol (H4385) approved by the James Cook University Human Research Ethics Committee.

4.2.2. Probing of a human protein microarray with recombinant Na-ASP-2

Purified, full-length recombinant *Na*-ASP-2 used for experiments in this chapter was a kind gift from Dr. Bin Zhan (Baylor College of Medicine, USA). The protein was expressed in *Pichia pastoris* and purified via two ion exchange chromatography steps followed by a final desalting step as described elsewhere [135]. *Na*-ASP-2 was biotinylated with NHS-LC-biotin according to the manufacturer's instructions (Thermo-Fisher) and used to probe a Human V5 ProtoArray (Invitrogen) [195] at a final concentration of 50 mg/mL. The ProtoArray contains over 9,000 human proteins

printed in duplicate, and includes more than 2,600 membrane proteins. Binding of biotinylated *Na*-ASP-2 to proteins on the array was detected with streptavidin Alexafluor 647 (Invitrogen) at a 1:1000 dilution in Protoarray blocking buffer (Invitrogen). The array was scanned using a GeneArray 4000B scanner (Molecular Devices) at 635 nm. Results were saved as a multi-TIFF file and analyzed using Genepix Prospector software, version 7.

4.2.3. Molecular Modelling

Comparative modelling was applied to generate a three-dimensional atomic model of human CD79A, using the crystal structure of human CD79B (PDB accession code 3kg5) as a template. A secondary structure-based amino acid sequence alignment of CD79A and CD79B was prepared with SBAL [196] and used to guide the comparative modelling calculations. Twenty independent models were calculated with MODELLER [197], and the one with the lowest energy was selected and superimposed onto one of the monomers in the CD79B homodimer structure (rigid body modelling). As previously described, the secondary structure topology and amino acid sequence of CD79A is compatible with the intermolecular interactions observed in the CD79 homodimer structure [198]. A minor manual adjustment was made to enable inter-molecular disulphide bond formation (CysA119-CysB136) in the resulting heterodimer. Both cysteine residues were in prime position for this bond, which is also observed in the CD79B homodimer. The crystal structure of *Na*-ASP-2 was docked to the CD79 heterodimer model, using a rigid body approach based on a multi-dimensional spherical polar Fourier correlation with shape and electrostatics expansion as implemented in Hex [199]. The rigid body docking calculations were carried out without preferred contacts to enable an unbiased complex formation. From the generated 100 independent

models the highest scoring one (energy score: -873, next highest -720) was chosen. The figures were generated with PyMOL [189].

4.2.4. Blood lymphocyte isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Lymphoprep^{TM}). Cells (1×10^6 cells/mL) were incubated for 4 or 24 h at 37°C in either 96-well plates (for flow cytometry) or 24-well plates (for sort purification) with *Na*-ASP-2 in DMEM with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 25mM HEPES (media).

4.2.5. Flow cytometry and cell sorting

Cells were stained with fluorophore-conjugated antibodies against CD19 (HIB19), CD20 (2H7), CD3 (OKT3), CD4 (OKT4) and CD14 (61D3) (all from eBioscience) following the manufacturer's recommended dilutions and protocols. In experiments using biotinylated *Na*-ASP-2, streptavidin-FITC was included in the staining panel. Cells were analyzed using a BD-FACSCantoII flow cytometer. Alternatively, or $CD3^-$ $CD14^-$ $CD19^+$ $CD20^+$ cells were sort-purified using a BD-FACSAria III cell sorter.

4.2.6. RNA isolation

RNA was extracted from sort-purified $CD19^+$ $CD20^+$ B cells for high-throughput RNA-Seq at BGI (Hong Kong) or real-time quantitative PCR (qPCR) analysis. Pelleted cells were lysed in RLT buffer (QIAGEN) + beta-mercaptoethanol and stored at -80°C until RNA was extracted. Total RNA was isolated from purified B cells that had been cultured in the presence or absence of *Na*-ASP-2 using the RNAeasy kit (QIAGEN)

and purified using the RNase-free DNAse Set kit (QIAGEN). The concentration and integrity of each RNA preparation were verified on a 2100 Bioanalyzer (Agilent) and each preparation was stored in RNAstable tubes (Biomatrica) at room temperature until subsequent use.

4.2.7. Illumina sequencing

RNA from duplicate experiments was reverse-transcribed to cDNA using the Illumina TruSeq™ sample preparation kit, according to the manufacturer's instructions. Briefly, 1.0 mg of total RNA from each cell preparation was used for poly-A mRNA selection using streptavidin-coated magnetic beads. Two rounds of poly-A mRNA enrichment were performed, followed by thermal fragmentation of the mRNA to a length of 100-500 bp. Each fragmented RNA sample was then reverse transcribed to cDNA using random primers, end-repaired and adaptor-ligated, according to the manufacturer's protocol (Illumina). Ligated products were PCR-amplified (15 cycles) and cleaned using a MinElute column (QIAGEN). Amplicons were paired-end sequenced on an Illumina HiSeq 2500 (Illumina), according to the manufacturers' instructions.

4.2.8. Bioinformatic analyses

For each sample, the 90 bp single-reads were first screened for the presence of adapter sequences and sequences with suboptimal read quality (sequences with >10% unknown bases and sequences with >50% low-quality bases). The remaining reads were splice-aligned to the human genome reference assembly GRCh37 (NCBI build 37.1) using TopHat software, which incorporates the Bowtie v0.11.3 algorithm [200] with up to 40 alignments and a maximum of five mismatches per read; subsequently, individual aligned read files were assembled into transcripts using Cufflinks [201]. Relative levels

of gene transcription were measured using the Reads Per Kilobase per Million reads algorithm (RPKM) [202] differences in levels of gene transcription were determined using a method developed for serial analysis of gene expression and applied to RNA-seq data [203]. The false discovery rate method (FDR) [204] was used to correct the errors associated with multiple pairwise comparisons (FDR $\leq$ 0.001) and a $|\log_2 \text{ratio}| \geq 1$ was set as the threshold for the significance of gene expression changes. Differentially expressed genes (DEGs) were mapped to Gene Ontology terms (according to the categories 'Biological Process', 'Cellular Component' and 'Molecular Function') and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways available in the GO and KEGG databases, respectively (www.geneontology.org; www.genome.jp/kegg/). A hypergeometric test was subsequently used to identify significantly enriched GO terms and KEGG pathways in DEGs compared to the transcriptome background using the GOTerm::Finder software [205]. The calculated *P*-values were subjected to Bonferroni correction using the corrected *P*-value of 0.05 as a threshold. Raw sequence data have been deposited in the Sequence Read Archive (SRA) of NCBI under accession number ID PRJNA259640.

4.2.9. Real-Time quantitative PCR

Transcription of *lyn* and *pi3k* was quantified by real-time quantitative PCR (qPCR). Total RNA was extracted from sort-purified B cells from four hookworm-naïve Caucasian human volunteers that were cultured for 24 h in the presence or absence of *Na*-ASP-2 as described above and DNase-treated. cDNAs were prepared using Superscript III reverse transcriptase (Invitrogen). SYBR Green mastermix (QIAGEN) was used with a Rotor-Gene q thermal cycler (QIAGEN) according to the manufacturer's protocol. The oligonucleotide primers for these genes were designed

manually and synthesized (Integrated DNA Technologies). Data of duplicate experiments were analysed using the ΔΔCT method whereby actin served as the endogenous gene and samples were normalized to media controls. Primer sequences 5'to 3' were as follows: *b-actin*- forward ATTGGCAATGAGCGGTTC and reverse GGATGCCACAGGACTCCAT; *lyn* forward GAGACTCGGGGAGCCATTG and reverse ACGCTGGGGATGTTATACTCT; *pi3kr5* (23533) forward CTTCCACGCTACGTGTTGTG and reverse TGAAGTTTGAAGAACCGTGTGAG.

4.2.10. Competitive inhibition binding assay

Human PBMCs were prepared identically to section 4.2.4, using lymphoprep and density centrifugation. Recombinant biotinylated *Na*-ASP-2 was pre-treated at 37°C for 1 hour with either a control 13-mer peptide (AGPKAQVEAIQKY) or SK3 peptide (ATHNHQHAGTTA) before incubation with PBMCs from a hookworm-naïve volunteer for 4 hours. B cells with Na-ASP-2 bound were detected by staining with FITC-labelled streptavidin and co-stained with anti-CD19. The experiment was done in duplicate.

4.2.11. Statistical Analyses

Statistical analyses of qPCR data were conducted using a paired *t* test where ΔΔCT values for each gene of interest were compared between test (*Na*-ASP-2 exposed) vs. control (media exposed) cells. *P* values of <0.05 were deemed statistically significant.

4.3 Results

4.3.1. Interaction between Na-ASP-2 and human CD79A revealed by probing of a human proteome microarray

Upon probing of the human proteome array with biotinylated *Na*-ASP-2, five primary interactions with human proteins were observed. The strongest of these was with propionyl-CoA carboxylase (PCCA), as expected as PCCA is a biotin-binding enzyme and would be anticipated to bind any biotinylated protein used to probe the array (**Figure 4.1** and **Table 4.1**) Other individuals in our research group have also noted consistent binding of other biotinylated recombinant proteins to PCCA (data not shown). The second most intense interaction was with CD79A, this forms a hetero-dimer with CD79B as a subunit of the B cell antigen receptor. The third and fifth most intense hits were also proteins involved in biotin metabolism, Methyl crotonoyl-Coenzyme A carboxylase 1 (MCCC1) and Enoyl Coenzyme A hydratase/3-hydroxylacyl Coenzyme A dehydrogenase (EHHADH). The fourth strongest association was cortactin, a protein involved in actin remodeling, however the intensity was 2.4 fold lower than CD79a, leading to the conclusion that it was unworthy of further investigation. A repeated probing of a second array under identical conditions resulted in consistent binding with the same 5 arrayed proteins. A table of the hits is shown below (**Table 4.1**).

Intensity	Protein	NCBI Accession	Description
1600	Propionyl coenzyme A carboxylase (PCCA)	NM_000282.1	Biotin binding enzyme
456.5	Immunoglobulin binding protein 1 (IGBP1/CD79a)	NM_001551.1	Co-receptor of B cell receptor complex

359.5	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha) (MCCC1)	NM_020166.2	Biotin metabolism
191.5	Cortactin (CTTN), transcript variant 2, intracellular	NM_138565.1	Actin remodelling enzyme
177.5	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (EHHADH)	BC000306.1	Biotin metabolism

Table 4.1 List of proteins on the Human V5 ProtoArray that interacted with recombinant *Na*-ASP-2.

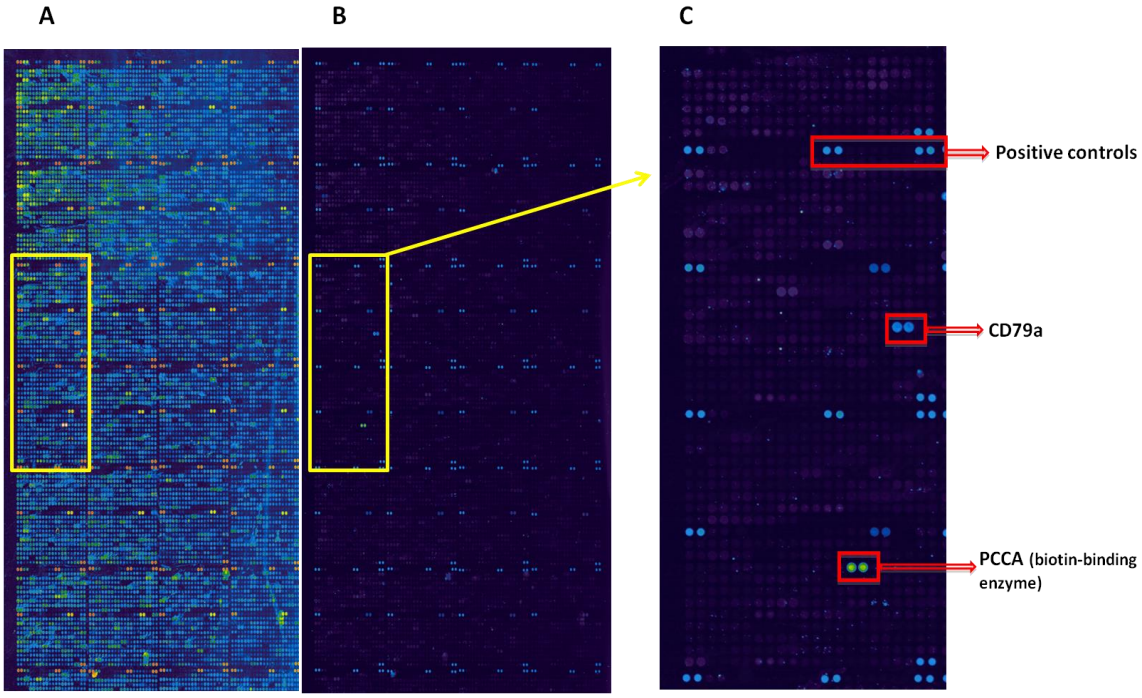

Figure 4.1 Binding of biotinylated *Na*-ASP-2 to CD79A and biotin-binding proteins on a Human V5 ProtoArray. The array was probed with biotinylated recombinant *Na*-ASP-2 followed by streptavidin Alexafluor 647 and scanned using a GeneArray 4000B scanner at 635 nm using laser intensity settings of 100% (**A**) and 66% (**B**). Yellow boxes denote the area of the array containing duplicate spots of CD79A and PCCA. Panel C shows a magnified view of the area contained within the yellow boxes. All other spots are positive control proteins spotted in duplicate to allow orientation on the array. Two separate arrays were probed independently, with representative results from one experiment displayed.

4.3.2. Modeling of Na-ASP-2 interactions with CD79

In chapter 4 we discussed the panning of random a 12-mer peptide phage display library with recombinant *Na*-ASP-2, and most bound peptides were enriched for Glutamine and Histidine residues [185]. The extracellular domain of CD79A contains a HXQXXH motif at residues 51-56; this peptide forms part of a large surface of CD79A which has previously been implicated as a co-receptor of the B cell surface receptor and integral in the B cell signaling pathway [198]. Rigid body docking of the crystal structure of *Na*-ASP-2 (conducted by L.Mason and A.Hoffman, Griffith University) and a model of heterodimeric CD79A/CD79B suggests that, based on shape and electrostatic charge, *Na*-ASP-2 may bind to the CD79 heterodimer (**Figure 4.2**).

Furthermore, this model indicates that *Na*-ASP-2 mainly interacts with the CD79A surface harboring the HXQXXH motif, however a protruding loop of CD79B (residues 79-86) may also engage in interactions with the protein.

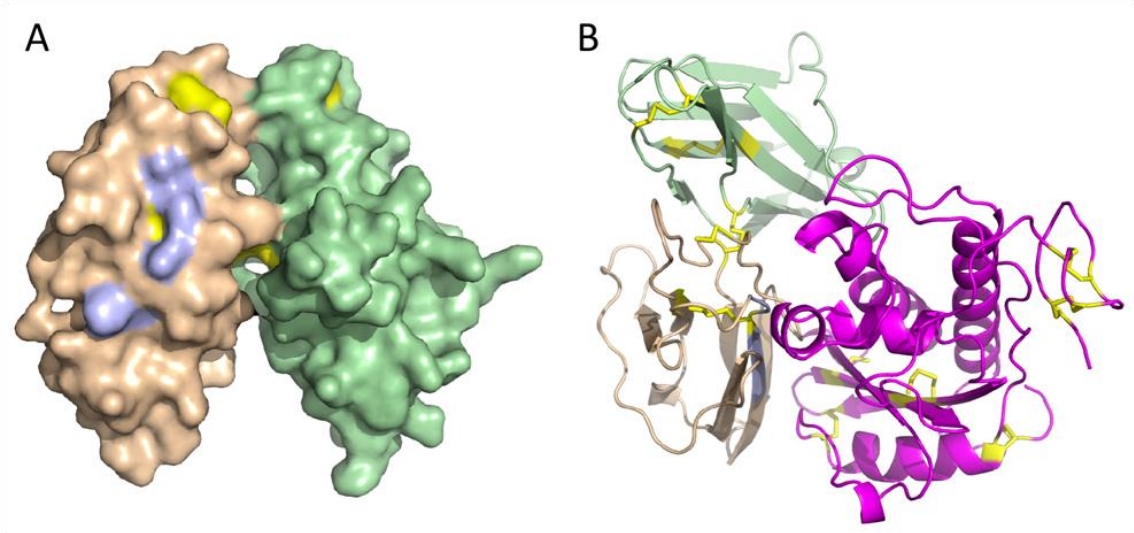

Figure 4.2 Rigid body models of the CD79A/CD79B heterodimer (A) and the CD79A/CD79B:*Na*-ASP-2 complex (B). CD79A - wheat, CD79B - pale green, and *Na*-ASP-2 - magenta. Cysteine residues/disulphide bonds are rendered as sticks in yellow. The CD79A peptide HFQCPH (residues 51-56) is coloured blue. Surface representation of the CD79 heterodimer model (A). Rendering of the modelled ternary complex of *Na*-ASP-2 and CD79 (B).

4.3.3. Na-ASP-2 binds selectively to human B cells.

The transmembrane protein CD79A is expressed exclusively on the surface of B lymphocytes. In order to verify the interaction between *Na*-ASP-2 and CD79A, we cultured human PBMCs from two healthy, hookworm-naïve human volunteers (2 biological replicates) *ex vivo* for 4 or 24 h with 0, 2, 10 or 20 µg/mL of biotinylated *Na*-ASP-2 and performed flow cytometry analysis. Cells were stained for CD19, CD20 B cell markers, T cell marker CD3, monocyte marker CD14, and Streptavidin-FITC for

detection of surface-bound, biotinylated recombinant *Na*-ASP-2. As expected, negligible frequencies of FITC$^+$ cells were detected when cells were treated with media alone or non-biotinylated *Na*-ASP-2, however treatment with increasing amounts of biotinylated *Na*-ASP-2 resulted in a dose-dependent increase in the frequency of *Na*-ASP-2/FITC$^+$ cells (**Figure 4.3A**). FITC$^+$ cells were subsequently gated and analyzed for various leukocyte surface markers. While only 8% of FITC$^+$ cells expressed CD3 or CD14, the vast majority (72%) of FITC$^+$ cells co-expressed CD20 and CD19, a phenotype consistent with human B lymphocytes (**Figure 4.3B**).

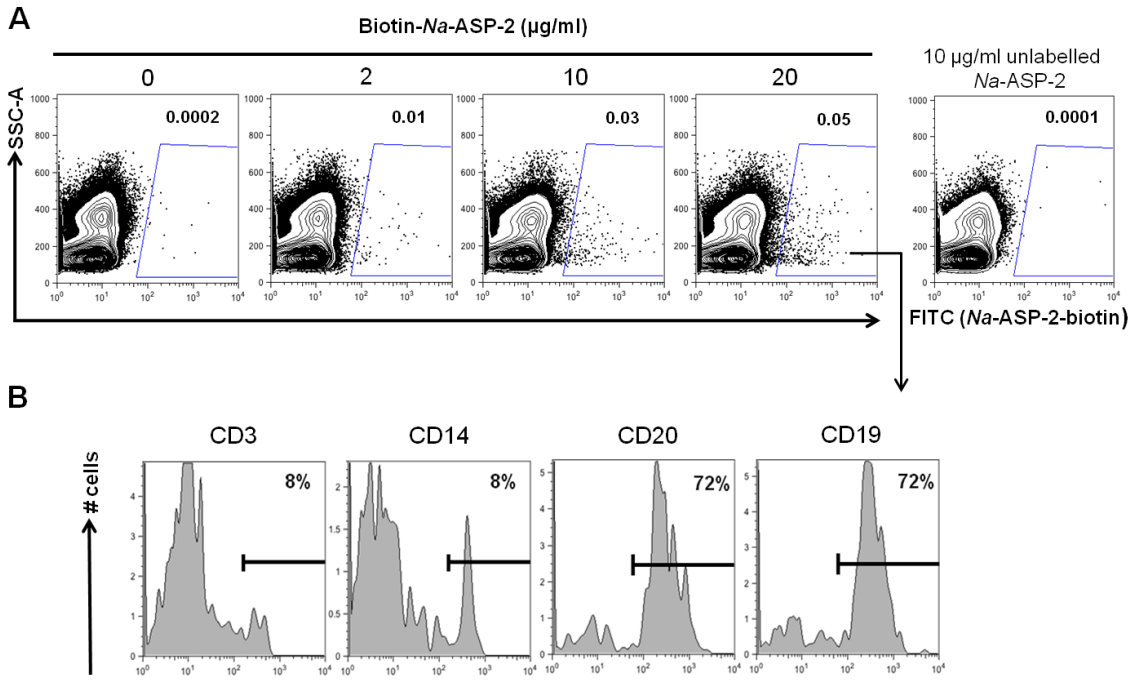

Figure 4.3 *Na*-ASP-2 binds selectively to human B cells shown via flow cytometry**.** PBMCs from hookworm-naïve donor volunteers were cultured for 24 h in the presence of increasing concentrations of biotinylated recombinant *Na*-ASP-2, or with unlabeled *Na*-ASP-2. (**A**) Frequencies of cells with biotinylated *Na*-ASP-2 on the surface were quantified by flow cytometry after incubation with FITC-conjugated streptavidin. (**B**) *Na*-ASP-2$^+$ FITC$^+$ cells were gated for analysis of the immune cell-surface markers

CD3, CD14, CD20 and CD19. Experiments were conducted twice, with two different blood donors; representative data are shown.

We undertook a competitive binding inhibition assay in duplicate, using the same commercially produced peptide as that used for the binding kinetics assay in chapter 4, containing a motif (HNHQH) similar to that present on the extracellular region of CD79A (and compared to an irrelevant control peptide) via pre-incubation for 1 hour with each peptide and the results are shown below in (**Figure 4.4**). A partial binding inhibition was achieved. The results demonstrated that while 2.5% of $CD19^{+}$ B cells bound *Na*-ASP-2 that had been treated with the control peptide, only 1.5% of B cells bound *Na*-ASP-2 when pre-treated with the SK3 derived peptide.

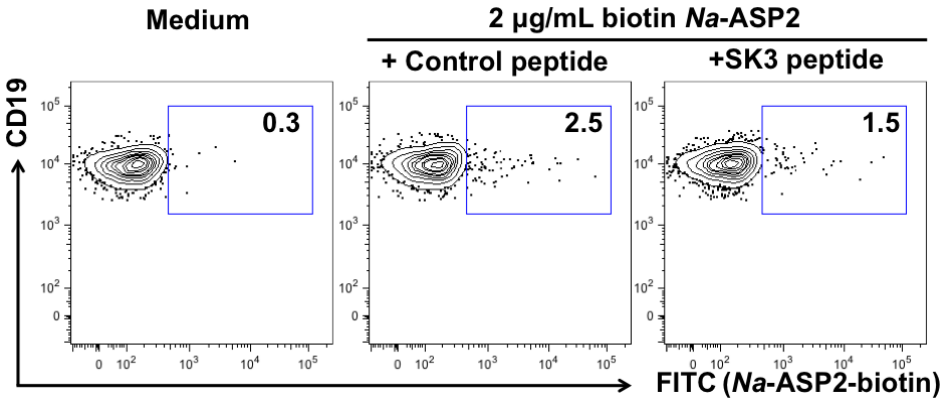

Figure 4.4 Partial blockade of *Na*-ASP-2 binding to human B cells following pre-treatment with SK3 peptide. Recombinant biotinylated *Na*-ASP-2 was pre-treated at 37°C for 1 hour with either a control 13-mer peptide (AGPKAQVEAIQKY) or SK3 peptide (ATHNHQHAGTTA) before incubation with PBMCs from a hookworm-naïve volunteer for 4 hours. *Na*-ASP-2 binding to B cells was detected by staining with FITC-labelled streptavidin and co-staining with anti-human CD19. Control cells received

medium only. Numbers represent frequency of $FITC^+$ cells. Experiment was done in duplicate.

4.3.4. Na-ASP-2 induces substantial changes in B cell gene expression.

In order to elucidate the cascade of molecular events upon binding of *Na*-ASP-2 to human B cells, we sort-purified $CD19^+$ $CD20^+$ B cells from one human volunteer's whole lymphocyte cell fraction, purified from PBMCs, that were treated with either *Na*-ASP-2 or media control and performed RNA-Seq and bioinformatics analyses. B cells cultured with *Na*-ASP-2 for 4 h underwent minimal changes in gene expression (37 up-regulated genes, 178 down-regulated genes compared to media control, **Figure 4.5A**). After 24 h of culture, however, there was a pronounced bias toward down-regulation of gene expression in B cells that were cultured with *Na*-ASP-2, with 1080 significantly down-regulated genes and only 95 up-regulated genes (**Figure 4.5B**).

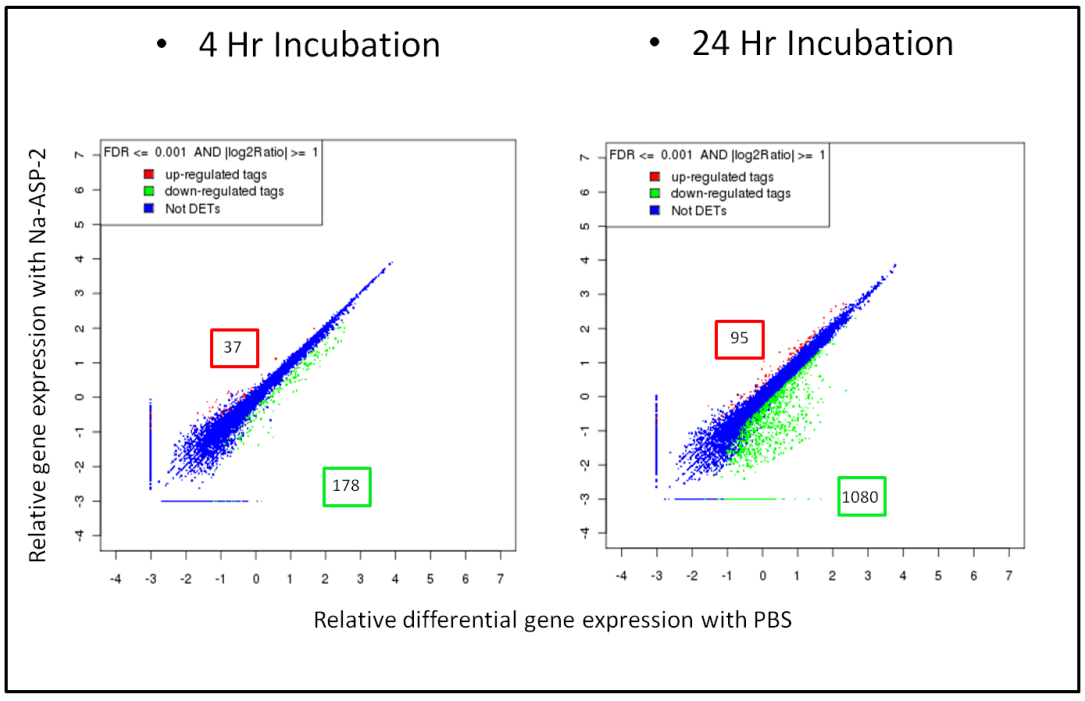

Figure 4.5 Changes in mRNA expression in B cells from a hookworm-naïve human volunteer after culture with *Na*-ASP-2. PBMCs were cultured for 4 or 24 h with either media or 2 μg/mL *Na*-ASP-2; $CD19^+$ $CD20^+$ B cells were sort purified and isolated RNA was subjected to RNA Seq analysis. Scatter plots comparing log_2 ratios of RPKM expression values for B cells from a hookworm-naïve human volunteer following *ex vivo* co-culture with *Na*-ASP-2 at 4 and 24 hours. Numbers indicate the numbers of up or down-regulated genes.

4.3.5. Na-ASP-2 down-regulates expression of genes involved in multiple biological pathways.

Genes whose expression was significantly altered following exposure to *Na*-ASP-2 were clustered according to their KEGG annotation in the form of a heatmap (**Figure 4.6**). In particular, the majority of down-regulated genes could be mapped to the 'cytokine-cytokine receptor interaction' (ko04060) and the 'leukocyte transendothelial migration' (ko04670) pathways, whereas the majority of genes whose expression was up-regulated by *Na*-ASP-2 binding could be mapped to the 'hematopoietic cell lineage' (ko04640) and 'chemokine signaling pathway' (ko04062), respectively.

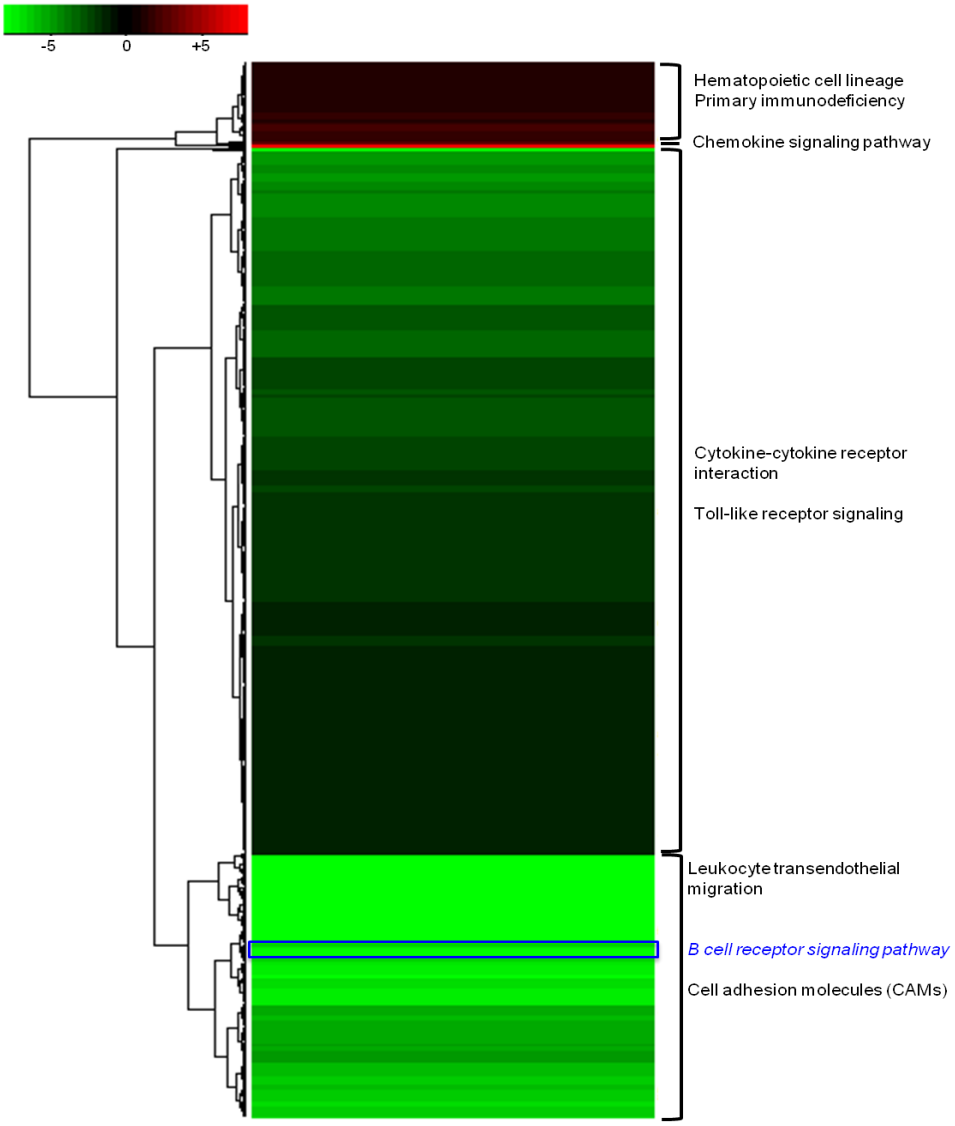

Figure 4.6 Differential gene expression following 24 h exposure of human B cells to *Na*-ASP-2. Heatmap analysis of differentially expressed genes, following 24 h culture of sort-purified human B cells with *Na*-ASP-2. Differentially expressed genes are clustered according to their KEGG annotation. The top KEGG pathways assigned to the majority of genes within each cluster are also reported.

4.3.6. Na-ASP-2 down-regulates expression of genes in the Ig receptor (CD79A) signaling pathway.

While *Na*-ASP-2 appears to affect the expression of genes involved in multiple biological pathways, we focused on potential consequences of interactions between *Na*-

ASP-2 and CD79A based on the protein array, protein-protein interaction studies. In particular, three genes mapping to the KEGG B cell receptor signaling pathway, i.e. *lyn*, spleen tyrosine kinase (*syk*) and phosphatidylinositol 3-kinase (*pi3k*) displayed significant down-regulation following exposure to *Na*-ASP-2 (**Figure 4.7**). Each of these gene products are involved in transmitting signals from the BCR and are crucial for essential B cell functions and ultimately, cell fates such as activation, development, proliferation, inhibition and cell death.

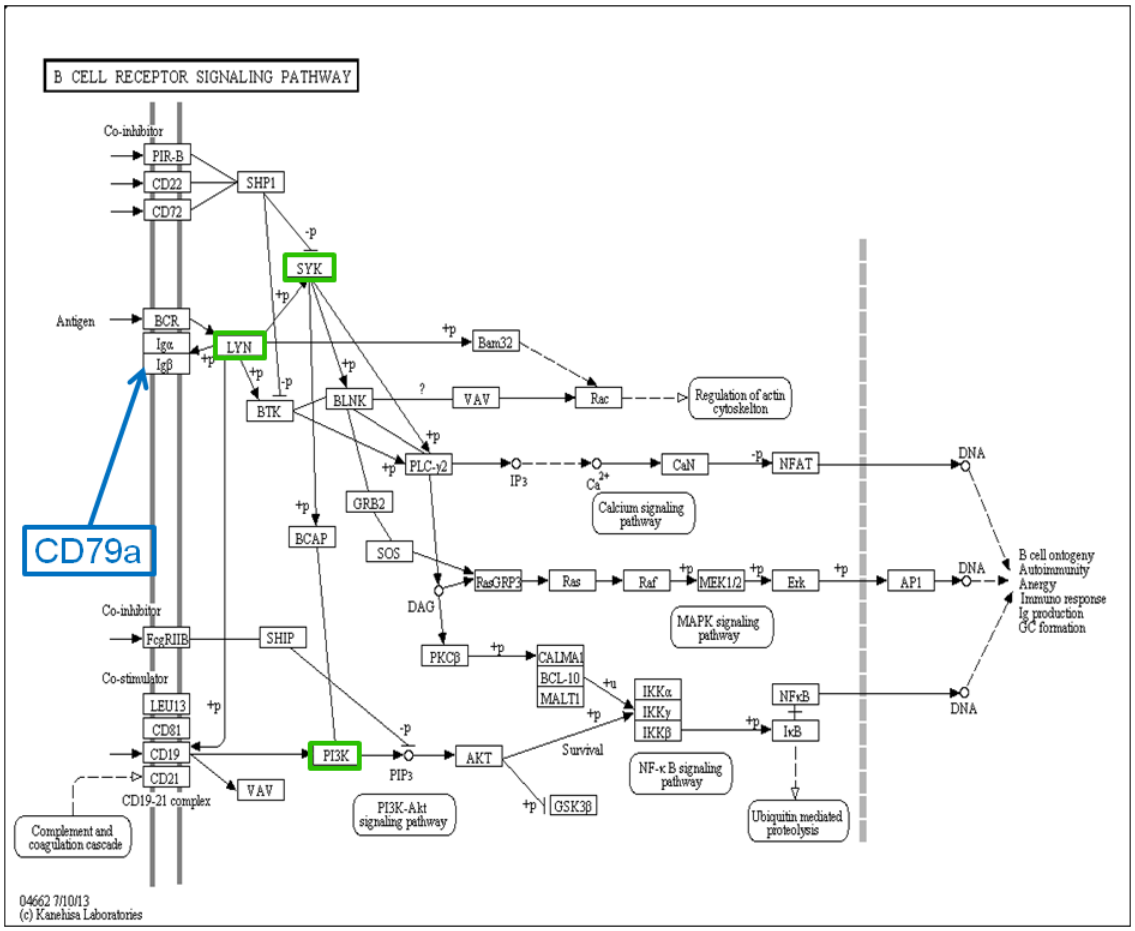

Figure 4.7 *Na*-ASP-2 causes B cell intrinsic reductions in expression of mRNAs immediately downstream of CD79A**.** Schematic representation of the B cell receptor signaling pathway (modified from http://www.genome.jp/kegg-bin/show_pathway?hsa04662). Genes whose expression was down-regulated following 24 h -co-culture of B cells with *Na*-ASP-2 are indicated by green boxes.

Follow-up studies involving the analysis of B cell-intrinsic gene expression induced by *Na*-ASP-2 from four hookworm-naïve blood donors were performed using qPCR. Consistent with the findings from RNA-seq, the expression levels of both *lyn* and *pi3k* were significantly reduced in B cells that were cultured for 24 h with *Na*-ASP-2 *ex vivo* relative to control cells (**Figure 4.8**).

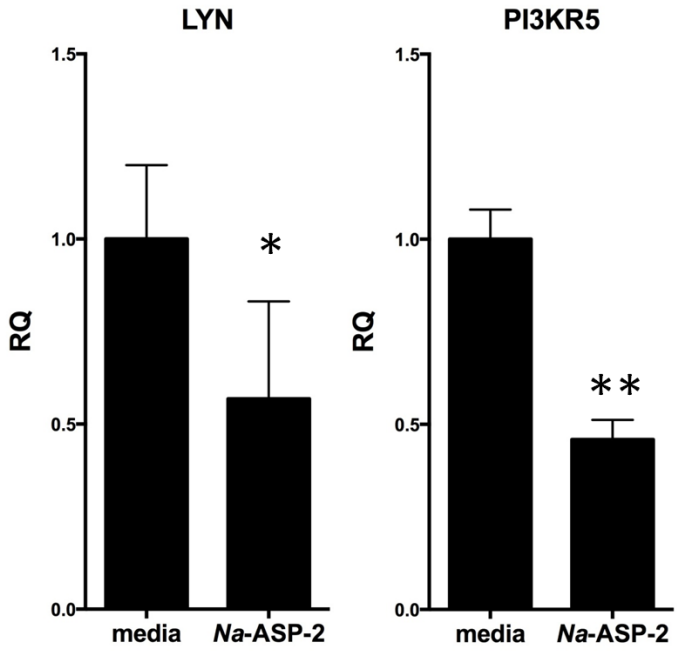

Figure 4.8 Down-regulated expression of *lyn* and *pi3k* in human B cells from four hookworm-naïve human volunteers after culture with *Na*-ASP-2. PBMCs from four hookworm-naïve donor volunteers were cultured for 24 h in the presence of medium alone or 2 μg/mL recombinant *Na*-ASP-2. qPCR analysis of *lyn* and *pi3kR5* was performed on RNA derived from sort-purified B cells. Data were analysed using the ΔΔCT method (Relative quantification, RQ), whereby actin served as the endogenous gene and samples were normalized to media controls. *P = 0.0295; **P = 0.0004. Error bars represent Standard Deviation (SD).

Cell surface activation markers were also examined for evidence of downstream phenotypic changes as a result of the interaction shown (**Figure 4.9**). There is evidence that *Na*-ASP-2 does marginally and reproducibly lower the expression of some cell surface markers (CD69, CD86, HLA-DR and CD80) on human B cells in the absence of exogenous stimulation (No Treatment). However, it is clearly evident that there was no substantial defect in the capacity for B cells to be directly activated by anti-IgM stimulation in the presence of *Na*-ASP-2.

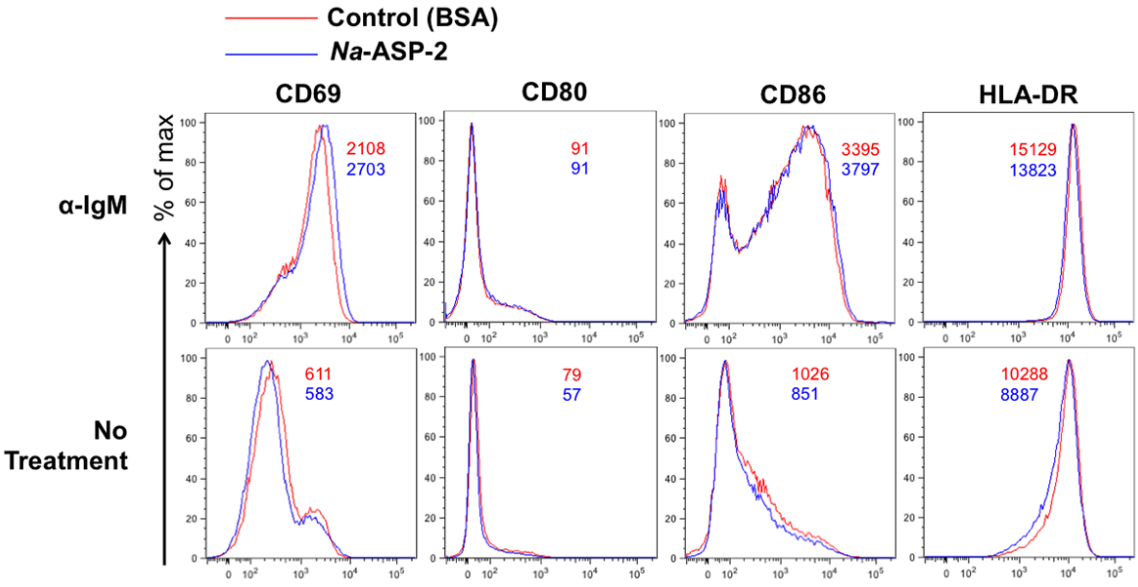

Figure 4.9 Impact of *Na*-ASP-2 on B cell expression of activation surface markers following anti-IgM-stimulation and in the steady-state. PBMCs from hookworm-naïve volunteers were pre-incubated for 4 h with 2 μg/mL of either a control protein (BSA) or *Na*-ASP-2 before incubation for 24 h with 10 μg/mL goat F(ab)2 anti-mouse IgM or media alone (No treatment). Surface marker expression on gated $CD19^+$ $CD20^+$ B cells was compared by flow cytometry. Colour-coded numbers in each inset represents mean fluorescence intensity for each marker. The experiment was performed twice, with a different blood donor each time; representative data are shown.

4.4 Discussion

N. americanus possesses the ability to modulate the human host's immune system to facilitate its migration from the skin to the lungs en route to the intestine [4]. Immuno-epidemiological studies from human populations have shown that protective acquired immunity to hookworm does not develop in most individuals; indeed, the elderly often have the heaviest worm burdens [33]. One of the primary means by which hookworms

modulate the host's immune system is via the production of ES proteins that divert or actively down-regulate different arms of the immune response [93, 192, 206, 207]. The results from this study are consistent with this theory, where it was demonstrated that *Na*-ASP-2 interacts with CD79A on human B lymphocytes, suppressing expression of genes that play key roles in multiple biological pathways, including the B cell receptor (BCR) signaling pathway. While the precise biological function of *Na*-ASP-2/B cell interactions *in vivo* remains unclear, my *ex vivo* data implies that *Na*-ASP-2 engages CD79A and results in suppression of signaling molecules involved in determining the activation state of B cells. If *Na*-ASP-2-exposed B cells become anergic, the generation of protective antibody responses against the parasite may be impaired, thereby promoting parasite survival.

The human BCR consists of a 1:1 stoichiometry of membrane-bound immunoglobulin and CD79A/CD79B. The immunoglobulin domain relies on a non-covalent association with the CD79A/CD79B heterodimer complex for triggering a signaling cascade [208]. Upon binding of antigen to membrane bound immunoglobulin, the receptor complex undergoes conformational changes, resulting in recruitment of LYN, phosphorylation of the relevant CD79 tyrosines, PI3K recruitment to CD19 and adaptor proteins, and generation of the potent lipid secondary messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). How interactions between CD79A and *Na*-ASP-2 result in down-regulated expression of *lyn* and *pi3k* is unclear, but it is possible that *Na*-ASP-2 may induce an anergic-like state in B cells by preventing BCR clustering and activation of the cell upon antigen binding. Anergic B cells are characterized by impaired propagation of activating signals that are triggered by CD79 engagement [209], substantially reduced PIP3 production [210] and have a dramatically reduced lifespan.

Hookworms are known to induce a state of immune hypo-responsiveness in chronic infections [33, 211], and by probing a hookworm protein microarray [96] we found that the most heavily infected individuals generated lower IgG responses than did individuals with low or moderate intensity infections [212]. These findings serve to highlight the altered B cell functions in individuals with heavy hookworm infection and offer a potential causative role for proteins such as *Na*-ASP-2; however this hypothesis requires rigorous testing.

In the absence of experimental three-dimensional structures of *Na*-ASP-2 in complex with host proteins, a rigid body model of the *Na*-ASP-2:CD79A:CD79B hetero-trimer complex was generated by colleagues at Griffith University (Qld), in the Hoffman Lab. This model is in agreement with two previously reported findings. First, the interaction interface between the hookworm protein and the CD79 hetero-dimer has a His/Gln-containing motif with similar properties to those identified by panning a random peptide phage display library with *Na*-ASP-2 [185]. Second, the vast majority of the interaction interface of *Na*-ASP-2 and CD79 is on the α-subunit, coinciding with the interaction interface of CD79 with membrane immunoglobulin [198]. Chapter 3 identified the human SK3 channel as a putative receptor of *Na*-ASP-2 based on the presence of a His/Gln-rich peptide that interacted with *Na*-ASP-2 [185]. Since this His/Gln peptide sequence is only present in one of the three isoforms of the SK3 channel (isoform 1), and the human V5 ProtoArray included isoform 2, no interactions with SK3 were observed in the present study.

The findings herein highlight the novel application of protein microarray technology to addressing host-hookworm interactions at the protein level, and provide a powerful and

rapid screening process that could be applied to the study and identification of interactions between other pathogens and host proteins. By coupling the probing of the human proteome array with *ex vivo* confirmation of binding to live cells and subsequent NGS to determine changes in gene expression, we demonstrated the utility of this pipeline for elucidating novel host-pathogen interactions and assigning functions to that enigmatic group of pathogen molecules that are frustratingly referred to as “proteins of unknown function”.

Chapter 5:

General Discussion:

With hundreds of millions of hookworm sufferers worldwide, there remains an urgent need for the development of a hookworm vaccine to limit the global disease burden, primarily in developing countries. The impact of hookworm disease is more than incidence alone, and includes the more immediate physical symptoms of abdominal pain/cramps/anaemia as well as the insidious impact of chronic anaemia on childhood mental and physical development. These pathophysiological effects have dramatic consequences, particularly in adulthood, such as reduced productivity, increased mortality for the immune suppressed, pregnant, elderly and those lacking in nutrition, and access to medical support.

Na-ASP-2 was both effective and immunogenic as a vaccine in animal models [172], and immunogenic in hookworm-naive human subjects in a phase 1A clinical trial in the US [138]. However, a phase 1b clinical trial showed the limitations of *Na*-ASP-2 as a vaccine in its native form [92], due to its immediate induction of allergic reactions (urticaria) in a subset of the trial subjects due to pre-existing *Na*-ASP-2-specific IgE. A

recombinant form of *Na*-ASP-2 incorporating human immunoglobulin Fc gamma region was engineered, and although it successfully lowered IgE reactivity and immediate type skin sensitivity reactions, it provided 23% reduction in hookworm burden versus controls in a hamster model and remains unproven in human trials [139]. Targeted mutagenesis of other allergens has been shown to effectively reduce IgE binding, with the birch pollen protein Bet v 1, the Der f 2 dust mite allergen and the Ves v 5 wasp venom protein as examples [158, 213, 214]. The implementation of *Na*-ASP-2 as a paediatric vaccine remains plausible. Nonetheless significantly reducing the allergenicity via targeted mutagenesis remains a desirable objective as it would enable the vaccine to be administered to adults.

After producing overlapping peptides spanning the length of the *Na*-ASP-2 protein, the peptides were probed with sera from the phase 1b clinical trial subjects from a hookworm-endemic area of Brazil. Through this methodology, I was able to accurately define the IgE and IgG binding epitopes [92], enabling further studies to target these areas of the protein for reduced IgE reactivity via mutagenesis.

Based on epitope mapping results, four mutations were selected: E137, K140, S149 and N150. The results of the targeted mutagenesis of *Na*-ASP-2 illustrated the potential of minimizing allergy via reduced IgE epitopes of *Na*-ASP-2 subsequent to epitope mapping. IgE binding was successfully lowered to varying degrees following the production of the 4 mutant *Na*-ASP-2 proteins, with minimal effects on the capacity to bind to IgG. Results showed that E140 was the best performing of the mutants in terms of maintaining IgG and reducing IgE reactivity by approximately 40%. The E140

mutant however expressed at lower yields than the other proteins. Moreover, mutating single residues had a noticeable difference in protein yield and stability, despite using identical expression and purification methods.

Further mutations combined into single construct are likely to significantly lower IgE binding via epitope disruption and may still allow *Na*-ASP-2 to be used as a vaccine in recombinant form. These studies are currently underway in my laboratory, where I have arranged for commercial production of a multiple mutant *Na*-ASP-2 incorporating all 4 of the mutations discussed above. Testing will be conducted once the protein has been produced and purified, with a focus on improved reduction in IgE reactivity and allergenicity with human subject sera, and then tested for efficacy in animal models of hookworm infection. In addition, recombinant *Na*-ASP-2 single point mutants may not be sufficient to completely disrupt the antibody-epitope interactions as shown in Chapter 3. Considering the incomplete reduction in IgE binding, additional mutations are likely required to disrupt binding. Indeed, this was the case with the Der f 2 dust mite allergen and the Ves v 5 wasp venom allergen [158, 213].

A major limiting factor in my experimental approach was the focus on linear epitopes as opposed to conformational epitopes. This is a major drawback of the overlapping peptide approach to epitope mapping, but not one that is easily dealt with in a high-throughput fashion [215]. Identifying conformational epitopes requires distinct labor-intensive approaches and was deemed beyond the scope of this project. Large banks of sera from human subjects in hookworm endemic areas are available [3, 6], so future mutants should be screened with as many sera as possible to obtain an accurate picture of even low level IgE reactivity in large populations. Information obtained from this

epitope mapping program might also result in epitopes that could form the basis of protective and safe peptide vaccines (typically in the form of multi-epitope constructs) that induce IgG but not IgE responses in exposed individuals. Chimeric peptide vaccines have been explored with mixed success for other helminth parasite vaccines, notably schistosomiasis [216].

There is also the possibility of co-administering a *Na*-ASP-2 derived mutant or peptide vaccine with omalizumab, an anti-IgE monoclonal antibody used in lowering serum levels of IgE and FcepsilonRI expression in both atopic and non-atopic asthma sufferers. Omalizumab is approved for use in humans [217], and while it may prove efficacious at driving selective IgG but not IgE responses, the cost of producing such a vaccine might well prove inhibitory for the development of NTD vaccines that need to be affordable given their target populations [218].

This study has several limitations; the bank of sera used to screen the IgE mutant peptides was very small. Sera from the phase Ib clinical trial was selected because it contained valuable information on anti-ASP-2 titres before and after a single immunization with *Na*-ASP-2 from subjects who responded safely as well as a small number who had adverse allergic responses to vaccination. Ideally, a follow-up experiment would have subjected the mutant proteins to immunoscreening with a larger bank of sera from people resident in hookworm-endemic areas, thereby providing robust IgE reactivity data. Pending a successful outcome showing lowered IgE reactivity, vaccine trials to assess efficacy of the mutant proteins in animal models would have proved informative prior to considering phase I clinical trials.

Group 2 ASPs, including *Na*-ASP-2, possess a putative peptide-binding groove in the centre of the molecule as revealed by crystal structures [219] prompting the assumption that ASP-2 interacts with either a human host protein or another hookworm protein/peptide. In pursuit of a potential ligand or interaction partner, I utilized phage display technology in the form of a recombinant 12-mer random peptide library expressed on the surface of the viral coat protein of the phage virion. The results of several rounds of panning yielded a consensus sequence of HXQQH from the sequenced, bound peptides. This sequence was then input into the BLAST online database algorithm and one of the primary hits was the SK3 or SKCa protein, a small conductance transmembrane, calcium activated potassium channel present on the surface of a variety of human cells that controls various physiological functions. Binding interaction studies were confirmed using differential scanning fluorimetry [185]. However with no further evidence of the interaction *in vivo* or any obvious biological relevance of such an interaction, a different technique to address protein-protein interactions was employed - protein array technology. Protein arrays have been constructed from mammalian species (hosts) and a number of distinct pathogens from viruses [220] and bacteria [221] to unicellular [222] and multicellular [96, 212] parasites. Typically arrays are probed with sera to assess antibody binding to pathogen or self proteins. However, a very powerful approach that had never been reported in the literature until now is the probing of a human proteome array with a pathogen protein to identify host-parasite molecular interactions.

Using such an approach, a reproducible protein-protein interaction was observed between *Na*-ASP-2 and CD79a, a cell surface/spanning protein involved in the B cell receptor complex and integral to the B cell receptor signaling pathway [223]. These

interactions were confirmed using human B cells *ex vivo*, and a detailed analysis of the downstream effects of this interaction were undertaken using next generation RNA sequencing. Critically, *Na*-ASP-2 had a predominately suppressive effect on B cell intrinsic gene expression, affecting a wide range of genes including those from the Cell Adhesion Molecules (CAMs), Leukocyte Transendothelial Migration and B cell signaling pathways. Importantly, *Na*-ASP-2 also reduced transcription of genes involved in the B cell receptor signaling pathway, potentially highlighting the biological importance of the interaction between CD79A and *Na*-ASP-2. Of particular interest were three genes: *lyn*, spleen tyrosine kinase (*syk*) and phosphatidylinositol 3-kinase (*pi3k*) as these displayed significant down-regulation following exposure to the *Na*-ASP-2 protein. These genes are integral to the B cell receptor signaling pathway and are responsible for transmitting signals responsible for a variety of cell fates such as proliferation, apoptosis, activation and anergy. In follow-on studies, I examined whether *Na*-ASP-2 induced B cell anergy (reduced activation marker expression) by employing commonly-used polyclonal cell stimulation procedures. However, under each of these conditions I did not observe a defect in the ability of B cells to be stimulated in the presence of *Na*-ASP-2, although I did detect a reproducible reduction in expression of traditional activation markers such as CD69, CD86, HLA-DR and CD80. Further studies are required to analyze levels of antibody and cytokine production by ASP-2-treated B cells. Moreover, given that ASP-2 has been shown by others to inhibit neutrophil recruitment [155], more extensive analyses of the cell types bound by ASP-2 are required, to determine the extent of its apparently pleiotropic functions.

Future studies might also address silencing of the *Na-asp-2* gene using RNA interference (RNAi) or gene knockout techniques such as CRISPR [224]. To date gene silencing and knockout techniques have yet to be successfully described for use with hookworms, but progress is being made with other g.i. nematodes [225]. If *Na-asp-2* could be silenced or knocked out the function of the protein could be further assessed in animal models and experimental human challenge studies [226]).

Previous studies have demonstrated that *Na*-ASP2 has different biological effects, depending on the experimental model employed. *Na*-ASP-2 has been shown to provoke neutrophil recruitment [155], and also induce Type 2 cytokine responses [227]. *Na*-ASP-2 is secreted by larval hookworms and may potentially function to suppress inflammatory responses and facilitate migration through tissues. This immunomodulation might take multiple forms, including the possibility of suppression of B cell activation, as well as recruitment of neutrophils via an unknown mechanism. While neutrophils are essential effector cells in acute infections, they also perform a less well-appreciated role in the suppression of T and B cell responses by competing for antigen with professional antigen presenting cells [228]. Moreover, neutrophils imprint on the quality of the interactions between dendritic cells and T cells despite a lack of direct contact between them [229]. Therefore, *Na*-ASP-2 might serve to suppress inflammatory responses via two distinct but convergent pathways aimed at reducing antibody (and other effectors) production to what is otherwise a large and antigenic/allergenic pathogen. Suppression of antibody production in the early phase of initial infection would facilitate migration of larvae from entry via the dermis to the lungs. As hookworm larvae mature they no longer express *Na*-ASP-2, instead relying on the secretion of other ES products (including distinct ASPs) [94]. Once established

in the gut, adult hookworms induce a regulatory response that promotes their long-term existence in the gut [207], and other adult-specific hookworm proteins have been associated with immunoregulation during this latter development phase of the parasite [230]. Any subsequent IgE resulting from class switching would only impact new larvae upon further hookworm exposure, and the patent infection of hookworms would be unaffected.

To my knowledge this is the first report of a pathogen protein that interacts with the BCR, or modulates expression of signaling molecules that determine the fate of B cells, namely Lyn and PI3K. Given the current interest in hookworm secreted proteins for treating autoimmune and allergic diseases [93], it is now apparent that *Na*-ASP-2 warrants testing as a novel biologic for treating B cell-mediated autoimmunity. Administration of anti-CD79 monoclonal antibody induced a transient, anergic-like state capable of preventing collagen-induced arthritis [231]. Anti-CD79B monoclonal antibodies have also been shown to ameliorate lupus in mice [232], and offers the potential for reduced side effects whilst maintaining B cells in a transient anergic state that does not require their specific depletion. Ultimately *Na*-ASP-2 could prove beneficial as a treatment for autoimmune B cell-mediated diseases such as multiple sclerosis or rheumatoid arthritis, or perhaps has potential as an immune suppressant for transplant patients. There has recently been an increasing interest in the potential for intestinal worms and worm-derived proteins to assist in treatment for inflammatory disorders caused by immune dysregulation [93, 192, 233, 234]. Along with several other hookworm proteins of interest that are in various stages of investigation [230], *Na*-ASP-2 can now also be considered for potential therapeutic immunomodulation. There are many potential uses of a B cell, anergy inducing protein for a variety of

common B cell mediated immune dysregulatory diseases, e.g. grave's disease, lupus, even arthritis, along with potential for transplant patient's immune-suppression. The potential increases more so when you consider the possibility of using certain hookworm proteins in tandem or concert with others of known function to improve or amplify the immunomodulatory effects of reducing inflammation in a therapeutic setting.

In this thesis I have generated important information on the allergenicity and structure-function of *Na*-ASP-2, a group 2 ASP. This now paves the way to explore the myriad other ASPs secreted by hookworms, and increase our understanding of why this gene family has undergone unprecedented expansion in the hookworm genome. Moreover, their developmental regulation throughout the hookworm's life cycle suggests critical roles in host-parasite interactions, with a particular emphasis on immunoregulation. Ultimately this knowledge will help researchers to make informed choices about vaccine antigen selection, as well as shed light on potential new avenues of treating inflammatory diseases.

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Appendix

Pepset sequences. Biotinylated peptides were 13 residues long, incorporated an SGSG spacer with an overlap of 2 residues and were plated onto streptavidin-coated 96 well plates. Numbers 1-90 correspond to the position of each peptide within the plate. See below.

- 1: SGSGGCPDNGMSEEARQ
- 2: SGSGPDNGMSEEARQKF
- 3: SGSGNGMSEEARQKFLE
- 4: SGSGMSEEARQKFLELH
- 5: SGSGEEARQKFLELHNS
- 6: SGSGARQKFLELHNSLR
- 7: SGSGQKFLELHNSLRSS
- 8: SGSGFLELHNSLRSSVA
- 9: SGSGELHNSLRSSVALG
- 10: SGSGHNSLRSSVALGQA
- 11: SGSGSLRSSVALGQAKD
- 12: SGSGRSSVALGQAKDGA
- 13: SGSGSVALGQAKDGAGG
- 14: SGSGALGQAKDGAGGNA
- 15: SGSGGQAKDGAGGNAPK
- 16: SGSGAKDGAGGNAPKAA
- 17: SGSGDGAGGNAPKAAKM
- 18: SGSGAGGNAPKAAKMKT
- 19: SGSGGNAPKAAKMKTMA
- 20: SGSGAPKAAKMKTMAYD
- 21: SGSGKAAKMKTMAYDCE
- 22: SGSGAKMKTMAYDCEVE
- 23: SGSGMKTMAYDCEVEKT
- 24: SGSGTMAYDCEVEKTAM
- 25: SGSGAYDCEVEKTAMNN
- 26: SGSGDCEVEKTAMNNAK
- 27: SGSGEVEKTAMNNAKQC
- 28: SGSGEKTAMNNAKQCVF
- 29: SGSGTAMNNAKQCVFKH
- 30: SGSGMNNAKQCVFKHSQ
- 31: SGSGNAKQCVFKHSQPN
- 32: SGSGKQCVFKHSQPNQR
- 33: SGSGCVFKHSQPNQRKG
- 34: SGSGFKHSQPNQRKGLG
- 35: SGSGHSQPNQRKGLGEN
- 36: SGSGQPNQRKGLGENIF
- 37: SGSGNQRKGLGENIFMS
- 38: SGSGRKGLGENIFMSSD
- 39: SGSGGLGENIFMSSDSG
- 40: SGSGGENIFMSSDSGMD
- 41: SGSGNIFMSSDSGMDKA
- 42: SGSGFMSSDSGMDKAKA
- 43: SGSGSSDSGMDKAKAAE
- 44: SGSGDSGMDKAKAAEQA
- 45: SGSGGMDKAKAAEQASK
- 46: SGSGDKAKAAEQASKAW
- 47: SGSGAKAAEQASKAWFG
- 48: SGSGAAEQASKAWFGEL
- 49: SGSGEQASKAWFGELAE
- 50: SGSGASKAWFGELAEKG
- 51: SGSGKAWFGELAEKGVG
- 52: SGSGWFGELAEKGVGQN
- 53: SGSGGELAEKGVGQNLK
- 54: SGSGLAEKGVGQNLKLT
- 55: SGSGEKGVGQNLKLTGG
- 56: SGSGGVGQNLKLTGGLF
- 57: SGSGGQNLKLTGGLFSR
- 58: SGSGNLKLTGGLFSRGV
- 59: SGSGKLTGGLFSRGVGH
- 60: SGSGTGGLFSRGVGHYT
- 61: SGSGGLFSRGVGHYTQM
- 62: SGSGFSRGVGHYTQMVW
- 63: SGSGRGVGHYTQMVWQE
- 64: SGSGVGHYTQMVWQETV
- 65: SGSGHYTQMVWQETVKL
- 66: SGSGTQMVWQETVKLGC
- 67: SGSGMVWQETVKLGCYV
- 68: SGSGWQETVKLGCYVEA
- 69: SGSGETVKLGCYVEACS
- 70: SGSGVKLGCYVEACSNM
- 71: SGSGLGCYVEACSNMCY
- 72: SGSGCYVEACSNMCYVV
- 73: SGSGVEACSNMCYVVCQ
- 74: SGSGACSNMCYVVCQYG
- 75: SGSGSNMCYVVCQYGPA
- 76: SGSGMCYVVCQYGPAGN
- 77: SGSGYVVCQYGPAGNMM
- 78: SGSGVCQYGPAGNMMGK
- 79: SGSGQYGPAGNMMGKDI
- 80: SGSGGPAGNMMGKDIYE
- 81: SGSGAGNMMGKDIYEKG
- 82: SGSGNMMGKDIYEKGEP
- 83: SGSGMGKDIYEKGEPCS
- 84: SGSGKDIYEKGEPCSKC
- 85: SGSGIYEKGEPCSKCEN
- 86: SGSGEKGEPCSKCENCD
- 87: SGSGGEPCSKCENCDKE
- 88: SGSGPCSKCENCDKEKG
- 89: SGSGSKCENCDKEKGLC
- 90: SGSGCENCDKEKGLCSA
- RACK LAYOUT FOR SYNTHESIS: **18899 : Rack A**
- DESCRIPTION: 90 peptides based on sequences provided.
- COMMENTS: Peptides despatched dried down.