Chapter 1

Introduction

1.1 Parasitic helminth infection

1.1.1 Characteristics of parasitic helminth

Parasitic helminth is a general term for parasitic worms that are divided into three main groups: tapeworms (Cestode), flukes (Trematode) and round worms (Nematode) (Baron, 1996). They can invade almost every niche, from intestinal lumen to intravascular and even intracellular spaces, within their mammalian hosts. Parasitic helminths are some of the commonest pathogens in the world. Human beings or livestock can have more than one type of parasitic helminth infection coexisting within the body. Parasitic worms are the pathogens with the most complex body structures. They have a well developed organ system, especially digestive system and reproductive organs (Baron, 1996).

Some of them, such as soil-transmitted helminths, have simple and direct life cycles, in which the larvae transfect the mammalian host without developing in an intermediate host. Others have complex life cycles which require more than one intermediate host. All of the parasitic helminths rely on the host to complete their development and reproductive life cycle; therefore they need to keep the host alive for their own benefit. Instead of simply taking nutrition from the host, parasitic helminths induce complex immune responses during the whole process of parasite-host interaction (MacDonald et al., 2002).

1.1.2 Parasitic helminths of medical importance

Parasitic helminth infection is still a worldwide problem. The overall prevalence is affected by climate, hygiene standards, exposure to vectors and availability of

efficacious drugs. It has been eradicated as a public health problem in most developed countries. However, due to the lack of insect vector control, unsafe disposal of human excrement and insufficient supply of medicines, parasitic helminth diseases still cause an enormous health burden in tropical developing countries (Awasthi et al., 2003). New problems such as the HIV epidemic also affect the course and control of some diseases (Karp and Neva, 1999). The population most infected are usually the poorest. They are the least favourite of the pharmaceutical industry and there is very limited funding for research and drug development (Murray et al., 2000).

More than a quarter of the world's population carry soil-transmitted intestinal helminths (such as *Ascaris lumbricoides*, the hookworms and *Trichuris trichiura*) and schistosomes (Awasthi et al., 2003). Disability-adjusted life-years (DALYs) lost for these three major intesitinal nematodes are estimated to be 39.0 million years (Stephenson et al., 2000). The other widespread helminthic infections include filariasis, trematodes and tapeworm infections. The main effects of these nematodes include malnutrition, retarded growth and anaemia (Stephenson et al., 2000).

Three-hundred million of the total infected population are estimated to have severe and life-threatening infections (Colley et al., 2001), with others only suffering from minor symptoms. It is more common for the helminth-host interplay to be harmonious and asymptomatic in the long term. They can negotiate host anatomy and tolerate complex host immune responses in terms of host protection and helminth survival (Maizels and Yazdanbakhsh, 2003). This tolerance between

parasites and human beings can be seen as a result of co-evolution (Cox, 2002). The same situation may happen in animal hosts as they have also been infected by parasitic helminths during evolution and migration.

1.1.3 "Seeking benefits from the worms"

As opposed to the developing tropical countries where helminth infections are prevalent, developed areas are thought to encounter health problems due to the "lack" of worm infection. There is a general inverse association between parasitic helminth infection and allergy, autoimmune diseases and some bacterial and viral infectious diseases. For example, areas with a high prevalence of parasitic helminth infection have a lower occurrence of allergy and autoimmune diseases than developed countries with higher hygiene standards (Wilson and Maizels, 2004). The figure below (Figure 1.1) is a world map showing the geographic epidemics of Type 1 diabetes and neglected infectious diseases (filariasis, leprosy, onchocerciasis, schistosomiasis, soil-transmitted helminths, and trachoma). These observations have led to the so-called "hygiene hypothesis" in which the increased cleanliness and westernized lifestyle that aims to wipe out helminth infections is associated with new problems such as allergy and autoimmune diseases (Strachan, 1989; Yazdanbakhsh et al., 2002).

It has been indicated that exposure early in life to parasitic helminths may protect the host in future from asthma and certain autoimmune diseases (Cooke et al., 2004). In brief, this exposure affects host immune responses resulting in the upregulation of regulatory cytokines that would be protective against both T helper type 1 (Th1) and T helper type 2 (Th2) inflammatory processes in

hypersensitivities, autoimmune diseases and infections (Wilson and Maizels, 2006). The possibility of controlling such diseases using parasitic helminths has attracted a considerable amount of research in immunology and clinical research. Experimental and animal models have provided evidence that inflammation can be regulated by parasitic helminth products. The preliminary clinical success of using whipworms for the treatment of inflammatory bowel disease (IBD) (Summers et al., 2003) has brought the future of such treatments to a new era.

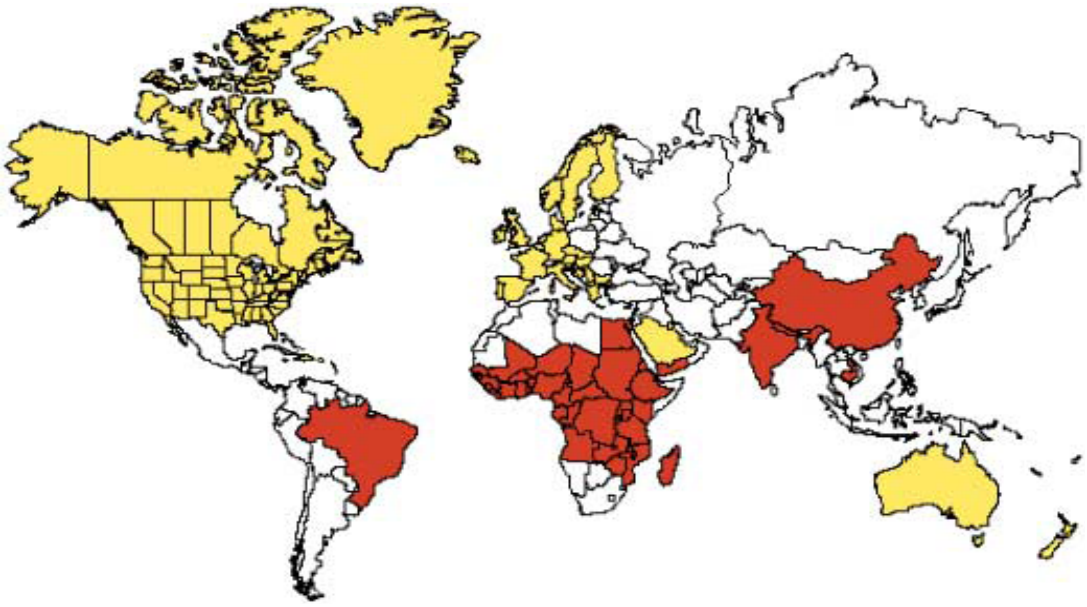


Figure 1.1 Geographic inverse relationship between Type 1 Diabetes (T1D) and 'neglected infectious diseases'. Red delineates areas which harbour six or more of the low mortality neglected diseases. Yellow delineates areas where there are relatively high incidences of T1D (> 8 per 100 000/year). Non coloured areas delineate where T1D < 8 per 100 000/year and where the 'neglected diseases' are not endemic (Adapted from Zaccone et al., 2006).

1.1.4 *Nippostrongylus brasiliensis*

Nippostrongylus brasiliensis is a rodent intestinal nematode. The main natural hosts are wild rats *Rattus norvegicus, Ratus rattus*, and the mouse, *Mus musculari*s (Haley, 1961). The definitive host is the rat and the rat strain of *N.brasiliensis* develops in mice and hamsters but not so successfully as in rats. About 14% of the L3 larvae reach maturity in mice and their adaptation in mice may alter their responses to the host immunity (Solomon and Haley, 1968). Infections in rats are therefore better than mouse models for studying immunity to *N.brasiliensis*. *N.brasiliensis* belongs to the superfamily *Strongyloidea* - a large group containing several important human parasites, such as hookworms. The host-parasite interaction in *N.brasiliensis* infection resembles that of nematodes of medical and veterinary importance. The life cycle of *N.brasiliensis* is simple and the strain currently used experimentally has been maintained in laboratory rats for at least 40 years. Rats are infected with large numbers of L3 larvae and faecal cultures that contain eggs are collected 6-9 days after infection for the production of L3 larvae (Ogilvie and Jones, 1971).

1.1.5 *N.brasiliensis* life cycle and lung stage

The life cycle of *N.brasiliensis* was first described by Haley (1962a) (Figure 1.2). After penetrating the skin, the third stage larvae (L3) migrate to the lungs via the bloodstream, making their way to the air sacs and trachea. Once there, the larvae moult to the fourth stage (L4). They then leave the lungs via the oesophagus and stomach, emerging in the small intestine. The larvae grow quickly in the intestine and then moult to the adult stage (L5). The adults pass eggs into the rat's faeces and after about one week most of the worms are expelled. The eggs (containing

L1) hatch to L2 and L3 stages outside the host and wait for a chance to infect the host again.

Before infesting the intestine from the third day, the L3 larvae pass through the lungs, where they are first found at about 15 hours after infection. The lymphatic channel may be another migratory route and some larvae are found in the peripheral lymph glands after infection (Haley, 1962). The pulmonary phase or lung stage is important for the process of maturity. The reason for this is unclear. Between 40% and 60% of injected larvae do not reach the lungs (Jarrett et al., 1968) and about 55% of the injected larvae reach maturity (Haley, 1962). The worm is normally found close to the mucosa, where oxygen tension is high (Rogers, 1949) and the parasites die quickly in the absence of oxygen (Roberts and Fairbairn, 1965). This suggests that the high oxygen tension in the lungs may attract the larvae during their long evolutionary development. During pulmonary stage, *N.brasiliensis* L3 larvae first encounter extensively with the host immune system. Our interest in the immune regulatory properties of *N.brasiliensis* lung stage larvae is evoked by the limited inflammation in the rat lungs, which is reviewed later.

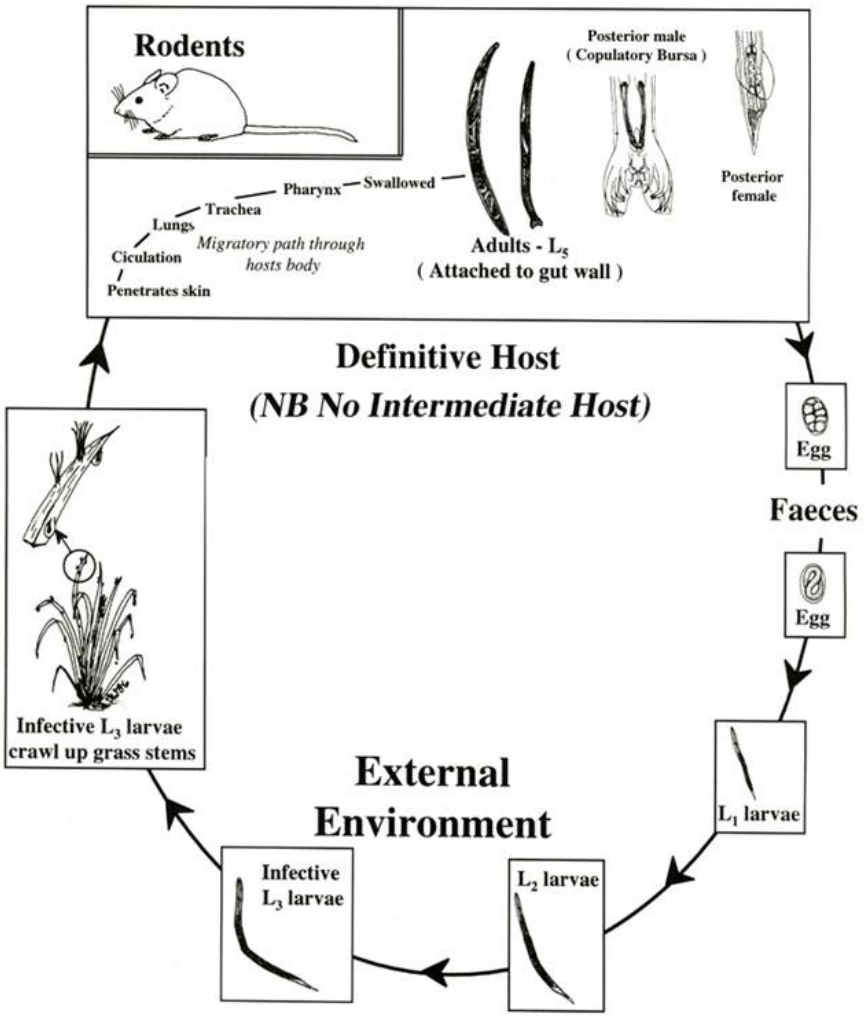


Figure 1.2 The life cycle of *Nippostrongylus brasiliensis* (University of Cambridge Teaching Server, 2008).

1.2 Immunology of parasitic helminth infection

One of the characteristics of many parasitic helminths is that the worms move extensively within the host. Immune responses initiate when infective larvae get into the host and continue to progress during the migration, moult and reproduction processes. Damage to the host tissues can be caused directly by their movement or metabolism. During our short history on earth, humans have acquired about 300 species of parasitic helminths among which about 90 species are still commonly found around the world (Cox, 2002). The application of animal models in helminth research sometimes means the adaptation of the immune responses.

Despite the differences across the species, immune responses to parasitic helminth infections are remarkably similar. These are usually in favour of host survival via the induction of Th2 cytokines including interleukin (IL)-4, IL-5 and IL-13, and T-regulatory (Treg) cytokines such as IL-10 and transforming growth factor (TGF)-β. Cellular responses are characterized by eosinophilia and mastocytosis. Immunoglobulin E (IgE) is usually dominant in most of helminth infections (MacDonald et al., 2002). Th2 and Treg cytokines have been demonstrated to be protective in several helminth infections. Th1 immune responses, on the other hand, have often been linked to susceptibility to parasitic helminths and responsible for the mortality during acute infections (Herbert et al., 2004). The outcome of infections depends on the individual balance of T cell subsets and antibody responses, which has been reviewed by Maizels and Yazdanbakhsh (2003). A detailed review of the immune responses to each species of infective

helminth is beyond the scope of this chapter, so the following information is mostly focused on *N.brasiliensis* induced immune responses.

1.2.1 Immune responses to invasive larvae

Skin penetration is one of the most common routes for helminth invasion. The animal model of *N.brasiliensis* infection initiates by subcutaneous inoculation of L3 under the skin. This route is similar to human hookworms, including *Ancylostoma duodenale* and *Necator americanus* (Loukas and Prociv, 2001). On penetrating, the infective larvae usually slough their outer cuticular sheath to release enzymes that facilitate the piercing process. Significant numbers of perished larvae could also release an extensive range of immune active molecules at this stage (Loukas and Prociv, 2001). There was a significant influx of eosinophils into the mouse skin air pouches 30 minutes after the primary infection of *N.brasiliensis*. This was associated with a significant increase of eosinophil peroxidase (EPO) activity which indicates eosinophil degranulation (Giacomin et al., 2008). In IL-5 transgenic mice, the eosinophil numbers in these air pouches were approximately 10-fold greater than in those of the wild type mice (Giacomin et al., 2008). Eosinophils mediate the killing of parasitic nematodes by the cell release of the toxic granule proteins including oxidative metabolic molecules (Bass and Szejda, 1979; Hamann et al., 1990). The trapping and killing of helminth larvae by eosinophils in the tissue has been found to be IL-5 dependent (Rainbird et al., 1998). IL-5 transgenic mice with lifelong eosinophilia have shown enhanced resistance to primary infection of *N.brasiliensis*, indicated by the recovery of fewer and less developed intestinal adult worms post infection (Dent et al., 1999). Most *N.brasiliensis* larvae failed to reach the lungs in IL-5 transgenic mice, 75-95% larvae injected into subcutaneous

air pouches for primary infection were trapped there for at least 24 hours, while less than 20% larvae were recovered from the skin 2 hours after infection (Daly et al., 1999). Complement fragments in serum have been demonstrated to be involved in eosinophil-mediated damage to *N.brasiliensis* L3 larvae (Giacomin et al., 2004; Shin et al., 2001). In a primary infection of *N.brasiliensis* in mice, L3 larvae had significantly higher complement C3 deposition and eosinophil-rich leukocyte binding than that of the lung stage larvae and adult worms, which suggested that *N.brasiliensis* acquired the ability to largely avoid complement-mediated eosinophil killing while developing within the host (Giacomin et al., 2005). Eosinophil-mediated larvae clearance was demonstrated to be complement-independent in the secondary infection of *N.brasiliensis* (Giacomin et al., 2008). To fight against the host reactions, tissue-invading helminths have been found to cause apoptosis of eosinophils (Min et al., 2004). The protective role of eosinophils has also been found in *Haemonchus contortus* (Rainbird et al., 1998), *Brugia pahangi*, *Brugia malayi* (Hamann et al., 1990) and *Trichinella spiralis* (Bass and Szejda, 1979). Mononuclear cells and neutrophils are more effective in killing newborn *T.spiralis* larvae (Ruitenberg et al., 1983), which suggest the effective cells are stage and species specific.

1.2.2 Immune responses to migrating larvae

The clearance of parasitic helminths by innate immunity at the site of the invasion in wild type hosts is usually very low. The majority of larvae end up migrating through the tissues of their mammalian host. The explanation for the migrating behaviour is not yet fully developed. It may resemble the process of natural

selection within the host, such that the worms survive migration show better development (Read and Skorping, 1995).

N.brasiliensis larval numbers peak in the lungs 48 hours after infection (Haley, 1961). In preparation for the existence in the intestine, the larvae in the bronchoalveolar space moult to the L4 stage and release more antigenic molecules that might have immune-regulatory properties (Haley, 1962). The significance of the 'lung phase' in host-parasite interactions has been reviewed by Bruschi (1992). Transition through the lungs enables completion of the life cycle such as in *N.brasiliensis* and *Toxocara* infections, or benefits the parasites by making the helminth more resistant to host defence systems, for example in *Schistosoma* infections (Bruschi, 1992).

Tissue damage in the host during parasite migration can be caused by the mixed effect of physical size of the helminths and local inflammation. Photographs of primary infected lungs showed haemorrhagic lungs 48 hours after *N.brasiliensis* infection, but the damage was soon self repaired 7 days post infection when most larvae have left the lungs (Keir, PhD thesis, Figure 1.3). Primary infection of *N.brasiliensis* causes an absolute increase in alveolar macrophages, eosinophils, neutrophils and lymphocytes (Egwang et al., 1984; Ramaswamy and Befus, 1989, Ramaswamy and Befus, 1993). However the infiltration of leukocytes has been described as "limited" in number (Finkelman et al., 1997). In the primary infection of *N.brasiliensis*, recruited neutrophil numbers are significant but small (Watkins et al., 1996, McNeil et al., 2002). The recruited immune cells are dominated by eosinophils and macrophages, which are linked to their capability to kill the

migrating larvae by disrupting the cuticular surface of some larvae (Ramaswamy and Befus, 1989; (Shin et al., 2001). Primary infection of *N.brasiliensis* is associated with an increase of the concentrations of total protein, albumin and immunoglobulin A (IgA), G (IgG) and M (IgM) in bronchoalveolar lavage (BAL) fluids of infected rats (Ramaswamy and Befus, 1989). This may also be caused by the damage to epithelial cells and microvascular (Ramaswamy and Befus, 1989). Secondary infection with *N.brasiliensis* induced a more significant infiltraction of leukocytes and formation of granulomatous lesions in the lungs (Ramaswamy et al., 1991). Mast cells also increased in numbers in granulomatous lesions (Matsuda et al., 2001). After primary and secondary infection of *N.brasiliensis*, eosinophil percentage in the BAL and the sizes of granulomatous lesions are both smaller in mast cell deficient mice than the wild type mice (Arizono et al., 1996). Continuous, low-level exposures to *N.brasiliensis* revealed a marked increase of macrophages and eosinophils by BAL (Ferens et al., 1994).

There is no TNF-α production in the lungs during a primary infection with *N.brasiliensis* (Benbernou et al., 1992, McNeil et al., 2002). IL-1 and IL-6 are induced at different times in primary infection but the levels are normal or ceased most of the time (Benbernou et al., 1992). The developed granulomatous lesions in the lungs in secondary infection are dominated by Th2 cytokine gene expression, including IL-4, IL-5 and IL-13 (Matsuda et al., 2001). The local oxidative stress level is low with an increase of anti-oxidant mechanisms (McNeil et al., 2002). The limited induction of Th1 inflammation to the lung stage *N.brasiliensis* larvae led us to believe that anti-inflammatory mechanisms may be

activated, and this developed into the main research question to be answered in this study.

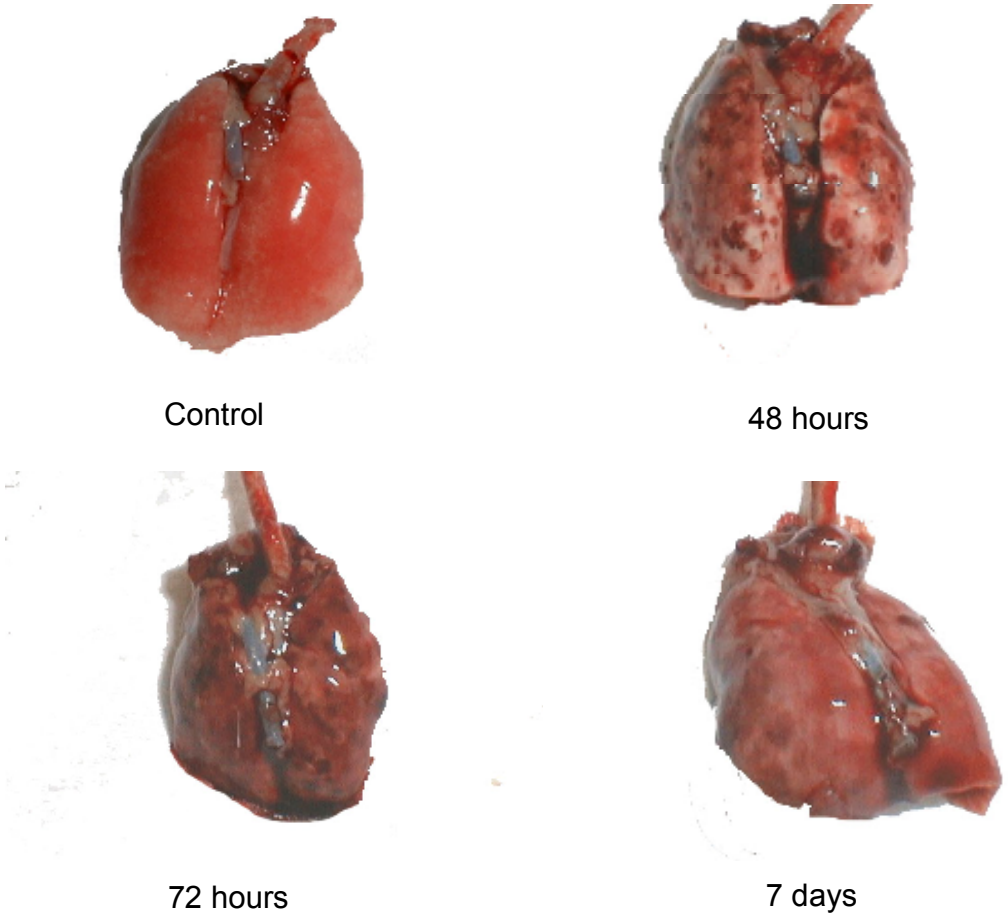


Figure 1.3 Photographs of lungs removed from rats following primary infection with 2000 L3 larvae of *N.brasiliensis*. (Adapted from Keir, PhD thesis)

Early immune response to *Trichinella spiralis* (Dzik et al., 2002)*, Ascaris suum* (Lewis et al., 2007), *Brugia pahangi* (Porthouse et al., 2006) and *Strongyloides stercoralis* (Rotman et al., 1996) migrating larvae are all characterized by the acute infiltration of inflammatory cells, including macrophages, neutrophils, eosinophils and lymphocytes. Establishment of a Th2 cytokine response

(Porthouse et al., 2006) and transient increase of oxide anions were also detected (Dzik et al., 2002).

1.2.3 Immune responses to adult worms

Immune responses to adult worms in the intestine are predominantly characterized by expansion of Th2 cells that produce cytokines such as IL-4, IL-5 and IL-13 (MacDonald et al., 2002). *N.brasiliensis* has been demonstrated to be a potent adjuvant that stimulates antigen-specific naïve T cells to differentiate to Th2 cells (Liu et al., 2002). Mouse mesenteric lymph node cells after primary infection with *N.brasiliensis* L3 larvae produce IL-4 and IL-5, which peak at day 7-9 after infection, shortly before the expulsion of adult worms from the intestine (Lawrence et al., 1996). T cells from IL-4 deficient mice infected with *N.brasiliensis* are unable to mount Th2 responses (Kopf et al., 1993). However, IL-4 deficient mice are still able to expel all the adult *N.brasiliensis* by day 10 post the primary infection, which is similar to the wild-type mice (Lawrence et al., 1996). IL-13 deficient mice develop a robust Th2 response but fail to expel *N.brasiliensis* adults efficiently, which highlights the importance of IL-13-mediated protection against *N.brasiliensis* (McKenzie et al., 1998).

Intestinal IgA response can be induced by intragastric administration of *N.brasiliensis* adults, but there is no correlation between the IgA level and degree of protection against a secondary infection with L3 larvae (Wedrychowicz et al., 1984). In rats primarily infected with *N.brasiliensis* L3 larvae, the major sites of IgE synthesis in the small intestine are the regional lymph nodes (Mayrhofer, 1977). Mast cell hyperplasia are frequently observed to occur at the onset of worm

expulsion and peak shortly after the expulsion complete (Woodbury et al., 1984). In mast cell deficient mice, the onset of worm expulsion happens 24 hours later than normal mice (Mitchell et al., 1983). In response to IgE, mucosal mast cells release a number of soluble mediators including prostaglandin E_2 (PGE_2) and histamine, which increase epithelial cell secretion (Schwartz and Austen, 1984). Th2 cytokines, IL-4 and IL-13, induce STAT6-dependent intestinal epithelial cell permeability and secretion in mice infected with *N.brasiliensis* (Madden et al., 2004). During the worm expulsion, goblet cell numbers increase two or four times when the expulsion happens (Levy and Frondoza, 1983) . Concluded from the above, Th2 cytokines, especially IL-4 and IL-13, promote *N.brasiliensis* expulsion by inducing an inhospitable environment for the adult worms which include increased mucus secretion, increased gut contraction and reduced sodium-linked glucose absorption (Levy and Frondoza, 1983);Shea-Donohue et al., 2001; Scales et al., 2007; Finkelman et al., 1997).

1.3 Parasitic helminth derived products

In parasitic helminth infections, immune responses are raised towards surface molecules, ES products and somatic components. These molecules play an important role in parasite development, invasion and immune regulation. Somatic components are only exposed to the immune system after the worms have died and therefore have less importance in host-parasite immune interactions, but may contribute to the chronic pathological processes. A better knowledge of the antigenic molecules and their functions is important for investigating the control of nematode infections as well as utilizing their immune regulatory properties.

1.3.1 Surface components of parasitic helminths

The outermost surface of a nematode is the external cuticle, which covers the parasite and protects it from environmental damage. The detailed structure of the cuticle has been discovered to include several layers. The outmost layer of the cuticle is a glycoprotein-rich surface coat, underneath which is a lipid-rich epicuticle (Maizels et al., 1993). *N.brasiliensis* L3 larvae epicuticles have been demonstrated to exhibit rapid changes in lipophilicity shortly after the larvae were exposed to tissue culture conditions which mimic the mammalian tissue environment (RPMI-1640 medium at 37ºC) (Proudfoot et al., 1990; Proudfoot et al., 1993). Some inner layers of nematodes have structures such as globular particles and collagen fibers which suggest a secretory function or nutrient absorbance as well as movement (de Moraes Neto et al., 2002; Cox et al., 1981). The inner layer of the cuticle and the oesophagus of the worms have been found to contain serine proteases which may facilitate the moulting and /or digestive functions (Trap et al.,

2006). The cuticle contains a range of antigenic molecules that have been demonstrated to change qualitatively during the moulting process (Philipp et al., 1980; Bruschi et al., 1992; Sakwe and Titanji, 1997). Common and stage-specific surface antigens have been identified by using antisera in combination with various separating and labelling techniques (Kasuga-Aoki et al., 2000). An ELISA method has been developed to detect specific IgG binding to the surface antigens of adult *N.brasiliensis* (Schroeder, 1985). Modification of the cuticle components may increase the antigenic capacity of helminths or their resistance to host defence mechanisms. It is of great therapeutic benefit to identify these antigenic molecules.

1.3.2 Excretory-secretory (ES) products of parasitic helminths

Helminth-host interactions involve the secretion of a variety of molecules into the host environment. These molecules can be excreted or secreted and are referred to as ES products. Parasitic helminth ES products have been extensively studied and a wide diversity of molecules including proteases (Williamson et al., 2003), protease inhibitors (Knox, 2007), antioxidants (Brophy et al., 1995) and host-like molecules (Maizels and Yazdanbakhsh, 2003) have been found to be included.

1.3.2.1 Proteases and protease inhibitors

Parasite proteases facilitate the invasion and migration of the helminths, mediate the moulting process and help the digestion of host proteins. Proteins are the most abundant nutrients in the blood and tissues of the host. A vast amount of proteases are expressed in the intestines of nematodes with the likely function of digesting their food. Digestive proteases of the blood-feeding nematodes, such as

the human hookworms, and the ruminant nematode *H.contortus* have been reviewed by Williamson et al (2003). Serine (Brown et al., 1999), cysteine (Caffery et al., 1994), aspartyl proteases (Brown et al., 1999) and metalloproteinases (Gamble et al., 1996) have been detected and characterized in parasite helminth ES products. Species- and stage-specificity of nematode proteases have been observed (Knox and Jones, 1990). A cysteine protease has been purified from adult *N.brasiliensis* somatic and ES products, and preferentially evokes IgG1 and IgE antibody response (Kamata et al., 1995). This suggested an antigenic role of *N.brasiliensis* cysteine protease.

Unregulated proteases can cause severe damage to both the parasite and the host (Armstrong, 2001). Protease inhibitors of the parasite origin are used by the parasites to protect themselves from the hostile proteolytic environment (Rawlings et al., 2004). Cystatins, which belongs to the cysteine protease inhibitor families, or cystatin-like molecules have also been widely characterized in various parasitic helmiths, such as *H. contortus* (Newlands et al., 2001), hookworm *Ancylostoma ceylanicum* (Milstone et al., 2000) and filarial nematodes *B.malayi* (Manoury et al., 2001), *O.volvulus* and *Acanthocheilonema viteae* (Schierack et al., 2003). Nippocystatin was firstly detected in adult *N.brasiliensis* ES products, and the cDNA was then sequenced and cloned. The mRNA of nippocystatin was demonstrated to express in both larvae and adults of *N.brasiliensis* (Dainichi et al., 2001). Recombinant nippocystatin has been confirmed to inhibit cysteine proteases, cathepsin L and B (Dainichi et al., 2001). *In vivo* treatment of ovalbumin (OVA)-immunized mice with recombinant nippocystatin significantly suppressed OVA specific proliferation of splenocytes and associated IL-4 and IFN-γ production,

as well as OVA-specific serum IgE level (Dainichi et al., 2001). In the same experiment, processing of OVA was inhibited by recombinant nippocystatin (Dainichi et al., 2001). Mice with anti-recombinant nippocystatin antibodies became partially resistant to subsequent *N.brasiliensis* infection, which suggested that *N.brasiliensis* evade host immune system by secreting nippocystatin (Dainichi et al., 2001).

1.3.2.2 Antioxidants

Copper/Zinc dependant superoxide dismutases (SODs) have been characterized in various parasite helminths, including *S.mansoni* (Hong et al., 1992), *Dictyocaulus viviparous* (Britton et al., 1994), *B.pahangi* (Tang et al., 1994), *H.contortus* (Liddell et al., 1998), *Fasciola hepatica* (Kim et al., 2000) and *Trichinella pseudospiralis* (Wu et al., 2006). SOD activities were observed in L3 and adult *N.brasiliensis* homogenates and ES (Knox and Jones, 1992; Batra et al., 1993). The induction of parasite-derived anti-oxidants may contribute to parasite survival by neutralizing oxidative stress generated by activated immune cells or oxygen-rich environment.

1.3.2.3 Acetylcholinesterases (AchEs)

AchEs have a primary function of mediating cholinergic neurotransmission in both vertebrates and invertebrates (Taylor, 1991). It is so far the best-defined molecule in *N.brasiliensis* ES products. Adult *N.brasiliensis* secrete three forms of AchEs, AchE A, B and C, as distinguished by their electrophoretic activities in non-denaturing gels (Ogilvie et al., 1973). These enzymes have similar molecular masses between 69 and 74 kDa (Grigg et al., 1997). They are encoded by three

separate genes with AchE A expressed in L4 larvae and AchE B and C expressed in adult *N.brasiliensis* (Blackburn and Selkirk, 1992; Edwards et al., 1971). Neither infective larvae nor larvae recovered from lungs 24 hours after infection secrete AchEs (Hussein et al., 2002). One other somatic form of AchE can be discriminated from secreted AchEs by its slower migration in non-denaturing polyacrylamide gels stained for AchE activity (Hussein et al., 1999). It is still unclear why *N.brasiliensis* expresses multiple forms of AchEs at different developmental stages. Rats immunised with recombinant AchE B via subcutaneous and intra-nasal routes showed protection to subsequent challenge with *N.brasiliensis* characterised by significantly less egg output compared to non-immunized rats (Ball et al., 2007). It is likely AchEs are involved in immune evasion, which requires further evidence regarding their physiological and immunological roles.

1.3.3 Parasite helminth products with immune regulatory functions

Immune regulation by parasites has become a general concept and there have been a large number of studies aimed at characterizing immune-active molecules across many species of parasitic helminths. Table 1.1 summarizes some of the helminth-derived molecules which are grouped according to their general effect on the immune system. However, given the complexity of the latter, it is possible that they may be found to have a broader effect in the host-parasite relationship. These molecules have been reviewed in detail by Maizels and Yazdanbakhsh (2003), Maizels et al (2004) and Proudfoot (2004).

Immune function	*Helminth products*	*Species*	*Description*	**Reference**
Modulation of antigen processing and presentation	Bm-CPI-2	*B. malayi*	Cystatin homologue	Manoury et al 2001
	Onchocystatin	*O. volvulus*	Cystatin homologue	Schönemeyer et al., 2001
	Nippocystatin	*N. brasiliensis*	Cystatin homologue	Dainichi et al., 2001a
	PGD2	*S. mansoni*	Prostaglandin	Angeli et al., 2001
	PGE2	*Taenia taeniaeformis.*	Prostaglandin	Leid and McConnell, 1983
	PGE2	*Wuchereria bancrofti*	Prostaglandin	Liu et al., 1992
	PGE2	*B. malayi*	Prostaglandin	Liu et al., 1992
	ES-62	*Acanthocheilonema viteae*	phosphorylcholine-containing glycoprotein	Goodridge et al., 2001
	Ov-ALT-1	*O. volvulus*	Abundant larval transcript	Joseph et al., 1998
Interference with effector mechanisms	Lewis x	*S. mansoni*	Carbohydrate antigen	Van Die et al., 2003
	Bm-SPN	*B. malayi*	Serine protease inhibitor	Zang et al., 1999
	PAF inhibitor	*N. brasiliensis*	Acetylhydrolase	Blackburn and Selkirk, 1992
	TES-70	*Toxocara canis*	C-type lectin	Loukas et al., 2000
	TES-32	*T.canis*	C-type lectin	Loukas et al., 1999
Interference or mimics of host cytokines	Bm-TGH1 Bm-TGH2	*B. malayi*	TGF-β mimic	Gomez-Escobar et al., 1998; Gomez-Escobar et al., 2000
	Bm-MIF	*B. malayi*	MIF mimic	Zang et al., 2002
	Sm-RK1	*S. mansoni*	TGF-β receptor mimic	Davies et al., 1998

Table 1.1 Examples of identified helminth-derived products that have immune-regulatory functions.

1.4 Anti-inflammatory properties of parasitic helminths

The limited recruitment of inflammatory neutrophils and the absence of TNF-α production in the rat lungs of *N. brasiliensis* infection, led us to the belief that anti-inflammatory mechanisms could be active in the lung stage. A variety of strategies have been developed by parasitic helminths to suppress excess inflammation for the purpose of their survival, and they are also demonstrated to be involved in the immune regulation by the parasites. Here, we focus on reviewing the strategies mostly likely to be utilized by the lung stage *N.brasiliensis* larvae to suppress early immune responses. These include, the regulation of leukocyte recruitment, modification of macrophage phenotype, suppression of pro-inflammatory molecules and induction of anti-inflammatory molecules. Due to the complexity of the immune system, immune regulation by parasitic helminths involves the participation of nearly every cellular and humoral component.

1.4.1 Lipopolysaccharide (LPS) inflammation

In this study, *Escherichia coli* (*E. coli*) LPS stimulated macrophages and rat lungs were used to establish an inflammation background. LPS is the principle glycolipid component of the outer membrane of Gram-negative bacteria (Rietschel et al., 1994). It is a highly potent activator of the innate immune system and its recognition by the host has been widely used as a major marker for invading Gram-negative bacteria. It has been demonstrated that in myeloid cells, at least four proteins are essential to mount a cellular response to LPS, and they are CD14, LPS binding protein (LBP), Toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD-2). CD14, which is expressed on monocytes, macrophages and

neutrophils, is a receptor for LPS (Wright et al., 1991). CD14 is also present as a soluble form, sCD14, which is involved in the activation of CD14 negative cells, such as epithelial cells, endothelial cells and smooth muscle cells (Pugin et al., 1993). Blockade of CD14 by monoclonal antibodies prevented LPS stimulated synthesis of TNF-α both *in vivo* and *in vitro* (Leturcq et al., 1996; Ulevitch and Tobias, 1995). The interaction of LPS and CD14 is significantly enhanced by soluble LBP in the serum (de Haas et al., 1998). CD14 is not a transmembrane protein, and the signal-transducing into the cells requires TLR4, which is a transmembrane receptor for LPS (Poltorak et al., 1998). MD-2 is another accessory protein that is essential for TLR4 to be fully functional (Nagai et al., 2002). Following the recognition of LPS by TLR4, MyD88, a cytoplasmic adaptor, associates with TIR domains of TLRs for intracellular signaling (Adachi et al., 1998), and subsequently activates NF-κB for the transcription of pro-inflammatory molecules. Inflammatory mediators released by these cells are essential for the early innate and the subsequent adaptive immune responses (Beutler et al., 2003). Excessive exposure to LPS leads to multiple pathophysiology effects such as endotoxic shock, tissue injury and death.

Most wild type bacteria, including *E. coli*, synthesize two forms of LPS molecules named Smooth (S) and Rough (R) form (Freundenberg et al., 2008). S-form LPS consists of the O-specific polysaccharide chain, the core oligosaccharide and a highly conserved hydrophobic lipid A. R-form LPS is lack of the O-polysaccharide chain. O-specific polysaccharides are strain specific and determine the serological identifications of many bacteria (Magalhães et al., 2007). In some strains such as *Salmonella*, the O-polysaccharide chain is important for bacteria survival by

protecting the bacteria from phagocytosis and complement mediated lysis (Rietschel et al., 1994). LPS is recognized by host cells through its Lipid A component. It has been suggested that the specificity of lipid A binding to its receptor is mediated by its hydrophilic backbone (Phosphorylated-D-glucosamine disaccharide) (Rietschel et al., 1994). The *E. coli* LPS strain (0111:B4) used in this study is characterized as S-LPS.

1.4.2 Neutrophil recruitment

Exposure to LPS triggers complex inflammatory responses that involve multiple inflammatory mediators and activation or recruitment of inflammatory cells, such as neutrophils. Neutrophils migrate out of the circulation to a desired site under the conditions of inflammation, infection and injury. It is well accepted that neutrophil migration is a multistep process that usually involves rolling, adhesion and transmigration. Due to the unique pulmonary alveolar/microvascular structure, neutrophils are more concentrated in the pulmonary capillaries (Downey et al., 1993) and their special biophysical migratory route has been reviewed in detail by Burns et al (2003). In general, neutrophil recruitment is under the control of cytokines, chemokines and adhesion molecules and their ligands on both leukocytes and endothelial cells (Wagner and Roth, 2000). The Gram-negative bacteria stimulus, LPS, elicits neutrophil migration largely dependent on CD18 (Mizgerd et al., 1999) and causes up-regulation of ICAM-1 in the lungs (Beck-Schimmer et al., 1997). LPS-induced pro-adhesive cytokines, such as TNF-α and IL-1, and chemokines including IL-8 or MIP-2 are all potent mediators of neutrophil migration (Wagner and Roth, 2000). Activated neutrophils limit inflammation via phagocytosis of pathogens and by releasing antimicrobial products such as

oxidants, microbicidal peptides and proteases (Bank and Ansorge, 2001; Quinn and Gauss, 2004). Uncontrolled neutrophil recruitment is destructive and anti-inflammatory agents have been developed to interfere with the process of neutrophil migration in certain diseases (Wagner and Roth, 2000).

In parasitic helminth infection, toxic molecules released by recruited neutrophils have also been found to be active in killing *S.mansoni* (Freudenstein-Dan et al., 2003), *O.volvulus* (Johnson et al., 1991), *B.malayi* (Chandrashekar et al., 1986) and *T.spiralis* (Ruitenberg et al., 1983) larvae. However, neutrophilia is generally not as dominant as eosinophila in parasitic helminth infections. Relatively small numbers of neutrophils are recruited to the lungs during *N.brasiliensis* infection (McNeil et al., 2002). It is possible that anti-inflammatory mechanisms are activated in *N.brasiliensis* infection to limit neutrophil recruitment in the lungs.

1.4.3 Alternatively activated macrophages (AAMs)

Macrophages are antigen presenting cells (APC) and play key roles in directing adaptive immune responses (Ma et al., 2003). They were first recognized for their phagocytic properties, although they have other important functions as major components in the innate immune system. In the pulmonary stage of *N.brasiliensis* infection, the majority of alveolar macrophages are activated and become helminthocidal (Egwang et al., 1985). Reviewed by Noël et al (2004), macrophages are activated in two ways. In an environment of LPS and Th1 cytokines, such as IFN-γ, IL-1, IL-12 and TNF-α, macrophages are classically activated. These cells produce inducible nitric oxide synthase (iNOS) that catalyses the production of NO from L-arginine and molecular oxygen.

Alternatively, extracellular parasitic helminths can activate macrophages to secrete significant amounts of Th2 and Treg cytokines, including IL-4, IL-5, IL-10, IL-13 and TGF-β. L-arginine is catalyzed by arginase to produce urea and L-ornithine in these cells. Raes et al (2002) have found that YM1 and FIZZ1 genes are abundantly expressed in *Trypanosoae brucei* elicited murine AAMs, and YM1/FIZZ1 and arginase expression have been established as markers for AAMs in many studies.

AAMs are believed to be an immune-regulatory tool of helminths. In mice, IL-4/IL-13 activated AAMs are essential for protection against organ injury through downregulation of Th1 inflammation (Herbert et al., 2004). There is evidence that helminth induced AAMs result in T cell hyporesponsiveness in the host by inhibition of cell-to-cell contact (Taylor et al., 2006; Loke et al., 2000). AAMs produce factors that are involved in extracellular matrix (ECM) construction, angiogenesis and wound repair (Kodelja et al., 1997), which may accelerate the recovery from inflammation. In the *N.brasiliensis* infected lungs, nearly all alveolar macrophages became YM1-producing AAMs as early as 2 days post-infection, which could be important in dampening inflammation in the lungs (Reece et al., 2006). Highly upregulated expression of YM1 and FIZZ1 genes has also been detected in lymph node macrophages in *N.brasiliensis* infection, which suggest a potential regulatory role for AAMs on lymphocyte development (Nair et al., 2005).

1.4.4 Toll-like receptors (TLRs)

The innate immune system uses a large collection of pattern recognition receptors (PRRs) to discriminate between self and non-self antigens (Janeway and

Medzhitov, 2002). Among all the PRRs, toll-like receptors (TLRs) recognize most of the microbial pathogens. Ten family members of TLRs have been identified. Reviewed by Takeda et al (2003), these TLRs share similar structures that contain an extracellular domain, a transmembrane domain and an intracellular Toll/Interleukin-1 receptor (TIR) domain for transducing signals to the inside of the cells. Generally, it appears that lipid and DNA components of bacteria are recognized by TLR 1, 2, 4, 5, 6 and 9. TLR 3, 7 and 8 recognize virus ligands whilst the ligand for TLR 10 remains unknown (Takeda et al., 2003). TLRs are among the most conserved receptor family, being found in a variety of cells such as macrophages, dendritic cells, neutrophils, lymphocytes, vascular endothelial cells and epithelial cells. Immune cells and other cells recognize LPS through TLR4, a process that requires the cooperation of CD14, LBP and MD-2 as introduced in section 1.4.1.

TLRs, especially TLR3 and TLR4, have been demonstrated to be involved in the recognition of *Caenorhabditis elegans* (Tenor and Aballay, 2008), filarial nematodes (Goodridge et al., 2005) and *Schistosome* antigens (Aksoy et al., 2005; Jenkins et al., 2005). Chronic helminth infected individuals have diminished TLRs (Babu et al., 2006), which may be responsible for their hyporesponsiveness to TLR ligands including LPS (van der Kleij et al., 2004). Helminth induced immune regulation though TLR has been reviewed by Van reit et al (2007). The response of dendritic cells to TLR ligands is re-directed by helminth antigens to activate Th2 cells or Treg cells; this is usually accompanied by a reduction in Th1 cytokines and down regulated signaling via MyD88 and NFκB (Kane et al., 2004; Tolouei et al., 2008; Pearce et al., 2006).

1.4.5 Nuclear factor-κB (NF-κB)

NF-κB activation is a central event leading to the activation of the Th1 inflammatory network (Caamano et al., 2002). NF-κB is an inducible transcription factor composed of subunits cRel, RelA (p65), RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). In most cells, NF-κB is a heterodimer comprised of RelA and p50 (Jobin and Sartor, 2000). In non-stimulated cells, NF-κB is found maintained within the cytoplasm by the inhibitory protein IκB, which masks the nuclear localization signal present within the NF-κB protein sequence. In response to pro-inflammatory stimulators, such as LPS, TNF-α and IFN-γ, IκB is phosphorylated, which allows NF-κB to translocate from the cell cytoplasm to the cell nucleus. This in turn activates the transcription of numerous genes that support an inflammatory response, which include TNF-α, IL-1, IL-12, NO, MIP-2 and ICAM-1 (Ghosh et al., 1998). Reviewed by Mizgerd (2002), NF-κB subunit RelA (p65) is essential for activating chemokines and adhesion molecules required for neutrophil recruitment (Figure 1.5). However, it has been reported that NF-κB1 expression in dendritic cells (DCs) is also required in promoting Th2 inflammation in *S.mansoni* infected hosts (Artis et al., 2005).

The inflammatory diseases that involve NF-κB activation include rheumatoid arthritis, atherosclerosis, multiple sclerosis, inflammatory bowel disease (IBD), asthma, *Helicobacter pylori*-associated gastritis and septic shock (Tak and Firestein, 2001). Therapeutic strategies have been developed aimed at blocking NF-κB activity (D'Acquisto and Ianaro, 2002). Interestingly, a recent study reported that a helminth glycan induced transient NF-kB activation only for the maturation of dendritic cells, which is different from the persistent NF-κB activation by LPS that

leads to Th1 inflammation (Thomas et al., 2005). The filarial nematode secreted product, ES-62, and *A.suum* product, PAS-1, have been reported to inhibit LPS-induced inflammation, possibly through inhibition of NF-κB activation (Harnett and Harnett, 2006; Oshiro et al., 2005). TNF-α-induced expression of adhesion molecules has also been observed to be reduced by interfering with NF-κB in *S.mansoni* infected lungs (Trottein et al., 1999). The role of NF-κB in *N.brasiliensis* induced inflammation remains unknown.

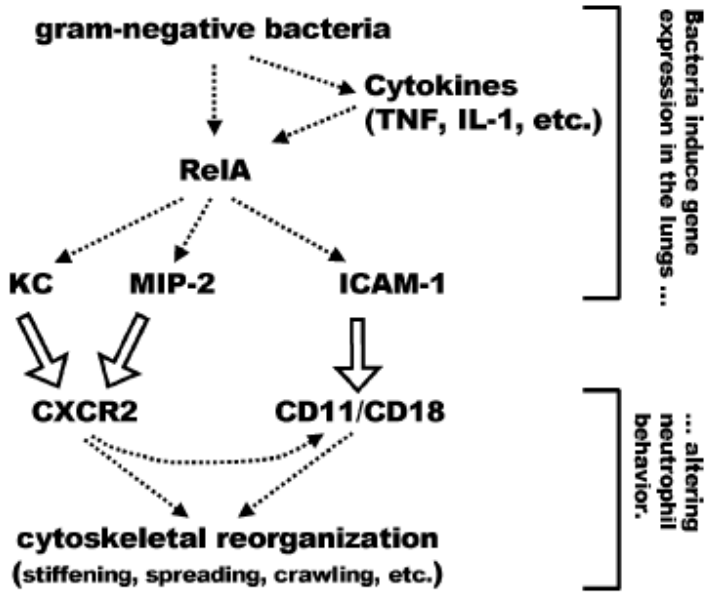


Figure 1.4 Molecular mechanisms by which neutrophil recruitment is stimulated by gram-negative bacteria through RelA in rodent lungs (reproduced from Mizgerd, 2000).

1.4.6 Macrophage inflammatory protein-2 (MIP-2)

Chemokines are small chemoattractant peptides with molecular weights of 6-14kDa. CXC chemokines are named because two cysteines nearest the N-terminus are separated by a single amino acid (Rollins, 1997). Among various

chemokines, CXC chemokines, which include IL-8 (CXCL8), MIP-2 (CXCL2) and KC (CXCL1), are the most potent for neutrophil recruitment (Kobayashi, 2006). However their effects on eosinophil, lymphocyte and macrophage recruitment are very limited (Driscoll et al., 1995). MIP-2 is produced by a variety of cells including monocytes, T lymphocytes, neutrophils, fibroblasts, endothelial cells and epithelial cells, and its production is stimulated by endotoxins such as LPS (Wolpe and Cerami, 1989). MIP-2 and KC induce all steps in the migration process of neutrophils, including rolling, adhesion and transmigration *in vivo* (Zhang et al., 2001).

MIP-2 is stimulated in the serum and tissue of *T.spiralis* (Frydas et al., 2001). In *S.mansoni* inflammation model, MIP-2 expression in the tissue is associated with type 1 immune responses (Park et al., 2001). However, a recent study reported a chemokine binding protein (smCKBP) encoded by *S.mansoni* eggs were able to bind to chemokines such as IL-8 and MIP-1α in mice, which inhibited their interaction with the chemokine receptors and their biological function (Smith et al., 2005). This again suggested that parasitic helminth products may be potential anti-inflammatory agents.

1.4.7 Intercellular adhesion molecule-1 (ICAM-1)

Leukocyte adherence to the endothelium is a key step in the inflammatory processes featured by leukocyte infiltration. ICAM-1 belongs to the immunoglobulin (Ig) gene superfamily and is involved in cellular adhesion. Other family members include ICAM-2, vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1 (PECAD-1) and the mucosal

addressin (MAdCAM-1) (Carlos and Harlan, 1994). Human and rat ICAM-1 have five extracellular Ig-like domains (Kita et al., 1992) that contain binding sites for CD11a/CD18 (LFA-1) (Staunton et al., 1990) and CD11b/CD18 (Mac-1) in leukocytes (Diamond et al., 1991). ICAM-1 is expressed on epithelial cells, endothelial cells, fibroblasts, as well as on recruited leukocytes. Its activation has been shown to be mediated by NF-κB (Chen et al., 1995). Neutrophil accumulation following airway instillation of LPS has been proved to be ICAM-1-dependent (Beck-Schimmer et al., 1997). Reviewed by Albelda et al (1994), blockade of ICAM-1 has effects in various models of inflammation, such as transplant rejection, ischemia, asthma and immune complex injury. In a mouse model of *O. volvulus*-mediated river blindness, IL-4 and IL-13 regulated ICAM-1 expression and eosinophil recruitment are related to parasitic infection in the eyes (Berger et al., 2002). ICAM-1 and LFA-1 expression in the liver and intestine were demonstrated to mediate granuloma formation in *S.mansoni* infection (Ritter et al., 1996; Jacobs et al., 1998). Serum levels of soluble ICAM-1 and other adhesion molecules in patients with *S.mansoni* infection have been associated with the disease severity (Esterre et al., 1998). It can be assumed that anti-ICAM-1 treatment would also benefit helminth infections. A study by Hassanein et al (2001) suggested that chronic *S.mansoni* infection may have reduced ICAM-1 expression that causes granuloma hyporesponsiveness. This suggested that helminths may produce molecules to suppress expression of adhesion molecules in the long term.

1.4.8 Tumor necrosis factor-alpha (TNF-α)

Monocytes and macrophages are the major sources of TNF-α *in vivo* and the receptors for TNF (TNFR1 and TNFR2) are present on almost all nucleated cell

types (Kollias and Kontoyiannis, 2002). The biosynthesis of TNF-α is highly regulated at multiple levels, transcriptional, translational and post-translational. Reviewed by Herbein and O'Brien (2000), TNF-α is expressed in two forms, a 26kDa transmembrane form and a 17kDa secreted form, which have high affinity for TNFR2 and TNFR1, respectively. It is believed that TNF-α and TNFR1 binding is responsible for the majority of biological activities of TNF-α through activation of NF-κB. Except for being cytotoxic to some tumor cell lines, TNF-α is a pro-inflammatory molecule which targets most cells of the immune system. TNF-α mediates endothelial leukocyte interaction by inducing enhanced surface expression of adhesion molecules, including ICAM-1 (Mulligan et al.,1993; Krunkosky et al., 2000) and VCAM-1 (Henninger et al., 1997). The blockage of TNF-α in patients with rheumatoid arthritis or inflammatory bowel disease has been shown to be beneficial for the majority of patients (Feldmann et al., 1996; Sandborn and Hanauer, 1999). Other pro-inflammatory cytokines are also inhibited if TNF-α is neutralized (Feldmann et al., 1996), which suggests that TNF-α is at the centre of the pro-inflammatory cytokine network.

A large amount of TNF-α biological activity is shared with interleukin-1 (IL-1), another important pro-inflammatory cytokine (Kollias and Kontoyiannis, 2002). Interestingly, limited TNF-α production is observed in many parasitic helminth infections including *T.suis* (Kringel et al., 2006*), B.malayi* (Babu et al., 2006) and *N.brasiliensis* (Matsuda et al., 2001). Some helminth products have been shown to down-regulate LPS-stimulated Th1 cytokine production, including TNF-α, IL-1 and IL-12 (Dirgahayu et al., 2002; Goodridge et al., 2001; Oshiro et al., 2005). The possibility that *N.brasiliensis* products perform a similar function will be

investigated in this project.

1.4.9 Interleukin-1 (IL-1)

Three members of the IL-1 gene family have been identified: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are antagonists, and IL-1β is a critical cytokine for inducing inflammatory events, while IL-1α may be a regulator of inflammation (Dinarello, 1996). Processing of IL-1β to its biologically activate mature form requires a protease, termed IL-1β converting enzyme (ICE) (Black et al., 1989). Binding of IL-1 to Type II IL-1 receptor (IL-1RII) does not transduce signals. Type I IL-1 receptor (IL-1RI) shares similarities to Toll-like receptors in their cytosolic region, and the binding to IL-1RI transduces signals via NF-κB and mitogen-activated protein kinase (MAPK) pathways (Dunne and O'Neill, 2003).

IL-1 affects nearly every cell type, often in concert with other Th1 cytokines or molecules. Controversially, while TNF-α is usually absent in helminth infection, parasitic helminths can provoke IL-1 production. For example, in *N. brasiliensis* infected rats, IL-1 levels in BAL transiently increase one day after infection (Benbernou et al., 1992) and two weeks after, a slight increase of IL-1β is still detectable in granulomatous lesions in rat lungs (Matsuda et al., 2001). Pre-exposure to microfilariae up-regulate *Mycobacterium tuberculosis* stimulated IL-1β production by DCs (Talaat et al., 2006). However, the *A.suum* product, PAS-1, has been found to reduce LPS-stimulated IL-1β production (Oshiro et al., 2005).

1.4.10 Interleukin-12 (IL-12)

IL-12 is produced by phagocytic cells (monocytes, macrophages and neutrophils)

and cells with antigen-presenting capabilities such as DCs. It acts as a pro-inflammatory cytokine in response to infectious agent such as LPS (Podlaski et al., 1992). IL-12 is a heterodimer cytokine composed of two covalently linked chains: a heavy chain of 40 kDa (p40) and a light chain of 35 kDa (p35). The biological activity of IL-12 and its importance in immunity has been reviewed by Romani et al (1997) in detail. The bioactive form of IL-12 has been demonstrated to be associated with the p70 heterodimer of p40 and p35 (Podlaski et al., 1992). IL-12 p40 is produced at 10- to 100- fold excess over the biologically active form IL-12 p70, but since they share the same receptor, it has been speculated that the production of IL-12 p40 may limit the activity of IL-12 p70 (Romani et al., 1997). The major cellular targets of IL-12 are T and NK cells, to which IL-12 is a potent signal for the development of Th1 cells and NK cell activity (Chensue et al., 1995).

Although the production of IL-12 during the early inflammatory responses to pathogens is important for microbicidal activity, uncontrolled production of IL-12 has pathological consequences, including endotoxic shock (Romani et al., 1997). TGF-β, IL-4, IL-10 and IL-13 are all potent inhibitors of IL-12 production (Wynn et al., 1994). However, in a collagen-induced arthritis model, deficiency of IL-12 has been observed to exacerbate the disease with elevated pro-inflammatory TNF-α, IL-1β, IL-6 and IL-17 (Murphy et al., 2003). Therefore, the role of IL-12 in inflammation is controversial. Administration of IL-12 during initiation of *N.brasiliensis* infection has been demonstrated to stimulate IFN-γ production, which encourages Th1 immune responses. Th2 cytokines, such as IL-4 and IL-5, mucosal mast cells, IgE production and eosinophilia were inhibited by IL-12 administration (Finkelman et al., 1994). Moderate production of IL-12 may be able

to down regulate Th2 cytokine-induced pulmonary granuloma formation in response to schistosome eggs (Wynn et al., 1994; Chensue et al., 1995). The regulation of IL-12 by parasitic helminths is usually associated with other Th1 cytokines, including TNF-α and IL-1β.

1.4.11 Nitric Oxide (NO)

NO is a major cytotoxic molecule produced by CAMs and is involved in many inflammatory conditions (Hibbs et al., 1988). NO is released by a variety of cells, including epithelial cells, endothelial cells, neutrophils and activated macrophages (Nussler and Billiar, 1993). NO production results from the oxidation of a guanidine nitrogen of L-arginine to produce L-citrulline (Figure 1.6). This process is catalyzed by the enzyme NO synthase (NOS) in mammalian cells (Nathan, 1992). Enhanced transcription of the gene for this enzyme, termed inducible NOS (iNOS), is observed in response to LPS and inflammatory cytokines IFN-γ and TNF-α (Drapier et al., 1988). NO is relatively short lived and is removed by oxyhaemoglobin. The direct toxicity of NO is moderate, but is greatly enhanced by reacting with superoxide to form peroxynitrite (ONOO-) (Beckman and Koppenol, 1996).

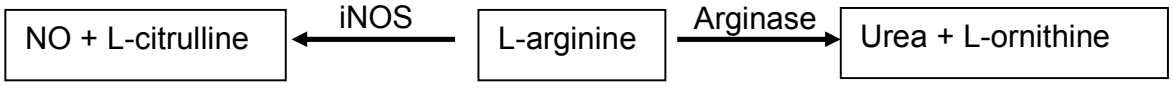


Figure 1.5 L-arginine metabolism.

Controversy exists over the assumed role of NO in extracellular helminth infections. NO of vascular origin was described as an important vasodilator, which may be necessary for parasite migration within the vascular system (Nathan, 1996). NO

was demonstrated to mediate intestinal pathology during *T. spiralis* infection (Lawrence et al., 2000). However, as a product of CAMs, its production has been found to be inhibited by helminth products that are potent activators of AAMs (Noël et al 2004). Th2 cytokines associated with helminth infection also downregulate NO production (Bogdan and Nathan, 1993). During *S. mansoni* infection, NO production is counteracted by Th2 responses to avoid deleterious hepatic damage (Brunet et al., 1999).

1.4.12 Arginase

Arginase catalyzes the hydrolysis of L-arginine to urea and L-ornithine, competing with the formation of NO via iNOS (Figure 1.5; Boucher et al., 1999). At least two distinct arginase genes have been reported coding for immunologically distinct isoforms, arginase I and arginase II. Arginase I is a cytoplasmic form located primarily in liver. Arginase II is a mitochondrial form expressed in extra hepatic tissues and cells, including macrophages (Spector et al., 1994).

In general, IL-4, IL-10 and TGF-β trigger arginase expression, which drive the reaction away from NO synthesis to the formation of urea and ornithine (Corraliza et al., 1995; Boutard et al., 1995). Exposure of cells to these cytokines may exhaust their ability to respond subsequently to inducers of NO synthase. The main role of arginase in the immune system is as a competitor of NOS. As has been discussed earlier in this thesis, the discrimination of CAMs and AAMs has mainly been demonstrated at the biochemical level, in the metabolism of L-arginine. Type 2 cytokine induced AAMs and arginase production is dominant in most parasitic helminth infections (Loke et al., 2002).

1.4.13 Interleukin-4 (IL-4) and Interleukin-13 (IL-13)

Parasitic helminth infection is usually associated with the production of Th2 cytokines, such as IL-4 and IL-13. IL-4 is a glycoprotein with a molecular weight of 14-19kDa, and is secreted by several cell types, such as T cells (Howard et al., 1982). The IL-4 R is expressed on a wide range of cell types. The presence of IL-4 is important for the differentiation of naive T helper cells to T helper 2 (Th2) cells that secrete IL-4, IL-5 and IL-13, which are named Th2 cytokines (Swain et al., 1990). Binding of IL-4 to the IL-4R leads to the activation of at least two distinct signaling pathways: STAT6 (signal transducers and activators of transcription 6) pathway and phosphorylation of IRS (insulin receptor substrate) (Keegan et al., 1994). It has been demonstrated that STAT6 is required for the development of Th2 cells (Kaplan et al., 1996).

IL-13 is a cytokine that is produced by different T-cell subsets, DCs and human basophils. It acts on monocytes, B cells and endothelial cells (Malefyt et al., 1995; Li et al., 1996). The biological activities of IL-13 are closely related to those of IL-4. This is due to the fact that IL-13 and IL-4 receptors share an alpha-chain, which is important for signal transduction. T cells do not express functional IL-13 receptors. This is the reason that IL-13 fails to induce Th2-cell differentiation, in contrast to IL-4 (McKenzie et al., 1998). Both IL-4 and IL-13 are involved in the induction of VCAM-1 (Bochner, et al., 1995). However, IL-13 itself is by far the most potent inducer of eotaxin (Li et al., 1999). Both VCAM-1 and eotaxin are key adhesion molecules for the accumulation of eosinophils. Neutrophil recruitment has not been observed to be induced by IL-13.

It is believed that IL-4 and IL-13 act together to suppress the production of Th1 inflammatory mediators, TNF-α, IFN-γ, IL-1, IL-12 and NO (Muchamuel et al., 1997; Doherty et al., 1993; Hart et al., 1989). This is mainly through the STAT6 signaling pathway (Takeda et al., 1996). Cytokines IL-4 and IL-13, IL-10 and TGF-β, upregulated during parasitic helminth infection have been reported to synergize the effect of each other to inhibit macrophage cytotoxic activity, which may be an important strategy to avoid macrophage mediated pathology (Oswald et al., 1992). IL-4 and IL-13 play critical roles in resistance to the intestinal nematode, *T. muris* (Bancroft et al., 1998). In *N. brasiliensis* infection, the expulsion of worms requires signalling via IL-4Rα and STAT6 (Finkelman et al., 2004). However it has been suggested that IL-13 may be more important than IL-4 as an inducer of the STAT6 signalling pathway, which leads to worm expulsion (Urban Jr et al., 1998).

1.5 Parasitic helminths and inflammatory diseases

Developing countries, where people are more likely to be infected with helminths, have a lower incidence of allergic and autoimmune diseases, such as asthma, arthritis, Type1 diabetes and IBDs. The hygiene hypothesis has been developed to explain the rise in the incidence of such diseases in developed countries, and states that this rise has coincided with the improvement in hygiene conditions since the 20th century. As has been reviewed in previous chapters (section 1.4), decades of research has provided evidence that parasitic worms regulate nearly every step in the inflammatory process. Studies in animal models have provided promising results supporting the potential application of parasitic helminths in the treatment of autoimmune and allergic diseases (Table 1.2).

The first outstanding achievement was an open clinical trial in controlling IBD (Summers et al., 2003). IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases of the gastrointestinal tract that may result from uncontrolled Th1 responses to normal gut flora (Podolsky, 2002). Weinstock's group used eggs of the pig whipworm, *Trichuris suis*, in a drink formulation to treat patients with UC and CD. This trial ended with clinical improvement with no adverse effects (Summers et al., 2003). Two years later, a larger scale open trial proved the effectiveness and safety of using *T. suis* in controlling Crohn's disease (Summers et al., 2005a). In the same year, the same group conducted a randomized, double-blind, placebo-controlled clinical trial, in which the patients with active ulcerative colitis received 2500 *T. suis* eggs or placebo orally at 2-week intervals for 12 weeks. It was concluded that the parasite

treatment was effective and safe (Summers et al., 2005b). Single dose administration of *T.suis* eggs had temporary benefit, and the prolonged effect was observed with maintenance therapy (Summers et al., 2003). This suggested that the modulation of both innate and adaptive immunity is the immunological basis of the helminth therapy. At a similar time, trials using human hookworm, *N. americanus,* to control Crohn's disease and asthma were being planned (Croese et al., 2006; Falcone and Pritchard, 2005).

Helminth or product	Disease	Reference
S. mansoni		
	Experimental autoimmune encephalomyelitis	(La Flamm et al., 2003)
	Graves' thyroiditis	(Nagayama et al., 2004)
	Allergic hypersensitivity	(Mangan et al., 2004)
S. mansoni eggs		
	Experimental autoimmune encephalomyelitis	(Sewell et al., 2003)
	Experimental colitis	(Elliott et al., 2003)
T. spiralis		
	Experimental colitis	(Khan et al., 2002)
T. suis		
	IBD	(Summers et al., 2003)
Heligmosomoides polygyrus		
	Experimental colitis	(Elliott et al., 2004)
	Food allergy	(Bashir et al., 2002)
ES-62 (*Filarial* product)		
	Collagen-induced arthritis	(McInnes et al., 2003)
	Allergic hypersensitivity	(Melendez et al., 2007)
N. brasiliensis ES products		
	Ovalbumin-specific allergy	(Trujillo-Vargas et al., 2006)

Table 1.2 Helminth or helminthic products that prevent autoimmunity and allergy in animal models (Adapted from Zaccone et al., 2006)

Although exciting success has been achieved by treating IBD patients with whipworm eggs, the drawbacks of using live helminths need to be considered.

Helminths have been observed to cause changes in intestinal physiology by inducing intestinal muscle hypercontractility and goblet cell hyperplasia (Khan and Collins, 2004). These effects could possibility result in abdominal symptoms such as diarrhoea. Most parasites are pathogenic in nature and persistent existence in the human body may cause chronic damage. Clinical trials that monitor the adverse effects of helminth treatment over extended periods are therefore required to confirm the safety of these potential therapies. Close examination of the biological behaviour of worms within the body may also be necessary. The other obvious drawback is that some patients may find that it is psychologically difficult to administer living organisms. Therefore, treatment with helminth molecules that have immune-regulatory activities may overcome these disadvantages (Ruyssers et al., 2008). With appropriate observations and strict monitoring, it could be anticipated that the anti-inflammatory properties of *N.brasiliensis* and its ES products, investigated in the current study, could be utilized for clinical treatment of inflammatory conditions in future.

1.6 Hypothesis and objectives

In this study, LPS stimulated macrophages or rat lungs were used as *in vitro* and *in vivo* inflammation models, respectively. It was hypothesised that firstly, *N.brasiliensis* lung stage larvae and/or their ES products could down regulate LPS-induced inflammation, both *in vitro* and *in vivo*. Secondly, there are active components in the ES products that are responsible for the anti-inflammatory properties.

The specific objectives arising from the above hypotheses were:

To observe the effect of *N.brasiliensis* L3 larvae and their ES products on LPS-stimulated inflammation in macrophage cell lines. The inflammatory mediators investigated include TNF-α, IL-1β and NO.

To investigate the potential active components in the ES products and to explore the inflammatory processes that are affected by *N.brasiliensis* ES *in vitro*. SDS-PAGE and lectin blot were carried out to characterise proteins in ES products. The inflammatory processes studied in LPS-stimulated inflammation include NF-κB translocation, inflammatory mediator gene transcription and post-translational modification.

To study the effects of *N.brasiliensis* larvae ES on neutrophil recruitment and inflammatory mediator gene transcription in LPS-instilled rat lungs. Real-time PCR was used to quantify the gene transcription of inflammatory mediators, including TNF-α, IL-1β, MIP-2, ICAM-1 and NO.

Chapter 2

Materials and methods

2.1 Reagents

Phosphate buffered saline (PBS) without calcium and magnesium, F-12K Nutrient Mixture Kaighn's Modification medium with L-glutamine, L-glutamine (L-G), penicillin/ streptomycin (P/S) and foetal bovine serum (FBS) were obtained from Gibco Invitrogen, Paisley, UK. Sterile H_2O and 0.9% NaCl (Saline) were obtained from Baxter, UK. All the remaining reagents were obtained from Sigma Chemical Company, Poole, UK, if not specified.

2.2 *N.brasiliensis* L3 larvae collection

This step was carried out in the laboratory of Professor Rick Maizels, University of Edinburgh, with the kind assistance of Yvonne Harcus. Sprague-Dawley rats were infected with approximately 4000 *N.brasiliensis* L3 larvae by subcutaneous injection. Faecal pellets were collected 6 days after infection, mixed with water and charcoal and incubated at 24°C. Six days to three months later, L3 larvae were collected from charcoal-faecal L3 cultures by Baermannization. Motile L3 larvae were then washed three times in PBS by centrifugation at 120g at room temperature (RT) for 10 seconds before being transported to the laboratory at Napier University, Edinburgh.

2.3 *N.brasiliensis* L3 larvae excretory-secretory products (NES) collection

ES collection medium (ES medium) was prepared as phenol-red free RPMI-1640 medium supplemented with 2mM L-glutamine, 100Units ml^{-1} penicillin, 100µg ml^{-1} streptomycin (P/S) and 125µg ml^{-1} gentamicin. *N.brasiliensis* L3 larvae collected as above, were washed five times in sterile PBS, then a further five times in ES medium by centrifugation at 130g at RT for 20 seconds. After the final wash,

larvae were incubated in ES medium at 37°C, 5% CO_2 for 48 hours, at a concentration of 2000 larvae ml^{-1} in a culture flask. After 48 hours, the larval suspension was centrifuged at 130g for 20 seconds before the supernatant was collected and filtered using a 0.2µm syringe filter (Whatman, London, UK) as NES. NES was then stored at -80°C before use.

2.4 NES concentrate preparation

Centrifugal concentrators are widely used for the concentration and/or purification of proteins bigger than the cut off size. The smallest molecular weight cut off size (MWCO) of 3000 Dalton (3kDa) was chosen in this study to avoid the loss of any proteins of interest. The retentate recovered after the concentration step was rich in proteins with molecular weight greater than 3KDa, and was named NES concentrate. L3 NES was subjected to a Vivaspin 6 concentrator with a 3kDa MWCO polyethersulphate (PES) membrane (Vivascience Sartoroius Group, Hannover, Germany). This was centrifuged at 3200g, 4°C for 60 minutes. NES concentrate was recovered from the bottom of the concentrate pocket with a pipette. For *in vitro* cell treatment, concentrated NES was resuspended to the same volume as the filtrate (NES filtrate) using ES medium, and this was used as concentrated NES. For biochemical analysis, NES concentrate was prepared by concentrating NES at least twice without resuspension with ES medium. Both NES concentrate and NES filtrate were stored at -80°C before use.

2.5 NR8383 cell culture

NR8383 is a rat alveolar macrophage cell line. NR8383 was generously given by Dr Ellen Drost at the Centre for Inflammation Research, Edinburgh University, and

were originally purchased from American Type Culture Collection (ATCC, Manassas, USA).

The cell culture medium was prepared as F-12K Nutrient Mixture Kaighn's Modification medium with 2mM L-G, 100Units ml^{-1} penicillin, 100µg ml^{-1} streptomycin and 15% (v/v) heat inactivated FBS. Cells were cultured in 40ml cell culture medium at 37°C, 5% CO_2 and culture was maintained by transferring floating cells to new flasks every two or three days. The culture flask was observed under an inverted microscope to assess the degree of confluence and confirm the absence of bacteria and fungal contaminants. The floating cells in the old cell medium were centrifuged at 380g for 2 minutes. The supernatant was discarded and 20ml of new medium added. The cell clumps were destroyed and 10ml of the cell suspension was reseeded to a new flask. A volume of 30ml of new medium was added into the cell suspension in the new flask. This was incubated at 37°C with 5% CO_2.

2.6 Cell count and cell viability

A volume of 100µl of filtered trypan blue solution (0.4% w/v trypan blue in saline) was added to 100µl of cell suspension. Only dead cells were stained blue because of leakage through damaged cell membranes. Unstained viable cells were counted using a haemocytometer (Weber Scientific, Middlesex, UK) and the cell concentrations were recorded. Cell viability was calculated as unstained cells x 100% / (unstained cells + stained cells). Cell viability was ascertained to be in excess of 95% before each experiment. The cells were centrifuged at 95g for 5

minutes, and fresh cell medium was added to obtain the correct concentration when necessary.

2.7 Cell preparation and stimulation

2.7.1 ELISA assays and cytotoxicity tests

Total number of $2x10^4$ cells were plated in each well of a 96-well plate and settled for 1 hour at 37°C, 5% CO_2 before any treatment. Cells treated with 25ng ml^{-1} LPS (*E.coli* 0111:B4) (Sigma, Poole, UK) were used as positive controls. Cells incubated with ES medium only were negative controls.

The cells were treated with 25% (v/v) NES, 50% (v/v) NES, NES concentrate (50% v/v) or NES filtrate (50% v/v) according to experimental designs. ES medium was always used to fill the total volume of each well to 200µl. The percentages of 25% and 50% NES both stand for the percentage of NES volume out of 200µl of total volume in each well. The 96-well plates were incubated at 37°C for 48 hours.

To investigate the heat and trypsin sensitivities of the immune active components, NES concentrate was heat-treated at 95°C for 8 minutes, or digested with 2% (m/m) trypsin for 18 hours before cell treatment.

To investigate the effects of live larvae, 20, 50 or 80 live *N.brasiliensis* L3 larvae on the day of collection were added to each well according to experimental designs. The volume in each well in a 96-well plate was made up to 200µl with ES medium.

2.7.2 Cytoplasmic, nuclear protein extraction and RNA extraction

Total number of 0.5-1x10^6 cells were incubated in each well of a 6-well plate at 37°C, 5% CO_2 for 1 hour before any treatment. Cells were then treated with ES medium, 25ng ml^{-1} LPS (*E.coli* 0111:B4) (Sigma, Poole, UK), or LPS with NES, NES concentrate or NES filtrate. NES, NES concentrate or NES filtrate were added to occupy 50% (v/v) of a total 2ml in each well.

2.8 Bradford protein assay

Bradford assay was used to determine the protein concentrations in NES, NES concentrate, NES filtrate, cellular extracts and BAL supernatants. The protein assay is a dye binding protein microassay based on the method of Bradford (Bradford, 1976). The Coomassie blue dye in the protein assay dye reagent binds to the basic and aromatic amino acid residues, especially arginine in the peptides (Compton and Jones, 1985). In response to various concentrations of protein, differential colour changes of the dye occur. This assay requires a minimum mass of protein to be present for the dye to bind. This protocol was optimised to produce a standard curve with consistent R-squared > 0.90 in this study.

The dye reagent was prepared by diluting 1 part of Protein Assay Dye Reagent Concentrate (Bio-Rad laboratories, Herts, UK) with 4 parts of distilled, deionised water. The diluted reagent was filtered through a Whatman #1 (Whatman, London, UK) paper filter and stored at 4°C before use. Bradford assay standards were prepared by double diluting of the top bovine serum albumin (BSA) protein standard (Sigma, Poole, UK) 40µg ml^{-1} in ES medium, giving standards in the range of 0.3125µg ml^{-1}-40µg ml^{-1}. ES medium only was used as a blank sample.

Fifty microliter of dye reagent were added to the test samples and standards in a 96 well plate and incubated at RT for 15 minutes. Following incubation, the plate was read in a Dynex MRX microplate reader at 595nm.

2.9 Enzyme-linked immunosorbant assay (ELISA)

ELISA is a technique based on the principle of immunoassay with enzyme labelled antibody (Lequin, 2005). The ELISA kits used in this project were based on the theory of "sandwich" ELISA.

2.9.1 TNF-α ELISA

The concentration of TNF-α in the culture supernatant was measured using a mouse TNF-α ELISA kit (RandD Systems, Oxon, UK). The mouse ELISA kits were shown to cross-react with rat TNF-α by the manufacturer. Briefly, the plate was coated with goat anti-mouse TNF-α overnight. Following the incubation, the unbound coating antibody was removed and the plate was washed three times with wash buffer (0.05% v/v Tween-20 in PBS). The plate was then blocked in blocking solution (1% w/v BSA in PBS) at RT for at least 1 hour. Afterwards, the plate was washed and incubated with samples and standards for 2 hours at RT. The standards were prepared by double diluting using reagent diluent (1% w/v BSA in PBS) in the range of 15.625pg ml^{-1} - 2000pg ml^{-1} mouse TNF-α. The plate was incubated for 2 hours at RT and washed as before. Biotinylated goat anti-mouse TNF-α 100µl at a concentration of 75ng ml^{-1} were then added to the plate and left at RT for another 2 hours before washing with wash buffer. Streptavidin-HRP 100µl were then added to all wells followed by an incubation of 20 minutes. The plate was then washed as before. Substrate reagent 100µl, made of equal

volume of hydrogen peroxide and tetramethylbenzidine (RandD Systems, Oxon, UK) were added to each well to develop the colour. The reaction was stopped with 2N H_2SO_4 and the plate was read at 450nm with a reference wavelength of 540nm using a Dynex MRX microplate reader.

2.9.2 IL-1β ELISA

The concentration of IL-1β in the culture supernatants was measured using a rat IL-1β ELISA kit (RandD Systems, Oxon, UK). A 96-well plate was coated with 0.8μg ml^{-1} of goat anti-rat IL-1β overnight at RT. Following the incubation, the unbound coating antibody was removed and the plate was washed three times with wash buffer. Three hundred microliter of blocking solution were added to each well and left at RT for at least 1 hour. After further washes, 100μl of sample, or standards ranging from 31.25pg ml^{-1} to 4000pg ml^{-1}, were added to the wells in duplicate. The plate was incubated for 2 hours at RT and washed as before. Biotinylated goat anti-rat IL-1β 100μl, at a concentration of 350ng ml^{-1} were added to the plate at 100μl per well and left at RT for another 2 hours. The plate was washed and 100μl of streptavidin-HRP were added to all wells. The plate was incubated at RT for 20 minutes. The plate was then washed as before and 100μl of substrate reagent made of equal volume of hydrogen peroxide and tetramethylbenzidine (RandD Systems, Oxon, UK) were added to the plate, followed by incubation at RT for 20 minutes. The reaction was stopped with 50μl of 2N H_2SO_4. The plate was read at 450nm with a reference wavelength of 540nm using a Dynex MRX microplate reader.

2.10 Griess reaction

The concentrations of NO in the culture supernatants were measured as the concentrations of its oxidised product nitrite (NO_2^-) by Griess assay (Green et al., 1982). As shown in Figure 2.1 below, the chemical reaction uses sulfanilamide and N-1 napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. The colormetric assay measures the azo compound after a coupling reaction with a diazonium salt.

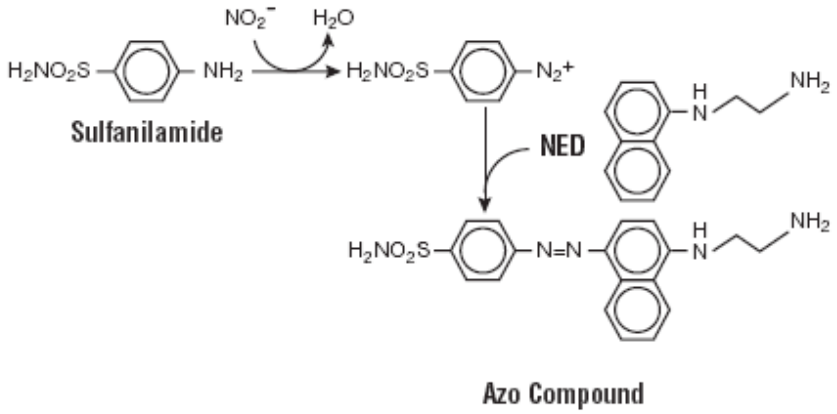


Figure 2.1 Chemical reactions involved in the measurement of NO_2^- using the Griess assay.

The Griess reagent was obtained from Sigma, Poole, UK. Standards were prepared by doubling dilution of sodium nitrite ($NaNO_2$) in ES medium, giving standards in the range of 2.25μM-144μM. ES medium was used as a blank. A volume of 50μl of Griess reagent were added to 50μl of standards and samples, followed by an incubation of at least 5 minutes at RT for the colour to develop. The plate was read in a Dynex MRX microplate reader at 550nm.

2.11 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT. The end products are dark blue formazan crystals that are largely accumulated within the healthy cells. By the addition of a detergent, the crystals are solubilised and can be quantified using a simple colorimetric assay.

Forty eight hours after cells were treated with LPS and/or NES as described in section 2.7.1, the plate was centrifuged at 140g for 5 minutes. After centrifugation, the supernatants were removed and replaced with 200µl fresh cell medium. A volume of 50µl of MTT (5 mg ml^{-1} in PBS) was also added to each well and the plate was incubated at 37°C for 4 hours. After 4 hours, the plate was centrifuged at 140g for 5 minutes. The supernatants were removed and replaced with 200µl iso-propanol. The liquid in each well was thoroughly mixed by pipetting up and down several times. The plate was again centrifuged at 140g for 10 minutes. A volume of 100µl of supernatants were transferred to a new 96-well plate and the OD was determined at 550nm using a Dynex MRX microplate reader. Cells incubated in cell medium or ES medium were used as a 100% cell viability control. Cell viability of each treatment group was determined as below:

Cell viability (%) = (OD treatment/ OD 100% cell viability control) x 100%

2.12 Movement of *N.brasiliensis* larvae

Sodium nitrite, urea or L-ornithine solutions were prepared by double diluting in ES medium to give a concentration range of 0.0625mM-72mM. A volume of 200µl of

each solution was added to each well in a 96-well plate and 200µl of ES medium were added to the first well as control. Forty *N.brasiliensis* larvae were also added to each well and incubated with the solutions for 18 hours at 37ºC. After 18 hours, each well was filmed for at least 1 minute in a random order under a dissection microscope which was connected with Ulead Photo Explorer 5.0. The movement of each larva was recorded as "thrash/minute" and a thrash was defined as a change in the direction of the bending at the mid-body of the larva (Miller et al., 1996). The movements of 5 to 8 larvae in each well were recorded.

2.13 NF-κB Western Blot

2.13.1 Cytoplasmic and nuclear protein extraction

NR8383 cells were grown and treated as described in section 2.7.2 for 1 hour before cytoplasmic and nuclear protein extraction. Cells were scraped from the 6-well plate and washed twice with ice-cold PBS. For cytoplasmic protein extracts, washed cells were lysed for 20 minutes on ice using 400µl buffer A (pH 7.6, 10mM Hepes, 10mM KCl, 2mM MgCl2, 1mM DTT, 0.1mM EDTA, 0.1% v/v NP-40) with protease inhibitor cocktail (Aprotinin 1µg ml^{-1}, Leupeptin 1µg ml^{-1}, Antipain 1µg ml^{-1}; Peptain A 1µg ml^{-1}; Sodium fluoride 1mM; orthovanadate 1mM and PMSF 1mM) to solubilize cytosolic proteins. After incubation on ice, the resulting supernatants representing cytoplasmic lysates were separated by centrifugation at 12000g for 20 minutes at 4°C. The pellets were resuspended in 50µl buffer B (pH 7.6, 10mM Hepes, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 10% v/v glycerol) with protease inhibitor cocktail (as described above) and incubated on ice for 30 minutes. Nuclear extracts were removed following centrifugation at 12000g for 20 minutes at 4°C. The protein concentrations in both cytoplasmic and nuclear

extractions were determined by Bradford protein assay. The samples were stored at -80°C until further use.

2.13.2 Western blot analysis of NF-κB p65

Western blot analysis of NF-κB p65 was conducted using nuclear and cytoplasmic protein samples as extracted above. Samples with equal amount of protein (10μg) were mixed with half volume of 2xSDS sample buffer consisting of 20% glycerol (v/v), 4% SDS (w/v), 125 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol (v/v), and 0.0025% bromophenol blue (w/v) and then boiled at 95ºC for 8 min before loading to a 5-10% polyacrylamide SDS-containing gel. Biotinylated molecular weight markers (Bio-Rad laboratories, Herts, UK) were prepared according to the manufacture's instructions and loaded onto the gel along with the samples. The recipes for 10% separating gel and 5% stacking gel are shown in Table 2.1 and Table 2.2. The SDS-PAGE gel was then run at 150V for 2 hours, and the gel-separated proteins were transferred to nitrocellulose membrane in transfer buffer (pH 8.5, 25mM Tris base, 0.2M Glycine and 20% v/v methanol) at 100V for 90 minutes. TBS buffer was prepared as 0.05M Tris base in 0.9% NaCl solution (pH 7.6). The membrane was blocked in blocking buffer (5% w/v dry milk powder in TBS, 0.1% v/v Tween-20) at RT for 1 hour. The membrane was washed in TBST (0.1% v/v Tween-20 in TBS) and incubated with primary rabbit antibodies against NFκB p65 subunit (1:1000 diluted with 3% w/v BSA in TBST) overnight at 4°C. After washing, the membrane was incubated with secondary anti-rabbit HRP conjugated antibody (1:5000 diluted with 3% w/v BSA in TBST) at RT for 1 hour. To detect the protein marker, an extra incubation step of the membrane in streptavidin-HRP (1:2000 diluted with 3% w/v BSA in TBST) for 1 hour was also

required. The membrane was then processed with enhanced chemiluminesescence (ECL) detection reagents (Amersham Biosciences, Buckinghamshire, UK). The processed membrane was then exposed to photographic films (Amersham Biosciences, Buckinghamshire, UK) for visualisation. The film was analysed using Genesnap software (Syngene, Cambridge, UK) and the band intensity measured using Genetools software (Syngene, Cambridge, UK).

2.14 Rat TNF-α western blot

Recombinant rat TNF-α (rrTNF-α, Immuno Tools, Friesoythe, Germany) was incubated with or without NES protein for 18 hours to study the possible post-translational degradation of rrTNF-α by NES protein. A total amount of 300ng rrTNF-α was incubated with 600ng trypsin as a positive degradation control and rrTNF-α in ES medium was used as a no degradation control. 5-15% SDS-acrylamide gels were prepared according to the recipes in Table 2.1 and Table 2.2. Eighteen hours after incubation, samples containing the same amount of rrTNF-α (300ng) were mixed with a half volume of 2xSDS sample buffer consisting of 20% glycerol, 4% SDS, 125 mM Tris-HCl (pH 6.8), 10% 2-Mercaptoethanol, and 0.0025% bromophenol blue and then boiled at 95ºC for 8 min before loading to the gel. Biotinylated molecular weight markers (Sigma, Poole, UK) were prepared according to the manufacturer's instructions and loaded to the gel along with the samples. The SDS-PAGE gel was then run at 300V for 80 minutes, and the gel-separated proteins were transferred to nitrocellulose membrane in transfer buffer at 100V for 90 minutes. The membrane was blocked in blocking buffer at RT for 1 hour. The membrane was washed in TBST and incubated with primary goat anti-

mouse TNF-α (1:1000 diluted with 3% BSA in TBST) overnight at 4°C. After washing, the membrane was incubated with secondary anti-goat HRP conjugated antibody (1:5000 diluted with 3% BSA in TBST) at RT for 1 hour. To detect the protein marker, an extra incubation step of the membrane in streptavidin-HRP (1:2000 diluted with 3% BSA in TBST) for 1 hour was also required. The membrane was then processed with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Buckinghamshire, UK). The processed membrane was then exposed to photographic films (Amersham Biosciences, Buckinghamshire, UK) for visualisation. The film was visualized by Genesnap software (Syngene, Cambridge, UK).

2.15 SDS-PAGE of NES concentrate and staining

NES was concentrated at least twice at 3200g for 60 minutes at 4°C to reach a protein concentration of 200μg ml^{-1}. A 5-12% polyacrylamide SDS-containing gel was cast in a Hoefer dual gel caster unit (Amersham Biosciences, Buckinghamshire, UK). The recipes for the gels are shown in Table 2.1 and Table 2.2. The gels were transferred to a Hoefer TE 260 mini-vertical electrophoresis tank powered by an EPS 301 power supply (Amersham Biosciences, Buckinghamshire, UK) and immersed in electrophoresis running buffer (24 mM Tris base, 190 mM glycine, 0.1% w/v SDS). A total of 2μg of concentrated NES were mixed with an equal volume of SDS sample buffer consisting of 20% glycerol, 4% SDS, 125 mM Tris-HCl (pH 6.8), 10% 2-Mercaptoethanol, and 0.0025% bromophenol blue and then boiled at 95ºC for 8 min before loading to the gel. Biotinylated molecular weight markers (Sigma, Poole, UK) were prepared according to the manufacturer's instructions and loaded along with the samples.

The SDS-PAGE gel was then run at 150V for 2 hours. After electrophoresis, the gel was immersed in Coomassie blue staining solution (40% v/v methanol, 10% v/v acetic acid, 50% v/v dH_2O and 0.025% w/v coomassie brilliant blue) for at least 90 minutes. After 90 minutes, the stained gel was destained in destain solution (10% acetic acid, 20% methanol and 70% dH_2O) until the bands were clearly visible with lowest background. The gel was then fixed in fixing solution (50% ethanol, 10% acetic acid and 50% dH2O) overnight in preparation for silver stain.

	10%	12%	15%
dH_2O	8.0 ml	6.4 ml	4.4
1.5M tris buffer pH 8.8	5.0 ml	5.2 ml	5.2
30% acrylamide/Bis-acrylamide	6.7ml	8ml	10ml
10% SDS	200µl	200µl	200µl
10% ammonium persulfate (APS)	200µl	200µl	200µl
TEMED	8µl	8µl	8µl

Table 2.1 Recipe for separating gels in SDS-PAGE

	5% Stacking gel
dH_2O	5.5ml
1.0M tris buffer pH 6.8	1.0ml
30% acrylamide/Bis-acrylamide	1.3ml
10% SDS	80µl
10% ammonium persulfate (APS)	80µl
TEMED	8µl

Table 2.2 Recipe for 5% stacking gel

Silver staining was carried out using the ProteoSilver™ silver stain kit purchased from Sigma (Poole, UK). Briefly, the fixing solution was decanted and the gel was washed with 100 ml of 30% v/v ethanol for 10 minutes while gentle shaking. The ethanol solution was then removed and the gel washed for 10 minutes in 100 ml of

ultrapure water. Following this, the water was decanted and the gel sensitised for 10 minutes with 100 ml of sensitiser solution. After removal of sensitiser solution, the gel was washed in 200 ml of ultrapure water for 10 minutes. This was repeated twice prior to equilibration in 100 ml of silver solution for 10 minutes. Following this, the silver solution was decanted and the gel washed in 200 ml of ultrapure water for 1 minute, after which the water was replaced with 100 ml of developer solution for 3-7 minutes until the bands were clearly visible with lowest background. To stop the developing reaction, 5 ml of stop solution were added and the gel allowed to incubate for 5 minutes. The solution was then replaced with 200 ml of ultrapure water and the gel washed for 15 minutes. The gel was analysed analysed using Genesnap software (Syngene, Cambridge, UK).

2.16 Lectin blot of NES concentrate

A lectin blot was employed to detect the presence of glycoproteins in concentrated NES and to estimate the linkage type between the sugar chain and amino acid residue of the polypeptide chain. Two lectins, biotinylated concanavalin A (ConA, from *Canavalia ensiformis*, Sigma, Poole, UK), which binds the N-linked sugar chain, and biotinylated peanut lectin (PNA, from *Arachis hypogaea*, Sigma, Poole, UK), which binds the O-linked sugar chain, were used to distinguish the linkage type of the glycoproteins. For lectin blotting, NES was concentrated as before to reach a protein concentration of 200µg ml^{-1}. A total of 4µg protein was separated by 5-12% SDS-PAGE as described in section 2.16. After electrophoresis, the gel was transferred to nitrocellulose membrane and blocked in blocking buffer (5% dry milk powder in TBS, 0.1% Tween-20). After blocking, the membrane was soaked in biotinylated ConA or PNA (0.0005% w/v in TBST) at 4°C overnight. Lectins

reacted with glycoproteins according to its selectivity at this stage. The membrane was washed five times in TBST and transferred to streptavidin-HRP (1:5000 diluted in 3% BSA in TBST). The membrane was then washed another five times and processed with enhanced chemiluminesescence (ECL) detection reagents (Amersham Biosciences, Buckinghamshire, UK). The processed membrane was then exposed to photographic films (Amersham Biosciences, Buckinghamshire, UK) for visualisation. The film was analysed as described above (section 2.13.2).

2.17 Rat experiment

2.17.1 Rat instillation

Care and animal procedures were approved by Napier University Ethics Committee and licensed by the Home Office. Female Sprague-Dawley rats aged between 8 and 12 weeks were anaesthetized with halothane and cannulated with a laryngoscope to expose the trachea. Animals were instilled with 0.5 ml ES medium, 0.5 ml NES, 0.5 ml *E. coli* LPS (*E. coli* 0111:B4, 100ng ml^{-1}) or both, LPS (100ng ml^{-1}) in 0.5 ml NES. Rats instilled with 100ng ml^{-1} LPS only were treated as positive controls here, in a manner consistent with the previous published observation (Keir et al., 2004) for the purpose of a follow on investigation.

2.17.2 Bronchoalveolar lavage (BAL)

Six hours after instillation, the rats were sacrificed with a single intraperitoneal injection of 2ml pentobarbitone. The lungs were then cannulated, removed, and lavaged with 8ml volumes of sterile saline. The procedure was repeated four times in total and the first BAL fluid was kept separately.

2.17.3 Cytospin and differential cell count

The BAL fluid was centrifuged at 380g for 2 minutes and the supernatant of the first BAL fluid was removed and kept at -80°C until further use. The resultant cell pellets in BAL fluids were resuspended in 1ml of RPMI-1640 and pooled. The white blood cells were counted and the cell concentrations were adjusted to $1x10^6$ cells ml^{-1}.

The corrected cell suspension was mixed with RPMI-1640 medium supplemented with 2mM L-glutamine, 100Unit ml^{-1} penicillin, 100µg ml^{-1} streptomycin and 10% (v/v) heat inactivated FBS. A volume of 100µl was added to an assembled sample chamber and the cells were centrifuged at 95g for 5 minutes in a Shandon Cytospin 3. Three cytocentrifuge slides were prepared for each treatment.

The cytocentrifuge smears were stained with Diffquick (Raymond A. Lamb, London, UK) for differential cell counts. A blind count was performed to count a total of 600 cells per slide. The numbers of macrophages, neutrophils, eosinophils and lymphocytes were recorded. The rest of the BAL cells were prepared for RNA extraction.

2.17.4 Albumin assay

The albumin content in the BAL supernatants was determined by albumin assay using bromocresol green as reagent substrate. At pH=4.2, albumin binds with bromocresol green to produce a blue-green complex. Bromocresol green 0.066mM was prepared in 100mM succinate buffer (pH 4.2). A range of albumin standards ranging from 6.25µg ml^{-1} to 800µg ml^{-1} were prepared by double diluting

the top standard. Standards (100µl) were then pipetted into triplicate wells in a 96-well plate. Samples (100µl) were also added to triplicate groups of wells. A total volume of 100µl Bromocresol green solution was added to each well and the plate was mixed and read using a Dynex MRX microplate reader at 630nm.

2.17.5 Reduced glutathione (GSH) measurement

GSH content in the BAL supernatants was measured based on the method of Hissin and Hilf (1976). This method utilises O-phthalaldehyde (OPT) as a fluorochrome which binds to sulphydryl groups (-SH found within cysteine) within GSH to yield a fluorescent complex that enables the detection of GSH. A total volume of 250µl BAL supernatant was mixed with 500µl extraction buffer (9mM Tetra Sodium EDTA in 14% Perchloric Acid, mixed with same volume of PBS on the day of experiment) and left on ice for 15 minutes. The solution was centrifuged at 2370g for 4 minutes at 4ºC. The supernatants were transferred to new tubes and mixed with 500µl neutralisation buffer (1M potassium hydroxide and 1M potassium bicarbonate in 250ml dH_2O) before centrifugation at 2370g for 5 minutes at 4ºC. GSH standards were prepared with concentrations ranging from 0.78µM to 12.5µM by double diluting from the top GSH standard with GSH buffer (0.1M sodium dihydrogen orthophosphate, 0.005M tetra sodium EDTA in 100ml dH_2O, pH 8). A volume of 180ul GSH Buffer was added to each well in a 96-we/ll clear bottomed plate (Porvair sciences, Middlesex, UK). At the same time, 10µl standard/sample and 10µl OPT (1mg ml^{-1} in methanol) were also added to the wells and the plate was incubated for 15 minutes at room temperature. The plate was then measured using a Fluostar optima microplate shaker with fluorescence at excitation at 350nm and emission 420 nm. To standardise the results, protein

measurements (mg) in the BAL supernatants were taken so that the expression of GSH concentration could be standardised as μM mg^{-1}.

2.18 Reverse transcriptase-polymerase chain reaction (RT-PCR)

2.18.1 Diethyl pyrocarbonate (DEPC) treated water (DEPC-H_2O)

DEPC inactivates RNases by covalent modification (Solymosy et al., 1968). A typical DEPC concentration of 0.1% is commonly used to inactivate RNases to create RNase-free solution or water (Ehrenberg et al., 1976). DEPC treated water was used throughout all relevant experiments. DEPC (0.1% v/v) was added to sterile dH_2O and shaken vigorously to bring the DEPC into the solution. This was incubated at 37°C overnight. The DEPC-water was then autoclaved for at least 15 minutes to destroy any trace of DEPC. DEPC-H2O was kept at 4ºC for further use.

2.18.2 RNA extraction

Six hours after treatment as described in section 2.7.2, cells were scraped from the 6-well plate and centrifuged at 380g for 2 minutes to obtain the pellet. For rat experiments, BAL cells were centrifuged at 380g for 2 minutes to obtain the pellet. The cells were then washed three times in ice-cold Ca^{2+} and Mg^{2+} free PBS by centrifuging at 380g for 2 minutes. After washing, cells were suspended in 1000μl of Tri-reagent (Sigma, Poole, UK), and stored at -80°C until required to process. A volume of 200μl of chloroform was added to each tube and vortexed vigorously. This was allowed to stand at RT for 15 minutes. The mixture was centrifuged at 12000g for 15 minutes at 4°C. After centrifugation, the colourless upper phase was transferred to fresh eppendorfs, before adding 450μl isopropanol. The mixed samples were allowed to stand for another 10 minutes at RT. Again, the

eppendorfs were centrifuged at 12000g for 10 minutes at 4°C. The supernatants were poured out and the white RNA pellets were washed in 1ml of 75% ethanol; this was vortexed briefly before centrifuge at 7500g for 5minutes at 4°C. The supernatants were poured out and the RNA pellets were air-dried for 10 minutes. The RNA was then suspended in 20µl DEPC water followed by DNAse treatment (section 2.18.3) or stored at -80°C until use.

2.18.3 DNAse treatment

Before real time PCR, RNA samples were further treated to remove genomic DNA contamination. DNA free™ (Ambion, Warrington, UK) is a kit that has been designed to remove unwanted DNA from preparations of RNA and to clean up the sample afterwards by removing any traces of DNase and divalent cations. A volume of 2µl of 10x buffer were added to each RNA sample. This was followed by 1µl of DNAse1 enzyme. Samples were then incubated at 37ºC for 30 minutes before the addition of 5µl of DNAse inactivation reagent. The inactivation reagent removes divalent cations such as calcium and magnesium from the sample. These cations catalyze the degradation of RNA at temperatures above 60ºC. Samples were then spun at 12000g for 2 minutes. The supernatants were removed to clean RNA-free tubes.

2.18.4 RNA quantification

A 1:100 dilution of the RNA sample was prepared in DEPC-H_2O. The RNA solution was then analysed at wavelengths of 260nm and 280nm in a spectrometer. DEPC-H_2O only was used as a blank. For each sample, the absorbance ratio of 260nm to 280nm was ensured to be 1.8 to 2.0 before the RNA

was used for future analysis. The following calculation was made to quantify the RNA concentration:

RNA in μg μl^{-1} = (40 x dilution factor x absorbance at 260nm) / 1000

2.18.5 Two step quantitative real time PCR (Q-PCR)

2.18.5.1 cDNA synthesis

cDNA synthesis was performed after DNase treatment as described in section 2.18.3. For NR8383 cell analysis, 1μg of RNA was used for cDNA synthesis. For rat instillation samples, 0.5μg of RNA was used for reverse transcription. To produce top-standard cDNA for Q-PCR standard curves, 2μg of RNA from LPS-treated NR8383 was used for reverse transciption and 1μg of RNA from LPS instilled rat BAL cells were used for cDNA synthesis.

The mixture for cDNA synthesis was prepared as Table 2.3, and the total volume for each reaction was made up to 25μl by DEPC-H_2O. This mixture was centrifuged briefly and placed in a/ thermal cycler (ThermoHybaid PCR express, Abgene Ltd., Epsom, UK). The reverse transcription programme was set up as Table 2.4. The synthesized cDNA was stored at -20°C until use.

2.18.5.2 Q-PCR primers

Gene specific PCR primers (MWG AG Biotech, Ebersberg, Germany) for Q-PCR were designed based on nucleotide sequences in GenBank database, and according to previously published primers or designed by Probefinder version 2.40 (Universal ProbeLibrary, Roche applied sciences, Burgess Hill, UK) (Table 2.5). The primers were prepared following the instructions provided by the manufacturer

by adding DEPC-H_2O to obtain 100pmol μl^{-1}. The sizes of the amplified products were confirmed by running Q-PCR products on a 2% agarose gel.

	Volume for one reaction (µl)
RNA	Volume for 1µg of RNA
Random Nonamers (50µM)	1µl
MgCl2 (25mM)	5µl
5xrecombinant Avian Myeloblastosis Virus (AMV)/Tfl reaction buffer	5µl
dNTP Mix (10mM each)	1µl
AMV reverse transcriptase (5 U μl^{-1})	1µl
DEPC-H2O	adjustable (µl)
Total volume	25µl

Table 2.3 Mixture for cDNA synthesis

cDNA synthesis	1 cycle	48°C	45 minutes
		94°C	2 minutes

Table 2.4 cDNA sythesis (reverse transcription) programme.

2.18.5.3 Q-PCR standards and coefficient files

cDNA reverse transcribed from 0.5µg or 1µg positive control (LPS treated) RNA was serial diluted ten-fold in triplicate to produce standard curves. The GAPDH gene was used as the reference gene throughout all experiments. The amplification coefficiency files were produced using RelQuant software (Roche

applied sciences, Burgess Hill, UK), and were used for efficiency correction for each run.

2.18.5.4 Q-PCR programme and relative quantification

The Q-PCR was performed using a Light-Cycler (Roche applied sciences, Burgess Hill, UK). Along with the cDNA samples, a calibrator which was 1:10 dilution of a positive control cDNA, was used throughout all experiments. PCR amplifications were performed in 20µl, containing 2µl of cDNA, 1µl of primer mix (2.5pmol μl^{-1} each), 10µl of SYBR green JumpStart Taq ready mix (Sigma, Poole, UK) and 7µl of DEPC-H_2O. The PCR mix was then added to glass capillaries and centrifuged in a capillary centrifuge (Roche applied sciences, Burgess Hill, UK).

The conditions for Q-PCR were one cycle of denaturation at 94ºC/30s followed by 45 PCR cycles as shown in Table 2.6. The melting curve analyses were performed for one cycle at 95ºC/0s, 65ºC/15s and 95ºC (temperature transition rate=0.1ºC/s) for denaturation, annealing and melting, respectively. Each gene was quantified from the standard curve with efficiency correction using RelQuant software (Roche applied sciences, Burgess Hill, UK). The quantification was determined as the ratio of target gene to GAPDH gene, and the results were expressed as the fold change to positive control gene levels.

Rat gene	*Primer sequence*	*Product size (bp)*	*References or design*
GAPDH	Forward 5'-ATG ACT CTA CCC ACG GCA AG-3' Reverse 5'-CCA CAG TCT TCT GAG TGG CA-3'	419	Caspersen et al., 2000
TNF-α	Forward 5'-TAC TGA ACT TCG GGG TGA TTG GTC C-3' Reverse 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'	294	Liu and Dubick, 2004
IL-1β	Forward 5'-GAA GCT GTG GCA GCT ACC TAT GTC T-3' Reverse 5'-CTC TGT TGA GAG GTG CTG ATG TAC-3'	520	Lin et al., 2000; Liu and Dubick, 2004
ICAM-1	Forward 5'-GGG CCC CCT ACC TTA GGA A-3' Reverse 5'-GGG ACA GTG TCC CAG CTT TC-3'	101	Hartner et al., 2005
MIP-2	Forward 5'- GCA AGG CTA ACT GAC CTG GA-3' Reverse 5'-CTT TGA TTC TGC CCG TTG AG-3'	64	Probefinder
iNOS	Forward 5'-GAT TTT CCC AGG CAA CCA G-3' Reverse 5'-GGC GAA GAA CAA TCC ACA AC-3'	69	Probefinder

Table 2.5 Rat primers for Q-PCR.

2.18.6 Agarose gels

Agarose gels (2% w/v) were used to confirm the product sizes of each gene after Q-PCR. Four grams of agarose were dissolved in 200ml of 1xTBE buffer (0.089M Tris, 0.089M boric acid and 0.002M EDTA in dH_2O, pH 8.0 adjusted with HCl) by heating in a microwave. Ten microliter of ethidium bromide (500μg ml^{-1}) were added into the agarose and TBE mix. After the gels were settled in a horizontal gel apparatus, 10μl of samples and Φ174 DNA/Hae III marker (Promega, Southampton, UK) were loaded to each well by 5:1 diluting with Blue/orange 6X loading dye (Promega, Southampton, UK). The gels were run at 100V for 90 minutes. After 90mnutes, the gels were visualized by Genesnap software (Syngene, Cambridge, UK).

Rat gene	***Denaturation (Temperature/ time)***	***Annealing (Temperature/ time)***	***Extension (Temperature / time)***	***Detection (Temperature / time)***
GAPDH	94ºC/0s	54°C/10s	72ºC/17s	83°C/5s
TNF-α	94ºC/0s	59°C/10s	72ºC/12s	85°C/5s
IL-1β	94ºC/0s	58°C/10s	72ºC/21s	82°C/5s
MIP-2	94ºC/0s	52°C/10s	72ºC/3s	77°C/5s
ICAM-1	94ºC/0s	56°C/10s	72ºC/5s	79°C/5s
iNOS	94ºC/0s	51°C/10s	72ºC/3s	80°C/5s

Table 2.6 PCR conditions for Q-PCR.

2.19 Statistical analysis

All values are expressed as the mean ± standard deviation from at least three independent experiments. The data was analysed using a general linear model of analysis of variance (ANOVA) with Tukey's pairwise comparisons. The significance level was set at 0.05.

.

Chapter 3

Downregulation of lipopolysaccharide-induced inflammation by *N.brasiliensis* larvae and *N.brasiliensis* larvae ES (NES) i*n vitro*

3.1 Introduction

Macrophages are the main cell types in the innate immune system and are involved in the first line of defence against invading microorganisms. As introduced in Chapter 1, the activation of macrophages differs according to the antigen types. Endotoxin lipopolysaccharide (LPS) is a major component of the gram-negative bacterial cell wall, which has been widely used to classically activate macrophages. Upon recognition of LPS, macrophages produce Th1 pro-inflammatory mediators to restrain the pathogen and enable efficient recruitment of other immune cells. These mediators include Th1 cytokines, such as TNF-α, IL-1β and IL-12, and nitrosative species including nitric oxide. In this chapter, macrophages were stimulated with LPS as an *in vitro* model of Th1 inflammation.

The pulmonary phase of *N.brasiliensis* infection has been associated with low levels of Th1 inflammation, characterised by a lack of TNF-α production and limited neutrophil recruitment (McNeil et al., 2002). A significant increase of IL-1 was detected 24 hours after infection but the production ceased thereafter (Benbernou et al., 1992). Instead, a strongly polarized Th2 immune response has been associated with the lung stage. This was supported by the evidences of an increased IL-4, IL-5 and IL-13 production in the BAL fluid 48 hours post infection (Keir, PhD thesis) and the dominant IL-4 and IL-13 expressing lymphoid cells in subsequent pulmonary granulomatous lesions developed two weeks after the infection (Matsuda et al., 2001). Eosinophils and alveolar macrophages are dominant in the pulmonary phase and both cell types have been demonstrated to be protective against migrating *N.brasiliensis* larvae (Egwang et al., 1984; Shin et

al., 1997; Knott et al., 2007). In addition, intranasal administration of *N.brasiliensis* L3 larvae ES products to presensitized mice induced a strong allergic airway immune response characterized by Th2 cytokine production, eosinophilia and IgE antibody production (Marsland et al., 2005). Due to the counter-regulatory effects of Th1 and Th2 cytokines, this highly polarized Th2 immune response provoked our interest in the ability of *N.brasiliensis* regulating the early immune responses towards the LPS induced Th1 inflammation in the lungs.

Various helminth ES products have been demonstrated to down-regulate Th1 immune responses (Maizels and Yazdanbakhsh, 2003). It has been recently discovered that Th1 inflammation associated with spontaneous arthritis in MRL/lrp mice was ameliorated in *N.brasiliensis* infection (Salinas-Carmona et al., 2009). However, as an established potent activator of Th2 immune response, the effect of *N.brasiliensis* larvae and their ES products (NES) on LPS-induced inflammation remains unrecognized. Here we proposed that the response of alveolar macrophages to LPS may be altered by *N.brasiliensis*. In contrast to LPS, *N.brasiliensis* alternatively activates alveolar macrophages when migrating through the lungs (Reece et al., 2006). The phenotype change of alveolar macrophages may play a role in the regulation of early immune responses by *N.brasiliensis*. NR8383 is a rat alveolar macrophage cell line, which has been characterized and widely used as a convenient, reliable substitute for freshly derived rat alveolar macrophages (Helmke et al., 1989). These cells were applied as a model for studying immune properties of rat alveolar macrophages in our experimental conditions. In this chapter, NR8383 cells were stimulated with LPS along with the increasing numbers of live *N.brasiliensis* larvae and increasing

amounts of NES. The effect of *N.brasiliensis* live larvae and NES on LPS-stimulated inflammation was determined by measurement of TNF-α, IL-1β and nitric oxide.

3.2 Results

3.2.1 Optimisation of LPS concentration for stimulation

LPS activates rat alveolar macrophages to release cytotoxic free radicals, including reactive oxygen species such as the superoxide anion ($O_2^{-}\cdot$) and nitrosative species such as nitric oxide (NO) and peroxynitrite ($ONOO^{-}\cdot$). The level of NO produced by LPS-stimulated macrophages was used to indicate inflammation levels in this experiment.

NR8383 macrophages were incubated with different concentrations of LPS (*E.coli* 0111:B4, 0-200ng ml^{-1}) in a 96-well plate for 48 hours, before the NO production in the supernatants was measured as nitrite by the Griess assay. Significant amount of NO was produced in response to 25ng ml^{-1} LPS and NO produced by 25-200ng ml^{-1} remained constant (Figure 3.1). 25ng ml^{-1} LPS stimulated macrophages were applied as the *in vitro* inflammation model throughout the whole study.

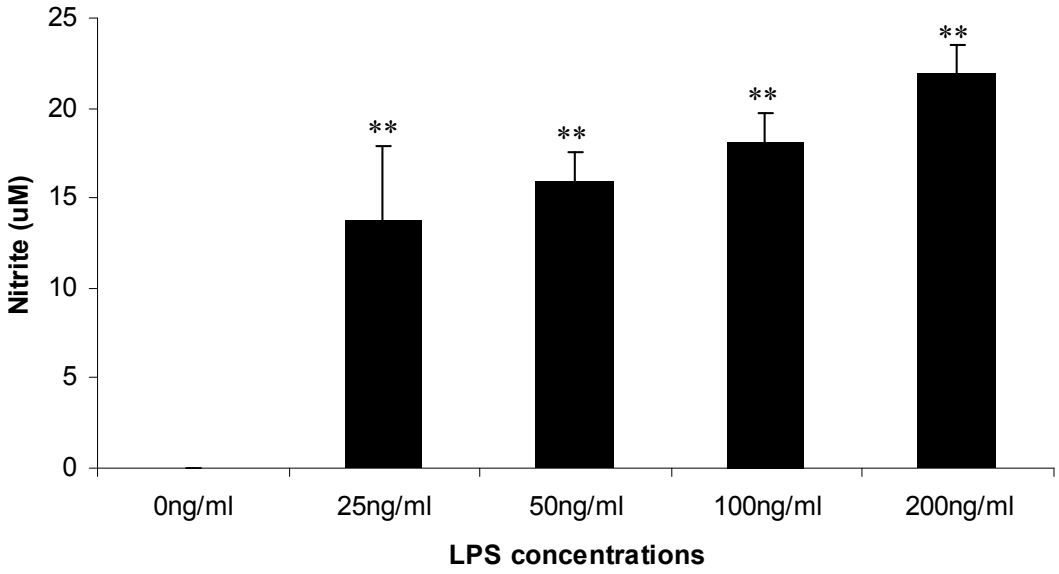


Figure 3.1 Nitric oxide productions in NR8383 cells stimulated with different concentrations of LPS. NR8383 cells ($2x10^4$ cells) were incubated with different concentrations of LPS in 96-well plates for 48 hours before NO levels in culture supernatants were measured as nitrite by Griess assay. The result represents the means of two independent experiments. All values are expressed as mean ± SD (n=2). **: $p<0.01$ compare to 0ng ml^{-1}.

3.2.2 Effects of live *N.brasiliensis* larvae and NES on TNF-α production

As shown in Figure 3.2, 25ng ml^{-1} LPS stimulated NR8383 cells to produce a significant amount of TNF-α which ranged at 2.48±1.00ng ml^{-1}. Live *N.brasiliensis* L3 larvae (L3s) alone, 20-80 larvae, did not stimulate any TNF-α production in NR8383 cells. Compared to LPS alone, TNF-α production in LPS-stimulated NR8383 cells was significantly reduced by adding 20, 50 or 80 (all $p<0.01$) larvae. Increasing larvae number from 20 to 50 ($p<0.05$) and to 80 ($p<0.01$) increased the reduction in TNF-α significantly. The reduction was more than 50% after incubation with 50 and 80 larvae compared to LPS alone.

NES alone did not stimulate TNF-α production in NR8383 cells (Figure 3.3). To further investigate the effect of NES, NR8383 cells were co-stimulated with LPS (25ng ml^{-1}) and 25% or 50% of NES for 48 hours. As shown in Figure 3.3, TNF-α production was significantly reduced by adding 25% ($p<0.01$) and 50% of NES ($p<0.01$) to the cells. The reduction ranged from 40-50%. Less TNF-α was released by cells when 50% NES was used but the levels measured here were not significantly different to those observed after incubation with 25% NES.

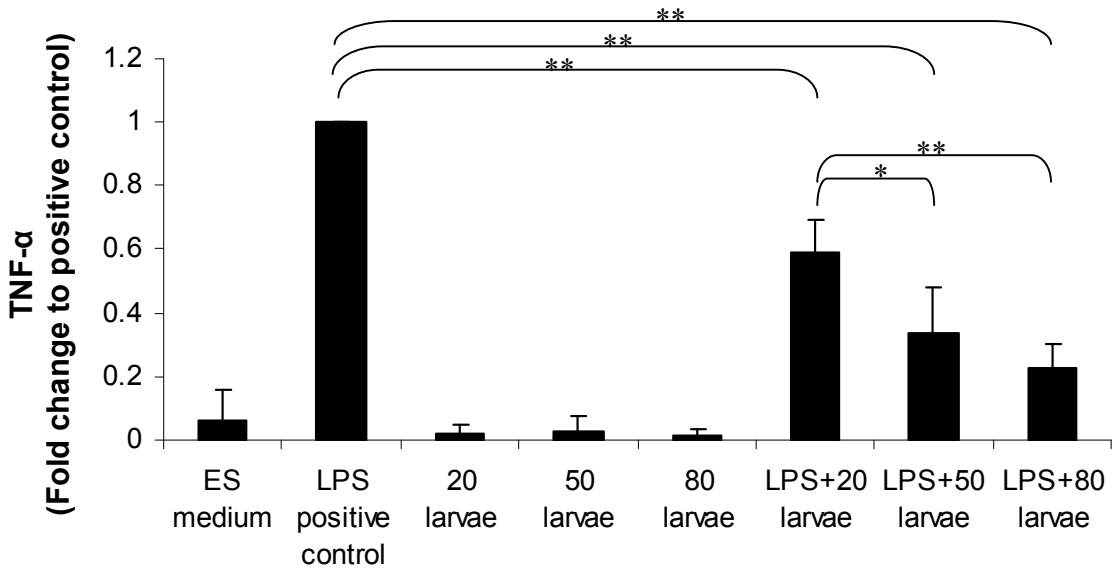


Figure 3.2 The fold change of TNF-α production in each treatment compare to TNF-α production in the positive control (2.48±1.00ng ml^{-1}), showing the effect of *N.brasiliensis* larvae. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3s (20, 50, 80) or LPS (25ng ml^{-1}) and L3s (20, 50, 80) in 96-well plates for 48 hours before TNF-α levels in culture supernatants were measured by ELISA. Cells treated with 25ng ml^{-1} LPS alone were used as the positive control. The result shows the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=3). *: $p<0.05$; **: $p<0.01$.

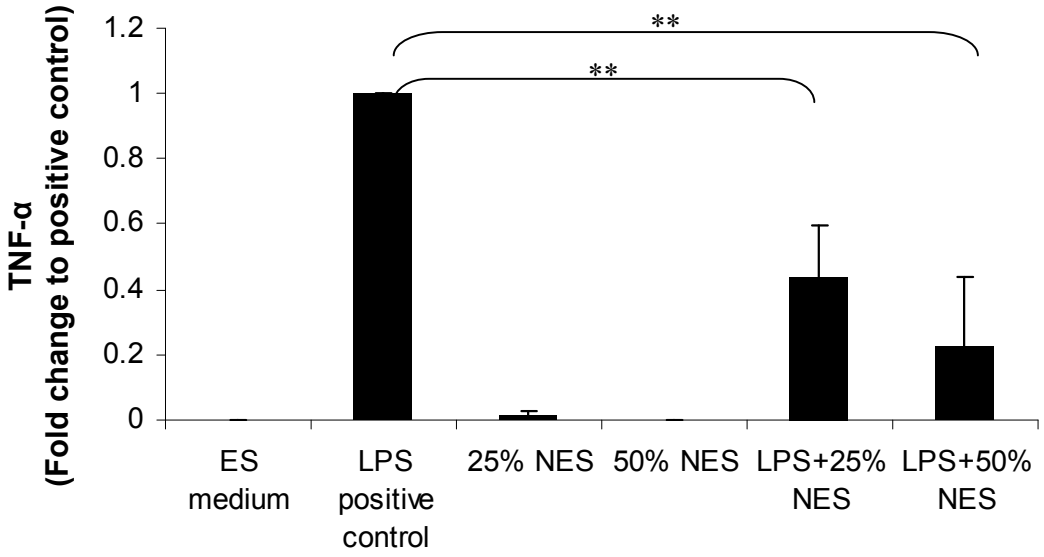


Figure 3.3 The fold change of TNF-α production in each treatment compared to TNF-α production in the positive control (range 1.62±0.38ng ml^{-1} TNF-α), showing the effect of NES. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3 NES (25%, 50%) or LPS (25ng ml^{-1}) with L3 NES (25%, 50%) in 96-well plates for 48 hours before TNF-α levels in culture supernatants were measured by ELISA. Cells treated with 25ng ml^{-1} LPS alone were used as the positive control. The result represents the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=3). **: $p<0.01$.

3.2.3 Effects of *live N.brasiliensis* larvae and NES on IL-1β production

As shown in Figure 3.4 and Figure 3.5, cells incubated in the ES medium produced some IL-1β. Live *N.brasiliensis* larvae did not stimulate IL-1β production in NR8383 cells, and they had no effect on LPS-stimulated IL-1β production (Figure 3.4). Similarly, both 25% and 50% NES did not induce IL-1β production. 25% or 50% of NES had no effect on IL-1β production compared to 25ng ml^{-1} LPS only (Figure 3.5).

3.2.4 Effects of *N.brasiliensis* larvae and NES on NO production

A small amount of NO (detected as nitrite) was produced by NR8383 cells incubated with live larvae or NES, which was not different from the cells in ES medium only (Figure 3.6 and Figure 3.7). As shown in Figure 3.6, NO production in LPS-stimulated macrophages was significantly reduced by co-incubation with 20, 50 and 80 larvae/well (all $p<0.01$). NO production in NR8383 cells was also inhibited significantly by 25% ($p<0.01$) and 50% L3 NES ($p<0.01$) compared to LPS-stimulation only (Figure 3.7). There was no observed relationship between the reduction level and the larvae number or the amount of NES.

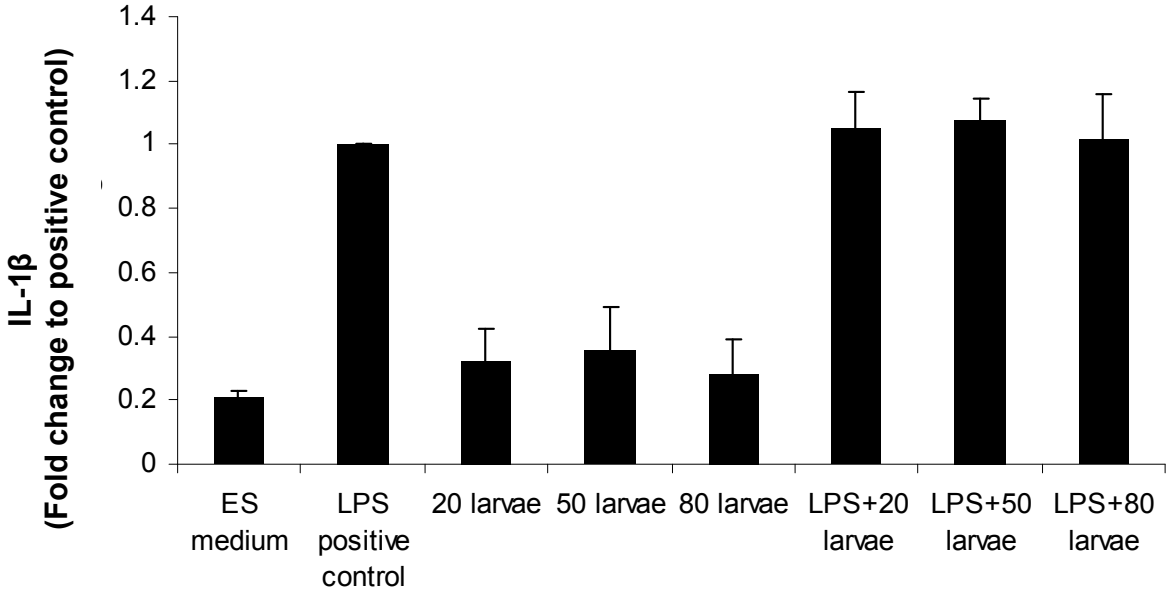


Figure 3.4 The fold change of IL-1β production in each treatment compared to IL-1β production in the positive control (range 1.03±0.08ng ml^{-1} IL-1β), showing the effect of *N.brasiliensis* larvae. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3s (20, 50, 80) or LPS (25ng ml^{-1}) and L3s (20, 50, 80) in 96-well plates for 48 hours before IL-1β levels in culture supernatants were measured by ELISA. Cells treated with 25ng ml^{-1} LPS alone were used as the positive control. The result shows the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=3).

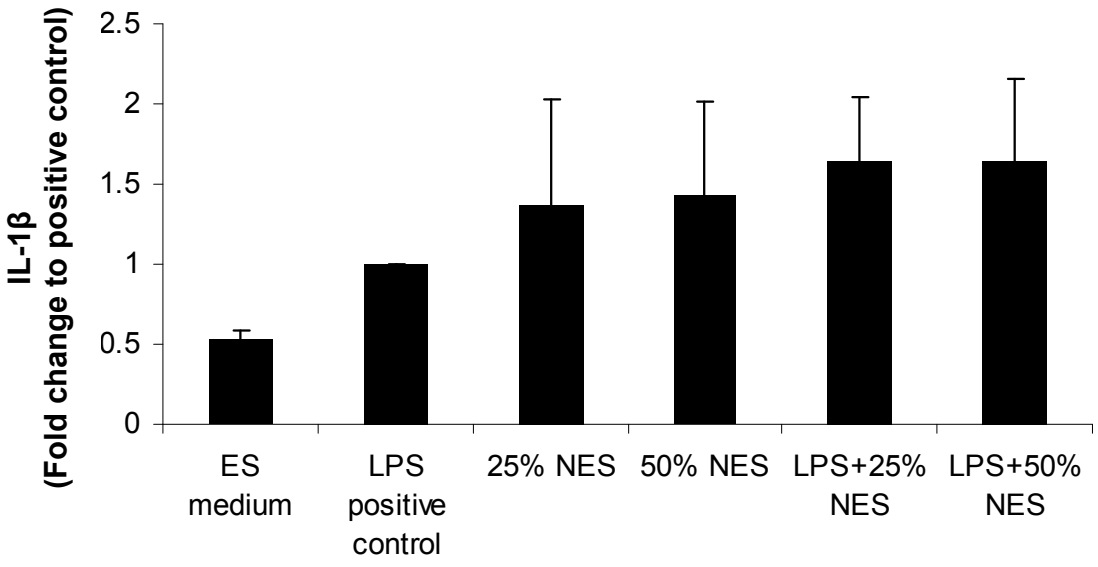


Figure 3.5 The fold change of IL-1β production in each treatment compared to IL-1β production in the positive control (range 1.18±0.10ng ml^{-1} IL-1β), showing the effect of NES. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3 NES (25%, 50%) or LPS (25ng ml^{-1}) with L3 NES (25%, 50%) in 96-well plates for 48 hours before IL-1β levels in culture supernatants were measured by ELISA. Cells treated with 25ng ml^{-1} alone were used as the positive control. The result represents the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=3).

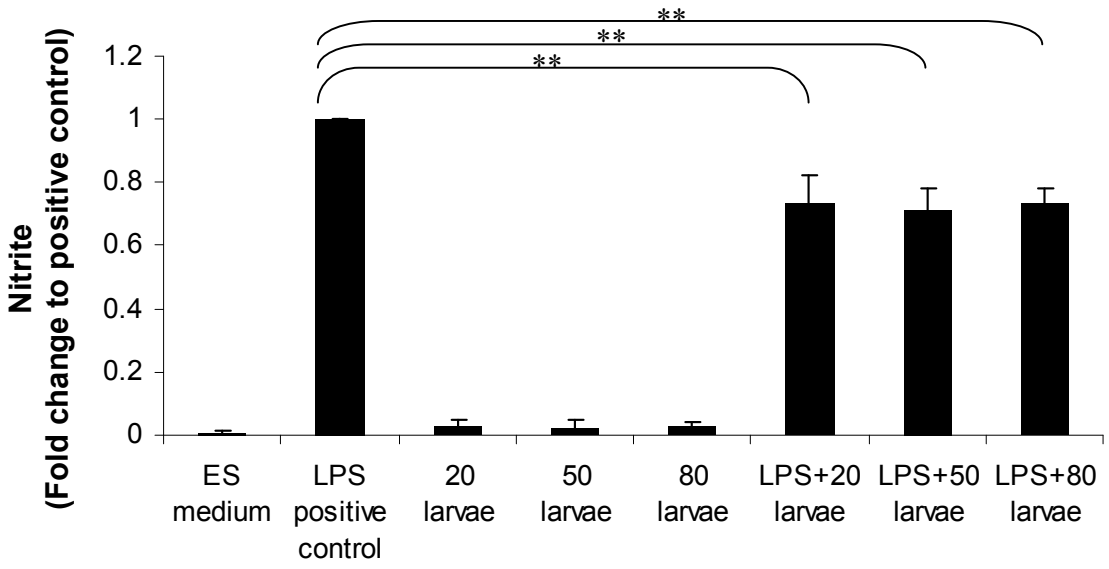


Figure 3.6 The fold change of nitric oxide production in each treatment compared to nitric oxide production in the positive control (range 26.44±8.96μM nitrite), showing the effect of *N.brasiliensis* larvae. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3s (20, 50, 80) or LPS (25ng ml^{-1}) and L3s (20, 50, 80) in 96-well plates for 48 hours before NO levels in culture supernatants were measured as nitrite by Griess assay. The result represents the means of five independent experiments each with three replicates. All values are expressed as mean ± SD (n=5). **: $p<0.01$.

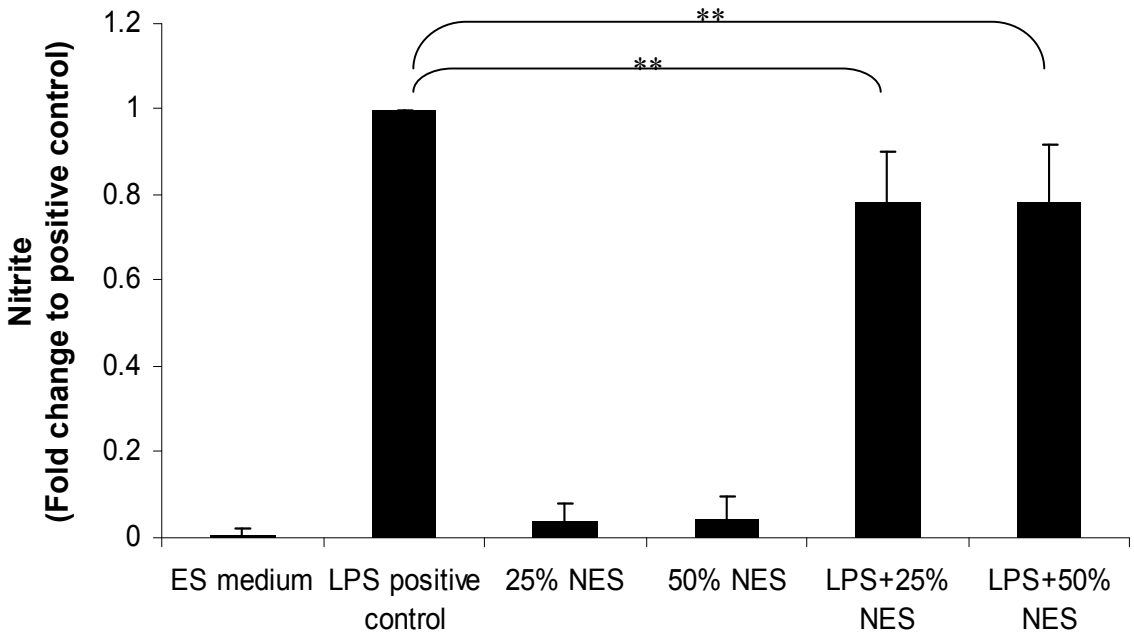


Figure 3.7 The fold change of nitrite production in each treatment compared to nitrite production in the positive control (range 17.07±6.10μM nitrite), showing the effect of NES. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}), L3 NES (25%, 50%) or LPS (25ng ml^{-1}) with L3 NES (25%, 50%) in 96-well plates for 48 hours before NO levels in culture supernatants were measured as nitrite by Griess assay. The result represents the means of eight independent experiments each with three replicates. All values are expressed as mean ± SD (n=8). **: $p<0.01$.

3.2.5 Evaluation of *N.brasiliensis* larvae and NES cytotoxicity

The toxicity of NES was evaluated by MTT cytotoxicity assay. Before incubation with NR8383 cells, NES was diluted 1/2, 1/10 and 1/100 with ES medium or without dilution. In figure 3.8, cells incubated in cell culture medium only were used as 100% viability controls. Cell viabilities were above 90% across all treatments, and there was no significant difference in viability levels between each treatment. Therefore, it would not appear that NES is toxic to NR8383 cells.

In Figure 3.9, cells incubated in ES medium were used as 100% viability controls. LPS (25ng ml^{-1})-treated cells showed high cell viability 48 hours after incubation. NR8383 cells co-incubated with LPS (25ng ml^{-1}) together with increasing amounts of NES did not vary significantly in their viability levels compared with the medium only or LPS only controls. This data showed that the inhibition of LPS-stimulated TNF-α and NO production by NES was not caused by cell death.

It was difficult to separate the cells and *N.brasiliensis* larvae after they were incubated together. The presence of the larvae has been suspected to affect the accuracy and sensitivity of MTT assay (Thomas et al., 1997). Lactate dehydrogenase (LDH) assay is another cell viability assay that measures the levels of LDH in the culture medium that are released from dead cells. Expressed sequence tags (ESTs) from *N.brasiliensis* L3 cDNA have been shown to include sequences (GenBank access number: EB390654, EB186419 and EB186418) that are homologues to rat LDH. LDH assay may be affected by the released LDH from the dead larvae and therefore considered to be inappropriate for cell viability test in this case.

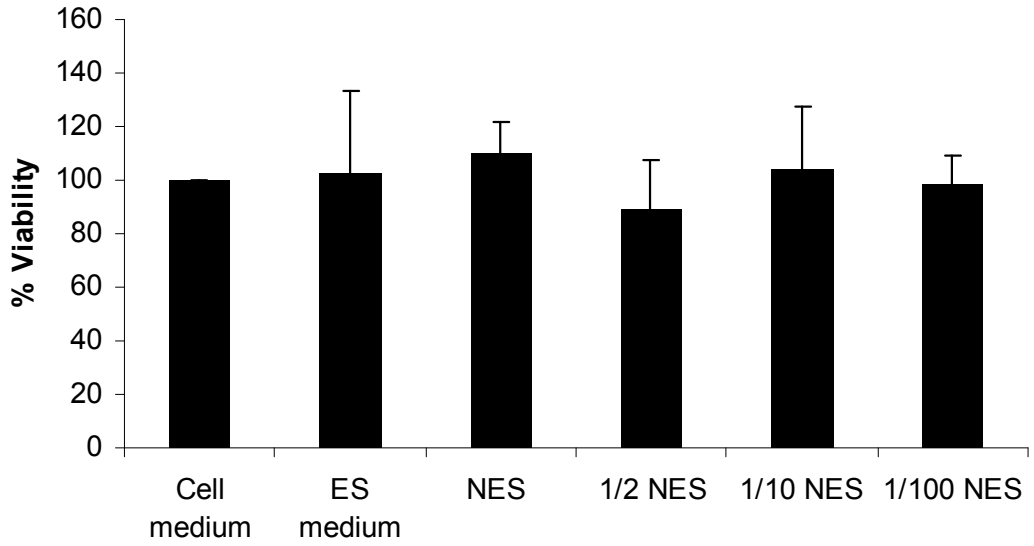


Figure 3.8: Viability of NR8383 cells after treatment with NES. NR8383 cells ($2x10^4$ cells) were incubated in cell culture medium, ES medium or in different dilutions of NES for 48 hours before cell viability was measured by MTT assay. Cells incubated in cell medium were used as 100% viability controls. Cell viability following each treatment was calculated as a percentage of the OD value compared to that of the 100% viability control. The result represents the means of three replicates in one experiment. All values are expressed as mean ± SD (n=3).

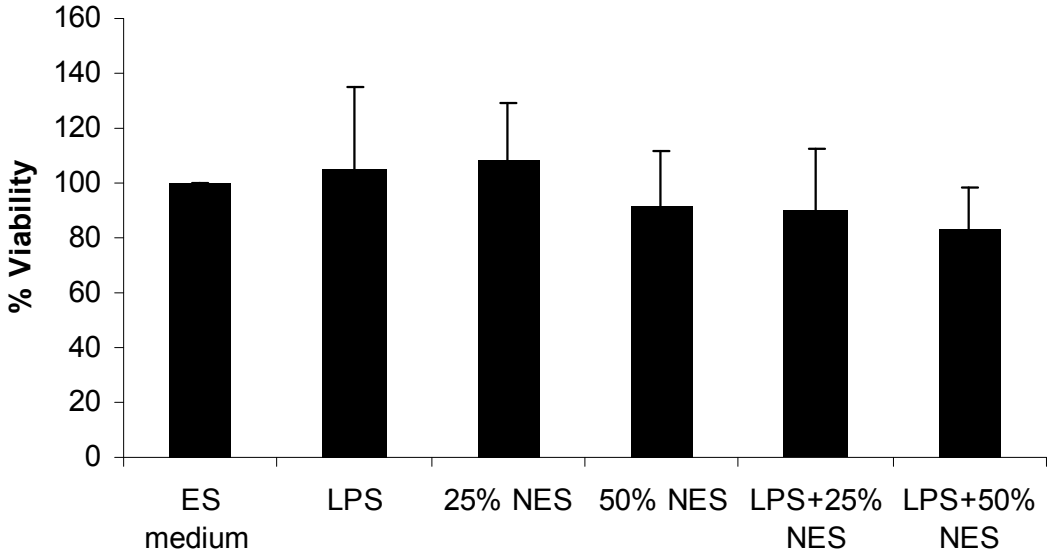


Figure 3.9: Cell viability of NR8383 cells after different treatments. NR8383 cells ($2x10^4$ cells) were treated with LPS (25ng ml^{-1}), NES (25%, 50%) or LPS (25ng ml^{-1}) with NES (25%, 50%) in 96-well plate for 48 hours before cell viability was measured by MTT assay. Cells incubated in ES medium were used as 100% viability control. Cell viability in each treatment was calculated as the percentage of the OD value compared to that of the 100% viability control. The result represents the means of six independent experiments each with three replicates. All values are expressed as mean ± SD (n=6).

3.2.6 Interaction between NR8383 cells and *N.brasiliensis* L3 larvae

48 hours after incubation, the wells in 96-well plates that contained both the larvae and macrophages were observed under an inverted microscope (400 × magnification). Different stages of cell-larvae interactions were detected under the microscope. However, this phenomenon was not dominant and there were still more than 95% of larvae moving freely in the culture. Representative fields that have visible cell-larvae binding were chosen and the photographs were taken with a Sony DSC-S70 digital camera. The cell-larvae interactions observed include the polarization, attachment and wrapping of macrophages to the larvae. There were also several “rods” formed by larvae surrounded by attached macrophages, and no movement of these larvae were observed. These ‘still’ larvae were only observed occasionally (Figure 3.10 A-C).

3.2.7 Movement of *N.brasiliensis* larvae in sodium nitrite, urea and L-ornithine

This experiment was conducted in order to assess the potential toxic effects of L-arginine metabolic products, including nitrite, urea and L-ornithine, which are associated with macrophage phenotypes. The results showed no significant difference of larval movement in different concentrations of sodium nitrite (figure 3.11A), urea (figure 3.11B) and L-ornithine (Figure 3.11C) as measured by thrashes per minute. The larvae were shown to be unaffected to even the highest concentrations (72mM) of each solution.

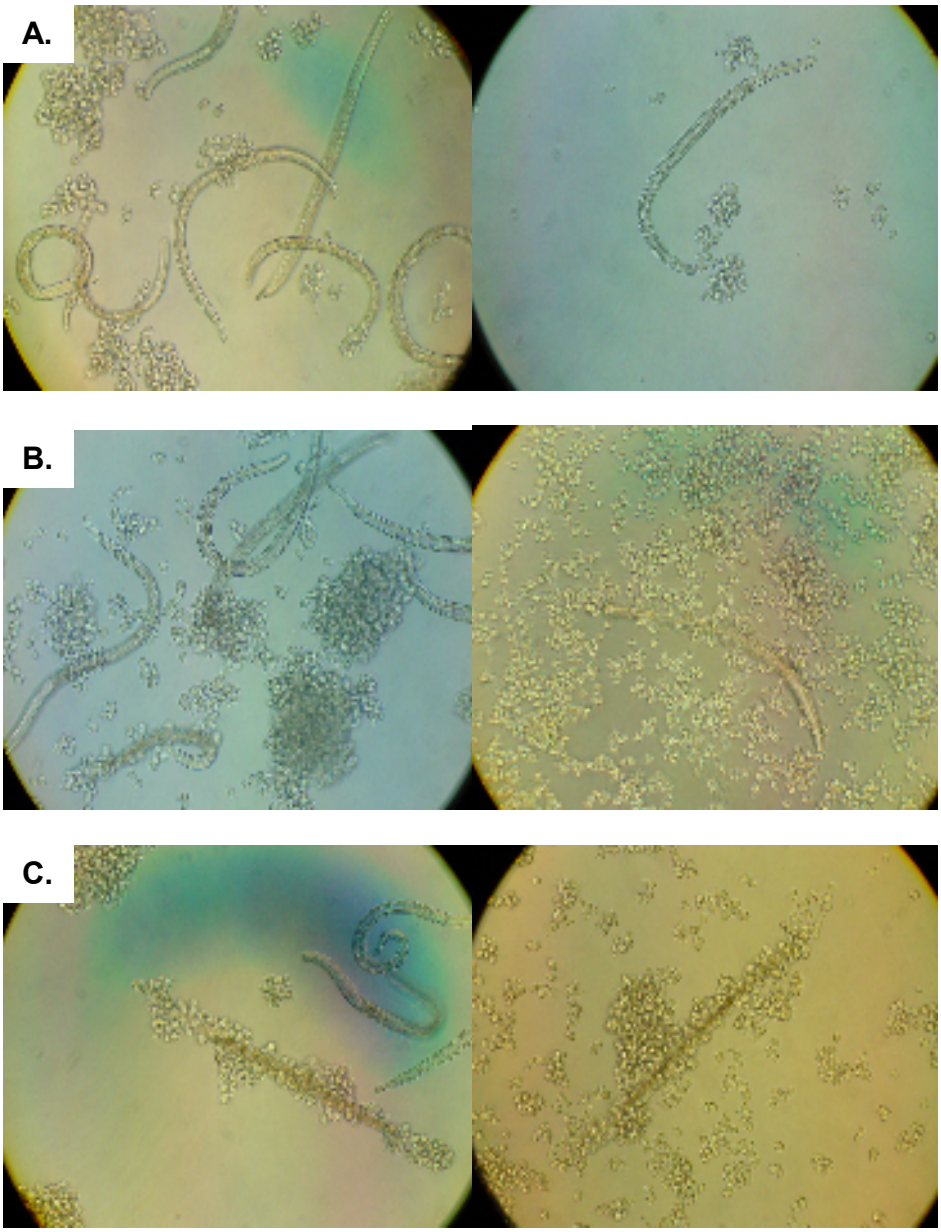
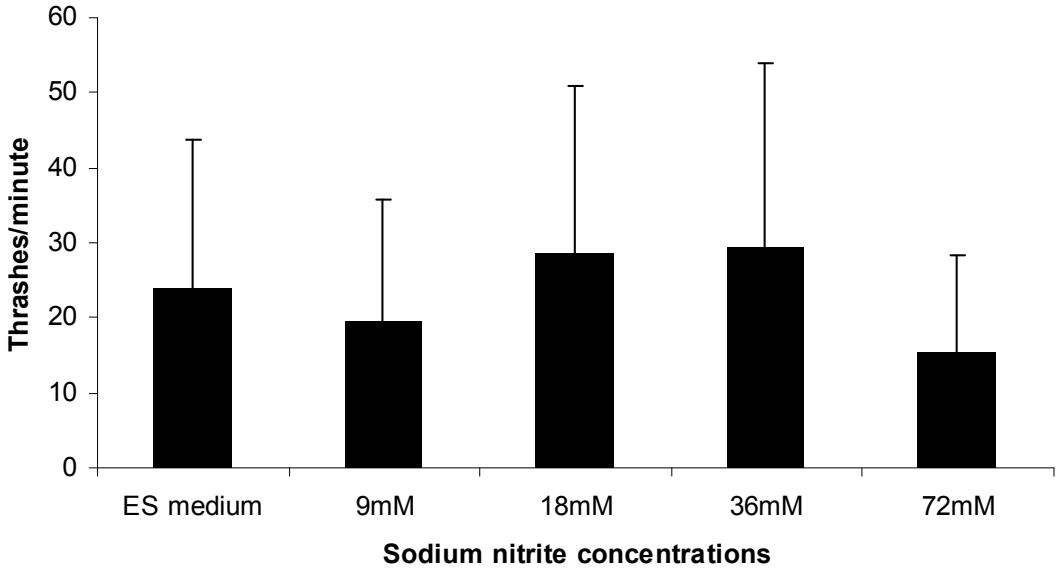
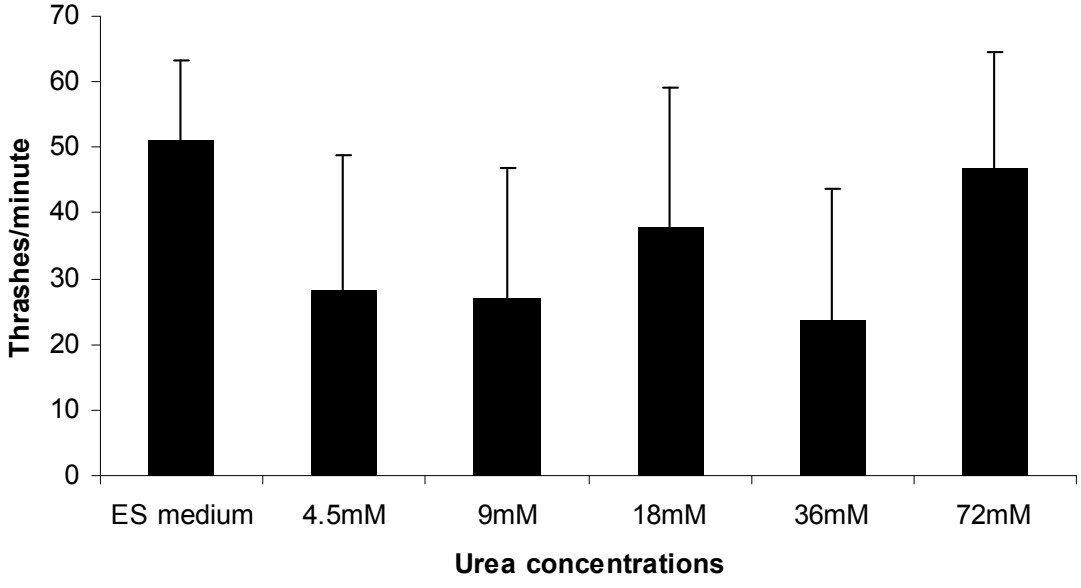


Figure 3.10 Interaction between NR8383 cells and *N.brasiliensis* L3 larvae. NR8383 cells ($2x10^4$ cells) were incubated with L3s (20, 50, 80) or LPS (25ng ml^{-1}) and L3s (20, 50, 80) in 96-well plates for 48 hours before representative fields that have visible cell-larvae binding were chosen and observed with 400x magnification. **A:** NR8383 cells polarize to *N.brasiliensis* L3 larvae. **B:** NR8383 cells attached to *N.brasiliensis* L3 larvae. **C:** *N.brasiliensis* L3 larvae surrounded by NR8383 cells.

A.


60
50
40
30
20
10
0
Thrashes/minute
ES medium
9mM
18mM
36mM
72mM
Sodium nitrite concentrations

B.


70
60
50
40
30
20
10
0
Thrashes/minute
ES medium
4.5mM
9mM
18mM
36mM
72mM
Urea concentrations

C.

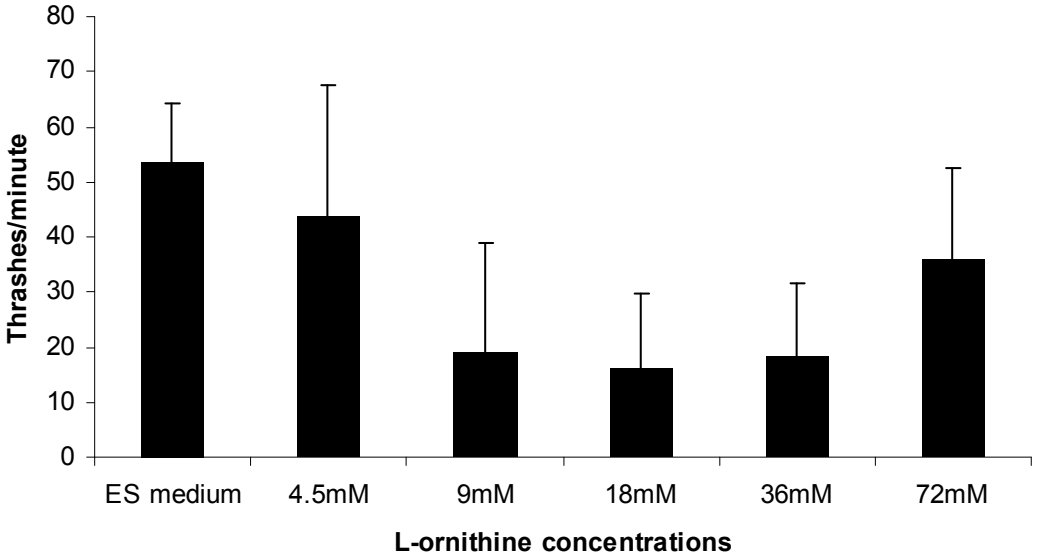


Figure 3.11 Movements of *N.brasiliensis* L3 larvae in different concentrations of sodium nitrite (A), urea (B) and L-ornithine. Twenty *N.brasiliensis* larvae were incubated with 4.5mM-72mM of sodium nitrite, urea or L-ornithine in 96-well plates for 18 hours at 37ºC. After 18 hours, each well was filmed for 1 minute under a dissection microscope. Movements of larvae were recorded as thrashes per minute. The results represent the means of thrashes/minute of five to eight larvae that were recorded in each well ± SD.

3.3 Discussion

N.brasiliensis infection has been demonstrated to induce a Th2 immune response, with only limited inflammation detectable in the lung stage (MacDonald et al., 2002; Keir, PhD thesis). Regulation of macrophage function may be one of the major strategies used by *N.brasiliensis* by analogy to other helminth studies mentioned previously (Section 1.4.3). Here, NR8383 cells stimulated with 25ng ml^{-1} LPS were demonstrated to produce sufficiently more NO than medium only controls and were therefore used as an *in vitro* inflammatory model. Both *N.brasiliensis* larvae and L3 NES alone did not induce any significant TNF-α, IL-1β and NO production in the rat alveolar cell line, NR8383 cells. The lack of pro-inflammatory cytokine production is consistent with previous *in vivo* studies demonstrated that TNF-α was not detectable in the lung spaces of infected rats and that IL-1 production ceased one day after the infection till day 5 during the early phase of *N.brasiliensis* infection (Benbernou et al., 1992; McNeil et al., 2002). Since TNF-α and IL-1β have the ability to induce upregulated adhesion molecules, such as ICAM-1 (Beck-schimmer et al., 1997), this data may provide an preliminary explanation for the limited recruitment of neutrophils in *N.brasiliensis* infected rat lungs (McNeil et al., 2002). The absence of TNF-α and IL-1β, potent activators for Th1 cell expansion, in the early immune responses may explain the dominance of Th2 rather than Th1 polarisation in the adaptive immune response to *N.brasiliensis* (MacDonald et al., 2002).

Previously, concentrated ES products from plerocercoids of *Spirometra erinaceieuropaei* (Miura et al., 2001) and purified ES protein including PAS-1 from

A. suum (Oshiro et al., 2005) and ES-62 from filarial nematode *A.viteae* (Goodridge et al., 2001), have been demonstrated to suppress the production of TNF-α in LPS stimulated murine peritoneal macrophages. Our data supplement these findings and revealed the first time that live *N.brasiliensis* larvae and crude NES inhibited LPS-stimulated production of a key pro-inflammatory cytokine, TNF-α, from alveolar macrophages *in vitro*. Our present study found no effect of *N.brasiliensis* larvae and NES on IL-1β production after LPS stimulated macrophages, which is in contrary to the inhibition of IL-1 by PAS-1 (Oshiro et al., 2005). This is not surprising giving that immunogenic properties of helminths are highly specific (MacDonald et al., 2002). In *N. brasiliensis* infected rats, IL-1 levels in BAL transiently increase one day after the infection (Benbernou et al., 1992) and two weeks after, a slight increase of IL-1β is detectable in granulomatous lesions in rat lungs (Matsuda et al., 2001). Certain amount of IL-1β may be required to counteract the potential excess production of Th2 cytokines in *N.brasiliensis* infection. In addition, the fact that *N.brasiliensis* inhibited LPS induced TNF-α and NO but not IL-1β production in NR8383 cells may suggest that these inflammatory mediators are regulated differently in LPS signalling pathways. Transcription of each pro-inflammatory gene involves the activation of numerous signal transduction pathways. It has been recognized for a long time that phosphorylation of I-κB kinase and translocation of NF-κB transcription factor plays a central role in the activation TNF-α, IL-1β and iNOS gene transcription (Ghosh et al., 1998; Li et al., 2002). However, NF-κB activity usually requires the assistance of another transcription factor, activator protein-1 (AP-1) family, which is activated via the phosphorylation of mitogen-activated protein kinases (MAPKs). The three most common distinct MAPK cascades are the extracellular signal–

regulated kinase (ERK), the Jun-N terminal kinase (JNK), and the p38 MAPK cascades (Karin, 1995). Interestingly, inhibiting p38 MAPK decreased 80% IL-1β gene transcription but had no effect on TNF-α transcription in LPS-stimulated murine monocyte cell lines (Baldassare et al., 1999). This suggested that gene transcriptions of IL-1β and TNF-α may not follow the same signalling pathways. However interestingly, concentrated ES products from plerocercoids of the tapeworm *S. erinaceieuropaei* suppressed the LPS induced IL-1β and TNF-alpha gene expression in peritoneal macrophages by reducing phosphorylation of ERK1/2 and p38 MAPK, but had no effect on NF-κB translocation (Dirgahayu et al., 2002; Dirgahayu et al., 2004). The role of each intracellular signalling pathway in the LPS-induced TNF-α, IL-1β and NO production requires specific investigation in different physiological conditions. The effect of NES on LPS-induced NF-κB translocation will be studied in Chapter 4, and the study of other elements involved in intracellular signalling pathways that may be regulated by *N.brasiliensis* are in our future plan. Moreover, TNF-α and IL-1β are translated as pro-TNF-α and pro-IL-1β within the cells, which need to be proteolytically processed to be secreted as biologically active mature forms of the proteins. Caspase 1 and 8 are believed to mediate the maturation of IL-1β (Maelfait et al., 2008) and metalloprotease have been demonstrated to cleave the 26-kDa pro-TNF-α to the secreted 17-kDa form (Solomon et al., 1997). It is possible that *N.brasiliensis* larvae and NES regulate TNF-α and IL-1β production via the modulation of different post-translational modification mechanisms. There was no detectable production of IL-12 p70 in 25ng ml^{-1} LPS treated NR8383 cells (data not show) using a mouse IL-12 p70 ELISA kit (RandD Systems, Oxon, UK) that cross link with rat. IL-12 p70 is known to be the only bioactive form of IL-12, but is produced 10-100-fold less than IL-12

p40 as mentioned previously. However the level of IL-12 p40 does not signify the level of bioactive IL-12, since it may even limit IL-12 p70 production (Del et al., 2007). Unfortunately, the effects of *N.brasiliensis* larvae and NES on LPS stimulated IL-12 p70 production remained unknown from present results.

NO is recognised as a major effector molecule of macrophage toxicity against a variety of invading pathogens, such as parasitic helminths. Its production is positively controlled by cytokines such as IFN-γ, TNF-α and IL-2 and negatively regulated by IL-4, IL-10 and TGF-β (Oswald et al., 1994). Investigations have shown that NO produced by macrophages is essential for killing parasites such as *S.mansoni* (Oswald et al., 1994) and *Brugia malayi* (Thomas et al., 1997), although such information about *N.brasiliensis* larvae is still absent. Our preliminary results showed that *N.brasiliensis* L3 larval movement remains relatively constant up until 72mM (5mg ml^{-1}) of $NaNO_2$. It is possible that the formation of peroxynitrite ($ONOO^{-\cdot}$) by nitrite and $O_2^{-\cdot}$ is essential for the toxic effect on the larvae. Sodium nitrite-originated nitrite may not be sufficient compare to that produced in physiological conditions. Alternatively, anti-oxidant mechanisms may exist in *N.brasiliensis* larvae to resist the oxidative damage from the host. Glutathione peroxidase has been found to be present in the cuticles of the filarial parasite *B.pahangi* (Tang et al., 1996), and CuZn superoxide dismutase was secreted by adult *B.malayi*, *Dirofilaria immitis* and *Onchocerca volvulus* (Selkirk et al., 1998). Also reviewed by Selkirk et al (1998), the cuticular structure and metabolic system of filarial nematodes may help to protect the parasite from oxidative stress. *N.brasiliensis* infection has been demonstrated to be associated with increased superoxide peroxidise and decreased lipid peroxidation in rat lungs

(McNeil et al., 2002). Potential anti-oxidant mechanisms and detoxification processes in *N.brasiliensis* larvae will be the subject of future work.

Antigens from several parasites have been demonstrated to trigger NO production by alveolar macrophages *in vivo* and *in vitro* (Andrade et al., 2005a; Andrade et al., 2005b; Andrade et al., 2005c; Espinoza et al., 2002a; Espinoza et al., 2002b). NO was also induced in *N.brasiliensis* infected lungs. In these cases, NO may benefit the movement and development of helminths as a vasodilator. Our data from this chapter demonstrated the inhibition of LPS stimulated NO production in macrophages by *N.brasiliensis* larvae and NES. Macrophages may respond differently to helminth antigens alone and in the presence of LPS. A tapeworm ES product induces iNOS mRNA in murine hepatocytes but suppresses LPS/IFN-γ induced hepatocyte iNOS mRNA expression (Wang et al., 1997). A similar inhibitory effect on iNOS mRNA was observed when incubating live plerocercoids of *Spirometra erinacei,* or ES, with LPS/IFN-γ stimulated peritoneal macrophages (Fukumoto et al., 1997). A study by Cuellar et al (1998) demonstrated that the co administration of crude extracts or ES products of *Anisakis simplex* inhibited NO accumulation in a dose-dependent manner in LPS-stimulated J774 cells, which was not due to the general cellular toxicity. Our data also proved that *N.brasiliensis* live larvae and NES did not induce NO production on their own but have inhibitory effects on 25ng ml^{-1} LPS-stimulated NO production *in vitro*. The induction of iNOS in alveolar macrophages by LPS was associated with NF-κB activation (Li et al., 2002). As discussed above, the different effects of *N.brasiliensis* larvae and NES on LPS induced NO and IL-1β production may be linked with the regulation of different intracellular signalling pathways.

There is evidence that alveolar macrophages are alternatively activated in reaction to *N.brasiliensis* (Nair et al., 2005, Reece et al., 2006). However, our results so far were not sufficient to identify macrophage phenotypes. Whether the inhibition of TNF-α and NO was caused by the down-regulation of classically activated macrophages (CAMs) alone, and/or associated with induction of alternatively activated macrophages (AAMs), represent one of our future research goals. Preliminary results in this chapter demonstrated that *N.brasiliensis* larvae were resistant *in vitro* to the metabolic products of both CAMs and AAMs, as evaluated by movement of the L3 larvae in relatively high concentrations of commercially available sodium nitrite, urea and L-ornithine. This suggested that induction of AAMs does not cause any damage to *N.brasiliensis larvae*. However, it is possible that our experimental conditions do not represent the toxicity of urea and L-ornithine produced by AAMs in physiological conditions. To investigate this further requires the adoption of more effective methods to evaluate the behaviour and viability of larvae in these conditions. It is known that the AAM metabolic product, urea, is capable of denaturing proteins, and is used extensively in industry to increase the solubility of proteins (Bennion and Daggett, 2004). Moreover, it was found that diets supplemented with urea reduced faecal egg output and burden of *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep (Knox and Steel, 1999). It remains to be determined whether urea is exclusively acting as an aid for food digestibility, or if there were other mechanisms by which this compound attributes to parasite resistance.

MTT tests confirmed that the suppression of TNF-α and NO by NES was not caused by cell death. The attempt of using LDH assay for L3 larvae toxicity

showed that live *N.brasiliensis* larvae were toxic to NR8383 cells (data not shown). However, as discussed in section 3.2.5, LDH may be released from dead larvae and interfere with the results.

50µl (25%) and 100µl (50%) of NES used with LPS to stimulate macrophages, were collected from 100 and 200 larvae respectively, based on the larval concentration of 2000 larvae ml^{-1}. However, the inhibitory effect of NES was in the similar range as for 50-80 larvae/well. ES production of *N.brasiliensis* larvae may be boosted when incubated with macrophages, which ensures the presence of an adequate amount of active ingredients. Another possibility is that live larvae may be more effective in interacting with macrophages than NES alone. These two possibilities involve the interaction between macrophages and the larvae. The microscopy photos (Figure 3.10) suggested that the larvae may attract macrophages. This is consistent with the *in vivo* observation that there is a steady increase in macrophages recovered from broncho-alveolar lavage following *N.brasiliensis* infection (Ramaswamy and Befus, 1989). The interaction of macrophages with nematode surfaces in the presence of complement or antibodies was first described by Mackenzie et al in 1981. They found that macrophages appeared to adhere permanently to the surface of all stages of *N.brasiliensis* without flattening and retain their integrity. In addition, the cytoplasmic inclusions appeared to decrease in size during culture. Toxic effects of activated macrophages have been observed on *Brugia malayi* that parasites of both microfilariae and adult stages showed a decline in viability over 48 hour of co-culture with LPS-stimulated J774 macrophages. This was associated with damage to the ultrastructure of the parasites (Thomas et al., 1997). In the current study,

macrophages were found occasionally surrounding dead larvae. Unfortunately, there is still no information available on the surface structural antigens and the signal transduction after macrophage-nematode binding within the helminths.

In conclusion, results from this chapter demonstrated that *N.brasiliensis* larvae and NES significantly reduced LPS-stimulated pro-inflammatory mediators (TNF-α and NO) production *in vitro*, and that this reduction was not caused by cell death. Immune active components in the NES may be responsible for the down regulation of pro-inflammatory mediators. The protein concentration in crude NES was not detectable by Bradford protein assay (sensitivity of 312.5ng ml^{-1}), however in Chapter 4, crude NES was concentrated and its immune active components were further explored.

Chapter 4

Active components of NES

and

Regulation of inflammatory process by NES

in vitro

4.1 Introduction

In Chapter 3, we observed that both live *N. brasiliensis* larvae and NES down-regulated the production of pro-inflammatory molecules, TNF-α and NO, in LPS-stimulated rat alveolar macrophages. It is of our interest to research into the nature of the molecules within NES that possess these potential immune-regulatory functions. NES components with such functions were viewed as the active components of NES. The properties of the active components of NES, which were investigated in this chapter, include the molecular size, heat lability and trypsin sensitivity.

Most studies regarding *N.brasiliensis* ES are done with the adult ES products since they are more abundant in quantity. In this chapter, concentrated NES (NES concentrate) was subjected to SDS-PAGE to examine its protein profile. Glycans have been demonstrated to modulate helminth infections through recognition by lectin receptors on cell surfaces (Van die and Cumming, 2006). Recently, mannose receptor on rat peritoneal macrophages has been demonstrated to be partially involved in *T.spiralis* crude somatic extract and L1 larval ES-stimulated NO production (Gruden-Movsesijan and Milosavljevic, 2006). The presence of glycoproteins in NES concentrate and the partial structures of glycoproteins were also studied in this chapter. Although knowledge on the feeding activities of *N.brasiliensis* larvae and adults is still limited, protein leakage in rat lungs and excess protein gastrointestinal loss (Haley, 1962) suggest that *N.brasiliensis* larvae and adults at least partly feed on proteins. The ingested proteins are usually digested by helminth proteases for the acquisition of nutrients (Knox and Jones,

1990). A previous study by Healer et al (1991) revealed that the major protease in *N.brasiliensis* larvae ES products was a 51 kDa metalloprotease. The proteolytic activity of NES protein and the protease types herein were examined using gelatin-acrylamide gels under different pH conditions in this chapter.

LPS is a major component of the outer membrane of gram-negative bacteria. The activation of macrophages by LPS involves the participation of a wide range of molecules within a complicated network. The pathway is initiated by the recognition of LPS by macrophage cell surface receptors, which include toll-like receptors (Takeda et al., 2003) and CD14 (Wright et al., 1990). LPS is usually trapped by LPS-binding protein (LBP) before being recognized by CD14 to form LBP-LPS-CD14 complex. The LPS-receptor binding leads to a cascade of intracellular signalling events that result in the translocation of transcription factors, such as NF-κB, into the nucleus of the macrophages (Caamano and Hunter, 2002). NF-κB promotes the transcription of pro-inflammatory genes, and the production of inflammatory molecules. These secreted pro-inflammatory molecules may undergo post-translational modifications in the physiological environment before being fully active, for example the formation of secondary and tertiary structures. In this chapter, it is hypothesized that the NES-regulated effect on LPS-stimulated inflammatory processes occurs in various levels, which include NF-κB translocation, gene transcriptions of pro-inflammatory molecules and post-translational modification. ICAM-1 is a key adhesion molecule elevated in inflammatory conditions to recruit leukocytes through binding to integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) (Alberlda et al., 1994). In the case of LPS-stimulated inflammation, ICAM-1 expressed on endothelial cells and

macrophages mediate neutrophil infiltration (Alberlda et al., 1994). The late immune response to *N.brasiliensis* in rats was marked by a substantial increase in the number of lymphocytes and eosinophils expressing ICAM-1 and integrin LFA-1 in the lungs and BAL (Watkins et al., 1996). In *O.volvulus* infection, ICAM-1 has also been demonstrated to play a significant role in recruiting eosinophils to the site of infection (Kaifi et al., 2001). A major chemokine secreted by LPS-stimulated macrophages is MIP-2, which is an extremely potent chemotactic factor for neutrophils (Wolpe and Cerami, 1989). It has been demonstrated in *O.volvulus* infection that MIP-2 expression in the cornea stroma was critical for neutrophil infiltration to the site (Hall et al., 2001). In this chapter, the effects of NES on LPS-induced ICAM-1 and MIP-2 gene transcription in macrophages were quantitatively measured by real-time PCR, along with TNF-α and IL-1β.

4.2 Results

4.2.1 The active components of NES

NES was fractionated into two components by concentrating with Vivaspin 6ml concentrator (3kD MWCO) at 3200g for 60 minutes at 4°C. The retentate was rich in molecules with MW bigger than 3kDa, potentially including proteins, polysaccharides and glycoproteins. After recovering with ES medium to the same volume as the NES filtrate, protein concentration in the retentate was approximately 20μg ml^{-1} as determined by the Bradford method. This fraction of NES was then named NES concentrate. Whereas both in the crude NES and NES filtrate, the protein concentrations were undetectable by Bradford assay with a sensitivity of 312.5ng ml^{-1}. NES filtrate may contain small molecules such as oligosaccharides, but this was not determined. Here *in vitro*, the effects of NES and its fractions, NES concentrate and NES filtrate, on LPS-stimulated inflammation were observed in the rat alveolar macrophage cell line, NR8383. Each independent experiment was carried out with a different batch of NES on different days.

4.2.1.1 Effects of NES, NES concentrate and NES filtrate on TNF-α production.

The effects of NES, NES concentrate and NES filtrate on TNF-α production were investigated with or without LPS (25ng ml^{-1}). Neither crude NES nor its fractions (NES concentrate and NES filtrate) were able to stimulate TNF-α production by NR8383 cells (Figure 4.1). In the background of LPS stimulation, the TNF-α production was reduced significantly by NES ($p<0.01$) and NES concentrate

($p<0.01$), but not NES filtrate. As indicated in Figure 4.1, the reductions by NES and NES concentrate were both more than 60%. Therefore the down-regulation of LPS-induced TNF-α production by NES was linked with NES concentrate.

4.2.1.2 Effects of NES, NES concentrate and NES filtrate on IL-1β production.

As shown in Figure 4.2, NR8383 cells incubated with ES medium produce a certain amount of IL-1β. Crude NES alone failed to stimulate any IL-1β production in NR8383 cells. Only when the cells were stimulated with LPS and NES concentrate together was there a significant amount of IL-1β ($p<0.05$) produced compared to cells treated with ES medium only, which may indicate an amplifying effect of NES concentrate on IL-1β production. However, this was not significant compared to LPS (25ng ml^{-1}) stimulation alone. NES or NES filtrate had no effect on LPS-induced IL-1β production.

4.2.1.3 Effects of NES, NES concentrate and NES filtrate on NO production.

Neither NES, NES concentrate nor NES filtrate were able to induce NO production in NR8383 cells, as presented in Figure 4.3. Similar to the data achieved in chapter 3 (Figure 3.7), NES significantly reduced NO production ($p<0.05$) in LPS-stimulated NR8383 cells by about 20%. However, such inhibitory effect was not retained by either NES concentrate or NES filtrate as shown in Figure 4.3.

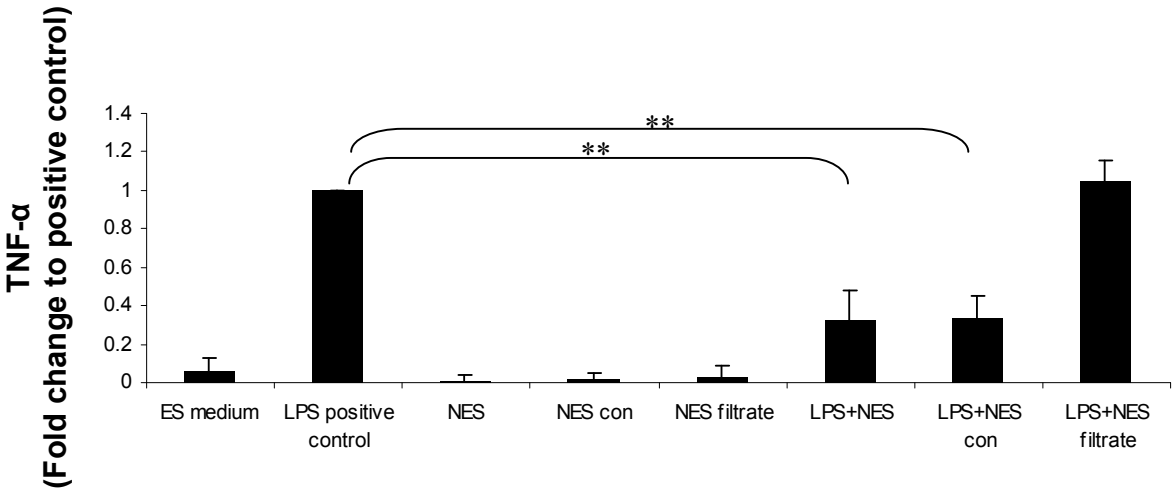


Figure 4.1 The fold change of TNF-α production in each treatment compared to TNF-α production in the positive control (range 1.54±0.55ng ml^{-1}). NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}) and/or NES, NES concentrate (NES con) or NES filtrate in a 96-well plate for 48 hours before TNF-α in culture supernatants was measured by ELISA. NR8383 cells incubated with LPS (25ng ml^{-1}) only were used as positive control. The result represents the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=3). **: $p<0.01$.

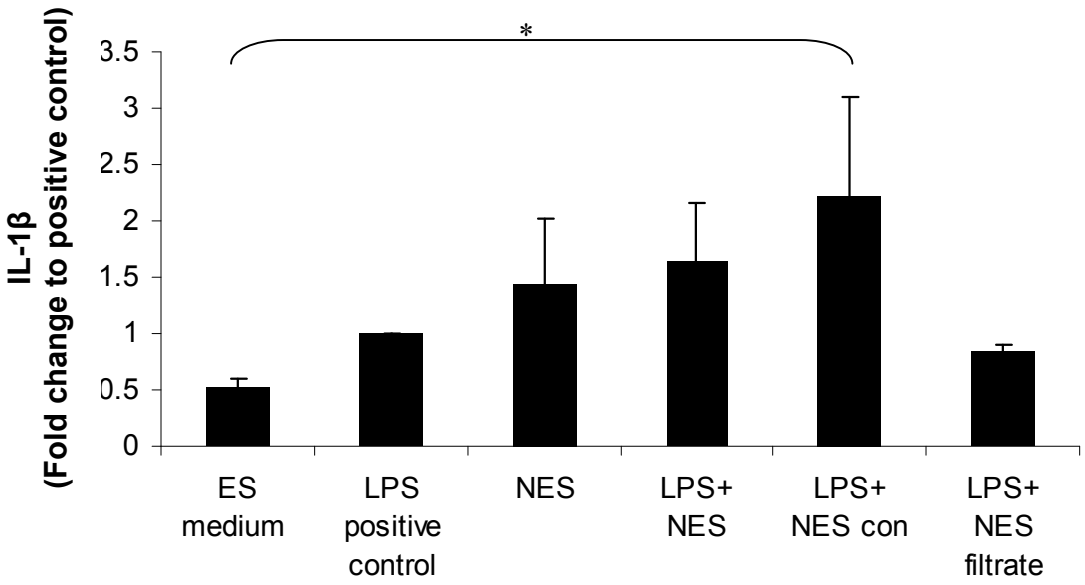


Figure 4.2 The fold change of IL-1β production in each treatment compared to IL-1β production in the positive control (range 1.18±0.10ng ml^{-1}). NR8383 cells ($2x10^4$ cells) were incubated with NES alone, LPS (25ng ml^{-1}) with or without NES, NES concentrate (NES con) or NES filtrate in a 96-well plate for 48 hours before IL-1β in culture supernatants was measured by ELISA. NR8383 cells incubated with LPS (25ng ml^{-1}) only were used as positive control. The result represents the means of three independent experiments each with three replicates. All values are expressed as mean ± SD (n=9). *: $p<0.05$.

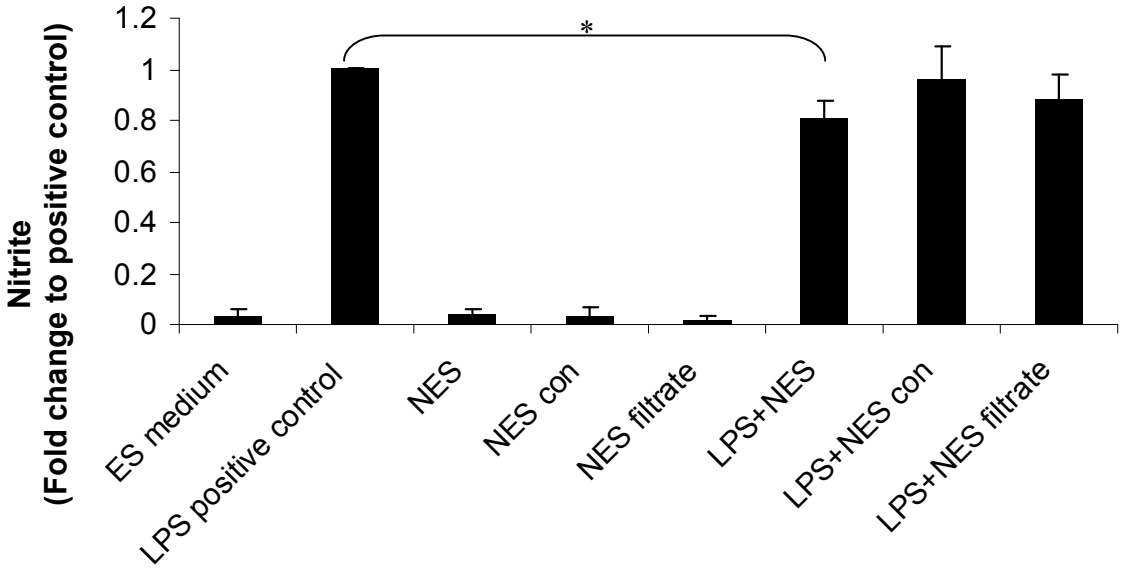


Figure 4.3 The fold change of nitric oxide production in each treatment compared to nitric oxide production in the positive control (range 26.62±13.13μM). NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}) and/or NES, NES concentrate (NES con) or NES filtrate in a 96-well plate for 48 hours before NO in culture supernatants was measured as nitrite by Griess assay. NR8383 cells incubated with LPS (25ng ml^{-1}) only were used as positive control. The result represents the means of four independent experiments each with three replicates. All values are expressed as mean ± SD (n=4). *: $p<0.05$.

4.2.1.4 Effects of heat-treatment and trypsin-digestion of NES concentrate on TNF-α production.

As shown in Figure 4.1, it is suggested that the active molecules were within NES concentrate and have molecular weights bigger than 3kDa. To further characterize the heat and trypsin sensitivities of these active components, NES concentrate was heat-treated at 95°C for 8 minutes (Figure 4.4) or digested with trypsin for 18 hours (Figure 4.5) before treating NR8383 cells along with LPS (25ng ml^{-1}). Similarly to results in Figure 4.1, NES concentrate reduced LPS-induced TNF-α production by more than 60% and this was highly significant ($p<0.01$) (Figure 4.4 and 4.5). Although heat-treated NES concentrate retained this inhibitory effect on TNF-α production, the reduction of TNF-α was only approximately 40% (Figure 4.4). NES concentrate digested with trypsin significantly reversed the effect of NES concentrate on LPS-stimulated TNF-α production ($p<0.05$) (Figure 4.5). However, there was still some suppression on TNF-α production by trypsin-digested NES concentrate. These results indicated that the inhibitory effect of NES concentrate on TNF-α production was at least partially due to its heat-labile and trypsin sensitive components.

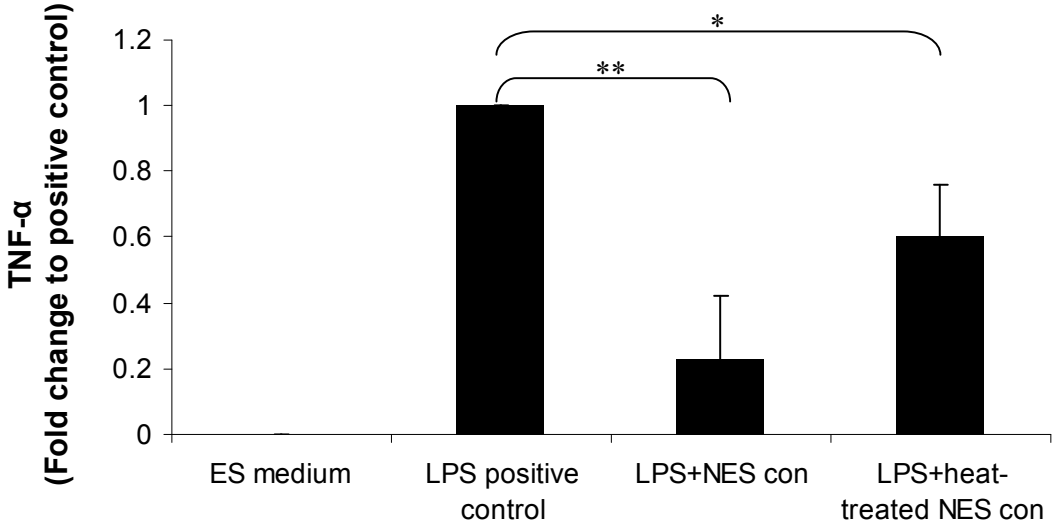


Figure 4.4 The fold change of TNF-α production in each treatment compared to TNF-α production in the positive control (range 2.40±1.01ng ml^{-1}), showing the effect of heat-treatment. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}) with or without NES concentrate (NES con) or heat-treated NES concentrate (heat-treated NES con) in a 96-well plate for 48 hours before TNF-α in culture supernatants was measured by ELISA. NR8383 cells incubated with LPS (25ng ml^{-1}) only were used as positive control. The result represents the means of four independent experiments each with three replicates. All values are expressed as mean ± SD (n=4). *: $p<0.05$, **: $p<0.01$.

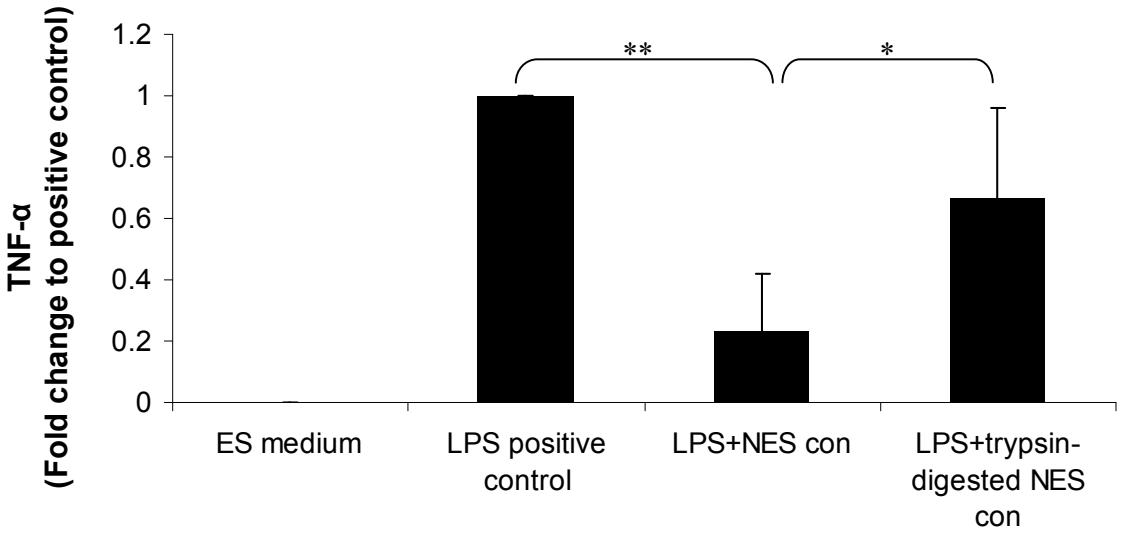


Figure 4.5 The fold change of TNF-α production in each treatment compared to TNF-α production in the positive control (range 2.40±1.01ng ml^{-1}), showing the effect of trypsin digestion. NR8383 cells ($2x10^4$ cells) were incubated with LPS (25ng ml^{-1}) with or without NES concentrate (NES con) or trypsin-digested NES concentrate (trypsin-digested NES con) in a 96-well plate for 48 hours before TNF-α in culture supernatants was measured by ELISA. NR8383 cells incubated with LPS (25ng ml^{-1}) only were used as positive control. The result represents the means of four independent experiments each with three replicates. All values are expressed as mean ± SD (n=4). *: $p<0.05$. **: $p<0.01$.

4.2.2 Characterisation of proteins present within NES concentrate

It was demonstrated in section 4.2.1 that NES concentrate included the active components which inhibited LPS-induced TNF-α production in macrophages (Figure 4.1). The characteristics of the proteins present within NES concentrate were explored in this section. SDS-PAGE and lectin blot (as described in section 2.15 and 2.16) were applied to investigate the protein profile, presence of glycans and proteases in NES concentrate. In this section, at least three batches of NES were pooled together and NES was concentrated at least twice in order to achieve the protein concentration of 200μg ml^{-1}.

4.2.2.1 SDS-PAGE and silver stain of NES concentrate.

As shown in Figure 4.6, abundant proteins with MW between 6kDa and 100kDa were detected in 2μg of NES concentrate by silver stain. A protein band with MW of approximately 34kDa may represent the most dominant proteins within NES concentrate.

4.2.2.2 Occurrence of glycoproteins in NES concentrate

Lectin blot with ConA or PNA was used to detect the presence of glycoproteins in NES concentrate and the linkage type with the sugar chains. As shown in Figure 4.7, most protein bands detected in Figure 4.6 were detected by lectins. This proved that most protein components in NES concentrate are glycoproteins. The sugar chain of a 116 kDa glycoprotein band reacted with ConA but not with PNA, indicating this glycoprotein had the *N*-linked sugar chain. The glycoproteins with molecular weight range from 18-80kDa reacted with both lectins, but only faintly reacted with PNA, indicating that the *N*-linked sugar chain was dominant in this

glycoprotein. The most abundant protein band with MW of approximately 34kDa was also proved to be rich in *N*-linked sugar chains.

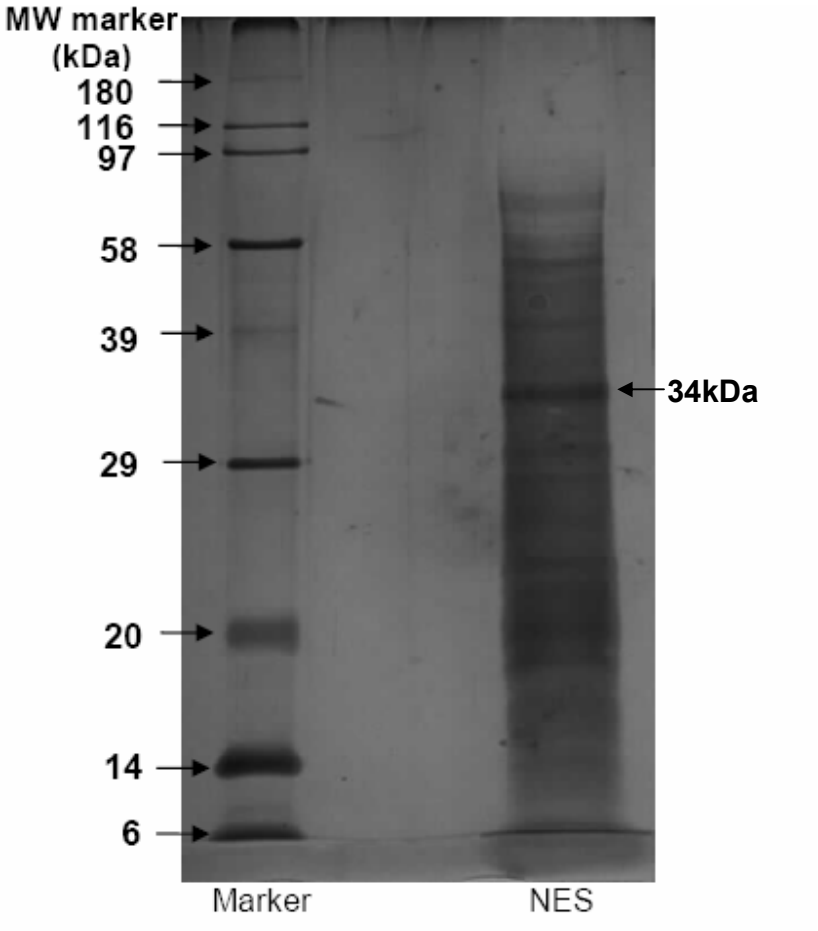


Figure 4.6 Silver stain of NES concentrate. NES concentrate (2μg) was subjected to 5-12% SDS-PAGE before silver stain.

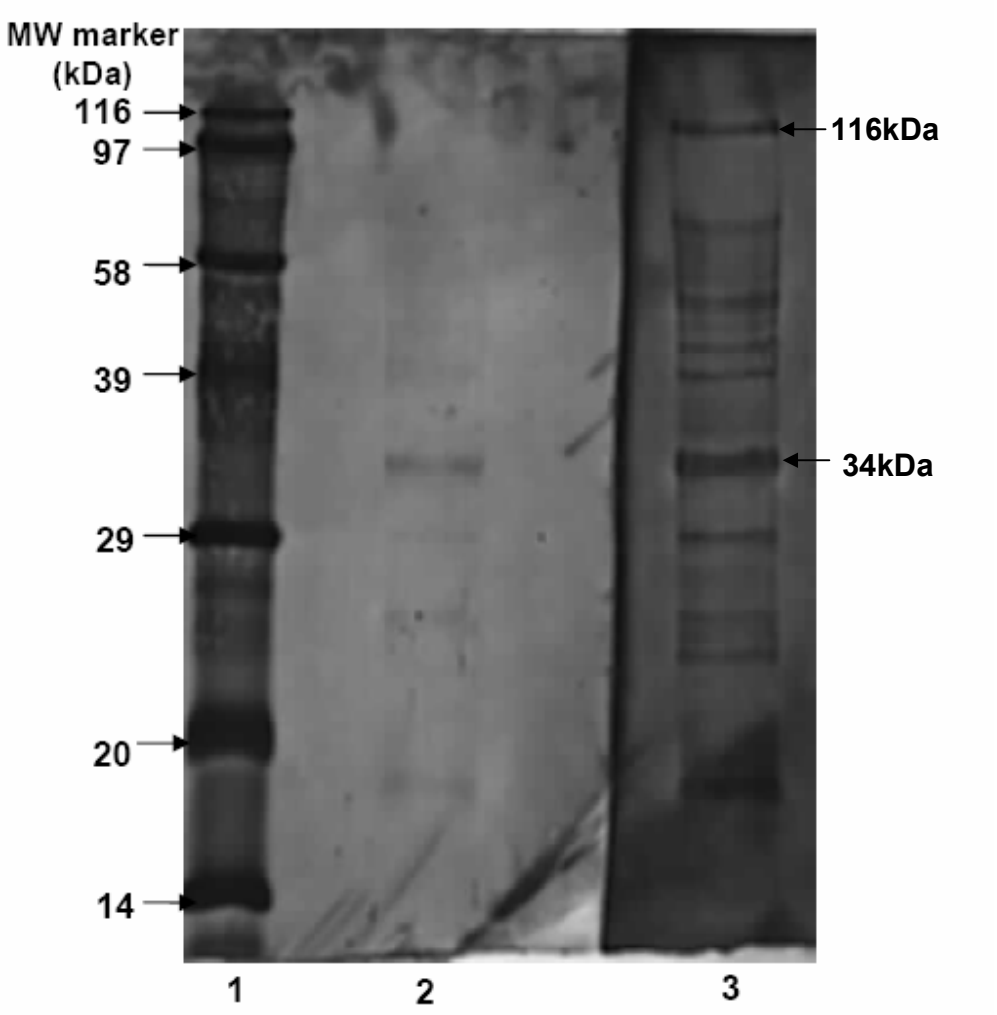


Figure 4.7 Lectin blot of NES concentrate. After 5-12% SDS-PAGE, NES concentrate (4μg) was transferred to nitrocellulose membrane and incubated with 0.0005% (w/v) biotinylated-PNA (lane 2) or -ConA (lane 3) overnight at 4ºC. The blotted membranes were detected by HRP-avidin and developed with ECL.

4.2.3 Effects of NES, NES concentrate and NES filtrate on the inflammatory processes

Data collected from Chapter 3 and section 4.1 indicated that macrophage responses to LPS could be modulated in various ways in the presence of NES, NES concentrate or NES filtrate. The following experiments were carried out to investigate the effects of NES, NES protein and NES filtrate on the LPS induced inflammatory processes, including NF-κB translocation, gene transcription and post-translational modification.

4.2.3.1 Effects of NES, NES concentrate and NES filtrate on NF-κB p65 translocation

The binding of LPS to its receptor triggers a series of intracellular signalling events, which lead to the translocation of NF-κB to the nucleus. The translocated NF-κB binds to the promoter or enhancer regions of targeted genes, and induces the gene transcription of many pro-inflammatory mediators, such as TNF-α, IL-1β, iNOS, ICAM-1 and MIP-2. The amount of NF-κB translocated from the cytoplasm to the nucleus reflects the level of macrophage stimulation to produce pro-inflammatory molecules. The most common activated form of NF-κB exists as a heterodimer containing p65 subunit. In this study, western blot was used to detect the level of NF-κB subunit p65. The translocation of NF-κB p65 was measured by comparing its level in the cytoplasm and nuclear extracts of activated NR8383 cells. As shown in Figure 4.8A, six hours after incubation, LPS stimulated the translocation of NF-κB p65 to the nucleus in NR8383 cells, which was detected by immuno-blot. With the addition of NES concentrate, less NF-κB p65 was translocated to the nucleus compared to the LPS positive control, and such

reduction was significant ($p<0.05$) (Figure 4.8B). However, the reduction of nuclear NF-κB p65 by NES concentrate was less than 15%, which did not match the inhibition of TNF-α production by NES protein in the protein level (~60%, Figure 4.1). Crude NES and NES filtrate did not have any effect on NF-κB p65 translocation. Across all the treatments, the amount of NF-κB p65 in the cytoplasm extracts remained as no change (Figure 4.9).

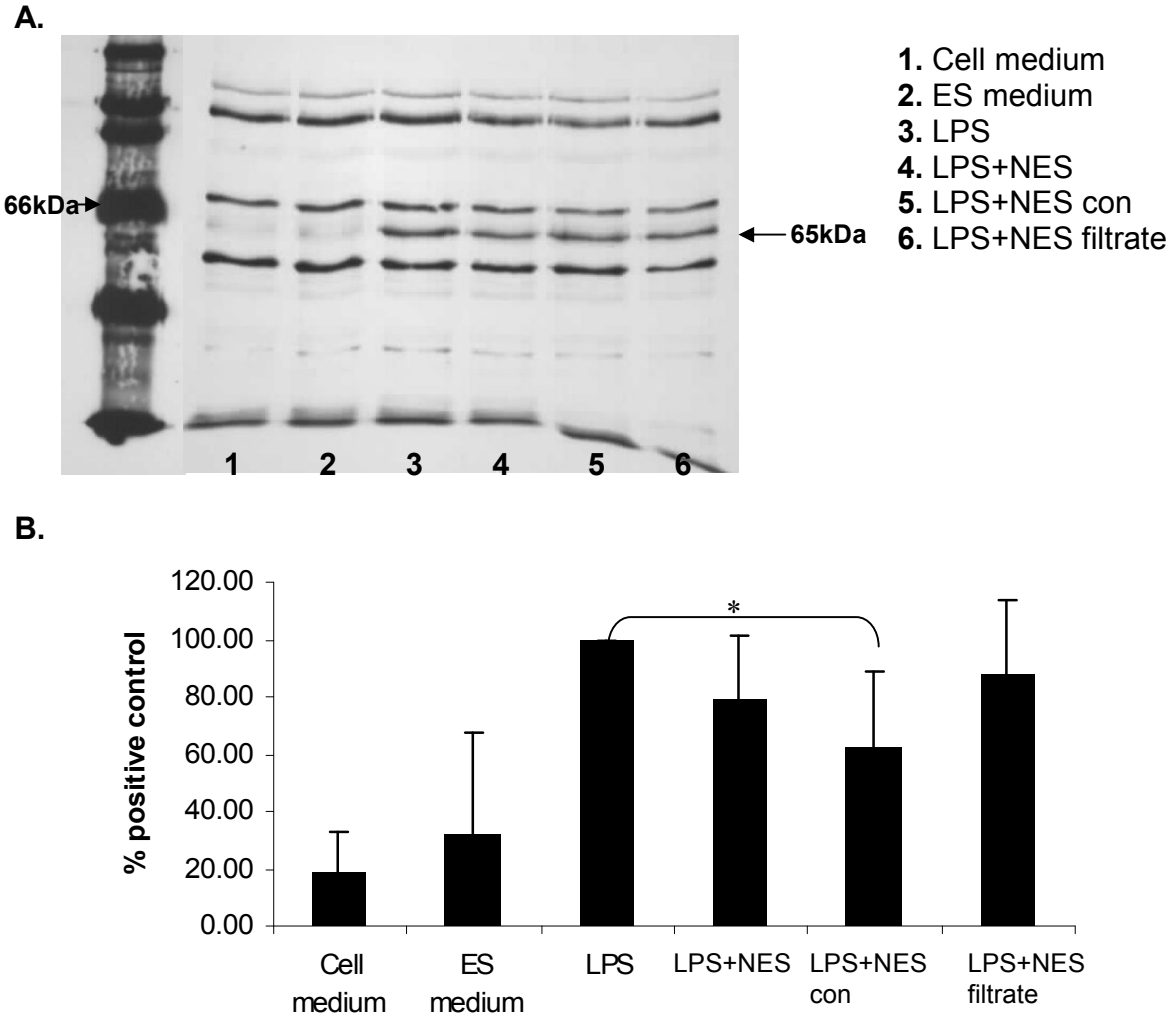


Figure 4.8 Western blot (**A**) and intensity (**B**) of NF-κB p65 in the nuclear extracts of NR8383 cell. NR8383 cells ($5x10^5$ cells) were treated with cell medium, ES medium, LPS (25ng ml^{-1}) with or without NES, NES concentrate (NES con) and NES filtrate for 6 hours before nuclear protein was extracted for NF-κB p65 western blot. Cells treated with LPS (25ng ml^{-1}) only were used as 100% positive control. The intensities of bands were measured and expressed as percentage to the positive control. The result represents three independent experiments. All values are expressed as the mean ± SD (n=3). *: $p<0.05$

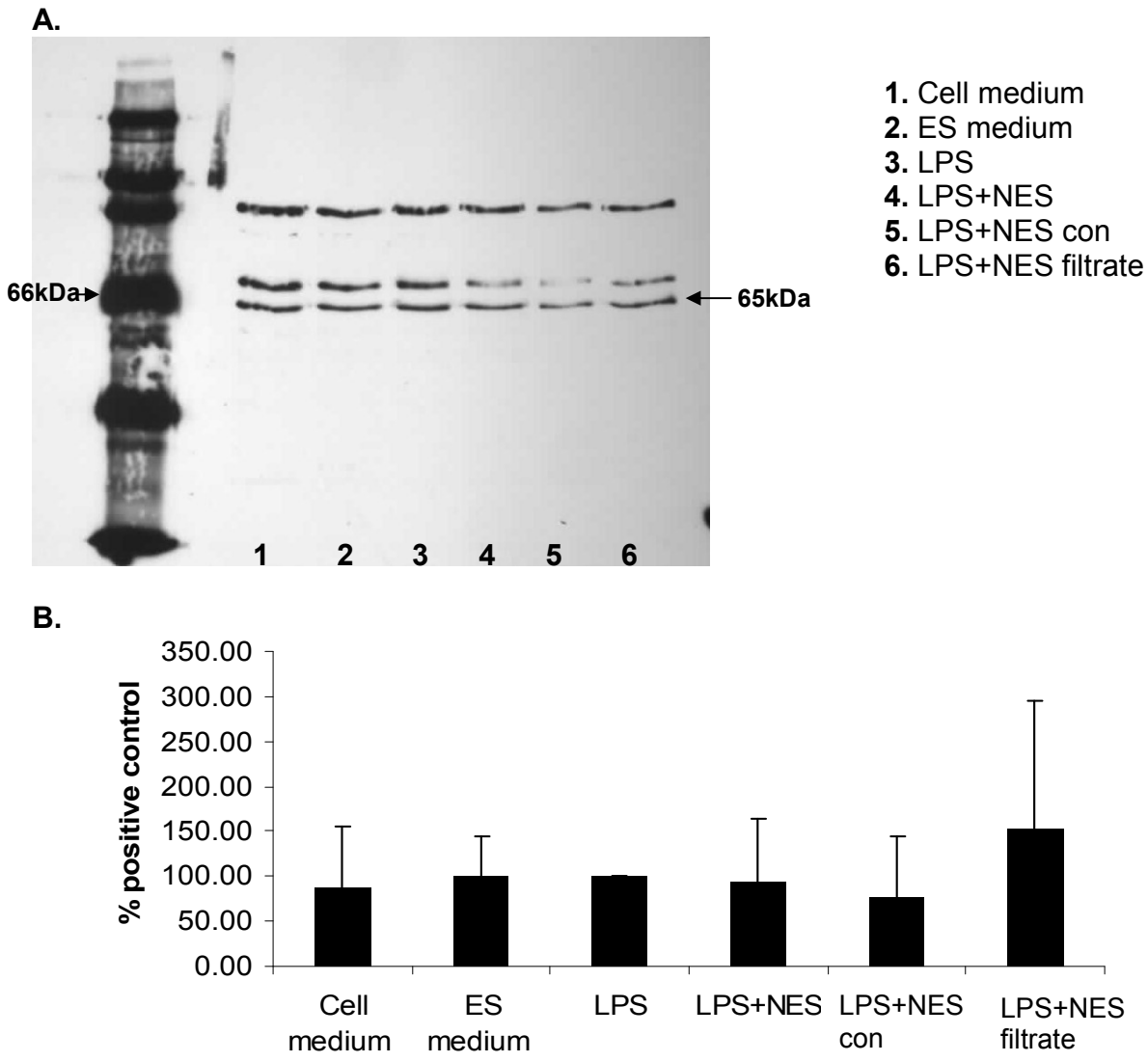


Figure 4.9 Western blot (**A**) and intensity (**B**) of NF-κB p65 in the cytoplasmic extracts of NR8383 cells. NR8383 cells ($5x10^5$ cells) were treated with cell medium, ES medium, LPS (25ng ml^{-1}) with or without NES, NES concentrate (NES con) and NES filtrate for 6 hours before cytoplasmic protein was extracted for NFκB p65 western blot. Cells treated with LPS (25ng ml^{-1}) only were used as 100% positive control. The intensities of bands were measured and expressed as percentage to the positive control. The result represents three independent experiments. All values are expressed as the mean ± SD (n=3).

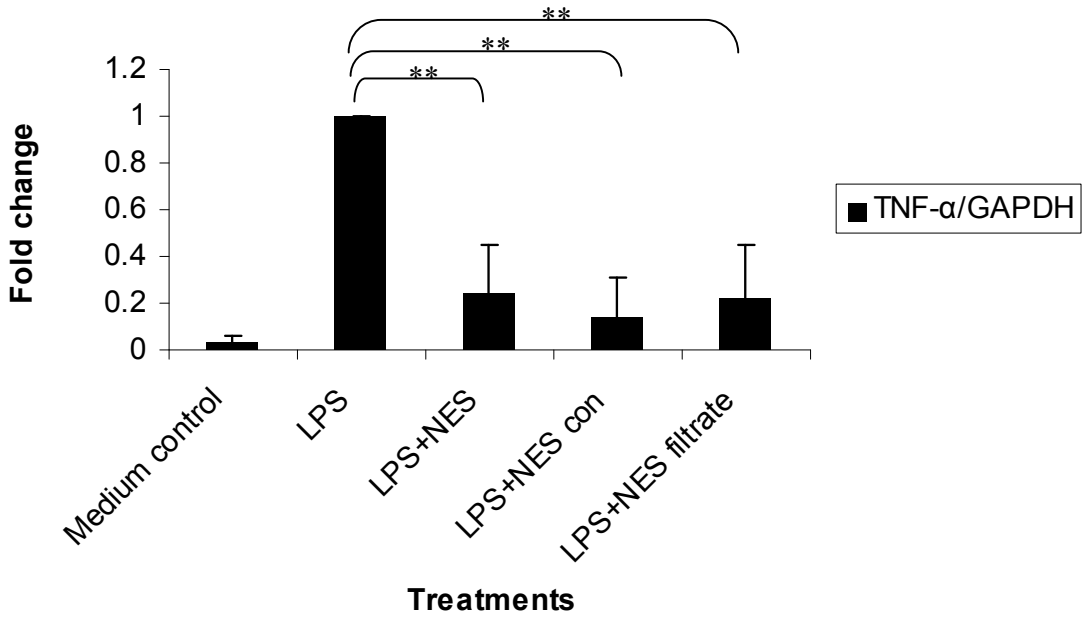
4.2.3.2 Effects of NES, NES concentrate and NES filtrate on the gene transcription of inflammatory molecules

Six hours after LPS stimulation, gene transcription of TNF-α, IL-1β, MIP-2 and ICAM-1 in NR8383 cells were significantly increased, which symbolises the activation of the pro-inflammatory mediators' network in NR8383 cells. Consistent with the significant inhibition of TNF-α protein by NES and NES concentrate (Figure 4.1), TNF-α gene transcription was significantly reduced by NES ($p<0.01$) and NES concentrate ($p<0.01$) (Figure 4.10A). Interestingly, NES filtrate also significantly inhibited TNF-α gene transcription ($p<0.01$). IL-1β is another important pro-inflammatory cytokine. Crude NES or its components, both NES concentrate and NES filtrate, had no significant effect on IL-1β expression induced by LPS (Figure 4.10B). There was no significant reduction of ICAM-1(Figure 4.10C), MIP-2 (Figure 4.10D) gene transcripts by NES, NES concentrate or NES filtrate.

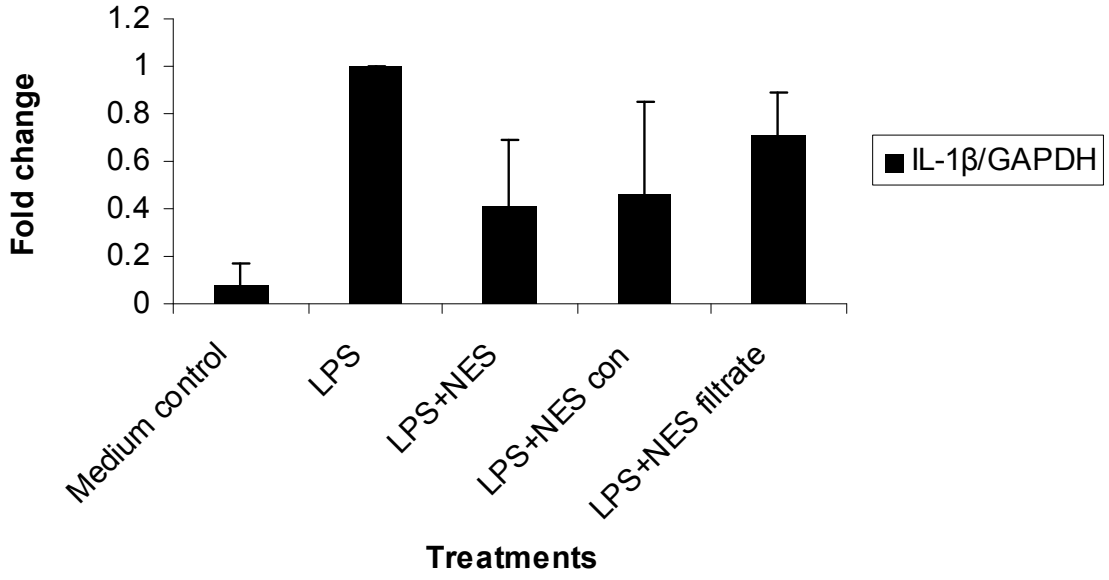
4.2.3.3 Effect of NES concentrate on recombinant rat TNF-α

This experiment was designed to investigate the possible post-translational degradation of TNF-α by NES protein. Recombinant rat TNF-α (rrTNF-α) incubated with trypsin was used as a 100% degradation control. We observed no degradation of rrTNF-α by NES concentrate (Figure 4.11).

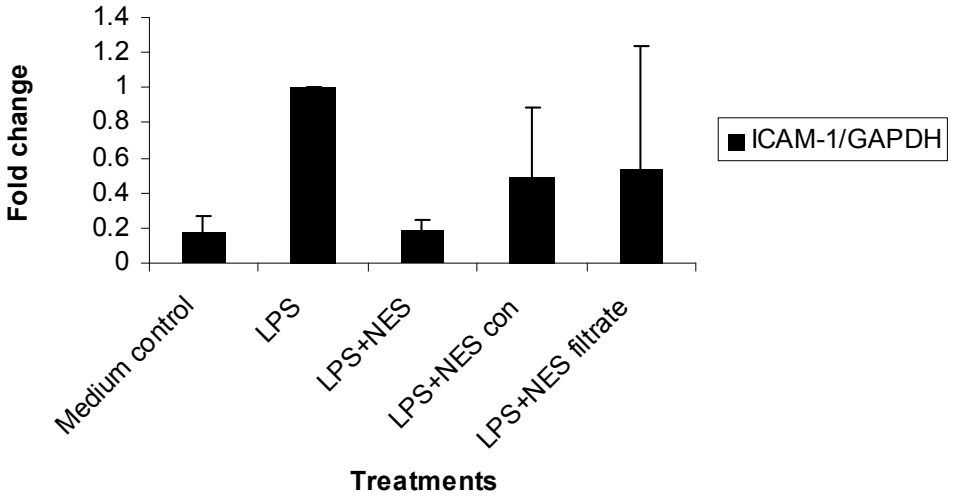
A.


**
**
**
1.2
1
0.8
0.6
0.4
0.2
0
Fold change
TNF-α/GAPDH
Medium control
LPS
LPS+NES
LPS+NES con
LPS+NES filtrate
Treatments

B.


1.2
1
0.8
0.6
0.4
0.2
0
Fold change
IL-1β/GAPDH
Medium control
LPS
LPS+NES
LPS+NES con
LPS+NES filtrate
Treatments

C.



D.

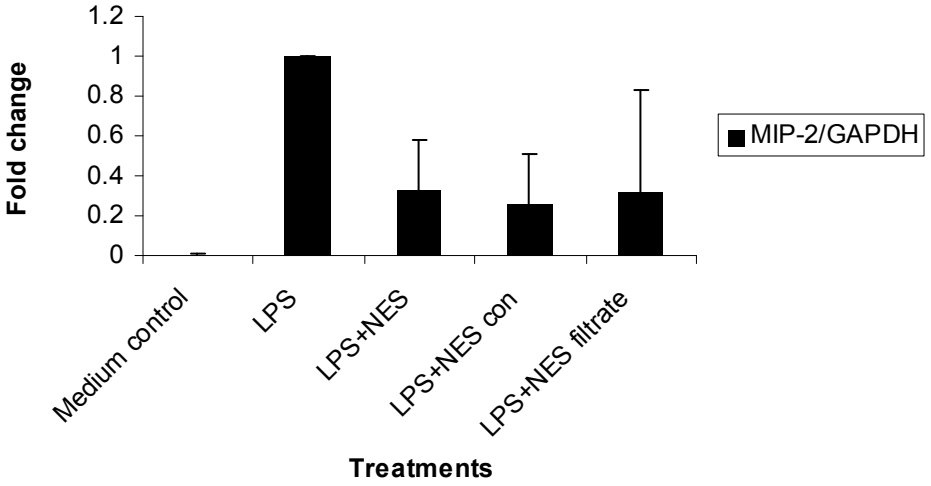


Figure 4.10 Quantitative real-time PCR in NR8383 cells ($1x10^6$ cells) 6 hours after cells incubated with ES medium control, LPS (25ng ml^{-1}) with or without NES, NES concentrate (NES con) or NES filtrate. Cells treated with LPS (25ng ml^{-1}) only were used as positive controls. TNF-α (**A**), IL-1β (**B**), ICAM-1 (**C**), MIP-2 (**D**) gene transcripts were quantified as the molecule/GAPDH ratio. The result is expressed as the mean fold change to positive control ± SD (n=5). **: $p<0.01$.

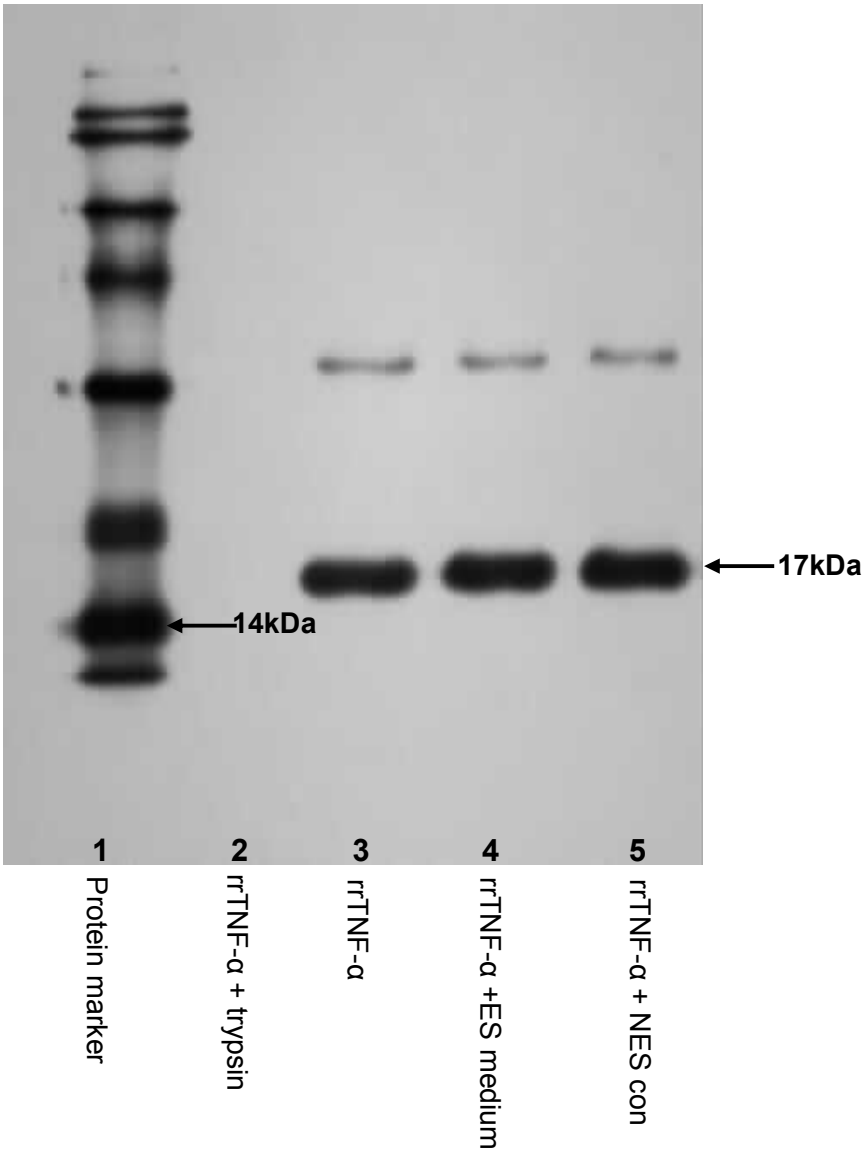


Figure 4.11 Degradation of recombinant rat TNF-α (rrTNF-α). 300ng rrTNF-α was incubated with 600ng trypsin (**lane 2**), none (**lane 3**), ES medium (**lane 4**) or NES concentrate (NES con) (~100ng, **lane 5**) at 37ºC for 18 hours before detecting by western blot. Protein molecular weight was indicated by the protein marker (**lane 1**). Result represents two independent experiments.

4.3 Discussion

In the case of parasitic helminth infection, host health and parasite survival rely mostly on a host immune system with a low level of pro-inflammatory molecules, which is associated with the active release of immune regulatory ES molecules by helminths (Maizels and Yazdanbakhsh, 2003). In chapter 3, we have observed the inhibition of LPS-induced TNF-α production by *N.brasiliensis* L3 larvae ES (NES) *in vitro*. In the first part of this chapter, we demonstrated that such an inhibitory effect was only retained by NES concentrate, but not NES filtrate. This indicates that the active components largely existed in NES concentrate. In contrast to the effect of crude NES, neither NES concentrate nor NES filtrate had effect on LPS-stimulated NO production. Therefore the regulatory function of NES concentrate or NES filtrate on NO production was limited compared to crude NES. This limitation could be both quantitative and qualitative, which remains unclear from the results available. There can be an assumption that some concentrated components in NES protein may have stopped the inhibitory effect. Previously in Chapter 3, we did not observe any effect of crude NES on IL-1β production. Reviewed by Dinerallo (1988), the biological effects of IL-1 include eosinophil degranulation and intestinal mucus production, which may have a protective role in terms of worm clearance and inflammation control. Data from this chapter showed an amplification of IL-1β production by NES concentrate, the reason may be that NES concentrate contains more concentrated active molecules that affected protective IL-1β production.

Although the active components of NES concentrate remain as unidentified mixture, at least part of the effect has been proved to be heat-labile and trypsin-sensitive. Results from this chapter (Figure 4.4) found that the inhibitory effect on TNF-α production by heat-treated NES concentrate was not as much as NES concentrate itself. A previous proteolytic study reported the presence of heat-labile proteases in NES (Healer et al., 1991). Although the immune activities of these proteases are still unknown, they may be related to the inhibition of LPS-stimulated TNF-α production. It has been revealed in *vivo* that heat-treated NES lost its ability to induce the production of IL-4 (Holland et al., 2000). The reason may be that heat-treated NES had reduced ability to induce IL-4 to counteract TNF-α. However, a full study of Th1 and Th2 cytokines is required to test this assumption. It is interesting that heat-treatment of NES concentrate did not totally demolish its inhibitory effect on TNF-α production, which suggested the existence of heat-stabilizing mechanisms in NES concentrate. A study by Wang et al (1996) found that deglycosylation decreased the thermal stability of the protein. This led us to believe that glycosylated proteins could remain as the active components in NES concentrate. In addition, the result showed that trypsin digestion of NES concentrate significantly reversed its inhibitory effect on TNF-α production. Trypsin hydrolyses peptide bonds specifically at the carboxyl side of arginine and lysine residues. It is reasonable to consider that the active sites of some active components in NES are rich in arginine and lysine residues. However, we can not exclude the possibility that trypsin may affect the function of NR8383 cells. An earlier study demonstrated that prior treatment of alveolar macrophages with trypsin severely decreased their capacity to bind ligands such as glycoproteins and synthetic glycoconjugates (Stahl et al., 1978). Further studies are necessary

to explore the roles of trypsin-sensitive molecules, both in NES concentrate and on the macrophage surface.

In this study, NES concentrate was collected by cutting off small molecules with MW less than 3kDa. Thus the active components within NES protein are molecules > 3 kDa, which include polypeptides (proteins), polysaccharides (glycans) and glycoproteins. Small molecules including small peptides or oligosaccharides had no effects in our experiments. Immunomodulatory molecules in helminth ES have been identified (Proudfoot, 2004) and belong to groups as proteases, glycoproteins, protease inhibitors, cytokine mimics, anti-oxidants and nucleotidases. Nearly all of these molecules have a MW greater than 3kDa. Future work will be focused on the identification and characterization of the specific active molecules in NES protein.

The protein profiles of NES concentrate were analyzed by SDS-PAGE. There were more protein bands detected in the larvae somatic extract compared to NES protein. The protein bands in NES concentrate were mostly between 6 and 100kDa, with the dominant bands of 18-25kDa and 34kDa. Lectin blot results in this chapter demonstrated that glycoproteins are abundant in NES concentrate. Inhibition of the lectin-binding of NES concentrate with specific sugars, for example mannose and glucose for ConA and galactose and lactose for PNA, would help to check the specificity of binding and identify sugar chains (Casaravilla et al., 2003; Gruden-Movsesijan et al., 2002). However, given the importance of glycoproteins as discussed below, our preliminary lectin-blot result showed the first

time the glyocoprotein profile in NES. Glycoproteins are known to contain covalently bound sugars, termed glycans that have been shown to play significant roles in complex biological responses (Winterburn and Phelps, 1972). It has become apparent that N-glycosylation of a protein can have a significant stabilising effect on large regions of the backbone structure (Wormald and Dwek, 1999). The oligosaccharide side chains of some glycoproteins have been found to be antigenic in *S.mansoni* (Grzych et al., 1987) and *T. spiralis* infections (Reason et al., 1994). Glycans in the soluble extracts of major helminths, such as *C. elegans* and *B.malayi*, trigger biased Th2 immune responses (Tawill et al., 2004). Oligosaccharide lacto-N-fucopentaose III (LNFP-III) of *S.mansoni* induces spleen cells to produce a large amount of IL-10 and prostaglandin E2, which are mediators that have a known function to downregulate Th1 inflammation (Velupillai and Harn, 1994). Galectins, which are glycan-binding proteins expressed by several cells of the immune system, have immune regulatory functions in several conditions including inflammatory diseases (Rabinovich et al., 2007). Interestingly, helminths express calcium-dependent (C-type) lectins that shares sequence and structure similarities with host galectins (Loukas and Maizels, 2000; Loukas et al., 1999). TES-70 is a C-type lectin secreted by *T. canis* that binds to host ligands (Loukas et al., 2000). The relationship between host and parasite glycans and lectins may be both competitive and collaborative in terms of immune responses. Glycosylation of NES protein components may be a vital modification for their immunomodulatory properties. Sugar chains in glycoproteins are usually located outside of the protein molecule (Tomiya et al., 1988). Due to the glutinous nature of sugar chains, the structure may benefit interactions between the glycoproteins in NES and other molecules on host cell surface. Oligosaccharide side chains

within the glycoprotein have been found to be resistant to some proteases (Kelly and Alpers, 1973). We can hypothesise that the glycosylation of NES concentrate may be important for *N.brasiliensis* to defend against the host proteases. PNA and ConA lectin blot results of this chapter suggested that most of the glycoproteins in NES contain both *O*-linked and *N*-linked chains, with the later dominant. The sugar binding specificity of ConA also suggested that the abundant N-linked sugar chains detected in NES protein contain α-mannose and/or α-glucose (Nishikawa et al., 1975). As introduced in section 4.1, mannose receptor on rat peritoneal macrophages has been demonstrated to be partially involved in *T.spiralis* crude somatic extract and L1 larval ES-stimulated NO production (Gruden-Movsesijan and Milosavljevic, 2006). The possible effects of these saccharides on the immune systems, especially mannose receptor binding, have not been fully revealed in *N.brasiliensis* infection. An 116kDa glycoprotein band seemed to only have *N*-linked sugar chains. This indicated that asparagine-linked oligosaccharide side chains are dominant in most of the glycoproteins synthesized and secreted by *N.brasiliensis* larvae. Suggested by Haslam et al (1999), N-linked glycan is conserved among filarial nematodes, including a well-studied immune modulator, ES-62. However, reviewed by Dell et al (1999), structures and molecular functions of helminthic glycoproteins of each nematode species can be highly specific. In order to study the immune functions of N- or O- linked glycoproteins in NES, specific N- or O- glycanases maybe used in future studies.

Originally indicated by the inverse relationship between helminth infection and some inflammatory diseases, helminth products have been proved to down-regulate inflammatory processes towards a second antigen, such as LPS (Maizels

and Yazdanbakhsh, 2003). In the second part of this chapter, we focused on investigating the LPS-stimulated inflammatory processes that are possibly interfered with by NES. LPS constitutes a major marker for the recognition of Gram-negative bacteria by the host. The interaction with LPS of cells, such as macrophages, leads to the production of a large spectrum of inflammatory mediators and the activation of the host immune response. The LPS strain used in this study, *E.coli* 0111:B4, belongs to S-form LPS molecules. S-form LPS consists of an O-polysaccharide chain, a core oligosaccharide and highly conserved lipid A. Activation of targeted cells by S-form LPS requires the cooperation of TLR4 and accessory proteins including MD-2, CD14 and LBP (Freudenberg et al., 2008). We used flow cytometry to investigate the binding pattern of FITC conjugated LPS to NR8383 cells, but no positive binding was observed with 25ng ml^{-1} FITC-LPS (data not shown). This may due to the low sensitivity of flow cytometry. The recognition of LPS by CD14 is usually amplified by LBP, which is constitutively synthesised in hepatocytes and is present in serum in low concentration *in vivo* (Wright et al., 1990). Without the presence of LBP, the FITC-LPS binding to monocytes were very poor as previously shown in other studies (Zweigner et al., 2001; De Haas et al., 1998). After binding to receptors, LPS induction of pro-inflammatory cytokines is dependant on two pathways, MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Uematsu and Akira, 2006). The roles of these pathways in *N.brasiliensis* induced immune regulation have not been studied in this project, but will be investigated in future.

In mammalian cells, five members of the NF-κB family, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), exist in the cytoplasm of un-

stimulated cells bound to IκB family proteins (Caamano and Hunter, 2002). Activation of TLR4 and MyD88 by LPS is among the pathways that are most commonly associated with the activation and translocation of NF-κB p65, which leads to the transcription of various pro-inflammatory genes, including TNF-α, IL-1, iNOS, chemokines and adhesion molecules (Hayden and Ghosh, 2004). Our results showed the translocation of NF-κB p65 to the nucleus of NR8383 cells after LPS stimulation. This translocation was significantly decreased by NES concentrate, which indicated that NES concentrate contains active components that down-regulate NF-κB p65 translocation. There was no difference between each treatment group in terms of cytoplasmic NF-κB p65, which usually serves as a reservoir of NF-κB protein. It is noticeable that the inhibition of LPS-stimulated NF-κB p65 translocation by NES concentrate (~15%) was not as dramatic as the inhibition of TNF-α production (~60%). In future studies, a more accurate NF-κB assay may be considered, such as electrophoretic mobility shift assay, which directly measures the ability of NF-κB to bind to DNA and the formation of NF-κB complex. However, it has been widely accepted that each NF-κB subunit has distinct and overlapping functions and feed-back loops (Baeuerle and Henkel, 1994; Hayden and Ghosh, 2004). The active components in NES concentrate may have prevented the activation of more than one NF-κB molecule, or the inhibition of NF-κB p65 translocation was amplified by positive feed back loops. Due to the complexity of the intracellular signalling network, more than one pathway and transcription factor is involved in the LPS signalling. Another transcription factor, AP-1, has been found to coordinate with NF-κB in the production of some pro-inflammatory cytokines such as TNF-α (Tak and Firestein, 2001) and they are simultaneously activated in some cases (Han et al., 1998). Therefore the reduction

of TNF-α production by NES may also be the consequence of the AP-1 inhibition, which remains unclear from the results of this study. Furthermore, with the presence of helminth products, the activation of NF-κB may be directed in other ways. It was suggested that a *S. mansoni*-related glycan activated transient, but not persistent, NF-κB translocation via TLR4, which is independent of IκB phosphorylation (Thomas et al., 2005). This transient NF-κB activation was demonstrated to be required for the maturation of dendritic cells to a DC2-type phenotype that is capable of driving naive T cells to differentiate into Th2 cells (Thomas et al., 2003). Adult *N.brasiliensis* ES has also been reported to drive DC2 dendritic cell maturation (Balic et al., 2004). However, further studies are required to examine the involvement of NF-κB transient translocation and identify its roles in the NES associated immune regulation.

Real-time PCR results indicated that the LPS-stimulated TNF-α gene transcription was also significantly reduced by NES, NES concentrate and NES filtrate. This was in contrast to the fact that NES filtrate did not change the production of TNF-α protein nor NF-κB translocation. The explanation for it is still unclear. IL-1β gene expression remained unchanged when NR8383 cells were co-incubated with LPS and NES, NES concentrate or NES filtrate, which was consistent with the results obtained on protein levels. The expression of MIP-2 and ICAM-1 increased in LPS stimulated macrophages, indicating that macrophages are effector cells in recruiting neutrophils and clearing out inflammation. However, NES or NES concentrate showed no effect on the LPS-stimulated expression of key pro-inflammatory mediators, MIP-2 and ICAM-1, *in vitro*. This is not consistent with the inhibition of NF-κB p65 translocation by NES protein. Results from this chapter

therefore suggested that TNF-α may be among the most sensitive gene targets of NF-κB p65, whereas the transcription of other genes may require larger changes of nuclear NF-κBp65 or associate with other transcription factors such as AP-1. In order to observe the effect of NES on LPS-induced inflammatory mediators gene transcription in physiological conditions, experiments were carried out *in vivo* in Chapter 5. Another possibility is that NES or NES concentrate may affect the post-translational modification or release of these pro-inflammatory mediators from the cells. In this chapter, a preliminary experiment showed that NES concentrate failed to degrade recombinant rat TNF-α. However, we can not exclude the possibility that NES concentrate may affect the secondary or tertiary structure of TNF-α protein, or destroy its active binding site with the TNFR. Such effects may not affect its protein size. A detailed study on the possible structural and biological modification of TNF-α by NES protein is needed to answer these questions.

In conclusion, results from this chapter showed that the active components that regulate LPS-induced pro-inflammatory cytokine release by macrophages are within NES protein. These components have molecular weights bigger than 3kDa and may be heat-labile and trypsin-sensitive. NF-κB p65 translocation and TNF-α gene transcription were significantly inhibited by NES protein. However, further *in vitro* studies and *in vivo* studies are required to fully explore the effects of NES on the LPS-induced inflammatory process.

Chapter 5

Effect of NES on LPS-induced inflammation in rat lungs

5.1 Introduction

The results from Chapter 3 and Chapter 4 suggested that *N.brasiliensis* larvae and NES are able to regulate the production of inflammatory mediators by LPS-stimulated rat alveolar macrophages *in vitro*. Experiments with primary cells and animals better represent the complicated immune response network than do cell lines. In this chapter, rats were instilled with 100ng ml^{-1} LPS to the lungs through the trachea as an inflammation model, in a manner consistent with the previous published observation (Keir et al., 2004) for the purpose of a follow on investigation. The effect of NES on LPS-induced lung inflammation was observed 6 hours after instilling the lungs with LPS (100ng ml^{-1}) along with NES. The early inflammatory status in the lungs will shape the following adaptive immune responses. Here we explored the ability of NES to regulate the early immune responses to LPS in the rat lungs.

Exposure to LPS triggers complex inflammatory responses that involve multiple inflammatory mediators and activation or recruitment of inflammatory cells. Uncontrolled inflammation causes damage to the lungs and even leads to chronic infection. Characterization of cells in the BAL is one of the most useful measurements for detecting pulmonary injury. In normal circumstances, more than 85% of BAL leukocytes are macrophages (Denicola et al., 1981; Mauderly, 1977). Any changes to the BAL cell profile can be indicated by the total, and differential, cell counts. In rodent species, an influx of polymorphonuclear cells (PMNs), especially neutrophils, is a sensitive indicator of an inflammatory response (Henderson et al., 1985). Our previous study showed that instillation of NES

inhibited the recruitment of neutrophils by 45% on a background of LPS induced inflammation (Keir et al., 2004). This experiment was repeated in this chapter.

Neutrophil recruitment involves the induction of a succession of inflammatory mediators including chemokines, cytokines and adhesion molecules. The mechanism of LPS induced neutrophil recruitment in rat lungs has been partially determined and involves induction of the cytokines TNF-α and IL-1β, and the CXC chemokine macrophage inflammatory protein – 2 (MIP-2, CXCL-2). A major player in the inflammatory process is ICAM-1, which is a ligand for CD11/CD18. Recruited inflammatory cells such as neutrophils and macrophages, and resident macrophages, can produce toxic radicals, such as NO, for the killing of invading pathogens. iNOS catalyses the production of NO. In this chapter, gene transcription of LPS-induce inflammatory mediators including TNF-α, IL-1β, MIP-2, ICAM-1 and iNOS, were analysed by quantitative real-time PCR as indicators for lung inflammation.

Biochemical indicators of lung injury were also measured in BAL supernatants after centrifugation. The total amount of protein and albumin in BAL were measured as signs of leakage of the alveolar/capillary barrier. Damaged cells release LDH into BAL, which was detected here by the LDH assay. Oxidative stress occurs when the production of free radicals is in excess of the anti-oxidant mechanisms in the respiratory tract. The major intracellular antioxidants in the airways include catalase, SOD and glutathione (Barnes et al., 2003). Reduced glutathione level was measured by GSH assay to indicate the oxidative stress/anti-oxidant balance in the lungs.

5.2 Results

5.2.1 Total protein and albumin concentrations in the BAL

Soluble protein concentration in the BAL fluid was measured by Bradford protein assay. More specifically, albumin concentration was measured to indicate the permeability of the alveolar/capillary barrier, which when damaged, results in more serum albumin leakage into the bronchoalveolar space. As shown in Figure 5.1A and Figure 5.1B, most leakage of protein happened in rats treated with 100ng ml^{-1} LPS, (average 80μg ml^{-1} of total protein and 60μg ml^{-1} of albumin). The protein concentrations in the BAL of the rats treated with 100ng ml^{-1} LPS in NES was very close to that with LPS only. No significant difference was observed between each treatment.

5.2.2 LDH concentrations in the BAL

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is released into the extracellular fluid by damaged cells. Figure 5.2 showed that LDH concentrations were below 300unit ml^{-1} across all the treatments, which indicated very limited cell damage. There was no significant difference between each treatment.

5.2.3 GSH concentrations in the BAL

GSH is a natural intracellular anti-oxidant, which is oxidised to GSSG in the presence of oxidants. Decrease of GSH usually indicates an increase inoxidative stress. Very low amounts of GSH were recovered in the BAL, and no difference was observed between each treatment (Figure 5.3).

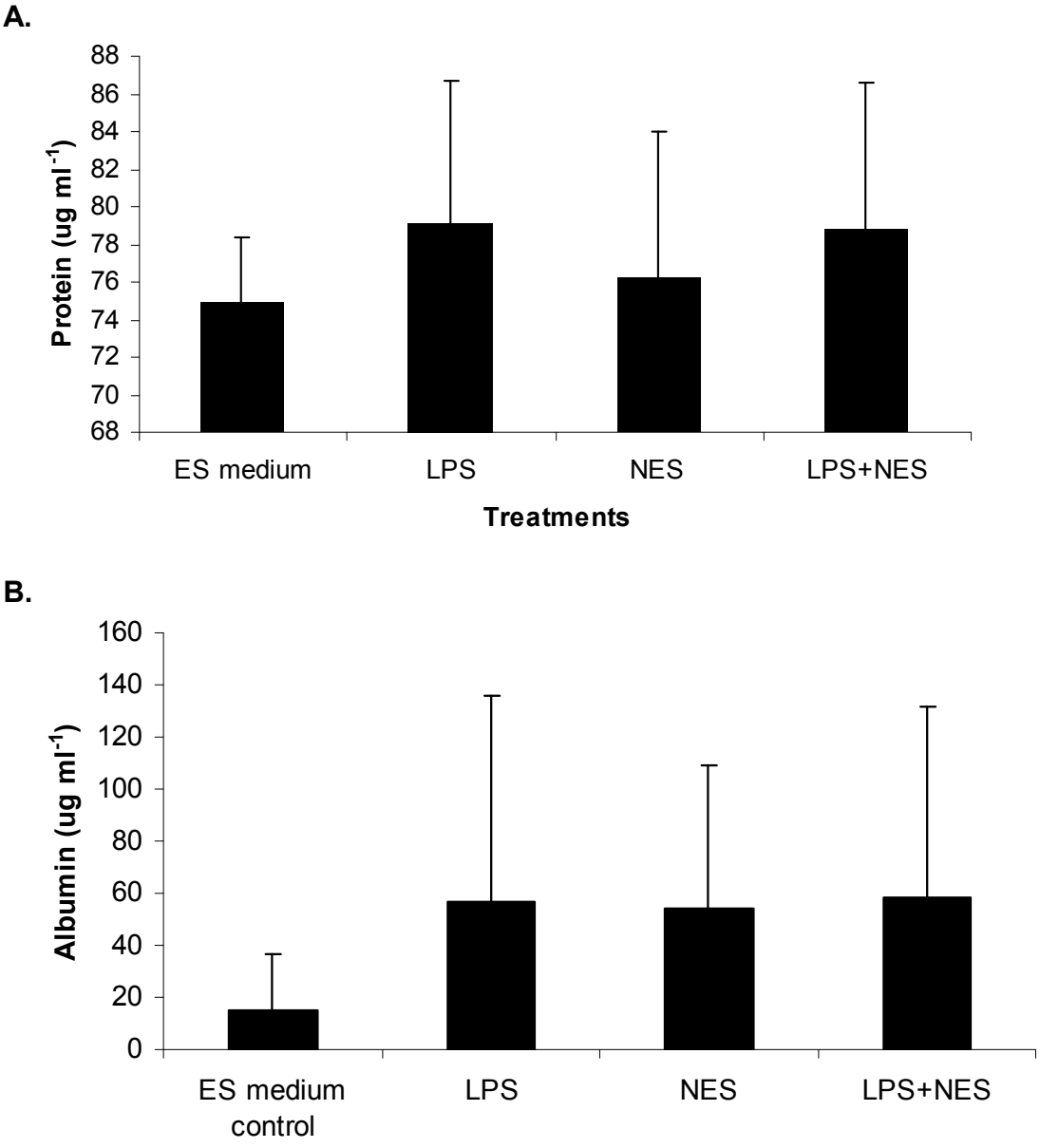


Figure 5.1 Protein (**A**) and albumin (**B**) concentrations in the BAL. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. Total protein and albumin concentrations in BAL were measured by Bradford assay and albumin assay respectively. Results are from five independent experiments. Data are present as means ± SD (n=5).

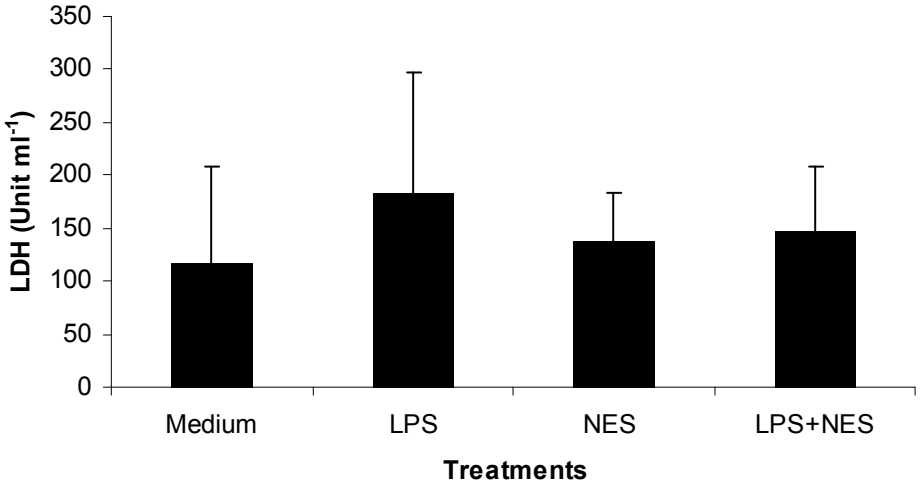


Figure 5.2 LDH concentrations in the BAL. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. LDH concentrations in the BAL were measured by LDH assay. Data are present as means ± SD (n=5).

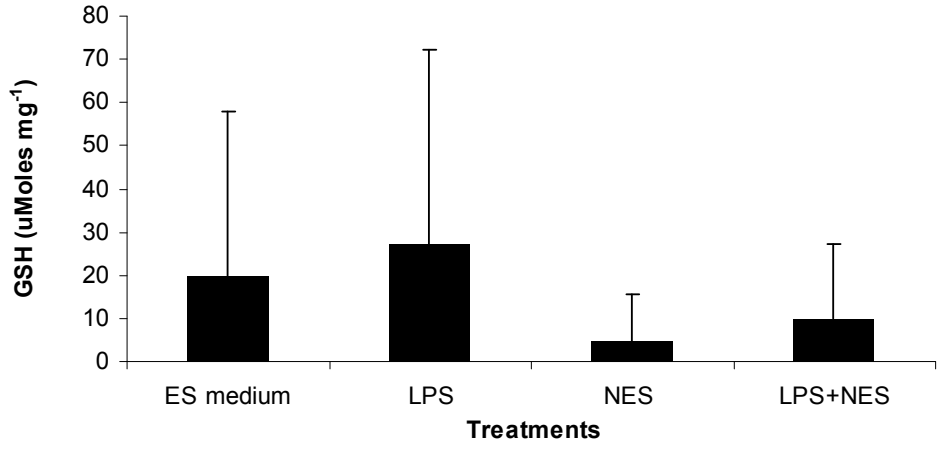


Figure 5.3 GSH concentrations in the BAL. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. GSH concentrations in BAL were measured by GSH assay and normalized with total protein concentration in each treatment. Data are present as means ± SD (n=5).

5.2.4 Leukocyte profile in the BAL cells

Figure 5.4 showed the total number of recruited leukocytes in each of five replicated, independent experiments. Instillation of 100ng ml^{-1} LPS caused the most leukocyte recruitment to the bronchoalveolar space apart from in the 5^{th} replicate of the experiment. The rat lungs instilled with LPS and NES had less leukocyte recruitment compared to LPS only. *N.brasiliensis* NES also caused enrolment of leukocytes. However, no significant difference was detected between each group in terms of total leukocyte numbers. The percentage of each cell type in BAL cells was shown in Figure 5.5, which also provides information for the leukocyte composition in the BAL cells that were used for real-time PCR analysis. The percentages of recruited lymphocytes and eosinophils in each group were all less than 1%. The BAL cells of rats instilled with medium or NES consisted of around 90% of macrophages. Compared to rats instilled with LPS only, the percentage of recruited neutrophils in the BAL of LPS and NES instilled rats was average 20% less. There was no significant difference in leukocyte composition between each group.

In Figure 5.6, taking into account of the total cell number and the BAL cell composition, each type of recruited leukocyte in the BAL was calculated as the fold change to the recruited numbers in the LPS instilled group (positive control). There were no significant differences between each treatment group in terms of recruited numbers of macrophages, lymphocytes and eosinophils (Figure 5.6.B). Rats instilled with LPS and NES had about 40% less recruited neutrophils in the BAL compared to LPS instillation only ($p<0.01$) (Figure 5.6.A), which was consistent with the previous result (Keir et al., 2004).

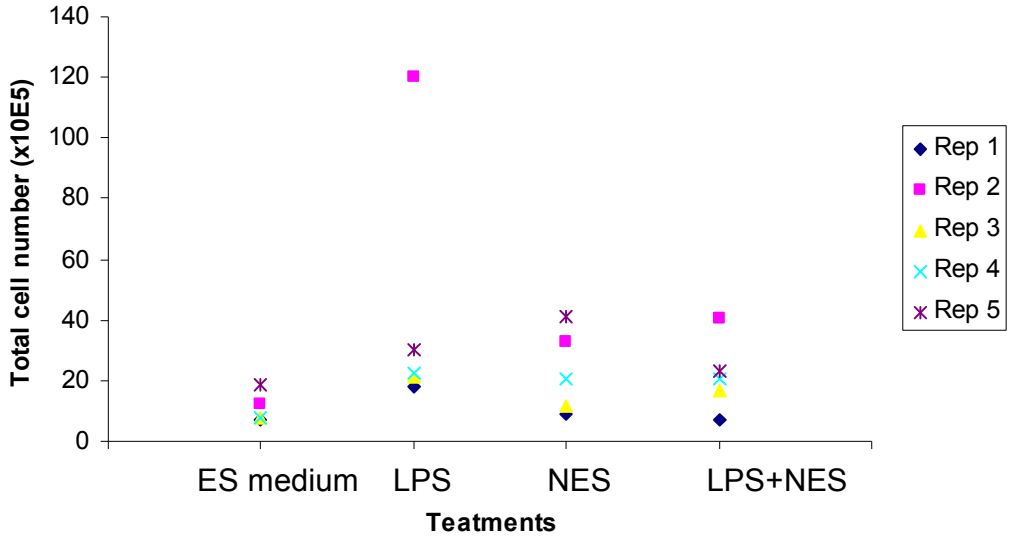


Figure 5.4 Total leukocyte number in the BAL. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. Results are from five independent experiments. Data are present as means ± SD (n=5).

BAL cells (%)	ES medium	LPS	NES	LPS+NES
Macrophages	97.9±0.8	34.2±15.5	91.8±9.9	52.3±21.8
Neutrophils	1.6±0.6	64.5±14.5	5.3±8.0	44.8±20.6

Figure 5.5 Percentage of each cell type in BAL cells. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. Results are from five independent experiments. Data are present as means ± SD (n=5). The percentages of lymphocytes and eosinophils were both less than 1% for all treatments.

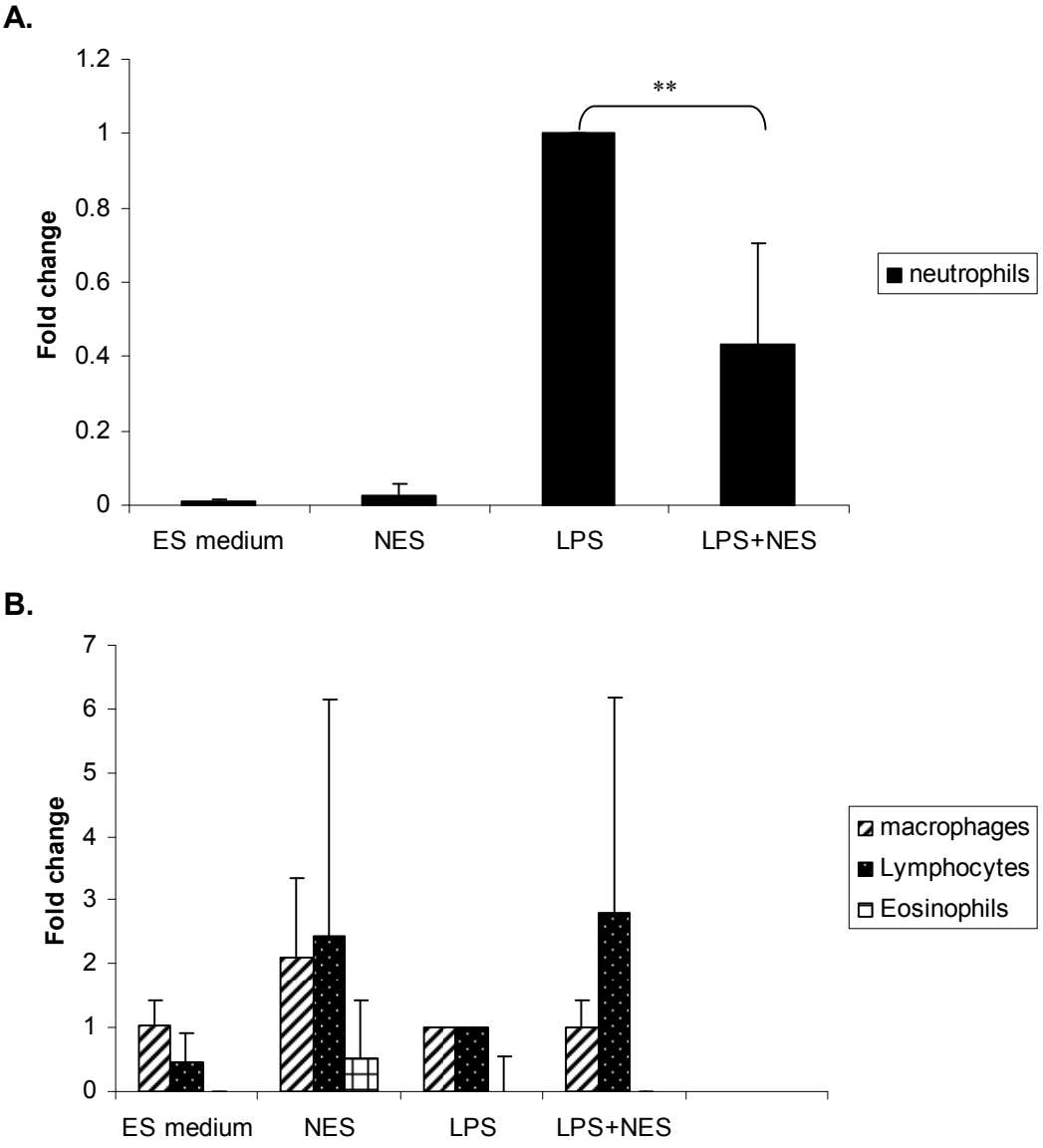
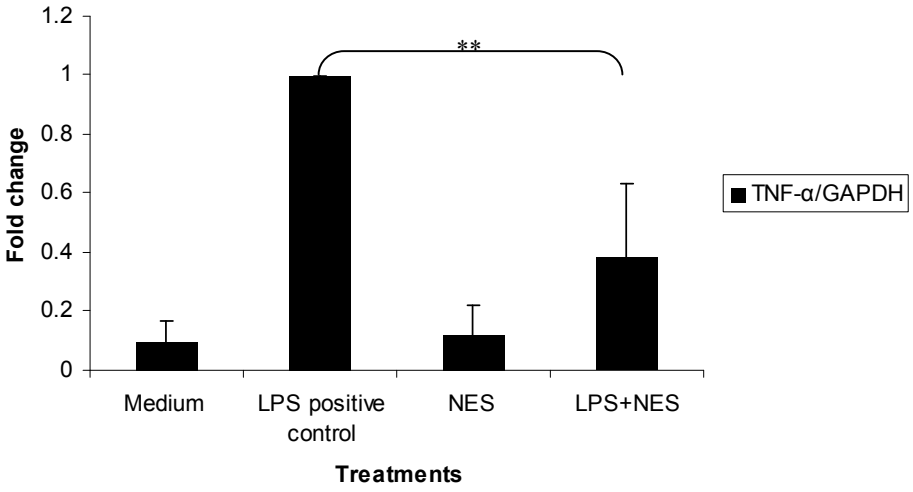


Figure 5.6 Differential cell counts in BAL cells. Rats were instilled with 0.5ml ES medium, 0.5ml LPS (100ng ml^{-1}), 0.5ml NES, or LPS (100ng ml^{-1}) in NES for 6 hours before the lungs were lavaged and collected for BAL. Rats instilled with LPS (100ng ml^{-1}) only were used as positive controls. **A:** Fold change of BAL neutrophils to the positive control. **B.** Fold change of BAL macrophages, lymphocytes and eosinophils to the positive control. All values are expressed as the mean fold change to positive control ± SD (n=5). **: $p < 0.01$.

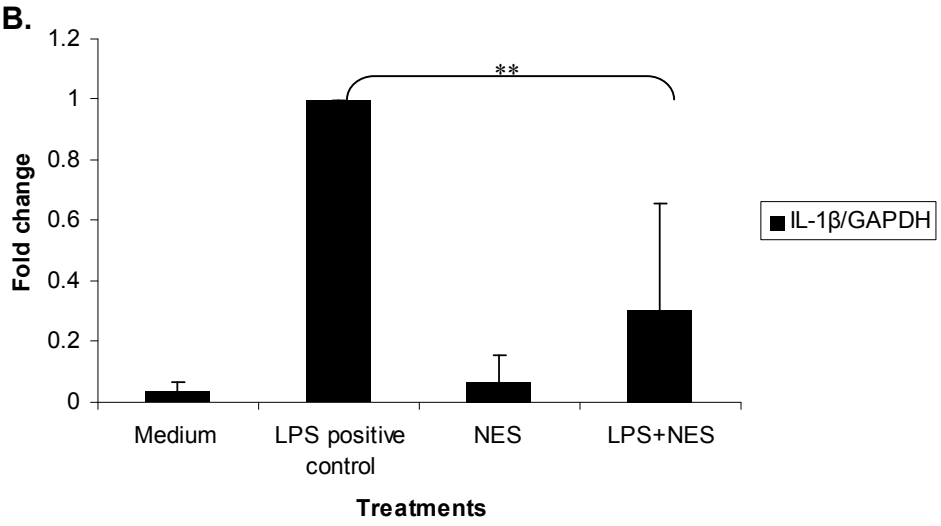
5.2.5 Gene transcription of inflammatory mediators in BAL cells

Quantitative real-time RT-PCR was used to measure the induction of inflammatory mediators in the BAL cells 6 hours after intratracheal instillation with LPS and/or L3 NES. Figure 5.7 A-E indicated a general inhibition of LPS-induced pro-inflammatory mediator gene transcription in BAL cells by NES. Gene transcription of the pro-inflammatory cytokines, TNF-α and IL-1β, were both significantly inhibited by LPS and NES compared to LPS alone (Figure 5.7A and Figure 5.7B; all $p<0.01$). The reduced neutrophil recruitment to the lung by NES suggested that inhibition of MIP-2 (CXCL-2), a neutrophil chemoattractant could be involved. We observed significant reduction in gene transcription of MIP-2 (Figure 5.7C) by NES and LPS compared to LPS alone ($p<0.01$). Induction of the adhesion molecule ICAM-1 (Figure 5.7D) was also significantly inhibited by NES and LPS compared to LPS alone ($p<0.01$). Gene transcription of iNOS, which catalyses the production of NO, was significantly down-regulated by L3 NES and LPS compared to LPS alone (Figure 5.7E).

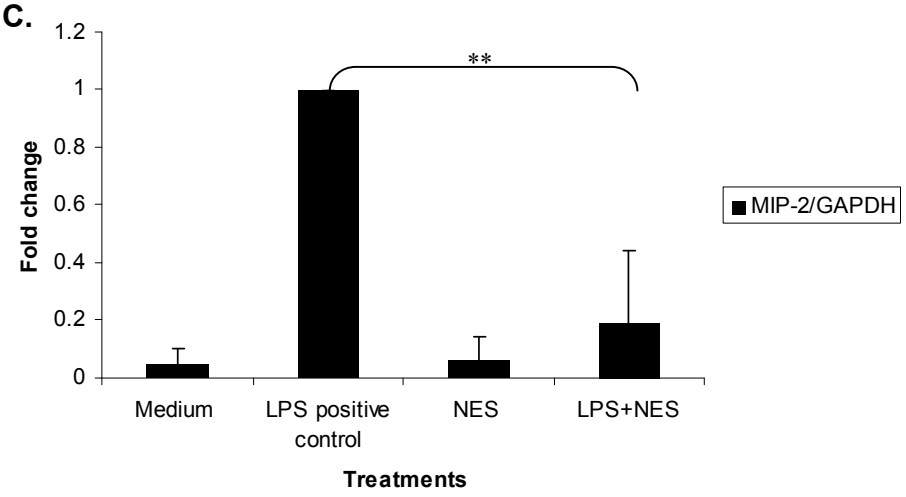
A.


1.2
1
0.8
0.6
0.4
0.2
0
Fold change
**
TNF-α/GAPDH
Medium
LPS positive control
NES
LPS+NES
Treatments

B.


1.2
1
0.8
0.6
0.4
0.2
0
Fold change
**
IL-1β/GAPDH
Medium
LPS positive control
NES
LPS+NES
Treatments

C.


1.2
1
0.8
0.6
0.4
0.2
0
Fold change
**
MIP-2/GAPDH
Medium
LPS positive control
NES
LPS+NES
Treatments

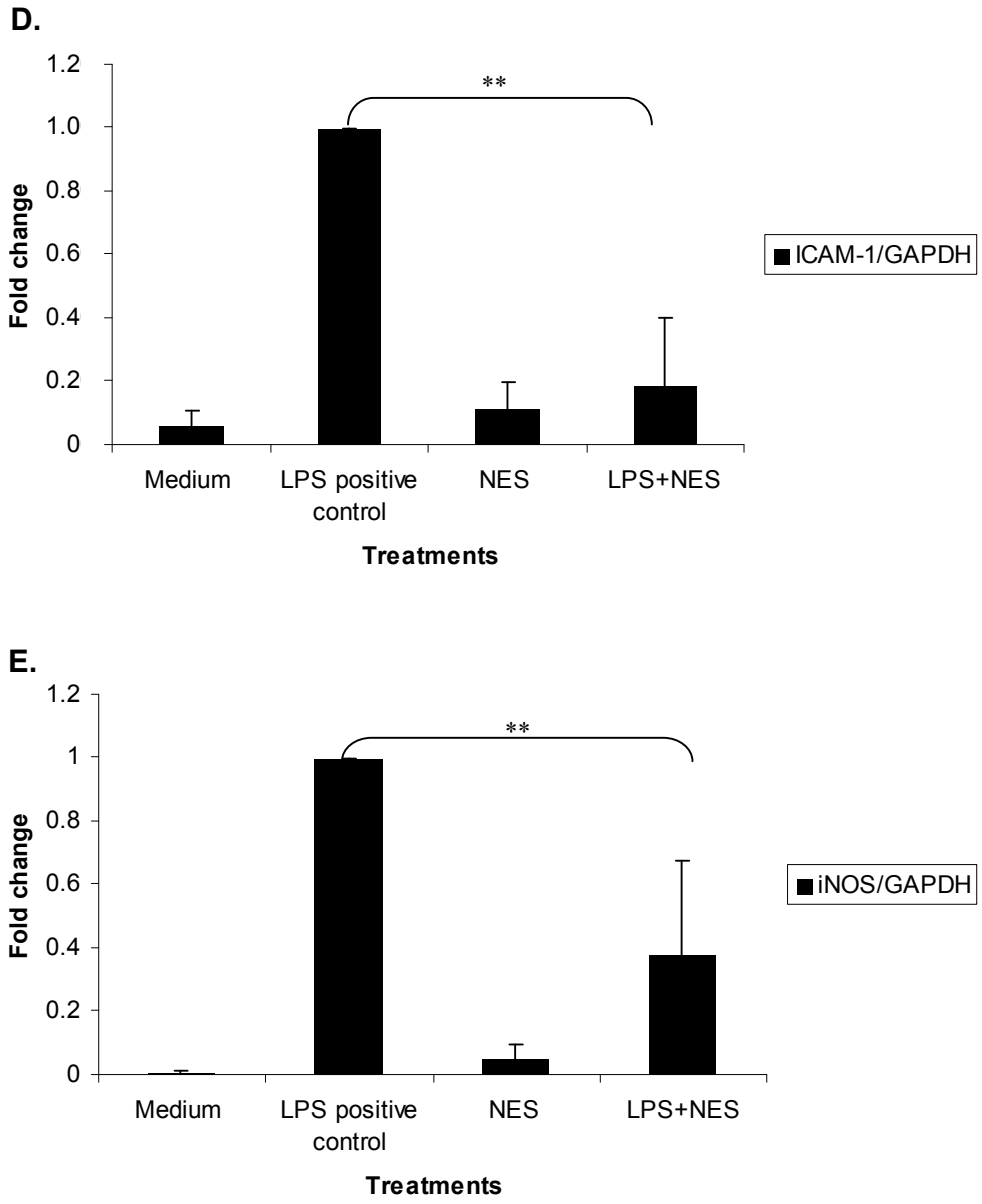


Figure 5.7 Quantitative real-time PCR in BAL cells 6 hours after rats instilled with ES medium, *E. coli* LPS (100ng ml^{-1}), NES or 100ng ml^{-1} LPS in NES. Rats instilled with 100ng ml^{-1} LPS only were used as positive controls. TNF-α (**A**), IL-1β (**B**), MIP-2 (**C**), ICAM-1 (**D**) and iNOS (**E**) gene transcription were quantified as the molecule/GAPDH ratio. The results are expressed as the mean fold change to positive control ± SD (n=5); **: $p< 0.01$.

5.3 Discussion

Bronchoalveolar lavage (BAL) fluid collected six hours after instillation is likely to indicate rapid changes in the lining of the respiratory tract and would be likely to show damage or inflammation in the lungs. The results from this chapter did not detect any significant indications of lung damage in BAL in all treatments as determined by LDH concentration. Total protein and more specifically albumin concentrations in BAL have been linked with physical damage to the alveolar bed and the intergrity of the alveolar/vascular barrier (Mauderly et al., 1977). A previous experiment reported that the average total protein level in the BAL from non *N.brasiliensis* infected rats was 200mg ml^{-1} (McNeil et al., 2002). In this study, lower concentrations of total protein and albumin were detected in the BAL. This indicated limited damage to the lung tissues by each type of instillation. Primary bronchial epithelial cells, alveolar macrophages and other lung cell lines have been shown to secrete glutathione peroxidase and maintain high levels of GSH in the alveolar epithelial lining fluid to protect against oxidative stress (Rahman et al., 2005). In *N. brasiliensis* infection, it has been demonstrated that GSH was consumed to counteract oxidative stress in the lungs (McNeil et al., 2002). Data from this chapter showed low levels of GSH across all the treatments, and no significant differences in GSH were observed between each treatment. GSH levels in the alveolar lining fluid may have been diluted via the alveolar lavage procedure. It is also possible that GSH was consumed to combat the oxidative stress, this now requires further proof from other oxidative stress assays, such as superoxide assay. In addition, many cellular proteins have a highly conserved cysteine sequence in their active sites, which are primary targets of oxidative modifications.

Emerging evidence suggests that intracellular GSH reacts with oxidants to form disulphide GSSG, to protect these proteins from peroxidation. The GSSG/GSH ratio is therefore a good cellular indicator of redox state (Valko et al., 2007). It will be interesting to examine GSSG/GSH ratio in BAL cells as an indication of cellular protein intergrity in future. Changes in BAL NO, detected as nitrite, were not identified here (data not shown), which suggests that there may be a lack of nitrosative stress 6 hours after instillation. By allowing time for transcription and translation of iNOS, there would only have been 1-2 hours to produce NO and then nitrite from the enzyme. Therefore, even with maximal induction of iNOS, little nitrite would have been detectable. There was an increase in gene transcription of iNOS in LPS (100ng ml^{-1}) instilled BAL cells, which was significantly reduced by instilling the rats with LPS and NES together. Although NO serves more complicated immune functions than the formation of free radicals, the reduced iNOS gene transcription could predict the inhibition of nitrosative stress by NES in a later stage.

An acute infection with *N. brasiliensis* normally results in rapid expulsion of adult parasites from the gut however, prior to this, larvae must survive lung migration. It would appear that host-parasite interactions in the lung have evolved to promote migration of the parasite and to minimise pathology. Previous studies have identified low numbers of neutrophils in the airways following infection with *N. brasiliensis* (McNeil et al., 2002). Considering the total recruited cells and cell composition together, the results from this chapter showed that instillation with LPS and NES significantly reduced neutrophil recruitment by approximately 40% compared to LPS alone, which was in agreement with the previous findings by

Keir et al (2004). Many pulmonary pathology processes, such as chronic obstructive pulmonary disease (COPD), initiates from the inflammation-induced accumulation of neutrophils. Recruited neutrophils produce serine proteases, matrix metalloproteinases (MMPs) as well as oxidants, that contribute to airway obstruction and destruction (Barnes et al., 2003). Results from this chapter suggested that NES is capable of minimizing the pro-inflammatory properties of LPS by inhibiting induction of neutrophil recruitment. The inhibition of neutrophil recruitment happened as early as 6 hours after instillation, and at which time point, the production of Th1 cytokine proteins (TNF-α and IL-1β) and NO were still undetectable. The inhibition of neutrophil recruitment was more likely to be caused by the immune active molecules of NES. As discussed before, a hookworm glycoprotein has been reported to inhibit neutrophil functions (Moyle et al., 1994). Similar mechanisms might be shared between these species. McNeil et al (2002) reported that *N.brasiliensis* infection is associated with reduced oxidative stress in the lungs as measured as superoxide and lipid peroxidation levels. As a result, it may protect the cell membrane integrity and prevent neutrophil infiltration.

On exposure to LPS, BAL cells (resident/ recruited macrophages and recruited neutrophils) are in the first line of defence and the first to respond to the LPS inflammatory stimulus. Any inflammatory mediators induced in these cells would reflect the later inflammatory status of the whole lung tissue. TNF-α and IL-1β are pro-inflammatory cytokines induced by LPS, which play important roles in promoting and amplifying lung inflammation through the release of chemoattractants and by up-regulating the expression of cell adhesion molecules (Ulich et al., 1991; Strieter and Kunkel, 1994). Down-regulation of TNF-α is

observed in many parasitic helminth infections including *T.suis* (Kringel et al., 2006*), B.malayi* (Babu et al., 2006) and *N.brasiliensis* (Matsuda et al., 2001; McNeil et al., 2002). We have shown previously that TNF-α was not detected during a primary infection with *N. brasiliensis*. A large amount of TNF-α activity is shared with interleukin-1 (IL-1) and transient IL-1 production was observed in *N.brasiliensis* infected rats (Benbernou et al., 1992). Some parasitic helminth derived products have been demonstrated to have a potent anti-inflammatory activity by suppressing pro-inflammatory cytokine production. The filarial nematode product ES-62 reduces LPS/IFN-γ induced IL-12p40, IL-6 and TNF-α production in macrophages (Goodridge et al., 2001). A protein component, PAS-1, from *A.suum*, significantly reduced LPS-stimulated TNF-α and IL-1β levels (Oshiro et al., 2005). Results from this chapter showed that LPS-induced TNF-α and IL-1β gene transcription in BAL cells were inhibited by NES as early as 6 hours after instillation, which is consistent with previous findings.

MIP-2 is the rat equivalent of human IL-8 and plays a crucial role in influencing early cell trafficking and neutrophil activation (Driscoll, 1995). MIP-2 has been defined as a potent neutrophil chemotactic factor in various parasitic helminth infections. MIP-2 upregulation has been linked to the intensity of inflammatory cell recruitment and inflammation in *S.mansoni* (Park et al., 2001). In a mouse model of *O.volvulus* mediated keratitis, targeted mutation of MIP-2 receptor significantly impaired neutrophil recruitment and development of corneal opacification (Hall et al., 2001). MIP-2 has been demonstrated to mediate neutrophil recruitment to the *T.spiralis* infected rat intestinal epithelium (Frydas et al., 2002). Depleting neutrophil and blocking neutrophil migration, both significantly reduced IL-1β gene

transcription in intestinal epithelial cells (Stadnyk et al., 2000). Although the role of MIP-2 in *N.brasiliensis* infection has not been defined, our results suggested that NES reduced LPS-induced MIP-2 production (Figure 5.7.C), which is in parallel with the inhibition of LPS-induced neutrophil recruitment in BAL (Figure 5.6.A).

On exposure to LPS, upregulated expression of ICAM-1 in endothelial cells and leukocytes causes the trafficking of neutrophils by recognition of their integrin receptors (Beck-schimmer et al., 1997; Yonekawa and Harlan, 2005). It should be noted that we also have preliminary results suggesting a reduction in ICAM-1 mRNA from lung tissue during a time course infection with *N.brasiliensis* (Keir, PhD thesis). A previous study reported that a glycoprotein from the canine hookworm *A.caninum* directly inhibits neutrophil function by blocking the CD11/ CD18 integrins, thereby preventing neutrophil infiltration to the sites of infection (Moyle et al., 1994). The possibility of *N.brasiliensis* ES components to hold such a function is yet to be determined. ICAM-1 is conventionally regarded as a molecule associated with the endothelium, however it is also important in activated macrophages and their interaction with other leukocytes, particularly in an accessory role in T cell activation (Wingren et al., 1995). Results from this chapter demonstrated that NES diminished ICAM-1 gene transcription to a level similar to that induced by ES medium or NES instillation only. The inhibition of ICAM-1 in BAL cells by ES could indicate a direct effect, but this could also be an indirect effect of TNF-α and IL-1β inhibition by ES as these cytokines would themselves induce ICAM-1 (Strieter and Kunkel, 1994).

In this experiment we also demonstrated an inhibition of iNOS transcription by NES. As mentioned earlier, our previous results (McNeil et al 2002) indicated that NO production would be increased by *N.brasiliensis* infection. This controversy suggests that the stimulation of NO production by *N.brasiliensis* in the rat lungs differs with and without the presence of LPS, which may be related to toll-like receptors on the cell surface in reference to other organisms (Doz et al., 2007). A reduction of NO in LPS-stimulated macrophages was demonstrated in the other parasite ES (Fukumoto et al., 1997; Wang et al., 1997). NO can be an anti- or pro-inflammatory molecule in different circumstances (Barnes and Liew, 1995). CAMs produce NO as a killing mechanism in response to bacterial LPS whereas later in the inflammatory process, NO could act as an inhibitor of neutrophil chemotaxis (Barnes and Liew, 1995). A main feature of helminth infection is the induction of AAMs, which are distinguished from others by elevated arginase I production and reduced NO production (Reece et al., 2006). Primary macrophages stimulated with *Fasciola hepatica* ES products together with LPS have reduced NO production, which is due to stimulation of AAMs (Flynn and Mulcahy, 2008). Whether the reduction of iNOS gene transcription was due to reduced CAMs or increased AAMs numbers in the airway will be one of the future questions to be answered.

Although the inhibition of inflammatory mediators was highly significant, the inhibition of neutrophil recruitment in this experiment was not as dramatic as expected and suggests that additional mechanisms may be responsible for neutrophil entry into the airways. Alternatively, there may be an alteration in neutrophil phenotypes. In addition to normal PMN-N, there are at least two other

subsets of PMNs, PMN-I and PMN-II, have been classified according to their cytokine and chemokine production, macrophage activation, TLR expression and surface antigen expression. PMN-I promotes CAM and NO, whereas PMN-II promotes AAM and reduced NO production (Tsuda et al., 2004). PMN-I and PMN-II have been described recently in mice with different susceptibilities to bacterial infection (Tsuda et al., 2004) and *L.major* infection (Charmoy et al., 2007). However, the PMN subsets have not been identified in helminth infections. Future research will focus on the phenotype of recruited macrophages and neutrophils and the mechanism of inhibition of inflammatory mediators by parasite ES in this model of lung inflammation.

In conclusion, results from this chapter showed a general inhibition of LPS stimulated pro-inflammatory mediator gene transcription by NES in BAL cells 6 hours after instillation. This was associated with significant reduction of neutrophil recruitment. Such down-regulation of the early inflammation by NES may be essential for the induction of an anti-inflammatory phenotype in the later immune response.

Chapter 6

General discussion and future studies

6.1 General discussion

In this study, both cell line and animal experiments provided promising findings on the potential anti-inflammatory properties of *N.brasiliensis*. Figure 6.1 is a schematic overview of LPS-induced inflammatory processes potentially affected by *N.brasiliensis* larvae and their ES products (NES) concluded from this study. Suppression of Type 1 inflammatory mediator production by macrophages has been an emerging theme of parasitic helminth infection research. In response to LPS, macrophages from *Toxocara canis* infected rats had diminished production of IL-12 and TNF-α, compared to LPS alone (Kuroda et al., 2001). Similar effects have been seen in murine macrophages exposed to filarial product ES-62 and *A.suum* product PAS-1 (Goodridge et al., 2001; Oshiro et al., 2005). As a supplement to these criteria, results from this study showed for the first time the inhibition of LPS-induced TNF-α production by live *N.brasiliensis* larvae, crude NES and concentrated NES *in vitro*. Receptor binding and intracellular signal transduction are among the key elements in the LPS-stimulated inflammatory process (Beutler et al., 2003). In this study, we did not detect any positive binding of 25ng ml^{-1} FITC-LPS to NR8383 cells by flow cytometry, which may be due to the low sensitivity of the equipment. Although such information remains unknown in *N.brasiliensis* infection, in reference to previous researches, helminth antigens were capable of modulating TLR-initiated dendritic cell (DC) activation (Kane et al., 2004). DCs exposed to soluble egg antigens (SEA) from *S.mansoni* had impaired response to TLR ligand such as LPS and CpG (Pearce et al., 2006). In a recent study by Tolouei et al (2008), live microfilariae (mf)-exposed monocyte-derived human DC had diminished TLR3 and TLR4 expression. Moreover, in this study, mf

interfered with LPS-induced MyD88 expression, which ultimately ends in disabled NF-κB activation and reduced production of IFN-α, MIP-1α, IL-12p40 and IL-1α (Tolouei et al., 2008). Our results showed inhibition of NF-κB translocation in macrophages by NES concentrate, which was followed by diminished TNF-α gene transcription by NES and NES concentrate. In our rat model of inflammation, NES caused a significant reduction in BAL cell gene transcription of pro-inflammatory molecules, including TNF-α, IL-1β, MIP-2, ICAM-1 and iNOS, as early as 6 hours after LPS instillation. This was closely linked with the significant inhibition of neutrophil recruitment, and also indicated an anti-inflammatory phenotype directed by NES. The general inhibition of LPS induced proinflammatory responses by *N.brasiliensis* L3s and NES highlights the possibility of utilizing *N.brasiliensis* in control of Th1 related inflammation, such as IBD, rheumatoid arthritis and type1 diabetes. Recently in a spontaneous arthritis mouse model, animals infected with *N.brasiliensis* had higher IL-4 production, which is associated with decreased tissue damage, such as cartilage erosion and bone destruction, shown by histopathology (Salinas-Carmona et al., 2009). This new study pushed the clinical value of *N.brasiliensis* products in the treatment of Th1 inflammation to an even brighter future.

Evidence from other studies has suggested that antigen presenting cells in the innate immune system may undergo significant and essential phenotype changes when they encounter parasitic helminths. As early as 1985, alveolar macrophages of rats infected with *N.brasiliensis* were demonstrated to be activated and became helminthocidal (Egwang et al., 1985). Not until recently, was a new form of macrophage in nematode infections reported, and classified as AAM (Loke et al.,

2000; Allen and Loke, 2001). Nematode induced AAMs have upregulated expression of arginase, YM1 and FIZZ1, of which YM1 is a chemo attractant of eosinophils and the function of FIZZ-1 was first reported as a novel cysteine rich protein that was abundant in BAL of a rat asthmatic model (Holcomb et al., 2000); Nair et al., 2005). Upregulation of YM-1 in mouse peritoneal macrophages in response to IL-4 was demonstrated to be STAT6-dependent (Welch et al., 2002). These macrophages play an important role in helminth-induced immune regulation by inhibiting T cell proliferation through cell-to-cell contact (Loke et al., 2000). In a previous study, nearly all alveolar macrophages were identified as AAMs two days after *N.brasiliensis* infection, and this was associated with up-regulation of IL-4, IL-10 and IL-13 expression (Nair et al., 2005; Reece et al., 2006). The induction of AAMs in the presence of *N.brasiliensis* could be involved in dampening the level of inflammation in the lungs provoked by LPS. In response to different microbial infection, human myeloid DCs develop into Th1-promoting (DC1) or Th-2 promoting (DC2) effector cells (de Jong et al., 2002). Helminth products usually induce development of DC2s that promote the expansion of Th2 cells via the upregulated expression of OX40 ligand (OX40L) (de Jong et al., 2002). Heat-labile components of *N.brasiliensis* ES have been reported to stimulate development of DC2s that prime Th2 responses (Balic et al., 2004). DCs pre-incubated with adult *N.brasiliensis* ES had reduced IL-12 p70 production in exposure to LPS compared to DCs exposed to LPS but with no pre-incubation to adult ES (Balic et al., 2004). It is reasonable to assume that the down-regulation of LPS-induced pro-inflammatory mediators by NES *in vivo* could involve regulation of DC phenotypes.

Recognition of pathogens by innate immune system and the associated up-regulation of ligands, cytokines and co-stimulatory factors, stimulate T cells differentiation in the adaptive immune system (McGuirk and Mills, 2002). Most helminths induce strong immune responses which are polarized towards the Th2 phenotype (McGuirk and Mills, 2002), within which *N.brasiliensis* is among the most potent activators of Th2 immune response (Lawrence et al., 1996; Mohrs et al., 2001; (Marsland et al., 2005). When mice were sensitised intranasally with 2µg NES and then challenged with 10µg NES on day 10, allergic airway disease developed which was characterized by airway eosinophilia, IgE antibody production and Th2 cytokine production by NES-specific T cells (Marsland et al., 2005). It has been demonstrated that the induction of Th2 biased response does not necessarily need live worms (Holland et al., 2000). In addition, NES immunization has been demonstrated to generate Th2 immune responses when administered alone or with complete Freund's adjuvant (CFA) which usually favours the development of Th1 responses, independent of mouse genotype (Holland et al., 2000). Resistance or susceptibility to most antigens has been demonstrated to be determined by the balance of Th1 vs Th2 cytokines produced during infection (Huang et al., 2001; Shinkai et al., 2002). The polarisation of Th2 cytokine production induced by *N.brasiliensis* may counteract the Th1 immune responses elicited by LPS. However, one limitation of the current study is the lack of data on Th2 immune responses. In addition, other emerging types of immune responses such as Treg and Th17 responses also need investigation in order to obtain a fuller picture of the anti-inflammatory mechanism (Bi et al., 2007). So far, there is still uncertainty on the induction of Treg responses in *N.brasiliensis* infection.

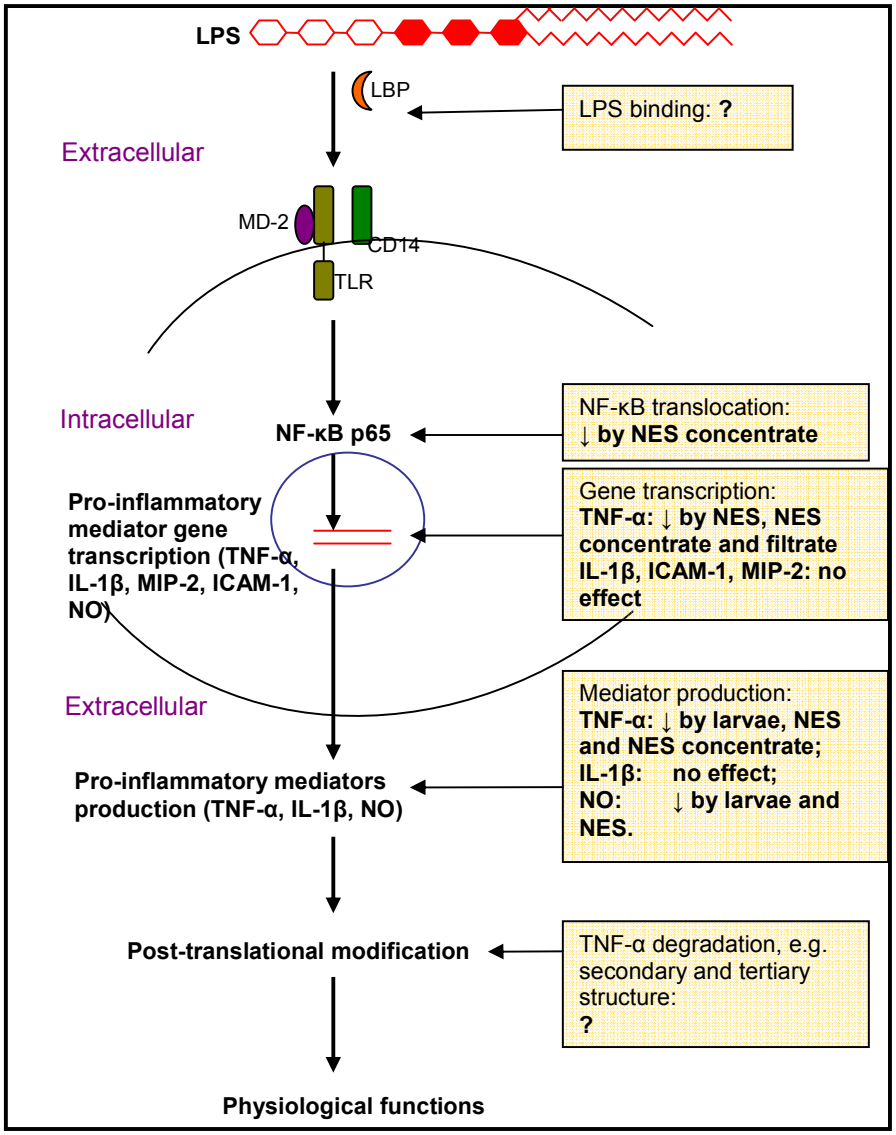


Figure 6.1 Summary of interactions of *N.brasiliensis* larvae and NES with LPS-induced inflammatory processes in macrophages *in vitro* as concluded from this study. The effects of *N.brasiliensis* on LPS binding to NR8383 cells remain unknown from current study. NES concentrate significantly inhibited LPS induced NF-κB p65 translocation into the nucleuses of NR8383 cells. LPS induced gene transcription of TNF-α was significantly decreased by NES, NES concentrate and NES filtrate. However, NES showed no effect on IL-1β, ICAM-1 and MIP-2 gene transcription. TNF-α and NO production by LPS stimulated NR8383 cells were significantly inhibited by live *N.brasiliensis* larvae and NES. TNF-α was also inhibited by NES concentrate but not NES filtrate. Recombinant rat TNF-α was not degraded by NES concentrate, yet the effect of NES on the secondary and tertiary structure of TNF-α is still not known from current study.

The future hypotheses derived from this work are: (1) is the observed reduction of LPS-induced Th1 inflammation a result of direct inhibition by *N.brasiliensis*, or is it

counteracted by Th2 responses induced by the worms; (2) is Treg immune response involved? To answer these questions, a full investigation into the Th2 and Treg immunity in the co-infection of LPS and *N.brasiliensis* is required. Another emerging query is whether inhibition of LPS-induced inflammation in the early stage of a primary infection will persist when the adaptive immunity takes over. Results from current study showed that instillation of NES and LPS together significantly suppressed transcription of pro-inflammatory molecules in rat lungs, compared to LPS alone. We have yet have no data to investigate if a transient exposure of rat lungs to NES via this intra-trachea route would be continually protective against further LPS challenge. It has been demonstrated that, during the early immune responses to *N.brasiliensis* in the lungs of primarily infected mice, transcription of Th2 cytokines, including IL-4 and IL-13, significantly elevated from day 4 to day 12 compared to that of uninfected controls (Reece et al., 2006). At 36 days post infection, transcription of IL-4 and IL-13 in mouse lungs sustained significantly higher than in uninfected mouse lungs (Reece et al., 2008). These experiments demonstrated that even transient pulmonary exposure to *N.brasiliensis* infection had sustained effect on the immunological environment of the lungs. When the mice were challenged with an allergen at 36 days after *N.brasiliensis* infection, there was a reduction in overall airway responsiveness and lung inflammation associated with significantly lower IL-4, IL-5 and IL-13 gene transcription in the lungs compared to allergen-challenged animals with no *N.brasiliensis* infection (Reece et al., 2008). Helminth infection has been associated with expansion of Th2 and Treg cells which provide protection against further infection and help sustain persistent changes to the immunological environment within the host (Raucsh et al., 2008). The roles of NES-induced Th2

or Treg cells in the sustaining of suppressed LPS-induced inflammation will be explored in future projects. Figure 6.2 summarizes the proposed effect of *N.brasiliensis* on LPS-stimulated innate and adaptive immune responses based on this study and those of others (Maizels and Yazdanbakhsh, 2003; Noël et al., 2004; Pearce et al., 2006; Wilson and Maizels, 2006).

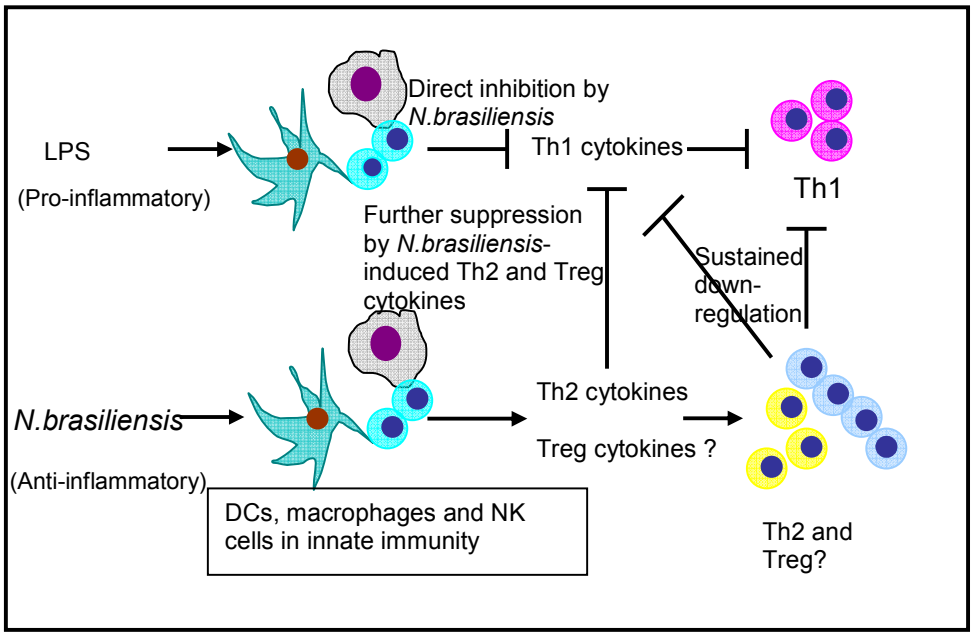


Figure 6.2 Proposed mechanisms involved in the down-regulation of LPS-induced Th1 inflammation by *N.brasiliensis*. In response to LPS, cells of the innate immune system (macrophages, dendritic cells, NK cells) produce pro-inflammatory Th1 cytokines (TNF-α, IL-1β), which might be directly inhibited by *N.brasiliensis* (larvae or NES). *N.brasiliensis* infection may stimulate the production of anti-inflammatory Th2 cytokines (IL-4, IL-5 and IL-13), or Treg cytokines (IL-10, TGF-β), which could also suppress any further induction of Th1 cytokines. Following the changes in the innate immune responses, T cells may differentiate into Th2 cells or Treg cells that help sustain the down-regulated Th1 inflammation.

One step forward is to characterize the active components in NES that may be responsible for the inhibitory effects on LPS-induced inflammation. Results from chapter 4 suggest that the molecules that inhibited TNF-α production are heat-labile and trypsin-sensitive, which suggested that the native protein structure may

be required for such an effect. This is comparable with the findings by Holland et al (2000) that immunizing mice with 50μg of NES stimulated IL-4 production by lymph node cells isolated 7 days after immunization and IL-4 production was abolished by heat-treatment and proteinase K digestion of NES. It has been suggested that both proteases and protease inhibitors are exist in parasitic helminth products. They may play vital roles in regulating the protease/protease inhibitor balance within the immune system (Knox, 2007). We had preliminary data suggested the presence of proteases in NES protein (data not shown). The roles of these proteases in the immune regulation need further exploration. Maizels and colleagues have analysed ESTs of *N.brasiliensis* adult cDNA, which clustered into 742 distinctive genes encoding proteases, enzymes, structural proteins and proteins for embryo, egg, mating, signalling and DNA transcription. However, they appeared to undergo accelerated evolution, which may be caused by selective pressure from the host (Harcus et al., 2004). Whether the evolution will have an impact on the modulatory function of *N.brasiliensis* remains a long term research subject.

In conclusion, this study demonstrated the down-regulation of LPS-induced inflammation by *N.brasiliensis* and their ES products. This study and planned future studies could be fundamental in developing anti-inflammatory agents from *N.brasiliensis*.

6.2 Future studies

Research projects in the near future will continue investigating the regulation of LPS-induced early immune responses by *N.brasiliensis* L3 larvae and NES. It is rationale to move from *in vitro* model to *in vivo* animal model for more supportive data before stepping into clinical studies. As a supplement to the current results, the future work will focus on the following areas.

Firstly, we would like to further explore the LPS-stimulated inflammatory processes interfered by *N.brasiliensis*. In this study, we failed to detect any positive LPS-binding *in vitro*. This could be corrected by using primary broncho alveolar macrophages from animals exposed to LPS and/or *N.brasiliensis* antigens. The level and expression of TLRs could be detected by flow cytometry, western blot and real-time PCR. The expression of MyD88, the adaptor molecule involved in TLR4 signalling in response to LPS, could be measured.

Secondly, the expression and production of Th2 and Treg cytokines, such as IL-4, IL-5, IL-10, IL-13 and TGF-β should be tested to further explore the mechanisms of Th1 inhibition. Phenotypes of macrophages and DCs would affect the outcome of the immune reaction. Expression and production of Ym1, FIZZ1 and arginase could be measured by PCR and immunohistochemistry as indicators of AAMs. CD86 and OX40L are DC maturation markers associated with the induction of Th2 cells (Balic et al., 2004), and they could be used to measure the DC2 population.

Lastly, proteomic techniques, such as mass spectrometry and MALDI protein identification will help to identify and characterise protein components in *N.brasiliensis*-derived products. These purified molecules could be further tested for their regulatory functions on LPS-induced inflammation both *in vivo* and *in vitro*. These are positive moves towards discovering *N.brasiliensis*-derived anti-inflammatory molecules that might be clinically beneficial.