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TRICHURIS SUIS EXCRETORY/SECRETORY PRODUCTS
MODULATE IMMUNE RESPONSES

presented by

Stacey Renee Wilder

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Linda L Mansfield
Major Professor's Signature

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TRICHURIS SUIS EXCRETORY/SECRETORY PRODUCTS MODULTE HOST IMMUNE RESPONSES

By

Stacey Renee Wilder

A THESIS

Submitted to
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ABSTRACT

TRICHURIS SUIS EXCRETORY/SECRETORY PRODUCTS MODULATE HOST IMMUNE RESPONSES

By

Stacey Renee Wilder

The intestinal whipworms *Trichuris suis* and *Trichuris muris* modulate the gastrointestinal environment allowing secondary bacterial infections to occur[1-3]. We tested the hypothesis that *T. suis* excretory/secretory products (TsESP) have an immunomodulatory role in mice challenged with a sublethal dose of lipopolysaccharide (LPS). TsESP can both exacerbate LPS-induced shock leading to death and mimic the phenomenon of endotoxin tolerance, depending on the timing of administration of TsESP and LPS. These two outcomes indicate a possible role for tumor necrosis factor (TNF)-α in TsESP-induced immunomodulation. We further hypothesized that TsESP can induce the production of TNF-α and this ability is due to an inherent component of TsESP and not to the presence of LPS in TsESP preparations. Two different batches of TsESP stimulated RAW 264.7 macrophages to secrete TNF-α; polymyxin B, a potent inhibitor of LPS activity, did not inhibit this induction. Furthermore, Batch 5 TsESP was able to stimulate TLR4; however, this stimulation was abrogated in the presence of polymyxin B. Batch 5 TsESP was also able to stimulate the TLR2 signal transduction pathway, while the LPS control did not. These results suggest that TsESP contains a substance that is not a lipopolysaccharide contaminant, but can elicit TNF-α production from macrophages.

Dedicated to my:

supportive and loving husband, Martin;

and supportive and encouraging parents, Neil and Norma Frodle

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LIST OF ABBREVIATIONS

Act D	actinomycin D
ADCC	antibody-dependent cell-mediated cytotoxicity
CHO	Chinese hamster ovary
cRPMI	concentrated RPMI
D-GalN	D-galactosamine
DMEM	Dulbecco's Modified Eagle's medium
ESP	excretory/secretory products
EU	endotoxin unit
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
HBSS	Hank's balanced salt solution
IEC	intestinal epithelial cell
IFN-γ	interferon-gamma
IL-#	interleukin-#
i.p.	intraperitoneal
kDa	kiloDalton
KDO	2-keto-3-deoxyoctulosonic acid
KO	knockout
LAL	Limulus amebocyte lysate assay
LGC	lymphoglandular complex
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MIF	macrophage migration-inhibitory factor
PAMP	pathogen-associated molecular pattern
PMB	polymyxin B
PRR	pattern recognition receptor
sBLP	synthetic bacterial lipoprotein
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TREM	triggering receptors expressed on myeloid cells
TsESP	*Trichuris suis* excretory/secretory products

I. Chapter 1

Literature Review

Introduction

Trichuris suis is a whipworm parasite that resides in the cecum and proximal colon of swine throughout the world and is a causative agent of bloody scours. Clinical signs of trichuriasis can include mucohemorrhagic diarrhea, anemia, anorexia and pronounced weight loss. Death can ensue as a result of these clinical manifestations. The presence of the whipworm facilitates secondary bacterial invasion by opportunistic, resident flora, which then exacerbates disease and pathology of trichuriasis. Bacterial invasion by opportunistic pathogens occurs throughout the colon, even in the distal portion away from the site of whipworm invasion. We hypothesized that the whipworms themselves, their excretory/secretory products (ESP) or both may contribute to secondary bacterial infections by modulating the immune response of the pig.

The scope of this thesis was to investigate the ability of *T. suis* ESP (TsESP) to modulate immune responses, which may contribute to the facilitation of opportunistic bacterial infections in the distal colon. We have demonstrated the ability of TsESP to modulate the immune response of female, BALB/c mice to endotoxic shock. Furthermore, we have demonstrated the ability of TsESP to induce tumor necrosis factor (TNF)-α secretion from RAW 264.7 macrophages.

TsESP preparations contain whipworm products, but may also contain contaminating bacterial structures, including lipopolysaccharide (LPS), that can contribute to immunomodulation. Therefore, we also investigated whether contaminating LPS has a role in the ability of TsESP to induce TNF-α from RAW 264.7 macrophages. This induction of TNF-α appears to be a result of an

inherent component of TsESP and not the presence of contaminating LPS in the preparations of TsESP.

This chapter reviews basic parasitology and epidemiology, disease and pathology, the host immune response, and the activity and composition of ESP of *Trichuris*, with emphasis on *T. muris* and *T. suis*. Additionally, this chapter reviews literature pertaining to the structure and biological relevance of LPS, Toll-like receptors, and LPS tolerance to provide insight into the design and outcome of the *in vivo* experiment in which mice were treated with TsESP and then challenged with a sublethal dose of LPS.

Trichuris suis

Basic Parasitology and Epidemiology

Trichuris is an intestinal nematode that has a thick posterior end and a longer slender anterior region giving it a characteristic "whip" shape. This whipworm parasite is grouped in the Trichuroidea superfamily with genera such as *Trichinella*. This superfamily is defined by the presence of a stichosome esophagus, which is a capillary tube surrounded by a column of gland cells called stichocytes[4,5]. *Trichuris* spp. are found throughout the world and infect several species of animals; *Trichuris* spp. include *T. ovis* (sheep and goat whipworm), *T. globulosa* (cattle whipworm), *T. vulpis* (dog whipworm), *T. muris* (mouse whipworm), *T. trichiura* (human whipworm), and *T. suis* (swine whipworm)[6].

There are 5 stages in the *T. suis* life-cycle with all development progressing in the mucosal layer of the cecum and proximal colon[7]. *Trichuris suis* is transmitted via the fecal-oral route. Eggs that are shed with the feces embryonate under the right conditions (especially temperature) in 1 to 2 months into infective eggs containing L1 larvae, which can remain infective for up to 11 years[8,9]. Pigs ingest the infective eggs containing L1 larvae from contaminated soil to become infected with *T. suis*. L1 larvae begin to hatch out within the distal small intestine and throughout the cecum and large intestine. They then penetrate the mucosal epithelial and goblet cells of the crypts of Lieberkühn[10]. On days 10 and 16, the L1 moult into L2 and then L3 larvae, respectively. At day 16, the posterior ends of the larvae (L3) begin to protrude into the lumen[10]. On

day 20, the L3 larvae moult into L4 larvae and then moult again on days 32 to 37 into the L5 (adult) stage[7,10]. The adult *T. suis* whipworms are 4 to 6 centimeters long with their posterior ends completely free within the lumen of the intestine and part of their anterior ends embedded in the mucosa called syncytial tunnels[9,11]. The prepatent period, when adult females excrete barrel-shaped eggs possessing bipolar plugs, is 41 to 47 days. Females can produce 3,000 to 10,000 eggs per day[12]. The *T. suis* whipworm has a life span of 4 months up to 2 years[7,8,12,13].

A study conducted in the early 1990s determined that 45% of the swine farms surveyed throughout the United States and 19% of pigs examined at slaughter were infected with *T. suis*[14]. Moderate whipworm infections have been shown to cause increased days to market, reduced carcass weights, or deaths[10,14]. Bliss reported in 1991 that on swine farms in the United States, veterinary care is needed for whipworm-induced bloody scours and deaths more than any other disease caused by intestinal worms[14]. Incidence of infection coupled with the ability of the parasite to cause debilitation can significantly affect the economics of the swine production industry.

T. suis infection is more severe in young weaned pigs than in adult pigs due to age-related resistance in adults[15,16]. *T. trichiura* infects approximately 900 million to 1 billion people worldwide and is also more prevalent in children than adults[17-19]. Both *T. suis* and *T. muris* are very similar to *T. trichiura* with respect to morphology, life-cycle and environmental niche in the large intestine[8,9,20]. In fact,

there is no visible morphological difference between *T. trichiura* and *T. suis* under phase contrast microscopy[21]. Furthermore, *T. suis* is able to infect humans[12,21].

Disease and Pathology

Clinical manifestations of trichuriasis include mucohemorrhagic diarrhea, anemia, anorexia, and severe weight loss[13,15,22]. In heavy infections, these manifestations can be so pronounced that death ensues[15]. Powers and colleagues reported that experimentally infected pigs weighed up to 81 pounds less than control pigs just before they died[13]. Pathology is present not only at the site of whipworm invasion throughout the cecum and proximal colon, but also in the distal colon away from the parasites. Mucosal edema, increased inflammatory cell penetration, bacterial accumulation, and destruction of both the colonic absorptive cells and the crypts of Lieberkühn all occur at the site of worm attachment in the cecum and proximal colon[1,23]. Lymphoglandular complexes (LGCs) are antigen processing centers that are an element of the gut-associated lymphoid tissue (GALT) located in the distal colon. In trichuriasis, LGCs enlarge due to infiltration of lymphocytes, macrophages, neutrophils, eosinophils and extracellular bacteria[1,2,23]. Resident, opportunistic bacteria that have been aseptically cultured from the inflamed LGCs include *Escherichia coli*, *E. fergusonii*, *Enterobacter intermedium*, *E. cloacae*, *Pseudomonas fluorescens*, *Lawsonia intracellularis*, *Campylobacter lari*, *C. coli*, and *C. jejuni*[23].

Disease and pathology observed in pigs affected with trichuriasis worsens with secondary bacterial infection. As early as the 1970s, Rutter and Beer

demonstrated that conventionally-reared pigs infected with *T. suis* develop severe clinical signs and pathology, while specific pathogen-free and gnotobiotic pigs only develop a mild catarrhal enteritis[15]. They concluded that the microbial component synergizing with *T. suis* was resident in the pig's intestinal flora and not transmitted by the embryonated eggs because specific pathogen-free and gnotobiotic pigs did not develop severe disease[15]. Furthermore, conventionally-reared pigs that are treated with broad spectrum antibiotics in tandem with *T. suis* eggs do not develop typical clinical manifestations of *T. suis* infection, including diarrhea and growth impairment[1,15,23]. *T. suis*-infected pigs administered antibiotics also have histologically normal LGCs with no invasive extracellular bacteria in the distal colon[1]. More recently, Mansfield et al. have shown that gnotobiotic pigs dually infected with *T. suis* and *C. jejuni* develop disease and pathology similar to that exhibited by conventionally-reared pigs experimentally infected with *T. suis* alone[2]. Together, these experimental findings demonstrate that the mucohemorrhagic enteritis following *T. suis* inoculation is a result of invasion and proliferation of resident, opportunistic bacteria present in the large intestine. Enhanced bacterial invasion in the distal colon leading to aggravated disease in pigs afflicted with trichuriasis is thought to result from the whipworms downregulating immunity to bacterial infections[1,24].

Immune Response Elicited by *Trichuris*

The classical immediate-type hypersensitivity response is the general immune response in mammals towards gastrointestinal nematodes[12,23]. It is

partially characterized by mucosal mastocytosis and eosinophilia, the production of IgE and IgG_1 antibodies, and the alteration of the composition and quantity of goblet cell mucins[12,23,25]. Researchers have studied the components of the immediate-type hypersensitivity response as it relates to *Trichuris* infection using a mouse model. Much has been discovered about the roles of mucosal mast cells, eosinophils, B cells, $CD4^+$ T cells and cytokines in trichuriasis.

Research has demonstrated that the classical immediate-type hypersensitivity response is not effective against *T. muris* infections. While mastocytosis and eosinophilia occur in response to *Trichuris* infections, they are neither necessary for resistance to *T. muris* nor are they involved in expulsion of the worm[17,26,27]. These conclusions are based on studies in which eosinophilia or mastocytosis were ablated within mice, or using genetically mast cell-deficient WBB6F1 mice[17,27]. Ablation of eosinophilia was obtained by injecting neutralizing anti-IL-5 antibodies into the mice, and ablation of mastocytosis was obtained by injecting neutralizing anti-c-*kit* antibodies that block stem cell factor[17]. Activated B cells are not required for expulsion of *T muris* in infected mice; however, they are required for resistance in the fact that they lead to the development of a Th2-type cytokine response[28]. *Trichuris muris*-specific IgE and IgG_1 are also not involved in worm expulsion after primary infection, but do play roles in resistance to subsequent challenges to *Trichuris*[28]. Furthermore, antibody-dependent cell-mediated cytotoxicity (ADCC) does not play a significant role in *T. muris* resistance or expulsions. FcγR-/- mice (C57BL/6 background) that were deficient in any effector function requiring high affinity IgE or IgG binding, behaved

similarly to the C57BL/6 controls, expelling the worms 21 to 24 days after infection and exhibiting cytokine profiles and antibody production consistent with resistant strains of mice[17].

$CD4^+$ T cells are very important in resistance to *Trichuris muris*[26,29]. Studies have shown that athymic BALB/c mice and mice where the $CD4^+$ T cell population has been ablated using neutralizing antibodies are susceptible to whipworm infections[28]. Furthermore, SCID mice (which lack B and T cells) reconstituted with purified $CD4^+$ T cells from infected BALB/c mice were resistant to whipworm infection[28]. The ability of the different inbred mice strains to expel the whipworm is based on genetic background, especially MHC-linked genes[30]. The majority of the inbred mouse strains are resistant to infection and expel the worms before patency is reached, which is approximately 32 days post-infection. Resistance and worm expulsion are associated with the $CD4^+$ Th2 cytokine response, in which interleukins (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are secreted by $CD4^+$ cells[12,24,26,27,31-39]. Th2-type cytokines promote differentiation of precursor Th cells into Th2 cells, class switching of B cells to IgE and IgG_1, eosinophilia, and mucosal mastocytosis[12,17,23,24,27,29,31,36,39]. Using IL-10- and IL-4/IL-10-knockout mice (C57BL/6 background) Schopf and colleagues have recently discovered that IL-10 is important in resistance to *T. muris* and in the survival of mice subject to the inflammatory process brought about by secondary bacterial infections[3]. More recently, tumor necrosis factor (TNF)-α has been demonstrated to play a role in Th2 cytokine-mediated *T. muris* immunity[40]. Artis et al. demonstrated that the expulsion is hindered by blocking TNF-α *in vivo*

using an anti-TNF-α antibody[40]. Additionally, intraperitoneal injections of recombinant TNF-α in *T. muris*-infected, BALB/c male IL-4 knockout (KO) mice, which cannot expel worms by day 35 post-infection, enhances the ability of these mice to clear worms by day 35 post-infection. It is important to note that the TNF-α treatments did not change levels of IL-5, IL-9 or IL-13 production[40].

Mice that are susceptible to *T. muris* infection mount a Th1-type response characterized by IL-18, IL-12 and interferon (IFN)-γ, and harbor adult worms even after patency[17,27,31,33,34,41-43]. The role of IL-18 in susceptibility is considered novel because it acts to downregulate Th2 cytokines such as IL-4 and IL-13 instead of acting as an inducer of IFN-γ[31,41,43]. Table I-1 details some of the susceptible and resistant strains of mice.

The cytokine profile in *T. muris*-infected mice is not always distinctly polarized to either the Th1 or Th2 response, and discrepancies among cytokine responses to experimental whipworm infections have been reported. Mice challenged repeatedly with low doses of *T. muris* embryonated eggs mount mixed cytokine responses and eventually expel the worm[44]. Also, mixed cytokine responses have been demonstrated in swine and human trichuriasis. Both IL-10 (Th2) and IL-12 (Th1) were shown to be elevated in swine exposed to *T. suis* on dirt lots. However, experiments have shown that only IL-10 is significantly elevated in *T. suis*-infected pigs kept in confinement, while IL-12 is not detectable[45]. A small percentage of mixed white blood cells from whole blood cultures from *T. trichiura*-infected humans secreted IL-4 (7%), IL-9 (5%), and IL-13 (17%) and a larger percentage secreted IL-10 (97%), TNF-α (93%) and IFN-γ

(32%) when stimulated with *T. trichiura* antigen *in vitro*[18]. Turner and colleagues have also shown using whole blood culture from *T. trichiura*-infected individuals that *Trichuris* ESP elicits a different cytokine response (IL-10 and TNF-α) than *Trichuris* somatic antigens (IL-4, IL-13 and proliferative responses of white blood cells). IFN-γ was not induced[19].

Trichuris excretory/secretory products (ESP)

Whipworms excrete and secrete various components from both the stichosome and the anal opening. Under *in vitro* conditions, each worm is able to produce approximately 10-100 μg protein/ml/day[46]. The idea that excretory/secretory products of nematodes are able to elicit immune responses was suggested as early as the 1930s. Chandler reported that precipitates formed at the orifices of nematodes incubated with immune sera, suggesting involvement of adaptive immunity in the host's response to proteins released by the nematode[47]. In fact, immunizing pigs with *T. suis* ESP can decrease worm burdens and pathology of subsequent infections[1]. More recently, Turner et al. reported that ESP of *T. trichiura* (human whipworm) and *T. muris* (mouse whipworm) preferentially stimulated secretion of TNF-α and IL-10 from whole blood cultures taken from *T. trichiura*-infected humans[19]. Known components of *Trichuris* spp. ESP function in parasite survival; either for the whipworms own physiological needs or in immunomodulation of the host. Components used for various physiological purposes include a zinc metalloprotease present in the stichosome of *T. suis* that is involved in feeding and tissue penetration to form

syncytial tunnels[46]. It is produced in large quantities at 10μg/worm/4days and was shown to degrade both fibrinogen and elastin[12,46]. This protease is also immunogenic. In fact, purified zinc metalloprotease used to vaccinate pigs has comparable efficacy to vaccination with crude ESP, reducing *Trichuris*-induced pathology and preventing growth impairment[12]. Thiol protease is present in the gut of *T. suis* and has been suggested to be involved in digestion and nutrient absorption[48]. Phenol oxidase has been localized to fertilized eggs found in the proximal portion of the female whipworm uterus and is involved in the tanning/hardening process of the egg[49,50]. Abner et al. have more recently described an antibacterial substance in *T. suis* ESP. The antibacterial substance was shown to be (1) less than 10 kiloDaltons (kDa) in size, (2) heat-stable, and (3) resistant to digestion with trypsin and pronase E. It killed both Gram-negative (*Campylobacter jejuni*, *C. coli*, and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria, and the probable mode of action is bacteriocidal. Abner and colleagues suggested that the antibacterial substance plays a role in the whipworms' immune system to defend against the bacteria present in the intestinal tract of swine[51].

Host immunomodulation is an important aspect of parasite survival, and several ESP constituents have been hypothesized to have an immunoregulatory function. Two of these constituents are (1) a serine protease inhibitor (6.2 kDa) that acts on chymotrypsin, neutrophil elastase, cathepsin G, and chymase, and (2) a chymotrypsin/elastase inhibitor (6.7 kDa)[52,53]. Protease inhibitors may act as part of the whipworm's defense mechanism against the host immune system,

and they have been hypothesized to modulate effector mechanisms of mast cells, neutrophils, and macrophages, which may facilitate secondary bacterial invasion into the cecum and colon of the swine[52]. A macrophage migration-inhibitory factor (MIF) and IFN-γ are two cytokine homologues that were discovered in *T. muris* ESP; they may function in immunomodulation[54,55]. In the host, MIF functions in initiating adaptive immune responses. Expression of the MIF homologue by parasitic organisms has been hypothesized to alter the immune response of the host[55]. Since IFN-γ is known to facilitate chronic *T. muris* infections, the IFN-γ homologue of ESP was suggested to be one mechanism that the whipworm uses to promote its own survival[54]. Grencis and Entwistle demonstrated that the homologue shares cross-reactive epitopes with mouse IFN-γ, and it can bind to IFN-γ receptors on lymphocytes to stimulate cellular changes analogous to those induced by the mouse IFN-γ cytokine[31,54]. It has been localized to the stichosome, cuticle, and bacillary band (the interface structure between whipworm and host that has a secretory function) of *T. muris* by immuno-gold staining using anti-IFN-γ antibodies[31].

There are several reasons it can be difficult to study the components of *Trichuris* ESP. First, the protein composition from each batch of worms from individual pigs may be different due to the life stage of the whipworm or the environment (e.g. bacterial population or genetic makeup of the pig) in which the whipworms reside (Figure I-1). Second, individual ESP components can vary per batch. This variation may be due to the worms from different batches producing differing amounts of ESP components or degradation of various proteins by

proteases. (Figure I-1). Third, there may be various bacterial components, from the bacteria either inhabiting the intestine of the pig or the whipworm itself, cultured with the ESP that cannot be removed during the washing or sterile filtering processes. The Gram-negative bacterial outer membrane moiety lipopolysaccharide (LPS) is a likely contaminant of ESP preparations.

Table I-1

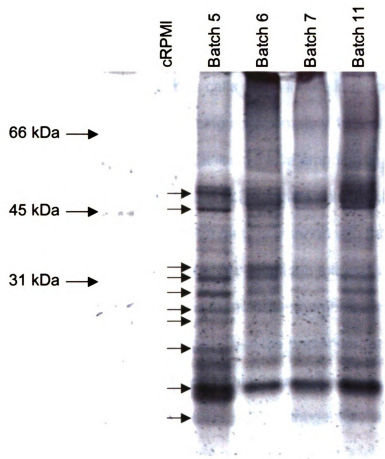
Strains of mice that are susceptible or resistant to *Trichuris muris* infection.

Mouse strain	Susceptible	Resistant	Reference:
AKR	x		17,56
BR10.BR	x		27,30
SCID	x		28
C57BL/6*		x	28
C57BL/10*		x	28
BALB/c		x	28,30
BALB/k		x	28,30
NIH		x	28,30
129		x	38,56

*Mount a mixed cytokine response, but the majority of the mice expel their worms via a Th2-mediated cytokine response between days 21 and 28 post-infection[28].

Figure I-1

SDS PAGE comparing four different batches of *Trichuris suis* ESP and concentrated RPMI-1640 (cRPMI; medium in which the whipworms are cultured to collect ESP). See page 43 for TsESP collection protocol.



Lipopolysaccharide

Structure

Pathogen-associated molecular patterns (PAMPs) are conserved molecular structures produced by microorganisms that are essential for either their survival or pathogenicity. PAMPs are recognized by pattern-recognition receptors (PRRs) displayed on various cells of the innate immune system[57,58]. Lipopolysaccharide is a PAMP comprising approximately 13% of the outer membrane of Gram-negative bacteria. It is a 10 kDa amphiphilic macromolecule composed of three distinct, covalently joined regions: O-antigen, core polysaccharide, and lipid A. The lipid A moiety is inserted into the outer leaflet of the outer membrane with the core, while the O-antigen extends away from the bacteria. Even though LPS is anchored to the outer membrane, small amounts may become solubilized during rapid growth of the bacterium (about 5% per cell division) or when the bacterial cell lyses within the host[59].

LPS is both immunogenic and toxic. Its immunogenicity is associated with the hydrophilic O-antigen polysaccharide and core regions, and its toxicity is generally associated with the hydrophobic lipid A moiety. Gram-negative bacteria can either lack the O-antigen completely (O-chains or O-specific polysaccharide chains) or have a polysaccharide chain made comprised of up to 50 repeating oligosaccharide subunits that are 3 to 5 sugars in length[59,60]. As the outermost fraction of LPS, O-antigen has many functions. First, the O-chains are highly variable and contribute to the serological specificity (serotype) of the bacterium. Second, bacteriophage recognize the conformation of more than one

of the repeating units to bind and infect a specific bacterium. Third, O-antigens can act as adhesins that are important in the initial stages of colonization and infection of a host. This phenomenon is observed in plant-nodulating bacteria binding to their proper legume and also in *Pseudomonas aeruginosa* colonizing the lungs of cystic fibrosis patients. Last, the bacterial lysing activity of the membrane-attack complex initiated via the classical and alternative complement pathways is inhibited by O-antigen. Ironically, the O-antigen can activate the alternative complement pathway[60]. Mucosal pathogens often produce lipooligosaccharide (LOS) instead of LPS. LOS lacks O-antigens, but contains a lipid A moiety indistinguishable from LPS, and an inner core[61].

The core polysaccharide can be separated into the outer core that is bound to the O-antigen and the inner core that is bound to the lipid A molecule. The outer core consists of glucose, galactose and *N*-acetylglucosamine giving this component a more neutral charge. Within bacterial species, structural differences occur mainly in the outer core whereas the inner core is more conserved. This conservation allows the inner core to maintain common epitopes amongst the species for various serum factors and antibodies contributing to cross-immunity and protection. Bacteria that are missing the core polysaccharide or the O-antigen are referred to as rough (R) strains because on agar media colonies appear drier and "rougher" than strains with normal LPS[59]. Modified 7-carbon heptose sugars and one to three 8-carbon 2-keto-3-deoxyoctulosonic acid (KDO) are the two unusual sugars that make up the negatively charged inner core[59,60]. KDO is essential for cell viability in that lipid A

linked to two KDO molecules is the smallest LPS structure that will sustain cell proliferation. This minimal LPS structure is referred to as Re LPS[60]. Re LPS strains are hypersensitive to both detergents and antibiotics[60].

Lipid A is a phosphoglycolipid that confers toxicity to LPS. This structure is the most conserved component within the LPS molecule[60]. It is generally a β(1-6)-linked disaccharide (*N*-acetylglucosamine) with six to eight moderate to long (C10 to C28) saturated fatty acids and one to three negatively charged, substituted phosphoryl groups[59,62]. Lipid A is necessary for outer membrane stability and proper fluidity making it a vital part of growth and survival for the bacterium. The toxicity of LPS is due to the ability of the lipid A moiety to interact with receptors on macrophages within the tissues and monocytes within the vascular system leading to inflammation and/or endotoxic shock. The core and O-antigen regions also contribute to the toxicity of LPS by allowing lipid A to become soluble in water and reactive within the vascular system[59].

Synthesis of each component of LPS occurs separately. Ligation of the individual components to form a mature LPS occurs on the inner membrane. The mature LPS then translocates from the inner membrane across the periplasm to the outer membrane[61]. Once LPS is constructed and deposited on the cell surface, it can be extracted and manipulated in the laboratory for various experiments. Extraction is a simple procedure involving water and phenol heated to about 68° C and centrifugation to remove any cellular debris. While the extraction procedure may be simple, it is not always stringent and the preparations can be contaminated with various membrane components.

Commercial LPS preparations have been shown to contain up to 60% protein, necessitating repurification prior to experimental use[63,64]. Numerous experiments require endotoxin-free solutions. Methods to eliminate LPS from solutions include distillation and reverse osmosis, but do not include sterilization via an autoclave[59]. Two different assays are available to detect LPS. The KDO structure can be detected via a colorimetric assay. Nanogram levels of endotoxin can be detected by clotting (gelatinizing) of the *Limulus* (horseshoe crab) hemolymph Limulus amebocyte lysate (LAL) assay[59].

Biological Relevance of LPS

LPS, specifically lipid A, is a potent stimulator of the innate immune system: it activates various cell types including B cells, granulocytes and mononuclear cells. Macrophages are one of the most important cell types responding to LPS because they initiate inflammation at the site of infection in tissues[57]. As little as 1 pg/ml of LPS can activate macrophages to secrete cytokines like IL-1, IL-6, IL-8, IL-12, MIF and TNF-α, as well as secondary mediators of inflammation like prostaglandins, leukotrienes, platelet-activating factor and nitric oxide[57,65,66]. Activated lymphocytes, NK cells, polymorphonuclear leukocytes, astrocytes, Langerhan cells, Kupffer cells, eosinophils, and mast cells can all also secrete TNF-α[59,67].

Of all the mediators released by macrophages, TNF-α is thought to be the "principal mediator of the response to endotoxin" with other mediators such as IL-1 and IFN-γ synergizing with TNF-α to increase its biological effects. [59,67]. TNF-α

can be found in two biologically active forms. It can be a 26 kDa membrane-bound prohormone that acts through paracrine activities of TNF-α in tissues. The predominant bioactive form in serum and other bodily fluids is a bell-shaped trimer consisting of the 17 kDa proteolytic product of the prohormone that is noncovalently associated[67].

During local infections, macrophages secrete TNF-α into the tissue which acts on blood vessels to enhance recruitment of lymphocytes and phagocytes to the site of infection. However, during systemic infections, macrophages activated in the spleen and liver release TNF-α into the bloodstream, which can then lead to endotoxic shock. Nanomolar amounts of TNF are induced by infusion of a lethal dose of LPS in rabbits, mice, and baboons[68]. TNF-α activates the clotting system leading to disseminated intravascular coagulation and impaired blood circulation. Other circulatory abnormalities include increased vascular permeability that leads to systemic edema and decreased blood volume. Eventually, multiple organ failure occurs and death can ensue[57,59,61]. After a lethal LPS challenge, nanomolar amounts of TNF in serum appearing in the serum will lead to death within minutes to hours[68]. Other manifestations of endotoxic shock include body temperature dysregulation (for example, fever in humans and hypothermia in mice), diarrhea, leukopenia, hyperglycemia, acidosis, and exhaustion of platelets and various clotting factors leading to internal bleeding [59,62,69].

TNF-α has been implicated as a pivotal mediator of endotoxic shock not only because it is tremendously overproduced during sepsis, but because of

various experimental outcomes. First, the physical manifestations of endotoxic shock can be reproduced by administering TNF-α alone[67,68]. For instance, an intra-arterial injection of TNF, with a dose that emulates an amount that could be produced endogenously during infection, in dogs will induce a syndrome of shock, tissue injury, capillary leakage syndrome, hypoxia, pulmonary edema, and multiple organ failure associated with a high mortality rate develops within minutes after administration[67]. Furthermore, Tracey and colleagues have also shown that 0.6 mg of TNF is lethal in rats with the pathophysiological and histological effects mimicking endotoxic shock[70]. Second, experiments have shown that septic shock can be prevented by injecting an animal with anti-TNF antibodies or soluble human TNF receptors[71-75]. For example, rats pretreated with an anti-TNF monoclonal antibody 1 hour before injection of TNF are fully protected against a challenge with a lethal dose of LPS[70]. Last, TNFR-I knockout mice are protected against endotoxic shock[67]. For instance, TNFRp55-/- mice coinjected with D-galactosamine (D-GalN) and LPS are resistant to doses of 100 μg LPS per mouse, while the wildtype mice are sensitive to doses as low as 0.1 μg LPS[76].

The amount of endotoxin necessary to produce shock and lead to death can vary depending on not only the species of animal, but also the model for endotoxic shock used. There are various "sensitizing agents" that increase the sensitivity of an animal to the lethal effects of LPS; therefore, decreasing the amount of LPS needed to induce shock and death. One group includes various bacteria (live or killed), viruses and protozoans[71]. In mice all ready challenged

with bacteria (*Propionibacterium acnes*, Bacillus Calmette Guérin, *Salmonella* Typhimurium, *Coxiella burnetti*), the subsequent administration of LPS leads to an enhanced production of TNF-α and an enhanced susceptibility to the lethal effects of TNF-α[71]. Another agent is IFN-γ. Galanos and colleagues have demonstrated that LPS-resistant mice injected with IFN-γ succumb to the lethal effects of LPS[71]. Other agents known to cause hypersensitivity to LPS are actinomycin D (Act D) and D-GalN[71,77]. For example, mice injected with 10 μg of LPS survive; however, mice injected with 1 μg of LPS together with 20 μg of Act D die within 6 to 7 hours[77]. Act D and D-GalN also sensitize BALB/c mice to the lethal effects of TNF-α and IL-1[71,77].

Toll-like Receptors (TLRs)

Toll-like receptors are microbial pattern-recognition receptors (PRRs) that function in cell signaling leading to the production of secondary mediators of inflammation, chemokines and cytokines. They are type I transmembrane proteins with an extracellular region containing leucine-rich repeats, a single transmembrane spanning region, and a cytoplasmic, signaling region containing a Toll/IL-1 receptor (TIR) domain[61,78]. Aderem and Ulevitch nicely detail the signaling transduction pathway in their paper titled "Toll-like receptors in the induction of the innate immune response":

> Ligation of a TLR promotes dimerization and results in the recruitment of MyD88, which contains two domains: a C-terminal Toll homology domain that interacts with the Toll homology domain of the receptor, and an N-terminal death domain. This death domain undergoes homophilic interaction with the death domain of a serine/threonine protein kinase known as IRAK; this leads to the

autophosphorylation of IRAK. Autophosphorylated IRAK then forms a complex with TRAF6 and this, in turn, results in the oligomerization of TRAF6. The oligomerization of TRAF6 then leads to the activation of the IκB kinases. These kinases, in turn, phosphorylate IκB, leading to its proteolytic degradation and the translocation of NF-κB to the nucleus[79].

MyD88 recruitment also leads to activation of the transcription factors c-Jun, Elk-1, and AP-1[80,81]. These transcription factors along with NF-κB lead to the production of various pro-inflammatory cytokines including IL-1, IL-8, IL-12, and TNF-α[79]. Furthermore, TLR signaling can trigger the production of some anti-inflammatory cytokines, like IL-6 and IL-10, linking the innate immune system to the adaptive immune system[82].

Currently, 10 toll-like receptors have been identified in the mouse and human genomes, but not all receptors have known ligands. Table I-2 summarizes TLRs and some of their known ligands. TLR2 and TLR4 are the two receptors that have been most studied. TLR2 is activated by several bacterial components including peptidoglycan, lipoteichoic acid, various lipoproteins, and LPS from *Porphyromonas gingivalis* and *Leptospira interrogans*[61,82-87]. *Escherichia* and *Salmonella* LPS were initially reported to bind TLR2 and mediate cell signaling; however, upon repurification of commercially purchased LPS to remove protein contaminants, Tapping et al. and Hirschfeld et al. demonstrated that TLR2 is not a true signaling receptor for the typical LPS[63,88,89].

TLR4 recognizes LPS complexed with CD14 and the lipopolysaccharide binding protein (LBP). LBP is not necessary for the interaction between CD14 and LPS, though it does hasten the binding process[90]. CD14, on the other hand, is an important component in LPS activation of cells. The sensitivity of cells to

LPS can be decreased 100 to 10,000-fold by removing or blocking CD14[91]. CD14 is a glycoprotein that is expressed on monocytes, macrophages and activated neutrophils and can be present in blood to facilitate activation of cells that do not express it[90,92]. CD14 is anchored to cells by glycosyl phosphatidylinositol and alone is incapable of inducing any signal transduction pathways due to the lack of a cytoplasmic domain[89]. The MD-2 protein, which is associated with the extracellular domain of TLR4, is also required for LPS activation of cells through the TLR4 signal transduction pathway[79]. The TLR4 complex recognizing LPS can detect picomolar amounts of lipid A[61]. However, TLR4 is not the only LPS recognition receptor. Macrophages and/or activated neutrophils also express L-selectin, macrophage scavenger receptor, β_2 integrins (CD11/CD18), and triggering receptors expressed on myeloid cells (TREM) that bind to or participate in LPS cell signaling[66,86,90,93,94].

Tolerance

Endotoxin tolerance is a state of hyporesponsiveness towards the effects of LPS that develops after repeated administration of sublethal doses of endotoxin[67,95-97]. Outcomes of endotoxin tolerance include protecting the host against (1) subsequent infections with Gram-negative and Gram-positive bacteria, fungi, parasites and viruses, (2) hemorrhagic shock, (3) myocardial ischemia/reperfusion injury, (4) and tumor development[98]. Hyporesponsiveness to the lethality of endotoxin is divided temporally and mechanistically into two phases. The early phase is a transient state that can develop within several

hours following one injection of a sublethal dose of LPS and can last several days. This is similar to the phenomenon in which animals can be desensitized to the lethal effects of TNF-α or IL-1 by prior injections with sublethal doses of the cytokines themselves. Desensitization was shown in BALB/c mice to rapidly develop after the initial, sublethal injection (30 minutes to 1 hour) and subside 24 to 48 hours after the lethal injection[77]. In the early phase of endotoxin tolerance, there is cross-protection against LPS from different bacterial species. Furthermore, this phenomenon is a relative state because tolerance can be overcome with increasing amounts of the secondary dose of endotoxin. The late phase may begin after 48 hours from the initial injection with a sublethal dose of LPS and can last several weeks. It is specific for the strain of LPS initially used and is mediated by O-specific antibodies; therefore, the tolerance is transferable with serum. Both phases of tolerance can coexist when repetitive injections of LPS are administered daily[71,77,98].

Tolerance to the pyrogenic, metabolic, and lethal effects of endotoxin can be induced in animals and tissue culture by treating with sublethal doses of LPS, IL-1, or TNF-α prior to challenge with a lethal dose of endotoxin[67,77,95,99]. The dose necessary to induce tolerance, when tolerance appears, the duration animals are hyporesponsive to lethal doses of LPS, and the threshold of subsequent doses of LPS the mice can tolerate are all aspects that can vary depending on the animal or tissue culture model employed[71]. For instance, lethal tolerance to LPS developed within one day and slowly dissipated over the succeeding two weeks when mice were administered *Serratia marcescens* daily

in progressively greater doses[98]. Also, in rabbit peritoneal macrophages, human monocytes, monomac 6 cells, mouse macrophages, and RAW 264.7 cells a decrease in the synthesis of TNF-α occurs upon subsequent treatment with a normal stimulatory dose of LPS after an initial low dose of LPS[98].

Through *in vitro* studies, researchers have demonstrated that hyporesponsiveness is characterized by a "reprogramming" of cells, in which some functions are suppressed while others are enhanced to result in a reduced inflammatory response[100]. Notably, the activation or expression of AP-1, NF-κB, IκB kinase, IRAK-1, TLR4, IL-1, IL-6, IL-12, and TNF-α, are all suppressed in tolerant macrophages[98,100,101]. Even though expression of TLR4 may be suppressed, CD14 expression is not downregulated and it has been shown that hyporesponsiveness is not due to loss of LPS recognition by macrophages[65,98]. It has recently been discovered that the p50p65 NF-κB heterodimer activates transcription of TNF-α, while the p50 NF-κB homodimer inhibits its transcription; and during tolerance, the p50 homodimer is predominately produced contributing the reduction in TNF-α production[82,98]. The release of endogenous corticosteroids in response to LPS may also contribute to the hyporesponsiveness to a subsequent dose of LPS because they inhibit the synthesis of IL-1, IL-6, and TNF-α; in fact, cortisone has been observed to protect mice against the lethality of LPS[98].

Table I-2

Summary of the toll-like receptors and some of their known ligands.

Toll-like Receptor	Ligands	Reference:
TLR1	Lipopeptide	102
TLR2	Lipoprotein Peptidoglycan Lipoteichoic Acid Lipoarabinomannan Zymosan Lipopolysaccharide?	61,78,79,82,83,86
TLR3	Viral dsRNA	103
TLR4	Lipopolysaccharide Heat shock protein 60 Extra domain A of fibronectin	86,89,104,105
TLR5	Flagellin	106
TLR6	Peptidoglycan Lipopeptides	107
TLR7	Imidazoquinolin	108,109
TLR8	Imidazoquinolin	109
TLR9	Unmethylated CpG dinucleotides	110
TLR10	?	

II. Chapter 2

Thesis Rationale

The goal of this thesis was to investigate the ability of *T. suis* ESP (TsESP) to modulate immune responses, which may contribute to the facilitation of opportunistic bacterial infections in the distal colon. The first specific aim involved testing modulation of the mouse immune response (in female BALB/c mice) to LPS-induced shock in the presence of TsESP. Our results suggest that modulation did occur and that TsESP is able to both sensitize mice towards the lethality of LPS and also promote endotoxin tolerance, depending on the timing of administration of TsESP and LPS. The second specific aim entailed testing whether the cellular response was consistent with known mechanisms of endotoxic shock and tolerance. Since TNF-α plays a central role in both shock and tolerance to LPS, we investigated TNF-α secretion from RAW 264.7 mouse macrophages treated with TsESP *in vitro*. Furthermore, since TsESP preparations may contain contaminating bacterial structures, including LPS, that can contribute to immunomodulation, the third specific aim entailed investigating whether contaminating LPS has a role in the ability of TsESP to induce TNF-α from RAW 264.7 macrophages. Our results suggest that the induction of TNF-α from RAW 264.7 macrophages treated with TsESP is a result of an inherent whipworm component and not to the presence of LPS in preparations of TsESP.

The effect of *Trichuris suis* excretory/secretory products on lipopolysaccharide-induced shock in female BALB/c mice

Disease outcome and the gastrointestinal immune response can be altered by the presence of various parasitic helminths when coinfected with other pathogens. A C57BL/6 mouse model for *Helicobacter*-induced gastric atrophy

demonstrated that the intestinal mouse nematode, *Heligmosomoides polygyrus*, can downregulate the Th1-type immune response associated with *Helicobacter* infection and lead to a reduction in gastritis[111]. *Schistosoma mansoni* can alter the disease outcome when coinfected with either *Trichuris muris*, in AKR mice, or *Toxoplasma gondii*, in C57BL/6, by shifting the cytokine environment to a Th2-dominant response[112,113]. *T. muris*-susceptible mice become resistant to the murine whipworm when the mice are coinfected with *S. mansoni*; however, disease and pathology are exacerbated when *Schistosoma mansoni*-infected mice are subsequently infected with *T. gondii*.

Previous experiments have also demonstrated that disease and pathology following *T. suis* (in swine) or *T. muris* (in mice) infections worsens due to invasion and proliferation of resident, opportunistic bacteria present in the large intestine[1-3,15]. Rutter and Beer demonstrated that conventionally-reared pigs infected with *T. suis* develop severe clinical signs and pathology, while specific pathogen-free and gnotobiotic pigs only develop a mild catarrhal enteritis[15]. Furthermore, conventionally-reared pigs that are provided broad spectrum antibiotics in tandem with *T. suis* eggs do not develop typical clinical signs of *T. suis* infection[1]. More recently, Mansfield et al. have shown that gnotobiotic pigs dually infected with *T. suis* and the food-borne bacterium *Campylobacter jejuni*, which is a commensal in swine, produce disease and pathology similar to disease and pathology exhibited by conventionally-reared pigs experimentally infected with *T. suis* alone[2]. Together, these experimental findings demonstrate that the mucohemorrhagic enteritis following *T. suis* inoculation is a result of

invasion and proliferation of resident, opportunistic bacteria present in the large intestine.

These secondary bacterial infections are likely initiated by the whipworm's excretory/secretory products (ESP), the worms themselves, or both, and may occur or be enhanced as a result of *T. suis* or TsESP modulating the gastrointestinal inflammatory response. One of our overall research goals is to determine how the whipworm ESP promote bacterial invasion within the distal colon of the pig during *T. suis* infection. Abner et al. have shown that while ESP contribute directly to intestinal epithelial cell (IEC) damage in *in vitro* model systems, ESP do not enhance bacterial, specifically *C. jejuni*, invasion within the IEC model system[114]. If disruption of the integrity of the intestinal epithelial barrier is not the mechanism whereby ESP affects the host leading to bacterial infection, then the mechanism may be related to the ability of ESP to alter the host's immune response.

Nedialkov and colleagues showed that V antigen from *Yersinia pestis* can modulate an inflammatory response by providing immediate resistance to a lethal dose of LPS[115]. LPS can stimulate an overwhelming production of pro-inflammatory cytokines (especially IL-1, and TNF-α) and secondary immune mediators (prostaglandins and nitric oxide), which in turn leads to multiple organ failure, shock, and sometimes death[57,65]. V antigen provides protection against the lethal affects of LPS when it is injected intraperitoneally (i.p.) either concomitantly with or 2 days prior to LPS challenge[115]. The mechanism of this protection appears to be the fact that V antigen immediately induces the

production of the anti-inflammatory cytokine IL-10, which plays an important role in downregulating pro-inflammatory cytokines and is known to protect mice from endotoxic shock[98,115,116]. Moderate amounts of TNF-α, which is a known inducer of IL-10, are also produced[115].

Since Mansfield et al. have shown that IL-10 expression is increased in *T. suis*-infected pigs and IL-10 is also important for resistance to *T. muris*, we set out to investigate the ability of TsESP to promote the production of an immediate anti-inflammatory immune response (similar to the response elicited by V antigen), thereby inhibiting a pro-inflammatory response[3,45]. We hypothesized that mice injected with TsESP would be protected from LPS-induced shock and/or death. Experiments were based on Nedialkov's mouse model for LPS resistance to determine the role of *Trichuris* ESP in immunomodulation against the pathophysiological effects of LPS by observing treated mice for signs of shock as a physical manifestation of a pro-inflammatory cytokine response. BALB/c mice were chosen for two reasons: first, because BALB/c mice were used in the experiments conducted by Nedialkov et al., and second, because BALB/c mice are known to be resistant to *T. muris* by a mechanism that is dependent upon the production of Th2-type cytokines, including IL-10[3,117].

In our experiments, mice were administered a sublethal dose of LPS, instead of a lethal dose, which was used in Nedialkov's experiments, as noted from the survival rate of the control mice injected with PBS prior to challenge with LPS. Therefore, we actually tested the hypothesis that TsESP would have an immunomodulatory role in mice challenged with a sublethal dose of LPS. This

hypothesis was tested by administering i.p. injections of increasing concentrations of TsESP to 10 week-old, female BALB/c mice and then challenging the mice with a sublethal dose (30,000 EU/ gram of body weight) of *Salmonella* Typhimurium LPS. By challenging the mice with a sublethal dose of LPS, we made an important discovery of the potential of TsESP as both a "sensitizing agent", like Act D and D-GalN, and an agent that can promote endotoxin tolerance in female, BALB/c mice, depending on the dose of TsESP and timing of administration of TsESP and LPS.

The effect of *Trichuris suis* excretory/secretory products on RAW 264.7 macrophages and Chinese hamster ovary cells expressing the human toll-like receptors 2 and 4.

Results from the mouse experiments demonstrated that there is a correlation between concentration of ESP and death. Clinical signs indicated that high concentrations of ESP in conjunction with a sublethal dose of LPS induced shock in the mice, which resulted in morbidity and mortality. Results also demonstrated the ability of TsESP to promote tolerance to the lethality of LPS-induced shock, depending on timing of administration of TsESP and LPS. The second specific aim entailed testing whether the cellular response was consistent with known mechanisms of endotoxic shock and tolerance. Since TNF-α plays a central role in both shock and tolerance to LPS, we investigated TNF-α secretion from RAW 264.7 mouse macrophages treated with TsESP *in vitro*.

Trichuris ESP could potentially contain contaminating LPS from bacteria that reside in the gut of either the whipworm or the pig, which may have

contributed to the effects on the mice. LPS can be an important mediator in the inflammatory disease process associated with nematode infections. For example, Taylor et al. discovered that LPS from the *Wolbachia* endosymbiont of the filarial nematode *Brugia malayi* is the essential mediator of the inflammatory disease associated with *B. malayi* in humans[118]. Since TsESP preparations may contain contaminating LPS that can contribute to immunomodulation the third specific aim entailed investigating whether contaminating LPS has a role in the ability of TsESP to induce TNF-α from RAW 264.7 macrophages.

Two different experimental approaches were taken to test for LPS activity. First, we tested the ability of TsESP to induce TNF-α secretion from RAW 264.7 macrophages in the presence of polymyxin B, a potent inhibitor of LPS. Second, we tested the ability of TsESP to bind and stimulate TLR2 and TLR4, two well characterized LPS receptors, using transfected Chinese hamster ovary cells. TLR2 binds a variety of Gram-positive bacterial components including lipoteichoic acid, peptidoglycan and various lipoproteins, as well as LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*. TLR4 recognizes LPS from Gram-negative bacteria that is complexed with mammalian CD14. (Table I-2, page 28). The signaling pathway for both TLR2 and TLR4 includes the translocation of NF-κB into the nucleus where it acts as a transcription factor for various pro-inflammatory cytokines (TNF-α, IL-1 and IL-6), chemokines, and co-stimulatory molecules[57,78,79,119].

III. Chapter 3

Materials and Methods

Mouse Experiments

Mice. Thirty-six 10 week-old, female BALB/c mice were purchased from Charles River Laboratories and were randomly separated into groups of 3 per cage with a total of 12 cages. Four cages were randomly assigned to Experiment 1, Experiment 2, or Experiment 3 as described in the Experimental Design section and Figures III-1, III-2, and III-3 (All images in this thesis are presented in color). The mice were allowed to acclimate to their environment for 1 week before starting the experiments. Pelleted food and water were provided *ad libitum*. All experiments were conducted according to animal use and care guidelines set by the University Laboratory Animal Resources at Michigan State University.

Reagents. Lot 58H4067 *Salmonella* Typhimurium LPS that was purified by gel filtration and sterilized by gamma irradiation was purchased from Sigma Inc. (St. Louis, Missouri). The potency of this lot of LPS as indicated by LAL assay was $3.0x10^6$ endotoxin units (EU)/mg. *Trichuris suis* Batch 36 ESP was a gift from Dr. Dolores Hill of the Agricultural Research Service, USDA, Beltsville, Maryland, USA[46]. Batch 36 ESP was diluted for experiments in sterile phosphate buffered saline. A LAL assay was performed and the amount of endotoxin present in undiluted Batch 36 ESP was about 145.2 EU/mg of total protein.

Experimental Designs. Experiment 1 was divided into 2 different temporal arrangements depending on the protein concentration of TsESP. The first arrangement consisted of 3 treatment groups injected intraperitoneally (i.p.) with

either phosphate buffered saline (PBS), 25 μg total TsESP protein, or 50 μg total TsESP protein daily for 7 days prior to and concomitantly with the LPS challenge (Figure III-1A). The second facet of Experiment 1 consisted of injecting one group of mice with 100 μg total TsESP protein 7 days prior to the LPS challenge (Figure III-1B). LPS challenge injections were administered i.p. as a sublethal dose in the amount of 30,000 EU/gram of body weight. LPS was injected into the right side of the peritoneal cavity, while TsESP or PBS was injected into the left side of the peritoneal cavity. All injections were less than or equal to 0.1 ml total volume. Mice were monitored every four hours for the first 48 hours and scored for clinical signs. Terminally ill mice, as indicated by clinical signs of hypothermia and cyanosis of extremities, were euthanized immediately using increasing concentrations of carbon dioxide in an unprimed chamber. All remaining mice were euthanized 7 days after the LPS challenge.

Experiment 2 was also divided into 2 different temporal arrangements depending on the concentration of TsESP. The first arrangement consisted of 3 treatment groups injected i.p. with either PBS, 25 μg total TsESP protein, or 50 μg total TsESP protein daily for 2 days prior to and concomitantly with the LPS challenge (Figure III-2A). The second facet of Experiment 2 consisted of injecting one group of mice with 100 μg total TsESP protein 2 days prior to the LPS challenge (Figure III-2B). Again, LPS challenge injections were administered i.p. in the amount of 30,000 EU/gram of body weight into the right side of the peritoneal cavity, while TsESP or PBS was injected into the left side of the peritoneal cavity. All injections were less than or equal to 0.1 ml total volume.

Mice were monitored, scored for clinical signs, and euthanized as described for Experiment 1.

Experiment 3 consisted of four treatment groups. The groups of mice were injected with either PBS, or 25 μg, 50 μg, or 100 μg total TsESP protein concomitantly with the LPS challenge (Figure III-3). LPS was administered in the amount of 30,000 EU/gram of body weight into the right side of the peritoneal cavity, while TsESP or PBS was injected into the left side of the peritoneal cavity. All injections were given i.p. in a volume less than or equal to 0.1 ml. Mice were monitored, scored for clinical signs, and euthanized as described for Experiment 1.

Figure III-1

Timeline of Experiment 1. (**A**) Mice were injected with PBS, 25 μg *T. suis* ESP, or 50 μg of *T. suis* ESP once a day for 7 days prior to LPS challenge. Arrows indicate injections of PBS or Batch 36 TsESP. On Day 0, treatments and LPS (★) were administered i.p. concomitantly. (**B**) Mice in Treatment Group #4 received only one injection of 100 μg *T. suis* ESP 7 days prior to LPS challenge because it was not known how mice would react to a high dose of *T. suis* ESP. On Day 0, mice received an i.p. injection of LPS (★). Arrow indicates injection of Batch 36 TsESP. All surviving mice were euthanized 7 days after LPS challenge.

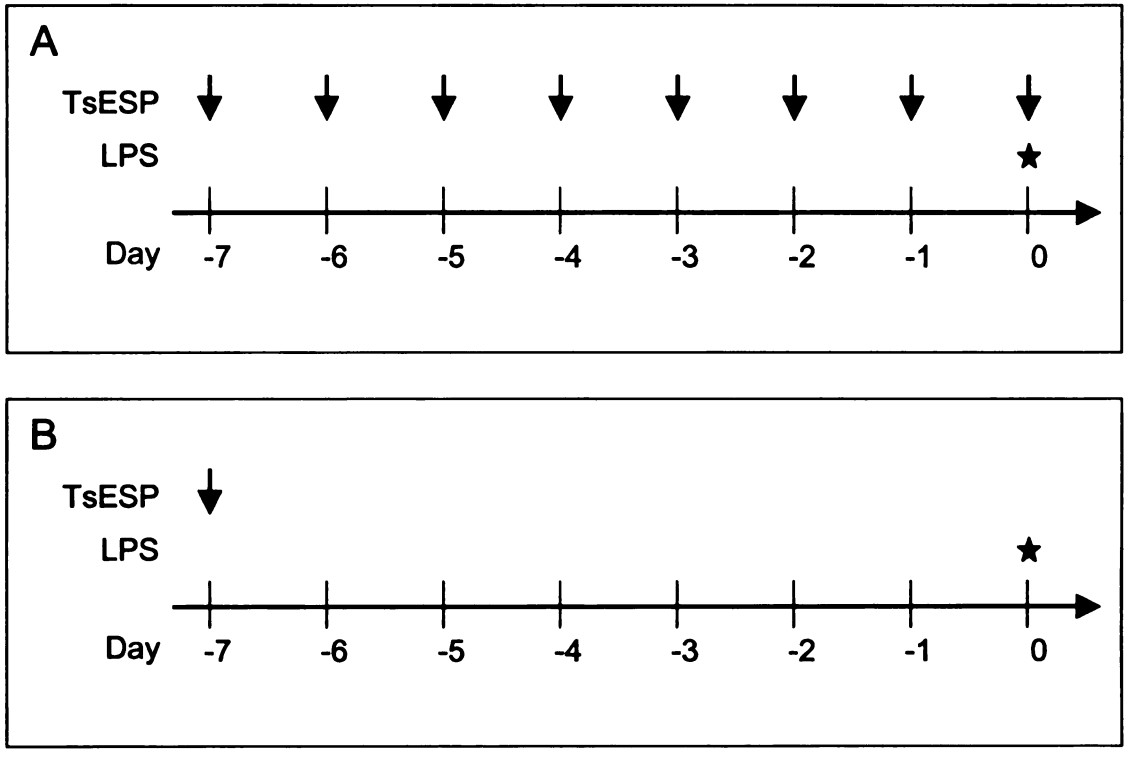


Figure III-2

Timeline of Experiment 2. (A) Mice were injected (i.p.) with PBS, 25 μg *T. suis* ESP, or 50 μg of *T. suis* ESP once a day for 2 days prior to LPS challenge. Arrows indicate injections of PBS or Batch 36 TsESP. On Day 0, treatments and LPS (★) were administered i.p. concomitantly. (B) Mice in Treatment Group #4 received only one injection of 100 μg *T. suis* ESP 2 days prior to LPS challenge because it was not known how mice would react to a high dose of *T. suis* ESP. On Day 0, mice received an i.p. injection of LPS (★). Arrow indicates injection of Batch 36 TsESP. All surviving mice were euthanized 7 days after LPS challenge.

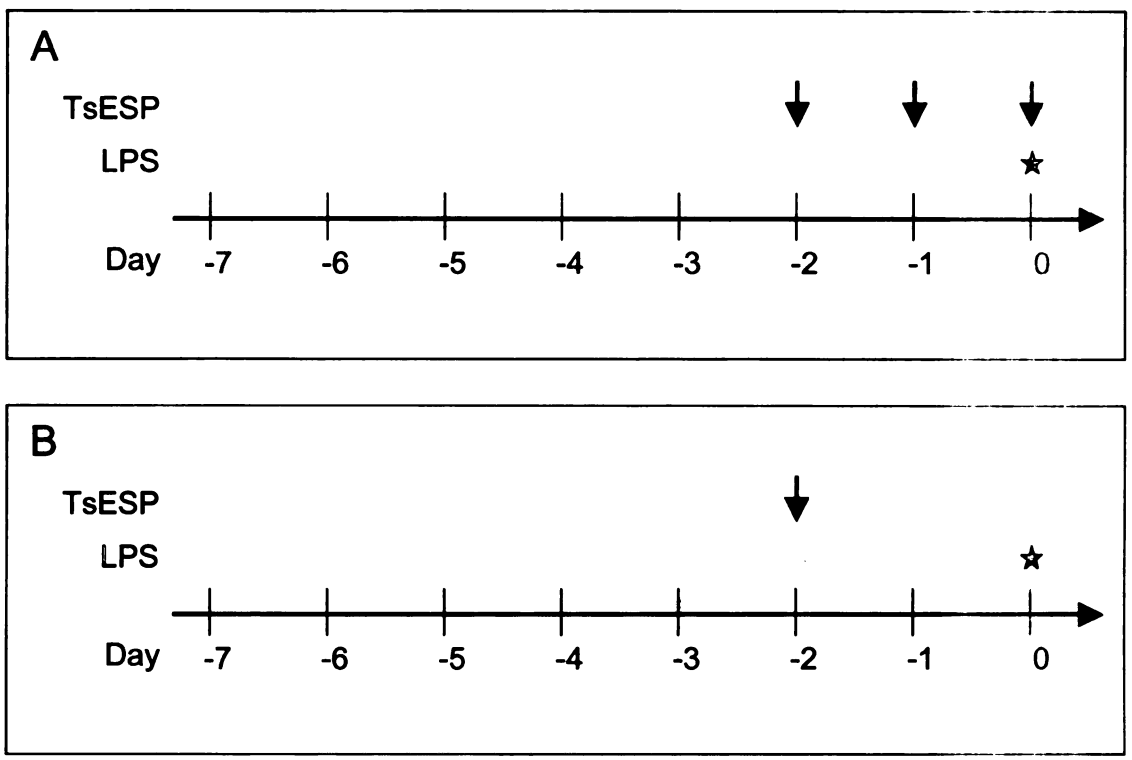
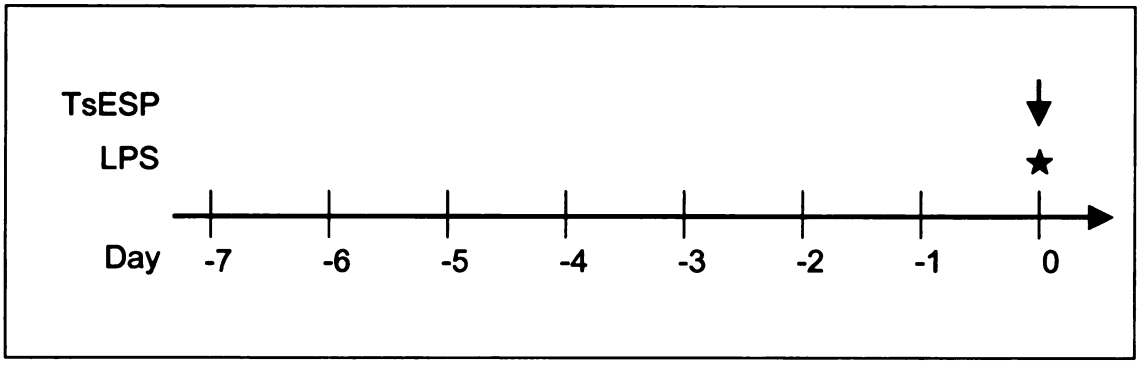


Figure III-3

Timeline of Experiment 3. All mice were injected concomitantly with either PBS, 25 μg Batch 36 *T. suis* ESP, 50 μg Batch 36 *T. suis* ESP, or 100 μg Batch 36 *T. suis* ESP (as indicated by the arrow) and LPS (★) on Day 0. All surviving mice were euthanized 7 days after LPS challenge.



Physical Examination of Mice. Physical examinations were performed on the mice every day prior to injections with LPS, and then every 4 hours after LPS challenge. Examinations included observations from a distance followed by physical examinations and scoring. Mice were scored (from a distance) to assess eating/drinking activity, socialization, respiration rate, coat condition, posture, and movement. Physical examinations included assessment of diarrhea, body temperature, ocular discharge, and color of mucus membranes, and extremities (nose, ears, feet, and tail).

RAW 264.7 Macrophage Experiments

T. suis ESP Preparation. Weaned pigs were experimentally infected by oral gavage with 4000 to 5000 *T. suis* eggs containing L1 larvae. Forty-five to 60 days after infection, pigs were anesthetized with intramuscular injections of 4.4 mg/kg Telazol (Fort Dodge, Overland Park, Kansas) and 2.2 mg/kg xylazine (Butler, Columbus, Ohio). They were then euthanized with an intravenous injection of 86 mg/kg sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, Michigan). TsESP was prepared based on techniques developed by Hill et al. (1993) and Abner et al. (2001)[46]. Adult *T. suis* worms were recovered from the cecum and proximal colon of each pig using forceps and placed into Petri dishes containing 0.85% sodium chloride prewarmed to 37°C to remove the majority of debris. The worms were then washed three to four times by passing to fresh Hank's Balanced Salt Solution prewarmed to 37°C to remove fine debris (HBSS; Sigma Inc., St. Louis, Missouri). After the washing process, the worms were

incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 in sterile RPMI-1640 medium supplemented with 5% Antibiotic-Antimycotic cocktail (Invitrogen, Carlsbad, California). The Antibiotic-Antimycotic cocktail contained 10,000 U/ml Penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B. A second overnight incubation in RPMI-1640 medium supplemented with 1% Antibiotic-Antimycotic cocktail was then performed. To collect the ESP, the sterile worms were incubated in RPMI-1640 medium containing 1% sterile glucose (4 worms/ml) for up to 10 days; collecting the culture fluid containing ESP every other day. The culture fluids were pooled into separate batches based on individual pigs; ESP Batches 5 and 6 were used in this study. Batches were concentrated by ultrafiltration using an Amicon stirred cell (Millipore, Bedford, Massachusetts). Total protein content of concentrated Batches 5 and 6 TsESP was determined by Bradford assay and was 0.25 and 0.4 mg protein/ml, respectively. LAL assays showed that Batch 5 TsESP contained 8.2 Endotoxin Units (EU)/ml and Batch 6 TsESP contained 6.4 EU/ml.

Cell cultures and media. RAW 264.7 mouse peritoneal macrophages were the gift of Dr. James Pestka, Michigan State University. The macrophages were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM final concentration of a MEM sodium pyruvate solution at 37°C in a humidified atmosphere containing 5% CO_2. All reagents used for culturing RAW 264.7 macrophages were purchased from Invitrogen (Carlsbad, California).

Stimulation of the RAW 264.7 macrophages and TNF-α measurements. RAW 264.7 macrophages were plated at a density of 7.5 x 10^5 cells/well in Costar® twelve-well, polystyrene tissue culture plates for overnight growth in a total volume of 1.0 ml per well (Corning Inc., Corning, New York). Cells were exposed in triplicate for 0, 1, or 3 hours to the following treatments: (1) DMEM only; (2) 10 μg/ml polymyxin B (Sigma Inc., St. Louis, Missouri); (3) 50 endotoxin units (EU)/ml *Salmonella* Typhimurium lipopolysaccharide (LPS; Sigma Inc., St. Louis, Missouri); (4) 50 EU/ml LPS plus 10 μg/ml polymyxin B (PMB); (5) 100 μg/ml total protein Batch 5 TsESP; (6) 90 μg/ml total protein Batch 5 TsESP plus 10 μg/ml polymyxin B; (7) 100 μg/ml total protein Batch 6 TsESP; (8) 80 μg/ml total protein Batch 6 TsESP plus 10 μg/ml polymyxin B; and (9 and 10) concentrated RPMI-1640 (cRPMI) medium with and without 10 μg/ml polymyxin B as controls for batches of TsESP (Table III-1). Treatments were prepared in DMEM medium without FBS, penicillin, streptomycin, and MEM sodium pyruvate, and a total of 0.6 mls of each treatment was added to the appropriate well. Polymyxin B and the various treatments were incubated for 15 minutes in DMEM (37°C, 5% CO_2) before addition to the macrophages. There was not enough TsESP to add the full amount to the media also containing polymyxin B, so the concentrations of the ESP plus polymyxin B treatments are lower. Treated macrophages were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were collected after each incubation time and centrifuged (3700 x g) for 10 minutes at 4°C to remove cell debris and then stored at -80°C. TNF-α production was quantified using an OptEIA Mouse TNF-α enzyme-linked immunosorbent

assay (ELISA) kit according to the manufacturer's instructions (BD PharMingen, San Diego, California).

The Scheffe's Test was used for all possible comparisons between treatment groups within each time point using a significance level of p=0.05.

Table III-1

Summary of treatment groups added to the RAW 264.7 macrophages for 0, 1, or 3 hours. After treatment, supernatants were collected, centrifuged at 3700 x g for 10 minutes at 4°C to remove cellular debris, and then stored at -80°C until the TNF-α production was quantified by ELISA.

Treatment	10 μg/ml PMB
DMEM	-
DMEM	+
50 EU/ml LPS	-
50 EU/ml LPS	+
100 μg/ml Batch 5 TsESP	-
90 μg/ml Batch 5 TsESP	+
100 μg/ml Batch 6 TsESP	-
80 μg/ml Batch 6 TsESP	+
cRPMI	-
cRPMI	+

Chinese Hamster Ovary Cell Experiments

Cell cultures and media. The Chinese hamster ovary fibroblasts used in these studies, 3E10 and 7.19/TLR2 were the gift of Dr. Douglas Golenbock (Section of Infectious Diseases, Boston Medical Center; and Boston University School of Medicine, Boston, Massachusetts).

3E10 is transfected with pCEP4 (Invitrogen, San Diego, California) containing the gene for human CD14 to confer responsiveness to LPS (Figure III-4). CD14 is transcribed from the cytomegalovirus (CMV) promoter for constitutive expression. 3E10 was subsequently transfected with the pUMS(ELAM)-Tac plasmid, which is constructed to transcribe CD25 when NF-κB binds to the promoter[120]. Therefore, if LPS stimulates endogenous TLR4 to activate and translocate NF-κB into the nucleus, CD25 will be expressed on the cell surface. Chinese hamster ovary fibroblasts express functional TLR4, but not functional TLR2[121].

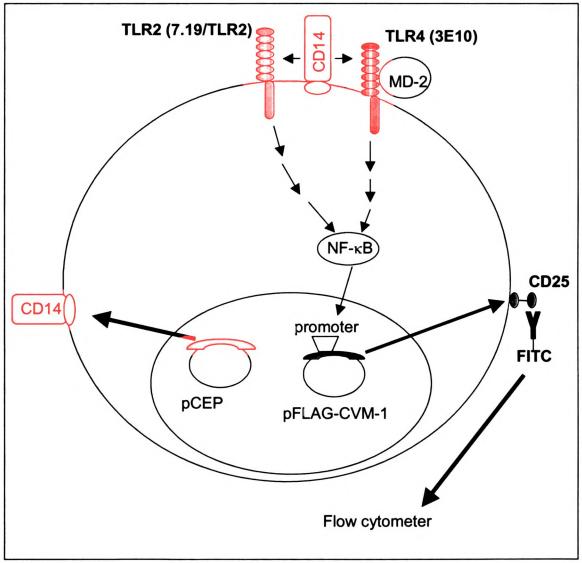
The cell line 7.19/TLR2 is a mutant of 3E10 that is hyporesponsive to LPS and is also transfected with the pFLAG-CMV-1 vector containing human TLR2 for constitutive expression[120,122-124]. The mutation carried by 7.19/TLR2 lies in the MD-2 protein that is necessary for LPS recognition and signal transduction via TLR4[124].

3E10 and 7.19/TLR2 cells were cultured in DMEM/F-12 (Invitrogen, Carlsbad, California) supplemented with 10% heat-inactivated FBS (hFBS), 400 U/ml Hygromycin B (Calbiochem, San Diego, California), and 10 μg/ml Ciprofloxacin (Bayer, Kankakee, Illinois) at 37°C in a humidified atmosphere

containing 5% CO_2. 7.19/TLR2 cells also required 0.5 mg/ml Geneticin (G418 Sulfate) (Invitrogen, Carlsbad, California) in the medium to maintain the plasmid expressing $TLR2^{120}$.

Figure III-4

Schematic diagram showing the signal transduction pathways of LPS-responsive 3E10 and LPS-nonresponsive 7.19/TLR2 Chinese hamster ovary cells. CD14 is constitutively expressed from the pCEP plasmid to function in TLR2 and TLR4 ligand recognition. LPS can bind to the TLR4/CD14 complex on 3E10 cells and sBLP can bind to the TLR2/CD14 complex on 7.19/TLR2 cells leading to the release of NF-κB from IκB. NF-κB then translocates into the nucleus where it binds to the promoter leading to transcription of the downstream CD25 cDNA fragment. CD25 protein is then expressed on the surface membrane where it can be detected with human FITC-conjugated anti-CD25 antibodies via flow cytometry.



Stimulation of the Chinese hamster ovary (CHO) cells and flow cytometry. 3E10 or 7.19/TLR2 cells were seeded at a density of 2.0 x 10^5 cells/well in Costar® twenty-four-well, polystyrene tissue culture plates (Corning Inc., Corning, New York) for overnight growth at 37°C in a humidified atmosphere containing 5% CO_2. Growth medium was removed, treatments were added, and the cells were incubated for 15 hours. All reagents were prepared in the DMEM:Ham's Nutrient Mixture F-12 (DMEM/F-12) medium with the appropriate supplements. The CHO cells were treated with the following reagents: (1) 12 ng/ml recombinant human interleukin 1-beta (rhIL-1β) (BD PharMingen, San Diego, California); (2) 1500 EU/ml *Salmonella* Typhimurium LPS; (3) 100 μg/ml Batch 5 TsESP; (4) 100 μg/ml Batch 6 TsESP; or (5) 100 ng/ml $Pam_3CysSerLys_4$ a synthetic bacterial lipoprotein (sBLP; purchased from Dr. Günther Jung, University of Tübingen, Germany). Reagents were incubated with or without 10 μg/ml polymyxin B for 15 minutes (37°C, 5% CO_2) before addition of 0.3 mls of the appropriate treatment per well (Table III-2). After the cells were incubated with the various treatments, they were detached from the plates using 1 ml Trypsin-EDTA (Invitrogen) for 3 minutes and transferred to 5 ml polystyrene round-bottom tubes designed for flow cytometry for the staining procedure. Cells were washed in 2 to 3 mls of an isotonic buffered diluent (Haema-Line 2; ABX Diagnostic Inc., Irvine, CA) to remove the Trypsin-EDTA, and then stained with 20 μl of either (1) fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD25 monoclonal antibody (IgG_1, κ), (2) FITC-conjugated mouse IgG_1, κ, isotype control for CD25, or (3) FITC-conjugated mouse anti-human CD14 monoclonal antibody (IgG_{2a}, κ). All

antibodies were purchased from BD PharMingen (San Diego, California). The stained CHO cells were counted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California).

Table III-2

Summary of various treatments added to either the 3E10 or 7.19/TLR2 Chinese hamster ovary cell lines for 15 hours. After treatment, the CHO cells were washed and then stained with 20 μl of either (1) FITC-conjugated anti-human CD25 monoclonal antibody (IgG_1, κ), (2) FITC-conjugated IgG_1, κ (isotype control for CD25), or (3) FITC-conjugated anti-human CD14 monoclonal antibody (IgG_{2a}, κ). Stained cells were counted using a FACSCalibur flow cytometer.

Treatment	10 μg/ml PMB
DMEM/F-12	-
DMEM/F-12	+
1500 EU/ml LPS	-
1500 EU/ml LPS	+
100 μg/ml Batch 5 TsESP	-
100 μg/ml Batch 5 TsESP	+
100 μg/ml Batch 6 TsESP	-
100 μg/ml Batch 6 TsESP	+
100 ng/ml sBLP	-
100 ng/ml sBLP	+

IV. **Chapter 4**

Results

Immunomodulatory Role of *T. suis* Excretory/secretory Products in BALB/c Mice

Several *Trichuris* ESP protein constituents have been hypothesized to have immunoregulatory functions, including a serine protease inhibitor, a chymotrypsin/elastase inhibitor, and an IFN-γ homologue[52-54] To test the hypothesis that TsESP would have an immunomodulatory role in mice, we challenged female, BALB/c mice with different concentrations of TsESP prior to and concomitantly with a sublethal dose of *Salmonella* Typhimurium LPS.

Table IV-1 summarizes the results of this series of experiments. The three mouse experiments are arranged generally according to the administration of TsESP in relation to the LPS challenge, with varying doses of TsESP used within each treatment schedule. Mice that received 100 μg TsESP are grouped together because they received only one injection of TsESP, instead of daily injections prior to the LPS challenge.

All control mice that received repeated injections of PBS prior to or a single injection of PBS concomitantly with the sublethal dose of LPS (without TsESP) survived, confirming that 30,000 EU/gram of mouse was a sublethal dose of LPS. However, within 4 to 6 hours of LPS challenge, all mice from the three experiments developed rough hair coats and hunched postures. By 12 hours after LPS challenge, all mice had developed diarrhea, mucopurulent ocular discharge, and a decrease in mobility (walking slowly or not at all when prompted). Three out of the 36 mice began to recover 18 hours after LPS injection. By 48 hours after LPS challenge, 24 out of the 36 mice had one or both

eyes clear of mucopurulent discharge and had regained most or all of their mobility.

Two of the 3 mice given 25 μg TsESP daily for 7 days prior to and concomitantly with the LPS challenge and 5 of the 9 mice administered TsESP concurrently with LPS developed hypothermia and/or cyanosis of the extremities in addition to the signs of shock previously described. These mice either died between times of monitoring or were euthanized. Twelve of the 36 mice in all three experiments died or were euthanized because clinical signs indicated a terminal stage of shock.

The crude preparation of TsESP is a complex mixture of whipworm antigens that includes various concentrations of proteins, carbohydrates, and lipids that can each influence the response of the mouse to a challenge with LPS. Accordingly, both the temporal administration of TsESP in relationship to the LPS challenge and the amount of total crude TsESP influenced the ability of the mice to survive endotoxic shock. Concomitant injections of TsESP and a sublethal dose of LPS increased frequency of death in a dose-dependent manner based on TsESP protein concentration, suggesting that TsESP acted as a sensitizing agent (Table IV-1, Figure IV-1). In contrast to the results suggesting that 100 μg TsESP promotes death in mice when given concurrently with a sublethal dose of LPS, all mice that were administered a single dose of 100 μg TsESP 7 or 2 days prior to or concurrently with LPS injection survived (Table IV-1B). Furthermore, while 50 μg TsESP can also act as a sensitizing agent when given concomitantly with the LPS challenge, it appeared to either act as either an immunoprotectant

agent or induce tolerance in the mice when it was administered daily for 7 or 2 days prior to the lethal combination of the concurrent injections of 50 μg TsESP and LPS (Table IV-1A, Figure IV-2). Mice responded differently to 25 μg TsESP than to 50 or 100 μg TsESP. Only 1 of the 3 mice died when given 25 μg TsESP daily for 2 days prior to and/or concomitantly with the sublethal LPS challenge. Interestingly, all 3 mice died when injected with 25 μg daily for 7 days prior to and concurrently with the sublethal dose of LPS (Table IV-1A, Figure IV-3).

Table IV-1

Ability of Batch 36 *Trichuris suis* excretory/secretory products (TsESP) to modulate the host response in BALB/c mice challenged with a sublethal dose of lipopolysaccharide (LPS). (**A**) Treatments were injected i.p. daily for the number of days indicated prior to and then concomitantly with a sublethal dose (30,000 EU/ gram of body weight) of *Salmonella* Typhimurium LPS. (**B**) TsESP was administered once prior to a sublethal dose (30,000 EU/gram of body weight) of *Salmonella* Typhimurium LPS. Surviving mice were euthanized 7 days after LPS challenge.

A

			Number of survivors on each day after challenge with 30,000 EU of LPS on Day 0.							
Treatment	**Days TsESP Administered**	**Amt. (μg) TsESP**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
PBS	Day -7, Day -6, Day -5, Day -4, Day -3, Day -2, Day -1, Day 0	-	3	3	3	3	3	3	3	3
TsESP		25	3	3	1	0	0	0	0	0
TsESP		50	3	2	2	2	2	2	2	2
PBS	Day -2, Day -1, Day 0	-	3	3	3	3	3	3	3	3
TsESP		25	3	3	2	2	2	2	2	2
TsESP		50	3	3	3	3	3	3	3	3
PBS	Day 0	-	3	3	3	3	3	3	3	3
TsESP		25	3	3	2	2	2	2	2	2
TsESP		50	3	1	0	0	0	0	0	0
TsESP		100	3	2	1	0	0	0	0	0

B

			Number of survivors on each day after challenge with 30,000 EU of LPS on Day 0.							
Treatment	**Days TsESP Administered**	**Amt. (μg) TsESP**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
TsESP	Day -7	100	3	3	3	3	3	3	3	3
TsESP	Day -2	100	3	3	3	3	3	3	3	3
TsESP	Day 0	100	3	2	1	0	0	0	0	0

Figure IV-1

Survival Curve of mice injected i.p. with Batch 36 *Trichuris suis* excretory/secretory products (TsESP) and then challenged concomitantly with a sublethal dose of *Salmonella* Typhimurium lipopolysaccharide (LPS). Mice received either 25, 50, or 100 μg of total TsESP protein and 30,000 EU LPS per gram of body weight. All mice that received PBS survived for seven days after LPS challenge, demonstrating a sublethal dose of LPS. All surviving mice were euthanized 7 days after LPS challenge.

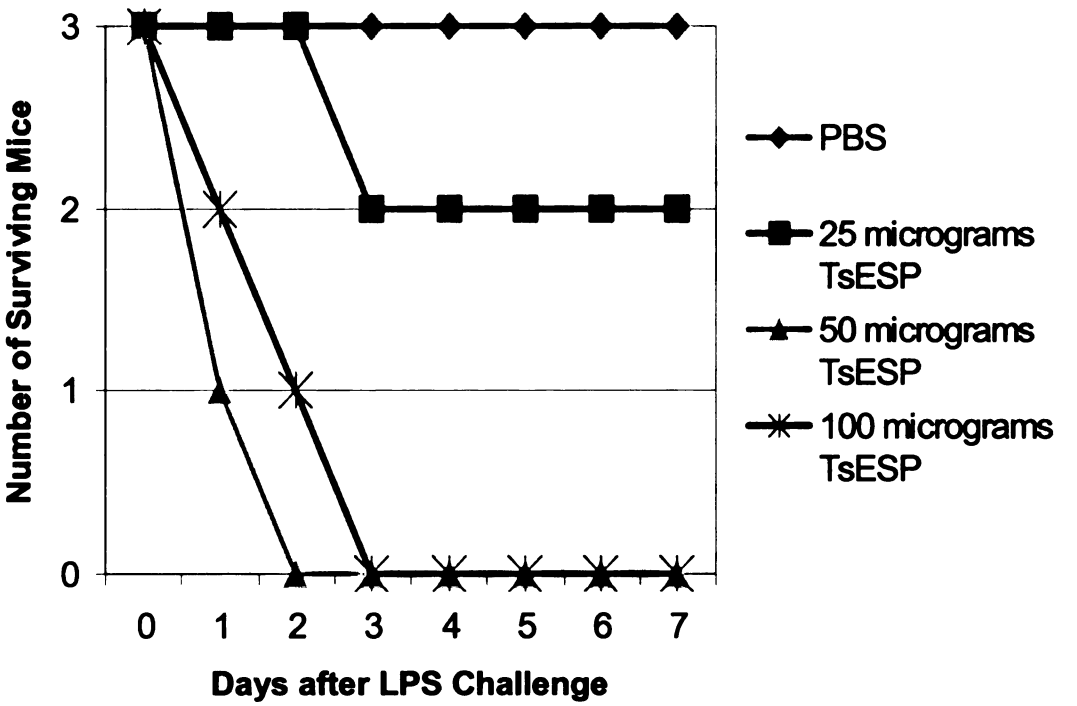


Figure IV-2

Survival Curve of mice injected i.p. with 50 μg Batch 36 *Trichuris suis* excretory/secretory products (TsESP) and then challenged with a sublethal dose of *Salmonella* Typhimurium lipopolysaccharide (30,000 EU LPS per gram of body weight). The TsESP was administered daily for 7 or 2 days prior to and concomitantly with the LPS challenge. All surviving mice were euthanized 7 days after LPS challenge.

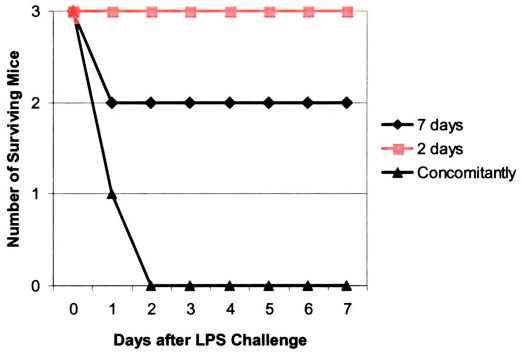
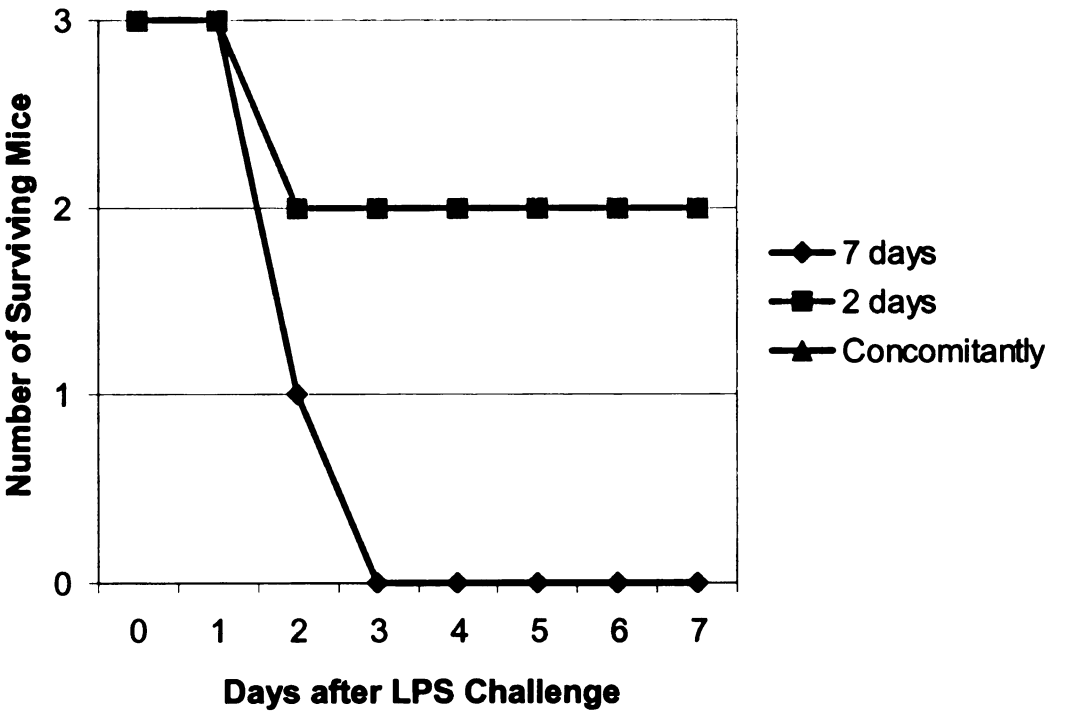


Figure IV-3

Survival Curve of mice injected i.p. with 25 μg Batch 36 *Trichuris suis* excretory/secretory products (TsESP) and then challenged with a sublethal dose of *Salmonella* Typhimurium lipopolysaccharide (30,000 EU LPS per gram of body weight). The TsESP was administered daily for 7 or 2 days prior to and concomitantly with the LPS challenge. All surviving mice were euthanized 7 days after LPS challenge.



Induction of TNF-α Production from RAW 264.7 Macrophages in Response to *T. suis* Excretory/secretory Products

The observation that TsESP can act as either a sensitizing agent or an agent that induces endotoxin tolerance depending on the dose and time of administration of TsESP in context to LPS led us to determine if an inherent component of TsESP can stimulate the secretion of TNF-α. This cytokine is known to play key roles in both sensitizing mice to LPS and inducing tolerance to LPS. Furthermore, *T. trichiura* and *T. muris* antigens, both somatic and excretory/secretory products, have been shown to stimulate production of TNF-α[19]. To test the hypothesis that TsESP will stimulate TNF-α production, we treated murine RAW 264.7 macrophages with two different batches of TsESP in the absence or presence of polymyxin B (PMB). LPS increasingly stimulated the production of TNF-α as determined by ELISA from 0 hour (data not shown), 1 hour (Figure IV-4) to 3 hours (Figure IV-5) of incubation. Both at 1 and 3 hours, TNF-α secretion stimulated by LPS is significantly increased when compared to the negative DMEM medium control or from macrophages treated with LPS and 6 μg of polymyxin B concurrently. There is no significant increase in TNF-α production, compared to the negative DMEM control, when RAW 264.7 macrophages are treated with polymyxin B only, concentrated RPMI (cRPMI) in the absence or presence of polymyxin B, or LPS in the presence of polymyxin B (Figures IV-4 and IV-5). Batches 5 (B5) and 6 (B6) TsESP significantly induced TNF-α secretion from the RAW 264.7 macrophages at both 1 and 3 hours of incubation. In contrast to LPS treatment, this triggering of TNF-α production was

not inhibited by polymyxin B at either time point. After 1 hour, there is no significant difference between TNF-α levels from cells treated with LPS, Batch 5 TsESP with and without polymyxin B, and Batch 6 TsESP with and without polymyxin B (Figure IV-4). However, macrophages stimulated with LPS for 3 hours secrete levels of TNF-α significantly different from that secreted by cells stimulated with the two batches of TsESP. Also, after 3 hours of incubation, TNF-α secretion from macrophages treated with Batch 5 was significantly greater than TNF-α secretion from macrophages treated with Batch 6 TsESP. At 0 hour, LPS and both Batches of TsESP do not significantly induce TNF-α as compared to the DMEM medium negative control (data not shown).

Figure IV-4

Mean TNF-α secretion (n = 6) from RAW 264.7 macrophages treated with DMEM, 50 EU/ml *Salmonella* Typhimurium LPS, 100 μg/ml Batch 5 *Trichuris suis* ESP (B5 TsESP), 100 μg/ml Batch 6 *Trichuris suis* ESP (B6 TsESP), and concentrated RPMI-1640 medium (cRPMI) all in the absence or presence of 10 μg/ml polymyxin B (PMB) for 1 hour. Supernatants were analyzed by sandwich ELISA. Scheffe's Test was used for all possible comparisons between treatment groups using a significance level of p=0.05. Statistically significant groups are marked as either A or B. *The amount of B5 TsESP incubated with PMB was 90 μg/ml and the amount of B6 TsESP incubated with PMB was 80 μg/ml.

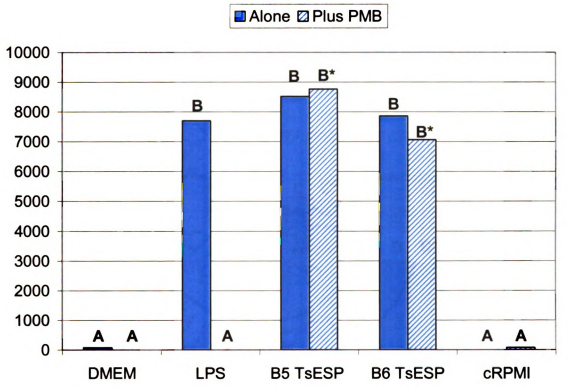
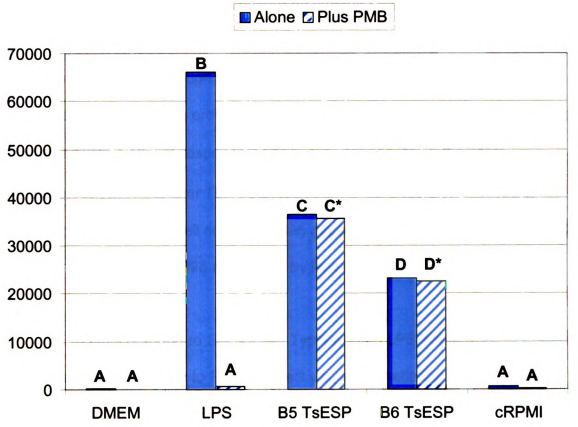


Figure IV-5

Mean TNF-α secretion (n = 6) from RAW 264.7 macrophages treated with DMEM, 50 EU/ml *Salmonella* Typhimurium LPS, 100 μg/ml Batch 5 *Trichuris suis* ESP (B5 TsESP), 100 μg/ml Batch 6 *Trichuris suis* ESP (B6 TsESP), and concentrated RPMI-1640 medium (cRPMI) all in the absence or presence of 10 μg/ml polymyxin B (PMB) for 3 hours. Supernatants were analyzed by sandwich ELISA. Scheffe's Test was used for all possible comparisons between treatment groups using a significance level of p=0.05. Statistically significant groups are marked as either A, B, C, or D. *The amount of B5 TsESP incubated with PMB was 90 μg/ml and the amount of B6 TsESP incubated with PMB was 80 μg/ml.



The Ability of *T. suis* Excretory/secretory Products to Stimulate Toll-like Receptor 2 and Toll-like Receptor 4

In addition to adding polymyxin B to the TsESP prior to stimulating the RAW 264.7 macrophages, we further tested for the presence of contaminating LPS in TsESP by evaluating the ability of Batches 5 and 6 to stimulate toll-like receptor 2 (TLR2) and/or TLR4 using the transfected Chinese hamster ovary cell lines 3E10 and 7.19/TLR2. TLR2 and TLR4 are the two receptors that have been most studied, and can be activated by various LPS moieties. TLR2 is specifically activated by LPS from *Porphyromonas gingivalis* and *Leptospira interrogans*, and TLR4 recognizes LPS complexed with CD14 and the lipopolysaccharide binding protein (LBP)[84,86,87,89]. 3E10 and 7.19T/TLR2 cells are transfected with a NF-κB-responsive CD25 reporter construct; therefore, stimulation of either TLR2 or TLR4 that leads to NF-κB activation will lead to CD25 expression on the cell surface. CD25 expression was detected using FITC-conjugated anti-human CD25 antibodies by flow cytometry (Figure III-4, page 50).

3E10 cells respond to *Salmonella* Typhimurium LPS through TLR4 (Figure IV-6A), but do not respond to $Pam_3CysSerLys_4$, a synthetic bacterial lipoprotein (sBLP; Figure IV-6B). The LPS stimulates 3E10 cells to display CD25 on the cell surface, thereby increasing the fluorescence intensity that is equated as a rightward shift in the curve. Polymyxin B was able to abrogate the response of 3E10 cells stimulated by LPS (Figure IV-6A). The human TLR2 is functional in the 7.19/TLR2 cell line as indicated by the increase in fluorescence when the cells are stimulated with sBLP (Figure IV-7B). Polymyxin B did not abrogate the

responsiveness of 7.19/TLR2 to sBLP. Furthermore, 7.19/TLR2 cells do not respond to the *Salmonella* Typhimurium LPS in the absence or presence of polymyxin B (Figure IV-7A).

Batch 5 TsESP is able to stimulate TLR4 in the absence of polymyxin B as demonstrated from the bimodal curve representing CD25 expression (Figure IV-8A). The second peak of the bimodal curve stimulates CD25 expression only slightly less than LPS in the absence of polymyxin B (Figure IV-8A). However, CD25 expression by 3E10 cells stimulated with Batch 5 TsESP was inhibited as much as LPS in the presence of polymyxin B suggesting that contaminating LPS is responsible for CD25 expression (Figure IV-8B). Limulus amebocyte lysate (LAL) assay results demonstrated that the endotoxin activity of Batch 5 TsESP is very low at 8.2 EU/ml, so each well of Chinese hamster ovary cells was treated with approximately 2.4 EU. Batch 5 TsESP also appears to stimulate TLR2 in the absence or presence of polymyxin B (Figure IV-9). Batch 6 TsESP appears to be unable to stimulate either TLR2 or TLR4 in the absence or presence of polymyxin B (Figure IV-10). The endotoxin activity of Batch 6 TsESP was also very low, 6.4 EU/ml. Each well of Chinese hamster ovary cells was treated with approximately 1.9 EU from Batch 6 TsESP.

Figure IV-6

Flow cytometry analysis of 3E10 cells stimulated with either (A) LPS, the positive control or (B) sBLP, the negative control. Cells were stimulated for 15 hours and then stained with either FITC-conjugated anti-IgG_1 isotype control antibody or FITC-conjugated anti-CD25 antibody and then analyzed on a FACSCalibur flow cytometer.

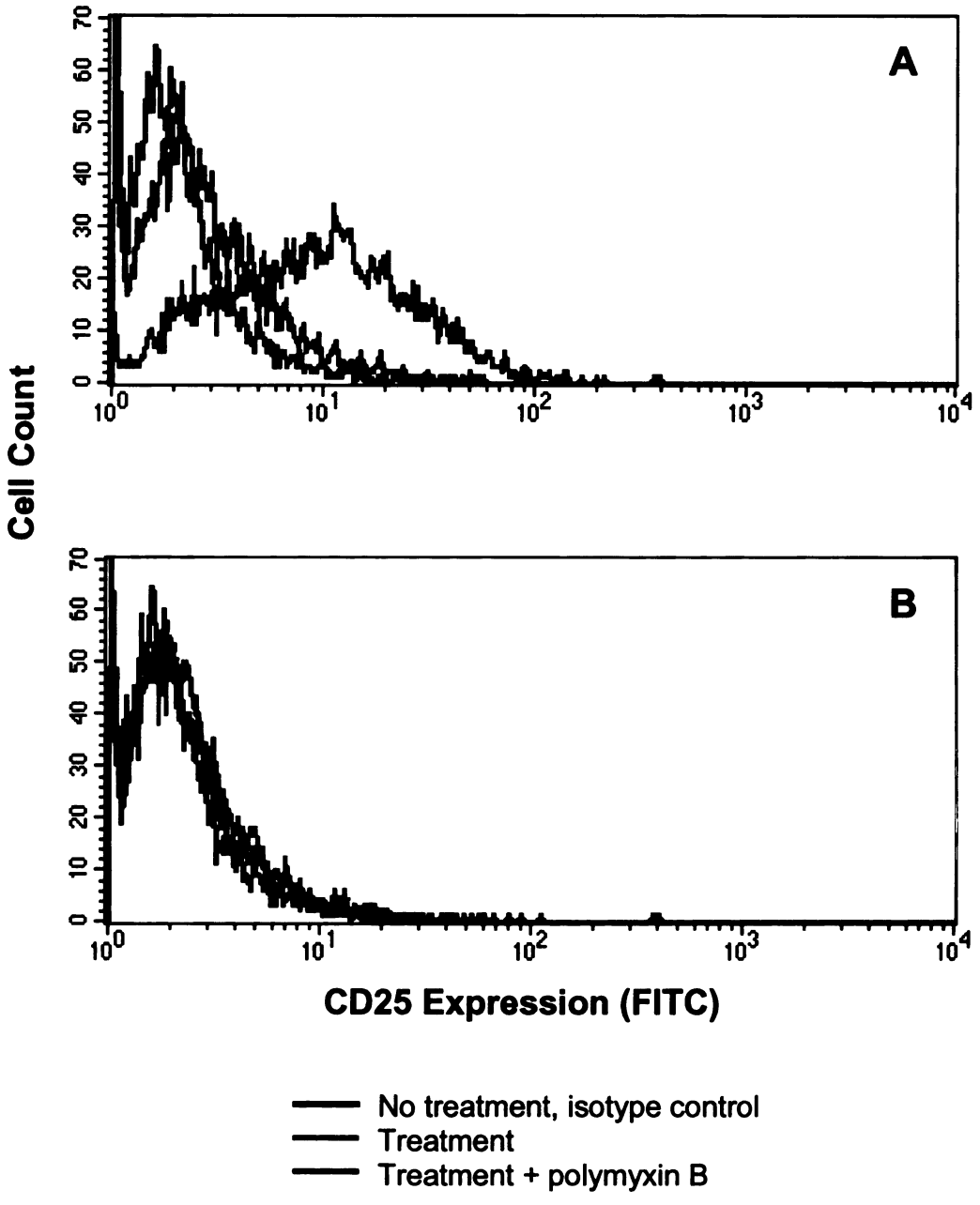


Figure IV-7

Flow cytometry analysis of 7.19/TLR2 cells stimulated with either (A) LPS, the negative or (B) sBLP, the positive control. Cells were stimulated for 15 hours and then stained with either FITC-conjugated anti-IgG_1 isotype control antibody or FITC-conjugated anti-CD25 antibody and then analyzed on a FACSCalibur flow cytometer.

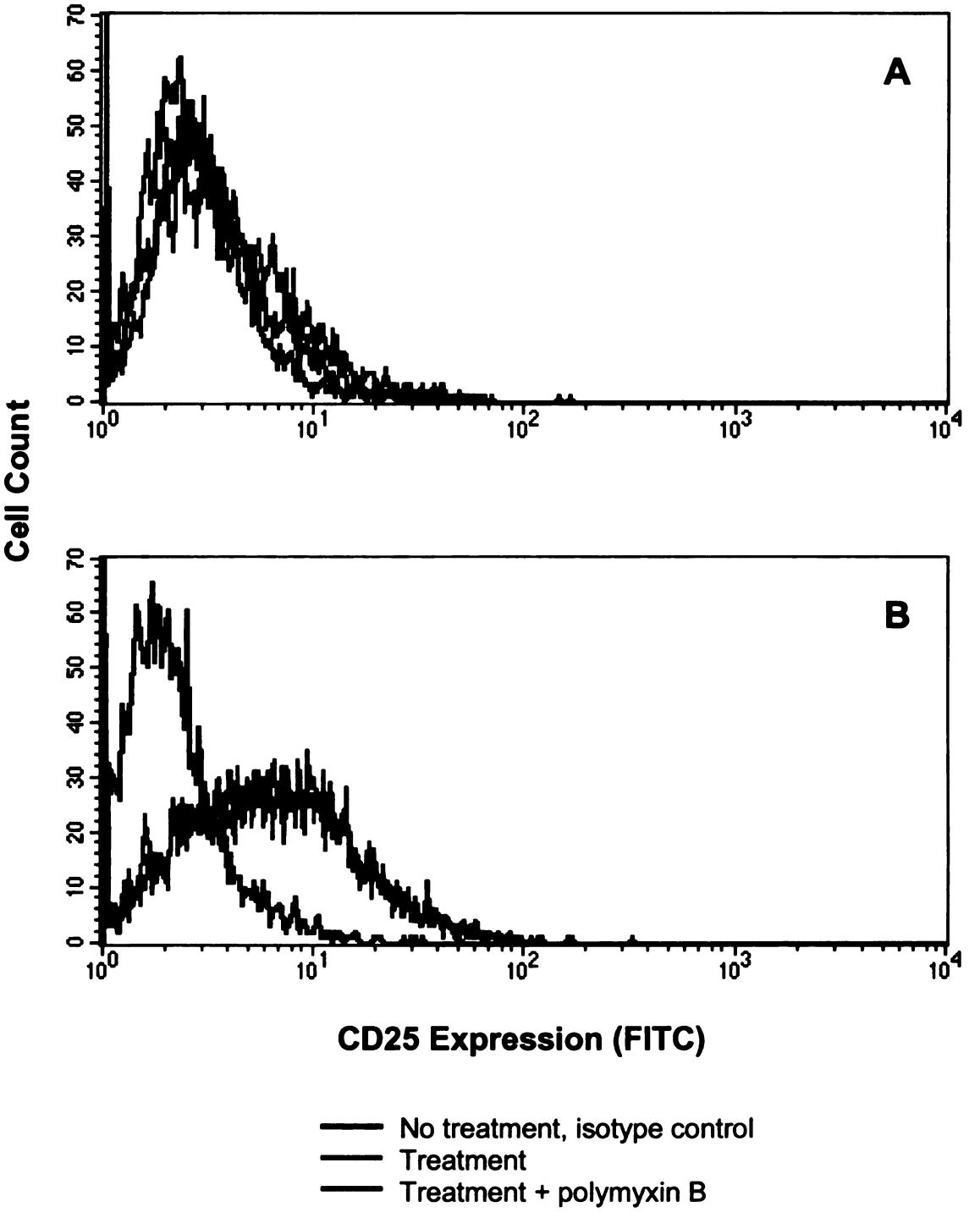


Figure IV-8

Flow cytometry analysis of 3E10 cells stimulated with Batch 5 TsESP in the (A) absence of polymyxin B or (B) presence of polymyxin B. Cells were stimulated for 15 hours and then stained with either FITC-conjugated anti-IgG_1 isotype control antibody or FITC-conjugated anti-CD25 antibody and then analyzed on a FACSCalibur flow cytometer.

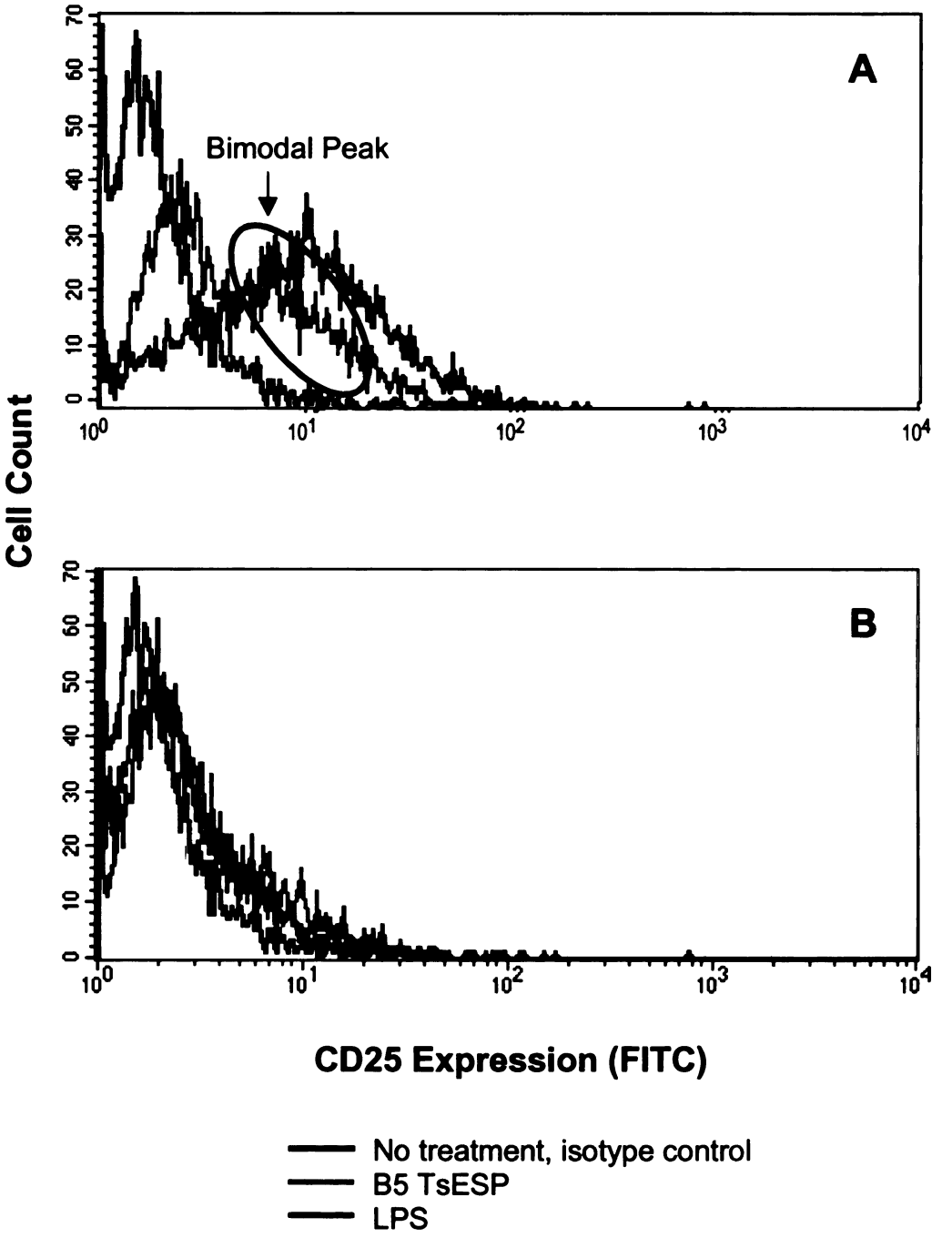


Figure IV-9

Flow cytometry analysis of 7.19/TLR2 cells stimulated with Batch 5 TsESP in the absence or presence of polymyxin B. Cells were stimulated for 15 hours and then stained with either FITC-conjugated anti-IgG_1 isotype control antibody or FITC-conjugated anti-CD25 antibody and then analyzed on a FACSCalibur flow cytometer.

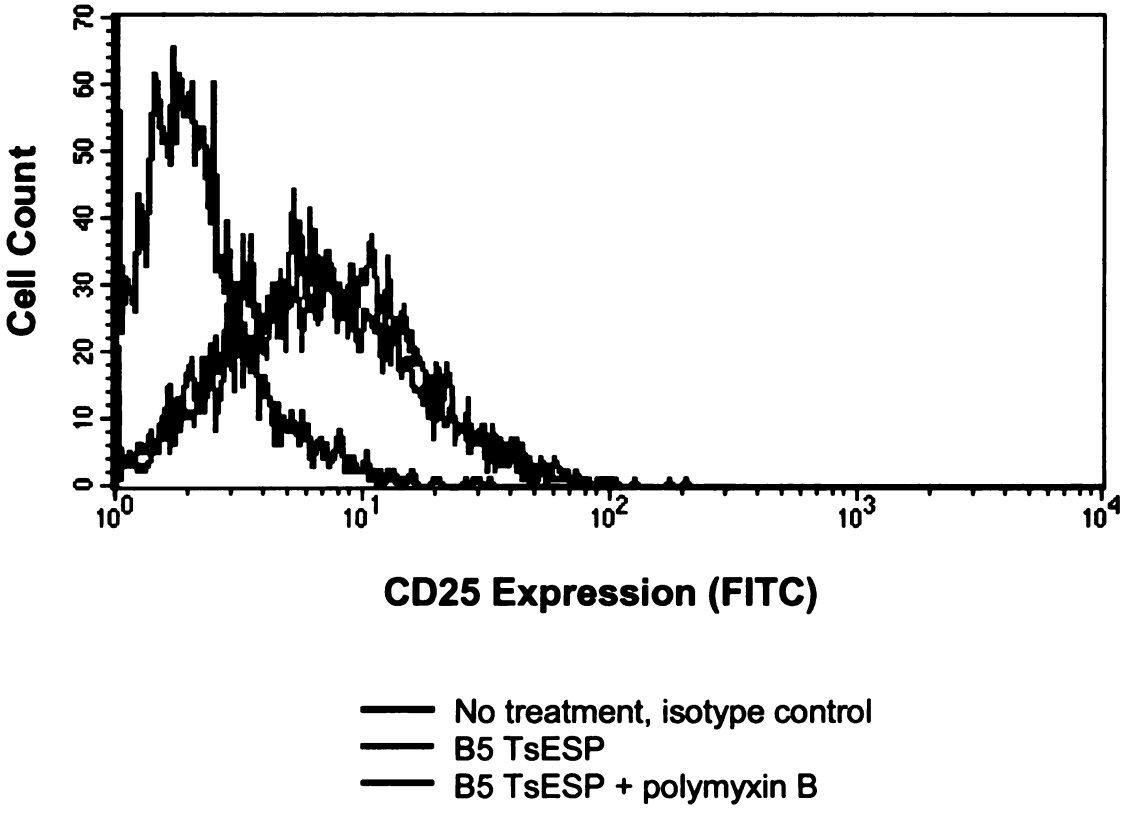
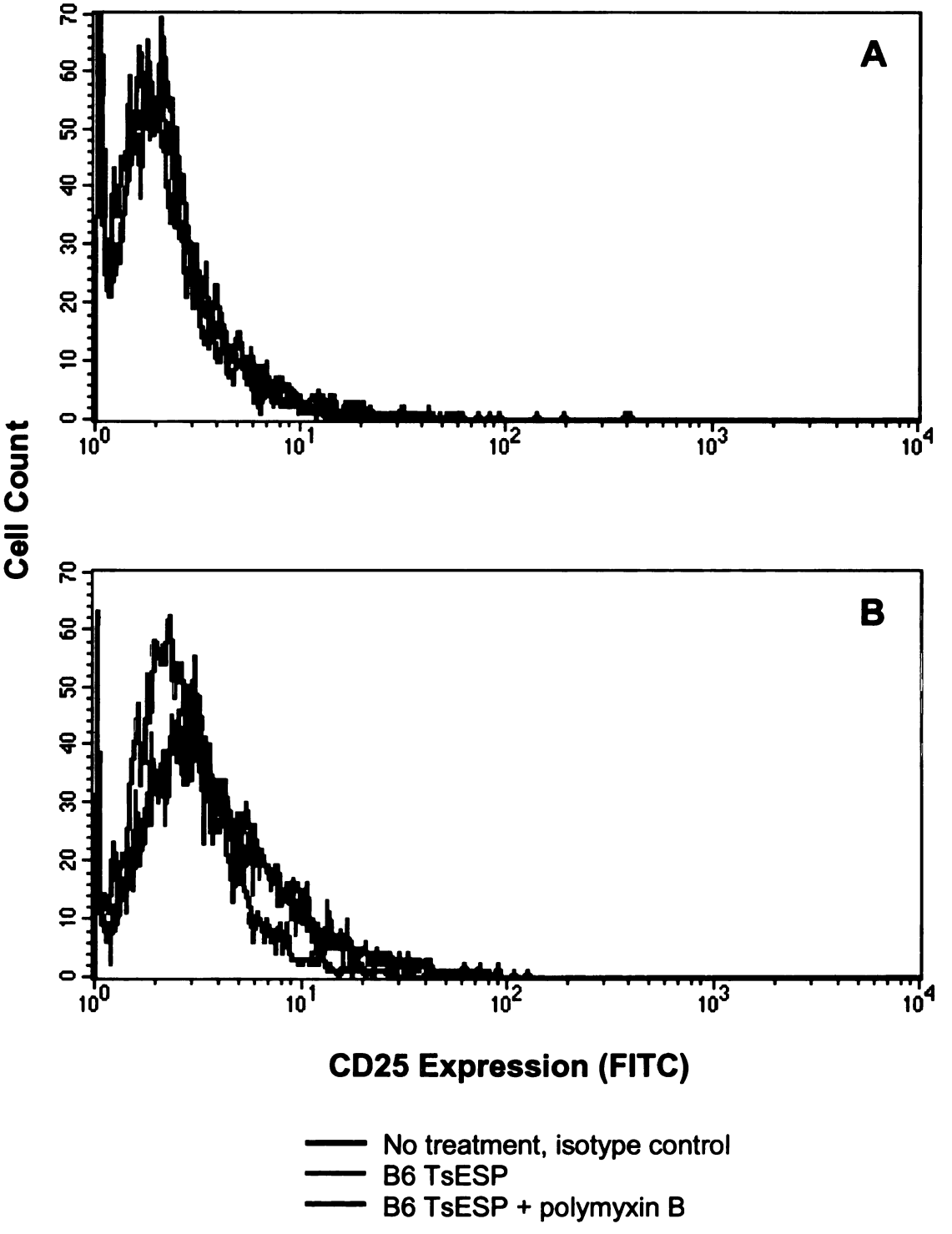


Figure IV-10

Flow cytometry analysis of (A) 3E10 cells and (B) 7.19/TLR2 cells stimulated with Batch 6 TsESP in the absence or presence of polymyxin B. Cells were stimulated for 15 hours and then stained with either FITC-conjugated anti-IgG_1 isotype control antibody or FITC-conjugated anti-CD25 antibody and then analyzed on a FACSCalibur flow cytometer.



V. **Chapter 5**

Discussion

The ability of *Trichuris* to promote secondary bacterial infections and the importance of these secondary infections in exacerbating disease in trichuriasis has been well documented[1-3,15]. One of the main research goals of our laboratory is to determine how the whipworm promotes bacterial infection. Our central hypothesis states that the whipworm prompts an anti-inflammatory immune response, thereby downregulating pro-inflammatory cytokine responses necessary for resistance to opportunistic bacterial pathogens. Turner et al. have demonstrated using a population of mixed white blood cells from *T. trichiura*-infected humans that *Trichuris* ESP elicits a different cytokine response from that elicited by *Trichuris* somatic antigens; therefore, discriminating between the effects of worm somatic antigens and ESP may prove to be beneficial in elucidating pathogenic mechanisms in trichuriasis[19]. The purpose of this thesis was to test the hypothesis that *T. suis* excretory/secretory products (TsESP) can modulate the immune system. We further hypothesized that TsESP can induce the production of TNF-α and this ability is due to an inherent component of TsESP and not to the presence of LPS in TsESP preparations.

The $CD4^+$ Th2 cytokine response, which includes the upregulation of IL-10, is associated with resistance and worm expulsion in *Trichuris* infections[12,24,26,27,31-39]. Using IL-10- and IL-4/IL-10-knockout mice of the C57BL/6 background, Schopf et al. have recently discovered that IL-10 is important in resistance to *T. muris* and in the survival of mice subject to the inflammatory process brought about by secondary bacterial infections[3]. IL-10 is also upregulated in humans and swine infected with *Trichuris*. Two different studies

have shown that IL-10 is secreted from a population of mixed white blood cells cultured from whole blood taken from *T. trichiura*-infected humans when these cells are stimulated with *T. trichiura* antigen *in vitro*[18,19]. IL-10 is elevated in experimentally infected pigs kept in confinement, as well as in swine exposed to *T. suis* on dirt lots[45].

Since IL-10 is induced in mouse, human, and swine trichuriasis and IL-10 induction also leads to resistance to endotoxic shock, the initial goal of these experiments was to investigate the ability of *T. suis* ESP (TsESP) to promote the production of an anti-inflammatory immune response (including IL-10) in mice, thereby inhibiting LPS-induced death[3,45,98]. We based our experimental design on the study conducted by Nedialkov et al. that proved *Y. pestis* V antigen was an inducer of LPS resistance[115]. (V antigen can immediately induce IL-10 in BALB/c mice as part of the innate immune response and this induction is dependent upon CD14 and the binding of V antigen to TLR2 to prevent NF-κB activation[116,125].) We hypothesized that mice injected with TsESP would be protected from LPS-induced shock and/or death.

In our experiment, mice were inadvertently administered a sublethal dose of LPS instead of a lethal dose as noted from the survival rate of the control mice injected with PBS and then challenged with LPS. The potency (endotoxin units/mg LPS) of the lot of *Salmonella* Typhimurium LPS used was very low. Therefore, the generally lethal dose of 10 μg of LPS per gram of mouse was sublethal. However, we were still able to gain insight into the immunomodulatory role of TsESP.

Even with the administration of a sublethal dose of LPS, we were able to reject our initial hypothesis that *T. suis* ESP induces anti-inflammatory cytokines in a manner similar to V antigen from *Y. pestis* and that this response would immediately protect BALB/c mice from LPS-induced death. Instead, we found that Batch 36 TsESP both exacerbated LPS-induced shock leading to death (nullifying our original hypothesis) and mimicked the phenomenon of endotoxin tolerance, depending on the amount of TsESP and the timing of administration of TsESP and LPS.

The two different actions of TsESP (both sensitizing and immunoprotective) reflect the complexity of the crude preparation of TsESP, which consists of a mixture including proteins, carbohydrates, and lipids. One possible model that could explain the varying actions of TsESP is that there are at least three different constituents of TsESP that can modulate the immune response of a mouse in response to a sublethal dose of LPS. The three components are two different sensitizing agents and one immunoprotective agent.

One sensitizing agent was apparent in low doses of TsESP (25 μg) and required greater than 2 days to take effect. All 3 mice that received 25 μg of Batch 36 TsESP daily for 7 days prior to and concomitantly with the LPS challenge suffered from increased morbidity and eventually died or were euthanized due to severe signs of shock (Table IV-1A, page 58). The immunoprotectant is also only effective when administered at least 2 days prior to the LPS. At least 50 μg TsESP was necessary to protect the mice from death

induced by concomitant injections of TsESP and a sublethal dose of LPS (Table IV-1A, page 58). This protection phenomenon mimics LPS tolerance, in which mammals are protected from LPS-induced death when they are subjected to nonlethal doses of LPS or TNF-α prior to challenge with a lethal dose of LPS[95,126,127]. The immunoprotective effect appeared to have a dominant function over the first sensitizing agent. While all mice that received 25 μg TsESP daily for 7 days prior to and concomitantly with the sublethal dose of LPS died, only 1 mouse died that received 50 μg TsESP daily for 7 days prior to and concomitantly with the sublethal dose of LPS (Table IV-1A, page 58). The second sensitizing agent acted acutely. When mice received Batch 36 TsESP and LPS concurrently, there was a positive correlation between increasing concentration of TsESP and death in mice. At least 50 μg TsESP was necessary to kill mice concomitantly injected with a sublethal dose of LPS (Table IV-1A). In conclusion, the dose necessary to act as a sensitizing or immunoprotective agent depended on the dose of TsESP, timing prior to LPS challenge, and duration of exposure to the TsESP.

All mice that were administered 100 μg TsESP at 7 or 2 days prior to the LPS challenge survived (Table IV-1B, page 58). We were not able to draw any conclusions relating to sensitizing or immunoprotective agents from this aspect of the mouse experiments because the mice did not also receive an injection of TsESP concomitantly with the sublethal dose of LPS. Future studies will incorporate administering 100 μg TsESP daily for 7 or 2 days prior to and concurrently with a sublethal dose of LPS.

All mice that received LPS developed signs of endotoxic shock, which included rough hair coats, hunched posture, diarrhea, mucopurulent ocular discharge, and a decrease in mobility. Many of the mice that died from the sensitizing agents also developed hypothermia and cyanosis in various extremities (mucus membranes, feet and tail). These clinical signs of shock developed only after LPS challenge. Mice that received 100 μg of Batch 36 TsESP once at 7 days or 2 days prior to the LPS challenge remained normal in appearance and behavior until 4 to 6 hours after the i.p. injection of a sublethal dose of LPS. Furthermore, mice injected with 25 or 50 μg Batch 36 TsESP daily for 7 or 2 days also remained free of clinical signs of shock until after the challenge with a sublethal dose of LPS. These results suggested that TsESP, alone, did not trigger signs of endotoxic shock in female, BALB/c mice.

Again, one model to explain the different activities of TsESP suggests that there are both sensitizing agents and immunoprotective agents in the TsESP. Interestingly, the contrasting activities of TsESP both indicate a possible role for TNF-α in TsESP-induced immunomodulation. TNF-α is pleiotropic and its behavior (Th1 or Th2) is dependent on the predominant cytokine milieu[3]. Its function is also dependent on timing, location, and duration of exposure to TNF-α[3]. Death resulting from endotoxic shock is associated with the acute production of excessive amounts of Th1-type cytokines, especially TNF-α[98,128]. TNF-α also has a central role in endotoxin tolerance, which is a state of hyporesponsiveness towards the effects of LPS that develops after repeated administration of sublethal doses of endotoxin or exogenous TNF-α[67,95-97,99].

Our investigation provided further support for different roles for TNF-α depending on the cytokine milieu. When TsESP and LPS were administered concomitantly, enough TNF-α may have been immediately stimulated by the presence of a sensitizing agent to have exacerbated the inflammatory capabilities of LPS, resulting in death. However, tolerance may have occurred when the immunoprotectant was administered prior to the LPS. Two possible mechanisms might account for the tolerance phenomenon; 1) BALB/c mice are generally resistant to *Trichuris* infection, and priming with TsESP may have skewed the cytokine response towards a Th2-type thereby suppressing the TNF-α-mediated pro-inflammatory effects and protecting mice from death when challenged with LPS, and 2) daily injections of TsESP at a concentration in which the immunoprotectant activity dominated may have induced enough TNF-α to produce a constant state of tolerance, so that TNF-α was not upregulated when LPS was injected. In other words, the LPS tolerance was TNF-α-induced.

The observation that TsESP can act as either a sensitizing agent or an agent that induces endotoxin tolerance depending on the dose and time of administration of TsESP in context to LPS led us to determine if an inherent component of TsESP can stimulate the secretion of TNF-α. This cytokine is known to play key roles in both sensitizing mice to LPS and inducing tolerance to LPS. Furthermore, *T. trichiura* and *T. muris* antigens, both somatic and excretory/secretory products, have been shown to stimulate production of TNF-α[19]. We confirmed that two different batches of *T. suis* ESP prepared by our laboratory (Batches 5 and 6) also has the ability to induce TNF-α secretion from

RAW 264.7 mouse macrophages *in vitro* (Figures IV-4 and IV-5, pages 64 and 65). RAW 264.7 macrophages were chosen because they are a cell line derived from mouse peritoneal macrophages, and they have been widely used in experiments involving both LPS and TNF-α[95,129-134].

The ability of TsESP to induce production of TNF-α, which could exacerbate shock or promote endotoxin tolerance depending on the situation, may also suggest that TsESP was contaminated with LPS from bacteria that reside in the gut of either the whipworms or the pig. Taylor et al. discovered that LPS from the *Wolbachia* endosymbiont of the filarial nematode *Brugia malayi* is an important mediator of the inflammatory disease associated with *B. malayi* in humans[118]. However, in this study, it is unlikely that the amount of endotoxin present in the Batch 36 TsESP (145.2 EU/ml) was sufficient to cause mortality in mice. 25 μg of Batch 36 ESP contained only 3.6 EU, while 100 μg TsESP contained only 14.5 EU. These concentrations of entotoxin are insignificant given that the experimentally administered dose of endotoxin from the LPS was 600,000 EU per mouse. Therefore the total amount of endotoxin (LPS + TsESP) administered to the mice ranged from 600,003.6 to 600,014.5 EU per mouse.

Nevertheless, to address the issue of potential LPS contamination of TsESP, Batches 5 and 6 TsESP were treated with polymyxin B before being administered to the RAW 264.7 macrophages. The data suggested that the ability of TsESP to induce TNF-α production from RAW 264.7 macrophages was due to an inherent component of ESP and not to any LPS that was present. The amount of LPS contamination in Batch 5 nor Batch 6 affected their ability to

stimulate TNF-α secretion from RAW 264.7 macrophages – the TsESP treated with polymyxin B did not significantly reduce the amount of TNF-α secreted by the macrophages (Figures IV-4 and IV-5, pages 64 and 65).

In addition to adding polymyxin B to the TsESP prior to stimulating the RAW 264.7 macrophages, we further tested for the presence of contaminating LPS in TsESP by evaluating the ability of Batches 5 and 6 to stimulate toll-like receptor 2 (TLR2) and/or TLR4 using the transfected Chinese hamster ovary cell lines 3E10 (TLR4) and 7.19/TLR2. TLR2 and TLR4 are the two receptors that have been most studied, and can be activated by various LPS moieties. Batch 5 TsESP stimulated TLR4 in the absence of polymyxin B (Figure IV-8A, page 70). However, polymyxin B abrogated the ability of Batch 5 TsESP to stimulate TLR4, indicating the presence of contaminating LPS (Figure IV-8B, page 70). Batch 6 did not stimulate TLR4 in the absence or presence of polymyxin B (Figure IV-10, page 72).

Batch 6 also did not stimulate TLR2. Interestingly, Batch 5 TsESP stimulated TLR2 in the absence and presence of polymyxin B; this stimulation may have led to the TNF-α secretion from RAW 264.7 macrophages. We expected that polymyxin B would not alter this ability because polymyxin B does not affect the outcome of CD25 expression on 7.19/TLR2 cells. Polymyxin B is specific for LPS, and the *Salmonella* Typhimurium LPS appeared not to bind and stimulate TLR2. While our data suggested that the LPS present in Batch 5 is not responsible for the stimulation of the 7.19/TLR2 cells, we could not rule out the presence of a different bacterial component in this preparation of TsESP. We

treated the 7.19/TLR2 cells with both *Staphylococcus aureus* lipoteichoic acid (LTA) and peptidoglycan and found that peptidoglycan can induce CD25 expression (data not shown). Surprisingly, the LTA did not stimulate TLR2 as expected from the literature[83]. Further studies are needed to test for the presence of peptidoglycan in Batch 5 TsESP.

MacDonald et al. suggested that TLRs are likely to recognize helminth surface glycoproteins and/or other helminth antigens[32]. It is therefore likely that TsESP can stimulate TNF-α production via a Toll-like receptor other than TLR2 or TLR4, either identified or unidentified. The behavior of Batch 6 TsESP supports MacDonald's statement. Batch 6 TsESP was shown to induce TNF-α production in macrophages without stimulating either TLR2 or TLR4 in the Chinese hamster ovary cells.

It is also possible that the TsESP could bind and stimulate other families of innate immune receptors, including L-selectin or TREM (triggering receptor expressed on myeloid cells)[66,94]. TREM-1 is a pattern recognition receptor that recognizes extracellular bacteria to promote an acute inflammatory response and shock in mice[66]. One could determine the receptor that recognizes TsESP by two different experiments. First, the 7.19 LPS-hyporesponsive cell line could be transfected with genes for known pattern recognition receptors and examined for CD25 expression. Second, antibody neutralizing experiments could be conducted on the RAW 264.7 macrophages or a human macrophage cell line inhibiting specific receptors before TsESP treatment.

The low amount of endotoxin present in the TsESP was not sufficient to explain the results of the mouse experiments; however, further studies are needed to determine if inherent components of TsESP can modulate the immune response as either a sensitizing agent or an immunoprotective agent in the absence of endotoxin contamination. The RAW 264.7 macrophages could be used as an *in vitro* screen to first determine the individual components of TsESP that induces TNF-α production. TNF-α is only one mechanism that explained the results of the mouse experiments. Nonetheless, it is a cytokine that has been extensively researched and there are many tools available to study the regulation and production of TNF-α in both *in vitro* and *in vivo* experiments.

Crude TsESP preparations were used in this thesis study. Total protein concentration was the way in which the preparations used in this study were standardized. Unfortunately, TsESP prepared from worms obtained from different pigs (different batches) exhibited differences in the amounts, combinations, and degradation products of total proteins as indicated by SDS PAGE gels (Figure I-1, page 16). For this reason, crude TsESP will not be used in future studies. There are several characterized and purified *Trichuris* ESP protein constituents that have been hypothesized to have an immunoregulatory function and may have played a role in promoting the shock-induced death or endotoxin tolerance observed in this study. These *Trichuris* products include a serine protease inhibitor, a chymotrypsin/elastase inhibitor, and an IFN-γ homologue[52-54]. These purified constituents will be the first components to be tested for their ability to induce TNF-α production using the RAW 264.7 macrophage screening assay.

The crude TsESP also contained other helminth components besides proteins, such as lipids, carbohydrates, or glycoproteins. Therefore, the TsESP constituents that induce TNF-α production may not be a protein. To determine the TNF-α-inducing components that have not been previously purified, crude TsESP will be fractionated using size columns and purified using High Performance Liquid Chromatography (HPLC). The fractions and/or purified unknown substance will then be used to treat the macrophages. To help characterize the individual components, treatments of freeze/thaw cycles, boiling, and digestions with proteases or amylases will be performed before adding the components to the macrophages for TNF-α stimulation.

The goal of these experiments was to determine the effect, if any, TsESP would have on the immune response against a challenge of LPS in female, BALB/c mice. The data suggested that TsESP can mimic the biological activities of LPS by promoting death due to shock and also producing tolerance in mice against endotoxic shock, depending on the dose and time of TsESP administration. One model that explains the contrasting functions of TsESP is that there are at least three different constituents of TsESP that can modulate the immune system of the mouse: two sensitizing agents and one immunoprotective agent. Interesting, both the exacerbation of endotoxic shock and the promotion of endotoxin tolerance can be explained as a function of the differing activities of the pleiotropic cytokine, TNF-α. Recent literature has shown that *T. muris* and *T. trichiura* ESP are capable of inducing TNF-α production; therefore, we hypothesized that the ability of TsESP to mimic endotoxin activity is due to the

ability of an inherent component TsESP to modulate TNF-α production. This hypothesis was tested by measuring TNF-α secretion from RAW 264.7 macrophages stimulated with two different batches of TsESP. TsESP stimulated the induction of TNF-α equally in the absence or presence of polymyxin B, suggesting that there is an inherent component responsible for the cytokine production. Furthermore, we have verified that this TNF-α induction is not necessarily due to the ability of TsESP to stimulate either TLR2 or TLR4. Batch 6 TsESP did not stimulate either Toll-like receptor, yet it promoted TNF-α secretion from the macrophages in the presence of polymyxin B.

In summary, this thesis demonstrated that the *Trichuris suis* excretory/secretory products modulated the immune system in the absence of the whipworm somatic antigens, and the ability to modulate the immune system is due to an inherent component of TsESP and not to the presence of LPS in the TsESP preparations.

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