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MECHANISMS OF INTESTINAL IMMUNITY TO NEMATODE PARASITES AND THE CONSEQUENCES TO INVASION BY OPPORTUNISTIC BACTERIA

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SUMMARY

Gastrointestinal nematode parasites can evoke dramatic and stereotypical changes in the intestinal milieu of the infected mammalian host. These changes may be inconsequential or result in protective immunity, pathology or an alteration in the immune response to opportunistic organisms that inhabit the intestine. The immune response is driven by the induction of a pattern of cytokines derived from $CD4^+$ helper T cells categorised as Th2. These cells, along with other T cell subsets and cells of the innate immune system, are initially stimulated by worm infections to produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. These cytokines evoke a type 2 immune response that is defined by an increase in mucosal mast cells, eosinophilia, and reaginic antibodies. There are also structural and physiological changes in the intestine that include increases in the quantity and composition of mucus secretions, a net accumulation of fluids into the lumen, smooth muscle contractility and alterations in lumenal content transit time, as well as changes in epithelial cell function and proliferation. Certain combinations of these effectors can limit parasite development or cure the host of worm infestation. This response can be quite polarised because of the additional feature of counter-regulation of some type 2 cytokines on the type 1 response. Production of IL-4 can limit the expansion of $CD4^+$ T helper cells of the Th1 type, and IL-10 can down-regulate macrophage activities that are largely responsible for expression of an IFN-γ-induced type 1 response directed at intracellular microorganisms and for delayed-type hypersensitivity responses. This interplay of cytokines can work in both directions so that intracellular parasites, bacteria and viruses that elicit a strong Th1 response can down regulate a type 2 response primarily through the growth limiting activity of IFN-γ on Th2 cells. Infections that strongly shift an immune response in one direction or another can predictably result in restricted immune flexibility that can be exploited by opportunistic infections. The current report describes changes in intestinal immunity induced by gastrointestinal nematode parasites and a spe-

cific situation where natural infection of pigs with *Trichuris suis* enhances susceptibility of colonic epithelial cells and gut-associated lymphoid tissue (GALT) in the distal colon to invasion by *Campylobacter jejuni*.

STEREOTYPICAL RESPONSE PATTERN TO GASTROINTESTINAL NEMATODE INFECTION

Mammals respond to gastrointestinal (GI) nematodes with a classical immediate-type hypersensitivity response where IgE antibody, mucosal mast cells, and tissue and blood eosinophils are markedly elevated (*Urban* et al., 1989), and the quantity and composition of goblet cell mucins are altered (*Ishiwata* et al., 1998). A general requirement for $CD4^+$ T cells in resistance to GI nematodes has been observed in several rodent models of infection (*Finkelman* et al., 1997). Neutralisation of $CD4^+$ T cells can either block worm expulsion, enhance worm fecundity by reducing immunity and inflammation or generally convert host resistance to susceptibility. Nematodes induce the development of $CD4^+$ Th2 cells that synthesise a cytokine pattern that includes IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (*Mosmann* and *Coffman*, 1989). Cytokines are pluripotent and, in the context of a parasitic infection, drive a generalised type 2 response. Production of IL-4 increases synthesis of IgE (*Finkelman* et al., 1990) and serves as a co-factor with IL-3 and IL-9 for development of intestinal mucosal mast cells (*Madden* et al., 1991); nematode-induced eosinophilia is dependent on the expression of IL-5 (*Sher* et al., 1990). Infective larvae stimulate IL-5 and IL-9 mRNA synthesis in duodenal Peyer's patch within 3 to 6 hr after per os inoculation (*Svetic* et al., 1993), but type 2 cytokine gene expression is not completely $CD4^+$ T cell-dependent until 6 days after inoculation (*Svetic* et al., 1993). Blocking of CD8 T cell function is generally without consequence in rodent models of GI nematode infections (*Katona* et al., 1991).

IL-4/IL-13 PROMOTE PROTECTIVE IMMUNITY TO GASTROINTESTINAL NEMATODES

There are shared aspects of a protective response to GI nematodes. Neutralisation of IL-4 that completely inhibits IgE production in *Nippostrongylus brasiliensis*-infected mice does not affect worm expulsion. In addition, expulsion is normal in IL-4 deficient gene knock-out (KO) mice (*Lawrence* et al., 1996), and treatment with anti-IL-4 receptor (R) mAb delays but does not interfere with expulsion (*Urban* et al., 1998a). Although IL-4 is not required for resistance to *N. brasiliensis*, it conditionally evokes a protective response. Adult *N. brasiliensis* do not self- cure in immunocompromised SCID or anti-CD4-treated mice, but the infection is cured by treatment with a long-lived formulation of IL-4-anti-IL-4 complexes (IL-4C) (*Urban* et al., 1995) which lengthens the half-life of IL-4 in vivo from <30 minutes to >24 hr (*Finkelman* et al., 1993). Blocking the IL-4R with a specific mAb prevents IL-4C-induced cure. However, IL-4 does not directly affect worms in situ because injection of IL-4C into Stat6-KO or IL-4R -chain-KO

mice, in which IL-4-induced cell signalling is decreased or absent, respectively, (*Shimoda* et al., 1996; *Takeda* et al., 1996) does not expel adult worms (*Urban* et al., 1998a). The observations that: 1) an intact IL-4R -chain and a functional Stat6 molecule is needed to activate expulsion of *N. brasiliensis*; and 2) IL-4 is not required to induce worm expulsion in immunocompetent mice, suggests that another cytokine that binds to the IL-4R -chainand activates Stat6, most likely IL-13, can also induce worm expulsion. This is supported by the fact that anti-IL-4R mAb can effectively block expulsion of *N. brasiliensis* in IL-4 KO mice, but not in normal IL-4 intact mice, and a specific antagonist for IL-13 blocks expulsion in normal and IL-4 KO mice (*Urban* et al., 1998a). In addition, IL-13 KO mice fail to efficiently clear *N. brasiliensis* adults even though a strong Th2 cytokine response is evident (*McKenzie* et al., 1998). Thus, expulsion of *N. brasiliensis* requires signalling via IL-4R -chain and Stat6, and IL-13 may be more important than IL-4 as an inducer of Stat6 signaling that precedes expulsion of *N. brasiliensis*.

Changes in the in vivo cytokine environment of the intestine of *Trichuris muris*-infected mice can reverse the outcome of an infection (*Else* et al., 1994). Chronic *T. muris* infection in susceptible mice is characterised by a predominant Th1 cytokine response pattern, but neutralisation of IFN-γ increases Th2 cytokines and the expulsion of worms. Conversely, the Th2-dominant cytokine response pattern of mice resistant to *T. muris* is reversed by treatment with anti-IL-4R mAb and results in continued worm development and a chronic infection. In addition, injection of IL-4C cures susceptible mice, while treatment of resistant mice with IL-12, during the second week of infection, induces an IFN-γ-dependent increase in host susceptibility (*Bancroft* et. al., 1997). There is redundancy in the protective response to *T. muris* because both IL-4 KO and IL-13 KO mice are susceptible to infection. (*Bancroft* et al., 1998).

The injection of IL-4C also effectively cures a chronic primary infection of mice with *Heligomosomoides polygyrus*, a nematode related to *N. brasiliensis*. Infection of immune system- deficient SCID mice is cleared less efficiently by injection of IL-4C (*Urban* et al., 1996). Protective immunity to a challenge *H. polygyrus* infection in mice previously drug-cured of a chronic primary infection is completely blocked by anti-IL-4R mAb in a way similar to the conversion of *T. muris*-resistant mice to susceptibility. IL-4 KO mice are susceptible to a secondary challenge infection with *H. polygyrus*, but the role of IL-13 in protective immunity has not been examined (*Urban* and *Finkelman*, unpublished results). Recent studies have shown, however, that Stat6 KO mice are susceptible not only to infections with *N. brasiliensis*, but also to *H. polygyrus*, *T. muris*, and *Trichinella spiralis* (*Urban* and *Finkelman*, unpublished results). Thus, there appears to be a common IL-4/IL-13-dependent protective mechanism to GI nematodes that proceeds through IL-4R -chain signalling via Stat6.

IL-4-DEPENDENT MECHANISM OF RESISTANCE TO GASTROINTESTINAL NEMATODES

Injection of IL-4C into either immunocompetent or immunodeficient mice infected with *H. polygyrus* alters the feeding behaviour of worms *in situ*. Rhodamine dye injected intravenously into mice with a primary *H. polygyrus* infection is ingested by the worm and appears in the intestine of explanted

worms viewed microscopically (*Urban* et al., 1998b). However, a single intravenous injection of IL-4C into an infected mouse greatly reduces uptake of the dye by the worm. A similar response in detected in IL-4C-treated mice infected with *N. brasiliensis.* The inhibition of feeding is probably related to the observed drop in *H. polygyrus* egg production (EPG) after IL- 4C treatment, suggesting that elevations in IL-4 can affect subtle changes in the intestine that affect worm metabolism. The mechanism remains largely unknown, but IL-4 induces changes in murine intestinal physiology that are partially mediated by products of mucosal mast cells (MMC) (*Goldhill* et al., 1997). Increased small intestinal smooth muscle contractility is observed during a secondary *H. polygyrus* challenge infection (*Goldhill* et al., 1997), but not during a primary infection when immunity is less intense. Contractility is induced in normal mice by injection with IL-4C, and blocked in mice challenged with *H. polygyrus* after injection with anti-IL-4R mAb. IL-4C-induced changes in contractility are also blocked by an inhibitor of leukotriene D_4 (LTD_4), and are not observed in SCID mice, in 5- lipoxygenase(LO)-deficient KO-mice, or in mast cell deficient W/W^v mice (*Goldhill* et al., 1998). SCID mice have virtually no gastrointestinal MMC following infection with *H. polygyrus*, but develop MMC after prolonged treatment with IL-4C (*Urban* et al., 1995). The MMC are a source of LTD_4 which is absent in 5-LO-KO mice. In addition, secondary *H. polygyrus*-challenge infection disrupts normal intestinal peristaltic activity, so that lumenal contents are evenly rather than segmentally distributed in the gut (*Goldhill* et al., 1997). However, this phenomenon does not appear to be completely IL-4-dependent. Nevertheless, IL-4-dependent changes in smooth muscle contractility can create a localised spastic action that could dislodge adult worms from their niche.

There are distinct IL-4-dependent differences in small intestine epithelial cell function during a primary *H. polygyrus* infection compared to a secondary challenge infection (*Sullivan* et al., 1998). Physiological measurements of segments of small intestine mucosa derived from mice with a secondary *H. polygyrus* infection, but not a primary infection, have significant increases in basal short circuit current (SCC) and tissue resistance, which measure net ion flux and changes in tissue permeability, respectively. Secretagogues like prostaglandin E_2 (PGE_2) enhance SCC in mucosa derived from mice infected with a secondary but not primary *H. polygyrus* infection. Similar changes in the response to PGE_2 were observed in normal mice treated with IL-4C over a 6 day period. In addition, injection of anti-IL-4R mAb at the time of a secondary challenge infection blocked *H. polygyrus*-induced changes in SCC, resistance, and responses to PGE_2. Thus, IL-4 appears to mediate increases in ion flux, decreased tissue permeability, and responses to secretagogues during a secondary infection with *H. polygyrus*. These results were substantiated by the observation that IL-4-KO mice, which are relatively susceptible to a secondary infection with *H. polygyrus* (*Shea-Donohue*, *Urban* and *Finkelman*, unpublished results), have unaltered SCC, resistance, and responses to secretagogues when challenge-infected with *H. polygyrus* (*Sullivan* et al., 1998). In addition, primary and secondary infections of mice with *H. polygyrus* and a single injection of IL-4C into mice with a primary *H. polygyrus* infection decrease glucose absorption. Thus, there are significant physiological differences in the host small intestine responding to a primary and secondary in-

fection with *H. polygyrus* that are attributable to IL-4. The ability of IL-4 to augment both intestinal secretion and contractility suggests that IL-4 can alter the concentration of critical nutrients in the intestinal lumen upsetting the microenvironment and provide host effectors that interfere with the metabolism and/or attachment of the infective organism (*Finkelman* et al., 1997).

THE COUNTER-REGULATORY PROPERTIES OF TH1/TH2 CYTOKINES CAN MODULATE IMMUNITY DURING AN INFECTION

A polarised cytokine response would inherently down regulate the reciprocal Th1/Th2 cytokine pattern (*Pearce* et al., 1991; *Acton* et al., 1993; *Scott* and *Kaufman*, 1991). Therefore, the cytokine status of the host could influence the process of active immunisation or immunity to an infection. Induction of a strong Th1 response would likely interfere with resistance to GI nematodes that are sensitive to Th2-dependent protective mechanisms. This was demonstrated experimentally when mice treated with exogenous rIL-12 (*Finkelman* et al., 1994), rIFN-γ or rIFN-α (*Urban* et al., 1993) became susceptible to *N. brasiliensis*. The stereotypical type 2 cytokine gene expression pattern was completely reversed by treatment with IL-12 via an IFN-γ-dependent down-regulation. As a result, there is a general decrease in effectors related to type 2 cytokines and an inhibition of the protective response. Once IL-12 treatment is terminated during the course of the infection, however, expulsion of worms follows after a period of increasing type 2 cytokine gene expression. Endogenous activators of IFNs can also down-regulate type 2 responses in *N. brasiliensis*-infected mice and temporarily inhibit parasite expulsion. Injection of mice with killed-*Brucella abortus* cells (*Urban* et al., 1993) or inoculation with live *Eimeria ferrisi* oocysts, a natural protozoan parasite of mice that invades the intestinal mucosa, will temporarily block parasite expulsion (*Urban* et al., 1993, 1996). This effect is reversible by neutralisation of IFN with specific mAb.

Worm-induced polarisation towards type 2 responses and down-regulation of Th1 immunity may be significant to both human and animal populations that have a propensity to acquire chronic worm infections. A normal in vitro Th1-like response to tetanus toxoid (TT) of PBMCs from vaccinated humans is more Th2-like when the PBMCs come from individuals infected with *Schistosoma mansoni* (*Sabin* et al., 1996). The amount of TT specific IFN-γ produced by PBMC from *S. mansoni*-infected individuals decreases inversely with the intensity of the infection compared to the response of PBMC from uninfected, TT-vaccinated controls. These results suggest that the type 2 milieu created by a *S. mansoni* infection affects the direction of a response to a non-parasite related antigen. Therefore, the parasite status of an individual may be of considerable importance in determining the cytokine pattern one is likely to achieve when an individual is exposed to a subsequent infection or to toxic materials.

The skewing of a cytokine pattern in response to an infectious agent could also have severe consequences when complex disease interactions exist. *Trichuris suis* in pigs interact with resident bacterial flora in the colon to induce mucohaemorrhagic enteritis (*Mansfield* and *Urban*, 1996). When the infected pigs are maintained in standard confinement housing indoors, they have a

parasite-induced 10-100 fold increase in IL-10 gene expression in the MLN draining the site of infection, but no increase in IL-12 gene expression (*Mansfield* et al., 1998). This is similar to the *T. muris*- and *H. polygyrus*-induced increases in Th2 cytokine gene expression that follows inoculation of mice (*Svetic* et al., 1993; *Else* et al., 1994). It is also of interest that increases in IL-12 gene expression in the MLN of *T. suis*-infected pigs are only observed when secondary bacterial invasion in colonic epithelial cells and GALT are evident (*Mansfield* et al., 1998). Interleukin-12 is induced as a consequence of infection with intracellular parasites and by exposure to microbial-derived products (*Biron* and *Gazzinelli,* 1995). Stimulation of IFN-γ synthesis by IL-12 leads to activation of macrophage accessory cell function through elevated expression of class II MHC antigen and the production of microbicidal products such as H_2O_2, proteases, and nitric oxide. This process can also activate non-immune cells such as intestinal epithelial cells to express cell surface ß$_2$-integrins that allow neutrophil adhesion and class II MHC molecules to mediate interactions with $CD4^+$ T-cells (*Colgan* et al., 1994). In contrast, IL-10 down regulates the production of inflammatory cytokines by mononuclear phagocytes and neutrophils and reduces or prevents macrophage activation by IFN-γ (*Cassatella* et al., 1994).

SPECIFIC INTERACTIONS BETWEEN *T. SUIS* AND *C. JEJUNI*

Confinement reared pigs inoculated with *T. suis* eggs exhibit diarrhoea, and mucosal oedema, inflammatory cell infiltration, and bacterial accumulation at the site of worm attachment in the proximal colon. There is also localised thickening of the muscularis and mucosa at the site of worm attachment, destruction of the absorptive cells on the surface of the colon, crypt destruction with loss of goblet cells, and an increase in inflammatory cells in the lamina propria. Bacteria are detected by Warthin-Starry stain in close proximity to adult worms. Notable also is the appearance of severe bacterial lesions in the lymphoglandular complexes (LGC) in the distal colon far from the site of worm attachment. Bacterial isolates from the LGC include *C. jejuni, C. coli, C. lari,* as well as single isolates of *Escherichia coli, E. fergusonii, Enterobacter intermedium, E. cloacae, Pseudomonas fluorescens,* and *Lawsonia intracellularis.* The most prominent change in LGC from *T. suis*-infected pigs is an increase in size. The LGCs are enlarged partly due to an increase in cells in germinal centres of the LGC nodule which include lymphocytes, macrophages, and neutrophils surrounded by eosinophils. There is a follicle-associated membrane overlying the follicle that contains cells with M cell morphology. Some of the bacteria in the LGCs are within the entrapped mucosal crypts that appear as crypt abcessation with purulent debris. Bacteria are in the submucosa and muscularis below the follicle suggesting the LGC is a route of invasion and dissemination of pathogenic organisms. Bacteria with morphology consistent with *C. jejuni* are within M cells of follicle-associated epithelium. No pathogenic bacteria were isolated from the tissues of pigs that were not *T. suis*-infected, although many of these pigs had *C. jejuni* in the stool with no clinical signs of disease. The severity of *T. suis*-induced mucohaemorrhagic enteritis is inhibited by broad spectrum antibiotics or by anthelmintic clearance of the worm infec-

tion (*Mansfield* and *Urban*, unpublished results). These observations demonstrate that *T. suis* alters conditions proximal and distal to worm attachment in the colon that affect susceptibility to opportunistic bacteria.

Supporting this concept are recent observations made in gnotobiotic pigs inoculated with either *T. suis* or *C. jejuni* alone or in combination (*Mansfield* et al., manuscript in preparation). Pigs derived germ-free by caesarean section of sows were placed in germ-free incubators and treated as four groups:

1) untreated and uninfected,
2) inoculated with *T. suis* eggs at 250 eggs/kg body weight,
3) inoculated with 10^6 colony forming units of log phase *C. jejuni* (ATTC strain 33292 isolated from a human with enteritis), or
4) inoculated with both *T. suis* eggs and *C. jejuni*.

Pigs with combined infections had significantly more frequent and severe signs of diarrhoea. All pigs inoculated with *C. jejuni* had transient fever, depression and diarrhoea for 24 hr after inoculation, and shed *C. jejuni* in the stool. However, severe clinical signs and pathology were present only in the colon of pigs inoculated with both *T. suis* and *C. jejuni*. In addition, only pigs inoculated with both *T. suis* and *C. jejuni* had bacterial invasion in the colonic epithelium and the epithelial cells of the follicles associated with LGCs in the distal colon. Macrophages stained immunohistochemically in the LGC also stained for intracellular *C. jejuni* with a specific stain suggesting that the macrophages were invaded by *C. jejuni*.

POTENTIAL MECHANISMS OF *T. SUIS*-FACILITATED *C. JEJUNI* COLONISATION OF PIG INTESTINAL CELLS

Campylobacter jejuni are attracted to an infection site, penetrate the mucus layer, and associate with the base of the crypt or adhere to the mucosal surface to initiate colonisation and infection (*Wallis*, 1994). *Campylobacter spp*. are opportunistic pathogens that multiply in the gastrointestinal tract of their hosts when immune defences are compromised (*Bernard* et al., 1989, *Sorvillo* et al., 1991). Pathogenicity of the organism is dependent on virulence traits and host susceptibility is secondary to agents that disrupt the host immune response or change the microenvironment of the mucosal surface. Nematodes are clearly able to modulate the immune response and alter the microenvironment with consequences that could affect bacterial colonisation. Penetration of the intestinal epithelial barrier by *C. jejuni* is dependent on adhesins binding to host substrates. Flagella (*McSweegan* and *Walker*, 1986; *Szymanski* et al., 1995), as well as other bacterial structures that bear adhesins (*Ofek* and *Doyle*, 1994) may be involved in the adhesive process. Receptors for bacterial adhesins are found on animal cell membranes (*Ofek* and *Doyle*, 1994) as well as extracellular matrix molecules including fibronectin, laminin, and collagens (*Gravis* et al., 1997). Glycoconjugates associated with glycoproteins and/or glycolipids can serve as receptors for bacterial adhesins. Nematode secretions that are important to parasite feeding and invasion mechanisms could modify bacterial adhesin/receptor interactions. Both a metalloprotease and cysteine protease has been identified in adult *T. suis* (*Hill* et al., 1993; *Hill* and *Sakanari*, 1997), and the metalloprotease is implicated in tissue breakdown during worm invasion of the mucosa. A serine protease inhibitor from secretions of *T. suis* has been shown to inhibit trypsin, chymotrypsin and elastase that

could alter the extracellular matrix during worm development (*Rhoads* et al., personal communication). Secreted products from *T. suis* (*Abner* et al., 1998), and a related parasite *Trichinella spiralis* (*Butcher* et al., 1998), have been shown to affect epithelial cell proliferation, and possibly enhance endocytosis or membrane permeability. Peroxidation of epithelial cell membrane lipids has been associated with host protective mechanisms against parasites in the gut, while parasite-derived catalase, glutathione reductase, and superoxide dismutase salvage oxygen radicals produced at the site of infection. Adhesive substances have recently been identified from nematodes that could facilitate epithelial cell attachment and invasion (*Maruyama* and *Nawa*, 1997). The nematode adhesins are heavily glycosylated proteins that have an affinity for mast cell proteoglycans like heparin and sulphated mucins secreted by goblet cells (*Ishiwata* et al., 1998). Changes in the sugar side chains of mucins present during the expulsion of *N. brasiliensis* suggest both a qualitative as well as quantitative alteration in mucus production coincident with goblet cell hyperplasia (*Oinuma* et al., 1995). Parasite-induced changes in intestinal epithelial cell permeability and especially the accumulation of glucose and other nutrients in the lumen could affect the adhesin quality of commensal bacteria. The impact of parasite product-induced changes of the intestinal microenvironment on bacterial adhesins and host receptor sites could be an intriguing target of facilitated bacterial adherence and invasion, especially at the site of worm attachment in the intestine.

Invasion of LGCs by *C. jejuni* distal to worm attachment sites in the proximal colon is more easily explained by immune modulation of GALT in the intestinal mucosa. Increases in pig IL-10 gene expression resulting from *T. suis*-induced stimulation of mucosal immunity maybe indicative of a more typical Th2 pattern (*Svetic* et al., 1993) that includes IL-4-induced changes in intestinal physiology (*Goldhill* et al., 1997) and down regulation of IFN-γ-dependent responses to intracellular organisms. It is therefore of considerable interest that macrophages in the LGCs of gnotobiotic pigs inoculated with both *T. suis* and *C. jejuni* were invaded by bacteria (*Mansfield* et al., 1998).

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