



Heligmosomoides polygyrus infection : the role of cytokines in regulation of mast cell development
by Luanne Hall-Stoodley

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in
Microbiology

Montana State University

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Abstract:

Bone marrow cultured mast cells (BMCMC) develop from bone marrow cells when cultured with conditioned medium (CM) made from splenocytes stimulated with Concanavalin A. BMCMC also develop when cultured with CM made from the spleens of mice infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*. These nematode infections induce an intestinal mastocytosis in vivo. In contrast, CM made from the spleens of mice infected with *Heligmosomoides polygyrus* (HpCM) fails to support the development of BMCMC. This nematode has been characterized as being a poor inducer of intestinal mastocytosis.

It is hypothesized that parasites induce two disparate immune responses in the host. One predisposes the host to a predominantly cell mediated immune response. The second predisposes the host to a predominantly antibody mediated response to the pathogen. These effector mechanisms appear to be regulated by distinct subsets of helper T cells called TH1 and TH2 subsets, respectively. T cell subsets produce mutually exclusive assortments of cytokines which function in the regulation of several immune responses.

One hypothesis that explained the inability of HpCM to support the development of BMCMC was that HpCM lacked the appropriate cytokines required for mast cell growth and differentiation. We hypothesized that HpCM lacked mast cell stimulatory cytokines and perhaps contained cytokines that could inhibit BMCMC development due to the expansion of TH1 cells.

Investigation of this hypothesis showed that HpCM contained very high concentrations of the mast cell stimulatory cytokines IL-3 and IL-4. Nevertheless HpCM had the ability to inhibit BMCMC when mixed with CM that normally supports BMCMC development. Cytokine removal from HpCM using anti-cytokine antibody treatment revealed that the removal of IL-4 from HpCM abrogated mast cell developmental inhibition. Since IL-4 has been characterized as only having stimulatory activity for mast cell development, this result is intriguing and may suggest a role for IL-4 in a negative feedback mechanism for mast cell development.

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A thesis submitted in partial fulfillment
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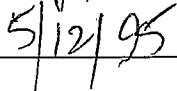
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ABSTRACT

Bone marrow cultured mast cells (BMCMC) develop from bone marrow cells when cultured with conditioned medium (CM) made from splenocytes stimulated with Concanavalin A. BMCMC also develop when cultured with CM made from the spleens of mice infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*. These nematode infections induce an intestinal mastocytosis *in vivo*. In contrast, CM made from the spleens of mice infected with *Heligmosomoides polygyrus* (HpCM) fails to support the development of BMCMC. This nematode has been characterized as being a poor inducer of intestinal mastocytosis.

It is hypothesized that parasites induce two disparate immune responses in the host. One predisposes the host to a predominantly cell mediated immune response. The second predisposes the host to a predominantly antibody mediated response to the pathogen. These effector mechanisms appear to be regulated by distinct subsets of helper T cells called TH1 and TH2 subsets, respectively. T cell subsets produce mutually exclusive assortments of cytokines which function in the regulation of several immune responses.

One hypothesis that explained the inability of HpCM to support the development of BMCMC was that HpCM lacked the appropriate cytokines required for mast cell growth and differentiation. We hypothesized that HpCM lacked mast cell stimulatory cytokines and perhaps contained cytokines that could inhibit BMCMC development due to the expansion of TH1 cells.

Investigation of this hypothesis showed that HpCM contained very high concentrations of the mast cell stimulatory cytokines IL-3 and IL-4. Nevertheless HpCM had the ability to inhibit BMCMC when mixed with CM that normally supports BMCMC development. Cytokine removal from HpCM using anti-cytokine antibody treatment revealed that the removal of IL-4 from HpCM abrogated mast cell developmental inhibition. Since IL-4 has been characterized as only having stimulatory activity for mast cell development, this result is intriguing and may suggest a role for IL-4 in a negative feedback mechanism for mast cell development.

INTRODUCTION

Mast cell growth and differentiation

Mast cells play a pivotal role in inflammation, particularly in immediate-type hypersensitivity disorders such as hayfever, certain types of asthma and anaphylaxis. As a prototypic secretory cell, mast cells contain numerous preformed mediators, including histamine, serotonin, proteoglycans and neutral proteases. These mediators are released upon activation of the cells, either by IgE-dependent or IgE-independent mechanisms. In addition to preformed mediators, several inflammatory mediators are synthesized *de novo* upon activation of mast cells. These include several membrane lipid metabolites such as prostaglandins and leukotrienes. Several of these mediators (namely the leukotrienes LTC₄, LTD₄, and LTE₄) induce long-term bronchial constriction and can lead to serious respiratory perturbation, particularly in asthma. Mast cells are also capable of secreting several cytokines that modulate the inflammatory response including: IL-1, IL-3, IL-4, IL-5, IL-6, GM-CSF and TNF- α (47,59,99). The release of cytokines upon mast cell activation suggests that mast cells play a role in the recruitment of leukocytes, thereby modulating the progression of the inflammatory response (44,46). Like other myeloid cells types, mast cells develop

from multipotent hemopoietic progenitors in the bone marrow (24,26,63). However, unlike other granulated myeloid cell types such as neutrophils, eosinophils and basophils, mast cells leave the bone marrow as undifferentiated progenitors, circulate in the blood and penetrate tissues before differentiating and proliferating (62). Mature mast cells then reside in tissue for weeks or months, compared with a life span of a few days for most other granulocytes (44).

Because mast cells are not terminally differentiated cells, they exhibit considerable morphological and biochemical heterogeneity depending upon which tissue they occupy (29,43,62). Mast cells are divided into two phenotypes; mucosal mast cells (MMC) and serosal or connective tissue mast cells (CTMC). This classification is based upon histological, biochemical and morphological criteria. Specifically, MMC are found in the gastrointestinal tract of mice and rats and contain chondroitin sulfate proteoglycans but lack heparin. CTMC, on the other hand, develop in skin and the peritoneal cavity and contain heparin but not chondroitin sulfate.

In an experimental system using mice infected with intestinal helminths, it was determined that MMC are T cell dependent while CTMC appear to be T cell independent (29). For example, nude mice possess CTMC, but fail to exhibit mucosal mastocytosis in response to helminth infection unless donor T cells are administered prior to infection

(55,91).

Much of what is known about the growth and differentiation of mouse mast cells comes from *in vitro* culture systems. These systems require a continuous supply of medium conditioned by mitogen-stimulated lymphoid cells (CM) to promote mast cell development, and it has been determined that the fundamental mast cell growth and differentiation factor present in CM is IL-3 (51,56). Cells developing from bone marrow cells cultured in a liquid suspension of IL-3-containing CM differ biochemically from CTMC and more closely resemble MMC (110). However, if bone marrow cultured mast cells (BMCMC) are co-cultured with 3T3 fibroblasts, the cultured mast cells have phenotypic characteristics consistent with CTMC (65). These results suggest either that BMCMC and MMC are relatively immature mast cells capable of differentiating further into CTMC or that mast cells have the ability to undergo "bidirectional transdifferentiation" (29,58).

There is, in fact, experimental evidence for each possibility. Nakano *et al.* (77) showed that BMCMC injected into different anatomical compartments in congenitally mast cell deficient W/W^v mice could give rise to either MMC or CTMC depending on the site of injection. This group also found that injection of a CTMC cell type into the gastric mucosa of W/W^v mice could elicit MMC (109). Thus, there is evidence that the tissue microenvironment plays an important

role in the maturation pathway of mouse mast cells *in vivo*.

The regulatory role exerted by the tissue microenvironment upon mast cell phenotypic differentiation is most likely mediated by cytokines. Mucosal mast cells are T cell dependent and can be induced in nude mice by repeated injection of IL-3 (1). Hamaguchi *et al.* (52) found that while CTMC are considered to be T cell independent, their development *in vitro* is considerably enhanced by both IL-3 and IL-4. The presence of both cytokines increases the number of CTMC in culture ten-fold compared with IL-3 alone. To date, several cytokines have been shown to act as mast cell growth and differentiation factors besides IL-3 and IL-4. These include: IL-9 (53), IL-10 (113), nerve growth factor (NGF) (72), and stem cell factor (SCF) or *c-kit* ligand (KL) (45). While IL-4, IL-9 and IL-10 synergize with IL-3 to enhance the mast cell proliferative effect of IL-3, NGF and SCF apparently promote a phenotypic switch from MMC to CTMC (44).

Intestinal nematodiasis has been extremely useful in studying the mucosal mast cell response, since MMC are observed in the intestine of humans or animals soon after infection with certain nematodes. For example, *Nippostrongylus brasiliensis* and *Trichinella spiralis* each induce pronounced increases in the numbers of mast cells in the intestinal tissue of rats and mice (4,51,54,66). Both nematodes also elicit a strong spontaneous cure response

characterized by the rapid expulsion of adult worms from the intestine within a few weeks of infection. After expulsion, the animals are refractory to challenge infections of *N. brasiliensis* or *T. spiralis*, respectively.

In contrast to these parasites, another intestinal nematode, *Heligmosomoides polygyrus*, has been widely characterized as a poor inducer of mucosal mastocytosis in several mouse strains (27,28,93). *H. polygyrus* produces a chronic infection in the mouse. Furthermore, studies of concomitant infections with *H. polygyrus* and *T. spiralis* or with *H. polygyrus* and *N. brasiliensis*, have demonstrated that *H. polygyrus* can depress the mast cell accumulation normally present in infections of the two latter nematodes alone (8,27,28). However, Behnke et al. (9) found that if infection with *H. polygyrus* coincides with the mucosal inflammatory response already elicited by *T. spiralis*, the majority, but not all, of *H. polygyrus* adults are eliminated. This observation suggests that at least NIH and C57BL/10 mice (moderate and low responder strains to *H. polygyrus* infection, respectively) have the capacity to eradicate *H. polygyrus*, but fail to do so presumably because of an immunomodulatory effect exerted by the worm. Behnke et al. propose that chronic infection with *H. polygyrus* is facilitated by the nematode's inhibition of host inflammatory effector cells such as mast cells.

In an *in vitro* system designed to examine more

thoroughly the mechanism of mucosal mast cell hyporesponsiveness in *H. polygyrus*-infected mice, Reed *et al.* cultured bone marrow cells with conditioned medium made from the spleens of either *T. spiralis*-infected mice (TsCM) or spleens from *H. polygyrus*-infected mice (HpCM) (93). Bone marrow cells cultured with TsCM produce cultures containing more than 95% mast cells by the end of the culture period. In marked contrast, when bone marrow cells are cultured with HpCM, less than 1% of the cells are mast cells by the end of the culture period. These results suggest that *H. polygyrus* might be a useful tool for investigating the mechanisms involved in the development of mucosal mastocytosis.

Cytokines and the T helper subset model

In 1986 Mosmann and Coffman (75) subdivided the CD4 T cell subpopulation, based on the differential secretion of cytokines from T cell clones. They found that some clones (designated TH1 cells) secreted IL-2, IFN- γ and TNF- β (lymphotoxin). These clones do not secrete IL-4, IL-5 or IL-6. Other clones (designated TH2 cells) exhibit the opposite cytokine secretion pattern, secreting IL-4, IL-5 and IL-6, but not IL-2, IFN- γ or TNF- β . Both types of clones secrete IL-3 and GM-CSF. This dichotomy, based on cytokine secretion patterns of cloned CD4 T cells, corresponds to a functional dichotomy that separates mature

T helper clones (42). TH1 cells are associated with delayed type hypersensitivity reactions and the development of cytolytic T lymphocytes (cell-mediated immune responses). TH2 cells, on the other hand, are associated with B cell proliferation and antibody production, especially IgE and IgG1 antibody production (humoral immune responses). Further analysis of CD4⁺ splenocytes, however, identified other subsets (designated TH0 cells). These cells were identified when clones were derived from antigen-primed lymphocytes and are characterized by the secretion of either IL-2, IL-4 and IL-5 (TH0-A) or IL-2, IFN- γ and IL-4 (TH0-B) (42). It is hypothesized that TH0 cells are TH1 and TH2 precursors that have not yet differentiated into mature cells. Other TH2 cytokines now include IL-9, IL-10 and IL-13. Although no new cytokines have been found to be secreted from TH1 cells, IL-12 has been identified as an important regulatory cytokine in promoting the development of TH1 type cells (114).

While to date no surface antigens (analogous to the CD4 and CD8 antigens) have been definitively identified with TH1 or TH2 subsets, these subset designations have gained widespread acceptance. The major reason for this acceptance has been the efficacy of the TH subset model in predicting

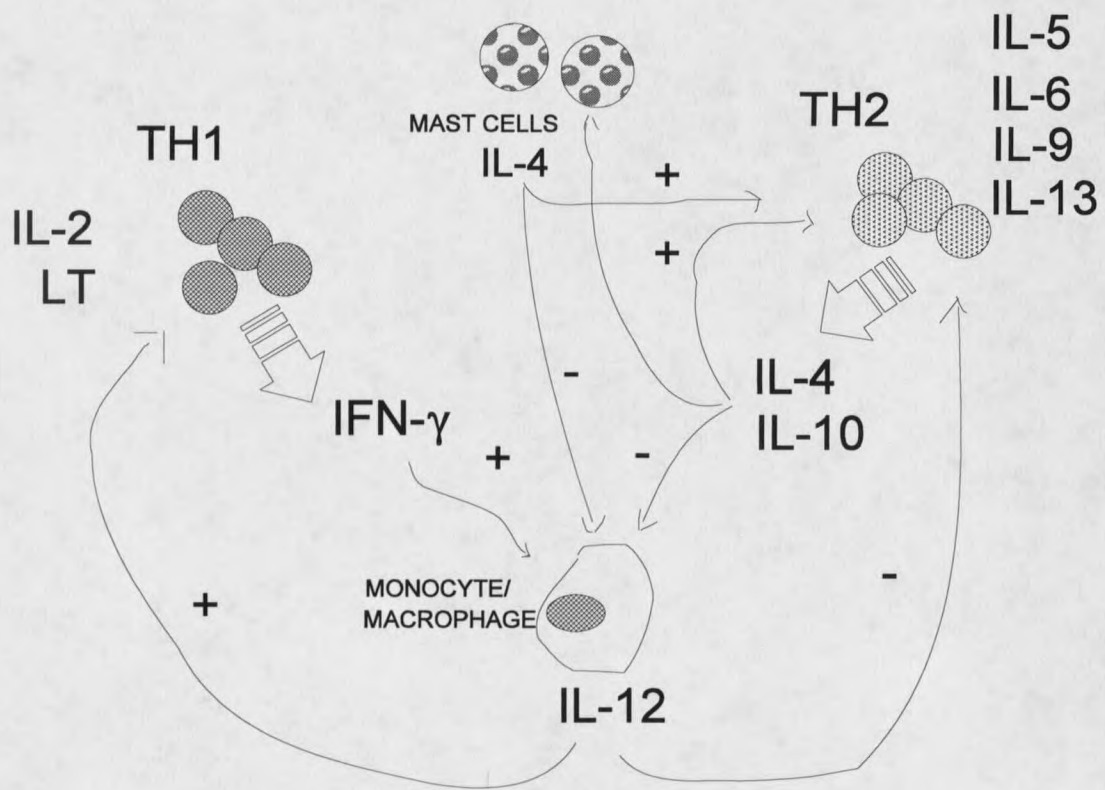


Figure 1. TH subsets and typical cytokine secretion profiles. Arrows and +/- symbols indicate positive and negative feedback mechanisms, respectively.

whether certain infections are associated with a susceptible or resistant pattern of host response. The best characterized infection involving cytokine analysis is *Leishmania major* infection in mice.

Analysis of CD4 T cells in mouse strains that display chronic lesions (the susceptible phenotype) and those that resolve infection with *L. major* (the resistant phenotype) show a distinct dichotomy (101). CD4 cells from susceptible BALB/c mice express higher levels of IL-4 mRNA and low levels of IFN- γ message. In contrast, resistant C57BL/10 mice express low levels of IL-4 and higher levels of IFN- γ transcripts. T cells from susceptible and resistant strains display the same dichotomy of cytokine secretion profiles when restimulated with antigen *in vitro*: susceptibility correlates with increased IL-4 secretion and resistance correlates with increased IFN- γ secretion from these cells. Ultimately it was observed that administration to susceptible mice of IFN- γ or of anti-IL-4 neutralizing antibody results in a switch to a TH1 type response, the healing of *L. major*-induced lesions, and disease remission. Administration of anti-IFN- γ neutralizing antibody to resistant mice has the opposite effect; a switch to a TH2 type response, the development of chronic lesions, and progression of the disease.

The association of disease susceptibility and resistance with specific TH subsets and their respective

cytokines has been observed in several other parasite models in mice and humans. TH1 cytokines correlate with immune responses to intracellular parasites such as *Listeria*, *Mycobacterium*, and certain viruses. TH2 cytokines, on the other hand, are associated with resistance to large, extracellular parasites such as *Trichuris muris*, an intestinal nematode (31,32). Furthermore, the TH1 cytokine IFN- γ interferes with both inflammatory responses and protective immunity to *N. brasiliensis* (118).

Specific TH1 and TH2 cytokines also appear to be important in cross-regulation of the TH1/TH2 response (figure 1). For example, the TH1 cytokine IFN- γ down-regulates the TH2 response by inhibiting the proliferation of TH2 cells (40). Furthermore, IFN- γ activates macrophages, which then release IL-12. IL-12 subsequently induces the proliferation of TH1 cells. The TH2 cytokine, IL-10 appears to decrease the production of both IL-2 and IFN- γ by TH1 cells. This effect is mediated via antigen presenting cells (APC) (38). IL-10 has also been shown to suppress both the release of TNF- α and reactive oxygen intermediates by activated macrophages (14,37). Without high levels of IFN- γ , TH2 cell development is favored. The presence of IL-4, another TH2 cytokine, promotes the development of CD4 clones into TH2 cells. IL-4 inhibits IL-12 production by macrophages, thereby effectively decreasing the amount of IFN- γ present and favoring

development of TH2 cells.

The hypothesis

In the research described herein we sought to explain the failure of mast cells to develop in bone marrow cell cultures grown in the presence of conditioned medium made from the spleens of mice infected with *H. polygyrus*. By using an *in vitro* culture system, the *in vivo* observation that *H. polygyrus* interferes with mucosal mastocytosis could be investigated. The hypothesis examined in this thesis is that *N. brasiliensis* and *H. polygyrus* each cause contrasting imbalances of TH1 and TH2 subsets. It is proposed that *N. brasiliensis* induces a predominantly TH2 immune response and *H. polygyrus* induces a predominantly TH1 immune response. Therefore, the cytokines IL-4 and IL-10 needed for mast cell differentiation and proliferation are expected to be abundant in conditioned medium made from the spleens of *N. brasiliensis*-infected mice (NbCM) (figure 2). IFN- γ a known inhibitor of TH2 cell development as well as mast cell development, is expected to be present in low levels. In contrast, the TH1 cytokines IFN- γ and IL-2 should be abundant in conditioned medium made from the spleens of *H. polygyrus*-infected mice (HpCM). Therefore mast cell development would not be favored due to the inhibitory effects of IFN- γ and the lack of TH2 cytokines. IFN- γ would directly inhibit mast cell development from progenitor cells

and indirectly inhibit mast cell development by down-regulating TH2 cells, thereby reducing the concentrations of mast cell proliferative cytokines like IL-4 and IL-10.

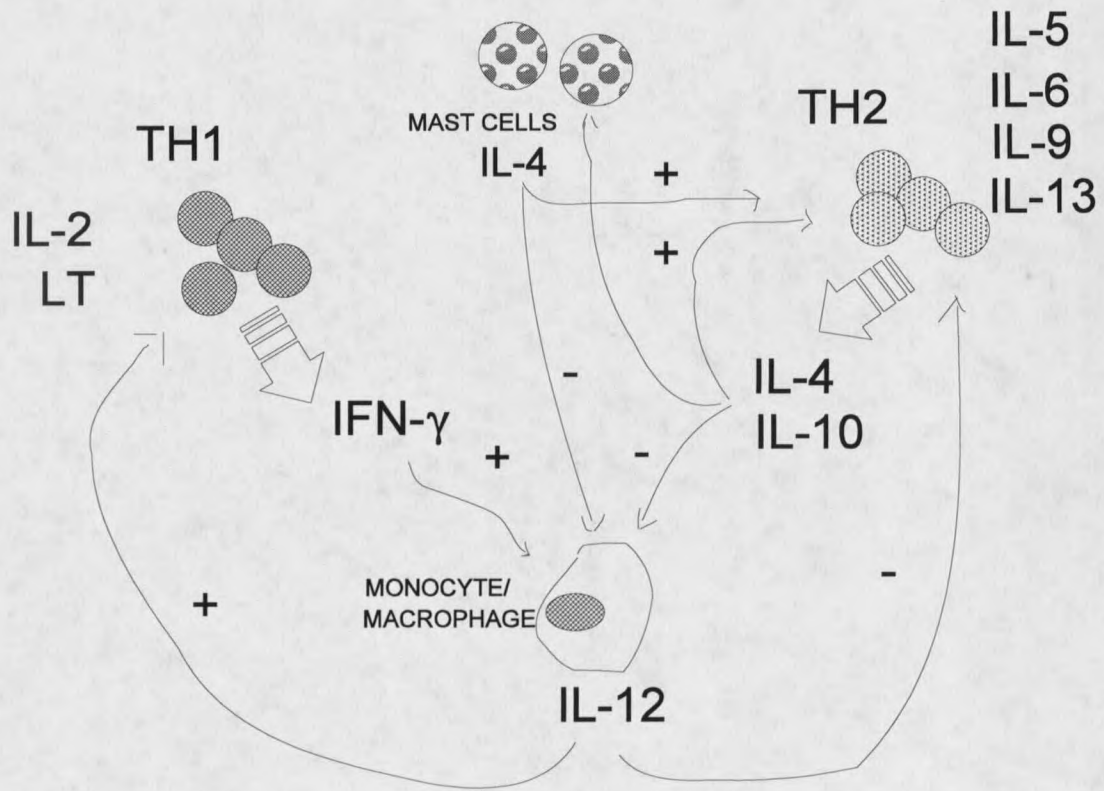


Figure 2. Diagram summarizing the hypothesis. *N. brasiliensis* infection is expected to induce the expansion of TH2 cells during infection. Conditioned medium made from the spleens of these mice is expected to be mastopoietic. In contrast, *H. polygyrus* infection, is expected to induce the expansion of TH1 cells during infection. CM made from the spleens of *H. polygyrus*-infected mice is expected to not support mast cell development.

MATERIALS AND METHODS

Animals

BALB/cBy female mice (8-12 weeks) were obtained from the MSU Animal Resources Center (ARC). Mice were routinely infected at 8 weeks of age and were used up to 13 weeks of age. CD-1 mice were used as a source for *Heligmosomoides polygyrus* eggs and BALB/cMSU *nu/nu* mice were used as a source for *Nippostrongylus brasiliensis* eggs. Both mouse strains were acquired from the MSU ARC. SJL and C57BL/10 mice were purchased from Jackson Laboratories, Bar Harbor, ME, and infected at 8 weeks of age.

Parasites

H. polygyrus L₃ larvae were obtained initially from the laboratory of D. Wakelin, University of Nottingham, Nottingham, England. *H. polygyrus* L₃ larvae were routinely cultured in our laboratory from eggs obtained from the feces of infected CD-1 source mice (19). Briefly, feces were collected and strained through double-layered cheesecloth and eggs were allowed to settle for 4 hr. The sediment was centrifuged and spread thinly over Whatman filter paper (No.1) supported by an inverted watch glass centered in 150 X 15 mm culture plates containing enough distilled water

(dH₂O) to keep the filter paper moist. L₃ stage larvae were collected after 168-200 hr of culture at room temperature. After collection, larvae were stored at 4°C for up to 6 weeks. Viability was monitored by observing motility in greater than 95% L₃ when quantifying larvae for inoculation into mice. The larvae were suspended in fresh dH₂O and stirred with a small stirring bar while three 50 µl aliquots were removed and quantified. A dose containing a suspension of 400 L₃ *H. polygyrus* in a volume of 0.2-0.3 ml was administered *per os* using a blunt ended feeding needle attached to a disposable 1 cc B-D syringe.

N. brasiliensis was obtained initially from J. Urban, USDA, Beltsville, MD. The third stage larvae were cultured from eggs collected in fecal pellets from *N. brasiliensis*-infected nude mice. The fecal pellets were stirred with a small amount of dH₂O and animal bone charcoal (BDH Chemicals Ltd., Poole, England) and placed in 15 X 100 mm culture dishes at room temperature. After 12-14 days of culture, the L₃ larvae were ready to harvest.

N. brasiliensis L₃ larvae were harvested by placing the charcoal culture material into a glass funnel fitted with plastic tubing and a clamp containing 0.85% saline warmed to 37°C. The larvae were allowed to migrate downward to the mouth of the tubing for 45 min and were collected in a small beaker, stirred with a stir bar and quantified as described for *H. polygyrus* larvae. The suspension of *N. brasiliensis*

L₃ larvae was adjusted so that 600 L₃ were administered in a single 0.2 ml dose injected subcutaneously behind the neck.

Preparation of Spleen Cell Conditioned Media

RPMT-1640 with bicarbonate was supplemented with 2 mM glutamine, 0.1 M sodium pyruvate, 10 mM HEPES, 7.5×10^{-5} M monothioglycerol, 100 U/ml penicillin and 100 µg/ml streptomycin (93). Fetal bovine serum (FBS) was added to 10% v/v. Spleens from uninfected (normal), *H. polygyrus*-infected or *N. brasiliensis*-infected mice were aseptically removed and disrupted through wire screens and dispersed through a 27 gauge needle to obtain a single cell suspension. Cells were placed on ice while viable cells were determined with fluorocein diacetate (FDA). Splenocytes were adjusted to 5×10^6 viable cells/ml and Concanavalin A (Type IV, Sigma) was added to a final concentration of 5 µg/ml. After 40-44 hr of incubation at 37°C in a 5% CO₂ humidified atmosphere, the supernatant was harvested by centrifugation (240 x g) for 10 min at 4°C. Conditioned media (CM) made from uninfected normal mice (NCM), *H. polygyrus*-infected mice (HpCM) or *N. brasiliensis*-infected mice (NbCM) were then filtered through 0.22 µm membranes and stored at -70°C until used in bone marrow cell cultures or ELISA's.

Bone marrow cell cultures

Bone marrow cells were collected from the femurs of BALB/c mice by flushing the femoral cavity with 2-3 ml of RPMI 1640 supplemented as described except with 15% defined FBS (low endotoxin, Intergen, Purchase, NY) (93). Cells were kept on ice while viability was determined using FDA. Cells were adjusted to 5×10^5 viable cells/ml and 1 ml of the cell suspension was placed into each well of 24 well cluster plates containing 1 ml of RPMI 1640S/15% FBS and 25% (final volume) of either NCM or HpCM or NbCM. The cultures were kept at 37°C in a water jacketed incubator in a 5% CO₂ atmosphere.

Cultures were harvested weekly up to 28 days and replenished with fresh RPMI 1640/15% FBS/25% CM every 3-4 days during the culture period. Cells were harvested by vigorous pipetting and placed on ice while viability was determined with FDA. Approximately 5×10^5 cells were centrifuged onto microscope slides in a Shandon Cytospin 2 (Shandon, Inc., Pittsburgh, PA) for 5 min at 500 rpm. The slides were allowed to air dry and immediately stained with Wright's stain. Mast cells were identified morphologically. In early experiments, toluidine blue was also used to stain mast cells. Because there was no difference in mast cell morphology with these two stains, Wright's stain was routinely used.

ELISA assays

CM were tested for cytokine content using the sandwich ELISA. Capture antibody and detecting antibody for GM-CSF, IL-2, IL-3, IL-6 and IL-10 were purchased from Pharmingen, San Diego, CA., as was the detecting antibody for IL-4. Monoclonal antibodies 11B11, TRFK-4, TRFK-5, XMG1.2 and R46A2 were generous gifts from Dr. Robert Coffman, DNAX. (See Table 1 for a summary of antibody specificities). Hybridoma cells secreting 11B11, TRFK-4 and 5, XMG1.2 and R46A2 were injected into nude mice and the resultant ascites fluid was purified using saturated ammonium sulfate (SAS) precipitation and a MAbTrap G column (Pharmacia LKB Biotechnology, Piscataway, NJ). After purification, protein content was determined using the BioRad Protein Assay (BioRad Laboratories, Richmond, CA). The monoclonal antibodies TRFK-4 and R46A2 were biotinylated using N-hydroxysuccinamido-biotin (Sigma). Recombinant cytokine standards were purchased from Pharmingen with the exception of GM-CSF (Intergen, Purchase, NY) and IFN- γ (Genzyme, Cambridge, MA).

The ELISA technique was carried out using the protocol provided by Pharmingen. Briefly, anti-cytokine capture antibodies were diluted in 0.1 M NaHCO₃ (pH 8.2) to 2 μ g/ml (or 4 μ g/ml for anti-IL-10). Fifty microliter aliquots of capture antibody solution were added to Corning 96-well Easy Wash modified flat bottom ELISA plates and incubated at 4°C

overnight. Wells were washed twice with PBS/Tween, blocked with 200 μ l PBS/10% FBS for 2 hr at room temperature and washed twice. Cytokine standards were diluted in PBS/10% FBS; the initial concentration for cytokine standard curves

Table 1. Cytokine antibody specificities including monoclonal antibody clone and isotype.

Cytokine	Capture antibody	Detecting antibody
IL-2	JES6-1A12 (Rat IgG2a)	JES6-5H4 (Rat IgG2b)
IL-3	MP2-8F8 (Rat IgG1)	MP2-43D11 (Rat IgG2a)
IL-4	11B11 (Rat IgG1)	BVD6-24G2 (Rat IgG1)
IL-5	TRFK5 (Rat IgG1)	TRFK4 (Rat IgG2a)
IL-6	MP5-20F3 (Rat IgG1)	MP5-32C11 (Rat IgG2a)
IL-10	JES5-2A5 (Rat IgG1)	SXC-1 (Rat IgM)
IFN- γ	R4-6A2 (Rat IgG1)	XMG1.2 (Rat IgG1)
GM-CSF	MP1-22E9 (Rat IgG2a)	MP1-31G6 (Rat IgG1)

for IL-2, IL-3, IL-4, IL-5 IL-6 and GM-CSF was 10 ng/ml; for IL-10 128 U/ml; and for IFN- γ , 200 ng/ml. Test conditioned media were analyzed neat and at 1:2 and 1:4 dilutions in PBS/10% FBS. Duplicate wells were set up for each dilution of sample CM and cytokine standards. Both standards and samples were incubated overnight at 4°C and washed four times with PBS/Tween. Appropriate biotinylated anti-cytokine second step antibody was diluted to 1 μ g/ml (or 2 μ g/ml for IL-10 because of the antibody isotype) and 100 μ l was added to each well, incubated at room temperature for 45 min and washed 6 times. Avidin-peroxidase (Sigma or Zymed, San Francisco, CA) was diluted in PBS/10% FBS and 100 μ l was

added per well; plates were incubated for 30 min. The wells were washed 8 times and ABTS substrate buffer (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) was added to each well and allowed to develop for 5-15 min before being read on a Biorad Plate Reader at 405 nm using a Bio-Rad Model 450 Microplate Reader (Richmond, CA).

From the six sample readings per experiment a concentration estimate was determined using the standard curves generated in three different experiments. In this way individual variation from assay to assay was integrated rather than being hidden by repeated averaging of sample values. The purpose of this treatment of the data was to give a more accurate representation of the range of cytokine concentrations present in CM so that cytokine neutralization and removal could then be carried out.¹

Flow cytometry analysis

Spleens or mesenteric lymph nodes from uninfected, *H. polygyrus*-infected or *N. brasiliensis*-infected mice were removed, placed in cold RPMI/10% FBS and a single cell suspension was obtained. Cells were washed and suspended in 1.0 ml cold RPMI/10%FBS. Erythrocytes were removed by hypotonic lysis. Cells were washed three times with DPBS/NaN₃ (0.01%)/10% rabbit serum (RS) (RS blocks Fc receptors)

¹ James Robinson Cox, Department of Mathematics, MSU, Bozeman, MT (personal communication).

Table 2. Cell surface antigens used for flow cytometry and fluorescent microscopy, including isotype.

<u>Cell specificity</u>	<u>Fluorescent-mAb</u>	<u>Clone & isotype</u>
T cell	FITC-anti-mouse CD3- ϵ^a	145-2C11 Hamster IgG
B cell	PE-CD45R/B220	RA3-6B2 Rat IgG2a, κ
B cell	FITC-rat-anti- mouse IgG (H+L)	NA ^b
CD4+ T cell	PE-L3T4	RM4-5 Rat IgG2a
CD8+ T cell	FITC-Ly-2	53-6.7 Rat IgG2a, κ
Granulocyte	FITC-Gr-1/Myeloid differentiation antigen	RB6-8C5 Rat IgG2b, κ
Granulocyte, Macrophage	FITC-Mac-1	M1/70 Rat IgG2b, κ
Isotype controls	FITC-Hamster IgG FITC-Rat IgG2a, κ FITC-RAT IgG2b, κ	UC8-4B3 R35-95 or R59-202 R35-38

^a all mAbs were mouse-specific

^b clone and isotype information not available

and suspended in trypan blue solution (0.4%) for viable cell counts. Cells (1×10^6) were incubated with anti-mouse CD3, L3T4, Ly-2, B220 (Pharmingen, San Diego, CA), rat anti-mouse IgG (H+L) Fab fragment (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or with the appropriate isotype controls or irrelevant antibody on ice for 30 min in DPBS/N₃/10%RS (Table 2). Antibodies were previously titrated by FACS analysis to determine optimal antibody concentrations for each antibody lot. All monoclonal

antibodies were directly conjugated. Cells were washed and in some experiments propidium iodide was added prior to centrifugation in order to stain dead cells. Cells were finally suspended in fresh DPBS/N₃/RS and retained on ice until analysis with a FACScan flow cytometer (Becton-Dickenson, San Jose, CA). Total cell numbers reflect the percentage of stained cells (with the percent of cells stained with irrelevant antibody or isotype control subtracted) multiplied by the total cell counts for spleen or MLN. Analysis of cell populations using orthogonal light scatter were used to gate out granulocytes and monocytes. In all experiments at least 10,000 events were analyzed.

Cells were also directly analyzed by Wright's staining after cytospin preparation. Briefly, cells were centrifuged directly onto glass slides in a Cytospin 2, air dried and stained with Wright's Stain. Duplicate slides were examined for cell morphology and differential counts were obtained.

Proliferation assays

BALB/c spleen or MLN cells from uninfected mice or mice infected with *H. polygyrus* or *N. brasiliensis* were pooled, washed and suspended in RPMI 1640S/2% FBS. Red blood cells were lysed, cells were washed and viable cell numbers determined with trypan blue. Cells (1×10^5) were added to 96 well-plates and cultured in triplicate in the presence of Con A at a concentration of 1.3 $\mu\text{g/ml}$, or medium alone for

72 hr at 37°C/5% CO₂. At 68 hr, the cells were pulsed with 1 µCi [³H] thymidine per well (Dupont NEN, Wilmington, DE). Cells were harvested onto glass-fiber filters using an automatic cell harvester (PHD, Cambridge, MA) and [³H] thymidine incorporation was quantified by liquid scintillation on a Packard scintillation counter. Results are expressed as the relative scintillation index (SI) [the ratio between counts per min (cpm) obtained with Con A to cpm without Con A].

Determination of serum immunoglobulin levels

Serum levels of polyclonal IgG1, IgG2a and IgE were obtained using the isotype-specific sandwich ELISA technique described by Maleszewski et al. (70). Briefly, 96-well flat-bottom Linbro plates (Flow Laboratories, Inc., McLean, VA) were coated with the first step isotype-specific antibody (Southern Biotechnology Associates) overnight and washed with PBS-Tween. The wells were rinsed six times with PBS. Non-specific sites were blocked by incubating wells for one hr with 150 µl of 5% nonfat dry milk. Isotype standards or sample sera from uninfected, *H. polygyrus*-infected or *N. brasiliensis*-infected mice were diluted in PBS/3% bovine serum albumin (BSA) (100 µl per well), incubated for one hr at room temperature and the wells were washed. One hundred microliters of horseradish peroxidase-conjugated second step antibody (Southern Biotechnology

Associates) appropriate for each isotype was added, incubated for 1 hr and washed. Wells were developed using the TMB Microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Plates were read on a Dynatech ELISA reader. Immunoglobulin concentrations in serum test samples were determined by comparing triplicate test values with the appropriate isotype control standard curve, using the Deltasoft 1.8 ELISA analysis program (Biometallics, Inc., Princeton, NJ).

For the IgG1 assay, unconjugated and horseradish peroxidase-conjugated affinity-purified goat anti-mouse isotype-specific reagents (Southern Biotechnology Associates) were used as capture and second step reagents, respectively. Standard curves for IgG1 were obtained with murine myeloma IgG1 (Southern). For the IgE assay, the EM95 anti-mouse IgE mAb was used as the capture step reagent and biotinylated rat anti-mouse IgE (Bioproducts for Science, Inc., Indianapolis, IN) was used as the second step reagent. Horseradish peroxidase-conjugated streptavidin (Zymed) was used in the third step. Standard curves were obtained with a murine anti-dinitrophenol-specific IgE myeloma antibody (TIB 141; American Type Culture Collection, Bethesda, MD). Both ELISA assays were found to be highly specific based upon cross-reactivity experiments using all individual mouse isotypes as controls. A 10 $\mu\text{g/ml}$ solution of nonspecific isotype gave a reading no higher than the lower limits of

detectibility for each assay.

ELISPOT assay for the detection of cytokines produced by mouse spleen cells

Day 1. Spleen cells were aseptically removed and monodispersed as for the preparation of CM. Cells were adjusted to 5×10^6 cells/ml in RPMI 1640S + 10% FBS and Con A was added at a concentration of $5 \mu\text{g/ml}$. The cells were incubated for 22-24 hr at 37°C in 5% CO_2 .

Cells producing IL-2, IFN- γ , IL-4 and IL-5 were assayed using a modification of the ELISPOT technique following Taguchi et al. (112). Briefly, 96-well nitrocellulose based plates (Millititer HA, Millipore Corp., Bedford, MA) were coated with $100 \mu\text{l}$ per well of anti-cytokine capture antibody at $5 \mu\text{g/ml}$ (anti-IL-2 (Pharmlingen, Inc., San Diego, CA), anti-IFN- γ (XMG1.2), anti-IL-4 (11B11) and anti-IL-5 (TRFK-5) (Table 1). Control wells were coated with PBS/5% FBS or with irrelevant goat anti-mouse immunoglobulin antibody. Plates were incubated overnight at 4°C .

Day 2. Plates were washed three times with PBS and blocked with RPMI 1640 + 5% FBS for one hr at room temperature. At 20-22 hr mitogen-stimulated spleen cells were added at a final concentration of 1×10^6 cells in the first well and diluted in a two-fold serial dilution across the row ending with approximately 4.9×10^2 cells per well. Each well contained $100 \mu\text{l}$ of cell suspension. Plates were

incubated for 20-22 hr at 37°C at 5% CO₂ and care was taken to not vibrate the plates during incubation. The cells were removed by washing the wells four times with PBS/Tween and 100 µl of biotinylated anti-cytokine antibody (anti-IL-4 or anti-IL-2 (Pharmingen) or anti-IFN-γ (RA46A2) or anti-IL-5 (TRFK-4) was added to each well and incubated overnight at 4°C. Wells were washed three times with PBS/Tween and 100 µl of avidin-peroxidase (Zymed), diluted in PBS/Tween, was added to each well and incubated for one hr at room temperature in the dark. The wells were washed with PBS and developed with 50 µl/well of 25 mg 3-amino-9-ethylcarbazole (PolySciences, Inc., Warrington, PA) in 2 ml N,N-dimethylformamide (Sigma). This solution was added to 95 ml 0.1 M citrate, pH 5.0) and the plates were immediately developed for 1-5 min. The plates were washed with tap water and ready for spot enumeration.

The principle behind this technique is that individual cells secreting cytokine will leave a spot where the cytokine has been captured and detected during the incubation period by the capture antibody/detecting antibody sandwich coating the nitrocellulose plate (figure 3). Spots were enumerated using image analysis enhancement of the wells (American Innovisions, Inc., San Diego, CA) and a stereo zoom dissecting microscope (Cambridge Instruments, Cambridge, MA). Spots were counted at dilutions exhibiting 100-200 discrete spots. Image analysis was used to display

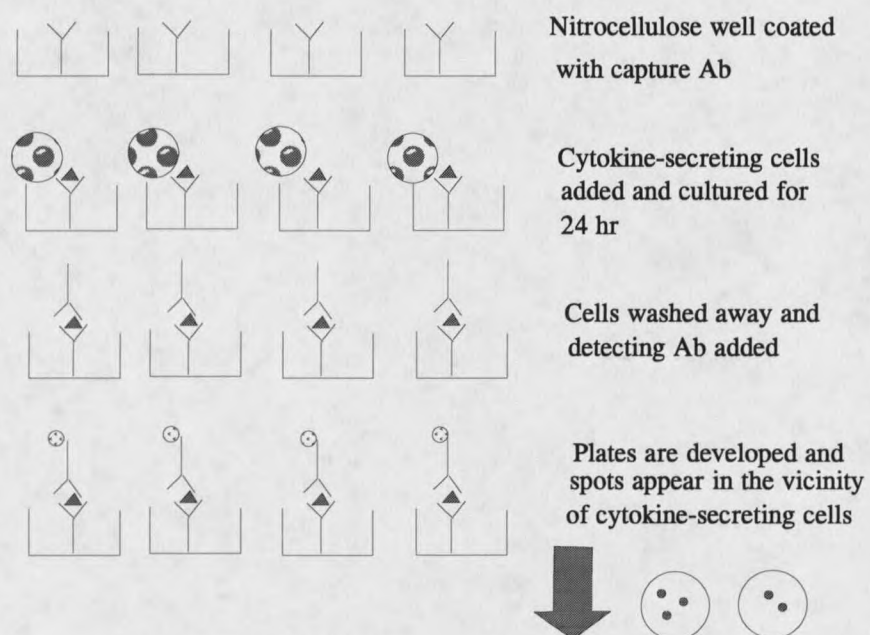


Figure 3. Diagram schematically outlining ELISPOT assay.

the spots on a computer screen so that they could be marked and manually counted on the screen to ensure that spots were not counted more than once. Duplicate wells were counted for each countable cell concentration (minimum of three) for each of three separate experiments. These data were used to construct a linear regression curve to assign the number of cytokine-secreting cells per million cells. This number was multiplied by the total number of cells per spleen to yield the number of cytokine secreting cells per spleen.

Separation of adherent splenocytes from non-adherent splenocytes

Flasks (75 cm²) (Costar) were coated with 15 ml of FBS for 2 hr at room temperature. Spleen cells were obtained as in CM materials and methods, adjusted to 5×10^6 cells/ml and incubated in the flasks for 4 hr at 37°C in a CO₂ incubator. Non-adherent cells were decanted and the flasks were washed with 15-25 ml RPMI 1640S/10% FBS by gentle rocking and non-adherent cells were decanted. Non-adherent cells were centrifuged, suspended in fresh RPMI/10% FBS and adjusted to 5×10^6 cells/ml. The non-adherent cells were added to appropriate flasks, Con A (5 µg/ml) was added to each flask and the cells were incubated for an additional 40 hr at 37°C. After incubation the CM were centrifuged and sterile filtered as for untreated CM.

Immunofluorescence staining of cultured bone marrow cells

Bone marrow cells cultured with HpCM for four weeks (Hp cells) developed an unusual morphology. To determine the phenotype of Hp cells, the cells were harvested and centrifuged for 10 min (110-170 x g) at 4°C. Cells were suspended and washed again. Cells were incubated on ice with FITC-conjugated Mac-1, RB8C5.1 (Gr-1) or CD45 (B cell exon) (Pharmlngen) for 30 min on ice. Following incubation with the antibodies, 3 ml of RPMI 1640/10% FBS was added to the cells. The cells were centrifuged, suspended in RPMI 1640/10% FBS and examined with a fluorescent microscope (Olympus BH-2).

Non-specific esterase staining

Hp cells were cytocentrifuged onto glass slides and fixed for 30 seconds at 4°C in 30 ml of 0.1 M phosphate buffer (pH 6.6) mixed with acetone and 25 ml formaldehyde solution. Slides were washed three times with dH₂O and allowed to air dry for 10-30 min. Immediately prior to staining, 6 ml of 4% w/v sodium nitrate was mixed with 6 ml pararosaniline stock solution (1 g pararosaniline in 5 ml concentrated HCL (10 M) and 20 ml dH₂O) and diluted to 200 ml with 0.067 M phosphate buffer (pH 5.0). The activated dye solution was adjusted to pH 5.8 with 0.1 M NaOH. Fifty mg of α -naphthyl acetate in 2 ml acetone was added to the

staining solution and the fixed slides were incubated for 4 hr at room temperature in the stain. The slides were counterstained for 1-2 min in a 0.4% aqueous solution of methyl green dye, washed with dH₂O and air dried.

Phagocytosis assays

Phagocytosis assays were done on Hp cells to determine if they were phagocytic cells (74). Briefly, Hp cells or thioglycollate-stimulated peritoneal cells were harvested and washed in Hanks' balanced salt solution (HBSS) pH 7, counted, pelleted and suspended in RPMI 1640 with 5% heat inactivated mouse serum. Cells (2×10^6) were applied to glass coverslips and incubated for one hr at 37°C/5% CO₂. The coverslips were rinsed with HBSS and 2×10^6 formalin-fixed *Candida albicans* cells in 0.5 ml RPMI 1640/5% mouse serum were added to the cells on the coverslips. After incubation for one hr, the coverslips were rinsed in HBSS, fixed in methanol and Giemsa stained.

Cytokine neutralization and removal from HpCM

The concentrations of GM-CSF and IL-4 were estimated in HpCM and NCM using the sandwich ELISA as previously described in the ELISA materials and methods. The highest concentration estimate was used to calculate the amount of cytokine needed to be neutralized and removed (3.0 ng/ml for GM-CSF and 12.0 ng/ml for IL-4). The volume of CM needed

for the bone marrow cultured mast cell (BMCMC) assay was calculated and multiplied by these concentration values to give the amount of antibody needed to neutralize enough cytokine per BMCMC assay. This amount was multiplied by 1000 in order to have neutralizing antibody present in excess.

Anti-IL-4 antibody (11B11) was a generous gift from Immunex, Corp., Seattle, WA. and anti-GM-CSF neutralizing antibody (MP1-22E9) was purchased from Pharmingen. Anti-IL-4 and anti-GM-CSF antibodies were added to two separate aliquots of HpCM and incubated overnight at 4°C. Goat anti-rat IgG (Jackson ImmunoResearch) was added to each HpCM aliquot containing anti-cytokine antibody. Goat anti-rat IgG was also added to a third HpCM aliquot that had not had anti-cytokine antibody added to it, to serve as a control for any non-specific antibody binding/neutralization that might occur. HpCM aliquots were incubated for 24 hr with slow rocking at 4°C. Protein G agarose beads (Pierce, Rockford, IL) were washed to remove sodium azide. Following incubation with goat anti-rat IgG, Protein G (approximately 1-2 mg) was added to each aliquot of HpCM and incubated for 20 hr at 4°C with agitation to remove antibody-complexed cytokine. The aliquots were inverted gently several times during this time to ensure that the beads did not settle out. After incubation, the beads were allowed to settle out and the supernatant was removed, centrifuged twice for 5 min

(40 x g) at 4°C to avoid breakage of the beads, filtered through a 0.22 μ m membrane and then centrifuged for one hr (20,000 x g) at 4°C to remove any antibody aggregates that might have remained after treatment with the Protein G beads. HpCM aliquots were sterile filtered and reserved for BMC MC assays and for cytokine ELISA. To test whether GM-CSF and IL-4 were neutralized and removed successfully, HpCM + anti-IL-4 (11B11), HpCM + anti-GM-SCF and HpCM + goat-anti-rat IgG alone were subjected to ELISA as described in the ELISA materials and methods. BMC MC assays were carried out as described in the bone marrow cell culture materials and methods.

Addition of recombinant IL-4 or GM-CSF to NCM

As a positive control for testing cytokine inhibition of BMC MC, recombinant (r) IL-4 (Pharmlngen) or rGM-CSF (Intergen) was added to NCM to concentrations commensurate with those estimated in HpCM. The aliquots of NCM were sterile filtered and reserved for BMC MC assays and ELISA. NCM with rIL-4 or NCM with rGM-CSF were also tested by ELISA to estimate the final amount of recombinant cytokine that appeared in NCM. BMC MC assays were carried out as described in the bone marrow cell culture materials and methods.

RESULTS

Conditioned medium made from the spleens of *Heligmosomoides polygyrus*-infected mice fails to support the development of cultured mast cells from bone marrow progenitors.

When bone marrow cells are cultured in the presence of Con A-stimulated splenocyte conditioned medium from uninfected animals (NCM) these cultures produced almost pure cultures (>95%) of bone marrow cultured mast cells (BMCMC) after four weeks of culture (figure 4). Total cell counts and differential cell counts were done each week during the four week culture period. The ability of NCM to induce mast cell development is due to the presence of mast cell growth and differentiation factors present in the conditioned medium.

In contrast, bone marrow cells cultured in the presence of conditioned medium made from spleens harvested at day fourteen post-infection with *H. polygyrus* (HpCM) did not develop into BMCMC by the end of the four week culture period (figure 4). Typically these cultures contained fewer than 1% cultured mast cells. Cultures containing HpCM produced low numbers of cells with an unusual morphology which we referred to as Hp cells.

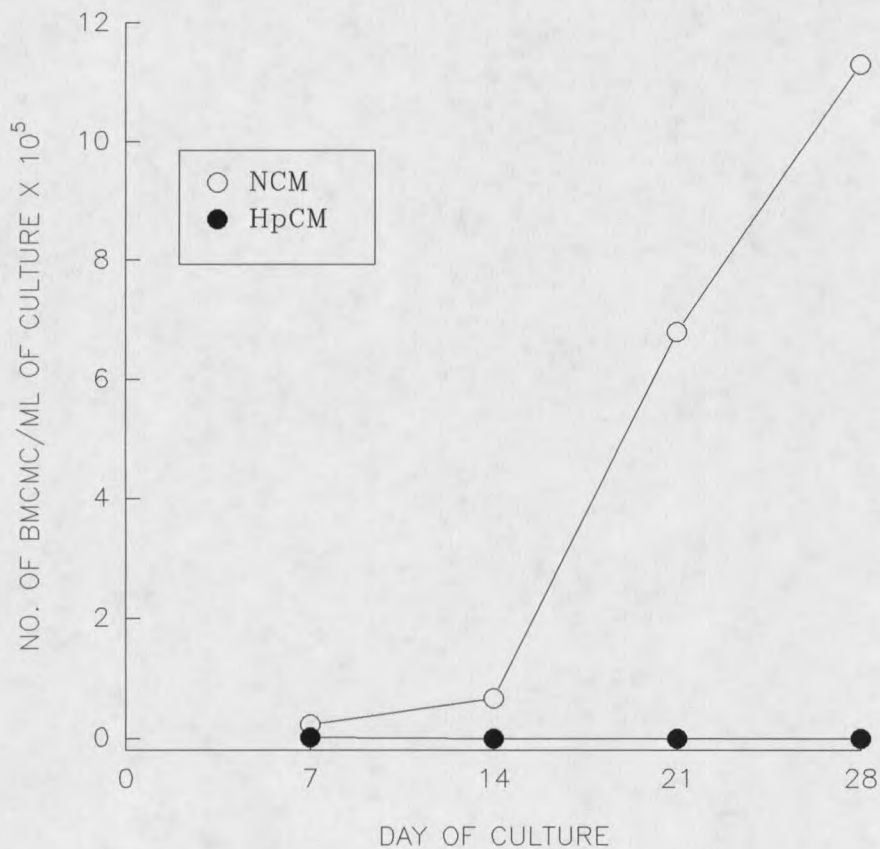


Figure 4. Bone marrow cultured mast cells (BMCMC) develop with NCM but not with HpCM. Bone marrow cells were cultured for four weeks in the presence of NCM made from the spleens of uninfected mice, or HpCM made from the spleens of mice infected with the intestinal nematode *H. polygyrus*. Cultured cells were harvested weekly, enumerated and stained with Wright's stain to monitor the development of BMCMC.

Failure of mast cell development in HpCM cultures is not a transient phenomenon during *H. polygyrus* infection.

Because *H. polygyrus* infection is chronic, we were interested in obtaining information about HpCM prepared at different times after infection. Consequently HpCM was made from the spleens of *H. polygyrus*-infected mice on different days post-infection. Data shown in figure 5 indicate that the mastopoietic potential of HpCM was reduced within three days of oral inoculation with L₃ *H. polygyrus*. HpCM made from mice infected with the nematode for nine days failed to support mast cell development. (Other experiments revealed that the mastopoietic potential of HpCM was abolished by six days post-infection.) The inability of HpCM to support BMCMC development lasted for several weeks during primary infection (figure 5).

CM made from spleens from *N. brasiliensis*-infected mice supports mast cell development.

It was possible that the failure of BMCMC to develop with HpCM was not unique to infection with *H. polygyrus*. Although it had been demonstrated that CM made from the spleens of *T. spiralis*-infected mice could support mast cells *in vitro* (93), it was possible that the development of BMCMC did not strictly correspond with mucosal mastocytosis *in vivo*. *N. brasiliensis* is an intestinal nematode of mice and rats that causes a short term, acute infection in mice

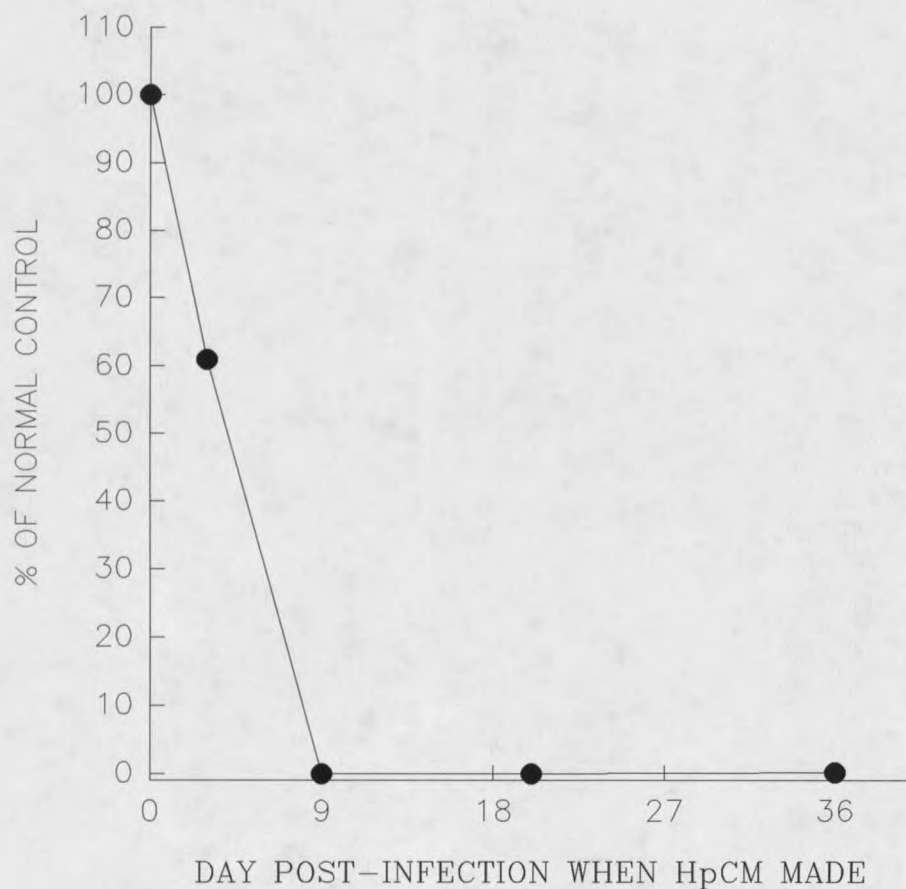


Figure 5. Failure of HpCM to develop BMCMC is not a transient phenomenon during *H. polygyrus* infection. Bone marrow cells were cultured for four weeks with NCM made from the spleens of uninfected mice (Day 0) or HpCM made from the spleens of mice infected with *H. polygyrus* for 3, 9, 20 or 36 days. BMCMC development was reduced with HpCM made on day 3 post-infection and failed to occur completely in cultures containing HpCM made on days 9, 20 or 36 post-infection. HpCM results are expressed as percent of NCM control.

and is typically characterized by an intense mucosal mastocytosis in the gut around the time of worm expulsion (92). BMCMC developed when bone marrow cells were cultured with CM made from *N. brasiliensis*-infected mice (NbCM) (day 14) (figure 6). Two nematode infections that induced MMC in the gut during infection *in vivo*, also produced spleen CM that supported BMCMC development *in vitro*. Therefore *H. polygyrus*-infection (and HpCM) appeared to be different from two other intestinal helminth infections in regard to the development of mast cells.

Splenocytes from mice infected with *H. polygyrus* proliferate in response to Con A.

One possibility that could account for the failure of HpCM to induce mast cell development in culture is that splenocytes used to generate CM from *H. polygyrus*-infected animals are unresponsive to Concanavalin A, the T cell mitogen used to prepare CM. Unresponsiveness would presumably result in inferior quantities of mast cell growth and differentiation factors in the conditioned medium and explain the inability of HpCM to produce BMCMC. There is some evidence for this. Several researchers have reported that infection with *H. polygyrus* correlates with an anergic response to several antigens and mitogens (3,21,68,84,86,88,103).

Proliferation assays were done on splenocytes and mesenteric lymph node cells from BALB/c mice that were

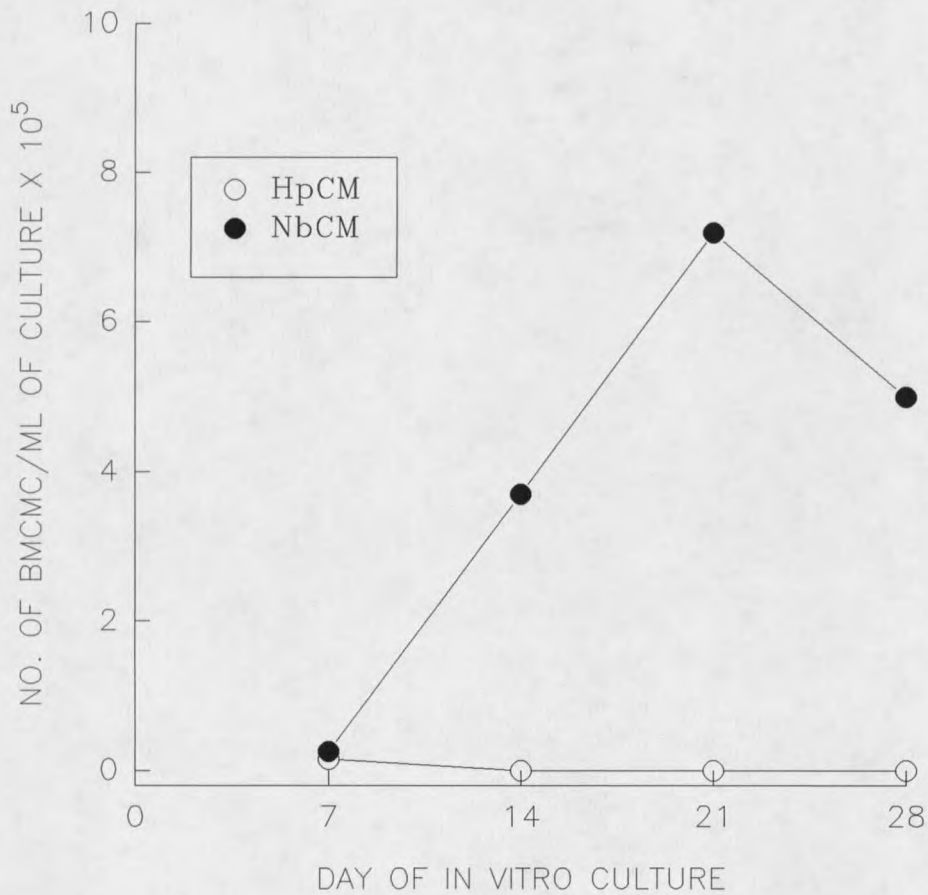


Figure 6. BMCMC develop with NbCM but not with HpCM. Bone marrow cells were cultured for four weeks in the presence of CM made from the spleens of either *H. polygyrus*-infected or *N. brasiliensis*-infected mice (day 14 p.i.). Cultured cells were harvested weekly, enumerated and stained with Wright's stain to monitor the development of BMCMC.

either uninfected, infected with *H. polygyrus*, or infected with *N. brasiliensis*. (*N. brasiliensis* infection correlates with a CM that has mastopoietic potential.) Data shown in figure 6A indicate that spleen cells from *H. polygyrus*-infected mice proliferated in response to Con A. Mesenteric lymph node cells also proliferated with Con A (figure 7B) demonstrating that T cells from *H. polygyrus*-infected mice were not anergic to this mitogen. Therefore unresponsiveness to Con A did not account for the failure of HpCM to produce mast cells.

Failure of HpCM to promote BMCMC development is not due to a decrease in the number of Con A responsive T cells in *H. polygyrus*-infected mice.

H. polygyrus infection is associated with a pronounced splenomegaly during the course of infection. This splenomegaly was characterized by increased cellularity (Table 3). It was therefore possible that non-T cell populations in the spleen were expanding preferentially in *H. polygyrus* infection, resulting in fewer T cells capable of responding to Con A compared with uninfected mice. This might result in inferior quantities of mast growth and differentiation factors in HpCM compared with NCM.

To address this possibility, splenocytes were characterized by morphological examination of Wright's-Giemsa stained cytopsin preparations and flow cytometric

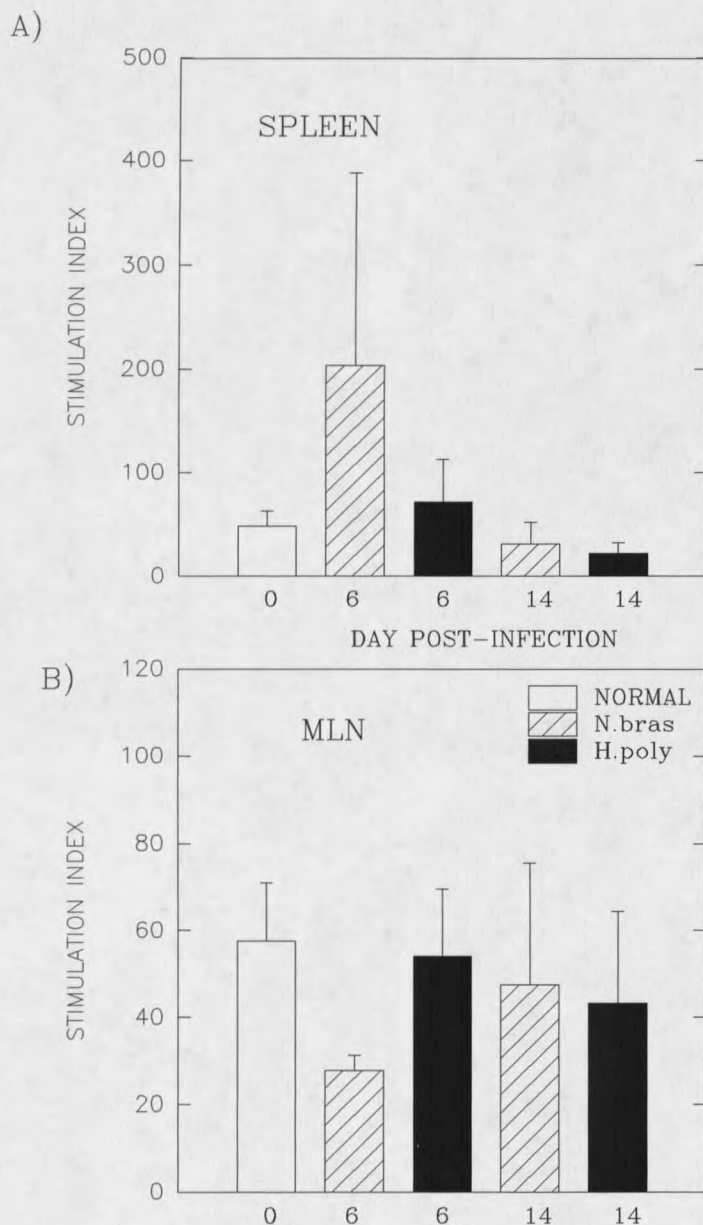


Figure 7. Spleen cells A) and MLN cells B) from *H. polygyrus*-infected mice respond to Con A. Cell proliferation was measured using ^3H -thymidine uptake. Cells were cultured in triplicate wells and mean scintillation counts per minute (cpm) were used to derive the stimulation index (SI). The SI represents the cpm of cells cultured with Con A divided by the cpm of cells cultured without the mitogen. Data points represent the average of three separate experiments (3-4 mice per experiment).

Table 3. Changes in leukocyte populations in mouse spleen and mesenteric lymph node (MLN) due to infections with either *H. polygyrus* or *N. brasiliensis*.

	Source of cells					
	Uninfected	<i>N. brasiliensis</i> -infected		<i>H. polygyrus</i> -infected		
	Day P.I.					
	DAY 6	DAY 14	DAY 6	DAY 14	DAY 6	DAY 14
	Spleen					
Lymphocytes	7.3 ^a (91.6)	9.8 (91.2)	8.5 (80.1)	8.4 (75.8)	18.2 (84.4)	15.2 (71.9)
Neutrophils	0.3 (4.1)	0.4 (3.8)	1.0 (9.2)	0.7 (6.0)	2.0 (9.2)	1.8 (8.4)
Eosinophils	0.1 (1.1)	0.1 (0.8)	0.2 (2.3)	1.2 (10.4)	0.5 (2.3)	2.2 (10.0)
Monocytes	0.3 (3.5)	0.5 (4.6)	0.9 (8.8)	1.0 (7.9)	1.9 (8.8)	2.0 (9.4)
Total cells	8.0 ^b	10.7	10.6	1.1	1.6	21.2
	MLN					
Lymphocytes	2.5 (96.8)	4.7 (97.5)	4.1 (94.3)	10.5 (90.6)	5.0 (90.0)	16.0 (87.5)
Neutrophils	0.02 (0.8)	0.01 (0.1)	0.03 (0.8)	0.03 (0.3)	0.02 (0.4)	0.02 (0.1)
Eosinophils	0.01 (0.5)	0.02 (0.4)	0.01 (0.2)	0.2 (2.0)	0.1 (1.6)	0.2 (1.0)
Monocytes	0.1 (2.3)	0.1 (2.0)	0.1 (2.0)	0.6 (5.0)	0.4 (7.3)	2.1 (11.5)
Total cells	2.6	4.8	4.3	11.6	5.6	18.3

^a Numbers represent the absolute number of leukocytes ($\times 10^7$) in uninfected, *H. polygyrus*-infected or *N. brasiliensis*-infected mice per organ. Numbers in parentheses are the percentage of leukocytes.

^b Total cell numbers determined from 6 separate experiments (3-4 mice per experiment).

Cells from spleen or MLN were prepared for cytospin and stained with Wright's-Giemsa stain. Differential cell counts were done based on cell morphology and a minimum of 200 cells were counted per slide. Duplicate slides were made for each experiment to give an average.

analysis. Data summarized in Table 3 show that in both spleen and mesenteric lymph node (MLN) compartments, infection with *H. polygyrus* resulted in at least a two-fold or greater increase in cellularity in these lymphoid organs. Infection with *N. brasiliensis*, which correlates with CM that supports mast cell development, showed an increase in cellularity largely confined to the MLN compartment.

Splenic neutrophil, eosinophil and monocyte cell populations all expanded in response to both nematode infections (Table 3). In fact, the gross cellular responses in the spleen were similar in both infections when analyzed by percentage even though *H. polygyrus* infection caused splenomegaly. However, when absolute numbers of cells were analyzed, *H. polygyrus* induced a dramatic increase in splenic lymphocytes. Splenic lymphocytes were not affected remarkably by *N. brasiliensis*.

The cellular response in MLN was largely confined to lymphocytes and monocyte-like cells in both infections, with MLN from *H. polygyrus*-infected mice displaying more monocyte-like cells than MLN from *N. brasiliensis*-infected mice.

Analysis of the lymphocyte population indicated that *N. brasiliensis* infection showed a consistent decrease in the percentage of lymphocytes throughout infection similar to that observed in *H. polygyrus*-infected mice (Table 3).

Further analysis using flow cytometry of the lymphocyte

population in each infection revealed two things. One, in BALB/c mice both nematode infections induced a B cell hyperplasia in spleen and MLN at both days 6 and 14 post-infection (figure 8). However the B cell hyperplasia was much more pronounced in response to *H. polygyrus* infection compared with the response to *N. brasiliensis* infection, reflecting the dramatic increase in organ size observed in *H. polygyrus* infection. Two, *H. polygyrus* elicited a splenic T cell hyperplasia in BALB/c mice whereas *N. brasiliensis* did not (figure 8). The T cell hyperplasia was most pronounced at day 6 post-infection. Both nematodes caused a T cell hyperplasia in MLN. These results suggest that fewer T cells are not likely to account for the incapacity of HpCM to support cultured mast cells.

However, the percentage of T cells in the spleens of *H. polygyrus*-infected mice did decline compared with uninfected mice and therefore it might be argued that the decreased ratio of T cells capable of responding to Con A might account for the failure of HpCM to produce BMCMC, presumably by resulting in HpCM containing more dilute cytokines necessary for mast cell development.

In order to address this possibility, splenic T cells from both uninfected and *H. polygyrus*-infected mice were purified using polymethacrylate beads coupled with anti-mouse immunoglobulin. Cells in the eluant (enriched for T cells) were counted, adjusted to 5×10^5 cells/ml and used

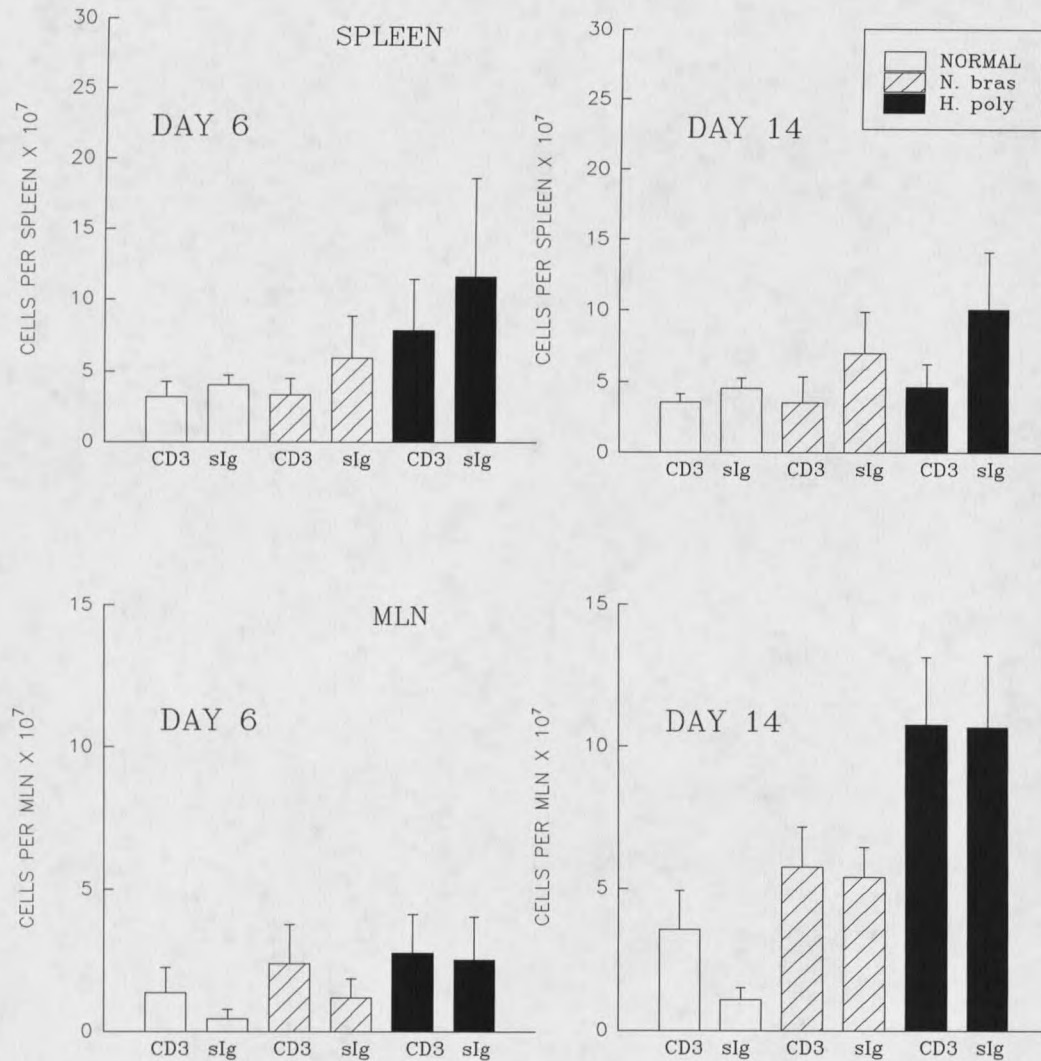


Figure 8. The effect of *H. polygyrus* and *N. brasiliensis* infection on mouse splenic and MLN lymphocyte populations. Spleen or MLN cells were removed on day 6 or 14 post-infection and compared with cells from uninfected (normal) age matched mice for each experiment. The percentage of T or B cells was determined by flow cytometric analysis and absolute numbers were determined by multiplying the percentage of positively stained cells by the total number of cells per organ (averaged between 3-4 mice). FITC-conjugated anti-CD3 was used to label T cells and FITC-conjugated goat anti-mouse IgG (H+L) Fab fragment was used to label surface immunoglobulin on B cells. Isotype matched irrelevant antibodies were used to control for nonspecific labelling. Propidium iodide was used to gate out dead cells. Data points represent the means of 4-5 separate experiments.

to make CM. Even though the same number of T cells was used to make both NCM and HpCM, mast cells still failed to develop in cultures containing HpCM (data not shown). A smaller ratio of T cells to other cells in the spleens of *H. polygyrus*-infected mice then, did not appear to explain the failure of HpCM to support BMCMC development.

HpCM made from the spleens of moderate and low responder mouse strains fails to support mast cell development; however HpCM from a high responder strain permits some mast cell development.

Several mouse strains have been shown to respond differently to *H. polygyrus* infection, prompting researchers to characterize inbred strains as either high, moderate or low responders to the parasite. This characterization is based primarily on the duration of the infection, with high responder strains expelling *H. polygyrus* in about two months and low responder strains harboring the nematode for up to a year or more. The responder status is also based upon how well a strain responds to challenge infections. High responder (refractory) strains show immunity after fewer challenge infections than do low responder (liable) strains (7,11,48,73,121).

Therefore we were interested in whether responder status correlated with the ability of mouse spleen conditioned medium to support the development of cultured

mast cells *in vitro*. HpCM made from low responder, C57BL/10 mice was not able to support the development of mast cells from syngeneic bone marrow progenitors *in vitro* (Table 4). The ability of HpCM to support BMCMC development was diminished at day 3 p.i. and dramatically reduced after day 6 post-infection. The reduction of mastopoietic potential of C57BL/10 HpCM was observed through 18 days post-infection.

HpCM made from the spleens of high responder SJL mice, on the other hand, permitted the development of some BMCMC from syngeneic bone marrow cells since cultures containing SJL HpCM made on day 12 p.i. had approximately 50% of the BMCMC response seen with SJL NCM (Table 4). SJL HpCM made at other times during *H. polygyrus* infection also showed more BMCMC in culture (8-10% BMCMC) compared with the other two strains (0-3% BMCMC with C57BL/10 or BALB/c HpCM).

C57BL/10 mouse bone marrow cells have been shown to be less capable of developing mast cells when cultured with conditioned media containing MCGFs (94). Therefore we wished to see if C57BL/10 HpCM failed to promote BMCMC development using allogeneic bone marrow cells. Data summarized in Table 5 show that C57BL/10 HpCM did not support the development of mast cells in culture using BALB/c bone marrow cells either. This suggested that the failure of BMCMC to develop with C57BL/10 HpCM was due to a

Table 4. The effect of HpCM made from different responder strains to *H. polygyrus* infection on syngeneic bone marrow cells.

	Uninf.	Day P.I.		9	12	18	20
		3	6				
<u>Mouse strain</u>							
SJL	2.2 ^a (100)	2.2 (98)	0.2 (9)	ND	1.0 (50)	0.2 (8)	ND
C57BL/10	2.9 (100)	1.2 (48)	0.04 (2)	ND	0.07 (3)	0.06 (3)	ND
BALB/c	6.3 (100)	3.9 (94)	ND	0 (0)	ND	ND	0 (0)

^a Number of BMCMC/ml of culture ($\times 10^5$) harvested after four weeks of culture. Numbers in parentheses reflect the percentage of cells that were mast cells in culture.

HpCM was made on different days p.i. with *H. polygyrus*. Syngeneic bone marrow cells were used that corresponded to the strain that was used to make HpCM. BMCMC failed to develop with *H. polygyrus* infection in liable strains, however the refractory strain, SJL, permitted some mast cell development. ND= not determined.

factor in C57BL/10 HpCM rather than due to a deficiency in C57BL/10 bone marrow cells. When SJL HpCM was applied to allogeneic bone marrow cells, mast cell development occurred except for slight reductions in cultures containing HpCM made from the spleens of mice infected with *H. polygyrus* for 3 and 12 days (Table 5). These results suggested that responder status did influence the capacity of HpCM to support the development of BMCMC, but this effect was more pronounced using allogeneic bone marrow cells. This implied that HpCM made from the spleens of different responder mouse strains exhibited a differential ability to promote mast

Table 5. The effect of HpCM made from different responder strains to *H. polygyrus* infection on allogeneic bone marrow cells.

<u>Mouse strain</u>	<u>DAY P.I.</u>				
	0	3	6	12	18
SJL	12.0 ^a (100)	7.9 (66)	10.0 (84)	8.1 (67)	12.0 (98)
C57BL/10	2.8 (100)	2.7 (94)	0.09 (3)	0 (0)	0.01 (0)

^a Numbers indicate the number of BMCMC/ml of culture ($\times 10^5$) harvested after 4 weeks of culture. Numbers in parentheses reflect the percentage of cells that were BMCMC in the culture.

HpCM made on different days p.i. with *H. polygyrus*. Allogeneic bone marrow cells (from BALB/c mice) were cultured with HpCM made from SJL or C57BL/10 mice. BMCMC failed to develop with HpCM made from C57BL/10 mice, however HpCM made from SJL mice permitted BMCMC development suggesting that comparison between HpCM made from these two mouse strains might be useful.

cell development and this difference could be useful in determining the mechanism of lack of mast cell development *in vitro*.

High responder SJL mice and low responder C57BL/10 mice exhibit converse lymphocyte responses to *H. polygyrus* infection.

We were interested in learning whether T cell numbers were grossly altered in response to *H. polygyrus* in other mouse strains than BALB/c. Therefore splenic lymphocytes were analyzed using flow cytometry in high and low responder

strains as well as moderate responder BALB/c mice. Results are shown in figure 9. All strains exhibited a B cell hyperplasia in response to infection with *H. polygyrus*. However, this response was more dramatic in the low responder C57BL/10 strain (figure 9A).

SJL mice, like BALB/c mice, displayed a T cell hyperplasia at day 6 p.i. (figure 9B). However, the T cell hyperplasia continued on day 14 p.i. in the high responder SJL strain and was more pronounced than the B cell expansion in these mice. Although low responder C57BL/10 mice did not exhibit a marked enlargement of the T cell population, absolute T cell numbers were nevertheless slightly above that of uninfected control mice. Thus, the high responder strain showed a T cell hyperplasia, whereas the low responder strain lacked this response and showed a more pronounced B cell hyperplasia in response to *H. polygyrus*.

The contrast between the high and low responder strains warranted further study. Hence, T and B cell numbers at several time points during infection with *H. polygyrus* were examined. B cell hyperplasia was most pronounced in the low responder C57BL/10 strain compared with the high responder SJL strain throughout the course of infection (except for day 28) (figure 10A). In contrast, high responder SJL mice exhibited a pronounced T cell hyperplasia throughout the course of infection. Although T cell numbers also increased during *H. polygyrus* infection in C57BL/10 mice, the T cell

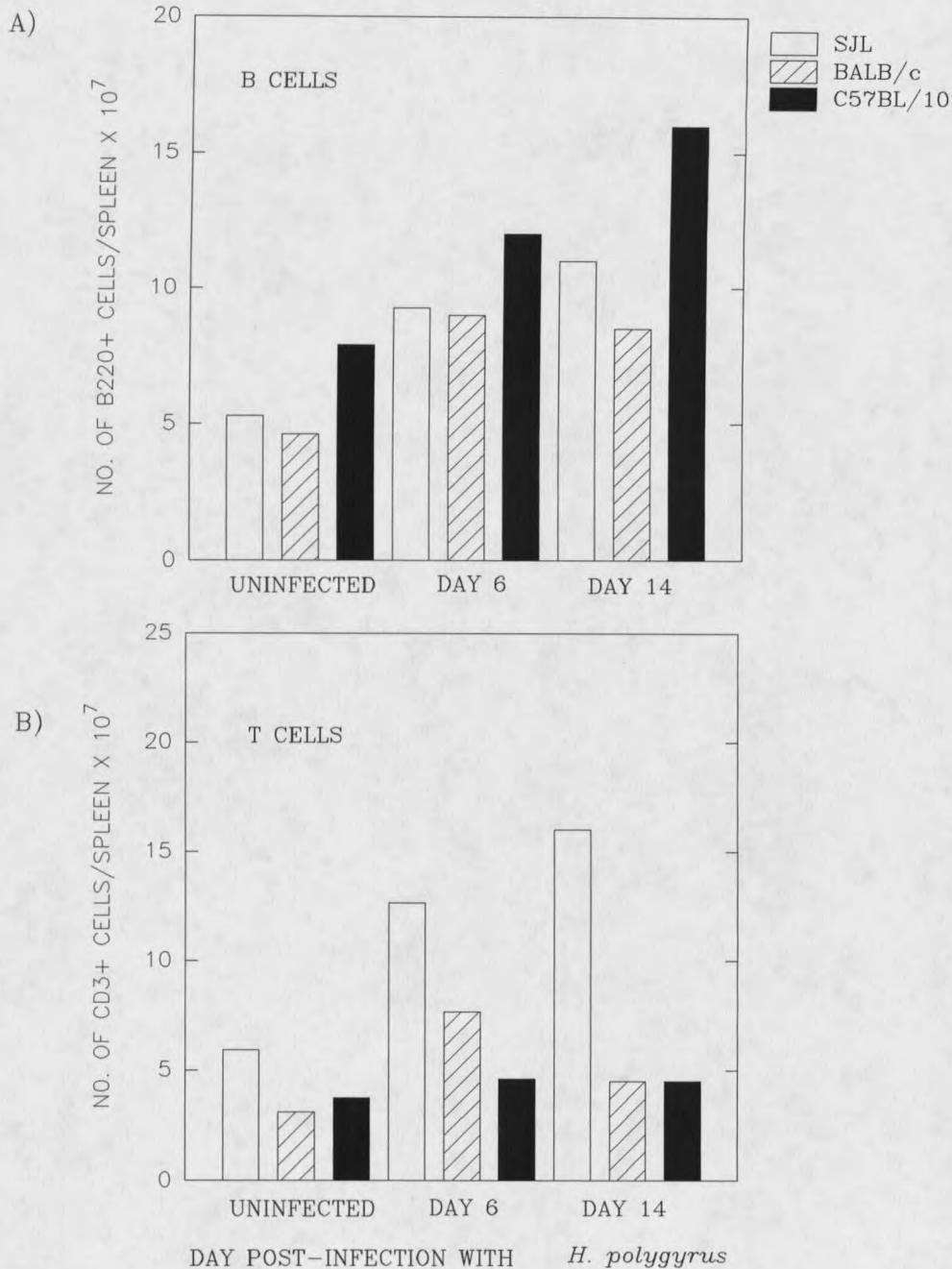


Figure 9. The effect of *H. polygyrus*-infection on splenic lymphocyte populations in high (SJL), moderate (BALB/c) and low (C57BL/10) responder mouse strains. Spleen cells were removed on day 6 or 14 post-infection and compared with cells from uninfected (Day 0) age matched mice for each experiment. Flow cytometric analysis proceeded as for figure 8, except that PE-conjugated B220 was used to label B cells. Data points represent the means of 3-4 separate experiments.

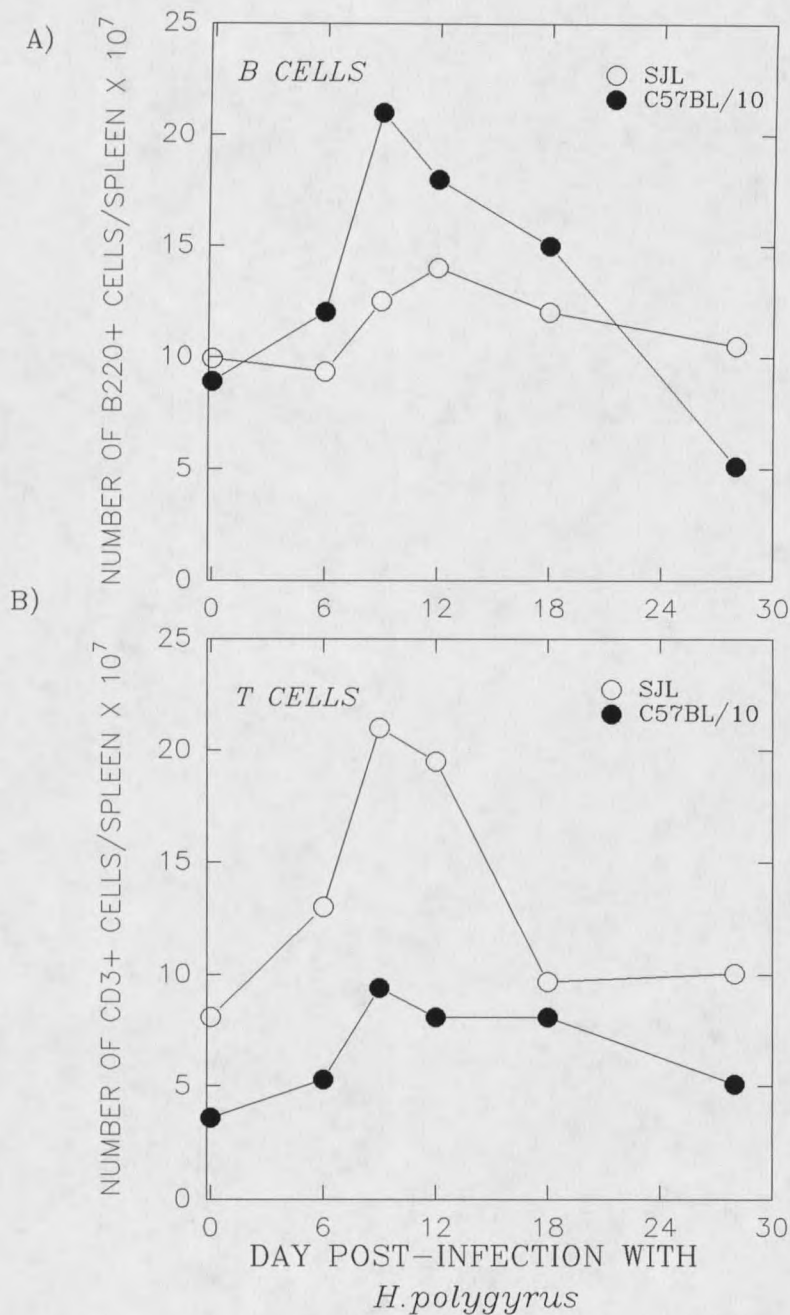


Figure 10. The effect of *H. polygyrus* infection on T and B cell populations in the spleen in low responder C57BL/10 and high responder SJL mice. Spleen cells were removed from SJL or C57BL/10 mice that were uninfected, or infected with *H. polygyrus* for 6, 9, 12, 18, or 28 days. Spleen cells from each strain were pooled (groups of 3-5 mice), labelled with FITC-conjugated anti-CD3 or PE-conjugated anti-B220 and fixed with 1% paraformaldehyde prior to flow cytometric analysis. Data points represent means of 2-3 experiments.

hyperplasia was much less dramatic in this susceptible strain (figure 10B).

***H. polygyrus* infection does not increase the ratio of CD8 T cells.**

It has been suggested that an increase in CD8 T lymphocytes might account for the chronicity of *H. polygyrus* infection (89). The notion that CD8⁺ T cells functioned largely as cytolytic T cells rather than cytokine secreting T helper cells suggested it was possible that a decrease in the ratio of CD4:CD8 cells might account for the inefficacy of HpCM to develop mast cells in culture. Specifically, one report had demonstrated that Ly-2⁺ (CD8) T cells did not produce IL-3 suggesting that a change in the CD4:CD8 ratio might explain the failure of HpCM to support BMCMC development (25).

Flow cytometric analysis of CD4⁺ and CD8⁺ T cell subpopulations, using monoclonal antibodies to mouse L3T4 (CD4) and Ly-2 (CD8), demonstrated that the ratio of CD4:CD8 T cells did not decrease in response to infection with *H. polygyrus* in BALB/c mice (figure 11). Rather, in the spleen, the CD4:CD8 ratio actually increased slightly with this nematode at day 6 p.i. and was commensurate with the ratio observed in uninfected mice at day 14 post-infection. *N. brasiliensis* infection also did not alter the ratio of CD4:CD8 cells in the spleen (figure 11). *H. polygyrus*

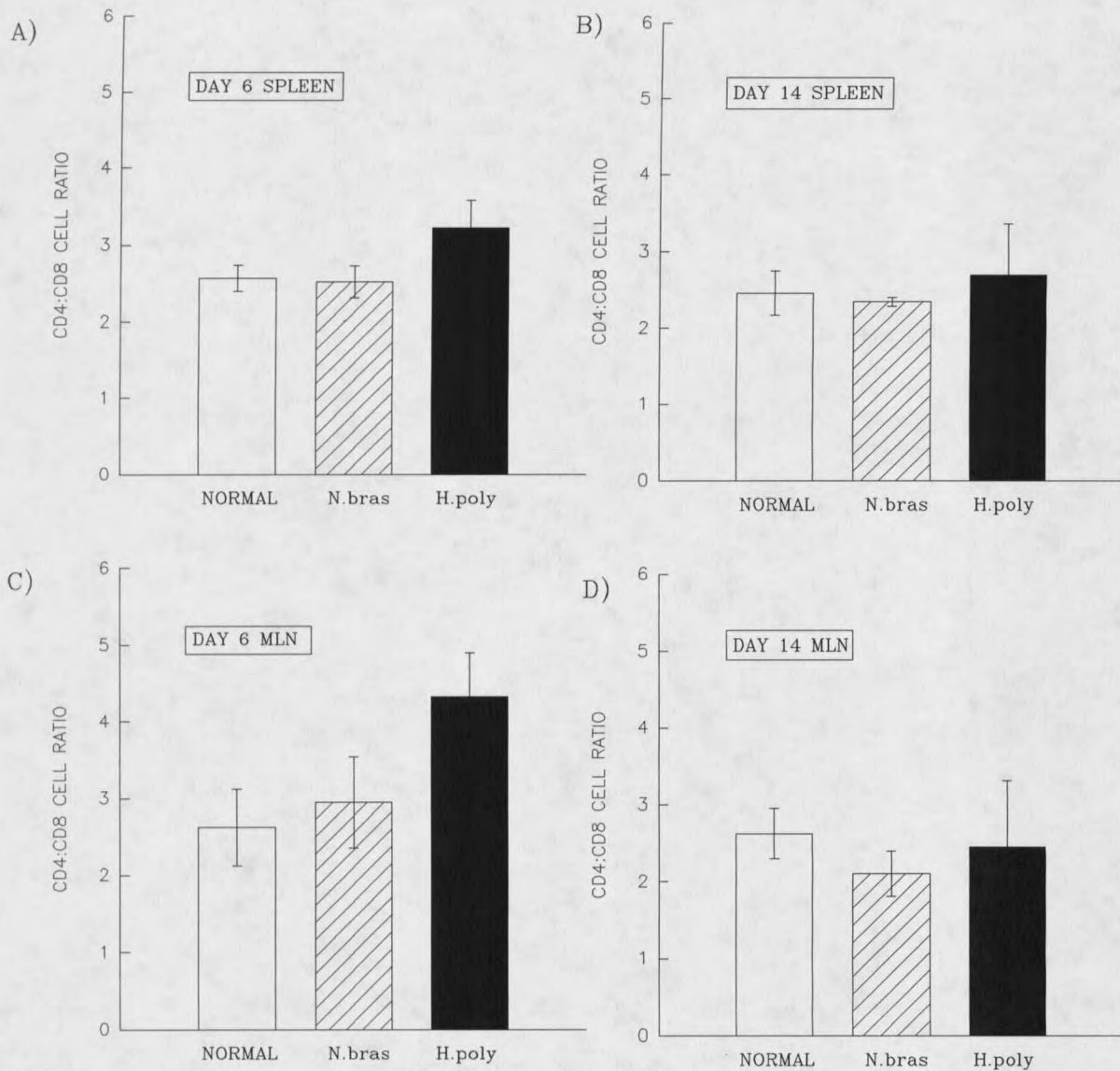


Figure 11. *H. polygyrus* infection is not associated with an increase in the number of CD8⁺ T cells in spleen or MLN. Spleen or MLN cells were removed on day 6 or 14 post-infection and compared with cells from uninfected (normal) age matched mice for each experiment. Cells were labelled with PE-conjugated L3T4 (CD4) or FITC-conjugated Ly-2 (CD8) (in some experiments cells were double labelled) and analyzed as in figure 8. Appropriate isotype control antibodies were used to control for nonspecific labelling and PI was used to gate dead cells (PI and PE signals were separate). Absolute numbers of L3T4⁺ and Ly-2⁺ cells in spleen or MLN were used to obtain a CD4:CD8 ratio for *H. polygyrus*-infected mice and compared to the ratios for both normal and *N. brasiliensis*-infected mice.

infection was also not associated with a decrease in the ratio of CD4:CD8 cells in the MLN compartment, but again resulted in an increase in this ratio at day 6 post-infection. *N. brasiliensis*, on the other hand, did not appear to obviously affect CD4:CD8 T cell ratios in MLN.

Comparison of T cell subsets in the spleens of high and low responder strains during infection with *H. polygyrus* also showed that the CD4:CD8 ratio was not consistently reduced. However, unlike BALB/c mice, SJL and C57BL/10 did show a decrease in the CD4:CD8 ratio at day 6 post-infection. Spleen cells examined on all other days post-infection with *H. polygyrus* exhibited increased CD4:CD8 ratios (figure 12).

It can be reasonably concluded therefore, that *H. polygyrus* infection did not induce a long-term imbalance in CD4 and CD8 T cell subsets in the mouse strains studied. The hypothesis that *H. polygyrus* induced an increase in the number of CD8 T cells that accounted for both the general immunosuppression associated with infection and the diminished mucosal mastocytosis observed *in vivo* was not supported by these data.

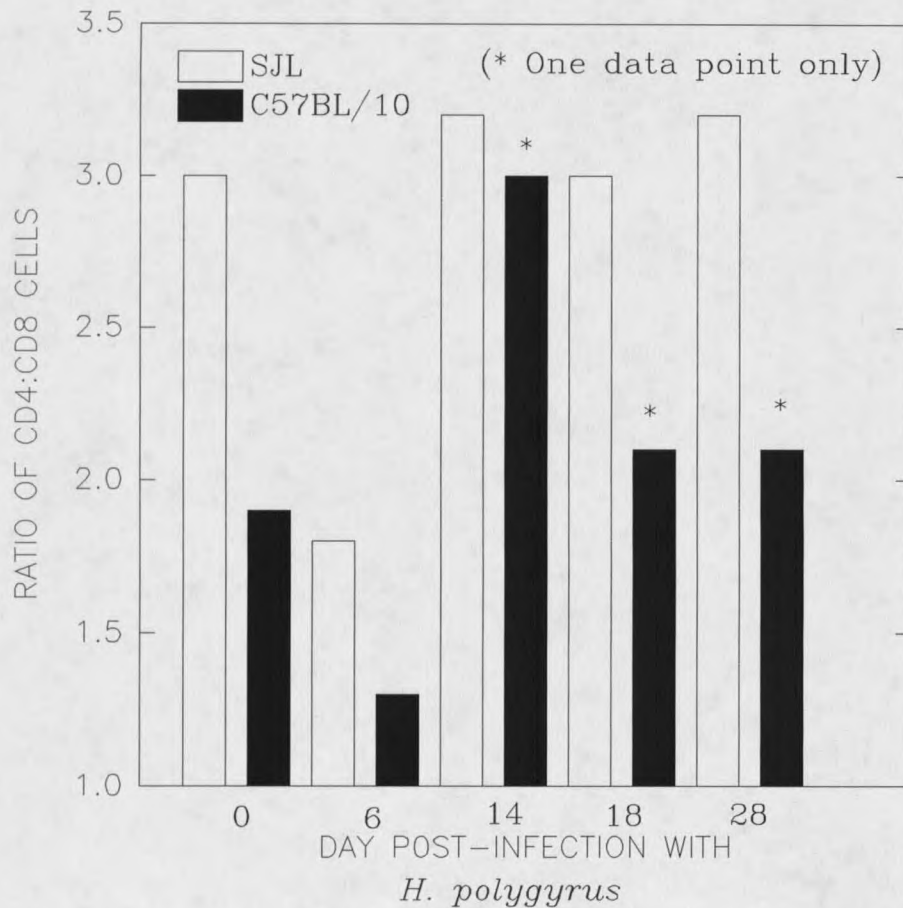


Figure 12. *H. polygyrus*-infection is not associated with a decrease in the number of CD8+ T cells in either high or low responder mouse strains. Spleen cells were removed as for figure 11 and labelled with PE-L3T4 (CD4) or FITC-Ly-2 (CD8) or the appropriate isotype control antibody and fixed with 1% paraformaldehyde prior to flow cytometric analysis. Data points represent 2-3 experiments except those denoted by an *, which represent only one experiment.

Failure of HpCM to promote mast cell development is not due to a lack of MCGFs: IL-3, IL-4 and IL-10 are present in greater concentrations in HpCM compared with NbCM.

While there was no evidence to support the notion that *H. polygyrus* infection caused an imbalance of CD4:CD8 T cell subsets, another possibility existed. CD4 T cell subsets have been divided into T helper subsets. These subsets have not been defined phenotypically by surface markers, but rather have been defined by distinct cytokine secretion profiles. TH1 and TH2 cells produce some common cytokines, but several mutually exclusive cytokines. TH1 subset cells have been associated with immune responsiveness to intracellular parasites. TH1 cells produce IL-2, IFN- γ and TNF- β , but not interleukins 4, 5, 6, 9, 10 and 13. TH2 subset cells, on the other hand, have been associated with the immune response to extracellular parasites. TH2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, but not IL-2, IFN- γ or TNF- β (35,101).

The lack of mucosal mastocytosis observed in several mouse strains infected with *H. polygyrus* could be explained by the lack of production of cytokines associated with mast cell development and growth, such as IL-4 and IL-10. It was possible that *H. polygyrus* infection induced an increase in TH1 cells which secreted IL-2 and IFN- γ . Infection with the nematode *N. brasiliensis*, which causes mucosal mastocytosis, would be expected to produce a TH2 response, characterized

by the plentiful production of IL-4 and IL-10 and low IL-2 and IFN- γ production.

Analysis of HpCM for IL-2, IFN- γ , IL-4, IL-5, IL-6 and IL-10 would help to establish whether *H. polygyrus* caused such an imbalance of these subset populations. NCM was analyzed to determine a normal level of cytokine production from naive splenocytes and NbCM (which was capable of supporting BMCMC development in culture) was analyzed to show a typical TH2 response.

While the analysis of cytokines generated in response to mitogen compared with stimulating with *H. polygyrus* antigen may be seen as artificial, experiments involving CD4 subset cytokine production using *Schistosoma mansoni* and *N. brasiliensis* antigen produced similar cytokine data when stimulated with mitogen, suggesting a generalized perturbation in these effector cells in infected mice (35). Since *H. polygyrus*-specific antigen is not available in a suitably purified form, mitogen stimulation was used to make HpCM.

Initial results with bioassays utilizing the indicator cell line HT-2 showed that NbCM contained less IL-2 and more IL-4 than did NCM, the expected result of a TH2 shift. HpCM, on the other hand, contained high levels of both IL-2 and IL-4. In fact, IL-4 levels in HpCM exceeded the level observed in *N. brasiliensis* conditioned medium (data not shown), a result that did not fit the working hypothesis of

a shift to TH1 cells.

Because bioassays are cumbersome and we wished to study the concentrations of several cytokines, ELISA assays were used to measure cytokine content in CM. A panel of ELISA assays was used to quantify IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF, TGF- β and INF- γ in spleen conditioned medium made from uninfected mice (NCM) or mice infected with either *N. brasiliensis* (NbCM) or *H. polygyrus* (HpCM) at two time points during the nematode infections (days 6 and 14 p.i.). Results are shown in figures 13 and 14.

Both nematode infections were associated with a decrease in the TH1 cytokine IFN- γ in CM made on both days 6 and 14 p.i. (figure 13). A difference was observed however, in the level of IL-2 between the two nematode infections. Where NbCM contained low levels of IL-2, HpCM contained higher levels of IL-2 that corresponded to levels observed in NCM. This agreed with earlier results indicating higher levels of IL-2 in HpCM compared with NbCM obtained using HT-2 cell bioassays.

When TH2 cytokine levels were quantified, NbCM displayed significantly higher levels of all TH2 cytokines IL-4, IL-5, IL-6 and IL-10 compared with NCM (figure 14). These results were predicted by the hypothesis that *N. brasiliensis* caused a TH2 shift. Surprisingly though, HpCM also had higher levels of all TH2 cytokines compared with NCM. In fact, analysis of HpCM revealed much higher levels

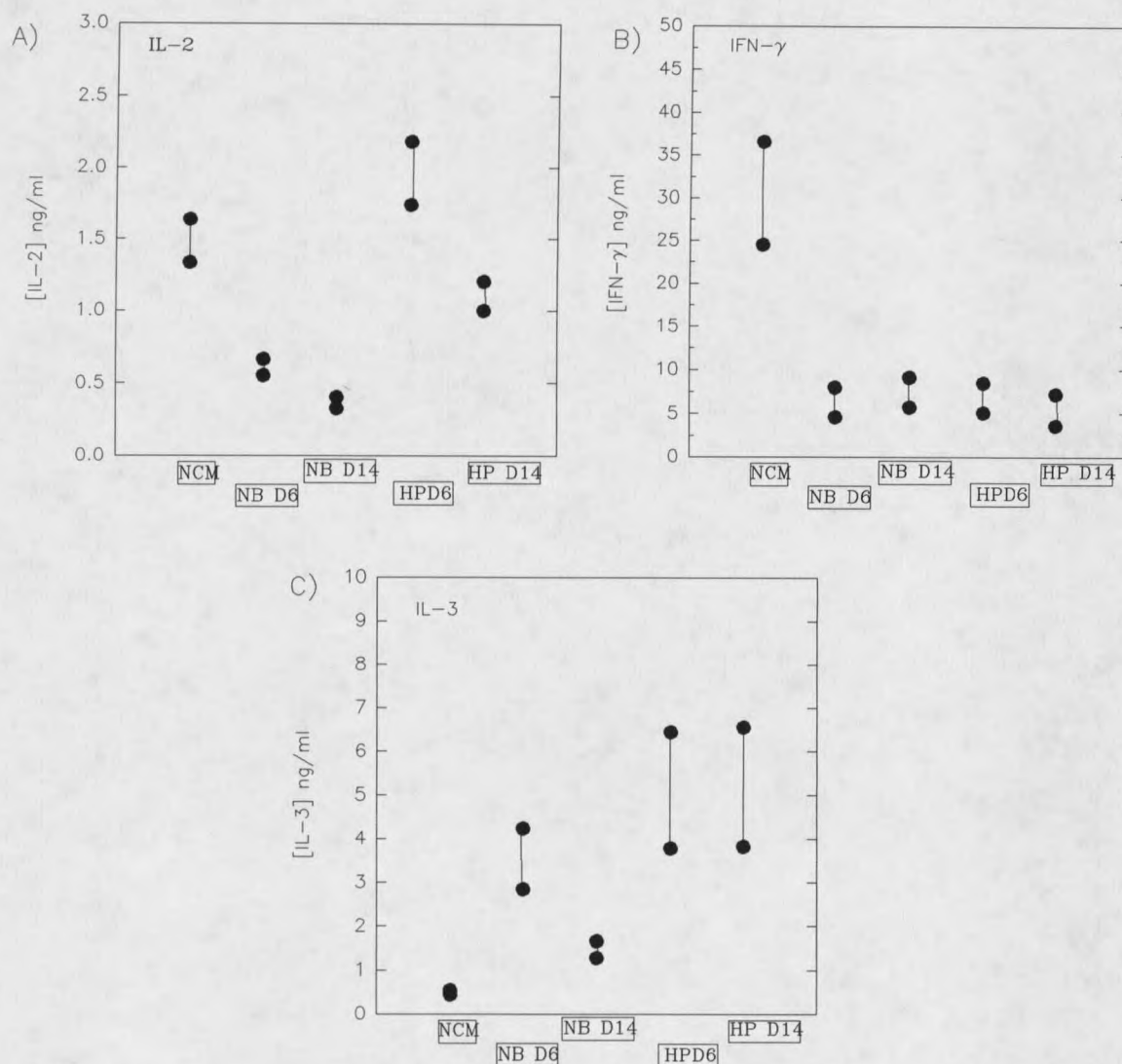


Figure 13. Concentration estimates of the TH1 cytokines, IL-2 and IFN- γ , and IL-3 as determined by ELISA in NCM and, HpCM or NbCM made on either day 6 or 14 post-infection. BALB/c CM were made from pooled spleen cells from 4-6 mice. Splenocytes were stimulated with Con A *in vitro*. Data points represent a maximum and minimum estimate of each cytokine concentration and depict the averaged concentration estimates for 3 separate ELISAs for each cytokine. NCM = normal CM, NbCM = CM from *N. brasiliensis*-infected mice, HpCM = CM from *H. polygyrus*-infected mice.

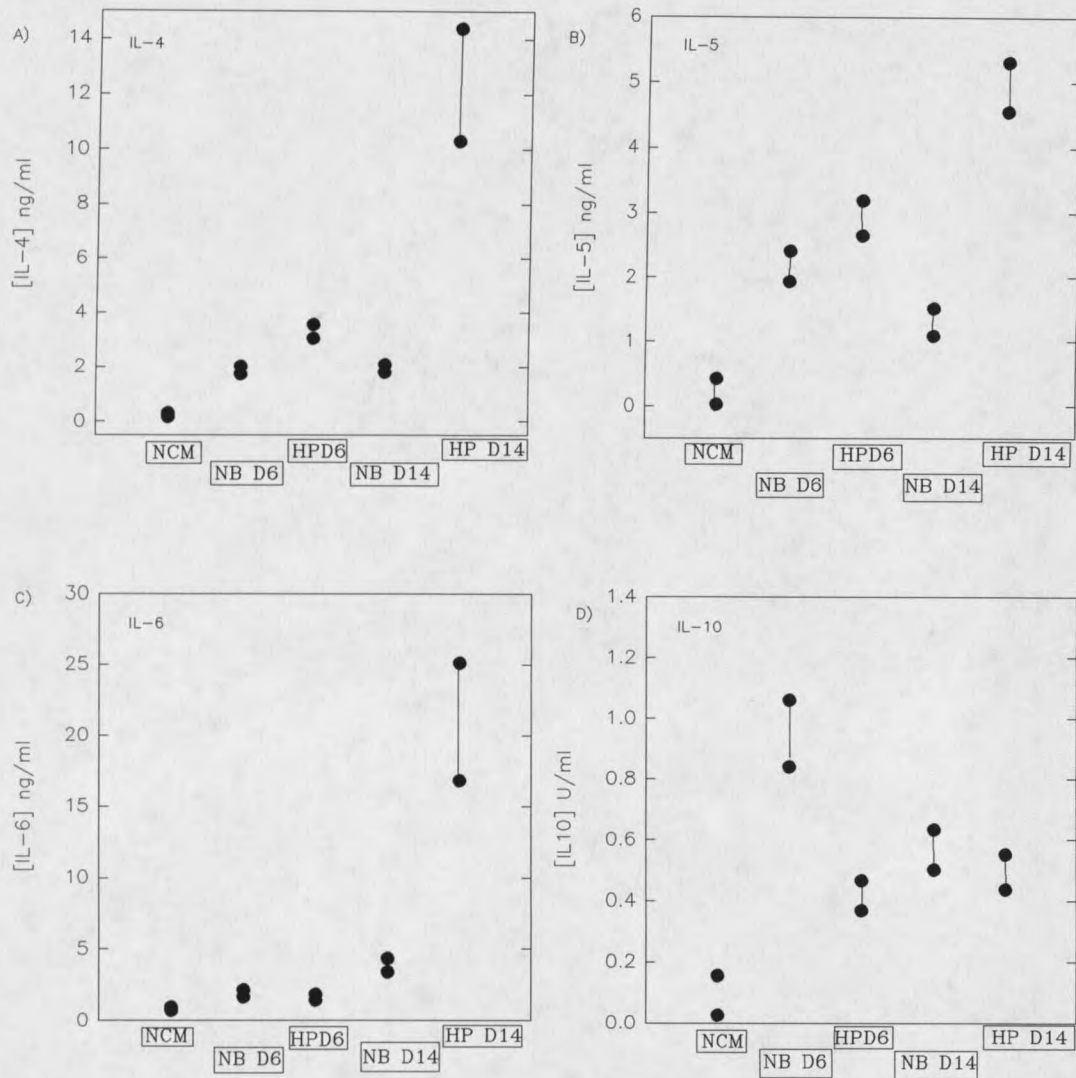


Figure 14. Concentration estimates of the TH2 cytokines, IL-4, IL-5, IL-6 and IL-10 as determined by ELISA in NCM and, HpCM or NbCM made on either day 6 or 14 post-infection. BALB/c conditioned media were made from pooled spleen cells from 4-6 mice. Splenocytes were stimulated with Con A *in vitro*. Data points represent both a maximum and minimum estimate of each cytokine concentration and depict the averaged concentration estimates for 3 separate ELISAs for each cytokine.

of IL-4, IL-5 and IL-6 compared with NbCM, particularly at day 14 post-infection. *This result did not support the hypothesis that H. polygyrus induced a TH1 shift.*

N. brasiliensis infection appeared to induce a TH2 shift associated with a TH1 down-regulation. *H. polygyrus* infection on the other hand, did not strictly follow this pattern. While HpCM had decreased levels of IFN- γ , it contained 2-3 times the IL-2 observed in NbCM. Consistent with a TH2 shift, HpCM did show elevations in all TH2 cytokines. IL-4, IL-5, IL-6 and IL-10 were all elevated above the levels observed in NCM. HpCM also had higher levels of IL-4, IL-5 and IL-6 compared with NbCM. However, the concentration of IL-10 in HpCM was approximately half of that found in NbCM on day 6 post-infection. Since IL-10 is thought to be a critical regulatory cytokine modulating TH1 down-regulation this result may be noteworthy. By day 14 p.i. however, both HpCM and NbCM had similar levels of IL-10. Therefore the significance of differential IL-10 levels in these two infections is not clear cut and should be investigated further.

Another possible explanation for the failure of mast cell development in culture was that levels of IL-3 were inadequate in HpCM. IL-3 is produced by both TH subsets and hence an imbalance of these subsets would not be expected to reduce levels of this cytokine. However, IL-3 is crucial for mast cell development in culture from bone marrow cells

and so IL-3 levels were measured. HpCM contained concentrations of IL-3 that were superior to concentrations of IL-3 observed in both NCM and NbCM (figure 13C).

Cytokine results suggested that *H. polygyrus*, although not displaying a classic TH2 shift accompanied by a TH1 cytokine down-regulation, nevertheless induced the production of plentiful amounts of IL-3, IL-4 and IL-10 at day 14 p.i. compared with NCM. These data suggested that, if anything, HpCM should be an exceptional source of mast cell growth and differentiation factors.

Levels of IL-3, IL-4 and IL-10 are higher in HpCM made from liable mouse strains compared with HpCM made from a refractory strain.

The above data suggested that a TH subset balance was not responsible for the failure of HpCM to generate cultured mast cells. The data so far had been derived from HpCM made only from BALB/c mouse spleens however, and BALB/c is a moderate responder strain to *H. polygyrus* infection. Comparison, then, between conditioned media made from high and low responder mouse strains might demonstrate more pronounced evidence of a TH imbalance. Data in figures 15 and 16 show quantitative cytokine determinations for conditioned media made from the high responder SJL strain, the low responder C57BL/10 strain and another batch of

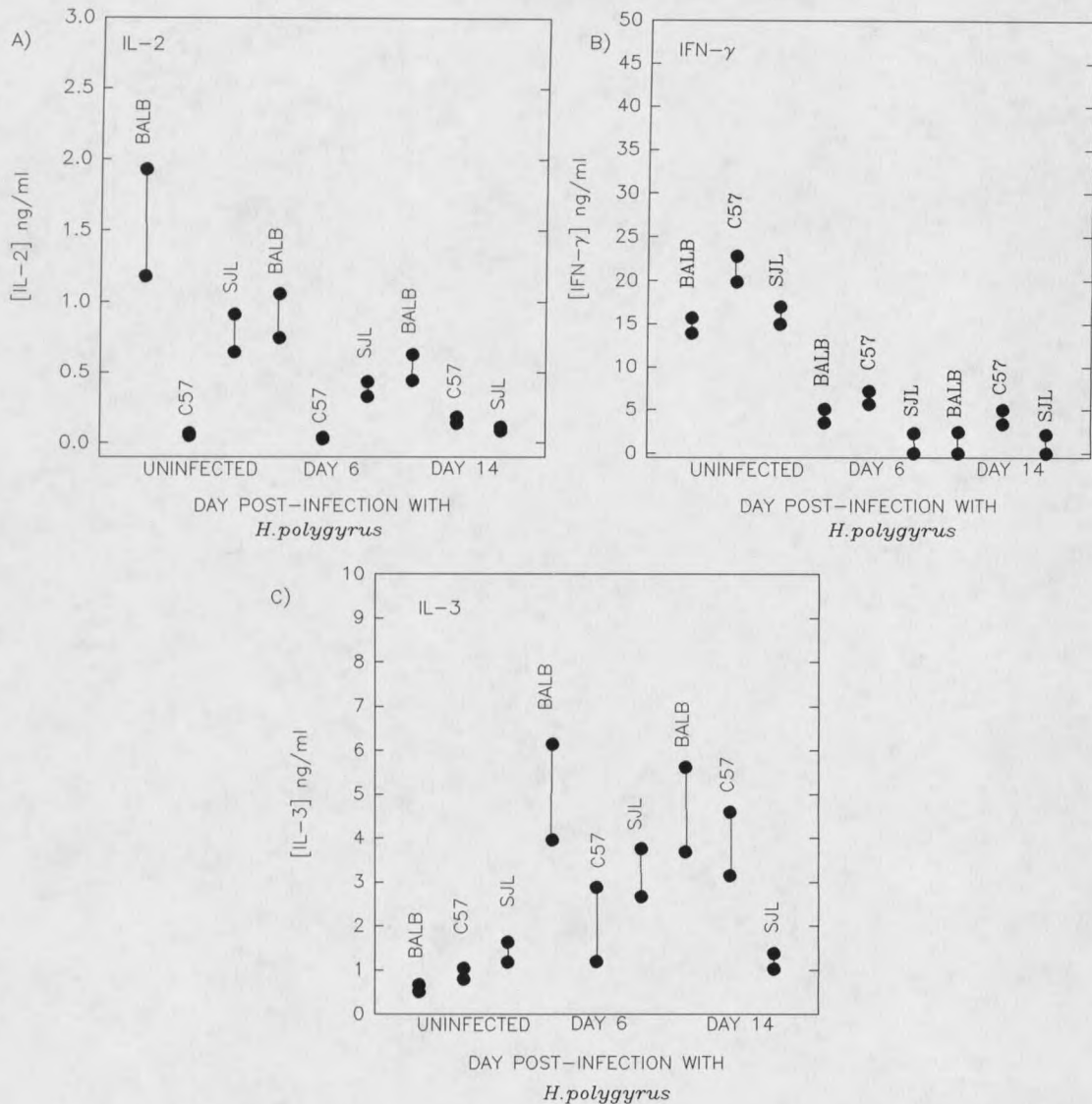


Figure 15. Concentration estimates for the TH1 cytokines, IL-2 and IFN- γ , and IL-3 in HpCM made on days 6 or 14 post-infection from the spleens of high, moderate and low responder mouse strains and their respective uninfected controls as determined by ELISA. Conditioned media were made with pooled spleen cells stimulated with Con A *in vitro* from 4-6 mice. Data points represent both a maximum and minimum estimate of each cytokine concentration and depict the averaged concentration estimates for 3 separate ELISAs for each cytokine.

conditioned medium made from the moderate responder strain, BALB/c.

The TH1 cytokine IFN- γ , similar to data represented in figure 12 which compared the two nematode infections, again was decreased in all three mouse strains after *H. polygyrus* infection (figure 15B). Down-regulation occurred more quickly in the high responder strain. The low responder strain showed higher levels of INF- γ at day 14 p.i. than the other strains perhaps indicating that it was not down-regulated as effectively as in the other strains or that down-regulation occurred more slowly in this strain. However, since the level of IFN- γ was higher to begin with in this strain, the decrease appeared to be commensurate with the other strains.

Analysis of IL-2 suggested a difference between the high responder and low responder phenotype, however. SJL HpCM made on day 14 p.i. contained much lower levels of IL-2 than NCM made from uninfected SJL mice (compared with the difference between day 14 HpCM and NCM in the other two strains) (figure 15A). For HpCM made from the moderate responder BALB/c strain, the dramatic decrease observed with IFN- γ was again not seen with IL-2. However, in C57BL/10 mice the level of IL-2 increased during the infection. C57BL/10 HpCM made on day 14 p.i. indicated that the level of this cytokine was elevated above the level of IL-2 observed in NCM in this low responder strain.

Analysis of TH2 cytokines also showed some differences between responder strains. HpCM derived from the high responder, SJL, strain did not exhibit the extraordinary increases in IL-4 and IL-5 observed in HpCM made from the other strains after infection with *H. polygyrus*. For example, SJL HpCM made on day 14 p.i. showed only a moderate increase in the amount of IL-4 over SJL NCM (figure 16A). In contrast, BALB/c and C57BL/10 HpCM made on day 14 p.i. showed a pronounced increase in IL-4 compared with NCM made from uninfected animals from either of these strains. IL-5 followed a similar trend (figure 16B).

All strains showed a striking increase in IL-6, consistent with the B cell hyperplasia observed in all strains in response to *H. polygyrus* (figure 16C). IL-10 was not dramatically elevated in HpCM derived from any of the strains, although BALB/c and C57BL/10 HpCM made on day 14 p.i. contained more IL-10 than SJL HpCM made on this day (figure 16D). These data suggest that the responder status of the animal does not appear to be dependent on a pronounced up-regulation of TH2 cytokines during infection. SJL HpCM consistently had lower levels of the TH2 cytokines, IL-4, IL-5, IL-6 and IL-10 compared with the other mouse strains. SJL mice did exhibit a more pronounced down-regulation of both IFN- γ and IL-2 than the other strains raising the possibility that down-regulation of a TH1 response is more important in the immune response to *H.*

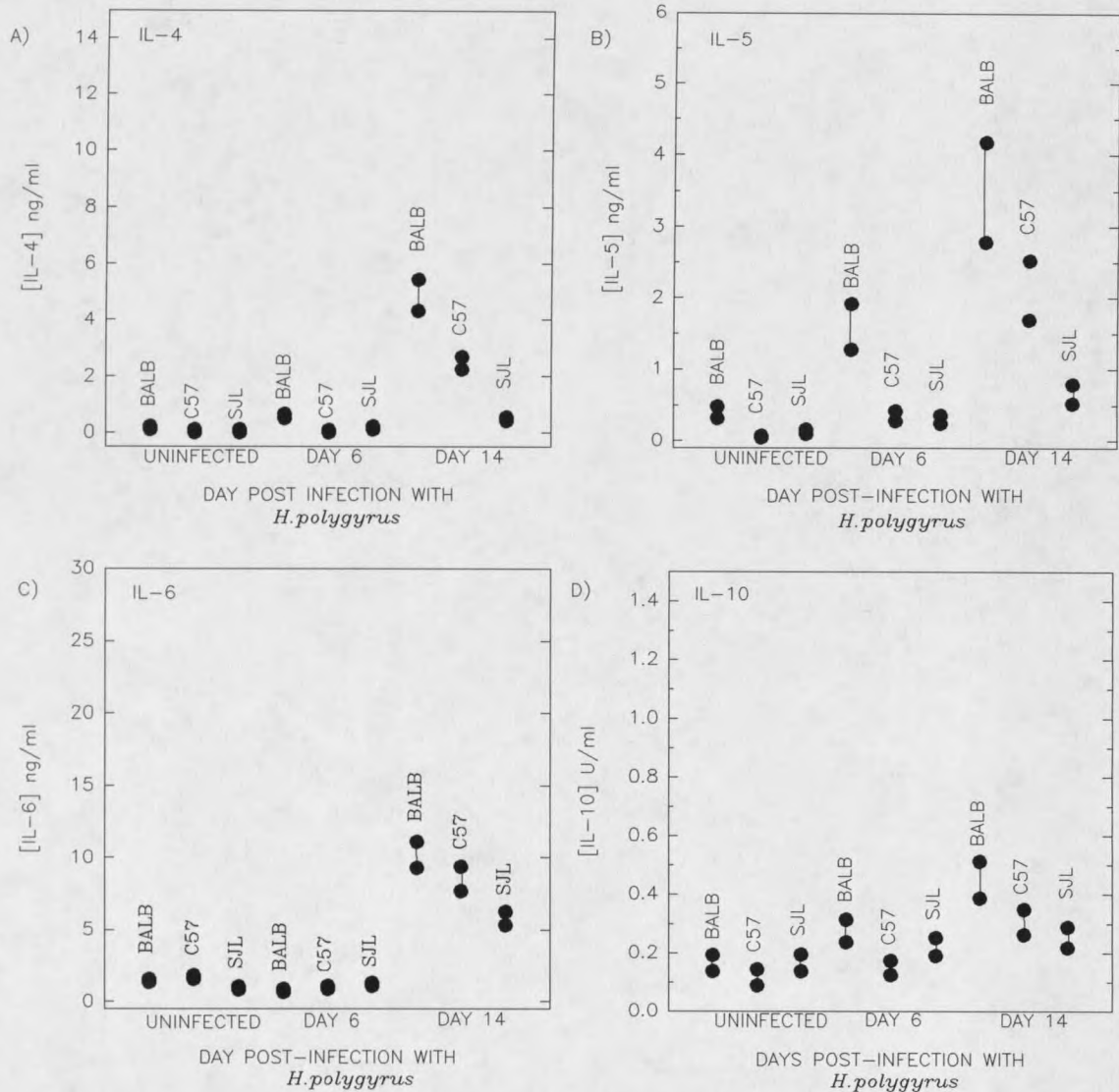


Figure 16. Concentration estimates for the TH2 cytokines, IL-4, IL-5, IL-6 and IL-10 in HpCM made on days 6 or 14 post-infection from the spleens of high, moderate and low responder mouse strains and their respective uninfected controls as determined by ELISA. Conditioned media were made with pooled spleen cells stimulated with Con A *in vitro* from 4-6 mice. Data points represent both a maximum and minimum estimate of each cytokine concentration and depict the averaged concentration estimates for 3 separate ELISAs for each cytokine.

polygyrus infection than dramatic up-regulation of the TH2 response.

SJL HpCM also had less IL-3 than HpCM from the other mouse strains. IL-3 levels were much higher in HpCM made from moderate and low responder strains (figure 15C). This was a curious result because both BALB/c and C57BL/10 mice produced HpCM that lacked mastopoietic activity, yet HpCM made from these strains contained higher levels of IL-3, IL-4 and IL-10 than SJL HpCM (which permitted some BMCMC development).

Immunoglobulin isotypes present during infection with *H. polygyrus* and *N. brasiliensis* give *in vivo* support to *in vitro* cytokine determinations.

One criticism of cytokine ELISA assays is that they may not reflect what is occurring *in vivo* in the experimental animal. In order to address this criticism, we looked at immunoglobulin isotypes present in the sera of uninfected mice and mice infected with either *H. polygyrus* or *N. brasiliensis*. Analysis of immunoglobulin isotypes has been used as evidence for a TH1/TH2 imbalance in other parasite infections (34).

Isotype switching involves the additional rearrangement of B cell heavy chain DNA where the $V_H D_H J_H$ segment combines with another C_H fragment. This class switch has been shown to be under the influence of discrete cytokines (36). For

example, IL-4 has been shown to induce a sequential class switch from IgM to IgG1 and IgE (71). IFN- γ , on the other hand, has been shown to induce an isotype switch to IgG2a. Therefore one way to demonstrate that the high levels of IL-4 observed in HpCM might also be present *in vivo* was to demonstrate elevated IgG1 and IgE in the sera of *H. polygyrus* infected mice.

Both *H. polygyrus* and *N. brasiliensis* elicited marked increases in circulating IgG1 and IgE in infected mice compared with uninfected mice (figure 17). *H. polygyrus* induced much higher levels of IgG1 and IgE than did *N. brasiliensis* however, suggesting that the higher levels of IL-4 observed in HpCM (compared with NbCM) corresponded with parallel elevations in these isotypes. In contrast, IgG2a levels did not dramatically change from the level observed in uninfected mice (data not shown). This supports the observation that the concentration of IFN- γ did not rise in response to either nematode infection.

When sera were examined from all three mouse strains after infection with *H. polygyrus*, IgG1 and IgE were again markedly elevated, consistent with the increased concentration of IL-4 present in HpCM made from the spleens of these mouse strains (figure 18). This result was observed in mice regardless of their responder status. SJL mice appeared to increase IgE production sooner (beginning at day 3) than C57BL/10 (beginning at day 6). However, the

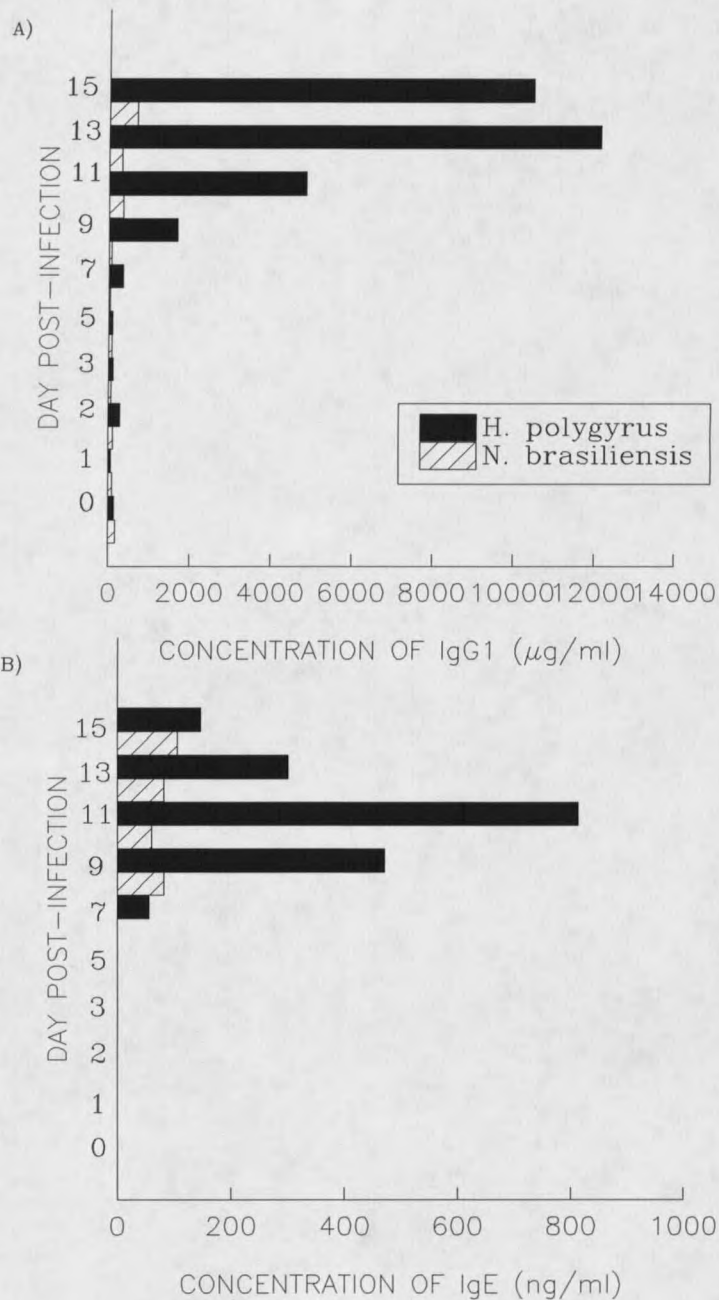


Figure 17. Concentrations of IgG1 and IgE in the sera of BALB/c mice on different days post-infection with either *H. polygyrus* or *N. brasiliensis* as determined by immunoglobulin isotype ELISA. Serum samples were obtained by retro-orbital bleeding from individual mice on each day post-infection and pooled for that day. Each data point represents a different group of 4-5 mice (i.e. mice were not bled repeatedly throughout the course of infection). Data points are averages of two separate ELISA determinations.

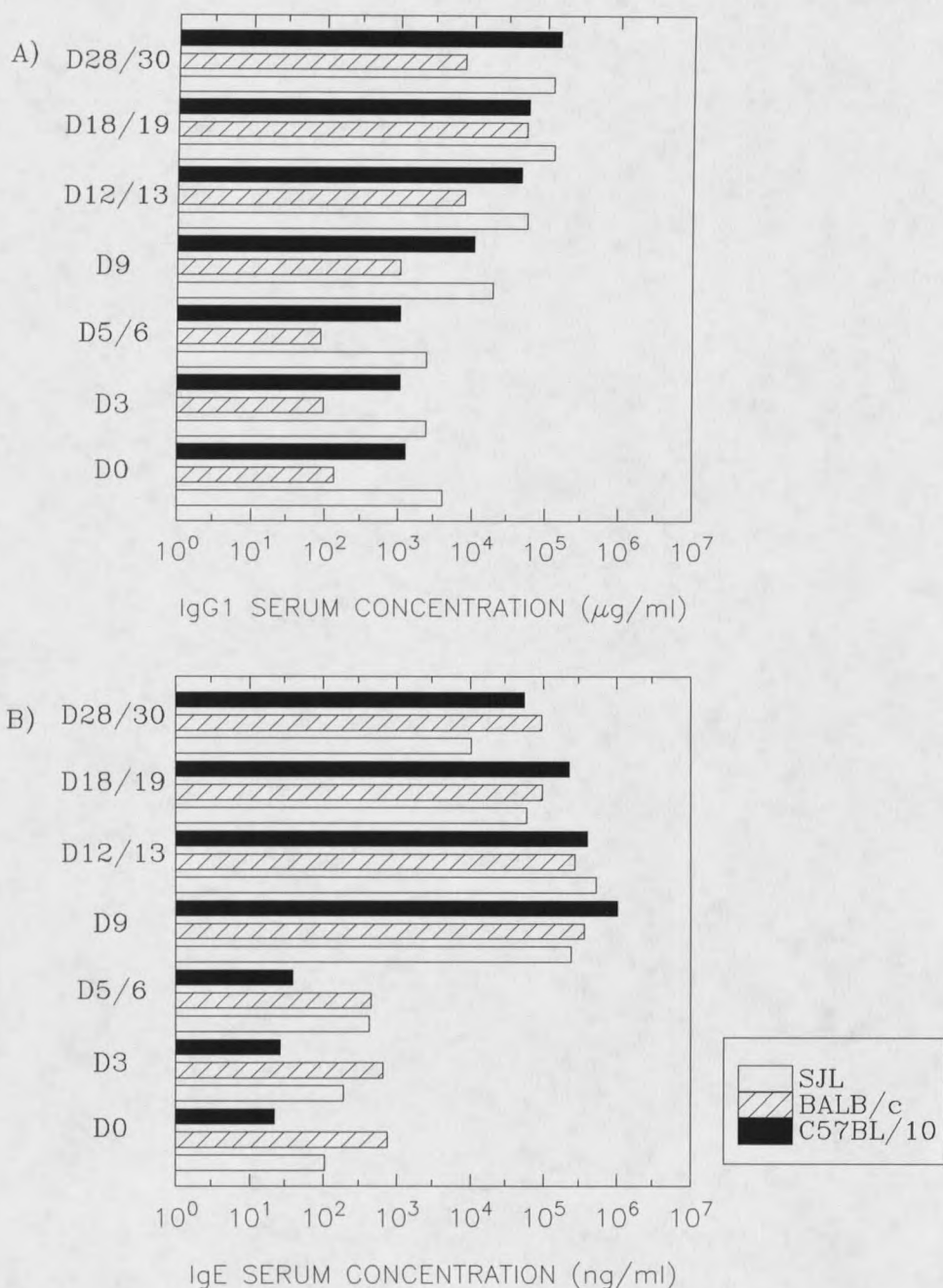


Figure 18. Concentrations of IgG1 and IgE in the sera of high, moderate and low responder mouse strains at different days post-infection with *H. polygyrus* as determined by immunoglobulin isotype ELISA. Serum samples were obtained by retro-orbital bleeding from individual mice on each day post-infection and then pooled for that day. Each data point represents a different group of 4-5 mice (i.e. mice were not bled repeatedly throughout the course of infection). Data points are averages of two separate ELISA determinations.

levels of both IgE and IgG1 were not dramatically different between SJL and C57BL/10 mice, suggesting that the lower levels of TH2 cytokines in SJL HpCM (particularly IL-4) are nevertheless capable of producing *in vivo* effects of the same magnitude as in C57BL/10 mice, at least in regard to immunoglobulin production. Both strains demonstrated a tapering back of IgE levels (but not IgG1) by day 28 of *H. polygyrus* infection.

ELISPOT assays indicate that *H. polygyrus* infection causes a proliferation of both IL-2 and IL-4 secreting cells.

The cytokine ELISA results showed that splenocytes from *H. polygyrus*-infected mice produced prodigious amounts of IL-4 and IL-5, and indicated they produced elevated amounts of IL-2. One problem with ELISA assays is that they fail to distinguish whether the same number of cells are producing more cytokine or more cells are producing the cytokine.

To address this question ELISPOT assays were done for IL-2, IL-4 and IL-5 in order to determine the number of cytokine secreting cells per spleen. Splenocytes from BALB/c mice that were either uninfected or infected with *H. polygyrus* for 14 days were compared. Spleen cells from *H. polygyrus*-infected mice displayed approximately a log-fold increase in the number of cells secreting IL-2 and IL-4 (Table 6). These data indicated that IL-2 did not appear to

be down-regulated as efficiently in *H. polygyrus* infection compared with *N. brasiliensis*-infection. These data also are consistent with flow cytometric data indicating an increase in the absolute number of CD3 cells during *H. polygyrus* infection (particularly on day 6 p.i.) since IL-2 acts as a T cell proliferative cytokine.

Table 6. Frequency of cells secreting cytokine as determined by ELISPOT assay.

	<u>Source of cells</u>	
	Uninfected	<i>H. polygyrus</i> -infected
<u>Cytokine</u>		
IL-2	4.36 X 10 ³	346 X 10 ³
IL-4	8.62 X 10 ³	328 X 10 ³
IL-5	8.8 X 10 ⁶	12.5 X 10 ⁶

^a Numbers indicate the frequency of cytokine-secreting cells per spleen in BALB/c mice.

Spleen cells were removed from uninfected mice or mice infected with *H. polygyrus* at day 14 post-infection. The frequency of cytokine secreting cells was determined by counting two-fold serial dilutions of cells and constructing a linear regression curve to extrapolate the frequency of cells per 10⁶ spleen cells. Total spleen cells were averaged (3-4 experiments) and then adjusted for 10⁶ cells to yield the frequency of cytokine secreting cells per spleen.

In contrast, the number of IL-5 secreting cells in the spleen did not appear to increase markedly with *H. polygyrus* infection. Since ELISA data showed that BALB/c HpCM had markedly elevated levels of IL-5, this result might suggest that the same number of cells produced more cytokine. The

ELISPOT data imply that IL-4 and IL-5, both TH2 cytokines, might be regulated differently. Limiting dilution experiments would be required to further explore this possibility, however.

The cytokine results and the ELISPOT data suggested two things. One, the hypothesis that *H. polygyrus* infection caused an imbalance in TH subsets was not in the direction proposed since IFN- γ levels were low in HpCM (although the level of IL-2 remained high in HpCM compared with NbCM). More importantly, TH2 cytokines were present in copious amounts, suggesting that *H. polygyrus* induced a significant TH2 response. Two, all known mast cell development and growth factors were present in HpCM in even greater amounts than in NCM and NbCM (except for IL-10). Since both NCM and NbCM could generate BMCMC, this result was puzzling. This second point led us to begin investigating the possibility that an inhibitor of mast cell development might be present in HpCM.

NCM does not support the development of mast cells in culture when mixed with HpCM.

The cytokine data had so far ruled out the possibility that a known growth factor, necessary for BMCMC development in culture, was absent from HpCM. Since new cytokines were being discovered on a regular basis though, it remained possible that HpCM could still be lacking a specific factor.

For example, both IL-9 and SCF, cytokines that are required for or stimulatory for mast cell development, had not been assayed.

Therefore, the design of the bone marrow culture assay was modified to test whether HpCM lacked a necessary factor for the development of BMCMC, or rather, contained an inhibitory factor to BMCMC development. HpCM was made using spleens from mice infected with *H. polygyrus* for 6 or 14 days (HpCM D6 and HpCM D14) as usual. To control for a dilution effect that would occur when two different CM were mixed together (particularly if one was inert), CM was also derived from athymic nude mice (NuCM).

Results demonstrate that, as expected, a dilution effect was observed when NuCM was added to NCM (figure 19). However when HpCM D14 was added to NCM (which contains all necessary factors for the development of BMCMC) mast cell development was not observed after four weeks in culture. This result indicated that HpCM D14 contained a factor that could *inhibit* the development of mast cells in culture. Furthermore, analysis of the HpCM D6 + NCM histogram in figure 19 indicates that HpCM D6 did not inhibit BMCMC development when mixed with NCM (although HpCM D6 alone inhibited BMCMC development) suggesting that the inhibitor was not present at a sufficient level to prevail over NCM until later in the infection. This result implied that the concentration of the inhibitor (and possibly the number of

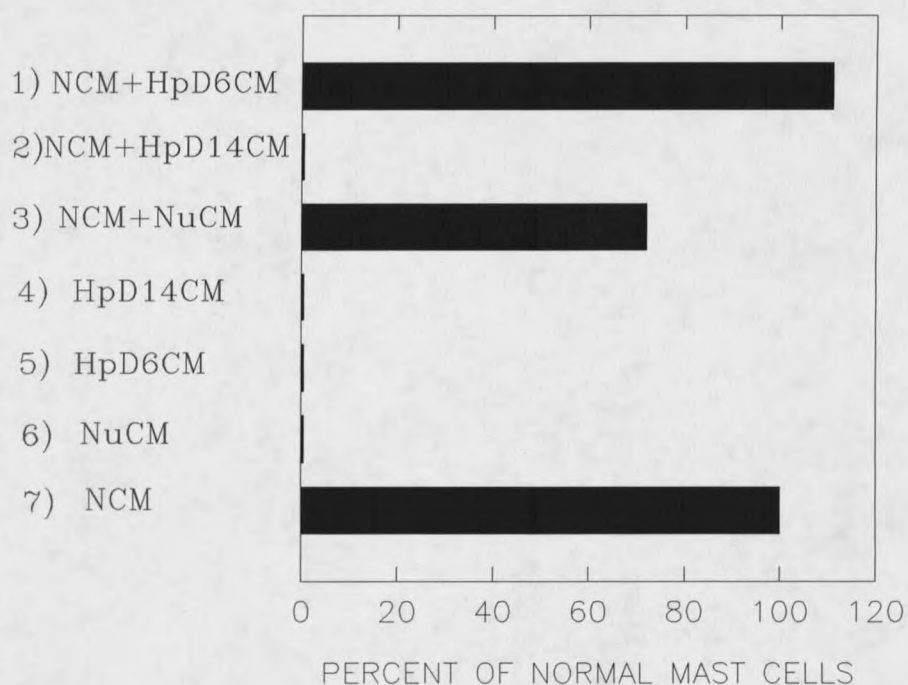


Figure 19. Evidence that HpCM contains an inhibitor of mast cell development. Bone marrow cells were cultured for 4 weeks with either NCM or HpCM made on day 6 or 14 post-infection. Conditioned medium made from nude mice (NuCM) was used to control for any effect that might be due to the dilution of mast cell growth and differentiation factors (MCGF) in mixtures of CM. NCM by itself supported BMC MC development (7) but failed to support BMC MC development when mixed with HpCM made on day 14 p.i. (2). When NCM was mixed with HpCM made on day 6 p.i. however, BMC MC developed (1), even though HpD6CM did not support BMC MC development by itself (5). When NCM was mixed with NuCM BMC MC developed, but the numbers were slightly decreased presumably due to MCGF being diluted (3). Thus, when NCM (which contains all MCGF necessary for the development of BMC MC) was present, BMC MC still did not develop with HpD14CM, suggesting that the failure of mast cell development with HpCM was not due to a lack of MCGF, but rather to an active inhibitor of mast cell development.

cells producing it) *increased* during infection with *H. polygyrus*.

These data strongly suggested that the failure of mast cells to develop in cultures containing HpCM was not due to a *lack* of mast cell growth and differentiation factors, but rather, was due to an active inhibitor. This was supported by the observation that HpCM D6+NCM promoted mast cell development whereas HpCM D14+NCM did not. This consequence was not due to a dilution effect since NCM+NuCM resulted in only a slight retardation of mast cell development in culture, as would be expected if growth factors in NCM were being diluted.

A non-adherent splenocyte produces the inhibitor.

Since the data summarized above strongly suggested the presence of an inhibitor of mast cell development in HpCM, we were interested in determining which cell type in the Con A activated spleen cell culture was producing the inhibitory factor. In order to show which type of spleen cell was producing the inhibitor, cell separation techniques were done on spleens from both uninfected and *H. polygyrus*-infected mice. Splenocyte fractionation of adherent and non-adherent cells was carried out by separating adherent and non-adherent cells by attachment to plastic. Spleen cells used to make CM were separated into two populations: 1) adherent to plastic, and 2) non-adherent to plastic.

This separation procedure was done for both uninfected spleens and *H. polygyrus*-infected spleens. Therefore, CM could be made from mixtures of adherent cells and non-adherent cells from uninfected mice and *H. polygyrus*-infected mice.

When *adherent* splenocytes from *H. polygyrus*-infected mice were mixed with normal non-adherent cells to make CM, BMCMC development occurred (figure 20). In contrast, when *non-adherent* splenocytes from *H. polygyrus*-infected mice were mixed with normal adherent cells to generate CM, BMCMC did not occur. These results indicated that non-adherent splenocytes from *H. polygyrus*-infected mice were responsible for manufacturing and secreting the inhibitory factor.

In an experiment designed to further characterize the cell type producing the inhibitor, nude mice were infected with *H. polygyrus* and HpCM was made on day 14 post-infection. When nude HpCM was used to culture bone marrow cells for 28 days it did not support the development of BMCMC by itself (figure 21). This was expected since the necessary cytokines for mast cell development would not be expected to be present. However, the inhibitory activity normally present in HpCM was also not present, since HpCM made from nude mice, unlike HpCM made from euthymic mice, did not suppress cultured mast cell development when mixed with NCM. These data strongly suggested that production of the inhibitor was T cell dependent.

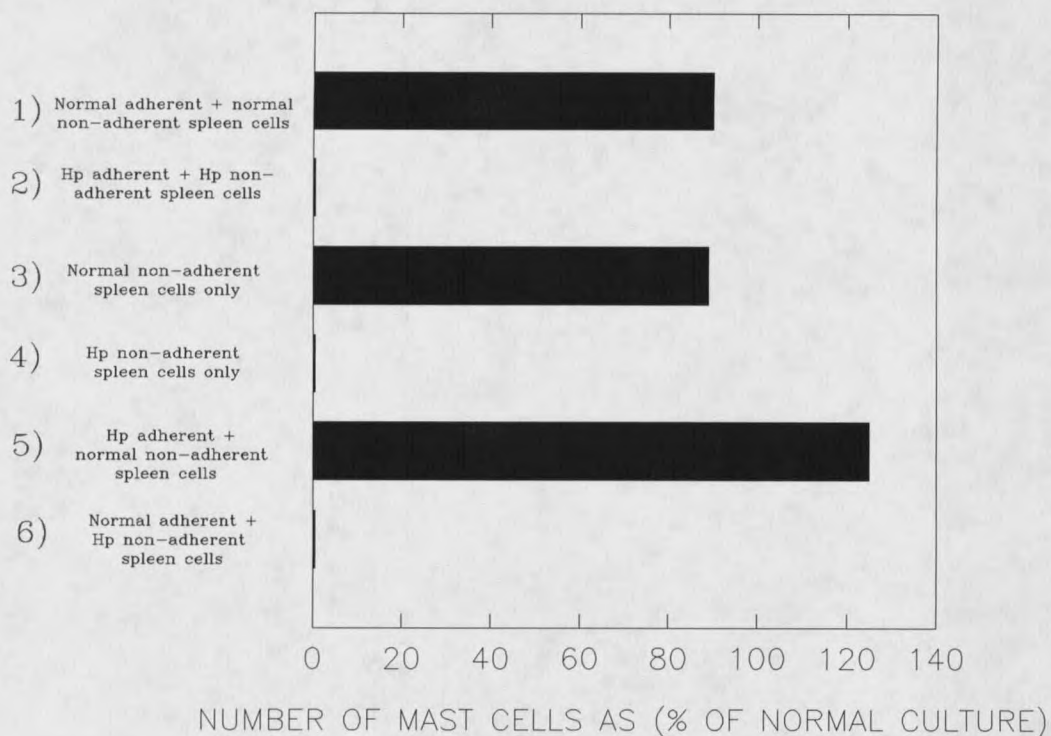


Figure 20. Evidence that non-adherent spleen cells from *H. polygyrus*-infected mice are responsible for producing the inhibitor of BMCMC. Bone marrow cells were cultured for 4 weeks with CM made from normal adherent and normal non-adherent spleen cells (1); normal non-adherent spleen cells only (3); adherent and non-adherent spleen cells from *H. polygyrus*-infected mice (2); non-adherent spleen cells from *Hp*-infected mice (4); or normal non-adherent cells and adherent spleen cells from *Hp*-infected mice (5) or, non-adherent spleen cells from *Hp*-infected mice and normal adherent spleen cells (6). Non-adherent spleen cells from *H. polygyrus*-infected mice inhibited the development of BMCMC whereas adherent spleen cells from infected mice did not.

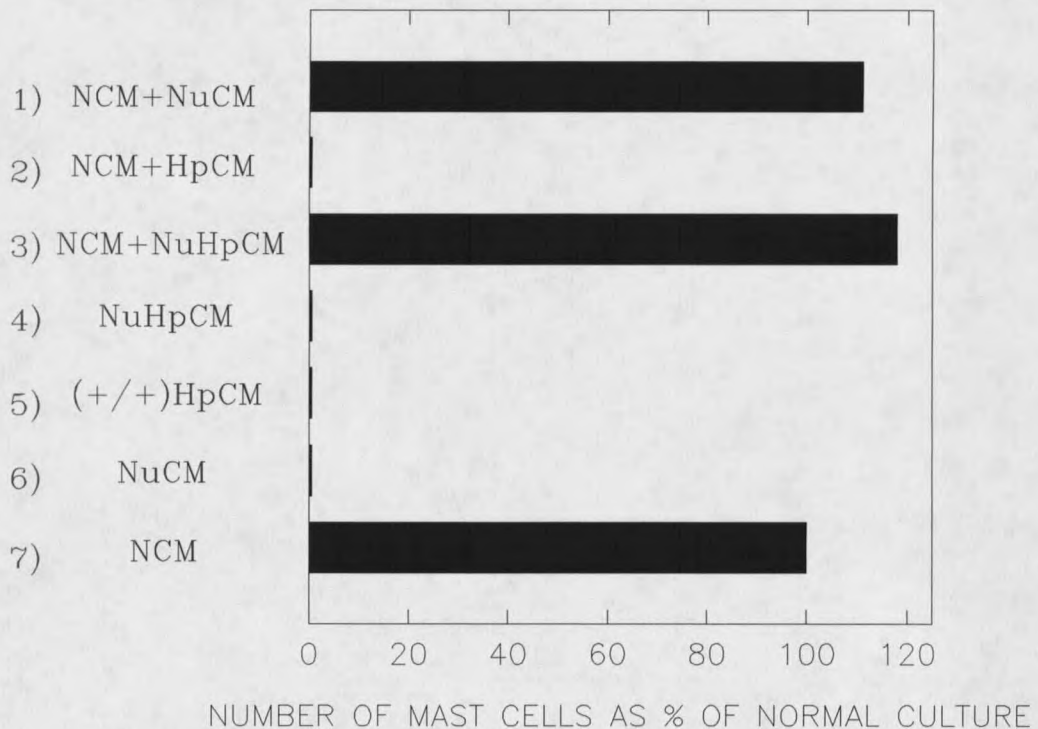


Figure 21. Evidence that HpCM made from the spleens of nude mice infected with *H. polygyrus* does not contain the mast cell inhibitor. Bone marrow cells cultured for 4 weeks with HpCM made from +/+ littermates produced the BMCMC inhibitor (2), whereas HpCM made from nude mice infected with *H. polygyrus* did not (3) since BMCMC developed when NuHpCM was mixed with NCM. Bone marrow cells were cultured for 4 weeks with CM. To control for a dilution effect when CM were mixed together, conditioned medium was made from uninfected nude mice (NuCM) and mixed with NCM (1). NuCM alone does not support the development of BMCMC due to a lack of MCGF (6). NuHpCM alone fails to produce BMCMC (4).

The inhibitor present in HpCM acts early in the development of cultured mast cells from bone marrow progenitors.

Given the growing evidence for the presence of an inhibitor of mast cell development in HpCM, it was important to establish when, during the culture period, the inhibitor was active. This would help determine the following: 1) whether mast cells could develop but simply not proliferate in culture, and 2) whether BMCMC inhibition was reversible. To investigate these questions, bone marrow cells were initially cultured with either NCM or HpCM. Normally, during the culture period, cultures are given fresh CM after 2 days and then every 4 days for the remaining period of culture. In these experiments, however, the CM was switched from NCM to HpCM or vice versa on day 2, 5 or 8 of the 28 day culture period. After substitution of CM, those cultures continued to receive the new type of CM for the remainder of the culture period.

Results shown in figure 22A indicate that if bone marrow cells were cultured with NCM initially for as little time as two days, mast cell progenitors differentiated despite being exposed to the inhibitor present in HpCM for the remainder of the 28 day culture period. This suggests that the inhibitor present in HpCM was not simply anti-proliferative. Conversely, data represented in figure 22B show that cultured mast cells developed if bone marrow cells

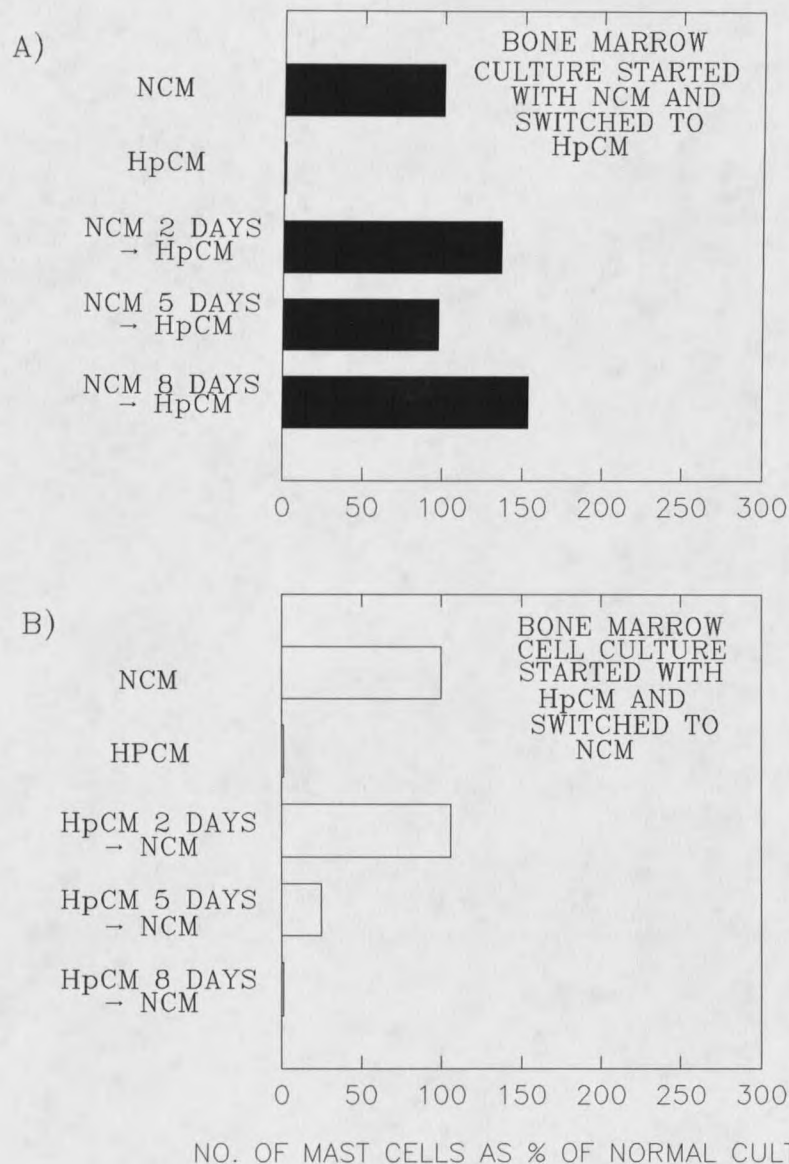


Figure 22. Evidence that HpCM inhibits mast cell development at a very early stage and does not simply inhibit proliferation. Bone marrow cells were cultured for 4 weeks with either NCM or HpCM A) and B) with the usual result: BMCMC develop with NCM but not with HpCM. However if bone marrow cells were cultured with NCM first for as few as 2 days, BMCMC developed even when HpCM was substituted for the remaining culture period (Fig. 22A). Conversely, if bone marrow cells were cultured first with HpCM, BMCMC could develop only if HpCM was present for the first 2-5 days before NCM substitution; if HpCM was present for 7-8 days BMCMC development was irreversibly inhibited despite the presence of NCM for the remaining 3 weeks of culture (Fig. 22B).

were only exposed initially (during the first few days of the four week culture period) to HpCM and then subsequently exposed to NCM. Exposure of bone marrow cells to HpCM for 7-8 days irreversibly shut down BMCMC development despite the presence of NCM for the remaining culture period.

These data indicate that commitment to a mast cell differentiation pathway occurred very early in culture and once that commitment was made, the inhibitor was unable to suppress further mast cell development. These data also indicate that the inhibitor was functional at a very early stage in mast cell differentiation and a narrow window for mast cell development appeared to exist before precursors were blocked from following a mast cell differentiation pathway. These results are consistent with observations indicating that HpCM is a superior source of growth factors for mature cultured mast cells (data not shown).

Semi-quantitative analysis of possible mast cell inhibitors suggests that GM-CSF may be a likely candidate for the inhibitor in HpCM.

Three cytokines have been identified with mast cell inhibitory activity; IFN- γ , TGF- β and GM-CSF. IFN- γ is the product of activated T cells and among the myriad effects elicited by this cytokine several regulatory actions have been described, including inhibition of cellular growth and differentiation. One report demonstrated that IFN- γ

inhibits proliferation of mouse bone marrow cells stimulated with IL-3 or other cytokines (41). Another report demonstrated an anti-proliferative effect on mast cell precursors but not on mature mast cells (76). This inhibitory effect is observed not only on mast cell progenitors but also on further differentiated precursors up to day 21 of culture.

IFN- γ was not considered further for two reasons. One, previous results from the cytokine analysis of HpCM indicated that IFN- γ was decreased in HpCM compared with NCM, making it an unlikely candidate for the cause of inhibition. Two, the experiments analyzing the kinetics of inhibition suggested that the inhibitor in HpCM acted early (during the first week of culture) on bone marrow progenitors rather than on more mature cells.

GM-CSF and TGF- β , two other cytokines associated with mast cell inhibition, were determined in HpCM, NbCM and NCM using ELISA. GM-CSF levels were also determined for high, moderate and low responder mouse strains. TGF- β_1 is a homodimer of 25 kD involved in modulating several cell types. TGF- β_1 is highly conserved among species; bovine, human and mouse TGF- β_1 are 99% homologous. TGF- β_1 opposes the mitogenic effects of PDGF on fibroblasts, FGF on endothelial cells, IL-2 on T cells, GM-CSF on hemopoietic cells and IL-3 on IL-3 dependent mast cells (17).

The results of TGF- β_1 analysis indicated that HpCM

contained approximately 1.3 ng/ml of TGF- β compared with about 1.0 ng/ml in NbCM and 1.1 ng/ml in NCM. These data suggested that TGF- β was not the likely inhibitor of BMCMC development since the amount of TGF- β was similar in all three samples of conditioned media. Furthermore, two groups had shown that TGF- β acted on mature mast cells. Keller et al. showed that TGF- β inhibited IL-3-dependent proliferation of bone marrow cells and that the effect was dose dependent (60). Approximately 50% inhibition was observed with 1 ng/ml of recombinant TGF- β_1 . Broide et al. confirmed this report and found that TGF- β inhibited IL-3 dependent mast cell proliferation but did not affect differentiation (17). These results are consistent with most of the biological effects of TGF- β that have been described. TGF- β inhibits the growth of several different cell lines, particularly epithelial cells but also T and B cells, primarily by suppressing proliferation (96). Therefore, TGF- β did not fit the profile of the inhibitor found in HpCM.

GM-CSF is a 22 kD protein that is important in hemopoiesis and angiogenesis. Although GM-CSF had been reported to act synergistically with IL-3 *in vivo* to produce myeloid cells, Bressler et al. reported that recombinant (r) GM-CSF inhibited mast cell proliferation and generated bone marrow cell cultures containing fewer than 1% mast cells (16). Moreover, when rGM-CSF was added to bone marrow cultures containing IL-3, GM-CSF inhibited mast cell

development in a dose dependent manner.

GM-CSF appeared to be a likely candidate for the inhibitory factor for several reasons. First, although there was more GM-CSF in NbCM compared with NCM, there was much more GM-CSF in HpCM. Specifically, there was approximately two-fold more GM-CSF in NbCM than in NCM (figure 23A). The concentration of GM-CSF in HpCM however, was 4-5 fold higher than the concentration of GM-CSF in NCM. Second, GM-CSF values followed the trends of responder status nicely, with low responder C57BL/10 mice having the highest concentration of GM-CSF and SJL having the lowest (figure 23B). Moderate responder BALB/c mice showed intermediate levels of GM-CSF. These data suggested a correlation between the quantity of GM-CSF in HpCM and lack of mast cell development in culture. The higher levels present in NbCM could be stimulatory to mast cell development since some evidence suggested that some GM-CSF was needed for mast cell growth and differentiation (73). Third, the cell type that was observed after four weeks of culture with HpCM morphologically resembled a macrophage. This last observation was consistent with a report by Bressler et al. showing that when mouse bone marrow cells were cultured with IL-3 for 3 weeks, mast cells comprised up to 95% of the cells present (16). However, when bone marrow cells were cultured with GM-CSF the cultures contained fewer than 1% mast cells and instead contained predominately

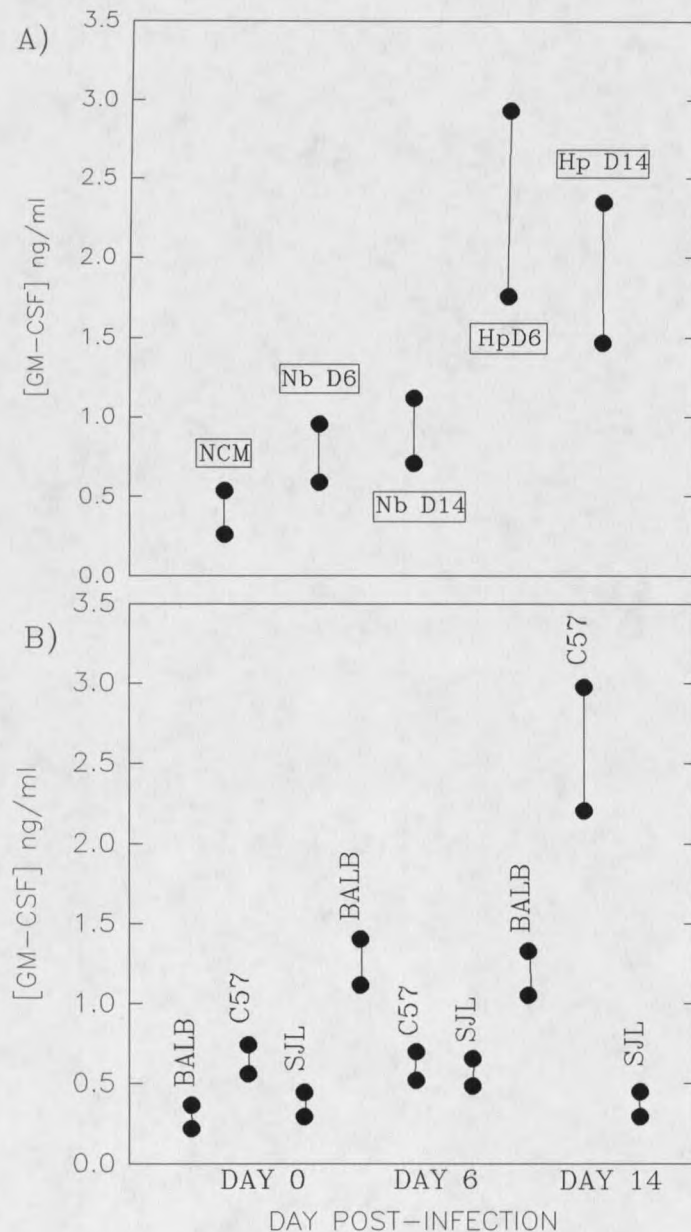


Figure 23. Concentration estimates of GM-CSF in NCM and HpCM or NbCM made from BALB/c spleens either day 6 or 14 post-infection (A) or in HpCM made on days 6 or 14 post-infection from the spleens of high, moderate and low responder mouse strains and their respective uninfected controls (B) as determined by ELISA. Conditioned media were made with pooled spleen cells from 4-6 mice stimulated with Con A *in vitro*. Data points represent both a maximum and minimum estimate of each cytokine concentration and depict the averaged concentration estimates for 3 separate ELISAs for each cytokine.

macrophages and granulocytes.

The GM-CSF data led to the development of a new hypothesis to explain the absence of mast cells with HpCM. Rather than specific inhibition of mast cells in HpCM cultures, there might be a shift to an alternative differentiation pathway occurring in HpCM cultures. This would be consistent with the nature of progenitor cells which are pluripotent cells. These cells must have the capacity to respond to several competing cytokine messages that are present in any given microenvironment; otherwise it would be necessary to postulate different microenvironments for the generation of each cell lineage. These pluripotent cells must be able to respond hierarchically to competing external messages, giving the host the flexibility to respond to different local conditions requiring different cell types. In fact, after the development of this hypothesis, Lopez et al. (46) reviewed evidence that GM-CSF, IL-3 and IL-5 could compete for receptors on human hemopoietic cells by using redundant receptor subunits.

Although this cross-competition had not yet been demonstrated in the mouse, it seemed a logical proposal in the context of our bone marrow cell cultures. When low concentrations of GM-CSF are present along with IL-3, as in NCM, mast cells can develop. However, when high levels of GM-CSF are present, even in the presence of high IL-3 (as in HpCM) GM-CSF competes for the common beta subunits shared

with IL-3, and macrophages develop rather than mast cells. In this way the culture microenvironment could promote the development of one inflammatory response over another. If GM-CSF was responsible for this alteration of the developmental pathway, it was likely that the type of cell present in HpCM cultures was a granulocyte or macrophage. In fact, in human bone marrow cultures GM-CSF had been shown to compete with IL-3 and IL-5 for receptor subunits leading to differentiation of monocytes rather than mast cells or eosinophils (46).

The predominant non-mast cell type observed in HpCM cultures is a macrophage.

Up to this point we had concentrated almost exclusively on the absence of mast cell development in bone marrow cell cultures incubated with HpCM. However, morphologically distinct cells were observed in cultures containing HpCM, albeit in greatly reduced numbers. Consequently we were interested in identifying the type of cell that developed in cultures containing HpCM.

Cytospin analysis of these cells showed large, vacuolar cells. Many of these cells appeared to be multinucleate giant cells. These cells also possessed numerous, prominent nucleoli. Based on morphological evidence alone, the cell appeared to be a macrophage, however it was also possible that the cell was a terminally differentiated B cell or

plasma cell. This was possible because HpCM also contained abundant amounts of IL-4 and IL-6, which are both known as B cell growth factors.

Several techniques were used to identify the type of cell (what we referred to loosely as Hp cells) in HpCM cultures. Because of the low numbers of Hp cells present after 28 days of culture, flow cytometry was not possible. Therefore Hp cells were stained with FITC-conjugated surface markers and observed using fluorescence microscopy. While these results were not quantitative, they provided at least a qualitative demonstration of the phenotype of the Hp cell. Preliminary results showed that the Hp cell stained brightly with RB8C5.1 (GR-1) and very brightly with Mac-1. Thioglycollate-stimulated mouse peritoneal cells and a macrophage cell line P388D1 were included as positive controls and a lymphocyte cell line, SP2/0, was included as a negative control. The results, shown in Table 7, demonstrate that cells that developed in HpCM were labelled more distinctly with Mac-1 and RB8C5.1 than were either of the positive controls. Hp cells were not labelled with B220, the B cell marker (data not shown).

Nonspecific esterase staining is another technique used as evidence of a macrophage cell type. Hp cells stained very well using this technique, even more strongly than peritoneal macrophages (Table 7). Finally to assay for phagocytosis, Hp cells were incubated with *Candida*

albicans. Data summarized in Table 7 show that Hp cells engulfed yeast.

Table 7. Phenotypic characteristics of bone marrow cells that developed in culture with HpCM.

<u>Treatment</u>	<u>Type of cell</u>			
	Peritoneal macrophages	P388D1 cells	Hp cells	SP2/0 cells
Non-specific esterase	++	-	++++	-
GR-1	++	+	+++	-
MAC-1	+++	+	+++++	+
Isotype control	+	-	++	-
None	-/+	-	-/+	-
Phagocytosis	+	ND	+	ND

Peritoneal macrophages and both a macrophage (P388D1) and a non-macrophage (SP2/0) myeloma cell line were used for comparison for several different types of treatment designed to characterize Hp cells. Peritoneal cells were stimulated with thioglycollate for 72 hours before removal by lavage. Hp cells were derived from bone marrow cells cultured for 4 weeks with HpCM made on day 14 post-infection with *H. polygyrus*. Phagocytosis was determined by ingestion of *Candida albicans* cells.

Taken together, these data strongly suggest that the cells that developed in the presence of HpCM were macrophages.

Cytokine removal from HpCM suggests that the mast cell inhibitor is IL-4.

Evidence to this point had strongly implicated an inhibitor of mast cell development that accounted for the absence of mast cells in cultures containing HpCM. Analysis of the concentrations in HpCM of three possible mast cell inhibitors had suggested that GM-CSF was the most likely candidate for the inhibitor. First, GM-CSF was present in HpCM in higher concentrations than in NCM or NbCM. Second, GM-CSF was present in higher concentrations in C57BL/10 and BALB/c mice compared with high responder SJL mice. It was possible then, that GM-CSF was responsible for shifting the direction of differentiation toward that of monocytes/macrophages and neutrophils.

While the discovery that the cell type present in cultures containing HpCM was a macrophage was consistent with GM-CSF acting as the inhibitor, there was one nagging inconsistency. Data shown in figure 23 indicate that the concentration of GM-CSF in HpCM was the same or higher on day 6 post-infection than on day 14 post-infection. This was puzzling because data derived from experiments in which HpCM was mixed with NCM showed that day 6 p.i. did not inhibit mast cell development when NCM was present, suggesting that the concentration of the inhibitor was lower at this time in the infection than at day 14. If we were to look for the inhibitor based on this observation, several

TH2 cytokines were implicated such as IL-4, IL-5 and IL-6. Due to the availability of reagents, IL-4 was chosen.

Anti-IL-4 antibody (11B11) or anti-GM-CSF antibody (MP1-22E9) was added to undiluted HpCM, followed by treatment with goat-anti-rat IgG and subsequent treatment with Protein G cross-linked to beaded agarose. HpCM was treated with anti-rat-IgG alone to control for any non-specific neutralization that might be caused by this antibody. Centrifugation removed antibody-cytokine complexes and the sterile-filtered, treated HpCM was used for; 1) *in vitro* bone marrow assay to test for the development of cultured mast cells, and 2) ELISA determination of the concentrations of IL-4 and GM-CSF after neutralization and removal. Results from the quantitative ELISAs were used to determine the amount of IL-4 and GM-CSF in NCM and removal of these cytokines in HpCM was judged complete if, following anti-cytokine antibody treatment and removal, ELISA results indicated that the IL-4 and GM-CSF levels had been reduced to the level of NCM or lower. Results of ELISA assays following antibody treatment and removal of GM-CSF and IL-4 are shown in figure 24. These data demonstrate that both IL-4 and GM-CSF were effectively removed from HpCM to levels at or below that of NCM.

To further test whether IL-4 or GM-CSF was acting as the inhibitor, recombinant IL-4 or GM-CSF was added to NCM to see if the capacity of NCM to support mast cell

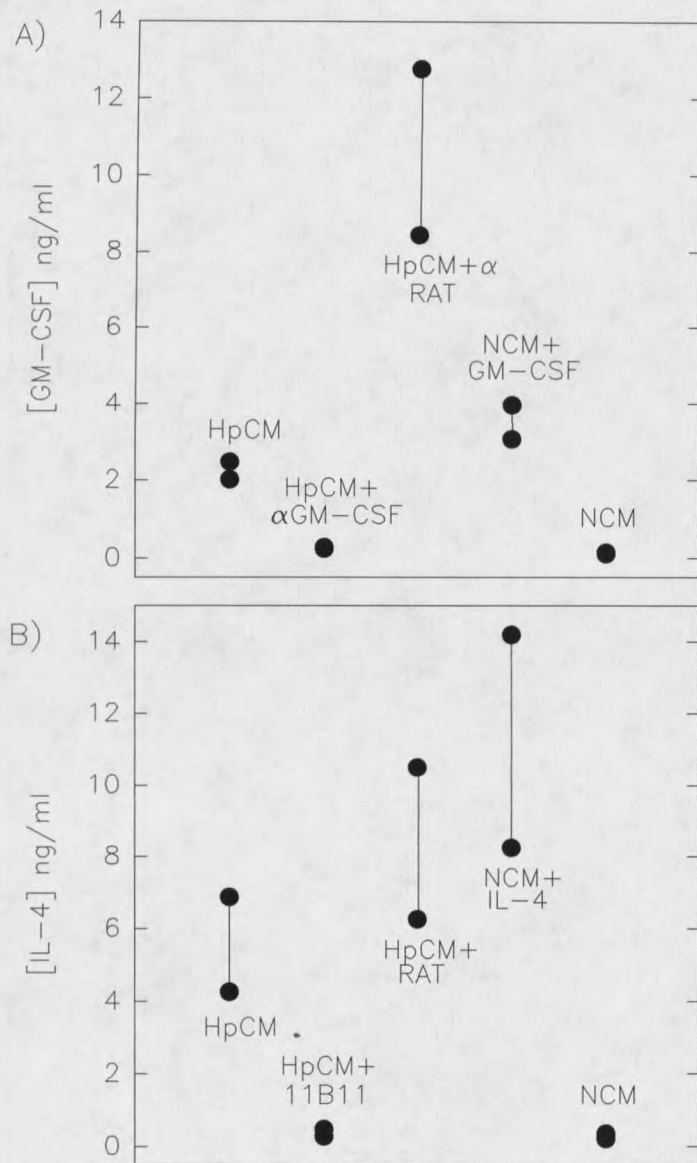


Figure 24. Concentration estimates of GM-CSF (A) and IL-4 (B) after treatment of HpCM with neutralizing antibodies MP1-22E9 (α -GM-CSF) and 11B11 (α -IL-4) or addition of rGM-CSF or rIL-4 to NCM as determined by ELISA. Anti-cytokine antibody treatment of GM-CSF or IL-4 succeeded in reducing the concentration of both cytokines in HpCM to levels commensurate with NCM. HpCM treated with goat anti-rat IgG alone as a control for non-specific binding of cytokine did not show a reduction in the concentration of either cytokine. Recombinant cytokines added to NCM increased the concentration of cytokine present in NCM to within the range of cytokine present in HpCM.

development could be abrogated with either cytokine. In these experiments the quantitative ELISA determinations of cytokine concentration in HpCM were used to calculate approximately how much IL-4 or GM-CSF was needed to make NCM similar to HpCM for these cytokines. GM-CSF (3.0 ng/ml) and IL-4 (12 ng/ml) was added to NCM and then assayed by ELISA to show that the cytokine concentration was indeed raised. Results are also shown in figure 24. These conditioned media, HpCM treated with anti-IL-4 or anti-GM-CSF or NCM treated with IL-4 or GM-CSF, were then used in bone marrow culture assays to determine if the mastopoietic potential of these CM were modulated.

The results of the bone marrow culture using both anti-IL-4 (11B11)-treated HpCM or anti-GM-CSF-treated HpCM, and NCM treated with rIL-4 or rGM-CSF are shown in figure 25. These results suggest that IL-4, not GM-CSF, was responsible for the inhibitory effect on the development of mast cells. HpCM treated with 11B11 produced copious amounts of BMCMC. Furthermore, when IL-4 was added to NCM, BMCMC production was suppressed.

Removal of GM-CSF from HpCM did not counteract mast cell inhibition in HpCM cultures. In fact, HpCM + anti-GM-CSF did not affect the inhibition of BMCMC by HpCM at all even though GM-CSF was reduced to the level of GM-CSF present in NCM (figure 25). While the addition of rGM-CSF to NCM did decrease mast cell development it did not inhibit

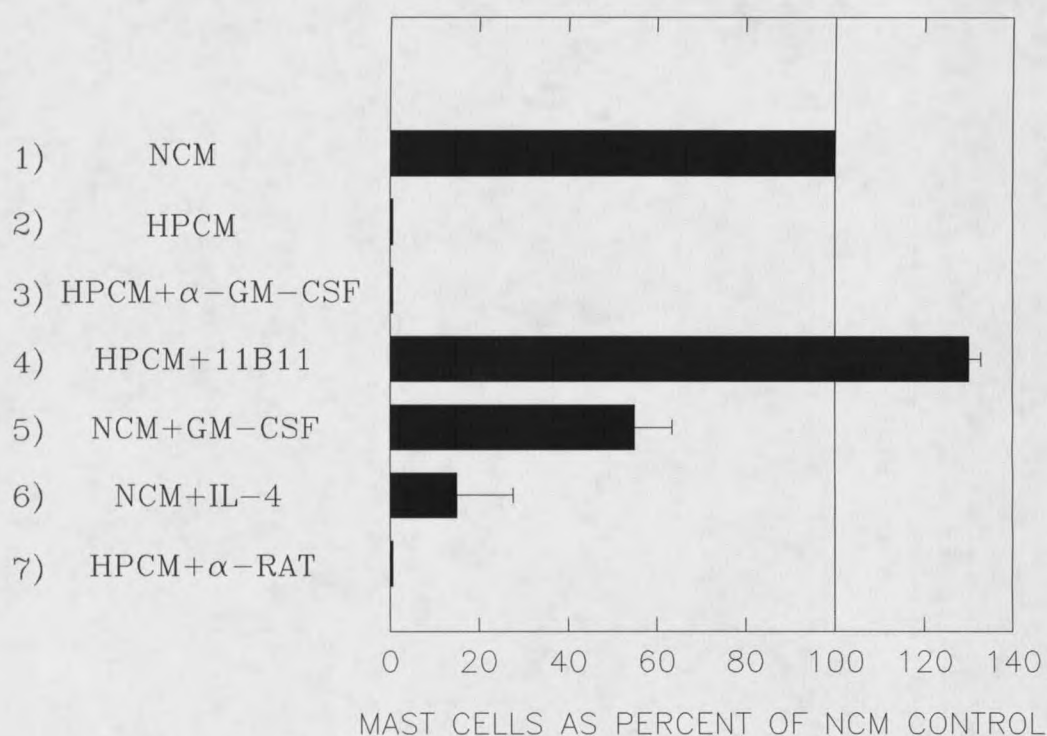


Figure 25. Removal of IL-4, but not GM-CSF, abrogates inhibition of BMCMC development *in vitro*. Bone marrow cells were cultured with NCM or HpCM (1) and (2) or HpCM treated with neutralizing antibody to GM-CSF (3) or IL-4 (4). NCM treated with rGM-CSF (5) or rIL-4 (6) was used to further test whether the cytokines were responsible for inhibition. HpCM treated with goat anti-rat IgG (7) did not affect BMCMC inhibition suggesting the effect is specific for IL-4. Data points are averages between two separate experiments using separate lots of NCM and HpCM and treated CM.

BMCMC development to the extent that IL-4 did. The inhibition observed with GM-CSF is likely due to an anti-proliferative effect on mast cells exerted by this cytokine (16).

DISCUSSION

Experiments outlined in the previous section show that:

(1) Bone marrow cultured mast cells (BMCMC) fail to develop from bone marrow cells when cultured with conditioned medium made from the spleens of *H. polygyrus*-infected mice (HpCM). Conditioned medium made from the spleens of uninfected mice (NCM), supports BMCMC development.

(2) The failure of HpCM to support BMCMC development is not a transient phenomenon during *H. polygyrus* infection, but rather lasts for at least four weeks during primary infection with *H. polygyrus*.

(3) Conditioned medium made from the spleens of mice infected with another nematode, *N. brasiliensis* (NbCM), supports BMCMC development.

(4) The failure of HpCM to promote BMCMC development is not due to an increase in the number of CD8⁺ T cells or a decrease in the number of CD4⁺ T cells in the spleens of infected mice.

(5) The failure of HpCM to support the development of BMCMC is not due to a lack of mast cell growth and differentiation factors: IL-3, IL-4 and IL-10 (cytokines necessary or enhancing for BMCMC development or growth) are

present in higher concentrations in HpCM than in NCM.

(6) HpCM inhibits the ability of NCM to promote mast cell development in culture and the inhibition is not due to a dilution of cytokines in NCM.

(7) Non-adherent cells in the spleen produce the inhibitor and HpCM made from nude mice infected with *H. polygyrus* does not have inhibitory activity.

(8) The inhibitor present in HpCM acts early in the development of BMCMC.

(9) HpCM supports the development of a macrophage-like cell rather than BMCMC.

(10) The inhibitory activity is not due to a cytokine with previously described mast cell inhibitory activity such as IFN- γ , TGF- β or GM-CSF.

(11) Cytokine clearing experiments suggest that the inhibitor of BMCMC development is IL-4, a cytokine known to enhance the growth of mast cells in culture.

The hypothesis that was initially investigated in this thesis was not supported by our research. *H. polygyrus* infection did induce a TH subset imbalance in the mouse, however, the infection did not induce a TH1 shift resulting in lower concentrations of several cytokines that are required or enhancing for mast cell growth and development. Rather, *H. polygyrus* caused a TH2 shift characterized by higher concentrations of IL-4, IL-5, IL-6 and IL-10 than that found in NCM made from uninfected mice.

However, the response to *H. polygyrus* infection differed from the "typical" TH2 response to *N. brasiliensis* infection in three important ways. One, IL-2 concentrations were higher in HpCM than in NbCM. Two, the concentration of IL-10 was lower in HpCM than in NbCM. And three, the concentrations of several cytokines, including IL-3, IL-4, IL-5, IL-6 and GM-CSF, were much higher in HpCM compared with NbCM.

The high concentration of IL-4 present in Day 14 p.i. HpCM (10.3-14.4 ng/ml) was particularly noteworthy, since this cytokine inhibited BMCMC development. Because the concentration of IL-4 present in NbCM (1.8-2.1 ng/ml) did not inhibit BMCMC development, we may have identified a threshold level of IL-4 that separates mast cell development from mast cell suppression. However, further dose response experiments are necessary to examine this possibility. The inhibition of BMCMC at high concentrations of IL-4 also suggests the presence of a negative feedback mechanism in mast cell development since mast cells themselves can secrete IL-4. The relevance of these findings is discussed.

Cytokine clearance suggests that the inhibitor of mast cell development is IL-4

Removal of IL-4 or GM-CSF from HpCM showed that IL-4, not GM-CSF, was the inhibitor of mast cell development in HpCM cultures. This conclusion is supported further by the

demonstration that recombinant IL-4 added to NCM markedly inhibited mast cell development. While rGM-CSF also reduced the number of mast cells that developed in culture with NCM, this effect was likely due to an inhibition of proliferation, since antibody treatment and removal of GM-CSF had no effect on the inhibition of mast cells in cultures containing HpCM (figure 25). This experiment has now been reproduced three times with unvarying results.

The most unanticipated feature of these data was that removal of IL-4 alone was so definitive, indicating very little, if any, synergistic inhibition with other cytokines. This was unexpected because to date, IL-4 has been identified as a mast cell stimulatory factor and the addition of IL-4 to mast cell cultures has been shown to result in the increased proliferation of mast cells (15,20,52,104,113).

However, a few reports in the literature have indicated that IL-4 has inhibitory effects on certain hemopoietic cells. Ashman et al. looked at conditions present in the MLN of *N. brasiliensis*-infected mice that could favor the development of mast cell committed progenitor cells (MCCP) and simulated these conditions *in vitro* (5). While IL-3 and, surprisingly, IgE immune complexes produce mast cell progenitors, IL-4 not only does not enhance production of mast cell progenitors, but appears to have a suppressive effect that is reproducible. Ashman

et al. speculate that while the mechanism for this effect is unknown, it may be important *in vivo* because IL-4 levels are low at the time when MSCP levels peak. This observation suggests that low levels of IL-4 correlate better with mast cell development than high levels *in vivo* during infection with *N. brasiliensis*. Indeed, the concentration of IL-4 was higher in NbCM (1.8-2.1 ng/ml) compared with NCM (0.2-0.3 ng/ml), but was not as high as the concentration of IL-4 in HpCM. Furthermore, HpCM made on day 6 p.i. contained a lower concentration of IL-4 (3.1-3.5 ng/ml) than HpCM made on day 14 (10.3-14.4 ng/ml). This is consistent with the observation that inhibition could be abrogated when day 6 HpCM was mixed with NCM, suggesting that the inhibitor may have been diluted below the inhibitory threshold. These data suggest that there may be a threshold between enhancement and inhibition of BMCMC development by IL-4.

Other research suggests a dual role for IL-4 in the regulation of growth and differentiation of pluripotent stem cells (67). IL-4 inhibits clonal growth of mouse bone marrow cells by 50% in cultures containing either IL-3 or GM-CSF. However, IL-4 enhances macrophage-colony stimulating factor (M-CSF)-induced colonies. These inhibitory and enhancing effects are both neutralized with anti-IL-4 antibody (11B11). The authors hypothesize that IL-4 inhibits early colony forming unit precursor cells, including stem cells, mixed colony-forming units and

multipotential precursor cells, but stimulates the growth of more mature cells. They further suggest that IL-4 is a major regulatory cytokine of normal hemopoietic growth and differentiation *in vivo*.

Two other groups have looked at the effects of myeloid colony formation mouse and human bone marrow cells. When mouse bone marrow progenitors are stimulated with various cytokines, IL-4 inhibits the IL-3-dependent proliferation of several colony forming cells (CFC) (95). Mature CFC growth is enhanced by IL-4 while the precursors of these mature cells are inhibited by IL-4. IL-4 does not enhance IL-3-dependent mast cell colony formation in short-term assays, but does appear to have a proliferative effect in long-term (21 day) assays. We have observed that HpCM was superior to NCM in maintaining the proliferation of mature BMCMC (unpublished observation). These results are also consistent with our data indicating that HpCM acted early on the development of mast cell precursor cells. Once mast cell precursors matured, the high concentration of IL-4 present in HpCM was not inhibitory and was even enhancing for BMCMC development (figure 22A).

In human bone marrow cells the inhibitory effects of IL-4 are observed only with higher concentrations of IL-4 (1000 U/ml); lower concentrations (400 U/ml) have stimulatory effects (18). This effect is not observed when recombinant human IL-4 is used on mouse bone marrow cells

rather than human bone marrow cells, suggesting that the suppressive effect is species-specific.

Another group has also demonstrated that IL-4 exerts effects on hemopoietic cells (80,81). Specifically, Pechel *et al.* found that IL-4 induces a reversible inhibitory activity in the adherent cells of mouse bone marrow (80). Inhibition of IL-3 dependent colony formation by IL-4 is observed only in the presence of stromal cells in bone marrow cultures. Furthermore, IL-4 has to be present for at least three days in order to obtain inhibition. If IL-4 is present in conjunction with IL-3, however, inhibition is observed with only a two day preincubation period. These results are consistent with our experiments analyzing the kinetics of inhibition (figure 22B). Finally, both IL-1 and IFN- γ , which inhibit hemopoietic cell development through the production of TNF and prostaglandins, fail to produce inhibition comparable to IL-4.

Pechel *et al.* show that stromal cells secrete a soluble factor that induces inhibition of hemopoietic cells because separation of the stromal cells and hemopoietic cells by an agar layer fails to prevent inhibition of colony forming cells. Inhibition of hemopoietic cells is not abrogated by the addition of indomethacin, indicating that the inhibition is not due to the generation of prostaglandins. Inhibition is, however, abrogated by anti-IL-4 antibody (11B11) or hydrocortisone. The cells present in the stromal layer of

cultured bone marrow cells are macrophages and fibroblasts, but attempts to determine which of these cell types are responsible for inhibition were not successful, although bone marrow macrophages alone induce partial inhibition. Even though the hemopoietic cell inhibitory activity apparently is due to a soluble factor, supernatants of the stromal cell layer do not cause inhibition. Nor is inhibition due to the production of several known cytokines including IL-1 and TNF.

Although Pechel *et al.* do not specifically mention mast cell inhibition, several findings reported by this group are consistent with our data. First, excess IL-3 fails to reverse the inhibition of hemopoietic cells. This agrees with our data indicating that HpCM contained very high levels of IL-3 (compared with NCM), yet mast cell development failed to occur with HpCM. Second, 11B11 abrogates the inhibition in Pechel's report which agrees with data from the cytokine neutralization and removal studies herein. Finally, Pechel *et al.* suggest that a macrophage is the most likely cell producing the inhibitor, since they, too, found that cells within the stromal layer were positive for Mac-1.

Pechel *et al.* conclude that it is likely that macrophages are directly modulated by IL-4 (80), since they found that the inhibitory effect is reversible when the stromal cell layer is removed. Production of IL-4 by other

myeloid cells such as mast cells also occurs and therefore they postulate that IL-4 may induce bone marrow cells to produce an inhibitory factor as part of a negative feedback mechanism (80).

Our results also support the hypothesis of a negative feedback mechanism since inhibition of mast cell development in culture with HpCM correlated with the observed suppression of mucosal mastocytosis *in vivo* in several mouse strains infected with *H. polygyrus*. Thus, our results are the first to correlate the phenomenon of IL-4 hemopoietic inhibition with an infection, lending considerably more support to the notion that IL-4 may function as a regulatory cytokine in hemopoiesis and inflammation *in vivo*. IL-4 could function to stimulate mast cell generation if held below a certain threshold. Once IL-4 levels exceeded that threshold, however, either by endogenous IL-4 production by mast cells, or by the production of exogenous IL-4 by TH2 cells (as in the case of *H. polygyrus* infection), mast cell production might be inhibited and macrophage production stimulated. This possibility is consistent with results in figure 18 showing that the inhibitory effects of day 6 HpCM were abrogated by NCM, suggesting that IL-4 was diluted below this threshold. A negative feedback mechanism mediated by macrophages might also explain many of the immunosuppressive effects of *H. polygyrus* noted others (3,8,10,13,21,22,27,28,57,82-88,103).

Price and Turner, in fact, demonstrate that peritoneal macrophages from *H. polygyrus*-infected C57BL/6J mice depress lymphoproliferative responses (82). A population of large, vacuolated, adherent cells exhibiting increased acid phosphatase activity develop in *H. polygyrus*-infected mice, but not in *N. brasiliensis*-infected mice. They postulate activated macrophages as a mechanism for several depressed immune responses that they reported in earlier papers.

In *N. brasiliensis* infection excess IL-4 production by mast cells could be responsible for the decrease in mast cells that follows expulsion of the nematodes (5). It is tempting to speculate that excess IL-4 is responsible for down-regulating mucosal mastocytosis early in *H. polygyrus* infection, perhaps allowing an opportunity for the nematode to establish a foothold in the host. However, it is more likely that IL-4 modulates the inflammatory response to *H. polygyrus* in a more global manner and the effect on MMC is only one consequence of this modulation.

It is also noteworthy that human mast cell SCF-dependent differentiation is suppressed by both IL-3 and IL-4 (120). Although the precise mechanism remains undetermined, it is suggested that suppression may occur by competitive recruitment of non-mast cell lineages from multipotent progenitors, through the down-regulation of KL (SCF)-receptor expression on hemopoietic progenitors (120). It is also proposed that because mast cells produce both

IL-3 and IL-4, the possibility for a negative feedback mechanism exists. This is encouraging in that despite the many differences between human and mouse mast cells, IL-4 could be a candidate for mast cell inhibition in humans as well as mice, although many other differences in mast cell biology exist between these species.

Our results not only explain why mast cells failed to develop with HpCM but provide some insight into the mechanisms of immune regulation and parasite evasion strategies. There is evidence that the macrophage-mediated inflammatory response induced by *H. polygyrus* may not be effective in controlling a primary infection. Schmitz et al. studied the different immune responses elicited by *H. polygyrus* in the rat, a resistant species to *H. polygyrus*-infection, and the mouse, a susceptible species (98). They observed that granulocytic cells with larvicidal activity develop rapidly in rats, whereas the larvicidal activity of macrophages against the parasite are weak. In contrast, mice exhibit a weak granulocytic response to the parasite during a primary infection, but immune mice show a "mobilization" of granulocytic cells. They postulate that a granulocytic response is important to resolution of *H. polygyrus* infection chiefly through the generation of toxic oxygen species.

H. polygyrus has apparently evolved resistant strategies for evading many of the effector functions of

macrophages and granulocytes, particularly free radicals (105). Smith and Bryant found that *N. brasiliensis* are much more susceptible to free radical damage compared with *H. polygyrus*. They suggest that this is due to the presence of enzymes in *H. polygyrus* capable of degrading oxygen metabolites. Specifically, *H. polygyrus* contains twice the amount of superoxide dismutase, three times the amount of catalase and four times the amount of glutathione reductase compared with *N. brasiliensis*. They hypothesize that *H. polygyrus* remains in the mouse host because of an effective enzymatic defense system in the worm. *N. brasiliensis*, on the other hand, has been shown by several groups to be more susceptible to damage by reactive oxygen intermediates (6,106-108). It is possible then, that *H. polygyrus* takes advantage of a putative IL-4-induced regulatory pathway in the host which induces the development of macrophages (but not activated macrophages) and aids the nematode in the establishment of a chronic infection.

**Are mast cells required for the expulsion of intestinal
nematodes?**

The *in vitro* observation that HpCM inhibited mast cell development agrees with several reports showing that *H. polygyrus* infection results in a depressed MMC response in several mouse strains compared with the MMC response elicited by either *T. spiralis* or *N. brasiliensis* infection

(8,27,28,93).

While HpCM made from both a moderate responder and a low responder mouse strain to *H. polygyrus* infection failed to support the development of BMCMC, HpCM made from the spleens of a high responder strain permitted some BMCMC development from syngeneic bone marrow cells. The high responder phenotype expels *H. polygyrus* more quickly (within two months) and is refractory to subsequent infections with the worm compared with low or moderate responder strains (48,73). Overall, SJL HpCM permitted as much as 10-times the BMCMC development observed in the other strains examined. The discovery that SJL HpCM had much lower concentration of IL-4, compared with the other two mouse strains, is consistent with the role of IL-4 as an inhibitor of mast cell development at higher concentrations.

Nevertheless, *H. polygyrus* appears to modulate mast cell development even in the high responder strain. In a preliminary histological examination of the mucosal mast cell response *in vivo* in both SJL and C57BL/10 mice we observed that *H. polygyrus* infection elicited a depressed MMC response compared with that observed with *N. brasiliensis* (unpublished observation). These cursory data agree with other reports demonstrating that *H. polygyrus* depresses the MMC response in both high and low responder mouse strains (2,3,8,9,27,28,89).

Histological reports of the mucosal mastocytosis

response to *H. polygyrus* in BALB/c mice, however, have suggested that these mice fail to exhibit a MMC depression compared with *N. brasiliensis* (119). Moreover, when mice are infected with *N. brasiliensis* and given antibodies to IL-3 and IL-4, 85-90% of the MMC response is abrogated without an observable effect on the expulsion of *N. brasiliensis* from the gut (69).

It is not surprising, however, that mucosal mastocytosis is not a definitive event in the expulsion of intestinal helminths. Although a role for MMC in intestinal helminth expulsion was an appealing hypothesis because it linked elevated IgE and the effusion of mast cells observed in the gut close to the time of worm rejection, previous experimental evidence suggested that it was not correct. W/W^v mice, which congenitally lack mucosal mast cells due to an inborn deficiency in *c-kit* ligand (the ligand for SCF), are still able to expel *N. brasiliensis* (23,90,115).

While the importance of MMC in intestinal helminth infections is currently debated it is generally agreed that *H. polygyrus* exerts an immunomodulatory effect upon the host that enables it to evade the host immune response. Concurrent infections with *H. polygyrus* and other nematodes result in the delayed expulsion of these other worms in addition to depression of mucosal mastocytosis (8,27,30). The chronic nature of *H. polygyrus* infection has led to the hypothesis that the nematode influences the host immune

response, enabling the worm to escape the characteristic expulsion mechanisms of the host (73). The early acquisition of the mast cell modulatory capacity shown in figure 4 and its long term effect most likely represents only one consequence of immunomodulation of the host response by *H. polygyrus*. It is possible that immunomodulation, like MMC suppression, is a consequence of the extraordinarily high levels of IL-4 induced by *H. polygyrus*.

***In vivo* observations**

While much of the research carried out in this thesis was accomplished in an *in vitro* culture system, several experiments were done comparing *H. polygyrus* infection with *N. brasiliensis* infection *in vivo* and comparing *H. polygyrus* infection in both a liable and refractory mouse strain. Therefore some conclusions may be drawn concerning *H. polygyrus* infection *in vivo*.

H. polygyrus and *N. brasiliensis* are often associated because they both induce a TH2 response. However, it is important to note that these intestinal nematodes cause very different infections; *H. polygyrus* infection is chronic and *N. brasiliensis* infection is acute. An obvious difference between infection with *N. brasiliensis* and *H. polygyrus* is the profound splenomegaly and mesenteric lymphadenopathy that is a well documented consequence of *H. polygyrus*

infection. Lymphocytes in the spleen (as determined by flow cytometry) increased much more dramatically in response to infection with *H. polygyrus* compared with *N. brasiliensis* infection (figure 8).

Determination of lymphocyte phenotypes in spleen and MLN of *H. polygyrus*- and *N. brasiliensis*-infected mice revealed that both nematode infections caused a B cell hyperplasia in the spleen. Analysis of the ratio of T:B cells in the spleen and MLN shows that the T:B cell ratio decreased in response to both nematode infections. However, unlike *N. brasiliensis*, *H. polygyrus* also caused a T cell hyperplasia in the spleen (figure 8). When the total number of splenic lymphocytes was evaluated, *H. polygyrus* caused a much more pronounced splenic B cell hyperplasia and a noticeable splenic T cell hyperplasia when compared with *N. brasiliensis* infection. The T cell hyperplasia was most profound at day 6 post-infection in BALB/c mice. These results agree with those of Parker and Inchley, who have shown that B cells expand in response to infection with *H. polygyrus* compared with uninfected mice (79).

Since different immune responses to infections are frequently observed in different mouse strains, we compared a more resistant strain to *H. polygyrus* infection with a more susceptible strain to see if any interesting patterns emerged. Low responder C57BL/10 mice showed a dramatic splenic B cell hyperplasia, but failed to display the

splenic T cell hyperplasia observed in BALB/c or SJL mice (figure 9A and 9B). High responder SJL mice, in contrast, showed a pronounced T cell hyperplasia in the spleen that was greater than the B cell hyperplasia observed in these mice. These trends continued throughout the course of *H. polygyrus* infection in these mouse strains (figure 10). The moderate responder strain, BALB/c showed T cell hyperplasia only on day 6 post-infection. These data suggest that T cell hyperplasia correlated with greater resistance to *H. polygyrus*, while B cell hyperplasia correlated with a susceptible phenotype. This result agrees with another report by Parker and Inchley, in which they found that another susceptible strain, CBA, exhibits the largest decrease in the T:B cell ratio in MLN (78).

The more exaggerated B cell response observed in the low responder strain is noteworthy, particularly since susceptible C57BL/10 mice did not show any obvious differences in circulating IgG1 or IgE (figure 18). Others have observed that low responder strains show higher titers of parasite-specific antibodies in the serum than do high responder strains (73). But when cells secreting parasite-specific antibody are measured in Peyer's patches and MLN, the resistant strains show higher levels of parasite-specific antibody compared with susceptible strains. Therefore it is possible that in mouse strains that are more susceptible to *H. polygyrus*, antibody is present

systemically, but is not able to cause damage to the worms *in situ*, perhaps because cell mediated effector mechanisms are not as efficient in these strains as in resistant strains. It is likely, then, that both humoral and cell mediated immunity are important in the immune response to *H. polygyrus* (73).

H. polygyrus infection caused a much more dramatic increase in circulating IgG1 and IgE compared with *N. brasiliensis* infection (figure 17). Although parasite-specific antibody was not measured, it is likely that the increased levels of IgE are not protective in *H. polygyrus* infection, since inhibition of IgE production *in vivo* fails to inhibit protective immunity in *H. polygyrus* infected mice (117). Therefore the higher level of circulating IgE observed in *H. polygyrus*-infected mice, compared with mice infected with *N. brasiliensis*, is probably due to the high levels of IL-4 induced by *H. polygyrus*.

Another hypothesis that explained the chronicity of *H. polygyrus* infection involved the role of suppressor T cells (89). This hypothesis failed to be substantiated by flow cytometric data, however. *H. polygyrus* infection, rather than causing a decrease in the CD4:CD8 ratio, caused an increase in this ratio on day 6 post-infection in both the spleen and MLN compared with *N. brasiliensis* infection (figure 11). Analysis of both splenic and MLN T cells on day 14 p.i. showed that *H. polygyrus*-infection did not

obviously affect this ratio in BALB/c mice. Instead, the CD4:CD8 ratio remained commensurate with that observed in uninfected mice. The only time that the CD4:CD8 ratio decreased in BALB/c spleen during the post-infection periods studied was at day 14 p.i. in the MLN of *N. brasiliensis*-infected mice, perhaps coinciding with a down-regulation of the host response following expulsion of the nematode.

Comparison of CD4:CD8 ratios in both high responder and low responder mice indicated that the low responder strain exhibited a greater increase in the CD4:CD8 ratio than the high responder strain. The ratio increased 50% in C57BL/10 mice compared with a very slight increase in SJL mice (figure 12). These results also agree with earlier results reported by Parker and Inchley (78). They found no evidence for an expansion of Ly-2⁺ (CD8) cells in mice infected with *H. polygyrus* although they confined their observations to MLN. They also observed a slight increase in the ratio of CD4:CD8 cells in moderate responder strains and a more protracted increase in L3T4⁺ (CD4) cells in a low responder strain. They conclude that responder status to *H. polygyrus* infection is not easily explained on the basis of CD4:CD8 analysis. However, cytokine data, which make it possible to examine TH subsets, together with flow cytometric data, suggested that susceptibility and resistance to *H. polygyrus* correlated with CD4⁺ T cells. These data are consistent with the observation that CD4⁺ T cell ablation increases

parasite egg production during a primary infection (116).

**Does *H. polygyrus* infection cause a TH2 shift and is it
beneficial to the host?**

H. polygyrus infection was associated with a dramatic increase in IL-4, IL-5 and IL-6 production from Con A-stimulated splenocytes. The levels of these cytokines in HpCM were not only higher compared with NCM, but also compared with NbCM (figure 14). These data indicated that *H. polygyrus* caused a TH2 shift, since the level of TH2 cytokines in HpCM (like NbCM) increased compared with the levels of these cytokines in NCM. There were three major differences between HpCM and NbCM however. First, the amount of IL-2 in HpCM was higher than in NbCM, suggesting that the modulation of TH1 cytokines observed with *N. brasiliensis* infection was different than that observed in *H. polygyrus* infection. Second, the level of IL-10 was lower in HpCM compared with NbCM. Third, the levels of IL-3, IL-4, IL-5, IL-6 and GM-CSF were much higher in HpCM compared with NbCM.

The presence of IL-2 in HpCM is interesting. In one report, IL-2 significantly enhances the production of IL-4 from T cells derived from mice that have been injected with anti-IgD (64). It is possible that sustained levels of IL-2 in *H. polygyrus* infection could contribute to the superior levels of IL-4 observed in HpCM compared with NbCM. It is

difficult to say if IL-2 elevation is unique to *H. polygyrus*, since in many parasite infections researchers have analyzed only IFN- γ and one other TH2 cytokine in looking for evidence that a TH2 shift has occurred.

Comparison of susceptible and resistant mouse strains to *H. polygyrus* infection revealed an interesting trend for IL-2. Where SJL HpCM exhibited reduced levels of IL-2 at day 14 post-infection, C57BL/10 HpCM exhibited more IL-2 compared with C57BL/10 NCM (figure 15). This suggests that IL-2 down-regulation may not occur as efficiently in this low responder strain in response to *H. polygyrus* infection. It is possible then that IL-2 modulation by the nematode influences the ability of the host to respond to *H. polygyrus*, perhaps in concert with IL-4.

Three different types of analysis, bioassay, ELISA and ELISPOT data, all indicated that *H. polygyrus* infection was not associated with decreased IL-2 secretion in the spleen. These data are consistent with reports indicating that high antigen concentrations are associated with elevated IL-2 production, a circumstance that would be expected in a parasitic infection (39). It is interesting, however, that IL-2 secretion did not seem to be a feature of *N. brasiliensis* infection since one would expect similarly high antigen concentrations with this parasite. It is tempting to speculate that the difference might lie with the chronic nature of *H. polygyrus* antigen exposure and/or the strictly

enteral location of this parasite.

Recently it has been shown that the homogenate of adult *H. polygyrus* stimulates naive T cells *in vitro* to proliferate and secrete IL-2 (97). Proliferation by naive T cells is independent of MHC restriction, suggesting the presence of a superantigen-like molecule. However, the adult *H. polygyrus* homogenate does not strictly adhere to the criteria specifying a superantigen, since presentation of the antigen requires metabolically active spleen accessory cells; paraformaldehyde fixation of these cells fails to elicit stimulation of target cells. These results are intriguing and suggest that the high levels of IL-2 present in HpCM may be due to adult *H. polygyrus*-induced T cell proliferation not subject to the usual MHC-restricted requirements of antigen presentation to T cells.

Gejewski et al. have observed that TH0 clones that are subjected to anergizing conditions dramatically reduce their secretion of IL-2 (42). TH2 cytokine (IL-4 and IL-5) secretion is not affected by the anergizing stimuli. They suggest that TH1 and TH2 precursors that are stimulated with antigen presenting cells (APC) lacking a co-stimulatory molecule (e.g. B-7) might down-regulate the IL-2 response and mature into TH2 cells. APC like B cells, which lack a co-stimulatory molecule, would therefore favor a TH2 response. This may be occurring in the case of *N. brasiliensis* infection where a mature TH2 response was

observed. *H. polygyrus* infection, on the other hand, might be associated with a delay in the development of a TH2 developmental pathway, evidenced by the lower concentration of IL-10 and the higher concentration of IL-2 present in HpCM. Thus, the cytokine data raise several questions regarding the kinetics of IL-2 production and/or the regulatory mechanisms involved in the response to *H. polygyrus* infection.

While our research was being completed Urban *et al.* also looked at cytokine production during *H. polygyrus* infection in BALB/c mice using a quantitative reverse transcriptase-polymerase chain reaction assay (RT-PCR) for IL-2, IFN- γ , IL-3, IL-4, IL-5, IL-6, IL-9 and IL-10 (111). They observed a predominantly TH2 response evidenced by increased RNA expression of IL-3, IL-4, IL-5, IL-6 and IL-9 in Peyer's patch cells and low expression of IL-2, IFN- γ and the TH2 associated cytokine IL-10. They found that cytokine gene expression is primarily localized to the enteric region with little change occurring in the spleen and conclude that the immune response to *H. polygyrus* remains localized to the gut.

These results are in contrast to our results showing that several cytokines are up-regulated in the spleens of infected mice. One important difference between the two studies is that Urban *et al.* analyzed lymphoid tissue only between days 1 and 12 post-infection. It is likely that the

effects of *H. polygyrus* infection occur sooner in Peyer's patch (PP) and MLN cells and occur later in the infection in the spleen due to the proximity of MLN and PP to the source of antigen stimulation (in the gut). It seems unlikely, however, that the spleen plays no role in *H. polygyrus* infection because of the dramatic splenomegaly observed with this nematode infection.

An interesting feature of this paper is that the early cytokine gene expression observed in *H. polygyrus* (within 24 hours) occurs prior to T cell activation. IL-3, IL-5 and IL-9 expression are all elevated by 12 hours post-infection. Administration of anti-CD4 and anti-CD8 antibodies does not affect the level of expression of these cytokines until after day 6 post-infection. They propose that mast cells, basophils and/or eosinophils could be possible sources of these cytokines and that early cytokine production then directs subsequent differentiation of a TH2 T cell subset and release of TH2 cytokines.

Our results, however, indicated that eosinophils were not observed in spleen or MLN until between days 6 and 14 post-infection during a primary infection (Table 3). Mast cells were not observed at all in the spleens or MLN during *H. polygyrus* infection. Furthermore, the generation of mucosal mast cells has been shown to be T cell dependent making these cells an unlikely source of increased cytokine expression prior to T cell activation (49,50). Thus, the

presence of these cytokines raises several questions about the early host response to *H. polygyrus*.

Urban *et al.* did note diminished levels of IL-10 message in both Peyer's patches and MLN. We, too, saw lower concentrations of IL-10 in HpCM compared with NbCM. IL-10 has been identified as important in the down-regulation of TH1 cytokine production in schistosome infections (102). Another group has also found that *H. polygyrus*-infection down-regulated IL-10 (and IL-9) production in mouse MLN compared with infection with *T. spiralis* (12). This group associated the lower concentrations of IL-9 and IL-10 with the inferior mucosal mastocytosis present during *H. polygyrus* infection compared with *T. spiralis* infection. Our data indicate that deficient IL-10 was not the cause of inferior mastocytosis, but rather excess IL-4 was the cause of decreased mastopoietic potential. It is more likely that the lower levels of IL-10 observed in *H. polygyrus* infection have other regulatory effects.

IL-4 and IL-10 are important regulatory cytokines. Both are involved in the differentiation of TH2 cells. IL-4 acts directly on TH2 cell proliferation whereas IL-10 appears to inhibit cytokine production by TH1 cells via antigen presenting cells (39). It is possible that the more limited IL-10 response induced by *H. polygyrus* infection compared with *N. brasiliensis* infection could influence IL-2 production by TH1 cells and thereby fail to effectively

down-regulate IL-2 production early in the infection. Another possibility is that the lower concentration of IL-10 in HpCM reflects one more facet of a delayed mature TH2 response in *H. polygyrus*-infected mice. Analysis of the concentration of IL-10 made on days post-infection further along in *H. polygyrus* infection might prove helpful.

Analysis of several TH2 cytokines also revealed a difference between high responder and low responder strains of mice. HpCM from all strains displayed a marked increase in the concentration of IL-6 at day 14 post-infection with *H. polygyrus*. This was consistent with the B cell hyperplasia observed in all strains. Similarly all strains exhibited an increase in IL-4, IL-5 and IL-10 compared with NCM made from respective uninfected controls. However, SJL HpCM consistently did not display the marked increases in IL-4 and IL-5 (nor in IL-3 and GM-CSF).

These data suggest that resistant SJL mice produced a less exaggerated TH2 response to *H. polygyrus* infection than BALB/c or C57BL/10 mice. HpCM made from resistant mice also contained less IFN- γ and less IL-2 suggesting that TH1 down-regulation may be more efficient in this strain compared with more susceptible strains. It is possible then that SJL mice, which are more refractory to infection with *H. polygyrus*, are able to fine tune the host response to the parasite more efficiently than other strains, perhaps by regulating the ratio of TH1:TH2 response differently.

The question of T helper subsets in protective immunity to parasites is controversial. Some have suggested that a TH1 response is beneficial regardless of the type of parasite infection and further suggest that TH2 cytokines are associated with immunopathology (100). They propose that intestinal helminths, rather than stimulating a TH2 response that functions in protection, stimulate a TH2 response as an evasion mechanism to down-regulate the protective TH1 response. Research with both *Leishmania* and *Schistosoma* has provided the foundation for this hypothesis. Others have suggested that a TH2 response is beneficial to the host in response to nematode parasites, while a TH1 response is beneficial to intracellular parasites (119).

Three major observations support the latter view. First, Urban *et al.* have shown that administration of IFN- γ to mice infected with *N. brasiliensis* interrupts the protective response to this nematode (118). However, although worm fecundity is increased and expulsion delayed (by 3 days), expulsion is not inhibited.

Second, this group has shown that IL-4 is important in protective immunity to *H. polygyrus* in secondary infections. Specifically, Urban *et al.* have demonstrated that 11B11 abrogates acquired resistance to *H. polygyrus* in mice given challenge infections with *H. polygyrus* (117). Neutralization of IL-4 during primary infection, however, does not appear to affect egg production where anti-CD4

treatment did, suggesting that IL-4 is not the only factor involved in protective immunity. These results suggest that the regulation of IL-4 by the host is important in the immune response to *H. polygyrus* during secondary infections, but is debatable during a primary infection. Urban *et al.* have also demonstrated that many of the cellular and humoral effectors induced by a TH2 response, such as mucosal mastocytosis, eosinophilia and elevated IgE are all apparently ineffective in protecting the host against *H. polygyrus*, since these responses can be eliminated *in vivo* by monoclonal antibody treatment with no apparent impact on expulsion of nematodes from the gut (69,117).

Third, it has recently been shown that resistance to the intestinal nematode *Trichuris muris* is associated with a TH2 response, while a TH1 response is associated with susceptibility (31-33). This definitive dichotomy has yet to be demonstrated for another helminth infection, however. Study of *T. spiralis* infection in the mouse suggests that a TH2 response is associated with worm expulsion from the intestine as well as inflammation and pathology, while resistance is associated with a TH1 response (120). These disparate results reflect the use of different strains. One interesting feature of the immune response to *T. spiralis* is a compartmentalization of TH subset responses, with a predominant TH2 response observed in the MLN and a TH1 response in the spleen (61). Taken together, the

experimental evidence of TH2 subsets in helminth-infected animals suggest that the hypothesis that a TH2 response in helminth infection is always associated with protection is an over-simplification. Finally, concurrent infection with *H. polygyrus* and *T. muris* have demonstrated that *H. polygyrus* impairs the expulsion of a primary *T. muris* infection, suggesting that the TH2 response elicited by *H. polygyrus* is not protective in the *T. muris* model (8,57). Since resistance is associated with a TH2 response by the host during infection with *T. muris*, it seems unlikely that the TH2 response elicited by *H. polygyrus* is protective.

Scott et al., on the other hand, have data that may be salient to what is occurring *in vivo* in an *H. polygyrus* infection. Specifically they have shown that IL-4 has a dual effect on macrophage activation mediated by IFN- γ (100). When macrophages from normal BALB/c mice are exposed to IFN- γ or TH2 supernatants for 18 hours and then infected with *L. major* amastigotes, IFN- γ -stimulated macrophages reduce the number of parasites present, whereas TH2 supernatants do not affect parasite growth. When macrophages are incubated with both IFN- γ and TH2 supernatants, however, macrophage activation is significantly inhibited compared with macrophages treated with IFN- γ alone.

IL-4, IL-3 and GM-CSF all down-regulate IFN- γ macrophage stimulation. When macrophages are exposed to

IL-4 following infection, however, IL-4 enhances IFN- γ mediated macrophage activation. IL-4 alone has no anti-leishmanial activity. Scott's group suggest that IL-4 has a dual effect on macrophage activation depending on the timing of the exposure to cytokine. IL-4 could be suppressive if macrophages are exposed to this cytokine prior to IFN- γ activation. After macrophage activation however, IL-4 is synergistic with IFN- γ . Interestingly, macrophages from immunized mice are capable of activation (and killing of *L. major* amastigotes) while those of unimmunized infected mice are not. If a similar state occurs in *H. polygyrus* infection, this might explain why anti-IL-4 antibodies affect the protective response with challenge infections, but do not affect primary infections. It would be interesting to give IFN- γ to *H. polygyrus*-infected mice to see if this treatment accelerated expulsion of the nematode from the intestine.

Scott et al. also found that IFN- γ production is associated with protective immunity to schistosomiasis (100). This helminth is associated with a chronic infection in mice and a pronounced granulomatous response. They observed that eosinophilia and elevated serum IgE correlates with a patent infection with *S. mansoni*, but these manifestations of TH2 cytokines are not observed in immunized mice. Splenocytes from mice infected with *S. mansoni* for 8 weeks show deficient IFN- γ production and

increased IL-5 production in response to either parasite antigen or Con A. These results suggest a dramatic TH2 shift during an acute infection similar to that of *H. polygyrus*. Mice vaccinated with irradiated cercariae display the opposite profile: elevated IFN- γ and low IL-5.

So far, our results suggest that the ability of *H. polygyrus* to overcome the host immune response may be due to an exaggerated TH2 response, since TH2 cytokines appear to be in lower concentrations in HpCM made from a refractory strain to *H. polygyrus* infection, SJL. This is not the only possible interpretation of our data however. SJL HpCM also showed a more pronounced decrease in the concentration of IL-2, suggesting that TH1 down-regulation might be a critical factor associated with resistance to *H. polygyrus* infection. Since IFN- γ was effectively down-regulated in susceptible strains however, this alternative seems unlikely. Nevertheless, further research is necessary to elucidate the importance of TH subsets in susceptibility or resistance to *H. polygyrus* infection.

Our finding that IL-4 may act as a mast cell inhibitor and that HpCM promoted the development of a macrophage in bone marrow cell cultures, however, provides some interesting data for speculation. IL-4 may be protective in subsequent *H. polygyrus* infections, but the extraordinary amounts that the nematode is capable of generating may be responsible for inducing an inflammatory response that

delays the appropriate T cell response. Inhibition of macrophage function might occur when high concentrations of IL-4 are present along with low concentrations of IFN- γ . This scenario would be similar to that observed with macrophages in *Leishmania* in BALB/c mice. It is possible then, that *H. polygyrus*-induced production of IL-4 stimulates the development of a macrophage with several host-suppressive effects. The bias toward TH2 cell development by IL-4 would further decrease IFN- γ production by the host in response to the infection and would result in the failure of macrophages to become activated. This would lead to the modulation of a cell type that is potentially lethal to the nematode. IL-4 could function in protective immunity to challenge infections with *H. polygyrus*, however by synergizing with IFN- γ and effectively eliminating L3 larvae attempting to encyst in the submucosa of the intestine.

Conclusions

In conclusion, our results have shown why mast cells failed to develop in bone marrow cultures exposed to HpCM. We have demonstrated that macrophages, rather than mast cells develop in the presence of HpCM. The factor responsible for mast cell inhibition appears to be IL-4. While evidence is now strong that mucosal mastocytosis is not sufficient in protection against *H. polygyrus* infection,

our results suggest a possible mechanism for the immunosuppression observed with this nematode and for the chronicity associated with the parasite. Moreover, our results suggest that mucosal mastocytosis might be regulated with increased amounts of IL-4, suggesting a possible therapeutic role for IL-4 in mast cell related inflammatory disease.

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