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

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 THl 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 THl 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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Luanne Hall-Stoodley

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

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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.

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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 LTC_4, LTD_4, and LTE_4) 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

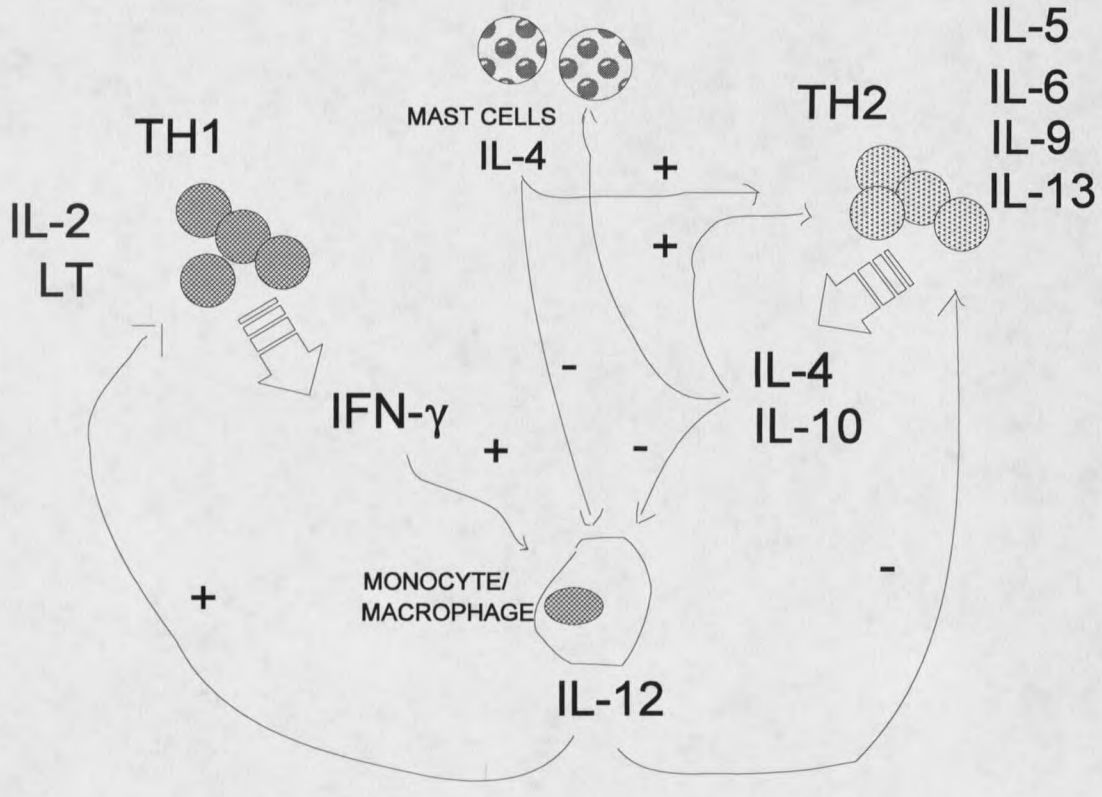

Figure 1. TH subsets and typical cytokine secretion profiles. Arrows and +/- symbols indicate positive and negative feedback mechanisms, respectively.

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.

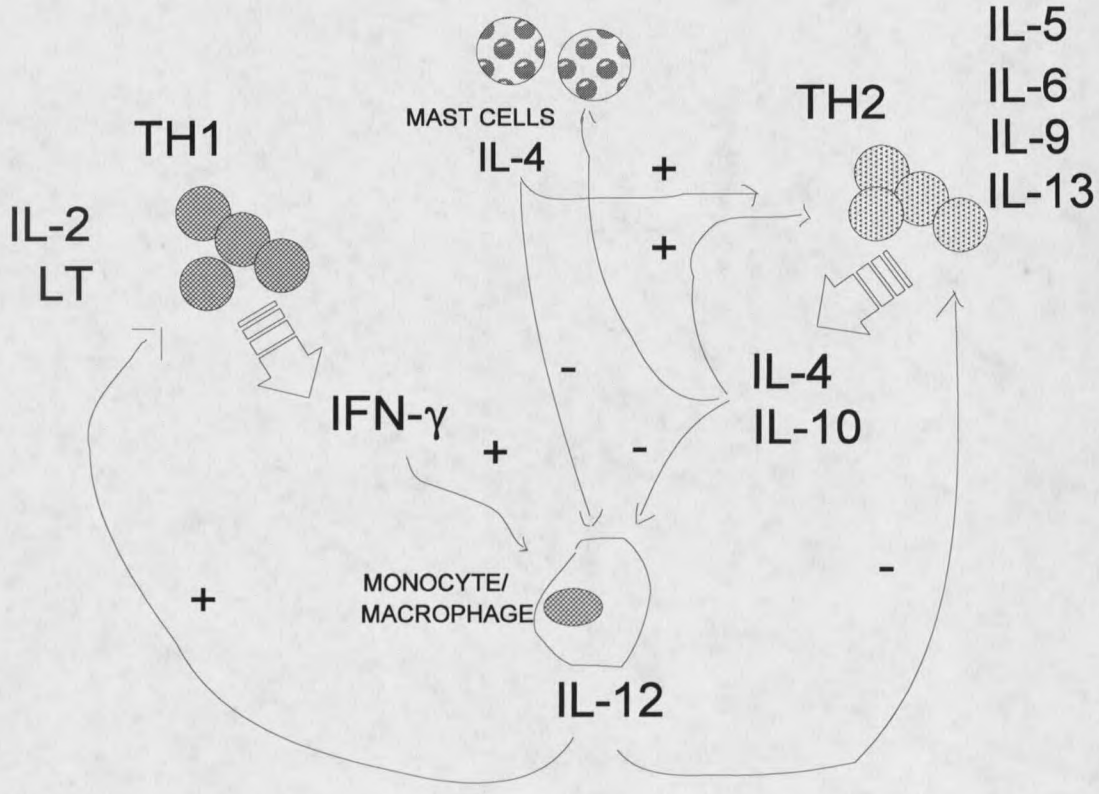

Figure 2. Diagram summarizing the hypothesis. *N. brasiliensis* infection is expected to induce the expansion of TH2 cells during infection. Conditioned medium made from the spleens of these mice is expected to be mastopoietic. In contrast, *H. polygyrus* infection, is expected to induce the expansion 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 L_3 larvae were obtained initially from the laboratory of D. Wakelin, University of Nottingham, Nottingham, England. *H. polygyrus* L_3 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

(dH_2O) to keep the filter paper moist. L_3 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% L_3 when quantifying larvae for inoculation into mice. The larvae were suspended in fresh dH_2O and stirred with a small stirring bar while three 50 µl aliquots were removed and quantified. A dose containing a suspension of 400 L_3 *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 dH_2O 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 L_3 larvae were ready to harvest.

N. brasiliensis L_3 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*

L_3 larvae was adjusted so that 600 L_3 were administered in a single 0.2 ml dose injected subcutaneously behind the neck.

Preparation of Spleen Cell Conditioned Media

RPMI-1640 with bicarbonate was supplemented with 2 mM glutamine, 0.1 M sodium pyruvate, 10 mM HEPES, 7.5 X 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 x 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% CO_2 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 x 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% CO_2 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 x 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 $NaHCO_3$ (pH 8.2) to 2 μg/ml (or 4 μg/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 μl 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 μg/ml (or 2 μg/ml for IL-10 because of the antibody isotype) and 100 μl 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 μl 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/ NaN_3 (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.

Cell specificity	Fluorescent-mAb	Clone & isotype
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 x 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/N_3/10%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/N_3/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 μg/ml, or medium alone for

72 hr at 37°C/5% CO_2. At 68 hr, the cells were pulsed with 1 μCi [^{3}H] thymidine per well (Dupont NEN, Wilmington, DE). Cells were harvested onto glass-fiber filters using an automatic cell harvester (PHD, Cambridge, MA) and [^{3}H] 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 μg/ml solution of nonspecific isotype gave a reading no higher than the lower limits of

detectiblity 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 X 10^6 cells/ml in RPMI 1640S + 10% FBS and Con A was added at a concentration of 5 μg/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 μl per well of anti-cytokine capture antibody at 5 μg/ml (anti-IL-2 (Pharmingen, 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 X 10^6 cells in the first well and diluted in a two-fold serial dilution across the row ending with approximately 4.9 X 10^2 cells per well. Each well contained 100 μl of cell suspension. Plates were

incubated for 20-22 hr at 37°C at 5% CO_2 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 discreet spots. Image analysis was used to display