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Modulation of T cell-mediated responses by *Nippostrongylus brasiliensis*

by

Robert Stefan Liwski

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
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DALHOUSIE UNIVERSITY

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Modulation of T cell-mediated responses by *Nippostrongylus brasiliensis*"

by Robert Stefan Liwski

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External Examiner

Research Supervisor

Examining Committee

Dalhousie University
Date: August 9, 1999
Author: Robert Stefan Liwski
Title: Modulation of T cell-mediated responses by *Nippostrongylus brasiliensis*
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For Asia, without whom this would be the last page.

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Abstract

Previous research from our laboratory demonstrated that infection of rats with the intestinal nematode *Nippostrongylus brasiliensis* (*Nb*), which induces strong Th2 responses, significantly prolongs the survival of fully allogeneic vascularized kidney grafts. The focus of this thesis is to investigate the manner in which *Nb* infection affects allo-reactivity to provide information to support or refute a hypothesis that these observed effects of *Nb* infection relate to type 2 dominant response. The importance of T cells in graft rejection focussed the research on the effects of *Nb* infection on T cell activation, T cell proliferation and differentiation, all aspects of T cell function critical to allo-reactivity.

It was found that T cells from mice infected with *Nb* prior to allo-immunization exhibit significantly reduced allo-specific CTL activity. In contrast, allo-specific proliferation in the MLR was not reduced by *Nb*, ruling out immunosuppression. Analysis of MLR culture supernatants by ELISA showed that *Nb* infection induced the production of type 2 cytokines and significantly inhibited production of type 1 cytokines. Further, staining of spleen T cells for CD8 and cytoplasmic IL-4 showed that *Nb* infection results in an 8-fold increase in the number of $CD8^+$, IL-4-producing Tc2 cells. These results are consistent with a hypothesis that, *Nb* mediates prolongation of allograft survival through induction of type 2 immunity, including the development of regulatory Tc2 cells.

The shift in the development of type 1 allo-specific responses towards type 2 immunity that occurred in the presence of *Nb* infection suggested that the nematode has immuno-modulatory effects on T cell activation. It was found that spleen cells from *Nb*-infected mice exhibited dramatically increased proliferation in response to Con A and anti-CD3. This hyper-proliferation could be transferred, *in vitro*, to naive splenocytes by co-culture with mitomycin C-treated cells from *Nb*-infected animals. The transfer was mediated by non-T accessory cells, and supernatants derived from Con A-activated non-T cells, suggesting the involvement of a soluble factor. The accessory cells secreted high levels of IL-6, and anti-IL-6 treatment abrogated the supernatant-induced hyper-proliferation, thus confirming that IL-6 was mediating the effect. Further, spleen cells from *Nb*-infected mice were more resistant to activation induced cell death (AICD) following mitogenic stimulation. Reduced AICD was also transferable and IL-6-dependent. Thus, the hyper-proliferation was, in large part, due to enhanced activated T cell survival.

These studies suggest that nematodes can induce powerful polyclonal activation of type 2 immunity by alteration of accessory cell function. It is postulated that one mechanism by which this type 2 switch might occur is by the inhibition of AICD in activated T cells allowing progression through sufficient cycling events to promote Th2/Tc2 phenotypes. The results of these studies have significant implications with respect to the regulation of immune responses to other antigens in countries where nematode infection is endemic.

List of abbreviations

ACK	ammonium chloride potassium
AICD	activation induced cell death
AP-1	activating protein-1
APC	antigen presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster determinant
CD28RE	CD28 responsive element
CD40L	CD40 ligand
CFA	complete Freund's adjuvant
Con A	concanavalin A
^{51}Cr	^{51}chromium
cRPMI	complete RPMI 1640 medium
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DPM	disintegration per minute
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
Fas	Fas receptor
FasL	Fas ligand
FCS	fetal bovine serum
GIC	graft infiltrating cells
h	hour
[^{3}H]-TdR	tritiated thymidine
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2ethanesulfonic acid
IFN-γ	interferon-gamma
IFN-γR	interferon-gamma receptor
IFN-γRα	interferon-gamma receptor alpha subunit
IFN-γRβ	interferon-gamma receptor beta subunit
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IL-12R	interleukin-12 receptor
IL-12Rb	interleukin-12 receptor beta subunit
IL-13R	interleukin-13 receptor
IL-2R	interleukin-2 receptor
IL-2Rα	interleukin-2 receptor alpha subunit
IL-2Rβ	interleukin-2 receptor beta subunit
IL-2Rγ	interleukin-2 receptor gamma subunit
IL-4R	interleukin-4 receptor

IL-4Rα	interleukin-4 receptor alpha subunit
IL-6R	interleukin-6 receptor
IL-6Rα	interleukin-6 receptor alpha subunit
IL-6Rβ	interleukin-6 receptor beta subunit
ip	intraperitoneal
IRF-1	interferon regulatory factor-1
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
KO	knock out
LFA-1	lymphocyte function in adhesion-1
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated kinase
MBP	myelin basic protein
Mc	mitomycin C
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
Min	minute
MLN	mesenteric lymph node
MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
Nb	*Nippostrongylus brasiliensis*
NFAT	nuclear factor of activated T lymphocyte
NK	natural killer
PBS	phosphate buffered saline
PKC	protein kinase C
PTK	protein tyrosin kinase
pv	portal venous
RBC	red blood cells
rIL	recombinant interleukin
sc	subcutaneous
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
STAT	signal transducers and activators of transcription
Tc	T cytotoxic cell
TCR	T cell receptor
Th	T helper cell
TNF	tumor necrosis factor
MBP	myelin basic protein

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1.0 Introduction

More than two thirds of world's population live in the regions of the world where infections with parasitic helminths are prevalent (Maizels *et al.* 1993). It has been estimated for example, that approximately one billion people are infected with the intestinal nematode *Ascaris lumbricoides* (Chan *et al.* 1994). Similar numbers have been reported for infections with other nematodes such as *Trichuris trichura* and hookworms (Chan *et al.* 1994). The immune responses mounted during these parasitic infections are characterized by dramatically increased production of IgE and IgG4 antibodies, blood eosinophilia and mastocytosis. It is of interest that although powerful immune responses are mounted, most of the residents of endemic areas harbor these parasites for life (Chan *et al.* 1994). This suggests that inappropriate immune responses are induced by the parasite through a variety of immunomodulatory mechanisms (Maizels *et al.* 1993).

This level of infection, taken together with studies of helminth infection of rodents, indicates that the response of infected individuals in endemic areas to antigenic challenge could be compromised. In fact, evidence from human studies suggests that exposure of the host to helminths can have a profound impact on the immune responsiveness towards unrelated antigens. Kilian and Nielsen (Kilian and Nielsen 1989) have reported, for example, that persons infected with the nematode *Onchocerca volvulus* exhibit dramatically reduced cutaneous reactivity following immunization with bacillus Calmette-Guerin (BCG), one of the most widely used vaccines in the world that protects against *Mycobacterium tuberculosis* (Nossal 1998). In addition, more recent studies have demonstrated that following vaccination with BCG, infants who's mothers

were infected with helminths tended to develop lower IFN-γ (type 1 cytokine) and increased IL-5 (type 2 cytokine) responses upon challenge (Malhotra *et al.* 1999). This suggests that nematode infection, or sensitization to helminths in utero, may interfere with the development of protective immune responses towards bacterial infection. The experimentation outlined in this thesis addresses the critical question of the nature of the immunomodulation by helminths, particularly nematodes, and the mechanisms by which that modulation could be mediated.

One of the most extensively characterized nematode infections is *Nippostrongylus brasiliensis* (*Nb*) infection of mice and rats. This infection leads to the development of potent type 2 responses, characterized by induction of type 2 cytokine expression, local mast cell hyperplasia, systemic eosinophilia, as well as the production of IgE and IgG1 (in mice; Urban *et al.* 1992, Urban *et al.* 1993) or IgG2a (in rats; Matsuda *et al.* 1995, Ushikawa *et al.* 1994). Therefore, infection with *Nb* in rodents provides a useful model with which to study the effects of nematodes on immune modulation upon challenge with clinically important unrelated antigens.

Previous studies from our laboratory (Ledingham *et al.* 1996) have demonstrated, for example, that infection of rats with *Nb* prior to transplantation with an allogeneic kidney leads to a significant prolongation of graft survival. This was associated with a marked reduction in numbers of graft infiltrating cells (GIC) and with the transcription of mRNA for IL-4 (type 2 cytokine).

There have been a number of reports suggesting that therapies which favor type 2 immunity have marked effects on the allograft survival. Because *Nb* infection leads to strong type 2 immunity, and since the allograft rejection process appears to depend on

type 1 T cell effector mechanisms, the hypothesis has been developed that *Nb*-induced allograft survival is associated with the development of type 2 allo-specific immunity and inhibition of type 1-induced effector responses. Therefore the first part of this thesis describes experimentation designed to examine this hypothesis.

The elaboration of this, and other, immuno-modulatory effects of nematodes is of great interest since a substantial part of the world's population encounters antigen, both naturally and in the form of vaccines, against a background of nematode infection (Maizels *et al.* 1993). However, little is known about the influences that nematodes may have on development of T cell-mediated responses. Thus the second part of this thesis investigates the effects of nematode infection on T cell activation.

In order to discuss the mechanisms by which *Nb* may modulate T cell responses to unrelated antigens, information regarding T cell activation, development of T cell effector mechanisms, T cell regulation, and mechanisms of allograft rejection must be introduced. In addition, the nature of immune responses induced during *Nb* infection has to be considered. The background section of this thesis summerizes the current understanding in these areas, which are necessary for the subsequent discussion.

2.0 Background

2.1 T cell activation: Two-signal hypothesis.

In 1890, Ehrlich and Morgenroth observed that animals could produce antibodies against xenogeneic or allogeneic red blood cells (RBC), but not against their own RBCs. This observation led to Ehrlich's description of "horror autotoxicus":

> "The organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins. These contrivances are naturally of the highest importance for the existence of the individual" (Silverstein and Rose 1997).

In order to explain these phenomena Bretscher and Cohn proposed a "two-signal" hypothesis (Bretscher and Cohn 1970). It stated that the recognition of foreign antigen by an antibody producing cell (signal 1) without signal 2, results in paralysis of the antibody producing cell. It is now well documented in the literature that signal 2 in B cell activation consists of T helper (Th) cell products released following recognition of an antigen epitope processed by the B cell. Therefore, as suggested by Bretcher and Cohn, in the absence of Th cell help, B cell unresponsiveness (anergy) results, thereby demonstrating that the lack of the second signal can provide a level of regulatory control for B lymphocyte activation.

Evidence from transplantation research suggested that T lymphocytes also require two signals in order to become activated. For example, it was found that the capacity of allogeneic cells to stimulate T cells requires metabolically active stimulators (Lafferty *et al.* 1983). It was further observed that cells of non-lymphoid origin, such as red blood cells, platelets or fibroblasts, fail to stimulate allogeneic lymphocytes *in vitro*, despite the

presence of MHC molecule expression (Lafferty *et al.* 1983). The demonstration by the same group that thyroid tissue cultured *in vitro* for 14 days prior to transplantation into allogeneic mice significantly extends allograft survival, suggests that alloantigen itself, provided by the *in vitro* cultured graft, was not sufficient to activate graft rejection. Furthermore, loss of antigenicity due to thyroid culture could not account for the extended survival of grafted thyroid tissue, since injection of the recipient with viable peritoneal donor cells at the time of transplantation resulted in prompt rejection of the thyroid tissue (Lafferty *et al.* 1976). A second "two-signal" hypothesis was proposed (Lafferty and Cunningham 1975) to explain these phenomena, which stated the following:

1) Two signals, antigen activity and co-stimulatory activity, are required for initiation of T cell activation. The resting T cell is converted to the activated state following antigen binding by the T cell receptor (delivery of signal 1) in the presence of co-stimulatory activity, which provides signal 2. Since co-stimulatory activity is a cellular product, a corollary of the first postulate is the idea that a stimulator cell is required for T cell activation.

2) A control structure on the stimulator cell regulates the release of co-stimulatory activity; co-stimulatory activity is only released when this structure is engaged by the potentially responsive T cell.

Slightly edited for clarity from (Lafferty and Cunningham 1975).

We now know that T cell recognition of antigen in the context of MHC molecule results in signal 1, while the second signal is provided by an antigen presenting cell (APC) in order for the T cell to become activated.

2.2 Signal 1 for T cell activation.

T cell receptor (TCR) is a heterodimer composed of disulfide-linked α and β, or γ and δ chains (Davies and Bjorkman 1988). These protein chains confer the antigen specificity of the T cell, and interact with the peptide-MHC complexes on the APC (Schwartz 1985). The TCR is co-expressed on the cell surface with a CD3 protein complex composed of four integral membrane proteins, γ, δ, ε, and ζ,which are involved in signal transduction to the T cell interior (Clevers *et al.* 1988). Two other proteins, namely CD4 and CD8, are co-expressed with the TCR complex in T helper (Th) and cytotoxic T cell precursors, respectively. These proteins interact with the non-polymorphic regions of the MHC class II and class I molecules, respectively and thus strengthen the TCR-MHC interaction (Doyle and Strominger 1987, Norment *et al.* 1988). Engagement of the TCR with the antigenic peptide-MHC complex results in a series of biochemical events, termed signal transduction, which are required for T cell activation (Weiss and Littman 1994). Signal transduction is thought to be initiated by activation of two members of the src family of protein tyrosine kinases (PTK), p56Lck (associated with the CD4 and CD8 molecules) and p59Fyn (Abraham *et al.* 1991, Barber *et al.* 1989, Samelson *et al.* 1990). This is accomplished through enzymatic activity of CD45, a protein phosphatase, which dephosphorylates tyrosine residues present on Lck and Fyn

(Koretzky 1993). Activation of these PTKs results in phosphorylation of the tyrosine residues present in the cytoplasmic portion of ε and ζ chains of the CD3 protein complex. CD3 chain phosphorylation allows for their association with Fyn and another PTK termed ZAP-70 (Koretzky 1993, Chan *et al.* 1991). These PTKs are responsible for tyrosine phosphorylation of phospholipase C (PLCγ1) (Weiss *et al.* 1991), which then cleaves a plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which are termed second messengers (Gardner 1989, Berridge 1993). IP_3 is responsible for the release of calcium ions from intracellular Ca^{2+} stores (Nishizuka 1992). Subsequently, calcium dependent activation of calmodulin results in activation of the phosphatase calcineurin (Clipstone and Crabtree 1992), which dephosphorylates the cytoplasmic component of nuclear factor of activated T lymphocyte (NFAT) (Crabtree and Clipstone 1994). This active form of NFAT translocates into the nucleus where it associates with AP-1 complex composed of two factors, c-Jun and c-Fos (Crabtree and Clipstone 1994, Karin *et al.* 1997). The NFAT –AP-1 transcriptional complex binds to a specific site within the promoter region of the IL-2 gene (Jain *et al.* 1995). The second signalling pathway initiated by DAG involves activation of protein kinase C (PKC) (Nishizuka 1992). This subsequently results in activation of another nuclear factor, NF-κB, which translocates to the nucleus where it binds to another site of the IL-2 promoter region (Jain *et al.* 1995). Production of IL-2 is a critical step in T cell activation since IL-2 is the main factor capable of inducing T cell proliferation (Karnitz and Abraham 1996).

Activation of the second messenger pathways that occurs upon interaction of TCR with peptide-MHC complex is not sufficient for T cell activation. It has been demonstrated, for example, that purified MHC class II molecules inserted into liposomes and loaded with processed peptide are unable to stimulate T cell division (Quill and Schwartz 1987). In addition, murine Th1 clones, when stimulated with a lectin mitogen concanavalin A (Con A), fail to proliferate or produce IL-2 in the absence of antigen presenting cells despite the fact that normal generation of second messengers occurs in these cells (Mueller *et al.* 1989). Similar findings were reported in studies using chemically-fixed APC and peptide to stimulate T cell lines. Interestingly, addition of allogeneic APC in this system (T cells + fixed APC + allogeneic APC), which were unable to provide signal 1 because of MHC disparity, conferred co-stimulatory activity necessary for IL-2 production and proliferation. This allogeneic APC-mediated T cell activation did not result in the increase in second messenger levels, suggesting that this co-stimulatory activity was not acting through the same pathway as the TCR mediated signal transduction (Mueller *et al.* 1989).

2.3 Signal 2 for T cell activation.

Cell surface receptors such as CD2, LFA-1 and VLA-4 have been implicated in T cell co-stimulation (Liu and Linsley 1992). However, the co-stimulatory molecule that has received the most attention in recent years is CD28. In 1992, it was shown that ligation of CD28 on T cells with anti-CD28 mAb was able to provide the co-stimulatory signal for proliferation and IL-2 production by purified $CD4^+$ T cells in response to suboptimal doses of plate bound anti-CD3 mAb (Harding *et al.* 1992), an artificial means

to mimic TCR signalling. This proliferation and IL-2 production could also be achieved by including syngeneic LPS blasts. However, this induction could subsequently be inhibited if an Fab fragment of anti-CD28 antibody was added to the culture (Harding *et al.* 1992), suggesting that LPS blasts also act upon CD28 to induce T cell activation. Subsequent studies by these authors demonstrated that fixation of syngeneic APCs rendered them incapable of activating antigen specific $CD4^+$ T cell clones. In fact, $CD4^+$ clones exposed to fixed APCs became anergic. Unresponsiveness of $CD4^+$ clones could, however, be prevented if allogeneic APC incapable of presenting specific antigen to these clones were included in the culture, suggesting that the allogeneic APCs were the source of co-stimulatory signal 2. Interestingly, addition of an anti-CD28 Fab fragment to this system inhibited the ability of allogeneic APCs to provide co-stimulation (Harding *et al.* 1992). These studies supported the hypothesis that a ligand for CD28, present on APC, is actively transported to the APC surface and is required for the activation of T cells.

In a separate study, Jenkins and colleagues confirmed the importance of CD28 as a co-stimulatory molecule (Jenkins *et al.* 1991). In their system, anti-CD28 antibody was able to provide the co-stimulatory signal necessary for proliferation and IL-2 production by human tetanus toxoid-specific $CD4^+$ T cells in response to the antigen. In addition, the ability of B-cell lines to provide the co-stimulatory signal in that study correlated with the expression of a molecule defined as B7.1 (Jenkins *et al.* 1991). The involvement of B7.1 in T cell costimulation was confirmed by transfection of chinese hamster ovary (CHO) cells with B7.1, which conferred the ability of transfected cells, unlike B7.1-void parental cells, to mediate T cell costimulation (Linsley *et al.* 1991a).

A second ligand for the CD28 was later described by Lenschow and colleagues (Lenschow *et al.* 1993), who demonstrated that neutralizing antibody to B7.1 was unable (less than 10% inhibition) to block T cell proliferation in mixed lymphocyte reaction using allogeneic cells enriched for dendritic cells as APCs. In contrast, a fusion construct of CTLA-4 with the immunoglobulin constant chain, referred to as CTLA-4Ig, was shown to be a potent inhibitor of B7.1-mediated co-stimulation (Linsley *et al.* 1991b). Taken together, these results suggested that there was an additional CD28 ligand expressed on dendritic APCs. This was subsequently demonstrated by FACS analysis. Although unlabeled anti-B7.1 mAb completely inhibited binding of FITC-labeled CTLA-4Ig to B7.1 transfected CHO cells, it was not able to abrogate the staining of dendritic cells with CTLA-4Ig, thereby confirming the presence of a second ligand for CTLA-4 on dendritic cells, later termed B7.2 (Lenschow *et al.* 1993).

The importance of CD28 mediated co-stimulation for T cell activation was demonstrated at the molecular level by Su and collegues (Su *et al.* 1994). This group found that simultaneous stimulation through the TCR and CD28 resulted in 30-fold increase in Jun kinase (JNK) activity. In contrast, triggering through TCR or CD28 alone resulted only in 1.5 and 2-fold increase in JNK activity, respectively. JNK is member of the mitogen-activated protein (MAP) kinase family. One of JNK's functions is to phosphorylate and activate the c-Jun transcription factor (Hibi *et al.* 1993), a component of the AP-1 complex that binds within the promoter region of the IL-2 gene to enhance transcription. An additional transcription factor, called CD28RC, results in five-fold augmentation of IL-2 transcription, and its expression is dependent on triggering through both TCR and CD28 molecules (Fraser *et al.* 1991). Therefore the main role of CD28

mediated costimulation seems to be the induction of IL-2 gene expression. However, recent studies have also shown that CD28 mediated co-stimulation results in upregulation of Bcl-x_L (survival factor) expression, thereby leading to extended T cell survival upon activation (Boise *et al.* 1995).

Subsequent studies performed *in vivo* provided more evidence supporting a critical role for CD28 as a co-stimulatory molecule in T cell activation. In 1992, Lenschow and co-workers demonstrated that diabetic C57BL/6 mice, treated with CTLA-4Ig, indefinitely tolerated human xenograft islets. (Lenschow *et al.* 1992). Furthermore, injection of CTLA-4 Ig resulted in antigen-specific tolerance, as a subsequent similar graft survived indefinitely, whereas the third-party xenografts were promptly rejected (Lenschow *et al.* 1992).

In addition, treatment of mice with the CTLA-4Ig construct at the time of immunization with sheep red blood cells or keyhole limpet hemocyanin (KLH) resulted in antigen-specific unresponsiveness, as assessed by specific antibody titres *in vivo* and T cell proliferative activity *in vitro* (Linsley *et al.* 1992a). Inhibition of the antigen-specific responses was not due to non-specific immunosuppression as mitogen-induced proliferation was unaltered following CTLA-4Ig treatment. These results confirm that CD28 is essential for T cell activation *in vivo*, and suggest that CD28 signaling is not merely an artifact of *in vitro* analysis.

2.4 Co-regulation of T cell activation.

a) Activation of APC: CD40/CD40L.

Another complexity was added to the co-stimulatory molecule story after an examination of the role of the receptor/counter receptor pair CD40-CD40L in immune responses. CD40 has been found to be expressed on dendritic cells, activated macrophages and B-cells whereas CD40L is expressed almost exclusively on T cells (Banchereau *et al.* 1994). This suggested that this receptor/ligand pair may be important in co-stimulation. In order to study the role of these molecules in costimulation *in vivo*, CD40L KO (knock out) mice were developed. Interestingly it was observed that following immunization with antigen such as KLH CD40L deficient mice exhibited profoundly reduced proliferative antigen-specific T cell recall responses *in vitro* (Grewal *et al.* 1995). In addition it was found that CD40L deficient, but not wild-type TCR transgenic T cells failed to expand *in vivo* in response to antigenic challenge (Grewal *et al.* 1995). This profound inability of CD40L deficient T cells to become activated was further confirmed by studies in an experimental autoimmune encephalomyelitis (EAE) model. In this study it was observed that CD40L deficient mice transgenic for the self-reactive TCR specific for myelin basic protein (MBP) failed to develop EAE even when immunized with MBP in complete Freund's adjuvant (CFA) (Grewal *et al.* 1996). The authors have hypothesized that the defect lay in the inability of these T cells to activate APCs *in vivo* and speculated that the role for the CD40-CD40L interaction may be to upregulate co-stimulatory molecules such as B7.1and/or B7.2 on the APCs which would, in turn, activate T cells. To test this hypothesis the authors injected CD40L deficient mice with APCs obtained from B7.1 transgenic mice prior to immunization with MBP in

CFA. As expected, CD40L deficient mice that were injected with B7.1^{+} APCs promptly developed severe EAE confirming that the defect involved an inability of T cells from these mice to activate APCs (Grewal *et al.* 1996). Further supporting this hypothesis were studies showing that administration of anti-CD40 mAb to CD40L deficient mice fully restored their cellular and humoral responses to antigens. This was associated with an increase in B7.2 expression on splenic APC and could be inhibited by administration of anti-B7.2 mAb (Yang and Wilson 1996). This confirmed that ligation of CD40 on APCs by CD40L present on T cells resulted in upregulation of B7.2.

b) Regulation of T cell responses by IL-6.

Besides upregulation of B7.2 expression, ligation of CD40 on APC results in the production of cytokines such as IL-1 and IL-6 (Rissoan *et al.* 1999). These cytokines were the first molecules thought to be able to provide a co-stimulatory signal necessary for T cell activation (Van Snick 1990). However, further studies demonstrated that T cell responses in the presence of IL-1 and IL-6 rapidly decreased with decreasing APC concentration, confirming that contact with the APC was required for T cell activation and IL-2 production (Holsti *et al.* 1994). As reviewed above this cell-cell contact involves engagement of CD28 and its ligands. However, recent evidence suggests that IL-1 and IL-6 are required for optimal T cell proliferation in response to antigen and anti-CD28 stimulation (Joseph *et al.* 1998). For example, in *in vitro* experiments with anti-CD28-mediated co-stimulation, the addition of IL-6 resulted in a dramatic increase in proliferation in response to anti-CD3. This was associated with augmented IL-2 production. These effects, mediated by IL-6, were synergistically augmented by

treatment with IL-1 and TNF-α. Further, during stimulation with anti-CD3 in an environment with minimal or absent co-stimulation, the addition of IL-6 did not induce IL-2 production but dramatically enhanced T cell proliferation if exogenous IL-2 was added to the culture. Thus, IL-6 can increase the sensitivity of T cells to receive an IL-2-induced signal. Taken together, these results suggested that CD28-mediated co-stimulation is necessary for IL-2 production and that IL-6 enhances IL-2 this production and responsiveness of the activated T cells to the IL-2-mediated signal.

The IL-2 receptor is composed of three chains: IL-2Rα IL-2Rβ and IL-2Rγ (Minami *et al.* 1993). IL-2Rβ and IL-2Rγ subunits both participate in signal transduction (Karnitz and Abraham 1996). These subunits are constitutively expressed on resting T cells and comprise a low affinity IL-2R (Nelson and Willerford 1999). However, the IL-2Rα subunit is necessary for high affinity binding of IL-2 to the receptor (Nelson and Willerford 1999). In fact, in mice deficient in the IL-2Rα subunit T cells are unable to use IL-2 and exhibit markedly reduced proliferative responses (Van-Parijs *et al.* 1997). The IL-2Rα subunit is not expressed on resting T cells (Minami *et al.* 1993, Akaishi *et al.* 1998, Nelson and Willerford 1999). Its expression is induced upon T cell activation and is at least in part dependent on IL-2 signaling through the low affinity IL-2R (Nakajima *et al.* 1997). This has been demonstrated by studies in STAT5a KO mice in which T cells were unresponsive to IL-2-mediated signals due to the inhibition of IL-2Rα expression (Nakajima *et al.* 1997). Signal transduction through the IL-2R is mediated by phosphorylation of transcription factors including STAT5a and STAT3 (Nelson and Willerford 1998) although STAT3 is phosphorylated to a lesser degree

(Takeda *et al.* 1998). Recent studies in mice that express non-functional STAT3 in T cells, demonstrated that T cell proliferation in these mice was severely compromised (Akaishi *et al.* 1998). This was due to inability to induce IL-2Rα expression upon activation with anti-CD3. Thus, activation of both STAT5a and STAT3 is important for transcription of IL-2Rα. Interestingly STAT3 is activated in T cells in response to IL-6 (Takeda *et al.* 1998). This provides a possible mechanism by which IL-6 signaling may augment expression of high affinity IL-2R expression, increase responsiveness to IL-2, and further enhance proliferation of activated T cells. In fact, recent studies in IL-6 KO mice demonstrated that T cells from these mice have a compromised ability to induce the IL-2Rα subunit upon activation and, as a result, they exhibit reduced proliferative responses to mitogenic stimulation (La Flamme *et al.* 1999).

The IL-6 receptor is composed of single 80 kDa IL-6Rα chain (gp80) and two 130 kDa IL-6Rβ (gp130) subunits (Yamasaki *et al.* 1988, Hibi *et al.* 1990). The interaction of IL-6 with the IL-6Rα subunit triggers the homo-dimerization of the gp130 chains (Murakami *et al.* 1993), which are the signal transducing subunits (Stahl *et al.* 1994, Heinrich *et al.* 1998). Besides IL-6, other cytokines such as IL-11, Leukemia Inhibitory Factor (LIF) and Ciliary Neurotrophic Factor (CNTF) utilize gp130 as a signaling subunit (Hibi *et al.* 1990, Kishimoto *et. al.* 1995). Thus gp80 is a subunit that confers specificity of the IL-6-mediated signal. Dimerization of gp130 leads to phosphorylation and activation of Jak1, Jak2 and Tyk2 kinases that are associated with the gp130 chain (Stahl *et al.* 1994, Stahl *et al.* 1995). These, in turn, phosphorylate gp130 at a number of tyrosine residues present in the cytoplasmic portion of the molecule

(Heinrich *et al.* 1998). These residues are important to enable docking and subsequent phosphorylation of STAT1 and STAT3 transcription factors as well as SHP2 molecules by the Jak kinases associated with the receptor (Heinrich *et al.* 1998). Upon phosphorylation STAT1 and STAT3 become activated, dimerize, and translocate to the nucleus where they induce transcription of a number of genes such as those important in the acute phase response, such as C-reactive protein, as well as AP-1 family transcription factors including c-Fos and Jun-B (Heinrich *et al.* 1998). Activation of SHP2, on the other hand, leads to signaling through the Ras-dependent MAPK pathway (Fukada *et al.* 1996, Ogata *et al.* 1997) leading to activation of the Erk-1 and Erk-2 kinases (Ogata *et al.* 1997) and the subsequent activation of transcription factors such as NF-IL-6 or c-Jun (Pulverer *et al.* 1991). The STAT3 and SHP2 pathway has recently been shown to be important in gp130 mediated induction of proliferation of a mouse pro-B cell line (Fukada *et al.* 1996). In these studies the activation of STAT3 led to resistance to cell death induced by growth factor deprivation. This was associated with the induction of expression of Bcl-2, a molecule capable of preventing apoptosis (Minn *et al.* 1998). In contrast, SHP2 was important for cell cycle progression and induction of mitogenesis in these cells. Thus, enhanced survival is yet another possible mechanism by which IL-6 could modulate T cell responsiveness.

Indeed, it has been demonstrated that the presence of IL-6, *in vitro*, led to a dramatic reduction in the death of resting T cells (Teague *et al.* 1997). This was associated with preservation of expression of anti-apoptotic molecules Bcl-2. Therefore IL-6 may be an important factor for maintenance of peripheral resting T cell populations. Recently, it has also been shown that IL-6 enhanced survival of mitogen-activated T cells

(Takeda *et al.* 1998, Ayroldi *et al.* 1998). This was not mediated through the induction of anti-apoptotic molecules such as Bcl-2, Bcl-x_L or BAG-1 (Takeda *et al.* 1998). The authors argued that this was mediated through direct IL-6-mediated induction of the STAT3 pathway, since IL-6 did not confer protection from death in STAT3-deficient T cells. However the studies described above (Akaishi *et al.* 1998), demonstrating that STAT3 KO mice are unable to respond to the IL-2 signal, suggest that this IL-6-mediated anti-apoptotic effect may be induced indirectly through IL-2. This, however, is unlikely since IL-2 signaling was found by a number of researchers to be a prerequisite for the induction of apoptosis in T cells through the upregulation of FasL expression (Esser *et al.* 1997, Ayroldi *et al.* 1998, Rafaeli *et al.* 1998). Interestingly, recent studies by Ayroldi and co-workers (Ayroldi *et al.* 1998) demonstrated that IL-6 dramatically reduced apoptosis in anti-CD3 activated T cells with concomitant downregulation of FasL expression. Further, this was not associated with a reduction in IL-2 or IL-2Rα expression, suggesting that IL-6 may prevent IL-2-induced apoptosis, but spare the IL-2 signaling pathway, leading to mitogenesis. Regardless of the mechanism, the presence of IL-6 during T cell stimulation can clearly lead to profound modulation of T cell survival.

The effects of IL-6 on T cell activation are likely to be important *in vivo*. For example, T cells from IL-6KO mice immunized with antigen exhibit reduced levels of activation, proliferation, and cytokine production during *in vitro* challenge (Samoilova *et al.* 1998). This inhibition of proliferative responses was associated with an inability to induce the effector functions necessary to mediate EAE in these mice. These results are strikingly similar to the studies described above that were performed in CD40L-deficient

mice. Those experiments showed that CD40L KO mice transgenic for MBP-specific TCR failed to develop EAE upon immunization with the antigen due to inability to activate the APC (Grewal *et al.* 1996).

2.5 Negative regulation of T cell responses: regulation at the level of activation/proliferation or effector cell death.

a) Inhibitory signal provided by CTLA-4.

Experiments investigating the inhibitory signals of T cell activation implicated CTLA-4. It was shown, for example, that cross-linking of CTLA-4 *in vitro*, results in inhibition of both proliferation and IL-2 production by T cells in response to stimulation with a combination of anti-CD3 and anti-CD28 mAb (Walunas *et al.* 1994). This suggests that cross-linking of CTLA-4 on the T cell surface results in a negative signal transduced into the cell. Subsequent studies showed that preventing the interaction of CTLA-4 with the B7.1 and/or B7.2 ligands by treatment with anti-CTLA Fab fragment could markedly enhance the proliferation and IL-2 production by anti-CD3/CD28 activated T cells (Krummel and Allison 1995). This further supported the notion that the interaction of CTLA-4 with its ligands is inhibitory for T cell activation.

The role of CTLA-4 was also investigated *in vivo*. Kearney and colleagues developed an adoptive transfer system, in which $CD4^+$ T cells that expressed transgenic TCR specific for OVA peptide in the context of $I\text{-}A^d$ were transfered into syngeneic mice. The expension of these transgenic cells in recipient mice following immunization with antigen was monitored using anti-clonotypic antibody, revealing that immunization with OVA resulted in a 10 fold increase in OVA-specific TCR transgene positive cells.

Treatment of these mice with CTLA-4 Ig, or the combination of anti-B7.1/B7.2 mAbs, at the time of immunization resulted in marked inhibition of the T cell expansion. However, when mice were treated with a Fab fragment of a CTLA-4 mAb at the time of immunization, the number of transgenic TCR expressing cells more than doubled as compared to mice treated with irrelevant antibody, confirming that CTLA-4 is involved in negative signaling (Kearney *et al.* 1995). The report that most clearly demonstrates the critical importance of CTLA-4 in regulation of T cell activation and maintenance of immunological homeostasis *in vivo* comes from studies in CTLA-4 KO mice (Tivol *et al.* 1995). These mice appear normal until 2 weeks of age at which time they begin to develop a severe lymphoproliferative disease culminating in multiorgan lymphocytic infiltration, followed by tissue destruction and death by 20-30 days of age. Lymph node T cells from these mice proliferate spontaneously *in vitro*, and their proliferation in response to anti-CD3 is four times higher than that exhibited by T cells from control mice. In addition, these cells secrete high levels of IL-4 and IFN-γ, and exhibit elevated expression of IL-2 receptor in the absence of stimulation, suggesting that they are activated *in vivo* prior to culture (Tivol *et al.* 1995).

It was postulated that CTLA-4 functioned to decrease proliferation or cytokine production at later time points following T cell activation. In support of this theory was work that demonstrated that while CD28 is constitutively expressed on T cells, CTLA-4 expression is only upregulated following T cell activation, reaching its peak of expression between 48 and 72h post-stimulation (Linsley *et al.* 1992b). In addition, upregulation of

CTLA-4 seems to be, in part, dependent on CD28-mediated co-stimulation, since CTLA-4 expression is greatly reduced in T cells from CD28KO mice (Walunas *et al.* 1994).

However, more recent studies have demonstrated that CTLA-4-mediated regulation of T cell activation may occur at early stages after stimulation with antigen. Ligation of CTLA-4 by anti-CTLA-4 mAb prior to activation, for example, induces potent inhibition of $CD4^+$ T cell proliferation and IL-2 production in response to stimulation with anti-CD3/CD28 mAbs (Krummel and Allison 1996). The inhibition of proliferation in those cultures persisted until the end of culture period (65 h), and proliferation reached only 25% of the control. In addition, IL-2 secretion was delayed by 20h, and reached only 20% of the control levels at its peak at 42h. Addition of the Fab fragment of an anti-CTLA-4 mAb to the anti-CD3/CD28 mAb stimulation culture resulted in 10-fold and 2-fold upregulation of IL-2 production and $CD4^+$ T cell proliferation, respectively.

Interestingly, CTLA-4Ig treatment, which presumably blocks the interaction between B7.1/B7.2 and CTLA-4, had the same effect, indicating that CTLA-4, although undetectable on the cell surface prior to T cell activation, is expressed in sufficient levels to exert inhibitory effects on T cell activation.

It was also suggested that low levels of B7.1 and B7.2 expressed on T cells or unactivated APCs may be sufficient for CTLA-4-mediated inhibition. Therefore, the role of CTLA-4 may be both to regulate the threshold necessary to activate T cell as well as controlling T cell activation at later time points. In support of the involvement of CTLA-4 in reducing the T cell activation threshold is the observation that the inhibitory effect observed upon ligation of the CTLA-4 molecule with specific antibody can be overcome

by increasing the amount of anti-CD3 mAb used for stimulation (Krummel and Allison 1995), or by addition of PMA (Chambers and Allison 1996). As treatment with PMA results in increased PKC activity, CTLA-4 may exert its effects by dephosphorylation and inactivation of certain mediators regulating T cell proliferation (Chambers and Allison 1996).

These studies suggest a possible role for other co-stimulatory molecules that contribute to T cell activation. For example coligation of CD3 and CD2 molecules results in marked augmentation of phospholipase C γ1 and PKC activity (Dietsch *et al.* 1994), and therefore may have an effect on CTLA-4-mediated inhibition. Ligation of LFA-1, which synergizes with CD28 in T cell activation (Damle *et al.* 1992), is also known to enhance PKC activity during anti-CD3 mediated activation (Kanner *et al.* 1992; Kanner *et al.* 1993).

b) Fas/FasL and effector T cell apoptosis.

As illustrated above, control of the expansion of T cells is crucial for effective immune function and lack of this control results in lymphoproliferative disorders and autoimmunity. A similar phenotype was observed in mice with mutations in Fas (*lpr*) or or FasL (*gld*) genes respectively (Watanabe-Fukunga *et al.* 1992, Takahashi *et al.* 1994). The interaction of Fas with FasL has been shown to induce programmed cell death or apoptosis in a variety of cells including activated T cells (Russell 1995). The lack of this activated T cell death is thought, in *lpr* and *gld* animals, to lead to exaggerated lymphoproliferation and autoimmunity. This is supported by evidence that *lpr* mice transgenic for a specific TCR do not exhibit deletion of antigen-specific T cells in vivo,

following injection with antigen (Van Parijs *et al.* 1998a). Indeed *in vitro* activated T cells from either *lpr* or *gld* mice fail to undergo activation induced cell death (AICD) by apoptosis and exhibit dramatically enhanced proliferation in response to stimulation with antigen or superantigen (Van Parijs *et al.* 1998b, Ettinger *et al.* 1995).

It is unclear at this point what the exact roles of the pro-apoptotic Fas/FasL pathway and the anti-apoptotic Bcl-2 pathway are in controlling T cell responses. However, recent evidence points towards a role for FasL in the termination of T cell responses to strong or chronic antigenic stimulation (Van Parijs *et al.* 1998a), and a role for Bcl-2 in homeostasis of the resting T cell repertoire (Teague *et al.* 1997), and elimination of the majority of responding T lymphocytes towards the end of the immune response, when antigen and growth factor concentrations are limited (Van Parijs *et al.* 1998a, Van Parijs *et al.* 1998b).

2.6 Immunomodulation.

Immune responses elicited by different pathogens and antigens exhibit different effector functions. Viral infections and some parasites such as *Toxoplasma gondii* or *Encephalitozoon cuniculi* are associated with strong cytotoxic T lymphocyte responses (Sharma *et al.* 1996, Actor *et al.* 1993, Cerwenka *et al.* 1998, Wizel *et al.*1997, Ely *et al.* 1999, Khan *et al.* 1999). Other intracellular parasites such as *Leishmania major* result in activation of $CD4^+$ T cells, which induce delayed type hypersensitivity-like response in infected tissues by recruitment and activation of macrophages, as well as inducing class switch towards the IgG2a isotype (in mice; Reiner and Locksley 1995). These protective responses are controlled by a subpopulation of $CD4^+$ Th cells called Th1 (Mosmann and

Sad 1996). These cells secrete cytokines such as IL-2, IFN-γ, and lymphotoxin (Mosmann and Sad 1996). They induce DTH reactions, development of CTL activity and provide help for B cells to undergo class switch and to produce IgG2a and IgG2b antibodies (Mosmann and Sad 1996). On the other hand, infection with intestinal nematodes result in the activation and differentiation of $CD4^+$ T cells called Th2 that produce cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13. This pathway promotes class switch in B cells towards IgG1 and IgE production, and the activation of mast cells and eosinophils (Urban *et al.* 1992).

Th2 responses are not protective against parasites such as *L. major* and the presence of Th2 cytokines during infection with this parasite leads to exacerbation of infection (Reiner and Locksley 1995). In contrast, the presence of Th1 cytokines during infection with the intestinal nematode *Trichuris muris* is associated with an inability to reject this parasite (Grencis 1996). Thus, protective immune responses against a given pathogen appear to be characterized by a dominant generation of one of the Th cell subsets and an inhibition of the other.

Such plasticity of the immune responses has been recognized for a long time. For example, it has been reported that i.v. injection of protein inhibits DTH reactions and induces protein-specific antibody formation (Asherson and Stone 1965, Asherson 1966). Asherson referred to this phenomenon as "immune deviation". Other groups have reported similar findings. However, the most important evidence for the inverse relationship between induction of antigen specific antibody production, and DTH, was provided by Parish. Parish investigated the various degrees of immunogenicity of

different forms of bacterial flagellin. He observed that polymerized flagellin induced a strong DTH response with no detectable antibody response, while acetoacetylated flagellin induced antibody but no DTH. Interestingly, other forms of flagellin or its fragments induced intermediate response phenotypes (Parish 1971a, Parish 1971b).

Subsequently, dose response showed that antigen doses inducing high antibody response induced low DTH, and vice-versa (Parish and Liew 1972). This suggested that apparent unresponsiveness may indicate a shift to a different type of an immune response. It was proposed that this shift could be mediated by two functionally different effector cell populations cross-regulating one another (Bretscher 1974).

2.7 Th1/Th2 paradigm.

In 1986, Timothy Mosmann and Robert Coffman reported cloning murine T-helper (Th) cells which, upon analysis of their cytokine production, could be subdivided into two subsets, termed Th1 and Th2. A number of $CD4^+$ Th clones were developed from mice immunized with Chicken red blood cells (CRBC) which gave a strong IgM response, and fowl γ-globulin (FGG) on alum, which induced IgE production. The analysis of the cytokine production revealed striking results. All CRBC-specific, but not FGG-specific clones, produced IL-2 and IFN-γ in response to either antigen or Con A. In contrast, FGG-specific, but not CRBC specific clones, secreted IL-4. The IL-2 and IFN-γ secreting clones were called Th1 while IL-4 secreting clones were designated Th2. Since it was known that T cells could exert their effects by secreting cytokines, the authors suspected that a distinction in cytokine patterns exhibited by those clones was

likely indicative of functionally different subsets. Indeed, further experiments demonstrated differences in helper activity provided by these clones to B-cells. Only Th1 cells provided help for IgG2a production, while Th2 were much better than Th1 clones at inducing *in vitro* IgG1. In addition, the help for IgE production could only be provided by Th2 clones (Mosmann *et al.* 1986). These results, together with the fact that only FGG immunization (Th2 generating) resulted in increased antigen specific serum IgE production, suggested that Th1 and Th2 population are relevant to the *in vivo* situation.

Further evidence for different functional capabilities of Th1 and Th2 came from an experiment by Cher and Mosmann. They found that when injected into the footpad together with the relevant antigen, all Th1, but not Th2 clones, caused DTH (Cher and Mosmann 1987). However, it was not until studies were performed in the model of *L. major* infection in mice that the relevance of Th1 and Th2 clones to the *in vivo* situation gained credibility. *L. major* infection causes a localized infection, which is efficiently cleared in most strains of mice, such as CBA or C57BL/6 (Howard *et al.* 1980). In contrast, susceptible strains of mice, such as BALB/c, develop a fatal infection. The inability to efficiently control the infection with *L. major* in BALB/c mice has been associated with the inhibition of parasite-specific cell-mediated immunity, as measured by DTH response (Howard *et al.* 1980). The inability of BALB/c mice to mount a DTH response was intriguing in the view of the fact that the *L. major* specific $CD4^+$ T cell proliferation was increased in these mice (Milon *et al.* 1986). With the recent description of Th1 and Th2 clones in mind, Heinzel and co-workers hypothesized that preferential development of cells of the Th2 phenotype in BALB/c should, in theory, render them

susceptible to the *L. major* infection. To test this hypothesis, they compared the IFN-γ and IL-4 mRNA levels expressed in the lymph nodes and spleens of BALB/c and C57BL/6 during the *L. major* infection. Indeed, the presence of IL-4 and absence of IFN-γ was observed in susceptible, but not resistant, mouse strains. Furthermore, treatment of BALB/c mice with anti-CD4 antibody (which has been previously shown to render them resistant to the infection), resulted in appearance of IFN-γ and loss of the IL-4 mRNA in the lymphoid tissues (Heinzel *et al.* 1989). Association of the reciprocal pattern of cytokine expression with resistance or susceptibility to infection clearly demonstrated the involvement of cytokines in regulation of the immune response. This work mirrored that of Parish in the flagellin model, thereby reintroducing the idea of immune deviation.

Was the reciprocal pattern of cytokine expression, observed by Heinzel and co-workers in the *Leishmania major* model and Mosmann and colleagues *in vitro*, a coincidence? Previous studies by Scott and co-workers demonstrated that the immunization with soluble *Leishmania* antigen (SLA) protected BALB/c mice from subsequent infection with the parasite. This immunity was associated with the recovery of *L. major* specific DTH response (Scott *et al.* 1987). Subsequently, $CD4^+$ T cell lines were prepared from BALB/c mice immunized with SLA. Both Th1 and Th2 cell lines were produced, and then injected into BALB/c recipients that were challenged with the parasite. Remarkably, injection of the Th1 cell line resulted in healing, while injection of the Th2 clones exacerbated the infection. This suggested that the susceptibility to infection was due to development of an inappropriate, in this case Th2, response. The

ability of the injected clones to reverse or exacerbate the susceptibility was thought to be due to the cytokines that they secreted. This was later supported by work of Sadick and colleagues who showed that injection of anti-IL-4 antibodies protected BALB/c from the infection while reversing the cytokine pattern from Th2 to Th1 (Sadick *et al.* 1990). These studies suggested that the two subsets cross-regulated one another through secretion of cytokines. In fact the early studies had shown that the production of IFN-γ inhibited proliferation of Th2 but not Th1 clones (Gajewski and Fitch 1988). On the other hand, another factor (later called IL-10) that was elaborated by Th2 clones was found to inhibit IFN-γ production from Th1 cells (Fiorentino *et al.* 1989).

It was soon noted however, that the Th1/Th2 paradigm could not alone explain a number of studies. Most notably, administration of recombinant IL-4 to *L. major*-infected BALB/c mice was found to reduce lesion size and parasitaemia (Carter *et al.* 1989), while adoptive transfer of *L. major*-specific Th1 cells could sometimes lead to exacerbation of the disease (Titus *et al.* 1991). In addition IFN-γR KO mice bred on a *Leishmania* resistant background succumbed to *L. major* infection even though T cells derived from draining lymph nodes or spleens of these mice produced IFN-γ and no IL-4 (Swihart *et al.* 1995). More recent studies also generated contradictory results. Most notably, IL-4 KO mice developed in 129/Sv mice later backcrossed onto a BALB/c background were resistant to *Leishmania* infection. However, when IL-4 deletion was performed on a BALB/c background, the mice were still susceptible to *L. major* infection even though they failed to develop Th2 responses both in response to *Leishmania* or when infected with the strong Th2 inducer, *Nippostrongylus brasiliensis*.

(Noben-Trauth *et al.* 1996). These studies suggest that other factors contribute to the increased susceptibility to this infection in BALB/c mice. In that context it is of interest that while IL-4 KO BALB/c mice are susceptible, the IL-4Rα KO BALB/c mice are resistant to infection with the same strain of *L. major* (Noben-Trauth *et al.* 1999). This suggests that another factor, which can signal through the IL-4Rα, can provide the signals necessary for inhibition of appropriate responses necessary to kill the parasite in BALB/c mice, and indicates that the cross-regulation by Th1 and Th2 cells is more complex than was originally thought.

2.8 Importance of IL-12 and IFN-γ in Th1/Th2 development.

a) IL-12.

IL-12 is a heterodimer composed of two subunits, termed p40 and p35, linked through a disulfide bond (Trinchieri 1995). This cytokine can be produced by dendritic cells (Macatonia *et al.* 1995) especially following activation via CD40/CD40L and ligation of class II MHC molecules (Koch *et al.* 1996). In addition IL-12 can be produced by macrophages activated with LPS (Trinchieri 1995), heat killed *Listeria monocytogenes* (Hsieh *et al.* 1993) or *Toxoplasma gondii* (Gazinelli *et al.* 1993) suggesting that innate immunity is involved in regulation of Th1 differentiation. IL-12 produced by these cells has been shown to induce IFN-γ production by NK cells and naive T cells (Guler *et al.* 1996, Hsieh *et al.* 1993, Guler *et al.* 1997). In fact it was shown that macrophages activated with heat-killed *L. monocytogeneses* primed naive T

cells for production of IFN-γ which could be blocked by anti-IL-12 treatment (Hsieh *et al.* 1993).

A direct role for IL-12 in the induction of Th1 effector functions was demonstrated using mice deficient for IL-12 expression. These mice, developed on a background resistant to *L. major* infection, exhibit both enhanced Th2 and reduced Th1 responses (Magram *et al.* 1996). As such they were now susceptible to *Leishmania* (Mattner *et al.* 1996). Moreover, genetic deletion of STAT4, which is phosphorylated upon interaction of IL-12 with the IL-12R (Trinchieri 1998) also led to abrogation of Th1 and enhanced Th2 cytokine production under conditions that would normally favor Th1 activation (Kaplan *et al.* 1996).

Recent studies in BALB/c mice demonstrated that injection of recombinant IL-12 during *L. major* infection conferred resistance to this parasite (Launois *et al.* 1997). However, the treatment with IL-12 was effective at blocking the development of the Th2 response, and inducing a healing phenotype, only if administered within the first 48h after infection (Launois *et al.* 1997). This suggests that newly activated T cells, from BALB/c mice, lose the ability to respond to IL-12 shortly after activation. This speculation was confirmed by studies in a *L. major* infection model (Himmelrich *et al.* 1998) and by *in vitro* experimentation (Guler *et al.* 1997) demonstrating that T cells from BALB/c mice downregulate their expression of the IL-12Rβ2 subunit soon after activation.

b) IFN-γ.

The downregulation of the IL-12Rβ2 subunit referred to above was mediated through IL-4, and anti-IL-4 treatment abrogated the effect (Himmerlich *et al.* 1998).

Interestingly, inhibition of IL-12Rβ2 expression due to Th2 polarizing conditions could be blocked *in vitro* by addition of IFN-γ at the time of activation (Szabo *et al.* 1995). Therefore, IFN-γ production from T cells activated in the presence of IL-12 is important in order to maintain the responsiveness to IL-12 signaling. This leads to further augmentation of IFN-γ production through a positive feedback loop and subsequently allows for full differentiation towards the Th1 phenotype (Szabo *et al.* 1997). Interference with responsiveness to IL-12 by enhancing IL-4 levels can therefore shift differentiation of naive T cells towards a Th2 phenotype.

IFN-γ has been shown to have a powerful enhancing effect on the production of IL-12 by human monocytes (Hayes *et al.* 1995) or murine macrophages (Levings and Schrader 1999). This further augments IL-12-mediated induction of Th1-associated effector functions. The critical role of this IL-12/IFN-γ positive feedback loop in the development of Th1-mediated responses has been demonstrated in an elegant set of experiments using mice deficient for a transcription factor termed interferon regulatory factor-1 (IRF-1) (Lohoff *et al.* 1997). IRF-1 is activated in cells upon IFN-γ signaling and binds to promoter sequences of many IFN-γ inducible genes such as inducible nitric oxide synthase (Kamijo *et al.* 1994) and IL-12 (Ma *et al.* 1996). IRF-1 KO mice generated in a C57BL/6 background were susceptible to *L. major* infection and developed strong parasite-specific Th2-associated responses including elevated serum levels of IgE and IgG1. Interestingly, the adoptive transfer of $CD4^+$ T cells from IRF-1 KO mice into RAG-1 KO (T and B cell-deficient) congenic recipients, which are highly susceptible to the *L. major*, protected these mice from infection. This was associated

with the development of IFN-γ-producing Th1, and the absence of IL-4-producing Th2 IRF-1 KO $CD4^+$ T cells (Lohoff *et al.* 1997). These results suggested that the defect in the IRF-1 KO mice lies in their inability to augment IL-12 production from APC due to absence of IFN-γ signaling. This was directly demonstrated by Taki and co-workers who showed that macrophages from IRF-1 KO mice are incapable of producing IL-12 in response to treatment with LPS and IFN-γ (Taki *et al.* 1997).

The role of IFN-γ in promoting the development of Th1 effector functions has been directly tested in mice which lack either IFN-γ or the IFN-γR. Deletion of the IFN-γ gene in normally resistant mice led to death upon injection with sublethal doses of Mycobacterium bovis (Dalton *et al.* 1993). Similarly, mice deficient in the IFN-γR failed to efficiently control infections with vaccinia virus or *Listeria monocytogenes* (Huang *et al.* 1993). In addition, antibody blocking of IFN-γ activity *in vivo* in C57BL/6 mice infected with *L. major* led to progressive disease and development of Th2 immunity (Heinzel *et al.* 1995).

IFN-γ positively regulates Th1 differentiation by promoting the maintenance of responsiveness to IL-12-mediated signaling. This however seems to be important only during the initial stages of Th1 activation since exposure of mature Th1 cells to an IFN-γ signal leads to inhibition of IFN-γRβ chain expression and subsequent unresponsiveness of these cells to the IFN-γ signaling (Bach *et al.* 1995). However, expression of the IFN-γR is not inhibited during development of Th2 cells (Bach *et al.* 1995). These results were also confirmed in studies of IFN-γRβ expression in established murine Th1 and Th2 clones (Pernis *et al.* 1995). Interestingly, IFN-γ is important in negative regulation of T

cell proliferation (Dalton *et al.* 1993, Konieczny *et al.* 1998) possibly acting by inducing apoptosis in activated T cells (Liu and Janeway 1990). Taken together the above studies explain previous data demonstrating that IFN-γ inhibits proliferation of Th2 but not Th1 cells (Gajewski and Fitch 1988) and provide a possible mechanism by which IFN-γ may negatively impact the development of Th2 responses.

2.9 Importance of IL-4, IL-10 and IL-13 in Th1/Th2 development.

a) IL-4.

IL-4 is the most important cytokine for the development Th2 cells. Numerous studies have demonstrated that production of IL-4 from naive T cells is dependent on IL-4. For example, using TCR-transgenic mice, researchers have demonstrated that the addition of IL-4 into *in vitro* cultures resulted in production of IL-4 and IL-5 upon restimulation (Hsieh *et al.* 1992, Seder *et al.* 1992). In contrast, anti-IL-4 treatment led to the absence of IL-4 and increased levels of IFN-γ production by T cells (Hsieh *et al.* 1992, Seder *et al.* 1992). IL-4 KO mice showed a complete absence of serum IgE and a greatly reduced ability to produce two Th2 cytokines (IL-5 and IL-10) in response to infection with the potent Th2-inducer *N. brasiliensis* (Kopf *et al.* 1993). Similar results were obtained in mice deficient for STAT6 expression (Takeda *et al.* 1996). This was not surprising since STAT6 is the transcription factor activated upon interaction of IL-4 with the IL-4R (Nelms *et al.* 1999). STAT6 is known to mediate the induction of transcription of a number of IL-4-inducible genes including CD23, IL-4Rα chain, MHC class II and germline ε Ig H chain (Nelms *et al.* 1999). In addition, the IL-4 promoter contains a

binding site for phosphorylated STAT6 and a multimer of this site can activate reporter gene transcription upon stimulation with IL-4 (Lederer *et al.* 1996). Nuclear STAT6 persists in T cells for prolonged periods of time after initial stimulation with IL-4 and is likely to be important in IL-4 gene transcription (Lederer *et al.* 1996).

Another transcription factor important for Th2 differentiation is GATA-3. Expression of this factor was found in newly activated naive Th2 cells and persisted in these cells. However, it was repressed in cells committed towards Th1 differentiation by 48h after activation (Zheng and Flavell 1997). It was found later that forced expression of GATA-3 during T cell differentiation in the presence of IL-12 and anti-IL-4 resulted in the complete inhibition of Th1 cells and the appearance of IL-4 producing cells (Ouyang *et al.* 1998). Interestingly, even though the expression of GATA-3 is induced in naive T cells by IL-4, the effects of this transcription factor on inhibition of IFN-γ production and enhancement of IL-4 production were not IL-4 dependent. It was further found that repression of GATA-3 in Th1 cells was dependent on IL-12-induced signals and required STAT4 activation (Ouyang *et al.* 1998). These experiments illustrate a dual role of IL-4 in Th2 differentiation. First, an IL-4 signal during T cell activation leads to the production of Th2 cytokines by inducing GATA-3 activation. Second, by causing downregulation of IL-12Rβ2 expression, IL-4 renders the cells committed towards a Th2 phenotype unresponsive to IL-12 signaling, and thus prevents inhibition of GATA-3 activity mediated by IL-12.

IL-4 may also have an effect on T cell differentiation indirectly through an action on the APC. For example, it was found that IL-4 inhibited the production of IL-12 from

mouse peritoneal and bone marrow derived macrophages in response to LPS alone or in combination with IFN-γ (Levings and Schrader 1999). Interestingly, studies using a murine macrophage cell line demonstrated that IL-4-induced STAT6 could bind to STAT1 binding elements in the IRF-1 promoter region. Further, STAT6 could out-compete STAT1 at high concentrations, and could inhibit IFN-γ-induced STAT1-mediated transcription (Ohmori and Hamilton 1997). Since IRF-1 genes are critical for IL-12 production by the APC (Lohoff *et al.* 1997) this provides a mechanism by which IL-4 may attenuate the generation of Th1 cells and promote Th2 differentiation.

b) IL-10.

IL-10 was initially shown to inhibit IFN-γ production from Th1 clones (Fiorentino *et al.* 1989). This was mediated indirectly through an action on the APC (Fiorentino *et al.* 1991). Indeed, studies using murine macrophages (Levings and Schrader 1999) and human monocytes (Aste-Amezaga *et al.* 1998) have demonstrated that IL-10 is a powerful inhibitor of IL-12 production from these cells in response to various treatments including LPS and LPS + IFN-γ. It has also been demonstrated that maturation of dendritic cells in the presence of IL-10 is either completely inhibited (Kalinski *et al.* 1998) or altered (De Smedt *et al.* 1997) such that their ability to produce IL-12 and polarize T cell differentiation towards the Th1 phenotype is completely abrogated. As a result, enhanced priming of Th2 cells was observed (De Smedt *et al.* 1997). Therefore, IL-10 could inhibit priming of Th1 responses. In addition, since production of IFN-γ from mature Th1 cells is greatly augmented by IL-12 (Kohno *et al.* 1997, Seder and Paul 1994), the presence of IL-10 could affect the generation of Th1-induced effector

responses such as DTH. Aside from Th2 cells, macrophages are also a source of IL-10 (Seder and Paul 1994). Production of IL-10 from LPS-activated murine macrophages can be strongly augmented in the presence of IL-4 (Kambayashi *et al.* 1996). This provides an additional amplification mechanism by which IL-4 may inhibit Th1 differentiation.

c) IL-13.

IL-13 is secreted by Th2 cells and has been found to share certain biological functions with IL-4 (Zurawski and de Vries 1994). This is not surprising since IL-13 can use the IL-4Rα chain as a signaling subunit of the IL-13 receptor and it leads to activation of STAT6 (Hilton *et al.* 1996, Lin *et al.* 1995). The importance of IL-13 in the polarization of Th cell responses has been recently verified with studies in IL-13-deficient mice. These mice were unable to control infection with the intestinal nematode *Trichuris muris* (Bancroft *et al.* 1998), which is normally rejected in association with Th2-mediated responses (Else *et al.* 1993, Else *et al.* 1994, Bancroft *et al.* 1997). Analysis of parasite antigen-specific T cell responses demonstrated profound inhibition of production of Th2 cytokines including IL-4, IL-5 and IL-10. Further studies assessed secretion of Th2 cytokines by T cells derived from IL-13 KO mice in response mitogenic stimulation. The results revealed a profound inhibition of production of Th2 cytokines following activation in the presence of IL-4 and anti-IL-12 (McKenzie *et al.* 1998).

These studies, illustrating the importance of IL-13 in protection against infection with *Trichuris muris*, are consistent with recent work performed by Urban and colleagues (Urban *et al.* 1998). These researchers demonstrated that while IL-4 KO mice reject another intestinal nematode *N. brasiliensis*, mice deficient in IL-4Rα, and STAT6

expression are not capable of rejecting it. These results suggested that in IL-4 KO mice another cytokine may be able to mediated some of the IL-4 activities by interacting with IL-4Rα and activating STAT6. Interestingly blocking of IL-13 activity *in vivo* in IL-4 KO mice infected with *N. brasiliensis* prevented the rejection of the parasite. These results indicate that IL-13 is an important factor for generation of Th2 cells.

2.10 Mechanisms of allograft rejection.

Upon transplantation of a vascularized allograft, donor dendritic cells migrate from the graft via the blood to the spleen (Austyn and Larsen 1990). These professional APC persist in the spleen for several days and associate primarily with $CD4^+$, but also with $CD8^+$ T cells of the host (Larsen et al. 1990). Host T cell recognition of class I and class II MHC molecules, expressed on foreign DCs, leads to T cell activation. This initiates the acute graft rejection process (Austyn and Larsen 1990). Both $CD4^+$ (class II MHC restricted) T-helper and $CD8^+$ (class I MHC restricted) cytotoxic T- lymphocytes are thought to be involved in acute allograft rejection by mechanisms involving DTH-like responses and T cell-mediated cytotoxicity respectively (Mason *et al.* 1984, Lowry *et al.* 1983). The relative importance of $CD4^+$ and $CD8^+$ T cells in allograft rejection has been the subject of much debate. Most adoptive transfer and cell depletion experiments indicate, however, that $CD4^+$ Th cells play a central role in this process (Hall 1991). Thus, while in most cases $CD4^+$ T cells reject allogeneic tissues irrespective of the presence of $CD8^+$ cells (Hall 1991, Shelton *et al.* 1992, Bishop *et al.* 1993, Morton *et al.* 1993) the reverse is not always true (Hall 1991, Bishop *et al.* 1992, Bishop *et al.* 1993).

For example, Bishop and co-workers (Bishop *et al.* 1992) have demonstrated that at early stages post heart transplantation, $CD4^+$ T cells are the dominant T lymphocyte population in the graft. This situation changes at later stages when $CD8^+$ T cells constitute the majority of the infiltrating cells. Interestingly, although graft destruction correlated with a strong $CD8^+$ CTL infiltration, this appears to be $CD4^+$ T cell dependent. Depletion of $CD4^+$ T cells from recipient mice prior to transplantation resulted in abrogation of the $CD8^+$ infiltrate and resulted in extended allograft survival. Similar results were obtained by others, using a different mouse strain combination (Krieger *et al.* 1996a). Later studies by the same group showed that injection of rIL-2 into recipients depleted of $CD4^+$ T cells, restored $CD8^+$ infiltration but did not result in the generation of CTL activity and graft rejection (Bishop *et al.* 1993). In addition, depletion of $CD8^+$ T cells in this system did not inhibit allograft rejection demonstrating that $CD4^+$ T cells were sufficient to destroy the allogeneic tissue. The authors argued that the development of CTL activity was dependent on $CD4^+$ T cells and that other factors in addition to IL-2 were important in activation of CTL. However, it was also possible that some kind of suppressor activity, which inhibited CTL generation, developed as a result of anti-CD4 mAb treatment. In order to avoid this problem subsequent studies were performed in CD4 KO mice. Cardiac allografts exhibited indefinite survival when transplanted into those mice (Krieger *et al.* 1996a). In addition adoptive transfer of $CD4^+$ T cells into CD4 KO mice restored the ability to reject allogeneic hearts. These results suggest that $CD8^+$ CTL function is not required for allograft rejection and that $CD8^+$ CTL require $CD4^+$ T cell help in order to mediate the rejection process.

These, and other, data suggest either that CTL activity is not important in allograft rejection or that alternative pathways of CTL induction are activated in the absence of $CD8^+$ T cells. A number of reports suggest that the latter possibility is correct. For example, transplantation of CD8 KO mice with class I MHC incompatible skin grafts led to prompt rejection of these grafts, which was associated with the development of cytotoxic population of $CD4^-$ $CD8^-$ TCR $\alpha\beta^{intermediate}$ cell population (Dalloul *et al.* 1996). Moreover, *in vitro* studies demonstrated that activation of $CD4^+$ T cells in the absence of $CD8^+$ T cells led to development of $CD4^+$ CTL capable of killing allogeneic targets by the classical perforin/granzyme B-dependent pathway (Williams and Engelhardt 1997), normally utilized by $CD8^+$ CTL for mediation of target cell destruction (Kagi *et al.* 1996). Recent studies in the small intestinal transplantation model (Krams *et al.* 1998) have shown that depletion of $CD8^+$ T cells did not reduce intragraft expression of granzyme B and perforin providing an *in vitro* correlate to the earlier in vitro experimentation. In this *in vivo* model graft rejection was not delayed and the extent of apoptosis induced in the cells of the graft was not reduced. Taken together these results suggest that in the absence of $CD8^+$ T cells other pathways are induced, which result in generation of alternate CTL activity capable of mediating graft destruction.

The studies above show that $CD8^+$ T cells are not required or even sufficient to reject grafts. However, they do not indicate that, under normal conditions $CD8^+$ T cells do not play a major role in graft rejection. Studies using adoptive transfer of $CD8^+$ T cells, previously sensitized with alloantigen have demonstrated a significant role for $CD8^+$ T cells in allograft rejection (Prowse *et al.* 1983, Hall *et al.* 1985). In addition,

$CD8^+$ T cells have been shown to mediate rejection of MHC class I mismatched grafts independently of $CD4^+$ T cells (Rosenberg *et al.* 1986). However this latter report must be considered in the face of the fact that it was performed with C57BL/6 mice, which seem to contain a population of $CD8^+$ T cells capable of conferring helper function including IL-2 production and thus leading to development of CTL in the absence of $CD4^+$ T cells (Roopenian *et al.* 1983, Andrus *et al.* 1984).

In humans the appearance of transcripts for granzyme B and perforin in kidney (Lipman *et al.* 1997, Strehlau *et al.* 1994) or heart (Legros-Maida *et al.* 1994) biopsies strongly correlates with acute rejection episodes. Expression of these markers is reduced following immunosuppressive therapy (Legros-Maida *et al.* 1994). Similarly, a correlation between acute rejection and granzyme A was found in biopsies of human cardiac allografts (Alpert *et al.* 1995). In an elegant experiment Wever and co-workers (Wever *et al.* 1998) developed infiltrating cell lines from human renal biopsies, and noted that these lines use the granzyme B/perforin-dependent pathway to efficiently kill proximal tubular epithelial cell lines derived from the corresponding biopsies. Similar results correlating the expression of CTL-specific cytotoxic molecule and allograft rejection have been obtained in mouse (McDiarmid *et al.* 1995, Chen *et al.* 1996) and rat (Chen *et al.* 1993, Krams *et al.* 1998) transplantation models. Therefore, although allograft rejection can occur in the absence of CTL under certain conditions induced in animal transplantation models, the presence and absence of CTL function appears to be a good predictor of allograft survival.

2.11 Th1/Th2 paradigm in transplantation.

Elegant studies from the *L. major* infection model, showing that induction of functionally different Th cell responses may have a significant impact on the outcome of infection, prompted researchers to investigate whether such modulation of immune responses could apply to transplantation models. Allograft rejection is thought to be primarily mediated by DTH and cytotoxic T-lymphocyte activity (Hall 1991), activities which are dependent on type 1 cytokines. Indeed, type 1 cytokines were often found in grafts undergoing acute rejection (Dallman 1993). Various therapies, such as treatment with cyclosporine A (Takeuchi *et al.* 1992), rapamycin (Ferraresso *et al.* 1994), anti-CD4 mAb (Takeuchi *et al.* 1992, Siegling *et al.* 1994, Mottram *et al.* 1995 and Binder *et al.* 1996), and CTLA-4Ig (Sayegh *et al.* 1995) as well as portal venous (pv) immunization (Gorczynski 1992 and Gorczynski 1995) and donor-specific transfusion (Takeuchi *et al.* 1992), result in prolongation of graft survival and are associated with downregulation of type 1, but preservation, or in some cases upregulation, of type 2 cytokine expression (IL-4 and IL-10). The involvement of regulatory T cells based on Th1/Th2 dichotomy has been shown to be responsible for induction and maintenance of tolerance in some of these systems. For example, experiments by Gorczynski and co-workers have demonstrated that mice receiving pre-transplant immunization via the portal vein show a delayed rejection of skin grafts (Gorczynski and Wojcik 1994). This effect was correlated with preferential expansion of Th2 cells (Gorczynski and Wojcik 1994; Gorczynski 1995). Interestingly, treatment with anti-IL-10 mAb alone (Gorczynski and Wojcik 1994) or in combination with rIL-12 (Gorczynski *et al.* 1995) was able to completely abrogate prolongation of graft survival and Th2 activation in that model, suggesting a role for IL-

10 in the establishment of the allo-specific Th2 response. In contrast, combination therapy with both recombinant IL-13 and anti-IL-12 mAb extended graft survival and enhanced the type 2 polarization afforded by pv immunization (Gorczynski *et al.* 1996). These results indicate a crucial role for cytokines in immunomodulation of transplant rejection.

Similarly to the studies in *L. major* infection model, there is evidence in the transplantation arena to suggest that immunomodulation towards allo-specific Th2 responses is not always associated with tolerance. It was demonstrated, for example, that depletion of $CD8^+$ T cells was associated with a rejection event characterized by intragraft expression of type 2 cytokines and by the infiltration of the graft with eosinophils and mononuclear cells (Chan *et al.* 1995). It has also been shown that treatment of mice with anti-IL-12, at the time of transplantation, exacerbated the rejection process despite the observed induction of type 2 cytokine expression within the graft (Piccotti *et al.* 1996). These results suggest that manipulation of the effector Th1 response, towards a tolerant phenotype, is associated, at least in some cases, with the development of a Th2 response. However, activation of type 2 immunity does not invariably protect from rejection. Thus, there may well be other elements of regulatory control necessary for allograft acceptance.

2.12 Role of IFN-γ in transplantation.

IFN-γ is known to activate dendritic cells and macrophages, enhance the production of IL-12 from these cells (Taki *et al.* 1997, Levings and Schrader 1999), direct the development of type 1 T cells (Lohoff *et al.* 1997, Szabo *et al.* 1997, Taki *et al.*

1997), and induce DTH activity (Belosevic *et al.* 1989, Wang *et al.* 1994) and CTL effector functions (Utermohlen *et al.* 1996, Gazzinelli *et al.* 1994, Ely *et al.* 1999). Because IFN-γ transcription has been consistently detected in cardiac and renal grafts undergoing acute rejection, and because in some cases long term allograft survival was associated with decreased IFN-γ expression, researchers have proposed that this cytokine mediates acute allograft rejection (Dallman 1993). However, recent data suggests that the role of IFN-γ in allograft rejection may be more complex than was previously thought. For example, Lakkis and co-workers have shown that IFN-γ transcripts persist in long surviving cardiac allografts in mice treated with CTLA4-Ig (Lakkis *et al.* 1997). Similar results were obtained in rats that were treated with monoclonal antibodies to CD2 at the time of transplantation (Krieger *et al.* 1996b). In addition it has been demonstrated that both IFN-γ KO (Saleem *et al.* 1996) and IFN-γR KO (Steiger *et al.* 1998) mice are capable of acutely rejecting cardiac and islet allografts, respectively, suggesting that this cytokine is not essential for allograft rejection.

Perhaps most surprising were the results obtained by Konieczny and co-workers, demonstrating that IFN-γ KO mice failed to accept cardiac and skin allografts following tolerizing therapy with CTLA4-Ig as well as combination treatment of CTLA4-Ig along with anti-CD40 mAb (Konieczny *et al.* 1998). Further, injection of anti-IFN-γ mAb into wild type allograft recipients undergoing the tolerizing treatment described above led to prompt allograft rejection, suggesting that in some situations IFN-γ may be required for tolerance induction (Konieczny *et al.* 1998). The mechanisms by which the presence of IFN-γ may mediate tolerance are not clear, however it has been suggested that IFN-

γ could be involved in negative feedback regulation of the expansion of allo-reactive T cells (Konieczny *et al.* 1998). In support of this are studies demonstrating that the presence of IFN-γ leads to decreased allo-specific T cell proliferation in response to alloantigen *in vitro* (Konieczny *et al.* 1998, Dalton *et al.* 1993).

2.13 Type 2 cytokines and transplantation.

IL-4 is recognized to be the most important factor in induction of type 2 immunity (Mosmann and Sad 1996). However, its role in transplantation tolerance is controversial. Maeda and colleagues were able to establish a foreign class II MHC-specific Th2 (IL-4 and IL-10 secreting) cell line (Maeda *et al.* 1994). Adoptive transfer of this cell line into naive mice markedly delayed rejection of skin graft bearing foreign class II MHC, but not third party MHC II. This was associated with inhibition of generation of allo-specific CTL activity (Maeda *et al.* 1994). In contrast Van Buskirk and co-workers had generated *in vitro* allo-specific IL-4 and IL-10 producing Th2 cells as well as IL-2 and IFN-γ producing Th1 cells. Both Th1 and Th2 cells were able to mediate cardiac allograft rejection within 7-10 days after adoptive transfer into congenic SCID recipients (Van Buskirk *et al.* 1996). There are problems associated with both of these studies. Maeda and co-workers, for example, did not show whether IL-4 and IL-10, secreted by the transferred cells, were actually involved in allograft-specific tolerance induction in the host. Van Buskirk et al, on the other hand, did not take into consideration the possibility that, in order to induce tolerance, Th2 cells may require other cells of the immune system such as B cells, or that a balance between type 1 and type 2 cytokine production,

impossible to obtain in T cell-deficient environment of SCID mice, may be required for tolerance induction. This point again is supported by the studies mentioned above that demonstrated that, in the complete absence of IFN-γ, tolerance could not develop in some transplantation models.

A recent study demonstrated that long term transplantation tolerance to allogeneic cardiac tissue can be induced in IL-4 KO mice if CTLA4Ig is used as tolerizing treatment (Lakkis *et al.* 1997). However, using infusion of anti-CD4 mAb (Sirak *et al.* 1998) or combination treatment with anti-CD2 and anti-CD3 (Punch *et al.* 1998) in IL-4 KO mice transplanted with allogeneic hearts, researchers have demonstrated that IL-4 is necessary for prolonged allograft survival. These studies suggest a differential requirement for IL-4 in tolerance induction using various treatments, or that other cytokines such as IL-13 may make up for the absence of IL-4 in some situations. They also suggest the possibility that the appearance of type 2 cytokines may not always be the cause, but rather the effect, of tolerance induction. The reverse may also be true. Thus, inability to detect type 1/type 2 shift may not mean that type 2 cytokines are not involved. This point has been suggested by studies using combination treatment with non-depleting anti-CD4 and anti-CD8 antibody in order to induce transplantation tolerance in mice (Cobbold *et al.* 1996). Treatment with these antibodies at the time of transplantation induces long-term allo-specific tolerance that can be transferred with T cells from tolerized animals into irradiated syngeneic hosts reconstituted with naive T cells (Davies *et al.* 1996). The analysis of allo-specific cytokine production failed to demonstrate the occurrence of type 2 switch in T cells from tolerant mice. In addition, once the tolerance was induced,

administration of anti-IL-4 mAb had no effect on allograft survival. However, anti-IL-4 treatment completely blocked the ability of tolerant T cells to transfer allograft-specific tolerance to naive T cells in secondary, irradiated hosts suggesting that IL-4 is important for tolerance induction (Davies *et al.* 1996). Further support for this hypothesis is provided by studies demonstrating: 1) prolonged survival of allogeneic hearts derived from IL-4 transgenic donors (Takeuchi *et al.* 1997); 2) prolonged heart allograft survival in rats upon infusion of rIL-4 (He *et al.* 1998); 3) abrogation of anti-CD4-induced tolerance with anti-IL-4 treatment in rats (He *et al.* 1998); 4) restoration of the ability of anti-CD2 and anti-CD3 combination treatment to induce graft-specific tolerance in IL-4 KO mice upon infusion of wild type T cells capable of producing IL-4 (Punch *et al.* 1998).

Recent studies have demonstrated that aside from IL-4, IL-6 may be important for induction of type 2 responses *in vivo* (Gorczynski *et al.* 1997, Anguita *et al.* 1998) and *in vitro* (Rincon *et al.* 1997, Gorczynski *et al.* 1997). Interestingly IL-6 was also found to downregulate activation of allo-specific type 1 responses, as assessed by inhibition of IFN-γ production, which was associated with prolongation of skin allograft survival in mice in MHC class II disparate strain combination (Tomura *et al.* 1997). Thus IL-6 may under certain conditions contribute to enhanced allograft survival.

2.14 Tc2 cells.

$CD8^+$ CTL have been suggested to destroy graft tissue mainly through direct cytotoxic effects against foreign class I MHC expressing cells by the granule exocytosis

pathway (Kagi *et al.* 1996). Many treatments that prolong allograft survival, including neonatal immunization (Donckier *et al.* 1995, Gao *et al.* 1995, Matriano *et al.* 1994), administration of anti-CD4 non-depleting mAb (Onodera et al. 1997), as well as portal venous inoculation with alloantigen (Gorczynski *et al.* 1998) are associated with decreased allo-specific CTL activity. In some cases (Donckier *et al.* 1995, Gao *et al.* 1995, Matriano *et al.* 1994, Gorczynski *et al.* 1998) inhibition of CTL activity was associated with the development of allo-specific type 2 immunity. IFN-γ is crucial for the development of CTL activity in response to viral (Sharma *et al.* 1996, Utermohlen *et al.* 1996) or *Toxoplasma gondii* (Ely *et al.* 1999) infection. In addition, experiments using an IL-4 transfected virus or *S. mansoni* to induce type 2 cytokine expression have shown a profound inhibition of virus-specific CTL activity and markedly delayed viral clearance (Sharma *et al.* 1996, Actor *et al.* 1993). These results suggest that IL-4 may interfere with the development of CTL activity. This has been shown directly by Erard and co-workers (Erard *et al.* 1993). In their studies these researchers demonstrated that $CD8^+$ T cells activated in the presence of IL-4 lost their cytotoxic potential and expression of cytolytic molecules including perforin and granzyme B. These cells also acquired the ability to produce IL-4 and not IFN-γ. These non-cytotoxic, IL-4-producing, $CD8^+$ T cells were called Tc2, to differentiate them from conventional IFN-γ producing cytotoxic Tc1 cells (Mosmann and Sad 1996). However, others have demonstrated that activation of $CD8^+$ T cells in type 2 polarizing conditions does not lead to loss of cytotoxic potential *in vitro* (Li *et al.* 1997, Matesic *et al.* 1998, Cerwenka *et al.* 1999, Dobrzanski *et al.* 1999). Interestingly, in most of these studies, when functional *in vivo* assays were performed

comparing efficacy of type 1 versus type 2 $CD8^+$ T cells at killing specific targets. Tc1 cells proved to be much more efficient CTLs (Matesic *et al.* 1998, Cerwenka *et al.* 1999, Dobrzanski *et al.* 1999).

2.15 Objectives.

The sections described above provide the reader with the information required in order to understand the result sections that follow. These sections are presented in a form of two manuscripts that were submitted for publication to *Transplantation* and *Journal of Immunology*. The first manuscript (chapter 3.0) is entitled "Prolongation of allograft survival by *N. brasiliensis* is associated with decreased allo-specific CTL activity and development of Tc2 cells" and addresses the mechanisms by which *N. brasiliensis* modulates allo-specific T cell responses. The second manuscript (chapter 4.0) is entitled "Nematode infection enhances survival of activated T cells by modulating accessory cell function" and investigates the effects of infection with *Nb* on T cell activation.

3.0 Prolongation of allograft survival by *N. brasiliensis* is associated with decreased allo-specific CTL activity and development of Tc2 cells.

This manuscript has been submitted to *Transplantation*.

Prolongation of Allograft Survival by *N. brasiliensis* is Associated with Decreased Allo-specific CTL Activity and Development of Tc2 cells.

Robert Liwski[1-2], Juan Zhou[1-2], Vivian McAlister[2] and Timothy D. G. Lee[1-3]

Departments of [1]Microbiology and Immunology and [2]Surgery, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4H7

Address correspondence for proofs to: Dr. Timothy D. G. Lee, Department of Microbiology and Immunology, Transplantation and Immunology Research Laboratory, Sir Charles Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7.

Footnotes:

1. Department of Microbiology and Immunology, Dalhousie University, Sir Charles Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7.

2. Department of Surgery, Dalhousie University, Sir Charles Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7.

3. Address correspondence and reprint requests to Dr. Timothy D. G. Lee, Department of Microbiology and Immunology, Transplantation and Immunology Research Laboratory, Sir Charles Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7.

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Abbreviations used in this paper: CTL, cytotoxic T lymphocyte; IFN-γ, gamma interferon; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; *Nb, Nippostrongylus brasiliensis*; Tc2, T cytotoxic cell type 2.

Key words: transplantation, T cell, type 1/type 2, Th1/Th2, cytokine, MLR, CTL, Tc2, nematode, *Nippostrongylus brasiliensis*.

Abstract

Background: We have demonstrated that infection with *Nippostrongylus brasiliensis* (*Nb*), which induces strong type 2 responses, prolongs kidney allograft survival in rats. Here we confirm that this effect is not species specific and address immune-modulation in allo-specific T cell responses mediated by nematode infection. ***Methods:*** C57BL/6 mice were injected with *Nb* or PBS. Four days later mice were transplanted with BALB/c hearts and graft survival was assessed. In other experiments *Nb*-infected mice were immunized with BALB/c spleen cells, and allo-specific T cell responses were determined *in vitro*. ***Results:*** In this study we show that *Nb* prolongs cardiac allograft survival in mice. Further, spleen T cells from *Nb*-infected, allo-immunized mice exhibit reduced allo-specific CTL activity. In contrast, allo-specific proliferation of T cells in the MLR was not reduced by *Nb*, ruling out immunosuppression as the mechanism of *Nb*-induced allograft survival. *Nb* infection induced IL-4 and IL-6 and inhibited IFN-γ production by T cells in response to alloantigen. Furthermore, anti-IL-4 treatment reduced allo-specific T cell proliferation from *Nb*-infected but not control mice, indicating that type 2 allo-specific T cells develop in the presence of *Nb*. We also double-stained T cells for CD8 and IL-4 and showed that *Nb* induces an 8-fold increase in Tc2 cell numbers. ***Conclusions:*** These results are consistent with a hypothesis that *Nb* mediates prolongation of allograft survival through induction of type 2 immunity, including the development of regulatory Tc2 cells, and subsequent inhibition of allo-specific CTL activity.

Introduction

Type 1 immunity, mediated primarily by $CD4^+$ Th1 cells, is currently thought to be the major effector mechanism of allograft rejection (reviewed in 1,2,3,4 and 5). The transcription of type 1 cytokines (IL-2, IFN-γ) is consistently observed in grafts undergoing acute rejection (6, 7, 8, reviewed in 9). Cytokines elaborated by type 2 cells (such as IL-4 and IL-10) have been demonstrated to inhibit generation of both delayed type hypersensitivity (10,11) and cytotoxic T lymphocyte (CTL) activities (12,13,14 and 15) leading to the conclusion that development of allo-specific type 2 immunity might suppress the graft rejection process.

There have been a number of reports suggesting that therapies, which favour type 2 immunity have marked effects on allograft survival. Cyclosporine A (7) rapamycin (16, 17), anti-CD4 mAb (7, 18,19, 20,21,22), CTLA-4Ig (23), as well as portal venous (13,24,25) and oral (13) immunization with alloantigen , all result in prolongation of graft survival and have all been associated with downregulation of type 1 immunity but preservation, or in some cases upregulation, of type 2 cytokine expression. The role of these type 2 responses in inhibition of graft rejection, however, is still controversial. Although VanBuskirk *et al.* (26) showed that allo-specific $CD4^+$ T-cells, polarized *in vitro* to produce either Th1 or Th2 cytokines, were both capable of mediating rejection of allogeneic heart grafts when transferred into congenic SCID recipients, Maeda *et al.* (27), demonstrated that allo-specific IL-4 and IL-10 secreting cell lines induced graft-specific tolerance upon adoptive transfer into skin allograft recipients. One manner in which type 2 responses might affect allo-specific effector function is by the presence of IL-4 during activation of $CD8^+$ CTL precursors. The presence of IL-4 during the

activation of $CD8^+$ T-cells has been shown to result in development of IL-4 producing Tc2 cells (14,28). Since these Tc2 cells exhibit compromised specific killing function (14,28) their generation could result in inhibition of allo-specific CTL activity. In support of this hypothesis, the generation of anti-viral Tc2 cells, due to expression of IL-4 (29) or *Schistosoma mansoni* infection (30), has been associated with marked reduction in virus-specific CTL function and decreased viral clearance.

Infection of mice and rats with the nematode parasite *Nippostrongylus brasiliensis* (*Nb*) leads to the development of strong type 2 responses, characterized by induction of type 2 cytokine expression, local mast cell hyperplasia, systemic eosinophilia, as well as the production of IgE and IgG1 (in mice: 32,33) or IgG2a (in rats: 34,35). It appears that polyclonal type 2 activation occurs during infection with *Nb*, since there is a dramatic increase in total (non-specific to *Nb*) IgE levels (34), potentiation of IgE production in response to superantigen (36), and a marked increase in the frequency of IL-4 producing $CD4^+$ T cells (37,38) observed during the infection. In addition, spleen cells from *Nb*-infected mice produce high levels of IL-4, IL-6 and IL-10 in response to mitogenic stimulation *in vitro* (manuscript in preparation, 36,39,40). This suggested to us that *Nb* may be a useful agent to modulate "bystander" allo-specific immune responses from type 1 towards type 2.

We have hypothesized (41) that robust allo-specific type 2 responses would interfere with the development of the allo-specific type 1 responses necessary for allograft rejection, and we have recently demonstrated (41) that infection of rats with *Nb* prior to transplantation with an allogeneic kidney leads to a significant prolongation of graft survival. This was associated with a marked reduction in numbers of graft

infiltrating cells (GIC) and with the transcription of significant levels of IL-4 mRNA by the GIC from *Nb*-treated recipients, whereas levels of IL-4 mRNA from control recipient GIC were below levels of detection. We here confirm that the ability of *Nb* infection to prolong solid organ allograft survival is not restricted to rat kidney, using a murine heterotopic cardiac transplantation model. Further we show that *Nb* reduces the ability to generate *in vivo* allo-specific CTL activity in mice. This was associated with the development of allo-specific type 2 responses and with the appearance in the spleens of *Nb*-infected mice of $CD8^+$ T-cells that produce IL-4, suggesting a bias towards Tc2 rather than Tc1 development.

Materials and Methods

Animals. Male 6-8 week old C57BL/6J (H-2^b), C57BL/6J perforin knockout (PKO), and BALB/cByJ (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the Medical Sciences animal care facility with food and water *ad libitum* for two weeks prior to experimentation.

Nippostrongylus brasiliensis. The life cycle of *Nb* was maintained in Sprague Dawley rats (Harlan, Indianapolis, IN) as we have previously described (41). Eight hundred third-stage larvae of *Nb*, in 200 µl of PBS, were used for inoculation of mice.

In vivo treatment. Groups of 8-12 week old male C57BL/6 mice (3 mice per group) were injected subcutaneously (s.c.) with *Nb*. Control mice were treated with PBS alone. Four days later all mice were immunized intraperitoneally (i.p.) with $5x10^7$ mitomycin C-treated BALB/cByJ spleen cells in 200 µl of PBS. On day 7 after immunization all mice were sacrificed by cervical dislocation and spleen cells were isolated for *in vitro* culture.

Heterotopic heart transplantation. Intra-abdominal heterotopic heart transplantation was performed as described by Corry and Russell (43). Briefly donor BALB/cByJ mice were anaesthetised with pentabarbitol and a midline abdominal incision was made. The heart was perfused with heparinized cold (4°C) saline through the vena cava and the aorta. After exposure of the heart, superior and inferior vena cava and pulmonary veins were ligated. The heart was removed and kept in cold saline while

the recipient C57BL/6 mouse was prepared. The donor heart was placed in a heterotopic position via end to side anastomosis of the donor aorta to the recipient abdominal aorta and donor pulmonary vein to recipient abdominal vena cava using 11-0 nylon sutures. Cold ischemia time was always less than 30 min. Transplanted heart function was monitored by ECG every 2-3 days.

Cell isolation. Single cell suspensions from spleens of C57BL/6 mice were prepared, as described previously (42), under aseptic conditions in RPMI 1640 (ICN, Aurora, OH) supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin (Gibco BRL, Burlington, ON), 10% FCS (Gibco BRL), 20 mM HEPES (GibcoBRL) and 50 μM 2-ME (Sigma, St Louis, MO). The cells were pooled within experimental groups. Erythrocytes were eliminated by lysis with ACK buffer for 2 min at RT. Effector T cells were enriched by passage through nylon wool columns (Polysciences, Warrington, PA) as described previously (42). The remaining B cells were eliminated by treatment with anti-B220 (rat IgM anti-mouse; clone RA3-2C2; ATCC, Rockwille, MD) for 1h at 4°C followed by treatment with rabbit low-tox complement (Cedarlane, Hornby, ON) for 1h at 37°C. The cells were washed three times and resuspended in RPMI for *in vitro* culture. The resulting enriched T cell population was 88 – 93% pure as assessed by flow cytometric analysis using anti-Thy1.2 FITC mAb (mouse $IgG2_b$ anti-mouse; clone 5a-8 : Cedarlane). In some experiments T cells were depleted from the spleen cell population by treatment with anti-Thy1.2 mAb complement. This treatment resulted in > 98% depletion of T cells as assessed by flow cytometry and by loss of proliferation in response to Con A (Sigma) or anti-CD3 mAb. CD8+ T-cells were enriched from the nylon wool

enriched spleen T cell population using CD8 immunocolumns (Cedarlane) according to the manufacturer's instructions. Passage through CD8 immunocolumns eliminated 98% of the CD4+ T cells as assessed by flow cytometry. Stimulator BALB/cByJ spleen cells were isolated as above and purged of T cells as above. Stimulator cells were inactivated by adding mitomycin C (Sigma; final concentration of 25 μg/ml) for 30 min prior to completion of the treatment with complement. Cells were washed three times and resuspended in RPMI for *in vitro* culture. For in vivo immunization of C57BL/6 recipient mice, donor (BALB/cByJ) whole spleen cells were isolated and treated with mitomycin C as above except that the isolation procedure was performed in PBS supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin (Gibco BRL).

Mixed lymphocyte reaction (MLR). To assess the proliferative response to alloantigen in the MLR, C57BL/6 effector T cells ($2x10^5$ cells/well) were cultured in triplicate wells of 96-well round-bottomed plates (Nunc; Gibco BRL) in the presence or absence of mitomycin C-treated BALB/cByJ stimulator cells ($5x10^4$ cells/well). After 72h of incubation at 37°C the cultures received 1 μCi/well of [^{3}H]thymidine ([^{3}H]TdR; ICN) and 18h later the contents of the wells were harvested onto filtermats (Skatron, Sterling, VA), using an automatic cell harvester (Skatron), for measurement of ^{3}H-thymidine ([^{3}H]TdR) incorporation. To assess the effects of neutralization of IL-4 on T cell proliferation in the MLR, supernatants of an anti-IL-4 producing hybridoma (rat IgG1anti-mouse; clone 11B11; ATCC) were added to the MLR cultures at a final concentration of 1:5. Control MLR wells received 10 μg/ml of rat IgG (Jackson

Laboratories). Proliferative responses to the T cell mitogens Con A (5 μg/ml) and anti-CD3 mAb (1:20 hybridoma supernatant) were also assessed.

Direct Cytotoxic T lymphocyte (dCTL) activity. To assess allo-specific dCTL activity C57BL/6 effector spleen T cells were cultured (total volume of 200 μl) in 96-well v-bottomed plates (Sarstedt, St-Leonard, QU) in the presence of $Na_2Cr^{51}O_4$ (Chromium release assay: ICN) or [^{3}H]TdR (JAM assay: 44) labelled allogeneic A20 (H-2^d: ATCC), P815 (H-2^d: kindly provided by Dr. D. Hoskin) or third-party BW5147.3 (H-2^k: ATCC) tumor cell targets ($1x10^4$ cells/well) at various effector to target ratios. The plates were incubated for 18h at 37°C. For the chromium release assay, plates were centrifuged at 200xg for 5 min and culture supernatants (100 μl) were collected from each well. Chromium levels in the supernatants were assessed using a γ-counter. Specific lysis was calculated after subtraction of spontaneous lysis. For the JAM assay (44), the contents of each well were harvested using an automatic cell harvester (Skatron). Specific apoptosis was calculated using the following formula: % specific apoptosis = ([^{3}H]TdR in targets at T_{18h} - [^{3}H]TdR in effector/targets at T_{18h}) / ([^{3}H]TdR in targets at T_{18h}) x 100%.

ELISA for detection of cytokines. Effector C57BL/6 T cells ($5.0x10^5$ cells/well) were activated in quadruplicate wells of 96-well round-bottomed plates with mitomycin C-treated BALB/cByJ stimulator cells ($1.25x10^5$ cells/well) for 48 h at 37°C. Supernatants were collected, pooled within quadruplicates, and stored at –70°C. Supernatants were analysed for presence of IFN-γ, IL-4 and IL-6 by ELISA according to

the manufacturer's instructions (Pharmingen, Mississauga, ON). All mAb and mouse recombinant cytokine standards used were purchased from Pharmingen; capture antibodies: anti-IL-4 (rat IgG_1 anti-mouse; clone 11B11), anti-IL-6 (rat IgG_1 anti-mouse; clone MP5-20F3), and anti-IFN-γ (rat IgG_1 anti-mouse; clone R4-6A2); biotinylated antibodies: anti-IL-4 (rat IgG_1 anti-mouse; clone BVD6-24G2), anti-IL-6 (rat IgG_{2a} anti-mouse; clone MP5-32C11), and anti-IFN-γ (rat IgG_1 anti-mouse; clone XMG1.2). Briefly, ELISA plates (Costar) were coated with anti-cytokine mAb in carbonate buffer (pH 9.6) at 4°C overnight. After overnight incubation and blocking with 2 mg/ml bovine serum albumin (BSA; Sigma) in Tris-buffered saline, test supernatants and recombinant cytokines were added to the plates and incubated overnight at 4°C. Cytokines were detected using biotinylated anti-cytokine mAb. Extravidin –Peroxidase (Sigma) and TMB substrate solution (Gibco BRL). Detection limits were 30 pg/ml for IFN-γ and 15 pg/ml for IL-4 and IL-6.

FACS analysis. Cells ($1x10^6$) were washed twice in PBS containing 1% BSA (Sigma). Cells were incubated in the dark with 2 μg/ml of anti-Thy1.2 FITC mAb (mouse $IgG2_b$ anti-mouse; clone 5a-8; Cedarlane), anti-CD4 mAb (rat $IgG2_b$ anti-mouse; clone YTS 191.1; Cedarlane), anti-CD8α mAb (rat $IgG2_b$ anti-mouse; clone YTS 169.4; Cedarlane) or isotype control (rat $IgG2_b$; Cedarlane) at 4°C for 30 minutes. Cells were then washed three times, fixed in PBS containing 1% paraformaldehyde and stored at 4°C overnight. FACS analysis was performed on FACscan (Beckton Dickinson) using Lysis II software.

Intracellular staining for IL-4. Enriched C57BL/6 T cells ($5.0x10^5$ cells/well) from *Nb*-infected allo-immunized and control allo-immunized mice were activated in quadruplicate wells of 96-well round-bottomed plates with mitomycin C-treated BALB/cByJ stimulator cells ($6.0x10^4$ cells/well) for 5 h at 37°C. A transport inhibitor solution IC Block™ (Biosource International, Camarillo, CA) was added to all the wells at a concentration of 1 µl/ml to prevent cytokine release from the cells. After an additional 3h of incubation at 37°C, cells from quadruplicate wells were pooled and transferred into 1.5 ml centrifuge tubes. Cells were washed 3 times in PBS 1% BSA pH 7.4 (PBS/BSA). Production of IL-4 by CD8+ cells was assessed using the IntraCellular Staining Kit (Biosource International) according to the manufacturer's instructions. Briefly cells were stained with anti-CD8 FITC mAb (rat $IgG2_b$ anti-mouse; Cedarlane) or isotype control FITC (rat $IgG2_b$; Cedarlane) for 30 min. at 4°C, washed twice with PBS/BSA and fixed with IC Fix™ for 10 min at 4°C, and washed twice more to remove excess IC-Fix™. Cells were permeabilized in IC Perm™ for 3 min at room temperature, centrifuged, resuspended in 50 µl of 1:5 dilution of anti-IL-4 R-PE mAb (rat $IgG2_b$ anti-mouse; clone 1D11; Biosource International) or isotype control R-PE (rat $IgG2_b$; Biosource International) and incubated for 30 min at 4°C. Cells were washed three times in PBS/BSA and fixed in PBS + 1% paraformaldehyde and stored at 4°C until analysis. Gates were set using cell populations stained with FITC-anti-CD8 mAb and PE-Rat $IgG2_b$ Ab. FACS analysis was performed on FACscan (Beckton Dickinson) using Lysis II software. The percentage of IL-4-producing $CD8^+$ cells was expressed as a percentage of the total $CD8^+$ cell population.

Statistical analysis. Statistical analysis was performed using either one-way or repeated measures ANOVA. In each case p values of differences between pairs of columns were calculated using Student-Newman-Keuls (multiple comparisons test) post-test. Significance of the allograft survival data was assessed using Mann-Whitney test.

Results

Cardiac allograft survival. We have shown previously that infection of rats with *Nb* prior to transplantation with fully allogeneic (major and minor disparate) kidney grafts resulted in significant prolongation of graft survival (41). To confirm that this is not a species (or organ) specific effect we performed cardiac allo-transplantation in mice. As shown in Figure 1, BALB/cByJ hearts transplanted heterotopically into fully allogeneic C57BL/6 recipients are rejected between day 9 and 12 after transplantation (n=5). In contrast, when mice were infected with *Nb* 4 days prior to transplantation, prolonged allograft survival was observed in most cases. Four of the infected mice exhibited moderate prolongation of graft survival ranging from 20 to 26 days. In two other mice the grafts survived for 41 and 84 days. The variability of the survival time as well as the fact that some of the mice did not exhibit prolonged graft survival (Fig. 1) may be due to the variations in the course of the *Nb* infection from animal to animal. Regardless of the variation in the response, it is clear that infection with *Nb* significantly ($p < 0.03$) prolongs survival of fully vascularized solid organ allografts in both mice and rats (41).

Model of allo-specific direct CTL activity. Our previous studies in the rat model demonstrated a significant reduction in the number of recipient T cells infiltrating the allografted kidneys in the *Nb* treated animals, five days post transplantation (41). This reduction was more pronounced for the $CD8^+$ compartment, suggesting that *Nb* may be interfering with the generation of allospecific CTLs. To test this hypothesis we developed a simple model to assess the *in vivo* generation of allo-specific CTL activity in

mice. C57BL/6 mice (H-2^b) were immunized with allogeneic mitomycin C-inactivated BALB/c (H-2^d) spleen cells. Seven days later T cells were isolated from these mice and their ability to kill H-2^d tumor targets (P815 mastocytoma) or H-2^k third party tumor targets (BW5147.3 thymoma) was verified in an 18 h direct CTL (dCTL) killing assay. For our purposes this direct CTL assay is superior to the conventional CTL assays since it does not involve the *in vitro* re-stimulation (expansion) step prior to the killing assay and thus measures the presence of *in vivo* activated allo-specific CTL in the harvested spleen. This gives a true measure of the generation of allo-specific CTL *in vivo*. Figure 2 shows that T cells from BALB/c-immunized C57BL/6 mice, but not from naive C57BL/6 mice, exhibit killing activity against allogeneic P815 cells. The killing was antigen specific since T cells from both naive and allo-immunized mice were unable to kill third party allogeneic BW5147.3 cells (Fig.2). The effector cells that are responsible for killing in dCTL are T cells, since deletion of Thy-1.2^+ cells from the effector population resulted in complete abrogation of killing activity (Fig.2). This killing is MHC class I restricted since MHC class II is not expressed on P815 cells (45). The killing follows the classical CTL pattern as verified by examination of apoptosis (JAM test; Fig.2 and 3B) and cytolysis (chromium release assay; Fig.3A) of target cells. Killing is presumably due to the granule exocytosis pathway since P815 tumors do not express Fas receptor and are TNF resistant (46,47). In addition, T cells from C57BL/6 perforin knock-out mice exhibited dramatically reduced ability to kill P815 targets, demonstrating that killing in the dCTL assay is largely dependent on perforin (data not shown).

Effects of Nb infection on development of allo-specific CTL responses in the spleen. In order to investigate the effects of *Nb* on the generation of allo-specific CTL activity, C57BL/6 mice were either injected with *Nb* or PBS. Mice were then immunized with allogeneic cells and spleen T cells were isolated 7 days later. As previously, dCTL activity against P815 targets was observed in both the chromium release assay and the JAM test (Figs 3A and 3B) with T cells from immunized mice. However, infection of mice with *Nb* prior to immunization significantly reduced CTL activity by 46-88% (at various effector to target ratios) as measured by both JAM and chromium release assays. Killing by T cells from both *Nb*-infected allo-immunized and uninfected allo-immunized mice was allo-specific as neither effector could kill BW5147.3 tumor targets (Figs 3A and 3B). These results strongly suggest that the generation of allo-specific CTL activity *in vivo* is compromised in *Nb*-infected mice.

While these experiments confirm that Class I MHC-restricted and FasL-independent CTL activity was reduced by *Nb* it is possible that other mechanisms of CTL killing may be upregulated, *in vivo*, to compensate for this reduction. Therefore we used A20 tumor targets which are H-2^d, and which express both Class I and II MHC molecules (48) as well as the Fas receptor (46), and are TNF sensitive (46). We found that T cells from infected animals exhibit significantly reduced ability to kill A20 targets as compared with uninfected controls (Fig 4A, 4B) suggesting that Class II MHC-restricted killing and Fas/FasL interactions are either not involved or equally affected by *Nb* infection.

Infection with *Nb* is known to induce changes in the cellular composition of the spleen in mice. It has been reported previously, for example, that at day 9 post infection

there is a dramatic increase in the non-B, non-T cell population in the spleen, from 6% in control mice to 21% in *Nb*-infected mice (49). Thus the decreased dCTL that we observed could result from a reduced number of T cells in the T cell enriched cell population derived from the spleens of of *Nb*-infected mice. To rule this out, enriched T cell populations isolated from infected and uninfected mice were assessed by flow cytometric analysis to compare the percentages of T cell subsets in the T cell enriched populations derived from the two groups of mice. Figure 5 depicts a representative of four experiments, showing that the percentage of the $CD8^+$ T cells (the classical CTL phenotype) is unaltered in the infected mice (30.5 +/- 4.7%; n=4) as compared to the controls (30.4 +/- 2.9%; n=4). This indicates that decreased Class I MHC-restricted allo-specific CTL activity in the cell preparation from infected mice is not due to reduction in numbers of $CD8^+$ T cells. Interestingly there was a significant decrease in $CD4^+$ T cell percentage in the *Nb*-infected mice (from 53.6 +/- 3.0% in control, to 40.9 +/- 4.1 in *Nb* infected mice; n=4; $p < 0.001$, repeated measures ANOVA). This suggests that the appearance of non-B, non-T cells observed by us (unpublished observations) and others (49) in the spleen cells of *Nb*-infected mice is associated with reduction in $CD4^+$ T cell numbers.

To further confirm the decrease in $CD8^+$ Class I MHC-restricted killing, $CD4^+$ T cells were depleted from T cell populations using CD8 immunocolumns. The resulting enriched $CD8^+$ T cell populations from the spleens of infected and uninfected mice were assessed for their ability to induce apoptosis in P815 and A20 targets. We found that $CD8^+$ T cells from *Nb*-infected mice exhibited significantly lower cytotoxic potential against allogeneic tumor targets as compared to the control T cells (Fig.6A, 6B). Taken

together these data indicate that *Nb* interferes with the generation of allo-specific $CD8^+$ CTLs in immunized mice.

Phenotype of allo-specific T cell responses, that develop in the spleen of Nb-infected mice. Evidence of type 2 immune activation by *Nb* suggests that the observed graft prolongation is not simply a result of suppression of allo-reactivity but a modulation of allo-reactive responses resulting in a bias towards type 2 effector cell development. To confirm that T cells from *Nb*-infected allo-immunized mice were equally capable of responding to allo-stimulation we performed MLR assays. Figure 7 demonstrates that T cell proliferation, in response to alloantigen, is greatly increased following allo-immunization, and that infection with *Nb* does not reduce this effect. This is consistent with our hypothesis that *Nb* infection leads to a shift in allo-reactivity rather than global suppression of allo-reactivity. To confirm this we examined the phenotype of the allo-specific T cell responses after infection with *Nb*. Figure 8 shows that T cells from control allo-immunized mice secrete IFN-γ but no IL-4 or IL-6 (as measured by ELISA) upon stimulation with allogeneic cells *in vitro*. This production of IFN-γ was alloantigen specific since no IFN-γ was secreted when 3rd party C3H/HeJ splenocytes were used as stimulators or when T cells from naive mice were activated in the MLR. In contrast T cells from *Nb*-infected allo-immunized mice secreted significant levels of IL-4 and high levels of IL-6 in response to allo-antigen but greatly reduced levels of IFN-γ (Fig. 8). Production of IL-4 and IL-6 was also specific to allo-antigen, although low levels of IL-4 and IL-6 were produced by spleen T cells from *Nb*-infected unimmunized mice (approximately three times lower as compared to T cells from *Nb*-infected allo-

immunized; Fig. 8). This interesting finding was expected since *Nb* is known to be a polyclonal activator of type 2 T cells (37). In addition there seemed to be a low level of cross-reactivity between BALB/c and C3H/HeJ allo-antigens since allo-immunization with BALB/c *in vivo*, slightly enhanced T cell production of IL-4 and IL-6 elicited by stimulation with C3H/HeJ spleen cells *in vitro* (Fig. 8). Taken together, these data strongly suggest that *Nb* biases allo-specific responses towards the type 2 phenotype, thus resulting in mixed type 1/type 2 allo-reactivity.

One of the characteristics of type 2 T cells is their dependence on IL-4 for antigen specific proliferation (50). Therefore, we examined the effect of anti-IL-4 mAb on allo-specific proliferation of T cells from allo-immunized *Nb*-infected mice. In a one way MLR, addition of anti-IL-4 did not reduce proliferation of T cells from allo-immunized mice (Fig. 9), but T cells from allo-immunized *Nb*-infected mice exhibited significantly reduced levels of allo-specific proliferation in the presence of the anti IL-4 (mean inhibition of 41.7% +/- SEM of 2.48%: n=4: $p < 0.01$, repeated measures ANOVA). This data suggests that proliferation of at least a subset of allo-reactive T cells was dependent on IL-4 (Fig.9).

Nb infection leads to the induction of Tc2 cells. There have been a number of reports demonstrating that activation of $CD8^+$ T cells in the presence of IL-4 *in vivo* leads to decreased antigen specific CTL activity. The $CD8^+$ T cells that develop under the influence of IL-4 have been found to elaborate type 2 cytokines including IL-4 (14,16,31). Because the infection with *Nb* leads to expression of high levels of type 2 cytokines in the spleen we investigated whether this would lead to the development of IL-

4 producing $CD8^+$ Tc2 cells. To this end, T cells from *Nb*-infected allo-immunized mice and allo-immunized controls were activated *in vitro* with BALB/c non-T spleen cells in the presence of Brefeldin A, which blocks cytokine secretion (51). The cells were double stained with fluorescent labeled antibodies specific for CD8 and for IL-4. Flow cytometric analysis showed that Tc2 cells were approximately 8 times more abundant in *Nb*-infected mice as compared to the controls (Fig.10). This data is consistent with our hypothesis that the reduced allo-specific CTL activity in *Nb*-infected mice results from the development of Tc2 cells.

Fig. 1 ***Nb infection prolongs cardiac allograft survival in BALB/c to C57BL/6 mouse strain combination.***

Mice were injected with *Nb* larvae (□, n = 9) or PBS (■, n = 5) vehicle 4 days prior to heterotopic transplantation of allogeneic hearts. Allograft survival was assessed using ECG. ($p < 0.03$, Mann-Whitney).

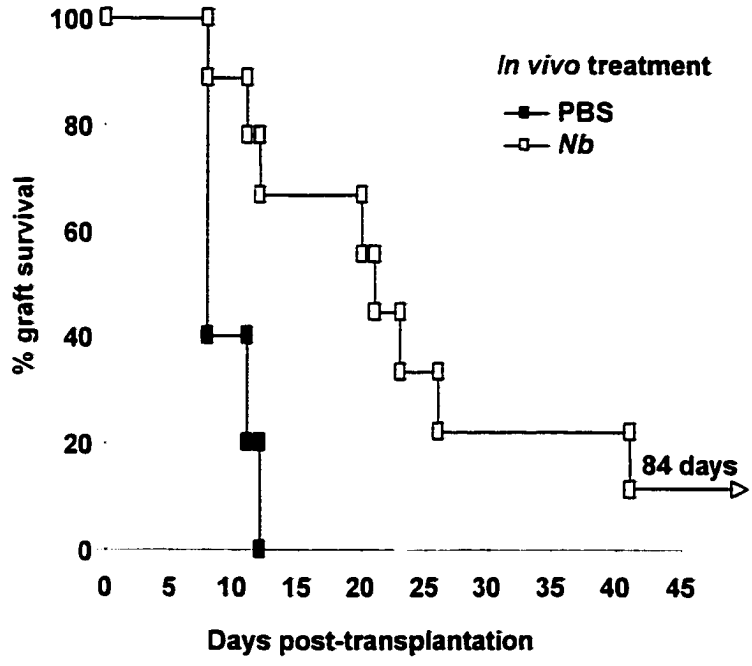

100
80
60
40
20
0
% graft survival
0
5
10
15
20
25
30
35
40
45
Days post-transplantation
In vivo treatment
PBS
Nb
84 days

Figure 1

Fig. 2 ***Direct CTL activity generated by immunization of mice with allogeneic spleen cells is allo-specific and T-cell dependent.***

Direct CTL activity of effector T-cells, from mice immunized with mitomycin C-treated allogeneic spleen cells (allo), or untreated controls (naive), was assessed by JAM assay. Splenocytes were isolated from mice seven days after immunization and enriched for, or depleted of, T-cells. Direct CTL activity by these effectors was measured against allogeneic P815 (H-2^d) (■,□,◆) or third-party BW5147.3 (H-2^k) (▲,△) targets at indicated effector to target ratios. Effector cells were naive T-cells (□,△) or T-cells from allo-immunized mice (■,▲). In addition non-T-cells from allo-immunized mice (◆) were tested. Data shown are expressed as mean ± SD of triplicate wells and are representative of 2-6 experiments.

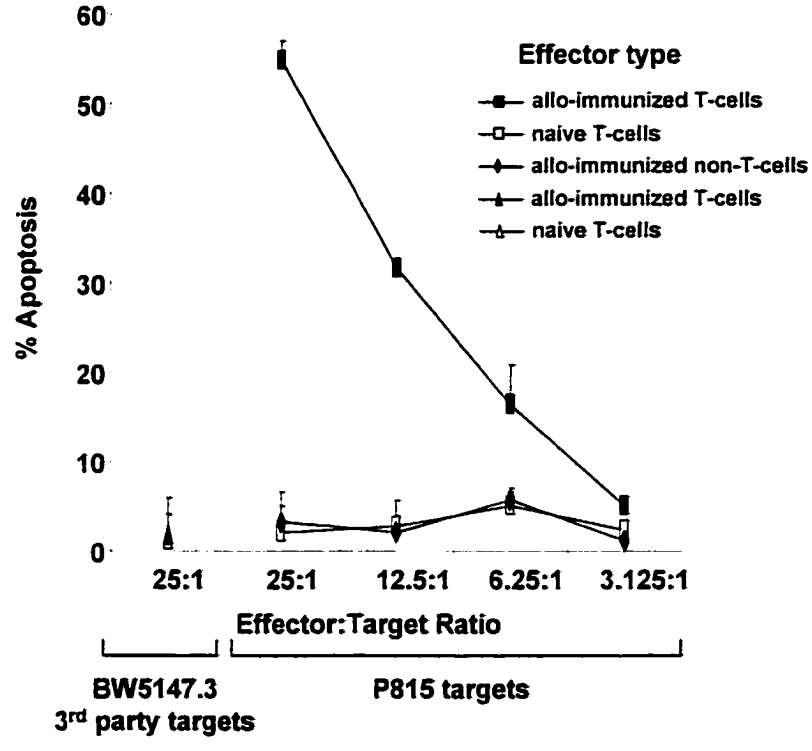
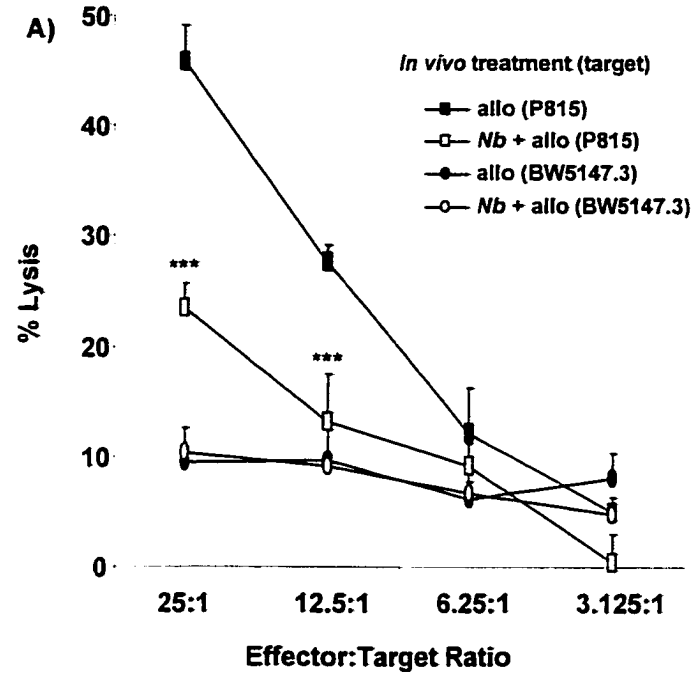

60
50
40
30
20
10
0
% Apoptosis
Effector type
allo-immunized T-cells
naive T-cells
allo-immunized non-T-cells
allo-immunized T-cells
naive T-cells
25:1
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio
BW5147.3
3rd party targets
P815 targets

Figure 2

Fig. 3 ***Nb infection downregulates allo-specific CTL activity against MHC class II-negative, Fas-negative , TNF-resistant P815 targets.***

Direct CTL activity of effector T-cells from mice infected with *Nb* (□,○), or injected with PBS vehicle (■,●) 4d prior to immunization with mitomycin C-treated allogeneic spleen cells, was assessed against MHC class II-negative, Fas-negative , TNF-resistant P815 targets. Effector T-cells were enriched 7 days post immunization, and dCTL activity was measured against allogeneic (P815;■,□), or third-party (BW5147.3:●,○) targets. In (A), cytolysis was measured by the chromium release assay. In (B), apoptosis was measured using the JAM assay. Data shown in (A) are expressed as mean ± SD of triplicate wells and are representative of 2 experiments (*** $p < 0.001$, one-way ANOVA). Data shown in (B) are expressed as mean ± SEM of 7 separate experiments (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, repeated measures ANOVA).


A)
50
40
30
20
10
0
% Lysis
In vivo treatment (target)
allo (P815)
Nb + allo (P815)
allo (BW5147.3)
Nb + allo (BW5147.3)
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25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio

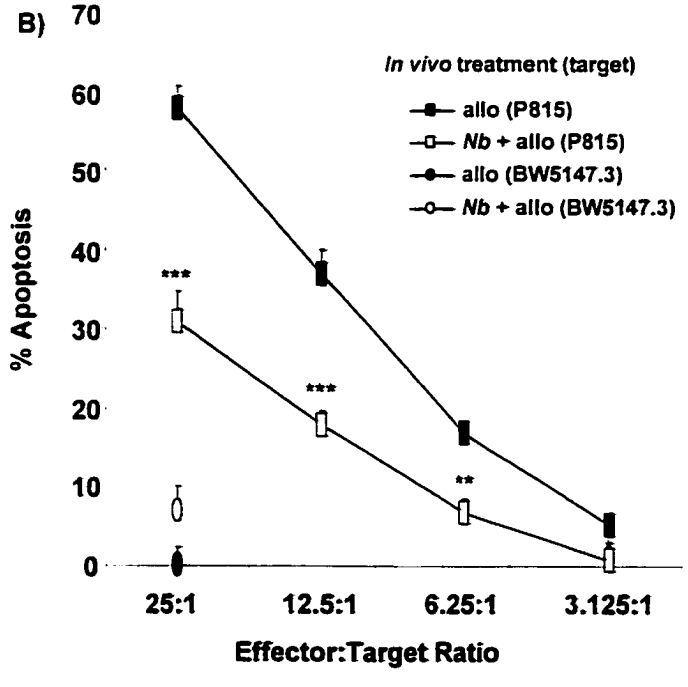

B)
70
60
50
40
30
20
10
0
% Apoptosis
In vivo treatment (target)
allo (P815)
Nb + allo (P815)
allo (BW5147.3)
Nb + allo (BW5147.3)
***
***
**
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio

Figure 3

Fig. 4 ***Nb infection downregulates allo-specific CTL activity against MHC class II-positive, Fas-positive , TNF-sensitive A20 targets.***

Direct CTL activity of effector T-cells from mice infected with *Nb* (□), or injected with PBS vehicle (■) 4d prior to immunization with mitomycin C-treated allogeneic spleen cells, was assessed against MHC class II-positive, Fas-positive, TNF-sensitive A20 targets. In (A), cytolysis was measured by the chromium release assay. In (B), apoptosis was measured using the JAM assay. Data shown are expressed as mean ± SEM of (A) 6, and (B) 5 separate experiments (*** $p < 0.001$, ** $p < 0.01$, repeated measures ANOVA).

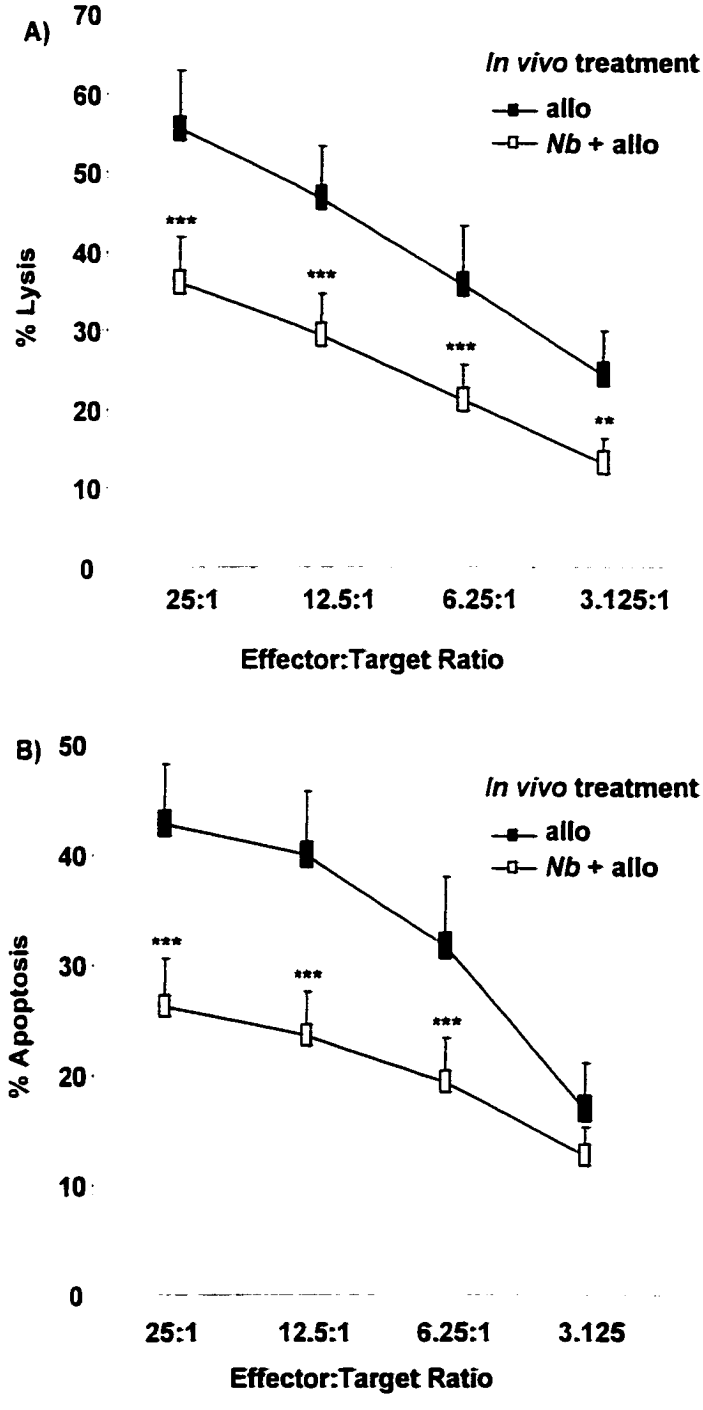

A)
70
60
50
40
30
20
10
0
% Lysis
In vivo treatment
allo
Nb + allo
***
***
***
**
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio
B)
50
40
30
20
10
0
% Apoptosis
In vivo treatment
allo
Nb + allo
***
***
***
25:1
12.5:1
6.25:1
3.125
Effector:Target Ratio

Figure 4

Fig. 5 ***Nb infection does not alter the percentage of CD8$^+$ T-cells in the spleen.***

Percentage of CD4$^+$ and CD8$^+$ T-cells, from mice injected with *Nb* (B and D), or PBS vehicle (A and C), that were later immunized with allogeneic spleen cells, was assessed using flow cytometric analysis. T-cells from treated mice were enriched and labeled with FITC-anti-CD4 (A and B), or FITC-anti-CD8 (C and D) mAbs. FITC-labeled isotypic control is shown in black. In this experiment the percentage of cells stained with specific mAbs (white) was 41.53% CD4$^+$ in *Nb*-infected vs 54.42% CD4$^+$ in control mice and 29.41% CD8$^+$ in *Nb*-infected vs 30.51% CD8$^+$ in control mice. This data is representative of 4 experiments.

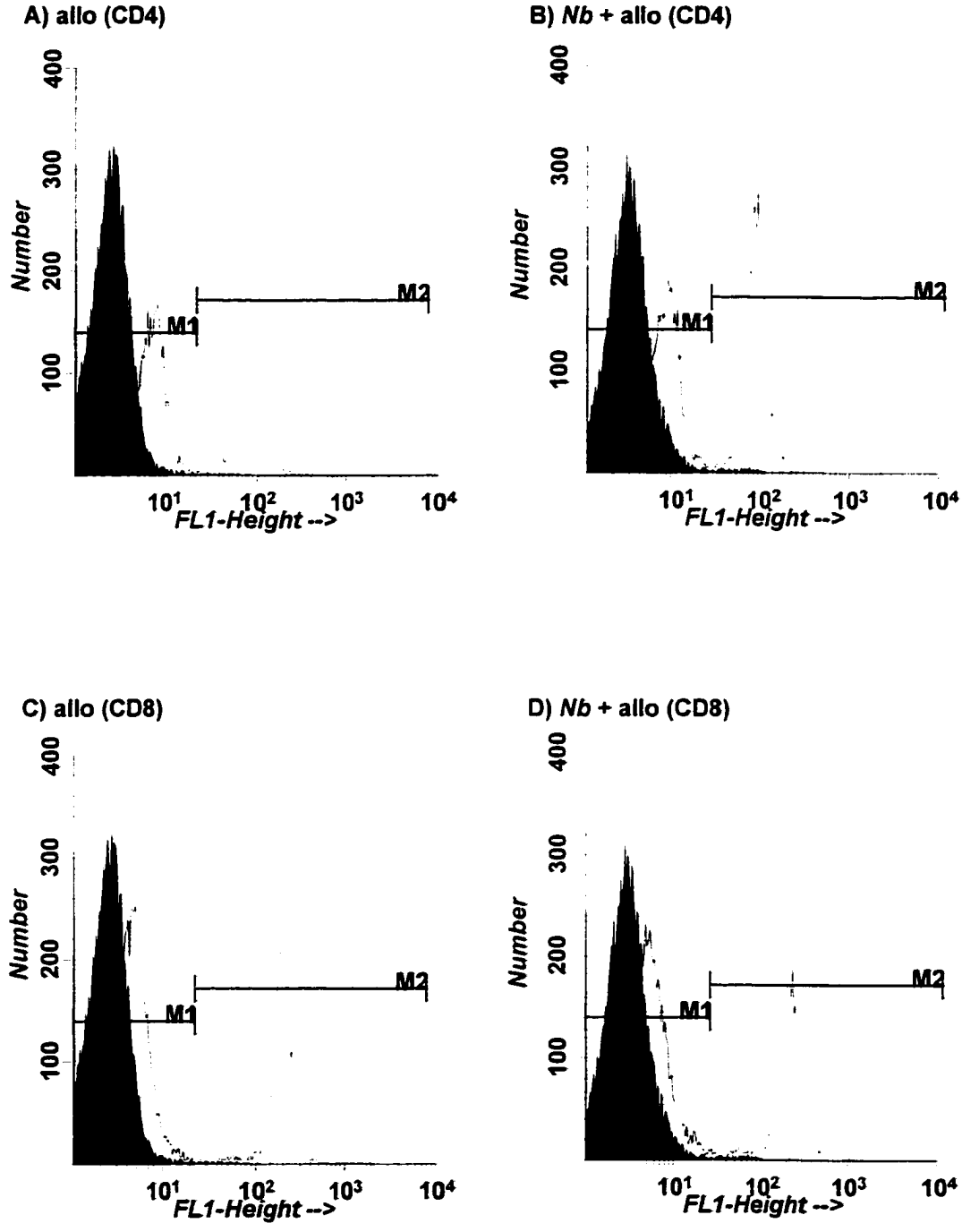
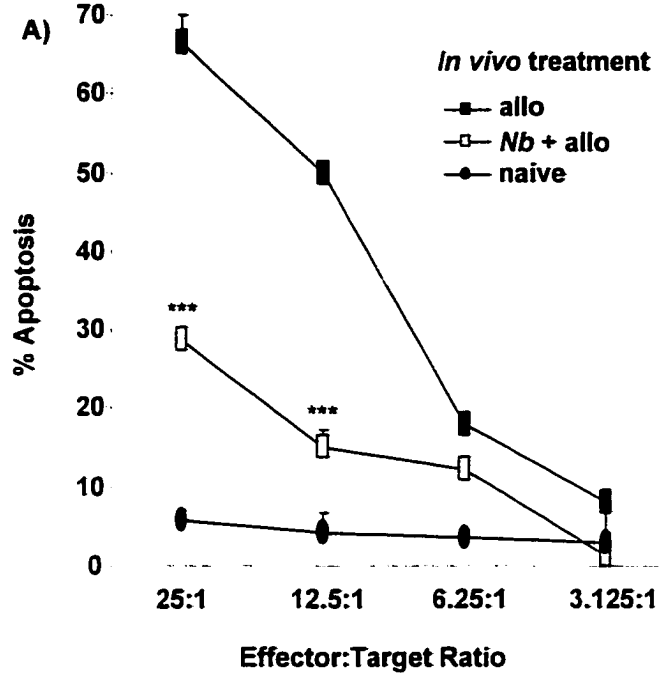

A) allo (CD4)
B) Nb + allo (CD4)
C) allo (CD8)
D) Nb + allo (CD8)
Number
400
300
200
100
M1
M2
10¹
10²
10³
10⁴
FL1-Height -->

Figure 5

Fig. 6 ***Nb infection downregulates allo-specific CTL activity mediated by CD8$^+$ cells.*** Direct CTL activity of effector CD8$^+$ T-cells from mice infected with *Nb* (□), or injected with PBS vehicle (■) 4d prior to immunization with mitomycin C-treated allogeneic spleen cells, was assessed against (A) P815, and (B) A20 targets. CD8$^+$ T-cells from naive mice (●) were used as a negative control. Effector CD8$^+$ T-cells were isolated 7 days after immunization, and dCTL activity was measured using the JAM assay. Data shown are expressed as mean ± SD of triplicate wells and are representative of 2 experiments (*** $p < 0.001$, one-way ANOVA).


A)
70
60
50
40
30
20
10
0
% Apoptosis
***
***
In vivo treatment
allo
Nb + allo
naive
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio

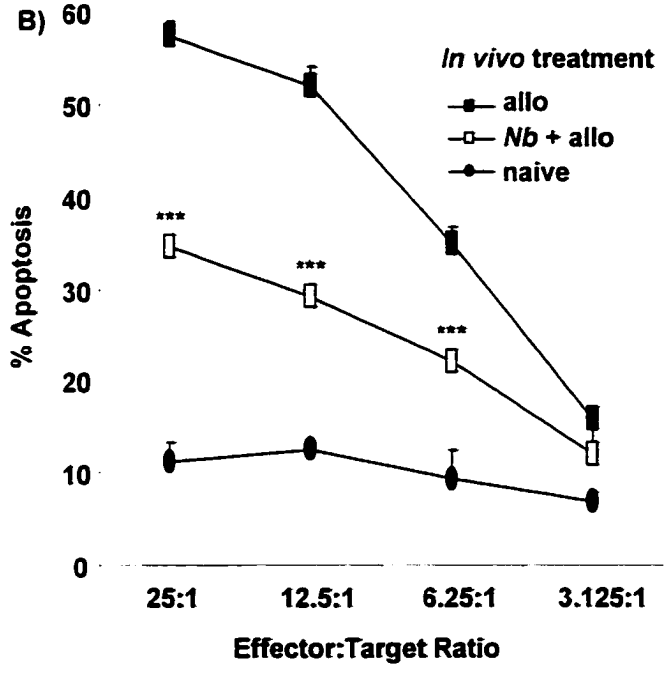

B)
60
50
40
30
20
10
0
% Apoptosis
***
***
***
In vivo treatment
allo
Nb + allo
naive
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio

Figure 6

Fig. 7 *Nb infection does not affect T-cell proliferation in 2° MLR.*

Allo-specific proliferation in 2° MLR of effector T-cells from mice that were infected with *Nb* (*Nb* + allo), or injected with PBS vehicle (allo) 4 days prior to allo-immunization, was assessed by ^{3}H-Thymidine incorporation. T-cells from unimmunized mice (naive) served as 1° MLR control. Enriched effector T-cells were activated, in the presence of mitomycin C-treated, T-cell-depleted, BALB/c (allogeneic) stimulator spleen cells, at a responder to stimulator ratio of 4:1. Data shown are expressed as mean ± SEM of 11 experiments (*** $p < 0.001$, repeated measures ANOVA).

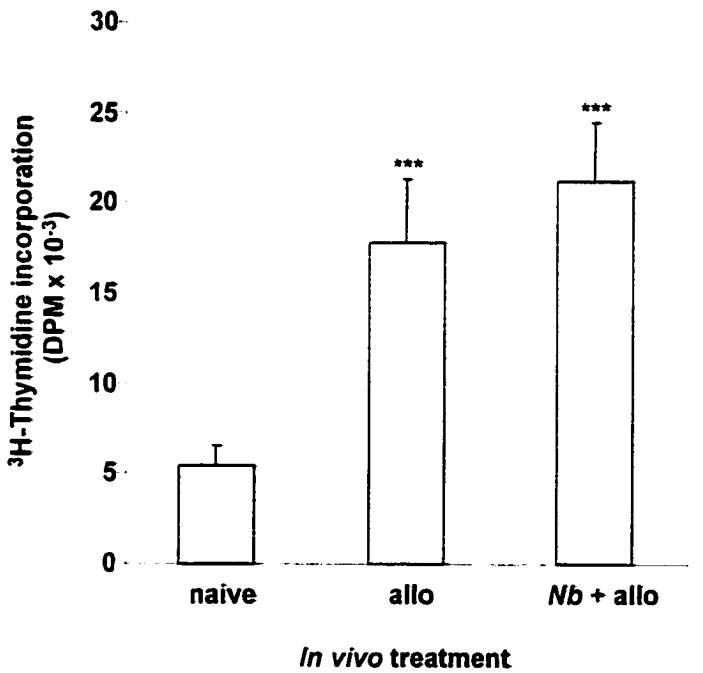

30
25
20
15
10
5
0
^{3}H-Thymidine incorporation
(DPM x 10^{-3})
***
***
naive
allo
Nb + allo
In vivo treatment

Figure 7

Fig. 8 ***Nb infection alters cytokine pattern secreted from T-cells in 2° MLR.***

Production of cytokines (in 2° MLR) by effector T-cells, from mice that were infected with *Nb* (□) or injected with PBS vehicle (■) 4 days prior to allo-immunization, was assessed by ELISA. T-cells from naive mice (▣) and mice infected with *Nb* (■) served as 1° MLR control. Effector T-cells were activated, in the presence of mitomycin C-treated, T-cell-depleted, BALB/c (allogeneic), or C3H/HeJ (3rd-party) stimulator cells. Supernatants were analyzed for the presence of (A) IFNγ, (B) IL-4 and (C) IL-6. Data shown are expressed as mean ± SD of triplicate wells and are representative of 3 experiments. In (A) statistical differences in comparison to "allo" *in vivo* treatment group are indicated. In (B) and (C) statistical differences in comparison to "*Nb* + allo" treatment group restimulated with BALB/c stimulators *in vitro* are indicated (*** $p < 0.001$, one-way ANOVA).

ND = not detectable.

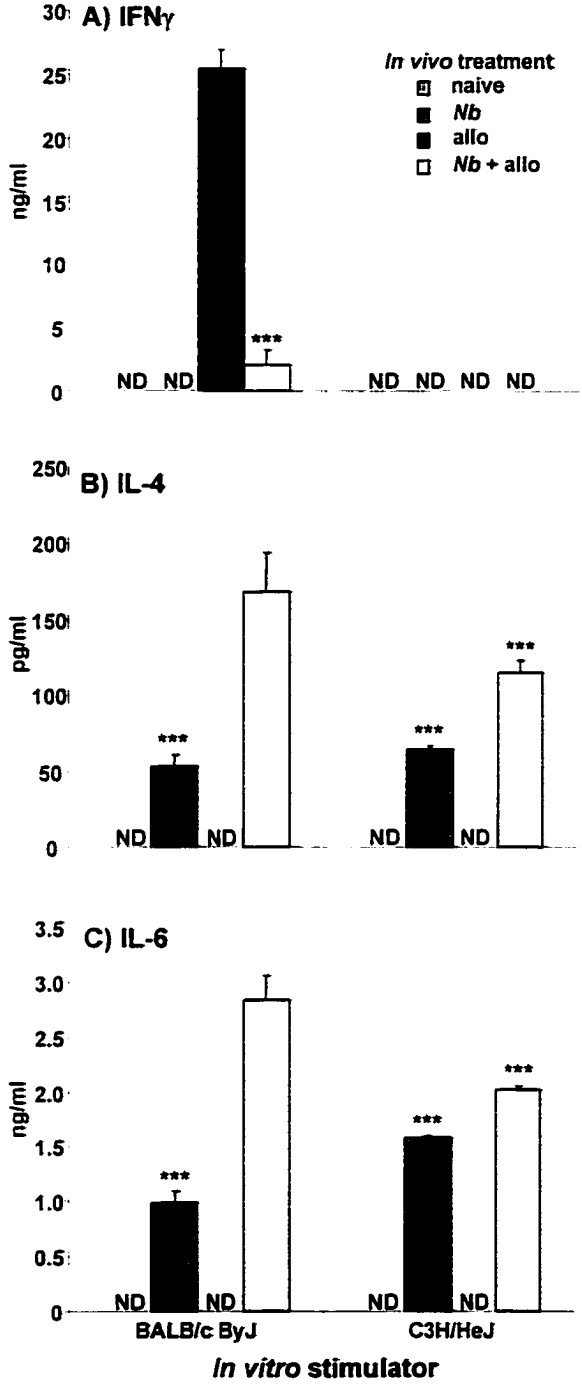

A) IFNγ
In vivo treatment
naive
Nb
allo
Nb + allo
ng/ml
30
25
20
15
10
5
0
***
ND ND
ND ND ND ND
B) IL-4
pg/ml
250
200
150
100
50
0
***
***
***
ND
ND
ND
ND
C) IL-6
ng/ml
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
***
***
***
ND
ND
ND
ND
BALB/c ByJ
C3H/HeJ
In vitro stimulator

Figure 8

Fig. 9 ***Proliferation of T-cells from Nb-infected allo-immunized mice is IL-4 dependent.***

Effects of *in vitro* anti-IL-4 treatment on allo-specific proliferation of effector T-cells from mice that were infected with *Nb* (*Nb* + allo), or injected with PBS vehicle (allo) 4 days prior to allo-immunization. T-cells were activated with mitomycin C-treated, T-cell-depleted, BALB/c stimulator spleen cells in the presence of anti-IL-4 mAb (■) or rat IgG control antibodies (□). Data shown are expressed as mean ± SD of quadruplicate wells and are representative of 4 experiments (*** $p < 0.001$, one-way ANOVA).

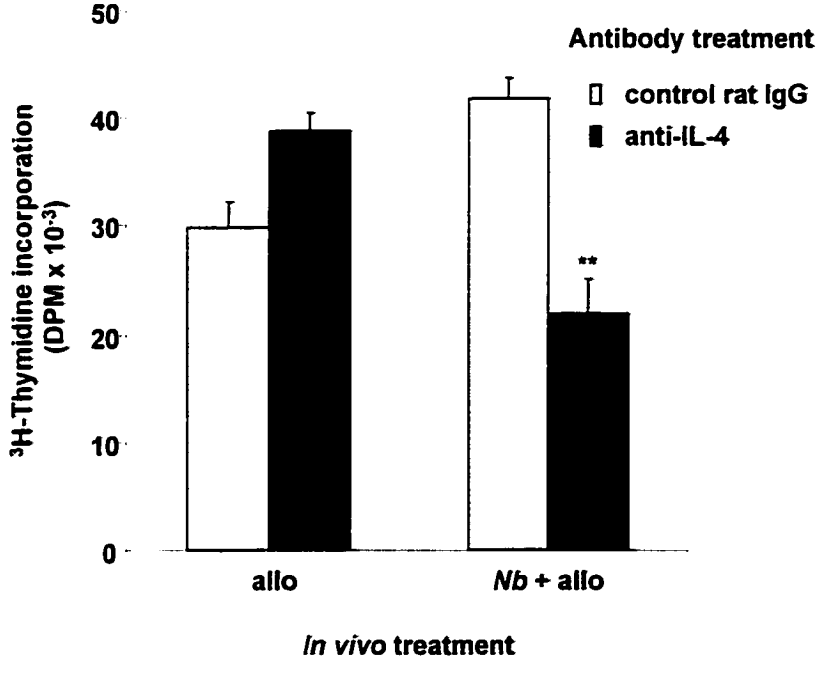

50
40
30
20
10
0
^{3}H-Thymidine incorporation (DPM x 10^{-3})
Antibody treatment
control rat IgG
anti-IL-4
**
allo
Nb + allo
In vivo treatment

Figure 9

Fig. 10 ***Nb infection leads to appearance of CD8$^+$, IL-4-producing cells.***

Percentage of $CD8^+$, IL-4-producing, T-cells, from allo-immunized mice previously injected with *Nb* (*Nb* + allo), or PBS vehicle (allo). T-cells were activated with mitomycin C-treated, T-cell-depleted, BALB/c stimulator in the presence of Brefeldin A. Cells were double stained with FITC-anti-CD8 and PE-anti-IL-4. Background fluorescence was determined using PE-labeled isotypic control. Data shown are expressed as mean ± SEM of 3 experiments (* $p < 0.02$; two-tailed unpaired Student's T-test).

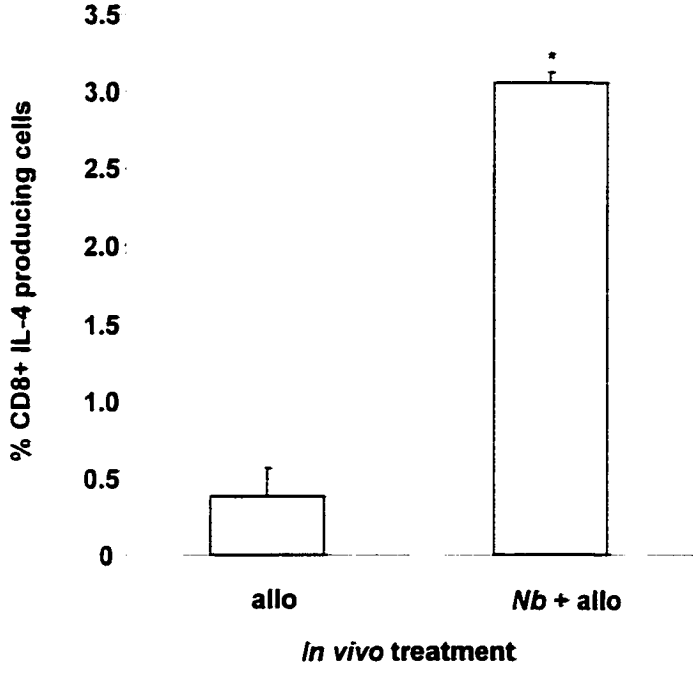

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
% CD8+ IL-4 producing cells
*
allo
Nb + allo
In vivo treatment

Figure 10

Discussion

In this study we have extended our previous findings that infection of rats with *Nb* prolongs kidney allograft survival in a fully disparate strain combination. Here we demonstrate a similar effect on heart allografts between C57BL/6 (H-2^b) and BALB/c (H-2^d) mice. It is important to note that *Nb* infection is self-limiting in rodents and that nematodes are present for only approximately 7 days after transplantation when they are vigorously expelled from the host (52). Graft prolongation thus outlasts the presence of the nematodes, indicating a role for immune-modulation during initial antigen perception. To address the mechanism of nematode induced graft prolongation we explored the effects of *Nb* infection on the development of allo-reactivity in the recipient C57BL/6 mouse. We established a simple model in which C57BL/6 mice were immunized with mitomycin C-treated BALB/c spleen cells. This immunization led to the development of potent allo-specific T cell responses. As expected, T cell proliferation in the MLR from such immunized mice (2^o MLR) was more than 3-fold higher than that seen with naive T cell responders. Similarly, IFN-γ production, while undetectable in the MLR cultures using naive responder T cells, was increased dramatically in the cultures using responder T cells from allo-immunized mice.

In vitro comparison of allo-specific responses mediated by T cells from *Nb*-infected allo-immunized and control allo-immunized mice allowed us to conclude that non-specific immunosupression does not play a role in *Nb*-induced prolongation of allograft survival. Allo-specific proliferation and cytokine production in the MLR by T cells from *Nb*-infected allo-immunized mice was intact as compared to the uninfected allo-immunized controls.

Previously (41) we have speculated that prolongation of allograft survival by *Nb* was due to its ability to stimulate powerful type 2 allo-immunity which would interfere with type 1 allo-responses important in graft rejection. Data in the current report support this hypothesis. For example, analysis of cytokine production in the MLR by T cells from allo-immunized mice that were infected with *Nb* clearly showed that type 2 cytokines (IL-4 and IL-6) were elaborated by these cells but not the cells from allo-immunized uninfected controls. Using flow cytometric analysis for intracellular expression of IL-4 we also show that a proportion of $CD8^+$ T cells from *Nb*-infected allo-immunized mice, but not control allo-immunized animals, are producing IL-4. The relatively low levels of IL-4 detected in the supernatants of the T cell cultures from *Nb*-infected allo-immunized animals may reflect the consumption of IL-4 by proliferating allo-specific T cells. In support of this we found that the addition of anti-IL-4 antibody decreased allo-specific proliferation of T cells from *Nb*-infected allo-immunized mice by approximately 40%. These results indicate that perception of alloantigen during the nematode infection leads to the development of type 2 allo-specific immunity, which interferes with generation of allo-specific type 1 cells.

Since anti-IL-4 treatment did not completely block the proliferation of T cells from *Nb*-infected allo-immunized animals in response to alloantigen, it is reasonable to suppose that the remaining T cells were of the type 1 phenotype. This is consistent with our observation that IFN-γ production is reduced, but not ablated, in allo-stimulated T cell cultures from *Nb*-infected allo-immunized mice. Taken together, these results suggest that both type 2 and type 1 allo-specific T cells develop in the presence of *Nb*. The development of both type 1 and type 2 allo-specific immunity might be expected

since it has been well documented (40) that production of type 2 cytokines is first observed in the spleens of *Nb* infected mice at day 5 post infection (40). In our model mice are exposed to alloantigen 4 days after infection with *Nb*, leaving approximately 24 hours in which type 1 cytokine activation could result in response to allo-challenge. *Nb* may thus cause increased allograft survival through attenuation of this developing type 1 allo-reactivity, through the emergence of a vigorous type 2 allo-specific response.

One way in which this type 2 shift is expressed is in the developing allo-specific CTL-response. $CD8^+$ CTL have been suggested to destroy graft tissue mainly through direct cytotoxic effects against foreign class I MHC expressing cells by the granule exocytosis pathway since perforin and granzyme B have been detected by *in situ* hybridization in grafts undergoing acute rejection (53, 54). Many treatments that prolong allograft survival, including neonatal immunization (55, 56, 57), administration of anti-CD4 non-depleting mAb (19), as well as portal venous inoculation with alloantigen (58) are associated with decreased allo-specific CTL activity. In some cases (55, 56, 57, 58) inhibition of CTL activity was associated with the development of allo-specific type 2 immunity. IFN-γ is crucial for the development of CTL activity in response to viral (29) or Toxoplasma gondii (59) infetion. In addition experiments using IL-4 transfected virus or *S. mansoni* to induce type 2 cytokine expression have shown a profound inhibition of virus-specific CTL activity and markedly delayed viral clearance (29, 30).

To assess the immunomodulatory effects of *Nb* on the development of allo-reactivity in the CTL compartment *in vivo*, it was essential to establish a model of direct CTL activity of *ex vivo* spleen cells without the normal 5 day expansion *in vitro*. We thus assessed CTL activity directly and found that killing of allogeneic tumor targets by

spleen T cells from allo-immunized but not naive mice in dCTL assay was detectable 4h after *ex vivo* incubation with P815 tumor targets (data not shown) and reached levels of up to 70% lysis within 18h of incubation.

In the experimentation described here the exposure of mice to *Nb* prior to immunization with alloantigen results in a marked decrease in MHC class I restricted dCTL activity against Fas-negative, TNF-resistant P815 (allogeneic) tumor targets. We observed significantly decreased killing by both the JAM assay (apoptosis) as well as the chromium release assay (cytolysis). However it was possible to argue that killing by other mechanisms, such as unconventional $CD4^+$ CTL (60, 61) or by Fas-mediated killing (by either $CD4^+$ or $CD8^+$ cells) may be intact or even upregulated in *Nb*-infected animals. Our experiments with Fas-negative P815 cells, which depend on perforin-mediated class I-restricted killing would not detect such killing activity. To address this point we used class II-bearing, Fas-positive, TNF-sensitive allogeneic tumor cells (A20) in order to assess killing by these other possible mechanisms. The results confirm that CTL activity is significantly decreased in *Nb*-infected allo-immunized mice rather than being shifted into a different killing phenotype.

There are number of explanations for the mechanism by which *Nb* may down-regulate the allo-specific CTL in mice. First, production of type 2 cytokines during *Nb* infection may interfere with the development of allo-specific CTL. Mosmann *et al.* (62) have shown that exposure of type 1 cytokine producing allo-specific $CD8^+$ CTL to IL-4 inhibits their ability to expand in response to stimulation. Second, a fraction of the allo-specific $CD8^+$ T cells activated during the infection with *Nb* may aquire a type 2 non-cytotoxic phenotype similar to that described by Erard *et al.* (14). Our flow cytometric

data showing the appearance of $CD8^+$ IL-4 secreting T cells in *Nb*-infected allo-immunized mice supports the hypothesis that *Nb* drives the development of $CD8^+$ T cells towards a Tc2 phenotype. Interestingly we found that $CD8^+$ T cells that stained positively for intracellular IL-4 expressed slightly lower levels of CD8; similar to the findings of Erard *et al.* (14) who observed down-modulation of CD8 following *in vitro* activation of naive $CD8^+$ T cells in the presence of IL-4. While most of the reports suggest that cytotoxic activity of Tc2 cells is compromised, at least one study shows otherwise. In this study Li and co-workers (31) found that allo-specific Tc2 cells were as efficient as Tc1 cells at killing allogeneic targets *in vitro*, and when injected into footpads of allogeneic mice were very effective at mediating DTH associated inflammatory reactions. In that study, however, Tc1 and Tc2 cells were differentiated *in vitro* using a macrophage like tumor cell line, therefore, the relation of such Tc2 cells to their *in vivo* activated counterparts is unclear.

Taken together the data reported here support a hypothesis, that the generation of allo-specific CTL is modulated by the type 2 responses induced by *Nb*. Although this modulation of CTL responses is likely to be an important part of the process that allows prolongation of allograft survival, additional mechanisms are likely to be involved. For example, we have previously reported that the number of T cells and macrophages infiltrating kidney allografts was dramatically reduced (by up to 90%) by *Nb* infection (41), suggesting that *Nb* may also interfere with cell migration and/or with the ability of allo-specific T cells in the graft to recruit additional leukocytes.

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4.0 Nematode infection enhances survival of activated T cells by modulating accessory cell function.

This manuscript has been submitted to *Journal of Immunology.*

The Full Title: Nematode infection enhances survival of activated T cells by modulating accessory cell function[1]

Robert S. Liwski[*†], and Timothy D. G. Lee[2*†]

Departments of [*]Microbiology and Immunology and [†]Surgery, Dalhousie University, Halifax, Nova Scotia, Canada. B3H 4H7

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Abstract

The type of immune response generated following exposure to antigen depends on a variety of factors including the nature of the antigen, the type of adjuvant used, the site of antigenic entry and the immune status of the host. We have previously shown that infection of rodents with *Nippostrongylus brasiliensis* (*Nb*)[3] shifts the development of type 1 allo-specific responses towards type 2 immunity, suggesting nematode modulation of T cell activation. In this report we explore the immuno-modulatory effects of *Nb* on T cell activation. We found that spleen cells from *Nb*-infected mice exhibited dramatically increased proliferation in response to concanavalin A (Con A) and anti-CD3. This hyper-proliferation could be transferred, *in vitro*, to naive splenocytes by co-culture with mitomycin C-treated cells from *Nb*-infected animals. The transfer was mediated by non-T accessory cells, and supernatants derived from Con A-activated non-T cells, suggesting the involvement of a soluble factor secreted by accessory cells. The accessory cells secreted high levels of IL-6, and anti-IL-6 treatment abrogated the supernatant-induced hyper-proliferation, thus confirming that IL-6 was mediating the effect. Further, spleen cells from *Nb*-infected mice were more resistant to activation induced cell death (AICD) following mitogenic stimulation. Reduced AICD was also transferable and IL-6-dependent. Thus, the hyper-proliferation was, in part, due to enhanced activated T cell survival. These phenomena mediated by accessory cells may contribute to the powerful polyclonal activation of type 2 immunity caused by nematode infection.

Introduction

Infection with the nematode parasite *N. brasiliensis* induces strong type 2 immune responses in mice and rats (reviewed in 1,2,3) which include mastocytosis (4,5), eosinophilia (6) and a dramatic increase in the production of IgE (7,8). These responses are similar to those noted in many nematode infections in humans and animals (9,10,11). As such, *Nb* has been used by many researchers as a model for immune effects mediated by nematode infection. These potent responses have been suggested to result from a marked activation of IL-4 producing T lymphocytes (4,6,7). *Nb* infection induces a strong proliferative T cell response *in vivo* (12,13,14), and exibits stable TCR Vβ chain usage (13,14). This data, taken together with the fact that most of the IgE induced by *Nb* is not specific to the worm (12,15), suggests polyclonal T cell activation. Indeed, *Nb* infection results in a four-fold increase in frequency of IL-4-secreting $CD4^+$ T cells (16), confirming the polyclonal nature of the immune hyper-activation of the T cell compartment of the immune responses during *Nb* infection. However, the mechanisms underlying this activation have not previously been described.

The development of type 2 immunity during nematode infection is dependent on both the cytokine milieu (17,18) and the type of co-stimulatory signals provided by the antigen presenting cells (19) during activation. The presence of IL-4 is necessary for the induction of Th2 responses associated with *Nb* infection since IL-4-deficient (20,21) or STAT6-deficient (22) mice do not exhibit blood eosinophilia (20) and do not produce IgE (20,21,22) or type 2 cytokines (20,22) in response to *Nb* infection. In addition to IL-4, recent evidence indicates that IL-6 is also an important factor for the induction of Th2 cells (23,24,25). Rincon *et al.* (23) for example, have shown that IL-6, secreted from

antigen presenting cells, stimulates IL-4 production from T cells in response to polyclonal T cell activators as well as specific peptide antigens. Interestingly, alveolar macrophages produce high levels of IL-6 (26,27) in the very early stages (starting from day 1) of *Nb* infection, suggesting a role for these APC in the early development of type 2 T cells.

Previous studies in our laboratory have demonstrated that infection with *Nb* prolongs allograft survival in rats (28) and mice (Liwski, R. L., *et al.* submitted). This was associated with immunomodulatory effects by *Nb* on developing allo-specific T cell responses induced by immunization with allogeneic cells. These effects included a depression of allo-specific CTL activity, a marked inhibition of IFN-γ production and a dramatic increase in secretion of IL-4 and IL-6 (Liwski, R. S., *et al.* submitted). Interestingly, spleen cells from *Nb*-infected mice, not previously immunized with alloantigen, also elaborated greater than control levels of IL-4 and very high levels of IL-6 upon primary activation with allogeneic cells further confirming that polyclonal type 2 activation occurs. Taken together these results suggested that infection with *Nb* had a dramatic effect on the development of immune responses to unrelated antigens and that these effects may be mediated through IL-6.

In this study we investigated the effects of *Nb* on *in vitro* T cell activation in response to polyclonal T cell activators. We found that spleen cells from *Nb*-infected mice exhibited proliferative hyper-responsiveness upon activation with Con A and anti-CD3. This effect could by transferred *in vitro* with accessory non-T cells from *Nb*-infected mice and was found to be mediated by IL-6 produced by these cells. In addition, this hyper-responsiveness was in large part due to induction of resistance to AICD. The

increased T cell proliferation, and reduced AICD, that occur in the type 2 cytokine environment induced by *Nb.* may explain how exaggerated type 2 T cell responses are generated and persist during nematode infection. The elaboration of this, and other, immuno-modulatory effects of nematodes is of great interest since a substantial part of the world's population encounters antigen, both naturally and in the form of vaccines, against a background of nematode infection (10).

Materials and Methods

Animals. Male 6-8 week old C57BL/6J (H-2^b) and BALB/cByJ (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the Medical Sciences animal care facility with food and water *ad libitum* for two weeks prior to experimentation.

Nippostrongylus brasiliensis. The life cycle of *Nb* was maintained in Sprague Dawley rats (Harlan, Indianapolis, IN) as we have previously described (28). Eight hundred third-stage larvae of *Nb*, in 200 µl of phosphate buffered saline, were used for inoculation of mice.

In vivo treatment. Groups of 8-12 week old male C57BL/6 mice were injected s.c. with *Nb*. Control mice were not treated. On day 11 after infection all mice were sacrificed by cervical dislocation and spleen cells were isolated for *in vitro* culture.

Cell isolation. Single cell suspensions from spleens of C57BL/6 mice were prepared (as described previously; 29), under aseptic conditions in RPMI 1640 (ICN, Aurora, OH) supplemented with 100 units/ml of penicillin, 100 µg/ml streptomycin (Gibco BRL, Burlington, ON), 10% FBS (Gibco BRL), 20 mM HEPES (Gibco BRL) and 50 µM 2-ME (Sigma, St Louis, MO). Cells were pooled within experimental groups. Erythrocytes were eliminated by lysis with ACK buffer for 2 min at room temperature. Cells were washed three times and resuspended in RPMI for *in vitro* culture. In co-culture experiments some cell populations were inactivated with 25 µg/ml of mitomycin

C (Sigma) for 30 min at 37°C. In some co-culture experiments T cells were depleted from the spleen cell population by treatment with anti-Thy1.2 mAb (mouse $IgG2_b$ anti-mouse; clone 5a-8 ; Cedarlane, Hornby, ON) for 1h at 4°C followed by treatment with rabbit low-tox complement (Cedarlane) for 1h at 37°C. This treatment resulted in > 98% depletion of T cells as assessed by flow cytometry (using anti-CD4 and anti-CD8 mAbs), and by abrogation of proliferation in response to Con A (Sigma) or anti-CD3 mAb. Non-T spleen cells were inactivated by adding mitomycin C (25 μg/ml) during the last 30 min of treatment with complement.

Production of conditioned supernatants. Whole spleen or T cell-depleted, mitomycin C-treated spleen cells from naive or *Nb*-infected mice ($5.0x10^5$ cells/well) were activated with Con A in 24 replicate wells of 96-well round-bottomed plates for 24 h at 37°C. Contents of each well were pooled within groups and centrifuged at 400x g for 5 min, supernatants were then transferred into fresh tubes and stored at –70°C.

Proliferation assay. Spleen cells ($1x10^5$/well) from *Nb*-infected (*Nb* spleen cells) or uninfected mice were cultured in RPMI with 5 μg/ml Con A or anti-CD3 mAb (1:20 hybridoma supernatant) in triplicate wells of 96-well round-bottomed tissue culture plates (Nunc; Gibco BRL). After 72h (unless stated otherwise) incubation at 37°C the cultures received 1 μCi/well of [^{3}H]thymidine ([^{3}H]TdR; ICN) and 18h later the contents of the wells were harvested onto filtermats (Skatron, Sterling, VA), using an automatic cell harvester (Skatron), for measurement of [^{3}H]TdR incorporation. For co-culture studies, $1x10^5$ responder cells were stimulated with 5 μg/ml Con A in the presence of $5x10^4$

mitomycin C-inactivated stimulator, whole or T cell-depleted spleen cells. To assess the immunomodulatory effect of conditioned supernatants on Con A-induced spleen cell proliferation naive spleen cells ($1x10^5$/well) were activated with Con A (5 μg/ml) in the presence of 50 μl of conditioned supernatant or media alone (final volume 200 μl/well). In order to assess the effects of mouse recombinant cytokines on Con A induced proliferation, varied concentrations of IL-4 (Endogen, Woburn, MA), IL-6 (Pharmingen, Mississauga, ON), or IL-10 (Pharmingen) were added at the beginning of culture. To block conditioned supernatant, or IL-6, mediated enhancement of spleen cell proliferation, varied concentrations (ranging from 10 ng/ml to 50 ng/ml) of anti-IL-6 mAb (rat IgG_1 anti-mouse; clone MP5-20F3; Pharmingen) were added to wells containing supernatant or mouse recombinant IL-6 and the plates were incubated at 37°C for 1 h prior to addition of spleen cells and the initiation of the proliferation assay.

Detection of AICD by JAM assay. Activation induced cell death was measured by using the JAM assay (30). Briefly, spleen cells ($2x10^5$/well) from *Nb*-infected or naive mice were activated with Con A (5 μg/ml) in 2 sets of replicate wells per group in 96-well round-bottomed plates. After 24 h incubation, 1 set of 6 replicates ("pulsed set") was pulsed with 1 μCi/well of [^{3}H]TdR, while the second set of 6 replicates was left untreated ("untreated set"). Eighteen h later the contents of the wells within each set were collected into centrifuge tubes. Cells from the "pulsed sets" were washed twice with RPMI to prevent further incorporation of [^{3}H]TdR into the cells and resuspended in the supernatant from the corresponding "untreated set" in order to preserve the culture conditions during subsequent culture. The cells were then re-plated in two sets of

triplicates. The first set was harvested (cell harvester) immediately (T_{0h}), while the second set was harvested after 24h incubation at 37°C (T_{24h}). The loss of radio-labeled DNA during 24 h incubation was used to measure the extent of DNA fragmentation in the cells that have proliferated during the pulsing period. Specific AICD was calculated using the following formula: % specific AICD = ([^{3}H]TdR in cells at T_{0h} – [^{3}H]TdR in cells at T_{24h}) / ([^{3}H]TdR in cells at T_{0h}) x 100%. Resistance to AICD was calculated using the following formula: % resistance= (% AICD of naive control group - % AICD of experimental treatment group)/(% AICD of naive control group) x 100%. In co-culture experiments $2x10^5$ responder cells were cultured in the presence of $1x10^5$, mitomycin C-treated stimulator cells. To assess the effects of conditioned supernatant or IL-6 on AICD the supernatant or the cytokine (at various concentrations) were added at the beginning of primary culture as for the proliferation assay. To block conditioned supernatant or IL-6-mediated resistance to AICD, anti-IL-6 mAb (final concentration 50 ng/ml) was added to the wells containing various supernatants or mouse recombinant IL-6 and the plates were incubated at 37°C for 1 h prior to addition of spleen cells and initiation of proliferation assay.

ELISA for detection of cytokines. Supernatants were analysed for presence of IFN-γ, IL-4, IL-6 and IL-10 by. All mAb and mouse recombinant cytokine standards used were purchased from Pharmingen; capture antibodies: anti-IL-4 (rat IgG_1 anti-mouse; clone 11B11), anti-IL-6 (rat IgG_1 anti-mouse; clone MP5-20F3), and anti-IFN-γ (rat IgG_1 anti-mouse; clone R4-6A2); biotinylated antibodies: anti-IL-4 (rat IgG_1 anti-mouse; clone BVD6-24G2), anti-IL-6 (rat IgG_{2a} anti-mouse; clone MP5-32C11), and

anti-IFN-γ (rat IgG_1 anti-mouse; clone XMG1.2). Briefly, ELISA plates (Costar) were coated with anti-cytokine mAb in carbonate buffer (pH 9.6) at 4°C overnight. After overnight incubation and blocking with 2 mg/ml bovine serum albumin (BSA; Gibco BRL) in Tris-buffered saline, test supernatants and recombinant cytokines were added to the plates and incubated overnight at 4°C. Cytokines were detected using biotinylated anti-cytokine mAb, Extravidin –Peroxidase (Sigma) and TMB substrate solution (Gibco BRL). Detection limits were 30 pg/ml for IFN-γ and 15 pg/ml for IL-4 and IL-6.

FACS analysis. Cells ($1x10^6$) were washed twice in PBS containing 1% BSA. Cells were incubated in the dark with 2 μg/ml anti-CD4 mAb (rat $IgG2_b$ anti-mouse; clone YTS 191.1; Cedarlane), anti-CD8α mAb (rat $IgG2_b$ anti-mouse; clone YTS 169.4; Cedarlane) or isotype control (rat $IgG2_b$; Cedarlane) at 4°C for 30 minutes. Cells were then washed three times, fixed in PBS containing 0.1% paraformaldehyde and stored at 4°C overnight. Flow cytometric analysis was performed on a FACscan (Beckton Dickinson) using Lysis II software.

Results

Spleen cells from Nb-infected mice exhibit enhanced responsiveness to Con A and anti-CD3 antibody. We have previously found that infection of mice with *Nb* inhibited allo-antigen-specific CTL activity if the immunization with allo-antigen took place 4 days after *Nb* infection (Liwski, R. S., *et al.* submitted). To confirm that *Nb* did not induce a non-specific suppresion of T cell responsiveness, we compared mitogen-induced proliferative activity of splenic T cells from *Nb*-infected, and uninfected mice. Spleen cells from *Nb*-infected C57BL/6 mice exhibited significantly ($p < 0.001$) enhanced proliferation in response to both Con A (Fig. 1A) and anti-CD3 (Fig. 1B) as compared to the cells from control uninfected mice. Similar results were obtained when BALB/c mice were used instead of C57BL/6 mice (129% increase over control for Con A stimulation; data not shown), confirming that *Nb*-induced hyper-responsiveness to the T cell mitogen is not a strain-specific phenomenon. There was no difference in the background proliferation exhibited by spleen cells isolated from infected or uninfected mice (data not shown). The increased proliferative response seen with spleen cells from *Nb*-infected mice did not result from an increase in the proportion of T cells in the spleen, since the percentages of $CD4^+$ and $CD8^+$ T cells in the spleens from *Nb*-infected mice (assessed by flow cytometry) were not different from those observed in uninfected controls (uninfected $CD4^+$ 15.63 ± 2.28 versus *Nb*-infected $CD4^+$ 14.08 ± 1.98; uninfected $CD8^+$ 15.03 ± 2.66 versus *Nb*-infected $CD8^+$ 15.23 ± 1.13; $n = 3$).

Non-T spleen cells from Nb-infected mice transfer increased responsiveness to naive spleen cells in vitro. To investigate the mechanism by which *Nb*-induced hyper-responsiveness to Con A is manifest, we assessed whether cells from *Nb*-infected mice could transfer the effect to naive spleen cells *in vitro*. Naive splenocytes were stimulated *in vitro* with Con A in the presence of spleen cells, which had been isolated from *Nb*-infected animals and inactivated with mitomycin C ("transferring cells"). Similarly treated "transferring cells" from uninfected mice were used as controls. Fig 2A shows that the addition of mitomycin C-inactivated transferring spleen cells from *Nb*-infected mice significantly ($p < 0.001$) enhanced Con A-induced proliferation of naive spleen cells (by approximately 260%). If the "transferring cells" were taken from uninfected mice, rather than *Nb*-infected mice, the proliferation was increased only mildly. These data indicate that the *Nb*-induced increase in proliferation can be transferred, by co-culture, to naive cells even if the "transferring cells" are mitomycin C-treated. Similar results were obtained in BALB/cByJ mice (data not shown).

Since accessory cell co-stimulation is necessary for the induction of T cell proliferation to Con A (31,32), the observed changes in proliferation could be due to modification of either T cell, or accessory cell, function. In order to address this question we examined whether a mitomycin C-inactivated, T cell-depleted, spleen cell population from *Nb*-infected mice could transfer the observed hyper-responsiveness onto naive spleen cells. Spleen cells were depleted of T cells by treatment with anti-Thy1.2 antibody and complement. The efficiency of T cell depletion was tested by inhibition of responsiveness to Con A, as well as by flow cytometry, and was always greater than 98% (data not shown). When added as co-culture cells, mitomycin-C-inactivated, T cell-

depleted "transferring" spleen cells from *Nb*-infected mice significantly augmented Con A-induced proliferation of naive syngeneic cells (by approximately 180%; Fig. 2B). Similar cells from uninfected animals did not show this dramatic effect (Fig. 2B).

Hyper-responsiveness to Con A induced by spleen cells from Nb-infected mice is mediated by a soluble factor. Accessory cells can enhance proliferative T cell responses through expression of membrane bound co-stimulatory molecules such as B7 (31,33) or ICAM-1 (33,34,35), or through secretion of soluble factors including IL-1(36,37), TNFα (36), and IL-6 (36,37,38). In order to ascertain whether a soluble factor (or factors) was responsible for the induction of hyper-responsiveness, we collected 24h supernatants from Con A-stimulated cultures of spleen cells from either *Nb*-infected or naive mice. We tested these supernatants for their ability to enhance Con A-induced proliferation of naive spleen cells. Figure 3 shows that supernatants from Con A-stimulated spleen cells from uninfected mice do not significantly enhance proliferation of naive spleen cells to Con A. In contrast, supernatants of Con A-stimulated spleen cells from *Nb*-infected mice markedly enhanced Con A-induced proliferation of naive spleen cells. This suggested that spleen cells from *Nb*-infected mice elaborated a factor (or factors), in response to Con A that enhanced proliferation. To investigate whether non-T cells were the source of this stimulatory factor, we generated supernatants from cultures of Con A-activated non-T cells, and assessed their ability to enhance proliferation of naive spleen cells in response to Con A. Figure 3 demonstrates that supernatants from stimulated non-T cells from *Nb*-infected mice effectively induced hyper-responsiveness to Con A.

In order to investigate the nature of the factor mediating this effect we analyzed the supernatants for cytokine content by ELISA. We found that levels of production of IFN-γ (Fig. 4A) were decreased in the cultures of Con A-stimulated spleen cells from *Nb*-infected mice. However, these same cultures showed a dramatic up-regulation of type 2 cytokine production, including IL-4 (Fig. 4B), IL-6 (Fig. 4C) and IL-10 (data not shown). This was not surprising since *Nb* infection is known to result in polyclonal activation of type 2 T cells (13,14,16). T cell depletion resulted in complete inhibition of IFN-γ (Fig. 4A) and IL-10 (data not shown) production suggesting that secretion of these cytokines in response to Con A is either mediated by, or dependent on, T cells. Secretion of IL-4 in response to Con A was T cell dependent to a large extent since T cell depletion resulted in approximately 83% inhibition of IL-4 production (Fig. 4B). In contrast, high levels of IL-6 were secreted by Con A-stimulated spleen cells from *Nb*-infected mice, even when T cell were depleted (Fig. 4C). This confirms that some (or most) of the IL-6 production in response to Con A was accessory cell-dependent.

To determine whether any of these cytokines could represent the factor responsible for the hyper-responsiveness seen above we tested recombinant murine IL-4, IL-6 and IL-10, at concentrations approximating those found in the supernatants, for their ability to mimic the effects of the "transferring" supernatant. Figure 5 confirms that IL-6, at a concentrations found in the stimulated culture supernatants of T cell-depleted spleen cells from *Nb*-infected mice, significantly enhances proliferation of naive spleen cells to Con A. In contrast, recombinant mouse IL-4 and IL-10 (data not shown) had no effect on Con A-induced proliferation. This strongly suggested that IL-6 was the accessory cell factor responsible for transferring the hyper-responsiveness to naive cells. To confirm

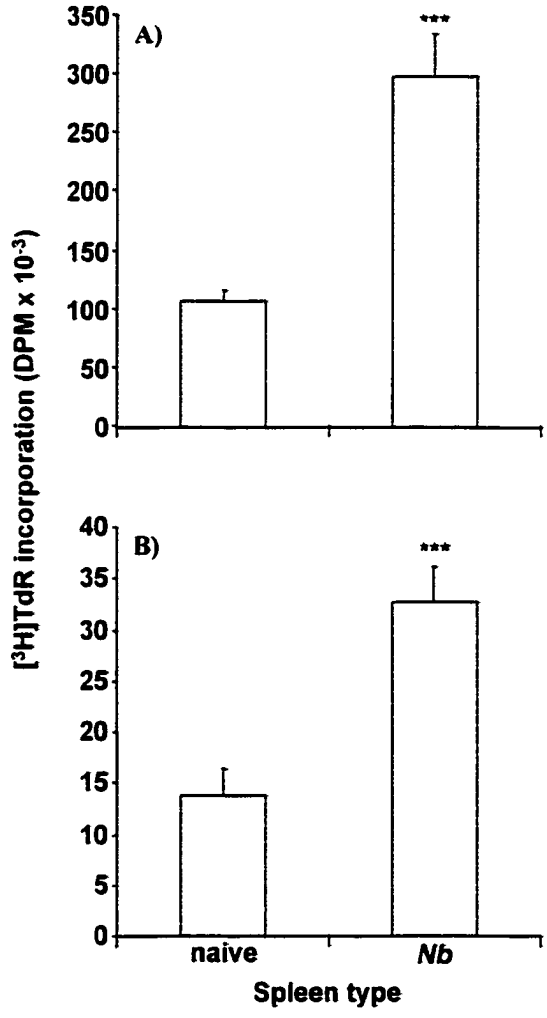
this, we inhibited this effect by treatment of the transferring supernatant with anti-IL-6 antibody (Fig. 6). When activated supernatants from uninfected mice were used, no enhanced proliferation was seen (as above) and this was not significantly effected by anti-IL-6 treatment; thus confirming the mAb had no non-specific inhibitory effects (Fig. 6). Moreover, as a further control, we confirmed that anti-IL-6 treatment completely abrogated the recombinant IL-6-mediated enhancement of spleen cell proliferation to Con A we demonstrated above, and that this blocking effect of antibody could be abolished by adding high levels of IL-6 (5 ng/ml) to the cultures (data not shown). Taken together these results confirm that IL-6, present in the supernatant, is the factor responsible for induction of proliferative hyper-responsiveness to Con A.

Infection with Nb results in decreased susceptibility of Con A-activated spleen T cells to activation induced cell death. *Nb*-induced proliferative hyper-responsiveness may be due either to an increase in the number of cells that are stimulated to enter the cell cycle, or to a reduction in the rate of activation induced cell death (AICD) of cells that have progressed through the cell cycle. A kinetic study of the Con A-induced proliferation demonstrated that naive spleen cells attain nearly maximal proliferation at 42 h. From that time on proliferation levels plateau out, exhibiting only a marginal increase over the next 48 h (Fig. 7A). In contrast, spleen T cells from *Nb*-infected mice, exhibited a dramatic increase in the rate of proliferation at each time point assessed, starting from 22 h until 90 h. Based on those results and the fact that IL-6 has been demonstrated to rescue T cells from undergoing apoptosis (38,39,40) we hypothesized that infection with *Nb* may lead to a decrease in susceptibility to AICD.

To test this hypothesis we activated spleen cells from control or *Nb*-infected mice with Con A and performed a JAM assay to assess apoptosis as a measure of AICD. As shown in figure 7B spleen cells from *Nb*-infected mice exhibited a significant (46%; n=10; $p < 0.001$) resistance to AICD as compared to cells from uninfected mice. Further, co-culture of naive spleen cells with mitomycin C-treated non-T-spleen cells from *Nb*-infected mice resulted in a significant increase in resistance to AICD after stimulation with Con A. Co-culture of naive spleen cells with non-T cells from uninfected mice did not show this effect (Fig. 7B). Since the hyper-proliferation could be transferred with supernatants we also assessed whether supernatants from Con A-stimulated spleen cells from *Nb*-infected mice would likewise reduce AICD. We found that supernatants from *Nb*-infected mice did confer resistance to AICD on naive spleen cells upon stimulation with Con A (Fig.8A). In support of our hypothesis that IL-6 in these supernatants is responsible for their effect we found that recombinant mouse IL-6 at levels matching those present in the supernatants was also able to induce resistance to AICD (Fig. 8A). Further, anti-IL-6 treatment abrogated both the supernatant (Fig. 8B), and recombinant IL-6 (from 39.8% to 5.0%; data not shown), induced resistance to AICD. These results suggest that *Nb*-induced hyper-responsiveness to Con A can be, in large part, attributed to decreased AICD after stimulation.

Fig. 1 ***Infection of mice with Nb results in enhance spleen cell proliferation in response to T cell mitogens.***

Proliferation of spleen cells from uninfected and *Nb*-infected mice in response to (A) Con A or (B) anti-CD3 mAb was assessed by ^{3}H-Thymidine incorporation. Splenocytes were isolated from mice eleven days after infection and stimulated with Con A (5 μg/ml) or anti-CD3 mAb (1:20 hybridoma supernatant) for 72 h before pulsing with ^{3}H-Thymidine. Data shown in (A) are expressed as mean ± SEM of 6 separate experiments (*** $p < 0.001$, two-tailed, paired Student's T-test). Data shown in (B) are expressed as mean ± SD of triplicate wells and are representative of 4 experiments (*** $p < 0.001$, two-tailed, paired Student's T-test).


A)
B)
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300
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200
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100
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40
35
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25
20
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5
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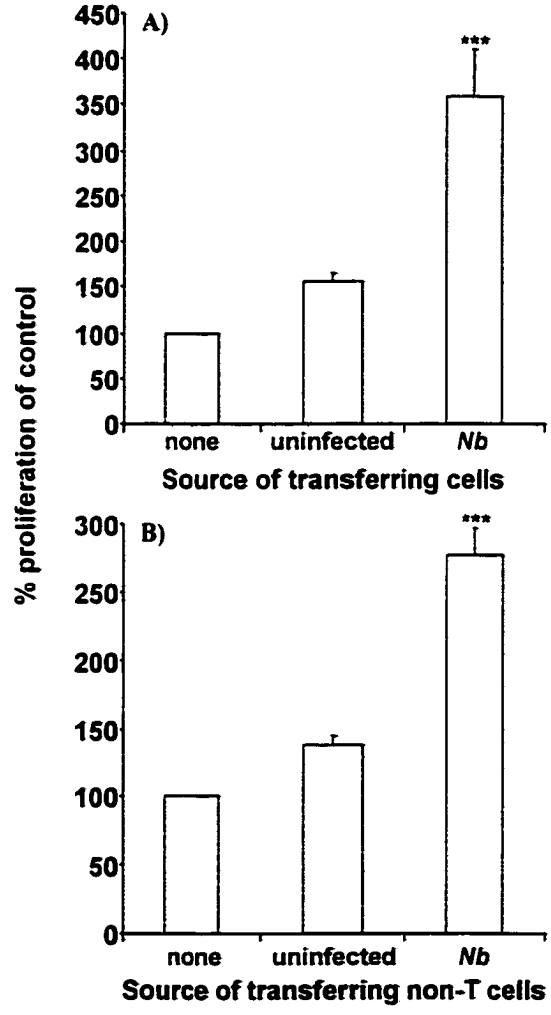
[³H]TdR incorporation (DPM x 10⁻³)
naive
Nb
Spleen type

Figure 1

Fig. 2 ***Naive spleen cells exhibit enhanced proliferation to Con A upon co-culture with spleen cells and T cell-depleted spleen cells from Nb-infected mice.***

The ability of cells from *Nb*-infected mice to transfer enhanced proliferation to Con A onto naive spleen cells was assessed by co-culture experiments. Naive spleen cells were activated with Con A in the absence of any transferring cells (none), or in the presence of mitomycin C-inactivated "transferring" cells from either *Nb*-infected (*Nb*) or uninfected (uninfected) mice. Mitomycin C-inactivated "transferring" cells were either spleen cells (A) or T cell-depleted spleen cells (B). All data are expressed as a percentage of the proliferation exhibited by naive (control) spleen cells (i.e. in the absence of transferring cells). Data shown in (A) are expressed as mean ± SEM of 9 separate experiments (*** $p < 0.001$, repeated measures ANOVA). Data shown in (B) are expressed as mean ± SEM of 12 separate experiments (*** $p < 0.001$, repeated measures ANOVA).


A)
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B)
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***
none
uninfected
Nb
Source of transferring non-T cells

Figure 2

Fig. 3 ***Supernatants of spleen cells or T cell-depleted spleen cells from Nb-infected mice enhance proliferation of naive spleen cells to Con A.***

Naive spleen cells were activated with Con A in the presence of supernatants derived from Con A-activated spleen cells from *Nb*-infected (*Nb*) or uninfected mice. Naive spleen cells were also activated in the presence of supernatants derived from Con A-activated, T cell-depleted spleen cells (uninfected non-T, *Nb* non-T). All supernatants were tested at a final culture concentration of 1:4. All data are expressed as a percentage of proliferation exhibited by naive spleen cells in the absence of any supernatant. Data are expressed as mean ± SEM of 5 (for whole spleen cell supernatants) and 9 (for non-T cell supernatants) separate experiments (*** $p < 0.001$, repeated measures ANOVA).

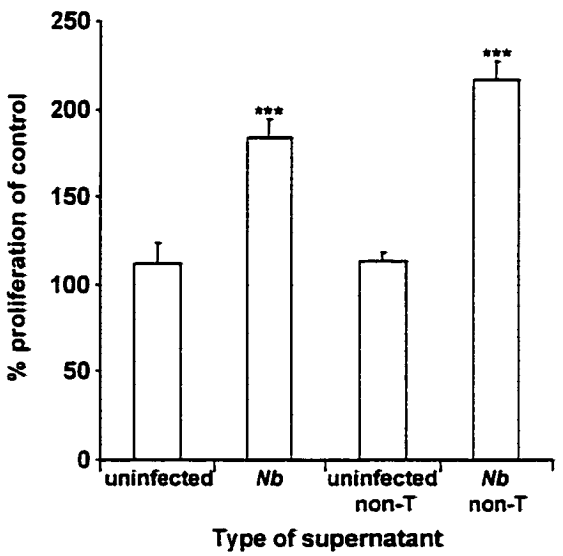
250
200
150
100
50
0
% proliferation of control
***
***
uninfected
Nb
uninfected non-T
Nb non-T
Type of supernatant

Figure 3

Fig. 4 ***Con A-stimulated spleen cells from Nb-infected mice produce enhanced levels of IL-4 and IL-6 but reduced levels of IFN-γ.***

Production of cytokines by spleen cells, or mitomycin C-treated T cell-depleted spleen cells, from uninfected or *Nb*-infected mice was assessed by ELISA. Cells were activated with Con A for 24h. Supernatants were analyzed for the presence of (A) IFN-γ, (B) IL-4 and (C) IL-6. Data shown are expressed as mean ± SEM of 4 (IFN-γ) or 9 (IL-4 and IL-6) experiments (*** $p < 0.001$, repeated measures ANOVA).

ND = not detectable.

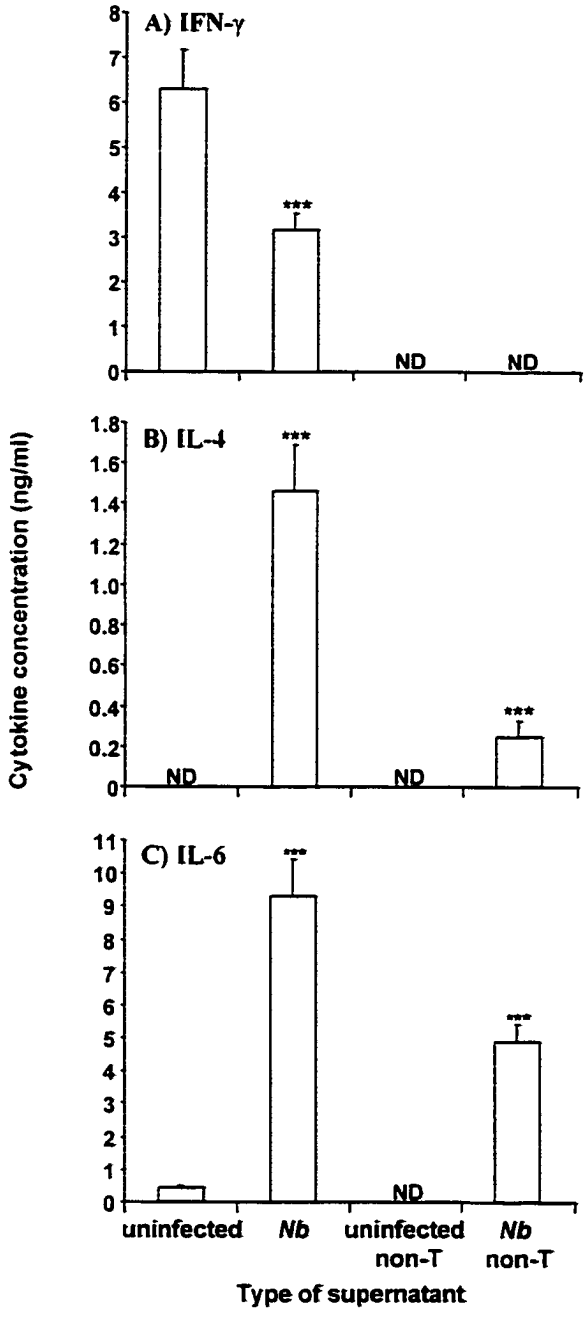
A) IFN-γ
B) IL-4
C) IL-6
Cytokine concentration (ng/ml)
***
ND
uninfected
Nb
uninfected non-T
Nb non-T
Type of supernatant

Figure 4

Fig. 5 ***Mouse recombinant IL-6 enhances spleen cell proliferation to Con A.***

Naive spleen cells were activated with Con A in the absence or presence mouse recombinant IL-6 at varying concentrations. All data are expressed as a percentage of proliferation exhibited by naive spleen cells in the absence of recombinant IL-6. Data are expressed as mean ± SEM of 3 (for 0.31 ng/ml of IL-6) or 8 (all other groups) separate experiments (*** $p < 0.001$, one-way ANOVA).

[a] 1.25 ng/ml is the average concentration of IL-6 in the test proliferation cultures containing 1:4 *Nb* non-T supernatant.

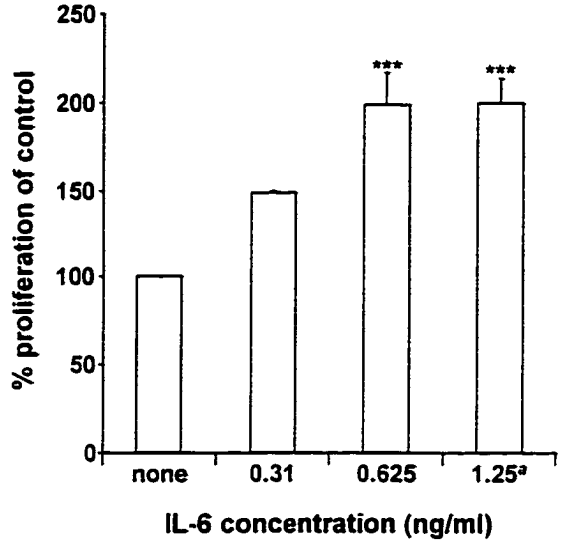
250
200
150
100
50
0
% proliferation of control
***
***
none
0.31
0.625
1.25[a]
IL-6 concentration (ng/ml)

Figure 5

Fig. 6 ***Anti-IL-6 treatment abrogates supernatant-induced hyper-responsiveness to Con A.***

Naive spleen cells were activated with Con A in the presence of a supernatant derived from Con A-stimulated T cell-depleted spleen cell cultures from *Nb*-infected (*Nb* non-T) or uninfected (uninfected non-T) mice (1:4 final concentration). Cultures received 10 ng/ml (▤), 25 ng/ml (□) or 50 ng/ml (■) anti-IL-6 antibody or no antibody (■). Data shown in are expressed as mean ± SEM of 3 separate experiments (*** $p < 0.001$, NS = not significant, repeated measures ANOVA).

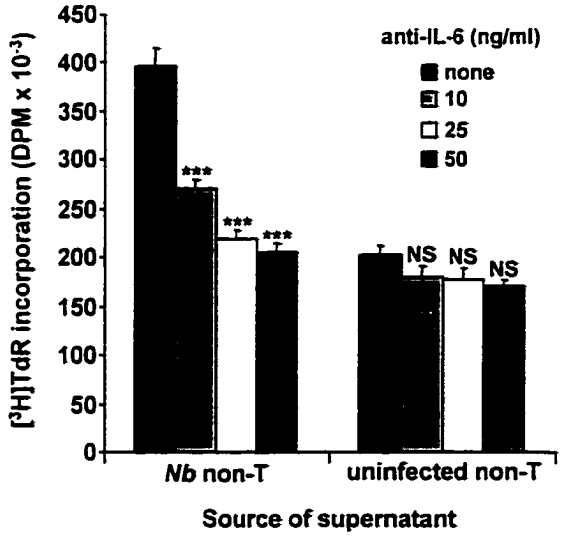
450
400
350
300
250
200
150
100
50
0
[³H]TdR incorporation (DPM x 10⁻³)
anti-IL-6 (ng/ml)
none
10
25
50
***
***
***
NS
NS
NS
Nb non-T
uninfected non-T
Source of supernatant

Figure 6

Fig. 7 ***Increased proliferation induced by Nb infection is associated with resistance to AICD, which can be transferred with non-T cells in vitro.***

In (A) Con A-induced proliferation of spleen cells from uninfected (■) and *Nb*-infected (□) mice was assessed by [^{3}H]TdR incorporation at various time points. [^{3}H]TdR was added 18h prior to termination of cultures. Data shown are expressed as mean ± SEM of 3 separate experiments (** $p < 0.01$, *** $p < 0.001$, NS = not significant, repeated measures ANOVA). In (B) the extent of AICD was assessed in spleen cell cultures from naive and *Nb*-infected mice as well as in co-cultures of naive spleen cells with non-T spleen cells from either *Nb*-infected or uninfected mice. AICD was assessed using the JAM test and % resistance in AICD relative to the AICD exhibited by naive spleen cells was determined as described in materials and methods. Data are expressed as mean ± SEM of 5-6 separate experiments (*** $p < 0.001$, one-way ANOVA).

[a] ND = not different from AICD exhibited by naive cells in the absence of any transferring cells.

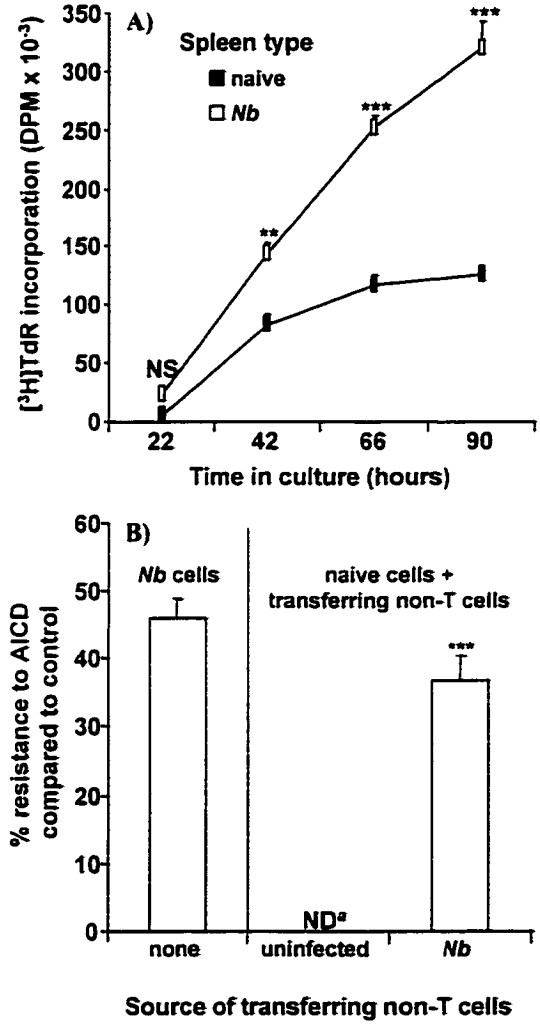
A)
Spleen type
naive
Nb
[³H]TdR incorporation (DPM x 10⁻³)
350
300
250
200
150
100
50
0
NS
**
***
***
22
42
66
90
Time in culture (hours)
B)
Nb cells
naive cells + transferring non-T cells
% resistance to AICD compared to control
60
50
40
30
20
10
0
***
ND[a]
none
uninfected
Nb
Source of transferring non-T cells

Figure 7

Fig. 8 ***Nb-induced resistance to apoptosis can be transferred with non-T cell supernatants and is mediated by IL-6.***

In (A) the extent of AICD was assessed in spleen cell cultures from naive mice stimulated in the absence or presence of supernatants derived from cultures of Con A-activated non-T cell from *Nb*-infected (*Nb* non-T sup) or uninfected (uninfected non-T sup) mice or recombinant IL-6. Data shown are expressed as mean ± SEM of 3 separate experiments (*** $p < 0.001$, repeated measures ANOVA). In (B) AICD was assessed on naive spleen cells that were activated with Con A in the absence or presence of a supernatant derived from Con A-activated T cell-depleted spleen cell cultures from *Nb*-infected (*Nb* non-T) or uninfected (uninfected non-T) mice (1:4 final concentration). In addition cultures received 50 ng/ml (■) anti-IL-6 antibody or no antibody (□). Data shown are expressed as mean ± SEM of 3 separate experiments (*** $p < 0.001$, NS = not significant, repeated measures ANOVA). In each case AICD was assessed using the JAM test and % resistance in AICD relative to the AICD exhibited by naive spleen cells was determined as described in materials and methods.

[a] 1.25 ng/ml is the average concentration of IL-6 in the test proliferation cultures containing 1:4 *Nb* non-T supernatant.

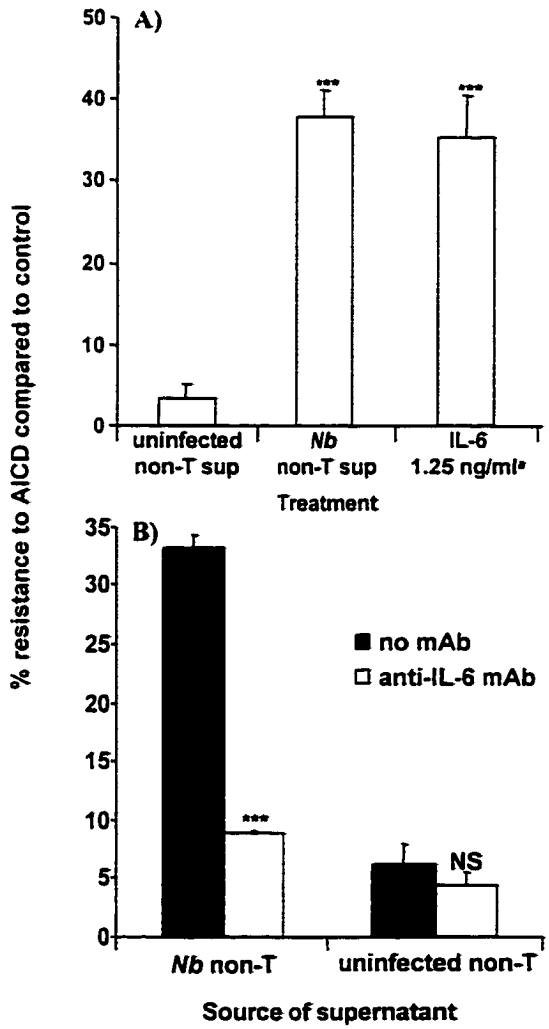
A)
50
40
30
20
10
0
***
***
uninfected non-T sup
Nb non-T sup
IL-6 1.25 ng/ml
Treatment
% resistance to AICD compared to control
B)
35
30
25
20
15
10
5
0
no mAb
anti-IL-6 mAb
***
NS
Nb non-T
uninfected non-T
Source of supernatant

Figure 8

Discussion

Nematode infection induces a marked type 2 T cell response (1,2,3), and considerable evidence points to this response being polyclonal in nature (12,13,14,15,16). Previously, we have shown that nematode infection (Liwski, R. S., *et al.* submitted, 28) as well as extracts (41) exacerbate type 2 responses to unrelated antigens, suggesting that nematode infection can modify the development of immune responses to infection or vaccination. The manner in which this immune deviation is mediated is unclear. The results of this study show that spleen cells from *Nb*-infected animals exhibit dramatic hyper-proliferation upon *in vitro* stimulation with the T cell activators Con A and anti-CD3 mAb. This modification of T cell-responsiveness could be transferred to naive spleen cells, *in vitro*, by co-incubation with spleen cells from *Nb*-infected mice. Interestingly, non-T-accessory cells from *Nb*-infected mice were sufficient in order to transfer this effect, suggesting that *Nb* infection induced an enhancement of co-stimulatory function in the accessory cell population.

Further to this, we demonstrated that a soluble factor, present in the supernatant of stimulated non-T cells from *Nb*-infected mice, could confer this hyper-responsiveness on naive T cells when added to naive T cell cultures along with Con A. Given the evidence in the literature regarding the enhancing effects of IL-6 on T cell proliferation (36,37,38), and our data, we hypothesized that the soluble factor could be IL-6. We found, for example, that IL-6 was secreted in response to Con A by the non-T cells from *Nb*-infected mice. In addition, mouse recombinant IL-6, at a concentration matching that found in the transferring supernatant, increased Con A-induced naive spleen cell proliferation to the same extent as the supernatant. We tested this hypothesis using a

monoclonal antibody to IL-6 to block IL-6 activity in the supernatant. Anti-IL-6 mAb treatment completely abrogated the supernatant-induced hyper-responsiveness, confirming that IL-6 was responsible for the effect. This anti-IL-6 mAb had an identical effect on the hyper-responsiveness induced by recombinant IL-6. The efficiency of transfer of hyper-proliferation to naive spleen cells was very similar for co-culture with non-T cells from *Nb*-infected mice (100% increase as compared with non-T cells from uninfected mice), non-T cell supernatants (92% increase as compared with uninfected non-T cell supernatants) and recombinant IL-6 (100% increase as compared with media control) suggesting that IL-6 elaboration by these accessory cells is the sole cause of this effect.

It is unclear at this point whether IL-6 mediates its effects by direct action on T cells or indirectly through accessory cells. Our recent experimentation (Liwski, R. S., and T. D. G. Lee, manuscript in preparation) has revealed that B7.2 is over-expressed on non-T cells from *Nb*-infected mice following activation with Con A. Increased availability of B7.2-CD28 interaction can in some cases lead to increased proliferative T cell activity (31,33). Whether induction of B7.2 expression was mediated through IL-6 was not addressed. However, this over-expression of B7.2 may partially contribute to *Nb*-induced hyper-responsiveness.

It is also possible that IL-6 exerts its effects through downregulation of IFN-γ activity. It has been demonstrated that IFN-γ is involved in down-regulation of proliferative T cell responses (42,43) possibly by induction of apoptosis through upregulation of Fas-L expression (44). Further, IFN-γ production from mitogen-activated T cells can be dramatically reduced by IL-6 (23). Our data clearly showed that spleen

cells from *Nb*-infected mice produced significantly lower levels of IFN-γ in response to Con A. However, addition of high levels of rIFN-γ did not diminish proliferation of naive spleen cells to Con A, blockage of IFN-γ activity by antibody treatment did not result in hyper-responsiveness, and co-incubation of naive spleen cells with non-T cells from *Nb*-infected mice did not decrease IFN-γ production in response to Con A (Liwski, R. S., and T. D. G. Lee, unpublished observations). Therefore, it is unlikely that IL-6 acted through downregulation of IFN-γ production to mediate hyper-responsiveness observed in our studies.

The type of accessory cell that is responsible for *Nb*-mediated hyper-responsiveness to Con A is unclear. Both macrophages (45,46) and B-cells (47) can become activated with Con A and contribute to hyper-responsiveness. Moreover, *Nb* infection leads to the appearance of an IgE receptor bearing non-B, non-T cell population in the spleen (48), which may also be able to become activated with Con A. Mitogen activation of these non-T cells is not required to induce hyper-responsiveness since spleen cells from *Nb*-infected mice show hyper-responsiveness to activation with anti-CD3. Moreover non-T cells from *Nb*-infected mice induce increased proliferation when used as stimulators in allogeneic mixed lymphocyte reaction (Liwski, R. S., and T. D. G. Lee, unpublished observations).

Our data suggest that the nematode reduces AICD in mitogen-activated cells. A kinetic study of the Con A-induced proliferation demonstrated that naive spleen cells attain nearly maximal proliferation at 42 h. From that time on thymidine uptake levels out, exhibiting only a marginal increase over the next 48 h. If all the cells that have gone through the cell cycle were capable of entering another cycle we would expect constantly

higher levels of proliferation at later time points. Since thymidine uptake by naive spleen cells was relatively constant, it suggests that a large proportion of the dividing T cells in the naive spleen cell population do not re-enter the cell cycle or are unable to complete the cycle. In contrast, spleen T cells from *Nb*-infected mice, exhibited a dramatic increase in the rate of proliferation at each time point assessed, starting from 22 h until 90 h. This progressive increase in thymidine uptake suggests that the majority of cells in spleen cell cultures from *Nb*-infected mice re-enter the cell cycle. Based on those results we hypothesized that infection with *Nb* leads to a decrease in susceptibility to AICD.

Assuming that all the cells that did not re-enter the cell cycle have died due to AICD, the estimated rate of AICD (based on data in Fig. 7a) between the 42h and 66h time points (assuming 24h-long cell cycle) was 56% for naive spleen cells and 25% for spleen cells from *Nb*-infected mice. Experimental assessment of AICD performed by the JAM assay showed that while 60.2% of naive spleen cells underwent AICD only 35.1% of spleen cells from *Nb*-infected mice died in that same 24 h period (46% reduction of AICD). These data were very similar to the estimation of AICD derived from figure 7A, suggesting that enhanced proliferation could be explained by reduction in AICD. Furthermore, the enhanced survival could be transferred in the same manner as the transfer of hyper-responsiveness as judged by proliferation. In addition, resistance to AICD conferred by supernatants could be blocked by anti-IL-6 treatment. Taken together these results confirm that the hyper-responsiveness induced by *Nb* is due to IL-6 mediation of resistance to AICD.

Our results are in agreement with the studies of Takeda *et al.* (38) who showed that IL-6 increased proliferation of T cells in response to both Con A and anti-CD3 by

inhibiting AICD through a Bcl-2-independent mechanism. It has been suggested that IL-6-induced inhibition of AICD may be mediated by down-regulation of Fas and Fas L expression on T cells (49). Since Fas L-mediated apoptosis plays a crucial role in the regulation of the survival of activated T cells *in vivo* (reviewed in 50 and 51), this link with IL-6 indicates that modulation of local IL-6 levels by nematodes could have profound effects on developing immune responses through mediating enhanced survival of activated T cells.

In this context, it is of interest that superantigen-activated T cells can be rescued from undergoing apoptosis by injection of LPS (52,53). This has been shown to be mediated through upregulation of pro-inflammatory cytokines (52,53). Similar effects were observed during infection with vaccinia virus (54). These results suggest that inflammatory reactions induced by microbial infection profoundly modulate development of other T cell-mediated responses. Interestingly, Rocken *et al.* (13,14) demonstrated that infection with *Nb* also reactivates superantigen-specific T cell in mice, previously tolerized with superantigen, and induces type 2 T cell responses towards the superantigen.

A recent report by Zhang *et al.* (55) showed that T cells activated *in vitro* in a type 2 polarizing environment were highly resistant to AICD. Similar resistance to AICD was observed in Th2 but not Th1 clones (56). Others (57) have suggested that this preferential susceptibility of Th1 cells to undergo AICD may explain the development of non-protective type 2 responses during HIV infection. Interestingly, Ranger *et al.* demonstrated that mice lacking both NFATp and NFAT4 exhibit a dramatic increase in type 2 responses (58). This has been shown to be associated with hyper-proliferation and

resistance to AICD of T cells stimulated with anti-CD3 antibodies (58), thus suggesting that uncontrolled overproduction of type 2 cytokines can lead to similar dramatic alterations in T cell responsiveness, as we have seen with *Nb* infection.

Our studies demonstrate that *in vivo* activation of type 2 T cells during *Nb*-infection results in resistance to AICD. We have identified the factor, IL-6, that we believe is responsible for this enhanced T cell survival and subsequent hyper-proliferation. Our studies suggest that modulation of IL-6 levels due to concurrent nematode infection could dramatically alter T cell-mediated responses to infections and/or vaccination in endemic areas.

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Footnotes:

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[2]Address correspondence for proofs to Dr. Timothy D. G. Lee, Department of Microbiology and Immunology, Transplantation and Immunology Research Laboratory, Sir Charles Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7. E-mail address: **Tim.Lee@dal.ca**

[3]Abbreviations used in this paper: AICD, activation induced cell death; *Nb. Nippostrongylus brasiliensis*;

5.0 Discussion

5.1 Modulation of allo-specific T cell responses by *N. brasiliensis*.

5.1.1 Effects of *N. brasiliensis* infection on allograft survival in rats.

Our laboratory has shown previously that infection of rats with *N. brasiliensis* four days prior to transplantation with fully allogeneic kidneys leads to prolonged survival of these grafts from 9 days in uninfected to 32 days in the *Nb*-infected animals (Ledingham *et al.* 1996). This suggested that generation of effector allo-specific immunity was either suppressed or altered in the presence of the nematode. Since *Nb* is known to induce marked polyclonal type 2 T cell activation, and to generate powerful type 2 effector mechanisms including mastocytosis, blood eosinophilia and IgE production (Urban *et al.* 1992), it is unlikely that *Nb* promotes allograft survival through a general suppression of T cell activation. Rather, it is likely that the allo-specific effector response that develops in the presence of the nematode infection is phenotypically different from the response that occurs in the absence of nematode infection.

Given the observations that *Nb* enhances type 2 responses, a possible mechanism for this enhanced allograft survival is by the induction of allo-specific type 2 cells. A shift from type 1 to type 2 T cell immunity has been associated with delayed allograft rejection (Takeuchi *et al.* 1992, Ferraresso *et al.* 1994, Takeuchi *et al.* 1992, Siegling *et al.* 1994, Mottram *et al.* 1995, Binder *et al.* 1996, Sayegh *et al.* 1995, Gorczynski 1995). Previous studies from our laboratory have supported such an explanation in that an increase in IL-4 mRNA has been demonstrated in the immune cells that infiltrated allogeneic kidneys in rats treated with *Nb* (Ledingham *et al.* 1996).

5.1.2 Prolongation of allograft survival by *N. brasiliensis* in mice.

The work described above was performed in a rat allo-transplantation model. However, much of the advancement in immunology has come from experimental work in murine systems. Detailed characterization of various aspects of the immune response has been facilitated by development of inbred strains of mice, including gene-knockout and transgenic mice, as well as monoclonal antibodies and recombinant cytokines. The majority of the experimental work investigating the mechanisms of graft rejection, or host responses to helminth parasites has been also performed in mice. Therefore, this research has adopted the murine model to assess the effects of *Nb* infection on the development of allo-specific T cell responses.

The first study was designed to confirm that *Nb* prolonged allograft survival in mice, as it did in the rat model. Since the life cycle of *Nb* is similar in rats and mice (Ogilvie and Jones 1971, Urban *et al.* 1992), a similar inoculation protocol was used such that C57BL/6 mice were inoculated with the nematode four days prior to transplantation with fully allogeneic hearts from allogeneic BALB/c donors. This treatment was chosen based on the fact that infection of mice with another nematode, *Trichinella spiralis*, led to prolongation of cardiac allograft survival only when the grafts were placed in the recipient during the stage at which the parasite resides in the gut (Ljungstrom and Huldt 1977). *Nb* larvae are present in the gut starting on day 4 of infection (Urban *et al.* 1992), thus transplantation at that time would ensure full exposure to the modulatory effects associated with the gut stage. The choice of injection of 800 larvae/male mouse in these experiments was based on the fact that in most studies researchers use smaller (female) mice and routinely inject them with 700-750 larvae. This dose is far from lethal since

researchers often inject male mice with 900-1000 larvae (Lawrence *et al.* 1996, Conrad *et al.* 1990, Seder *et al.* 1991). The data in this thesis shows that nematode infection resulted in a significant ($p < 0.03$) prolongation of allograft survival in mice. However, this enhanced survival was not as pronounced as it was in the rat model. While all of the infected rats exhibited prolonged graft survival, three of nine mice treated with *Nb* rejected grafts normally.

There are a number of possible explanations for this discrepancy. First, *Nb* is a natural parasite of rats (Haley 1961). In the rat, *Nb* exhibits 70-80% efficiency of infection (the number of larvae that are able to mature to the adult stage; Ogilvie and Jones 1971) while in a mouse this efficiency drops to approximately 35% (Wescott and Todd 1966). The ability to establish a more efficient infection may have a greater effect on the immunity of the host. Second, allogeneic kidney grafts seem to be less immunogenic than cardiac allografts. Zhang and co-workers, for example, have demonstrated that while all allogeneic hearts are rejected relatively quickly in mice (surviving 8-20 days), some (20-50%) kidney allografts are retained spontaneously in certain strain combinations without any immunosuppression (Zhang *et al.* 1996). Third, it has been demonstrated in a variety of transplantation models using various anti-rejection treatments that the Brown Norway to Lewis strain combination, that was used in the experimentation described above, is particularly susceptible to tolerance induction (Murase *et al.* 1990, Murase *et al.* 1993). Fourth, in most transplant models, including the one used in the rat experiments above, the second native kidney is removed a few days after transplantation and the survival of the recipient is used to assess graft survival. In the absence of the native kidney, graft rejection episodes, which compromise kidney

function lead to the accumulation of toxic molecules in the circulation. This may have effects on the immune system and may slow down the rejection process. Consistent with this hypothesis Grau and co-workers (Grau *et al.* 1997) have demonstrated that allogeneic kidneys from hosts in which the second native kidney was not removed exhibited earlier and more pronounced signs of acute rejection. There are, however, a number of possible interpretations of this data only one of which associates it with immune function.

Nonetheless, our results clearly show that *Nb* infection prolongs allograft survival in mice, and that the effects of the nematode are not tissue- or species-specific. It is important to note that *Nb* infection is self-limiting in rodents with the parasite present for only approximately 7 days after transplantation (day 11 after infection) when they are vigorously expelled from the host (Urban *et al.* 1995). Graft prolongation thus outlasts the presence of the nematodes, indicating a role for immune-modulation during initial antigen perception. To address the mechanism of nematode-induced graft prolongation we explored the effects of *Nb* infection on the development of allo-reactivity in the recipient C57BL/6 mouse.

5.1.3 Development of allo-immunization model.

In order to investigate the effects of the nematode on the generation of graft-specific effector mechanisms one could assess the proliferative and CTL activities as well as cytokine production by graft infiltrating cells activated *ex vivo* with alloantigen. While physiologically relevant, this would be technically difficult and prone to artifact considering the small numbers of GIC that can be recovered from murine cardiac allografts. Further, since the immunity towards grafts could be, in large part, directed

towards tissue-specific antigens, assessment of recall responses of GIC using allogeneic spleen stimulator cells may not yield results that are representative of the total GIC population. Others have shown, for example, that CTL generated against allogeneic renal epithelial cell lines efficiently lysed these cell targets but failed to kill Con A-activated allogeneic spleen cell blasts which are commonly used in CTL assays (Hadley *et al.* 1996). *In vivo* evidence showing that tolerance induced to certain tissues may not prevent rejection of other tissue types from the same donor strain (Sirak *et al.* 1997), suggests that matching of 1^{o} stimulus and 2^{o} stimulus is required for *in vitro* assays. Indeed, most of the splenic CTL precursors that can be activated with allogeneic spleen stimulators *in vitro* do not become activated *in vivo* following cardiac transplantation (Bishop *et al.* 1993, Bishop *et al.* 1994).

To avoid these potential problems, and to investigate the immunomodulatory effects of *Nb* infection on the development of T cell responses to alloantigen, a model was developed in which C57BL/6 mice were immunized with allogeneic mitomycin C-inactivated BALB/c spleen cells by injection into peritoneal cavity. The allo-reactive response to similar allogeneic spleen cells was later measured *in vitro*. This model was selected because intraperitoneal injection has been previously shown to stimulate powerful antigen-specific proliferative and cytokine responses followed by the generation of memory cells in the spleen (Yip *et al.* 1999). We determined recall responses of splenic T cells seven days after immunization using mitomycin C-treated donor spleen non-T cells as stimulators for assessment of *in vitro* allo-specific proliferation and cytokine production. T cells were eliminated from the *in vitro* allogeneic stimulator cell population because mitomycin C treatment of cells blocks proliferation but not cytokine

responses (data not shown). Without such T cell elimination, a 2-way MLR would result, at the cytokine production level, which would interfere with the analysis of the function of responder T cells.

This experimentation revealed that immunization led to the development of potent allo-specific T cell responses. T cell proliferation in the MLR from allo-immunized mice (2^o MLR) was more than 3-fold higher than that seen with naïve T cell responders, suggesting that *in vivo* expansion of allo-reactive cells occurred. This difference in proliferation between T cells from naïve and allo-immunized mice was easier to discern at a 4:1 effector to stimulator ratio (3.3-fold difference) than at a 1:1 ratio (1.5-fold difference; Fig. 1; section 7.0). In these kinds of experiments, it is important to choose the culture conditions which best reflect the effects of immunization on the expansion of allo-specific T cells. A number of studies have shown, for example, that proliferation of T cells in the MLR is not altered by tolerizing treatments applied during antigen exposure *in vivo*. However, in many of these studies researchers failed to optimize the conditions, and were not able to detect differences in proliferation between T cells from immunized (or transplanted) animals and naïve animals (Pearce *et al.* 1989, Qin *et al.* 1994, Dahmen *et al.* 1994, Zeng *et al.* 1996). The results obtained from such models are uninterpretable. Thus, we chose to perform MLR at 4:1 ratio for all the subsequent studies investigating the effects of *Nb* on recall proliferation in 2^o MLR.

5.1.4 Effects of allo-immunization on allo-specific cytokine production.

In this experimentation, IFN-γ production, while undetectable in the MLR cultures using naïve responder T cells, was increased dramatically in the cultures using T

cells from allo-immunized mice. This was expected, since the frequency of IFN-γ producing cells increases slowly with successive cell cycles (Bird *et al.* 1998). Therefore, a very small proportion of activated T cells from naïve mice would be found to secrete this cytokine within 48h of activation in culture. In contrast, T cells from allo-immunized mice presumably have progressed through a number of division cycles *in vivo*, thus acquiring the ability to produce IFN-γ, which was observed upon activation with alloantigen *in vitro*. The production of IFN-γ but not IL-4 or IL-6 in the MLR, confirmed that allo-immunization initiates type 1 allo-specific T cell development.

5.1.5 Effects of *Nb* on T cell activation by allo-immunization.

In vitro comparison of allo-specific responses mediated by T cells from *Nb*-infected allo-immunized and control allo-immunized mice demonstrated that *Nb* does not induce non-specific immunosuppression to alloantigen. Allo-specific proliferation in the MLR by T cells from *Nb*-infected allo-immunized mice was not decreased compared to the uninfected allo-immunized controls. In fact, although mean proliferation data obtained from eleven experiments showed no significant differences between infected and uninfected mice, in eight of those eleven experiments allo-specific proliferation was increased in T cells from *Nb*-infected allo-immunized mice as compared with control allo-immunized animals. The difference is more pronounced if expressed per T cell, since the data showed that the percentage of $CD4^+$ T cells is significantly reduced in enriched T cell populations from *Nb*-infected mice as compared to controls. Therefore, compensating for the reduction in the numbers of T cells available to proliferate further

increases the observed effect. Whether this trend is of significance is highly speculative but *Nb* infection may result in enhanced proliferation to alloantigen.

If there is enhanced proliferation to alloantigen, it may result from an *in vitro* or *in vivo* expansion of allo-specific T cells. Spleen cells from *Nb*-infected mice hyper-proliferate when stimulated with Con A or anti-CD3 mAb. Hyper-responsiveness to Con A was demonstrated in this thesis to be due to increased IL-6 expression by non-T cells, which induced resistance to AICD of activated T cells. Indeed, we have observed that stimulators derived from *Nb*-infected BALB/c mice were more effective in inducing proliferation from responder C57BL/6 T cells (data not shown). Therefore, large amounts of IL-6 produced in the MLR cultures of T cells from *Nb*-infected, allo-immunized mice may explain the tendency to hyper-proliferation exhibited by allo-specific T cells from *Nb*-infected animals. Finally, allo-specific T cells from *Nb*-infected mice that have <u>not</u> been allo-immunized, and presumably have <u>not</u> expanded *in vivo*, proliferated better in the MLR as compared to T cells from uninfected non-immunized mice suggesting that *Nb* enhanced the *in vitro* and not the *in vivo* expansion of allo-specific T cells (Fig. 2; section 7.0). However, *Nb* infection results in T cell activation and therefore some of the T cells that are activated *in vivo* by nematode antigens could be cross-reactive with alloantigen. Alternatively, polyclonal activators associated with *Nb* infection could activate cells, which react with alloantigen.

The enhanced proliferation of T cells from *Nb*-infected allo-immunized mice, however, was not solely due to non-specific effects induced by *Nb*. The data confirm that allo-specific *in vitro* proliferation of T cells from *Nb*-infected mice was markedly increased if the animals were exposed, *in vivo*, to alloantigen (Fig. 2; section 7.0).

5.1.6 Central hypothesis: type 2 shift as a mechanism of *Nb*-induced prolongation of allograft survival.

The hypothesis that this thesis is based on is that prolongation of allograft survival by *Nb* is due to the nematode's ability to stimulate powerful type 2 allo-immunity, which would interfere with type 1 allo-responses important in graft rejection. Data in this thesis support this hypothesis. For example, analysis of cytokine production in the MLR by T cells from allo-immunized mice that were infected with *Nb* clearly showed that type 2 cytokines (IL-4 and IL-6) were elaborated by these cells, but not the cells from uninfected allo-immunized controls. In addition, the ability to produce IFN-γ in response to alloantigen *in vitro* was greatly inhibited (65-90%) by the nematode infection. The relatively low levels of IL-4 detected in the supernatants of the T cell cultures from *Nb*-infected allo-immunized animals may reflect the consumption of IL-4 by proliferating allo-specific T cells. In support of this, is the experimentation showing that the addition of anti-IL-4 antibody decreased allo-specific proliferation of T cells from *Nb*-infected allo-immunized mice by approximately 40%. These results indicate that IL-4 secreted by allo-specific T cells contributed to the proliferation in the MLR and that the perception of alloantigen during the nematode infection leads to the development of type 2 allo-specific immunity.

Interestingly, IL-4 and IL-6 were secreted in response to alloantigen by T cells from both *Nb*-infected, allo-immunized, and *Nb*-infected, unimmunized mice, although allo-immunization significantly augmented production of these cytokines. In addition, T cells from infected, allo-immunized mice secreted IL-4 and IL-6 in response BALB/c and 3rd-party C3H/HeJ alloantigen, although again donor-type alloantigen stimulated

significantly higher levels of these cytokines. These results, taken together with the fact that T cells from uninfected allo-immunized mice did not produce any IFN-γ in response to C3H/HeJ stimulators (confirming allo-specificity of the response in the absence of the infection), suggest that some activation of allo-reactive T cells occurs during *Nb* infection in the absence of alloantigen. This is in agreement with the studies by Street *et al.*, who demonstrated that infection with *Nb* causes some T cells to develop into type 2 allo-specific clones *in vitro* (Street *et al.* 1990).

Since anti-IL-4 treatment did not completely block the proliferation of T cells from *Nb*-infected allo-immunized animals in response to alloantigen, it is reasonable to suppose that the remaining T cells were of the type 1 phenotype. This is consistent with our observation that IFN-γ production is reduced (by 65-90%), but not ablated, in allo-stimulated T cell cultures from *Nb*-infected allo-immunized mice. Taken together, these results suggest that both type 2 and type 1 allo-specific T cells develop in the presence of *Nb*. The development of both type 1 and type 2 allo-specific immunity might be expected since it has been shown that production of type 2 cytokines in the spleen of infected C57BL/6 mice is weak at day 5 post infection but attains high levels at day 7 post infection (Lawrence *et al.* 1996). These results were confirmed at the level of IgE transcription, which although apparent at day 5 post infection reaches peak levels in the spleen only seven days after infection (Moravetz *et al.* 1996). In our model, mice are exposed to alloantigen 4 days after infection with *Nb*, leaving approximately 24 hours in which type 1 cytokine activation could result in response to allo-challenge. *Nb* may thus cause increased allograft survival by attenuation of this developing type 1 allo-reactivity, through the emergence of a vigorous type 2 allo-specific response, rather than by

substitution of a type 1 with a type 2 response. This finding is consistent with our previous observations in the rat model showing the presence of both IL-4 and IFN-γ mRNA transcription by GIC in animals transplanted with allogeneic kidneys that were infected with *Nb* (Ledingham *et al.* 1996) or were orally tolerized (Carr *et al.* 1998) prior to transplantation.

5.1.7 Role of IFN-γ.

Given the evidence provided in section 2.12 regarding the complex roles of IFN-γ in graft rejection and tolerance, the secretion of some IFN-γ by allo-specific T-cells from *Nb*-infected allo-immunized mice as well as the IFN-γ elaborated in the kidney transplants in rats infected with the nematode (Ledingham *et al.* 1996) may have a tolerizing, rather than allo-stimulatory effect, mediated through the control of expansion of allo-specific T cells. Type 2 T cells express IFN-γ receptor (Bach *et al.* 1995, Pernis *et al.* 1995) and are susceptible to IFN-γ-induced inhibition of proliferation (Gajewski and Fitch 1988). Thus it is possible that the *in vitro* treatment with anti-IL-4 mAb that led to a partial inhibition of allo-specific proliferation in the MLR of T cells from *Nb*-infected allo-immunized mice was mediated by inhibition of IL-4-induced repression of IFN-γ production.

An alternative possibility for *Nb*-induced allograft survival is that the remaining allo-specific type 1 T cells, generated in *Nb*-infected mice, retain the ability to produce IFN-γ but that *Nb* interferes with the ability of various cells to respond to IFN-γ signaling by an IL-4-dependent mechanisms, thus attenuating development of effector functions necessary for graft destruction and preventing IFN-γ-mediated inhibition of Th2

activities. Inhibition of IFN-γ signaling could be mediated either through downregulation of the IFN-γ receptor or downstream blocking of molecules involved in the signal transduction pathway that leads to the expression of IFN-γ inducible genes. It is unlikely that *Nb* infection results in a downregulation of the expression of the IFN-γ receptor, since Th2 cells have been shown to express this receptor (Bach *et al.* 1995, Pernis *et al.* 1995). However, it has been demonstrated recently that IL-4 can suppress IFN-γ – induced expression of the IRF-1 gene (Ohmori *et al.* 1997), which is necessary for the induction of type 1 gene expression including IL-12 (Ma *et al.* 1996). This was mediated through STAT6 binding to the SBE site in the IRF-1 promoter region normally bound by STAT1 (Ohmori *et al.* 1997). Thus, STAT6 can physically out-compete binding of IFN-γ-activated STAT1 to IRF-1 promoter and render the cell unresponsive to signaling through the IFN-γ receptor. In addition, others have shown that IL-4 blocks the production of IL-12 and TNF-α from LPS activated macrophages in a STAT6-dependent fashion, and that IFN-γ–dependent augmentation of IL-12 and TNF-α production from these cells can be blocked by IL-4 in a STAT6-independent fashion without effecting IFN-γ receptor expression (Levings *et al.* 1999). These results further support that IL-4 can interfere with IFN-γ-mediated signaling. Since production of IFN-γ can be greatly augmented by IL-12, the ability of IL-4 to interfere with IFN-γ-induced IL-12 secretion would in turn inhibit optimal IFN-γ expression and inhibit type 1 effector function.

As mentioned above, the role of IFN-γ in allograft rejection is complex. Thus, IFN-γ may activate the effector mechanisms necessary for allograft rejection, but also may be involved in subsequent negative regulation of effector function. To test the role

of IFN-γ in *Nb*-mediated graft prolongation, experiments could be performed investigating the effects of *Nb* on allograft survival in IFN-γ KO or anti-IFN-γ-treated mice. In addition it would be interesting to determine whether more complete blockage of IFN-γ production and possibly further enhancement or abrogation of extended allograft survival would occur if immunization or transplantation were delayed until day 7 post infection when type 2 cytokine expression becomes dominant in the spleen.

5.1.8 Role of type 2 cytokines in allo-specific T cell proliferation.

It is of interest to note that induction of allo-specific type 2 T cell responses was not associated with decreased proliferation in the MLR. A number of studies in the transplantation arena have demonstrated that various tolerizing treatments associated with alteration of cytokine pattern from type 1 towards type 2 secretion resulted in inhibition of allo-specific proliferative responses *in vitro* (Gorczynski 1992, Chen *et al.* 1996, Lehmann *et al.* 1997). In contrast, numerous studies investigating proliferation of type 1 vs type 2 T cells did not find proliferative depression in association with the type 2 phenotype. Recent evidence (Zhang *et al.* 1997, Varadhachary *et al.* 1997), including the experiments in this thesis, suggests that type 2 cytokine expression can lead to a profound enhancement of T cell proliferation and resistance to AICD. Data in this thesis show that IL-4 and IL-6 can potentiate proliferation and/or survival of naïve and activated T cells *in vitro* (see discussion section 5.2). In addition, the data show that addition of rIL-4 to MLR cultures dramatically enhances responses of T cells from allo-immunized, and *Nb*-infected allo-immunized animals, suggesting that type 1, type 2 and possibly type 0 T cells are capable of proliferation in the presence of IL-4 (Fig. 3; section 7.0). Thus, it is

likely that type 2 cytokine-associated suppression of T cell proliferation observed by others is mediated through mechanisms that are either independent of type 2 cytokines or require other signals in addition to those provided by type 2 cytokines.

5.1.9 Effect of type 2 cytokines on CTL development.

One way in which a type 2 shift can have an effect on allograft survival is through modulation of development of allo-specific CTL-response. To assess the effects of *Nb* on the development of allo-reactivity in the CTL compartment *in vivo*, it was essential to establish a model of direct CTL activity, which would measure spleen cell CTL activity *ex vivo* without the expansion *in vitro*. Conventional assessment of CTL activity involves expansion of CTL *in vitro* prior to assessment of cytotoxicity (Wunderlich and Canty 1970). It has been demonstrated that a significant proportion of allo-specific T cell precursors that become activated *in vitro* are not activated *in vivo* (Bishop *et al.* 1993, Bishop *et al.* 1994) even if the same type of allo-antigen is used both *in vivo* and *in vitro*. Therefore, cells that have not encountered allo-antigen in the animal under the influence of immunomodulating treatment may retain the ability to develop into functional CTL *in vitro*, which would lead to misinterpretation of the events that occurred *in vivo*. Moreover, functional changes in allo-responsive cells, generated *in vivo*, may occur during *in vitro* expansion. For example, $CD8^+$ T cells from animals with kidney allografts surviving for more than 3 weeks have been shown to downmodulate TCR expression (Mannon *et al.* 1998). However 4-day stimulation in culture in the presence of allo-antigen led to recovery of TCR expression and allo-specific CTL activity by those $CD8^+$ T cells (Mannon *et al.* 1998). This clearly illustrates the artifacts that may arise

during *in vitro* reactivation. In addition to this, potential differences between responder groups in the rate of proliferation, cytokine production, growth factor exhaustion and other phenomena specific to the *in vitro* system may influence the resulting frequency of CTL that develop following such expansion. Therefore, the standard CTL assay performed after the *in vitro* expansion step does not reflect the true generation of allo-specific CTL *in vivo*.

A number of researchers, including myself, have demonstrated that CTL activity can be detected from GIC directly, without *in vitro* expansion (Mottram *et al.* 1995, Bishop *et al.* 1993, Zhou *et al.* submitted). One complication for our experiments, using spleen cell responders rather than GIC, is that Bishop *et al.* have shown that the frequency of allo-specific CTL found in the spleens of mice transplanted with allogeneic hearts was substantially lower (approximately 57 times) than that observed in the GIC (Bishop *et al.* 1993). However, I reasoned that immunization i.p. with allogeneic spleen cells rather than with a transplanted heart would generate a substantial allo-specific CTL response in the spleen, since the immunizing population contains more professional APC and the spleen is the major lymphatic organ responding to such immunization. In addition, passage through nylon wool and anti-B220 treatment results in approximately 2-fold enrichment of $CD8^+$ T cells, thus augmenting the resulting CTL frequency. Further, virus-specific CTL responses of spleen T cells from infected mice can be readily detected in CTL assays without *in vitro* expansion (Sharma *et al.* 1996, Butz and Bevan 1998, Murali-Krishna *et al.* 1998). Therefore, I speculated that immunization of mice with a powerful antigenic stimulus such as allogeneic spleen cells would lead to activation and expansion of a large number of CTL and that further *in vitro* expansion would not be

necessary in order to detect killing activity. Indeed, my experiments demonstrated that allogeneic P815 target cell death was readily detectable 6h after initiation of a CTL assay, and killing of 50-70% of the targets was seen by 18h in culture (Fig. 4; section 7.0).

Passage of cells through the nylon wool column results in enrichment of both T cells and NK cells. Activated NK cells are known to be capable of killing P815 targets under certain circumstances (Nakajima *et al.* 1998, Carbone *et al.* 1997) Allo-specific NK activity can be activated following organ transplantation (Petersson *et al.* 1997a) as well as by i.p. injection of allogeneic spleen cells (Petersson *et al.* 1997b). To investigate whether T cells played a primary role in mediating cytotoxic activity deletion of Thy1.2 positive T cells was performed using treatment with mAb and complement. This treatment completely abrogated the ability to kill allogeneic tumor targets, suggesting that T cell, and not NK cell, mediated cytotoxicity was involved in this model. In addition, an enriched T cell population, which contained NK cells from naïve mice, did not exhibit any killing activity against allogeneic P815 tumors further supporting the position that NK cells were not involved.

In the experiments described the exposure of mice to *Nb* prior to immunization with alloantigen resulted in a marked decrease in MHC class I restricted dCTL activity against Fas-negative, TNF-resistant P815 (allogeneic) tumor targets. We observed significantly decreased killing by both the JAM assay (apoptosis) as well as the chromium release assay (cytolysis). However, it was possible to argue that killing by other mechanisms, such as unconventional $CD4^+$ CTL (Williams and Engelhard 1996, Williams and Engelhard 1997) or by Fas-mediated killing (by either $CD4^+$ or $CD8^+$ cells) may be intact or even upregulated in *Nb*-infected animals. Our experiments with Fas-

negative MHC class II-negative P815 cells, which depend on perforin/granzyme mediated class I-restricted killing, would not detect such killing activity. To address this point, class II-bearing, Fas-positive, TNF-sensitive allogeneic tumor cells (A20) were used in order to assess killing by these other possible mechanisms. The results confirm that CTL activity is significantly decreased in *Nb*-infected allo-immunized mice rather than being shifted into a different killing phenotype.

There are number of explanations for the mechanism by which *Nb* down-regulates the allo-specific CTL response. First, production of type 2 cytokines during *Nb* infection may interfere with the development of allo-specific CTL. Sad *et al.* (1997) have shown that exposure of type 1 cytokine producing allo-specific $CD8^+$ CTL to IL-4 inhibits their ability to expand in response to stimulation, thus limiting the number of effector CTL. This was further supported by recent studies in IL-4-deficient mice. Virus-specific CTL in these mice have dramatically increased cytolytic capability and expansion of $CD8^+$ CTL precursors is increased (Villacres and Bergmann 1999). This was correlated with increased numbers of virus-specific IFN-γ-producing $CD8^+$ T cells in IL-4 KO mice. In addition, these effects could be abrogated by addition of exogenous IL-4 *in vitro*, confirming that IL-4 can limit the expansion and cytolytic activity of type 1 $CD8^+$ T cells (Villacres and Bergmann 1999). Thus, enhanced IL-4 production during nematode infection could decrease the numbers of allo-specific type 1 CTL.

Second, a decreased frequency of CTL precursors in the spleens of *Nb*-infected animals, and an inhibition of CTL activity, could also be caused by migration of a proportion of the activated cells into the mucosal sites where *Nb*-induced inflammation

occurs. This possibility is unlikely, however, since the decrease in CTL activity is not accompanied by a similar inhibition of allo-specific proliferative responses.

Third, a large fraction of the allo-specific $CD8^+$ T cells activated during the infection with *Nb* may acquire a type 2 non-cytotoxic phenotype similar to that described by Erard and co-workers (1993). Flow cytometric data showed the appearance of $CD8^+$ IL-4 secreting T cells in *Nb*-infected allo-immunized mice, supporting the hypothesis that *Nb* drives the development of $CD8^+$ T cells towards a Tc2 phenotype. Interestingly, I found that $CD8^+$ T cells that stained positively for intracellular IL-4 expressed slightly lower levels of CD8 (data not shown). This is similar to the findings of Erard and colleagues (Erard *et al.* 1993), who observed down-modulation of CD8 following *in vitro* activation of naïve $CD8^+$ T cells in the presence of IL-4. While numerous reports suggest that cytotoxic activity of $CD8^+$ T cells is compromised by the presence of IL-4 (Actor *et al.* 1992, Erard *et al.* 1993, Sharma *et al.* 1996, Villacres and Bergmann 1999), a number of recent studies show otherwise. For example, Li and co-workers (Li *et al.* 1997) found that allo-specific Tc2 cells were as efficient as Tc1 cells at killing allogeneic targets *in vitro*. When injected into footpads of allogeneic mice, these Tc2 cells were effective at mediating DTH associated inflammatory reactions. In this study, however, Tc1 and Tc2 cells were differentiated *in vitro* using a macrophage like tumor cell line. Therefore, the relation of such Tc2 cells to their *in vivo* activated counterparts is unclear. Two other factors may contribute to this apparent discrepancy. 1) In all of the *in vitro* studies, showing that Tc1 and Tc2 cells kill with the same efficiency, generation of the polarized populations is performed in the absence of $CD4^+$ T cells. In contrast, the *in vivo* studies showing decreased CTL activity in the presence of IL-4 were performed under conditions

in which both $CD4^+$ and $CD8^+$ T cells were present. In fact, Villacres and Bergmann (1999) observed downregulation of CTL activity in the presence of IL-4 when whole spleen cells were activated with antigen *in vitro*. 2) The studies in which cytotoxic Tc2 cells were generated *in vitro* were performed in the presence of IL-4 and anti-IFN-γ mAb. In contrast, the *in vivo* studies (Sharma *et al.* 1996, Actor *et al.* 1993) or the *in vitro* studies demonstrating that IL-4 inhibits CTL function, either during priming of naïve $CD8^+$ T cells (Erard *et al.* 1993) or during restimulation of type 1 $CD8^+$ effector cells (Sad *et al.* 1997, Villacres and Bergmann 1999), did not include anti-IFN-γ treatment. Therefore, it is possible that IL-4-mediated inhibition of CTL function is dependent on the presence of some endogenous IFN-γ. In this thesis, inhibition of CTL activity mediated by infection of *Nb* occurred *in vivo* in the presence of 1) $CD4^+$ T cells and 2) significant levels of IFN-γ. This may explain the discrepancy between results obtained in this thesis and those from some of the *in vitro* studies described above.

Fourth, *Nb* infection may lead to development of mixed Tc1/Tc2 or Tc0 phenotype. Although activation of $CD4^+$ T cells *in vitro*, under type 2 polarizing conditions, leads to clear Th2 development, (Matesic *et al.* 1998), in most systems $CD8^+$ T cells activated under identical conditions produce both type 1 and type 2 cytokines (Matesic *et al.* 1998, Cerwenka *et al.* 1999, Dobrzanski *et al.* 1999). However, it is not clear whether this is a result of the development of one homogenous population capable of producing both type 1 and type 2 cytokines, or a heterogenous population containing cells which produce cytokines of one phenotype exclusively. Although, $CD8^+$ T cells that developed under the influence of type 2 polarizing conditions (IL-4 + anti-IFN-γ mAb) are capable of killing antigen-bearing targets *in vitro*, their ability to mediate

effector function *in vivo* was compromised. For example, 1) transfer of allo-specific type 2 $CD8^+$ T cells into syngeneic SCID mice resulted in delayed allograft rejection in comparison to transfer of naïve $CD8^+$ T cells (Matesic *et al.* 1998); 2) tumor-specific Tc1 cells were five time more efficient at reducing tumor metastasis *in vivo* than Tc2 cells (Dobrzanski et al. 1999); 3) transfer of influenza-specific type 2 clones offered only partial (60%) protection against the infection as compared with the transfer of type 1 antigen-specific clones (Cerwenka *et al.* 1998). This suggests that the generation of Tc2 cells in these studies may actually result in the generation of a mixed population of cells in which only a subpopulation exhibits cytotoxic activity *in vivo*. In fact, contrary to the results with influenza noted above which showed partial protection against infection, Tc2 cells derived from IFN-γ KO mice, conferred no protection at all (Cervenka *et al.* 1998). This suggests that a mixed Tc1/Tc2 population, or a population containing Tc0 cells was present in the population arising from type 2 polarization, since in the absence of IFN-γ production by these cells the effector function was dramatically altered.

The data presented in this thesis provide evidence to support three of these four alternatives. Indeed, more than one of these alternative mechanisms is probably active. It is likely that the production of IL-4 does downregulate effector functions and expansion of allo-specific Tc1 cells and leads to the development of some Tc2 cells. The most likely possibility, based on the fact that both type 1 and type 2 cytokines are produced by T cells from *Nb*-infected, allo-immunized mice, and that *Nb* only partially inhibits development of CTL activity, is that *Nb* infection leads to a mixed population of cytotoxic Tc1 and non-cytotoxic Tc2 cells.

An important point to address in future experimentation would be to determine the proportion of the Tc2 cells from *Nb*-infected, allo-immunized mice that are allo-specific. This could be attempted by comparing the level of intracellular staining for IL-4 expression in T cells from *Nb*-infected unimmunized and *Nb*-infected allo-immunized mice with or without subsequent allo-stimulation *in vitro*. However, because of the fact that significant allo-specific IL-4 and IL-6 production was observed without prior *in vivo* administration of alloantigen, and because a relatively small fraction (approximately 3%) of $CD8^+$ T cells from *Nb*-infected allo-immunized mice produced IL-4, it may be difficult to determine the size of the antigen-specific fraction in this system. This problem could be avoided by using mice expressing a transgenic TCR of known allo-specificity. A suitable model could be generated using 2C mice (Sha *et al.* 1988). These mice are of a C57BL/6 background and are transgenic for both the α and β-TCR chains that recognize the MHC class I L^d molecule present in BALB/c mice (Sha *et al.* 1988). In these mice, approximately 85% of the T cells are $CD8^+$, and express this transgenic TCR which can be recognized by clonotypic antibody 1B2 (Sha *et al.* 1988). These transgenic T cells could be adoptively transferred to wild type C57BL/6 mice such that they constitute a significant and easily detectable proportion (i.e. 20-30%) of the $CD8^+$ repertoire. These chimeric mice would, however, retain an intact immune system, especially in the $CD4^+$ compartment, which is likely to be important for *Nb*-mediated effects. Subsequently, effects of *Nb*-infection and/or allo-immunization on activation, differentiation and CTL function, of these allo-specific, transgenic TCR^+, $CD8^+$ cells, could be examined *ex vivo*. Importantly, by performing 3-color flow cytometric analysis using antibodies specific to

the transgenic TCR, IL-4 and IFN-γ it could be established whether *Nb* infection leads to development of mixed Tc1/Tc2 or Tc0 population.

5.2 Modulation of proliferative T cell responses by nematode infection.

5.2.1 Induction of hyper-proliferation by nematode infection.

Nematode infection induces a marked type 2 T cell response (Urban *et al.* 1992, Sher and Coffman 1992), and evidence points to this response being polyclonal in nature (Jarrett and Haig 1976, Finkelman *et al.* 1986, Rocken *et al.* 1992). Data presented in this thesis and experimentation performed by others have shown that nematode infection (Ledingham *et al.* 1996) as well as nematode extracts (Lee and McGibbon 1993, Ehigiator *et al.* unpublished observation) enhance type 2 responses to unrelated antigens. Given this evidence, and the trend towards increased T cell activation in the 1^{o} MLR using cells from *Nb*-infected animals (Fig. 2; section 7.0), it seemed possible that *Nb* might be enhancing the responsiveness of T cells to antigen stimulation. While this might seem beneficial, the ability to control the extent of expansion of antigen-specific T cells is very important in the development of appropriate immune responses. Failure to control this expansion may lead to an exaggerated immune response, undesirable pathology and, potentially, autoimmunity.

In experimentats to investigate this phenomenon, the effects of *Nb* on T cell proliferation were assessed using the polyclonal T cell activators concanavalin A and anti-CD3. The results showed that spleen cells from *Nb*-infected animals show hyper-proliferation upon *in vitro* stimulation with Con A or anti-CD3 mAb. Both anti-CD3 and Con A-induced proliferative responses were similarly affected by *Nb*. Since anti-CD3

mAb was in the form of hybridoma supernatant, Con A was used for all of the subsequent studies in order to avoid possible experimental variation resulting from preparation of the supernatant or the influence of other factors secreted by the hybridoma.

5.2.2 Transfer of hyper-proliferation with cells.

Three potential explanations for this *Nb*-mediated enhancement of T cell proliferation came to mind. First, the increased proliferation could be due to increased numbers of activated T cells as a result of the infection. Second, *Nb* may somehow alter the activation threshold level necessary to stimulate T cells (perhaps through regulation of CTLA4 expression). Third, *Nb* could alter the activation environment such that all T cells (including naïve cells) would respond with increased proliferation. To differentiate among these possibilities, experimentats were performed to determine whether hyper-proliferation could be transferred, *in vitro*, to naïve spleen cells in co-culture. In other words whether co-culture of naïve spleen cells together with mitomycin C-treated splenocytes from *Nb*-infected mice would result in enhanced Con A-induced proliferation of the naïve spleen cells. We found that Con A-induced proliferation of naïve spleen cells could indeed be significantly augmented with "transferring cells" from *Nb*-infected animals. This data confirms that the increased proliferation seen in the splenocyte population derived from *Nb*-infected mice was not due to increased numbers of activated T cells, and was not an inherent property of the T cells from *Nb*-infected animals. This suggests that the *Nb* infection creates an environment in which naïve T cell responsiveness to antigen is altered.

A number of parasite infections have been demonstrated to alter T cell responsiveness through effects mediated by antigen presenting cells, although these effects were usually inhibitory (Ahvazi *et al.* 1995, Motran *et al.* 1996, Kadian *et al.* 1996, Rakha *et al.* 1996). It was therefore of interest to explore the possibility that accessory cells were responsible for the phenomenon described above. To address this question, T cells were deleted from the mitomycin C-inactivated "transferring cell" population and these T cell-depleted "transferring cells" were used in co-culture experiments as above. T cell depletion was very efficient (>98%) as determined by FACS. This depletion was confirmed in that Con A or anti-CD3-induced proliferation was completely abrogated in these cells, and the production of IFN-γ in response to stimulation was abolished. The results showed that non-T accessory cells from *Nb*-infected mice were sufficient to transfer this effect, strongly suggesting that *Nb* infection induces an enhancement of co-stimulatory function in the accessory cell population. This is of significant interest, since it points towards a mechanism where nematodes may be capable of modulating responses to other antigens by their effects on accessory cell function.

5.2.3 Transfer of hyper-proliferation by supernatants.

Accessory cells can modulate T cell proliferation either by secretion of soluble factors and/or expression of surface molecules. In order to investigate the mechanism by which the transfer of hyper-proliferation was mediated we determined whether cell-cell contact between responder and transferring cells was required for the effect, which would be indicative of a role for surface accessory molecules. The data demonstrate that

incubation of naïve spleen cells with a 24h supernatant, derived from Con A –activated non-T cells from *Nb*-infected mice, led to a dramatic increase in proliferation of naïve cells stimulated with Con A. Thus, a soluble factor, present in the supernatant of stimulated non-T cells from *Nb*-infected mice, could confer this hyper-responsiveness on naive T cells when added to naive T cell cultures along with Con A, indicating that cell-cell contact is not required for the transfer of this phenomenon.

5.2.4 Role of IL-6.

There are a number of known factors that are capable of enhancing T cell proliferation, including type 2 cytokines such as IL-4 and IL-6. Because *Nb* infection leads to dramatic upregulation of these cytokines, it was possible that they mediated the induction of hyper-proliferation observed in the experiments described above. Given the evidence in the literature regarding the enhancing effects of IL-6 on T cell proliferation (Holsti *et al.* 1994, Joseph *et al.* 1998, Takeda *et al.* 1998), and data generated in this experimentation, the prime candidate was IL-6. It was observed, for example, that large quantities of IL-6 were secreted in response to Con A stimulation by the non-T cells from *Nb*-infected mice. IL-4 secretion from these non-T cells was limited. In addition, mouse recombinant IL-6 but not IL-4 (Fig. 5: section 7.0), at a concentration matching that found in the transferring supernatant, increased Con A-induced naive spleen cell proliferation to the same extent as the supernatant derived from non-T accessory cells from *Nb*-infected animals. Thus, a hypothesis was developed that IL-6 was the factor in the supernatant which was responsible for these T cell enhancing effects. This hypothesis was tested using a monoclonal antibody to IL-6 to block IL-6 activity in the active

supernatant. Interestingly, this treatment completely abrogated the supernatant-induced hyper-responsiveness, confirming that IL-6 was responsible for the effect. Further support for this hypothesis came from the fact that the efficiency of transfer of hyper-proliferation to naive spleen cells was very similar for co-culture with cells (100%) from *Nb*-infected mice, supernatants (92% increase) from these cells and recombinant IL-6 at a level matching the IL-6 concentration in the supernatants (100% increase). These data corroborate the mAb experiments and confirm that IL-6 elaboration by these accessory cells is the sole cause of this effect.

5.2.5 Role of T cells in the hyper-responsiveness.

Non-T accessory cells were able to transfer hyper-responsiveness to Con A. However, in these experiments, non-T accessory cells from *Nb*-infected mice were slightly less effective as "transferring cells" than whole spleen cells (compare Fig. 2B and 2A; section 4.0; 100% versus 130% enhancement) at enhancing of the proliferative response of naive cells. This difference may be due to the fact that only T-cell-depleted spleen cell populations were treated with complement, which could result in a non-specific toxicity to the cells rendering them less effective. This is unlikely, however, since non-T cells exhibited a similar increased proliferation in response to LPS as compared to untreated whole spleen cells (data not shown) indicating intact B cell and accessory cell function. Another possibility is that the presence of T cells in the "transferring" population contributes directly and/or indirectly to the overall increased production of IL-6. We have shown that higher levels of IL-6 were produced by whole spleen cells than by non-T-cell populations in response to Con A. However, enhanced

proliferation mediated by recombinant IL-6 reached maximal levels at a similar concentration to that found in the supernatants derived from Con A-stimulated non-T-cells from *Nb*-infected mice. Further, supernatants derived from non-T cells were equally effective at inducing hyper-responsiveness as those derived from whole spleen cells. Thus it is unlikely that increased production of IL-6 in the presence of T-cells from *Nb*-infected mice is responsible for the additional boost. Alternatively, T-cells from *Nb*-infected mice may be producing another factor, independent of accessory cells, that further enhances the IL-6 effect. For example, it was found that IL-4 at doses of 2.5 ng/ml or higher also induced increased proliferation to Con A (Fig. 5; section 7.0). Although the levels of IL-4 present in the supernatant either from whole spleen cells or non-T cells derived from *Nb*-infected animals were too low to induce the hyper-responsiveness, the presence of IL-4 producing cells in the co-culture may create high local concentrations of IL-4 and subsequently slightly increase the IL-6 mediated hyper-proliferation. This may explain the fact that whole spleen cells were somewhat more efficient at inducing hyper-proliferation than non-T cells in co-culture experiments.

Con A-induced proliferation of spleen cells from *Nb*-infected mice was 180% higher than that attained by naïve spleen cells. This suggests that 1) the transfer of the hyper-proliferation is not completely efficient; 2) that T-cells from *Nb*-infected mice are more sensitive to stimulatory signals provided by accessory cells; or 3) T cells from *Nb*-infected mice are less sensitive to some factors that may inhibit their proliferation.

To address the first point, both autocrine and paracrine consumption of IL-6 by T cells from *Nb*-infected mice can occur, whereas only a paracrine pathway is active during transfer to naive T cells. Regarding the second possibility, T cells from *Nb*-infected mice,

for example, may express higher levels of the IL-4 receptor or IL-6 receptor and thus be sensitive to activation by this cytokine. In addition, sensitivity to IL-6 can be regulated by varying the ratio of surface gp130 to gp80 expressed on the target cells (Peters *et al.* 1998). Thus, T cells from *Nb*-infected mice could express the two subunits at a ratio that favors stronger activation. Alternatively, given the fact that the level of IFN-γ produced by spleen cells from *Nb*-infected mice was significantly reduced, and that IFN-γ has been shown to have inhibitory effects on T-cell proliferation (Konieczny *et al.* 1997, Dalton *et al.* 1993), the decreased levels of IFN-γ (and/or possibly reduced sensitivity to IFN-γ signal) could be responsible for the increase in T cell reactivity. In order to address these questions, one could assess the effects of anti-IL-4, rIL-6 and rIFN-γ on Con A-induced proliferation of spleen cells derived from *Nb*-infected mice.

5.2.6 Nature of the IL-6 effect.

It is unclear at this point whether IL-6 mediates its effects by direct action on T cells or indirectly through accessory cells. My recent experimentation has revealed that B7.2 is over-expressed on non-T cells from *Nb*-infected mice following activation with Con A (data not shown). Increased B7.2-CD28 interaction can in some cases lead to increased proliferative T cell activity (Perrin *et al.* 1997). Whether the induction of B7.2 expression is mediated through IL-6 was not addressed in my studies (unpublished). However, this over-expression of B7.2 may partially contribute to *Nb*-induced hyper-responsiveness. One could address this point by first investigating whether naïve non-T cells pre-treated with IL-6 and extensively washed would acquire the ability to induce hyper-responsiveness to Con A in purified naïve T cells. If so, then one could test

whether this ability was due to the induction of expression of surface bound molecules such as B7.2.

It is also possible that IL-6 exerts its effects through downregulation of IFN-γ activity. It has been demonstrated that IFN-γ is involved in down-regulation of proliferative T cell responses (Dalton *et al.* 1993, Konieczny *et al.* 1998) possibly by induction of apoptosis through upregulation of FasL expression (Novelli *et al.* 1997). Further, IFN-γ production from mitogen-activated T cells can be dramatically reduced by IL-6 (Rincon *et al.* 1997). The data in this thesis clearly showed that spleen cells from *Nb*-infected mice produced significantly lower levels of IFN-γ in response to Con A. Therefore, it is possible that IL-6 exerted its effects on naïve T cell proliferation through downregulation of IFN-γ activities. To test this possibility high concentrations of exogenous IFN-γ could be added to the cultures of naïve spleen cells activated in the presence of IL-6.

5.2.7 Nature of the accessory cell.

The type of accessory cell that is responsible for *Nb*-mediated hyper-responsiveness to Con A is unclear. Both macrophages (Takashima *et al.* 1993, Maldonado *et al.* 1994) and B-cells (Lenschow *et al.* 1993) become activated with Con A and could contribute to hyper-responsiveness. Moreover, *Nb* infection leads to the appearance of an IgE receptor bearing non-B, non-T cell, a so called "null" population in the spleen (Conrad *et al.* 1990). This null cell population may be able to become activated with Con A since Con A is known to bind IgE and activate IgE receptor bearing mast cells and basophils (Haas *et al.* 1999, Miller *et al.* 1997). It is important to note,

however, that mitogen activation of the non-T accessory cells is not required to induce hyper-responsiveness. This conclusion is based on the data showing that spleen cells from *Nb*-infected mice exhibit hyper-responsiveness to activation with anti-CD3 and in the MLR. Moreover, non-T cells from *Nb*-infected mice induce increased proliferation when used as stimulators in allogeneic mixed lymphocyte reaction (data not shown). Future studies will be required to identify the cell(s) involved, and the activation requirements for the production of IL-6 by these cells.

5.2.8 AICD.

The data presented in this thesis suggests that *Nb* reduces AICD in mitogen-activated cells. A kinetic study of the Con A-induced proliferation demonstrated that naive spleen cells attain nearly maximal proliferation at 42h. From that time on, thymidine uptake plateaus, exhibiting only a marginal increase over the next 48h. If all the cells that have gone through the cell cycle were capable of entering another cycle we would expect constantly higher levels of proliferation at later time points. Since thymidine uptake by naive spleen cells was relatively constant, it suggests that a large proportion of the dividing T cells in the naive spleen cell population do not re-enter the cell cycle or are unable to complete the cycle. In contrast, spleen T cells from *Nb*-infected mice exhibited a dramatic increase in the rate of proliferation at each time point assessed, starting from 22h until 90h. This progressive increase in thymidine uptake suggests that the majority of cells in the spleen cell cultures from *Nb*-infected mice re-enter the cell cycle. Based on these results, a reasonable hypothesis would be that infection with *Nb* leads to a decrease in susceptibility to AICD.

Assuming that all the cells that did not re-enter the cell cycle have died due to AICD, the estimated rate of AICD (based on time course proliferation data) between the 42h and 66h time points (assuming 24h-long cell cycle) was 56% for naive spleen cells and 25% for spleen cells from *Nb*-infected mice. Experimental assessment of AICD performed by the JAM assay showed that while 60.2% of naive spleen cells that have proliferated until 42h in culture underwent AICD in the next 24h, only 35.1% of spleen cells from *Nb*-infected mice died in that same 24h period (a 46% reduction of AICD). These data were very similar to the estimation of AICD derived above from time course proliferation results, suggesting that the enhanced thymidine uptake observed above could be explained by a reduction in AICD. Furthermore, the enhanced survival could be transferred in the same manner (non-T cells, non-T cell supernatants and IL-6) as the transfer of hyper-responsiveness as judged by proliferation. In addition, resistance to AICD conferred by supernatants could be abrogated by anti-IL-6 treatment. Taken together, these results confirm that the hyper-responsiveness induced by *Nb* is due, in large part, to IL-6-mediated resistance to AICD.

The JAM assay, following restimulation with Con A, was chosen because it enabled us to measure the extent of cell death in the T cell population that was actively synthesizing DNA in the last 18h of primary stimulation, and not in the non-proliferating T cells or B cells, which represent the majority of the spleen cell population. This was very important, considering the fact that IL-6 has been demonstrated to enhance survival of resting T cells *in vitro* (Teague *et al.* 1997). However, one could argue that AICD measured by the JAM assay does not reflect cell death during the proliferation assay, since in the JAM assay AICD was measured after the cells were harvested, washed, and

re-stimulated with Con A. Thus, death may be a result of reactivation in the secondary culture rather than death that is induced in the course of the primary culture period. Indeed, studies by Radvanyi and co-workers (Radvanyi *et al.* 1993) have suggested that re-ligation of TCR by antigen or mitogen can lead to AICD. However, others have clearly shown that T cells undergo apoptosis after a number of cycles in the primary culture (Ayroldi *et al.* 1998). In addition, other studies demonstrate that AICD occurs *in vivo* after a discrete number of cycles and that secondary stimulation is not required for this T cell death (Renno *et al.* 1999, Van Parijs *et al.* 1998). This discrepancy between some *in vitro* data and the *in vivo* situation may be due to differences in the original activation conditions used *in vitro*. In fact, Radvanyi and colleagues (Radvanyi *et al.* 1993) found that if enriched T cells were used in the primary culture, instead of whole spleen cells, AICD occurred in the absence of re-stimulation. Data in this thesis also suggest that re-ligation of TCR was not responsible for T cell death in the JAM assay since comparable T cell death was observed irrespective of the addition of Con A during the secondary culture (Fig. 6; section 7.0). This suggests that the AICD model in this thesis is similar to the *in vivo* situation.

The results presented here are in agreement with the studies of Takeda and co-workers (Takeda *et al.* 1998), who showed that IL-6 increased thymidine uptake by T cells in response to both Con A and anti-CD3 by inhibiting AICD. This effect of IL-6 was dependent on STAT3 signaling since T cells that were STAT3-deficient were not rescued from apoptosis and, as a result, proliferated poorly even in the presence of IL-6. However, recent data suggests that signaling through the IL-6Rβ (gp130) subunit leads to two distinct signals (Fukada *et al.* 1996). One of these signals is SHP2-dependent,

STAT3-independent and leads to activation of MAP kinase and subsequent mitogenesis (Fukada *et al.* 1996). The other signal results in STAT3 activation and leads to an anti-apoptotic signal, including upregulation of Bcl-2 (Fukada *et al.* 1996). STAT3 is also phosphorylated in T cells upon stimulation with IL-2 (Akaishi *et al.* 1998). This event is crucial for IL-2-induced IL-2Rα expression, which is necessary for optimal T cell proliferation. Therefore STAT3 activation by IL-6 may similarly induce IL-2Rα expression and hence contribute to enhanced proliferation in an IL-2-dependent fashion. Thus IL-6 can induce hyper-proliferation through three potential mechanisms.

The data presented here are consistent with the hypothesis that *Nb* induced hyper-response is due, for the most part, to an IL-6-mediated resistance to AICD. It is possible to postulate a number of mechanisms to explain this finding. First, upregulation of Bcl-2 or Bcl-x_L may play a role in our model. This could be investigated by looking at mRNA expression of these factors. Second, IL-6 may induce mitogenesis, such that a large percentage of the cells are in the S phase of the cell cycle. Cells in S phase have been reported to be resistant to apoptosis while those in G1 phase are susceptible (Dao *et al.* 1997, Lissy *et al.* 1998). Thus, IL-6 may allow T cell survival by preventing the arrest or shortening the time in G1 phase. Third, IL-6 may be regulating the expression of Fas or FasL expression on T cells or by modulating T cell susceptibility to FasL-mediated lysis.

The involvement of Bcl-2 expression is unlikely. Even though IL-6 can cause Bcl-2 expression in non-T cells (Fukada *et al.* 1996), and preserve its expression in the resting T cell population (Teague *et al.* 1997), activation of T cells with Con A in the presence of IL-6 led to resistance in AICD without alteration of Bcl-2 levels (Takeda *et al.* 1998). Increased mitogenesis is also less likely since activation of T cells in the

presence of IL-6 does not promote progression into the S phase of the cycle (Takeda *et al.* 1998). I predict, that interference with FasL-induced apoptosis is involved, since it has been demonstrated that IL-6-induced inhibition of FasL expression on T cells has been associated with inhibition of AICD (Ayroldi *et al.* 1998). Since FasL-mediated apoptosis plays a crucial role in the regulation of the survival of activated T cells *in vivo* (Russell 1995, Krammer 1999), this link with IL-6 indicates that modulation of local IL-6 levels by nematodes could have profound effects on developing immune responses, through mediating enhanced survival of activated T cells.

In this context, it is of interest that superantigen-activated T cells can be rescued from undergoing apoptosis *in vivo* by an injection of LPS (Vella *et al.* 1995, Vella *et al.* 1997). This was mediated by an upregulation of pro-inflammatory cytokines (Vella *et al.* 1995, Vella *et al.* 1997) which interfered with the deletion of superantigen-reactive T cells. Similar effects were observed during infection with vaccinia virus (Mitchell *et al.* 1999). Vaccinia was shown to exert its effects by enhancing superantigen –activated T cell survival through novel mechanisms not involving the upregulation of Bcl-2 or Bcl-x_L expression. These results suggest that inflammatory reactions induced by microbial infection profoundly modulate the development of other T cell-mediated responses through enhanced survival of activated T cells. Interestingly, Rocken and co-workers (Rocken *et al.* 1992, Rocken *et al.* 1994) demonstrated that infection with *Nb* reactivates superantigen-specific T cells in mice previously tolerized with superantigen, and induces type 2 responses towards the superantigen. Because enhanced polyclonal proliferation induced by *Nb* was important for breaking of this tolerance, it would be interesting to investigate the role of accessory non-T cells and IL-6 in this phenomenon in the context

of results presented in this thesis.

How may IL-6 be involved in reactivation of superantigen-induced tolerance? An interesting possibility is provided by recent experimentation in mice transgenic for Vα11, Vβ3 TCR (Cauley *et al.* 1997). Injection of SEA into these mice activates virtually all the $CD4^+$ T cells due to expression of Vβ3 transgene. As is the case in non-transgenic mice, SEA-responsive T cells exhibit rapid clonal expansion (Miethke *et al.* 1993) followed by apoptosis, such that the numbers of superantigen-responsive $CD4^+$ T cells are reduced compared to the initial state (Kawabe and Ochi 1991). The remaining $CD4^+$ T cells, that have not undergone apoptosis, become unresponsive to subsequent stimulation with superantigen *in vitro* (Rocken *et al.* 1994, Vella *et al.* 1995, Vella *et al.* 1997). The recent studies in transgenic mice have addressed the mechanisms by which this unresponsiveness in the remaining T cell population occurs. It turns out that unresponsiveness is maintained by a population of $CD4^-$ $CD8^-$ T cells through the secretion of high levels of IFN-γ. This was demonstrated by showing that anti-IFN-γ treatment or elimination of these double negative T cells *in vitro* resulted in reversal of unresponsiveness in $CD4^+$ cells (Cauley *et al.* 1997). IL-6 has been suggested to interfere with IFN-γ production both *in vivo* (Mizuhara *et al.* 1996, Yamamoto *et al.* 1995, Tomura *et al.* 1997) and *in vitro* (Rincon *et al.* 1997). Research in this thesis has demonstrated that *Nb* infection results in downregulation of IFN-γ and enhancement of IL-6 production. These results suggest a possible role for IL-6 in the *Nb*-induced reactivation of tolerant cells observed by others (Rocken *et al.* 1992, Rocken *et al.* 1994). In order to confirm the role of IL-6 in this system one could determine whether anti-IL-6 treatment *in vivo* would abrogate the effect of *Nb* infection on superantigen responsiveness. Further it

would be interesting to investigate whether the non-T cells from *Nb*-infected mice, which in our model induced hyper-proliferation *in vitro* through IL-6-mediated effects, would reactivate the superantigen specific T cells *in vivo*.

Rocken and co-workers did not investigate whether administration of *Nb* concurrently with the injection of a tolerizing dose of superantigen would lead to abrogation of tolerance induction. However, based on the results obtained in the studies utilizing LPS and vaccinia virus, and our data showing that *Nb* infection leads to hyper-proliferation and reduced AICD, it is likely that *Nb* would promote the expansion and prevent deletion of the superantigen-reactive T cells, and that the infection would interfere with the induction of tolerance to superantigen.

A recent report by Zhang and co-workers (Zhenh *et al.* 1997) showed that T cells activated *in vitro* in a type 2 polarizing environment were highly resistant to AICD. The major pathway of AICD induction in that study was demonstrated to be FasL-mediated apoptosis. However, the factors involved in mediating resistance to AICD were not identified. Similar resistance to AICD was observed in Th2 but not Th1 clones (Varadhachary *et al.* 1997). Others (Estaquier *et al.* 1995) have suggested that this preferential susceptibility of Th1 cells to undergo AICD may explain the development of the non-protective type 2 responses during HIV infection. Experiments presented in this thesis demonstrate that *in vivo* activation of type 2 T cells during *Nb*-infection likewise results in resistance to AICD. In addition, the results demonstrate that IL-6 induced by the infection with *Nb* is responsible for this enhanced T cell survival and subsequent hyper-proliferation. Taken together these studies suggest that modulation of IL-6 levels due to nematode infection could dramatically alter T cell-mediated responses to

infections and/or vaccination in endemic areas.

5.2.9 Type 2 switch.

The studies described above demonstrate that induction of cytokines, such as IL-6, may profoundly change the outcome of T cell activation. But can IL-6 also influence the switch from type 1 towards type 2 responses? There is substantial evidence for a role of IL-6 in T cell subset shift (Rincon *et al.* 1997, Gorczynski *et al.* 1997). However this cytokine does not appear to be essential in the induction of type 2 cytokine production (Kopf *et al.* 1995, La Flamme and Pearce 1999). The mechanism by which IL-6 may be involved in this polarization of T cell responses is unclear. However, an interesting possibility is provided by a number of recent studies, including this thesis. Bird and colleagues (Bird *et al.* 1998) showed that type 1 and type 2 phenotype development is associated with the cell cycle. These authors have demonstrated that expression of the type 2 phenotype does not occur in newly activated T cells. Only 4th generation daughter T cells acquired the ability to produce IL-4, and only after the 6th generation did the percentage of IL-4 producing cells rise significantly. Acquisition of the type 1 phenotype (as assessed by IFN-γ production) occurs earlier (after the 1st cell division). However, the type 1 phenotype is also cell cycle dependent since more cells acquire the ability to produce IFN-γ with increased numbers of cell divisions. These data correlate well with other studies showing that while mRNA for IFN-γ is already detectable within 6h of CD4^{+} T cell activation, IL-4 mRNA is not detectable until 48h in culture (Lederer *et al.* 1996). Although the percentage of IL-4 producing T cells was increased when exogenous IL-4 and anti-IL-12 were included in the culture, the onset of acquisition of

the ability to produce IL-4 was independent of IL-4 and completely dependent on the number of cell cycle progressions (Bird *et al.* 1998). The same was true for IFN-γ production upon stimulation in type 1 polarizing conditions. The authors also suggested that Th cell differentiation is controlled epigenetically and a specific number of cell divisions are required in order to remodel the DNA configuration before transcription from the relevant gene sequences is possible (Bird *et al.* 1998). Thus, the presence of IL-6, and the resulting increase in activated T cell proliferation (through decreased AICD), may lead to rapid expansion of T cells, and prompt polarization of the responses towards the type 2 phenotype, in the presence of IL-4. In contrast, if the proliferation rate is limited, IFN-γ producing cells would predominate early and produce significant levels of this cytokine to reduce the proliferation of the subset of T cells remodeling their DNA towards type 2 differentiation. In this context it is of interest to note that Th0 and Th2 cells, but not Th1 cells, express IFN-γ receptor and thus IFN-γ may preferentially downregulate development of type 2 immunity through inhibition of proliferation of Th0 cells (Bach *et al.* 1995, Pernis *et al.* 1995). Taken together, IL-6 may be controlling Th cell differentiation through modulation of proliferative responses.

This hypothesis is based on the speculation that IL-6 mediated cell division is a pre-requisite for Th cell differentiation. Strong expansion leads to Th2 prominence but some division is needed for Th1 development. With this in mind it is not surprising that T cells from IL-6 KO mice, immunized *in vivo*, displayed profoundly reduced levels of both type 1 and type 2 cytokine production upon antigenic challenge *in vitro* (Samoilova *et al.* 1998). Interestingly, T cells from IL-6 KO mice proliferate well to optimal concentrations of Con A and under these activation conditions they are capable of

producing both IL-4 and IFN-γ (Samioilova *et al.* 1998). Thus, the requirement for IL-6 in Th cell differentiation may be overridden by potent T cell activators.

5.2.10 Role of NFAT in type 1/type 2 switch: correlation with proliferative activity.

Deletion of the NFATc isoform from lymphocytes leads to profound inhibition in IL-4 and IL-6 production from T cells, even if the activation occurs in a type 2 polarizing environment (Yoshida *et al.* 1998). Differentiation towards the type 1 phenotype was not affected by this mutation (Yoshida *et al.* 1998). These NFATc KO T cells exhibited a reduced ability to proliferate during stimulation (Yoshida *et al.* 1998). This was further confirmed by Ranger and co-workers (Ranger *et al.* 1998), who showed in addition that type 2 antibody (IgE and IgG1) levels in these mice were generally reduced whereas IgG2a levels were enhanced as compared to the wild type counterparts. In contrast, mice deficient in the NFATp isoform exhibited profound inhibition of type 1 and concomitant enhancement of type 2 cytokine development (Kiani *et al.* 1997, Hodge *et al.* 1996). This skewing towards type 2 T cell development led to abrogation of type 1 effector function necessary for efficient clearance of *Leishmania* infection (Kiani *et al.* 1997). In agreement with the data presented in this thesis, T cells from NFATp KO mice, in which preferential type 2 switch occurs, exhibited hyper-proliferative responses towards mitogenic and antigen stimulation *in vitro* (Hodge *et al.* 1996). Even more dramatic effects were observed in mice deficient in both NFATp and NFAT4 expression. In addition to profound upregulation of type 2 cytokine production, and dramatically enhanced (up to 10,000 fold) levels of circulating IgE and IgG1, these mice exhibited greatly augmented antigen-driven proliferation, and resistance to AICD, (Ranger *et al.*

1998). These data, taken together, demonstrate that NFAT transcription factors regulate T cell proliferation (or susceptibility to apoptosis) and differentiation into type 1 and type 2 effector T cells. NFATc seems to be important for induction of high levels of proliferation and type 2 responses whereas NFATp reduces proliferation and preferentially leads to type 1 switch.

It is unclear how these NFAT factors regulate proliferation. However, it is possible that they regulate proliferation through modulation of expression of FasL on T cells upon activation. In fact, T cells from both NFATp KO and NFATp + NFAT4 double KO mice failed to upregulate FasL expression upon activation (Hodge *et al.* 1996, Ranger *et al.* 1998). Furthermore, the promoter region of FasL contains NFAT binding sequences (Latinis *et al.* 1997). Interestingly, overexpression of NFATc in Jurkat cells led to inhibition of FasL expression, which suggests that NFATc negatively regulates AICD (Latinis *et al.* 1997). Since NFATc and NFATp seem to have opposing roles in regulating AICD, this may explain why NFATp KO mice, which presumably express NFATc, fail to undergo AICD. Since T cell activation in the presence of IL-6 results in downregulation of FasL expression, it is tempting to speculate that this effect is mediated through regulation of NFAT expression, or function and may subsequently lead to a preferential type 2 switch. Alternatively, since NFATp seems to be important for IFN-γ expression, and since IFN-γ has been demonstrated to induce AICD in Th2 cells by inducing FasL expression (Novelli *et al.* 1997), type 2 cytokine expression could be enhanced in NFATp-deficient mice due to the absence of IFN-γ and the resulting inability to control Th2 cell differentiation. Therefore, in our model, expression of IL-6 which can downregulate IFN-γ production, may result in a type 2 switch through decreased

apoptosis in cells differentiating towards type 2 phenotype. Interestingly, it has recently been demonstrated that FasL-deficient mice, when infected with the protozoan parasite *Trypanosoma cruzi*, are unable to control the infection (Lopes *et al.* 1999). This was associated with the dramatic upregulation of type 2 cytokine production (IL-4 and IL-10) by $CD4^+$ T cells in those mice. This susceptibility to *Trypanosoma* was reversed upon anti-IL-4 treatment. These results suggest that FasL-mediated apoptosis may be an important regulator of T cell differentiation, supporting a role for the regulation of FasL expression in *Nb*-induced hyper-proliferation and subset switch.

In this context it is worth noting that cells from BALB/c mice are extremely susceptible to a type 2 switch partly because of low levels of IFN-γ production and failure to maintain the responsiveness to IL-12 (Szabo *et al.* 1997, Hsieh *et al.* 1995, Himmerlich *et al.* 1998). Interestingly, spleen cells from BALB/c mice proliferate much more strongly in response to Con A stimulation, than splenocytes derived from C57BL/6 animals (Fig. 5: section 7.0). Thus, vigorous proliferation of BALB/c spleen cells could be due to the downregulation of IFN-γ production, and decreased AICD, due to potential failure of the BALB/c cells to upregulate FasL expression.

5.2.11 Nematode –mediated effects.

Based on the studies described above it is hypothesized that *Nb* infection induces dysregulation of T cell function at a number of levels. Most importantly, *Nb* infection appears to affect the ability of accessory cells to produce effector cytokines, such as IL-6, which have profound effect on T cell proliferation, survival and differentiation. Nematode induced cytokines likely modulate surface expression on T cells of key

molecules, such as FasL, in a manner which mimics the artificial NFATp mutant mice, although possibly through disparate mechanisms. The result, however, is similar – increased proliferation, decreased AICD and increased switch to type-2 responses. Indeed, mice that over-express IL-6 appear to exhibit similar T cell dysregulation as seen in *Nb*-infected and NFATp KO mice (Screpanti *et al.* 1995), including splenomegaly, hyperproliferation, failure to produce type 1 cytokines (especially IL-12) and mount DTH reaction as well as preferential induction of type 2 responses.

5.3 Summary and conclusions.

The evidence presented in this thesis is consistent with the hypothesis that *N. brasiliensis* modulates the response to alloantigen by inducing type 2 immunity. T cells from *Nb*-infected allo-immunized mice secreted greatly elevated levels of type 2 and reduced levels of type 1 cytokines upon antigenic challenge. The infection also resulted in an inhibition of allo-specific CTL activity. This was not a result of parasite induced immunosuppression because proliferative allo-specific T cell responses were not compromised by the infection. A premise is put forward that this effect could be due, at least in part, by modifying $CD8^+$ T cell responses such that a substantial fraction of allo-specific $CD8^+$ T cell precursors develop the non-cytotoxic Tc2 phenotype in the context of nematode infection. The inhibition of effector functions, such as CTL, is suggested as a likely reason for enhanced graft survival by *N. brasiliensis*. These results suggest that perception of antigen in the context of nematode infection results in development of altered effector functions towards unrelated antigens.

Type 2 cytokine production in response to alloantigen, observed in the context of

Nb infection, suggested that the nematode predisposes T cells to progress towards a type 2 phenotype upon antigenic stimulation. This indicated that T cell activation is altered in the environment of *Nb* infection. Indeed, it was found that spleen cells from *Nb*-infected mice hyper-proliferate when activated with T-cell mitogens *in vitro*. By using a co-culture system it was found that this hyper-activation was transferable to naïve T cells and was mediated by secretion of high IL-6 levels from an accessory cell population in *Nb*-infected animals. A suggestion is put forward that this hyper-activation is due, for the most part, to a reduced susceptibility of T cells to AICD. A further suggestion is put forward that this reduced susceptibility to AICD provides the opportunity for increased cell cycle progression, leading to a type 2 phenotype.

The observations described above demonstrate that nematode infection has profound effects on the immune response, which develops subsequent to antigen recognition. Clearly, T cell activation, proliferation, and differentiation are altered in the presence of nematode infection. These data suggest that individuals in areas where nematode infections are prevalent will react differently to antigenic challenge in comparison to individuals who are non-infected. This has important implications for vaccine strategies and therapy in developing countries.

6.0 Appendix: additional figures and disclosure letters

Fig. 1 ***Immunization of mice with allogeneic spleen cells leads to enhanced proliferation in MLR.***

Allo-specific proliferation in MLR of C57BL/6 effector T-cells from unimmunized (□) or allo-immunized (■) mice, was assessed 7 days post immunization by ^{3}H-Thymidine incorporation. Enriched responder T-cells ($2x10^{5}$ per well) were activated, in the presence of mitomycin C-treated, T-cell-depleted, BALB/c (allogeneic) stimulator spleen cells, at indicated responder to stimulator ratios. Data shown are expressed as mean ± SD of triplicate wells and are representative of 3 separate experiments.

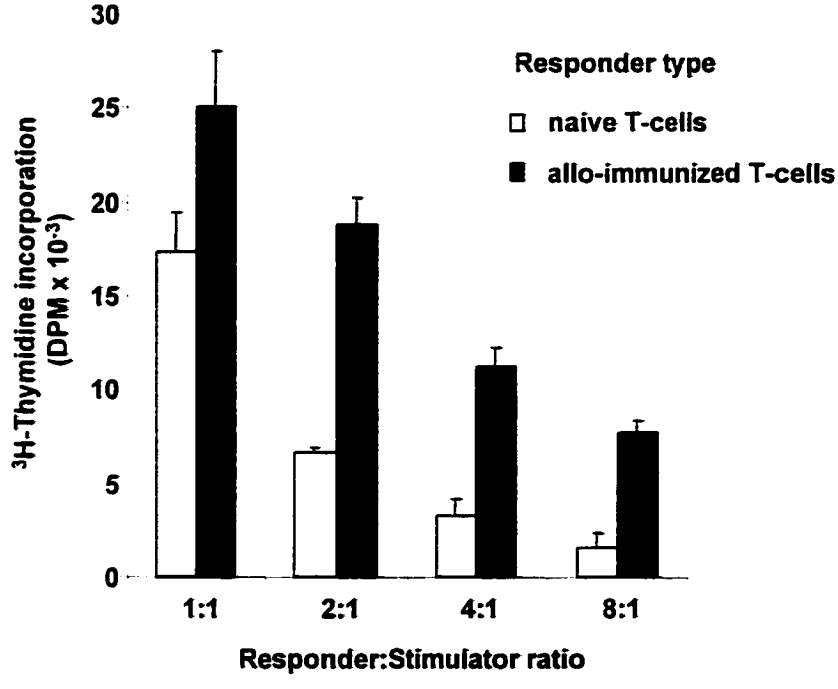
30
25
20
15
10
5
0
^{3}H-Thymidine incorporation (DPM x 10^{-3})
Responder type
naive T-cells
allo-immunized T-cells
1:1
2:1
4:1
8:1
Responder:Stimulator ratio

Figure 1

Fig. 2 ***Allo-immunization in Nb-infected mice leads to enhanced proliferation in MLR.*** Allo-specific proliferation in MLR of effector T-cells from mice that were infected with *Nb* and either left untreated (*Nb*) or allo-immunized (*Nb* + allo) was assessed 7 days post immunization by ^{3}H-Thymidine incorporation. T-cells from uninfected, unimmunized mice (naive) served as 1^{o} MLR control. Enriched effector T-cells were activated, in the presence of mitomycin C-treated, T-cell-depleted, BALB/c (allogeneic) stimulator spleen cells, at a responder to stimulator ratio of 4:1. All data are expressed as a percentage of allo-specific proliferation exhibited by naive T cells. Data shown are expressed as mean ± SEM of 3 experiments (*** $p < 0.001$, comparing *Nb* + allo and *Nb* treatments; repeated measures ANOVA).

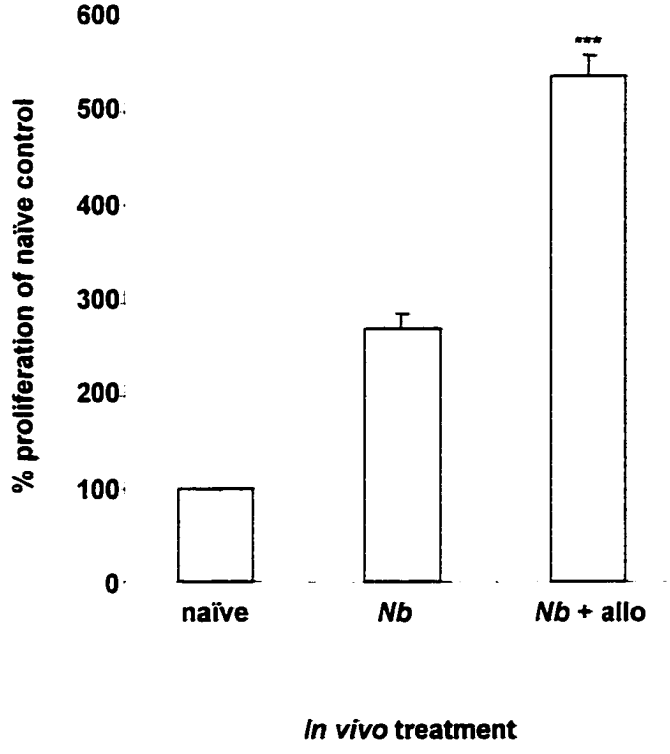
600
500
400
300
200
100
0
% proliferation of naïve control
***
naïve
Nb
Nb + allo
In vivo treatment

Figure 2

Fig. 3 ***Recombinant IL-4 enhances T cell proliferation in 2° MLR.***

Effects of rIL-4 on allo-specific proliferation in 2° MLR of effector T-cells from mice that were infected with *Nb* (*Nb* + allo), or injected with PBS vehicle (allo) 4 days prior to allo-immunization, was assessed by ^{3}H-Thymidine incorporation. Enriched effector T-cells were activated with mitomycin C-treated, T-cell-depleted, BALB/c (allogeneic) stimulator spleen cells, in the absence (■) or presence of 2.5 ng/ml mouse rIL-4 (□) at a responder to stimulator ratio of 4:1. Data shown are expressed as mean ± SD of triplicate wells and are representative of 2 experiments.

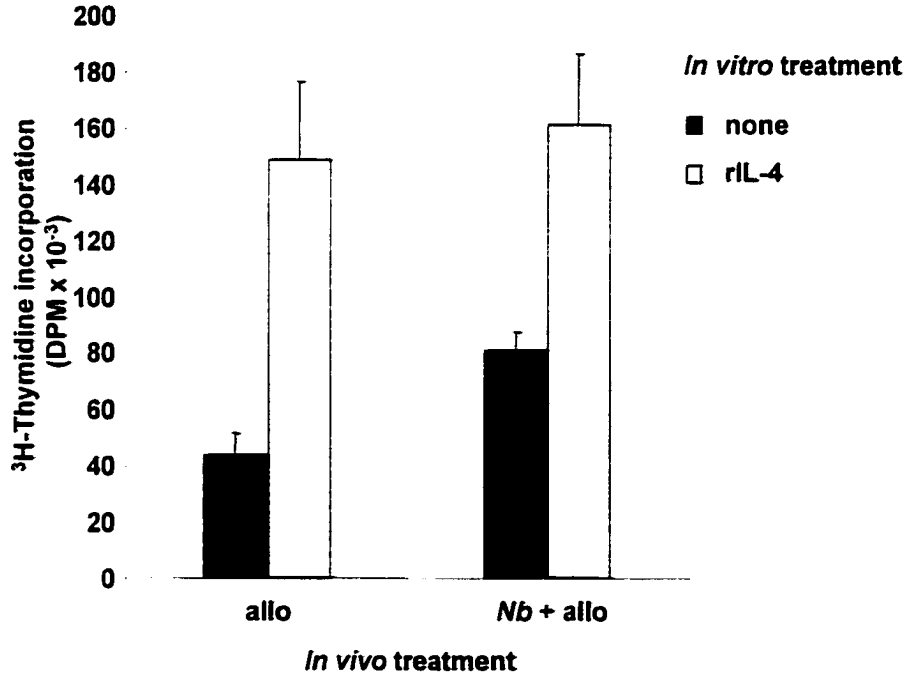
200
180
160
140
120
100
80
60
40
20
0
^{3}H-Thymidine incorporation (DPM x 10^{-3})
allo
Nb + allo
In vivo treatment
In vitro treatment
none
rIL-4

Figure 3

Fig. 4 ***Time course of direct CTL activity generated by immunization of mice with allogeneic spleen cells.***

Direct CTL activity of effector T-cells, from mice immunized with mitomycin C-treated allogeneic spleen cells was assessed by JAM assay. Splenocytes were isolated from mice seven days after immunization and enriched for T cells. Effector T cells were incubated with allogeneic P815 ($H\text{-}2^d$) targets at a 25:1 effector to target ratio, and CTL activity was measured at indicated time points. Data shown are expressed as mean ± SD of triplicate wells and are representative of 2 experiments.

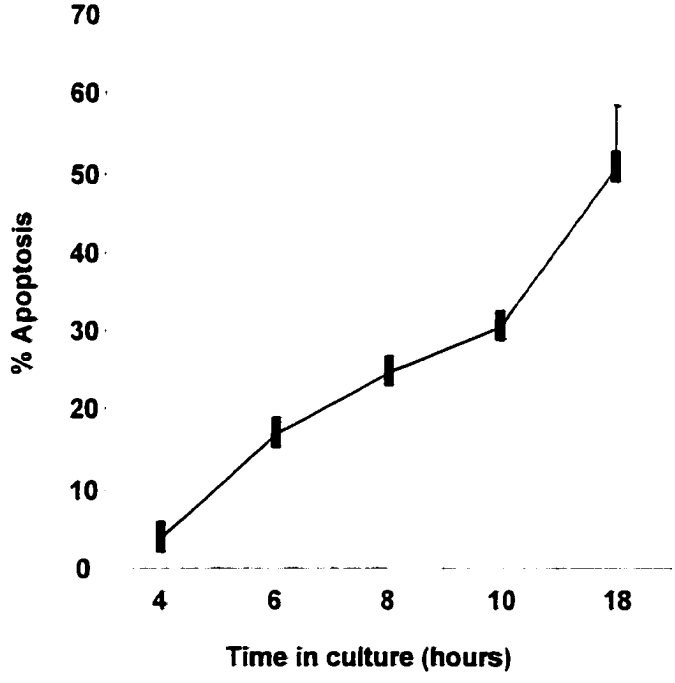

Figure 4

Fig. 5 ***Mouse recombinant IL-4 enhances spleen cell proliferation to Con A only at high IL-4 concentrations.***

Naive spleen cells were activated with Con A in the absence or presence mouse recombinant IL-4 at varying concentrations Data are expressed as mean ± SD of triplicate wells and are representative of 2 separate experiments.

[a] 0.31 ng/ml is the average concentration of IL-4 in the test proliferation cultures containing 1:4 *Nb* spleen supernatant.

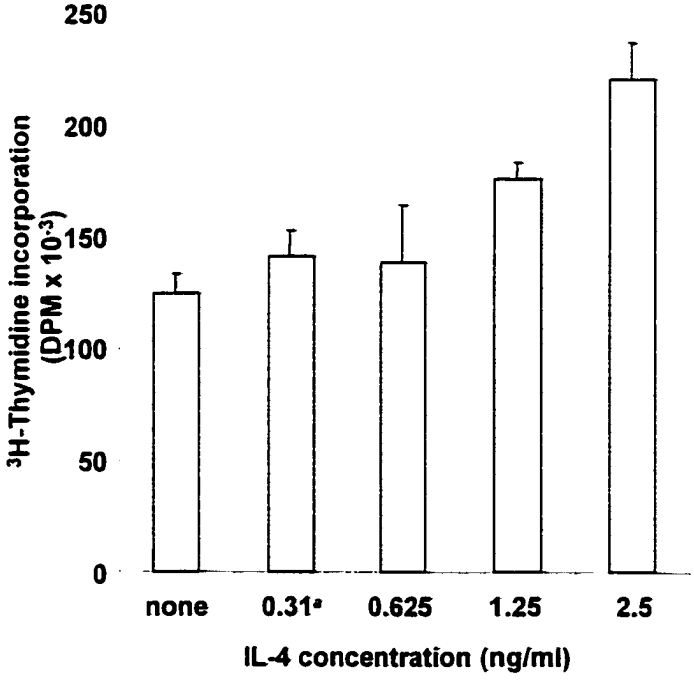
250
200
150
100
50
0
^{3}H-Thymidine incorporation (DPM x 10^{-3})
none
0.31[a]
0.625
1.25
2.5
IL-4 concentration (ng/ml)

Figure 5

Fig. 6 ***AICD is not induced by re-crosslinking of TCR.***

Spleen cells from naive animals were activated with Con A in the primary culture and were left untreated (□) or restimulated with Con A (■) in the secondary culture. AICD was assessed by JAM assay after 24h. Data shown are expressed as mean ± SD of triplicate wells.

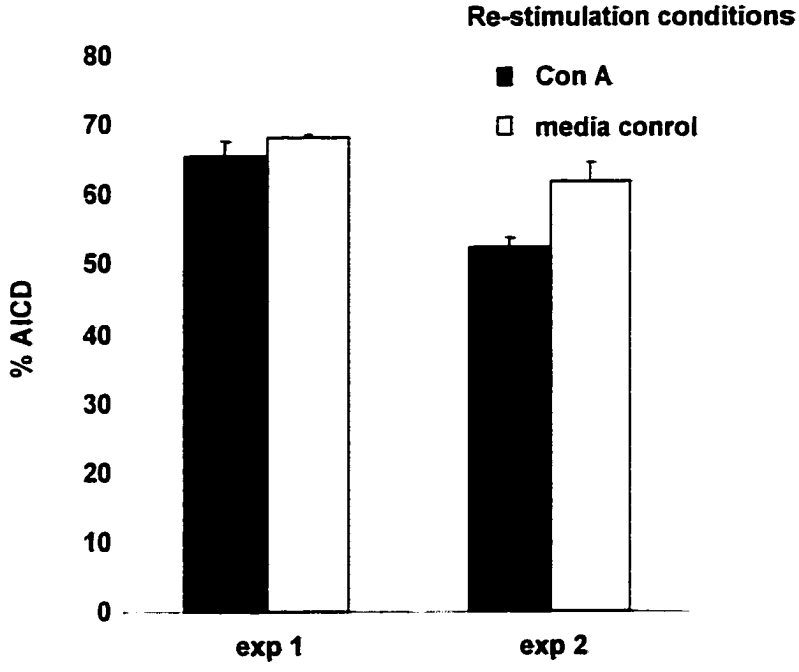
Re-stimulation conditions
Con A
media conrol
% AICD
80
70
60
50
40
30
20
10
0
exp 1
exp 2

Figure 6

Fig. 7 ***BALB/c spleen cells exhibit enhanced proliferation to Con A.***

Con A-induced proliferation of BALB/c and C57BL/6 spleen cells was assessed by ^{3}H-Thymidine incorporation. Splenocytes were isolated and stimulated with Con A (5 μg/ml) for 72 h before pulsing with ^{3}H- Thymidine. Data shown are expressed as a mean ± SD of triplicate wells and are representative of 3 separate experiments.

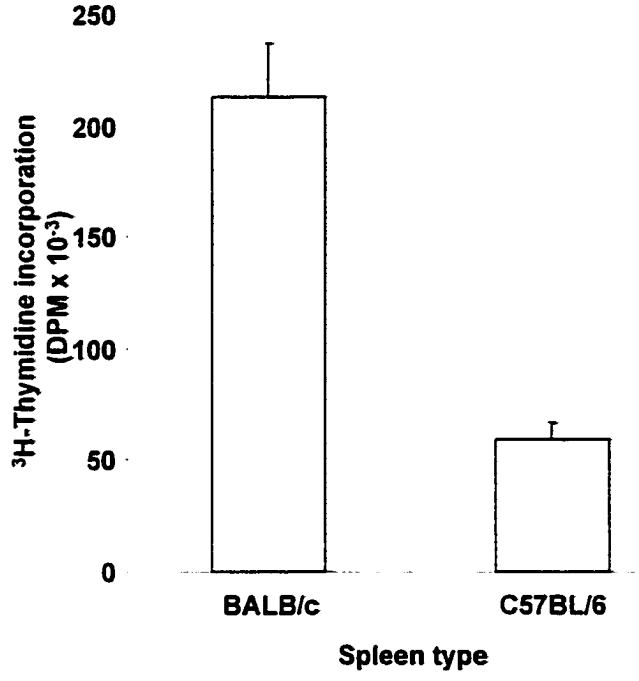
250
200
150
100
50
0
^{3}H-Thymidine incorporation
(DPM x 10^{-3})
BALB/c
C57BL/6
Spleen type

Figure 7

Dalhousie University

Timothy D.G. Lee, Ph.D.
Director of Research
Transplantation and Immunology
Research Laboratory
Tupper Medical Building
Halifax, Nova Scotia
Canada B3H 4H7
(902) 494-3882
FAX (902) 494-5125

July 8, 1999

Thesis Committee
Department of Microbiology and Immunology

RE: Rob Liwski

This letter is to confirm that the paper entitled "Prolongation of Allograft Survival by Nippostrongylus brasiliensis is Associated with Decreased Allo-specific CTL activity and Development of Tc2 Cells", on which we are joint authors with Rob Liwski, was work that was entirely of his conception and was his experimentation. In addition he interpreted the data and wrote the paper. Our role in this project was as mentors during the planning stages and throughout the data interpretation stages to provide input and discuss his ideas. The only part of this work that is not his own was the technical assistance given to him by Juan Zhou, who completed the heart transplants mentioned in this paper.

Timothy D.G. Lee, Ph.D.
Professor
Departments of Microbiology & Immunology and Surgery

Vivian McAlister, MD
Associate Professor
Department of Surgery

Dalhousie University

Department of Microbiology
and Immunology
Faculty of Medicine
Sir Charles Tupper Medical Building
Halifax, Nova Scotia
Canada B3H 4H7

(902) 494-3587
Fax (902) 494-5125

July 8, 1999

Thesis Committeee
Department of microbiology and Immunology

RE: Rob Liwski

This letter is to confirm that my input into the experimentation contained in the manuscript entitled "Prolongation of Allograft Survival by Nippostrongylus brasiliensis is Associated with Decreased Allo-specific CTL activity and Development of Tc2 Cells", on which I am a joint author with Rob Liwski, was limited to the technical help I providede by completing the heart transplant reffered to in this manuscript.

Sincerely

Juan Zhou M.Sc.
Department of Microbiology and Immunology

Dalhousie University

Timothy D.G. Lee, Ph.D.
Director of Research
Transplantation and Immunology
Research Laboratory
Tupper Medical Building
Halifax, Nova Scotia
Canada B3H 4H7
(902) 494-3882
FAX (902) 494-5125

July 8, 1999

Thesis Committee
Department of Microbiology and Immunology

RE: Rob Liwski

This letter is to confirm that the paper entitled "Nematode Infection Enhances Survival of Activated T cells by Modulating Accessory Cell Function", on which I was a joint authors with Rob Liwski, was work that was entirely of his conception and was his experimentation. In addition he interpreted the data and wrote the paper. My role in this project was as a mentor during the planning stages and throughout the data interpretation stages to provide input and discuss his ideas.

Timothy D.G. Lee, Ph.D.
Professor
Departments of Microbiology & Immunology
and Surgery

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