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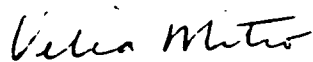


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ABSTRACT

Title of Dissertation: The function of CTLA4 during the *in vivo* immune response to infectious disease.

Velia Mitro. Doctor of Philosophy, 2000.

Dissertation directed by: Dr. William C. Gause, Professor, Department of Microbiology and Immunology.

CD4⁺ T cells play a key role in the adaptive immune response to foreign antigens. For T cells to be activated, two signals are required. The first signal is delivered through antigen recognition by the T cell receptor. A second, or costimulatory, signal is also required for optimal activation of T cells. CD28 ligation by B7 is a potent mediator of positive costimulation. In contrast, B7 ligation of CTLA4 (CD152), a homologue of CD28, provides a critical downregulatory signal. Recent data has suggested that CTLA4 may also share some stimulatory functions with CD28. Because costimulatory molecule interactions are critical for many immune responses, a greater understanding of CTLA4 function may promote development of immunotherapies where enhancement or inhibition of the immune response would be clinically beneficial. This research was directed at developing a greater understanding of CTLA4 function in the immune response to infectious disease. A murine model of gastrointestinal nematode infection, *Heligmosomoides polygyrus*, was utilized in this research to investigate the role of CTLA4 after onset of infection, once naïve T cells have differentiated to effector T cells.

These data support a negative regulatory role for CTLA4 late in the response. Blockade of CTLA4 by *in vivo* administration of anti-CTLA4 antibody enhanced the polarized Th2 response to *H. polygyrus*, resulting in increased serum concentrations of immunoglobulins, IL-4 secretion, and T and B cell activation. Further evidence of enhanced immune response upon CTLA4 blockade was provided in another nematode model, *Trichuris muris*. Anti-CTLA4 antibody treatment increased serum immunoglobulin concentrations and T and B cell activation. The treatment also caused immune deviation from Th1 to Th2, as evidenced by decreased IFN γ and increased IL-4 secretion. These data are consistent with a model for Th1 versus Th2 cell differentiation which describes the decision as based on the balance between strength of signal and innate response. On a molecular level, the phosphorylation events following CTLA4 blockade were examined, and intracellular binding partners for CTLA4 and CD28 were identified. The dependence of effector T cells upon continued combined CTLA4/CD28 signaling was also explored.

**THE FUNCTION OF CTLA4 DURING THE *IN VIVO* IMMUNE RESPONSE TO
INFECTIOUS DISEASE**

by

Velia Mitro

**Dissertation submitted to the Faculty of the Program in Molecular and Cell Biology of
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DEDICATION

This work is dedicated to my husband, Matthew Olson, and to my family. Thank you for your love and support.

QUOTATIONS

The world of learning is so broad, and the human soul is so limited in power! We reach forth and strain every nerve, but we seize only a bit of the curtain that hides the infinite from us. -Maria Mitchell

What is there that confers the noblest delight? What is that which swells a man's breast with pride above that which any other experience can bring to him? Discovery! To know that you are walking where none others have walked . . . -Mark Twain

Go confidently in the direction of your dreams. Live the life you've imagined. -Henry David Thoreau

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LIST OF ABBREVIATIONS

3AT, 3-aminotriazole; 5FOA, 5-fluoroorotic acid; APC, antigen presenting cell; BSA, bovine serum albumin; CTLA4, cytotoxic T lymphocyte antigen #4; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FACS, fluorescent activated cell sort; FCS, fetal calf serum; FITC, fluorescein-5-isothiocyanate; FS, forward light scatter; His, histidine; HP, *Heligmosomoides polygyrus*; HPRT, hypoxanthine-guanine ribosyl transferase; HRP, horse radish peroxidase; IFN γ , interferon-gamma; IL, interleukin; IL2R, interleukin-2 receptor; JNK, jun n-terminal kinase; kDa, kilodalton; LCMV, lymphocytic choriomeningitis virus; Leu, leucine; LiAc, lithium acetate; MFI, mean fluorescence intensity; MHCII, major histocompatibility complex II; MLN, mesenteric lymph node; NaCl, sodium chloride; NaOH, sodium hydroxide; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PEG, polyethylene glycol; PI-3K, phosphatidylinositol-3 kinase; PP, Peyer's patch; RT-PCR, reverse transcription-polymerase chain reaction; SA-PE, streptavidin-phycoerythrin; SC, synthetic complete; SDS, sodium dodecyl sulfate; SE, standard error; SEM, standard error of the mean; SH2, src homology 2; SH3, src homology 3; TCR, T cell receptor; Th1, T helper 1; Th2, T helper 2; TM, *Trichuris muris*; Trp, tryptophan; Ura, uracil.

I. Introduction

A. The two-signal model of T cell activation

Understanding the biochemical and cellular events which regulate the immune response against foreign antigens is an important goal of research in immunology. CD4⁺ helper T cells play a key role in these events. At the initiation of the response to a specific antigen, naïve Th cells must recognize the antigen expressed on the surface of professional antigen presenting cells (APCs) such as macrophages, dendritic cells, or B cells. APCs continually sample the extracellular milieu by endocytosis or phagocytosis of microbes, particulates or soluble matter. Once internalized, the proteins in these antigenic materials are proteolytically degraded into smaller fragments and bound to MHCII molecules. The antigen/MHCII complexes are transported to the surface of the APC. T cells become activated through cell-cell interaction with APCs, when the antigen-specific T cell receptor (TCR) recognizes the antigen/MHCII complex displayed on the surface of the APC. Activated T cells amplify and regulate the effector functions of the immune response. This regulation occurs through the release of cytokines and cell-cell interactions with immunoglobulin-producing B cells, mast cells, cytotoxic T cells, and other cell types that serve to limit infection. Research is needed into the activation of T cells, to aid in the development of therapies to promote protection against infection and prevent autoimmune disease.

Substantial research shows that T cell activation requires two signals from the APC (Bretscher, 1992). The first is the antigen-specific interaction between the TCR and the antigen/MHCII complex. The second signal, or costimulatory signal, is not antigen-

specific. Numerous receptor/ligand pairs expressed on the surface of T cells and APCs have been suggested to function as costimulatory receptors. The most well-characterized of these is the interaction between CD28 (expressed on the surface of T cells) and B7-1 (CD80) or B7-2 (CD86) (expressed on the surface of APCs). In the absence of costimulation, TCR-mediated delivery of signal 1 can result in incomplete activation of T cells, anergy (unresponsiveness), or cell death. CD28 ligation by B7 molecules aids in the complete activation of T cells upon antigen recognition by increasing T cell proliferation, IL-2 secretion and IL-2R expression, and Bcl-xl expression. Costimulatory interactions are critical for many immune responses, including response to infection, autoimmunity, transplant rejection, and tumor immunity.

This doctoral dissertation research focuses on the role of cytotoxic T lymphocyte antigen #4 (CTLA4), a homologue of CD28 (Harper et al., 1991). CTLA4 is expressed on T cells, and binds B7-1 and B7-2 (Linsley et al., 1991). At first, it was thought that CTLA4 ligation by B7 molecules would also function as a positive costimulatory signal (Linsley et al., 1992). However, subsequent studies showed that CTLA4 interactions with B7 molecules provide a critical downregulatory signal for T cells (McCoy and Le Gros, 1999). Mice lacking the CTLA4 gene developed massive polyclonal expansion of T cells with multiorgan tissue infiltration and destruction. These mice died within a few weeks after birth with myocarditis and pancreatitis. *Ex vivo*, T cells from CTLA4-deficient mice proliferated spontaneously, indicating that the T cells had been activated *in vivo* (Tivol et al., 1995; Waterhouse et al., 1995). The lymphoproliferation *in vivo* is initiated by CD4⁺ T cells, as anti-CD4 antibody treatment of CTLA4-deficient mice abrogates this phenotype (Chambers et al., 1997). Blocking all B7-ligand interactions

prevents the severe phenotype of these mice, suggesting that CD28 costimulation is required for the lymphoproliferation and tissue destruction (Tivol et al., 1997). These experiments indicate that CTLA4 supplies a critical negative signal to CD4⁺ T cells.

While the downregulatory role of CTLA4 is consistent with these and other *in vivo* and *in vitro* experiments, recent data have suggested that CTLA4 may substitute for CD28 in providing a positive signal in some cases. CD28-deficient mice have T cell-dependent responses that are in most aspects impaired; however, these mice are capable of mounting strong immune responses in some model systems (Gause et al., 1997a; Shahinian et al., 1993; Kawai et al., 1996). Because CTLA4 is the only known receptor for B7 molecules other than CD28, these results suggest that CTLA4/B7 interactions can provide positive costimulation. While these data do not refute the conclusions that CTLA4 supplies a negative signal in the other systems examined, these data do raise the question of whether CTLA4 may also share some stimulatory functions with CD28.

Understanding how CTLA4 functions in infectious disease may provide new tools to optimize an individual's immune response against a pathogen. The purpose of this doctoral dissertation research is to characterize the role of CTLA4 in regulating T cell-mediated immune responses to infectious disease models, and to address the molecular mechanism of CTLA4 signaling.

In this chapter, I will discuss CTLA4 structure and expression, and address their relevance to the function of CTLA4 in comparison with CD28. Next, I will discuss previous reports that support an inhibitory role for CTLA4, and some conflicting data that remain to be resolved. Current knowledge of the mechanism of CTLA4 inhibitory action and CTLA4-mediated signal transduction events will also be presented. The chapter

concludes with an introduction of the experimental models of infectious disease used in this dissertation, and an outline of the experimental design of the work presented in this volume.

B. CTLA4 and CD28 structure and expression

CTLA4 is a glycosylated T cell surface receptor and a member of the immunoglobulin superfamily (Brunet et al., 1987). Homodimers of CTLA4 molecules are linked by a single disulfide bond in the extracellular domain (Figure 1) (Linsley et al., 1995; Walunas et al., 1994). The sequences of murine and human CTLA4 are well conserved (76% amino acid identity), and the cytoplasmic domains are 100% conserved (Dariavach et al., 1988; Harper et al., 1991). The complete conservation of cytoplasmic domains is notable as it may indicate conservation of critical function in the cytoplasmic domain. CTLA4 also shares ~30% identity overall with CD28, another immunoglobulin superfamily member expressed in the surface of T cells (Figure 2) (Harper et al., 1991). The genes encoding CTLA4 and CD28 colocalize on mouse chromosome 1 band C and on human chromosome 2q33-34 (Howard et al., 1991; Lafage-Pochitaloff et al., 1990). The human genes are estimated at only 25-150 kb apart (Balzano et al., 1992). The proximity of the genes, along with similar genomic structure, suggests that these genes arose by duplication (Harper et al., 1991). CTLA4 and CD28 also share an extracellular motif, MYPPPY, and it is through this region that they bind B7-1 and B7-2 (Peach et al., 1994). CTLA4 binds B7 molecules with 10-fold higher affinity than does CD28, with the difference in K_d due to a faster dissociation rate for CD28 (Linsley et al., 1991; van der

Figure 1. Structure of CTLA4. CTLA4 disulfide-linked homodimer is illustrated.

Indicated in the figure are the transmembrane domain and the domain homologous to an immunoglobulin variable region.

Structure of CTLA4

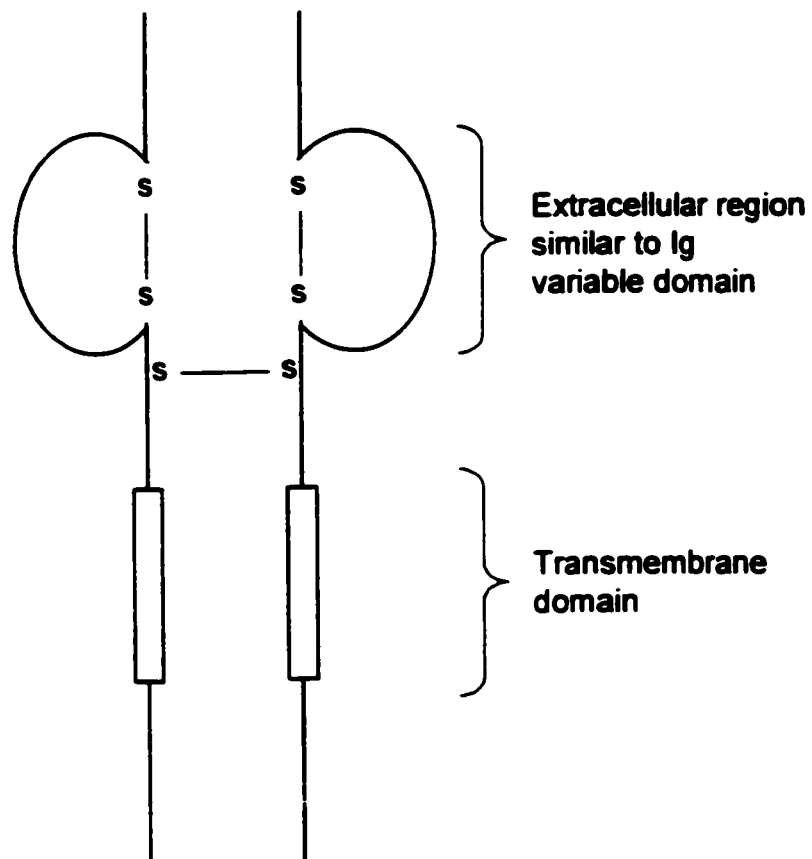
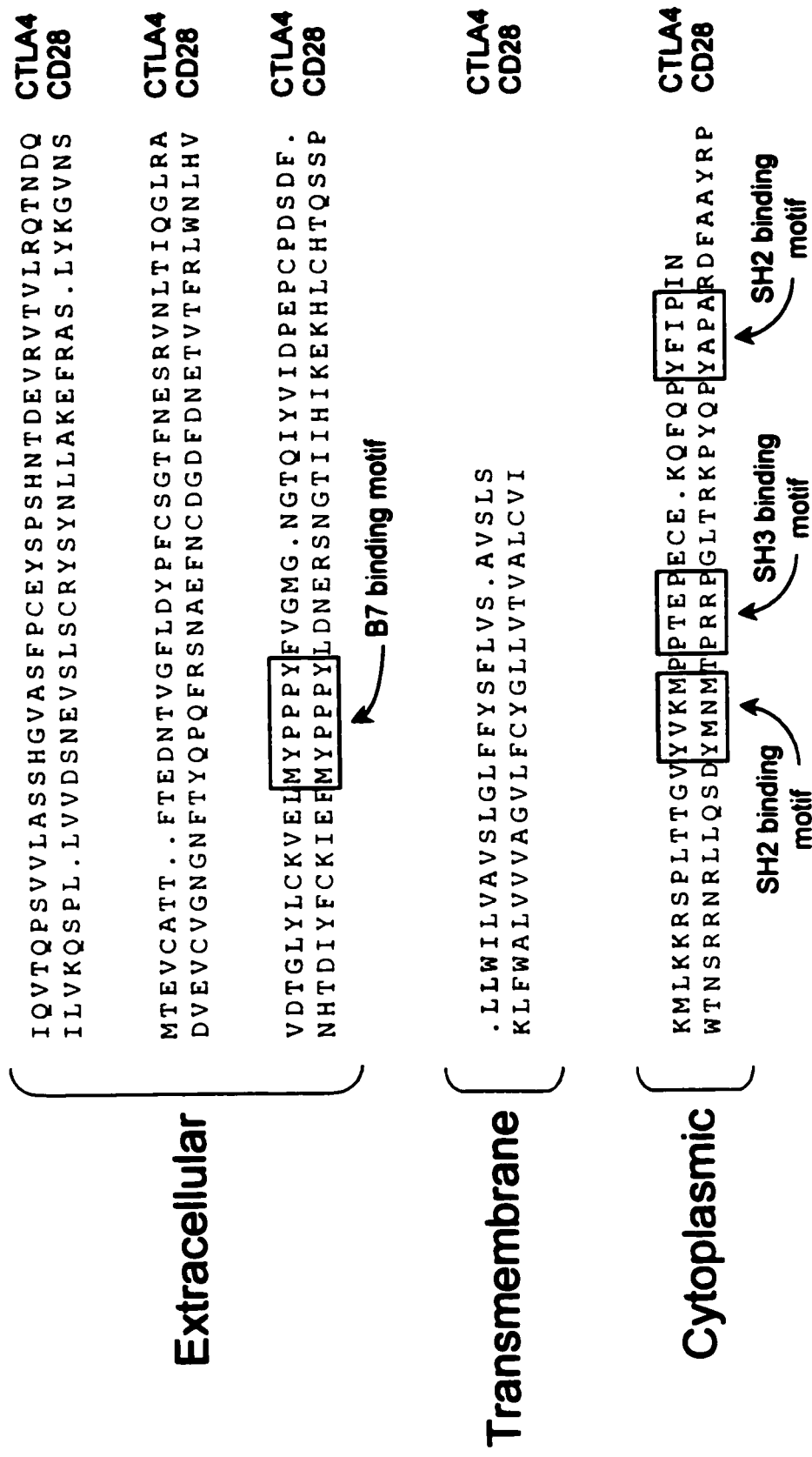


Figure 2. Sequence alignment of murine CTLA4 and CD28. The sequences encoded by the murine CTLA4 (Brunet et al., 1987) and CD28 (Harper et al., 1991) genes are compared. The leader sequences have been omitted. The shown sequences correspond to the mature polypeptides, beginning with amino acid 38 for CTLA4 and amino acid 22 for CD28 (when counted from the methionine start codon). The extracellular, transmembrane, and cytoplasmic domains are indicated by brackets. The B7, SH2, and SH3 binding motifs are boxed.

Sequence alignment of murine CTLA4 and CD28



Merwe et al., 1997; Greene et al., 1996). This difference may produce preferential binding of B7 molecules to CTLA4.

CTLA4 and CD28 expression appears to be restricted primarily to CD4⁺ and CD8⁺ T cells, though expression of CTLA4 on B cells activated *in vitro* has also been reported (Kuiper et al., 1995; Brunet et al., 1987). The temporal patterns of CTLA4 and CD28 are very different. Unlike CD28, which is abundantly expressed on both naïve and effector T cells, CTLA4 expression is not detectable on the surface of naïve T cells (Linsley et al., 1992; Freeman et al., 1992; Scheipers and Reiser, 1998). It is rapidly upregulated upon T cell activation, with CTLA4 mRNA transcripts becoming detectable by 1 hour after T cell activation (Lindsten et al., 1993). TCR signaling, CD28 costimulation, and IL-2 are required for maximal expression of CTLA4 (Alegre et al., 1996). In addition to increasing the transcription of the CTLA4 gene, CD28 costimulation also increases the stability of CTLA4 mRNA (Finn et al., 1997). CTLA4 protein is expressed on the cell surface by 24 hours, and peaks 48-72 hours after T cell activation. Even at maximal expression, the majority of CTLA4 protein is not found on the T cell surface, but in a perinuclear Golgi compartment (Linsley et al., 1992; Leung et al., 1995). CTLA4 protein is continually and rapidly shuttled between intracellular stores and the plasma membrane (Linsley et al., 1996; Alegre et al., 1996). Tight control of CTLA4 gene transcription, mRNA stability, and intracellular localization all serve to limit the surface expression of CTLA4 to low levels.

Several papers have addressed the mechanism of CTLA4 intracellular trafficking (Shiratori et al., 1997; Bradshaw et al., 1997; Chuang et al., 1997; Zhang and Allison, 1997; Linsley et al., 1996). CTLA4 is endocytosed by a clathrin-mediated mechanism, as

CTLA4 internalization is prevented by conditions that prevent the assembly of clathrin-coated pits. CTLA4 is then shuttled to an intracellular site that co-localizes with the transferrin receptor. It is from this site that CTLA4 is continually shuttled to and from the plasma membrane. *In vitro*, the cytoplasmic domain of CTLA4 binds AP50 (also called $\mu 2$), which is the component of clathrin adapter AP-2 that mediates the interaction with tyrosine-based internalization motifs of target proteins. The interaction between CTLA4 and AP50 is dependent on the membrane-proximal tyrosine (Y165). *In vivo*, a six amino-acid motif from CTLA4 including Y165 has been shown to confer the ability to be internalized upon a fusion protein that without the motif remains on the cell surface, indicating that the region that binds AP50 is functionally responsible for internalization. Phosphorylation of Y165 inhibits the binding between CTLA4 and AP50 *in vitro*, and mutation of Y165 to phenylalanine prevents internalization of CTLA4 *in vivo*, suggesting that phosphorylation of this amino acid regulates the movement of CTLA4 between intracellular stores and the plasma membrane.

CD28 is not trafficked intracellularly in the same manner as CTLA4 (Chambers and Allison, 1997; Lenschow et al., 1996). On resting and effector T cells, the majority of CD28 is expressed on the cell surface, and there is no evidence that CD28 directly binds components of clathrin adapter complexes (Schneider et al., 1999; Chuang et al., 1997). However, the expression of CD28 on the surface is not static. A portion of the CD28 molecules undergo clathrin-mediated endocytosis, but only upon receptor ligation (Cefai et al., 1998). The endocytosed CD28 is sorted to two different compartments. About half is returned to the cell surface, and half is degraded in a lysosomal compartment. Mutations in the cytoplasmic domain of CD28 that inhibit internalization

block costimulation, suggesting that the internalization is important for the costimulatory function of CD28. One potential explanation is that CD28 interacts with intracellular signaling proteins after internalization to transduce the costimulatory signal. The mechanism of internalization is unknown, but it is clear that CD28 localization is not analogous to that of CTLA4.

Another important aspect of CTLA4 expression is its polarized localization. Both intracellular and surface CTLA4 are focused towards the site of TCR engagement (Linsley et al., 1996). This effect can be mediated by alloantigen-bearing cells or by anti-CD3 stimulation, and is not dependent on CD28 stimulation or CTLA4 interaction with B7 molecules. The polarized localization occurs within two hours, and the increased number of CTLA4 molecules at the cell surface at the site of TCR engagement come from existing intracellular stores. The increased steady state concentration of surface CTLA4 is a result of increased rate of transport to the cell surface, rather than a decreased rate of uptake. In addition to limiting its surface expression to low levels, intracellular trafficking of CTLA4 provides a potential mechanism for regulating the proximity of CTLA4 to the TCR and CD28. The dynamic regulation of CTLA4 localization may control the ability of CTLA4 to interact with the signaling events that mediate T cell activation. Recently, the importance of focal receptor organization for the activation of T cells has become apparent. The formation of glycolipid-enriched microdomains (GEMs or rafts) is required for efficient TCR signal transduction (Xavier et al., 1998), and CD28 signaling contributes to the recruitment of kinase-rich rafts to the site of site of TCR engagement (Viola et al., 1999). A model for an "immunological synapse" has been proposed, wherein T cell activation is dependent of the formation of a specific pattern of

receptors at the site of T cell contact with the APC. In this model, efficient TCR signaling only occurs when a central cluster of T cell receptors and associated signaling molecules are surrounded by a ring of specific cell-cell adhesion molecules (Grakoui et al., 1999; Dustin and Shaw, 1999; Monks et al., 1998; Wulfig and Davis, 1998). It has been proposed that costimulation is mediated in part by recruitment of the TCR and other molecules to the immunological synapse (Viola et al., 1999; Yashiro-Ohtani et al., 2000). This recent work highlights the potential importance of cellular transport and localization in CTLA4 function.

C. CTLA4 function

As discussed above, CTLA4-deficient mice provide dramatic evidence for the critical downregulatory role of CTLA4. These mice display massive proliferation of peripheral T cells, tissue destruction resulting from lymphocytic infiltration, and early death. These defects have been linked to B7-dependent activation of CD4⁺ T cells. These results have been interpreted to mean that in normal mice, CTLA4 counters the activation induced by antigenic stimulation plus CD28-mediated costimulation. In addition to these results, there is considerable *in vitro* and *in vivo* evidence supporting the downregulatory role of CTLA4.

In vitro, when anti-CTLA4 antibody is added to T cells being stimulated with allogeneic APCs, the proliferation of the T cells is augmented (Walunas et al., 1994). The effect is dependent on stimulation through the TCR because anti-CTLA4 antibody had no effect when syngeneic APCs were used. It was determined that the soluble antibody was blocking a negative CTLA4 signal (instead of providing a positive signal)

because Fab fragments of the antibody also augment proliferation. Furthermore, crosslinking the antibody enabled it to transduce a negative signal, inhibiting proliferation. In another *in vitro* study, blockade of CTLA4 with antibody or Fab fragments increased IL-2 production (Krummel and Allison, 1995).

One avenue of *in vivo* investigation that has shed light on the roles of CD28 and CTLA4 has been experiments with CD28-deficient mice (Green et al., 1994). The T cells of these mice have greatly diminished ability to respond. When immunized with protein antigen, the proliferation of the T cells is reduced (although not absent). *Ex vivo*, CD28-deficient T cells were shown to have reduced proliferation in response to B7 molecules. Immunoglobulin responses were also reduced (Shahinian et al., 1993). While T cell-dependent responses are not universally abrogated by loss of CD28, these experiments confirm that CTLA4 and CD28 do not have completely redundant roles.

Blockade of CTLA4 signaling *in vivo* by administration of anti-CTLA4 antibody has also been used to demonstrate the negative regulatory role of CTLA4. Anti-CTLA4 antibody administration increases clonal expansion of antigen-specific TCR-transgenic T cells (Kearney et al., 1995). The first study that demonstrated enhancement of T cell effector function by CTLA4 blockade *in vivo* was in a tumor rejection model (Leach et al., 1996). Normal mice were injected with colon carcinoma cells, which grew rapidly in size in mice that received no further treatment, until euthanasia was required 35 days later. Anti-CTLA4 treatment of these mice enabled them to reject the tumors within 20-30 days. Blockade of CTLA4 also caused increased protection during secondary challenge, indicating increased immunological memory. It is interesting that these results were obtained with wild-type tumors, as opposed to tumor cells which had been

engineered to enhance antitumor immunity. However, there was little information in this report to suggest how anti-CTLA4 antibody treatment induced the increased antitumor immunity.

Blockade of CTLA4 has also been used to increase protection in an infectious disease model (McCoy et al., 1997). Mice infected with the intestinal nematode parasite *Nippostrongylus brasiliensis* and treated with anti-CTLA4 antibody had decreased intestinal worm burden by seven days after inoculation and decreased parasite egg production by day eight after infection. The enhanced protection was associated with increased IL-4 and IL-5 production, and with increased lymphocyte numbers in the draining lymph nodes. It should be noted that these experiments were conducted in a strain of mouse that is not susceptible to chronic *N. brasiliensis* infection, and the increased protection was observed as an increase in kinetics of parasite expulsion. If it could be shown that CTLA4 blockade could induce resistance in mice that normally develop a chronic infection, then this research may have clinical application. In addition, these mice were treated with anti-CTLA4 antibody beginning on the day of infection. A more clinically relevant model would be one in which intervention was begun after infection had been established. Both of these issues are addressed in my research, which utilized a model system wherein mice were inoculated with a pathogen that normally establishes a chronic infection. In addition, the role of CTLA4 after infection has been established was investigated by blocking CTLA4 function late after inoculation.

Although these and other experiments supply a preponderance of evidence that CTLA4 supplies a negative signal (Oosterwegel et al., 1999a; Saito, 1998; Greenfield et al., 1998; Scheipers and Reiser, 1998), there are a few exceptions. As mentioned earlier,

CD28-deficient mice have T cell responses that are in most aspects impaired, but in some cases the T cells are still able to respond. The cytotoxic response to LCMV is intact (Shahinian et al., 1993), as is the rejection of skin allografts (Kawai et al., 1996). CD28-deficient mice and wild-type mount comparable responses to *Heligmosomoides polygyrus*, another intestinal nematode parasite (Gause et al., 1997a). Mice resistant to *Leishmania major* mount a normal T cell response and control the infection in the absence of CD28 (Brown et al., 1996). One potential explanation is that other non-B7-dependent compensatory pathways exist that mediate activation when CD28 is absent from ontogeny (Saito et al., 1998). However, CD28-deficient T cells can be induced to proliferate *ex vivo* by anti-CD3 antibody plus B7 stimulation (although to a lesser extent than wild-type cells), and the proliferation is blocked by anti-CTLA4 antibody or Fab fragments (Wu et al., 1997). These results can be interpreted to mean that in the absence of CD28, CTLA4 ligation by B7 can give a positive signal. In addition to data generated from CD28-deficient *ex vivo* T cells, evidence for a positive role for CTLA4 comes from a model using B7 with a point mutation that no longer binds CD28, but still binds CTLA4 (Zheng et al., 1998). Expression of this mutant B7 on thymoma or plasmacytoma cells enhances the antitumor immunity. Data like these have prompted some to take a cautious view of the role of CTLA4 in downregulation of the immune response (Liu, 1997). An understanding of the signal transduction pathways and mechanism of CTLA4 action is needed to resolve these conflicting results.

D. CTLA4 mechanism

How does CTLA4 provide a negative signal? Recent work has shed light on its mechanism, as well as supporting its downregulatory role. There are three models for how CTLA4 functions:

1. CTLA4 could outcompete CD28 for B7 molecules, due to its higher affinity for B7.
2. CTLA4 could antagonize TCR-mediated signals.
3. CTLA4 could antagonize CD28-mediated signals.

These three potential mechanisms are not mutually exclusive, and there is evidence supporting each of them.

The preferential binding of B7 for CTLA4 over CD28 fits well with their expression patterns. At the initiation of a response, CTLA4 expression on the surface is extremely low, allowing B7 to interact with CD28. Surface expression of CTLA4 is upregulated upon T cell activation, preventing CD28 from interacting with B7, terminating the costimulation. However, the strict conservation of the cytoplasmic domain of CTLA4 suggests that a critical signaling function is also conserved. The conservation argues against outcompetition as a sole mechanism of CTLA4 action, though it may have some functional importance.

Experiments using CD28-deficient mice have supported CTLA4 antagonism of TCR-mediated signals. Cardiac allograft survival is significantly prolonged in CD28-deficient mice, with associated decreases in cellular infiltration, destruction of cardiomyocytes, and expression of inflammatory cytokines when compared with wild-type mice (Lin et al., 1998). When B7 ligand interactions are blocked in CD28-deficient mice (by either CTLA4Ig or combined anti-B7-1 and anti-B7-2 antibodies), the rejection

of the cardiac allografts is accelerated. Treatment with anti-CTLA4 antibody also accelerated rejection, confirming that CTLA4 was the B7 receptor mediating the inhibition in CD28-deficient mice. In the absence of CD28, CTLA4 is still able to inhibit T cell activation. Another study also used CD28-deficient mice, but in this case they were bred into a TCR transgenic/Rag-2-deficient background, so that all of the T cells would be specific for one alloantigen, Ld (Fallarino et al., 1998). These mice rejected tumors that expressed Ld alloantigen. *Ex vivo*, their T cells also responded to Ld alloantigen in the absence of B7. The response of these cells was strongly inhibited by costimulation by B7, which was blocked by anti-CTLA4 antibody. This model also indicates that CD28 antagonism is insufficient to explain CTLA4 negative signals, and suggest that CTLA4 may inhibit T cell activation by counteracting TCR-mediated signals.

There are two lines of evidence supporting CTLA4 inhibition of CD28 costimulatory signals as its mechanism for inhibiting T cell activation. The first comes from a paper in which the effects of CTLA4 signaling induced by crosslinked antibody were most pronounced under conditions where T cells received optimal CD28 costimulation (Walunas et al., 1996). Because sensitivity to negative control by CTLA4 depends on the strength of CD28 costimulation, the data suggest that CTLA4 ligation is blocking CD28-dependent activation. The second line of evidence concerns the role of CD28 in IL-2 upregulation. Induction of IL-2 production is a key feature of CD28-mediated costimulation of T cells activated *in vitro*. The ability to oppose CD28-induced elevations in IL-2 is an important function of CTLA4 (Krummel and Allison, 1996; Blair et al., 1998; Walunas et al., 1996; Vandenborre et al., 1999). Because TCR signaling

alone is unable to stimulate high levels of IL-2 secretion, these results indicate that CTLA4 negative signals are blocking a function which is CD28-specific.

However, not all CD28-specific signals are inhibited by CTLA4. CD28 induction of IL-2 is caused by both increased transcription of the IL-2 gene and increased stability of IL-2 mRNA. CTLA4 does not inhibit CD28-mediated stabilization of IL-2 mRNA, only the transcriptional activity of the IL-2 gene (Brunner et al., 1999). The ability of CTLA4 ligation to inhibit cell cycle progression was thought to be due to reduction in IL-2 (which in turn was CD28-dependent), but in fact addition of exogenous IL-2 does not fully reverse inhibition of proliferation by CTLA4, and CD28-dependent proliferation has been found to have both IL-2-dependent and IL-2-independent aspects (Brunner et al., 1999; Appleman et al., 2000). CD28 ligation also induces expression of Bcl-xl, a gene necessary for long-term survival of effector T cells, and CTLA4 ligation does not reduce expression of this gene (Sperling et al., 1996; Blair et al., 1998). While the three models for CTLA4 action presented here provide a framework for discussion, CTLA4-mediated signals are likely to interact with TCR and CD28 signals in multiple, complex ways to inhibit T cell activation. Dissecting the signal transduction events that occur within the T cell upon CTLA4 ligation would aid in understanding how CTLA4 influences T cell stimulation.

E. CTLA4 signaling

As mentioned earlier, human and murine CTLA4 molecules are 100% conserved in their cytoplasmic domains, suggesting that a critical function is mediated by those sequences. While there is 30% overall identity between CTLA4 and CD28, the

conservation is primarily in the extracellular domains. There is very limited conservation between CTLA4 and CD28 cytoplasmic domains, opening the door for differential signaling. Neither cytoplasmic domain exhibits any intrinsic kinase activity, but CTLA4 and CD28 contain several potential phosphorylation sites. The current model is that TCR stimulation and B7 ligation directs tyrosine and/or serine/threonine phosphorylation of CD28. The phosphorylation would allow association of CD28 with intracellular signaling proteins that are recruited to specific phosphorylated sites on the cytoplasmic domain of CD28. CTLA4 is upregulated and brought to the surface, perhaps in close proximity to the TCR, where TCR-associated tyrosine kinases would associate with and phosphorylate CTLA4, again allowing recognition by intracellular signaling proteins.

The kinases that initiate the signaling cascades by CD28 and CTLA4 have not been firmly established, but there is evidence that Src-family kinases may be involved. Specifically, Lck and Fyn have been implicated in CD28 phosphorylation (Leung et al., 1999; Slavik et al., 1999). Lck, Fyn, and Lyn have been shown to associate with and phosphorylate CTLA4 since this project was begun (Miyatake et al., 1998; Chuang et al., 1999). The association was identified in cells that had been transiently co-transfected with the kinase and with CTLA4, and experimentation is needed to show that Src-family kinases phosphorylate CTLA4 in the T cell, and that Src-family phosphorylation of CTLA4 is necessary for CTLA4 signal transduction.

A report that examined the downstream signal transduction events utilized T cells from CTLA4-deficient mice (Marengere et al., 1996). These mice, which have constitutively activated Lck and Fyn kinases, display increased tyrosine phosphorylation of TCR CD3 ζ , ZAP-70 kinase, and other intracellular proteins. The Ras pathway was

also found to be activated. To investigate the cause of increased phosphorylation, *in vitro* experiments were conducted with CTLA4 and SHP-2 protein tyrosine phosphatase. CTLA4 binds SHP-2 in immunoprecipitation assays from *ex vivo* activated normal T cells, and CTLA4-associated SHP-2 has phosphatase activity toward p52-SHC, a regulator of the Ras pathway. The association between CTLA4 and SHP-2 was later confirmed and found to be dependent on tyrosine phosphorylation of CTLA4 by Lck or Fyn in cotransfection experiments (Miyatake et al., 1998; Chuang et al., 1999). These data suggest that the mechanism of CTLA4 negative regulation is mediated by recruitment of phosphatases which dephosphorylate intracellular proteins involved in TCR and CD28 signal transduction, thereby antagonizing the positive signal. While the paper that first identified the association with SHP-2 indicated that CTLA4 inhibition occurred somewhat downstream, in the Ras pathway (Marengere et al., 1996), a subsequent paper showed that CTLA4 associates with the TCR, that the association is enhanced by phosphorylation by Lck, and that CTLA4 could recruit SHP-2 to dephosphorylate CD3 ζ directly (Lee et al., 1998). SHP-2 dephosphorylation of CD3 ζ also prevented its association with CTLA4. Thus, CTLA4 also may interfere with the TCR signal at an upstream site.

SHP-2 was seen to specifically associate with the same motif in CTLA4 as does AP50. The phosphorylation state of this motif (YVKM) determines whether SHP-2 or AP50 binds to the peptide sequence. When the sequence is tyrosine phosphorylated, SHP-2 binds and presumably transduces the CTLA4 signal; when the sequence is not phosphorylated, AP50 binds and mediates endocytosis (Shiratori et al., 1997; Miyatake et al., 1998). This was the first time a single phosphorylation site differentially regulated

endocytosis and activation of signal transduction. Since this discovery, another molecule, TGN38, was also found to be subject to differential endocytosis/signal transduction through phosphorylation of a single site (Stephens and Banting, 1997). These tyrosine motifs may play an important role in the integration of localization and activation signals. Subsequent experimentation has also revealed that SHP-2 may not directly associate with CTLA4, as had been previously indicated, but the association may occur through an intermediate, perhaps PI-3K (Schneider and Rudd, 2000).

Downstream of the initial signal transduction events, CTLA4 ligation inhibits TCR/CD28 signaling at the level of extracellular signal-regulated kinase (ERK) and jun n-terminal kinase (JNK) activation (Calvo et al., 1997). It was reported that CD3 ζ phosphorylation was unaffected by CTLA4 signaling, and the reason for the discrepancy between the results of Calvo and Lee is unclear. The identification of ERK and JNK as points where CTLA4 can affect T cell activation fits with the observed downregulation of IL-2 by CTLA4, as ERK and JNK activation is required for IL-2 transcription. Likewise, there have been reports that CTLA4 reverses CD28-mediated activation of NF-kappaB, AP-1 and NFAT, transcription factors essential for IL-2 gene expression (Olsson et al., 1999; Fraser et al., 1999; Pioli et al., 1999). Taken together, CTLA4 inhibition of TCR/CD28 signaling has been demonstrated at several stages, from limiting phosphorylation at a membrane proximal site, to limiting activity of important transcription factors.

CTLA4 interacts with PI-3K, and ligation with anti-CTLA4 antibody results in increased PI-3K activity (Hutchcroft and Bierer, 1996; Saito, 1998). A considerable body of work has demonstrated an association between CD28 and PI-3K, and it may be

important for IL-2 production and Bcl-xl upregulation (Slavik et al., 1999). If PI-3K is important for CD28 to deliver a positive signal, what is the functional consequence of PI-3K recruitment by CTLA4? One possibility is that CTLA4 sequesters PI-3K away from CD28, by nature of its primarily intracellular localization, thereby preventing signal transduction by CD28. Or another possibility is that CTLA4 and CD28 have common intracellular binding partners, indicating a partial overlapping of function. This hypothesis provides a basis for the ability of CTLA4 to provide costimulation under some circumstances.

Recently, two reports provided data that call into question whether activation-induced tyrosine phosphorylation of CTLA4 initiates recruitment of SHP-2 or other molecules that inhibit TCR/CD28 activation. Both showed that tyrosine phosphorylation is not required for the downregulatory signal. Inhibition of ERK activation and IL-2 production occurs through mutant CTLA4 lacking both tyrosines, and also in the absence of Lck and ZAP-70 (Baroja et al., 2000). It was suggested that phosphorylation of CTLA4 is required only in that it allows CTLA4 to be retained on the cell surface (and to interact with B7). Association with signal transduction molecules would then occur independently of tyrosine phosphorylation. The second study also demonstrated CTLA4 inhibition of IL-2 production through mutants lacking tyrosine residues, and also localized the region important for signal transduction to 11 amino acids in the n-terminal half of the cytoplasmic domain (Cinek et al., 2000). This region includes the YVKM motif that had been identified as the binding site for AP50, PI3-K and SHP-2. Its does not include the adjacent proline-rich SH3 binding motif, or the c-terminal YFIP SH2 binding motif. It is possible that CTLA4 recruits signaling molecules in both

phosphotyrosine-dependent and -independent interactions. Consistent with this model, IL-2 production was less efficiently inhibited by the CTLA4 construct lacking tyrosines than by wild-type CTLA4. Also, other downstream effects of CTLA ligation (for example, proliferation) were not quantitated with the mutant CTLA4 construct, and may have a greater dependence on tyrosine phosphorylation than does IL-2 production. A more complete understanding of the signal transduction pathways utilized by CTLA4 would also promote understanding of its mechanism and function. The research presented in this dissertation investigates the signal transduction events following CTLA4 ligation, by looking at tyrosine phosphorylation events, and by searching for proteins which bind the cytoplasmic domain of CTLA4.

F. Th1 and Th2 differentiation

Helper T cells (Th) can be separated into two different categories, depending on the cytokines they secrete (Mosmann and Coffman, 1989). Th1 cells secrete interferon- γ , IL-2 and IL-12, and Th2 cells secrete IL-4, IL-5, IL-6, IL-9, and IL-13. Th1 and Th2 responses also differ in the activities of non-T cell populations, in the isotype of immunoglobulin produced, and against which type of infection it protects. Th1 responses are characterized by: natural killer cell and macrophage activity; serum IgG2a production; protection against bacteria, viruses and intracellular parasites. Th2 responses include: mast cell activation and eosinophilia; serum IgE and IgG1 production; protection against extracellular parasites.

Often, the Th1 versus Th2 cell differentiation decision can determine whether the immune response to a pathogen is protective or not. A well-documented example of this

dichotomy is the response to *Leishmania major* infection. Different strains of mice respond with either Th1 or Th2 characteristics, depending on their genetic background. Strains of mice that develop a Th1 response to the protozoan parasite *L. major* resolve the infection, and develop resistance to reinfection. On the other hand, a Th2 response is not protective, and strains of mice that develop a Th2 response are unable to control the infection. The importance of the cytokine milieu in determining whether a response promotes clearance of the infection is illustrated in mice that have received antibody intervention to alter their cytokine profiles. When resistant mice are depleted of IFN γ , by administration of anti-IFN γ antibody, they respond to *L. major* with Th2 characteristics and they are no longer able to control the infection (Belosevic et al., 1989). Conversely, susceptible mice depleted of IL-4 by anti-IL-4 antibody treatment develop a Th1 response that allows them to resolve the infection (Chatelain et al., 1992).

In the case of nematode parasites, often a Th2 response is protective, and a Th1 response leads to susceptibility (Finkelman et al., 1997). Effector functions associated with protective responses to nematode parasites (IgE production, mastocytosis, eosinophilia) are dependent on Th2 cytokines (Urban, Jr. et al., 1992). Th2 responses are effective in controlling *Trichuris muris* infection, and whether or not an appropriate response is generated varies with the genetic background of the mouse. As with responses to *L. major*, a Th2 response can be converted to a Th1 response and vice versa by manipulating the cytokine microenvironment. Experiments utilizing cytokine depletion, blockade of cytokine receptors, and gene disruption have demonstrated the importance of IL-4, IL-9 and IL-13 for development of a protective Th2 response to *T. muris*, and of IFN γ and IL-12 for the development of an ineffective Th1 response (Else et

al., 1994; Richard et al., 2000; Faulkner et al., 1998; Bancroft et al., 1998; Bancroft et al., 1997).

As it does with immune responses to infectious pathogens, the decision to develop a Th1 versus a Th2 response to environmental or self antigens also plays an important role in autoimmune disease and allergy. Th1 versus Th2 decisions are important in the immunopathology of multiple sclerosis, autoimmune diabetes, rheumatoid arthritis, other autoimmune disorders, and allergy (Singh et al., 1999). In particular, Th2 responses are associated with allergy, asthma and some systemic autoimmune diseases such as lupus. Th1 responses are associated with multiple sclerosis and other organ-specific autoimmune disorders. The identification of mechanisms that cause autoimmune disease may provide an opportunity to intervene and redirect the immune system to provide clinical relief. An understanding of the factors involved in the Th1/Th2 cell fate decision is necessary to develop therapies. Especially important is an evaluation of the ability of differentiated T cells to deviate from Th1 to Th2 (or vice versa).

Several factors influence Th1 versus Th2 differentiation. As discussed, the cytokine microenvironment in which the T cells are activated and the genetic background of the immunized individual contribute to this decision. Also important are the strength of TCR signaling, the strength of costimulation, and the nature of the antigen. CTLA4 is expressed on both Th1 and Th2 cells, so it has the potential to influence both compartments (Alegre et al., 1998). It has been observed that blockade of CTLA4 negative signaling can enhance both Th1 and Th2 responses, without immune deviation to the opposite type (McCoy and Le Gros, 1999). These data support a model in which increased costimulation serves to amplify either response, whichever is already present.

However, substantial research has shown that Th2 responses are more dependent than Th1 responses on B7 costimulation (Harris and Ronchese, 1999). A model that is consistent with these data describes the Th1/Th2 decision as based on the balance between strength of signaling and the innate response (Gause et al., 1999). The Th2 response is favored by increasing strength of signal (both TCR signal and costimulatory signal) relative to the innate response. This model is tested in my dissertation. It was tested whether blockade of CTLA4 increased the strength of signal, enabling a Th1 response to shift to a Th2 response.

Recently, a number of reports have been published that are consistent with our model. The data have supported a role for CTLA4 signaling in limiting the development of Th2 responses (Oosterwegel et al., 1999b; Kato and Nariuchi, 2000). Likewise, CD28 signaling has been linked to promotion of Th2 responses (Khattari et al., 1999; Kubo et al., 1999). A mechanistic explanation may be that Th2 cytokine gene transcription is dependent on signal transduction molecules associated with synergistic activation of T cells by CD28/TCR ligation (Hehner et al., 2000). CTLA4 negative signaling may oppose these same pathways. Manipulation of CTLA4 signaling may provide an avenue to alter the Th1/Th2 balance of immune responses (Anderson et al., 1999).

G. Infectious disease models of the immune response: *H. polygyrus* and *T. muris*

Gastrointestinal nematodes are one of the most widespread sources of infection in humans (Finkelman et al., 1997). Endemic in much of the developing world, infection rates approach 100% in some areas. Rodent models of nematode infection provide a valuable tool for analyzing immune responses to these pathogens. Experiments with

these models have revealed that immunity to gastrointestinal nematodes is dependent on CD4⁺ T cells, and Th2 responses are protective while Th1 responses are not.

H. polygyrus, a native murine parasite, has been used extensively in our lab (Greenwald et al., 1999; Gause et al., 1997a; Greenwald et al., 1997; Gause et al., 1996; Lu et al., 1994; Svetic et al., 1993). Infection is initiated by ingestion of third-stage larvae. The larvae invade the wall of the small intestine, where they mature into adults eight days after inoculation. Upon maturation, the adults enter the lumen of the small intestine. They inhabit the lumen for varying lengths of time, depending on the strain of mouse. In BALB/c mice, the strain used in these studies, a chronic infection develops. The infection persists in spite of the polarized Th2 immune response that is mounted in response to the pathogen. The highly polarized nature of the response to *H. polygyrus* allows the opportunity to study the mechanisms involved in the development of Th2 immune responses. Although Th2 responses provide resistance to nematode infections, they are also associated with allergy and some autoimmune disorders. Understanding the development of Th2 responses may uncover ways to increase the response to nematode parasites to promote clearance of infection, and may also uncover ways to decrease the response against environmental or self antigens to provide relief for allergy or autoimmune diseases.

Another parasite utilized in our lab is *T. muris*, which is closely related to the human parasite *T. trichiura* (Finkelman et al., 1997). Like *H. polygyrus*, infection begins with ingestion of infective *T. muris* larva. The infective form of *T. muris* is a first-stage larva encased in an egg. Following ingestion, the larva imbeds in the epithelium of the cecum and colon. While most nematode parasites elicit Th2 immune responses, *T. muris*

is unusual in that it elicits either a Th1 or Th2 response, depending on the strain of mouse infected, with the former leading to a chronic infection and the latter successfully eliminating the pathogen (Else et al., 1994). AKR mice, utilized in this doctoral research, develop a chronic infection, with both Th1 and Th2 characteristics present in their immune responses, and Th1 being more prominent. This unusual property affords the opportunity to examine the Th1/Th2 decision in the activation of the T cell. As Th1/Th2 differentiation is important in the development of effective responses to infection, and also in the development of autoimmunity, an understanding of the mechanisms involved may lead to the ability to redirect immune responses for the clinical benefit of the patient.

H. Experimental design and specific goals of the project

The focus of this doctoral dissertation research is on the role of costimulation in the development of T cell-dependent Th2 immune responses, with particular emphasis on CTLA4 as a mediator of downregulation. The first hypothesis tested here is that delayed inhibition of CTLA4 interactions during the Th2 immune response can enhance the response. As discussed previously, blockade of CTLA4 function by administration of anti-CTLA4 antibody can enhance the response to *N. brasiliensis*, when given concurrently with onset of infection (McCoy et al., 1997). These data support CTLA4 as a negative modulator of the immune response to infectious disease. However, whether blockade of CTLA4 could enhance the response once infection had already been established had not been investigated. Also, these data should be reconciled with the ability of CD28-deficient mice to mount polarized Th2 immune responses to *H. polygyrus*. To test whether CTLA4 functions as a negative regulator late in the immune

response. BALB/c mice were inoculated with *H. polygyrus*, and treated with anti-CTLA4 antibody or control antibody beginning eight days after inoculation, when infection had been well established. Changes in the immune response were quantitated by analyzing serum immunoglobulin concentrations, cytokine secretion, expression of activation markers and size of T and B cells, and egg production of the pathogen. The data generated in these experiments further understanding of the role CTLA4 plays in the response to infectious disease. It is important to examine its role late in the response, a more clinically relevant timepoint of intervention than concurrently with inoculation.

The second hypothesis tested in this work is that blocking CTLA4 interactions can promote deviation toward a Th2 response. The Th1 versus Th2 nature of the response to *T. muris* determines whether the response is curative or inappropriate. A Th1 response can be deviated to a Th2 response, and vice versa, by manipulating the cytokine environment (Else et al., 1994). Recent data generated in our lab has indicated that strength of costimulation is also important in determining which kind of response is elicited. BALB/c mice normally respond to *T. muris* with Th2 characteristics, and clear the infection rapidly. Reducing the strength of costimulation by CTLA4Ig administration causes immune deviation toward a Th1 response, resulting in loss of protection (Urban et al., 2000). To test whether increasing the strength of costimulation can cause immune deviation toward a Th2 response, AKR mice were infected with *T. muris*. Beginning eight days after inoculation, the mice were treated with anti-CTLA4 antibody or control antibody. The nature of the immune response was monitored to determine whether the treatment resulted in immune deviation.

The third hypothesis tested is that changes in the intracellular events caused by blockade of CTLA4 in wild-type mice are similar to the changes in intracellular events caused by loss of the CTLA4 gene. CTLA4-deficient mice display severe uncontrolled lymphoproliferation, resulting in death at an early age. Molecularly, increased phosphorylation of signaling proteins has been observed in CTLA4-deficient mice, leading to the model that CTLA4 mediates negative signaling in normal mice by recruiting and activating SHP-2 phosphatase (Marengere et al., 1996). To investigate the intracellular events following CTLA4 blockade, BALB/c mice were inoculated with *H. polygyrus* and treated with anti-CTLA4 antibody or control antibody beginning eight days after infection. The proteins from mesenteric lymph nodes were analyzed for tyrosine phosphorylation thirteen days after infection. The phosphotyrosine patterns from anti-CTLA4 antibody treated animals were also compared with those from CTLA4-deficient mice. These experiments were designed to investigate whether CTLA4 blockade by antibody and loss of the CTLA4 gene result in similar alterations of intracellular events.

The fourth hypothesis tested is that signaling proteins that bind CTLA4 and/or CD28 may be identified by screening a novel cDNA library with the CTLA4 and CD28 cytoplasmic domains. A novel cDNA library was constructed from the mesenteric lymph nodes of mice eight days after *H. polygyrus* inoculation, to ensure that the library was representative of genes expressed during a strong immune response. The library was screened for binding to the cytoplasmic domains of CTLA4 and CD28. Identifying the intracellular proteins that bind costimulatory receptors may shed light on their mechanisms.

The fifth hypothesis tested is that blockade of all B7/CD28/CTLA4 interactions after initiation of the immune response can inhibit the immune response. CTLA4Ig is a fusion protein constructed from the extracellular portion of CTLA4 fused to an immunoglobulin Fc region. This construct binds both B7-1 and B7-2, preventing their interactions with both CD28 and CTLA4. CTLA4Ig treatment is effective in blocking the response to *H. polygyrus* when administered at the initiation of infection (Lu et al., 1994). However, effector T cells may be less sensitive to loss of costimulation than naïve T cells. In addition, CTLA4 is upregulated upon activation of T cells, and CTLA4/B7 interactions may predominate over CD28/B7 interactions after the response has been initiated. To test this hypothesis, both *T. muris*-infected AKR mice and *H. polygyrus*-infected BALB/c mice were treated with CTLA4Ig or control fusion protein late after inoculation. Parameters of the immune response were measured, to determine whether delayed CTLA4Ig treatment altered the strength or type of immune response. Immunotherapies aimed at ameliorating autoimmunity will be utilized in patients whose self-reactive T cells have already been activated, so it is important to evaluate manipulation of costimulatory pathways as a means to inhibit effector T cells.

II. Materials and Methods

A. Animals

Eight to 12 week old female BALB/c and AKR mice were used for experiments involving *H. polygyrus* and *T. muris* infection. All animals were purchased from Charles River Laboratories, National Cancer Institute, Frederick, MD. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals." Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare.

B. Antibodies

In vivo antibody intervention experiments included the use of hamster anti-CTLA4 antibody (4F10), control normal Syrian hamster IgG (Pierce, Rockford, IL), the murine fusion protein CTLA4Ig, and its control, murine L6. CTLA4Ig and L6 were provided by Dr. Robert Peach (Bristol-Myers Squibb, Princeton, NJ).

Quantitation of serum IgE by ELISA used a pair of anti-IgE antibodies, provided by Dr. Fred Finkelman (Division of Immunology, University of Cincinnati College of Medicine, Cincinnati OH): clone EM95 was used for capture, and biotinylated RIE4 was used for detection. Streptavidin alkaline phosphatase (Pharmingen, San Diego, CA) was used to detect biotinylated antibody. To quantitate serum IgG1 and IgG2a, goat anti-mouse IgG1 and goat anti-mouse IgG2a polyclonal antibodies (The Binding Site, Birmingham, UK) were used for capture. Alkaline phosphatase-conjugated goat anti-

mouse IgG1 and goat anti-mouse IgG2a polyclonal antibodies (Southern Biotechnology Associates, Birmingham, AL) were used for detection .

For ELISPOT, pairs of monoclonal antibodies that bind to different epitopes of IL-4 (BVD4-1D11.2 and biotinylated-BVD6-24G2.3) and IFN γ (R46A2 and biotinylated-XMG-6) were used (Chatelain et al., 1992). Streptavidin-conjugated alkaline phosphatase (Jackson Immuno Research, West Grove, PA) was used as a second step for biotinylated antibodies. To stain for FACs analysis, FITC anti-CD4 (GK1.5), biotin anti-IL-2R (7D4), cychrome anti-B220 (RA3-6B2), and biotin anti-Ia^d (MHC class II, MKD6) antibodies were used (Pharmingen, San Diego, CA). Streptavidin-phycoerythrin (Becton Dickinson, Franklin Lakes, NJ) was used as a second step to detect biotinylated antibodies.

Anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) and anti-CD3 ζ (3A1, Transduction Laboratories, San Diego, CA) were used for immunoprecipitation and western blotting. Horseradish peroxidase-conjugated anti-mouse IgG (polyclonal, Transduction Laboratories) was used to detect blotting antibody.

C. Chemicals and reagents

Quantitation of serum immunoglobulins by ELISA required the following reagents: p-nitrophenylphosphate, methylumbelliferyl phosphate (Sigma, St. Louis, MO); IgG1 standard, IgG2a standard (The Binding Site); IgE standard (Pharmingen); powdered non-fat milk; fetal calf serum (HyClone, Logan, UT); and Immulon II 96-well polystyrene flat-bottomed plates (Dynex, Chantilly, VA).

ELISPOT and FACs analysis used the following reagents: RPMI 1640 media (BioWhittaker, Walkersville, MD); phosphate buffered saline (Quality Biological, Gaithersburg, MD); fetal calf serum (HyClone); 5-bromo-4-chloro-3-indoyl phosphate, 2-amino-2-methyl-1-propanol (Sigma); paraformaldehyde (Fischer, Pittsburgh, PA); SeaPlaque low-melt agarose (FMC Bioproducts, Rockland, ME); and Immulon II 96-well polystyrene flat-bottomed plates (Dynex).

Immunoprecipitation used the following reagents: sodium pyrophosphate, sodium fluoride, sodium orthovanadate, phenylmethylsulfonylfluoride, aprotinin, pepstatin, leupeptin (Sigma); Bradford assay kit (Biorad, Hercules, CA); and Protein A sepharose (Pharmacia, Piscataway, NJ). The following reagents were used for western blotting: 4-12% NuPage Bis-Tris gels, NuPage LDS sample buffer, MOPS running buffer (Novex, San Diego, CA); Immobilon-P membrane (Millipore, Bedford, MA); Multiphor NovaBlot (semi-dry) electro-blot system (Pharmacia); and ECL reagents (Pierce).

The following reagents were required for yeast two hybrid procedures. Amino acids, nucleic acids, 3-aminotriazol, dextrose, 5-fluoroorotic acid, lithium acetate, magnesium sulfate, polyethylene glycol 3350 (Sigma); bacto-peptone, bacto-yeast extract, yeast nitrogen base without amino acids and ammonium sulfate (Difco, Detroit, MI); Biotin A neutral nylon, X-gal (Life Technologies, Gaithersburg, MD); ammonium sulfate (JT Baker, Phillipsburg, NJ); dimethylformamide (Aldrich, Milwaukee, WI); sheared salmon sperm DNA (5Prime 3Prime, Boulder, CO); potassium chloride, monobasic sodium phosphate, dibasic sodium phosphate (Mallinckrodt, Phillipsburg, NJ); and a replica plating block and velveteen squares (ReplicaTech, Princeton, NJ).

Several chemicals were obtained from Sigma that were used in multiple procedures and experiments: sodium azide, Tween-20, Tris-HCl, Tris 7-9, magnesium chloride, sodium bicarbonate, boric acid, sodium chloride, EDTA, Triton X-100, and bovine serum albumin.

D. Parasites

BALB/c mice were inoculated orally with 200 infective, ensheathed, third-stage *H. polygyrus* larvae by using a ball-tipped feeding tube (Svetic et al., 1993). *H. polygyrus* larvae were propagated on vermiculite-fecal cultures, separated using a Bearmann apparatus, washed in saline, and stored at 4°C until use (specimens on file at the U.S. National Parasite Collection, U.S. National Museum Helminthological Collection, No. 81930, Beltsville, MD). Adult worm and egg numbers were quantitated as previously described (Urban, Jr. et al., 1991).

AKR mice were inoculated orally with 500 *T. muris* eggs containing first-stage larvae (Wakelin, 1967). *T. muris* eggs were originally provided by Dr. Richard Grencis (University of Manchester, Manchester, UK). Worm numbers were quantitated as previously described (Else et al., 1990). To propagate *T. muris*, eight to ten week old female AKR mice were inoculated orally with 500 infective eggs, and adult worms were recovered from the cecum and proximal colon by removal with insect forceps at day 35 after inoculation. Worms were washed ten times in sterile saline and placed in 24-well tissue culture plates containing RPMI 1640 medium with 100 units penicillin, 100 µg streptomycin, and 2.5 µg /gentamycin per ml at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Worms were transferred to fresh media at 24 and 48 hours. The

conditioned media and excreted eggs were centrifuged to recover supernatant (containing parasite products as a source of antigens) and the eggs in the pellet. The eggs were washed three times in sterile saline, and incubated at room temperature for 35-40 days in filtered tap water with intermittent shaking and water replenishment. The eggs were adjusted to 5,000 eggs per ml filtered tap water and stored at 4°C until use.

E. Anti-CTLA4 antibody administration

After oral inoculation with *H. polygyrus* or *T. muris*, mice were administered anti-CTLA4 monoclonal antibody by tail vein injection. Mice were given 0.2-1.2 mg antibody as indicated. Normal hamster IgG was given at the same dose as a control.

F. CTLA4Ig administration

H. polygyrus- or *T. muris*-inoculated mice were administered 0.1-0.2 mg murine CTLA4Ig. The fusion protein L6 was given at the same dose as a control.

G. Quantitation of serum immunoglobulins

Serum immunoglobulin concentrations were determined by micro-ELISA assay. For serum IgE quantitation, Immulon II 96-well plates were coated with anti-IgE capture antibody (EM95) at 10 µg/ml, and blocked with 5% milk buffer (5% powdered milk, 100 mM Tris, 0.2% sodium azide). Serial dilutions of serum samples and IgE standard were added and incubated overnight at 4°C. The plates were washed with MBTA (0.25% powdered milk, 100 mM Tris, 0.2% sodium azide, 0.5% Tween). IgE was detected with the addition of biotinylated-anti-IgE detection antibody (RIE4) at 2 µg/ml. The plates

were washed with MBTA, incubated with streptavidin-alkaline phosphatase, washed, and developed with p-nitrophenylphosphate as substrate at 1 mg/ml in 1 M Tris, 0.3 mM magnesium chloride. Results were read at 405 nm with a microplate ELISA reader (Finkelman et al., 1987).

To quantitate serum IgG concentrations, Immulon II 96-well plates were coated with goat anti-mouse IgG1 or goat anti-mouse IgG2a polyclonal capture antibody at 10 µg/ml, and blocked with 1% fetal calf serum (FCS) in borate buffered saline. Alternatively, to quantitate antigen-specific IgG, plates were coated with *H. polygyrus* extract or *T. muris* excretory-secretory antigen (ES), and blocked with 10% milk buffer. *H. polygyrus* extract was prepared by homogenizing adults worms, and clarifying the supernatant by centrifugation. *T. muris* ES was produced as previously described (Bancroft et al., 1998). Serial dilutions of serum samples and IgG standard were added and incubated overnight at 4°C. The plates were washed with 0.02% Tween/borate buffered saline, and IgG was detected with the addition of goat anti-mouse IgG1 or goat anti-mouse IgG2a polyclonal antibody conjugated with alkaline phosphatase. The plates were washed with 0.02% Tween/borate buffered saline, and developed with methylumbelliferyl phosphate as substrate at 0.5 mg/ml in 50 mM sodium bicarbonate, 1 mM magnesium chloride. Results were detected with a microplate ELISA fluorescence reader.

H. ELISPOT

The frequencies of IL-4- and IFN γ -producing cells were determined by ELISPOT, as previously described (Morris et al., 1994). Immulon II 96-well plates were coated

overnight at 4°C with anti-IL-4 (BVD4-1D11.2) or anti-IFN γ (R46A2) capture antibody at 10 μ g/ml in phosphate buffered saline (PBS). The plates were washed three times with 0.05% Tween in PBS, three times with PBS, and then blocked with 5% FCS in RPMI 1640 a minimum of 1 hour at 37°C. Single cell suspensions were prepared from mesenteric lymph nodes by gently pressing the tissue against a fine wire mesh while immersed in cold RPMI + 5% FCS, filtered through plastic mesh, and gently centrifuged to collect the cells. The volumes of the cell pellets were adjusted with cold RPMI + 5% FCS to five million cells per ml. Serial dilutions of the cell suspensions from each mouse were added in duplicate to the coated plates and incubated for three hours or overnight at 37°C. The plates were washed three times with PBS and three times with 0.05% Tween in PBS. Biotinylated anti-IL-4 (BVD6-24G2.3) or anti-IFN γ (XMG-6) was added and incubated one hour at 37°C. After washing three times with PBS and three times with 0.05% Tween in PBS, streptavidin-conjugated alkaline phosphatase was added at 1:1000 dilution in 0.05% Tween + 5% FCS + PBS and incubated one hour at 37°C. Following a final wash five times with PBS, the plates were developed with 5-Bromo-4-chloro-3-indoyl phosphate as substrate at 1 mg/ml in 0.1 M 2-amino-2-methyl-1-propanol buffer, with 0.6% SeaPlaque agarose. The plates were incubated overnight and the number of blue spots per well were counted with a dissecting microscope. Each spot represents a single cytokine secreting cell.

I. Cell labeling and analysis

Single cell suspensions from mesenteric lymph nodes were prepared as described for ELISPOT, and washed in 2% FCS in PBS. The concentration of cells was adjusted to

ten million cells per ml. One million cells were used for each sample. Before staining for surface markers, non-specific binding was blocked by adding anti-Fc γ RII (24G2) (Unkeless, 1979) at 1 μ g per sample. Cell suspensions were then dual stained with FITC anti-CD4 and biotin anti-IL-2R antibodies or cychrome anti-B220 and biotin anti-MHC class II antibodies, and incubated for 30 minutes on ice. The cells were washed with 2% FCS in PBS, centrifuged to collect the cells, and resuspended in 2% FCS in PBS. To detect the biotinylated antibodies, the cells were stained with streptavidin-PE, incubated and washed as above. The cell pellet was resuspended in 2% paraformaldehyde in PBS and stored at 4°C in the dark. Dual-color flow cytometric analysis was performed with an Epics ELITE flow cytometer (Coulter Electronics) with an argon laser as the excitation source (Svetic et al., 1991).

J. Quantitation of cytokine gene expression

1. Isolation and purification of RNA

Cytokine gene expression was quantitated as previously described (Svetic et al., 1991; Svetic et al., 1993). Tissues were homogenized in RNazol at 50 mg tissue per ml immediately after collection, frozen in liquid nitrogen, and stored at -70°C for later analysis. To extract the RNA, the samples were warmed at 37°C for 5 minutes and transferred to 2 ml tubes. 200 μ l chloroform was added per 2 ml homogenate, vortexed for 15 seconds, and incubated in ice for 15 minutes. The suspension was centrifuged at 4°C at 12,000g for 15 minutes. The aqueous phase was transferred to a fresh tube, and precipitated with isopropanol. The resultant RNA precipitate was washed twice with

75% ethanol, and suspended in 50 μ l RNase-free water. The concentration and OD_{260/280} were determined for each sample.

2. Reverse transcription

For each reverse transcription reaction, 1.8 μ g RNA was diluted to 11.8 μ l RNase-free water. This solution was added to 13.2 μ l reaction mix, consisting of: 2.5 μ l 10 mM dNTPs; 5 μ l 5X RT buffer (260 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂); 2 μ l 100 mM dithiothreitol; 2 μ l random hexamers (0.5 U/ μ l); 0.5 RNasin (40 U/ μ l); 1.2 μ l reverse transcriptase (200 U/ μ l). The samples were incubated at 37°C for one hour, denatured at 90°C for 5 minutes, and cooled on ice for 5 minutes. The samples were stored at -20°C.

3. Polymerase chain reaction

Each reaction consisted of 2.5 μ l cDNA from the reverse transcription reaction, 4.0 μ l dNTPs (2.5 mM), 5.0 μ l 10X Taq polymerase buffer, 3.0 μ l 25 mM MgCl₂, 0.2 μ l Taq polymerase (5 U/ μ l), 2.0 μ l sense oligo primer (0.2 μ g/ μ l), 2.0 μ l anti-sense oligo primer (0.2 μ g/ μ l), and 31.1 μ l water. The solution was mixed, and centrifuged briefly. Mineral oil was added to each tube. After an initial incubation at 95°C for 5 minutes, temperature cycling was initiated. Each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 53°C for one minute, and extension at 72°C for 2 minutes. An additional extension at 72°C for 7 minutes was carried out upon completion of the cycles. The samples were stored at 4°C. For each gene product, the optimum number of cycles was determined experimentally, and was defined as that number of cycles that would achieve a detectable concentration that was well below saturating conditions. To verify that equal amounts of undegraded RNA were added to each PCR reaction within an

experiment, hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal standard. HPRT values did not vary more than two- or three-fold. Each cytokine expression level was normalized to the expression of HPRT.

4. Southern blotting

A total of 10 µl of the final PCR reaction was run on a 1% agarose gel at 120V for 3 hours. The gel was denatured by soaking for 45 minutes in several volumes 1.5 M NaCl, 0.5 N NaOH with constant, gentle agitation. The gel was rinsed briefly in deionized water, and neutralized by soaking for 30 minutes in several volumes 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant agitation. The DNA was transferred to a Nytran membrane, and cross-linked using the UV Stratalinker 1800 (Stratagene, La Jolla, CA). Blots were prehybridized at 42°C for 6 hours in a solution containing 6XSSPE, 10X Denhardt's, 1% SDS and 50 µg/ml ssDNA. The blots were hybridized with ³²P-labeled oligoprobes at 49°C for 16 hours in a solution containing 6X SSPE and 1% SDS. After hybridization, the blots were washed for 15 minutes in 6X SSPE, 0.1% SDS and then 3-4 minutes in 2X SSPE or 1X SSPE at 49°C. Amplified PCR product was quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

K. Immunoprecipitation and western blotting

Proteins from mesenteric lymph nodes were solubilized, immunoprecipitated and western blotted as described (Kirken et al., 1997; Marengere et al., 1996). Mesenteric lymph nodes were homogenized and incubated at 4°C for one hour in lysis buffer containing 10 mM Tris, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50

mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, and 2 µg/ml leupeptin. The lysate was cleared by centrifugation, protein concentration was determined by Bradford assay, and the concentrations of each group were normalized. Precipitating antibody (anti-phosphotyrosine or anti-CD3ζ, as indicated) was incubated with the lysate for two hours at 4°C. Protein A sepharose was added and incubated for one hour at 4°C. Precipitated proteins were washed three times with cold lysis buffer. After the addition of NuPage LDS sample buffer, samples were stored at -70°C until further analysis.

Protein samples were resolved on Novex 4-12% NuPage Bis-Tris gels with MOPS running buffer, according to the manufacturer's protocol. Proteins were transferred to Millipore Immobilon-P membrane with a semi-dry electro-blot system (Pharmacia NovaBlot). Membranes were incubated for one hour at room temperature with a blocking buffer containing 1% BSA, 50 mM Tris and 150 mM NaCl. Membranes were incubated with blotting antibody (anti-phosphotyrosine or anti-CD3ζ, as indicated) at 1 µg/ml for 90 minutes at room temperature and washed (wash buffer 0.25% Tween 20, 50 mM Tris, 150 mM NaCl). Blotting antibody was detected with anti-mouse IgG-HRP, and developed with Pierce ECL reagents.

L. Yeast two hybrid system

The GeneQuest system (Life Technologies) was used for the yeast two hybrid experiments described here. GeneQuest is based on the system initially described by Fields and Song (Fields and Song, 1989), with more recent modifications (Durfee et al.,

1993; Vojtek et al., 1993; Chevray and Nathans, 1992; Vidal et al., 1996a; Vidal et al., 1996b).

1. Plasmids

Plasmids pDBLeu and pPC86 were used in the yeast two hybrid procedures. These plasmids are low-copy ARS/CEN-based vectors which allow for consistent expression of fusion proteins at levels closer to physiological conditions, when compared with more common 2 micron-based vectors (Futcher and Cox, 1984).

pDBLeu contains the following sequences: the DNA binding domain of the GAL4 transcription factor (GAL4-DB) under the constitutive, moderate strength promoter of the yeast ADH1 gene (alcohol dehydrogenase); the ARS4/CEN6 sequence for replication in yeast; the LEU2 gene for selection in yeast in media lacking leucine; a pUC-based origin of replication for maintenance in *E. coli*; the kanamycin resistance gene for selection in *E. coli* in medium containing kanamycin at 25 µg/ml; a multiple cloning site to allow for in-frame insertion of a gene to be expressed as a fusion with GAL4-DB (Chevray and Nathans, 1992). pPC86 contains the following sequences: the activation domain of the GAL4 transcription factor (GAL4-AD) fused to the SV40 large T antigen nuclear localization signal, under the constitutive, moderate strength promoter of the yeast ADH1 gene; the ARS4/CEN6 sequence for replication in yeast; the TRP1 gene for selection in yeast in medium lacking tryptophan; a pUC-based origin of replication for maintenance in *E. coli*; the ampicillin resistance gene for selection in *E. coli* in media containing ampicillin at 100 µg/ml; a multiple cloning site to allow for in-frame insertion of a gene to be expressed as a fusion with GAL4-AD.

The sequence of a known protein is inserted into pDBLeu (the bait). A cDNA library to be screened is inserted into pPC86. Alternatively, a known protein is inserted into pPC86 and is assayed for its interaction with the bait. Both approaches were used in this work. The intracellular portions of CTLA4 and CD28 were used as bait to screen a cDNA library. In addition, AP50 was tested for its interaction with CTLA4, and Lck was tested for interaction with CTLA4 and CD28. These genes were amplified from an activated lymphocyte cDNA library (described in the following section) using the primers listed in Table A. Purified PCR products were digested and ligated into NotI and SalI sites in the multiple cloning site of the indicated plasmid. In-frame fusions of the inserted genes with GAL4-DB and GAL4-AD were confirmed by sequencing in both directions using the pDBLeu and pPC86 sequencing primers listed in Table B.

2. Construction of activated lymphocyte library

BALB/c mice were infected with *H. polygyrus*, and mesenteric lymph nodes were harvested at day eight. RNA was purified using RNAsol B (Tel-Test, Friendswood, TX) and checked for quality on an ethidium-stained agarose gel. The RNA was reverse transcribed using random primers. The cDNA inserts were ligated into pPC86. The library contains 1.2×10^8 primary clones with an average insert size of 1.7kb. This library was produced in collaboration with Life Technologies, and is currently commercially available (Life Technologies cat# 11289014).

3. Media

For growth of untransformed yeast host, YPAD rich medium was used (contains bacto-yeast extract, bacto-peptone, dextrose, and adenine sulfate). In other yeast procedures, synthetic complete (SC) dropout media were used (Lundblad et al., 1989).

Table A. Primer sequences for amplification of constructs from activated lymphocyte library for yeast two hybrid screen.

Gene	PCR Primer Sequence	Direction	Restriction	Plasmid
CTLA4	5'TACTGAGTCGACAAAGATCCTCTTACAAC	Sense	Sall	pDBLeu
CTLA4	5'TAGTATGCGGCCCGCCCTTTCAGTTGATGGG	Antisense	NotI	pDBLeu
CD28	5'AAGGAAGTCGACAAAGTAGAAGGAACAGA	Sense	Sall	pDBLeu
CD28	5'AACGTAGCGCGGGCGTCAGGGGCGGTACG	Antisense	NotI	pDBLeu
AP50	5'AACCAAGTCGACCCACACAGAACTCAGAGAC	Sense	Sall	pPC86
AP50	5'AACGTAGCGGCCCGCACTAGCACCGGTTTCATAAATG	Antisense	NotI	pPC86
Ick	5'TAGTATGTCGACCATGGCTTGTGTCTGC	Sense	Sall	pPC86
Ick	5'TACTGTGCGGCCGCATCAAGGCTGGGGC	Antisense	NotI	pPC86

Table B. Sequencing primers used to confirm in frame fusion of inserted genes in yeast two hybrid system.

Plasmid	Primer sequence	Direction
pDBLeu	5'GAATAAGTGCGACATCATC	Sense
pDBLeu	5'GTAAATTTCTGGCAAGGTACAG	Antisense
pPC86	5'TATAACGCGTTTGAATCACT	Sense
pPC86	5'GCAAATTTCTGGCAAGGGATAC	Antisense

The basic SC medium consists of yeast nitrogen base (without amino acids and ammonium sulfate), dextrose, ammonium sulfate, all amino acids and nucleic acids except for leucine, tryptophan, histidine and uracil. Depending on the auxotrophy to be tested, leucine, tryptophan, histidine and/or uracil were added to the basic SC medium. For example, to test for dependence on leucine and tryptophan, only histidine and uracil were added (SC-Leu-Trp).

4. Transformation of CTLA4 and CD28 constructs into yeast host

To transform pDBLeuCTLA4 and pDBLeuCD28 (bait constructs) into MaV203 (the yeast host, (Vidal et al., 1996a; Vidal et al., 1996b)), competent cells were prepared in the lab (Lundblad et al., 1989). MaV203 was streaked from glycerol stocks stored at -70°C onto a YPAD plate and incubated for 24 hours at 30°C. Several isolated colonies were suspended in 50 µl sterile distilled deionized water. Suspended cells were spread onto the center of a YPAD plate and incubated for 24 hours at 30°C. The resulting cell mass was scraped from the plate and suspended in 5 ml sterile distilled deionized water. Suspended cells were added to 100 ml liquid YPAD medium to produce an OD₆₀₀ of approximately 0.1, and incubated with shaking at 30°C until the OD₆₀₀ reached 0.4. The culture was spun down by centrifugation at 3000g for five minutes, resuspended in 40 ml sterile distilled deionized water, and spun down again. The cell pellet was resuspended in 20 ml freshly prepared and filtered TE/LiAc (0.1M lithium acetate pH 7.5 in TE), and spun down again. Following final resuspension in 350 µl TE/LiAc, the yeast cells were ready for immediate transformation.

For each bait construct (pDBLeuCTLA4 and pDBLeuCD28), five small-scale transformations were performed, and transformants were plated on appropriate selective

media as described in Table C. For each transformation, 50 µl competent cells were mixed with 100 ng each plasmid and 5 µl freshly boiled carrier DNA (sonicated herring sperm or salmon sperm DNA 10 mg/ml). To the cell DNA mixture, 300 µl PEG/LiAc (40% PEG 3350, 0.1 M lithium acetate in TE) was added and mixed gently. The transformations were incubated 30 minutes at 30°C and heat-shocked for 15 minutes at 42°C. The cells were spun down at 8000g for 30 seconds, and the cell pellet was resuspended in 500 µl sterile distilled deionized water. The transformed cells were plated on the indicated selection plates and incubated 2 to 3 days at 30°C. Typically, 1,000 colonies are obtained per cotransformation, 10,000 per transformation with a single plasmid.

5. Titration of basal HIS3 expression

To correctly identify proteins which interact and induce HIS3 reporter gene expression once the library had been co-transformed with the bait construct, it was necessary to determine the concentration of 3-aminotriazole (3AT) required to inhibit basal expression levels of HIS3 (Durfee et al., 1993; Kishore and Shah, 1988). This basal expression of HIS3 varies with the presence of different bait constructs and plasmids, and was performed independently for each bait (pDBLeuX) plus pPC86. Once the threshold of resistance to 3AT was determined, this concentration of 3AT was included in all selection plates lacking histidine. This allowed slight increases in HIS3 expression to be detected, so that weak interactions could be identified.

Four isolated pDBLeuX + pPC86 transformed colonies were patched onto an SC-Leu-Trp plate. Also patched onto the same plate were two colonies from control strains A-E (Chevray and Nathans, 1992; Vidal et al., 1996a). The plate was incubated 18 hours

Table C. Plasmids, purposes and selection media of five transformations performed in yeast two hybrid system.

Transformation	Plasmids	Purpose	Selection Media
1	pDBLeuX	Sequential transformation of library	SC-Leu
2	pDBLeuX + pPC86	Test for self-activation of pDBLeuX	SC-Leu-Trp
3	pDBLeu + pPC86	Transformation control	SC-Leu-Trp
4	pDBLeu	Transformation control	SC-Leu
5	none	Transformation control	SC-Leu + SC-Leu-Trp

at 30°C. The growth on the plate was replica plated onto SC-Leu-Trp-His+3AT plates plus 0, 10, 25, 50, 75, 100 mM 3AT, and immediately replica cleaned (Lundblad et al., 1989). The plates were incubated for 24 hours at 30°C, and replica cleaned again. Following incubation for 2 days at 30°C, the growth on the 3AT plates was assessed. The lowest concentration of 3AT where no growth was seen for the pDBLeuX – pPC86 transformed colonies was the concentration used in all plates lacking histidine. For both pDBLeuCTLA4 and pDBLeuCD28, 10 mM 3AT was used.

6. Transformation of library into yeast host

The library was sequentially transformed into yeast cells already containing the bait construct (pDBLeuX). Several isolated pDBLeuX transformed colonies were suspended in 100 µl sterile distilled deionized water and spread onto the centers of two SC-Leu plates. The plates were incubated overnight at 30° C. From this point, the transformation procedure was the same as outlined above (in *Transformation of CTLA4 and CD28 constructs into yeast host*) except that it was scaled up to produce one million co-transformants. The cell mass from the SC-Leu plates was suspended in 10 ml sterile distilled deionized water, and a sufficient volume was added to YPAD broth to produce 500 ml with an OD₆₀₀ of 0.1. Once the culture reached an OD₆₀₀ of 0.4, the cells were spun down and resuspended in 200 ml sterile distilled deionized water, and spun down again. The cells were resuspended in 100 ml freshly prepared and filtered TE/LiAc, and spun down. The cells were resuspended in a final volume of 2.5 ml TE/LiAc.

The prepared cells were split into 25 aliquots. To each aliquot, 0.5 µg cDNA library and 5 µl freshly boiled carrier DNA was added and mixed. 600 µl PEG/LiAc was added and mixed gently. The cells were incubated 30 minutes at 30°C, heat shocked 15

minutes at 42°C, and spun down. Each cell pellet was suspended in 400 µl sterile distilled deionized water. A small volume (20 µl) was set aside, to be used to estimate the total number of transformants by plating on SC-Leu-Trp plates. The remaining transformation mixtures were plated on 15-cm SC-Leu-Trp-His+3AT plates (one plate per transformation, 25 plates total), and incubated for 60 hours at 30°C. Each plate was replica cleaned, and incubated for an additional 2 to 3 days at 30°C. Resulting colonies were streaked onto SC-Leu-Trp and incubated 48 hours at 30°C.

7. Analysis of reporter gene induction

Three reporter genes (HIS3, URA3, and lacZ) were used to reduce the occurrence of false positives (Durfee et al., 1993; Vojtek et al., 1993; Vidal et al., 1996a; Bartel et al., 1993). These genes are stably integrated at different loci of the yeast genome, and have unrelated promoters. Growth on SC-Leu-Trp-His+3AT and SC-Leu-Trp-Ura plates indicated expression of the HIS3 gene and URA3 gene, respectively. LacZ activity was detected by growing yeast cells on a nylon membrane placed on a YPAD plate and then performing an X-Gal assay on the membrane. The URA3 gene also allowed for a second means to detect its expression: induction of the URA3 gene results in conversion of 5-fluoroorotic acid (5FOA) to a toxic compound, 5-fluorouracil. Growth inhibition resulted from induction of the URA3 gene on medium containing 5FOA.

To characterize potentially interacting transformants, single colonies were first purified on SC-Leu-Trp for 48 hours at 30°C. Four isolated colonies were then patched onto an SC-Leu-Trp plate, along with two colonies from each control strain A-E. This plate was incubated for 18 hours at 30°C. The growth was replica plated onto four kinds of plates: SC-Leu-Trp-His+3AT, SC-Leu-Trp-Ura, SC-Leu-Trp+0.2% 5FOA, and a

nylon membrane placed on a YPAD plate. The 3AT and 5FOA plates were replica cleaned immediately. All plates were incubated for 24 hours at 30°C. An X-Gal assay was then performed on the membrane, described in the following section. The remaining three kinds of plates were replica cleaned, and incubated for another two days at 30°C. The phenotypes of the potentially interacting transformants were then compared to control strains A-E.

8. X-Gal assay

For each membrane analyzed, 10 ml of X-Gal solution was made (0.1% X-Gal, 1% N,N-dimethyl formamide, 0.6% 2-mercaptoethanol in 60 mM sodium phosphate [dibasic], 40 mM sodium phosphate [monobasic], 10 mM potassium chloride, 1 mM magnesium sulfate). A stack of three round 125 mm Whatman 541 filters was soaked with the X-Gal solution. The membrane was removed from the surface of the YPAD plate with forceps and placed onto a Whatman filter to dry for approximately three minutes. The dried membrane was immersed in liquid nitrogen for 30 seconds and placed on top of the soaked filters. Blue color, indicative of LacZ gene activity, developed after 24 hours incubation at 30°C.

9. Purification, amplification, and re-transformation of candidate clone DNA

The sizes of the cDNA inserts of candidate clones were checked by PCR before proceeding to purification. Clone inserts were amplified by PCR directly from yeast using the Whole Cell Yeast PCR Kit (Bio 101, Vista, CA). A pipet tip was touched to a fresh colony or cell pellet from a liquid culture, and the small amount of cells adhering to it was resuspended in 5 µl cell lysis solution. The suspension was incubated for 3 hours at 37°C. The suspended, digested cells were used as the PCR template.

Purification of vectors encoding potentially interacting proteins (pPC86Y) was accomplished by shuttle into *E. coli*. Three different methods were used. The first was as described previously (Robzyk and Kassir, 1992). Yeast cells were grown in liquid SC-Leu-Trp, spun down and resuspended in STET (8% sucrose, 50 mM Tris, 50 mM EDTA, 5% Triton X-100). Glass beads (425-600 micron, acid-washed, Sigma) were added to the resuspended cells and vortexed at maximum speed for at least five minutes. The suspension was placed in a boiling water bath for three minutes, chilled on ice, and spun down. The resulting supernatant was precipitated with 7.5 M ammonium acetate, spun down, and the cleared supernatant was decanted to a new tube. The cleared supernatant was precipitated with 100% ethanol, washed with 70% ethanol, dried, and resuspended in water. The resulting solution was used to transform chemically competent *E. coli* (Life Technologies Max Efficiency DH5 α).

Some clones were refractory to shuttle by this method, so one of two additional methods were employed. In some cases, the RPM Yeast Plasmid Isolation Kit (Bio 101) was used. A small volume of SC-Leu-Trp culture (1-2 ml) was pelleted, decanted, and the cell mass was vortexed with lysis matrix and alkaline lysis solution for at least five minutes. The solution was neutralized and spun down. The supernatant was transferred to a spin filter along with glassmilk spin buffer, and spun through to the catch tube. The DNA, adhering to the filter, was washed two times, and eluted with water. The resulting solution was transformed into *E. coli* (Life Technologies ElectroMax DH10B) by electroporation (2.5 kV, 25 μ F, 200 ohms, 0.2 cm gap). A second shuttle method was performed as outlined in the GeneQuest manual. Yeast cells were grown in liquid SC-Leu-Trp, pelleted, resuspended in freshly prepared 3% SDS, 0.2 M NaOH, and incubated

at room temperature for 15 minutes. TE, 3 M sodium acetate, and phenol:chloroform:isoamyl alcohol were added, and the solution vortexed at maximum speed for at least two minutes. The phenol extraction was repeated with the upper aqueous layer. The aqueous layer was precipitated with cold isopropanol, the pellet washed with 70% ethanol and resuspended in TE. This solution was transformed into *E. coli* by electroporation.

All interacting clones (pPC86Y) were reconfirmed by retransforming back into the yeast host strain. Plasmid DNA was purified from *E. coli* using Promega Wizard minipreps, and co-transformed into yeast along with pDBLeuX with Bio 101 EZ Yeast Transformation Kit. A small volume of SC-Leu-Trp was cultured until the $OD_{600} = 0.8$ - 0.9 . The culture was spun down and decanted. Plasmid DNA, carrier DNA, and transformation buffer were mixed with the cells. The cells were incubated overnight at room temperature without mixing, allowing the cells to settle to the bottom of the liquid. The cells were transferred from the bottom of the tube, plated on SC-Leu-Trp, and reporter gene induction was characterized. Cotransformants consisting of the interacting clone (pPC86Y) plus the empty bait vector (pDBLeu) were also tested for self-activation of the interacting clone in the absence of the bait. Clones which reproduced the reporter gene induction (and also tested negative for self-activation) were sequenced.

M. Data analytic strategies

For ELISPOT and egg production, the data were calculated as the mean $\pm$ SEM. For quantitation of serum immunoglobulin concentration, the geometric means and SEM were calculated. RT-PCR analysis of cytokine gene expression was conducted as

previously described (Peterson et al., 1994). A log transform was made of the ratio of the corrected densities for cytokine mRNA measurements to the corrected densities for the housekeeping gene, HPRT. For each treatment group, the mean of these values was calculated and the mean of the log transformed ratio for untreated values was subtracted from these means. The antilog values of these differences were then plotted along with their standard error. A proportion of "1" indicates that the value for a treated group equals the value for the untreated control group.

One-way analysis of variance was utilized to compare treatment groups. If the results were significant, then two-tailed Tukey-HSD tests were conducted to determine statistical significance of pairwise combinations. A p value less than or equal to 0.05 was considered statistically significant. In experiments with two treatment groups, Student's t test was conducted to determine statistical significance. SigmaStat 2.03 software was used for all statistical comparisons.

III. Results.

A. Studies on the effects of CTLA4 blockade during a polarized Th2 immune response to an infectious pathogen.

1. Administration of anti-CTLA4 antibody to *H. polygyrus*-infected BALB/c mice does not enhance the immune response.

Previous reports have demonstrated that T cell activation can be enhanced by CTLA4 blockade at the initiation of an immune response (Leach et al., 1996; McCoy et al., 1997). CTLA4 expression in naïve T cells is restricted mainly to intracellular pools, and expression on the cell surface rapidly increases within 24 hours upon activation of the T cell (Chuang et al., 1997). This suggests that CTLA4 would be more available for inhibitory signaling after initiation of the immune response, when T cells have differentiated to effector T cells and CTLA4 is more fully expressed on the cell surface. However, it may be that in some systems effector T cells are less sensitive to B7 ligand interactions than naïve cells (Gause et al., 1997b); if so, then CTLA4 blockade late in the response may not be effective. These experiments were designed to determine whether blockade of CTLA4 negative signals by anti-CTLA4 antibody administration during an ongoing Th2 response to an infectious pathogen can enhance T cell activation.

BALB/c mice (5 per group) were orally inoculated with 200 third-stage *H. polygyrus* larvae. Anti-CTLA4 antibody or control antibody was administered by i.v. injection at 200 µg doses at days 9, 14, and 19 after *H. polygyrus* inoculation. Serum immunoglobulin concentrations were assessed at day 14 and day 24 after *H. polygyrus*

inoculation. *H. polygyrus*-inoculated mice treated with control antibody had increased concentration of serum IgE. No further elevation was induced by anti-CTLA4 antibody treatment (Figure 3). Similarly, IgG1 serum concentrations were comparable between anti-CTLA4 antibody and control antibody treated mice inoculated with *H. polygyrus*, with both groups being elevated above untreated control levels (Figure 4).

T and B cell activation were evaluated by FACS analysis at day 24. These data show no evidence of increased T or B cell activation. Forward scatter profiles of CD4⁺ cells and B220⁺ cells were comparable in *H. polygyrus*-inoculated anti-CTLA4 antibody and control antibody treated mice (Figure 5). There also was no change in expression of activation markers IL-2R and MHCII on CD4⁺ and B220⁺ cells (Figure 6).

The mRNA expression of IL-3, IL-4, IL-9 and IL-13 in mesenteric lymph node and Peyer's patch tissues were quantitated by RT-PCR. In mesenteric lymph node tissue, IL-3 expression in anti-CTLA4 antibody treated mice was increased three-fold over control antibody treated mice (Figure 7), but this difference was not statistically significant. IL-4, IL-9, and IL-13 were not increased in the mesenteric lymph nodes of anti-CTLA4 antibody treated animals, when compared with control antibody treated animals. Likewise, there were no increases in gene expression in Peyer's patch tissues (Figure 7). Together, these data show that anti-CTLA4 antibody, given in this administration regimen, is not effective in enhancing the immune response to *H. polygyrus*. This protocol was not repeated.

An additional, similar experiment was completed, with 200 µg anti-CTLA4 antibody injected four times instead of three times, and beginning at timepoints earlier than the previous experiment, at days 6, 10, 14, and 16 after *H. polygyrus* inoculation. T

Figure 3. Administration of anti-CTLA4 antibody does not enhance *H. polygyrus*-induced elevations in serum IgE. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 9, 14, and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 14 and 24 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.

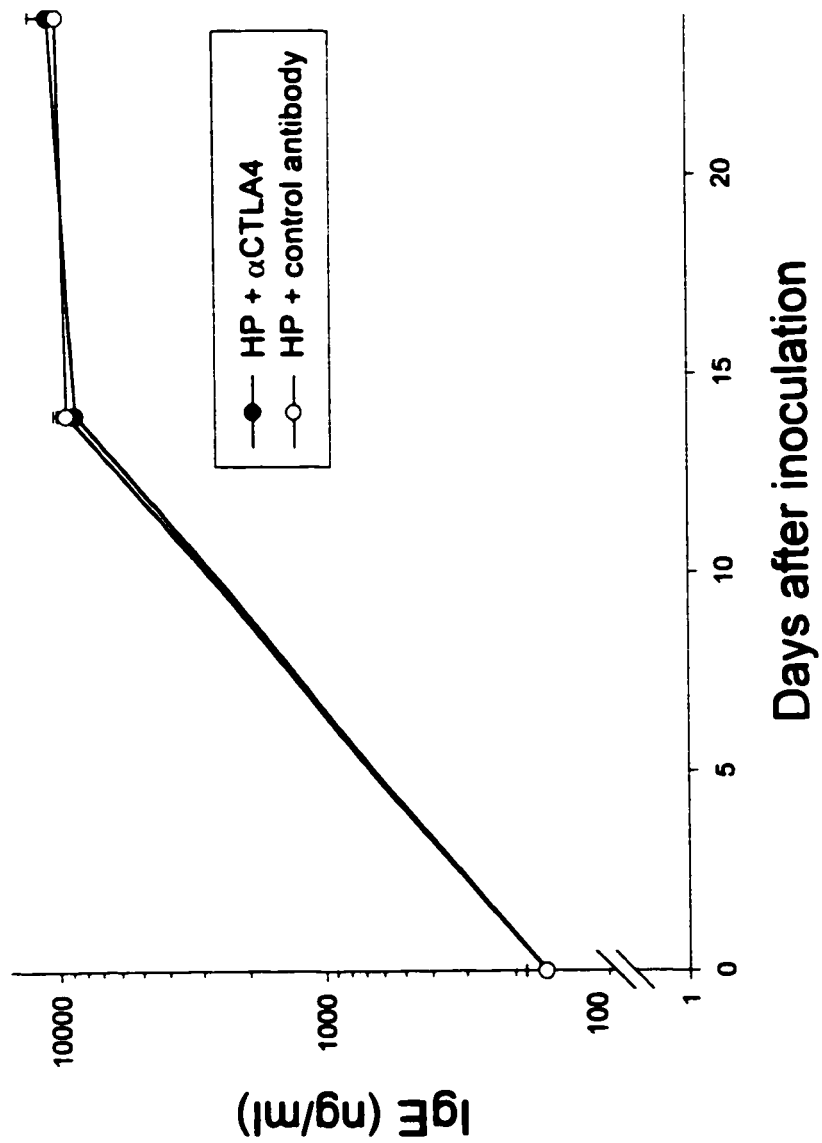


Figure 4. Administration of anti-CTLA4 antibody does not enhance *H. polygyrus*-induced elevations in serum IgG1. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 9, 14, and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 14 and 24 after inoculation, and serum IgG1 levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.

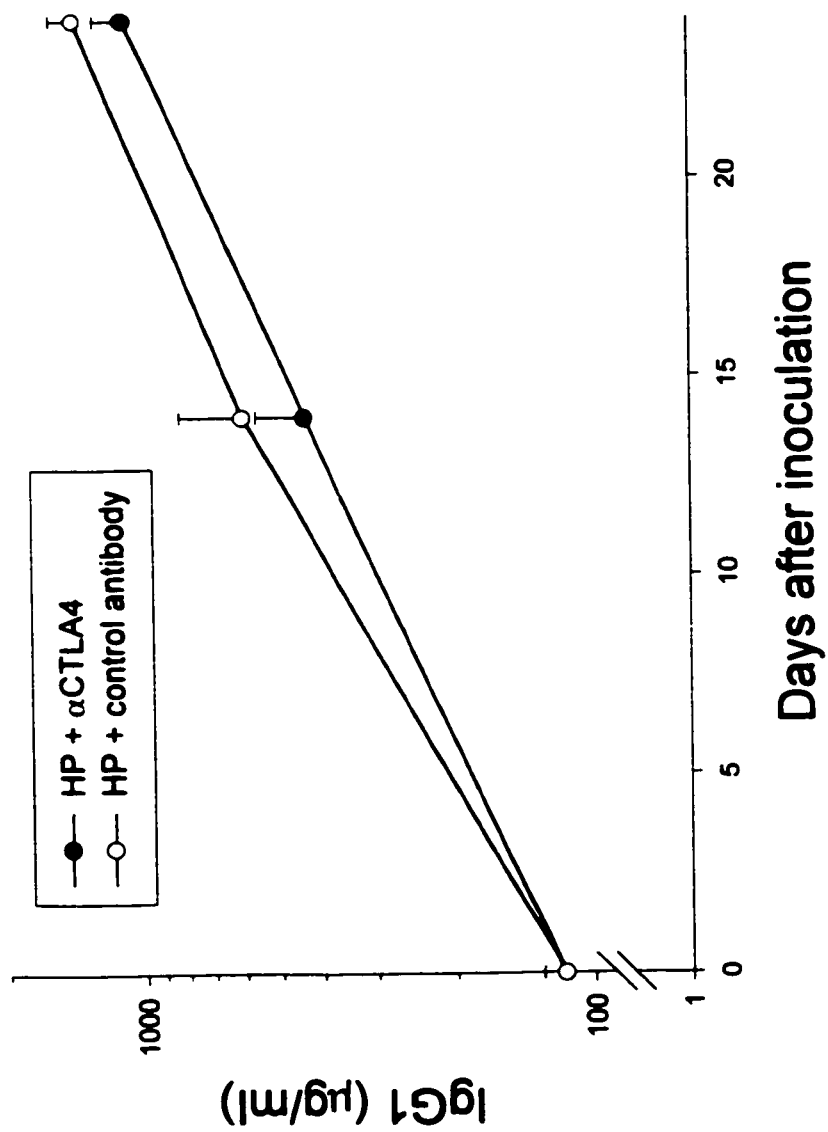


Figure 5. Administration of anti-CTLA4 antibody does not increase CD4⁺ T cell or B220⁺ B cell size at day 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 9, 14, and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with FITC-anti-CD4 and biotin-anti-IL-2R or cyochrome-anti-B220 and biotin-anti-MHCII followed by streptavidin-PE. Single histogram analyses of CD4⁺ T cell size and B220⁺ B cell size (forward scatter) are shown.

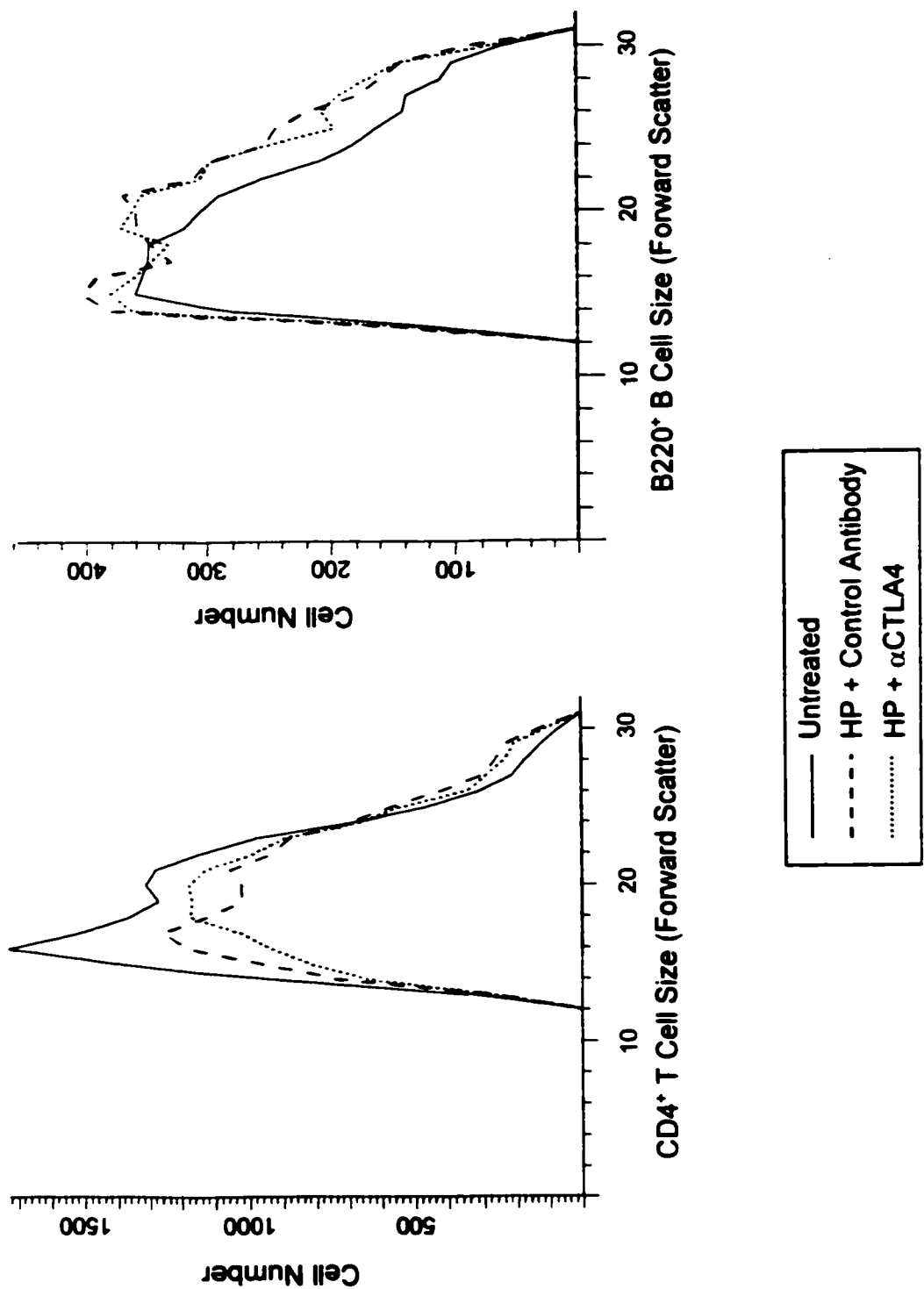


Figure 6. Administration of anti-CTLA4 antibody does not increase expression of IL-2R or MHCII on CD4⁺ or B220⁺ cells. BALB/c mice were inoculated with *H. polygyrus* and treated with anti-CTLA4 antibody or control antibody, and mesenteric lymph node cells were pooled and stained as described in the legend of Figure 5 (five mice per treatment group). Single histogram analyses of IL-2R and MHCII expression on CD4⁺ and B220⁺ cells are shown. The IL-2R and MHCII expression scales are logarithmic.

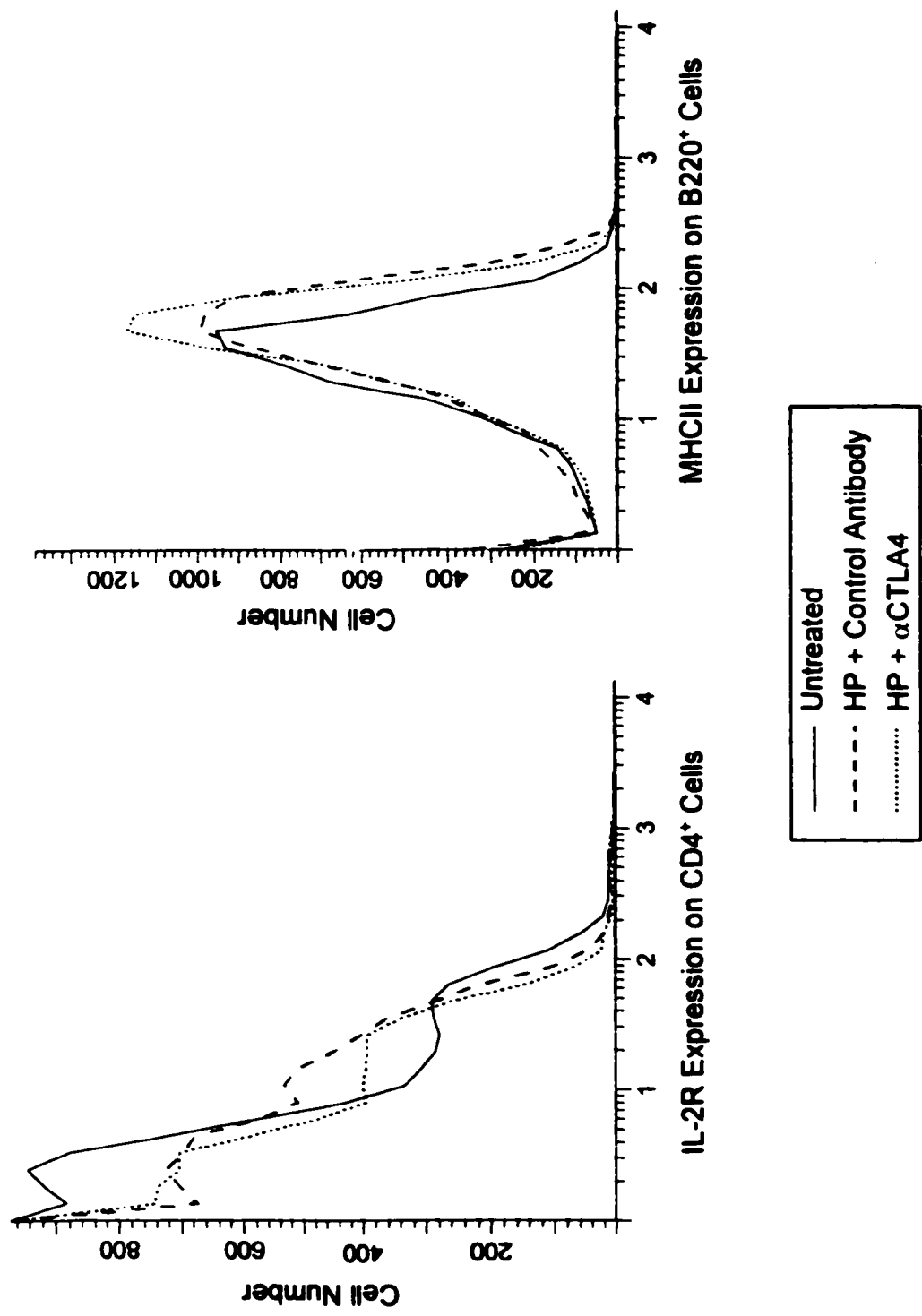
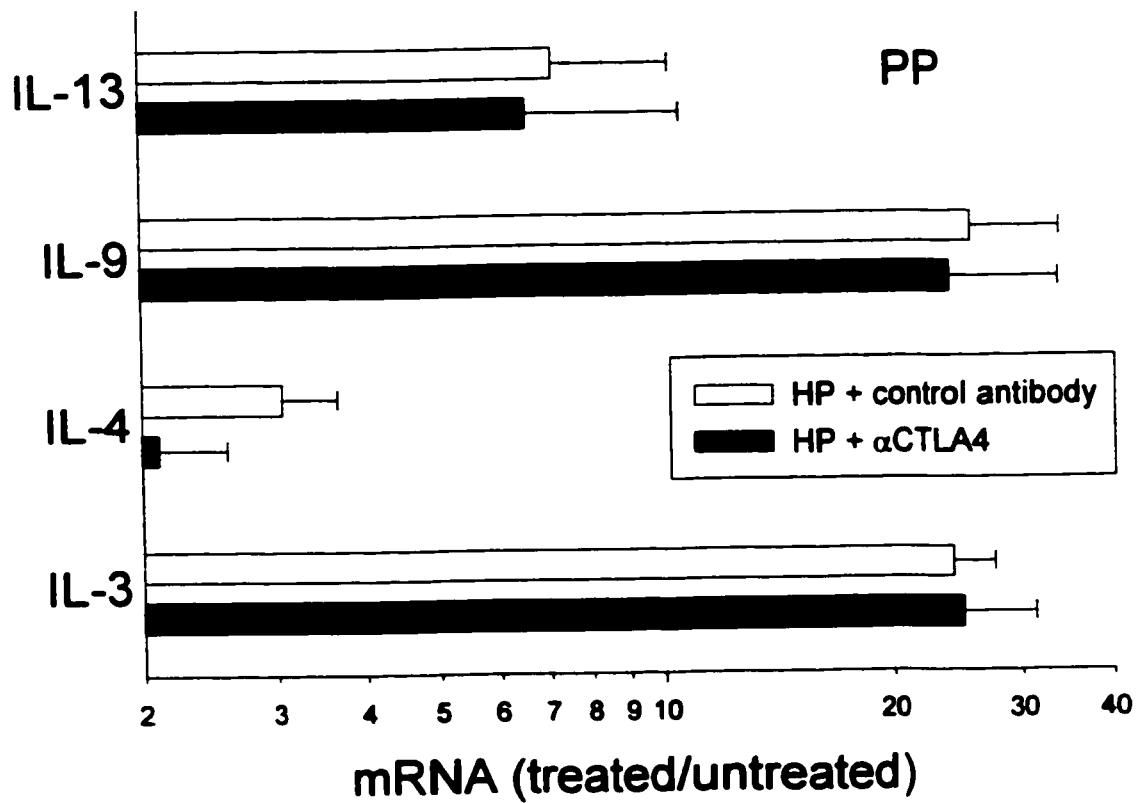
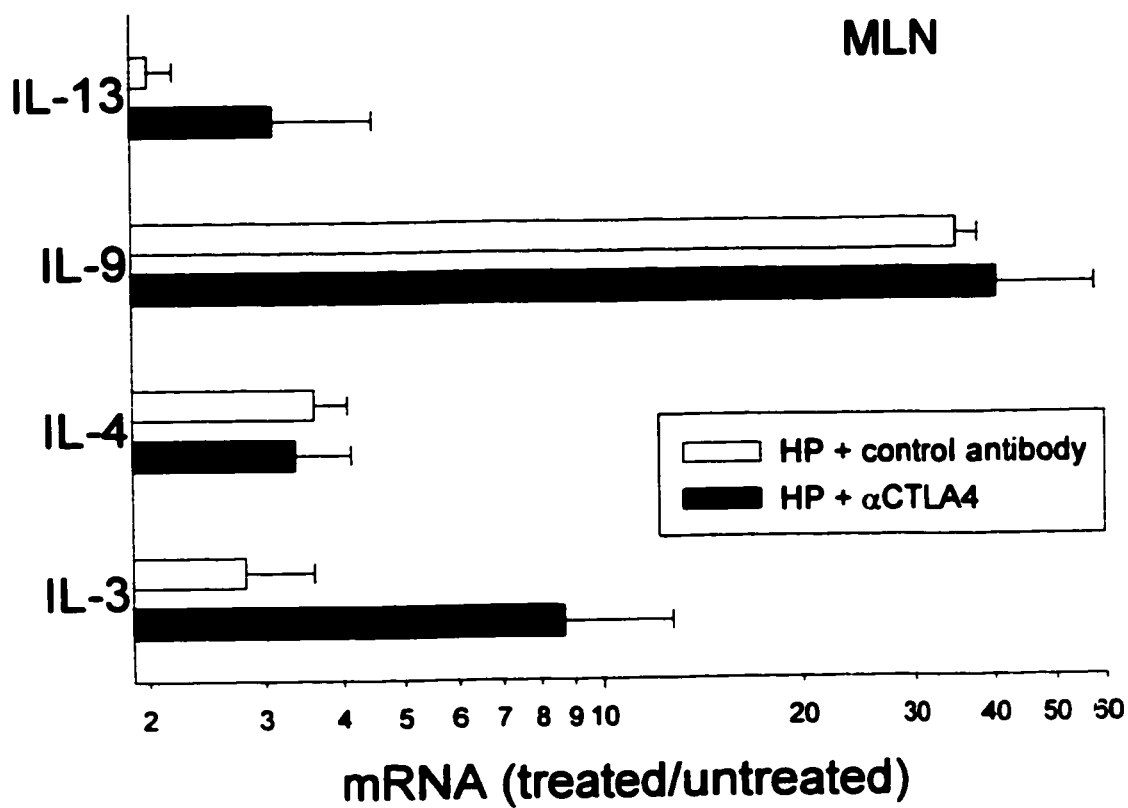


Figure 7. Blocking CTLA4 interactions with anti-CTLA4 antibody may enhance IL-3 gene expression in the mesenteric lymph node on day 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 9, 14, and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mesenteric lymph node and Peyer's patch tissues were collected on day 24 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and standard error are derived from RNA preparations from the mesenteric lymph node and Peyer's patch tissues of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to three-fold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1.



cell activation was evaluated at day 20. Again, serum concentrations of IgE and IgG1 were not elevated (data not shown). FACS analysis showed no evidence of T or B cell activation, as forward scatter and expression of activation markers IL-2R and MHCII were comparable in anti-CTLA4 and control antibody treated groups (data not shown). Gene expression of IL-3, IL-4, IL-5 and IL-9 were quantitated in Peyer's patch tissues. In this experiment, IL-5 gene expression was significantly decreased (58%) in anti-CTLA4 antibody treated animals when compared with control antibody treated animals (Figure 8). IL-9 was also reduced (66%), although the difference was not statistically significant (Figure 8). In fact, in this experiment, there was a statistically significant increase in the *H. polygyrus* egg production in the anti-CTLA4 antibody treated group (Figure 9). Also, serum IgE concentration was significantly reduced (Figure 10). These results indicate that anti-CTLA4 antibody treatment was ineffective in enhancing the response in BALB/c mice infected with *H. polygyrus*. Whether these data represent an actual inhibition of the response to *H. polygyrus* is uncertain, and additional experiments would be required to determine whether anti-CTLA4 antibody can have an inhibitory effect on the immune response in this system. This protocol was not repeated.

2. Anti-CTLA4 antibody specifically enhances Th2-associated serum immunoglobulin concentrations when administered to BALB/c mice after onset of *H. polygyrus* infection.

In the previous experiments, anti-CTLA4 antibody given at 200 µg doses was not effective in enhancing the immune response to *H. polygyrus*. While anti-CTLA4 antibody has been used effectively in other *in vivo* antibody intervention experiments in

Figure 8. Blocking CTLA4 interactions with anti-CTLA4 antibody does not enhance Th2 cytokine gene expression in Peyer's patch tissues on day 20 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 6, 10, 14, and 16 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Peyer's patch tissues were collected on day 20 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and standard error are derived from RNA preparations from Peyer's patch tissues of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to three-fold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1.

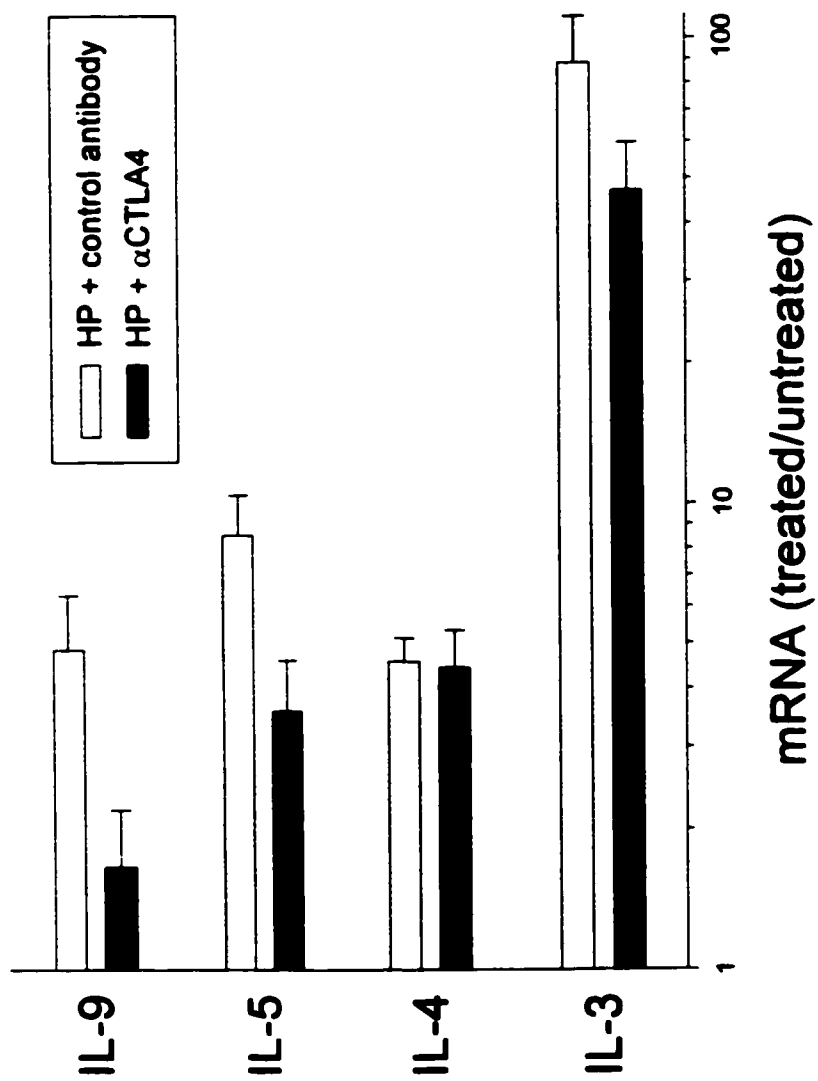


Figure 9. Blocking CTLA4 interactions with anti-CTLA4 antibody increases *H. polygyrus* egg production on day 20 after inoculation. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 6, 10, 14, and 16 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Total eggs were enumerated from the contents of the cecum and large intestine on day 20 after inoculation. The data are expressed as the mean and standard error of five individual mice per treatment group.

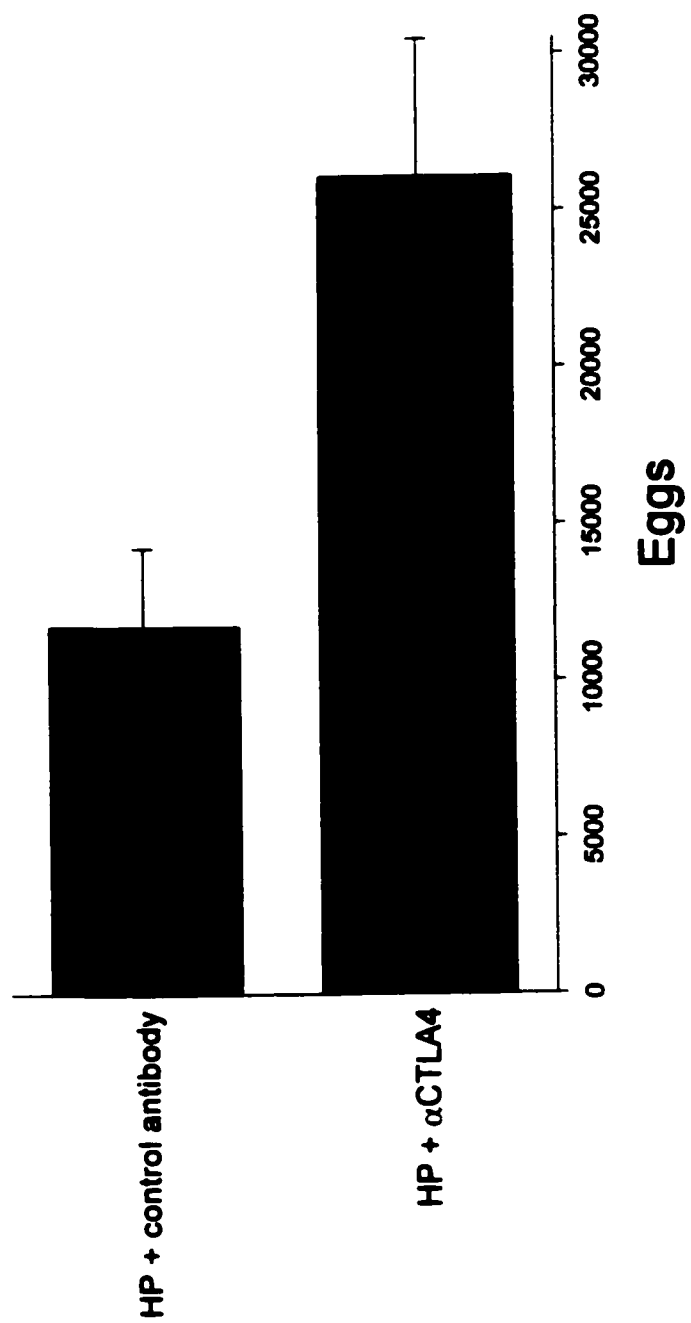
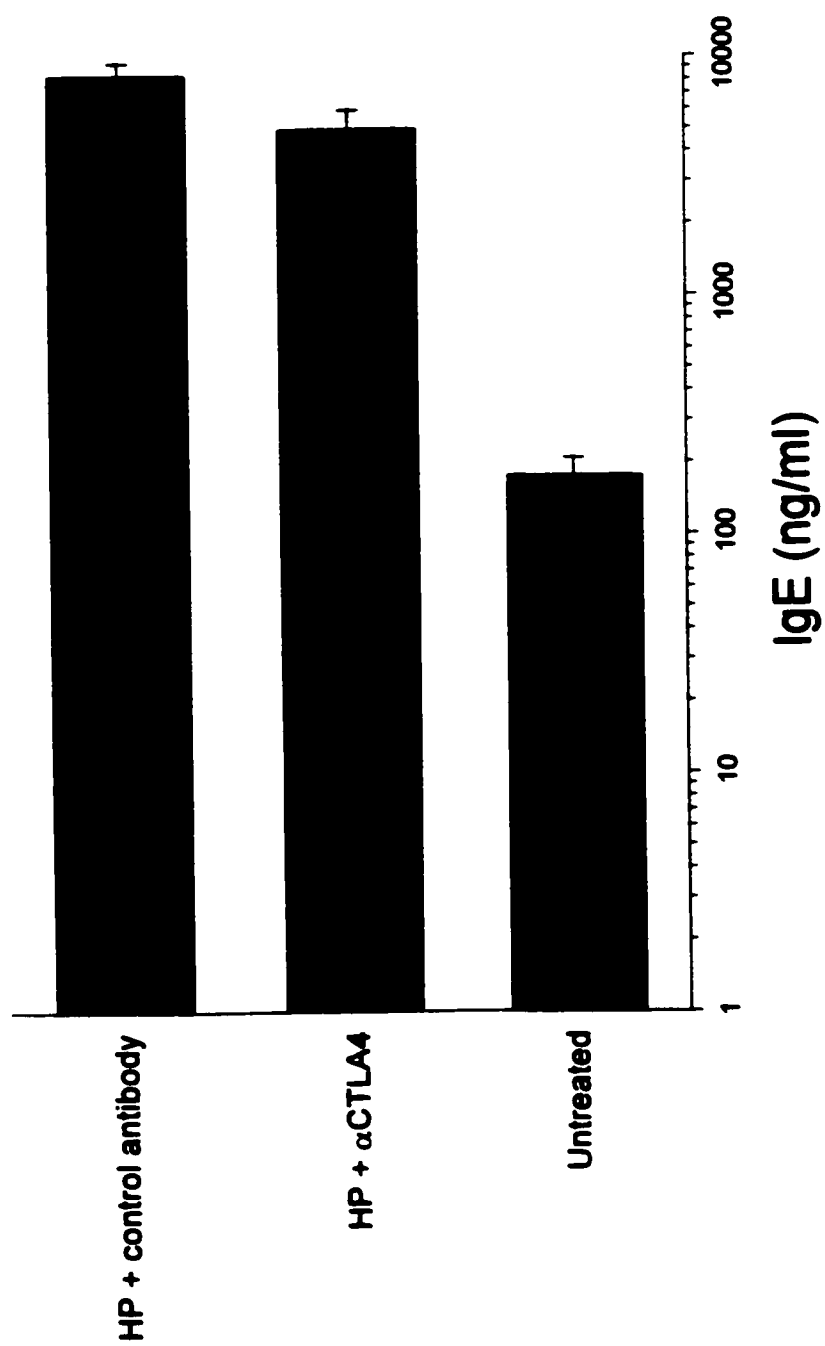


Figure 10. Blocking CTLA4 interactions with anti-CTLA4 antibody reduces *H. polygyrus*-induced elevations in serum IgE at day 20 after inoculation. BALB/c mice were injected i.v. with 200 µg anti-CTLA4 antibody or control antibody on days 6, 10, 14, and 16 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on day 20 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.



similar dosages (Leach et al., 1996; Walunas and Bluestone, 1998; Perez et al., 1997; Luhder et al., 1998), higher doses (up to 1 mg) have also been used (McCoy et al., 1997). Administration of high dose anti-CTLA4 antibody was tested. BALB/c mice (5 per group) were orally inoculated with 200 third-stage *H. polygyrus* larvae, and injected i.v. on days eight and ten with 1 mg anti-CTLA4 antibody or control antibody. On days 15 and 20, serum IgE, IgG1 and IgG2a concentrations were evaluated. The serum from untreated mice contained very little IgE, less than 1 µg/ml (Figure 11). In *H. polygyrus*-inoculated mice treated with control antibody, IgE levels rose by day 15, and remained elevated at day 20. In infected mice treated with anti-CTLA4 antibody, the concentration rose significantly above mice administered control antibody by day 15. This elevation was sustained at day 20.

These results are similar to those seen in serum concentrations of IgG1 (Figure 12). In infected animals treated with control antibody, the serum concentration of IgG1 rose by day 15, and rose further by day 20. Anti-CTLA4 antibody treatment of infected mice resulted in the serum concentration of IgG1 rising significantly higher at days 15 and 20 than in control antibody treated inoculated mice. In addition to polyclonal IgG1, antigen-specific IgG1 was also quantitated (Figure 13). The elevation in antigen-specific IgG1 concentration in anti-CTLA4 antibody treated animals was even more pronounced than the elevation seen in polyclonal IgG1. Anti-CTLA4 antibody treatment increased the antigen-specific IgG1 titer more than 40 fold over the concentration in control antibody treated animals; in comparison, polyclonal IgG1 was increased 2.4 fold.

Serum IgG2a concentrations were quantitated at days 15 and 20 (Figure 14). Unlike the Th2-associated isotypes IgE and IgG1, the serum concentration of IgG2a was

Figure 11. Blocking CTLA4 interactions enhances *H. polygyrus*-induced elevations in serum IgE at days 15 and 20 after inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 15 and 20 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

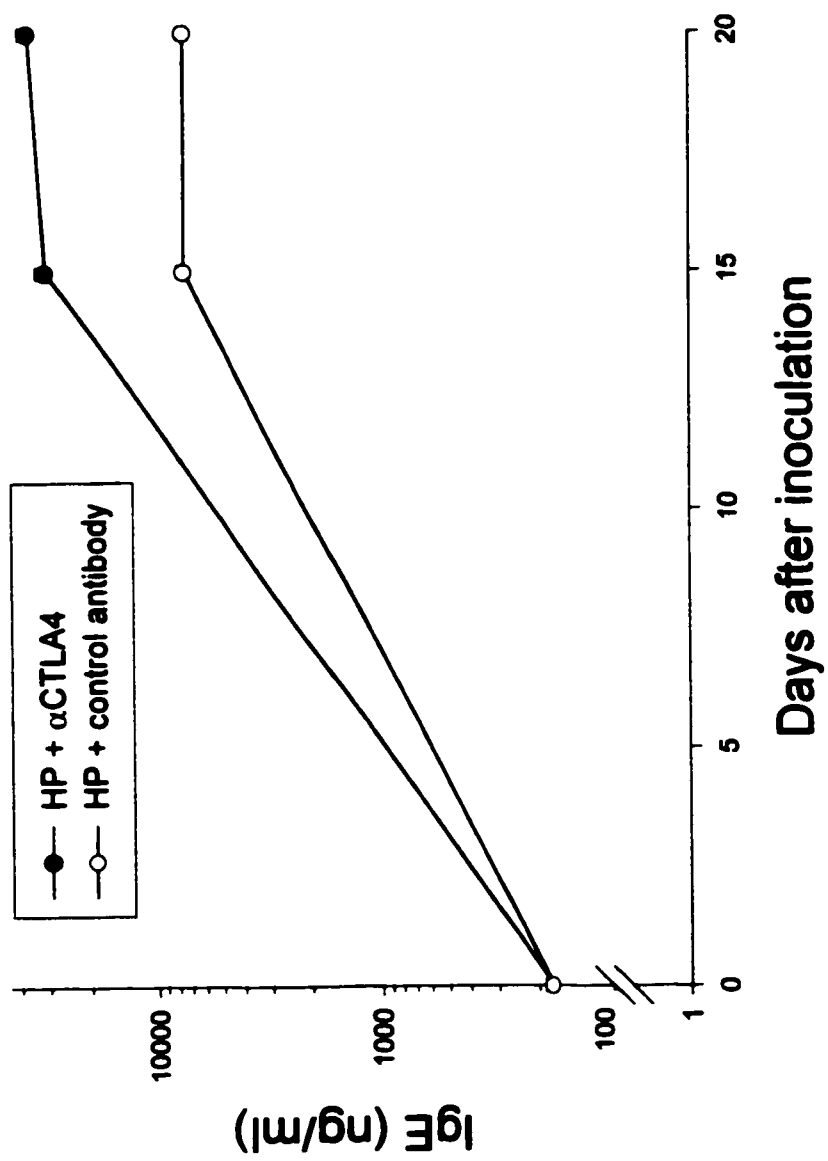


Figure 12. Blocking CTLA4 interactions enhances *H. polygyrus*-induced elevations in polyclonal serum IgG1 at days 15 and 20 after inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 15 and 20 after inoculation, and serum IgG1 levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

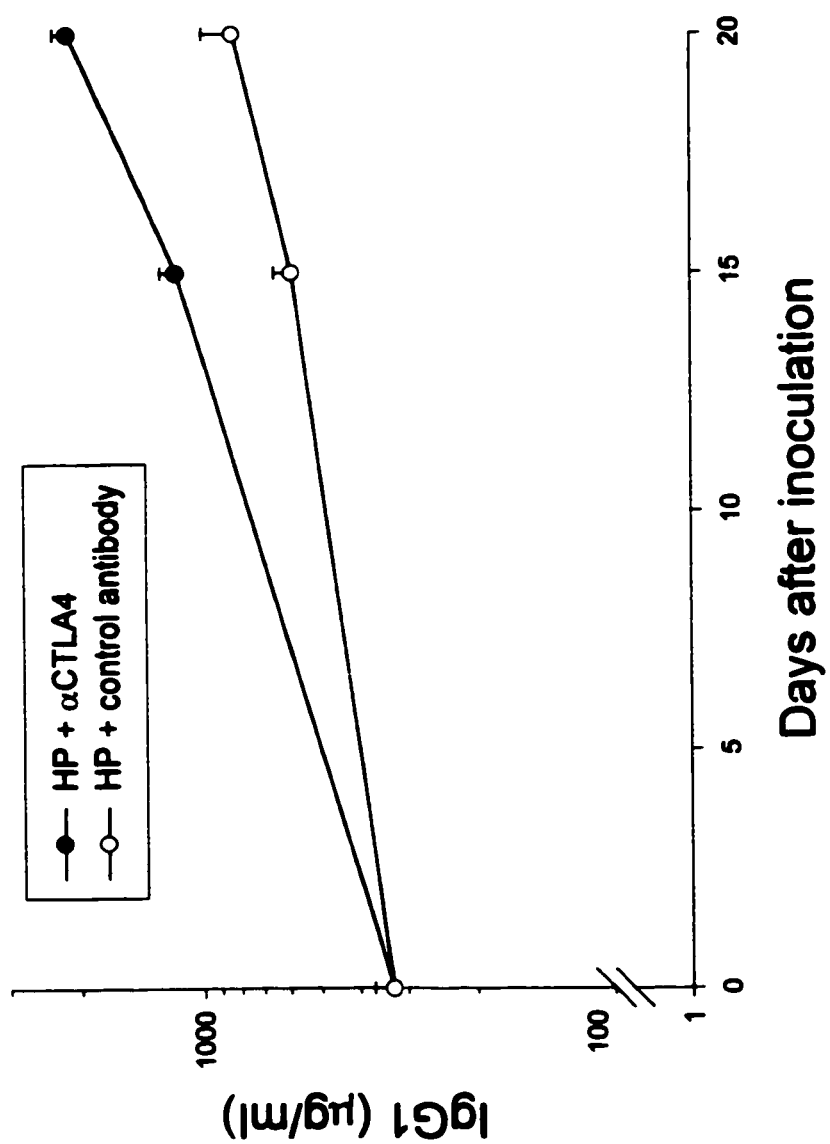


Figure 13. Blocking CTLA4 interactions enhances *H. polygyrus*-induced elevations in antigen-specific serum IgG1 at days 15 and 20 after inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 15 and 20 after inoculation, and serum IgG1 levels were determined by antigen-specific ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

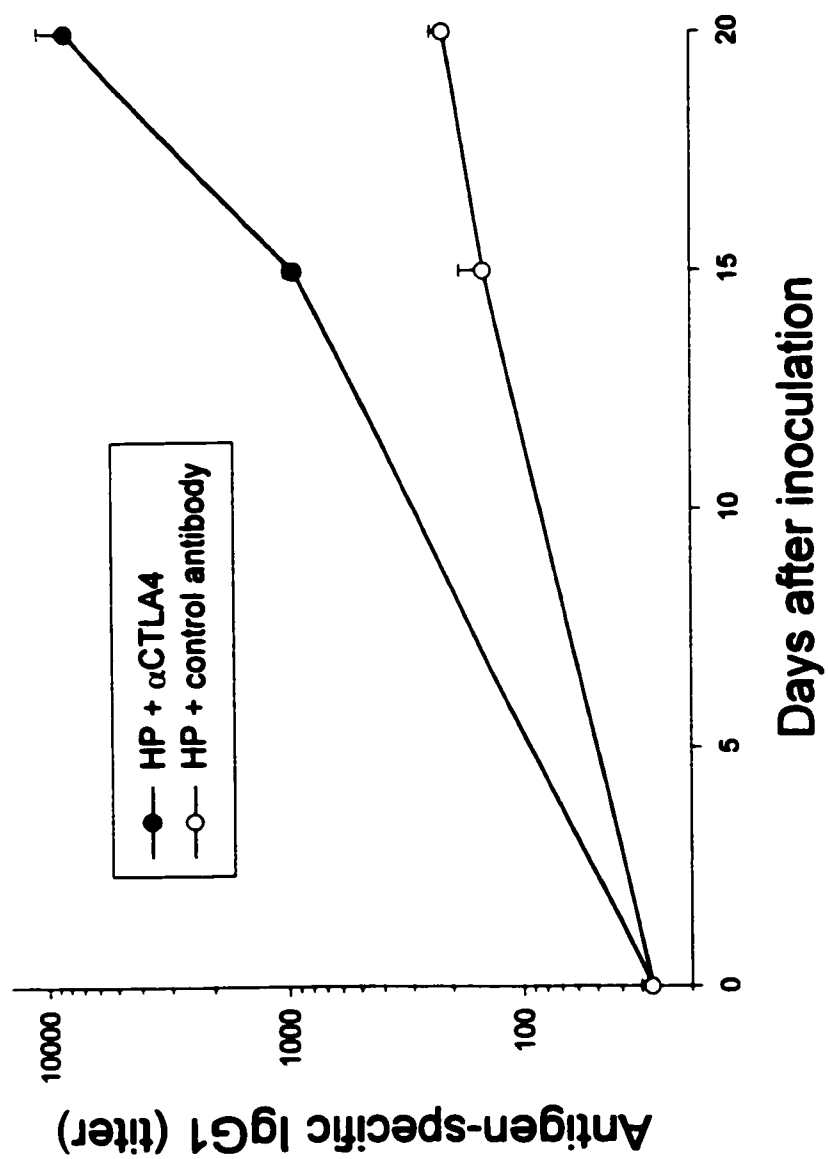
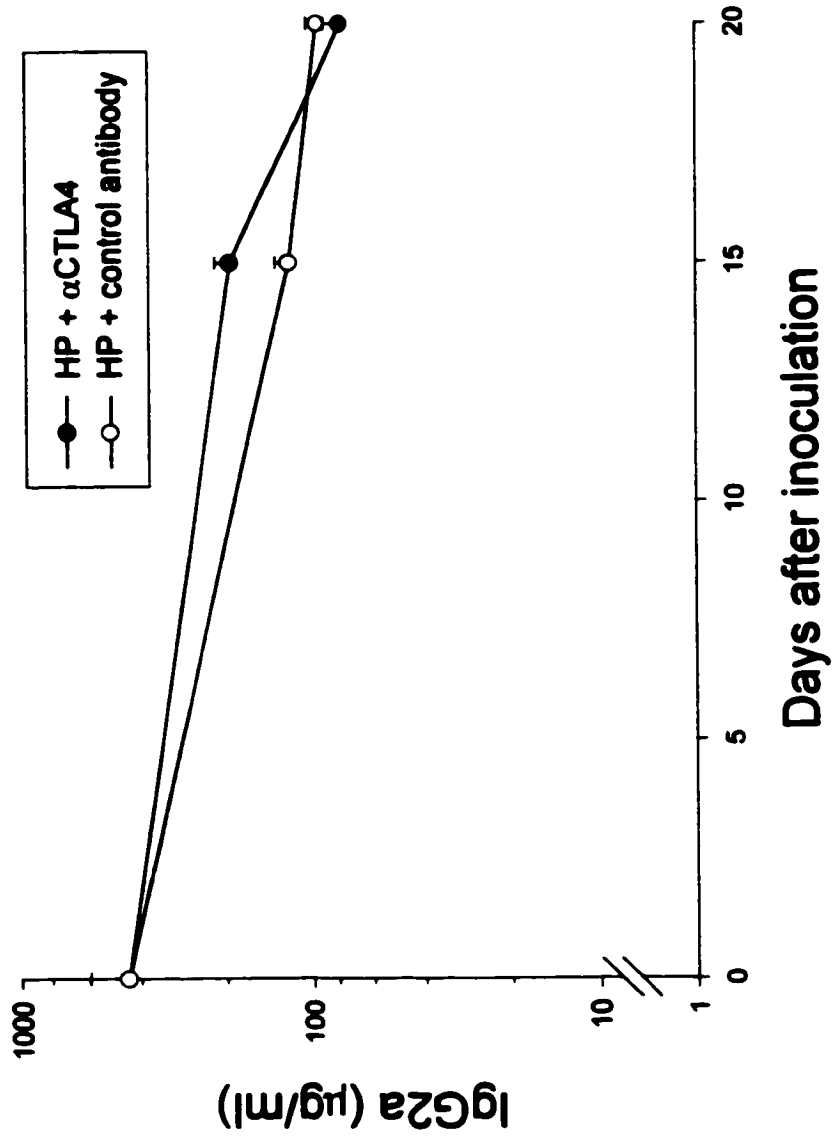


Figure 14. Blocking CTLA4 interactions does not inhibit *H. polygyrus*-induced reductions in serum IgG2a at days 15 and 20 after inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 15 and 20 after inoculation, and serum IgG2a were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.



not elevated by *H. polygyrus* inoculation, in either control antibody or anti-CTLA4 antibody treated mice. In fact, all inoculated groups exhibited a decreased concentration of IgG2a when compared with uninfected controls. This is consistent with the strongly polarized Th2 immune response that *H. polygyrus* elicits. The elevation of IgG1 and IgE, but not IgG2a, indicates a specific increase in the Th2, but not Th1, response in inoculated mice treated with anti-CTLA4 antibody when compared with control antibody treated mice (Mosmann and Coffman, 1989). These experiments were conducted two times with similar results.

3. Administration of anti-CTLA4 antibody to *H. polygyrus*-infected BALB/c mice increases T and B cell activation, resulting in increased cell size, IL-2R expression on CD4⁺ T cells, and number of B220⁺ B cells.

As an additional means to evaluate T cell activation in this system, T and B cell size and surface activation markers were evaluated at days 15 and 20. Single cell suspensions were made of mesenteric lymph nodes, dual stained with anti-CD4 vs. anti-IL-2R antibodies and anti-B220 vs. anti-MHCII antibodies, and quantitated by FACS analysis. At day 15, CD4⁺ T cells from *H. polygyrus*-inoculated mice treated with anti-CTLA4 antibody displayed increased forward scatter compared to those from control antibody treated mice, indicating larger size and a greater degree of activation (Figure 15). A greater percentage of CD4⁺ T cells from anti-CTLA4 antibody treated mice was also IL-2R⁺ compared with CD4⁺ cells from control antibody treated mice at day 15 (Figure 16). However, the intensity of expression of IL-2R on CD4⁺ cells was not increased in anti-CTLA4 antibody treated mice. In fact, CD4⁺ T cells from all *H.*

Figure 15. Blocking CTLA4 interactions increases CD4⁺ T cell size on day 15, but not day 20, after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with FITC-anti-CD4 and biotin-anti-IL-2R followed by streptavidin-PE. Single histogram analysis of CD4⁺ cell size (forward scatter) is shown. This experiment was conducted two times with similar results.

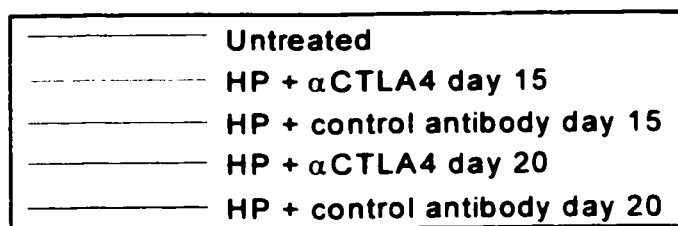
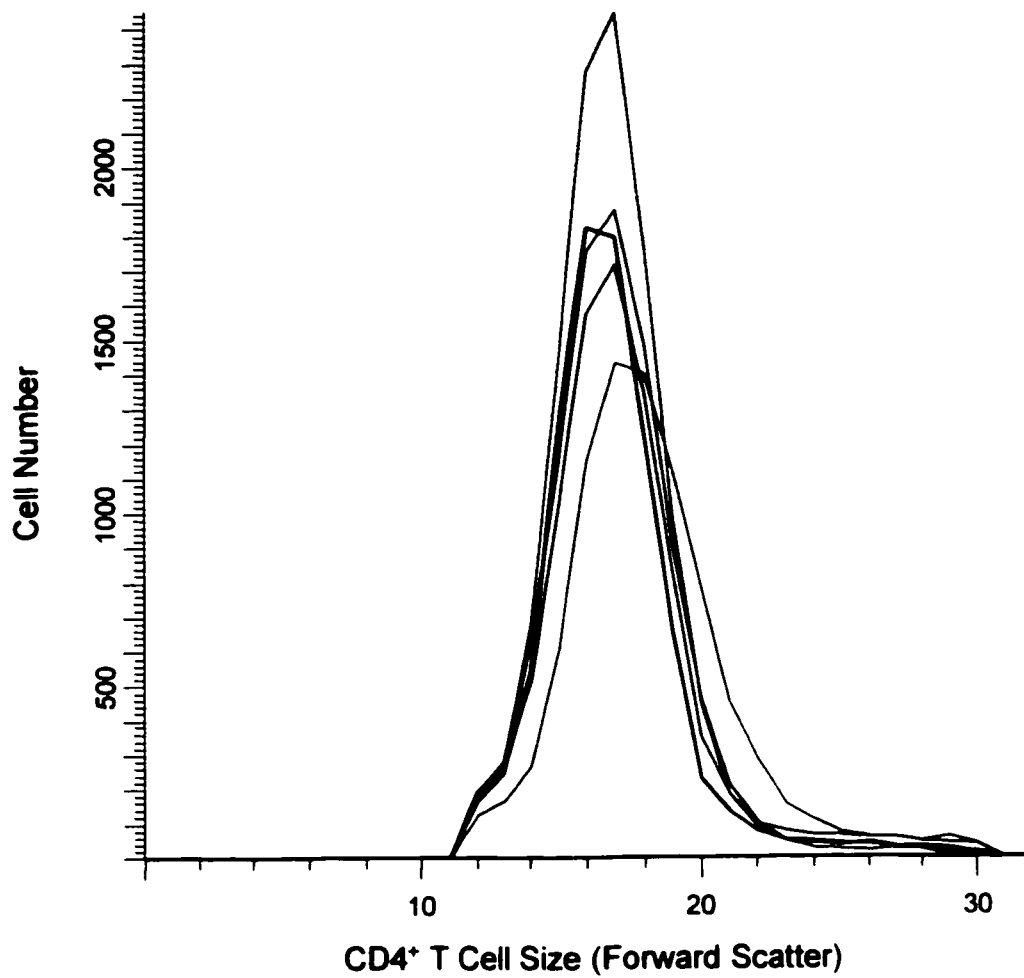
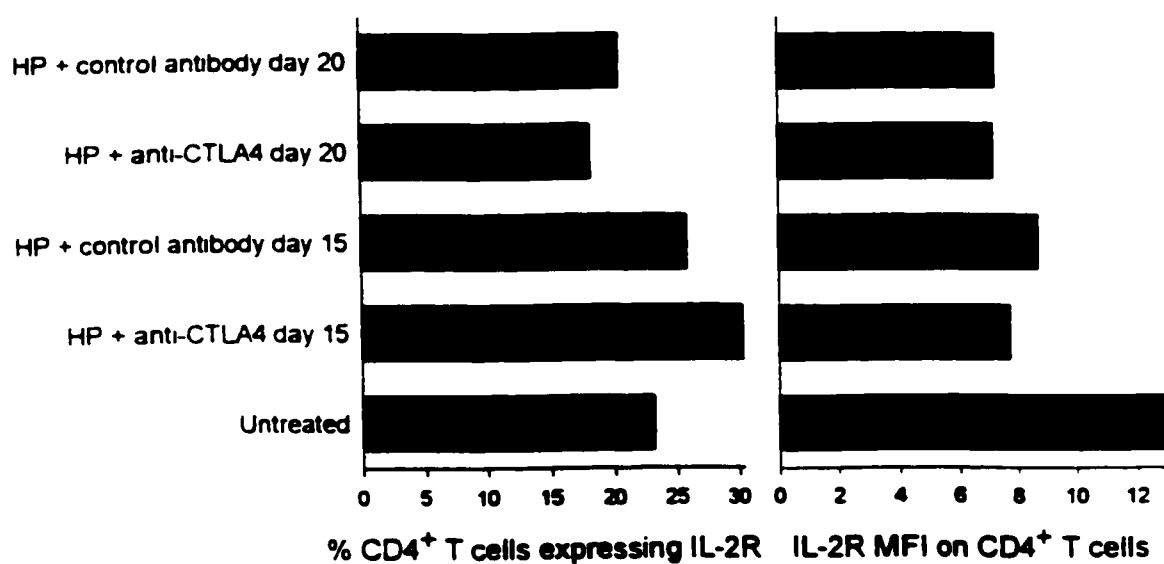
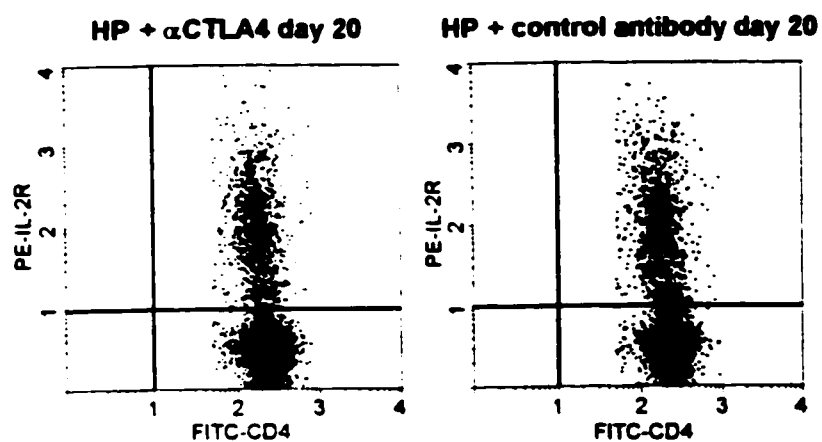
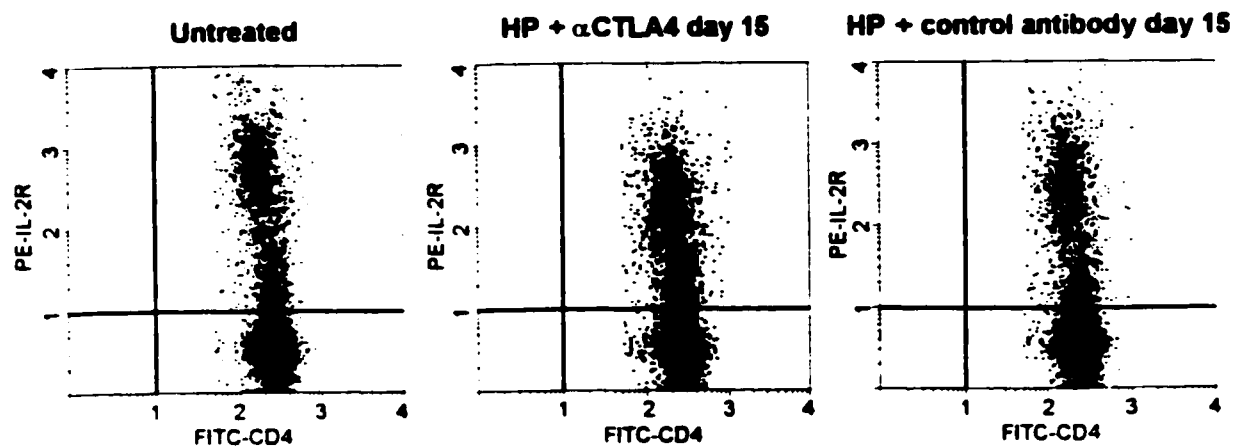


Figure 16. Blocking CTLA4 interactions increases the percentage of CD4⁺ cells expressing IL-2R on day 15, but not day 20, after *H. polygyrus* inoculation. BALB/c mice were inoculated with *H. polygyrus* and treated with anti-CTLA4 antibody or control antibody, and mesenteric lymph node cells were pooled and stained as described in the legend of Figure 15 (five mice per treatment group). Cells were gated on CD4 expression, and IL-2R expression was quantitated. PE-IL-2R versus FITC-CD4 fluorescence scatter plots are displayed on a log scale. The percentage of CD4⁺ T cells expressing IL-2R and the IL-2R mean fluorescence intensity (MFI) on CD4⁺ cells are shown. This experiment was conducted two times with similar results.



polygyrus-inoculated groups had lower IL-2R mean fluorescence intensity when compared with untreated animals. By day 20, the increase in forward scatter of CD4⁺ cells and the number of CD4⁺IL-2R⁺ double positive cells was no longer evident. The percentage of CD4⁺ cells that were also IL-2R⁺ at day 20 was slightly lower in anti-CTLA4 antibody treated animals when compared with the control antibody treated group.

B cells also showed evidence of increased activation in anti-CTLA4 antibody treated inoculated mice, when compared with control antibody treated mice. At day 15, B220⁺ B cells from *H. polygyrus*-inoculated mice treated with anti-CTLA4 antibody displayed increased forward scatter (Figure 17). By day 20, the increased forward scatter was still evident (though somewhat less pronounced than at day 15), in contrast to the return to control levels seen in CD4⁺ cells. The number of B220⁺ B cells was increased at day 15 in anti-CTLA4 antibody treated animals when compared with control antibody treated mice (Figure 18). The number returned to control antibody treated levels by day 20. The MHCII mean fluorescence intensity on B220⁺ B cells was reduced at day 20 in anti-CTLA4 antibody treated animals when compared with control antibody treated mice. This is in contrast to forward scatter, which is still elevated at this timepoint. Together, the FACS data and quantitation of immunoglobulin concentrations are consistent with increased T cell activation, resulting in increased help for B cells in the production of immunoglobulins. These experiments were repeated two times with similar results.

Figure 17. Blocking CTLA4 interactions increases B220⁺ B cell size on days 15 and 20 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with cychrome-anti-B220 and biotin-anti-MHCII followed by streptavidin-PE. Single histogram analysis of B220⁺ cell size (forward scatter) is shown. This experiment was conducted two times with similar results.

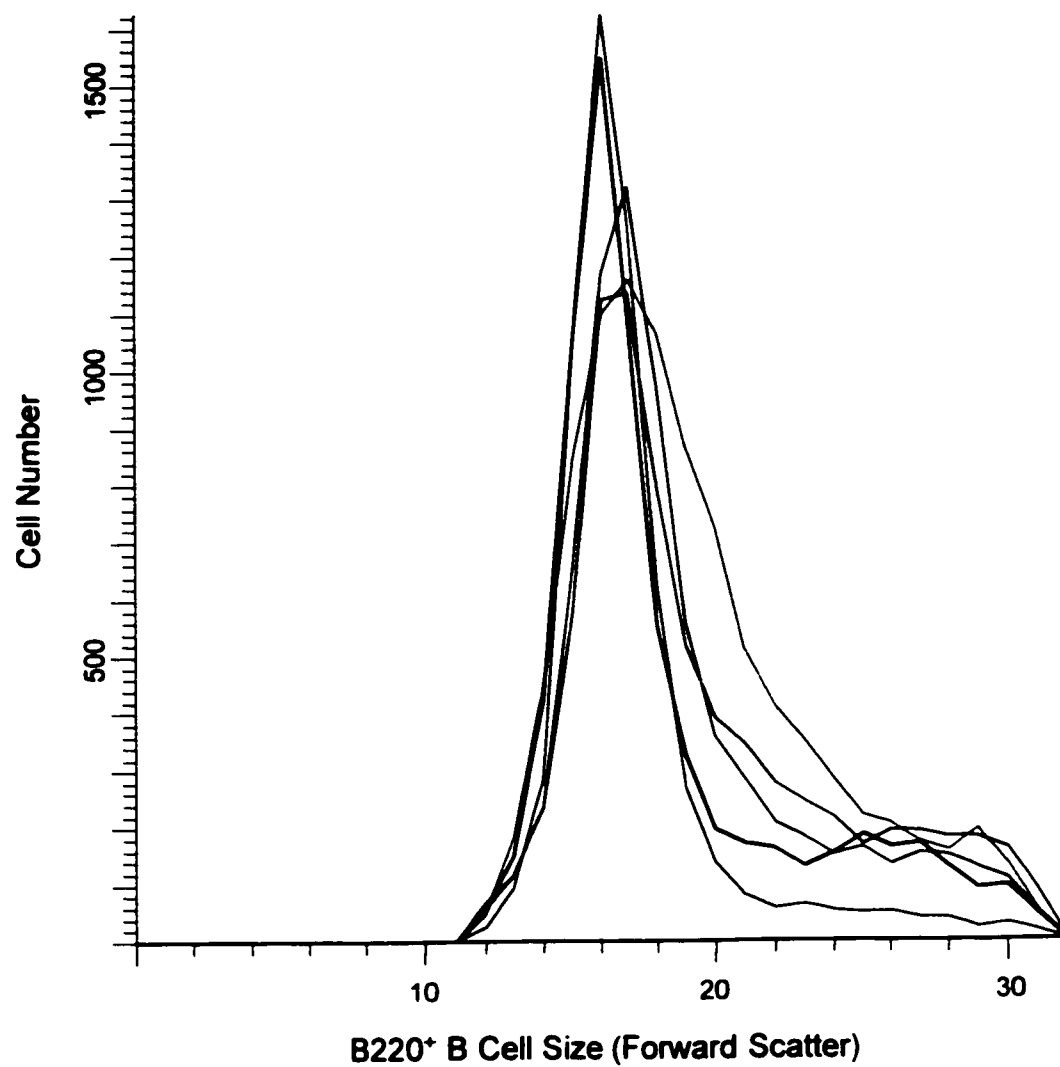
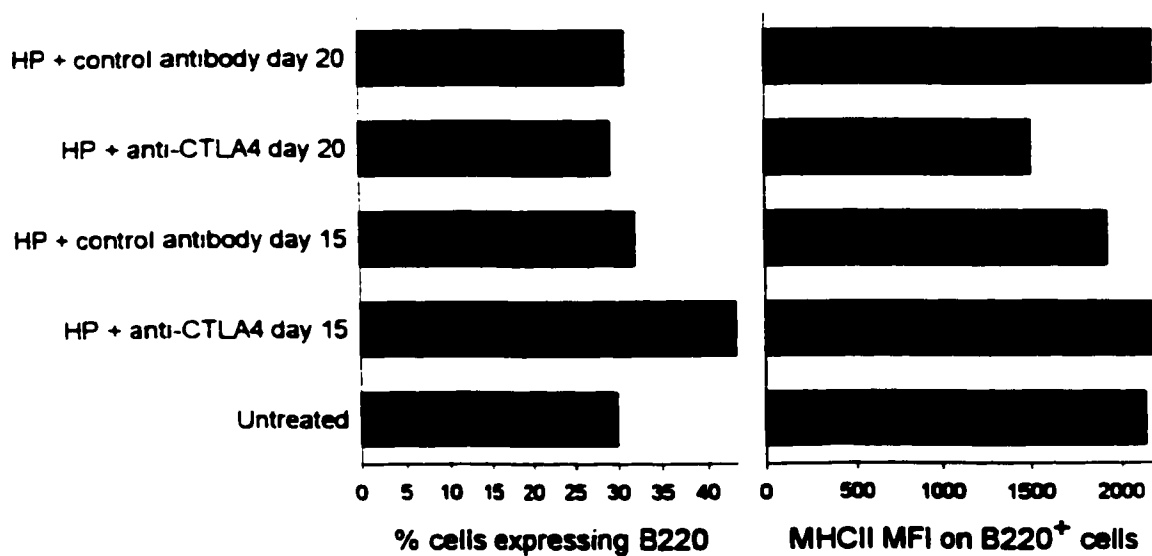
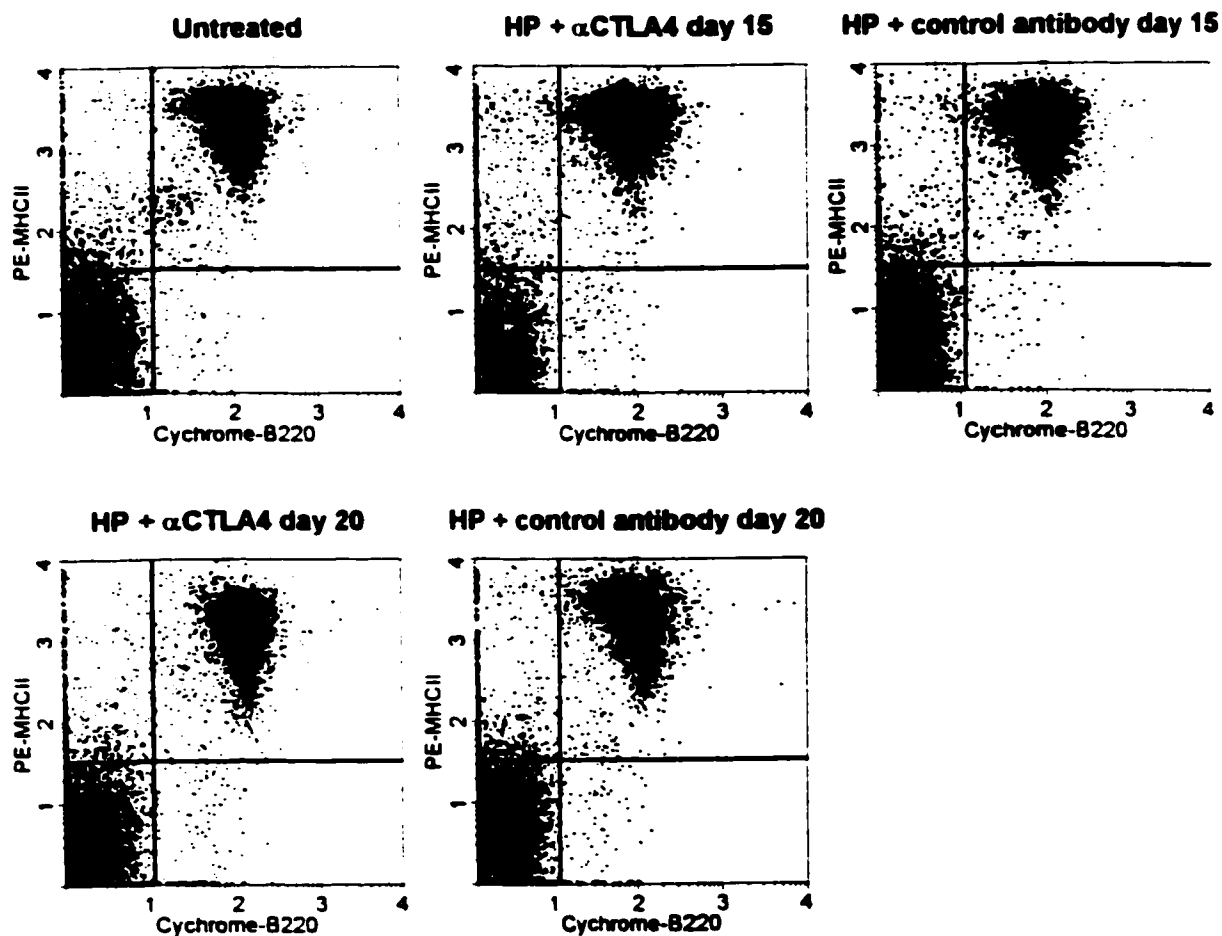


Figure 18. Blocking CTLA4 interactions increases the number of B220⁺ B cells at day 15 and reduces the expression of MHCII on B220⁺ B cells at day 20 after *H. polygyrus* inoculation. BALB/c mice were inoculated with *H. polygyrus* and treated with anti-CTLA4 antibody or control antibody, and mesenteric lymph node cells were pooled and stained as described in the legend of Figure 17 (five mice per treatment group). All live cells were included in the analysis. PE-MHCII versus cychrome-B220 fluorescence scatter plots are displayed on a log scale. The percentage of cells expressing B220 and the MHCII mean fluorescence intensity (MFI) on B220⁺ cells are shown. This experiment was conducted two times with similar results.



4. Anti-CTLA4 antibody treatment of *H. polygyrus*-infected BALB/c mice may increase IL-4 and IL-3 cytokine production.

IL-4 secretion by mesenteric lymph node cells was quantitated in an ELISPOT assay. In untreated control mice, IL-4 secretion was very low (Figure 19). After *H. polygyrus* inoculation, control antibody treated mice exhibited an increase in the number of IL-4 producing cells at both day 16 and day 20. At day 20, anti-CTLA4 antibody treatment significantly increased the number of IL-4 producing cells above the number seen in control antibody treated mice. IL-4 is a potent mediator of the protective response to *H. polygyrus* (Urban et al., 1998; Finkelman et al., 1997). Consistent with this, egg production was found to be significantly reduced in anti-CTLA4 antibody treated mice at day 20 in this experiment (Figure 20). This suggests that in this experiment host protection was enhanced by CTLA4 blockade. However, in a subsequent experiment egg production was found to be unaffected.

In a separate, independent experiment, an RT-PCR assay was utilized to quantitate IL-3 and IL-4 mRNA expression in mesenteric lymph node tissues as an additional means to evaluate cytokine production (Figure 21). At day 15 after *H. polygyrus* inoculation, IL-3 gene expression increased 3.8 fold in anti-CTLA4 antibody treated mice, when compared with control antibody treated mice. By day 20, IL-3 gene expression in anti-CTLA4 antibody treated mice dropped below the expression level seen in control antibody treated mice. However, these differences were not statistically significant. No difference was seen in IL-4 mRNA expression at either day 15 or day 20 in this experiment. In Figure 19, IL-4 secretion started to rise by day 16, becoming significant by day 20. The cytokine gene expression data in Figure 21 suggest that the

Figure 19. Blocking CTLA4 interactions enhances IL-4 secretion on day 20 in the mesenteric lymph node of *H. polygyrus*-inoculated mice. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mesenteric lymph node tissues were collected on days 16 and 20 after inoculation, and the number of IL-4 secreting mesenteric lymph node cells was determined in an ELISPOT assay without restimulation. Cell suspensions were assayed from five individual mice per treatment group, and the mean and standard error are shown.

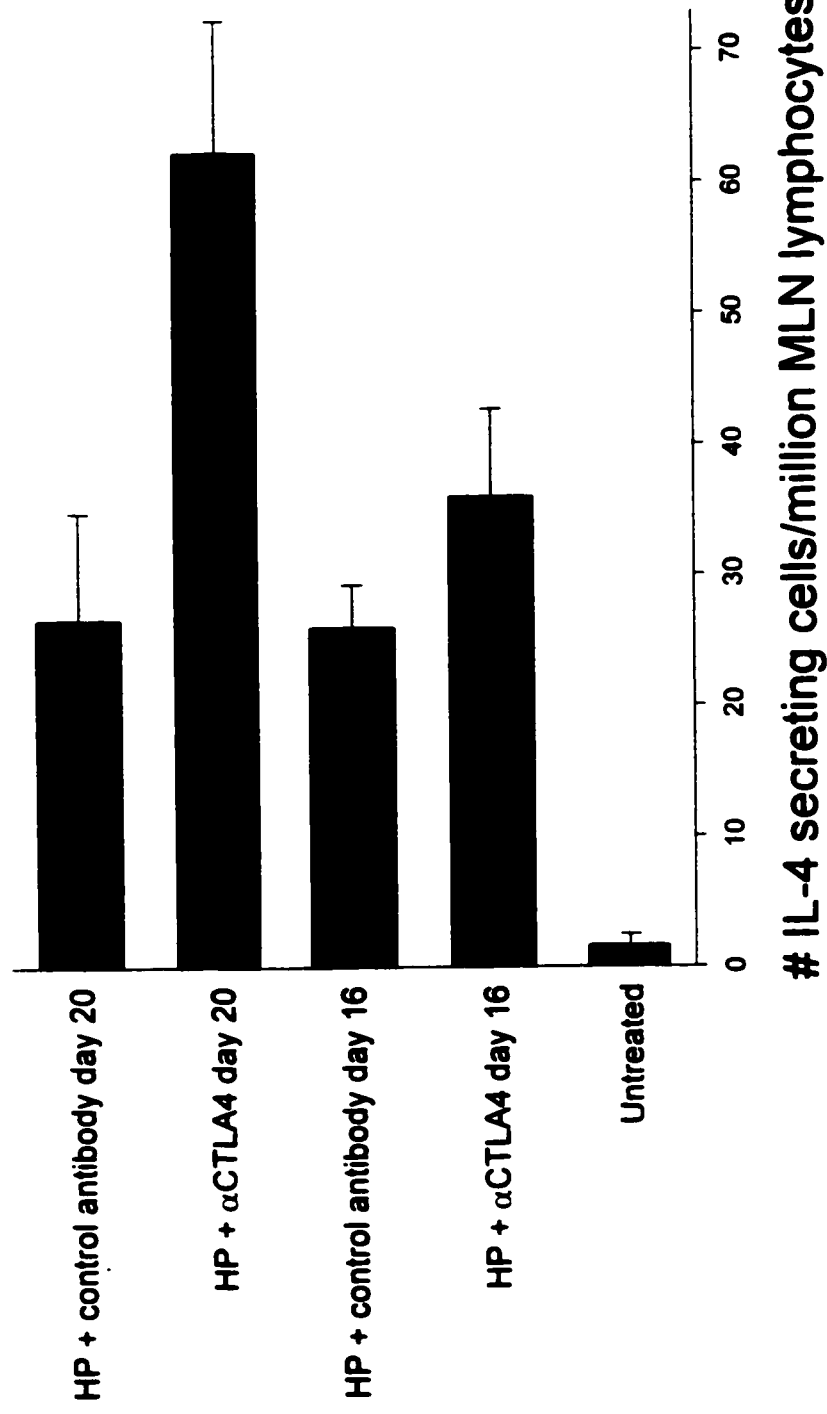


Figure 20. Blocking CTLA4 interactions reduces egg production in *H. polygyrus*-inoculated mice. BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae. Total eggs were enumerated from the contents of the cecum and large intestine on days 16 and 20 after inoculation. The data are expressed as the mean and standard error of five individual mice per treatment group.

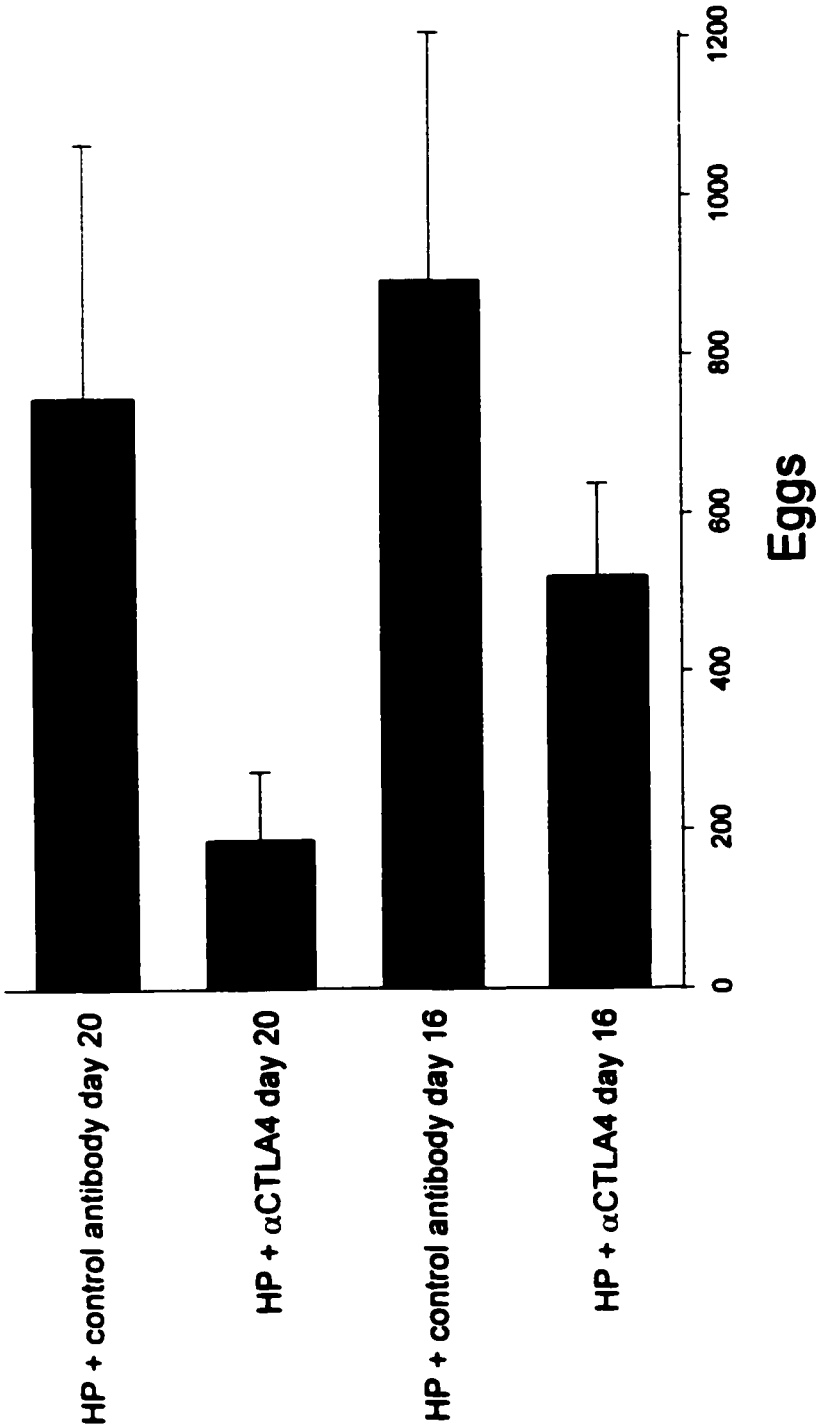
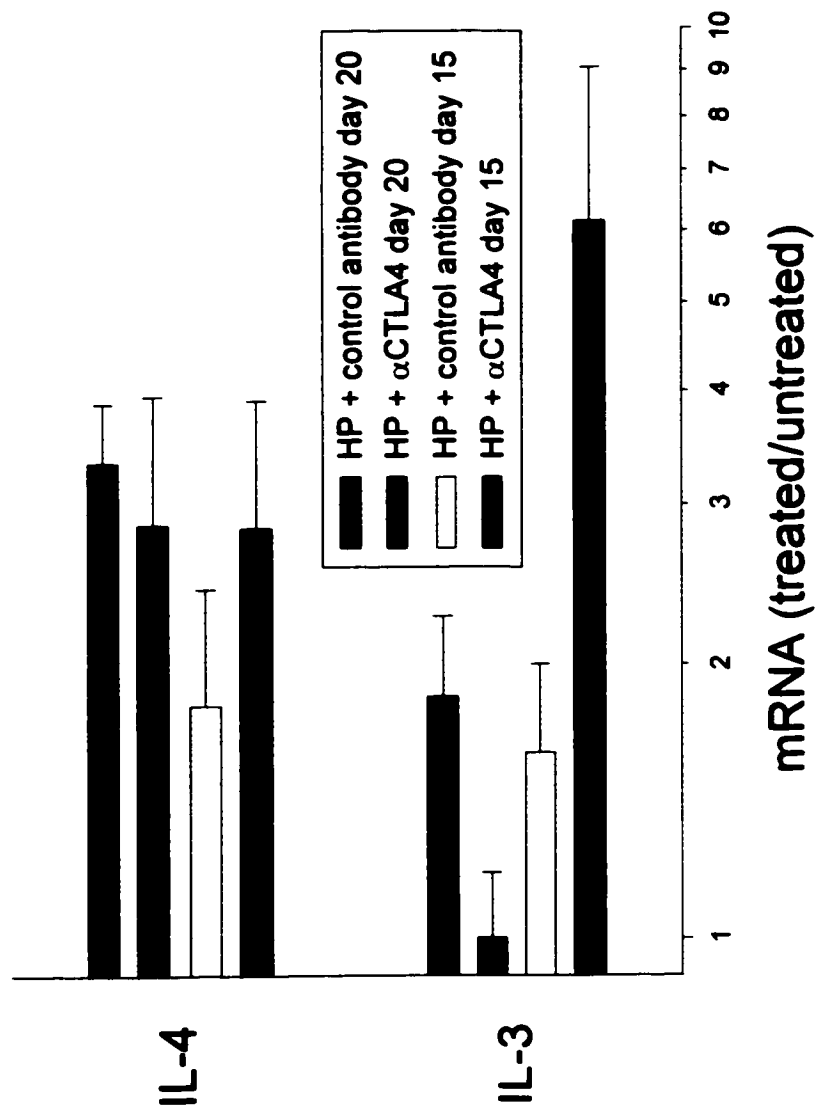


Figure 21. Blocking CTLA4 interactions may enhance IL-3 gene expression in the mesenteric lymph node on day 15 after *H. polygyrus* inoculation.

BALB/c mice were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten after oral inoculation with 200 third-stage *H. polygyrus* larvae.

Mesenteric lymph node tissues were collected on days 15 and 20 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and standard error are derived from RNA preparations from the mesenteric lymph node tissues of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to three-fold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1.



increase in secretion of IL-4 is not reflected in a corresponding increase in mRNA at this timepoint, and may be due to other factors such as increased translation.

B. Studies on the effects of CTLA4 blockade during a mixed Th1/Th2 immune response to an infectious pathogen.

1. Anti-CTLA4 antibody specifically enhances IL-4 secretion, and inhibits IFN γ secretion, when administered to AKR mice after onset of *T. muris* infection.

Whether an immune response against *T. muris* is effective in protecting against infection depends largely on the type of response that develops. Th2 responses are effective in controlling *T. muris* infection, and Th1 responses are not host protective (Else et al., 1992). AKR mice normally mount a response to *T. muris* that is not protective and has both Th1 and Th2 components, but the response can be shifted to a protective Th2 response by depletion of IFN γ or administration of exogenous IL-4 (Else et al., 1994). It has been hypothesized that the Th1/Th2 decision is based on the balance between strength of signaling and the ability of the pathogen to stimulate the innate response (Gause et al., 1999). A Th2 response is favored by increased strength of signal relative to the innate response. This experiment was designed to test this hypothesis. By blocking CTLA4 signaling with anti-CTLA4 antibody, the overall strength of signal should be increased relative to the innate response, which may result in immune deviation toward a Th2 response.

AKR mice were infected with *T. muris* and administered 600 μ g anti-CTLA4 antibody or control antibody at days 8 and 16 after inoculation. At day 21, the number of IFN γ and IL-4 producing mesenteric lymph node cells were quantitated with an ELISPOT assay. These experiments were conducted two times with similar results. The number of IFN γ producing mesenteric lymph node cells was increased upon *T. muris*

inoculation (Figure 22). *T. muris* infection of control antibody treated mice induced a four-fold increase in the number of IFN γ producing cells, when compared with uninfected controls. Anti-CTLA4 antibody treatment significantly inhibited the *T. muris*-induced increase in the number of IFN γ producing cells, to close to the number in the uninfected control group (57% inhibition). In contrast with IFN γ , the number of IL-4 producing cells was not increased by *T. muris* inoculation of control antibody treated animals, when compared with uninfected controls (Figure 23). Anti-CTLA4 antibody treatment of *T. muris*-infected mice resulted in a significant increase in the number of IL-4 producing cells (57% increase). These results indicate that anti-CTLA4 antibody treatment specifically inhibited the secretion of a Th1 cytokine (IFN γ), while enhancing a Th2 cytokine (IL-4).

In addition to these experiments, the mesenteric lymph node cells were restimulated with conA, and the secretion of IFN γ was quantitated by ELISPOT in one experiment, as an additional method to evaluate Th1 cytokine secretion. *T. muris* inoculation of control antibody treated mice induced an increase in the number of IFN γ -secreting conA stimulated mesenteric lymph node cells (Figure 24). Anti-CTLA4 antibody treatment inhibited *T. muris*-induced IFN γ secretion, by 44%. These results also support inhibition of Th1 cytokine secretion by anti-CTLA4 antibody treatment.

Cytokine message was quantitated by PCR in one experiment. Surprisingly, anti-CTLA4 antibody treatment enhanced IFN γ mRNA expression in both Peyer's patch and mesenteric lymph node tissues (Figure 25). In Peyer's patch tissues, expression was significantly increased, by more than three-fold over control antibody treated mice. In mesenteric lymph node tissues, the message was increased to a lesser degree and the

Figure 22. Blocking CTLA4 interactions reduces *T. muris*-induced IFN γ secretion in the mesenteric lymph node on day 21 after inoculation. AKR mice were injected i.v. with 600 μ g anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mesenteric lymph node tissues were collected on day 21 after inoculation, and the number of IFN γ secreting mesenteric lymph node cells was determined in an ELISPOT assay without restimulation. Cell suspensions were assayed from five individual mice per treatment group, and the mean and standard error are shown. This experiment was conducted two times with similar results.

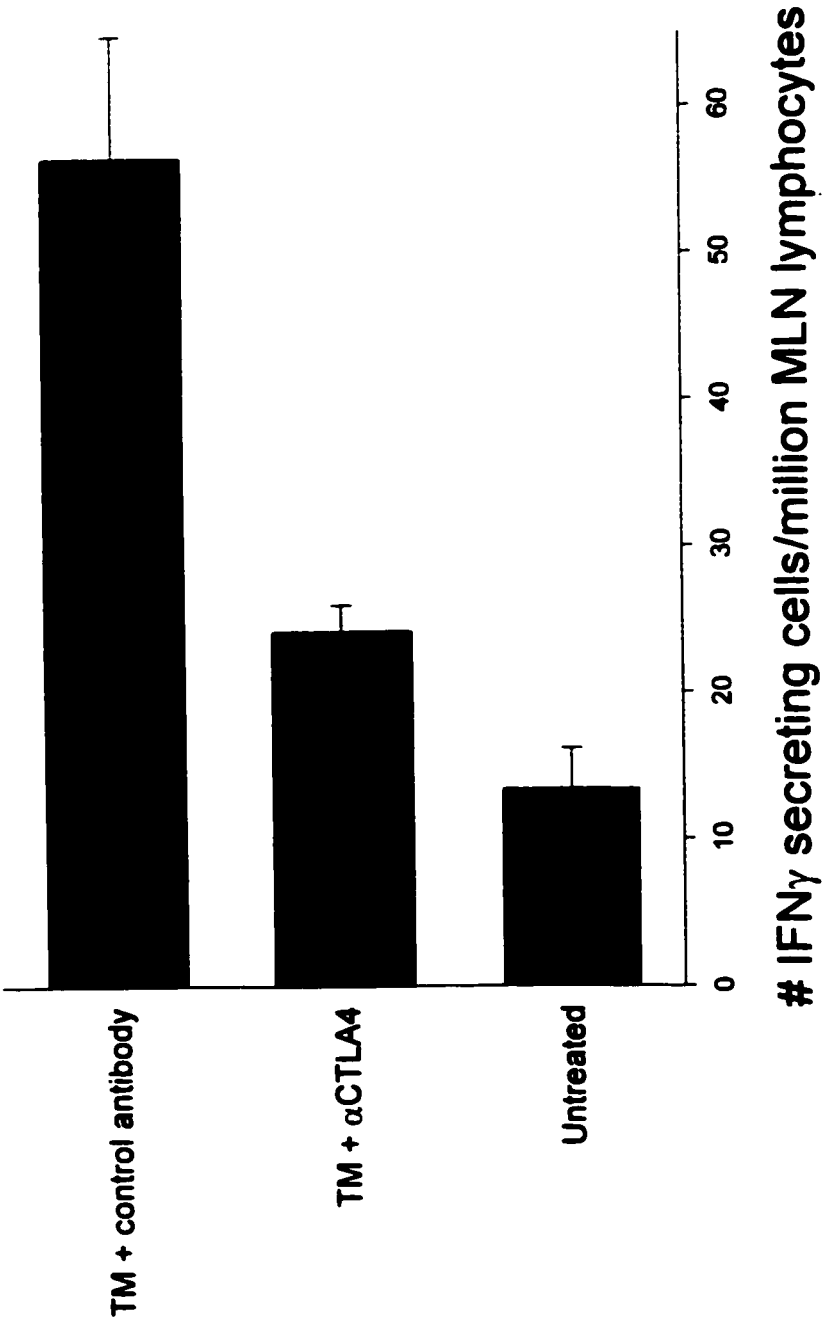


Figure 23. Blocking CTLA4 interactions increases *T. muris*-induced IL-4 secretion in the mesenteric lymph node on day 21 after inoculation. AKR mice were injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mesenteric lymph node tissues were collected on day 21 after inoculation, and the number of IL-4 secreting mesenteric lymph node cells was determined in an ELISPOT assay without restimulation. Cell suspensions were assayed from five individual mice per treatment group, and the mean and standard error are shown. This experiment was conducted two times with similar results.

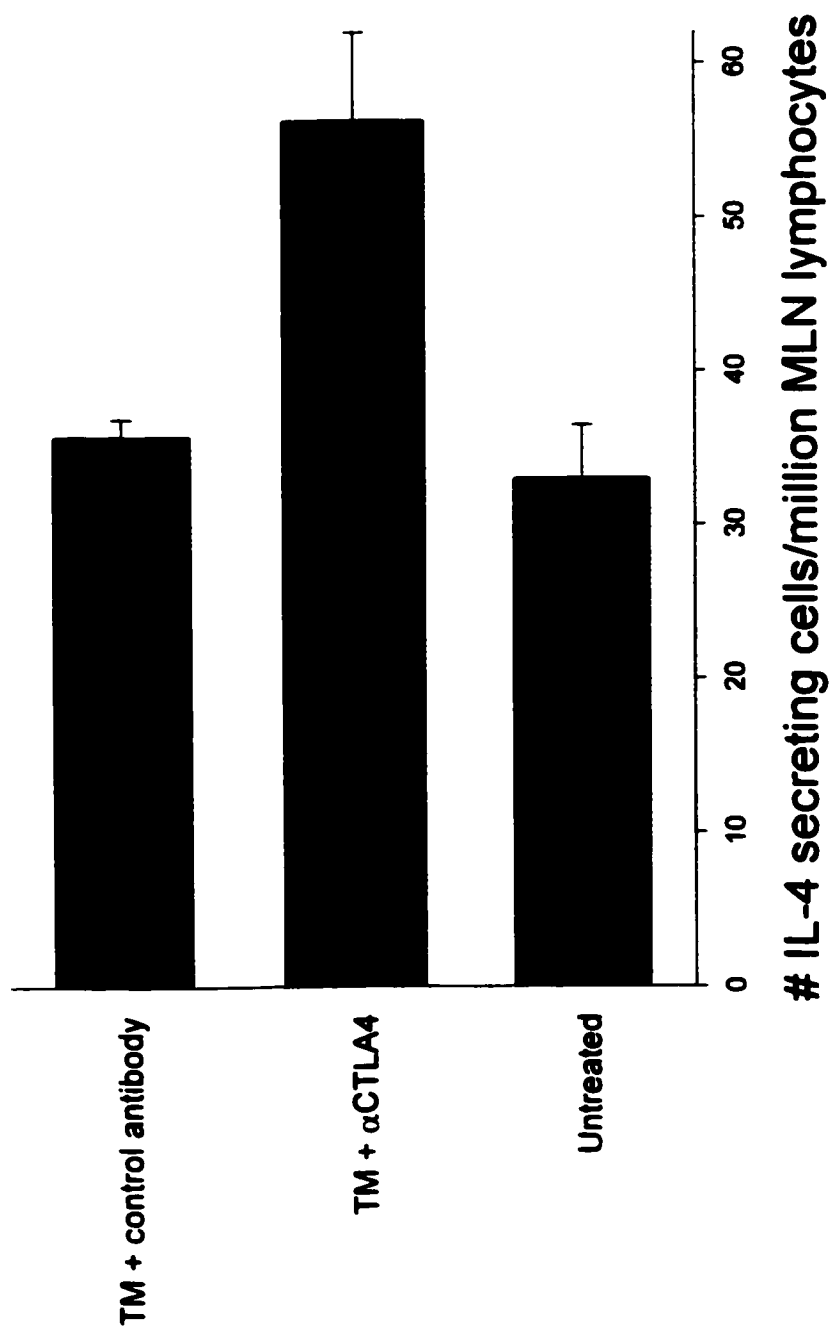


Figure 24. Blocking CTLA4 interactions reduces *T. muris*-induced IFN γ secretion in conA-restimulated mesenteric lymph node cells on day 21 after inoculation. AKR mice were injected i.v. with 600 μ g anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mesenteric lymph node tissues were collected from five individual mice on day 21 after inoculation, pooled, and the number of IFN γ secreting mesenteric lymph node cells was determined in an ELISPOT assay with conA restimulation.

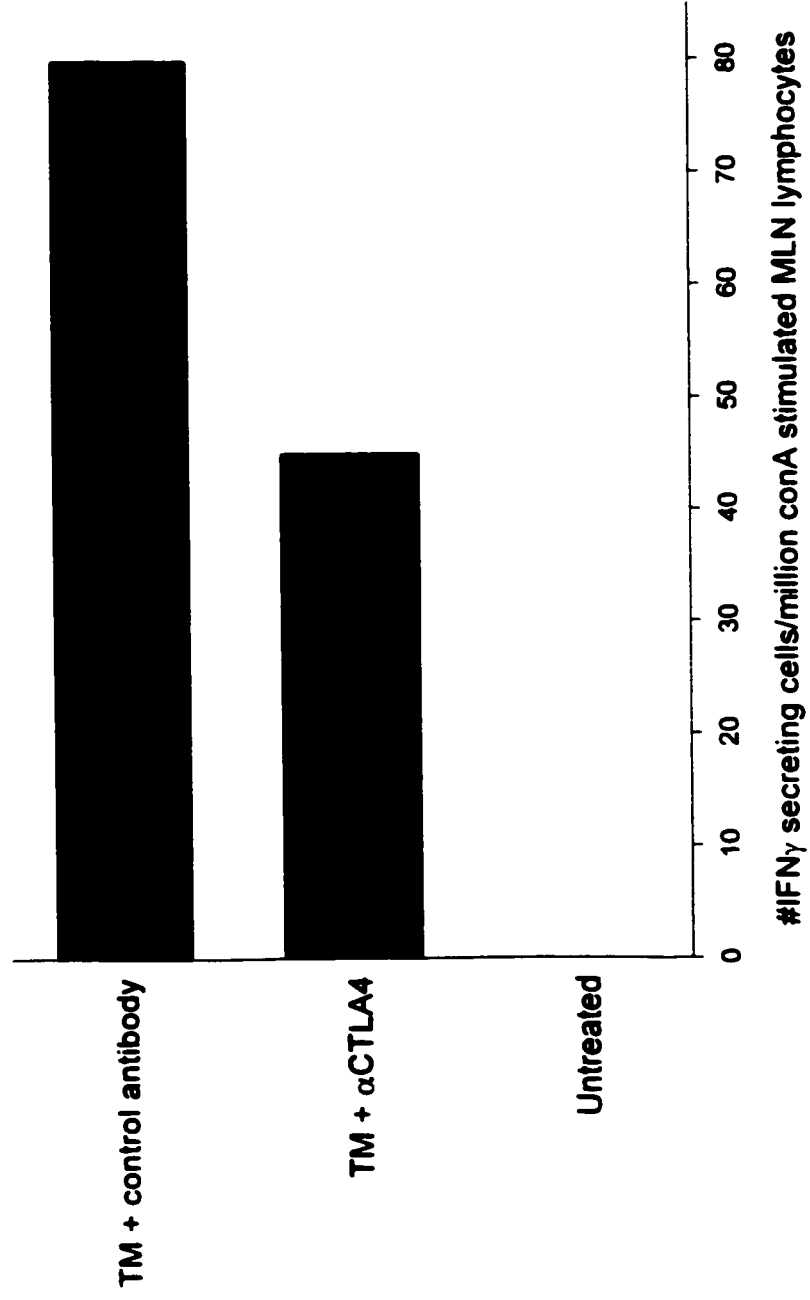
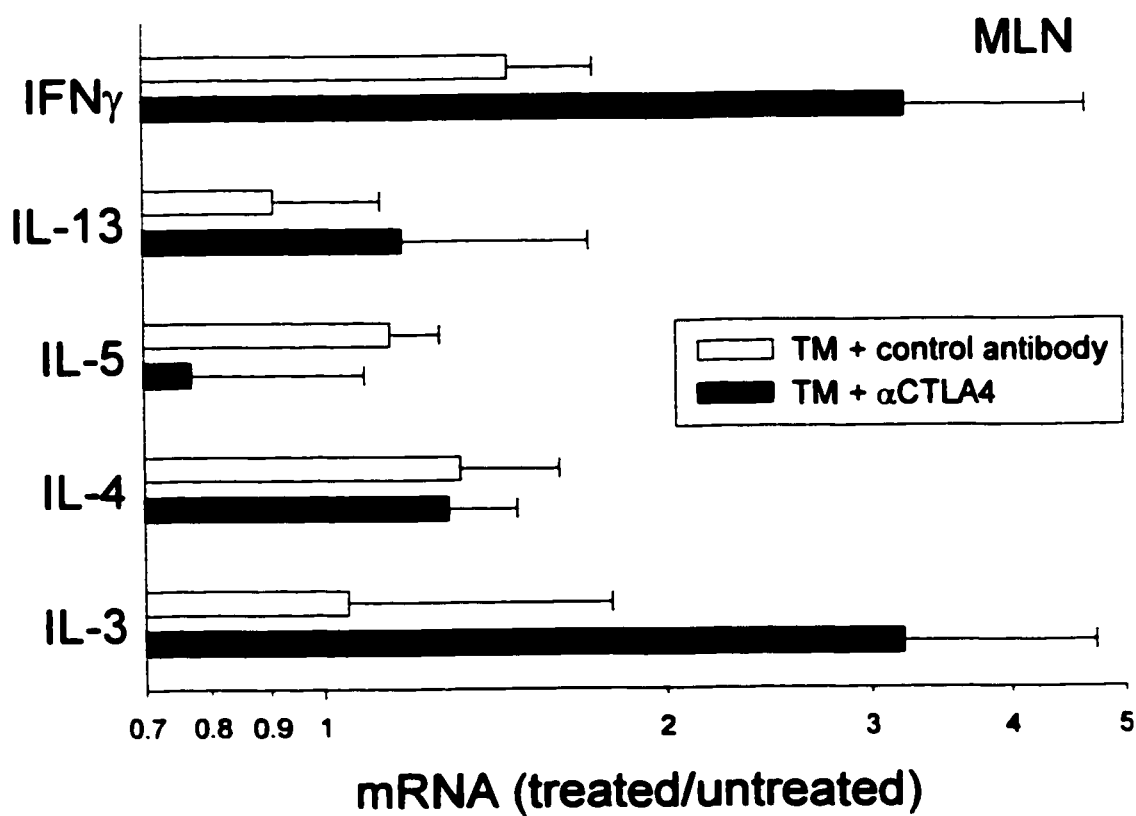
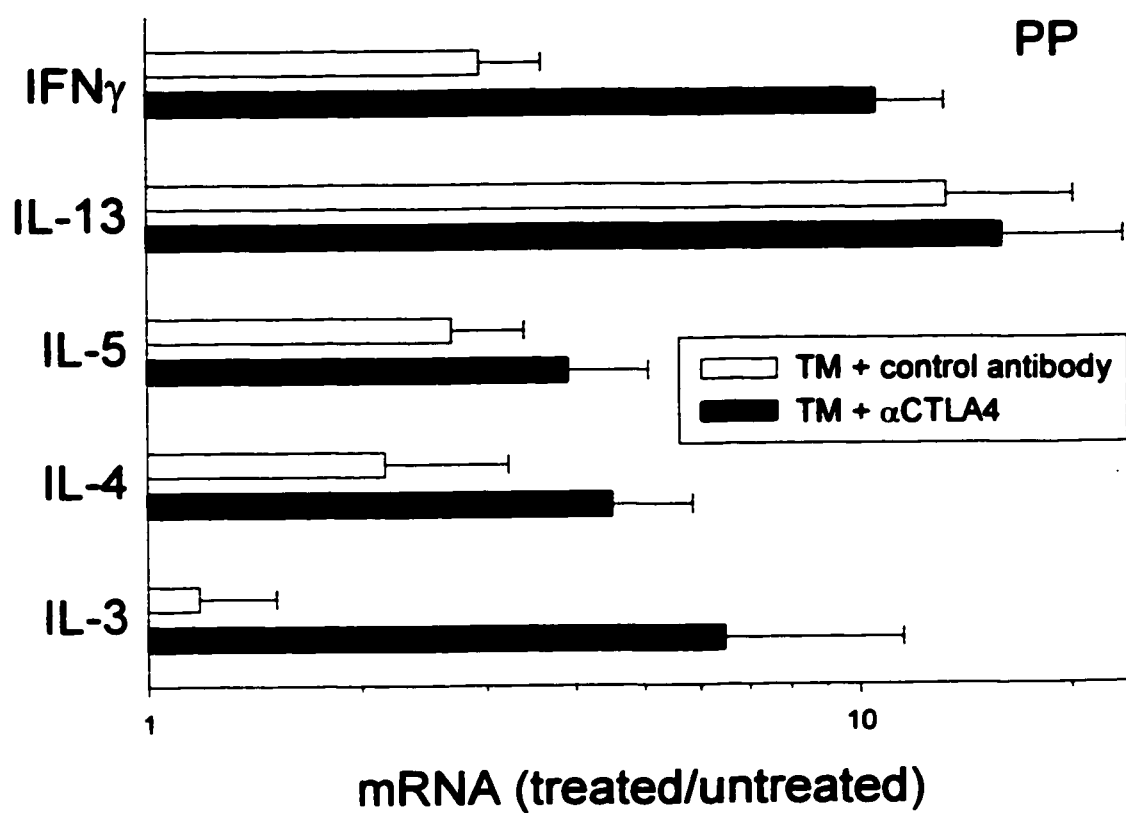


Figure 25. Blocking CTLA4 interactions enhances IFN γ gene expression in mesenteric lymph node and Peyer's patch tissues on day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 600 μ g anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mesenteric lymph node and Peyer's patch tissues were collected on day 21 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and standard error are derived from RNA preparations from the mesenteric lymph node and Peyer's patch tissues of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to three-fold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1.



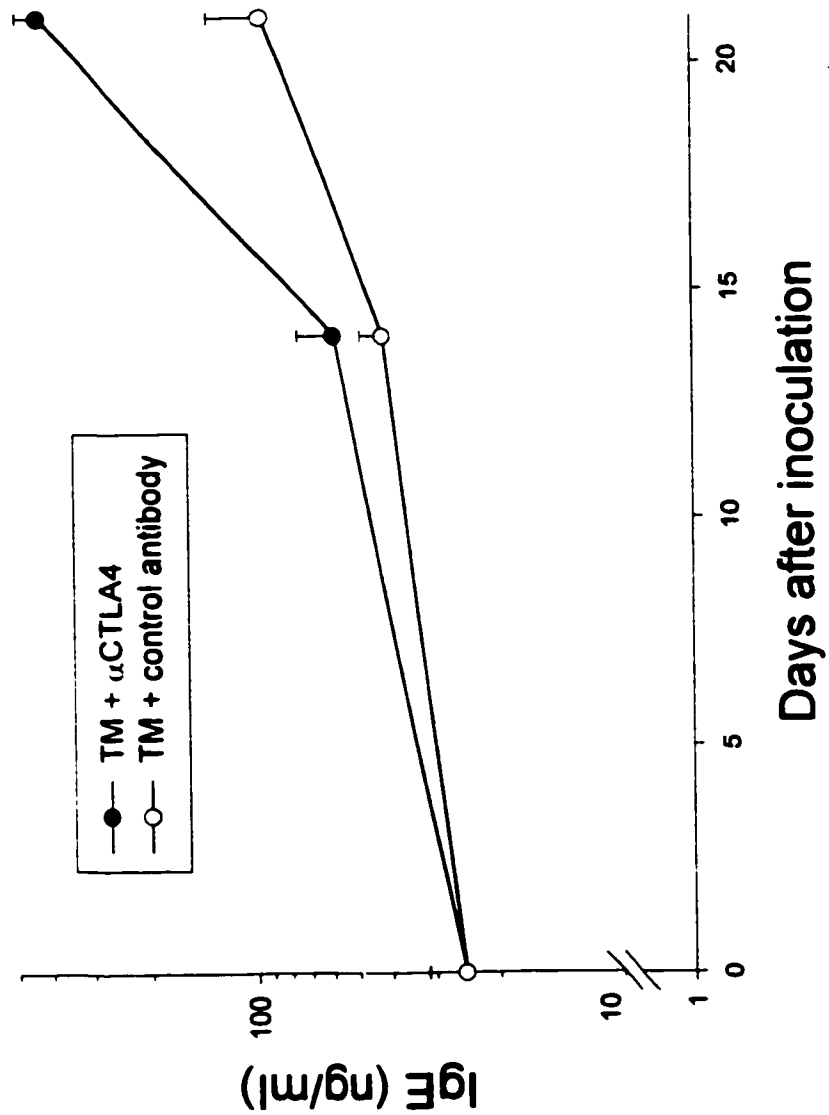
difference was not statistically significant. No statistically significant changes were seen in IL-3, IL-4, IL-5 and IL-13 mRNA expression in either Peyer's patch or mesenteric lymph node tissues. However, two other trends were noted. IL-3 message was increased in both Peyer's patch and mesenteric lymph node tissues (by five- and three-fold, respectively), and IL-4 message was increased in Peyer's patch tissues (two-fold over control antibody treated mice). Perhaps the increase in gene expression of IFN γ would be reflected in an increase in secretion at a later time point. Additional experiments would be required to determine whether anti-CTLA4 antibody treatment of *T. muris*-infected AKR mice results in an initial decline and then a later rise in the secretion of IFN γ .

2. Immunoglobulin isotypes associated with both Th1 and Th2 cytokine responses are increased by anti-CTLA4 antibody administration during the immune response to *T. muris* in AKR mice.

Serum IgE concentrations were quantitated at days 14 and 21 (Figure 26). *T. muris*-inoculated mice exhibited an increase in serum IgE concentration by day 14. At this timepoint there was no difference between anti-CTLA4 antibody and control antibody treated groups. At day 21, serum IgE concentration rose further, with anti-CTLA4 antibody treated animals having significantly greater concentrations than control antibody treated mice. This experiment was conducted two times with similar results.

Serum IgG1 and IgG2a concentrations were also quantitated at day 21. Serum IgG1 concentrations were higher in *T. muris*-inoculated mice treated with control antibody when compared with uninfected controls, though the difference was not statistically significant. Anti-CTLA4 antibody treatment resulted in significantly

Figure 26. Blocking CTLA4 interactions enhances *T. muris*-induced elevations in serum IgE on day 21 after inoculation. AKR mice were injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on day 21 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.



increased serum concentration of IgG1, when compared with inoculated mice treated with control antibody (Figure 27). The concentration of IgG2a, which was not elevated in *T. muris*-inoculated mice treated with control antibody, was significantly increased by treatment with anti-CTLA4 antibody (Figure 27). This experiment was conducted two times with similar results. Antigen-specific IgG1 and IgG2a concentrations were also quantitated. *T. muris* inoculation resulted in increases in both antigen-specific IgG1 and IgG2a titers, in both anti-CTLA4 antibody and control antibody treated groups (Figure 28). Surprisingly, the difference between anti-CTLA4 antibody and control antibody treated groups was not as great as for polyclonal IgG1 and IgG2a, and was below the level of statistical significance. In fact, in a subsequent experiment anti-CTLA4 antibody treated groups had reduced antigen-specific IgG1 and IgG2a when compared with control antibody treated groups.

3. Anti-CTLA4 antibody treatment during *T. muris* infection of AKR mice enhances expression of IL-2R on CD4⁺ T cells.

The effect of anti-CTLA4 antibody treatment of *T. muris*-infected AKR mice on T and B cell activation was assessed by FACS analysis at day 21. *T. muris* inoculation of control antibody treated mice decreased the proportion of CD4⁺ cells that were also IL-2R⁺, when compared with untreated control mice (Figure 29). Anti-CTLA4 antibody treatment increased the proportion of CD4⁺ T cells that were also IL-2R⁺, when compared with control antibody treated animals. The expression of IL-2R on CD4⁺ T cells was increased by anti-CTLA4 antibody treatment (Figure 30). Forward scatter of CD4⁺ T cells was unchanged by anti-CTLA4 antibody treatment (data not shown). *T.*

Figure 27. Blocking CTLA4 interactions enhances polyclonal serum IgG1 and IgG2a at day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on day 21 after inoculation, and serum IgG1 and IgG2a levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

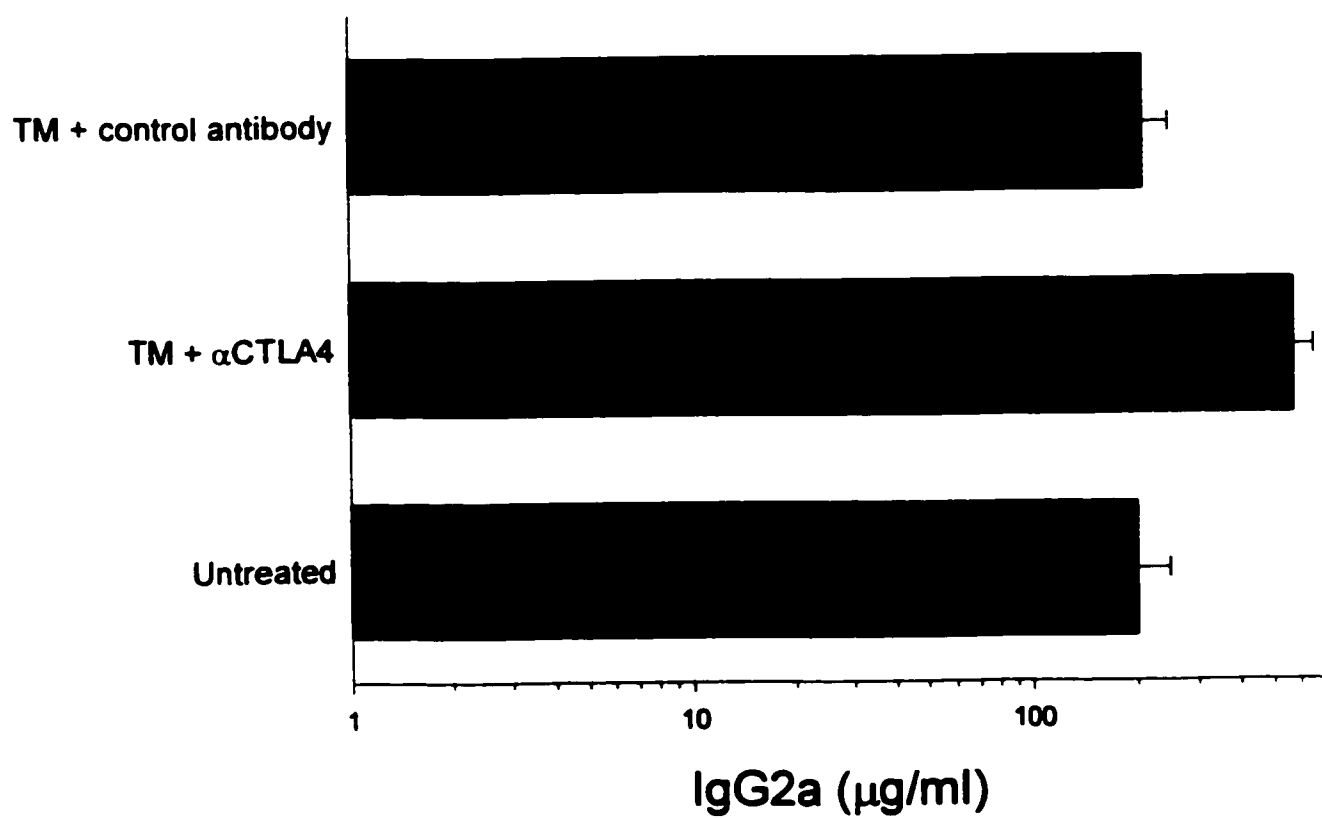
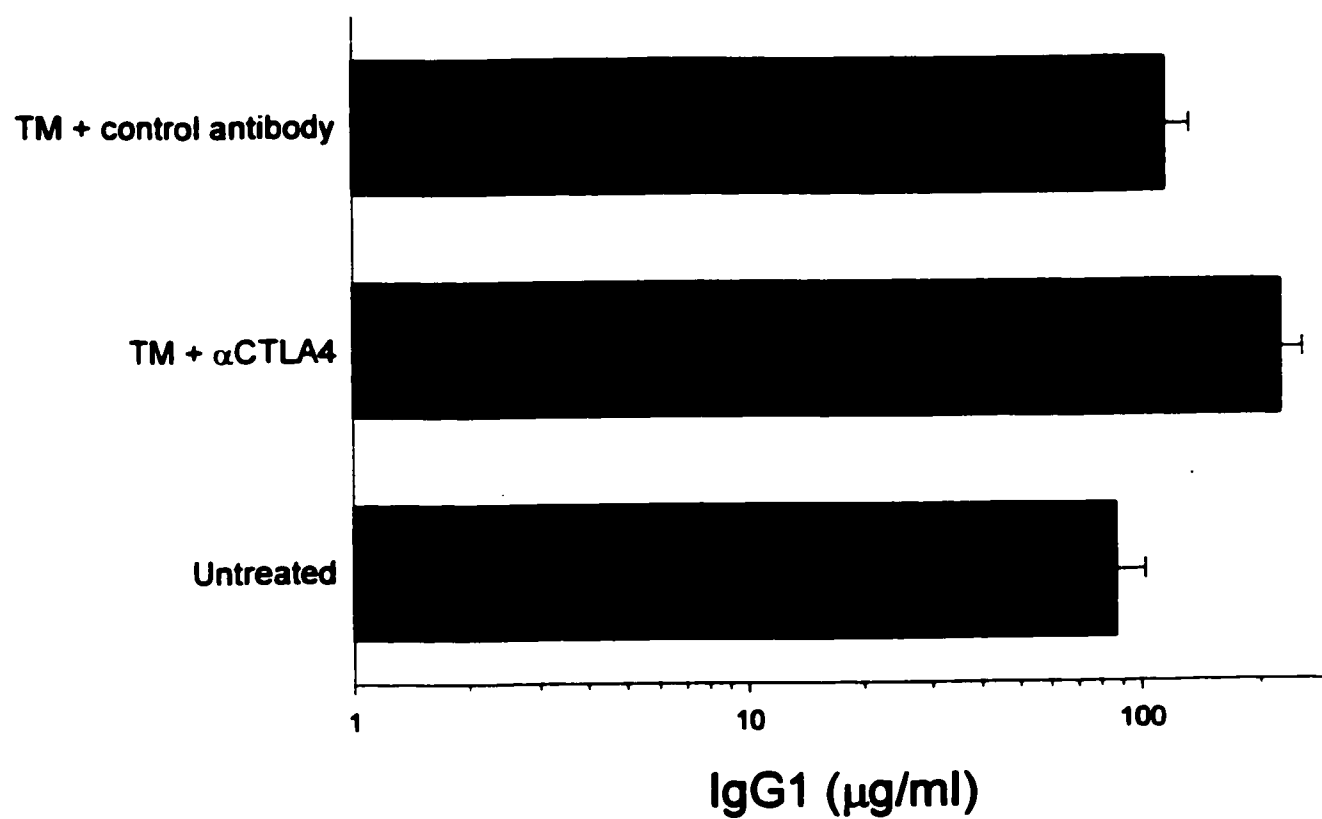


Figure 28. Blocking CTLA4 interactions does not enhance antigen-specific serum IgG1 and IgG2a at day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on day 21 after inoculation, and serum IgG1 and IgG2a levels were determined by antigen-specific ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

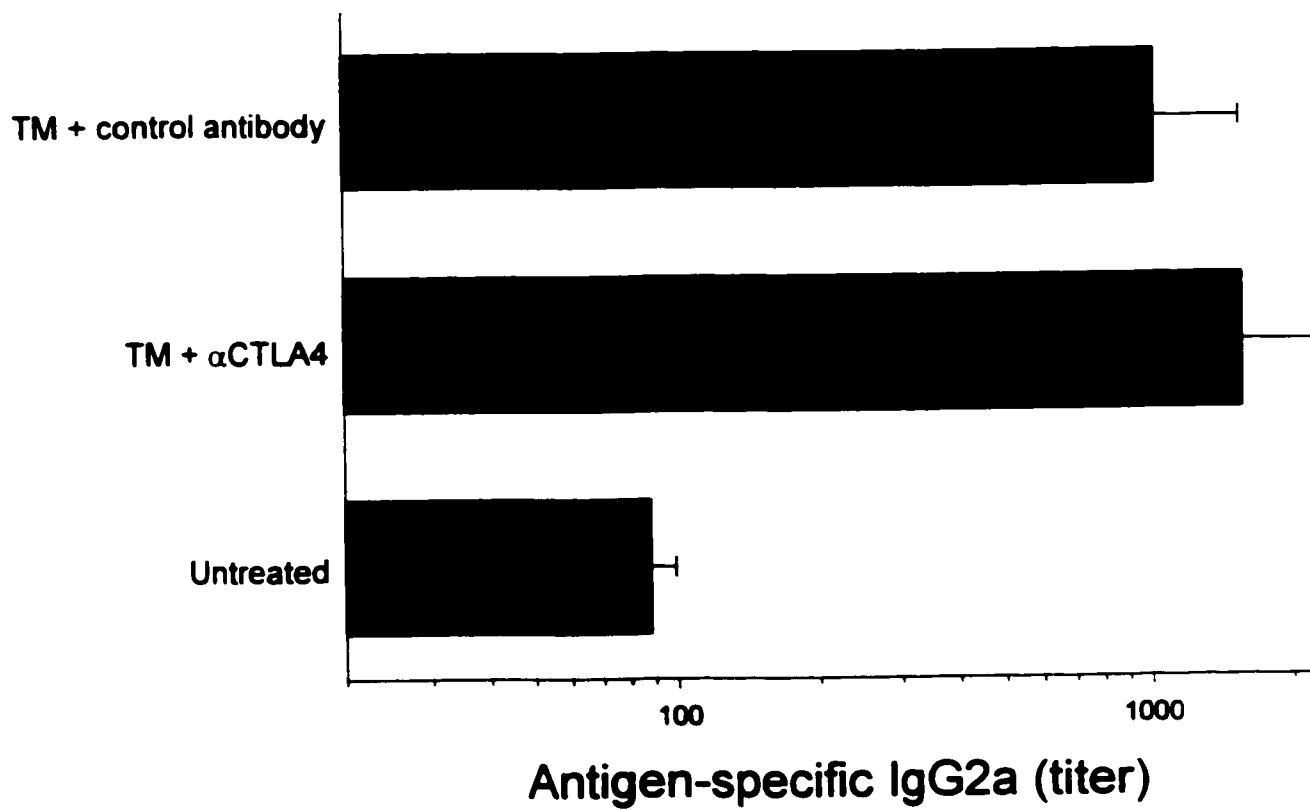
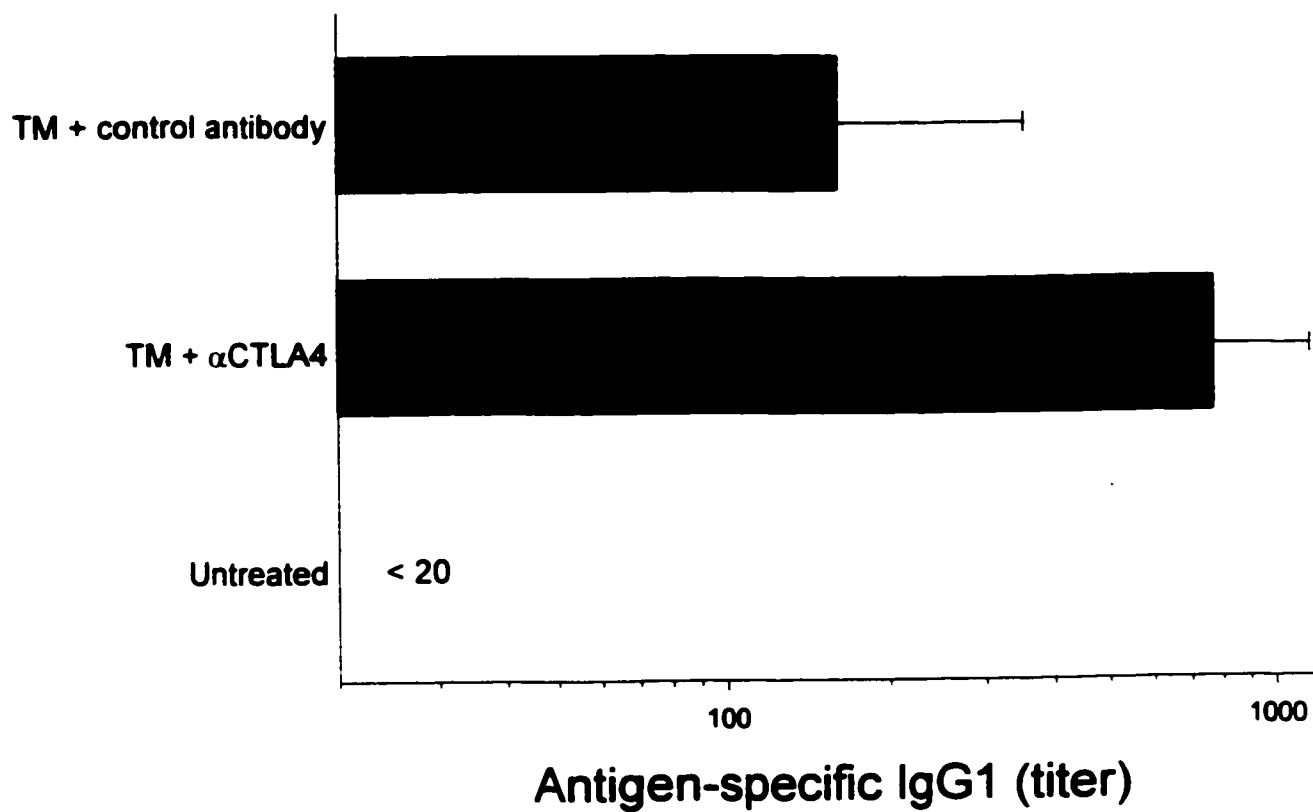


Figure 29. Blocking CTLA4 interactions increases the percentage of CD4⁺ T cells expressing IL-2R on day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with FITC-anti-CD4 and biotin-anti-IL-2R followed by streptavidin-PE. Cells were gated on CD4 expression, and IL-2R expression was quantitated. PE-IL-2R versus FITC-CD4 fluorescence scatter plots are displayed on a log scale. The percentage of CD4⁺ T cells expressing IL-2R is shown.

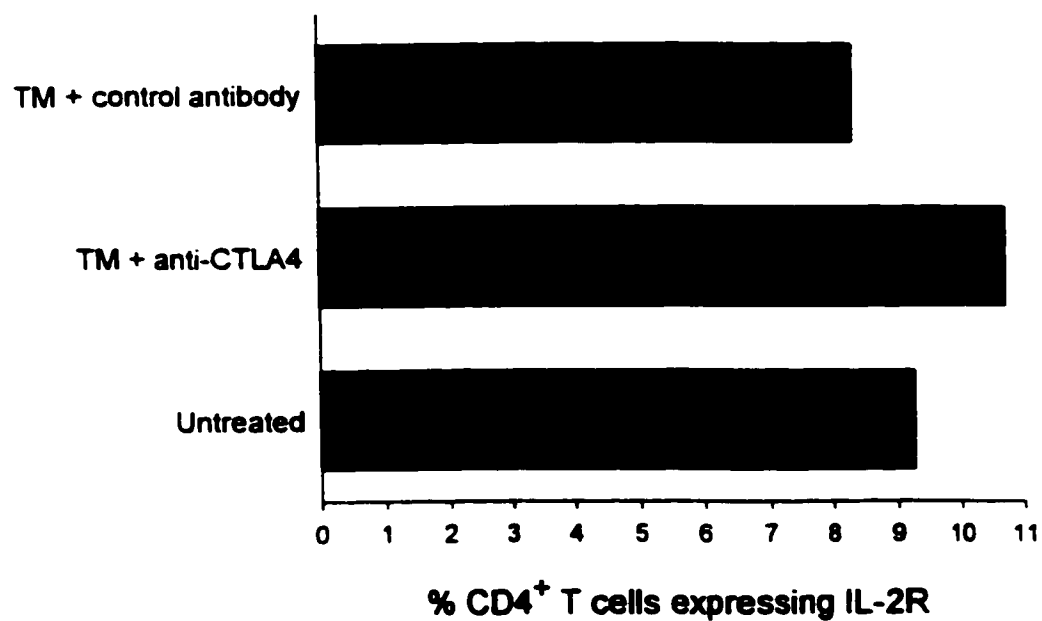
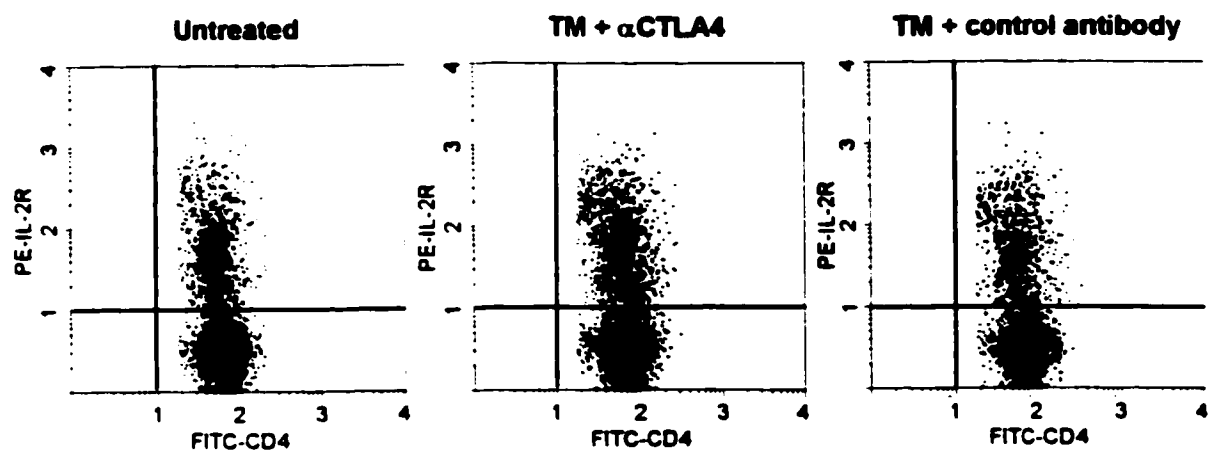
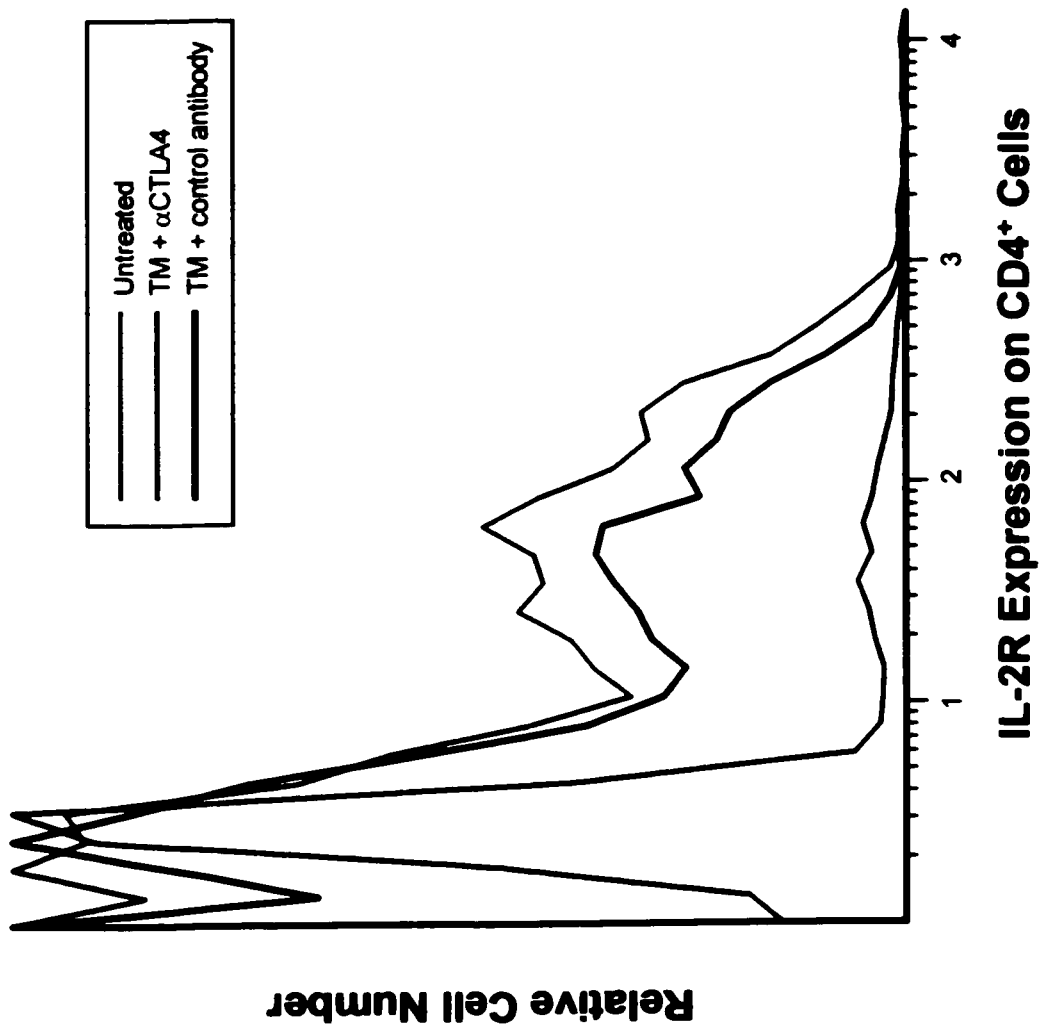


Figure 30. Blocking CTLA4 interactions increases the intensity of expression of IL-2R on CD4⁺ T cells on day 21 after *T. muris* inoculation. AKR mice were inoculated with *T. muris* and treated with anti-CTLA4 antibody or control antibody, and mesenteric lymph node cells were pooled and stained as described in the legend of Figure 29 (five mice per treatment group). Single histogram analysis of IL-2R expression on CD4⁺ T cells is shown. The IL-2R expression scale is logarithmic.



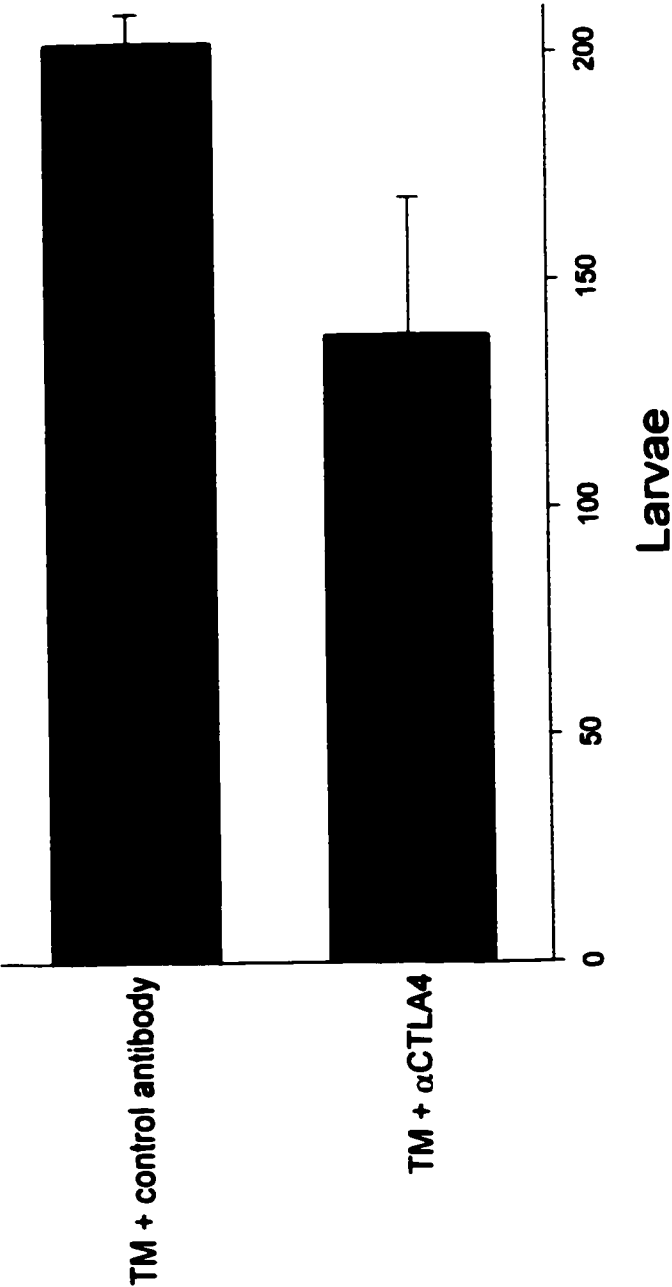
muris inoculation resulted in an increase in the percentage of B220⁺ B cells, but there was no difference between anti-CTLA4 and control antibody treated groups. There also was no change in forward scatter of B220⁺ B cells (data not shown). These data indicate that anti-CTLA4 antibody treatment may result in increased activation of T cells; however, the FACS data do not support increased B cell activation by anti-CTLA4 antibody treatment.

4. Protection against *T. muris* is not increased by anti-CTLA4 antibody treatment.

The numbers of *T. muris* larvae were quantitated at day 21, as a means to ascertain whether anti-CTLA4 antibody treatment increased protection against the pathogen. There was no statistical difference between anti-CTLA4 antibody and control antibody treated groups (Figure 31). This experiment was conducted two times with similar results.

The shift from Th1 to Th2 cytokine secretion and increases in serum immunoglobulin concentration and T cell activation marker expression suggest that anti-CTLA4 antibody had an effect on the immune system that may facilitate protection against the pathogen. However, protection was not increased. Higher doses of anti-CTLA4 antibody were used, to see if there was a threshold that had not yet been crossed in the previous experiments. AKR mice were inoculated with *T. muris*, and injected i.v. with anti-CTLA4 antibody or control antibody at doses of either 600 µg or 1.2 mg at days 8, 12 and 16. At these higher doses, anti-CTLA4 antibody treatment still did not reduce the number of larvae (data not shown).

Figure 31. Blocking CTLA4 interactions does not reduce the number of *T. muris* larvae. AKR mice were infected with *T. muris* and injected i.v. with 600 µg anti-CTLA4 antibody or control antibody on days 8 and 16. Larval worm burden was quantitated at day 21. The data are expressed as the mean and standard error of five individual mice per treatment group. This experiment was conducted two times with similar results.



C. A comparison of the phosphorylation status in anti-CTLA4 antibody treated mice and CTLA4-deficient mice.

Studies with CTLA4-deficient mice provide dramatic evidence of the negative regulatory role of CTLA4 (Waterhouse et al., 1995; Tivol et al., 1995). These mice display severe uncontrolled lymphoproliferation, organ infiltration by lymphocytes, resulting in early death. T cells from CTLA4-deficient mice were used to suggest a mechanism for CTLA4 (Marengere et al., 1996). CD3 ζ and p52SHC were found to be constitutively hyperphosphorylated in coimmunoprecipitated complexes in T cells from CTLA4-deficient mice but not wild-type mice. Also, a tyrosine phosphorylated motif in the CTLA4 cytoplasmic tail was shown to bind SHP2, and CTLA4-coimmunoprecipitated SHP2 has phosphatase activity toward p52SHC. This suggests that CTLA4 negatively regulates the phosphorylation status of CD3 ζ and p52SHC, perhaps through SHP2 activation. These experiments were designed to determine whether the increased activation of T cells by anti-CTLA4 antibody intervention in an infectious disease model is mediated by the same mechanism as in mice where CTLA4 is absent. If anti-CTLA4 antibody intervention enhances T cell activation by the same mechanism as in CTLA4-deficient mice, then we may observe similar changes in phosphorylation status in anti-CTLA4 antibody treated but not control antibody treated mice.

1. CD3 ζ is phosphorylated in CTLA4-deficient mice, in *H. polygyrus*-inoculated mice treated with anti-CTLA4 antibody, and in *H. polygyrus*-inoculated mice treated with control antibody.

BALB/c mice (3 to 5 per group) were injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days 8 and 10 after *H. polygyrus* inoculation. In addition to untreated controls, two additional groups were included, which had been treated with anti-CTLA4 antibody or control antibody but not infected with *H. polygyrus*.

At day 13, serum was collected and assayed for IgE and IgG1 concentrations. As expected, uninfected mice had very little IgE or IgG1 (Figure 32). There was no difference between uninfected mice treated with anti-CTLA4 antibody and control antibody. *H. polygyrus* infection in mice treated with control antibody induced elevated IgE and IgG1. Serum IgE concentration was further elevated in infected mice treated with anti-CTLA4 antibody. Serum IgG1 was not yet enhanced by anti-CTLA4 antibody treatment at this early timepoint. The increase in IgE by anti-CTLA4 antibody treatment at day 13 indicates that by this early timepoint blockade of CTLA4 has had an effect in this model. Changes in signal transduction events induced by CTLA4 blockade may be observed at this timepoint.

Mesenteric lymph nodes were collected at day 13. Mesenteric lymph nodes were also collected from one CTLA4-deficient mouse (CTLA4^{-/-}). The lymph node tissues were homogenized in lysis buffer, and the protein concentrations of clarified supernatants were normalized. CD3 ζ was immunoprecipitated, separated by SDS-PAGE, transferred and immunoblotted with anti-phosphotyrosine antibody (Figure 33). A band of approximately 19 kDa is present in the CTLA4^{-/-} lysate, but not in the lysate from

Figure 32. Anti-CTLA4 antibody treatment enhances elevation in serum IgE concentration by day 13 after *H. polygyrus* inoculation. BALB/c mice (3 – 5 per group) were inoculated with 200 third-stage *H. polygyrus* larvae and injected i.v. with 1 mg anti-CTLA4 antibody or control antibody on days eight and ten. In addition to untreated controls, uninfected anti-CTLA4 antibody and control antibody treated groups were included. Mice were bled on day 13 after inoculation, and serum IgE and IgG1 concentrations were determined by ELISA. The mean and standard error derived from serum samples of three to five individual mice are shown for each treatment group.

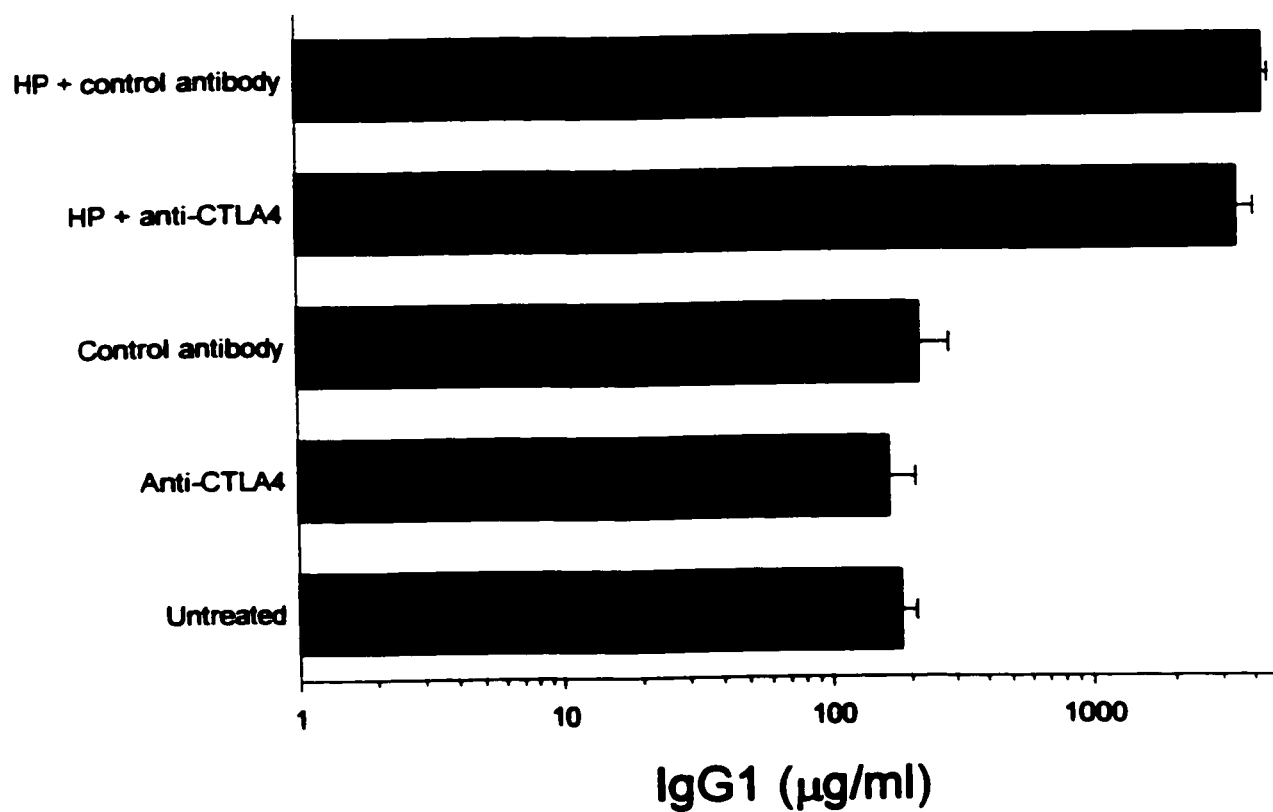
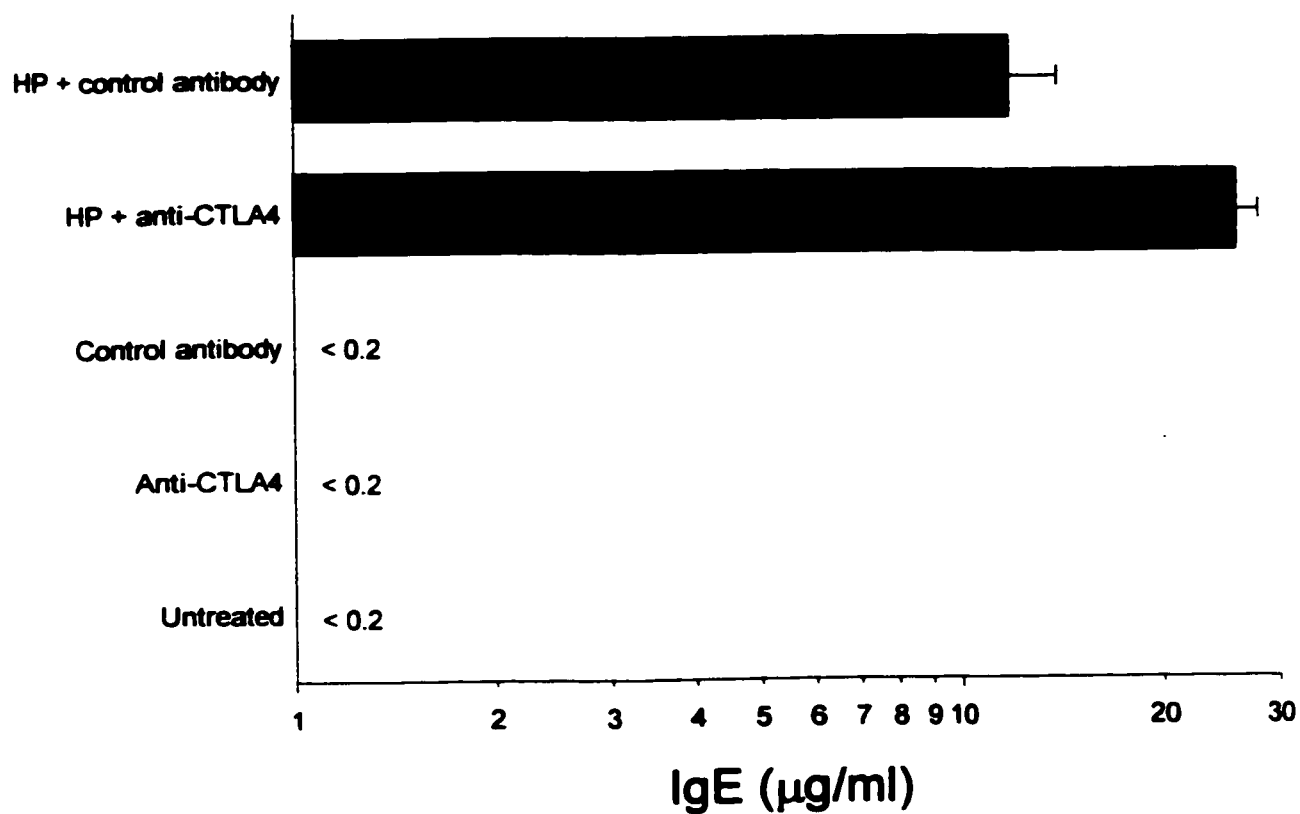
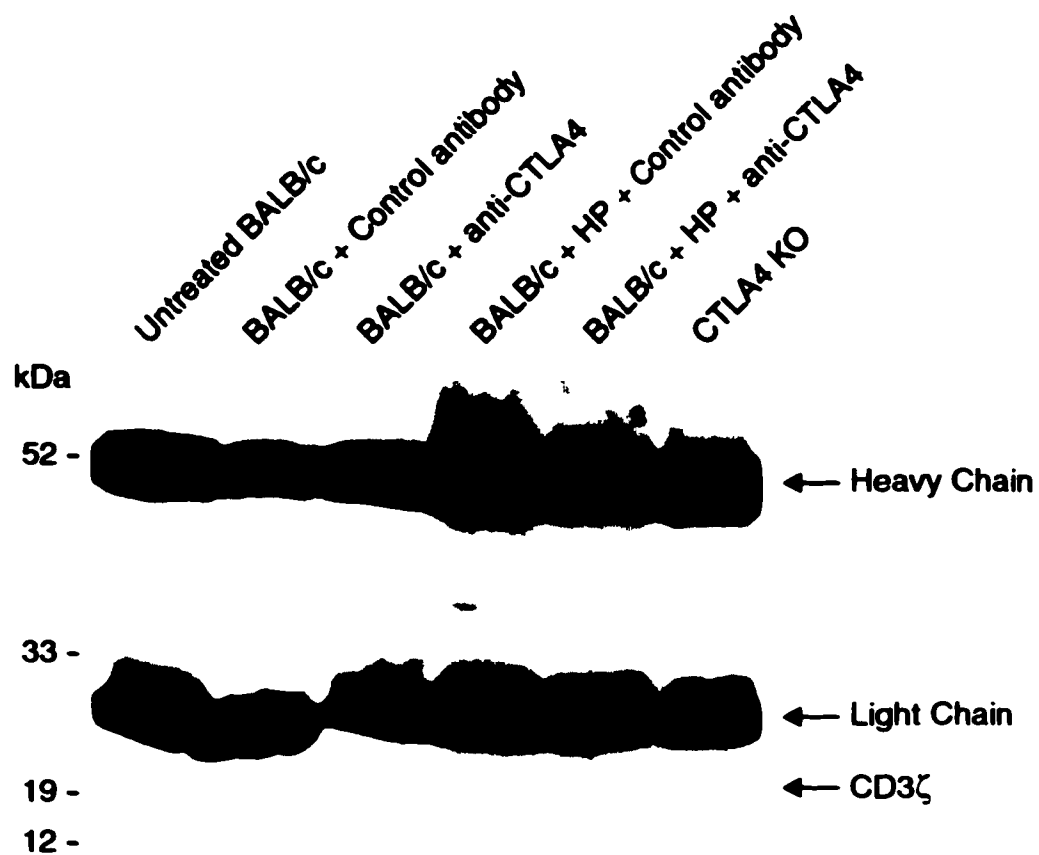
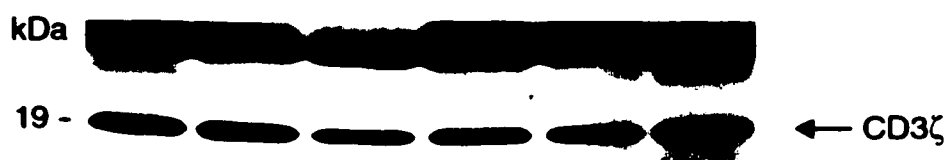


Figure 33. *H. polygyrus* inoculation induces increased CD3 ζ phosphorylation in both anti-CTLA4 antibody- and control antibody-inoculated mice, similar to the increased phosphorylation in CTLA4-deficient mice. BALB/c mice were treated with or without *H. polygyrus* inoculation, anti-CTLA4 antibody or control antibody, as described in Figure 32 (3 – 5 per group). Mesenteric lymph nodes were collected from these mice and pooled. Mesenteric lymph nodes were also collected from one CTLA4-deficient mouse. Mesenteric lymph nodes were homogenized in lysis buffer, and immunoprecipitated with anti-CD3 ζ antibody. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (top blot) and anti-CD3 ζ antibody (bottom blot). The molecular weight markers are indicated on the left side. CD3 ζ is marked by arrows on the right side. The endogenous immunoglobulin, which bound the second step HRP-conjugated anti-mouse IgG antibody, is also indicated on the right side.



Anti-CD3 ζ IP, anti-phosphotyrosine blot



Anti-CD3 ζ IP, anti-CD3 ζ blot

untreated BALB/c mice. This band is also not present in uninfected mice treated with either anti-CTLA4 antibody or control antibody. This indicates that CD3 ζ is phosphorylated in CTLA4-deficient mice, but not in uninfected BALB/c mice. Both lanes from *H. polygyrus*-infected groups treated with anti-CTLA4 antibody and with control antibody contained the 19 kDa band. There was no difference in phosphorylation of CD3 ζ between anti-CTLA4 antibody and control antibody treated infected groups. The anti-CD3 ζ immunoprecipitate was also blotted with anti-CD3 ζ , which confirmed that equal amounts of CD3 ζ were present in all lanes (Figure 33). In the anti-CD3 ζ IP anti-phosphotyrosine blot there appears to be a stronger band at 52 kDa in the *H. polygyrus*-infected and CTLA4^{-/-} lanes than in the uninfected lanes. Total protein samples blotted only with anti-mouse IgG showed the same pattern, so the 52 kDa bands probably represent increased heavy chain (endogenous immunoglobulin) and not p52SHC in these lanes. If p52SHC has been coimmunoprecipitated with CD3 ζ , this band has been obscured by the endogenous immunoglobulin.

While anti-CTLA4 antibody treated infected mice had similar phosphorylation of CD3 ζ compared with CTLA4-deficient mice, this does not explain the increased T cell activation in anti-CTLA4 antibody treated mice compared with control antibody treated mice, as control antibody treated infected mice also had a similar level of phosphorylation of CD3 ζ .

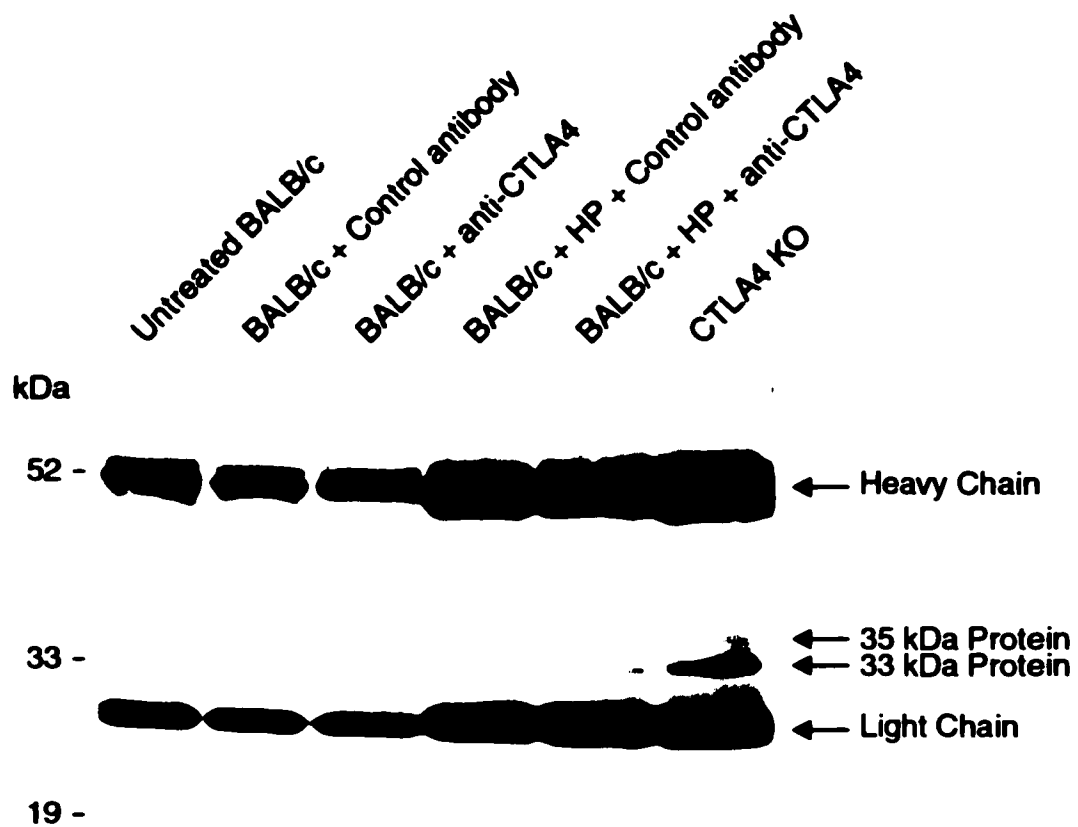
2. CTLA4-deficient mice have increased phosphorylation of 33 kDa and 35 kDa unknown proteins, and may have decreased electrophoretic mobility of phosphorylated CD3 ζ , when compared with *H. polygyrus*-infected BALB/c mice.

The lymph node lysates were also immunoprecipitated with anti-phosphotyrosine antibody, separated by SDS-PAGE, transferred and blotted with anti-phosphotyrosine antibody (Figure 34). A 33 kDa band is present in all lanes but much stronger in CTLA4^{-/-}. The band is of approximately the same intensity in all BALB/c lysates. There is also another band, of approximately 35 kDa, present in CTLA4^{-/-} lysates but not in BALB/c lysates. Neither of these bands are likely to represent dimers of CD3 ζ , as these bands were not present in the anti-CD3 ζ immunoprecipitate.

Total protein lysates were blotted with anti-phosphotyrosine antibody (Figure 34). There is a doublet visible in both *H. polygyrus*-infected lanes (anti-CTLA4 antibody and control antibody treated) that is approximately the same size as CD3 ζ . This doublet has increased separation in the CTLA4^{-/-} lane. The upper band of the doublet may be shifted to a higher apparent molecular weight due to more complete phosphorylation of CD3 ζ in CTLA4-deficient mice. There was no difference between anti-CTLA4 antibody and control antibody treated infected mice. These results do not support a similar mechanism for increased T cell activation in anti-CTLA4 antibody treated mice and mice lacking CTLA4.

Figure 34. CTLA4-deficient mice have increased phosphorylation of 33 and 35 kDa unknown proteins, and decreased electrophoretic mobility of a 19 kDa protein.

Mesenteric lymph nodes were collected, pooled and homogenized as described in Figure 33 (3 – 5 BALB/c mice per group, 1 CTLA4-deficient mouse). Anti-phosphotyrosine immunoprecipitated (top blot) and total protein lysates (bottom blot) were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. The molecular weight markers are indicated on the left side. Proteins of apparent molecular weights of 19, 33, and 35 kDa and marked on the right side with arrows. The endogenous immunoglobulin, which bound the second step HRP-conjugated anti-mouse IgG antibody, is also indicated on the right side.



Anti-phosphotyrosine IP, anti-phosphotyrosine blot



Total protein lysate, anti-phosphotyrosine blot

D. Identification of intracellular binding partners of costimulatory receptors

CTLA4 and CD28.

The yeast two hybrid system, originally developed by Fields and Song in 1989, provides a powerful method to identify molecular interactions (Fields and Song, 1989). It is an important improvement over classical biochemical techniques because the interaction being tested occurs within a living yeast cell, which more closely approximates the natural *in vivo* environment of the components being tested. This technique was employed to screen a library for proteins which interact with the cytoplasmic domains of CTLA4 and CD28. Identifying the intracellular binding partners of costimulatory receptors would aid in understanding the signal transduction events that occur upon costimulation.

For use in the yeast two hybrid screen, a novel cDNA library was constructed. It was important to use a library whose components were representative of those present in an effector T cell, because CTLA4 is maximally expressed in effector T cells. Mesenteric lymph nodes were harvested from mice eight days after infection with *H. polygyrus*. It has been shown that mice infected with *H. polygyrus* display:

1. increased IL-4, IL-5 and IL-13 secretion by CD4⁺ T cells.
2. increased IL-4 gene expression.
3. increased T cell size.
4. increased IgE and IgG1 serum concentration.
5. increased germinal center formation.

These features are consistent with a strong T cell-dependent immune response characterized by elevations in Th2 cells (Svetic et al., 1993; Lu et al., 1994; Greenwald et al., 1997). RNA was purified from the mesenteric lymph nodes, reverse transcribed, and ligated into the yeast two hybrid library expression vector. This library contains 1.2×10^8 primary clones with an average insert size of 1.7kb.

1. Two novel truncated forms of AP50 and phospholipase C-alpha are identified as binding the intracellular portion of CTLA4 in a screen of an activated lymphocyte library.

The library was co-transformed into the yeast host along with the expression vector containing the CTLA4 cytoplasmic sequence. The cells were plated on selective media lacking histidine and containing 3AT, to select for HIS3 expression. A total of 7,420,000 co-transformants were screened. Fifty-five candidates were analyzed for reporter gene induction. Of these, the phenotypes of four potential interactors (T1, T2, 5-1, 5-2) were identified as warranting further inspection (Table D).

The vectors encoding the potential interactors were shuttled into *E. coli*, purified, and sequenced. BLAST searches identified two of them (T1 and T26) as novel, truncated forms of AP50. This protein had already been identified as binding to CTLA4 in a yeast two hybrid screen (Shiratori et al., 1997), but there had been no previous report of these truncated forms. Clones T1 and T26 encode the N-terminal 273 and 250 amino acids of AP50, compared with its full-length of 435 amino acids. BLAST searches identified the two remaining potential interactors (5-1 and 5-2) as phospholipase C-alpha, and Sca-2. The Sca-2 sequence was found to be ligated out of frame. All four clones were re-

Table D. Phenotypes of clones identified in yeast two hybrid screen of CTLA4 cytoplasmic region.

Clone	Selective media phenotype					Gene identified	
	10mM 3AT ^a	50mM 3AT ^b	xgal ^c	URA ^d	5FOA ^e	Name	Accession #
T1	+	+	+	+	Insensitive	AP50	U27106
T26	+	+	-	+	Insensitive	AP50	U27106
5-1	+	+	-	-	Insensitive	mus PLC α	M73329
5-2	+	-	-	-	Insensitive	mus Sca2	U04268

Reporter gene induction is indicated by +, absence of reporter gene induction is indicated by -.

Selective media :

- (a) SC-Leu-Trp-His + 10mM 3AT
- (b) SC-Leu-Trp-His + 50mM 3AT
- (c) YPAD + nylon membrane followed by xgal assay
- (d) SC-Leu-Trp-Ura
- (e) SC-Leu-Trp + 0.2% 5FOA

transformed into the yeast host along with the CTLA4 expression vector, and the induction of reporter genes was reassessed. The phenotypes of the two truncated forms of AP50, and of phospholipase C-alpha were confirmed. As expected, the phenotype of the Sca-2 transformant was not reproduced upon re-transformation with the CTLA4 expression vector.

2. N-methyltransferase, homer-3, and TAFII250 transcription factor are identified as binding the intracellular portion of CD28 in a screen of an activated lymphocyte library.

A screen was also performed with the cytoplasmic domain of CD28. A total of 2,998,000 co-transformants were screened. Seventy-eight candidates were analyzed further for reporter gene induction. Based on the phenotypes displayed, 11 were chosen for purification and sequencing (Table E). Of these, four were found to be ligated out of frame or to have an intervening stop codon between the activation domain and cDNA sequences. The seven in-frame potential interactors were identified through BLAST searches as NF-kappaB, ARF3, arginine n-methyltransferase, homer-3, and three copies of TAFII250 transcription factor. In the case of NF-kappaB and ARF3, the phenotypes displayed were extremely weak, no stronger than the clones that were found to be out of frame. The clones encoding arginine n-methyltransferase, homer-3 and TAFII250 were re-transformed into the yeast host with the CD28 expression vector, and reporter gene induction was confirmed.

Table E. Phenotypes of clones identified in yeast two hybrid screen of CD28 cytoplasmic region.

Clone	Selective media phenotype					Gene identified	
	10mM 3AT ^a	25mM 3AT ^b	50mM 3AT ^c	xgal ^d	URA ^e	Name	Accession #
3D1	+	ND	ND	-	ND	gallus px19	U31977
3D2	+	ND	ND	-	ND	mus inv. chain	X07129
3F1	+	ND	ND	-	ND	homo NF-κB	X61498
3F2	+	ND	ND	-	ND	mus ARF3	D87900
3F4	+	ND	ND	-	ND	mus EF-Tu	M22432
3K2	+	ND	ND	+	+	rattus EF-2	X80774
4-1	+	+	-	+	-	rattus meth transf	U60882
4-2	+	+	+	-	-	mus TAFII250	AF022178
4-3	+	+	-	-	+	mus homer3	AF093261
4-4	+	+	+	-	-	mus TAFII250	AF022178
4-5	+	+	-	-	-	mus TAFII250	AF022178

Reporter gene induction is indicated by +, absence of reporter gene induction is indicated by -. ND indicates that reporter gene induction was not tested.

Selective media :

(a) SC-Leu-Trp-His + 10mM 3AT

(b) SC-Leu-Trp-His + 25mM 3AT

(c) SC-Leu-Trp-His + 50mM 3AT

(d) YPAD + nylon membrane followed by xgal assay

(e) SC-Leu-Trp-Ura

3. Two novel truncated forms of AP50 bind the cytoplasmic portion of CTLA4 more strongly than does full-length AP50.

The identification of the truncated forms of AP50 (T1 and T26) prompted the question of whether there was any difference in the strength of binding between full-length and truncated AP50 with CTLA4. The yeast two hybrid system provides a means of estimating strength of binding, by comparison with control strains on different types of selective media. For example, the HIS3 gene can be induced by very weakly interacting partners, but induction of the URA3 gene usually requires a stronger interaction. To address this issue, full length AP50 was cloned from the cDNA library and co-transformed with the CTLA4 expression vector. The reporter gene induction phenotype of this co-transformant was compared with the phenotypes CTLA4 co-transformed with T1 and T26. The truncations were found to interact with CTLA4 more strongly than did full-length AP50. While full-length AP50 showed growth only on plates lacking histidine and including 10 mM 3AT, the truncations showed growth on plates lacking histidine and including 3AT at a concentration up to 50 mM, and on plates lacking uracil. The truncations also were positive in an X-gal assay (Table F).

4. Neither CTLA4 nor CD28 bind Src-family tyrosine kinase Lck, as assessed in the yeast two hybrid system.

The kinases responsible for phosphorylation of CTLA4 and CD28 to initiate signaling upon ligation have not been identified. Lck has been proposed as a candidate for phosphorylating CD28 (King et al., 1997). Likewise, Src family tyrosine kinases (of which Lck is a member) have been reported to phosphorylate CTLA4 in vitro (Miyatake

Table F. Comparison of phenotypes of full-length AP50 and truncations identified in screen of CTLA4 cytoplasmic region.

Clone	Selective media phenotype				
	10mM 3AT ^a	50mM 3AT ^b	xgal ^c	URA ^d	5FOA ^e
AP50	+	-	-	-	Insensitive
T1	+	+	+	+	Insensitive
T26	+	+	+	+	Insensitive

Reporter gene induction is indicated by +, absence of reporter gene induction is indicated by -.

Selective media :
 (a) SC-Leu-Trp-His + 10mM 3AT
 (b) SC-Leu-Trp-His + 50mM 3AT
 (c) YPAD + nylon membrane followed by xgal assay
 (d) SC-Leu-Trp-Ura
 (e) SC-Leu-Trp + 0.2% 5FOA

et al., 1998). Determining whether CTLA4 and CD28 bind overlapping sets of signal transduction components may promote understanding of whether CTLA4 and CD28 have overlapping functions. To address this issue, Lck was tested in the yeast two hybrid system for binding to CTLA4 and CD28.

The sequence encoding Lck was cloned from the cDNA library. A single residue near the 5'-end of Lck was mutated from glycine to alanine to eliminate myristoylation. The Lck construct was co-transformed into the yeast host along with the CTLA4 expression vector. A second co-transformant was made with the Lck construct and the CD28 expression vector. The reporter gene induction phenotypes of both co-transformants were assessed. Neither co-transformant induced the HIS3, URA3, or beta-galactosidase reporter genes (data not shown); from this it can be concluded that Lck interacts with neither CTLA4 nor CD28 in the yeast two hybrid system.

E. Studies on the effects of blockade of both CD28 and CTLA4 during the immune response to infectious pathogens.

1. Both Th2 and Th1 components of the immune response to *T. muris* are reduced by blockade of both CD28 and CTLA4 by CTLA4Ig treatment during the immune response.

T. muris-infected AKR mice mount an immune response with both Th1 and Th2 components that is associated with chronic infection, but can be shifted to a Th2-dominant protective response by depleting IFN γ or administering IL-4 (Else et al., 1994). CTLA4Ig, a fusion protein which binds B7 molecules thereby preventing their binding to both CD28 and CTLA4, has been administered at the onset of infection with *H. polygyrus* in BALB/c mice to block the immune response (Lu et al., 1994). Whereas this demonstrates the requirement for B7-mediated costimulation for naïve T cells, effector T cells may be less sensitive to loss of costimulation. Also, late after initiation of the response, CTLA4/B7 interactions may predominate over CD28/B7 interactions, due to the upregulation of CTLA4 surface expression. If delayed administration of CTLA4Ig favors inhibition of CTLA4/B7 interactions, then the treatment may result in an increase in strength of signal and immune deviation toward a Th2 response. In the experiments described here, *T. muris*-inoculated AKR mice were treated with CTLA4Ig after onset of infection, when T cells have already been activated. These experiments were conducted to determine whether delayed blockade of B7 interactions would inhibit the immune response or promote deviation toward a Th2 response.

T. muris-infected AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Serum immunoglobulin concentrations were quantitated at days 14 and 21. *T. muris* inoculation induced an elevation in serum IgE concentration at day 21 in control antibody treated mice (Figure 35). CTLA4Ig treatment significantly inhibited this elevation, and in fact reduced the serum concentration of IgE to below untreated control levels at both days 14 and 21. Serum IgG1 concentrations were elevated to a lesser degree by *T. muris* inoculation, but significant inhibition by CTLA4Ig treatment was still evident (Figure 36). Serum IgG1 concentrations were reduced to about one third of the concentration seen in uninfected controls. The serum concentration of IgG2a was also reduced by CTLA4Ig treatment of *T. muris*-inoculated mice when compared to control antibody treated mice (Figure 37). These experiments were conducted two times with similar results.

Antigen-specific IgG1 and IgG2a serum titers were quantitated. Antigen-specific IgG1 titers in uninfected controls were below the detection limit of the assay (Figure 38). Mice infected with *T. muris* and treated with control antibody displayed elevated antigen-specific IgG1. CTLA4Ig treatment inhibited this elevation; the concentration in CTLA4Ig treated mice was reduced to below the detection limit of the assay. Due to the large standard error, the difference was not statistically significant, though it was consistent in two independent experiments. Similar results were seen for antigen-specific IgG2a (Figure 38). In uninfected control mice, there was a very low level of antigen-specific IgG2a. *T. muris* inoculation resulted in an elevation in antigen-specific IgG2a.

Figure 35. Blocking B7-ligand interactions with CTLA4Ig reduces serum IgE concentrations at days 14 and 21 after *T. muris* inoculation. AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on days 14 and 21 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

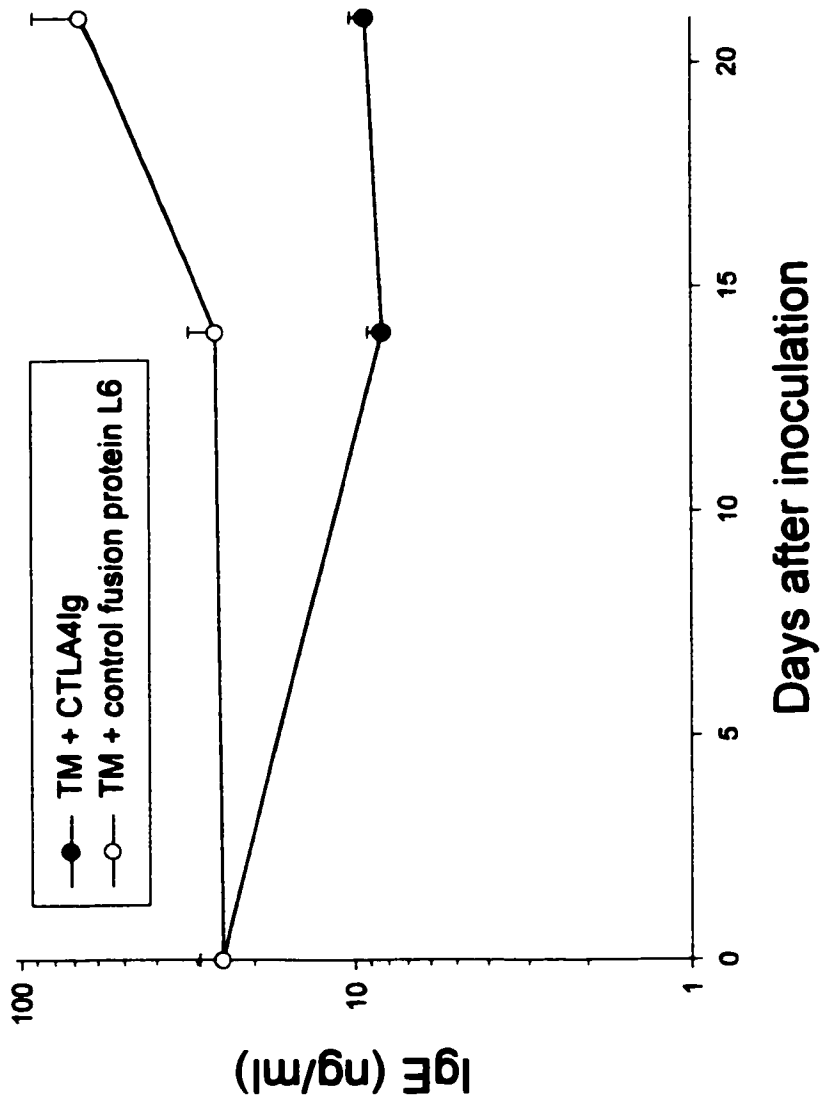


Figure 36. Blocking B7-ligand interactions with CTLA4Ig reduces serum IgG1 concentrations at days 14 and 21 after *T. muris* inoculation. AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on days 14 and 21 after inoculation, and serum IgG1 levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

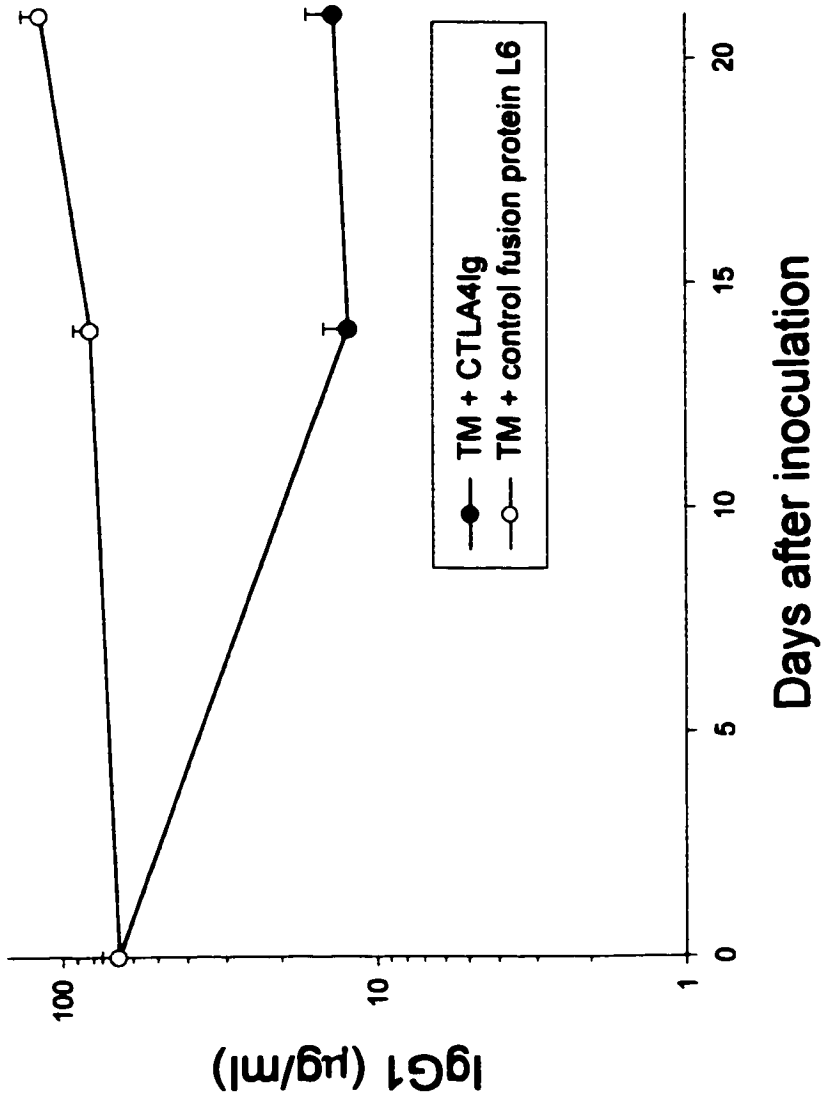


Figure 37. Blocking B7-ligand interactions with CTLA4Ig reduces serum IgG2a concentrations at days 14 and 21 after *T. muris* inoculation. AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on days 14 and 21 after inoculation, and serum IgG2a levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.

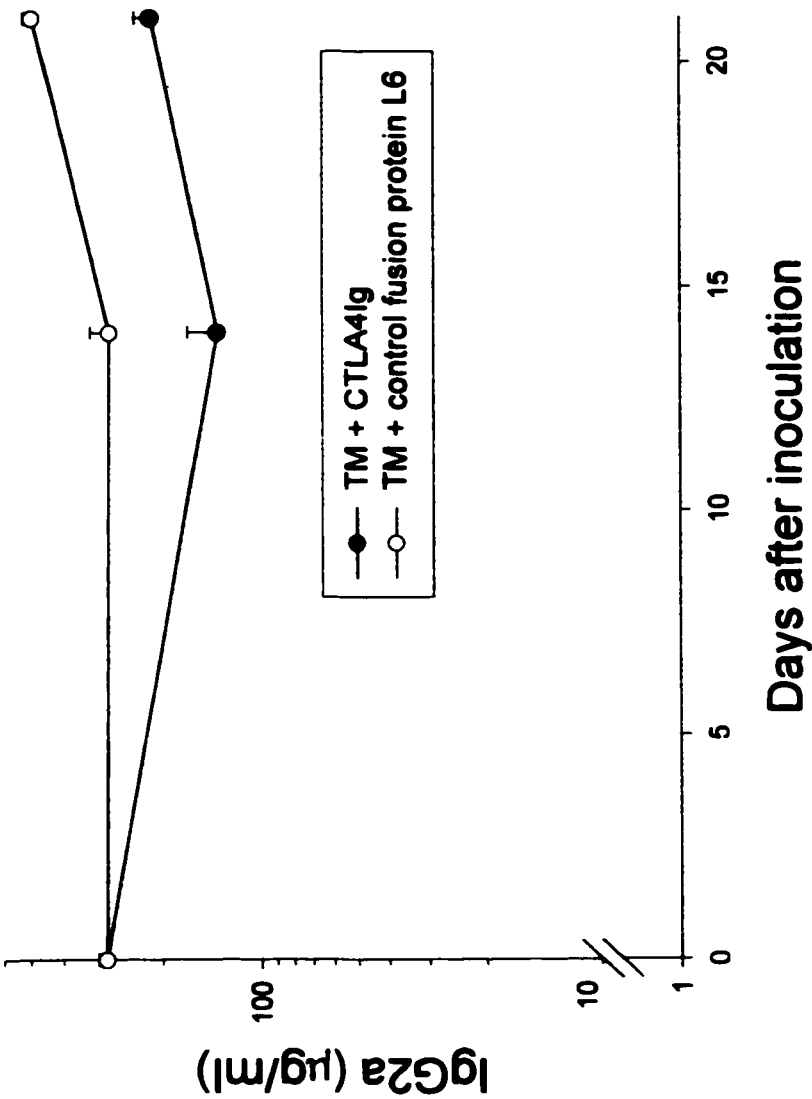
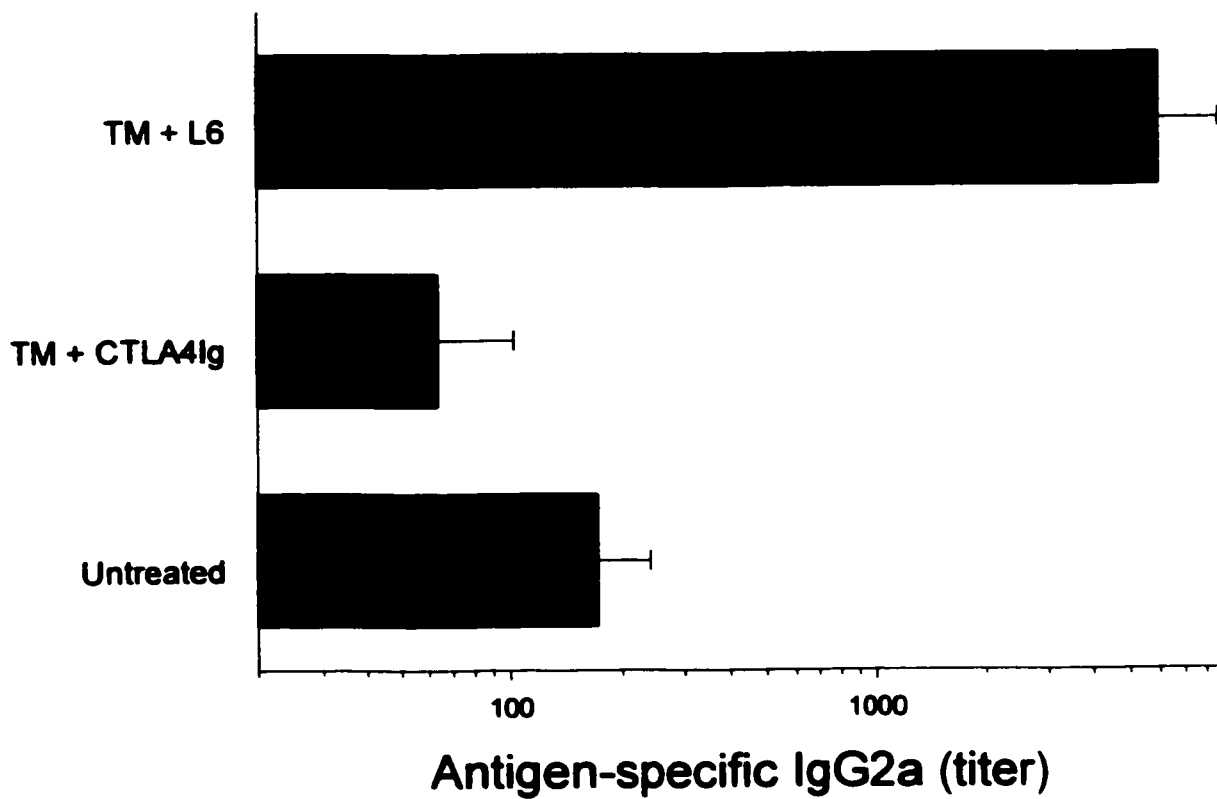
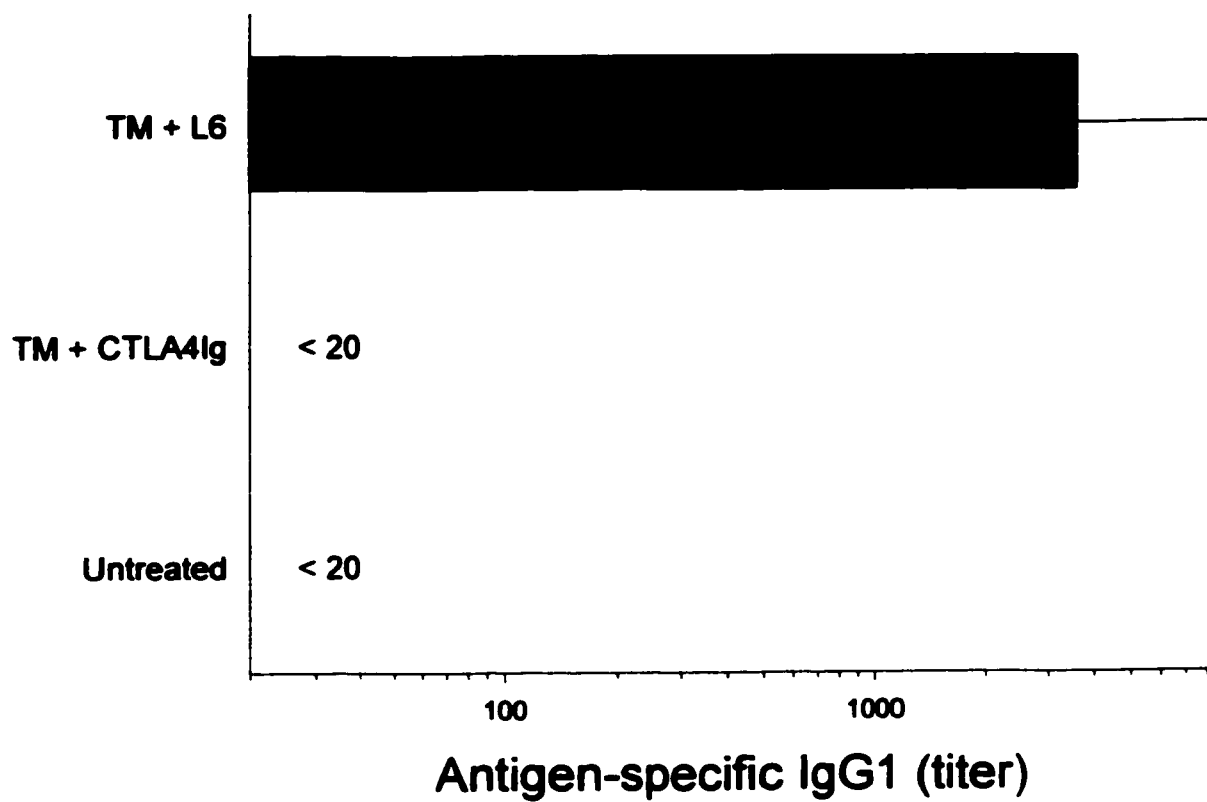


Figure 38. Blocking B7-ligand interactions with CTLA4Ig reduces antigen-specific serum IgG1 and IgG2a at day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mice were bled on day 21 after inoculation, and serum IgG1 and IgG2a levels were determined by antigen-specific ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group. This experiment was conducted two times with similar results.



which was significantly reduced by CTLA4Ig treatment, to untreated control levels.

These experiments were conducted two times with similar results.

In one experiment, T and B cell activation were examined by FACS analysis. CTLA4Ig treatment of *T. muris*-inoculated mice reduced the proportion of CD4⁺ cells that also express IL-2R when compared with control antibody treated mice, indicating a lesser degree of T cell activation in CTLA4Ig treated animals (Figure 39). The intensity of IL-2R expression and forward scatter of CD4⁺ T cells was comparable between the two groups (data not shown). B cells also showed a decrease in activation. There were fewer B220⁺ B cells in CTLA4Ig treated animals when compared with control antibody treated animals, and forward scatter of B220⁺ B cells was reduced by CTLA4Ig treatment (Figure 40).

The number of IFN γ and IL-4 producing mesenteric lymph node cells were quantitated at day 21 in an ELISPOT assay in one experiment. *T. muris* inoculation induced a three-fold increase in the number of IFN γ producing cells in mice treated with control antibody (Figure 41). CTLA4Ig treatment significantly inhibited the *T. muris*-induced increase in the number of IFN γ producing cells, down to untreated control levels. In contrast to IFN γ , *T. muris* inoculation induced a decrease in the number of IL-4 producing mesenteric lymph node cells in control antibody treated mice (Figure 41). The number of IL-4 producing cells was reduced further by CTLA4Ig treatment of *T. muris*-inoculated mice (although the difference between CTLA4Ig and L6 treatment groups was not statistically significant). These results indicate that both Th1 and Th2 parameters of the immune response to *T. muris* may be sensitive to inhibition by CTLA4Ig.

Figure 39. Blocking B7-ligand interactions with CTLA4Ig reduces the percentage of CD4⁺ T cells that express IL-2R in *T. muris*-inoculated mice. AKR mice were injected i.v. with 100 µg CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. At day 21 cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with FITC-anti-CD4 and biotin-anti-IL-2R followed by streptavidin-PE. Cells were gated on CD4 expression, and IL-2R expression was quantitated. PE-IL-2R versus FITC-CD4 fluorescence scatter plots are displayed on a log scale. The percentage of CD4⁺ T cells expressing IL-2R is shown.

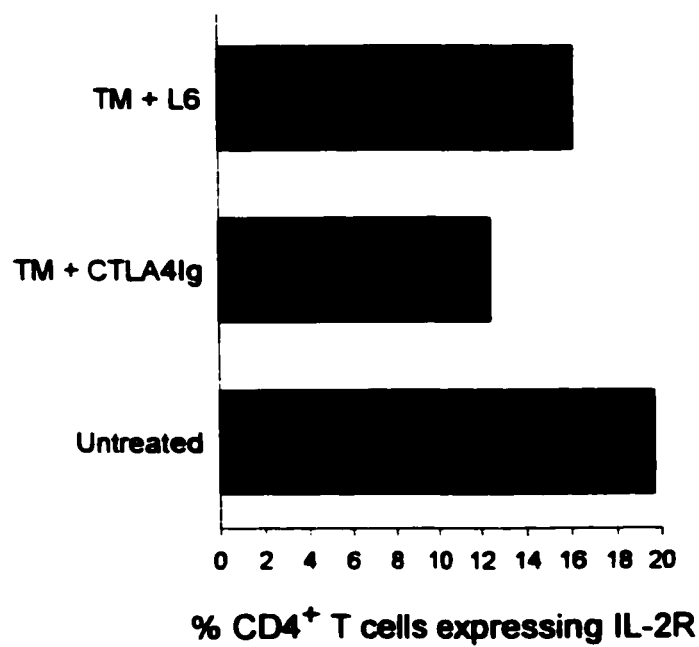
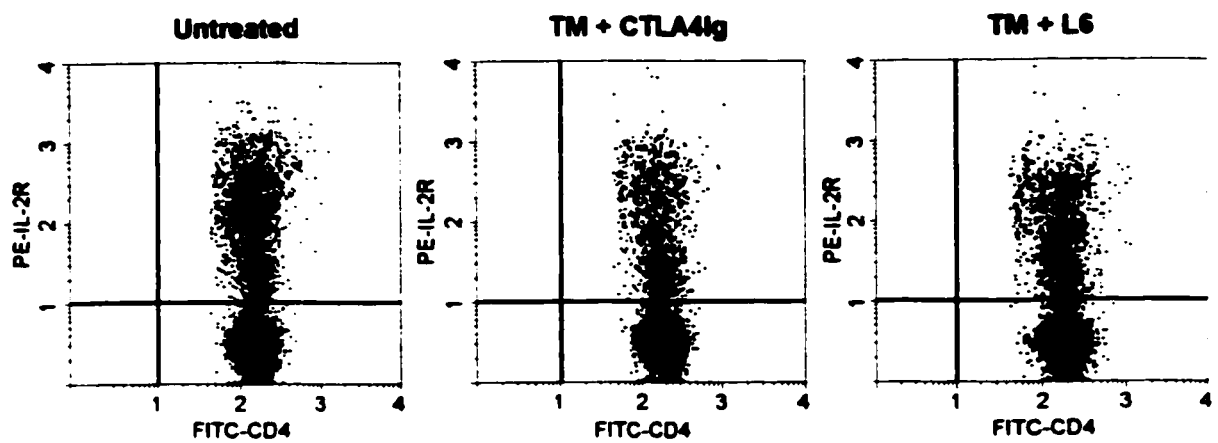


Figure 40. Blocking B7-ligand interactions with CTLA4Ig reduces B220⁺ B cell size and the number of B220⁺ B cells in *T. muris*-inoculated mice. AKR mice were inoculated with *T. muris* and treated with CTLA4Ig or control fusion protein L6 as described in Figure 39. Cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were stained with cychrome-anti-B220. Single histogram analysis of B220⁺ B cell size (forward scatter) and the percent of cells expressing B220⁺ are shown.

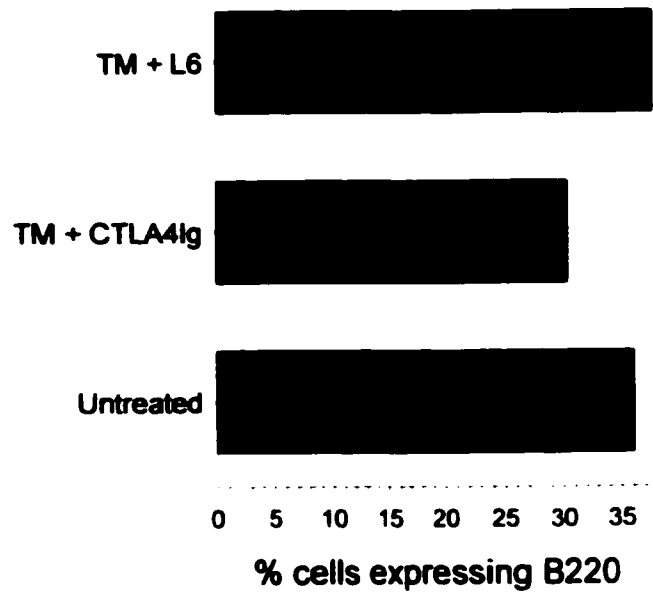
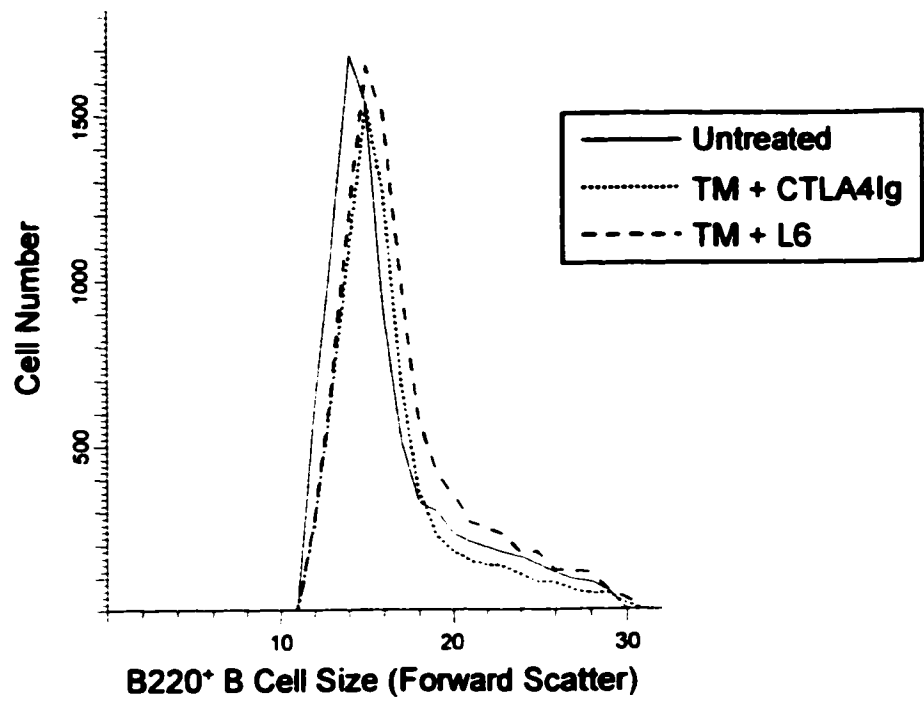
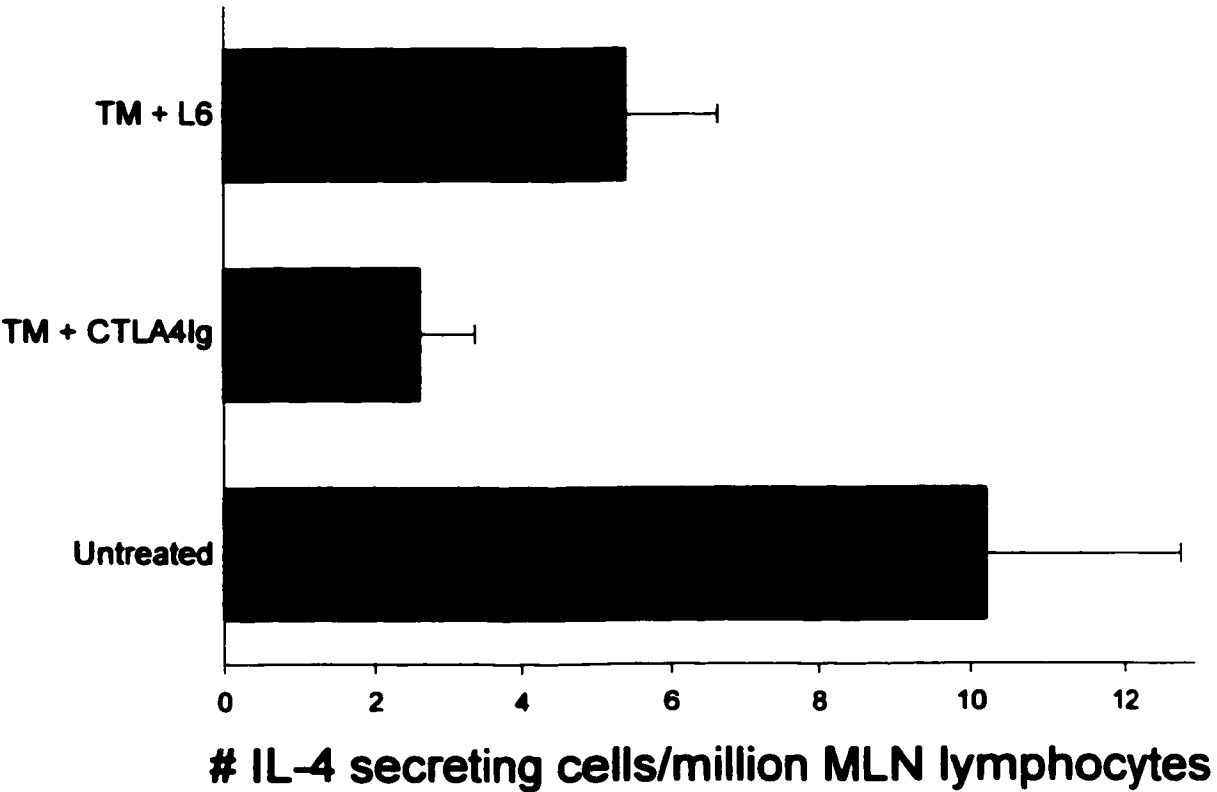
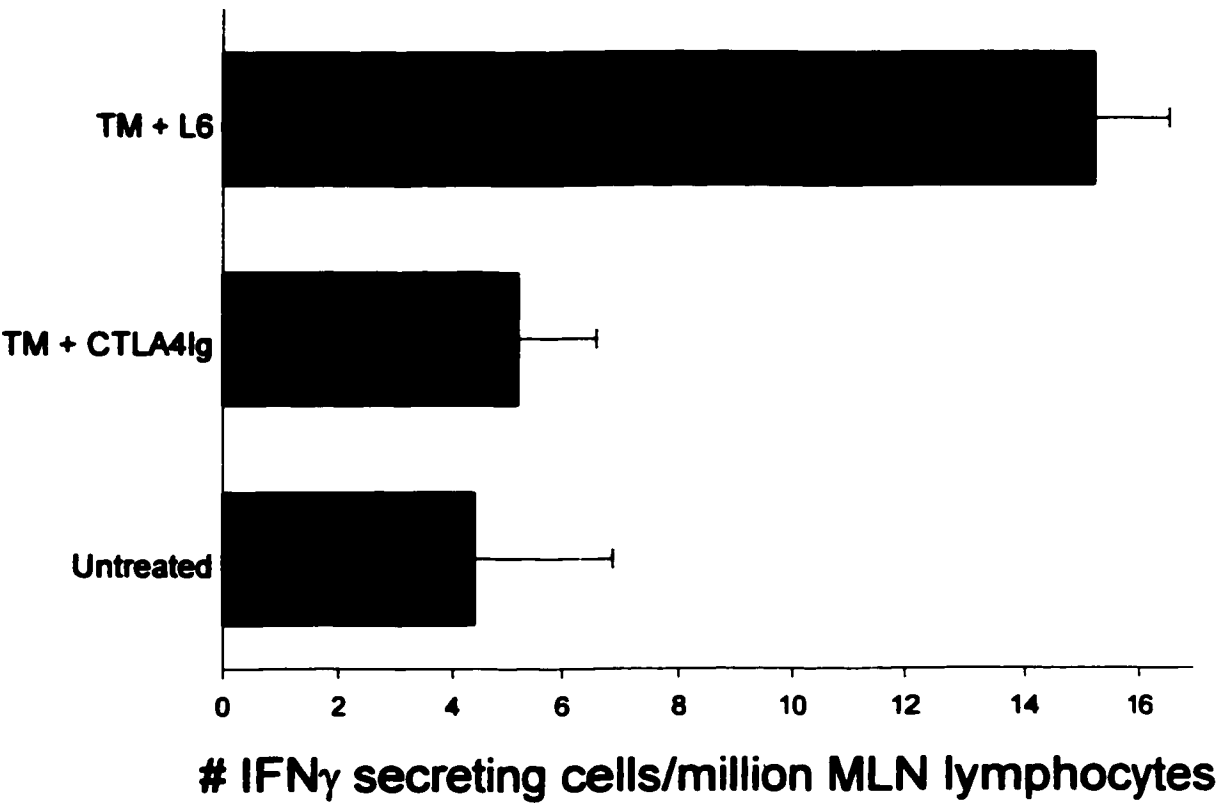


Figure 41. Blocking B7-ligand interactions with CTLA4Ig reduces IFN γ and IL-4 secretion on day 21 after *T. muris* inoculation. AKR mice were injected i.v. with 100 μ g CTLA4Ig or control fusion protein L6 at days 8 and 16 after oral inoculation with 500 *T. muris* eggs. Mesenteric lymph node tissues were collected on day 21 after inoculation, and the number of IFN γ and IL-4 secreting mesenteric lymph node cells were determined in an ELISPOT assay without restimulation. Cell suspensions were assayed from five individual mice per treatment group, and the mean and standard error are shown.



The number of *T. muris* larvae were quantitated, as a means to assess whether CTLA4Ig treatment altered the level of protection against the pathogen. The numbers of larvae were unchanged by CTLA4Ig treatment when compared with L6 control fusion protein treatment (Figure 42). Large numbers of larvae were present in both groups. This experiment was conducted two times with similar results.

2. The polarized Th2 immune response to *H. polygyrus* is partially reduced by blockade of both CD28 and CTLA4 by CTLA4Ig treatment during the immune response.

Blockade of both CD28 and CTLA4 interactions with B7 molecules has been shown to be effective in preventing activation of the immune response to *H. polygyrus* when given concurrently with the onset of infection (Lu et al., 1994). To determine whether delayed CTLA4Ig administration is still effective in blocking a polarized Th2 immune response when given at a later time point after T cells have been activated, *H. polygyrus*-inoculated BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. On days 14 and 24 after *H. polygyrus* inoculation, serum immunoglobulin levels were quantitated. As in previous experiments, *H. polygyrus*-infected mice treated with control antibody exhibited increased serum concentration of IgE when compared with uninfected controls, at both days 14 and 24 (Figure 43). CTLA4Ig treatment significantly reduced IgE serum concentration at day 14 when compared with control antibody treated mice. At day 24, the serum level of IgE was reduced even further by CTLA4Ig treatment, to approximately one third of the

Figure 42. CTLA4Ig treatment does not alter the number of *T. muris* larvae. AKR mice were infected with *T. muris* and injected i.v. with 100 µg CTLA4Ig or L6 at days 8 and 16. Larval worm burden was quantitated at day 21. The data are expressed as the mean and standard error of five individual mice per treatment group. This experiment was conducted two times with similar results.

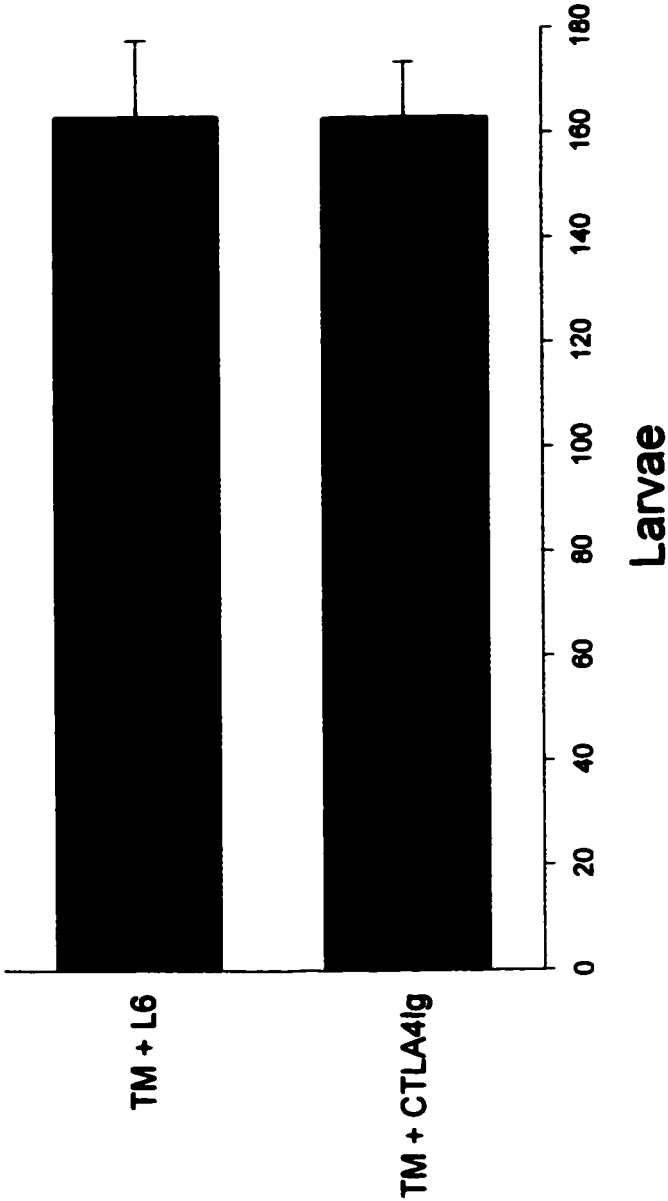
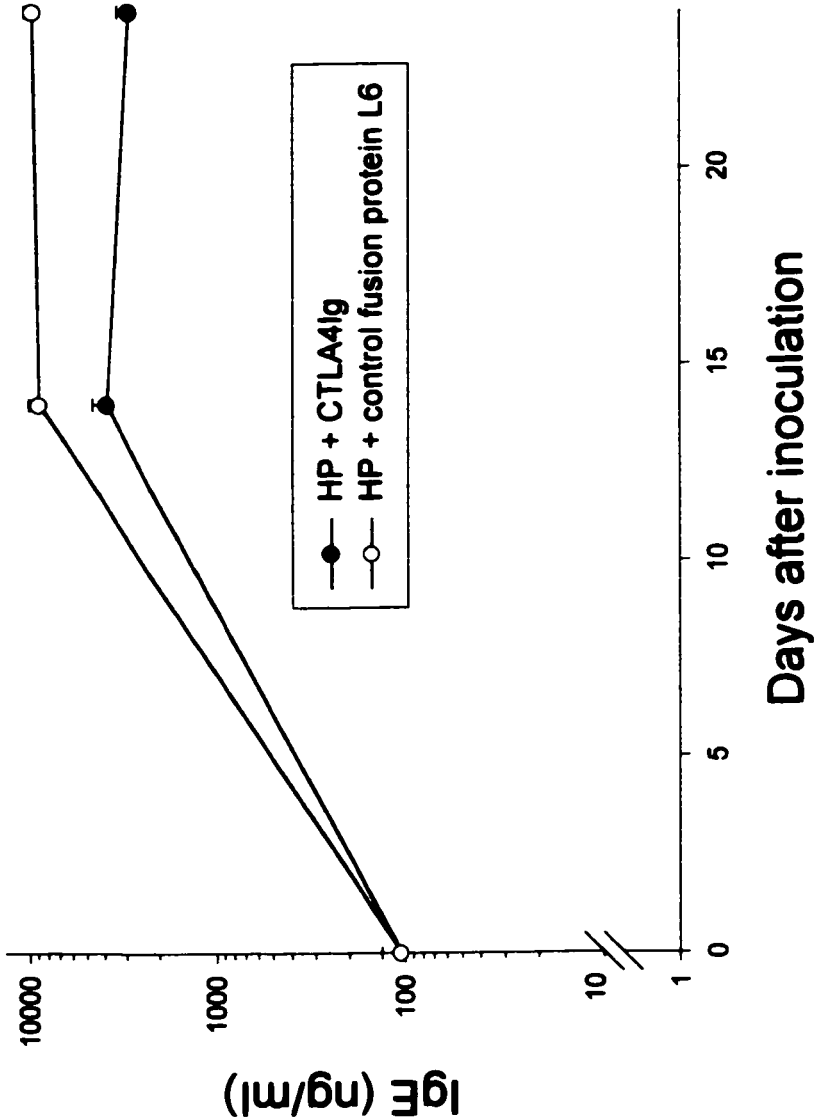


Figure 43. Blocking B7-ligand interactions with CTLA4Ig reduces serum IgE concentrations at days 14 and 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 14 and 24 after inoculation, and serum IgE levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.



concentration seen in control antibody treated animals. A similar pattern was seen in the serum concentration of IgG1 (Figure 44). CTLA4Ig treatment significantly inhibited *H. polygyrus*-induced elevations in IgG1 by 24. At this timepoint, IgG1 serum levels were reduced to about half of the concentration seen in control antibody treated animals. Serum concentrations of IgG2a were also quantitated (Figure 45). *H. polygyrus* infection induced a modest decrease in IgG2a concentration. No statistically significant difference was seen in the serum IgG2a concentration between CTLA4Ig or control antibody treated groups. These changes in immunoglobulin concentrations are consistent with induction of a Th2 response by *H. polygyrus*, and inhibition of the Th2 immune response by CTLA4Ig treatment, without deviation to a Th1 response.

Th2 cytokine expression was evaluated by RT-PCR of IL-4, IL-9, IL-3 and IL-13 message in mesenteric lymph node and Peyer's patch tissues. IL-4, IL-9 and IL-3 mRNA expression levels were significantly inhibited both in mesenteric lymph nodes and Peyer's patches in CTLA4Ig-treated animals (Figure 46). IL-13 gene expression was unaffected. Inhibition of IL-4 expression may underlie the reduction in IgE serum immunoglobulin concentration, as class-switching to IgE is IL-4 dependent (Finkelman et al., 1986). On the other hand, another mechanism is responsible for the reduction in IgG1 levels, as IgG1 production is IL-4 independent.

T and B cell activation was also examined by FACS analysis. *H. polygyrus* inoculation of control antibody treated mice increased forward scatter of B220⁺ B cells (Figure 47). CTLA4Ig treatment inhibited the *H. polygyrus*-induced increase in forward scatter, indicating a reduction in size. CTLA4Ig treatment also inhibited the *H. polygyrus*-induced increase in MHCII expression on B220⁺ B cells (Figure 48). There

Figure 44. Blocking B7-ligand interactions with CTLA4Ig reduces serum IgG1 concentrations at day 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 14 and 24 after inoculation, and serum IgG1 levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.

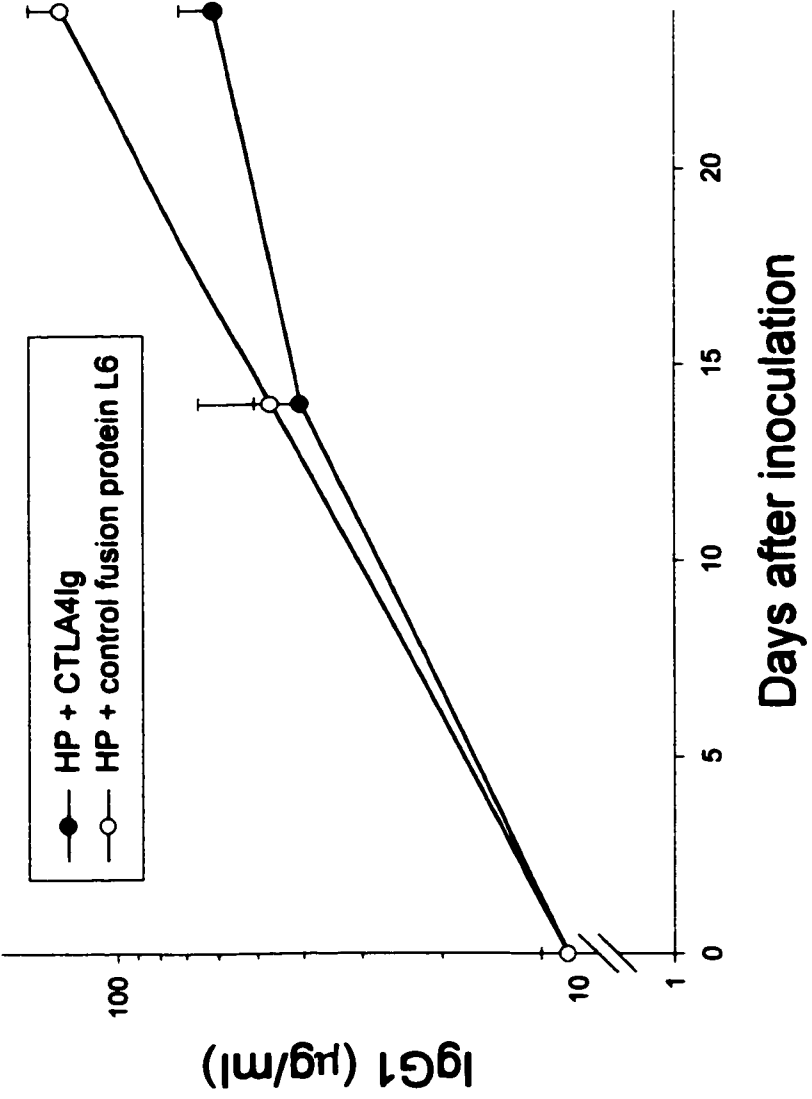


Figure 45. Blocking B7-ligand interactions with CTLA4Ig does not reduce serum IgG2a concentrations at days 14 or 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on days 14 and 24 after inoculation, and serum IgG2a levels were determined by ELISA. The mean and standard error derived from serum samples of five individual mice are shown for each treatment group.

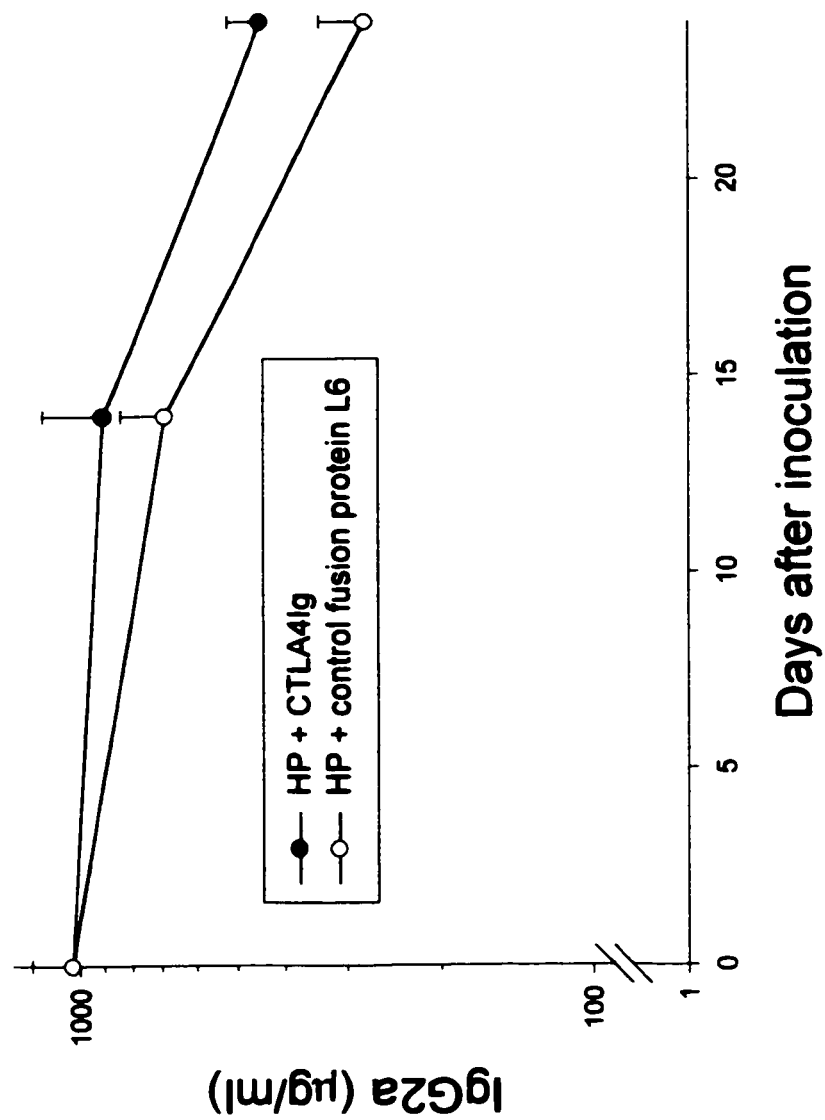


Figure 46. Blocking B7-ligand interactions with CTLA4Ig reduces IL-3, IL-4, and IL-9 gene expression in mesenteric lymph node and Peyer's patch tissues on day 24 after *H. polygyrus* inoculation. BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mesenteric lymph node and Peyer's patch tissues were collected on day 24 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and standard error are derived from RNA preparations from the mesenteric lymph node and Peyer's patch tissues of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to three-fold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1.

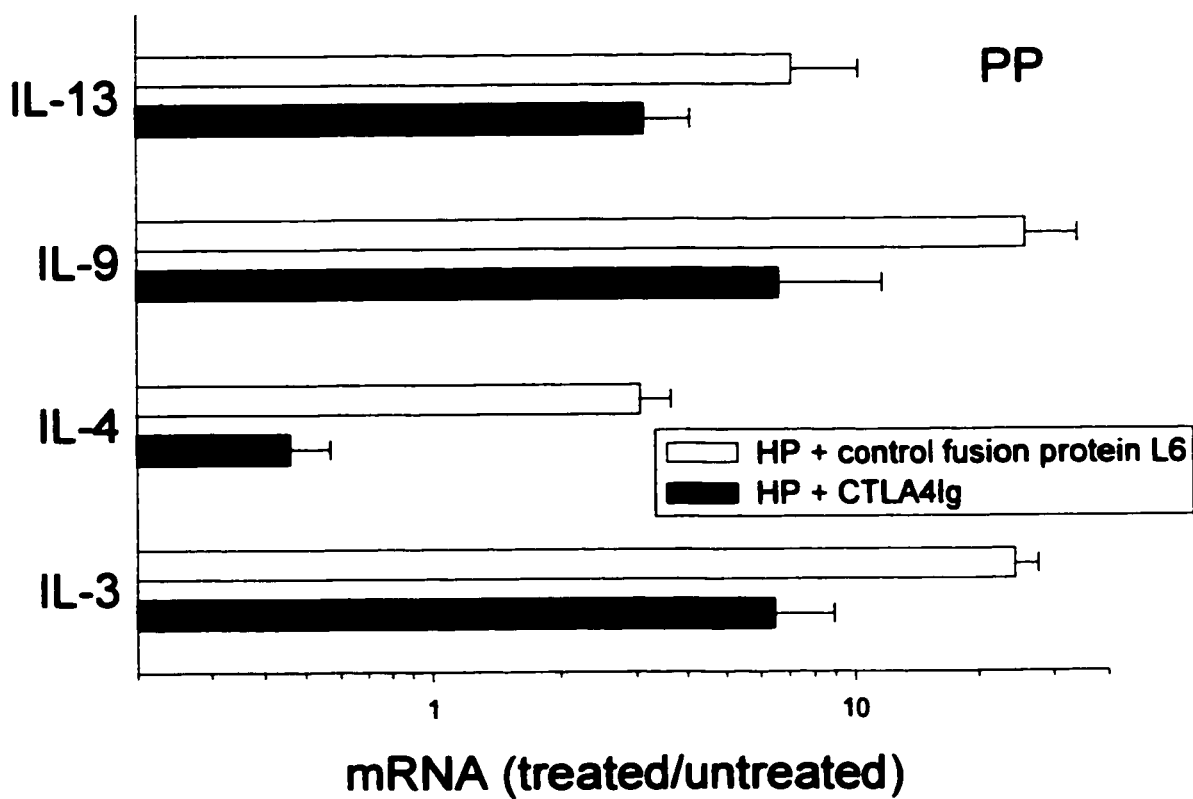
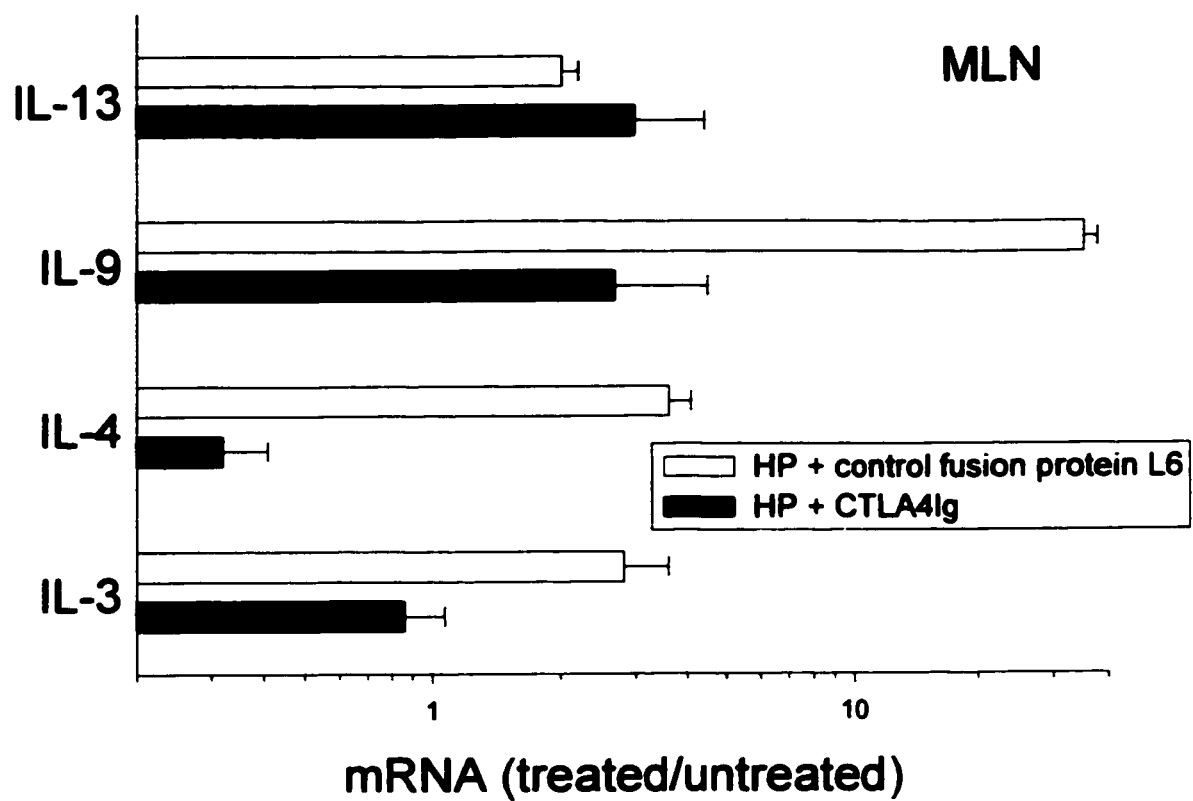


Figure 47. Blocking B7-ligand interactions with CTLA4Ig inhibits the *H. polygyrus*-induced increase in B220⁺ B cell size. BALB/c mice were injected i.v. with 200 µg CTLA4Ig or control fusion protein L6 at days 9, 14 and 19 after oral inoculation with 200 third-stage *H. polygyrus* larvae. At day 24 cell suspensions were prepared from mesenteric lymph nodes from five individual mice per treatment group, and pooled. Cells were dual stained with cychrome-anti-B220 and biotin-anti-MHCII followed by streptavidin-PE. Single histogram analysis of B220⁺ B cell size (forward scatter) is shown.

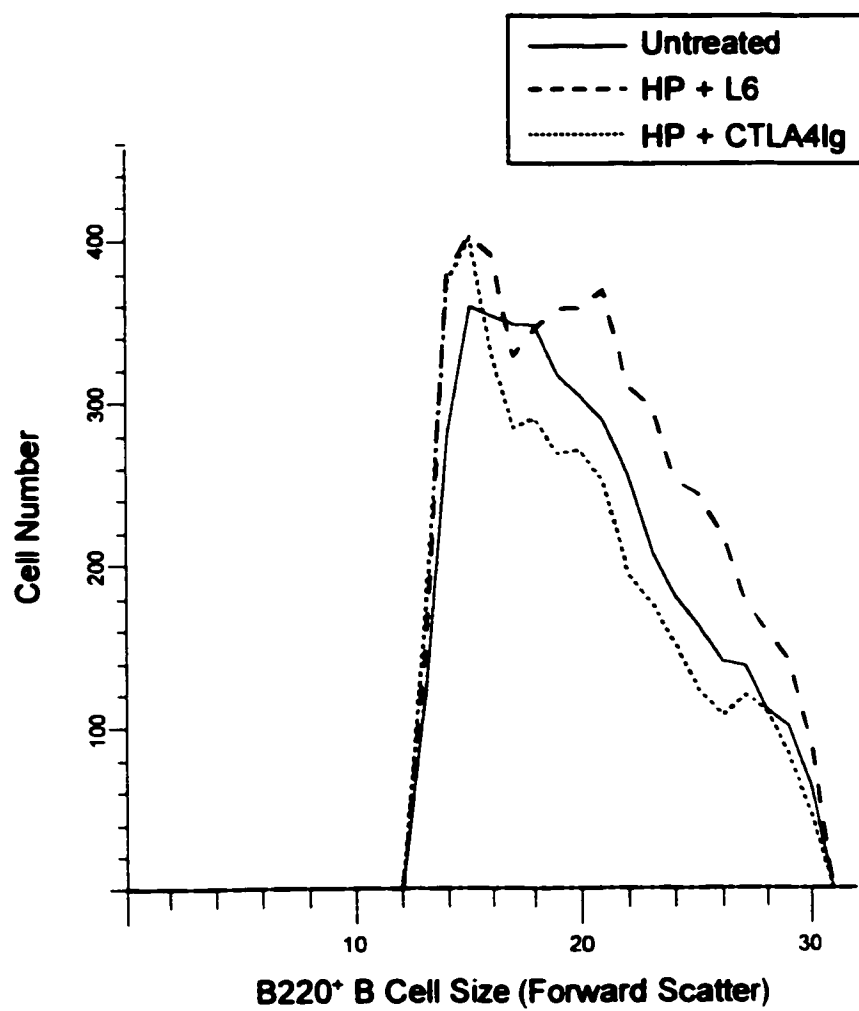
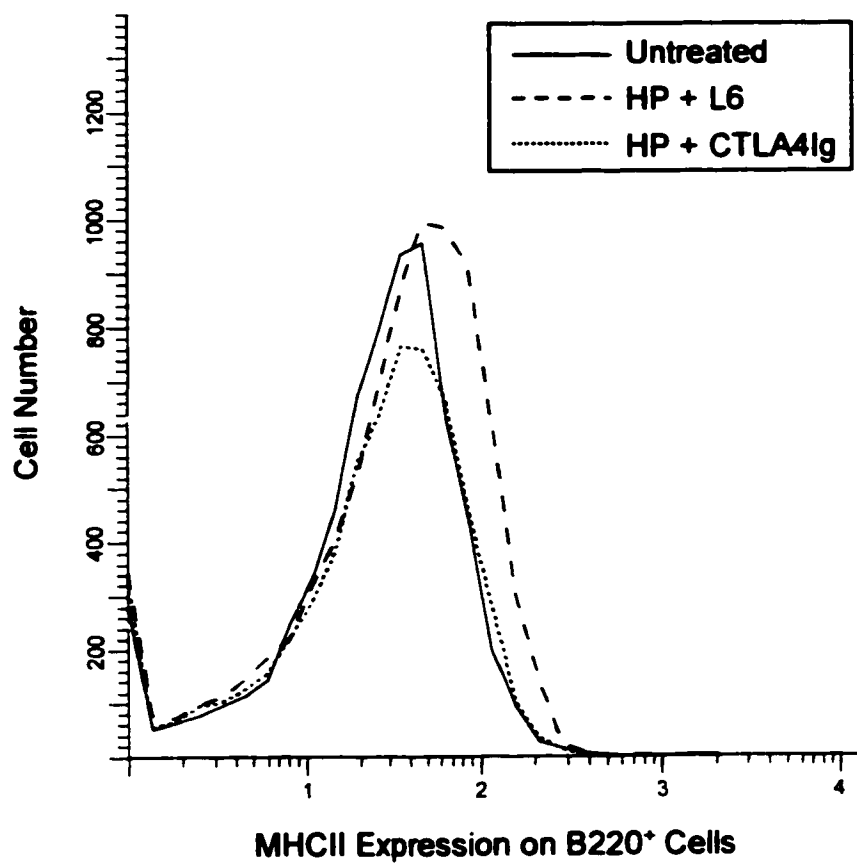


Figure 48. Blocking B7-ligand interactions with CTLA4Ig inhibits the *H. polygyrus*-induced increase in MHCII expression on B220⁺ B cells. BALB/c mice were inoculated with *H. polygyrus* and treated with CTLA4Ig or control fusion protein L6, and mesenteric lymph node cells were pooled and stained as described in the legend of Figure 47 (five mice per treatment group). Single histogram analysis of MHCII expression on B220⁺ B cells is shown. The MHCII expression scale is logarithmic.



was no change in forward scatter of CD4⁺ T cells or expression of IL-2R on CD4⁺ T cells by CTLA4Ig treatment (data not shown).

As a parameter of host protection against the pathogen, the production of *H. polygyrus* eggs was also measured. CTLA4Ig treatment did not increase the number of eggs produced (data not shown). It should be noted that a large number of eggs were being produced in control antibody treated mice, so that even if CTLA4Ig treatment significantly inhibited T cells activation, this may not result in increased egg production in a host that was already failing to control the infection. Together, the immunoglobulin, cytokine, and FACS data suggest that blockade of B7 interactions by CTLA4Ig is effective at reducing the activation of T cells that have been stimulated for nine days by a potent Th2-associated infectious disease.

IV. Discussion

This dissertation has focused on the role of CTLA4 in T cell activation and the immune response to infectious disease. CTLA4 plays a critical role in homeostasis of the immune system (Oosterwegel et al., 1999a; Saito, 1998; Greenfield et al., 1998; Scheipers and Reiser, 1998). CTLA4 also may be a promising target for manipulation to achieve therapeutic benefit, by controlling the strength or nature of the immune response. This research provides data concerning the function of CTLA4 in effector T cells and the molecular mechanism of CTLA4 signaling. The model systems of infectious disease used here (*H. polygyrus* and *T. muris* nematode parasites) have important clinical relevance, as they are similar to pathogens that are endemic in much of the developing world (Finkelman et al., 1997). The immune responses elicited by these parasites are also similar to the clinical manifestations of allergy. The results presented here may aid in the development of therapies to combat these two widespread medical conditions. Interpretations of my results are presented in several sections in this chapter. First, this discussion will address the experiments utilizing *in vivo* anti-CTLA4 antibody intervention. Next, the studies examining the molecular mechanism of CTLA4 will be discussed. An interpretation of the experiments with CTLA4Ig blockade of B7 ligand interactions is also included.

A. CTLA4 blockade during a polarized Th2 immune response to an infectious pathogen

The purpose of these experiments was to provide evidence on whether CTLA4 signaling delivers a negative signal late in the immune response to *H. polygyrus*. Specifically, these experiments were designed to assess whether the response to *H. polygyrus* can be increased by blocking CTLA4 with anti-CTLA4 antibody intervention. Initial experiments using moderate antibody doses did not indicate enhancement of the immune response by CTLA4 blockade. However, additional experiments using high dose administration of anti-CTLA4 antibody showed that CTLA4 blockade was effective in enhancing the immune response to *H. polygyrus*. Several parameters indicated an increased immune response. Serum IgE, IgG1 and antigen-specific IgG1 concentrations were increased by CTLA4 blockade by day 15 after *H. polygyrus* inoculation. Serum IgG2a, a Th1 response-associated isotype, was unaffected. These data are consistent with the polarized Th2 immune response that *H. polygyrus* elicits, and indicates that the enhanced response was specific to Th2-associated isotypes (Mosmann and Coffman, 1989). The increase in the Th2 response was also reflected in enhanced IL-4 secretion by day 16. IL-4 is a potent mediator of protection against *H. polygyrus* (Urban et al., 1998; Finkelman et al., 1997). In one experiment, egg production was lower at day 20 in anti-CTLA4 antibody treated mice compared with control antibody treated mice, suggesting that an increased Th2 response can reduce the fecundity of infection. At day 15, CD4⁺ T cell and B220⁺ B cell size was increased (as measured by forward scatter analysis) in anti-CTLA4 antibody treated mice. Increased cell size is an indication of blastogenesis and proliferation (Greenwald et al., 1997). A greater percentage of CD4⁺ T cells

expressed IL-2R, an activation marker, and there was a greater percentage of B220⁺ B cells, consistent with expansion of activated T and B cells. These data support a negative signaling role for CTLA4 late in the immune response to infectious disease.

CTLA4 blockade had previously been examined in a model of antitumor immunity (Leach et al., 1996). Anti-CTLA4 antibody administration inhibited the growth of subcutaneously injected tumor cells. Increased antitumor immunity was observed with B7-transfected colon carcinoma cells, wild-type colon carcinoma cells, and wild-type fibrosarcoma cells. In some mice, CTLA4 blockade resulted in complete rejection of the tumor, while mice treated with control antibody exhibited rapid tumor growth and required euthanasia. However, this report was limited to measurements of tumor growth, and did not address the mechanism of increased immunity. The mechanism of enhanced response by CTLA4 blockade was examined in a subsequent paper, which demonstrated increased protective immunity to *N. brasiliensis* (McCoy et al., 1997). Administration of anti-CTLA4 antibody concurrently with onset of infection reduced worm colonization of the small intestine and reduced parasite egg output. The enhanced protection was associated with increased lymphocyte numbers in the draining lymph nodes and increased secretion of IL-4 and IL-5.

It should be noted that in McCoy et al. (1997), *N. brasiliensis*-infected mice that were treated with control antibody cleared the infection, and the increased protection induced by CTLA4 blockade was observed as an increase in the kinetics of clearance. Whether CTLA4 blockade could induce increased protection against a parasite that normally establishes chronic infection had not been investigated. The model used in this dissertation research addresses this issue, as *H. polygyrus* establishes a chronic infection

in BALB/c mice (Finkelman et al., 1997). In addition, the previous experiments with *N. brasiliensis* utilized administration of anti-CTLA4 antibody concurrently with inoculation. The effects of blocking CTLA4 later in the immune response to infectious disease had not been explored. CTLA4 is not expressed on the surface of naïve T cells, and is only upregulated upon activation (Chuang et al., 1997). This suggests that CTLA4 may be more available for ligation by B7 molecules later after initiation of the response. However, effector T cells may be less dependent on B7-mediated costimulation than naïve T cells (Gause et al., 1997b). The results presented here are consistent with CTLA4 providing a negative signal late after onset of *H. polygyrus* infection, and enhancement of the immune response by blockade of the negative signal.

Although CTLA4 blockade caused upregulation of the response against *H. polygyrus*, protection was not consistently increased. In one experiment anti-CTLA4 antibody treatment reduced the number of eggs produced by the pathogen, but this result was not reproduced in a separate, independent experiment. The chronic nature of the *H. polygyrus* response and the acute nature of *N. brasiliensis* infection may be important. It is possible that the threshold for increased protection is lower in *N. brasiliensis*-infected mice treated with anti-CTLA4 antibody. The treatment protocol used here may have enhanced the immune response near the threshold required to overcome *H. polygyrus* infection, resulting in similar results between experiments when parameters of the immune response were measured (for example, immunoglobulin concentrations), but inconsistent results between experiments when protection was measured (egg production). The timepoint at which egg production was measured (day 20) may not have been optimal. More consistent results may be obtained at a later timepoint.

especially if antibody administration is extended later than day ten. This approach may not be practical, however, because the large amounts of antibody required may be prohibitive. Chronic administration of the hamster-derived anti-CTLA4 antibody may cause an immune response directed against the antibody, inhibiting its ability to block CTLA4 signaling.

It is unclear why very high doses of antibody were required to enhance the immune response. This antibody has been used effectively at moderate doses in the past (Leach et al., 1996; Walunas and Bluestone, 1998; Perez et al., 1997; Luhder et al., 1998). High dose anti-CTLA4 antibody administration was utilized in the *N. brasiliensis* report (McCoy et al., 1997), which was published while this dissertation research was in progress. Not only were more conventional doses ineffective in enhancing the response, in one experiment the immune response appeared to be diminished by moderate dose anti-CTLA4 antibody treatment. In this experiment, serum IgE concentrations were reduced in anti-CTLA4 antibody treated mice, and *H. polygyrus* egg production was increased when compared with control antibody mice. If partial blockade of CTLA4 results in a decreased immune response, then does this indicate that there is a positive component of CTLA4 signaling that was selectively blocked in this experiment? While it is generally accepted that CTLA4 supplies a negative signal under most circumstances (Oosterwegel et al., 1999a; Saito, 1998; Greenfield et al., 1998; Scheipers and Reiser, 1998), some studies have suggested that CTLA4 can also mediate positive costimulation (Liu, 1997; Wu et al., 1997; Zheng et al., 1998). Because the decrease in the strength of the immune response was seen in only one experiment with moderate dose anti-CTLA4 antibody administration, these experiments would need to be repeated to see if this effect

is consistently observed. Whether or not this is the case, it is clear that moderate dose anti-CTLA4 antibody treatment is ineffective in enhancing the response in this model system. The requirement for higher doses of CTLA4 may be due to the use of a potent infectious agent. The pathogen may induce a greater degree of costimulation when compared with peptide antigens used in previous studies that utilized CTLA4 blockade (Perez et al., 1997; Walunas and Bluestone, 1998). This requirement should be kept in mind if experiments addressing enhancement of the response by CTLA4 blockade are conducted in the future.

B. CTLA4 blockade during a mixed Th1/Th2 immune response to an infectious pathogen

The purpose of these experiments was to examine whether blockade of CTLA4 can increase the strength of signal and promote deviation toward a Th2 response. Chronic *T. muris* infection of AKR mice produces an immune response that is not so strongly polarized as the response of BALB/c mice to *H. polygyrus*. The response of AKR mice to *T. muris* is characterized by both Th1 and Th2 components (Else et al., 1994), providing the opportunity to investigate the role of CTLA4 in the Th1/Th2 developmental decision in a model of chronic nematode infection. The experiments presented in this dissertation provide data that are consistent with a negative signaling role for CTLA4 and enhanced strength of signal upon CTLA4 blockade. Anti-CTLA4 antibody treatment of *T. muris*-infected AKR mice was effective in raising serum concentrations of IgE, IgG1 and IgG2a. Antibody treated mice also had a greater percentage of CD4⁺ T cells that also expressed IL-2R, and the intensity of expression of

this activation marker was increased. The strength of signal received by the T cell during activation may be an important factor in guiding Th1/Th2 differentiation (Gause et al., 1999). The data presented here suggest that increasing the strength of signal by blocking the negative signal provided by CTLA4 deviated cytokine secretion from Th1 to Th2. *T. muris*-induced IFN γ secretion was inhibited and IL-4 secretion was increased by anti-CTLA4 antibody treatment of *T. muris*-infected mice. The reciprocal shift in cytokine secretion supports the model for T cell differentiation wherein increased strength of signal favors the development of a Th2 response.

A number of reports have addressed the roles of CTLA4 and CD28 in Th1/Th2 differentiation. It has been proposed that individual B7 molecules, through their interactions with CD28, differentially regulate the development of Th1 and Th2 phenotypes. In particular, it has been suggested that CD28 ligation by B7-2 promotes Th2 differentiation, and CD28 ligation by B7-1 allows Th1 differentiation (Freeman et al., 1995; Ranger et al., 1996; Corry et al., 1994). Recently, the current view has shifted away from selective regulation of cytokines by individual molecules, to integration of multiple signals received by the TCR, costimulatory receptors and cytokine receptors (Rogers and Croft, 2000). In the current model, Th2 development is favored by high strength of signal; reducing the strength of signal while maintaining comparable stimulation of the innate response can lead to immune deviation toward a Th1 phenotype. Indeed, when costimulation was prevented by CTLA4Ig administration, the Th2 response to *T. muris* mounted by BALB/c mice was shifted toward a Th1 response (Urban et al., 2000). CTLA4Ig treatment of *T. muris*-infected BALB/c mice resulted in increased IFN γ gene expression and IgG2a serum concentration, and decreased IL-4 gene expression and

IgE serum concentration, consistent with deviation from a Th2 toward a Th1 response. The cytokine secretion data presented in this dissertation represents the reverse situation, where increased strength of signal promotes Th2 cytokine secretion (IL-4), and inhibits Th1 cytokine secretion (IFN γ). It is unclear, however, why the decrease in Th1 response does not extend to serum concentration of the Th1 response-associated isotype IgG2a. These experiments demonstrated elevation of both Th1 and Th2 isotypes.

An unexpected outcome was seen in the concentrations of antigen-specific IgG1 and IgG2a, in comparison with polyclonal IgG1 and IgG2a. Effector T cells can provide help both for antigen-specific B cells and antigen non-specific B cells, in a process known as the “bystander effect” (Whalen et al., 1988; Lipsky, 1990). Normally, antibodies directed at immunogenic determinants would be expected to predominate, with the bystander effect contributing some non-specific immunoglobulins to a lesser extent. Therefore, it had been expected that antigen-specific IgG1 and IgG2a would be increased at least as much as polyclonal IgG1 and IgG2a, as had been seen in the *H. polygyrus* model system. It was surprising that in *T. muris*-infected mice, anti-CTLA4 antibody treatment increased polyclonal IgG1 and IgG2a, but there was not a statistically significant enhancement of antigen-specific IgG1 and IgG2a. Blocking CTLA4 signaling may preferentially upregulate B7-dependent activation of bystander B cells. This may indicate that CTLA4 antagonist treatment may not be effective in enhancing protection when the humoral response is necessary to promote resolution of infection. Alternatively, the pathogen may be influencing the host in a way that inhibits increased pathogen-specific antibodies. Data from other nematode model systems have shown that pathogens can influence the nature of B cell responses. A glycoprotein secreted by

Acanthocheilonema viteae has been shown to desensitize some but not all B cells, with the residual response being skewed toward Th2-associated isotypes (Harnett et al., 1999). The body fluid of *Ascaris* has been shown to contain a B cell mitogen that stimulates polyclonal B cell activation (Lee and Xie, 1995), and the DNA from nematodes also can cause polyclonal activation and proliferation of B cells (Sun et al., 1997). In either case, antibody responses may not be required for resistance to *T. muris*. Although the induction of B cell responses has been described in a strain of mouse that is resistant to *T. muris* (Koyama et al., 1999), two experiments have demonstrated that antibody-dependent mechanisms are not necessary for parasite expulsion. SCID mice, which lack endogenous B and T cells, are able to expel *T. muris* larvae upon adoptive transfer of CD4⁺ T cells (Else and Grencis, 1996). Fcγ-R-deficient mice also maintain resistance, despite the lack of functional antibody-dependent cell-mediated cytotoxicity (Betts and Else, 1999).

While the results of the experiments presented here support increased strength of signal and associated immune deviation, increased protection against *T. muris* was not observed. Previous experiments have shown that administration of IL-4 or depletion of IFNγ in susceptible mice induced worm expulsion (Else et al., 1994). The reason for this discrepancy is not clear. It may be that in these experiments the shift was not sustained long enough to promote expulsion. Although IFNγ secretion was inhibited, IFNγ gene expression was not decreased at this timepoint, and this may be reflected in a later rise in IFNγ secretion. This suggests that continuing CTLA4 blockade for a longer duration of time and measuring larval counts at a later timepoint may be more likely to reveal an effect on protection. In the previous report, larvae were counted at day 35, compared

with day 21 in the research presented here. Also, if the later timepoint is used, egg production can be measured, which can be a more sensitive measure of protection.

The strength of signal hypothesis is an attractive one to explain Th1/Th2 differentiation, but conflicting results remain to be resolved. T cells from CD28-deficient mice that were primed *in vivo* and restimulated *in vitro* displayed reduced IL-4 secretion, consistent with the decreased strength of signal caused by the lack of costimulation provided by CD28. However, CD28-deficient mice of the BALB/c background were able to express IL-4 mRNA and secrete IL-4 protein when infected with *L. major* (Brown et al., 1996). This indicates that CD28 is not required for the development of the Th2 response. Similarly, there is data concerning the role of CTLA4 that is difficult to reconcile. In wild-type BALB/c mice, which are susceptible to *L. major*, blocking CTLA4 signaling with Fab fragments of anti-CTLA4 antibodies ameliorated disease by immune deviation toward a Th1 response (Saha et al., 1998). It is possible that CTLA4 supplies a positive signal in this system, and blockade of the positive signal reduces the overall strength of signal. The Th1/Th2 decision is likely to be governed by a complex array of factors, and more research is needed to determine how costimulatory signals contribute to this decision.

C. Phosphorylation status in anti-CTLA4 antibody treated mice and CTLA4-deficient mice

The intracellular events following CTLA4 ligation remain poorly understood. The molecular mechanism of CTLA4 signaling was investigated in this dissertation research. In CTLA4-deficient mice, several proteins in mesenteric lymph node cells were

hyperphosphorylated, including CD3 ζ chain of the TCR (Marengere et al., 1996). The identification of hyperphosphorylated proteins in mesenteric lymph node cells from CTLA4-deficient mice led to the development of a model for the mechanism of CTLA4 inhibitory signals. This model suggests that ligation of CTLA4 recruits SHP-2 phosphatase, which dephosphorylates proteins involved in transduction of the antigen-dependent activation signal, thereby inhibiting the activation signal (Lee et al., 1998). Experiments described in this dissertation investigate whether blocking CTLA4 by antibody intervention *in vivo* induces changes in phosphorylation of intracellular proteins, and whether these changes are similar to those seen in CTLA4-deficient mice. The purpose of these experiments was to provide evidence for the mechanism of enhanced immune response in anti-CTLA4 antibody treated mice.

As expected, these experiments demonstrate CD3 ζ phosphorylation in cells from CTLA4-deficient mice, but not in untreated mice, as had previously been published (Marengere et al., 1996). There were similar levels of phosphorylation of CD3 ζ in CTLA4-deficient mice and in *H. polygyrus*-infected mice treated with anti-CTLA4 antibody. However, *H. polygyrus*-infected mice treated with control antibody also had a similar level of CD3 ζ phosphorylation. Therefore, these experiments do not provide an explanation for the increased immune response induced by anti-CTLA4 antibody treatment. No other changes in phosphorylation of intracellular proteins were observed in both CTLA4-deficient mice and in *H. polygyrus*-infected mice treated with anti-CTLA4 antibody.

The report from Marengere et al. (1996) was the first to identify association between CTLA4 and SHP-2, and to provide evidence for CTLA4-induced phosphatase

downregulation of the TCR signal transduction pathway. Since that report, there have been several to support antagonism of TCR signals as the mechanism of CTLA4 action. CTLA4 ligation by B7 was able to inhibit T cell activation in the absence of CD28 (Lin et al., 1998; Fallarino et al., 1998). Phosphorylation-induced association between CTLA4 and SHP-2 has been independently demonstrated (Miyatake et al., 1998; Chuang et al., 1999). A direct involvement of CTLA4 in downregulation of TCR signals has also been demonstrated (Lee et al., 1998). When primary T cells were activated *ex vivo*, CD3 ζ was tyrosine phosphorylated: ligation of CTLA4 inhibited the phosphorylation. CD3 ζ coprecipitated with CTLA4 in *ex vivo* activated T cells. The association between CTLA4 and CD3 ζ did not require any additional T cell-specific proteins, because the interaction persisted in transfected human embryonic kidney epithelial cells. Finally, SHP-2 participated in a multimolecular complex with CTLA4 and CD3 ζ , and dephosphorylated CTLA4-associated CD3 ζ .

In light of the previously published data, it is unclear why CTLA4-dependent increased CD3 ζ phosphorylation was not observed in the experiments presented here. Several limitations were encountered in these experiments. The western analysis of phosphorylation was limited by the low protein amounts available. Only one CTLA4-deficient mouse was available to us, due to the difficulty in generating these mice. The lymph nodes of this mouse were at the lower limit of the mass needed for immunoprecipitation. For this reason, the western blots did not have the desired sensitivity. The changes induced by anti-CTLA4 antibody treatment may have been very subtle, so more material may have been needed to detect a difference between the experimental group and the mice treated with control antibody. Another issue was the

uncertainty in the timepoint after antibody administration to proceed with immunoprecipitation. At day 13, or three days after the second antibody administration, IgE was elevated in *H. polygyrus*-infected mice treated with anti-CTLA4 antibody, above the level in control antibody treated mice. This indicates that by this timepoint, antibody intervention has had an effect. It is possible, though, that by day 13 the intracellular events mediating the enhanced immune response have passed. If more materials had been available, it would have been optimal to repeat the experiment at an earlier timepoint. On a technical level, one aspect of phosphorylation status was not available for inspection. A secondary antibody, anti-mouse IgG conjugated HRP, was used in western detection. This antibody recognized endogenous mouse IgG in addition to the primary western antibody, anti-phosphotyrosine. The heavy chain of the endogenous mouse IgG ran with electrophoretic mobility similar to that of p52 SHC, obscuring p52 SHC that may have been coimmunoprecipitated.

Technical issues may have contributed to the negative results; it is also possible that the regulation of CD3 ζ by CTLA4 varies with the T cell stimulation conditions. The previous experiments that documented inhibition of CD3 ζ phosphorylation by CTLA4 were conducted with T cells that had been activated *ex vivo*, and CTLA4 was crosslinked *in vitro* by antibody (Lee et al., 1998). This differs substantially from the experimental conditions used in this dissertation research, where T cells were activated *in vivo*, and CTLA4 is ligated by B7 *in vivo*. There has also been a report that did not find evidence of CD3 ζ regulation by CTLA4 (Calvo et al., 1997). In this case, the conditions of ligation of CTLA4 differed from the experiments by Lee et al. (1998), in that soluble

antibody was used instead of membrane bound anti-CTLA4 antibody. Differences in experimental conditions may explain the conflicting results of these experiments.

D. Intracellular binding partners of CTLA4 and CD28

While it is generally agreed that CTLA4 supplies a negative signal to T cells under most circumstances, the possibility remains that CTLA4 can also give a positive costimulatory signal. This possibility is supported by research that has shown that positive costimulation can be delivered in the absence of CD28 (Gause et al., 1997a; Shahinian et al., 1993; Kawai et al., 1996). B7-dependent proliferation in CD28-deficient T cells can be inhibited by blocking CTLA4, indicating that CTLA4 is promoting proliferation (Wu et al., 1997). Research presented in this dissertation addresses whether CTLA4 and CD28 have common intracellular binding partners, because common signal transduction pathways may also indicate common functions. In addition to investigating potential common function, the identification of novel intracellular binding partners for CTLA4 and/or CD28 could lead to a greater understanding of how these molecules transduce their negative and positive signals, respectively.

The yeast two hybrid screen using the cytoplasmic domain of CTLA4 a bait identified two clones of AP50. This protein, a subunit of the clathrin adapter complex, had previously been shown to bind CTLA4 (Shiratori et al., 1997; Bradshaw et al., 1997; Zhang and Allison, 1997); however, this was the first identification of two novel truncated forms of AP50. The clones identified in the screen encode the N-terminal 273 and 250 amino acids of AP50 (the full-length form is 435 amino acids). The screen also revealed a weak association between CTLA4 and phospholipase C- α . The binding

was somewhat stronger than apparently non-specific interactions, in that the interaction persisted at 50 mM 3AT, but should still be considered very weak, because the moderately sensitive β -galactosidase reporter gene was not induced. This is the first report of association between CTLA4 and phospholipase C-alpha.

Three proteins were found to bind CD28: homer-3, arginine N-methyltransferase, and TAFII250. It can be inferred that homer-3 binds CD28 with moderate strength, as it induced the URA3 gene. Likewise, the arginine N-methyltransferase/CD28 interaction induced the β -galactosidase reporter gene, also indicating an association of moderate strength. However, TAFII250 did not induce either β -galactosidase or URA3 reporter genes, and only persisted at low concentrations of 3AT. From these data it can be concluded that TAFII250 interacted only weakly with CD28.

The yeast two hybrid system as a means to screen for unidentified binding partners for known proteins has shortcomings that apply to the experiments described in this dissertation. False positives are a common problem encountered with the yeast two hybrid system. An interaction taking place in the artificial environment of the yeast nucleus does not always indicate a functionally relevant interaction in the native mammalian cytoplasmic environment. Aside from AP50, the identities of the proteins that bound CTLA4 and CD28 in this assay do not immediately suggest how they may play a role in costimulation. Phospholipase C-alpha, which bound CTLA4 in the screen, is expressed in T cells, and is upregulated upon T cell activation (Goldfien et al., 1991). However, this molecule was originally misidentified as having phospholipase activity, and is actually a protein disulfide isomerase (Charnock-Jones et al., 1996; Hirano et al., 1994). It probably is involved in peptide loading and folding of MHC class I molecules

(Hughes and Cresswell, 1998), and seems an unlikely candidate for participation in signal transduction. Homer-3, which bound CD28, interacts with metabotropic glutamate receptors and modulates their synaptic properties (Brakeman et al., 1997; Soloviev et al., 2000). There are no data concerning the function of homer family members outside of neural systems. The two remaining proteins identified as binding CD28, arginine N-methyltransferase and TAFII250, are expressed in the nucleus. Arginine N-methyltransferase, also known as PRMT1, methylates nuclear proteins that interact with RNA (Gary and Clarke, 1998; Tang et al., 1998; Nichols et al., 2000). Arginine methylation has also been proposed to modulate protein-protein interactions important for signal transduction (Bedford et al., 2000). While other methyltransferases may function in this manner, the nuclear localization of PRMT1 argues against a role in signal transduction proximal to the plasma membrane, where CTLA4 is located when it is ligated by B7. TAFII250 is a component of the basal transcription factor IID (Hampsey and Reinberg, 1997). There is little to suggest why a plasma membrane-bound receptor should bind a transcription factor located in the nucleus, despite the intrinsic kinase activity of TAFII250 (O'Brien and Tjian, 1998). Further research would be needed to determine whether any of the interactions identified here are important for the function of CTLA4 or CD28.

Lck has previously been identified as interacting with both CTLA4 and CD28 (Miyatake et al., 1998; Leung et al., 1999). Lck was specifically tested in the yeast two hybrid system for interaction with both CTLA4 and CD28. No interaction was detected. These results could be interpreted in different ways. Other kinases may be responsible for phosphorylating CTLA4 and CD28. It is also possible that Lck phosphorylates

CTLA4 and/or CD28, but the interaction was not detected with this system. The interactions that transduce the signals of CTLA4 and CD28 may be weak and transient, which would make them difficult to distinguish from irrelevant false positives. It also creates the possibility that critical associations may escape detection entirely (false negatives). Very weak interactions identified in the yeast two hybrid system are difficult to confirm with independent, less sensitive techniques such as western blotting. The results presented here do not supply evidence of common functions between CTLA4 and CD28.

E. Blockade of both CD28 and CTLA4 during the immune response to infectious pathogens

Previous research has shown that blockade of both CTLA4 and CD28 with CTLA4Ig administration concurrent with onset of infection with *H. polygyrus* is effective in inhibiting the response (Lu et al., 1994). As with the research demonstrating enhancement of the response to *N. brasiliensis* by CTLA4 blockade, the effect upon an established infection remains to be explored. In addition, late blockade of both CD28 and CTLA4 may influence Th1/Th2 differentiation. Because CTLA4 is upregulated in effector T cells (Linsley et al., 1992), and because CTLA4 has higher affinity for B7 than does CD28 (Linsley et al., 1991; van der Merwe et al., 1997; Greene et al., 1996), CTLA4/B7 interactions may predominate over CD28/B7 interactions late after initiation of the response. Preferential blockade of CTLA4 interactions by CTLA4Ig administration may increase the overall strength of signal, promoting deviation toward a Th2 response. Alternatively, effector T cells may still be dependent on CD28-mediated

positive costimulation, and CTLA4Ig administration may block the ongoing response. The purpose of this research was to gain insight into the costimulatory dependence of ongoing immune responses.

The results from the experiments with CTLA4Ig treatment of *T. muris*-infected AKR mice indicate that delayed blockade of B7 interactions inhibited the response. As described earlier, *T. muris* infection of these mice produces a response with Th1 and Th2 components, and provides an opportunity to examine both the strength of the immune response and the Th1/Th2 balance (Else et al., 1994). CTLA4Ig administration inhibited *T. muris*-induced elevations in serum IgE, IgG1, and IgG2a. CTLA4Ig treatment also reduced antigen-specific IgG1 and IgG2a. When CD4⁺ T cells and B220⁺ B cells were examined, it was found that a lesser percentage of CD4⁺ T cells expressed IL-2R, that there was a lesser percentage of cells expressing B220, and that B220⁺ B cells were smaller in CTLA4Ig treated mice than in mice treated with the control fusion protein L6. The immune response to *T. muris* was also inhibited at the level of cytokine secretion. There were fewer IFN γ and IL-4 secreting cells in CTLA4Ig treated mice. These results are consistent with global inhibition of the response, without immune deviation (Mosmann and Coffman, 1989). These data stand in contrast with previous experiments that demonstrated immune deviation by delayed CTLA4Ig blockade of B7 molecules in other experimental models. When CTLA4Ig was administered to renal allograft recipients two days after transplantation, the cytokine secretion profile was shifted from Th1 to Th2, preventing rejection of the allografts (Sayegh et al., 1995). Similarly, CTLA4Ig administration ten days after induction of experimental autoimmune encephalomyelitis specifically inhibited Th1 cytokines but not Th2 cytokines in the

central nervous system, resulting in suppression of clinical disease (Khoury et al., 1995). Whether CTLA4Ig can block ongoing immune responses has important consequences for the development of immunotherapies for allergy and autoimmunity (Djukanovic, 2000; Anderson et al., 1999; Khoury et al., 1999). Because Th1/Th2 differentiation plays an important role in the pathogenesis of allergy and autoimmunity, it is important to understand how inhibition of B7 interactions would influence this aspect of T cell activation (Singh et al., 1999).

Similar results were obtained with *H. polygyrus*-infected BALB/c mice, although it should be pointed out that the experiments with CTLA4Ig were only conducted once in this model system, and should be considered preliminary. Delayed CTLA4Ig treatment inhibited *H. polygyrus*-induced elevations in IgE and IgG1. IgG2a, which was not elevated in L6 treated mice, was not affected by CTLA4Ig treatment. When B220⁺ B cells were examined, they were found to be smaller in CTLA4Ig treated mice, and also had less intense expression of MHCII activation marker than in L6 treated mice. It can be concluded from these data that CTLA4Ig is effective in reducing an ongoing polarized Th2 immune response. CTLA4Ig may prove to be a useful tool for modulation of the immune response. Phase I clinical trials have been conducted, to evaluate CTLA4Ig as a treatment for psoriasis vulgaris (Abrams et al., 1999). The trials have met with some success: 46% of patients achieved improvement in clinical disease activity. CTLA4Ig may be used in the treatment of a wide range of T cell-dependent diseases, and more research is needed to fully understand its effects on immune responses.

F. Future directions

There are many experiments that could expand upon the work presented here. First, to improve upon the CTLA4 blockade experiments in both *H. polygyrus* and *T. muris*-infected mice, later timepoints could be examined. If a sufficient quantity of antibody were available, sustained longer doses may be more effective. Another approach would be to utilize Fab fragments in place of whole antibody. This would remove the possibility (though remote) of whole antibody inducing signaling through CTLA4 instead of blocking its signal. CTLA4 blockade could be used in conjunction with signaling antibodies directed against "alternative" costimulatory molecules. There have been a number of receptors described that cannot substitute for CD28 in promoting IL-2 production but still are able to augment TCR signals (Watts and DeBenedette, 1999). The roles of these receptors are largely unexplored.

The number of *T. muris* eggs that are used in infection has been shown to influence the type of response that results (Bancroft et al., 1994). Mice that normally mount a Th2 response when infected with a moderate number of eggs mount a Th1 response when infected with very few *T. muris* eggs. This is consistent with our model of the Th1 versus Th2 decision: decreasing the number of infecting eggs could decrease the strength of signal, resulting in immune deviation to Th1. The reverse approach could also be used. In conjunction with CTLA4 blockade, the strength of signal could be augmented by using repeated inoculation, or combining infectious inoculation with administration of *T. muris* antigens. Alternatively, strength of signal could be augmented with combined blockade of CTLA4 and treatment with crosslinked anti-CD28 antibody. Experiments could also be conducted to address the open question of polyclonal versus

antigen-specific immunoglobulin responses to *T. muris*. The development of transgenic mice expressing a TCR specific for ovalbumin (OVA) has allowed investigators to track the activation and differentiation of small populations of antigen-specific T cells. T cells from the TCR-transgenic mice are adoptively transferred to syngeneic recipients, which are immunized with either OVA peptide (Ingulli et al., 1997) or bacteria that expresses an OVA construct (Chen and Jenkins, 1998). This approach could be used in the *T. muris* system: mice that have received adoptive transfer of OVA-specific T cells could be immunized with OVA peptide concurrently with *T. muris* inoculation. In this manner, the response to a specific antigen could be followed.

In the experiments that examine the phosphorylation status of intracellular proteins upon CTLA4 blockade, day nine would be an appropriate timepoint for immunoprecipitation. The intracellular molecular events may be transient, so a timepoint one day after antibody administration would be of interest. Proceeding with these experiments would be dependent on the availability of CTLA4-deficient mice, preferably a minimum of five so that a stronger signal could be generated in the western blot assay. The Ras pathway was found to be activated in CTLA4-deficient mice, so components of the Ras pathway could also be investigated using kinase assays, assuming sufficient material were available.

To search for intracellular binding partners of CTLA4 and CD28, the use of phosphorylated bait constructs may yield information about the interactions that occur after the initial phosphorylation step. Confirming the relevance of identified interactions can be difficult, when the interaction is not of sufficient affinity to be detected by

immunoprecipitation. One method that could be used would be to overexpress the identified proteins in a T cell line, and monitor the ability to stimulate these cells.

The inhibition of an ongoing immune response by late blockade of both CTLA4 and CD28 was demonstrated here in experiments using both the *T. muris* and *H. polygyrus* systems. However, the CTLA4Ig experiments in the *H. polygyrus* system were conducted only once, and must be considered preliminary until they were repeated. In addition, CTLA4Ig administration could be compared with the combination of anti-CTLA4 and anti-CD28 antibodies. This would test whether there are additional B7 ligands that are active late in the immune response.

G. Conclusions

The research in this dissertation has focused on the role of CTLA4 in *in vivo* models of infectious disease. Specifically, the experiments presented here have explored the function of CTLA4 during ongoing immune responses, after the development of effector T cells. In addition, experiments were conducted to elucidate the molecular mechanism of CTLA4 function. It can be concluded from these experiments that CTLA4 blockade can enhance the immune response after onset of infection. More generally, it can also be inferred from the antibody intervention experiments that CTLA4 gives a negative signal late in the immune responses to infectious disease. Results from the *T. muris* system indicate that CTLA4 blockade, and resulting increased strength of signal, can shift the cytokine secretion profile from Th1 to Th2.

Despite the ability of CTLA4 blockade to enhance the immune response, no intracellular phosphorylation events were identified that may mediate this effect. The

changes induced by CTLA4 blockade may be very subtle, compared with the complete loss of the gene in CTLA4-deficient mice. No common binding partners were identified for CTLA4 and CD28. The binding events that mediate CTLA4 and CD28 signals may be weak and transient, so these results do not rule out the possibility of common functions for CTLA4 and CD28.

The ability to enhance immune responses may have clinical applications in the treatment of infectious disease, and may be useful in the development of cancer immunotherapies. In addition, it would be advantageous for the treatment of allergy and autoimmune disease to develop means of downregulating ongoing immune responses. This dissertation has also addressed the sensitivity of effector T cells to blockade of CD28 and CTLA4. Results presented here have shown that ongoing immune responses can be inhibited with CTLA4Ig. The results of this dissertation may aid in the development of therapies that utilize manipulation of costimulation as a means to modulate immune responses.

V. References

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