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Abstract

Title of dissertation:

Functional Analysis of CD28/B7 and CD40/CD40L Costimulation During the *in vivo* Type 2 Immune Response

Pin Lu

Doctor of Philosophy, 1995

Dissertation directed by:

William C. Gause, Ph.D.

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Cytokine production and other effector functions of $CD4^+$ T helper (Th) cells are crucial for the initiation of a primary immune response. The activation of naive $CD4^+$ Th cells requires two signals delivered from antigen presenting cells (APCs). The engagement of the T cell surface receptor (TCR) by antigenic peptides presented in the context of major histocompatibility complex (MHC) class II molecules on APCs provides the primary signal, but this Ag-specific signal alone is not sufficient to activate naive $CD4^+$ Th cells. A second or costimulatory signal from APCs, which is independent of T cell receptor signaling, is required for optimal activation, proliferation, and cytokine production by naive $CD4^+$ Th cells. Recent studies have shown that T cell surface

molecule CD28, and its homologue CTLA-4, can provide costimulatory signals to Th cells when they interact with their ligands, B7-1/B7-2 on APCs. Interactions between Th cell surface CD40 ligand (CD40L) and B cell CD40 molecules have also been shown to costimulate Th cell activation. In this investigation, the role of CD28/CTLA4-ligand costimulation in naive Th cell activation and T cell cytokine production was examined by blocking CTLA-4-ligand interactions in two *in vivo* type 2 immune response models. These studies demonstrated that costimulation through CD28/ CTLA4 is required for Th cell priming leading to IL-4 cytokine production, B cell activation, and IgE secretion during an *in vivo* type 2 mucosal immune response to a nematode parasite, *Heligmosomoides polygyrus* (HP). Activation of naive T cells, elevations in IL-4 cytokine gene expression and secretion, and increased levels of serum Ig isotypes all require CD28/ CTLA4 costimulation during the *in vivo* IL-4 "dominant" immune response to foreign anti-mouse IgD antibody. Furthermore, the possible differential costimulation of B7-1 vs B7-2 was examined by blocking either B7-1 or B7-2 signaling with specific antibodies during the *in vivo* type 2 immune response to HP inoculation. We found that blocking both B7-1 and B7-2 signaling simultaneously was required for inhibition of elevated Th cell-derived IL-4 gene expression, B cell activation, elevations in serum IgG1 levels, and increases in the number of blood eosinophils and mucosal mast cells. Blocking either B7-1 or B7-2 signaling alone had no effects on the course of the immune response to HP. These results suggest that B7-1 and B7-2 deliver similar

costimulatory signals and can substitute for each other in this type 2 *in vivo* mucosal immune response. Finally, the *in vivo* effect of blocking CD40-CD40L interactions by anti-CD40L antibody administration during the immune response to HP was examined. Blocking CD40-CD40L interactions inhibited HP-induced elevations in serum IgG1 levels, blood eosinophils and intestinal mastocytosis. However, elevations in T cell cytokine gene expression and IL-4 secretion, as well as B cell MHC class II expression, were unaffected. Increases in B cell surface B7-2 expression was maximally inhibited only by blocking both CD40 and IL-4 receptor signaling. These results indicate that CD40-CD40L interactions are not required for Th cell activation and cytokine production, but are required for B cell activation and the proliferation of other effector cells associated with the type 2 immune response to HP inoculation.

Dedication

To my parents and my fiancee, Yue, for their endless love and support and for all the help they have provided to me.

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Functional Analysis of CD28/B7 and CD40/CD40L Costimulation During the *in vivo* Type 2 Immune Response

by

Pin Lu

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1995

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List of Abbreviations

aa, amino acid; Ab, antibody; Ag, antigen; ADP, adenosine diphosphate; APC, antigen presenting cell; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; bp, base pair; CD40L, CD40 ligand; CHO, Chinese hamster ovary; Con A, Concanavalin A; CTL, cytotoxic T lymphocyte; CTRL, control; Cy5, cyanine-chrome 5; dNTP, deoxynucleoside triphosphate; EAE, experimential allergic encephalomyelitis; EBV, Epstein-Barr Virus; FACS, fluorescent activated cell sorter; FBS, fetal bovine serum; FSC, forward light scatter; FITC, fluorescein-5-isothiocyanate; GaMIgD, goat anti-mouse IgD; GBM, glomerular basement membrane; GM-CSF, granulcyte macrohpage colony stimulating factor; GVHD, graft-versus-host disease; HBSS, Hank's balanced salt solution; HIM, hyper IgM; HP, *Heligmosomoides polygyrus*; *H. polygyrus*, *Heligmosomoides polygyrus*; HPRT, hypoxanthine-guanine phosphoribosyl transferase; ICAM, intercellular adhesion molecule; IFN-γ, interferon-γ; IgV, immunoglobin variable; IL-2R, IL-2 receptor; IL-4R, IL-4 receptor; KLH, keyhole lympet hemocyanin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLN, mesenteric lymph node; MLR, mixed lymphocyte reaction; NOD, nonobese diabetic; NZB, New Zealand black; NZW, New Zealand white; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PE, phycoeryrin; PP: Peyer's patch; RT, reverse transcriptase; RT-PCR, reverse

transcription-polymerase chain reaction; SA-PE, streptavidin-phycoeryrin; SE, standard error; SRBC, sheep red blood cell; TCR, T cell receptor; Th, T helper cell; Th1, T helper 1; Th2, T helper 2; TNFα, tumor necrosis factor α; UNTR, untreated; wk, week.

I. Introduction

A. Overviews of type 1 and type 2 immune responses

Initiation of an immune response against foreign antigens (Ag) requires an elaborate scenario of cell-cell interactions and activation events. Antigen presenting cells (APC) such as macrophages and dendritic cells are among the first cells responding to foreign antigens and efficiently process antigens and present antigenic peptides in the context of MHC class II molecules on their cell surface. Naive $CD4^+$ T helper (Th) cells contact APCs and become activated by recognizing the Ag-MHC complex through the surface antigen-specific T cell receptor (TCR) and also through other signals from APCs. Activated $CD4^+$ Th cells proliferate and exert their helper functions that serve to amplify the immune response and also influence its nature. The end result can be a specific immune response which includes the activation of effector cells that can mediate a protective response against a particular pathogen (Paul, 1993). Understanding the processes that initiate Th cell differentiation may provide insights into the development of therapies that favor host protective and prevent disease-causing immune responses.

Cytokines produced by activated $CD4^+$ Th cells play a crucial role in the development of the immune response. Activated $CD4^+$ Th cell clones maintained *in vitro* have been classified into two distinct subsets by their patterns of cytokine production (Mosmann *et al.*, 1986). T helper 1 (Th1) T cells secrete IL-2, IFN-γ, and lymphotoxin selectively, while T helper 2 (Th2) T cells selectively produce IL-4, IL-5, IL-6, IL-9 and

IL-10. In addition, IL-3, GM-CSF and TNF-α are produced by both subsets and naive Th cells produce both IL-2 and IL-4 (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). Various studies of T cell cytokine production following immunization with antigens or pathogens suggest that similar T cell cytokine patterns are also generated *in vivo*. The Th1-like or "type 1" immune response is associated with increased T cell-derived IL-2 and IFN-γ, macrophage activation and elevated IgG2a production, whereas the Th2-like or "type 2" response is associated with T cell-derived IL-4, IL-5, IL-9, and IL-10, eosinophil activation, and elevated serum IgG1 and IgE levels (Mosmann and Coffman, 1989). IFN-γ produced by Th1 cells inhibits Th2 cell development, IL-4 secretion, IgG, and IgE production, while IL-10 produced by Th2 cells can, under some conditions, inhibit Th1 cell functions (Mosmann and Moore, 1991; Mosmann and Coffman, 1989). The development of a type 1 or a type 2 immune response to a pathogen is strongly influenced by the genetic background of the individual. For example, C57BL/6 and C3H mice infected with *Leishmania major* initiate a strong host protective type 1 response with IFN-γ production and macrophage activation. These mice are able to contain the infection and, thus, can successfully rid themselves of the parasite. In contrast, BALB/c mice infected with *Leishmania major* generate a type 2 response, with high IL-4 production and serum IgE levels which is associated with a disseminated disease. They eventually succumb to the infection (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991).

Activation of naive Th cells is one of the key steps in initiating an immune response. Engagement of Th cell surface TCR with specific antigenic peptides presented

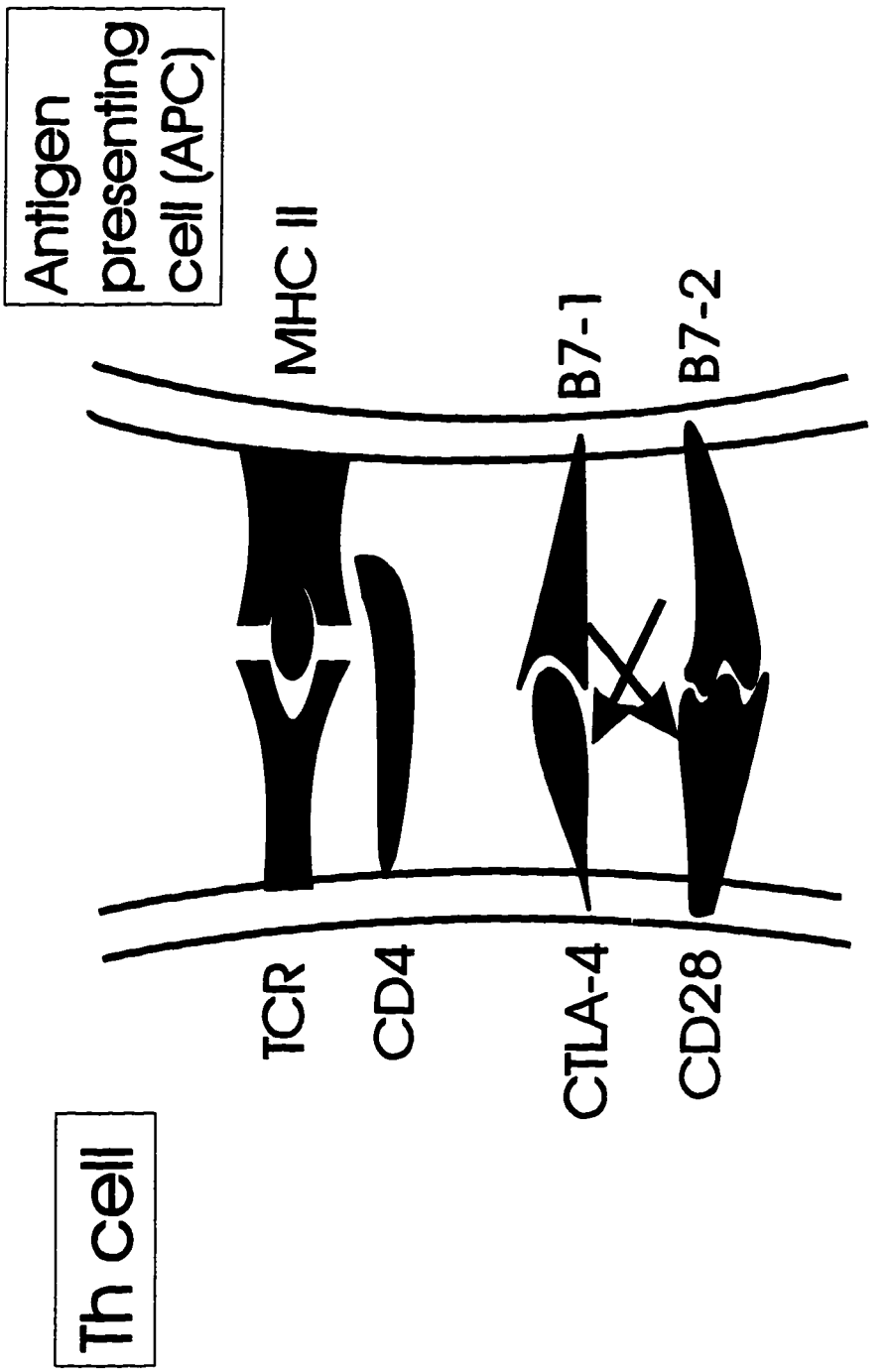
by MHC class II molecules on APCs is a primary requirement for activation. A second or "costimulatory" signal delivered by APCs through close cell-to-cell contact with Th cells that is distinct from TCR signaling is also required. Together these two kinds of signals stimulate Th cells to undergo clonal expansion and to develop into either Th1 or Th2 effector cells. Naive Th cells that lack proper costimulation undergo only partial activation and eventually these cells become unresponsive or may even undergo apoptosis (Harding *et al.*, 1992). A variety of cell surface receptor-ligand pairs between APC and Th cells have been proposed to have costimulatory functions; the best-substantiated are CD28/CTLA-4 on T cells and B7-1/B7-2 on APCs (Figure 1).

B. CD28 and CTLA-4

1. Gene and molecular structure of CD28

CD28 was first identified as the human T cell surface antigen, T90/44, that is recognized by mAb 9.3, and expressed on peripheral T cells and thymocytes. It is a disulfide-bonded homodimer with a molecular weight of 90 kDa consisting of two 44 kDa identical glycoproteins (Hansen *et al.*, 1980). Human (Aruffo and Seed, 1987), murine (Gross *et al.*, 1990), rat (Clark and Dallman, 1992), and chicken (Young *et al.*, 1994) CD28 genes have been cloned. It is a single copy gene with four exons that compasses about 36 kb in the human genome (Lee *et al.*, 1990a): Exon 1 encodes the 5' untranslated region and signal peptide, exon 2 contains most of the extracellular domain, exon 3 comprises the rest of the extracellular domain and the transmembrane region, and exon 4 encodes the intracellular domain and 3' untranslated region (Lee *et al.*, 1990a).

Figure 1. Two signals are required for naive Th cell activation, cytokine production, and effector Th cell function. For Th cell activation, the first signal is transduced through T cell receptor conjugation with an MHC class II antigen plus antigenic peptides on APC, and through CD4 co-receptor binding to MHC class II molecule. The second or costimulatory signal is hypothesized to be provided through interactions of CD28 and, perhaps also CTLA-4, with B7-1/B7-2 molecules expressed on APCs.


Antigen presenting cell (APC)
MHC II
B7-1
B7-2
TCR
CD4
CTLA-4
CD28
Th cell

The location of the CD28 gene is mapped to the q33 region of chromosome 2 in human (Dariavach *et al.*, 1988; Lafage-Pochitaloff *et al.*, 1990) and bands C of chromosome 1 in the mouse (Brunet *et al.*, 1987; Harper *et al.*, 1991). Four mRNA transcripts of CD28 have been detected in human T cells and plasma cell lines: a large pair (3.7- and 3.5- kb) and a small pair (1.5- and 1.3- kb) (Lee *et al.*, 1990b). All four mRNA transcripts are upregulated during T cell activation, but whether protein products from all these transcripts are expressed on the cell surface is still unclear (Kozbor *et al.*, 1987). An alternate spliced form of CD28 mRNA has also been found and it encodes a truncated CD28 molecule lacking most of the immunoglobin variable (IgV)-like domain (Lee *et al.*, 1990b). Analyses of the predicted amino acid sequence of CD28 genes shows that human, mouse, and rat CD28 molecules share 68% sequence identity overall (June *et al.*, 1994). Structural studies indicate that CD28 belongs to the immunoglobulin supergene family and contains an extracellular Ig V-like domain, a transmembrane domain and a short intracellular domain (Williams and Barclay, 1988).

2. Cell surface expression of CD28

CD28 is expressed on most human $CD4^+$ T cells and 50% of $CD8^+$ peripheral T cells (Yamada *et al.*, 1985) and also on plasma cells (Kozbor *et al.*, 1987). It has been reported that most human thymocytes have cytoplasmic CD28 (Zocchi *et al.*, 1990). In mice, all mature $CD4^+$ and $CD8^+$ T cells express CD28. Murine $CD4^-$, $CD8^+$ and $CD4^+$, $CD8^+$ thymocytes have two to four-fold higher surface CD28 expression than peripheral

T cells (Gross *et al.*, 1992). CD28 is also expressed on murine medullary thymic epithelial cells and dendritic cells in the cortico-medullary and medullary areas of the thymus (Verwilghen *et al.*, 1994). T cell CD28 expression can be enhanced by PMA, PHA, and anti-CD3 mAb stimulation (Lesslauer *et al.*, 1986; Baroja *et al.*, 1989). Reports showed that engagement of CD28 in activated T cells by its ligands B7-1/B7-2 transiently reduces CD28 mRNA synthesis, surface expression and signaling (Linsley *et al.*, 1993). Moreover, addition of IL-2 into culture media causes down-regulation of T cell surface CD28 expression (Shindo *et al.*, 1990).

3. Costimulatory function of CD28

The involvement of CD28 in T cell activation was discovered by findings that anti-CD28 mAb 9.3 and its Fab fragments blocked autologous mixed lymphocyte reactions (Damle *et al.*, 1981; Damle *et al.*, 1988). It also inhibits Ag-specific T cell activation (Lesslauer *et al.*, 1986), while at the same time 9.3 has strong co-stimulatory effects on the proliferative response of resting human T cells. The early contradictory results may be due to differences in surface CD28 cross-linking by 9.3 (Lesslauer *et al.*, 1986). Later studies showed that: 1) anti-CD28 mAb greatly increases T cell proliferative responses to polyclonal activators such as PMA, PHA, anti-CD3 and anti-CD2 mAbs (Gmunder and Lesslauer, 1984; Ledbetter *et al.*, 1985; Hara *et al.*, 1985; Weiss *et al.*, 1986; Pierres *et al.*, 1988; van Lier *et al.*, 1988; Moretta *et al.*, 1985); 2) induces T cell IL-2 production and surface IL-2 receptor expression in conjunction with phorbol esters

(Clark and Dallman, 1992; Brunet *et al.*, 1987); 3) prevents induction of anergy in T cell clones (Harding *et al.*, 1992); 4) augments Th1 type cytokine production through mRNA stabilization (Lindsten *et al.*, 1989). Th1 cells and freshly isolated $CD4^+$ T cells have clearly been shown to be dependent on CD28 co-stimulation for their activation, proliferation, and production of IL-2 and other cytokines (Linsley *et al.*, 1991a; Thompson *et al.*, 1989; Hans *et al.*, 1992). Whether Th2 cell activation or T cells activated during a type 2 response require CD28 costimulation is still controversial. Some reports have shown that exogenously supplied IL-1 can costimulate Th2 cell activation (Lichtman *et al.*, 1988), and that Th2 cell IL-4 production does not require CD28 co-stimulation, but that CD28 engagement increases Th2 cell proliferative responses to IL-4 (McArthur and Raulet, 1993). This increased responsiveness to IL-4 was hypothesized to occur through elevated T cell IL-2 production via CD28 costimulation (Seder *et al.*, 1994).

$CD8^+$ T cells can be activated by anti-CD3 and anti-CD28 mAbs in the presence of self APC (Carabasi *et al.*, 1991). Ligation of CD28 by monoclonal antibodies or by its ligand, B7-1, together with anti-CD3 mAb, promotes the generation of $CD8^+$ cytotoxic T cells from purified resting human T cells (Van Gool *et al.*, 1993; Azuma *et al.*, 1992b). Activation of $TCR\gamma\delta^+$ T cells also requires CD28 co-stimulation, since addition of the soluble fusion protein homolog of CD28, CTLA4-Ig, significantly inhibits the activation of $TCR\gamma\delta^+$ T cells from both the spleen and the intestinal epithelium. Anti-CD28 mAb strongly increases $TCR\gamma\delta^+$ T cell proliferation and both IFN-γ and IL-2 production when

cultured with suboptimal concentrations of immobilized anti-pan TCRγδ mAb or Ag-bearing fixed stimulator cells (Sperling *et al.*, 1993). Anti-CD28 mAb also synergizes with PMA to induce strong proliferation and IL-2 production in *lpr* and *gld* $B220^+$, $CD4^-$, $CD8^-$ functionally anergic T cells (Giese *et al.*, 1993). Stimulation through CD28 also induces proliferation of tumor-infiltrating lymphocytes from tumor tissue and enhances their CTL activity (Nijhuis *et al.*, 1990). A bispecific hybrid antibody against the pancarcinoma antigen and CD28 directs tumor specific cytotoxicity (Moller and Reisfeld, 1991). Moreover, the CD28 costimulatory pathway has been shown to be involved in superantigen-induced T cell activation (Ohnishi *et al.*, 1993; Goldbach-mansky *et al.*, 1992), thymocyte proliferation (Turka *et al.*, 1990; Lucas *et al.*, 1995) and T cell apoptosis (Punt *et al.*, 1994). Finally, reports have shown that anti-CD28 mAb inhibits NK cell proliferation, cytokine secretion, and MHC-unrestricted cytotoxicity (Azuma *et al.*, 1992a; Nandi *et al.*, 1994), suggesting that CD28-B7 costimulation may also be important for NK cell activation.

4. Cytokine production enhanced by CD28 costimulation

Although anti-CD28 mAb alone does not affect T cell cytokine production, it greatly enhances anti-CD3 mAb induced IL-2, TNFα, GM-CSF, and IFN-γ gene expression and protein secretion (June *et al.*, 1987; van Kooten *et al.*, 1991). T cell monokine production, such as IL-1α and IL-6, is also upregulated by CD28 costimulation (van Kooten *et al.*, 1991). CD28 stimulation in combination with anti-CD2 or anti-CD3

mAb not only increases IL-2 and IL-2 receptor (IL-2R) gene expression, but also prolongs the half-lives of IL-2 and IL-2R mRNAs (Cerdan *et al.*, 1992). It has been reported that the signal from CD28 costimulation induces a nuclear factor that binds to an NF-κB-like CD28 responsive element in the 5' enhancer region of the IL-2 gene and subsequently promotes IL-2 gene transcription (Verweij *et al.*, 1991). Stimulation of CD28 together with TCR signaling significantly increases the transcription of GM-CSF, IL-3 and IFN-γ promoters (Fraser and Weiss, 1992).

5. Signal transduction of CD28 costimulation

Signal transduction through CD28 stimulation is believed to be different from TCR/CD3 signaling. Stimulation through TCR/CD3 activates tyrosine kinase and phospholipase C, causes phosphatidylinositol hydrolysis, increases intracellular free calcium concentration, activates protein kinase C and elevates cytoplasmic cyclic AMP (cAMP) concentrations (Wegner *et al.*, 1992). Cross-linking CD28, however, does not increase intracellular calcium concentration or activate protein kinase C, but does elevate cytoplasmic cyclic GMP (cGMP) levels (Ledbetter *et al.*, 1987; Ledbetter *et al.*, 1986). A recent report showed that cross-linking CD28 induces intracellular calcium elevations in $CD4^+$ T cells but that the patterns of calcium mobilization are different between CD28 and TCR stimulation (Abe *et al.*, 1995b). Protein kinase C inhibitors and cyclosporin A effectively block anti-CD3 mAb-induced T cell proliferation and IL-2 secretion, but have no effects on the same events enhanced by anti-CD28 mAb (Van Lier *et al.*, 1991; Moller

and Reisfeld, 1991). Stimulation with anti-CD28 mAb plus PMA induces rapid tyrosine phosphorylation of multiple cellular substrates which is independent of TCR signaling. Recent studies demonstrate that both CD28 and CTLA-4 interact with the intracellular lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) by a specific motif within their cytoplasmic tail (Prasad *et al.*, 1994; Schneider *et al.*, 1995), and CD28/CTLA-4 ligation induces rapid increases in PI 3-kinase activity (Schneider *et al.*, 1995; Ward *et al.*, 1993). Another report showed that cross-linking CD28 increases c-jun but not c-fos mRNA synthesis and that this elevation is both protein kinase C and cytosolic calcium independent (Chatta *et al.*, 1994). Recently, a report showed that anti-CD28 mAb plus PMA induces increases in intracellular calcium concentration in activated $CD4^+$ but not $CD8^+$ T cells (Abe *et al.*, 1995b). Also, the *in vitro* activation of human $CD8^+$, but not $CD4^+$, T cells was completely inhibited by cyclosporine A (Carabasi *et al.*, 1991). These findings suggest that the signal transduction pathways through CD28 between these two T cell subsets may be different.

6. Immune responses in mice lacking CD28 costimulation

Studies of CD28-B7 costimulation have been facilitated by the generation of genetically altered mice deficient in CD28. In CD28 knockout mice, T and B cell development and phenotypes appear to be normal, but basal serum Ig levels are reduced and Ig subclass patterns were altered. In addition, T cell proliferation, IL-2 production, and cell surface IL-2R expression are reduced but cytotoxic T cell activation seemed to

be normal (Shahinian *et al.*, 1993). Although CTLA-4 is expressed at similar levels by both normal and $CD28^-$ T cells, the Ag-specific proliferative response of $CD28^-$ T cells is still markedly reduced, suggesting CD28 is the major B7-binding costimulatory ligand on T cells (Green *et al.*, 1994). Another study on CD28 knockout mice showed that CD28-deficient T cells have normal initiation of Ag-specific activation but are unable to maintain the response (Lucas *et al.*, 1995). Also, $CD28^-$ T cells proliferate poorly to the stimulation of allotype MHC antigens and superantigens with low IL-2 and IFN-γ production. However, $CD28^-$ T cells generate a strong proliferative response in secondary allotype MHC antigen stimulation with high IL-4 and IL-5 production, suggesting that CD28 costimulation may favor Th1 cell generation (Abe *et al.*, 1995a). In another model, transgenic mice that overexpress a soluble murine CTLA4-Ig protein, where *in vivo* CTLA-4-ligand interactions (including CD28-B7 interactions) are blocked, has extensive defects in B cell function such as low serum IgG isotypes, impaired T-dependent Ab response, and reduced B cell IgV region somatic mutation; however, T cell activation and cytokine production are normal (Ronchese *et al.*, 1994). These results suggested that although CD28 costimulation is important for optimal naive T cell activation, other costimulatory signals may still be utilized by naive T cells to initiate T cell activation in the absence of CD28.

7. Gene and molecular structure of CTLA-4

CTLA-4 was discovered as the fourth cDNA screened from a subtracted cDNA library of the KB5C20 murine cytotoxic T-cell clone during the search for "cytolytic T lymphocyte associated antigen" (abbreviated as "CTLA-4") (Brunet *et al.*, 1987). The genomic organization of murine and human CTLA-4 is very similar to that of CD28. They all have four exons separated by three introns and the lengths and compositions of exons are well conserved (Harper *et al.*, 1991). Human and mouse CTLA-4 genes are single copy and co-locate with the CD28 gene in both the human chromosome 2q33 and the mouse chromosome 1C (Balzano *et al.*, 1992). There are high interspecies sequence homologies between murine and human CTLA-4 (70% overall) at both the DNA and protein level, particularly in the cytoplasmic domain which contains two potential phosphorylation sites (Harper *et al.*, 1991). Like CD28, the CTLA-4 molecule is also a member of the immunoglobulin gene superfamily composed of an IgV-like extracellular domain, a transmembrane region and a cytoplasmic tail. The overall amino acid identity between CD28 and CTLA-4 is only 30% in both the murine and human genome (Harper *et al.*, 1991). CTLA-4 is expressed as a 41- to 43-kDa monomer on the surface of activated T cells, and unlike CD28, it does not have disulfide bonds to form homo- or heterodimers on the cell surface (Lindsten *et al.*, 1993).

8. CTLA-4 gene expression

CTLA-4 mRNA is undetectable in inactivated peripheral lymphocytes, NK cells, macrophages and mast cells (Brunet *et al.*, 1987). It is only co-expressed with CD28 in activated $CD4^+$ and $CD8^+$ T cells (Brunet *et al.*, 1987; Lindsten *et al.*, 1993). Two CTLA-4 mRNA transcripts have been found in PHA-stimulated peripheral blood cells: 1.8 kb and 0.8 kb (Harper *et al.*, 1991). It is still unclear whether both or just one of the mRNA transcripts are translated. PHA and PMA stimulation as well as TCR cross-linking rapidly induces CTLA-4 gene expression in T cells (Lindsten *et al.*, 1993). Peripheral blood cells exhibit peak CTLA-4 mRNA expression at 48 hr after PHA stimulation and expression begins to decline after 72 hr (Harper *et al.*, 1991). Anti-CD3 mAb induces CTLA-4 gene expression at 1 hr and peaks at 6 hr in *in vitro* cultured T cell (Lindsten *et al.*, 1993). CD28 costimulation plus TCR cross-linking strongly upregulates CTLA-4 gene expression (Lindsten *et al.*, 1993). Studies showed that protein kinase C activation was required for the CTLA-4 gene expression and that intracellular calcium mobilization also enhanced the transcription (Lindsten *et al.*, 1993).

9. Cellular expression of CTLA-4

CTLA-4 expression is undetectable on the resting T cell surface. Following T cell activation, surface CTLA-4 expression can be upregulated by several hundred-fold (Linsley *et al.*, 1992a). Both murine $CD4^+$ and $CD8^+$ T cells have surface CTLA-4

expression after anti-CD3 Ab stimulation, but $CD8^+$ T cells have higher levels of surface CTLA-4 than $CD4^+$ cells (Walunas *et al.*, 1994). Kinetic studies showed that T cell surface CTLA-4 expression is elevated at 24 hr, peaks at 48 hr, and is barely detectable at 96 hr following anti-CD3 stimulation (Walunas *et al.*, 1994). Although one report showed that $CD28^-$ T cells from CD28 knockout mice have normal surface CTLA-4 expression, another report showed that CTLA-4 surface expression on $CD28^-$ T cells is greatly reduced but can be partially restored by the addition of IL-2 (Walunas *et al.*, 1994), suggesting that IL-2 production enhanced by CD28 signaling is required for optimal CTLA-4 expression on the T cell surface. Recently, studies showed that CTLA-4 is also expressed on activated B cells (Kuiper *et al.*, 1995); yet it is still unknown if B cell surface CTLA-4 is involved in T-B cell interactions.

10. Comparison of costimulatory functions between CD28 and CTLA-4

Although the expression of CTLA-4 is much lower than CD28 on the T cell surface, CTLA-4 has a much higher affinity (20- to 100- fold) for both B7-1 and B7-2 than CD28 and is responsible for much of the B7-1/B7-2 binding on activated T cells (Linsley *et al.*, 1992a; Linsley *et al.*, 1991b). Early studies demonstrated that anti-CTLA-4 mAb costimulates the activation of anti-CD3 primed-$CD4^+$ T cells, but not as effectively as anti-CD28 mAb (Linsley *et al.*, 1992a). Anti-CTLA-4 and anti-CD28 mAbs cooperatively costimulate the proliferation of anti-TCR mAb primed-$CD4^+$ T cells, and synergistically inhibit the adhesion of activated $CD4^+$ T cells to B7 transfected CHO

cells and primary mixed lymphocyte reaction (MLR) (Linsley *et al.*, 1992a; Lindsten *et al.*, 1993). These reports indicated that CD28 and CTLA-4 have similar costimulatory functions and act cooperatively during T cell activation. Recently, differential costimulatory effects between CD28 and CTLA-4 were observed. One report showed that while blocking CD28 signaling greatly inhibits the T cell proliferative response in an allogeneic MLR, blocking CTLA-4 signaling significantly augments T cell proliferation. Also, stimulation through the CTLA-4/B7 pathway by anti-CTLA-4 mAb inhibited anti-CD3 and anti-CD28 induced T cell activation (Walunas *et al.*, 1994). Another report showed that antigen-specific $CD4^+$ T cell clonal expansion *in vivo* is enhanced by blocking CTLA-4/B7 interactions (Keamey *et al.*, 1995). Finally, cross-linking of CTLA-4 with the TCR or CD28 strongly inhibits T cell proliferation and IL-2 production (Krummel and Allison, 1995). These observations suggest that CTLA-4 may act as a negative regulator antagonizing the positive signal through CD28, so as to balance the magnitude of T cell activation and the immune response.

B. CTLA4-Ig

1. Introduction

In vivo studies of CD28 costimulation have been facilitated by CTLA4-Ig, a soluble chimeric fusion protein composed of the extracellular domain of CTLA-4 and the immunoglobulin Cγ2a chain (Linsley *et al.*, 1991b). CTLA4-Ig has a high affinity for both B7-1, B7-2 and other possible ligands on APCs and therefore prevents

CD28/CTLA-4 on T cells from binding to their ligands both *in vitro* and *in vivo* (Linsley *et al.*, 1992b). Murine or human CTLA4-Ig itself has no apparent toxic effect on animals when given *in vivo*, but murine CTLA4-Ig has higher affinity for murine B7 and a longer serum half life in mice than human CTLA4-Ig (Wallace *et al.*, 1995).

2. CTLA4-Ig and the immune response

In vivo administration of CTLA4-Ig strongly inhibits the proliferation and Ab responses of antigen-specific T cells in mice immunized with sheep red blood cells (SRBC) or a soluble protein Ag, keyhole limpet hemocyanin (KLH) (Linsley *et al.*, 1992b). Elevations of splenic IL-2 and IL-4 gene expression from both T and non-T cells are also inhibited (Ranheim and Kipps, 1993a). Likewise, secondary Ab responses are inhibited by CTLA4-Ig, although not as effectively as primary responses (Linsley *et al.*, 1992b). The inhibitory effect of CTLA4-Ig is Ag-specific; mice immunized with SRBC and treated with CTLA4-Ig are unresponsive to SRBC challenge, but they can still show a strong Ab response to KLH (Ranheim and Kipps, 1993a). The unresponsiveness is long-lasting but reversible by repetitive SRBC challenge (Ranheim and Kipps, 1993a). The Ag-specific unresponsive T or B cells from CTLA4-Ig treated mice mount a strong Ab response when they are adoptively transferred into nonlethally irradiated mice (Ranheim and Kipps, 1993a). These findings suggest that the T or B cell anergy resulting from CTLA4-Ig treatment is not complete.

3. CTLA4-Ig and transplantation

CTLA4-Ig administration induces long-term survival of human pancreatic islet grafts in mice and this tolerance is donor-specific (Lenshow *et al.*, 1992). Histological studies show that human pancreatic islets in CTLA4-Ig treated mice are devoid of any lymphocyte infiltration, while an intensive lymphocyte invasion and an extensive destruction of the islet are found in mice treated with the control fusion protein L6 (Lenshow *et al.*, 1992). CTLA4-Ig administration plus donor-specific transfusion induces long-term acceptance of cardiac allografts in mice, although CTLA4-Ig given alone only delays the rejection (Lin *et al.*, 1993). A combination of anti-CD4 mAb and CTLA4-Ig treatment induces indefinite survival of heart allografts in high responder Lewis rats and the tolerance is donor-specific (Yin and Fathman, 1995). Early high dose CTLA4-Ig treatment completely aborts the development of acute graft-versus-host disease (GVHD). Immunal abnormalities such as expansion of donor lymphocytes, increased IL-2 production, and subsequent immune deficiency are abolished (Hakins *et al.*, 1995). Other transplantation studies also showed that blocking CD28 costimulation significantly prolongs allograft survival and reduces the incidence and the lethality of GVHD (Baliga *et al.*, 1994; Blazar *et al.*, 1994; Chahine *et al.*, 1994; Tepper *et al.*, 1994).

4. CTLA4-Ig and autoimmune disease

Murine CTLA4-Ig treatment of lupus-prone NZB/NZW F1 mice blocks autoantibody formation, delays the onset of disease and prolongs life (Finck *et al.*, 1994). In rat experimental anti-glomerular basement membrane (GBM) autoimmune glomerulonephritis, CTLA4-Ig given every other day from day 0 to day 14 after bovine GBM protein immunization greatly reduces disease severity, while CTLA4-Ig is less effective when given at day 14 to day 35 (Nishikawa *et al.*, 1994). In a chronic relapsing experimental allergic encephalomyelitis (EAE) model, CTLA4-Ig treatment significantly diminishes clinical disease and IL-2 and IL-4 production by encephalitogenic T cells. CTLA4-Ig treatment during the *in vitro* activation of encephalitogenic T cells markedly reduces their proliferation and IL-2 production and attenuates the severity of the adoptively transferred EAE (Perrin *et al.*, 1995). Interestingly, another report showed that while a single injection of CTLA4-Ig at an early time point inhibits the development of EAE, repetitive injections exacerbate the disease (Abe *et al.*, 1995b). In addition, early CTLA4-Ig treatment prevents the development of diabetes in the nonobese diabetes mouse (NOD) model (Lenschow *et al.*, 1995). These reports indicate that CD28-B7 costimulation is essential for the onset and maintaining of many autoimmune diseases .

5. CTLA4-Ig and infection

CTLA4-Ig treatment during the first week of *Leishmania major* infection completely abrogates the progression of the disease in susceptible BALB/c mice. Elevations in IL-2 and IL-4 gene expression, as well as increased serum IgE and IgG1 levels are inhibited following CTLA4-Ig administration. In contrast, IFN-γ gene expression and serum IgG2a levels are unaffected. Meanwhile, CTLA4-Ig treatment does not alter the protective immune response in C57BL/6 mice. These data suggest that blocking CD28-B7 interactions during the initiation of leishmaniasis selectively inhibit the type 2 response without affecting the type 1 response (Corry *et al.*, 1994). However, because a number of cell populations besides T cells (macrophages, NK cells) mediate the immune response at an early time point and T cells become activated at a later stage (>2 wks) in leishmaniasis, it is possible that early *in vivo* CTLA4-Ig administration may not directively affect T cells.

6. Summary of the effects of CTLA4-Ig

In all of the preceding studies, CTLA4-Ig shows maximal blocking effects when it is given at the onset of the immune response or at the time of transplantation. CTLA4-Ig administration two days after allocardiac graft transplantation does not prolong graft survival (Baliga *et al.*, 1994) and CTLA4-Ig treatment does not change the cause and the severity of the adoptive transfer EAE after the transfer of myelin basic protein activated T

cells to recipient mice (Perrin *et al.*, 1995). Also, CTLA4-Ig given one week after *Leishmania major* inoculation does not protect BALB/c mice from lethal infection (Corry *et al.*, 1994). Although CTLA4-Ig effectively suppresses the development of chronic relapsing EAE when given in the initiation of the autoimmune disease and at the stage of *in vitro* encephalitogenic T cell activation, it does not affect the progression of the established EAE (Perrin *et al.*, 1995). Finally, CTLA4-Ig only blocks the development of diabetes in female NOD mice when mice are treated at an early age (<10 wk) (Lenschow *et al.*, 1995). All of these reports indicate that costimulation via CD28/CTLA-4 is essential only at the early stages of naive T cell activation. Once naive T cells are activated, CD28/CTLA-4 costimulation may no longer be required to amplify or sustain the immune responses. Similarly, memory T cell activation is also much less CD28-dependent and can be activated in vitro by anti-CD3 mAb alone without any costimulatory signals or by resting B cells or macrophages, both of which have very low surface B7 expression (Croft *et al.*, 1994).

C. B7-1 and B7-2

1. Molecular structure of B7-1

B7-1(CD80) was first discovered as a B cell surface activation antigen which appeared on activated human B cells (Freeman *et al.*, 1987). The human B7-1 gene is located on chromosome 12 (Freeman *et al.*, 1989). Sequence analysis showed that B7 is a 44 to 54 kDa glycoprotein with 262 amino acids (Freeman *et al.*, 1989). Its structure

consists of an extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic domain with only 19 amino acids (Freeman *et al.*, 1989). The extracellular domain of B7-1 has a typical IgV, C1 and C2 structure: a characteristic of the Ig superfamily (Freeman *et al.*, 1989). Gene studies indicate that B7-1 is encoded by a single gene which encompasses a large genomic region (Freeman *et al.*, 1989). Four major B7-1 mRNA transcripts are observed but the 2.9 kb band is predominant in activated B cells (Freeman *et al.*, 1989).

2. Cellular expression of B7-1

Resting B cells do not express B7-1 mRNA or surface B7-1. However, B7-1 mRNA is detectable as early as 4 h after B cell activation by anti-Ig, peaks at 12 h and decreases to undetectable levels after 48 h (Freeman *et al.*, 1989). B cell surface B7-1 expression is increased at 24 h and peaks between 48 and 72 hr after B cell activation with LPS (Freeman *et al.*, 1987). Later studies found B7-1 is also expressed on other professional APCs, such as macrophages and dendritic cells (Freeman *et al.*, 1991), as well as B cell tumors and rheumatoid synovial T cells (Verwilghen *et al.*, 1994). Recently studies have shown that some resting T cells have low level surface B7-1 and that B7-1 surface expression is upregulated following activation (Prabhu Das *et al.*, 1995). B7-1 expression on resting B cells can be upregulated by MHC cross-linking or signaling through the MHC class II cytoplasmic domain. IL-4 and IL-2 have been

reported to be able to upregulate B cell surface B7-1 expression (Valle *et al.*, 1991). Cyclic AMP is also reported to be required for B7-1 elevation (Nabavi *et al.*, 1992).

3. Costimulatory functions of B7-1

B7-1 can provide a potent costimulatory signal for naive Th cell activation. B7-1 transfected cell lines synergize with anti-CD3 mAb or lectin to activate naive T cell proliferation, IL-2 or IL-4 secretion and IL-2R expression (Gimmi *et al.*, 1991; Linsley *et al.*, 1991a; Hans *et al.*, 1992). B7-1 is also efficient at initiating responses of purified $CD4^+$ T cells to allogeneic MHC molecules (Koulova *et al.*, 1991). Adhesion of activated B cells to CD28-transfected chinese hamster overy (CHO) cells is inhibited by anti-B7-1 mAb, and B7-1 expression on B cells correlates closely with CD28-mediated adhesion (Linsley *et al.*, 1990). Anti-B7-1 mAb shows similar inhibitory effects on Th cell activation and B cell differentiation as anti-CD28 mAb (Damle *et al.*, 1991; Koulova *et al.*, 1991). Immobilized B7-1-Ig and B7-1-transfected CHO cells bind strongly to CD28 and efficiently costimulate T cell activation (Linsley *et al.*, 1991a). CHO cells co-transfected with MHC class II and B7-1 are able to induce MLR and present peptide Ags to specific T cells, while neither CHO-MHC class II nor CHO-B7-1 single transfectant has any effect (Galvin *et al.*, 1992). B7-1 also costimulates Th2 cell clone activation and IL-4 secretion (Galvin *et al.*, 1992). Rheumatoid synovial T cells expressing B7-1 can act as APCs to induce primary allogenic MLR and this response is inhibited by CTLA4-Ig addition (Verwilghen *et al.*, 1994). Furthermore, B7-1 and MHC class II-transfected

sarcoma cells induce tumor rejection in sarcoma-bearing mice (Baskar *et al.*, 1995). Studies on B7-1-deficient mice show that naive B7-1$^{-/-}$ mice have normal components of B and T cell compartments, normal proliferative response to B cell mitogen LPS and the T cell mitogen Con A, and normal serum Ig levels, but a 70% reduction in the response to alloantigen. The overall characterization of the B7-1-deficient mice indicate that the lack of B7-1 results in only a minor defect in the immune response (Freeman *et al.*, 1993a).

4. Molecular structure of B7-2

B7-2 (CD86) was discovered through the observation that APCs from B7-1-deficient mice can still bind to CTLA4-Ig and costimulate T cell proliferation which is inhibited by CTLA4-Ig (Freeman *et al.*, 1993a). Also, anti-B7-1 mAb does not block the binding of CTLA4-Ig to LPS-activated murine B cells, and mAb GL1 identifies a non-B7-1 CTLA-4 counter-receptor on activated human B cells (Hathcock *et al.*, 1993). Mature B7-2 protein has a 222 amino acid extracellular region containing Ig superfamily V and C-like domains, a transmembrane domain of 20 aa and a 44 aa cytoplasmic domain (Freeman *et al.*, 1993b). Like B7-1, B7-2 is also a member of the Ig gene superfamily. It is heavily glycosylated and has a molecular weight about 34 kDa after deglycanization. Murine and human B7-2 have 50% amino acid identity but they have only about 25% overall identity with murine and human B7-1(Freeman *et al.*, 1993b). The cytoplasmic domain of B7-2 contains three potential phosphorylation sites and shows the least identity with B7-1 (Freeman *et al.*, 1993b). The human B7-2 gene has been located on

chromosome 3, the same region as the B7-1 gene (Fernandez-Ruiz *et al.*, 1995). Two transcripts of B7-2 (3.1 and 1.8 kb) are detected in murine B cell lines and they can be upregulated at 8 hr after cAMP stimulation (Freeman *et al.*, 1993b).

5. Cellular expression of B7-2

B7-2 is expressed on multiple cell types, which include: B cells, dendritic cells, macrophages, and T cells (Hathcock *et al.*, 1994). Resting B cells exhibit low-level surface B7-2, but its expression is upregulated in less than 6 h when stimulated by IL-5, cAMP, and Con A *in vitro* (Lenschow *et al.*, 1993). Reports have shown that B cells and macrophages upregulate their surface B7-2 during *in vitro* culture without any specific stimuli and LPS stimulation can further elevate their surface B7-2 expression (Inaba *et al.*, 1994). B7-2 is also abundant on dendritic cells and accounts for most of the CTLA4-Ig binding, but in contrast to B cells and macrophages, LPS does not upregulate dendritic cell B7-2 expression (Inaba *et al.*, 1994). Resting T cells have low level B7-2 on their surface and both $CD4^+$ and $CD8^+$ T cells upregulate B7-2 expression after anti-CD3 or Con A stimulation (Prabhu Das *et al.*, 1995). *In vivo* B7-2 is also expressed on liver Kupffer cells, the interstitial cells of the heart and the lung (Inaba *et al.*, 1994). Dendritic cells in the spleen and epidermis show the highest B7-2 expression (Inaba *et al.*, 1994). The B7-2-rich regions include the base of the lamina propria, submucosa of the small intestine, thymic medulla, splenic marginal zone and periarterial sheaths, and lymph node subcapsular cortex (Inaba *et al.*, 1994).

6. Costimulatory function of B7-2

As mentioned above, B7-2 was discovered by its non-B7-1-mediated costimulatory function and CTLA4-Ig binding ability on APCs (Freeman *et al.*, 1993a; Hathcock *et al.*, 1993). The costimulatory effects of B7-2 are inhibited by CTLA4-Ig and anti-B7-2 mAb GL-1, but not by anti-B7-1 mAb (Freeman *et al.*, 1993b). COS cells transfected with B7-2 induce strong T cell proliferation and IL-2 production. Both murine and human B7-2 are able to costimulate either murine or human T cell proliferation (Freeman *et al.*, 1993b). Anti-B7-2 mAb substantially inhibits primary allogeneic MLR and blocks CTLA-4 binding (Azuma *et al.*, 1993).

7. Comparison of B7-1 and B7-2 distribution and function

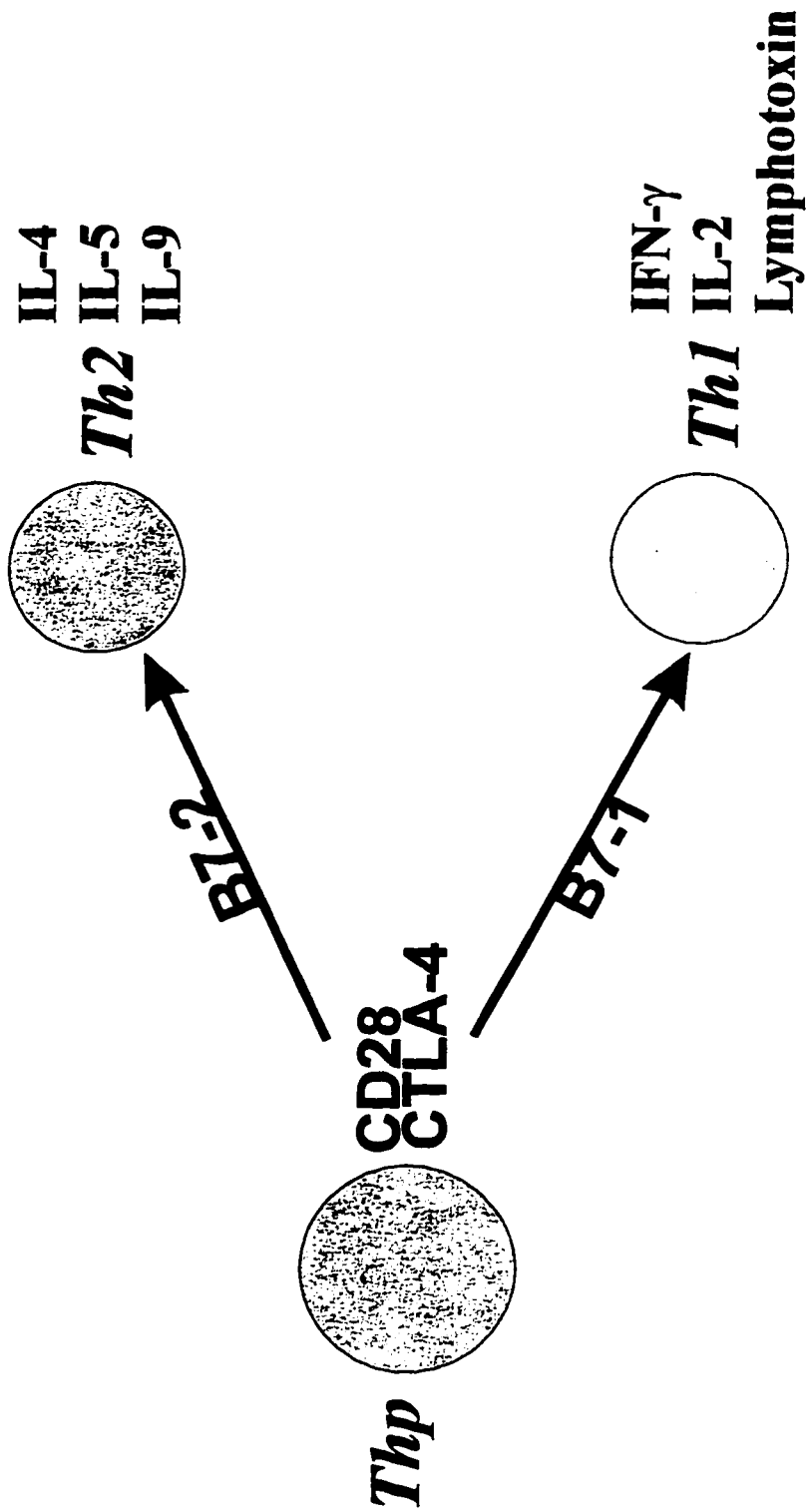
Several reports suggest that the costimulatory signals provided by B7-1 and B7-2 are redundant. Both B7-1 and B7-2 are co-expressed on a variety of APCs, including activated B cells, macrophages, and dendritic cells (Hathcock *et al.*, 1994). B7-1 and B7-2 have similar binding affinity to CD28 or CTLA-4, but CTLA-4 binds B7-1 and B7-2 100-fold better than CD28 (Linsley *et al.*, 1994). Early studies showed that these two ligands provide similar costimulatory signals for T cell activation, cytokine production and generation of CTL (Lanier *et al.*, 1995). Both anti-B7-1 and anti-B7-2 Abs are required to optimally block CD28-dependent T cell proliferation (Lanier *et al.*, 1995). However, the difference in the temporal expression of B7-1 and B7-2 on APCs raises

some doubt as to whether these two molecules serve identical functions. Only B7-2 is constitutively expressed on dendritic cells, macrophages and resting B cells *in vivo* (Hathcock *at al.*, 1994) and B7-2 surface expression is upregulated 24 hr earlier than B7-1 after B cell activation by LPS (Inaba *et al.*, 1994). Moreover, B7-2 expression is > 10-times higher than B7-1 on activated B cells and dendritic cells and thus may account for most of the CTLA-4/CD28 ligand activity (Hathcock *et al.*, 1994). It is also reported that expression of B7-1 and B7-2 is differentially regulated as GM-CSF stimulation increases both B7-1 and B7-2 expression whereas IFN-γ increases B7-2, but decreases B7-1 expression on splenic dendritic cells (Larsen *et al.*, 1994). Binding studies showed B7-1 and B7-2 bind on different sites of the CTLA-4 molecule. Y100A, a mutate form of CTLA4-Ig with a mutation in B7-1 and B7-2 binding site, binds B7-1 200-times stronger than B7-2 (Linsley *et al.*, 1994). Kinetics of B7-1 and B7-2 binding to CTLA-4 are also different. B7-2 has a faster rate of dissociation from CTLA-4 than B7-1 (Linsley *et al.*, 1994).

Some recent B7-1 and B7-2 functional studies have suggested that the two ligands deliver different costimulatory signals to modulate naive T cell activation. Mice transgenically overexpressing B7-1 on their B cells exhibit reduced antigen-specific T-dependent antibody production, and blocking B7-1 signaling by administration of anti-B7-1 mAb restores this response (Sethna *et al.*, 1994). Reports on the murine experimental allergic encephalomyelitis (EAE) model, which is induced by autoreactive Th1 cells, showed that anti-B7-1 mAb treatment reduces the incidence of EAE by

increasing IL-4- producing Th2 cell frequency, while anti-B7-2 mAb treatment exacerbates the disease (Kuchroo *et al.*, 1995). This result has been interpreted to mean that costimulation through B7-1 preferentially promotes Th1 cell generation while B7-2 signaling enhances the development of Th2 cells (Figure 2) (Kuchroo *et al.*, 1995). Another study on EAE also showed that while a single anti-B7-1 mAb treatment greatly inhibits the onset of clinical disease as does the combination of anti-B7-1 and anti-B7-2 mAbs, the injection of anti-B7-2 mAb increases the severity of the disease (Abe *et al.*, 1995b). In addition, neither anti-B7-1 nor anti-B7-2 mAb alone inhibits the proliferative response of encephalitogenic T cells *in vitro*, although the combination of both mAbs blocks proliferation (Abe *et al.*, 1995b). Another *in vitro* study on the activation of anti-CD3 mAb primed $CD4^+$ T cells demonstrated that both B7-1 and B7-2 are equivalent in inducing IL-2 and IFN-γ production and IL-2R expression. B7-2 costimulation also induces the production of another Th1 cytokine, TNF-β (Freeman *et al.*, 1995). Although B7-2 induces higher levels of IL-4 production than B7-1 in anti-CD3 mAb stimulated naive $CD4^+$ T cells, both B7-1 and B7-2 costimulate IL-4 production in memory $CD4^+$ T cells (Freeman *et al.*, 1995). Reports from the treatment of murine autoimmune diabetes in the nonobese diabetic (NOD) mouse show that CTLA4-Ig and anti-B7-2 mAb administration effectively prevents the development of diabetes in female NOD mice, whereas anti-B7-1 mAb or the combination of anti-B7-1 and anti-B7-2 mAb treatment accelerates the disease. Furthermore, anti-B7-1 mAb induces diabetes in normally resistant male NOD mice (Lenschow *et al.*, 1995).

Figure 2. T cell differentiation towards Th1 or Th2 cytokine production may be influenced by whether B7-1 or B7-2 is interacting with CD28/CTLA-4. In this model, costimulation through B7-1 favors a type 1 response while costimulatory signaling through B7-2 favors a type 2 response.


IL-4
IL-5
IL-9
Th2
IFN-γ
IL-2
Lymphotoxin
Th1
B7-2
B7-1
CD28
CTLA-4
Thp

Although contradictory, these reports suggest that B7-1 and B7-2 may have distinct costimulatory properties and play different roles in T cell activation and Th cell differentiation depending on the particular immune response.

D. Other molecules and receptor-ligands that deliver putative costimulatory signals for T cell activation

A third putative ligand for CD28/CTLA-4 was recently reported to be expressed on activated B cells and was named B7-3 (Boussiotis *et al.*, 1993); as yet, B7-3 has not been cloned. Besides CD28-B7 costimulatory ligand, a variety of other surface receptor-ligands on T cells and APCs have been reported may exert costimulatory functions. Anti-intercellular adhesion molecule-1 (ICAM-1) mAb has been shown to enhance both resting and Ag-primed T cell proliferation (Damle *et al.*, 1992b). ICAM-2, a second counter-receptor for CD11a/CD18 (Damle *et al.*, 1992a), CD44 (Huet *et al.*, 1989), heat-stable antigen (Liu *et al.*, 1992), and vascular cell adhesion molecule-1 (VCAM-1) (Damle *et al.*, 1993) have all been reported to have a costimulatory effect on $CD4^+$ T cells. Likewise, CD43 has also been suggested to be a T cell costimulatory molecule and it functions independently of CD28 (Sperling *et al.*, 1995). Recently, another receptor-ligand pair CD40-CD40L has also been reported to be able to deliver costimulatory signals for both T and B cell activation (Fanslow *et al.*, 1994b). Some cytokines are also assumed to have costimulatory functions. Studies have shown that IL-6 strongly augments the proliferation of anti-TCR mAb stimulated purified T cells (Garman *et al.*,

1987) and IL-1 acts synergistically with IL-6 to promote T cell activation and IL-2 production in the same system (Holsti and Raulet, 1989).

E. CD40 and CD40 ligand

1. Molecular structure of CD40

CD40 and CD40L are another important costimulatory receptor-ligand pair involved in the initiation of an immune response. CD40 was first discovered as a 48 kDa glycoprotein expressed on Burkitt's lymphoma and normal B cells (Paulie *et al.*, 1985). The human CD40 gene is mapped to chromosome 20q11-2-q13-2 (Lafage *et al.*, 1994). Its complete structure consists of 277 aa with a 193 aa extracellular domain, a 22 aa transmembrane domain, and a 62 aa intracellular domain (Stamenkovic *et al.*, 1989). Homology studies of CD40 showed that it belongs to the tumor necrosis factor receptor superfamily, which share a common domain with tandemly repeated cysteines in their cytoplasmic tails (McDonald and Hendrickson, 1993). Human and murine CD40 molecules have high sequence homology: 62% aa identity in the extracellular domain and 78% identity in the cytoplasmic domain (Torres and Clark, 1992).

2. Cellular surface expression of CD40

CD40 is expressed on B cells at all developmental stages and was initially identified as a "pan-" B cell antigen (Ling *et al.*, 1987). CD40 is also expressed on all chronic lymphocytic leukemia B cells, non Hodgkin's lymphoma cells and EBV-

transformed B cell lines (Paulie *et al.*, 1985). Dendritic cells isolated from peripheral blood and in the T cell-rich areas of peripheral lymph nodes have high surface CD40 expression (Ling *et al.*, 1987). Moreover, a variety of cell types on many different tissues, such as medullary thymic epithelial cells (Galy and Spits, 1992a), endothelial cells (Moller and Mielke, 1989), follicular dendritic cells (Schriever *et al.*, 1989), activated monocytes (Galy and Spits, 1992a), basophils (Valent *et al.*, 1990), and even some T cells have surface CD40 expression (Hasbold *et al.*, 1994).

3. Molecular structure of CD40-ligand

CD40-ligand (CD40L, gp39) was identified by the binding of CD40-Ig fusion protein with EL-4 thymoma cells (Hansen *et al.*, 1980). The CD40L cDNA was thus isolated and found to encode a 260 aa polypeptide, consisting of a 214 aa extracellular domain, a 24 aa transmembrane domain and a 22 aa cytoplasmic tail (Armitage *et al.*, 1992). CD40L belongs to the TNF cytokine superfamily. Human and murine CD40L have 78% aa identity (Banchereau *et al.*, 1991).

4. Cells expressing CD40 ligand

CD40L is primarily expressed on activated mature $CD4^+$ Th cells, although a small population of $CD8^+$ T cells also have surface CD40L after activation (Armitage *et al.*, 1992; Burdin *et al.*, 1993). Although resting Th cells do not express CD40L, its expression is upregulated shortly (1-2 hr) after Th cell activation (Burdin *et al.*, 1993).

CD40L is also expressed on EL-4 thymoma cells, human thymocytes, eosinophils, lung mast cells and blood basophils (Burdin *et al.*, 1993; Clark and Shu, 1990).

5. Functions of CD40-CD40L interaction

Most of the functions associated with CD40-CD40L interactions involve B cells. Cross-linking of CD40 with anti-CD40 mAb or cells expressing CD40L induces DNA replication, cell size increase, and upregulation of surface MHC class II and B7 expression on resting B cells (Defrance *et al.*, 1992; Durie *et al.*, 1994a; Foy *et al.*, 1994; Ganchat *et al.*, 1993). Stimulation through CD40 alone does not promote B cell multiplication and Ig secretion. However, B cells cultured with anti-CD40 mAb plus IL-4 or IL-10 results in sustained proliferation (Gordin *et al.*, 1988), development of memory B cells (Foy *et al.*, 1994), and multiple Ig isotype secretion (Graf *et al.*, 1992; Hermann *et al.*, 1993). CD40 cross-linking also induces B cell IL-6 and IL-10 production (Lederman *et al.*, 1992; Macchi *et al.*, 1995). Anti-CD40L (anti-gp39) mAb together with IL-4 prevents B cell apoptosis due to surface IgM or IgD hypercross-linking (Renshaw *et al.*, 1994).

Both cytokines produced by activated T cells and T-B cell contacts are required for T cell-dependent B cell activation. The functional properties of CD40-CD40L interactions indicate that this receptor-ligand pair plays a key role in the process (Mohan *et al.*, 1995).. Reports showed that the CD40-Ig fusion protein strongly blocks B cell activation induced by activated T cells and their membranes (Noelle *et al.*, 1992).

Moreover, anti-CD40 mAb markedly inhibits tonsillar B cell proliferation and Ig secretion when co-cultured with anti-CD3-activated T cells (Notrarngelo *et al.*, 1992). Stimulation through CD40 also activates monocytes, dendritic cells, and thymic epithelial cells to secrete certain cytokines, suggesting that CD40-CD40L interactions also affects other cell populations besides T and B cells (Wagner *et al.*, 1994; Caux *et al.*, 1994; Galy and Spits, 1992b).

6. *In vivo* functional studies of CD40-CD40L interaction

In vivo experiments demonstrated that CD40L expression on activated $CD4^+$ helper T cells is required for the generation of a specific Ab response to T-dependent Ags. Mice treated with anti-gp39 mAb show diminished antibody production in both primary and secondary immune responses to T-dependent Ag, but not to T-independent Ag (Parker, 1993). Germinal center formation and antigen-specific memory B cell generation are inhibited in anti-gp39 mAb treated mice (Parker, 1993). Short term treatment of lupus-prone NZB mice with anti-gp39 mAb results in a long lasting reduction of serum autoantibody levels and marked delays in the occurrence of lupus nephritis (Parry *et al.*, 1994). Anti-gp39 mAb also blocks autoantibody production in chronic and acute graft-versus-host-disease (GVHD) and the inhibitory effect extends long after the termination of anti-gp39 mAb administration (Ranheim and Kipps, 1993a). Recently, defects in functional CD40L have been shown to be responsible for a rare human congenital immunodeficiency disorder: X-linked hyper-IgM (HIM) syndrome.

Patients with HIM have increased serum IgM but very low levels of other Ig isotypes and without germinal center formation. They are unable to initiate T cell-dependent Ab responses to produce other Ig isotypes, except IgM, and to generate memory B cells. HIM patients are very susceptible to opportunistic bacterial infections (Rousset *et al.*, 1991). Activated T cells from HIM patients either lack CD40L expression or express mutated CD40L that does not bind CD40. Genetic studies have shown a variety of mutations and deletions on the CD40L gene in these patients, resulting in frameshift and/or premature termination of CD40L mRNA (Splawski *et al.*, 1993). CD40L gene knockout mice exhibit similar defects as in HIM patients, although they do not show elevated serum IgM levels (Valle *et al.*, 1989). These reports clearly show that CD40-CD40L interactions are essential for the initiation of T-dependent B cell activation, Ig isotype switching, Ag specific antibody production and memory B cell generation.

7. Costimulatory function of CD40-CD40L interaction on T cells

Recent studies indicate that CD40-CD40L interactions exert potent costimulatory effects on T cells. CD40L, together with a suboptimal concentration of Con A, induces the proliferation of resting $CD4^+$ and $CD8^+$ T cells, increases in T cell surface IL-2R and CD69 expression and IL-2 production (Fanslow *et al.*, 1994b). CD40 expressed on cell transfectants substantially increases anti-CD3 induced $CD4^+$ T cell proliferation and the generation of cytotoxic T cells. Both naive and memory T cells are equally responsive to CD40 stimulation (Cayabyab *et al.*, 1994). It is also reported that CD40L promotes

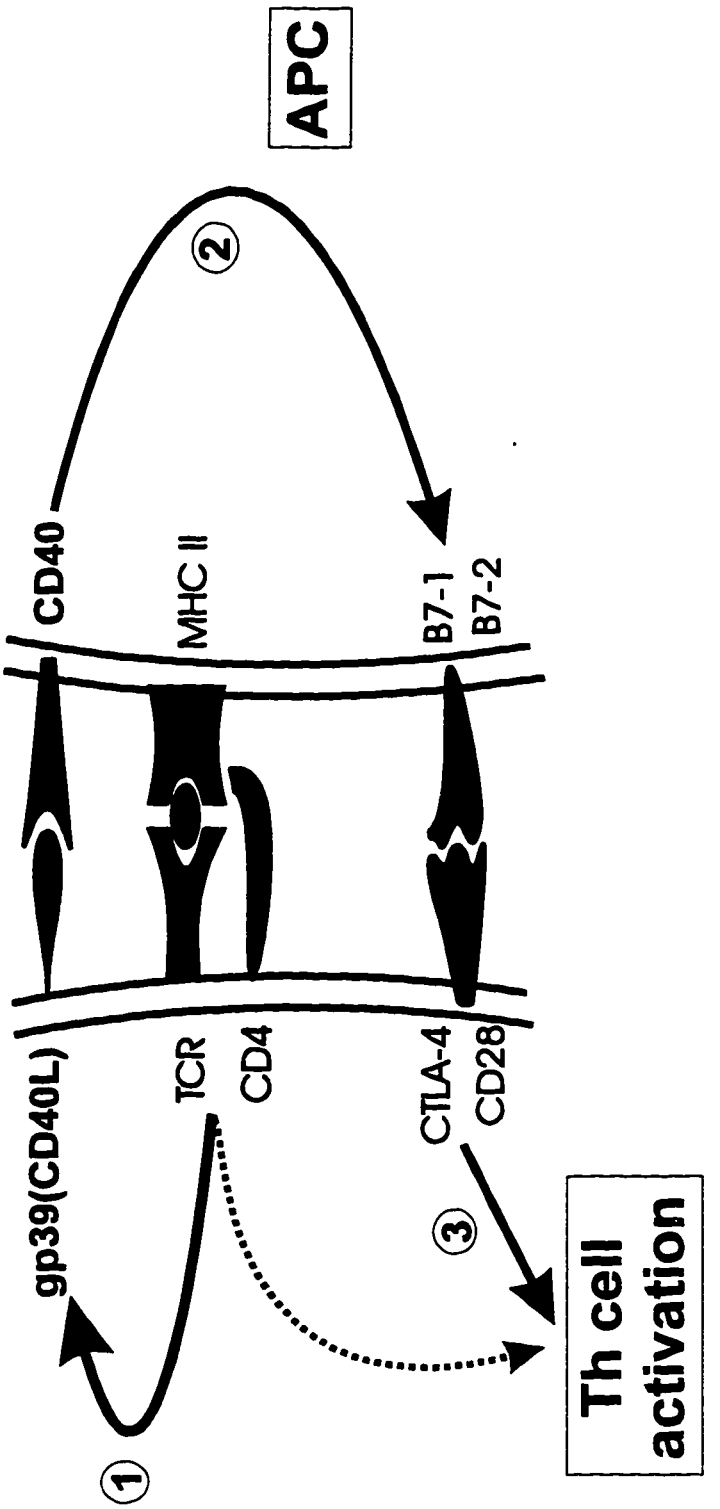
TCR$\gamma\delta^+$ T cell activation (Ramsdell *et al.*, 1994). All these observations are based on *in vitro* cell culture studies; whether CD40-CD40L is involved in T cell activation *in vivo* is unclear. Studies on chronic GVHD and acute GVHD models show that anti-gp39 mAb administration blocks allogeneic CTL activation (Ranheim and Kipps, 1993a), whereas results from murine lupus studies clearly show autoreactive T cells are not affected by anti-gp39 mAb treatment (Parry *et al.*, 1994).

8. The interdependence of expression and function between CD40-CD40L and CD28-B7 costimulatory ligand pair

Both CD40-CD40L and CD28-B7 receptor-ligand pairs actively participate in T-B cell mutual activation during the initiation of an antigen-specific immune response. To activate naive T cells, efficient costimulatory signals through CD28/CTLA-4 are maintained by upregulation of B7-1/B7-2 expression on activated B cells. Activated T cells increase surface CD40L expression and promote B cell activation through binding to B cell surface CD40. Recent evidence has shown an interdependence of expression and function between these two receptor-ligand pairs. Stimulation through CD28 increases T cell surface CD40L expression and enhances T cell-dependent B cell activation (Klaus *et al.*, 1994). Cross-linking CD40 on B cell and dendritic cell surfaces by anti-CD40 mAb promotes B7-1/B7-2 expression (Ranheim and Kipps, 1993a; Caux *et al.*, 1994). Addition of anti-gp39 mAb strongly inhibits the increase of B7-1 expression and partially blocks B7-2 upregulation, and the combination of anti-gp39 and anti-IL-4 mAbs totally

blocks Ag-induced B7-2 expression on B cells *in vitro* (Roy *et al.*, 1995). In contrast, another report showed that CD40L elevation on activated T cells is not affected by the addition of CTLA4-Ig or anti-B7-1&-B7-2 mAbs, but is blocked by anti-MHC class II mAb (Roy *et al.*, 1995), suggesting that CD40-CD40L interactions may function earlier than CD28-B7. Currently, two hypotheses have been proposed for the reciprocal regulation of CD40-CD40L and CD28-B7 expression during an Ag-specific immune response. The first proposed scenario is that TCR engagement by the antigenic peptide plus MHC class II Ag upregulates T cell CD40L expression, which binds to B cell surface CD40 and triggers B cell activation and increased surface B7-1/B7-2 expression. The subsequent CD28-B7 interaction between T and B cells provides the necessary costimulatory signals for T cell activation and differentiation (Figure 3) (Durie *et al.*, 1994b). A similar sequence of events may also occurs with other $CD40^+$ APCs. An alternative proposal is that the initial TCR signaling and CD28-B7 costimulation is enough to trigger T cell activation without the requirement of CD40-CD40L interactions. Activated T cells upregulate CD40L expression and then promote B cell activation. Increased B7-1/B7-2 expression on activated B cells subsequently amplifies T cell proliferation. Thus, CD40-CD40L interactions are not required for initial T cell activation but may be important in maintaining the T cell response (Hollenbaugh *et al.*, 1994). Currently, there is little evidence from *in vivo* studies to support either of these hypotheses. Functions of CD40-CD40L interaction on other CD40 or CD40L expressing cell types, such as eosinophils and mast cells, are still under investigation.

Figure 3. T cell activation leading to cytokine production may involve CD40-CD40L interactions as well as interactions between CD28/CTLA-4 and B7. In one model, TCR signaling alone may rapidly upregulate CD40L which, upon ligating CD40 on APCs, stimulates increased expression of B7-1 and/or B7-2. B7 molecules then trigger CD28/CTLA-4 costimulation and T cell activation. Alternatively, CD40L may not be a major player at this early stage of the response because B7 molecules are either already expressed at sufficient levels to mediate costimulation or are upregulated by other factors.


APC
CD40
MHC II
B7-1
B7-2
gp39(CD40L)
TCR
CD4
CTLA-4
CD28
Th cell activation
1
2
3

F. The mouse immune response induced by foreign anti-mouse IgD antibody administration *in vivo*.

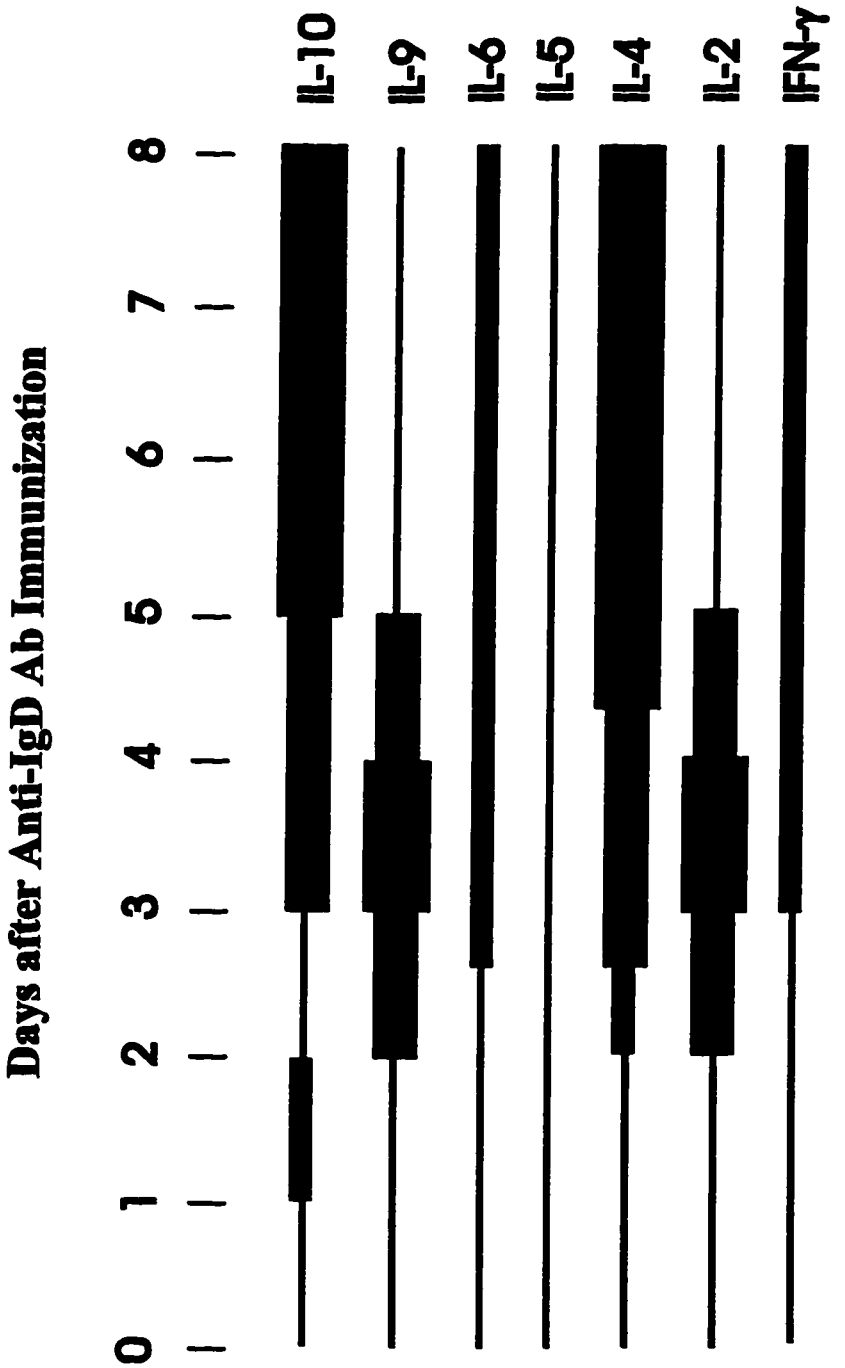
In vivo administration of the foreign anti-mouse IgD antibody induces a strong polyclonal activation of the murine immune system. Foreign anti-mouse IgD Ab binds to mouse B cell surface IgD molecules, and through cross-linking, induces polyclonal B cell activation. Activated B cells internalize the antibody, process it, and present the antigenic peptides on the B cell surface conjugated with MHC class II (Ia) molecules (Finkelman *et al.*, 1982b). T cells with TCR specific for these peptides are subsequently activated, proliferate, secrete cytokines, and, in turn, promote further B cell proliferation and antibody production. Two distinct waves of lymphocyte proliferation have been observed during the *in vivo* immune response: the initial T-independent B cell proliferation and a secondary T and B cell proliferation which is T-dependent (Finkelman *et al.*, 1982b; Muul *et al.*, 1983). In the first wave of B cell activation, increases in splenic B cell size and DNA synthesis begin at 24 hr. Nearly all splenic B cells are activated and surface Ia expression is increased by day 2 after anti-IgD Ab administration (Finkelman *et al.*, 1982b; Cambier and Monroe, 1984). Splenic T cell DNA synthesis begins to increase by day 2 and T cell surface IL-2R expression is elevated after day 3 (Finkelman *et al.*, 1986b). Serum IgE levels increase ~100 fold and significant increases in serum IgG1, IgG2a, and IgG3 are detectable by day 8 (Finkelman *et al.*, 1982a; Finkelman *et al.*, 1987). A sequential elevations on cytokine gene expression in the spleen in anti-IgD Ab immunized mice have been showed. IL-2 and IL-9 gene expression are elevated by day 2

but decreased after day 4. Marked increases in IL-4 and IL-10, as well as a smaller elevations in IFN-γ gene expression, can be detected by day 3 and these elevations are sustained until day 8 (Figure 4) (Svetic' *et al.*, 1991). Thus, the type 2 response predominates during the later stage of this response since the Th2 cytokines, IL-4 and IL-10, and serum IgE and IgG1 are particularly elevated. Studies on sorted splenic cells on day 3 and day 5 after anti-IgD mAb immunization have shown that $CD4^+$ T cells are the only source of increased Th2 cytokine gene expression, with the exception of IL-6 (Svetic' *et al.*, 1991).

G. The mouse immune response to *Heligmosomoides polygyrus* inoculation

Heligmosomoides polygyrus (HP) is a common rodent parasite and has a strict enteral life cycle in mice. When infective HP third-stage larvae are ingested, they invade the gut mucosa within 24 hr, molt twice in 8 days, then return to the intestinal lumen as adult worms and persist there for several weeks (Bryant, 1973). Mice infected with HP mount an effective secondary immune response that results in quick worm expulsion and the prevention of repeated infections (Cambier and Monroe, 1984). HP-infected mice show significant elevations of serum IgE (over 100 folds) and eosinophilia by day 14 (Urban, Jr. *et al.*, 1991). A highly consistent sequential elevation in cytokine gene expression in the mesenteric lymph node (MLN) has been observed in HP-infected BALB/c mice in previous studies in our lab (Figure 5). IL-3, IL-5, and IL-9 gene

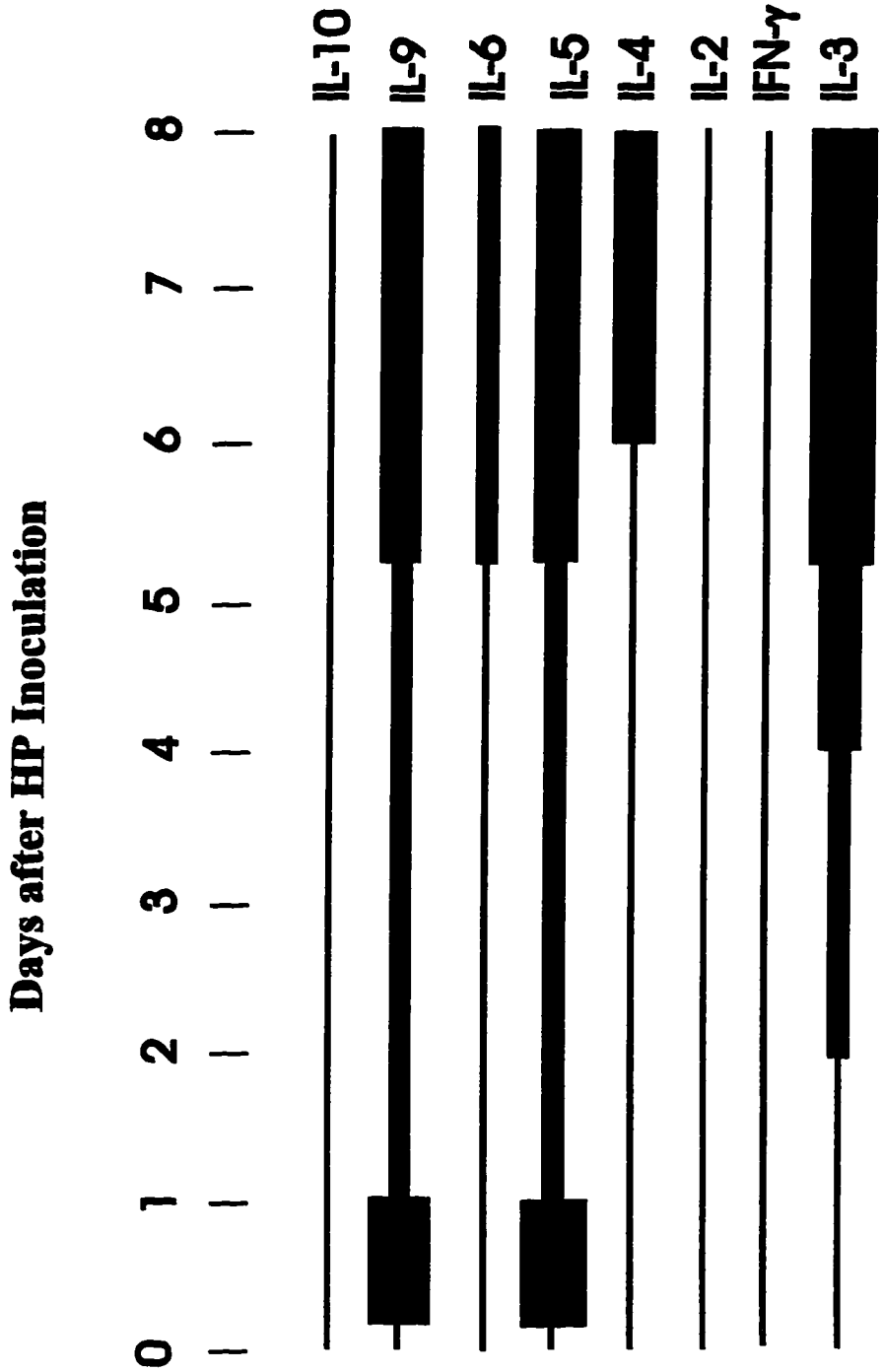
Figure 4. Kinetics of elevations of cytokine gene expression in spleen during the course of the immune response to foreign anti-mouse IgD Ab in BALB/c mice. Mice (5 per group) were immunized with 800 μg Goat-anti-mouse IgD (GaMIgD) antibody *i.v.* at day 0. Spleen samples were collected at each day after immunization until day 8 for kinetic study. Cytokine gene expression was investigated by a quantitative RT-PCR, and was quantitated on a PhosphoImager (Materials and Methods). All data were individually normalized to the internal gene expression standard, HPRT, and the mean of each cytokine gene expression was derived from five individual mice in each group. The mean of a specific cytokine gene expression through the time course was graphed as the width of the band, which generally reflects the relative intensity (Svetic, *et al.*, 1991).


Days after Anti-IgD Ab Immunization
0
1
2
3
4
5
6
7
8
IL-10
IL-9
IL-6
IL-5
IL-4
IL-2
IFN-γ

expression are elevated very early after HP inoculation but reduced by day 3 (Svetic *et al.*, 1993b). These early elevations in cytokine mRNA are believed to be derived from non-T/non-B cells because similar elevations are detected in T cell-depleted BALB/c mice, T cell deficient nude and T/B cell deficient SCID mice (Svetic *et al.*, 1993b). IL-4 gene expression is elevated by day 2, peaks by day 8 and continues until day 10. A second peak of IL-5 gene expression starts at day 5 and continues until day 10. IL-2, IFN-γ, and IL-10 gene expression are not elevated during the response. A similar sequential elevations in cytokine gene expression have also been shown in the Peyer's patch (PP) (Svetic *et al.*, 1993b). Unlike anti-mouse IgD immunization, elevations in cytokine gene expression induced by HP inoculation are limited to the PP and the MLN, indicating that the immune response is localized to the gut mucosal region (Svetic *et al.*, 1993b). Cell sorting studies also show that the increases in IL-4 gene expression are exclusively from $CD4^+$ T cells (Svetic *et al.*, 1993c). IL-4 production is important for serum IgE and IgG1 elevations and for protective immunity, whereas IL-5 is only responsible for HP-induced mucosal eosinophilia (Urban *et al.*, 1991; Urban, Jr. *et al.*, 1991). Overall, previous investigations showed that HP inoculation in BALB/c mice induces a strong type 2 mucosal immune response.

Although these two models are both type 2 dominant *in vivo* immune responses, many characteristics of these two responses are different. Foreign anti-IgD Ab is a homogeneous, soluble and T-dependent antigen, while HP is a large whole worm with a variety of distinct T-dependent and T-independent antigens. The APCs in the anti-IgD

Figure 5. Kinetics of cytokine gene expression in the MLN during the course of the immune response to HP. Mice (5 per group) were orally infected with 200 third stage HP larvae at day 0. For kinetic study of cytokine gene expression in the MLN during the immune response to HP, MLN samples were collected each day after HP inoculation till day 8. A quantitative RT-PCR was performed to study the cytokine gene expressio in the MLN and data was quantitated on a PhosphoImager (Materials and Methods). All data were individually normalized to the internal gene expression standard, HPRT, and the mean of each cytokine gene expression was derived from five individual mice in each group. The mean of a specific cytokine gene expression through the time course was graphed as the width of the band, which generally reflects the relative intensity.


Days after HP Inoculation
0
1
2
3
4
5
6
7
8
IL-10
IL-9
IL-6
IL-5
IL-4
IL-2
IFN-γ
IL-3

model are activated B cells in which surface costimulatory molecules such as B7-1, B7-2, and CD40 expression are upregulated. In contrast, macrophages and dendritic cells in the PP and the MLN are most likely the APCs in anti-HP response. Also, the anti-IgD response is systemic, whereas the anti-HP response is localized to the intestinal mucosa region. Elevations in cytokine gene expression are also different between these two models. The similarities and differences between these two immune response models are useful in the investigation in T cell co-stimulation, activation, and differentiation studies *in vivo*. Both of the immune responses are strong, consistent, and develop rapidly. Together they can be used to identify common and unique pathways of T cell costimulation and differentiation *in vivo*.

H. Specific aims of the project

The first part of this project investigates whether B7-dependent costimulation is required for *in vivo* naive T cell activation and generation of the type 2 immune response in both the anti-foreign IgD and HP inoculation models. In particular, the effects of blocking CTLA-4 ligand costimulation on the generation of IL-4 producing $CD4^+$ T cells are investigated. The second part of the project examines the possibility of differential signaling between B7-1 and B7-2 during the activation of naive Th cells and their maturation into Th2 cytokine production. Finally, the role of another costimulatory receptor-ligand pair, CD40-CD40L, and the interdependence of B7- and CD40-signaling during the *in vivo* type 2 immune response is examined.

II. Materials and Methods

Animals. BALB/c female mice were purchased from the Small Animal Division of the National Institutes of Health, Bethesda, MD., and were used at age 8-12 weeks. The experiments were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animals, National Research Council, Department of Health, Education and Welfare (NIH) 85-23.

Antibodies. Antibodies and reagents used for the *in vivo* experiments and for FACS analysis included: anti-CD4 (GK1.5) (Dialynas *et al.*, 1983), anti-B220 (6B2) (Morse *et al.*, 1982), anti-IL-2 receptor (7D4) (Cheever *et al.*, 1993), anti-MHC class II (Ia^d, MKD6) (Kappler *et al.*, 1981), anti-IL-4 receptor (M1) (Finkelman *et al.*, 1991), rat IgG2a (GL117) and anti-FcγRII (24G2) (Unkeless, 1979) antibodies were the gifts of Dr. F.D. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD). Anti-TCRα/β (H57-597) antibody was purchased from Pharmingen, (San Diego, CA). Anti-B7-1 (1G10) (Xu *et al.*, 1994), anti-B7-2 (GL-1) (Hathcock *et al.*, 1994) were the gift of Dr. P.J. Perrin (Naval Medical Research Institute, Bethesda, MD). For anti-mouse IgD Ab immunization, goat-anti-mouse IgD Ab (GaMIgD) and rat-anti-mouse IgD mAb $H\delta^a1$ (Zitron and Clevinger, 1980) and FF1-4D5 (Sawada *et al.*, 1993) were the gifts of Dr. F.D. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD) (Finkelman *et al.*, 1982a; Finkelman *et al.*, 1987).

Armenian hamster IgG anti-gp39 antibody (MR1) hybridoma cell line was the gift of Dr. R.J. Noelle (Yale University, New Haven, CT) (Roy *et al.*, 1993). Syrian hamster IgG was used as the control antibody in all experiments because control Armenian hamster polyclonal or monoclonal IgG is not availabl. For the ELISPOT assay, anti-IL-4 antibodies BVD41D112 and BVD6.24g2.3 (Chatelain *et al.*, 1992), anti-IL-5 antibodies TRFK-4 and TRFK-5 (Abrams *et al.*, 1992), and anti-IL-10 antibodies SXC2 (Mosmann *et al.*, 1990) and SXC1 (Mosmann *et* al., 1990) were kindly provided by Dr. F.D. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD).

Chemicals and reagents. RPMI-1640 and HBSS media were purchased from BioWhittaker (Walkersville, MD); fetal bovine serum (FBS) was purchased from HyClone (Logan, UT); sodium azide, 2-amino-2-methyl-1-propanol, Tween 20, mineral oil, 5-bromo-4-chloro-3-indolyl-phosphate and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO); immunolon II plates were purchased from Dynatech Laboratories, Inc. (Chantilly, VA); Seaplaque agarose was from FMC BioProducts (Rcokland, ME); streptavidin alkaline phosphatase was from Jackson Immuno Reseach Lab Inc. (West Grove, PA); pyrantel pamoate was purchased from Strongid T, Pfizer Diagnostics (New York, NY); RNAzol was purchased from Cinna/biotecx (Friendswood, TX); superscript RT was purchased from Life Technologies (Gaithersburg, MD); Taq polymerase was purchased from Promega (Madison, WI); gamma ATP-^{32}P was purchased from ICN (Irvine, CA); T4 kinase and dNTP set were

purchased from Pharmacia Biotech (Piscataway, NJ); human CTLA4-Ig fusion protein and human chimeric mAb L6 as well as murine CTLA4-Ig and the control murine L6 were provided by Dr. P.S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) (Linsley et al., 1991b)(Wallace et al., 1994). Cellect^{TM} plus CD4 immunocolumns were purchased from Biotex Lanoratories Inc. (Edmonton, Alberta, Canada).

Immunization of mice with anti-IgD antibody. Individual mice were injected *i.v.* via the tail vein with 800 μg of affinity purified GaMIgD in 100 ml 0.15 M NaCl (Svetic' *et al.*, 1991; Finkelman *et al.*, 1987) or with a combination of 100 μg each of the two monoclonal alloantibodies (Hδ^{a}1 and FF1-4D5) that are specific for IgD of the rat IgG2a allotype in 100 ml 0.15 M NaCl solution.

***H. polygyrus* infection.** Infective, ensheathed third-stage larvae of *H. polygyrus* (specimens on file at the U.S. National Parasite Collection, U.S. National Museum Helminthological Collection, U.S. National Museum Helmintholgical Collection No. 81930, Beltsville, MD) were propagated on vermiculite-fecal cultures, seperated from culture by using a Bearmann apparatus, washed in saline, counted, and stored at 4° C until used (generally within four weeks). Mice were inoculated orally with 200 larvae by using a ball-tipped feeding tube (Svetic *et al.*, 1993c).

***H. polygyrus* challenge inoculation.** Mice (5 per group) were inoculated orally with 200 third stage *H. polygyrus* larvae by using a ball-tipped feeding tube. Fourteen days later mice were given an oral dose of 2 mg of pyrantel pamoate, an anti-helminth drug, administered through a feeding tube to cure the infection. A second *H. polygyrus* oral infection was performed at day 60 after primary inoculation.

CTLA4-Ig treatment. After immunization, mice were injected *i.v.* via the tail vein at day 0, 1, and 2 with 100 μg of human CTLA4-Ig, or at day 0 and day 1 with 100 μg of murine CTLA4-Ig. The control fusion protein L6 (100 μg) was administered in the same way.

Anti-gp39 antibody treatment. Mice were injected *i.v.* via the tail vein with 1 mg of hamster anti-gp39 antibody or the control hamster antibody on day 0 and day 7 after inoculation with *H. polygyrus*. The control hamster IgG was administered in the same way.

Preparation of single cell suspension. Spleen samples were collected 6 days after GaMIgD immunization. Mesenteric lymph node (MLN) tissue was collected 8 or 14 days after *H. polygyrus* inoculation. Cell suspensions were made by grinding tissue samples in 5 ml HBSS + 0.1% BSA + 0.1% sodium azide (for FACS analysis) or RPMI-1640 + 5% FBS (for ELISPOT assay and cell sorting) media on a metal sieve

with a 6 cc syringe plunger. After filtering through the nylon mesh (Martin Supply Co., Baltimore, MD) to remove tissue debris, the cell suspensions were centrifuged at 200 × g in a Sorvall RC-3B refrigerated centrifuge (Du Pont Co., Willmington, DE). The supernatant was descanted after centrifugation and the cell pellets were resuspended in 5 ml HBSS + 0.1% BSA + 0.1% sodium azide (for FACS analysis) or RPMI-1640 + 5% FBS (for ELISPOT assay and cell sorting) media. Cell concentration was determined by Coulter Counter (Coulter electronics, Hialeah, FL).

Enrichment of $CD4^+$ T cells by Cellect™ plus CD4 Immunocolumn. CD4 immunocolumns were assembled and activated one day before the experiment. For column activation, the column was washed with 15 ml of PBS + 2% FBS followed by 1.5 ml of "Column Reagent" reconstituted in PBS. After the "Column Reagent" get into the column bed, the eluting tube was closed and the column was placed at 4°C overnight. A lymphocyte suspension was prepared as described above and the cell concentration was adjusted to about 8×10^7 cells/ ml. The "Cell Reagent" was reconstituted in 1.5 ml PBS and added to 1.0 to 1.25×10^8 cells. The subsequent cell suspension was incubated on ice for 30 min. Cells were then washed with 10 ml of PBS + 2% FBS twice after the incubation and resuspended to a final concentration of 5×10^7 cells/ml. Columns were washed with 10 ml PBS + 2% FBS and the flow rate was adjusted to 6 to 8 drops per minute. The cell suspension was added to the column just before the last washing solution entered the column bed and the elusion was immediately collected in 15 ml conical tubes on ice. When the sample reached the top

of the column, more buffer was loaded and the eluant was collected to a total volume of 10 to 15 ml. The eluant was centrifuged at 200 × g for 5 min and the supernatant was discarded. The cell pellet was resuspended in 2 ml RPMI1640 + 5% FBS.

Immunofluorescent staining, FACS analysis and cell sorting. 2×10^6 cells in 50 ml HBSS + 0.1% BSA + 0.1% sodium azide were add into each tube for immunofluorescent staining. Anti-FcγRII mAb (24G2, 1μg per 2×10^6 cells) was added into each sample before stained with primary mAbs in order to block non-specific binding. Samples were incubated at 4° C for 30 min after primary mAbs were added. After incubation, 1 ml HBSS + 0.1% BSA + 0.1% sodium azide was added into each tube and samples were centrifuged at 200 × g for 5 min. The supernatant was discarded and cell pellets were resuspended. If a second step reagent was needed, samples were re-incubated and re-washed as above. In the final step cells were suspended in 0.5 ml of 2% paraformaldehyde and stored at 4° C in the dark. FACS analysis was performed on the next day with an ELITE flow cytometer (Coulter Electronics, Miami, FL). An argon laser was used as the excitation source for FITC, Cy5 and PE.

For cell sorting, 1×10^8 cells in 5 ml RPMI-1640 + 5% FBS (without phenol red) were pretreated with blocking Ab (24G2, 1 μg per 2×10^6 cells) and then stained with FITC-anti-CD4 (GK1.5, 0.2 μg per 1×10^6 cells) and PE-anti-TCRα/β (H57-597, 0.2 μg per 1×10^6 cells) mAbs. After incubation at 4° C for 30 min, 5 ml RPMI-1640 + 5% FBS (without phenol red) was added and samples were centrifuged at 200 × g for 5 min.

Cell pellets were resuspended in 2 ml RPMI-1640 (without phenol red)+ 5% FBS and loaded on the ELITE flow cytometer. Both the $CD4^+$, $TCR\alpha/\beta^+$ and $CD4^-$ cells were sorted and analyzed for purity after completion of the sort. The $CD4^+$, $TCR\alpha/\beta^+$ population was >97% pure and the $CD4^-$ population was >98% pure in all sorts presented.

ELISPOT. Individual wells of Immulon II polystyrene 96 well flat-bottomed plates were precoated with one of two anti-cytokine "capture" antibodies (BV41D122 for IL-4, TRFK5 for IL-5, and SXC2 for IL-10) and incubated at 4° C overnight. After 3 PBS-Tween 20 (0.05%) and 3 PBS washes, plates were blocked with RPMI-1640 + 5% FBS for 1 hr at 37° C. A 0.1 ml of spleen or mesenteric lymph node single cell suspension (5×10^6 cells/ml) in RPMI-1640 + 5% FBS was added to the coated plates in serial 5-fold dilutions and incubated for 3 hr at 37° C in humidified CO_2-incubator. For a positive control, recombinant IL-4 (100 units/ml), IL-5 (100 units/ml), and IL-10 (10 units/ml) were added into wells and serial dilutions were performed. For a negative control, RPMI-1640 + 5% FBS alone was added. Plates were washed 3 times with PBS and 3 times with PBS-Tween 20 after 3 hr and then a biotinylated secondary anti-cytokine antibody (BVD6.24G2 for IL-4, TRFK4 for IL-5, and SXC1 for IL-10) was added to the wells at a concentration of 4 μg/ml (BVD6.24G2 and TRFK4) or 0.8 μg/ml (SXC1). After incubation for 1 hour at 37° C, the plates were washed 3 times with PBS, then 3 times with PBS-Tween 20, after which streptavidin-alkaline phosphatase, diluted 1:2000 in PBS-Tween 20 + 5% FBS, was added to the

wells. Plates were incubated for 1 hr at 37° C and were then washed 5 times in PBS and a solution of 0.6% low-melting Seaplaque agarose containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 0.15 M 2-amino-2-methyl-1-propanol was added to individual wells. After overnight incubation in a humidified chamber, the number of blue spots in an individual well (where each spot represents a single cytokine-secreting cell) was counted by examining wells under a dissecting microscope.

Isolation and purification of RNA. RNase-free plastic and water were used throughout the assay. Tissues were homogenized in 2 ml RNAzol (Cinna/Biotecx, Friendswood, TX) at 50 mg of tissue/ml in 70 × 100mm 2006 FALCON polypropylene tubes (Becton Dickinson Labware, Lincoln Park, NJ). Samples were then snap-frozen in liquid nitrogen and stored at -70° C for future extraction. Upon RNA extraction, samples were thawed in 37° C waterbath and transferred into 2 ml conical tubes. An aliquot of 0.2 ml chloroform was added per 2 ml of homogenate, the samples were tightly covered, shaken vigorously for 15 sec and then incubated on ice for 15 min. The suspension was centrifuged at 12,000 × *g* (4° C) in an Eppendorf centrifuge 4515 C (Brinkman Instruments, Westbury, NY) for 15 min. The aqueous phase was transferred to a fresh tube, and an equal volume of isopropanol was added. After mixing, the samples were incubated for 45 min at -20° C. Samples were then centrifuged for 30 min at 12,000 × *g* (4° C) in the Eppendorf centrifuge 4515 C. The supernatant was discarded and the resultant RNA pellets were washed twice with 75% ethanol, dried under vacuum by a Speed Vac Concentrator (Savant, Hicksville, NY). The dried RNA pellets were

dissolved in 20-50 µl of ultra-pure RNase-free water and stored in -70° C. For RNA quantification, 1 µl was taken from each sample and diluted in 59 µl distilled, deionized water. The concentration and OD 260/280 ratio were determined for each sample. To ascertain whether the RNA was undegraded and whether the concentration had been correctly determined, the purified RNA (10 µg) was electrophoresed on a 2% formaldehyde gel containing ethidium bromide. The gel was photographed, and each individual lane was examined for the presence of the 18s and 28s ribosomal bands to assess whether equal loading of RNA on each lane had occurred.

Reverse transcription. Reverse transcription of RNA was carried out in a 25 µl final volume for 3.6 µg RNA. For each sample, 3.6 µg RNA was transfered into a 0.5 ml tube and double distilled H_2O was added to make a final volume of 11.8 µl. Aliquoted RNA samples were heated at 70° C for 5 min and then chilled on ice. A master mix was prepared based on the number of samples with components in the following concentrations: 2.5 µl of a 10 mM mix of all four deoxynucleotide triphosphates; 5 µl of 5 × RT buffer (259 mM Tris-HCl, pH8.3, 375 mM KCl, 15 mM $MgCl_2$); 2 µl dithiothreitol (0.1 M); 2 µl random hexamers (0.5 U/25 µl); 0.5 µl RNasin (40 U/µl); and 1.2 µl reverse transcriptase (200 U/µl). The master mix was well mixed and 13.2 µl was aliquoted into each tube. The samples were incubated at 37° C for 60 min, denatured at 90° C for 5 min, and quenched on ice for 5 min. The samples were stored at -20° C.

Polymerase chain reaction

Primer and probe design. The selection of primers for each cytokine for PCR amplification was accomplished by downloading a cytokine cDNA sequence into PC/GENE, (IntelliGenetics, Inc., Mountain View,CA), a computer program specifically deviced for PCR primer design (Lowe *et al.*, 1990). Generally, Primers were selected that ranged from 18 to 25 bp, have a 50% GC content, and amplified a region ranging from 200 to 400 bp. Additionally, a selected primer set had to span an intron so large that the gene itself could not be amplified. If this is not possible, then the primer set should at least span an intron large enough that products from the gene and the cDNA could be easily distinguished on an agarose gel. Probes for detecting each cytokine PCR product followed similar criteria as primers except that the position of probe sequences should be in the middle of the PCR amplified sequence. Sequence of Primers for amplification of cytokine cDNA and probes for detection of amplified DNA product on Southern blot are shown in Table I.

PCR master mix preparation. The volumes of each component needed for each PCR reaction were listed as follows: dNTP (2.5 mM each) 4.0 µl; 10 × Taq DNA polymerase buffer 5.0 µl; 25 mM $MgCl_2$ 3.0 µl; Taq polymerase (5 U/µl) 0.2 µl; sense oligo primer (0.2 µg/µl) 2.0 µl; antisense oligo primer (0.2 µg/µl) 2.0 µl; ultra pure H_2O 31.3 µl. Master mix was prepared by multiplying the volume by the sample number.

Table I. Primer sequences for amplification of cytokine cDNA during PCR and probe sequences for detection of amplified DNA product on Southern blot (Svetic *et al.*, 1991)

Cytokines	Sense, Antisense Primer and Probe Sequences	Bases Spanned	No. of PCR Cycles
IL-2	Sense GAG TCA AAT CCA GAA CAT GCC	122-140	
	Antisense TCC ACT TCA AGC TCT ACA G	349-369	26
	Probe CTC CCC AGG ATG CTC ACC TTC	256-277	
IL-3	Sense ACT GAT GAT GAA GGA CC	1000-1016	
	Antisense TTA GCA CTG TCT CCA GAT C	2485-2504	28
	Probe TCG GAG AGT AAA CCT GTC CA	2030-2049	
IL-4	Sense CTC AGT ACT ACG AGT AAT CCA	110-130	
	Antisense GAA TGT ACC AGG AGC CAT ATC	472-493	23
	Probe AGG GCT TCC AAG GTG CTT CGC ATA TTT	271-298	
IL-5	Sense GAC AAG CAA TGA GAG ACG ATG AGG	129-150	
	Antisense GAA CTC TTG CAG GTA ATC CAG G	342-363	26
	Probe GGG GGT ACT GTG GAA ATG CTA T	240-262	

Table I. (continued)

IL-6	Sense TTC CAT CCA GTT GCC TTC TTG G	73-94	
	Antisense CTT CAT GTA CTC CAG GTA G	414-432	21
	Probe ACT TCA CAA GTC CGG AGA	127-144	
IL-9	Sense TGA TGA TTG TAC CAC ACC GTG	1573-1593	
	Antisense CCT TTG CAT CTC TGT CTT CTG G	2873-2894	28
	Probe GCC TGT TTT CCA TCG GGT GAA A	1648-1669	
IL-10	Sense CGG GAA GAC AAT AAC TG	147-163	
	Antisense CAT TTC CGA TAA GGC TTG G	315-333	20
	Probe GGA CTG CCT TCA GCC AGG TG	209-227	
	A AGA CTT TCT TT		
IFN-γ	Sense AAC GCT ACA CAC TGC ATC TTG G	73-94	
	Antisense GAC TTC AAA GAG TCT GAG G	291-310	20
	Probe GGA GGA ACT GGC AAA AGG A	229-248	
HPRT	Sense GTT GGA TAC AGG CCA GAC TTT GTT G	514-538	
	Antisense GAT TCA ACT TGC GCT CAT CTT AGG	652-678	12
	Probe GTT GTT GGA TAT GCC CTT GAC	562-582	

A 2.5 μl aliquot of cDNA from the reverse transcription product of each sample was transfered into a 0.5 ml PCR tube (NOVEL, San Diego, CA). An aliquot of 47.5 μl master mix was added into each reaction tube and mixed. The PCR mixture was centrifuged and 50 μl mineral oil was added into the tube.

PCR cycle conditions. PCR reactions were performed on a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The program for PCR reaction was set so that after an initial incubation at 95° C for 5 min, temperature cycling was started with each cycle as follows:denaturation at 94° C for 45 sec; annealing at 53° C for 1 min; and extension at 72° C for 2 min. After all cycles were finished, a final extension at 72° C for 7 min was followed and samples were keeped at 4° C. The optimal number of PCR cycles for each cytokine was determined experimentally and was defined as that the number of cycles would achieve a detectable concentration and was well below saturating conditions (Table I).

To verify that equal amounts of RNA were added in each PCR reaction within an experiment, primers for a "housekeeping gene", hypoxanthine-guanine phosphoribosyl transferase (HPRT) were used to amplify the cDNA that was from the total RNA.

Southern blot. An aliquot of 10 μl of final PCR reaction mix sample was transfered to a new 0.5 ml tube and 2 μl gel loading buffer was added. Samples were then denatured by at 65° C for 5 min and chilled on ice before loading on 1% agarose-TBE gel. The gel was run at 120V for about 30 min in 1 × TBE buffer. After electrophoresis was

was finished, the gel was soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH at pH 13) for 25 min and the solution was decanted and repeated with fresh solution. The gel was then rinsed twice with distilled water and soaked in a 1.5 M NaCl, 1 M Tris-HCl solution at pH 7.5 for 15 min twice. After rinsing twice with distilled water, the gel was saturated in 20 × SSPE for 30 min. During this time, a Nytran nylon membrane (pore size: 0.2 μm, Schleicher & Schuell. Inc., Keene, NH) was placed in distilled water with two pieces of GB002 thin blotting paper (Schleicher & Schuell. Inc., Keene, NH) in 10 × SSPE and one piece of GB004 thick blotting paper (Schleicher & Schuell. Inc., Keene, NH) in 5 × SSPE for 30 min. The gel was removed after saturation and inverted "bottom up" on bench top covered with a layer of plastic membrane. The Nytran was then placed on top, air bubbles removed, and any excess agarose gel was cut away. Presoaked GB002 blotting paper was then placed on the top of the Nytran membrane, and followed by the presoaked BG004 paper. Two to three inches of dry GB004 blotting paper was then added and a hard metal or plastic tray was placed on the blotting paper, followed by two 1-liter bottles of water. The cappillary transfer was completed overnight.

Prehybridization. After transfer to Nytran was completed, the blot was placed in a UV Stratalinker 1800 (Stratagene, Menasha, WI). The crosslinking of the DNA product to the Nytran membrane was finished by preset "auto-crosslink" program. Prehybridization solution was made as follows: 20 × SSPE 30 ml; 100 × Denhardt's 10 ml; 10% SDS 10 ml; and distilled H_2O 50 ml. Denatured Salmon Sperm DNA (Promega, Madison,WI) was added to a final concentration of 50 μg/ml and prewarmed to 42° C. The blot was

placed on a piece of nylon mesh, rolled to fit in a hybridization bottle, and 10 ml of prewarmed prehybridization solution was added into the bottle for each blot (up to five blots could be put into each hybridization bottle). The bottle was returned to the hybridization oven (Bellco Glass, Inc., Vineland, NJ) at 42° C for 5 hr.

Probe labeling. The oligonucleotide probe was end-labelled with [γ-^{32}P]-dATP. First, the probe buffer was prepared as follows: 1 M Tris (pH 7.6) 5.0 µl; 2 M $MgCl_2$ 0.5 µl; 0.5 M dithiothreitol 1.0 µl; and distilled H_2O 3.5 µl. Second, the probe reaction mix was prepared as follows: probe buffer 2.5 µl; oligonucleotide probe (0.2 µg/µl) 2.0 µl; T4 polynucleotide kinase 1.0 µl; distilled water 9.5 µl; and [γ-^{32}P] dATP (10 mCi/ml) 10 µl. The probe reaction mix was incubated at 37° C for 40 min. Prior to the end of the 40 min incubation, a G-25 Sephadex spin column (5 prime-3 prime, Inc., Boulder, CO) was removed from 4° C, beads were mixed by inverting several times, the cap and stopper of the column were removed, and the column was drained for 5 min. The collected buffer in the receiving tube was discarded and the column was spun in Sorvall RC-3B refrigerated centrifuge at 2000 rpm for 2 min. After centrifuge, buffer in the receiving tube was discarded and the probe reaction mix was loaded into the column. The column was centrifuged again at 2000 rpm in Sorvall RC-3B refrigerated centrifuge for 4 min. The labled probe was collected in the receiving tube after the centrifugation. An aliquot of 1 µl of probe solution was placed into 5 ml scintillation fluid, and the ^{32}P incorporation was determined by a Beckman Liquid Scintillation System (Beckman Inc., Irvine, CA).

Hybridization. Recipe for 100 ml hybridization solution is listed below: 20 × SSPE 30 ml; 10% SDS 10 ml; and distilled H_2O 60 ml. The solution was preheated to 49° C. After prehybridization was complete, the hybridization bottle was removed from the oven and the prehybridization solution was discarded. A aliquot of 15×10^6 cpm probe was mixed with 10 ml of hybridization solution and added to the hybridization bottle. The bottle was put in the oven and rotated at 49° C overnight.

Blot washing. After overnight hybridization, the blot was removed from the bottle and washed in low-stringency washing solution at 49° C for 15 min. The recipe of the solution was as follows: 20 × SSPE 180 ml; 10% SDS 6 ml; and distilled H_2O 414 ml. The blot was then washed again in high-stringency washing solution at 49° C for 45 sec. The recipe of the solution was as follows: 20 × SSPE 30 ml; 10% SDS 6 ml; and distilled H_2O 564 ml.

PhosphorImager analysis. After washing the blot was covered with saran wrap and inserted into phosphor storage screen (Molecular Dynamics, Sunnyvale, CA) overnight. The screen was scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) the next day. The intensity of each band was quantitfied using a PhosphorImager quantitation program (Molecular Dynamics, Sunnyvale, CA) and the quantity of cytokine gene expression of each sample was normalized by relating gene expression to the expression of its corresponding HPRT measment.

Statistical evaluation. For cytokine gene expression studies, a log transform was made of the ratio of the corrected densities for cytokine mRNA measurement to the corrected densities for the housekeeping gene, HPRT. Arithmetic mean and standard error (SEM) were calculated within each experimental group. The mean of the log transformed ratio for untreated values was subtracted from means of treated groups. The anti-log values of these differences were then plotted along with their standard errors. A proportion of the untreated control of "1" indicates that the value for a treated group equals the value for the untreated group. For serum Ig isotype evaluations, geometric means and SEM were calculated. Arithmetic means and SEM were calculated in studies of ELISPOT, eosinophilia, and mastocytosis studies. All data shown in following experiments is representative of at least 2 independent experiments.

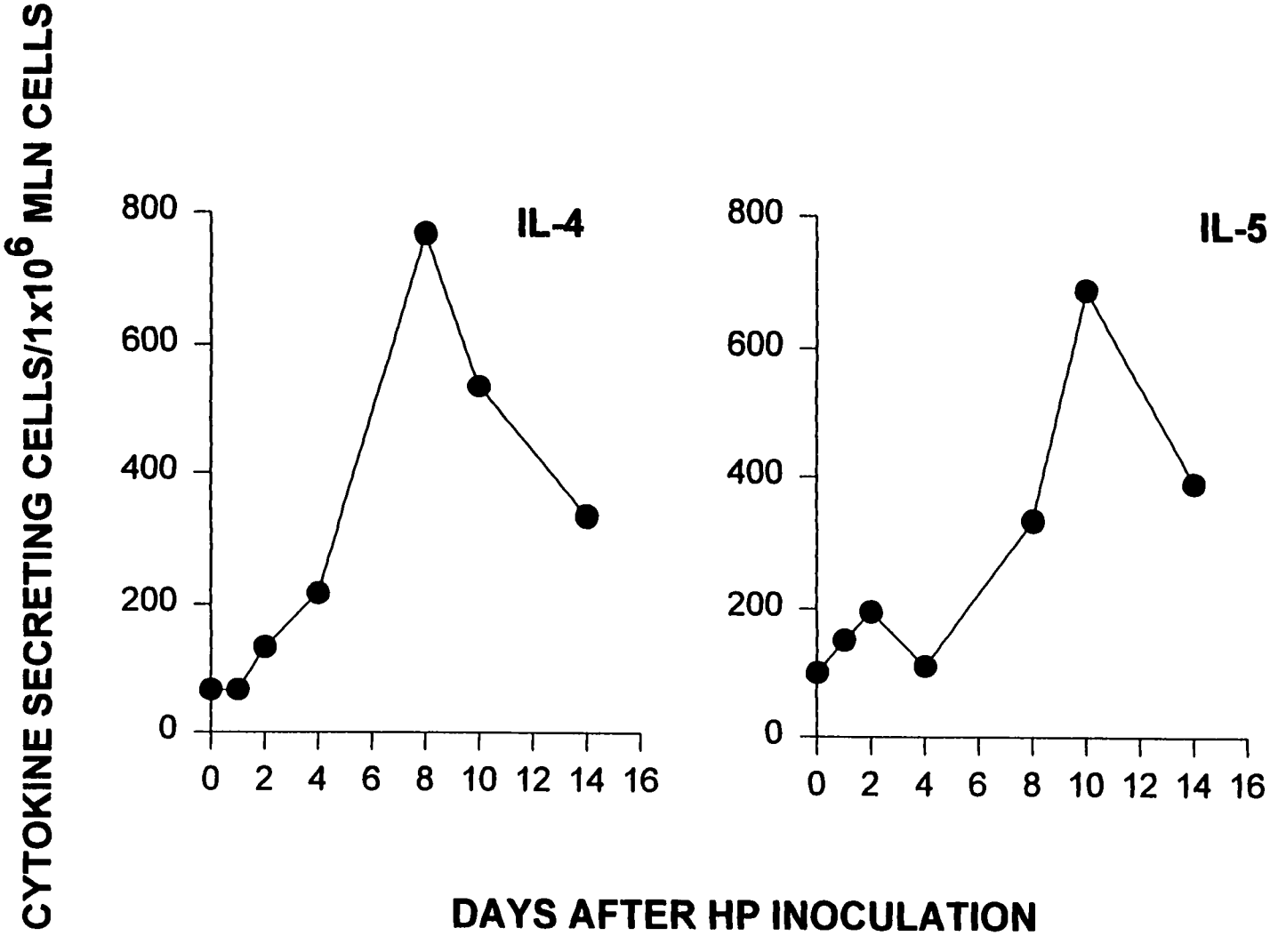
III. Results

A. Studies of T cell cytokine production and B cell activation during the primary immune response to HP inoculation

1. Kinetics of IL-4 and IL-5 secretion in the MLN during the course of immune response to HP

Previous studies in our laboratory have shown a unique pattern of elevation in IL-4 and IL-5 gene expression in both the Peyer's patch (PP) and mesenteric lymph node (MLN) during the *in vivo* immune response that occurs after oral HP inoculation of BALB/c mice. IL-4 gene expression started to rise at day 2 and peaked from day 6 to day 8 in both the PP and the MLN. IL-5 gene expression showed a slight increase at day 2, followed by more marked elevations by day 5 in the PP and day 6 in the MLN and was still elevated at day 12 (Svetic *et al.*, 1991). To examine whether a similar pattern occurred at the cytokine protein secretion level, ELISPOT assays were developed to study IL-4 and IL-5 secretion in the MLN during the primary immune response. Mice were orally infected with 200 third stage HP larvae, MLN samples were collected at day 0, 1, 4, 8, 10, and 14 after HP inoculation, and ELISPOT assays were performed to detect changes in the numbers of IL-4 and IL-5 secreting cells in the MLN during the course of the response (Figure 6). The number of IL-4 secreting cells in the MLN increased by day 2, peaked at day 8 (where a 10-fold increase was observed), then subsequently

Figure 6. Kinetics of elevations in the number of IL-4 and IL-5 secreting cells in the MLN during HP primary infection. BALB/c mice (3 per group) were orally infected with 200 third stage HP larvae and MLN samples were collected on day 0, 1, 4, 8, 10 and 14. Single cell suspensions were made from pooled MLN and the number of IL-4 and IL-5 secreting cells /10^6 MLN cells were determined in an ELISPOT assay as described in Materials and Methods. Data shown here is representative of 2 independent experiments.

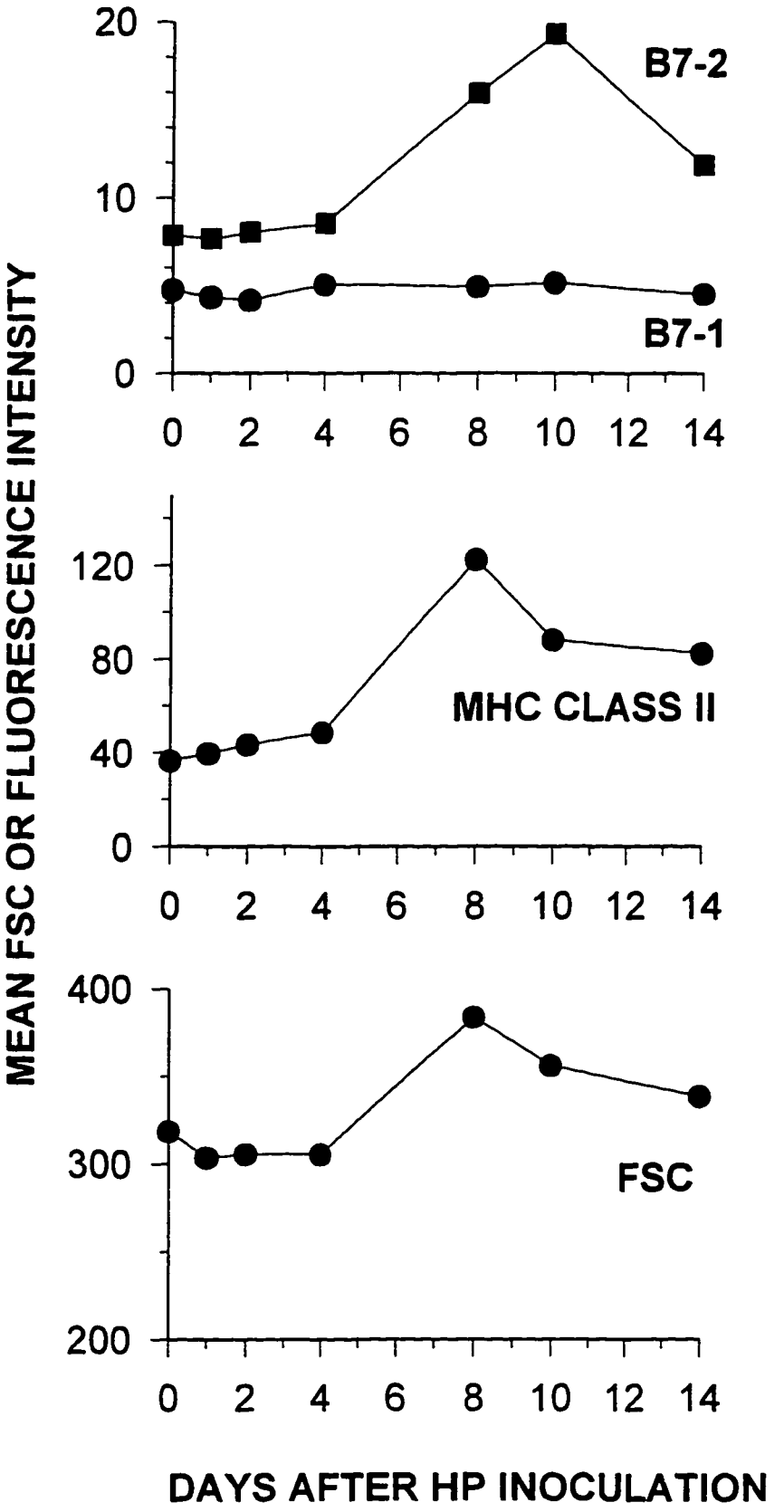

CYTOKINE SECRETING CELLS/1x10^6 MLN CELLS
IL-4
IL-5
800
600
400
200
0
0 2 4 6 8 10 12 14 16
DAYS AFTER HP INOCULATION

decreased. Meanwhile, IL-5 secretion in the MLN exhibited a slight rise around day 2, a large increase by day 10, and a steady decrease by day 14. Both the changes in the number of IL-4 and IL-5 secreting cells in the MLN highly correlated with their gene expression patterns (Svetic *et al.*, 1991).

2. Kinetics of MLN B cell activation in the primary immune response to HP

The marked elevation of serum IgE levels, previously detected in HP infected mice (Urban, Jr. *et al.*, 1991; Urban *et al.*, 1991), suggested that extensive B cell activation had occurred. To study further B cell activation events during the response, the kinetics of surface expression of certain B cell activation antigens were investigated. Pooled MLN cells from HP-infected mice were stained with anti-B7-1, anti-B7-2 and anti-MHC Class II mAbs at different time points. Surface expressions of these activation markers on B cells and changes in B cell size (forward light scatter) were analyzed by flow cytometry (Figure 7). B cell surface B7-1 expression was unchanged throughout the course of the response, but B7-2 expression started to increase at day 5 and reached its maximum by day 10 before decreasing. Elevations in both B cell size and surface MHC class II expression peaked at day 8. These findings demonstrate that maximum B cell activation occurred by days 8-10 in the MLN during the HP primary response.

Figure 7. Kinetics of B cell activation in the MLN during primary HP infection. Mice (3 per group) were orally inoculated with 200 third stage HP larvae. MLN samples were collected at days 0, 1, 2, 4, 8, 10 and 14 after inoculation. Pooled MLN cells from each treatment group were dual stained with B cell specific Cy5-anti-B220 (6B2) and biotin-anti-B7-1(1G10)/-B7-2 (GL-1) mAbs plus SA-PE, or FITC-anti-MHC class II(MDK6) mAb. The mean fluorescence intensity of B7-1(CD86), B7-2(CD80), MHC class II staining and cell size (forward light scatter, FSC) in the $B220^{+}$ cell population in different treatment groups were measured by flow cytometry and graphed over time. Data shown here is representative of 2 independent experiments.


20
10
0
B7-2
B7-1
0 2 4 6 8 10 12 14
120
80
40
0
MHC CLASS II
0 2 4 6 8 10 12 14
400
300
200
FSC
0 2 4 6 8 10 12 14
MEAN FSC OR FLUORESCENCE INTENSITY
DAYS AFTER HP INOCULATION

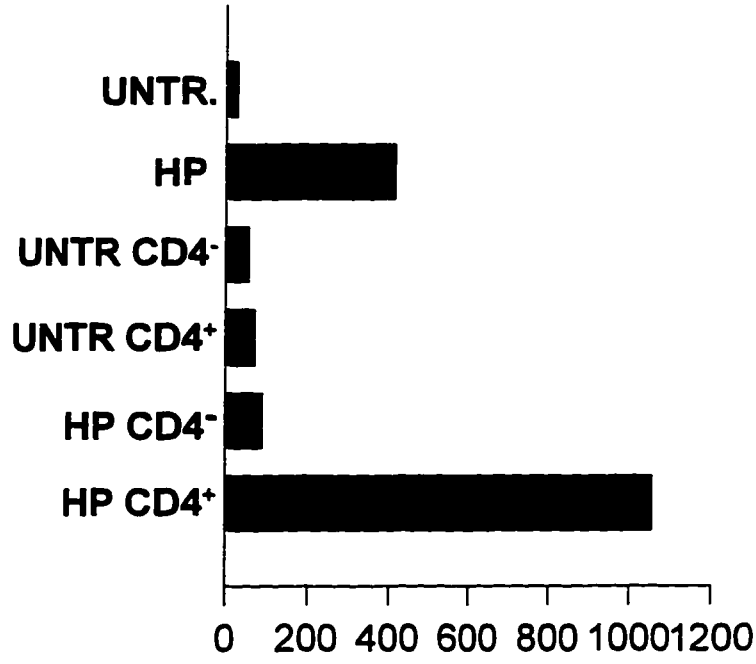
3. $CD4^+$, $TCR\alpha/\beta^+$ T cells are the source of elevated IL-4 but not IL-5 protein secretion during the HP primary immune response

Early studies have demonstrated that $CD4^+$ T cell population was the source for elevated IL-4, but not IL-5 gene expression in the MLN by day 6 after HP infection (Svetic *et al.*, 1993b). However, it was not yet confirmed whether IL-4 and IL-5 proteins were also secreted by the same T cell subpopulations. To address this question, $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted from a MLN cell suspension at day 8 in HP-inoculated mice and IL-4 and IL-5 ELISPOT assays were performed with the sorted cells (Figure 8). Increases in the number of IL-4 secreting cells in the HP-infected group are mostly from $CD4^+$, $TCR\alpha/\beta^+$ T cells, whereas increases in IL-5 secretion are from other cell populations. These results are highly consistent with our previous gene expression data (Svetic *et al.*,1993).

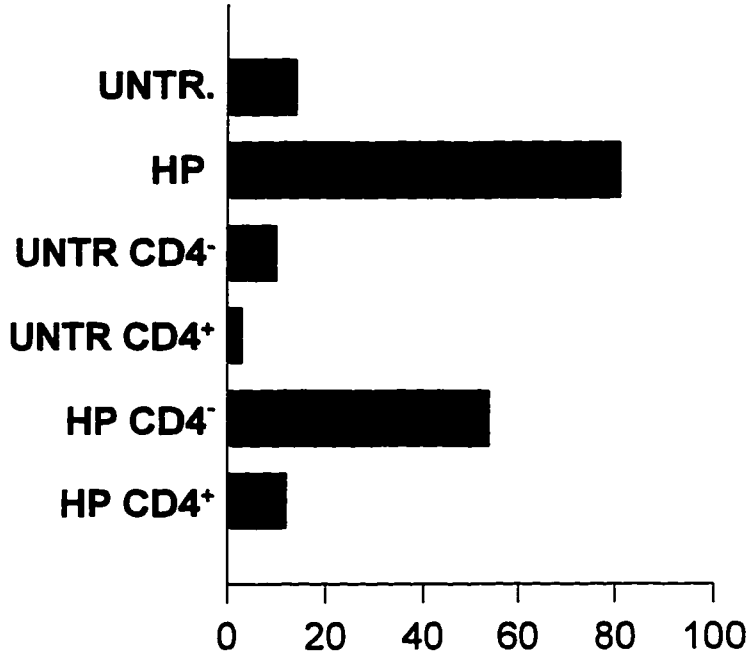
B. Effect of CTLA4-Ig administration on cytokine gene expression and protein secretion in unimmunized mice

CTLA4-Ig is a fusion protein in which the extracellular domain of the CTLA-4 molecule is hybridized to the IgG2a Fc region. It has a high affinity for B7-1, B7-2 and other possible ligands for CD28/CTLA4 both *in vitro* and *in vivo*. *In vitro* studies have shown that CTLA4-Ig has no effect on normal unstimulated lymphocytes in culture (Ranheim and Kipps, 1993a). The *in vivo* effect of CTLA4-Ig administration has not been thoroughly investigated. Although previous studies have demonstrated that

Figure 8. $CD4^+$, $TCR\alpha/\beta^+$ T cells are the source of elevated IL-4, but not IL-5 secretion in the MLN at day 8 after HP inoculation. Mice (5 per group) were either orally infected with 200 third stage HP larvae or given PBS as controls. MLN samples were collected and pooled on day 8. MLN cells from both groups were stained with FITC-anti-CD4 (GK1.5) and PE-anti-TCRα/β (H57-597) mAbs. $CD4^+$, $TCR\alpha/\beta^+$ cells were sorted from both groups on the same day using an Epics Elite cell sorter and were shown to be >95% pure. The number of IL-4 and IL-5 secreting cells per 10^6 MLN cells was determined in an ELISPOT assay. UNTR: untreated. Data shown here is representative of 2 independent experiments.



IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES



IL-5 SECRETING CELLS/10^6 MLN LYMPHOCYTTES

CTLA4-Ig had no obvious toxic effects *in vivo* (Wallace *et al.*, 1995), it is still unknown whether cytokine production in unimmunized mice might be affected by blocking CTLA-4 ligand interaction. Thus, we investigated the effect of *in vivo* CTLA4-Ig administration on cytokine production in unimmunized mice. Murine CTLA4-Ig or the control fusion protein, L6, was administered to BALB/c mice (100 μg/mouse, 5 mice per group) for two consecutive days. Tissue samples from the spleen and the MLN were collected after 6 days for IL-4 and IL-5 secretion studies. Samples of the PP, MLN and spleen were also collected for cytokine gene expression studies. ELISPOT results showed that *in vivo* CTLA4-Ig administration did not affect IL-4 and IL-5 secretion in the spleen (Figure 9) and MLN (data not shown), nor did it have any significant effect on cytokine gene expression in the PP, MLN and the spleen, compared with the group given the control protein L6 or the untreated control (Figure 10). The inhibition of IL-9 gene expression in the MLN by CTLA4-Ig administration was not observed in another independent experiment (data not shown).

Figure 9. ***In vivo*** **CTLA4-Ig administration did not affect the number of IL-4 and IL-5 secreting cells in the spleen.** Murine CTLA4-Ig or the control fusion protein, L6, was administered *i.v.* (100 μg per mouse; 5 mice per group) on day 0 and 1. Spleen samples were taken at day 6 and the number of IL-4 and IL-5 secreting cells per 10^6 lymphocytes were determined by an ELISPOT assay. The mean and SEM derived from values of five individual BALB/c mice are shown for each treatment group. UNTR: untreated. Data shown here is representative of 2 independent experiments.

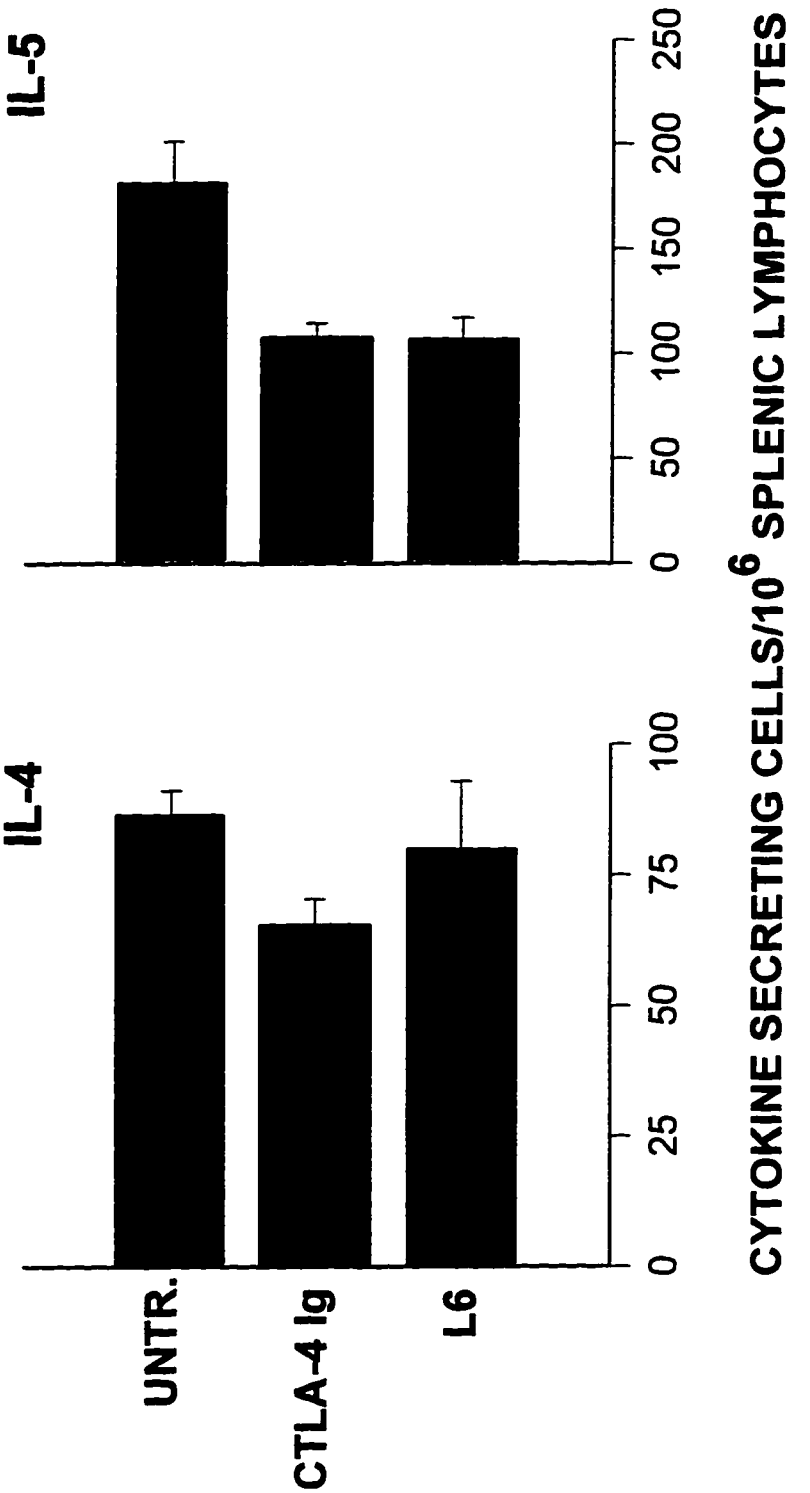
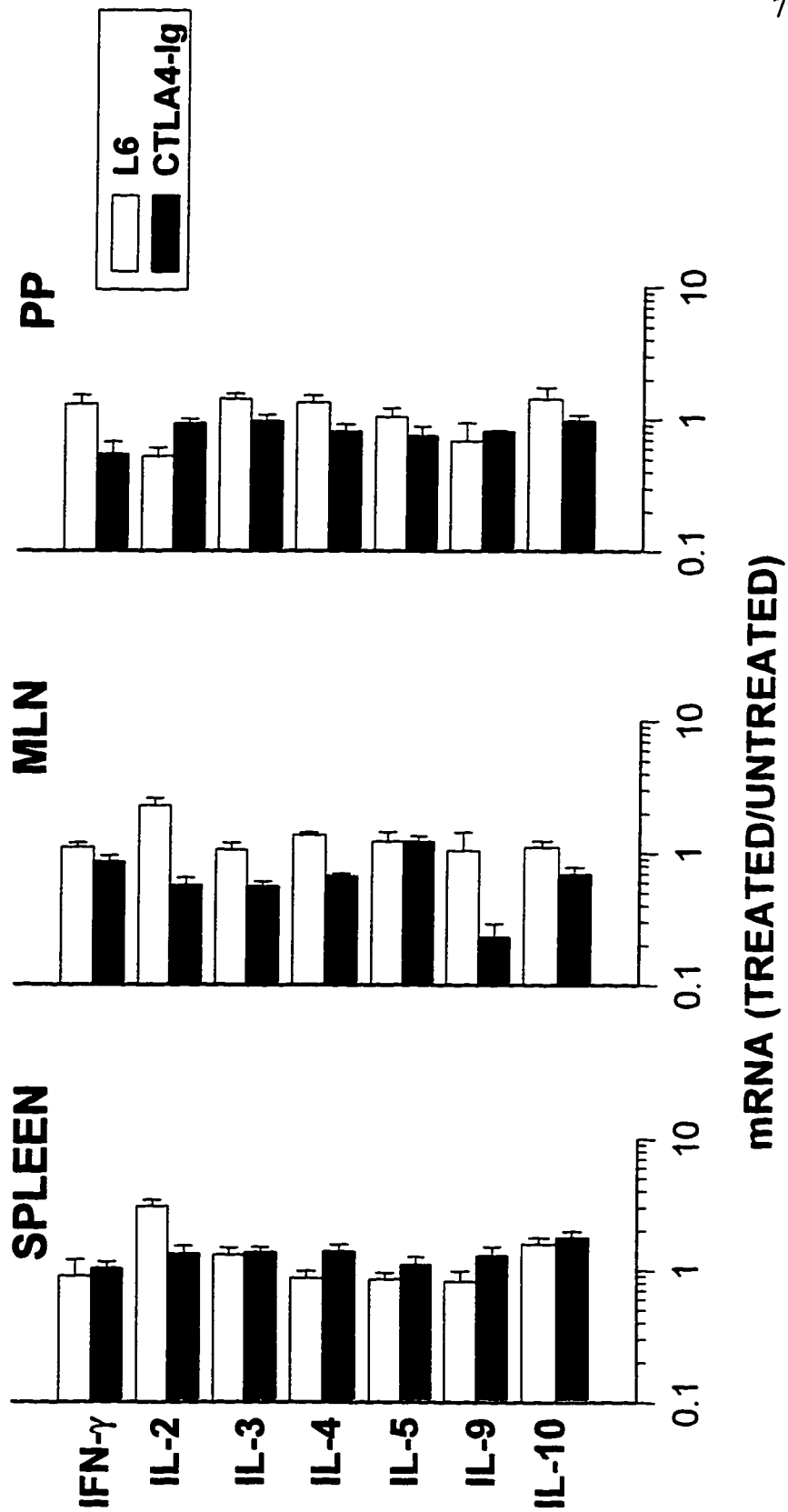

IL-5
IL-4
0
50
100
150
200
250
0
25
50
75
100
UNTR.
CTLA-4 Ig
L6
CYTOKINE SECRETING CELLS/10^6 SPLENIC LYMPHOCYTES

Figure 10. ***In vivo*** **CTLA4-Ig administration has no effect on cytokine gene expression in normal unstimulated mice.** CTLA4-Ig (100 μg) or the control fusion protein, L6, (100 μg) was administered *i.v.* at day 0 and 1. Samples from the PP, MLN and the spleen were collected for cytokine gene expression studies using a quantitative RT-PCR. The arthmetic mean and SEM derived from data of five individual BALB/c mice are shown for each treatment group. All data were individually normalized to the endogenous internal standard, HPRT, which did not show greater then a two- to three-fold change throughout the experiment. The means of the normalized data are expressed relative to the mean of the untreated control, which was arbitrarily given a value of 1. Results shown here is representative of 2 independent experiments.


SPLEEN
MLN
PP
L6
CTLA4-Ig
IFN-γ
IL-2
IL-3
IL-4
IL-5
IL-9
IL-10
0.1
1
10
mRNA (TREATED/UNTREATED)

C. Blocking CD28-B7 costimulation inhibits serum Ig isotype elevation, IL-4 but not IL-10 production, and T cell activation in the immune response against immunogenic anti-mouse IgD antibodies

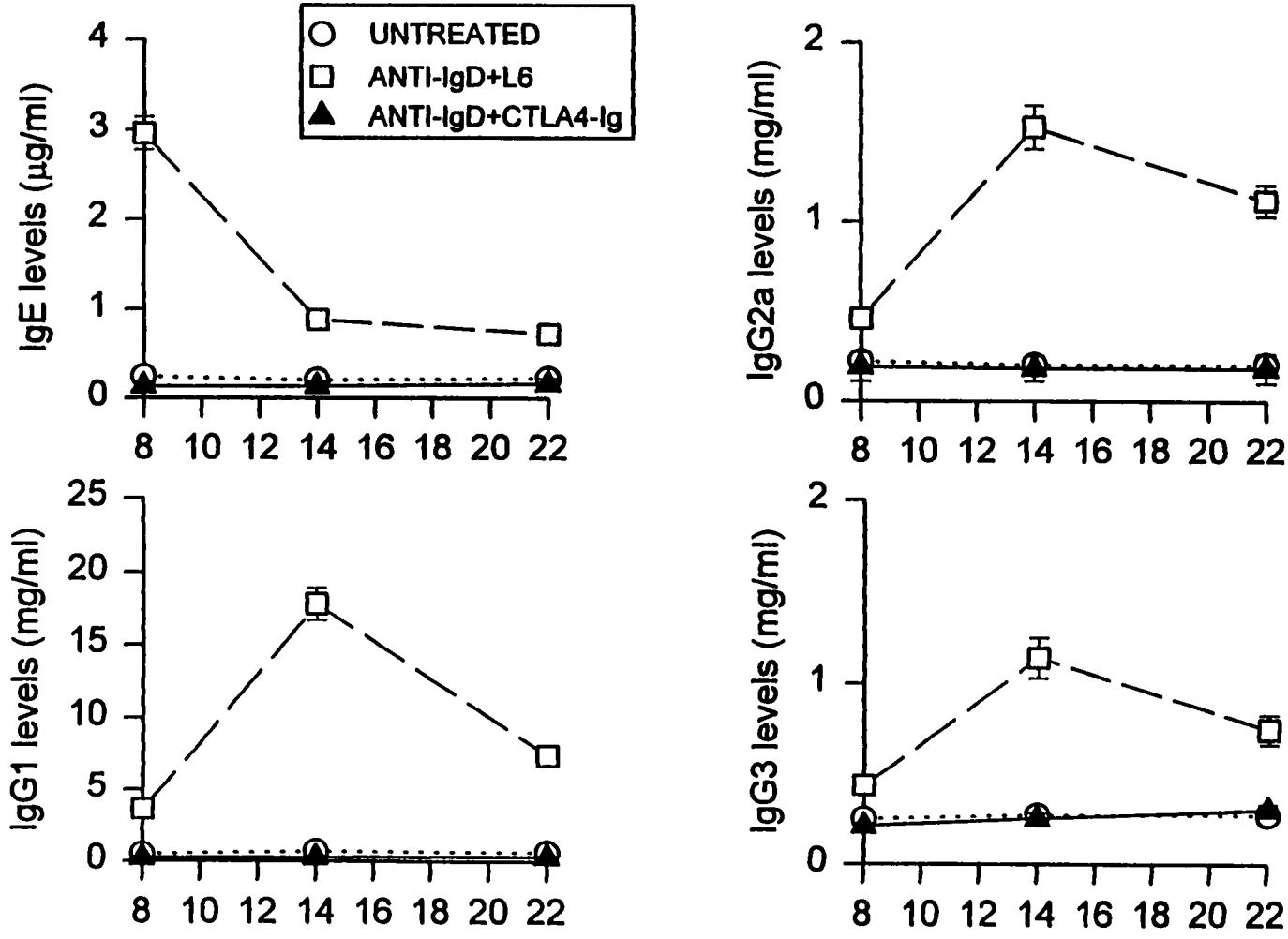
1. Administration of CTLA4-Ig suppresses serum Ig isotype elevations in anti-mouse IgD Ab immunized mice

Previous results have shown significant increases in serum IgE, IgG1, IgG2a, and IgG3 8 days after immunization with goat-anti mouse IgD (GaMIgD) antibody or mouse anti-IgD alloantibodies (Finkelman *et al.*, 1982b). To address whether blocking CD28-B7 costimulation at an early time points could affect the elevations in serum Ig isotypes, human CTLA4-Ig or the control fusion protein, L6, was administered at day 0, 1, and 2 after alloanti-IgD Ab immunization. Results of serum Ig levels showed that administration of CTLA4-Ig blocked elevations of IgE, IgG1, IgG2a, and IgG3 at day 8, 14, and 22 (Figure 11).

2. CTLA4-Ig administration selectively inhibits elevations in cytokine gene expression in spleens in GaMIgD Ab immunized mice

The immune response against foreign anti-mouse IgD antibodies is an IL-4 dominant T cell dependent systemic response in which IL-2, IL-4 and IL-9 gene expression is elevated at day 3, and IL-4 and IL-10 gene expression is greatly elevated at

Figure 11. Early CTLA4-Ig administration inhibits elevations in serum IgE, IgG1, IgG2a, and IgG3 at day 8, 14, and 22 after mouse alloanti-IgD mAb immunization. Mice (5 per group) were administered with human CTLA4-Ig (100 μg) or the control fusion protein, L6, (100 μg) at day 0, 1, and 2 after *i.v.* injection of the combination of anti-IgD alloantibodies Hδ^{a}1 (100 μg) and FF1-4D5 (100 μg). Serum IgE, IgG1, IgG2a and IgG3 levels were determined as described in Materials and Methods. Similar results were obtained at day 8 after immunization with GaMIgD. Results shown here is representative of 3 independent experiments.


UNTREATED
ANTI-IgD+L6
ANTI-IgD+CTLA4-Ig
IgE levels (µg/ml)
IgG2a levels (mg/ml)
IgG1 levels (mg/ml)
IgG3 levels (mg/ml)
8 10 12 14 16 18 20 22
DAYS AFTER IMMUNIZATION

day 6 in the spleen after anti-IgD administration (Svetic *et al.*, 1991). It would be interesting to know whether CD28-B7 costimulation is required for elevated cytokine production during the response. Mice were immunized with GaMIgD antibody and treated with either human CTLA4-Ig or the control fusion protein, L6, on the day 0, 1 and 2. Spleen samples were collected at day 3 and 6 to investigate cytokine gene expression. Three days after GaMIgD injection, CTLA4-Ig blocked elevations in IL-2, IL-4 and IL-9 mRNA; 6 days after immunization, CTLA4-Ig inhibited elevations in IL-4 but not IL-10 gene expression (Figure 12).

3. CTLA4-Ig administration inhibits elevations in IL-4 but not IL-10 production in splenic $CD4^+$, $TCR\alpha/\beta^+$ T cells in GaMIgD immunized mice

The surprising finding that increased IL-10 gene expression after GaMIgD immunization was not blocked by CTLA4-Ig led us to question whether the source of this cytokine was $CD4^+$ T cells. Previous studies (Takahashi *et al.*, 1992) have demonstrated that the primary source of elevated IL-4 and IL-10 production was $CD4^+$ T cells in GaMIgD-immunized mice. However, other cell types, such as macrophage and B cells, can produce IL-10 in response to some stimuli and might be the source in mice treated with GaMIgD plus CTLA4-Ig (Fiorentino *et al.*, 1991; O'Garra *et al.*, 1990). To address this question, $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted from GaMIgD-immunized, CTLA4-Ig- or L6-treated mice at day 6. CTLA4-Ig blocked elevations in IL-4 mRNA, but had little effect on IL-10 in $CD4^+$, $TCR\alpha/\beta^+$ T cells (Figure 13). An ELISPOT assay was used to

Figure 12. CTLA4-Ig administration inhibits elevations in IL-2, IL-4 and IL-9 at day 3, IL-4 but not IL-10 gene expression in the spleen at day 6 after GaMIgD immunization. Mice (5 per group) were administered with human CTLA4-Ig (100 μg) or the control fusion protein, L6, (100 μg) at day 0, 1, and 2 after *i.v.* injection of GaMIgD (800 μg). Spleen samples were collected at day 3 and 6 for cytokine gene expression, which was done by a quantitative RT-PCR. All data were quantitated on a PhosphorImager and normalized to the internal standard HPRT. The final data were graphed as in Figure 10. Data shown here is representative of 3 independent experiments.

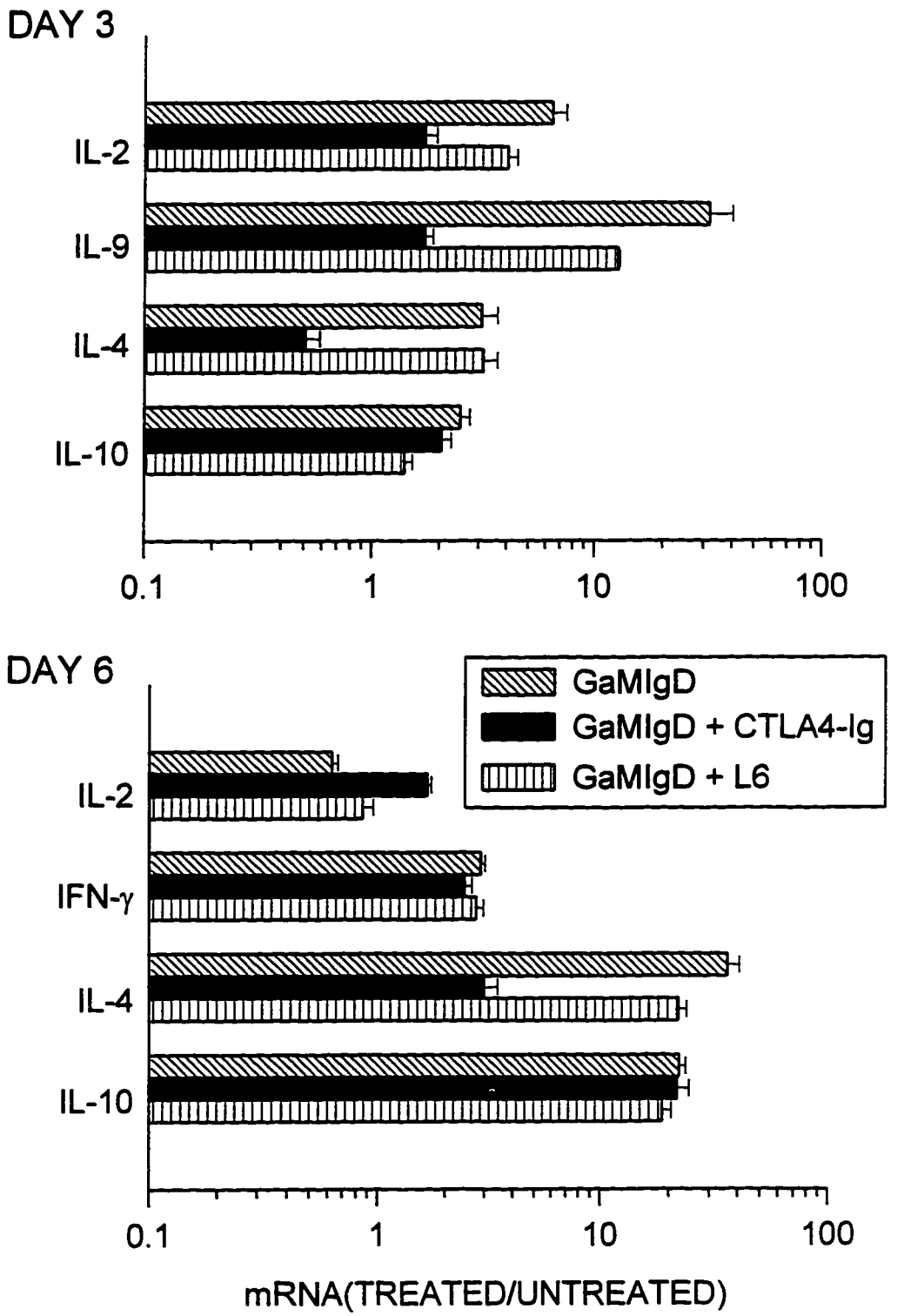
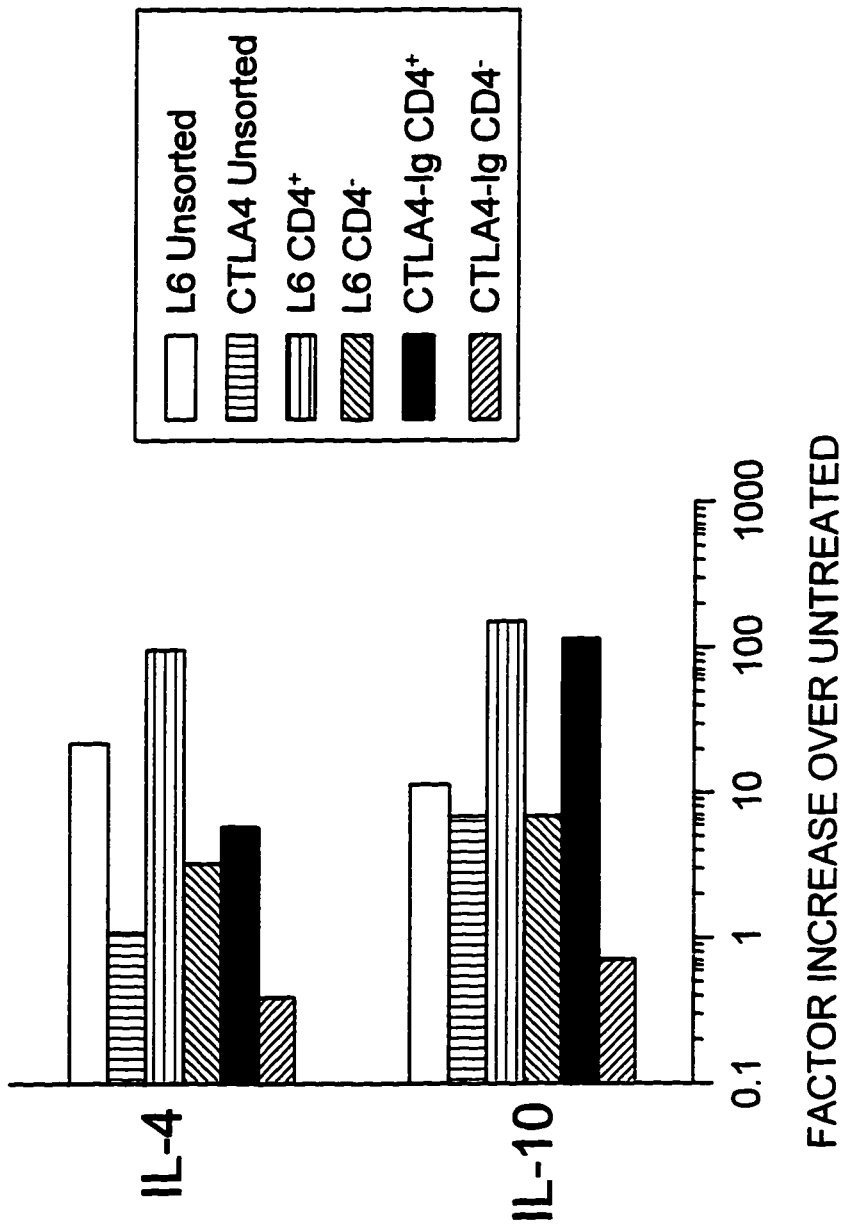

DAY 3
IL-2
IL-9
IL-4
IL-10
0.1
1
10
100
DAY 6
GaMIgD
GaMIgD + CTLA4-Ig
GaMIgD + L6
IL-2
IFN-γ
IL-4
IL-10
0.1
1
10
100
mRNA(TREATED/UNTREATED)

Figure 13. $CD4^+$, $TCR\alpha/\beta^+$ T cells from GaMIgD-immunized mice administered CTLA4-Ig show marked inhibition of IL-4 but minimal change in IL-10 cytokine gene expression at day 6 after immunization. Mice (3 per group) injected *i.v.* with 800 μg of affinity-purified GaMIgD and administered either human CTLA4-Ig (100 μg) or L6 (100 μg) at days 0, 1, and 2 were sacrificed 6 days after immunization. Pooled spleen cells from mice in the same group were first passed through Cellect™ plus mouse CD4 immuncolumn to enrich for $CD4^+$ cells. Cells eluted from the column were simutaneously stained with FITC-anti-CD4 (GK1.5) and PE-anti-TCRα/β (H57-597) mAbs. $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted from both groups on the same day using an Epics Elite cell sorter and were shown to be >95% pure. IL-4 and IL-10 gene expression were determined by quantitative RT-PCR. All data were individually normalized to the endogenous internal standard HPRT, which did not show greater then a two- to three-fold changes throughout the experiment, and are expressed relative to the mean of the untreated control, which was arbitrarily given a value of 1. Data shown here is representative of 4 independent experiments.


L6 Unsorted
CTLA4 Unsorted
L6 CD4+
L6 CD4-
CTLA4-Ig CD4+
CTLA4-Ig CD4-
IL-4
IL-10
0.1
1
10
100
1000
FACTOR INCREASE OVER UNTREATED

determine the number of IL-4 and IL-10-secreting cells for both the unsorted splenic cells and the sorted $CD4^+$, $TCR\alpha/\beta^+$ T cells from GaMIgD-immunized mice treated with either CTLA4-Ig or L6. CTLA4-Ig treatment blocked the increase in the number of IL-4-secreting cells, but not the increase in the number of IL-10-secreting cells in the sorted $CD4^+$, $TCR\alpha/\beta^+$ T cells from GaMIgD-immunized mice (Figure 14).

4. Blocking CD28-B7 interactions by CTLA4-Ig administration inhibits $CD4^+$, $TCR\alpha/\beta^+$ T cell activation in GaMIgD immunized mice

The reduction in IL-4 but not IL-10 production by $CD4^+$, $TCR\alpha/\beta^+$ T cells in GaMIgD-immunized mice given CTLA4-Ig suggests that $CD4^+$, $TCR\alpha/\beta^+$ T cells could still be partially activated without CD28-B7 costimulation. To determine whether CTLA4-Ig blocked $CD4^+$ T cell activation, spleen cells from GaMIgD-immunized mice treated with CTLA4-Ig or L6 were dual stained with anti-CD4 and anti-IL-2 receptor (IL-2R) mAbs. Single histogram analysis of $CD4^+$ T cells demonstrated that CTLA4-Ig blocked elevations in both cell size (forward light scatter) and surface IL-2R expression, both of which are characteristic of $CD4^+$ T cell activation in GaMIgD-immunized mice (Figure 15) (Finkelman *et al.*, 1986b).

Figure 14. $CD4^+$, $TCR\alpha/\beta^+$ T cells from GaMIgD-immunized mice administered CTLA4-Ig show marked inhibition in the number of IL-4-, but not IL-10-secreting cells, at day 6 after immunization. GaMIgD immunization, CTLA4-Ig treatment, $CD4^+$ cell enrichment, and cell sorting are as described in the legend of Figure 13. The number of IL-4 and IL-10 secreting cells/10^6 cells were determined in an ELISPOT assay as described in Materials and Methods. UNTR: untreated. Data shown here is representative of 3 independent experiments.

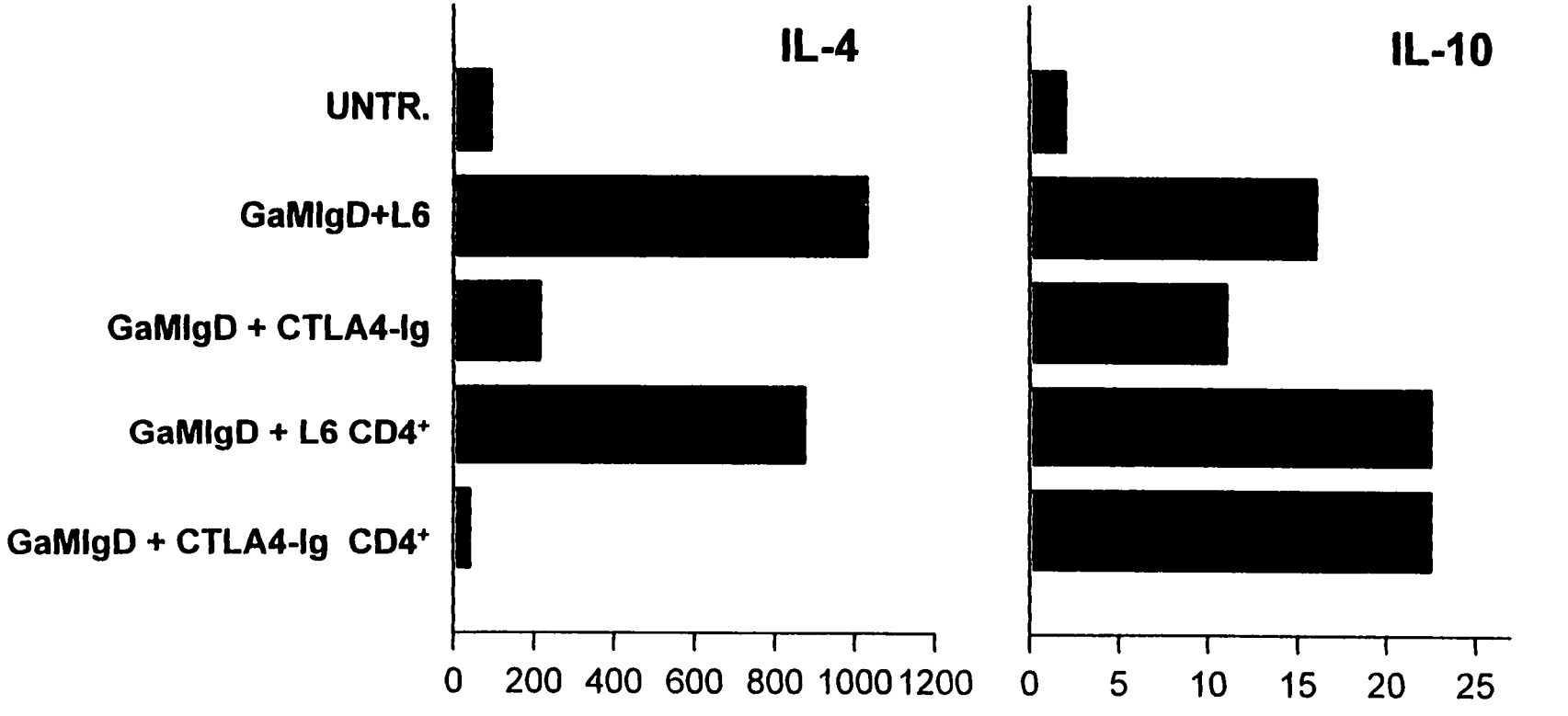
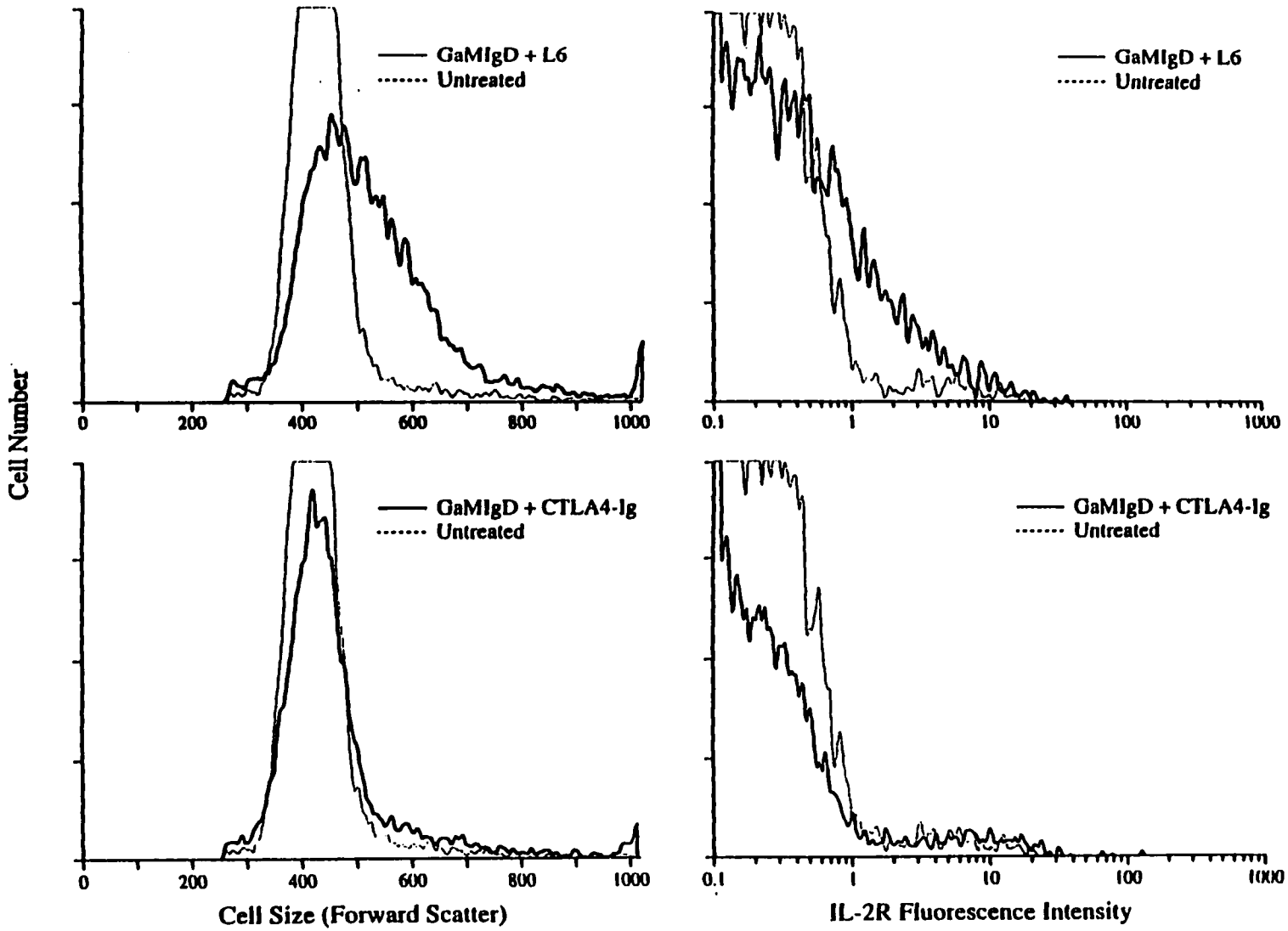

IL-4
IL-10
UNTR.
GaMIgD+L6
GaMIgD + CTLA4-Ig
GaMIgD + L6 CD4+
GaMIgD + CTLA4-Ig CD4+
0 200 400 600 800 1000 1200
0 5 10 15 20 25
CYTOKINE SECRETING CELLS PER 10^6 SPLENIC LYMPHOCYTES

Figure 15. Increases in $CD4^+$ T cell IL-2R expression and cell size are inhibited by blocking CD28-B7 costimulation during the immune response to GaMIgD. GaMIgD immunization and CTLA4-Ig treatment are as described in the legend of Figure 13. Spleen cells were collected at day 6 after immunization and cell suspensions from five individual BALB/c mice per treatment group were pooled and dual stained with FITC-anti-CD4 (GK1.5) and biotinylated anti-IL-2R (7D4) mAbs followed by SA-PE. Single histogram analysis of $CD4^+$ T cell cell size and IL-2R expression are shown. Data shown here is representative of 3 independent experiments.


GaMIgD + L6
Untreated
GaMIgD + CTLA4-Ig
Untreated
Cell Number
0
200
400
600
800
1000
0.1
1
10
100
1000
Cell Size (Forward Scatter)
IL-2R Fluorescence Intensity

5. Blocking CTLA-4 ligand costimulation at a later stage of the immune response to GaMIgD does not inhibit elevations in IL-4 gene expression

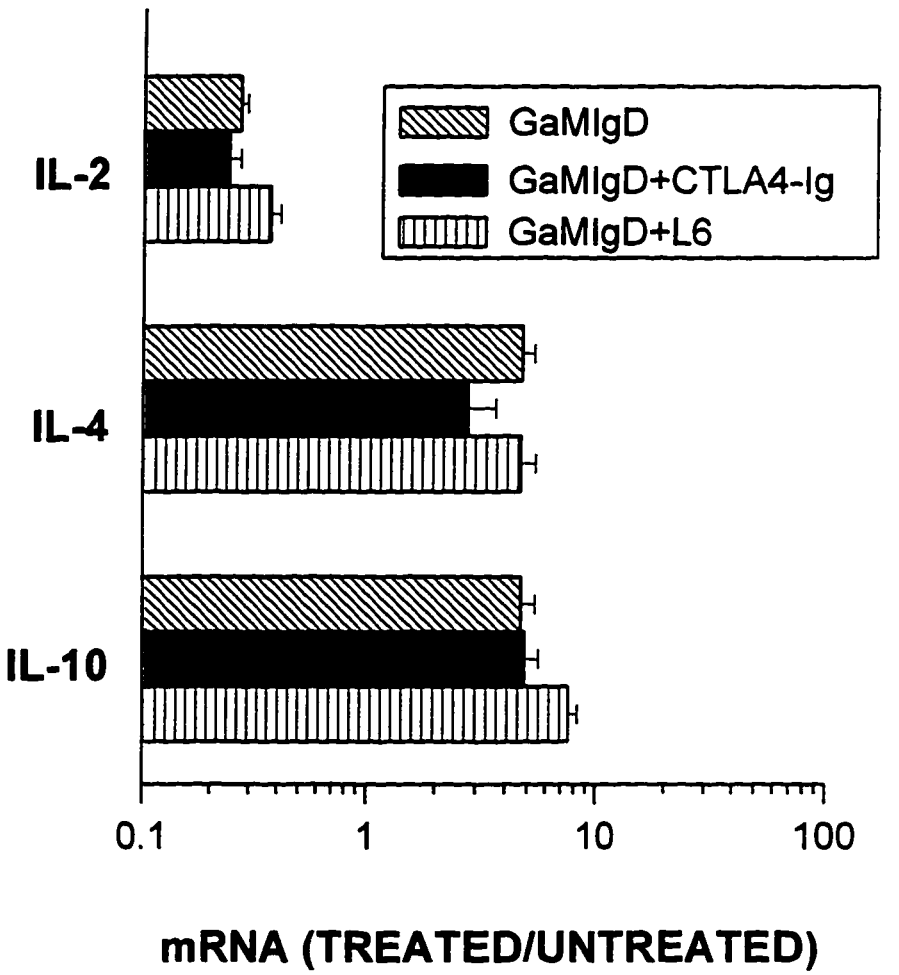
The inhibitory effects of CTLA4-Ig on IL-4 gene expression and $CD4^+$ T cell activation when given at day 0, 1, and 2 indicate that CTLA-4 ligand interactions are required at a very early stage of the immune response to trigger T cell activation. Once T cells become activated, costimulatory signals through CTLA-4 ligand interactions may not be necessary for further T cell proliferation and effector T cell differentiation. To test whether CTLA-4 ligand interactions were involved in the later stage of the immune response to GaMIgD, mice were immunized with GaMIgD and treated with human CTLA4-Ig or L6 at day 3, 4, and 5. At day 6 after immunization, no difference in elevations in IL-4 gene expression in the spleen were detected between CTLA4-Ig- and L6-treated groups (Figure 16).

D. Blocking CTLA-4 ligand interactions inhibit T cell Th2 cytokine production and B cell activation during the *in vivo* type 2 primary immune response to HP

1. CTLA4-Ig inhibits HP-induced cytokine gene expression in the PP and the MLN

Previous studies have shown that elevations in Th2 cytokine gene expression such as IL-3, IL-4, IL-5, and IL-9 in the PP, and IL-3, IL-4, and IL-5 in the MLN were prominent by day 6-8 after primary HP inoculation (Svetic *et al.*, 1993b). To determine whether CTLA-4 ligand interactions were required for increases in Th2-

Figure 16. Administration of CTLA4-Ig at later stage of GaMIgD immunization does not affect elevations in IL-4 gene expression. Mice (5 per group) were injected with 800 μg GaMIgD and human CTLA4-Ig or L6 (100 μg) was administered at day 3, 4, and 5. Spleen samples were collected at day 6 and cytokine gene expression was performed using a quantitative RT-PCR. The data were quantitated and represented as described in Figure 10. Data shown here is representative of 3 independent experiments.

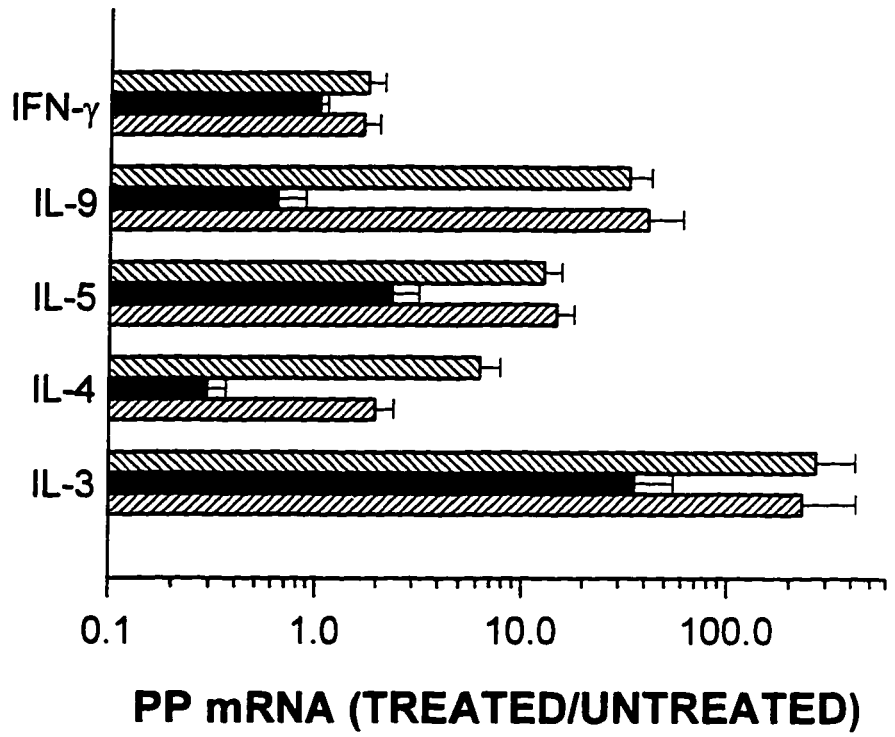

GaMIgD
GaMIgD+CTLA4-Ig
GaMIgD+L6
IL-2
IL-4
IL-10
0.1
1
10
100
mRNA (TREATED/UNTREATED)

associated cytokine mRNA, mice were injected *i.v.* with 100 μg of either murine CTLA4-Ig or the control fusion protein L6 at day 0 and 1 after oral inoculation with 200 third stage HP larvae. On day 8, PP and MLN samples were removed from mice (5 per group) and analyzed individually for cytokine gene expression. CTLA4-Ig blocked elevation in IL-4 gene expression in both the PP and the MLN (Figure 17). Increases in IL-3 gene expression were completely inhibited by CTLA4-Ig in the MLN and partially blocked in the PP, whereas increases in IL-5 gene expression were only partially blocked in both the PP and the MLN. IL-9, usually elevated in the PP but not in the MLN, was also inhibited by CTLA4-Ig. IFN-γ was not elevated in either the PP or the MLN.

2. Blocking CTLA-4 ligand costimulation by CTLA4-Ig administration completely suppresses HP-induced increases in IL-4 but not IL-5 secreting MLN cells

The complete inhibition of HP-induced IL-4, but not IL-5 gene expression by CTLA4-Ig suggests that CTLA4-Ig might have similar effects on cytokine secretion. IL-4 and IL-5 ELISPOT assays were performed to determine the number of IL-4 and IL-5 secreting cells in the MLN from HP-infected, CTLA4-Ig or the control fusion protein L6 treated mice. The number of IL-4 secreting cells in the MLN was markedly increased at day 8 in the HP-infected, L6 treated group. CTLA4-Ig treatment inhibited the number of IL-4 secreting cells to levels close to those of uninfected mice (Figure 18). Although a marked increase in IL-5 secreting cells was also detected in the MLN of HP-infected

Figure 17. CTLA4-Ig administration inhibits elevations in IL-4 and IL-9 and partially inhibits elevations in IL-3 and IL-5 in the PP, and IL-4 and IL-3, but not IL-5, in the MLN at day 8 after HP-inoculation. Mice were administered with either murine CTLA4-Ig (100 μg) or the control L6 (100 μg) at day 0 and 1 after oral inoculation with 200 third stage HP larvae. PP and MLN samples were collected at day 8 and cytokine gene expression levels were determined by a quantitative RT-PCR. Data were presented as in Figure 15. IFN-γ was not consistently elevated in this response and IL-9 was not consistently elevated in the MLN. Data shown here is representative of 3 independent experiments.


IFN-γ
IL-9
IL-5
IL-4
IL-3
0.1
1.0
10.0
100.0
PP mRNA (TREATED/UNTREATED)

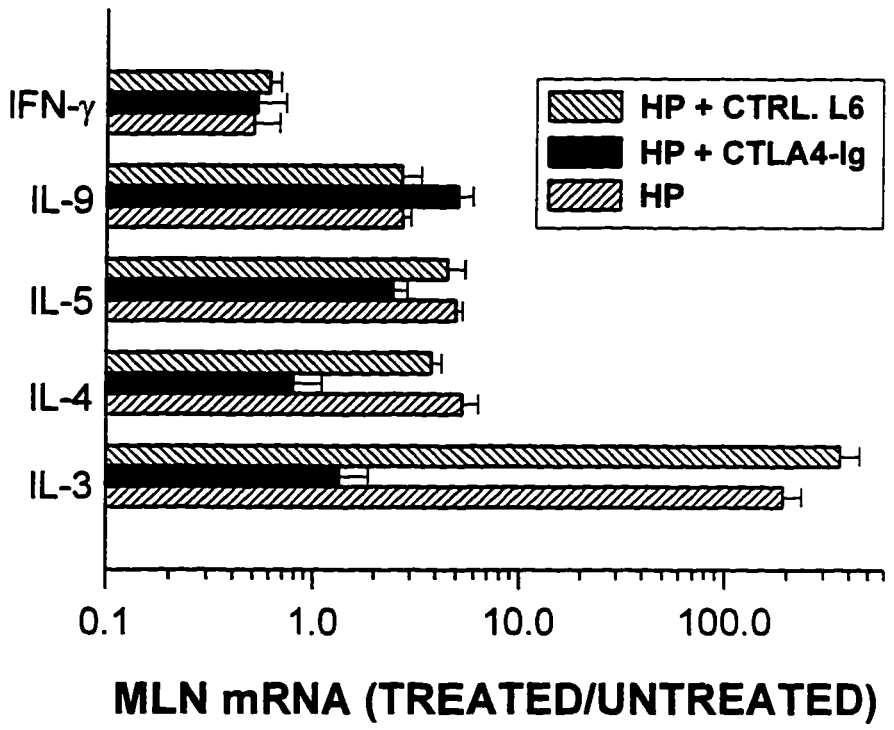
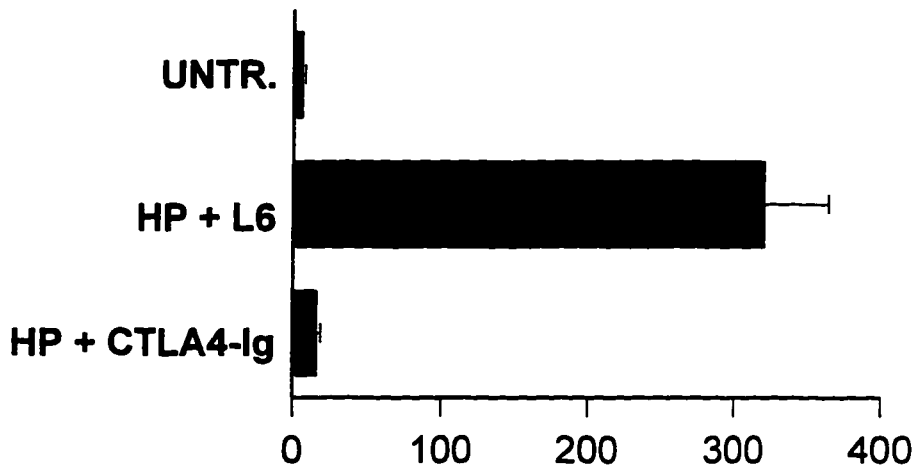
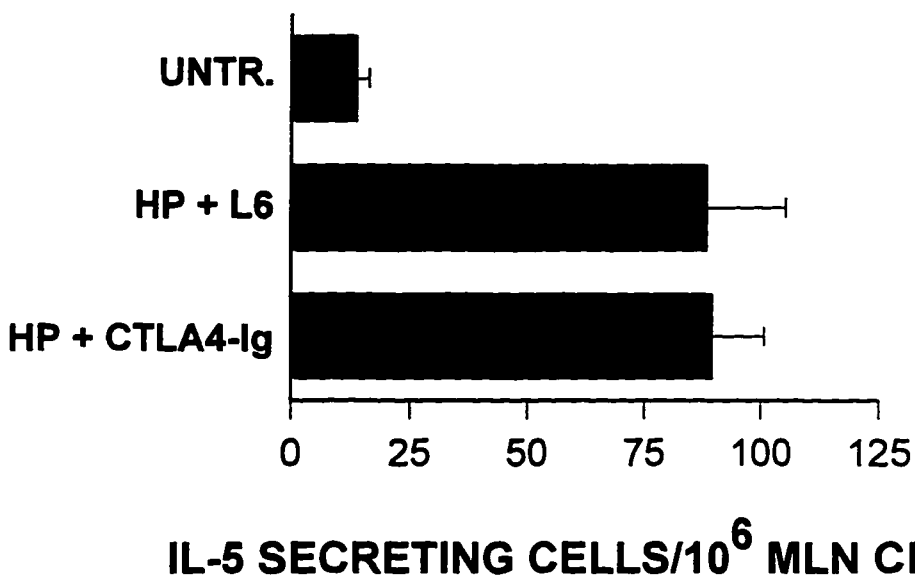

IFN-γ
IL-9
IL-5
IL-4
IL-3
HP + CTRL. L6
HP + CTLA4-Ig
HP
0.1
1.0
10.0
100.0
MLN mRNA (TREATED/UNTREATED)

Figure 18. Elevations in the number of IL-4- but not IL-5-secreting cells from the MLN of HP-inoculated mice are blocked by CTLA4-Ig administration. CTLA4-Ig treatment and HP inoculation are as described in Figure 17. The number of IL-4 and IL-5 secreting cells/10^6 MLN cells were determined in an ELISPOT assay without restimulation. Data were presented as in Figure 9. UNTR: untreated. Data shown here is representative of 3 independent experiments.


UNTR.
HP + L6
HP + CTLA4-Ig
0
100
200
300
400
IL-4 SECRETING CELLS/10^6 MLN CELLS

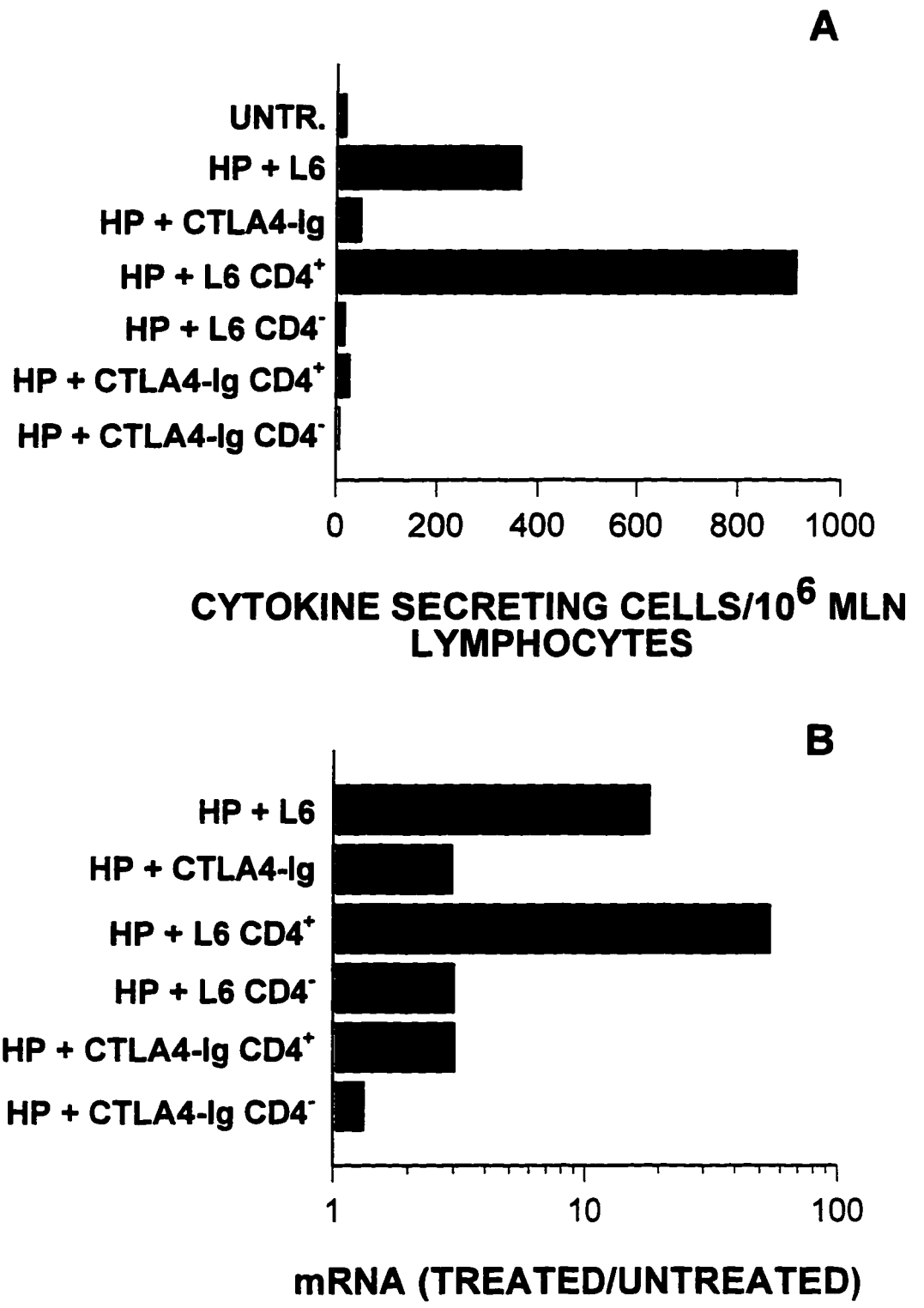

UNTR.
HP + L6
HP + CTLA4-Ig
0
25
50
75
100
125
IL-5 SECRETING CELLS/10^6 MLN CELLS

mice, blocking CTLA-4 ligand costimulation by CTLA4-Ig administration did not affect the increases in the number of IL-5 secreting cells (Figure 18), which is consistent with the less than two-fold inhibition by CTLA4-Ig of IL-5 cytokine gene expression in HP-inoculated mice (Figure 17).

3. Blocking CTLA-4 ligand interactions by CTLA4-Ig inhibits increases in IL-4 protein secretion and gene expression in $CD4^+$, $TCR\alpha/\beta^+$ T cells in the MLN in HP-inoculated mice

Cell sorting studies have shown that $CD4^+$, $TCR\alpha/\beta^+$ T cells are the source of increased IL-4 secretion and gene expression during HP primary infection (Figure 8). The strong inhibitory effect of CTLA4-Ig on IL-4 production in the MLN suggests it may act directly on the $CD4^+$, $TCR\alpha/\beta^+$ T cell population by blocking B7 costimulatory signals from APCs, which is required for naive T cell activation. To test this hypothesis, MLN cells from HP-inoculated, CTLA4-Ig- or control L6-treated mice were dual stained with anti-CD4 and anti-TCRα/β mAbs, and $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted. The number of IL-4 secreting cells and IL-4 mRNA levels were determined by an ELISPOT assay and a quantitative RT-PCR. IL-4 ELISPOT data showed that the elevation in the number of IL-4 secreting cells in $CD4^+$, $TCR\alpha/\beta^+$ T cells population were totally blocked by CTLA4-Ig administration (Figure 19A), and IL-4 gene expression in this population was also inhibited by CTLA4-Ig (Figure 19B). These data indicate costimulatory signals

Figure 19. CTLA4-Ig treatment inhibits elevations in the number of IL-4-secreting cells and IL-4 gene expression in $CD4^+$, $TCR\alpha/\beta^+$ T cells in the MLN from HP-inoculated mice at day 8. CTLA4-Ig treatment and HP infection are as described in the legend of Figure 17. MLN cells from five BALB/c mice in the same group were pooled and stained with FITC-anti-CD4 (GK1.5) and PE-anti-TCRα/β (H57-597) mAbs. $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted from both groups on the same day. A: The number of IL-4 secreting cells per 10^6 MLN lymphocytes was determined by an ELISPOT assay. B: IL-4 gene expression was studied by a quantitative RT-PCR. Data from B were then normalized to the internal standard HPRT and expressed as relative to the uninfected control, which were arbitrarily given a value of 1. UNTR: untreated. Data shown here is representative of 3 independent experiments.

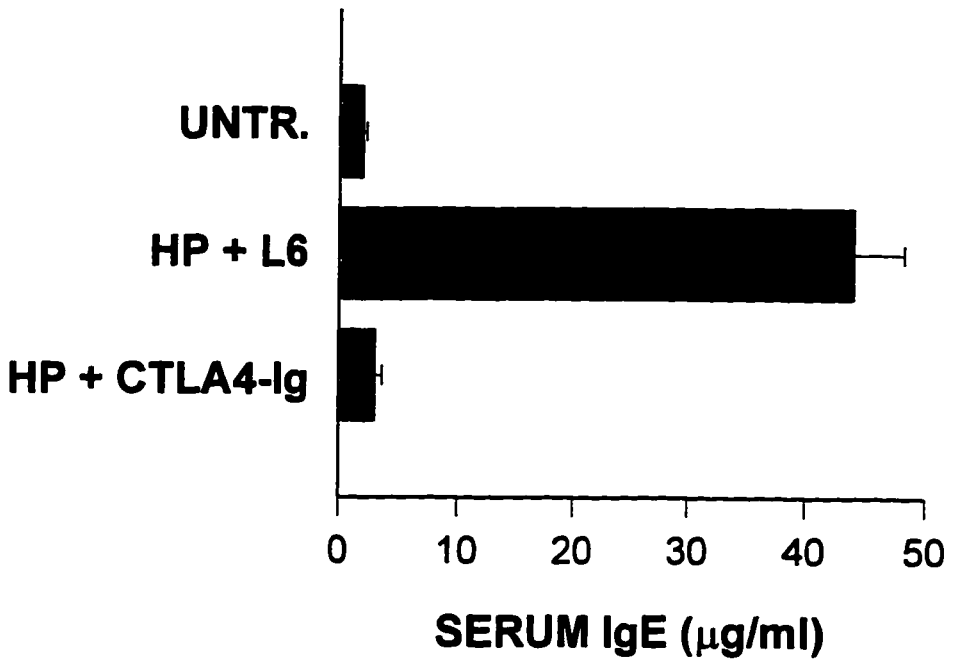

A
UNTR.
HP + L6
HP + CTLA4-Ig
HP + L6 CD4+
HP + L6 CD4-
HP + CTLA4-Ig CD4+
HP + CTLA4-Ig CD4-
0
200
400
600
800
1000
CYTOKINE SECRETING CELLS/10^6 MLN LYMPHOCYTES
B
HP + L6
HP + CTLA4-Ig
HP + L6 CD4+
HP + L6 CD4-
HP + CTLA4-Ig CD4+
HP + CTLA4-Ig CD4-
1
10
100
mRNA (TREATED/UNTREATED)

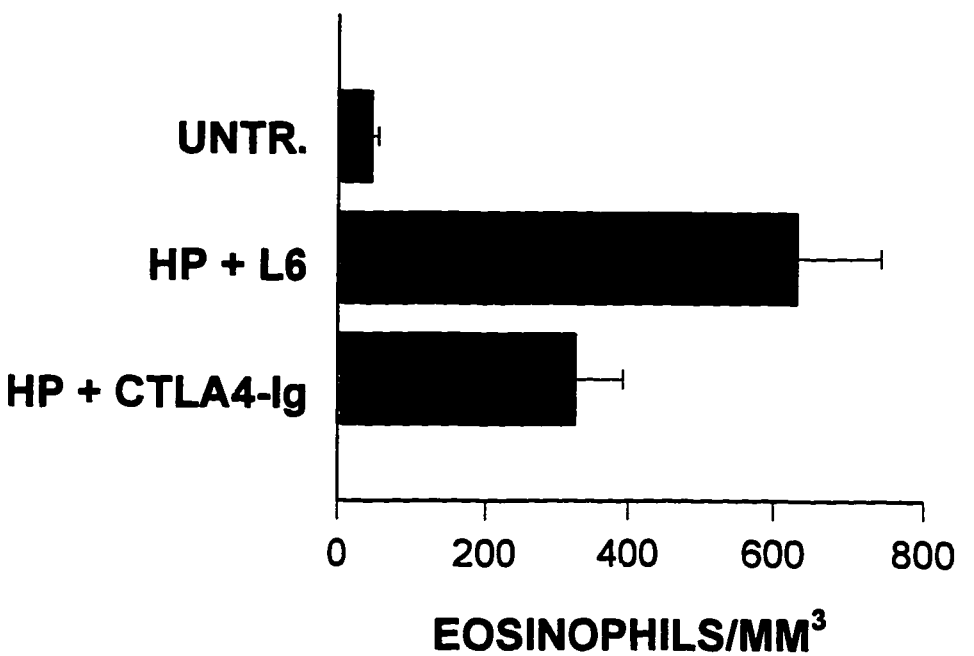
through CD28/CTLA-4 and B7-1/B7-2 interaction are required for increased IL-4 gene expression and secretion in $CD4^+$, $TCR\alpha/\beta^+$ T cells during HP primary infection.

4. CTLA4-Ig administration blocks serum IgE elevations during the immune response to HP

The decreases observed in both IL-4 gene expression and protein secretion suggest that IgE production, which is IL-4 dependent (Finkelman *et al.*, 1990), might be induced by blocking CTLA-4 ligand interactions during the immune response to HP. Because circulating IgE levels are not consistently elevated until 2 wk after HP inoculation, mice were administered with CTLA4-Ig or L6 at day 0 and 1, and blood samples were collected at day 14 after the infection for the determination of serum IgE level and blood eosinophil counts. Mice treated with L6 after HP infection showed marked increases in serum IgE levels, whereas serum IgE levels in HP-inoculated mice treated with CTLA4-Ig were only slightly greater than those of uninfected control (Figure 20). In the same experiment, the HP-induced increases in blood eosinophil counts were, like the increase in the PP and the MLN IL-5 gene expression (Figure 17), only partially inhibited by CTLA4-Ig treatment (Figure 20).

Figure 20. Blocking CTLA-4 ligand costimulation inhibits elevations in serum IgE levels but only partially inhibits blood eosinophil elevations at day 14 after HP inoculation. HP inoculation and CTLA4-Ig treatment are as described in the legend of Figure 17. Mice were bled at day 14 after HP inoculation. Serum IgE levels and blood eosinophil counts were determined as described in Materials and Methods. UNTR: untreated. Data shown here is representative of 3 independent experiments.


UNTR.
HP + L6
HP + CTLA4-Ig
0
10
20
30
40
50
SERUM IgE (μg/ml)


UNTR.
HP + L6
HP + CTLA4-Ig
0
200
400
600
800
EOSINOPHILS/MM3

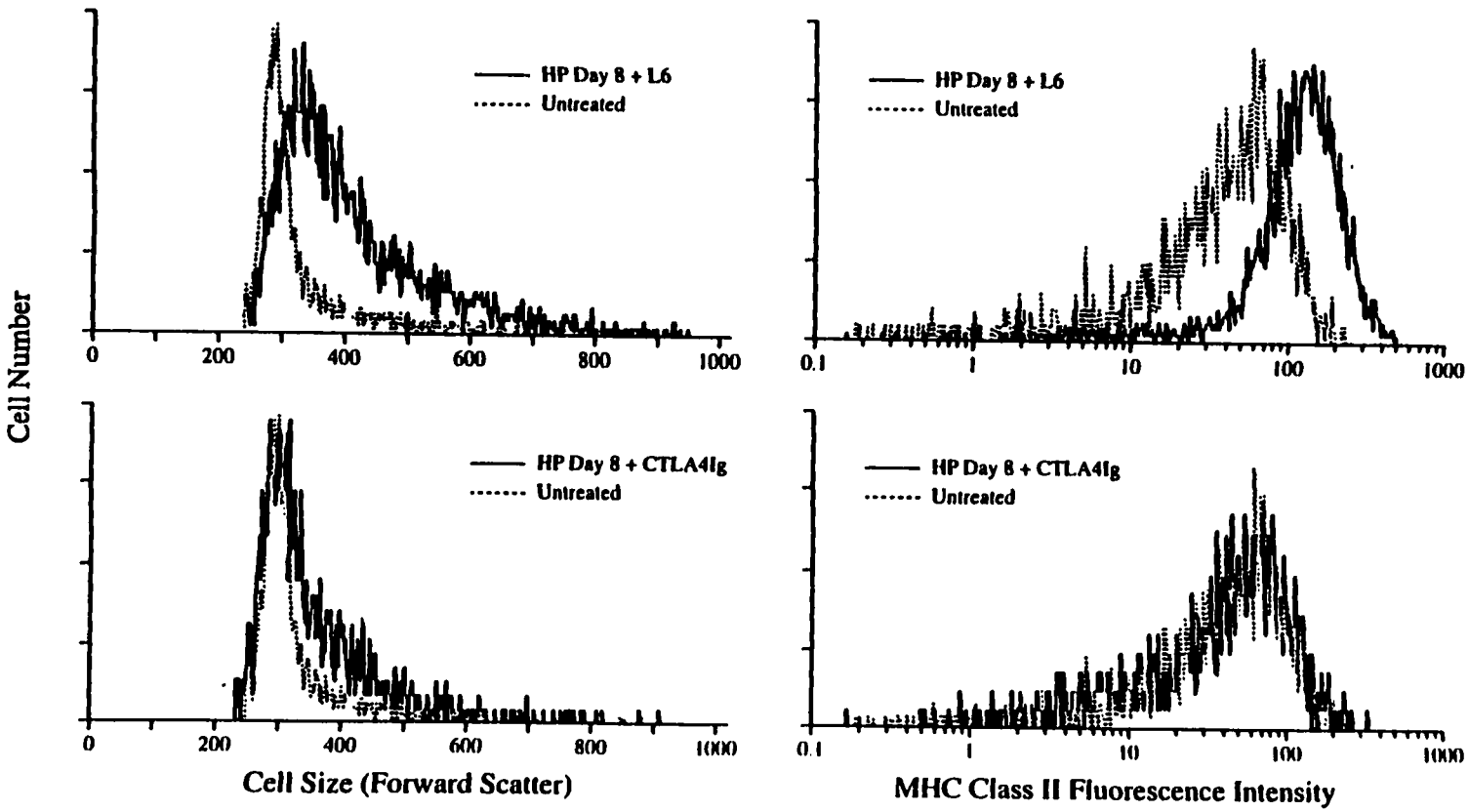
5. B cell activation in primary immune response against HP is also inhibited by CTLA4-Ig administration

The inhibition of serum IgE levels 14 days after HP inoculation suggested that blocking CTLA-4 ligand may affect B cell activation or differentiation to Ig-secreting cells. To examine whether B cell activation was affected by CTLA4-Ig administration, MLN cells from HP-inoculated, CTLA4-Ig- or control L6-treated mice were stained simultaneously with anti-MHC class II and anti-B220 mAbs. Flow cytometric analysis showed that MHC class II expression and cell size (forward light scatter) on $B220^+$ cells were markedly increased in HP-inoculated mice given L6, whereas B cells from HP-inoculated mice given CTLA4-Ig showed surface MHC class II expression and cell size profiles similar to B cells from untreated mice (Figure 21).

6. CTLA-4 ligand costimulation is not required to initiate memory T cell activation during the challenge immune response to HP inoculation

The results thus far strongly indicate that the interaction between CD28/CTLA-4 on T cells and B7-1/B7-2 on APCs are required to initiate the activation of naive $CD4^+$ T cells in Th2-dominate immune responses. *In vitro* studies have shown that contrary to naive $CD4^+$ T cells, memory $CD4^+$ T cells were far less dependent on CD28-B7 costimulation for their activation, while they could be activated by a variety of non-professional APCs which have very low surface B7-1/B7-2 expression (Croft *et al.*, 1994). To reaffirm these observations in our *in vivo* type 2 response model, a challenge

Figure 21. Increases in B cell MHC class II expression and B cell size are inhibited by blocking CD28-B7 interaction during the primary immune response to HP. CTLA4-Ig (100 μg) or the control fusion protein L6 (100 μg) were administered at day 0 and 1 after oral inoculation with 200 third stage HP larvae. MLN tissues were collected at day 8 after inoculation and cell suspension from five individual BALB/c mice in each treatment group were pooled and dual stained with FITC-anti-MHC class II (MDK.6) and Cy5-anti-B220 (6B2). Single histogram analysis of B cell ($B220^+$) size (forward light scatter) and MHC class II expression are shown. Control Ab staining was similar in all groups (data not shown). Data shown here is representative of 3 independent experiments.


Cell Number
HP Day 8 + L6
Untreated
HP Day 8 + CTLA4Ig
Untreated
0
200
400
600
800
1000
0.1
1
10
100
1000
Cell Size (Forward Scatter)
MHC Class II Fluorescence Intensity

immune response against HP was established to test if CTLA4-Ig could block memory T cell activation. BALB/c mice (5 per group) were inoculated with HP and treated with an anti-helminthic drug (pyrantel pamoate) to cure the infection 14 days later. After 60 days, these mice were challenged with a secondary HP inoculation plus either CTLA4-Ig or L6 administration. Both the elevation in the number of IL-4-secreting MLN lymphocytes at day 8 and the serum IgE level were unaffected by CTLA4-Ig administration (Figure 22). Also, there were similar elevations in cytokine gene expression in the PP and the MLN in HP-challenged mice treated with either CTLA4-Ig or L6 (Figure 23). Furthermore, purified $CD4^+$, $TCR\alpha/\beta^+$ T cells from the MLN of both CTLA4-Ig- and L6-treated, HP-challenged mice showed similar increases in the number of IL-4 secreting cells and in IL-4 mRNA levels (Figure 24).

E. Investigation of the differential costimulatory effects of B7-1 and B7-2 during the *in vivo* immune response to HP inoculation

The requirement of signaling through CTLA-4 ligands during the early stage of naive T cell activation in the HP-induced immune response has been demonstrated. However, CTLA4-Ig binds both B7-1, B7-2 and perhaps other unidentified CTLA-4 ligands. We were interested in directly examining whether B7-1 and/or B7-2 signaling was required to costimulate naive T cells and promote the type 2 immune response. Both B7-1 and B7-2 have been shown to deliver similar costimulatory signals for T cell proliferation and IL-2 production (Lanier *et al.*, 1995). But recent *in vivo* studies showed

Figure 22. CTLA4-Ig administration does not block elevations in serum IgE and IL-4 secretion in a challenge response against HP. BALB/c mice (5 per group) were orally inoculated with 200 third stage HP larvae. Fourteen days later these mice were treated with pyrantel pamoate (2 mg per mouse), an anti-helminthic drug which causes rapid worm expulsion and cures the infection. After 60 days, mice were orally inoculated again with 200 third stage HP larvae and treated with *i.v.* injection of 100 μg murine CTLA4-Ig or L6 at day 0 and 1 after the challenge. MLN samples were collected at day 8 after the challenge infection for the IL-4 ELISPOT assay and blood samples were collected at day 14 for serum IgE detection. UNTR: untreated. Results shown here is representative of 3 independent experiments.

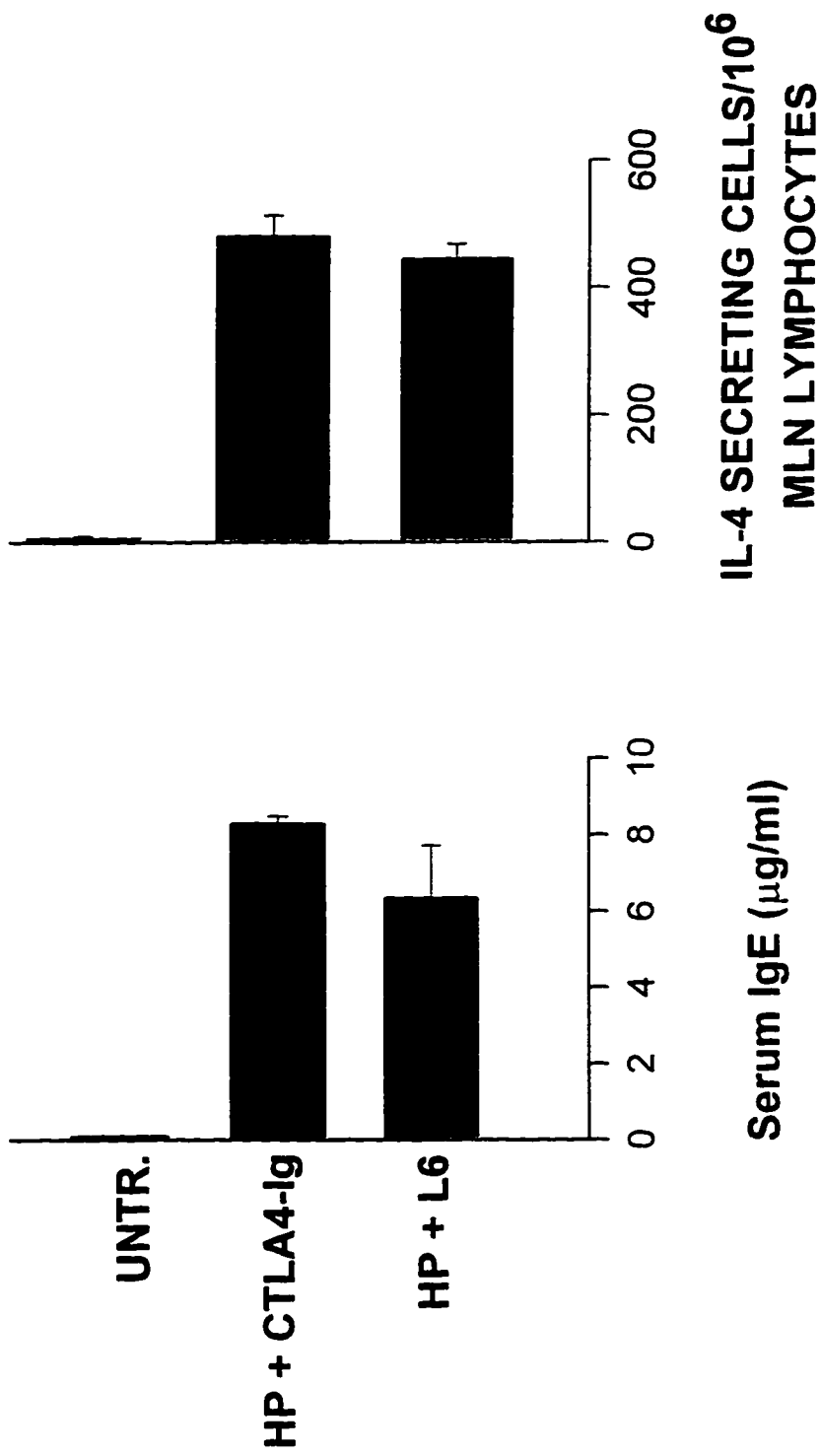

UNTR.
HP + CTLA4-Ig
HP + L6
0
2
4
6
8
10
Serum IgE (μg/ml)
0
200
400
600
IL-4 SECRETING CELLS/10^6
MLN LYMPHOCYTES

Figure 23. CTLA4-Ig administration does not affect elevations in cytokine gene expression in the PP and the MLN in a challenge response against HP. HP challenge inoculation (HP 2^{o}) and CTLA4-Ig treatment were described in the legend of Figure 22. PP and MLN samples were collected at day 8 after the challenge infection. Cytokine mRNA levels were determined by a quantitative RT-PCR. Data were presented as described in Figure 10. Results shown here is representative of 2 independent experiments.

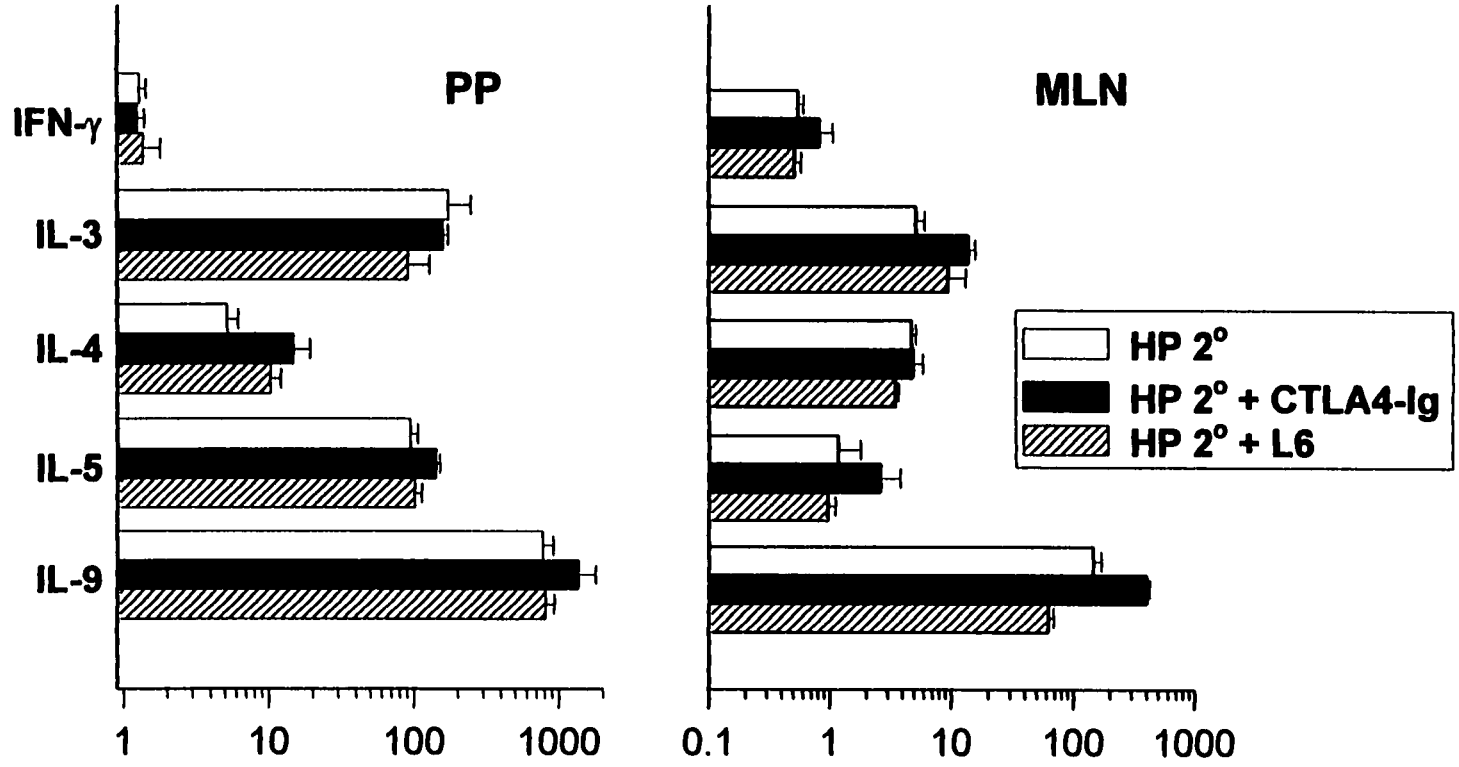
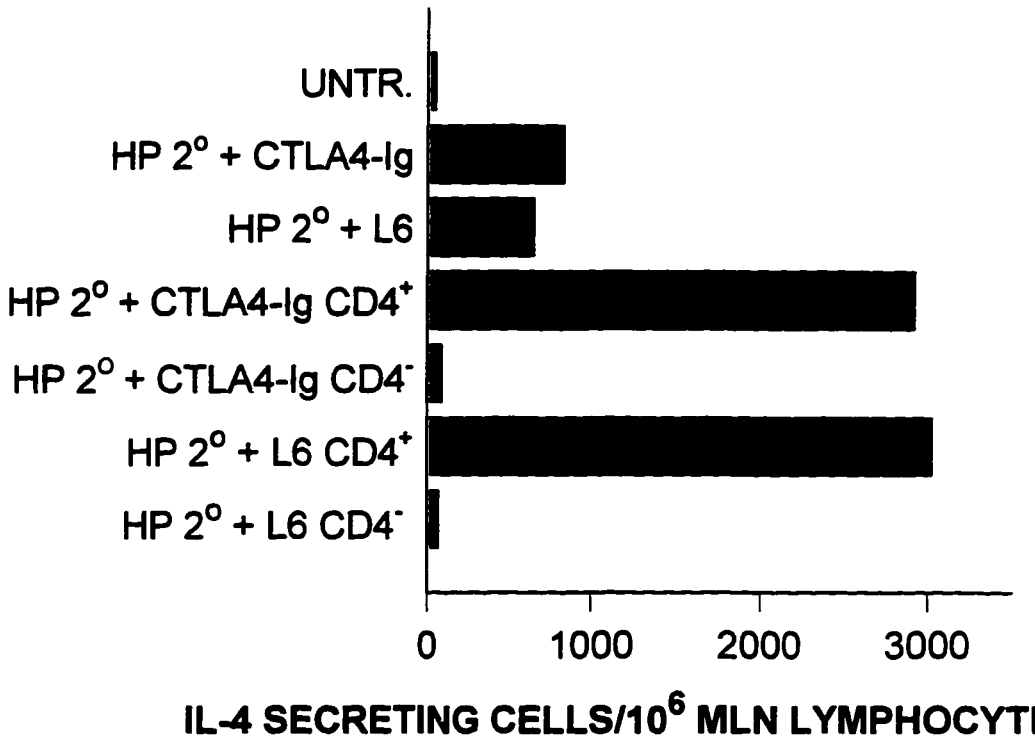
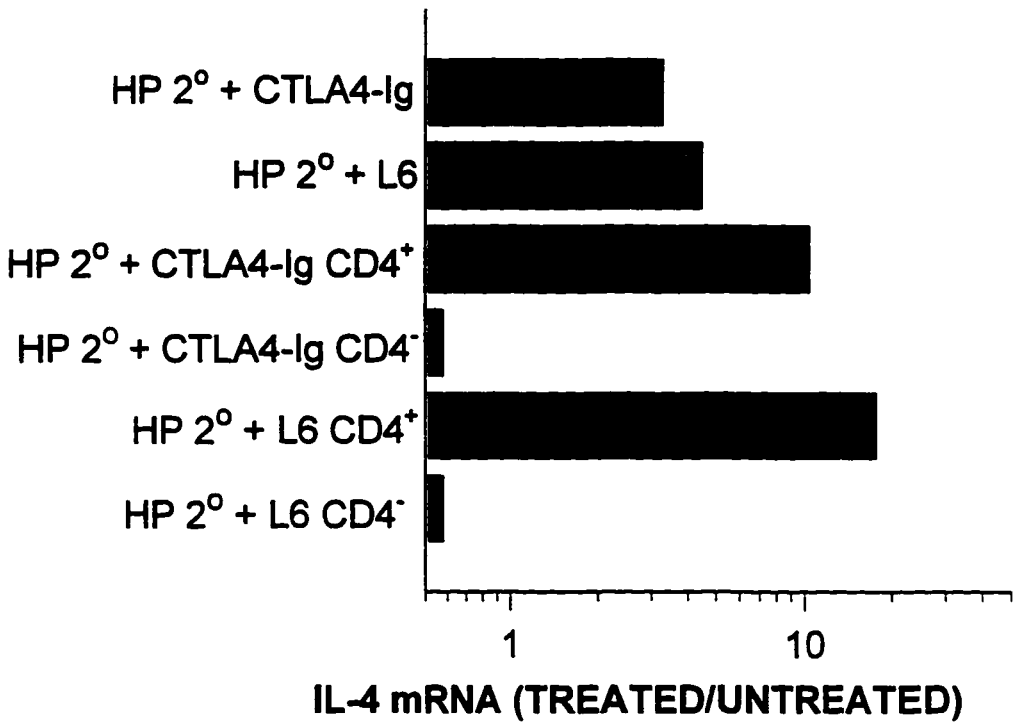

PP
MLN
IFN-γ
IL-3
IL-4
IL-5
IL-9
HP 2°
HP 2° + CTLA4-Ig
HP 2° + L6
1
10
100
1000
0.1
1
10
100
1000
mRNA (TREATED/UNTREATED)

Figure 24. CTLA4-Ig administration does not block increases in the number of IL-4-secreting cells and IL-4 gene expression in $CD4^+$, $TCR\alpha/\beta^+$ T cells in the MLN during the challenge immune response to HP. HP challenge inoculation (HP 2^o) and CTLA4-Ig treatment were as described in the legned of Figure 22. MLN samples were collected at day 8 after the challenge infection and pooled from 5 individual mice in the same group. MLN cells were dual stained with FITC-anti-CD4 (GK1.5) and PE-anti-TCRα/β (H57-597) mAbs. $CD4^+$, $TCR\alpha/\beta^+$ T cells were sorted from both groups on the same day using an Epics Elite cell sorter and were shown to be >95% pure. The number of IL-4 secreting cells per 10^6 MLN lymphocytes was determined by an ELISPOT assay and IL-4 gene expression was detected by a quantitative RT-PCR. UNTR: untreated. Results shown here is representative of 3 independent experiments.


UNTR.
HP 2° + CTLA4-Ig
HP 2° + L6
HP 2° + CTLA4-Ig CD4+
HP 2° + CTLA4-Ig CD4-
HP 2° + L6 CD4+
HP 2° + L6 CD4-
0
1000
2000
3000
IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES


HP 2° + CTLA4-Ig
HP 2° + L6
HP 2° + CTLA4-Ig CD4+
HP 2° + CTLA4-Ig CD4-
HP 2° + L6 CD4+
HP 2° + L6 CD4-
1
10
IL-4 mRNA (TREATED/UNTREATED)

different effects of anti-B7-1 and anti-B7-2 mAb treatment in experiental autoimmune diseases such as EAE and NOD, and *Leishmania* infection models (Kuchroo *et al.*, 1995; Lenschow *et al.*, 1995). In fact, some findings suggested that B7-1 costimulation may favor a type 1 T cell responses while B7-2 costimulation favors a type 2 responses (Kuchroo *et al.*, 1995). In order to elucidate the roles of B7-1 and B7-2 in the HP infection model, anti-B7-1 and anti-B7-2 mAbs were administered alone or in combination to HP-inoculated mice and their effects on cytokine production, serum Ig levels, blood eosinophil numbers, and B cell activation were investigated.

1. MLN IL-4 and IL-5 secretion are inhibited only when both B7-1 and B7-2 are blocked in the immune response to HP inoculation

To investigate the possible differential costimulation of B7-1 and B7-2 during naive T cell activation, the effect of blocking B7-1 and/or B7-2 costimulation on IL-4 and IL-5 secretion in the MLN in mice infected with HP at day 8 and 14 were examined. Anti-B7-1 and anti-B7-2 mAbs were administered to HP-inoculated mice either alone or in combination. Results from the IL-4 and IL-5 ELISPOT assay showed that anti-B7-1 and anti-B7-2 mAbs given alone had no effect on the elevations in IL-4 and IL-5 secretions; however, the combination of the two mAbs markedly inhibited the increases in IL-4 secretion at both day 8 and 14 after HP inoculation (Figure 25, 26). Elevations in IL-5 secretion are not affected at day 8, but were blocked at day 14 in the group administered both anti-B7-1 and -B7-2 mAbs (Figure 25, 26).

Figure 25. Blocking both B7-1 and B7-2 costimulation is required to inhibit increases in IL-4 secretion at day 8 after HP inoculation. Mice (5 per group) were orally inoculated with 200 third stage HP larvae. Anti-B7-1 (100 μg) and/or anti-B7-2 (100 μg) mAbs were injected *i.v.* at day 0 and 4 after HP inoculation. For the control group, hamster IgG (100 μg) and rat IgG2a (100 μg) were injected the same time. MLN samples were collected at day 8 and the number of IL-4 and IL-5 secreting cells per 10^6 MLN lymphocytes were determined by an ELISPOT assay. The arithmetic mean and SEM derived from data of five individual BALB/c mice are shown for each treatment group. UNTR: untreated. Results shown here are the representative of 3 independent experiments.

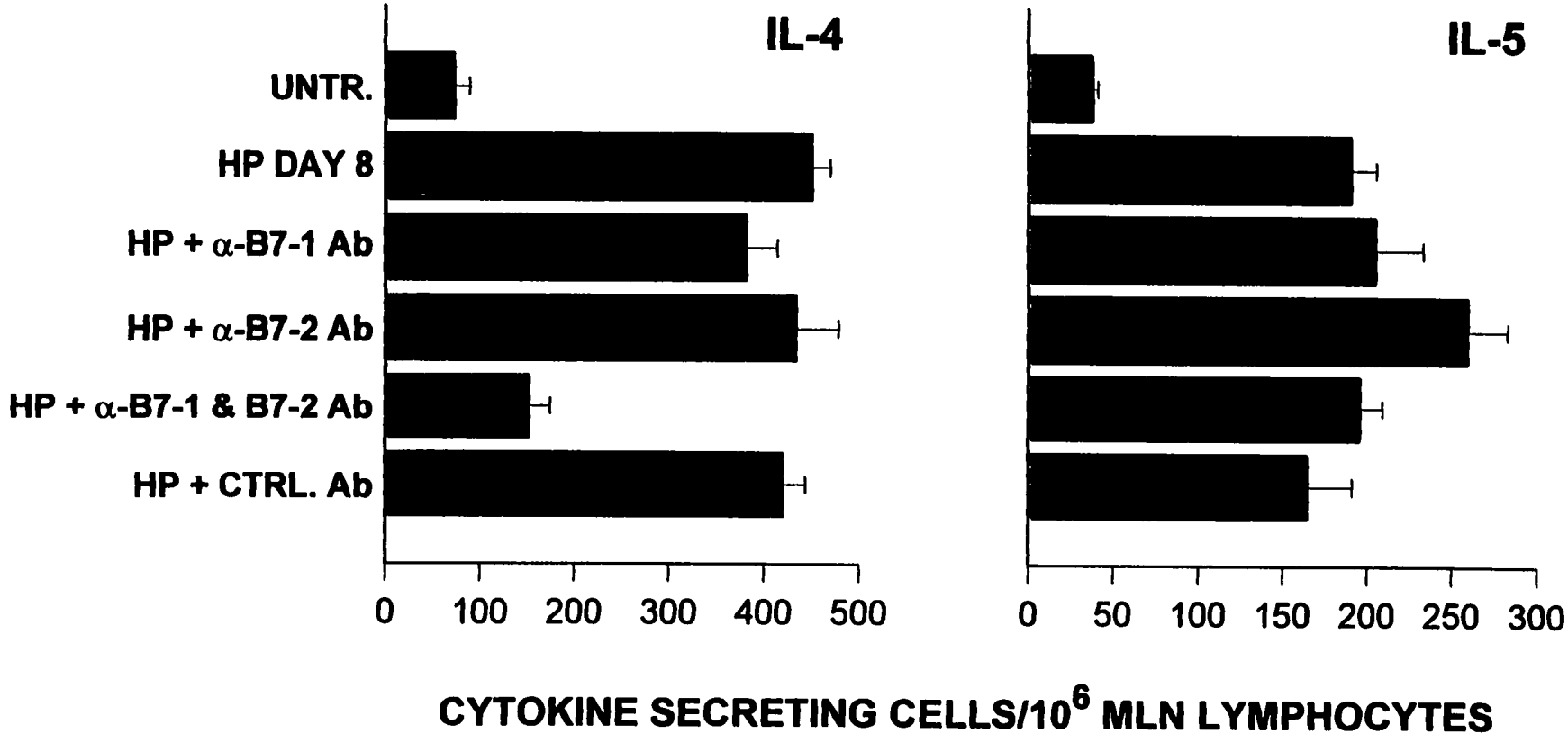
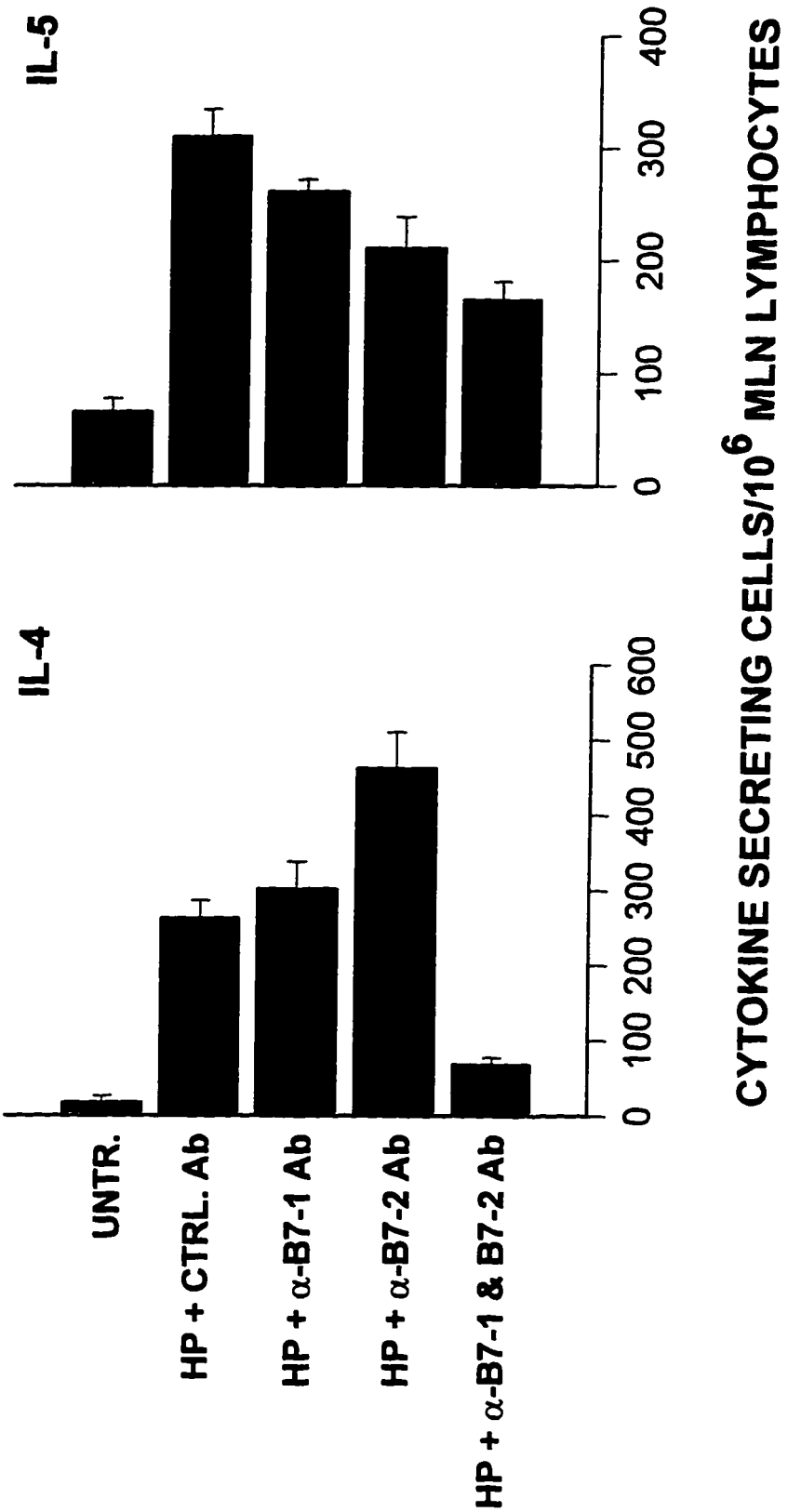

IL-4
IL-5
UNTR.
HP DAY 8
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1 & B7-2 Ab
HP + CTRL. Ab
0
100
200
300
400
500
0
50
100
150
200
250
300
CYTOKINE SECRETING CELLS/10^6 MLN LYMPHOCYTES

Figure 26. Blocking both B7-1 and B7-2 costimulatory signaling inhibits IL-4 and IL-5 secretion at day 14 after HP inoculation. Mice were orally inoculated with 200 third stage HP larvae. Anti-B7-1 (100 μg) and/or anti-B7-2 (100 μg) mAb were injected *i.v.* at day 0, 4, 8, and 12 after HP inoculation. Mice in the control Ab group were administered with hamster IgG (100 μg) and rat IgG2a (100 μg) at the same time. IL-4 and IL-5 ELISPOT assays are as described in Materials and Methods. The mean and SE derived from data of five individual BALB/c mice are shown for each treatment group. UNTR: untreated. Results shown here is representative of 2 independent experiments.


IL-5
IL-4
0
100
200
300
400
500
600
UNTR.
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1 & B7-2 Ab
CYTOKINE SECRETING CELLS/10^6 MLN LYMPHOCYTES

2. The combination of anti-B7-1 and-B7-2 mAbs inhibits HP-induced IL-4 gene expression in MLN at day 14 after HP inoculation

The blockage of HP-induced increases in IL-4 and IL-5 secretion only by anti-B7-1 and-B7-2 mAbs in combination implies that cytokine gene expressions in the MLN are also affected. MLN and PP samples were taken from each mouse at day 8 and day 14 in the above experiments and cytokine gene expressions were analyzed. Although none of the treatment groups shows reduced cytokine gene expression at day 8 after HP infection(data not shown), IL-4 and also IL-9 gene expression were greatly inhibited in the MLN and the PP in the group given both anti-B7-1 and-B7-2 mAbs at day 14. However, IL-5 gene expression was not affected (Figure 27).

3. The combination of anti-B7-1 and anti-B7-2 mAbs is required to block elevations in serum IgG1 level, blood eosinophilia, and mucosal mastocytosis

Results from previous studies have shown that costimulatory signals mediated by CTLA-4 ligand interactions were critical for serum IgE elevations. The inhibition of IL-4 secretion and gene expression, with partial inhibition of IL-5 secretion following blocking both B7-1 and B7-2 signaling suggested that serum Ig levels eosinophilia and mucosal mastocytosis may also be affected. To examine this possibility, mice were infected with HP and treated with anti-B7-1 and/or anti-B7-2 mAbs at day 0, 4, 8, and 12. Mice were bled at day 14, IgG1 levels, blood eosinophil and mucosal mastocytosis were

Figure 27. Combination of anti-B7-1 and anti-B7-2 mAbs blocks elevations in IL-4 and IL-9 gene expression in the PP and the MLN at day 14 after HP inoculation. HP infection, administration of anti-B7-1/anti-B7-2 and control (CTRL.) Abs for the day 14 experiment were performed as described in the legend of Figure 26. PP and MLN samples were collected at day 14 and cytokine mRNA levels were determined by a quantitative RT-PCR. The data were quantitated by using a PhosphorImager (Molecular Dynamics) and were individually normalized to the endogenous standard HPRT gene expression. The mean and SE were derived from five individual BALB/c mice. The means are expressed relative to the mean of the uninfected control group, which was arbitrarily given a value of 1. Data shown here is representative of 3 independent experiments.

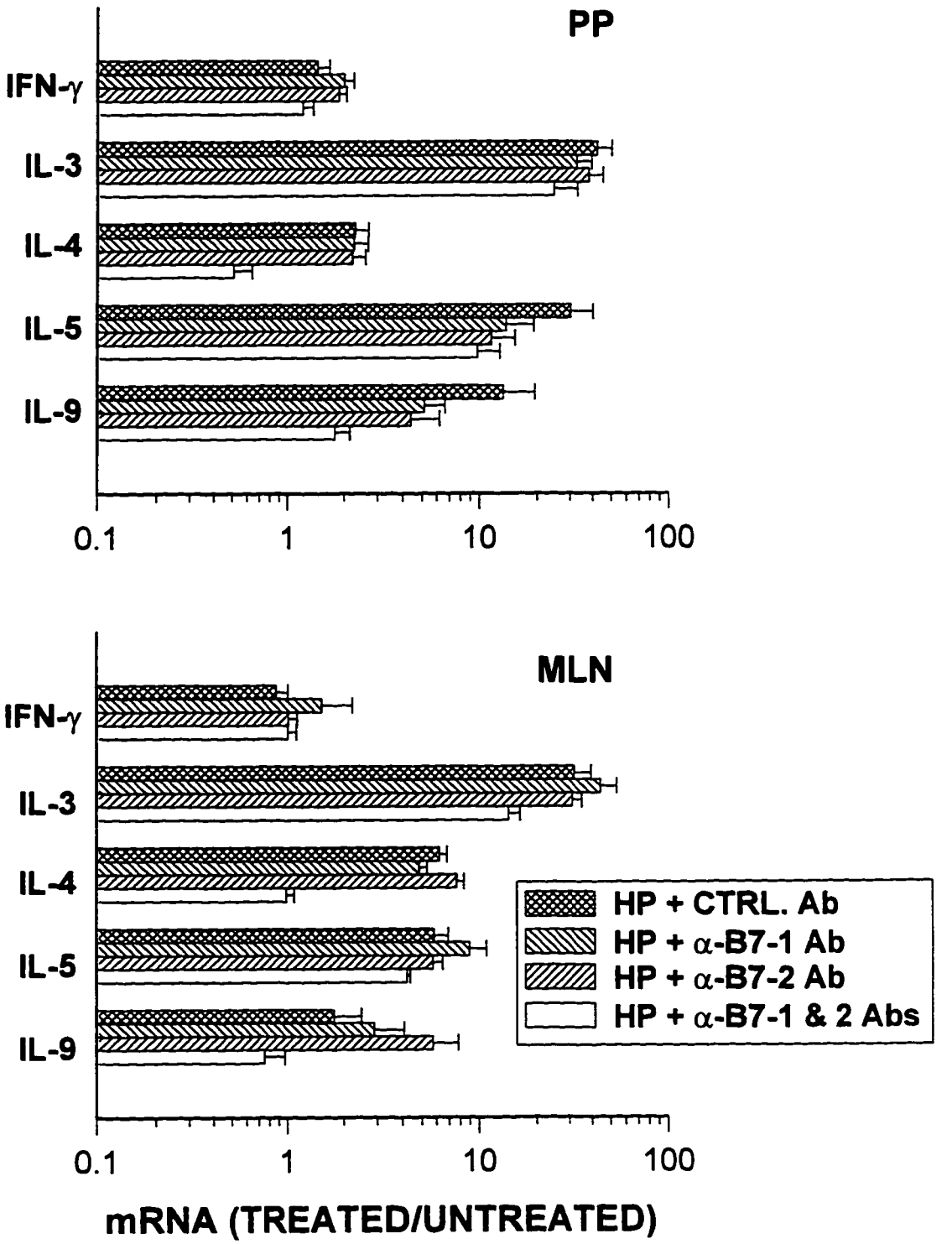
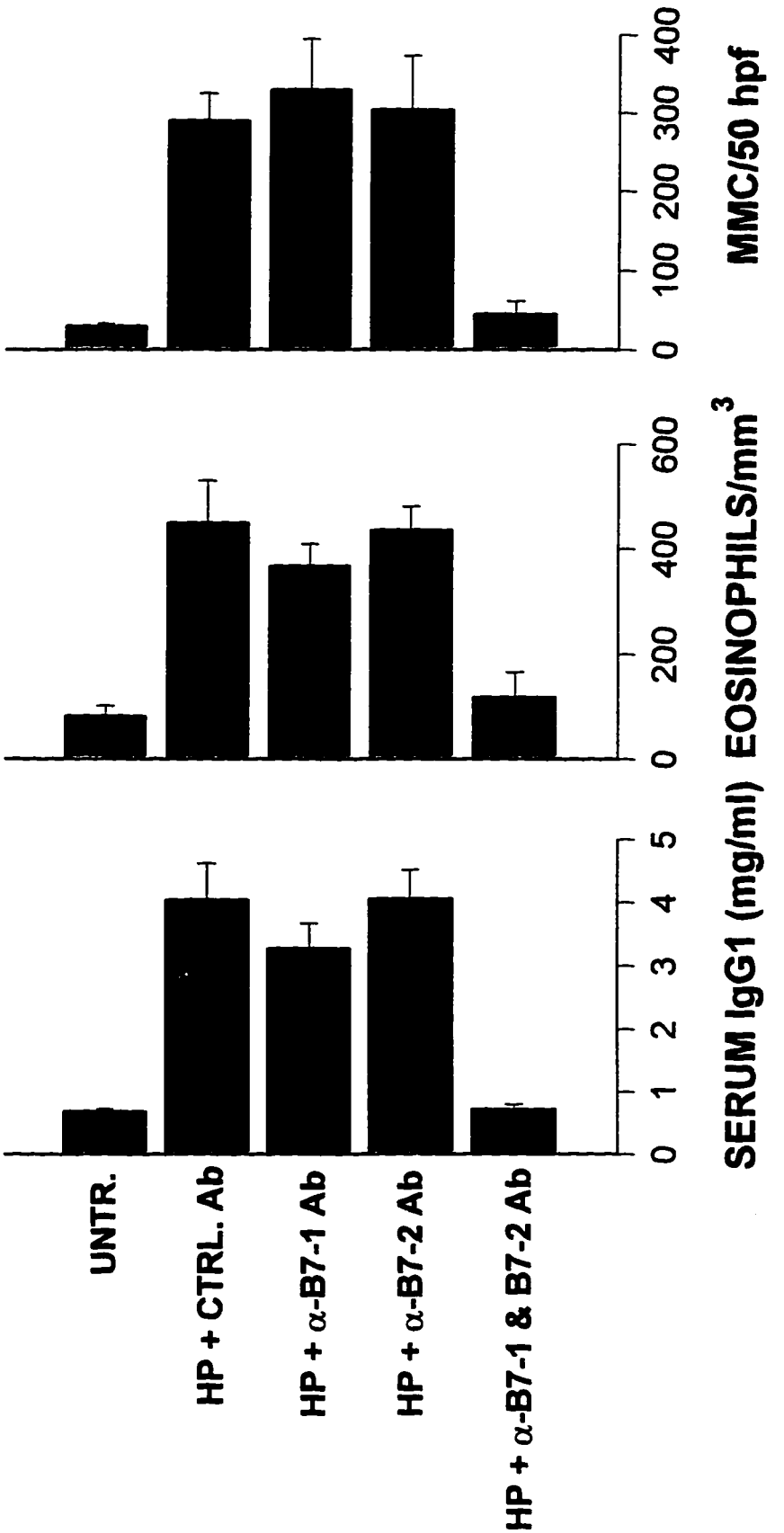

PP
IFN-γ
IL-3
IL-4
IL-5
IL-9
0.1
1
10
100
MLN
IFN-γ
IL-3
IL-4
IL-5
IL-9
0.1
1
10
100
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1 & 2 Abs
mRNA (TREATED/UNTREATED)

Figure 28. Blocking both B7-1 and B7-2 costimulation inhibits elevations in serum IgG1 levels, blood eosinophilia and mastocytosis at day 14 after HP inoculation. HP infection, anti-B7-1 / anti-B7-2 and control (CTRL.) Ab administration were described in Figure 25. Mice were bled at day 14 and serum IgE levels were determined by a micro-ELISA. The data are expressed as the geometric mean and SE for serum IgG1 levels and the arithmetic mean and SE for eosinophil and mucosal mast cell number. Each treatment group includes five individual BALB/c mice. MMC/ 50 hpf: mucosal mast cells/50 high-powered microscope fields. UNTR: untreated. Data shown here is representative of 2 independent experiments.

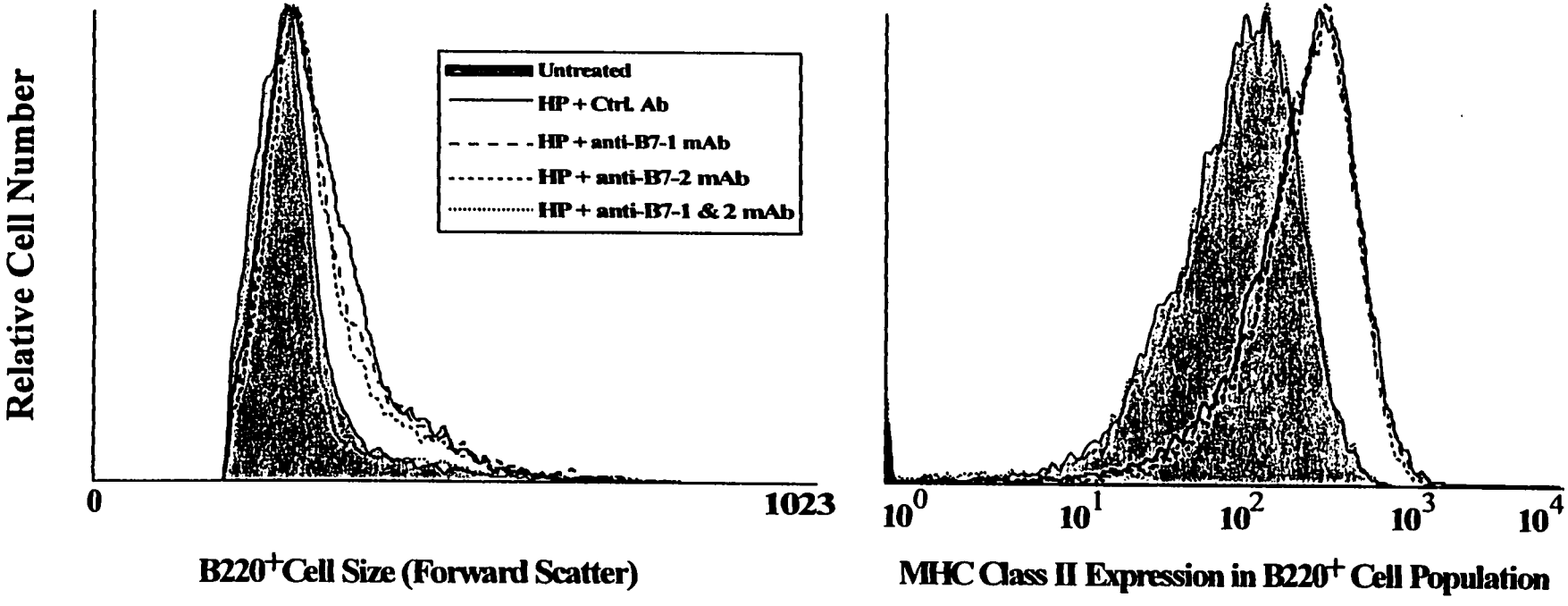

UNTR.
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1 & B7-2 Ab
SERUM IgG1 (mg/ml)
0 1 2 3 4 5
EOSINOPHILS/mm3
0 200 400 600
MMC/50 hpf
0 100 200 300 400

determined (Figure 28). HP infection caused significant elevations in serum IgE levels, the number of blood eosinophils and intestinal mucosal mast cells by day 14. Treatment with either anti-B7-1 or B7-2 mAb alone did not alter these elevations. However, administration of the combination of both mAbs totally inhibited elevations in serum IgE levels, blood eosinophilia and mucosal mastocytosis.

4. B cell activation is blocked by administration of the combination of anti-B7-1 and anti-B7-2 antibodies

The inhibition of elevations in serum IgG1 levels 14 days after HP inoculation suggested that blocking B7-ligand interactions may affect B cell activation or differentiation to Ig-secreting cells. To examine whether B cells were activated in HP-inoculated mice administered either anti-B7-1 mAb, anti-B7-2 mAb or both, MLN cell suspensions from these mice were stained simultaneously with anti-MHC class II and B cell-specific anti-B220 mAbs at day 14. MHC class II expression and forward light scatter (a correlate of cell size) on $B220^+$ cells were markedly increased in HP-inoculated mice administered the control antibodies, whereas HP-inoculated mice given the combination of anti-B7-1 and anti-B7-2 mAbs showed MHC class II and forward light scatter profiles similar to B cells from untreated mice (Figure 29). The administration of either anti-B7-1 or anti-B7-2 mAb alone showed a slight, but reproducible decrease in B cell size, but no appreciable decrease in MHC class II expression compared to levels observed in HP-inoculated mice given control antibodies. Increase in B cell number in

Figure 29. The combination of anti-B7-1 and anti-B7-2 mAb treatment blocks increases in B cell surface MHC class II expression and cell size at day 14 after infection. HP inoculation, anti-B7-1 and/or anti-B7-2 mAb treatment, and control (Ctrl.) Ab administration are as in the legend of Figure 26. MLN samples were collected and pooled from five individual mice in each treatment group. Cells were simultaneously stained with Cy5-anti-B220 (6B2) and FITC-anti-MHC class II (MDK6) mAbs. FACS analysis was performed on an Epics Elite flow cytometer. Single histogram analysis of B cell ($B220^+$) size and MHC class II expression are shown. Data shown here is representative of 3 independent experiments.


Relative Cell Number
Untreated
HP + Ctrl. Ab
HP + anti-B7-1 mAb
HP + anti-B7-2 mAb
HP + anti-B7-1 & 2 mAb
0
1023
B220+Cell Size (Forward Scatter)
10^0
10^1
10^2
10^3
10^4
MHC Class II Expression in B220+ Cell Population

the MLN were also blocked by the combination of anti-B7-1 and anti-B7-2 mAb but not by either mAb alone (data not shown).

F. Study of the costimulatory function of CD40-CD40L (gp39) during the *in vivo* type 2 immune response to HP inoculation

CD40-CD40L (gp39) is another important receptor-ligand pair during the initiation of the immune response. Previous reports showed CD40-CD40L costimulation was important for B cell activation, Ig class switching, and secretion (Defrance *et al.*, 1992; Hermann *et al.*, 1993). However, recent studies indicate that CD40-CD40L interaction might also be required for optimal T cell activation (Fanslow *et al.*, 1994b). It has been proposed that CD40-CD40L interactions are a prerequisite for CD28-B7 costimulation to activate T cells to produce cytokines. In order to test this hypothesis, anti-gp39 mAb was applied to block the interaction of CD40-CD40L during the *in vivo* immune response to HP inoculation. The effects on T cell cytokine production, B cell activation and Ig production, blood eosinophilia, and mastocytosis were investigated.

1. Administration of anti-gp39 Ab suppresses elevations in serum IgG1, blood eosinophilia, and intestinal tissue mastocytosis

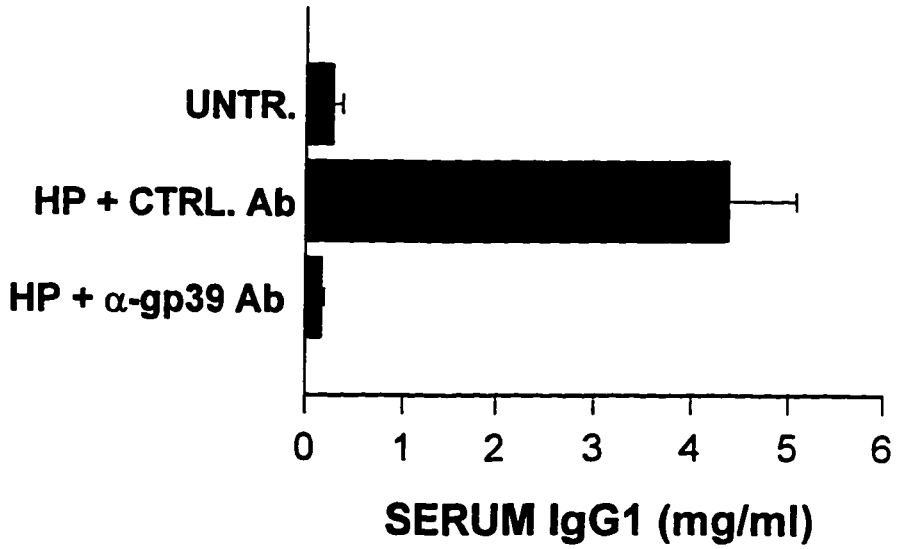
Previous studies have shown that blocking CD40/CD40L interactions profoundly inhibit humoral immunity (Defrance *et al.*, 1992; Hermann *et al.*, 1993). To determine whether blocking CD40L interactions affect increases in serum immunoglobulin levels

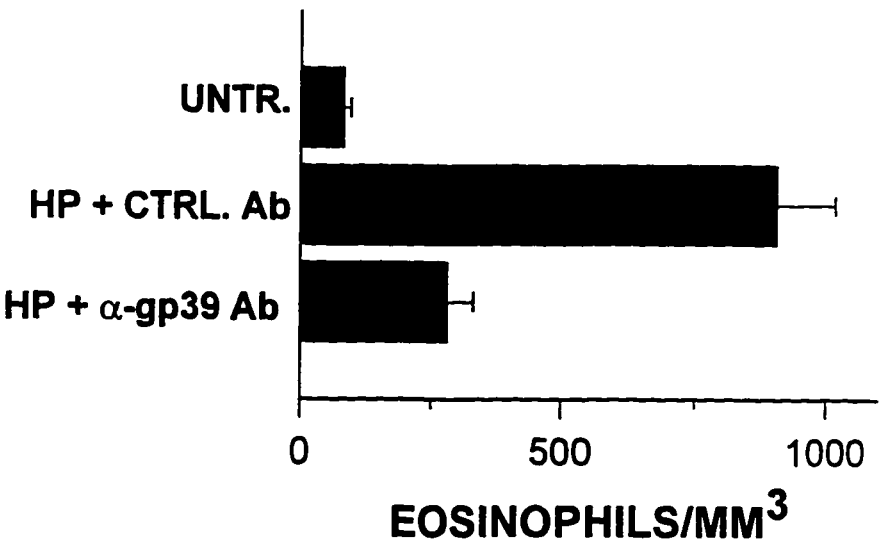
and allergy-associated responses during a primary type 2 immune response, mice were inoculated with 200 third stage HP larvae and, at days 0 and 7, injected *i.v.* with 2 mg of either hamster anti-gp39 (MR1) mAb or control hamster Ig. Fourteen days after HP inoculation, blood samples were taken and serum IgG1 levels, blood eosinophil levels, and intestinal mucosal mastocytosis were evaluated. As shown in Figure 30, mice administered control hamster antibodies at day 0 and 7 after HP inoculation showed marked increases in serum IgG1, whereas IgG1 levels in anti-gp39 mAb-treated HP-inoculated mice were comparable to those of uninfected control mice. Blocking CD40L interactions also partially inhibited blood eosinophilia and mucosal mastocytosis in three independent experiments.

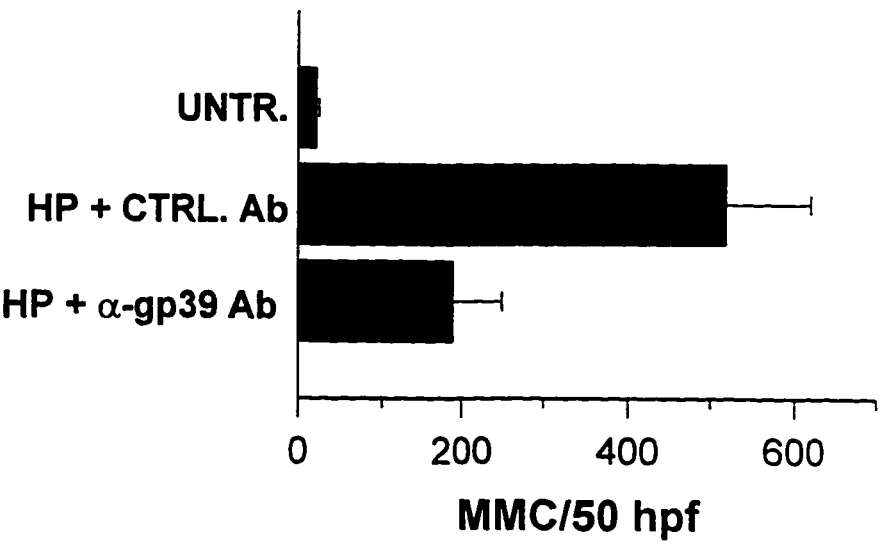
2. Anti-gp39 antibody differentially inhibits B cell activation in the *in vivo* immune response to HP

Blocking CD40/CD40L interactions might either affect initial B cell activation or later differentiation of B cells to Ig-secreting cells. To examine whether anti-gp39 mAb blocks events in initial B cell activation in HP-infected, anti-gp39 mAb-treated mice, MLN cell suspensions from these mice were stained simultaneously with anti-MHC II mAb and B cell-specific anti-B220 (6B2) mAb 8 days after inoculation, when B cells exhibit a maximum increase in cell size and MHC class II expression (Figure 7).

Figure 30. Blocking CD40/CD40L interactions suppresses IgG1 production, blood eosinophilia, and intestinal mucosal mastocytosis during a primary HP infection. Anti-gp39 mAb (2 mg) or control hamster Ig (CTRL. Ab; 2 mg) was administered on day 0 and day 7 after oral inoculation with 200 third stage HP larvae. Mice were bled and tissue sample were taken 14 days after inoculation. Serum IgG1 levels, blood eosinophil counts, and numbers of intestinal mucosal mast cells/50 high-powered microscope fields (MMC/50hpf) were determined as described in Materials and Methods. Geometric means and standard errors are shown for IgG1 levels; arithmetic means and standard errors are shown for eosinophil and mast cell counts. UNTR: untreated. Data shown here is representative of 3 independent experiments.


UNTR.
HP + CTRL. Ab
HP + α-gp39 Ab
0
1
2
3
4
5
6
SERUM IgG1 (mg/ml)


UNTR.
HP + CTRL. Ab
HP + α-gp39 Ab
0
500
1000
EOSINOPHILS/MM3

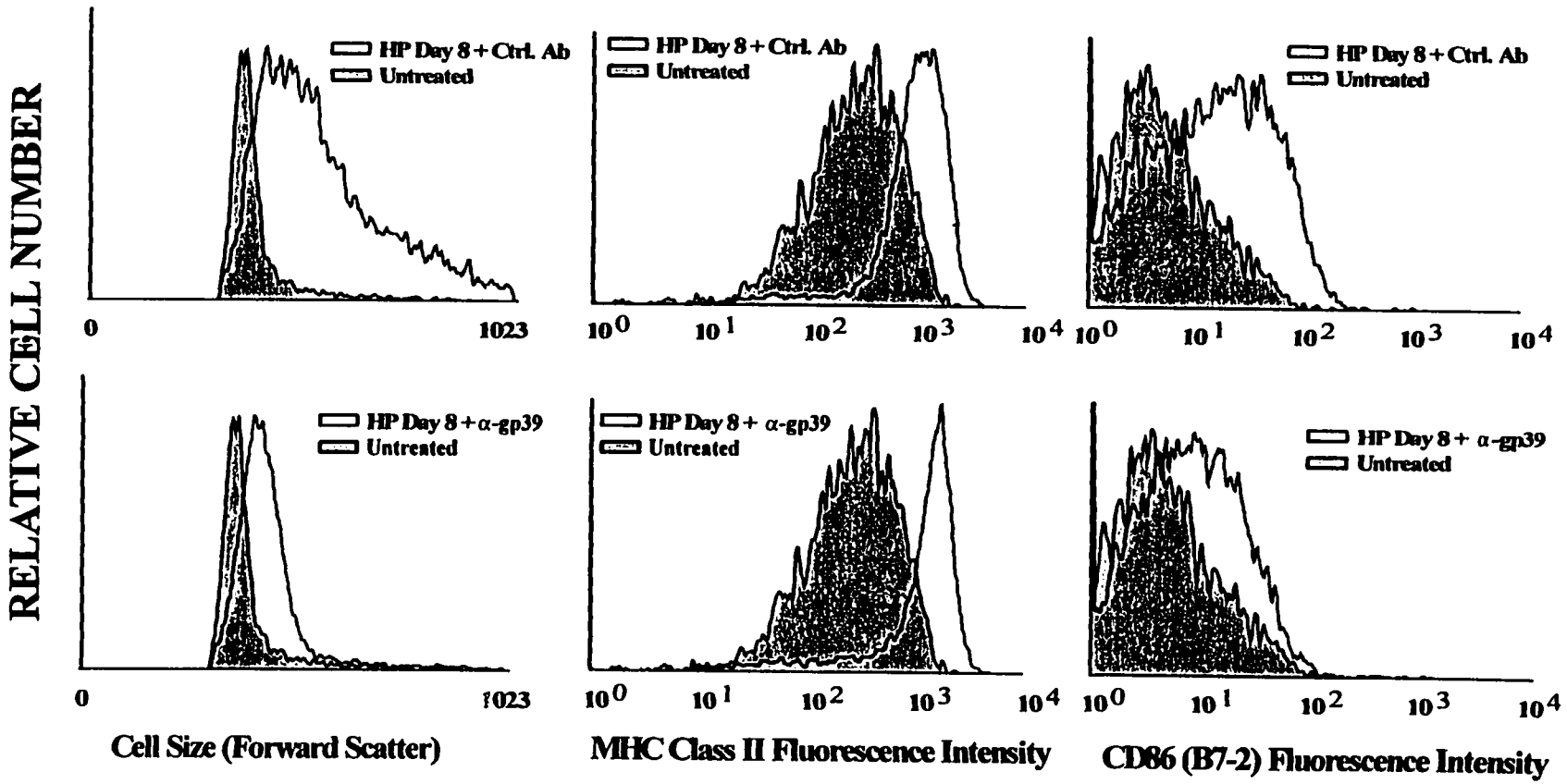

UNTR.
HP + CTRL. Ab
HP + α-gp39 Ab
0
200
400
600
MMC/50 hpf

MHC class II expression on $B220^+$ cells was as elevated in HP-inoculated mice given anti-gp39 Ab as it was in HP-inoculated mice administered the control antibody. In contrast, increases in B cell size were clearly inhibited in HP-inoculated mice given an anti-gp39 antibody as compared to HP-inoculated mice administered control antibody, although B cell size in the anti-gp39 mAb-treated group was still elevated compared to untreated controls (Figure 31). B cell surface B7-2 expression was also examined since previous studies have suggested that B cells may require CD40 costimulation for CTLA-4 ligand elevations (Ranheim and Kipps, 1993a). Treatment of HP-inoculated mice with an anti-gp39 antibody partially suppressed elevations in B7-2 cell surface expression compared to inoculated mice administered the control Abs (Figure 31).

3. Anti-gp39 mAb administration does not affect elevated cytokine gene expression in HP-inoculated mice

Previous studies have shown that by day 8 after HP inoculation, elevations in IL-4 production are exclusively from $CD4^+$, TCR-α/β^+ T cells and blocking CTLA-4 ligand interactions completely blocks elevations in IL-4 and partially blocks elevations in IL-3 and IL-5 gene expression in the PP and the MLN. To determine whether blocking CD40 ligand interactions could also inhibit increases in type 2 cytokine production, mice were injected with 2 mg of either hamster anti-gp39 (MR1) mAb or control hamster Ig on day 0 and 7 after HP inoculation. PP and MLN samples were removed from mice and

Figure 31. Increases in B cell size and surface B7-2, but not MHC class II expression are inhibited by blocking CD40/CD40L interaction during the immune response to HP inoculation. Anti-gp39 mAb (2 mg) or a control hamster Ig (Ctrl. Ab; 2 mg) was administered *i.v.* at day 0 and 7 after HP-inoculation. MLN samples were collected at day 8 and cell suspensions from five individual BALB/c mice in each treatment group were pooled and dual stained with either FITC-anti-MHC class II (MDK6) and Cy5-anti-B220 (6B2) mAbs or Cy5 anti-B220 (6B2) and biotinylated anti-B7-2 (GL-1) mAbs followed by SA-PE. Single histogram analysis of B cell ($B220^+$) size, MHC class II expression, and B7-2 expression are shown. Data shown here is representative of 3 independent experiments.


RELATIVE CELL NUMBER
HP Day 8 + Ctrl. Ab
Untreated
HP Day 8 + α-gp39
Untreated
0
1023
10^0
10^1
10^2
10^3
10^4
Cell Size (Forward Scatter)
MHC Class II Fluorescence Intensity
CD86 (B7-2) Fluorescence Intensity

individually analyzed for cytokine gene expression at day 8. Anti-gp39 mAb did not inhibit increases in gene expression of the T cell-derived cytokines IL-3, IL-4, IL-5, IL-9 in either the PP or the MLN (Figure 32).

4. Anti-gp39 Ab does not inhibit HP-induced increases in IL-4-secreting MLN T cells at day 8 after HP inoculation

To determine whether blocking CD40-CD40L interaction can inhibit T cell cytokine production, ELISPOT assays were performed to determine whether HP-induced cytokine secretion, as well as RNA expression, was inhibited by blocking CD40-CD40L interaction. The numbers of IL-4 secreting cells/10^6 MLN cells were individually determined from five mice per treatment group at 8 days after HP inoculation. Elevations in IL-4 secretion were comparable in HP-inoculated mice given either anti-gp39 mAb or the control hamster Ig (Figure 33A). Similarly, sorted $CD4^+$, TCR-α/β^+ T cells from HP-inoculated mice given anti-gp39 mAb did not exhibit any decreases in the number of IL-4-secreting cells compared to sorted $CD4^+$, TCR-α/β^+ T cells from HP-inoculated mice given the control Ig (Figure 33B). The number of IL-4 secreting cells from $CD4^+$, TCR-α/β^+ T cells was not significantly elevated compared to unsorted cells, probably due to the sorting process causing decreased levels of cytokine secretion (Figure 33B).

Figure 32. Anti-gp39 antibody treatment does not affect elevations in type 2 cytokine gene expression in the PP and the MLN at day 8 after HP-inoculation. Anti-gp39 mAb (2 mg) or a control hamster Ig (CTRL.Ab; 2 mg) was administered at days 0 and 7 after HP-inoculation. PP and MLN samples were collected at day 8 and cytokine gene expression levels were determined by a quantitative RT-PCR assay. The arithmetic mean and SE derived from the PP or the MLN of five individual BALB/c mice are shown for each treatment group. All data were individually normalized to the endogenous internal standard, HPRT, which did not show greater than two- to threefold variation throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1. Data shown here is representative of 3 independent experiments.

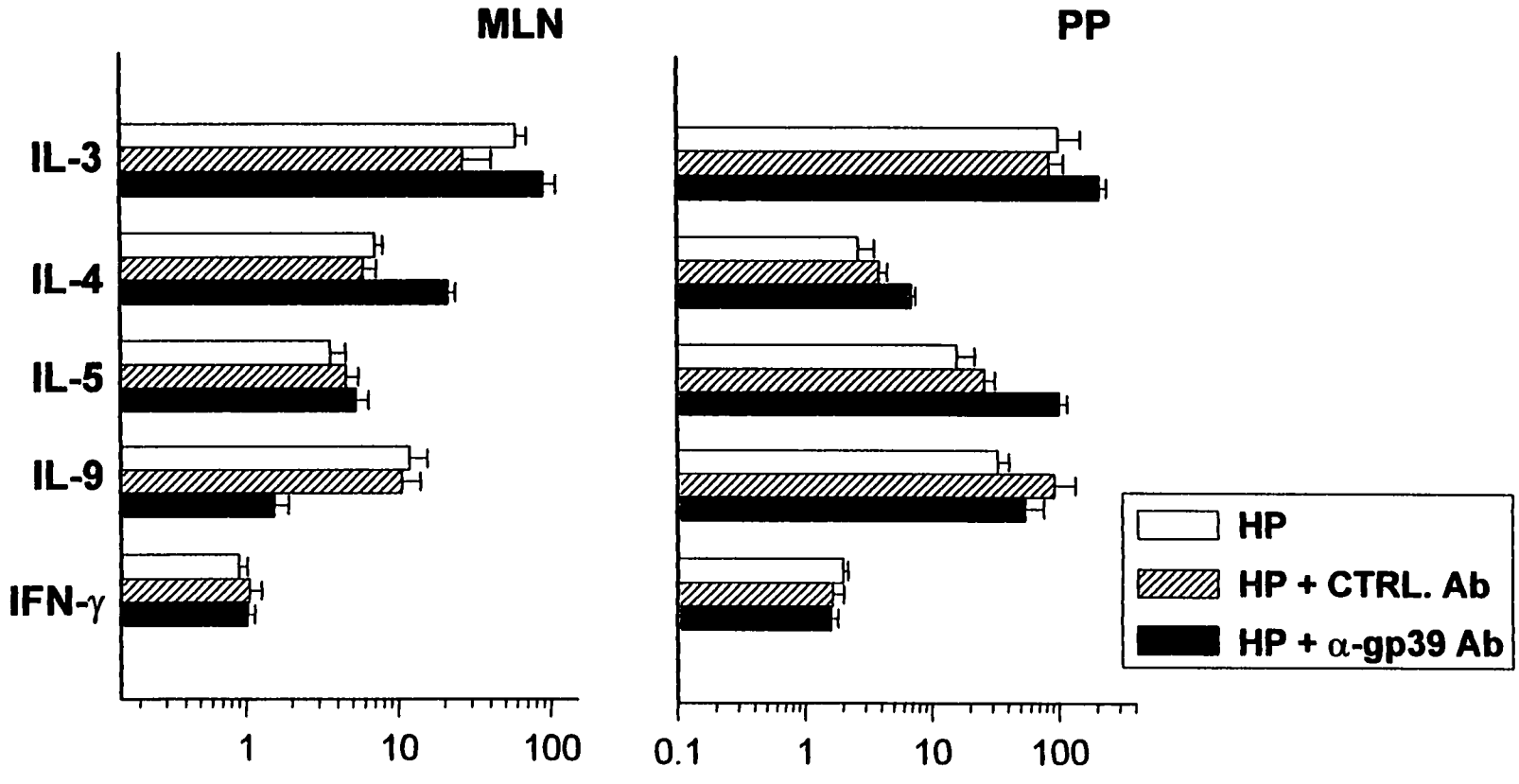
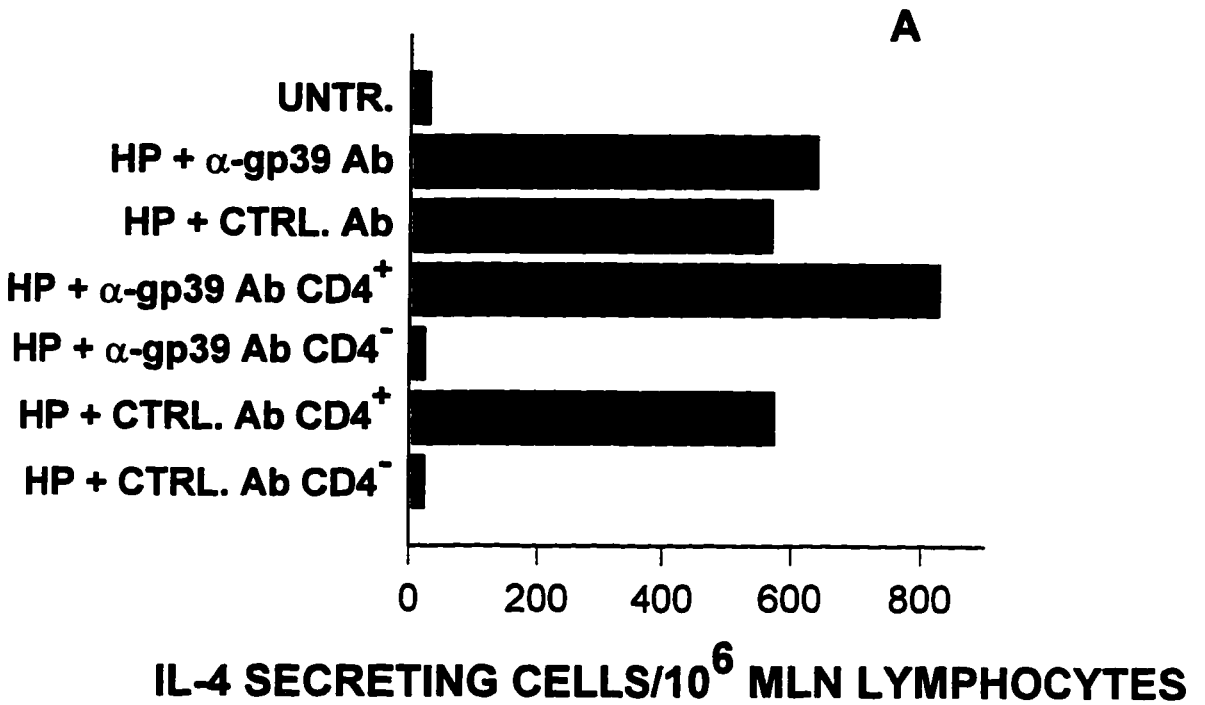
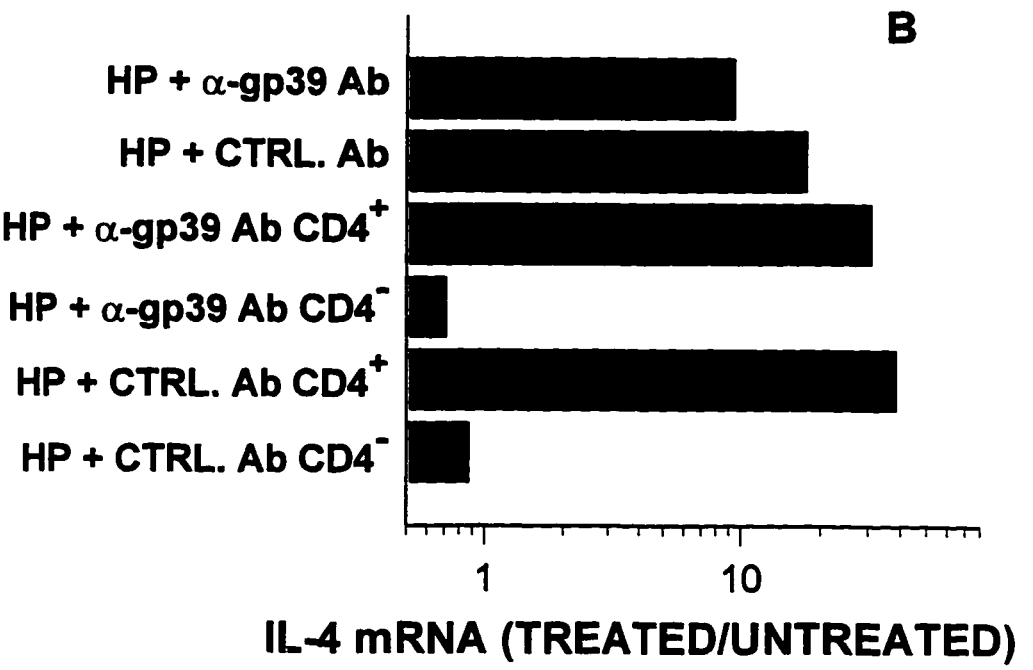

MLN
PP
IL-3
IL-4
IL-5
IL-9
IFN-γ
1
10
100
0.1
1
10
100
HP
HP + CTRL. Ab
HP + α-gp39 Ab
mRNA (TREATED/UNTREATED)

Figure 33. Elevations in the number of IL-4-secreting $CD4^+$, TCR-α/β^+ cells from the MLN of HP-inoculated mice are refractory to blocking CD40/CD40 ligand interaction at day 8 after HP inoculation. Anti-gp39 mAb (2 mg) or a control hamster Ig (CTRL.Ab; 2 mg) was administered at days 0 and 7 after HP inoculation. The number of IL-4-secreting cells/10^6 MLN cells was determined in an ELISPOT assay at day 8. MLN samples were collected at day 8 after HP inoculation and cell suspensions from five individual BALB/c mice per treatment group were either individually assayed (A) or pooled and dual stained with PE-anti-TCR-α/β (H57-597) and FITC-anti-CD4 (GK1.5) mAbs. $CD4^+$, TCR-α/β^+ cells were then sorted from both the control Ab- and anti-gp39 mAb-treated HP-inoculated mice on the same day, using an Epics Elite cell sorter, and were shown to be >95% pure. Both positively and negatively sorted cells were then assayed by ELISPOT for IL-4 secretion (B). UNTR: untreated. Data shown here is representative of 3 independent experiments.

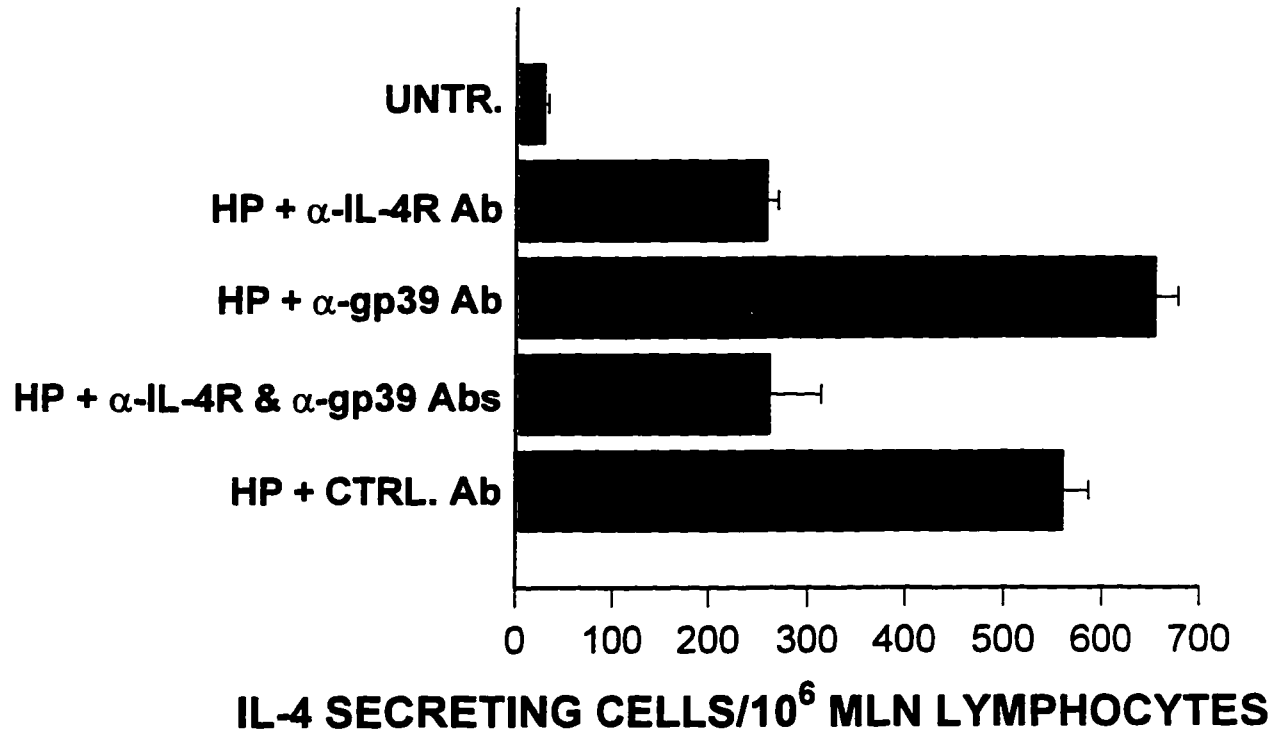

A
UNTR.
HP + α-gp39 Ab
HP + CTRL. Ab
HP + α-gp39 Ab CD4+
HP + α-gp39 Ab CD4-
HP + CTRL. Ab CD4+
HP + CTRL. Ab CD4-
0
200
400
600
800
IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES

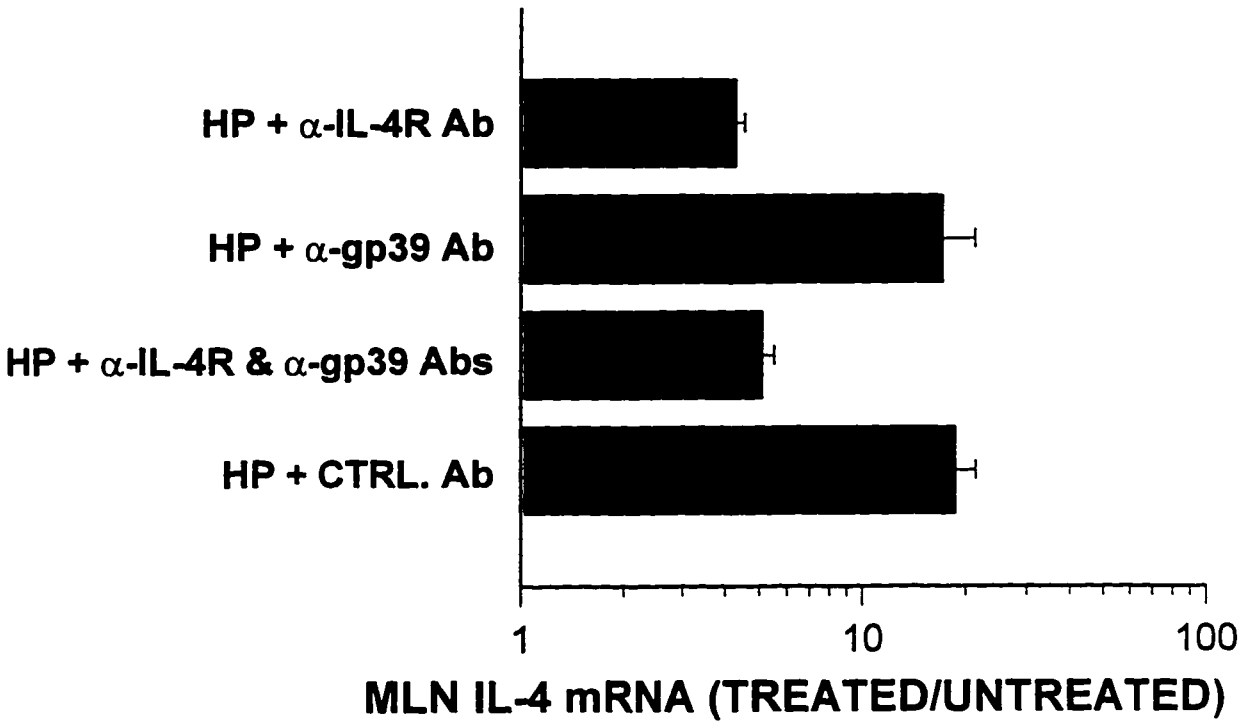

B
HP + α-gp39 Ab
HP + CTRL. Ab
HP + α-gp39 Ab CD4+
HP + α-gp39 Ab CD4-
HP + CTRL. Ab CD4+
HP + CTRL. Ab CD4-
1
10
IL-4 mRNA (TREATED/UNTREATED)

5. Anti-IL-4 receptor (IL-4R) mAb treatment blocks elevations in CD40-CD40L independent T cell IL-4 production

The observation that blocking CD40L interactions did not inhibit IL-4 elevations suggested that T cell IL-4 production did not require CD40L signaling. Since IL-4 can act as an autocrine growth factor for T cell proliferation and IL-4 production (Seder *et al.*, 1992), we would like to examine whether IL-4 or the combination of IL-4 and CD40L signaling was required for optimal T cell IL-4 production. HP-inoculated mice were injected with 5 mg of anti-IL-4R mAb on day 0 and 4 and 1 mg anti-gp39 mAb on day 0 after inoculation. IL-4 secretion and gene expression in the MLN were examined at day 8. As shown in Figure 34, although anti-gp39 mAb did not affect IL-4 elevations, anti-IL-4R mAb treatment alone blocked increases in both IL-4 secretion and gene expression, suggesting that IL-4 signaling in the absence of CD40L signaling is sufficient for the expansion of IL-4-producing T cells.

Figure 34. IL-4, but not CD40L stimulation, enhances T cell IL-4 production in the MLN at day 8 after HP inoculation. BALB/c mice (5 per group) were orally inoculated with 200 third stage HP larvae. Anti-gp39 mAb (2 mg/mouse) or control hamster Ig (CTRL. Ab; 2 mg/mouse) was administered *i.v.* at day 0 and 8, whereas anti-IL-4R mAb (M1, 5 mg/mouse) or control Ab (GL117, 5 mg/mouse) was given *i.v.* at day 0 and 4. MLN samples were collected at day 8 after HP inoculation and single cell suspensions were prepared for IL-4 ELISPOT assay. The arithmetic mean and SE derived from data of five individual BALB/c mice are shown for each treatment group. MLN cytokine gene expression levels were determined by a quantitative RT-PCR assay. The arithmatic mean and SEM derived from the PP or the MLN of five individual BALB/c mice are shown for each treatment group. All data were individually normalized to the endogenous internal standard, HPRT, which did not show greater than two- to threefold variation throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1. UNTR: untreated. Data shown here is representative of 3 independent experiments.


UNTR.
HP + α-IL-4R Ab
HP + α-gp39 Ab
HP + α-IL-4R & α-gp39 Abs
HP + CTRL. Ab
0
100
200
300
400
500
600
700
IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES


HP + α-IL-4R Ab
HP + α-gp39 Ab
HP + α-IL-4R & α-gp39 Abs
HP + CTRL. Ab
1
10
100
MLN IL-4 mRNA (TREATED/UNTREATED)

IV. Discussion

A. Studies of the requirement of CTLA-4 ligand costimulation for the immune response to anti-mouse IgD Ab immunization

In this study, the requirement of CTLA-4 ligand costimulation during the early stage of the *in vivo* type 2 systemic immune response was investigated by CTLA4-Ig administration. The response after foreign anti-mouse IgD Ab immunization is associated with early increases in IL-2 mRNA, marked elevations in IL-4 and IL-10, and a smaller elevation in IFN-γ gene expression. Splenic $CD4^+$ T cells are the sources of elevated IL-4 and IL-10 gene expression (Svetic *et al.*, 1991). Our studies demonstrate that B7-1/B7-2 plays a key role in costimulating naive $CD4^+$ T cells to differentiate to IL-4 producing cells and help B cells to differentiate into IgG and IgE secreting cells. CTLA4-Ig administration totally blocked T cell cytokine production (Figure 12-14), surface IL-2R and cell size increases (Figure 15), and serum Ig isotype elevation in anti-IgD Ab immunized mice (Figure 11). All these results indicate that CD28-B7 costimulation is required for the initiation of the *in vivo* type 2 dominant immune response to anti-mouse IgD Ab.

Previous studies have demonstrated that CD28 signaling, in addition to TCR/CD3 signaling, is required to induce long-term T cell clones and freshly purified T cells to produce IL-2 (Harding *et al.*, 1992; Linsley *et al.*, 1991a; Fanslow *et al.*, 1994a; Hans *et al.*, 1992) and that signaling through the TCR in the absence of CD28

costimulation can induce anergy (Harding *et al.*, 1992). In contrast, some murine IL-4-producing Th2 cell clones can produce IL-4 and proliferate in response to IL-4 in the absence of CD28 costimulation, apparently by utilizing other costimulatory signals, including IL-1 (Kurt-Jones *et al.*, 1987; Greenbaum *et al.*, 1988; Weaver and Unanue, 1990; June *et al.*, 1990; McArthur and Raulet, 1993). Also CTLA4-Ig inhibits alloantigen-specific responses and IL-2 and IFN-γ gene expression in mixed lymphocyte cultures, but increased IL-4 gene expression and some proliferation persists (Tan *et al.*, 1993). As yet, however, few studies examining the requirement of CD28/CTLA-4 for T cell activation during an *in vivo* immune response have been reported. CTLA4-Ig administration blocked elevations in serum Ab levels following SRBC-immunization, but T cell activation and differentiation to cytokine-producing cells were not examined (Linsley *et al.*, 1992b). Although serum Ig elevations in mice transgenic for soluble CTLA4-Hγ1 were also inhibited in response to immunization with a T-dependent antigen, analysis of T cell function revealed normal priming and Th1/Th2 cytokine production (Ronchese *et al.*, 1994). These findings have been interpreted to show that co-stimuli other than B7 may be sufficient *in vivo* to induce T cell activation and cytokine production, but that B7 signaling is required to induce T cells to provide help for B cell activation and differentiation (Linsley and Ledbetter, 1993). Results here in, in contrast, demonstrate that the B7-CD28/CTLA-4 interaction is required for either T cell IL-2 or IL-4 production during the primary *in vivo* systemic

immune response to GaMIgD and that, in this system, other costimulatory signals cannot substitute for its absence.

Two different assay systems to measure IL-4 expression in these studies, both of which provided corroborating data. Each assay has distinct advantages and disadvantages. The RT-PCR technique measures changes in gene expression *in situ* with essentially no manipulation of tissue required; however, it does not provide direct information on protein production. The ELISPOT assay provides protein secretion data and in many ways is a marked improvement over other *in vitro* assays which require restimulation or extended cultures, but still requires some *in vitro* culture (three hours) which might induce production of cytokines not being made *in vivo*.

The nature of the systemic immune response to anti-mouse IgD Ab may favor a critical role for CD28/CTLA-4 costimulation since B cells are the principal APC in this system (Morris *et al.*, 1994). If B cells rely on B7-1/B7-2 as the principal costimulatory ligand for naive T cell activation, immunization favoring B cell antigen presentation may be particularly sensitive to CTLA4-Ig. *In vivo*, other APCs, such as dendritic cells or activated macrophages, may be more permissive to an absence of CD28/CTLA-4 signaling because they express a greater repertoire of cell surface or secreted costimulatory molecules.

The possibility existed in the anti-IgD model that blocking the B7-CD28/CTLA-4 interaction inhibited IL-2 production that was required for later IL-4 production as has been demonstrated *in vitro* (Lane *et al.*, 1994; Ronchese *et al.*, 1994). However,

IL-2 gene knockout mice have been shown to have constitutively high levels of IL-4 production and serum IgG1 which usually requires IL-4 production (Gause *et al.*, unpublished data; Ben-Sasson *et al.*, 1990). Furthermore, we have been unable to block elevations in IL-4 gene expression in GaMIgD-immunized mice with anti-IL-2 and anti-IL-2R mAbs (unpublished data). Taken together, these results suggest that a strong immunogen favoring a Th2-like response can rapidly induce previously resting T cells by a CD28-dependent pathway to produce IL-4 in the absence of initial increases in IL-2 or the appearance of a Th0-like pattern.

The observation that CTLA4-Ig did not block the T cell response when given 3 days after immunization (Figure 16) suggests that unlike naive T cells, effector T cells do not require CD28/CTLA-4 signaling and that T cell-B cell interactions occurring at later stages of the immune response may not require such signaling. The effectiveness of CTLA4-Ig treatments only at the onset of the immune response also suggests that in this system resting T cells but not already activated cells are initially stimulated by Ag-presenting B cells, consistent with increasing evidence that activated B cells can present Ag to and activate resting T cells (Linsley and Ledbetter, 1993; Schorle *et al.*, 1992).

A notable exception to the inhibition of T cell cytokines by CTLA4-Ig was the sustained elevation of IL-10. Results in this paper (Figure 12-14) suggests that IL-10 gene expression and protein secretion are regulated differently than other Th2 cytokines *in vivo*. In other immunization systems, IL-10 gene expression has also been found not to be coordinately elevated with other Th2 cytokines (Finkelman *et al.*, 1986a; Ho *et*

al., 1994; Svetic *et al.*, 1993b). Differential regulation of IL-4 and IL-10 has also been shown in activated T cell clones where elevations in IL-4 gene expression are cyclosporin-sensitive, while IL-10 elevations are cyclosporin-resistant (Wang *et al.*, 1991). IL-10 has been shown to affect a variety of cell types including B cells, macrophages, and T cells (Alfons *et al.*, 1994; Fiorentino *et al.*, 1991; O'Garra *et al.*, 1990). Moreover, IL-10 can selectively inhibit the upregulation of B7 on macrophages thereby inhibiting macrophage co-stimulatory activity (Fiorentino *et al.*, 1989). The sustained elevation of IL-10 in the absence of the CD28 costimulatory signal may suggest that either other costimulatory molecules can substitute for CD28 *in vivo* or that signaling through the TCR is sufficient for upregulating IL-10 gene expression and protein production.

Although studies here in demonstrated that the systemic anti-IgD response was dependent on CTLA-4 ligand signaling, it is quite possible that this is the exception and that a type 2 immune response to a whole pathogen, with all its associated antigens and microbial structures, may not require these signals. The mucosal immune response to HP is strong and is associated with allergy responses, including the activation of IL-4 producing cells, elevations in serum IgE and IgG1, mucosal mastocytosis, and eosinophilia. We hypothesized that this markedly different *in vivo* immune response to a whole pathogen, with which the host has co-evolved, might show more plasticity including signalings that might substitute for CTLA-4 ligand costimulation.

B. Studies of the requirement of CTLA-4 ligand costimulation in the *in vivo* mucosal type 2 immune response to HP inoculation

Studies of HP-inoculated mice treated with CTLA4-Ig demonstrate that CTLA-4 ligand costimulation plays a key role in the development of the type 2 enteric immune response to the nematode parasite, *H. polygyrus*. Administration of CTLA4-Ig at early stages of the infection inhibits development of both the T cell and the B cell response in this system.

Previous studies have not been performed on the role of CD28-B7 costimulation in the development of an IL-4 "dominant" *in vivo* immune response. However, *in vitro* studies have suggested that Th2 cells can utilize costimulatory signals besides CD28 (Kim *et al.*, 1985; Lichtman *et al.*, 1988; Weaver *et al.*, 1988). One report has shown that either IL-1 or CD28 can serve as costimulatory signals mediating responsiveness to IL-4 in Th2 clones, but that CD28 is not required for IL-4 production (McArthur and Raulet, 1993). Furthermore, anti-CD28 antibodies have been shown to induce human T cell responsiveness to IL-4 *in vitro* (Seder *et al.*, 1994). Studies here in extend these findings to an *in vivo* model where IL-4 is produced in the early stages of a primary response by T cells in the absence of increased IL-2 gene expression (Svetic *et al.*, 1993b). Results from this study show that CTLA-4 ligand interactions are necessary for elevations in IL-4 gene expression and IL-4 production in this system and suggest that other costimulatory signals, such as IL-1, the heat stable

antigen (Liu *et al.*, 1992), or LFA-1 and CD2 (Springer, 1990; Bierer *et al.*, 1989), are either not available or cannot substitute for CTLA-4 ligand interactions.

In contrast to results in this study, mice transgenic for soluble murine CTLA-4-Hγ1 have been reported to exhibit normal T cell priming and cytokine production in response to immunization with a T cell-dependent antigen (Lane *et al.*, 1994; Ronchese *et al.*, 1994). One possible reason for this difference from this study are that these transgenic mice lack CD28/CTLA-4 costimulatory signals from birth and may have consequently compensated during ontogeny by utilizing other signals. Alternatively, mCTLA-4-Hγ1 may not be produced at sufficient levels to block CTLA-4 ligand interactions required for the induction of T cell responses. The results in Figure 19 clearly shows that blocking CTLA-4 ligands by administration of CTLA4-Ig in normal mature animals completely inhibits elevations in T cell-derived cytokine gene expression and IL-4 protein secretion.

Although very high circulating levels of IgE are detected 14 days after HP inoculation, CTLA4-Ig administration at day 0 and 1 after inoculation strongly inhibited serum IgE levels. Previous studies have also demonstrated CTLA4-Ig inhibition of a primary *in vivo* antibody response to sheep red blood cells and KLH (Linsley *et al.*, 1992b; Lane *et al.*, 1994; Ronchese *et al.*, 1994). The further observation that increases in B cell size and MHC class II expression are blocked by CTLA4-Ig administration is consistent with a requirement for T cell help for B cell activation during this in vivo mucosal response (Figure 21). Cytokine production by

non-T cells and costimulatory signals provided to B cells by mast cells and basophils expressing CD40L (Gauchat *et al.*, 1993) are either themselves T cell dependent or cannot substitute for the T helper cell stimulation that is mediated by CTLA-4 ligand interactions. In contrast, findings from this study shows that blocking CTLA-4 ligand interactions only partially inhibited elevations in IL-5 gene expression, suggesting partial T cell-independence of these responses and consistency with the prior observations that anti-CD4 mAb only partially inhibits blood eosinophilia (Urban, unpublished data).

Studies from other *in vitro* systems have suggested that IL-2 may be required for IL-4 production and that blocking CTLA-4 ligands, in the absence of exogenous IL-2, may inhibit IL-4 production by initially blocking IL-2 production (Ben-Sasson *et al.*, 1990; Swain *et al.*, 1988; Seder *et al.*, 1994). However, IL-2 gene knockout mice have been shown to have constitutively high levels of IgG1, which is at least partially IL-4-dependent, and to make in vivo T cell dependent immune responses to some pathogens that are comparable to those of control mice, suggesting that IL-2 is not required in in vivo T cell-mediated immune responses (Schorle *et al.*, 1992; Kundig *et al.*, 1993). It is also unlikely that IL-2 plays a major role in the HP system since no increase is detected in IL-2 gene expression in the PP or the MLN during the course of the response to HP (Svetic *et al.*, 1993b) and administration of anti-IL-2 antibodies to HP-infected mice does not affect serum IgE production (Finkelman and Urban, unpublished observations), although the same anti-IL-2 antibodies block immune

responses in another system (Via and Finkelman, 1993). Thus the findings presented here in provide evidence for the requirement of a CTLA-4 ligand interaction that is important in the generation of an IL-4 response even in an IL-2-independent system.

A more likely explanation for the discrepancy observed between results from this *in vivo* study and other *in vitro* studies may be related to the state of activation of the responding T cells. Th1 or Th2 cell clones are continually restimulated and are probably more similar to *in vivo* activated T effector or memory cells, which are far less dependent of CD28/CTLA-4-B7 signalling than naive T cells (Croft *et al.*, 1994). In this study since CTLA4-Ig administration completely inhibits T cell cytokine production, the cell populations involved in the immune response to HP are probably naive T helper cells, a population which has been shown to be more dependent on costimulatory signals than effector or memory T helper cells (Croft *et al.*, 1994).

The finding that CTLA4-Ig administration during a secondary immune response to HP affects neither T cell cytokine nor serum IgE levels confirms that memory T cells do not require CD28-B7 costimulation (Croft *et al.*, 1994). Stimulation through the TCR alone could not initiate naive T cell proliferation and IL-2 production, whereas memory T cells proliferated very well with high levels of IL-2 production (Croft *et al.*, 1994). The addition of anti-CD28 mAb or APC in the above system greatly increased naive T cell proliferation and IL-2 production, but memory T cell IL-2 production only increased sightly. Moreover, naive T cells responded poorly to Ag presented by resting B cells and macrophages, which lack surface B7. They could only be activated by Ag on dendritic

cells and activated B cells, which had high surface B7-2 expression. Memory T cells, however, could respond to Ag on a wider variety of APCs, but proliferated best when stimulated by dendritic cells and activated B cells(Croft *et al.*, 1994). Results from *in vivo* study in this thesis further confirm these *in vitro* observations that memory T cells are less dependent on costimulation than naive T cells..

Findings from this study are also important from a therapeutic viewpoint since they suggest that *in vivo* the T cell developmental pathway that leads to IL-4 production in the mucosal region can be inhibited by blocking CTLA-4 ligand interactions. This is particularly relevant to the control of immediate hypersensitivity reactions, which are also characterized by marked elevations in IL-4 production and serum IgE levels. Although blocking CTLA-4 ligands cannot inhibit activation of allergen-specific memory T cells, the newly recruited naive T cells could be made anergic by blocking CD28/CTLA-4 costimulation, so that the maintenance and the severity of the allergy response would be reduced. This may also be the mechanism underlying successful CTLA4-Ig treatment of a murine model of lupus-like autoimmune disease (Finck *et al.*, 1994). In this study, low-dose CTLA4-Ig treatment of NZB/NZW mice at a late stage of lupus blocked further increases in serum autoantibody and prolonged life (Finck *et al.*, 1994), suggesting that established and even pathological immune response can still be suppressed by blocking CTLA-4 ligand interactions. Another potential therapeutic approach relies on the premise that blocking CTLA-4 ligand interactions during an immune response to an allergen or other antigen might induce tolerance resulting in

unresponsiveness to subsequent challenges. Findings that the primary T cell response to HP is dependent of B7-costimulation suggests an experimental *in vivo* model for future studies directed towards determining whether T cell anergy will occur in the absence of CTLA-4 ligand costimulatory signals during a type 2 response.

C. The investigation of differential costimulatory effects of B7-1 versus B7-2 during the *in vivo* immune response to HP inoculation

Results in this study showed that administration of both anti-B7-1 and anti-B7-2 mAbs at early stages of the infection inhibits the development of both T and B cell responses, eosinophilia, and mastocytosis, but administration of either anti-B7-1 or anti-B7-2 mAb alone has little effect. These findings demonstrate that B7 molecules play a key role in the development of the enteric immune response to HP and that either B7-1 or B7-2 alone can provide the necessary costimulatory ligand interactions that lead to the development of effector T helper cells and other effector populations characteristic of this primary type 2 mucosal response.

Results from previous parts of this thesis show that blocking CTLA-4 ligand interactions by CTLA4-Ig administration inhibits T cell differentiation to IL-4 production and B cell activation and Ig secretion during the primary immune response to HP. These findings are extended here by demonstrating that in this system, blocking both B7-1 and B7-2 with specific monoclonal antibodies yields very similar results, suggesting that blocking both B7-1 and B7-2 interactions is sufficient to cause the immunosuppressive

effects observed with CTLA4-Ig. Although other CTLA-4 ligands may still exist (Boussiotis *et al.*, 1993), apparently B7-1 and B7-2 are the primary ones required to mediate T cell costimulation resulting in cytokine production and B cell differentiation during this primary type 2 response. These studies also corroborate those undertaken with CTLA4-Ig, suggesting that B7-CD28/CTLA-4 interactions are necessary for T cell costimulation leading to effector function *in vivo* and that other costimulatory signals, such as IL-1, the heat stable antigen (Korsmeyer, 1992), or LFA-1 and CD2 (van Seventer *et al.*, 1991) do not substitute for them.

Although the combination of anti-B7-1 and anti-B7-2 mAbs was quite effective in blocking T cell costimulation during the immune response to HP, administering these antibodies individually had little effect, suggesting that costimulation through B7-1 and B7-2 deliver similar activation signals for naive T cells, and B7-1 can substitute for B7-2 and vice versa during the course of this type 2 response. A number of different effects have been observed following administration of these antibodies during other *in vivo* immune responses. For example, NOD mice treated with anti-B7-1 mAbs showed significantly accelerated diabetes while anti-B7-2 mAb treatment blocked the development of disease (Lenschow *et al.*, 1995). In contrast, anti-B7-2 mAb-treated SJL mice immunized with proteolipid protein exhibit a more severe development of EAE while anti-B7-1 mAb treatment significantly inhibited EAE induction and increased the frequency of IL-4-producing type 2 T cells (Kuchroo *et al.*, 1995). Recent *in vitro* studies of anti-CD3-stimulated T cells provided either B7-1 or B7-2 costimulation showed that

although B7-2 stimulation promotes the production of the Th2 cytokine, IL-4, it also favors the Th1 cytokine, TNF-β, while B7-1 engagement favors IFN-γ and IL-2 production but also promotes some IL-4 production (Freeman *et al.*, 1995). One possible explanation for these differences is that during some immune responses effector T cell development and cytokine production may be influenced by whether B7-1 or B7-2 is providing the costimulating signal at a time point critical to disease development. Alternatively, differential expression of B7-1 and B7-2 may have a subtle effect on cytokine gene expression that modulates cytokine selection in systems that are not heavily biased in a type 1 or type 2 direction, but that cannot deviate a normally strong type 2 response such as that induced by HP infection.

The differential effects of B7-1 and B7-2 on T cell development may therefore be particularly important with those antigens that can promote the development of both Th1-like and Th2-like T cells. The EAE model appears to have this characteristic and one might predict that other antigens, such as *Leishmania*, which can elicit either a type 1 or a type 2 response depending on the mouse strain (C57BL/6 or BALB/c) infected, may also have this characteristic and, hence, may be more easily influenced by the particular B7 molecule costimulating the T cell. However, other antigens appear to evoke a less easily influenced type 1 or type 2 response. For example, HP elicits a strong type 2 response in both BALB/c and C57BL/6 mice while killed *Brucella abortus* induces a type 1 mucosal immune response in both of these two strains (Svetic *et al.*, 1993a). Cytokines such as IL-12, which can promote T cell differentiation to IFN-γ production (Chan *et al.*, 1991),

or IL-4, which can influence T cell development towards type 2 cytokine production (Swain *et al.*, 1990), may be more important in determining whether these type 1 or type 2 responses develop than the particular B7 costimulatory signal involved.

Another possibility is that the localization of the response to the mucosal immune system may predispose naive T cells to differentiate towards type 2 cytokine production. Some studies have suggested that unique dendritic cells (Hathcock *et al.*, 1994) or the hormonal environment (Daynes and Araneo, 1992) may favor type 2 cytokine production by T cells during the mucosal immune response. However, oral infection with *Salmonella typhimurium* induces a strong type 1 mucosal immune response characterized by marked elevations in IFN-γ (Svetic *et al.*, 1993d) and other studies have shown that exogenous administration of IL-12 blocks IL-4 production and induces marked elevations in IFN-γ production in the MLN of HP-inoculated mice, demonstrating that the mucosal, as well as the systemic immune response can be easily influenced when exposed to agents that strongly promote either a type 1 or a type 2 response (Gause *et al.*, unpublished data).

Differential expression of both B7-1 and B7-2 on APCs may also influence what cytokines will be produced by the effector T cells. LPS-stimulated B cells rapidly express B7-2 with later upregulation of B7-1 (Lenschow *et al.*, 1993), but it is unlikely that these are the major APCs involved in initiating the immune response to HP since B cell null mice also support T cell differentiation to IL-4 production following HP-inoculation (data not shown). Dendritic cells are more likely APC candidates, but studies with this population are limited partly because some dendritic cell populations are not

readily released into suspension preventing effective flow cytometric analysis (Hathcock *et al.*, 1994). *In situ* studies suggest that B7-2, but not B7-1, is constitutively expressed on dendritic cells in splenic tissue (Hathcock *et al.*, 1994), but as yet *in situ* studies have not examined changes in B7 expression during the course of an *in vivo* immune response. Our findings that either B7-1 or B7-2 can support effector T cell development suggests that both molecules must be expressed on APCs at sufficient levels to provide required costimulatory signals in the MLN, but whether this is a consequence of rapid upregulation following their activation or due to constitutive expression is undetermined.

In summary, results in this section demonstrate that B7 signaling is required for the development of cytokine producing effector T helper cells and other effector cell populations during the mucosal type 2 immune response. However, either B7-1 or B7-2 can provide the requisite signaling suggesting that in this primary response to an enteric pathogen these two molecules do not play a major role in influencing which cytokines T helper cells will secrete.

D. Studies of the roles of CD40-CD40L interaction during the *in vivo* type 2 response to HP inoculation

Earlier studies in this thesis have shown that the CTLA-4 ligand costimulation is required for Th2 cytokine production, B cell activation, and Ig isotype production during the type 2 immune response to HP. Also, we have shown that the signaling through either B7-1 or B7-2 alone can promote the development of effector T cells and the

immune response. Since recent studies have shown that signaling through another receptor-ligand pair between T cells and APCs, CD40L-CD40, also has costimulatory effects on naive T cells (Fanslow *et al.* 1994b; Caybyab *at al.*, 1994.), the role of CD40-CD40L interactions in the *in vivo* immune response to HP inoculation were studied. Results from this study demonstrate that CD40-CD40L interactions play a key role in the development of the Type 2 enteric immune response to the nematode parasite, *H. polygyrus*. This vigorous immune response is enterically centered and is characterized by marked elevations in serum IgG1 levels, blood eosinophils, intestinal mucosal mast cells, and type 2 cytokines including IL-3, IL-4, IL-5, and IL-9 (Svetic *et al.*, 1993b). Results from Figure 30-34 show that although elevations in IL-4-dependent T cell IL-4 gene expression and protein secretion are comparable in mice administered anti-gp39 antibody, significant impairment of other effector cell populations still occurs, resulting in the abrogation of elevated serum IgG1 levels and a marked reduction in blood eosinophils and intestinal mucosal mastocytosis.

These results differ markedly from the data of earlier sections of this thesis showing that blocking CD28/CTLA-4 signaling in HP-inoculated mice resulted in the inhibition of T cell cytokine production but less marked inhibition of eosinophilia. Cognate interactions required for T helper cell activation leading to cytokine production apparently do not require CD40L stimulation or CD40-dependent elevations in APC surface B7 expression during this response. Previous studies have suggested that signals through the CD40L may be required to upregulate B7-1/B7-2 expression on B cells and

other APCs to initiate or amplify the T cell response (Durie *et al.*, 1994b). Results from Figure 30-33 are instead more consistent with the model that CD40-CD40L interactions are not required for activation of T cells to cytokine-secreting cells or B cells to a stage at which they increase somewhat in size and express increased MHC class II. Furthermore, these results suggest the absence of a requirement for CD40-CD40L interactions to maintain the T cell cytokine response as late as 8 days after HP inoculation. These findings further suggest that the expansion of IL-4-producing T cells in HP-infected mice is IL-4-dependent, since the adminstration of anti-IL-4R mAb inhibits the increase in IL-4 producing T cell in HP-inoculated mice (Figure 34). Moreover, because IL-4 is the last type 2 cytokine to be elevated and $CD4^+$, TCR-α/β^+ T cells are the sole detectable source of IL-4 in the immue response to HP inoculation (Svetic *et al.*, 1993), other signals are probably important in triggering the initial differentiation to IL-4 production.

Although elevations in B cell MHC class II expression were unaffected in HP-inoculated mice given anti-gp39 mAb, increases in B cell size were markedly diminished. Thus, CD40-CD40L interactions are not required for the IL-4-dependent elevations in B cell surface MHC class II (Finkelman *et al.*, 1986b; Noelle *et al.*, 1984), but are required for B cell blastogenesis. Signaling through CD40 has previously been shown to be required for B cell proliferation during a T-dependent response (Van den Eertwegh *et al.*, 1993). The requirement of CD40L interactions for B cell blastogenesis but not increased MHC class II surface expression may explain the observation that during an immune response, bystander cells, that would not interact with CD40L on T cells, show elevated

MHC class II expression, but only slight increases in cell size, whereas B cells participating in cognate interactions show elevations in both (Morris *et al.*, 1992).

Consistent with previous studies in which humoral immunity was inhibited by anti-gp39 mAb administration (Foy *et al.*, 1994; Foy *et al.*, 1993), the marked elevations in serum IgG1 characteristic of the immune response to HP were completely blocked by anti-gp39 mAb treatment (Figure 30). Previous *in vitro* studies have shown that signaling through CD40 or IL-4R can induce expression of B7 by B cells cultured *in vitro* (Ranheim and Kipps, 1993b; Stack *et al.*, 1994). Results from Figure 31 demonstrate that B cell surface B7-2, but not B7-1 is elevated in HP-inoculated mice and that the elevations in B7-2 are diminished but still markedly increased compared to untreated controls in HP-inoculated mice administered anti-gp39 mAb or anti-IL-4R mAb. Results from Figure 31 also suggest that increased B7-2 cell surface expression, like MHC class II expression, can occur prior to increased B cell size. Whether the limited elevations in B cell surface B7-2 in HP-inoculated mice given anti-gp39 or anti-IL-4R mAb alone can provide sufficient T cell costimulation for cytokine production is not known, because other APCs, in particular dendritic cells, may well be the major presenting cells, at least during the initial stages of this response. In another study, it has been shown that wild type and B cell null mice express comparable elevations in T cell-derived IL-4 at day 8 after HP-inoculation (Gause *et al.*, data not shown). At later stages of the immune response, the possibility remains that B cell B7-dependent signaling is required for effector T helper cell function.

The inhibition of both blood eosinophilia and mucosal mastocytosis, despite marked elevations in T cell-derived IL-3, IL-4, IL-5 production in HP-inoculated mice given anti-gp39 mAb, suggests that these two effector populations are dependent on CD40-signaling for their activation and proliferation. Since both eosinophils and mast cells express CD40 and CD40L (Gauchat *et al.*, 1993; R.S. Mittler, pers.comm.), the possibility exists that these populations, like B cells, require a CD40 or CD40L signal from T helper cells or from other cell types for their maximal production or survival. The observation that mast cell CD40L surface molecules can trigger B cell CD40, resulting in Ig class switching to IgE in the presence of IL-4 (Gauchat *et al.*, 1993), for example, raises the possibility that reciprocal signals may be provided to mast cells via CD40L.

Studies in this section suggest a model by which $CD4^+$ T cells use costimulatory molecules during their differentiation to IL-4 production in response to a live pathogen. Shortly after infection, HP Ags are presented to naive T cells by dendritic cells and other APCs that express sufficient B7 molecules to provide B7-dependent costimulation. Activated T cells stimulate B cells to secrete immunoglobulin and express high levels of cell-surface B7-2 through the combination of cytokine production and CD40L expression. Thus, unlike CD28/CTLA-4 signals, CD40-CD40L interactions are not required for naive T cell activation, but are required to induce and/or support other effector cell activity, including the development of eosinophilia and mastocytosis.

E. Summary

In these studies, the requirement of CD28/CTLA-4 costimulation during two *in vivo* type 2 immune responses was first investigated. Two different models were used: the response to the intestinal nematode parasite *H. polygyrus*, which elicited a localized mucosal immune response, and the systemic response to foreign anti-mouse IgD Ab immunization. Furthermore, the role of B7-1 vs B7-2 costimulation was studied in the response to HP-inoculation and, finally, the effects of blocking CD40-CD40L interactions in the HP-inoculation model were examined.

By blocking CTLA-4 ligand interactions with CTLA4-Ig administration during the immune response to foreign anti-mouse IgD Abs, results showed that CD28/CTLA-4 costimulation is required for: 1) increased IL-2, IL-4, and IL-9, but not IL-10 gene expression; 2) elevations in the number of IL-4-, but not IL-10-secreting cells in both unsorted splenic cells and sorted $CD4^+$, TCR-α/β^+ T cells; 3) increases in T cell size and surface IL-2R expression; 4) elevated serum IgG1, IgE, IgG3, and IgG2a. These effects were dependent on early administration of CTLA4-Ig since, as with the HP system, elevations in cytokine production or serum Ig levels were not affteced when CTLA4-Ig was administered at days 3-4 after immunization. These results indicate that CTLA-4 ligand interactions are required for the activation of naive $CD4^+$ T cells to differentiate to IL-4-producing cells and for B cells to differentiate into IgG1- and IgE-secreting cells during a primary systemic type 2 "dominant" immune response.

Blocking CTLA-4 ligand interactions in the primary immune response to oral HP inoculation inhibits: 1) increased IL-3, IL-4, and IL-9 gene expression in the PP and the MLN, as measured by RT-PCR at day 8 after inoculation; 2) increased IL-4 cytokine protein secretion by $CD4^+$, TCR-α/β^+ cells, as determined by ELISPOT of FACS-sorted cells; 3) increased B cell MHC class II expression and size; 4) increased serum IgE levels. The complete absence of detectable B cell activation by blocking CTLA-4 ligand interactions, as measured by increased B cell size, MHC II expression and serum Ig levels, suggests that B cells cannot be initially activated by the antigens and other molecular structures characteristic of this live pathogen without the help of T cells activated by B7-CD28/CTLA-4 interactions. These findings demonstrate the requirement of CTLA-4 ligands for the development of the type 2 enteric immune response to HP inoculation. Together with the results from the anti-IgD response, the key role of B7-CD28/CTLA-4 interactions in costimulating naive $CD4^+$ T cell activation and IL-4 production in the *in vivo* type 2 immune response is clearly established. In contrast, CTLA4-Ig administration during the challenge response to HP inoculation does not affect the elevations in IL-4 gene expression and protein secretion in unsorted MLN lymphocytes and sorted $CD4^+$, TCR-α/β^+ T cells, indicating that memory T cell activation is much less dependent on CD28/CTLA-4 costimulation than the activation of naive T cells.

The role of B7-1 vs B7-2 in the development of the *in vivo* type 2 immune response to HP inoculation has been examined by blocking B7-1 and/or B7-2 ligand

interactions with specific mAbs. Administration of both anti-B7-1 and anti-B7-2 mAbs is required to inhibit: 1) HP-induced elevations in IL-4 gene expression and secretion; 2) elevations in serum IgG1, blood eosinophilia, and mucosal mastocytosis; 3) increases in B cell size, surface MHC class II expression. Treatment of HP-inoculated mice with either anti-B7-1 or anti-B7-2 mAb alone has no effect on this effector cell activity. These results suggest that in this mucosal immune response to a live pathogen, the B7-1 and B7-2 molecules can substitute for each other to deliver necessory costimulatory signals required for naive T cell activation. It also shows that either B7-1 or B7-2 is required for the development of the T cell response and that, if there are additional B7 molecules, they cannot substitute for the combined absence of these two signals.

Finally, the *in vivo* effect of blocking CD40-CD40L interactions on the type 2 mucosal immune response to HP was investigated. Administration of anti-gp39 mAb blocked HP-induced elevations in serum IgG1 levels, blood eosinophils, and intestinal mastocytosis. However, elevations in B cell surface MHC class II expression were unaffected and elevations in B7-2 expression were maximally inhibited by blocking both CD40 and IL-4R signaling. Furthermore, elevations in T cell cytokine gene expression and IL-4-secreting cells were unaffected by anti-gp39 mAb treatment. These results suggest that CD40-CD40L interactions are not required for T cell activation and cytokine production but for the activation and proliferation of other effector cells associated with the type 2 response.

Other reports have shown that proliferation and IL-4 production by Th2 cell clones (Kurt-Jones *et al.*, 1987) could be induced by other costimulatory signals besides CD28-B7, and that CTLA4-Ig treatment inhibited elevations in Th1 cytokines but not IL-4 gene expression (Kurt-Jones *et al.*, 1987; Greenbaum *et al.*, 1988; Weaver and Unanue, 1990; McArthur and Raulet, 1993). Results herein showed that despite the differences in the forms of the immunogen, the patterns and the kinetics of elevated cytokines, and the tissues involved in the immune response to anti-IgD Ab vs HP, blocking CTLA-4 ligand interaction showed similar inhibitory effects. These data suggest that CTLA-4 ligand costimulation is required for increased T cell IL-4 production, B cell activation, and Ig isotype secretion, as well as for the development of other effector cells in primary type 2 immune responses *in vivo*. The difference between our findings and previous reports that Th2 cells do not require CD28/CTLA-4 costimulatory signals may be that T cells activated in the two *in vivo* response models are naive phenotypes while Th2 cell clones that have undergone differentiation after prolonged *in vitro* culture and Ag restimulation may have become more similar to activated or memory T cell phenotypes in their costimulatory requirements.

Although recent studies have demonstrated differential costimulatory effects between B7-1 and B7-2, our studies showed that blocking either B7-1 or B7-2 costimulation did not affect the progression of the *in vivo* type 2 response. IL-4 production and other effector cell functions were inhibited only when both B7-1 and B7-2 signals were blocked. These data suggest that B7-1 and B7-2 can provide similar

costimulatory signals during the immune response to HP. Other systems where differential effects of B7-1 and B7-2 signaling on T cell activation are observed may be due to the nature of the response itself. An immune response which can be easily switched to either type 1 or type 2 may be more sensitive to the differences between B7-1 and B7-2 signaling, such as the EAE model and the *Leishmania* infection model. Other responses, such as the strong type 2 immune response following HP-inoculation, may not be able to be influenced by B7-1 vs B7-2 costimulation.

Results from Figure 30-34 show that CD40-CD40L interactions are not involved in the development of effector cells and T cell IL-4 production, but are required for certain B cell activation events, elevations in serum Ig isotype, and the activation of other effector cells (mast cells and eosinophils) in the *in vivo* type 2 immune response to HP inoculation. These data are more consistent with those studies suggesting that CD40-CD40L interactions predominantly promote humoral immunity and memory B cell generation rather than the initial T cell response (Foy *et al.*, 1994; Foy *et al.*, 1993). Results herein favor the hypothesis that initial T cell activation during an immune response is triggered by TCR engagement and the CD28-B7 costimulation between T cells and APCs. Activated T cells upregulate surface CD40L expression and secret IL-4. These cells, in turn, promote B cell activation and differentiation through CD40-CD40L and the effect of IL-4.

F. Conclusion

Results from these studies suggest a model for T cell differentiation to cytokine production and effector cell function during the course of a primary *in vivo* type 2 immune response. Because most of the studies were performed in the HP inoculation system, this model is most applicable to animal immune response to a nematode parasite. Antigen presenting cells capture and process foreign antigens and present antigenic peptides conjugated with MHC class II molecules on their cell surface. Naive Th cells are activated through TCR engagement with the antigen-MHC class II complex on APCs and CD28/CTLA-4-B7-1/B7-2 interactions. CD28/CTLA-4 ligation with either B7-1 or B7-2 on APCs can support Th cell activation and differentiation to IL-4 production (Figure 35). Activated Th cells begin to produce cytokines and upregulate their surface CD40L expression. CD40L on activated Th cells binds CD40 molecules on B cell through T-B cell contact, and together with T cell-derived cytokines, promotes B cell activation and differentiation to Ig secreting cells. B cells activated by CD40 signaling also upregulate surface B7-2 expression which may further promote Th cell activation through increased CD28-B7 ligation. In addition, CD40-CD40L interactions promote type 2-associated responses including increased blood eosinophils and mucosal mastocytosis (Figure 36). Activated Th effector cells (TE) rapidly lose their requirement for CD28/CTLA-4-B7 costimulation and can progress to IL-4 production in its absence. Memory Th cells similarly do not require CD28/CTLA-4-

B7 costimulatory signals for their activation to IL-4 production during the *in vivo* challenge response.

Figure 35. Either B7-1 or B7-2 can deliver necessary costimulatory signals for the activation of naive Th cells to develop into IL-4 producing effector Th cells during the *in vivo* type 2 immune response to HP. Further effector Th cell clonal expansion does not require B7 costimulation.

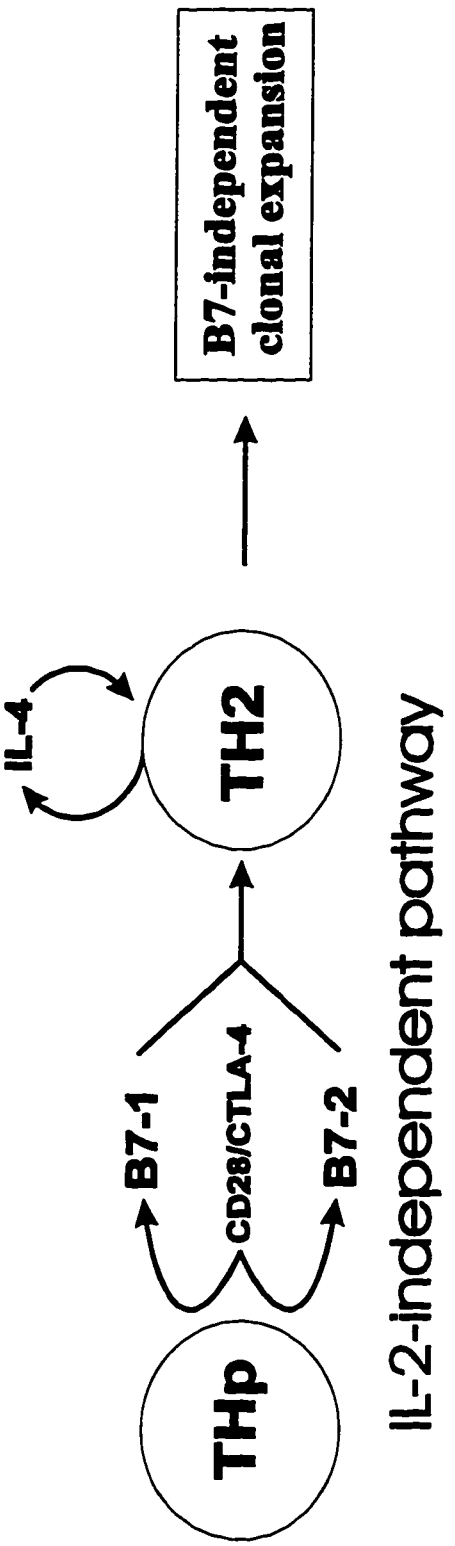
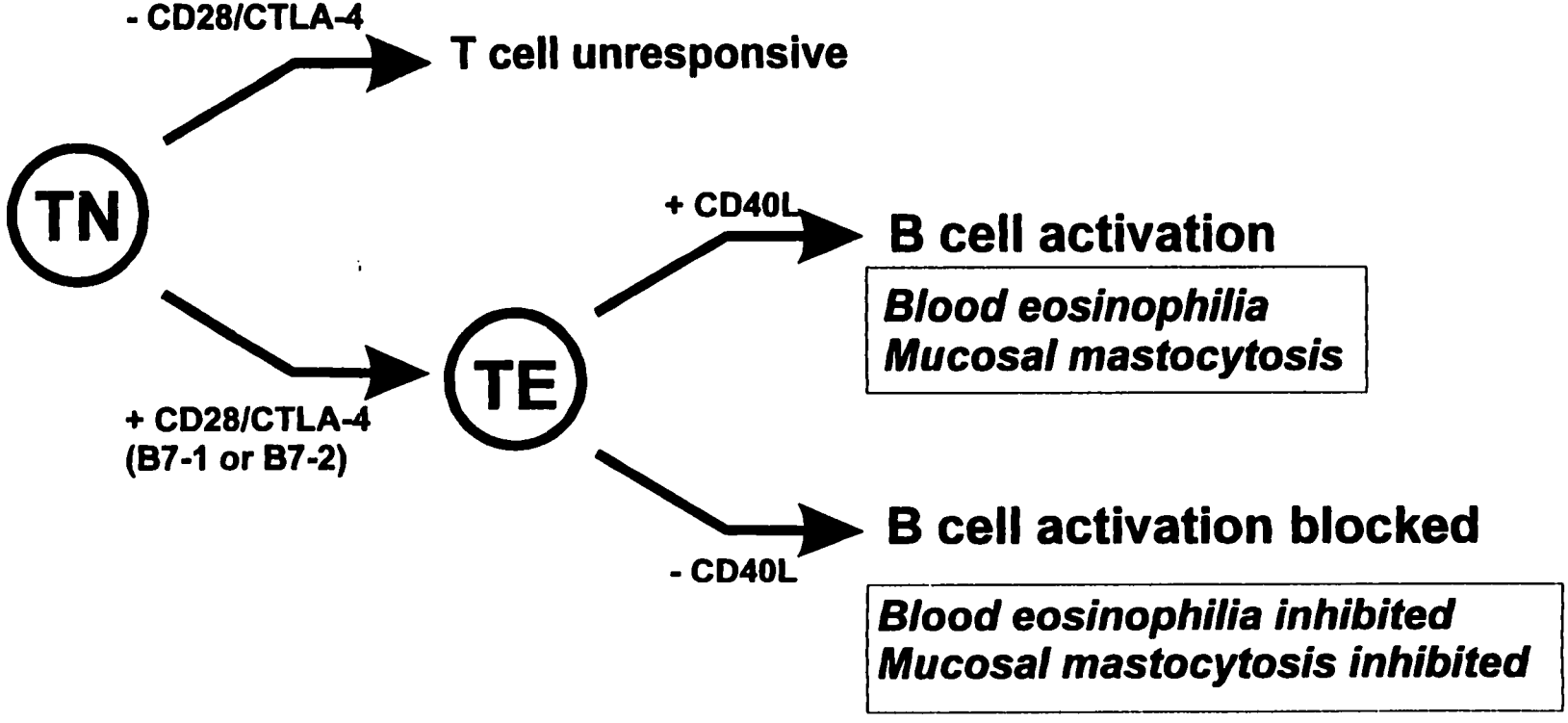

B7-independent clonal expansion
IL-4
TH2
B7-1
CD28/CTLA-4
B7-2
THp
IL-2-independent pathway

Figure 36. CD28/CTLA-4 costimulation and CD40-CD40L interactions are both required for the development of the mucosal type 2 immune response. Costimulatory signals through CD28/CTLA4 are important for naive Th cell (TE) activation. Blocking CD28/CTLA4 signaling will cause T cell unresponsiveness. CD40-CD40L interactions are not required for Th cell differentiation to cytokine producting effector Th cells (TE) but are important for the activation of B cell and other effector cells. Eosinophils and mast cells are enclosed in a box since their CD40L dependence may ont be T cell-mediated.


- CD28/CTLA-4
T cell unresponsive
TN
+ CD40L
B cell activation
Blood eosinophilia
Mucosal mastocytosis
TE
+ CD28/CTLA-4
(B7-1 or B7-2)
B cell activation blocked
- CD40L
Blood eosinophilia inhibited
Mucosal mastocytosis inhibited

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adhesion mo[illegible] CD[illegible]

post-transcr[illegible]

Chahine, A.[illegible] [illegible]u, M[illegible]

Local CTLA[illegible] synerg[illegible]

pancreatic isl[illegible]

Chan, S.H., [illegible] B[illegible]

S.F., Young, [illegible] Clark[illegible]

production b[illegible] ki[illegible]

and synergy [illegible] i[illegible]

Chatelain, R[illegible] [illegible]a, [illegible]

Leishmania [illegible]

Chatta, G.S., [illegible], A.G[illegible]

Morris, D.R. [illegible]. [illegible]

lympnocytes [illegible] th[illegible]

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