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Title of Dissertation: "The Role of Costimulatory Molecules in the Development of Memory and Effector T helper 2 Cells During an In Vivo Immune Response to the Murine Gastrointestinal Parasite *Heligmosomoides polygyrus*"

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ABSTRACT

Title of Thesis: The Role of Costimulatory Molecules in the Development of Memory and Effector T helper 2 Cells During an *in vivo* Immune Response to the Murine Gastrointestinal Parasite *Heligmosomoides polygyrus*

Name: Melinda Ekkens

Thesis directed by: William C. Gause, Ph.D. Director, Molecular and Cell Biology, Vice Chair, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences

Previous studies have demonstrated the importance of costimulatory interactions for effector $CD4^+$ T helper (Th) cell development during the primary immune response. However, the role of costimulatory molecules in memory $CD4^+$ T cell differentiation is not well understood. One model used to study the Th immune response involves oral infection of mice with the gastrointestinal nematode parasite *Heligmosomoides polygyrus*. Although the primary immune response to *H. polygyrus* is a chronic infection, challenge immunization triggers a T-dependent memory response that impairs adult worm maturation. In the studies presented herein, the effects of costimulatory molecule blockade on T helper effector cell function during the memory response were examined.

Effector T cell development was inhibited during the primary response to *H. polygyrus* in B7-1/B7-$2^{-/-}$ mice; however, memory Th cells developed that produced IL-4 and mediated effective reductions in adult worm egg production, but did not provide effective Ag-specific B cell help or support increased germinal center (GC) formation. Parallel studies in *H. polygyrus*-challenged $CD28^{-/-}$ mice demonstrated similar IL-4 elevations and decreases in adult worm egg production. However, Ag-specific Ab

responses and increased GC formation were significantly restored in *H. polygyrus*-inoculated $CD28^{-/-}$ mice.

Although elevations in serum IgG1 and GC formation were intact in *H. polygyrus*-challenged $OX40L^{-/-}$ mice, elevations in IL-4 and serum IgE were partially inhibited, and associated with decreased worm expulsion and increased egg production. To further examine the role of OX40L in Ag-specific $CD4^{+}$ T cell IL-4 production following priming, adoptively transferred OVA-specific DO11.10 T cells were analyzed in the context of the *H. polygyrus* response. Following immunization with OVA plus *H. polygyrus*, Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, and migration were comparable in $OX40L^{+/+}$ and $OX40L^{-/-}$ mice; however, Ag-specific T cell IL-4 production was reduced in $OX40L^{-/-}$ mice, suggesting a preferential role for OX40L costimulation in IL-4 production.

These studies extend our understanding of the role of costimulatory molecules in the development of memory Th2 cells during infectious disease. They also suggest that B7-1/B7-2 antagonists may be particularly effective in the treatment of chronic diseases where continuous renewal of effector populations from naïve precursor T cells mediates pathogenesis.

The Role of Costimulatory Molecules in the Development of Memory and Effector T helper 2 Cells During an *in vivo* Immune Response to the Murine Gastrointestinal Parasite *Heligmosomoides polygyrus*

by

Melinda Ekkens

Dissertation submitted to the Faculty of the
Molecular and Cell Biology Graduate Program of the
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in partial fulfillment of the requirements
for the degree of Doctor of Philosophy, 2002

DEDICATION

To Jaime—for your unending support and understanding.
Thank you for believing in me, and for always being there.
Thank you for the laughter and the joy you bring to my life.

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INTRODUCTION

A. General background

An understanding of the development of long-lasting memory cells is of particular importance when designing vaccines, or strategies to treat cancer, autoimmune diseases, or parasitic infections. Gastrointestinal parasitic infections play a major role in overall health in developing countries where sanitation and nutrition are poor, allowing easy transmission of infectious parasites. Changing economic and political environments, and limited financial resources, compound the difficulty in obtaining appropriate treatment for parasitic infections. According to the World Health Organization, approximately 3.5 billion people are infected worldwide with intestinal parasites and protozoa, including the soil-transmitted nematodes: *Ascaris lumbricoides* (roundworm); *Trichuris trichiura* (whipworm); and the hookworms, *Ancylostoma duodenale* and *Necator americanus*. In 1993, WHO ranked intestinal helminths as the main cause of disease burden in school-aged children (1), while other studies have repeatedly demonstrated *A. lumbricoides* infection rates of 75-90% in children aged 0-11 years-old (2,3). Infection with *A. lumbricoides*, *T. trichiura*, and the hookworms (*A. duodenale*) results in an estimated loss of 39 million disability-adjusted life-years (4), which represents a significant loss of disability-adjusted life-years in developing countries.

Mortality from soil-transmitted helminths is low; however, morbidity is high and chronic infection results in anemia, malnutrition—malabsorption of, and competition for, nutrients, as well as reduced food intake—and intestinal bleeding, which cause impaired physical and intellectual development in the young (1,2,5). The most dramatic effects of intestinal parasitic infections are seen in young children (2); however, the resulting

anemia and malnutrition can have a significant impact on quality of life, as well as on the overall health and productivity of a community. An immune response that results in adult worm expulsion is generally slow to develop, and since repeated infections increase the worm burden, resulting in more severe health effects, it is important to develop treatment strategies which allow long-term protection against chronic re-infection. This dissertation will focus on the development of effector and memory T cells following infection with a murine model for hookworm infection, the gastrointestinal helminthic parasite *Heligmosomoides polygyrus*. Basic aspects of the immune response will first be discussed followed by an overview of the immune response to this specific nematode parasite.

B. General introduction to in vivo immune responses

The immune response can be divided into innate (natural) and adaptive (acquired) immune responses (Figure 1). The innate immune response is the first line of defense against pathogens and consists of physiological barriers such as the skin and mucous membranes, phagocytic cells (neutrophils and macrophages), natural killer cells, and soluble factors such as complement and interferons. Adaptive immunity is the second line of defense and involves the Ag-specific response to pathogens mediated by Ag-specific lymphocytes generated by clonal selection. Adaptive immunity is also characterized by the ability to develop immunological memory, which allows generation of a rapid, strong response following repeated exposure to the same Ag. The adaptive immune response can be further sub-divided into the cellular immune response—mediated by T lymphocytes, and the humoral immune response—mediated by antibodies (6).

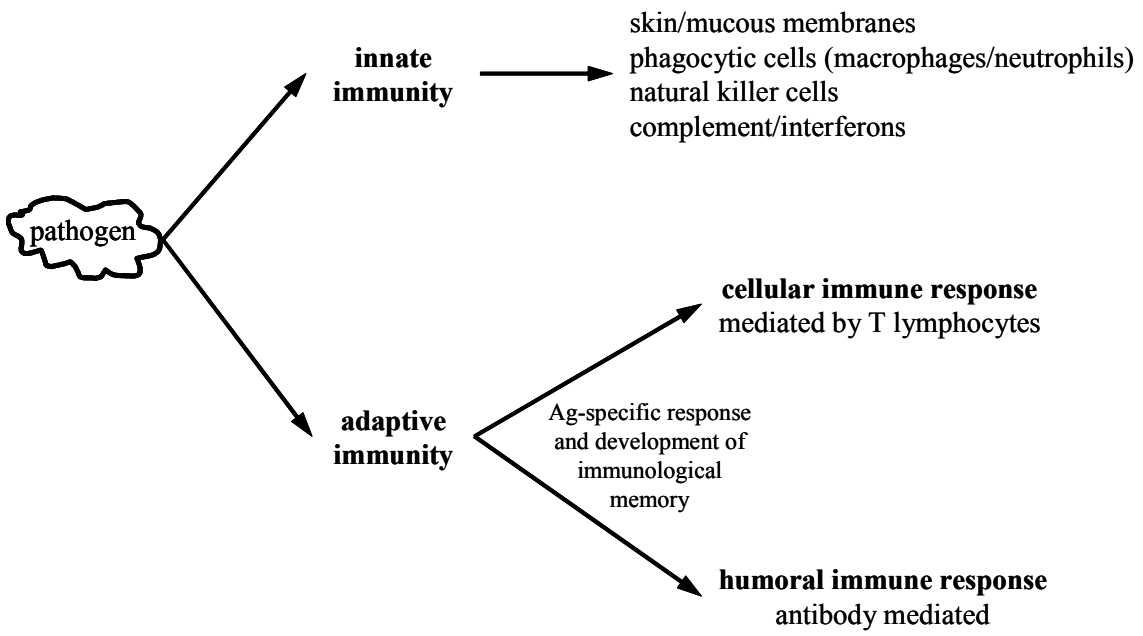
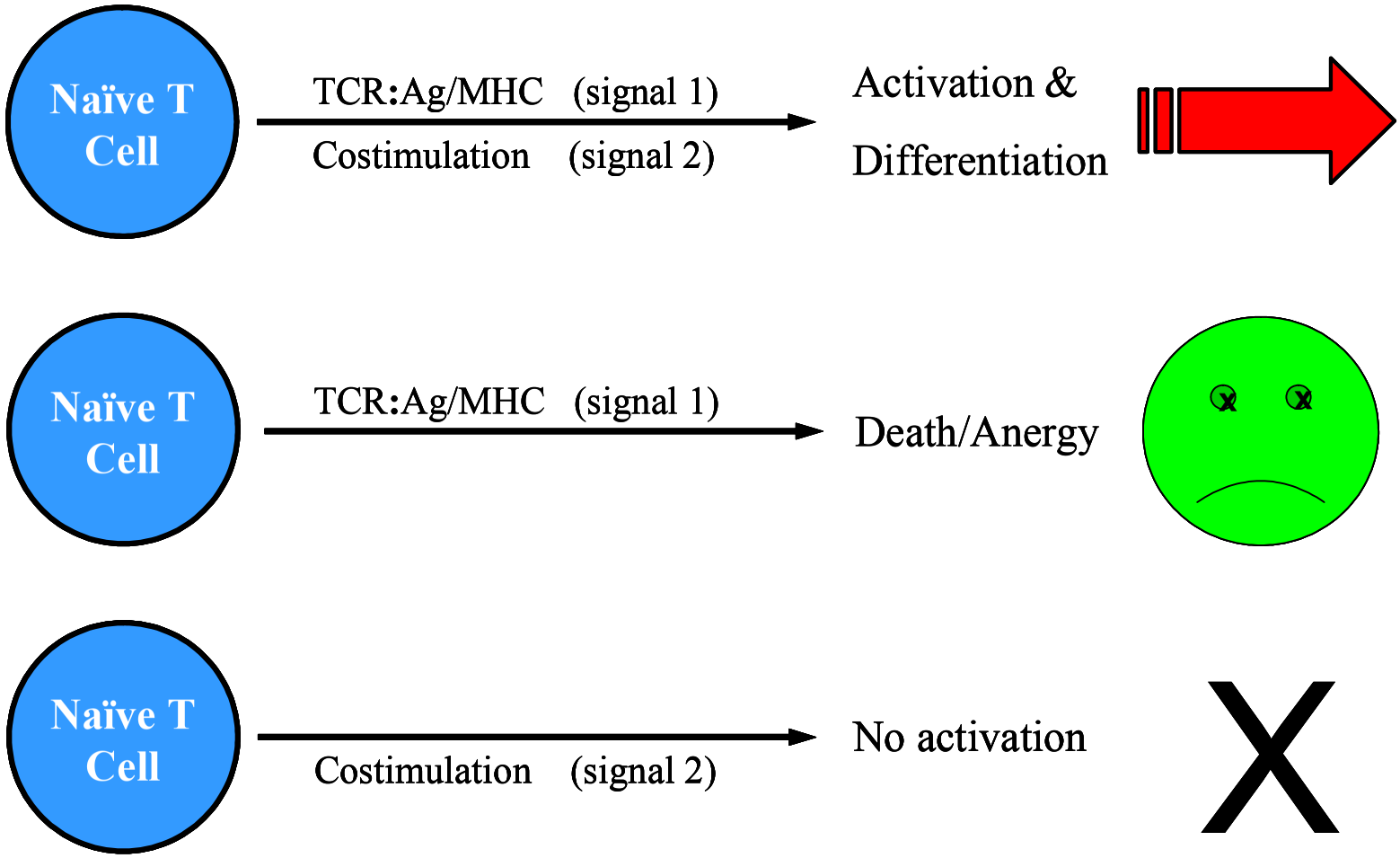


Figure 1. Protective immunity is mediated by both innate and adaptive immune responses. The innate immune response is the first line of defense, and consists of physiological barriers such as the skin and mucous membranes, phagocytic cells, natural killer cells, and soluble factors. Adaptive immunity is the second line of defense and involves the Ag-specific response to pathogens mediated by Ag-specific lymphocytes generated by clonal selection. The adaptive immune response can be further sub-divided into the cellular immune response—mediated by T lymphocytes, and the humoral immune response—mediated by antibodies.

Naïve T lymphocytes are activated by signals through the T cell receptor (TCR) following recognition of specific antigenic peptides displayed by the MHC on antigen presenting cells (APC) such as dendritic cells (DCs), B cells, and macrophages. Costimulatory molecules present on both the APC and the T lymphocyte provide a second, non-Ag-specific, signal (Figures 2) (6). In the absence of costimulatory molecule interactions, Ag binding to the T cell receptor can result in inactivation of the T lymphocyte through induction of anergy or cell death; both signals are usually required for effective activation of T cells (6,7).

Figure 2. Two signal model for naïve T cells. Naïve T cells require two signals for their activation and differentiation to effector T helper cells. Signal 1 is provided by the T cell receptor binding the Ag-MHC complex, while signal 2 is provided by costimulatory molecules present on both APCs and T cells. If only signal 1 is present, the T cell will undergo initial inactivation, followed by anergy or apoptosis. If only signal 2 is present, the T cell will not respond—it will not be activated or inactivated.


Naïve T Cell
TCR:Ag/MHC (signal 1)
Costimulation (signal 2)
Activation &
Differentiation
Naïve T Cell
TCR:Ag/MHC (signal 1)
Death/Anergy
Naïve T Cell
Costimulation (signal 2)
No activation

Following appropriate activation, the naïve T lymphocyte differentiates into a T helper (Th) effector cell—either a Th1 or Th2 cell (Figure 3). Th1 cells are characterized by increased production of interleukin (IL)-2, IFN-γ, and lymphotoxin, while Th2 cells secrete IL-4, IL-5, IL-9, and IL-13 (8,9). IL-10, although initially considered a Th2 cytokine, is also elevated in Th1 responses and appears to have a downregulatory effect in both responses (10-16). The Th1 immune response is associated with an inflammatory response characterized by activation of natural killer cells and macrophages, and elevations in serum IgG2a levels; this immune response is important for protection against intracellular pathogens such as viruses and bacteria. The Th2 immune response is characterized by eosinophilia, mast cell activation, and elevations in serum IgE and IgG1 levels. The IL-4 dominant Th2 immune response is important for protection against extracellular pathogens such as intestinal parasites, and also plays a primary role in mediating immediate hypersensitivity responses (17).

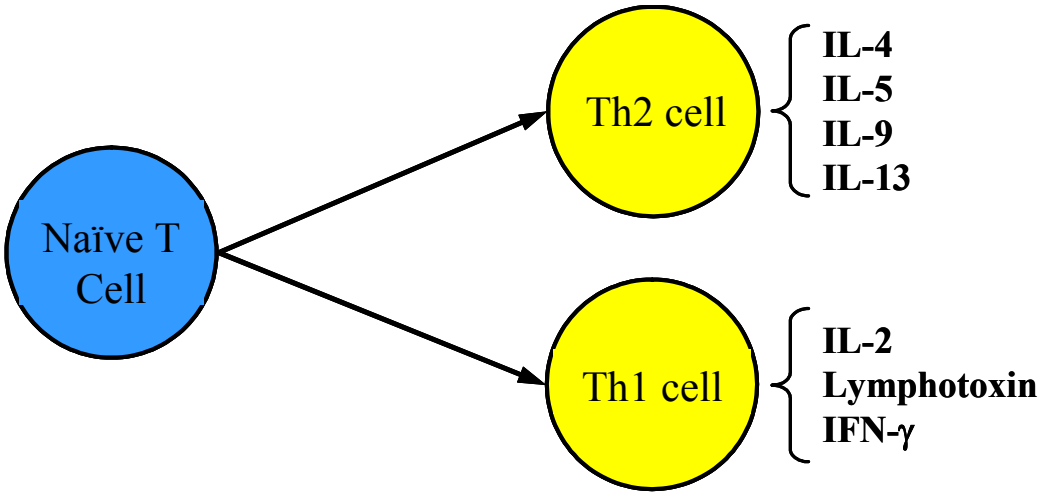
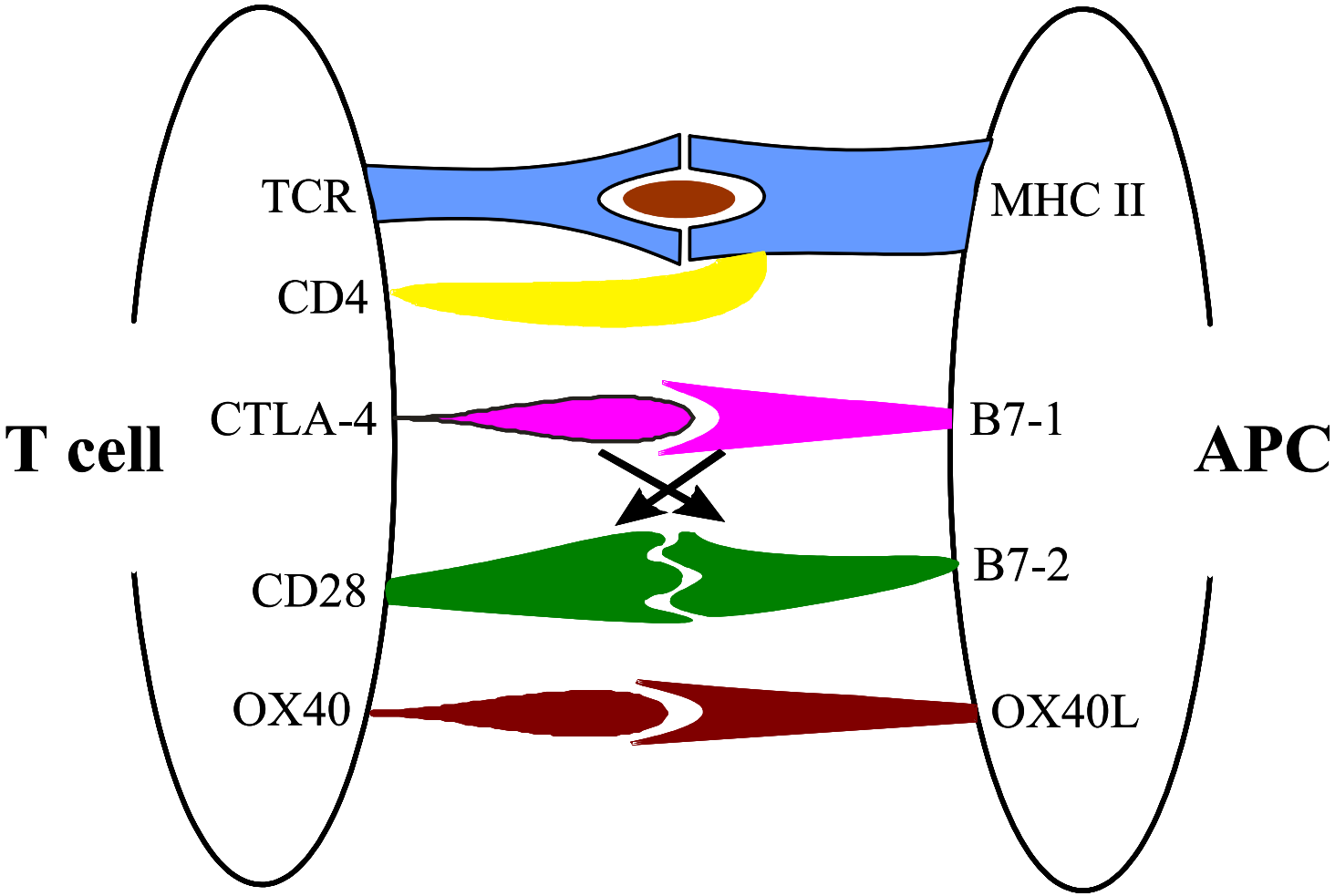


Figure 3. Following activation, naïve T cells can differentiate into either Th1 or Th2 cells. Th1 cells secrete IL-2, IFN-γ, and lymphotoxin, while Th2 cells secrete IL-4, IL-5, IL-9, and IL-13.

Costimulatory molecule interactions are important not only because they allow appropriate activation of naïve T cells, but also because they represent important clinical immunotherapy targets. T cell activation can be inhibited with Ag-specific antagonists—which often require labor-intensive identification and analysis—or through manipulation of costimulatory molecule interactions by Abs or genetically engineered fusion proteins. Blocking costimulatory molecule interactions selectively affects T lymphocytes which have already received signals through the TCR; bystander T cells which have not received the Ag-specific stimulus will not be affected by blockade of costimulatory molecules. Therefore, targeting costimulatory molecules allows selective manipulation of an Ag-specific immune response, without knowing exactly which Ag is involved (18).

Several studies have demonstrated that a particularly potent costimulatory signal is provided by the interaction between CD28 on T lymphocytes and B7-1 or B7-2 on APCs (19-27). Of more recent interest is the costimulatory signal provided by OX40 on T lymphocytes and OX40L on APCs (28-32) (see Figure 4). Previous studies have suggested that these costimulatory molecules are involved in the development of Th2 immune responses (21,23-26,30-34); however, their importance during the development of memory Th2 cells is unclear. Understanding the role of these costimulatory molecules during the development and activation of effector and memory Th2 cells could aid in the development of vaccines and other strategies that promote the development of long-lasting immunological memory, or—alternatively—the development of immunotherapies aimed at blocking the development of memory cells during allergy, autoimmune disease, or transplantation.

Figure 4. Interactions between costimulatory molecules present on both APCs and T cells are required for appropriate T cell activation, in addition to the Ag-specific TCR:Ag-MHC interaction. CD28 and CTLA-4 on the T cell bind either B7-1 or B7-2 on the APC; CD28/B7 interactions provide a potent costimulatory signal, while CTLA-4 provides a negative signal. An additional signal may be provided by OX40 which is expressed on the T cell, and binds OX40L which is expressed on the APC; both OX40 and OX40L are up-regulated following T cell activation.


T cell
TCR
CD4
CTLA-4
CD28
OX40
MHC II
B7-1
B7-2
OX40L
APC

C. Overview of B7/CD28 costimulatory molecule interactions

B7-1/B7-2

B7-1 (CD80) and B7-2 (CD86) are expressed on the surface of APCs—particularly DCs, B cells, macrophages, and T cells (35-37), and they bind CD28 and CTLA-4 on the surface of T lymphocytes (21,22,35). B7-1 and B7-2 are members of the immunoglobulin (Ig) gene superfamily, and contain an Ig variable-like region and an Ig constant-like region in their extracellular domain (22,38). The amino acid homology between murine B7-1 and B7-2 is approximately 25%, with greater homology in the extracellular domain than in the cytoplasmic domain (7,39); murine and human B7 molecules share greater than 40% amino acid homology, with the greatest similarities in the extracellular domains (40). This lack of homology in cytoplasmic domains between species suggests that the cytoplasmic domains of B7-1 and B7-2 may not provide important cell signaling functions.

Although B7-1 and B7-2 are expressed on the same cells, they have different kinetics of expression on APCs during an immune response. B7-2 is constitutively expressed on APCs, and is rapidly up-regulated following activation, while expression of B7-1 is more slowly up-regulated following activation of APCs (7,35,36,39,41-46). Additionally, B7-1 and B7-2 have a higher affinity for CTLA-4 than CD28 (47), while B7-1 has a slower dissociation ("off") rate than B7-2 during both CD28 and CTLA-4 interactions (48). It has been suggested that the different expression patterns, kinetics, and binding affinities of B7-1 and B7-2 mediate different costimulatory functions during in vivo immune responses (7,39). In vivo studies have suggested that blocking B7-2 interactions favors development of a Th1 immune response, while blocking B7-1 favors

development of Th2 immune responses (26,49-51). However, recent cardiac transplantation studies have demonstrated that graft rejection is dependent on the presence of B7 molecules—either B7-1 or B7-2—in the recipient, but not the donor (52), suggesting that either B7-1 or B7-2 can mediate allograft rejection. In agreement, studies utilizing the intestinal nematode parasite *H. polygyrus* have demonstrated that either B7-1 or B7-2 can mediate induction of the Th2 immune response to *H. polygyrus* (24); however, B7-2 is required to sustain the Th2 immune response at later time points (53). These studies suggest that during an in vivo immune response, B7-1/B7-2 costimulation is required for the initiation of a primary immune response. However, few studies have examined the role of B7 molecules in the development and activation of a memory immune response during infectious disease.

CD28/CTLA-4

CD28 and CTLA-4 are members of the Ig superfamily (54-56), and they are expressed on the surface of T lymphocytes (21,22,35). Although CD28 and CTLA-4 both bind B7-1 and B7-2 (7,21,22,39,45), they have limited amino acid sequence homology—approximately 25-30%, with most of the homology occurring in the extracellular region (19,55). Mouse and human CD28 and CTLA-4 amino acid sequences share approximately 68% homology (57); the cytoplasmic domain is highly conserved between species—particularly the phosphotyrosine motif Tyr-X-X-Met—suggesting that CD28 and CTLA-4 are involved in T lymphocyte signaling. CD28 is constitutively expressed on T cells, while expression of CTLA-4 is rapidly up-regulated following T cell activation (58,59).

Following CD28 engagement by B7-1/B7-2 or anti-CD28 Abs, the cytoplasmic domain of CD28 is phosphorylated at the Tyr-X-X-Met motif and binds the SH2 domain of the 85-kDa regulatory subunit of the heterodimer phosphatidylinositol 3-kinase (PI3-K) (60,61). Through the same Tyr-X-X-Met motif, CD28 also recruits and binds the SH2 domain of the intracellular protein growth factor receptor-bound protein-2 (GRB-2), although GRB-2 binds with lower avidity than PI3-K. GRB-2 is an adaptor protein which binds CD28 through an SH2 domain and binds the guanine nucleotide exchange protein Son of sevenless (SOS) through one of two SH3 domains (60,62,63). CD28 binding of GRB-2/SOS anchors this complex to the plasma membrane, which allows GRB-2/SOS to interact with membrane-associated $p21^{ras}$, allowing SOS to activate $p21^{ras}$ by exchanging GDP for GTP. Activation of $p21^{ras}$ by SOS then activates the downstream MAPK (mitogen-activated kinase) and JNK (Jun kinase) kinases; JNK in turn phosphorylates c-Jun and increases AP-1 transcriptional activity which is required, along with NFAT, for IL-2 gene transcription/regulation (60,63). The interaction between CD28 and GRB-2/SOS links CD28 with the Ras pathway, although it's unclear whether SOS acts as a guanine nucleotide exchange protein during CD28 signal transduction. An interaction between PI3-K and $p21^{ras}$ has also been suggested (60,63). CD28 can also activate downstream kinases by binding the T-cell specific protein-tyrosine kinase Itk (also known as Emt and Tsk) through a proline-rich domain (60); however, the functional importance of this signaling pathway is unclear (63).

The signaling pathway for CTLA-4 is poorly understood. Several studies have demonstrated that CTLA-4 can bind PI3-K and the protein tyrosine phosphatase SRC homology protein 2 domain-containing tyrosine phosphatase 2 (SHP-2), however, the

physiological relevance is unclear (64,65). It has also been demonstrated that CTLA-4 engagement directly inhibits TCR signaling by reducing tyrosine phosphorylation of ZAP-70 (ζ-associated protein of 70 kDa) and inhibiting the association of ZAP-70 and the tyrosine kinase $p56^{lck}$ (65). CTLA-4 has been shown to regulate cell cycle progression by inhibiting the production of cyclin D3, and the cyclin-dependent proteins, cdk4 and cdk6 (66). CTLA-4 also antagonizes the downregulation of the cell cycle inhibitor $p27^{kip}$ by CD28/B7 (66-68). Therefore, CTLA-4 exerts negative effects on cell activation through multiple pathways.

Although early in vitro studies suggested that both CD28 and CTLA-4 mediate a positive costimulatory signal (19,20,22,69), in vivo studies have demonstrated that CTLA-4 provides a negative signal to the T cell, independent of CD28 (70-74,74-76). Mice genetically deficient for CTLA-4 develop a spontaneous fatal lymphoproliferative disorder, resulting in multiorgan damage, and wasting death by 3 months of age (77-80). Recent studies have also demonstrated a role for CTLA-4 signaling during the induction of tolerance; blocking CTLA-4 signaling with CTLA-4Ig, anti-CTLA-4 Abs (81-83), or CTLA-4-deficient mice (67) inhibited induction of tolerance to specific Ag. These studies support a negative, regulatory role for CTLA-4. However, few studies have examined the role of CTLA-4 in the development and activation of memory T lymphocytes during infectious disease.

CD28 engagement by B7-1 and B7-2 has been shown to mediate a positive costimulatory signal to T lymphocytes, resulting in enhanced proliferation and survival of activated T cells, and increased production of IL-2 and IL-4 (19,20,22,71,84-86). Blockade of CD28/B7 interactions with the fusion protein CTLA-4Ig has been shown to

inhibit the T-dependent responses to sheep erythrocytes and keyhole limpet hemocyanin (KLH) (87), as well as the IL-4 dominant Th2 immune responses to goat anti-mouse IgD (34), the protozoan parasite *Leishmania major* (25), and the gastrointestinal parasite *H. polygyrus* (23,33). These studies suggested that CD28/B7 interactions were of particular importance for the development of Th2 immune responses. However, studies using mice genetically deficient for CD28 have suggested that CD28/B7 costimulation is not an absolute requirement for all T helper effector cell functions. In vitro studies demonstrated that T cells from CD28-deficient mice had impaired proliferative responses to alloantigens, KLH, ConA, and stimulation with anti-CD3 antibodies (88,89). In vivo, CD28-deficient mice demonstrated reduced basal Ig levels, impaired Ig class-switching in response to vesicular stomatitis virus (VSV) (89), impaired germinal center formation in response to nitrophenyl-chicken γ-globulin (90), and an impaired Th2 response following infection with the helminthic parasite *Schistosoma mansoni* (91). However, CD28-deficient mice also demonstrated normal generation of Ag-specific cytotoxic T lymphocytes (CTL) in response to lymphocytic choriomeningitis virus (LCMV) (89), and developed normal Th2 immune responses to *H. polygyrus* (92) and *L. major* (93). These studies suggested that CD28 is not always required for T cell priming in vivo.

D. CD28/B7 costimulation and development of memory immune responses

Previous studies have demonstrated the importance of the interaction between CD28 and its ligands, B7-1 and B7-2, for the initiation and maintenance of effector T cells during the primary immune response to intestinal pathogens (23,53,85). However, the role of CD28/B7 interactions in the development of memory cells, which also occurs during the primary immune response, has been more difficult to ascertain.

The secondary immune response to antigen occurs more rapidly, has a larger magnitude, and is more efficient at clearing antigen than the primary immune response (94,95). These characteristics may be due to an increased frequency of antigen-specific cells, or to differences in the way naive and memory cells respond to antigenic stimulation. Early studies suggested that memory T cells could respond to a wider range of antigen-presenting cells than naive T cells (96), and that activation of memory T cells was less dependent on costimulatory interactions than activation of naive T cells (96,97). These findings suggested that, once developed, memory T cells were more permissive than naive T cells.

In vitro studies using antigen-presenting cells from mice lacking both B7-1 and B7-2 demonstrated that the absence of both B7-1 and B7-2 did not inhibit the activation of memory T cells, as measured by the ability of memory cells to proliferate (95) and produce IL-4 (95,98), IL-5, or IFN-γ (95) following restimulation with OVA antigen. However, priming of naive T cells was B7-dependent (98). This was in agreement with previous studies, which demonstrated that blocking CD28/B7 interactions with CTLA-4Ig at the time of challenge with the intestinal parasite *H. polygyrus* did not inhibit memory T cell activation or effector function (85,99). Additional studies also demonstrated that blocking CD28/B7 interactions during the primary immune response with either anti-B7-2 antibodies (100) or the murine fusion protein CTLA-4Ig (87,101) inhibited development of a memory response to T-dependent Ags. These studies suggested that the development of memory T cells was dependent on CD28/B7 costimulatory interactions, while the activation of memory cells was CD28/B7-independent.

In contrast, other studies have demonstrated that blocking B7 interactions during a secondary immune response with CTLA-4Ig or anti-B7-2 (102), or a combination of anti-B7-1 and anti-B7-2 (103), partially inhibited activation of memory T cells. Consistent with these studies, mice deficient for CD28 alone, or for both CD28 and HSA, demonstrated that either B7 or HSA costimulation could induce immunological memory to influenza virus, but that B7 interactions were essential for the activation of effector $CD8^+$ CTLs from either naive or memory $CD8^+$ T cells (104). The authors proposed that naive T cells may integrate distinct, but overlapping, costimulatory signals, such that a strong overall signal (i.e. B7/CD28) would induce production of effector cells, while a weaker signal (i.e. HSA) would be sufficient to induce development of memory cells (104). This study suggested that other costimulatory molecules (such as OX40/OX40L, HSA, or ICOS) might be able to substitute for B7 interactions during the development of memory T cells, but not for their ultimate differentiation to effector cells.

Interestingly, while CD28-deficient mice were resistant to chronic infection with a nonvirulent strain of *Toxoplasma gondii*, chronically infected CD28-deficient mice were not able to mount a host-protective response to rechallenge with a virulent strain of *T. gondii* (105). This study suggested that other costimulatory molecules may substitute for CD28 during development of a primary immune response, but that CD28 plays an important role in either the development or maintenance of memory T cells (105).

In contrast, a recent study using CD28-deficient mice demonstrated that CD28 was not required for the development or activation of memory $CD8^+$ LCMV-specific CTLs (106), while another study demonstrated that blocking CD28/B7 interactions with CTLA-4Ig during both the primary and secondary immune responses did not inhibit the

host protective memory response to *Nippostrongylus brasiliensis* (107). Interestingly, treatment with CTLA-4Ig during the primary immune response to *N. brasiliensis* decreased Th2 cell cytokine production following in vitro restimulation and reduced IgE levels during both the primary and secondary immune responses, but did not inhibit worm expulsion following either primary or secondary immunization, suggesting that in vivo T helper effector functions required for host protection were not blocked by treatment with CTLA-4Ig (107). The reduced levels of IgE in this study suggested that the ability of T cells to provide cognate B cell help might be more sensitive to B7 costimulation than the development and activation of other T effector cell functions (107). This was consistent with data from CTLA-4Ig transgenic mice; T cells from these mice were unable to provide cognate help to B cells in vivo, even though the T lymphocytes were not anergic (108).

However, although the primary response to *N. brasiliensis* is CD4-dependent, the secondary, host-protective immune response is CD4-independent (107,109). Furthermore, the primary host protective response to *N. brasiliensis,* resulting in CD4-dependent worm expulsion, is B7-independent (107). In contrast, the primary immune response to *H. polygyrus* is B7-dependent, while the memory response is $CD4^+$ T cell-dependent (23,24,110). Furthermore, the memory, but not the primary immune response, is associated with impaired adult worm maturation. Therefore, the *H. polygyus* memory immune response is a particularly good model for examining Th2 memory cell development; more specifically, studies of the *H. polygyrus* memory immune response can address whether memory Th2 cells can develop in the absence of B7-dependent effector T cell development during the primary immune response.

In summary, few studies have examined the role of B7-1/B7-2 interactions in the development and maintenance of memory Th2 cells, particularly during parasitic infection. Additionally, few studies have examined whether tolerance is induced in the absence of CD28 during infectious disease, when CTLA-4 is presumably the only B7-1/B7-2 ligand. Therefore, the studies presented in this thesis will focus on the development of memory Th2 cells during the in vivo CD4-dependent response to *H. polygyrus*, in the absence of both B7-1 and B7-2, or CD28.

E. OX40/OX40L interactions and development of primary and memory immune responses

OX40 (CD134) and its ligand (OX40L; CD134L) are members of the tumor necrosis factor (TNF) receptor/ligand superfamily, and have been shown to play an important role in antigen-specific T cell activation and costimulation. OX40 is expressed on activated T cells (28,29), while OX40L is expressed on activated B cells (28,29), activated DCs (111,112) and activated endothelial cells (113,114). OX40/OX40L interactions have been shown to enhance T cell activation and proliferation (31,115,116), cytokine and Ig production (30-32,116-118), and T cell survival (31,119,120). They also play a role in DC:T cell interactions, enhancing DC maturation (111) and Ag-presenting functions in a CD40-dependent manner (116,121,122).

Although initial studies suggested that OX40/OX40L interactions might be particularly important during development of a Th2 immune response (30-32), more recent studies have demonstrated that OX40/OX40L interactions enhance production of both Th1 and Th2 cytokines (116,122,123). Studies using genetically deficient mice have demonstrated that OX40/OX40L interactions are not required for the development

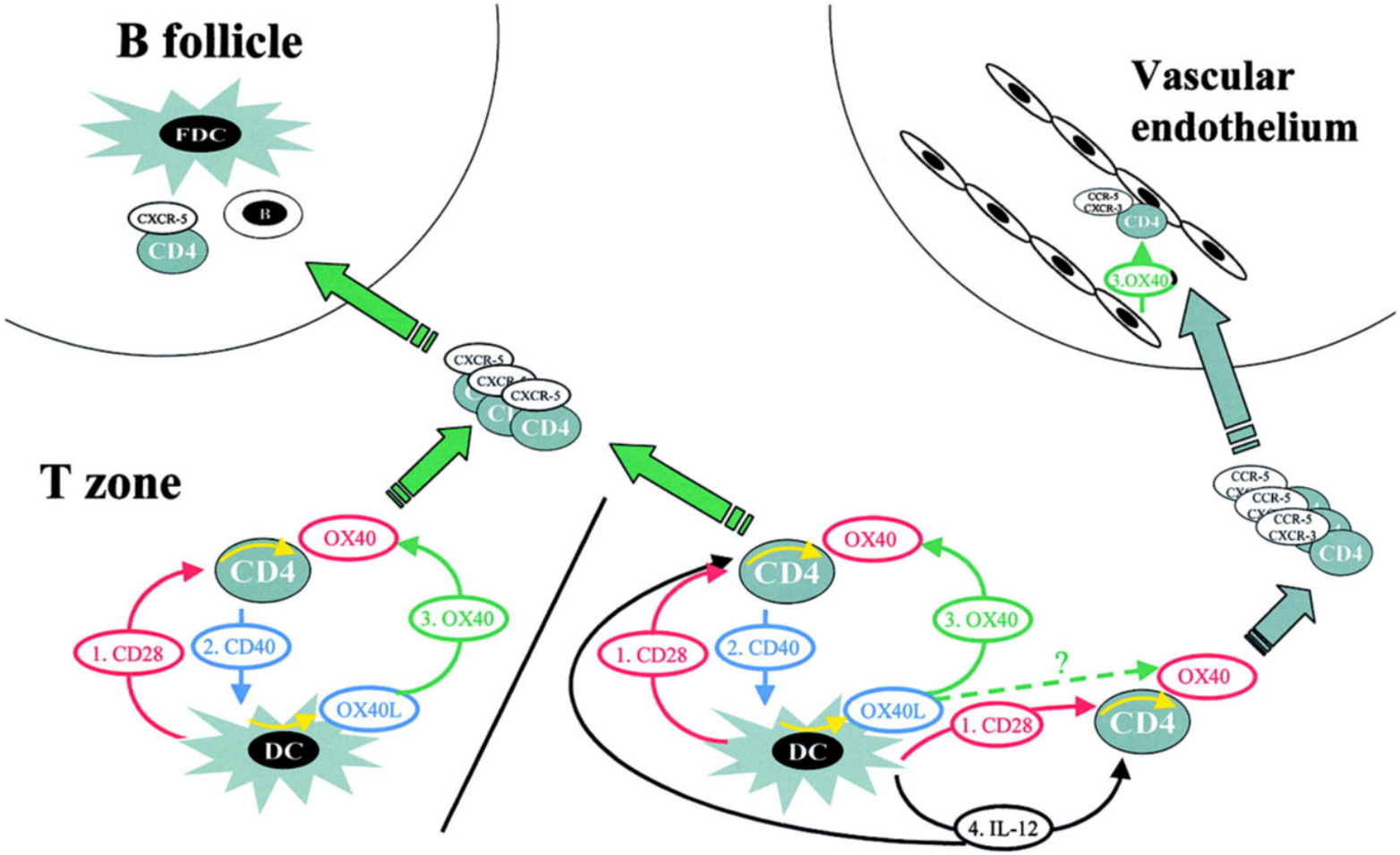
of germinal centers (GCs) or Ab responses to Ags or infectious agents that trigger IFN-γ-dominant, Th1 responses (115,121,123), while anti-OX40L mAb administered during the immune response to *L. major* caused a decrease in Th2 cytokines and antibody production with a slight increase in Th1 cytokine production associated with enhanced protection (112). In contrast, OX40-deficient mice made normal Ab responses to trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) and nitrophenyl-OVA, induced normal delayed-type hypersensitivity (DTH) responses, and demonstrated normal Th2-mediated protection to *N. brasiliensis*, Th1-mediated protection to *L. major*, and CTL-mediated clearance of Theiler's murine encephalomyelitis virus (123).

Several studies have also suggested a role for OX40/OX40L interactions during migration and homing of T cells. Studies of autoimmune diseases have identified $CD4^+$ $OX40^+$ T cells at sites of inflammation (124-127), and several studies have demonstrated binding of $OX40^+$ T cells to endothelial cells expressing OX40L, suggesting a role for these costimulatory molecules during inflammatory lymphocytic infiltration (113,114). Blocking OX40/OX40L interactions has been shown to inhibit T cell proliferation, cytokine secretion, and cellular infiltration during chronic inflammatory diseases such as experimental allergic encephalomyelitis (126,128,129), inflammatory bowel disease (124,130), and rheumatoid arthritis (127), resulting in amelioration of disease. Treatment with anti-OX40L mAb inhibited acute graft-versus-host disease following allogeneic bone marrow transplantation by decreasing organ inflammation and production of IFN-γ, while inducing hyporesponsiveness to alloantigens (131). The Th2 asthma response is also dependent on OX40/OX40L interactions, since OX40-deficient mice demonstrate reduced cellular infiltration and inflammation, reduced production of Th2 cytokines,

decreased mucous production, and enhanced airway responses (132). OX40/OX40L interactions may also play a role in T cell homing within secondary lymphoid organs. CD28-dependent OX40 signaling has been correlated with up-regulation of CXCR5 on $CD4^+$ T cells, which results in homing of these T cells toward the B cell-rich follicles, where they provide the necessary signals for germinal center formation (see Figure 5) (30,133,134). Taken together, these studies suggested that OX40/OX40L interactions may regulate migration, homing, and retention of activated $CD4^+$ T cells within secondary lymphoid organs, and at the site of inflammation (125,135,136).

In addition to regulating T cell trafficking, recent studies have suggested that OX40 interactions may also function to sustain immune responses, maintaining T cell survival. An in vitro study demonstrated that T cells from OX40-deficient mice produce IL-2 and proliferate normally, but as the response proceeds, T cell expansion and associated increased cytokine production is not sustained (119). In addition, the frequency of Ag-specific T cells decreased in the absence of OX40, during both the primary and secondary immune responses, suggesting that OX40/OX40L interactions were important for sustaining long-lived T cell responses, resulting in the generation of greater numbers of memory cells (31,119). More recently, reduced levels of the anti-apoptotic proteins Bcl-xL and Bcl-2 were observed in OX40-deficient mice (120). These levels were similar to those observed in CD28-deficient mice (86), and suggested that CD28 and OX40 may act sequentially, with CD28/B7 interactions initiating cell proliferation and expansion, while OX40/OX40L interactions act later to prolong cell division and enhance survival by suppressing apoptosis (120).

Figure 5. Proposed model for the role of OX40/OX40L interactions during migration of activated T cells to the B cell follicle and/or the site of inflammation [adapted from Lane (2000) (136)]. *A.)* CD28, expressed on $CD4^+$ T cells, interacts with B7-2 on DCs, and upregulates expression of OX40 on antigen activated $CD4^+$ T cells. CD40 is expressed on activated $CD4^+$ T cells, and interacts with CD40L on activated DCs to upregulate expression of OX40L on DCs. CD28 and OX40 costimulatory signals synergize to induce rapid expansion of Ag-specific Th2 cells, which upregulate expression of IL-4 and the chemokine receptor CXCR5. Expression of CXCR5 results in homing of these Ag-specific Th2 cells to the B cell follicle where they can provide cognate B cell help. *B.)* CD28-dependent OX40 signaling also upregulates CXCR5 expression, when inflammatory Ags are encountered, along with subsequent migration to the B cell follicles. However, in this scenario, $CD4^+$ T cells are also signaled by IL-12 produced by DCs, resulting in differentiation of Th1 cells which upregulate expression of inflammatory chemokine receptors and migrate to the site of inflammation where OX40L expressed on activated endothelial cells directs migration of $OX40^+$ $CD4^+$ T cells. Therefore, following activation by inflammatory Ags, $CD4^+$ T cells can provide help for both B cells and inflammatory responses, depending on expression of CXCR5 or other chemokine receptors. Yellow arrows indicate intracellular signaling, while red color indicates CD28 dependence, and blue color indicates CD40L dependence. Green indicates rapid expansion of Ag-activated $CD4^+$ T cells. Black arrows indicate IL-12 signaling.



A. Antibody alone B. Inflammatory+Antibody

In summary, these primarily in vitro studies suggested that OX40/OX40L interactions may be important at later stages of the primary immune response, and may be particularly important during development and maintenance of a memory immune response. However, few studies have examined the role of OX40/OX40L interactions during in vivo priming and activation of $CD4^+$ T lymphocytes, particularly during the course of an infectious disease. This thesis will focus on the role of OX40L interactions in the development of effector Th2 cells during the primary and memory immune responses to *H. polygyrus* infection.

F. *H. polygyrus* as a model for the development of memory T cells during Ag priming

The murine intestinal nematode parasite *H. polygyrus* has been used extensively as a model for human intestinal parasite infection. It is especially useful because it is a natural parasite of mice and therefore the immune response is a product of host-pathogen coevolution, and because it shows a number of similarities to human infection with hookworms, including both *Necator americanus* and *Ancylostoma duodenale* which are highly host specific, making animal studies difficult. *H. polygyrus* is also an important model for studying the development of the Th2 memory response during infectious disease, as the primary host response is a chronic infection, while the secondary immune response is a host protective response, resulting in worm expulsion (Figure 6) (137). This difference between the effectiveness of the primary and secondary immune response makes it possible to functionally determine whether a memory response has occurred.

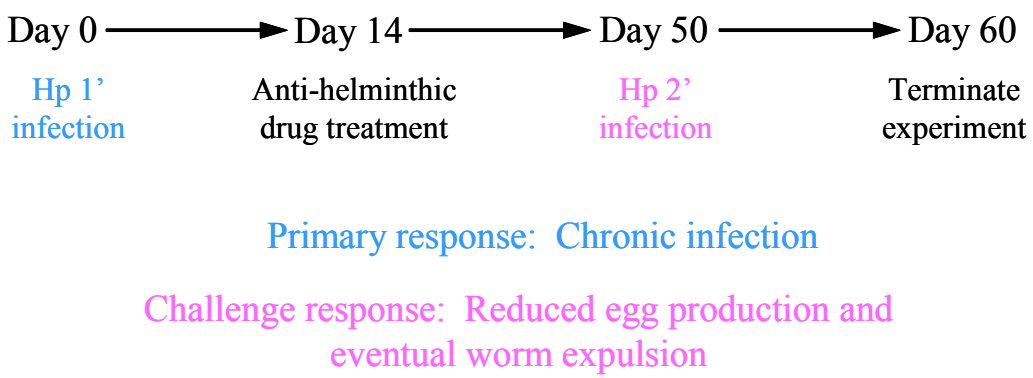


Figure 6. A model for the development of in vivo Th2 memory immune responses. *H. polygyrus* is a useful model for investigating the development and activation of memory Th2 cells, since it is easy to distinguish between the primary, chronic immune response, and the memory, host-protective response which impairs adult worm maturation, resulting in reduced egg production and eventual worm expulsion. The same general protocol was used for all memory studies presented in this thesis: Wildtype and genetically deficient mice were infected with 200 L3 *H. polygyrus* larvae, followed by treatment of all mice at approximately two weeks post-infection with the anti-helminthic pyrantel pamoate. After two to four weeks, the mice were given a challenge dose of *H. polygyrus*, while separate control groups of wildtype and genetically deficient mice were given an initial dose of *H. polygyrus*, allowing comparison of primary and memory immune responses to *H. polygyrus* in the same experiment.

H. polygyrus has a strictly enteral life cycle in which infective third stage larvae invade the intestinal mucosa after ingestion and develop there into mature adults that enter the gut lumen approximately eight days later (138). During the primary Th2 response, adult worms mature and produce eggs, inhabiting the gut lumen for at least several months. However, after clearance of worms from the gut with an anti-helminthic drug, a secondary challenge inoculation triggers a CD4-dependent, IL-4-dependent memory response that effectively limits adult worm maturation and egg production (110,137). The host response is characterized by elevations of Th2 cytokines, with $CD4^+$ $TCR\alpha/\beta^+$ T cells being the primary source of IL-4 elevations during the primary (33) and the secondary immune responses (99). Pronounced $CD4^+$ T cell-dependent elevations in serum IgE and IgG1 and increased GC formation in the mesenteric lymph node (MLN)

are also observed (33,53). Although a substantial memory humoral response is generated, previous studies have shown that protective immunity is primarily mediated by direct effects of Th2 cytokines on the gut (139-141). The results presented in this dissertation will focus on the development of memory T lymphocytes during the primary *H. polygyrus* immune response in the absence of the costimulatory molecules B7-1/B7-2, CD28, and OX40L.

G. Specific goals of this study

This dissertation is focused on the role of the costimulatory molecules B7-1 and B7-2, CD28, and OX40L, in the development of effector and memory T cells following infection with the intestinal helminthic parasite, *Heligmosomoides polygyrus*. The initial studies will examine the role of B7/CD28 costimulatory molecules in the development of memory effector $CD4^+$ T cells following priming with *H. polygyrus*. The purpose of these studies is two-fold: a) to investigate whether blocking B7-1 and B7-2 interactions during the primary immune response to *H. polygyrus* will inhibit the development of memory effector $CD4^+$ T cells following *H. polygyrus* challenge, and b) to investigate whether blocking CD28, while leaving CTLA4 interactions intact, will result in the induction of tolerance. Later studies will examine the role of OX40/OX40L costimulatory interactions in the development of effector and memory $CD4^+$ T cells following immunization with *H. polygyrus*. These studies will investigate whether a) OX40L blockade will inhibit the development of memory effector Th2 cells following immunization with *H. polygyrus*, and b) OX40L blockade will inhibit the development and trafficking of Ag-specific effector $CD4^+$ Th2 cells and the associated immune response following primary inoculation with *H. polygyrus*.

PAPER 1

Memory Th2 effector cells can develop in the absence of B7-1/B7-2, CD28 interactions, and effector T helper cells after priming with an intestinal nematode parasite. (*J. Immunol,* 2002, 168**:**6344-6351.)

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Please see the citation above for the information

PAPER 2 (submitted)

The role of OX40L interactions in the development of the Th2 response to the gastrointestinal nematode parasite *Heligmosomoides polygyrus*.

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Please see **J Immunol. 2003 Jan 1;170(1):384-93** for the information

DISCUSSION

This dissertation has focused on the role of costimulatory molecules, including B7-1/B7-2 CD28, and OX40L, in the development of effector memory Th2 cells following priming with the murine gastrointestinal helminthic parasite *Heligmosomoides polygyrus*. The results of these studies demonstrate that the development of IL-4 producing memory Th2 cells can occur in the absence of the costimulatory molecules B7-1/B7-2, CD28, and OX40L, following priming with *H. polygyrus*. However, OX40/OX40L interactions are required for T helper effector function during the primary and memory immune responses to *H. polygyrus*. These results extend our understanding of the role of costimulatory molecules in the development of memory Th2 cells, and should provide a framework for designing immunotherapies aimed at manipulating the development of memory cells during infectious diseases.

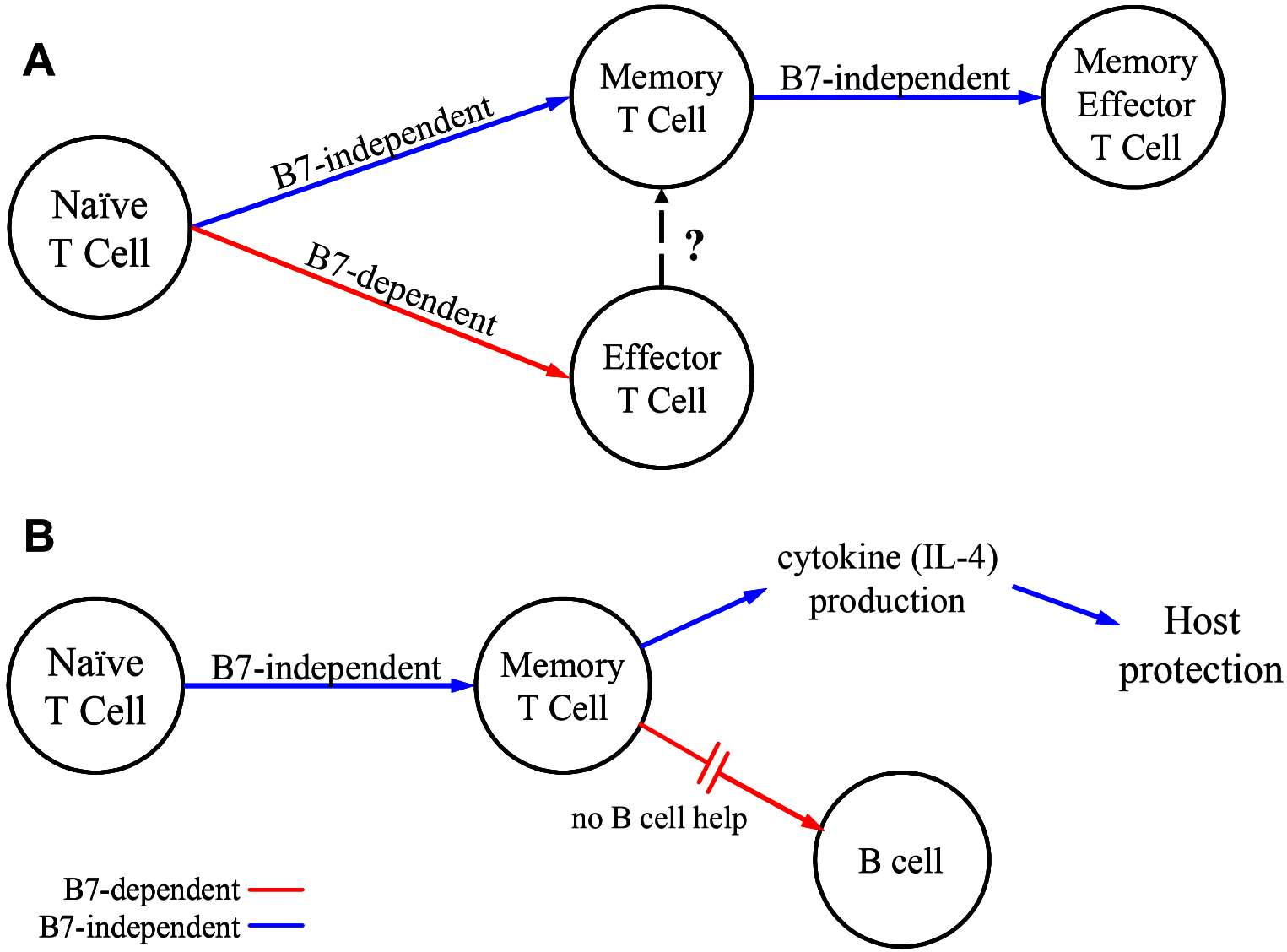
Discussion of results

These studies focused on the role of the costimulatory molecules B7-1/B7-2, CD28, and OX40L, in the development of effector and memory T cells during infectious disease. In order to directly examine the role of costimulatory interactions in the development of memory $CD4^+$ T cells, we utilized the murine nematode parasite, *H. polygyrus*, which induces a non-protective primary immune response, but a host-protective secondary immune response in mice. The host response to *H. polygyrus* is a Th2 immune response characterized by blood eosinophilia, intestinal mastocytosis, pronounced $CD4^+$ T cell-dependent elevations in IL-4 production and serum IgE and IgG1 levels, and increased GC formation in the mesenteric lymph node. This strong Th2 response allows one to investigate aspects of both cell-mediated and humoral immunity.

Previous studies in our lab have demonstrated that blocking B7 interactions with CTLA-4Ig at the time of *H. polygyrus* challenge does not inhibit activation of memory T cells (85,99), although the primary immune response to *H. polygyrus* is B7-dependent (23). Therefore, studies of the *H. polygyrus* memory immune response can address whether memory $CD4^+$ Th2 cells can develop in the absence of B7-dependent effector T cell development during priming. The recent availability of mice genetically deficient for both B7-1 and B7-2 (142) allows continuous blockade of B7/CD28 interactions after priming, and avoids the possibility of incomplete blockade by CTLA-4Ig, or the generation of murine blocking Abs to the fusion protein CTLA-4Ig during prolonged experiments addressing the development of the memory response.

In the studies presented in this thesis, we utilized B7-1/B7-$2^{-/-}$ mice to directly examine the development of memory $CD4^+$ T cells when B7 interactions were blocked during both the primary and secondary immune responses to *H. polygyrus*. These studies demonstrated that IL-4-producing memory Th2 cells that inhibit adult worm egg production following challenge *H. polygyrus*-inoculation were able to develop in the absence of B7-1/B7-2 costimulatory molecules and effector Th2 cell development during priming (Figure 7A). However, the T-dependent memory Ab response was impaired, with complete inhibition of Ag-specific serum IgE and IgG1 and GC formation. These findings demonstrated that B7/CD28 interactions were not required for the development of memory Th2 effector cells important in host protection, but were required for delivery of cognate B cell help in vivo (Figure 7B).

Figure 7. Memory T cells which produce IL-4 and mediate reductions in adult worm egg production are able to develop directly from naïve T cells in the absence of both B7-1 and B7-2. *A.)* Memory T cells are able to develop directly from naïve T cells in a B7-independent manner, and without going through an intermediate effector stage. Activation of primary effector T cells is B7-dependent. *B.)* Memory T cells which develop in the absence of B7-1 and B7-2 are able to produce IL-4 in sufficient quantities to mediate reductions in adult worm egg production, but require B7/CD28 interactions for optimal delivery of cognate B cell help.


A
Naïve
T Cell
B7-independent
B7-dependent
Memory
T Cell
?
Effector
T Cell
B7-independent
Memory
Effector
T Cell
B
Naïve
T Cell
B7-independent
Memory
T Cell
cytokine (IL-4)
production
Host
protection
no B cell help
B cell
B7-dependent
B7-independent

Although B7-1 and B7-2 are the only known ligands for CD28 and CTLA-4 (80), several studies have suggested that CTLA-4, or an as yet unidentified B7 ligand, may also provide a positive costimulatory signal, in addition to CD28 (89,93,143-146). Additional studies have suggested that CD28 is not required for all T helper cell responses in vivo (89,92,93), while CTLA-4 has been implicated in the induction of tolerance (67,81-83,147). Therefore, we also utilized mice genetically deficient for CD28 to investigate whether the absence of CD28, but not CTLA-4, during infectious disease would result in tolerance or development of a functional memory response. In these studies, *H. polygyrus*-inoculated CD28-deficient mice developed IL-4-producing memory Th2 cells that were able to mediate reductions in adult worm egg production similar to those observed in wildtype mice. Total and Ag-specific serum Ig levels and GC formation were reduced in *H. polygyrus*-inoculated CD28$^{-/-}$ mice as compared to *H. polygyrus*-inoculated wildtype mice during both the primary and secondary immune responses. However, *H. polygyrus*-infected CD28$^{-/-}$ mice demonstrated marked increases in Ag-specific serum Ig levels and GC formation, as compared to B7-1/B7-2$^{-/-}$ mice inoculated with *H. polygyrus*. Consistent with the studies performed in *H. polygyrus*-challenged B7-1/B7-2$^{-/-}$ mice, these findings suggested that B7/CD28 interactions were required for optimal delivery of cognate B cell help, but were not required for IL-4 production during the memory response to *H. polygyrus*.

Other studies (105,107,108,142) have also suggested that the ability of CD4^{+} T cells to provide B cell help is impaired in the absence of B7/CD28 interactions. However, our studies and those of others (108), suggest that although B cell help is inhibited, the CD4^{+} T cells are not anergic in the absence of B7/CD28 interactions, as

indicated by their ability to produce IL-4 and mediate a memory response to *H. polygyrus*. Further studies will be required to determine the role of B7 interactions during optimal delivery of B cell help. However, an inherent defect in GC formation in the absence of B7/CD28 interactions (90,142,148) may inhibit the ability of T cells to provide cognate help to B cells (149-152). Additionally, the absence of CD40/CD40L interactions has been shown to inhibit T cell helper functions—specifically Ig class-switching and GC formation (153-156). Since CD28/B7 interactions are required to induce CD40L expression (157-159) on activated T cells (160,161), this suggests that impaired CD40/CD40L signaling may also play a role in the inability of T cells to provide optimal B cell help in the absence of B7-1 and B7-2 (162).

Our finding that Ag-specific serum Ig production and GC formation were differentially regulated in mice genetically lacking B7-1/B7-2 or CD28, these studies suggested the possibility that a third B7 ligand—which is neither CD28 nor CTLA-4—might be providing a positive costimulatory signal. This possibility would explain the complete inhibition of Ag-specific serum IgE and IgG1 production in the absence of both B7-1 and B7-2, while CD28-deficient mice demonstrated increased, although reduced relative to wildtype mice, production of Ag-specific serum Igs following infection with *H. polygyrus*. In the absence of CD28, B7-1 and B7-2 may still bind the alternate costimulatory receptor, allowing an increase in Ag-specific Ig production. These findings are consistent with the differential survival patterns observed in B7-1/B7-2$^{-/-}$ mice and CD28$^{-/-}$ mice following allogenic cardiac transplantation; graft survival is prolonged in B7-1/B7-2$^{-/-}$ mice when B7 costimulation is completely blocked, while CD28$^{-/-}$ mice reject cardiac allografts nearly as rapidly as wildtype mice (52,72,146).

Although B7/CD28 interactions were not required for the development of memory IL-4-producing Th2 cells, it was possible that other costimulatory molecules were important during the development and activation of memory T cells. Several studies have suggested that OX40/OX40L interactions promote long-term survival of T cells during a primary response, leading to enhanced generation of memory cells (31,119,120,123,125,126,136,163). Mice genetically deficient for OX40L were inoculated with primary and challenge doses of *H. polygyrus* to directly examine the role of OX40/OX40L interactions in the development of a memory response during infectious disease. During both the primary and secondary immune response, elevations in IL-4 production and total and Ag-specific serum IgE were inhibited in *H. polygyrus*-inoculated OX40L$^{-/-}$ mice, while elevations in serum IgG1 levels and GC formation were primarily intact. An inhibition in memory effector T cell function, which mediates adult worm expulsion and decreased egg production, was also observed (see Figure 8).

These studies suggested a preferential role for OX40/OX40L interactions in the development of memory IL-4-producing Th2 cells following inoculation with *H. polygyrus*. In order to further investigate the priming of IL-4-producing Th2 cells in the absence of OX40L interactions, OVA-specific DO11.10 transgenic T cells were transferred into wildtype and OX40L$^{-/-}$ mice. Recipient mice were then immunized intracutaneously in the ear with OVA (Ag peptide) plus *H. polygyrus* (adjuvant). This approach provides a useful model system to examine the requirements for OX40/OX40L costimulation during the development and trafficking of Ag-specific Th2 cells (Figure 9).

Figure 8. OX40/OX40L interactions are required for optimal activation of Th2 cells during infectious disease. Following inoculation with *H. polygyrus*, OX40/OX40L interactions are required for T cell IL-4 production and host protection, but not GC formation and IgG1 production. An inhibition in memory effector T cell function, which mediates adult worm expulsion and decreased egg production, was also observed. OX40L-independent pathways are indicated in blue, while OX40L-dependent pathways are outlined in red.

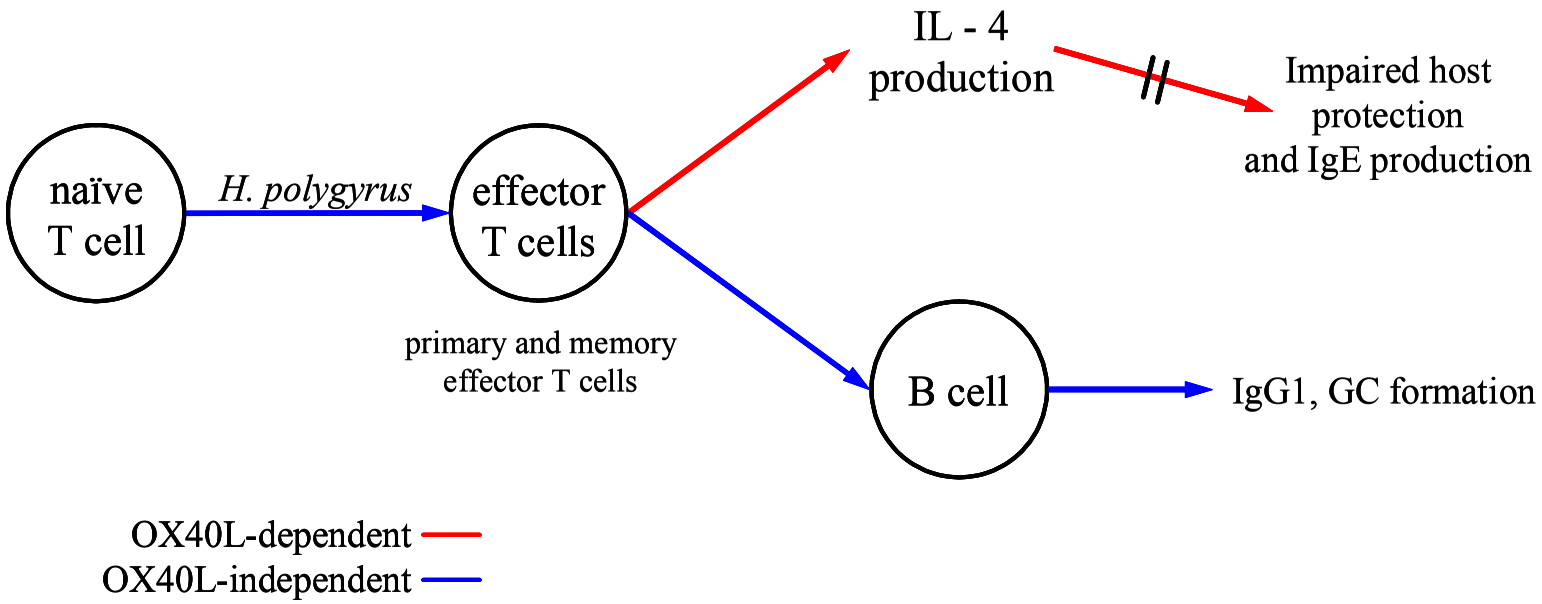
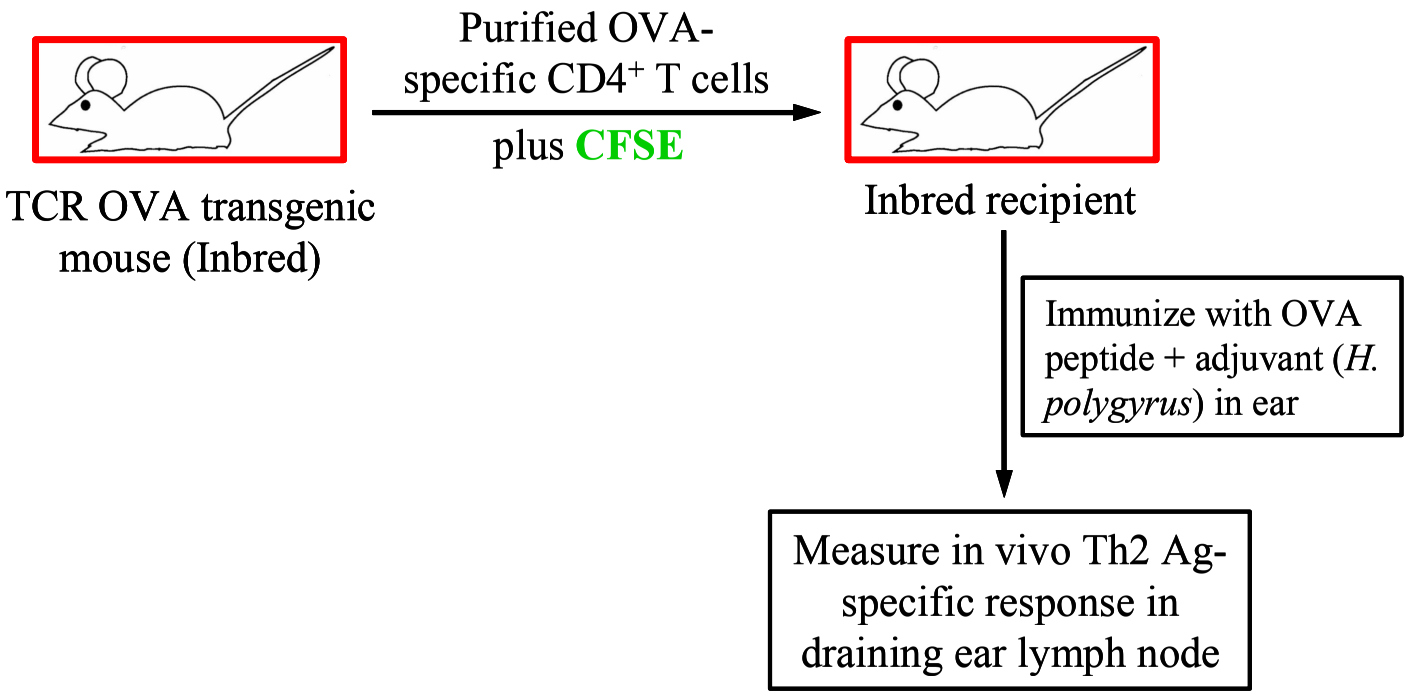

naïve
T cell
H. polygyrus
effector
T cells
primary and memory
effector T cells
IL - 4
production
Impaired host
protection
and IgE production
B cell
IgG1, GC formation
OX40L-dependent
OX40L-independent

Figure 9. DO11.10 TCR transgenic mice can be used to study the development and trafficking of Ag-specific T cells. DO11.10 transgenic T cells express a TCR specific for a class II MHC-restricted chicken ovalbumin (OVA) peptide [323-339], with the sequence I-S-Q-A-V-H-A-A-H-A-E-I-N-E-A-G-R-COOH. OVA-specific $CD4^+$ T cells were purified from DO11.10 transgenic mice and labeled with the cytoplasmic dye CFSE prior to transfer into age- and sex-matched recipient wildtype and OX40L-deficient mice. Two days later, the recipient mice were immunized in the ear with OVA peptide alone (Ag), *H. polygyrus* alone (adjuvant), or a combination of OVA and *H. polygyrus*. The in vivo Th2 Ag-specific response can then be measured in the draining cervical lymph node.


Purified OVA-specific CD4+ T cells
plus CFSE
TCR OVA transgenic mouse (Inbred)
Inbred recipient
Immunize with OVA peptide + adjuvant (H. polygyrus) in ear
Measure in vivo Th2 Ag-specific response in draining ear lymph node

In these studies, adoptively transferred DO11.10 transgenic T cells demonstrated a reduction in Ag-specific T cell IL-4 production following primary immunization of OX40L$^{-/-}$ mice with OVA plus *H. polygyrus*; however, Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, and migration were comparable between wildtype and OX40L$^{-/-}$ mice primed with OVA and *H. polygyrus* (Figure 10). These findings were in contrast to previous studies which demonstrated a CD28-dependent OX40-dependent up-regulation of CXCR5 (see Figure 5) (30,133,136), as well as a requirement for OX40/OX40L costimulation in order to sustain cell cycling and proliferation (31,115,119,120).

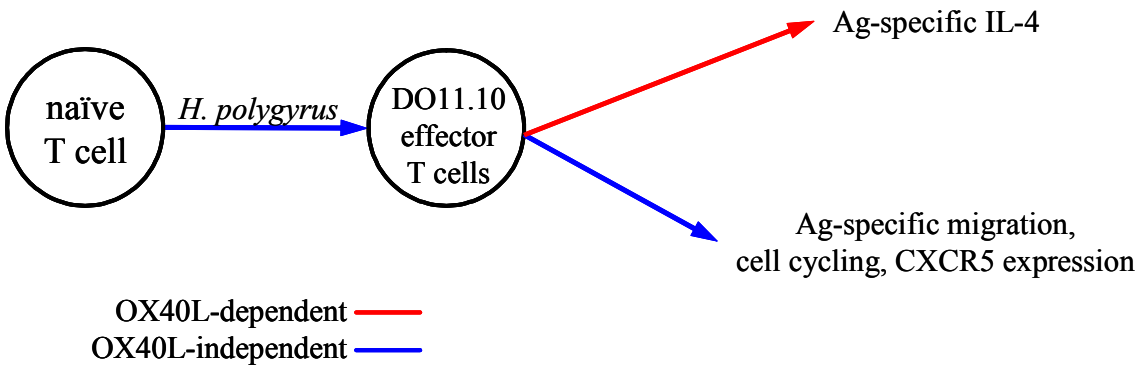


Figure 10. OX40/OX40L costimulation is required for optimal IL-4 production, but not Ag-specific T cell expansion, cell cycle progression, or CXCR5 expression. Adoptively transferred DO11.10 transgenic T cells demonstrated a reduction in Ag-specific T cell IL-4 production following primary immunization of OX40L-deficient mice with OVA plus *H. polygyrus*. However, Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, and migration were comparable between wildtype and OX40L-deficient mice inoculated with OVA and *H. polygyrus*. OX40L-independent pathways are indicated in blue, while OX40L-dependent pathways are outlined in red.

Further studies are required to investigate the specific impairment in IL-4 production and associated serum IgE elevations in the absence of OX40/OX40L interactions. However, possible explanations include impaired expression of the IL-4

receptor, and impaired IL-4-dependent signaling in the absence of OX40/OX40L costimulation. The findings presented in this thesis indicate that OX40/OX40L interactions are not required for Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, or migration to the T:B zone following priming with *H. polygyrus*.

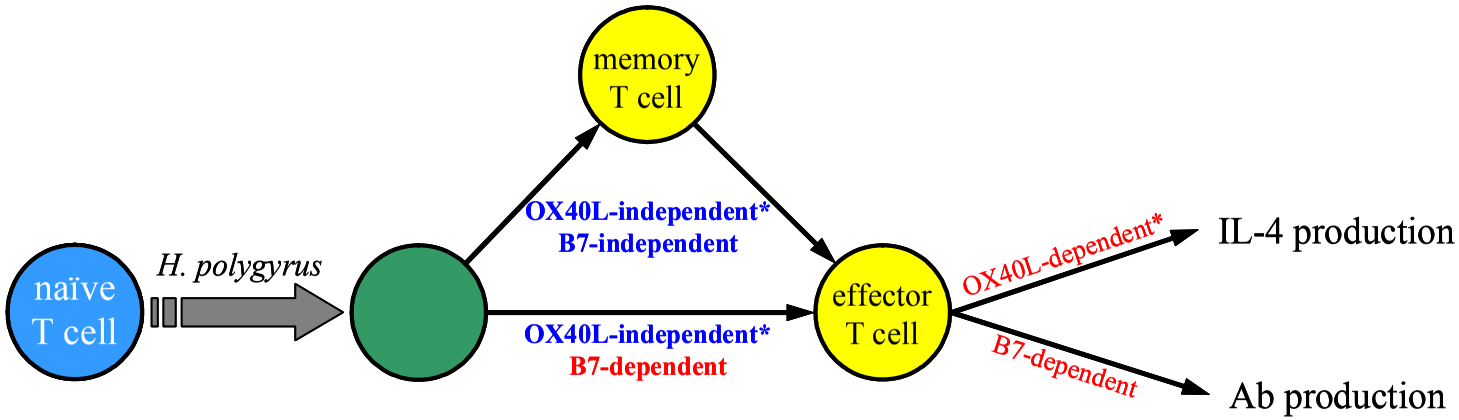
Taken together, the studies presented in this thesis suggest differential roles for costimulatory molecules in the development of memory T cells (Figure 11). Although the development of memory Th2 cells does not require CD28/B7 interactions, OX40/OX40L costimulation is required for optimal development of memory cells following infection with the intestinal pathogen *H. polygyrus*. Additionally, blockade of costimulatory molecules during priming, and the associated inhibition of T helper effector cell function, does not inhibit the development of memory cells during the Th2 immune response. The studies presented here provide a better understanding of the role of costimulatory molecules in the development of memory T cells during infectious disease, which should aid in designing novel immunotherapies, including improved vaccine development.

Conclusions

This dissertation has focused on the role of the costimulatory molecules B7-1 and B7-2, CD28, and OX40L in the development of IL-4-producing effector and memory Th2 cells following priming with the murine gastrointestinal nematode parasite *H. polygyrus*. Previous studies investigating the role of B7/CD28 costimulatory molecules in the development of memory cells have predominately examined $CD8^+$ T cells, or have blocked B7/CD28 interactions with CTLA-4Ig or blocking Abs. These studies have provided useful insights into the development of memory T cells.

Figure 11. OX40/OX40L interactions are required for optimal IL-4 production, while B7/CD28 costimulation is required for Ag-specific Ab production during the memory response to *H. polygyrus*. These studies suggest that memory T cells capable of producing IL-4 and mediating decreased adult worm fecundity can develop when B7 interactions are blocked during priming, but that B7 is required for delivery of cognate B cell help. In contrast, OX40L is required for optimal activation of Th2 cells resulting in IL-4 production, which mediates increases in serum IgE levels and impairs *H. polygyrus* adult worm fecundity; however, OX40L interactions are not required for cognate B cell help resulting in elevated serum IgG1 levels and GC formation. B7- or OX40L-independent pathways are indicated in blue, while B7- or OX40L-dependent pathways are indicated in red.

* partial dependence on OX40L costimulation


naïve
T cell
H. polygyrus
memory
T cell
OX40L-independent*
B7-independent
OX40L-independent*
B7-dependent
effector
T cell
OX40L-dependent*
B7-dependent
IL-4 production
Ab production

However, they have also demonstrated that the generation of primary and memory $CD8^+$ CTLs is less dependent on B7/CD28 costimulatory interactions than the development of $CD4^+$ T helper cells (89,104,106,164), making comparisons between the development of memory $CD4^+$ and $CD8^+$ T cells difficult. Studies which have investigated the role of costimulatory molecules during the in vivo development of memory $CD4^+$ T cells have used blocking anti-B7-1 and anti-B7-2 Abs or CTLA-4Ig to block B7/CD28 interactions. In these studies, treatment with CTLA-4Ig at the time of challenge did not inhibit the secondary host-protective response to *H. polygyrus* (85,99,107), but did inhibit the secondary Ab response to KLH, as well as in vitro lymphocyte proliferation in response to sheep red blood cells (87). Treatment with anti-B7-1 or anti-B7-2 blocking Abs inhibited development (100) or activation (102,103) of memory T cells, depending on whether the antibodies were administered during the primary or secondary immune responses, respectively. However, the possibility remained that incomplete blockade by Abs or CTLA-4Ig allowed development of effector or memory T cells in these studies. The recent availability of mice genetically deficient for both B7-1 and B7-2, or CD28, allowed us to directly examine the role of B7/CD28 interactions during the generation of memory $CD4^+$ T cells when B7/CD28 interactions were blocked during both the primary and secondary immune responses to the intestinal pathogen *H. polygyrus*.

These studies represent the first analysis of the role of OX40/OX40L costimulation in the development of memory T cells during infectious diseases. Previous studies have suggested that OX40/OX40L interactions play an important role in the long-term survival of lymphocytes, and in the Th2-mediated allergy response (31,119,120,123-126,132,136,163). However, it was unclear whether these costimulatory molecules were

important for the development of memory T cells during infectious disease. Therefore, the studies reported here allowed direct examination of the role of OX40/OX40L interactions during the development of the primary and memory immune responses to *H. polygyrus*.

H. polygyrus, as a model system for investigating the development of memory cells, has the added advantage that it is easy to distinguish between the chronic primary immune response and the host-protective secondary response which inhibits adult worm maturation, resulting in reduced egg production and eventual worm expulsion. This is particularly beneficial because there is no expression marker that is consistently used to identify memory cells. In addition, although *H. polygyrus* is a murine parasite, it provides a useful in vivo model for human nematode infections—particularly hookworm infections, which have significant morbidity due to the anemia and malnutrition resulting from chronic infection.

Examination of the role of costimulatory molecules during memory T cell development is particularly relevant to the treatment of autoimmune diseases and the development of vaccines. Recent clinical trials have used CTLA-4Ig to block B7/CD28 costimulatory interactions in the T-dependent disease, psoriasis, resulting in an improvement of clinical disease activity, as indicated by a reduction in epidermal hyperplasia, correlated with quantitative reductions in T cell infiltration to lesion sites (165). This study suggested that treatment with CTLA-4Ig mediated clinical improvement of psoriasis due to inhibition of T cell activation and proliferation. Although these results hold great potential for clinical treatment of T cell mediated diseases, our studies suggest that blocking costimulatory interactions during clinical

disease should be carefully tailored to the specific disease and stage of disease. The studies presented in this thesis demonstrated that the development of the Th2 memory response to *H. polygyrus* was able to occur when a functional primary immune response was blocked in the absence of costimulation through B7-1 and B7-2 (see paper 1 & Figure 7), suggesting that the memory Th2 cells were developing directly from naïve T cells, without going through an intermediate effector stage (166,167). Therefore, blocking B7/CD28 interactions at the time of priming may block the primary immune response, while allowing development of a memory response. This effect may be desirable during the development of vaccines, where the desired end-result is development of a protective memory response without development of a pathogenic primary response. However, when developing treatments for allergic diseases, the goal is to block the development of memory responses to Ag; this may require blocking multiple costimulatory interactions—for example, B7/CD28 and OX40/OX40L costimulatory interactions. Although further studies are required, the studies presented in this thesis should provide a framework for the development of vaccines and other immunotherapies for Th2-mediated diseases. Traditionally, the antigenic complexity of extracellular parasites has hindered conventional vaccine development based on cloning of a few protective antigens. Therefore, the ability to regulate the immune response in an Ag-specific manner by targeting costimulatory molecules may work particularly well against complex antigenic targets such as intestinal worms.

In conclusion, the studies presented in this thesis have examined the role of costimulatory molecules in the development of memory Th2 cells during infection with the nematode *H. polygyrus*. These studies have demonstrated that 1) development of

IL-4-producing memory Th2 cells can occur in the absence of B7-1 and B7-2, or CD28; 2) memory Th2 cells can develop directly from naïve T cells when a functional primary immune response is blocked; 3) the absence of CD28 does not result in tolerance induction during infectious disease; 4) Ag-specific serum Ig production and GC formation are differentially regulated in mice genetically lacking B7-1/B7-2 or CD28, suggesting the possibility that a third B7 ligand—which is neither CD28 or CTLA-4—may be providing a positive signal; 5) OX40L is required for optimal Th2 cell activation, resulting in IL-4 production, class-switching to IgE, and host protection; and 6) OX40L is not required for Ag-specific T cell cycle progression, expression of CXCR5, or migration to the B cell zone. Taken together, these studies suggest differential roles for costimulatory molecules in the development of memory T cells; development of IL-4-producing T cells is OX40L-dependent, while Ig production is B7-dependent (see Figure 11). The studies presented in this thesis provide a clearer understanding of the role of costimulatory molecules in the development of memory Th2 cells during infectious disease, and should aid in the development of immunotherapies which manipulate the development of immunological memory.

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Appendix 1

Abbreviations:

Ab	antibody;
Ag	antigen;
APC	antigen presenting cell;
ConA	concanavalinA;
CTL	cytotoxic T lymphocytes;
DC	dendritic cell;
DTH	delayed-type hypersensitivity;
GC	germinal center;
GDP	guanine diphosphate;
GTP	guanine triphosphate;
HSA	heat stable antigen;
Ig	immunoglobulin;
KLH	keyhole limpet hemocyanin;
LCMV	lymphocytic choriomeningitis virus;
MLN	mesenteric lymph node;
NP-OVA	nitrophenyl-OVA;
OVA	ovalbumin peptide;
TCR	T cell receptor;
TNP-KLH	trinitrophenyl-keyhole limpet hemocyanin;

Appendix 2

Statement of Author's Contribution:

The author was personally involved in the planning and execution of all experiments described in this dissertation. The author also developed the immunofluorescence technique described in these experiments, and was directly involved in the additional experimental assays used for these studies. The author was directly responsible for writing both papers presented here, as partial fulfillment of the dissertation requirements.